



Published in final edited form as:

*Development*. 2006 May ; 133(10): 1901–1910. doi:10.1242/dev.02328.

## Retinoic acid guides eye morphogenetic movements via paracrine signaling but is unnecessary for retinal dorsoventral patterning

Andrei Molotkov, Natalia Molotkova, and Gregg Duester\*

Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.

### Abstract

Retinoic acid (RA) is required for patterning of the posterior nervous system, but its role in the retina remains unclear. RA is synthesized in discrete regions of the embryonic eye by three retinaldehyde dehydrogenases (RALDHs) displaying distinct expression patterns. Overlapping functions of these enzymes have hampered genetic efforts to elucidate RA function in the eye. Here, we report *Raldh1*, *Raldh2* and *Raldh3* single, double and triple null mice exhibiting progressively less or no RA synthesis in the eye. Our genetic studies indicate that RA signaling is not required for the establishment or maintenance of dorsoventral patterning in the retina, as we observe normal expression of *Tbx5* and ephrin B2 (*Efnb2*) dorsally, plus *Vax2* and *Ephb2* ventrally. Instead, RA is required for the morphogenetic movements needed to shape the developing retina and surrounding mesenchyme. At early stages, *Raldh2* expressed in mesenchyme and *Raldh3* expressed in the retinal pigmented epithelium generate RA that delivers an essential signal to the neural retina required for morphogenetic movements that lead to ventral invagination of the optic cup. At later stages, *Raldh1* expressed in dorsal neural retina and *Raldh3* expressed in ventral neural retina (plus weaker expression of each in lens/corneal ectoderm) generates RA that travels to surrounding mesenchyme, where it is needed to limit the anterior invasion of perioptic mesenchyme during the formation of corneal mesenchyme and eyelids. At all stages, RA target tissues are distinct from locations of RA synthesis, indicating that RALDHs function cell-nonautonomously to generate paracrine RA signals that guide morphogenetic movements in neighboring cells.

### Keywords

Retinoic acid; Morphogenetic movements; *Raldh1*; *Raldh2*; *Raldh3*; *Tbx5*; *Vax2*; EphB2; ephrin B2; Eye; Optic cup; Retina; Perioptic mesenchyme; Mouse

## INTRODUCTION

Congenital defects of optic cup morphogenesis represent an important cause of childhood blindness or vision impairment (Gregory-Evans et al., 2004). Many ocular developmental defects are of unknown etiology and some may be caused by environmental influences such as vitamin A deficiency in humans (Hornby et al., 2002) or animals (Wilson et al., 1953; Marsh-Armstrong et al., 1994; Dickman et al., 1997; Reijntjes et al., 2005). One important function of vitamin A (retinol) is to serve as the precursor of retinoic acid (RA), an important developmental signaling molecule (Duester, 2000). Some studies suggest a connection between impaired retinol or RA function and developmental eye defects in humans and mice. For instance, humans with missense mutations in the gene encoding serum retinol binding protein have been shown to exhibit retinal dystrophy (Seeliger et al., 1999). Also, mice with

\*Author for correspondence (duester@burnham.org).

reduced RA receptor signaling give rise to offspring with ocular defects (Lohnes et al., 1994).

RA serves as a ligand for three nuclear RA receptors (RAR) that bind DNA as heterodimers with retinoid X receptors (RXR) and directly regulate gene expression. Binding of all-trans-RA to the RAR component of RAR/RXR heterodimers is necessary and sufficient to rescue signaling in RA-deficient embryos, whereas the isomer 9-cis-RA (which can bind RXR) is unnecessary and is undetectable under physiological conditions (Mic et al., 2003). RARs are expressed in overlapping patterns in the eye during development, and mice carrying single null mutations of RARs have relatively normal eye development except for RAR $\beta$  null mice, which exhibit a retrolenticular membrane in the vitreous body (Ghyselinck et al., 1997). Genetic elimination of two RARs results in microphthalmia, ventral shortening of the retina, and abnormalities of the cornea, eyelids and conjunctiva (Lohnes et al., 1994). Thus, RARs mediate the ocular functions of vitamin A, as the defects observed are essentially the same as those seen during gestational vitamin A deficiency.

Based upon the expression patterns of RA-synthesizing enzymes, RA was proposed to control dorsoventral patterning of the retina (Wagner et al., 2000). However, functional studies on these enzymes have not supported a role for RA in the establishment of retinal dorsoventral patterning (Fan et al., 2003; Matt et al., 2005) or cell fate determination (Mic et al., 2004). A recent study in chick, using overexpression of dominant-negative RA receptors has suggested that RA is needed to maintain retinal dorsoventral patterning at later stages (Sen et al., 2005), but this has not been examined genetically. By contrast, RA has been clearly defined as a signaling molecule needed for patterning of other regions of the central nervous system, including the hindbrain and the spinal cord (Maden, 2002). Although it is clear that RA controls eye development, its mechanism may be different from that of other eye signaling molecules that control patterning or cell fate determination, such as fibroblast growth factor, which is released by the surface ectoderm to stimulate neural retina differentiation (Russell, 2003), or activin, which is released by perioptic mesenchyme to stimulate retinal pigment epithelium differentiation (Fuhrmann et al., 2000). A recent genetic study in mice suggests that RA controls eye development by acting within the neural crest-derived perioptic mesenchyme (Matt et al., 2005). The mechanism of RA action during eye development remains unclear largely because of an incomplete understanding of the complex spatiotemporal properties of RA synthesis in this organ and of the target tissues upon which RA acts.

The first step of RA synthesis (the oxidation of retinol to retinaldehyde) occurs ubiquitously through the action of several overlapping alcohol dehydrogenases and short-chain dehydrogenase/reductases (Molotkov et al., 2002). By contrast, the second step of RA synthesis (oxidation of retinaldehyde to RA) is limited to specific tissues by retinaldehyde dehydrogenases (i.e. RALDH1, RALDH2, RALDH3) expressed in non-overlapping patterns in the developing mouse eye (Mic et al., 2002; Matt et al., 2005). Despite their unique expression patterns, analyses of *Raldh1*, *Raldh2* and *Raldh3* null mice have not resulted in clearly defined ocular defects, probably because of the cell-nonautonomous action of RA generated by the remaining enzymes (Niederreither et al., 1999; Fan et al., 2003; Dupé et al., 2003; Mic et al., 2004). Here, RALDH single and compound null mutant mice have been analyzed to provide further insight into the role of RA in eye development, and to sort out the individual contributions of each enzyme. We find that RA signaling guides the morphogenetic movements of the retina and perioptic mesenchyme, rather than playing a role in retinal dorsoventral patterning. Furthermore, our studies reveal several paracrine mechanisms whereby RA is released from RALDH-expressing cells and guides morphogenetic movements in neighboring cells, thus demonstrating that RALDHs function cell-nonautonomously in eye development.

## MATERIALS AND METHODS

### Generation of *Raldh3* null mice

A gene targeting vector for *Raldh3* was designed to generate a floxed allele of *Raldh3* with the ultimate goal of eliminating exon 11, similar to a *Raldh1* gene targeting vector that generated a null phenotype in that case (Fan et al., 2003). The *Raldh3* gene targeting vector thus included a *loxP* site upstream of exon 11, followed by exon 11, a *PGK-neo* positive-selection gene downstream of exon 11 (transcribed in same direction as *Raldh3*), and another *loxP* site just downstream of *PGK-neo*. In order to generate a null allele, matings were performed between the initial *Raldh3*-targeted chimeric males and wild-type females expressing Cre recombinase in the germline in order to remove exon 11 (i.e. about 600 bp of *Raldh3*) and *PGK-neo* in all cells. PCR genotyping of tail or yolk sac DNA was performed using the primers 5'-GCCATAAAAGCTGGGGTGTCTG-3' (located in intron 10) and 5'-TGGATGGATGGATGGGTGATG-3' (located in intron 11) which produce a wild-type product of ~950 bp and a mutant product of ~350 bp (primer annealing temperature was 63°C). Initial matings of *Raldh3*<sup>+/-</sup> heterozygous null mice resulted in 70 adult offspring with the following genotypes: 0 -/- (0%), 45 +/- (64%), 25 +/+ (36%). The absence of surviving homozygous mice indicated that the *Raldh3* knockout leads to a lethal phenotype. The description of another *Raldh3*<sup>-/-</sup> mouse line indicated the existence of a newborn lethal defect due to blockage of the nasal passages, leading to respiratory stress at birth (Dupé et al., 2003).

### Generation of compound *Raldh* null embryos

Several *Raldh* mutant mice have been previously described, including *Raldh1*<sup>-/-</sup> mice, which survive to adulthood (Fan et al., 2003), *Raldh2*<sup>-/-</sup> embryos, which exhibit midgestation lethality (Mic et al., 2002), and *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> double null embryos, which also exhibit midgestation lethality (Mic et al., 2004). Generation of compound RALDH null mice was facilitated by independent assortment of the three RALDH genes on separate chromosomes and by the ability to obtain adult mice homozygous for the *Raldh1* null allele. Matings were performed between the above mice and *Raldh3*<sup>-/-</sup> mice to obtain *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> and *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null embryos, as well as *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> triple null embryos. Embryos derived from timed matings were genotyped by PCR analysis of yolk sac DNA. Following mating, noon on the day of vaginal plug detection was considered as embryonic day 0.5 (E0.5). Embryos were staged according to somite number.

### Rescue of eye defects with dietary RA supplementation

The rescue of *Raldh2*<sup>-/-</sup> embryos by maternal dietary RA supplementation was performed similar to a previous description (Mic et al., 2004), with RA dose demonstrated to be in the normal physiological range (Mic et al., 2003). Briefly, all-trans-RA (Sigma) was dissolved in corn oil and mixed with powdered mouse chow to provide a final concentration of 0.1 mg/g for treatment from E6.75–E8.5 (limited rescue), or 0.2 mg/g for treatment from E8.5–E14.5. In some cases, embryos were analyzed when the mother was still on the RA-supplemented diet, but in other cases the mother was returned to standard mouse chow and embryos were analyzed at a later time point. Such food was prepared fresh twice daily (morning and evening) and provided ad libitum.

### Detection of retinoic acid

RA activity was detected as previously described in embryos carrying the *RARE-lacZ* RA-reporter transgene, which places *lacZ* (encoding β-galactosidase) under the transcriptional control of a retinoic acid response element (RARE) (Rossant et al., 1991). Following genetic crosses of *RARE-lacZ* mice with RALDH mutant mice, the *RARE-lacZ* transgene segregated

independently from *Raldh1* and *Raldh2*, but not *Raldh3*, null alleles, suggesting that *RARE-lacZ* is located close to *Raldh3* on mouse chromosome 7. In order to detect *RARE-lacZ* expression in *Raldh3*<sup>-/-</sup> embryos, 20 male mice obtained from *RARE-lacZ*×*Raldh3*<sup>+/-</sup> crosses were screened for a crossover event by mating to wild-type females, and one male was identified that carried *RARE-lacZ* linked to the *Raldh3* null allele. The *RARE-lacZ-Raldh3* crossover male was also mated to *Raldh1* and *Raldh2* mutant mice to enable the analysis of RA activity in double and triple *Raldh* mutants. Stained embryos were embedded in 3% agarose and sectioned 50 μm with a vibratome.

### In situ hybridization, proliferation and apoptosis assays

Whole-mount in situ hybridization was performed to detect expression *Raldh1*, *Raldh2* and *Raldh3*, as described previously (Mic et al., 2002). Probes for *Tbx5* (Chapman et al., 1996) and *Vax2* (Mui et al., 2002) were also examined. Histological examination was performed on paraffin-sectioned tissues stained with hematoxylin/eosin, as previously described (Fan et al., 2003). Cell proliferation assays were performed on paraffin tissue sections by staining for histone-3 phosphorylation using anti-phosphohistone-3 antibodies (Upstate Cell Signaling Solutions, Lake Placid, NY) as previously reported (Molotkova et al., 2005); cells counts were averages of six sections from comparable ocular regions of two embryos (three sections per eye). Apoptosis was examined in paraffin tissue sections using a TUNEL assay, as described (Hyer et al., 2003).

## RESULTS

### Lack of RA results in morphogenetic movement defects

Our previous studies demonstrated that *Raldh1*<sup>-/-</sup> mice survive to adulthood and exhibit no noticeable defects in eye development, although they completely lose RA synthesis in the dorsal neural retina (Fan et al., 2003). Other studies have recently shown that *Raldh3*<sup>-/-</sup> mice die at birth as a result of nasal defects and exhibit a mild delay in ventral retina growth (Dupé et al., 2003). We generated an independent line of *Raldh3*<sup>-/-</sup> mice, as well as *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null mice, and examined the effect on ocular development. Compared with wild-type and *Raldh1*<sup>-/-</sup> embryos, which appeared identical at E14.5, *Raldh3*<sup>-/-</sup> embryos initiated optic cup formation but exhibited completely penetrant ocular defects consisting of excessive thickening of the central neural retina, loss of the vitreous body, and a prominent retrolenticular membrane between the retina and lens (Fig. 1A–C; *n*=5 out of 5). Our *Raldh3*<sup>-/-</sup> null mutant exhibits more of a central retina defect than that of another *Raldh3*<sup>-/-</sup> null mutant that was reported to exhibit a shortening of the ventral retina (Matt et al., 2005). This could be due to strain differences, especially differences in how efficiently *Raldh2* acts in the two strains during early optic cup formation (see below). Together, the findings from both strains suggest that RALDH3 synthesizes the RA required to complete optic cup and vitreous body formation.

Unexpectedly, *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null embryos exhibited not only the same ocular defects as *Raldh3*<sup>-/-</sup> embryos, but also excessive invasion of periocular mesenchyme anterior to the retina, resulting in abnormal thickening of the corneal stroma and eyelid folds (Fig. 1D; *n*=3 out of 3). This observation reveals that *Raldh1* and *Raldh3* both function to control the invasion of perioptic mesenchyme around the retina that normally occurs during anterior eye formation (Cvekl and Tamm, 2004). We find that loss of both *Raldh1* and *Raldh3* is required to observe the defect because of functional redundancy. Another recent study also observed excessive perioptic mesenchyme in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null embryos (Matt et al., 2005). As *Raldh1* and *Raldh3* are expressed in different regions of the retina but not in the periocular mesenchyme (Fig. 2G,I), this suggests that RA synthesized in the retinal epithelium

travels as a signal to the surrounding mesenchyme to control morphogenetic movements of mesenchymal ocular tissues.

The location of RA signaling activity in the developing eye at E10.5 was examined in whole-mount wild-type and *Raldh* mutant mice carrying the *RARE-lacZ* RA-reporter transgene (Rossant et al., 1991). *Raldh3*<sup>-/-</sup> embryos exhibited a large decrease in eye RA activity compared with wild-type embryos, whereas *Raldh1*<sup>-/-</sup> embryos exhibited only a modest decrease (Fig. 1E–G). *Raldh3*<sup>-/-</sup> embryos completely lost RA activity in the ventral retina and olfactory pit, tissues that express *Raldh3* but not *Raldh1* or *Raldh2* at E10.5 (Fig. 2G–I). E10.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos carrying *RARE-lacZ* exhibited almost a total loss of RA activity in the eye, demonstrating that *Raldh1* is primarily responsible for the RA activity remaining in the eye of *Raldh3*<sup>-/-</sup> embryos (Fig. 1H); residual RA activity in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> eyes is due to RA synthesized by *Raldh2* up to the point when its expression ends near E9.75 (see below). These results confirm that *RARE-lacZ* is a specific marker for eye RA signaling, as its expression is totally dependent upon RA-synthesizing enzymes.

### Rescue of retinal and mesenchymal ocular defects by maternal RA treatment

As early embryonic defects in *Raldh2*<sup>-/-</sup> embryos can be rescued by maternal dietary RA supplementation (Mic et al., 2004), we used this method to rescue eye development in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos. Following RA treatment from E8.5–E14.5, we found that E14.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos exhibited eye development that was very similar to normal with a vitreous body now clearly observable, a neural retina of close to normal thickness, a diffuse retrolenticular membrane, and normal perioptic mesenchyme growth (Fig. 2A,B; *n*=3 out of 4). Examination of E11.5 rescued *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos carrying *RARE-lacZ* revealed that maternally-derived RA had reached the neural retina/vitreous body junction and dorsal perioptic mesenchyme (Fig. 2D; *n*=4 out of 4), although such RA activity was significantly less than that observed in *Raldh1*<sup>-/-</sup> littermates, where *Raldh3* is generating RA locally (Fig. 2C). As an unrescued E11.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> eye completely lacked RA activity (Fig. 7E), maternal RA was responsible for the RA detected in rescued mutants. Interestingly, the RA activity detected in the neural retina of rescued *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos was not uniformly distributed, but was found along the periphery of the neural retina closest to the vitreous body (Fig. 2D). It is also apparent that such RA activity is not detected in the dorsal-most or ventral-most neural retina where *Raldh1* and *Raldh3* are expressed (Fig. 2G,I) and where RA activity is normally found at high levels (Fig. 2C), but instead is observed in the central neural retina (Fig. 2D). Analysis of E12.5 rescued *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos demonstrated high RA activity in the vitreous body and ventral perioptic mesenchyme, and RA was more uniformly distributed throughout the central neural retina but was still not detected in the dorsal-most or ventral-most regions of the neural retina, which normally have highest levels of RA activity (Fig. 2E,F; *n*=2 out of 2). These results reveal that the targets of RA action during eye development (inferred from the action of rescuing levels of maternal RA) are distinct from the normal sites of RA synthesis.

Our observations with the RA-reporter transgene demonstrate that the low dose of maternal dietary RA used to rescue *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> eye development is evidently not stimulating RA signaling in all portions of the eye. One possible explanation for this observation is that RA may be preferentially degraded in certain embryonic tissues, as has been observed for rhombomere 4 of the hindbrain, which induces a RA-degrading enzyme encoded by *Cyp26c1* to create an RA boundary (Sirbu et al., 2005). Although *Cyp26a1* and *Cyp26c1* function in RA degradation in the central retina during late eye development (E15.5), no such role has been found for earlier stages, and certainly no role has been found in the dorsal or ventral retina at any stage (Sakai et al., 2004). Another possibility for the selective appearance of RA activity in rescued eyes is that incoming RA may be preferentially sequestered by cellular



RA-binding proteins (CRABP) that facilitate RA signaling (Noy, 2000). In support of this possibility, we found that *Crabp1* is preferentially expressed in the vitreous body (and weakly in perioptic mesenchyme), and that *Crabp2* is expressed in the dorsal and ventral perioptic mesenchyme (Fig. 2J,K). These mesenchymal sites of CRABP expression correspond to the regions in which the highest RA activity is found during the rescue.

### RA generated by *Raldh1* controls perioptic mesenchyme invasion

Our experiments indicate that either *Raldh1* or *Raldh3* alone generates sufficient RA to control anterior invasion of perioptic mesenchyme both dorsally and ventrally in *Raldh3*<sup>-/-</sup> embryos. However, at E10.5, we detected RA activity in the dorsal but not ventral perioptic mesenchyme of *Raldh3*<sup>-/-</sup> embryos (Fig. 1G). This discrepancy was resolved by examination at a later stage (E11.5), at which point *Raldh3*<sup>-/-</sup> embryos did exhibit RA activity in both dorsal and ventral mesenchyme (Fig. 3A,B). Thus, RALDH1 evidently generates increasing levels of RA in the dorsal retina during development that are sufficient to reach the dorsal and ventral perioptic mesenchyme by E11.5 to fulfill RA function in this tissue.

### RA is required for selective cell death in perioptic mesenchyme

In order to investigate RA function in the perioptic mesenchyme, we examined the effect of a loss of RA on cell proliferation and apoptosis. Ocular cell proliferation, examined in E11.5 embryos by the detection of phosphohistone-3 (H3P), was not significantly different between *Raldh1*<sup>-/-</sup> embryos that maintain relatively normal RA activity and *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> littermates that lose RA activity (Fig. 4A,B). The number of H3P-positive cells in *Raldh1*<sup>-/-</sup> perioptic mesenchyme was 99±7.3, whereas in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> littermates the number was 95±7.6 (*n*=6). For retina, the number of H3P-positive cells in *Raldh1*<sup>-/-</sup> embryos was 26.3±2.8, whereas in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> littermates the number was 27.5±1.1 (*n*=6). Thus, loss of RA does not effect cell proliferation in either perioptic mesenchyme or retina. However, a loss of RA does have an effect on apoptosis, as described in a recent study where dorsal and ventral regions of apoptosis detected in the perioptic mesenchyme of wild-type embryos were missing in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos (Matt et al., 2005). We found that these dorsal and ventral regions of perioptic mesenchyme apoptosis were still present in E11.5 *Raldh1*<sup>-/-</sup> embryos, but that almost no apoptotic cells were detectable in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> littermates (Fig. 4C,D; *n*=3 out of 3). The pattern of apoptosis in the retina did not appear to be significantly different. These findings suggest that RA derived from either RALDH1 or RALDH3 functions in the perioptic mesenchyme to limit cell numbers by stimulating apoptosis in selective regions.

### RA is unnecessary to establish or maintain dorsoventral patterning of the retina

The unique expression patterns of *Raldh1* in the dorsal retina and *Raldh3* in the ventral retina led to the hypothesis that RA generated differentially in these two regions may somehow control dorsoventral patterning of the retina (Wagner et al., 2000). We examined this possibility by examining mutant embryos for the expression of *Tbx5* expressed dorsally (Koshiba-Takeuchi et al., 2000) and *Vax2* expressed ventrally (Mui et al., 2002; Barbieri et al., 2002), both of which play crucial roles in the establishment of retinal dorsoventral patterning (McLaughlin et al., 2003). We found that *Tbx5* and *Vax2* were expressed in their correct retinal positions in E10.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos that lack eye RA activity (Fig. 5A–C; *n*=3). As it is possible that *Raldh2* may have supplied RA for dorsoventral patterning in *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos, we also examined an *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> triple mutant embryo at E10.5. In this embryo, *Vax2* expression was still detectable in the ventral optic vesicle, which had not invaginated to form a complete optic cup (Fig. 5D). Previous studies had already shown that *Tbx5* is still expressed in *Raldh2*<sup>-/-</sup> embryos, and appears prior to the ocular expression of *Raldh1* or *Raldh3* (Mic et al., 2002).

RA signaling in chick embryos has been reported to be necessary for expression of the retinal topographic guidance molecules ephrin B2 (dorsally) and EPHB2 (ventrally) that maintain dorsoventral patterning at later stages (Sen et al., 2005). However, we found that ephrin B2 (*Efnb2* – Mouse Genome Informatics) and *Ephb2* were still expressed in their correct locations in E14.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> mouse embryos that lack retinal RA activity (Fig. 5E–H; *n*=3). Thus, the previous studies that had relied upon the introduction of a dominant-negative RA receptor may have been artifactual (Sen et al., 2005). Combined with our previous observations, these findings suggest that RA generated by *Raldh1* and *Raldh3* in the neural retina does not function cell-autonomously to establish or maintain dorsoventral patterning of the neural retina, but instead functions cell-nonautonomously to control survival of the adjacent perioptic mesenchyme.

### RA is necessary for ventral invagination of the optic cup

The observation that *Raldh3*<sup>-/-</sup> and *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos can initiate optic cup formation indicates that RA is either unnecessary for initial optic cup formation or that *Raldh2* expressed during the optic vesicle stage can provide RA for this function. We previously reported that E10.5 *Raldh2*<sup>-/-</sup> embryos and *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos develop an optic vesicle that fails to invaginate into an optic cup and fails to express *Raldh3*, but that both of these defects could be rescued by maternal dietary RA supplementation limited to E8.5 when *Raldh2* expression is first observed in the optic vesicle (Mic et al., 2004). However, it is unclear whether the maternal RA introduced during such a limited rescue had a direct effect on optic vesicle invagination, which does not begin until E9.5, or was simply needed to remove the impairment of trunk development caused by the loss of *Raldh2*, which leads to a growth defect at E8.5 and lethality by E10.5 (Mic et al., 2002). In order to resolve this issue, we examined limited-rescue *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos carrying *RARE-lacZ* at E8.5 and found that RA activity was detected in the trunk, but no activity was observed in the developing eye or anywhere in the head (Fig. 7L). Thus, maternal RA administered only to E8.5 does not stimulate RA signaling in the developing eye, suggesting that maternal RA that enters the embryo during this limited-rescue procedure acts posteriorly to rescue the survival of *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos, which then allows optic vesicle development to continue to the stage when *Raldh3* expression begins.

In order to determine whether RA generated by *Raldh3* in rescued *Raldh2*<sup>-/-</sup> embryos functions in initial optic cup formation, we generated *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null embryos. *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos examined at E10.5–E11.5 following maternal dietary RA supplementation to E8.5 exhibited a failure in the ventral invagination of the optic vesicle that defines the junction between the ventral retina and optic stalk (Fig. 6A–D; *n*=3 out of 3). In these mutants, lens invagination had occurred, as well as dorsal invagination of the optic vesicle, which generates the junction between the retinal pigment epithelium (RPE) and the neural retina, thus resulting in an incomplete optic cup (Fig. 6A–D). As the ventral optic cup defect was still observed later at E11.5, this demonstrated a true blockage in ventral invagination rather than simply a delay (Fig. 6C,D). These results show that RA is required for ventral folding during initial optic cup formation in addition to the role described above for morphogenetic movements that limit neural retina thickness. Also, it is now clear that *Raldh2* can provide this initial function in unrescued *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos that develop an optic cup, and that *Raldh3* can provide this function in limited-rescue *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos.

### RA is not required for dorsal invagination of the optic cup

We noticed that limited-rescue *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos carrying *RARE-lacZ* still exhibited *Raldh1* expression (Fig. 6A,B) and RA activity (Fig. 6C,D) dorsally, where optic vesicle invagination had occurred to generate an incomplete optic cup. In order to determine

whether dorsal RA activity generated by *Raldh1* was necessary for dorsal invagination of the optic vesicle, we examined *Raldh1<sup>-/-</sup>;Raldh2<sup>-/-</sup>;Raldh3<sup>-/-</sup>* triple null embryos carrying *RARE-lacZ*. *Raldh1<sup>-/-</sup>;Raldh2<sup>-/-</sup>;Raldh3<sup>-/-</sup>* embryos examined at E10.5 following maternal dietary RA supplementation to E8.5 exhibited no RA activity in the eye; *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* embryos had low RA activity (Fig. 6E–G; also compare with Fig. 1H). The triple mutant confirms that the low RA activity observed in the eye of *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* embryos is in fact due to *Raldh2*, and demonstrates the persistence of RA activity in some neural and heart tissues in the absence of *Raldh1*, *Raldh2* and *Raldh3*. Tissue sections revealed that rescued *Raldh1<sup>-/-</sup>;Raldh2<sup>-/-</sup>;Raldh3<sup>-/-</sup>* embryos carrying *RARE-lacZ* do retain dorsal invagination of the optic vesicle despite a complete loss of RA activity (Fig. 6H). These important observations in double and triple mutants demonstrate that RA is not required for dorsal invagination of the optic vesicle, and that expression of *Raldh1* in the dorsal optic vesicle does not generate sufficient RA for ventral invagination.

### Contribution of each RALDH gene to eye development

The three RALDHs are expressed in unique tissues during mouse eye development, and RA activity can be detected in those tissues in mice carrying the *RARE-lacZ* RA-reporter transgene (Wagner et al., 2000; Mic et al., 2002). As RA synthesized in one region of the developing eye can travel to other regions, it has previously been impossible to determine the extent to which each enzyme can supply RA to the eye and the importance of that RA. However, analysis of *RARE-lacZ* expression in each of the RALDH double null mutants now makes this possible, and comparison of the locations of such RA activity with expression of the single remaining enzyme allows conclusions to be made concerning the spatiotemporal relationship between sites of RA synthesis and RA target tissues. The insights obtained from each RALDH double mutant are described below.

### Contribution of *Raldh2*

RA activity generated by *Raldh2* in *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* eyes was initially detected throughout the optic vesicle epithelium (but not the surface epithelium) prior to invagination, whereas when ventral optic vesicle invagination was underway at E9.75 RA activity was much higher in the anterior optic vesicle fated to become neural retina, and after optic cup formation at E10.5 only a low level of RA activity remained in the most anterior and central portion of the neural retina adjacent to the vitreous body (Fig. 7A–D). At E11.5, no RA was detectable in *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* eyes (Fig. 7E). The location of RA synthesis in *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* eyes (determined by *Raldh2* expression) was initially throughout the optic vesicle epithelium prior to invagination (Fig. 7F,G), matching well with the RA activity at this stage (Fig. 7B). However, *Raldh2* expression in the optic vesicle was lost by E9.0 and replaced by expression in the mesenchyme adjacent to the temporal portion of the optic vesicle from E9.0–E9.75 (Fig. 7H–K), and by E10.5 this expression was also lost (Fig. 2H). Thus, during the stage when ventral optic vesicle invagination occurs (E9.5–E9.75), RA is being synthesized by *Raldh2* in the temporal mesenchyme and is traveling to the adjacent optic vesicle where it acts. Our studies further suggest that early expression of *Raldh2* throughout the optic vesicle epithelium prior to invagination is unnecessary for eye development. This is consistent with studies in chick embryos demonstrating that *Raldh2* is never expressed in the optic vesicle epithelium but is expressed in the temporal mesenchyme (Blentic et al., 2003). As *Raldh2* expression in mouse temporal mesenchyme ends before E10.5, *Raldh2* does not synthesize the RA needed for later morphogenetic movements that limit neural retinal thickness and peri-optic mesenchyme growth.



### Contribution of *Raldh3*

RA activity generated by *Raldh3* in limited-rescue *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> eyes was detected at E9.25 in the eye field surface ectoderm but not the optic vesicle epithelium (Fig. 7L–N); however, when ventral invagination had begun RA activity was detected in the optic vesicle with the highest activity dorsally and lower activity ventrally, at the site where ventral invagination was occurring (Fig. 7O,P). The site of RA synthesis in *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> eyes inferred from the *Raldh3* expression pattern was initially limited to the surface ectoderm prior to ventral optic vesicle invagination (Fig. 7Q,R), thus matching well with the RA activity at this early stage (Fig. 7N). However, when ventral invagination begins at E9.75, *Raldh3* expression was also detected in the dorsal RPE (Fig. 7S,T), and by E10.5, when the optic cup has formed, expression was observed in the entire RPE (Fig. 7S,T), and by E10.5, when the optic cup has formed, expression was observed in the entire RPE and ventral neural retina, but was absent in the central/dorsal neural retina (Fig. 7U, Fig. 2I). Our previous studies of unrescued *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos (which do not form optic cups as late as E10.5) demonstrated that *Raldh3* expression and RA activity is observed in the pre-lens surface ectoderm but not the optic vesicle as late as E10.5, indicating that RA synthesized by *Raldh3* in the surface ectoderm does not travel to the optic vesicle and does not stimulate invagination (Mic et al., 2004). Studies in chick have demonstrated that optic cup formation requires signaling from pre-lens ectoderm (Hyer et al., 2003), but evidently RA is not this signal as the optic cup develops in *Raldh3*<sup>-/-</sup> embryos lacking RA synthesis in the pre-lens ectoderm (Fig. 1C). Thus, the early expression domain of *Raldh3* in the surface ectoderm is an insufficient source of RA for optic cup formation, making it clear that *Raldh3* expression in the RPE is the source of RA that acts on the neural retina for ventral invagination. After RA from the RPE has stimulated ventral invagination, *Raldh3* expression initiates in the ventral neural retina and RA travels throughout the optic cup and into the surrounding mesenchyme, where it functions to limit perioptic mesenchyme growth (Fig. 2C).

### Contribution of *Raldh1*

RA activity was not detected in limited-rescue *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> eyes at E9.5 (Fig. 7V,W), but low RA activity generated by *Raldh1* was observed at E10.0 (Fig. 7X) and high RA activity was seen at E11.5 (Fig. 6D). The location of RA synthesis in *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> eyes inferred from the *Raldh1* expression pattern was limited to the dorsal neural retina at both the optic vesicle stage, E9.5 (Fig. 7Y), and the optic cup stage, E10.5 (Fig. 2G). These findings indicate that RA synthesized by *Raldh1* accumulates too late to play a role in ventral invagination of the optic vesicle or the control of neural retina expansion. Our studies above have demonstrated that, later in development, *Raldh1* does not provide sufficient RA for vitreous body morphogenesis, but does generate sufficient RA for the control of perioptic mesenchyme growth both dorsally and ventrally (Fig. 1C,D). As RALDH1 has a 10-fold lower enzyme activity for RA synthesis than RALDH2 or RALDH3 (Grün et al., 2000), the relative inefficiency of RALDH1 as an RA source for eye development may be understandable.

## DISCUSSION

### RA controls retinal morphogenetic movements rather than dorsoventral patterning

The genetic studies presented here enable us to generate a new model for RA action during eye development. Previously it was hypothesized that RA may be involved in dorsoventral patterning of the retina based upon the dorsal and ventral expression patterns of *Raldh1* and *Raldh3* (Wagner et al., 2000). *Tbx5* and *Vax2* encode two transcription factors playing key roles in establishing dorsoventral patterning of the retina (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Barbieri et al., 2002). However, we demonstrate here that E10.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos exhibit correct dorsoventral expression of *Tbx5* and *Vax2* in the retina. We further show that *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> triple mutants that lack RA throughout the optic vesicle stage still express *Vax2*, thus providing a completely conclusive

genetic result. In addition, our studies here indicate that RA function during eye development does not commence until E9.5, after *Tbx5* and *Vax2* expression has already been established (Chapman et al., 1996; Mui et al., 2002). Thus, genetic studies indicate that RA is not required for the establishment of retinal dorsoventral patterning. A role for RA in maintaining retinal dorsoventral patterning was suggested from studies in chick embryos where dominant-negative RA receptors were found to interfere with the expression of ephrin B2 and EPHB2, which function as dorsoventral topographic guidance molecules (Sen et al., 2005). However, our genetic studies demonstrate conclusively that this is not the case, as *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos completely lack RA activity in the retina, but maintain ephrin B2 and *Ephb2* expression. We suggest that the introduction of dominant-negative RA receptors may have wider effects on gene expression than was expected, possibly because of the ability of RA receptors to heterodimerize with RXR receptors that regulate not only RA signaling but also signaling by numerous other nuclear receptors (Chawla et al., 2001). RA signaling is also unnecessary for retinal determination, as *Raldh2*<sup>-/-</sup> embryos lacking RA activity in the optic vesicle still express early retinal determinants such as *Pax6*, *Six3*, *Rx* and *Mitf* (Mic et al., 2004). Instead, our findings here indicate that RA signaling occurring within the retina itself is required to control the morphogenetic movements that result in optic cup formation.

In addition to optic cup formation, neural tube closure represents another major morphogenetic movement in the central nervous system. However, RA is unnecessary for neural tube closure (Wilson et al., 2003). Also, RA has not previously been associated with neural morphogenetic movements, although it has been reported to play a role in ventral retina formation (Marsh-Armstrong et al., 1994; Lohnes et al., 1994; Dickman et al., 1997; Reijntjes et al., 2005). Thus, our studies define a novel role for RA in neural development, through its ability to stimulate the morphogenetic movements needed for retinal invagination. Our studies on *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos completely lacking eye RA activity demonstrate that RA is not required for dorsal invagination of the optic vesicle that defines the junction between the dorsal retina and pigment epithelium. However, our studies on these triple mutants have revealed that RA is required for ventral invagination of the optic vesicle that defines the junction between the ventral retina and optic stalk. Also, our studies on *Raldh3*<sup>-/-</sup> single mutants suggest that, as the neural retina expands during later stages of optic cup formation, RA is required for the morphogenetic movements that restrict its thickness and allow space for the vitreous body to form.

### RA controls morphogenetic movements in retina and perioptic mesenchyme

This investigation has revealed the existence of two distinct phases of RA signaling required for eye development, including an early phase for optic cup formation and a late phase for anterior eye formation (Fig. 8). In both cases, cells expressing RALDH genes provide an RA signal that functions to control morphogenetic movements in neighboring cells. Also, in both cases two RALDH genes function as RA sources, providing functional redundancy.

During optic cup formation the sources of RA are *Raldh3* expressed in the future RPE and *Raldh2* expressed in mesenchyme on the temporal side of the optic vesicle; the RA target tissue is the neural retina. The outcome of this early phase of RA signaling is the stimulation of morphogenetic movements leading to ventral invagination of the optic vesicle to form a complete optic cup. Although *Raldh2* provides sufficient RA to initiate ventral invagination of the neural retina, it alone is an incomplete source of RA, as its expression ends too early to provide the RA needed for proper distribution of neural retinal cells as the optic cup forms. However, *Raldh3* expression continues and it alone can provide RA that is sufficient for both the initiation of ventral invagination and the proper distribution of neural retinal cells leading to formation of a normal vitreous body cavity. Our studies suggest that RA control of neural

retina morphology does not involve the regulation of cell proliferation or apoptosis. Thus, RA may control cell movements within the retina.

During anterior eye formation the sources of RA are *Raldh1* expressed in the dorsal neural retina and *Raldh3* expressed in the ventral neural retina, and the RA target tissue is the perioptic mesenchyme that is migrating past the optic cup. As *Raldh1* and *Raldh3* are also expressed at lower levels in the lens and cornea ectoderm (Matt et al., 2005), this could be another source of RA that enters the perioptic mesenchyme. The outcome of this late phase of RA signaling is the limitation of perioptic mesenchyme morphogenetic movements that provide tissue contributing to the corneal mesenchyme and eyelids (Cvekl and Tamm, 2004). *Raldh1* and *Raldh3* can each alone function as sufficient sources of RA for the control of perioptic mesenchyme invasion. Our studies, and those of others (Matt et al., 2005), suggest that RA may perform this function by stimulating apoptosis in specific regions of the perioptic mesenchyme, which would reduce cell numbers. However, a restrictive effect of RA on mesenchymal cell migration cannot be ruled out. The identification of perioptic mesenchyme as a target of RA synthesized in ocular ectodermal tissues is consistent with other studies demonstrating the need for perioptic mesenchyme to interact with retina and lens for proper eye morphogenesis (Cvekl and Tamm, 2004).

### **RALDHs function cell-nonautonomously during eye development**

Our studies with rescued RALDH mutant mice carrying an RA-reporter transgene have revealed that the target of RA action changes during eye morphogenesis. The initial target is the neural retina at the optic vesicle stage, then the target switches to the perioptic mesenchyme after optic cup formation. These targets are distinct from but adjacent to locations of RA synthesis, thus demonstrating that RA does not need to function in the cells that synthesize RA but instead functions in a paracrine fashion to guide the morphogenetic movements of neighboring cells. Thus, all three RALDHs function cell-nonautonomously. This has also been observed for posterior neural development, where it has been determined that RA synthesized in the somitic mesoderm by RALDH2 functions in the adjacent neuroectoderm but not in the somites themselves (Molotkova et al., 2005). Thus, in the neural tube, paracrine RA signaling stimulated by RALDH2 occurs by mesenchymal to epithelial signaling. In the eye, we find the existence of three paracrine RA signaling mechanisms: RALDH2 stimulates mesenchymal to epithelial signaling for optic cup formation; RALDH3 stimulates epithelial to epithelial signaling for optic cup formation; RALDH1 and RALDH3 both stimulate epithelial to mesenchymal signaling for anterior eye formation.

Interestingly, for both neural tube and optic cup development, RALDH2 stimulates mesenchymal to epithelial signaling. This was not initially suspected for optic cup development, as mouse *Raldh2* is expressed early in the optic vesicle epithelium just after its budding from the forebrain, and only later exhibits expression in the mesenchyme adjacent to the optic vesicle, just prior to optic cup formation. However, our studies here on RA-rescued *Raldh1<sup>-/-</sup>;Raldh2<sup>-/-</sup>* embryos carrying *RARE-lacZ* have demonstrated that a dose of RA sufficient to rescue overall embryonic development and optic cup formation does not stimulate RA signaling in the early optic vesicle epithelium. Thus, *Raldh2* expression in the early optic vesicle is unnecessary for optic cup formation, a finding that is consistent with the observation that *Raldh2* is expressed in the temporal mesenchyme but not in the optic vesicle epithelium of chick embryos (Blentic et al., 2003). It is unclear whether *Raldh2* expression in the mouse optic vesicle serves any function or whether it is an evolutionary relic.

### **RALDH3 provides sufficient RA to control morphogenetic movements**

The investigations reported here illustrate the usefulness of compound RALDH null mice in revealing the spatiotemporal role of RA signaling in tissues where more than one RALDH

contributes RA. Our findings make it clear that each of the three RALDH genes contributes to eye morphogenesis. *Raldh1* can provide RA only for the control of perioptic mesenchyme growth. *Raldh2* is able to supply RA only for initial optic cup formation, as its expression ends too early to provide RA for later morphogenetic movements. *Raldh3* alone can supply all of the RA needed for eye morphogenetic movements in the mouse. Knowledge of the ocular RA target tissues gained in these studies will facilitate the identification of RA-regulated genes to further reveal the mechanisms by which RA controls morphogenetic movements.

## Acknowledgments

We thank Ozgene Pty Ltd (Bentley, Australia) for help in generating the *Raldh3* null mouse, V. Papaioannou for the *Tbx5* probe, G. Lemke for the *Vax2* probe, N. Gale for the ephrin B2 probe, T. Pawson for *Ephb2* probe, and O. Sirbu and X. Zhao for helpful discussions. This work was funded by National Institutes of Health grant EY013969.

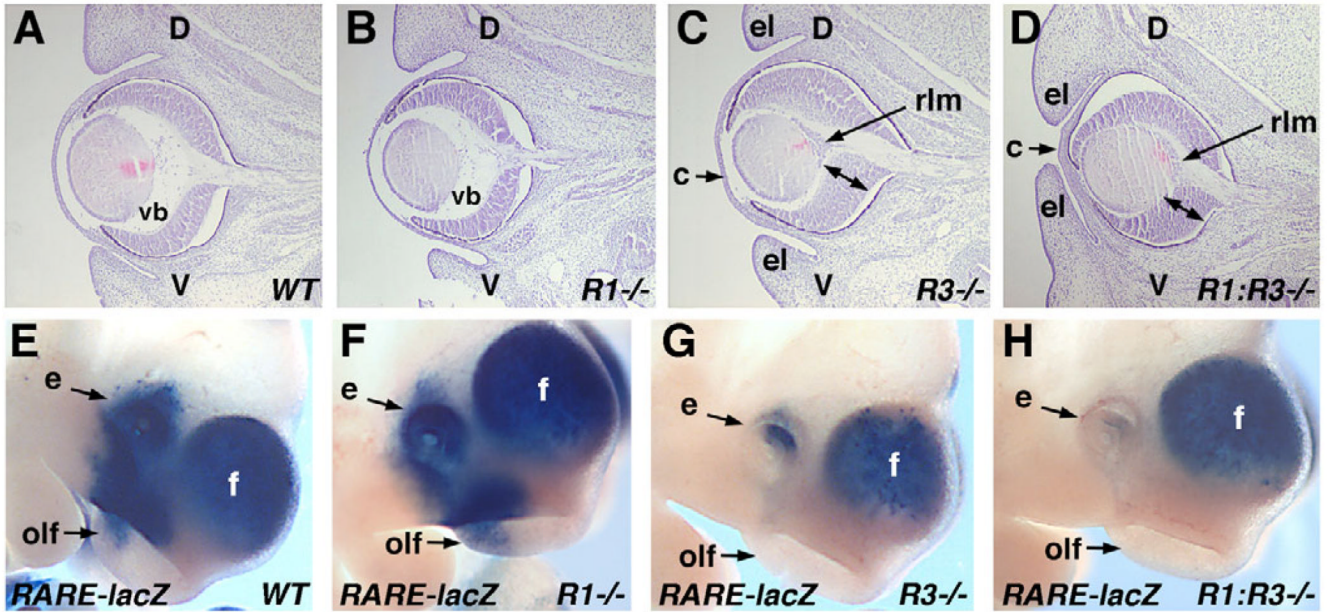
## References

- Barbieri AM, Broccoli V, Bovolenta P, Alfano G, Marchitello A, Mocchetti C, Crippa L, Bulfone A, Marigo V, Ballabio A, et al. *Vax2* inactivation in mouse determines alteration of the eye dorsal-ventral axis, misrouting of the optic fibres and eye coloboma. *Development* 2002;129:805–813. [PubMed: 11830579]
- Blentic A, Gale E, Maden M. Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. *Dev. Dyn* 2003;227:114–127. [PubMed: 12701104]
- Chapman DL, Garvey N, Hancock S, Alexiou M, Agulnik SI, Gibson-Brown JJ, Cebra-Thomas J, Bollag RJ, Silver LM, Papaioannou VE. Expression of the T-box family genes, *Tbx1–Tbx5*, during early mouse development. *Dev. Dyn* 1996;206:379–390. [PubMed: 8853987]
- Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: Opening the X-files. *Science* 2001;294:1866–1870. [PubMed: 11729302]
- Cvekl A, Tamm ER. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. *BioEssays* 2004;26:374–386. [PubMed: 15057935]
- Dickman ED, Thaller C, Smith SM. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* 1997;124:3111–3121. [PubMed: 9272952]
- Duester G. Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur. J. Biochem* 2000;267:4315–4324. [PubMed: 10880953]
- Dupé V, Matt N, Garnier J-M, Chambon P, Mark M, Ghyselinck NB. A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. *Proc. Natl. Acad. Sci. USA* 2003;100:14036–14041. [PubMed: 14623956]
- Fan X, Molotkov A, Manabe S-I, Donmoyer CM, Deltour L, Foglio MH, Cuenca AE, Blauer WS, Lipton SA, Duester G. Targeted disruption of *Aldh1a1* (*Raldh1*) provides evidence for a complex mechanism of retinoic acid synthesis in the developing retina. *Mol. Cell. Biol* 2003;23:4637–4648. [PubMed: 12808103]
- Fuhrmann S, Levine EM, Reh TA. Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Development* 2000;127:4599–4609. [PubMed: 11023863]
- Ghyselinck NB, Dupé V, Dierich A, Messaddeq N, Garnier J-M, Rochette-Egly C, Chambon P, Mark M. Role of the retinoic acid receptor beta (RARβ) during mouse development. *Int. J. Dev. Biol* 1997;41:425–447. [PubMed: 9240560]
- Gregory-Evans CY, Williams MJ, Halford S, Gregory-Evans K. Ocular coloboma: a reassessment in the age of molecular neuroscience. *J. Med. Genet* 2004;41:881–891. [PubMed: 15591273]
- Grün F, Hirose Y, Kawachi S, Ogura T, Umesono K. Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem* 2000;275:41210–41218. [PubMed: 11013254]
- Hornby SJ, Ward SJ, Gilbert CE, Dandona L, Foster A, Jones RB. Environmental risk factors in congenital malformations of the eye. *Ann. Trop. Paediatr* 2002;22:67–77. [PubMed: 11926054]

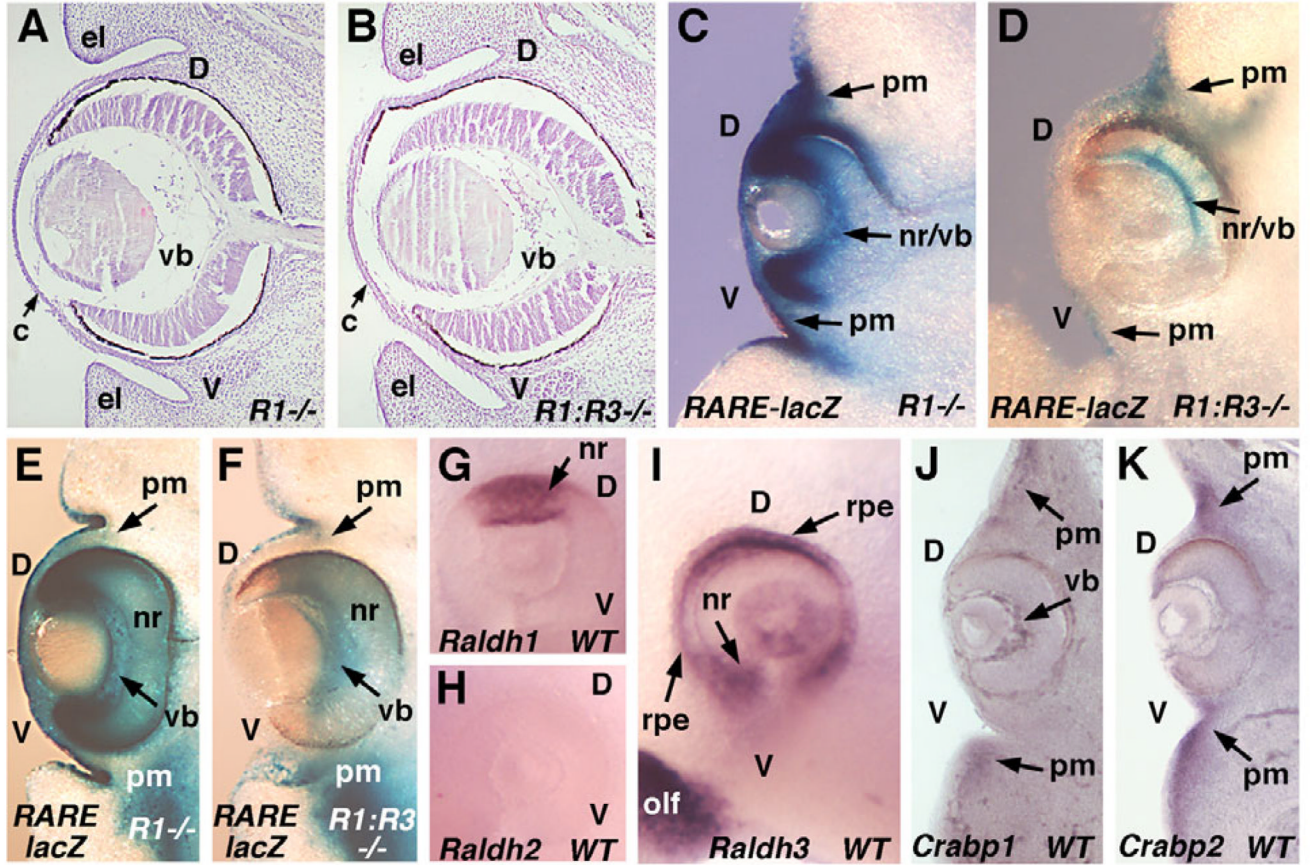
- Hyer J, Kuhlman J, Afif E, Mikawa T. Optic cup morphogenesis requires pre-lens ectoderm but not lens differentiation. *Dev. Biol* 2003;259:351–363. [PubMed: 12871706]
- Koshiba-Takeuchi K, Takeuchi JK, Matsumoto K, Momose T, Uno K, Hoepker V, Ogura K, Takahashi N, Nakamura H, Yasuda K, et al. *Tbx5* and the retinotectum projection. *Science* 2000;287:134–137. [PubMed: 10615048]
- Lohnes D, Mark M, Mendelsohn C, Dollé P, Dierich A, Gorry P, Gansmuller A, Chambon P. Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. *Development* 1994;120:2723–2748. [PubMed: 7607067]
- Maden M. Retinoid signalling in the development of the central nervous system. *Nat. Rev. Neurosci* 2002;3:843–853. [PubMed: 12415292]
- Marsh-Armstrong N, McCaffery P, Gilbert W, Dowling JE, Dräger UC. Retinoic acid is necessary for development of the ventral retina in zebrafish. *Proc. Natl. Acad. Sci. USA* 1994;91:7286–7290. [PubMed: 8041782]
- Matt N, Dupé V, Garnier J-M, Dennefeld C, Chambon P, Mark M, Ghyselinck NB. Retinoic acid-dependent eye morphogenesis is orchestrated by neural crest cells. *Development* 2005;132:4789–4800. [PubMed: 16207763]
- McLaughlin T, Hindges R, O’Leary DDM. Regulation of axial patterning of the retina and its topographic mapping in the brain. *Curr. Opin. Neurobiol* 2003;13:57–69. [PubMed: 12593983]
- Mic FA, Haselbeck RJ, Cuenca AE, Duester G. Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of *Raldh2* null mutant mice. *Development* 2002;129:2271–2282. [PubMed: 11959834]
- Mic FA, Molotkov A, Benbrook DM, Duester G. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc. Natl. Acad. Sci. USA* 2003;100:7135–7140. [PubMed: 12782789]
- Mic FA, Molotkov A, Molotkova N, Duester G. *Raldh2* expression in optic vesicle generates a retinoic acid signal needed for invagination of retina during optic cup formation. *Dev. Dyn* 2004;231:270–277. [PubMed: 15366004]
- Molotkov A, Fan X, Deltour L, Foglio MH, Martras S, Farrés J, Parés X, Duester G. Stimulation of retinoic acid production and growth by ubiquitously-expressed alcohol dehydrogenase *Adh3*. *Proc. Natl. Acad. Sci. USA* 2002;99:5337–5342. [PubMed: 11959987]
- Molotkova N, Molotkov A, Sirbu IO, Duester G. Requirement of mesodermal retinoic acid generated by *Raldh2* for posterior neural transformation. *Mech. Dev* 2005;122:145–155. [PubMed: 15652703]
- Mui SH, Hindges R, O’Leary DDM, Lemke G, Bertuzzi S. The homeodomain protein *Vax2* patterns the dorsoventral and nasotemporal axes of the eye. *Development* 2002;129:797–804. [PubMed: 11830578]
- Niederreither K, Subbarayan V, Dollé P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet* 1999;21:444–448. [PubMed: 10192400]
- Noy N. Retinoid-binding proteins: mediators of retinoid action. *Biochem. J* 2000;348:481–495. [PubMed: 10839978]
- Reijntjes S, Blentic A, Gale E, Maden M. The control of morphogen signaling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. *Dev. Biol* 2005;285:224–237. [PubMed: 16054125]
- Rossant J, Zirngibl R, Cado D, Shago M, Giguère V. Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev* 1991;5:1333–1344. [PubMed: 1907940]
- Russell C. The roles of Hedgehogs and Fibroblast Growth Factors in eye development and retinal cell rescue. *Vision Res* 2003;43:899–912. [PubMed: 12668059]
- Sakai Y, Luo TL, McCaffery P, Hamada H, Dräger UC. CYP26A1 and CYP26C1 cooperate in degrading retinoic acid within the equatorial retina during later eye development. *Dev. Biol* 2004;276:143–157. [PubMed: 15531370]
- Seeliger MW, Biesalski HK, Wissinger B, Gollnick H, Gielen S, Frank J, Beck S, Zrenner E. Phenotype in retinol deficiency due to a hereditary defect in retinol binding protein synthesis. *Invest. Ophthalmol. Vis. Sci* 1999;40:3–11. [PubMed: 9888420]



- Sen J, Harpavat S, Peters MA, Cepko CL. Retinoic acid regulates the expression of dorsoventral topographic guidance molecules in the chick retina. *Development* 2005;132:5147–5159. [PubMed: 16251210]
- Sirbu IO, Gresh L, Barra J, Duester G. Shifting boundaries of retinoic acid activity control hindbrain segmental gene expression. *Development* 2005;132:2611–2622. [PubMed: 15872003]
- Wagner E, McCaffery P, Dräger UC. Retinoic acid in the formation of the dorsoventral retina and its central projections. *Dev. Biol* 2000;222:460–470. [PubMed: 10837133]
- Wilson JG, Roth CB, Warkany J. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am. J. Anat* 1953;92:189–217. [PubMed: 13030424]
- Wilson L, Gale E, Maden M. The role of retinoic acid in the morphogenesis of the neural tube. *J. Anat* 2003;203:357–368. [PubMed: 14620376]



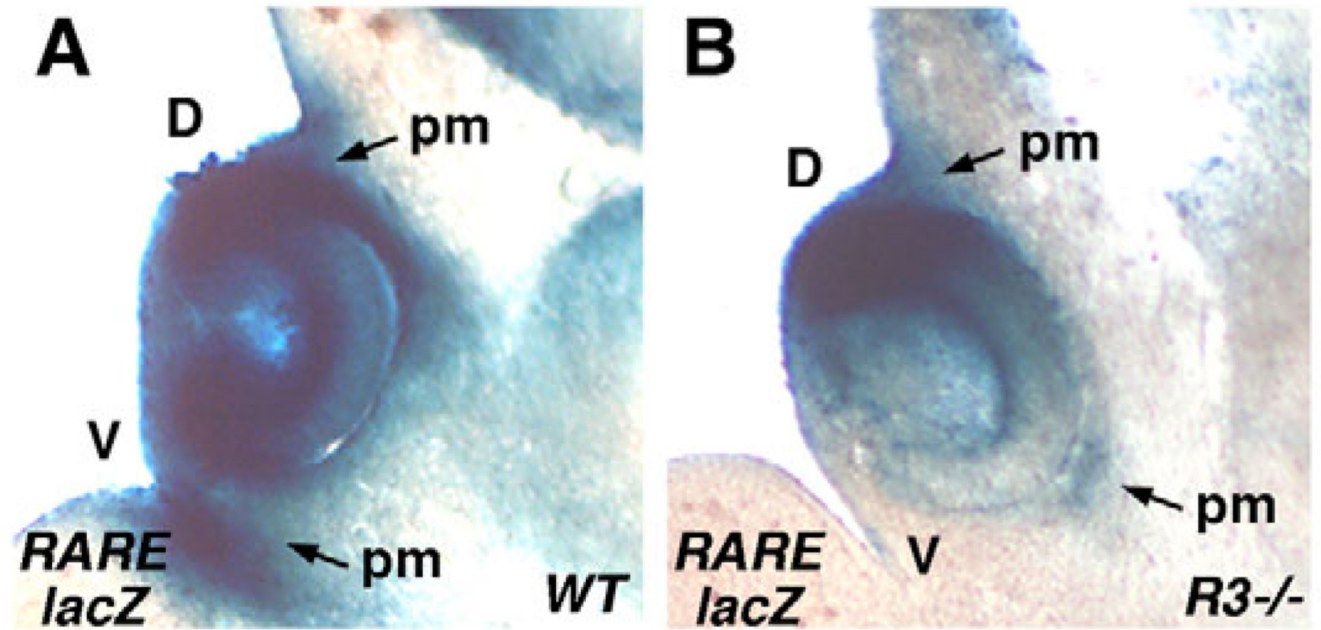
**Fig. 1. Morphogenetic movement defects in developing mouse eyes deficient for RA synthesis**  
 (A–D) Hematoxylin and eosin staining of frontal sections through the eye of E14.5 wild-type (WT), *Raldh1*<sup>-/-</sup> (*R1*<sup>-/-</sup>), *Raldh3*<sup>-/-</sup> (*R3*<sup>-/-</sup>) and *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> double null (*R1;R3*<sup>-/-</sup>) embryos. The double-arrow indicates thickening of the neural retina in *R3*<sup>-/-</sup> and *R1;R3*<sup>-/-</sup> embryos; notice the lack of a vitreous body and the presence of a retrolenticular membrane in these mutants. (E–H) Expression of *RARE-lacZ* (an RA-reporter transgene) in whole-mount E10.5 wild-type and mutant embryos. Note the near complete loss of *RARE-lacZ* expression in eyes of *R1;R3*<sup>-/-</sup> embryos. Olfactory pit staining is completely dependent upon *Raldh3*, but forebrain staining is not dependent upon *Raldh1* or *Raldh3*. c, cornea; D, dorsal; e, eye; el, eyelid; f, forebrain; olf, olfactory pit; pm, perioptic mesenchyme; rlm, retrolenticular membrane; V, ventral; vb, vitreous body.



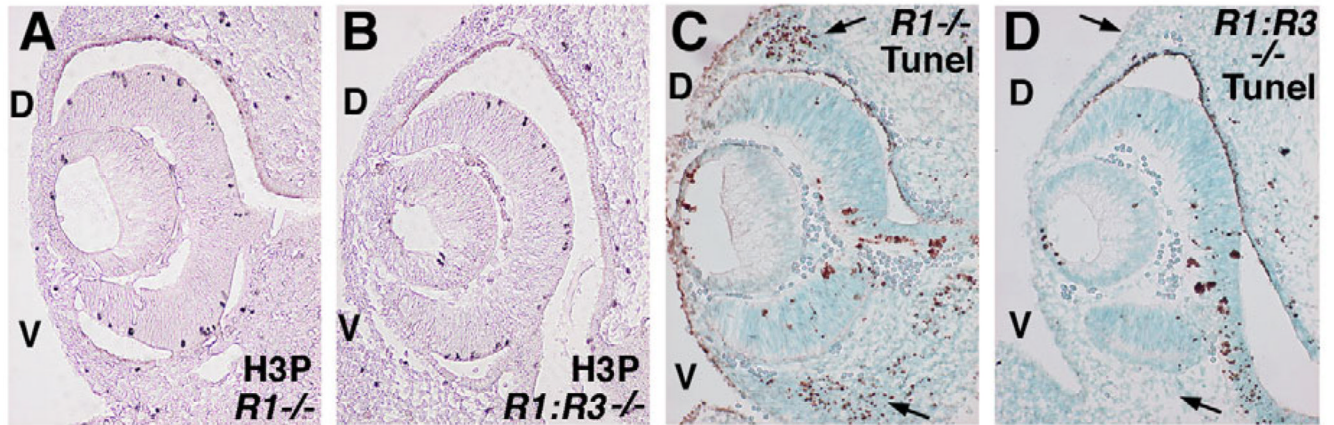
**Fig. 2. Rescue of *Raldh1*<sup>-/-</sup>; *Raldh3*<sup>-/-</sup> eye morphogenetic movements by maternal dietary RA supplementation**

(A–F) *Raldh1*<sup>-/-</sup> and *Raldh1*<sup>-/-</sup>; *Raldh3*<sup>-/-</sup> littermates were subjected to maternal dietary RA supplementation from E8.5 until the point of analysis, then analyzed by hematoxylin-eosin staining of frontal sections through the eye of E14.5 embryos (A,B), or *RARE-lacZ* expression in frontal sections of E11.5 (C,D) and E12.5 (E,F) eyes. (G–K) Whole-mount in situ hybridization to detect the expression of *Raldh1*, *Raldh2* and *Raldh3* in E10.5 whole-mount eyes (G–I), and *Crabp1* and *Crabp2* in E11.5 eyes following frontal sectioning (J,K). c, cornea; D, dorsal; el, eyelid; nr, neural retina; nr/vb, neural retina/vitreous body junction; olf, olfactory pit; pm, perioptic mesenchyme; rpe, retinal pigment epithelium; V, ventral; vb, vitreous body.





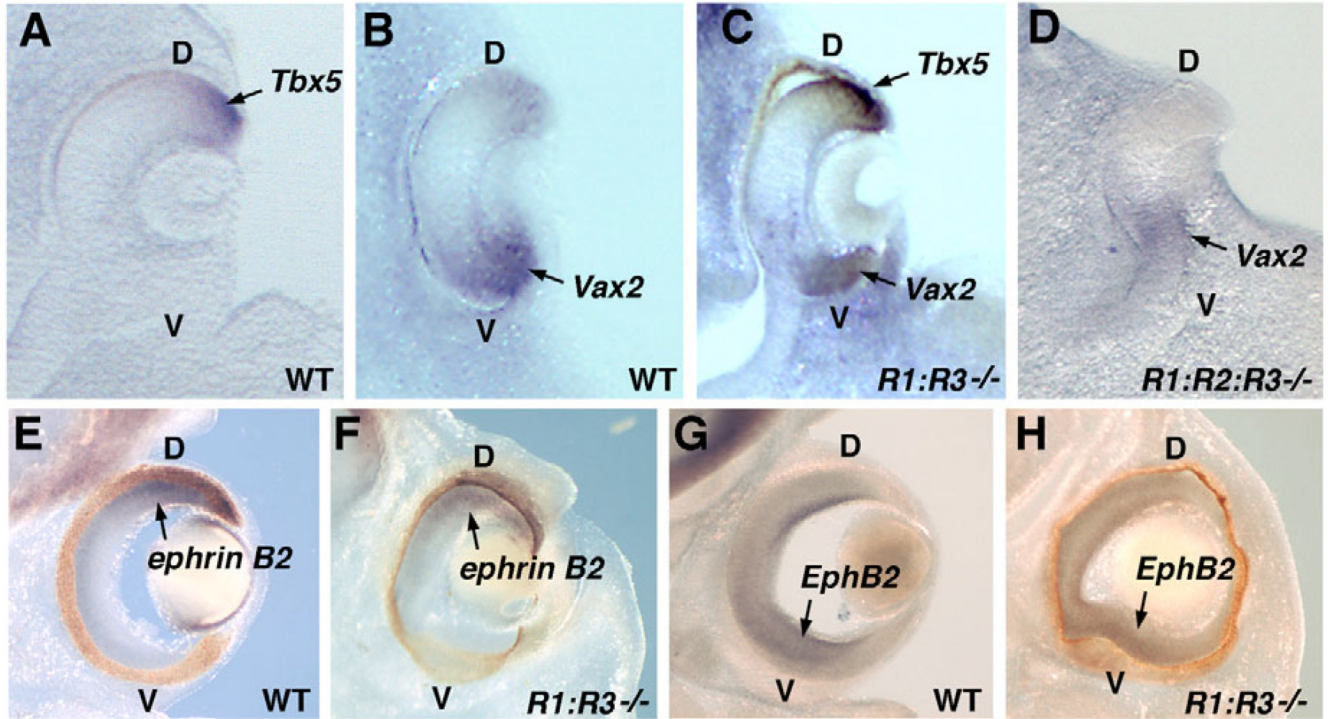
**Fig. 3. *Raldh1*-mediated RA signaling to perioptic mesenchyme**  
(A,B) *RARE-lacZ* expression in frontal sections of E11.5 wild-type (A) and *Raldh3*<sup>-/-</sup> (B) eyes.  
D, dorsal; pm, perioptic mesenchyme; V, ventral.



**Fig. 4. Loss of RA curtails apoptosis in perioptic mesenchyme**

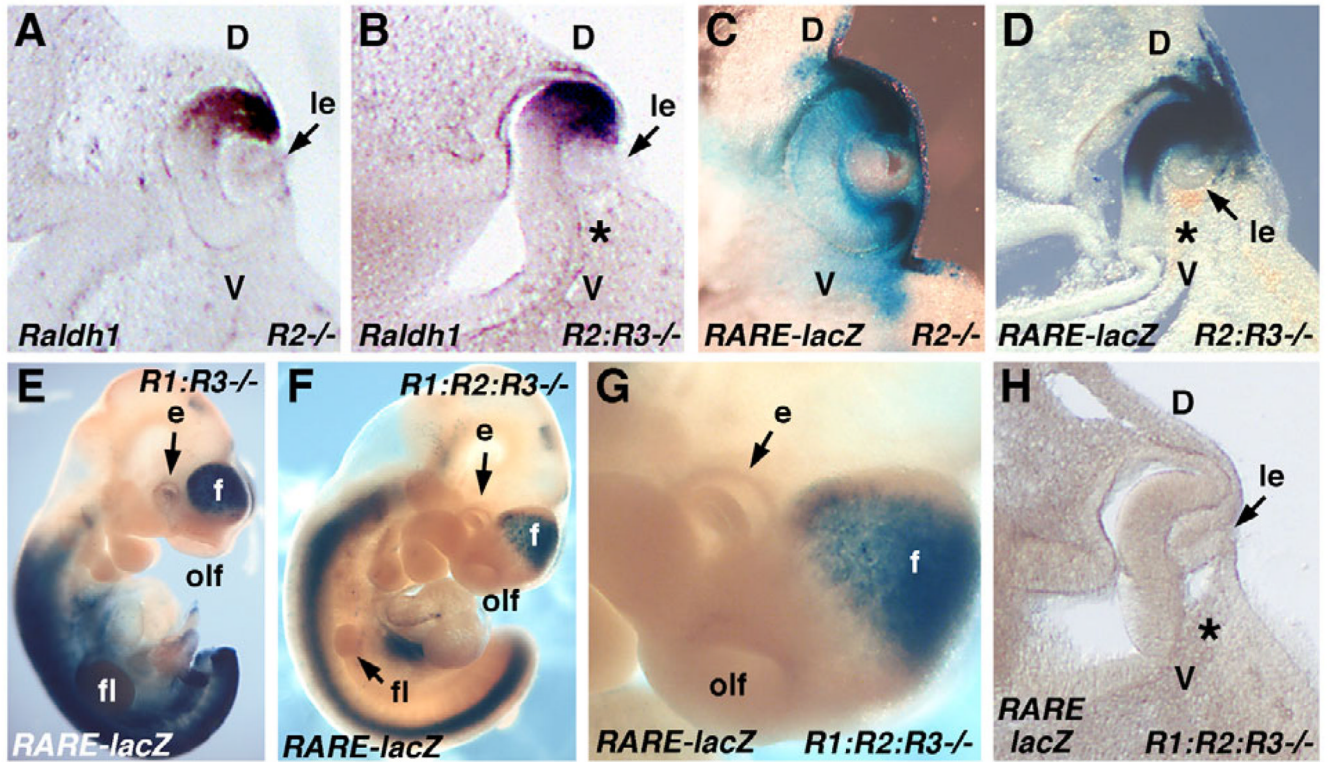
(A,B) Detection of phosphohistone 3 (H3P) in frontal sections of E11.5 *Raldh1<sup>-/-</sup>* (A) and *Raldh1<sup>-/-</sup>;Raldh3<sup>-/-</sup>* (B) eyes reveals no difference in cell proliferation. (C,D) TUNEL assay in frontal sections of E11.5 *Raldh1<sup>-/-</sup>* (C) and *Raldh1<sup>-/-</sup>Raldh3<sup>-/-</sup>* (D) eyes reveals mesenchymal regions of apoptosis that are lost in the double mutant (arrows). D, dorsal; V, ventral.





**Fig. 5. *Raldh1* and *Raldh3* are not required to establish or maintain dorsoventral patterning of the retina**

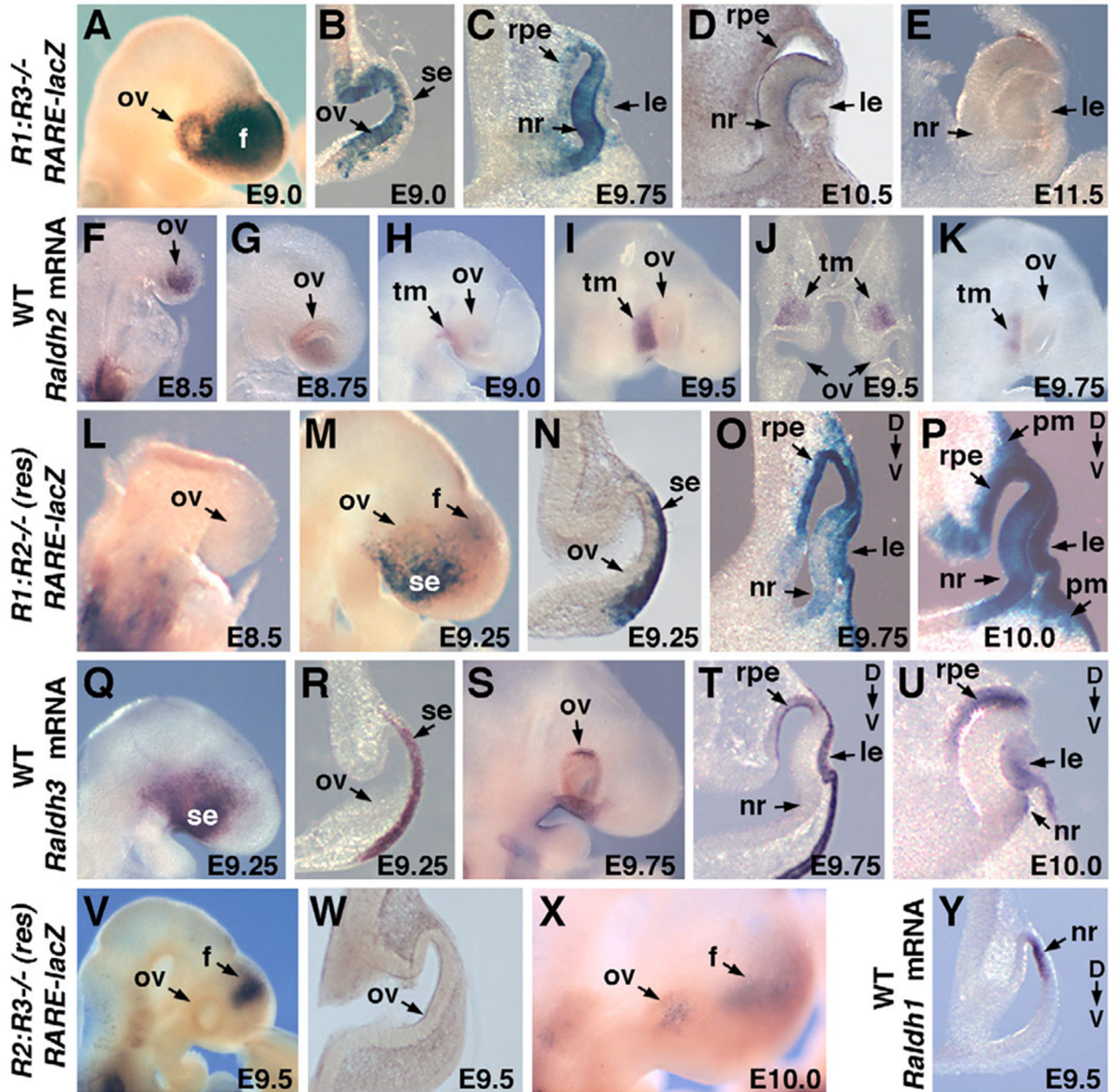
All panels consist of frontal sections of eyes at either E10.5 (A–D) or E14.5 (E–H). (A) *Tbx5* mRNA in a wild-type embryo. (B) *Vax2* mRNA in a wild-type embryo. (C) Double in situ hybridization showing both *Tbx5* and *Vax2* mRNA in a *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryo. (D) *Vax2* mRNA in limited-rescue *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> triple mutant embryo. (E–H) Detection of ephrin B2 mRNA (E,F) and *EphB2* mRNA (G,H) in wild-type and *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos reveals that the double mutant still expresses both genes at relatively normal levels in their correct dorsoventral positions. D, dorsal; V, ventral.



**Fig. 6. Severe ventral optic cup invagination defects in embryos lacking RA synthesis**

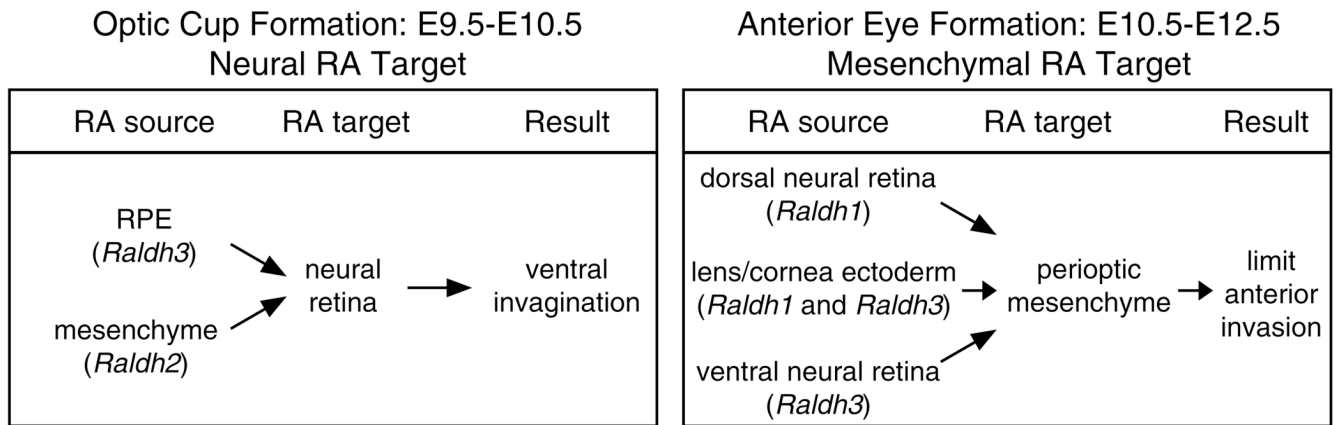
All embryos (except that shown in E) were subjected to maternal dietary RA supplementation from E6.75–E8.5, then returned to a normal diet until the point of analysis (limited-rescue). (A–D) *Raldh2*<sup>-/-</sup> and *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> littermates were analyzed for *Raldh1* expression at E10.5 (frontal sections; A,B), and for *RARE-lacZ* expression at E11.5 (frontal sections; C,D); asterisks mark the region failing to undergo ventral invagination. (E) *RARE-lacZ* expression in unrescued E10.5 *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryo. (F,G) *RARE-lacZ* expression in limited-rescue E10.5 *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> triple mutant embryo; note the complete lack of staining in the eye compared with the double mutant (E), the lack of olfactory staining (confirming the *Raldh3*<sup>-/-</sup> genotype), and the reduced trunk staining and forelimb bud growth (confirming the *Raldh2*<sup>-/-</sup> genotype). (H) Frontal section through a triple mutant optic vesicle (from embryo in F), demonstrating a complete loss of *RARE-lacZ* expression and a failure of ventral invagination (asterisk), but persistence of dorsal invagination in the absence of RA activity. D, dorsal; e, eye; f, forebrain; fl, forelimb bud; le, lens; olf, olfactory pit; V, ventral.





**Fig. 7. Sources of RA and essential target tissues for RA signaling during optic cup formation** (A–E) *RARE-lacZ* expression in unrescued *Raldh1*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos. (F–K) *Raldh2* expression in wild-type embryos. (L–P) *RARE-lacZ* expression in limited-rescue *Raldh1*<sup>-/-</sup>;*Raldh2*<sup>-/-</sup> embryos. (Q–U) *Raldh3* expression in wild-type embryos. (V–X) *RARE-lacZ* expression in limited-rescue *Raldh2*<sup>-/-</sup>;*Raldh3*<sup>-/-</sup> embryos. (Y) *Raldh1* expression in a wild-type embryo. In all panels, dorsal is at the top and ventral at the bottom, and sections are frontal except for J, which is a horizontal section that has temporal at the top and nasal at the bottom. Embryo stages are indicated on each panel. D, dorsal; f, forebrain; le, lens; nr, neural retina; ov, optic vesicle; pm, perioptic mesenchyme; res, limited rescue (maternal RA from

E6.75–E8.5); rpe, retinal pigment epithelium; se, surface ectoderm; tm, temporal mesenchyme; V, ventral.



**Fig. 8. Model for RA action during eye development**

The two phases of RA signaling during eye development, both of which occur through the cell-nonautonomous actions of RALDH genes that generate paracrine RA signals affecting the morphogenetic movements of neighboring cells. See text for a more complete description.