

Retinoic acid-dependent eye morphogenesis is orchestrated by neural crest cells

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Summary

Using genetic approaches in the mouse, we show that the primary target tissue of retinoic acid (RA) action during eye morphogenesis is not the retina nor the corneal ectoderm, which both express RA-synthesizing retinaldehyde dehydrogenases (RALDH1 and RALDH3), but the neural crest cell-derived periocular mesenchyme (POM), which is devoid of RALDH. In POM, the effects of the paracrine RA signal are mediated by the nuclear RA receptors heterodimers RXR α /RAR β and RXR α /RAR γ . These heterodimers appear to control: (1) the remodeling of the POM through activation of *Eya2*-related apoptosis;

(2) the expression of *Foxc1* and *Pitx2*, which play crucial roles in anterior eye segment development; and (3) the growth of the ventral retina. We additionally show that RALDH1 and RALDH3 are the only enzymes that are required for RA synthesis in the eye region from E10.5 to E13.5, and that patterning of the dorsoventral axis of the retina does not require RA.

Key words: Retinoic acid receptor, Retinaldehyde dehydrogenase, Periocular mesenchyme, Somatic mutagenesis, Axenfeld-Rieger's syndrome

Introduction

Vertebrate eye development is a complex process that involves multiple inductive interactions between the forebrain neuroectoderm, the ectoderm and the neural crest cell (NCC)-derived periocular mesenchyme (Johnston et al., 1979; Trainor and Tam, 1995; Cvekl and Tamm, 2004). Vitamin A (retinol), which is indispensable for eye development (Hale, 1933; Warkany and Schraffenberger, 1946; Wilson et al., 1953), exerts its biological functions through its metabolite, retinoic acid (RA). Two families of nuclear receptors, retinoid receptor (RAR) α , β and γ (binding all trans- and 9cis-RA), and RXR α , β and γ (binding 9cis-RA only), transduce the RA signal (Chambon, 1996). They act in the form of RXR/RAR heterodimers, and all RAR and RXR isoforms are expressed in the developing eye (Ghyselinck et al., 1997; Mori et al., 2001). Genetic ablation of RARs and RXRs yields a variety of severe congenital eye defects, including agenesis of the lens, coloboma of the retina or of the optic disc, shortening of the ventral retina, ventral rotation of the lens, severe malformations of the anterior segment of the eye (including absence of the iris stroma, corneal stroma and anterior chamber), agenesis of the sclera, open eyelids and absence of the Harderian gland (Lohnes et al., 1994; Kastner et al., 1994; Kastner et al., 1997; Ghyselinck et al., 1997; Mascres et al., 1998). Importantly, all of these defects have also been described in vitamin A and/or RA-deficient fetuses (Warkany and Schraffenberger, 1946; Wilson et al., 1953; Dickman et al., 1997; Dupé et al., 2003).

RA is not produced by all cells of the body at all stages of development, but is generated in a unique spatio-temporal

pattern (Rossant et al., 1991). The local conversion of vitamin A to RA involves two sequential oxidation steps: (1) the generation of retinaldehyde from retinol, essentially carried out by the ubiquitously expressed alcohol dehydrogenase type 3 (Molotkov et al., 2002); and (2) the synthesis of RA from retinaldehyde, catalyzed by retinaldehyde dehydrogenases (RALDH) (Duester, 2000). At least four isoforms (RALDH1 to RALDH4, encoded by mouse genes *Aldh1a1*, *Aldh1a2*, *Aldh1a3* and *Aldh8a1*, respectively) are able to synthesize RA in vitro (Duester, 2000; Lin et al., 2003). RALDH1 is less efficient than RALDH2 and RALDH3 at synthesizing all trans-RA (Haselbeck et al., 1999; Grün et al., 2000), whereas RALDH4 is much more active for 9cis-RA synthesis (Lin et al., 2003). Only RALDH1, RALDH2 and RALDH3 are expressed in the mouse developing eye, with non-overlapping patterns. RALDH2 is transiently present at embryonic day (E) 8.5 in the optic vesicle (Wagner et al., 2000; Mic et al., 2004), while from E9.5 onwards RALDH1 and RALDH3 are mainly detected in the dorsal and ventral fields of the retina, respectively (McCaffery et al., 1999; Grün et al., 2000; Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000). Their expression domains are separated by a stripe expressing the RA-degrading enzymes CYP26A1 and CYP26C1 (Fujii et al., 1997; Sakai et al., 2004). This spatial arrangement of RALDH1, RALDH3, CYP26A1 and CYP26C1 expression generates three areas along the dorsoventral (DV) axis of the developing retina: a ventral one with high RA levels, a dorsal one containing lower amounts of RA and an intermediate one free of RA; altogether, these areas are thought to play a crucial

role in DV patterning (Wagner et al., 2000; Dräger et al., 2001; Peters and Cepko, 2002; Sakai et al., 2004).

RALDH2, whose genetic ablation kills embryos at E9.5 (Niederreither et al., 1999; Mic et al., 2002), is required between E8.5 and E9.5 for optic cup formation and for setting up the correct expression of RALDH3 at later stages in the forming eye (Mic et al., 2004). RALDH1-null mice are viable and do not exhibit ocular defects, even though they lack RA-dependent activity in the dorsal retina and in the corresponding axonal projections at E16.5 (Fan et al., 2003). RALDH3-null mice, which die at birth from respiratory distress due to choanal atresia, display only discrete ocular malformations, namely a retrolenticular membrane and a mild shortening of the ventral retina (Dupé et al., 2003). Furthermore, neither RALDH1-null nor RALDH3-null mice exhibit an altered DV patterning of the retina (Fan et al., 2003; Dupé et al., 2003). These observations raised the question as to whether the loss of RALDH1 or RALDH3 could be functionally compensated by RALDH3 or RALDH1, respectively, or by other RALDH proteins expressed in the eye region. To gain further insight into the roles of RA signaling pathways in the developing eye, we have analyzed the ocular phenotype of mutant mice (1) lacking both RALDH1 and RALDH3, and (2) lacking RAR β and RAR γ selectively in the NCC-derived periocular mesenchyme (POM). We demonstrate that RA produced in the neural retina, the retinal pigmented epithelium and the corneal ectoderm by RALDH1 and RALDH3 acts in a paracrine manner to selectively control the expression of several genes in POM cells, whereas it is dispensable for the DV patterning of the retina.

Materials and methods

Mice

All mice, with a mixed C57BL/6 \times 129/Sv (50:50) genetic background, were housed in an animal facility licensed by the French Ministry of Agriculture (Agreement Number B67-218-5), and all animal experiments were supervised by N.B.G. (Agreement Number 67-205), who is qualified for experimenting with mice, in compliance with the European legislation on care and use of laboratory animals. The breeding diet (D03) contained 25000 UI of vitamin A per kg (UAR, Villemoisson sur Orge, France) and was provided ad libitum. Heterozygous mice were mated overnight, and animals with a vaginal plug at noon of the next day were considered as embryonic day (E) 0.5. Mice bearing loxP-flanked (floxed) RAR β (*Rarb*^{+L2}) and RAR γ (*Rarg*^{+L2}) genes (Chapellier et al., 2002a; Chapellier, 2002b), as well as the *Rare-hsp68-lacZ* (Rossant et al., 1991), *R26R* (Soriano, 1999) and *Wnt1-Cre* (Danielian et al., 1998) transgenic mice were genotyped as described. Note that mutant fetuses lacking both RALDH1 and RALDH3 (hereafter designated as *Aldh1a1/3*-null mice) were obtained at a Mendelian ratio (11 mutants obtained out of 173 littermates), but that newborns died at birth from respiratory distress, as did *Aldh1a3*-null mutants (Dupé et al., 2003).

Generation and genotyping of mice

To construct the *Aldh1a1* targeting vector for homologous recombination in embryonic stem (ES) cells (see Fig. 1A), a 6 kb-long fragment isolated from a 129/Sv mouse genomic DNA library and containing exons E7 and E8, was inserted into pBluescript II SK+ (Stratagene). A loxP-flanked neomycin resistance cassette (*neo*) was cloned into the *Bam*HI site located upstream of exon 8. Oligonucleotides 5'-ATACTTCGTATAATGTATGCTATACGAA-GTTATCCGCGG-3' and 5'-CCGCGGATAACTTCGTATAGCAT-ACATTATACGAAGTTAT-3', containing loxP and *Sac*II sites, were

then inserted 22 bp downstream of the *Ppu*MI site located on the 3' side of exon 8. This exon (amino acid residues 249-283) encodes the aldehyde dehydrogenases glutamic acid active site (E at position 268 in the LELGGKSP motif) in RALDH1. A diphtheria toxin A (*DT-A*) expression cassette was inserted at the 3' side of the genomic DNA. This targeting vector was linearized with *Kpn*I and electroporated into ES cells. One clone, targeted as expected (out of 146), was identified by Southern blot analysis (NO65, L3 allele, *Aldh1a1*^{+L3}). Transient transfection of NO65 ES cells with a Cre-expressing plasmid allowed excision of the *neo* gene, yielding cells bearing the conditional loxP-flanked L2 allele. One such clone (NO65.23, L2 allele, *Aldh1a1*^{+L2}) was injected into C57BL/6 blastocysts and the chimeras transmitted the conditional allele to their germ line. Homozygous mice (*Aldh1a1*^{L2/L2}) were indistinguishable from their wild-type littermates. They were crossed with *CMV-Cre* transgenic mice (Metzger and Chambon, 2001), and the resulting mice bearing the excised allele (*Aldh1a1*^{+L-}) were identified by Southern blot and PCR analysis. Tail DNA was genotyped by PCR using primers 1 (5'-GATTCCAGCA-AACGGTAGGA-3') and 2 (5'-ACAGGATCAGGCATCAGGAG-3'; see Fig. 1A) to amplify the wild-type (871 bp long), the loxP-flanked L2 (1030 bp long), and the excised, null, L⁻ (298 bp long) alleles. Conditions were 30 cycles, with denaturation for 15 seconds at 92°C, annealing for 30 seconds at 61°C and elongation for 30 seconds at 72°C.

Western blot analysis

Cytosolic protein extracts (50 μ g) were resolved on 12% SDS-PAGE gels and blotted onto nitrocellulose membranes (Schleicher & Schuell). RALDH1 and RALDH3 were detected using purified IgG from rabbit polyclonal antisera (gift from Dr J. L. Napoli) at a dilution of 1/500. Immunoreactions were visualized using protein A coupled to horseradish peroxidase (dilution 1/10,000), followed by chemiluminescence according to the manufacturer's protocol (Amersham).

Histology, staining and in situ RNA analysis

For histology, samples were fixed in Bouin's fluid for 5 days, embedded in paraffin, serially sectioned, and stained with Groat's hematoxylin and Mallory's trichrome. For β -galactosidase activity detection, staining for was performed as described (Rossant et al., 1991). In situ RNA hybridization was carried out as described (Dupé et al., 2003). The digoxigenin-labeled antisense riboprobes were synthesized using cDNA as templates (references upon request). In situ hybridizations were performed on serial histological sections along the entire anteroposterior axis of the embryo head. All of the sections through the eye region (i.e. about 40) were systematically analyzed. To ensure reproducibility, in situ hybridizations were repeated on both eyes from at least two embryos of each genotype. Terminal transferase-mediated dUTP-Nick-End-Labeling (TUNEL) was performed using the Apoptag kit (Chemicon International) on 7 μ m-thick serial frontal sections from paraffin-embedded embryos. Note that the entire eye region, which spans 40 sections, was analyzed and that TUNEL-positive cell clusters (see Results) were detected on six to seven consecutive sections. Cell proliferation was assessed on serial histological sections using the antibody NCL-Ki-67p (Novocastra Laboratories) as described by the manufacturer. The number of Ki-67-positive POM cell nuclei was determined by counting more than 2000 cells in at least four eyes of each genotype by the manufacturer.

Results

Ablation of RALDH1 is not life-threatening

To assess the role of RALDH1 in vivo, we generated an *Aldh1a1*-null allele in the mouse through homologous recombination in embryonic stem cells (Fig. 1A-C). Heterozygous mating ($n=35$; mean of eight pups per litter) yielded 25% ($n=74$) wild-type (*Aldh1a1*^{+/+}), 50% ($n=143$)

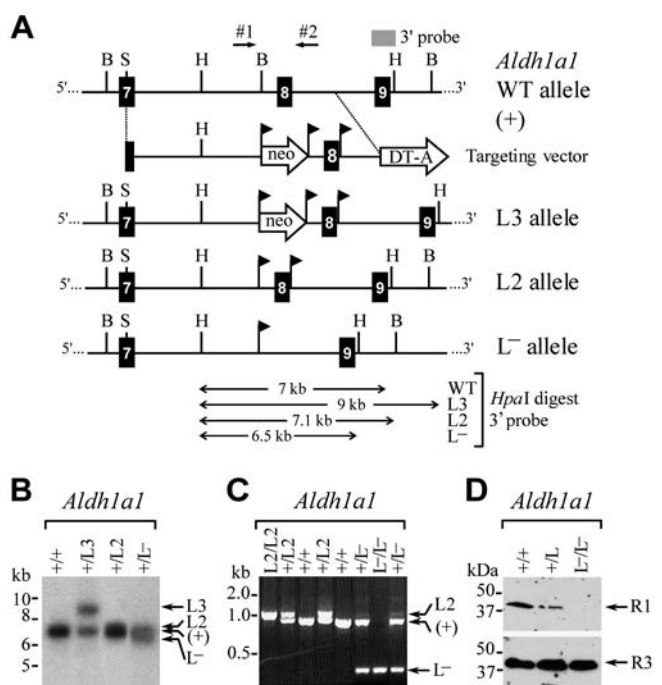


Fig. 1. Targeted disruption of the *Aldh1a1* gene encoding RALDH1. (A) Structure of the targeting vector and partial restriction map of the *Aldh1a1* locus before [wild-type (WT) allele, +] and after homologous recombination (L3 allele) and Cre-mediated recombination (L2 and L- alleles). Black boxes (labeled 7-9) stand for exons. The location of restriction sites (B, *Bam*HI; H, *Hpa*I; S, *Sa*II) and of the 3' external probe is indicated. Arrowhead flags represent loxP sites. Arrows indicate the location of primers 1 and 2 used for PCR genotyping. Sizes of the restriction fragments obtained for each allele are shown below and are in kilobases (kb). (B) Southern blot analysis of *Hpa*I-digested genomic DNA from ES cell clones with the indicated *Aldh1a1* genotype, using the 3' probe. (C) PCR analysis of tail genomic DNA from mice with the indicated *Aldh1a1* genotype. The identities of the different alleles are indicated on the right. (D) Western blot analysis of liver proteins (50 μ g) from adult mice with the indicated *Aldh1a1* genotype, using anti-RALDH1 (upper panel) and anti-RALDH3 (lower panel) polyclonal antibodies, showing the absence of RALDH1 (R1) and a normal amount of RALDH3 (R3) in *Aldh1a1*^{L-/L-} mice.

heterozygous (*Aldh1a1*^{+L-}) and 25% ($n=72$) homozygous (*Aldh1a1*^{L-/L-}, hereafter designated as *Aldh1a1*-null) mice. To check that *Aldh1a1* gene disruption was efficient, RALDH1 expression was analyzed by western blotting. RALDH1 (~54 kDa) was readily detected in extracts from wild-type and heterozygous livers, but not in *Aldh1a1*-null mice (Fig. 1D, upper panel), whereas the levels of RALDH2, RALDH3 and RALDH4 were identical in wild-type and *Aldh1a1*-null liver extracts (Fig. 1D, lower panel; and data not shown). *Aldh1a1*-null males and females grew normally, were fertile, healthy and indistinguishable from wild-type littermates. Additional analysis of serial histological sections of E14.5 ($n=2$) and E18.5 ($n=2$) mutant fetuses did not reveal any abnormalities (data not shown). Altogether, these results indicate that RALDH1 is dispensable for development in utero, as well as for post-natal life.

To demonstrate that RA synthesis was actually impaired, *Aldh1a1*-null mutants were crossed with a RA-responsive

transgenic line (*RARE-lacZ*) (Rossant et al., 1991), and the reporter activity in tissues that normally contain high levels of *Aldh1a1* transcripts, such as the dorsal field of the retina at E13.5 (Li et al., 2000), was analyzed. When compared with wild-type fetuses, the reporter activity was dramatically reduced in this part of the retina in E13.5 *Aldh1a1*-null fetuses, establishing that RALDH1 is responsible for the generation of RA. However, *Aldh1a1*-null eyes did not display histological abnormalities (see below).

RALDH1 and RALDH3 activities account for all RA synthesis in the developing eye region from E10.5 onwards

Vitamin A-deficient (VAD) fetuses, as well as fetuses carrying retinoid receptor ablations, display severe ocular abnormalities that highlight the crucial roles played by the RA-liganded RAR/RXR heterodimers in eye development (see Introduction). By contrast, *Aldh1a1*-null mutants have normal eyes, and fetuses lacking RALDH3 (hereafter designated *Aldh1a3*-null fetuses) display mild ocular defects (Dupé et al., 2003) (see below). These observations raised the question as to whether the losses of RALDH1 or RALDH3 could be functionally compensated by the other RALDH proteins expressed in the eye region, even though such a functional compensation appeared to be unlikely in the light of published data showing the non-overlapping expression patterns of the genes encoding the three RALDHs. Indeed, it has been reported that *Aldh1a1* and *Aldh1a3* transcripts are confined to the dorsal and ventral retinal fields, respectively (Li et al., 2000; Mic et al., 2000; Grün et al., 2000), whereas *Aldh1a2* (encoding RALDH2) is transiently expressed in the optic vesicle at E8.5 and thereafter in the pericocular mesenchyme (POM), but never in the neural retina (Niederreither et al., 1997; Wagner et al., 2000; Mic et al., 2004).

To determine whether a functional compensation between RALDHs may nevertheless exist, we (1) re-investigated the expression profiles of *Aldh1a1*, *Aldh1a2* and *Aldh1a3* during normal eye development, and (2) analyzed the activity of the *RARE-lacZ* transgene in the ocular region of *Aldh1a1*-null mutants, *Aldh1a3*-null mutants and *Aldh1a1/3*-null mutants (lacking both RALDH1 and RALDH3; see Materials and methods). At E10.5, E11.5 and E13.5, *Aldh1a1* was expressed in the dorsal retina and the lens as well as in the corneal ectoderm (Fig. 2A,D,G), in accordance with previous results (Li et al., 2000; Suzuki et al., 2000; Mic et al., 2000; Grün et al., 2000). We additionally detected *Aldh1a1* transcripts in the temporal side of the ventral retina, not only at E13.5 as described (Li et al., 2000), but also at E11.5 (Fig. 2D,G). This discrepancy might be accounted for by a higher sensitivity of in situ hybridizations on histological sections (present report) as compared with whole mounts (Li et al., 2000). At E10.5, *Aldh1a2* expression was absent in the eye region (Fig. 2B), while, at E11.5 and E13.5, it was localized in developing muscles present around the eye, but was not detected in the POM per se (Fig. 2E,H). At E10.5 and E11.5, *Aldh1a3* was expressed in the ventral retina (Fig. 2C,F), as reported (Grün et al., 2000; Mic et al., 2000; Li et al., 2000; Suzuki et al., 2000). However, we also detected *Aldh1a3* expression in the peripheral portion of the dorsal retina (arrowhead; Fig. 2C,F), throughout the retinal pigment epithelium, in the optic nerve anlage and in the corneal ectoderm (Fig. 2C,F). The expression

of *Aldh1a3* was similar at E13.5, except in the retinal pigment epithelium where the transcripts were no longer detected (Fig. 2I).

Expression of the *RARE-lacZ* in the ocular region of E10.5 and E11.5 wild-type fetuses was identical to that seen in *Aldh1a1*-null mutants: a strong β -galactosidase activity was detected at these developmental stages in the retina, optic nerve, retinal pigment epithelium, corneal ectoderm and POM, whereas a faint β -galactosidase activity was detected at the dorsal side of the lens (compare Fig. 3A,E with 3B,F). At E13.5, the transgene activity was undetectable in the dorsal retina of *Aldh1a1*-null fetuses, except in its most peripheral portion (compare Fig. 3I with 3J). These results indicate that RALDH3 expressed at the peripheral portion of the dorsal retina, the dorsal retinal pigment epithelium and/or the dorsal corneal ectoderm is likely to compensate at E10.5 and E11.5 for the loss of RALDH1 by producing RA that may diffuse to the dorsal retina. This compensation does not operate any longer at E13.5. As for *Aldh1a3*-null mutants, the RA-dependent β -galactosidase activity was always maintained in the dorsal retina, the dorsal corneal ectoderm and the dorsal POM (Fig. 3C,G). These results indicate that RALDH1 in the dorsal retina and corneal ectoderm probably compensates for the loss of RALDH3, by producing RA that may diffuse to the dorsal POM. By contrast, such a compensation does not occur in the retinal pigment

epithelium and in the ventral side of the eye, as β -galactosidase activity was abolished at E10.5 and E11.5 in the retinal pigment epithelium, the ventral retina, the ventral corneal ectoderm and the ventral POM (compare Fig. 3C,G with 3A,E). At E13.5, a robust reporter activity appeared in the temporal side of the ventral retina of *Aldh1a3*-null mutants (Fig. 3K), where *Aldh1a1* transcripts were detected at this stage (see Fig. 2G). Thus, at E13.5, RALDH1 compensates for the loss of RALDH3 in the temporal side of the ventral retina, but not in other ventral ocular tissues. Altogether, these results indicate that the absence of eye malformations in *Aldh1a1*-null mutants (Fan et al., 2003) (and the present report), and the mild, ventral, ocular defects of *Aldh1a3*-null fetuses (Dupé et al., 2003) (and see below), may indeed be accounted for by a functional compensation between RALDH1 and RALDH3.

Most importantly, in E10.5, E11.5 and E13.5 *Aldh1a1/3*-null fetuses, the β -galactosidase activity was abolished in all eye structures (Fig. 3D,H,L), indicating that RALDH1 and RALDH3 are the only enzymes that synthesize RA in the neural retina, the retinal pigment epithelium and the corneal ectoderm at E10.5, E11.5 and E13.5.

Absence of RALDH1 and RALDH3 yields severe eye abnormalities

The outcome of the lack of RA synthesis in the ventral region of the eye of *Aldh1a3*-null mutants was investigated. At E14.5, these mutants exhibited mild defects, including shortening of the ventral retina, ventral rotation of the lens, persistence of the primary vitreous (retrolenticular membrane) and thickening of the ventral periorcular mesenchyme (Dupé et al., 2003) (see also below). Apart from the retrolenticular membrane, which is normally present in E12.5 wild-type embryos (Ghyselinck et al., 1997), these defects were much more apparent at E12.5 (compare Fig. 4A with 4D). We have previously suggested that similar ventral ocular defects in mutants lacking either RXR α or RAR β and RAR γ could be related to the thickened mesenchyme interposed between the retina and the ectoderm (Mark et al., 1998). To investigate the cause of the POM thickening in *Aldh1a3*-null mutants, apoptosis was analyzed at E10.5 (Fig. 4B) and E11.5 (not shown) by performing TUNEL assays on serial histological sections along the anteroposterior axis of the eye region. In the POM of wild-type embryos, we identified two clusters of TUNEL-positive cells, a dorsal one located in the posterior region and a ventral one located in the anterior region, both at E10.5 ($n=3$) (Fig. 4B) and at E11.5 (see below, Fig. 7A,D). These two clusters may be instrumental to the physiological remodelling of the POM, especially because TUNEL-positive cells represent only a small fraction of the cells committed toward apoptosis (Gavrieli et al., 1992). In *Aldh1a3*-null eyes, the ventral cluster was not detected at E10.5 ($n=2$) (brackets, Fig. 4E), and was of a reduced size at E11.5 ($n=2$) (not shown). It has been proposed that the expression

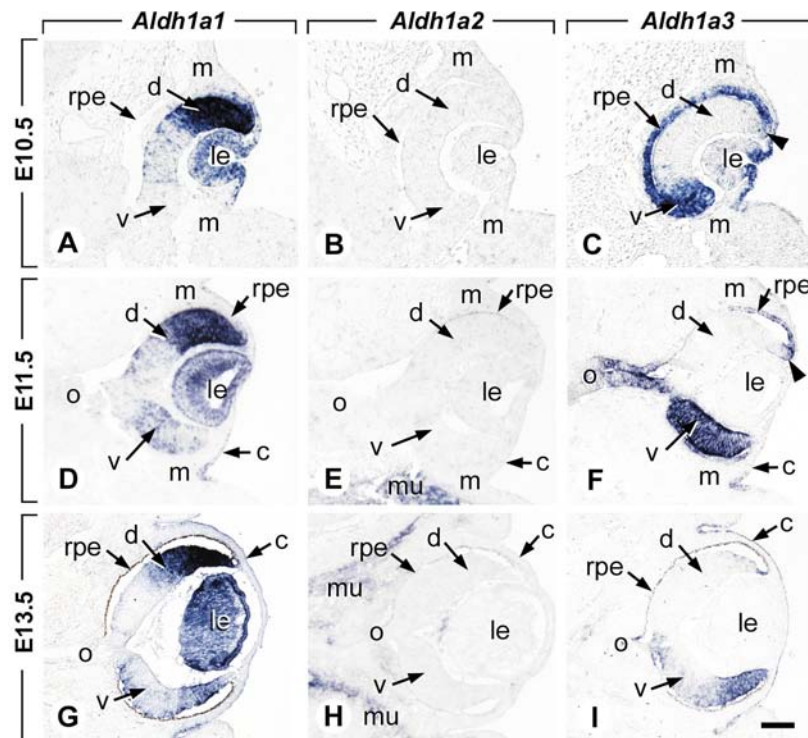
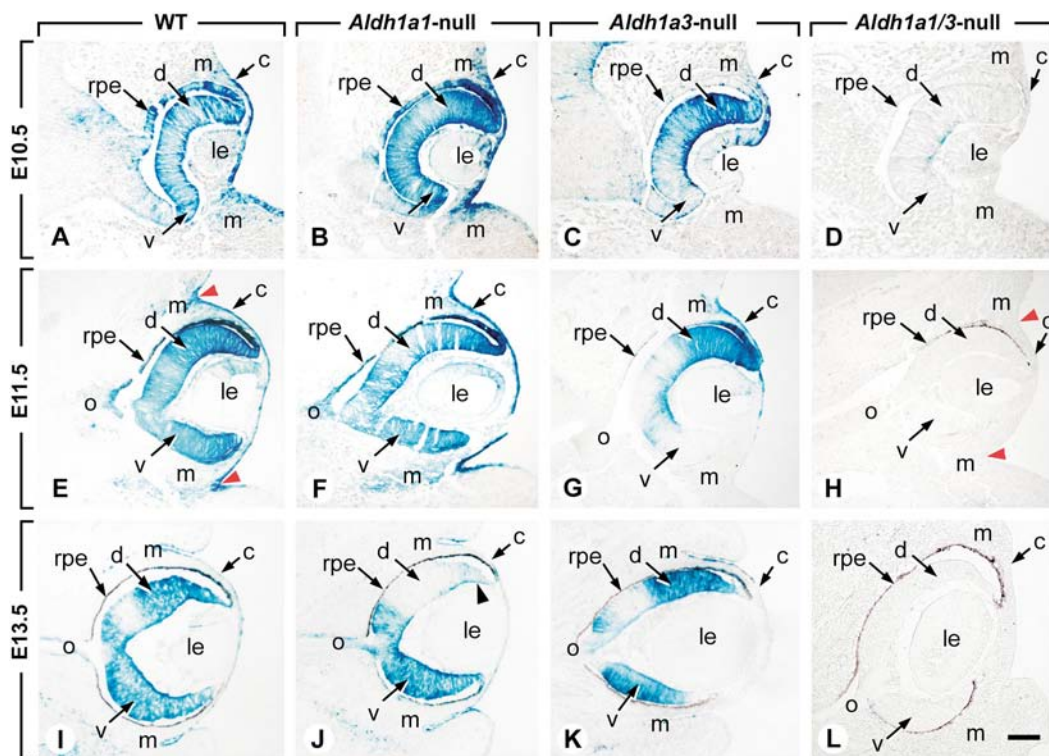


Fig. 2. Expression patterns of RALDH1, RALDH2 and RALDH3 during eye development. In situ hybridization using digoxigenin-labeled *Aldh1a1* (A,D,G), *Aldh1a2* (B,E,H) and *Aldh1a3* (C,F,I) antisense riboprobes at E10.5 (A-C), E11.5 (D-F) and E13.5 (G-I). Arrowheads (C,F) indicate the peripheral portion of the dorsal retina. c, presumptive corneal ectoderm; d, dorsal retina; le, lens; m, periorcular mesenchyme; mu, muscle; o, optic nerve; rpe, retinal pigment epithelium; v, ventral retina. Scale bar in I: 40 μ m for A-C; 50 μ m for D-F; 80 μ m for G-I.

Fig. 3. Effects of RALDH1 and RALDH3 inactivation, alone or in combination, on the RA-dependent activity of a reporter transgene.

Distribution of β -galactosidase activity driven by the *RARE-lacZ* transgene in wild-type (A,E,I), *Aldh1a1*-null (B,F,J), *Aldh1a3*-null (C,G,K) and *Aldh1a1/3*-null (D,H,L) mutants at E10.5 (A-D), E11.5 (E-H) and E13.5 (I-L). Red arrowheads (E,H) indicate dorsal and ventral eyelid grooves. Black arrowhead (J) indicates the most peripheral portion of the dorsal retina where *RARE-lacZ* activity is not abolished in *Aldh1a1*-null fetuses. The faint staining observed at E10.5 in the retina of *Aldh1a1/3*-null mutants (D) reflects a residual activity of the β -galactosidase synthesized at an earlier stage, as no *lacZ* mRNA can be detected

using in situ hybridization. Note also that the β -galactosidase activity detected at E13.5 in the ventral retina of the *Aldh1a3*-null fetus (K) results from expression of *Aldh1a1*. c, presumptive corneal ectoderm; d, dorsal retina; le, lens; m, periocular mesenchyme; o, optic nerve; rpe, retinal pigment epithelium; v, ventral retina. Scale bar in L: 40 μ m for A-C; 50 μ m for D-F; 80 μ m for G-I.



level of *Eya2* correlates with programmed cell-death during eye development (Xu et al., 1997; Clark et al., 2002). In E11.5 wild-type embryos, *Eya2* was strongly expressed in the POM, both in the ventral and the dorsal sides (Fig. 4C; and data not shown, see below). In E11.5 *Aldh1a3*-null mutants ($n=1$), *Eya2* expression was specifically reduced in the ventral POM (Fig. 4F). This observation indicates that the decreased programmed cell-death in the ventral POM of *Aldh1a3*-null mutants, caused by impairment of RA synthesis in the ventral eye region, correlates with a decreased *Eya2* expression in ventral POM cells.

Aldh1a1/3-null fetuses displayed multiple and severe ocular malformations affecting the eyelids, the cornea, the anterior chamber, the lens, the primary vitreous and the retina (Fig. 5D,I,L). At E11.5 ($n=5$), the dorsal eyelid groove failed to invaginate, whereas the ventral groove was formed (compare Fig. 3E with 3H) and yielded a rudimentary lower eyelid fold. The latter fused at E14.5 ($n=3$) with the presumptive corneal ectoderm to generate a small and abnormal conjunctival sac (Fig. 5I), whereas an abnormal, thick layer of mesenchyme interposed between the lens and the surface ectoderm replaced the normal eyelids and cornea (Fig. 5I). Moreover, in these mutant fetuses, the retrolenticular membrane, the shortening of the ventral retina and the ventral rotation of the lens were all much more severe than in *Aldh1a3*-null mutants (compare Fig. 5H with 5I). At E18.5 ($n=4$), *Aldh1a1/3*-null mutants exhibited the persistent hyperplastic vitreous body and the agenesis of the Harderian gland that are hallmarks of RALDH3 inactivation (data not shown) (Dupé et al., 2003). In addition, they

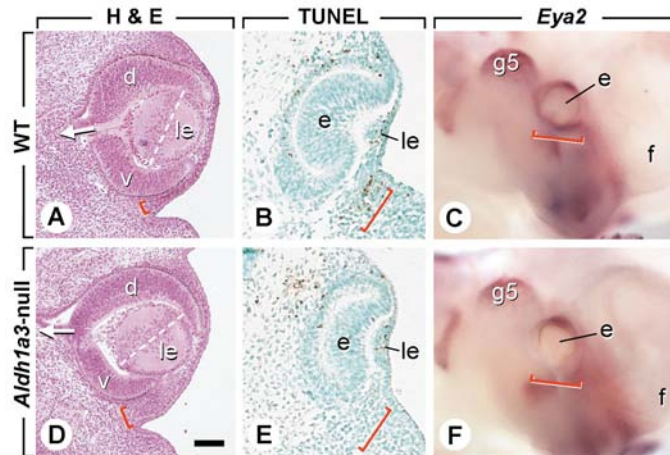
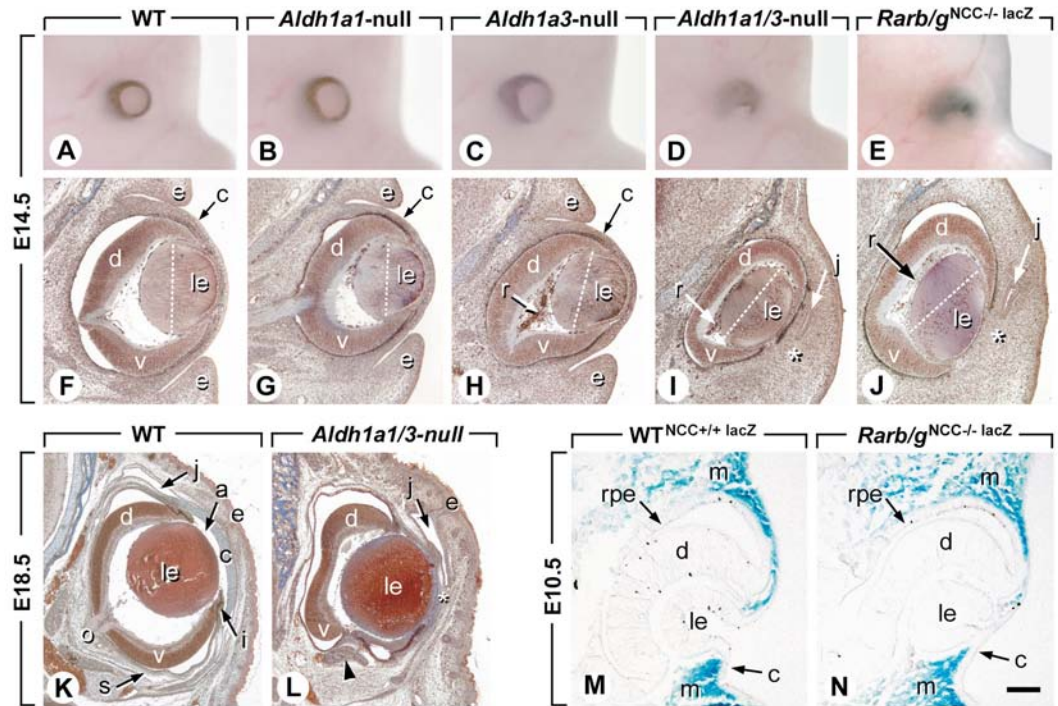


Fig. 4. Ablation of RALDH3 alters development of the ventral periocular mesenchyme. (A,D) Frontal histological sections through the head of E12.5 wild-type (A) and *Aldh1a3*-null (D) fetuses. (B,E) Distribution of apoptotic cells (assessed by TUNEL assays) in E10.5 wild-type (B) and *Aldh1a3*-null (E) fetuses. (C,F) Whole mount in situ hybridization with a digoxigenin-labeled *Eya2* antisense riboprobe on E11.5 wild-type (C) and *Aldh1a3*-null (F) fetuses. Note that the histological sections illustrated have been selected to provide the best match between mutant and wild-type embryos. Arrows indicate the optic nerve exit point; red brackets indicate the periocular mesenchyme. d, dorsal retina; e, eye cup; f, forebrain; g5, ganglion of the Vth nerve; le, lens or lens placode; v, ventral retina. Scale bar in D: 80 μ m for A,D; 25 μ m for B,E.

Fig. 5. Absence of RA signaling in the periocular mesenchyme results in severe ocular defects. (A-E) External views of the ocular region of E14.5 fetuses (genotypes as indicated). (F-L) Frontal histological sections through heads of E14.5 (F-J) and E18.5 (K,L) fetuses. (M,N) Distribution of β -galactosidase activity driven by the *R26R* transgene in E10.5 embryos bearing the *Wnt1-Cre* transgene either in a wild-type genetic background (M, $WT^{NCC+/+ lacZ}$), or in a genetic background containing loxP-flanked *Rarb* and *Rarg* genes (N, $Rarb/g^{NCC-/- lacZ}$). In both $WT^{NCC+/+ lacZ}$ and $Rarb/g^{NCC-/- lacZ}$ embryos, the β -galactosidase staining is identical, indicating that ablation of RAR β and RAR γ does not alter the migration of neural crest cells (NCCs)



in the periocular mesenchyme. Asterisks (I,J,L) indicate undifferentiated mesenchyme replacing the eyelids and cornea; dotted lines (F-J) indicate the equatorial plane of the lens; arrowhead (L) points to the coloboma of the retina. a, anterior chamber; c, cornea; d, dorsal retina; e, eyelid; i, iris; j, conjunctival sac; le, lens; m, periocular mesenchyme; o, optic nerve; rpe, retinal pigment epithelium; r, retrolenticular membrane; s, sclera; v, ventral retina. Scale bar in N: 120 μ m for F-J; 250 μ m for K,L; 25 μ m for M,N.

displayed several malformations of the anterior segment of the eye, including agenesis of the corneal and iris stroma, agenesis of the anterior chamber and hypoplasia of the conjunctival sac (compare Fig. 5K with 5L). The sclera was consistently absent, and a coloboma of the retina was observed in one (out of four) eyes (Fig. 5L). By contrast, the retinal pigment epithelium was unaffected, and the layers of the neural retina were normally organized (data not shown).

It is noteworthy that the nasal defects characteristic of the *Aldh1a3*-null phenotype were not worsened in *Aldh1a1/3*-null fetuses; all of the other organs in these fetuses had a normal histological appearance (data not shown).

The periocular mesenchyme is the primary target of RA action

The eye defects of *Aldh1a1/3*-null fetuses are similar to those observed in mutant fetuses lacking both RAR β and RAR γ (Ghyselinck et al., 1997). RAR β and RAR γ , which both act in a heterodimer with RXR α (Kastner et al., 1994; Kastner et al., 1997), are normally expressed in the cells surrounding the developing optic cup (i.e. in the POM), but not in the retina (Ghyselinck et al., 1997; Mori et al., 2001). Therefore RA synthesized by RALDH1 and RALDH3 in the neural retina may control eye development through the activation of RXR α /RAR β and RXR α /RAR γ heterodimers in POM cells. To test this possibility, we performed Cre-mediated somatic ablation of the *Rarb* and *Rarg* genes (Metzger and Chambon, 2001) in POM cells, which largely originate from the neural crest (Johnston et al., 1979; Trainor and Tam, 1995). To this end, we used a transgenic line (*Wnt1-Cre*^{tg/0}), expressing Cre

under the control of the *Wnt1* promoter (Danielian et al., 1998), which is active in neural crest cells (NCCs). We also used a mouse line bearing a conditional reporter transgene (*R26R*^{tg/0}), which expresses β -galactosidase only upon Cre-mediated recombination of a loxP-flanked (floxed) intervening DNA sequence (Soriano, 1999). This reporter continues to be expressed in all progeny of the NCCs that have undergone recombination, even though they are no longer expressing Cre (Jiang et al., 2000). In E10.5 *Wnt1-Cre*^{tg/0}/*R26R*^{tg/0} fetuses (hereafter designated $WT^{NCC+/+ lacZ}$), reporter activity was detected in POM cells (Fig. 5M), including those forming the stroma of the cornea (data not shown), but not in other ocular structures. Therefore, *Wnt1*-driven expression of Cre allows efficient recombination in POM cells.

Mice in which both alleles of the genes encoding RAR β and RAR γ are floxed (*Rarb/g*^{L2/L2} mice) were then crossed with *Wnt1-Cre*^{tg/0}/*R26R*^{tg/0} transgenic mice, to generate fetuses in which RAR β and RAR γ ablation was restricted to NCCs and to their derivatives, including the POM (hereafter designated *Rarb/g*^{NCC-/- lacZ} mutants). Importantly, the pattern of β -galactosidase activity identifying NCC derivatives was identical in E10.5 *Rarb/g*^{NCC-/- lacZ} and $WT^{NCC+/+ lacZ}$ fetuses, demonstrating that ablation of RAR β and RAR γ in NCCs did not alter their migration from the neuroectoderm into the periocular region (compare Fig. 5N with 5M). Similarly to *Aldh1a1/3*-null (Fig. 5I) and *Rarb/g*-null mutants (Ghyselinck et al., 1997), E14.5 *Rarb/g*^{NCC-/- lacZ} fetuses ($n=2$) exhibited an abnormal, thick layer of mesenchyme replacing the eyelids and the cornea (Fig. 5J), and a small and abnormal conjunctival sac (Fig. 5J), as well as a severe shortening of the ventral retina,

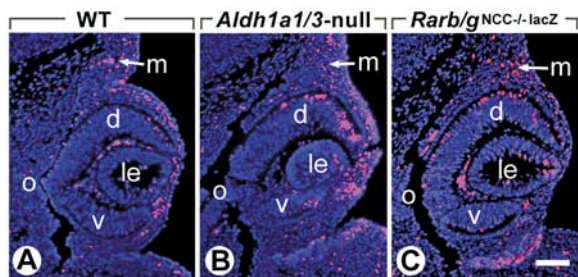


Fig. 6. Cell proliferation in the pericocular mesenchyme is not altered upon ablation of RALDH1 and RALDH3, or of RAR β and RAR γ . (A–C) Distribution of proliferating cells (assessed by Ki-67 expression) in E11.5 wild-type (A), *Aldh1a1/3*-null (B) and *Rarb/g^{NCC-/-lacZ}* (C) fetuses. The histological sections illustrated have been selected to provide the best match between mutant and wild-type embryos. d, dorsal retina; le, lens; m, pericocular mesenchyme; o, optic nerve; v, ventral retina. Scale bar: 50 μ m.

ventral rotation of the lens, and persistence and hyperplasia of the primary vitreous body (Fig. 5J). These results demonstrate that selective ablation of RAR β and RAR γ in the POM yields eye defects identical to those displayed by mutants lacking RALDH1 and RALDH3.

Ablation of RALDH1 and RALDH3 impairs apoptosis and alters gene expression in pericocular mesenchymal cells

As RA-liganded RAR and RXR can be involved in cell

proliferation and apoptosis (Altucci and Gronemeyer, 2002), we tested whether one of these two processes could be altered upon RALDH1 and RALDH3 ablation. Cell proliferation was assessed at E11.5 by immunodetection of the cell proliferation-associated nuclear antigen Ki-67 (Schlüter et al., 1993). In the POM of wild-type eyes ($n=6$), the number of Ki-67-positive cells was 489 ± 70 versus 448 ± 82 in *Aldh1a1/3*-null mutant eyes ($n=4$) (compare Fig. 6A with 6B). Therefore cell proliferation in the POM was not significantly affected by ablation of RALDH1 and RALDH3. As for apoptosis, the POM of E11.5 wild-type embryos ($n=5$) displayed, similarly to E10.5 embryos (see above, Fig. 4B), two clusters of TUNEL-positive cells: one in the posterior dorsal region and one in the ventral anterior region (Fig. 7A,D). Out of 40 histological sections through the entire eye, these two clusters were consistently observed on six consecutive sections. These two clusters, which together span one fourth of the length of the ocular region, may be instrumental to the remodeling of the POM (see above). In *Aldh1a1/3*-null eyes ($n=4$), these clusters were not detected at either E10.5 (not shown) or at E11.5 (Fig. 7D,E). It is noteworthy that apoptosis was not impaired in other ocular regions of the *Aldh1a1/3*-null fetuses, for instance around the optic nerve (compare Fig. 7B with 7E). In E11.5 wild-type embryos, *Eya2* was strongly expressed in the POM and in the central portion of the retina (Fig. 7C), notably in POM areas displaying apoptotic cells (Fig. 7C). Expression of *Eya2* appeared to be selectively reduced in the POM of E11.5 *Aldh1a1/3*-null mutant eyes ($n=4$), both on the dorsal and the ventral sides, Fig. 7F). This observation indicates that the decreased programmed cell-death, caused by the impairment of RA synthesis in the retina through RALDH1 and RALDH3 ablations, correlates with a decreased *Eya2* expression in POM cells.

Several transcription factors are required for eye development (Cvekl and Tamm, 2004). These include FOXC1 and PITX2 expressed in POM cells (Kume et al., 1998; Kitamura et al., 1999), as well as PAX6 expressed in the retina (Gehring and Ikeyo, 1999; Ashery-Padan and Gruss, 2001). Interestingly, the expression of *Foxc1* (Fig. 7G,K) and *Pitx2* (Fig. 7H,L) appeared to be reduced,

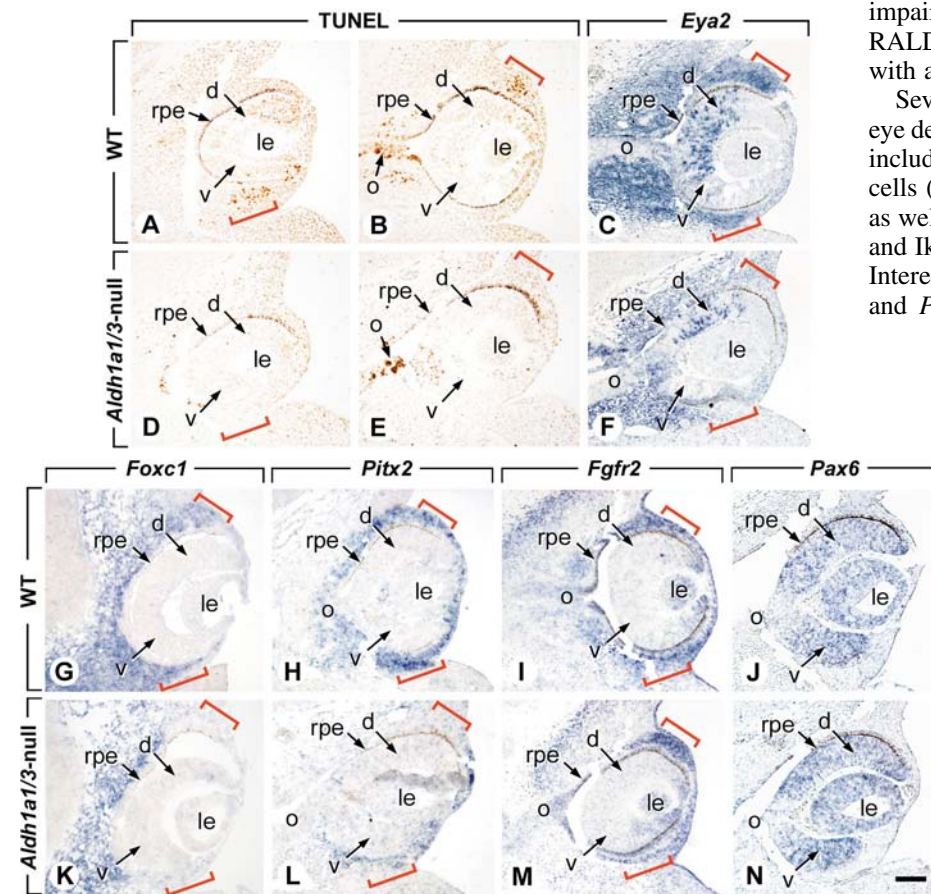


Fig. 7. Ablation of RALDH1 and RALDH3 impairs programmed cell death and differentiation in the pericocular mesenchyme. (A,B,D,E) Distribution of apoptotic cells (assessed by TUNEL assays) in E11.5 wild-type (A,B) and *Aldh1a1/3*-null (D,E) fetuses. (C,F,G–N) In situ hybridization with digoxigenin-labeled *Eya2* (C,F), *Foxc1* (G,K), *Pitx2* (H,L), *Fgfr2* (I,M) and *Pax6* (J,N) antisense riboprobes on frontal sections through the eye of E11.5 wild-type (C,G,H,I,J) and *Aldh1a1/3*-null (F,K,L,M,N) fetuses. Note that the histological sections illustrated have been selected to provide the best match between mutant and wild-type embryos. Red brackets indicate the pericocular mesenchyme. d, dorsal retina; le, lens; o, optic nerve; rpe, retinal pigment epithelium; v, ventral retina. Scale bar: 50 μ m.

when compared to wild-type eyes ($n=4$), in the POM of E11.5 *Aldh1a1/3*-null mutant eyes ($n=4$; compare Fig. 7G with 7K, and Fig. 7H with 7L), whereas *Pax6* was normally expressed in the retina (compare Fig. 7J with 7N). POM cells also express *Crabp1* and *Fgfr2* (Maden et al., 1992; Wilke et al., 1997). The expression of these two genes was not altered in *Aldh1a1/3*-null mutants (Fig. 7I,M; and data not shown), indicating that the impairment of RA synthesis, through RALDH1 and RALDH3 ablation, does not globally disrupt gene expression in POM cells, but selectively decreases *Foxc1* and *Pitx2*.

Ablation of *Rarb* and *Rarg* in neural crest cells impairs apoptosis and alters gene expression in perioocular mesenchymal cells

In the POM of *Rarb/g^{NCC-/-lacZ}* eyes ($n=2$), the number of Ki-67-positive cells (406 ± 34) was similar to that in wild-type eyes (see above) (compare Fig. 6A with 6C). Therefore, as in *Aldh1a1/3*-null mutants, cell proliferation in the POM appeared to be unaffected by the ablation of *Rarb* and *Rarg* in NCCs. Moreover, and similarly to *Aldh1a1/3*-null mutants, the normal ventral and dorsal clusters of apoptotic POM cells (Fig. 8A,B; and see above) were not detected in *Rarb/g^{NCC-/-lacZ}* mutant eyes ($n=4$; Fig. 8C,D). Along these lines, expression of *Eya2* appeared to be reduced in the POM in *Rarb/g^{NCC-/-lacZ}* mutant eyes ($n=2$; compare Fig. 8E with 8G). Finally, it is noteworthy that expression of both *Pitx2* (data not shown) and *Foxc1* appeared to be also decreased in the POM in *Rarb/g^{NCC-/-lacZ}* mutant eyes ($n=4$; compare Fig. 8F with 8H). Therefore, similar cellular and molecular defects are generated in the POM upon the ablation of RALDH1 and RALDH3, or of *Rarb* and *Rarg*.

Dorsoventral patterning of the retina does not depend upon RA

It has been proposed that RA is important for the dorsoventral (DV) patterning of the retina (McCaffery et al., 1999; Peters and Cepko, 2002). Thus, we analyzed whether impairing RA synthesis, through the combined ablation of RALDH1 and RALDH3, alters the DV patterning of the retina. The identity of progenitor cells along the retina DV axis is specified by the expression of *Tbx5*, *Vax2* and *Pax2* genes (reviewed by McLaughlin et al., 2003), while the stereotyped distribution of apoptotic cells during retina development provides a reliable landmark of DV axis patterning (Laemle et al., 1999). The distribution patterns of *Tbx5* and *Vax2* transcripts at E10.5 (Fig. 9A-D), and of *Pax2* transcripts at E11.5 (compare Fig. 9E with 9F), as well as the distribution of TUNEL-positive cells at E10.5 (compare Fig. 9G with 9H), were undistinguishable in *Aldh1a1/3*-null and wild-type fetuses. Altogether, our results show that impairing RA synthesis in the retina from E10.5 onwards does not alter the formation of its DV axis.

Discussion

RA produced by RALDH1 is instrumental in eye development

The targeted ablation of either *Aldh1a2* or *Aldh1a3* in the mouse has demonstrated that RALDH2 and RALDH3 actually generate RA in vivo, as activity of the RA-responsive *RARE-lacZ* transgene is abolished in tissues lacking these enzymes (Niederreither et al., 1999; Dupé et al., 2003). In contrast to

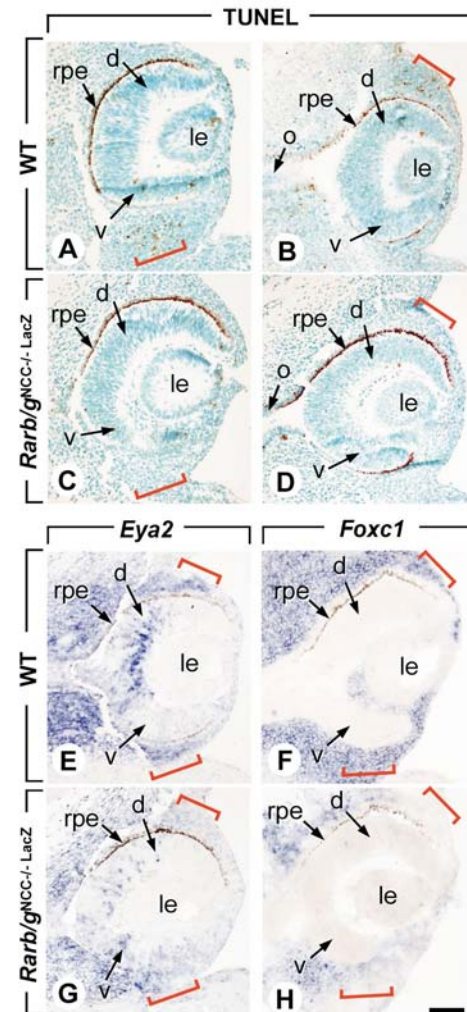


Fig. 8. Ablation of *Rarb* and *Rarg* in neural crest cells impairs apoptosis and gene expression in the perioocular mesenchyme. (A-D) Distribution of apoptotic cells (assessed by TUNEL assays) in E11.5 wild-type (A,B) and *Rarb/g^{NCC-/-lacZ}* (C,D) fetuses. (E-H) In situ hybridizations with digoxigenin-labeled *Eya2* (E,G) and *Foxc1* (F,H) antisense riboprobes on frontal sections through the eye of E11.5 wild-type (E,F) and *Rarb/g^{NCC-/-lacZ}* (G,H) fetuses. The histological sections illustrated have been selected to provide the best match between mutant and wild-type embryos. (A-D) Sections are counterstained with methyl green. Red brackets indicate the perioocular mesenchyme. d, dorsal retina; le, lens; o, optic nerve; rpe, retinal pigment epithelium; v, ventral retina. Scale bar: 50 μ m.

RALDH2 and RALDH3, RALDH1 has a low affinity for retinaldehyde and is about 10-fold less efficient at producing RA (Haselbeck et al., 1999; Grün et al., 2000). As amongst the tissues expressing RALDH1, only few stain positively for *RARE-lacZ* activity (Niederreither et al., 2002; Mic et al., 2002), it was speculated that RALDH1 is not, or is only marginally, involved in RA synthesis in vivo, and may rather function as a general detoxifying enzyme (Niederreither et al., 2002). Contrary to this expectation, the activity of *RARE-lacZ* is lost in the dorsal retina of E13.5 (the present study) and E16.5 (Fan et al., 2003) *Aldh1a1*-null fetuses, demonstrating that during development RALDH1 is actually instrumental in

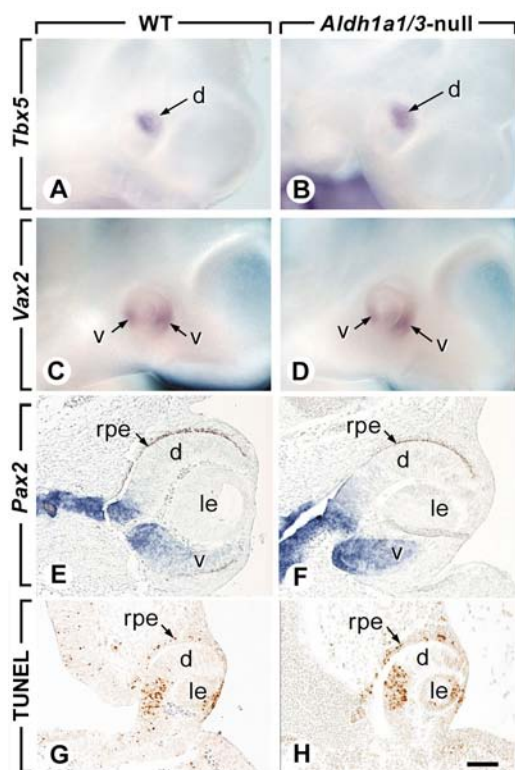


Fig. 9. RA is not required for establishing the dorsoventral axis of the retina. (A–D) Whole mount in situ hybridization of E10.5 wild-type (A,C) and *Aldh1a1/3*-null (B,D) fetuses using digoxigenin-labeled antisense riboprobes for *Tbx5* (A,B) and *Vax2* (C,D). (E,F) In situ hybridization with a digoxigenin-labeled *Pax2* (E,F) antisense riboprobe on frontal sections through the eye of E11.5 wild-type (E) and *Aldh1a1/3*-null (F) fetuses. (G,H) Distribution of apoptotic cells (assessed by TUNEL assays) in E10.5 wild-type (G) and *Aldh1a1/3*-null (H) fetuses. d, dorsal retina; le, lens; rpe, retinal pigment epithelium; v, ventral retina. Scale bar: 50 μm .

RA synthesis in the eye. In other tissues, RALDH1 seems to be dispensable, as the developmental defects exhibited by *Aldh1a1/Aldh1a2*-null (Mic et al., 2004) and *Aldh1a1/Aldh1a3*-null (the present study) mutants are identical to those displayed by *Aldh1a2*-null and *Aldh1a3*-null mutants, respectively. Note that in adult mice, RALDH1 appears to be dispensable with the exception of the liver, where the amount of free and stored vitamin A is increased, while the conversion of retinol to RA is reduced (Fan et al., 2003).

RA produced by RALDH1 and RALDH3 controls apoptosis and gene expression in periorbital mesenchymal cells

The present comparison of the RA-responsive *RARE-lacZ* activity in wild type and *Aldh1a1/3*-null mutants with the expression patterns of RALDH proteins allows us to conclude that: (1) RALDH1 and RALDH3 are the only enzymes that synthesize RA in the neural retina, the retinal pigment epithelium and the corneal ectoderm at E10.5, E11.5 and E13.5; and (2) an RA-dependent signal, which is abolished in *Aldh1a1/3*-null mutants, nevertheless exists in POM cells where RALDH1 and RALDH3 are not detected from E10.5 to E13.5. Furthermore, we show that *Aldh1a1/3*-null mutants

display eye defects that are identical to those generated upon somatic genetic ablation of *RAR β* and *RAR γ* in the NCC-derived POM. We conclude therefore that, from E10.5 onwards, RA synthesized by RALDH1 and RALDH3 in the neural retina, the retinal pigment epithelium and the corneal ectoderm diffuses towards POM cells, where it determines eye morphogenesis through the activation of *RXR α /RAR β* and *RXR α /RAR γ* heterodimers that act in several ways.

Different mechanisms, including cell proliferation, apoptosis and the extent of extracellular matrix deposition, may control the thickness of the POM. On the one hand, we show that cell proliferation is not affected upon ablation of either RALDH1 and RALDH3, or *RAR β* and *RAR γ* . On the other hand, we show, in POM cells of both *Aldh1a1/3*-null and *Rarb/g^{NCC-/- lacZ}* mutants, a decreased expression of *Eya2* that may account for the impaired apoptosis (Xu et al., 1997; Clark et al., 2002), and thus for the thickening of the POM, especially as our result also indicates that eye morphogenesis in the wild-type situation is accompanied by cell-death in the POM. However, we cannot exclude the possibility that other mechanisms, such as changes in the composition of the extracellular matrix, may also participate in the thickening of the POM. In any event, we propose that RA synthesized by RALDH1 and RALDH3 in the retina, the retinal pigment epithelium and/or the corneal ectoderm activates *RXR α /RAR β* and *RXR α /RAR γ* heterodimers in POM cells, which in turn control the *Eya2*-related programmed cell death (Fig. 10).

We also found that losses of RALDH1 and RALDH3, and the selective ablation of *Rarb* and *Rarg* in NCCs, apparently decrease, in POM cells, the expression of *Pitx2* and *Foxc1*, which code for transcription factors involved in eye morphogenesis (Cvelk and Tamm, 2004). RA treatment has been shown to induce *Pitx2* expression in embryonic stem cells (Lindberg et al., 1998). Furthermore, mutations in *PITX2* and *FOXC1* generate the human Axenfeld-Rieger's syndrome, which includes ocular anterior segment defects (reviewed by Lines et al., 2002). Haploinsufficiency or deletion of either *Pitx2* or *Foxc1* in the mouse (Kume et al., 1998; Kitamura et al., 1999) induces an anterior segment dysgenesis, characterized by an absence of the anterior chamber, a thickening of the mesenchymal component of the cornea, an agenesis of the iris, the persistence of the primary vitreous body and a ventral rotation of the eye, all of which closely resemble the ocular defects displayed by *Aldh1a1/3*-null and *Rarb/g^{NCC-/- lacZ}* mutant mice. Thus, the malformations induced by the loss of RALDH1 and RALDH3, and the ablation of *Rarb* and *Rarg*, most probably result from a reduced *Pitx2* and *Foxc1* expression. We propose therefore that RA synthesized by RALDH1 and RALDH3 in the retina, the retinal pigment epithelium and/or the corneal ectoderm activates *RXR α /RAR β* and *RXR α /RAR γ* heterodimers in POM cells, which in turn control the expression of *Pitx2* and *Foxc1* required for normal morphogenesis of the eye anterior segment (Fig. 10).

We found that fetuses lacking RALDH1 and RALDH3 in the retina, the retinal pigment epithelium and the corneal ectoderm (*Aldh1a1/3*-null mutants), or selectively missing *RAR β* and *RAR γ* in the POM (*Rarb/g^{NCC-/- lacZ}* mutants), displayed an identical shortening of the ventral retina. Thus, it appears that RA synthesized by RALDH1 and RALDH3 activates *RXR α /RAR β* and *RXR α /RAR γ* heterodimers in

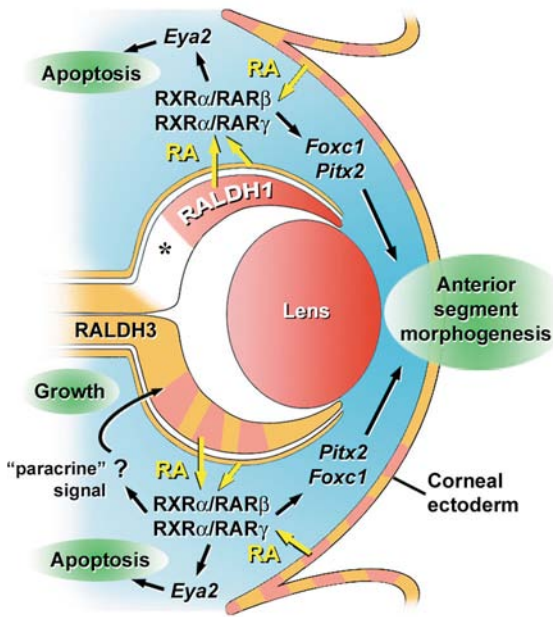


Fig. 10. RA-dependent eye morphogenesis is orchestrated by the NCC-derived periocular mesenchyme. RALDH1 (red) and RALDH3 (orange), expressed in the retina, the retinal-pigmented epithelium, the lens and the corneal ectoderm, are the only RA-synthesizing enzymes required in the eye region from E10.5 onwards. The RA synthesized in these locations (yellow arrows) diffuses towards the periocular mesenchyme (POM, blue), where it determines eye morphogenesis through the activation of RXR α /RAR β and RXR α /RAR γ heterodimers. The latter may control (1) the *Eya2*-dependent apoptosis involved in POM remodeling; (2) the expression of *Foxc1* and *Pitx2* transcription factors that are required for the eye anterior segment morphogenesis; and (3) the production of an unknown signal (question mark) necessary for the growth of the ventral retina. Asterisk indicates the RA-depleted zone expressing the catabolizing enzymes CYP26A1 and CYP26C1. This drawing is representative of the E11.5 condition.

POM cells, which in turn may produce a 'paracrine' signal necessary for the proper growth of the ventral retina (Fig. 10).

Lastly, it has been suggested that *Pax6* expression could be regulated by retinoids (Gajovic et al., 1997; Cvelk and Tamm, 2004), and *Pax6* haploinsufficiency in the mouse yields defects of the ocular anterior segment, similar to those exhibited by *Aldh1a1/3*-null and *Rarb/g^{NCC-/-lacZ}* mutants (Baulmann et al., 2002; Collinson et al., 2003). However, we found that *Pax6* expression was normal in E10.5 *Aldh1a1/3*-null fetuses. Therefore, the ocular defects generated through impairing RA-dependent signaling pathway are not causally related to an altered *Pax6* expression.

A sequential role for RALDH1, RALDH2 and RALDH3 during eye morphogenesis

From E9.0 to E16.5, eye morphogenesis involves the coordinated development of forebrain neuroectoderm, surface ectoderm and NCC-derived mesenchyme (Johnston et al., 1979). The panel of ocular malformations induced by vitamin A-deficiency (Warkany and Schraffenberger, 1946; Wilson et al., 1953; Dickman et al., 1997), or upon impairment of RA signaling (Kastner et al., 1994; Kastner et al., 1997; Lohnes et

al., 1994; Ghyselinck et al., 1997; Mascrez et al., 1998), indicates that RA-liganded RAR/RXR heterodimers are required at all stages of eye development. Mutant fetuses lacking RALDH2 fail to form an optic cup (Mic et al., 2004), but their rescue through maternal administration of RA before E9.5 prevents the occurrence of ocular malformations and restores a normal expression pattern of *RARE-lacZ* in the eye (Mic et al., 2002; Niederreither et al., 2002), suggesting an involvement of RALDH2 in eye morphogenesis until E9.5, but not at later stages. In the present study, we show that (1) the activity of *RARE-lacZ* is abolished in the eye region of *Aldh1a1/3*-null fetuses from E10.5 to E13.5, and (2) the ocular defects reported for *Aldh1a1/3*-null mutants all reflect a loss of RA signaling occurring from E10.5 onwards. Altogether, these data indicate that RALDH1 and RALDH3, expressed in the retina, retinal pigment epithelium and corneal ectoderm, are actually the only RA-synthesizing enzymes required in the eye region from E10.5 onwards.

The spectrum of ocular defects in *Aldh1a1/3*-null and *Rarb/g^{NCC-/-lacZ}* mutants recapitulates that induced by vitamin A-deficiency and/or loss-of-function mutations of RAR and RXR genes, with the exceptions of open eyelids and agenesis of the lens (Lohnes et al., 1994; Ghyselinck et al., 1997; Kastner et al., 1997; Mascrez et al., 1998). The RA-degrading enzyme CYP26B1 is expressed in the mesenchyme underlying the palpebral fissure just prior to eyelid fusion (Abu-Abed et al., 2002), and *Cyp26b1*-null mutants display open eyes at birth (Yashiro et al., 2004). Therefore, eyelid fusion is likely to require unliganded RA-receptors, but not RALDH1 and RALDH3. Agenesis of the lens in *Rara/Rarg*-null mutants has been ascribed to a default in lens induction, a process that depends upon interactions between the optic vesicle and the surface ectoderm, and which occurs before E10.5 (Lohnes et al., 1994). A recent study shows that RA synthesized by RALDH2 in the optic vesicle between E8.5 and E9.5 is actually required for optic cup and lens formation (Mic et al., 2004). Altogether, these data add to the notion that RALDH2 is the RA-synthesizing enzyme needed during the early steps of ocular morphogenesis (before E9.5), while RALDH3 and RALDH1 are required for the subsequent steps of eye development (from E10.5 onward).

RA is not required for the dorsoventral patterning of the retina

During early eye morphogenesis, RALDH1 and RALDH3 generate RA-rich regions in the dorsal and ventral fields of the retina, separated by a RA-depleted zone expressing the catabolizing enzymes CYP26A1 and CYP26C1 (Fujii et al., 1997; McCaffery et al., 1999; Grün et al., 2000; Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000; Sakai et al., 2004). Several authors have proposed that the combined activities of these enzymes set up a RA gradient in the retina that is important for its dorsoventral (DV) patterning (McCaffery et al., 1999; Peters and Cepko, 2002). Neither the ablation of RALDH1 (Fan et al., 2003) nor that of RALDH3 (Dupé et al., 2003) alters the retina DV axis. However, the actual role of these two enzymes in setting this DV axis could have been masked by a functional, reciprocal compensation of RALDH1 by RALDH3. In the present study, we show that impairing RA synthesis in the retina from E10.5 onwards, through RALDH1 and RALDH3 ablation, does not alter the formation of its DV axis.

Therefore, contrary to previous expectations (McCaffery et al., 1999; Peters and Cepko, 2002), RA is actually not required to set up the DV axis of the retina. However, our results do not exclude a role for RA in setting up the other retinal axes, or in retinal histogenesis (Grondona et al., 1996).

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References

- Abu-Abed, S., MacLean, G., Fraulob, V., Chambon, P., Petkovich, M. and Dollé, P. (2002). Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. *Mech. Dev.* **110**, 173-177.
- Altucci, L. and Gronemeyer, H. (2001). Nuclear receptors in cell life and death. *Trends Endocrinol. Metab.* **12**, 460-468.
- Ashery-Padan, R. and Gruss, P. (2001). Pax6 lights-up the way for eye development. *Curr. Opin. Cell Biol.* **13**, 706-714.
- Baulmann, D. C., Ohlmann, A., Flügel-Koch, C., Goswami, S., Cvekl, A. and Tamm, E. R. (2002). Pax6 heterozygous eyes show defects in chamber angle differentiation that are associated with a wide spectrum of other anterior eye segment abnormalities. *Mech. Dev.* **118**, 3-17.
- Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940-954.
- Chapellier, B., Mark, M., Bastien, J., Dierich, A., LeMeur, M., Chambon, P. and Ghyselinck, N. B. (2002a). A conditional floxed (loxP-flanked) allele for the retinoic acid receptor beta (RARbeta) gene. *Genesis* **32**, 91-94.
- Chapellier, B., Mark, M., Garnier, J. M., Dierich, A., Chambon, P. and Ghyselinck, N. B. (2002b). A conditional floxed (loxP-flanked) allele for the retinoic acid receptor gamma (RARgamma) gene. *Genesis* **32**, 95-98.
- Clark, S. W., Fee, B. E. and Cleveland, J. L. (2002). Misexpression of the eyes absent family triggers the apoptotic program. *J. Biol. Chem.* **277**, 3560-3567.
- Collinson, J. M., Quinn, J. C., Hill, R. E. and West, J. D. (2003). The role of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. *Dev. Biol.* **255**, 303-312.
- Cvekl, A. and Tamm, E. R. (2004). Anterior eye development and ocular mesenchyme, new insights from mouse models and human diseases. *BioEssays* **26**, 374-386.
- Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.
- Dickman, E. D., Thaller, C. and Smith, S. M. (1997). Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* **124**, 3111-3121.
- Dräger, U. C., Li, M., Wagner, E. and McCaffery, P. (2001). Retinoic acid synthesis and breakdown in the developing mouse retina. *Prog. Brain Res.* **131**, 579-587.
- Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function, production of visual pigment and retinoic acid. *Eur. J. Biochem.* **267**, 4315-4324.
- Dupé, V., Matt, N., Garnier, J. M., Chambon, P., Mark, M. and Ghyselinck, N. B. (2003). A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. *Proc. Natl. Acad. Sci. USA* **100**, 14036-14041.
- Fan, X., Molotkov, A., Manabe, S., Donmoyer, C. M., Deltour, L., Foglio, M. H., Cuenca, A. E., Blaner, W. S., Lipton, S. A. and Duester, G. (2003). Targeted disruption of *Aldh1a1* (*Raldh1*) provides evidence for a complex mechanism of retinoic acid synthesis in the developing retina. *Mol. Cell. Biol.* **23**, 4637-4648.
- Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163-4173.
- Gajovic, S., St-Onge, L., Yokota, Y. and Gruss, P. (1997). Retinoic acid mediates Pax6 expression during in vitro differentiation of embryonic stem cells. *Differentiation* **62**, 187-192.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.
- Gehring, W. J. and Ikeo, K. (1999). Pax 6, mastering eye morphogenesis and eye evolution. *Trends Genet.* **15**, 371-377.
- Ghyselinck, N. B., Dupé, V., Dierich, A., Messaddeq, N., Garnier, J. M., Rochette-Egly, C., Chambon, P. and Mark, M. (1997). Role of the retinoic acid receptor beta (RARbeta) during mouse development. *Int. J. Dev. Biol.* **41**, 425-447.
- Grondona, J. M., Kastner, P., Gansmuller, A., Decimo, D., Chambon, P. and Mark, M. (1996). Retinal dysplasia and degeneration in RARbeta2/RARgamma2 compound mutant mice. *Development* **122**, 2173-2188.
- Grün, F., Hirose, Y., Kawauchi, S., Ogura, T. and Umesono, K. (2000). Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem.* **275**, 41210-41218.
- Hale, F. (1933). Pigs born without eyeballs. *J. Hered.* **24**, 105-127.
- Haselbeck, R. J., Hoffmann, I. and Duester, G. (1999). Distinct functions for *Aldh1* and *Raldh2* in the control of ligand production for embryonic retinoid signaling pathways. *Dev. Genet.* **25**, 353-364.
- Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616.
- Johnston, M. C., Noden, D. M., Hazelton, R. D., Coulombre, J. L. and Coulombre, A. J. (1979). Origins of avian ocular and periocular tissues. *Exp. Eye Res.* **29**, 27-43.
- Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Décimo, D., Vonesch, J. L., Dollé, P. and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function, convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003.
- Kastner, P., Mark, M., Ghyselinck, N., Krezel, W., Dupé, V., Grondona, J. M. and Chambon, P. (1997). Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development* **124**, 313-326.
- Kitamura, K., Miura, H., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R., Ohuchi, H., Suehiro, A., Motegi, Y., Nakahara, Y. et al. (1999). Mouse *Pitx2* deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. *Development* **126**, 5749-5758.
- Kume, T., Deng, K. Y., Winfrey, V., Gould, D. B., Walter, M. A. and Hogan, B. L. (1998). The forkhead/winged helix gene *Mfl* is disrupted in the pleiotropic mouse mutation congenital hydrocephalus. *Cell* **93**, 985-996.
- Laemle, L. K., Puzkarczuk, M. and Feinberg, R. N. (1999). Apoptosis in early ocular morphogenesis in the mouse. *Dev. Brain Res.* **112**, 129-133.
- Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A. and Dräger, U. C. (2000). A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.* **95**, 283-289.
- Lin, M., Zhang, M., Abraham, M., Smith, S. M. and Napoli, J. L. (2003). Mouse retinal dehydrogenase 4 (RALDH4), molecular cloning, cellular expression, and activity in 9-cis-retinoic acid biosynthesis in intact cells. *J. Biol. Chem.* **278**, 9856-9861.
- Lindberg, C., Wunderlich, M., Ratliff, J., Dinsmore, J. and Jacoby, D. B. (1998). Regulated expression of the homeobox gene, *rPtx2*, in the developing rat. *Dev. Brain Res.* **110**, 215-226.
- Lines, M. A., Kozlowski, K. and Walter, M. A. (2002). Molecular genetics of Axenfeld-Rieger malformations. *Hum. Mol. Genet.* **11**, 1177-1184.
- Lohnes, D., Mark, M., Mendelsohn, C., Dollé, P., Dierich, A., Gorry, P., Gansmuller, A. and Chambon, P. (1994). Function of the retinoic acid

- receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**, 2723-2748.
- Maden, M., Horton, C., Graham, A., Leonard, L., Pizze, J., Siegenthaler, G., Lumsden, A. and Eriksson, U.** (1992). Domains of cellular retinoic acid-binding protein I (CRABPI) expression in the hindbrain and neural crest of the mouse embryo. *Mech. Dev.* **37**, 13-23.
- Mark, M., Ghyselinck, N. B., Kastner, P., Dupé, V., Wendling, O., Krezel, W., Mascrez, B. and Chambon, P.** (1998). Mesectoderm is a major target of retinoic acid action. *Eur. J. Oral. Sci.* **106**, 24-31.
- Mascrez, B., Mark, M., Dierich, A., Ghyselinck, N. B., Kastner, P. and Chambon, P.** (1998). The RXRalpha ligand-dependent activation function 2 (AF-2) is important for mouse development. *Development* **125**, 4691-4707.
- McCaffery, P., Wagner, E., O'Neil, J., Petkovich, M. and Dräger, U. C.** (1999). Dorsal and ventral retinal territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. *Mech. Dev.* **82**, 119-130.
- McLaughlin, T., Hindges, R. and O'Leary, D. D.** (2003). Regulation of axial patterning of the retina and its topographic mapping in the brain. *Curr. Opin. Neurobiol.* **13**, 57-69.
- Metzger, D. and Chambon, P.** (2001). Site- and time-specific gene targeting in the mouse. *Methods* **24**, 71-80.
- Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E. and Duester, G.** (2000). RALDH3, a retinaldehyde dehydrogenase that generates retinoic acid, is expressed in the ventral retina, otic vesicle and olfactory pit during mouse development. *Mech. Dev.* **97**, 227-230.
- Mic, F. A., Haselbeck, R. J., Cuenca, A. E. and Duester, G.** (2002). Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of Raldh2 null mutant mice. *Development* **129**, 2271-2282.
- Mic, F. A., Molotkov, A., Molotkova, N. and Duester, G.** (2004). Raldh2 expression in optic vesicle generates a retinoic acid signal needed for invagination of retina during optic cup formation. *Dev. Dyn.* **231**, 270-277.
- Molotkov, A., Fan, X., Deltour, L., Foglio, M. H., Martras, S., Farres, J., Pares, X. and Duester, G.** (2002). Stimulation of retinoic acid production and growth by ubiquitously expressed alcohol dehydrogenase Adh3. *Proc. Natl. Acad. Sci. USA* **99**, 5337-5342.
- Mori, M., Ghyselinck, N. B., Chambon, P. and Mark, M.** (2001). Systematic immunolocalization of retinoid receptors in developing and adult mouse eyes. *Invest. Ophthalmol. Vis. Sci.* **42**, 1312-1318.
- Niederreither, K., McCaffery, P., Dräger, U. C., Chambon, P. and Dollé, P.** (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67-78.
- Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P.** (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.
- Niederreither, K., Vermot, J., Fraulob, V., Chambon, P. and Dollé, P.** (2002). Retinaldehyde dehydrogenase 2 (RALDH2)- independent patterns of retinoic acid synthesis in the mouse embryo. *Proc. Natl. Acad. Sci. USA* **99**, 16111-16116.
- Peters, M. A. and Cepko, C. L.** (2002). The dorsal-ventral axis of the neural retina is divided into multiple domains of restricted gene expression which exhibit features of lineage compartments. *Dev. Biol.* **251**, 59-73.
- Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguère, V.** (1991). Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.
- Sakai, Y., Luo, T., McCaffery, P., Hamada, H. and Dräger, U. C.** (2004). CYP26A1 and CYP26C1 cooperate in degrading retinoic acid within the equatorial retina during later eye development. *Dev. Biol.* **276**, 143-157.
- Schlüter, C., Duchrow, M., Wohlenberg, C., Becker, M. H., Key, G., Flad, H. D. and Gerdes, J.** (1993). The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *J. Cell Biol.* **123**, 513-22.
- Soriano, P.** (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
- Suzuki, R., Shintani, T., Sakuta, H., Kato, A., Ohkawara, T., Osumi, N. and Noda, M.** (2000). Identification of RALDH-3, a novel retinaldehyde dehydrogenase, expressed in the ventral region of the retina. *Mech. Dev.* **98**, 37-50.
- Trainor, P. A. and Tam, P. P.** (1995). Cranial paraxial mesoderm and neural crest cells of the mouse embryo, co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. *Development* **121**, 2569-2582.
- Wagner, E., McCaffery, P. and Dräger, U. C.** (2000). Retinoic acid in the formation of the dorsoventral retina and its central projections. *Dev. Biol.* **222**, 460-470.
- Warkany, J. and Schraffenberger, S.** (1946). Congenital malformations induce in rats by maternal vitamin A deficiency. I. Defects of the eye. *Arch. Ophthalm.* **35**, 150-169.
- Wilke, T. A., Gubbels, S., Schwartz, J. and Richman, J. M.** (1997). Expression of fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3) in the developing head and face. *Dev. Dyn.* **210**, 41-52.
- Wilson, J. G., Roth, C. B. and Warkany, J.** (1953). 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.* **92**, 189-217.
- Xu, P. X., Woo, I., Her, H., Beier, D. R. and Maas, R. L.** (1997). Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. *Development* **124**, 219-231.
- Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y. and Hamada, H.** (2004). Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. *Dev. Cell* **6**, 411-422.