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Impairing retinoic acid signalling in the neural crest cells is sufficient to alter entire eye morphogenesis

Nicolas Matt[3], Norbert B. Ghyselinck[2], Isabelle Pellerin[1] and Valérie Dupé[1*]

[1]Institut de Génétique et Développement, CNRS UMR6061, Université de Rennes 1, IFR140 GFAS, Faculté de Médecine, 2, avenue du Professeur Léon Bernard, Rennes 35043 Cedex, France.

[2]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Institut Clinique de la Souris (ICS), CNRS/INSERM/ULP, Collège de France, Illkirch 67404, CU de Strasbourg, France.

[3]UPR9022 du CNRS, IBMC, 15 rues Descartes, 67084 Strasbourg, France.

*Author for correspondence (e–mail: valerie.dupe@univ-rennes1.fr; phone: +33(0)223233807; fax: +33(0)223234478).

Abbreviations: E(n), embryonic day; NCC, neural crest cell; POM, periocular mensenchyme; RA, retinoic acid; ALDH1A, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RPE, retinal pigmented epithelium; RXR, 9cis–RA receptor, VAD, vitamin A-deficient.

Key words: retinoic acid receptor; neural crest cells; eye development; PITX2; Extraocular muscle.

ABSTRACT

Retinoic acid (RA) is known to be required at various levels of eye patterning via Retinoic Acid Receptors (RAR); however the molecular and cellular mechanisms triggered by these nuclear receptors are still obscure. The genetic studies performed here enable us to present a new model to study RA action during eye development. By inactivating the three RARs, specifically in the periocular mesenchyme, we discriminate the individual contribution of each RAR during eye development and describe a new function for RARs during the formation of the optic nerve. We demonstrate that RARα is the only receptor that mediates RA signalling in the neurectoderm during ocular development. Surprisingly, and despite a sophisticated pattern of RA-activity in the developing retina, we observed that RA signalling is not autonomously required in this tissue for eye formation. We show that the action of RA during eye morphogenesis is occurring specifically in neural crest-derived periocular mesenchyme and is mediated by all three RARs. Furthermore, we point out that *Pitx2*, which encodes a homeodomain transcription factor, is a key RA-responsive gene in neural crest cells during eye development. Interestingly, we observed that RA is required in the neural crest cells for normal position of the extraocular muscle.

INTRODUCTION

During vertebrate development, the eye is constructed from neural and surface ectoderm as well as from the periocular mesenchyme (POM). This latter tissue derives from neural crest cells (NCCs) and mesoderm. The primary function of ocular mesenchyme is to provide multiple mature cell lineages that are necessary for the development of normal anterior segments (including the corneal endothelium, anterior chamber and iris stroma). A second essential function is that cells originating from the surface epithelium need to interact with POM cells for proper eyelid development (Le Lievre and Le Douarin, 1975). Finally, the POM provides essential signals for the specification of retinal pigmented epithelium (RPE) and the differentiation of the optic stalk, both structures arising from neural ectoderm (Evans and Gage, 2005; Fuhrmann et al., 2000). Failure of proper interactions between these ectodermal tissues and the POM results in developmental disorders such as Peters' anomaly, Axenfeld-Riegers's syndrome or aniridia (Lines et al., 2002).

Vitamin A (retinol) is known to be critical for vertebrate eye development as demonstrated by severe ocular defects occurring after gestational vitamin A deficiency, such as microphthalmia and coloboma of the retina (Dickman et al., 1997). Administration of Retinoic acid (RA), the active metabolite of vitamin A, can rescue these eye defects indicating that RA mediates the developmental actions of vitamin A (Dickman et al., 1997). RA signalling is transduced by specific nuclear retinoic acid receptor heterodimers (RXRα/RARα, RXRα/RARβ and RXRα/RARγ) that act as transcription factors and regulate specific target genes during development. During the last decade, extensive work has been done to decipher the molecular mechanisms triggered by RA during vertebrate development and notably in eye patterning (for a review see (Mark et al., 2006). In particular, numerous gene knockout studies have demonstrated that the three RARs (RARα, RARβ and RARγ) play important and overlapping roles during eye development (Ghyselinck et al., 1997; Lohnes et al., 1994). Embryos carrying a null mutation of only one RAR display relatively minor defects, but loss of function of two RARs results in several ocular abnormalities, including malformation of the eyelids, shortening of the ventral retina, coloboma and severe malformation of the anterior segment (absence of the iris stroma, the corneal stroma and the anterior chamber).

Although obvious redundancy between RARs has been documented, accurate comparison of phenotype between various combinations of RAR knockout mice has led to the attribution of specific function to a given RAR. For instance, while all three RARs are expressed in developing ocular structures (Ghyselinck et al., 1997; Mori et al., 2001), several lines of evidence suggest that RXRα/RARβ and RXRα/RARγ are the main heterodimers which are instrumental in ocular morphogenesis (Kastner et al., 1997; Zhou et al., 2001). Therefore, a clearly established function for RARα in the eye has not yet emerged.

During development, RA is generated by specific cells in a unique spatio-temporal pattern (Rossant et al., 1991). The first enzymatic step, the oxidation of retinol to retinal, is performed by the retinol dehydrogenase RDH10 (Sandell et al., 2007). Then, three RA–synthesizing enzymes (ALDH1A1, ALDH1A2 and ALDH1A3) are involved in RA synthesis during mouse eye development (Matt et al., 2005; Mic et al., 2004). They have distinct tissue-specific expression patterns from the optic vesicle to the optic cup as ocular formation proceeds. The distribution of ALDH1As and the RA-degrading enzymes within the retina produces a dorsal and a ventral domain of RA separated by a central zone lacking RA activity

(Wagner et al., 2000). This particular dorso-ventral pattern of RA activity observed during neural retina formation was initially thought to be crucial for eye development (Wagner et al., 2000). However, so far no conclusive data has been reported to support such a role for RA (Halilagic et al., 2007; Matt et al., 2005; Molotkov et al., 2006). ALDH1A2 which is the first enzyme expressed during ocular development is required between E8.5 and E9.5 for the initial formation of the optic pit (Mic et al., 2004; Niederreither et al., 1999; Ribes et al., 2006). *Aldh1a3*-null embryos display only discrete ocular malformations despite a loss of ventral RA signalling (Dupé et al., 2003) and partial loss of dorsal RA signalling in *Aldh1a1*-null mice does not induce any abnormal eye development (Fan et al., 2003; Matt et al., 2005). Finally, despite a non-overlapping expression pattern of their genes, a functional compensation between ALDH1A1, ALDH1A2 and ALDH1A3 has been demonstrated (Halilagic et al., 2007; Matt et al., 2005; Molotkov et al., 2006). To date, it has been established that during eye morphogenesis, RA is provided by the ALDH1A1 and ALDH1A3 enzymes expressed in the epithelial compartments of the eye (*i.e.,* the retina, retinal pigment epithelium and corneal ectoderm) (Matt et al., 2005; Molotkov et al., 2006). This RA signal diffuses in the mesenchymal compartment (*i.e.,* the POM) to activate RXRα/RARβ and RXRα/RARγ heterodimers. Thus, RA acts in a paracrine process to pattern the anterior segment. It has also been shown that RXRα/RARβ and RXRα/RARγ heterodimers control the extent of cell–death involved in POM remodelling and the expression of *Foxc1* and *Pitx2* genes (Matt et al., 2005), which are crucial genes for the development of the anterior eye segment in mice and humans (Cvekl and Tamm, 2004). Beside this relatively good knowledge of RA action during anterior segment development, its function during optic cup formation is still largely obscure.

The data obtained with various RA-deficient mouse models demonstrate unambiguously that RA is required for several morphogenetic processes during eye organogenesis. By using conditional mutations to inactivate all three RARs in the NCCs, we have generated a new mouse model where RA activity is entirely absent in the NCC-deriving POM. Not only does our work allow us to interpret the disparity of eye phenotypes previously observed in various models of RA deficiency, but definitely demonstrates that RA signalling plays key roles in eye development exclusively through the POM.

METHODS

Mice. Mice, with a mixed C57BL/6–129/Sv (50%:50%) genetic background, were housed in an animal facility licensed by the French Ministry of Agriculture (agreement N°B67–218–5) and all animal experiments were supervised by NBG who is qualified to experiment with mice, in compliance with the European legislation on care and use of laboratory animals (agreement N°67–205). 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. The generation of *lox*P-flanked (floxed) *Rara* (*Rara*$^{+/L2}$), *Rarb* (*Rarb*$^{+/L2}$), *Rarg* (*Rarg*$^{+/L2}$) and *Rara*-null mice have been previously described (Chapellier et al., 2002a; Chapellier et al., 2002b; Chapellier et al., 2002c; Lufkin et al., 1993). *Wnt1-Cre* mouse which carrying a transgene containing a *Cre* cassette under the control of the *Wnt1* promoter was obtained from A. McMahon (Danielian et al., 1998). The *R26R* transgenic mice have been previously described (Soriano, 1999). The *Wnt1-Cre* and *Rara*$^{+/L2}$, *Rarb*$^{+/L2}$, *Rarg*$^{+/L2}$ parental lines were intercrossed to generate mutant foetuses lacking RARα, RARβ and RARγ in NCCs (hereafter designated *Rara*/*b*/*g*$^{NCC-/-}$ mice). The resulting embryos were genotyped for *Cre*, *Rara*, *Rarb* and *Rarg* using PCR-based methods. The embryos of a littermate who does not carry the *Wnt1-Cre* transgene were used as controls. *Rara*/*b*/*g*$^{NCC-/-}$ mice were obtained at a mendelian ratio, but newborns died at birth from respiratory distress.

Histology, staining and *in situ* RNA analysis. For histology experiments, 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 was performed as described (Rossant et al., 1991). In situ RNA hybridization was carried out as described (Dupé et al., 2003). Briefly, 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 head. Terminal transferase–mediated dUTP-Nick-End-Labeling (TUNEL) was performed using the Apoptag® kit (Chemicon International).

RESULTS

RARα is the only mediator of RA signalling in the retina

During early eye development, immunolocalization experiments suggest that *Rara*, *Rarb* and *Rarg* genes are all expressed in the cells surrounding the developing optic cup (or POM for Peri Ocular Mesenchyme) whereas RARα would be the only receptor located in the developing retina and the retinal pigmented epithelium (RPE) (Mori et al., 2001).
In order to verify this observation, we examined the RA signalling activity in absence of RARα by crossing a mouse carrying a *RAR-lacZ RA-reporter* (Rossant et al., 1991) gene with the *Rara*-null mutant. The resulting mouse allowed us to follow the RA signalling activity spatiotemporally. We have tested *RARE-lacZ* activity at E10.5, when RA activity is strong in the retina, RPE and corneal ectoderm and present as a weaker signal in the POM (Fig. 1A,C). Compared to this characteristic *lacZ* expression, *Rara*-null mutant mice carrying the *RARE-lacZ RA-reporter* transgene exhibited an alteration of the reporter activity. On external view, RA signalling activity in the developing *Rara*-null eye was slightly reduced in ocular area whereas a significant decrease was observed at the level of the forebrain (Fig. 1A and B). To further explore *lacZ* activity in the developing eye, these embryos were sectioned. We discovered that RA activity was completely lost specifically at the level of the neural retina, the RPE and lens whereas RA activity was normal at the level of the surface ectoderm and the POM. Identical lacZ activity was observed at E11.5 and E13.5 (data not shown). These observations strongly support that *Rara* was the only RAR implicated in the transduction of RA activity in the neurectoderm of the developing eye.

Retinal morphology in *Rara*-null mouse is normal

The previous experiment revealed that *Rara* was the only receptor capable of transducing RA signalling in the retina. Consequently, *Rara*-null mutant gives a unique model to study the role of an autonomous-RA signal during retina development. We thus undertook an histological analysis of the retina in newborn and adult *Rara*-null mutants. As shown in Fig. 1E, newborn mouse retina have a defined ganglion cell layer and the development of other layers is still under way. There were no significant morphological differences in the retina of control and *Rara*-null newborn mice, either in terms of retinal thickness or with respect to the presence of a ganglion cell layer (Fig. 1E, F). In addition, no morphological differences were

observed in the adult retina, as *Rara*-null retina developed normally, displaying the expected lamination (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer and photoreceptors segments) with the same degree of thickness as that of control mice (Fig. 1G,H). Furthermore, the expression of *Tbx5 and Vax2*, that are respectively dorsal and ventral neural retina markers (McLaughlin et al., 2003), was tested and no difference was found between E10.5 *Rara*-null mutant and control (data not shown). Therefore, loss of *Rara* does not prevent normal lamination of the retina and survival. These data suggest that the absence of RA signalling in the retina does not give rise to an obvious defect in eye organization. Consequently, RA is not needed autonomously in the neural retina and RPE during overall eye morphogenesis. However, to date, no direct functional study and quantitative analysis have been performed on *Rara*-null adult mice. Therefore RARα could be implicated in more subtle developmental processes or in physiological aspects of the vision that we do not have analysed in this work.

$Rara/b/g^{NCC-/-}$ triple mutants exhibit more severe ocular defects than $Rarb/g^{NCC-/-}$ double mutants

In a previous work, by inactivating *Rarg* and *Rarb* specifically in the NCCs of the POM, we demonstrated a functional redundancy between RARβ and RARγ during the formation of the anterior segment, the eyelids and the ventral retina (Matt et al., 2005). Considering the severity of the eye phenotype observed in these mutant mice, we hypothesized at this point that the function of RA in POM was mediated by RARβ and RARγ. Nevertheless, in order to determine whether RARα may also function in the POM during ocular development, we removed all three RARs from the NCCs (mutant so called $Rara/b/g^{NCC-/-}$). Mice with the alleles of the genes encoding RARα, RARβ and RARγ floxed (*Rara/b/g L2/L2* mice) were crossed with the *wnt1-Cre* transgenic mouse line (Chapellier et al., 2002a; Chapellier et al., 2002b; Chapellier et al., 2002c; Danielian et al., 1998; Matt et al., 2005). In parallel, all combinations of double mutants for RAR in the NCCs were similarly obtained (*i.e.* $Rara/b^{NCC-/-}$, $Rarb/g^{NCC-/-}$ *and* $Rara/g^{NCC-/-}$).

Unpredictably, we observed that $Rara/b/g^{NCC-/-}$ mutant displayed a much more severe eye phenotype compared to $Rarb/g^{NCC-/-}$ (Matt et al., 2005). Indeed, in contrast to $Rarb/g^{NCC-/-}$, $Rara/b/g^{NCC-/-}$ mutant eyes were not visible externally at E18.5 (Fig. 2A-D). However,

examination of histological sections revealed that the two eyes were present in *Rara/b/g*$^{NCC-/-}$ mutants but buried within the skull, near the midline, directly beneath the hypothalamus and at the level of the optic chiasma (Fig. 2K). The presence of globes that were not externally visible indicated that *Rara/b/g*$^{NCC-/-}$ mutants were clinically anophthalmic. Interestingly, histological examination revealed that the *Rara/b/g*$^{NCC-/-}$ mutant eyes were attached to the ventral hypothalamus rather than connected through an extended optic nerve. Therefore, the *Rara/b/g*$^{NCC-/-}$ mutant eye was characterized by the presence of the lens and retina deep within the head mesenchyme with no evidence of optic nerve differentiation (Fig. 2H-K).

Notably, *Rara/b/g*$^{NCC-/-}$ embryos had normal lens vesicles at E14.5 suggesting that lens induction and invagination had proceeded normally in all mutants. However, their lens was rotated ventrally and distant from the surface. At E18.5, the lens was generally hypoplasic, probably due to its deep location during organogenesis and the presence of a persistent retrolenticular membrane that completely filled the space between the retina and the lens (Fig. 2H-K). In E14.5 *Rara/b/g*$^{NCC-/-}$ embryos, the dorsal retina was well developed, and both neural and pigment epithelium layers were present. At the opposite, the ventral retina was absent or poorly developed, resulting in a complete coloboma (Fig. 2H,I). Moreover, *Rara/b/g*$^{NCC-/-}$ mutant eyes display eversion of the remaining ventral retina that arises from the transformation of RPE into neural epithelium. As a consequence, ventral RPE was never observed in the ventral side (Fig. 2K). The dorsal retina and the remnant ventral retina of the *Rara/b/g*$^{NCC-/-}$ mutant appeared to be normally multilayered and, despite the absence of optic nerve, the extend axons entered the ventral hypothalamus and formed the optic chiasma (Fig. 2H-K). Both the optic nerve and ventral retina arise from neural ectoderm, indicating that one essential function of RA in the periocular NCCs is to influence the development of the neural ectoderm.

Multiple structures partially or totally arising from neural crest are also absent in *Rara/b/g*$^{NCC-/-}$ eyes. Similarly to *Rarb/g*$^{NCC-/-}$ mutants (Matt et al., 2005) (Fig. 2F and Table 1), the corneal endothelium and stroma, anterior chamber and eyelids which receive contributions from neural crest and mesoderm, were absent in *Rara/b/g*$^{NCC-/-}$ mutant eyes and were replaced by a thick layer of loosely organized mesenchyme (Fig. 2H,I). The sclera surrounding the eye deriving from the neural crest was completely absent (Fig. 2K). Interestingly, *Rara/g*$^{NCC-/-}$ mutants (Fig. 2G and Table 1) and *Rara/g*-double null mutants both display a similar phenotype: the eye was buried, the optic nerve was present but shorter and the ventral retina was underdeveloped (Lohnes et al., 1994). Importantly, in *Rara/g*$^{NCC-/-}$ mutants and *Rara/g*-

double null mutants the anterior segment and eyelids were malformed but not completely absent compared to *Rara/b/g*$^{NCC-/-}$ and *Rarb/g*$^{NCC-/-}$ mutants. To resume, whereas the *Rara/b*$^{NCC-/-}$ eye phenotype was mild, consisting only in a persistent retrolenticular membrane, both *Rara/g*$^{NCC-/-}$ and *Rarb/g*$^{NCC-/-}$ mutants presented a severe eye phenotype (Table 1). Nonetheless, these phenotypes are milder compared to the *Rara/b/g*$^{NCC-/-}$ eyes. Interestingly, the elongation of the optic nerve is affected in *Rara/g*$^{NCC-/-}$ mutants but not in *Rarb/g*$^{NCC-/-}$ embryos whereas the anterior segment was more affected in *Rarb/g*$^{NCC-/-}$ embryos compared to *Rara/g*$^{NCC-/-}$. Altogether, these data suggest that the 3 RARs were implicated in the POM with specific function for each receptor.

Morphological defects in *Rara/b/g*$^{NCC-/-}$ eye primordia begin at E10.5

To identify the origin of the accumulated eye defects, we examined *Rara/b/g*$^{NCC-/-}$ embryos after NCCs migration (*i.e.* E9.5). We used the 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 line was crossed with the *Rara/b/g*$^{NCC-/-}$ mice and resulting embryos (designated *Rara/b/g*$^{NCC-/-lacZ}$) were taken at E9.5. First, we observed the pattern of β-galactosidase activity identifying NCC derivatives and concluded that they were identical in eye area of E9.5 *Rara/b/g*$^{NCC-/-lacZ}$ and *WT*^{lacZ} embryos (Fig. 3A, B). Thus, we demonstrate that ablation of all 3 RARs in NCCs does not alter their migration into the periocular region. These data allowed us to verify that the morphological defects observed in *Rara/b/g*$^{NCC-/-}$ mutants did not result from alteration of NCCs migration. Importantly, we observed that mutant and control optic vesicles were histologically indistinguishable until E10.5 (Fig. 3A-D). Then, from this stage, *Rara/b/g*$^{NCC-/-}$ eye phenotype is characterized by a systematic absence of the ventral eyelid groove. In contrast, the optic stalk and optic cup were similar in both control and mutant (arrowhead in Fig. 3E,F).

By E11.5, the eye defects of the *Rara/b/g*$^{NCC-/-}$ mutant became very drastic and were recognizable externally (data not shown) as pigmented cells were not observed on the ventral side of the eye (Fig. 3G,H). The initial specification of the RPE appeared to occur normally in *Rara/b/g*$^{NCC-/-}$ embryo, since the mutant dorsal RPE was indistinguishable from the control at E10.5 (Fig. 3E, F). However, at E11.5, when the RPE becomes pigmented, no pigment-

containing cells were observed in the remnant ventral RPE of the *Rara/b/g*$^{NCC-/-}$ mutant (data not shown), suggesting that the expansion of the ventral RPE was blocked in the mutant eyes. As a consequence, an eversion of the ventral retina is observed at E14.5 and E18.5 (Fig. 2H, K). In addition, the optic stalk in *Rara/b/g*$^{NCC-/-}$ mouse remained a thick neuroblastic structure whereas in control embryos, the optic stalk had begun to extend and thin down, leading to the normal distance between the optic cup and the ventral diencephalon (Fig. 3G, H). As a result, the mutant eye remains closely associated with the ventral diencephalon by E14.5 (Fig. 2H).

It has been shown previously that RA triggers apoptosis in the POM during eye formation. Indeed, the 2 physiological apoptotic clusters observed in the POM during eye development are not found in *Rarb/g*$^{NCC-/-}$ E11.5 mutants (Matt et al., 2005). To investigate the RA-dependent physiological function of programmed cell death during *Rara/b/g*$^{NCC-/-}$ eye formation, a TUNEL assay was performed on serial histological sections along the anteroposterior axis of the eye region at E10.0 and E11.5 (Fig. 3C,D and G,H). As expected, the normal apoptotic clusters were consistently not detected in the POM of *Rara/b/g*$^{NCC-/-}$ E11.5 embryos (Fig. 3G, H and data not shown). Interestingly, as illustrated in Figures 3 (G and H) and previously shown by others (Cuadros and Rios, 1988; Ulshafer and Clavert, 1979), optic nerve formation is also associated with a marked apoptotic cell death. However, this wave of dying cells in the forming optic nerve was similarly observed in the E10.0 and E11.5 mutant and control embryos (Fig. 3C,D and G,H). Therefore, our data show that the abnormal development of optic nerve observed in *Rara/b/g*$^{NCC-/-}$ mutant mice is not correlated with an abnormal regulation of apoptosis. Although the undifferentiated mesenchyme in the POM area of the E14.5 *Rara/b/g*$^{NCC-/-}$ mutants suggested that the anterior segment was hypercellular, we were unable to find evidence of increased proliferation in the POM of *Rara/b/g*$^{NCC-/-}$ mutant mice at E10.5 and E11.5 (data not shown).

Pitx2 is not expressed in periocular mesenchyme of *Rara/b/g*$^{NCC-/-}$ mutants

Amongst the genes involved in ocular development, the homeodomain transcription factor PITX2 has been designated as a key protein, playing essential functions during anterior segment patterning (Evans and Gage, 2005). At E11.5 this gene is expressed in the POM, especially in the presumptive anterior segment. Interestingly, we have previously reported that in E11.5, *Rarb/g*$^{NCC-/-}$ embryos, *Pitx2* expression is reduced in the POM dorsal region (Matt et al., 2005). Subsequent to this work, a mutant mouse in which *Pitx2* expression is

specifically inactivated in NCCs (*Pitx2*$^{NCC-/-}$) has been described (Evans and Gage, 2005). This mutant is characterized by an abnormal anterior segment differentiation, an absence of eyelids and exhibited a failure to maintain the optic nerve. Interestingly, the same defects are observed in *Rara/b/g*$^{NCC-/-}$ mutant. We therefore tested the expression of *Pitx2* in *Rara/b/g*$^{NCC-/-}$ mutant. Importantly, we observed that *Pitx2* expression was totally absent in the periocular area of the *Rara/b/g*$^{NCC-/-}$ E11.5 embryos (n=3, Fig. 4A, B) whereas its expression in the pharyngeal ectoderm was not altered, demonstrating that the expression of *Pitx2* in the POM required RA signalling. Therefore, the specific absence of *Pitx2* expression in *Rara/b/g*$^{NCC-/-}$ demonstrates that *Pitx2* is a target of RA signalling in the POM.

Among the other genes involved in ocular development and potential target of RA, *Gas1* (Growth arrest specific gene 1) is a relevant candidate. Indeed, *Gas1*-null mouse present microphthalmia, anterior segment defects and eversion of the ventral retina (Lee et al., 2001). Furthermore, microarray experiments using F9 embryonic carcinoma cells exposed to RA suggest that *Gas1* would be a RA-responsive gene (Eifert et al., 2006). As shown on figure 4C, *Gas1* expression is detected at E11.5 in the dorsal and ventral tip of the retina and in the mesenchyme surrounding the eye (i.e POM). However, this expression of *Gas1* in the POM is similar in control mouse as in *Rara/b/g*$^{NCC-/-}$ mutant (Fig. 4C, D). These data indicate that Gas1 is not a target of RA signalling in the POM, and also that the absence of RA signalling in the NCCs does not globally disrupt gene expression in POM cells, but selectively decreases *Pitx2*.

Initial patterning of retina and optic nerve is not affected in *Rara/b/g*$^{NCC-/-}$ mutants

To this date, only a few mutant mouse strains display a failure of morphogenetic extension of the optic nerve. This severe defect in optic nerve development was described in *Pitx2*$^{NCC-/-}$ (Evans and Gage, 2005). Importantly, it has been shown that the absence of the optic nerve in this mutant was not due to a lack of initial specification, since the expression of an early marker of the optic stalk like *Pax2* is normal (Torres et al., 1996). On the basis of a similar severe optic nerve phenotype in the *Rara/b/g*$^{NCC-/-}$ mutant and a down-regulation of *Pitx2* in this mutant, we have tested *Pax2* expression at E11.0. Similarly to *Pitx2*$^{NCC-/-}$ mutant, we observed that *Pax2* was normally expressed in the optic stalk of both control and *Rara/b/g*$^{NCC-/-}$ mutant (Fig. 4G,H), establishing that a lack of initial specification cannot account for the

optic stalk defects. In addition, and considering the absence of ventral retina in E11.5 *Rara/b/g*$^{NCC-/-}$ mutant, we have evaluated the dorsoventral polarity of the *Rara/b/g*$^{NCC-/-}$ mutant retina by studying the expression of *Tbx5* and *Vax2* (McLaughlin et al., 2003). We did not find any difference in the expression patterns of *Vax2* and *Tbx5* between *Rara/b/g*$^{NCC-/-}$ mutant and control eye at E10.5 (Fig. 4I, J and data not shown). Since the polarity of the neural retina is presumed to be determined at E9.5, it indicates that initial patterning of the retina was not significantly affected.

Extraocular muscles are ectopic in *Rara/b/g*$^{NCC-/-}$ mutants

The extraocular muscles, which contain mainly mesoderm-derived myocytes but also neural crest-derived fascia cells, are normally present as mesenchymal condensations lateral to the optic cup and are characterized by the expression of *Pitx2* at E11.5 (Fig. 4A). During our histological analysis of *Rara/b/g*$^{NCC-/-}$ mutant, we observed that extraocular muscle was not present in the periocular area (Fig. 2E, H-K). We thus extended our analysis to the whole head using serial sections. From this work, we detected a muscle-like tissue between the trigeminal ganglion and the pituitary (Fig. 4F). In addition, this muscle-like tissue exhibited specific mesodermal expression of *Pitx2* (Fig. 4F) (Kitamura et al., 1999). These ectopic muscle-like structures were still present at E14.5 between the trigeminal ganglion and the pituitary (data not shown). Altogether, these observations demonstrated that muscles were properly formed in mutant mice but localized more caudally compared to its normal position lateral to the optic cup. This result strengthens a recent report suggesting that early craniofacial myogenesis is NCC-independent whereas migration of head muscle cells would be regulated by cranial NCCs (Rinon et al., 2007). Interestingly, this new model of total RA-deficiency in NCCs has highlighted an unexpected role for RA signalling in ocular muscle positioning. We therefore propose that RA in the NCCs provide guidance cues to allow muscle precursor cells to migrate to the correct position in the embryonic head.

DISCUSSION

Sophisticated experimental genetics in mice provides a powerful approach to understand developmental process by allowing precise molecular, cellular and temporal dissection of gene function. Tissue-specific knockouts are particularly useful when trying to understand the

function of a gene that is expressed in multiple cell lineages of a developing organ. The triple *Rara/b/g*$^{NCC-/-}$ mutants generated here give a new model to study RA function during eye development and the data presented in this manuscript provide essential new information toward the understanding of mammalian eye patterning. In particular, our data demonstrate definitely the essential role of RA in the POM to pattern the optic vesicle.

Eye development results from complex cell interactions between neurectoderm (which give rise to the retina and optic nerve), the surface ectoderm (which give rise to the lens, epithelia of the cornea, conjunctiva and eyelids) and NCC-derived mesenchyme (which forms the choroids, sclera, stroma of the cornea and iris, anterior chamber and vitreous body) (Le Douarin et al., 1993). Previous studies using either vitamin A deficiency models, *Rdh10, Aldh1a* or *Rar* mutants have demonstrated that RA signalling is crucial for the formation of all these ocular structures (Dickman et al., 1997; Halilagic et al., 2007; Lohnes et al., 1994; Mark et al., 2006; Matt et al., 2005; Sandell et al., 2007). Consequently, it was thought that RA was required for various morphogenetic processes in various eye tissues and at several stages to pattern eye formation.
In contrast to this general assessment, we show that the complete removal of RA activity only in the NCCs recapitulates all eye malformations previously described in the various RA-deficient models (Halilagic et al., 2007; Lohnes et al., 1994; Mark et al., 2006). We have thereby demonstrate for the first time that the only target of RA signalling is the NCC-derived mesenchyme and that impairing this signal in this tissue is sufficient to alter the entire eye morphogenesis.

RA is not required in the retina during its lamination

In the present study, we show that RA activity was absent from the developing retina and RPE in a *Rara*-null mutant between E10.5 and E13.5. By this experiment, we demonstrated that *Rara* is the only RAR capable to efficiently mediate RA activity in the developing retina and RPE.
Previously it was hypothesized that RA maybe involved autonomously in dorsoventral patterning of the retina based upon the dorsal and the ventral expression patterns of *Aldh1a1* and *Aldh1a3* respectively in the retina from E10.5. However, studies on *Aldh1a1*$^{-/-}$*Aldh1a3*$^{-/-}$ embryos have shown that a lack of RA signalling in the retina from E10.5 does not affect expression of dorso-ventral key determinants like *Tbx5* and *Vax2* (Matt et al., 2005; Molotkov

et al., 2006). Additionally, the absence of RA-signalling in *Rara*-null retina did not alter the expression of *Vax2* and *Tbx5*. This indicates that RA does not have an autonomous function in the dorso-ventral patterning of the retina. Furthermore, the fact that *Tbx5* and *Vax2* are normally expressed in the *Rara/b/g*$^{NCC-/-}$ retina indicates that initial dorsoventral patterning is also not affected and that RA does not regulate these genes by a cell non-autonomous process.

Moreover, a later autonomous function for RA in the retina or RPE should have been revealed by the *Rara*-null mutant. However, no detectable eye defects were observed in adult *Rara*-null mutant eye. Thus, our genetic studies demonstrate conclusively that RA signalling has no autonomous function during retinal lamination. Thus RA is dispensable within the retina for the formation of the eye. However, an autonomous role for RA in more elaborate specialization of the retina is still conceivable as our histological study cannot rule out eventual more subtle defects in adult retina.

RARα, RARβ and RARγ are differently required in the periocular mesenchymal NCCs

Since RARβ and RARγ are essential in the POM (Matt et al., 2005), it had been postulated that these 2 receptors were the main receptors involved in RA signalling in this tissue. In this study, we found major ocular defects in *Rara/g*$^{NCC-/-}$ and *Rarb/g*$^{NCC-/-}$ but not in *Rara/b*$^{NCC-/-}$. Simultaneously, using the same approach, we also demonstrated that RARγ was crucial for normal differentiation of the craniofacial NCCs (V.D, unpublished). Altogether, it suggests that RARγ is the most important RAR implicated in cranial NCCs differentiation. Besides highlighting the role of RARγ in NCCs differentiation, our data gives a better understanding of the role of RARα during eye development. Although comparison between phenotypes of RAR double null mutants has suggested a role for RARα in eye development (Lohnes et al., 1994), no studies were available to attribute a specific function to this receptor. Here, we observed a strong genetic interaction in the POM between *Rara, Rarb* and *Rarg*, demonstrating clearly a function of RARα in this tissue. Precisely, complete absence of the optic nerve has never been observed in the various combinations of *Rar*-double null mutants.

Furthermore, our data demonstrate that the role of each RAR is not equivalent during ocular morphogenesis and that they are specialized in patterning different NCCs-dependent structures. *Rarb*/$g^{NCC-/-}$ mutants have the anterior segment and eyelids preferentially affected whereas the optic nerve is normal and the ventral retina is present while slightly shorter. In contrast, the couple of RARα and RARγ is more important for optic disc invagination and nerve optic differentiation since their inactivation in the POM produces an hypoplasic optic nerve whereas the anterior segment and eyelid formation is less severely affected compared to those of *Rarb*/$g^{NCC-/-}$ mutants. Thus, RARβ is specifically involved cell-autonomously within the anterior segment whereas RARα is rather implicated cell-non autonomously during optic disc invagination and optic nerve maintenance.

During eye formation, RA signalling emerges in specific regions while the eye undergoes a dramatic remodelling. In this sophisticated process, the specific function of each ALDH1A and RAR were not clear until now. Here, we clarify the sequential contribution of each actor of the RA signalling to eye morphogenesis. By inactivating all 3 RARs in the POM, we largely recapitulated the spectrum of eye phenotype of *Aldh1a2*$^{-/-}$*Aldh1a3*$^{-/-}$ mutant that have been RA supplemented between E7.5 and E8.5 to rescue heart failure (Halilagic et al., 2007). This *Aldh1a2*$^{-/-}$*Aldh1a3*$^{-/-}$ mutant supplemented before E8.5 shows no more RA-signal in the ocular region at E9.5 (Halilagic et al., 2007; Molotkov et al., 2006) suggesting that ALDH1A2 and ALDH1A3 are the main RA-producing enzyme in the POM at this stage. These data, as well as the results described herein, demonstrate that the absence of RA signalling in the ocular region from E9.5 is implicated in the apparition of the *Rara*/*b*/$g^{NCC-/-}$ eye phenotypes. Thus, at this stage, RA produced by ALDH1A2 and ALDH1A3 in the POM stimulates the invagination of the optic vesicle and the formation of the optic nerve (Molotkov et al., 2006). Here, we have shown that this event is mainly mediated by RARα and RARγ in the POM and is therefore cell-non autonomous. Subsequently, no additional RA-signal is found in the ocular region of the *Aldh1a1*$^{-/-}$*Aldh1a3*$^{-/-}$ mutant from E10.5 (Matt et al., 2005; Molotkov et al., 2006). Thus, RA production is exclusively performed by ALDH1A1 and ALDH1A3 from this stage. As the phenotype of *Aldh1a1*$^{-/-}$*Aldh1a3*$^{-/-}$ mutant is similar to *Rarb*/$g^{NCC-/-}$ phenotype (Matt et al., 2005), this RA mainly activates RARβ and RARγ to pattern the anterior segment, eyelids and late invagination of the retina. A schematic presentation of these overall data is given in figure 5.

RA in the POM is crucial for optic nerve maintenance

The ocular defects observed in *Rara/b/g*$^{NCC-/-}$ mutants such as abnormalities of the cornea, eyelids, anterior chamber, and presence of PHPV, all correlate with the abnormal differentiation of cranial NCCs (Cvekl and Tamm, 2004). This part of the phenotype has been described in *Rarb/g*$^{NCC-/-}$ mutants and was found to be associated to a decrease of *Pitx2*, *Foxc1* and *Eya2* expression in POM cells (Matt et al., 2005). Here, by additionally removing RARα in the NCCs, we have found that *Pitx2* expression is fully abolished in the POM. Interestingly, recent data have demonstrated that specific inactivation of *Pitx2* in the NCCs induces an anterior segment dysgenesis but also defects in the maintenance of the optic nerve, which closely resemble the ocular defects displayed by *Rara/b/g*$^{NCC-/-}$ mutant mice (Evans and Gage, 2005).

As RA and PITX2 are both required in the periocular NCCs and as PITX2 is absent in the POM of *Rara/b/g*$^{NCC-/-}$ mutant mouse, RA and *Pitx2* are involved in a simple linear genetic pathway. In this genetic cascade, we propose that RARs are upstream of this pathway and control the expression of *Pitx2,* which in turn is required for anterior segment differentiation and optic nerve maintenance. While the involvement of *Pitx2* in RA signalling is clear from our experiments, a direct action of RA on *Pitx2* promoter remains unclear. We therefore performed an *in silico* analysis of the *Pitx2* gene and found no RARE sequence in its promoter. In addition, the analysis of microarray experiments did not identify *Pitx2* as a direct RA inducible gene. Therefore RA would regulate *Pitx2* indirectly through a transcription intermediary (Balmer and Blomhoff, 2002; Eifert et al., 2006).

In various models of RA-deficiency, including the *Rara/b/g*$^{NCC-/-}$ mutant, anterior segment defect was associated with reduced apoptosis (present data; (Matt et al., 2005). Since, the same anterior segment defects are observed in *Rara/b/g*$^{NCC-/-}$ and *Pitx2*$^{NCC-/-}$ mutants, it can be suggested that *Pitx2* triggers cell apoptosis in a linear genetic pathway regulated by RA. However, since cell death was not studied in the *Pitx2*$^{NCC-/-}$ mutant, we cannot conclude firmly that this linear genetic pathway leads to cell apoptosis. In this process, *Eya2* (Clark et al., 2002) could also be involved since its decreased expression in the POM was described for *Rarb/g*$^{NCC-/-}$ mutants (Matt et al., 2005).

Other genes could also be included in this cascade. The transcription factors *Lmx1b* and *Foxc1* are notably required for normal anterior segment development. These genes are co-expressed within the POM and their inactivation results in anterior segment defects, although

less severe compared to those of *Pitx2*$^{NCC-/-}$ and *Rara/b/g*$^{NCC-/-}$ mutants (Kidson et al., 1999; Pressman et al., 2000). Furthermore, the regulation of *Foxc1* by RA (Matt et al., 2005) strengthens the hypothesis that these genes are involved in the RA-dependent molecular mechanism leading to anterior segment morphogenesis.

The striking new phenotype of the *Rara/b/g*$^{NCC-/-}$, as compared to the other RARs loss-of-function models, is the absence of optic nerves. These new data demonstrate that the POM is instrumental for optic nerve maintenance in a RA-dependent process. Most interestingly, *Rara/b/g*$^{NCC-/-}$ as *Pitx2*$^{NCC-/-}$ mutants displayed the same absence of optic nerve (present data and (Evans and Gage, 2005). Indeed, both mutants exhibit normal initial specification of the optic stalk as testified by the expression of *Pax2,* followed by a failure to maintain the optic nerve. Since these conditional mutants (*Rara/b/g*$^{NCC-/-}$ and *Pitx2*$^{NCC-/-}$) are specifically inactivated in the NCCs, the defect of the optic stalk observed is the result of an impairment of an extrinsic signal arising from the POM. We thus propose that RA in the POM controls *Pitx2* expression which, in turn, activates a yet unknown paracrine factor that subsequently conditions the morphogenetic extension of the optic stalk. We also point out that a cell-autonomous (formation of the anterior segment) and non-autonomous (optic cup morphogenesis) mechanisms, both RA-dependent, involve the same target gene, i.e. *Pitx2*.

Altogether, our work clearly demonstrates that RA is not cell-autonomously required in the neurectoderm to pattern eye morphogenesis. In contrast, ocular development required RA producing ectodermal cells, sending this RA signal to the POM, which in turn acts on retina. It thus questions the presence of sophisticated *Aldh1as* expression in the retina during eye development as previously described (Wagner et al., 2000). It could be speculated that such timely and regionally regulated RALDH expression could be an evolutionary relic of an important mechanism present in lower vertebrates. Another hypothesis is that such ALDH1As expression is indispensable for retinal differentiation in order to acquire an elaborate retina with high-acuity.

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FIGURES LEGENDS

Table 1: Eye abnormalities present in *Rara/b*$^{NCC-/-}$, *Rarb/g*$^{NCC-/-}$, *Rara/g*$^{NCC-/-}$ and *Rara/b/g*$^{NCC-/-}$. Note that the single abnormality observed in *Rara/b*$^{NCC-/-}$ mutant, consisting of a persistent retrolenticular membrane, was also observed in *Rarb*$^{NCC-/-}$ single mutant.

Figure 1: Effects of *Rara* inactivation on the RA-dependent activity of a *RARE-lacZ* reporter transgene. Distribution of β−galactosidase activity driven by the *RARE-lacZ* transgene in WT (A,C) and *Rara*-null mutants (B,D) at E10.5 (A-D). *RARE-lacZ* activity is abolished in *Rara*-null in the retina, RPE and lens but not in the surface ectoderm and mesenchymal cells. C and D are sections from A and B respectively. Note the reduced RA activity at the level of the forebrain. Aspects of retinal histogenesis in WT and *Rara-null* mutant at birth (E,F) and adulthood (G,H). c, presumptive corneal ectoderm; ch, choroids; e, eye; f, forebrain; gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; le, lens; mx, maxillary part of first branchial arch; n, optic nerve; onl, outer nuclear cell layer; opl, outer plexiform layer; pom, periocular mesenchyme; ps, photoreceptor segments; r, neural retina; rpe, retinal pigmented epithelium.

Figure 2: Partial or complete absence of RA signalling in the POM results in severe ocular defects. (A-D) External view of E18.5 foetus eye areas. (E-K) Frontal histological sections through heads of E14.5 (E-I) and E18.5 (J,K) foetuses. In order to illustrate the shortening of the optic nerve, the orientation of the eye in G is more transversal, this section does not allow illustration of the shortening of the ventral retina. Asterisks (F-I, K) indicate undifferentiated mesenchyme replacing the eyelids and cornea. Arrowhead (A, B and C) indicates the eye. c, cornea; d, dorsal retina; e, ear; el, eyelid; er, eversion of the retina; le, lens; m, extraocular muscle; n, optic nerve; oc, optic chiasma; p, persistent retrolenticular membrane; rpe, retinal pigment epithelium; v, ventral retina.

Figure 3: Defects in *Rara/b/g*$^{NCC-/-}$ eye primordial begin at E10.5. Frontal histological sections through head of E9.5 (A,B), E10.0 (C,D), E10.5 (E,F), E11.5 (G,H) genotypes as indicated. (A, B) distribution of β-galactosidase activity driven by the R26R transgene in (A) WT and (B) *Rara/b/g*$^{NCC-/-}$ E9.5 embryos that are histologically similar. The β-galactosidase staining is identical, indicating that ablation of all three RARs does not alter the migration of NCCs in the POM. (C,D and G,H), Distribution of apoptotic cells (assessed by TUNEL assays). Note the absence of the ventral eyelid groove (arrowhead in F). Square brackets (C and D) indicate physiological apoptosis in optic stalk. Asterisks (H) indicate undifferentiated mesenchyme replacing the eyelids and cornea. a, apoptotic cluster in the POM. b, brain; d, dorsal retina; le, lens; m, muscle; n, optic nerve; n*, undifferentiated optic nerve; os, optic stalk; ov, optic

vesicle; pom, periocular mesenchyme; r, neural retina; rpe, retinal pigmented epithelium; v, ventral retina.

Figure 4: Ablation of *Rara, Rarb* and *Rarg* in neural crest cells impairs gene expression in the POM but not in the retina. (A-J). In situ hybridization with digoxigenin-labeled *Pitx2*, *Gas1*, *Vax2* and *Pax2* on frontal sections. Note the persistent expression of *Pitx2* in the pharyngeal ectoderm of the *Rara/b/g*$^{NCC-/-}$ mutants. b, brain; d, dorsal retina; g5, trigeminal ganglion; le, lens; m, muscle; n, optic nerve; n*, undifferentiated optic nerve; o, oral cavity; p, pituitary; pe, pharyngeal ectoderm; pom, periocular mesenchyme; rpe, retinal pigmented epithelium; v, ventral retina.

Figure 5: ALDH1A1, ALDH1A2, ALDH1A3, RARα, RARβ and RARγ participate sequentially to eye morphogenesis. At E9.5, RARα and RARγ are activated by RA produces by ALDH1A2 and ALDH1A3 to determine non-autonomously the RA-dependent optic nerve morphogenesis and invagination of the retina. Subsequently, from E10.5, RARβ and RARγ are activated by RA produces by ALDH1A1 and ALDH1A3 to determine non-autonomously the late retinal invagination process and to control autonomously anterior segment morphogenesis. Light blue indicates that the contribution of this specific RAR to the formation of the corresponding eye structure is weaker. Thick arrow represents the progressive RA-dependent stage of the various eye structures.

Eye defects	***Rara/b*$^{NCC-/-}$**	***Rarb/g*$^{NCC-/-}$**	***Rara/g*$^{NCC-/-}$**	***Rara/b/g*$^{NCC-/-}$**
E14.5 E18.5	n = 4 n = 2	n = 4 n = 2	n = 3 n = 3	n = 6 n = 4
Shorter ventral retina	0%	100%	100%	100%
Complete agenesis of the ventral retina	0%	0%	0%	**80%**
Lens rotation	0%	100%	100%	100%
Persistent retrolenticular membrane	100%	100%	100%	100%
Absence of the optic nerve	0%	0%	0%	**100%**
Corneal lenticular stalk	0%	100%	100%	100%
Anterior segment defects	0%	100%	100% (partial)	100%
Eversion of the retina	0%	0%	100%	100%
Coloboma	0%	0%	100%	100%
Agenesis of the eyelid	0%	100%	100% (partial)	100%

Table1

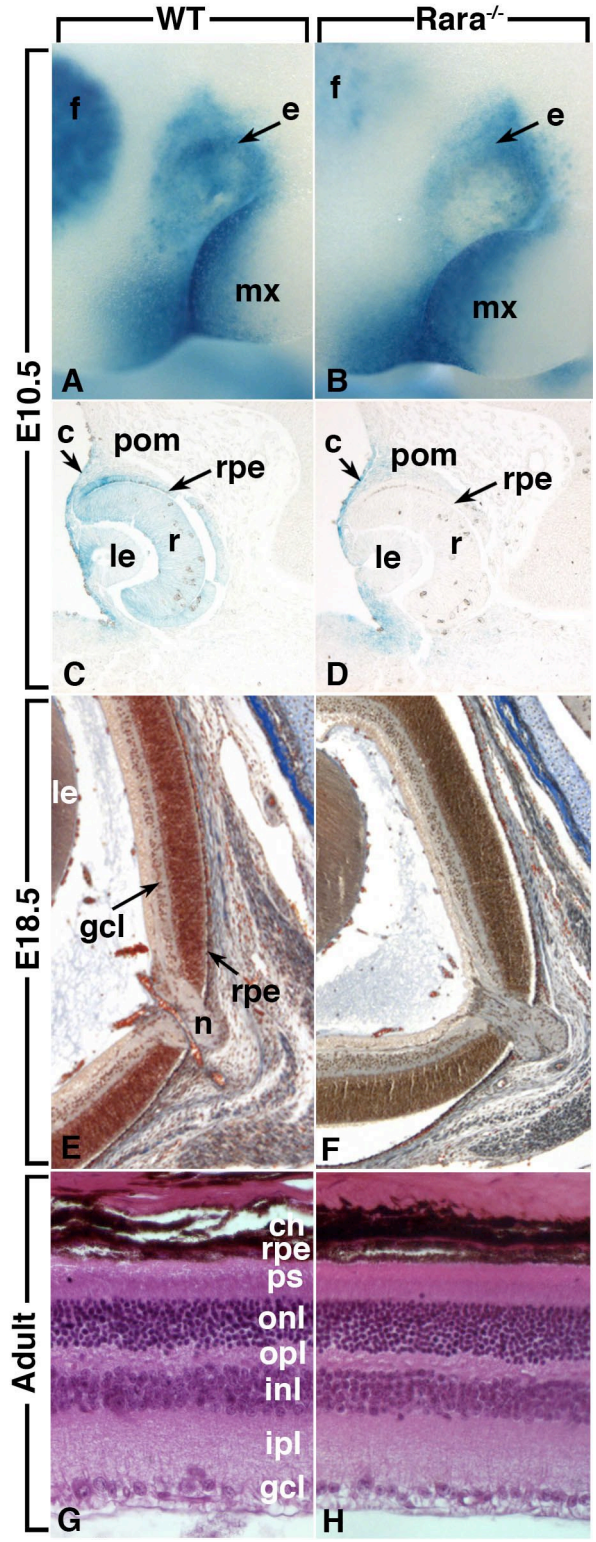
WT
Rara-/-
E10.5
E18.5
Adult
f
e
mx
A
B
c
pom
rpe
le
r
C
D
gcl
n
E
F
ch
ps
onl
opl
inl
ipl
G
H

Figure 1

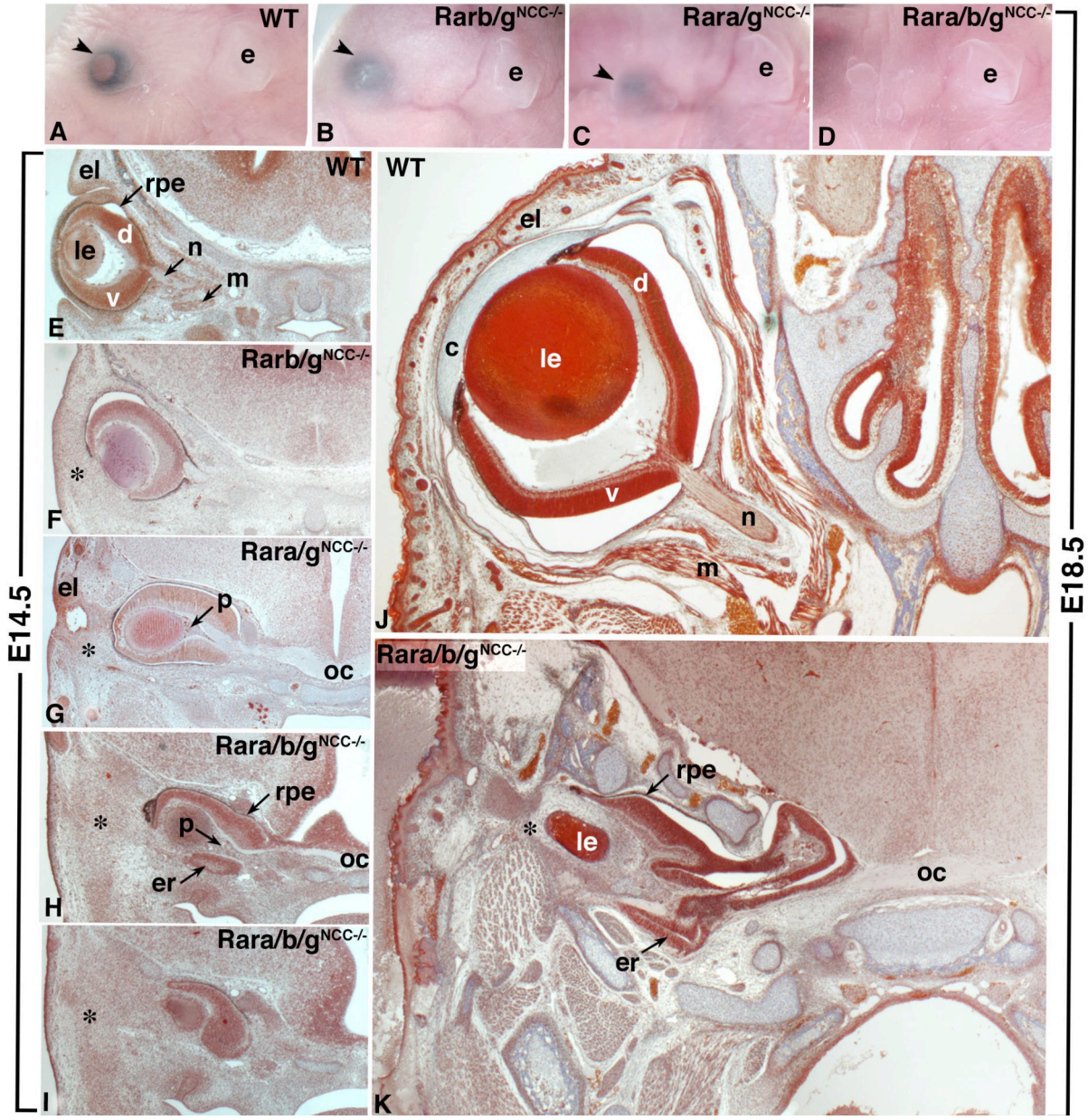
WT
e
A
Rarb/g^NCC-/-
e
B
Rara/g^NCC-/-
e
C
Rara/b/g^NCC-/-
e
D
E14.5
E18.5
el
rpe
WT
le
d
n
m
v
E
Rarb/g^NCC-/-
F
Rara/g^NCC-/-
el
p
oc
G
Rara/b/g^NCC-/-
rpe
p
oc
er
H
Rara/b/g^NCC-/-
I
WT
el
d
c
le
v
n
m
J
Rara/b/g^NCC-/-
rpe
le
oc
er
K

Figure 2

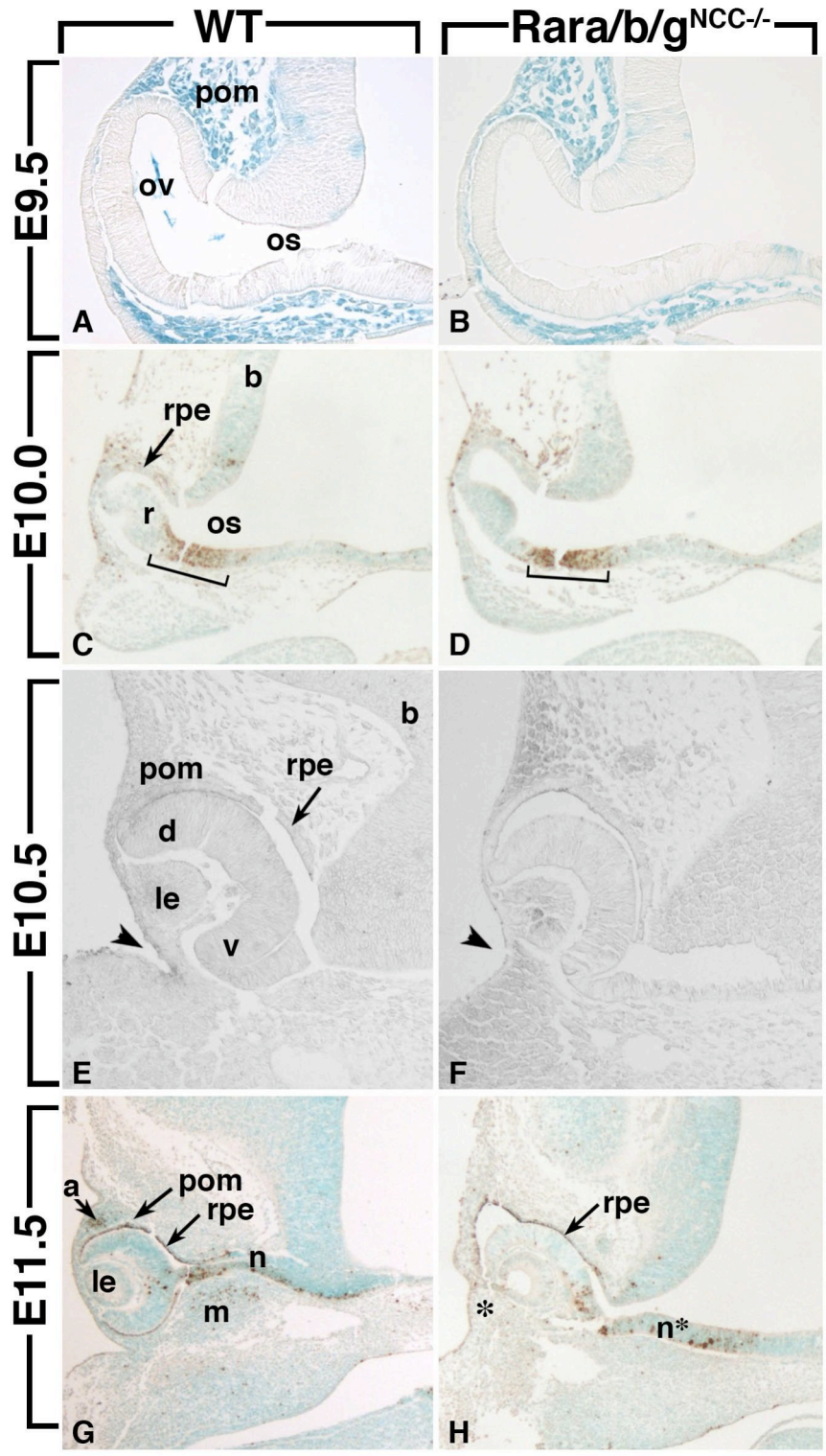
WT
Rara/b/g NCC-/-
E9.5
E10.0
E10.5
E11.5
pom
ov
os
A
B
b
rpe
r
os
C
D
b
pom
rpe
d
le
v
E
F
a
pom
rpe
n
le
m
G
rpe
*
n*
H

Figure 3

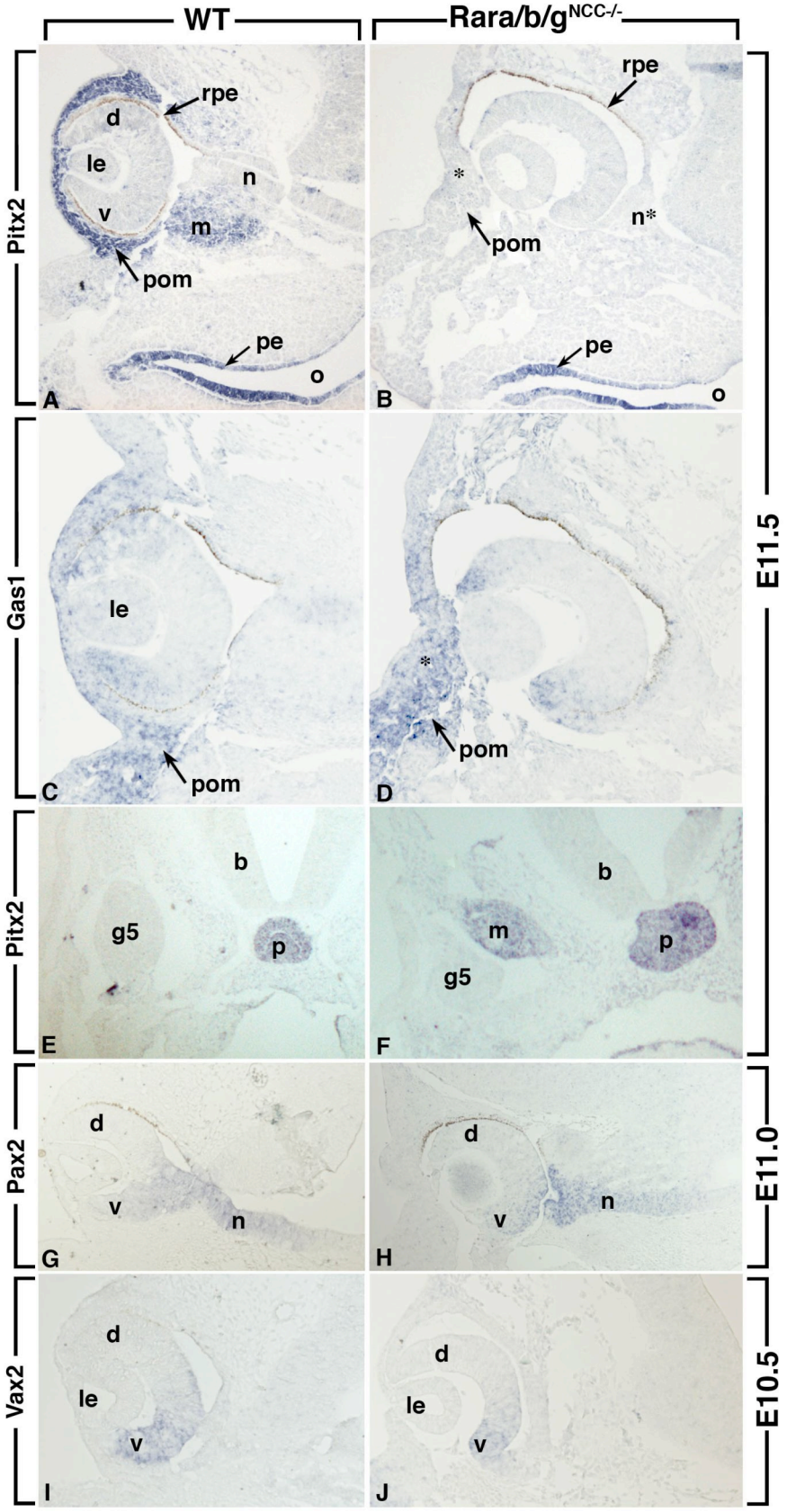
WT
Rara/b/g NCC-/-
Pitx2
Gas1
Pitx2
Pax2
Vax2
E11.5
E11.0
E10.5
rpe
d
le
n
v
m
pom
pe
o
A
B
n*
*
C
D
b
g5
p
E
F
G
H
I
J

Figure 4

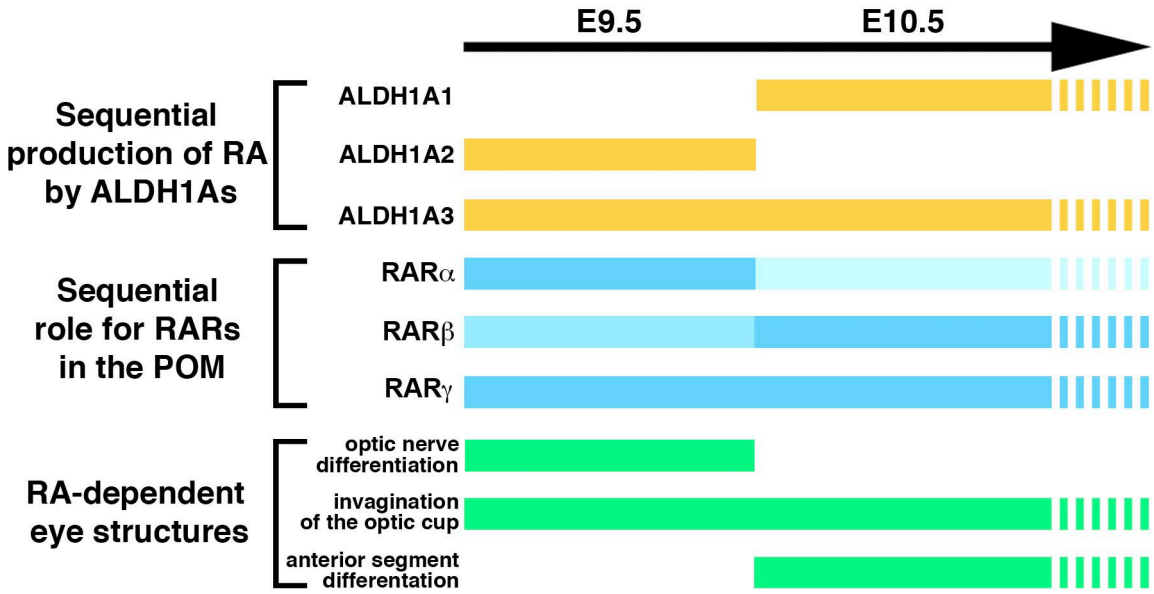
E9.5
E10.5
Sequential production of RA by ALDH1As
ALDH1A1
ALDH1A2
ALDH1A3
Sequential role for RARs in the POM
RARα
RARβ
RARγ
RA-dependent eye structures
optic nerve differentiation
invagination of the optic cup
anterior segment differentation

Figure 5