**Vax2 regulates retinoic acid distribution and cone opsin expression in the vertebrate eye**

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**SUMMARY**

Vax2 is an eye-specific homeobox gene, the inactivation of which in mouse leads to alterations in the establishment of a proper dorsoventral eye axis during embryonic development. To dissect the molecular pathways in which Vax2 is involved, we performed a transcriptome analysis of Vax2−/− mice throughout the main stages of eye development. We found that some of the enzymes involved in retinoic acid (RA) metabolism in the eye show significant variations of their expression levels in mutant mice. In particular, we detected an expansion of the expression domains of the RA-catabolizing enzymes Cyp26a1 and Cyp26c1, and a downregulation of the RA-synthesizing enzyme Raldh3. These changes determine a significant expansion of the RA-free zone towards the ventral part of the eye. At postnatal stages of eye development, Vax2 inactivation led to alterations of the regional expression of the cone photoreceptor genes Opn1sw (S-Opn) and Opn1mw (M-Opn), which were significantly rescued after RA administration. We confirmed the above described alterations of gene expression in the Oryzias latipes (medaka fish) model system using both Vax2 gain- and loss-of-function assays. Finally, a detailed morphological and functional analysis of the adult retina in mutant mice revealed that Vax2 is necessary for intraretinal pathfinding of retinal ganglion cells in mammals. These data demonstrate for the first time that Vax2 is both necessary and sufficient for the control of intraretinal RA metabolism, which in turn contributes to the appropriate expression of cone opsins in the vertebrate eye.

**KEY WORDS:** Vax2, Retinoic acid, Cone opsins, Mouse, Oryzias latipes (medaka)

**INTRODUCTION**

Vertebrate eye development is a complex phenomenon that requires a precise series of morphogenetic and differentiation events. A large number of signaling molecules and transcription factors that control key events underlying this process have been identified (Jean et al., 1998). Among them, Vax2 and Vax1 (Hallonet et al., 1999) belong to a subfamily of homeobox genes highly related to the *Drosophila empty spiracles* and to its vertebrate homologues, the Emx genes (Dalton et al., 1989; Simeone et al., 1992). In particular, Vax2 is specifically expressed in the ventral region of the prospective neural retina in vertebrates and it is required for ventral eye specification (Barbieri et al., 1999; Ohsaki et al., 1999; Schulte et al., 1999). Overexpression of Vax2 in *Xenopus* and of εVax in chick was found to determine a molecular ventralization of the developing eye (Barbieri et al., 1999) and a profound alteration of the retinotectal projection on the dorsal-ventral (DV) axis (Schulte et al., 1999). However, the genetic inactivation of Vax2 in mouse produces: (1) an incomplete closure of the choroidal fissure that results in an incompletely penetrant ocular coloboma (Barbieri et al., 2000); (2) incorrect establishment of the DV axis of the eye; and (3) alteration of ventral retinal ganglion cell (RGC) axonal projections to the superior colliculus (Barbieri et al., 2002; Mui et al., 2002). Altogether, these data point to a crucial role for Vax2 in regulating the proper development of the ventral region of the eye, although the molecular pathways underlying this process are still poorly understood.

Secreted molecules are also known play a key role in the development of the ventral eye, and among these is retinoic acid (RA), an active vitamin A derivative (Luo et al., 2006; Marsh-Armstrong et al., 1994; Matt et al., 2005; Molotkov et al., 2006; Sen et al., 2005; Wang and Montell, 2005). RA is asymmetrically distributed along the DV axis in the retina, suggesting that it may play a patterning role. The presence of a dorsal and of a ventral zone of RA activity in the retina, separated by an intermediate zone that is devoid of RA (RA-free zone), is conserved among vertebrate species such as zebrafish, chick and mice (Marsh-Armstrong et al., 1994; McCaffery et al., 1999; Mey et al., 1997). The two zones of RA activity are determined by the expression of two RA-synthesizing enzymes, *Raldh1* (*Aldh1a1* – Mouse Genome Informatics) in the dorsal retina (Hyatt et al., 1996; McCaffery et al., 1992) and *Raldh3* (*Aldh1a3* – Mouse Genome Informatics) in the ventral retina (Applebury et al., 2000; Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000). On the contrary, the mid-zone is free of RA owing to the expression of members of the Cyp26 family of RA-degrading enzymes, including Cyp26a1 and Cyp26c1 (Abu-Abed et al., 2002; Fujii et al., 1997; McCaffery et al., 1999; Ray et al., 1997; Reijntjes et al., 2003; Sakai et al., 2001). The molecular events leading to these specific expression domains, and hence to the asymmetric distribution of RA in the developing eye, are poorly understood. In vivo studies in chicken involving εVax...
overexpression suggest that \( Vax2 \) is able to influence \( Raldh1 \), \( Raldh3 \) and \( Cyp26a1 \) expression (Golz et al., 2008; Sen et al., 2005).

Here, based on studies performed both in mouse and in medaka, we report that \( Vax2 \) is necessary for the correct expression in the eye of the genes encoding the enzymes involved in RA and, consequently, for the proper local distribution of endogenous RA. We also found an alteration in the expression patterns of the cone opsin genes, which display regional expression domains in the vertebrate retina. We were able to obtain a significant rescue of the cone opsin expression alterations in newborn \( Vax2 \) knockout mice by RA administration to pregnant females at early stages of eye development. Finally, we carried out a detailed functional and morphological analysis of the retina of adult \( Vax2 \) mutant mice, which revealed a disorganization of the nerve fiber layer within the retina, in the region corresponding to the altered RA distribution. Our data indicate that \( Vax2 \) plays a key role in the establishment of a physiological asymmetric expression of genes not only at early stages of development but also at adult stages and in mature photoreceptors. We suggest that the misexpression of cone markers determined by \( Vax2 \) is mediated, at least in part, by the abnormal distribution of RA at early stages of eye development.

**MATERIALS AND METHODS**

**RNA preparation and microarray experiments**

Total RNA was extracted using TRIzol extraction kit (Gibco BRL) and subsequently used to prepare cRNA for hybridization to the Affymetrix Mouse 430A 2.0 array platform. For each developmental stage, we analyzed at least two biological duplicates, i.e. RNAs obtained from two different litters. Microarray hybridizations were performed at the Coriell Genotyping and Microarray Center (Coriell Institute for Medical Research, Camden, NJ, USA). Microarray results are available from the GEO database (Accession Number GSE19626). A false discovery rate (FDR) <0.1 was used to assess significant gene differential expressions.

Real-time reverse transcriptase (RT)-PCR experiments for the validation of microarray results were performed as previously described (Gennarino et al., 2009).

**RNA in situ hybridization and immunohistochemistry**

RNA in situ hybridization on mouse sections were performed according to previously published protocols (Bulfone et al., 2000; Conte et al., 2002). The mouse probes for \( Raldh1 \), \( Raldh3 \), \( Cyp26b1 \) and \( Cyp26c1 \) have been previously described (MacLean et al., 2001; Niederreither et al., 2002; Tahayato et al., 2003).

Whole-mount RNA in situ hybridization on medaka fish (\( Oryzias latipes \), \( ol \) ) embryos was performed, and the fish were photographed and sectioned as described previously (Conte and Bovolenta, 2007). In situ hybridization probes for the murine \( Cyp26a1 \), \( Opn1sw \) and \( Opn1mw \), and for the medaka \( ol\text{Opn}1s2w \), \( ol\text{Opn}1s1m \) and \( ol\text{Opn}1s1w1 \) genes were generated by reverse transcription (RT)-PCR on embryonic and/or adult eye cDNA using the oligonucleotide primers listed in Table S1 in the supplementary material.

For immunohistochemistry experiments, cryosections (10 \( \mu m \)) were blocked and hybridized with PBS containing 5% goat serum, 6% BSA and 0.3% Tween 20. A rabbit S-Opsin antibody (1:400, generous gift by Thierry Leveillard) and a secondary antibody AlexaFluor594 goat anti-rabbit (1:1000) were used. Slides were viewed on a Carl Zeiss AxioscplanA2 microscope.

**Luciferase assays**

A pcDNA3-\( Vax2 \) expression construct (100 ng) was co-transfected with the previously described \( Cyp26a1-2.6 \) kb (Loudet et al., 2005) (a generous gift from Martin Petkovich, Queen’s University, Kingston, ON, Canada), the \( Opn1sw \) (Srinivas et al., 2006) promoter, and the pRaldh3-1Tk-luc, pRaldh3-2Tk-luc and pRaldh3-3Tk-luc luciferase constructs (100 ng) into HeLa cells. The Raldh3-1, Raldh3-2 and Raldh3-3 are three DNA fragments containing putative \( Vax2 \)-binding sites (Mai et al., 2005), which were localized upstream the marine \( Raldh3 \) gene. As a control experiment, we also co-transfected a pcDNA3-Crx expression construct (100 ng) with the \( Opn1sw \) promoter construct (Srinivas et al., 2006). Luciferase activity was monitored by using the Dual Luciferase Reporter System Assay kit (Promega).

**Mouse crosses and \( \beta \)-galactosidase staining**

To evaluate RA distribution in \( Vax2 \) mutant mice we took advantage of a \( RARE\text{hsplacZ} \) (\( RARE\text{-lacZ} \)) transgenic mouse line (Rossant et al., 1991). \( Vax2^{\text{–/–}} \) mice were mated to \( RARE\text{-lacZ} \) mice to generate \( Vax2^{\text{–/–}} \) male mice carrying one copy of the \( RARE\text{-lacZ} \) transgene as assessed by PCR analysis using the oligonucleotide primers listed in Table S1 in the supplementary material.

\( \beta \)-Galactosidase staining of either whole-mount optic cups or cryostat-sections (18 \( \mu m \)) from RA-reporter mice were obtained using 5 mM K-Ferricyanide, 2 mM MgCl\(_2\), and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactopyranoside (X-gal) in 1 \( \times \) PBS buffer.

**Retinoic acid (RA) administration**

Aliquots from a stock solution of 0.1 M all-trans RA (Sigma) in dimethyl sulfoxide (DMSO) were emulsified in 0.5 ml tissue culture medium containing 10% fetal calf serum and injected intraperitoneally to pregnant mice. RA doses were adjusted to the animal body weight at a final 60 \( \mu \)g/ml. Administration of RA to medaka embryos was performed as described previously (Prabhudesai et al., 2005).

**Medaka stocks and mRNA injections**

The Cab-strain of wild type medaka fish were kept and staged as described (Conte and Bovolenta, 2007). In vitro synthesis of the medaka \( Vax2 \) mRNAs was performed as described (Conte et al., 2010). \( ol\text{Vax}2 \) mRNAs were injected at 50-200 ng/\( \mu l \), which induced a dose-dependent phenotype. The selected working concentration was 150 ng/\( \mu l \). Control embryos were injected with 15 ng/\( \mu l \) of eGFP mRNA. A morpholino (Mo; Gene Tools, OR, USA) was designed against the 5'UTR of \( ol\text{Vax}2 \) (Mo-Vax2), whereas a control Mo carrying five mismatches (mmMo-Vax2) was used as a control (see sequences in Table S1 in the supplementary material). The specificity and inhibitory efficiencies of Mo-Vax2 were determined as described previously (Conte et al., 2010). Mo-Vax2 was injected at 90 \( \mu \)M into one blastomere at the two-cell stage.

**Animal preparation for in vivo studies**

All experiments were performed in accordance with the regulations of the local authorities and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Electroretinography (ERG)**

ERGs were recorded as previously described (Seeliger et al., 2001) using a PC-based Ganzfeld system (Multiliner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). Mice were dark-adapted overnight and anaesthetized with ketamine/xylazine (66.7/11.7 mg/kg). Pupils were dilated and single-flash ERG recordings were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Single white-flash stimulation ranged from –4 to 1.5/–2 to 1.5 log cd*s/m\(^2\) under scotopic/photopic conditions (i.e. recorded following 10 minutes of light adaptation with 30 cd/m\(^2\)), divided into ten and eight steps, respectively. Ten responses were averaged with an inter-stimulus interval (ISI) of either five seconds or 17 seconds (for 0, 0.5, 1 and 1.5 log cd*s/m\(^2\)). Band-pass filter cut-off frequencies were 0.3 and 300 Hz. We also performed single white-flash ERGs at very dim intensities from –5.8 to –3.0 log cd*s/m\(^2\) in steps of 0.2 logarithmic units under scotopic conditions. For this recording, fifty responses were averaged with an ISI of two seconds. Band-pass filter cut-off frequencies were 0.3 and 70 Hz.

**Confocal scanning-laser ophthalmoscopy (cSLO)**

cSLO is a non-invasive technique providing retinal images comparable with a whole mount. cSLOs were obtained according to previously reported procedures (Seeliger et al., 2005). Briefly, cSLO imaging was performed with a commercial scanning-laser ophthalmoscope (HRA I,
Heidelberg Engineering, Germany). The HRA features two short argon wavelengths (488 nm/514 nm) and two infrared diode lasers (795 nm/830 nm). A setting of 20°/10° was used for a detailed view of confocal planes of the fundus.

Spectral domain optical coherence tomography (SD-OCT)

SD-OCT imaging was carried out in the same session as ERG and cSLO using the Spectralis device and the proprietary software package Eye Explorer version 3.2.1.0 from Heidelberg Engineering as described previously (Fischer et al., 2009). The optical depth resolution is about 7 μm, with digital resolution reaching 3.5 μm (Huber et al., 2009). Resulting data were exported as 24-bit color image files and processed in Corel Draw 11 graphics software.

RESULTS

Eye transcriptome analysis of Vax2 knockout mice

To gain insight into the molecular pathways regulated by the Vax2 gene, we carried out a transcriptome analysis on Vax2 homozygous mutant mice versus wild-type animals. The dynamic expression pattern of Vax2 suggests that it may play diverse roles at different stages of eye development (Barbieri et al., 1999). Therefore, we performed the transcriptome analysis at three different embryonic stages: E10.5, E12.5 and E16.5. We also analyzed two postnatal stages: postnatal day (P) 8 and the fully differentiated eye (P60). We hybridized Affymetrix chips (Mouse 430A 2.0 platform) comparing wild-type and Vax2–/– total RNA from whole eyes, except for the E10.5 stage in which we used RNA from the entire embryo head. As a result, we identified a set of transcripts that were upregulated or downregulated with a false discovery rate (FDR) value <0.1 in at least one of the developmental stages analyzed (see Table S2 in the supplementary material). Among these genes, we focused our attention on two specific subgroups, the first one including genes involved in RA metabolism and the second one including the cone photoreceptors markers Opn1sw and Opn1mw.

Vax2 inactivation leads to an altered expression of RA-metabolizing genes

Microarray analysis and subsequent validation assays by real-time RT-PCR (data not shown) revealed an upregulation and a downregulation in the expression levels of Cyp26a1 and Raldh3, respectively, in Vax2-null mice eyes. RNA in situ hybridization assays revealed an upregulation of Cyp26a1 in Vax2–/– developing eyes, from early embryonic to postnatal stages compared with wild-type mice (Fig. 1A-L). In Vax2–/– mice at early stages of eye development, from E10.5 to E14.5, Cyp26a1 displayed a significant expansion of its expression domain into the ventronasal part of the retina (Fig. 1C-H and data not shown). Later on, from E16.5 to P8 (Fig. 1K-L and data not shown), Cyp26a1 was markedly upregulated across the entire ventral retina, including both the nasal and the temporal quadrants. Consistent with previous data (Sakai et al., 2004), we did not detect any Cyp26a1 signal in...
the adult retina in either control or mutant mice. We extended our analysis to the other two members of the Cyp26 gene family, which were not represented on the chip platform used for microarray assays. As previously reported (Luo et al., 2006; Sakai et al., 2004), we detected Cyp26c1 expression in a few cells within the Cyp26a1 expression domain in the retina of E16.5 control animals (Fig. 1M,N), whereas, in the retina of Vax2−/− mice, this gene displayed a wider expression domain, which extended into the ventral retina (Fig. 1P-R). However, contrary to previous reports (Luo et al., 2006), we were able to detect Cyp26b1 expression in retinal ganglion cells in embryonic as well as in newborn retina of control mice but we detected a downregulation of its expression in Vax2-null retinas (Fig. 1S-T and data not shown).

We also analyzed, by RNA in situ hybridization, the expression of the genes encoding the RA-metabolizing enzymes Raldh1 and Raldh3. In agreement with the microarray data, our assays revealed no differences in Raldh3 expression between wild-type and Vax2 mutant mice at E10.5 (Fig. 2A,B). However, we found a strong downregulation of this gene at E12.5 in mutant mice at E10.5 (Fig. 2A,B). However, we found a strong downregulation of this gene at E12.5 in mutant mice at E10.5 (Fig. 1P-R). However, contrary to previous reports (Luo et al., 2006), we were able to detect Cyp26b1 expression in retinal ganglion cells in embryonic as well as in newborn retina of control mice but we detected a downregulation of its expression in Vax2-null retinas (Fig. 1S-T and data not shown).

Overall, our data indicate that Raldh3 was comparable with wild type at all stages analyzed (Fig. 2K-N and data not shown). Overall, our data indicate that Vax2 is involved in expression of RA-metabolizing enzymes during eye development in mouse. Finally, to verify whether Vax2 could regulate in a direct manner the expression of the above mentioned RA-metabolizing genes, we carried out in vitro transactivation assays by co-transfecting HeLa cells with a Vax2 expression construct and either a Cyp26a1 promoter-driven luciferase reporter (Loudig et al., 2005) or three luciferase constructs containing candidate cis-regulatory elements localized upstream the murine Raldh3 gene. However, we could not detect any significant change in luciferase activity (data not shown), which suggests that Vax2 does not seem to be able to directly control the expression of the Cyp26a1 and Raldh3 genes.

Vax2 inactivation leads to the ventral expansion of the RA-free zone in the developing retina

To assess whether the upregulation of the Cyp26a1 and Cyp26c1 genes, and the concomitant downregulation of Raldh3 led to an alteration of RA distribution in the retina of Vax2−/− mice, we took advantage of the RARE-lacZ transgenic line (Rossant et al., 1991). We generated RARE-lacZ; Vax2−/− transgenic mice and we used them to study the local distribution of RA in the developing eye, both by β-galactosidase staining and by RNA in situ hybridization using a lacZ probe (Fig. 3 and data not shown). In Vax2 mutant eyes, at E12.5, we observed a moderate expansion of the RA-free zone into the ventronasal retina (data not shown), whereas from E16.5 to P0, Vax2 mutant animals displayed a remarkable expansion of the RA-free zone in the ventral retina (Fig. 3A-H and data not shown), which was consistent with the above-described Cyp26a1 upregulation. We also performed a β-galactosidase staining coupled with a Cyp26a1 RNA in situ hybridization assay, which confirmed that the expansion of the RA-free zone in Vax2−/− mouse eyes largely corresponds to the expanded domain of Cyp26a1 expression (Fig. 3I-L). The expansion of the RA-free zone was detectable also in adult eyes (data not shown). In summary, these data indicate that the distribution of RA is altered in Vax2−/− eyes, thus confirming a role for this gene in ensuring a proper RA distribution in the developing eye.

Vax2 mutant mice display altered expression of cone photoreceptor genes

The cone opsin genes Opn1sw and Opn1mw normally display an asymmetric expression along the dorsal-ventral axis in the mouse retina (Applebury et al., 2000; Corbo et al., 2007). We observed, using microarray analysis, a downregulation of Opn1sw and an upregulation of Opn1mw in Vax2−/− mice. We validated all the above data using real time RT-PCR assays (data not shown). We then carried out RNA in situ hybridization experiments on the Opn1sw and Opn1mw transcripts. In Vax2 mutant eyes, Opn1sw displayed a notable reduction of its high ventral to low dorsal expression gradient in comparison with the developing eye of control mice (Fig. 4A-L). These results were also confirmed at the protein level using immunofluorescence assays (Fig. 5A-P). However, Opn1mw was upregulated in Vax2 adult eyes in comparison with wild-type eye (Fig. 4M-P). In particular, we detected a strong upregulation in the Vax2−/− ventral retina with a significant decrease in its normal high dorsal to low ventral gradient (Fig. 4N,P). A staining for peanut-agglutinin (PNA), which specifically labels cone photoreceptor cells, revealed no differences in the overall number of cone photoreceptors between

\[ \text{Fig. 2. Vax2 inactivation determines } \text{Raldh3 downregulation in the developing eye.} \]

(A-N) RNA in situ hybridization on frontal eye sections of wild-type and Vax2−/− mice, with the Raldh3 (A-J) and Raldh1 (K-N) probes. At E10.5, Raldh3 expression in Vax2−/− mice (B) is comparable with that in wild-type mice (A), whereas it is clearly downregulated both at E12.5 (E,F) and E16.5 (I,J) compared with wild-type animals (C,D,G,H). Raldh1 expression does not display any difference between wild-type (K,L) and Vax2−/− mice (M,N). C,E,G,I,K,M are representative of anterior (nasal) retinal sections; D,F,H,J,L,N are representative of more posterior (temporal) retinal sections. Mouse genotypes and developmental stages are indicated. le, lens; re, retina; on, optic nerve; D→V, dorsal versus ventral retina. Scale bars: 100 μm in A-F; 200 μm in G-N.
is expressed in the dorsal retina (Fig. 6J). Injections of Mo-Vax2 closest homolog of the murine promoter (data not shown). These data indicate that Vax2 does not reported (Srinivas et al., 2006) to be an activator of the construct expressing the enzymes, we tested in vitro a possible direct effect of Vax2 on the promoter of the opsin genes in the mouse retina.

Similar to what described is above for RA-metabolizing enzymes, we tested in vitro a possible direct effect of Vax2 on the promoter of the Opn1lw gene. We could not find any significant change in luciferase activity when a Vax2 expression construct was co-transfected with an Opn1sw promoter-driven luciferase construct (Srinivas et al., 2006) in HeLa cells (data not shown). By contrast, we observed a significant increase in luciferase activity when we co-transfected the Opn1sw promoter construct with a construct expressing the Crx gene, which has previously been reported (Srinivas et al., 2006) to be an activator of the Opn1sw promoter (data not shown). These data indicate that Vax2 does not seem to be able to control Opn1sw expression directly by interacting with its promoter.

Vax2 loss- and gain-of-function lead to RA-metabolizing enzyme and cone opsin gene expression alterations in the medaka fish retina

To further validate the ability of Vax2 to control RA-metabolizing enzymes and cone opsin expression in the retina, we carried out Vax2 gain- and loss-of-function studies in the medaka fish (Oryzias latipes, ol) model system. We evaluated the consequences of olVax2 misexpression by analyzing the expression patterns of olCyp26a1, olRaldh2, olOpn1mw and olOpn1sws1 in the medaka retina. The olCyp26a1 and the olOpn1mw retinal expression domains are very similar to those observed in mouse (Fig. 6A,G). However, the olRaldh2, the only medaka ortholog of the mammalian Raldh subfamily of genes, shows two distinct domains of expression in the developing medaka retina, one in the dorsal region and one in the ventral region (Fig. 6D), thus combining the expression domains of the Raldh1 and Raldh3 mouse genes (Canestro et al., 2009). Finally, the medaka olOpn1sws1 gene, the closest homolog of the murine Opn1sw (Matsumoto et al., 2006), is expressed in the dorsal retina (Fig. 6I). Injections of Mo-Vax2 morpholinos caused upregulation of olCyp26a1 and downregulation of the ventral expression domain of olRaldh2 in the medaka retina at stage 24 (Fig. 6C,F). Moreover, we found that in Mo-Vax2-morphants, at stage 40, olOpn1sws1 expression was reduced while olOpn1mw was ventrally expanded (Fig. 6G,I,J,L). Overall, the above-described effects (present in 60±5% of Mo-Vax2-injected embryos; n=1800) were concordant with those observed in Vax2–/– mice. Conversely, after injections of the olVax2 mRNA (at a dose of 150 ng/μl), we observed, at stage 24, an expansion of olRaldh2 expression in the retina, particularly in its ventral domain (Fig. 6D,F) and a concomitant reduction of the olCyp26a1 expression domain in most of the central retinal neuroepithelium (Fig. 6A,B). Furthermore, Vax2 overexpression led, at stage 40, to a reduction of the olOpn1mw expression domain, which was restricted to the dorsalmost retinal region (Fig. 6H). Finally, olVax2 overexpression was also accompanied by a modest reduction of the olOpn1sws1 expression domain (Fig. 6K). The above-described effects of olVax2 overexpression were observed in 85% of the injected embryos (n=2000). Overall, these data indicate that Vax2 is both necessary and sufficient to control the proper expression of RA-metabolizing enzymes and cone opsin genes in the vertebrate eye.

Notably, we also found that exposure of medaka embryos to exogenous RA induced cone opsin expression defects similar to those observed in olVax2-overexpressing embryos (see Fig. S1 in the supplementary material), suggesting the possibility that the control exerted by Vax2 on cone opsin expression could be mediated, at least in part, by the correct distribution of RA in the retina.

Rescue of the Opn1sw defect with RA supplementation

RA has previously been shown to be involved both in rod and cone differentiation, as well as in cone opsin expression (Fujieda et al., 2009; Hyatt and Dowling, 1997; Hyatt et al., 1996; Osakada et al., 2008; Prabhudesai et al., 2005; Roberts et al., 2006). In order to understand whether the cone opsin misexpression observed in Vax2 mutant mice is indeed linked to the altered distribution of RA in the developing eye, we
administered exogenous RA (60 mg/kg) by intraperitoneal injections to Vax2–/– pregnant females. Cone precursor commitment occurs at early stages of eye development (Cepko et al., 1996) before opsin gene onset of expression. In mice, Opn1sw expression starts at late embryonic stages (Applebury et al., 2007; Ng et al., 2001), whereas Opn1mw expression is induced during the second postnatal week (Fei, 2003). We carried out single RA injections at either E10.5 or E12.5, and double RA injections (at E12.5 and E16.5) and sacrificed the pups at birth (n=39 from six different litters). By analyzing the expression of Opn1sw, we observed a significant increase of Opn1sw expression in about half of the Vax2–/– injected mice (18 out of 39) (Fig. 7C,F) compared with Vax2–/– non-injected mice (Fig. 7B,E). Interestingly, we observed rescue of the Opn1sw expression in all the newborn mice (n=8) whose mother underwent double RA injection. These data suggest that RA may indeed mediate the effect of Vax2 on cone opsin expression regulation in the mouse eye.

Vax2 mutant mice display a disorganization of nerve fiber bundles in the retina

The cone opsin expression alterations observed as a consequence of Vax2 inactivation prompted us to perform a detailed functional and morphological analysis of the adult retina in Vax2 mutant mice.
To achieve this, we carried out ERG to assess retinal function, as well as cSLO and OCT analyses, which allow to obtain a detailed in vivo retinal fundus imaging, in Vax2 mutant mice. Both cSLO and OCT revealed a striking difference of the nerve fiber layer of Vax2–/– mice when compared with wild-type mice. cSLO with the 514 nm short wavelength laser allows the specific imaging of the retinal nerve fiber layer (Fig. 8A-D). In wild-type mice, the nerve fibers converge evenly distributed from all parts of the retina toward the optic disc (Fig. 8A), whereas in Vax2 mutant eyes this regular nerve bundle distribution is disrupted (Fig. 8B-D). In the temporal retina, irregular bundle-free regions were detected whereas, in the opposite direction, in the nasal-ventral zone of the retina, overly aggregated bundles are present (Fig. 8D). In the dorsal part of the retina, however, the nerve fiber bundles seem to be regularly distributed. These patterns of irregular distributed nerve fiber bundles were observed in all Vax2 mutant animals (n=6) but in none of the controls (Fig. 8D). OCT scans (Fig. 8E-H) revealed the sites of aggregated fiber bundles as a distinct local thickening of the nerve fiber layer. All the other retinal layers down to the RPE and choroid appear to be unaffected by Vax2 inactivation in OCT imaging.

To assess alterations in retinal neuronal function of Vax2–/– mice, flash ERGs were recorded from wild-type and Vax2–/– mice under scotopic and photopic conditions (see Fig. S2 in the supplementary material). At the higher intensities used, there was a mild tendency towards shortened and possibly reduced cone system responses in Vax2–/– mice versus wild-type mice, although these changes did not reach the threshold of statistical significance. Except for the latter, retinal function was normal in Vax2–/– mice under both scotopic and photopic conditions (see Fig. S2A-C in the supplementary material), indicating that the altered expression of cone photoreceptor genes described above presumably alters local spectral sensitivity but not overall functionality.
Based on the defective nerve fiber bundle pattern in Vax2–/– mice, we further checked for possible functional consequences of Vax2 deficiency on ganglion cell function. For this purpose, we further analyzed ERG components, which are thought to derive from third order retinal cells (Saszik et al., 2002), e.g. ganglion cells, using very dim light stimuli (see Fig. S2D in the supplementary material). No remarkable difference could be observed between wild-type and Vax2–/– mice in these measurements, indicating that: (1) ganglion cell function itself is not affected; and that (2) the disorganization of nerve fiber bundles observed in the in vivo morphological examinations either did not cause functional deficits or that the latter may not be detectable using this assay.

DISCUSSION

In this report, we have demonstrated, for the first time, the existence of a specific relationship between the control of RA metabolism in the mammalian eye and the Vax2 gene. In particular, we found that Vax2 inactivation in mouse leads to an altered expression of all members of the Cyp26 gene family, which are responsible for RA catabolism, and to a significant downregulation of Raldh3, which is implicated in RA synthesis. These variations lead to a notable expansion of the RA-free zone into the ventral retina of Vax2 mutant mice (Fig. 3). Our data clearly demonstrate that Vax2 is both necessary and sufficient to control RA metabolism in the developing eye in vertebrates.

We also sought to determine whether or not Vax2 might directly control the expression of the Cyp26a and of the Raldh subfamily of genes in the retina. To achieve this, we tested in vitro the effect of Vax2 on the activity of both the well-characterized Cyp26a1 promoter (Loudig et al., 2005) and three putative cis-regulatory elements localized upstream the murine Raldh3 gene. However, we did not find any significant effect of Vax2 transfections, suggesting that this protein may exert an indirect transcriptional control on the retinal expression of RA-metabolizing enzymes. Obviously, we cannot exclude the possibility that Vax2 may interact with additional, yet unidentified, cis-regulatory elements controlling the expression of the above genes.

The functional role of RA in eye development, as well as its peculiar topographic distribution within this tissue, is still poorly understood. Two distinct phases of RA signaling during murine eye development have been recognized, an early phase (E9.5-E10.5, optic vesicle stage) for optic cup formation and a late phase (E10.5-E13.5) for the development of anterior structures. In particular, the lack of both the Raldh1 and Raldh3 enzymes, which leads to a complete absence of RA in the optic cup determines a mesenchymal overgrowth in the cornea and eyelids (Duester, 2008; Matt et al., 2005; Molotkov et al., 2006). The latter alterations are accompanied by a marked downregulation of Pitx2, a potential target of ocular RA signaling, in the periocular mesenchyme (POM) (Duester, 2008; Matt et al., 2005). We believe that the changes of RA distribution observed in the eye of Vax2–/– mice do not cause significant alterations of RA signaling in the POM of mutant mice.

Fig. 8. Vax2–/– mouse eyes display a disorganization of optic nerve fiber bundles throughout the expanded RA-free zone in the ventral retina. (A-H) In vivo imaging of mouse eyes using confocal scanning-laser ophthalmoscopy (A-D) at 514 nm (whole-mount-like surface view) and spectral domain optical coherence tomography (E-H) for histology-analogue sections. The SLO examination in Vax2–/– animals reveals a disruption of the regular nerve fiber bundle distribution (A versus B; detail in C). The arrows indicate areas of overly aggregated bundles, whereas the arrowheads indicate irregular bundle-free regions. Topographically, the changes were not random, as shown in three different knockout animals (D). Major bundle aggregates were always present at 6-7 and 9-10 o’clock (arrows). No or only minor changes were found in the dorsal part of the retina (color-marked areas in D), which coincides with the presence of RA during development. (E-H) The SD-OCT scans reveal the extent of nerve fiber layer thickening at the sites of aggregate formation (E versus F; detail in G versus H). The arrows indicate sites of massively increased NFL thickness (black layer). wt, wild type; KO, Vax2–/–; D, dorsal; V, ventral; N, nasal; T, temporal.
This assumption is based on both the absence of any suggestive phenotypic abnormalities that affect the anterior eye chamber and the lack of any significant change in Pitx2 expression in the POM of Vax2-null mice (data not shown).

\textit{cVax} overexpression has previously been shown to influence the proper topographic distribution of rod photoreceptors in the chicken retina (Schulte et al., 2005). We found that \textit{Vax2} is required for the establishment of the physiological asymmetric expression of cone marker genes in the vertebrate retina (Figs 4-6). The control of the proper expression of cone-specific genes has been the subject of a number of studies (Deeb et al., 2006; Fujieda et al., 2009; Li et al., 2008; Liu et al., 2008; Ng et al., 2001; Oh et al., 2007; Raine and Hawryshyn, 2009; Roberts et al., 2005; Srinivas et al., 2006; Veldhoen et al., 2006), although the precise molecular mechanisms underlying this process have not been completely elucidated. However, there is evidence to suggest that RA signaling plays an important role in cone photoreceptor development. For example, the retinoid X receptor $\gamma$ (RXR$\gamma$) and the thyroid hormone receptor $\beta$2 (Thrb) act in concert to repress the expression of the protein product of the \textit{Opn1sw} gene (S-Opsin) in the dorsal retina. ROR$\beta$, a member of the retinoic acid receptor-related orphan receptor (ROR) family, was shown to act as a transcriptional activator of S-opsin in concert with the transcription factor Gcn4 (Srinivas et al., 2006). ROR$\alpha$, another member of the ROR family, was reported to play a crucial role in cone development by regulating \textit{Opn1sw}, \textit{Opn1mw} and \textit{Arr3} expression, and ROR$\alpha$ mutant mice display reduced transcript levels of these genes. Finally, it has been shown that RA treatment in zebrafish embryos was able to influence opsin transcription in photoreceptors (Prabhudesai et al., 2005).

Based on both the above considerations and on the data described in this report, i.e. the evidence of an altered RA distribution in the eye of \textit{Vax2} null mice and the similarity between the effects of \textit{Vax2} overexpression and RA administration in medaka embryos (see Fig. S1 in the supplementary material), we hypothesized that the altered expression of cone markers in our mutant mice could be, at least in part, due to the alteration in the RA distribution affecting the ventral developing retina. This hypothesis was further supported by the results of RA intraperitoneal administration experiments, at early embryonic stages, in \textit{Vax2} mutant mice. We found a significant \textit{Opn1sw} upregulation in about half of \textit{Vax2}-injected null mice (Fig. 7C,F) in comparison with non-injected mutant mice (Fig. 7B,E). The fact that not all RA-injected embryos displayed a rescue of \textit{Opn1sw} expression can be to some extent explained by the high variability related to peritoneal injections, which makes it difficult to predict the amount of RA that effectively reaches the retina. The evidence that 100% of the pups whose mother underwent a double RA injection showed a rescue supports this assumption. However, there are alternative possibilities, and, in particular, (1) a direct transcriptional control of \textit{Vax2} on cone opsin gene expression or (2) a contribution of other gene and pathways to the \textit{Vax2}-mediated control of opsin expression. We consider the first possibility less likely as we did not detect any significant effect of \textit{Vax2} transfection on the action of the previously characterized \textit{Opn1sw} promoter (Srinivas et al., 2006), although we cannot exclude the possibility that \textit{Vax2} may interact with additional, yet unidentified, expression control elements. On the contrary, the second possibility cannot be ruled out. To explore it further, we analyzed, by both microarray and real time RT-PCR the expression levels of the \textit{Thrb} gene, previously shown to be required for proper cone opsin expression and cone photoreceptor development (Ng et al., 2001), in the retina of \textit{Vax2} mutant mice but we did not find any significant variations versus normal controls (data not shown). Nevertheless, we cannot exclude the role of other genes and pathways in the modulation of opsin gene expression in which \textit{Vax2} seems to play a basic role. In any case, our data, both those derived from the analysis of the medaka \textit{Vax2} misexpression models and those obtained after RA administration to \textit{Vax2} mutant mice, indicate that RA signaling may play an important, although non exclusive, role in mediating the function exerted by \textit{Vax2} in controlling cone opsin expression in the vertebrate eye.

We previously reported the presence of a non completely penetrant eye coloboma as one of the major phenotypic hallmarks of \textit{Vax2} mutant mice during development (Barbieri et al., 2002). We also confirmed the presence of this anomaly in this study, even though to a slightly smaller extent (50% of analyzed mice) (data not shown). However, the impact of \textit{Vax2} on the correct regionalization of gene expression in cones prompted us to carry out a detailed morphological and functional analysis of the mature eye in \textit{Vax2} mutant mice, which was not previously reported. We did not find significant aberrations of photoreceptor function (neither involving rods nor cones) by means of ERG analysis, which suggest that the functional consequences of cone opsin expression changes in \textit{Vax2} mutant mice lead to alterations in local spectral sensitivity rather than to overall cone functionality, at least within the range detectable by ERG. However, cSLO and OCT analyses revealed a profound disorganization of the nerve fiber layer organization in the retina of knockout mice (Fig. 8). These data indicate that \textit{Vax2} is necessary for the correct ganglion cell axon intraretinal pathfinding in the mammalian eye (but not ganglion cell functionality itself) and are consistent with previous findings obtained after \textit{cVax} overexpression in chicken (Muhleisen et al., 2006). Strikingly, the retinal areas most severely characterized by irregular distribution of nerve fibers seem to coincide with the expanded RA-free zone observed in the retina of \textit{Vax2} mutant eyes (Fig. 3), which may also underline a specific role for RA in the migration of retinal ganglion cells during eye development.

Altogether, our study contributes to shed light on the role of \textit{Vax2} in eye development in vertebrates. \textit{Vax2} is confirmed to play a crucial role not only in the development of the ventral retina and in the correct establishment of the DV axis at early stages of eye development (Barbieri et al., 2002) but its action also extends to the mature retina and, in particular, to ensure appropriate gene expression in cone photoreceptor cells and to ensure a correct ganglion cell intraretinal axonal pathfinding. Therefore, \textit{Vax2} may play a role in the generation of central structures such as the visual streak or the macula, which are present in many mammalian species. Finally, we hypothesize that \textit{Vax2} may exert part of its actions through regulation of RA metabolism within the eye.

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\textbf{Competing interests statement}

The authors declare no competing financial interests.
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