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DEVELOPMENT AND DISEASE

Vax2 inactivation in mouse determines alteration of the eye dorsal-ventral axis, misrouting of the optic fibres and eye coloboma

Anna Maria Barbieri[1,*], Vania Broccoli[1,†], Paola Bovolenta[2], Giovanna Alfano[1], Anna Marchitiello[1], Cristina Mocchetti[1], Luca Crippa[3], Alessandro Bulfone[1,*], Valeria Marigo[1], Andrea Ballabio[1,4] and Sandro Banfi[1,‡]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Via Pietro Castellino 111, Naples, Italy
[2]Departamento de Neurobiologia del Desarrollo, Instituto Cajal, CSIC, Madrid, Spain
[3]Novuspharma, Monza, Italy
[4]Istituto di Patologia Generale ed Oncologia, Facolta' di Medicina e Chirurgia, Seconda Universita degli Studi di Napoli, Naples, Italy

*Present address: Molecular Histology Unit, Department of Biological and Technological Research (DIBIT), San Raffaele Biomedical Science Park, Milan, Italy
†Present address: Stem Cell Research Institute (SCRI), Department of Biological and Technological Research (DIBIT), San Raffaele Biomedical Science Park, Milan, Italy
‡Author for correspondence (e-mail: banfi@tigem.it)

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SUMMARY

***Vax2* is a homeobox gene whose expression is confined to the ventral region of the prospective neural retina. Overexpression of this gene at early stages of development in *Xenopus* and in chicken embryos determines a ventralisation of the retina, thus suggesting its role in the molecular pathway that underlies eye development. We describe the generation and characterisation of a mouse with a targeted null mutation of the *Vax2* gene. *Vax2* homozygous mutant mice display incomplete closure of the optic fissure that leads to eye coloboma. This phenotype is not fully penetrant, suggesting that additional factors contribute to its generation. *Vax2* inactivation determines dorsalisation of the expression of mid-late (*Ephb2* and *Efnb2*) but not early (*Pax2* and *Tbx5*) markers of dorsal-ventral polarity in the developing retina. Finally, *Vax2* mutant mice exhibit abnormal projections of ventral retinal ganglion cells. In particular, we observed the almost complete absence of ipsilaterally projecting retinal ganglion cells axons in the optic chiasm and alteration of the retinocollicular projections. All these findings indicate that *Vax2* is required for the proper closure of the optic fissure, for the establishment of a physiological asymmetry on the dorsal-ventral axis of the eye and for the formation of appropriate retinocollicular connections.**

Key words: Eye development, Coloboma, Optic fibres, Homeobox, Mouse

INTRODUCTION

Eye development is a multistep process requiring a series of specific inductive signals and precise morphogenetic movements. An initial phase of formation of the main eye structures (the lens, the neural retina and the optic nerve) is followed by a differentiation step by which these structures acquire their mature forms. A third phase is represented by the formation of the appropriate connections between the retina and the higher neural centres involved in the visual pathways, such as the superior colliculus and the geniculate nucleus.

One of the key events in eye development is represented by the establishment of asymmetries, both in terms of gene expression and of cell type distribution (Flanagan and Vanderhaeghen, 1998; Huh et al., 1999; Szel et al., 1996), along the anteroposterior (AP) and the dorsoventral (DV) axes of the neural retina. The main outcomes of such asymmetries are the appropriate outgrowth of retinal ganglion cells (RGC) along the visual pathway and the establishment of their correct connections with their neuronal targets located in the optic tectum (the superior colliculus in mammals). The projections of the RGC onto the neurones of the superior colliculus are spatially ordered, determining precise topographic maps. The formation of such maps relies on the acquisition by retinal cells of positional identity, which, in turn, dictates both the behaviour of the corresponding axons within the chiasm and their projections to the appropriate targets in the superior colliculus. According to Sperry's chemoaffinity theory (Sperry, 1963), these positional identity values may be conferred by the graded distribution of surface molecules in the RGC, the optic chiasm and the superior colliculus. Recently, the Eph family of receptor protein-tyrosine kinases, and their ephrin ligands have been recognised as some of the surface markers involved in

these processes (reviewed by Wilkinson, 2001; Flanagan and Vanderhaeghen, 1998).

The mechanisms leading to the establishment of both the AP and the DV asymmetries in the developing eye and consequently to the graded expression of surface molecules involved in axonal projections are likely to be controlled by transcription factor genes. This assumption has already been confirmed in the case of the winged helix *Bf1* (*Foxg1* – Mouse Genome Informatics) and *Bf2* (*Foxd1* – Mouse Genome Informatics) transcription factors, which display complementary patterns of expression on the AP axis in the retinas of chicken and mouse, with *Bf1* found in the nasal retina and *Bf2* in the temporal retina (Hatini et al., 1994). Ectopic expression of *Bf1* and *Bf2* in temporal and nasal retinas, respectively, leads to a disturbance of the retinotectal projection on the AP axis (Yuasa et al., 1996).

Positional control of the DV axis of the eye seems to require the activity of two recently identified transcription factors, the T-box5 (*Tbx5*) and the ventral anterior homeobox 2 (*Vax2*) genes. *Tbx5*, a member of the T-box transcription factor family, is expressed in the dorsal part of the developing eye in mouse and chicken (Gibson-Brown et al., 1998a; Gibson-Brown et al., 1998b). Its misexpression in chicken induced dorsalisation of the ventral region of the eye and altered projections of RGC axons to the tectum (Koshiba-Takeuchi et al., 2000). *Vax2*, together with *Vax1* (Hallonet et al., 1998), belongs to a novel subfamily of homeobox genes, highly related to the *Drosophila empty spiracles* and to its vertebrate homologous Emx genes (Dalton et al., 1989; Simeone et al., 1992). We identified this gene in the course of a systematic project aimed at the isolation of novel mammalian genes highly conserved during evolution (Banfi et al., 1997; Banfi et al., 1996). *Vax2* has a remarkable expression domain that is confined to the ventral portion of the prospective neural retina in mouse, human, chicken and *Xenopus laevis* (Barbieri et al., 1999; Ohsaki et al., 1999; Schulte et al., 1999). We found that overexpression of both the human *VAX2* and the corresponding *Xenopus* gene in frog embryos led to an aberrant eye phenotype and, in particular, determined a molecular ventralisation of the developing eye (Barbieri et al., 1999). Similarly, misexpression in chick of both the murine *Vax2* and of its chicken counterpart (*Vax*) was found to determine a ventralisation of the developing retina and a profound alteration of the retinotectal projection on the DV axis (Schulte et al., 1999).

To determine the role of *Vax2* in mammalian eye development, we generated a loss-of-function mutation of *Vax2* in mouse. The characterisation of this mutation confirmed that *Vax2* plays a role in eye development as revealed by the presence of a developmental malformation, such as eye coloboma, in *Vax2* knockout mice. Furthermore, *Vax2* mutant mice also showed anomalies of the establishment of the correct DV axis of the eye, as revealed by the alteration of the expression domains of transcripts asymmetrically expressed along the DV axis (*Efnb2* and *Ephb2*) and by the alteration of the ventral RGC axon pathway.

MATERIALS AND METHODS

Gene targeting

Two genomic clones spanning the last two coding exons of the *Vax2* gene were obtained by screening a λ-DASH II mouse (129Ssv strain) genomic DNA library, a generous gift from Andras Nagy (Mount Sinai Hospital, Toronto, Canada) using a mouse *Vax2* cDNA as a probe. Eight and a half kilobases (kb) of sequence upstream of exon 2 and 2.2 kb of sequence downstream of exon 2 were cloned into the pPNT plasmid (Tybulewicz et al., 1991), containing both a neomycin resistance gene and a herpes simplex virus thymidine kinase (hsv-tk) gene, which permitted selection against random integration.

Linearisation of the vector was performed using a *Sal*I unique site. Electroporation in ES cells was as previously described (Soriano et al., 1991). *Vax2* mutant cells derived from two homologously recombined clones were used to produce chimaeric animals by both injection and aggregation techniques (Joyner, 1993; Wood et al., 1993). Chimaeras were then mated to CD1 and C57/Bl6 mice for germline transmission. Genotypes of ES cells and mouse tails were analysed by Southern blotting after *Hin*dIII and *Bam*HI digestions. Both the 5′ and 3′ probes were located outside the flanking regions used in the targeting vector and consisted, respectively, of a 1000 bp *Eco*RI fragment and a 440 bp PCR fragment in exon 3 of *Vax2*. The PCR fragment used as 3′ probe was generated using oligonucleotide primers TO-8371 (5′- GAGCAGAGACCTGGAGAAG-3′) and TO-8372 (5′- GTAGTGCTCTGCTGGGGAC-3′).

Histology and in situ hybridisation

Age-matched mouse embryos and tissues were dissected, fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin wax for microtome sectioning. Haematoxylin and Eosin was used for staining. Eyes from 4% paraformaldehyde perfused postnatal mice were carefully dissected, dehydrated and included in Tecnovit 7100 (Kulzer Histo-Technik, Hearaeus Kulzer, Wehrheim, GmbH). Sections, cut by a glass knife (1 μm) were then stained with Haematoxylin and Eosin.

In situ hybridisation was performed as described (Rugarli et al., 1993) with antisense RNA probes transcribed from plasmids containing fragments of *Vax2* (Barbieri et al., 1999), *Pax2* (Torres et al., 1996), *Tbx5* (Chapman et al., 1996), *Ephb2* (Birgbauer et al., 2000), *Efnb2* (ephrin B2) (Birgbauer et al., 2000), *Vax1* (Hallonet et al., 1998), *Epha5* (BMAP clone UI-M-BH1-anm-a-08-0-UI, Research Genetics) and *Efna5* (ephrin-A5) (PCR product).

DiI labelling

Wild type and *Vax2*$^{-/-}$ postnatal animals (129/C57Bl6 strain) were deeply anaesthetised by an injection of sodium pentobarbital and perfused transcardially with phosphate-buffered saline (PBS) followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were carefully removed with their eyes attached. Glass micropipettes with tip diameters of 5-10 μm were used to deposit crystals of the lipophylic dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyllindocarbocyanine perchlorate, Molecular Probes), previously dissolved in DMSO (2.5 mg/ml). Crystals were deposited either in the ventral retina or in the optic nerve head of the right eye after removal of the lens. The tissue was then stored in PBS containing 0.5% paraformaldehyde in the dark at 37ºC for 5-7 days. In the case of adult animals DiI application was performed in alive animals anaesthetised by an injection of sodium pentobarbital. After 5-7 days of survival, animals were anaesthetised and perfused transcardially, as described above. Brains were then carefully removed and stored in PBS. The animals analysed after DiI injections were: four wild type, three heterozygous mutant (Vax2$^{+/-}$) and four homozygous mutant (Vax2$^{-/-}$) adult mice; and eight wild type and five Vax2$^{-/-}$ pups of 1 day of age. In both adult and postnatal animals, transport of the dye was observed and photographed in the intact brains using a stereomicroscope equipped with epifluorescence optics (Leica). Brains were then embedded in agar-gelatine solution and sectioned in the frontal plane using a vibratome (Leica) to analyse projections in the superior colliculus. The preparations were mounted on a glass slide, and viewed and photographed using a Nikon microscope.

RESULTS

Generation of *Vax2* mutant mice

To investigate the function of *Vax2*, we generated a targeted mutation of *Vax2* by homologous recombination using embryonic stem (ES) cell technology. A 2 kb *Bam*HI fragment containing the second coding exon of the murine *Vax2* gene was replaced with a PGK-*neo* cassette (Fig. 1A). This exon encodes the first two helices and part of the third helix of the Vax2 homeodomain, which are essential for the function of all known homeodomain transcription factors. Southern blot analysis was used to detect the appropriate targeting events (Fig. 1B). Mice heterozygous for the *Vax2* mutation ($Vax2^{+/-}$) were obtained from two independently mutated ES clones and grown in the mixed genetic backgrounds 129/CD1 and 129/C57Bl6; they were fertile, viable and indistinguishable from their wild-type (WT) littermates. $Vax2^{+/-}$ mice were intercrossed to obtain homozygous *Vax2* mutant ($Vax2^{-/-}$) mice. Genotype analysis of both embryos and adult mice obtained from these heterozygous matings showed no deviation from the expected Mendelian ratio. RT-PCR (Fig. 1C) and RNA in situ hybridisation experiments showed that residual *Vax2* mRNA was present in $Vax2^{-/-}$. However, sequence analysis of *Vax2* RT-PCR products indicated that the second coding exon of *Vax2* was not present in knockout mice and that the transcript detected was the result of an aberrant splicing between exon 1 and exon 3 (Fig. 1C). This splicing event leads to a shift in the *Vax2* open reading frame that prevents the inclusion of the remaining part of the homeodomain in the hypothetical aberrant protein produced. Based on this evidence, we conclude that the *Vax2* mutant allele described in this paper is very likely to represent a null allele.

Phenotypic analysis of $Vax2^{-/-}$ mice

$Vax2^{-/-}$ mice are viable, fertile and have normal life span and somatic development. As *Vax2* is expressed almost exclusively in the ventral part of the developing eye, we focused our attention to the morphological examination of this organ. Two main morphogenetic events take place in the ventral part of the developing eye, namely the beginning of the invagination of the optic vesicle and the formation and subsequent closure of the optic fissure, the latter event occurring by E12.5. While the invagination of the optic vesicle was found to be indistinguishable in wild-type and mutant mice, a significant percentage of $Vax2^{-/-}$ mice examined at several stages of development (from E12.5 to postnatal stages) showed coloboma, caused by a non-closure of the optic fissure (Fig. 2). This phenotype had different degrees of severity and was observed both bilaterally and unilaterally in both the genetic backgrounds of $Vax2^{-/-}$ mice but with a higher penetrance in 129/CD1 $Vax2^{-/-}$ mice (Table 1). In particular, the most remarkable difference between the two genetic backgrounds of $Vax2^{-/-}$ mice concerned the frequency of bilateral coloboma, observed in 46% of 129/CD1 while in 129/C57Bl6 was detected in only 17% of cases (Table 1). In summary, coloboma (either unilateral or bilateral) was detected in 70% (82/116) of the mice specifically analysed for this phenotype, in three successive generations (Table 1).

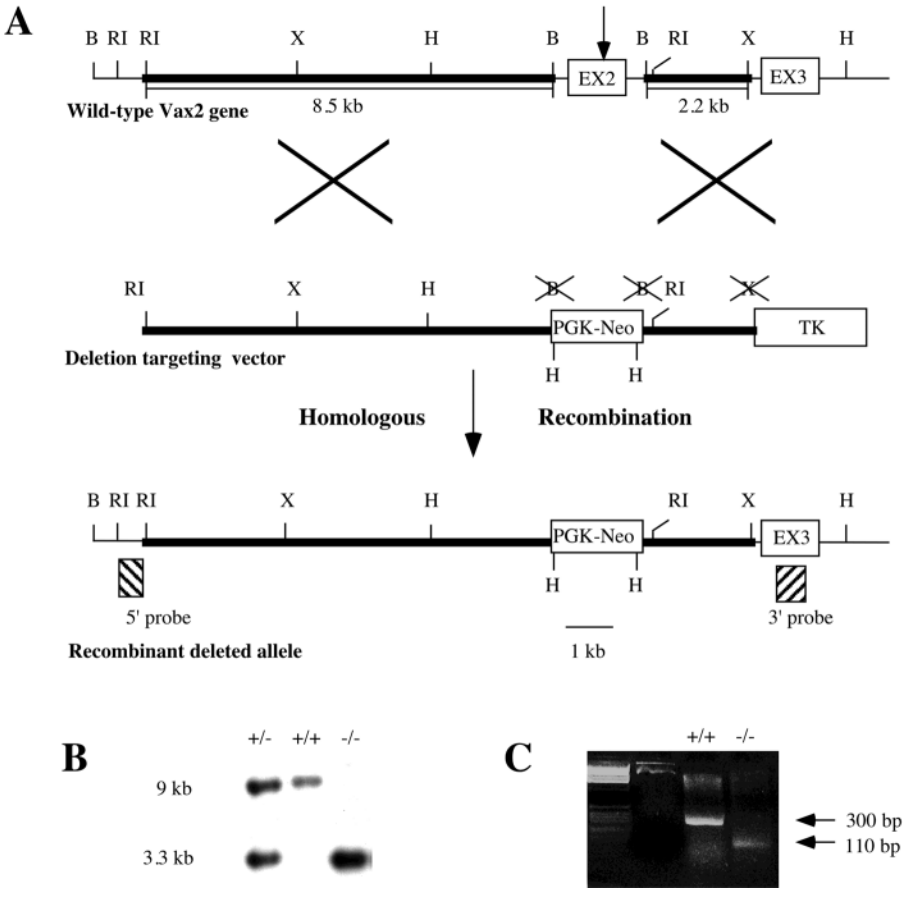

Fig. 1. Generation of *Vax2* mutant mice. (A) Targeted deletion of exon 2 (EX2) of the *Vax2* gene by homologous recombination in ES cells. The PGK-Neo expression cassette introduces two novel *Hin*dIII sites and removes two *Bam*HI restriction sites. The probes used for Southern-blot analysis are also shown (hatched boxes). Tk, thymidine kinase; B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI; X, *Xba*I. (B) Southern blot analysis of genomic DNA from wild-type (+/+), heterozygous (–/+) and homozygous (–/–) mutant mice, hybridised with the 3′ probe indicated in A. (C) RT-PCR analysis of total retina RNA from wild-type and $Vax2^{-/-}$ mice using primers 32F and 7R located, respectively, in the first and in the third coding exons of the *Vax2* gene. The amplified RT-PCR fragment in the $Vax2^{-/-}$ lane does not contain exon 2 (see the text for more details). Lanes 1 and 2, molecular weight markers.

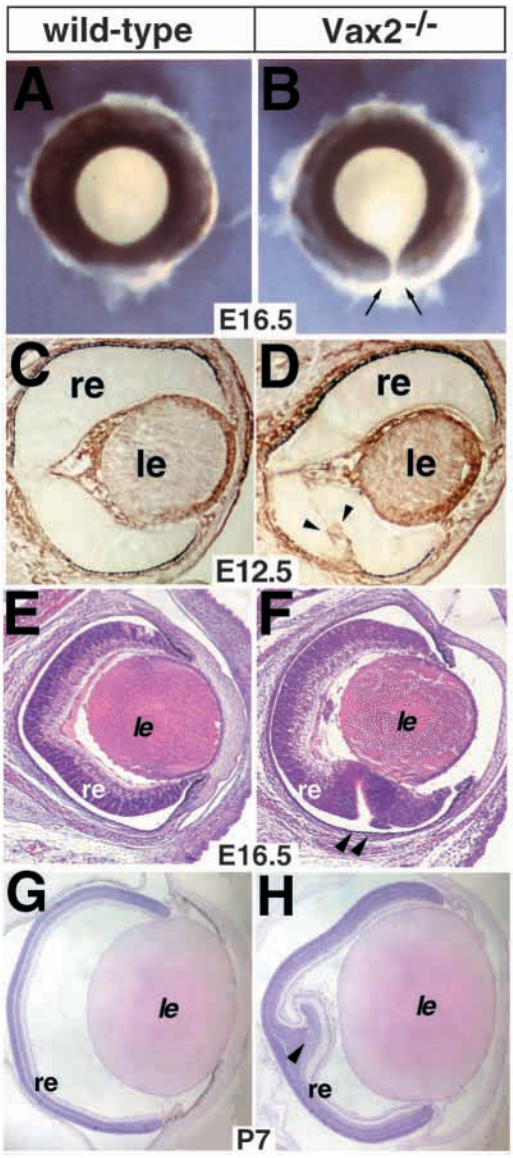

Fig. 2. Homozygous *Vax2* mutant mice show coloboma in a significant percentage of cases. (A,B) Whole-mount eyes dissected from E16.5 foetuses. (A,B) Frontal views of wild-type (A) and mutant (B) eyes. The open optic fissure in the mutant is indicated by arrows in B. (C,D) Detection of laminin by immunohistochemistry in order to identify the basal lamina in frontal sections of wild-type (C) and *Vax2*$^{-/-}$ (D) E12.5 embryos. Note how the basal lamina still persists in the mutant eye in the contact regions of the converging lips of the optic fissure (arrowheads), while in the wild-type eye it has completely dissolved. (E-H) Frontal sections, stained with Haematoxylin and Eosin, of wild-type (E,G) and *Vax2* mutant (F, H) eyes at E16.5 (E,F) and P7 (G,H). No differences, apart from the presence of coloboma (arrowheads) can be detected in *Vax2* mutant mice compared with wild-type animals in the organisation of retinal cell layers. re, retina; le, lens.

Apart from the presence of coloboma, no other morphological or histological alterations were found in the retina and optic nerve of *Vax2*$^{-/-}$ mice examined at several developmental stages (Fig. 2E,F and data not shown). In particular, the retina appeared normally stratified at all developmental stages analysed with no obvious alterations in the cell type composition (data not shown). In addition, we observed no defect in either proliferation or survival of retinal cells in *Vax2*$^{-/-}$ mice, as assessed by BrdU- and TUNEL staining carried out at E12.5 and at E14.5 (data not shown). In summary, *Vax2* represents the first gene identified to date whose inactivation is responsible for an isolated form of eye coloboma in mammals.

Molecular studies

The inactivation of other transcription factor genes in mouse, such as *Vax1* and *Pax2*, is also responsible for eye coloboma in the context of more complex phenotypes affecting both the CNS and other tissues (Bertuzzi et al., 1999; Torres et al., 1996). The inhibition of the contact-dependent dissolution of the basal lamina at the contacting neuroepithelial lips of the optic fissure has been found to be the cause of eye coloboma in *Pax2*$^{-/-}$ mice (Torres et al., 1996). To verify if this event could also underlie the coloboma present in *Vax2*$^{-/-}$ mice, we tested the basal lamina in E12.5 embryos for the presence of laminin. As in the case of *Pax2*$^{-/-}$ mice, we observed that in *Vax2*$^{-/-}$ mice, the basal lamina persists, thus preventing the closure of the optic fissure (Fig. 2C,D). This analogy prompted us to determine whether either the *Pax2* or the *Vax1* genes could play any role in the eye coloboma observed in *Vax2*$^{-/-}$ mice. However, we found that both *Pax2* and *Vax1* expression domains in the optic cup and in the optic stalk were indistinguishable between wild-type and mutant mice at E12.5, a developmental stage in which the closure of the optic fissure is normally completed (Fig. 3A,B and data not shown). These findings indicate that the coloboma is a direct consequence of *Vax2* inactivation and that *Vax2* does not control the expression of either *Pax2* or *Vax1* in the optic cup and optic stalk.

Overexpression of *Vax2* was previously shown to determine, in both *Xenopus laevis* and chicken, a ventralisation of the expression domains of several markers of retinal DV asymmetry, including the transcription factors Pax2, Xvent2 and Tbx5 (Barbieri et al., 1999; Schulte et al., 1999), as well as the receptor tyrosine kinases EphB2 and EphB3 and their ligands ephrin B1 and ephrin B2 (Schulte et al., 1999).

To test the possibility that *Vax2* loss of function could determine a molecular dorsalisation of the developing eye, we examined the expression of some of the above mentioned transcripts by RNA in situ hybridisation experiments in *Vax2*$^{-/-}$ mice. The expression of the genes *Pax2* and *Tbx5*, which are early markers of ventral retina and optic stalk (*Pax2*) and dorsal retina (*Tbx5*) showed no differences between mutant and wild-type mice, from E9.5 to E16.5 (Fig. 3A-F, and data not shown). By contrast, the expression domains of the *Efnb2* mRNA and of its receptor *Ephb2* differed in *Vax2*$^{-/-}$ mice and wild-type animals. In particular, the *Efnb2* transcript, whose localisation is normally confined to the dorsal retina at E16.5, was also strongly detected in the ventral retina in *Vax2*$^{-/-}$ mice (Fig. 3G,H). In addition, the *Ephb2* mRNA, which is expressed with a high ventral to low dorsal gradient in the neural retina of E16.5 wild type (Birgbauer et al., 2000) appeared to be uniformly expressed and only barely detectable throughout the retina of *Vax2*$^{-/-}$ mice (Fig. 3I,J). However, we could not detect any significant alteration in the expression of markers of temporonasal asymmetry, such as the transcription factors BF1 and BF2, the receptor tyrosine kinase EphA5 (expressed with a high temporal to low nasal gradient) and its ligand ephrin A5

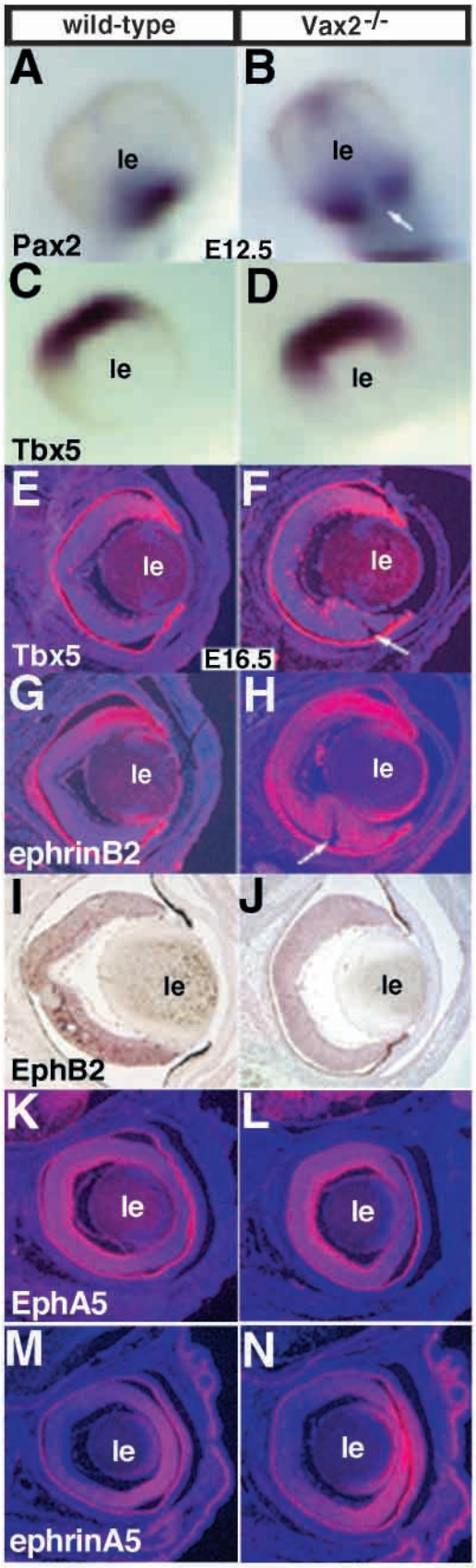

Fig. 3. Effects of *Vax2* inactivation on the expression of markers of DV and of temporo-nasal asymmetry in the eye, as determined by RNA in situ hybridisation. *Pax2* expression at E12.5, which is normally restricted (within the optic cup) to the lips of the optic fissure, was the same in wild-type (A) and *Vax2* mutant (B) mice. The white arrow in B indicates the persistence of the optic fissure in *Vax2*$^{-/-}$ mice. Similarly, the expression of *Tbx5*, a marker of the dorsal retina, was comparable between normal mice at E12.5 (C) and E16.5 (E), and mutant animals at the same stages (D,F, respectively). (G,H) Frontal sections of a wild-type (G) and of a *Vax2*$^{-/-}$ (H) mouse embryos at E16.5 hybridised with the probe for *Efnb2* (ephrin B2) probe. Expression of *Efnb2*, which is normally restricted to the dorsal retina (G), is extended to the ventral retina of *Vax2*$^{-/-}$ mice. (I,J) Frontal sections of a wild-type (I) and of a *Vax2*$^{-/-}$ (J) mouse embryos at E16.5 hybridised with the probe for *Ephb2*. The *Ephb2* mRNA at E16.5 is expressed with a high ventral to low dorsal gradient in wild-type mice, while in *Vax2*$^{-/-}$ mice, it is expressed uniformly, and at lower levels, throughout the retina. (K-N) Sagittal sections of wild-type (K,M) and of *Vax2*$^{-/-}$ (L,N) mouse embryos at E16.5 hybridised with the probes for *Epha5* (K,L) and *Efna5* probes (M,N). No differences could be detected in the expression of the latter marker between wild-type and *Vax2*$^{-/-}$ mice. The white arrows in F,H indicate the presence of coloboma in *Vax2*$^{-/-}$ mice. le, lens.

(high nasal to low temporal gradient) (Fig. 3 and data not shown). These findings indicate that *Vax2* inactivation results in dorsalisation of the expression of mid-late (*Ephb2* and *Efnb2*) but not early (*Pax2* and *Tbx5*) markers of DV asymmetry in the developing retina.

Retinal ganglion cell axon pathway is altered in *Vax2* mutant mice

The establishment of the retinotectal projections in the DV axis appears to require the activity of EphB2 and ephrin B2 (Holash and Pasquale, 1995). The altered expression of these molecules in *Vax2*$^{-/-}$ mice could therefore be associated with changes in the visual projections. The idea that *Vax2* is important for correct RGC axon navigation was reinforced by the observation that in chick ectopic expression of either chick *Vax* or mouse *Vax2* in the dorsal retina causes topographic targeting errors of dorsal RGC axons (Schulte et al., 1999). To test whether *Vax2* inactivation could also lead to alterations in the projections of RGC axons, we filled either the ventral retina or the optic nerve head of both newborn and adult wild type and *Vax2*$^{-/-}$ mice (129/C57Bl6 strain) with lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate (DiI). In mice, RGC axons grow along the optic nerve towards the ventral midline of the diencephalon, where the majority of the axons cross at the chiasm to invade the contralateral optic tract, while a small proportion of fibres project into the ipsilateral optic tract. Ipsilaterally projecting RGC axons (approximately 5% of the total) derive from the ventrotemporal region of the retina, while the contralaterally projecting axons originate from the entire retina (Guillery et al., 1995). Complete filling of the optic nerve head of wild-type newborn animals with DiI confirmed this normal behaviour of RGC axons at the chiasm (Fig. 4A). Strikingly, similar filling in *Vax2*$^{-/-}$ mutants revealed the complete absence of axons invading the ipsilateral optic tract (Fig. 4B). This observation was further confirmed by the restricted DiI labelling of RGC of the ventral retina (Fig. 4C,D) and by the absence of labelled projections in the ipsilateral superior colliculus of neonatal

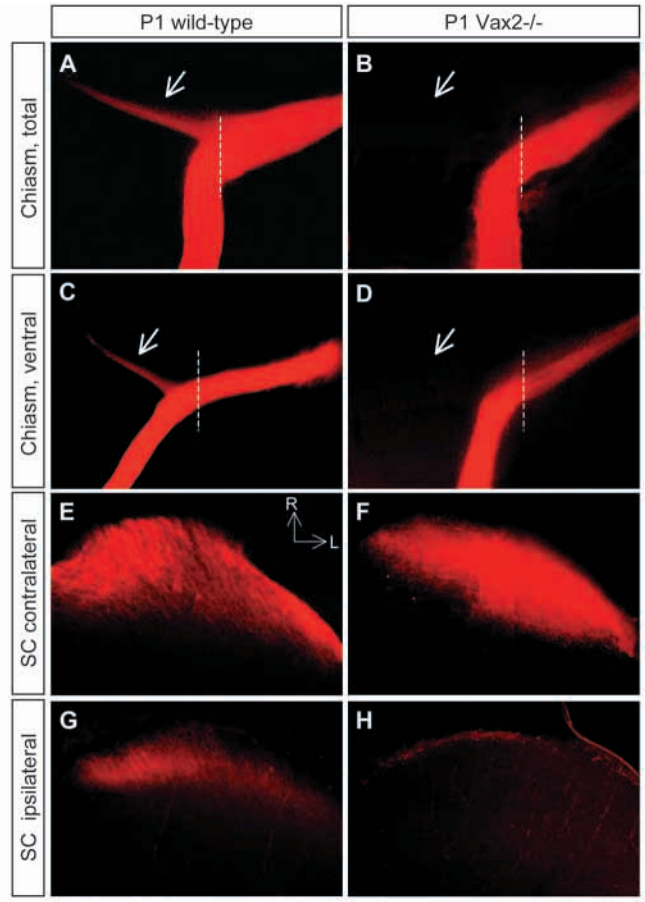

Fig. 4. Retinal ganglion cell axon pathway and projections are altered in *Vax2* mutant mice. The ventral retina (C,D) or the right optic nerve head (A,B; E,H) of new-born wild-type (WT) or *Vax2* mutant mice were filled with DiI to label the retinal ganglion cell axon trajectory. (A-D) Ventral views of the brain at the chiasm region (dotted lines indicate the midline). (E,H) Coronal sections through the contralateral (E,F) and ipsilateral superior colliculus (G,H). All sections are oriented rostral (R) towards the top and lateral (L) towards the right, as indicated in E. Note how retinal ganglion cell axons normally projecting in the ipsilateral optic tract (arrows in A,C) are totally absent in the mutant mice (B,D). Furthermore, note how, in mutant mice, projections to ipsilateral superior colliculus are absent (compare G with H), while those to the contralateral side terminate predominantly in the lateral region (compare E with F), where normally the majority of axons arising from the dorsal retina project.

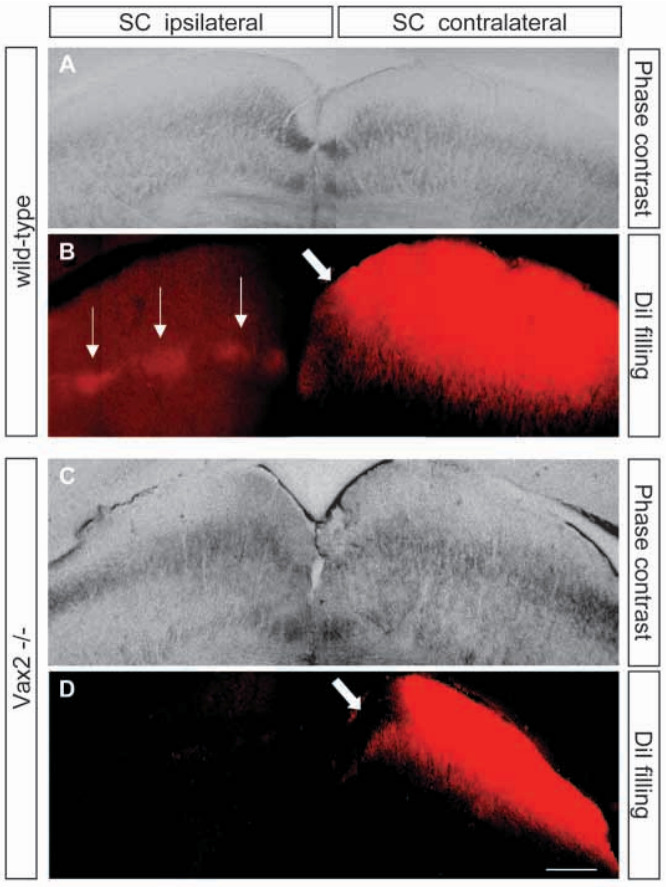

Fig. 5. Visual projections are altered in adult mutant mice. Frontal sections of adult wild type (A,B) and Vax2$^{-/-}$ (C,D) brains at the mid-superior colliculus. (A,C) Phase contrast views of the sections presented in B,D, respectively. DiI was injected into the ventral retina of adult wild-type and mutant mice. (B) In wild-type mice, DiI-labelled axons project to the entire medial region (thick arrow) of the contralateral superior colliculus and to patches of the stratum opticum of the ipsilateral superior colliculus (thin arrows). (D) Note the absence of the ipsilateral and most medial contralateral projections in the superior colliculus of mutant mice. SC, superior colliculus. Scale bar: 150 μm.

mutant mice (Fig. 4H) when compared with wild-type littermates (Fig. 4G). These alterations were not the result of a delayed development of the uncrossed projections, as absence of ipsilateral projecting fibres was still clearly visible both in the chiasm (not shown) and in the superior colliculus of adult animals. Thus, the clusters of fibres described in the stratum opticum of the ipsilateral superior colliculus of wild-type animals (Godement et al., 1984) were absent in adult *Vax2*$^{-/-}$ mice (Fig. 5). Furthermore, analysis of DiI labelling distribution in the contralateral superior colliculus of both newborn and adult animals indicated further alterations in RGC projections. In newborn mutant animals, a reduced number of labelled projections terminated in the medial region of the colliculus, which normally constitutes the main target of the axons originating from the ventral retina. Concomitant with this decrease there appeared to be an increase in the number of labelled fibres terminating in the lateral region of the colliculus, where the majority of axons arising from the dorsal retina normally project (Fig. 4, compare E with F). This abnormal distribution of the RGC projections to the contralateral superior colliculus persisted at adult stages (Fig. 5). Altogether, the changes in the axon projections of *Vax2*$^{-/-}$ mice indicate that *Vax2* inactivation is sufficient to prevent RGC projection to the ipsilateral optic tract, with the consequent formation of totally crossed visual projections. Furthermore, molecular analysis of *Vax2*$^{-/-}$ mice indicates that this change is probably determined by the alteration of the retina expression domains of EphB2 and of its ligand ephrin B2, which leads also to a topographic rearrangement of the projection in the contralateral superior colliculus.

Table 1. Coloboma in *Vax2*$^{-/-}$ mice

Mouse strain	Bilateral coloboma	Unilateral coloboma	No coloboma	Total	% of animals with coloboma
129/CD1	27	19	13	59	78%
129/C57Bl6	10	26	21	57	63%
Total	37	45	34	116	70%

The mice analysed derived from three successive generations obtained by interbreeding *Vax2*$^{-/-}$ littermates.

DISCUSSION

Vax2 is a homeobox gene that, by virtue of both its expression pattern and functional studies carried out in *Xenopus laevis* and chicken (Barbieri et al., 1999; Schulte et al., 1999), has been thought to play an important role in eye development. In this report, the generation and the analysis of a mouse with a targeting mutation of *Vax2* demonstrates that the inactivation of this gene causes both eye defects and abnormal pathfinding of RGC axons.

A significant number of *Vax2*$^{-/-}$ mice (about 70%) have eye coloboma (Fig. 2, Table 1) owing to a failure of the optic fissure to close. The incomplete penetrance of the phenotype suggests that additional factors, both genetic, i.e. related to the mixed genetic background of the mice analysed, and non-genetic, may contribute to its generation. Moreover, the coloboma in *Vax2*$^{-/-}$ mice has a wide extent of expression, even in the same litter, ranging from severe bilateral cases to mild unilateral cases (Table 1). This phenotype is isolated and is not accompanied by other gross anomalies in either the CNS or other tissues and organs. Therefore, *Vax2* represents the first gene responsible for isolated coloboma in mouse identified to date. In fact, mutations in several other murine genes [mostly transcription factors expressed in the developing eye such as Pax2 (Favor et al., 1996; Torres et al., 1996), Vax1 (Bertuzzi et al., 1999; Hallonet et al., 1999), Pitx2 (Gage et al., 1999) and Bf1 (Huh et al., 1999)] have already been associated with coloboma. In contrast to the *Vax2* knockout, however, the coloboma observed in those mutant mice is part of more complex phenotypes affecting not only the brain but also other tissues.

Isolated coloboma in humans is a relatively frequent condition with frequency estimates ranging from about 5 (Bermejo and Martinez-Frias, 1998) to 26 per 100,000 (Traboulsi, 1998). From a genetic point of view, isolated eye coloboma is heterogeneous as both autosomal dominant and autosomal recessive modes of inheritance have been described (see OMIM at http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM). In most cases, however, a hereditary pattern cannot be established (Onwochei et al., 2000). Similar to the phenotype observed in *Vax2*$^{-/-}$ mice, variable expressivity and incomplete penetrance are commonly observed in families with eye coloboma. To date, the genetic basis of this condition remains largely unknown: with the exception of *PAX2*, no other human gene has been found to be responsible for the pathogenesis of ocular coloboma. However, mutations in the *PAX2* gene are responsible for a syndromic form of coloboma, the renal-coloboma syndrome (OMIM 120330), characterised by coloboma of the optic nerve and renal disease (Sanyanusin et al., 1995). Considering all the similarities between the phenotype observed in *Vax2* mutant mice and the clinical features of isolated colobomata in human, *Vax2* represents an excellent candidate for this human condition. The extent of variable expressivity and incomplete penetrance may be higher in human than in mouse, and this may also explain the predominance of apparently sporadic forms of eye coloboma observed in humans. Mutation analysis of this gene in large collections of individuals with isolated eye coloboma will help to clarify whether this gene also plays any role in the pathogenesis of eye developmental anomalies in humans.

The eye coloboma in *Vax2*$^{-/-}$ mice reflects the presence of anomalies in the specification of ventral eye structures. In fact, these mice are characterised by profound alterations in the axonal projections of ventral RGC. This is evident from the complete absence of ipsilaterally projecting axons at the level of the optic chiasm and from abnormal terminations in the contralateral superior colliculus (Fig. 4; Fig. 5), where retinal fibres project preferentially to the lateralmost region that is the main target of the axons originating from the dorsal retina. These findings are consistent with the topographic targeting errors observed in the dorsal RGC axons after misexpression of either the chicken *Vax* or the murine *Vax2* gene in the dorsal retina of chick embryos (Schulte et al., 1999). A molecular explanation for the abnormalities observed in *Vax2*$^{-/-}$ mice may reside in the alterations of the expression in the retina of the EphB2 receptor and of its ligand ephrin B2. The EphB2 receptor is normally expressed with a high ventral to low dorsal gradient in the mouse retina. Analogous to the well-established role of EphA receptors in the formation of the appropriate retinocollicular (or retinotectal) projection on the anteroposterior axis (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001), this asymmetric expression of EphB2 suggests that it has a role in retinocollicular mapping along the DV axis. The notable reduction of the *Ephb2* retinal gradient observed in *Vax2*$^{-/-}$ mice adds support to the hypothesis formulated by Schulte et al. (Schulte et al., 1999) that this gene is indeed involved in the retinocollicular mapping on the DV axis. In contrast to *Vax2* overexpression experiments in both chicken and *Xenopus laevis* (Barbieri et al., 1999; Schulte et al., 1999), we observed no variation in the expression levels of *Pax2* and *Tbx5*, early molecular markers of, respectively, ventral and dorsal developing retina. These differences may indicate either that the molecular pathway underlying the establishment of the DV axis in the developing eye is different in mammals from that in birds and amphibia or, more likely, that *Vax2* is sufficient (as shown in overexpression studies) but not required for the correct expression of *Tbx5* and *Pax2* in retina. However, based on the results of this study, we conclude that *Vax2* is required and sufficient for controlling *Ephb2* and *Efnb2* levels of expression in the ventral retina.

In *Vax2*$^{-/-}$ mice we observed the almost complete absence of RGC axons projecting into the ipsilateral optic tract at the level of the optic chiasm (Fig. 4). As ipsilaterally projecting RGC axons derive from the ventrotemporal region of the retina,

this finding is consistent with the hypothesis that the inactivation of *Vax2*, and the subsequent reduced expression of *Ephb2* in the ventral retina, determine an alteration in the projection of ventral RGC to the higher visual sensory centres of the brain. This is also consistent with the recent observation that the expression of ephrin B2 at the chiasm plays a key role in directing the growth of EphB2-expressing ventral retinal axons into the ipsilateral brain in *Xenopus* (Nakagawa et al., 2000). The reduced levels of expression of *Ephb2* in the ventral RGC axons in Vax2$^{-/-}$ mice may alter the EphB2/ephrin B2 interaction at the optic chiasm, contributing to the absence of ipsilaterally projecting fibres. Furthermore, the change of the expression domain of *Efnb2* in the retina of *Vax2*$^{-/-}$ mice may further contribute to the establishment of the abnormal axonal projections of ventral RGC, both in the optic chiasm and in the superior colliculus. It has recently been observed in chicken that the misexpression of ephrin A2 and ephrin A5 in temporal RGCs alters the function of the EphA receptor, causing topographic targeting errors of temporal axons (Hornberger et al., 1999). Similarly, the aberrant expression of *Efnb2* in ventral RGC in *Vax2*$^{-/-}$ mice may reduce the capability of EphB2 receptors to interact with their ligands both in the optic chiasm and in the superior colliculus, contributing to the abnormal axonal projections of these cells.

In summary, the characterisation of *Vax2*$^{-/-}$ mice confirms the prominent role played by *Vax2* in the development of ventral eye territories in mammals. In particular, this gene is required for the proper closure of the optic fissure, for the establishment of a physiological asymmetry on the DV axis of the eye and for the formation of appropriate retinocollicular connections.

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REFERENCES

Banfi, S., Borsani, G., Bulfone, A. and Ballabio, A. (1997). *Drosophila*-related expressed sequences. *Hum. Mol. Genet.* **6**, 1745-1753.

Banfi, S., Borsani, G., Rossi, E., Bernard, L., Guffanti, A., Rubboli, F., Marchitiello, A., Giglio, S., Coluccia, E., Zollo, M. et al. (1996). Identification and mapping of human cDNAs homologous to *Drosophila* mutant genes through EST database searching. *Nat. Genet.* **13**, 167-174.

Barbieri, A. M., Lupo, G., Bulfone, A., Andreazzoli, M., Mariani, M., Fougerousse, F., Consalez, G. G., Borsani, G., Beckmann, J. S., Barsacchi, G. et al. (1999). A homeobox gene, vax2, controls the patterning of the eye dorsoventral axis. *Proc. Natl. Acad. Sci. USA* **96**, 10729-10734.

Bermejo, E. and Martinez-Frias, M. L. (1998). Congenital eye malformations: clinical-epidemiological analysis of 1,124,654 consecutive births in Spain. *Am. J. Med. Genet.* **75**, 497-504.

Bertuzzi, S., Hindges, R., Mui, S. H., O'Leary, D. D. and Lemke, G. (1999). The homeodomain protein vax1 is required for axon guidance and major tract formation in the developing forebrain. *Genes Dev.* **13**, 3092-3105.

Birgbauer, E., Cowan, C. A., Sretavan, D. W. and Henkemeyer, M. (2000). Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. *Development* **127**, 1231-1241.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev. Dyn.* **206**, 379-390.

Dalton, D., Chadwick, R. and McGinnis, W. (1989). Expression and embryonic function of empty spiracles: a Drosophila homeo box gene with two patterning functions on the anterior-posterior axis of the embryo. *Genes Dev.* **3**, 1940-1956.

Favor, J., Sandulache, R., Neuhauser-Klaus, A., Pretsch, W., Chatterjee, B., Senft, E., Wurst, W., Blanquet, V., Grimes, P., Sporle, R. et al. (1996). The mouse Pax2(1Neu) mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. *Proc. Natl. Acad. Sci. USA* **93**, 13870-13875.

Flanagan, J. G. and Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. *Annu. Rev. Neurosci.* 309-345.

Gage, P. J., Suh, H. and Camper, S. A. (1999). Dosage requirement of Pitx2 for development of multiple organs. *Development* **126**, 4643-4651.

Gibson-Brown, J. J., Agulnik, S. I., Silver, L. M., Niswander, L. and Papaioannou, V. E. (1998a). Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. *Development* **125**, 2499-2509.

Gibson-Brown, J. J., S, I. A., Silver, L. M. and Papaioannou, V. E. (1998b). Expression of T-box genes Tbx2-Tbx5 during chick organogenesis. *Mech. Dev.* **74**, 165-169.

Godement, P., Salaun, J. and Imbert, M. (1984). Prenatal and postnatal development of retinogeniculate and retinocollicular projections in the mouse. *J. Comp. Neurol.* **230**, 552-575.

Guillery, R. W., Mason, C. A. and Taylor, J. S. (1995). Developmental determinants at the mammalian optic chiasm. *J. Neurosci.* **15**, 4727-4737.

Hallonet, M., Hollemann, T., Pieler, T. and Gruss, P. (1999). Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system. *Genes Dev.* **13**, 3106-3114.

Hallonet, M., Hollemann, T., Wehr, R., Jenkins, N. A., Copeland, N. G., Pieler, T. and Gruss, P. (1998). Vax1 is a novel homeobox-containing gene expressed in the developing anterior ventral forebrain. *Development* **125**, 2599-2610.

Hatini, V., Tao, W. and Lai, E. (1994). Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. *J. Neurobiol.* **25**, 1293-1309.

Holash, J. A. and Pasquale, E. B. (1995). Polarized expression of the receptor protein tyrosine kinase Cek5 in the developing avian visual system. *Dev. Biol.* **172**, 683-693.

Hornberger, M. R., Dutting, D., Ciossek, T., Yamada, T., Handwerker, C., Lang, S., Weth, F., Huf, J., Wessel, R., Logan, C. et al. (1999). Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. *Neuron* **22**, 731-742.

Huh, S., Hatini, V., Marcus, R. C., Li, S. C. and Lai, E. (1999). Dorsal-ventral patterning defects in the eye of BF-1-deficient mice associated with a restricted loss of shh expression. *Dev. Biol.* **211**, 53-63.

Joyner, A. L. (1993). *Gene Targeting: A Practical Approach*. Oxford: IRL Press.

Koshiba-Takeuchi, K., Takeuchi, J. K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. et al. (2000). Tbx5 and the retinotectum projection. *Science* **287**, 134-137.

Nakagawa, S., Brennan, C., Johnson, K. G., Shewan, D., Harris, W. A. and Holt, C. E. (2000). Ephrin-B regulates the Ipsilateral routing of retinal axons at the optic chiasm. *Neuron* **25**, 599-610.

Ohsaki, K., Morimitsu, T., Ishida, Y., Kominami, R. and Takahashi, N. (1999). Expression of the Vax family homeobox genes suggests multiple roles in eye development. *Genes Cells* **4**, 267-276.

Onwochei, B. C., Simon, J. W., Bateman, J. B., Couture, K. C. and Mir, E. (2000). Ocular colobomata. *Surv. Ophthalmol.* **45**, 175-194.

Rugarli, E. I., Lutz, B., Kuratani, S. C., Wawersik, S., Borsani, G., Ballabio, A. and Eichele, G. (1993). Expression pattern of the Kallmann syndrome gene in the olfactory system suggests a role in neuronal targeting. *Nat. Genet.* **4**, 19-25.

Sanyanusin, P., Schimmenti, L. A., McNoe, L. A., Ward, T. A., Pierpont, M. E., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the PAX2 gene in a family with optic nerve colobomas, renal anomalies and vesicoureteral reflux. *Nat. Genet.* **9**, 358-363.

Schulte, D., Furukawa, T., Peters, M. A., Kozak, C. A. and Cepko, C. L. (1999). Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. *Neuron* **24**, 541-553.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli,

E. (1992). Nested expression fomains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.

Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693-702.

Sperry, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Natl. Acad. Sci. USA* **50**, 703-710.

Szel, A., Rohlich, P., Caffe, A. R. and van Veen, T. (1996). Distribution of cone photoreceptors in the mammalian retina. *Microsc. Res. Tech.* **35**, 445-462.

Torres, M., Gomez Pardo, E. and Gruss, P. (1996). Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-3391.

Traboulsi, E. I. (1998). Colobomatous microphthalmia, anophthalmia, and associated malformation syndromes. In *Genetic Diseases of the Eye*. Chap. 2 (ed. E. I. Traboulsi), pp. 51-80. New York: Oxford University Press.

Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. *Cell* **65**, 1153-1163.

Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat. Rev. Neurosci.* **2**, 155-164.

Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A. and Nagy, A. (1993). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365**, 87-89.

Yuasa, J., Hirano, S., Yamagata, M. and Noda, M. (1996). Visual projection map specified by topographic expression of transcription factors in the retina. *Nature* **382**, 632-635.