

The homeodomain protein *Vax2* patterns the dorsoventral and nasotemporal axes of the eye

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SUMMARY

The vertebrate retina is highly ordered along both its dorsoventral (DV) and nasotemporal (NT) axes, and this order is topographically maintained in its axonal connections to the superior colliculus of the midbrain. Although the graded axon guidance cues that mediate the topographic mapping of retinocollicular connections are increasingly well understood, the transcriptional regulators that set the DV and NT gradients of these cues are not. We now provide genetic evidence that *Vax2*, a homeodomain protein expressed in the ventral retina, is one such regulator. We demonstrate that in *Vax2* mutant mice, retinocollicular projections from the ventral

temporal retina are dorsalized relative to wild type. Remarkably, however, this dorsalization becomes systematically less severe in progressively more nasal regions of the ventral retina. *Vax2* mutants also exhibit flattened DV and NT gradients of the EphA5, EphB2, EphB3, ephrin-B1 and ephrin-B2 axon guidance cues. Together, these data identify *Vax2* as a fundamental regulator of axial polarization in the mammalian retina.

Key words: Retina, Homeobox, *Vax* genes, *Emx* genes, Dorsoventral axis, Axon guidance, Mouse

INTRODUCTION

Although axial polarization of the retina is essential to perception of the visual world, the molecules that specify the retinal axes during development have not been intensively studied. For the most part, analyses of embryonic retinal organization and topography have instead focused on one important read-out of retinal polarization – the cell surface receptors and ligands that guide the precise wiring of retinal ganglion cell (RGC) neurons to their synaptic targets in the superior colliculus (SC) (O'Leary et al., 1999). These receptors and ligands act in concert to organize retinocollicular connections into a topographic map – a spatial ordering of axonal connections in which the Cartesian coordinates of a two-dimensional sheet of projecting RGC neurons are mapped onto the coordinates of a second two-dimensional sheet of target neurons in the SC. In the retinocollicular map, axons from RGCs in the nasal retina project to and form synapses in the caudal end of the SC, while RGCs in the temporal retina project to targets in the rostral SC. RGCs located at intermediate NT retinal positions project to correspondingly intermediate caudal-rostral positions in the SC. The dorsoventral (DV) axis of the retina is similarly mapped onto the lateral-medial axis of the SC.

The proteins most closely tied to the topography of retinocollicular mapping are the receptor tyrosine kinases of the EphA family, together with their ligands, the ephrin-A

proteins (Flanagan and Vanderhaeghen, 1998; O'Leary and Wilkinson, 1999). Two key lines of recent genetic evidence (Frisén et al., 1998; Brown et al., 2000; Feldheim et al., 2000), together with a large body of earlier in vitro membrane stripe and in vivo misexpression studies, have demonstrated that a low-nasal-to-high-temporal retinal EphA receptor gradient, combined with a reciprocal low-rostral-to-high-caudal collicular ephrin-A gradient, serve to order the mapping of the NT axis of the retina onto the caudal-rostral axis of the SC. The cell-surface molecules that mediate mapping of the orthogonal retinocollicular axes are less well understood. Although clear DV gradients of EphB2 and EphB3 receptor expression have been observed in the vertebrate retina, and medial-lateral gradients of ephrin-B ligands have been detected in the SC (Marcus et al., 1996; Braisted et al., 1997; Holash et al., 1997; Schulte et al., 1999), these gradients are not reciprocally configured, as would be required for a chemorepellent action of the ephrin-Bs, and genetic tests of the importance of the EphB/ephrin-B signaling system to retinocollicular mapping have yet to be reported.

Less clear still are the transcriptional control mechanisms through which the NT and DV retinal gradients of the EphA and EphB receptors are established during development. Recently, two candidates for transcriptional regulators of DV polarization of the retina – *Tbx5* and *Vax2* – have been identified (Barbieri et al., 1999; Koshiba-Takeuchi et al., 2000; Schulte et al., 1999). *Vax2* (for ventral anterior homeobox 2)

is one of two vertebrate-specific homeobox genes that are structurally related to, and that have almost certainly evolved from the duplication of, the vertebrate *Emx* genes (Barbieri et al., 1999; Hallonet et al., 1998; Ohsaki et al., 1999). Loss-of-function experiments for *Vax1* have demonstrated that its product plays essential roles in axon guidance and major tract formation in the developing forebrain (Bertuzzi et al., 1999; Hallonet et al., 1999). *Vax2*, which carries a homeodomain identical to that of *Vax1*, is a candidate regulator of the retinal DV axis for two reasons. First, it is steeply graded in its expression along this axis in chick and frog embryos, with highest expression ventrally. And second, dominant gain-of-function studies in these embryos have shown that, when misexpressed in the dorsal retina, *Vax2* is capable of ventralizing this tissue, as assessed by (1) the altered expression of DV marker genes, both putative guidance cues such as *EphB2/B3* and putative transcriptional regulators such as *Tbx5*, and (2) the altered projection of RGC axons to the midbrain. These observations have been interpreted as indicating that *Vax2* may function as a global 'ventralizing' regulator of the developing eye. In this report, we describe the generation of *Vax2*^{-/-} mice, and the use of these mutants to perform loss-of-function tests of this hypothesis.

MATERIALS AND METHODS

Gene inactivation

The mouse *Vax2* gene was cloned from a strain 129/sv genomic library and was inactivated by deletion/replacement (see Fig. 1G). *Vax2*^{-/-} mice were generated by standard procedures (Tybulewicz et al., 1991). Electroporated W95 embryonic stem (ES) cell clones were doubly selected with G418 and FIAU, and were screened for homologous recombination by Southern blot, using the 230 bp 3'-external probe indicated in Fig. 1. Four positive ES cell clones were microinjected into C57/Bl6 blastocysts to generate chimeric mice, which were then mated with C57/Bl6 to produce heterozygous knockouts, all according to standard procedures (Capecchi, 1989). Although most *Vax2*^{-/-} mice did not exhibit overt postnatal phenotypes, ~7% of homozygotes were abnormal, in that they either were unusually small in size, exhibited a trembling phenotype or displayed small eyes.

Expression studies

In situ hybridization with ³³P-radiolabeled or digoxigenin-labeled riboprobes were performed as described previously (Braisted et al., 1997; Zhadanov et al., 1995). Dissected mouse embryos were anesthetized and immersion fixed in 4% paraformaldehyde/PBS overnight at 4°C, followed by overnight infiltration with 20% sucrose. Cryoprotected tissues were then embedded in OCT medium (Miles). Hybridization was performed with 20 µm coronal and transverse sections of retina at birth (P0), and with embryos at E9.5, E11.5 and E14.

Axon tracing

In vivo anterograde labeling was performed between postnatal day 7 (P7) and P9 by focal injection of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes; 8% w/v in N,N-dimethylformamide) into the left eye, as described previously (Brown et al., 2000; Simon and O'Leary, 1992). Mice were sacrificed 1 day later, and termination zone (TZ) number and location in the contralateral superior colliculi were analyzed using a Zeiss LSM510 confocal microscope. The size and location of focal retinal DiI injection sites were identified post hoc.

Intraocular injection of fluorescently tagged cholera toxin B-

subunit tracers (CTB) into one eye allowed for visualization of both ipsilateral and contralateral retinal projections. For anterograde tracing of the RGC axons, CTB conjugated to a fluorescein (FITC) fluorophore (List Biological Laboratories, Campbell, CA) was made in a 0.2% solution in 1% DMSO. Whole eye fill injections were carried out at P8 and the animals were perfused with 4% paraformaldehyde 24 hours later. Brains were cut coronally in 200 µm sections and subsequent imaging was performed using a confocal microscope (Zeiss).

RESULTS

Dual gradients of *Vax2* expression in the mouse retina

Although the *Vax2* homeodomain is identical to that of *Vax1* (Barbieri et al., 1999; Schulte et al., 1999), the two *Vax* genes nonetheless exhibit distinct and largely complementary patterns of expression during embryogenesis in the mouse. At embryonic day 10.5 (E10.5) and thereafter, *Vax1* mRNA is expressed in the ventral diencephalon and telencephalon, and in the optic stalk and disk, but is not prominently expressed in the developing neural retina (Bertuzzi et al., 1999; Hallonet et al., 1999). By contrast, the expression of mouse *Vax2* mRNA is largely confined to the ventral half of the developing eye itself (Fig. 1A). These distinct embryonic expression domains in the mouse contrast with the situation in the chick, where a single gene (*VAX*) exhibits a hybrid *Vax1/Vax2*-like expression profile throughout the ventral diencephalon, optic stalk, and ventral retina (Schulte et al., 1999). At E11.5, mouse *Vax2* mRNA is expressed in all cells of the ventral neural retina (Fig. 1B), but by birth becomes restricted to ventral RGCs (Fig. 1E). In addition to the previously reported high-ventral-to-low-dorsal retinal gradient of *Vax2* mRNA (Fig. 1B,E), we have observed a shallower *Vax2* gradient along the NT axis, with highest expression nasally (Fig. 1B-D,F). This dually polarized expression is evident throughout the neural retina at E14 (Fig. 1C,D), when RGC axons first reach the SC, and persists at least until birth in the mouse, when the DV patterning of RGC projections is well under way. The double *Vax2* retinal gradient in the mouse is similar in orientation and configuration to the recently described dual retinal expression gradient of the bone morphogenetic protein 4 (BMP4) antagonist ventroptin in the chick (Sakuta et al., 2001).

Inactivation of the mouse *Vax2* gene

In order to test the hypothesis that *Vax2* may specify positional identity in the retina along the DV axis (Barbieri et al., 1999; Schulte et al., 1999), we generated *Vax2* mutant mice (Fig. 1G-I). We replaced exon 2 of the mouse *Vax2* gene, which encodes the first two essential α helices of the *Vax2* homeodomain, with a G418 resistance (PGK-neo) cassette. In addition to deleting exon 2, this mutation introduces a frame shift that eliminates all *Vax2*-coding sequence downstream of exon 2 (see Fig. 1G, Materials and Methods).

Retinocollicular mapping in the *Vax2* mutants

We first analyzed the projection of RGC axons from the retina to the SC in the *Vax2* mutants. Normally, the DV and NT axes of the retina are precisely mapped onto the correspondingly orthogonal lateral-medial and caudal-rostral axes of the SC (Brown et al., 2000). If *Vax2* is required for ventral patterning

of the retina, then ventral RGCs that lack *Vax2* should be dorsalized in terms of their projection to the SC; that is, they should project to lateral rather than to medial SC. In mice, the topography of the retinocollicular map is mature by postnatal day 7 (P7) (O'Leary et al., 1986). We found that most of the *Vax2*^{-/-} mice (94 out of 101) were healthy and superficially normal for many months after birth; in marked contrast to *Vax1* mutants (Bertuzzi et al., 1999), no postnatal colobomata were observed. We therefore labeled discrete loci of RGCs and their projecting axons across the full extent of the NT and DV axes of the mutant retina at P7-9. We performed focal injections of the lipophilic axon tracer DiI (Fig. 2), and then analyzed labeled projections to the contralateral SC 1 day later (see Materials and Methods). In total, we analyzed 40 injections (from 40 mutant mice). The *Vax2* mutant retinæ that we selected for study were histologically indistinguishable from wild type in terms of retinal lamination, cellular density, organization of plexiform layers and closure of the optic disk (data not shown).

Given that *Vax2* is not expressed in the extreme dorsal retina, axon projections from dorsal RGCs should not be altered in the *Vax2* mutants. This was the case. The dorsal RGC axons of mutant mice ($n=8$ dorsal injections, at varying NT positions, in 8 mice) entered the SC at its rostral lateral edge and formed tight termination zones (TZs) at expected locations in the lateral SC (Fig. 2S, and data not shown). In marked contrast, axon projections from most *Vax2*^{-/-} ventral RGCs did not, with RGC axons from the ventral temporal retina being the most strongly affected. These ventral temporal axons entered the SC at the lateral rostral edge, and always formed single, well-circumscribed TZs in the lateral rostral SC ($n=10/10$) (Fig. 2A, lower panel, Fig. 2D-F). As both wild-type and *Vax2*^{+/-} ventral temporal axons project to *medial* rostral SC (Fig. 2A, middle panel), the projection pattern of these *Vax2*^{-/-} RGCs represents a complete retinal dorsalization of their mapping behavior. This dorsalization was robust and fully penetrant (Fig. 2D-F), and was reflected not only in the position of the TZs, but also in the site at which RGC axons entered the rostral colliculus (compare middle and lower panels in Fig. 2A).

Unexpectedly, however, the dorsalization of RGC projections became progressively less severe as DiI injections were moved to increasingly nasal regions of the ventral retina. At 80-85% of the retinal NT axis (where extreme temporal=100%), ventral RGC TZs remained aberrantly lateralized in their SC projection, but more than one lateral TZ was typically observed (Fig. 2G,H). As injections were moved to 60-80% of the NT axis, the size of ectopic lateralized TZs became progressively smaller, and an appropriate TZ appeared very near the expected medial position (Fig. 2I-K). For mid-ventral injections into the *Vax2*

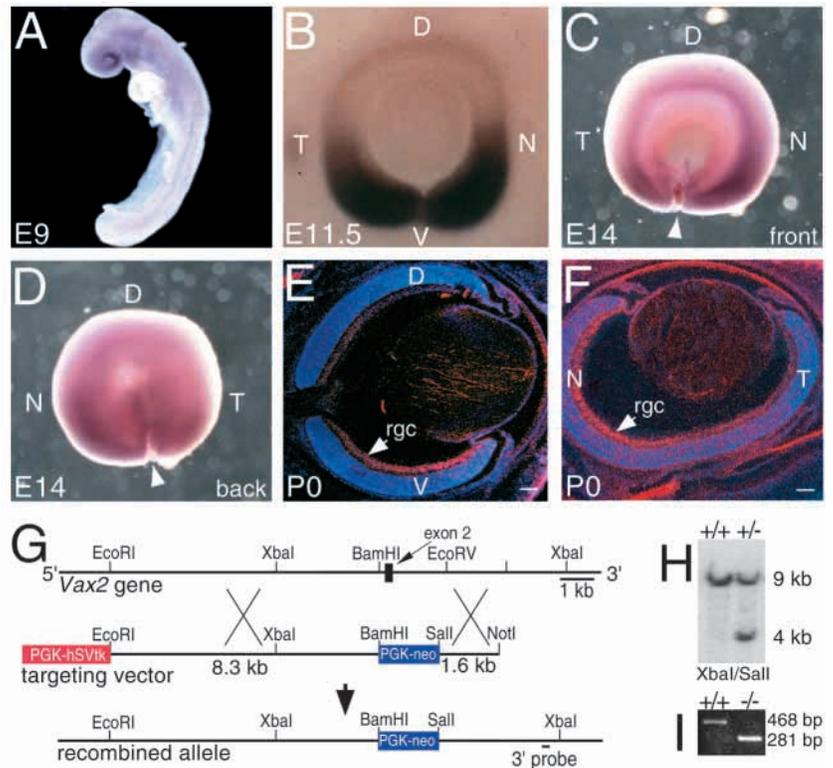


Fig. 1. *Vax2* mRNA expression in the mouse and inactivation of the mouse *Vax2* gene. (A) Whole-mount in situ hybridization for *Vax2* mRNA in an E9 mouse embryo. (B) High-power view of an E11.5 mouse eye, illustrating the pronounced dorsal-ventral (DV) *Vax2* gradient. D, V, N, and T indicate dorsal, ventral, nasal and temporal poles of the retina. (C,D) Front (C) and back (D) views of a whole-mount in situ hybridization for *Vax2* mRNA (purple reaction product) in a dissected mouse retina at E14. Arrowhead denotes a ventral cut made before the eye was dissected from the embryo. (E) In situ hybridization of a coronal section through a wild-type P0 mouse eye, demonstrating high expression of *Vax2* mRNA in the retinal ganglion cells (rgc) of the ventral (V) but not the dorsal (D) retina. (F) In situ hybridization of a transverse section through a wild-type P0 mouse eye, demonstrating slightly higher expression of *Vax2* mRNA in the retinal ganglion cells (rgc) of the nasal (N) than the temporal (T) retina. (G) Targeting construct for inactivation of the mouse *Vax2* gene, and structure of the inactivated allele after homologous recombination in mouse embryonic stem cells. A *Bam*HI/*Eco*RV genomic fragment containing exon 2 of the *Vax2* gene was replaced by a PGK-neo cassette (see Materials and Methods). Exon 2 encodes amino acid residues 83-145; these residues include the first two α helices of the *Vax2* homeodomain, which are essential to the function of all known homeodomain transcription factors. The *Bam*HI/*Eco*RV deletion also introduces a shift in the *Vax2* reading frame. (H) Southern blot of *Xba*I and *Sal*I-digested genomic DNA from wild-type (+/+) and heterozygous (+/-) ES cell clones probed with the external 3' probe indicated in G and described in Materials and Methods. (I) RT-PCR analysis of mRNA isolated from wild-type (+/+) and *Vax2* mutant (-/-) retinæ at P0. The primers for the PCR reaction are located in exons 1 and 3, and correspond to nucleotides 127-148 and 586-606 of the mouse *Vax2* cDNA sequence (Genbank Accession Number, Y17792). The size of the -/- fragment is consistent with the clean excision of exon 2, which was confirmed by DNA sequence analysis of the 468 and 261bp bands, and by the loss of *Vax2* immunoreactivity in the homozygous mutant retina (data not shown). Scale bar: 0.1mm in E,F.

mutant retinæ (30-60% of the NT axis), collicular TZs typically appeared near the expected medial location, occasionally as single, well-formed TZs (Fig. 2M). When ectopic lateral TZs were observed, they were most frequently small (Fig. 2B, lower panel and Fig. 2N-R). Finally, ventral DiI injections into the most nasal regions of the *Vax2* mutant retina

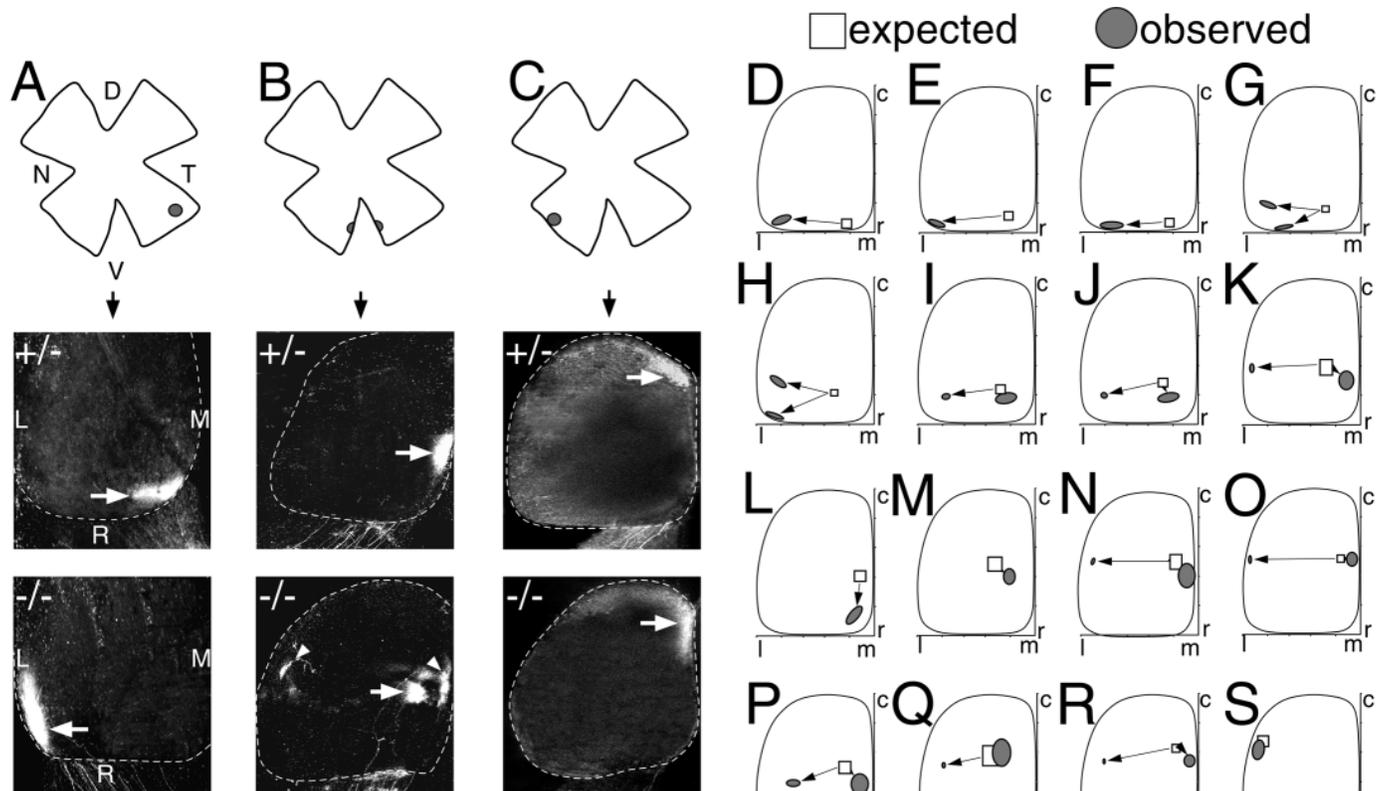


Fig. 2. Aberrant projections of retinal ganglion cell (RGC) axons from the retina to the superior colliculus (SC) in *Vax2* mutants, as assessed by anterograde axonal tracing with DiI. (A) A focal DiI injection into the ventral temporal (VT) quadrant of the retina (indicated by the circle on the upper panel retinal flatmount schematic) labels RGC axons that terminate at the medial-rostral (MR) border of the SC in wild-type and heterozygous mice [+/-, middle panel, arrow indicates termination zone (TZ)], but at the rostral-lateral border of the SC in the mutant [-/-, lower panel, arrow]. Note that for all anterograde labeling we analyzed, the projections of heterozygous *Vax2*^{+/-} axons were indistinguishable from the previously described projections of wild-type RGCs. (B) A mid-ventral injection (split circle on schematic) labels RGC axons that terminate near the medial border of the SC at the midpoint of the collicular RC axis in both heterozygous (+/-, middle panel, arrow) and mutant mice (-/-, lower panel, arrow). Arrowheads indicate additional multiple ectopic TZs in the mutant, some of which are lateralized. (C) A focal injection into the ventral nasal quadrant of the retina (circle, upper panel) labels RGC axons that terminate in the medial-caudal SC in both heterozygous (+/-, middle panel) and *Vax2* mutant mice (-/-, lower panel). (D-W) SC diagrams depicting expected (squares) and observed collicular TZs (gray) from focal DiI injections into the ventral retinae of *Vax2* mutants, illustrating the progressive change in misprojection phenotype from extremely strong for ventral temporal injections (D-F) to extremely weak for ventral nasal injections (U-W). The position of the expected TZ in the SC is plotted from the observed position of the retinal injection site, assuming a linear map from extreme ventral retina=extreme medial SC to extreme dorsal retina=extreme lateral SC. Squares and gray shapes are scaled to indicate relative sizes of retinal injection sites and collicular TZs, respectively. With the exception of S, all panels illustrate results from ventral retinal injection between 60–85% of the DV axis, with extreme dorsal defined as 0%. c, caudal; l, lateral; m, medial; r, rostral.

(0–30% of the retinal NT axis) almost always yielded single tight TZs at the expected wild-type location in the medial SC (Fig. 2C, Fig. 2T–W). Innervation space in the rostral medial quadrant of the *Vax2*^{-/-} SC vacated by lateralized ventral temporal RGC axons appeared to be occupied by TZs from RGCs whose axons would normally occupy extreme medial locations near the midpoint of the RC axis (Fig. 2L). Thus, the *Vax2*^{-/-} axon projection phenotype progressed from extremely strong and fully penetrant in the ventral temporal retina (Fig. 2A, D–F) to almost non-existent in the ventral nasal retina (Fig. 2C, U–W). Note that this NT phenotypic progression is

the inverse of the shallow gradient of *Vax2* mRNA (see Discussion).

Maintenance of ipsilateral projections

At maturity, the vast majority (>95%) of RGC axons in the mouse cross at the optic chiasm and innervate the contralateral SC; the rare ipsilateral projections originate primarily in the ventral temporal quadrant of the retina (Sretavan and Kruger, 1998). Although ventral temporal RGC projections are those that are most strongly perturbed in the contralateral SCs of the *Vax2* mutants, we nonetheless consistently detected ipsilateral

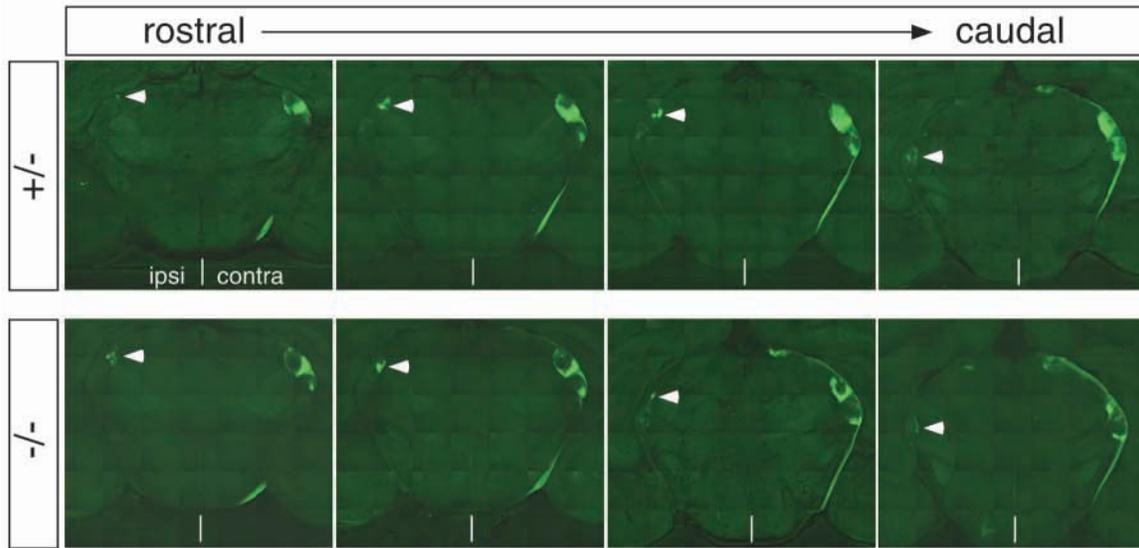


Fig. 3. Maintained ipsilateral projections in the *Vax2* mutants. Whole single eye injections with fluorescent cholera toxin B subunit label abundant contralateral (contra) and rare ipsilateral (ipsi) RGC projections in both *Vax2*^{+/-} (upper panels) and *Vax2*^{-/-} (lower panels) mice at P7. Arrowheads indicate ipsilateral termination sites of RGCs in successive rostral to caudal serial coronal sections, spaced at 200 μ m, which traverse the thalamus into the SC. The ventral midline is indicated by the vertical lines.

RGC projections after full eye fills with fluorescent anterograde axonal tracers in these mice (Fig. 3; $n=5$). Indeed, in some mice, these ipsilateral projections appeared to be atypically abundant.

Flattened DV gradients of axon guidance cues

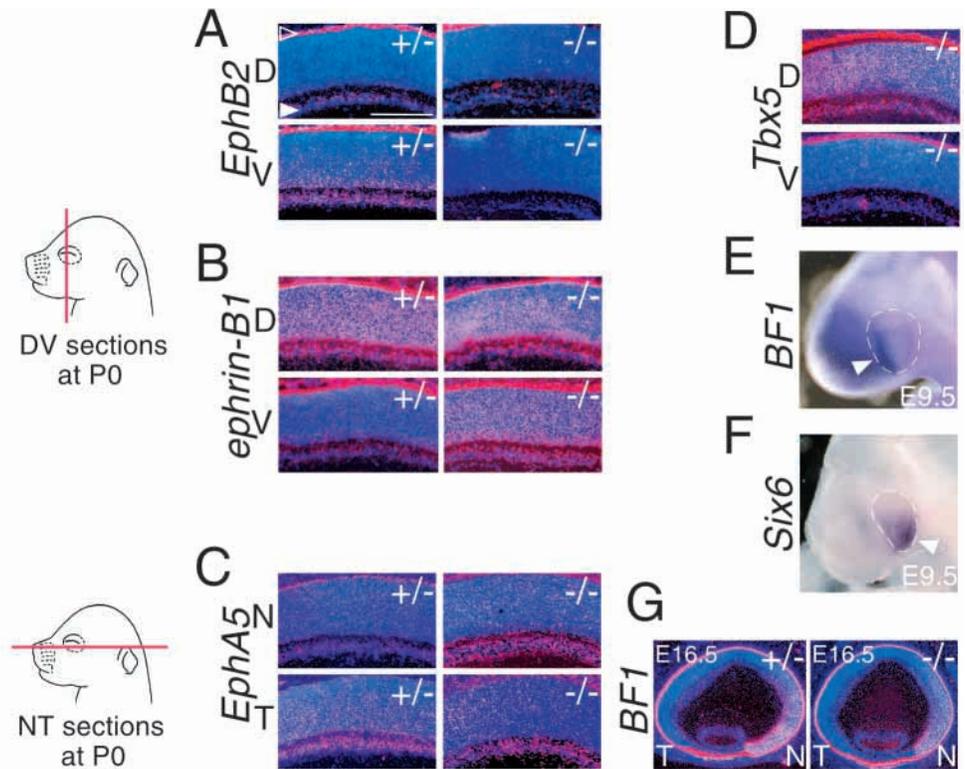
The aberrant RGC projections seen in the *Vax2* mutants suggest that this transcription factor normally patterns the mouse retina beginning around E10, and thereby directly or indirectly controls expression of later position-dependent axon guidance cues. *Vax2* misexpression in the dorsal chick and frog retina results in (1) the upregulated expression of mRNAs encoding the EphB2 and EphB3 receptor tyrosine kinases, which are normally ventrally restricted, and (2) the downregulated expression of mRNAs encoding their ligands ephrin-B1 and ephrin-B2, which are normally dorsally restricted (Barbieri et al., 1999; Schulte et al., 1999). Similarly, mRNA encoding a dorsally restricted transcription factor and presumed DV regulator – *Tbx5* – is downregulated by these gain-of-function manipulations. Consistent with a subset of these earlier observations, we found that ventral expression of both the *Ephb2* and *Ephb3* mRNAs was lost in the *Vax2*^{-/-} ventral retina (Fig. 4A and data not shown), and that conversely, ventral expression of the ephrin-B1 and ephrin-B2 mRNAs (*Efnb1* and *Efnb2* – Mouse Genome Informatics) was acquired (Fig. 4B and data not shown). In addition to these DV alterations, and consistent with our detection of a shallow NT *Vax2* gradient, we also observed a partial reordering of the retinal NT axis in the ventral retina of the *Vax2*^{-/-} mutants. For example, *Epha5* mRNA, which is normally distributed in a low-nasal-to-high-temporal gradient throughout the DV axis (Brown et al., 2000), was upregulated in the ventral nasal mutant retina (Fig. 4C). Thus, each of the above changes leads to flattened or abolished gradients of guidance cue mRNAs that are normally graded along both the DV and NT axes (compare left and right panels in Fig. 4A-C). This global regulation of multiple guidance cues

is apparently crucial, as preliminary analysis of mice doubly mutant for the *Ephb2* and *Ephb3* genes indicates that medial-lateral RGC axon targeting defects in these double EphB mutants are much less pronounced than those detailed above for the *Vax2* single mutants (R. H., T. McLaughlin and D. D. M. O., unpublished).

Maintained DV gradients of transcriptional regulators

While *Vax2* orchestrates the expression of several genes that encode graded axon guidance cues, the gradients of transcription factors that have been hypothesized to define retinal axial polarity, and thereby set up the guidance cue gradients, remained unchanged in the *Vax2* mutants. Notably, the expression of *Pax2*, an early embryonic marker of the ventral retina (Koshiba-Takeuchi et al., 2000; Schulte et al., 1999) remained graded, low-dorsal-to-high-ventral, in early *Vax2* mutant embryos (data not shown). Similarly, the inverse high-dorsal-to-low-ventral gradient of mRNA encoding the T box transcription factor *Tbx5*, which is the earliest known dorsal retinal marker (Koshiba-Takeuchi et al., 2000), was, unlike ephrin-B1 and -B2, unchanged in the *Vax2* mutants at E10.5 and P0 (Fig. 4D, and data not shown). Although gain-of-function *Vax2* misexpression experiments have suggested that *Tbx5* is normally repressed by *Vax2* (Schulte et al., 1999), our results demonstrate that this is not the case: loss of *Vax2* in the ventral retina does not lead to *Tbx5* upregulation. As *Tbx5* misexpression experiments have similarly suggested that *Vax2* may be repressed by *Tbx5* (Koshiba-Takeuchi et al., 2000), analysis of retinal polarity in *Tbx5* loss-of-function mutants will be required in order to clarify the relationship between these transcription factors. Nonetheless, our results indicate that the highly polarized expression of *Tbx5* does not require *Vax2*. Similarly, the homeodomain protein *Six6* and the winged-helix transcription factor *Bf1* (*Foxg1* – Mouse Genome Informatics) (Huh et al., 1999; Lopez-Rios et al., 1999), which

Fig. 4. Flattened gradients of axon guidance cues and maintained gradients of transcription factors in the retinae of *Vax2* mutants at birth. (A) *Ephb2* mRNA (visualized as pink/red silver grains) is expressed in a high ventral (V)/low dorsal (D) gradient in both the wild-type and heterozygous retina (+/-, lower and upper left panels, respectively), and is lost in the ventral retina of the mutants (-/-). The positions of the retinal ganglion cell and pigment epithelial layers are denoted by filled and open arrowheads, respectively, in the upper left panel. (The intense signal seen in the pigment epithelial layer of nearly all panels is an artifact caused by refraction of pigment when viewed with dark field optics. The image used for C was, by chance, generated from an unpigmented mutant.) Essentially identical results were obtained for mRNA encoding the closely related and similarly graded receptor EphB3. (B) mRNA encoding ephrin-B1, a ligand for EphB2/B3, is expressed in a high dorsal (D)/low ventral (V) gradient, the inverse of EphB2/B3, in the heterozygous retina (+/-, left panels), and this mRNA is upregulated in the ventral retina of the mutants (-/-, right panels). Essentially identical results were obtained for mRNA encoding the closely related and similarly graded ligand ephrin-B2. (C) *Epha5* mRNA is expressed in a high temporal (T)/low nasal (N) gradient in the heterozygous retina (+/-, left panels), and is upregulated in the ventral nasal mutant retina of the mutant (-/-, right panels). (D) mRNA encoding the transcription factor *Tbx5* exhibits a pronounced high-dorsal-to-low-ventral gradient of expression in wild-type P0 mouse retinae (Koshiba-Takeuchi et al., 2000), and this gradient is not changed in the *Vax2* mutants (-/-). (E) Expression of *Bfl1* mRNA (dark purple reaction product) at E9.5 in wild type is most pronounced in the ventral nasal quadrant (arrowhead) of the optic vesicle (circled). Anterior is towards the left. (F) Expression of *Six6* mRNA at E9.5 in wild type is most pronounced in the ventral temporal quadrant (arrowhead) of the optic vesicle (circled). Anterior is towards the left. (G) Radioactive in situ hybridization to transverse sections of heterozygous (+/-) and mutant (-/-) retinae at E16.5 demonstrate unaltered expression of *Bfl1* mRNA, which remains preferentially expressed in the nasal (N) as opposed to temporal (T) side of the eye. Scale bar: 0.1 mm.



are also hypothesized regulators of retinal axial polarity, and which at E9.5 exhibit expression in the ventral nasal (Bf1) and ventral temporal (Six6) mouse retina, respectively (Fig. 4E,F), were unchanged in their expression domains in the *Vax2* mutants (Fig. 4G, and data not shown). These domains are dynamic for both Bf1 and Six6 from E9.5 to birth: Bf1 is first expressed in the ventral nasal retina (Fig. 4E) and by E12.5 moves to the dorsal nasal retina; and Six6 is first detected in the ventral temporal retina (Fig. 4F), but later occupies the entirety of the retina. These dynamic expression domains were unaffected in the *Vax2* mutants, as assessed by in situ hybridization at E10.5, E12.5, E14.5, E16.5 and P0 (Fig. 4G, and data not shown).

DISCUSSION

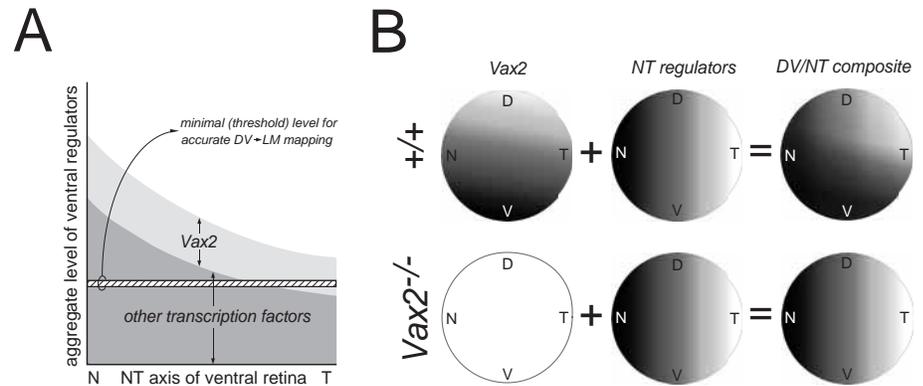
Our loss-of-function analyses definitively demonstrate that *Vax2* controls DV polarization of the mouse retina. This transcription factor directly or indirectly activates expression of ventral axon guidance cues such as EphB2 and EphB3, and at the same time represses dorsal cues such as ephrin-B1 and ephrin-B2. In the temporal regions of the ventral retina, the loss of *Vax2* results in RGC projections to the contralateral SC

that are completely dorsalized relative to their wild-type counterparts.

These results notwithstanding, our analyses also demonstrate that the ventralizing influence of *Vax2* is in two respects incomplete. First, although *Vax2* expression normally extends across the entirety of the ventral retina, the dorsalization of RGC projections in the *Vax2* mutant retina does not. As detailed above (Fig. 2), dorsalization is robust only in the extreme ventral temporal retina, and becomes progressively less severe in progressively more nasal regions of the retina. In the extreme ventral nasal retina, mutant RGC projections are indistinguishable from wild type. And second, some of the phenotypes seen upon loss of *Vax2* are not consistent with those reported for *Vax2* gain-of-function studies. Most notably, loss of *Vax2* does not lead to the activation of *Tbx5* expression in the ventral retina, or to any other obvious perturbation in the high-dorsal-to-low-ventral gradient of *Tbx5* expression. These latter observations suggest that transcriptional specifiers of vertebrate retinal polarity, like many transcriptional specifiers of retinal identity (Marquardt et al., 2001), operate independently.

The surprising finding that the axonal targeting errors of ventral temporal RGCs are much more severe than those of ventral nasal RGCs suggests that either: (1) *Vax2* interacts with

Fig. 5. Two models for the concerted combinatorial action of *Vax2* and other transcriptional regulators along the nasal-temporal (NT) axis of the ventral retina. (A) Ventral regulators are normally expressed at higher levels in the nasal than the temporal ventral retina, and a minimum aggregate level of the regulators (indicated by the striped bar) is necessary for proper retinocollicular mapping. When one of the regulators (e.g. *Vax2*) is removed by mutation, the aggregate level becomes limiting (falls below the threshold bar) only in the ventral temporal retina. (B) *Vax2* (left discs), which is normally steeply graded along the DV axis, genetically interacts with one or more genes that are graded along the NT axis (middle discs), to form a composite wild-type map of axon guidance regulators (right discs). With the loss of *Vax2*, the map reverts to that of the NT genes alone, which again most severely affects ventral temporal RGCs. See text for details.



one or more additional transcriptional regulators that are similarly graded along the DV and NT axes, such that a 'limiting concentration effect' of these nuclear proteins is detected in the mutants only in the retinal quadrant where their aggregate level is normally lowest (Fig. 5A); or (2) the *Vax2* gene, which is steeply graded along the retinal DV axis, normally interacts genetically with one or more genes that are steeply graded along the NT axis (Fig. 5B). With regard to the second possibility, other homeodomain transcription factor genes, such as *SOHO1* and *GH6* in the chick (Schulte and Cepko, 2000), have been shown to be graded along the retinal NT axis, and have been hypothesized to control the expression of NT guidance cues such as EphA receptors and their ephrin-A ligands. The homologs of *SOHO1* and *GH6* remain to be analyzed in the mouse, but the transcription factor *Bf1* is specifically expressed, from E8.5-E9.5, in the mouse ventral nasal retina (Fig. 4E). Although the EphA/ephrin-A guidance cues have been analyzed almost exclusively in terms of RGC mapping along the rostral-caudal axis of the SC (Brown et al., 2000; Feldheim et al., 2000), it is interesting to note that medial-lateral mapping anomalies have been consistently observed in ephrin-A2/A5 double mutants (Feldheim et al., 2000). If, as schematized in Fig. 5B, *Vax2* normally acts in concert with one or more transcription factors that are graded along the NT axis, then RGCs in the ventral temporal retina would again be those most sensitive to the loss of *Vax2*. Direct tests of these and related models must await the generation and analysis of a set of single and compound mutants of *Vax2* with *Bf1*, *SOHO1*, *GH6*, ephrin-A2 and ephrin-A5, among others.

Together, our results identify *Vax2* as an essential specifier of both DV and NT axial polarity in the developing mammalian eye. They demonstrate that the steeply graded expression of *Vax2* that first appears near the midpoint of mouse embryogenesis is essential to the ventral specification of the retina, most prominently in its ventral temporal quadrant. Importantly, they further demonstrate that this specification is independent of more widely expressed transcription factors that have also been thought to act during axial determination of the eye, and suggest that these regulators normally act in concert with *Vax2*.

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