Foxd1 is required for proper formation of the optic chiasm

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Summary

In animals with binocular vision, retinal ganglion cell (RGC) axons from each eye sort in the developing ventral diencephalon to project to ipsi- or contralateral targets, thereby forming the optic chiasm. Ipsilaterally projecting axons arise from the ventrotemporal (VT) retina and contralaterally projecting axons primarily from the other retinal quadrants. The winged helix transcription factor Foxd1 (previously known as BF-2, Brain Factor 2) is expressed in VT retina, as well as in the ventral diencephalon during the formation of the optic chiasm. We report here that in embryos lacking Foxd1, both retinal development and chiasm morphogenesis are disrupted. In the Foxd1 deficient retina, proteins designating the ipsilateral projection, such as Zic2 and EphB1, are missing, and the domain of Foxg1 (BF-1) expands from nasal retina into the VT crescent. In retina-chiasm co-cultures, VT RGCs from Foxd1 deficient retina are not repulsed by chiasm cells, and in vivo many VT RGCs aberrantly project contralaterally. However, even though the ipsilateral program is lost in the retina, a larger than normal uncrossed component develops in Foxd1 deficient embryos. Chiasm defects include axon stalling in the chiasm and a reduction in the total number of RGCs projecting to the optic tract. In addition, in the Foxd1 deficient ventral diencephalon, Foxg1 invades the Foxd1 domain, Zic2 and Islet1 expression are minimized, and Slit2 prematurely expands, changes that could contribute to axon projection errors. Thus, Foxd1 plays a dual role in the establishment of the binocular visual pathways: first, in specification of the VT retina, acting upstream of proteins directing the ipsilateral pathway; and second, in the patterning of the developing ventral diencephalon where the optic chiasm forms.

Key words: Foxd1, Foxg1, Brain Factor 1, Brain Factor 2, BF-1, BF-2, Retinal axon divergence, Zic2, EphB1

Introduction

During visual system development, retinal ganglion cell (RGC) axons leave the retina via the optic disc and navigate through the optic nerve until they reach the optic chiasm. There they diverge to form the optic tracts, and project to ipsi- or contralateral targets, the lateral geniculate nucleus (LGN) and the superior colliculus. This decussation of retinal axons at the optic chiasm is essential for the establishment of binocular vision.

Foxg1 (also named Brain Factor 1, BF-1) and Foxd1 (also named Brain Factor 2, BF-2) are winged helix transcription factors characterized by a DNA-binding motif originally identified in HNF3 and found in *Drosophila* forkhead proteins (Hatini et al., 1994). Foxg1 and Foxd1 are expressed in adjacent domains in the neural tube at the time the optic vesicles evaginate. Foxg1 is expressed in the nasal optic cup and Foxd1 is expressed in a complementary pattern temporally (Clark et al., 1993). As misexpression of Foxg1 and Foxd1 in chick retina results in projection errors of retinal axons along the anteroposterior axis in the tectum (Takahashi et al., 2003; Yuasa et al., 1996), this pair of proteins has been proposed to determine the regional specificity of axon projection along the nasotemporal retina through the regulation of downstream targets. Thus, for example, misexpression of Foxg1 in the temporal retina represses the tyrosine kinase receptor EphA3 and the expression of Foxd1, and induces the expression of members of the ephrin-A family (Takahashi et al., 2003).

In mouse, both Foxg1 and Foxd1 are also expressed in the developing ventral diencephalon, with the Foxd1 domain including the region in which early retinal axons establish the optic chiasm, whereas Foxg1 is located more rostrally. Both genes are therefore well positioned to play a role in the regionalization of the optic chiasm (Marcus et al., 1999). However, despite the recent advances in understanding the role of Foxg1 and Foxd1 in retinal axial polarity and retino-tectal projections, and in understanding their relationship to the Eph/ephrin-A family, the extent to which these transcription factors regulate aspects of retinal specification and axon navigation in other portions of the retinal axon pathway, such as in the optic chiasm, remained unexplored. Because the

retinofugal projection in the chick lacks an uncrossed projection at the optic chiasm (Thanos and Mey, 2001), additional studies are needed to determine whether Foxg1 and Foxd1 are required for patterning the visual projection in animals with binocular vision such as the mouse. Recent analyses in mice lacking Foxg1 show an increase in the number of ipsilateral axons, suggesting a role for Foxg1 in the formation of the optic chiasm (Pratt, 2004).

Mice that lack Foxd1 (Foxd1$^{lacZ/lacZ}$ mice) die at birth due to kidney malformations but appear to have grossly normal eye development (Hatini et al., 1996). In this study we used mice lacking Foxd1 to further study the role of Brain Factor genes in retinofugal pathway development during the period of chiasm formation (E14-E18). RGCs that project ipsilaterally are located in the ventrotemporal (VT) crescent in the mouse retina, whereas axons projecting contralaterally arise from RGCs throughout the retina. The decision of RGCs from the VT retina to project ipsilaterally at the optic chiasm is regulated by the zinc-finger transcription factor Zic2 (Herrera et al., 2003), and is subsequently mediated by the pair of tyrosine kinase factors implicated in axon guidance, EphB1/ephrin-B2, with EphB1 acting as a receptor in ipsilateral RGC axons, and ephrin-B2 expressed in midline glial cells at the optic chiasm and functioning as a ligand (Williams et al., 2003). Other guidance factors, such as Slit2 (Erskine et al., 2000; Plump et al., 2002), chondroitin sulfate proteoglycans (Chung et al., 2000) and ephrin-As (Marcus et al., 1996a; Marcus et al., 2000) that have been localized in specialized populations of neurons and glia at the chiasmatic midline, function to pattern the overall organization of the optic chiasm, rather than in the decussation of RGC axons. At present, whether any of these, or other, factors are involved specifically in crossing the midline is unknown.

We report here that the absence of Foxd1 leads to disruption in the shape of the optic chiasm and in the proportion of fibers projecting ipsi- versus contralaterally, and to stalling or misrouting of retinal axons at the optic chiasm. Although eye development and RGC differentiation appear to be normal in the Foxd1 deficient retina, later-expressed genes specifying the ipsilateral RGC axon projection in retina are lost, and regionalization of the ventral diencephalon is altered, both aspects that are important for proper chiasm formation.

Materials and methods

Animals

Foxd1$^{lacZ/+}$ and Foxd1$^{lacZ/lacZ}$ mice have been previously described; in these mice, the *lacZ* gene was substituted for the coding region of *Foxd1*, allowing *lacZ* expression to serve as a marker of *Foxd1* expression (Hatini et al., 1996). Foxd1$^{lacZ/lacZ}$ embryos, also referred to as Foxd1 deficient, were obtained from heterozygote crosses. E0 was defined as midnight of the night before a plug was found. Embryos were removed from anesthetized mothers by caesarian section.

Anterograde and retrograde labeling of RGC axons

Anterograde labeling of the whole retinal projection with DiI, and retrograde tracing using rhodamine-dextran applied to the optic tract, were performed as described (Erskine et al., 2000; Herrera et al., 2003; Rachel et al., 2002).

To count retrogradely labeled RGCs in the retina ipsilateral to optic tract labeling in Foxd1$^{lacZ/lacZ}$ embryos, a region was delineated in the VT crescent in flattened retinal wholemounts of Foxd1$^{+/+}$ embryos by two perpendicular lines: one at the central boundary of the ventrotemporal crescent (defined as the region containing all of the dextran-labeled RGCs); and the second perpendicular to this line and to the border of the retina, 300 μm in length. We superimposed these lines onto the ventrotemporal region of Foxd1$^{lacZ/lacZ}$ flattened retinal wholemounts, taking as a reference point the labeled cells closest to the peripheral border of the retina. All of the labeled cells inside the region delimited by these lines were then counted.

Co-culture assays

E14.5 retina and chiasms from embryos from Foxd1$^{+/+}$×Foxd1$^{lacZ/lacZ}$ crosses were dissected. Foxd1$^{+/+}$ or Foxd1$^{lacZ/lacZ}$ retinal explants were co-cultured with either Foxd1$^{+/+}$ or Foxd1$^{lacZ/lacZ}$ dissociated chiasm cells, as previously described (Herrera et al., 2003; Marcus and Mason, 1995; Marcus et al., 1996b; Wang et al., 1995; Williams et al., 2003).

X-gal histochemistry and immunohistochemistry

Retinal wholemounts were dissected, treated with 3% H_2O_2 in PBS to block endogenous peroxidase activity, washed in PBS, fixed in 2% paraformaldehyde (PFA) and then incubated with 1 mg/ml X-gal diluted in 30 mM $K_3Fe(CN)_6$, 30 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ to detect *lacZ* expression. The tissue was then processed for immunohistochemistry with anti-Zic2 antibodies (Brown et al., 2003) and anti-rabbit-peroxidase antibodies.

For immunohistochemistry on sections, 4% PFA-fixed cryosections were incubated with 10% normal goat serum (NGS)-1% Triton X-100 in PBS for 1 hour at room temperature. Sections were then incubated in anti-Brn3b, anti-Islet1/2 (K4, gift of Dr T. Jessell, Columbia University), anti-SSEA (Marcus et al., 1995) or anti-Zic2, for 1 hour at room temperature; washed six times for 30 minutes each wash in PBS at room temperature; and then incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch) for 1 hour at room temperature.

In situ hybridization

In situ hybridization, using digoxigenin-labeled riboprobes, was performed on 20 μm cryosections (Schaeren-Wiemers and Gerfin-Moser, 1993). Rat cDNA clone IMAGE 1003496 was used as a template to detect Islet2 mRNA. Ephrin-B2 and EphB1 specific probes were generated as described by Williams et al. (Williams et al., 2003). A Slit2 probe was generated as described by Erskine et al. (Erskine et al., 2000). The specific probe used to detect *Foxg1* mRNA was similar to that previously described by Hatini et al. (Hatini et al., 1994).

Antibody fusion protein binding

The distributions of ephrin-A were visualized by receptor-antibody fusion protein binding on unfixed frozen sections. Receptor antibody fusion proteins consisting of the extracellular portions of the EphA receptors fused to the human IgG1 Fc domain were used. Briefly, 15-μm-thick sections collected on subbed slides, then blocked for 30 minutes in a solution containing 10% normal goat serum, 2% BSA and 0.02% Na Azide. Slides were then incubated in 2 μg/ml of receptor fusion protein in 0.5×blocking solution for 1 hour. The tissue was then fixed in fresh 4% PFA, washed in PBS, heat treated at 70°C for 1 hour to destroy endogenous phosphatase activity, and incubated for 1 hour in goat anti-human IgG alkaline phosphatase-conjugated secondary antibody (diluted 1:1000 in 0.5× blocking solution). Sections were color developed using NBT/BCIP and mounted (Marcus et al., 1996a; Marcus et al., 2000).

Results

The absence of Foxd1 leads to aberrant optic chiasm formation

To investigate whether Foxd1 is required for the normal

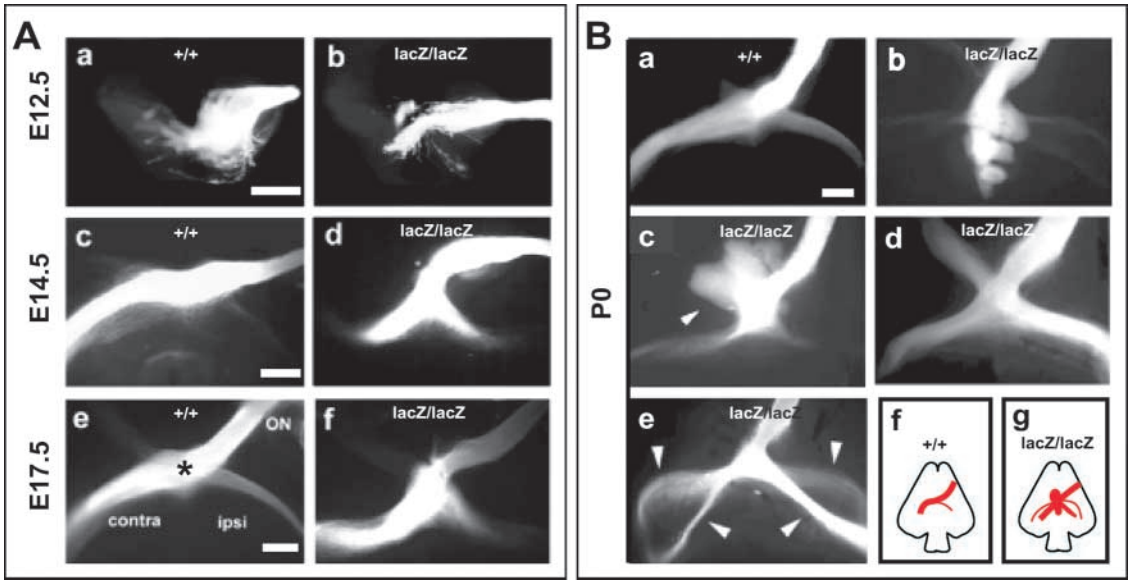

Fig. 1. The optic chiasm is perturbed in Foxd1 deficient embryos. (A) Anterograde DiI labeling in E12.5, E14.5 and E17.5 Foxd1$^{+/+}$ (a,c,e) and Foxd1$^{lacZ/lacZ}$ (b,d,f) embryos reveals that the characteristic X-shape of the optic chiasm becomes elongated and splayed, and that the ipsi- to contralateral ratio of retinal axons increases. Contra, contralateral projection; ipsi, ipsilateral projection; ON, optic nerve; asterisk, midline. (B) P0 embryos lacking Foxd1 (Foxd1$^{lacZ/lacZ}$, b-e) display four aberrant phenotypes; compare with Foxd1$^{+/+}$ embryos (a,f). (b) Most retinal axons are arrested at the midline and end in 'nodules'. (c) Retinal axons reach the midline in a more caudal position than in Foxd1$^{+/+}$ littermates and project contra- or ipsilaterally. Axons are arrested as the optic nerves converge (asterisk), adding to the increased breadth of the chiasmatic crossing point; some axons are routed into the optic tracts and grow in an aberrant ipsi- versus contralateral ratio. (d) The position of the crossing site is shifted caudally and the RGC project ipsi- or contralaterally with a greatly increased uncrossed component. (e) Retinal axons take four different routes (arrows) after leaving the optic chiasm, forming two optic tracts on each side rather than one. (f,g) Diagrams summarizing the chiasm phenotypes in Foxd1$^{+/+}$ and Foxd1$^{lacZ/lacZ}$ mice. Scale bars: 200 μm.

establishment of the optic chiasm, we assessed the retinofugal pathway in Foxd1$^{+/+}$ and Foxd1$^{lacZ/lacZ}$ embryos from E12.5, when RGCs axons are growing toward the midline, to P0, when chiasm formation is completed.

During the establishment of the optic chiasm in Foxd1$^{+/+}$ mice, pioneer axons arising from dorsocentral retina at E12.5 leave the optic cup through the optic disc and navigate into the optic stalk to the developing ventral diencephalon. There, they grow in close relationship to the inverted V-shaped array of CD44/SSEA neurons, postulated to be important for the establishment of the correct position of the X-shaped optic chiasm (Marcus and Mason, 1995; Mason and Sretavan, 1997). A small proportion of these early axons turn to the same side of the brain, distant from the midline, forming an early uncrossed projection that is thought to be transient (Guillery et al., 1995; Mason and Sretavan, 1997) (Fig. 1A, part a).

In E12.5-E13.5 Foxd1$^{lacZ/lacZ}$ embryos, RGC axons grow as in Foxd1$^{+/+}$ embryos from the time they leave the retina and enter the optic stalk, but their behavior is abnormal when they approach the midline. In Foxd1 mutant mice, all RGC axons traverse the diencephalon by growing straight towards the midline. Once there, they turn sharply ventrally and project either contra- or ipsilaterally (Fig. 1A, parts a,b).

By E14.5, many axons have reached the midline area in wild-type embryos, and the typical X-shape of the chiasm is apparent. In addition, the first permanent retinal axons project ipsilaterally from the VT retina (Fig. 1A, part c). In Foxd1$^{lacZ/lacZ}$ embryos, however, the X-shape of the chiasm is more elongated, and the ipsi- and contralateral components seem to be comparable in size (Fig. 1A, part d), in contrast to the predominantly contralateral pattern of the Foxd1$^{+/+}$ optic chiasm (Fig. 1A, part c).

At E17.5, the altered crossed/uncrossed RGC axon ratio and the difference in the chiasm shape in Foxd1$^{lacZ/lacZ}$ embryos are more evident than at E14.5 (Fig. 1A, parts e,f). At P0, when the development of the optic chiasm is complete in Foxd1$^{+/+}$ mice (Fig. 1B, part a), four distinctive abnormal optic chiasm phenotypes were observed in the Foxd1$^{lacZ/lacZ}$ embryos. (1) Most RGC axons terminate at the midline of the optic chiasm and end in one or more nodules (Fig. 1B, part b). (2) Some RGC axons traverse the midline and enter the optic tracts, but in an altered crossed/uncrossed ratio, and others terminate in nodules in the optic nerves, rostral to the

Table 1. Optic chiasm phenotypes in Foxd1 deficient mice

Genotype	1	2	3	4	Total
Foxd1$^{+/+}$	0	0	0	0	23
Foxd1$^{LacZ/LacZ}$	3 (15%)	10 (50%)	1 (5%)	6 (30%)	20

1, axons stall at the optic chiasm.
2, some axons stall in different parts of the chiasm and enter the optic tracts, with an altered contra- versus ipsilateral ratio.
3, axons project ipsi- or contralaterally but with an increased proportion of ipsilateral axons.
4, axons split into two optic tracts.
The number of embryos in each phenotypic group is indicated in each column.
The percentage of each phenotype is shown in parentheses.

chiasm and lateral to the midline (Fig. 1B, part c). (3) All RGC axons project ipsi- or contralaterally in an abnormal crossed/uncrossed ratio (Fig. 1B, part d). (4) Most RGC axons project ipsi- or contralaterally, but they split, creating two routes on each side rather than one (Fig. 1B, part e). In some cases, a combination of these phenotypes was observed. The penetrance of each of these phenotypes is shown in Table 1. The major phenotype observed in the Foxd1 deficient chiasm is an increase in the proportion of axons that project ipsilaterally relative to the contralateral component, observed in 85% of the cases. Thus, in the absence of Foxd1, the shape and position of the optic chiasm change, and the behavior of the retinal axons is affected at the midline, with many axons misprojecting or stalling at the midline.

Interestingly, in Foxd1-deficient embryos, although many RGC axons never grow past the chiasm, some extend through the optic tract to innervate the lateral geniculate nucleus (LGN) (data not shown).

RGCs that project ipsilaterally arise from the entire retina, rather than only from VT retina

To determine the retinal origin of the increased proportion of RGC axons that aberrantly project to the ipsilateral optic tract of Foxd1$^{lacZ/lacZ}$ mice, we retrogradely labeled RGCs in E17.5 Foxd1$^{lacZ/lacZ}$ and Foxd1$^{+/+}$ embryos with dextran, applied to one optic tract. In the retina contralateral to the labeled optic tract, dextran-labeled RGCs were found across the entire retina in Foxd1$^{+/+}$ embryos except in the peripheral VT crescent that presumably contains the RGCs that project ipsilaterally from this eye (Fig. 2B, part a). In the Foxd1$^{lacZ/lacZ}$ embryos, labeled cells were likewise located across the entire retina, but were also found in the most peripheral VT crescent (Fig. 2B, part b, asterisk). Moreover, overall there were far fewer labeled cells in the Foxd1$^{lacZ/lacZ}$ retina compared with the Foxd1$^{+/+}$ retina, although this reduction was variable from embryo to embryo (Table 2).

In the retina ipsilateral to dextran application in Foxd1$^{+/+}$ mice, retrogradely labeled RGCs were found exclusively in the VT region (Fig. 2C, part a). This region is wider in the central-to-peripheral axis than the cell-free region found in the retina contralateral to dye application (compare Fig. 2B, part a and 2C, part a). The explanation for this difference is that at the time of backfilling, E17.5, the most central boundary of the ipsilateral RGCs and the most peripheral limit of the contralaterally projecting cells partially overlap. This is in contrast to the situation at E14.5-E16.5, when both populations are segregated from one another (Guillery et al., 1995). Strikingly, in Foxd1$^{lacZ/lacZ}$ embryos, RGCs retrogradely labeled from the ipsilateral optic tract were found across the entire retina (Fig. 2C, part b). In addition, there were far fewer RGCs that projected ipsilaterally in the VT crescent of Foxd1$^{lacZ/lacZ}$ retina compared with in the equivalent VT crescent of Foxd1$^{+/+}$ retina (Fig. 2C, part b). Again, this reduction in the number of cells that project ipsilaterally from the VT quadrant in the mutant compared with the Foxd1$^{+/+}$ retina is variable from animal to animal, but in most cases is comparable to the reduction in the number of cells projecting

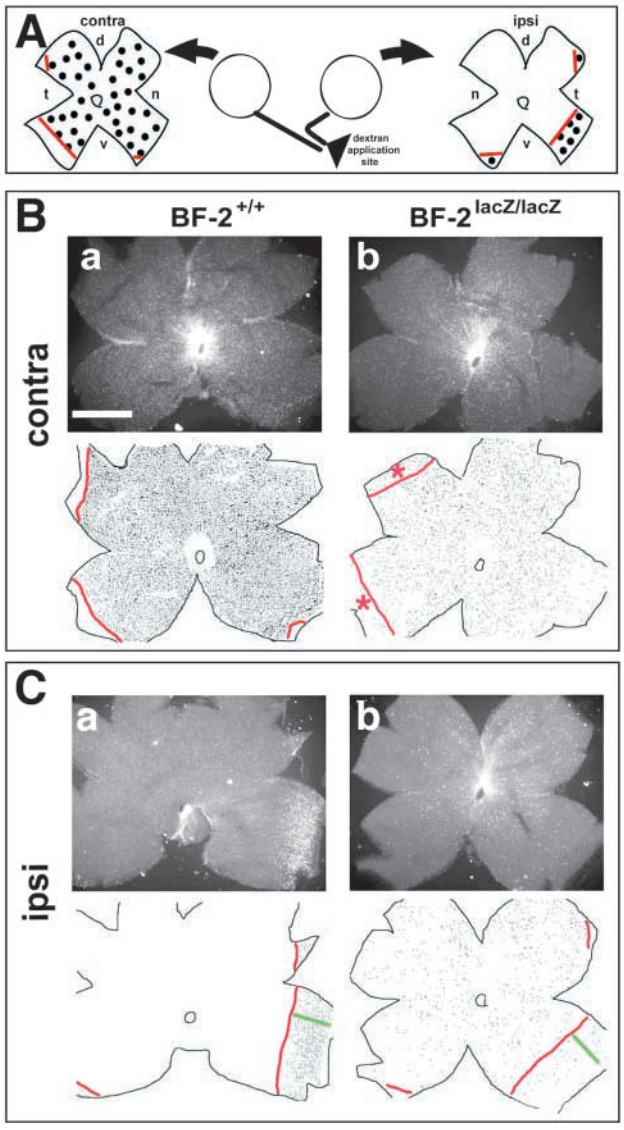

Fig. 2. In Foxd1 (BF-2 in figure) deficient embryos, RGCs that project ipsilaterally arise from the entire retina, rather than exclusively from the ventrotemporal quadrant. (A) Schematic indicates that after retrograde labeling from one optic tract at E17.5, RGCs in Foxd1$^{+/+}$ embryos are labeled over the entire contralateral retina (left), whereas in the ipsilateral eye, RGCs are labeled only in the peripheral ventrotemporal retina (right). (B) Wholemounts of retina after retrograde labeling from the contralateral optic tract at E17.5. (a) In Foxd1$^{+/+}$ retina, contralateral-projecting RGCs come from all retinal quadrants except the VT crescent. (b) In Foxd1 deficient retina contralateral RGCs also arise from all the quadrants, including the VT crescent (asterisks). Overall, there are fewer contralateral labeled cells in Foxd1$^{lacZ/lacZ}$ retina than in Foxd1$^{+/+}$ retina, probably because many axons stall at the optic chiasm. (C) Retinal wholemounts after retrograde labeling from the ipsilateral optic tract at E17.5. In Foxd1 deficient embryos (b), RGCs that project ipsilaterally arise from all retinal quadrants, not only from the VT crescent (a). Red lines in the contralateral retina delimit the VT crescent, where contralateral RGCs are not found in wild-type retina. Red lines in the ipsilateral retina mark the VT crescent, where ipisilateral RGCs are located. Note that both sectors are not equivalent in size; this is because at E17.5, when the backfill labeling is performed, both populations (ipsi- and contralaterally-projecting RGCs) partially intermingle, and the boundary segregating both populations is more peripheral at this time than at E14.5-E16.5. Scale bar: 500 μm.

Table 2. Ipsi- and contralateral projection phenotype in wild-type and Foxd1 deficient mice

Genotype*	Ipsilateral + contralateral RGCs	Total contralateral RGCs	Total ipsilateral RGCs	Ipsilateral RGCs within the VT crescent	Ipsilateral RGCs outside the VT crescent	Percentage ipsis among total labeled RGCs
$Foxd1^{+/+}$	9854	9251	600	603	0	6.1
$Foxd1^{+/+}$	8917	8317	603	600	0	6.7
$Foxd1^{LacZ/LacZ}$	2394	1788	606	46	560	25.3
$Foxd1^{LacZ/LacZ}$	3959	2050	1009	103	906	32.9
$Foxd1^{LacZ/LacZ}$	1811	1011	800	85	715	44.1
$Foxd1^{LacZ/LacZ}$	1500	752	748	48	700	49.8

*Counts from individual mice, based on retrograde labeling via the optic tract.

contralaterally (in Foxd1 deficient versus $Foxd1^{+/+}$ retina, about 15-25% of the normal number; see Table 2).

Unfortunately, due to technical limitations of forward- and backfilling in the same embryo, is not possible to test whether there is a correspondence between the phenotypes observed by DiI-forward filling and the percentage reduction in the number of retrogradely labeled RGCs. However, we hypothesize that the overall reduction in the number of RGCs observed in mutant compared with in $Foxd1^{+/+}$ retina reflects the failure of many RGC axons to project past the optic chiasm into the optic tract. Alternatively, the decrease in the RGCs that are backfilled from the optic tract could be a consequence of a reduction in the number of RGCs in the mutant retina. We favor the first possibility, because (1) the optic nerve is robustly labeled upon forward filling from the optic nerve head, and (2) many axons accumulate before and in the chiasm, reflected by axon tangles and knots, which is evidence of a failure of RGC axons to project further than the chiasm (Fig. 1B, parts b and c).

Thus, in the absence of Foxd1, fewer axons reach the optic tracts, probably because many axons are unable to pass through the optic chiasm. Those axons that do traverse the optic chiasm and that project ipsi- or contralaterally arise from the entire retina rather than from their respective topographic locations in the retina. These results indicate that Foxd1 is important for proper routing at the level of the optic chiasm.

Factors mediating the inhibition of uncrossed retinal axons are lost in the Foxd1 deficient retina

To address the question of whether the misrouting of retinal fibers at the optic chiasm is a consequence of alterations in the retina, in the chiasm, or in both, we co-cultured $Foxd1^{+/+}$ and $Foxd1^{lacZ/lacZ}$ retinal explants with cells dissociated from either $Foxd1^{+/+}$ or $Foxd1^{lacZ/lacZ}$ chiasm. Under normal conditions, axons that extend from VT retinal explants are inhibited by dissociated chiasm cells, whereas axons from the remainder of the retina grow well on chiasm cells (Fig. 3A, part a) (Herrera et al., 2003; Marcus et al., 1995; Marcus and Mason, 1995; Marcus et al., 1996b; Wang et al., 1995). Given the stalling and misrouting seen in contra- and ipsilateral projections in

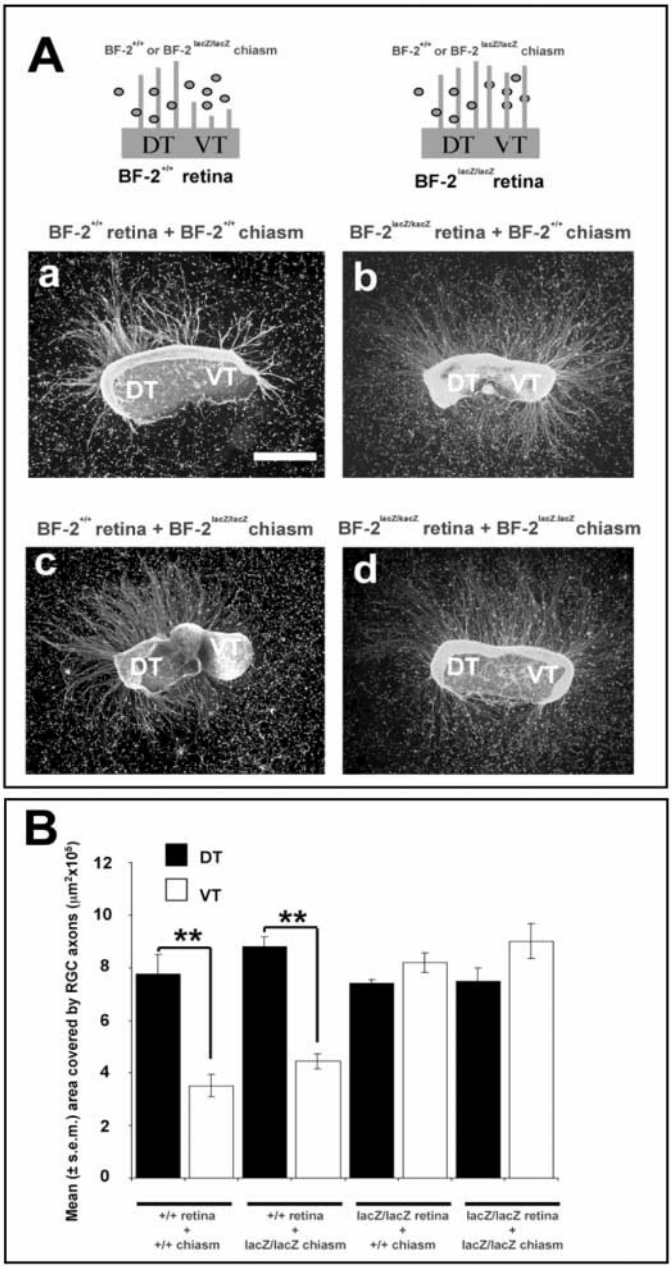

Fig. 3. Neurite growth from Foxd1 (BF-2 in figure) deficient VT explants is not inhibited by chiasm cells. (A) Retinal explants from E14.5 $Foxd1^{+/+}$ (a,c) or $Foxd1^{lacZ/lacZ}$ (b,d) embryos co-cultured with dissociated chiasm cells from $Foxd1^{+/+}$ (a, b) or $Foxd1^{lacZ/lacZ}$ (c,d) embryos. Drawings at the top indicate the two different combinations: (left) RGC axons from $Foxd1^{+/+}$ VT but not DT retina are inhibited by $Foxd1^{+/+}$ or $Foxd1^{lacZ/lacZ}$ chiasm cells, whereas (right) RGC axons from the VT retina of $Foxd1^{lacZ/lacZ}$ embryos are not inhibited by $Foxd1^{+/+}$ or $Foxd1^{lacZ/lacZ}$ chiasm cells. (B) Quantification of mix-and-match experiments shows that RGC axons from $Foxd1^{+/+}$ VT retina are inhibited by chiasm cells from either $Foxd1^{lacZ/lacZ}$ or $Foxd1^{+/+}$ embryos. However, there is a loss of significant difference between VT and DT axon behaviors when retina from $Foxd1^{lacZ/lacZ}$ embryos are co-cultured with $Foxd1^{+/+}$ or $Foxd1^{lacZ/lacZ}$ chiasm cells. $^{**}P<0.005$. Scale bars: 500 μm.

Foxd1$^{lacZ/lacZ}$ embryos in vivo (forward and backfill experiments), we expected a general inhibition of all RGC axons, and/or that axons from contralateral retina would act as ipsilateral axons and vice versa in this co-culture assay. Our results indicate that in Foxd1$^{lacZ/lacZ}$ explants, dorsotemporal (DT) axons behaved like Foxd1$^{+/+}$ RGCs, e.g. they were not inhibited by Foxd1$^{+/+}$ or Foxd1$^{lacZ/lacZ}$ chiasm cells. By contrast, RGCs from Foxd1 deficient VT retina grew well on chiasm cells from Foxd1$^{+/+}$ or Foxd1$^{lacZ/lacZ}$ embryos, rather than being repulsed (Fig. 3A, parts b,d). Interestingly, Foxd1$^{lacZ/lacZ}$ chiasm cells were able to inhibit Foxd1$^{+/+}$ VT retinal axons, suggesting that the chiasm signals that normally repel ipsilateral RGC axons are present in Foxd1 nulls (Fig. 3A, part c).

The co-culture results argue for perturbation of factors within the VT retina that normally mediate RGC inhibition at the chiasm midline, but also suggest that DT retina may not be perturbed in terms of response to midline cues. However, this conclusion is at odds with the results obtained from the apparent chiasm phenotypes observed by anterograde and retrograde labeling, in which both ipsi- and contralateral axons stall or misproject (above). The simplest explanation for these seemingly contradictory results is that the in vitro assay is limited to testing the repulsive response on the part of ipsilateral axons, and is not informative on the behavior of axons from the DT crescent toward chiasmatic cells. This is because: first, the assay detects growth or inhibition only in a two dimensional plane (the Petri dish); and second, chiasm cells are dissociated, losing their original structure and the three-dimensional architecture in which the axons are guided. Thus, these experiments seem to be useful for detecting loss of the ability of RGC axons to respond to repellant cues from dissociated chiasm cells, but do not provide insights as to whether retinal cells from DT retina are mispecified or whether there is a defect in the three-dimensional organization of Foxd1 deficient chiasm cells.

Gene expression is altered in the Foxd1 deficient retina

Differentiation genes

Retrograde labeling experiments showed that fewer axons traverse the chiasm in absence of Foxd1. To test the possibility that the total number of RGCs or that RGC differentiation are affected in Foxd1 deficient embryos, we analyzed the expression of two markers of differentiated RGCs, Islet1 and Brn3b in the Foxd1 deficient retina. Islet1 is a LIM-homeodomain transcription factor expressed in RGCs when they are in S phase of their terminal division (Brown et al., 2000). Brn3b is a POU transcription factor involved in the guidance of RGC axons as they exit from the retina (Liu et al., 2000). Brn3b has also been implicated in other axon guidance events along the retinofugal pathway (Wang et al., 2002; Xiang et al., 1995). Islet1 and Brn3b expression was unchanged in Foxd1 deficient retina, including in the VT crescent (Fig. 4A,B, and data not shown). These results indicate that in the absence of Foxd1, RGC number is normal and RGC neurons are normally differentiated, in accordance with the robust DiI staining observed in the optic nerve of Foxd1 deficient mice in forward filling experiments.

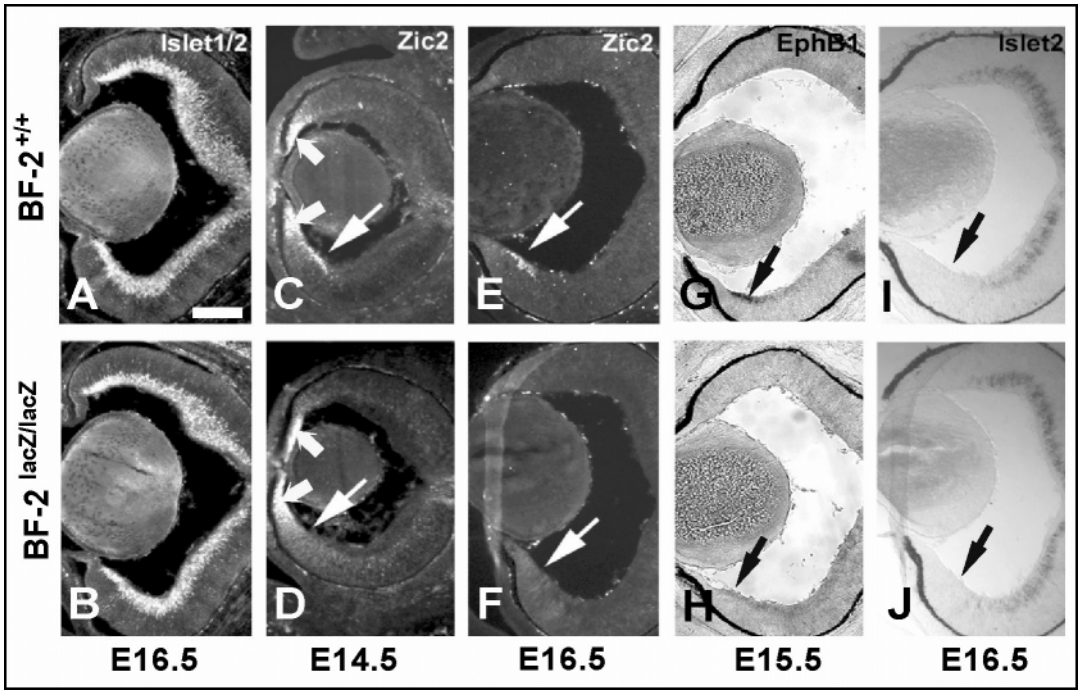

Fig. 4. Zic2 and EphB1 are not expressed in VT retina in Foxd1 (BF-2 in figure) deficient embryos. (A,B) Cryosections of E16.5 Foxd1$^{+/+}$ (A) and Foxd1$^{lacZ/lacZ}$ (B) embryos showing similar Islet1/2 expression in both wild type and mutants. (C-F) Zic2, detected by anti-Zic2 antibodies, in frontal sections from wild-type and Foxd1$^{lacZ/lacZ}$ embryos. (C,D) At E14.5, Zic2 is not detected in Foxd1$^{lacZ/lacZ}$ VT retina (elongated arrows, D; compare wild type in C), but it is detected in the ciliary margin zone (short arrows). (E,F) At E16.5, Zic2 expression seen in wild-type VT retina (E) is absent in the mutant (F). (G,H) In situ hybridization for EphB1 in frontal cryosections of E15.5 Foxd1$^{+/+}$ (G) and Foxd1$^{lacZ/lacZ}$ (H) embryos reveals that EphB1, like Zic2, is absent in embryos lacking Foxd1. (I,J) mRNA for Islet2 is similarly expressed Foxd1$^{lacZ/lacZ}$ and Foxd1$^{+/+}$ retina. Scale bars: 100 μm.

Genes regulating the ipsilateral decision

Because our in vitro results indicated that Foxd1 function is needed in the retina for normal behavior of VT axons, we next investigated whether VT RGCs are specified correctly in Foxd1 deficient embryos. We focused on the expression patterns of regulatory genes, and on axon guidance factors in the retina that have been implicated in the determination of RGC axonal routing at the optic chiasm. We previously reported that the decision of RGC axons to project ipsilaterally at the midline appears to be controlled by the zinc-finger transcription factor Zic2. At E14.5, Zic2 is expressed in progenitor cells at the ciliary margin zone (CMZ) in the entire retina, but it is also detected in a small population of postmitotic cells in the VT crescent of the neural retina. Zic2 expression in the CMZ disappears at about E15.5, but expression continues in the postmitotic RGCs that project ipsilaterally until E17.7-E18.5, and then is downregulated once all the retinal axons have passed through the optic chiasm (Herrera et al., 2003). Strikingly, in Foxd1$^{lacZ/lacZ}$ embryos, although expression in the CMZ seems to be normal at E14.5 (compare panels C and D in Fig. 4), Zic2 expression is nearly lost in Foxd1 deficient VT retina (compare panels E and F in Fig. 4). We did not detect Zic2-positive cells outside of the VT retina in Foxd1$^{lacZ/lacZ}$ mice. This loss of Zic2 expression is consistent with our in vitro results showing that axons from Foxd1$^{lacZ/lacZ}$ VT retinal explants are not inhibited by Foxd1$^{+/+}$ or Foxd1$^{lacZ/lacZ}$ chiasm cells.

The tyrosine kinase receptor EphB1 is expressed in the same VT domain as Zic2 and mediates the response of ipsilateral RGC axons to inhibitory signals emanating from chiasm cells (Williams et al., 2003). We have previously postulated that Zic2 might regulate the tyrosine kinase receptor EphB1 (Williams et al., 2004). In Foxd1$^{lacZ/lacZ}$ mice, as with Zic2, EphB1 is not expressed in VT retina (Fig. 4G,H), suggesting a role for Foxd1 in the regulation of these two proteins that modulate the ipsilateral pathway.

Islet2 is a LIM homeodomain transcription factor expressed in a pattern complementary to Zic2 in the developing retina. Because Islet2 is expressed in contra- but not in ipsilateral RGCs, it has been suggested that Islet2 could be involved in the identity and navigation of crossed retinal axons (Pak et al., 2004). To test whether changes in Islet2 expression contribute to the misrouting of contralateral RGCs from contra- to ipsilateral optic tracts in Foxd1$^{lacZ/lacZ}$ embryos, we performed in situ hybridization with specific probes to Islet2 in Foxd1$^{lacZ/lacZ}$ and Foxd1$^{+/+}$ retinal sections. Islet2 expression is normal in the Foxd1 deficient retina. It is important to note that despite the loss of Zic2 in the VT crescent of Foxd1 deficient retina, Islet2 maintains its VT-negative pattern (Fig. 4I,J), demonstrating that there is a population of cells, now Zic2 negative, that are still present in the VT crescent of the Foxd1 deficient retina. This result argues that there is a specific downregulation of Zic2 and EphB1, rather than cell loss at this region. Moreover, the normal Islet2 expression in the retina suggests that its expression is not repressed by Zic2 or regulated by Foxd1.

Genes regulating early retinal patterning

Foxd1 was previously reported to be expressed in the temporal half of the retina at E12.5 (Hatini et al., 1994). We next investigated whether Foxd1 expression is maintained at later times; specifically, at E14.5 and E16.5, the period in which Zic2 and EphB1 are expressed in the VT crescent. To do this, we performed X-gal staining in Foxd1$^{+/lacZ}$ embryos. Rather than being expressed in the entire temporal half of the retina, Foxd1 is restricted to the VT quadrant at E14.5 and E16.5 (Fig. 5A, part a,e).

The restriction of Foxd1 expression to the VT quadrant, together with the downregulation of Zic2/EphB1, in the Foxd1$^{lacZ/lacZ}$ retina suggests a relationship between Foxd1 and Zic2. We performed X-gal staining in Foxd1$^{+/lacZ}$ embryos to detect *lacZ* expression, followed by immunohistochemistry with anti-Zic2 antibodies, in order to compare the expression patterns of these two regulatory genes. At both ages, the Zic2 expression domain is mostly included within the Foxd1-positive area, and the ventral edge of Zic2 expression falls within the Foxd1 expression domain, except for a few Zic2-positive cells outside the ventral edge of the Foxd1 expression domain. Foxd1 expression extends more centrally, whereas Zic2 is restricted to the peripheral retina (Fig. 5A, parts b-d,f-h). Therefore, most Zic2-positive cells are located in the Foxd1 expression domain – although they do not seem to co-localize – consistent with a role for Foxd1 acting upstream of Zic2.

Because Foxd1 and Foxg1 show adjacent expression domains in the retina (Hatini et al., 1994), a pattern suggesting that these two winged helix transcription factors (Foxg1 and Foxd1) might repress each other, we next studied the Foxg1 expression pattern in Foxd1$^{lacZ/lacZ}$ and Foxd1$^{+/+}$ embryos. In the Foxd1 deficient retina, the Foxg1 expression domain expands into the VT retina, occupying the Foxd1 domain (compare Fig. 5B, parts a-c with parts d-f), but does not expand into the dorsotemporal retina, in agreement with previous data suggesting that Foxd1 represses Foxg1 in retina (Huh et al., 1999).

Foxg1 induces ephrin-A2/A5 expression when is misexpressed in the temporal retina (Takahashi et al., 2003). Because Foxg1 expression expands into the domain of Foxd1 in the Foxd1 deficient retina, we investigated the pattern of ephrin-A in the Foxd1 deficient retina. In Foxd1$^{+/+}$ retina, ephrin-A was found in a high-nasal low-temporal pattern, whereas in Foxd1$^{lacZ/lacZ}$ retina ephrin-A was highly expressed in both halves of the retina (Fig. 5B, parts g,h).

In summary, the absence of Foxd1 in retina leads to the loss of genes known to directly control the uncrossed pathway in the optic chiasm, specifically, Zic2 and EphB1, and to an expansion of Foxg1 and ephrin-A (Fig. 5C).

Expression of regulatory genes and axon guidance factors is altered in the optic chiasm of Foxd1 deficient embryos

Co-cultures of Foxd1$^{+/+}$ and Foxd1$^{lacZ/lacZ}$ retina and chiasm cells suggested that RGC axons in the VT retina of Foxd1$^{lacZ/lacZ}$ embryos display 'contralateral axon-like phenotypes', as they are less repulsed by chiasmatic cells than RGCs in Foxd1$^{+/+}$ VT retina. This finding is supported by the loss of two genes designating the uncrossed projection, Zic2 and EphB1, in the Foxd1 deficient VT retina. However, in vivo, early retinal axons show altered pathfinding errors as early as E12.5 (Fig. 1A, part b), a finding that cannot be explained by Zic2 or EphB1 downregulation in E14.5 Foxd1 deficient retina, as the first RGCs that form the optic chiasm derive from dorsocentral retina (Guillery et al., 1995). One possible explanation for the early misrouting is that contralateral RGCs are also de-specified. Unfortunately, to date, only one molecule

has been suggested to be involved in contralateral RGC specification, Islet2 (Pak et al., 2004), but this transcription factor shows normal expression in Foxd1 mutant retina. No other proteins have been directly implicated in the establishment of the contralateral projection, making it difficult to test for perturbations in the mechanisms for crossing.

It is also possible that in the absence of Foxd1, the ventral diencephalon is altered and causes axon misrouting. To investigate this possibility, we analyzed the expression pattern of transcription factors and axon guidance molecules implicated in retinal axon divergence or interactions with Foxd1 in the optic chiasm region.

Patterning genes in the ventral diencephalon

Combinatorial domains of regulatory gene expression in the ventral diencephalon have been implicated in the patterning of retinal axon projections in the optic chiasm (Marcus et al., 1999), either by regulating the expression of molecules involved in axon guidance or by specifying development of the specialized cell groups that provide cues for guidance (Marcus et al., 1999; Williams et al., 2003).

The transcription factor Foxg1 is expressed in the pre-optic area, in a pattern complementary to Foxd1, in a domain where the optic chiasm is formed (Marcus et al., 1999). The boundary between these two transcription factors has been proposed to play a role in the establishment of the optic chiasm. We tested the expression pattern of Foxg1 in Foxd1$^{lacZ/lacZ}$ embryos and consistently found that the zone of Foxg1 expression is expanded compared with the pattern in Foxd1$^{+/+}$ embryos, invading the supraoptic area normally occupied by Foxd1. The expansion can be viewed in sagittal (Fig. 6A, parts a-d) and frontal sections of Foxd1$^{lacZ/lacZ}$ embryos (Fig. 6B, parts a,b).

Zic2 is expressed in a pattern partially overlapping the Foxd1 expression domain in the ventral diencephalon (E.H. and C.M., unpublished). Moreover, the absence of Zic2 in Foxd1$^{lacZ/lacZ}$ retina suggests that Zic2 might be downstream

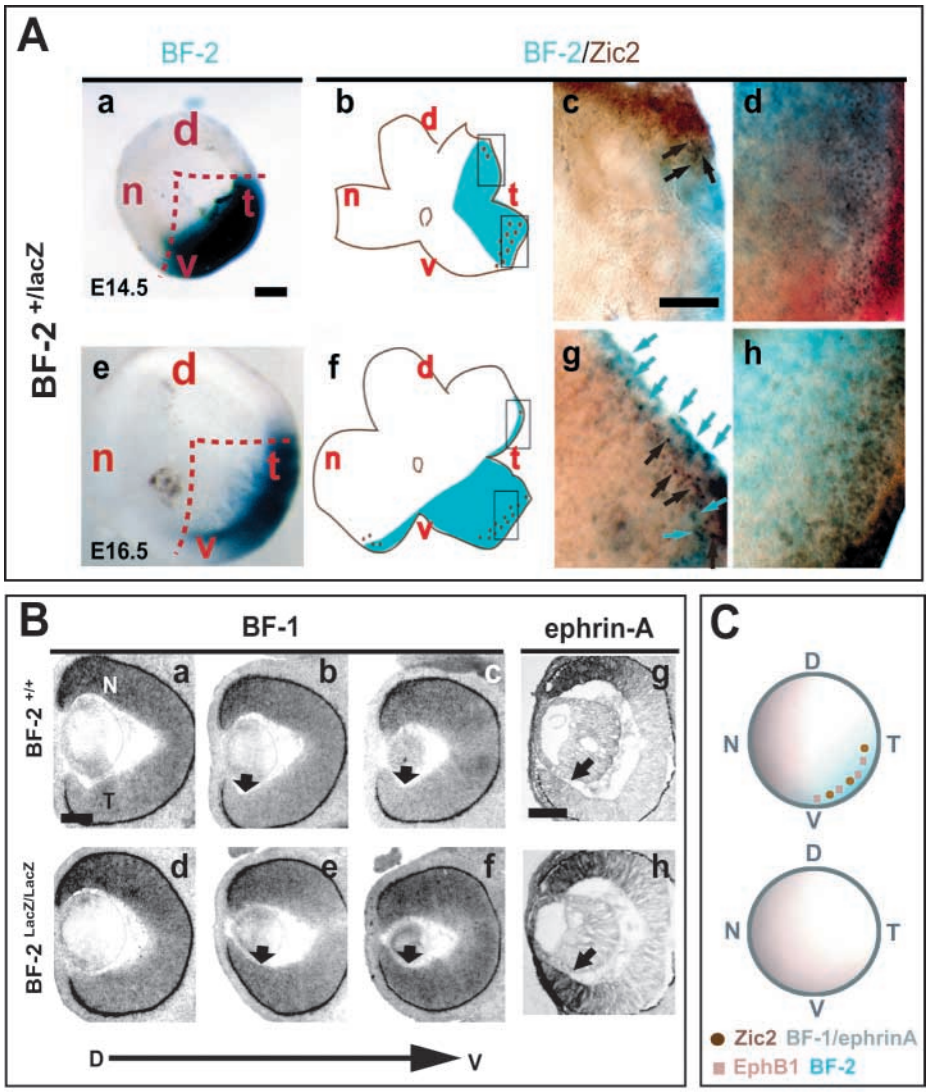

Fig. 5. Foxd1 (BF-2 in figure) is expressed in VT retina and its absence leads to expansion of the Foxg1 (BF-1) and ephrin-A domains. (A) X-gal staining (blue) of retinal wholemounts from Foxd1$^{+/lacZ}$ embryos at E14.5 (a) and at E16.5 (e) indicates that Foxd1 is highly expressed in VT retina at these ages. Flattened retinal wholemounts at E14.5 (b) and E16.5 (f) summarize the relationship between Foxd1 expression domain and Zic2-positive cells over the entire retina. Blue indicates Xgal-Foxd1 staining and dark brown speckles represent Zic2-positive cells. The cartoon shows that the borders of Zic2 and Foxd1 expression overlap, although the Foxd1 expression domain extends more centrally than that of Zic2. Moreover, some Zic2-positive cells are found outside the most ventral lateral border of the Foxd1 expression domain. Higher magnification views of the two boxed regions in b and f are shown in c and d, and g and h, respectively. These pictures show that most Zic2-positive cells (dark brown speckles) are located in the Foxd1-positive domain (blue area) in the VT retina (d,h). c and g show that the peripheral temporal borders of the Zic2 and Foxd1 domains overlap. Scale bars: 100 μm. (B) (a-f) In situ hybridization for Foxg1 in serial horizontal sections from the middle of the retina (a,d) and through the most ventral retina (c,f) in E15.5 Foxd1$^{+/+}$ and Foxd1$^{lacZ/lacZ}$ embryos, indicating that Foxg1 expands into VT retina in the absence of Foxd1. Arrows in b-f indicate that Foxg1 is expressed in the ventrotemporal quandrant of Foxd1 deficient retina but not in normal retina. (g,h) Antibody fusion protein localization in horizontal sections of E12.5 embryos shows that in the absence of Foxd1, ephrin-A is also expressed in the temporal retina (h, arrow), which is in contrast to the normal high-nasal-low-temporal expression in wild-type embryos (g, arrow). Scale bars: 100 μm. (C) Schematic showing that Zic2 and EphB1, thought to regulate the uncrossed projection, are missing in the retina of Foxd1 deficient embryos, and that Foxg1 and ephrin-A proteins expand their territory into VT retina. Top, wild type; bottom, mutant.

of Foxd1. Islet1 is expressed in the most posterior aspect of the chiasmatic region, as well as in a few cells at the optic chiasm midline. The function of these regulatory genes in the optic chiasm region is unknown, but, because of their particular location with respect to Foxd1 in the chiasm region, we analyzed their expression pattern (see cartoon, Fig. 7). In sagittal sections of Foxd1$^{+/+}$ embryos, Zic2 is highly expressed in the supraoptic area, above the retinal fibers, in a pattern complementary to that of Foxg1 (Fig. 6A, part e). By contrast, in Foxd1$^{lacZ/lacZ}$ embryos, there are no Zic2-positive cells in the supraoptic area, which is instead occupied by Foxg1 (Fig. 6A, part f). Moreover, the number of Islet1-positive cells at the optic chiasm midline was greatly reduced in Foxd1$^{lacZ/lacZ}$ compared with Foxd1$^{+/+}$ embryos (Fig. 6A, parts e-h). In frontal sections, concomitant with the expansion of Foxg1, fewer Zic2-positive cells were seen on either side of the midline compared with Foxd1$^{+/+}$ embryos (Fig. 6B, parts c,d). Interestingly, Zic2 expression in more dorsal aspects of the brain appeared to be normal (Fig. 6B, part c,d; arrows).

Axon guidance factors in the ventral diencephalon

We next wished to determine whether the alterations of Foxg1, Zic2 and Islet1 expression in the ventral diencephalon of Foxd1 deficient embryos are associated with aberrant expression of axon guidance factors important for the navigation of RGCs. We first examined the expression of ephrin-B2, responsible for the inhibition of ipsilateral RGC axons at the chiasm midline (Nakagawa et al., 2000; Williams et al., 2003). In the absence of Foxd1, this tyrosine kinase protein is expressed at the chiasm midline (Fig. 7A, parts a and b). Thus, this major signal for axon divergence at the midline is intact, consistent with the co-culture results that both Foxd1 deficient and Foxd1$^{+/+}$ chiasm cells elicit inhibition in Foxd1$^{+/+}$ VT RGCs.

Slit2, a member of the secreted Slit chemorepellent family

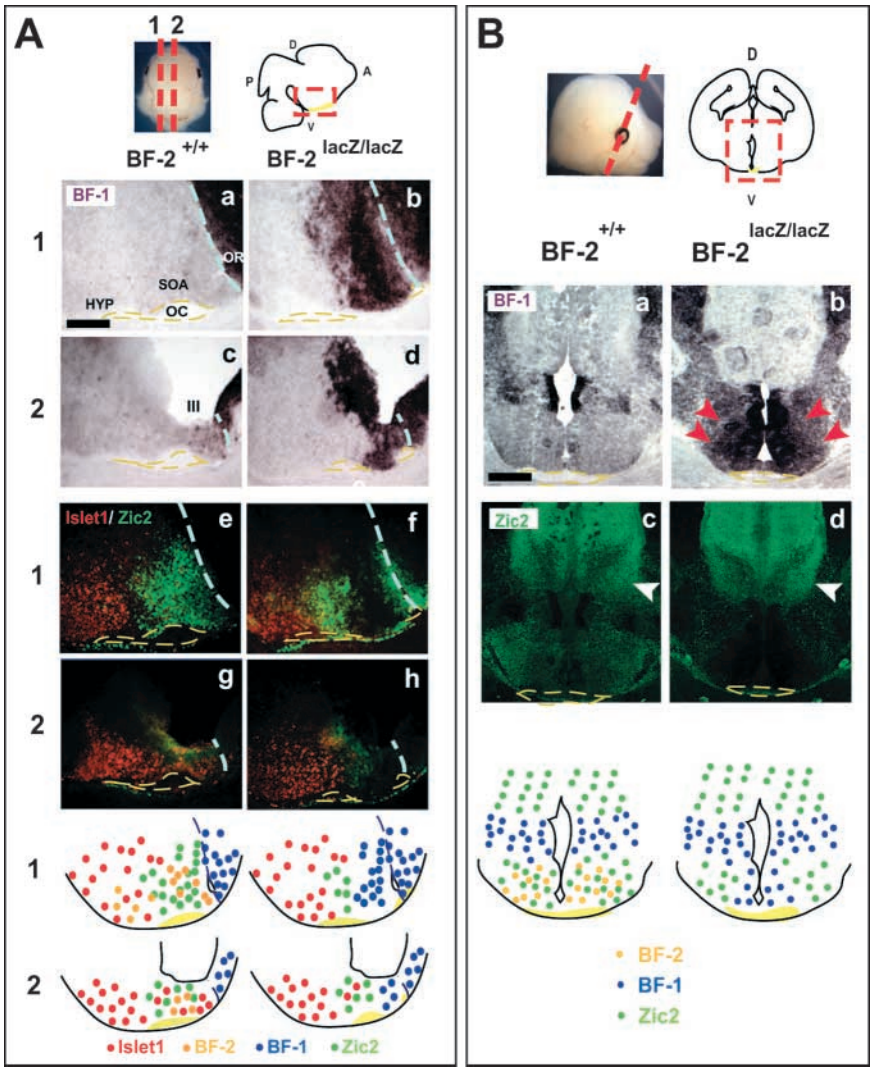

Fig. 6. Foxg1 (BF-1) expression is expanded, and Zic2 and Islet1 zones are reduced in the Foxd1 (BF-2)-deficient ventral diencephalon. (A) Top left panel is a dorsal view of an E15.5 embryo head with dashed red lines (1, 2) indicating the plane and level of section shown in panels a-h. Top right panel is a drawing of a sagittal section; red-dashed square indicates the area shown in panels a-h. (a-h) Sagittal cryosections of E15.5 Foxd1$^{lacZ/lacZ}$ (b,d,f,h) and Foxd1$^{+/+}$ embryos (a,c,e,g), hybridized for Foxg1 (a-d), or immunostained (e-h) against Zic2 (green) and Islet1 (red). Dashed yellow lines indicate where the axons are located, in different positions in the Foxd1$^{+/+}$ embryo compared with in the Foxd1 deficient embryo. The light blue-dashed lines represent the posterior boundary of Foxg1 domain in Foxd1$^{+/+}$ embryos. Note that in the most lateral sections (level 1) of Foxd1 deficient embryos, the Foxg1 expression domain is expanded posteriorly behind the optic recess, the Zic2 domain is reduced, and Islet1 appears to be normal. In more medial sections (level 2), the Foxg1 expression domain is expanded caudally, there is no Zic2 expression, and Islet1 expression is restricted to the most posterior aspect of the chiasmatic region. Bottom panels, schematics comparing the expression patterns of Foxd1, Foxg1, Zic2 and Islet1 in Foxd1$^{+/+}$ (left) and Foxd1$^{lacZ/lacZ}$ (right) embryos, as viewed in sagittal sections. OC, optic chiasm; OR, optic recess; SOA, supraoptic area; HYP, hypothalamus; III, third ventricle. Scale bars: 200 μm. (B) Top left panel is a lateral view of an E15.5 embryo head, and the red-dashed line indicates the plane and level of section showed in panels a-d. Top right panel is a drawing of a frontal section; red-dashed square indicates the area shown in panels a-d. (a,b) In situ hybridization for Foxg1 in frontal cryosections of E15.5 Foxd1$^{lacZ/lacZ}$ (b) and Foxd1$^{+/+}$ (a) embryos. (c,d) Immunostaining against Zic2 (green) in the same sections as in a and b, respectively. Bottom panels are summary cartoons comparing the expression patterns of Foxd1, Foxg1, Zic2 and Islet1 in Foxd1$^{+/+}$ (left) and Foxd1$^{lacZ/lacZ}$ (right) embryos, in frontal view. Note that Foxg1 expression is expanded ventrally and Zic2 expression is restricted to a few cells in the most lateral aspect of the chiasm; however, Zic2 expression is normal more dorsally. Scale bars: 200 μm.

that is necessary for channeling RGC axons during chiasm formation (Plump et al., 2002), was next examined in the Foxd1 deficient chiasm. In Foxd1$^{+/+}$ embryos, Slit2 is strongly expressed immediately dorsal to the optic chiasm and is also weakly detected ventroposterior to the chiasm, in a patch on either side of the ventral diencephalon (Fig. 7D, cartoon) (Erskine et al., 2000; Plump et al., 2002). At E13.5, Slit2 expression appears to be normal in the most dorsal area of the Foxd1 deficient chiasm. However, its ventroposterior expression is stronger than in the Foxd1$^{+/+}$ chiasm (Fig. 7B, parts a-d). At E15.5, although less intense, this altered pattern is maintained (Fig. 7B e-h).

As CD44/SSEA-positive neurons are believed to define the midline and the posterior border of the future chiasm (Marcus et al., 1995; Mason and Sretavan, 1997; Sretavan et al., 1994), we also investigated the position of this population of early neurons in the Foxd1 deficient chiasm. CD44/SSEA neurons are located ventral to the optic stalks and dorsal to the postoptic recess (Mason and Sretavan, 1997). At E13.5 (Fig. 7C, parts a,b) and at E15.5 (Fig. 7C, parts c,d), the organization of these early neurons appears to be normal in Foxd1 deficient embryos. However, at E15.5, RGCs axons enter the CD44/SSEA domain rather than avoid it, as occurs normally in Foxd1$^{+/+}$ chiasms (Fig. 7C, parts c,d).

Thus, Foxd1 deficient embryos exhibit aberrant expression of Foxg1, Zic2 and Islet1 transcription factors in the ventral diencephalon, providing evidence for mis-regionalization of the ventral diencephalon. Ephrin-B2, which is repulsive ipsilateral axons, appears to be normal at the Foxd1 deficient chiasm, but Slit2, which mediates inhibition of all RGC axons,

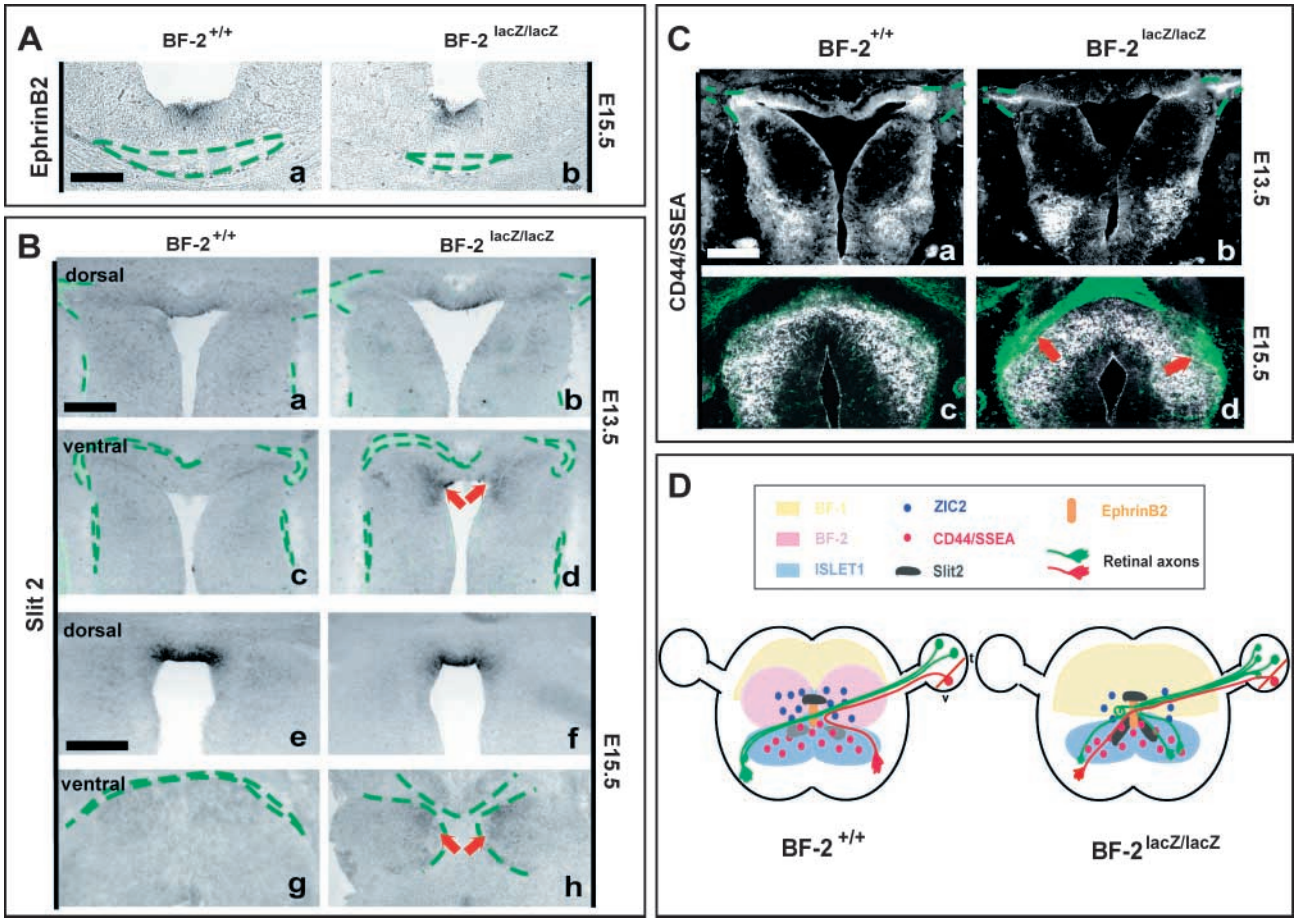

Fig. 7. Axon guidance factors are relatively unchanged in the optic chiasm of Foxd1 (BF-2) deficient embryos. (A) In situ hybridization for ephrin-B2 in frontal cryosections of E15.5 Foxd1$^{+/+}$ (a) and Foxd1 mutant (b) embryos, revealing similar expression pattern in cell bodies at the floor of the third ventricle, thought to belong to the midline radial glia. (B) In situ hybridization for Slit2 in horizontal cryosections at E13.5 (a-d) and E15.5 (e-h). Comparison of Foxd1$^{+/+}$ (left panels) and Foxd1$^{lacZ/lacZ}$ (right panels) chiasms at E13.5 show that although Slit2 expression appears normal in more dorsal sections (a,b), Slit2 levels are increased in the region ventrocaudal (c,d) to the chiasm in Foxd1 deficient embryos (red arrows, d). At E15.5, the same pattern is maintained (e-h). Green dashed lines indicate the position of retinal axons in each section. (C) Immunostaining with anti-CD44/SSEA antibodies (red) and anti-neurofilament (green) in horizontal cryosections at E13.5 (a,b) and E15.5 (c,d). Comparison of Foxd1$^{+/+}$ (left panels) and Foxd1$^{lacZ/lacZ}$ (right panels) chiasms indicates that although CD44/SSEA expression appears normal in Foxd1 deficient embryos, retinal axons enter the CD44/SSEA-positive area (d), in contrast to the wild type in which axons never transgress the CD44/SSEA zone (c). Green indicates retinal axons labeled with neurofilament antibodies. Red arrows indicate that retinal axons invade the CD44/SSEA expression zone. Scale bars: 200 μm. (D) Schematic indicating that in the chiasm, the Foxg1 territory expands in the absence of Foxd1, as in retina, and retinal axons aberrantly course through the CD44/SSEA zone. Islet1 and Zic2 expression territories are reduced around the chiasmatic midline, and Slit2 expression is expanded ventrocaudally. Ephrin-B2 and CD44/SSEA expression appear unaltered in the Foxd1 deficient chiasm; however, in contrast to wild-type axons, Foxd1 deficient RGCs trespass the CD44/SSEA domain.

is more strongly expressed in the ventral diencephalon in the absence of Foxd1.

To recapitulate, as a consequence of the absence of Foxd1, Zic2 and EphB1 are lost in the VT retina, and Foxg1 ectopically expands into this retinal sector. Accordingly, VT RGCs are less inhibited by chiasm cells in vitro, and the number of ipsilaterally-projecting RGCs from the VT crescent is drastically reduced in the Foxd1 deficient embryos. Moreover, the number of ipsilaterally projecting RGCs from the retinal quadrants outside of the VT crescent increases, and only 10-15% of the normal number of RGCs project past the chiasm into the optic tracts in the Foxd1 deficient embryo, when compared with the wild-type embryo. These data suggest that in Foxd1 deficient embryos, those RGC axons that are able to project into the ipsilateral or contralateral optic tracts do so in a stochastic manner. Our analyses also demonstrate perturbations in the regionalization of the ventral diencephalon, including expansion of Foxg1 and Slit2, and reduction in Zic2 and Islet1. In summary, regionalization and specification of both the retina and ventral diencephalon are disrupted in Foxd1 deficient embryos, and although the relative contribution of alterations of each site is not identified by the present study, these disruptions produce a misallocation of ipsi- to contralateral axons, malformation of the optic chiasm, and reduced retinal projection into the optic tracts.

Discussion

Previous studies have implicated the winged helix transcription factor Foxd1 in the control of retinotectal pathways during visual system development in chick (Yuasa et al., 1996). Here we report for first time that Foxd1 is also involved in an earlier phase of development of the retinofugal pathway, the establishment of the optic chiasm. Foxd1 is important for the regulation of genes involved in the specification of ipsilateral RGCs, and also for regionalization of the ventral diencephalon in which the chiasm forms.

Foxd1 as a pre-patterning gene of ventrotemporal retinal identity

It has been previously reported that Foxd1 is expressed in the temporal half of the retina at the time that optic vesicles evaginate (Hatini et al., 1994). At E14.5, Foxd1 is confined to the VT quadrant rather than the temporal half of the retina, suggesting a role for Foxd1 in the specification of the ventrotemporal region of the retina and, in turn, ipsilateral RGC identity. The expression domain of Foxd1 includes the peripheral VT retina where Zic2 is expressed. As Zic2 and EphB1, both essential for the specification and guidance of ipsilateral RGCs, show similar expression in VT RGCs (Herrera et al., 2003; Williams et al., 2003), we presumed that the Foxd1 expression domain also includes the EphB1-positive area. Strikingly, Zic2 and EphB1 are missing in the VT quadrant of Foxd1 deficient mice, indicating that Foxd1 is required for the expression of these two proteins in this area of the retina. Our data strongly suggests, for the first time, that Zic2 and EphB1 are linked. Moreover, in accordance with the loss of Zic2 and EphB1 in this region of the retina, retrograde labeling shows that there are fewer cells projecting ipsilaterally from the VT retina in Foxd1 nulls compared with $Foxd1^{+/+}$ mice. Instead, VT RGCs project contralaterally, if they do not stall in the chiasm in Foxd1 deficient mice.

Mix-and-match co-culture assays also support a de-specification of the ipsilateral phenotype, as in the absence of Foxd1, axons from VT retina are no longer inhibited by chiasm cells. Therefore, based on expression patterns and loss-of-function studies, we propose that Foxd1 expression in the retina is essential for regulation of the molecular cascade directing the ipsilateral retinal projection, specifically in directing VT RGCs to recognize inhibitory signals such as ephrin-B2 at the chiasm midline (Williams et al., 2003). At present, whether Foxd1 directly regulates Zic2 is not known. Foxd1 might regulate Zic2 and EphB1 through a non-cell autonomous mechanism or, alternatively, Foxd1-expressing cells may represent progenitors that will later express Zic2.

Previous results indicate that Foxg1 regulates ephrin-A2/A5, because misexpression of Foxg1 in nasal retina induces ephrin-A2/A5 regulation (Takahashi et al., 2003). In the case of Zic2 and EphB1, whether the loss of these molecules is a consequence of the expansion in Foxg1 or the absence of Foxd1 is not known, but further experiments will address this issue.

Foxd1 function is essential for correct regionalization of the optic chiasm

We observed that at E12.5, pioneer axons of Foxd1 deficient embryos display an abnormal course once they exit the optic stalk, growing straight to the optic chiasm midline instead of turning rostrally and ventrally. The loss of proteins that direct the ipsilateral projection in older VT retina (e.g. Zic2 and EphB1) does not explain the misrouting of the early pioneer axons or the subsequent increase in RGCs in Foxd1 deficient embryos that aberrantly project ipsilaterally or are arrested in their growth at the midline. One hypothesis is that early RGCs and/or contralateral RGCs are mis-specified. Islet2 is expressed in contra- but not ipsilateral RGCs in both the Foxd1 deficient and wild-type retina, but it is possible that other molecular mechanisms underlying RGC axon crossing may be defective.

Another, but not mutually exclusive, hypothesis underlying the chiasm phenotype in the Foxd1 deficient embryos is that the morphogenesis and specification of the ventral diencephalon is perturbed. Supporting this view, the boundary between Foxg1 and Foxd1 is disrupted in the ventral diencephalon of Foxd1 deficient embryos. This boundary was previously proposed to define naso-temporal identity (Hatini et al., 1994) and to mark where the optic chiasm forms (Marcus et al., 1999). Associated with this disruption is the fact that Zic2- and Islet1-expressing cells are decreased in number and misplaced. Moreover, Slit2 expression expands at an earlier time than in Foxd1 wild-ype embryos, consistent with the prevalent stalling and misrouting of RGC axons at the midline. However, the continued presence of ephrin-B2, important for retinal axon divergence, is in agreement with the in vitro results indicating that chiasm cells from $Foxd1^{lacZ/lacZ}$ mice are still able to inhibit $Foxd1^{+/+}$ VT axons.

The absence of Foxd1 in the ventral diencephalon may affect the formation of the optic chiasm in two ways. (1) Foxg1 controls the number and type of cells produced in the cortex (Xuan et al., 1995; Hanashima et al., 2002; Hanashima et al., 2004). Thus, it is possible that the Foxg1 expansion in the ventral diencephalon that results from the loss of Foxd1 affects the number and/or fate of specific cell types in the midline, thereby indirectly affecting the organization of the expression

pattern of guidance factors they express, such as Slit2. (2) Foxd1 might directly regulate the expression of transcription factors and guidance molecules important for RGC growth across the Foxd1-expressing zone.

Specificity of Foxd1 function in optic chiasm formation

We observed expansion of the Foxg1 expression zone into the Foxd1 domain in retina, and an induction of ephrin-A in nasal retina. These data are in agreement with previous reports showing that Foxg1 missexpression in temporal retina in chick induces ephrin-A5, probably leading to the missprojection of retinal axons in the tectum (Takahashi et al., 2003). To study whether the genetic manipulation of Foxd1 affects the retinocollicular projection in mice, and to clarify whether Foxd1 plays a role in the specification of contralateral RGCs, conditional or tissue-specific mice will be needed. However, in addition to new information about other genes that act downstream of Foxd1 (i.e. Zic2, EphB1), the total removal of Foxd1 has revealed that Foxd1 is essential for the correct formation of the optic chiasm at the midline, but is not required for the guidance of retinal axons to exit the retina, navigation through the optic nerves or tracts, or projection to the lateral geniculate nucleus.

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References

Brown, A., Yates, P. A., Burrola, P., Ortuno, D., Vaidya, A., Jessell, T. M., Pfaff, S. L., O'Leary, D. D. and Lemke, G. (2000). Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. *Cell* **102**, 77-88.

Brown, L. Y., Kottman, A. H. and Brown, S. (2003). Immunolocalization of Zic2 expression in the developing forebrain. *Gene Expr. Patterns* **3**, 361-367.

Chung, K. Y., Shum, D. K. and Chan, S. O. (2000). Expression of chondroitin sulfate proteoglycans in the chiasm of mouse embryos. *J. Comp. Neurol.* **417**, 153-163.

Clark, K. L., Halay, E. D., Lai, E. and Burley, S. K. (1993). Co-crystal of the HNF-3/forkhead DNA-recognition motif resembles histone H5. *Nature* **364**, 412-420.

Erskine, L., Williams, S. E., Brose, K., Kidd, T., Rachel, R. A., Goodman, C. S., Tessier-Lavigne, M. and Mason, C. A. (2000). Retinal ganglion cell axon guidance in the mouse optic chiasm: expression and function of robos and slits. *J. Neurosci.* **20**, 4975-4982.

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

Hanashima, C. L. S., Shen, L., Lai, E. and Fishell, G. (2004). Foxg1 suppresses early cortical cell fate. *Science* **303**, 56-59.

Hanashima, C. S. L., Li, S. C. and Lai, E. (2002). Brain factor-1 controls the proliferation and differentiation of neocortical progenitors cells through independent mechanisms. *J. Neurosci*. **22**, 6526-6536.

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.

Hatini, V., Huh, S. O., Herzlinger, D., Soares, V. C. and Lai, E. (1996). Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. *Genes Dev.* **10**, 1467-1478.

Herrera, E., Brown, L. Y., Aruga, J., Rachel, R. A., Dolen, G., Mikoshiba, K., Brown, S. and Mason, C. A. (2003). Zic2 patterns binocular vision by specifying the uncrossed retinal projection. *Cell* **114**, 545-557.

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.

Liu, W., Khare, S. L., Liang, X., Peters, M. A., Liu, X., Cepko, C. L. and Xiang, M. (2000). All Brn3 genes can promote retinal ganglion cell differentiation in the chick. *Development* **127**, 3237-3247.

Marcus, R. C. and Mason, C. A. (1995). The first retinal axon growth in the mouse optic chiasm: axon patterning and the cellular environment. *J. Neurosci.* **15**, 6389-6402.

Marcus, R. C., Blazeski, R., Godement, P. and Mason, C. A. (1995). Retinal axon divergence in the optic chiasm: uncrossed axons diverge from crossed axons within a midline glial specialization. *J. Neurosci.* **15**, 3716-3729.

Marcus, R. C., Gale, N. W., Morrison, M. E., Mason, C. A. and Yancopoulos, G. D. (1996a). Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. *Dev. Biol.* **180**, 786-789.

Marcus, R. C., Wang, L.-C. and Mason, C. A. (1996b). Retinal axon divergence in the optic chiasm: midline cells are unaffected by the albino mutation. *Development* **122**, 859-868.

Marcus, R. C., Shimamura, K., Sretavan, D., Lai, E., Rubenstein, J. L. and Mason, C. A. (1999). Domains of regulatory gene expression and the developing optic chiasm: correspondence with retinal axon paths and candidate signaling cells. *J. Comp. Neurol.* **403**, 346-358.

Marcus, R. C., Matthews, G. A., Gale, N. W., Yancopoulos, G. D. and Mason, C. A. (2000). Axon guidance in the mouse optic chiasm: retinal neurite inhibition by ephrin "A"-expressing hypothalamic cells in vitro. *Dev. Biol.* **221**, 132-147.

Mason, C. A. and Sretavan, D. W. (1997). Glia, neurons, and axon pathfinding during optic chiasm development. *Curr. Opin. Neurobiol.* **7**, 647-653.

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.

Pak, W., Hindges, R., Lim, Y.-S., Pfaff, S. L. and O'Leary, D. D. M. (2004). Magnitude of binocular vision controlled by Islet-2 repression of a genetic program that specifies laterality of retinal axon pathfinding. *Cell* (in press).

Plump, A. S., Erskine, L., Sabatier, C., Brose, K., Epstein, C. J., Goodman, C. S., Mason, C. A. and Tessier-Lavigne, M. (2002). Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. *Neuron* **33**, 219-232.

Pratt, T., Tian, N. M., Simpson, T. I., Mason, J. O. and Price, D. J. (2004). The winged helix transcription factor Foxg1 facilitates retinal ganglion cell axon crossing of the ventral midline in the mouse. *Development* **131**, 3773-3784.

Rachel, R. A., Dolen, G., Hayes, N. L., Lu, A., Erskine, L., Nowakowski, R. S. and Mason, C. A. (2002). Spatiotemporal features of early neuronogenesis differ in wild-type and albino mouse retina. *J. Neurosci.* **22**, 4249-4263.

Schaeren-Wiemers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.

Sretavan, D. W., Feng, L., Pure, E. and Reichardt, L. F. (1994). Embryonic neurons of the developing optic chiasm express L1 and CD44, cell surface molecules with opposing effects on retinal axon growth. *Neuron* **12**, 957-975.

Takahashi, H., Shintani, T., Sakuta, H. and Noda, M. (2003). CBF1 controls the retinotectal topographical map along the anteroposterior axis through multiple mechanisms. *Development* **130**, 5203-5215.

Thanos, S. and Mey, J. (2001). Development of the visual system of the chick. II. Mechanisms of axonal guidance. *Brain Res. Brain Res. Rev.* **35**, 205-245.

Wang, L. C., Dani, J., Godement, P., Marcus, R. C. and Mason, C. A. (1995). Crossed and uncrossed retinal axons respond differently to cells of the optic chiasm midline *in vitro*. *Neuron* **15**, 1349-1364.

Wang, S. W., Mu, X., Bowers, W. J., Kim, D.-S., Plas, D. J., Crair, M. C.,

Federoff, H. J., Gan, L. and Klein, W. H. (2002). *Brn3b/Brn3c* double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth. *Development* **129**, 467-477.

Williams, S. E., Mann, F., Erskine, L., Sakurai, T., Wei, S., Rossi, D. J., Gale, N. W., Holt, C. E., Mason, C. A. and Henkemeyer, M. (2003). Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. *Neuron* **39**, 919-935.

Williams, S. E., Mason, C. and Herrera, E. (2004). The optic chiasm as a midline choice point. *Curr. Opin. Neurobiol.* **14**, 51-60.

Xiang, M., Zhou, L., Macke, J. P., Yoshioka, T., Hendry, S. H., Eddy, R. L., Shows, T. B. and Nathans, J. (1995). The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. *J. Neurosci.* **15**, 4762-4785.

Xuan, S., Baptista, C. A., Balas, G., Tao, W., Soares, V. C. and Lai, E. (1995). Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14**, 1141-1152.

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.