Zic2 promotes axonal divergence at the optic chiasm midline by EphB1-dependent and -independent mechanisms

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Axons of retinal ganglion cells (RGCs) make a divergent choice at the optic chiasm to cross or avoid the midline in order to project to ipsilateral and contralateral targets, thereby establishing the binocular visual pathway. The zinc-finger transcription factor Zic2 and a member of the Eph family of receptor tyrosine kinases, EphB1, are both essential for proper development of the ipsilateral projection at the mammalian optic chiasm midline. Here, we demonstrate in mouse by functional experiments in vivo that Zic2 is not only required but is also sufficient to change the trajectory of RGC axons from crossed to uncrossed. In addition, our results reveal that this transcription factor regulates the expression of EphB1 in RGCs and also suggest the existence of an additional EphB1-independent pathway controlled by Zic2 that contributes to retinal axon divergence at the midline.

KEY WORDS: EphB1, Zic2, Binocular vision, Midline axon divergence, Mouse

INTRODUCTION

Most metazoans are bilaterally symmetric and many features of mature neural function, including the interpretation of sensory information and the coordination of locomotion, depend on the coherent communication between the two halves of the nervous system. The mechanism underlying this communication is established by axons of neurons located on one side of the nervous system growing towards, recognizing and then choosing to transverse or avoid the embryonic midline. Thus, alterations in the decision to cross or not to cross the midline may perturb the proper establishment of nervous system circuitry. For instance, in albino animals or in organisms that have abnormalities in the pyramidal tract, perturbations in fiber decussation cause problems in stereoscopic vision and in motor coordination, respectively (Beg et al., 2007; Jeffery, 2001; Kullander et al., 2003).

The crossing of the midline has frequently been employed as a model to understand axonal guidance. Studies on how this process occurs have resulted in the identification of a considerable number of specific axon guidance ligand-receptor pairs such as netrins and DCC, Slits and Robos, semaphorins and neuropilins/plexins and ephrins and Ephs, to cite some examples (Erskine et al., 2000; Keleman et al., 2002; Kidd et al., 1998; Lustig et al., 2001; Nakagawa et al., 2000; Oster et al., 2003; Plump et al., 2002; Stein et al., 2001; Williams et al., 2006; Williams et al., 2003; Zou et al., 2000). A new level of analysis of the mechanisms of axonal navigation has revealed that combinations of transcription factors expressed in neuronal subpopulations control diverse trajectories to specific targets (Jessell, 2000; Shirasaki and Pfaff, 2002). How the activity of a specific transcription factor dictates axon guidance choices is still an open question, but in recent years it has become clear that transcription factors provide a molecular drive by which growth cones can respond appropriately and differentially to the cues they encounter through the activation and inhibition of a balance of target axon guidance molecules. Thus, the transcription factor Even-skipped (Eve), expressed in motoneurons, dorsalizes ventral axons in part through upregulation of the netrin receptor Unc-5, which can act with the additional netrin receptor DCC (Labrador et al., 2005). The cell adhesion molecule Fasciclin III might be a potential target for Nkx6 (HGTX – FlyBase) in a subset of ventral motoneurons that require this molecule to exit the central nervous system (Broihier et al., 2004). In vertebrate systems, Lim1 (Lhx1) controls motoneuron projections to the limb in the dorsoventral axis by inducing the expression of EphA4, enabling dorsal projecting axons to respond to ephrin A expressed by the ventral limb mesenchyme (Kania and Jessell, 2003), whereas Islet2 seems to be upstream of Slit-induced fasciculation of sensory neurons (Yeo et al., 2004). In the murine visual system, Irx4 regulates Slit proteins to control axonal navigation within the retina (Jin et al., 2003). The guidance cue Shh and the cell adhesion molecule L1 have been identified as targets of the Pou transcription factor Brn3b (Pou4f2), which is essential for retinal pathfinding at several points along the retinofugal pathway (Erkman et al., 2000; Pan et al., 2005). Misexpression and genetic manipulation of the transcription factors FoxG1, FoxD1, Vax2 or Tbx5 alter regional specification of visual paths in the targets by regulation of axon guidance molecules such as Ephs and ephrins (Herrera et al., 2004; Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Pratt et al., 2004; Schulte and Cepko, 2000; Takahashi et al., 2003). These and other findings describe links between transcription factor expression and regulation of axon guidance proteins in different scenarios of the developing nervous system (for a review, see Erskine and Herrera, 2007). However, to date, there have been no reports on transcriptional regulation of axon guidance molecules acting on laterality at the vertebrate midline.

The optic chiasm, a structure essential for establishing binocular vision, is a good model for analyzing axon guidance and midline crossing because at this juncture, retinal fibers arising from each retina diverge to project to the higher visual targets in the same (ipsilateral) or the opposite (contralateral) side of the brain. The turn at the midline executed by ipsilateral axons has been suggested to be mediated by EphB1/ephrin B2 signaling. Ephrin B2 is expressed by glial cells located at the midline, whereas EphB1 is highly expressed in the ventrotemporal (VT) retina where ganglion cells giving rise

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to uncrossed axons are located (Williams et al., 2003). In addition to the receptor tyrosine kinase EphB1 and its ligand ephrin B2, the zinc-finger transcription factor Zic2 has also been implicated as a determinant of axonal laterality at the chiasmatic midline. Zic2, which is essential for a wide array of other developmental programs, such as patterning the neural plate and tube (Aruga et al., 2002; Brown et al., 2001), is differentially expressed in the uncrossed but not crossed retinal pathway. Moreover, reduced levels of Zic2 in vivo lead to a near absence of the ipsilateral projection (Herrera et al., 2003). Zic2 expression in the VT retina correlates with the degree of binocularity through evolution. In mice, which have poor binocular vision, about 3% of retinal ganglion cell (RGC) axons do not cross the midline and the number of Zic2-expressing cells during the period of retinal axon outgrowth reflects this proportion of ipsilateral retinal axons. Animals with a greater extent of binocular vision than mice, such as the ferret, and, concomitantly, a greater number of ipsilateral retinal axons, display an equivalent number of Zic2-positive cells. Species with panoramic vision, such as chick and zebrafish, have no ipsilateral axons and, accordingly, their RGCs lack Zic2 expression (Herrera et al., 2003; Seth et al., 2006). In addition, *Islet2*, another regulatory gene from the LIM homeodomain family, has been proposed to affect laterality of the late-born crossing RGCs in mouse VT retina, by repressing Zic2, EphB1 or both (Pak et al., 2004), and/or by putatively regulating expression of NrCAM, which is crucial for the midline crossing of this late-born population (Williams et al., 2006).

Since Zic2 and EphB1 expression patterns overlap spatiotemporally during the formation of the ipsilateral projection, and because in vivo loss-of-function manipulation for one or the other protein dramatically reduces the ipsilateral projection (Herrera et al., 2003; Williams et al., 2003), we wondered whether EphB1 might be a target of Zic2 in this process. Here, we first demonstrate that in mammals, Zic2 expression in RGCs is not only essential but is sufficient to switch the trajectory of retinal axons from crossed to uncrossed at the chiasm midline. This control of the ipsilateral projection by Zic2 is in large part mediated through EphB1, implicating transcriptional regulation of axon guidance receptors that, in turn, trigger an axonal response in an intermediate target such as the midline. In addition, we also report that Zic2 is able to switch axonal laterality at the midline by an EphB1-independent mechanism.

MATERIALS AND METHODS
Mice and DNA constructs
Embryos from C57Bl/6J *EphB1*^{−/−}, *EphB1*[lacZ] mice (Williams et al., 2003) and *Zic2*[+/kd] and *Zic2*[kd/kd] mice (Nagai et al., 2000) kept in a timed-pregnancy breeding colony at the Instituto de Neurociencias were removed from anesthetized mothers by caesarean section and staged with E0 defined as midnight before a plug was found. Animals were treated according to Spanish, European Union and NIH guidelines for Care and Use of Laboratory Animals. Animal protocols were approved by the Instituto de Neurociencias Animal Care and Use Committee.

For electroporation experiments, human ZIC2 and EGFP coding sequences were cloned in a modified version of the pCAGGS vector (Borrell et al., 2005; García-Frigola et al., 2007) that includes a greater number of restriction sites 3′ to the CAG promoter. The CAG promoter contains a chicken β-actin/rabbit β-globin hybrid promoter (AG) and the human CMV-IE enhancer. The AG promoter sequence consists of the chicken β-actin promoter, the first exon and part of the first intron (that seems to have a strong enhancer-like activity) linked to a rabbit β-globin fragment, consisting of a 3′ part of the second intron (inclusive of a branch point that is required for normal splicing reactions) and a 5′ part of the third exon (Niwa et al., 1991). Plasmid DNA was purified using a conventional Midiprep Kit (Qiagen, Valencia CA) and resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA).

In situ hybridization and immunohistochemistry
The in situ hybridization protocol was modified from reported methods (Schaeren-Wiemers and Gerfin-Moser, 1993) to perform fluorescent instead of the colorimetric reaction [using the TSA system (Perkin Elmer), following the manufacturer's instructions].

Immunohistochemistry using anti-Zic2 antibodies (gift of S. Brown, University of Vermont) was performed as described (Herrera et al., 2003).

In utero electroporation and axon quantification
Timed-pregnant mouse females were anesthetized with sodium pentobarbital (0.625 mg per 10 g body weight) and Rytodrine (0.1 ml of a 14 mg/ml solution) intraperitoneally, the abdomen cut open and the uterine horns exposed. A DNA solution (1 μg/μl CAG-EGFP plasmid alone, or in combination with 1 μg/μl CAG-Zic2 plasmid and 0.03% Fast Green in PBS) was injected into one eye of each embryo using a pulled glass micropipette. The head of each embryo was placed between tweezer-type electrodes (CUY650-P5 Nepa GENE, Chiba, Japan) and five square electric pulses (50 milliseconds) were passed at 1-second intervals using an electroporator (CUY21E, Nepa GENE). Voltage conditions depended on the developmental stage of the embryo (38V and 48 V were applied to electroporate E13 and E14 embryos, respectively). The abdominal cavity was suture-closed and embryos allowed to develop normally. Three days after electroporation, pregnant mothers were sacrificed and embryos removed and fixed in 4% paraformaldehyde overnight at 4°C. Brains were dissected and the optic chiasm viewed en face in whole-mount with a fluorescence dissecting scope.

Quantification of the degree of co-localization of markers after co-electroporation of two plasmids into retinal cells was performed as follows. Retinal sections from E16 embryos (n=4) that were co-electroporated at E13 with CAG-Zic2 and CAG-EGFP plasmids were analyzed. A total of 25 sections and 914 cells were included in the study. Each given percentage was calculated as the average percentage ±s.e.m. Quantification of the crossed and uncrossed projection was performed as described (Herrera et al., 2003) in whole-mounts of the optic chiasm area.

Quantitative RT-PCR
Retinas of E13.5 wild-type embryos were electroporated in utero with CAG-GFP alone or together with CAG-Zic2 plasmids. Embryos were allowed to develop for 2 days and then their retinas were removed. Each electroporated retina was observed under a fluorescence microscope and the electroporated GFP-positive area was dissected out and kept for RNA extraction. An average of six retinal fragments was used for each experiment. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), DNaseI digested and retrotranscribed using the Reverse Transcription System (Promega) the following manufacturer's recommendations. Quantitative (q) PCR was performed using an ABI PRISM 7000 sequence detection system with the SYBR Green method. Primers were designed using Primer Express (software v2.0, Applied Biosystems): mouse EphB1 forward, 5′-CGC-TCTATCCCAGACTTCACG-3′; mouse EphB1 reverse, 5′-GTGAAG-CCTGCGGTGAGG-3′; mouse Gapdh forward, 5′-CTTCACCACCA-TGGAGAAGGC-3′; mouse Gapdh reverse, 5′-CATGGACTGTGGTC-ATGAGCC-3′. Transcript levels were calculated using the comparative Ct method normalized to *Gapdh*. The final results were expressed relative to calibrator (control embryos electroporated with CAG-GFP) using the $2^{-(\Delta\Delta Ct)}$ ±s.e.m. formula.

RESULTS
Zic2 expression in vivo is sufficient to change axonal laterality at the mammalian optic chiasm midline
We previously reported that Zic2 is expressed in differentiated RGCs that project ipsilaterally during the period when ipsilateral axons turn away from the midline, and that mutant mice expressing low levels of Zic2 show a remarkable reduction of ipsilateral axons (Herrera et al., 2003). However, Zic2 is also expressed in the chiasmatic region (Brown et al., 2003; Herrera et al., 2004) as well as in cycling cells at the ciliary margin zone (Herrera et al., 2003), and our experiments
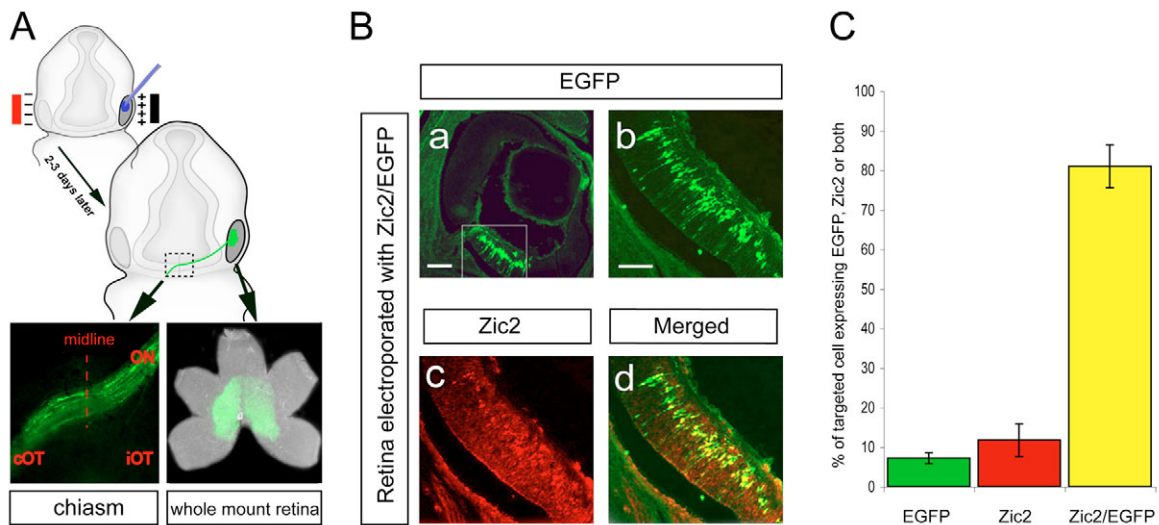
Fig. 1. Ectopic expression of Zic2 in mouse RGCs by in utero electroporation. (**A**) Schematic summarizing electroporation procedures. Plasmids (blue) were microinjected into the eye cup at embryonic stages. Two or 3 days later, embryos were analyzed to visualize EGFP-targeted cells in retinal whole-mounts (right) and axons in the optic chiasm (left). (**Ba**) Retinal section of a E16.5 embryo co-electroporated at E13.5 with plasmids bearing the coding sequences of EGFP and Zic2. Green cells in the center of the retina are targeted cells after the electroporation process. Scale bar: 100 μm. (**Bb-d**) Higher magnification of the boxed area in Ba showing Zic2/EGFP-targeted cells. EGFP is visualized in green and ectopic Zic2 is visualized in red by anti-Zic2 labeling. In the merged image (d), all of the targeted cells are Zic2-positive. Scale bar: 30 μm. (**C**) Quantification of the proportion of cells that co-express EGFP, Zic2 or both, in embryonic retinas as compared with the total number of transfected cells after electroporation.

with Zic2 hypomorphic mutants could not distinguish whether the reduced ipsilateral projection was attributable to the decreased expression of Zic2 in RGCs, in resident cells at the optic chiasm region or in cycling cells in the ciliary margin zone.

To definitively address this issue and to investigate whether Zic2 expression in the RGCs is sufficient to change laterality at the midline in vivo, we ectopically expressed Zic2 in RGCs that normally project contralaterally by in utero electroporation in living embryos. Whereas this technique has been previously used to express genes in the developing cortex (Borrell et al., 2005; Saito and Nakatsuji, 2001) and postnatal retina (Matsuda and Cepko, 2004), we have adapted it for gene delivery in embryonic retina (García-Frigola et al., 2007). Plasmids bearing EGFP alone or together with plasmids containing the Zic2 coding sequence under the regulation of the CAG promoter (Niwa et al., 1991) (see Materials and methods) were monocularly delivered to embryonic retinas (Fig. 1 and see Materials and methods). From previous work, we know that as little as 1 day after electroporation, EGFP expression can be detected in targeted cells (García-Frigola et al., 2007). Two or 3 days after electroporation, pregnant mothers were sacrificed and embryos analyzed. In embryos co-electroporated with

CAG-EGFP and CAG-Zic2 plasmids, Zic2 was expressed in around 85-90% of the EGFP-expressing cells (Fig. 1C), indicating that EGFP can be considered as a surrogate indicator of neurons that express ectopic Zic2 when both plasmids are injected together. In flattened whole-mount retinas, targeted cells can be visualized in the center of the retina and axons of targeted cells can be followed by EGFP fluorescence up to and through the optic chiasm (Fig. 1).

In the mouse retina, the wave of differentiation and subsequent axonogenesis occur in a central-to-peripheral manner. Thus, axons from the central retina reach the midline sooner than axons from peripheral retinal regions. Because many axons from the central retina reach the midline at E13.5 (Colello and Guillery, 1990; Godement et al., 1990; Marcus et al., 1995; Sretavan, 1990; Sretavan, 1993), gene delivery was performed at this age. Pregnant mothers were sacrificed 3 or 4 days later and embryos analyzed to check the site of electroporation into the retina. Embryos electroporated only in the central retina were chosen. In E16.5 or E17.5 embryos electroporated at E13.5 with EGFP, nearly all the green axons projected contralaterally (Table 1). By contrast, in embryos electroporated with Zic2 and EGFP, a large proportion of axons changed their behavior at the midline to project ipsilaterally (Table 1, Fig. 2).

Table 1. Quantification of the retinal labeled-axon projection phenotype at the optic chiasm after in utero electroporation

| Row	Genotype	DNA injected	Number of embryos	Age at electroporation	Age at sacrifice	Cip	Ccp
1	Wild type	EGFP	7	E13	E16	0.03±0.01**	0.41±0.07
2	Wild type	Zic2+EGFP	8	E13	E16	0.24±0.04**,***	0.27±0.05
3	Wild type	EGFP	3	E14	E17	0.04±0.02	0.38±0.03
4	Wild type	Zic2+EGFP	4	E14	E17	0.12±0.03	0.32±0.03
5	EphB1KO	EGFP	7	E13	E16	0.03±0.02*	0.47±0.04
6	EphB1KO	Zic2+EGFP	9	E13	E16	0.09±0.01*,***	0.37±0.02

For statistical analysis (Student's unpaired *t*-test) of data from: rows 5 and 6, *$P<0.05$; rows 1 and 2, **$P<0.001$; rows 2 and 6, ***$P<0.004$.
Cip, coefficient of ipsilaterally projecting axons.
Ccp, coefficient of contralaterally projecting axons.
EphB1KO, *EphB1*-knockout.
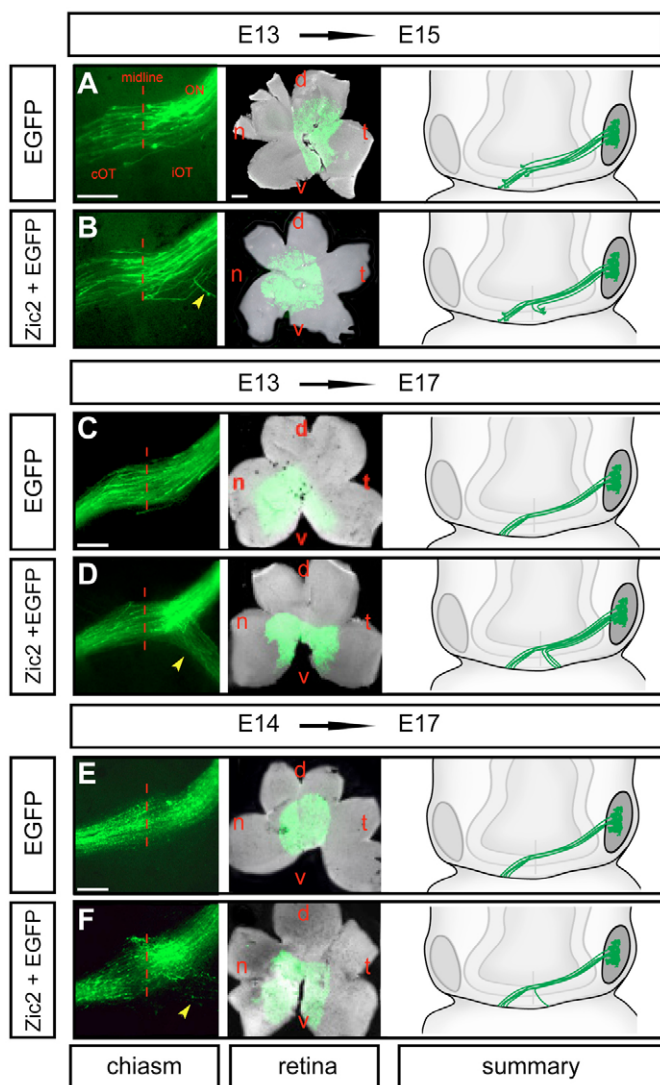
Fig. 2. Zic2 is sufficient to change axonal behavior at the midline. Left panels show axonal trajectories of targeted RGCs at the optic chiasm. Middle panels show the location of the targeted RGCs (green) in retinal whole-mounts (gray). Right panels are cartoons summarizing the results for each particular case. (**A**) In mouse embryos electroporated only with plasmids bearing EGFP, many green axons originating from the central retina have reached or crossed the midline (dashed line). (**B**) In embryos ectopically expressing Zic2/EGFP in the central retina, many targeted axons have crossed the midline but in this case some growth cones are positioned at the midline and a few axons are also observed turning to the ipsilateral side. (**C**) In embryos electroporated only with plasmids bearing EGFP at E13.5 and sacrificed at E17.5, all green axons coming from the central retina cross the optic chiasm midline. (**D**) By contrast, embryos ectopically expressing Zic2 plus EGFP in the central retina show a significant increase in the number of axons that project ipsilaterally at the chiasm. (**E**) In embryos electroporated at E14.5 with plasmids bearing only EGFP and sacrificed at E17.5, all green axons coming from the central retina cross the midline at the optic chiasm. (**F**) The increase in the proportion of axons projecting ipsilaterally after Zic2 ectopic expression is modest when electroporation is performed at E14.5, as compared with electroporation at E13.5. Yellow arrowheads point to the ectopic ipsilateral projection. ON, optic nerve; cOT, contralateral optic tract; iOT, ipsilateral optic tract; d, dorsal; n, nasal; t, temporal; v, ventral. Scale bars: chiasm panels, 200 μm; retina panels, 500 μm.

Although a very significant proportion of RGCs targeted with Zic2/EGFP changed laterality, we observed that many GFP-positive axons still projected contralaterally. These axons cannot be completely attributed to cells that have been targeted with the EGFP plasmid only and not with the Zic2 plasmid. Thus, we hypothesize that by the time that Zic2-driven genes are activated, many of the labeled axons have already passed the optic chiasm region. To test this, we repeated the experiment but sacrificed the mother 1 day earlier, at E15.5. We found that 2 days post-electroporation there were many axons that had already crossed the midline or their growth cones were positioned at the level of the optic chiasm region, both in EGFP- and in Zic2/EGFP-electroporated embryos (Fig. 2A). In embryos electroporated with Zic2/EGFP, many growth axons were positioned at or near the midline and a few axons were in the process of turning. In EGFP-electroporated embryos, no turning axons were detected.

To further investigate the timing of Zic2's ability to switch RGC projection laterality, we also electroporated EGFP or Zic2/EGFP 1 day later, at E14.5, when many more axons from the central retina have passed through the chiasm region. In E14.5 embryos electroporated with Zic2/EGFP in the central retina, the proportion of axons projecting ipsilaterally was smaller than in embryos electroporated at E13.5. In control E14.5 embryos electroporated with EGFP alone, almost all the axons projected contralaterally (Fig. 2C). Thus, in order for Zic2 to change behavior during interactions with the chiasm midline, it must be delivered to RGCs before axons reach the chiasm.

The quantification of these results (Table 1) confirms that: (1) There is a significant increase in axons that project ipsilaterally after ectopic expression of Zic2 in central retina at E13.5, as compared with ectopic expression of EGFP in the same area; (2) this increase is much lower when Zic2 ectopic induction is performed 1 day later at E14.5. Together, these results demonstrate that in vivo Zic2 expression is sufficient to switch the trajectory of axons that have not yet reached the midline, but cannot alter the trajectory of axons that have already passed the midline region.

EphB1 as a candidate effector of Zic2

In the mouse, RGCs whose axons do not cross the midline are located exclusively in the VT region of the developing retina, whereas the cell bodies giving rise to crossing RGC axons are found across the entire retina. We have previously reported that the zinc-finger transcription factor Zic2, and the receptor tyrosine kinase EphB1, are expressed in the VT portion of embryonic retina and that each protein is essential for the formation of the ipsilateral projection (Herrera et al., 2003; Williams et al., 2003). Zic2 expression in VT retina is initiated at E14.5 and it is maintained until E17.5, the period when uncrossed RGCs traverse the midline (Herrera et al., 2003). EphB1 is dynamically expressed in the embryonic retina, commencing at E13.5 in the dorsocentral retina. One day later, dorsocentral expression of EphB1 disappears, but then EphB1 is highly expressed in the VT region from E14.5 until approximately E17.5. Subsequently, EphB1 expression spreads throughout the entire retina but not in RGCs (Williams et al., 2003) (Fig. 3). Thus, based on temporal and spatial expression, EphB1 is a good candidate for a downstream target of Zic2. Based on the above findings, we examined more closely the spatiotemporal coincidence of Zic2 and EphB1 expression by utilizing immunohistochemistry against Zic2 combined with in situ hybridization for *EphB1*. At E15.5, Zic2 alone is expressed by a group of RGCs at the edge of the neural retina, whereas both Zic2 and EphB1 are expressed in
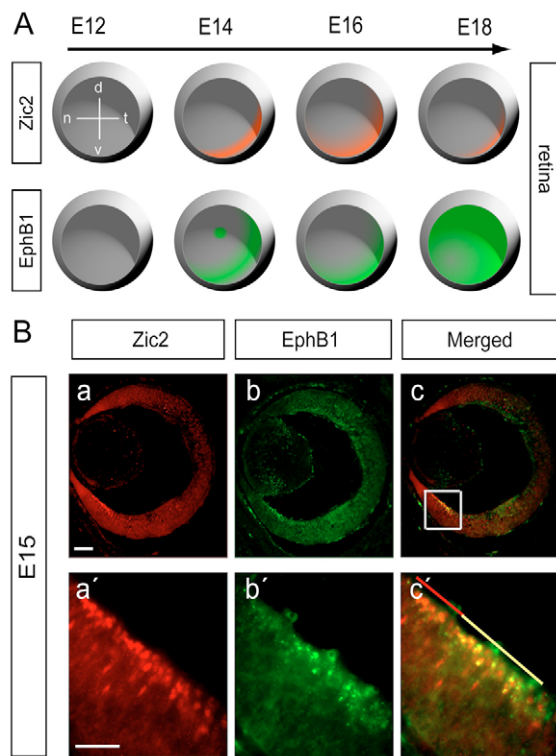
Fig. 3. Zic2 and EphB1 are expressed in the same RGCs in the ventrotemporal (VT) crescent. (**A**) Spatiotemporal representation of Zic2 (red) and EphB1 (green) in mouse retina (gray). Zic2 expression is first seen in VT retina at E14.5. Expression peaks at E16.5 and then Zic2 is downregulated by E17.5-18.5. EphB1 expression in the VT retina commences at E14.5, lasting until E17.5-18.5. EphB1 expression then expands into the entire retina in retinal cells other than RGCs. EphB1 expression is also observed in the central retina from E13.5-15.5. (**Ba-c'**) Immunostaining against Zic2 in E15.5 retinas (a,a', red) combined with in situ hybridization for *EphB1* (b,b', green) in the same section, indicates that Zic2 is expressed in the most-peripheral (younger) cells, whereas older and more-central cells co-express Zic2 and EphB1. (a',b',c') Higher magnification of the boxed area in c. Red line in c' indicates cells positive only for Zic2, which are presumably younger cells. Yellow line indicates the Zic2 and EphB1-positive region, in which more-mature cells are located. Scale bars: in a, 100 μm for a,b,c; in a', 30 μm for a',b',c'.

RGCs located more centrally (Fig. 3B). The wave of RGC generation progresses from central to peripheral retina and the youngest cells are located more peripherally.

Thus, Zic2 is detected first in the most-peripheral VT retina and, as RGCs mature, EphB1 is expressed along with Zic2. The sequential spatiotemporal expression of Zic2 and EphB1 make this guidance receptor an excellent candidate for a downstream target of Zic2.

Zic2 is required for EphB1 expression in VT retina

Since Zic2 expression precedes EphB1 induction in VT RGCs in the developing retina, we wondered whether this transcription factor is required for EphB1 expression.

As Zic2 expression is barely detectable in the VT retina of *Zic2*-knockdown mice ($Zic2^{kd/kd}$) (Herrera et al., 2003), we used these mice to perform in situ hybridization for *EphB1*. At E16.5, RGCs uniquely expressing Zic2 are not found in wild-type embryos and the most-peripheral cells co-expressed Zic2 and EphB1. By contrast,

$Zic2^{kd/kd}$ RGCs in VT retina did not exhibit EphB1 expression. In mice heterozygous for the *Zic2* mutation ($Zic^{+/kd}$) in which very low levels of *Zic2* expression are detected in VT retina, EphB1 expression was accordingly reduced (Fig. 4A). These data show that Zic2 expression in the developing neural retina is essential for the expression of EphB1 in VT RGCs.

In E16.5 $Zic2^{kd/kd}$ embryos, however, EphB1 was detected in other brain areas in a pattern very similar to that in wild-type embryos. Both $Zic2^{+/+}$ and $Zic2^{kd/kd}$ show high expression levels of EphB1 in entorhinal cortex, hippocampus and lateral globus pallidus (Fig. 4B), suggesting that Zic2 selectively regulates EphB1 expression in RGCs.

Ectopic expression of Zic2 in non-VT retina upregulates *EphB1* levels

To test whether EphB1 is induced after ectopic expression of Zic2 in vivo, we compared *EphB1* mRNA levels after ectopic electroporation of Zic2 in the center of embryonic retinas. Two days after electroporation of CAG-Zic2/CAG-EGFP or CAG-EGFP plasmids in E13.5 embryo retinas, the EGFP-positive central portions of the retinas were dissected. Quantitative RT-PCR (qRT-PCR) was performed to detect *EphB1* mRNA levels in these samples, as well as in non-electroporated central regions of retina. Segments from the center of the retina electroporated with Zic2/EGFP showed a statistically significant increase of 1.4±0.116-fold in the levels of *EphB1* mRNA as compared with non-electroporated retinal segments or those electroporated with EGFP alone. These results demonstrate that in vivo, Zic2 is sufficient to enhance EphB1 expression (Fig. 5A).

Zic2 is sufficient to induce axon turning at the midline in the absence of EphB1

Since our in vivo assays indicated that Zic2 can regulate EphB1 expression, we decided to test whether the ipsilateral phenotype induced by Zic2 in wild-type embryos depends on EphB1 expression. If Zic2 controls ipsilaterality through EphB1 expression, ectopic expression of Zic2 in the absence of the *EphB1* gene should not result in an increased ipsilateral projection in vivo. To test this hypothesis, we repeated the in utero electroporation experiments expressing Zic2 ectopically in RGCs of E13.5 mutant mouse embryos lacking EphB1. In $EphB1^{-/-}$ embryos electroporated with CAG-EGFP plasmids in the central retina at E13.5 and sacrificed 3 days later, almost no ipsilateral axons were seen at the chiasm level, similar to what was observed for wild-type embryos electroporated with EGFP (Fig. 5B). When co-electroporation of Zic2/EGFP was performed in *EphB1*-null embryonic retinas, a statistically significant increase in the proportion of ipsilateral axons was observed, as compared with electroporation of EGFP alone. Nevertheless, the amount of axons projecting ipsilaterally after Zic2/EGFP introduction was significantly smaller in *EphB1*-null embryos than when wild-type retinas were electroporated with Zic2/EGFP (Fig. 5B). The quantification of these experiments is shown in Table 1.

These results demonstrate that Zic2 can change retinal axon laterality in vivo primarily through upregulation of EphB1, and also indicate that Zic2 is able to change axonal laterality through an undetermined pathway that is independent of EphB1 signaling.

DISCUSSION

During development, distinct neuronal subtypes establish highly specific projection patterns by responding differentially to spatially discrete attractive and repulsive molecular cues. In the past few years, the mechanisms of action of many axon guidance molecules
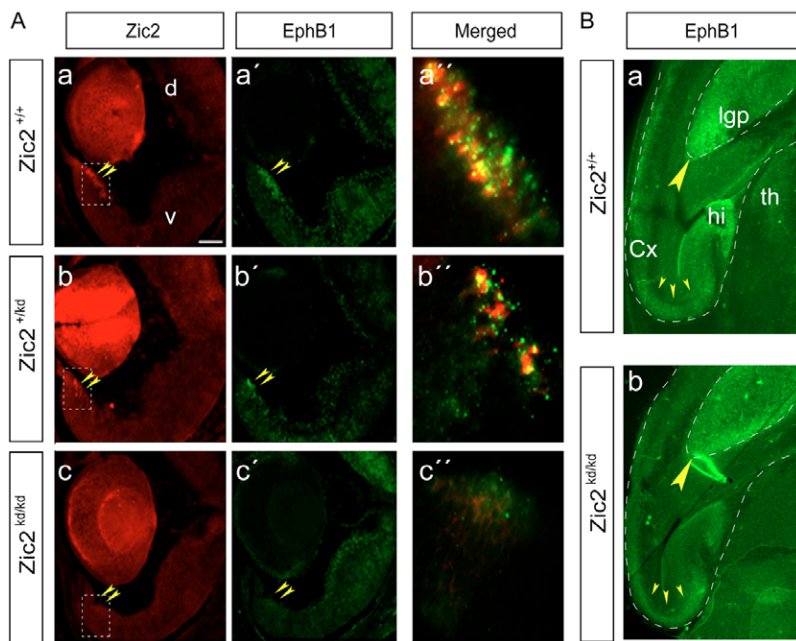
Fig. 4. Zic2 is essential for EphB1 expression in the VT retina. (**A**) White dashed lines delineate retinal sections from wild-type (**a-a″**), heterozygous (**b-b″**) and homozygous (**c-c″**) E16.5 littermate embryos. Immunostaining shows that Zic2 (red) is expressed in VT retina (yellow arrows) in $Zic2^{+/+}$ mice, but is not detected in $Zic2^{kd/kd}$ embryos. Some Zic2-positive cells are detected in $Zic2^{+/kd}$ embryos. (**a′,b′,c′**) In situ hybridization for $EphB1$ (green) in the same retinal sections. (**a″,b″,c″**) Higher-magnification of the respective boxed areas. These images reveal that EphB1 is expressed in the same area as Zic2 in wild-type retinas. In $Zic2^{+/kd}$ retinas, EphB1 expression is reduced compared with the wild type in accordance with a downregulation of Zic2. In $Zic2^{kd/kd}$ retinas, in which Zic2 is not expressed, levels of EphB1 are undetectable. d, dorsal; v, ventral. Yellow arrowheads indicate high expression of Zic2 (a,b,c) and EphB1 (a′,b′,c′). Scale bar: 100 μm. (**B**) In situ hybridization for $EphB1$ in horizontal sections from E17.5 $Zic2^{+/+}$ (**a**) and $Zic2^{kd/kd}$ (**b**) brains. $EphB1$ is extensively expressed in a very similar pattern in both genotypes. Lgp, lateral globus pallidus; Cx, cortex; hi, hippocampus; th, thalamus. Yellow arrows indicate high expression of $EphB1$.

have been uncovered, but very little is known about how the expression of these molecules is regulated during axonal navigation in different scenarios during nervous system development. The activity of specific transcription factors appears not only to correlate with the cell identity of different neuronal subtypes, but also with their growth towards specific targets (Butler and Tear, 2006). Transcriptional regulation of axon guidance receptor expression could provide a means for modulating the interpretation of the myriad of guidance cues faced by navigating axons. Here we report the induction of EphB1 by Zic2 as the first described link between an axon guidance molecule and a transcription factor controlling laterality at the vertebrate midline.

Zic2 is a highly regulated transcription factor, the expression of which is necessary and sufficient to change the behavior of axons at the chiasm midline

Based on spatiotemporal expression patterns and loss-of-function experiments in vivo, previous reports pinpointed Zic2 as a regulatory protein functioning in one of the earliest decisions that optic fibers make during their journey: to cross or not to cross the midline (Herrera et al., 2003). However, because Zic2 is also expressed in the optic chiasm region, in the retinal ciliary margin zone and in the early optic cup (Herrera et al., 2004; Nagai et al., 1997), it was unclear whether postmitotic expression of Zic2 in VT retina is responsible for determining axonal behavior at the midline. Our in vivo electroporation experiments at E13.5 show that misexpression of Zic2 in the central retina leads to the development of an ectopic ipsilateral projection, but when Zic2 is introduced 1 day later the number of axons that switch behavior at the midline from a crossed to uncrossed trajectory was very modest. These findings demonstrate that expression of Zic2 in RGCs is sufficient, in a cell-autonomous and temporally restricted manner, to determine an ipsilateral projection at the chiasmatic midline. We also observed that when wild-type embryos are electroporated with Zic2/EGFP, a percentage of targeted cells still project contralaterally. It is likely that these remaining contralateral axons represent axons from the few targeted RGCs that express EGFP but do not co-express Zic2, together with fibers that had already passed through the chiasm region when the ectopic Zic2 protein began to induce its target genes.

Electroporation experiments performed at earlier stages might more definitively demonstrate that Zic2 can change the behavior of the contralateral axons before their growth cones reach the midline. Unfortunately, the efficiency of the electroporation technique is very low at stages earlier than E13.5. In addition, because the reported ligand for EphB1, ephrin B2, is not expressed at the midline until E13.5-14.5, the outcome of such experiments would be difficult to interpret. Despite this, our observations present compelling evidence that Zic2 is sufficient to control laterality at the midline and that its regulation is highly coordinated with the navigation of retinal axons through the optic chiasm midline.

Recent studies have suggested that the VT region of retina is the domain uniquely competent to project ipsilaterally and that RGCs in this region are genetically distinct from those in the remainder of the retina (Herrera et al., 2003; Herrera et al., 2004; Pak et al., 2004; Pratt, 2004; Williams et al., 2006; Williams et al., 2003). Our results confirm that Zic2, a protein uniquely expressed in the VT quadrant, is in fact responsible for the repulsion of retinal axons at the midline. Since Zic2 is essential for the ipsilateral projection to form and ectopic expression of Zic2 in the central retina is sufficient to change axonal behavior at the chiasm, it is likely that all of the upstream dissimilarities between the VT and the remainder of the retina converge at Zic2 expression and that once this molecule is expressed, other differences are not relevant for determining laterality of axonal projection at the midline.

Transcriptional regulation of expression of a guidance receptor that functions at the mammalian midline

Zic2 is expressed in brain regions that do not express EphB1 and, conversely, EphB1 is expressed in areas of the developing nervous system negative for Zic2 expression. In fact, EphB1 expression is not affected in the cortex or in the globus pallidus in the absence of Zic2. Thus, in addition to Zic2, other transcription factors might participate in the control of EphB1 expression. Moreover, our electroporation experiments on the $EphB1$-knockout background show that a reduced proportion of Zic2-positive RGCs are still able to direct their axons ipsilaterally, supporting the view that Zic2
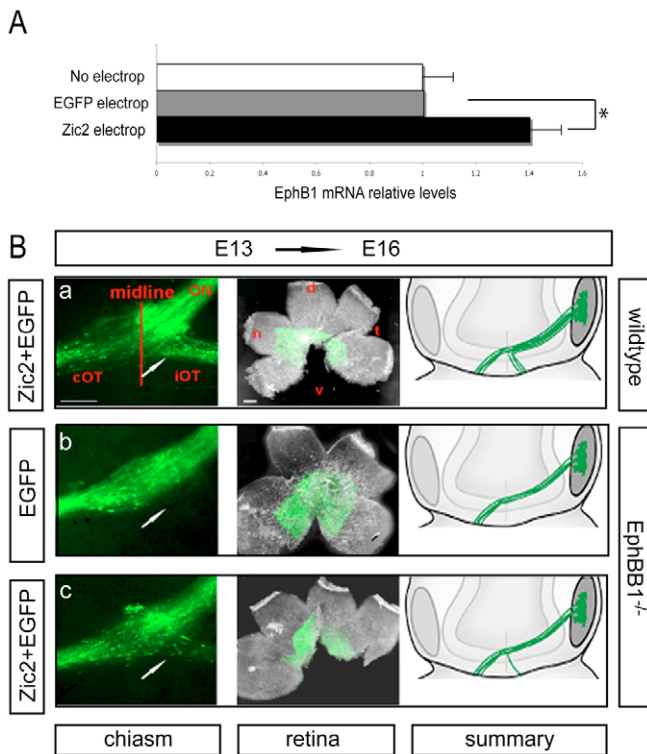
Fig. 5. Zic2-induced turning of ipsilateral retinal axons away from the midline is mediated by both EphB1-dependent and -independent pathways. (**A**) Quantitative RT-PCR for *EphB1* performed on non-electroporated or electroporated mouse retinas at E13.5 with CAG-EGFP or with CAG-Zic2/CAG-EGFP plasmids. *EphB1* levels significantly increase after ectopic expression of Zic2 in the center of the retina by in utero electroporation, as compared with control retinas. More than ten retinas for each condition were pooled per experiment. Data from seven different experiments are shown. Error bars indicate s.e.m.; *$P<0.005$. (**B**) Left panels show axonal trajectories of targeted RGCs at the optic chiasm. Middle panels show the location of the targeted RGCs (green) in retinal whole-mounts (gray). Right panels are cartoons summarizing the results in each particular case. (**a**) In wild-type embryos electroporated at E13.5 with Zic2/EGFP, many of the green axons originating from the central retina turn at the midline. (**b**) In *EphB1⁻/⁻* embryos ectopically expressing only EGFP in the central retina, no ipsilateral axons are seen at the chiasm. (**c**) When Zic2/EGFP are ectopically expressed in the central retina of *EphB1*-null embryos at E13.5, the number of axons projecting ipsilaterally does not change as dramatically as when wild-type embryos are electroporated with Zic2/EGFP, but a significant proportion of axons that project ipsilaterally can be still detected. White arrows indicate ipsilateral projections. ON, optic nerve; cOT, contralateral optic tract; iOT, ipsilateral optic tract; d, dorsal; n, nasal; t, temporal; v, ventral. Scale bars: chiasm panels, 200 μm; retina panels, 500 μm.

might regulate other proteins in addition to EphB1 during retinal axonal decisions at the midline. It is possible that Zic2 regulates EphB1 in VT retina, but when Zic2 is expressed in non-VT regions other molecules replace EphB1 to promote ipsilaterality. Our findings, together with previous observations that *EphB1*-null mice still have a remaining ipsilateral projection (Williams et al., 2003), whereas almost no ipsilateral axons are detected in the *Zic2* hypomorphic mutants (Herrera et al., 2003) (our unpublished observations), strongly suggest that EphB1 is not the only target for Zic2 in the VT retina.

In the peripheral nervous system of both vertebrates and invertebrates, as well as in the visual system of vertebrates, a limited number of transcription factors have been described that modulate axon guidance receptors (Broihier et al., 2004; Herrera et al., 2004; Jin et al., 2003; Kania and Jessell, 2003; Labrador et al., 2005; Pan et al., 2005; Pratt, 2004; Schulte and Cepko, 2000; Takahashi et al., 2003; Yeo et al., 2004). In many of these cases, whether the transcription factor plays a role in direct regulation of axon guidance cues or in general patterning in early neural differentiation is not clear. At the invertebrate midline, Longitudinals lacking (Lola) appears to be an example of a class of transcription factor that controls axon targeting by coordinating the regulation of multiple (Slit, as well as its axonal receptor Robo) guidance genes, ensuring that they are co-expressed at the correct time, place and relative level (Crowner et al., 2002). Lola homologs have not been described in vertebrates, but we have found a transcription factor that precisely regulates one particular axon guidance decision by the expression of at least one axon guidance receptor, EphB1, to specifically control laterality at the vertebrate midline.

Previous studies have suggested that the LIM homeodomain transcription factor Islet2 might be part of the program that controls laterality at the midline. In the embryonic retina, crossed projections originate from RGCs located over the entire retina except for the VT region, up to E17.5-18.5. Ganglion cells born later in the VT segment project contralaterally and intermingle with already differentiated ipsilaterally projecting RGCs in the VT retina (Guillery, 1995). Islet2 is expressed in RGCs that project contralaterally, including the late-born crossed RGCs in the VT crescent, but not in those projecting ipsilaterally. The role of Islet2 in the rest of the retina is not clear yet, but it appears to affect the laterality of the later-born crossing RGCs arising from the VT segment. Mice deficient for Islet2 show an increase in the number of ipsilateral axons originating in the VT retinal segment, which is accompanied by increased levels of Zic2 and EphB1 expression in that segment of the retina (Pak et al., 2004). Thus, Islet2 might repress an ipsilateral axon pathfinding program exclusively in the VT retina, which involves repression of Zic2 or EphB1 expression, or both. Although we cannot rule out the possibility that Islet2 represses EphB1, our results suggest that determination of ipsilaterality is, at least partially, a consequence of direct activation of EphB1 by Zic2, the latter potentially repressed by Islet2, rather than Islet2 repressing EphB1.

Transcription events can affect axon guidance decisions at distant choice points

Several transcriptional mechanisms have been suggested for regulating axonal pathways at intermediate targets. At the nuclear level, transcriptional regulation at early stages of differentiation might provide the complete repertoire of mRNAs that are required by a growth cone to navigate its entire pathway. According to this notion, transcriptional regulation could specify the pathway, whereas post-transcriptional regulation at the level of the axon or growth cone (local translation, protein modification, etc.) would control growth and directionality along the path, priming the axon with the cellular components necessary for 'on-site' post-transcriptional responses (Butler and Tear, 2006; Dickson and Gilestro, 2006; Polleux et al., 2007). Alternatively, specific receptors might be directly activated by different transcription factors (controlled by a master gene specifying the entire pathway) expressed in a hierarchical manner as the axon grows. Whether or not transcription factors directly activate guidance receptors in a sequential manner at different points along the journey of an axon is not clear. Nevertheless, because the midline is not the first choice-point encountered by the RGC axons, as they must first

orient and navigate towards the optic disc to then exit the retina, our work clearly supports the idea that initial transcriptional events can affect guidance decisions at distant choice points without disturbing previous axon decisions.

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