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Zic2 Patterns Binocular Vision by Specifying the Uncrossed Retinal Projection

Eloísa Herrera,[1] Lucia Brown,[2] Jun Aruga,[3] Rivka A. Rachel,[1,4] Gül Dolen,[1] Katsuhiko Mikoshiba,[3] Stephen Brown,[2] and Carol A. Mason[1,*]

[1]Department of Pathology and
Center for Neurobiology and Behavior
[2]Department of Obstetrics and Gynecology
Columbia University
College of Physicians and Surgeons
630 West 168th Street
New York, New York 10032
[3]Laboratory for Developmental Neurobiology
RIKEN Brain Science Institute
Saitama 351-0198
Japan

Summary

During CNS development, combinatorial expression of transcription factors controls neuronal subtype identity and subsequent axonal trajectory. Regulatory genes designating the routing of retinal ganglion cell (RGC) axons at the optic chiasm to the appropriate hemisphere, a pattern critical for proper binocular vision, have not been identified. Here, we show that the zinc finger transcription factor Zic2, a vertebrate homolog of the *Drosophila* gene *odd-paired*, is expressed in RGCs with an uncrossed trajectory during the period when this subpopulation grows from the ventrotemporal retina toward the optic chiasm. Loss- and gain-of-function analyses indicate that Zic2 is necessary and sufficient to regulate RGC axon repulsion by cues at the optic chiasm midline. Moreover, Zic2 expression reflects the extent of binocularity in different species, suggesting that Zic2 is an evolutionarily conserved determinant of RGCs that project ipsilaterally. These data provide evidence for transcriptional coding of axon pathfinding at the midline.

Introduction

The decussation of retinal ganglion cell (RGC) projections at the optic chiasm is essential for the normal mapping of visual information in higher visual centers. This projection pattern ensures that each relay receives information from both eyes, a pattern critical for proper binocular vision.

The proportion of uncrossed to crossed retinal fibers varies according to the extent of binocular vision across species. The uncrossed component ranges from 40% of all RGCs in humans (Kandel et al., 2000) to less than 15% in ferrets (Cucchiaro, 1991; Thompson and Morgan, 1993) and about 3%–5% in mice, depending on the strain (Rice et al., 1995). RGCs with an uncrossed trajectory are located in the temporal retina, but in less binocular species, ganglion cells that project ipsilaterally reside in the ventrotemporal (VT) retina. In *Xenopus,* a subpopulation of ganglion cells in VT retina projects ipsilaterally only at metamorphosis (Hoskins and Grobstein, 1985; Mann and Holt, 2001). Finally, adult birds and fish do not have an uncrossed projection (O'Leary et al., 1983; Polyak, 1957) and thus lack binocular vision.

How the development of the binocular pathway is determined has been a longstanding enigma. Most recent work has focused on the localization of guidance factors that function in other species and settings, especially in axon navigation at the CNS midline, and their mechanism of action (Mason and Erskine, 2000). Among these are Slit2 (Erskine et al., 2000; Plump et al., 2002), Nr-CAM (Lustig et al., 2001), chondroitin sulfate proteoglycans (Chung et al., 2000), and ephrin-A's (Marcus et al., 2000), all of which have been localized to the specialized populations of neurons and glia at the chiasmatic midline and modulate different aspects of RGC axon guidance. The only molecule expressed at the chiasm midline that is directly involved in RGC divergence is ephrin-B2, which prevents midline crossing of VT retinal axons in both *Xenopus* and mouse (Nakagawa et al., 2000; Williams et al., 2003). Moreover, EphB1, a receptor for ephrin-B2, is expressed in a restricted pattern in VT retina during RGC divergence at the midline and is essential for the formation of the uncrossed retinal pathway (Williams et al., 2003). Despite these advances in elucidating mechanisms of RGC axon divergence, it remains unknown whether there might be regulatory genes within the retina that determine the binocular projection, perhaps by encoding expression of guidance factor receptors.

Combinatorial codes of transcription factor expression define cell subtypes in the spinal cord (Tsuchida et al., 1994; Lee and Jessell, 1999; Arber et al., 2000; Pierani et al., 2001; for review, see Jessell, 2000; Shirasaki and Pfaff, 2002). Several of these transcription factors direct projections of subsets of motoneurons to specific peripheral targets (Lin et al., 1998; Kania et al., 2000). Members of the LIM protein family would be good candidates for specifying RGC subtype and, in turn, axon guidance in the retina, because at least two of them, Islet1 (Thor et al., 1991) and Islet2 (Brown et al., 2000), are expressed in RGCs. Other transcription factors, such as SOHo (Deitcher et al., 1994), BF-1 and BF-2 (Hatini et al., 1994), Brn.3b (Xiang et al., 1995), and Vax2 (Schulte et al., 1999) are expressed along the nasotemporal or dorsoventral retinal axes. However, none of these proteins are expressed in patterns that reflect the retinal axon decussation.

The *Zic* genes, a family of zinc finger transcription factors homologous to the *Drosophila* pair rule gene odd-paired (*opa*) (Benedyk et al., 1994; Aruga et al., 1996), are apt candidates for specification of axon projection relative to the midline, since *Zic* genes are involved in the left-right asymmetry of the body plan (Brown et al., 1998; Kitaguchi et al., 2000; Purandare et al., 2002), and they are expressed in the developing

*Correspondence: cam4@columbia.edu
[4]Present Address: Mouse Cancer Genetics Program, National Cancer Institute, Frederick, Maryland, 21702-1201.

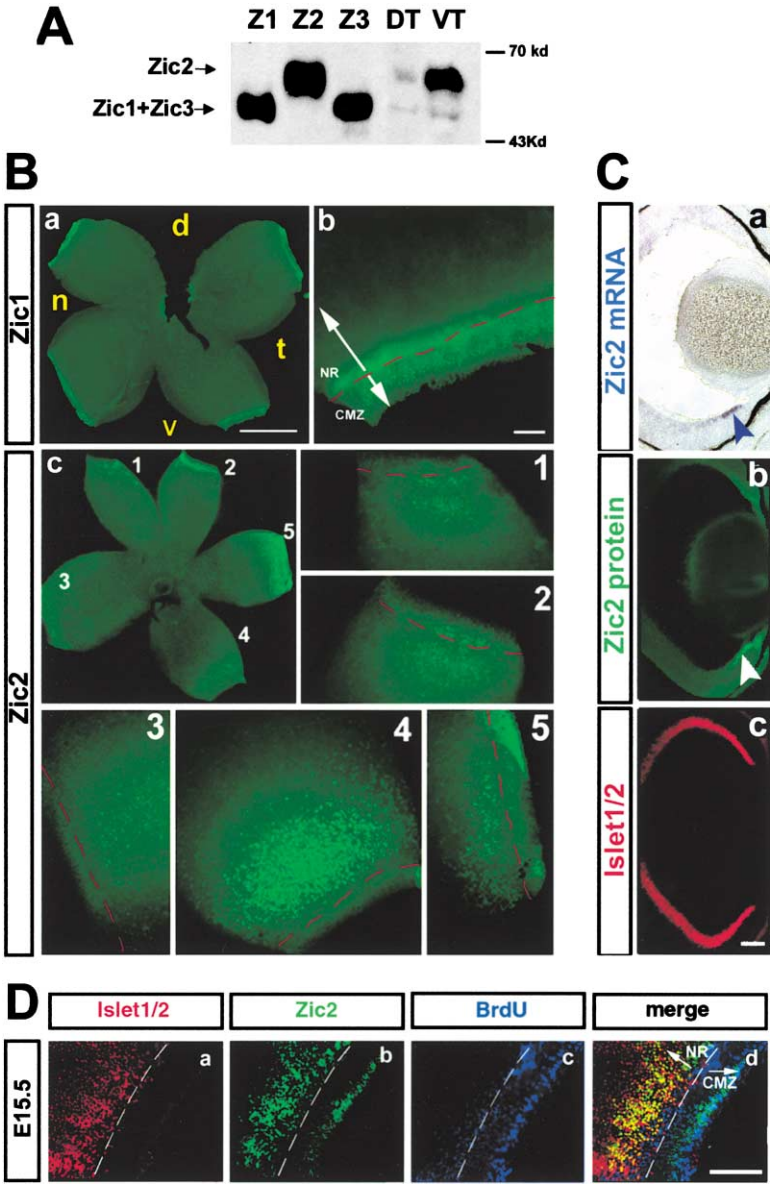

Figure 1. Zic1, Zic2, and Zic3 Expression in Developing Retina

(A) Peripheral ventrotemporal (VT) and dorsotemporal (DT) lysates from E16.5 retinae, immunoblotted with α-pan-Zic. 293-Zic1, -Zic2, and -Zic3 transformed cell lines are shown as controls. A band corresponding to Zic2 is detected in the VT retinal lysate but is barely detectable in the DT lysate. The weak bands observed in VT and DT lysates at the Zic1 and Zic3 molecular weight level are probably due to Zic1 expression in the ciliary margin (CMZ) at this age.

(B) E16.5 retinal whole mount stained with α-Zic1 (a and b) or α-Zic2 (c). (b) Higher magnification of (a). (1–5) Higher magnification of each retinal segment in (c). Note that only the VT segment shows Zic2+ cells. Abbreviations: N, nasal; D, dorsal; T, temporal; V, ventral; NR, neural retina; and CMZ, ciliary margin. Scale bar: 500 μm.

(C) Horizontal sections of E16.5 retina. (a) *Zic2* mRNA detected in ventral retina by in situ hybridization (blue). (b) Zic2 in ventral retina (green, white arrows) detected by α-Zic2. (c) RGC layer (red) visualized with α-Islet1/2 in the same section as (b). Scale bar: 100 μm.

(D) Zic2 is expressed in postmitotic cells in VT retina. (a–d) Confocal sections of the VT region labeled with α-Islet1/2 (a, red), α-Zic2 (b, green) or α-BrdU (c, blue). (d) merged image; Zic2+ cells in the neural retina are also Islet1/2+ (yellow), but rarely BrdU+ (turquoise), as in the CMZ. Abbreviations: NR, neural retina; CMZ, ciliary margin zone. Scale bar: 100 μm.

eye. At embryonic day (E)9.5, Zic1, Zic2, and Zic3 are expressed in the optic vesicle and stalk. After E10.5, the expression of the three genes converges in the ciliary margin zone (CMZ) of the retina (Nagai et al., 1997) where retinal precursors reside.

Here, we show that in addition to Zic2 expression in proliferating retinal cells in the CMZ, Zic2, but not Zic1 or Zic3, is upregulated in differentiated ganglion cells in VT retina when the ipsilateral projection is formed (E14–E17). Genetically modified mice that express low levels of Zic2 (Nagai et al., 2000) display a reduced ipsilateral projection. Moreover, in gain-of-function experiments in vitro, Zic2 is sufficient to switch the outgrowth behavior of retinal axons from crossed to uncrossed patterns in response to inhibitory cues from chiasm cells. Finally, the proportion of Zic2-expressing cells correlates with the degree of binocular vision across species, implicating Zic2 function in patterning binocular vision throughout evolution. These results indicate that Zic2 contributes to RGC subtype identity by directing the retinal axon projection at the optic chiasm midline.

Results

Zic2 Is Expressed in the Ventrotemporal Retina during the Outgrowth of the Uncrossed Retinal Projection

To investigate the possibility that Zic family members are expressed differentially in RGCs with an uncrossed or crossed trajectory, we first examined Zic protein expression in retina at E16.5, since this stage is within the principal period of axon divergence near the optic chiasm. In addition, at this age, contra- and ipsilaterally projecting RGCs are located in distinct areas of the retina (Guillery et al., 1995). Extracts of peripheral VT retina, where the uncrossed population resides, and dorsotemporal retina (DT), where only crossed RGCs are

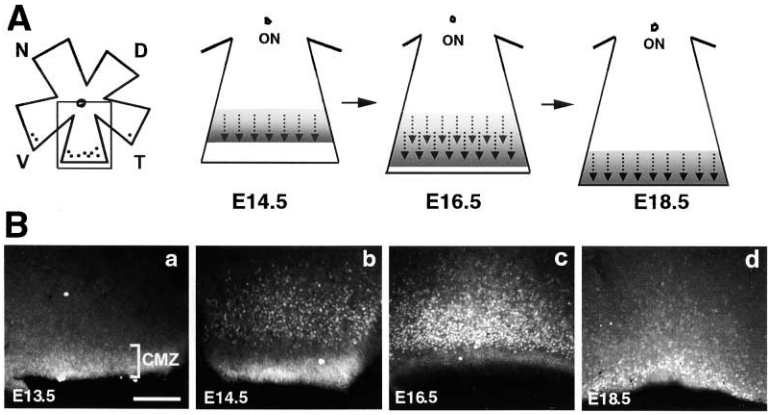

Figure 2. Zic2 Is Expressed from E14.5–E18.5

(A) Schematic representation of the VT segment of the retina, indicating the central to peripheral location of Zic2 over time. ON, optic nerve.

(B) In (a)–(d), VT retinae from E13.5-E18.5 mouse embryos, stained with α-Zic2. (a) At E13.5, no Zic2+ cells are detected in the VT neural retina. Note weak staining at the ciliary margin (CMZ) at this age, detected only until E14.5–15.5. (b) At E14.5 Zic2 is upregulated in VT neural retina. (c) A peak in the number of Zic2+ cells in VT retina is observed at E16.5, but by E18.5, few Zic2-expressing cells are seen (d). Scale bar: 100 μm

located, were immunoblotted using an anti-pan-Zic antibody. 293T cells transfected with Zic1, Zic2, or Zic3 were used as controls. While VT retinal lysates revealed a strong Zic2 band, this protein was barely detectable in DT retinal lysates. Immunohistochemistry using specific antibodies against Zic1 or Zic2 (Brown et al., 2003) on E16.5 retinal whole mounts revealed that Zic2, but not Zic1, is expressed specifically in VT retina. In situ hybridization and immunohistochemistry on retinal sections confirmed that *Zic2* mRNA and protein are selectively localized in neural retina in the peripheral VT segment (Figures 1B and 1C). Moreover, RGCs are the only retinal cells that express Islet at this age (Galli-Resta et al., 1997), and double immunostaining of Zic2 and of Islet1/2 demonstrated that Zic2 is located in the ganglion cell layer (Figure 1C). Thus, Zic2, but not other Zic family members, is expressed in RGCs at E16.5 in the peripheral VT neural retina, the source of the uncrossed retinal projection.

In mouse, RGCs are generated from E11.5 to birth. The first RGCs to be born and project axons both ipsi- and contralaterally derive from dorsocentral retina, but this uncrossed RGC population is thought to be transient. The permanent ipsilaterally projecting RGCs arise from VT retina, and these are generated from E14.5–E17.5. In contrast, contralaterally projecting cells differentiate at E11.5 and continue proliferating until the end of embryogenesis (Drager, 1985; Colello and Guillery, 1990). To determine whether the timing of Zic2 expression relates to generation and formation of the ipsilateral projection, E13.5–E18.5 retinal whole mounts were immunostained with anti-Zic2 antibodies. At E13.5, no Zic2-positive cells were found in any retinal quadrant except for weak staining in the CMZ around the entire retina (Figure 2Ba) (Nagai et al., 1997). Strong expression of Zic2 in the neural retina was first detected in the VT segment at E14.5 (Figure 2Bb), when axons from the VT retina begin to grow ipsilaterally to form the permanent uncrossed projection. For the next 2 days, the number of Zic2-positive cells increases as additional ipsilaterally-projecting RGCs in VT retina grow into the optic stalk. From E17.5–E18.5, when the generation of ipsilaterally projecting RGCs terminates, Zic2 expression wanes (Figure 2Bd). Zic2 is thus expressed in RGCs in a highly restricted spatiotemporal pattern, precisely at the time the uncrossed RGC projection from VT neural retina is established.

Zic2 Expression in Ventrotemporal Retina Is Postmitotic

Neuronal differentiation is controlled by transcription factors acting either during neuronal proliferation to specify neuronal fate (Cepko et al., 1996) or in postmitotic neurons to specify neuronal subtype (for review, see Shirasaki and Pfaff, 2002). Zic2-expressing RGCs are always located peripherally at a gradually increasing distance from central retina, in a pattern mirroring RGC genesis (Figure 2A). To investigate whether Zic2 is expressed in neural VT retina in differentiated RGCs or in proliferating cells, we labeled cycling cells with BrdU and then colabeled retinal whole mounts with antibodies against Islet 1/2 and Zic2. Islet1 and Islet2 are expressed by postmitotic RGCs as they leave the cell cycle (Rachel et al., 2002). At all ages studied (E14.5, E16.5, and E18.5), Zic2-positive cells within the RGC layer in VT retina were Islet1/2 positive but rarely BrdU labeled (Figure 1D). Thus, Zic2 is expressed in postmitotic RGCs in VT neural retina, supporting a role for Zic2 in the specification of the uncrossed RGC subtype.

Zic2 Is Expressed Exclusively in Uncrossed Retinal Ganglion Cells

To investigate whether Zic2 is expressed exclusively in ganglion cells that project ipsilaterally, RGCs were retrogradely labeled with rhodamine dextran from one optic tract in E16.5–E18.5 embryos, and retinal whole mounts were immunostained with anti-Zic2. In the retina ipsilateral to the labeled optic tract, retrogradely labeled cells were located in the VT retinal segment (Figure 3A) but positioned more centrally than Zic2-positive cells. This pattern indicates that the ganglion cells labeled from the optic tract are more mature than Zic2-expressing ganglion cells, since the wave of RGC generation progresses from central to peripheral retina. However, even though the majority of retrogradely labeled and Zic2-positive cells are not in exact register, at E16.5, a region exists where both populations overlap (Figure 3A); these double-labeled cells represent approximately 5% of all retrogradely labeled RGCs.

In the retina contralateral to the labeled optic tract, dextran-labeled cells were found throughout the retina with the exception of the peripheral VT retina (Figure 3B), where cells double-labeled with Zic2 and dextran were never observed (Figure 3B). This Zic2- and dextran-label-free gap in the contralateral retina presumably contains the RGCs that project ipsilaterally from this

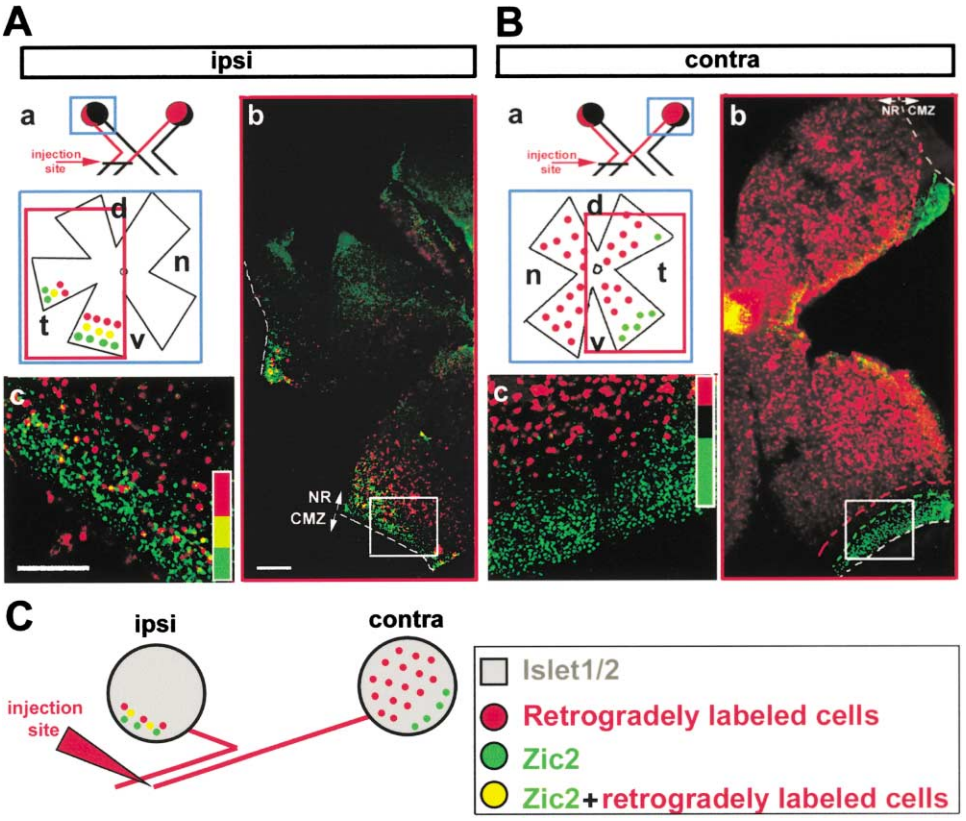

Figure 3. Zic2-Positive Cells and RGCs Retrogradely Labeled from the Ipsilateral Optic Tract Are Located in the Same Zone but Do Not Always Colabel

(A and B) Retrogradely labeled retinae, stained with α-Zic2. (A) Retina ipsilateral to the labeled optic tract. (B) Retina contralateral to the labeled optic tract. (a) Diagram representing cells labeled retrogradely from the optic tract (OT) (red). (b) View of half of a retinal whole mount showing retrogradely labeled cells (red) and Zic2+ cells (green). (c) Higher magnification of (b); note that only in the ipsilateral, but not in the contralateral, retina Zic2+ cells (green) are intermixed with dextran+ cells (red), and occasionally some cells are double labeled (yellow). Abbreviations: NR, neural retina; CMZ, ciliary margin zone. Scale bar: 100 μm.

(C) Schema summarizing the spatial aspects of Zic2 expression (green dots) with respect to zones occupied by Islet1/2+ RGCs (blue) and RGCs retrogradely labeled from the optic tract (red dots). In both eyes, Islet1/2 is expressed by RGCs in the entire retina, whereas Zic2 is observed in the peripheral VT retina. In the eye ipsilateral to the dextran label, RGCs are labeled only in VT retina and are located more centrally than Zic2+ cells. Yellow dots mark double-labeled cells. In the contralateral retina, dextran+ cells are found in the entire retina except in the region in VT retina where uncrossed RGCs from this eye should be located. Instead, there is a gap between dextran-labeled and Zic2+ populations. Cells located in the gap are Islet1/2+, indicating that they are RGCs.

eye. By E17.5, however, the gap between contralaterally projecting dextran-labeled cells and Zic2-positive RGCs is almost imperceptible (data not shown). This latter finding is consistent with previous data showing that up to E17.5–18.5, crossed projections originate from RGCs over the entire retina except for the VT region; RGCs born after this time are only contralaterally projecting and intermingle with already differentiated ipsilaterally projecting RGCs in VT retina (Guillery et al., 1995).

These results on the time course of Zic2 expression coinciding with the generation of the ipsilateral projection, together with the colocalization of Zic2 and retrograde labeling in the ipsilateral, but not contralateral retina, argue that Zic2 is exclusively expressed in RGCs that project ipsilaterally.

Zic2 Expression Is Downregulated As RGCs Extend toward Chiasmatic Midline

The retrograde labeling experiments described above show that at E17.5, only a small proportion of cells within VT retina that were labeled from the ipsilateral optic tract express Zic2 (<5% of all ipsilateral cells, Figure 3A). One explanation for this observation is that Zic2 is initially expressed in RGCs that will ultimately project ipsilaterally but is downregulated once axons reach the optic chiasm. Alternatively, it may not have been possible to retrogradely label many of the Zic2-positive cells that project ipsilaterally because their axons have not yet reached the optic tract. To distinguish between these possibilities, we applied dextran to the optic tract at E15.5, E16.5, and E17.5 and compared the total number of retrogradely labeled ganglion cells with the number of Zic2-positive cells in the retina ipsilateral to the injected optic tract. At E15.5, the Zic2-expressing population was larger than the number of dextran-labeled cells (578 ± 96 Zic2-positive cells/retina compared to 285 ± 24 dextran-positive cells/retina), and both numbers nearly doubled by E16.5. However, by E17.5, the number of cells dextran-labeled cells exceeded the number of Zic2-positive cells (668 ± 86 Zic2-positive cells/retina and 1284 ± 88 dextran-positive cells/retina) (Figure 4A).

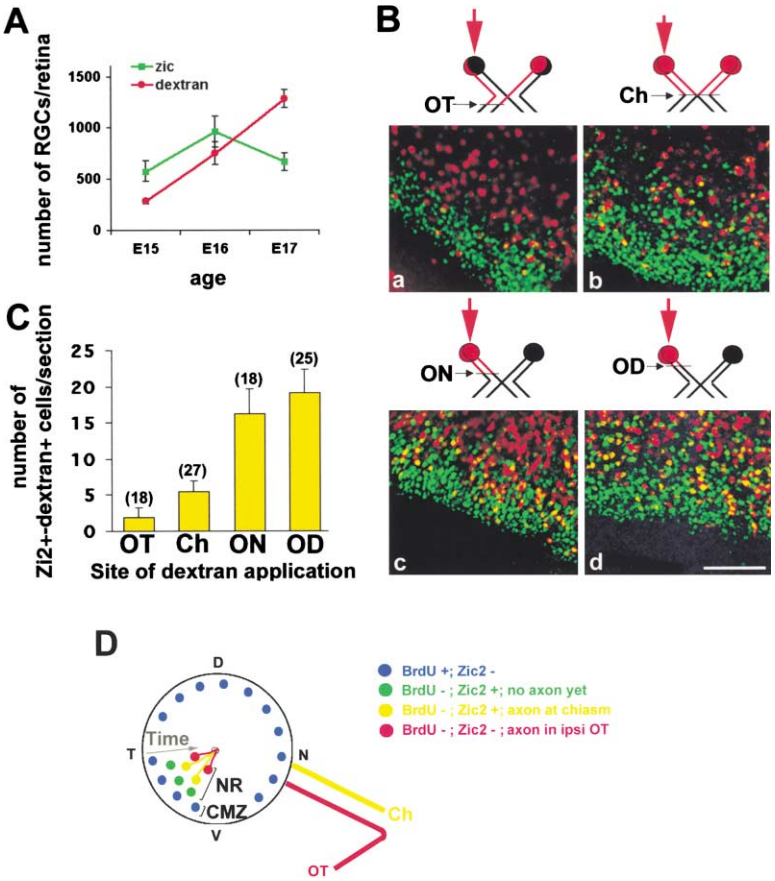

Figure 4. Zic2 Is Downregulated before Axons Enter the Optic Tract

(A) Quantification of Zic2+ cells (green line) and RGCs retrogradely labeled from the ipsilateral optic tract (red line) at E15.5, E16.5, and E17.5 in retinal whole mounts. At earlier ages, more Zic2+ cells are detected relative to the number of dextran-labeled uncrossed cells. In contrast, at E17.5, the number of ipsilateral cells labeled with dextran exceeds the number of Zic2+ cells (n = 5 retinal whole mounts for each age).

(B) In (a)–(d), confocal sections of VT retinal whole mounts retrogradely labeled with rhodamine dextran at E17.5 (red) and stained with α-Zic2 (green). The application point of dextran is indicated above each picture. Red arrow indicates the eye used to count RGCs. Scale bar: 100 μm.

(C) Quantification of double-labeled RGCs (yellow cells in Figure 4B) after retrograde labeling and α-Zic2 staining. The number of double-labeled cells was counted in confocal sections. As the site of dextran application became more distal to the retina, fewer RGCs were double-labeled. Number above bar indicates number of sections from five different retinae. Abbreviations: OD, optic disc; ON, optic nerve; Ch, optic chiasm; and OT, optic tract.

(D) Schema summarizing the spatiotemporal features of Zic2 expression in the developing neural retina. Zic2 is expressed postmitotically, through RGC extension to and within the chiasm and then is downregulated in the optic tract.

These data suggest that at E17.5, when many more uncrossed fibers have reached the optic tract compared to E15.5, Zic2 expression is downregulated in the retina.

To determine where along the route Zic2 expression might be downregulated, RGC axons were retrogradely labeled via four points along the retinal axon trajectory: (1) the optic tract, after RGC axons exit the chiasm; (2) the optic chiasm; (3) the optic nerve, just before the axons reach the chiasm; and (4) the optic disc, as axons are leaving the retina. Retinal whole mounts were incubated with anti-Zic2 and the number of double-stained cells counted in the eye ipsilateral to the dextran application. It should be noted that dextran application into the optic chiasm, nerve, or disc labels RGCs that project both ipsi- and contralaterally. However, based on the Zic2/dextran-free gap observed in the retina contralateral to optic tract labeling (see above), we assumed that any double-labeled cells in this zone must be RGCs that project ipsilaterally.

As described above, Zic2 is expressed only in a few cells retrogradely labeled from the ipsilateral optic tract (1.75 ± 1.30 double-labeled cells/section or 5% of all retrogradely labeled ipsilateral cells) (Figure 4Ba). Retrograde labeling from the chiasm yielded a greater number of dextran-positive cells that expressed Zic2 compared to labeling from the optic tract (5.3 ± 1.7 double-labeled cells/section, representing approximately 15% of all retrogradely labeled cells that projected ipsilaterally) (Figures 4Bb and 4C). Application of dextran to the optic nerve resulted in yet more cells double labeled for Zic2 and dextran (11.4 ± 2.1 double-labeled cells/section or 32% of all retrogradely labeled RGCs projecting ipsilaterally) (Figures 4Bc and 4C). When axons were labeled from the optic disc, the number increased to 19.0 ± 3.02 double-labeled cells/section (54% of all retrogradely labeled ipsilaterally projecting RGCs) (Figures 4Bd and 4C). Thus, the closer to the eye the label was applied, the more dextran-labeled cells were observed that expressed Zic2. These findings favor the hypothesis that Zic2 is expressed in RGCs that will project ipsilaterally during the early axonogenesis but is downregulated as axons navigate through the optic nerve and chiasm and is reduced to very low levels when RGCs reach the optic tract.

Zic2 Expression Reflects Different Degrees of Binocularity across Species

We hypothesized that if Zic2 specifies the ipsilateral projection, then the proportion of ganglion cells expressing Zic2 would reflect the relative size of the uncrossed projection in organisms having a larger or smaller ipsilateral retinal projection. We used four models to test this hypothesis: (1) albino mouse, where the proportion of uncrossed axons is reduced compared to the uncrossed component in pigmented mouse; (2) ferret, in which the uncrossed RGC zone is expanded compared to that in mouse; (3) *Xenopus,* in which an ipsilateral projection develops only after metamorphosis (about stage 54); and (4) chick, which lacks a permanent ipsilateral projection.

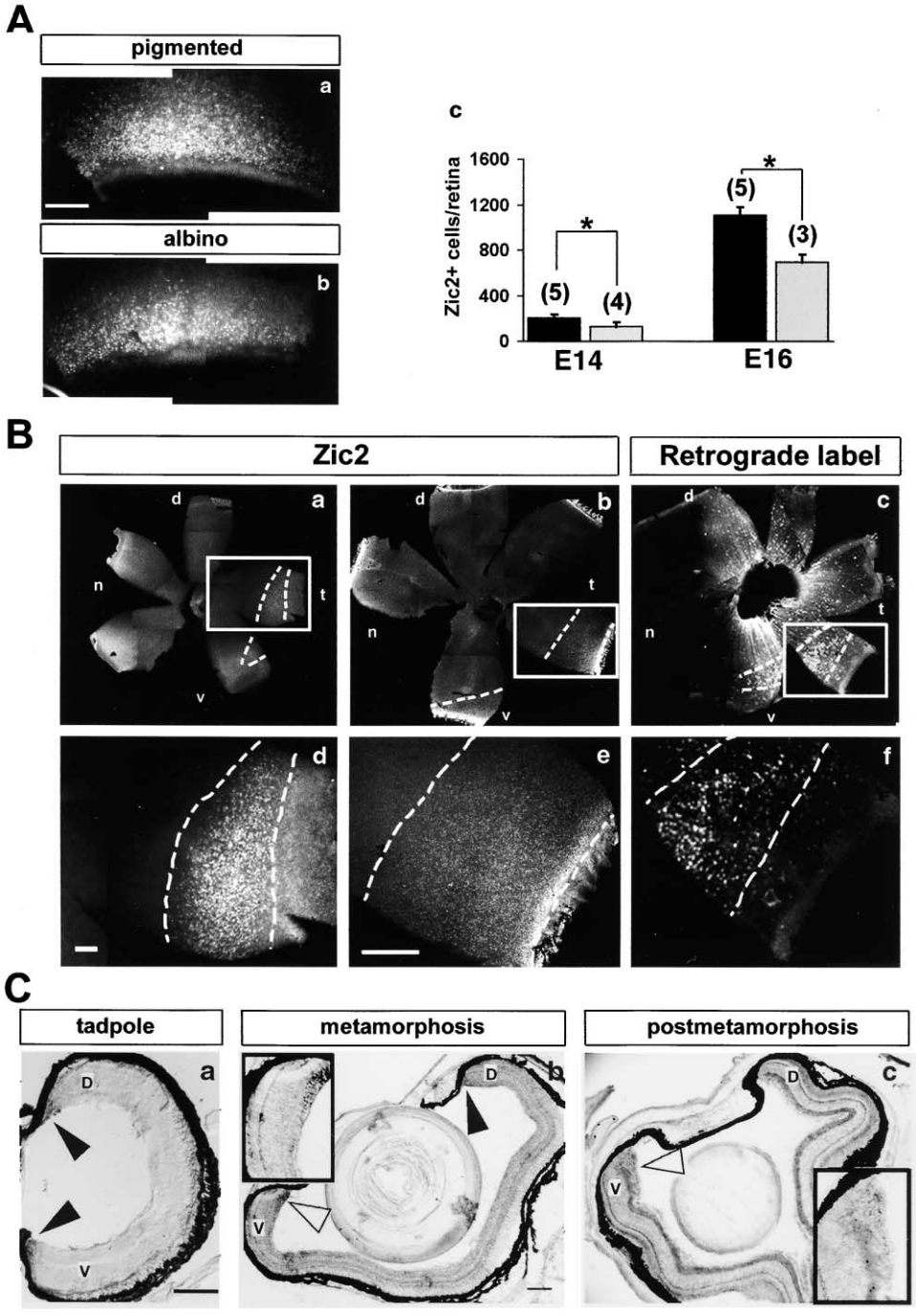

Figure 5. The Number of Cells Expressing Zic2 in Different Species Reflects the Degree of Binocular Vision

(A) Albino mouse. Retinal whole mounts from littermates of E16.5 pigmented (a) and albino mouse embryos (b) stained with α-Zic2. Scale bar: 100 μm. (c) Quantification of Zic2+ cells in pigmented (black columns) versus albino retinae (gray columns) at E14.5 and E16.5. Number above bars indicates number of retinal whole mounts. *$p < 0.05$ compared to pigmented retinae (Student's unpaired t-test).

(B) Ferret. Retinal whole mounts from E27.5 (a) and E34.5 (b) embryos labeled with α-Zic2. Dashed white lines delineate the area in which Zic2 is expressed. (c) Retinal whole mount from an E34.5 embryo retrogradely labeled with DiI from the optic tract. Dashed white lines delineate retrogradely labeled cell location. (d–g) High magnification of selected areas in (a), (b), and (c), respectively. Scale bars: (d), 100 μm; (e and f), 500 μm.

(C) *Xenopus*. In situ hybridization of frontal sections using Zic2-specific probes in premetamorphic (a), metamorphic (stage 54) (b), and postmetamorphic *Xenopus* (c). Black arrows, *Zic2* mRNA in the ciliary margin; white arrows, *Zic2* mRNA in the neural retina. Abbreviations: D, dorsal; V, ventral; T, temporal; and N, nasal. Scale bar: 100 μm.

In albino mice, fewer Zic2-positive cells were observed compared to pigmented retinae at E14.5 (125 ± 38 cells/retina in albino and 207 ± 32 cells/retina in pigmented littermates) and at E16.5 (700 ± 66 cells/retina in albino and 1108 ± 77 cells/retina in pigmented) (Figure 5A). This decrease in the number of Zic2-positive cells in albino retina (a reduction of about 37%) closely parallels the reduction in uncrossed RGCs compared to pigmented adult retinae (Rice et al., 1995).

In ferret, E27.5 and E34.5 are ages that correspond to E15.5 and E17.5, respectively, in mouse in terms of uncrossed RGC production and axon growth to the chiasm (Chalupa and Snider, 1998). At both of these ages, Zic2-positive cells occupy an area in temporal retina larger than in mouse retina that reflects the proportion and location of uncrossed RGCs. At E27.5, Zic2 expression was more central than at E34.5 (compare Figures 5Bd and 5Be). In ferret retina, Zic2-positive and retrograde-labeled zones overlap but are not in register, suggesting as in mouse that Zic2 is expressed in younger ganglion cells, likely prior to or during the early phases of axonogenesis.

In *Xenopus* tadpoles, *Zic2* mRNA was detected in the CMZ and not VT neural retina. At metamorphosis, in contrast, when the ipsilateral projection develops, Zic2 is expressed in VT retina. In adults, *Zic2* mRNA was present in VT retina but at lower levels than at metamorphosis, corresponding to the continued but slowed production of uncrossed ganglion cells (Figure 5C).

Finally, *Zic2* mRNA was not detected in chick neural retina at two different stages, St24 and St30, when axons reach the chiasm area (Thanos and Mey, 2001), even though Zic2 was localized in spinal cord, ventral diencephalons, and other structures (data not shown).

Thus, the number of retinal cells expressing Zic2 correlates with the spatiotemporal features of the formation of the uncrossed projection in the albino and pigmented mouse, ferret, *Xenopus,* and chick, and reflects the degree of binocularity in each of these species and strains.

Loss and Gain of Zic2 Function in Retinal Ganglion Cells

Reduced Levels of Zic2 In Vivo Perturb the Development of the Ipsilateral Projection

To test whether Zic2 is required for the formation of the ipsilateral projection, we analyzed embryos from genetically modified mice that have significantly reduced levels of Zic2 (knockdown mice), both heterozygotes ($Zic2^{+/kd}$) and homozygotes ($Zic2^{kd/kd}$) (Nagai et al., 2000).

$Zic2^{kd/kd}$ embryos exhibit varying defects in neural tube closure (Nagai et al., 2000). Some $Zic2^{kd/kd}$ embryos also show defects in the eye, in agreement with the proposed role of Zic2 in early eye development (Nagai et al., 1997), but in most $Zic2^{kd/kd}$ embryos, the eyes appeared normal (Figure 6A, Table 1). Immunohistochemistry using the Islet1/2 antibody showed normal patterns of RGCs in $Zic2^{kd/kd}$ or $Zic2^{+/kd}$ mice with normal eyes, even in those embryos with the most severe phenotype in the spinal cord, suggesting that RGC positioning is unaltered in these animals (Figure 6B). $Zic2^{kd/kd}$ embryos also exhibited anatomical malformations in the ventral diencephalon and concomittantly altered expression of molecules important for axon divergence at the chiasm, such as ephrin-B2 (Nakagawa et al., 2000; Williams et al., 2003) (Figure 6B). Importantly, $Zic2^{+/kd}$ mice have an apparently normal chiasm (including normal expression of ephrin-B2) (Figure 6B), making the heterozygote knockdown a more suitable model for this study.

To investigate whether the ipsilateral projection was reduced in embryos with decreased levels of Zic2, the retinal projection was labeled with DiI at E16.5–E17.5 in $Zic2^{+/+}$, $Zic2^{+/kd}$, and $Zic2^{kd/kd}$ embryos. $Zic2^{+/kd}$ embryos displayed a reduced ipsilateral projection compared to $Zic2^{+/+}$ littermates (Figures 6C and 6D, and Table I). Most $Zic2^{kd/kd}$ embryos lacked an ipsilateral projection, but there were a few cases in which the uncrossed projection was apparent (Table 1). In both $Zic2^{kd/kd}$ and $Zic2^{+/kd}$ genotypes, a subset of axons consistently projected in aberrant directions distal to the optic nerve but before the chiasmatic midline, conferring a frayed appearance to the caudal aspect of the chiasm (Figure 6DI).

$Zic2^{+/kd}$ embryos showed a reduction in the ipsilateral projection, but there was variability in the phenotype (Figure 6C). To test whether this variability in the uncrossed projection was a consequence of differing levels of Zic2, we compared expression of Zic2 in VT retina with the size of the ipsilateral projection in each embryo by measuring fluorescence in the ipsilateral optic tract and comparing this value to fluorescence in the optic nerve (coefficient of the ipsilateral projection [Cip]; see Experimental Procedures). We found that the number of cells expressing Zic2 in VT neural retina in $Zic2^{+/kd}$ embryos closely correlates with the proportion of fibers that project ipsilaterally ($n = 8$ for $Zic2^{+/+}$, $n = 13$ for $Zic2^{+/kd}$, Figure 6D; Table 1). It was not possible to make this correlation in $Zic2^{kd/kd}$ embryos because Zic2 levels were not detectable in VT retina. The phenotype of each $Zic2^{kd/kd}$ embryo is described in Table 1.

Zic2 Modulates the Behavior of RGC Axons in Response to Chiasm Cues

To investigate whether Zic2 is sufficient to switch the response of RGCs to inhibitory cues at the optic chiasm midline, we utilized in vitro assays previously established by our lab that reconstitute the interactions of retinal axons with chiasm cells. In these assays, retinal explants are cultured in contact with cells dissociated from the midline region of the ventral diencephalon surrounding the optic chiasm, the native cue-presenting cells that direct the bilateral retinal projection. Neurites from VT retinal explants are more strongly inhibited by chiasm cells than neurites from other retinal regions (Marcus et al., 1995; Wang et al., 1995).

First, we confirmed that Zic2 is not expressed at E14.5 in explants from DT retina after 18–24 hr in coculture with chiasm cells (data not shown). Next, prior to coculture with dissociated chiasm cells, E14.5 DT retinal explants were infected with a recombinant Sindbis virus expressing EGFP alone (EGFP), Zic2 (Zic2-EGFP), or an inactive Zic2 mutant (Zic2Δ-EGFP). Neurite outgrowth from DT retinal explants was not affected by the introduction of EGFP or Zic2Δ-EGFP, but when Zic2 was ectopically misexpressed, neurites from DT explants grew 30% shorter compared to those explants in which EGFP ($p < 0.02$) or Zic2Δ-EGFP ($p < 0.04$) were overexpressed (Figure 7B). To test the possibility that Sindbis virus or overexpression of Zic2 itself was toxic, we in-

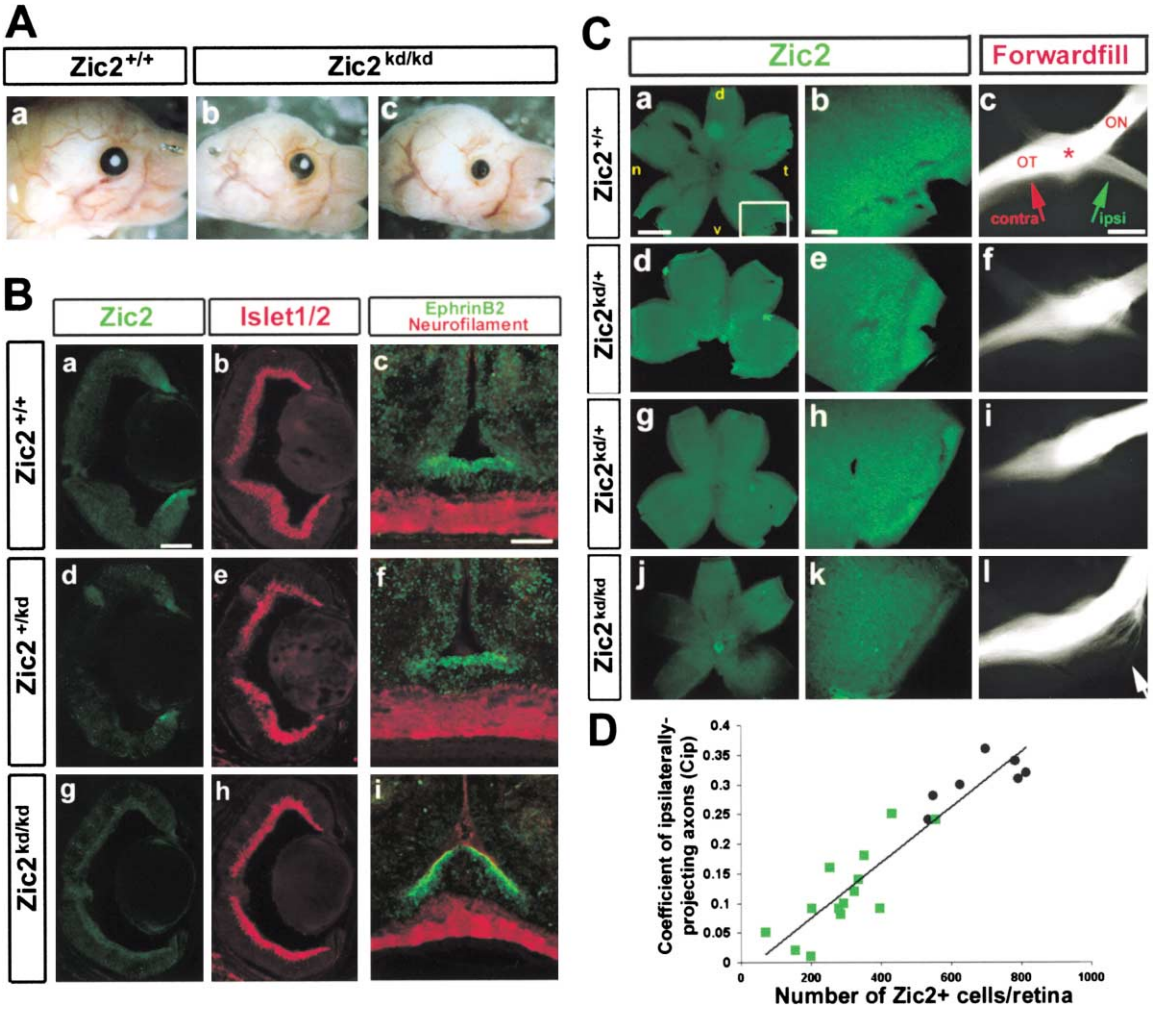

Figure 6. Zic2 Is Required for the Proper Development of the Ipsilateral Projection In Vivo

(A) Lateral views of E17.5 littermate embryos: *Zic2*$^{+/+}$ (a) and *Zic2*$^{kd/kd}$ embryos (b and c). *Zic2*$^{kd/kd}$ embryos usually have normal-appearing eyes (b) although occasionally display an underdeveloped eye (c).

(B) E16.5 *Zic2*$^{+/+}$, *Zic2*$^{+/kd}$, and *Zic2*$^{kd/kd}$ retinal sections (cut frontally) stained with α-Zic2 and Islet1/2. Zic2 is expressed in VT retina in *Zic2*$^{+/+}$ embryos (a) but was not detected in *Zic2*$^{kd/kd}$ embryos (g). Some cells express Zic2 in the *Zic2*$^{+/kd}$ retina (d). Islet1/2 expression was similar in *Zic2*$^{+/+}$, *Zic2*$^{+/kd}$, and *Zic2*$^{kd/kd}$ embryos (b, e, and h). Scale bar: 100 μm. (c, f, and i) Ephrin-B2 expression (green) is similar in *Zic2*$^{+/+}$ and *Zic2*$^{+/kd}$ embryos in the chiasm region but is altered in *Zic2*$^{kd/kd}$, along with the conformation of the ventral diencephalons. Scale bar: 200 μm.

(C) In (a), (d), (g), and (j), Zic2 expression in E16.5 retinal whole mounts from *Zic2*$^{+/+}$, *Zic2*$^{+/kd}$, and *Zic2*$^{kd/kd}$ embryos. Scale bar: 500 μm. In (b), (e), (h), and (k), higher magnification of the VT region. In (c), (f), (i), and (l), DiI anterograde tracing of the retinal projection in the chiasm in the same embryos as in (a), (d), (g), and (j), respectively. Scale bar: 100 μm. (c) Chiasm of a *Zic2*$^{+/+}$ embryo. Abbreviations: ON optic nerve; OT, optic tract. Green arrowhead, ipsilateral projection; red arrowhead, contralateral projection; the asterisk marks the chiasmatic midline. (f) Chiasm of a *Zic2*$^{+/kd}$ embryo with a dramatic reduction of ipsilateral fibers. (i) Chiasm of a *Zic2*$^{+/kd}$ embryo having no visible axons in the ipsilateral pathway. (l) Chiasm of a *Zic2*$^{kd/kd}$ embryo with no visible axons growing into the ipsilateral pathway, but fibers stray from the optic nerve and grow into the diencephalon caudal to the chiasm (arrowhead). Scale bar: 200 μm.

(D) Correlation between Zic2+ cell number and ipsilaterally projecting axons in *Zic2*$^{+/+}$ and *Zic2*$^{+/kd}$ embryos. Black circles, *Zic2*$^{+/+}$; Green squares, *Zic2*$^{+/kd}$. *Zic2*$^{kd/kd}$ were not represented because they have no Zic2+ cells. Data on *Zic2*$^{kd/kd}$ are listed in Table 1.

fected DT and VT retinal explants with Zic2-EGFP Sindbis virus and plated them without chiasm cells. Axonal outgrowth under these conditions was similar to or better than uninfected DT or VT explants grown without chiasm cells, indicating that the amount of Zic2-Sindbis virus that we added to the explants did not perturb axonal outgrowth (Figure 7B).

At E14.5, because Zic2 expression has commenced in many cells in retina, it is possible that RGCs in DT retina have already been specified to project contralaterally by other regulatory genes putatively determining the crossing axonal phenotype and thus are less responsive to the introduction of Zic2. We therefore performed the same experiment on DT retinal explants but taken from younger retinae. The misexpression of Zic2-EGFP in DT explants from E13.5 rather than E14.5 retinae led to a dramatic reduction in the extent of neurite outgrowth (about 67%) compared to controls (EGFP [$p < 0.001$] or Zic2Δ-EGFP [$p < 0.003$]) (Figures 7A and 7B). In addition, neurite outgrowth from VT retinal explants in which Zic2

Table 1. Retinal Axon Projection Phenotype at the Optic Chiasm of *Zic2* Knockdown Embryos

Genotype	Number of Embryos	Age	Overall Phenotype	Zic2+ Cells in Retina	Cip
+/+	8	E16–E17	normal	685 ± 116*	0.30 ± 0.03*
+/kd	14	E16–E17	normal	295 ± 122*	0.11 ± 0.07*
kd/kd	1	E16	NTM, one eye not developed	ND	0.16, AP
kd/kd	1	E16	NTM, one eye not developed	35	0.21, AP
kd/kd	1	E16	NTM, normal eyes	10	UI, AP
kd/kd	1	E16	NTM, normal eyes	0	UI, AP
kd/kd	1	E17	NTM, normal eyes	0	UI, AP
kd/kd	1	E17	NTM, normal eyes	0	UI, AP
kd/kd	1	E17	NTM, normal eyes	0	UI, AP
kd/kd	1	E17	NTM, normal eyes	ND	UI, AP

Zic2-positive cells were counted in retinal whole mounts for each individual embryo (Zic2$^+$cells). Cip, coefficient of ipsilaterally projecting axons, an index of the size of the projection (see Experimental Procedures and Figure 6). In *Zic2*$^{+/+}$ and *Zic2*$^{+/kd}$ embryos, Zic2-positive cell number and Cip are expressed as an average, with data pooled from ten different litters. In *Zic2*$^{kd/kd}$ embryos, data are shown for each individual embryo. Abbreviations: ND, not determined; NTM, neural tube malformation; UI, undetectable ipsilateral projections; and AP, aberrant projections straying from the chiasm.
*Represented in graphic form in Figure 6D.

was overexpressed was severely inhibited by chiasm cells (65% reduction in neurite outgrowth compared to EGFP-expressing VT explants) ($p < 0.001$) (Figure 7B). In sum, gain-of-function assays in vitro indicate that Zic2 activity is sufficient to switch the projection phenotype of RGCs with respect to cues provided by chiasmatic cells, from extension to neurite inhibition, and suggest that this behavior is determined in a narrow time interval.

Using this in vitro assay, we also tested whether Zic2 is required to switch the behavior of RGCs toward chiasm cells. Zic2 function was blocked with decoy oligos bearing the Zic DNA binding sequence (*Zic2* decoy). As a negative control, we used the same sequence only with point mutations that abolish *Zic2* binding (*Zic2M* decoy). The outgrowth from VT retina transfected with *Zic2*-decoy oligos was significantly enhanced compared to explants incubated with the lipofection agent alone or explants transfected with *Zic2M* decoy). In contrast, outgrowth from DT explants transfected with *Zic2* decoy was unchanged and was similar to outgrowth in explants transfected with the lipofection agent alone or with *Zic2M* decoy (see Supplemental Data online at http://www.cell.com/cgi/content/full/114/5/545/DC1).

Together, the loss- and gain-of function analyses implicate Zic2 as an essential determinant of the ipsilateral retinal projection at the optic chiasm.

Discussion

In this study, we show that in the mouse retina, ganglion cells having an ipsilateral projection can be distinguished from the more numerous contralaterally projecting ganglion cells by expression of the transcription factor Zic2. Zic2 expression in VT neural retina is limited to the period when the ipsilateral projection is established at the optic chiasm. Three additional lines of evidence implicate Zic2 as a determinant of RGC identity with respect to laterality of the retinal projection. (1) The spatiotemporal expression of Zic2 reflects the extent of binocularity in four distantly related species. (2) A reduction in Zic2 levels in vivo leads to a decrease in the number of axons that project ipsilaterally at the optic chiasm. (3) Gain-of-function experiments convert the response of DT retinal ganglion cell neurites to cells of the chiasm midline, from extension to inhibition.

Spatiotemporal Aspects of Zic2 Expression in the Retina

During the development of the RGC projections to the brain, Zic2 expression is restricted temporally, from E14.5 to about E17.5, after neurogenesis and during the period in which the permanent ipsilateral RGC axon projection is established, and spatially, exclusively in the region from which the uncrossed projection arises. The finding that Zic2 is more likely to be expressed in RGCs with axons in the optic nerve than in RGCs that have projected into the optic tract suggest that this gene is downregulated as axons transverse the optic chiasm.

The formation of the optic chiasm occurs in two phases. First, early-generated RGC axons originating from dorsal-central retina reach the optic chiasm at E12.5 and project ipsi- or contralaterally. This early ipsilateral projection from dorsocentral retina is thought to be transient (Guillery et al., 1995). In a second phase, at E14.5, RGCs differentiate in a central to peripheral wave to produce the permanent uncrossed projection from peripheral VT retina. Zic2 is not detected in dorsocentral retina at E11.5–E13.5, suggesting that Zic2 exclusively specifies the permanent RGC projection from VT retina.

Together, these data are consistent with the current idea that postmitotically expressed transcription factors have key functions in activating programs for specific pathway choices and target recognition (for review, Shirasaki and Pfaff, 2002) here, with respect to the midline.

Zic2 As a Candidate Transcriptional Regulator of the Uncrossed RGC Projection

The *Zic* family was originally identified as a group of genes encoding zinc finger proteins expressed in adult mouse cerebellum (Aruga et al., 1996). Zic2 collaborates with Zic1 in cerebellar folial and areal patterning (Aruga et al., 2002) and marks later stages of granule cell differentiation (Rubin et al., 2002). The three best studied members of the Zic family, Zic1, Zic2, and Zic3, are also

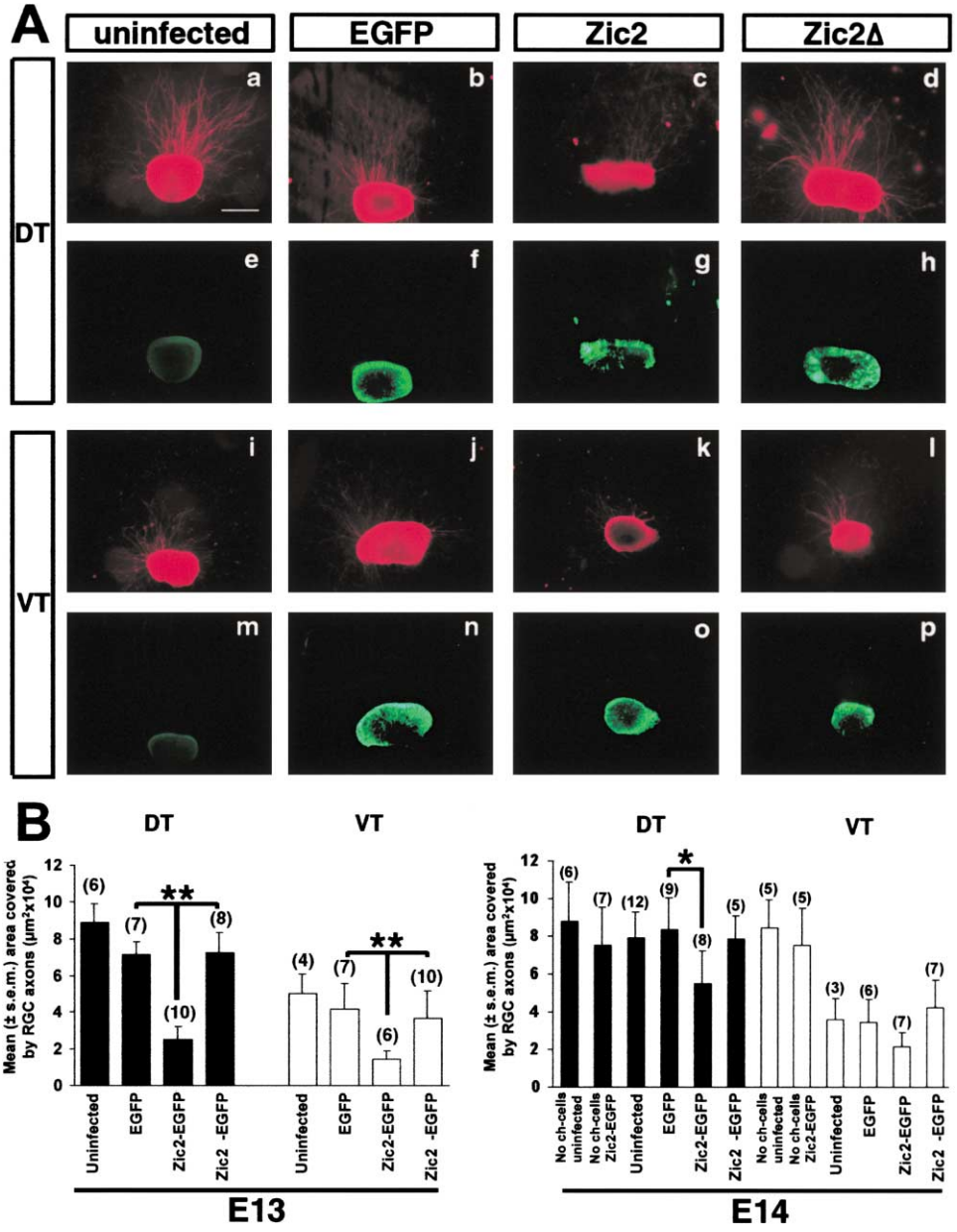

Figure 7. Zic2 Is Sufficient to Switch the Response of RGCs to Midline Cues from Extension to Inhibition

(A) Representative DT (a–h) or VT (I–p) retinal explants from E13.5 mouse embryos, uninfected (a, e, i, and m), missexpressing EGFP (b, f, j, and n), Zic2-EGFP (c, g, k, and o), or Zic2Δ-EGFP (d, h, l, and p), cocultured with dissociated chiasm cells. Axons are visualized with α-neurofilament (a–d and i–l). (e–h) and (m–p) represent the same explants as (a)–(d) and (i)–(l), respectively, but show EGFP fluorescence. Scale bar: 500 μm.

(B) Quantification of the area occupied by neurites from DT (filled bars) or VT (open bars) retinal explants at E13.5 (left graph) or E14.5 (right graph); uninfected; expressing EGFP, Zic2-EGFP, or Zic2Δ-EGFP; and cocultured with dissociated chiasm cells. Explants that were uninfected or infected with Zic2-EGFP-Sindbis virus and grown without chiasm cells were included as a control for possible toxicity from the virus. **$p < 0.001$ relative to EGFP infected explants and *$p < 0.02$ relative to EGFP infected explants (Student's unpaired t- test). Number above bars indicates number of explants. Data were pooled from nine independent experiments.

expressed in the early embryo, including the eye, playing essential roles in CNS development as "prepatterning" genes (Nagai et al., 1997; Brown et al., 1998, 2001; Aruga et al., 2002). Here, we show that Zic2, but not Zic1 or Zic3, is implicated in an additional developmental process, the specification of a subtype of retinal ganglion cell that have uncrossed axon projections with respect to the CNS midline. Thus, it appears that Zic2 plays multiple roles in neural development, as a neuronal inducer in undifferentiated cells, and as a postmitotic factor, acting during later differentiation in VT retina.

Zic2$^{kd/kd}$ mice show variability in the RGC projection phenotype, from a severe reduction to an approximately normal number of uncrossed axons; in all of these vari-

ants, a subset of axons strays from the optic chiasm. Zic2 is also highly expressed in the developing optic chiasm (Brown et al., 2003), and accordingly, *Zic2*$^{kd/kd}$ embryos show anatomical malformations at the chiasm region. The random unraveling of a subset of axons in *Zic2*$^{kd/kd}$ embryos could be a consequence of perturbations of the ventral diencephalon on which the chiasm forms. In fact, the disposition of ephrin-B2, a major inhibitory cue for VT axons in the optic chiasm midline and thus key to retinal axon divergence in the optic chiasm (Williams et al., 2003), appears to be altered in *Zic2*$^{kd/kd}$ mice. Thus, the possibility that Zic2 also regulates axon guidance factors at the optic chiasm cannot be excluded and will be investigated in the future. However, the finding that *Zic2*$^{+/kd}$ embryos have a normal chiasm, together with our gain-of-function experiments in vitro, argue for a role for Zic2 in designating the ipsilateral optic projection, acting primarily within the retina.

Our functional experiments in vitro demonstrate that postmitotic expression of Zic2 is able to switch axon projection phenotype within 24 hr and more potently when it is ectopically expressed at E13.5 than at E14.5, likely before the expression of transcription factors that might specify the contralateral RGC phenotype. This suggests that Zic2 may operate over a relatively short period, in accordance with transcription factor function.

The LIM code expressed in postmitotic neurons has been proposed to participate in cell fate and axon trajectory of neuronal subtypes throughout the CNS (Shirasaki and Pfaff, 2002). Although Zic2 does not belong to the LIM family of homeodomain transcription factors, it seems to act similarly, inducing postmitotic RGC axons to differentially respond to extrinsic guidance cues. However, the present findings point to a novel transcriptional program for axon pathfinding with respect to the midline of the CNS. It will be interesting to investigate the generality of this program, particularly whether Zic family members are involved in determining axon projections in other bilateral pathways in the CNS. Intriguingly, Zic2 has recently found in a subset of cells in the periotic mesenchyme (Warner et al., 2003), potentially relating to the several uncrossed pathways in the acoustic nuclei-brainstem projection.

The experiments presented here suggest that Zic2 controls downstream events that may direct the avoidance of chiasm midline cells by RGCs with an uncrossed trajectory. Eph receptors and their ephrin ligands mediate axonal divergence at the optic chiasm (Nakagawa et al., 2000; Williams et al., 2003). Notably, the EphB1 receptor is expressed in retina in a spatiotemporal pattern identical to Zic2 and loss-of-function analyses in vivo indicate that EphB1 is necessary for formation of the uncrossed pathway at the optic chiasm (Williams et al., 2003). Thus, EphB1 is a prime candidate for regulation by Zic2, a hypothesis in line with LIM homeodomain protein regulation of ephrinA-EphA interactions in dorsoventral selection of motor axon pathways in the limb (Kania and Jessell, 2003).

Zic2 Expression Reflects the Degree of Binocularity

Zic2 expression matches the extent of binocularity across several species, including mouse, *Xenopus*, and ferret. Thus, although mammals and amphibians use different strategies to establish binocular vision during their lifetime, the fact that Zic2 expression correlates with degree of binocularity suggests a conserved function for Zic2 in modulating the uncrossed RGC axon projection. Moreover, Zic2 is absent in chick retina, again supporting a role for Zic2 in the development of the ipsilateral retinal pathway, since chicks lack a noticeable uncrossed projection.

The findings on the albino retina underscore the hypothesis that Zic2 determines the uncrossed retinal projection. Albino mice display a reduction in the number of uncrossed fibers and a concomitant reduction in Zic2-expressing cells. This reduction might arise because the tempo of retinal neurogenesis is accelerated in the albino (Rachel et al., 2002). A slight alteration in the numbers of cells born on each day of retinal neurogenesis could bias the production of RGCs in favor of one population or the other by affecting the postmitotic expression of Zic2.

In summary, the spatiotemporal expression pattern of Zic2, across species and strains with varying sizes and developmental onset of the binocular projection, along with gain- and loss-of-function studies, implicate Zic2 in the patterning of binocular vision.

Experimental Procedures

Animals

All mouse experiments were performed using C57BL/6 mice except for experiments involving albino embryos for which albino mice (Tyr^{c}/Tyr^{c}) of the C57BL/6 strain were crossed with heterozygous pigmented mice (Tyr^{+}/Tyr^{c}). The *Zic2* knockdown embryos were obtained from mice as described in Nagai et al. (2000). Noon of the day on which a plug was found was considered embryonic day (E)0.5. Ferret embryos were purchased at Marshall Farms USA, Inc. *Xenopus* tadpoles and frogs were obtained from *Xenopus*, Inc.

Immunoblotting, Immunohistochemistry, and In Situ Hybridization

HEK293 cells and peripheral DT and VT segments of retinae were pooled, lysed, loaded on a gel, and immunoblotted using a pan-Zic antibody (Brown et al., 2003). Fixed retinal whole mounts or vibratome sections were processed for immunohistochemistry with α-Zic1 (AbCAM), α-Zic2 (Brown et al., 2003), or α-Islet1/2 (K4, provided by Tom Jessell, Columbia University). For BrdU staining, BrdU (100 mg/kg) was injected intraperitoneally into pregnant females, and 1 hr later embryos were anesthetized and removed. Fixed retinas were treated with 2 N HCl, 1% Triton X-100, and 0.1 M borate to denature DNA and processed for inmunohistochemistry with α-BrdU antibodies. In situ hybridization was performed according to reported methods (Schaeren-Wiemers and Gerfin-Moser, 1993). A 603 bp StuI-EcoRI segment from *Zic2* mouse cDNA and the *Xenopus* cDNA clone IMAGE: 3378215 were used as templates for mouse and *Xenopus* Zic2 riboprobe synthesis, respectively.

Anterograde and Retrograde Dye Tracing and Quantification of the Ipsilateral Projection

Anterograde labeling of normal and mutant mice was accomplished as described (Plump et al., 2002) but with the exception that the projection was labeled by placing dye crystals on the optic nerve head of one eye after removing the retina for immunolabeling with anti-Zic2. After DiI labeling, brains were removed and the optic chiasm viewed en face in whole mount with a fluorescence dissecting microscope. To quantify the uncrossed projection, squares were superimposed on the width of the labeled optic nerve proximal to the chiasm and on the ipsilateral optic tract. The fluorescence within the square covering the optic nerve (ONF) and optic tract ipsilateral to the dye labeling (OTF) was measured using Openlab

Software. ONF and OTF were normalized with respect to the area in each case, and the coefficient of the ipsilateral projection (Cip) was determined for each individual embryo by applying the following formula: Cip = OTF/ONF + OTF. Retrograde labeling with DiI was performed according to standard methods. Retrograde tracing combined with immunohistochemistry was performed as described (Rachel et al., 2002).

Culture Assay

Retinal explants and dissociated chiasm cells from E14.5 and E13.5 embryos were cocultured as described previously (Marcus et al., 1996) and immunostained with α-neurofilament (3A10, provided by Tom Jessell, Columbia University). The extent of axon outgrowth was quantified using Openlab software to measure the area covered by RGC axons, expressed as mean area (±SEM) occupied by RGC axons ($\mu m^2 \times 10^4$) (Aa). The amount of axon growth was normalized in each case with respect to the explant size: (Aa/Ae) $\times 10^4$, where Ae = area covered by each retinal explant.

Sindbis Virus Synthesis and Infection

Sindbis virus encoding EGFP, Zic2-EGFP, or Zic2-Δ-EGFP was generated according to the Sindbis Expression System, Version E (Invitrogen) but using a modified version of the virus with decreased cytotoxicity (gift of Dr. Osten, Max-Planck, Heidelberg). The Zic2 mutant was generated by deletion of an internal SmaI fragment, leading to a nonfunctional protein (Sin-Zic2Δ-IRES-EGFP) that lacks 100 amino acids from the zinc finger domain (Brown et al., unpublished). Sindbis virus was incubated with retinal explants for 1 hr at 37°C in serum-free medium. Excess virus was removed by washing with serum-free medium, explants were plated, and dissociated chiasm cells added.

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