

Disruption of gradient expression of *Zic3* resulted in abnormal intra-retinal axon projection

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Summary

The targeting of retinal ganglion axons toward the optic disc is the first step in axon pathfinding in the visual system. The molecular mechanisms involved in guiding the retinal axons to project towards the optic disc are not well understood. We report that a gene encoding a zinc-finger transcription factor, *Zic3*, is expressed in a periphery-high and center-low gradient in the retina at the stages of active axon extension inside the retina. The gradient expression of *Zic3* recedes towards the periphery over the course of development, correlating with the progression of retinal cell differentiation and axonogenesis. Disruption of gradient expression of *Zic3* by retroviral overexpression resulted in mis-targeting of retinal axons and some axons

misrouted to the sub-retinal space at the photoreceptor side of the retina. Misexpression of *Zic3* did not affect neurogenesis or differentiation inside the retina, or grossly alter retinal lamination. By stripe assay, we show that misexpression of *Zic3* may induce the expression of an inhibitory factor to the retinal axons. *Zic3* appears to play a role in intra-retinal axon targeting, possibly through regulation of the expression of specific downstream genes involved in axon guidance.

Key words: *Zic3*, Gradient expression, Intra-retinal axon projection, Chick

Introduction

During development of the visual system, ganglion cell axons from all radial positions within the cup-shaped retina project to optic disc, a small opening in the center of the retina. From optic disc, the axons exit the eye to form the optic nerve (Fig. 1) (Halfter, 1985; Thanos and Mey, 2001). Ganglion cells located at central positions of the retina differentiate and extend their axons first, followed by cells at more peripheral positions, in a wave-like fashion. In chicken, the first ganglion axons appear in the dorsal central retina shortly after secondary invagination of the optic vesicles at Hamburger-Hamilton stage 15 (E2 to E3) (Goldberg and Coulombre, 1972; Horder and Mashkas, 1982; Kahn, 1973; Rager et al., 1993). By E8, most of the retinal axons have reached the optic disc except those at the very periphery of the retina.

Despite the recent discovery of a number of conserved families of axon guidance cues, the molecular mechanisms underlying the process of axon projection toward the optic disc remain unclear (Oster and Sretavan, 2003). By intraretinal grafting experiment, it has been shown that the optic disc does not provide detectable chemotrophic activity to direct the retinal axon projection (Halfter, 1996). In *netrin1/DCC* or the *EphB* ligand-deficient mouse embryos, RGC axon pathfinding defects have been observed near the optic disc (Birgbauer et al., 2000; Deiner et al., 1997), suggesting that these molecules may not be involved in long-range intra-retinal guidance (Deiner et al., 1997).

A number of molecules have been implicated to be involved

in centrally directed projection of the retinal axons toward the optic disc. Chondroitin sulfate proteoglycans (CSPGs), a major component of the ECM, are suggested to act as inhibitory molecules to prevent the growth of retinal axons toward the periphery of the rat retina (Brittis et al., 1992; Snow et al., 1991). The CSPGs recognized by a monoclonal antibody CS-56 are expressed in a gradient in the rat retina, low in the center and high in the periphery (Brittis et al., 1992). However, the distribution of CSPGs in the retina of chick, quail and cat embryos has been shown to colocalize with the growing optic axons, suggesting a different role in retinal axon guidance (McAdams and McLoon, 1995; Ring et al., 1995).

Axonal growth toward the optic fissure also requires expression of cell adhesion molecules (CAM) of the immunoglobulin superfamily like L1, neural cell adhesion molecule (NCAM) and neurolin (DM-GRASP). Intravitreal injection of the antibodies to NCAM disrupted the orderly formation of the fissure and resulted in massive overshooting of axons which failed to exit at the optic disc (Thanos and Bonhoeffer, 1984). Injection of Fab fragments to L1 resulted in defasciculation of axons (Giordano et al., 1997), whereas injection of Fab fragments to neurolin caused both defasciculation and aberrant intraretinal axonal trajectory (Ott et al., 1998). In addition, a number of receptor tyrosine phosphatases have been shown to play a role in intraretinal axon guidance (Ledig et al., 1999).

We report that a gene encoding a zinc-finger transcription factor, *Zic3*, is involved in patterning the retina for intraretinal

axon guidance. *Zic* genes are the vertebrate homologues of the *Drosophila* pair-rule gene, *odd-paired* (*opa*) and are key regulators of neural and neural crest development (Aruga et al., 1998; Brewster et al., 1998; Nakata et al., 1997). Recently, *Zic2* has been shown to pattern binocular vision that it is both necessary and sufficient to regulate RGC axon repulsion by cues at the optic chiasm (Herrera et al., 2003). Mutations in *Zic* genes are known to cause holoprosencephaly and situs abnormalities in human (Brown et al., 1998; Gebbia et al., 1997).

By in situ hybridization, we have shown that *Zic3* is expressed in a periphery-high, center-low gradient in the developing retina, and the gradient expression of *Zic3* recedes toward the periphery of the retina correlating with the progression of retinal cell differentiation and axonogenesis. Disruption of gradient expression of *Zic3* by retroviral overexpression resulted in mis-targeting of the retinal axons and some axons misrouted to the subretinal space at the photoreceptor side of the retina. Misexpression of *Zic3* did not affect neurogenesis or differentiation inside the retina, or grossly alter retinal lamination. By stripe assay, we have further shown that *Zic3* may play a role in intra-retinal axon guidance by regulation of the expression of an inhibitory factor(s) to drive the axons to project to the optic disc.

Materials and methods

In situ hybridization and immunofluorescent staining

The procedures of in situ hybridization on cryosections, whole-mount and flat-mount samples were essentially as described previously (Bao and Cepko, 1997; Jin et al., 2003). The chicken *Zic3* and *Netrin-1* probes were kindly provided by Dr Cepko (Lin and Cepko, 1998) and Dr Tabin (K. Vogan and C. Tabin, unpublished) at Harvard Medical School.

Immunofluorescent staining on flat-mounts or sections of retina was essentially as described previously (Jin et al., 2003). Axons were stained with monoclonal antibody 270.7, a generous gift from Dr Virginia Lee (University of Pennsylvania Medical School). Viral infection was confirmed by immunofluorescent staining with either a monoclonal antibody 3C2 (diluted 1:5) or a polyclonal antibody p27 (SPAFAS, Norwich CT, diluted 1:10,000). Mouse monoclonal antibodies against Islet-1 (39.4D5), collagen IX (2C2) and visinin were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), and used at 1:50, 1:5 and 1:10, respectively. The monoclonal antibody CS-56 was obtained from Sigma (St Louis, MO) and used at 1:200 dilutions.

Retrovirus and chick embryo injection

A 1.5 kb fragment of cDNA containing the entire coding region of the human *ZIC3* gene (Gebbia et al., 1997) was cloned into the *Clal* site of an avian replication-competent retroviral vector (RCAS) (Hughes et al., 1987; Morgan and Fekete, 1996). RCAS-*Zic3* virus was prepared by transfection of a chicken fibroblast line, DF1, as previously described (Bao et al., 1999). All viral stocks were harvested and concentrated to titers of approximately 5×10^8 cfu/ml.

Standard specific pathogen-free white Leghorn chick embryos from closed flocks were provided fertilized by Charles River Laboratories (North Franklin, Connecticut). Eggs were incubated inside a moisturized 38°C incubator and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992).

TUNEL assay

Cryosections (20 µm) were prepared from the E7 or E10 retinas injected with either the control RCAS-GFP or RCAS-*Zic3* virus. The DeadEnd Colorimetric TUNEL system from Promega was used to detect programmed cell death. The assay was carried out according

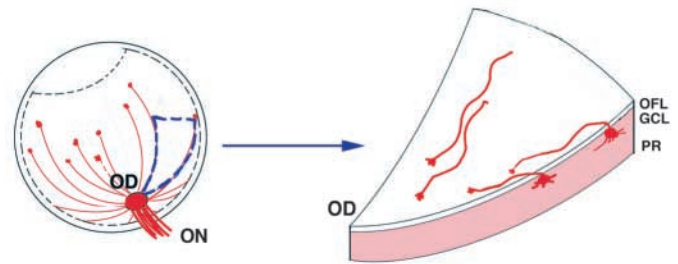


Fig. 1. Intraretinal axon guidance. Ganglion axons at all positions inside the retina travel in the optic fiber layer (OFL) and project to the center of the retina, the optic disc (OD). At the optic disc, the ganglion axons exit the retina and join the optic nerve (ON). GCL, ganglion cell layer (where the cell bodies of the ganglion cell reside). PR, photoreceptor layer (where the photoreceptor cells are localized at the other side of the retina).

to the manufacturer's instruction except that the treatment of proteinase K was changed to 3 µg/ml of concentration for 10 minutes at room temperature to preserve tissue integrity. After TUNEL assay was completed, the tissue sections were further co-stained with anti-neurofilament antibody 270.7 and anti-P27 antibodies, to visualize the axons and the viral antigens, respectively.

Stripe assay

The stripe assay was carried out essentially as described previously (Walter et al., 1987). For some experiments, uninjected E8 chick eyecups were dissected out and cut along at one half radial distance to the center, in order to obtain the central and peripheral halves of the retina. For other experiments, optic vesicles were injected with either the RCAS-*Zic3* or a control RCAS-GFP virus at HH stage 10 and incubated until E8. Only the central halves of the virus-injected retinas were collected and homogenized by pressing through 1 ml syringe needles multiple times in a buffer containing 10 mM Tris-HCl, pH 7.4, 1.5 mM CaCl₂, 1 mM spermidine and protease inhibitor cocktail (Roche). Nuclei and cytoplasm were removed by centrifugation in step sucrose gradient for 10 minutes at 50,000 g. The membrane fragments were collected and washed in PBS and vacuumed onto a polycarbonate filter (Waterman, pore size 0.1 µm) following the manufacturer's instruction (Max-Planck-Institute at Tübingen). One type of membrane fragments were vacuumed on first to form the first stripes and the second type of membrane fragments mixed with green fluorescent beads (505/515) (Molecular Probes) were filled in to form the second stripes. After the stripes were formed, the membranes were coated with 50 µg/ml of laminin (BD Biosciences) for 1–4 hours at room temperature.

E6 chick retinas were dissected and flat-mounted with the ganglion cell side up onto a nitrocellulose filter (Sartoris). Thin stripes of retina were cut and laid onto the polycarbonate filter with the ganglion cell side facing the polycarbonate filter. Retinal explants were cultured in DME/F-12 medium (1:1) containing 10% fetal calf serum, 2% chicken serum and 0.4% methyl cellulose. 40–44 hours later, the explants with the membranes were fixed in 4% paraformaldehyde and stained with an anti-neurofilament antibody (270.7) followed by a Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories).

The fluorescent images of axons and stripes were photographed with a digital camera (SPOT camera). Green images of stripes and red images of anti-neurofilament staining were merged using the SPOT Advanced software. To quantify the results, axons were traced and scanned to obtain digital images. We quantified only those patches that had axons covering eight or more stripes. The number of black pixels of the tracings on the stripes with the fluorescent beads (second stripes) and those on the stripes with no beads (first stripes) was calculated by a public domain NIH Image program. The 'preference'

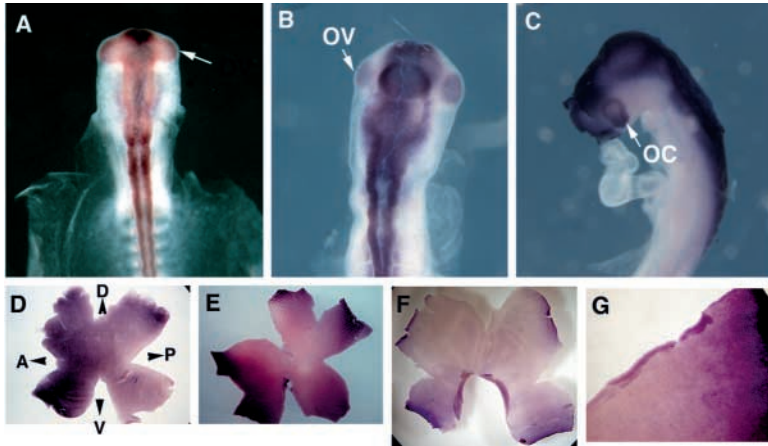


Fig. 2. Expression of *Zic3* mRNA in whole-mount chicken embryos (A–C) and flat-mount retinas (D–G) by in situ hybridization. (A) HH stage 10; (B) HH stage 12; (C) HH stage 17. *Zic3* is expressed in early optic vesicle (arrows in A and B) and optic cup (arrow in C). (D) E5.5 retina; (E) E7 retina; (F) E9 retina; (G) high-magnification view of E7 retina. *Zic3* is expressed in a periphery-to-center gradient with higher expression at the periphery. All the flat-mount retinas were shown with the ganglion cell side upwards, oriented with the anterior side (A) towards the left, the posterior side (P) towards the right, the dorsal side (D) upwards and the ventral side (V) downwards.

of axonal growth was calculated based on the ratio (s/f) of the number of black pixels on the second stripes to that on the first stripes and normalized to the width of the stripes. Control stripe assay experiments were performed to assess the variation of the experimental data by laying down same membrane preps from the central halves of the uninjected E8 retinas in both stripes. Axonal growth preference between two membrane fragments (ratio s/f) was compared with the control stripe assay data (ratio s/f) and statistical significance was analyzed by using the unpaired Student's *t*-test.

Results

Expression of *Zic3* mRNA in the embryonic chicken eye

By in situ hybridization, we have analyzed the expression of

the *Zic3* gene in embryonic chicken eye. At HH stage 10 (E1.5), *Zic3* is already expressed in the lateral regions of the diencephalon which are designated to form optic vesicles (Fig. 2A). *Zic3* expression is retained in the optic vesicle and optic cup at later stages (Fig. 2B,C). On flat-mount retinas (Fig. 2D–G), we observed that *Zic3* is expressed in a graded fashion with lower expression at the center and higher expression at the periphery. The graded expression recedes toward the retinal periphery over the course of development. At E5.5, high level of *Zic3* mRNA is detected at the periphery, although some expression at the center remains (Fig. 2D). At E7, the expression is much decreased in the center, while at E9, the expression of *Zic3* is mostly confined to the ventral periphery of the retina (Fig. 2E,F). In addition, *Zic3* expression is not completely even in the four quadrants of the retina. At E5.5, higher expression was detected in the anterior side of the retina than the posterior side (Fig. 2D). At E7 and E9, *Zic3* expression was detected at higher levels in the ventral than in the dorsal retina (Fig. 2E,F).

The dynamic changes in expression patterns of the *Zic3* gene appear to correlate with the progression of neurogenesis and axonogenesis inside the retina. It is known that the ganglion cells at the center of the retina differentiate and extend their axons first, followed by cells at more peripheral locations. The earliest axonal extension is in the dorsal central region of the retina around E3 in chicken, whereas the axons at the peripheral ventral retina initiate the extension at E7–E8. The process of axonal extension is also more advanced in dorsal than in ventral, and in nasal than in temporal retina (Goldberg and Coulombre, 1972;

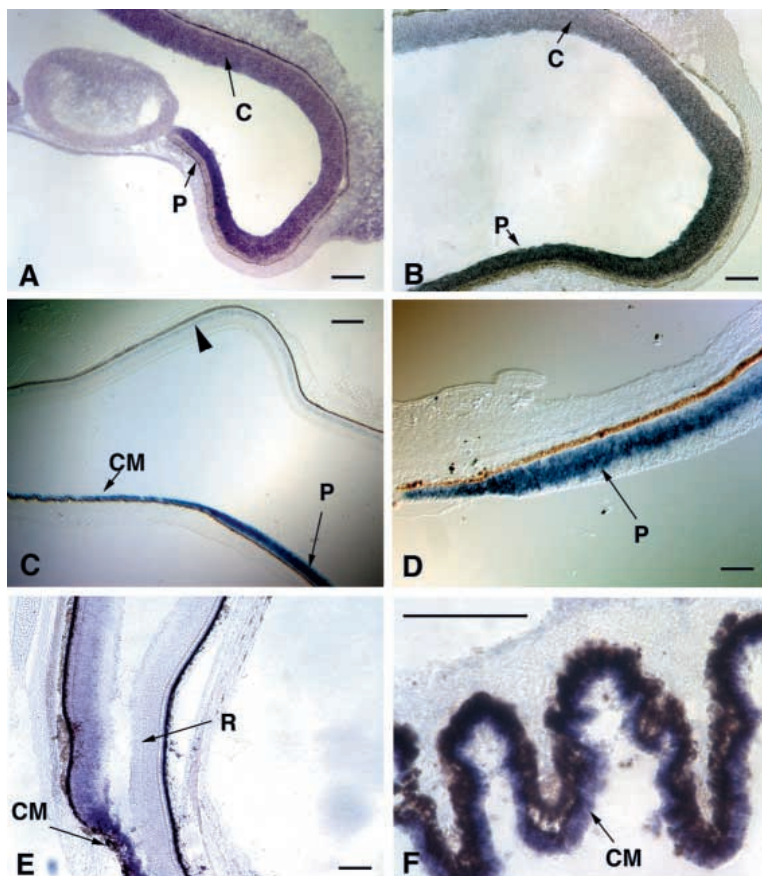


Fig. 3. Expression of *Zic3* mRNA on cross sections of embryonic chick eye. In situ hybridization experiments were performed on the cryosections of embryonic chick eye at various stages: (A) E4.5; (B) E6; (C,D) E9; (E,F) E16. Note that *Zic3* mRNA expression is in a periphery-high, center-low gradient at E4.5 and E6 (A,B). At E9, most of *Zic3* expression is confined to the retinal periphery (labeled 'P' in C,D). *Zic3* is also highly expressed in the ciliary margin area (C,E,F). At E16, *Zic3* expression can be barely detected inside the retina while at a high level at the ciliary margin (E,F). C, center of the retina; P, periphery of the retina; R, retina; CM, ciliary margin. Arrowhead in C indicates the remaining undifferentiated cells located in the inner nuclear layer that are weakly expressing *Zic3* mRNA at the retinal center. Scale bars: 100 μ m in A,B,D–F; 400 μ m in C.

Horder and Mashkas, 1982; Kahn, 1973; Rager et al., 1993).

The graded expression pattern of the *Zic3* gene was confirmed by in situ hybridization on the cross sections of the embryonic chicken eye (Fig. 3). In addition, we found that *Zic3* is highly expressed in the ciliary margin region (Fig. 3C,E,F), similar as reported in mouse (Nagai et al., 1997). Within the retina, *Zic3* appears to be expressed in the undifferentiated progenitor cell population. The graded expression of *Zic3* is consistently periphery-high and center-low in the undifferentiated cell population at all developmental stages analyzed. At E16, *Zic3* expression is no longer detected inside the retina and is only retained in the ciliary margin (Fig. 3E,F).

Misexpression of *Zic3* resulted in abnormal intraretinal axon targeting

To understand the role of *Zic3* gene in eye development, we subcloned the full-length *Zic3* gene into a retroviral vector, RCAS (Fig. 4A). This viral construct can produce replication-competent viral particles that propagate among the proliferating cells and express *Zic3* protein in the infected cells. A similar viral construct with a GFP gene (RCAS-GFP) was used as a control. Injected at HH stage 10-11 (~E1.5) with the viral stocks, the infected eyes were analyzed at various ages including E6, E7, E10, E12, E16 and E18. Because *Zic3* is highly expressed in the ciliary margin region, we examined the structure of the anterior chamber of the eye. We did not detect any obvious defect in eye anatomy, including in the ciliary margin area (data not shown).

Because the retinal axons project from periphery to retinal center, in the same direction as the graded expression of *Zic3*, we analyzed the axonal projection in the retinas infected with the RCAS-*Zic3* virus. The infected retinas were flat-mounted and co-stained with an anti-neurofilament antibody (270.7) and an anti-viral GAG antibody (P27) for visualizing the axons and the infected area, respectively. In the retinas injected with a control virus RCAS-GFP, the axons appeared completely normal as in the uninjected wild-type samples, projecting straight toward the center of the retina where the optic disc was located (Fig. 4B). In the RCAS-*Zic3* injected samples, however, the axons appeared abnormal in some areas. Most of the areas with axonal phenotype appeared somewhat long and thin in shape and were strongly stained by anti-viral GAG antibody (Fig. 4F,G).

First observed at E6, the number of areas with axonal abnormality increased over time but peaked around E10 (Table 1). In two cases, we observed that a small group of axons turned back 180° to project toward the periphery of the retina (Fig. 4D). In another case, the axons appeared to go around in a circle (Fig. 4C). More commonly (>150 areas), however, the axons had the appearance of being blocked from projecting toward the center of the retina and piled up into a line (Fig. 4E,F). By adjusting the focal plane of the microscope, we found that some axons penetrated the entire thickness of the retina and misrouted to the sub-retinal space (Fig. 5A-F). There, the axons initially followed the same aberrant trajectories as in the ganglion cell side (Fig. 5A,B). Farther away from the site where the axons crossed over, the axons appeared much more disorganized (arrowhead, Fig. 5D).

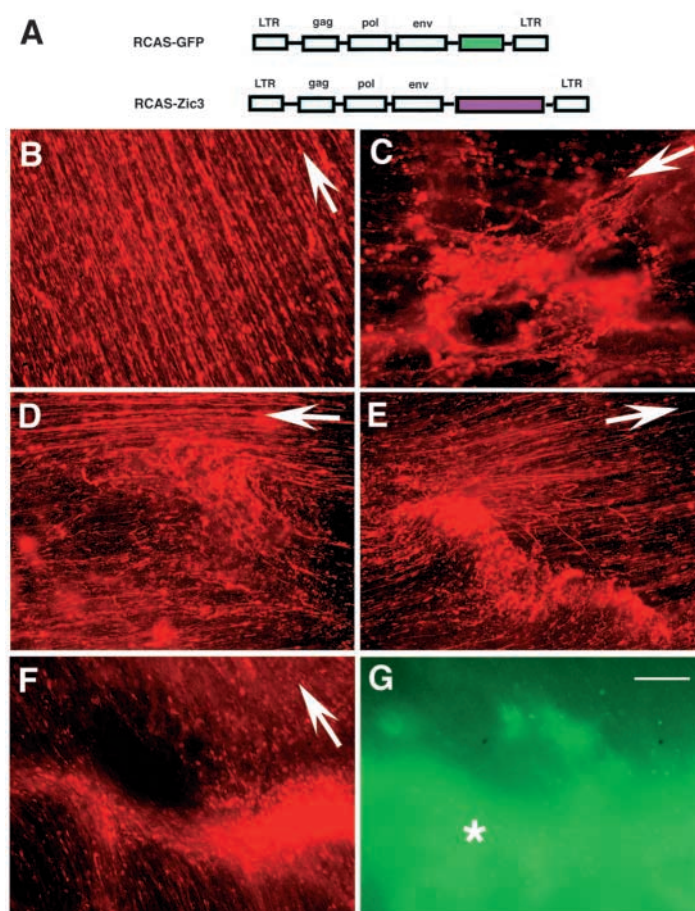


Fig. 4. Retroviral expression of *Zic3* caused abnormality in retinal axon projection toward the optic disc. (A) Retroviral constructs. LTR, long terminal repeat; gag, gene encoding the viral capsid proteins; env, gene encoding viral envelop protein; pol, gene encoding viral reverse transcriptase. Colored boxes indicate the inserted GFP (green) and *Zic3* (pink) cDNAs. (B-G) Infected at HH stage 10-11, the retinas were analyzed at E12 by co-staining with an anti-neurofilament antibody (B-F) and an anti-viral GAG antibody (G). The axons were viewed from the optic fiber side of the flat-mount retinas. White arrows indicate the direction towards the optic disc. (B) In control virus RCAS-GFP-injected retinas, axons appeared normal, projecting straight toward the center of the retina. In the RCAS-*Zic3*-injected retinas, however, some axons showed abnormal trajectories (C-G). Although the infected area identified by anti-viral GAG antibody (P27) was much larger than the area with axonal phenotype, the area with phenotype appeared to be more intensely stained by anti-p27 (* in G). Note the whole area shown in G is infected. Images in F and G are from the same field. Scale bar: 50 μ m.

Table 1. Retinal ganglion axons were affected in more areas by *Zic3* misexpression at later stages

Age	E6	E7	E8	E10	E12
Total number of areas with phenotype	6	13	24	108	38
Number of retinas	5	6	7	19	8
Affected areas per retina	1	2	3	6	5

The misrouted axons did not project to the optic disc, indicating that the molecular cues for directing the axons to the center of the retina are not present at the photoreceptor side.

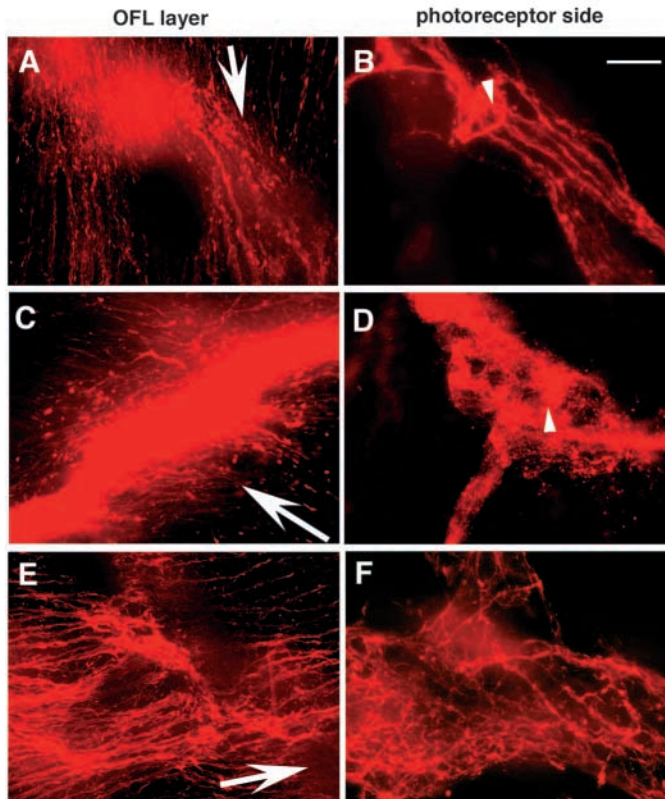


Fig. 5. The most common axonal phenotype of *Zic3* misexpression is that the axons misrouted to the sub-retinal space at the photoreceptor side. The axons were photographed at the focal plane of the optic fiber layer (A,C,E). White arrows indicate the direction toward the optic disc. The same area in A,E was also photographed with the focal plane at the photoreceptor side to show that some axons were present at the sub-retinal space (B and F, respectively). The area shown in D at the photoreceptor side is a little distance away from the area shown in C. Note that the misrouted axons in the subretinal space were very disorganized without clear direction. Scale bar: 50 μ m.

Misexpression of *Zic3* did not interfere with neurogenesis in the retina

To verify that the axonal phenotype was not secondary because *Zic3* altered the normal course of cell differentiation and maturation, we examined retinal cell marker expression in the samples infected with RCAS-*Zic3* virus. Incomplete infection allowed us to compare marker expression in the infected areas with that in the adjacent wild-type areas (Jin et al., 2003; Schulte and Cepko, 2000). Expressed by differentiated retinal neurons, islet 1 is mostly expressed by ganglion cells at early stages such as E7. We did not observe any difference in either the number or distribution of the islet 1-expressing cells in the areas infected with the RCAS-*Zic3* virus compared with the uninfected areas (Fig. 6A,B). The expression of the marker for amacrine cell (Pax6) and photoreceptor cell (visinin) also remained unchanged (data not shown). These results suggest that misexpression of *Zic3* did not grossly alter the course of neurogenesis or cell differentiation in the retina.

Next, we examined whether the axonal phenotypes of *Zic3* misexpression was indirectly caused by induction of ciliary margin formation inside the retina. Morphologically, we did

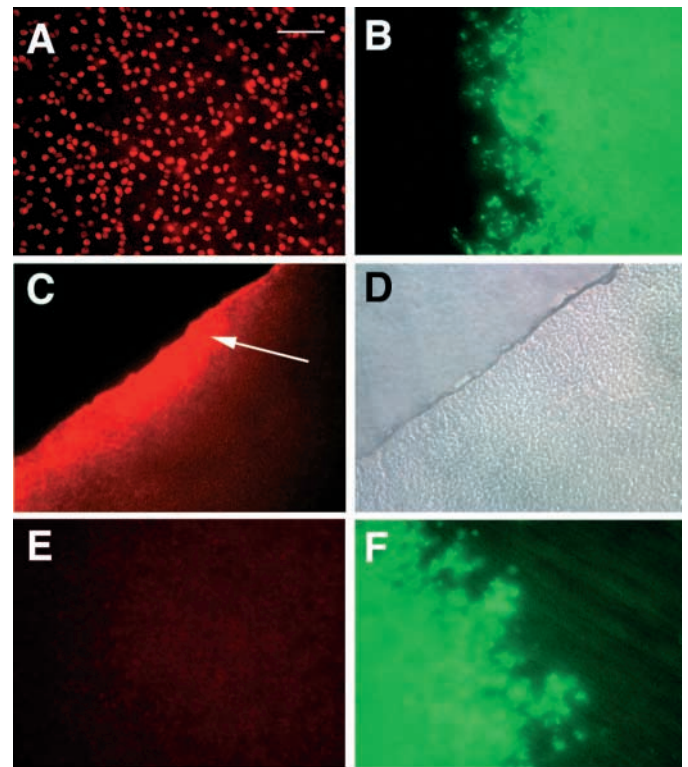


Fig. 6. Misexpression of *Zic3* did not alter the expression of islet 1 or induce the expression of a ciliary margin marker, collagen IX. RCAS-*Zic3* virus-infected retinas were analyzed at E7. Samples were double stained with an anti-viral GAG antibody (B,F) and an anti-islet 1 antibody (A), or an anti-collagen IX antibody 2C2 (C,E). (D) A bright-field image is shown. The same areas are shown in A and B, C and D, and E and F, respectively. Note that there was no apparent difference in either the number or distribution of islet 1-expressing cells in the infected area (A,B) compared with the uninfected wild-type area. Collagen IX is highly expressed in the very periphery of the retina, the prospective ciliary margin region (arrow in C), but not inside the retina, identical to the patterns in the uninjected control retina (not shown). Scale bar: 50 μ m.

not observe any change in the RCAS-*Zic3*-infected retinas that might suggest ectopic formation of ciliary margin. Furthermore, a marker of the ciliary margin, collagen IX (Liu et al., 1993), showed identical patterns in the RCAS-*Zic3*-infected retinas as in the uninjected wild-type retinas (Fig. 6C-F). These results suggest that the axonal phenotype was not due to ectopic formation of ciliary margin inside the neural retina.

Misexpression of *Zic3* did not grossly affect retinal lamination

To examine whether *Zic3* misexpression affects retinal lamination, E10 retinas infected with the RCAS-*Zic3* virus were sectioned and stained with various cell type-specific antibodies. Similar to what is observed in flat-mount retinas, the axons crossed over to the sub-retinal space at the photoreceptor side (Fig. 7A-C). However, the optic fiber layer appeared largely normal, although some protrusions of the axons were observed (arrows, Fig. 7A,B). By contrast, the photoreceptor layer often appeared broken, possibly by misrouted axons (Fig. 7D,G). After examining all sections

from two infected retinas containing 15 areas of abnormal axonal crossings, we did not observe any gross defect in lamination. However, some disturbances of retinal layers were observed, at the immediate vicinity of axon misrouting. Cells in GCL and inner nuclear layer (INL) were seen to disperse into the deeper layers of the retina toward the photoreceptor side (Fig. 7H,I). Within the sites of axon crossing over, the anti-viral GAG antibody staining appeared much brighter than the adjacent infected areas (Fig. 7F). Consistent with the results obtained on flat-mount retina, we also did not observe any significant change in the number of cells expressing islet 1, Pax6 or visinin in retinal sections. These results support the idea that *Zic3* misexpression appears to affect intra-retinal axon targeting specifically without grossly altering cell differentiation and lamination in the retina.

To understand the cause of the increased anti-GAG staining at the crossing-over sites, we next analyzed programmed cell death by TUNEL assay followed by staining with anti-neurofilament and anti-GAG antibodies. As shown in Fig. 8, these sites had a substantial increase of cell death compared with the adjacent tissues that were also infected. The increase of TUNEL-positive cells coincided with the increase of intensity of anti-GAG staining, suggesting that the apoptotic cells may release a higher concentration of viral proteins in the area. However, the increased cell death coincided only with the sites of axon crossing over, not viral infection. Although the samples used for the TUNEL assays were infected in over 90% of the total area, increased cell death was restricted to the vicinity of axon crossing over. In addition, the apoptotic cells

tended to accumulate at the photoreceptor side, not the ganglion side (Fig. 8A,D-F).

Results of stripe assay suggest that *Zic3* may activate the expression of an inhibitory factor to the retinal axons

Because *Zic3* encodes a zinc-finger transcription factor, its role in axon guidance is most probably indirect, through regulation of the expression of secreted or membrane-bound molecules. To test whether the axonal phenotype by *Zic3* misexpression is caused by activation of the expression of a negative factor, we performed stripe assays. Membrane fragments were prepared from the E8 central retinas (one half radial distance to the center) injected with either the RCAS-*Zic3* or the control RCAS-GFP virus. We chose to use only the central retina because it has little endogenous *Zic3* gene expression and may have a relatively low level of other negative guidance factors.

As shown in Fig. 9B, axons appeared to 'prefer' the stripes prepared from the control RCAS-GFP-injected retinas to the stripes prepared from the RCAS-*Zic3*-injected samples. To quantify the results, the distribution of the axons on the stripes was digitalized and calculated by a NIH Image software and the preference of the axons were accessed by the ratio of axonal growth on the second stripes (RCAS-GFP-injected) to that on the first stripes (RCAS-*Zic3*-injected) (s/f) normalized to the width of the stripes (see Materials and methods). A total of 15 sample areas (from five independent experiments) were quantified and a mean ratio (s/f) was 5.74.

Fig. 7. Misexpression of *Zic3* did not grossly alter retinal lamination. Cryosections of E10 retinas infected with RCAS-*Zic3* were stained with anti-neurofilament (A-C) and various cell type-specific antibodies including: anti-visinin (G), anti-islet 1 (H) and anti-Pax6 (I). (C-F) Same RCAS-*Zic3*-infected retinal section was shown stained with anti-neurofilament (C), DAPI (D), anti-viral GAG antibody (F) and in phase contrast (E). Note some axons in the RCAS-*Zic3*-infected retinas misrouted to the subretinal spaces at the photoreceptor side of the retina (arrowheads, A-C). However, the laminated structure of the retina was largely normal (D-I), except some dispersion of cells towards the photoreceptor side (arrowheads, H and I). The optic fiber layer was largely normal, except for some protrusions of the axons (arrows in A,B). Frequently, however, the photoreceptor layer was broken (arrowheads, D and G). Note that the anti-viral GAG antibody staining was much more intense at the sites of axon crossing-over than the nearby infected region (arrowhead in F). PR, photoreceptor layer; OFL, optic fiber layer; GCL, ganglion cell layer; INL, inner nuclear layer.

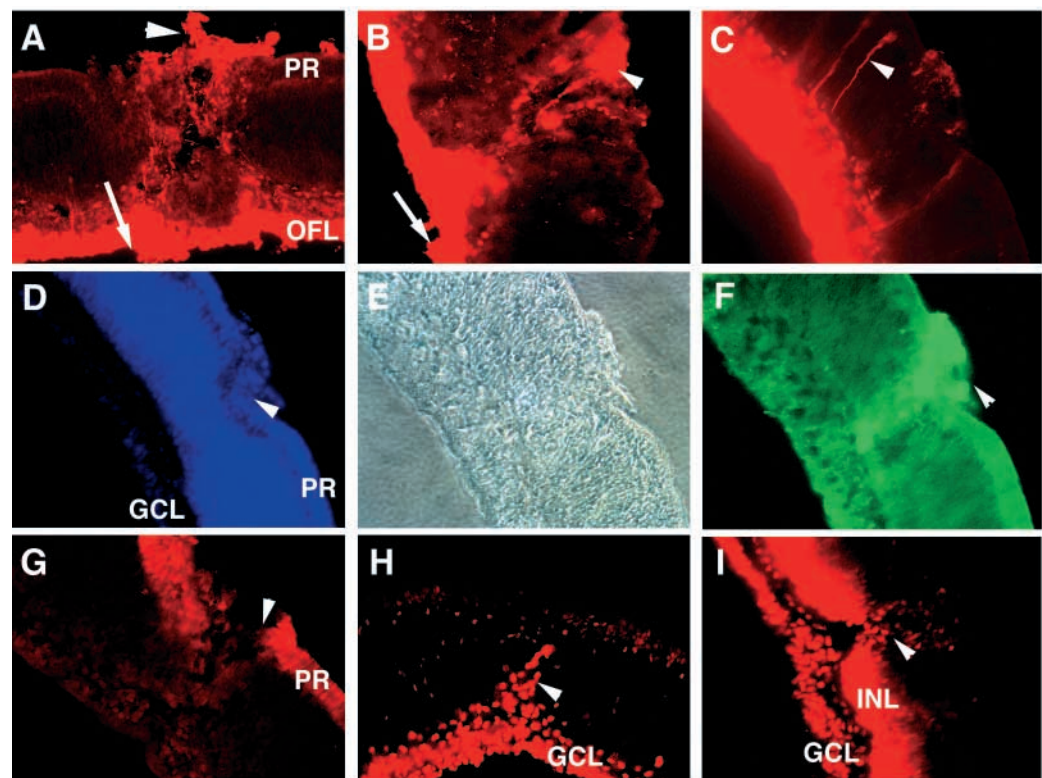
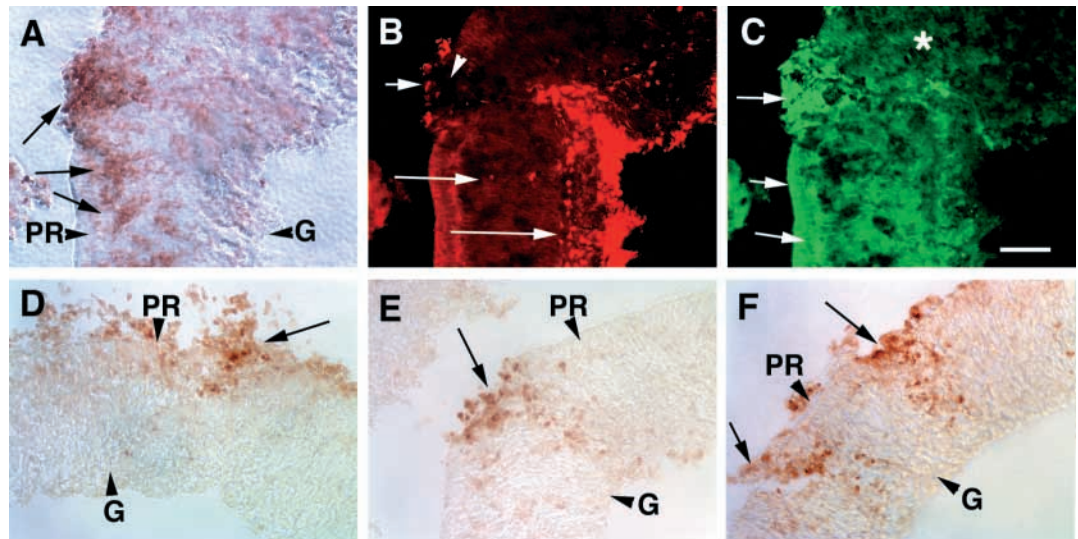


Fig. 8. Apoptosis was increased at the sites of axon crossing-over. E10 retinas infected with RCAS-Zic3 were analyzed by TUNEL assay (A,D-F), followed by staining with anti-neurofilament (B) and anti-viral GAG (P27) antibodies (C). At the sites with axon crossing-over (arrows in B), there was an increase in the number of apoptotic cells (arrows in A) and intensity of anti-viral GAG staining (arrows in C). Note that the adjacent area was also completely infected (marked with *), and some of the fluorescent signals

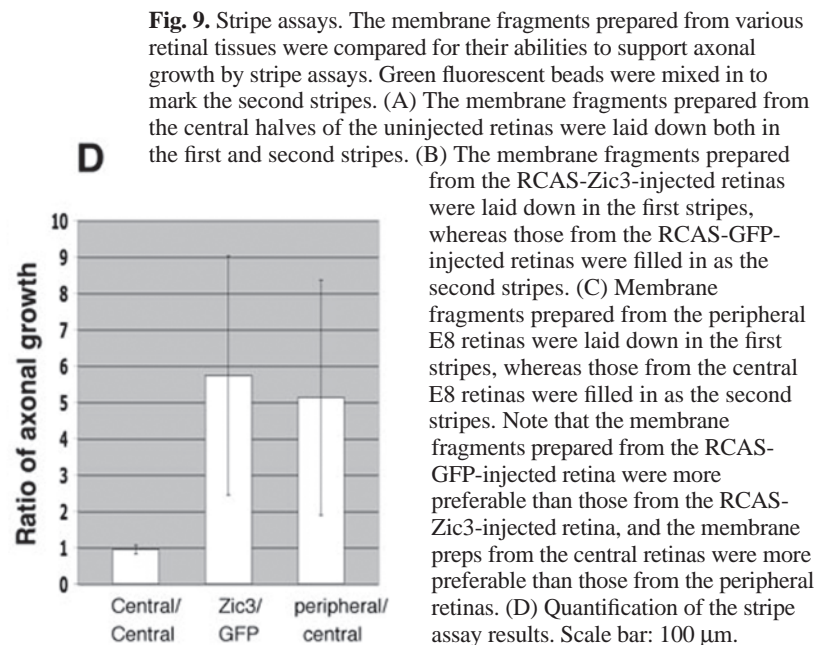
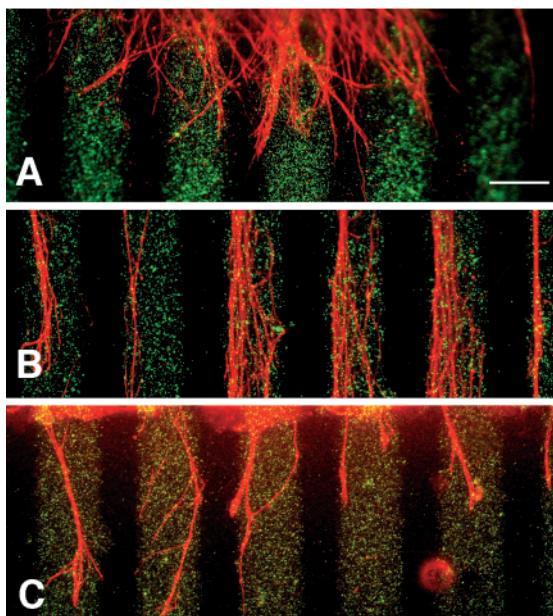
were blocked by the DAB precipitates from the TUNEL assay. In addition, the apoptotic cells tended to concentrate at the photoreceptor side, not the ganglion side (A,D-F). PR, photoreceptor layer; G, ganglion cell layer. Scale bar: 50 μ m.



To assess the variation in the assay, we laid down the membrane fragments from the E8 uninjected central retinas both as the first and as the second stripes and similarly analyzed the results. As shown in Fig. 9A, the axons appeared to grow randomly on the stripes and the mean ratio of the axonal growth (s/f) was 0.953 ($n=15$, three independent experiments). Student's unpaired *t*-test was performed to compare the ratio (s/f) in the Zic3/GFP experiments with the ratio (s/f) in the control central/central experiments with $P < 0.001$, indicating that the RCAS-Zic3-injected membrane preps was significantly less preferable by the retinal axons than the RCAS-GFP-injected membrane preps.

To determine whether an endogenous inhibitory factor is present at higher concentration in the periphery than in the retinal center, we performed stripe assays by laying down the

membrane fragments prepared from the peripheral E8 retina as the first stripes and those from the central E8 retina as the second stripes. As shown in Fig. 9C, the axons appeared to grow preferentially on the stripes prepared from the central retina. The results were similarly quantified (mean ratio s/f=5.13, $n=15$, from five independent experiments). Statistical analysis by the Student's unpaired *t*-test indicates that the stripes prepared from the central membrane fragments were significantly more preferable to the retinal axons than those from the peripheral membrane fragments ($P < 0.001$). These results suggest that an endogenous negative factor(s) is present at a higher concentration in the peripheral than in the central retina, and Zic3 misexpression may activate the expression of an inhibitory factor to the retinal axons.



Misexpression of *Zic3* may activate the expression of a currently unknown guidance factor

We next tested a few candidate molecules that might be downstream of *Zic3*. In chicken, *Netrin1* is also expressed in the optic fissure and optic disc. No change in *Netrin1* expression was observed in the retinas infected with the RCAS-*Zic3* virus compared with the control-uninjected retinas (Fig. 10A,B). Similar to previous reports (McAdams and McLoon, 1995; Ring et al., 1995), we also observed that the staining of an anti-chondroitin sulfate proteoglycans (CSPGs) antibody (CS-56) persists in the center of the retina in chicken, albeit at a much lower level than in the periphery. This suggests that CSPGs may colocalize with the growing optic axons and play a different role in retinal axon guidance. Furthermore, we did not detect any obvious changes in CS-56 staining, in the areas infected with the RCAS-*Zic3* virus compared with that in the uninfected wild-type areas, suggesting that the expression of CSPGs is not subjected to the regulation by *Zic3* (Fig. 10C-F).

Further experiments have shown that the expression of neither *Slit1* nor *Slit2* was altered in the samples with

misexpression of *Zic3* (data not shown). *Ephb5* (*cek11*) mRNA is reported to be expressed in the center-high and periphery-low patterns inside the retina (Sefton et al., 1997). However, we found that the expression of *Ephb5* mRNA was not subjected to the regulation by *Zic3* by in situ hybridization (data not shown). These results confirm the notion that *Zic3* misexpression does not alter retinal cell differentiation grossly and further suggest that *Zic3* may regulate a currently uncharacterized pathway to influence intra-retinal axon pathfinding.

Discussion

In this paper, we have shown that disruption of *Zic3* gradient expression by retroviral misexpression resulted in aberrant intraretinal axon projection. *Zic3* is expressed in a periphery-to-center gradient inside the retina with higher expression at the periphery, correlating with the directed retinal axon projection from periphery to retinal center. The gradient expression of *Zic3* appears to be important for intraretinal axon targeting. Misexpression of *Zic3* caused specific axonal phenotypes, possibly through regulation of the expression of specific down stream genes involved in axon guidance.

Because cell differentiation and axonogenesis initiates from the cells at the center of the retina followed by cells at the more peripheral positions in a wave-like fashion, many genes involved in cell growth and differentiation exhibit dynamic expression patterns from center to periphery. However, the expression of these genes usually begins at the central retina, where the retinal cells are actively differentiating at the early stages such as E4.5. The expression of these genes gradually moves toward retinal periphery correlating with the wave of retinal differentiation. By contrast, *Zic3* gene is consistently expressed at higher levels at the periphery, even at E4.5. *Zic3* did not appear to affect cell differentiation grossly because misexpression did not alter the expression of islet 1, Pax6 and visinin. Furthermore, *Zic3* misexpression did not alter the expression of many axon guidance molecules expressed in the retinal cells, including *Slit1*, *Slit2*, *Robo1*, *Ephb5* and CSPGs, suggesting that *Zic3* misexpression did not change the properties of the retinal cells substantially. The axonal phenotype caused by *Zic3* may, therefore, be due to more specific changes in expression of molecules involved in axon guidance.

Members of the Eph family of receptor protein-tyrosine kinases, together with their ephrin ligands have been shown as graded molecular tags in establishment of retinotectal map (Flanagan and Vanderhaeghen, 1998; O'Leary and Wilkinson, 1999). A number of studies have also shown that the graded expression of Eph family molecules and ephrins is set up by graded expression of various transcription factors. Expressed in opposing gradient to *Epha3*, two homeobox genes, *Sohol* and *Gh6* are shown to specifically repress *Epha3* expression in the retina (Schulte and Cepko, 2000). At the D/V axis, a homeobox gene *Vax/Vax2* expressed in a gradient in the ventral retina has been shown to specify the D/V axis of the retina to influence retinotectal mapping (Mui et al., 2002; Schulte and Cepko, 2000). In mesencephalon, a gradient of engrailed gene expression defines the rostral/caudal axis of the tectum and set up the gradient expression of the axon guidance cues, *Elfl* and *Rags* (Friedman and O'Leary, 1996; Itasaki and Nakamura,

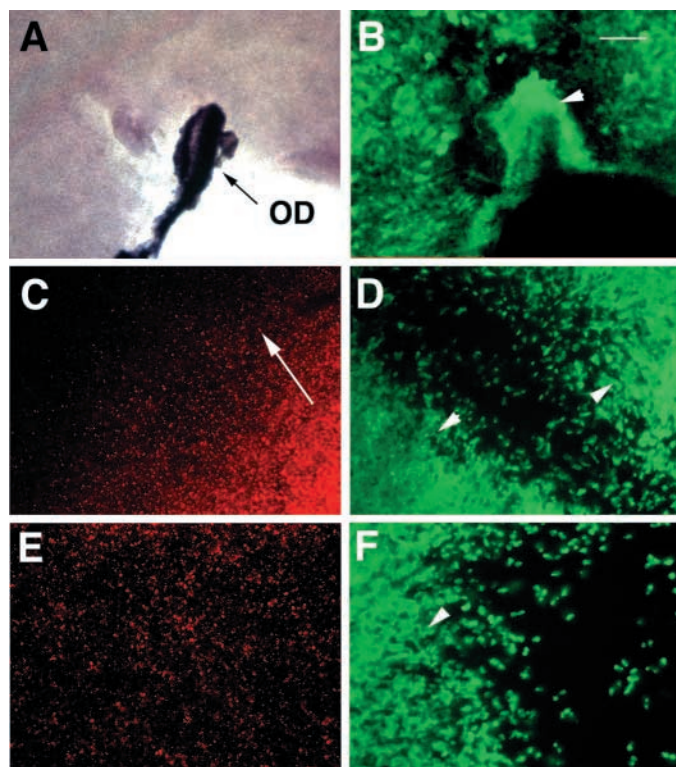


Fig. 10. *Zic3* misexpression did not alter the expression of netrin 1 and CSPGs. The RCAS-*Zic3*-infected samples were hybridized with the netrin 1 probe (A) and then immunostained with an anti-GAG antibody (p27) (B). Netrin 1 is highly expressed in the optic disc (OD, in A) and optic fissure, similar to wild-type uninjected retinas. The RCAS-*Zic3* virus-infected retinas were also double stained with an anti-chondroitin sulfate antibody (CS-56) (C,E) and an anti-viral GAG antibody (P27) (D,F). CS-56 staining is intense at the periphery of the retina. However, a lower level of CS-56 staining is also visible in the center of the retina (E). Comparing the RCAS-*Zic3* virus-infected area (arrowheads in D,F) with the adjacent uninfected areas, there is no difference in the level of CS-56 expression (C-F). Identical fields are shown in A and B, C and D, E and F, respectively. Scale bar: 200 μ m.

1996; Logan et al., 1996; Retaux et al., 1996). However, the role of graded expression of axon guidance cues established by transcriptional regulation in direction of relatively short-range axonal projection, has not been fully characterized.

Although the identity of the downstream target gene of Zic3 is currently unknown, the results of stripe assay suggest that misexpression of Zic3 upregulates the expression of a negative axon guidance cue. This is consistent with the axonal phenotype we observed by in vivo retroviral misexpression, and with the fact that *Zic3* is normally expressed in a periphery-high, center-low gradient. A receding periphery-to-center gradient of repellent under the control of Zic3 may be involved in directing the retinal axon projection toward the optic disc. Previous studies have shown that the optic disc is not necessary for centrally directed optic axon growth (Halfter, 1996; Harris, 1989). Removal of the host optic disc or grafting of an additional optic disc in a host retina in intraretinal grafting experiment had no obvious effect on the global navigation of retinal axons. Netrin 1, a known axon guidance molecule that is expressed in optic disc, has also been shown not directly involved in long-range guidance of the retinal axons to the optic disc (Deiner et al., 1997). These findings suggest that local environment may play a prominent role in intraretinal guidance instead of long-range attraction from the optic disc. Graded expression of attractants or repellents inside the retina is thus likely to be part of the mechanism that underlies the centrally directed retinal axon projection. Although our result is consistent with the previous findings that staining of CS-56 antibody persists in the center of the retina in chicken (McAdams and McLoon, 1995; Ring et al., 1995), we cannot rule out that a particular constituent of the CSPGs may still be involved in intraretinal axon targeting or subjected to the regulation by Zic3. The identities of the core proteins of the CSPGs have remained undefined.

At the sites of axon crossing-over to the photoreceptor side in the RCAS-Zic3-infected retinas, we have shown that there were disturbances in cell distribution and increased cell death. It is possible that the misrouting of axons is due to physical anomalies generated by increased cell death. However, it is also possible that the abnormal trajectories of the axons disrupted normal organization of the retina, which has resulted in increased cell death. Although we currently do not have definitive evidence to distinguish these models, several lines of evidence appear to support the latter. First, the results of stripe assay demonstrate that a negative guidance cue is induced by misexpression of Zic3. The induction of an inhibitory molecule in vivo can inhibit the normal centrally directed axon projection and cause misrouting of the axons. Results of stripe assays comparing the membrane fragments prepared from the central retina with the peripheral retina further suggest that an endogenous negative guidance factor is present in higher concentration in the periphery than in the center. Second, the increase in cell death did not coincide with viral infection, hence Zic3 misexpression. Despite the fact that the retinas were infected in over 90% of the total area, the areas with increased cell death were restricted to the immediate vicinity of the sites where axons crossed over. If increased cell death were resulted directly from Zic3 misexpression, a much wider spread of cell death would be expected. Third, similar phenotypes of axon misrouting to sub-retinal spaces have been reported for netrin 1 and sonic hedgehog mutant mice (Dakubo et al., 2003;

Deiner et al., 1997). Because both netrin 1 and sonic hedgehog proteins are known to act as axon guidance cues (Charron et al., 2003; Deiner et al., 1997), the misrouting of axons to sub-retinal spaces are likely resulted directly from mis-targeting of the axons. Further study is needed to determine whether there is a common molecular basis for these similar phenotypes.

It is also intriguing that the phenotype with the axons turning around constituted only a minor fraction of the total number of the phenotypes. One possibility is that increased anti-GAG staining intensity was used in most of the experiments to identify the sites with axonal phenotypes. A small fraction of the sites with axons turning but no crossing-over might remain undetected. Second, it is also possible that additional factors are present to keep the axons in the peripheral-central pathways. It has been shown previously by intraretinal grafting experiments that the axons are unlikely to grow in the directions other than the peripheral-central pathways (Halfter, 1996).

We have previously shown that Slit1 is involved in definition of retinal axon trajectories by providing intermediate targets to the retinal axons. In the low *Slit1* expression area resulted from overexpression of *Irx4*, the axons appeared to turn to avoid the low Slit1 area and were excessively fasciculated (Jin et al., 2003). However, the axons still managed to project to the optic disc, suggesting that Slit1 does not provide overall direction for the retinal axons to project to the optic disc. In this paper, we have further shown that *Slit1* expression is not under the control of Zic3 regulation, suggesting that Slit1 and Zic3 are in two separate pathways.

Interestingly, it has recently been reported that Zic2 patterns binocular vision by specifying the uncrossed retinal projection (Herrera et al., 2003). It is common that transcription factors within one gene family play distinct roles in biological processes by regulating different downstream target genes. In human, mutations in *Zic2* gene have been shown to cause holoprosencephaly, whereas mutations in *Zic3* have been associated with X-linked situs abnormalities (Brown et al., 1998; Gebbia et al., 1997). Moreover, *Zic2* and *Zic3* are expressed in overlapping but distinct patterns during embryonic development (Nagai et al., 1997). In contrast to *Zic2*, which is expressed specifically in postmitotic ganglion cells in ventrotemporal mouse retina but not in chick (Herrera et al., 2003), *Zic3* is found only in undifferentiated progenitor cells in chick retina. Future experiments aimed at identification of downstream targets of the Zic family transcription factors may thus provide further insights in the molecular mechanisms of the retinal axon guidance.

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