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EMBRYONIC EXPRESSION AND EXTRACELLULAR SECRETION OF *XENOPUS* SLIT

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Abstract—The *slit* genes have recently been found to encode proteins with a conserved chemorepulsive activity for axons in invertebrates and vertebrates. We have determined the expression pattern of a *slit* gene in *Xenopus* embryos. In the neural tube, *slit* is expressed at the ventral and dorsal midlines, and the motor neurons. *slit* is also expressed in a changing pattern in the retina. The full-length *Xenopus* Slit protein is secreted extracellularly, whereas its receptor Roundabout can not be secreted. Using a myc-tagged secreted Slit protein, we confirmed the binding of Slit to Roundabout expressed on the cell surface.

These results confirm Slit–Roundabout interactions and the biochemical properties of Slit and Roundabout proteins, and further support the idea that Slit may guide axon projections in multiple regions of the embryo. © 2000 IBRO. Published by Elsevier Science Ltd.

Key words: Slit, Roundabout, olfactory bulb, axon guidance, chemorepulsion.

Correct projection of axons to their targets is essential for the formation and function of the nervous system. Identification of axon guidance molecules and determination of their expression patterns and functional properties are, therefore, important for our understanding of neural development. Work in the past few years has revealed the function of two families of secreted long-range chemoattractants and chemorepellents, Netrins and Semaphorins (Sema).[12,28,38,42,57] Recently, Slit proteins have been found to be a new family of long-range chemorepellents.[6,25,33,40]

Netrins were identified as attractants existing in the floor plate for axons from commissural interneurons located in the dorsal part of the vertebrate spinal cord.[22,49,56] It is functionally conserved in *C. elegans* and *Drosophila*.[8,16,18,30] Netrins can also be repulsive to specific axons.[9,18] There are two types of receptors for netrins: UNC40/DCC/neogenin,[7,18,21] and UNC5.[1,18,31,32] Sema was identified in chick embryos as an axon collapsing molecule[34,35] and in *Drosophila* as an axon guidance molecule.[27] It was soon shown to be an axon repellent.[36] The Sema proteins are now known to be a large family with repulsive and attractive activities.[2–5,14,41,43,50,51,58,61] Transmembrane receptors for Sema include neuropilins and plexins.[10,11,17,20,26,29,54,55,60]

The *slit* gene was initially found in *Drosophila* to affect larval cuticular patterning.[39] *slit* cDNAs were cloned by Rothberg *et al*. and shown to encode a large protein with leucine-rich repeats (LRRs) and epidermal growth factor (EGF) repeats.[44] *slit* was thought to play roles in the differentiation of midline glial cells and the separation of longitudinal axonal tracts in the ventral nerve cord of *Drosophila* embryos.[44–46] Vertebrate *slit* genes have been identified from rats and humans.[19,37] Recent studies of *Drosophila* and vertebrate Slit proteins have shown that they are chemorepellents.[6,25,33,40,59] A receptor for Slit is the transmembrane protein Roundabout (Robo),[6,25,33] encoded by the *robo* genes in invertebrates and vertebrates.[23,24,48,65] Most recently, Slit has been directly shown to be guide the direction of migrating neurons.[63,66]

We have isolated cDNAs for *slit* genes from *Xenopus*, the chicken and the mouse.[33,64] We show here the pattern of *slit* expression in *Xenopus* embryos. We also present evidence for extracellular secretion of *Xenopus* Slit protein. We have carried out experiments to confirm the binding of a Slit protein to Robo, and the chemorepulsive activity of Slit. Results from these studies are complementary to the recent findings of a ligand–receptor relationship between Slit and Robo and a repellent role for Slit.[6,25,33,40,59]

EXPERIMENTAL PROCEDURES

In situ *hybridization*

Whole-mount *in situ* hybridization was performed as described previously with minor modifications.[33,62] The color reaction was carried out in 34 μg/ml NBT and 340 μg/ml BCIP. To detect *slit* mRNA in early embryos such as chick stages 4, 5, 6 and *Xenopus* stages 12, 13, 14, 15, the color reaction was carried out at 4°C, which increased the signal-to-noise ratio. Sections were obtained after whole-mount *in situ* hybridization; stained embryos were embedded in 7.5% low-melting-point agarose and 50-μm sections were cut with a Vibratome.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells or 293T cells were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM; Gibco). Cells were grown to 70% confluence on 10-cm tissue culture dishes and transfected with approximately 25 μg plasmid DNA per plate using calcium phosphate for 16–24 h. Plasmids expressing a myc-tagged *Xenopus* Slit and HA-tagged rat Robo-1 were used in transient transfections described in Fig. 4.

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Abbreviations: AP, alkaline phosphatase; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; LRR, leucine-rich repeats; Robo, Roundabout; Sema, Semaphorins.

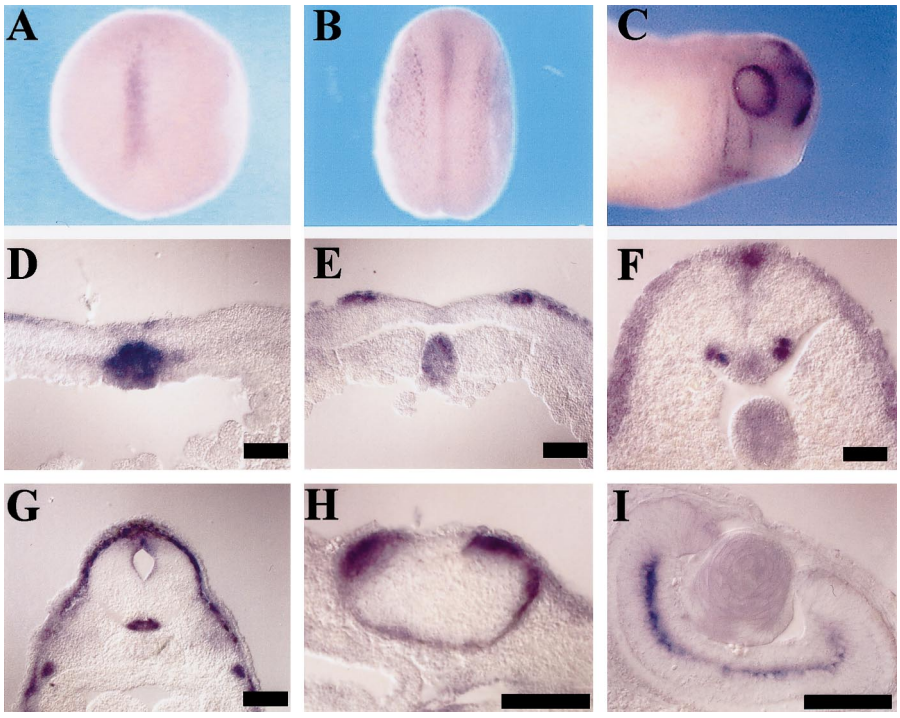

Fig. 1. Expression of *slit* in *Xenopus* embryos. Results of *in situ* hybridization are shown here. Scale bars = 100 μm. (A) A dorsal view of a stage 12 embryo showing *slit* expression at the midline. (B) A dorsal view of a stage 17 embryo showing expression at the midline and the neural fold. (C) A lateral view of a stage 26 embryo showing expression in the retina and branchial clefts. (D) A transverse section of a stage 12 embryo showing expression in the notochord. (E) A transverse section of a stage 17 embryo showing expression in the notochord, and the neural fold. The expression in the floor plate is weak in this section, but is clearly detectable in sections of multiple embryos. (F) A transverse section of a stage 20 embryo showing expression in the notochord, floor plate, the motor neurons, and the roof plate. (G) A transverse section of a stage 35 embryo at the hindbrain level, showing expression in the floor plate and the roof plate. (H) A transverse section of a stage 28 embryo showing expression in the ciliary margin and the retina pigment epithelium layers. (I) A transverse section of a stage 45 embryo showing expression in the amacrine cells in the retina.

A stable cell line expressing xSlit-myc and a stable cell line transfected with a control vector were also used in the experiments.

Cell surface binding and immunocytochemistry

HEK293 cells grown in 10-cm dishes were transfected with Robo-HA or vector plasmids. Approximately 30 h after transfection, cells were suspended by pipetting up and down several times and then seeded on to 6-well or 24-well dishes to 50% confluence. Cells were grown for another 12–18 h before incubation with Slit-myc. After 1 h of incubation with the conditioned media followed by three to four washes in HBHA buffer (Hanks' balanced salt solution [HBSS], 0.5 mg/ml bovine serum albumin, 20 mM HEPES, pH 7.0), cells

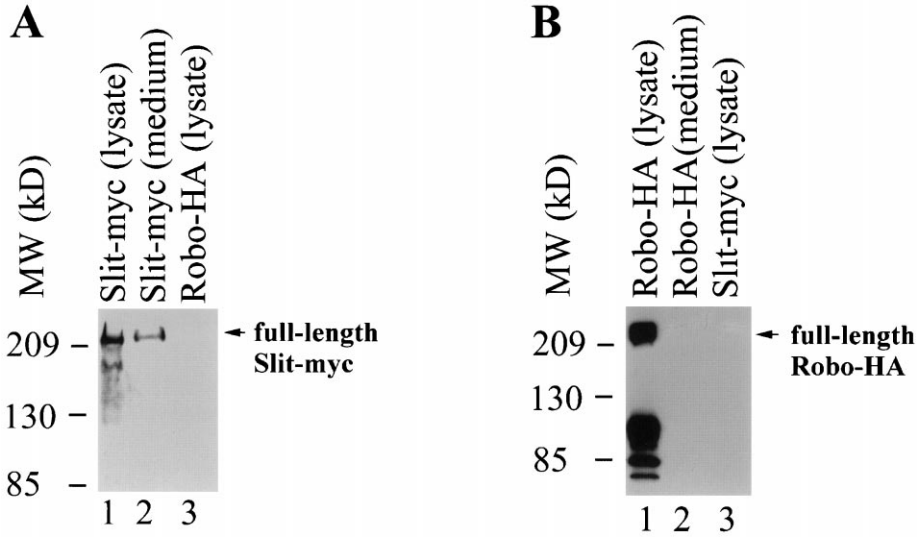

Fig. 2. Extracellular secretion of Slit, but not Robo. Shown here are results from western blots. (A) The monoclonal anti-myc antibody detected Slit-myc in both the conditioned medium (lane 1) and lysates (lane 2) of cells transfected with *slit-myc*, but not the lyate (lane 3) of cells transfected with *robo-HA*. The upper band is equivalent to the band produced by *in vitro* translation of an mRNA encoding the full-length Slit-myc. Multiple bands of Slit in addition to the full-length Slit-myc were observed. (B) The monoclonal anti-HA antibody detected Robo-HA in the lysates (lane 1), but not in the medium (lane 2), of cells transfected with *robo-HA*. It did not recognize Slit-myc (lane 3). The uppermost band is equivalent to the band produced by *in vitro* translation of an mRNA encoding the full-length Robo-HA. Multiple bands of Robo-HA in addition to the full-length were observed.

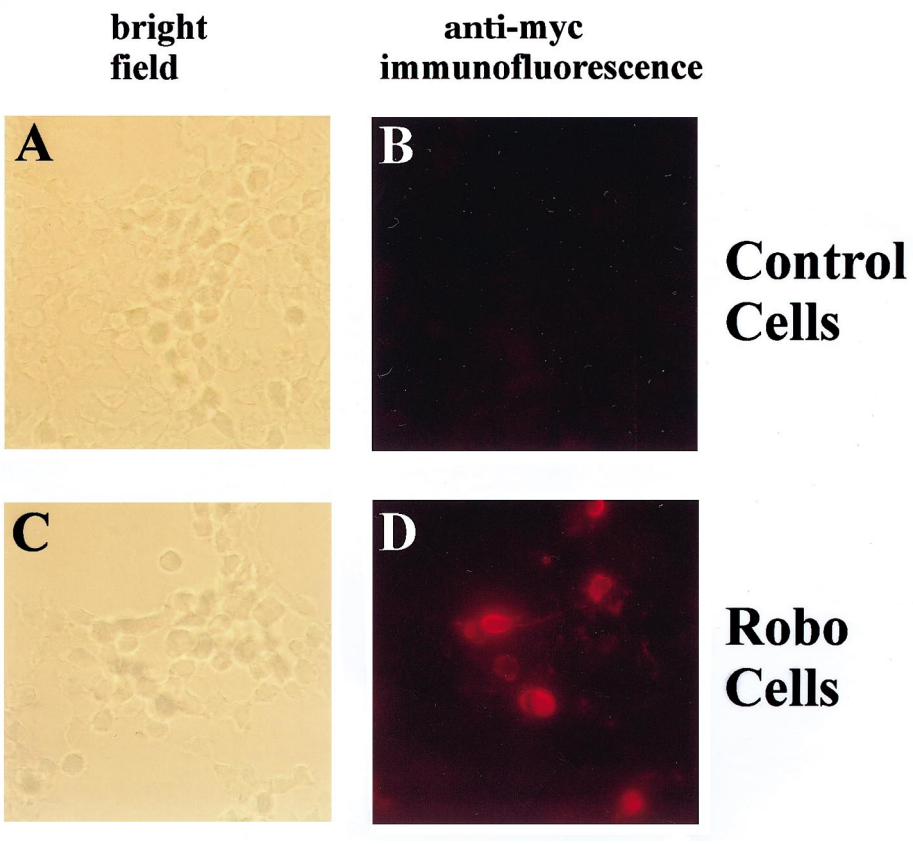

Fig. 3. Binding of the soluble Slit-myc protein to *robo*-transfected cells. Slit-myc was added to cells transfected either with the vector (A, B) or *robo-HA* (C, D). The anti-myc antibody and Cy3-conjugated anti-mouse secondary antibody were used to detect Slit-myc immunofluorescent staining (B, D). (A) A bright-field view of cells transfected with the vector plasmid. No immunofluorescence was detected after anti-myc staining. (B) The same cells as those shown in A, but viewed under a fluorescence filter. No fluorescent signal was visible to indicate anti-myc staining. (C) A bright-field view of cells transfected with *robo-HA*. (D) The same cells are shown in C, but viewed under a fluorescence filter. Some of the cells were found to stain for the anti-myc antibody, indicating the binding of Slit-myc to these cells.

were fixed for 30 s in acetone–formaldehyde fixative (60% acetone, 3% formaldehyde, 20 mM HEPES pH 7.0). Cells were then washed three times in HBSS (150 mM NaCl, 20 mM HEPES, pH 7.0). Following three washes, mouse anti-myc and anti-mouse conjugated to Cy3 were used to visualize Slit-myc binding. Green fluorescent protein expression indicated similar transfection efficiencies in vector and Robo-HA transfected cells.

Olfactory bulb axon guidance assay

Collagen gel matrices were prepared according to Guthrie and Lumsden.[15] Cell aggregates were prepared according to Fan and Tessier-Lavigne.[13] Co-culture of olfactory bulb–telencephalon were carried out according to Sugisaki *et al*.[53] The telencephalic hemisphere and the olfactory bulb were dissected out from E12.5 mice and placed on a collagen gel. Aggregates of cells stably transfected with either vector alone or with *slit-myc* cDNA were put on top of the telencephalon region, but not on the olfactory bulb. Whole-mount preparations were cultured with DMEM containing 10% FBS at 37°C with 5% CO_2. Forty hours later, small crystals of lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI; Molecular Probes) were inserted into the olfactory bulbs. Eight hours later, the specimens were fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline and kept at 4°C before microscopic examination. All experiments were carried in accordance with local and national ethical guidelines on the use and welfare of animals.

RESULTS

Determination of slit *expression patterns in* Xenopus *and chick embryos by* in situ *hybridization*

There are three mammalian homologs of the *Drosophila slit* gene.[6,19,33,37,40,59,64] We have recently isolated cDNAs encoding a full-length *Xenopus* Slit protein.[33] The *Xenopus slit* is an ortholog of the mouse *slit-2* (*mslit-2*). In the subsequences, an LRR in a Slit from one species is closest to the corresponding LRR in a Slit of another species, whereas the sequence of an EGF repeat in one species is not necessarily closest to the corresponding EGF in another species. The sequence of the chick *slit* is partial, appears to be closest to the *Xenopus* sequence.[33] Although we have shown the expression pattern of chick *slit*, the uncertainty in comparing partial sequences made it unclear whether the expression of the *Xenopus slit* would be similar to that of chick *slit*. We present here results of *in situ* hybridization showing the embryonic distribution of *Xenopus slit* mRNA.

Xenopus slit expression begins at the midline in the late gastrula at stage 12 (Fig. 1A, D), initially in the midline of the dorsal mesoderm including the prechordal plate and the notochord (Fig. 1A, D). *slit* can be detected in the floor plate and the neural fold in neurula at stage 17 (Fig. 1B, E). After the completion of neural tube closure by stage 19, *slit* is expressed in the motoneuron columns as well as in the roof plate and the floor plate (see Fig. 1F for a stage 20 embryo). *slit* is expressed in the eyes of the tailbud after stage 22; the expression pattern in the eyes changes over time: *slit* is initially expressed in the retina pigment epithelium as well as the ciliary margin (Fig. 1C, H), but later it is expressed in the amacrine cell layer (Fig. 1I). At stage 26, *slit* expression in the mesodermal midline including the prechordal plate and

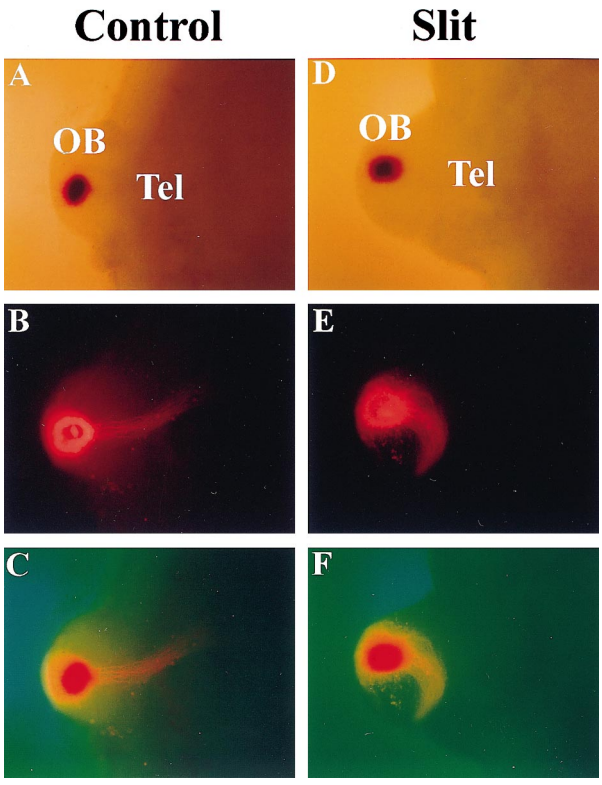

Fig. 4. Repulsion of olfactory bulb axons by Slit. A–C show different views of the same co-culture with control HEK cells laid on top of the telencephalon. D–F show different views of the same co-culture with Slit expressing cells laid on top of the telencephalon. (A) A bright-field view of the olfactory bulb–telencephalon co-culture. Control cells were laid on top of the telencephalon and DiI was inserted into the olfactory bulb. OB indicates the olfactory bulb and Tel indicates the telencephalon. (B) A fluorescent view of the same co-culture as that shown in A; note that axons from the olfactory bulb projected into the telencephalon. (C) A superimposition of A and B. (D) A bright-field view of the olfactory bulb–telencephalon co-culture. Slit expressing cells were laid on top of the telencephalon. (E) A fluorescent view of the same co-culture as that shown in D; note that axons from the olfactory bulb turned away from the telencephalon. (F) A superimposition of D and E.

the notochord begins to disappear, first from the rostral regions and later from caudal regions. Its expression in the floor plate, the roof plate and the motor neurons persists to the latest stage examined (stage 45).

Extracellular secretion of Slit, but not Robo, protein

To test whether Slit and Robo proteins can be secreted extracellularly, we expressed Slit and Robo proteins in cultured cells. cDNAs expressing the full-length *Xenopus* Slit protein tagged with the myc epitope (Slit-myc) and the full-length rat Robo-1 protein tagged with the hemagglutinin (HA) epitope (Robo-HA) were separately transfected into HEK-derived 293T cells. Conditioned media and lysates of cells transfected with Slit-myc or Robo-HA were collected. A monoclonal anti-myc antibody specifically detected and immunoprecipitated the Slit-myc protein in both the culture medium and the lysate of cells transfected with *slit-myc* cDNA (Fig. 2A). The detection of Slit in the extracellular medium indicated that the full-length *Xenopus* Slit is a secreted protein. By contrast, Robo-HA could be detected only in the lysate, but not in the conditioned medium of cells transfected with *robo-HA* (Fig. 2B). This is consistent with the prediction that the full-length Robo protein is a membrane protein.[23] Multiple bands lower than the full-length Slit and Robo proteins were observed (Fig. 2A–D), suggesting possible proteolytic cleavage of these proteins.

Binding of soluble Slit-myc protein to cell surface Robo protein

We have shown that an alkaline phosphatase (AP)-tagged Slit protein (Slit-AP) could bind to Robo expressed on the cell surface.[33] Since that fusion protein contained a large AP tag, one question is whether the binding is mediated by Slit part of the fusion protein. We, therefore, tested the ability of Slit-myc, which has only a small myc tag, to bind to the membrane of cells transfected with *robo*.

The soluble Slit-myc protein was collected from the conditioned medium of cells transfected with *slit-myc* cDNA and added to the media culturing control cells or cells transfected with *robo*. After incubation, Slit-myc was detected by the monoclonal anti-myc antibody and a Cy3-conjugated anti-mouse secondary antibody. Slit-myc did not bind to cells transfected with the vector (Fig. 3A, B), whereas its presence was observed after cells were transfected with *robo* (Fig. 3C, D). Taken together with results from Slit-AP binding to Robo, these results suggest that the binding of Slit fusion proteins to Robo is mediated through the Slit part, not the tags.

Repulsion of olfactory bulb axons by Slit

Using aggregates of cells expressing Slit pre-labeled with the lipophilic dye DiO, we have found that these cells could repel axons from the olfactory bulb.[33] To ensure that dye labeling of the cultured cells does not change the properties of the Slit expressing cells, we have repeated these experiments with unlabeled control or Slit expressing cells.

We used the whole-mount co-culture of the olfactory bulb and telencephalon.[33,53] During normal development, olfactory bulb axons grow into the telencephalon, forming the lateral olfactory tract.[47,52] In the co-culture, two days after the telencephalon region was covered with aggregates of control HEK cells, olfactory bulb axons grew into the telencephalon ($n = 24$) (Fig. 4A–C). If the telencephalon was covered with Slit expressing HEK cells, olfactory bulb axons could not grow into the telencephalon and instead avoided the telencephalon ($n = 12$) (Fig. 4D–F). These results confirm that Slit cells can indeed repel olfactory bulb axons projecting to the telencephalon.

DISCUSSION

We have examined the expression pattern of *slit* in *Xenopus* embryos, demonstrated the secretion of Slit protein into the extracellular medium, shown the binding of Slit-myc to cells expressing Robo, and confirmed the repulsive activity of the Slit protein.[6,25,33,40,59,64]

That Slit is a ligand for Robo has been previously suggested by genetic interactions of *slit* and *robo* mutants,[25] by direct binding of Slit and Robo proteins,[6,33,64] and by the binding of Slit-AP to Robo expressed on the cell surface.[33] Results presented here show that Slit-myc can also bind to Robo-expressing cells. This rules out the possibility that the large AP tag in the Slit-AP fusion plays a significant role in binding to Robo cells.

The patterns of *slit* expression in *Xenopus* and chick

embryos are not identical, but quite similar. In chick embryos,[33] expression was observed in Hensen's node at stage 4+. Equivalent expression of *Xenopus slit* in the Spemann organizer has not been observed. However, most of the other aspects of expression are similar in these two species, including the changing patterns in the notochord and the retina. In both species, the expression patterns suggest that Slit is likely to play roles in multiple regions of the embryo. Slit expression in the ventral midline of the neural tube suggests functions in axon guidance around the midline. For olfactory bulb axons, Slit may drive them to grow laterally on the same side of the midline. For commissural axons in the spinal cord, *slit* expression in the floor plate may prevent commissural axons that have crossed the floor plate from re-crossing the floor plate. *slit* expression in the motor neurons may force these commissural axons to turn longitudinally. *slit* expression in the roof plate suggests that Slit may guide axons around the dorsal midline of the neural tube. *slit* expression in the retina suggests that Slit functioning is not limited to axon guidance at the midline. Moreover, the changing pattern of *slit* expression in the retina does not correlate simply with projection of axons from the retina ganglion cells, suggesting possible function in guiding the formation of local circuits within the retina, or functions other than axon guidance.

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