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Overexpression of a Slit Homologue Impairs Convergent Extension of the Mesoderm and Causes Cyclopia in Embryonic Zebrafish

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Slit is expressed in the midline of the central nervous system both in vertebrates and invertebrates. In *Drosophila*, it is the midline repellent acting as a ligand for the Roundabout (Robo) protein, the repulsive receptor which is expressed on the growth cones of the commissural neurons. We have isolated cDNA fragments of the zebrafish *slit2* and *slit3* homologues and found that both genes start to be expressed by the midgastrula stage well before the axonogenesis begins in the nervous system, both in the axial mesoderm, and *slit2* in the anterior margin of the neural plate and *slit3* in the polster at the anterior end of the prechordal mesoderm. Later, expression of *slit2* mRNA is detected mainly in midline structures such as the floor plate cells and the hypochord, and in the anterior margins of the neural plates in the zebrafish embryo, while *slit3* expression is observed in the anterior margin of the prechordal plate, the floorplate cells in the hindbrain, and the motor neurons both in the hindbrain and the spinal cord. To study the role of Slit in early embryos, we overexpressed Slit2 in the whole embryos either by injection of its mRNA into one-cell stage embryos or by heat-shock treatment of the transgenic embryos which carries the *slit2* gene under control of the heat-shock promoter. Overexpression of Slit2 in such ways impaired the convergent extension movement of the mesoderm and the rostral migration of the cells in the dorsal diencephalon and resulted in cyclopia. Our results shed light on a novel aspect of Slit function as a regulatory factor of mesodermal cell movement during gastrulation.

Key Words: **zebrafish; Slit; cyclopia; floor plate; mesoderm; convergent extension.**

INTRODUCTION

The midline of the embryonic *Drosophila* central nervous system (CNS) comprises a small number of neuronal and glial cells with unique morphological properties. The midline glial cells express several molecules which play crucial roles for axonal pathfinding by the commissural neurons (see review, Nambu *et al.*, 1993). For the commissural axons to successfully cross the midline, their growth cones must first grow toward the midline and then leave it to extend into the contralateral side. Such complex behavior is accomplished by the precise temporal regulation of attractive and repulsive activities exerted by the midline glial cells. Netrin protein, secreted by the midline glial cells, plays an attractive role (Kennedy *et al.*, 1994; Serafini *et al.*, 1996; Mitchell *et al.*, 1996). Commissureless (Comm) protein is a transmembrane protein expressed on the sur-

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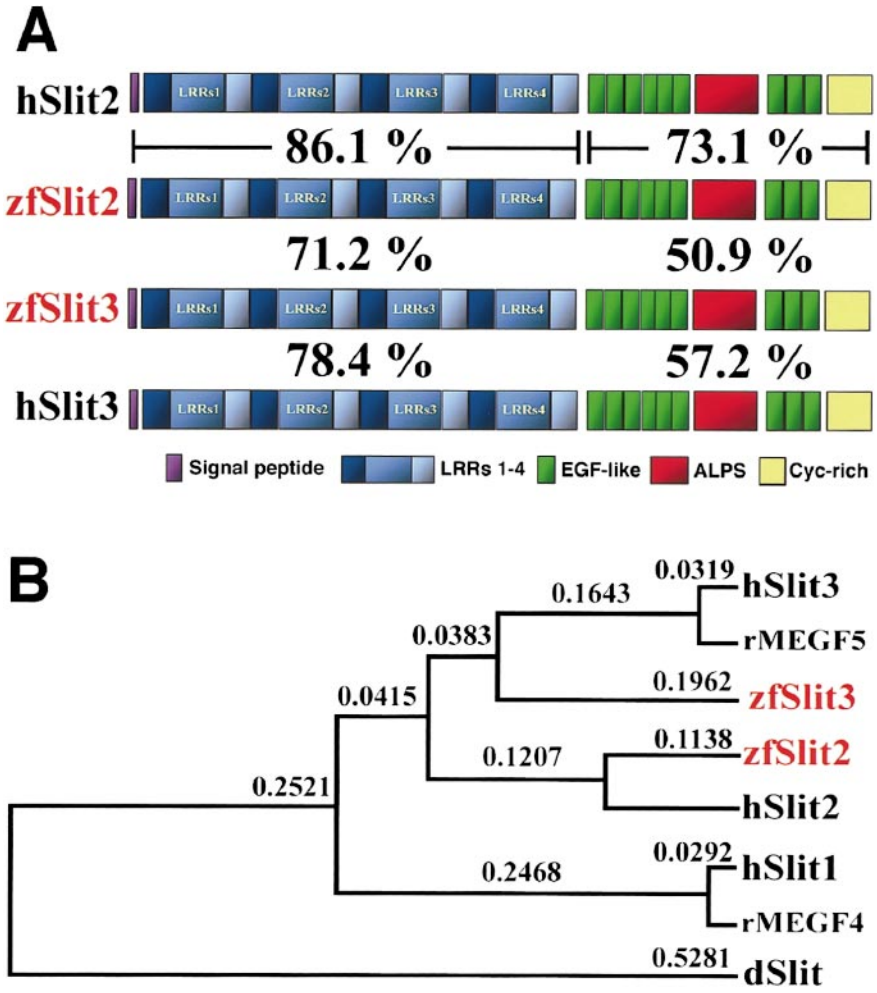

FIG. 1. Structural motifs within zebrafish Slit2 and Slit3 in comparison to Slit proteins of other species. (A) Comparison of zebrafish and human Slit proteins. Amino acid identities within the amino-terminal or carboxyl-terminal halves are indicated by the percentage. Each Slit sequence contains a putative signal peptide, four regions (LRRs1–4) containing four to six tandem arrays of a typical 24-amino-acid leucine-rich-repeats (blue boxes), each of which is flanked by amino- and carboxy-terminal conserved flanking regions (LRR-NR1–4, LRR-CR1–4). The following 9 units of EGF-like motifs (green boxes) are interrupted by an Agrin-Laminin-Perlecan-Slit (ALPS) domain (red box). Carboxyl-terminal to this repeat is a cysteine-rich (Cys-rich) carboxyl-terminal domain (yellow box). (B) Evolutionary relationship between *Drosophila*, zebrafish, rat, mouse, and human Slit proteins. By comparison of amino acid sequences, evolutionary distances were calculated using the UPGMA algorithm. The length of the lines are proportional to the evolutionary distances (numbers indicated above each line) from branch points.

face of the midline glial cells and prevents the growth cones of the commissural neurons from being repulsed before they reach the midline (Tear *et al.*, 1996). Comm does so by counteracting the activity of Roundabout (Robo) protein, the repulsive receptor which is expressed on the growth cones of the commissural neurons (Kidd *et al.*, 1998).

Slit is another protein expressed by the midline glial cells. The *slit* gene was originally identified as a gene whose mutation affects the external midline structure in the *Drosophila* embryo. Mutations in the *slit* locus cause the collapse of the ladderlike scaffold of the commissural and longitudinal axon bundles in the CNS to a single longitudinal tract at the midline (Rothberg *et al.*, 1988). Recent genetic and biochemical evidence has shown that Slit is the midline repellent acting as a ligand for the Robo receptor (Kidd *et al.*, 1999; Brose *et al.*, 1999; Li *et al.*, 1999). Slit guides the growth cones of the commissural axons so that they are repulsed away from the midline after they cross it, thereby preventing them from recrossing the midline. The CNS axonal scaffold collapses in the *slit* mutant embryo because the commissural axons are unable to leave the midline. Slit also functions as a chemorepellent controlling mesoderm migration away from the midline (Kidd *et al.*, 1999). The predicted protein encoded by *slit* contains four

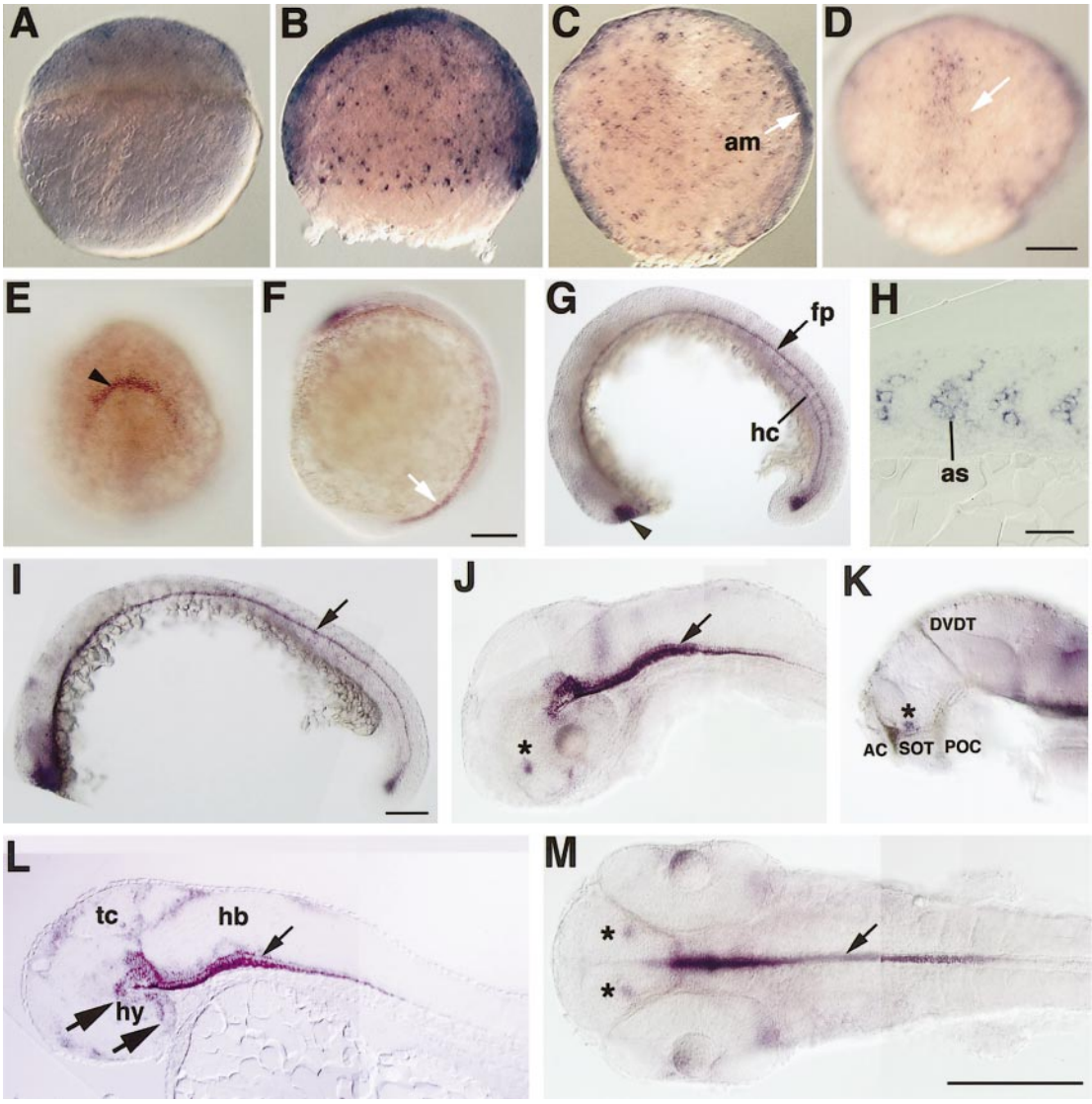

FIG. 2. Expression patterns of zebrafish *slit2* mRNA. (A) Expression of *slit2* mRNA at the midblastula stage (4 h). (B) Expression of *slit2* mRNA at 70% epiboly. Lateral view. (C, D) Expression of *slit2* mRNA at 80% epiboly. Expression in the axial mesoderm (am, white arrows) is indicated. C, lateral view; D, dorsal view. (E, F) Expression of *slit2* mRNA in 10-h embryos. E, dorsal view; F, lateral view. *slit2* mRNA is expressed in the anterior margin of the neural plate (arrowheads), the axial mesoderm (white arrow). (G, H) Expression of *slit2* mRNA in 16-h embryos. G, lateral view; H, a close-up view of the somites in a parasagittal section. *slit2* mRNA is expressed in the anterior margin of the forebrain (arrowhead), floor plate cells (fp and thin arrow), the hypochord (hc), and the anterior margin of the somites (as). (I) Expression of *slit2* mRNA in 18-h embryo. (J–M) Expression of *slit2* mRNA in the head region of 48-h embryos. J and K, lateral views; M, dorsal view; L, a sagittal section. In K, the axons in the initial scaffold of the brain are immunohistochemically stained with an antibody for acetylated α-tubulin (brown signals). *slit2* mRNA is expressed in the floor plate cells (thin arrows) and their anterior equivalents in the ventral forebrain (thick arrows), and in a small number of cells (asterisks) dorsally adjacent to the supraoptic tract (SOT). tc, tectum; hb, hindbrain; hy, hypothalamus; n, notochord; AC, anterior commissure; DVDT, dorsoventral diencephalic tract; POC, postoptic commissure. Bars, 200 μm (A–D, E and F, G and I, J–M), 50 μm (H).

leucine-rich repeats (flank-LRR-flank), seven EGF-like repeats (Rothberg *et al.*, 1990), and an Agrin-Laminin-Perlecan-Slit (ALPS) motif (Rothberg and Artavanis-Tsakonas, 1992).

The mammalian homologues of Slit and Robo have been identified (Holmes *et al.*, 1998; Itoh *et al.*, 1998). They are coordinately expressed in the developing CNS of vertebrate (Yuan *et al.*, 1999). Slit proteins (Slit1, Slit2, and Slit3) share a common domain structure and show a high degree of sequence homology with *Drosophila* Slit. As in *Drosophila*,

the vertebrate Slit proteins exert pleiotropic functions. Slit acts as a repellent on the axons from the olfactory bulb, the dentate gyrus in the hippocampus, and the spinal motor neurons (Li *et al.*, 1999; Brose *et al.*, 1999; Nguyen Ba-Charvet *et al.*, 1999). Slit is proteolytically cleaved, and its N-terminal cleavage product promotes branching of the axons from the dorsal root ganglion (Wang *et al.*, 1999).

Recently, Enabled (Ena), a factor regulating actin polymerization, has been shown to act downstream of the Slit-Robo signaling cascade in the growth cone guidance of *Drosophila* embryos (Bashaw *et al.*, 2000). The fact that its vertebrate homologues play major roles in the control of cell migration (see review, Machesky, 2000) suggests that the Slit-Robo signaling cascade may also be involved in the control of cell migration. In fact, Slit repulses the migrating neurons from the lateral ganglionic eminence, the olfactory bulb, choroid plexus, and the septum (Wu *et al.*, 1999; Zhu *et al.*, 1999; Hu, 1999).

In the present study, we identified two zebrafish homologues, *slit2* and *slit3*, of the *slit* gene family. We found that both genes start to be expressed by the midgastrula stage well before the axonogenesis begins in the nervous system, both in the axial mesoderm, and *slit2* in the anterior margin of the neural plate and *slit3* in the polster at the anterior end of the prechordal mesoderm. We showed that overexpression of zebrafish Slit2 either by injecting *in vitro* synthesized RNA encoding Slit2 into the one-cell stage embryos or by inducing ubiquitous overexpression of Slit2 in the embryos transgenic for the *slit2* gene under the control of the heat-shock promoter (Halloran *et al.*, 2000) brings the convergent extension movement of the mesoderm to a complete halt. Subsequently, the embryos suffer from cyclopia. Furthermore, the mesodermal cell migration is significantly retarded in response to the local and ectopic expression of Slit2 in the mosaic embryos. These data demonstrate that Slit2 acts negatively on the mesodermal movement. During gastrulation, the mesodermal cells show drastic changes in behavior and morphology (see review, Keller *et al.*, 1992). They become bipolar and are mediolaterally oriented with protrusions actively extending on both ends. By mediolaterally intercalating on each other, these cells achieve the convergent extension movement. On contact with the notochord-somite boundary, however, the side of the cells facing the boundary becomes inactive and flat. Our observation suggests that Slit may be involved in control of this complex behavior by the mesodermal cells during convergent extension movement of the mesoderm.

MATERIALS AND METHODS

Zebrafish Maintenance and Mutant Strains

Zebrafish were maintained as described in Westerfield (1995). The embryos were staged according to Kimmel *et al.* (1995). *cyclops* (*cyc*^{tf219}) was obtained from Drs. M. Furutani-Seiki and H. Takeda.

Isolation of Zebrafish slit2 and slit3 cDNA and Sequencing

By using approximately 0.7 kilo base-pair (kb) *Apa* I-*Sac* I fragment which contains the region encoding the ALPS motif of human SLIT2 (Holmes *et al.*, 1998; Genebank Accession No. AF055585), we screened λgt10 cDNA library derived from 18–24 h embryos (Inoue *et al.*, 1994). Hybridization was performed at low stringency (Sambrook *et al.*, 1989). Screening of the 10^6 pfu (plaque-forming units) of phages gave 29 hybridization-positive clones. Restriction mapping showed that these clones were classified into five groups. Sequence analysis revealed that 25 clones putatively encoded zebrafish homologue of human *SLIT2* and the remainders were false-positive clones. The longest cDNA clone, designated clone15, was approximately 4.5 kb and was subcloned into *Bam*H I site of pBluescript II SK (Stratagene). We screened λgt11 cDNA library derived from 22–24 h embryos at high stringency by using the *Bgl* II fragment (about 1.5 kb) of the clone 15 encoding the regions from LRRs2 to the middle of LRRs3 as a probe. From the latter library, 62 positive clones were identified, purified, and analyzed by restriction fragment mapping and partial DNA sequencing. Restriction fragment mapping showed that there were two independent groups of clones. One group of these clones, represented by clone 9, contained an approximately 5 kb cDNA fragment with the entire coding region of zebrafish *slit2*, and the others contained an approximately 6 kb cDNA fragment with an open reading frame of zebrafish *slit3*. These cDNA clones were sequenced on both strands using Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech). Sequence assembly, identification of the signal peptide, and prediction of the cleavage site were carried out by GENETYX-MAC 9.01 program (Software Development Ltd.). Homology searches were performed using BLAST algorithm at the NCBI (Altschul *et al.*, 1990). The evolutionary tree was constructed using UPGMA method (Nei *et al.*, 1985). *Drosophila* (Rothberg *et al.*, 1998), mouse (Holmes *et al.*, 1998; Yuan *et al.*, 1999), rat (Nakayama *et al.*, 1998), and human (Holmes *et al.*, 1998; Itoh *et al.*, 1998) *slit* gene sequences were used for comparison. The accession numbers for the zebrafish *slit2* and *slit3* sequences are AF210321 and AF210320, respectively.

Whole-mount in Situ Hybridization, Tissue Sections, and Immunohistochemistry

Whole-mount *in situ* hybridization was performed according to the standard method (Westfield, 1995). The 2.2-kb fragment of *slit2* cDNA and the 2.7-kb fragment of *slit3* cDNA were used as templates for synthesizing antisense probes. Antisense RNA probes were synthesized using the digoxygenin-RNA labeling kit (Roche Diagnostics). Antisense probes for markers were synthesized from cDNAs of *Islet-1* (Inoue *et al.*, 1994), *shh* (Krauss *et al.*, 1993), *dlx3* (Akimenko *et al.*, 1994), *zfz8b* (Kim *et al.*, 1998), *hlx1* (Fjose *et al.*, 1994), *pax2* (Krauss *et al.*, 1991), *ntl* (Schulte-Merker *et al.*, 1992), *fkh5*, and *opl* (Grinblat *et al.*, 1998). The *ntl* cDNA probe was synthesized by RT-PCR. Sections of *in situ* hybridized embryos were made by refixing the hybridized embryos, embedding them in JB-4 (Polysciences), and sectioning at 5–10 μm intervals with a microtome (MICROM HM330, Mcbain Instruments). For the double staining, immunohistochemistry using the monoclonal antibody for acetylated α-tubulin (Sigma) was first performed, followed by *in situ* hybridization for *slit2* mRNA. Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS)

overnight at 4°C and washed in PBS containing 0.4% Triton. Embryos were blocked for 1 h in PBS containing 0.4% Triton, 50 μg/ml heparin, 250 μg/ml tRNA (Sigma), and 20 U/ml RNase inhibitor (Amersham Pharmacia Biotech), and incubated overnight at 4°C in 1/2000 diluted primary antibody, washed, preincubated again, and incubated overnight at 4°C in 1/100 diluted biotin-conjugated anti-mouse IgG antibody (Vectastain). Chromogenic reaction was performed in PBS containing 300 μg/ml diaminobenzidine (Sigma) and 0.0003% hydrogen peroxide. The double staining using rabbit antiserum for Green Fluorescent Protein (Santa Cruz Biotechnology) was performed in a similar manner except that biotin-conjugated anti-rabbit IgG antibody was used as the secondary antibody. After immunostaining, embryos were further processed for the whole-mount *in situ* hybridization reaction as described above. Digital images of all embryos were captured using a differential interference contrast microscope (Axioplan2, Zeiss) and a CCD camera (DKC 5000, Sony).

Plasmid Construction for RNA Injection

The Slit expression vector was generated in the following manner. The backbone plasmid was pBluescript II SK. The *Sal* I-*EcoR* I fragment of clone 9 and *EcoR* I-*Xba* I fragment of clone 15 were isolated and inserted between the *Sal* I and *Xba* I sites of the pBluescript II SK to create pSK-zfSlit2. A *SnaB* I-*Not* I fragment containing an SV 40 poly(A) signal was isolated from the pCS2+ plasmid (Turner and Weintraub, 1994; Rupp *et al*., 1994) and was further inserted between the blunted *Xba* I site and the *Not* I site of the pSK-zfSlit2 to create pSK-zfSlit2-pA. This plasmid contained the full-length zebrafish *slit2* cDNA and SV 40 poly(A) signal under the control of the promoter for T7 RNA polymerase. To create pSK-zfSlit3-pA containing the full-length of zebrafish *slit3* cDNA, pSK-zfSlit2-pA was cut with *Xho* I and *Spe* I to remove the zebrafish *slit2* cDNA and to replace it with the corresponding *Xho* I-*Spe* I fragment of zebrafish *slit3* cDNA. *In vitro* synthesis of capped mRNA from the linearized pSK-zfSlit2-pA and pSK-zfSlit3-pA was carried out using the mMessage mMachine T7 kit (Ambion), according to the manufacture's instruction. The RNA concentration was determined spectrophotometrically. The mRNA solution at concentrations of 0.5, 0.8, 1, and 2 mg/ml was injected into the one-cell stage embryos using an air-pressure microinjector (Eppendorf, Transinjector 5246) as described previously (Kikuchi *et al*., 1997). For the experiments presented in Figs. 4 and 5, 0.8 mg/ml of *slit2* mRNA was injected. For mosaic analysis, *slit2* mRNA (0.8 mg/ml) was coinjected with *GFP* mRNA (2 mg/ml).

Generation of the Transgenic Zebrafish for Overexpression of slit2

The plasmid for the transgenic overexpression of *slit2* was constructed in the following manner. pCS2-zfSlit2 plasmid was made by inserting zfSlit2 cDNA between the *Cla* I and *Xba* I sites of pCS2+. The *Sal* I-*Cla* fragment of the zebrafish heat-shock 70 promoter (Halloran *et al*., 2000), the *BstX* I-*BamH* I fragment encoding C-terminal region of zebrafish Slit2, and the *BamH* I-*Xba* I fragment encoding GFP were performed using pHSP70/4-EGFP (Halloran *et al*., 2000), *slit2* cDNA, and pEGFP-C1 (Clontech) as templates, respectively, with the following sets of oligonucleotide primers to introduce appropriate restriction enzyme sites into the 5′- and 3′-ends of each DNA fragment; hspSal, 5′-*GTC GAC* CAG GGG TGT CGC TTG GTT-3′ and hspCla, 5′-*ATC GAT* AAA AAA AAA CAA TTA GAA TTA-3′; slit2BstX, 5′-CTC ACC CAG TTT TTG CTG-3′ and slit2BamH, 5′-*GGA TCC* GGA TGG ACA TTT TGT GCA-3′; gfpBamH, 5′-*GGA TCC* ATG GTG AGC AAG GGC-3′ and gfpXba, 5′-*GAT ATC* TAG ATT ACT TGT ACA GCT C-3′. The amplified DNA fragments were subcloned into pCR2.1 vector (Novagen) to generate pCR-hsp, pCR-zfSlit2^c, and pCR-gfp, respectively. The *BstX* I-*BamH* I fragment of pCR-zfSlit2^c and *BamH* I-*Xba* I fragment of pCR-gfp were isolated and inserted between the *BstX* I and *Xba* I sites of the pCS2-zfSlit2 to create pCS-zfSlit2:GFP. To create phsp-zfSlit2:GFP containing the zebrafish heat-shock promoter, the *Sal* I-*Cla* I fragment of pCS-zfSlit2:GFP containing the sCMV IE94 promoter of pCS2+ was replaced with the corresponding *Sal* I-*Cla* I fragment of the zebrafish heat-shock promoter of pCR-hsp. In addition, *BamH* I-*Xba* I fragment of pCR-gfp was introduced into the *BamH* I-*Xba* I site of the pCS2 to create pCS2-gfp.

phsp-zfSlit2:GFP was purified using QIAprep Spin Miniprep kit (Qiagen). Then, linearized DNA was extracted using the GeneClean II kit (Bio 101). A DNA solution of 25 μg/ml in distilled water was injected into the cytoplasm of one-cell stage zebrafish embryos with its chorion intact under the dissecting microscope as described in Higashijima *et al.* (2000). The injected embryos were raised to sexual maturity and crossed with each other to identify the founder fish pairs which bear the progeny transgenic for hsp-zfSlit2:GFP. Transgenic embryos were identified by their expression of GFP fluorescence in the whole body following heat-shock treatment at 39°C. for 1 h. Four out of 56 pairs turned out to bear positive progeny. Other clutches of embryos were collected from each positive founder pairs and raised to adulthood without heat-shock treatment. Transgenic individuals were identified by incrossing the siblings and examining whether their progeny are transgenic or not in the manner described above and used for establishing the transgenic lines. Two lines, termed HS2E-1S and HS2E-4S, in which Slit2/GFP fusion protein was induced to a similar level, were used for further analysis. Since we always obtained the same results with these lines, we do not indicate which line we used for obtaining the individual pictures shown in the text. Photos of GFP fluorescence of the transgenic embryos were taken using a confocal laser scanning microscope (LSM510, Carl Zeiss) and were superimposed with a differential interference contrast image.

RESULTS

Molecular Cloning of the Zebrafish slit2 and slit3 Genes and Domain Analysis of Their Gene Products

To identify zebrafish *slit* homologues, we used a cDNA fragment of the human *Slit2* (Holmes *et al*., 1998) as a probe to screen a zebrafish cDNA library under low-stringency hybridization conditions. We obtained a cDNA clone containing an open reading frame (ORF) of 4539 base pairs (bp) which putatively encoded 1513 amino acid polypeptides. Sequence analysis indicated that the putative protein included four units (LRRs1–4) containing four to six tandem arrays of a typical 24-amino-acid leucine-rich-repeat (LRR) motifs, each of which is flanked by amino- and carboxy-

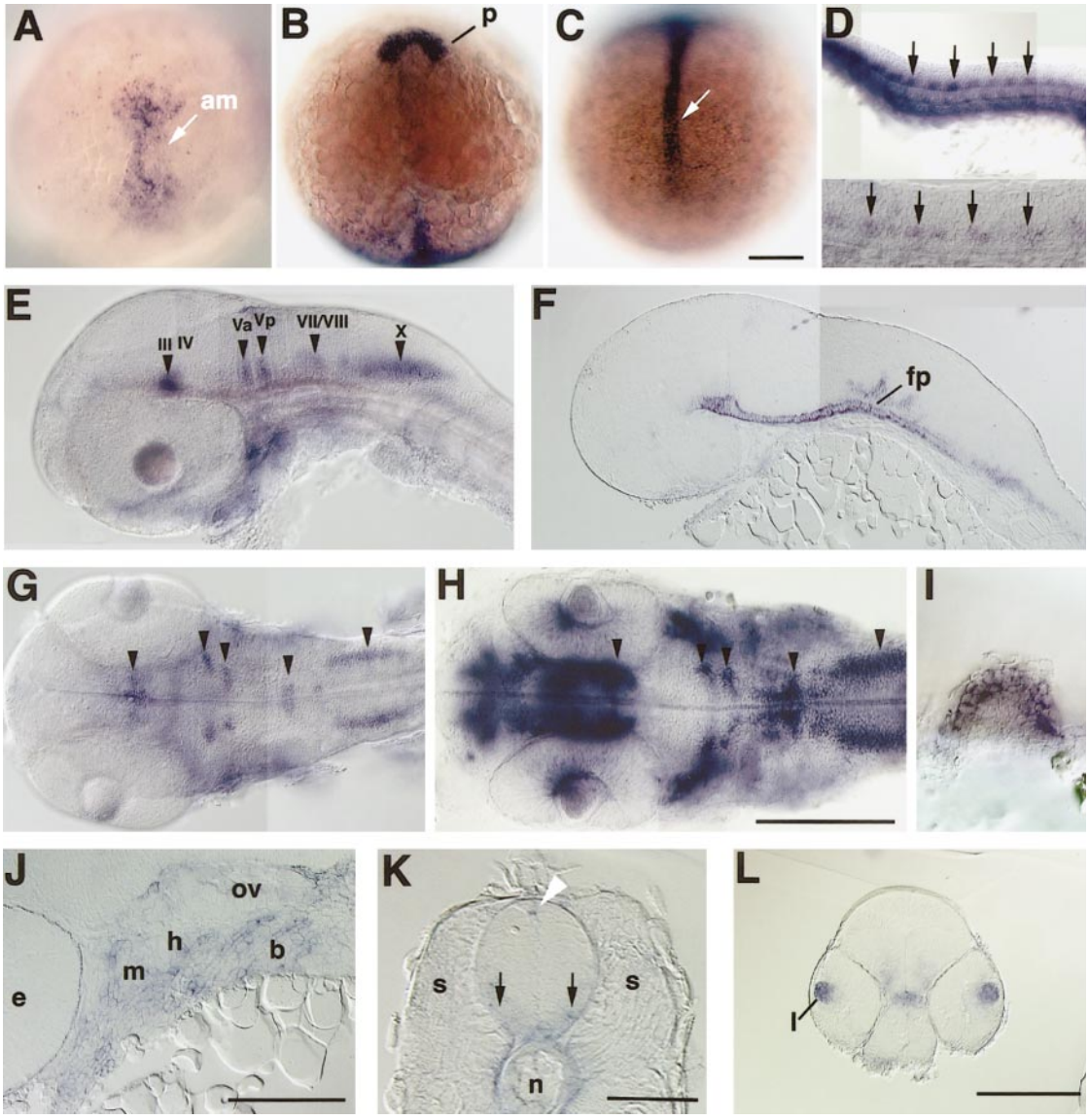

FIG. 3. Expression patterns of zebrafish *slit3* mRNA. (A) Expression of *slit3* mRNA at 70% epiboly in the axial mesoderm (am, white arrow). Dorsal view. (B, C) Expression of *slit3* mRNA in 10-h embryos. Dorsal views of the rostral (B) and caudal (C) parts of the embryo. *slit3* mRNA is expressed in the polster (p) as well as in axial mesoderm cells (white arrow). (D) Expression of *slit3* mRNA in 26-h embryo. *slit3* mRNA is expressed in the motor neurons in the ventral spinal cord (arrows). The lower inset shows the close-up view of the motor neurons. (E–G) Expression of *slit3* mRNA in the head region of 48-h embryos. E, lateral view; F, a sagittal section; G, dorsal view. *slit3* mRNA is expressed in the cranial motor neurons (arrowheads) and the floor plate cells (fp) in the hindbrain. (H) Expression of *Islet-1* mRNA in the head region of 48-h embryos. *Islet-1* mRNA is also expressed in the cranial motor neurons (arrowheads). III, IV, Va, Vp, VII/VIII, X, oculomotor, trochlear, anterior trigeminal, posterior trigeminal, facial/octavolateralis, and vagus motor neurons, respectively. (I–L) Expression of *slit3* mRNA in various parts of 48-h embryo. *slit3* mRNA is expressed in the pectoral fin bud (I), the pharyngeal arches (m, h, b in J), spinal motor neurons, and the roof plate (arrows and white arrowhead in K, respectively) and the lens (l in L). I, lateral view; J, a parasagittal section; K, a cross section of the anterior spinal cord; L, a frontal section of the head. m, h, and b, the mandibular arch, the hyoid arch, and the posterior set of branchial arches, respectively; e, eye; ov, otic vesicle; s, somites; n, notochord. Bars, 200 μm (A–D, E–H, L), 100 μm (I–K).

terminal conserved flanking regions (LRR-NR1–4, LRR-CR1–4), and nine units of epidermal-growth-factor (EGF) -like motifs (EGF1–9), Agrin-Laminin-Perlecan-Slit (ALPS) conserved domain, and a cysteine-rich (Cys-rich) carboxy-terminal domain. The deduced amino acid sequence of this cDNA fragment had approximately 40.9, 62.6, 79.7, and

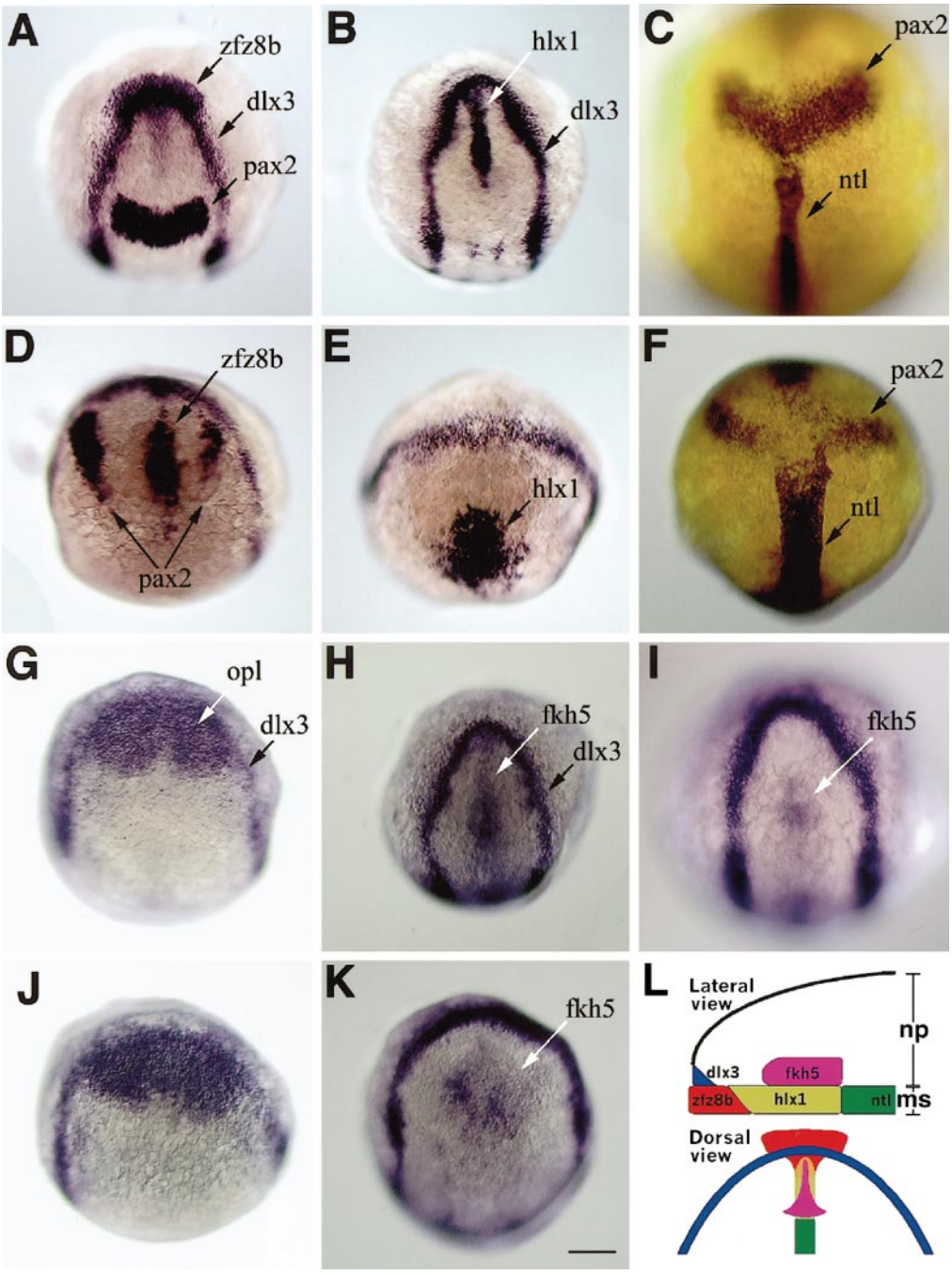

FIG. 4. Overexpression of *slit2* mRNA impairs migration of the prechordal mesodermal cells and the ventral diencephalic cells in the zebrafish embryo. (A–F) The retarded migration of the prechordal plate cells in the *slit2*-overexpressing embryos (D–F) in comparison to normal embryos (A–C) at 100% epiboly. The positions of the mesodermal cells were examined by comparing the expression patterns of the prechordal plate mesoderm markers, *zfz8b* (A, D) and *hlx1* (B, E), and the chordal mesoderm marker *ntl* (C, F) relative to the neural plate markers *dlx3* (A, B, D, E), and *pax2* (A, C, D, F), which are expressed in the anterior margin of the neural plate and in the midbrain/hindbrain boundary (MHB), respectively. In the *slit2*-overexpressing embryos, the *zfz8b*- and *hlx1*-positive prechordal mesodermal cells are prominently displaced caudally and are improperly located between the anterior margin of the neural plate and MHB. The chordal mesoderm is wider and flatter in the *slit2*-overexpressing embryo than in the normal embryo. (G–K) The retarded migration of the ventral diencephalic cells both in the *slit2*-overexpressing and cyc^{tz219} mutant embryos at 100% epiboly. The position of the *fkh5*-positive diencephalic cells relative to the *dlx3*-positive anterior margin of the neural plate is displaced caudally in the *slit2*-overexpressing embryo (K) in comparison to the normal embryo (H), as in the cyc^{tz219} mutant (I). The *opl*-positive cells are normally distributed in the *slit2*-overexpressing embryo (J) as in the normal embryo (G). (L) Schematic of the normal expression patterns of the marker genes used in this figure. np, neural plate; ms, chordal and prechordal mesoderm. Bars, 200 μm.

64.8% similarity to *Drosophila* Slit (dSlit) (Rothberg *et al.*, 1988) and human Slit1 (hSlit1), Slit2 (hSlit2), and Slit3 (hSlit3) (Itoh *et al.*, 1998), respectively. Therefore, we termed the gene zebrafish *slit2*.

During further screening, we also isolated a distinct cDNA clone putatively encoding a protein similar to Slit2 protein. Sequence analysis indicated that this cDNA fragment contained an ORF of 4548 bp which encoded a 1516-amino-acid polypeptide. The amino acid sequence of this cDNA fragment had about 39.5, 62.6, 62.4, and 68.7% similarity to dSlit, hSlit1, hSlit2, and hSlit3, respectively. Therefore, we termed the gene zebrafish *slit3*. Both of the predicted amino acid sequences of zebrafish *slit2* and *slit3* gene products contained the same motif structures (Fig. 1A). These motifs are found in a number of intracellular and extracellular proteins and function in such diverse biological aspects as protein–protein interaction, signal transduction, ligand recognition, and cell adhesion in development (Kobe and Deisenhofer, 1994; Rebay *et al.*, 1991; Perez-Vilar *et al.*, 1996). The overall amino-acid sequences were well conserved from zebrafish to human. In contrast to the high degree of similarity in the region of LRRs motifs and EGF-like motifs between zebrafish Slit2 and hSlit2, the region which contains EGF-like motifs of zebrafish Slit3 was less similar to that of hSlit3 (Fig. 1A). A phylogenetic tree shows sequence similarities among *Drosophila*, zebrafish, rat, mouse, and human Slit proteins (Fig. 1B).

Zebrafish slit2 and slit3 Start to Be Expressed in the Axial Mesoderm Much Earlier Than Initiation of Neurogenesis

A very low level of *slit2* mRNA started to be detected ubiquitously in the embryos at the midblastula stage (4 h after fertilization) (Fig. 2A). In the midgastrula stage (70% epiboly), the mRNA of *slit2* was detected in the cells which were randomly dispersed in the embryonic body (Fig. 2B). By 80% epiboly, this salt and pepper-like expression of *slit2* mRNA continued only on the ventral side of the embryo (Fig. 2C). In contrast, it was downregulated on the dorsal side, where *slit2* expression was detected only in the axial region of the mesoderm (Fig. 2D). The mRNA of *slit2* was concentrated in the anterior margin of the neural plate (Fig. 2E) and in the axial mesodermal cells (Fig. 2F) by the late gastrula stage (10 h). The ventral midline cells in the neuroectoderm including the floor plate cells and their anterior equivalents started to express *slit2* mRNA at around this stage (Fig. 2G). This expression in the midline neuroectodermal cells continued to be observed at 16 and 18 h (Figs. 2G and 2I). At 16 h, it was detected in the hypocord (Fig. 2G) and in the anterior margins of the somites (Fig. 2H). At 48 h, expression of the *slit2* mRNA at the ventral midline of the CNS extended anteriorly to the level of the hypothalamus in the forebrain (Figs. 2J, 2L, and 2M). *slit2* mRNA was also expressed by a small number of cells located dorsally adjacent to the supraoptic tract (SOT) at the level between the anterior commissure and the postoptic commissure (POC) (Figs. 2J, 2K, and 2M) (Chitnis and Kuwada, 1990; Wilson *et al.*, 1990).

By the midgastrula (70% epiboly), *slit3* expression was evident in the axial region of the mesoderm (Fig. 3A). At the late gastrula stage (10 h), mRNA of *slit3* was detected in the axial mesodermal cells, and in the anterior edge of the prechordal plate, i.e., the polster which gives rise to the hatching gland cells later in development (Figs. 3B and 3C). At 24 h, *slit3* mRNA was expressed at low levels in the motor neurons in the spinal cord (Fig. 3D) and the floor plate cells of hindbrain (data not shown). On the second day of development (48 h), *slit3* expression was specifically detected in the cranial motor neurons of the hindbrain coinciding with the cells expressing *Islet-1* mRNA (Inoue *et al.*, 1994; Chandrasekhar *et al.*, 1997; Higashijima *et al.*, 2000) (Figs. 3E, 3G, and 3H) and the floor plate cells of the hindbrain (Fig. 3F). At this stage, *slit3* mRNA was also detected in the spinal motor neurons and in the roof plate (Fig. 3K), the margin of the fin mesoderm (Fig. 3I), the pharyngeal arches (Fig. 3J), and the lens (Fig. 3L).

Overexpression of slit2 mRNA Causes Defects in Convergent Extension of the Mesoderm and in Migration of the Diencephalic Cells

Our observation that expression of both *slit2* and *slit3* mRNA starts well before initiation of neurogenesis suggested that they may be involved in control of early embryogenesis in an aspect distinct from control of axonal pathfinding in which they are involved later in development. As a first step to analyze the role of Slit function in early embryogenesis, we overexpressed Slit2 by injecting its mRNA into the cytoplasm of the one-cell stage embryos. The injected embryos were fixed at the late gastrula stage and analyzed by *in situ* hybridization with several markers (Fig. 4L).

The expression pattern of *dlx3* which highlights the margin of the neural plate (Fig. 4A) (Akimenko *et al.*, 1994) revealed the broadening of the neural plate in the embryos overexpressing *slit2* mRNA (Fig. 4D).

During late gastrulation, zebrafish *frizzled 8b* (*zfz8b*) mRNA is expressed in the anterior-most prechordal plate, including the polster (Kim *et al.*, 1998) (Fig. 4A), while expression of the H2.0-like-homeobox 1 (*hlx1*) gene is detected in the posterior prechordal region (Fjose *et al.*, 1994) (Fig. 4B). The prechordal plate is derived from the hypoblast cells that migrate anteriorly in zebrafish embryos (Stachel *et al.*, 1993). Although prechordal plate mesoderm existed in the embryos overexpressing *slit2* mRNA, *zfz8b*- and *hlx1*-positive cells were improperly located. The *zfz8b*-positive cells were caudally shifted, distributing broadly between the levels of the *dlx3*-positive anterior margin of the neural plate and the *pax2*-positive stripe at the midbrain/hindbrain boundary (Krauss *et al.*, 1991) (Fig. 4D).

Furthermore, the rostral migration of the *hlx1*-positive cells was also severely retarded (Fig. 4E).

To assess whether *slit2* overexpression also affects the formation of the chordal mesoderm, embryos were double labeled at the same stage for *pax2* and *no tail* (*ntl*). *ntl* is expressed in the notochord precursors at the bud stage (Schulte-Merker *et al.*, 1992) (Fig. 4C). The notochord was significantly widened and flattened, although its anterior end reached the same rostrocaudal level as the *pax2*-positive cells in the neuroectoderm (Fig. 4F).

The defects observed in the embryos overexpressing *slit2* mRNA, i.e., the broadening of the neural plate, the retardation of the anterior migration of the prechordal mesoderm, and the widening and flattening of the notochord, resembled precisely the morphological abnormalities of the mutant embryos which have defects in the convergence and extension of the mesoderm and ectoderm during gastrulation (Marlow *et al.*, 1998; Heisenberg *et al.*, 2000). We have previously overexpressed several extracellular matrix proteins such as F-spondin1, F-spondin2, Mindin1, and Mindin2 in zebrafish embryos by injecting their mRNA into one-cell stage embryos (Higashijima *et al.*, 1997). However, none of them caused defects in convergent extension movement of the mesoderm (unpublished data).

To investigate whether the neuroectodermal cells are also affected by overexpression of *slit2* mRNA at the late gastrula stage, we performed *in situ* hybridization analysis for expression of *odd-paired-like* (*opl*) and *fkh5,* a forkhead domain protein (Grinblat *et al.*, 1998). Zebrafish *opl* is expressed in the anterior neural plate which includes the presumptive telencephalon (Fig. 4G). The *opl*-expressing cells were not affected from 90% epiboly stage through 14-somite stage in the embryos overexpressing *slit2* mRNA (compare Figs. 4G and 4J, Figs. 5H and 5J). In contrast, the cells expressing *fkh5*, a marker for the ventral diencephalon and mesencephalon (Fig. 4H), were widely dispersed and did not reach the anterior margin (Fig. 4K).

Varga *et al*. (1999) have recently shown that the diencephalic cells migrate anteriorly at 90% epiboly into the ventral anterior diencephalon, separating the single eye field into two. They also showed that such anterior migration by the diencephalic cells was impaired in the *cyclops* (*cyc*) mutant, which shows severe defects in the development of the prechordal plate mesoderm and the ventral midline neruoectoderm including the floor plate (Hatta *et al.*, 1991). Consequently, *fkh5* expression was missing in the presumptive anterior forebrain region at 100% epiboly in this mutant (Fig. 4I). The abnormalities observed in the *slit2* overexpressing embryos resembled those in the *cyc* mutants.

Overexpression of slit2 mRNA Causes Cyclopia

As expected from the resemblance of the early defects in the *slit2*-overexpressing embryos with those observed in the *cyc* embryos, the *slit2*-overexpressing embryos frequently became cyclopic with some variation in the degree of abnormality (Table 1, Figs. 5B and 5C). The cyclopic phenotype becomes more prominent when pigmentation of the eyes starts on the second day of development (Figs. 5E and 5F). Overexpression of *slit3* mRNA had weaker effects on induction of cyclopia than that of *slit2* mRNA (Table 1).

To analyze possible neural defects underlying the cyclopic phenotype in the embryos overexpressing *slit2* mRNA, we fixed the injected embryos at 16 h and processed them for *in situ* hybridization using two markers. We analyzed the expression of *opl* and *sonic hedgehog* (*shh*) to test for defects in the anterior dorsal and ventral neuroectoderm in the embryos overexpressing *slit2*. In normal embryos, *shh* was expressed in the floor plate cells and their anterior equivalents in the ventral forebrain (Krauss *et al.*, 1993) (Fig. 5G). In injected cyclopic embryos, expression of *shh* in the ventral forebrain ended at the level of the diencephalon (Fig. 5I), and the number of the cells expressing *shh* in the diencephalon increased. This result was consistent with our earlier observation of the absence of *fkh5* expression in the anterior most neural plate and further supported that the anterior migration of the diencephalic cells had been stalled in the *slit2*-overexpressing embryos. In contrast to *shh*, *opl*, a marker for dorsal neural tissue, was normally expressed in both wild-type and cyclopic embryos (Figs. 5H and 5J).

The Convergent Extension Movement of the Mesoderm is Suppressed by Transient Activation of the Transgenically Integrated slit2 Gene Throughout the Entire Period of Gastrulation

To exclude the possibility that the apparent defects in the convergent extension movement during gastrulation in the *slit2*-overexpressing embryos were caused by the earlier defects, such as those in early pattern formations, we generated transgenic zebrafish lines in which Slit2 protein fused with Green Fluorescent Protein (GFP) can be transiently induced in the whole body of embryos (Fig. 6A). The zebrafish hsp70 promoter was used to activate the transgene encoding this recombinant fusion protein so that it can be induced throughout the entire embryo following an increase in ambient temperature (Halloran *et al.*, 2000).

The vertebrate Slit2 protein is proteolytically processed into the amino- and carboxyl-terminal fragments (Slit2-N and Slit2-C), of which Slit2-N is responsible for the biological activities of Slit2 (Brose *et al.*, 1999). By Western blot analysis using *slit2*-overexpressing transgenic embryos, we confirmed that zebrafish Slit2 was also proteolytically cleaved between EGF5 motif and EGF6 motif *in vivo* as in other species (data not shown). Therefore, in the *slit2*-overexpressing transgenic embryos, the biologically active Slit-N is cleaved off from inactive Slit2-C, which is fused with GFP.

Heat shock at 80% epiboly brought the convergent extension movement of the mesoderm to a complete halt.

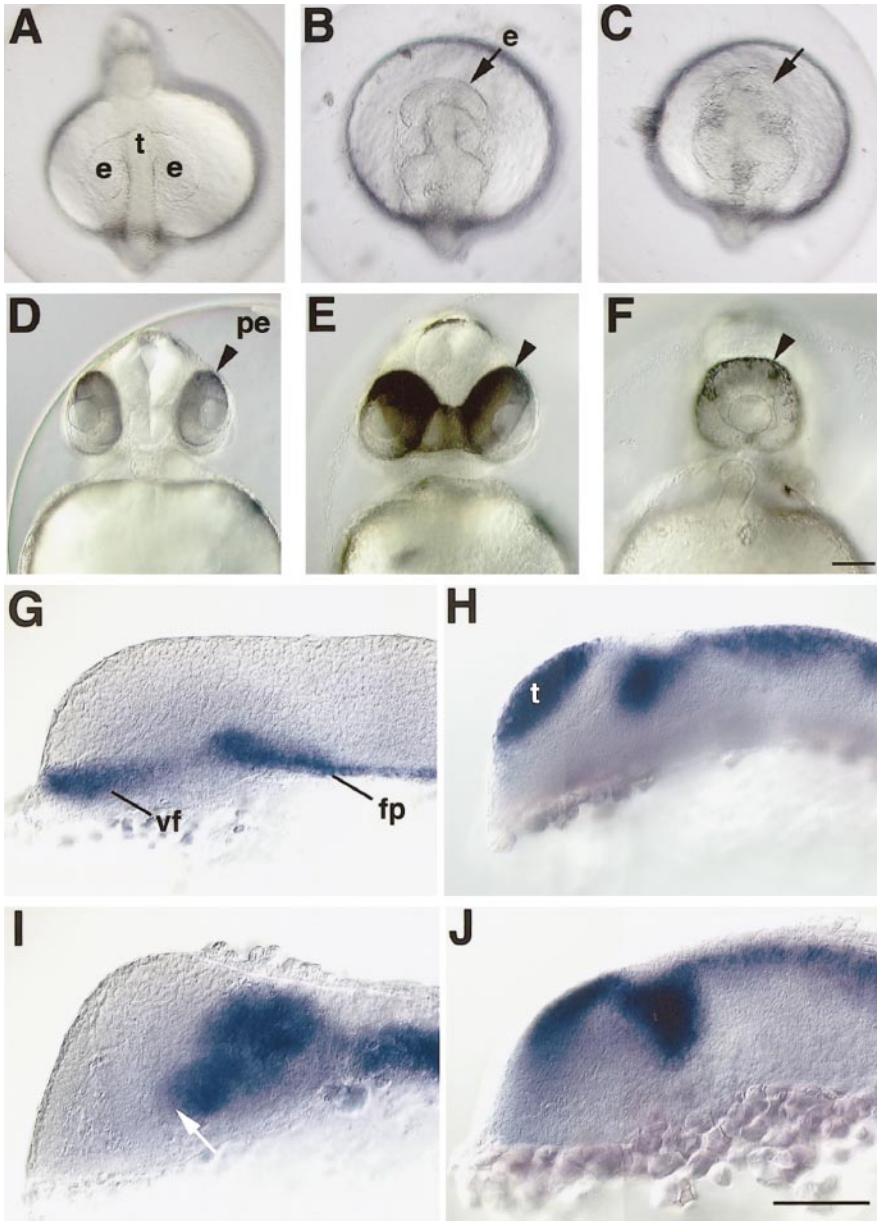

FIG. 5. Overexpression of *slit2* mRNA induces cyclopia in the zebrafish embryo. (A–F) Various degrees of cyclopia were observed in the *slit2*-overexpressing embryos at 16 h (B, C) and 48 h (E, F). A and D, normal embryos. Embryos are shown in dorsal views (A–C) and in frontal views (D–F). (G–J) Defect in the ventral forebrain of 16-h zebrafish embryo overexpressing *slit2* mRNA. (G, H) Expression of *shh* (G) and *opl* (H) mRNA in normal embryos. (I, J) Expression of *shh* (I) and *opl* (J) mRNA in embryos overexpressing *slit2* mRNA. In the *slit2*-overexpressing embryo, the anterior edge of the *shh*-positive domain in the ventral forebrain (vf) is caudally shifted as indicated by the arrow in (I). In contrast, *opl* mRNA is expressed indistinguishably in the dorsal forebrain of the normal embryo (H) and the embryo overexpressing *slit2* mRNA (J). e and arrows, eyes; pe and arrowheads, the retinal pigment epithelium; t, telencephalon; fp, floor plate cells. Bars, 200 μm (A–F, G–J).

This was evident because, during 2 h following heat shock, the *hlx1*-positive cells of the prechordal mesoderm rarely changed their positions relative to the margin of the neural plate which were demarcated by the *dlx3* expression (compare Fig. 6G with 6J and 6K). The notochord was also widened and flattened as observed by the *ntl* expression (Fig. 6H). Subsequently, the anterior extension of the *fkh5*-positive cells of the diencephalon was impaired (Fig. 6I), and

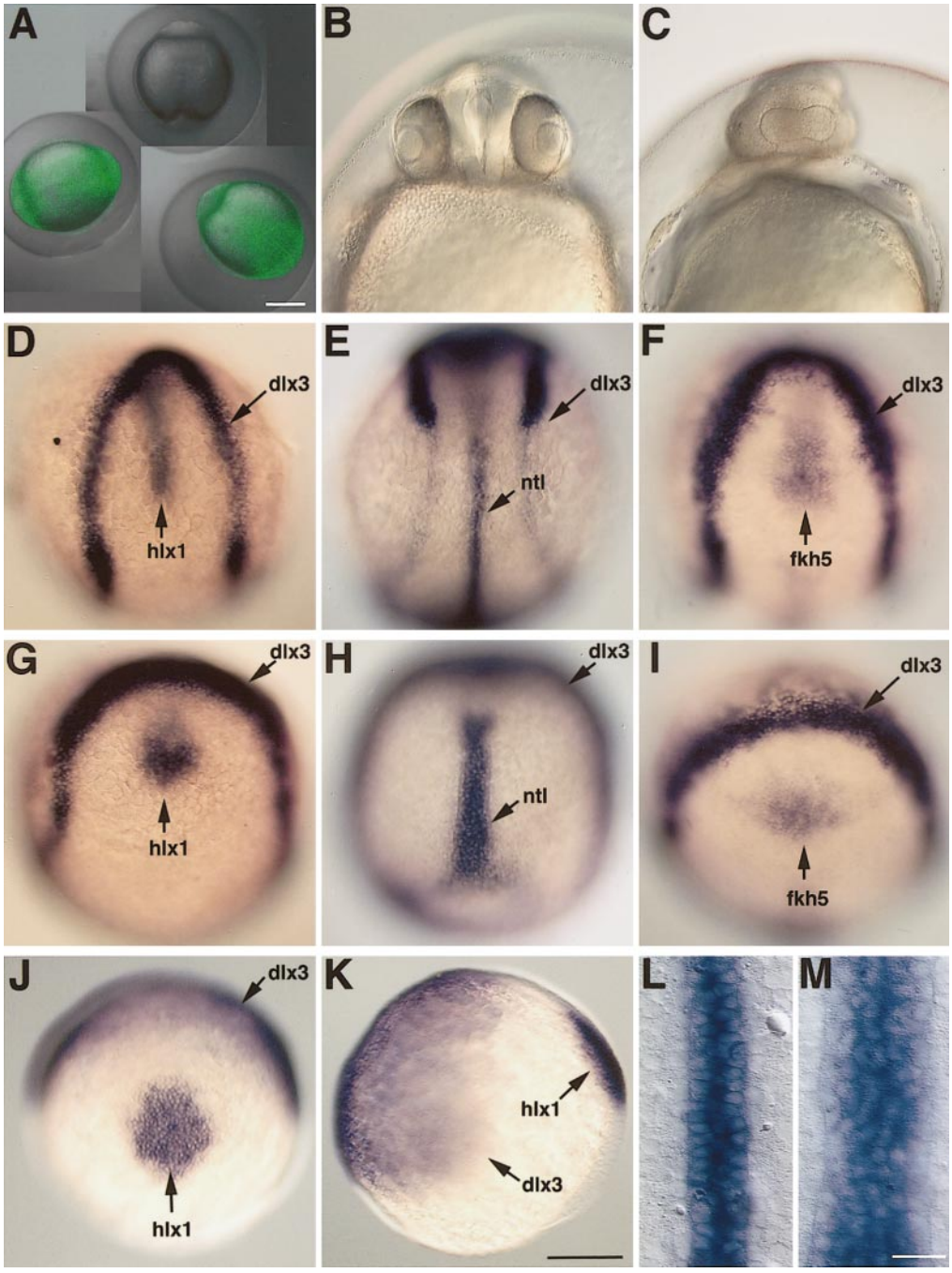

FIG. 6. Overexpression of *slit2* at the late gastrula stage suppressed the convergent extension of the mesoderm. (A) The transgenic embryos (green) overexpressing the Slit2/GFP fusion protein and a wild-type sibling (dark) at 12 h which had been heat-shocked at 100% epiboly (10 h). (B, C) The *slit2*-overexpressing transgenic embryo which had been heat shocked at 80% epiboly suffered from severe cyclopia at 28 h. Frontal views of the wild-type sibling (B) and the *slit2*-overexpressing transgenic embryo (C). (D–I) Heat-shock induction of Slit2 at 80% epiboly impaired the convergent extension movement of the mesoderm and the anterior migration of the diencephalic cells. dorsal views, anterior to the top. The positions of the axial cells were examined by comparing the expression of axial mesoderm markers, *hlx1* (D, G) and *ntl* (E, H), and the ventral forebrain marker, *fkh5* (F, I), relative to the marker for anterior neural plate margin, *dlx3* (D–I). Compared to the wild-type siblings (D–F), the transgenic embryos (G–I) overexpressing Slit2 by heat shock at 80% epiboly showed impaired convergent extension of the mesoderm and defect in the anterior migration of the diencephalic cells. (J, K) Normal embryos at 80% epiboly for comparisons. (L, M) Close-up views of the notochord of the normal embryo shown in E and the heat-shocked transgenic embryo shown in H, respectively. Bars, 250 μm (A, B–K), 50 μm (L and M).

TABLE 1
Frequency of Cyclopia and Flattened Notochord by Overexpression of Zebrafish *slit2* and *slit3* mRNA in 48-h Embryos

Type of injected mRNA (concentration)	Total number of injected embryos	Normal	Cyclopia with flattened notochord	Normal eyes with flattened notochord	Dead
slit2 (0.5 mg/ml)	69	26 (37.7%)	19 (27.5%)	12 (17.4%)	12 (17.4%)
slit2 (0.8 mg/ml)	119	6 (5%)	66 (55.5%)	34 (28.6%)	13 (10.1%)
slit3 (1.0 mg/ml)	96	87 (90.6%)	1 (1%)	3 (3.1%)	5 (5.2%)
slit3 (2.0 mg/ml)	124	91 (73.4%)	14 (11.3%)	10 (8.1%)	9 (7.2%)

the heat-shocked transgenic embryos suffered from severe cyclopia (Fig. 6C). Similar defects were induced by heat shock at 90% epiboly, and heat shock near the completion of gastrulation (95% epiboly) caused only mild cyclopia (data not shown). However, subsequent heat shock caused no abnormality in eye morphology.

These results indicate that the convergent extension movement is susceptible to transient overexpression of Slit2 until near the end of gastrulation. The impairment of the convergent extension movement in the heat-shocked transgenic embryos was further supported by the observation that the notochord of normal embryos appeared like a column of compactly piled square bricks, while the notochord of the heat-shocked transgenic embryos appeared like a pavement covered with randomly distributed round cobblestones (Figs. 6L and 6M).

Localized Ectopic Expression of Slit2 Impairs the Mesodermal Movement

To identify the types of cells whose migratory behaviors are affected by Slit2 expression during gastrulation, we misexpressed Slit2 in restricted subpopulations of embryonic cells by injecting a mixture of *gfp* and *slit2* mRNA into an arbitrarily chosen single cell within 16- to 64-cell stage embryos. The embryos were fixed at 10 h and stained by immunohistochemistry for GFP and *in situ* hybridization for *hlx1* and *dlx3*.

When the GFP-positive cells were broadly distributed in the anterior region of the neural plate, the *hlx1*-positive cells of the prechordal mesoderm failed to reach the anterior margin of neural plate which was demarcated by *dlx3* expression (Fig. 7B; n = 3). When the GFP-positive cells were incorporated on one side of the neural plate and the adjacent ectoderm, the *hlx1*-positive cells were bent toward the same side (Fig. 7C; n = 17), indicating unilateral retardation in the convergent extension movement of the nonaxial mesoderm on the side overexpressing Slit2.

These results revealed that the migratory movement of both the prechordal mesoderm and the nonaxial mesoderm are impaired by ectopic expression of Slit2.

DISCUSSION

We cloned two cDNAs encoding zebrafish Slit2 and Slit3 which have the closest similarity to human Slit2 and Slit3, respectively. Structural comparison has shown that the *Slit* gene family is highly conserved between *Drosophila* and vertebrates. Like *Drosophila slit*, which is expressed in the midline glial cells, zebrafish *slit2* and *slit3* mRNA are expressed in the floor plate cells of the ventral midline of the neuroectoderm. In mouse and rat, *Slit2* was expressed during CNS development in the floor plate, roof plate, and developing motor neurons (Holmes *et al*., 1998; Brose *et al.*, 1999; Wang *et al.*, 1999), while zebrafish *slit2* is not expressed in the developing motor neurons. During development, the expression of mouse and rat *Slit3* in the motor column was present but reduced, with expression largely restricted to the floor plate (Holmes *et al*., 1998; Brose *et al.*, 1999; Yuan *et al*., 1999), while zebrafish *slit3* was consistently expressed in the motor neurons during zebrafish development. Although the structural conservation strongly suggests the preserved functions of Slit homologues during evolution, the roles and the spatio-temporal expression patterns of the individual subtypes may have shuffled among themselves.

Defective Convergent Extension of the Mesoderm, a Possible Cause of Cyclopia

In the embryo overexpressing *slit2*, the anterior prechordal mesoderm tissue expressing *zfz8b* mRNA was significantly displaced posteriorly, compared to the *dlx3*-positive neural plate margin. The anterior prechordal mesoderm normally stretched beyond the margin of the neural plate. The *hlx1*-positive posterior prechordal mesoderm tissue was also shifted caudally.

In the normal embryo, the single coherent eye field in the

forebrain is split into two (Li *et al*., 1997). This process is accompanied by rostroventral migration of the diencephalic cells near the end of epiboly as revealed by a fate mapping study (Woo and Fraser, 1995) and by the anterior extension along the midline of the *fkh5*-positive region (Fig. 4H). A recent study showed that the ventral diencephalon separates the eye field into two regions, and that Cyclops signaling is responsible for this process (Varga *et al.*, 1999). Such anterior extension was similarly impaired in the *slit2*-overexpressing cyclopic embryo. This is likely to be the cause of the absence of *shh*-positive tissue at the rostral end of the ventral midline of the forebrain and the abnormal accumulation of *shh*-positive cells in the dorsal diencephalic region in the *slit2*-overexpressing embryo. Shh is known to prevent the cells in the eye field from becoming retinal cells and to induce them to become the optic stalk cells (Ekker *et al*., 1995; Macdonald *et al*., 1995). Mice with a disrupted *shh* gene show holoprocencephalic syndrome including cyclopia (Chiang *et al*., 1996).

The ventral midline cells of the forebrain migrate anteriorly and ventrally following migration of the prechordal plate mesoderm in chick embryos (Dale *et al*., 1999), implicating the prechordal plate in the anterior migration of the forebrain cells and the separation of the eye field. Involvement of the anterior prechordal mesoderm in the separation of the eye field has also been confirmed by the observations that physical or genetic removal of the prechordal mesoderm induces cyclopia (Li *et al.*, 1997; Schier *et al*., 1997; Feldman *et al*., 1998; Sampath *et al*., 1998; Rebagliati *et al*., 1998). These data support the view that displacement of the anterior prechordal mesoderm away from the eye field and the accompanying defect in the rostroventral migration of the diencephalic cells are the primary causes of cyclopia in embryos overexpressing *slit2*.

A similar cyclopic phenotype is observed in the mutant embryos with defects in convergent extension, such as the double mutant of the *knypek* (*kny*) and the *trilobite* (*tri*) genes or the *silberblick* (*slb*) mutant (Marlow *et al.*, 1998; Heisenberg *et al.*, 2000). In these embryos, the anterior prechordal mesoderm is also severely shifted caudally.

Implication of Slit2 in Regulation of Convergent Extension Movement of the Mesodermal Cells

We have shown that Slit2 and Slit3 start to be expressed in the axial mesoderm around the midgastrula stage. Furthermore, responses of the mesoderm to ubiquitously or ectopically expressed Slit2 have demonstrated that Slit2 acts negatively on convergent extension movement of the mesoderm. This observation is intriguing especially when we consider the possible role of the axial mesoderm in convergent extension and the recent data which links the Slit-Robo signaling cascade with the regulatory machinery of actin polymerization.

During gastrulation, the axial and paraxial mesoderm undergoes convergent extension and differentiates into the notochord and the somites, respectively. Behaviors of the mesodermal cells in convergent extension movement have been most thoroughly analyzed in *Xenopus laevis* embryos (see review, Keller *et al*., 1992). They take bipolar shapes, extending protrusions medially and laterally, and appear to exert traction on one another, using these protrusions (Keller and Tibbetts, 1989; Shih and Keller, 1992a). As a result, the cells elongate, align parallel to one another and parallel to the mediolateral axis, and intercalate along the mediolateral axis, thus achieving convergent extension. After the notochord-somite boundary is formed, the boundary has been shown to exert a distinctive effect on the mediolateral intercalation behavior of the mesodermal cells (Keller *et al.*, 1989; Shih and Keller, 1992b). Upon contact with the boundary, the bipolar cells change to monopolar activity. The surfaces of the cells impinging on the boundary region become flat and quiescent and make stable filiform protrusions overlapping other boundary cells. Meanwhile, protrusive activity continues at the opposite surfaces of the boundary cells. These changes result in pulling more cells into the boundary region.

Bashaw *et al.* (2000) have recently shown that Ena interacts with Robo both physically and genetically and may at least partly mediate the Slit-Robo signaling (also see review, Machesky, 2000). Mammals also contain a family of Ena-related proteins (the Ena/VASP family), including VASP (vasodilator-stimulated phosphoprotein), EVL (Ena-VASP like), and Mena (Mammalian Enabled). They are thought to play a universal role in the control of cell motility by dynamic regulation of actin polymerization. Overexpression of Ena/VASP slowed cells down to less than half of their wild-type speed, while sequestration of all of the Ena/VASP proteins from the leading edges of the cells accelerates the cells to move faster than the wild-type cells, strongly suggesting that the Ena/VASP proteins regulate cell migration negatively (Bear *et al*., 2000).

The repulsive role of Slit in cell migration has been reported both in fly and vertebrates. Slit also functions as a chemorepellent controlling mesoderm migration away from the midline in *Drosophila melanogaster* (Kidd *et al.*, 1999). Slit also repulses the migrating neurons from the lateral ganglionic eminence, the olfactory bulb, choroid plexus, and the septum in mammals (Wu *et al.*, 1999; Zhu *et al.*, 1999; Hu, 1999).

In zebrafish embryos, the mediolateral intercalation of the mesodermal cells start by 7 h (60–70% epiboly) prior to the morphological emergence of the notochord-somite boundary approximately at 80% epiboly (Warga and Kimmel, 1990; Kimmel *et al*., 1995). Our results in ubiquitous and ectopic overexpression of Slit2 suggest that Slit proteins expressed in the axial mesoderm may be critically involved in the change of the mediolateral intercalation behavior of the mesodermal cells. Overexpression of Slit2 may impair the convergent extension movement of the mesoderm by prematurely or ectopically inducing the behavioral change to the mediolaterally intercalating meso-

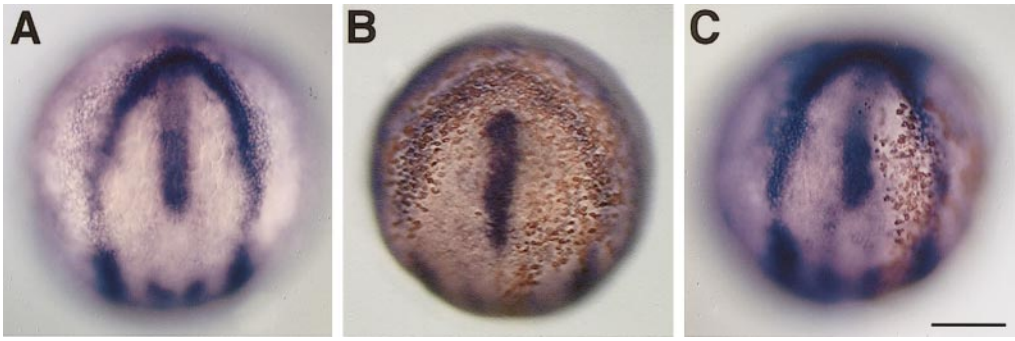

FIG. 7. Localized ectopic expression of Slit2 impairs the mesodermal movement. The embryos expressing *slit2* and *gfp* mRNA in a mosaic pattern were fixed at 10 h and stained for *hlx1* and *dlx3* expression by *in situ* hybridization and for GFP by immunohistochemistry. Dorsal views, anterior to the top. (A) Control embryo. (B) The embryos (n = 3) in which the GFP-positive cells were broadly distributed in the anterior region of the neural plate. The *hlx1*-positive cells of the prechordal mesoderm failed to reach the anterior margin of neural plate. (C) The embryos (n = 17) in which the GFP-positive cells were incorporated on one side of the neural plate and the adjacent ectoderm. The *hlx1*-positive cells were bent toward the side which incorporated the GFP-positive cells. Bar, 250 μm.

dermal cells which should normally be induced only upon their contact with the notochord-somite boundary.

Several zebrafish mutants of the convergent extension movement of the mesoderm have been reported, such as the double mutant of the *knypek* (*kny*) and the *trilobite* (*tri*) genes or the *silberblick* (*slb*) mutant (Marlow *et al.*, 1998; Heisenberg *et al.*, 2000). The identification of the *silberblick* locus as the *wnt11* gene has elucidated the role of Wnt11 in driving the convergent extension movement during gastrulation by activating a similar signaling cascade as the noncanonical *wingless* signaling cascade for the control of the cellular polarity in *Drosophila* (Heisenberg *et al.*, 2000). Implication of small GTPase such as RhoA and Rac1 in this signaling cascade, which are also involved in the control of cell and growth cone motility, suggests that the morphogenetic signaling cascade of Wnt and the Slit-Robo signaling cascade may interact with each other (Axelrod *et al.*, 1998; also see reviews, Lin and Greenberg, 2000; McEwen and Peifer, 2000).

Although we have localized *slit2* mRNA in the axial mesoderm, we have not examined whether Slit2 protein is concentrated at the boundary between the axial and paraxial mesoderm. Further study on the localization of Slit2 protein is hence essential to examine the validity of our hypothesis. Recently, it has been suggested that proteins in the Slit family interact with Glypican-1, a member of a family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans (Liang *et al.*, 1999). Other heparan sulfate proteoglycans secreted by the axial mesoderm into the surrounding extracellular matrix may function to trap Slit proteins at the notochord-somite boundary.

The homologues of Slit are also expressed in the axial mesoderm in the late gastrula embryos of various other species than zebrafish such as *Xenopus laevis* and chick (Chen *et al.*, 2000; Li *et al.*, 1999), where convergent extension of the mesoderm has been thoroughly studied. Use of such animals may facilitate our understanding of the role of Slit in regulation of convergent extension at the cellular and molecular level.

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