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Slit-like 2, a novel zebrafish slit homologue that might involve in zebrafish central neural and vascular morphogenesis

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Abstract

Nervous and vascular systems grow as parallel networks, indicating common cues in distal targets. We have identified a novel zebrafish gene slit-like 2 (slitl2) that might involve in zebrafish central neural and vascular morphogenesis. Whole-mount in situ hybridization of zebrafish embryo detected distinct signals of slitl2 transcripts in zebrafish midline structure of central nervous system similar to that of slits. Strong expression is also observed in zebrafish vasculature. Zebrafish slitl2 shares amino acid sequence identity of 41% with *Homo sapiens* slitl2 (vasorin) and *Mus musculus* slitl2, and 35%, 33% with *Danio rerio* slit3, slit2. Analysis of zebrafish slitl2 cripto growth factor domain, extracellular matrix protein slit domain, and putative signal peptide confirms that as a secreted and cell-surface protein slitl2 may be essential in axon guidance, vessel development, and axis patterning. These results provide evidence that slitl2 may play important roles in zebrafish central nervous system and vascular morphogenesis.

Keywords: Slit-like 2; Slit; Vasorin; Zebrafish (*Danio rerio*); Whole-mount in situ hybridization

Nervous and vessel systems often follow parallel ways, which suggests that common cues exist in distal targets to induce vascularization and innervation [1].

The Slit gene, encoding a 170–190 kDa secreted protein [2], expresses in the commissural and longitudinal axon tracts from the midline glia [3]. Slit genes form an evolutionary conserved group in vertebrates and invertebrates from *Drosophila* [4], mammalian [5] to zebrafish [6]. It contains conserved protein–protein interaction domains including four leucine-rich repeats (flank-LRR-flank), seven to nine EGF-like repeats [3] divided by insertion of an agrin-laminin-perlecan-slit (ALPS) motif [7], and a cysteine-rich carboxy-terminal domain. Slit is a pivotal component in central neural system midline formation and axon pathfinding. It acts as a chemorepellent for growth cone navigation away from the midline [8] during convergent extension movement [9], thus preventing the commissural axons from crossing the midline multiple times. By contrast, it also promotes branching and elongation of sensory axons [10]. Slit acts negatively on the axons from the olfactory bulb, the dentate gyrus in the hippocampus, lateral ganglionic eminence, choroid plexus, the septum, and spinal motor neurons [11]. And it functions as a ligand for the Roundabout (Robo) [2], the repulsive transmembrane receptor expressing on the growth cones of the commissural neurons. Robo4 is expressed in the vascular endothelium during murine embryonic development and may provide a repulsive cue to migrating endothelial cells during vascular development [12]. Robo4 is also found to express in embryonic zebrafish vasculature and is essential for coordinated symmetric and directed sprouting of intersomitic vessels [13].

The *Homo sapiens* homologue of slit, slitl2 (Vasorin), is a TGF-β binding protein predominantly expressing in vascular smooth muscle cells (VSMCs) that generate extracellular

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matrix proteins. TGF-β plays an important role in vascular pathophysiology, involves in differentiation, extracellular matrix accumulation, immune system, cell signaling, cardiovascular disorders, and wound repair responds [14]. TGF-β1 signaling contributes to the development of yolk sac vascular and smooth muscle cells from embryonic stem cells [15]. The expression of vasorin increases gradually in parallel with the differentiation of VSMCs in aortas, kidney, and placenta [16]. As a secreted and cell-surface typical type I membrane protein, vasorin modulates the vascular response to injury by attenuating TGF signaling in vivo and plays crucial roles in vascular cells growth, migration, differentiation, and survival. Vasorin is composed of a putative hydrophobic signal sequence, 10 tandem arrays of a characteristic leucine-rich repeat motif, an epidermal growth factor-like motif, and a fibronectin type III-like motif at the extracellular domain [16].

Although slit was well known to express in zebrafish midline of central nervous system, slit-like gene and its expression pattern during zebrafish embryogenesis have never been reported. In this paper, we report the identification, functional characterization, and expression of a zebrafish slit-like 2 (slitl2) gene that might involve in zebrafish central neural and vascular development. Since the zebrafish embryos are optically transparent with externally fertilization, this system may cast light on the function of slitl2.

Materials and methods

Zebrafish and embryo maintenance. Zebrafish were raised and maintained under standard laboratory conditions at 28 °C, as described by Westerfield [17]. To facilitate visualization of RNA during in situ hybridization, 0.003% phenylthiourea (PTU) (Sigma) was added to the collected embryos before 24 h post-fertilization (hpf) to block pigment formation. The embryo stages were identified by morphological features and the corresponding embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) as required [18].

Cloning of zebrafish slitl2 cDNAs. A zebrafish cDNA clone RK080A2H04 containing part of slitl2 open-reading frame (ORF) was isolated from the zebrafish adult kidney marrow cDNA library by large-scale cDNA sequencing. The library was provided by Zon, L.I. lab and was large-scale sequenced by Song et al. [19]. Total zebrafish RNA was extracted from the kidney tissues of mature zebrafish. The poly(A)$^+$ RNA fraction was separated from total RNA by oligo(dT) cellulose chromatography. The zebrafish cDNA library was initially constructed in the lambda ZAP Express vector (Stratagene) and in vivo excised into pBK-CMV expression vector with two inserted enzyme sites *Eco*RI and *Xho*I, respectively.

The remaining part of the slitl2 ORF was predicted by the expressed sequence tag (EST) (CK 698091) on National Center for Biotechnology Information (NCBI) blast server 2.0 (http://www.ncbi.nlm.nih.gov), and was identified by the rapid amplification of cDNA end 5′UTR (5′ RACE) with its upper primer from Clontech smart cDNA Library (www.clontech.com) and lower primer chemically synthesized. Their sequences were 5′-aagcagtggtatcaacgcagagt-3′ and 5′-gcctttctaggttaacaagtccaatg-3′, respectively. PCR amplification proceeds with single-strand cDNA as template, which is the reverse transcript from 36 hpf embryo mRNA. A total of 30 cycles proceeded at an annealing temperature of 68 °C. The amplified product was cloned into the pGEM-T Easy vector (Promega).

To confirm the full-length ORF of slitl2 cDNA, PCR amplification with its upper primer 5′-attttgtacagggattgatcac-3′ and lower primer 5′-accaacccaacagagtctatcta-3′ was carried out. PCR amplification proceeds with single-strand cDNA reverse-transcribed from 36 hpf embryos mRNA as template. The PCR proceeds for 30 cycles (annealing temperature 60 °C). The amplified product was cloned into the pGEM-T Easy vector (Promega).

Sequencing and Bioinformatics analysis of zebrafish slitl2. After sequencing, the zebrafish slitl2 nucleotide sequence, exon–intron position, amino acid sequence, and conserved domains were analyzed with NCBI blast server 2.0 and Sanger zebrafish genomic sequence project database (http://www.sanger.ac.uk/Projects/D_rerio/blast-server). Signal peptide was predicted with SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP-2.0) [20]. Multiple alignments and phylogenetic trees were performed with clustalw version 1.82 (http://www.ebi.ac.uk/clustalw/) and the graphics were presented with GeneDoc software.

Whole-mount in situ hybridization. To study the expression pattern of zebrafish slitl2 gene during embryogenesis, whole-mount in situ hybridization procedure was carried out as described by Dongwang Wei [21]. Zebrafish embryos of different stages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight and washed in PBS containing 0.1% Tween 20 (PBST) after dehydration, rehydration, and Proteinase K (Sigma) digestion. Embryos were hybridized with 1 ng/μl probe, 50 μg/ml heparin, 0.5 mg/ml yeast RNA overnight at 68 °C, blocked for 1 h with 10% lamb serum (Sigma), and incubated overnight at 4 °C in 1/5000 diluted Anti-digoxigenin-AP Fab fragments (Roche). Chromogenic reaction was performed with BM Purple AP Subctrate (Roche) and levamisol (Sigma).

The zebrafish cDNA clone RK080A2H04 was used as the template for synthesizing antisense probes. By using the T7 RNA polymerase, the antisense RNA probes were synthesized in vitro with T7 promoters in pBK-CMV expression vector, and labeled with digoxigenin (Roche Diagnostics).

Digital images of all embryos were captured using a differential interference contrast microscope (Nikon) and a digital camera (Olympus).

Results and discussion

Isolation of zebrafish slitl2 cDNA sequence

Large-scale cDNA sequencing isolated 2.4 kb zebrafish cDNA clone RK080A2H04 containing 1.7 kb portion of slitl2 ORF from the zebrafish adult kidney marrow cDNA library.

Predicted by EST on NCBI (CK 698091), the remaining part of the slitl2 ORF was identified by 5′ RACE. The

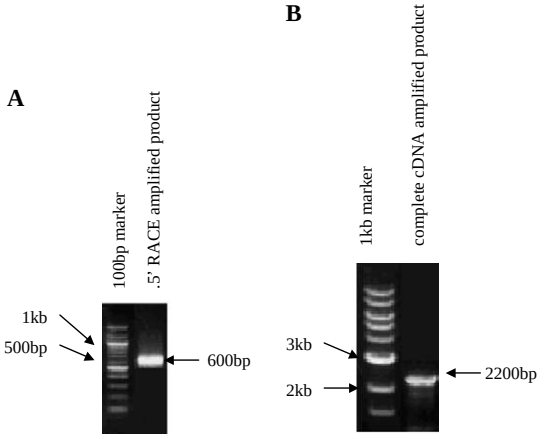

Fig. 1. (A) 5′ RACE amplified product of 600 bp, fragment containing part of 5′ ORF of zebrafish slitl2. (B) PCR amplified product of 2200 bp, fragment containing complete ORF of zebrafish slitl2.

resulting 600 bp fragment which contains 400 bp portion of slitl2 ORF was resolved on 1.0% agarose gel (Fig. 1A).

The full-length ORF of slitl2 cDNA was confirmed by PCR amplification. The resulting 2200 bp fragment containing 2067 bp full-length slitl2 ORF was resolved on 1.0% agarose gel (Fig. 1B). Sequence analysis revealed that its 5′ and 3′ORF regions are complete. The sequences above added up to 3001 bp, which is the full-length of zebrafish slitl2 cDNA.

We named the gene zebrafish slit-like 2 (slitl2) in agreement with HUGO Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature).

```
1    ggacgcaacctatacgcaccaggagactccgtatataacagtgatgtgttaaagcttgcattcaggagaaatgattcctgcttaaggaat
91   gaagtagcttttaagcgttgtctcttgtggataaaacaacgcgcgcttgcttgacgtttcgtattttgtacagggattgatcacaaaaga
181  aaaaagacaccaattgaca

200  atgcggccattatctcccctgtctcatctcattttgcttcagttactttgtgggtgcctgagcagcagatgtcctcatgattgcacatgc
       M  R  P  L  S  P  L  S  H  L  I  L  L  Q  L  L  C  G  C  L  S  S  R  C  P  H  D  C  T  C
290  ctccccagtaataacatcttctgcgtccagaggaacttgatctacatgcctcgtggccttccgtccactggaaaacagctttatgtcttt
       L  P  S  N  N  I  F  C  V  Q  R  N  L  I  Y  M  P  R  G  L  P  S  T  G  K  Q  L  Y  V  F
380  cagaacaaaatcaatatcttgcaacagcaggactttgtggagcttggcgaacttgaaatgttggacttaagtcagaattccctcagtgaa
       Q  N  K  I  N  I  L  Q  Q  Q  D  F  V  E  L  G  E  L  E  M  L  D  L  S  Q  N  S  L  S  E
470  atccccgatggtgtattcagcccactctcctctctacacaatctagatctctcctcaaactacattactcacatttccaaagacagtttc
       I  P  D  G  V  F  S  P  L  S  S  L  H  N  L  D  L  S  S  N  Y  I  T  H  I  S  K  D  S  F
560  attggacttgttaacctagaaaggctgtatctctacagtaacatcatccagaacattcacccagctgcctttgagggactggagaacctt
       I  G  L  V  N  L  E  R  L  Y  L  Y  S  N  I  I  Q  N  I  H  P  A  A  F  E  G  L  E  N  L
650  ttggagctgaagcttcaggggaatcagattagtgtgttgcctgctctgcagctgcccagactgctccacttggaccttagttatataatagt
       L  E  L  K  L  Q  G  N  Q  I  S  V  L  P  A  L  Q  L  P  R  L  L  H  L  D  L  S  Y  N  S
740  atcccacctcttgtagctcaagatttacagacaccacatcttgaatctcttaaaatagctggattggggctgaccagtttggatgaggag
       I  P  P  L  V  A  Q  D  L  Q  T  P  H  L  E  S  L  K  I  A  G  L  G  L  T  S  L  D  E  E
830  ctgttaggcagcctagtaaatctgcatgttctcgatgtttctcagaaccagcttgtagatatacaacctacactaaagtcaatgggagga
       L  L  G  S  L  V  N  L  H  V  L  D  V  S  Q  N  Q  L  V  D  I  Q  P  T  L  K  S  M  G  G
920  ctacgaaaccttaatttaactggcaacccttgggatctctgaaacatgaagacttccaaaatttggtcaatctccttgagcttgattta
       L  R  N  L  N  L  T  G  N  P  L  G  S  L  K  H  E  D  F  Q  N  L  V  N  L  L  E  L  D  L
1010 agcaaccttaatttgcaaggcttccctgaaggattcttcaacctttccccaaacttgaaaagctcactgcagctgaaaaccccttttaac
       S  N  L  N  L  Q  G  F  P  E  G  F  F  N  L  F  P  K  L  E  K  L  T  A  A  E  N  P  F  N
1100 tgcctctgcccactagcctggttcccagcatggctaaaagatgtacgtgtggagctgttgaggacggaggagacctcgctgccactttccg
       C  L  C  P  L  A  W  F  P  A  W  L  K  D  V  R  V  E  L  L  R  T  E  E  T  R  C  H  F  P
1190 ccaataaactcaggaaaggttttggaaaagttggaacacaaagattttggctgtcccacaacaaccattgagctaacaagtgcagggaca
       P  I  N  S  G  K  V  L  E  K  L  E  H  K  D  F  G  C  P  T  T  T  I  E  L  T  S  A  G  T
1280 agtagtaccacaagcaaacctaaaaactcctcgacacaattaggcacaacacacatcgttcctccagcaccaccaagtgacatatcctca
       S  S  T  T  S  K  P  K  N  S  S  T  Q  L  G  T  T  H  I  V  P  P  A  P  P  S  D  I  S  S
1370 gcagacgcagacaacttcccagtttatcagaccactgctttccccagcagaatcatggaagactctgaaggagaaggaattatgtgccct
       A  D  A  D  N  F  P  V  Y  Q  T  T  A  F  P  S  R  I  M  E  D  S  E  G  E  G  I  M  C  P
1460 cccaatatctgtctaaatggaggaacatgcatatttaagtcaaatggggtgattgtttgcttgtgtccaccttcaatgtcaggaaactac
       P  N  I  C  L  N  G  G  T  C  I  F  K  S  N  G  V  I  V  C  L  C  P  P  S  M  S  G  N  Y
1550 tgtgagattcagaacgaagctatgcttccccgccatcaccaagagtttctctagagaccatcgctacagtccagcccaacacaatcagt
       C  E  I  Q  N  E  A  M  L  P  P  P  S  P  R  V  S  L  E  T  I  A  T  V  Q  P  N  T  I  S
1640 tcccatcacataaccagcacttccatttccttagaccttcatcgttacatacaaacaagaccacacatacgtggaatccgtctgacgtac
       S  H  H  I  T  S  T  S  I  S  L  D  L  H  R  Y  I  Q  T  R  P  H  I  R  G  I  R  L  T  Y
1730 agtaacttgtcaggtcctgaccgccgaccattacagctgagtgtgcctccaagttaccctgaatacacacttagagggttacaaccaaac
       S  N  L  S  G  P  D  R  R  P  L  Q  L  S  V  P  P  S  Y  P  E  Y  T  L  R  G  L  Q  P  N
1820 agcacttattctgtatgtgctagccctctaggagagcctgtccatgcctcggttagtgcctgcatggaggctcgcacagcaggaatccca
       S  T  Y  S  V  C  A  S  P  L  G  E  P  V  H  A  S  V  S  A  C  M  E  A  R  T  A  G  I  P
1910 ccctcctcccatgagcccagcgttgacaggaccgaaccttcttcctctcttacacccattgtggtagcggtggcagtggtgatggtggtt
       P  S  S  H  E  P  S  V  D  R  T  E  P  S  S  S  L  T  P  I  V  V  A  V  A  V  V  M  V  V
2000 gccataatagctactgtggttgtcatcagccgcaggagaagacccaaggctcctgtagacatggacttacatgagacatctcctttagag
       A  I  I  A  T  V  V  V  I  S  R  R  R  R  P  K  A  P  V  D  M  D  L  H  E  T  S  P  L  E
2090 atggaaggagtgaaaaccaacccagagaatggactgacacatccaaaacaacctgacatcacacttgctcatcattagcaccaaacagc
       M  E  G  V  K  T  N  P  E  N  G  L  T  H  P  K  Q  P  D  I  T  P  C  S  S  L  A  P  N  S
2180 ttggagtatgatgtactttaatacaaggacaaggacagtgtccagccaacaacaatatagattgtacaaaagctttatatgtgtaa 2266
       L  E  Y  D  V  L  L  I  Q  G  Q  G  Q  C  P  A  N  N  N  I  D  C  T  K  A  L  Y  V  *

2267 aagctctattgcgcaattcatacctcgagctcacagagggcaatgtgctccgtgactcctgcaggcgggtgtgatgcagacactagatag
2357 actctgttgggttggtgaacatttaccgcatcccagccaaacgcttaaactgactgccagtatttaaaccagatgcatttgccatcttt
2447 acctttttacagatacaacttaagtgcctttatagccatagttatcagagaatttctctcttaaaaggtggccagacattatgcagtac
2537 acagtgttgctagcctggaaaacaggtcgctttaaaggtagctggctctggaactggtagacagaagtcagaaattgtgacctggacgcc
2627 tggactgttttgttgtccagaaaaaaaaaattgcactgatcagagacactgggactgtgcctaaaagaggaactgaaagtgttcagtctc
2717 ttagaagatgcttcaaataggtcagcatcaaagcatcccactgtccctctaccccatctatcctgcacgggagattatttgctgtgaaaa
2807 acacaatgaagagacatttttttttactttgtctagtttactctgttttagctatgtaattataatgttttttgttttgttttttgtattt
2897 ttaaagtaaattgtttaacttggagattaattctgttgtttactcactctcaaagtgttcaagaactgtgtgaatctgtattaaagaatg
2978 tcaaacaccaaaaaa  3001
```

Fig. 2. Nucleotide and deduced amino acid sequences of zebrafish slitl2. The amino acid sequence is shown below the nucleotide sequence in single letter code. The translation initial and stop codons are marked as bolded M and asterisk (*), respectively. The in-frame stop condon and the poly(A) signature are marked with underlines. The extracellular matrix protein slit domain and the Cripto growth factor domain are marked in shade, respectively. The signal peptide is marked in-frame.

Exon				Splicing acceptor	Splicing donor	Intron
No.	Length (bp)	Genomic position	cDNA position			Length (bp)
1	163	36632-36794	1-163		TTTGTACAG**gt**aagcagtg	26513
2	2832	63306-66137	164-2995	ttttctattgc**ag**GGATTGA		

Fig. 3. Exon–intron analysis of zebrafish slitl2 gene. Intron and exon nucleotide sequences are shown in lowercase and uppercase letters, respectively. Bold letters indicate donor and acceptor splice sites.

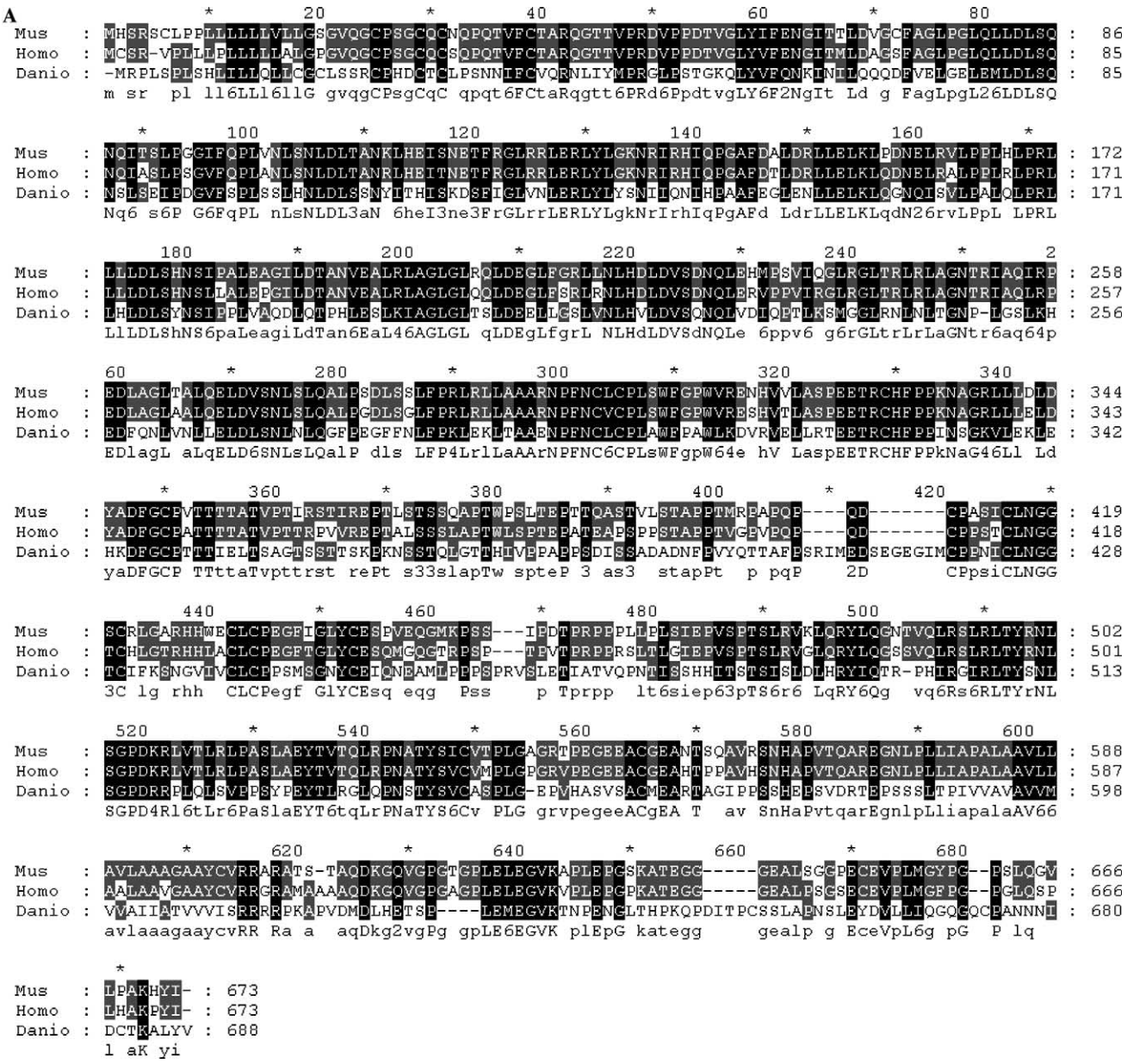

Fig. 4. (A) Alignments of *M. musculus* slit-like 2, *H. sapiens* Slit-like 2 (vasorin), and *D. rerio* slit-like 2. Amino acids are shaded according to the degree of conservation using GeneDoc: black (100% similarity), gray (80% similarity), and light gray (60% similarity). The amino acid sequences of *M. musculus* slit-like 2 and *H. sapiens* slit-like 2 (vasorin) were obtained from the GenBank database with Accession Nos. NP_647468 and AAO27704. (B) Alignments of *D. rerio* slit-like 2, slit2, and slit3. Amino acids are shaded according to the degree of conservation using GeneDoc: black (100% similarity), gray (80% similarity), and light gray (60% similarity). The amino acid sequences of *D. rerio* slit2, slit3 were obtained from the GenBank database with Accession Nos. AF210321.1 and AF210320.1.

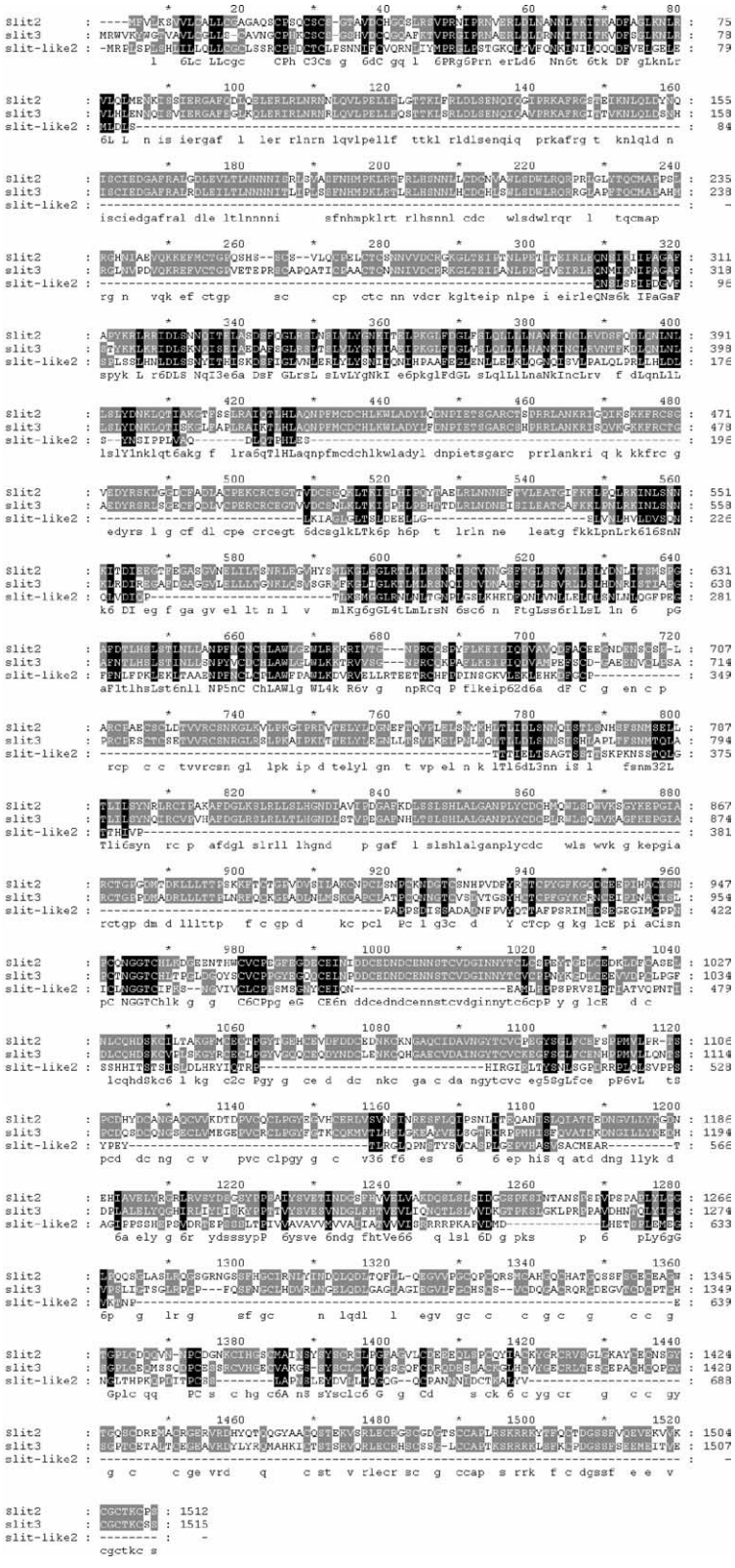

Fig. 4. (*continued*)

Nucleotide sequence analysis of zebrafish slitl2

The 3001 bp zebrafish slitl2 cDNA spans an open-reading frame (ORF) from nucleotide 201–2267, encoding a putative protein of 688 amino acids (GenBank Accession No. AY838878). The calculated molecular weight and isoelectric point of the deduced protein are predicted to be 75.3 and 5.55 kDa, respectively.

An in-frame stop condon TAA presents at nucleotide 122–124, the initial ATG conforms to Kozak's consenses sequence, and a poly(A) signature ATTAAA locates at the 3′ terminus from nucleotide 2976–2981. These data suggest that the nucleotide sequence is a full-length cDNA. The zebrafish slitl2 nucleotide sequence and deduced amino acid sequence are shown in Fig. 2.

Sequence blast in Sanger zebrafish genomic sequence project database shows that zebrafish slitl2 genomic sequence spans 29.5 kb, containing two exons and one intron. Its first exon is as large as 163 bp, the translation initial codon ATG is located at the second exon and its intron spans 26.5 kb. The putative splice junctions conform to donor/acceptor consensus sequences (Fig. 3).

Amino acid sequence analysis of zebrafish slitl2

Analyzing zebrafish slitl2 deduced product with NCBI blast server 2.0 and the clustalw version 1.82 online, we found that zebrafish slitl2 protein is conserved sharing 41% identity in amino acid sequence with *H. sapiens* slitl2 (vasorin) (GenBank Accession No. AAO27704), *Mus musculus* slit-like 2 (GenBank Accession No. NP_647468), and 35%, 33% with *Danio rerio* slit3 (GenBank Accession No. AF210320.1), slit2 (GenBank Accession No. AF210321.1) (Figs. 4A and B).

In addition, a phylogenetic tree was constructed by comparison with the amino acid sequences of proteins above (Fig. 5). Evolutionary distances were calculated using the UPGMA algorithm. The lengths of the lines are proportional to the evolutionary distances from branch points. The result shows that *H. sapiens* slitl2 (vasorin) shares the closest evolutionary relationship with zebrafish slitl2, next is *M. musculus* slitl2, *D. rerio* slit2, and *D. rerio* slit3.

To figure out the structural features of the zebrafish slitl2 protein, two of its conserved domains were analyzed with NCBI blast server 2.0 Conserved Domain Database (Fig. 2):

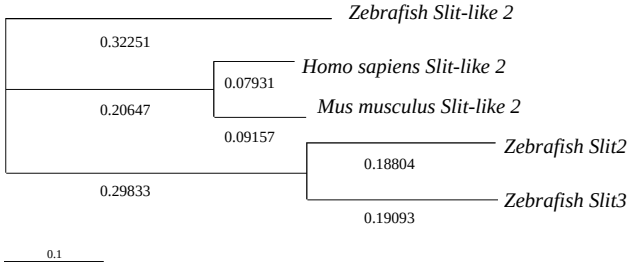

Fig. 5. Phylogenetic tree of evolutionary relationship between *D. rerio*, slit-like 2, *H. sapiens* slit-like 2, (vasorin), *M. musculus* slit-like 2, *D. rerio* slit2, and *D. rerio* slit3 proteins. The sequences are available under Accession Nos. AY838878 (*D. rerio* slit-like 2), AAO27704 (*H. sapiens* slit-like 2, vasorin), NP_647468 (*M. musculus* slit-like 2), AF210320.1 (*D. rerio* slit3), and AF210321.1 (*D. rerio* slit2). Evolutionary distances were calculated using the UPGMA algorithm. The lengths of the lines are proportional to the evolutionary distances from branch points (numbers indicated below each line).

Cripto growth factor domain is located from amino acid sequence 422–452. Cripto growth factor gene is responsible for left–right axis formation. All vertebrates display a characteristic asymmetry of internal organs with the cardiac apex, stomach and spleen towards the left, and the liver and gall bladder on the right, and this domain is conserved from fish to humans [22]. Left–right (L–R) axis abnormalities or laterality defects also affect the cardiovascular system. Absence of cripto results in a defective precardiac mesoderm, unable to differentiate into functional cardiomyocytes [23].

Extracellular matrix protein slit domain, which contains leucine-rich and EGF-like repeats, is located from amino acid sequence 25–348. Extracellular matrix protein is essential for extracellular structures and signal transduction mechanisms, and plays critical roles in cell migration and differentiation.

By using predictive algorithms in SignalP 3.0 Server, we demonstrate that zebrafish slitl2 protein includes a signal peptide from amino acid sequence 1–22 (Fig. 2).

Leucine-rich repeat domain (LRR) and Epidermal growth factor-like domain (EGF) domains are characteristic motifs involved in protein–protein interactions [24], and can be found among secreted and cell-surface molecules, such as extracellular matrix proteins and adhesion molecules. These domains are also found in zebrafish slitl2, which indicates that zebrafish slitl2 might be a secreted protein that is likely bound to the cell surface by interactions with extracellular proteins.

These structure patterns are consistent with those of the slit-like genes and slits, which may suggest that they have similar developmental regulated means.

All these indicate that as a secreted and cell-surface protein, zebrafish slitl2 may execute axon formation and vascularization during zebrafish embryo development, and may be released from midline cells and serve to regulate the expression of other genes in vascularization and innervation.

Spatiotemporal expression pattern of zebrafish slitl2 gene

To analyze the spatiotemporal expression of slitl2 transcripts during early embryonic development, we performed whole-mount in situ hybridization on zebrafish embryos using digoxigenin-labeled riboprobe.

Whole-mount in situ hybridization results showed that the slitl2 transcripts were detected in the germ ring by the midgastrula stage before the axonogenesis and vascularization take place. A lateral view reveals that the shield, which later forms the dorsal–ventral axial hypoblast, is locally thickened. (Fig. 6A) The position along the dorsal–ventral axis of the gastrula generally corresponds to a later position along the anterior–posterior axis of the pharyngula [17]. The stained cells migrate to the polster with the epiboly movement and along with the prechordal plate up to the tail bud. Expression emerged strong at the six-somite stage with obvious convergence towards the dorsal midline,

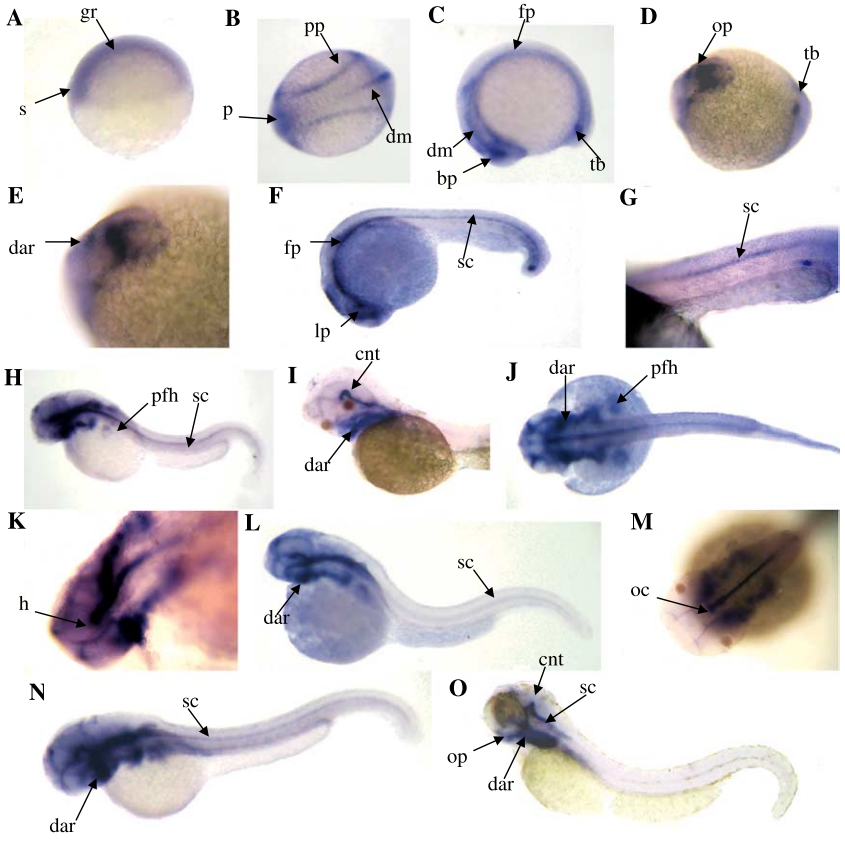

Fig. 6. Expression patterns of slitl2 in zebrafish embryo. Lateral view (A, C–I, K, L, N, and O), dorsal view (B, J, and M), anterior to left. (E,G) are close-up views of (D, F, and K) (M) are close-up views in the head region. (A) Shield stage; (B, C, and D) (E) 6-somite stage; (F,G) 26-somite stage; (H,I) prim-22 stage; (J,K) long-pec stage; (L,M) pec-fin stage; (N,O) protruding-mouth stage. *Abbreviations:* s, shield; gr, germ ring; p, ploster; pp, prechordal plate; dm, dorsal midline; tb, tail bud; bp, brain primordium; fp, floor plate; op, optic primordial; dar, dorsal aortic root; sc, spinal cord; lp, len primordial; pfh, pectoral fin hold; cnt, central neural tube; h, hypothalamus; and oc, optic chiasm.

which sustained throughout all the subsequent developmental stages. The adjacent signals along the midline can also be discerned, such as in brain primordium, optic primordia; dorsal aortic roots, floor plate, and tail bud (Figs. 6B–E). At 26-somite, the stained spinal chord becomes morphologically distinctive. The signals in len primordial appeared together with the strengthening of brain primordium signals. (Figs. 6F and G) Signals in pectoral fin hold were made visible from prim-22 stage to protruding-mouth stage, which might indicate the capillary vessel in pectoral fin hold. In prim-22 stage, expression of slitl2 mRNA is also detected in the central neural tube and dorsal aortic roots. (Figs. 6H and I) The long-pec stage, pec-fin stage, and protruding-mouth stage showed detailed signals in the central neural system, just like optic chiasm and hypothalamus. (Figs. 6J–O).

Result of the whole-mount in situ hybridization provides evidence that the zebrasfish slitl2 protein expresses developmentally in zebrafish central neural system, from dorsal midline, tail bud, brain primordium, and optic primordia to spinal chord, hypothalamus, and optic chiasm. Slitl2 also expresses in vascular system such as dorsal aortic roots and capillary vessel in pectoral fin hold.

Since the zebrafish slit2 expresses in hypothalamus of the forebrain, optic commissure, and floor plate, zebrafish slit3 in spinal cord, floor plate cells of hindbrain, prechordal plate, fin mesoderm, and lens [9], and *H. sapiens* slit-like 2 (vasorin) in aortas, kidney, and placenta [16], the consistency with zebrafish slitl2 expressions sheds light on the fact that slitl2 may play important roles in axon guidance and vascular plasticity during zebrafish embryonic development.

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