Dual roles of zygotic and maternal Scribble1 in neural migration and convergent extension movements in zebrafish embryos

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Summary
In the developing vertebrate hindbrain, the characteristic trajectory of the facial (nVII) motor nerve is generated by caudal migration of the nVII motor neurons. The nVII motor neurons originate in rhombomere (r) 4, and migrate caudally into r6 to form the facial motor nucleus. In this study, using a transgenic zebrafish line that expresses green fluorescent protein (GFP) in the cranial motor neurons, we isolated two novel mutants, designated landlocked (llk) and off-road (ord), which both show highly specific defects in the caudal migration of the nVII motor neurons. We show that the landlocked locus contains the gene scribble1 (scrb1), and that its zygotic expression is required for migration of the nVII motor neurons mainly in a non cell-autonomous manner. Taking advantage of the viability of the llk mutant embryos, we found that maternal expression of scrb1 is required for convergent extension (CE) movements during gastrulation. Furthermore, we show a genetic interaction between scrb1 and trilobite(stbm) in CE. The dual roles of the scrb1 gene in both neuronal migration and CE provide a novel insight into the underlying mechanisms of cell movement in vertebrate development.

Key words: Zebrafish, landlocked, scribble1, facial motor neuron, neuronal migration, convergent extension

Introduction
Among the mammalian cranial nerves, the facial (nVII) motor nerve shows a very characteristic trajectory within the hindbrain. The axons first project medially and anteriorly, and then make a turn around the abducent (nVI) nucleus. This projection is generated by caudal migration of the nVII motor neurons during embryonic development. This developmental process is also conserved in zebrafish (Higashijima et al., 2000; Bingham et al., 2002) (summarized in Fig. 1). The first-born nVII motor neurons appear in rhombomere (r) 4 at 16 hours post-fertilization (hpf) at the ventral surface of the hindbrain near the floor plate, followed by continuous production of the neurons up to 36 hpf. Soon after their birth, the nVII motor neurons start migrating caudally into r5, resulting in a row of the migrating neurons in the ventral hindbrain. At the same time, the migrating neurons extend axons anteriorly then laterally to exit the hindbrain at r4. These peripheral axons project to the branchial arches and anterior/posterior lateral lines. In 24 hpf, the first-born nVII motor neurons reach r6, where they turn laterally to form the facial nucleus. The later-born neurons follow the same pathway in serial order. After 48 hpf, most of the nVII motor neurons are localized in the r6 region.

Several mechanisms have been implicated in the caudal migration of r4-derived nVII motor neurons. In hoxb1 knockout mice, r4-derived nVII motor neurons fail to migrate caudally (Studer et al., 1996). In chick hindbrain, they fail to migrate caudally and form a nucleus at r4. However, replacement of r5 or r6 with that of mouse restored caudal migration of the nVII motor neurons in chick hindbrain, indicating that in mice, r5 or r6 may emanate guidance cues to which chick nVII motor neurons can respond (Studer, 2001). Other molecules that regulate this migration have been recently and unexpectedly identified in zebrafish in studies on the convergent extension (CE) movements during gastrulation. The trilobite/stbm (tri/stbm) and prickle1 (pk1) gene products were shown to regulate both CE and migration of the nVII motor neurons (Bingham et al., 2002; Jessen et al., 2002; Carreira-Barbosa et al., 2003). In Drosophila, both Stbm and Prickle are involved in planar cell polarity (PCP) in epithelial cells in a Frizzled (Fz)/Dishevelled (Dsh)-dependent manner, and this pathway is referred to as the PCP pathway (reviewed by Strutt, 2003). These suggest that CE and neuronal migration may share common mechanisms that are associated with the PCP pathway.

However, in zebrafish, there is evidence that CE may also be regulated by other PCP signaling molecules encoded by knypek(kny)/glypicanc4/6, silverblick(slb)/wnt11 and pipetail(ppt)/wnt5a (Topezewski et al., 2001; Heisenberg et al., 2000; Kilian et al., 2003), but disruption of these genes does
Materials and methods

Fish strains and mutagenesis
Zebrafish (Danio rerio) were maintained according to standard procedures (Westerfield, 1995). The Is1-GFP line (Higashijima et al., 2000) was derived from the RIKEN wild-type strain. The WIK strain was used for the genetic mapping (Shimoda et al., 1999). Mutagenesis was carried out as described previously (Masai et al., 2003; Solnica-Krezel et al., 1994). Mutations were induced in the male germ cells of the Is1-GFP fish using N-ethyl-N-nitrosourea (ENU, Sigma). To isolate mutants deficient in migration of the nVII neurons, we observed peripheral axons from donor cells labeled with rhodamine-conjugated dextran (Molecular Probes) was carried out as described previously (Moens et al., 1996). Mosaic embryos were analyzed alive observing expression of GFP. At least 20 embryos were stained and heterozygous parents and identified mutant homozygous embryos by restriction analysis (RDA) was carried out as described previously (Lisitsyn et al., 1993; Sato and Mishina, 2003; Matsuda and Mishina, 2004). Genomic DNA was extracted from pools of 20 homozygous mutant embryos (2054 meioses) were collected from F2 families and five wild-type siblings at 30 dpf. Amplicons were prepared from genomic DNA (4 µg) and pooled wild-type genomic DNA (4 µg) with XbaI, EcoRI, BamHI, SpeI and NcoI. The interactive hybridization-amplification step was repeated three times. The resulting RDA products were cloned and their flanking genomic sequences were obtained from the Sanger Centre genome database. Specific primers were designed, and PCR products amplified from DNA of each mutant embryo of the mapping F2 panel were digested with the appropriate enzymes to detect restriction enzyme length polymorphisms. Four RDA products (NcoI-10, Xbal-1, Xbal-4, and EcoRI-46) were successfully mapped near the llk locus (see text). The following primers and enzymes were used:

Immunohistochemistry and in situ hybridization
Standard protocols were used for immunohistochemistry with a zn-5 antibody (Oregon Monoclonal Bank, 1:100 dilution) (Trevarrow et al., 1990), anti-acetylated α-tubulin antibody (Sigma, 1:1000) and a secondary antibody conjugated to Alexa Fluor 533 (Santa Cruz Biotechnology, 1:500). The samples were viewed by confocal microscopy (Zeiss LSM 510). In situ hybridization using RNA probes was carried out as described previously (Westerfield, 1995). Digital images of the embryos were captured using a differential interference contrast (DIC) microscope (Zeiss Axioplan2) with a CCD camera (Olympus DP50). In each experiment involving comparison between wild-type and mutant embryos, we used embryos obtained from heterozygous parents and identified mutant homozygous embryos by observing expression of GFP. At least 20 embryos were stained and observed in each experiment.

Restrograde labeling and cell transplantation
Retrograde labeling of reticulospinal neurons with rhodamine-conjugated dextran (Molecular Probes) was carried out as described previously (Moens et al., 1996). Retrograde labeling of putative octavolaterals efferent (OLe) neurons with Dil (Molecular Probes) was also performed as described previously (Higashijima et al., 2000). The putative OLe neurons extend axons to the anterior and posterior lateral lines. The OLe axons exit the hindbrain at the r4 and r6 level at 24 hours post-fertilization (hpf) and extend anteriorly or posteriorly at 28 hpf (Higashijima et al., 2000). The Dil was applied, at 30 hpf, to the anterior or posterior lateral line ganglion regions, through which the OLe axons extend. Co-localization of Dil and GFP signals in the cell bodies was confirmed in each optical section of confocal microscopy (see Fig. S1 in supplementary material). From a total of 20 embryos, six wild-type embryos (three anterior and three posterior lateral line ganglia) and six llk<sup>Wt</sup> homozygous embryos (four anterior and two posterior lateral line ganglia) were successfully labeled.

Cell transplantation was carried out according to standard protocols (Westerfield, 1995). llk<sup>Wt</sup> homozygous embryos were produced by crossing llk<sup>Wt</sup> homozygous parents. Cells from dome-stage (4-5 hpf) donor embryos injected with rhodamine-conjugated dextran were transplanted into shield-stage (6 hpf) host embryos as described previously (Moens et al., 1996). Mosaic embryos were analyzed alive at 36 hpf. To ensure that the transplanted donor cells were nVII motor neurons, we observed peripheral axons from donor cells labeled with rhodamine. In all of the mosaic embryos examined (three wild type<sup>+</sup>mutant and two mutant<sup>+</sup>wild type), a part of the facial motor axons bundle was rhodamine labeled, confirming that these donor cells were nVII motor neurons.

Mapping the mutant locus
In total, 1027 llk homozygous embryos (2054 meioses) were collected from parents derived from a llk<sup>Wt</sup> homozygous fish × WIK cross. Genomic DNA was extracted from individual embryos at 3 dpf. PCR analysis with SSLP markers (Shimoda et al., 1999) was carried out to assign the llk locus to the linkage group. Representational differential analysis (RDA) was carried out as described previously (Lisitsyn et al., 1993; Sato and Mishina, 2003; Matsuda and Mishina, 2004). Genomic DNA was extracted from pools of 20 homozygous mutant fish and five wild-type siblings at 30 dpf. Amplicons were prepared by digesting pooled mutant genomic DNA (4 µg) and pooled wild-type genomic DNA (4 µg) with XbaI, EcoRI, BamHI, SpeI and NcoI. The interactive hybridization-amplification step was repeated three times. The resulting RDA products were cloned and their flanking genomic sequences were obtained from the Sanger Centre genome database. Specific primers were designed, and PCR products amplified from DNA of each mutant embryo of the mapping F2 panel were digested with the appropriate enzymes to detect restriction enzyme length polymorphisms. Four RDA products (NcoI-10, XbaI-1, XbaI-4, and EcoRI-46) were successfully mapped near the llk locus (see text). The following primers and enzymes were used:
Neol-10: amplified with 5′CAGGGAGGAGCCTATAGTTT3′ and 5′TGCAGACCTTGGTTAAGCT3′, digested with MspI.
Xbal-1: amplified with 5′GGAGAACATCCGGTGTTACAA3′ and 5′CTGACTTTGTGCTCAGT3′, digested with HaeIII.
Xbal-4: amplified with 5′TGGTGTAACAGTCTGTCAC′ and 5′ACCTTCACAAACCTACCG3′, digested with DraI.
EcoRI-46: amplified with 5′TGAACAAATGCTCTAAGTGTTGTTGTTGTCCTGCAGT3′, digested with EcoRI.

**Identification of the gene**

A zebrafish PAC library (BUSUMP, RZPD) was screened by PCR using standard procedures. Specific primers from the EcoRI-46 flanking genome sequence were used for the amplification step (5′TAAAGGCAACAGGGAGTGAGATCAAC′ and 5′ACCTGTGATGAGGAGTATCACC′). Both ends of the resulting PCR products were sequenced, and consistency with the database was confirmed. The scrb1 genomic region was covered by a PAC clone (BUSUM#149G1) and the database contigs (AL772146 and Z06003613). To isolate the scrb1 gene, total RNA was extracted from 1.5, 10, 18, and 24 hpf embryos. Primers were designed for the database genomic sequences of nine partial cDNAs. To exclude nucleotide changes derived from genomic regions covered by a PAC clone, one-cell stage embryos were selected for the reverse transcription PCR (RT-PCR) analyses. The lkrw468 product. Total RNAs extracted from 1.5, 10, 18, and 48-hpf embryos were used. Specific primers designed in the database genomic sequences of nine partial cDNAs. To exclude nucleotide changes derived from polymorphisms, genomic DNA from male grandsparents of the family containing the lkrw468 and lkrw468 mutations was also sequenced. Six alternatively used exons 16, 28, 31, 34, 40, and 43 (see text in detail) were identified and RT-PCR analyses were performed to show the predominant scrb1 product. Total RNAs extracted from 1.5, 10, 18, 24, 36, and 48-hpf embryos were used. Specific primers designed in the flanking regions of each exon are as follows:

exon 16: 5′CTAGATGCAGCAGAGCTAGA3′ and 5′XbaI-1: amplified with 5′ATTGGTGTTGGAGAGGGTG3′
exon 28: 5′GTCGACAGAGACCTGAGTCC3′ and 5′XbaI-4: amplified with 5′GTGGTAGTGGAGATCAAC′
exon 31: 5′CTACTGTTGGCATACT3′ and 5′XbaI-3: amplified with 5′ACCTTCACAAACCTACCG3′
exon 34: 5′ACTAAACCTGGTGCCATCC3′ and 5′XbaI-2: amplified with 5′AGATAAGTTTGCTCAGAT3′
exon 40: 5′CTGTGCCTAC3′ and 5′XbaI-1: amplified with 5′CAGGGAGGGAAGCTTAGGTTT3′
exon 43: 5′CAGGGAGGGAAGCTTAGGTTT3′ and 5′XbaI-1: amplified with 5′CAGGGAGGGAAGCTTAGGTTT3′

For in situ hybridization, we used a partial cDNA fragment from the genomic region was covered by a PAC clone (BUSUM#149G1) and the database contigs (AL772146 and Z06003613). To isolate the scrb1 gene (157-418 aa, corresponding to the N-ethyl-nitrosourea (ENU). Two novel mutagenized with ENU. Mutations that showed perturbed migration of the nVII derived of the nVII motor neurons is impaired in the llk embryos [tri/llk]. Subsequent experiments further characterized the llk mutation.

**Results**

**landlocked and off-road are novel mutants with disrupted migration of the nVII motor neurons**

The llk-GFP transgenic line expresses GFP in the branchial motor neurons of the hindbrain (Higashijima et al., 2000). Using this line, we screened a total of 1816 haploid genomes mutagenized with N-ethyl-N-nitosourea (ENU). Two novel mutants that showed perturbed migration of the nVII derived of the nVII motor neurons compared to wild-type were isolated. These were designated landlocked (llk; Fig. 2B,F,J, compare with the wild-type embryos shown in A,E,I) and off-road (ord; Fig. 2C,G,K). We also identified a novel allele for the trilobite (tri) mutant (Fig. 2D,H,L), in which CE movements were also impaired (Fig. 2D; Bingham et al., 2002; Jessen et al., 2002). Subsequent experiments further characterized the llk mutation.

**Migration, but not differentiation, of the nVII motor neurons is impaired in the llk embryos**

In wild-type embryos, the nVII motor neurons originated and began to express GFP in r4 at 16 hpf, after which they started to migrate caudally through r5 into r6 (Fig. 3A,C) (Chandrasekhar et al., 1997; Higashijima et al., 2000). The nVII motor neurons form the facial motor nucleus exclusively in r6 at 2 dpf (Fig. 3E). We examined two alleles of llk (lkrw468 and lkrw468) that caused equivalent disruption of migration of the GFP-positive cells. All of the homozygous embryos (n=211...
for \(llkrw_{16}\) and \(n=121\) for \(llkrw_{68}\) showed complete loss of migration of r4-derived GFP-expressing cells (Fig. 3B,D,F).

Although r6-derived GFP-expressing neurons (putatively octavolateralis efferent (OLE) neurons) also failed to migrate to r7 in the \(llk\) embryos (Fig. 3M-P, see also Fig. S1 in supplementary material), all the other migratory cell types were unaffected. The mutant embryos had normal tangential and radial migration of the trigeminal (nV) and vagus (nX) motor neurons (Fig. 2F), migration and positioning of the pigment cells derived from the neural crest (data not shown), and migration of lateral line neuromast cells derived from placode cells (data not shown). Thus, we concluded that the \(llk\) embryos had specific impairment of migration of the nVII motor neurons.

Tag-1 is a specific marker for migrating nVII motor neurons (Fig. 3G) (Warren et al., 1999). The non-migratory cells in the \(llk\) embryos still expressed tag-1 mRNA (Fig. 3H), suggesting that these cells retained the potential to differentiate normally into nVII motor neurons. Consistent with this, these non-migratory cells extended the GFP-positive peripheral axons normally (Fig. 3J). The axons in the \(llk\) embryos projected to the correct specific target muscles with the same pattern as normally (Fig. 3I,J). The axons in the migratory cells extended the GFP-positive peripheral axons into nVII motor neurons. Consistent with this, these non-migratory cells still expressed tag-1 mRNA (Fig. 3H), suggesting that these cells retained the potential to differentiate normally into nVII motor neurons. Thus, we concluded that the \(llk\) embryos had specific impairment of migration of the nVII motor neurons.

**Patterning of the hindbrain is unaffected in the \(llk\) embryos**

Each rhombomere shows differential expression of several genes which are essential for the fate determination of that specific rhombomere. hoxb1a, krox20 and valentinoneuromast/mafB are expressed in r4, r3/5 and r5/6, respectively, in the developing zebrafish hindbrain (Prince et al., 1998; Oxtoby and Jowett, 1993; Moens et al., 1998). The patterns of expression of hoxb1a (Fig. 4A,B), krox20 (Fig. 4C,D) and val/mafB (Fig. 4E,F) were identical between the \(llk\) and wild-type embryos, suggesting that the segmental patterning of the rhombomeres was normal in the mutant embryos.

The zn-5 antibody specifically labels segmentally repeated commissural axons in the zebrafish hindbrain (Trevorrow et al., 1990). The formation of zn-5-immunoreactive axons appeared normal in the \(llk\) embryos (Fig. 4G,H). Furthermore, labeling of the reticulospinal neurons by injecting a tracer dye into the spinal cord (Metcalfe et al., 1986; Moens et al., 1996) revealed the overall patterning and differentiation of the hindbrain neurons was identical to that in the wild-type embryos (Fig. 4I,J). Together, these results suggest that the overall patterning and differentiation of the hindbrain neurons were unaffected by the \(llk\) mutation.

**\(llk\) encodes zebrafish scribble1**

The \(llk\) locus was genetically mapped to linkage group 7 between the SSLP markers, Z11545 and Z62080 (Shimoda et al., 1999) (Fig. 5A). To isolate DNA fragments closely associated with the \(llk\) locus, a representational differential analysis (RDA) (Lisitsyn et al., 1993; Sato and Mishina, 2003; Matsuda and Mishina, 2004) was performed. Four RDA products were closely linked to the \(llk\) locus and one of them, EcoRI-46, showed no recombination per 2054 meioses in F2 crosses (Fig. 5A). The DNA fragments carrying the EcoRI-46 sequence were obtained by screening a PAC library together with a search of the Sanger Center genome database. The EcoRI-46 site was located in the first intron of a gene (Fig. 5B) that is highly homologous to mouse ScrB (Fig. 5F) (Murdoch et al., 2003). Sequence analyses of cDNA revealed that at least exons 16, 28, 31, 34, 40 and 43 were differentially used by alternative splicing (Fig. 5C). Two of them (exons 16 and 43) corresponded to those used in mouse ScrB (exons 16 and 36 in mouse (Murdoch et al., 2003)), RT-PCR was performed and the most predominant transcript that putatively encoded a 1724...
zebrafish *scribble1* regulates neuronal migration and convergent extension

Amino acid protein was identified (encompassing exon 16, but no other alternatively used exons; Fig. 5C,D). We refer to this gene product as the wild-type *scrb1* gene in the following experiments. *Scrb1* is a cytoplasmic protein carrying a set of 16 leucine-rich repeats (LRR) and four PDZ (for PSD-95/Discs-large/ZO-1) domains (Fig. 5F). Sequence analyses showed that each of the two alleles of the *llk* locus carries a point mutation in the *scrb1* gene. The allele *llk*<sup>rw16</sup> carries a mis-sense amino acid substitution in the first PDZ domain (I734D), and *llk*<sup>rw468</sup> carries a stop codon in the LRR domain (K310Stop; Fig. 5E,F).

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**Fig. 3.** Migration of the nVII motor neurons is specifically impaired in *llk* embryos. A-F, Isl1-GFP expression in the wild-type (A,C,E) and *llk*<sup>rw16</sup> homozygous embryos (B,D,F) at 18 hpf (A,B), 24 hpf (C,D), and 48 hpf (E,F). In the wild-type siblings, the nVII motor neurons arise in r4, migrate caudally through r5 into r6 and form the nucleus in r6 (E, arrowhead). In contrast, in the *llk*<sup>rw16</sup> embryos the GFP-expressing cells that arise in r4 fail to migrate and form an ectopic nucleus in r4 (F, arrowhead). Asterisks (D,E,F) indicate r6-derived putative OLe neurons, which migrate into r7 in the wild-type embryos. These neurons also fail to migrate in the mutant embryos and remain in r6 (D,F). G,H *tag-1* mRNA expression in the wild-type (G) and *llk*<sup>rw16</sup> (H) embryos at 24 hpf. *tag-1*-positive cells are located in r4 in the *llk*<sup>rw16</sup> embryo (H, arrowhead). Dorsal views. The position of the ears are indicated by the broken lines. Va, Vp, anterior and posterior trigeminal nuclei, respectively; VII, facial nucleus; X, vagus nucleus; Allg, Pllg, anterior and posterior lateral line ganglion, respectively.

(I-L) Isl1-GFP expression in the wild-type (I,K) and *llk*<sup>rw16</sup> (J,L) embryos at 5 dpf. Arrowheads indicate the facial motor nucleus. The trajectories of facial motor axons are normal in the *llk*<sup>rw16</sup> embryo (arrows). The axons reach the target organs (K,L, higher magnifications of the boxed regions in I and J). Lateral line organs and the cranial muscles are indicated by dots and broken lines, respectively. Lateral views; anterior is to the left. M-N The putative OLe neurons (arrows) projecting to the lateral lines are retrogradely labeled (red) in the wild-type (M,O) and *llk*<sup>rw16</sup> (N,P) embryos. DiI was applied to the anterior (M,N) or posterior lateral line ganglion (O,P). Sites of DiI application are indicated by triangles. Small arrows indicate the vagal (X) motor neurons, which were labeled with DiI that diffused from the application site (P). Single-channel images of the labeled neurons are shown in M’, N’, O’ and P’. These neurons extend dendrites to the contralateral side of the brain. Inset in M’ shows another example in which dendritic processes were clearly labeled. Asterisks indicate r6-derived r7-located neurons, which fail to migrate and remain in r6 in the *llk* embryos. Arrowheads indicate the facial motor nuclei. Dorsal views. Scale bar: 50 µm.

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**scrib1** mRNA is expressed in the whole brain

In situ hybridization was performed using the RNA probe that detects all of the spliced variants. The *scrib1* mRNA was expressed maternally during the early embryonic stages. Expression was initially detected throughout the embryo (Fig. 5G-I), but then became restricted to the brain region (Fig. 5J-M). At 18 hpf, when migration of the nVII motor neurons is initiated, *scrib1* mRNA was expressed throughout the neural tube (Fig. 5L,M). However, *scrib1* mRNA was very weakly expressed in the ventral neural tube region, where the
migrating nVII motor neurons were located (Fig. 5M,M'). Strong expression continued to be observed in the brain during and after the migration of the nVII motor neurons in 22- to 48-hpf embryos (Fig. 5J,K).

Functional knock-down of scrb1 recapitulates defects of migration of the nVII motor neurons

To confirm that loss of function of the scrb1 gene is responsible for the llk phenotype, antisense morpholino oligos (MO) were designed to specifically disrupt scrb1 gene function. MO/ATG was designed to abolish translation of the gene zygotically, while the MO/2e2i abolishes splicing of the gene maternally and zygotically. Normal migration of the nVII motor neurons was completely lost in both the resulting morphants compared with the wild-type embryo (5 ng of MO per embryo, Fig. 6B,C, and A, respectively; 100% of MO/ATG-injected embryos (n=82) and 98% of MO/2e2i-injected embryos (n=61)]. Injection of each control MO (MO/ATG-5mis and MO/2e2i-5mis) did not impair migration of nVII motor neurons (0%, n=22 and n=35, respectively), confirming the specificity of the antisense MOs.

The llk gene acts mainly in a non cell-autonomous manner during migration of the nVII motor neurons

Mosaic experiments were performed to determine the cell autonomy of the llk mutation. Wild-type-derived nVII motor neurons failed to migrate caudally in MZ-llk host embryos (Fig. 7A). We observed peripheral axons of these cells in each mosaic embryo to ensure that the donor cells were nVII motor neurons. In all of the mosaic embryos examined, a part of the facial motor axons bundle was labeled with rhodamine-dextran (Fig. 7A'). M. Mauthner’s cell. Asterisks indicate r6-derived putative OLe neurons. Arrowheads indicate the facial motor nuclei. Dorsal views.

Injection of scrb1 mRNA into one-cell-stage llk embryos restores migration of the nVII motor neurons

To confirm the role of the scrb1 gene in the migration of nVII motor neurons, we generated a wild-type scrb1 cDNA (Fig. 6D), mutated scrb1 cDNAs encoding the llk<sup>rw16</sup>, llk<sup>rw468</sup> alleles (scrb1<sup>lw16</sup> and scrb1<sup>lw468</sup>) and a truncated scrb1 gene encoding only the LRR domain (scrb1<sup>APDZ2</sup>) (Fig. 6D). Maternal-and-zygotic (MZ) llk<sup>rw468</sup> embryos were injected with 0.5 ng of each mRNA. MZ-llk<sup>rw468</sup> embryos showed slight CE defects (described in detail below), but these defects were restricted to the tail regions (see below), and the migration phenotype was not affected by the maternal depletion of the gene. When wild-type scrb1 mRNA was injected into MZ-llk<sup>rw468</sup> homozygous eggs (n=162), 61% of embryos had migration of nVII motor neurons restored (Fig. 6E,F); in 24% this migration was only into r5, and in 37%, migration was fully restored so that neurons moved through r5 into r6. Injection of scrb1<sup>rw16</sup> mRNA (n=175) also rescued the migration phenotype, but only in 10.2% of embryos (5.1% with partial migration to r5, 5.1% with fully restored migration through to r6; Fig. 6G). In contrast, injection of scrb1<sup>lw468</sup> mRNA (n=41) or scrb1<sup>APDZ2</sup> mRNA (n=42) failed to rescue the migration phenotype in any embryos. Thus, both loss-of-function and gain-of-function experiments confirmed that the llk locus encompasses the scrb1 gene.

The llk gene acts mainly in a non cell-autonomous manner during migration of the nVII motor neurons

Mosaic experiments were performed to determine the cell autonomy of the llk mutation. Wild-type-derived nVII motor neurons failed to migrate caudally in MZ-llk host embryos (Fig. 7A). We observed peripheral axons of these cells in each mosaic embryo to ensure that the donor cells were nVII motor neurons. In all of the mosaic embryos examined, a part of the facial motor axons bundle was labeled with rhodamine-dextran (Fig. 7A'), showing that they were indeed nVII motor neurons. These results suggest that the llk gene acts in a non cell-autonomous manner during migration of the nVII motor neurons, which is consistent with the observation that scrb1 mRNA is strongly expressed in the dorsal neural tube cells surrounding the migrating nVII motor neurons (Fig. 5M). In contrast, most of the MZ-llk-derived nVII motor neurons migrated normally through r5 into r6 in wild-type host embryos (Fig. 7B). Since some of the late-born neurons remained in r4
at the time of observation, we could not completely exclude
the autonomous involvement of the scrb1 gene in migration of
the nVII motor neurons.

**Maternal scrb1 is required for convergent extension
movements**

Zygotic llk embryos do not show any defects in convergent
extension (CE) movements and these mutant embryos were
homozygously viable (Fig. 2B; 96% of 211 zygotic llk<sup>rw16</sup>
embryos and 97% of 121 zygotic llk<sup>rw468</sup> embryos survived to
larval stages), suggesting that zygotic llk/scrb1 function is not
essential for CE. The tri/stbm and pk1 genes regulate both
migration of the nVII motor neurons and CE movements
during gastrulation (Jessen et al., 2002; Carreira-Barbosa et al.,

Fig. 5. Identification of the llk
gene. (A) Genetic map for the llk
locus. The llk locus mapped to
linkage group (LG) 7. The RDA
marker, EcoRI-46 is located at 0
cM map-distance from the llk
locus (0 per 2054 meioses). (B)
Genomic structure of the
zebrafish scrb1. The scrb1 gene
is encoded by 45 exons spanning
120 kb in the genome. EcoRI-46
is located in the first intron of the
gene. Each of the two mutant
alleles, llk<sup>rw16</sup> and llk<sup>rw468</sup>
carries a nucleotide substitution in exons
11 and 17, respectively. (C)
Schematic drawing for the
putative cDNA encoding 45
exons shown in B. Six
alternatively used exons, 16, 28,
31, 34, 40 and 43 are shown as
red boxes. Exon numbers and
first ATG site are indicated
above. Regions encoding LRR
domain and four PDZ domains
are indicated below. (D) RT-PCR
analyses were performed to
identify the predominant gene
product. Primers were designed
in the flanking exons
encompassing each exon of
interest. Total RNA was
extracted from 1.5, 10, 18, 24, 36
and 48-hpf embryos.
Arrowheads in each panel
indicate the predominant RT-
PCR product expressed during
migration of the nVII motor
neurons at 18-24 hpf. The
predominant RT-PCR products
contain exon 16, but no other
exons (exons 28, 31, 34 and 43).
The lesser RT-PCR products
contain exons 28, 31, 34 and 43,
but not exon 16 (indicated by
arrows). 100 bp-interval
molecular markers (bp) are
shown in each panel. (E)
Sequence diagrams of the
mutation sites for the llk<sup>rw16</sup>
and llk<sup>rw468</sup> alleles compared to
the wild-type allele. (F) Schematic
drawings of the wild-type (zScr1) and mutant Scr1 proteins (Scr1<sup>rw16</sup>
and Scr1<sup>rw468</sup>). Percentage identity of the amino acid sequences (%) to
the mouse Scrb (mScr1) is shown for each domain. The allele llk<sup>rw16</sup>
carries a miss-sense amino acid substitution in the first PDZ domain, while
llk<sup>rw468</sup> carries a stop codon in the LRR domain. (G-M) Lateral views of wild-type embryos stained with the scr1 RNA probe in the 8-cell
stage (G), dome-stage (H), 18 hpf (I), 22 hpf (J) and 48 hpf (K) embryos. (L,M) scr1 mRNA expression in the brain at 20 hpf (L, dorsal view)
and (M) cross section at r5 (indicated by the broken line in L). M' shows the cross section at r5 of the 24 hpf-Isl1-GFP embryo stained
with anti-acetylated α-tubulin antibody. Arrowheads indicate medial longitudinal fascicles (MLF); nc indicates notochord. Scale bars: 50 µm.
Since scrb1 mRNA is strongly expressed maternally (Fig. 5G,H), this gene may also be involved in CE at early stages. Taking advantage of the normal viability of the zygotic llk mutants, we were able to generate MZ-llk embryos and examine this possibility. Indeed, MZ-llk embryos showed slight CE defects during early gastrulation (Fig. 8B,E). They had slightly curled tails in 24-48 hpf (Fig. 8H,J), although some recovered to a normal shape by 4 dpf (4.4% of 113 MZ-llkrw468 embryos and 53% of 102 MZ-llkrw16 embryos). To further clarify the role of maternal scrb1 in CE, llkrw468 homozygous females were crossed with heterozygous males (+/llkrw468). 56% of the resulting embryos (n=123) showed normal nVII motor neuron migration, indicating that they were zygotically heterozygous with no maternal contribution of scrb1. The remaining embryos showed loss of the neuron migration, and were MZ-llk<sup>rw468</sup>. Only 28% of the zygotically heterozygous embryos were morphologically normal despite their normal nVII motor neuron migration (n=69). In contrast, 9.3% of the MZ-llk<sup>rw468</sup> embryos were morphologically normal (n=54). These results indicate that maternal scrb1 is required for CE movements but dispensable for migration of the nVII motor neurons. Moreover, the zygotic scrb1 expression can compensate for loss of the maternal scrb1, but only incompletely. Furthermore, scrb1 MO/ATG also induced CE defects that were similar to those of MZ-llk embryos in all 82 embryos injected (Fig. 8C,F), but injection of scrb1 MO/2e<sup>2i</sup> only affected CE in a small proportion

**Fig. 7.** The llk gene is required for migration of the nVII motor neurons in a non cell-autonomous manner. Mosaic experiments were performed to determine the cell autonomy of the llk gene. A total of 8 wild-type embryo-derived nVII motor neurons (arrows) all failed to migrate caudally in 3 llk<sup>rw16</sup> host embryos (A). (A’) The peripheral axons (arrowheads) of these cells were comprised in a part of the facial motor axons bundle. (B) In contrast, a total of 21 llk<sup>rw16</sup> embryo-derived nVII motor neurons (arrows) migrated normally through r5 into r6 in 2 wild-type host embryos. (A,B) Dorsal views; (A’) lateral views, anterior is to the right, 2 dpf. Scale bar: 50 µm.
(7.6%, n=92) of embryos, confirming that maternal scrb1 is essential for CE in early development.

Next, we analyzed whether injection of scrb1 mRNA could rescue CE defects in MZ-llk<sup>rw468</sup> embryos. Injection of 0.5 ng of wild-type scrb1 mRNA into MZ-llk<sup>rw468</sup> embryos induced recovery of CE defects in 40% of 168 embryos. Injection of 0.5 ng of scrb1<sup>rw16</sup> mRNA into MZ-llk<sup>rw468</sup> embryos also induced recovery of the CE phenotype at a lower frequency (31%, n=130). However, injection of 0.5 ng of scrb1<sup>rw468</sup> mRNA (n=41) or scrb1<sup>dpdz5</sup> mRNA (n=42) failed to rescue the CE phenotype in any embryos. These results indicate that the scrb1 gene is essential for CE, and that the first PDZ domain of the Scrb1 protein is important for this activity.

**Subcellular localization of Scrb1 and mutated proteins**

To analyze the subcellular localization of Scrb1 protein, we injected mRNA from expression vectors encoding wild-type or mutated Scrb1 fused with GFP (Scrb1:GFP) into one-cell stage embryos. Overexpression of the wild-type Scrb1:GFP also rescued the migration of the nVII motor neurons (in 62% of embryos; n=45), suggesting that the GFP fusion does not abolish normal function of the original Scrb1. Wild-type Scrb1:GFP protein was localized to the plasma membranes of all cells in which they were overexpressed (5 embryos; Fig. 6H). The mutated Scrb1<sup>rw16</sup>:GFP and Scrb1<sup>dpdz5</sup>:GFP proteins were both similarly localized to the plasma membrane (5 embryos; Fig. 6L). However, mutated Scrb1<sup>rw468</sup> protein was not associated with the cell membrane, but was localized to the cytoplasm (5 embryos; Fig. 6J). These suggest that the LRR domain is sufficient for the membrane-associated localization of Scrb1 protein. Although Scrb1<sup>rw16</sup> was localized to the plasma membrane, injection of this construct restored migration of the nVII motor neurons and CE movements in MZ-llk<sup>rw468</sup> embryos only at a lower frequency. Therefore, the membrane-associated localization of Scrb1 by way of the LRR domain is sufficient, but the first PDZ domain is required for the normal functions of Scrb1.

**Genetic interaction between llk/scrb1 and tri/stbm**

To determine whether there is an epistatic interaction between the scrb1 and stbm genes in the regulation of migration of the nVII motor neurons, we performed some rescue experiments.

We confirmed that only 0% (n=45) and 30% (n=37) of embryos injected with 5 ng and 0.5 ng of stbm MO, respectively, showed the normal migration of the nVII motor neurons (Fig. 8K). We also confirmed the activity of stbm mRNA by using the tri/stbm mutant embryos. The tri<sup>rw75</sup> homozygous embryos show the nVII motor neurons migration defects with strong CE defects (Fig. 2D). Sequencing analyses showed that the tri<sup>rw75</sup> allele carries a stop codon (Y342Stop), which results in deletion of the C-terminal intracellular domain of Stbm, and is likely to be a loss-of-function mutation. 22% of embryos obtained from heterozygous tri<sup>rw75</sup> parents, only 7.9% of embryos showed the normal migration of the nVII motor neurons. 22% of embryos obtained from heterozygous tri<sup>rw75</sup> parents show slight CE defects with strong CE defects (Fig. 8D). Sequencing analyses showed that the tri<sup>rw75</sup> allele carries a stop codon (Y342Stop), which results in deletion of the C-terminal intracellular domain of Stbm, and is likely to be a loss-of-function mutation. 22% of embryos obtained from heterozygous tri<sup>rw75</sup> parents, only 7.9% of embryos showed the nVII migration defects as expected (n=96). When 0.5 ng of stbm mRNA was injected into eggs obtained from heterozygous tri<sup>rw75</sup> parents, only 7.9% of embryos showed the nVII migration defects (n=139). These results indicate that 0.5 ng of stbm mRNA has activity enough to rescue loss of stbm gene function. Similarly, 0.5 ng of scrb1 mRNA restored the nVII motor neuron migration in MZ-llk<sup>rw468</sup> embryos efficiently as described (Fig. 8F). In contrast, injection of 0.5 ng of scrb1 mRNA into the MZ-llk<sup>rw468</sup> embryos did not restore the migration (0%, n=70). Similarly, injection of 0.5 ng of scrb1 mRNA with 5 ng of stbm MO did not restore the neuronal migration (0%, n=67). Injection of 0.5 ng of scrb1 mRNA with 0.5 ng of stbm MO also did not restore the neuronal migration (25% of embryos showed the normal
mRNA into wild-type embryos induced CE defects resembling tri mutant phenotypes without affecting migration of the nVII motor neurons as judged by the defects in extension of the tail. Overexpression of 0.5 ng of scrb1 mRNA in wild-type embryos also induced slight CE defects without affecting migration of the nVII motor neurons (63%, n=51, Fig. 8M). Injection of 5 ng of stbm MO into MZ-ilk/wg68 embryos slightly enhanced CE defects (21% of embryos showed enhanced phenotype, n=39, Fig. 8L). Injection of 0.5 ng of stbm mRNA in the MZ-ilk/wg68 embryos significantly enhanced CE defects (14%, n=70). More strikingly, co-injection of 5 ng of stbm MO together with 0.5 ng of scrb1 mRNA markedly enhanced CE defects (91% of embryos showed severe defects, n=67, Fig. 8N; Table 1). Co-injection of 5 ng of stbm MO together with 0.5 ng of scrb1 mRNA also enhanced CE defects, but at a lower frequency (33% of embryos showed severe defects, n=98).

We also carried out additional experiments on genetic interaction between scrb1 and stbm at a suboptimal dose of stbm MO. Wild-type embryos were injected with 0.1 ng stbm MO and only 3% of resulting embryos showed tri-like phenotypes (n=263), indicating that this dose is suboptimal. When 0.1 ng of stbm MO was injected with 0.5 ng of scrb1 mRNA, 17% of embryos showed tri-like phenotypes (n=334). Although the CE defects were not as severe as in embryos injected with 5 ng of stbm MO and 0.5 ng of scrb1 mRNA, the enhancement of CE defects was detected (Table 1).

In conclusion, overexpression of scrb1 or stbm induced the similar CE phenotypes as loss of function of these genes. Moreover, CE was affected most severely when scrb1 was overexpressed in the absence of stbm.

**Migration of the nVII motor neurons is not associated with CE movements**

It is shown that CE movements of the midline cells are required for neural tube closure in *Xenopus* (Wallingford and Harland, 2002). In mouse embryos, Crc/Scribble is required for neural tube closure (Murdock et al., 2003). Therefore, we wondered if the causal migration of the nVII motor neurons in normal embryos could be a consequence of any uneven morphogenetic movements of the hindbrain neuroepithelial tissues. For examples, if CE movements proceed more slowly near the ventral midline than in the more lateral region of the r4 tissue, then the medial part including the nVII motor neurons may be left behind by the rest of the r4 tissue and appear to have migrated out from the other r4 tissue. To address this possibility, we labeled the r4 region by uncaging the caged fluorescein-conjugated dextran and traced the cell movements during development (Kozlowski and Weinberg, 2000). We showed that the nVII motor neurons were the only population which came out of the labeled r4 tissue (3 embryos; Fig. 9). These results indicate that the nVII motor neurons migrate completely independently of the rest of the r4 tissues. Thus, we conclude that the uncoordinated CE movements between the tissue surrounding the nVII motor neurons and the rest of the hindbrain is not the cause of the posterior displacement of the nVII motor neurons from r4.

**Discussion**

We have isolated zebrafish mutants with highly specific defects in the caudal migration of the nVII motor neurons, one cause of which is a zygotic defect in scrb1 function. Taking advantage of the normal viability of the zygotic mutants, we were able to further analyze the role of Scr1 in early embryogenesis by depletion of maternal transcripts. Our results suggest that scrb1 plays dual roles in the regulation of cell migration and CE movements, which are differentially controlled by maternal and zygotic expression of scrb1, and that scrb1 interacts with tri/stbm gene to regulate CE.

**Localization of Scribble to the plasma membrane is mediated by the LRR domain**

We showed that overexpressed Scribble protein is associated with the plasma membrane. Moreover, the LRR domain alone is sufficient for the targeting of this protein to the membrane, which is consistent with previous results (Legouis et al., 2003). *Drosophila* Scribble and the *C. elegans* ortholog LET-413...
localize to the basolateral membranes of epithelial cells (Bilder and Perrimon, 2000; Bilder et al., 2000; Legouis et al., 2000). The LRR domain may be required for primary targeting of the protein to the membranes, and then the PDZ domains may be important for precise localization of the protein to specific sites on the membrane, via interaction with other membrane proteins.

**Llk/Scrb1 and Tri/Stbm may constitute a functional complex**

Recent studies reported that a mammalian homologue circletail(Crc)/Scrb is required for neural tube closure and the orientation of sensory cells in the cochlea (Murdoch et al., 2003; Montcouquiol et al., 2003). The defects in the Crc embryos are very similar to that in loop-tail(Lp) mutants which are the result of mutations in Van Gogh2(Vangl2)/stbm, and Crc/Scrb interacts with Lp/Vangl2/stbm genetically (Kiber, 2001; Murdoch et al., 2003; Montcouquiol et al., 2003). Thus, in vertebrates, Scrb may act together with Stbm in morphogenesis of neural tissues.

In this study, we showed that injection of llk/scrb1 mRNA did not rescue migration of the VII motor neurons in tri/stbm MO-injected embryos. Similarly, injection of tri/stbm mRNA also failed to rescue neuronal migration in the llk embryos, suggesting that the llk/scrb1 and tri/stbm genes do not act in a simple linear pathway, but rather that they function by forming a functional complex.

Although our results and previous studies have suggested that there is a genetic interaction between scrb1 and stbm (Murdoch et al., 2003; Montcouquiol et al., 2003), it is not known whether the PDZ domains of Scrb directly interact with the PDZ-binding domain of Stbm. In Drosophila, the second PDZ domain of Scrb interacts with Dlg via GUKH (guanylate kinase holder protein) to form a scaffolding complex at synaptic junctions (Mathew et al., 2002). Furthermore, Dlg interacts with Stbm and this complex is required for plasma membrane formation in epithelial cells (Lee et al., 2003). These results suggest that Scrb, Stbm and Dlg may constitute a functional complex during the formation of membrane structures.

If Tri/Stbm and Llk/Scrb1 form a functional complex, this complex would probably have two sites that associate with membranes: the transmembrane domain of Tri/Stbm and the LRR domain of Llk/Scrb1. In this study, we showed that knock-down of Tri/Stbm with overexpression of Llk/Scrb1 led to the most severe impairment of CE. These results indicate that Tri/Stbm may be required for localization of Llk/Scrb1 protein to the specific site of the membrane where they are anchored and function together. Release of membrane-associated Llk/Scrb1 from such positional constraint in the absence of Stbm may have more markedly perturbed the functional protein complexes controlling CE than simple overexpression of Scrb1 in the presence of Stbm.

We also demonstrated that the Scrb1<sup>rw16</sup> protein, which has a single amino acid substitution in the first PDZ domain, has lower activity than the wild-type protein to rescue migration of the nVII motor neurons in the llk mutation. Similarly, overexpression of Scrb1<sup>rw16</sup> induced CE defects to a lesser extent than that of wild-type Scrb1 protein. These results indicate that the first PDZ domain is also essential for Scrb1 activity. The first PDZ domain of Llk/Scrb1 may interact with another, as yet unidentified, component to establish a multi-protein complex required for its function.

**Possible roles of Llk/Scrb1 in migration of the nVII motor neurons**

We showed that the llk/scrb1 gene functions mainly in a non cell-autonomous manner in migration. We also showed that the uncoordinated CE movements between the medial r4 tissue surrounding the nVII motor neurons and the rest of the hindbrain is not likely to be the cause of the posterior displacement of the nVII motor neurons relative to r4. One possibility may be the involvement of the Llk/Scrb1 protein (or the protein complex) in establishing a concentration gradient of attractive cues in the hindbrain. For example, the Llk/Scrb1 protein may interact with a transmembrane protein to capture and display the attractive cues on the surface of cells in the migratory pathway of the nVII motor neurons. Alternatively, the Llk/Scrb1 protein may be required by the neuroepithelial cells to prevent the migrating nVII motor neurons from veering away from the normal migratory pathway as is the case in the llk and ord embryos (see Fig. 2J,K).

In zebrafish, we showed that several putative OLe neurons are born in r6 and migrate into r7, and that this migration is also impaired in the llk embryos. The glossoophynggeal (nIX) motor neurons also failed to migrate from r6 to r7 in the tri embryos (Bingham et al., 2002). These results show that there are at least two cell populations that migrate, one from r4 to r6 (nVII motor and r6-located OLe neurons), and the other from r6 to r7 (nIX motor and r7-located putative OLe neurons). The fact that both r4-derived cells and r6-derived cells failed to migrate in the llk and tri embryos may indicate that the migrations of these cells are regulated by a common mechanism in different rhombomeres. If they are both guided by a common attractive cue emanating from the caudal end of the hindbrain, as was suggested in mouse embryos (Studer, 2001), this cue may have been accumulated to saturation at r6 at which level an effective gradient may have been lost, by the time the r4-derived nVII neurons had arrived at r6.

**Similarity and diversity in mechanisms regulating CE and migration of the nVII motor neurons**

It has now been shown that llk/scrb1 (present study), tri/stbm (Bingham et al., 2002; Jessen et al., 2002) and pki (Carreira-Barbosa et al., 2003) are required for both CE and neuronal migration. However, the possible PCP signaling molecules kny/glypican4/6, slb/wnt11 and ppt/wnt5a regulate CE (Topczewski et al., 2001; Heisenberg et al., 2000; Kilian et al., 2003), but do not regulate neuronal migration (Bingham et al., 2002; Jessen et al., 2002). Moreover, overexpression of a dominant-negative Dishevelled (Dsh), which blocks CE movements (Heisenberg et al., 2000), does not affect the neuronal migration (Jessen et al., 2002). These results suggest that genetic cascades, which regulate the VII motor neuron migration, may not coincide completely with those regulating CE movements.

In this study, we isolated a second mutant, ord, in which the nVII motor neurons are misguided away from the normal pathway. Preliminary results showed that the MZ-ord embryos did not have any defects in CE movements and are viable. These results suggest that the ord gene is only required for neuronal migration, and not for CE. Identification of the gene
responsible for the *ord* mutation may provide us with clues to the mechanisms of neuronal migration, e.g. molecules regulating the attractive guidance cues.

**Differentiation and migration of the nVII motor neurons occurs independently**

The functions of the nVII motor neurons located ectopically in r4 in morphants or mutants have not been analyzed. In hoxb1a knock-down embryos, the non-migratory nVII motor neurons extend peripheral axons normally (McCIntock et al., 2002). However, OLe neurons innervating the ear fail to extend axons, indicating that differentiation of these neurons is deficient in these morphants (McCIntock et al., 2002). In the *tri* embryos, although all of the non-migratory nVII motor neurons appear to extend axons normally, it is not known whether they are functional because of embryonic lethality (Bingham et al., 2002).

In this study, we were able to address this question, because the *llk* mutation exclusively affects neuronal migration zygotically, and the resultant embryos remain viable. We showed that the nVII motor neurons in the zygotic *llk* embryos failed to migrate and remained at r4, but had normal morphological development. Moreover, the *llk* homozygous larvae showed apparently normal foraging behavior, and the jaw muscles appeared to contract normally. The *llk* homozygous embryos were viable and developed into fertile adults. Therefore, these non-migratory motor neurons must function relatively normally despite their aberrant localization. Since many genes have been implicated in migration of the VII motor neurons and this process has been conserved in evolution (Studer et al., 1996; Garel et al., 2000; Ohshima et al., 2002; Muller et al., 2003), it is unlikely that correct migration of nVII motor neurons has been maintained without any survival advantage. Thus, it is rather more likely that mislocation of the nVII motor neurons in the *llk* embryos may be epigenetically compensated for by reorganization of neural networks during development. This innate developmental plasticity may have laid the basis for accommodating the loss of migration of the nVII motor neurons during evolution to avian species (Studer, 2001).

**Possible functional redundancy within LAP family genes**

In *Drosophila scribble* and *C. elegans* *let-413* mutants, cell junctions are not positioned properly, resulting in embryonic death with severe apicobasal polarity defects in epithelial cells (Bilder and Perrimon, 2000; Bilder et al., 2000; Legouis et al., 2000). However, in mice (Murdoch et al., 2003; Montcouquiol et al., 2003) and in zebrafish (this study), *scrb1* mutant embryos appear to have normal epithelial cells. Four LAP family genes (*scrb1*, *erbin*, *densin-180* and *lano*) have been identified in mice (reviewed by Santoni et al., 2002). Therefore, it is possible that other LAP family genes may have overlapping or redundant functions in epithelial formation in vertebrate species. In zebrafish, at least four LAP family genes were also identified in the genome database (corresponding to *llk/scrb1*, *erbin*, *densin-180* and *lano*, data not shown). Putative zebrafish *erbin* and *lano* mRNA was strongly expressed maternally (data not shown), thus these genes are good candidates to compensate for loss of *Scribble1* function in epithelial polarity formation in vertebrates.

In *Crc/Scrb* mutant mice embryos neural tube closure is severely deficient (Murdoch et al., 2003). In contrast, there is no neural tube defect in zygotic or *MZ-llk* embryos in zebrafish. It is possible that unidentified zebrafish *scribble1* homologs may regulate neurulation independently of *llk/scrb1* function. Alternatively, neurulation in zebrafish may be achieved by mechanisms different from that in mice (reviewed by Lowery and Sive, 2004). In mice, a neural tube with an open ventricle lumen forms by folding of the neural plate epithelium. In contrast, in zebrafish, the neural plate forms a solid neural keel, then a lumen opens in its midline to form the tube (reviewed by Lowery and Sive, 2004). Thus, it is possible that *llk/scrb1* function may not be required for the teleost-specific neurulation steps.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/10/2273/DC1

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