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Slit and robo: expression patterns in lung development

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Abstract

First described as an axonal guidance cue through its repulsive effect on neurons expressing its receptor Roundabout (Robo), the Slit ligand has effects on cell migration, axon branching and elongation. Indirect evidence implicates Slit and Robo in lung development. We now demonstrate that *Slit-2* and *Slit-3* are developmentally regulated in embryonic murine lung. Immunohistochemistry demonstrates Slit-2 and Slit-3 expression by the pulmonary mesenchyme and airway epithelium. Robo-1 and Robo-2 are also expressed by the developing mesenchyme and airway epithelium. As lung development progresses, Robo-1 and Robo-2 expression localizes to only the airway epithelium. We conclude Slit/Robo are expressed in temporo-spatially adjacent domains suggesting interactive roles in pulmonary bronchiolar development.

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Keywords: Slit; Robo; Branching morphogenesis; Lung development

1. Results and discussion

The mammalian lung must provide a large surface area for gas exchange, yet maintain minimal volume and moisture loss. To achieve this dichotomy, the embryonic lung undergoes branching morphogenesis, a reproducible reiterative process of stereotypic branching culminating in alveolar development (Hogan, 1999).

The mechanism of branching morphogenesis is complex and involves migration of the pulmonary epithelium in response to mesenchymal signaling (Cardoso, 2001; Hogan, 1999; Rajagopal and Kinane, 2000; Warburton et al., 2000). Similar paradigms are seen in the developing nervous system. Neuronal migration and axonal projection are directed by cues from the midline (Bagri et al., 2002; Nguyen-Ba-Charvet and Chedotal, 2002; Plump et al., 2002; Wu et al., 1999). Akin to the mammalian nervous system, the developing lung undergoes extensive yet specific repetitive branching and growth (Cardoso, 2001; Hogan, 1999; Warburton et al., 2000). Slit/Robo interactions are responsible for axon guidance and it is plausible that they have a similar role in lung development.

First described in Drosophila, the secreted Slit ligand and its receptor (Robo) prevent developing neurons from inappropriately crossing the midline during patterning of the larval cuticle (Kidd et al., 1999; Rothberg et al., 1990; Seeger et al., 1993). Drosophila *Slit* mutants have longitudinal and commissural axons converging and coalescing at the midline (Kidd et al., 1999). *Robo* mutants have axons which cross the midline multiple times (Seeger et al., 1993). Homologs to *Slit* and *Robo* have been described in the mammalian central nervous system (Li et al., 1999).

There are three identified Robo trans-membrane receptors in mammals, Robo-1, Robo-2 and Rig-1 (Nguyen-Ba-Charvet and Chedotal, 2002). The Robo proteins have been shown biochemically and genetically to interact with the secreted Slit ligands (Kidd et al., 1998; Rajagopalan et al., 2000; Simpson et al., 2000). Three Slit homologs exist in mammals.

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Slit-2 is a 190 kDa protein composed of 1531 amino acids and murine *Slit-3* encodes a 1523 amino acid protein (Rothberg and Artavanis-Tsakonas, 1992; Yuan et al., 1999). *Slit-1* has extensive functional consequence for the developing brain; however, it is not expressed in murine lung (Nguyen-Ba-Charvet and Chedotal, 2002; Wu et al., 2001).

Although Slit-2 repulses migrating neurons and olfactory bulb axons, it also inhibits leukocyte migration induced by chemotactic factors (Hu, 1999; Li et al., 1999; Wu et al., 1999, 2001). Recent evidence exemplifies cell specific responses to Slit stimulation in that exogenous Slit-2 can either enhance or reduce axon growth and branching depending on affected cell type (Erskine et al., 2000; Ozdinler and Erzurumlu, 2002; Ringstedt et al., 2000; Wang et al., 1999; Whitford et al., 2002).

There is also mounting evidence that Slit–Robo interactions may be involved in non-neuronal morphogenesis. *Slit* and *Robo* expression has been demonstrated in developing somatic tissues (Holmes and Niswander, 2001; Yuan et al., 1999). *Slit-2*, *Slit-3*, and *Robo-2* are expressed in murine lung, some at levels equal to or greater than in adult brain (Wu et al., 2001). Mice homozygous for a deletion of the first Ig domain of Robo-1 frequently died of respiratory failure due to immature lungs and histology demonstrated thickened mesenchyme and reduced airspace (Xian et al., 2001). Robo-1 has been localized to murine pulmonary mesenchyme in embryonic lungs and to the epithelium in newborn and adult mice (Clark et al., 2002; Xian et al., 2001). Recently, several targets for unicellular tracheal branches in *Drosophila* have been shown to express Slit, resulting in either branch attraction or repulsion (Englund et al., 2002). Additionally, Robo and Robo-2 receptors were expressed in different branches and were required for correct orientation of the branch outgrowth (Englund et al., 2002). Therefore, present evidence implicates Slit–Robo interactions in mammalian airway development.

1.1. Transcription of Slit-2 and Slit-3 is developmentally regulated in the germinating murine lung

Key regulatory molecules are expressed throughout lung development at specific times and locations (Cardoso, 2000; Cardoso, 2001; Hogan, 1999; Warburton et al., 2000). We expect to obtain significant insight about the function of the *Slit* family by understanding the profile of expression. *Slit-2* transcription is developmentally regulated in the murine embryonic lung from a baseline level of transcription in the early pseudoglandular stage (e12–e16.6) (Fig. 1A). Transcription increases steadily through the canalicular stage (e16.6–e17.4), reaching a peak in the early saccular stage (e17.5–postnatal day 5) of lung development. Expression of *Slit-2* in day e18 lung is much greater than that of adult brain (Fig. 1A). Although *Slit-2* is transcribed during branching morphogenesis, its transcription level is highest during the saccular stage in which branching is superceded by terminal bud growth and differentiation. A profile for *Slit-3* was also

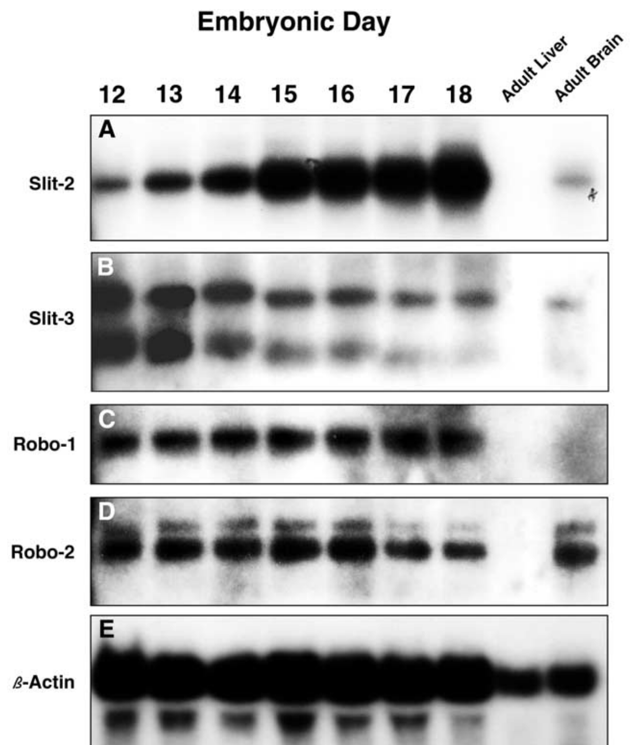

Fig. 1. Slit-2 and Slit-3 are developmentally regulated in murine embryonic lung development. Expression of *Slit-2* increases from day e12 until e18 (A). *Slit-3*, however, peaks early in lung development and decreases until birth (B). *Robo-1* and *Robo-2* transcription is uniform throughout development (C,D). Adult brain total RNA served as positive control and adult liver total RNA served as negative control. Equal loading was confirmed by probing with *β-Actin* (E).

obtained (Fig. 1B). A splice variant of *Slit-3* was expressed during lung development. The expression profile of *Slit-3* is highest at day e13, and it displays a downward trend until day e18 (Fig. 1B). Whereas *Slit-2* expression increases in mid to late gestation, *Slit-3* is dominant early in branching morphogenesis. This overlapping expression of *Slits* has been previously seen in other organ systems, including chick limb development (Vargesson et al., 2001). Robo-1 and Robo-2 have been determined as mammalian receptors for Slit-2 and Slit-3. The expression profiles of the receptors were uniform from days e12 to e18 with respect to *β-Actin* (Fig. 1C–E).

1.2. Slit-2 is expressed in the mesenchyme and on the apical aspect of the epithelium

The precise location of critical developmental factors often provides functional information. An antibody to the N-terminal cleavage fragment of Slit-2 was used for immunohistochemistry and illustrated expression throughout the mesenchyme on days e12 and e14 (Fig. 2A–D). Interestingly, the epithelium also demonstrated an immunofluorescent ring on the cell apices adjacent to the bronchiolar lumen, suggesting Slit-2 secretion into the airway lumen (Fig. 2B, D). By day e16, Slit-2 is expressed

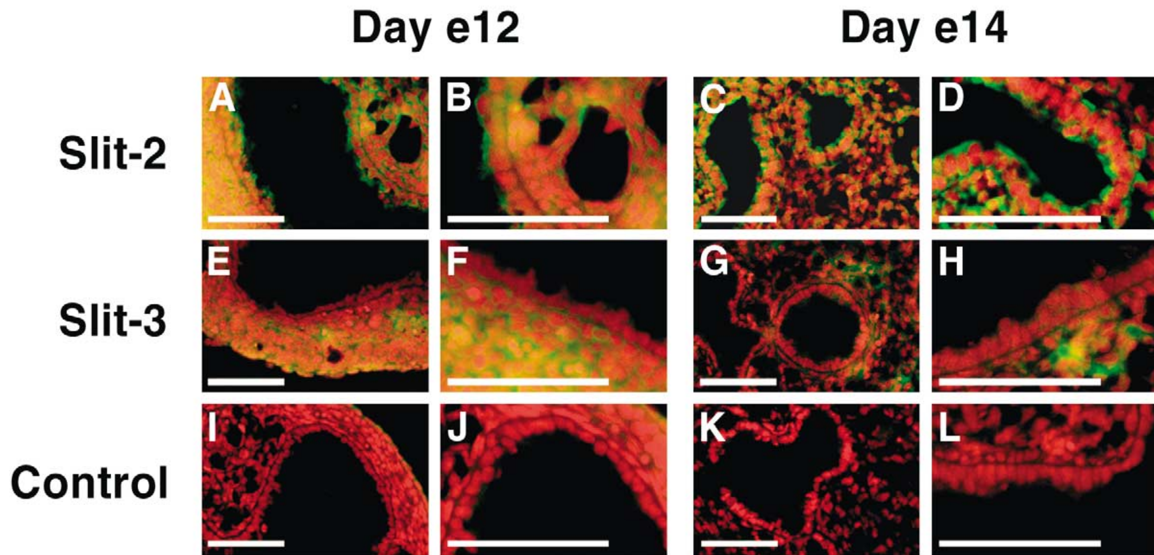

Fig. 2. On days e12 and e14, Slit-2 and Slit-3 are expressed in the mesenchyme, although Slit-2 is highly expressed by the bronchiolar epithelium with respect to Slit-3. Immunolocalization of Slit-2 demonstrates protein expression in the mesenchyme (A–D). It is also expressed on the apical surface of the bronchiolar epithelium (A–D). Slit-3 is expressed by the mesenchyme (E–H) and epithelium in a limited manner (G,H). Goat IgG served as primary antibody in the control group with FITC-labeled secondary antibody (Green) and nuclei stained with propidium iodide (Red) (I–L). Scale bar equals 0.1 mm.

in the mesenchyme and in the airway lumen (Fig. 3A, B). On day e18, one day before birth, Slit-2 is expressed in the saccular mesenchyme but a peri-luminar array is the dominant pattern (Fig. 3C, D).

1.3. Slit-3 expression is expressed primarily by the mesenchyme

Slit-3 localization was performed with an antibody recognizing an internal region of the protein. Slit-3 is expressed in the mesenchyme and surrounds the primordial airways of the early pseudoglandular stage (e12–e14) (Fig. 2E–H). By the late pseudoglandular stage (e16), there is limited expression of Slit-3 protein on the luminal aspect of the bronchiole (Fig. 3E, F). Slit-3 fluorescent signal is relatively weaker by the saccular stage (e18), although it is still expressed in the saccular interstitium (Fig. 3G, H). Control sections were incubated with Goat IgG as primary antibody and displayed low amounts of background signal in the mesenchyme (Figs. 2 and 3A–D).

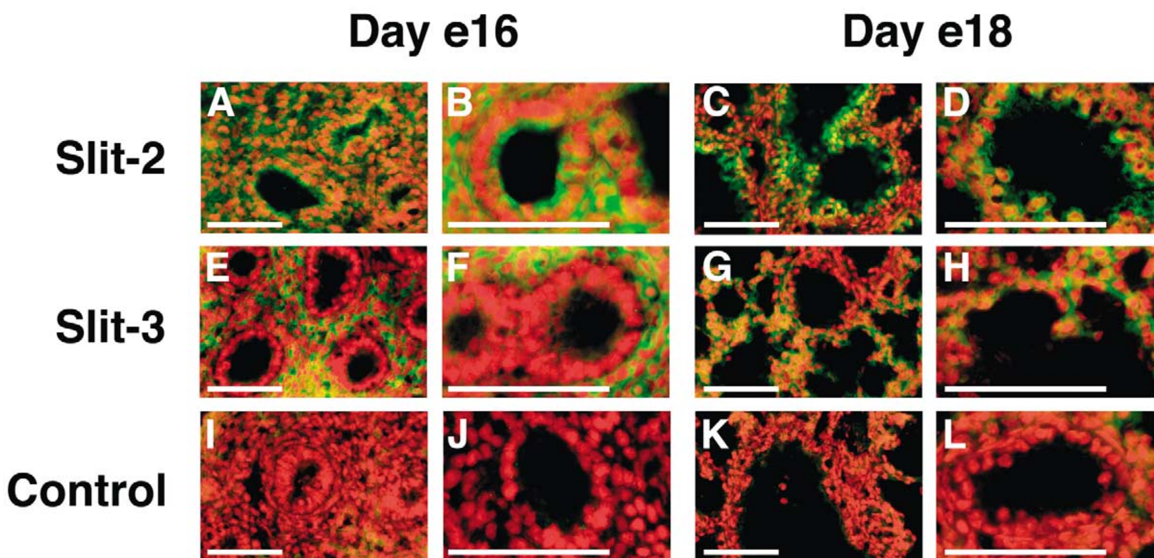

Fig. 3. In later gestation Slit-2 and Slit-3 continue to be expressed in the mesenchyme, although Slit-2 is highly expressed by the airway epithelium (A–D) as compared to Slit-3 (E–H). Immunolocalization of Slit-2 on day e16 demonstrates protein expression in the mesenchyme and on the apical surface of the bronchiolar epithelium (A,B). By day e18, Slit-2 is expressed by the epithelium surrounding the large airways (C,D). Slit-3 is expressed in the mesenchyme and in the bronchiolar lumen in a limited manner (E–H). Goat IgG served as primary antibody in the control group with FITC-labeled secondary antibody (Green) and nuclei stained with propidium iodide (Red) (I–L). Scale bar equals 0.1 mm.

1.4. Robo-1 and Robo-2 are expressed by both the mesenchyme and the epithelium during the late embryonic and early pseudoglandular stages

Using an antibody that recognizes the amino terminus of the Robo-1 protein, expression was localized to the mesenchyme and apical aspect of the pulmonary epithelium on days e12 and e14 (Fig. 4A–D). Robo-2 is similarly expressed by the mesenchyme; however, the epithelial fluorescence demonstrated by Robo-2 on day e12 is more intense than Robo-1 (Fig. 4A, B, E, F). Robo-2 is expressed in the mesenchyme and epithelium on day e14 (Fig. 4G, H). Control sections were incubated with goat IgG as the primary antibody and FITC-tagged donkey anti-goat antibody as the secondary and demonstrated little background (Fig. 4I–L).

1.5. The pattern of expression of Robo-1 and Robo-2 changes into the saccular stage of lung development

On day e16, Robo-1 and Robo-2 are expressed in the mesenchyme (Fig. 5A, B, E, F). More evident, however, was the expression pattern provided by the bronchiolar epithelium. The apical and basal aspects of the airway epithelium demonstrated strong expression of the Robo receptors (Fig. 5A, B, E, F). By the saccular stage of development (day e18), Robo-1 and Robo-2 expression change in that the proteins localize only to the luminal aspects of the epithelium (Fig. 5C, D, G, H). Such a shift in location of Robo-1 receptor with development has been documented in the developing murine kidney (Piper et al., 2000). In metanephric explant cultures using in situ hybridization, *Robo-1* expressing cells were found initially throughout the metanephric mesenchyme. Expression shifted to the pretubular aggregates and finally to the distal end of the comma and S-shaped bodies (Piper et al., 2000). A similar model seems to exist in the developing lung. Therefore, in late gestation, the Robo receptors are expressed on the apical aspects of the pulmonary epithelium adjacent to the ligand Slit-2. Goat IgG served as the primary antibody in the control group (Fig. 5I–J).

The patterns of expression of Slit/Robo in mammalian lung are indicative of a role in branching morphogenesis and airway development.

2. Experimental procedures

2.1. Isolation of lung RNA

Timed pregnant Swiss-Webster mice (Taconics Laboratories, NY) were sacrificed according to the animal protocol for the institution. Documentation of the vaginal plug was determined as embryonic day 0. Embryonic lungs were retrieved under a dissecting microscope and stored in RNAlater solution (Qiagen, Inc., CA). Total RNA was extracted using RNeasy kit (Qiagen, Inc., CA) and pooled samples representing each day from e12 to e18 as well as adult brain and liver were isolated. RNA integrity was assessed from appearance of ethidium bromide-stained ribosomal bands following fractionation on a 1.2% (wt/vol) agarose–formaldehyde gel (Buch et al., 2000).

2.2. PCR-generated molecular probes

A murine *Slit-2* cDNA fragment 1.3 kb in length cloned

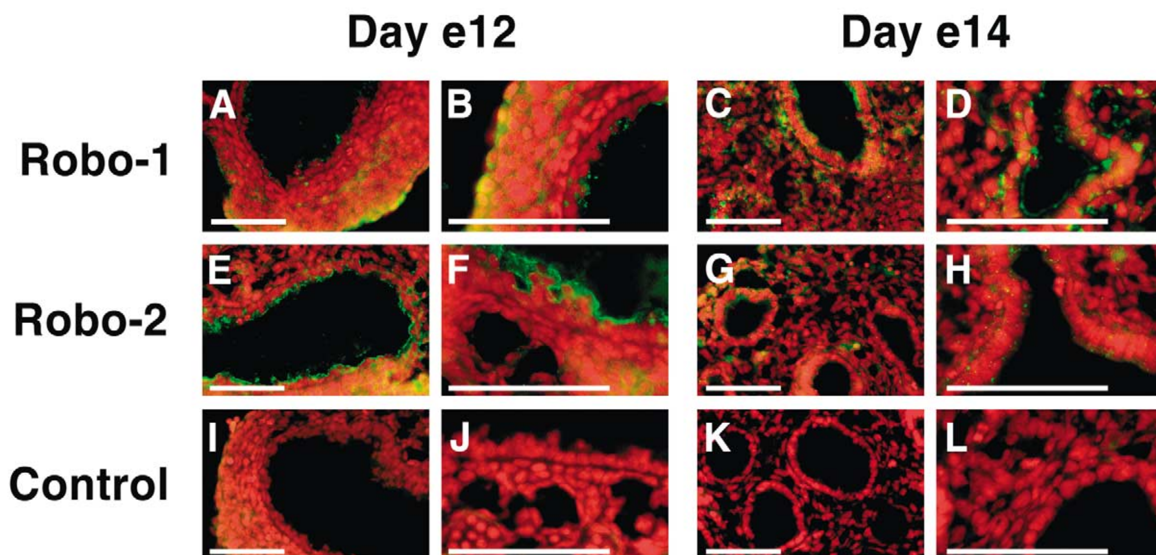

Fig. 4. Robo-1 and Robo-2 are expressed in the mesenchyme and by the epithelium in early lung development. Robo-1 and Robo-2 are expressed in the mesenchyme and by main bronchus epithelium on day e12 (A,B,E,F). By day e14, Robo-1 and Robo-2 continue to be expressed by the epithelium, however, the fluorescent intensity seen in the mesenchyme is less pronounced (C,D,G,H). Goat IgG served as primary antibody in the control group with FITC-labeled secondary antibody (Green) and nuclei stained with propidium iodide (Red) (I–L). Scale bar equals 0.1 mm.

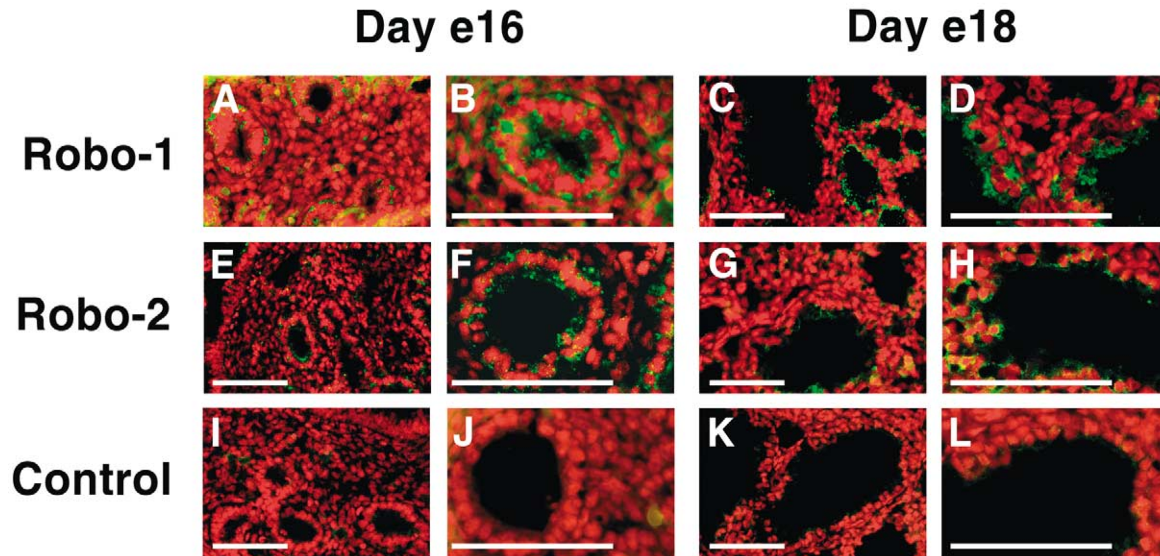

Fig. 5. Robo expression pattern changes during the transition into the saccular stage. On day e16, Robo-1 and Robo-2 are expressed by the airway epithelium on both the basal and apical aspects of the cell (A,B,E,F). However, by day e18, Robo-1 and Robo-2 expression is restricted to the apical aspects of the epithelium (C,D,G,H). Control sections were incubated with Goat IgG as the primary antibody and FITC-labeled secondary antibody (Green). Nuclei were stained with propidium iodide (Red) (I–L). Scale bar equals 0.1 mm.

into a pGEM-T vector (Promega, WI) and a murine *Slit-3* cDNA fragment (1.9 kb) cloned into a pBluescript SK vector (Stratagene, CA) served as templates for generation of probes for *Slit-2* and *Slit-3*. Separate pBluescript SK vectors (Stratagene, CA) containing rat cDNAs for *Robo-1* (1.0 kb) and *Robo-2*(1.7 kb) served as templates for Robo-1 and Robo-2 probe generation. All four vectors were supplied as gifts from M. Tessier-Lavigne (Stanford). The pUC/M13 forward and reverse sequencing primer sites were utilized to construct primers flanking the multiple cloning sites of both vectors. Polymerase chain reaction (PCR) was performed separately for each vector using the following primers: 5′-ATTCGCCATTCAGGCTGCG-3′ and 5′GTCCTTTGTCGATACTGGTACTAATGC-3′. The flanking primers added approximately 0.3 kb to the probe size. Therefore, the PCR product using the *Slit-2* pGEM-T vector provided a 1.6 kb product. The product of the reaction using the *Slit-3* pBluescript SK vector was 2.2 kb in length. PCR of the *Robo-1* containing pGEM-T vector provided a 1.3 kb fragment and PCR of the *Robo-2* containing vector provided a 2.0 kb fragment. The PCR-generated fragments were labeled with [^{32}P]dCTP (NEN Life Science, MA) by random hexamer priming with High Prime (Roche Diagnostics, Switzerland), and used as hybridization probes.

2.3. Northern blot hybridization

RNA was separated by electrophoresis as above and transferred to GeneScreen Plus membranes (DuPont NEN, MA). Membranes were prehybridized for 1 h at 42°C in the presence of ULTRAhyb (Ambion, Inc., TX). Hybridization was performed under similar conditions for 24 h in the presence of labeled *Slit-2*, *Slit-3*, *Robo-1* or *Robo-2* probes in separate experiments. After hybridization, membranes were washed twice for 30 min each in 2 × SSC, 0.1% SDS, then twice for 30 min each in 0.1 × SSC, 0.1% SDS at 48°C. The membranes were dried and autoradiographed with Kodak XAR film at −80°C for 6–96 h with Cronex Lightning Plus intensifying screens. Equal loading was confirmed by probing with *β-actin* as control and each study was repeated three times.

2.4. Immunohistochemistry

Lungs extracted as above from days e12, e14, e16 and e18 were fixed in 4% paraformaldehyde for 4 h at 4°C followed by four washes in 7% sucrose in PBS at 4°C. The fixed tissue was then washed in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) for 5 min. Tissue was then placed in Cryomolds (Miles, Elkhart, IN) which were then filled with tissue freezing medium and then frozen at −70°C. Sections of 6 μm were cut with a Minotome cryostat (Damon/IEC Division, Needham Heights, MA), and sections were air dried and stored at 4°C. Air-dried sections were permeabilized with 0.2% Triton X-1-00 in PBS, washed three times, and blocked with 5% donkey serum (Jackson ImmunoResearch, PA) for 2 h. All primary antibodies were purchased from Santa Cruz Biotechnology, CA. Slit-2 (sc-16619), Slit-3 (sc-16624), Robo-1 (sc-16611) and Robo-2 (sc-16615) goat anti-mouse antibodies were added at a dilution of 1:50 in PBS containing 2% BSA (PBS/2% BSA) at 4°C in a humidified chamber overnight while experimental negative controls were incubated in PBS/2% BSA with Goat IgG (1:50) (Santa Cruz Biotechnology, CA). After three washes in

PBS, the secondary antibody (FITC donkey anti-goat (Jackson ImmunoResearch, PA) was added at a dilution of 1:200 for 1 h. After three washes with PBS, sections were incubated with RNAse A for 5 min before addition of nuclear stain, propidium iodide (Sigma, MO) diluted 1:1000 in PBS for 5 min. Sections were washed three more times in PBS. Sections were analyzed with a Nikon microscope and photographed with a Spot digital camera (Diagnostic Instruments, MI). Images were imported into Adobe Photoshop (Adobe Inc, CA) (Kinane et al., 1999; Kling et al., 2002).

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