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Robo4 is a vascular-specific receptor that inhibits endothelial migration

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Abstract

Guidance and patterning of axons are orchestrated by cell-surface receptors and ligands that provide directional cues. Interactions between the Robo receptor and Slit ligand families of proteins initiate signaling cascades that repel axonal outgrowth. Although the vascular and nervous systems grow as parallel networks, the mechanisms by which the vascular endothelial cells are guided to their appropriate positions remain obscure. We have identified a putative Robo homologue, Robo4, based on its differential expression in mutant mice with defects in vascular sprouting. In contrast to known neuronal Robo family members, the arrangement of the extracellular domains of Robo4 diverges significantly from that of all other Robo family members. Moreover, Robo4 is specifically expressed in the vascular endothelium during murine embryonic development. We show that Robo4 binds Slit and inhibits cellular migration in a heterologous expression system, analogous to the role of known Robo receptors in the nervous system. Immunoprecipitation studies indicate that Robo4 binds to Mena, a known effector of Robo-Slit signaling. Finally, we show that Robo4 is the only Robo family member expressed in primary endothelial cells and that application of Slit inhibits their migration. These data demonstrate that Robo4 is a bona fide member of the Robo family and may provide a repulsive cue to migrating endothelial cells during vascular development.
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Introduction

Intraembryonic vascular development begins when endothelial cells differentiate and coalesce into tubes that form the central axial vessels, the dorsal aortae, and cardinal veins. Subsequently, in a process termed angiogenesis, endothelial tubes sprout from these central vessels to form an intricate pattern that is highly conserved within and between vertebrate species. Molecular signals that regulate endothelial sprouting and maturation have been identified and include vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), and angiopoietins (Yancopoulos et al., 2000). Recently, it has been reported that the vasculature not only provides an efficient transport system for nutrients and waste, but also provides inductive signals required for the formation of organs such as the pancreas and liver (Lammert et al., 2001; Matsumoto et al., 2001). Thus, the timing and the specific trajectories of the vessels are critical for orchestrating organogenesis. Though it is not currently understood how vessels choose specific paths to reach their final destinations, the regimented and conserved pattern of the vascular network suggests that specific genetic programs coordinate its formation (Weinstein, 1999).

Similar to the vascular system, the peripheral nervous system of the trunk forms from central structures such as the neural tube and dorsal root ganglia. The neurons of these

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structures sprout axons to distal targets to form a complex and highly reproducible network. Nerves often follow paths parallel to blood vessels, as illustrated by the neurovascular bundles that course toward the distal limbs (Martin and Lewis, 1989; Mukouyama et al., 2002). The formation of the nervous system is regulated by a series of molecular programs that guide growth cones using a combination of attractive and repulsive cues. Major classes of neuronal guidance molecules include the Netrins, Semaphorins, Ephrin, and Slits, and these ligands interact with cognate families of receptors (Chisholm and Tessier-Lavigne, 1999; Yu and Bargmann, 2001). The three Slit ligands identified to date provide chemorepulsive signals for several classes of axons and neuronal cells via members of the Roundabout (Robo) receptor gene family. In vitro experiments show that Slit-Robo signaling can have a potent effect on axonal branching, extension, and repulsion (Brose and Tessier-Lavigne, 2000; Wang et al., 1999). In vivo experiments in *Drosophila* and zebrafish demonstrate that the *Slit* and *Robo* genes are highly expressed in the nervous system and are required for neural guidance and migration (Challa et al., 2001; Fricke et al., 2001; Kidd et al., 1998; Lee et al., 2001; Rajagopalan et al., 2000a,b; Rothberg et al., 1988). In addition to their expression and function in the nervous system, Slits and Robos may function in invertebrate muscle development and provide chemotactic signals to muscle cells and leukocytes (Holmes and Niswander, 2001; Kidd et al., 1999; Kramer et al., 2001; Vargesson et al., 2001; Wu et al., 2001). Thus, the Slit-Robo signaling pathway is important for both neuronal and nonneuronal cell migration.

Insight into whether analogous guidance programs exist for the vasculature can be gained by examining mutants in which the paths of sprouting blood vessels are disrupted. Normally, arteries and veins sprout from the primitive vasculogenic vessels to form parallel but distinct vascular networks that only interconnect at the capillary beds of the target organs (Gale and Yancopoulos, 1999). Patients afflicted with hereditary hemorrhagic telangiectasia (HHT) suffer from multiple arteriovenous malformations: direct and abnormal connections between arterial and venous vascular beds. This autosomal dominant vascular dysplasia is caused by loss-of-function mutations in the *activin receptor-like kinase 1 (Alk1)* gene that encodes a member of the transforming growth factor beta superfamily of receptors (Johnson et al., 1996). We have previously demonstrated that mice lacking *Alk1* lose the morphological, molecular, and functional distinctions between arteries and veins. These mice die during midgestation due to angiogenic defects characterized by aberrant fusion of sprouting arterial and venous endothelial tubes (Urness et al., 2000). These observations led us to hypothesize that genes responsible for guiding endothelial tubes to their appropriate destinations might be differentially expressed in $Alk1^{+/+}$ and $Alk1^{-/-}$ embryos.

Here, we have identified a putative Robo homologue, Robo4, based on its differential expression in these vascular sprouting mutants. In silico database mining previously identified Robo4 as a gene expressed in endothelial cell lines, yet no functional characterization of Robo4 during development has been undertaken (Huminiecki et al., 2002). Unlike the previously characterized Robo receptors that are expressed and function in nervous system, we show that Robo4 expression is restricted to the vasculature during embryonic development. Furthermore, although Robo4 shares similar domains with known neuronal Robo receptors, the number and spatial localization of these domains diverge significantly. Despite its unusual expression pattern and divergent ligand-binding domain, we provide evidence that Robo4 shares functional characteristics with other Robo receptors based on ligand-binding and cell biological assays. Analogous to the roles played by other Robo receptors in the nervous system, we show that Robo4 mediates inhibition of vascular endothelial cell migration. Together, our data suggest that Robo4 is a vascular-specific Robo receptor that plays a role in angiogenesis and vascular patterning.

Materials and methods

Expression analysis of Robo4

Differential display was performed on total RNA that was isolated from $Alk1^{+/+}$ and $Alk1^{-/-}$ mice embryos and yolk sacs (E9.0, E9.5, and E10.0) using TRIZOL (Gibco-BRL). The RNA was reverse-transcribed with the RETROscript kit (Ambion), and DD-PCR was performed by using the GenHunter Kit (Ambion). Staging of embryos, in situ hybridization, paraffin section, and whole-mount immunohistochemistry were performed as described previously (Urness et al., 2000). Immunohistochemistry on mouse adult sections was performed as described previously (Murphy et al., 2001). For Northern blot analysis, 20 μg of total RNA was loaded per lane. The multiple tissue and murine embryonic Northern blots were purchased from Clontech. The *Robo4* probe spanned nucleotides 1634 and 2143 from the start codon of the mouse Robo4 cDNA.

Cloning of the zebrafish Robo4 gene and phylogenetic analysis of the Robo family members

A BLAST search of the zebrafish expressed sequences tag (EST) database originally yielded three clustered clones: fc58b09, fa16f09, and fb97a08. fc58b09 was obtained from the Resource Center for the German Human Genome Project (RZPD). Sequencing revealed that this clone comprises the complete coding sequence for *Robo4*, interrupted by an unspliced exon and a premature stop codon (presumably a Reverse-Transcriptase error). This was confirmed by comparison with the genome sequence database (http://www.sanger.ac.uk) and RT-PCR from wild-type zebrafish embryos using primers spanning the questionable regions, followed by sequencing. The fa16f09 clone has

been mapped to LG10 both by radiation hybrid mapping and meiotic mapping (http://zfin.org). Multiple sequence alignment of *Robo4* with known *Robo* family members using the CLUSTALW algorithm was done by using the biology Workbench site (http://workbench.sdsc.edu).

HEK cell culture and transfection

HEK 293 cells were maintained in Dulbecco's modified eagle's medium (DMEM) (Gibco-BRL) with 10% fetal bovine serum (FBS). Cells were grown to 70% confluency on six well plates and transfected with 2 μg plasmid/well by using FuGene-6 (Roche) according to the manufacturer's instructions. The transfected plasmids contained mouse *Robo4* tagged at the carboxy terminus with hemaglutinin (HA) (Robo4-HA) or the N-terminal extracellular domain of *Robo4* tagged with HA (NRobo4-HA), or control vector, pcDNA3 (Invitrogen). Stable cell lines were selected with 300 μg/ml of G418 for 3 weeks. HEK cells expressing human Slit2-myc and the extracellular domain of rat Robo1 fused to HA (N-Robo1) were described previously (Wu et al., 2001). Conditioned media from stable cell lines were collected 48 h after adding serum-free media, and then concentrated by using Biomax-30K ultrafree-15 filters (Millipore). A NGF-responsive chimeric Robo4 plasmid was constructed by fusing the cytoplasmic and transmembrane domains of mouse Robo4 with the ectodomain of trkA receptor tyrosine kinase, a receptor for nerve growth factor (NGF). Stable cell lines were selected with 200 μg/ml of hygromycin for 4 weeks and confirmed by Western blotting.

Generation of polyclonal antibodies, immunoprecipitation, and Western blotting

Rabbit polyclonal antibodies, raised against amino acids 964–981 of the mouse Robo4 protein, were affinity purified (Biosource International). Immunoprecipitation was carried out by using anti-HA antibody (Babco) and Protein A/G beads (Santa Cruz Biotech) with the same amounts of cell lysates or conditioned media from each cell. Conditioned media were collected in the same manner as above. Lysates from Robo4-HA, Slit-myc cells, and control cells were prepared with lysis buffer [0.5% NP-40, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and protease inhibitor cocktail (Roche)]. Precipitated proteins were detected by Western blotting with anti-myc antibody (Santa Cruz biotech) with enhanced chemiluminescence (Amersham). Immunoprecipitation studies were performed to assess a Robo4:Mena interaction. Cell lysates of chimeric Robo4-HA-expressing cells (chimeric Robo4-HA) and empty-vector transfected cells (vector) were immunoprecipitated with an anti-HA affinity matrix (Roche). Robo4-Mena complexes were detected by Western blot analysis using an anti-Mena antibody (Transduction Laboratories).

Demonstration of Robo4 antibody specificity by peptide blocking

Anti-Robo4 antibodies were incubated with 10-fold excess of either epitope peptide (EWRPDWLEDAEISHTQRL) or control peptide (EQAMEQSARDPRKHV) for 24 h at 4°C. Immune complexes were removed by centrifugation (15,000 rpm) at 4°C, and the resultant supernatant was analyzed by Western blot analysis to verify effective absorption of antibodies specific for Robo4.

Detection of cell surface binding

Stable cell lines expressing Robo4-HA (Robo4-HEK), or the pcDNA3 vector alone (Control-HEK), were seeded in six well culture dishes precoated with 100 μg/ml poly-L-lysine. Cells were incubated with HEK CM or Slit-myc CM at 37°C. After 1 h incubation with conditioned media, followed by three washes in PBS, cells were fixed in 4% paraformaldehyde for 20 min. Cells were then washed three times with PBS and incubated with mouse *anti*-myc primary antibody (Santa Cruz Biotech) and *anti*-mouse Alexa 594-conjugated secondary antibody (Molecular Probes).

Chemotaxis

Slit-myc CM and HEK CM were collected and concentrated as described above. Twenty-four well, 8-μm (Falcon) pore transwell inserts were used for chemotaxis. Robo4-HEK or control-HEK cells were seeded at 20,000 cells/upper chamber, and HEK-CM, or Slit-myc CM, or 10 ng/ml of fibroblast growth factor (FGF) were added to the lower chamber. After incubation at 37°C for 2 h, filters were fixed with Zamboni's fixative and stained with a Hema 3 Kit (Fisher). All migrated cells were counted by reviewers blinded to the experimental conditions. The migrated cell numbers were expressed as the fold increase in migration over control per five fields at 400× magnification. Migration data are the average of at least three independent experiments each performed in triplicate. Migration of Chimeric Robo4 and control cells was measured by using 2% FBS (fetal bovine serum) as a positive factor. HMVEC migration was carried out in the same manner as above, except that vascular endothelial growth factor (VEGF, 10 ng/ml) was used as the positive control. Two independent HMVEC clones were used for chemotaxis. For depleting Slit-myc protein, Slit-myc CM was incubated with N-Robo1-HA CM or HEK CM for 30 min with anti-HA antibodies and protein A/G beads (preincubated with HEK CM) at 4°C. Similarly, Slit-myc CM was incubated with protein A/G beads (Santa Cruz Biotech), preincubated with HEK CM, and with anti-myc antibodies for 30 min at 4°C. N-Robo1-Slit and anti-myc-Slit complexes were removed by centrifugation. This process was repeated twice before applying the depleted conditioned media to cells. Anti-HA antibodies (Babco) were used as a mock control in anti-myc

depletion experiments. Effective depletion of conditioned media was confirmed by Western blotting using anti-myc antibodies.

Results

Robo4 was identified on the basis of differential expression in Alk1$^{-/-}$ mice

To identify genes that may regulate endothelial sprouting, we screened for differential gene expression in *Alk1*$^{+/+}$ and *Alk1*$^{-/-}$ embryos during midgestation (E8.5–E10.0). This screen utilized differential display, reverse transcriptase-polymerase chain reaction (RT-PCR), Northern blot analysis, and in situ hybridization. One gene, *Robo4*, was upregulated fivefold in *Alk1*$^{-/-}$ tissues and encoded a protein with sequence and structural similarities to members of the Roundabout family of neural guidance receptors (Fig. 1A and Suppl. Fig. 1). In parallel, we also identified the zebrafish *Robo4* in a bioinformatic search for zebrafish *Roundabout* homologs (Suppl. Fig. 1). The human ortholog was identical to Unigene cluster HS.111518 that others, using serial analysis of gene expression, found to be expressed in endothelial cell lines and tumor vessels (Huminiecki et al., 2002). However, the expression profile during embryonic development is completely unknown.

To further investigate the differential expression of Robo4 in wild-type and Alk1$^{-/-}$ embryos, in situ hybridization was performed. In wild-type murine embryos, *Robo4* is moderately expressed in the dorsal aorta (red arrowhead) and well-organized intersomitic (white arrowhead) and capillary vessels (Fig. 1B). *Robo4* is highly expressed in the disorganized and dilated intersomitic and capillary vessels in *Alk1*$^{-/-}$ embryos (Fig. 1C). Immunofluorescence studies using an anti-PECAM antibody (green) revealed that the intersomitic vessels in *Alk1*$^{-/-}$ embryos anomalously dilate and sprout into the somites rather than remaining confined to the intersomitic boundaries as shown in *Alk1*$^{+/+}$ embryos (white arrowhead) (Fig. 1D and E).

The domain structure of Robo4 protein diverges from other Robo family members

There are important similarities and differences between Robo4 and other Robo family members. The previously identified members of the Robo family, Robo1, Robo2, and Robo3, share approximately 45–50% amino acid identity and 50–60% similarity with one another. In the cytoplasmic region of Robo receptors, there are four conserved domains, CC0–CC3, and these are implicated in signal transduction (Bashaw et al., 2000; Kidd et al., 1998; Wong et al., 2001). Robo4 lacks CC1 and CC3 but encodes CC0 and CC2. The extracellular regions of Robo1, Robo2, and Robo3 are conserved among human, mice, zebrafish, *Drosophila*, and *Caenorhabditis elegans*, and these ligand-binding regions are characterized by five immunoglobulin (IgG) and three fibronectin (FN) domains (Kidd et al., 1998; Lee et al., 2001; Sundaresan et al., 1998; Yuan et al, 1999a,b; Zallen et al., 1998). In contrast, the Robo4 extracellular, or ligand-binding domain, diverges significantly from other Robo family members; murine and human *Robo4* encode two IgG and two FN domains, while the zebrafish *Robo4* encodes three IgG and two FN domains (Fig. 1F). A molecular phylogenetic analysis using the first two IgG domains of all the Robos and *CDO* genes (the closet known IgG superfamily genes that are not *Robo*) suggests that human, mouse, and zebrafish *Robo4* genes are more closely related to the *Robo* superfamily than the *CDO* genes (Fig. 1G). However, this analysis also reveals that the IgG domain sequences of Robo4 significantly diverge from those of the other three Robo family members. Given these marked differences in the extracellular region of the putative Robo4 subfamily, further investigation was needed to determine whether *Robo4* is a true *Robo* family member.

Robo4 is specifically expressed in the embryonic vasculature

To provide the framework for understanding the role for Robo4 in development, we began by investigating the expression of *Robo4* during embryonic development. In situ hybridization detected *Robo4* mRNA in the vascular endothelium. *Robo4* shows a dynamic expression pattern within vessels, with expression starting in the larger axial vessels and intersomitic vessels at earlier ages, and changing to intersomitic vessel and capillary expression at later stages. At E9.0, *Robo4* is expressed in the central vessels, the dorsal aorta (DA, red arrowhead), and intersomitic vessels (ISV, white arrowhead) in both whole-mount preparations and cross-sections (Fig. 2A and D). At E9.5, although *Robo4* mRNA was still primarily detected in the intersomitic vessels (ISV), some staining of capillaries was observed (Fig. 2B). In cross-section at E9.5, *Robo4* is highly expressed in intersomitic vessels with little expression remaining in dorsal aortae (Fig. 2D). By E10.0, *Robo4* is detected in capillary vessels, the capillary plexus of the limb buds (LB), and throughout the endothelium as microvessels sprout from the dorsal aortae (Fig. 2C and F). This expression profile suggests that Robo4 activity may be important in the process of sprouting angiogenesis. As a comparison with a known marker of the vascular endothelium, the distribution of *Robo4* mRNA in the trunk region at E9.5 is similar to the expression pattern of *Flk1*, a VEGF receptor (Fig. 2G). Importantly, no expression of *Robo4* was detected in the neural tube at E9.0 and E9.5. However, Robo4 mRNA is detected within the capillaries sprouting into the neural tube, as well as in the adjacent perineural capillary plexus at E10.0 (Fig. 2F). In contrast, *Robo1, Robo2*, and *Robo3* are highly expressed in the nervous system of developing mice, chick, and zebrafish, consistent with their roles in neuronal migration and axonal guidance (Holmes and Niswander,

2001; Lee et al., 2001; Sundaresan et al., 1998; Vargesson et al., 2001; Yuan et al., 1999a,b; Zallen et al., 1998). To confirm that the observed transcript distribution reflects the expression pattern of Robo4 protein, we raised polyclonal antibodies against murine Robo4 (see Fig. 6A) and performed immunohistochemistry. We detected Robo4 protein in endothelial cells of the intersomitic vessels (ISV) (Fig. 2H). Northern blot analysis indicated that *Robo4* is transcribed as an approximately 4-kb mRNA throughout embryogenesis (Fig. 2I). These results demonstrate that murine *Robo4* expression is confined to the endothelium during early vascular development and that this expression does not overlap with that of the other *Robo* family members.

Robo4 is predominantly expressed in the vasculature of adult tissues

To further investigate the expression pattern of Robo4 in adult tissues, we performed immunohistochemistry on heart, lung, and brain of adult mice using our Robo4 antibody. In the heart, Robo4 is expressed in both the vascular endothelium and the surrounding medial layers of blood vessels (Fig. 3B). Similarly, the Robo4 antibody stained blood vessels (V) and smooth muscle cells surrounding the bronchi (Br) in the lung (Fig. 3E). In the brain, Robo4 is expressed strongly in the vascular smooth muscles cells and little in vascular endothelial cells (Fig. 3H). Unlike the other Robo family members, Robo4 is not expressed in the brain parenchyma. We used two controls to verify the specificity of Robo4 antibodies. Control experiments using preimmune serum (Fig. 3A, D, and G) and antibody blocked with epitope peptide (Fig. 3C, F, and I) completely abrogate Robo4 staining in all cases. Both control peptide-blocked and epitope peptide-blocked antibodies were verified by Western blotting to assess the resultant specificity of the antibody preparation. Control peptide-blocked antibodies detected Robo4 protein, whereas epitope peptide-blocked antibodies did not (data not shown). Finally, Northern blot analysis indicates that *Robo4* expression is highest in the heart, intermediate in the liver, kidney, and lung, and low or undetectable in the brain, skeletal muscle, spleen, and testis (Fig. 2J). In contrast, Northern blot analysis of *Robo1* mRNA expression demonstrated prominent brain and skeletal muscle expression consistent with previous reports (data not shown; Sundaresan et al., 1998). These data, in conjunction with the embryonic expression profile, indicate that Robo4 is predominantly expressed in vasculature.

Robo4 binds Slit2, a known ligand of the Robo receptor family

The Robo receptors are defined by both their sequence similarities and their common ligands, the Slit family of secreted proteins (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Simpson et al., 2000; Wu et al., 1999; Yuan et al., 1999b). Robo4 has similar domains in its cytoplasmic region; however, the arrangement of two IgG and two FN domains in the ligand-binding region is quite different from the five IgG and three FN of the canonical Robo family. Despite similarities in sequence between Robo1 and Robo4 (Suppl. Fig. 1), the marked differences in the structure of the extracellular, or ligand-binding domain, and dramatically different expression profile, suggested that Robo4 may have unique biochemical and functional characteristics relative to other Robo family members. To determine whether Robo4 can function as a canonical Robo receptor, as defined by interaction with Slit ligands, we performed immunoprecipitation experiments to investigate whether Robo4 binds Slit. cDNAs encoding full-length hSlit2 tagged with a myc-epitope (Slit-myc), full-length Robo4 tagged with a hemagglutinin epitope (Robo4-HA), and the soluble ligand-binding ectodomain of Robo4 (NRobo4-HA) were each transfected into human embryonic kidney-293 (HEK) cells. The expression of Robo4-HA was confirmed by Western blotting using our polyclonal anti-Robo4 antibodies and anti-HA antibodies (see Fig. 6A). Cell lysates of Slit-myc-expressing cells were incubated with cell lysates of Robo4-HA-expressing cells (Robo4-HEK), and Robo4-HA receptor complexes were immunoprecipitated with an anti-HA antibody. Western blot analysis, using an anti-myc antibody, detected coimmunoprecipitated Slit-myc protein (Fig. 4A, lane 6). To further confirm this interaction, we predicted that the extracellular region (NRobo4-HA) should be able to bind secreted Slit protein. Indeed, Slit-NRobo4-HA complexes were detected when serum-free-conditioned media from Slit-myc-expressing cells (Slit-myc CM) were incubated with serum-free-conditioned media from NRobo4-HA cells (Fig. 4B, lane 6). In control experiments, with either cell lysates from HEK cells transfected with vector alone (Control-HEK) or conditioned media (HEK CM), Slit-Robo complexes were not detected. Finally, to test whether Slit can bind Robo4 on intact cell membranes, we incubated Robo4-HEK cells or Control-HEK cells with Slit-myc CM. Indirect immunofluorescence detected Slit-myc protein bound to the surface of Robo4-HEK cells but not Control-HEK cells (Fig. 4C–F). When cells were incubated with either HEK CM or secondary antibody alone, no signal was detected (data not shown). Taken together, these immunoprecipitation and immunofluorescence data demonstrate that, despite its unique extracellular domain structure, Robo4 is a Slit-binding receptor.

Robo4-Slit interaction inhibits cellular migration in a heterologous expression system

Previous work demonstrated that Slit inhibits migration of Robo1-expressing HEK cells (Wu et al., 2001). To determine whether Robo4 shares functional similarities with the other Robo receptors, we examined its effect on cellular

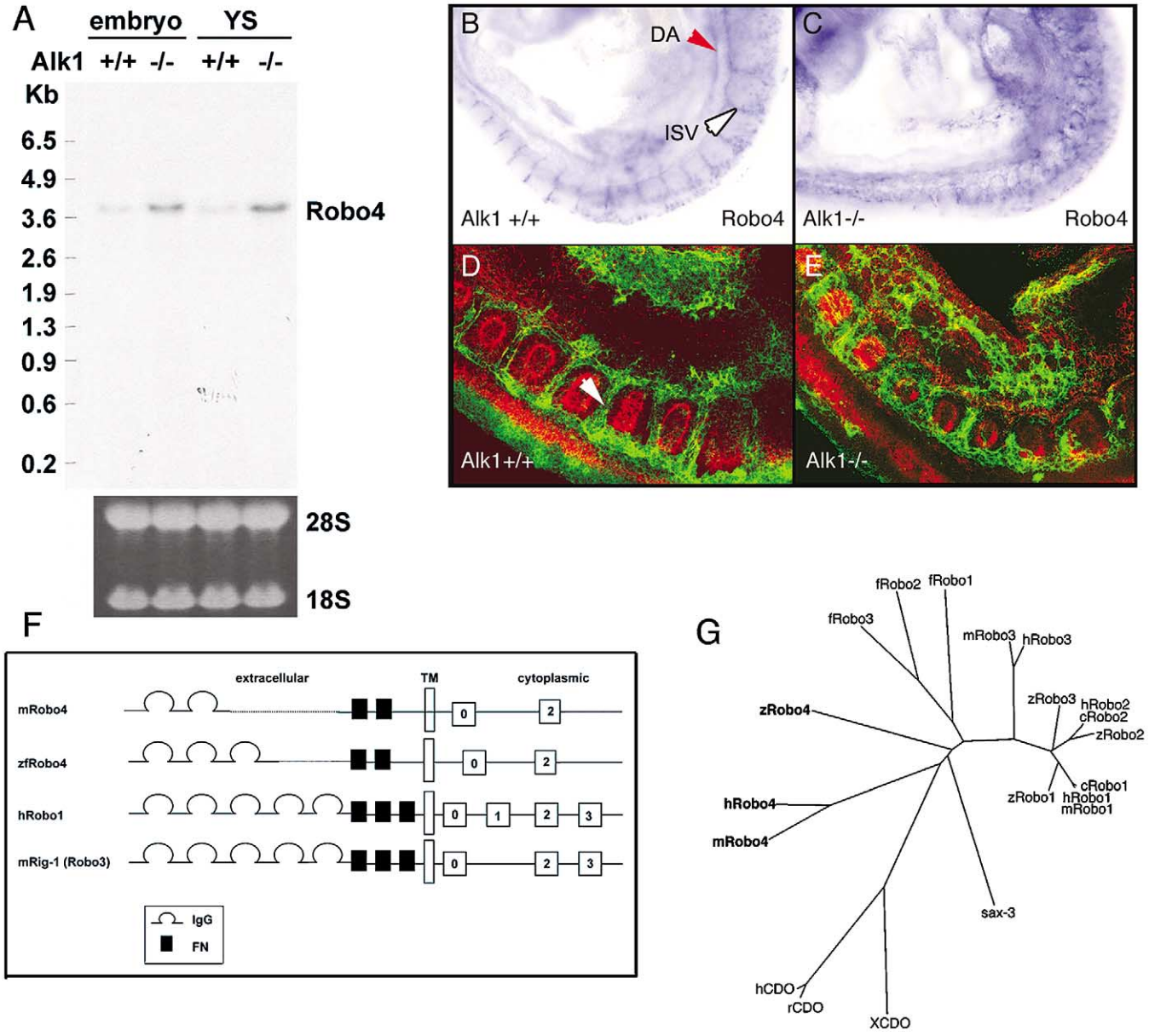
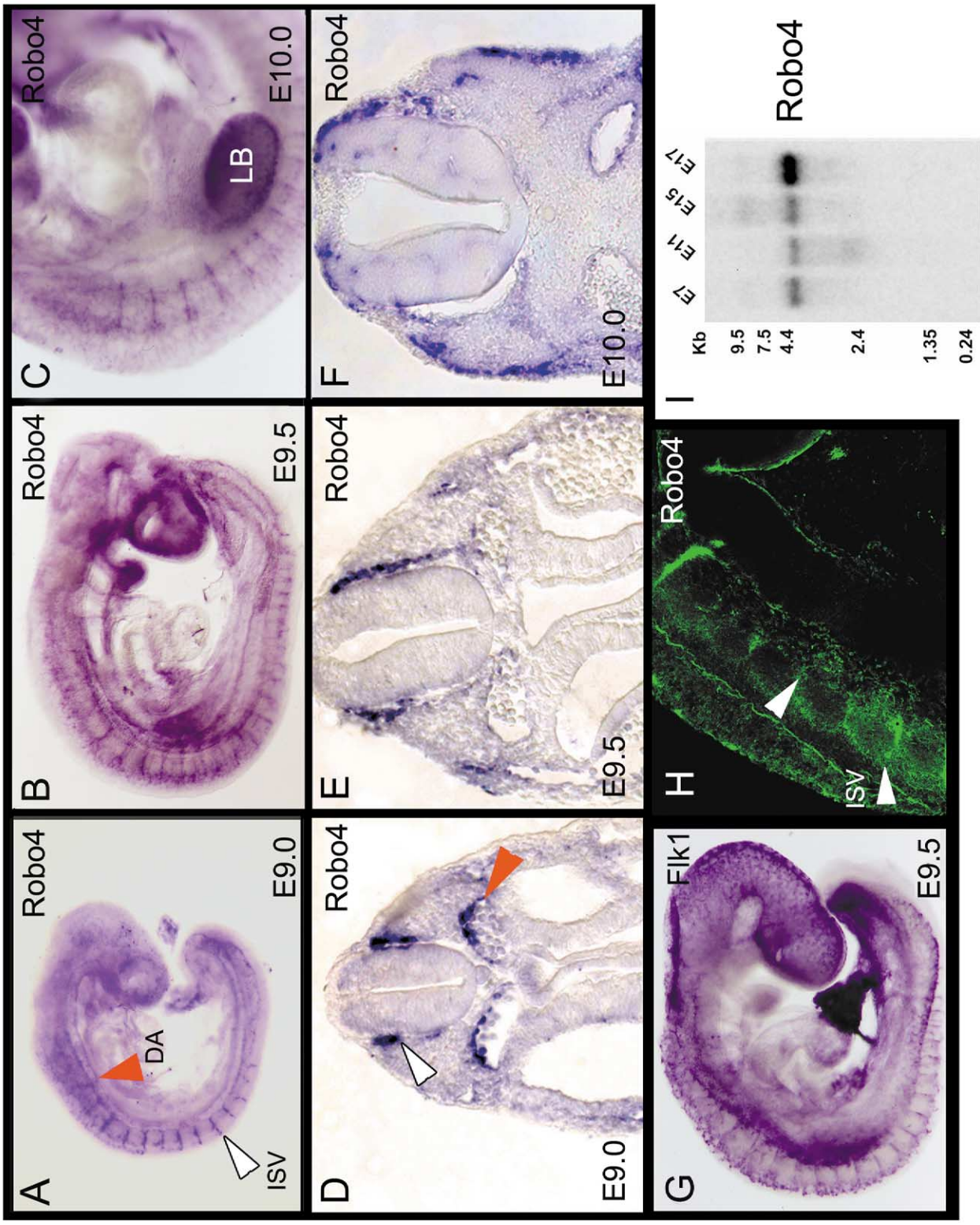


Fig. 1. *Robo4* was identified on the basis of differential expression in *Alk1* mutant embryos. (A) Northern blot analysis of *Robo4* expression in $Alk1^{+/+}$ and $Alk1^{-/-}$ tissues. *Robo4* mRNA levels are upregulated in $Alk1^{-/-}$ embryos and yolk sacs at E9.5. The bottom panel shows loading controls, 28S and 18S RNA. (B, C) Whole-mount *in situ* hybridizations of wild-type and $Alk1^{-/-}$ embryos reveal the differential vascular expression of Robo4. Robo4 is moderately expressed in well-organized intersomitic vessels (ISV, white arrowhead), capillary vessels, and in the dorsal aortae (DA, red arrowhead) in wild-type embryos (B), while Robo4 is highly expressed in the disorganized and dilated intersomitic and capillary vessels of $Alk1^{-/-}$ embryos (C). (D, E) Confocal microscopy utilizing an anti-PECAM antibody that highlights vessels (green), and Phalloidin, which stains somites (red), reveals the well-organized vasculature wherein vessels sprout normally between the somitic boundaries in wild-type embryos (white arrowhead) (D). In contrast, the intersomitic vessels of $Alk1^{-/-}$ embryos are disorganized and sprout abnormally into the somites (E). (F) Comparison of the domain organization of Robo4 with that of other members of the Robo family of guidance receptors. In all other species, members of the *Roundabout* family have five immunoglobulin (IgG) and three fibronectin (FN) domains in the extracellular region. In contrast, murine *Robo4* encodes two IgG and two FN domains (human Robo4 also encodes two IgG and two FN domains, not shown), and zebrafish Robo4 has three IgG and two FN domains. There is also conservation of specific domains within the cytoplasmic regions of Robo4 (CC0 and CC2) and other Robo family members. (G) Unrooted phylogeny of vertebrate and invertebrate *Robo* and *CDO* gene families, based on alignment of the first two IgG domains. The *Robo4* genes form a subfamily within the *Robo* superfamily. c, chicken; f, fruitfly; h, human; m, mouse; r, rat; x, Xenopus; z, zebrafish.

Fig. 2. *Robo4* is expressed in the embryonic vascular endothelium. (A–C) In situ hybridization using a *Robo4* riboprobe. At E9.0 (A), *Robo4* is expressed in the dorsal aortae (DA, red arrowhead) and intersomitic vessels (ISV, white arrowhead). By E9.5 (B) and E10 (C), *Robo4* expression extends to the capillary vessels, capillary plexus of the limb bud (LB), and is clearly detected in the intersomitic vessels, though its expression becomes less prominent in the central vessels. (D–F) Cross-sections of these embryos confirmed the vascular endothelial cell-specific expression of *Robo4*. At E9.0, strong expression is apparent in the dorsal aortae (red arrowhead) and intersomitic vessels (white arrowhead) (D). From E9.5 to E10, signal in the dorsal aortae diminishes as transcription predominates in smaller caliber vessels (E, F). In situ hybridization to *Flk1* is included for comparison to an established vascular-specific marker (G). Immunohistochemistry with a Robo4 antibody detects the expression of Robo4 protein predominantly in the intersomitic vessels at E9.5 (H, transverse section). Northern analysis reveals a Robo4 transcript present in embryos from E7 to E17 (I).


A
Robo4
DA
ISV
E9.0
B
Robo4
E9.5
C
Robo4
LB
E10.0
D
Robo4
E9.0
E
Robo4
E9.5
F
Robo4
E10.0
G
Flk1
E9.5
H
Robo4
ISV
I
Robo4
E7
E11
E15
E17
Kb
9.5
7.5
4.4
2.4
1.35
0.24

migration in this heterologous expression system. For these studies, we utilized a modified Boyden chamber assay in which test factors were placed in the lower chamber and cells were placed in the upper chamber. We quantified the number of cells that migrated to the lower chamber. We examined the migration of Robo4-HEK and Control-HEK cells to media collected from Slit-myc-expressing HEK cells (Slit-myc CM), as well as to media collected from control HEK cells (HEK CM). As expected, fibroblast growth factor and HEK CM induced both Robo4-HEK and Control-HEK cells to migrate at a rate three to fourfold greater than untreated cells (None). Slit-myc CM induced high levels of migration on Control-HEK cells. However, when applied to Robo4-HEK cells, Slit-myc CM inhibited migration to baseline levels (Fig. 5A and B). To show that this inhibitory effect of Slit-myc CM is specific to the Slit protein, we preincubated the Slit-myc CM with conditioned media from HEK cells that expressed the soluble ligand-binding ectodomain of Robo1 (NRobo1-HA) (Fig. 5C–F). The binding of NRobo1 to Slit-myc has been shown to be an effective method for specifically depleting Slit protein from conditioned media (Whitford et al., 2002; Wu et al., 1999, 2001). This depletion abolished the inhibitory effect of Slit-myc CM on the migration of Robo4-HEK cells (Fig. 5C and D). Similarly, Slit-myc CM, depleted of Slit protein by pretreatment with an anti-myc antibody, lost its inhibitory effect on Robo4-HEK cell migration (Fig. 5E and F). Depletion of Slit protein was confirmed by Western blotting (data not shown). Mock depletion with an anti-HA antibody did not reduce the inhibitory effect of Slit-myc CM. Therefore, similar to its effect on Robo1-expressing HEK cells (Wu et al., 2001), Slit specifically inhibited the migration of Robo4-expressing HEK cells.

Because purified Slit is unavailable, we adopted a strategy used by others to specifically induce activation of Robo1 using chimeric receptors fused to the nerve growth factor receptor (TrkA), as previously described (Stein and Tessier-Lavigne, 2001). We expressed a chimeric Robo4 receptor composed of the extracellular region of TrkA and the transmembrane and cytoplasmic regions of Robo4. Commercially available NGF completely abrogated the promigratory effect of FBS (fetal bovine serum, 2%) in cells that synthesize TrkA-Robo4 receptor (Fig. 5G and H); however, NGF treatment of control HEK cells had no effect on cellular migration. Therefore, as previously shown for Robo1 (Stein and Tessier-Lavigne, 2001; Bashaw and Goodman, 1999), the role of Robo4 in inhibiting migration is mediated by the cytoplasmic region of the activated receptor. These studies indicate that activation of Robo4 alone is sufficient to inhibit migration in response to various promigratory factors. Together, these cell biological experiments indicate that, similar to the other Robo family members, Slit is capable of inhibiting the migration of cells expressing Robo4. These data suggest that Robo4 is a functional Robo receptor that can inhibit cellular migration when stimulated by Slit ligand.

In vitro experiments are just beginning to delineate the signaling pathways activated by the neuronal Robo family members. Sequence comparison of the cytoplasmic domains of Robo1-3 reveals four conserved motifs, CC0–CC3. Alignment of Robo4 and Robo1-3 demonstrate that Robo4 only contains CC0 and CC2 (Suppl. Fig. 1). Previous studies have shown that the cytoskeletal modifying protein, Ena, binds to CC2 of *Drosophila* Robo, although the ligand dependence of this association has not been characterized (Bashaw et al., 2000). Ena is the only signaling molecule known to interact with either CC0 or CC2 of Robo, and we sought to determine whether Robo4 could physically associate with mammalian Ena, Mena. Immunoprecipitation experiments were performed to assay for Robo4:Mena interaction. Cell lysates from chimeric Robo4-HA- expressing cells (chimeric Robo4-HA) and cells transfected with vector alone (vector) were immunoprecipitated with an anti-HA antibody. Western blot analysis, using an anti-Mena antibody, detected coimmunoprecipitated, endogenous Mena protein in lysates from chimeric Robo4-HA HEK cells (Fig. 5I). Mena:Robo complexes were not detected in cell lysates from vector only transfected HEK cells. We also detected the Mena and wild type Robo4 interaction using Robo4-HEK cells (data not shown). Moreover, yeast two-hybrid screening of an aortic cDNA library with CC0 and CC2 of Robo4 as bait identified Mena multiple times. These data indicate that Robo4 shares a cytoplasmic signaling pathway known to mediate the anti-migratory effects of neuronal Robo family members.

Slit-Robo4 signaling inhibits endothelial migration

The observation of vascular-specific expression of Robo4 led us to investigate the function of endogenous Robo4 in primary human endothelial cells. We first tested whether Robo4 was present in endothelial cells by using an anti-Robo4 polyclonal antibody raised against a region that is highly conserved between human and murine Robo4, yet absent in Robo1, Robo2, and Robo3. Western blot analysis of extracts from Robo4-HA-expressing cells using an anti-HA antibody or anti-Robo4 polyclonal antibody detected the same size protein, suggesting that the anti-Robo4 antibody is specific for Robo4. We detected Robo4 protein and mRNA in human microvascular endothelial cells (HMVEC) by Western blot analysis and RT-PCR (Fig. 6A and B). Consistent with our in situ hybridization analysis of *Robo4* expression during embryogenesis, we did not detect expression of *Robo1*, *Robo2*, or *Robo3* in HMVEC by RT-PCR, although these primers amplified the expected products from human brain cDNA (data not shown). These data suggest that *Robo4* is the only Robo family member that is expressed in HMVEC. To investigate the function of endogenous Robo4 in HMVECs, a modified Boyden chamber assay was used. While HEK CM induced HMVEC migration at a level comparable to 10 ng/ml vascular endothelial growth factor (VEGF), Slit-myc CM inhibited HM-

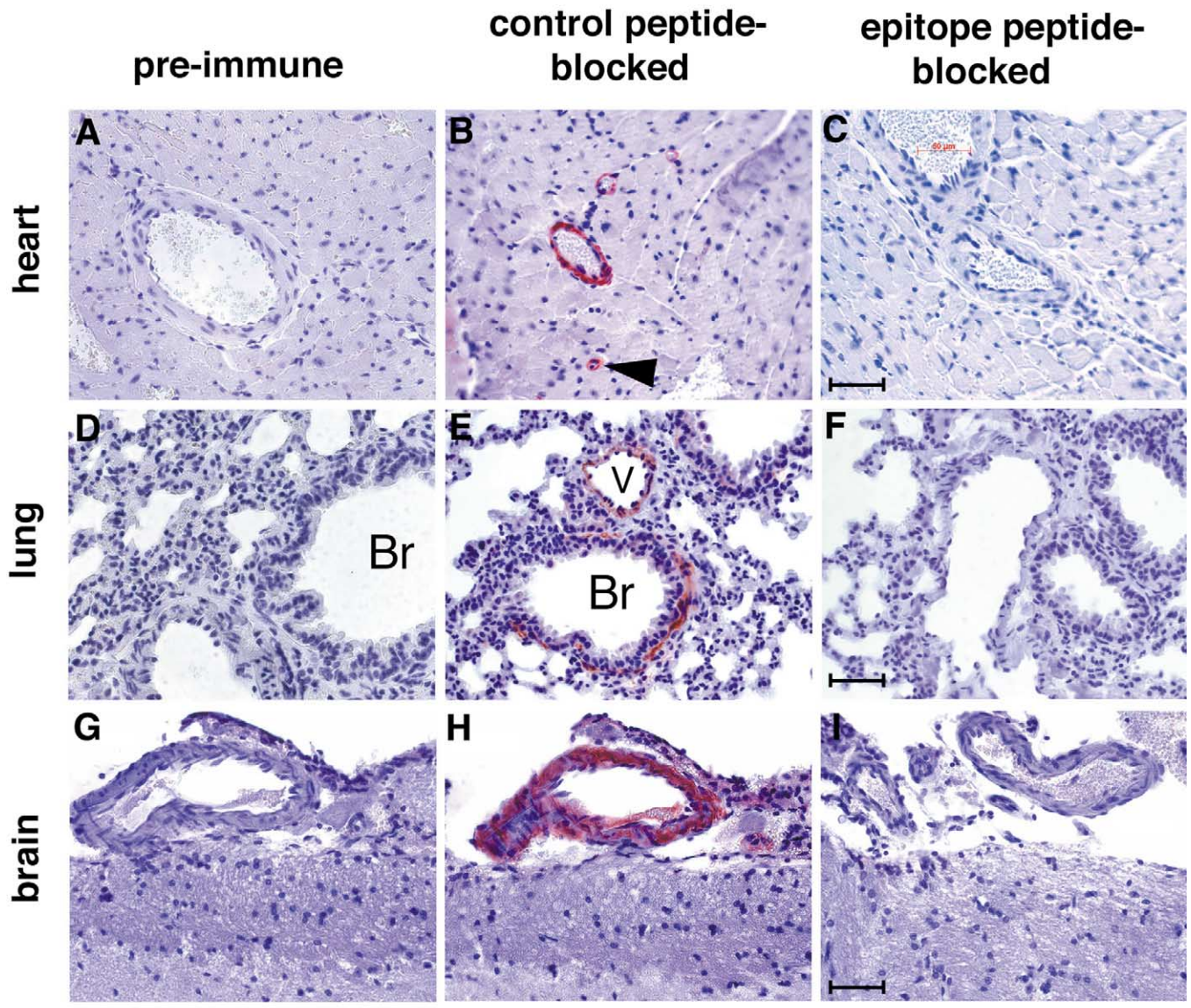
VEC migration to a level comparable to untreated cells (None) (Fig. 6C). Depletion of Slit from Slit-myc CM with either anti-myc antibody, or NRobo1, blocked the inhibitory effect of Slit-myc CM on endothelial cell migration. Depletion with anti-HA antibody had no effect (Fig. 6C). In parallel experiments, we found that Slit-conditioned media had no apparent pro- or anti-proliferative effects on endothelial cells (data not shown). To determine whether Slit-myc CM also inhibits endothelial cell migration in the presence of VEGF, we performed endothelial cell migration assays using conditioned media containing VEGF. Slit-myc CM did inhibit the VEGF-induced migratory effect, whereas control HEK CM did not. These data suggest that Robo4 can inhibit endothelial cell migration induced by a specific migratory factor, VEGF. We also observed a Slit-mediated inhibitory effect on FBS-induced endothelial cell migration (data not shown). In summary, the robust migratory response of HEK cells to FGF and FBS, and of HMVECs to VEGF and FBS, can be inhibited by Slit-Robo4 signaling. These findings, in conjunction with data from our heterologous and chimeric receptor expression systems, strongly suggest that Robo4 mediates the inhibition of primary human endothelial cell migration by Slit.

Discussion

We have identified *Robo4*, a putative member of the Robo receptor family, in a screen for genes that are differentially expressed in mice with vascular sprouting and vessel patterning defects. Whereas Robo1, Robo2, and Robo3 are highly expressed in the nervous system and undetectable in the vascular system, Robo4 paradoxically exhibits an exclusively vascular endothelial expression pattern during embryonic development. Moreover, the divergent extracellular domain structure suggests that Robo4 may have distinctly different ligand-binding specificities and signaling functions relative to the neuronal receptor family. No data have been reported to functionally link Robo4 with other members of the canonical Robo family. Here, we provide five lines of evidence that Robo4 is a functional member of the Robo family of receptors. First, indirect immunofluorescence and immunoprecipitation studies indicate that Robo4 is capable of binding the same ligands, the Slit proteins. Second, in accordance with the effects of Slit ligands on the neural Robo receptors (Wu et al., 2001), Slit activation of Robo4 inhibits cellular migration in a heterologous expression system. In order to confirm the specificity of this effect, we generated a chimeric Robo4 receptor that could be activated by NGF. As demonstrated for Robo1 (Stein and Tessier-Lavigne, 2001), the migration of chimeric Robo4 (trkA-Robo4) transfected cells was inhibited by NGF treatment. These data also indicate that the cytoplasmic region of Robo4 mediates the cellular effect as previously shown for Robo1 (Bashaw and Goodman, 1999). Third, we show that primary endothelial cells express Robo4, but not Robo1, Robo2, or Robo3, and that Slit inhibits migration of these cells. Fourth, Robo4, like other Robo family members, interacts with a cytoplasmic signaling molecule, Mena. Finally, there is an evolutionarily conserved chromosomal colocalization and pairing of Robo4 with Robos 1, 2, and 3 from zebrafish to human (Suppl. Fig. 2). This organization of gene pairs suggests that the Robo genes were derived through a series of gene duplication events. In summary, Robo4 is a bona fide Robo receptor that specifically functions in the vasculature to modulate endothelial cell migration. Our data suggest that, analogous to the role of Robo1, Robo2, and Robo3 in neuronal guidance (Challa et al., 2001; Fricke et al., 2001; Kidd et al., 1998, 1999; Lee et al., 2001; Rajagopalan et al., 2000a,b; Rothberg et al., 1988; Wang et al., 1999), Robo4 may provide a chemorepulsive cue to sprouting endothelial cells during angiogenesis.

The only known ligands for the Robo family of receptors are the three Slit proteins. *Slit1*, *Slit2*, and *Slit3* have overlapping expression patterns in neuronal and nonneuronal tissues (Vargesson et al., 2001; Whitford et al., 2002; Wu et al., 2001; Yuan et al., 1999b). In both zebrafish and mouse, the Slit genes are expressed in the developing somites but not within the intersomitic boundary (Yuan et al., 1999b; Hutson and C-B.C., unpublished data). These observations suggest that the Slit protein could interact with Robo4 to demarcate the path that intersomitic vessels take as they sprout from central vessels. Although no angiogenic defects have been described in Slit-deficient mice, the potential roles for these proteins might not be revealed due to their redundant expression (Bagri et al., 2002; Plump et al., 2002). Our work defines Slit2 as a biochemical and functional ligand; however, it remains an open question whether this protein is the physiologic partner for Robo4. The promiscuous binding between the Slit and Robo family members suggest that all three Slit proteins will be able to bind and activate Robo4. However, caution must be exercised in presuming that Robo4 shares the same spectrum of ligands

Fig. 3. Robo4 is expressed in blood vessels and smooth muscle cells of adult organs. (B, E, H) Immunohistochemical analysis of adult murine tissues using a Robo4 polyclonal antibody preparation that was mock-preabsorbed with a nonspecific peptide. Marked expression of Robo4 protein is evident in the vasculature of the heart (B). Expression extends to the smooth muscle medial layer of larger vessels, in addition to staining of the endothelial layer that is apparent in smaller caliber vessels (arrowhead). Similarly, expression was detected in vessels (V) and in the smooth muscle component of bronchioles (Br) in the lung (E). Expression in the brain is restricted to blood vessels (H); no staining was detected in the nonvascular tissues. No staining was apparent in preimmune sera (A, D, G) or epitope peptide-blocked polyclonal antibody (C, F, I) controls. Northern analysis indicates that *Robo4* mRNA is strongly expressed in the heart, liver, lung, and kidney of adult mice (J). Scale bar, 50 μm.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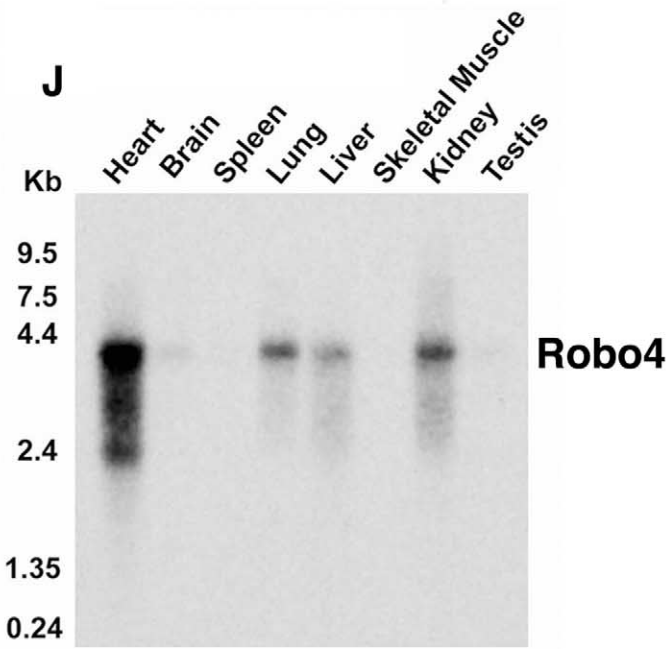
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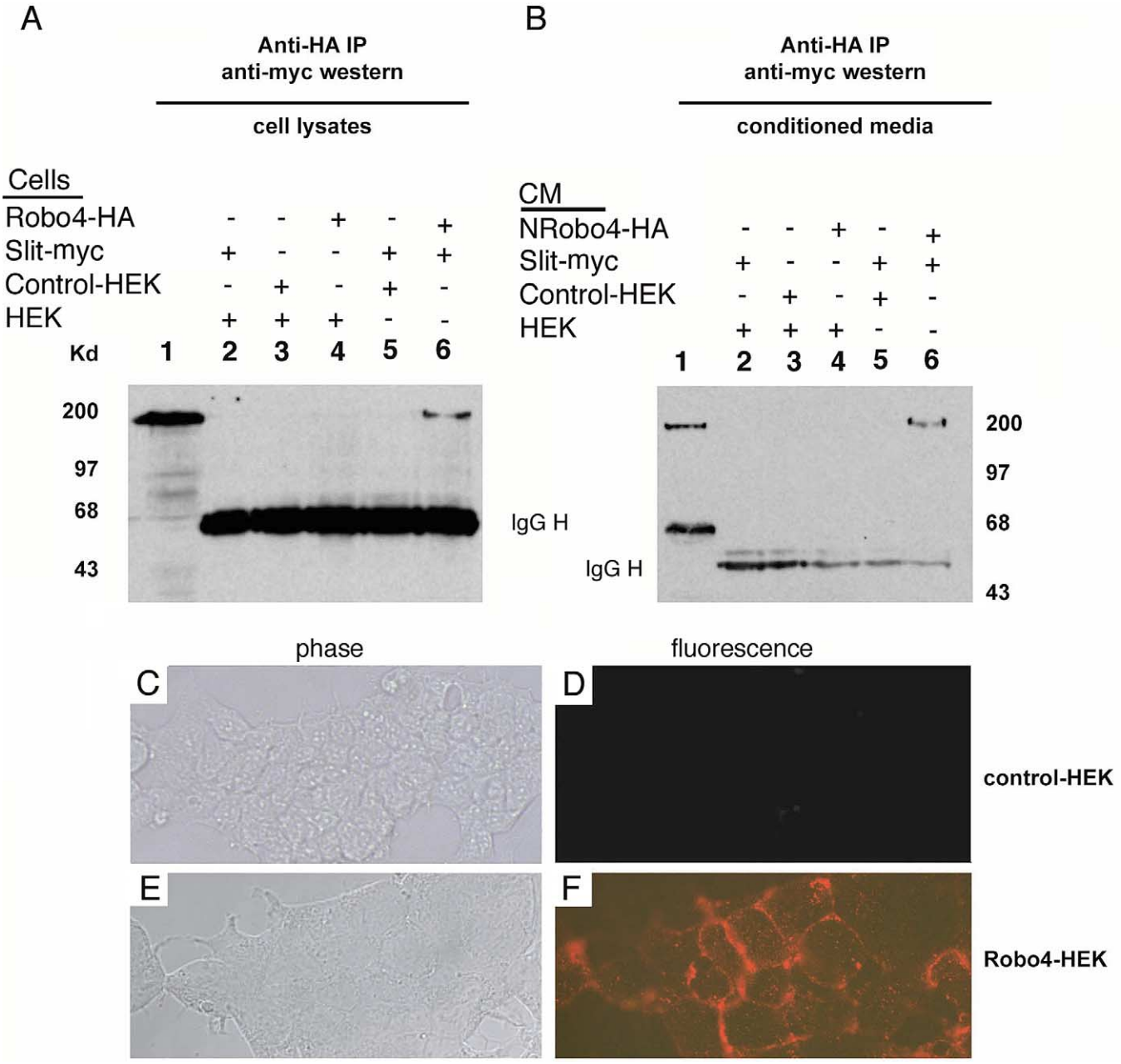

Fig. 4. Robo4 interacts with Slit ligand. (A, B) Immunoprecipitation demonstrates an interaction between Slit ligand and the Robo4 receptor. (A) Immunoprecipitation of cell lysates from untransfected HEK cells (HEK), HEK cells transfected with Slit-tagged with a myc epitope (Slit-myc), HEK cells transfected with Robo4-tagged with a HA epitope (Robo4-HA), and HEK cells transfected with a control vector (Control-HEK). Western blot analysis of the Slit-myc cell lysates serves as a control and demonstrates that the Slit protein has a mass of approximately 200 kDa, as previously reported (lane 1). Slit-myc protein is also detected by Western blot with an anti-myc antibody after Slit-myc and Robo4-HA cell lysates were combined and immunoprecipitated with an anti-HA antibody (lane 6). The specificity of this interaction is confirmed by the absence of detectable Slit protein with all other combinations of lysates. The same amount of lysate was used in each experiment. The lower bands in lanes 2–6 correspond to immunoglobulin heavy chains. (B) Immunoprecipitation of conditioned media from untransfected HEK cells (HEK CM), HEK cells transfected with Slit tagged with a myc epitope (Slit-myc CM), HEK cells transfected with the N-terminal soluble ectodomain of Robo4 tagged with the HA epitope (NRobo4-HA CM), and HEK cells transfected with control vector (Control-HEK CM). The full-length Slit-myc protein (200 kDa) and its C-terminal proteolytic fragment (60 kDa) are detected in Slit-myc CM by an anti-myc antibody (lane 1). As in (A), Slit-myc protein is also detected by Western blot after Slit myc and Robo4-HA conditioned media are combined and immunoprecipitated with an anti-HA antibody (lane 6). The specificity of this interaction is confirmed by the absence of Slit protein with all other combinations of conditioned media. (C–F) Slit protein binds to the plasma membrane of cells expressing *Robo4*. Binding of Slit-myc protein was detected by using an anti-myc antibody and an Alexa 594-conjugated anti-mouse antibody. Binding is detected on the surface of Robo4-HEK cells (F) but not Control-HEK cells (D).

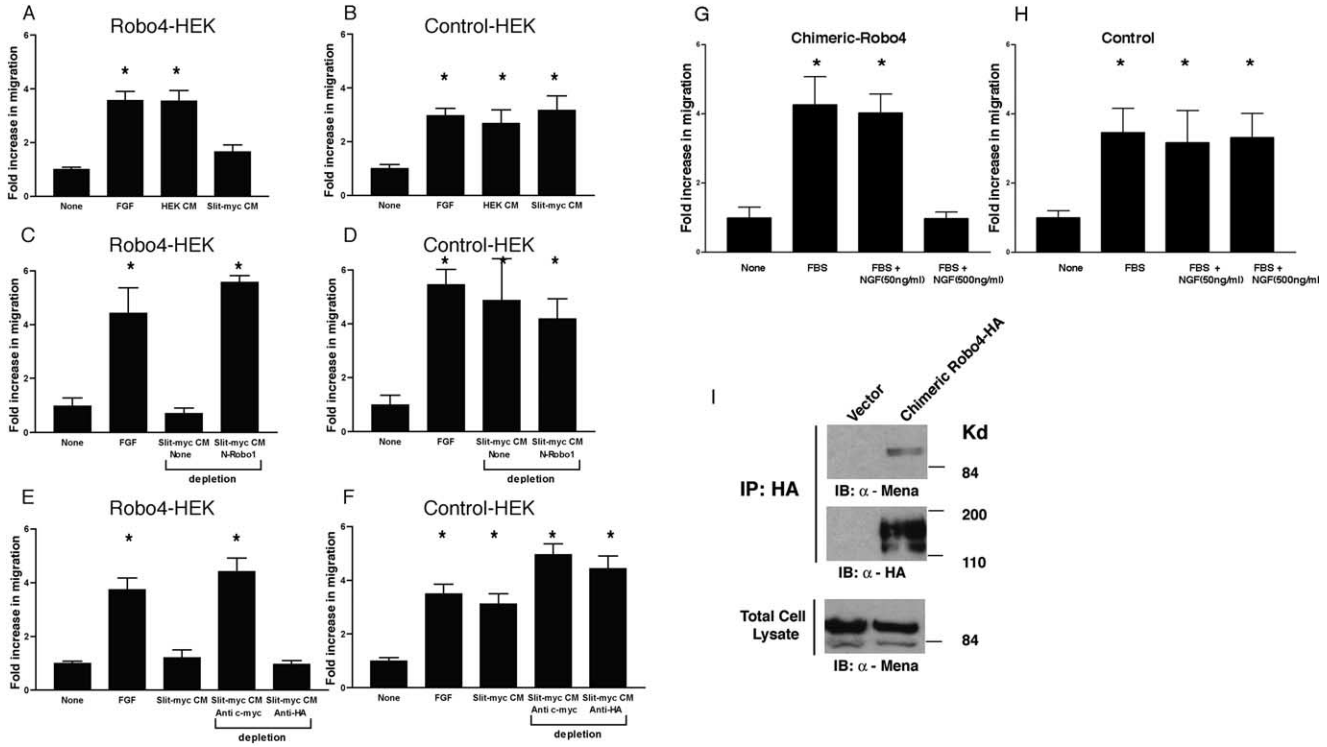

Fig. 5. Slit-activated Robo4 inhibits cellular migration in a heterologous expression system. Migration of Robo4-HEK cells and Control-HEK cells to basic fibroblast growth factor (FGF), HEK CM, and Slit-myc CM is measured by a modified Boyden chamber assay. (A, B) Treatments with Slit-myc CM inhibit migration to the level of untreated cells only in Robo4-HEK cells, not Control-HEK cells. Robo4-HEK cells migrated toward FGF and HEK CM. However, Slit-myc CM inhibits the migration of Robo4-HEK cells. Slit-myc CM does not inhibit the migration of Control-HEK cells. (C–F) Slit protein is responsible for the inhibitory effect of Slit-myc CM on Robo4-HEK cells. The pretreatment of Slit-myc CM with a soluble N-terminal ectodomain of Robo1 (N-Robo1) (C) or anti-myc antibody (E) blocks the inhibitory effect of Slit on migration of Robo4- HEK cells. (D, F) The migration of Control-HEK cells to Slit-myc CM is not affected by either N-Robo1 or anti-myc depletion. (E, F) Mock depletions of Slit- myc CM with anti-HA antibody do not affect cellular migration. (G, H) Chimeric Robo4 (trkA-Robo4) induced by nerve growth factor (NGF) mimics the inhibition of migration. Migration of Chimeric-Robo4 cells and Control cells to 2% FBS (fetal bovine serum) with or without NGF is measured. (G) The migration of Chimeric-Robo4 cells to FBS is inhibited by 500 ng/ml NGF but not 50 ng/ml NGF. (H) The migration of Control cells is not affected by either treatment of NGF. [*represent treatments that give statistically significant increases ($P < 0.05$, Student's t test) in migration, compared with untreated cells (None); CM, conditioned media]. (I) Immunoprecipitation experiments show Robo4:Mena interaction. Cell lysates from chimeric Robo4-HA-expressing cells (chimeric Robo4-HA), and empty vector transfected cells (vector) were immunoprecipitated with an anti-HA antibody. Western blot analysis, using an anti-Mena antibody, detected coimmunoprecipitated, endogenous Mena protein in lysates from chimeric Robo4-HA HEK cells, whereas Mena:Robo complexes were not detected in cell lysates from empty vector transfected HEK cells.

as the other Robo receptors given its divergent extracellular structure. If mice lacking all three Slit genes fail to show angiogenic defects, it will be important to reassess the potential for other peptides and cytokines to bind and activate Robo4. Interestingly, Robo4-expressing cells appear to have a rounder shape than cells transfected with empty vector or unrelated proteins. One potential explanation for our findings is that Robo4 can mediate a cellular response through ligand-independent dimerization. Hivert et al., (2002) described such an interaction for Robo1 and Robo2 which promotes axonal outgrowth through mechanisms of homophilic or heterophilic binding that does not require ligand activation. Therefore, Robo family members might have both ligand-dependent and -independent functions.

The importance of the Robo signaling pathway to axonal guidance has been well-documented. *Robo* was initially identified in a large-scale *Drosophila* screen as a mutant affecting axonal projection in the central nervous system (Guthrie, 2001; Seeger et al., 1993). In *Drosophila*, the Robo guidance receptor recognizes Slit that is produced by the midline, and this interaction prevents axons from crossing the midline (Brose and Tessier-Lavigne, 2000). Mutations in the homologous *C. elegans* gene, *sax-3*, produce a defect in axon guidance at the ventral midline that is similar to the *Drosophila Robo* mutant (Kidd et al., 1998; Seeger et al., 1993; Zallen et al., 1998). In the zebrafish visual system, recognition of surrounding domains of Slit expression by Robo2 appears to be critical for restricting retinal axons to the appropriate pathway, thus enabling formation of the optic chiasm (Fricke et al., 2001; Hutson and Chien, 2002). In vitro studies in rat and chick show that activation of the Robo signaling pathway by Slit proteins repel motor, olfactory, and retinal ganglia axons (Brose et al., 1999; Li et al., 1999; Niclou et al., 2000). Mice deficient in *Slit* genes show

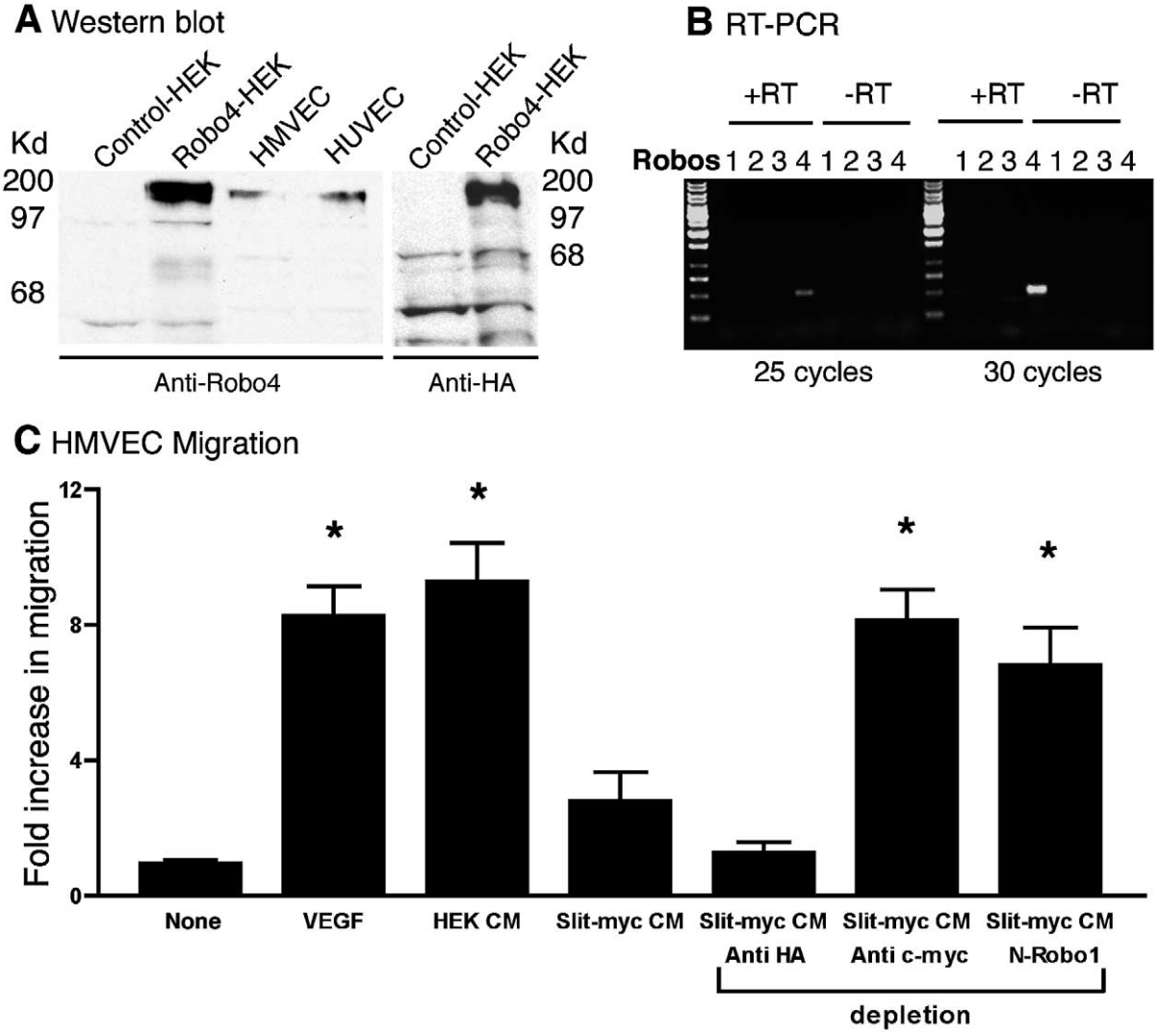

Fig. 6. Slit inhibits the migration of primary human endothelial cells. (A) Western blot analysis demonstrates that primary endothelial cells express Robo4. Robo4 protein is detected in the cell lysates of Robo4-HEK cells, human umbilical vein endothelial cells (HUVEC), and human microvascular endothelial cells (HMVEC) using a polyclonal anti-Robo4 antibody. Robo4 is not detected in Control-HEK cells. Western blot analysis using an anti-HA antibody was performed to confirm the expression of Robo4-HA protein in Robo-HEK cells. (B) *Robo4* is the only member of the Robo family expressed in HMVEC. RT-PCR experiments detect the *Robo4* transcript, yet fail to detect expression of the other three members of the *Robo* family. Negative control experiments indicate that no product is made in the absence of Reverse Transcriptase (− RT). (C) Slit inhibits the migration of HMVEC. HMVEC migration to vascular endothelial growth factor (VEGF), HEK CM, and Slit-myc CM is measured by a modified Boyden chamber assay. HMVECs migrate toward VEGF and HEK CM; however, Slit-myc CM inhibits the migration of HMVEC. The inhibitory effect of Slit- myc CM on HMVEC migration is blocked by depleting the conditioned media with either N-Robo1 or anti-myc antibody. [*represent treatments that give statistically significant increases ($P < 0.05$, Student's t test) in migration compared with untreated cells (None)].

significant axon guidance errors in a variety of pathways, including the corticofugal, callosal, and thalamocortical tracts (Bagri et al., 2002; Plump et al., 2002). Together, these genetic and cell biologic data suggest that the Robo family of receptors will play a key role in neuronal guidance and patterning during murine embryogenesis. However, at present, there have been no published reports confirming a critical role for Robo receptors in mammalian neurogenesis.

A recent body of evidence suggests that molecular pathways guiding axonal pathfinding also guide endothelial sprouting and angiogenesis. This is supported by a recent study reporting that sensory nerves instruct arterial differentiation and blood vessel patterning in the skin (Mukouyama et al., 2002). In addition, there is an expanding list of neural guidance gene families that also regulate migration and proliferation of endothelial cells, suggesting that the developmental similarities and physical associations between the vascular and nervous systems are based on the use of common molecular mechanisms. First, the *Ephrin, Eph, Neuropilin*, and *Semaphorin* families of genes were initially iden-

tified based on their fundamental roles in neural guidance and development. Later, these genes were demonstrated to regulate vascular cell biology and angiogenesis (Shima and Mailhos, 2000). Second, the Eph receptors and ephrin ligands play a key role in the nervous system by mediating cell-contact-dependent repulsion. These genes may also play a similar role in the vascular system where ephrinB2 is expressed in arterial endothelial cells while its cognate receptor, EphB4, is expressed in veins. Gene-targeting experiments have demonstrated that both ephrinB2 and EphB4 are essential for angiogenesis and suggest that they are important for distinguishing arterial and venous domains (Adams and Klein, 2000; Wang et al., 1998). The neuropilins are receptors for the axon guidance factors belonging to the class-3 semaphorin subfamily. In addition to their role in the nervous system, neuropilins are capable of binding certain splice forms of vascular endothelial growth factor (VEGF), indicating that neuropilins also function in the vascular system (Kawasaki et al., 1999; Miao et al., 1999; Soker et al., 1998; Takashima et al., 2002). Our data imply that Slit-Robo4 signaling could be an additional example of a co-opted neuronal guidance mechanism applied to vascular development. Taken together, these findings suggest that neural guidance signaling molecules may have a similar function in vascular development and patterning (Childs et al., 2002; Fouquet et al., 1997; Mukouyama et al., 2002).

In situ hybridization on whole-mount embryos and cross-sections indicate that Robo4 expression shifts from the dorsal aorta to intersomitic vessels as development proceeds. By E10.0, Robo4 is predominantly detected in capillary vessels with little expression in intersomitic vessels and dorsal aortae (Fig. 2). This expression profile suggests that Robo4 activity may be important in the process of sprouting angiogenesis during embryonic development. The expression pattern of Robo4 in adult tissues shows that Robo4 is highly expressed in tissues that have complex and well-organized vascular networks such as lung, liver, kidney, and heart. The expression pattern of Robo4 suggests that Robo4 might be important for vascular patterning (i.e., the formation of tubular structures) in adult tissues. Robo4 may be important for guiding and maintaining the highly reproducible vascular patterning observed in embryonic and adult tissues. Understanding the in vivo role of the Robo4 gene in both embryonic and adult tissues is presently being addressed by the characterization of a conditional Robo4 knockout mouse.

We previously showed that mutations in *Alk1* produced sprouting arterial and venous endothelial tubes that failed to follow distinct paths and ultimately fused with one another (Urness et al., 2000). Subsequent immunofluorescence studies indicated that the intersomitic vessels of *Alk1*$^{-/-}$ embryos are disorganized and sprout abnormally into the somites instead of remaining between them. In order to show that the upregulation of Robo4 expression is specific to Alk1$^{-/-}$ mutants, we examined Robo4 expression in two other vascular mutants, endoglin$^{-/-}$ and Ccm1$^{-/-}$ (Li et al., 1999; Whitehead et al., unpublished observations). Both mutants show significant vascular developmental defects, and yet Robo4 is not differentially expressed in either mice lacking endoglin or Ccm 1. This suggests that the differential expression of Robo4 in Alk1$^{-/-}$ embryos is specific. Although the identification of *Robo4* is based on its differential expression in *Alk1* mutant mice, we do not at this time have any direct evidence that the vascular phenotype observed in these mutants is a consequence of increased expression of Robo4. It is possible that this may be a secondary, or indirect effect of *Alk1* deficiency or, alternatively, may represent an effort to compensate for the loss of guidance mechanisms.

Knockdown experiments in zebrafish using morpholinos demonstrate that Robo4 is essential for embryogenesis. However, the dual expression of zebrafish Robo4 in both the neural and vascular systems prevents us from making any definitive conclusions regarding the function of Robo4 in vessel development (B. Weinstein, personal communication). In contrast, mice express Robo4 in their developing vascular systems and without potential redundancies with other known Robo family members. We anticipate that future experiments designed to ablate Robo4 signaling by gene targeting in mice will allow us to specifically define its role in angiogenesis.

Acknowledgments

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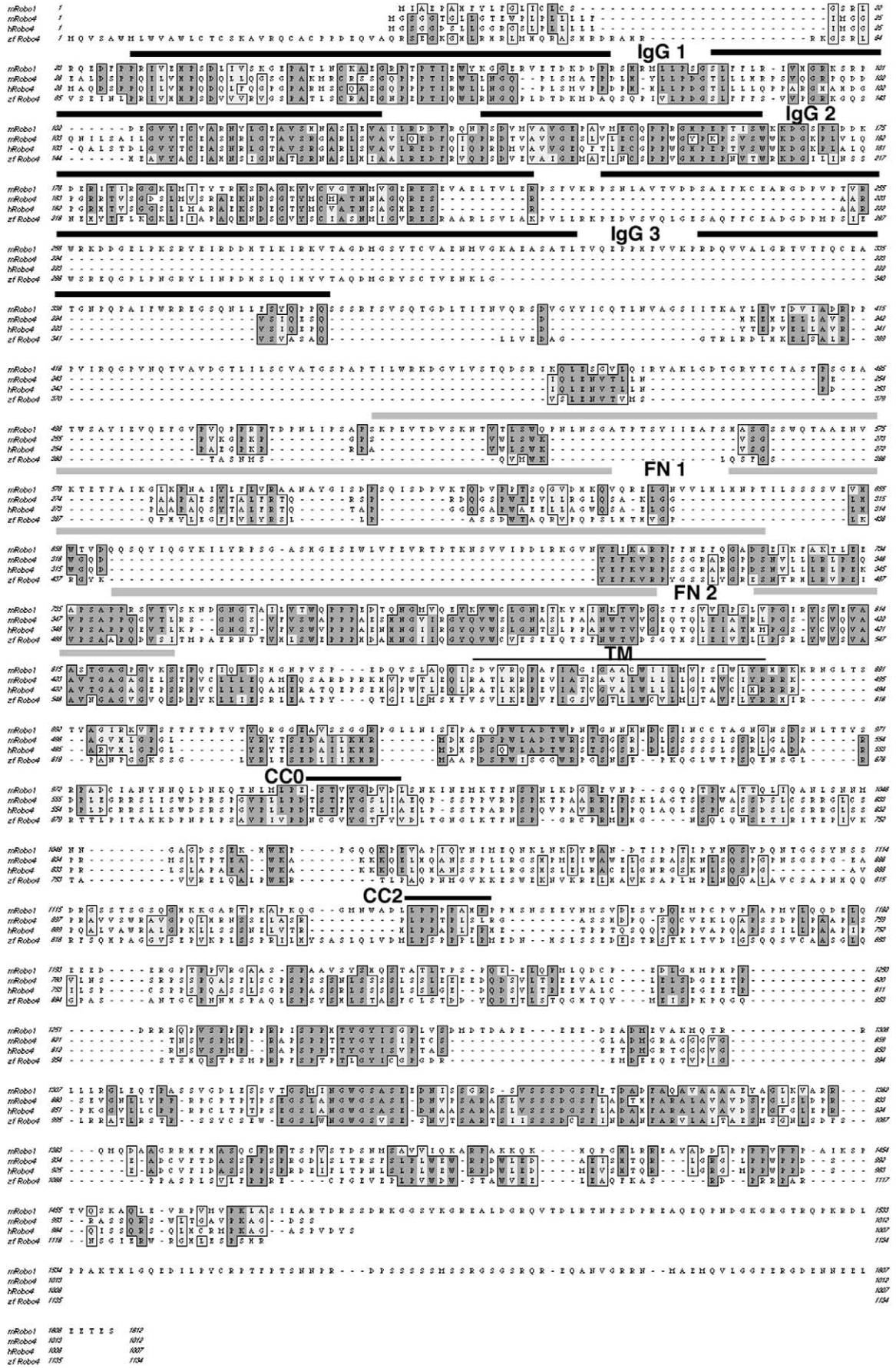
Appendix Fig. 1 Alignment of zebrafish, murine and human *Robo4* with murine *Robo1*. The Robo receptor family exhibits five immunoglobulin (IgG) domains and three fibronectin type III (FN) domains, a single transmembrane (TM), and four conserved cytoplasmic domains (CC0-CC3). Murine and human Robo4 encode two IgG, two FN, a single TM, and two cytoplasmic conserved domains (CC0 and CC2), while zebrafish Robo4 encodes three IgGs, two FNs, a single TM, and CC0 and CC2 domains. Dark and pale grey boxes represent, respectively, identical and similar amino acids.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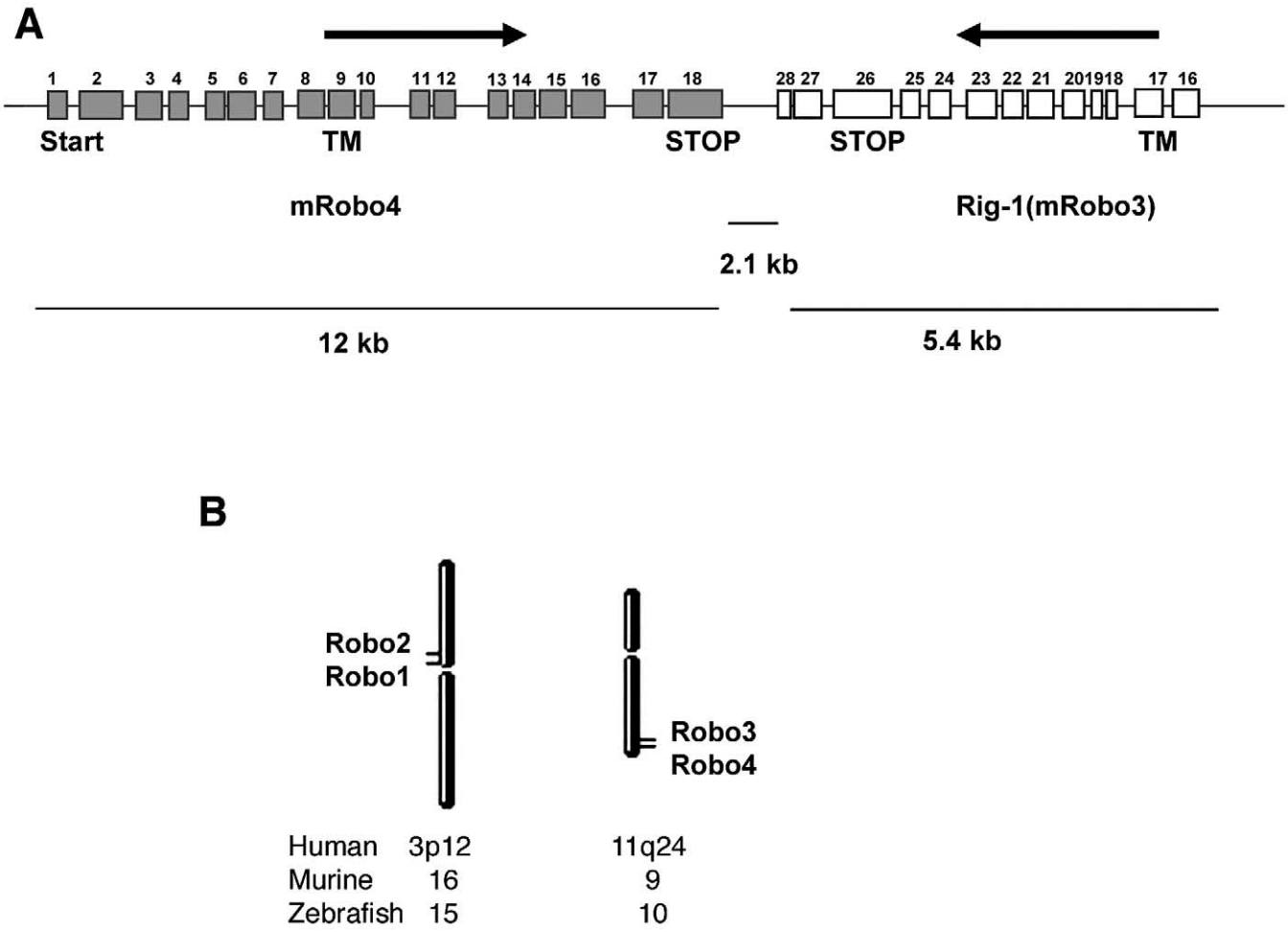

Appendix Fig. 2 Chromosomal locations of Robo family members are evolutionary conserved. (A) m*Robo4* is encoded by 18 exons and is located adjacent to m*Robo3* in a tail-to-tail orientation on chromosome 9. (B) The closely linked organization of *Robo1* and *Robo2* on human chromosome 3, and *Robo3* and *Robo4* on chromosome 11, suggests that this gene family results from a series of duplication events. The pairing of *Robo1* and *Robo2*, as well as *Robo3* and *Robo4*, is conserved in mice and zebrafish.

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