Robo4 Signaling in Endothelial Cells Implies Attraction Guidance Mechanisms\(^*\)(\(^{1,5}\))

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Sukhiber Kaur\(^1,2\), Maria Domenica Castellone\(^4,1\), Victoria M. Bedell\(^6\), Martha Konar\(^4\), J. Silvio Gutkind\(^1\), and Ramani Ramchandran\(^4,3\)

From the \(^1\)Laboratory of Pathology, NCI, and the \(^5\)Cell Growth Regulation Section, Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Rockville, Maryland 20850

Roundabouts (robo) are cell-surface receptors that mediate repulsive signaling mechanisms at the central nervous system midline. However, robo may also mediate attraction mechanisms in the context of vascular development. Here, we have performed structure-function analysis of roundabout4 (Robo4), the predominant robo expressed in embryonic zebrafish vasculature and found by gain of function approaches in vitro that Robo4 activates Cdc42 and Rac1. To study signaling mechanisms triggered by Robo4 in the vasculature, we initially designed four structural mutants tagged with GFP. Overexpression analysis in zebrafish in vivo and in HEK-293T or porcine aortic endothelial (PAE) cells in vitro was used to determine the functionality of the constructs, and signaling pathways triggered by the mutant proteins. Pull-down analysis in transfected HEK-293T and endothelial cells show that full-length Robo4 activate Cdc42 and Rac1 but not Robo4 and that this activation was dependent on the presence of the extracellular ligand-binding region in Robo4. Furthermore, Cdc42DN blocks Robo4-induced filopodia, as well as cell adhesion and migration, which suggest an important role for Cdc42 and Rac activation in Robo4-mediated signaling events. Overall, this study implies Robo4-GTPases in vertebrate vascular guidance.

MATERIALS AND METHODS

Zebrafish Stocks and Reagents—Zebrafish were grown and maintained at 28.5 °C. Mating was routinely carried out at 28.5 °C, and the embryos were staged according to established protocols (12). Zygogen Inc. (Atlanta) provided the Tg(vegf2: G-RCFP) fish (13). The dominant negative and constitutive active Rho GTPase constructs have been reported before (14). All zebrafish studies were performed under NCI and National Institutes of Health animal institutional guidelines (animal protocol no. LP-020). Antibodies used in this study include GFP (Santa Cruz Biotechnology, Santa Cruz, CA), Cdc42 and Rac (BD Transduction Laboratories), Rho (Santa Cruz), Actin (Sigma), Tubulin (abcam), anti-rabbit horseradish peroxidase (Amersham Biosciences), pan-cadherin (abcam), and anti-rabbit fluorescent isothiocyanate (Jackson ImmunoResearch). Mounting media with 4',6-diamidino-2-phenylindole were purchased from Vector Laboratories Inc.

Cloning and Constructs—For fusion constructs F, N, C, C1, C2, and C3, fragments were amplified from previously cloned full-length Robo4 construct using upstream CCACCATGAGTTGTCATGATG- GTGTG (F and N), CCACCATGAGTTGTCATGATGTCGTGC (C), CCACCATGAGTTGTCATTAGGAGGTGTGT (C1, C2, and C3), and downstream GTGGCTGAAGGAGGCTTTTCTCCAGATGTC- CCTC (F, C, and C1), GACCTGCAAGGACTGATGTTAGCTC (C2), GCCTTTTAGGGTTGAATGGGaCAGG (C3), and TGCCACCAGGGTTGCGGTACTGATGTC (N) primers using cycling parameters: 94 °C 2 min, 94 °C 30 s, 58 °C 30 s, 72 °C 1 min (34 cycles), and 72 °C 5 min.
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as described before. The PCR products were cloned in-frame into CTA-GFP fusion Topo TA expression vectors (Invitrogen), and all clones were sequenced. Expression constructs were as follows. PAKN: N-terminal 150 aa non-catalytic domain of PAK1, which contains the Cdc42 and Rac binding regions was cloned into pcCFLP backbone containing an an 1 tag. PAKNL2: same region of PAK1 with mutations in aa residues Leu-83 and Leu-86 (15). WASP: 201–321 aa residues of WASP were cloned into pcCFLP backbone.

**Cell Lines and Transfections**—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B. Tissue culture plates were treated with phosphate-buffered saline containing 20 μg/ml poly-D-lysine for 15 min before seeding the cells to prevent them from detaching from the plates when in serum-free conditions. HEK-293T cells were transiently transfected with 5 μg of plasmid in a 10-cm plate using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. Porcine aortic endothelial cells (PAE) were maintained in Ham’s F-12 media (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin. Transient transfections in PAE cells were performed using the Lipofectamine Plus reagent (Invitrogen) in conjunction with Magnetofection™ CombiMag-2000 (OZ biosciences) to increase transfection efficiency. For co-transfection experiments, equal amounts of the two plasmids were used.

**Western Blots**—Cells were lysed at 4°C in radioimmune precipitation assay buffer containing 25 mM Tris (pH 8.0), 150 mM KCl, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. For pull-down analysis Rho lysis buffer was used. For GFP westerns, radioimmune precipitation assay buffer, or Rho lysis buffer were used. Solubility of the different mutant proteins varies in radioimmune precipitation assay buffer and Rho lysis buffers.

**In Vivo Cdc42 and Rac1 Guanine Nucleotide Exchange Assay**—In vivo Rac1 and Cdc42 activity was assessed by a modified method described before (16). Briefly, after serum starvation for 24 h, the cells were lysed with ice-cold Rho-lysis buffer containing 20 mM HEPES, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM MgCl₂, 1 mM NaVO₄, 1 mM dithiothreitol, mixture of protease inhibitors, and 1 mM phenylmethylsulfonyl fluoride. The lysates were incubated for 20 min with a purified, bacterially expressed GST fusion protein containing the CRIB domain of PAK1 (p21 activated kinase), previously bound to glutathione-Sepharose beads, followed by three washes with Rho-lys buffer. The GTP-bound forms of Rac1 or Cdc42 associated with GST-CRIB were quantified by Western blot analysis using the appropriate antibody against Rac1 or Cdc42. For Rho pull-down assays, GST-Rhotekin-RBD beads were used and assay was performed as described before (17). For in vivo embryo pull-down assays, most of the procedure remained the same except that lysates were made from 75 dechorionated, deyolked, MO injected or un.injected 24-hour post fertilization embryos per sample using 500 μl of Rho-lys buffer.

**Fluorescent Confocal Microscopy**—Endothelial cells transfected with relevant plasmid were grown on coverslips and serum-starved for 8 h prior to washing twice with PBS and fixing with 2% paraformaldehyde in 1× PBS for 20 min. The fixed cells were permeabilized with 0.5% Triton X-100 in 1× PBS for 10 min, blocked with 0.5% bovine serum albumin (BSA), and incubated for 20 min with phalloidin conjugated to Texas Red (Molecular Probes, Inc.). For membrane staining, transfected cells were stained with primary anti-pan cadherin followed by secondary anti-rabbit fluorescein isothiocyanate antibody. The coverslips were mounted, and confocal images were acquired at room temperature using a Zeiss LSM 510 confocal system mounted on a Zeiss Axiovert 100M microscope equipped with an oil immersion Plan-Apochromat 63×/1.4 differential interference contrast (DIC) objective lens.

**Morpholino and RNA Microinjections**—Microinjections of one-cell stage zebrafish embryo with RNA or MO were carried out as described before (11). MOs were purchased from Gene Tools, Inc. and reconstituted in nuclease-free water to a 2 mM stock concentration (16 ng/ml). Appropriate dilutions were made in 5× injection dye (100 mM HEPES, 1 M KCl, 1% phenol red, and ~2–3 nL of MOs (8–12 ng) were injected at the one-cell stage. Sequences of MO1 and MO2 were described previously (9) except that MO1 and control MO (CTCTTTACCCCTGAT- TACAATTATA) used in this study were fluorescein-tagged. For RNA injections, 150 pg was injected per embryo of wt-Robo4, untagged zebrafish Robo4 (zfR4), and YFP-capped RNA transcribed by T7 polymerase from linearized vectors containing the respective inserts.

**Embryo Dissociation Protocol**—Embryos from Tg(vegfr2: G-RCFP) were dechorionated manually at 21 som in egg water. Dechorionated embryos were washed three times in 1× PBS before incubating the embryos in Tm1 buffer (100 mM NaCl, 5 mM KCl, 5 mM HEPES, 1% polyethylene glycol 20000). The cells were dissociated in 1× Tryptsin containing Proteinase K (0.08 mg/ml) with a narrow bore pipette tip and observed under a stereomicroscope until a single cell suspension was obtained. The dissociated cells were filtered with 70-μm nylon cell strainer and immediately transferred into a LabTek glass chamber along with media containing 10% sheep serum.

**Ex Vivo Time-lapse and Image Analysis**—Dissociated cells from 21 som embryos were observed for GFP and RFP fluorescence using argon gas laser for GFP at 488 nm and helium neon laser for Rhodamine at 546 nm. Once a GFP+/RFP− cell was detected, initial confocal time series acquisitions was collected one frame (sequentially) every 5 s for 30 min using the Zeiss AIM software version 3.2 sp2 (Carl Zeiss GmbH, Heidelberg, Germany). All original confocal time series datasets were of frame size 532 pixels by 532 pixels, scan zoom of 3 and ~94 to 394 megabytes. Post-processing of the time series datasets was analyzed using Bitplane’s Imaris software version 4.1.1 (Bitplane AG, Zurich, Switzerland). Datasets were cropped to an approximate frame size of 225 pixels by 190 pixels and resampled using a subsampling factor of 2 for x and y. Each dataset was then volume-rendered with a 5-μm grid, and coordinate axes were calculated from the image properties used during image acquisition. After all modifications were completed, the time series datasets were recorded using MPEG file format at 25 frames per second and with a compression factor of 50. For time track analysis, each time series was collected using a Plan-Apochromat 63×/1.4 numerical aperture differential interference contrast oil immersion objective with a scan zoom ranging from 2.5 to 5. For panels B–I in Fig. 2, Imaris 3D (Bitplane AG, Basel, Switzerland) software (version 4.2.0) was used to create a volume rendering of each time series where a spot with a diameter of 4–5 μm represented the cell and a time track reflecting the movement of the spot was generated with the auto-regressive motion gap close function. Time tracks were color-coded with blue for early time points and white for late for time points (see color-coded time bar, bottom right of each image). For panels F–I in Fig. 2, a 40-frame subset of each time series was made with the Zeiss AIM software, keeping the midpoint the same as the full 360 frame (5 s/frame) time series. Regions of interest were drawn around the cell perimeter using Image-Pro Plus (MediaCybernetics Inc., Silver Spring, MS) software (version 5.1.2). The green and/or red fluorescence plus phase contrasts were used as a guide to draw regions of interest around each cell at each time point. The new tif series was imported into the Zeiss AIM software to modify the z-step size from 1 mm to 15 mm to create the cylindrical effect for visual clarity. Using Imaris 3D software, the modified z-step
tiff series was imported into the surpass module and volume rendered. Once rendered, the green and red fluorescence plus phase was switched off leaving only the regions of interest outline of the cell with or without the cellular projections intact.

**Migration Assay**—Migration assay was performed on HEK-293T cells transfected with GFP, wt-Robo4, Cdc42DN, and wt-Robo4 plus Cdc42DN constructs using the BD Falcon insert system. Approximately \( 2 \times 10^5 \) cells/well were placed on the upper chamber, and Dulbecco’s modified Eagle’s medium containing 10% serum was used as the stimulus in the lower chamber. The cells were incubated for 5 h at a 37 °C incubator, and all treatments were carried out in triplicates. The cells from the upper membrane side were removed by cotton tip. The migrated cells on the bottom side facing the stimulus were stained by Diff Quick stain kit (Dade Behring). The stained cells on the membrane were counted manually using an inverted microscope using a 60× objective (Leica).

**Adhesion Assay**—Transfected HEK-293T cells with relevant constructs were washed thrice with PBS, trypsinized, resuspended to a concentration of \( 10^6 \) cells/well, and plated in 0.5% bovine serum albumin (BSA)- or fibronectin (5 μg/ml)-coated wells. After 5 h, cells were washed thrice with PBS, fixed, stained with Diff Quick stain kit, and counted manually.

**Statistical Analysis and Quantitation**—A two-way \( \chi^2 \) test was performed to determine \( p \) value of RNA injected samples in Fig. 1. The RNA injection experiments showed statistical significance across samples for YFP and wt-Robo4 or zfr4 \( (p < 0.0005) \), whereas no significance was attributed between WT and YFP or wt-Robo4 and zfR4 samples. Statistical significance for Fig. 8 was determined by Student two-sample t test assuming equal variances. For Fig. 8 (A and B), \( p \) values for GFP and wt-Robo4 or wt-Robo4 and wt-Robo4 plus Cdc4DN are \( p < 0.0005 \) in migration assay (serum stimulus), and \( p < 0.0005 \) for adhesion assay (fibronectrin). For Fig. 8C, \( p = 0.16 \) for wt-Robo4 and re-wt-Robo4 plus WASH, \( p < 0.001 \) for wt-Robo4 and wt-Robo4 plus PAKN. For Fig. 8D, \( p < 0.003 \) for c-Robo4 and GFP, \( p = 0.63 \) for n-Robo4 and GFP, \( p = 0.75 \) for C1-Robo4 and GFP. For Fig. 8E, \( p = 3.9e-08 \) for GFP and c-Robo4, \( p = 0.05 \) for GFP and c1-Robo4, and \( p = 0.009 \) for GFP and n-Robo4. Quantitation of Western blots was performed using Quantity One software. A volumetric analysis tool was used to generate values and, after background subtraction, percentage activation was calculated by GTP pull-down values divided by total lysate values multiplied by 100. The fold activation was determined by dividing all values to untransfected or GFP values.

**RESULTS**

Roundabouts are a class of cell-surface receptors originally discovered to mediate repulsion guidance decisions that target axons to their final destination (2). Robo1 and Robo4 are the only two Robos of the robo family that are implicated to play a role in vascular signaling (Fig. 1A) (5, 6, 18). Previously, we have shown that removing endogenous robo4 in zebrafish results in temporal and spatial disruption of ISVs sprouting from dorsal aorta (9). To investigate structure-function correlation for Robo4, we initially generated four deletion mutants tagged with GFP at their C terminus. A full-length zebrafish Robo4 tagged with GFP (wt-Robo4: 1–1134AA), N-terminal that lacks the entire C terminus (n-Robo4: 1–624AA), and two C-terminal GFP fusion constructs with one lacking partial N terminus (c-Robo4: 531–1134AA) while another lacking the N terminus completely (c1-Robo4: 585–1134AA) were generated (Fig. 1B). The constructs were transiently transfected into HEK-293T cells and lysates were immunoblotted with GFP antibody. Western blot showed protein above 160 kDa for wt-Robo4 (Fig. 1C, lane F), slightly above 105 kDa for n-Robo4 (Fig. 1C, lane N), and below 105 kDa for c-Robo4 (Fig. 1C, lane C) and c1-Robo4 (Fig. 1C, lane C1) constructs. Interestingly, wt-Robo4 and n-Robo4 fusion proteins migrate higher in molecular mass than their expected size (wt-Robo4: 150 kDa, n-Robo4: 91 kDa) reflecting probable post-translational modification in the extracellular domain of Robo4. The difference between c-Robo4 and c1-Robo4 is 54 aa, which amounts to a ~6-kDa size difference that is observed in gradient gel (data not shown).

To check if addition of GFP tag altered the function of the native protein, we used RNA overexpression readouts that was previously shown to phenocopy gene knockdown experiments. To observe vessels, we utilized Tg(vegfr2:GFP) transgenic fish carrying a 6.5-kb
vegfr2-promoter fragment driving GRCFP (green reef coral fluorescent protein) expression in angioblasts. We injected capped RNA for untagged zebrafish Robo4 (zfR4), wt-Robo4, and control yellow fluorescent protein (YFP) into Tg(vegfr2:GRCFP) embryos and checked for ISVs sprouting from dorsal aorta at 22–24 som (Fig. 1, D–G). Both untagged zebrafish Robo4 (zfR4) (Fig. 1F) and wt-Robo4 RNA (Fig. 1G) disrupted ISVs sprouting from dorsal aorta when compared with YFP (Fig. 1E) or uninjected embryos (Fig. 1D) suggesting that the GFP tag did not alter the native functionality of Robo4. Quantitation shows a 3-fold increase in defective embryos in wt-Robo4 and zfR4 injected samples when compared with wild type (WT) un.injected or YFP-injected embryos (Fig. 1H) and are statistically significant (p < 0.0005).

**Robo4 Knockdown Angioblasts ex Vivo Search Actively for Guidance Cues**—Because robos knockdown zebrafish embryos display ISVs sprouting defects (9), we checked the behavior of angioblasts isolated from these embryos. We developed an *ex vivo* approach (Fig. 2A) where fluorescein-tagged splicing MO was injected at one-cell stage into Tg(vegfr2:GRCFP) embryos. The embryos were developed until desired stage, and angioblasts were isolated by the embryo dissociation protocol explained under “Materials and Methods.” Two different splicing MOs were used that were previously shown to target robos transcript (9). Live time-lapse pictures were captured of angioblasts plated on glass cover chambers, and all movies were made during the period from 24 to 26 som stage where robos expression is seen in the vasculature (9). Unexpectedly, angioblasts isolated from robos MO1 (supplemental movie 3) and MO2 (movie 4) embryos show movement that resemble cells actively searching for guidance and continue rolling (movie 4) in a non-directional manner. On the other hand, control MO injected (movie 2) or uninjected (movie 1) angioblasts display less activity and appear stationary. Moreover, cells that were not endothelial in origin (GFP–) but were injected with MO did not show any difference in behavior when compared with control MO or uninjected embryos (data not shown). Time tracks in the still images (Fig. 2, B–E) show the track of angioblast movement. The MO-injected embryos (Fig. 2, C and D) show extended time tracks from the original position in comparison to uninjected (Fig. 2B) or control MO-injected embryos (Fig. 2E), which show compacted tracks. In addition to time tracks, four different cylindrical projections (Fig. 2, F–I) showing different cell topology angle were generated for each sample. Comparing the F panels, the MO sample (F) shows numerous deviations from concentric patterns seen in angioblast from uninjected (F) and control MO (F) injected samples. Moreover, the well defined path traversed by the angioblast in both uninjected (G–I) and control MO (G’–I’) is lost in random movements seen in MO (G’–I’) cylindrical projections. These results suggest that angioblast in the absence of Robo4 seem to search and move in a random trajectory.

To determine whether the absence of Robo4 had any correlation to the angioblast behavior and movements, we investigated whether Rho GTPases that play a role in actin cytoskeletal rearrangement leading to directional migration were perturbed in the presence of Robo4.

**Robo4 Fusion Proteins Activate Rho GTPases**—Structure-function fusion constructs described previously were transfected into endothelial cells (Fig. 3, A–L), and protein expression was visualized under GFP.
fluorescent microscopy. Because all deletion proteins contained the transmembrane domain we checked for membrane targeting for fusion proteins by performing immunostaining with pan-cadherin marker on the transfected cells. Confocal analysis shows co-localization patterns for wt-Robo4 (Fig. 3, A–C), n-Robo4 (Fig. 3, D–F), c-Robo4 (Fig. 3, G–I), and c1-Robo4 (Fig. 3, J–L) proteins with cadherin suggesting that all the proteins are appropriately targeted to the plasma membrane. We also checked for co-localization with markers for nucleus, golgi, ER, mitochondria, and lysosome, and none of them co-localized with the fusion proteins (data not shown).

Initially, we performed overexpression analysis in HEK-293T cells, because biochemical studies are relatively easier to perform due to their high transfection efficiency. We transfected the fusion constructs into HEK-293T cells and performed an endogenous Rho GTPase pull-down assay with PAK beads that recognized both activated Cdc42 and Rac1, followed by Western blot with specific antibodies to Cdc42 (Fig. 4A, top gel) or Rac1 (Fig. 4D, bottom gel). Again, similar to HEK-293T cells, wt-Robo4 and c-Robo4 (Fig. 4, A and B, lane R1) also activated Cdc42 and Rac1. In contrast, Rho pull-down assays with lysates from wt-Robo4, n-Robo4, mRobo4, or rRobo1 (Fig. 4C, lanes F, C, M, and R1, respectively) showed no active Rho. Quantitation of the blots show 70–80% activation for Cdc42 in wt-Robo4, and rRobo1 HEK 293 cells, and 80% Cdc42 or Rac activation for mRobo4-transfected cells. Cross-species activation differences were not statistically significant (Cdc42: p=0.2, Rac: p=0.3). Taken together, these results suggest that extracellular ligand binding region of Robo4 is necessary for activation of Cdc42 and Rac1 but not Rho and that this activation is mediated by the cytoplasmic region, and is conserved across family members, and in evolution.

Robo4 Activates Cdc42 and Rac1 in Endothelial Cells and Induces Actin Bundles That Resemble Filopodia and Lamellipodia—To determine if the Rho GTPase activation was conserved in endothelial cells, we repeated the pull-down experiments in PAE cells. Endothelial cells were transfected with wt-Robo4, n-Robo4, and c-Robo4 constructs by magnetofection method, and pull-down assays were subjected to western with Cdc42 (Fig. 4D, top gel) or Rac1 (Fig. 4D, bottom gel) specific antibody. Again, similar to HEK-293T cells, wt-Robo4 and c-Robo4 (Fig. 4D, lanes F and C) activate Cdc42 when compared with untransfected
lysates (Fig. 4D, lane -) in PAE cells. However, in the case of Rac1, wt-Robo4 activates Rac1 (Fig. 4D, lane F, bottom gel) but c-Robo4 does not (Fig. 4D, lane C, bottom gel), which suggests that probable differences in affinities between wt-Robo4 and c-Robo4 for ligand in endothelial cell lysates could account for this discrepancy. Quantitation of the blots show 80% activation for Cdc42 and Rac in wt-Robo4- transfected endothelial cells, slightly higher than 293 cells. Robo1 lysates also showed robust Rac1 and Cdc42 activation in endothelial cells (data not shown), which implicates roles for Rac1 and Cdc42 in Robo1-induced endothelial cell migration (5).

To determine if variance in mutant protein production could explain the Rho GTPase activation results in overexpression experiments, we checked Cdc42 and Rac-GTP levels in lysates containing approximately equal amount of fusion proteins (Fig. 4, E and F, GFP panel). The results are similar in that wt-Robo4 lysates have more Cdc42-GTP or Rac-GTP levels when compared with n-Robo4 lysates (Fig. 4E), and c-Robo4 shows more Rho GTPase levels when compared with c1-Robo4 (Fig. 4F). These results support the conclusion that wt-Robo4 and c-Robo4 activate Cdc42 and Rac, whereas n-Robo4 and c1-Robo4 do not, and suggest that Rho GTPase activation by these fusion constructs is not the result of variation in levels of wild-type and mutant protein expression.

Phenotypic cell morphology analysis of dominant active Rho GTPases transfected in endothelial cells show distinct filamentous actin phenotypes. Active Rac1 induces lamellipodia (Fig. 5B), whereas active Cdc42 induces filopodia (Fig. 5C) when compared with untransfected cells (Fig. 5A). wt-Robo4 (Fig. 5D) or c-Robo4 (Fig. 5E)-transfected endothelial cells showed both filopodia (Fig. 5, D and E, white arrowhead) and lamellipodia (Fig. 5D, gray arrowhead) similar to mouse Robo4-transfected cells (data not shown). These results when taken together suggest that Robo4-induced Cdc42- and Rac1-activated endothelial cells show morphological readouts of this activation state, namely filopodia and lamellipodia.

Robo4 Mediates Cdc42 Activation Partially via CC0 Domain—To further localize the intracellular domain in Robo4 responsible for Rho GTPase activation, we designed two additional mutants, namely c2-Robo4 and c3-Robo4, that are similar to c-Robo4 in that it contains...
the small extracellular region that is required for Rho GTPase activation but they lack the CC2 domain in c2-Robo4 and both CC0 and CC2 domains in c3-Robo4 (Fig. 6A). Essentially, c2-Robo4 and c3-Robo4 encompass 531–807 aa and 531–688 aa in Robo4 and, when transfected into endothelial cells, localize to the plasma membrane (data not shown) and produce proteins of expected sizes (data not shown). Further, Rho GTPase pull-down assays in HEK-293T cells show that both c2-Robo4 and c3-Robo4 activate Cdc42 and Rac when compared with c1-Robo4 (Fig. 6B, lanes C2 and C3). However, the c3-Robo4 lysate shows attenuated levels (50%) of Cdc42-GTP when compared with c2-Robo4 (80%) and c-Robo4 (80%). Rac activation, however, was not much affected with 80% for c2-Robo4 and 70% for c3-Robo4. These results when taken together with previous deletion mutants suggest that Robo4 mediates Rho GTPase activation through the intracellular C-terminal domain and that the 54-amino acid extracellular ligand region is necessary for this activation. Further, the intracellular CC0 domain partially mediates Cdc42 activation but is not necessary for Rac activation. Fig. 6C shows quantitative differences in Cdc42 and Rac activation between all the mutants. wt-Robo4, c-Robo4, c2-Robo4, and c3-Robo4 show comparable Rac activation levels when compared with n-Robo4- and c-Robo4-expressing 293 cells. For Cdc42, a marked decrease in activation levels is noted for c3-Robo4-expressing cells, although it is still higher than n-Robo4- and c1-Robo4-expressing cells.

Gene Knockdown of Robo4 in Zebrafish Embryos Results in Lower Amounts of Active Rho GTPases in Vivo—Based on the findings that overexpression of Robo4 activates Cdc42 and Rac in HEK-293T and endothelial cells, we asked whether knockdown of Robo4 in vivo in zebrafish embryos would lower the activation state of Rho GTPases. For these experiments, we performed pull-down assays with lysates from MO-injected embryos (Fig. 6D). robo4 knockdown embryos showed 80% decrease in Cdc42-GTP levels (Fig. 6D, top gel, lane MO) when compared with un injected (Fig. 6D, top gel, lane U). Control MO (Fig. 6D, top gel, lane CMO) injected embryos, whereas the Rac-GTP pull-down assays showed a decrease of 40% in MO samples when compared with uninjected. No difference was observed in Rho pull-down assays across uninjected and MO samples (Fig. 6D, bottom gel). The in vivo pull-down results suggest that robo4 knockdown in zebrafish embryos results in lesser amounts of active Cdc42 and Rac but no difference in Rho and reciprocates with gain of function analysis in endothelial cells.

Robo4-induced Morphological Effects in Endothelial Cells Are Blocked by Cdc42 Dominant Negative—Dominant negative (DN) Rho GTPase constructs are extensively used in Rho GTPase biology to address whether blocking specific Rho GTPase activation results in changes in downstream functional consequences measured by phenotypic readouts (19). Initially, we determined by biochemical pull-down assays in HEK-293T cells whether the Cdc42DN and Rac1DN constructs did indeed block Robo4-activated Cdc42 and Rac1 proteins. Interestingly, only Cdc42DN blocks the Robo4-activated Cdc42-GTP (Fig. 7A, top gel, compare lanes F and F+DN), whereas Rac1DN did not (Fig. 7A, bottom gel, compare lanes F and F+DN). One explanation for this result is that Robo4 might activate Rac indirectly by activating Cdc42, and precedence for this cascade mechanism exists at least in fibroblasts (20). To investigate whether activation of Rac is a consequence of Cdc42 activation by Robo4, we checked Rac-GTP levels in wt-Robo4 plus Cdc42DN-transfected cells (Fig. 7B). The pull-down assays show that Rac-GTP level is indeed affected (compare lanes F and F+DN) and suggest that Robo4-induced Rac activation is not direct. However, whether Robo4-mediated Rac activation is exclusively indirect is an open question.

Because Robo4 also activates both Cdc42 and Rac1 in endothelial cells, we determined whether Robo4-induced Rac activation was indirect in endothelial cells by confocal microscopy of phalloidin-stained actin cytoskeletal changes in endothelial cells transfected with wt-Robo4, wt-Robo4 plus Cdc42DN, and wt-Robo4 plus RacDN. wt-Robo4-transfected cells show thick actin bundles and filopodia that are short and thick (Fig. 7C) in comparison to wt-Robo4 plus Cdc42DN (Fig. 7D) co-transfected PECs that show minimal filopodia and loss of actin bundles in the cytoskeleton. In high power, the outer edges of the wt-Robo4-transfected cells clearly shows filopodia and membrane protrusions (Fig. 7D) that disappear when cells are co-transfected with Cdc42DN (Fig. 7F), and instead the outer edge appears smooth and uniform. When wt-Robo4 plus Cdc42DN was compared with wt-Robo4 plus RacDN, Cdc42DN blocked all actin structures induced by Robo4 (Fig. 7E), and Rac1DN did not (Fig. 7G), suggesting that Robo4-induced Rac activation is most likely indirect in endothelial cells as well. In addition to Robo4, we had earlier shown that Rob1 also activates Cdc42 and Rac in HEK cells (4A, lane R1) and endothelial cells (data not shown). Interestingly, Robo1-transfected endothelial cells were constricted (Fig. 7H) and showed retraction fibers that are long and thin suggesting a level of specificity in the actin cytoskeleton rearrangement induced by robos.

Robo4-induced Phenotypic Effects Are Rescued by Dominant Negative Cdc42—To investigate the biological consequence of Rho GTPase activation, we performed migration (Fig. 8A) and adhesion (Fig. 8B) assays in HEK-293T cells, because interference with endogenous Robo4 in endothelial cells could hinder interpretation of results. wt-Robo4-expressing cells showed a 2-fold increase in migration to serum when compared with GFP-transfected cells (Fig. 8A). We have also performed migration in HEK-293T cells in response to epidermal growth factor and found no difference between GFP- and Robo4-transfected cells (data not shown) suggesting either epidermal growth factor is not the ligand for Robo4 or that some factor in the serum induces the pro-migratory signal. The angioblast movies (supplemental movie 4) and the structural features of Robo4s extracel-

**FIGURE 7.** Robo4-mediated morphological effects can be blocked by co-transfecting dominant negative Cdc42. A, Western blot with specific antibodies for Cdc42 (upper) and Rac1 (lower) using pull-down lysates from G- and F-transfected cells, and F+DN: full-length Robo4 plus respective dominant negative transfected cells. B, pull-down assays for Rac-GTP in lysates from cells transfected with respective constructs, C-DN, Cdc42DN, F and G nomenclature is same as in other figures. C-H, confocal microscopy of endothelial cells transfected with Robo4 alone (C and D), Robo4 plus Cdc42DN (E and F), Robo4 plus RacDN (G), and Robo1 (H). C, E, G, and H are low power (20×); whereas D, F, and A are high power (40×). pd, pull-down; total, total lysates loaded per lane.
lular domain (Fig. 1B) suggest adhesion properties for Robo4. We performed adhesion assay in the presence of BSA or fibronectin, because Robo4 contains fibronectin type III domains in its extracellular region. wt-Robo4-transfected HEK-293T cells adhere 4-fold better when compared with GFP-transfected HEK-293T cells (Fig. 8B) on fibronectin matrix, but no significant change was observed on BSA. Because adhesion is one component of chemotaxis, these data together suggest that increased adhesion of Robo4-transfected cells may contribute to the enhanced migratory activity of these cells.

We next investigated whether blocking Robo4-induced filopodia with Cdc42DN would also block the migration and adhesion readouts. Cdc42DN alone does not alter the basal migration status of the cells but blocked Robo4-induced HEK-293T migration to serum (Fig. 8A, panel F+cdcDN). In addition, Robo4-induced adhesion in HEK-293T cells was also partially blocked by Cdc42DN (Fig. 8B, panel F+cdcDN). Because Cdc42DN blocks Robo4-induced Rac and Cdc42 activation and their downstream effector functions, we asked whether blocking only downstream events from Cdc42 activation specifically by WASP (21) would rescue Robo4-mediated migration responses. WASP-mediated blocking of serum-induced migration of wt-Robo4 co-transfected cells (Fig. 8C, panel F+WASP) is not statistically significant (p = 0.16) when compared with wt-Robo4-transfected cells (Fig. 8C, panel F). Further, PAKN (another blocker of downstream effectors of both Cdc42 and Rac) blocked serum-induced migration of Robo4-transfected 293 cells (Fig. 8C, compare black bars, F+PAKN and F). Also, PAKNL2, a control for PAKN, was unable to block the activation, and this state indeed promoted migration of HEK293T cells to serum (Fig. 8C, panel PAKNL2). Taken together, these results suggest that blocking downstream effector activation from both activated Rac and Cdc42 is necessary for attenuating Robo4-mediated phenotypic responses.

To determine whether Robo4-induced phenotypic responses are ligand-dependent, we checked for the phenotypic responses of c-Robo4- and c1-Robo4-transfected 293 cells to serum. As expected, wt-Robo4- and c-Robo4-transfected cells migrated in response to serum, but c1-Robo4- and n-Robo4-transfected cells did not (Fig. 8D). In addition, the trend in adhesion assays is similar to the migration assay (Fig. 8E). Previously, we have shown that c-Robo4 activates Rho GTPase while c1-Robo4 did not (Fig. 4A). The differing activities of c-Robo4 and c1-Robo4 mutant in Rho GTPase activation along with their expected phenotypic readouts suggest that Robo4-induced Rho GTPase activation is ligand-dependent.

**DISCUSSION**

Robos in general have been reported to transduce negative guidance cues (22). However, in the case of Robo4, removal of Robo4 in vivo, a presumptive negative regulator, results in fewer sprouts. We had previously proposed two mechanisms to explain this finding (9). First, a non-productive sprouting mechanism where in the absence of guidance a vascular cell throws many sprouts in different directions and eventually regresses and dies and second, an attraction mechanism, where robos mediate attractive cues and removal of such cues results in collapse of vessels. Here, we provide evidence that suggests both mechanisms are...
not mutually exclusive and implies that intracellular Rho GTPase signaling molecules play a role in Robo4-mediated attractive guidance mechanism in vertebrates.

In general, axon guidance molecules are bi-functional. Slits were originally discovered as attractants (23) and more recently have been implicated in repulsion signaling (22). Whether Robo mediates attraction or repulsion cues in terms of endothelial guidance has remained controversial (5, 6, 24). Our study reports a pathway that can be explained by both attraction and repulsion mechanisms and suggests, perhaps, that this pathway may be dynamically utilized to relay both signals arising from different ligands.

Robo4 mediate signals through four conserved cytoplasmic (CC) domains (25). Robo4 has diverged from other members of the Robo family in that it contains two of the four CC domains, namely CC0 and CC2 (Fig. 1A). We initially made four deletion mutants of Robo4 and found that the extracellular ligand-binding region is essential for Robo4-mediated signaling. A minimal region of 54 amino acids in the extracellular region is sufficient to activate intracellular signaling pathways, and deletion of this region in construct c1-Robo4 results in no signal transmission. Additional mutants c2-Robo4 and c3-Robo4 focusing on the CC0 and CC2 domains suggest that CC0 domain is partially involved in Robo4-mediated signaling events.

One of the well studied signaling mechanisms induced by robo in neurons involves the Rho family of GTPases (including Cdc42, Rac1, and RhoA) (25). Rho family GTPases regulate several actin-dependent cellular processes such as cell migration, adhesion, morphogenesis, and axon guidance in addition to other aspects of cell biology such as cell polarity, microtubule cytoskeleton, gene expression, and vesicle formation (19). Most small Rho GTPases are regulated by the combined activity of guanine nucleotide exchange factors (GEFs) that exchanges GDP for GTP and GTPase-activating proteins (GAPs) that hydrolyze GTP. Robo1 is established as a repulsive guidance cue for axons and slit binding to Robo1 recruits srGAP1 (GTPase-activating protein) to the CC3 domain, which results in local inactivation of Cdc42 (26). Recently, Vilse, a RhoGAP, has been implicated in robo-mediated repulsion mechanisms through Rac1 (27, 28). The result of inactivation of Rho GTPases is to reorganize the cytoskeletal components such as actin and microtubules leading to directed cell migration (29). The absence of CC3 domain in Robo4 would suggest that either Robo4 inactivates Cdc42 through alternate mechanisms or that Robo4 does not inactivate Cdc42.

Here, we find that Robo4 when overexpressed, activates both Cdc42 and Rac1 in HEK-293T and endothelial cells, and this activation results in induction of filopodia and lamellipodia in endothelial cells, consequently enhancing cell adhesion and migration. Further, lysates from Robo4 knockdown embryos show lower amounts of Cdc42 and Rac in vivo with no difference in Rho levels. Moreover, angioblasts isolated from the knockdown embryos display behavior characteristic of cells searching for guidance. Taken together, results from this study imply a role for attraction mechanisms for Robo4 in vascular guidance.

Most of the phenotypic and morphological readouts observed in this study can be explained by a dominant negative mechanism where overexpressing a receptor leads to sequestering components through the intracellular C-terminal domain such as a Cdc42GAP and hence leading to Cdc42 activation. However, the strongest argument against this mechanism arises from evidence that two mutants made in this study, namely c-Robo4 and c1-Robo4, share the entire C terminus domain, but only c-Robo4 activates Rho GTPases and c1-Robo4 does not. This suggests that simply sequestering GAPs may not be an exclusive mechanism of Robo4-mediated signaling and argues that ligand-mediated activation of Rho GTPase occurs either by recruitment of GEFs that activate Cdc42/Rac or of GAPs that inactivate Cdc42/Rac.

This brings us to the ligand question. Slits are ligands for Robos (1). However the slit2-Robo4 interaction remains controversial (6, 30). Further, at least three independent lines of evidence suggest that ligands other than slits may be involved in Rho GTPase activation. First, c-Robo4 activates Rho GTPases in the absence of N-terminal Ig domains, which are known interacting domains for slits (24), and HEK-293T cells do express slit2 and slit3 (microarray data not shown). Second, Robo4 dimerizes via the cytoplasmic tail independent of slit binding. Third, during development none of the slits are temporally or spatially expressed during Robo4 expression in vascular development (31, 32). Because most assays here were performed in the absence of serum, we suggest here that ligand is secreted in a cell autonomous manner, and a recent report by Görrn et al. (33) suggests this possibility in cell culture supernatants of proliferating endothelial cells.

Overexpression experiments can also be interpreted as ligand-independent signaling, and recent reports suggest that Robo4 overexpression in endothelial cells can trigger other signaling cascades, such as FAK (10). In addition, overexpression in cells has been previously proposed for Robo4 (6) to induce ligand-independent dimerization and for Robo1 and robo2 to promote axonal outgrowth through homophilic and heterophilic mechanisms (34). Unpublished data show that Robo4 does dimerize through intracellular C-terminal domain, but whether dimerization is ligand-dependent or necessary for Rho GTPase activation is not known. However, in our study the differential Rho GTPase activation observed when overexpressing c-Robo4 and c1-Robo4 argues that Rho GTPase activation by Robo4 is ligand-dependent.

Until now, relatively little has been known about the role of cell adhesion in the function of axon guidance molecules. Our data suggest that, in the absence of adhesion, Rho GTPase is not activated and, consequently, cells do not move (compare C and C1 in Figs. 6B, 8D, and 8E). Further, because n-Robo4-expressing cells do not adhere, the extracellular Ig domains in Robo4 do not exclusively mediate adhesion. Moreover, wt-Robo4- and c-Robo4-expressing cells adhere well to fibronectin suggesting that inside-out signaling is in effect with Robos.

Angioblast movies suggest that Robo4 knockdown cells appear lost and continue to actively search for guidance cues in several directions. Although the ex vivo data seem counterintuitive at first, it suggests either that alternate mechanisms exist for filopodia generation or that Cdc42 in vivo or simply that we are observing the end stage of a confused angioblast in the movies. The ex vivo data correlate well with in vivo data where vessels collapse in Robo4 knockdown embryos suggesting that, when the guidance cue is lost, cells do not have a general direction to migrate and resort to a default mechanism of collapse.

Experiments with Cdc42DN dominant negative construct suggest that Rac activation is not direct but is mediated through activated Cdc42. Confocal microscopy of endothelial cells stained for actin structures confirm this observation, because RacDN does not abrogate all actin structures while Cdc42DN does. Indirect Rac activation in endothelial cells may also explain why the c-Robo4 construct in Fig. 4D did not show robust Rac activation in endothelial cells. One explanation is that binding affinities of wt-Robo4 and c-Robo4 for putative ligand are different. However, it is also possible that a minimum baseline amount of Cdc42 activation is required for Rac activation, and this was not achieved due to transfection efficiencies. Robo4-induced Rac activation is also not exclusively indirect as suggested by WASP experiments. By selective blocking of downstream effectors of Cdc42 activation pathway using WASP, we were unable to rescue Robo4 phenotypic responses but were able to rescue by PAKN, which blocks effectors of both Cdc42 and

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2 D. Li, University of Utah, personal communication.
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Rac activation. Also, the WASP data suggest that a parallel Robo4-mediated direct Rac activation pathway does exist, which is independent of Cdc42-mediated Rac activation. Further, c3-Robo4 mutant activation of Rac (80%), even when the Cdc42 activation is attenuated (50%), is indicative of parallel direct Rac activation pathways from Robo4. Thus, attenuating Robo4 phenotypic readouts requires blocking downstream events from both activated Cdc42 and Rac.

The consequence of Rho GTPase activation could be many (19), but morphology and migration readouts have direct implications for vascular guidance. Previous reports on slit-robo system in endothelial migration has reported conflicting results with reports suggesting slit2 promoting migration in human umbilical vein endothelial cells through Robo1 (5, 24) and inhibiting migration of human umbilical vein endothelial cells through Robo4 (6, 10). Our result shows that both Robo1 and Robo4 activate Cdc42 and Rac in both endothelial (Robo1: data not shown) and 293 cells. The Robo1 result in HEK293T cells is conflicting with published reports that Robo1 inactivates Cdc42 (26). However, microarray gene expression data on 293 cells shows down-regulation of srGAP1 (data not shown), which is known to inactivate Cdc42. Also, 293 cells do not express Robo4 but express Robo1, slit-2, and slit-3 (data not shown). These results support the notion that Robo1 and Robo4 might be co-expressed in varying amounts on endothelial cells (10), and regulation of signals from each could determine the ultimate path of the endothelial growing tip. In fact, precedence for different combinations of robo receptors mediating attractive versus repulsive signals does exist in Drosophila tracheal branching network (35) and is suggestive in studies where slit promotes branching and elongation of neurites of selective neuron while inhibiting others (36). Our study extends this observation to perhaps vascular guidance as well and whether these signals arise from common or separate ligand is unclear.

Vascular guidance involves integrating multiple signaling cues both positive and negative to direct the endothelial tip through complex environment. Based on results here, we suggest Robo4 functions as a one of the molecular rheostats in endothelial cells that regulate critical signals for steering the cell to its appropriate target. Because the decisions to move away or toward a target have to be made temporally and spatially in a rapid fashion, the robustos have evolved an exquisite cross-talk mechanism with Rho GTPases (Cdc42 and Rac1), which results in an active competition for molecular rheostats in endothelial cells that regulate critical signals for vascular guidance.

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