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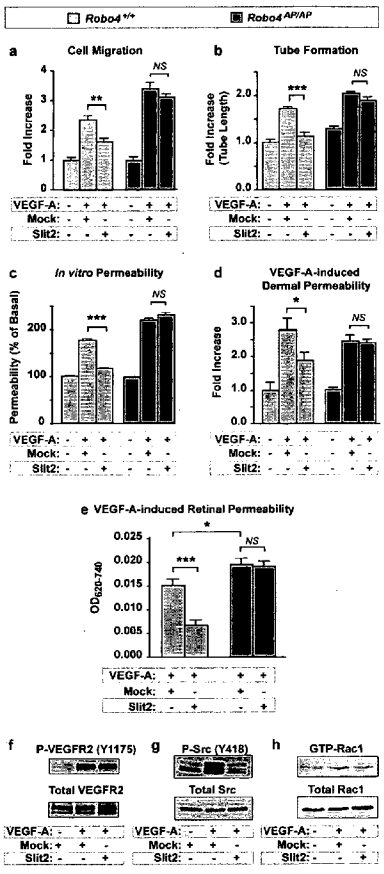

(57) Abstract: Compounds, compositions and methods for inhibiting vascular permeability and pathologic angiogenesis are described herein. Methods for producing and screening compounds and compositions capable of inhibiting vascular permeability and pathologic angiogenesis are also described herein. Pharmaceutical compositions are included in the compositions described herein. The compositions described herein are useful in, for example, methods of inhibiting vascular permeability and pathologic angiogenesis, including methods of inhibiting vascular permeability and pathologic angiogenesis induced by specific angiogenic, permeability and inflammatory factors, such as, for example VEGF, bFGF and thrombin. Methods for treating specific diseases and conditions are also provided herein.

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COMPOSITIONS AND METHODS FOR TREATING PATHOLOGIC ANGIOGENESIS AND VASCULAR PERMEABILITY

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant 1R01 HL77671-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Though, the formation of the vertebrate vasculature of any organ system is a complex process that is orchestrated by a constellation of growth factors and guidance cues (Jain et al., 2003), recent studies have dramatically increased our understanding of the signaling cascades that regulate angiogenesis. For example, it is increasingly clear that molecular programs, which direct trajectory of axons and the formation of the neural network, have important roles in generating the highly stereotypical pattern of the mature vascular network (Carmeliet et al., 2005; Urness et al., 2004; and Jones et al., 2007).

During the initial phase of vascular development in mammals, which is referred to as vasculogenesis, endothelial cells differentiate, migrate and coalesce to form the central axial vessels, the dorsal aortae and cardinal veins. The second phase, called angiogenesis, is characterized by the sprouting of new vessels from the nascent plexus to form a mature circulatory system. VEGF (or VPF) is critical for both of these first two phases: the differentiation and survival of endothelial cells during vasculogenesis as well as proliferation and permeability during angiogenesis. Following this angiogenic remodeling, the endothelium secretes platelet-derived growth factor (PDGF), which induces the recruitment and differentiation of vascular smooth muscle cells. Subsequently, the vascular smooth muscle cells secrete angiopoietins, which ensure proper interaction between endothelial and vascular smooth muscle cells. Finally, the vascular smooth muscle cells deposit matrix proteins, such as elastin, that inhibit vascular smooth muscle cell proliferation and differentiation, thereby stabilizing the mature vessel. Thus, to establish and maintain a mature vascular network, the endothelial and smooth muscle compartments of a vessel must interact via autocrine and paracrine signaling. The gaps between endothelial cells (cell junctions) forming the vascular endothelium are strictly regulated depending on the type and

physiological state of the tissue. For example, in a mature vascular bed, endothelial cells do not behave independently of one another; rather, they form a monolayer that prevents the movement of protein, flud and cells from the endothelial lumen into the surrounding tissue.

Even after development, the vascular system is continually exposed to events, conditions or pathogens that cause injury, ischemia, and inflammation, which typically result in the release of cytokines and angiogenic factors, such as vascular endothelial growth factor (VEGF). Initially, VEGF was described, purified and cloned as vascular permeability factor (VPF), based on its ability to induce blood vessels to leak. VEGF destabilizes endothelial cell-cell junctions, leading to endothelial permeability, stimulates endothelial proliferation and migration, and promotes vascular sprouting and edema. These functions serve to deconstruct a stable vascular network producing leaky new blood vessels. In many contexts, the release of cytokines and angiogenic factors in response to injury, ischemia and inflammation is desirable, in that such a response leads initiates a restorative or healing processes. However, excessive angiogenesis and vascular leak (e.g., endothelial hyperpermeability) underscore the pathologies of several diseases and pathologic conditions.

For example, in the developed world, pathologic angiogenesis and endothelial hyperpermeability in the retinal or choroidal vascular beds are the most common causes of catastrophic vision loss. New and dysfunctional blood vessels leak, bleed or stimulate fibrosis that in turn precipitates edema, hemorrhage, or retinal detachment compromising vision. The major diseases sharing this pathogenesis include proliferative diabetic retinopathy (DR), non-proliferative diabetic macular edema (DME), and age-related macular degeneration (AMD) (Dorrell et al., 2007; Afzal et al., 2007). Approximately 15 million Americans over the age of 65 suffer from AMD, and 10% of these patients will experience visual loss as a result of choroidal neovascularization. Further, more than 16 million Americans are diabetic, and over 400,000 new patients suffer from retinal edema or neovascularization. Given that the current number of 200 million diabetics worldwide is likely to double in the next 20 years, and that over 8% of such patients suffer from microvascular complications, the number of patients that will experience vision loss from diabetic eye disease is unfortunately set to increase rapidly. Though less prevalent than DR, DME and AMD, retinopathy of prematurity (ROP) and ischemic retinal vein occlusion (IRVO) are also associated with pathologic angiogenesis and endothelial hyperpermeability in the retinal or choroidal vascular beds and lack effective treatment.

In addition to diseases of the eye, pathologic angiogenesis is also associated with tumor formation and growth. Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. With angiogenesis tumor growth proceeds, without it, it stops. Tumor angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels. Angiogenesis is regulated by both activator and inhibitor molecules. Under normal conditions, the inhibitors predominate, blocking growth. However, during tumor formation and growth, tumor cells release angiogenesis activators, causing such activators to increase in number/concentration. Such an increase in angiogenesis activators results in the growth and division of vascular endothelial cells and, ultimately, the formation of new blood vessels.

More than a dozen different proteins, as well as several smaller molecules, have been identified as "angiogenic." Among these molecules, two proteins appear to be the most important for sustaining tumor growth: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF and bFGF are produced by many kinds of cancer cells and by certain types of normal cells. VEGF and bFGF are first synthesized inside tumor cells and then secreted into the surrounding tissue. When they encounter endothelial cells, they bind to specific proteins, called receptors, sitting on the outer surface of the cells. The binding of either VEGF or bFGF to its appropriate receptor activates a series of relay proteins that transmits a signal into the nucleus of the endothelial cells. The nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth. The activation of endothelial cells by VEGF or bFGF sets in motion a series of steps toward the creation of new blood vessels. First, the activated endothelial cells produce matrix metalloproteinases (MMPs), a special class of degradative enzymes. These enzymes are then released from the endothelial cells into the surrounding tissue. The MMPs break down the extracellular matrix--support material that fills the spaces between cells and is made of proteins and polysaccharides. Breakdown of this matrix permits the migration of endothelial cells. As they migrate into the surrounding tissues, activated endothelial cells begin to divide and organize into hollow tubes that evolve gradually into a mature network of blood vessels.

Additional diseases and disorders characterized by undesirable vascular permeability include, for example, edema associated with brain tumors, ascites associated with

malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion, pleural effusion, acute lung injury, inflammatory bowel disease, ischemia/reperfusion injury in stroke, myocardial infarction, and infectious and non-infectious diseases that result in a cytokine storm. Though a cytokine storm is the systemic expression of a healthy and vigorous immune system, it is an exaggerated immune response caused by rapidly proliferating and highly activated T-cells or natural killer (NK) cells and results in the release of more than 150 inflammatory mediators (cytokines, oxygen free radicals, and coagulation factors). Both pro-inflammatory cytokines (such as Tumor Necrosis Factor-alpha, InterLeukin-1, and InterLeukin-6) and anti-inflammatory cytokines (such as interleukin 10, and interleukin 1 receptor antagonist) are elevated in the serum, and it is the fierce and often lethal interplay of these cytokines is referred to as a "cytokine storm."

Cytokine storms can occur in a number of infectious and non-infectious diseases including, for example, graft versus host disease (GVHD), adult respiratory distress syndrome (ARDS), sepsis, avian influenza, smallpox, and systemmic inflammatory response syndrome (SIRS). In the absence of prompt intervention, a cytokine storm can result in permanent lung damage and, in many cases, death. Many patients will develop ARDS, which is characterized by pulmonary edema that is not associated with voume overload or depressed left ventricular function. The end stage symptoms of a disease precipitating the cytokine storm may include one or more of the following: hypotension; tachycardia; dyspnea; fever; ischemia or insufficient tissue perfusion; uncontrollable hemorrhage; severe metabolism dysregulation; and multisystem organ failure. Deaths from infections that precipitate a cytokine storm are often attributable to the symptoms resulting from the cytokine storm and are, therefore, not directly caused by the relevant pathogen. For example, deaths in severe influenza infections, such as by avian influenza or "bird flu," are typically the result of ARDS, which results from a cytokine storm triggered by the viral infection.

Because of its involvement in angiogenesis and vascular permeability, much attention has been focused on vascular endothelial growth factor (VEGF). Products that that reduce VEGF mediated angiogenesis and vascular edema are now marketed and available to patients. For example, the anti-VEGF antibody Ranibizumab (Lucentis), an antibody

fragment of Bevacizumab (Avastin), which is itself a VEGF antibody (Rosenfeld et al., 2006; Brown et al., 2006) is commercially available for the treatment of AMD. The development and success of this product has triggered enormous commercial interest in alternative strategies for the treatment of diseases and conditions associated with pathlogic angiogenesis or enthothelial hyperpermeability. Other approaches for inhibiting VEGF signaling include, for example, anti-VEGF aptamer, a soluble VEGF receptor ectodomain, receptor tyrosine kinase inhibitors, and siRNA against either VEGF or its receptors. With respect to AMD, such strategies have shown promise. However, there remains tremendous interest in a similar approaches for treating other conditions associated with pathologic angiogenesis and vascular leak. Moreover, as VEGF is only one of many angiogenic, permeability and inflammatory factors that contribute to angiogenesis and vascular permeability, there is continued value in identifying pathways and developing methods that affect VEGF functionality as well as the functionality of other angiogenic, permeability, or inflammatory factors.

SUMMARY

Generally, compounds, compositions and methods for inhibiting vascular permeability and pathologic angiogenesis are described herein. Methods for producing and screening compounds and compositions capable of inhibiting vascular permeability and pathologic angiogenesis are also described herein. Pharmaceutical compositions are included in the compositions described herein.

Compositions according to the present description can be used in, for example, methods of inhibiting vascular permeability and pathologic angiogenesis, including methods of inhibiting vascular permeability and pathologic angiogenesis induced by specific angiogenic, permeability and inflammatory factors, such as, for example VEGF, bFGF and thrombin. Methods for treating specific diseases and conditions are also provided herein.

Additional aspects of the specification provided herein will become apparent by reference to the Detailed Description, including the Examples and Materials and Methods, the Claims, and the Figures, including the Brief Description of the Drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the disclosed method and compositions and

together with the description, serve to explain the principles of the disclosed method and compositions. As it is used herein, the term "Mock" indicates a sham preparation that does not include active Slit protein.

FIG. 1 shows Robo4-mediated vascular guidance requires the cytoplasmic tail of the receptor. Shown is the results of confocal microscopy of 48 hpf *TG(fli:egfp)y1* embryos (A) un-injected, (B) injected with *robo4* morpholino, (C) *robo4* morpholino and wild-type murine *robo4* RNA, and (D) *robo4* morpholino and *robo4Δtail* RNA. Quantification is shown in FIG. 7. FIG. 1E shows model of defective vascular guidance in *robo4* morphant embryos. 5X and 20X images are shown in the left and right panels, respectively. DLAV = dorsal longitudinal anastomosing vessel. PAV = parachordal vessel. DA = dorsal aorta. PCV = posterior cardinal vein.

FIG. 2 shows Robo4-dependent inhibition of haptotaxis requires the aminoterminal half of the cytoplasmic tail. FIG. 2A shows schematic representation of cDNA constructs used in the haptotaxis migration assays. TM represents the transmembrane domain. CC0 and CC2 are conserved cytoplasmic signaling motifs found in Robo family members. HA = hemagglutinin epitope. FIG. 2B and FIG. 2C show HEK 293 cells were co-transfected with GFP and the indicated constructs and 36 hours later subjected to haptotaxis migration on membranes coated with 5μg/ml fibronectin and either Mock preparation or Slit2. Expression of Robo4 constructs was verified by Western blotting (Inset). Results are presented as the mean±SE.

FIG. 3 shows Robo4 interacts with Hic-5 and paxillin in HEK 293 cells. FIG. 3A shows HEK 293 cells were co-transfected with the Robo4 cytoplasmic tail-HA and Hic-5-V5, or empty vector (pcDNA3) and Hic-5-V5. Robo4 was immunoprecipitated with HA antibodies and Hic-5 was detected by western blotting with V5 antibodies. FIG. 3B shows total cell lysates from Cho-K1, HEK 293 and NIH 3T3 cells were probed with antibodies to Hic-5 and paxillin. FIG. 3C shows HEK 293 cells were co-transfected with paxillin-V5 and Robo4 cytoplasmic tail-HA or empty vector (pcDNA3). Robo4 was immunoprecipitated from cell lysates with HA antibodies and paxillin was detected by western blotting with V5 antibodies. FIG. 3D shows HEK 293 cells were transfected with full length Robo4-HA and paxillin-V5, and stimulated with Slit2 for 5 minutes. Robo4 was immunoprecipitated from cell lysates with HA antibodies and paxillin was detected by western blotting with V5 antibodies.

FIG. 4 shows paxillin interacts with Robo4 through a novel motif that is required for Robo4-dependent inhibition of haptotaxis. FIG. 4A shows schematic representation of GST-Robo4 fusion proteins used in pull down assays shown in panel B. FIG. 4B shows GST-Robo4 fusion proteins were purified form *E. coli* and incubated with recombinant purified paxillin. Paxillin was detected by western blotting with paxillin-specific monoclonal antibodies. FIG. 4C shows schematic representation of GST-Robo4 fusion proteins used in pull down assays described in panel D. FIG. 4D shows GST-Robo4 fusion proteins were purified form *E. coli* and incubated with recombinant purified paxillin. Paxillin was detected by western blotting with a paxillin-specific monoclonal antibodies. FIG. 4E shows GST-Robo4 wild-type or GST-Robo4ΔPIM were purified from *E. coli* and incubated with recombinant purified paxillin or *in vitro* transcribed/translated Mena-V5. Paxillin and Mena were detected with paxillin-specific monoclonal antibodies and V5 antibodies, respectively. FIG. 4F shows HEK 293 cells were transfected with GFP and the indicated constructs and 36 hours later subjected to haptotaxis migration on membranes coated with 5μg/ml fibronectin and either Mock preparation or Slit2. Expression of Robo4 constructs was verified by western blotting (Inset). Results are presented as the mean±SE.

FIG. 5 shows Robo4 suppresses cell spreading through inactivation of Rac. FIG. 5A, FIG. 5D, and FIG. 5G show HEK 293 cells were transfected with GFP and the indicated constructs and 36 hours later subjected to cell spreading assays on coverslips coated with 5μg/ml fibronectin and either Mock preparation or Slit2. Results are presented as the mean±SE. FIG. 5B and FIG. 5E show HEK 293 cells were transfected with the indicated constructs and 36 hours later plated onto dishes coated with 5μg/ml fibronectin and either Mock preparation or Slit2. Following a 5-minute incubation, cells were lysed and GTP-Rac was precipitated with GST-PBD. Rac was detected by western blotting with a Racspecific monoclonal antibody. FIG. 5H shows HUVEC were incubated for 60 minutes with Slit2, stimulated with 25 ng/ml VEGF for 5 minutes, lysed and GTP-Rac was precipitated with GST-PBD. Rac was detected by western blotting with a Rac-specific monoclonal antibody. Slit2-dependent inhibition of (C) and (F) adhesion induced- and (I) VEGF-induced Rac activation was quantified by densitometry. Results are presented as mean±SE.

FIG. 6 shows a paxillinΔLim4 mutant does not interact with Robo4, or support Slit2-Robo4-mediated inhibition of cell spreading. FIG. 6A shows a schematic representation of paxillin constructs used in panels B, C and D. FIG. 6B shows HEK 293 cells were co-

transfected with the Robo4 cytoplasmic tail-HA and paxillin-V5, or empty vector (pcDNA3) and paxillin-V5. Robo4 was immunoprecipitated from cell lysates with HA antibodies, and paxillin was detected by western blotting with V5 antibodies. FIG. 6C shows HEK 293 cells were co-transfected with the Robo4 cytoplasmic tail-HA and either wild-type paxillin-V5 or paxillinΔLim4-V5. Robo4 was immunoprecipitated with HA antibodies, and paxillin was detected by western blotting with V5 antibodies. FIG. 6D shows Endogenous paxillin was knocked down in HEK 293 cells using siRNA and reconstituted with either wild-type chicken paxillin or chicken paxillinΔLim4. Knock down and reconstitution were visualized by western blotting with paxillin antibodies and quantified by densitometry. Paxillin expression was determined to be 35% of wild-type levels. FIG. 6E shows HEK 293 cells subjected to knock down/reconstitution were subjected to spreading assays on coverslips coated with 5μg/ml fibronectin and either Mock preparation or Slit2. Results are presented as the mean±SE.

FIG. 7 shows the paxillin interaction motif is required for repulsive vascular guidance. FIG. 7A shows Quantification of vascular pattering defects in uninjected (n=66), *robo4* morpholino (n=56), *robo4* morpholino and wild-type murine *robo4* RNA (n=60), *robo4* morpholino and *robo4Δtail* RNA (n=17), and *robo4* morpholino and *robo4ΔPIM* RNA (n=45) injected *TG(fli:egfp)y1* embryos. Representative images are shown in FIG. 1. FIG. 7B shows a model of a Slit2-Robo4 signaling axis that inhibits cell migration, spreading and Rac activation.

FIG. 8 shows splice-blocking morpholinos suppress expression of *robo4* in zebrafish embryos. FIG. 8A shows a schematic representation of the *robo4* locus in *Danio rerio* and the encoded Robo4 protein. The exon targeted with the splice-blocking morpholino is indicated, as is the location of the primers used to amplify *robo4* cDNA. FIG. 8B shows RNA from uninjected embryos and embryos injected with *robo4* spliceblocking morpholinos was isolated and used to reverse transcribe cDNA. The cDNA was then used to amplify *robo4* and the resulting fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

FIG. 9 shows Hic-5 is a Robo4-interacting protein. FIG. 9A shows a schematic representation of full-length Hic-5 and the cDNA clones recovered from the yeast two-hybrid screen. FIG. 9B shows *S. cerevisiae* strain PJ694-A was transformed with the indicated plasmids and plated to synthetic media lacking Leucine and Tryptophan, or Leucine,

Tryptophan, Histidine and Alanine. Colonies capable of growing on nutrient deficient media were spotted onto the same media, replica plated, and either photographed or used for the beta-galactosidase assay.

FIG. 10 shows the paxillin interaction motif lies between CC0 and CC2 in the Robo4 cytoplasmic tail. Schematic representation of the murine Robo4 protein and identification of the amino acids comprising the paxillin interaction motif.

FIG. 11 shows the Robo4 cytoplasmic tail does not inhibit Cdc42 activation nor interact with srGAP1. FIG. 11A shows HEK 293 cells expressing Robo4 were plated onto bacterial Petri dishes coated with 5 μg/ml fibronectin and either Mock preparation or Slit2. Following a 5-minute incubation, cells were lysed, and GTP-Cdc42 was precipitated with GST-PBD. Cdc42 was detected by western blotting with a Cdc42-specific monoclonal antibody. FIG. 11B shows HEK 293 cells were transfected with the indicated plasmids, and Robo1/Robo4 were immunoprecipitated with HA antibodies. srGAP1 was detected by western blotting with Flag M2 antibodies.

FIG. 12 shows slit reduces retinopathy of prematurity, which is an FDA standard for factors that affect diabetic retinopathy, retinopathy of prematurity, and age related macular degeneration. FIG. 12A shows percent neovascularization of the retina in wildtype mice receiving Mock preparation compared to those receiving Slit protein. There was a 63% reduction in neovascularization in mice treated with Slit treated mice as compared to wildtype mice. N=6, P<0.003. FIG. 12B shows percent neovascularization of the retina in wildtype mice receiving Mock preparation compared to those receiving saline control. N=5, P<0.85. FIG. 12C shows percent neovascularization of the retina in knockout mice compared to slit. N=1.

FIG. 13 shows slit and netrin can reduce VEGF-induced dermal permeability.

FIG. 14 shows slit can reduce VEGF mediated retinal permeability.

FIG. 15 shows semaphorin like VEGF increases dermal permeability.

FIG. 16 shows that Robo4 blocks Rac-dependent protrusive activity through inhibition of Arf6. CHO-K1 cells stably expressing αIIb or αIIb-Robo4 cytoplasmic tail were plated on dishes coated with fibronectin or fibronectin and fibrinogen, lysed and GTP-Arf6 was precipitated with GST-GGA3. Arf6 was detected by western blotting with an Arf6-specific monoclonal antibody (See, FIG. 16A). CHO-K1 cells stably expressing αIIb or αIIb-Robo4 cytoplasmic tail were cotransfected with GFP and either an empty vector or the

GIT1-PBS, and subjected to spreading assays on coverslips coated with fibronectin or fibronectin and fibrinogen. The area of GFP-positive cells was determined using ImageJ, with error bars indicating SEM (See, FIG. 16B). HEK 293 cells were co-transfected with GFP and the indicated constructs and 36 h later were subjected to spreading assays on fibronectin and either Mock preparation or a Slit2 protein (See, FIG. 16C). In all panels, error bars indicate mean±SE. Expression of Robo4 and ARNO was verified by western blotting (data not shown). HEK 293 cells were co-transfected with GFP and the indicated constructs and 36 h later were plated on dishes coated with fibronectin and either Mock preparation or a Slit2 protein. GTP-Rac was precipitated with GST-PBD and Rac was detected with a Rac1-specific monoclonal antibody (See, FIG. 16D).

FIG. 17 illustrates the results of immunoprecipitation reactions that demonstrate the Robo4 receptor binds to the Slit ligand. FIG. 17A shows the results of immunoprecipitation of cell lysates from untransfected human embryonic kidney 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 210 kD, 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. FIG. 17B shows the results of 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 (210 KD) and its C-terminal proteolytic fragment (70 KD) are detected in Slit-myc CM by an anti-myc antibody (lane 1). As in FIG. 17A, 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. As shown in FIG. 17C – FIG. 17F,

Slit protein binds to the plasma membrane of cells expressing Robo4. Binding of Slit-myc protein was detected using an anti-myc antibody and an Alexa 594 conjugated anti-mouse antibody. Binding is detected on the surface of Robo4-HEK cells (FIG. 17F) but not Control-HEK cells (FIG. 17D).

FIG. 18 illustrates that Robo4 expression is endothelial-specific and stalk-cell centric. FIG. 18A illustrates retinal flatmounts prepared from P5 *Robo4*$^{+/AP}$ mice and stained for Endomucin (endothelial cells), NG2 (pericytes) and Alkaline Phosphatase (AP; Robo4). The top-most arrow pointing to the right in the upper left panel indicates a tip cell, and the remaining arrows indicate pericytes (NG2-positive). "T" also indicates tip cells. FIG. 18B illustrates retinal flatmounts prepared from adult *Robo4*$^{+/AP}$ mice and stained for NG2 (pericytes) and AP (Robo4), with the arrows included in FIG. 18B indicating pericytes (NG2-positive). FIG. 18C shows the results of quantitative RT-PCR (qPCR) performed on the indicated samples using primers specific for PECAM, Robo1 and Robo4. As used in FIG. 18C: "HAEC" represents Human Aortic Endothelial Cells; "HMVEC" represents Human Microvascular Endothelial Cells; and "HASMC" represents Human Aortic Smooth Muscle Cells. FIG. 18D illustrates the results of probing total cell lysates from HMVEC and HASMC with antibodies to Robo4, VE-Cadherin, Smooth Muscle Actin and ERK1/2.

FIG. 19 illustrates that Robo4 signaling inhibits VEGF-A-induced migration, tube formation, permeability and Src family kinase (SFK) activation. Lung endothelial cells (ECs) isolated from *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were used in endothelial cell migration (FIG. 19A), tube formation (FIG. 19B), *in vitro* permeability (FIG. 19C), Miles assay (FIG. 19D) and retinal permeability assay (FIG. 19E). Human microvascular endothelial cells were stimulated with VEGF-A in the presence of a Mock preparation or a Slit2 protein for 5 minutes, lysed and subjected to western blotting with phospho-VEGFR2 antibodies (FIG. 19F), western blotting with phospho-Src antibodies (FIG. 19G) and Rac activation assays (FIG. 19H). In all panels, * represents $p<0.05$, ** represents $p<0.005$, *** represents $p<0.0005$, NS indicates "not significant" and error bars represent SEM.

FIG. 20 illustrates that Robo4 signaling inhibits pathologic angiogenesis in an animal model of oxygen-induced retinopathy ("OIR") and in an animal model of choroidal neovascularization ("CNV"). Neonatal *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were subjected to oxygen-induced retinopathy and perfused with fluorescein isothiocyanate (FITC)-dextran (green). Retinal flatmounts were prepared for each condition and analyzed by fluorescence

microscopy. Arrows indicate areas of pathological angiogenesis (FIG. 20A through FIG. 20D). Quantification of pathologic angiogenesis observed in FIG. 20A through FIG. 20D is provided in FIG. 20 E. In the CNV model, 2-3 month old *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were subjected to laser-induced choroidal neovascularization. Choroidal flatmounts were prepared, stained with *isolectin* and analyzed by confocal microscopy (FIG. 20F through FIG. 20I). Quantification of pathologic angiogenesis observed in FIG. 20F through FIG. 20I is provided in FIG. 20J. In all panels, * represents $p<0.05$, ** represents $p<0.005$, *** represents $p<0.0005$, NS indicates "not significant" and error bars represent SEM.

FIG. 21 illustrates that Robo4 signaling inhibits bFGF-induced angiogenesis and thrombin-stimulated endothelial hyperpermeability. In carrying out the experiments that provided the results illustrated in FIG. 21A, murine lung endothelial cells were subjected to tube formation assays on matrigel in the presence of bFGF and Mock preparation or a Slit2 protein. In carrying out the experiments that provided the results illustrated in FIG. 21B, muring lung endothelial cells were subjected to thrombin-induced permeability assays on fibronectin-coated Transwells.

FIG. 22 illustrates that Robo4 signaling reduces injury and inflammation in a model of acute lung injury. Mice were exposed to intratracheal LPS and treated with either Slit protein or a Mock preparation. The concentrations of inflammatory cells and protein in bronchoalveolar lavages (BAL) were significantly reduced by treatment with Slit protein.

FIG. 23 illustrates different constructs for Slit proteins and shows that recombinant Slit peptides as small as Slit2-D1 (40kD) are active. In FIG. 23A, different constructs for the Slit protein are depicted. The four leucine rich domains (LRR), the epidermal growth factor homology region (EGF) and the c-terminal tags (MYC/HIS) are indicated. Inhibition of VEGF mediated endothelial cell migration by the different Slit construts (2nM) is shown in FIG. 23B.

FIG. 24 shows the effect of administering Slit protein on the survival of mice infected with Avian Flu Virus in accordance with a mouse model of avian flu.

FIG. 25 illustrates the genomic traits of knockout mice described in Example 14.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed

method and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a polypeptide is disclosed and discussed and a number of modifications that can be made to a number of molecules including the polypeptide are discussed, each and every combination and permutation of polypeptide and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, is this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, and that each such combination is specifically contemplated and should be considered disclosed.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the method and compositions described herein. Such equivalents are intended to be encompassed by the following claims.

It is understood that the disclosed method and compositions are not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the meanings that would be commonly understood by one of skill in the art in the context of the present specification.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a polypeptide" includes a plurality of such polypeptides, reference to "the polypeptide" is a reference to one or more polypeptides and equivalents thereof known to those skilled in the art, and so forth.

"Optional" or "optionally" means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units

are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

As used herein, the term "subject" means any target of administration. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. A patient refers to a subject afflicted with a disease or disorder. The term "patient" includes human and veterinary subjects.

"Inhibit," "inhibiting," and "inhibition" mean to decrease an activity, response, condition, disease, or other biological parameter. This can include but is not limited to the complete ablation of the activity, response, condition, or disease. This may also include, for example, a 10% reduction in the activity, response, condition, or disease as compared to the native or control level. Thus, the reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between the specifically recited percentages, as compared to native or control levels.

"Promote," "promotion," and "promoting" refer to an increase in an activity, response, condition, disease, or other biological parameter. This can include but is not limited to the initiation of the activity, response, condition, or disease. This may also include, for example, a 10% increase in the activity, response, condition, or disease as compared to the native or control level. Thus, the increase in an activity, response, condition, disease, or other biological parameter can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or more, including any amount of increase in between the specifically recited percentages, as compared to native or control levels.

The term "therapeutically effective" means that the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

The term "carrier" means a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

The term "regulatory sequences" refers to those sequences normally within 100-1000 kilobases (kb) of the coding region of a locus, but they may also be more distant from the coding region, which affect the expression of the gene. Such regulation of expression comprises transcription of the gene, and translation, splicing, and stability of the messenger RNA.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. The term "operably linked" may refer to functional linkage between a nucleic acid expression control sequence (*e.g.*, a promoter, enhancer, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

"Isolated," when used to describe biomolecules disclosed herein, means, e.g., a peptide, protein, or nucleic acid that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the isolated molecule(s), and may include enzymes, hormones, and other proteinaceous or non-proteinaceous materials. Methods for isolation and purification of biomolecules described herein are known and available in the art, and one of ordinary skill in the art can determine suitable isolation and purification methods in light of the material to be isolated or purified. Though isolated biomolecules will typically be prepared using at least one purification step, as it is used herein, "isolated" additionally refers to, for example, peptide, protein, antibody, or nucleic acid materials *in-situ* within recombinant cells, even if expressed in a homologous cell type.

Further, where the terms "isolated", "substantially pure", and "substantially homogeneous" are used to describe a monomeric protein they are used interchangeably herein. A monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide sequence. A substantially pure protein can typically comprise about 60 to 90% W/W of a protein sample, and where desired, a substantially pure protein can be greater than about 90%, about 95%, or about 99% pure. Protein purity or homogeneity can be indicated by a number of means well known in the art, such as

polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band upon staining the gel. For certain purposes, higher resolution can be provided by using HPLC or other means well known in the art which are utilized for purification.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

As used herein, "vascular permeability" refers to the capacity of small molecules (e.g., ions, water, nutrients), large molecules (e.g., proteins and nuceic acids) or even whole cells (lymphocytes on their way to the site of inflammation) to pass through a blood vessel wall.

The terms "pathologic" or "pathologic conditions" refer to any deviation from a healthy, normal, or efficient condition which may be the result of a disease, condition, event or injury.

PROTEINS & PEPTIDES

As the terms are used herein, "protein" and "peptide" are simply refer to polypeptide molecules generally and are not used to refer to polypeptide molecules of any specific size, length or molecular weight. Protein variants and derivatives are well understood to those of skill in the art and can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA

encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 1 and are referred to as conservative substitutions.

TABLE 1: Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions, others are known in the art.
Ala	Ser
Arg	Lys; Gln
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn, Lys
Glu	Asp
Gly	Pro
His	Asn;Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone

in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

It is understood that one way to define the variants and derivatives of the proteins and peptides disclosed herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. MoL Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. Science 244:48-52, 1989, Jaeger et al. Proc. Natl. Acad. Sci. USA 86:7706-7710, 1989, Jaeger et al. Methods Enzymol. 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e., all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence.

It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed compositions. For example, there are numerous D amino

acids or amino acids which have a different functional substituent then the amino acids shown in Table 1. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., Methods in Molec. Biol. 77:43-73 (1991), Zoller, Current Opinion in Biotechnology, 3:348-354 (1992); Ibba, Biotechnology & Genetic Enginerring Reviews 13:197-216 (1995), Cahill et al., TIBS, 14(10):400-403 (1989); Benner, TIB Tech, 12:158-163 (1994); Ibba and Hennecke, Bio/technology, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include CH_2NH--, --CH_2S--, --CH_2--CH_2 --, --CH=CH-- (cis and trans), --$COCH_2$ --, --$CH(OH)CH_2$--, and --CHH_2SO—(These and others can be found in Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley, Trends Pharm Sci (1980) pp. 463-468; Hudson, D. et al., Int J Pept Prot Res 14:177-185 (1979) (--CH_2NH--, CH_2CH_2--); Spatola et al. Life Sci 38:1243-1249 (1986) (--CH H_2--S); Hann J. Chem. Soc Perkin Trans. I 307-314 (1982) (--CH--CH--, cis and trans); Almquist et al. J. Med. Chem. 23:1392-1398 (1980) (--$COCH_2$--); Jennings-White et al. Tetrahedron Lett 23:2533 (1982) (--$COCH_2$--); Szelke et al. European Appln, EP 45665 CA (1982): 97:39405 (1982) (--$CH(OH)CH_2$--); Holladay et al. Tetrahedron. Lett 24:4401-4404 (1983) (--$C(OH)CH_2$--); and Hruby Life Sci 31:189-199 (1982) (--CH_2--S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is --CH_2NH--. It is understood that peptide analogs can have more than one atom between the bond atoms, such as b-alanine, g-aminobutyric acid, and the like.

Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

NUCLEIC ACIDS

There are a variety of molecules disclosed herein that are nucleic acid based. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantagous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymin-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose. The phosphate moiety of a nucleotide is pentavalent phosphate. An non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-GMP (5'-guanosine monophosphate).

A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T/U as well as different purine or pyrimidine bases, such as uracil-5-yl (.psi.), hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine,

2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex formation. Often time base modifications can be combined with for example a sugar modifcation, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability. There are numerous United States patents such as 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, which detail and describe a range of base modifications. Each of these patents is herein incorporated by reference.

Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety would include natural modifications of the ribose and deoxy ribose as well as synthetic modifications. Sugar modifications include but are not limited to the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10}, alkyl or C_2 to C_{10} alkenyl and alkynyl. 2' sugar modiifcations also include but are not limited to $-O[(CH_2)_n O]_m CH_3$, $-O(CH_2)_n OCH_3$, $-O(CH_2)_n NH_2$, $-O(CH_2)_n CH_3$, $-O(CH_2)_n -ONH_2$, and $-O(CH_2)_nON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10.

Other modifications at the 2' position include but are not limted to: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties

of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars would also include those that contain modifications at the bridging ring oxygen, such as CH_2 and S. Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. There are numerous United States patents that teach the preparation of such modified sugar structures such as 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate moieties include but are not limited to those that can be modified so that the linkage between two nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkage between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Numerous United States patents teach how to make and use nucleotides containing modified phosphates and include but are not limited to, 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

It is understood that nucleotide analogs need only contain a single modification, but may also contain multiple modifications within one of the moieties or between different moieties.

Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

Nucleotide substitutes are nucleotides or nucleotide analogs that have had the phosphate moiety and/or sugar moieties replaced. Nucleotide substitutes do not contain a standard phosphorus atom. Substitutes for the phosphate can be for example, short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones;formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts. Numerous United States patents disclose how to make and use these types of phosphate replacements and include but are not limited to 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

It is also understood in a nucleotide substitute that both the sugar and the phosphate moieties of the nucleotide can be replaced, by for example an amide type linkage (aminoethylglycine) (PNA). United States patents 5,539,082; 5,714,331;and 5,719,262 teach how to make and use PNA molecules, each of which is herein incorporated by reference. (See also Nielsen et al., Science, 1991, 254, 1497-1500).

It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA,

1989, 86:6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937. Numerous United States patents teach the preparation of such conjugates and include, but are not limited to U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

A Watson-Crick interaction is at least one interaction with the Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide analog, or nucleotide substitute.

A Hoogsteen interaction is the interaction that takes place on the Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH2 or O) at the C6 position of purine nucleotides.

i. Nucleic Acid Sequences

A variety of sequences are provided herein, with some of these sequences available from Genbank at www.pubmed.gov. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences. Primers and/or probes can be designed for any sequence given the information disclosed herein and known in the art.

ii. Hybridization/selective hybridization

The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the Tm (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the Tm. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring

Harbor, New York, 1989; Kunkel et al. Methods Enzymol. 1987:154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their k_d, or where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their k_d.

Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

iii. Functional Nucleic Acids

Functional nucleic acids are nucleic acid molecules that have a specific function, such as binding a target molecule or catalyzing a specific reaction. Functional nucleic acid molecules can be divided into the following categories, which are not meant to be limiting. For example, functional nucleic acids include antisense molecules, aptamers, ribozymes, triplex forming molecules, RNAi, and external guide sequences. The functional nucleic acid molecules can act as affectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional nucleic acid molecules can possess a de novo activity independent of any other molecules.

Functional nucleic acid molecules can interact with any macromolecule, such as DNA, RNA, polypeptides, or carbohydrate chains. Thus, functional nucleic acids can interact with the mRNA, genomic DNA, or polypeptide for any of the herein disclosed guidance cues or receptors therefor. Often functional nucleic acids are designed to interact with other nucleic acids based on sequence homology between the target molecule and the functional nucleic acid molecule. In other situations, the specific recognition between the functional nucleic acid molecule and the target molecule is not based on sequence homology between the functional nucleic acid molecule and the target molecule, but rather is based on the formation of tertiary structure that allows specific recognition to take place.

Antisense molecules are designed to interact with a target nucleic acid molecule through either canonical or non-canonical base pairing. The interaction of the antisense molecule and the target molecule is designed to promote the destruction of the target molecule through, for example, RNAseH mediated RNA-DNA hybrid degradation.

Alternatively the antisense molecule is designed to interrupt a processing function that normally would take place on the target molecule, such as transcription or replication. Antisense molecules can be designed based on the sequence of the target molecule. Numerous methods for optimization of antisense efficiency by finding the most accessible regions of the target molecule exist. Exemplary methods would be *in vitro* selection experiments and DNA modification studies using DMS and DEPC. It is preferred that antisense molecules bind the target molecule with a dissociation constant (k_d)less than or equal to 10^{-6}, 10^{-8}, 10^{-10}, or 10^{-12}. A representative sample of methods and techniques which aid in the design and use of antisense molecules can be found in the following non-limiting list of United States patents: 5,135,917, 5,294,533, 5,627,158, 5,641,754, 5,691,317, 5,780,607, 5,786,138, 5,849,903, 5,856,103, 5,919,772, 5,955,590, 5,990,088, 5,994,320, 5,998,602, 6,005,095, 6,007,995, 6,013,522, 6,017,898, 6,018,042, 6,025,198, 6,033,910, 6,040,296, 6,046,004, 6,046,319, and 6,057,437.

Aptamers are molecules that interact with a target molecule, preferably in a specific way. Typically aptamers are small nucleic acids ranging from 15-50 bases in length that fold into defined secondary and tertiary structures, such as stem-loops or G-quartets. Aptamers can bind small molecules, such as ATP (United States patent 5,631,146) and theophiline (United States patent 5,580,737), as well as large molecules, such as reverse transcriptase (United States patent 5,786,462) and thrombin (United States patent 5,543,293). Aptamers can bind very tightly with k_ds from the target molecule of less than 10^{-12} M. It is preferred that the aptamers bind the target molecule with a k_d less than 10^{-6}, 10^{-8}, 10^{-10}, or 10^{-12}. Aptamers can bind the target molecule with a very high degree of specificity. For example, aptamers have been isolated that have greater than a 10000 fold difference in binding affinities between the target molecule and another molecule that differ at only a single position on the molecule (United States patent 5,543,293). It is preferred that the aptamer have a k_d with the target molecule at least 10, 100, 1000, 10,000, or 100,000 fold lower than the k_d with a background binding molecule. It is preferred when doing the comparison for a polypeptide for example, that the background molecule be a different polypeptide. Representative examples of how to make and use aptamers to bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,476,766, 5,503,978, 5,631,146, 5,731,424 , 5,780,228, 5,792,613, 5,795,721, 5,846,713,

5,858,660 , 5,861,254, 5,864,026, 5,869,641, 5,958,691, 6,001,988, 6,011,020, 6,013,443, 6,020,130, 6,028,186, 6,030,776, and 6,051,698.

Ribozymes are nucleic acid molecules that are capable of catalyzing a chemical reaction, either intramolecularly or intermolecularly. Ribozymes are thus catalytic nucleic acid. It is preferred that the ribozymes catalyze intermolecular reactions. There are a number of different types of ribozymes that catalyze nuclease or nucleic acid polymerase type reactions which are based on ribozymes found in natural systems, such as hammerhead ribozymes, (for example, but not limited to the following United States patents: 5,334,711, 5,436,330, 5,616,466, 5,633,133, 5,646,020, 5,652,094, 5,712,384, 5,770,715, 5,856,463, 5,861,288, 5,891,683, 5,891,684, 5,985,621, 5,989,908, 5,998,193, 5,998,203, WO 9858058 by Ludwig and Sproat, WO 9858057 by Ludwig and Sproat, and WO 9718312 by Ludwig and Sproat) hairpin ribozymes (for example, but not limited to the following United States patents: 5,631,115, 5,646,031, 5,683,902, 5,712,384, 5,856,188, 5,866,701, 5,869,339, and 6,022,962), and tetrahymena ribozymes (for example, but not limited to the following United States patents: 5,595,873 and 5,652,107). There are also a number of ribozymes that are not found in natural systems, but which have been engineered to catalyze specific reactions de novo (for example, but not limited to the following United States patents: 5,580,967, 5,688,670, 5,807,718, and 5,910,408). Preferred ribozymes cleave RNA or DNA substrates, and more preferably cleave RNA substrates. Ribozymes typically cleave nucleic acid substrates through recognition and binding of the target substrate with subsequent cleavage. This recognition is often based mostly on canonical or non-canonical base pair interactions. This property makes ribozymes particularly good candidates for target specific cleavage of nucleic acids because recognition of the target substrate is based on the target substrates sequence. Representative examples of how to make and use ribozymes to catalyze a variety of different reactions can be found in the following non-limiting list of United States patents: 5,646,042, 5,693,535, 5,731,295, 5,811,300, 5,837,855, 5,869,253, 5,877,021, 5,877,022, 5,972,699, 5,972,704, 5,989,906, and 6,017,756.

Triplex forming functional nucleic acid molecules are molecules that can interact with either double-stranded or single-stranded nucleic acid. When triplex molecules interact with a target region, a structure called a triplex is formed, in which there are three strands of DNA forming a complex dependant on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind target regions with high affinity and

specificity. It is preferred that the triplex forming molecules bind the target molecule with a k_d less than 10^{-6}, 10^{-8}, 10^{-10}, or 10^{-12}. Representative examples of how to make and use triplex forming molecules to bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,176,996, 5,645,985, 5,650,316, 5,683,874, 5,693,773, 5,834,185, 5,869,246, 5,874,566, and 5,962,426.

External guide sequences (EGSs) are molecules that bind a target nucleic acid molecule forming a complex, and this complex is recognized by RNase P, which cleaves the target molecule. EGSs can be designed to specifically target a RNA molecule of choice. RNAse P aids in processing transfer RNA (tRNA) within a cell. Bacterial RNAse P can be recruited to cleave virtually any RNA sequence by using an EGS that causes the target RNA:EGS complex to mimic the natural tRNA substrate. (WO 92/03566 by Yale, and Forster and Altman, Science 238:407-409 (1990)).

Similarly, eukaryotic EGS/RNAse P-directed cleavage of RNA can be utilized to cleave desired targets within eukarotic cells. (Yuan et al., Proc. Natl. Acad. Sci. USA 89:8006-8010 (1992); WO 93/22434 by Yale; WO 95/24489 by Yale; Yuan and Altman, EMBO J 14:159-168 (1995), and Carrara et al., Proc. Natl. Acad. Sci. (USA) 92:2627-2631 (1995)). Representative examples of how to make and use EGS molecules to facilitate cleavage of a variety of different target molecules be found in the following non-limiting list of United States patents: 5,168,053, 5,624,824, 5,683,873, 5,728,521, 5,869,248, and 5,877,162.

Gene expression can also be effectively silenced in a highly specific manner through RNA interference (RNAi). This silencing was originally observed with the addition of double stranded RNA (dsRNA) (Fire,A., et al. (1998) Nature, 391, 806 811) (Napoli, C., et al. (1990) Plant Cell 2, 279 289) (Hannon, G.J. (2002) Nature, 418, 244 251). Once dsRNA enters a cell, it is cleaved by an RNase III –like enzyme, Dicer, into double stranded small interfering RNAs (siRNA) 21-23 nucleotides in length that contains 2 nucleotide overhangs on the 3' ends (Elbashir, S.M., et al. (2001) Genes Dev., 15:188-200) (Bernstein, E., et al. (2001) Nature, 409, 363 366) (Hammond, S.M., et al. (2000) Nature, 404:293-296). In an ATP dependent step, the siRNAs become integrated into a multi-subunit protein complex, commonly known as the RNAi induced silencing complex (RISC), which guides the siRNAs to the target RNA sequence (Nykanen, A., et al. (2001) Cell, 107:309 321). At some point the siRNA duplex unwinds, and it appears that the antisense strand remains bound to RISC and

directs degradation of the complementary mRNA sequence by a combination of endo and exonucleases (Martinez, J., et al. (2002) Cell, 110:563-574). However, the effect of siRNA or siRNA or their use is not limited to anytype of mechanism.

Also disclosed are nucleic acids can be used for RNAi or RNA interference. It is thought that RNAi involves a two-step mechanism for RNA interference (RNAi): an initiation step and an effector step. For example, in the first step, input double-stranded (ds) RNA (siRNA) is processed into small fragments, such as 21–23-nucleotide 'guide sequences'. RNA amplification appears to be able to occur in whole animals. Typically then, the guide RNAs can be incorporated into a protein RNA complex which is cable of degrading RNA, the nuclease complex, which has been called the RNA-induced silencing complex (RISC). This RISC complex acts in the second effector step to destroy mRNAs that are recognized by the guide RNAs through base-pairing interactions. RNAi involves the introduction by any means of double stranded RNA into the cell which triggers events that cause the degradation of a target RNA. RNAi is a form of post-transcriptional gene silencing. Disclosed are RNA hairpins that can act in RNAi. For description of making and using RNAi molecules see See, e.g., Hammond et al., Nature Rev Gen 2: 110-119 (2001); Sharp, Genes Dev 15: 485-490 (2001), Waterhouse et al., Proc. Natl. Acad. Sci. USA 95(23): 13959-13964 (1998) all of which are incorporated herein by reference in their entireties and at least form material related to delivery and making of RNAi molecules.

RNAi has been shown to work in a number of cells, including mammalian cells. For work in mammalian cells it is preferred that the RNA molecules which will be used as targeting sequences within the RISC complex are shorter. For example, less than or equal to 50 or 40 or 30 or 29, 28, 27, 26, 25, 24, 23, ,22, 21, 20, 19, 18, 17, 16 , 15, 14, 13 , 12, 11, or 10 nucleotides in length. These RNA molecules can also have overhangs on the 3' or 5' ends relative to the target RNA which is to be cleaved. These overhangs can be at least or less than or equal to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 nucleotides long. RNAi works in mammalian stem cells, such as mouse ES cells.

Short Interfering RNA (siRNA) is a double-stranded RNA that can induce sequence-specific post-transcriptional gene silencing, thereby decreasing or even inhibiting gene expression. In one example, an siRNA triggers the specific degradation of homologous RNA molecules, such as mRNAs, within the region of sequence identity between both the siRNA and the target RNA. For example, WO 02/44321 discloses siRNAs capable of sequence-

specific degradation of target mRNAs when base-paired with 3' overhanging ends, herein incorporated by reference for the method of making these siRNAs. Sequence specific gene silencing can be achieved in mammalian cells using synthetic, short double-stranded RNAs that mimic the siRNAs produced by the enzyme dicer (Elbashir, S.M., et al. (2001) *Nature*, 411:494 498) (Ui-Tei, K., et al. (2000) *FEBS Lett* 479:79-82). siRNA can be chemically or *in vitro*-synthesized or can be the result of short double-stranded hairpin-like RNAs (shRNAs) that are processed into siRNAs inside the cell. Synthetic siRNAs are generally designed using algorithms and a conventional DNA/RNA synthesizer. Suppliers include Ambion (Austin, Texas), ChemGenes (Ashland, Massachusetts), Dharmacon (Lafayette, Colorado), Glen Research (Sterling, Virginia), MWB Biotech (Esbersberg, Germany), Proligo (Boulder, Colorado), and Qiagen (Vento, The Netherlands). siRNA can also be synthesized *in vitro* using kits such as Ambion's *SILENCER* siRNA Construction Kit. Disclosed herein are any siRNA designed as described above based on the sequences for the herein disclosed inflammatory mediators.

The production of siRNA from a vector is more commonly done through the transcription of a shRNA. Kits for the production of vectors comprising shRNA are available, such as for example Imgenex's GeneSuppressor Construction Kits and Invitrogen's BLOCK-iT inducible RNAi plasmid and lentivirus vectors. Disclosed herein are any shRNA designed as described above based on the sequences for the herein disclosed inflammatory mediators.

iv. Vectors

Transfer vectors can be any nucleotide construction used to deliver genes into cells (e.g., a plasmid), or as part of a general strategy to deliver genes, e.g., as part of recombinant retrovirus or adenovirus (Ram et al. Cancer Res. 53:83-88, (1993)).

As used herein, plasmid or viral vectors are agents that transport the disclosed nucleic acids, such as those encoding scFvs into the cell without degradation and include a promoter yielding expression of the gene in the cells into which it is delivered. Viral vectors are, for example, Adenovirus, Adeno-associated virus, Herpes virus, Vaccinia virus, Polio virus, AIDS virus, neuronal trophic virus, Sindbis and other RNA viruses, including these viruses with the HIV backbone. Also preferred are any viral families which share the properties of these viruses which make them suitable for use as vectors. Retroviruses include Murine Maloney Leukemia virus, MMLV, and retroviruses that express the desirable properties of MMLV as a vector. Retroviral vectors are able to carry a larger genetic payload, i.e., a

transgene or marker gene, than other viral vectors, and for this reason are a commonly used vector. However, they are not as useful in non-proliferating cells. Adenovirus vectors are relatively stable and easy to work with, have high titers, and can be delivered in aerosol formulation, and can transfect non-dividing cells. Pox viral vectors are large and have several sites for inserting genes, they are thermostable and can be stored at room temperature. A preferred embodiment is a viral vector which has been engineered so as to suppress the immune response of the host organism, elicited by the viral antigens. Preferred vectors of this type will carry coding regions for Interleukin 8 or 10.

Viral vectors can have higher transaction (ability to introduce genes) abilities than chemical or physical methods to introduce genes into cells. Typically, viral vectors contain, nonstructural early genes, structural late genes, an RNA polymerase III transcript, inverted terminal repeats necessary for replication and encapsidation, and promoters to control the transcription and replication of the viral genome. When engineered as vectors, viruses typically have one or more of the early genes removed and a gene or gene/promotor cassette is inserted into the viral genome in place of the removed viral DNA. Constructs of this type can carry up to about 8 kb of foreign genetic material. The necessary functions of the removed early genes are typically supplied by cell lines which have been engineered to express the gene products of the early genes in trans.

v. Retroviral Vectors

A retrovirus is an animal virus belonging to the virus family of Retroviridae, including any types, subfamilies, genus, or tropisms. Retroviral vectors, in general, are described by Verma, I.M., Retroviral vectors for gene transfer. In Microbiology-1985, American Society for Microbiology, pp. 229-232, Washington, (1985), which is incorporated by reference herein. Examples of methods for using retroviral vectors for gene therapy are described in U.S. Patent Nos. 4,868,116 and 4,980,286; PCT applications WO 90/02806 and WO 89/07136; and Mulligan, (Science 260:926-932 (1993)); the teachings of which are incorporated herein by reference.

A retrovirus is essentially a package which has packed into it nucleic acid cargo. The nucleic acid cargo carries with it a packaging signal, which ensures that the replicated daughter molecules will be efficiently packaged within the package coat. In addition to the package signal, there are a number of molecules which are needed in cis, for the replication, and packaging of the replicated virus. Typically a retroviral genome, contains the gag, pol,

and env genes which are involved in the making of the protein coat. It is the gag, pol, and env genes which are typically replaced by the foreign DNA that it is to be transferred to the target cell. Retrovirus vectors typically contain a packaging signal for incorporation into the package coat, a sequence which signals the start of the gag transcription unit, elements necessary for reverse transcription, including a primer binding site to bind the tRNA primer of reverse transcription, terminal repeat sequences that guide the switch of RNA strands during DNA synthesis, a purine rich sequence 5' to the 3' LTR that serve as the priming site for the synthesis of the second strand of DNA synthesis, and specific sequences near the ends of the LTRs that enable the insertion of the DNA state of the retrovirus to insert into the host genome. The removal of the gag, pol, and env genes allows for about 8 kb of foreign sequence to be inserted into the viral genome, become reverse transcribed, and upon replication be packaged into a new retroviral particle. This amount of nucleic acid is sufficient for the delivery of a one to many genes depending on the size of each transcript. It is preferable to include either positive or negative selectable markers along with other genes in the insert.

Since the replication machinery and packaging proteins in most retroviral vectors have been removed (gag, pol, and env), the vectors are typically generated by placing them into a packaging cell line. A packaging cell line is a cell line which has been transfected or transformed with a retrovirus that contains the replication and packaging machinery, but lacks any packaging signal. When the vector carrying the DNA of choice is transfected into these cell lines, the vector containing the gene of interest is replicated and packaged into new retroviral particles, by the machinery provided in cis by the helper cell. The genomes for the machinery are not packaged because they lack the necessary signals.

vi. Adenoviral Vectors

The construction of replication-defective adenoviruses has been described (Berkner et al., J. Virology 61:1213-1220 (1987); Massie et al., Mol. Cell. Biol. 6:2872-2883 (1986); Haj-Ahmad et al., J. Virology 57:267-274 (1986); Davidson et al., J. Virology 61:1226-1239 (1987); Zhang "Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis" BioTechniques 15:868-872 (1993)). The benefit of the use of these viruses as vectors is that they are limited in the extent to which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infectious viral particles. Recombinant adenoviruses have been shown to

achieve high efficiency gene transfer after direct, *in vivo* delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma and a number of other tissue sites (Morsy, J. Clin. Invest. 92:1580-1586 (1993); Kirshenbaum, J. Clin. Invest. 92:381-387 (1993); Roessler, J. Clin. Invest. 92:1085-1092 (1993); Moullier, Nature Genetics 4:154-159 (1993); La Salle, Science 259:988-990 (1993); Gomez-Foix, J. Biol. Chem. 267:25129-25134 (1992); Rich, Human Gene Therapy 4:461-476 (1993); Zabner, Nature Genetics 6:75-83 (1994); Guzman, Circulation Research 73:1201-1207 (1993); Bout, Human Gene Therapy 5:3-10 (1994); Zabner, Cell 75:207-216 (1993); Caillaud, Eur. J. Neuroscience 5:1287-1291 (1993); and Ragot, J. Gen. Virology 74:501-507 (1993)). Recombinant adenoviruses achieve gene transduction by binding to specific cell surface receptors, after which the virus is internalized by receptor-mediated endocytosis, in the same manner as wild type or replication-defective adenovirus (Chardonnet and Dales, Virology 40:462-477 (1970); Brown and Burlingham, J. Virology 12:386-396 (1973); Svensson and Persson, J. Virology 55:442-449 (1985); Seth, et al., J. Virol. 51:650-655 (1984); Seth, et al., Mol. Cell. Biol. 4:1528-1533 (1984); Varga et al., J. Virology 65:6061-6070 (1991); Wickham et al., Cell 73:309-319 (1993)).

A viral vector can be one based on an adenovirus which has had the E1 gene removed and these virons are generated in a cell line such as the human 293 cell line. In another preferred embodiment both the E1 and E3 genes are removed from the adenovirus genome.

vii. Adeno-associated viral vectors

Another type of viral vector is based on an adeno-associated virus (AAV). This defective parvovirus is a preferred vector because it can infect many cell types and is nonpathogenic to humans. AAV type vectors can transport about 4 to 5 kb and wild type AAV is known to stably insert into chromosome 19.

Adeno-associated virus (AAV) is a member of the Parvoviridae, a virus family characterized by a single stranded linear DNA genome and a small icosahedral shaped capsid measuring about 20nm in diameter. AAV was first described as a contamination of tissue culture grown simian virus 15, a simian adenovirus and was found dependent on adenovirus for measurable replication. This lead to its name, adeno-associated virus, and its classification in the genus Dependovirus (reviewed in Hoggan, M.D. Prog Med Virol 12 (1970) 211-39). AAV is a common contaminant of adenovirus samples and has been isolated from human virus samples (AAV2, AAV3, AAV5), from samples of simian virus-15 infected

cells (AAV1, AAV4) as well as from stocks of avian (AAAV) (Bossis, I. and Chiorini, J.A. J Virol 77 (2003) 6799-810), bovine, canine and ovine adenovirus and laboratory adenovirus type 5 stock (AAV6). DNA spanning the entire rep-cap ORFs of AAV7 and AAV8 was amplified by PCR from heart tissue of rhesus monkeys (Gao, G.P., et al. Proc Natl Acad Sci U S A 99 (2002) 11854-9). With the exception of AAVs 1 and 6, all cloned AAV isolates appear to be serologically distinct. Nine isolates have been cloned, and recombinant viral stocks have been generated from each isolated virus.

AAV2 is the best characterized adeno-associated virus and will be discussed as an AAV prototype. The AAV2 genome consists of a linear single stranded DNA of 4,780 nucleotides. Both polarities of DNA are encapsulated by AAV with equal efficiency. The AAV2 genome contains 2 open reading frames (ORF) named rep and cap. The rep ORF encodes the non-structural proteins that are essential for viral DNA replication, packaging and AAV integration. The cap ORF encodes the capsid proteins. The rep ORF is transcribed from promoters at map units P5 and P19. The rep transcripts contain an intron close to the 3' end of the rep ORF and can be alternatively spliced. The rep ORF is therefore expressed as 4 partially overlapping proteins, which were termed according to their molecular weight Rep78, 68, 52 and 40. The cap ORF is expressed from a single promoter at P40. By alternative splicing and utilization of an alternative ACG start codon, cap is expressed into the capsid proteins VP1-3 which range in size from 65-86 kDa. VP3 is the most abundant capsid protein and constitutes 80% of the AAV2 capsid. All viral transcripts terminate at a polyA signal at map unit 96.

During a productive AAV2 infection, unspliced mRNAs from the p5 promoter encoding Rep78 are the first detectable viral transcripts. In the course of infection, expression from P5, P19 and P40 increase to 1:3:18 levels respectively. The levels of spliced transcripts increased to 50% for P5, P19 products and 90% of P40 expressed RNA (Mouw, M.B. and Pintel, D.J. J Virol 74 (2000) 9878-88).

The AAV2 genome is terminated on both sides by inverted terminal repeats (ITRs) of 145 nucleotides (nt). 125 nt of the ITR constitute a palindrome which contains 2 internal palindromes of 21 nt each. The ITR can fold back on itself to generate a T-shaped hairpin with only 7 non-paired bases. The stem of the ITR contains a Rep binding site (RBS) and a sequence that is site and strand specifically cleaved by Rep – the terminal resolution site

(TRS). The ITR is essential for AAV2 genome replication, integration and contains the packaging signals.

The single-stranded AAV2 genome is packaged into a non-enveloped icosahedral shaped capsid of about 20-25 nm diameter. The virion consists of 26% DNA and 74% protein and has a density of 1.41 g/cm3. AAV2 particles are extremely stable and can withstand heating to 60°C for 1 hour, extreme ph, and extraction with organic solvents.

Rep proteins are involved in almost every step of AAV2 replication including AAV2 genome replication, integration, and packaging. Rep78 and Rep68 possess ATPase, 3'-5' helicase, ligase and nicking activities and bind specifically to DNA. Rep52 and Rep40 appear to be involved in the encapsidation process and encode ATPase and 3'-5' helicase activities. Mutational analysis suggests a domain structure for Rep78. The N-terminal 225 aa are involved in DNA binding, DNA nicking and ligation. Rep78 and Rep68 recognize a GCTC repeat motif in the ITR as well as in a linear truncated form of the ITR (Chiorini, J.A., et al. J Virol 68 (1994) 7448-57) with similar efficiencies. Rep78 and Rep68 possess a sequence and strand specific endonuclease activity, which cleaves the ITR at the terminal resolution site (TRS). Rep endonuclease activity is dependent on nucleoside triphosphate hydrolysis and presence of metal cations. Rep 78 and 68 can also bind and cleave single stranded DNA in a NTP independent matter. In addition, Rep78 catalyzes rejoining of single stranded DNA substrates originating from the AAV2 origin of replication – i.e., sequences containing a rep binding and terminal resolution element.

The central region of AAV2 Rep78, which represents the N-terminus of Rep52 and Rep40, contains the ATPase and 3'-5' helicase activities as well as nuclear localization signals. The helicase activity unwinds DNA-DNA and DNA-RNA duplexes, but not RNA-RNA. The ATPase activity is constitutive and independent of a DNA substrate. The C-terminus of Rep78 contains a potential zinc-finger domain and can inhibit the cellular serine/threonine kinase activity of PKA as well as its homolog PRKX by pseudosubstrate inhibition. Rep68 which is translated from a spliced mRNA that encodes the N-terminal 529 amino acids (aa) of Rep78 fused to 7 aa unique for Rep68, doesn't inhibit either PKA or PRKX. In addition to these biochemical activities, Rep can affect intracellular conditions by protein-protein interactions. Rep78 binds to a variety of cellular proteins including transcription factors like SP-1, high-mobility-group non-histone protein 1 (HMG-1) and the oncosuppressor p53. Overexpression of Rep results in pleiotrophic effects. Rep78 disrupts

cell cycle progression and inhibits transformation by cellular and viral oncogenes. In susceptible cell lines, overexpression of Rep resulted in apoptosis and cell death. Several of Rep78 activities contribute to cytotoxicity, including its constitutive ATPase activity, interference with cellular gene expression and protein interactions.

The first step of an AAV infection is binding to the cell surface. Receptors and coreceptors for AAV2 include heparan sulfate proteoglycan, fibroblast growth factor receptor-1, and αvβ5 integrins whereas N-linked 2,3-linked sialic acid is required for AAV5 binding and transduction (Walters, R.W., et al. J Biol Chem 276 (2001) 20610-6). In HeLa cells, fluorescently labeled AAV2 particles appear to enter the cell via receptor-mediated endocytosis in clathrin coated pits. More than 60% of bound virus was internalized within 10 min after infection. Labeled AAV particles are observed to have escaped from the endosome, been trafficked via the cytoplasm to the cell nucleus and accumulated perinuclear, before entering the nucleus, probably via nuclear pore complex (NPC). AAV2 particles have been detected in the nucleus, suggesting that uncoating takes place in the nucleus (Bartlett, et al. J Virol 74 (2000) 2777-85; Sanlioglu et al. J Virol 74 (2000) 9184-96). AAV5 is internalized in HeLa cells predominantly by clathrin coated vesicles, but to a lesser degree also in noncoated pits. AAV particles can also be trafficked intercellularly via the Golgi apparatus (Bantel-Schaal, U., et al. J Virol 76 (2002) 2340-9). At least partial uncoating of AAV5 was suggested to take place before entering the nucleus since intact AAV5 particles could not be detected in the nucleus (Bantel-Schaal et al., 2002) After uncoating, the single stranded genome is converted into duplex DNA either by leading strand synthesis or annealing of input DNA of opposite polarity. AAV replication takes place within the nucleus.

During a co-infection with a helper virus such as Adenovirus, herpes simplex virus or cytomegalovirus, AAV is capable of an efficient productive replication. The helper functions provided by Adenovirus have been studied in great detail. In human embryonic kidney 293 cells, which constitutively express the Adenovirus E1A and E1B genes, the early Adenovirus gene products of E2A, E4 and VA were found sufficient to allow replication of recombinant AAV. Allen et al. reported that efficient production of rAAV is possible in 293 cells transfected with only an E4orf6 expression plasmid (Allen, J.M., et al. Mol Ther 1 (2000) 88-95). E1A stimulates S phase entry and induces unscheduled DNA synthesis by inactivating the pRB checkpoint at the G1/S border by interaction with pRB family proteins which results in the release of E2F (reviewed in (Ben-Israel, H. and Kleinberger, T. Front Biosci 7 (2002)

D1369-95). This leads to either induction or activation of enzymes involved in nucleotide synthesis and DNA replication. Since unscheduled DNA synthesis is a strong apoptotic signal, anti-apoptotic functions are required. E1B-19k is a Bcl-2 homolog and E1B-55k is a p53 antagonist. Both proteins have anti-apoptotic functions. E4orf6 forms a complex with E1B-55k and results in degradation of p53. It is also reported to cause S-phase arrest (Ben-Israel and Kleinberger, 2002). E2A encodes a single strand DNA binding protein, which appears to be non-essential for DNA replication but effects gene expression (Chang and Shenk. J Virol 64 (1990) 2103-9). The VA transcription unit affects AAV2 RNA stability and translation (Janik et al., Virology 168 (1989) 320-9). E1A has a more direct effect on AAV2 gene expression. The cellular transcription factor YY-1 binds and inhibits the viral P5 promoter. E1A relieves this transcriptional block. None of the late Ad gene products have been found to be essential for AAV2 replication. The main function of the helper virus appears to be the generation of a cellular environment with active DNA replication machinery and blocked pro-apoptotic functions that allows high-level AAV replication rather than a direct involvement in AAV replication.

viii. Large payload viral vectors

Molecular genetic experiments with large human herpesviruses have provided a means whereby large heterologous DNA fragments can be cloned, propagated and established in cells permissive for infection with herpesviruses (Sun et al., Nature genetics 8: 33-41, 1994; Cotter and Robertson,.Curr Opin Mol Ther 5: 633-644, 1999). These large DNA viruses (herpes simplex virus (HSV) and Epstein-Barr virus (EBV), have the potential to deliver fragments of human heterologous DNA > 150 kb to specific cells. EBV recombinants can maintain large pieces of DNA in the infected B-cells as episomal DNA. Individual clones carried human genomic inserts up to 330 kb appeared genetically stable The maintenance of these episomes requires a specific EBV nuclear protein, EBNA1, constitutively expressed during infection with EBV. Additionally, these vectors can be used for transfection, where large amounts of protein can be generated transiently *in vitro*. Herpesvirus amplicon systems are also being used to package pieces of DNA > 220 kb and to infect cells that can stably maintain DNA as episomes.

Other useful systems include, for example, replicating and host-restricted non-replicating vaccinia virus vectors.

ix. Non-nucleic acid based systems

The disclosed compositions can be delivered to the target cells in a variety of ways. For example, the compositions can be delivered through electroporation, or through lipofection, or through calcium phosphate precipitation. The delivery mechanism chosen will depend in part on the type of cell targeted and whether the delivery is occurring for example *in vivo* or *in vitro*.

Thus, for example, the compositions can comprise lipids, such as liposomes, such as cationic liposomes (e.g., DOTMA, DOPE, DC-cholesterol) or anionic liposomes. Liposomes can further comprise proteins to facilitate targeting a particular cell, if desired. Administration of a composition comprising a compound and a cationic liposome can be administered to the blood afferent to a target organ or inhaled into the respiratory tract to target cells of the respiratory tract. Regarding liposomes, see, e.g., Brigham et al. Am. J. Resp. Cell. Mol. Biol. 1:95-100 (1989); Felgner et al. Proc. Natl. Acad. Sci USA 84:7413-7417 (1987); U.S. Pat. No.4,897,355. Furthermore, the compound can be administered as a component of a microcapsule that can be targeted to specific cell types, such as macrophages, or where the diffusion of the compound or delivery of the compound from the microcapsule is designed for a specific rate or dosage.

In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), delivery of the compositions to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the disclosed nucleic acid or vector can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem., 2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br.

J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425, (1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol, 42:2062-2065, (1991)). These techniques can be used for a variety of other speciifc cell types. Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

Nucleic acids that are delivered to cells which are to be integrated into the host cell genome, typically contain integration sequences. These sequences are often viral related sequences, particularly when viral based systems are used. These viral intergration systems can also be incorporated into nucleic acids which are to be delivered using a non-nucleic acid based system of deliver, such as a liposome, so that the nucleic acid contained in the delivery system can be come integrated into the host genome.

Other general techniques for integration into the host genome include, for example, systems designed to promote homologous recombination with the host genome. These systems typically rely on sequence flanking the nucleic acid to be expressed that has enough homology with a target sequence within the host cell genome that recombination between the vector nucleic acid and the target nucleic acid takes place, causing the delivered nucleic acid

to be integrated into the host genome. These systems and the methods necessary to promote homologous recombination are known to those of skill in the art.

x. In Vivo/Ex Vivo

As described above, the compositions can be administered in a pharmaceutically acceptable carrier and can be delivered to the subject's cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, liposome fusion, intramuscular injection of DNA via a gene gun, endocytosis and the like).

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art. The compositions can be introduced into the cells via any gene transfer mechanism, such as, for example, calcium phosphate mediated gene delivery, electroporation, microinjection or proteoliposomes. The transduced cells can then be infused (e.g., in a pharmaceutically acceptable carrier) or homotopically transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

xi. Expression systems

The nucleic acids that are delivered to cells typically contain expression controlling systems. For example, the inserted genes in viral and retroviral systems usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements.

a. Viral Promoters and Enhancers

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., Nature, 273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenway, P.J. et al., Gene 18:

355-360 (1982)). Of course, promoters from the host cell or related species also are useful herein.

Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., Proc. Natl. Acad. Sci. 78: 993 (1981)) or 3' (Lusky, M.L., et al., Mol. Cell Bio. 3: 1108 (1983)) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., Cell 33: 729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., Mol. Cell Bio. 4: 1293 (1984)). They are usually between 10 and 300 bp in length, and they function in *cis*. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, -fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

In certain embodiments the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In certain constructs the promoter and/or enhancer region be active in all eukaryotic cell types, even if it is only expressed in a particular type of cell at a particular time. A preferred promoter of this type is the CMV promoter (650 bases). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTR.

It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

b. Markers

The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Preferred marker genes are the *E. Coli* lacZ gene, which encodes ß-galactosidase, and green fluorescent protein.

In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR- cells and mouse LTK- cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth

requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. Science 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

GUIDANCE CUES

Cell migration is involved in diverse morphogenetic programs, including patterning of the vascular and neural networks (Lauffenburger and Horwitz, 1996, Ridley et al., 2003). To execute these developmental programs, a migrating cell must reorganize its actin cytoskeleton in response to positive and negative guidance cues present in the extracellular milieu. The influence of these cues on cell migration is dictated by the complement of transmembrane receptors on the surface of the cell, and the diverse intracellular signal transduction cascades that are activated by specific cues.

The formation of neural and vascular networks share common molecular cues that reduce the complex task of projecting long distances to the simpler task of navigating a series of short segments based on these specific cues in the extracellular environment. Guidance cues come in four varieties: attractants and repellents, which may act either at short range (being cell- or matrix-associated) or at longer range (being diffusible). Intermediate targets are often the source of long-range attractive signals that lure axons, and of short- or long-range repellent signals that expel axons that have entered the target, or prevent their entry altogether. In between intermediate targets, axons and vessels are often guided through tissue corridors by attractive cues made by cells along the corridors, and by repulsive signals that prevent them from entering surrounding tissues.

As used herein, a "guidance cue" is a molecule that can act to attract or repulse neuron or blood vessel navigation or formation. Guidance cues, such as axonal guidance

cues, are often categorized as "attractive" or "repulsive." However, this is a simplification, as different axons will respond to a given cue differently. Furthermore, the same axonal growth cone can alter its responses to a given cue based on timing, previous experience with the same or other cues, and the context in which the cue is found. Thus, in one aspect, the guidance cue can be an attractive guidance cue for a specific cell. In another aspect, the guidance cue can be a repulsive guidance cue for a specific cell. As disclosed herein, "guidance cues" can be proteins that act extracellularly on cell receptors. However, also disclosed are molecules, including nucleic acids and small molecules, that can act either extracellularly or intracellularly to attract or repulse neuron or blood vessel navigation. Thus, as an example, where a ligand of a guidance cue receptor is disclosed herein, also disclosed are molecules that can modulate the activity or expression of said receptor. Thus, for example, disclosed are compositions, such as functional nucleic acids, that can alter gene expression of a receptor of a guidance cue disclosed herein or signaling molecule thereof. In one aspect, these molecules affect the same cell receptors and intracellular signaling pathways as the traditional protein guidance cues disclosed herein. In another aspect, these molecules can be identified by the screening methods disclosed herein.

Guidance cues can be identified based on the ability to guide axons. Growing axons have a highly motile structure at the growing tip called the growth cone, which "sniffs out" the extracellular environment for signals that instruct the axon which way to grow. These signals, called guidance cues, can be fixed in place or diffusible; they can attract or repel axons. With respect to axons, growth cones contain receptors that recognize these guidance cues and interpret the signal into a chemotropic response. The general theoretical framework is that when a growth cone "senses" a guidance cue, the receptors activate various signaling molecules in the growth cone that eventually affect the cytoskeleton. If the growth cone of the axon senses a gradient of guidance cue, the intracellular signaling in the growth cone happens asymmetrically, so that cytoskeletal changes happen asymmetrically and the growth cone turns toward or away from the guidance cue.

A combination of genetic and biochemical methods has led to the discovery of several important classes of guidance molecules and their receptors. Netrins and their receptors, DCC and UNC5, are secreted molecules that can act to attract or repel axons. Slits are secreted proteins that normally repel neural growth cones by engaging Robo (Roundabout) class receptors. Ephrins are cell surface molecules that activate Eph receptors on the surface

of other cells. This interaction can be attractive or repulsive. In some cases, Ephrins can also act as receptors by transducing a signal into the expressing cell, while Ephs act as the ligands. Signaling into both the Ephrin- and Eph-bearing cells is called "bi-directional signaling." The many types of Semaphorins are primarily axonal repellents, and activate complexes of cell-surface receptors called Plexins and Neuropilins. In addition, many other classes of extracellular molecules are used by growth cones to navigate properly, including developmental morphogens, such as BMPs, Wnts, Hedgehogs, and FGFs; extracellular matrix and adhesion molecules, such as NCAM, L1, and laminin; growth factors like NGF; and neurotransmitters and modulators like GABA. Thus, as disclosed herein, a repulsive cue can be, for example, a ligand of a roundabout receptor or a ligand of a netrin receptor.

xii. Unc5 and Netrin

Netrins were identified as chemoattractants that guide axons to the midline by binding receptors of the DCC (deleted in colorectal carcinoma) family. Netrins have also been implicated in axon repulsion, an effect mediated by receptors of the Unc5 family acting alone or with DCC receptors. In addition, DCC–Unc5 heterodimers can mediate repulsion at longer range than Unc5 receptors alone. Netrin1 and Unc5b, one of four mammalian Unc5 receptors, also regulate blood vessel guidance. Unc5b is expressed in endothelial tip cells. Loss of Unc5b in mice results in aberrant extension of tip cell filopodia and excessive branching of many vessels. Treatment of cultured endothelial cells or growing vessels in vivo with netrin1 induces filopodial retraction. A role for Unc5b in mediating endothelial cell repulsion was confirmed by analysis of the developing intersegmental vessels (ISV) in zebrafish embryos.

Netrins comprise a phylogenetically conserved family of guidance cues related to the extracellular matrix molecule laminin. Four secreted netrins have been identified in vertebrates: netrin-1 in chickens, mice, zebrafish and humans; netrin-2 in chickens; netrin-3 in mice and humans; and netrin-4 in mice and humans. All netrins are structurally related to the short arms of laminin and contain the laminin VI and V domains. All netrins also contain positively charged C-terminal domains, termed NTR modules. Netrin-1, -2, and -3 are more closely related to the laminin gamma chain. In contrast, netrin-4 is more closely related to the laminin beta chain.

Two families of netrin receptors have been identified that dictate the direction of migration. Both families belong to the immunoglobulin (Ig) superfamily of receptors. In

vertebrates, the Deleted in Colorectal Cancer (DCC) family has two members, DCC and neogenin, that contain six, extracellular fibronectin type III repeats in addition to four Ig domains and three regions of intracellular homology (P1, P2 and P3) that mediate interactions with other receptors such as UNC5b (P1) and Robo1 (P3). The UNC5 family has four members, UNC5a (UNC5H1), UNC5b (UNC5H2), UNC5c (UNC5H3), that contain two Ig and two thrombospondin type I (TspI) domains extracellularlly and ZU-5, DCC binding and C-terminal death domains intracellularly. Functionally, the DCC family mediates attraction to netrin-1 while the UNC5 family mediates repulsion by forming a netrin-1 dependent complex with DCC. Members of both families have been shown to act as dependence receptors and induce apoptosis in the absence and not the presence of ligand.

xiii. Semaphorins and Neuropilins/ Plexins

As disclosed herein, some semaphorins can act through plexins to increase vascular permeability. Thus, in some aspects of the disclosed compositions and methods, the repulsive guidance cue is not a semaphorin. In some aspects of the disclosed compositions and methods, the repulsive guidance cue is not a ligand of a plexin or neuropilin.

However, as disclosed herein, semaphorin 3E acts through plexin D1 to inhibit vascular permeability. Thus, in some aspects, the repulsive guidance cue can be semaphorin 3E. In some aspects, the repulsive guidance cue can be a ligand of plexin D1.

Semaphorins are guidance signals that are secreted and capable of long range diffusion (class 3) but can, in some contexts, have restricted diffusion, or are membrane-bound and function as short range guidance cues. Semaphorins are best known as repellents, but semaphorin 3A (Sema3A) can also function as a chemoattractant, depending on the intracellular level of cyclic nucleotides. Semaphorins signal through multimeric receptor complexes: membrane-bound semaphorins bind plexins, whereas secreted class 3 semaphorins bind neuropilins, which function as non-signalling co-receptors with plexins. An exception to this rule is the secreted Sema3E, which binds plexinD1 (Plxnd1) directly. Furthermore, the membrane-anchored Sema7A stimulates axon extension by activating integrins. Semaphorins and their receptors also regulate vessel guidance and branching. Endothelial cells express various neuropilin and plexin receptors. Sema3A inhibits formation of endothelial lamellipodia and vessels. Neuropilin2 is expressed in veins and lymph vessels, and Neuropilin1 is expressed widely in the developing vasculature. Neuropilins have also been implicated in vessel patterning, but this can reflect their role in modulating VEGF rather

than semaphorin signaling, since neuropilins are also receptors for specific VEGF isoforms (VEGF165) and modulate the activity of VEGF receptors. Moreover, VEGF165 competes with Sema3A for binding to neuropilins.

As disclosed herein, semaphorin 3E acts through plexin D1 to inhibit vascular permeability. Thus, in some aspects, the repulsive guidance cue can be semaphorin 3E. In some aspects, the repulsive guidance cue can be a ligand of plexin D1.

xiv. Ephrins and Ephs

Another principal class of shortrange axon guidance molecules is the Eph receptor tyrosine kinases and their ephrin ligands. The 13 Eph receptors in mammals are categorized into A (EphA1–8) and B (EphB1–4 and EphB6) subfamilies. The eight ephrin ligands comprise ephrinA1–5, which are tethered to the membrane via a glycosyl-phosphatidylinositol anchor, and ephrinB1–3, which contain transmembrane and cytoplasmic regions. EphrinA ligands bind EphA receptors, and ephrinB ligands bind EphB receptors; only a modest degree of cross-reactivity between the families has been observed; for example, EphA4 binds some B class ephrins. Eph receptors and ephrins initiate bidirectional signaling in cells expressing Eph receptors (forward signaling) or ephrinB ligands (reverse signalling). Ephrins were first identified as repellent axon guidance molecules through studies on topographic retinotectal projections, and subsequently have been implicated as both negative and positive cues in other wiring processes. Eph–ephrin signals also control vascular development. Some of these guidance molecules were among the first factors found to be expressed selectively in either arteries or veins. Historically, haemodynamic pressure differences were presumed to regulate the differentiation of high-pressure vessels into arteries and low-pressure vessels into veins. Expression analysis and loss-of-function studies in mice indicated, however, that EphB4 and ephrinB2 are expressed in developing veins and arteries, respectively, and are critical for their maintenance. These studies indicated that repulsive ephrinB2–EphB4 signaling—both forward and reverse—can prevent intermixing of venous and arterial endothelial cells, secure assembly of 'like' endothelial cells and demarcate arterial–venous cell boundaries. Repulsive ephrin–Eph signals provide short-range guidance cues for vessels to navigate through tissue boundaries. For instance, ephrinB2 repels EphB3/EphB4-expressing ISVs from entering somites. However, ephrin–Eph interactions can also provide attractive cues and induce capillary sprouting in other contexts. For instance, juxtacrine expression of ephrinB ligands and EphBs on adjacent endothelial cells or

smooth muscle cells in the same vessel may provide bidirectional signals for establishing contact-dependent communication, and promote vessel assembly, sprouting and maturation. For example, EphrinA ligands may also function as positive regulators of vascular morphogenesis.

EphA2/ephrinA1 signaling has been shown to inhibit VEGF-induced retinal vascular permeability and has been implicated in the treatment of neovascularization and vasopermeability abnormalities in diabetic retinopathy (Ojima et al, 2006). Thus, in some aspects of the disclosed compositions and methods for inhibiting vascular permeability, the repulsive cue is not a ligand of an Eph or ephrin receptor. In other aspects, the disclosed compositions comprise at least one guidance cue in addition to a ligand of an Eph or ephrin receptor.

xv. Slits and Roundabouts

A well-known example of a repulsive guidance cue is the Slit family of extracellular matrix proteins. Slit was originally identified in a genetic screen for axon guidance defects at the midline of *Drosophila* embryos (Seeger et al., 1993; Kidd et al, 1998; Battye et al., 1999; Kidd et al., 1999). Subsequently, three evolutionarily conserved Slit genes were cloned in vertebrates and their encoded proteins repel axons (Brose et al., 1999; Li et al., 1999) and promote sensory axon arborization (Wang et al., 1999).

Genetic and biochemical studies have demonstrated that the Robo family of transmembrane proteins function as receptors for Slit proteins. Like *slit*, *robo* was discovered in a genetic screen for defective axon guidance in *Drosophila* (Seeger et al., 1993). Four Robos have been identified in vertebrates, and Robo1-3 are predominantly expressed in the nervous system (Marillat et al., 2002). In contrast, Robo4, also known as Magic Roundabout, is exclusively expressed in the vasculature of embryonic mice (Park et al., 2003), placental arteries (Huminiecki et al., 2002) and in the tumor endothelium of a variety of human malignancies (Huminiecki et al., 2002; Seth et al., 2005). Robo4 is further distinguished from Robo1-3 by its divergent sequence: the ectodomain of the neuronal Robos contains five immunoglobulin (Ig) domains and three fibronectin type III (FNIII) repeats, while Robo4 contains two Ig domains and two FNIII repeats (Huminiecki et al., 2002; Park et al., 2003). In addition, Robo1-3 possess four conserved cytoplasmic (CC) motifs, CC0, CC1, CC2 and CC3 (Kidd et al., 1998; Zallen et al., 1998), of which, only CC0 and CC2 are present in Robo4 (Huminiecki et al., 2002; Park et al., 2003).

The ability of Robo to facilitate guidance decisions in the nervous system is dependent upon activation of specific biochemical programs downstream of the Slit-stimulated receptor. Analysis of Slit-dependent repulsion in *Drosophila*, *C. elegans*, and mammals has identified key mediators of Robo signaling in the nervous system. In *Drosophila*, the Abelson (Abl) tyrosine kinase and the actin binding protein Enabled (Ena) are involved in regulating Robo's repulsive activity (Bashaw et al., 2000). Additional studies in *Drosophila* identified a Rac GTPase activating protein (GAP) that is involved in Robo-mediated repulsion of tracheal cells and axons (Lundstrom et al., 2004; Hu et al., 2005). In *C. elegans*, a direct role for Ena in modulating Slit signaling has emerged from genetic analyses (Yu et al., 2002). In mammalian neurons, the Robo1-interacting protein srGAP1 is essential for Slit-dependent repulsion of precursor cells migrating from the anterior subventricular zone (Wong et al., 2001). Not only have these mechanistic studies begun to elucidate the signaling pathways downstream of neuronal Robos, but such studies have provided an explanation for the receptor's repulsive activity.

In contrast to the nervous system, little is known about Slit-Robo signal transduction in the vasculature, and despite the preponderance of evidence that Slit-Robo signaling inhibits the migration of both neuronal and non-neuronal cell types, including endothelial cells (Wu et al., 1999; Zhu et al., 1999; Wu et al., 2001; Park et al., 2003; Seth et al., 2005), several recent reports have proposed that Robos can promote angiogenesis in both Slit-dependent and Slit-independent ways. For example, it was reported that Slit2 stimulation of Robo1 induced migration and tube formation *in vitro*, and promoted tumor angiogenesis *in vivo* (Feng et al., 2004). Moreover, a recent study showed blocking Robo4 activity with a soluble Robo4 ectodomain inhibited migration and tube formation *in vitro*, consistent with a positive role for Robo4 during angiogenesis. Further, this study reported that Slit proteins do not bind to Robo4, thereby implicating an unknown ligand for the receptor (Suchting et al., 2004). The notion that Robo4 is proangiogenic has also emerged from recent data showing that overexpression of Robo4 augments endothelial cell adhesion and migration independently of Slit (Kaur et al., 2006). These seemingly incongruous observations emphasize the need to define both the functional significance and mechanism of Slit-Robo signaling in endothelial cells.

As disclosed herein, Slit2 is a ligand of Robo4, and Slit2-Robo4 signaling negatively regulates cell motility and inhibits vascular permeability. In particular, the teachings

provided herein establish that Slit2 elicits a repulsive cue in the endothelium via activation of Robo4, defining a novel signal transduction cascade responsible for such activity. As described herein Slit2 activation of Robo4 inhibits Rac activation and, hence, Rac initiated or mediated cell motility and cell spreading. The teachings provided herein further establish a Slit2-dependent association between Robo4 and the adaptor protein paxillin, with the experimental data detailed herein providing biochemical and cell biological evidence that this interaction is critical for Robo4-dependent inhibition of cell migration, spreading and Rac activation. In particular, as is taught herein, Robo4 activation initiates paxillin activation of GIT1 and, in turn, GIT1 inhibition of ARF6. Robo4 activation preserves endothelial barrier function, blocks VEGF signaling downstream of the VEGF receptor, and reduces vascular leak and pathologic angiogenesis. Of significance, Robo4 activation not only blocks VEGF signaling, but inhibits signaling from multiple angiogenic, permeability and inflammatory factors, including thrombin and bFGF. As is also disclosed herein, Robo4-paxillin signaling is essential for proper embryonic vascular development in zebrafish.

These disclosed relationships and results associated with Robo4 activation allow for new targets for modulation and for cellular manipulation as discussed herein. "Modulation" as used herein includes changing the activity of a target, and "manipulation" as used herein includes a change in the cellular state.

VASCULAR PERMEABILITY

Diseases and disorders characterized by undesirable vascular permeability include, for example, edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nepbrotic syndrome, pericardial effusion and pleural effusion. Thus, provided is a method of treating or preventing these or any other disease associated with an increase in vascular permeability or edema. For example, inhibiting edema formation should be beneficial to overall patient outcome in situations such as inflammation, allergic diseases, cancer, cerebral stroke, myocardial infarction, pulmonary and cardiac insufficiency, renal failure, and retinopathies, to name a few. Furthermore, as edema is a general consequence of tissue hypoxia, it can also be concluded that inhibition of vascular leakage represents a potential approach to the treatment of tissue hypoxia. For example, interruption of blood flow by pathologic conditions (such as thrombus formation) or medical intervention

(such as cardioplegia, organ transplantation, and angioplasty) could be treated both acutely and prophylactically using inhibitors of vascular leakage.

Ischemia/reperfusion injury following stroke and myocardial infarction is also characterized by vascular permeability and edema. A deficit in tissue perfusion leads to persistent post-ischemic vasogenic edema, which develops as a result of increased vascular permeability. Tissue perfusion is a measure of oxygenated blood reaching the given tissue due to the patency of an artery and the flow of blood in an artery. Tissue vascularization may be disrupted due to blockage, or alternatively, it may result from the loss of blood flow resulting from blood vessel leakage or hemorrhage upstream of the affected site. The deficit in tissue perfusion during acute myocardial infarction, cerebral stroke, surgical revascularization procedures, and other conditions in which tissue vascularization has been disrupted, is a crucial factor in outcome of the patient's condition. Edema can cause various types of damage including vessel collapse and impaired electrical function, particularly in the heart. Subsequent reperfusion, however, can also cause similar damage in some patients, leading to a treatment paradox. While it is necessary, to unblock an occluded blood vessel or to repair or replace a damaged blood vessel, the ensuing reperfusion can, in some cases, lead to further damage. Likewise, during bypass surgery, it is necessary to stop the heart from beating and to have the patient hooked to a heart pump. Some patients who undergo bypass surgery, for example, may actually experience a worsening of condition ("post-pump syndrome"), which may be the result of ischemia during cessation of cardiac function during surgery. An arterial blockage may cause a reduction in the flow of blood, but even after the blockage is removed and the artery is opened, if tissue reperfusion fails to occur, further tissue damage may result. For example, disruption of a clot may trigger a chain of events leading to loss of tissue perfusion, rather than a gain of perfusion.

Additional diseases and disorders characterized by undesirable vascular permeability include, for example, infectious and non-infectious diseases that may result in a cytokine storm. A cytokine storm can be precipitated by a number of infectious and non-infectious diseases including, for example, graft versus host disease (GVHD), adult respiratory distress syndrome (ARDS), sepsis, avian influenza, smallpox, and systemmic inflammatory response syndrome (SIRS).

PATHOLOGIC ANGIOGENESIS

Angiogenesis and angiogenesis related diseases are closely affected by cellular proliferation. As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The term "endothelium" is defined herein as a thin layer of flat cells that lines serous cavities, lymph vessels, and blood vessels. These cells are defined herein as "endothelial cells." The term "endothelial inhibiting activity" means the capability of a molecule to inhibit angiogenesis in general. The inhibition of endothelial cell proliferation also results in an inhibition of angiogenesis.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

New blood vessels may also also form in part by vasculogenesis. Vasculogenesis is distinguished from angiogenesis by the source of the endothelial cells. Vasculogenesis involves the recruitment of endothelial progenitor cells known as angioblasts. These angioblasts can come from the circulation or from the tissue. Vasculogenesis is regulated by similar signaling pathways as angiogenesis. Thus, the term "angiogenesis" is used herein interchangeably with vasculogenesis such that a method of modulating angiogenesis can also modulate vasculogenesis.

Pathologic angiogenesis, which may be characterized as persistant, dysregulated or unregulated angiogenesis, occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological damage seen in these conditions. The diverse disease states in which pathologic angiogenesis is present have been grouped together as angiogenic-dependent, angiogenic-associated, or angiogenic-related

diseases. These diseases are a result of abnormal or undesirable cell proliferation, particularly endothelial cell proliferation.

Diseases and processes mediated by abnormal or undesirable endothelial cell proliferation, including, but not limited to, hemangioma, solid tumors, leukemia, metastasis, telangiectasia psoriasis scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy (DR), retrolental fibroplasia, non-proliferative diabetic macular edema (DME), arthritis, diabetic neovascularization, age-related macular degeneration (AMD), retinopathy of prematurity (ROP), ischemic retinal vein occlusion (IRVO), wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, and placentation.

COMPOSITIONS

Provided herein are compositions for inhibiting vascular permeability and pathologic angiogenesis in a tissue.

In one embodiment, such a composition comprises a ligand of a Unc5 or Deleted in Colorectal Cancer (DCC) receptor. In one such embodiment, a ligand of Unc5 or DCC can be any composition or molecule that can act through an Unc5 or DCC receptor to inhibit Rac activation by VEGF. As it is used herein the term "act through" a receptor refers to the binding of a composition to a receptor that promotes an activity by the receptor. For example, the composition may comprise a ligand of Unc5 or DCC that acts through an Unc5 or DCC receptor to activate Git1 inhibition of ARF6. In another example, the composition may comprise a ligand of Unc5 or DCC that acts through an Unc5 or DCC receptor to activate paxillin activation of Git1. In yet another example, the composition described herein may comprise a composition or molecule that mimics an Unc5 or DCC receptor to activate paxillin activation of Git1.

In one embodiment, the composition described herein includes a ligand of Unc5, wherein the ligand is a netrin, such as human netrinl, netrin2, netrin4, netrin Gl, or netrin G2 and rodent (e.g., mouse or rat) netrinl, netrin3, netrin4, netrin Gl, or netrin G2, or a fragment or variant thereof that binds and activates Unc5b inhibition of ARF6. For example, the netrin ligan can comprise an amino acid sequence selected from SEQ ID NO:17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25 or a variant or fragment of such amino

acid sequences that binds Unc5b. A fragment of such amino acid sequences can be at least about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids long. In another embodiment, the netrin ligand of Unc5b can comprise an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% sequence identity to an amino acid sequence selected from SEQ ID NO:17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, or a fragment thereof that binds Unc5b.

In another embodiment, a composition as described herein may include a ligand of Eph. In one such embodiment, the composition comprises a ligand of Eph that can act through an Eph receptor to inhibit Rac activation by VEGF. In another such embodiment, the composition comprises a ligand of Eph that can act through an Eph receptor to activate Git1 inhibition of ARF6. In yet another embodiment, a composition according to the present description may comprise any composition or molecule that can act through an Eph receptor to activate Eph activation of Git1. In still a further embodiment, a composition as described herein may include any composition or molecule that mimics an Eph receptor to activate Paxillin activation of Git1.

In another embodiment, the composition provided herein comprises a ligand of a Robo4 receptor. In one such embodiment, the ligand of Robo4 can be any composition or molecule that can act through Robo4 to negatively regulate cell motility. In another such embodiment, the ligand of Robo4 can be any composition or molecule that can act through Robo4 to inhibit vascular permeability. In yet another such embodiment, the ligand of Robo4 can be any composition or molecule that can act through Robo4 to inhibit Rac activation by VEGF. In still a further embodiment, a composition as described herein includes a ligand of a Robo4 receptor, wherein the ligand can act through Robo4 to initiate paxillin activation of GIT1. In another embodiment, a composition as described herein includes a ligand of a Robo4 receptor, wherein the ligand can act through Robo4 to activate Git1 inhibition of ARF6. In a further embodiment, a composition as described herein includes a ligand of a Robo4 receptor, wherein the ligand can act through Robo4 in a manner that results in one or more of the following preservation of endothelial barrier function, blocking of VEGF signaling downstream of the VEGF receptor, inhibition of vascular leak, inhibition of pathologic angiogenesis, signal inhibition of multiple angiogenic, permeability and inflammatory factors.

Where the composition of the present invention includes a ligand of Robo4, the ligand be any composition or molecule that binds the extracellular domain of Robo4. Alternatively, a ligand of Robo4 can be any composition or molecule that acts through the Robo4 receptor to inhibit Rac activation by VEGF. Even further, a ligand of Robo4 can be any composition or molecule that acts through the Robo4 receptor to activate Git1 inhibition of ARF6. Still further, a ligand of Robo4 can be any composition or molecule that acts through the Robo4 receptor to activate Paxillin activation of Git1. In another aspect, a ligand of Robo4 can be any composition or molecule that mimics the Robo4 receptor to activate Paxillin activation of Git1. In one embodiment, a ligand of Robo4 included in a composition according to the present description comprises an isolated polypeptide of about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400 amino acids in length.

Where a composition as described herein includes a ligand of Robo4, such ligand can be a Slit, such as Slit2, or a fragment or variant thereof that binds and activates Robo4. In specific embodiments, the Slit ligand, or fragment or variant thereof, binds to and activates Robo4 in a manner that results in one or more of the following: inhibition of ARF6; preservation of endothelial barrier function; blocking of VEGF signaling downstream of the VEGF receptor; inhibition of vascular leak; inhibition of pathologic angiogenesis; and signal inhibition of multiple angiogenic, permeability and inflammatory factors. For example, the ligand of Robo4 can comprise an amino acid sequence selected from SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, and any of SEQ ID NO: 36 through SEQ ID NO: 47 or a fragment thereof that binds Robo4. For example, a fragment of such amino acid sequences can be at least about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids long. The ligand of Robo4 can comprise an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% sequence identity to and amino acid sequence selected from an amino acid sequence selected from SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, and any of SEQ ID NO: 36 through SEQ ID NO: 47, or a fragment thereof that binds Robo4. The fragment of Slit can comprise the N-terminal region of a Slit. For example, the ligand of Robo4 can comprise amino acids 1-1132 of Slit1 (SEQ ID NO:36), amino acids 1-1121 of Slit2 (SEQ ID NO:37), amino acids 1-1118 of Slit3 (SEQ ID NO:38), or any of the n-terminal fragments illustrated in FIG. 23 and detailed SEQ ID NO: 39 through SEQ ID NO: 47. In particular embodiments, the ligand of Robo4 can comprise a polypeptide consisting essentially of an

amino acid sequence selected from any one of SEQ ID NO: 36 through SEQ ID NO: 47. In some embodiments, as reflected in the amino acid sequences of SEQ ID NO: 39 through SEQ ID NO: 47, a Slit fragment included in a composition of the present invention does not comprise the N-terminal most amino acides. For example, the amino acid sequence may lack about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 N-terminal amino acids of a natural Slit. In other embodiments, the Slit fragment may not comprise the C-terminal most about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids of a natural Slit.

For example, the ligand of Robo4 can comprise a polypeptide consisting essentially of amino acids 281-511 (SEQ ID NO:15) of Slit1 or amino acids 271-504 of Slit2 (SEQ ID NO:16). Thus, the ligand of Robo4 can comprise SEQ ID NO:15 or SEQ ID NO: 16 or a fragment thereof that binds Robo4. The ligand of Robo4 can comprise an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% sequence identity to SEQ ID NO:15 or SEQ ID NO:16 or a fragment thereof that binds Robo4.

In yet another embodiment, a composition according to the present invention may include a fragment of Robo4 that can activate Paxillin activation of Git1. Thus, provided herein is an isolated polypeptide comprising the paxillin binding sequence of Robo4, wherein the polypeptide does not comprise full-length Robo4. In one such embodiment, the paxillin binding sequence may comprise the amino acid sequence SEQ ID NO:27 or a fragment or variant thereof of that binds paxillin. For example the fragment can be at least about 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids long. A fragment or variant of the amino acid sequence of SEQ ID NO:27 can comprise an amino acid sequence having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% sequence identity to SEQ ID NO:27 or a fragment thereof that binds paxillin.

In yet a further embodiment, a composition as described herein comprises and isolated polypeptide comprising the paxillin binding sequence (PBS) of Robo4, wherein the polypeptide is defined by the formula:

$$\mathbf{R^1 - PBS - R^2}$$

wherein R^1 and R^2 are, independently, H, acyl, NH_2, an amino acid or a peptide, wherein the polypeptide does not comprise full-length Robo4. The PBS can consist of an amino acid

sequence having at least 80% sequence homology to SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.

Also provided herein is an isolated nucleic acid encoding any of the herein disclosed polypeptides. Thus, provided is an isolated nucleic acid encoding a polypeptide comprising the paxillin binding sequence of Robo4, wherein the polypeptide does not comprise full-length Robo4. Also provided is an isolated nucleic acid comprising SEQ ID NO: 2 or a fragment thereof of at least 30 residues in length, wherein the nucleic acid does not encode full-length Robo4.

PHARMACEUTICAL COMPOSITIONS

The compositions disclosed herein, e.g, the ligands, proteins and peptides disclosed herein, can be formulated in a pharmaceutical composition. For example, such compositions can be combined with a pharmaceutically acceptable carrier to provide a formulation that is suitable for therapeutic administration. As used herein, "pharmaceutically acceptable" refers to a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the desired composition (e.g., a desired ligand, protein, peptide, nucleic acid, small molecule therapeutic, etc.), without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem., 2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br. J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425, (1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol, 42:2062-2065, (1991)). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific

therapeutic retroviral targeting of murine glioma cells in vivo. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

Suitable carriers and their formulations are described in Remington: The Science and Practice of Pharmacy (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable..

Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

METHODS

Methods of screening for, or evaluating, an agent that inhibits vascular permeability or pathologic angiogenesis are provided herein. In one embodiment, the method comprises

determining the ability of said agent to affect Robo4-mediated activation of Git1. For example, Robo4-mediated activation of Git1 can be determined by the steps comprising: contacting a first cell expressing Robo4 with a candidate agent, contacting a second cell essentially identical to the first cell but substantially lacking Robo4 with the candidate agent, and assaying for Git1 activation in the first and second cells, wherein detectably higher Git1 activation in the first cell as compared to the second cell indicates Robo4-mediated Git1 activation by said agent.

As disclosed herein, Robo4-mediated Git1 activation results in ARF6 inactivation. ARF6 is involved in VEGF-mediated activation of Rac, which activates Pak, which activates MEK, which activates ERK, which promotes vascular permeability. Thus, as disclosed herein Git1 activation can be assayed by detecting any of the components of the signaling pathway that is either activated or inactivated. Thus, Robo4-mediated Git1 activation can be assayed by detecting ARF6 inactivation, Rac inactivation, Pak inactivation, MEK inactivation, or ERK inactivation. It is understood that any other known or newly discovered method of monitoring this signaling pathway can be used in the disclosed methods.

Also provided is a method of screening for, or evaluating, an agent that inhibits vascular permeability, comprising determining the ability of said agent to inhibit ARF6, Rac, Pak, MEK, or ERK. For example, Robo4-mediated inhibition of ARF6, Rac, Pak, MEK, or Erk is determined by the steps comprising: contacting a first cell expressing Robo4 with a candidate agent, contacting a second cell essentially identical to the first cell but substantially lacking Robo4 with the candidate agent, assaying for inhibition of ARF6, Rac, Pak, MEK, ERK, or a combination thereof, in the first and second cells, wherein detectably lower ARF6, Rac, Pak, MEK, or ERK activation in the first cell as compared to the second cell indicates Robo4-mediated ARF6, Rac, Pak, MEK, or ERK inhibition by said agent.

Activation of signaling proteins such as Rac, Pak, MEK, ERK can be assayed by detecting the phosphorylation of said proteins. Cell-based and cell-free assays for detecting phosphorylation of proteins are well known in the art and include the use of antibodies, including, for example, anti-Phosphoserine (Chemicon® AB1603) (Chemicon, Temecula, CA), anti-Phosphothreonine (Chemicon® AB1607), and anti-Phosphotyrosine (Chemicon® AB1599). Site-specific antibodies can also be generated specific for the phosphorylated form of DDX-3. The methods of generating and using said antibodies are well known in the art.

The herein disclosed assay methods can be performed in the substantial absence of VEGF, TNF, thrombin, or histamine. Alternatively, the disclosed assay methods can be performed in the presence of a biologically active amount of VEGF, TNF, thrombin, or histamine.

"Activities" of a protein include, for example, transcription, translation, intracellular translocation, secretion, phosphorylation by kinases, cleavage by proteases, homophilic and heterophilic binding to other proteins, ubiquitination.

In one embodiment, the method of screening described herein is a screening assay, such as a high-throughput screening assay. Thus, the contacting step can be in a cell-based or cell-free assay. For example, vascular endothelial cells can be contacted with a candidate agent either in vivo, ex vivo, or in vitro. The cells can be on in monolayer culture but preferably constitute an epithelium. The cells can be assayed in vitro or in situ or the protein extract of said cells can be assayed in vitro for the detection of activated (e.g., phosphorylated) Rac, Pak, MEK, ERK. Endothelial cells can also be engineered to express a reporter construct, wherein the cells are contacted with a candidate agents and evaluated for reporter expression. Other such cell-based and cell-free assays are contemplated for use herein.

For example, the effect of small molecule, amino acid or nucleic acid mimetics on vascular permeability or pathologic angiogenesis can be evaluated in endothelial cells expressing Robo4 and compared to endothelial cells lacking Robo4.

In general, candidate agents can be identified from large libraries of natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, polypeptide- and nucleic acid-based compounds. Synthetic compound libraries are commercially available, e.g., from Brandon

Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, Fla.), and PharmaMar, U.S.A. (Cambridge, Mass.). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods. In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their effect should be employed whenever possible.

When a crude extract is found to have a desired activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having an activity that stimulates or inhibits vascular permeability. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using animal models for diseases or conditions in which it is desirable to regulate vascular permeability.

Methods for inhibiting vascular permeability in a subject are also provided herein. As is detailed herein, activation of Robo4 inhibits vascular permeability, inhibits Rac activation by VEGF, preserves endothelial cell barrier function, blocks of VEGF signaling downstream of the VEGF receptor, inhibits vascular leak, and inhibits multiple angiogenic, permeability and inflammatory factors. As determined herein, activation of Robo4 signaling achieves such effects through initiation of paxillin activation of GIT1, which, in turn, leads to GIT1 inhibition of ARF6. Therefore, in one embodiment, the method for inhibiting vascular permeability provided herein comprises administering a therapeutically effective amount of a ligand of Robo4, wherein such ligand results in GIT1 inhibition of ARF6. In another

embodiment, the ligand administered is a Slit protein as described herein. In specific embodiments, the vascular permeability experienced by the subject and treated by administration of a therapeutically effective amount of a ligand of Robo4 is associated with a disease state selected from infectious and non-infectious diseases that may result in a cytokine storm, including, for example, graft versus host disease (GVHD), adult respiratory distress syndrome (ARDS), sepsis, avian influenza, smallpox, and systemmic inflammatory response syndrome (SIRS), ischemia/reperfusion injury following stroke or myocardial infarction, edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion and pleural effusion, inflammation, allergic diseases, cancer, cerebral stroke, myocardial infarction, pulmonary and cardiac insufficiency, renal failure, and retinopathies.

Methods for inhibiting pathologic angiogenesis in a subject are provided herein. As is detailed herein, activation of Robo4 inhibits the effect of multiple inflammatory, permeability and angiogenic factors. Again, as determined herein, activation of Robo4 signaling initiates paxillin activation of GIT1, which, in turn, leads to GIT1 inhibition of ARF6. Therefore, in one embodiment, the method for inhibiting pathologic angiogenesis provided herein comprises administering a therapeutically effective amount of a ligand of Robo4, wherein such ligand results in GIT1 inhibition of ARF6. In another embodiment, the ligand administered is a Slit protein as described herein. In specific embodiments, the pathologic angiogenesis experienced by the subject and treated by administration of a therapeutically effective amount of a ligand of Robo4 is associated with a disease state selected from hemangioma, solid tumors, leukemia, metastasis, telangiectasia psoriasis scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy (DR), retrolental fibroplasia, non-proliferative diabetic macular edema (DME), arthritis, diabetic neovascularization, age-related macular degeneration (AMD), retinopathy of prematurity (ROP), ischemic retinal vein occlusion (IRVO), wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, and placentation.

In another embodiment, a method of treating or preventing avian flu is provided, wherein the method comprises identifying a subject having or at risk of having said avian flu,

and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing adult respiratory distress syndrome (ARDS) is provided, wherein the method comprises identifying a subject having or at risk of having said ARDS, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing systemic inflammatory response syndrome (SIRS) is provided, wherein the method comprises identifying a subject having or at risk of having said SIRS, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing graft versus host disease (GVHD) is provided, wherein the method comprises identifying a subject having or at risk of having said RDS, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing tumor formation or growth is provided, wherein the method comprises identifying a subject having or at risk of having said tumor formation or growth, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing respiratory distress syndrome (RDS) is provided, wherein the method comprises identifying a subject having or at risk of having said RDS, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventin ischemic retinal vein occlusion (IRVO) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said IRVO, and and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing non-proliferative diabetic macular edema (DME) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said DME, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing retinopathy of prematurity (ROP) is provided, wherein the method comprises identifying a subject having or at

risk of having said ROP, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing diabetic retinopathy (DR) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said DR, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing wet macular degeneration (AMD) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said AMD, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing ischemia in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said ischemia, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing hemorrhagic stroke in a subject is provided, wherein the methods comprises identifying a subject having or at risk of having said hemorrhagic stroke, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing reperfusion injury, such as that observed in myocardial infarction and stroke, in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said reperfusion injury, and administering to the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing a dermal vascular blemish or malformation in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said blemish, and administering to the skin of the subject a therapeutically effective amount of a ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing avian flu is provided, wherein the method comprises identifying a subject having or at risk of having said avian flu, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing adult respiratory distress syndrome (ARDS) is provided, wherein the method comprises identifying a subject having or at risk of having said ARDS, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing systemic inflammatory response syndrome (SIRS) is provided, wherein the method comprises identifying a subject having or at risk of having said SIRS, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing graft versus host disease (GVHD) is provided, wherein the method comprises identifying a subject having or at risk of having said RDS, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing tumor formation or growth is provided, wherein the method comprises identifying a subject having or at risk of having said tumor formation or growth, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing respiratory distress syndrome (RDS) is provided, wherein the method comprises identifying a subject having or at risk of having said RDS, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventin ischemic retinal vein occlusion (IRVO) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said IRVO, and and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing non-proliferative diabetic macular edema (DME) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said DME, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing retinopathy of prematurity (ROP) is provided, wherein the method comprises identifying a subject having or at risk of having said ROP, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing diabetic retinopathy (DR) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said DR, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing wet macular degeneration (AMD) in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said AMD, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing ischemia in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said ischemia, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing hemorrhagic stroke in a subject is provided, wherein the methods comprises identifying a subject having or at risk of having said hemorrhagic stroke, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing reperfusion injury, such as that observed in myocardial infarction and stroke, in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said reperfusion injury, and administering to the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

In another embodiment, a method of treating or preventing a dermal vascular blemish or malformation in a subject is provided, wherein the method comprises identifying a subject having or at risk of having said blemish, and administering to the skin of the subject a therapeutically effective amount of a repulsive guidance cue, such as ligand of roundabout-4 (Robo4) receptor.

Ligands suitable for use in conjunction with the methods described herein include, for example, those ligands described herein. For example, in particular embodiments, the

compositions described herein in relation to Robo receptors, including the Robo4 receptor, and in relation to the Unc5 or Deleted in Colorectal Cancer (DCC) receptor may be used as ligands in the methods of the present invention. Even more specifically, for example, the slit compounds described herein may be used as ligands for activating Robo4 and achieving the therapeutic benefits of the methods described herein.

In some aspects, subjects are identified by medical diagnosis. For example, subjects with diabetic retinopathy and macular degeneration can be identified by visualization of excess blood vessels in the eyes. Acute lung injury can be diagnosed by lung edema in the absence of congetive heart failure. Ischemic stroke can be diagnosed by neurologic presentation and imaging (MRI and CT). Other known or newly discovered medical determinations can be used to identify subjects for use in the disclosed methods.

In addition, subjects can be identified by genetic predisposition. For example, genes that predispose patients to age related macular degeneration have been identified (Klein RJ, et al, 2005; Yang Z, et al. 2006; Dewan A, et al. 2006). Likewise, genetic mutations that predispose patients to vascular malformations in the brain have been identified (Plummer NW, et al., 2005). Other known or newly discovered genetic determinations can be used to identify subjects for use in the disclosed methods.

The nucleic acid and polypeptide molecules disclosed herein, as well as any compositions necessary to perform the disclosed methods, can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta et al., Ann. Rev. Biochem. 53:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang et al., Methods Enzymol.,

65:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen et al., Bioconjug. Chem. 5:3-7 (1994).

One method of producing the disclosed proteins described herein is to link two or more peptides or polypeptides together by protein chemistry techniques. For example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert* -butyloxycarbonoyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) Synthetic Peptides: A User Guide. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) Principles of Peptide Synthesis. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., Biochemistry, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. Synthesis of Proteins by Native Chemical Ligation. Science, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate

undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) FEBS Lett. 307:97-101; Clark-Lewis I et al., J.Biol.Chem., 269:16075 (1994); Clark-Lewis I et al., Biochemistry, 30:3128 (1991); Rajarathnam K et al., Biochemistry 33:6623-30 (1994)).

Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. Science, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., Techniques in Protein Chemistry IV. Academic Press, New York, pp. 257-267 (1992)).

Disclosed are processes for making nucleic acids disclosed herein as well as for making nucleic acids useful for expressing the protein and peptide moledules described herein. There are a variety of methods that can be used for making these compositions, such as synthetic chemical methods and standard molecular biology methods. It is understood that the methods of making these and the other disclosed compositions are specifically disclosed.

Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid comprising the sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, or SEQ ID NO: 28 and a sequence controlling the expression of the nucleic acid.

Also disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence having 80% identity to a sequence set forth in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, or SEQ ID NO: 28, and a sequence controlling the expression of the nucleic acid.

Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence that hybridizes under stringent hybridization conditions to a sequence set forth SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, or SEQ ID NO: 28 and a sequence controlling the expression of the nucleic acid.

Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a peptide set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or any of SEQ ID NO: 36 through SEQ ID NO: 47 and a sequence controlling an expression of the nucleic acid molecule.

Disclosed are nucleic acid molecules produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a peptide having 80% identity to a peptide set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or any of SEQ ID NO: 36 through SEQ ID NO: 47 and a sequence controlling an expression of the nucleic acid molecule.

Disclosed are nucleic acids produced by the process comprising linking in an operative way a nucleic acid molecule comprising a sequence encoding a peptide having 80% identity to a peptide set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or any of SEQ ID NO: 36 through SEQ ID NO: 47, wherein any change is a conservative changes and a sequence controlling an expression of the nucleic acid molecule.

THERAPEUTIC ADMINISTRATION

The herein disclosed compositions, including pharmaceutical composition, may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. For example, the disclosed compositions can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally orally, parenterally (e.g., intravenously), intratracheally, ophthalmically, vaginally, rectally, intranasally, topically or the like, including topical intranasal administration or administration by inhalant.

Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as

emulsions. A revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

The compositions disclosed herein may be administered prophylactically to patients or subjects who are at risk for vascular permeability or pathologic angiogenesis. Thus, the method can further comprise identifying a subject at risk for vascular permeability or pathologic angiogenesis prior to administration of the herein disclosed compostions.

The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. For example, effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are effected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example, guidance in selecting appropriate doses for antibodies can be found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noges Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of a peptide or protein therapeutic used alone might range from about 1 μg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above. For example, the concentration of the herein disclosed ligands, proteins, peptides and guidance cues can be in the range of about 1pM to 100μM, including about 1pM, 2pM, 3pM, 4pM, 5pM, 6pM, 7pM, 8pM, 9pM, about 10pM, about 20nM, about 30nM, about 40nM, about 50nM, about 60nM,

about 70nM, about 80nM, about 90nM, or about 100nM, about 1μM, 2μM, 3μM, 4μM, 5μM, 6μM, 7μM, 8μM, 9μM, about 10μM, about 20μM, about 30μM, about 40μM, about 50μM, about 60μM, about 70μM, about 80μM, about 90μM, or about 100μM in the body of the subject.

EXAMPLES

The Examples that follow are offered for illustrative purposes only and are not intended to limit the scope of the compositions and methods described herein in any way. In each instance, unless otherwise specified, standard materials and methods were used in carrying out the work described in the Examples provided. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art (See, *e.g.*, Maniatis, T., et al. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Ausubel, F. M., et al. (1992) Current Protocols in Molecular Biology, (J. Wiley and Sons, NY); Glover, D. (1985) DNA Cloning, I and II (Oxford Press); Anand, R. (1992) Techniques for the Analysis of Complex Genomes, (Academic Press); Guthrie, G. and Fink, G. R. (1991) Guide to Yeast Genetics and Molecular Biology (Academic Press); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Jakoby, W. B. and Pastan, I. H. (eds.) (1979) Cell Culture. Methods in Enzymology, Vol. 58 (Academic Press, Inc., Harcourt Brace Jovanovich (NY); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And

Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Hogan et al. (eds) (1994) Manipulating the Mouse Embryo. A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. A general discussion of techniques and materials for human gene mapping, including mapping of human chromosome 1, is provided, e.g., in White and Lalouel (1988) Ann. Rev. Genet. 22:259 279. The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, and immunology. (See, e.g., Maniatis et al., 1982; Sambrook et al., 1989; Ausubel et al., 1992; Glover, 1985; Anand, 1992; Guthrie and Fink, 1991).

Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of publications are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

EXAMPLE 1

Robo4 is Required for Vascular Guidance in vivo: During the past decade, the zebrafish has become an attractive model for analysis of vascular development (Weinstein, 2002), and was chosen to investigate the biological importance of Robo4 *in vivo*. To suppress Robo4 gene expression, a previously described splice-blocking morpholino that targets the exon10-intron10 boundary of Robo4 pre-mRNA (Bedell et al., 2005) was used. To verify the efficacy of the Robo4 morpholino, RNA was isolated from un-injected and morpholino-injected embryos, and analyzed by RT-PCR with primers flanking the targeted exon (FIG. 8A). Injection of the Robo4 morpholino resulted in complete loss of wild-type RNA when compared to the un-injected control, indicating that morphant zebrafish are functionally null for Robo4 (FIG. 8B).

TG(fli1:egfp)^{y1} zebrafish embryos, which express green fluorescent protein under the control of the endothelialspecific *fli1* promoter, and permit detailed visualization of the developing endothelium *in vivo* were utilized to evaluate the consequence of morpholino-

mediated knockdown of Robo4 on vascular development (FIG. 1A; Lawson and Weinstein, 2002). At 48 hpf, Robo4 MO-injected embryos exhibited wild-type formation of the primary axial vessels (dorsal aorta and posterior cardinal vein), as well as the dorsal longitudinal anastomotic vessel and parachordal vessel, indicating that vasculogenesis and angiogenesis, respectively, are not affected by reduction of Robo4 levels (FIG. 1B, right panel). However, a striking degree of abnormality was observed in the architecture of the intersegmental vessels in Robo4 morphants. In wild-type embryos, the intersegmental vessels arise form the dorsal aorta and grow toward the dorsal surface of the embryo, tightly apposed to the somitic boundary. It is this precise trajectory between the somites that defines the characteristic chevron shape of the intersegmental vessels (FIG. 1A, right panel). Rather than adopting this stereotypical pattern, the intersegmental vessels of Robo4 morphant embryos grew the wrong direction (FIG. 1B, right panel: white arrows indicate abnormal vessels). At 48 hpf, 60% of embryos injected with the Robo MO exhibited this defect, compared to 5% in wild-type embryos. Importantly, Robo4 morphants were indistinguishable from control embryos by phase microscopy, indicating that the observed vascular patterning defects were not a result of gross morphological perturbation. Together, these data demonstrate a requirement for Robo4 during zebrafish vascular development and suggest that functional output from the receptor elicits a repulsive guidance cue.

EXAMPLE 2

The Robo4 Cytoplasmic Tail is required for Vascular Guidance in vivo: It was next determined whether the vascular defects observed in Robo4 morphants could be suppressed by reconstitution of *robo4*. *robo4* MO and wildtype murine Robo4 RNA, which is refractory to the morpholino, were injected into *TG(fli1:egfp)y1* embryos and vascular patterning was analyzed at 48 hpf. Robo4 RNA restored the stereotypic patterning of the trunk vessels in approximately 60% of morphant embryos, confirming the specificity of gene knockdown (FIG. 1B and C, right panels).

The ability of the *robo4* to regulate vascular development is likely a consequence of its ability to transmit cytoplasmic signals. To substantiate this notion, Robo4 MO and a mutant form of murine Robo4 lacking the portion of the receptor that interacts with cytoplasmic components (robo4Δtail) were co-injected and vessel architecture evaluated at 48 hpf. Unlike wild-type Robo4 RNA, robo4Δtail was unable to rescue patterning defects in

morphant embryos (FIG. 1B and D, right panels). These data demonstrate that information contained in the cytoplasmic tail of Robo4 is critical for vascular guidance during zebrafish embryogenesis. All together, these *in vivo* analyses indicate that Robo4 activity is required for precisely defining the trajectory of the intersegmental vessels during vertebrate vascular development (FIG. 1E).

EXAMPLE 3

The Robo4 Cytoplasmic Tail is required for Inhibition of Haptotaxis: Slit2-Robo4 signaling inhibits migration of primary endothelial cells towards a gradient of VEGF, and of HEK 293 cells ectopically expressing Robo4 towards serum (Park et al., 2003; Seth et al., 2005). In addition to soluble growth factors, immobilized extracellular matrix proteins such as fibronectin play a critical role in cellular motility (Ridley et al., 2003), and gradients of fibronectin can direct migration in a process called haptotaxis. Indeed it was recently shown that fibronectin is deposited adjacent to migrating endothelial cells in the early zebrafish embryo (Jin et al., 2005). The observation that Robo4 is required for proper endothelial cell migration *in vivo* (FIG. 1), indicated the ability of Slit2-Robo4 signaling to modulate fibronectin-induced haptotaxis. HEK 293 cells were transfected with Robo4 or Robo4ΔTail (FIG. 2A) and subjected to haptotaxis migration assays on membranes coated with a mixture of fibronectin and Slit2. Slit2 inhibited fibronectin-induced migration of cells expressing Robo4, but not Robo4ΔTail, demonstrating that the Robo4 cytoplasmic tail is critical for repulsive activity of the receptor (FIG. 2B).

The region of the Robo4 cytoplasmic tail that is required for inhibition of cell migration was next defined. HEK 293 cells were transfected with Robo4 deletion constructs (FIG. 2A) and subjected to haptotaxis migration assays. Fibronectin-dependent migration of cells expressing Robo4-NH2, but not Robo4-COOH was inhibited by Slit2 (FIG. 2C), demonstrating that the N-terminal half of the Robo4 cytoplasmic tail is necessary and sufficient for modulation of cell motility.

EXAMPLE 4

Paxillin Family Members are Robo4-interacting Proteins: Identification of the region of the Robo4 cytoplasmic tail that confers functional activity allowed the search for cytoplasmic components that might regulate Robo4 signal transduction. Using the N-

terminal half of the Robo4 tail as a bait, a yeast two-hybrid screen of a human aortic cDNA library was performed, which identified a member of the paxillin family of adaptor proteins, Hic-5, as a potential Robo4-interacting protein (FIG. 8). To verify this interaction, Hic-5 plasmids were isolated and re-transformed into yeast with Robo4 or empty vector. Only strains co-expressing Robo4 and Hic-5 were competent to grow on nutrient deficient medium and induce robust betagalactosidase activity (FIG. 8B). To further confirm this interaction, co-immunoprecipitation experiments were performed using mammalian cells co-transfected with Hic-5 and the Robo4 cytoplasmic tail. Hic-5 was found in anti-Robo4 immunoprecipitates of HEK 293 cells expressing Robo4 and Hic-5, but not Hic-5 alone (FIG. 3A). Collectively, these data demonstrate that Hic-5 specifically interacts with the Robo4 cytoplasmic tail in both yeast and mammalian cells.

Hic-5 and its paralog, paxillin, can exhibit cell-type specific expression (Turner, 2000; Yuminamochi et al., 2003). For this reason, it was determined which of these proteins were expressed in HEK 293 cells, the cell line used in the haptotaxis migration assays. Western blotting of cell lysates from CHO-K1, HEK 293 and NIH3T3 cells with antibodies to Hic-5 or paxillin detected paxillin in all cell lines, whereas Hic-5 was only found in CHO-K1 and NIH3T3 cells (FIG. 3B). This not only suggested that Hic-5 and paxillin could interact with Robo4 to regulate cell migration, but that paxillin was the likely binding partner in HEK 293 cells. With this latter idea in mind, co-immunoprecipitation experiments were performed using mammalian cells expressing paxillin and the Robo4 cytoplasmic tail. As was observed with Hic-5, paxillin was identified in anti-Robo4 immunoprecipitates of HEK 293 cells expressing paxillin and Robo4, but not paxillin alone (FIG. 3C).

Since Slit2 is a physiological ligand of Robo4 (Park et al., 2003; Hohenester et al., 2006), it was determined whether Slit2 stimulation regulated the interaction between Robo4 and paxillin. HEK 293 cells expressing Robo4 were incubated in the presence or absence of Slit2. In the presence of Slit2, endogenous paxillin was detected in Robo4 immunoprecipitates. In sharp contrast, in the absence of Slit2, no paxillin was detected in the immunoprecipitates (FIG. 3E). Thus, engagement of Robo4 by Slit2 stimulated its association with paxillin.

EXAMPLE 5

Identification of the Paxillin Interaction Motif of Robo4: To precisely define the region of Robo4 that is required for interaction with paxillin a series of GST-Robo4 fusion proteins spanning the entire length of the cytoplasmic tail were created (FIG. 4A). *In vitro* binding assays with purified recombinant paxillin demonstrated that the amino terminal half of the Robo4 tail (494-731) is necessary and sufficient for direct interaction with paxillin (FIG. 4B). Four additional GST-Robo4 fusion proteins encompassing approximately 70 amino acid fragments of the amino terminal half of the cytoplasmic tail were then generated (FIG. 4C). *In vitro* binding assays revealed that paxillin selectively interacts with a fragment of the Robo4 tail residing between the CC0 and CC2 motifs (604-674; FIG. 4D). To determine whether this region of Robo4 was necessary for interaction with paxillin amino acids 604-674 were deleted from the cytoplasmic tail and this mutant GST-Robo4 fusion protein subjected to *in vitro* binding assays. While interaction with paxillin was attenuated, so was interaction with a known Robo4-binding protein, Mena, indicating that elimination of amino acids 604-674 affects the conformation of the Robo4 tail. To circumvent this issue, smaller deletions were created within this 70 amino acid stretch and additional *in vitro* binding assays performed. Using this approach a mutant GST-Robo4 fusion protein was identified lacking 36 amino acids (604-639; FIG. 9) that lost binding to paxillin, but retained binding to Mena (FIG. 4E). This region of Robo4 is heretofore referred to as the paxillin interaction motif (PIM).

EXAMPLE 6

The Paxillin Interaction Motif is required for Robo4-dependent Inhibition of Haptotaxis: It was next determined whether the paxillin interaction motif of Robo4 is important for functional activity of the receptor. A mutant form of full length Robo4 lacking amino acids 604-639 (Robo4ΔPIM) was generated by site directed mutagenesis and used in haptotaxis migration assays. Robo4ΔPIM failed to mediate Slit2-directed inhibition of migration towards a gradient of fibronectin (FIG. 4F), demonstrating that the region of the Robo4 tail necessary for paxillin binding is likewise required for Robo4-dependent inhibition of cell migration.

EXAMPLE 7

Slit2-Robo4 Signaling Inhibits Cell Spreading and Adhesion-dependent Rac Activation: The ability of immobilized Slit2 to inhibit the migration of cells expressing Robo4 on fibronectin could potentially result from negative regulation of adhesion and/or spreading on this ECM protein. To determine whether Slit2-Robo4 signaling influences these processes, HEK 293 cells were transfected with Robo4 or empty vector (pcDNA3) and subjected to adhesion and spreading assays on fibronectin. Although cells expressing Robo4 adhered normally to coverslips coated with fibronectin and Slit2, they were significantly less spread than cells transfected with pcDNA3 (FIG. 5A). These data indicate that Slit2-Robo4 signaling modulates intracellular pathways that control cell spreading.

The ability of a cell to spread on an ECM protein, such as fibronectin, is regulated by activation of the Rho family of small GTPases, which include Rho, Cdc42 and Rac migration (Nobes and Hall, 1995; Nobes and Hall, 1998). Of these proteins, Rac plays an essential role in promoting the actin polymerization that leads to cell spreading and migration (Nobes and Hall, 1995; Nobes and Hall, 1998). This established relationship between Rac and cell spreading indicated that Slit2-Robo4 signaling might inhibit adhesion-dependent activation of Rac. To evaluate this possibility, HEK 293 cells were transfected with Robo4 or pcDNA3, plated onto dishes coated with fibronectin and Slit2 and Rac-GTP levels were assayed using GST-PBD pull down assays. Cells expressing Robo4 exhibited significantly less adhesion-stimulated Rac activation when compared to cells transfected with pcDNA3 (FIG. 5B and C). To confirm the specificity of this effect, Cdc42 activation was also examined in cells expressing Robo4, which was unaltered by exposure to Slit2 (FIG. 11A). This result is supported by the observation that Robo4 does not interact with the Robo1 binding-protein srGAP1, a known GTPase activating protein for Cdc42 (FIG. 11B). Together, these data demonstrate that Slit2-Robo4 signaling specifically inhibits adhesion-induced activation of Rac.

EXAMPLE 8

The Paxillin Interaction Motif is required for Robo4-dependent Inhibition of Cell Spreading and Rac Activation: Whether Robo4ΔPIM was competent to inhibit fibronectin-induced cell spreading and Rac activation was next evaluated. HEK 293 cells were transfected with Robo4ΔPIM, plated onto fibronectin and Slit2 coated surfaces and subjected to spreading or Rac assays. This mutant form of the receptor was incapable of inhibiting cell

spreading and adhesion-dependent Rac activation (FIG. 5D, E and F), demonstrating that the paxillin interaction motif is essential for functional activity of Robo4 *in vitro*.

To confirm that Robo4-dependent inhibition of cell spreading was due principally to suppression of Rac activation, HEK 293 cells were co-transfected with Robo4 and a dominant active form of Rac, Rac (G12V), and subjected to spreading assays. Cells expressing Rac (G12V) were refractory to Robo4-dependent inhibition of cell spreading (FIG. 5G), demonstrating that Slit2-Robo4 signaling blocks spreading by inhibiting Rac activity.

EXAMPLE 9

Slit2 Inhibits VEGF-induced Rac Activation in Primary Human Endothelial Cells: Slit2 inhibits VEGF-stimulated migration of several primary human endothelial cell lines (Park et al., 2003), and Rac plays an essential role for in VEGF-induced cell motility (Soga et al., 2001a; Soga et al., 2001b). It was therefore determined whether Slit2-Robo4 signaling could inhibit Rac activation in an endogenous setting. Human Umbilcal Vein Endothelial Cells (HUVEC) were stimulated with VEGF in the presence and absence of Slit2, and GTP-Rac levels were analyzed using GST-PBD pull down assays. Slit2 treatment completely suppressed VEGF-stimulated Rac activation (FIG. 5H and I), demonstrating that endogenous Slit2-Robo4 signaling modulates Rac activation.

EXAMPLE 10

Lim4 of Paxillin is required for Interaction with Robo4 and Robo4-dependent Inhibition of Cell Spreading: Although Robo4ΔPIM maintains its interaction with Mena (FIG. 4E), it is possible that this mutation perturbed interaction of Robo4 with proteins other than paxillin. To address this issue definitively, paxillin mutants were generated that disrupt association with Robo4. Paxillin is a modular protein composed of N-terminal leucine/aspartic acid (LD) repeats and C-terminal Lim domains (FIG. 6A). Analysis of the clones recovered from the yeast two-hybrid screen (see FIG. 9A) indicated that the Lim domains, particularly Lim3 and Lim4, are important for interaction with Robo4. To validate this notion, co-immunoprecipitation experiments were performed using HEK 293 cells co-transfected with the Robo4 tail and either paxillin-LD or paxillin-Lim. Paxillin-Lim, but not paxillin-LD was found in Robo4 immunoprecipitates (FIG. 6B), demonstrating that the Lim

domains of paxillin are necessary and sufficient for interaction with Robo4. To clarify which Lim domain is required for binding to Robo4, serial deletions were made from the carboxy terminus of paxillin, cotransfected with the Robo4 tail into HEK 293 cells, and coimmunoprecipitation experiments performed. Deletion of the Lim4 domain of paxillin completely abrogated binding to Robo4 (FIG. 6C), demonstrating that this region of paxillin is critical for its ability to interact with Robo4.

Delineation of the Robo4 binding site on paxillin allowed direct evaluation of the role of paxillin in Robo4-dependent inhibition of cell spreading. Endogenous paxillin was knocked-down in HEK 293 cells using siRNA and reconstituted with wild type chicken paxillin (Ch-paxillin) or Ch-paxillin ΔLim4 (FIG. 6D). These cells were then subjected to spreading assays on coverslips coated with fibronectin and Slit2. Cells expressing Ch-paxillin ΔLim4 were refractory to Robo4-dependent inhibition of cell spreading, while cells expressing Ch-paxillin exhibited the characteristic reduction in cell area (FIG. 6E). These data confirm that interaction of paxillin with the Robo4 enables Slit2-Robo4 signaling to suppress cell spreading.

EXAMPLE 11

The Paxillin Interaction Motif is required for Vascular Guidance in vivo: The requirement of the paxillin interaction motif of Robo4 during zebrafish vascular development was assessed. As described previously, injection of *robo4* MO into *TG (fli1:egfp)*^{y1} embryos caused disorganization of the intersegmental vessels (see FIG. 1B). Co-injection of *robo4ΔPIM* RNA exacerbated the defects caused by the *robo4* MO, while wild-type *robo4* RNA suppressed these defects (FIG. 7A). The inability of both *robo4Δtail* and *robo4ΔPIM* RNA to rescue vascular patterning defects in morphant embryos demonstrates that the 36 amino acid paxillin interaction motif is a critical signal transduction module in the Robo4 cytoplasmic tail. Further, these data indicate that the interaction between paxillin and Robo4 is essential for proper patterning of the zebrafish vasculature.

EXAMPLE 12

Our determination that Robo4 interacts with paxillin and inhibits protrusive activity prompted us to determine whether Robo4 impinges upon the Arf6 pathway. Cells expressing αIIb-Robo4:β3 were plated on fibronectin alone, or fibronectin and fibrinogen, and Arf6-

GTP levels were analyzed using a GST-GGA3 affinity precipitation technique. While fibronectin stimulated activation of Arf6, fibrinogen reduced Arf6-GTP levels in cells expressing αIIb-Robo4:β3 (FIG. 16A). This result demonstrated that Robo4 signaling inhibits Arf6 activation and suggested that Robo4's ability to block Rac activity stems from its regulation of Arf6.

Next we analyzed the requirement of a paxillin-GIT1 complex in Robo4-dependent inhibition of protrusive activity. The paxillin binding sequence (PBS) on GIT1 is found at the carboxy-terminus of the protein and has been shown to prevent interaction of GIT1 and paxillin (Uemura et al., 2006). Cells were transfected with αIIb-Robo4:β3 and either an empty vector or the GIT1-PBS and subjected to spreading assays on fibronectin or fibronectin and fibrinogen. As described previously, cells expressing αIIb-Robo4:β3 displayed a decrease in cell area when plated on fibrinogen, but this was lost in cells transfected with the GIT1-PBS (FIG. 16B). We repeated this experiment in cells expressing full length Robo4 plated on fibronectin or fibronectin and Slit2, and similar to the chimeric receptor experiment, the GIT1-PBS prevented the Slit2-dependent decrease in cell area (FIG. 16C). These data demonstrate that a functional paxillin-GIT1 complex is required for Slit2-Robo4 signaling.

To determine whether Slit2-Robo4 signaling inhibits protrusive activity by inactivating Arf6, we co-expressed the Arf6 guanine nucleotide exchange factor ARNO with Robo4 and performed spreading assays. Overexpression of ARNO blocked the ability of Slit2 to reduce cell area, indicating that a principal effect of Slit2-Robo4 signaling is to prevent GTP-loading of Arf6 (FIG. 16C). If ARNO restored the ability of Robo4-expressing cells to spread on Slit2, we reasoned that it should likewise re-establish Rac activation in response to fibronectin. Indeed, overexpression of ARNO led to normal levels of GTP-Rac in cells plated on fibronectin and Slit2 (FIG. 16D). Together these experiments demonstrate that Slit2-Robo4 signaling inactivates Arf6, which leads to the local blockade of Rac activation and the subsequent inhibition of the membrane protrusion necessary for cell spreading and migration.

EXAMPLE 13

Immunoprecipitation Demonstrates Interaction Between Slit Ligand and Robo4 Receptor: Cell lysates from untransfected human embryonic kidney 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) were immunopreciptated. Slit-myc protein was 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 (FIG. 17A, lane 6). The specificity of this interaction was confirmed by the absence of detectable Slit protein with all other combinations of lysates (FIG. 17A, lanes 2-5). The same amount of lysate was used in each experiment. A Western blot analysis of the Slit-myc cell lysates served as a control and demonstrated that the Slit protein has a mass of approximately 210 kD in accordance with previous reports (FIG. 17A, lane 1). The lower bands shown in lanes 2-6 of FIG. 17A correspond to immunoglobulin heavy chains.

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) was also immunoprecipitated. The full-length Slit-myc protein (210 KD) and its C-terminal proteolytic fragment (70 KD) were detected in Slit-myc CM by an anti-myc antibody (FIG. 17B, lane 1). Slit-myc protein was also detected by Western blot after Slit-myc and Robo4-HA conditioned media were combined and immunoprecipitated with an anti-HA antibody (FIG. 17B, lane 6). The specificity of this interaction was confirmed by the absence of Slit protein with all other combinations of conditioned media.

As is shown in FIG. 17C through FIG. 17F, Slit protein binds to the plasma membrane of cells expressing Robo4. Binding of Slit-myc protein was detected using an anti-myc antibody and an Alexa 594 conjugated anti-mouse antibody. As can be seen in FIG. 17D and FIG. 17F, binding was detected on the surface of Robo4-HEK cells (FIG. 17F) but not Control-HEK cells (FIG. 17D).

EXAMPLE 14

Robo4 Knockout Mouse: To ascertain the functional significance of Robo4 *in vivo,* knockout mice were produced using standard techniques. To produce the knockout mice, exons one through five of the gene expressing Robo4 were replaced with an alkaline phosphatase (AP) reporter gene using homologous recombination. This allele, $Robo4^{AP}$,

lacked the exons encoding the immunoglobulin (IgG) repeats of the Robo4 ectodomain, which are predicted to be required for interaction with Slit proteins. The *Robo4*$^{+/AP}$ animals were intercrossed to generate mice that were homozygous for the targeted allele. An illustration of the genomic structure of the mice is provided in FIG. 25. *Robo4*$^{AP/AP}$ animals were viable and fertile, and exhibited normal patterning of the vascular system. These data indicate that Robo4 is not required for sprouting angiogenesis in the developing mouse, and point to an alternate function for Robo4 signaling in the mammalian endothelium. Alkaline phosphatase activity was detected in these animals throughout the endothelium of all vascular beds in the developing embryos and in the adult mice, which confirmed that the Robo4^{AP} allele is a valid marker of Robo4 expression.

EXAMPLE 15

Robo4 Activation Stabilizes Mature Vessels: The central region of the murine retinal vascular plexus, comprised specifically of stalk cells, is an example of the differentiated/stabilized phenotype characteristic of a mature, lumenized vascular tube. We reasoned, therefore, that Robo4 expression in the stalk might maintain this phenotype by inhibiting processes that are stimulated by pro-angiogenic factors, such as VEGF-A. The effect of Robo4 signaling on processes stimulated by VEGF-A was evaluated using a VEGF-A endothelial cell migration assay and a VEGF-A tube formation assay. Both such assays are routinely used to investigate angiogenesis *in vitro*.

In order to conduct the endothelial cell migration and tube formation assays, endothelial cells from the lungs of *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were isolated and their identity confirmed using immunocytochemistry and flow cytometry. These cells were then utilized in VEGF-A-dependent endothelial cell migration and tube formation assays. The Slit2 molecule used in these assays was Slit2N (SEQ ID NO: 39). As is shown in FIG. 19A and FIG. 19B, Slit2 inhibited both migration and tube formation of *Robo4*$^{+/+}$ endothelial cells. However, the inhibitory activity of Slit2 was lost in *Robo4*$^{AP/AP}$ endothelial cells. These results demonstrate that Slit2 inhibits endothelial cell migration and tube formation in a Robo4-dependent manner, and indicate that activation of Robo4 by Slit2 serves to stabilize the vascular endothelium of mature vessels.

EXAMPLE 16

Robo4 Activation Preserves Endothelial Barrier Function: In a mature vascular bed, endothelial cells do not behave independently of one another; rather they form a monolayer that prevents the movement of protein, fluid and cells from the endothelial lumen into the surrounding tissue. This barrier function was modeled *in vitro* using a Transwell assay to analyze the transport of horseradish peroxidase (HRP), across confluent cell monolayers of endothelial cells taken from the lungs of *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice. Stimulation of *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ endothelial cells with VEGF-A, a known permeability-inducing factor, enhanced the accumulation of HRP in the lower chamber of the Transwell. As is shown in FIG. 19C, however, pre-treatment of the cell monolayers with a Slit2 protein (Slit2N (SEQ ID NO: 39)) prevented this effect in *Robo4*$^{+/+}$, but not *Robo4*$^{AP/AP}$ endothelial cells.

Next, the influence of Slit2 on endothelial barrier function *in vivo* was evaluated. A Miles assay was performed by injecting Evans Blue into the tail vein of *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice. VEGF-A in the absence and presence of a Slit2 protein (Slit2N (SEQ ID NO: 39)) was subsequently injected into the dermis. Analogous to the *in vitro* assay, VEGF-A-stimulated leak of Evans Blue into the dermis could be prevented by concomitant administration of Slit2 protein in *Robo4*$^{+/+}$, but not in *Robo4*$^{AP/AP}$ mice (shown in FIG. 19D). These observations were extended by evaluating the ability of Slit2 to suppress VEGF-A induced hyperpermeability of the retinal endothelium. In particular, it was found that intravitreal injection VEGF-A in *Robo4*$^{+/+}$ mice induced leak of Evans Blue from retinal blood vessels. However, such VEGF-A induced leak of Evans Blue from the retinal blood vessels was suppressed in *Robo4*$^{+/+}$ mice by co-injection of the Slit2 protein Slit2N (SEQ ID NO: 39) (FIG. 19E). This experiment was repeated in retinas of *Robo4*$^{AP/AP}$ mice, and it was found that *Robo4*$^{AP/AP}$ were refractory to treatment with Slit2N (SEQ ID NO: 39). These data demonstrate that Robo4 mediates Slit2-dependent inhibition of VEGF-A-induced endothelial hyperpermeability *in vitro* and *in vivo.*

EXAMPLE 17

Robo4 Blocks VEGF Signaling Downstream of the VEGF Receptor: The ability of VEGF-A to promote angiogenesis and permeability is dependent upon activation of VEGFR2, which occurs by autophosphorylation following ligand binding. Subsequently, a number of non-receptor tyrosine kinases, serine/threonine kinases and small GTPases are

activated to execute VEGF-A signaling in a spatially and temporally specific manner. To determine where Slit2-Robo4 signaling intersects the VEGF-A-VEGFR2 pathway, VEGFR2 phosphorylation following stimulation with VEGF-A and Slit2 was analyzed using Slit2N (SEQ ID NO: 39). Slit2N (SEQ ID NO: 39) had no effect on VEGF-A-induced VEGFR2 phosphorylation (FIG. 19F), indicating that the Slit2-Robo4 pathway must intersect VEGF-A signaling downstream of the receptor. Attention was then focused on the Src family of non-receptor tyrosine kinases, Fyn Yes and Src, due to their well-documented role in mediating VEGF-A-induced angiogenesis and permeability (Eliceiri et al., 2002; Eliceiri et al., 1999). Treatment of endothelial cells with Slit2N (SEQ ID NO: 39) reduced VEGF-A-stimulated phosphorylation of c-Src (FIG. 19G). Recently, several reports have shown that Src-dependent activation of the Rho family small GTPase, Rac1, is essential for VEGF-A-induced endothelial cell migration and permeability (Gavard et al., 2006; Garrett et al., 2007). Treatment of endothelial cell monolayers with Slit2N (SEQ ID NO: 39) prevented VEGF-A-dependent Rac1 activation (FIG. 19H). These biochemical experiments indicate that the Slit2-Robo4 pathway suppresses VEGF-A-induced endothelial migration and hyperpermeability via inhibition of an Src-Rac1 signaling axis.

EXAMPLE 18

Activation of Robo4 Reduces Vascular Leak and Pathologic Angiogenesis in CNV and OIR Models: A murine model of oxygen-induced retinopathy (OIR) that mimics the ischemia-induced angiogenesis observed in both diabetic retinopathy and retinopathy of prematurity was used to investigate the effect of Robo4 signaling on retinal vascular disease. In this model, P7 mice were maintained in a 75% oxygen environment for five days and then returned to 25% oxygen for an additional five days. The perceived oxygen deficit initiates a rapid increase in VEGF-A expression in the retina, leading to pathological angiogenesis (Ozaki et al., 2000; Werdich et al., 2004. *Robo4*$^{+/+}$ mice and *Robo4*$^{AP/AP}$ mice were evaluated using this model. Intravitreal administration of Slit2N (SEQ ID NO: 39). markedly reduced angiogenesis in *Robo4*$^{+/+}$ mice, but not in *Robo4*$^{AP/AP}$ mice (FIG. 20A – FIG. 20E, where arrows indicate areas of pathological angiogenesis). Furthermore, *Robo4*$^{AP/AP}$ mice displayed more aggressive angiogenesis than *Robo4*$^{+/+}$ mice following exposure to hyperoxic conditions (See, e.g., FIG. 20A and 20C).

In addition to the described OIR model, laser-induced choroidal neovascularization, which mimics age-related macular degeneration, is commonly used to study pathological angiogenesis in the mouse (Lima et al., 2005). In this model, a laser is used to disrupt Bruch's membrane, which allows the underlying choroidal vasculature to penetrate into the subretinal pigment epithelium. To discern the effect of Robo4 signaling on this pathological process, 8-12 week old *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were subjected to laser-induced choroidal neovascularization followed by intravitreal injection of Slit2N (SEQ ID NO: 39). Similar to the results achieved in the mouse model of oxygen-induced retinopathy, intravitreal administration of Slit2N reduced angiogenesis in *Robo4*$^{+/+}$ mice, but not in *Robo4*$^{AP/AP}$ mice (See FIG. 20F – FIG. 20J). Together, the oxygen-induced retinopathy and choroidal neovascularization models indicate that two vascular beds with distinct characteristics, one a tight blood-brain barrier and the other a fenestrated endothelium, are protected from pathological insult by activation of Slit2-Robo4 signaling.

EXAMPLE 19

Robo4 Inhibits Signaling From Multiple Factors That Destabilize the Mature Vessel: The effect of Robo4 activation by a Slit2 molecule on the activity of bFGF, and angiogenic factor, and thrombin, the endothelial permeability factor, was evaluated. As shown in FIG. 21, Slit2N (SEQ ID NO: 39) blocked bFGF-induced endothelial tube formation and thrombin-induced permeability. These studies demonstrate that Slit-Robo4 signaling is capable of inhibiting the signaling induced by multiple angiogenic and permeability factors and support the concept that the Slit-Robo4 pathway protects the mature vascular beds from multiple angiogenic, permeability and cytokine factors.

To reinforce that Robo4 signalizing protects vasculature from multiple angiogenic, permeability and cytokine factors, the effect of Robo4 activation by Slit2N (SEQ ID NO: 39)was evaluated in a mouse model of acute lung injury. In this model, the bacterial endotoxin LPS was dosed to the mice via intratracheal administration. Exposure to the bacterial endotoxin leads to a cytokine storm that causes catastrophic destabilization of the pulmonary vascular bed and results in non-cardiogenic pulmonary edema (Matthay et al., 2005). Following intratracheal administration of LPS, the mice were treated with Slit2N (SEQ ID NO: 39)or Mock preparation, which was a sham protein extract that served as a control. As shown in FIG. 22, the concentrations of inflammatory cells and protein in

bronchoalveolar lavages (BAL) from mice treated with Slit2N (SEQ ID NO: 39) were significantly lower than in the mice treated with the Mock preparation. These results demonstrate that activating Robo4 under these circumstances provides potent vascular stabilization and suggest that Slit2-Robo4 is a potent vascular stabilization pathway that works to preserve the integrity of the mature endothelium and maintain vascular homeostasis against an extreme form of cytokine storm.

EXAMPLE 20

Administration of Slit2 Protein Reduces Mortality in Mouse Model of Avian Flu: In the following example, the effect of Slit protein on the survival of mice infected with Avian Flu Virus was analyzed. A total of 120 female BALB/c mice were inoculated intranasally with 50 µl of a 1:400 dilution of the Avian Flu Virus, strain H5N1/Duck/Mn/1525/81. The mice used in this example were obtained from Charles River and had an average weight ranging from 18-20 grams. With reference to Table 2, the mice were randomly divided into 6 cages of 20 mice each, and each group were subjected to daily treatments for 5 days. Survivorship (death) and body weight were observed during and after treatment.

TABLE 2

# mice /Cage	Group #	Infected y or n	Compound	Dosage	Treatment Schedule
20	1	Y	PSS	50 µl volume	Qd X 4 or 5 (5 if possible) beg -4 before virus exposure, I.V.
20	2	Y	SLIT "Mock" 1	15.625 µl SLIT/Mock + 34.375 µl PSS per mouse	Same as # 1
20	3	Y	SLIT "Mock" 2	1.5625 µl SLIT/Mock + 48.44 µl PSS per mouse	Same as # 1

20	4	Y	SLIT - Conc. 1	15.625 µl of 800 µg/ml SLIT + 34.375 µl PSS per mouse	Same as # 1
20	5	Y	SLIT - Conc. 2	1.5625 µl of 800 µg/ml SLIT + 48.44 µl PSS per mouse	Same as # 1
20	6	Y	Ribavirin	75 mg/kg/day	0.1 ml I.P. BID X 5 days

Briefly, as shown in Table 2, Group 1 was treated with physiological saline solution (PSS) a negative control. Groups 2 and 3 were treated with a Mock preparation. Groups 4 and 5 were treated with different concentrations of a Slit protein (Slit2N (SEQ ID NO: 39)). As a positive control, the 20 mice of group 6 were treated with intraperitoneally with 75 mg/kg/day of Ribavirin brought up in a total volume of 0.1mL PSS.

The results of the analysis are illustrated in FIG. 24 and detailed in Table 3. After 23 days, the mice treated with Slit protein in Groups 4 and 5 had a lower mortality than those mice that did not receive Slit protein in Groups 1, 2, and 3. The Group 4 mice, treated with 12.5 µg of Slit per dose, had a 25% survivability rate. The Group 5 mice, treated with 1.25 µg of Slit per dose, had a 50% survivability rate. In contrast to the survivorship of Groups 4 and 5, only 5% (1/20) of the negative control mice in Group 1, treated with PSS, survived past 23 days.

Table 3 shows that at 14 days after inoculation, the average body weights of the survivors in Groups 1, 2, and 3 were significantly lower than the Slit treated survivors in Groups 4 and 5. Moreover, 10/20 mice in Group 5, which was the lower of the Slit treatment concentrations, survived with body weights averaging 17.6 grams at 21 days, nearly as high as the starting average body weight of 17.7 grams. Therefore, those infected mice treated with Slit protein were able to maintain their body weights better than the untreated mice.

TABLE 3

	Day	0	1	2	3	4	5	6	7	8	9
Cage #1	Alive	20	20	20	20	20	19	17	11	8	3
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.6									
Cage #2	Alive	20	20	20	20	20	20	19	14	7	3
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.6									
Cage #3	Alive	20	20	20	20	20	20	19	12	8	6
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.6									
Cage #4	Alive	20	20	20	20	20	20	17	13	10	7
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.4									
Cage #5	Alive	20	20	20	20	20	20	20	17	12	11
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.7									
Cage #6	Alive	20	20	20	20	20	20	20	20	20	20
	Total	20	20	20	20	20	20	20	20	20	20
	Av. Wt.	17.5									

TABLE 3 (continued)

		10	11	12	13	14	15	16	17	18	19	20	21	22	23
Cage	Alive	2	2	1	1	1	1	1	1	1	1	1	1	1	1
# 1	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					12.5							16.0		
Cage	Alive	2	2	2	2	2	2	2	2	2	2	2	2	2	2
# 2	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					12.5							15.3		
Cage	Alive	5	4	4	4	4	3	3	3	3	3	3	3	3	3
# 3	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					13.0							16.1		
Cage	Alive	6	5	5	5	5	5	5	5	5	5	5	5	5	5
# 4	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					16.0							18.5		
Cage	Alive	10	10	10	10	10	10	10	10	10	10	10	10	10	10
# 5	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					15.4							17.6		
Cage	Alive	20	20	20	20	20	20	20	20	20	20	20	20	20	20
# 6	Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Av. Wt.					17.2							18.3		

EXAMPLE 21

Fragments of Slit Proteins Work to Activate Robo4:. FIG. 23 illustrates various constructs of the Slit2 protein. As has already been described herein, the 150kD protein Slit2N (SEQ ID NO: 39), has been found to be effective in *in vitro* and *in vivo* models, including Miles assays, assays for retinal permeability, tube formation and endothelial cell migration and in OIR and CNV models of ocular disease. Moreover, as is shown in FIG. 23, the (40kD) protein SlitD1 (SEQ ID NO: 42) and Slit2N (SEQ ID NO: 39) constructs exhibits similar activity to full length Slit2 (SEQ ID NO: 40) in a VEGF-induced endothelial cell migration assay.

Materials and Methods

Reagents: HEK 293 and COS-7 cells, and all IMAGE clones were from ATCC. SP6 and T7 Message Machine kits were from Ambion. HUVEC, EBM-2 and bullet kits were from Cambrex. Yeast two-hybrid plasmids and reagents were from Clontech. FBS was from Hyclone. Anti-HA affinity matrix, Fugene6 and protease inhibitor cocktail were from Roche. Goat Anti-Mouse-HRP and Goat Anti-Rabbit-HRP secondary antibodies were from Jackson ImmunoResearch. Anti-V5 antibody, DAPI, DMEM, Lipofectamine 2000, Penicillin-Streptomycin, Superscript III kit, Trizol and TrypLE Express were from Invitrogen. Anti-Flag M2, Phosphatase Inhibitor Cocktails, Soybean Trypsin Inhibitor and Fatty acid-free Bovine Serum Albumin (BSA) were from Sigma. Human fibronectin was from Biomedical Technologies and Invitrogen. Costar Transwells and Amicon Ultra-15 Concentrator Columns were from Fisher. Rosetta2 *E. coli* were from Novagen. Glutathione-Sepharose 4B, parental pGEX-4T1 and ECL PLUS were from Amersham-Pharmacia. Coomassie Blue and PVDF were from BioRad. Quick change site-directed mutagenesis kit was from Stratagene. Normal Rat IgGagarose conjugate was from Santa Cruz. *Robo4* morpholinos were from Gene Tools. Oligonucleotides for PCR were from the University of Utah Core Facility. Alexa564-Phalloidin, Anti-GFP and Goat Anti-Rabbit Alex488 were from Molecular Probes. Low melt agarose was from NuSieve. T7 *in vitro* transcription/translation kit was form Promega.

Molecular Biology: The Robo4-HA, Slit2-Myc-His and chicken paxillin plasmids have been previously described (Park et al., 2003; Nishiya et al., 2005). Robo4-NH2 was

amplified from Robo4-HA and cloned into EcoRV/NotI of pcDNA3-HA. Robo4-COOH was amplified from Robo4-HA by overlap-extension PCR and cloned into EcoRV/NotI of pcDNA3-HA. The amino terminal half of the human Robo4 cytoplasmic tail (AA 465-723) was amplified by PCR and cloned into (EcoRI/BamHI) of pGBKT7. Murine Robo4 fragments were amplified by PCR and cloned into BamHI/EcoRI of pGEX-4T1. Murine Hic-5, Mena and paxillin (including deletions) were amplified from IMAGE clones by PCR and cloned into EcoRV/NotI of pcDNA3-V5. GST-Robo4ΔPIM and full-length Robo4ΔPIM were generated by site-directed mutagenesis of relevant wild-type constructs using Quick Change. The integrity of all constructs was verified by sequencing at the University of Utah Core Facility.

Embryo Culture and Zebrafish Stocks: Zebrafish, *Danio rerio*, were maintained according to standard methods (Westerfield, 2000). Developmental staging was carried out using standard morphological features of embryos raised at 28.5°C (Kimmel et al., 1995). The *Tg (fli:EGFP)*^{y1} transgenic zebrafish line used in this study was described in Lawson and Weinstein, 2002. Imaged embryos were treated with 0.2mM 1-phenyl-2-thio-urea (PTU) after 24 hpf to prevent pigment formation.

Antisense Depletion of robo4: Antisense morpholino oligonucleotides (MO) directed against the exon 10 / intron 10 splice site of *robo4* (5'- tttttagcgtacctatgagcagtt-3', SEQ ID NO:28) were dissolved in 1X Danieau's Buffer at a concentration of 5 ng/nl, respectively. Before injection, the morpholino was heated at 65°C for 5 minutes, cooled briefly, mixed with a negligible amount of dye to monitor injection efficiency, and approximately 1nl was injected into the streaming yolk of 1-2 cell stage embryos.

Reverse Transcription (RT) PCR: RNA was extracted from 20 uninjected and 20 *robo4* MO-injected embryos using Trizol, reagent and subsequent cDNA synthesis was performed using Superscript III primed by a mixture of both random hexamers and oligo dT primers. *robo4* was amplified from cDNA by PCR with a forward primer in exon 8 (5'-caacaccagacacttacgagtgcc -3', SEQ ID NO:29) and a reverse primer in exon 12 (5'-ttcgaaggccagaattctcctggc -3', SEQ ID NO:30) using the following parameters: (94°C for 4', 94°C for 30", 58°C for 30", 68°C for 45", 68°C for 1'). To identify the linear range of the

PCR reaction, cDNA was amplified for 23, 25, 27 and 30 cycles. β-actin was amplified using a forward primer (5'-cccaaggccaacagggaaaa, SEQ ID NO:31) and a reverse primer (5'-ggtgcccatctcctgctcaa-3', SEQ ID NO:32) from all samples to control for cDNA input.

Whole-Mount Indirect Immunofluorescence: Briefly, age-matched 24 and 48 hpf embryos were dechorionated and fixed in 4% PFA / 4% sucrose / PBS overnight at 4°C. The embryos were then washed in PBS / 0.1% Tween-20, dehydrated to absolute methanol, re-hydrated back to PBS-Tween 20, further permeabilized in PBS / 1% Triton-X, rinsed in PBS / 1% Triton-X / 2% BSA, blocked at room temperature in PBS / 1% Triton-X / 2% BSA / 10% Sheep Serum / 1% DMSO, then incubated in IgG purified anti-GFP (1:400) in blocking solution overnight at 4°C. The following day embryos were washed vigorously in PBS / 1% Triton-X / 2% BSA, then incubated in goat-anti-Rabbit Alexa 488 conjugated secondary antibody (1:200) in blocking solution overnight at 4°C. The following day the embryos were washed extensively in PBS / 1% Triton-X / 2% BSA, then embedded in 1% low melt agarose in PBS and photographed on Leica confocal microscope and processed using Adobe Photoshop software.

Cell Culture: HEK 293 and COS-7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin / streptomycin. Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 supplemented with 10% FBS. HUVEC were routinely used between passages 2 and 5.

Transfection: HEK293 and COS-7 cells were transfected with Fugene6 or Lipofectamine2000 according to the manufacturer's protocol.

Preparation of Concentrated Slit2 Protein: COS-7 cells were transiently transfected with empty pSECTAG2 or pSECTAG2::hSlit2. Forty-eight hours later, the cells were washed twice with PBS and incubated with 6ml salt extraction buffer (10mM HEPES, pH 7.5, 1M NaCl and 1X protease inhibitors) for 15 minutes at 25°C. Salt extraction was repeated and the samples were centrifuged at 10,000 rpm for 20 minutes to pellet cell debris. The supernatant was loaded on Amicon Ultra-15 concentrator columns/100 kDa cutoff and centrifuged until 12ml of salt extracts was reduced to approximately 500µl. The concentrated protein

preparations were analyzed by Coomassie Blue staining, and stored at 4°C for up to one week. Using this protocol, Slit2 concentrations of 20-50 μg/ml were routinely obtained. In addition to preparing concentrated protein from cells transfected with Slit2 plasmid, the identical protocol was performed on cells transfected with an empty vector (pSECTAG2). This resulting preparation was referred to as a "Mock" preparation, and it was used as a control in all experiments analyzing the effect of Slit2.

Haptotaxis Migration Assay: Transfected HEK 293 cells were removed from tissue culture dishes with TrypLE Express, washed once with 0.1% trypsin inhibitor, 0.2% fatty acid-free BSA in DMEM or EBM-2, and twice with 0.2% BSA in the relevant media. The washed cells were counted and resuspended at 0.3×10^5 cells / ml. 1.5×10^5 were loaded into the upper chamber of 12μm Costar transwells pre-coated on the lower surface with 5μg/ml fibronectin. The effect of Slit2 on haptotaxis was analyzed by co-coating with 0.5 μg/ml Slit2 or an equivalent amount of Mock preparation. Cell migration was allowed to proceed for 6 hours, after which cells on the upper surface of the transwell were removed with a cotton swab. The cells on the lower surface were fixed with 4% formaldehyde for 5 minutes and washed three times with PBS. For HEK 293 cells, the number of GFP-positive cells (HEK 293) on the lower surface was enumerated by counting six 10X fields on an inverted fluorescence microscope. The number of migrated cells on fibronectin/Mock-coated membranes was considered 100% for data presentation and subsequent statistical analysis. At least two independent experiments in duplicate were performed.

Yeast Two Hybrid Assay: pGBKT7::hRobo4 465-723 was transformed into the yeast strain PJ694A, creating PJ694A-Robo4. A human aortic cDNA library was cloned into the prey plasmid pACT2 and then transformed into PJ694A-Robo4. Co-transformed yeast strains were plated onto SD -Leu-Trp (-LT) to analyze transformation efficiency and SD -Leu-Trp-His-Ade (-LTHA) to identify putative interacting proteins. Yeast strains competent to grow on SD –LTHA were then tested for expression of β-galactosidase by the filter lift assay. Prey plasmids were isolated from yeast strains capable of growing on SD –LTHA and expressing β -galactosidase, and sequenced at the University of Utah Core Facility.

Immunoprecipitation: Cell lysates were prepared in 50mM Tris-Cl, pH 7.4, 50mM NaCl, 1mM DTT, 0.5% Triton X-100, phosphatase and protease inhibitors, centrifuged at 14K for 20 minutes to pellet insoluble material, cleared with normal IgG coupled to agarose beads for 60 minutes, and incubated for 2 hours at 4°C with relevant antibodies coupled to agarose beads. The precipitates were washed extensively in lysis buffer and resuspended in 2X sample buffer (125mM Tris-Cl, pH 6.8, 4% SDS, 20% Glycerol, 0.04% bromophenol blue and 1.4M 2-mercaptoethanol).

GST Pull Down Assay: Rosetta2 *E. coli* harboring pGEX-4T1::mRobo4 were grown to OD600 of 0.6 and induced with 0.3mM IPTG. After 3-4 hours at 30°C, 220rpm, the cells were lysed by sonication in 20mM Tris-Cl pH 7.4, 1% Triton X-100, 1 μg/ml lysozyme, 1mM DTT and protease inhibitors. The GST-fusion proteins were captured on glutathione-Sepharose 4B, washed once with lysis buffer without lysozyme and then twice with binding/wash buffer (50mM Tris-Cl, pH 7.4, 150mM NaCl, 1mM DTT, 1% Triton X-100, 0.1% BSA and protease inhibitors). The GST-fusion proteins were incubated with 60nM purified recombinant paxillin overnight at 4°C, washed extensively in binding/wash buffer, and resuspended in 2X sample buffer.

Western Blotting: Immunoprecipitates and GST-fusion proteins were incubated for 2 minutes at 100°C, separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldifluoride (PVDF) membrane. PVDF membranes were incubated with 5% nonfat dry milk in PBS + 0.1% Tween20 (PBST) (PBST-M) for 60 minutes at 25°C. Blocked membranes were incubated with primary antibody (anti-Flag M2 at 1:2000; anti-HA at 1:10,000; anti-Hic-5 at 1:500; anti-paxillin at 1:10,000; anti-Rac at 1:1,000 and anti-Cdc42 at 1:500) in PBST-M for 60 minutes at 25°C, or overnight at 4°C. Membranes were washed 3 x 10 minutes in PBST and then incubated with secondary antibody (goat anti-mouse or goat anti-rabbit horseradish peroxidase at 1:10,000) for 60 minutes at 25°C. Membranes were washed 3 x 10 minutes in PBST and visualized with ECL PLUS.

In vitro Transcription/Translation: Mena-V5 was synthesized with the T7 Quick Coupled *in vitro* Transcription/Translation system according to the manufacturer's protocol.

Spreading Assay: Transfected HEK 293 cells were plated onto coverslips coated with 5μg/ml fibronectin. Following a 30 minute incubation at 5% CO_2 and 37°C, the cells were washed three times with ice-cold PBS and fixed with 3.7% formaldehyde for 10 minutes at room temperature. The cells were then peremabilized with 0.2% Triton X-100 for three minutes, washed three times with PBS + 0.1% Tween20 (PBST) and incubated with 10 μg/ml Rhodamine-Phalloidin for one hour at room temperature. Following three more washes in PBS-T, the coverslips were mounted in Pro-Long Gold and analyzed by confocal microscopy. The total area of 150 cells in three independent experiments was determined using ImageJ.

siRNA-mediated knockdown of paxillin: HEK 293 cells were transfected with 100nM siRNA duplexes (5'-CCCUGACGAAAGAGAAGCCUAUU-3', SEQ ID NO:33 and 5'-UAGGCUUCUCUUUCGUCAGGGUU-3', SEQ ID NO:34) using LipofectAMINE 2000, according to the manufacturer's instructions. 48 h after transfection, cells were processed for biochemical analysis or cell spreading assays. Paxillin reconstitution was accomplished by transfection with an expression vector encoding chicken paxillin, which has the nucleotide sequence 5'-CCCCTACAAAAGAAAAACCAA-3' (SEQ ID NO:35) within the siRNA target site. Knockdown and reconstitution were visualized by western blotting with paxillin antibodies and quantified by densitometry.

Rac and Cdc42 Activation Assay: Transfected HEK 293 cells were detached from cell culture dishes, held in suspension for one hour in DMEM + 0.2% BSA, and plated onto bacterial Petri dishes coated with 5 μg/ml fibronectin for five minutes. The cells were then washed twice with ice-cold PBS and lysed in 50mM Tris pH 7.0, 500mM NaCl, 1mM MgCl2, 1mM EGTA, 1mM DTT, 0.5% NP-40, 1X protease inhibitors, 1X phosphatase inhibitors and 20 μg/ml GST-PBD. The lysate was centrifuged for five minutes at 14,000 rpm and the supernatant was incubated with 30 μl of glutathione agarose for 30 minutes at 4°C. Following three washes with lysis buffer, bound proteins were eluted with 2X sample buffer. Rac and Cdc42 were detected by western blotting with antibodies specific to each protein. Rac activation levels were normalized to total Rac and the highest value in each experiment was assigned a value of 1.

Generation of Robo4 $^{AP/AP}$ mice and genotyping: The Robo4 targeting vector was electroporated into embryonic stem (ES) cells. ES cells heterozygous for the targeted allele were injected into blastocysts and then transferred to pseudopregnant females. Chimeric males were identified by the presence of agouti color and then mated to C57BL/6 females to produce ES-cell derived offspring. Genotype was confirmed by Southern blot analysis of tail DNA. Genomic DNA from ear punch or tail samples was used for PCR genotyping under the following conditions; denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds, 40 cycles. The following two primers were used for genotyping of *Robo4:* 5' cccttcacagacagactctcgtatttcc 3' (forward) and 5'cccagacctacattacctttgccg 3'(reverse) and for *AP:* 5'ggcaacttccagaccattggcttg 3'(forward) and 5' ggttaccactcccactgacttccctg 3' (reverse).

Embryos and expression analysis: Staging of embryos, *in situ* hybridization, paraffin sectioning and whole-mount PECAM-1 immunohistochemistry were performed as previously described[1]. For Northern Blot analysis, 20 μg of total RNA was loaded per lane after isolation with TRIZOL. ^{32}P-labelled probe was generated using prime It II Random-Primer labeling kit (Stratagene). Lung lysates were prepared with lysis buffer [1% NP-40, 150mM NaCl, 50mM Tris-Cl (pH 7.5), 1 mM EDTA and protease inhibitor cocktail (Roche)]. Robo4 protein from the lung lysates was detected by Western blot analysis using a polyclonal *anti-*Robo4 antibody as previously described.

Alkaline phosphatase (AP) staining: Embryos or tissues were fixed in 4% paraformaldehyde and 2mM $MgCl_2$ in PBS overnight at 4°C with shaking. Samples were washed three times for 15 min in PBST (PBS, 0.5% Tween 20). Endogenous alkaline phosphatase was inactivated at 65°C for 90 min in PBS with 2mM $MgCl_2$, then washed in AP buffer (100mM Tris-Cl, pH9.5, 100mM NaCl, 50mM $MgCl_2$,, 0.1% Tween 20, 2mM Levamisole) twice for 15 minutes. Staining was carried out in BM purple substrate (Boehringer Mannheim) for embryos (Boehringer Mannheim) or NBT/BCIP for adult tissues. Staining was stopped in PBS, with 5mM EDTA.

*Whole mount immunohistochemistry after AP staining:*Alkaline phosphatase (AP) staining on fixed and dissected retinas was performed as described above. Staining was

stopped in PBS -5mM EDTA. Retinas were washed twice in PBS and post-fixed 5 minutes in 4% paraformaldehyde, phosphate-buffered saline at RT, then washed twice in PBS. After 2h hours incubation in PBlec (PBS, pH 6.8,1% Triton-X100, 0.1 mM CaCl 0.1 mM MgCl 0.1 mM MnCl), retinas were incubated with antibodies overnight at 4°C. Pericytes were labeled using rabbit anti-NG2 antibody (1:200; Chemicon) and endothelial cells were labeled using rat anti-endomucin (Clone V.7C7 kindly provided by Dietmar Vestweber; diluted 1:20). After 3 washes in PBS-T (PBS, pH 7.4,1% Triton-X100), samples were incubated with secondary antibodies conjugated with the appropriate fluorochrome—Alexa Fluor 488 or 568 (Molecular Probes; Invitrogen) in PBS. After washing and a brief postfixation in 4% PFA, the retinas were flat mounted and coversliped using Mowiol/DABCO (Sigma-Aldrich) Samples were analyzed by conventional light and fluorescence microscopy using a Zeiss Stereomicroscope Stemi SV 11 Bioquad equipped with a Zeiss Axiocam HRc digital camera and by confocal laser scanning microscopy using a Zeiss LSM Meta 510. AP staining was visualized using the 633nm HeNe laser and reflection settings. Digital images were processed using Volocity (4.0 Improvision) and compiled in Adobe Photoshop CS2.

Immunohistochemistry: Whole-mount triple immunofluorescence confocal microscopy was performed as previously described[3]. Briefly, antibodies to PECAM, NP1, CX40, 2H3, BFABP and αSMA were used to label the limb skin of Robo4 +/+ or Robo4 -/- embryos at E15.5.

Construction of expression vectors for recombinant Slit fragments: The proposed expression vectors are depicted in FIG. 23. DNA encoding all fragments was cloned into the pSECTAG2 vector (Invitrogen) and shared the following features: a CMV promoter, a Kozak consensus sequence, a myc/his tag in-frame fusion, and a bovine growth hormone polyA sequence. The Fc fusions were generated by replacing the myc/his epitope with a recombinant form of the Fc domain of human IgG1 in which the complement activating and effector cell interaction domains have been replaced with IgG4 and IgG2 sequences respectively (Katoh et al., 2005; Armour et al., 1999). The recombinant Slit fragments and Slit fragment-Fc fusion proteins were isolated from transiently transfected cells. The desired construct was stably transfected into CHO cells by selection for Zeocin resistance.

Binding and activity of Robo4 agonists on Robo4 expressing HEK cells: Stable cell lines expressing Robo4-HA (Robo4-HEK), or the pcDNA3 vector alone (Control-HEK), were seeded in 6-well culture dishes precoated with 100μg/ml poly-L- lysine. Cells were incubated with HEK CM or Slit-myc CM at 37°C. After 1hr 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 antibody (Santa Cruz Biotech) and *anti*-mouse Alexa 594-conjugated secondary antibody (Molecular Probes). The ability of those agonists, which bind to Robo4 to inhibit migration, was performed according to Park KW, Morrison CM, Sorensen LK, et al., "Robo4 is a vascular-specific receptor that inhibits endothelial migration," Dev Biol 2003;261(1):251-67.

Isolation of murine lung endothelial cells: Isolation of murine endothelial cells has been previously described[4]. Sheep anti-rat IgG Dynal beads (Dynal Biotech) were conjugated with either anti-PECAM-1 or anti-ICAM-2 monoclonal antibody (BD Pharmingen) at 5 μg of antibody per 100μL of beads. The beads were precoated and stored at 4°C ($4x10^8$ beads/mL of PBS with 0.1% BSA) for up to 2 weeks. The lungs from three adult mice were harvested. The lung lobes were dissected from visible bronchi and mediastinal connective tissue. The lungs were washed in 50mL cold isolation medium (20% FBS-DMEM) to remove erythrocytes, minced with scissors and digested in 25mL of pre-warmed Collagenase (2mg/mL, Worthington) at 37°C for 45 minutes with gentle agitation. The digested tissue was dissociated by triturating 12 times through a 60 cc syringe attached to a 14 gauge metal cannula and then filtered through sterile 70μm disposable cell strainer (Falcon). The suspension was centrifuged at 400 x *g* for 10 minutes at 4°C. The cell pellet was resuspended in 2ml cold PBS and then incubated with PECAM-1 coated beads (15μL/mL of cells) at room temperature for 10 minutes. A magnetic separator was used to recover the bead-bound cells, which were washed in isolation medium, and then resuspended in complete medium (EGM-2 MV, Lonza). The cells were plated in a single fibronectin-coated 75-cm^2 tissue culture flask and nonadherent cells were removed after overnight incubation. The adherent cells were washed with PBS and 15 ml of complete medium was added. Cultured cells were fed on alternate days with complete medium. When the cultures reached 70 to 80% confluency, they were detached with trypsin-EDTA, resuspended in 2 ml

PBS and sorted for a second time using ICAM-2 conjugated beads (15 μL/mL of cells). The cells were washed and plated as above. Passages 2 to 5 were used for functional assays.

Cell Culture: Human dermal microvascular endothelial cells (HMVEC, Cambrex) were grown in EGM-2 MV, and used between passages 3 and 6.

Immunocytochemistry: 8 well chamber slides (Lab-Tek) were coated with 1.5μg/cm^2 fibronectin for two hours prior to plating cells. Murine lung endothelial cells were plated overnight at 37°C (100,000 cells/well) in complete medium, EGM-2 MV. The cells were then washed three times in PBS, and fixed in 4% paraformaldehyde for 10 minutes at room temperature. After three additional washes in PBS, the cells were washed in 1% Triton X-100 in PBS for 15 minutes at room temperature followed by three washes in PBST (0.1% Triton X-100 in PBS). The cells were then blocked in 2% BSA in PBS for 20 minutes at room temperature and incubated with primary antibody in 2% BSA: rat anti-PECAM-1 (Pharmigen), rabbit anti-Von Willebrand Factor (vWF) (DAKO) for 1 hour at room temperature. After incubation with primary antibody, the cells were washed in PBST and incubated with secondary antibody in 2% BSA: Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes) for 1 hour at room temperature. The cells were washed once in PBST, once in PBS, mounted in Vectashield mounting media (Vector Laboratories), and photographed by a confocal microscopy.

Fluorescence-Activated Cell Sorting (FACS): Murine lung endothelial cells were detached from the culture dish by brief trypsinization (no more than 2 minutes) at 37°C. Proteolysis was arrested by the addition of trypsin inhibitor in EBM-2 + 0.1% BSA. The cells were washed twice in FACS buffer (PBS without Ca2+ and Mg2+ + 0.1% BSA) and then resuspended in 1mL FACS buffer. Analysis of the expression of cell surface markers was performed with two-step immunofluorescence staining. The cells were incubated for 30 minutes at 4°C with purified monoclonal antibodies: rat anti-PECAM -1, rabbit anti-vWF. The cells were then washed two times in FACS buffer and resuspended in 1mL FACS buffer. The cells were then incubated for 30 minutes at 4°C with fluorescent secondary antibody: Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (Molecular

Probes). The cells were again washed twice, resuspended in 1mL FACS buffer and analyzed with the FACS.

Cell migration assay: Cells were labeled with CellTracker Green CMFDA (Molecular Probes) for 1 hour, washed and then starved overnight in EBM-2 supplemented with 0.1% BSA. Cells were trypsinized, washed and resuspended to 300,000 cells/mL. 100μL of cell suspension (30,000 cells) was loaded onto 8-μm HTS FluoroBlock filters (BD Falcon) that had been previously coated on both sides with 5μg/mL human fibronectin. Test factors were diluted in EBM-2 /0.1% BSA and placed in the lower chamber. After incubation at 37° C for 3 hours, two 5X fields from each well were photographed on an inverted fluorescence microscope (Axiovert 200). The number of migrated cells was enumerated by counting fluorescent cells. Basal migration of *Robo4*$^{+/+}$ cells was set at 1. Data are presented as mean ± S.E. of three independent experiments in triplicate.

Tube formation assay: Tube formation was performed as previously described[5]. In brief, lung endothelial cells isolated from *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were plated onto matrigel-coated wells of a 48-well dish, and starved overnight in 0.5% serum. The cells were then stimulated with 0.48nM VEGF-A in the absence or presence of Slit2 for 3.5 hours, and then photographed. Average tube length was determined using ImageJ software. Data are presented as mean ± S.E. of three independent experiments in duplicate.

In vitro permeability assay: Lung endothelial cells (ECs) isolated from *Robo4*$^{+/+}$ and *Robo4*$^{AP/AP}$ mice were plated onto 3.0μm Costar transwells pre-coated with 1.5μg/cm^2 human fibronectin and grown to confluency. Cells were starved overnight, pre-treated with 0.3nM Slit2 for 30-60 minutes and then stimulated with 2.4nM VEGF-A for 3.5 hours. Horseradish peroxidase (HRP) was added to the top chamber at a final concentration of 100μg/ml, and 30 minutes later the media was removed from the lower chamber. Aliquots were incubated with 0.5 mM guaiacol, 50 mM Na_2HPO_4, and 0.6 mM H_2O_2, and formation of *O*-phenylenediamine was determined by measure of absorbance at 470 nm. Basal permeability of monolayers was set at 100%. The data is presented as mean ± S.E. of three independent experiments in triplicate.

VEGF Induced Retinal Permeability: Retinal permeability was assessed as described in[53]. In brief, 8-10 week old mice were anesthetized with Avertin (2-2-2 Tribromoethanol, 0.4 mg/g; Acros Organics, Morris Plains, NJ). Mice were given an intraocular injection of 1.4uL of 35.7ug/mL VEGF-A (R&D Systems Inc. Minneapolis, MN) with 50ng Slit2N (SEQ ID NO: 39). An injection with equivalent volume of Mock preparation was given in the contralateral eye. As indicated, other conditions of 1.4uL of saline, Mock preparation, or slit were administered. Six hours later, mice were given an I.V. injection via the tail vein of 50uL Evans Blue 60mg/mL. After two hours, mice were sacrificed and perfused with citrate-buffered para-formaldehyde to remove intravenous Evans Blue. Eyes were enucleated and retinas dissected. Evans Blue dye was eluted in 0.3mL formamide for 18 hours at 70°C. The extract was ultra-centrifuged through a 5kD filter for 2 hours. Absorbance was measured at 620nm. Background absorbance was measured at 740nm and subtracted out.

Adenoviral expression of Robo4: Robo4 was expressed via adenovirus as previously described.

Miles Assay: Evans Blue was injected into the tail vein of 6-8 week old mice, and thirty minutes later either saline, or 10ng of VEGF-A in the absence and presence of 100ng Slit2 was injected into the dermis. After an additional thirty minutes, punch biopsies were preformed and Evans Blue was eluted from the dermal tissue in formamide for 18 hours at 60°C. Following centrifugation, the absorbance was measured at 620nm. The amount of dermal permeability observed in saline injected animals was set at 1. Data are presented as mean ± S.E. of five individual mice with each treatment in duplicate (six total injections per animal).

Retinal permeability: Retinal permeability was assessed as previously described[8]. In brief, 8-10 week old mice were anesthetized with Avertin (2-2-2 Tribromoethanol, 0.4 mg/g; Acros Organics, Morris Plains, NJ). Mice were given an intraocular injection of 1.4μL of 35.7μg/mL VEGF-A (R&D Systems Inc. Minneapolis, MN) with 50ng Slit2. An equivalent volume of Mock was injected into the contralateral eye. As indicated, other conditions were administered. Six hours later, 50μL of 60mg/mL Evans Blue solution was administered via the femoral vein. After two hours, mice were sacrificed and perfused with citrate-buffered

formaldehyde to remove intravenous Evans Blue. Eyes were enucleated and retinas dissected. Evans Blue dye was eluted in 0.4mL formamide for 18 hours at 70°C. The extract was ultra-centrifuged through a 5kD filter for 2 hours. Absorbance was measured at 620nm. Background absorbance was measured at 740nm and subtracted out. Data are presented as mean ± S.E. of five individual mice per genotype.

Biochemical assays: HMVEC were grown to confluence on fibronectin-coated dishes and starved overnight in EBM-2 + 0.2% BSA. The next day, the cells were stimulated with 50ng/mL VEGF-A for 5 minutes, washed twice with ice-cold PBS and lysed in 50mM Tris pH 7.4, 150mM NaCl, 10mM $MgCl_2$, 1mM DTT, 10% Glycerol, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1X protease inhibitors, 1X phosphatase inhibitors. Lysates were combined with 2X sample buffer, separated by SDS-PAGE and probed with antibodies to phospho-VEGFR2, phospho-p42/44 and phospho-Src (Cell Signaling) at 1:1000. For Rac activation assays, crude membrane preps were generated[9] and GTP-Rac was precipitated with 20 μg/ml GST-PBD. Following three washes with lysis buffer, bound proteins were eluted with 2X sample buffer. Rac1 was detected by western blotting with monoclonal antibodies (BD Biosciences).

Oxygen Induced Retinopathy: In brief, P7 pups along with nursing mothers were placed in 75% oxygen, which was maintained by a Pro-OX oxygen controller (BioSpherix, Redfield, NY). Pups were removed on P12 and given an intraocular injection of Slit2N (SEQ ID NO: 39) agonist or Mock preparation, which served as a control condition. Mice were sacrificed on P17 and perfused via the left ventricle with 1ml 50mg/ml FITC-Dextran (Sigma, St. Louis, MO). Eyes were enucleated, fixed for 30 minutes in 4% paraformaldehyde, and retinal flatmounts generated. Images were taken using Axiovert 200 fluorescence microscopy (Carl Zeiss, Thornwood, NY). Neovascularization was quantified using AxioVision software, which calculates the amount of vascularization per area (Carl Zeiss, Thornwood, NY). Data are presented as mean ± S.E. of five individual mice per genotype.

Laser Induced Choroidal Neovascularization: Two-three month old mice were anesthetized with Avertin (2-2-2 Tribromoethanol, 0.4 mg/g; Acros Organics, Morris Plains,

NJ) and the pupils dilated with 1% tropicamide (Alcon, Fort Worth, TX). An Iridex OcuLight GL 532 nm laser photocoagulator (Iridex, Mountain View, CA) with slit lamp delivery system was used to create three burns 3 disc diameters from the optic disc at 3, 6, and 9 o'clock with the following parameters: 150mW power, 75um spot size, and 0.1 second duration. Production of a bubble at the time of laser indicating rupture of Bruch's membrane was an important factor in obtaining CNV; therefore, only burns in which a bubble was produced were included in this study. Immediately after laser treatment and 3 days later, mice were given an intravitreal injection of 50ng Slit2N (SEQ ID NO: 39). An equal volume of Mock preparation was given by intravitreal injection in the other eye. One week after laser treatment, mice were sacrificed and choroidal flat mounts generated. Biotin conjugated isolectin (Sigma, St. Louis, MO) and Texas red conjugated streptavidin (Sigma, St. Louis, MO) were used to stain CNV. Flat mounts were examined using a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY) and CNV quantified using ImageJ software (NIH, Bethesda, MD).

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CLAIMS

What is claimed is:

1. A method of inhibiting vascular permeability in tissue, comprising administering to the tissue a repulsive guidance cue of axons, blood vessels, or a combination thereof.
2. The method of claim 1, wherein the repulsive guidance cue is a ligand of a roundabout receptor or Unc5 receptor.
3. The method of claim 2, wherein the repulsive guidance cue is a ligand of roundabout-4 (Robo4) receptor.
4. The method of claim 3, wherein the repulsive guidance cue is slit2 or a fragment thereof that binds Robo4.
5. The method of claim 2, wherein the repulsive guidance cue is a ligand of a Unc5b receptor.
6. The method of claim 5, wherein the repulsive guidance cue is netrin-1 or a fragment thereof that binds a Unc5b receptor.
7. A method of screening for, or evaluating, an agent that inhibits vascular permeability, comprising determining the ability of said agent to affect Robo4-mediated activation of Git1.
8. The method of claim 7, wherein Robo4-mediated activation of Git1 is determined by the steps comprising:
   (a) contacting a first cell expressing Robo4 with a candidate agent,
   (b) contacting a second cell essentially identical to the first cell but substantially lacking Robo4 with the candidate agent,
   (c) assaying for Git1 activation in the first and second cells,
   (d) wherein detectably higher Git1 activation in the first cell as compared to the second cell indicates Robo4-mediated Git1 activation by said agent.
9. The method of claim 8, wherein Git1 activation is assayed by detecting ARF6 inactivation.
10. The method of claim 9, wherein ARF6 inactivation is assayed by detecting Rac inactivation.

11. A method of screening for, or evaluating, an agent that inhibits vascular permeability, comprising determining the ability of said agent to inhibit ARF6, Rac, Pak, Mek, or Erk.

12. The method of claim 11, wherein Robo4-mediated inhibition of ARF6, Rac, Pak, Mek, or Erk is determined by the steps comprising:

    (a) contacting a first cell expressing Robo4 with a candidate agent,

    (b) contacting a second cell essentially identical to the first cell but substantially lacking Robo4 with the candidate agent,

    (c) assaying for inhibition of ARF6, Rac, Pak, Mek, Erk, or a combination thereof, in the first and second cells,

    (d) wherein detectably lower ARF6, Rac, Pak, Mek, or Erk activation in the first cell as compared to the second cell indicates Robo4-mediated ARF6, Rac, Pak, Mek, or Erk inhibition by said agent.

13. The method of claim 7 or 11, wherein the method is performed in the substantial absence of VEGF, TNF, thrombin, or histamine.

14. The method of claim 7 or 11, wherein the assay is performed in the presence of a biologically active amount of VEGF, TNF, thrombin, or histamine.

15. A method of treating or preventing respiratory distress syndrome (RDS) in a subject, comprising:

    (a) identifying a subject having or at risk of having said RDS, and

    (b) administering to the lung of the subject a repulsive guidance cue that binds to neuronal receptors and endothelial cell.

16. A method of treating or preventing retinopathy of pre-maturity (ROP) in a subject comprising

    (a) identifying a subject having or at risk of having said ROP, and

    (b) administering to the retina of the subject a repulsive guidance cue that binds to neuronal receptors and endothelial cell.

17. A method of treating or preventing diabetic retinopathy in a subject comprising

    (a) identifying a subject having or at risk of having said diabetic retinopathy, and

    (b) administering to the retina of the subject a repulsive guidance cue that binds to neuronal receptors and endothelial cell.

18. A method of treating or preventing wet macular degeneration in a subject comprising

    (a) identifying a subject having or at risk of having said wet macular degeneration, and

    (b) administering to the retina of the subject a repulsive guidance cue that binds to neuronal receptors and endothelial cell.

19. A method for treating subjects with repulsive cues or mimetics in a subject comprising

    (a) identifying a subject who have indications for treatment with VEGF blockers, TNF blockers, histamine blockers, or thrombin blockers, and

    (b) administering to the the subject a repulsive guidance cue that binds to neuronal receptors and endothelial cell.

20. The method of claim 15, 16, 17, 18, or 19 wherein the repulsive guidance cue is a ligand of a roundabout receptor, Unc5 receptor, DCC receptor, neogenin receptor, DSCAM receptor, or ICAM-2 receptor.

21. The method of claim 20, wherein the ligand is Slit2.

22. An isolated polypeptide comprising the paxillin binding sequence of roundabout-4 (Robo4), wherein the polypeptide does not comprise full-length Robo4.

23. The isolated polypeptide of claim 22, wherein the paxillin binding sequence consists of SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.

24. An isolated polypeptide of 10 to 400 amino acids comprising SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.

25. An isolated polypeptide of 10 to 400 amino acids comprising an amino acid sequence having at least 80% sequence homology to SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.

26. An isolated polypeptide comprising the paxillin binding sequence (PBS) of roundabout-4 (Robo4), wherein the polypeptide consists of the formula:

$$R^1 - PBS - R^2$$

wherein R^1 and R^2 are, independently, H, acyl, NH_2, an amino acid or a peptide, wherein the polypeptide does not comprise full-length Robo4.

27. The isolated polypeptide of claim 26, wherein the PBS consists of an amino acid sequence having at least 80% sequence homology to SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.

28. An isolated polypeptide consisting essentially of an amino acid sequence having at least 80% sequence homology to SEQ ID NO:27 or a fragment thereof of at least 10 residues in length.
29. An isolated nucleic acid encoding the polypeptide of claim 22, 24, 26, or 28.
30. An isolated nucleic acid encoding a polypeptide comprising the paxillin binding sequence of roundabout-4 (Robo4), wherein the polypeptide does not comprise full-length Robo4
31. An isolated nucleic acid comprising SEQ ID NO:2 or a fragment thereof of at least 30 residues in length, wherein the nucleic acid does not encode full-length roundabout-4 (Robo4).
32. A vector comprising the isolated nucleic acid of claim 29, 30, or 31.
33. A method of promoting angiogenesis in a tissue, comprising delivering into endothelial cells of the tissue a composition comprising the polypeptide of claims 22, 24, 26, or 28.
34. A method of promoting angiogenesis in a tissue, comprising delivering into endothelial cells of the tissue a composition comprising the nucleic acid of claims 29, 30, or 31.
35. A method of promoting angiogenesis in a tissue, comprising administering to the tissue a composition comprising the vector of claim 32, wherein the vector transduces an endothelial cell.

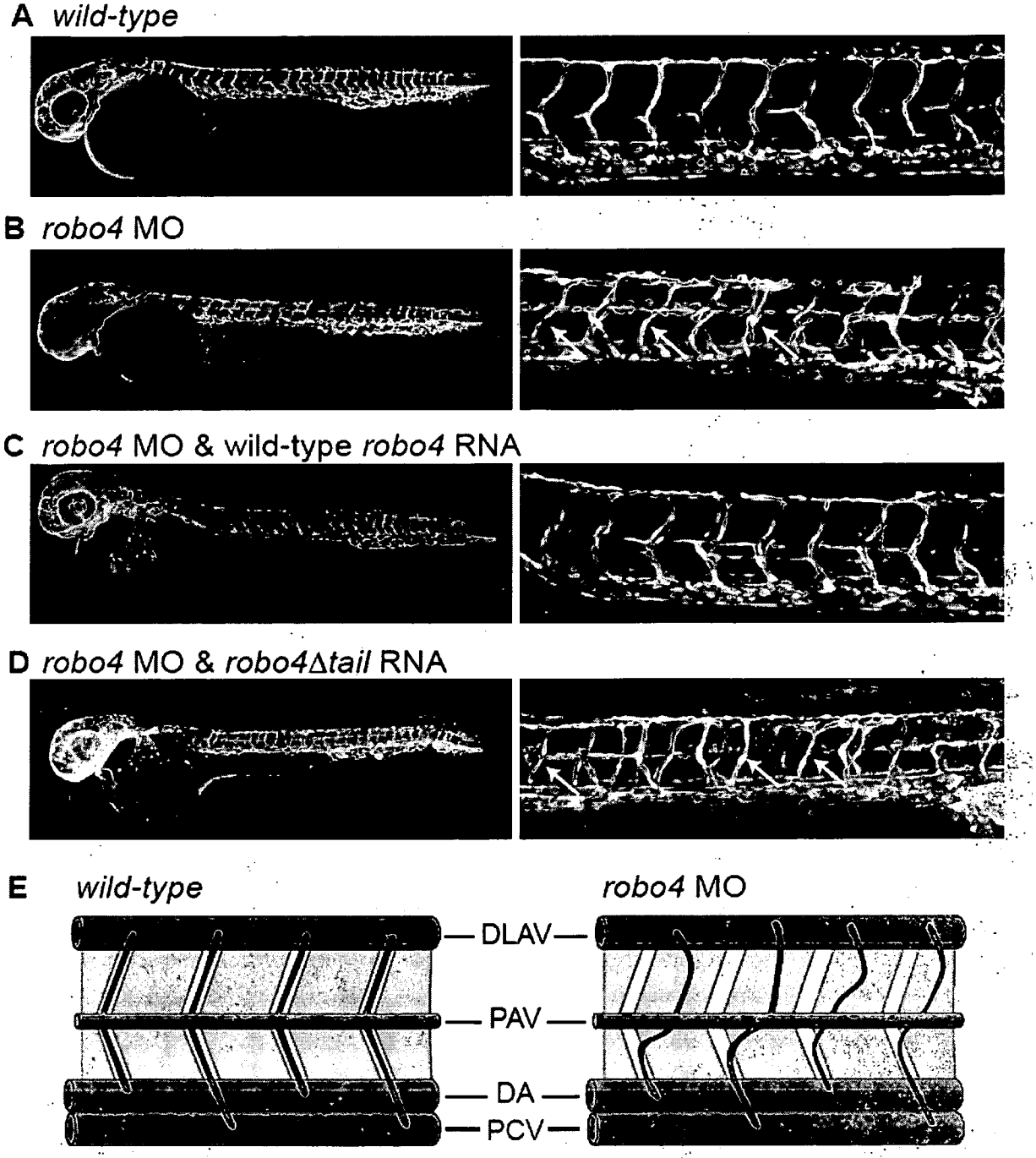

A wild-type
B robo4 MO
C robo4 MO & wild-type robo4 RNA
D robo4 MO & robo4Δtail RNA
E wild-type
robo4 MO
DLAV
PAV
DA
PCV

FIG. 1

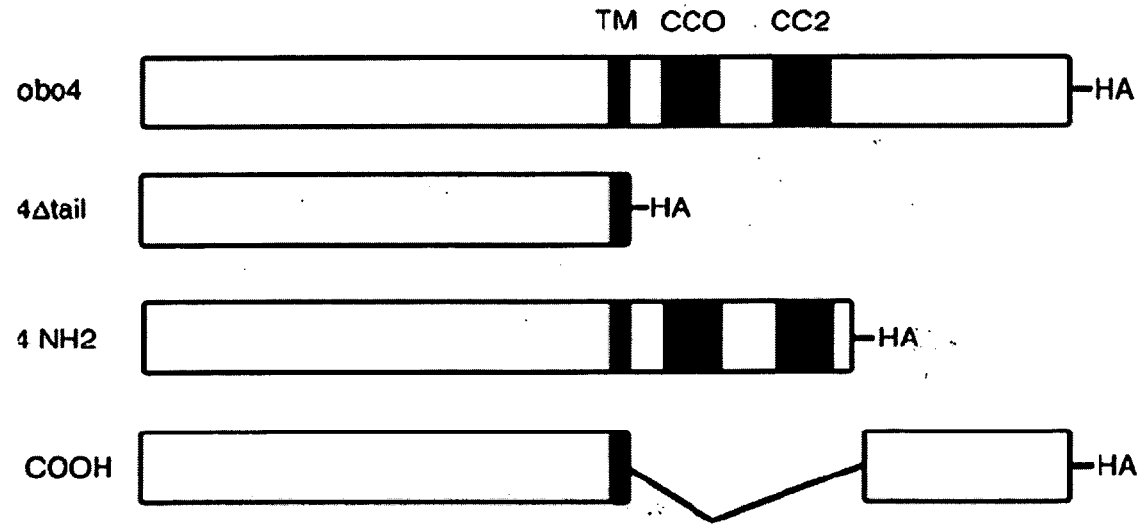

TM CCO CC2
obo4
HA
4Δtail
HA
4 NH2
HA
COOH
HA

FIG. 2A


B
Robo4
Robo4ΔTail
Blot: Robo4

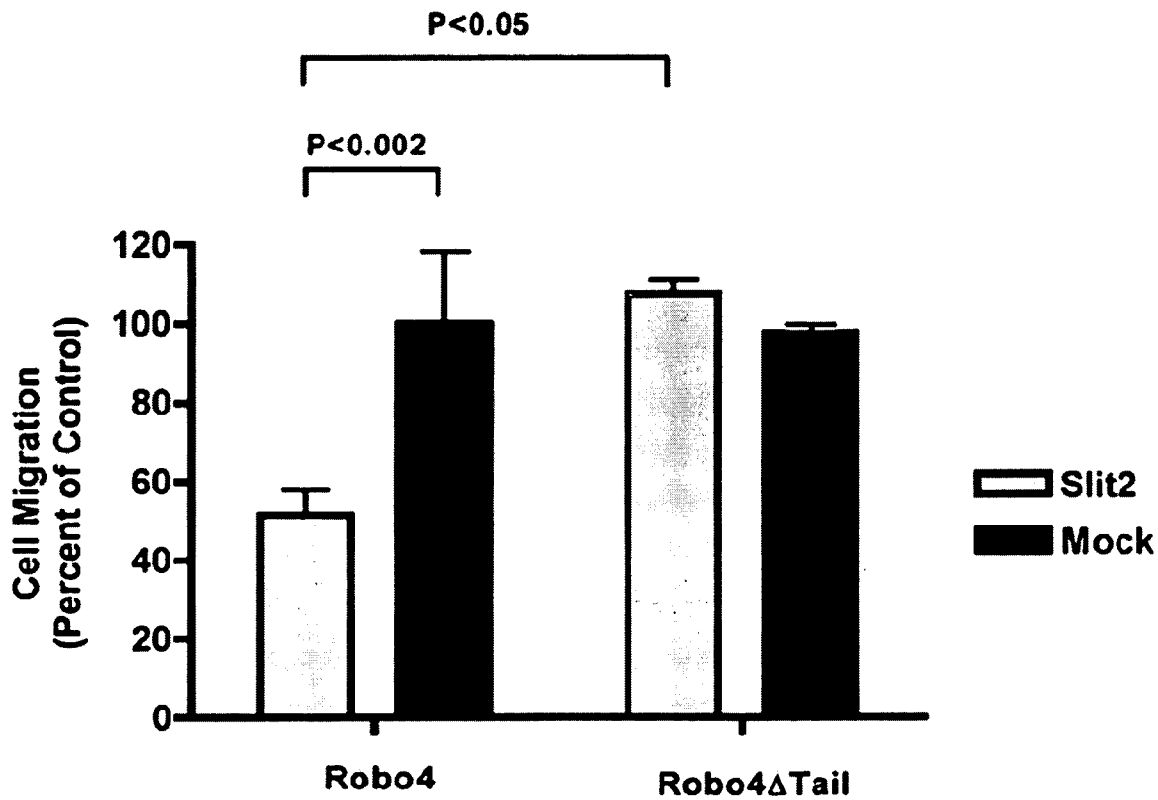
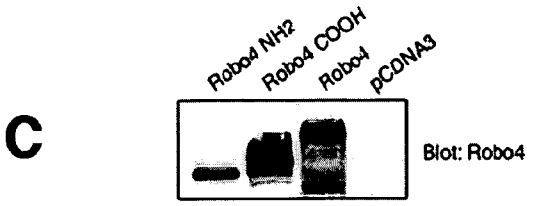

P<0.05
P<0.002
Cell Migration (Percent of Control)
120
100
80
60
40
20
0
Robo4
Robo4ΔTail
Slit2
Mock

FIG. 2B


C
Robo4 NH2
Robo4 COOH
Robo4
pCDNA3
Blot: Robo4

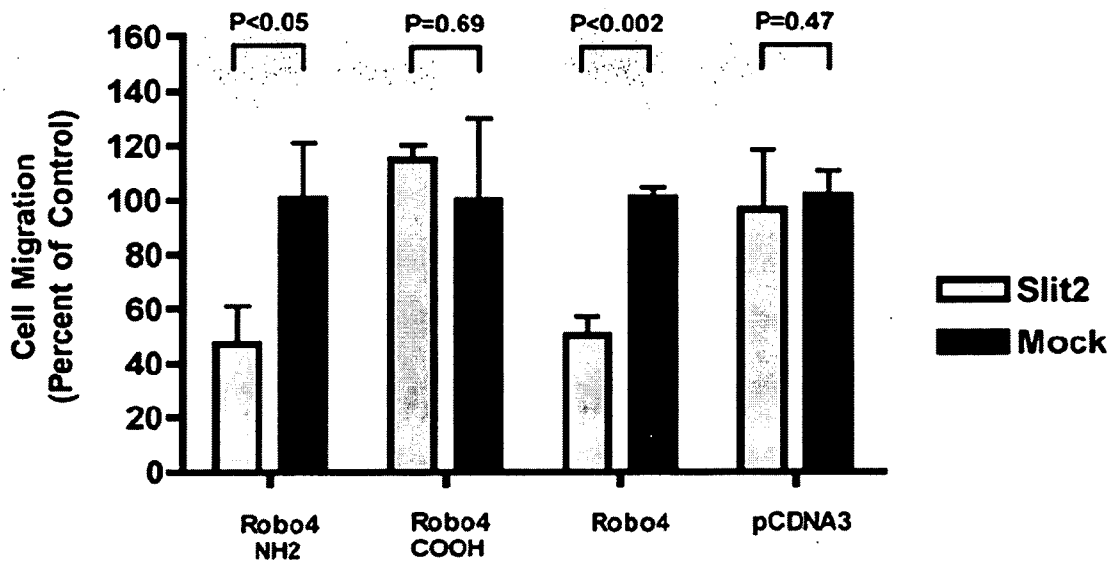

P<0.05
P=0.69
P<0.002
P=0.47
Cell Migration (Percent of Control)
160
140
120
100
80
60
40
20
0
Robo4 NH2
Robo4 COOH
Robo4
pCDNA3
Slit2
Mock

FIG. 2C

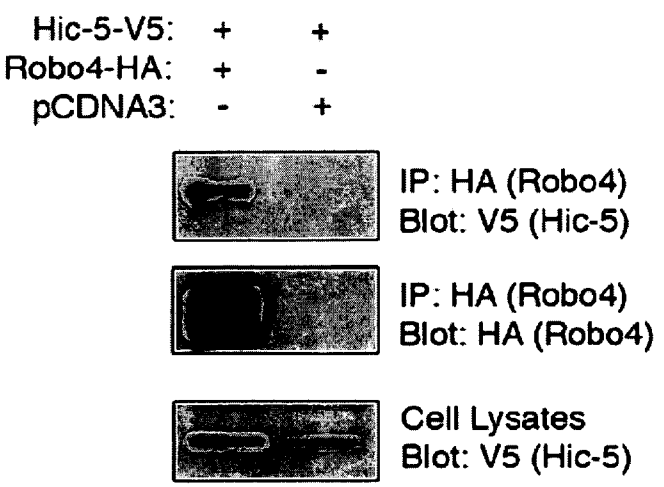

Hic-5-V5: + +
Robo4-HA: + -
pCDNA3: - +
IP: HA (Robo4)
Blot: V5 (Hic-5)
IP: HA (Robo4)
Blot: HA (Robo4)
Cell Lysates
Blot: V5 (Hic-5)

FIG. 3A

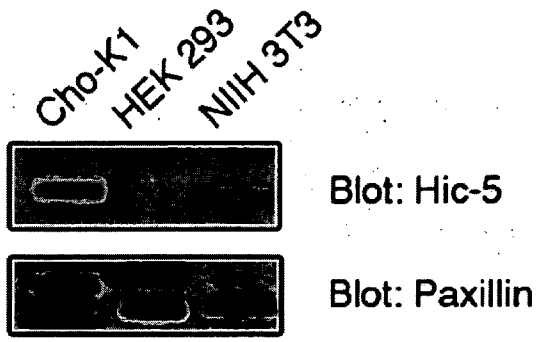


FIG. 3B

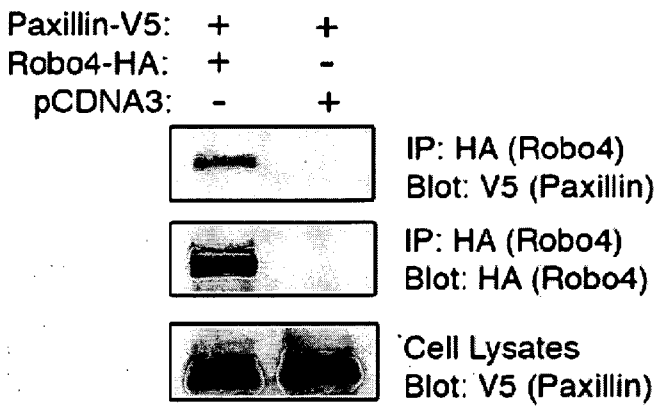


FIG. 3C

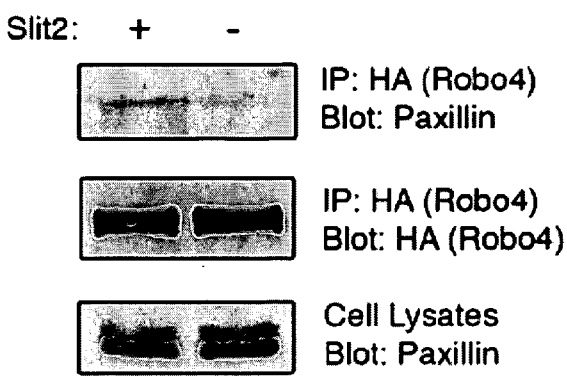


FIG. 3D

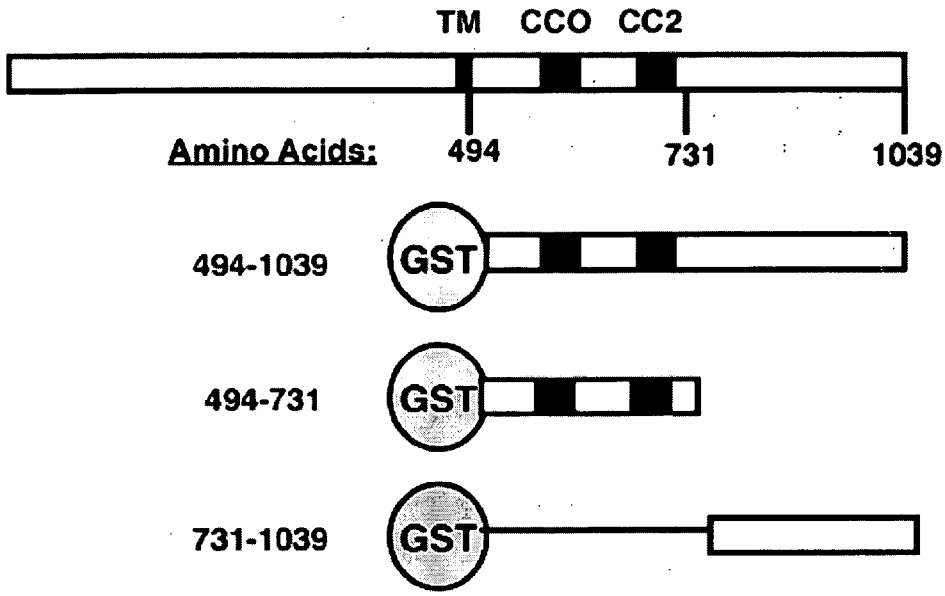

TM
CCO
CC2
Amino Acids:
494
731
1039
494-1039
GST
494-731
GST
731-1039
GST

FIG. 4A

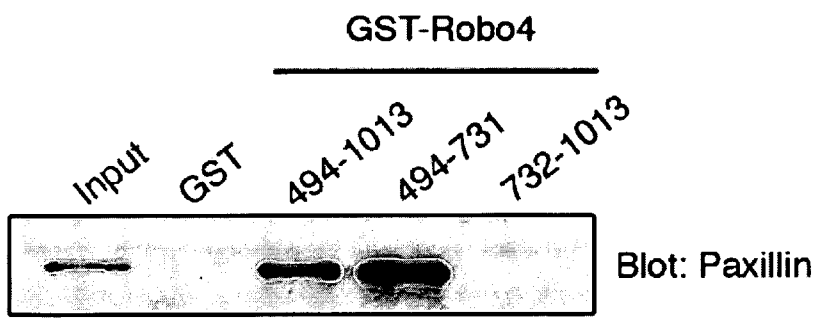

GST-Robo4
Input
GST
494-1013
494-731
732-1013
Blot: Paxillin

FIG. 4B

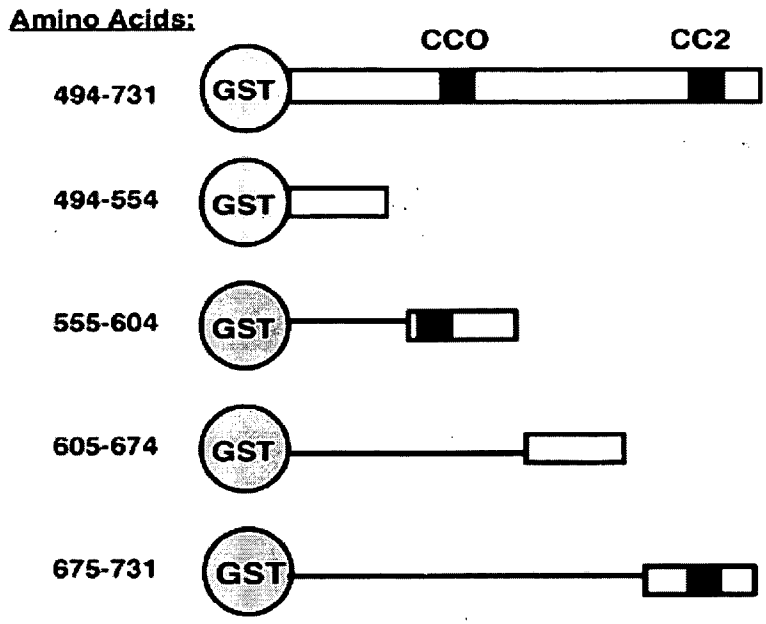

Amino Acids:
CCO
CC2
494-731
GST
494-554
GST
555-604
GST
605-674
GST
675-731
GST

FIG. 4C

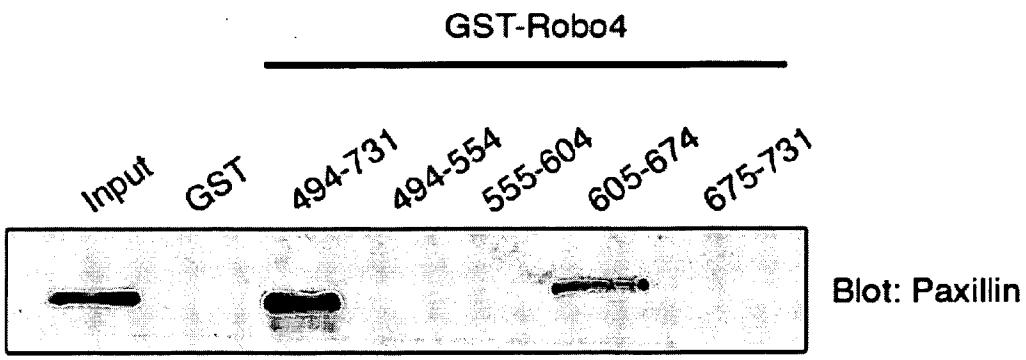

GST-Robo4
Input
GST
494-731
494-554
555-604
605-674
675-731
Blot: Paxillin

FIG. 4D

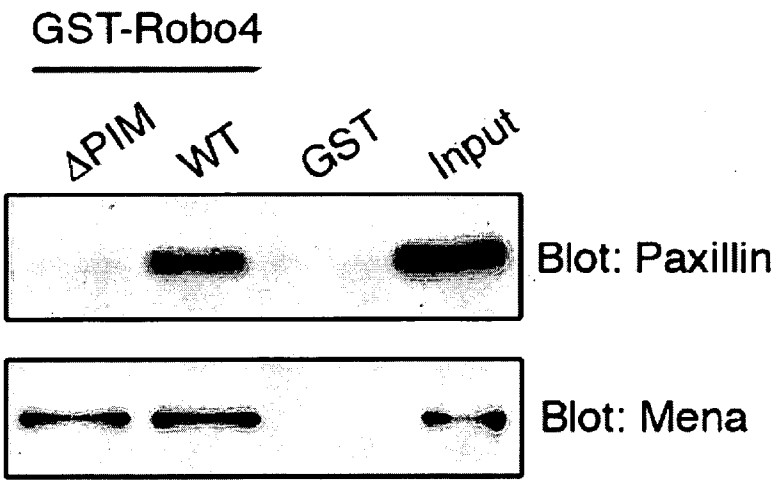
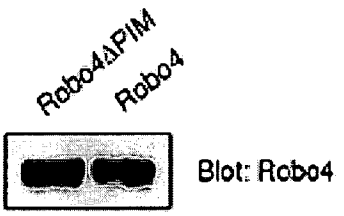


FIG. 4E

F



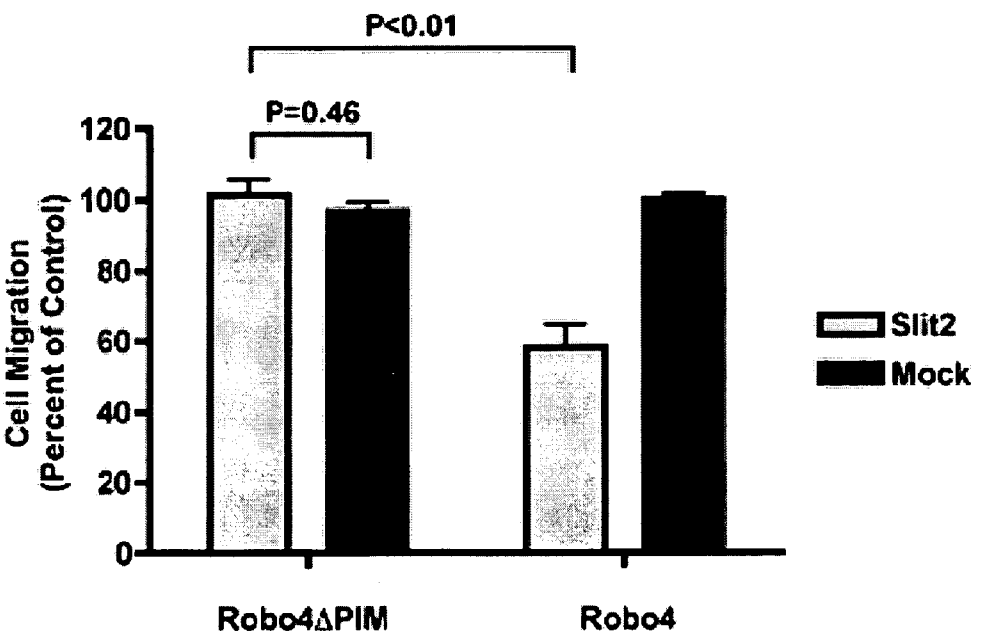


FIG. 4F

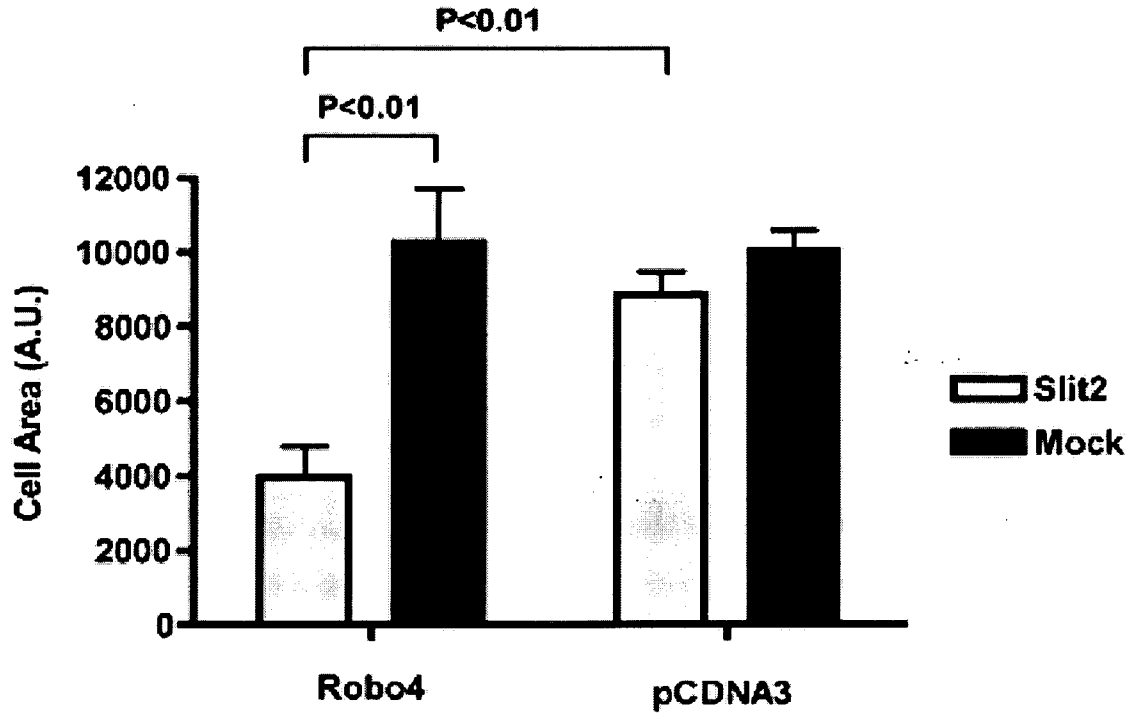

P<0.01
P<0.01
12000
10000
8000
6000
4000
2000
0
Cell Area (A.U.)
Slit2
Mock
Robo4
pCDNA3

FIG. 5A

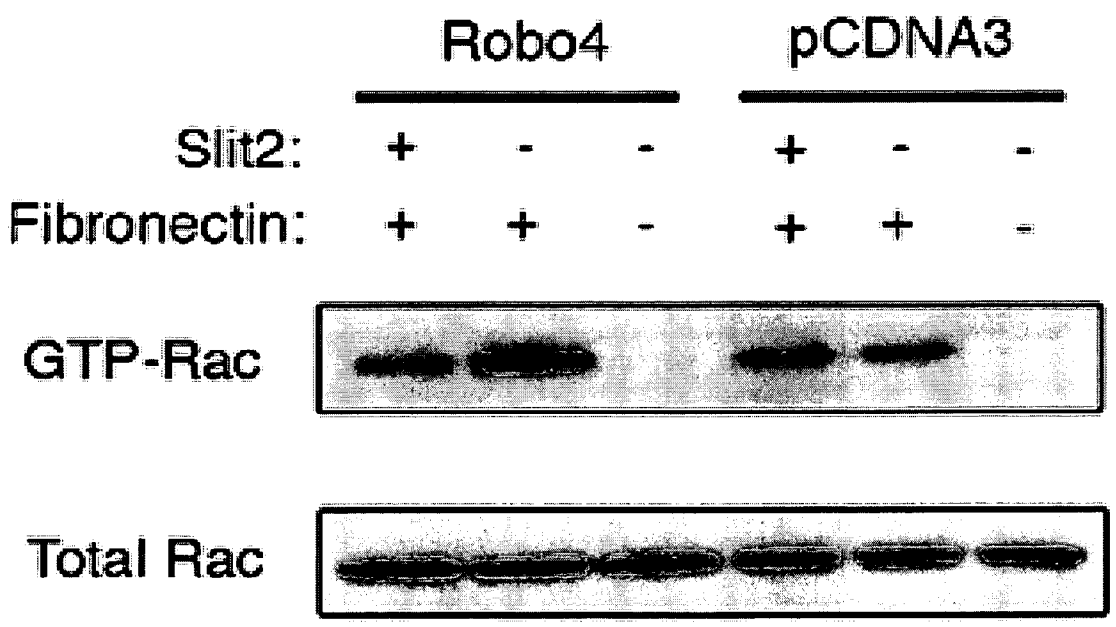

Robo4
pCDNA3
Slit2: + - - + - -
Fibronectin: + + - + + -
GTP-Rac
Total Rac

FIG. 5B

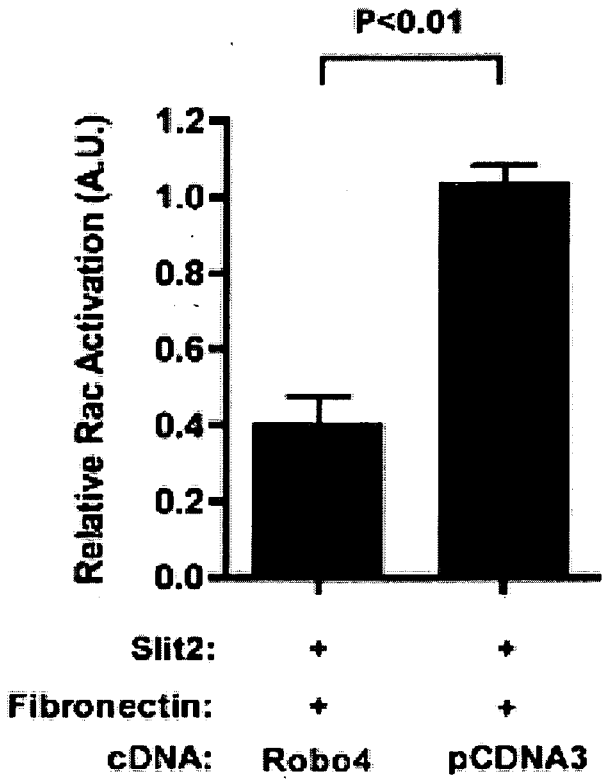

P<0.01
Relative Rac Activation (A.U.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Slit2: + +
Fibronectin: + +
cDNA: Robo4 pCDNA3

FIG. 5C

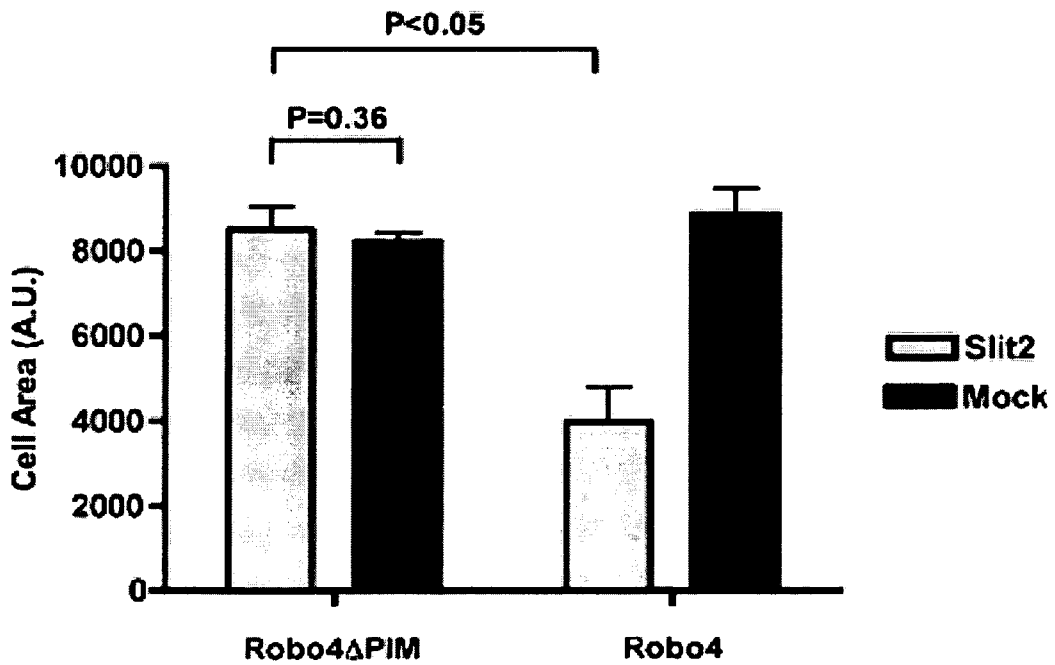

P<0.05
P=0.36
Cell Area (A.U.)
10000
8000
6000
4000
2000
0
Robo4ΔPIM
Robo4
Slit2
Mock

FIG. 5D

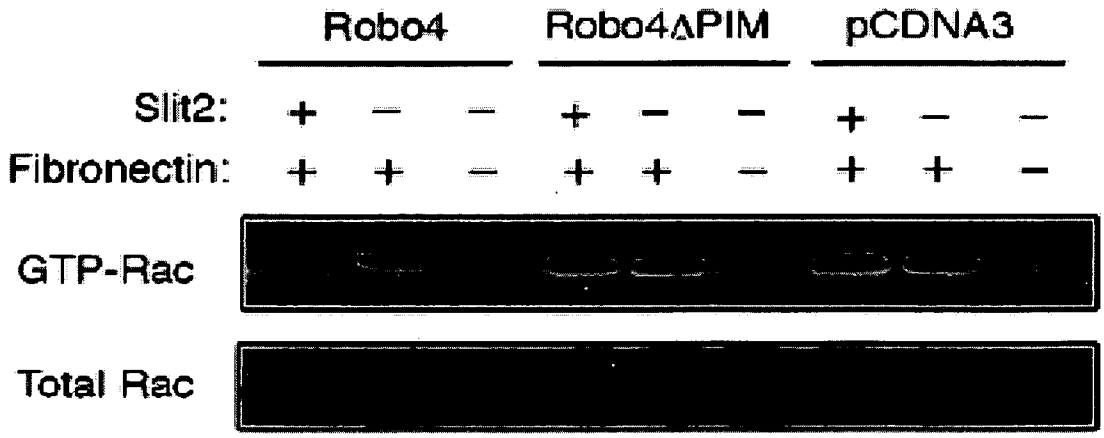

Robo4
Robo4ΔPIM
pCDNA3
Slit2:
Fibronectin:
GTP-Rac
Total Rac

FIG. 5E

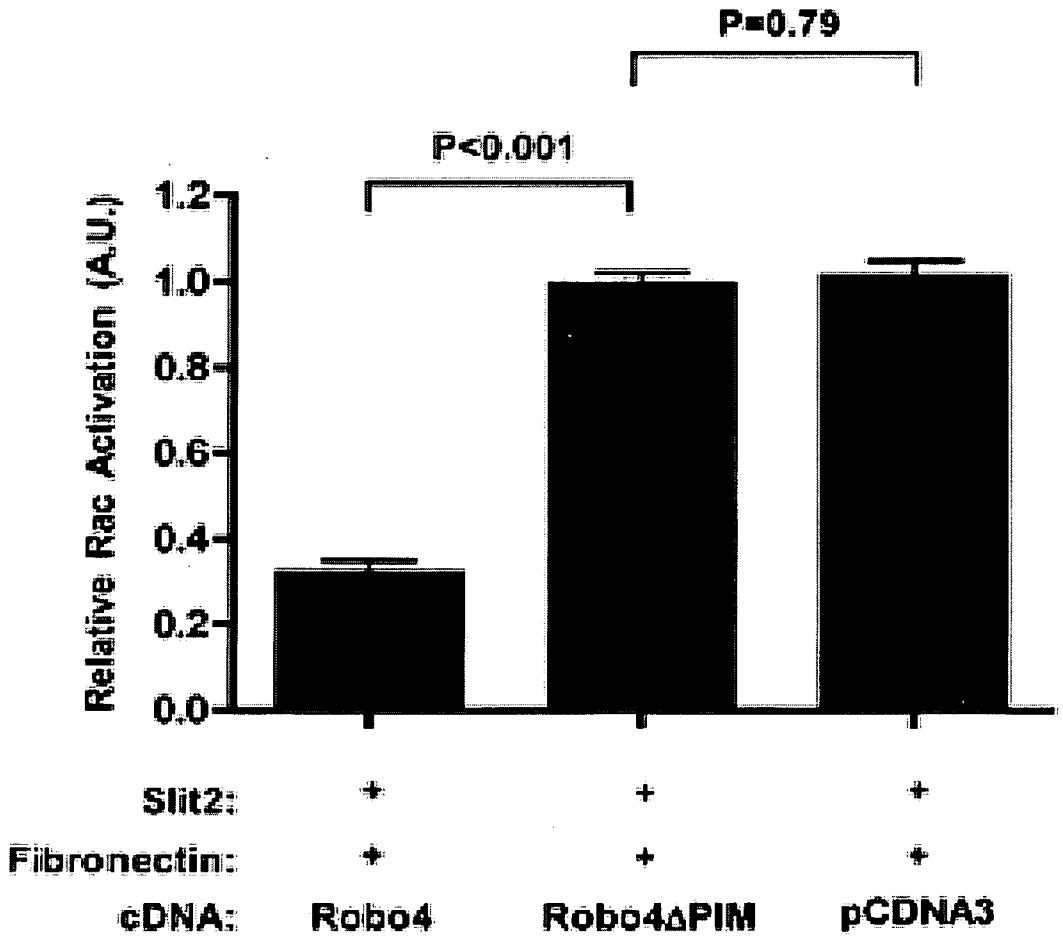

P=0.79
P<0.001
Relative Rac Activation (A.U.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Slit2:
Fibronectin:
cDNA:
Robo4
Robo4ΔPIM
pCDNA3

FIG. 5F

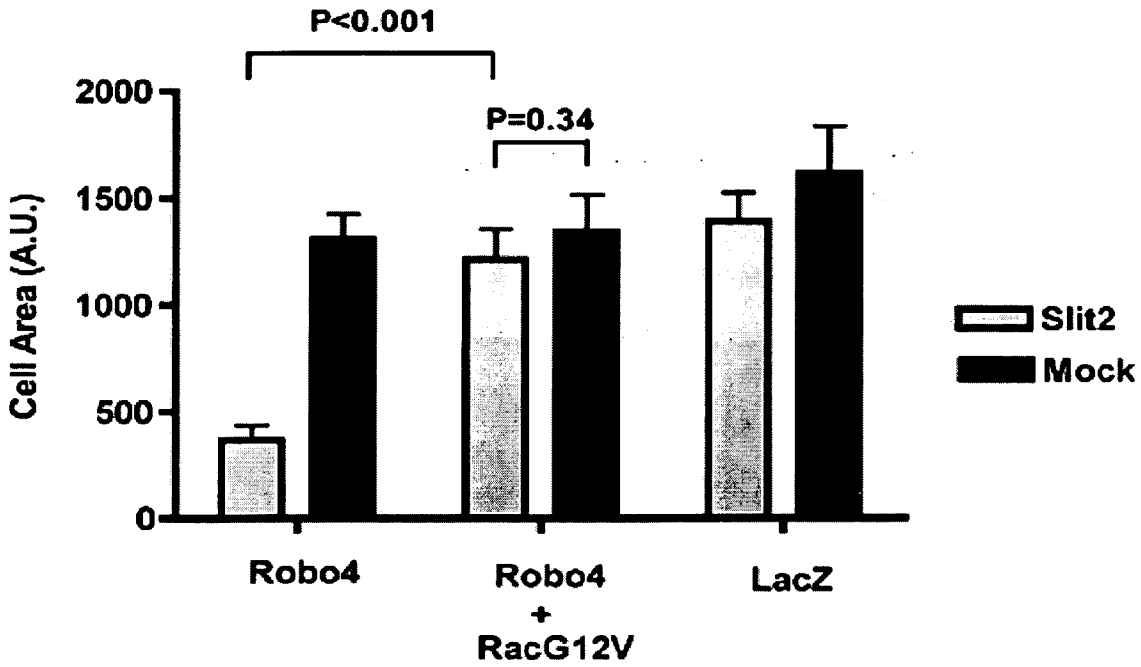

P<0.001
P=0.34
2000
1500
1000
500
0
Cell Area (A.U.)
Slit2
Mock
Robo4
Robo4
+
RacG12V
LacZ

FIG. 5G

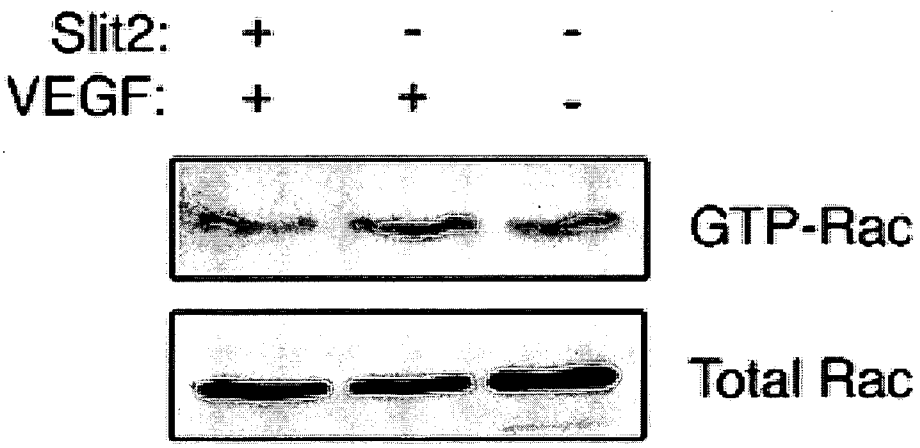

Slit2: + - -
VEGF: + + -
GTP-Rac
Total Rac

FIG. 5H

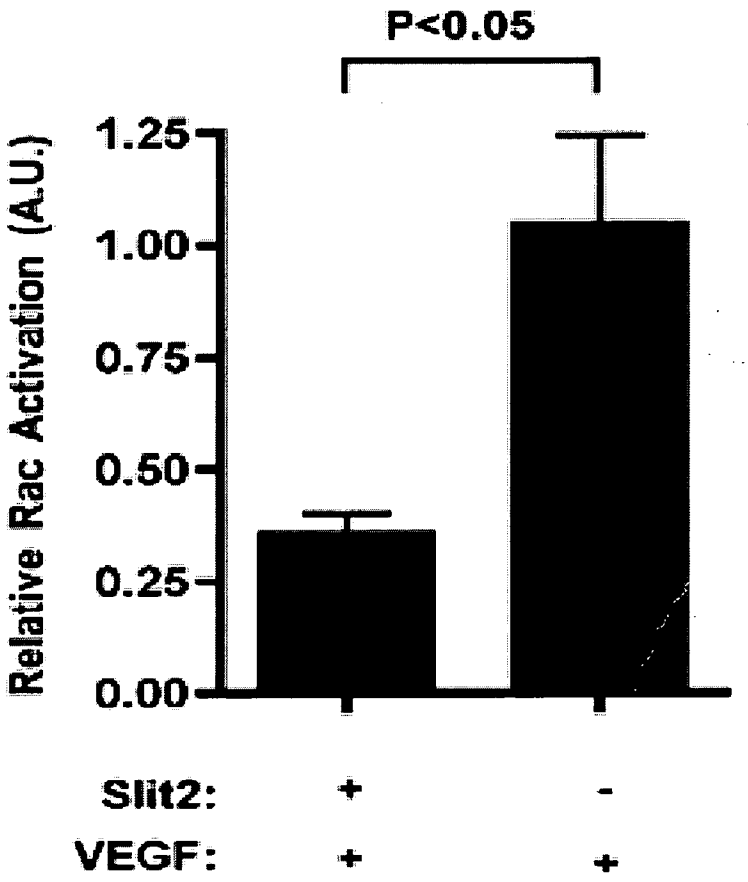

P<0.05
Relative Rac Activation (A.U.)
1.25
1.00
0.75
0.50
0.25
0.00
Slit2: + -
VEGF: + +

FIG. 5I

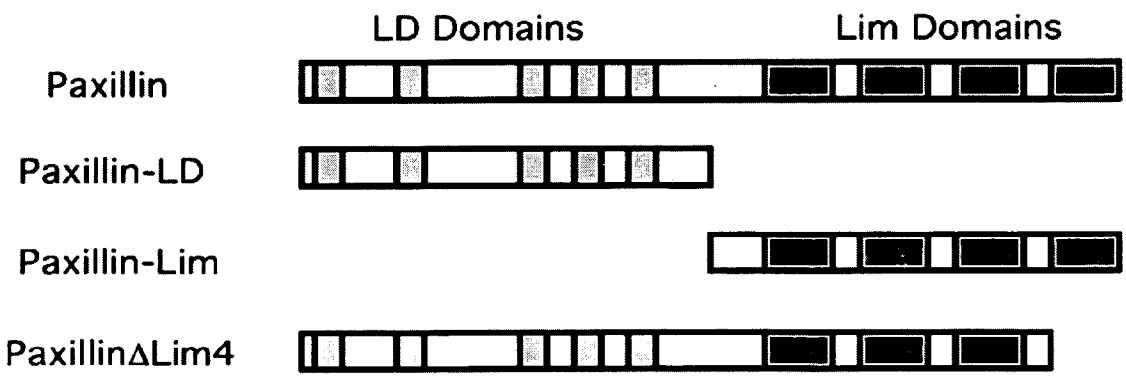

LD Domains
Lim Domains
Paxillin
Paxillin-LD
Paxillin-Lim
PaxillinΔLim4

FIG. 6A

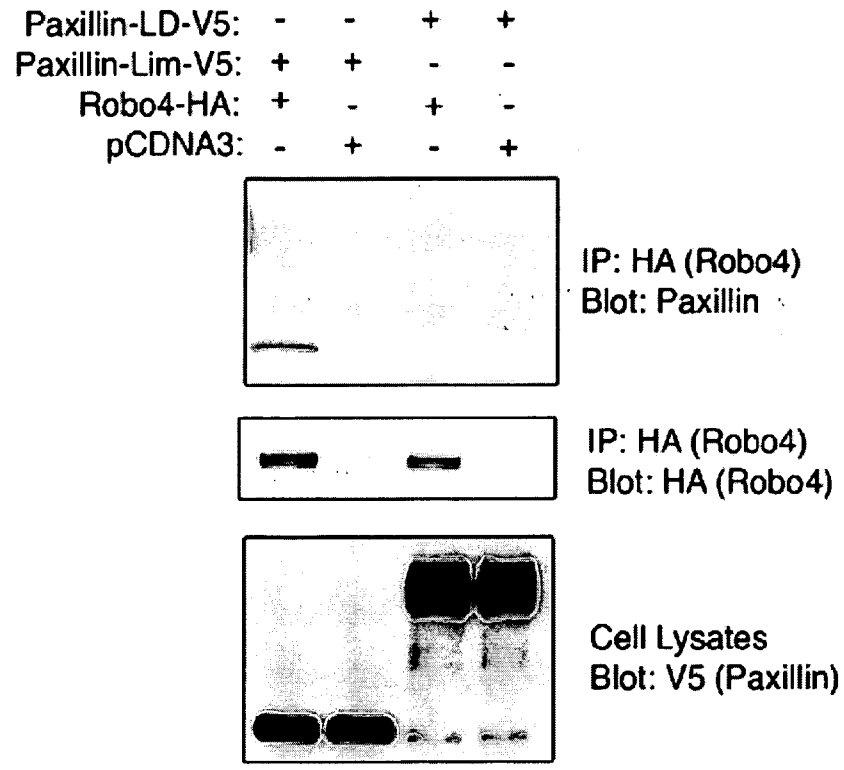

Paxillin-LD-V5: - - + +
Paxillin-Lim-V5: + + - -
Robo4-HA: + - + -
pCDNA3: - + - +
IP: HA (Robo4)
Blot: Paxillin
IP: HA (Robo4)
Blot: HA (Robo4)
Cell Lysates
Blot: V5 (Paxillin)

FIG. 6B

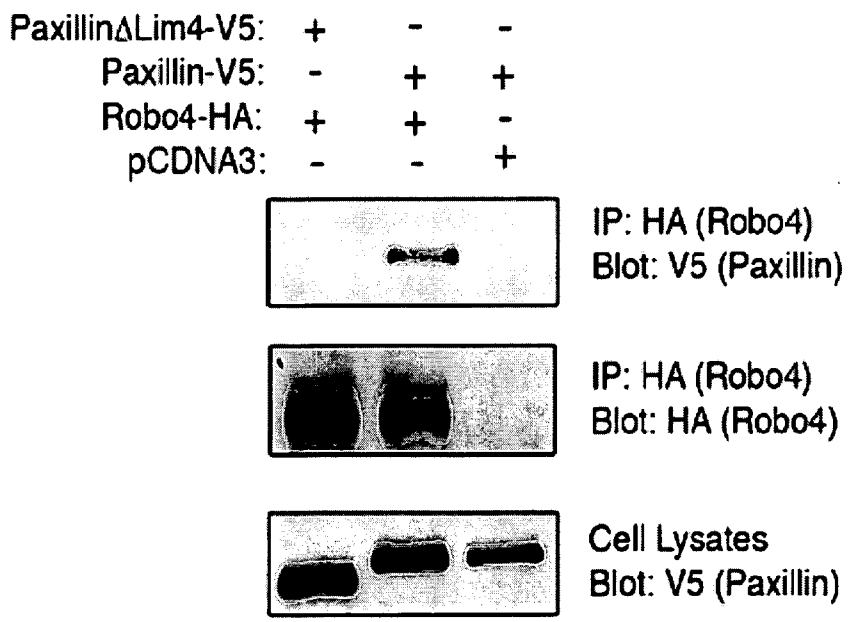

PaxillinΔLim4-V5: + - -
Paxillin-V5: - + +
Robo4-HA: + + -
pCDNA3: - - +
IP: HA (Robo4)
Blot: V5 (Paxillin)
IP: HA (Robo4)
Blot: HA (Robo4)
Cell Lysates
Blot: V5 (Paxillin)

FIG. 6C

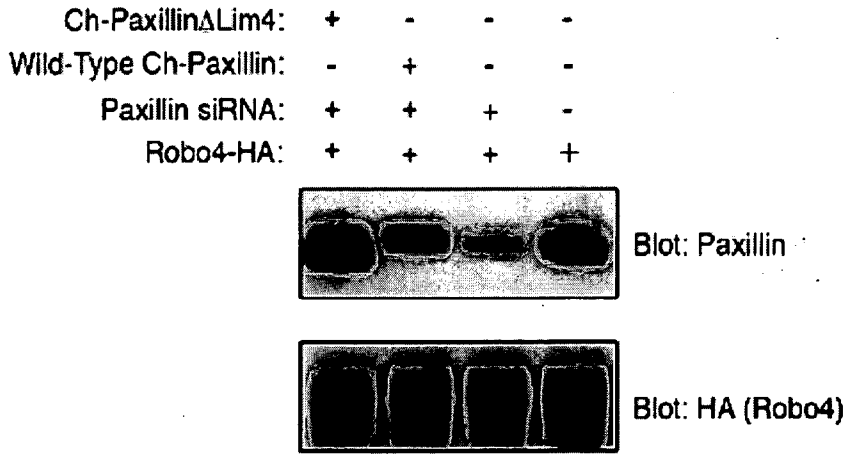

Ch-PaxillinΔLim4: + - - -
Wild-Type Ch-Paxillin: - + - -
Paxillin siRNA: + + + -
Robo4-HA: + + + +
Blot: Paxillin
Blot: HA (Robo4)

FIG. 6D

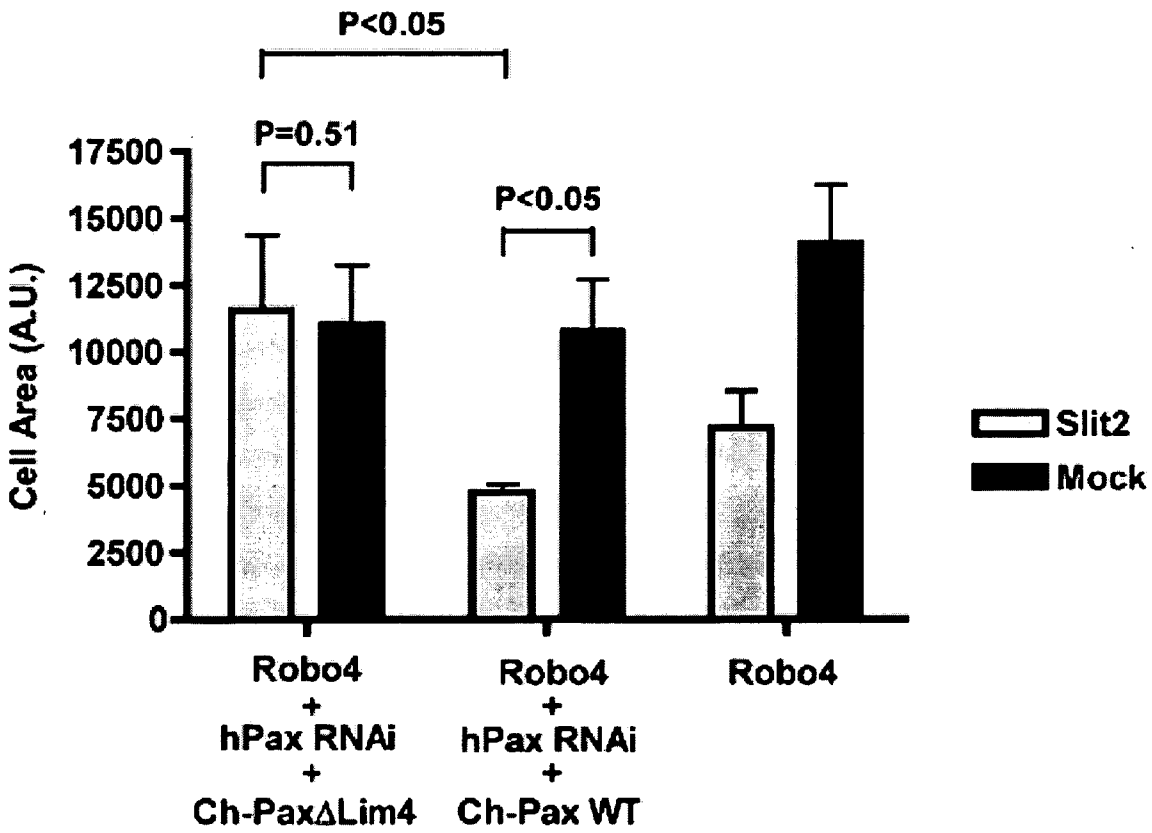

P<0.05
P=0.51
P<0.05
17500
15000
12500
10000
7500
5000
2500
0
Cell Area (A.U.)
Slit2
Mock
Robo4 + hPax RNAi + Ch-PaxΔLim4
Robo4 + hPax RNAi + Ch-Pax WT
Robo4

FIG. 6E

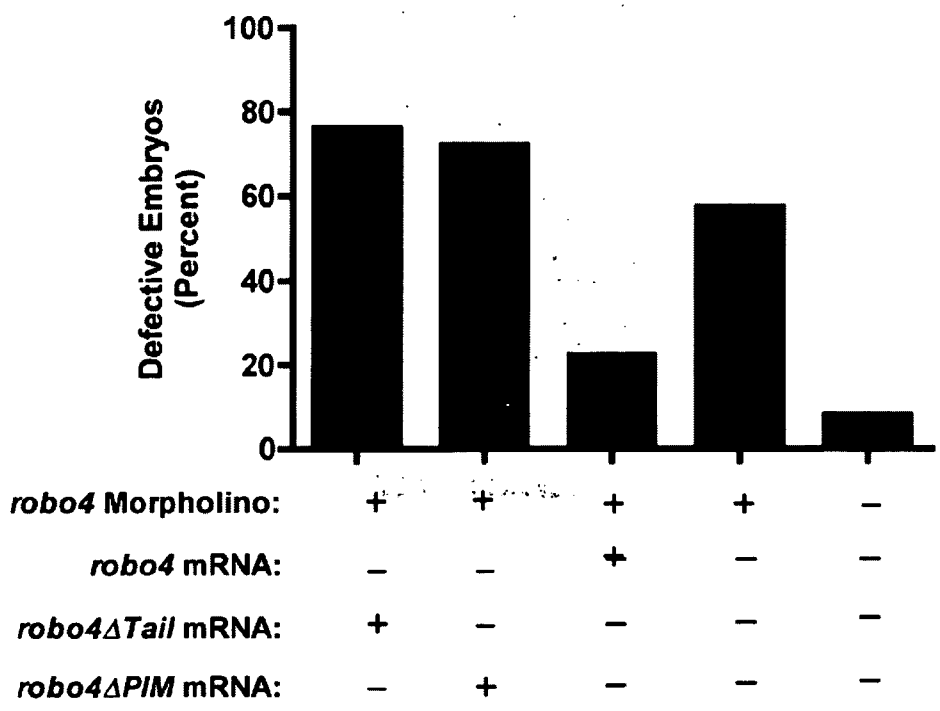

Defective Embryos (Percent)
100
80
60
40
20
0
robo4 Morpholino: + + + + –
robo4 mRNA: – – + – –
robo4ΔTail mRNA: + – – – –
robo4ΔPIM mRNA: – + – – –

FIG. 7A

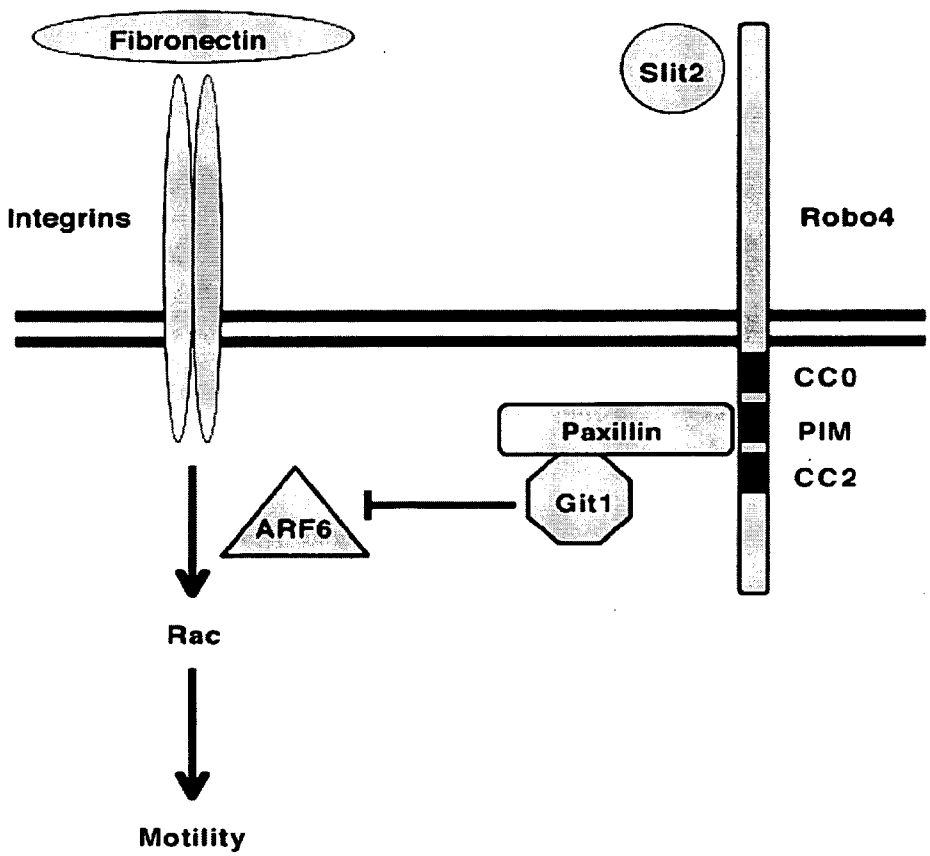

Fibronectin
Slit2
Integrins
Robo4
CC0
Paxillin
PIM
CC2
Git1
ARF6
Rac
Motility

FIG. 7B

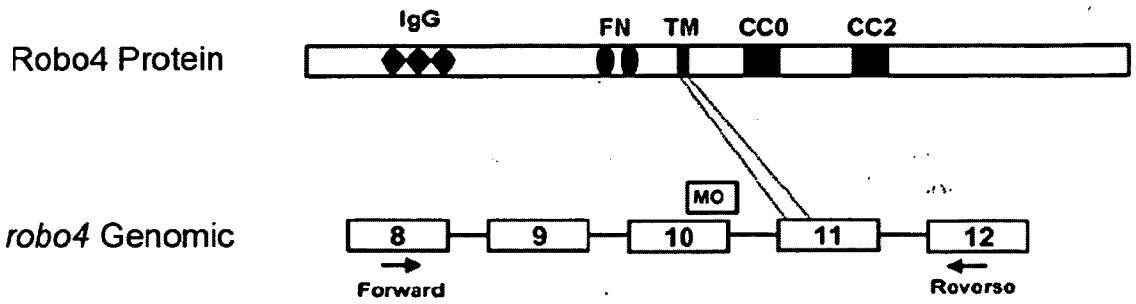

Robo4 Protein
IgG
FN
TM
CC0
CC2
robo4 Genomic
MO
8
9
10
11
12
Forward
Reverse

FIG. 8A

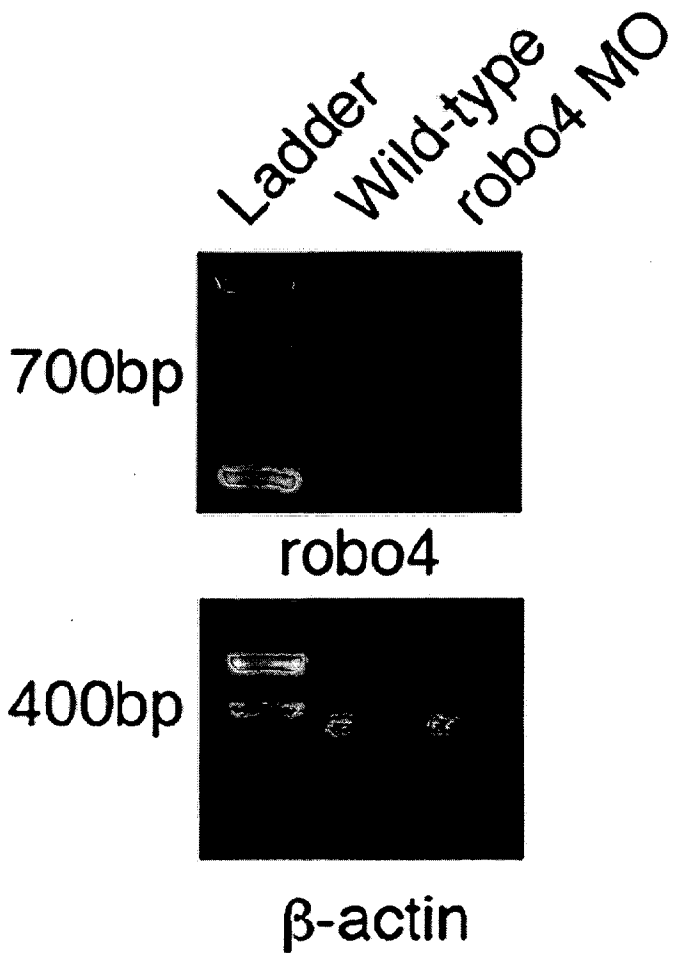

Ladder
Wild-type
robo4 MO
700bp
robo4
400bp
β-actin

FIG. 8B

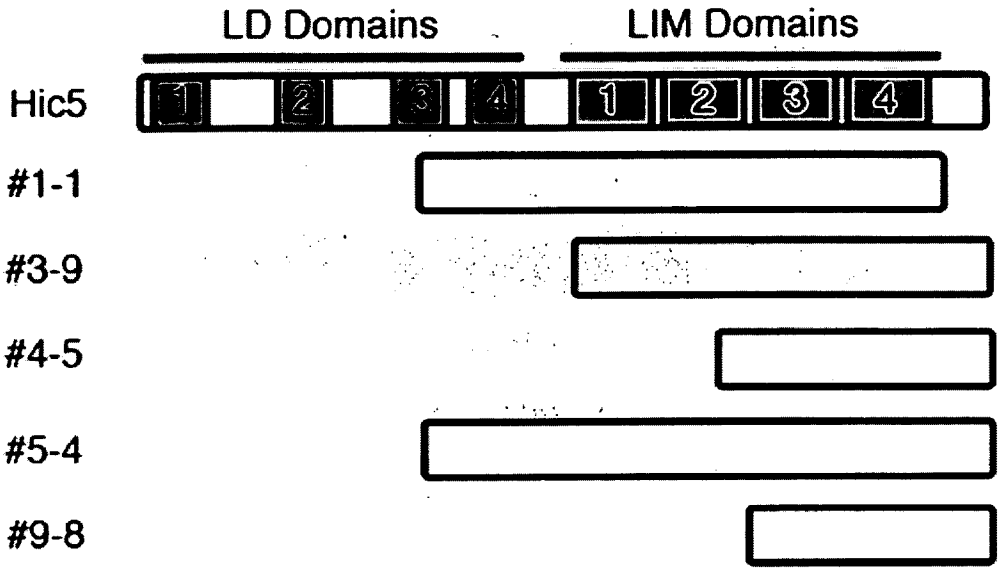


FIG. 9A

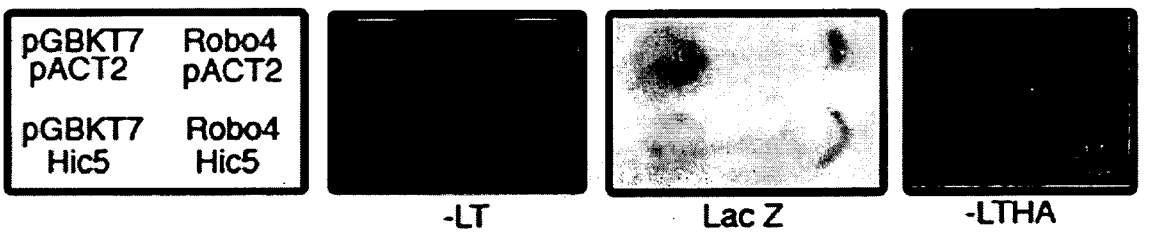


FIG. 9B

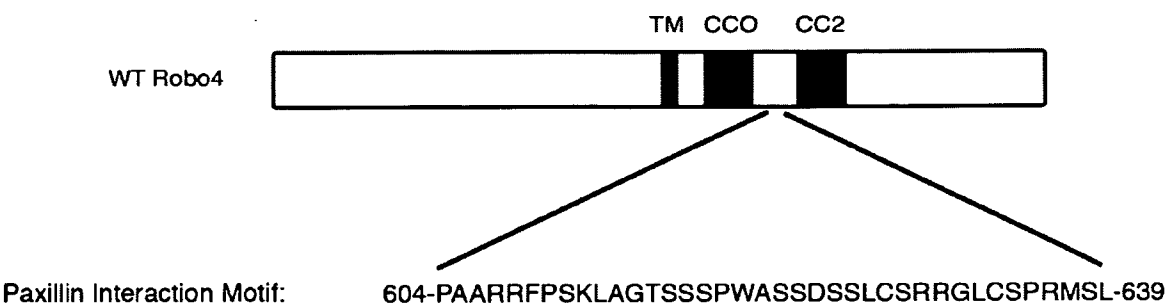


FIG. 10

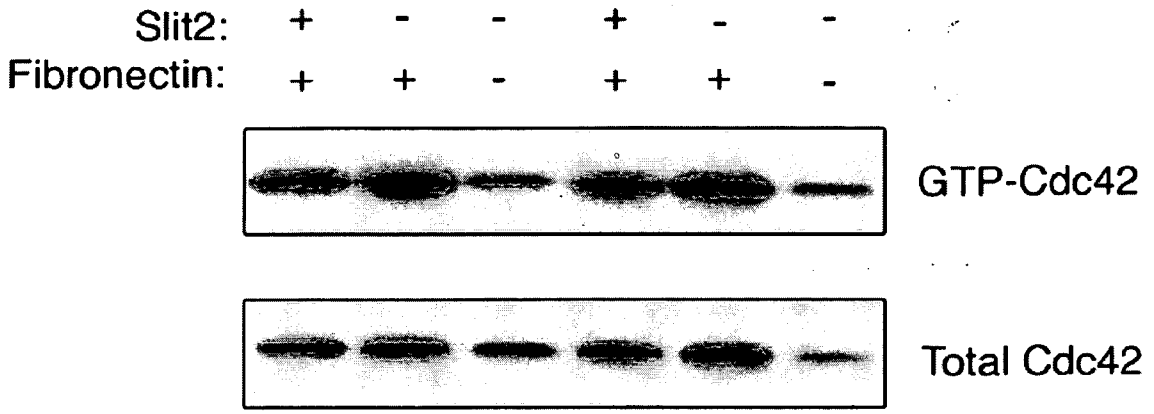


FIG. 11A

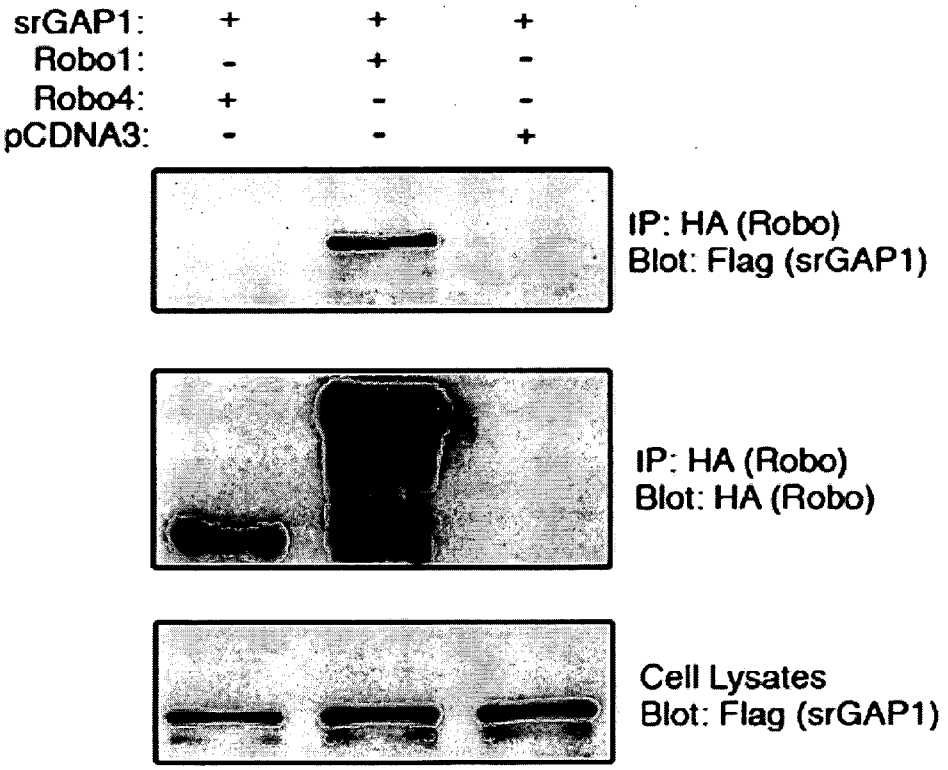


FIG. 11B

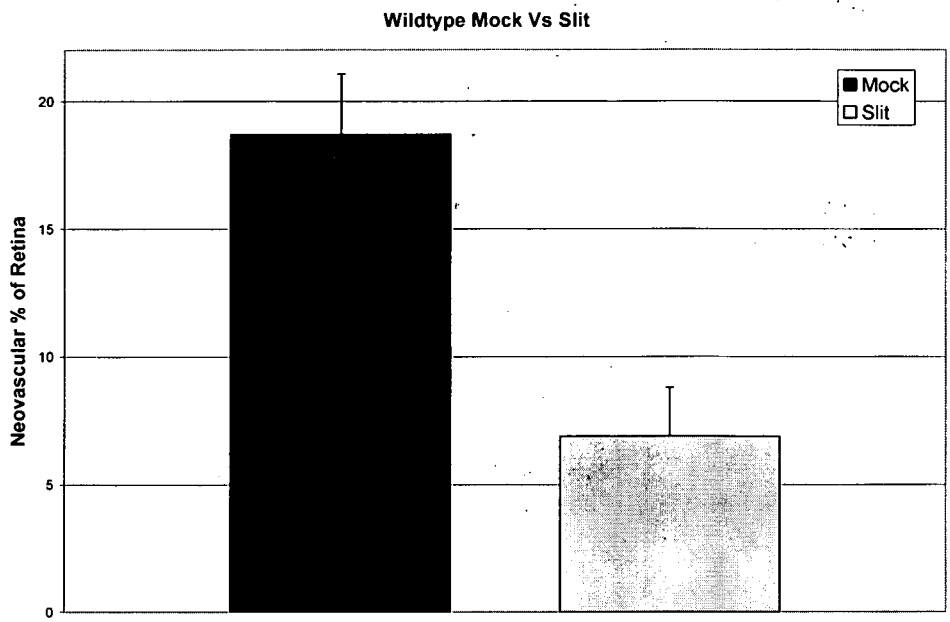


FIG. 12A

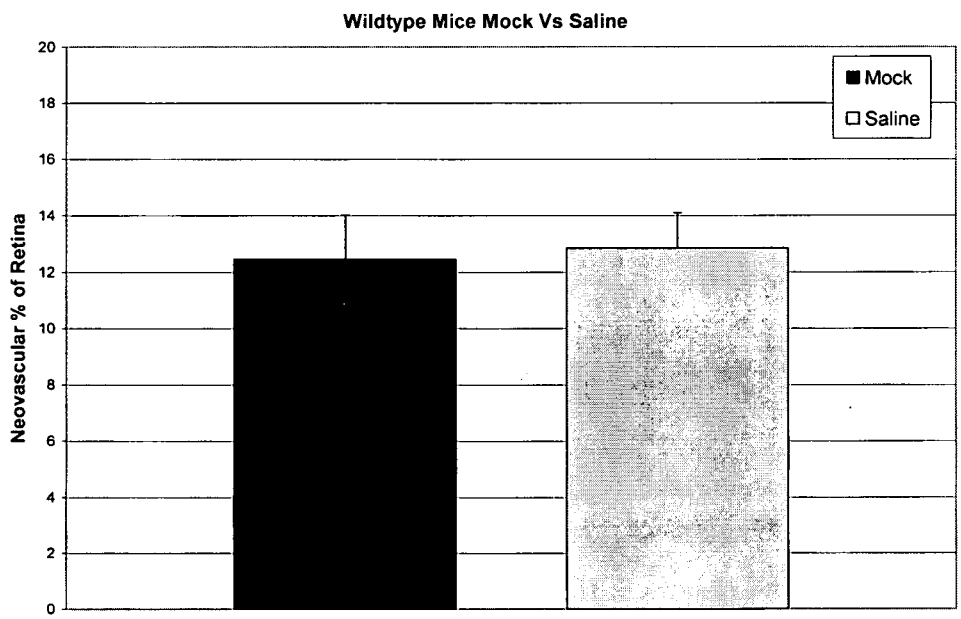


FIG. 12B

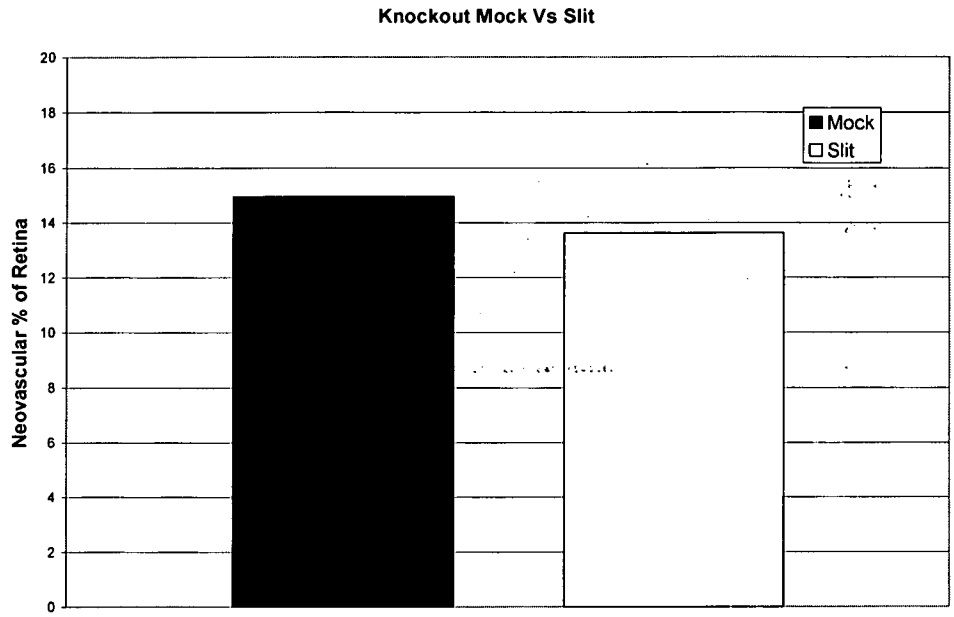


FIG. 12C

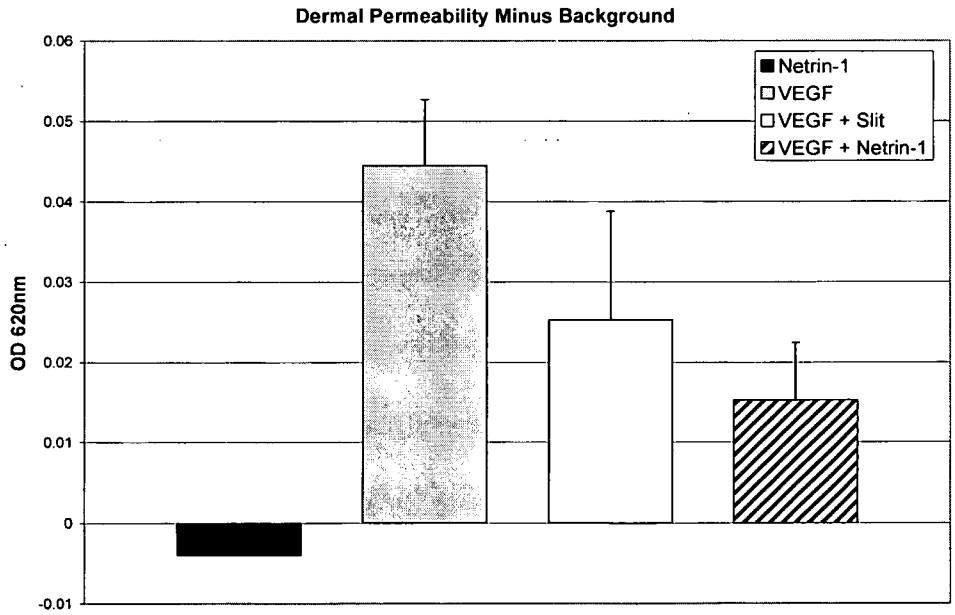


FIG. 13

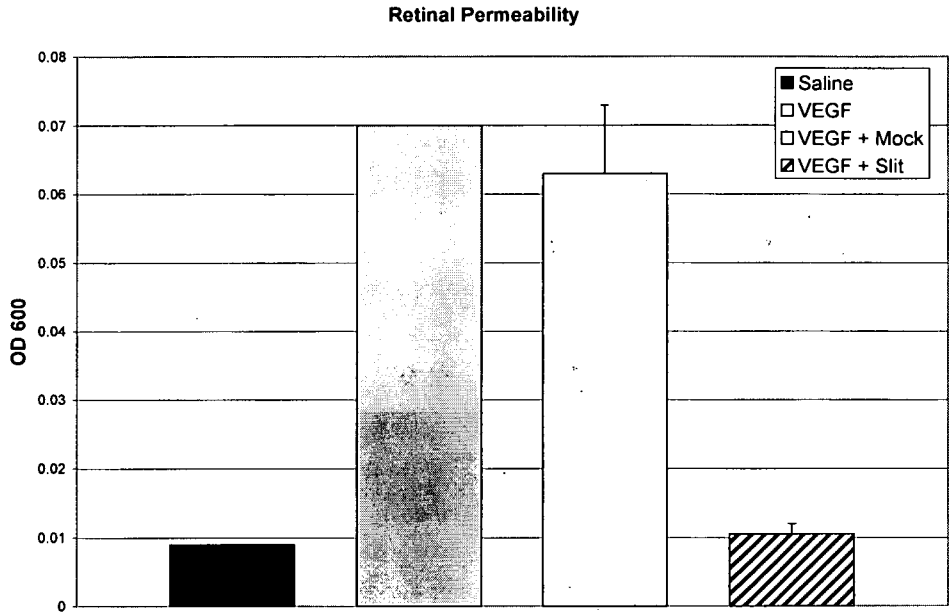

Retinal Permeability
OD 600
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Saline
VEGF
VEGF + Mock
VEGF + Slit

FIG. 14

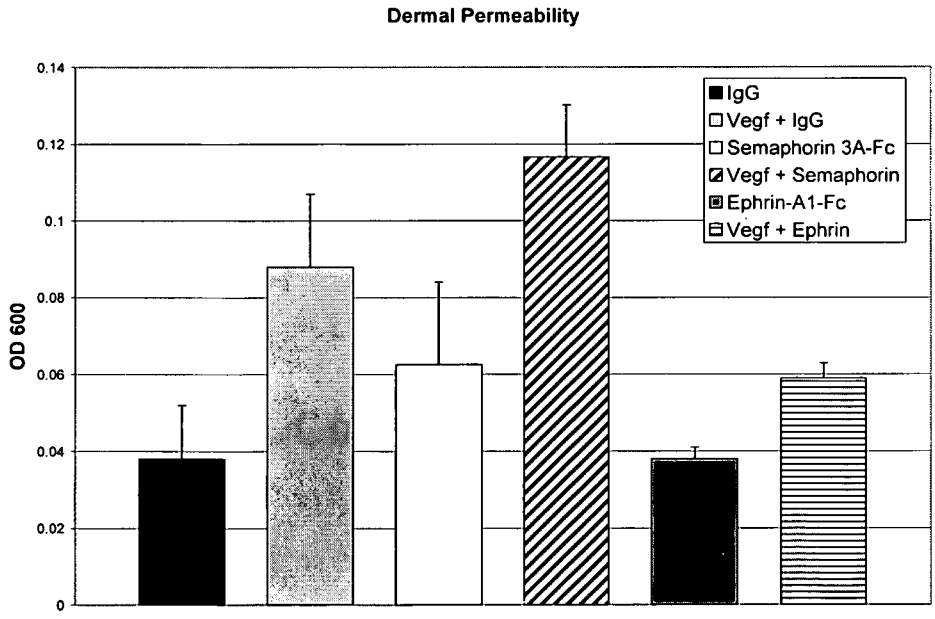

Dermal Permeability
OD 600
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
IgG
Vegf + IgG
Semaphorin 3A-Fc
Vegf + Semaphorin
Ephrin-A1-Fc
Vegf + Ephrin

FIG. 15

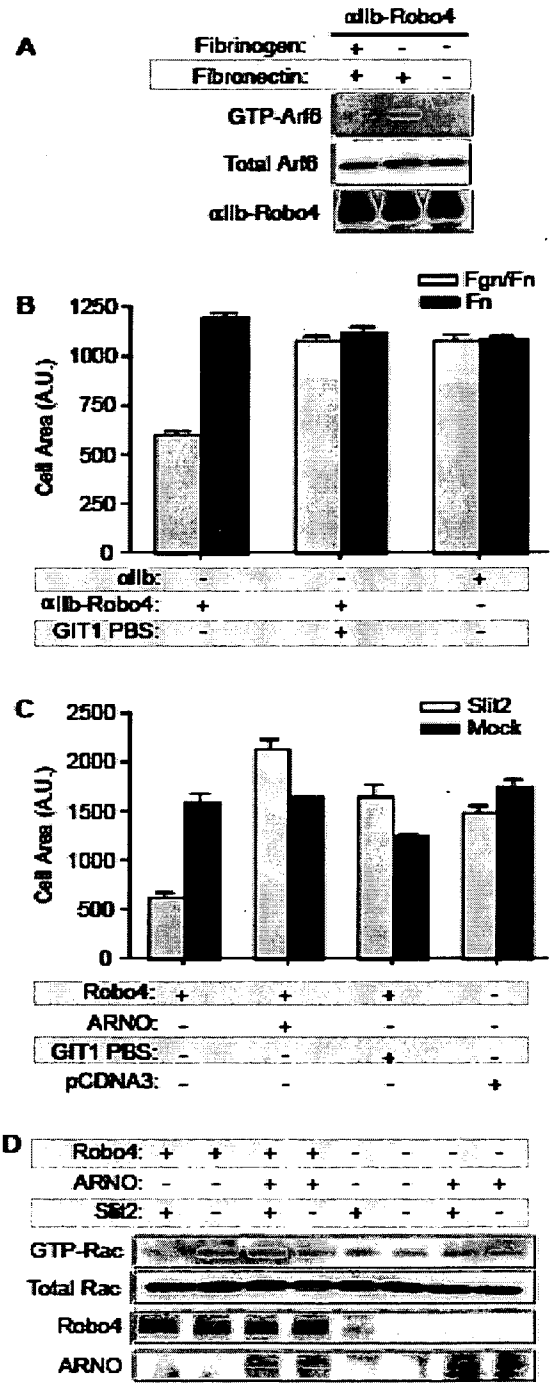

A
αIIb-Robo4
Fibrinogen: + - -
Fibronectin: + + -
GTP-Arf6
Total Arf6
αIIb-Robo4
B
Fgn/Fn
Fn
Cell Area (A.U.)
1250
1000
750
500
250
0
αIIb: - - +
αIIb-Robo4: + + -
GIT1 PBS: - + -
C
Slit2
Mock
Cell Area (A.U.)
2500
2000
1500
1000
500
0
Robo4: + + + -
ARNO: - + - -
GIT1 PBS: - - + -
pCDNA3: - - - +
D
Robo4: + + + + - - - -
ARNO: - - + + - - + +
Slit2: + - + - + - + -
GTP-Rac
Total Rac
Robo4
ARNO

FIG. 16

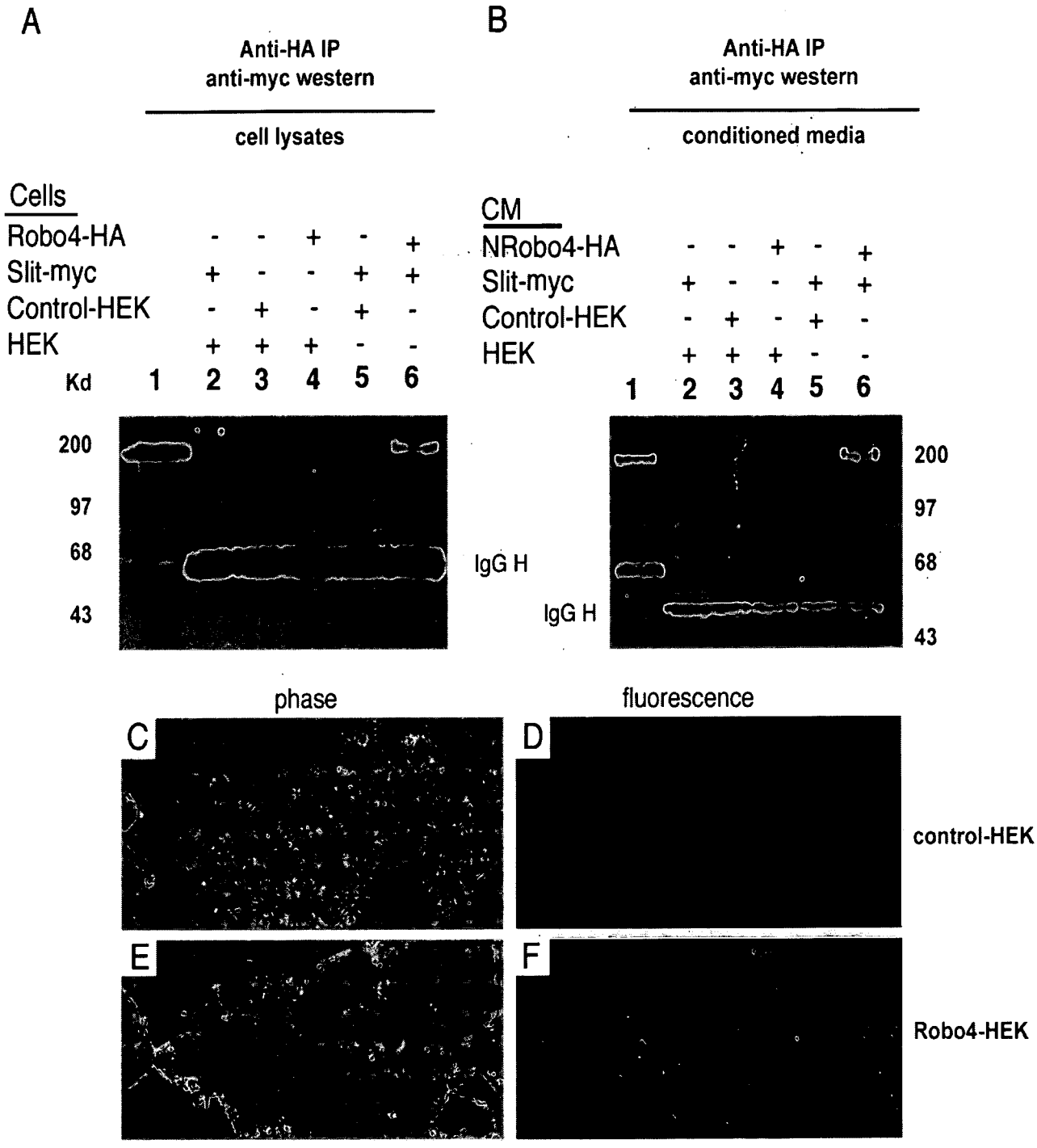

A
Anti-HA IP
anti-myc western
cell lysates
Cells
Robo4-HA - - + - +
Slit-myc + - - + +
Control-HEK - + - + -
HEK + + + - -
Kd 1 2 3 4 5 6
200
97
68
43
B
Anti-HA IP
anti-myc western
conditioned media
CM
NRobo4-HA - - + - +
Slit-myc + - - + +
Control-HEK - + - + -
HEK + + + - -
1 2 3 4 5 6
IgG H
IgG H
200
97
68
43
phase
fluorescence
C
D
control-HEK
E
F
Robo4-HEK

FIG. 17

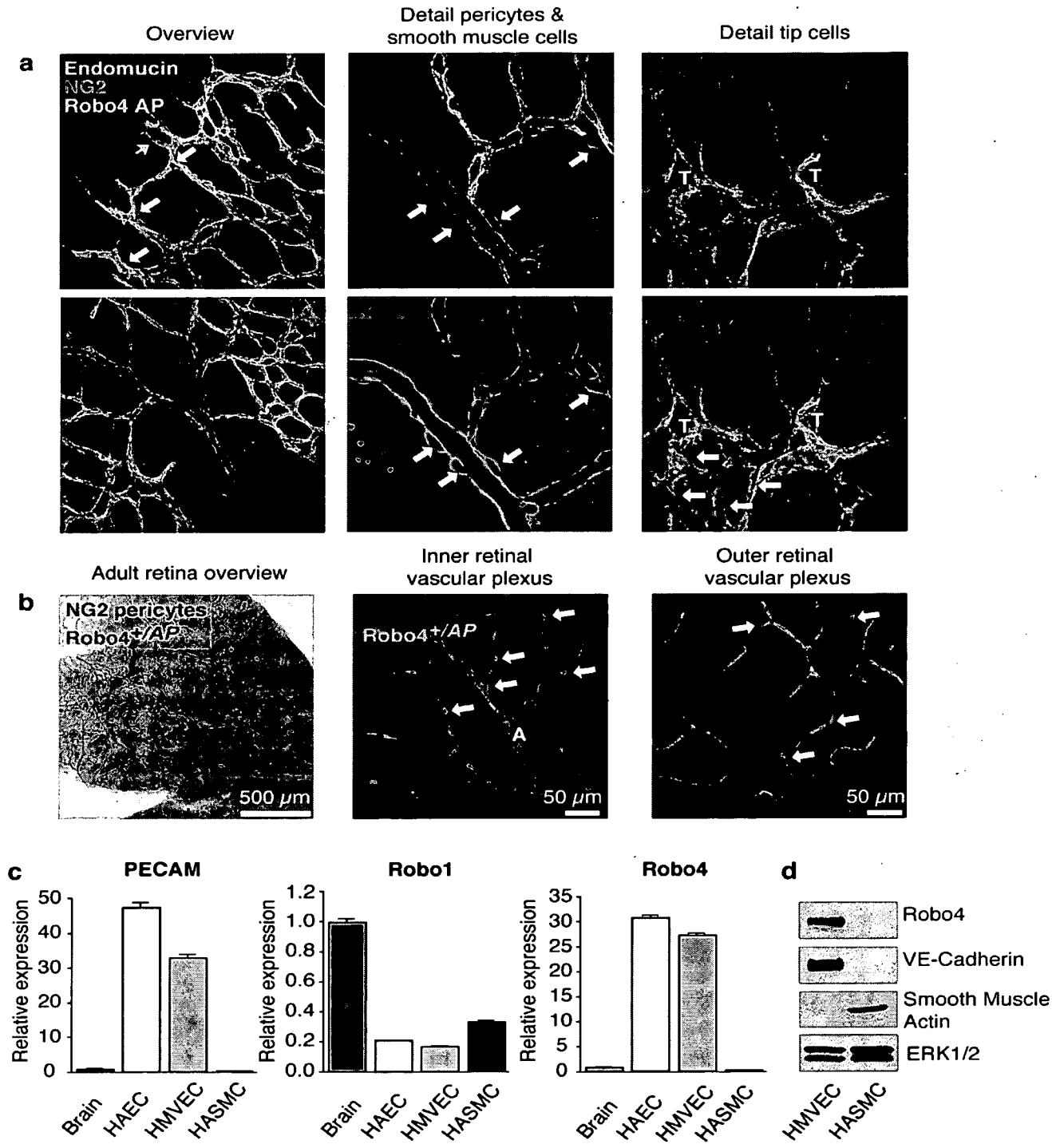

Overview
Detail pericytes & smooth muscle cells
Detail tip cells
a
Endomucin
NG2
Robo4 AP
T
T
T
T
Adult retina overview
Inner retinal vascular plexus
Outer retinal vascular plexus
b
NG2 pericytes
Robo4+/AP
Robo4+/AP
A
500 μm
50 μm
50 μm
c
PECAM
Robo1
Robo4
Relative expression
Brain
HAEC
HMVEC
HASMC
d
Robo4
VE-Cadherin
Smooth Muscle Actin
ERK1/2

FIG. 18

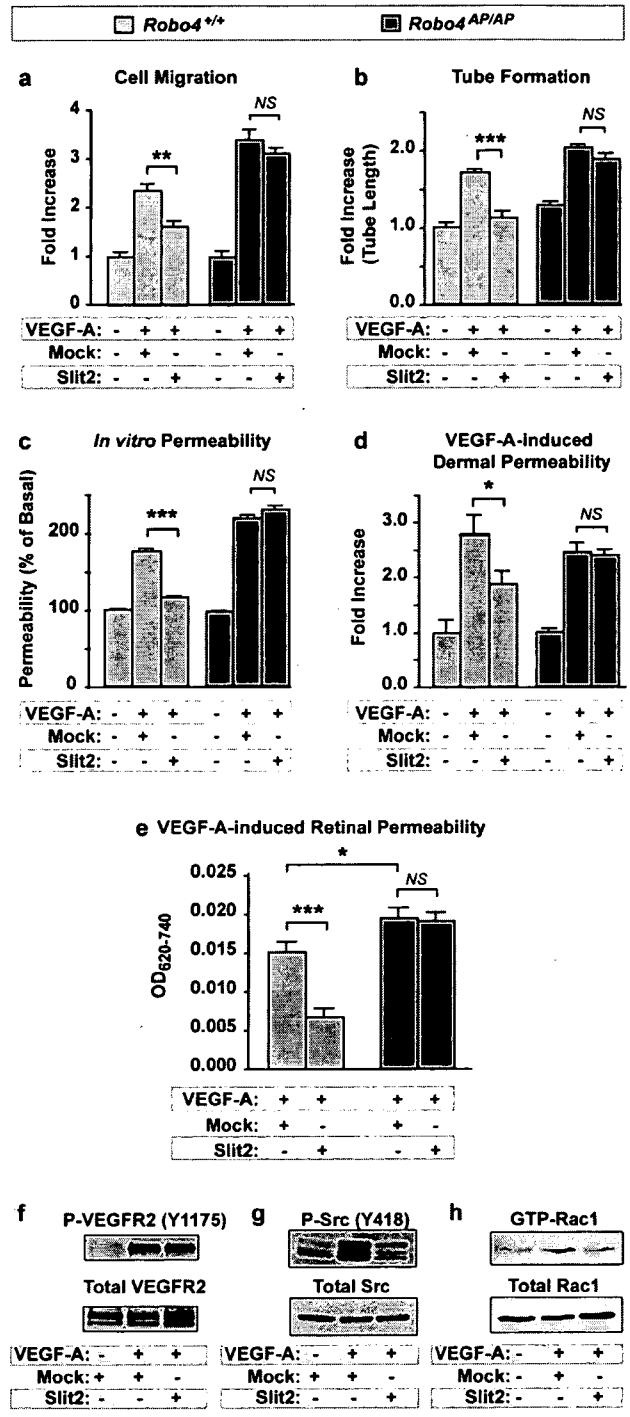

Robo4 +/+
Robo4 AP/AP
a
Cell Migration
Fold Increase
NS
**
VEGF-A:
Mock:
Slit2:
b
Tube Formation
Fold Increase (Tube Length)
***
NS
c
In vitro Permeability
Permeability (% of Basal)
d
VEGF-A-induced Dermal Permeability
Fold Increase
*
e VEGF-A-induced Retinal Permeability
OD620-740
f
P-VEGFR2 (Y1175)
Total VEGFR2
g
P-Src (Y418)
Total Src
h
GTP-Rac1
Total Rac1

FIG. 19

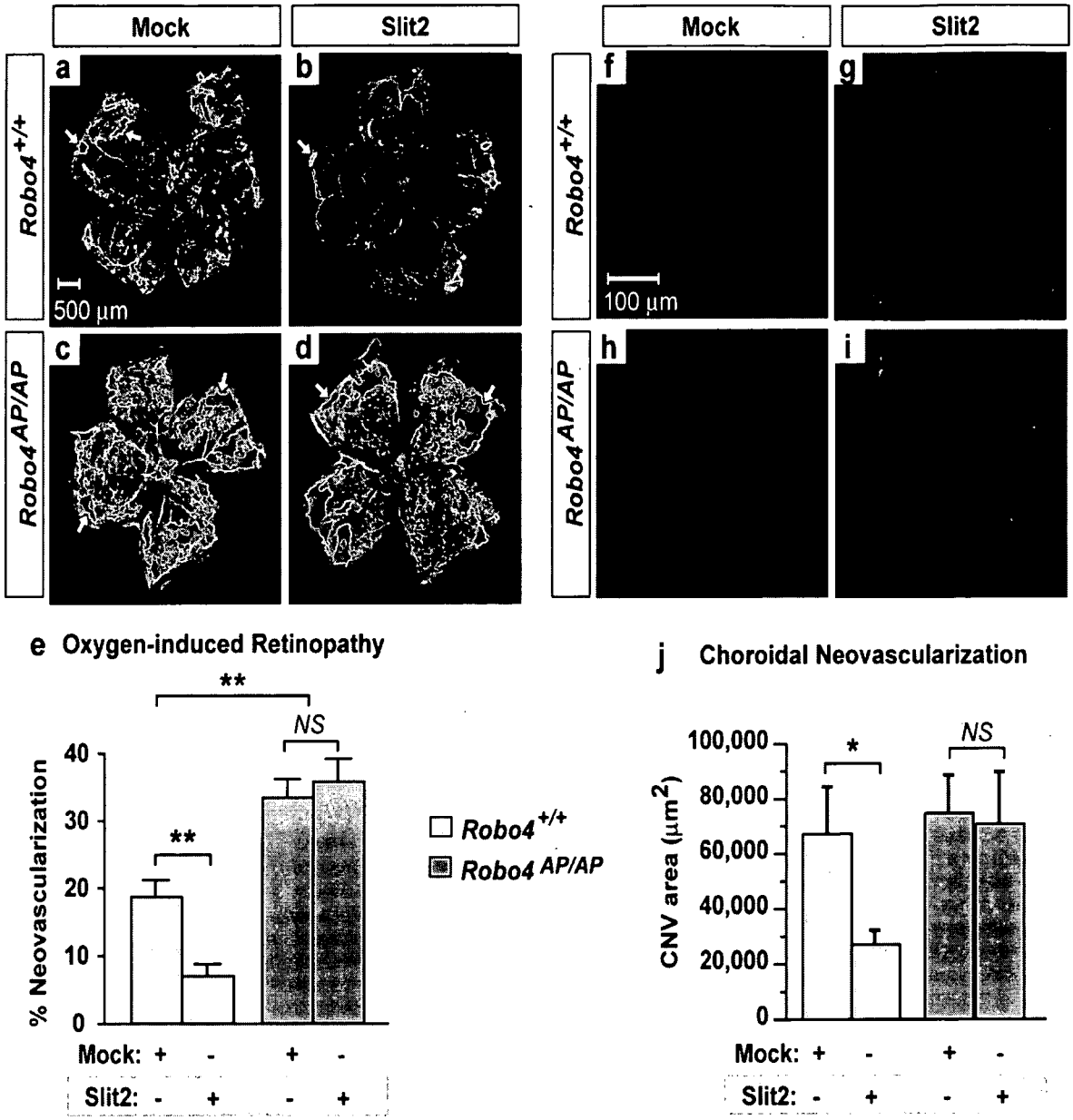

Mock
Slit2
Mock
Slit2
a
b
f
g
Robo4+/+
Robo4+/+
500 µm
100 µm
c
d
h
i
Robo4AP/AP
Robo4AP/AP
e Oxygen-induced Retinopathy
% Neovascularization
40
30
20
10
0
**
**
NS
Mock: + - + -
Slit2: - + - +
Robo4+/+
Robo4 AP/AP
j Choroidal Neovascularization
CNV area (µm2)
100,000
80,000
60,000
40,000
20,000
0
*
NS
Mock: + - + -
Slit2: - + - +

FIG. 20

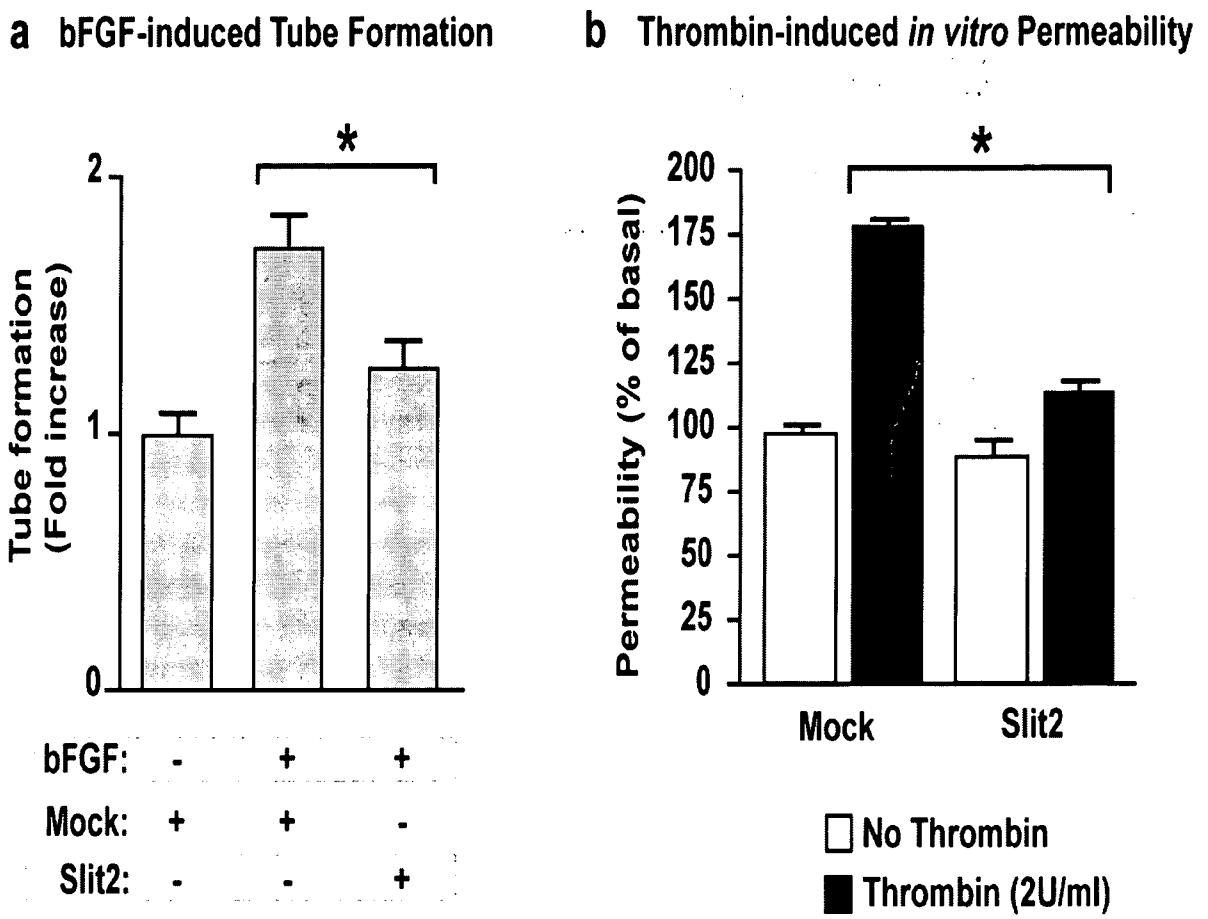

a bFGF-induced Tube Formation
Tube formation (Fold increase)
2
1
0
*
bFGF: - + +
Mock: + + -
Slit2: - - +
b Thrombin-induced in vitro Permeability
Permeability (% of basal)
200
175
150
125
100
75
50
25
0
*
Mock
Slit2
No Thrombin
Thrombin (2U/ml)

FIG. 21

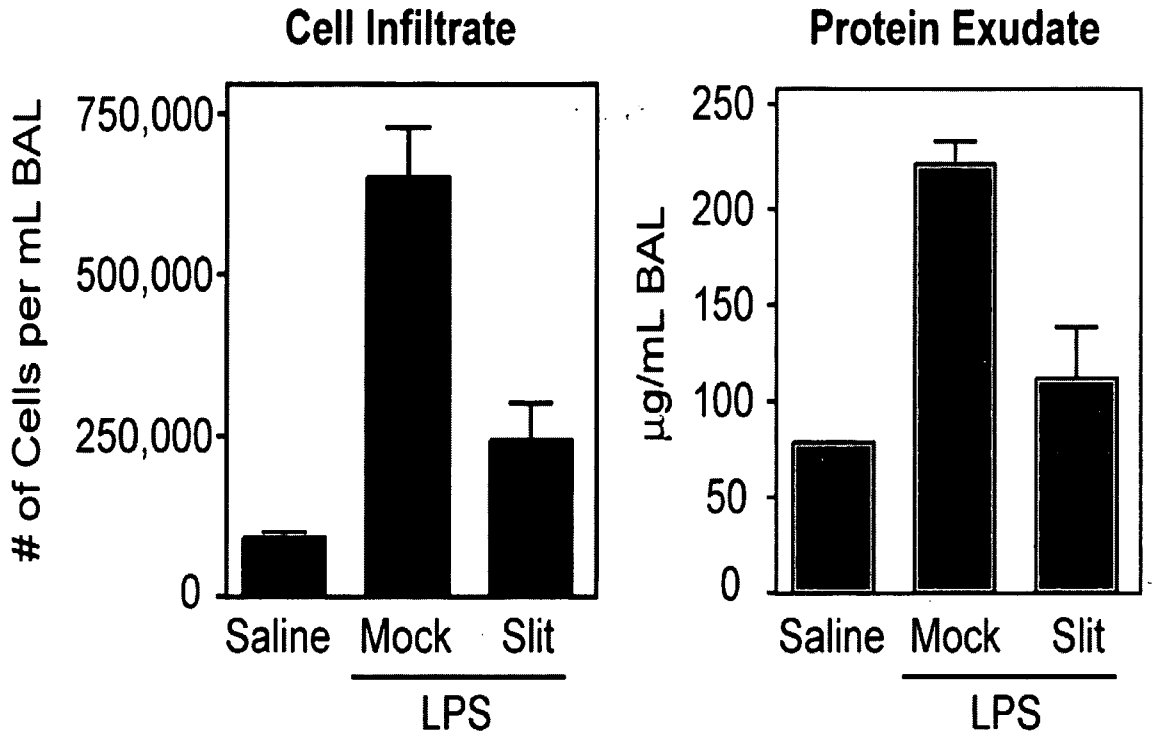

Cell Infiltrate
# of Cells per mL BAL
750,000
500,000
250,000
0
Saline
Mock
Slit
LPS
Protein Exudate
µg/mL BAL
250
200
150
100
50
0
Saline
Mock
Slit
LPS

FIG. 22

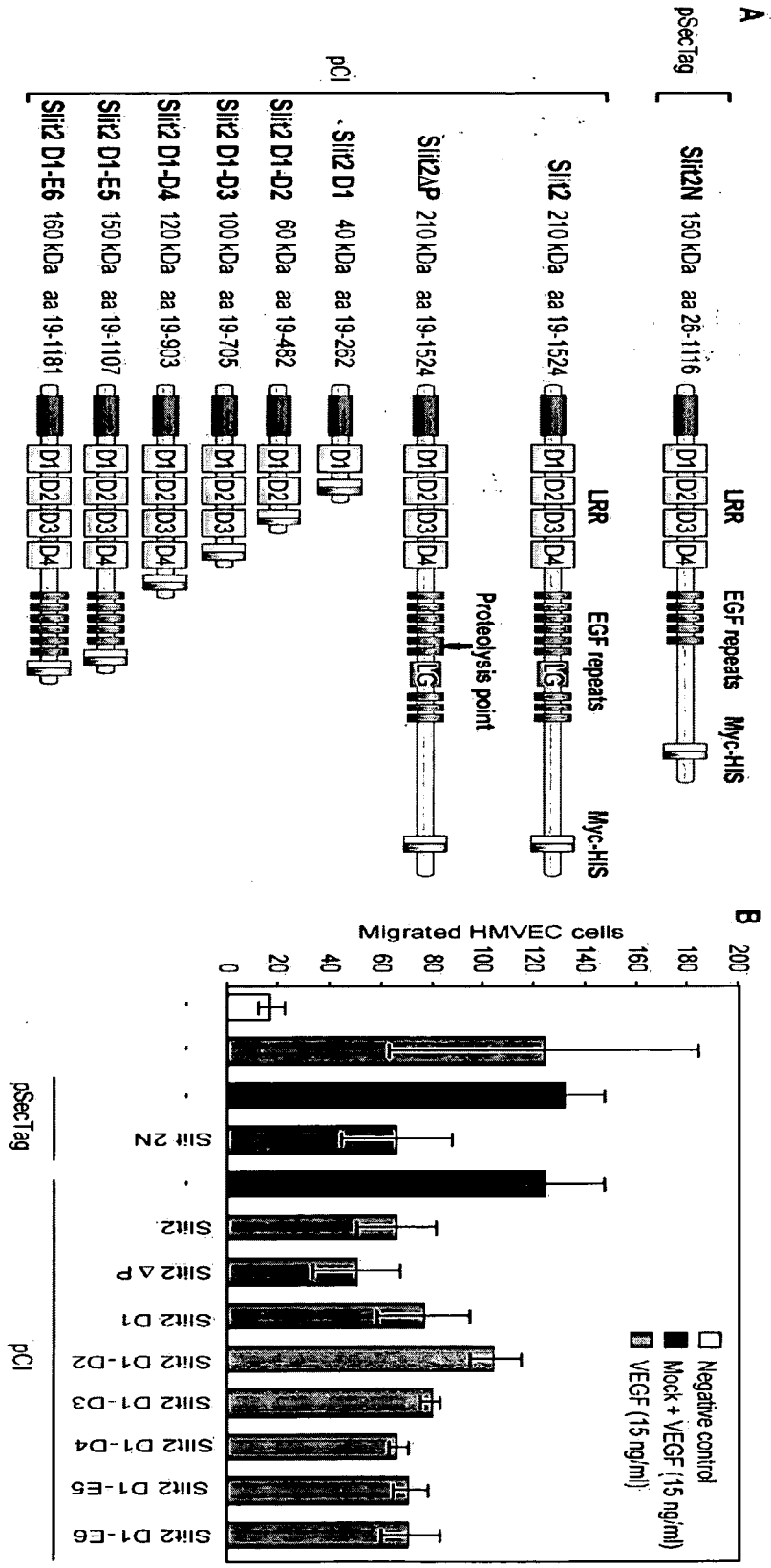

A
pSecTag
Slit2N 150 kDa aa 26-1116
LRR
EGF repeats
Myc-HIS
pCI
Slit2 210 kDa aa 19-1524
Slit2ΔP 210 kDa aa 19-1524
Proteolysis point
Slit2 D1 40 kDa aa 19-262
Slit2 D1-D2 60 kDa aa 19-482
Slit2 D1-D3 100 kDa aa 19-705
Slit2 D1-D4 120 kDa aa 19-903
Slit2 D1-E5 150 kDa aa 19-1107
Slit2 D1-E6 160 kDa aa 19-1181
B
Migrated HMVEC cells
0
20
40
60
80
100
120
140
160
180
200
Negative control
Mock + VEGF (15 ng/ml)
VEGF (15 ng/ml)
pSecTag
pCI
Slit 2N
Slit2
Slit2 ΔP
Slit2 D1
Slit2 D1-D2
Slit2 D1-D3
Slit2 D1-D4
Slit2 D1-E5
Slit2 D1-E6

FIG. 23

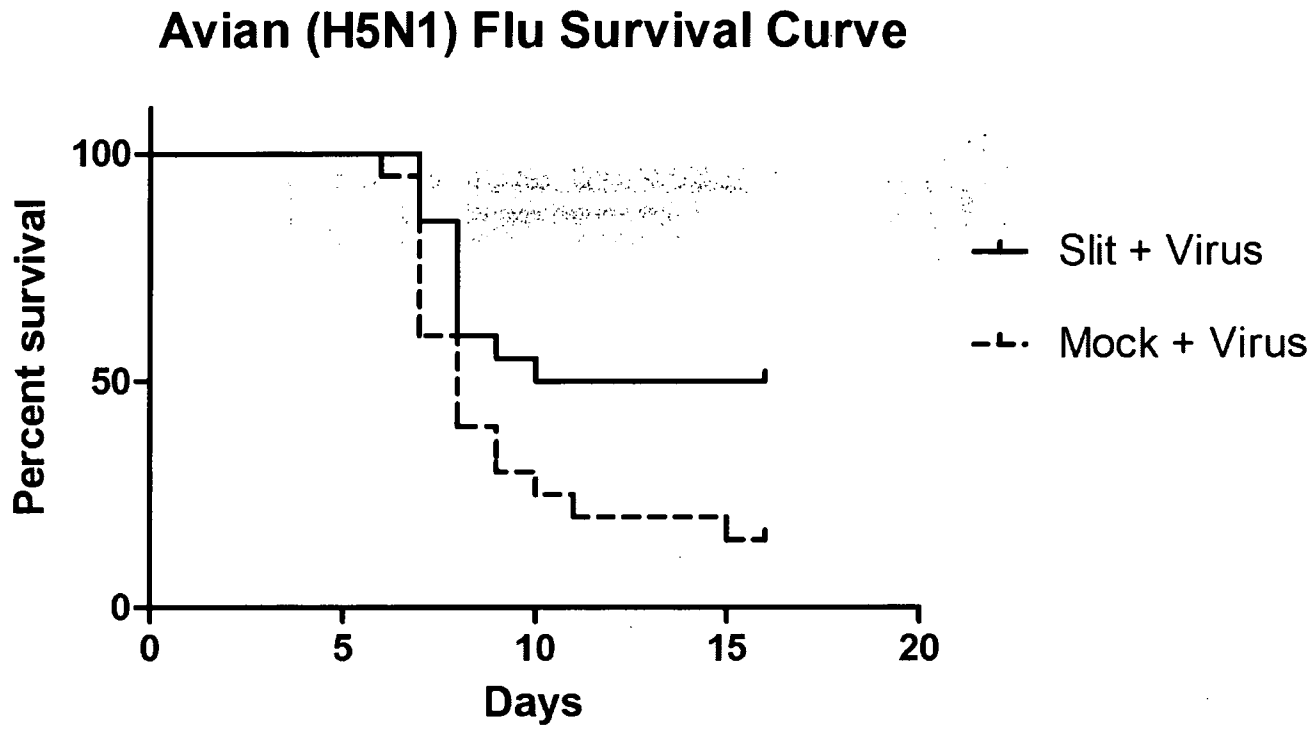
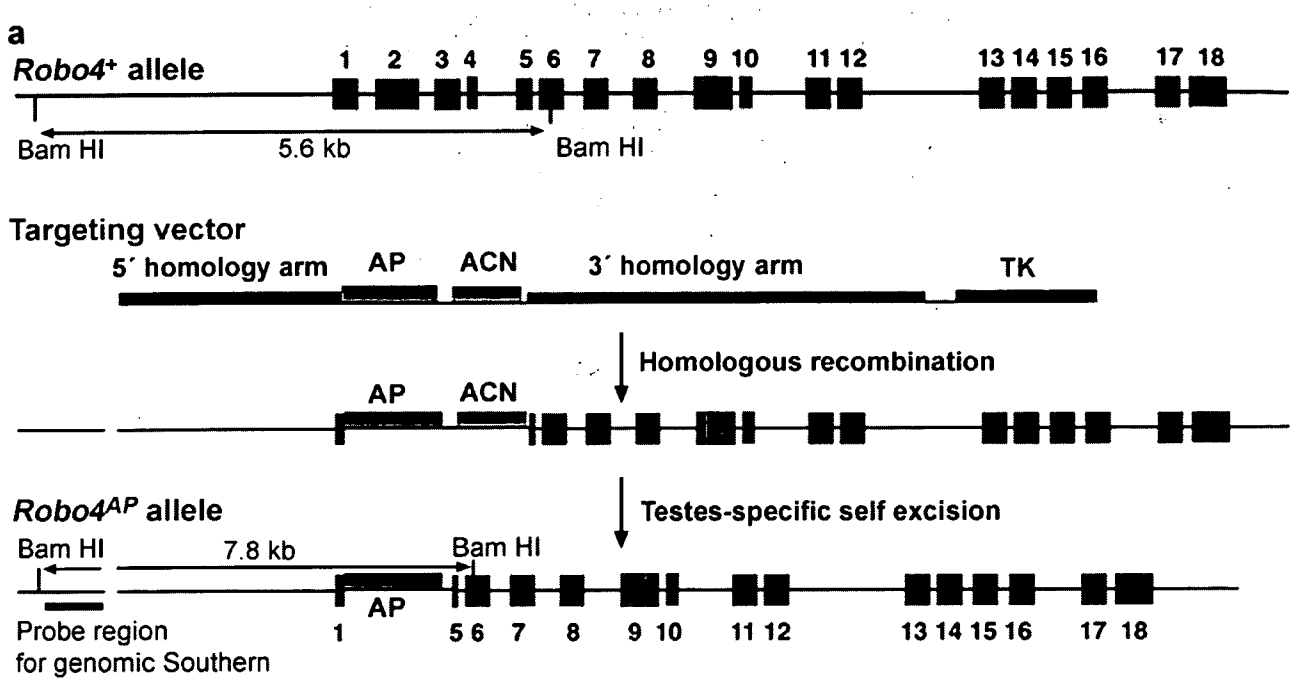

Avian (H5N1) Flu Survival Curve
Percent survival
100
50
0
0
5
10
15
20
Days
Slit + Virus
Mock + Virus

FIG. 24


a
Robo4+ allele
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Bam HI
5.6 kb
Bam HI
Targeting vector
5′ homology arm
AP
ACN
3′ homology arm
TK
Homologous recombination
AP
ACN
Robo4AP allele
Testes-specific self excision
Bam HI
7.8 kb
Bam HI
AP
Probe region
for genomic Southern
1 5 6 7 8 9 10 11 12 13 14 15 16 17 18

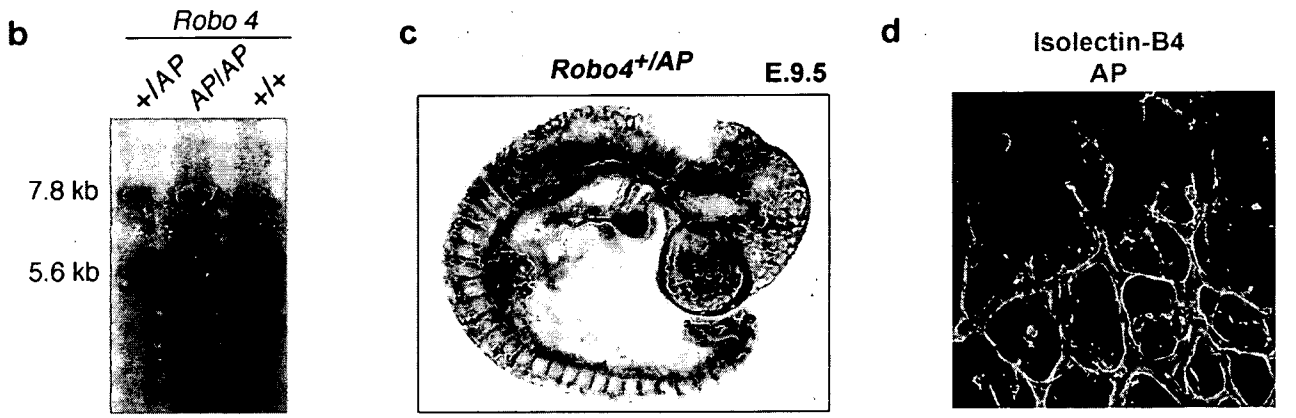

b
Robo 4
+/AP
AP/AP
+/+
7.8 kb
5.6 kb
c
Robo4+/AP
E.9.5
d
Isolectin-B4
AP

FIG. 25