SLIT-ROBO SIGNALING FOR DIAGNOSIS AND TREATMENT OF KIDNEY DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Serial No. 61/583,379 filed on 5 January 2012, the contents of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The field of the invention relates to methods for the treatment of chronic kidney disease and proteinuria and for the diagnosis of chronic kidney disease and monitoring the effects of treatment on the progression of chronic kidney disease and proteinuria.

GOVERNMENT SUPPORT

[0003] This invention was made with Government Support under Contract No. DK078226 awarded by the National Institutes of Health. The Government has certain rights in the invention.

SUMMARY OF THE INVENTION

[0004] Provided herein are novel methods for the treatment of chronic kidney disease and proteinuria and for the diagnosis of chronic kidney disease, and monitoring the effects of treatment on the progression of chronic kidney disease and proteinuria based, in part, on the inventors' discovery of a novel and unexpected role for the SLIT-ROBO signaling pathway in the regulation of podocyte F-actin cytoskeleton and foot process structure in the kidney.

[0005] Accordingly, in some aspects, provided herein are methods for the treatment of chronic kidney disease in a subject in need thereof, the methods comprising administering to a subject having or at risk for a chronic kidney disease a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.

[0006] Also provided herein, in some aspects, are method for the reduction of proteinuria in a subject in need thereof, comprising administering to a subject having or at risk for proteinuria a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.

[0007] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

[0008] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

[0009] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

[0010] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

[0011] In some embodiments of these methods and all such methods described herein, the subject having or at risk for a chronic kidney disease has diabetic nephropathy or high blood pressure.

[0012] In some embodiments of these methods and all such methods described herein, the method further comprises administering to the subject an additional therapeutic agent.

[0013] In some embodiments of these methods and all such methods described herein, the additional therapeutic agent is an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin II receptor blocker (ARB).

[0014] Also provided herein, in some aspects, are methods comprising:

a. assaying a biological test sample from a subject to determine an expression level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide;
b. determining whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in the biological test sample is above a reference threshold level; and
c. diagnosing the subject as in need of treatment or therapy for chronic kidney disease.

[0015] In some embodiments of these methods and all such methods described herein, assaying the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.

[0016] In some embodiments of these methods and all such methods described herein, assaying the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.

[0017] In some embodiments of these methods and all such methods described herein, the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.

[0018] In some embodiments of these methods and all such methods described herein, the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.

[0019] In some embodiments of these methods and all such methods described herein, the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.

[0020] Also provided herein, in some aspects, are assays comprising:

a. contacting a biological test sample isolated from a subject with a reagent that detects ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide; and
b. measuring the level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide,

wherein an increased level of said ROBO2 polypeptide or said RNA encoding a ROBO2 polypeptide, relative to a normal biological sample, identifies a subject having chronic kidney disease and/or progression of chronic kidney disease or proteinuria.

[0021] In some embodiments of these assays and all such assays described herein, detecting the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.

[0022] In some embodiments of these assays and all such assays described herein, detecting the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.

[0023] In some embodiments of these assays and all such assays described herein, the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.

[0024] In some embodiments of these assays and all such assays described herein, the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.

[0025] In some embodiments of these assays and all such assays described herein, the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.

[0026] In some embodiments of these assays and all such assays described herein, the subject has been diagnosed with diabetes or high blood pressure.

[0027] In some aspects, provided herein are systems for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:

a. a measuring module configured to determine the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in a biological sample obtained from a subject;
b. a comparison module configured to receive said expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide determined by the measuring module and perform at least one analysis to determine whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is greater than a pre-determined reference level or ratio, and to provide a retrieved content; and
c. a display module for displaying a content based the data output from said comparison module, wherein the content comprises a signal indicative that the expression level or ratio of ROBO2 polypeptide or RNA is greater than the pre-determined reference level

or ratio, or a signal indicative that the level or expression ratio of ROBO2 is not greater than the reference level or pre-determined ratio.

[0028] In some embodiments of these systems and all such systems described herein, the content displayed on the display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.

[0029] In some aspects, provided herein are systems for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:

a. a determination module configured to receive at least one test sample obtained from a subject and perform at least one analysis on said at least one test sample to determine the presence or absence of either of the following conditions:
    i. an expression ratio of ROBO2 greater than a pre-determined ratio, or
    ii. an expression level of ROBO2 greater than a pre-determined level
b. a storage device configured to store data output from said determination module; and
c. a display module for displaying a content based on the data output from said determination module, wherein the content comprises a signal indicative that the expression ratio of ROBO2 is greater than the pre-determined ratio or level of ROBO2 greater than a pre-determined level, or a signal indicative that the expression ratio of ROBO2 is not greater than the pre-determined ratio or not greater than a pre-determined level.

[0030] In some embodiments of these systems and all such systems described herein, the content displayed on the display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.

[0031] Also provided herein, in some aspects, are methods for treating a human subject with a risk of chronic kidney disease or proteinuria, comprising administering a treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria to a human subject who is determined to have a level of ROBO2 protein above a reference threshold level.

[0032] In some embodiments of these methods and all such methods described herein, the level of ROBO2 protein is at least 20% above the reference level.

[0033] In some embodiments of these methods and all such methods described herein, the level of ROBO2 protein is at least two standard deviations above the reference level.

[0034] In some embodiments of these methods and all such methods described herein, the treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria comprises a ROBO2 inhibitor.

[0035] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

[0036] In some embodiments of these methods and all such methods described herein, ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

[0037] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

[0038] In some embodiments of these methods and all such methods described herein, the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

[0039] Also provided herein, in some aspects, are ROBO2 inhibitors for use in treating a chronic kidney disease, and ROBO2 inhibitor for use in treating proteinuria.

[0040] In some embodiments of these uses and all such uses described herein, the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

[0041] In some embodiments of these uses and all such uses described herein, the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

[0042] In some embodiments of these uses and all such uses described herein, the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

[0043] In some embodiments of these uses and all such uses described herein, the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

[0044] In some embodiments of these uses and all such uses described herein, the chronic kidney disease or proteinuria is caused by diabetic nephropathy or high blood pressure.

[0045] In some embodiments of any of these aspects and all such aspects described herein, ROBO2 refers to human ROBO2 having the amino acid sequence of SEQ ID NO: 1 encoded by the mRNA sequence of SEQ ID NO: 2. In some embodiments of any of these aspects and all such aspects described herein, ROBO2 refers to human ROBO2 having the amino acid sequence of SEQ ID NO: 3 encoded by the mRNA sequence of SEQ ID NO: 4.

Definitions

[0046] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular

embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0047] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[0048] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[0049] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0050] As used in this specification and the appended claims, the singular forms “a,” "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0051] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used in connection with percentages can mean ±10%, ±5%, or ±1%.

[0052] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. 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 is defined solely by the claims. Definitions of common terms in immunology, and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 18th Edition, published by Merck Research Laboratories, 2006 (ISBN 0-911910-18-2); Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006. Definitions of common terms in molecular biology are found in Benjamin Lewin, Genes IX, published by Jones & Bartlett Publishing, 2007 (ISBN-13: 9780763740634); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.),

Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1982); Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1989); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987); Current Protocols in Molecular Biology (CPMB) (Fred M. Ausubel, *et al.* ed., John Wiley and Sons, Inc.), Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.) and Current Protocols in Immunology (CPI) (John E. Coligan, et. al., ed. John Wiley and Sons, Inc.), which are all incorporated by reference herein in their entireties.

[0053] It is understood that the following detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

BRIEF DESCRIPTION OF THE DRAWINGS

[0054] **FIGS. 1A-1R** demonstrate that Robo2 is expressed and localized to the basal cell surface of podocytes. All immunostainings in (FIGS. 1A-1Q) are performed at mouse E16.5 days at 600X magnification (see FIGS. 5A-5M for immunostainings in adult mouse glomeruli). (FIGS. 1A-1C) Robo2 co-localizes with podocyte slit-diaphragm protein nephrin. (FIGS. 1D-1F) Robo2 co-localizes with podocyte slit-diaphragm protein podocin. (FIGS. 1G-1I) Robo2 co-localizes with adaptor protein Nck in glomeruli. (FIGS. 1J-1L) Robo2 is expressed on the basal podocyte surface adjacent to glomerular basement membrane protein nidogen. (FIGS. 1M-1O) Robo2 does not co-localize with glomerular endothelial cell protein marker Pecam1. (FIG. 1P) The enlarged region boxed in (FIG. 1L) shows that Robo2 is expressed predominantly on the basal cell surface (arrow) of podocytes (p) adjacent to glomerular basement membrane marker nidogen. Robo2 is weakly expressed in the apical and lateral cell surfaces (arrowheads) of podocytes. (FIG. 1Q) Robo2 is expressed predominantly on the basal cell surface (arrows) of podocytes (p) and is weakly expressed on the apical or lateral cell surfaces (arrowheads). (FIGS 1R) Immunogold electron microscopy shows localization of gold partials (arrows)

conjugated to Robo2 antibody in the foot process (fp) of a podocyte from a 3-week old mouse. GBM, glomerular basement membrane. Magnification: 50,000X. See also FIGS. 5A-5M.

[0055] **FIGS. 2A-2J** demonstrate that Robo2 interacts with the adapter protein Nck and forms a complex with nephrin. (FIG. 2A) Yeast two-hybrid assays show a positive interaction between Robo2 intracellular domain (Robo2-ICD) and Nck1. LacZ reporter (X-gal): +++, yeast turned dark; ++, light ; -, white in 24 hours. Leucine reporter (-Leu): +, yeast grew; -, yeast did not grow. CC, cytoplasmic conserved region. Numbers indicate residue positions in the full-length protein. (FIG. 2B) Yeast two-hybrid assays show the first two SH3 domains of Nck1 are required for its interaction with Robo2-ICD. (FIG. 2C) Yeast two-hybrid assays show potential binding domains that mediate Robo2 and Nck1 interaction. The sequence is the potential binding region in Robo2 for Nck1. Proline-rich regions are highlighted. (FIG. 2D) Co-precipitation of Robo2 and Nck. Cell lysates in lane 5 are collected from His-myc-Robo2 transfected cells (used in lanes 1 and 2); Cell lysates in lane 6 are collected from His-myc-Robo2-ΔNBD transfected cells (used in lanes 3 and 4). (FIG. 2E) Co-precipitation of Robo2, Nck, and nephrin. (FIG. 2F) A similar co-precipitation as (FIG. 2E) except that His-myc-nephrin is pulled-down instead of His-myc-Robo2. (FIG. 2G) Co-immunoprecipitation of kidney endogenous Robo2, Nck, and nephrin. (FIG. 2H) A similar assay as (FIG. 2G) except that precipitates are prepared using mouse anti-nephrin antibody. (I) Slit2 enhances Robo2-Nck-nephrin complex formation. His-myc-Robo2, nephrin, and Fyn are expressed in HEK cells that are stimulated with Slit2 conditioned medium (lanes 1, 3) or control conditioned medium (lane 2, 4). (FIG. 2J) Intensity quantification of (FIG. 2I). Data are represented as mean ± SEM; n=7, *$p < 0.05$, **$p < 0.01$ compared with the control, paired student's t-test. See also FIGS. 6A-6F'.

[0056] **FIGS. 3A-3G** demonstrate that Slit2-Robo2 signaling inhibits nephrin-mediated actin polymerization. (FIG. 3A) CD16/7-NCD is co-expressed with Robo2 in HEK cells, which are treated with anti-CD16 antibody and rhodamine-conjugated anti-IgG antibody in the presence of Slit2 conditioned medium (Slit) or control conditioned medium (CTL). Cells are then fixed and stained with FITC-conjugated phalloidin to reveal F-actin. Scale bar, 5 μm. NCD: nephrin cytoplasmic domain. (FIG. 3B) A similar assay as (FIG. 3A) except that CD16/7-NCD is replaced by CD16/7-HA and is used as a control assay. (FIG. 3C) The percentage of cells with F-actin tails over total cells with CD16/7 clusters in each group is quantified. Data are represented as mean ± SEM, *$p < 0.01$, n=5. (FIG. 3D) CD16/7-NCD in (FIG. 3A) is immunoprecipitated by anti-CD16 antibody after Slit2 conditioned medium stimulation (lanes1 and 3) or control conditioned medium (lanes 2 and 4). Note reduced F-actin in lane 1. CD16/7-HA is used as a negative control. (FIG. 3E) Intensity quantification of (FIG. 3D). Data are represented as mean ± SEM; n=4, *$p < 0.05$ compared with the control, paired student's t-test. (FIG. 3F) Immunoprecipitation of nephrin from Robo2 knockout homozygous (Robo2-/-), heterozygous (Robo2+/-), and wild-type (Robo2+/+) mouse kidneys using the anti-nephrin antibody. Note increased F-actin in lane 3. (FIG. 3G) Intensity quantification of (FIG. 3F). Data are represented as

mean ± SEM; n=4, *p < 0.05 compared with the wild-type and heterozygous, ANOVA analysis. See also FIGS. 7A-7C.

[0057] **FIGS. 4A-4W** demonstrate podocyte structural phenotypes in the Robo2 homozygous null, Robo2 podocyte specific knockout, and Robo2 and Nphs1 double knockout mice. (FIGS. 4A and 4B) Representative images of newborn kidneys show podocyte bodies (arrowheads) and Bowman's capsule (arrows) in wild-type (FIG. 4A) and Robo2 homozygous null mice (FIG. 4B). (FIGS. 4C and 4D) High magnification images of (FIGS. 4A and4 B) show podocyte foot processes (arrows) in the newborn kidney. Scale bar, 1 µm. (FIGS. 4E and 4F) Representative images of 3-week kidneys at low magnification show podocyte cell body (arrowheads) in a Robo2 homozygous null mouse (FIG. 4F) compared to an age-matched control (FIG. 4E). (FIGS. 4G and 4H) Higher magnification images of (FIGS. 4E and 4F) show disorganized shorter meandering foot processes (arrow) in a 3-week Robo2 homozygous null mouse (FIG. 4H) compared to well-organized zip-like foot processes in the age-matched control (FIG. 4G). Scale bars: 2 µm. (FIGS. 4I and 4J) Representative transmission electron microscopy images (magnification at 5000x) depict the focal segmental podocyte foot process effacement (arrow in FIG. 4J) in a one month old Robo2 podocyte-specific knockout mouse and the normal phenotype in the control (FIG. 4I). Abbreviations: gc: glomerular capillary; us: urinary space. (FIGS. 4K and 4L) Higher magnification transmission electron microscopy images (40000x) show broader podocyte foot processes (arrow in FIG. 4L) in a two months old Robo2 podocyte-specific mutant mouse compared with the control (FIG. 4K). Abbreviations: fp, podocyte foot process; GBM, glomerular basement membrane. (FIG. 4M) Quantification of podocyte foot process width in one month old $Robo2^{del5/flox}$;$Tg^{Nphs2\text{-}Cre+}$ podocyte specific knockout mice (Robo2 KO) and the wild-type littermate controls (WT). Data are represented as mean ± SEM, n=333, *p < 0.01. (FIG. 4N) ELISA assay of spot urine shows an elevated albumin/creatinine ratio in $Robo2^{del5/flox}$; Nphs2-Cre+ (KO) adult mice compared with control wild-type (WT). Data are represented as mean ± SEM, n=20, *p < 0.01. (FIG. 4O) Western blot analysis shows the presence of albumin in urine; 1µl urine was loaded on each well, 0.2µg albumin was used as a positive control. WT, three wild-type littermates; Robo2 KO, three individual Robo2del5/flox;Nphs2-Cre+ mice. (FIGS. 4P and 4Q) Representative scanning electron microscope images show disrupted interdigitating podocyte foot processes that resemble disorganized cellular protrusions (arrows) in the Nphs1-/- single homozygous newborn mouse kidney. Scale bars: 1 µm. (FIGS. 4R and 4S) Glomeruli from Nphs1-/-Robo2-/- double homozygous newborn mice exhibit restored interdigitating foot processes (arrows), indicating alleviation of nephrin null phenotype by knocking out Robo2. (FIGS. 4T and 4U), Glomeruli from Robo2-/- single homozygous newborn mice display irregular and broader foot processes but extensive interdigitating pattern formation (arrows). (FIGS. 4V and 4W), Glomeruli from newborn wild type mice with normal regular interdigitating pattern of the foot process (arrows). See also FIGS. 8A-8Z and Tables 1-4.

[0058] **FIGS. 5A-5M** demonstrate that Robo2 is expressed in the developing and adult glomeruli. (FIGS. 5A and 5B) In situ hybridization analysis shows that *Robo2* transcripts are expressed in

developing glomeruli (arrows) at E16.5. Magnification: 60X (FIG. 5A) and 200X (FIG. 5B). (FIGS. 5C-5F) Immunohistochemistry (IHC) studies reveal that Robo2 is expressed during developing glomeruli from E14.5 to E17.5. Magnification: 600X. (FIG. 5G) IHC shows that Robo2 is specifically expressed in adult mouse glomeruli at 5 weeks of age (FIG. 5G). DAPI marks cell nuclei in the kidney. Magnification: 400X. (FIG. 5H) IHC co-localization stainings of 5w kidney show Robo2 is co-expressed in the glomerulus with podocyte marker Wt1. Magnification: 600X. (FIGS. 5I-5K) Robo2 and WT1 are co-expressed in the mouse glomerulus at E16.5. Magnification: 600X. (FIGS. 5L and 5M) IHC co-localization stainings of 5w kidney show Robo2 is co-expressed in the glomerulus with mesangial cell marker Pdgfrb (FIG. 5L), and endothelial cell marker Pecam1 (FIG. 5M). Magnification: 600X.

[0059] **FIGS. 6A-6F'** demonstrate that Robo2 interacts with Nck and forms a complex with nephrin, which is enhanced by Slit2 stimulation. (FIG. 6A) Co-IP of Robo2 and nephrin with endogenous Nck. Robo2, nephrin, and Fyn are expressed in HEK cells and stimulated by Slit2. The endogenous Nck is immunoprecipitated by an anti-Nck antibody. The mouse IgG is used as a control. The complex formation with nephrin is enhanced by Slit2 and Fyn expression. (FIGS. 6B and 6C) Slit2 is expressed in the newborn mouse glomeruli by Immunoperoxidase staining (FIG. 6B) and is co-expressed in the glomerulus with the podocyte marker Synaptopodin (FIG. 6C). Magnification: 600X. (FIGS. 6D and 6D') CD16/7-NCD is co-expressed with Robo2 in HEK cells in the presence of Slit2, treated with anti-CD16 antibody and rhodamine-conjugated anti-IgG antibody, then fixed and stained with anti-Robo2 antibody. CD16/7-NCD clusters co-localize with Robo2 (FIG. 6D) but no colocalization is observed in control CD16/7-HA clusters (FIG. 6D'). Scale bar: 5 μm. NCD: nephrin cytoplasmic domain. (FIGS. 6E and 6E') Deletion of Nck binding domain (NBD) in Robo2 impairs its co-localization with CD16/7-NCD in the presence of Slit2. CD16/7-NCD clusters co-localize with Robo2 (FIG. 6E) but no colocalization is observed in Robo2-ΔNBD clusters (FIG. 6E'). Scale bar: 5 μm. (FIGS. 6F and 6F') Slit2 stimulation enhances CD16/7-NCD and Robo2 co-localization in HEK cells. CD16/7-NCD clusters co-localize with Robo2 in the presence of Slit2 (FIG. 6F) but not with control conditioned medium (FIG. 6F'). Scale bar: 5 μm.

[0060] **FIGS. 7A-7C** demonstrate deletion of Nck binding domain in Robo2 compromises Slit2-Robo2 inhibition on nephrin-induced actin polymerization. (FIG. 7A) CD16/7-NCD and Robo2 were co-expressed in HEK cells, clustered with anti-CD16 antibody and rhodamine-conjugated anti-IgG antibody in the presence of Slit2 conditioned medium (Slit2) or control conditioned medium (CTL). Cells were then fixed and stained with FITC-conjugated phalloidin to reveal F-actin fibers. Clusters of CD16/7-NCD and F-actin fibers were examined using confocal microscopy. Scale bar, 5μm. NCD, nephrin cytoplasmic domain. (FIG. 7B) CD16/7-NCD and Robo2-ΔNBD were co-expressed in HEK cells. Scale bar, 5μm. NBD, Nck binding domain. (FIG. 7C) The percentage of cells with F-actin tails over total cells with CD16/7-NCD clusters in each group was quantified. Data are represented as mean ± SEM, *p = 1.436×10^{-5}, **p = 6.32×10^{-5}, n=5, *ANOVA*.

[0061] **FIGS. 8A-8Z** demonstrate glomerular phenotype in the *Robo2* homozygous null, *Robo2* podocyte specific knockout, *Robo2* and *Nphs1* double knockout mice, and a proposed model of Robo2-Nephrin signaling. (FIGS. 8A-8F) Transmission electron microscopy analysis of glomerular ultrastructure in newborn (NB) $Robo2^{del5/del5}$ mutant mice kidney. (FIGS. 8A, 8C, 8E) Glomerular ultrastructure from a newborn heterozygous *Robo2* control mouse at low (FIG. 8A, 2200X), medium (FIG. 8C, 15500X) and high (FIG. 8E, 52000X) magnifications. (FIGS. 8B, 8D, 8F) Glomerular ultrastructure from a newborn homozygous Robo2 (-/-) (*i.e.*, $Robo2^{del5/del5}$) mutant mouse at low (FIG. 8B), medium (FIG. 8D) and high (FIG. 8F) magnifications. Arrows indicate focal foot process effacement. Abbreviations: gc: glomerular capillary; us: urinary space; GBM: glomerular basement membrane. (FIGS. 8G-8N) Abnormal podocyte foot process patterns in *Robo2* podocyte-specific knockout mice. (FIGS. 8G-8J) Representative scanning electron microscopy images of glomeruli from one-month old $Robo2^{del5/flox}$;*Nphs2-Cre*$^{+}$ podocyte-specific knockout mice and aged matched $Robo2^{flox/+}$ control mice. Mild irregularities of the interdigitating podocyte foot processes were found in a one month old *Robo2* podocyte-specific knockout mouse (FIGS. 8K and 8N). At seven months old, *Robo2* podocyte-specific knockout mice developed markedly irregular foot processes (FIGS. 8L and 8N). Scale bars: 10 μm (FIGS. 8G, 8H, 8K, 8L at 2000x magnification) and 2 μm (FIGS. 8I, 8J, 8M ,8N at 13000x magnification). (FIGS. 8O-8T) Glomerular morphology in *Robo2* podocyte-specific knockout mice. (FIGS. 8O-8R) Periodic acid-Schiff (PAS) staining showed mesangial matrix expansion in the glomeruli from 2-month and 6-month old *Robo2* podocyte-specific knockout mice (FIGS. 8P, 8R) compared to age-matched controls (FIGS. 8O, 8Q). (FIG. 8S) Quantitative analysis of glomeruli shows mesangial matrix expansion in 12-month old *Robo2* podocyte-specific knockout mice (MU) compared to age matched wild-type (WT) controls. Data are represented as mean ± SEM, n=5, *$p < 0.01$. (T) *Robo2* podocyte specific knockout does not affect podocyte numbers. Podocyte cells were identified using WT-1 staining. The number of podocytes per glomerular cross section was counted in four one-year old $Robo2^{del5/flox}$;$Tg^{Nphs2\text{-}Cre+}$ podocyte specific knockout mice (MU) compared to four age-matched wild-type mice (WT). Data are represented as mean ± SEM, $p = 0.645$, t-test; mutant: n=165 glomeruli; control: n=166 glomeruli. (FIGS. 8U-8Y) Glomerular phenotype in *Robo2* and *Nphs1* double knockout mice. (FIG. 8U) H&E staining shows glomeruli with characteristic dilatations of the Bowman's space (asterisks) in a $Nphs1^{-/-}$ single homozygous newborn mouse, 400x. (FIG. 8V) Glomeruli from a $Robo2^{-/-}$ single homozygous newborn mouse show absence of Bowman's space dilatations; 400x. (FIG. 8W) Normal looking glomeruli without significant Bowman's space dilatations (arrows) are shown in a $Robo2^{-/-}$;$Nphs1^{-/-}$ double homozygous newborn mouse indicating alleviation of $Nphs1^{-/-}$ glomerular phenotype; 400x. (FIG. 8X) H&E staining of normal kidney and glomeruli from an age-matched wild-type newborn mouse control; 400x. (FIG. 8Y) Quantification of glomeruli with dilated Bowman's space in newborn mice show significant reduction of glomeruli with the characteristic dilatation phenotype of the Bowman's space in $Robo2^{-/-}$;$Nphs1^{-/-}$ double homozygous compared to $Nphs1^{-/-}$ single homozygous ($Robo2^{+/-}$;$Nphs1^{-/-}$). Data are represented as mean ± SEM, *p <

0.01. (FIG. 8Z) A model of inhibitory effects of Slit2-Robo2 signaling on nephrin to influence podocyte foot process structure: Under physiological conditions (e.g., during foot process development), nephrin intracellular phosphorylated tyrosine domains (YDxV-p) recruit Nck through its interaction with the SH2 domain. Nck in turn recruits cytoskeleton regulators through its SH3 domains to promote actin polymerization. Slit2 binds Robo2 to increase Robo2 intracellular domain interaction with SH3 domains of Nck, which would prevent binding of Nck to cytoskeletal regulators and result in an inhibition of nephrin-induced actin polymerization. Balanced actin polymerization is maintained during podocte development for a normal foot process structure. In the absence of Slit2-Robo2 signaling (e.g., when *Robo2* is knocked out), the inhibitory effects of Robo2 on nephrin induced polymerization is lost. The SH3 domains of Nck are able to interact with downstream cytoskeletal regulators to increase actin polymerization, which may explain the altered podocyte foot process structure in *Robo2* mutant mice. Abbreviations: Ig: Immunoglobulin domain; FN3: Fibronectin type 3 domain; SH2: Src homolog 2 domain; SH3: Src homolog 3 domain; CC0, CC1, CC2, CC3: Cytoplasmic Conserved region 0, 1, 2, 3.

DETAILED DESCRIPTION

[0062] Robo2 has been previously shown to be the cell surface receptor for the repulsive guidance cue Slit and to be involved in axon guidance and neuronal migration in the nervous system. Nephrin is a podocyte slit-diaphragm protein that functions in the kidney glomerular filtration barrier. We demonstrate herein that Robo2 is expressed at the basal surface of podocytes, such as mouse podocytes, and co-localizes with nephrin. Biochemical studies indicate that Robo2 forms a complex with nephrin in the kidney through adaptor protein Nck. In contrast to the role of nephrin that promotes actin polymerization, we show herein that Slit2-Robo2 signaling inhibits nephrin-induced actin polymerization. For example, the amount of F-actin associated with nephrin is increased in Robo2 knockout mice that develop an altered podocyte foot process structure and microalbuminuria. Genetic interaction studies further reveal that loss of Robo2 alleviates the abnormal podocyte phenotype in nephrin null mice. The results provided herein show that Robo2 signaling acts as a negative regulator on nephrin to influence podocyte foot process architecture.

[0063] In addition, it has been shown that a patient having vesicoureteral reflux (VUR) has a chromosome translocation that disrupts the ROBO2 gene and produces dominant negative ROBO2 fusion proteins that abrogate the SLIT2-ROBO2 signaling pathway. Normally, VUR is a disease characterized by the retrograde flow of urine from the bladder into the ureters and kidney and VUR patients can present with reflux nephropathy, a condition that manifests with severe proteinuria. It has been shown that dominant negative ROBO2 fusion proteins produced by a VUR patient blocks the SLIT2-ROBO2 signaling pathway and protects the patient from reflux nephropathy and proteinuria, thus confirming and further supporting the inventors results in animal models of the therapeutic value of targeting the SLIT2-ROBO2 signaling pathway for the treatment of chronic kidney disease.

[0064] In the normal kidney, the trilaminar glomerular capillary wall, composed of fenestrated endothelial cells, basement membrane and podocytes, restricts the permeability to plasma proteins. Podocytes are specialized epithelial cells that extend primary and secondary processes to cover the outer surface of the glomerular basement membrane. The actin-rich interdigitating secondary processes, or foot processes, from neighboring podocytes create filtration slits bridged by a semi-porous slit-diaphragm that forms the final barrier to protein permeation. Whereas genetic mutations of podocyte slit-diaphragm proteins such as nephrin and others are associated with hereditary forms of proteinuric kidney disease (Tryggvason et al., 2006), it has become evident that the proteins that make up and associate with the slit-diaphragm are more than a simple structural barrier. These proteins form a balanced signaling network that can influence podocyte foot process structure and function through interaction with the F-actin cytoskeleton (Faul et al., 2007; Jones et al., 2006; Verma et al., 2006).

[0065] Roundabout (Robo) family proteins, Robo1, Robo2, Robo3 and Robo4 are cell surface receptors for the secreted ligand Slit (Dickson and Gilestro, 2006). Slit1, Slit2, and Slit3 were originally found as repulsive guidance cues for axon pathfinding and migrating neurons during nervous system development (Guan and Rao, 2003). The transmembrane protein Robo contains five Ig motifs and three fibronectin type III (FNIII) repeats in its extracellular domain (Dickson and Gilestro, 2006). While both immunoglobulin (Ig) motifs 1 and 2 interact with Slit, the first Ig1 motif of Robo is the primary binding site for Slit (Dickson and Gilestro, 2006). The intracellular domain of Robo has four cytoplasmic conserved (CC) sequences named CC0, CC1, CC2, and CC3 (Bashaw et al., 2000; Kidd et al., 1998; Morlot et al., 2007; Zallen et al., 1998). CC0 and CC1 contain tyrosine, while CC2 and CC3 are proline-rich stretches. The repulsive activity of Slit-Robo signaling inhibits actin polymerization (Guan and Rao, 2003) or induces F-actin depolymerization (Piper et al., 2006).

[0066] Slit-Robo signaling also plays crucial roles during early kidney induction and ureteric bud outgrowth. Mouse mutants that lack *Slit2* or *Robo2* develop supernumerary ureteric buds, which lead to a broad-spectrum of urinary tract phenotype including duplex kidneys, abnormal ureterovesical junctions and hydronephrosis (Grieshammer et al., 2004; Lu et al. 2007). Disruption of *ROBO2* in humans causes congenital anomalies of the kidneys and urinary tracts (CAKUT), and point mutations of *ROBO2* have been identified in patients with vesicoureteral reflux (VUR) (Lu et al., 2007). Our recent study demonstrates that *Robo2* is crucial for the formation of a normal ureteral orifice and for the maintenance of an effective anti-reflux mechanism (Wang et al., 2011).

[0067] Herein we demonstrate that Robo2 is a novel podocyte protein expressed at the basal surface of glomerular podocytes in the kidney and is co-localized with nephrin and podocin. Robo2 interacts directly with adaptor protein Nck SH3 domains and forms a complex with nephrin. Whereas *Robo2* knockout mice develop altered podocyte foot processes, the loss of Robo2 alleviates the foot process structural abnormalities that are seen in nephrin null mice. These results described herein indicate that Robo2 signaling acts as a negative regulator on nephrin signaling to influence podocyte foot process architecture. In addition, as demonstrated herein, it has been discovered that the dominant

negative ROBO2 fusion proteins produced by a patient blocks the SLIT2-ROBO2 signaling pathway and protects the patient from reflux nephropathy and proteinuria, thus confirming and further supporting the results described herein in animal models of the therapeutic value of targeting the SLIT2-ROBO2 signaling pathway for the treatment of chronic kidney disease.

[0068] Accordingly, in some aspects, provided herein are methods for the treatment of chronic kidney disease in a subject in need thereof, such method comprising administering to a subject having or at risk for a chronic kidney disease a therapeutically effective amount of a composition comprising a SLIT2-ROBO2 signaling pathway inhibitor.

[0069] Also provided herein, in some aspects, are methods for the reduction of proteinuria in a subject in need thereof, comprising administering to a subject having or at risk for proteinuria a therapeutically effective amount of a composition comprising a SLIT2-ROBO2 signaling pathway inhibitor.

[0070] In other aspects, provided herein are methods for preventing kidney diseases or promoting prophylaxis of kidney diseases in a subject in need thereof, comprising administering to a subject a therapeutically effective amount of a composition comprising a SLIT2-ROBO2 signaling pathway inhibitor so as to prevent or promote prophylaxis of kidney disease in the subject.

[0071] Also provided herein, in some aspects, are methods for mitigating the effects of kidney disease, reducing the severity of kidney disease, reducing the likelihood of developing kidney disease and/or slowing the progression of kidney disease in a subject in need thereof.

[0072] As used herein, "ROBO2" refers to the polypeptide having the amino acid sequence of:
MARRHERVTRRMWTWAPGLLMMTVVFWGHQGNGQGQGSRLRQEDFPPRIVEHPSDVIVSK
GEPTTLNCKAEGRPTPTIEWYKDGERVETDKDDPRSHRMLLPSGSLFFLRIVHGRRSKPDEGS
YVCVARNYLGEAVSRNASLEVALLRDDFRQNPTDVVVAAGEPAILECQPPRGHPEPTIYWKK
DKVRIDDKEERISIRGGKLMISNTRKSDAGMYTCVGTNMVGERDSDPAELTVFERPTFLRRPI
NQVVLEEEAVEFRCQVQGDPQPTVRWKKDDADLPRGRYDIKDDYTLRIKKTMSTDEGTYM
CIAENRVGKMEASATLTVRAPPQFVVRPRDQIVAQGRTVTFPCETKGNPQPAVFWQKEGSQ
NLLFPNQPQQPNSRCSVSPTGDLTITNIQRSDAGYYICQALTVAGSILAKAQLEVTDVLTDRPP
PIILQGPANQTLAVDGTALLKCKATGDPLPVISWLKEGFTFPGRDPRATIQEQGTLQIKNLRIS
DTGTYTCVATSSSGETSWSAVLDVTESGATISKNYDLSDLPGPPSKPQVTDVTKNSVTLSWQ
PGTPGTLPASAYIIEAFSQSVSNSWQTVANHVKTTLYTVRGLRPNTIYLFMVRAINPQGLSDPS
PMSDPVRTQDISPPAQGVDHRQVQKELGDVLVRLHNPVVLTPTTVQVTWTVDRQPQFIQGY
RVMYRQTSGLQATSSWQNLDAKVPTERSAVLVNLKKGVTYEIKVRPYFNEFQGMDSESKTV
RTTEEAPSAPPQSVTVLTVGSYNSTSISVSWDPPPPDHQNGIIQEYKIWCLGNETRFHINKTVD
AAIRSVIIGGLFPGIQYRVEVAASTSAGVGVKSEPQPIIIGRRNEVVITENNNSITEQITDVVKQP
AFIAGIGGACWVILMGFSIWLYWRRKKRKGLSNYAVTFQRGDGGLMSNGSRPGLLNAGDPS
YPWLADSWPATSLPVNNSNSGPNEIGNFGRGDVLPPVPGQGDKTATMLSDGAIYSSIDFTTKT
SYNSSSQITQATPYATTQILHSNSIHELAVDLPDPQWKSSIQQKTDLMGFGYSLPDQNKGNNG

GKGGKKKKNKNSSKPQKNNGSTWANVPLPPPPVQPLPGTELEHYAVEQQENGYDSDSWCPP
LPVQTYLHQGLEDELEEDDDRVPTPPVRGVASSPAISFGQQSTATLTPSPREEMQPMLQAHLD
ELTRAYQFDIAKQTWHIQSNNQPPQPPVPPLGYVSGALISDLETDVADDDADDEEEALEIPRP
LRALDQTPGSSMDNLDSSVTGKAFTSSQRPRPTSPFSTDSNTSAALSQSQRPRPTKKHKGGRM
DQQPALPHRREGMTDEEALVPYSKPSFPSPGGHSSSGTASSKGSTGPRKTEVLRAGHQRNAS
DLLDIGYMGSNSQGQFTGEL (*Homo sapiens* roundabout homolog 2 isoform ROBO2a; **SEQ ID NO: 1**), as described by, *e.g.*, NP_001122401.1 and encoded by NM_001128929.2 (**SEQ ID NO: 2**); or
MSLLMFTQLLLCGFLYVRVDGSRLRQEDFPPRIVEHPSDVIVSKGEPTTLNCKAEGRPTPTIE
WYKDGERVETDKDDPRSHRMLLPSGSLFFLRIVHGRRSKPDEGSYVCVARNYLGEAVSRNA
SLEVALLRDDFRQNPTDVVVAAGEPAILECQPPRGHPEPTIYWKKDKVRIDDKEERISIRGGK
LMISNTRKSDAGMYTCVGTNMVGERDSDPAELTVFERPTFLRRPINQVVLEEEAVEFRCQVQ
GDPQPTVRWKKDDADLPRGRYDIKDDYTLRIKKTMSTDEGTYMCIAENRVGKMEASATLTV
RAPPQFVVRPRDQIVAQGRTVTFPCETKGNPQPAVFWQKEGSQNLLFPNQPQQPNSRCSVSP
TGDLTITNIQRSDAGYYICQALTVAGSILAKAQLEVTDVLTDRPPPIILQGPANQTLAVDGTAL
LKCKATGDPLPVISWLKEGFTFPGRDPRATIQEQGTLQIKNLRISDTGTYTCVATSSSGETSWS
AVLDVTESGATISKNYDLSDLPGPPSKPQVTDVTKNSVTLSWQPGTPGTLPASAYIIEAFSQSV
SNSWQTVANHVKTTLYTVRGLRPNTIYLFMVRAINPQGLSDPSPMSDPVRTQDISPPAQGVD
HRQVQKELGDVLVRLHNPVVLTPTTVQVTWTVDRQPQFIQGYRVMYRQTSGLQATSSWQN
LDAKVPTERSAVLVNLKKGVTYEIKVRPYFNEFQGMDSESKTVRTTEEAPSAPPQSVTVLTV
GSYNSTSISVSWDPPPPDHQNGIIQEYKIWCLGNETRFHINKTVDAAIRSVIIGGLFPGIQYRVE
VAASTSAGVGVKSEPQPIIIGRRNEVVITENNNSITEQITDVVKQPAFIAGIGGACWVILMGFSI
WLYWRRKKRKGLSNYAVTFQRGDGGLMSNGSRPGLLNAGDPSYPWLADSWPATSLPVNNS
NSGPNEIGNFGRGDVLPPVPGQGDKTATMLSDGAIYSSIDFTTKTSYNSSSQITQATPYATTQI
LHSNSIHELAVDLPDPQWKSSIQQKTDLMGFGYSLPDQNKGNNGGKGGKKKKNKNSSKPQK
NNGSTWANVPLPPPPVQPLPGTELEHYAVEQQENGYDSDSWCPPLPVQTYLHQGLEDELEED
DDRVPTPPVRGVASSPAISFGQQSTATLTPSPREEMQPMLQAHLDELTRAYQFDIAKQTWHIQ
SNNQPPQPPVPPLGYVSGALISDLETDVADDDADDEEEALEIPRPLRALDQTPGSSMDNLDSS
VTGKAFTSSQRPRPTSPFSTDSNTSAALSQSQRPRPTKKHKGGRMDQQPALPHRREGMTDEE
ALVPYSKPSFPSPGGHSSSGTASSKGSTGPRKTEVLRAGHQRNASDLLDIGYMGSNSQGQFTG
EL (*Homo sapiens* roundabout homolog 2 isoform ROBO2b; **SEQ ID NO: 3**), as described by, *e.g.*, NP_002933.1 and encoded by NM_002942.4 (**SEQ ID NO: 4**), together with any naturally occurring allelic, splice variants, and processed forms thereof. Typically, ROBO2 refers to human ROBO2. The ROBO2 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, and *C.elegans*. Specific residues of ROBO2 can be referred to as, for example, "ROBO2(30)."

[0073] Specific domains of ROBO2 can be referred to by such nomenclature as well. The N-terminal or "extracellular domain of ROBO2", comprising the five immunoglobulin motifs and three

fibronectin type III (FNIII) repeats can be referred to as ROBO2(46-848) of SEQ ID NO: 1 or ROBO2(30-832) of SEQ ID NO: 3, for example. The immunoglobulin (Ig) motifs 1 and 2 that interact with Slit2, or the "Ig SLIT binding domain" can be referred to as ROBO2(46-145) and ROBO2(151-237) respectively of SEQ ID NO: 1, and ROBO2(30-129) and ROBO2(135-221) respectively of SEQ ID NO: 3. Similarly, the "intracellular domain" comprising the "Nck intracellular binding domain," which comprises the four intracellular proline rich motifs, described herein, can be referred to as ROBO2(881-1378) of SEQ ID NO: 3.

[0074] As used herein, the terms "ROBO2 inhibitor," "ROBO2 antagonist," "ROBO2 inhibitor agent," and "ROBO2 antagonist agent" refer to a molecule or agent that significantly blocks, inhibits, reduces, or interferes with ROBO2 (mammalian, such as human, ROBO2) biological activity *in vitro*, *in situ*, and/or *in vivo*, including activity of downstream pathways mediated by ROBO2 signaling, such as, for example, ROBO2 interaction with the adaptor protein Nck and/or complex formation with nephrin, SLIT2-ROBO-2 mediated inhibition of nephrin-mediated actin polymerization, and/or elicitation of a cellular response to ROBO2. The term "agent" as used herein in reference to a ROBO2 inhibitor means any compound or substance such as, but not limited to, a small molecule, nucleic acid, polypeptide, peptide, drug, ion, etc. An "agent" can be any chemical, entity, or moiety, including, without limitation, synthetic and naturally-occurring proteinaceous and non-proteinaceous entities. In some embodiments of the aspects described herein, an agent is a nucleic acid, a nucleic acid analogue, a protein, an antibody, a peptide, an aptamer, an oligomer of nucleic acids, an amino acid, or a carbohydrate, and includes, without limitation, proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, antisense RNAs, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof etc. Compounds for use in the therapeutic compositions and methods described herein can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds, using screening methods known to one of ordinary skill in the art.

[0075] Exemplary ROBO2 inhibitors contemplated for use in the various aspects and embodiments described herein include, but are not limited to, anti-ROBO2 antibodies or antigen-binding fragments thereof that specifically bind to ROBO2; anti-sense molecules directed to a nucleic acid encoding ROBO2 (*e.g.*., ROBO2a or ROBO2b or both); short interfering RNA ("siRNA") molecules directed to a nucleic acid encoding ROBO2 (*e.g.*., ROBO2a or ROBO2b or both); RNA or DNA aptamers that bind to ROBO2, and inhibit/reduce/block ROBO2 mediated signaling; ROBO2 structural analogs; and soluble ROBO2 proteins, inhibitory polypeptides, *e.g.*, dominant negative polypeptides, or fusion polypeptides thereof. In some embodiments of these aspects and all such aspects described herein, a ROBO2 inhibitor (*e.g.*, an antibody or antigen-binding fragment thereof) binds (physically interacts with) ROBO2, targets downstream ROBO2 signaling, and/or inhibits (reduces) ROBO2 synthesis, production or release. In some embodiments of these aspects and all such aspects described herein, a ROBO2 inhibitor binds and prevents its binding a SLIT protein ligand, such as SLIT2. In some embodiments of these aspects and all such aspects described herein, a ROBO2 inhibitor

specifically reduces or eliminates expression (*i.e.*, transcription or translation) of one or more ROBO2 isoforms.

[0076] As used herein, a ROBO2 inhibitor or antagonist has the ability to reduce the activity and/or expression of ROBO2 in a cell (*e.g.*, podocytes) by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95 %, at least 98%, at least 99%, or more, relative to the activity or expression level in the absence of the ROBO2 inhibitor.

[0077] Accordingly, in some embodiments of the compositions and methods described herein, the ROBO2 inhibitor inhibits ROBO2 mediated signal transduction. In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor targets ROBO2 interaction with the adaptor protein Nck and/or complex formation with nephrin, SLIT2-ROBO-2 mediated inhibition of nephrin-mediated actin polymerization, and/or elicitation of a cellular response to ROBO2.

[0078] In some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitors, such as an antibody or antigen-binding fragment thereof, are directed against a ROBO2 ligand interaction site, such as a SLIT2 ligand interaction site. In some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitor, such as an antibody or antigen-binding fragment thereof, are directed against a ROBO2 adaptor interaction site such as an Nck interaction site or the NCK intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2. In some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitors are directed against a site on a target in the proximity of the ligand interaction site, in order to provide steric hindrance for the interaction of the receptor (e.g., ROBO2) with its ligand (e.g., SLIT2). By binding to a ROBO2 ligand interaction site, a ROBO2 inhibitor described herein can reduce or inhibit the activity or expression of ROBO2, and downstream ROBO2 signaling consequences (e.g., ROBO2 interaction with the adaptor protein Nck and/or complex formation with nephrin, SLIT2-ROBO-2 mediated inhibition of nephrin-mediated actin polymerization, and/or elicitation of a cellular response to ROBO2). For example, in some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitors block or target at least the Ig1, and preferably both the Ig1 and Ig2 sites, on ROBO2, i.e., ROBO2(46-145) and ROBO2(151-237) respectively of SEQ ID NO: 1, and ROBO2(30-129) and ROBO2(135-221) respectively of SEQ ID NO: 3, for example. In some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitors block or target the ROBO2 intracellular domain comprising the Nck intracellular binding domain, i.e., ROBO2(881-1378) of SEQ ID NO: 3. In some embodiments of the compositions and methods described herein, the binding sites of the ROBO2 inhibitors block or target the ROBO2 Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2. This can

be accomplished by a variety of means well known in the art, such as antibodies and antigen-binding fragments thereof, inhibitor RNAs, etc., and as described herein.

[0079] Accordingly, in some embodiments of the compositions and methods described herein, the ROBO2 inhibitor is an antibody or antigen-binding fragment thereof that selectively binds or physically interacts with ROBO2. In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor is an antibody or antigen-binding fragment thereof that binds to ROBO2 and inhibits and/or blocks and/or prevents interaction with Nck and/or complex formation with nephrin. In some embodiments of the compositions and methods described herein, the antibody or antigen-binding fragment thereof binds to the Ig SLIT binding domain of ROBO2. In some embodiments of the compositions and methods described herein, the antibody or antigen-binding fragment thereof binds to the Ig1SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2, i.e., ROBO2(46-145) and ROBO2(151-237) respectively of SEQ ID NO: 1, and ROBO2(30-129) and ROBO2(135-221) respectively of SEQ ID NO: 3. In some embodiments of the compositions and methods described herein, the antibody or antigen-binding fragment thereof binds to or blocks the ROBO2 intracellular domain, i.e., ROBO2(881-1378) of SEQ ID NO: 3. In some embodiments of the compositions and methods described herein, the antibody or antigen-binding fragment thereof binds to or blocks the Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2.

[0080] Antibodies specific for or that selectively bind ROBO2, suitable for use in the compositions and for practicing the methods described herein are preferably monoclonal, and can include, but are not limited to, human, humanized or chimeric antibodies, comprising single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and/or binding fragments of any of the above. Antibodies also refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain antigen or target binding sites or "antigen-binding fragments." The immunoglobulin molecules described herein can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule, as is understood by one of skill in the art.

[0081] Accordingly, in some embodiments of the compositions and methods described herein, a ROBO2 inhibitor as described herein is a monoclonal anti-ROBO2 antibody or antigen-binding fragment.

[0082] In some embodiments of the compositions and methods described herein, a ROBO2 inhibitor as described herein is a ROBO2 antibody fragment or antigen-binding fragment. The terms "antibody fragment," "antigen binding fragment," and "antibody derivative" as used herein, refer to a protein fragment that comprises only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the terms antibody fragment or antigen-binding fragment include: (i) the Fab fragment, having V_L, C_L, V_H and C_H1 domains; (ii) the Fab' fragment, which is a Fab fragment

having one or more cysteine residues at the C-terminus of the C_H1 domain; (iii) the Fd fragment having V_H and C_H1 domains; (iv) the Fd' fragment having V_H and C_H1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the V_L and V_H domains of a single arm of an antibody; (vi) a dAb fragment (Ward *et al.*, Nature 341, 544-546 (1989)) which consists of a V_H domain or a V_L domain ; (vii) isolated CDR regions; (viii) $F(ab')_2$ fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (*e.g.* single chain Fv; scFv) (Bird *et al.*, Science 242:423-426 (1988); and Huston *et al.*, PNAS (USA) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (see, *e.g.*, EP 404,097; WO 93/11161; and Hollinger *et al.*, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata *et al.* Protein Eng. 8(10):1057-1062 (1995); and U.S. Pat. No. 5,641,870); and modified versions of any of the foregoing (*e.g.*, modified by the covalent attachment of polyalkylene glycol (*e.g.*, polyethylene glycol, polypropylene glycol, polybutylene glycol) or other suitable polymer).

[0083] In some embodiments of the compositions and methods described herein, a ROBO2 inhibitor or antagonist is a chimeric antibody derivative of a ROBO2 antagonist antibody or antigen-binding fragment thereof.

[0084] The ROBO2 inhibitor or antagonist antibodies and antigen-binding fragments thereof described herein can also be, in some embodiments, a humanized antibody derivative.

[0085] In some embodiments, the ROBO2 inhibitor or antagonist antibodies and antigen-binding fragments thereof described herein include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody, provided that the covalent attachment does not prevent the antibody from binding to the target antigen, e.g., ROBO2.

[0086] In some embodiments of the compositions and methods described herein, completely human antibodies are used, which are particularly desirable for the therapeutic treatment of human patients.

[0087] In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor comprises at least one antisense molecule capable of blocking or decreasing the expression of a particular functional ROBO2 by targeting nucleic acids encoding ROBO2, *e.g.*, SEQ ID NO: 2 or SEQ ID NO: 4 or both, or relevant domains thereof. In some embodiments of the compositions and methods described herein, the at least one antisense molecule targets nucleic acids encoding the Ig SLIT binding domain of ROBO2. In some embodiments of the compositions and methods described herein, the at least one antisense molecule targets nucleic acids encoding theIg1 SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2. In some embodiments of the compositions and methods described herein, the at least one antisense molecule targets nucleic acids

encoding the ROBO2 intracellular domain. In some embodiments of the compositions and methods described herein, the at least one antisense molecule targets nucleic acids encoding the Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2.Methods are known to those of ordinary skill in the art for the preparation of antisense oligonucleotide molecules that will specifically bind ROBO2 mRNA without cross-reacting with other polynucleotides. Exemplary sites of targeting include, but are not limited to, the initiation codon, the 5' regulatory regions, including promoters or enhancers, the coding sequence, including any conserved consensus regions, and the 3' untranslated region. In some embodiment of these aspects and all such aspects described herein, the antisense oligonucleotides are about 10 to about 100 nucleotides in length, about 15 to about 50 nucleotides in length, about 18 to about 25 nucleotides in length, or more. In certain embodiments, the antisense oligonucleotides further comprise chemical modifications to increase nuclease resistance and the like, such as, for example, phosphorothioate linkages and 2'-O-sugar modifications known to those of ordinary skill in the art.

[0088] In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor comprises at least one short interfering RNA (siRNA) molecule capable of blocking or decreasing the expression of functional ROBO2 by targeting nucleic acids encoding or both isoforms of ROBO2, *e.g.*, SEQ ID NO: 2 or SEQ ID NO: 4, or relevant domains thereof. In some embodiments of the compositions and methods described herein, the at least one siRNA molecule targets nucleic acids encoding the Ig SLIT binding domain of ROBO2. In some embodiments of the compositions and methods described herein, the at least one siRNA molecule targets nucleic acids encoding theIg1 SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2. In some embodiments of the compositions and methods described herein, the at least one siRNA molecule targets nucleic acids encoding the ROBO2 intracellular domain. In some embodiments of the compositions and methods described herein, the at least one siRNA molecule targets nucleic acids encoding the Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2. It is routine to prepare siRNA molecules that will specifically target ROBO2 mRNA without cross-reacting with other polynucleotides. siRNA molecules for use in the compositions and methods described herein can be generated by methods known in the art, such as by typical solid phase oligonucleotide synthesis, and often will incorporate chemical modifications to increase half-life and/or efficacy of the siRNA agent, and/or to allow for a more robust delivery formulation. Alternatively, siRNA molecules are delivered using a vector encoding an expression cassette for intracellular transcription of siRNA.

[0089] In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor is an RNA or DNA aptamer that binds to one or more isoforms of ROBO2. In some embodiments of the compositions and methods described herein, a ROBO2 inhibitor or antagonist is an RNA or DNA aptamer that binds or physically interacts with ROBO2, and blocks interactions between ROBO2 and a ligand or adaptor molecule, for example, SLIT2 or Nck, respectively. In some

embodiments of the compositions and methods described herein, a ROBO-2 inhibitor or antagonist is an RNA or DNA aptamer that binds or physically interacts with ROBO2, and reduces, impedes, or blocks downstream ROBO2 signaling, such as SLIT2-ROBO-2 mediated inhibition of nephrin-mediated actin polymerization, and/or elicitation of a cellular response to ROBO2. In some embodiments of the compositions and methods described herein, the RNA or DNA aptamer binds to or physically interacts with the Ig SLIT binding domain of ROBO2. In some embodiments of the compositions and methods described herein, the RNA or DNA aptamer binds to or physically interacts with the Ig1SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2, i.e., ROBO2(46-145) and ROBO2(151-237) respectively of SEQ ID NO: 1, and ROBO2(30-129) and ROBO2(135-221) respectively of SEQ ID NO: 3. In some embodiments of the compositions and methods described herein, the RNA or DNA aptamer binds to or physically interacts with the ROBO2 intracellular domain, i.e., ROBO2(881-1378) of SEQ ID NO: 3. In some embodiments of the compositions and methods described herein, the RNA or DNA aptamer binds to or physically interacts with or blocks the Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2.

[0090] In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor is a small molecule compound or agent that targets or binds to ROBO2, including, but not limited to, small peptides or peptide-like molecules, soluble peptides, and synthetic non-peptidyl organic or inorganic compounds. As used herein, the term "small molecule" refers to a chemical agent which can include, but is not limited to, a peptide, a peptidomimetic, an amino acid, an amino acid analog, a polynucleotide, a polynucleotide analog, an aptamer, a nucleotide, a nucleotide analog, an organic or inorganic compound (e.g., including heterorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. Exemplary sites of small molecule binding include, but are not limited to, the portion of ROBO2 that binds to SLIT2 or to the adaptor Nck, i.e., the Ig1SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2, the ROBO2 intracellular domain or the Nck intracellular binding domain comprising the four intracellular proline rich motifs of ROBO2.

[0091] In some embodiments of the compositions and methods described herein, a ROBO2 inhibitor or antagonist comprises a small molecule that binds to ROBO2 and inhibits ROBO2 biological activity.

[0092] In some embodiments of the compositions and methods described herein, the ROBO2 inhibitor or antagonist comprises at least one ROBO2 structural analog, such as a dominant negative ROBO2 polypeptide. The term ROBO2 structural analogs, as used herein, refers to compounds that

have a similar three dimensional structure as part of that of ROBO2 and which bind to SLIT2 and/or to Nck under physiological conditions *in vitro* or *in vivo*, wherein the binding at least partially inhibits a ROBO2 biological activity, such as SLIT2-ROBO2 mediated inhibition of nephrin-mediated actin polymerization, and/or elicitation of a cellular response to ROBO2. Suitable ROBO2 structural analogs can be designed and synthesized through molecular modeling of ROBO2-SLIT2 binding, for example. The ROBO2 structural analogs can be monomers, dimers, or higher order multimers in any desired combination of the same or different structures to obtain improved affinities and biological effects.

[0093] In some embodiments of the compositions and methods described herein, a ROBO2 inhibitor or antagonist comprises at least one soluble ROBO2 receptor or fusion polypeptide thereof, such as, for example, a ROBO2 inhibitory polypeptide. In some such embodiments, the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein. In some embodiments of the compositions and methods described herein, the ROBO2 inhibitory polypeptide comprises the ROBO2 extracellular domain, for example, the Ig1SLIT binding domain of ROBO2 or both the Ig1 and Ig2 SLIT binding domains of ROBO2, with no intracellular ROBO2 domains.

[0094] ROBO2 inhibitors or antagonists for use in the compositions and methods described herein can be identified or characterized using methods known in the art, such as protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which are well known in the art, including, but not limited to, those described herein in the Examples.

[0095] For example, to identify a molecule that inhibits interaction between ROBO2 and its ligand, e.g., SLIT2, binding assays can be used. For example, ROBO2 or SLIT is immobilized on a microtiter plate by covalent or non-covalent attachment. The assay is performed by adding the non-immobilized component (ligand or receptor), which can be labeled by a detectable label, to the immobilized component, in the presence or absence of a test agent. When the reaction is complete, the non-reacted components are removed and binding complexes are detected. If formation of binding complexes is inhibited by the presence of the test agent, the test agent can be deemed a candidate antagonist that inhibits binding between ROBO2 and SLIT2, for example. Cell-based or membrane-based assays can also be used to identify ROBO2 inhibitors. In other embodiments, by detecting and/or measuring levels of ROBO2 gene expression, ROBO2 inhibitor molecules that inhibit ROBO2 gene expression can be tested. ROBO2 gene expression can be detected and/or measured by a variety of methods, such as real time RT-PCR, enzyme-linked immunosorbent assay ("ELISA"), Northern blotting, or flow cytometry, and as known to one of ordinary skill in the art.

[0096] Such identified ROBO2 inhibitors can further be tested using *in vivo* animal models of chronic kidney disease, such as glomerular and interstitial injury models (e.g., animal models of lupus nephritis, including mice of the NZB, (NZB x NZW) F1 hybrid (termed NZB/W), and congenic derivatives thereof, MRL/lpr and BXSB strains), animal models of aging (e.g., aged Sprague Dawley rats and aged C57BL/6 mice); spontaneously hypertensive rats (SHR); Buffalo/mna rats, which are a model of human idiopathic nephrotic syndrome; Munich Wistar Frömter (MWF) rats, which are a

genetic model related to a congenital deficit in nephron number being predisposed to the development of hypertension and salt sensitivity in adulthood; primary podocyte-specific genetic FSGS models; HIV-associated nephropathy (HIVAN) transgenic mouse models; animal models of Alport syndrome, which comprise mutations of the α3, α4, or α5 chains of type IV collagen (COL4A3, COL4A4, and COL4A5); immune-induced models, such as the Thy-1 nephritis model, which is an experimental rat model of mesangioproliferative glomerulonephritis (MsPGN), anti-glomerular basement membrane (GBM) models; and non-immune induced models.

[0097] As used herein, in regard to a ROBO2 inhibitor, "selectively binds" or "specifically binds" or "specific for" refers to the ability of a ROBO2 inhibitor as described herein , such as, for example, a ROBO2 antagonist antibody or ROBO2 antigen-binding fragment thereof, to bind to a target, i.e., ROBO2, with a K_D 10^{-5} M (10000 nM) or less, *e.g.*, 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, or 10^{-12} M or less. For example, if a ROBO2 inhibitor/antagonist described herein binds to ROBO2 with a K_D of 10^{-5} M or lower, but not to a related molecule, such as, for example, other ROBO family members, then the agent is said to specifically bind ROBO2. Specific binding can be influenced by, for example, the affinity and avidity of, for example, the ROBO2 inhibitor/antagonist antibody or antigen-binding fragment thereof and the concentration of polypeptide agent. The person of ordinary skill in the art can determine appropriate conditions under which the polypeptide agents described herein selectively bind the targets using any suitable methods, such as titration of a polypeptide agent in a suitable cell binding assay.

[0098] In regard to the methods of treating chronic kidney disease by inhibiting ROBO2 activity, the term "chronic kidney disease" or CKD refers to renal diseases that slowly and progressively worsen over time due to the progressive loss of nephrons and consequent loss of renal function. In the early stages, there may be no symptoms. The loss of function usually takes months or years to occur. It may be so slow that symptoms do not appear until kidney function is less than one-tenth of normal. The final stage of chronic kidney disease is called end-stage renal disease (ESRD). At this stage, the kidneys are no longer able to remove enough wastes and excess fluids from the body. The patient needs dialysis or a kidney transplant. Diabetes, which leads to diabetic nephropathy, and high blood pressure are the two most common causes of chronic kidney disease and account for most cases. Other diseases and conditions that can damage the kidneys and lead to chronic kidney disease, include: autoimmune disorders (such as systemic lupus erythematosus and scleroderma); birth defects of the kidneys (such as polycystic kidney disease); certain toxic chemicals; glomerulonephritis; injury or trauma; kidney stones and infection; problems with the arteries leading to or inside the kidneys; some pain medications and other drugs (such as cancer drugs); reflux nephropathy (in which the kidneys are damaged by the backward flow of urine into the kidneys); etc. As used herein, "proteinuria" refers to the presence of an excess of serum proteins in the urine. Proteinuria can, in some embodiments, be indicative of kidney disease, but, by itself, is not conclusive.

[0099] Accordingly, in some embodiments of these aspects and all such aspects described herein, the subject having or at risk for a chronic kidney disease has diabetic nephropathy.

[00100] By "reduce" or "inhibit" in terms of the chronic kidney disease and proteinuria treatment methods described herein is meant the ability to cause an overall decrease preferably of 20% or greater, 30% or greater, 40% or greater, 45% or greater, more preferably of 50% or greater, of 55% or greater, of 60 % or greater, of 65% or greater, of 70% or greater, and most preferably of 75% or greater, 80% or greater, 85% or greater, 90% or greater, or 95% or greater, for a given parameter or symptom of a chronic kidney disease. Reduce or inhibit can refer to, for example, symptoms of the disorder being treated, for example, high blood pressure, protein in the urine, etc.

[00101] High blood pressure is almost always present during all stages of chronic kidney disease. A nervous system exam may show signs of nerve damage. The health care provider may hear abnormal heart or lung sounds when listening with a stethoscope. The early symptoms of chronic kidney disease are also symptoms of other illnesses. These symptoms can be the only signs of kidney disease until the condition is more advanced. Symptoms of chronic kidney disease can include: appetite loss; general ill feeling and fatigue; headaches; itching (pruritus) and dry skin; nausea; weight loss without trying to lose weight; etc. Other symptoms that can develop, especially when kidney function has gotten worse, include: abnormally dark or light skin; bone pain; brain and nervous system symptoms; drowsiness and confusion; problems concentrating or thinking; numbness in the hands, feet, or other areas; muscle twitching or cramps; breath odor; easy bruising, bleeding, or blood in the stool; excessive thirst; frequent hiccups; low level of sexual interest and impotence; stopping of menstrual periods (amenorrhea); shortness of breath; sleep problems, such as insomnia, restless leg syndrome, and obstructive sleep apnea; swelling of the feet and hands (edema); vomiting, typically in the morning.

[00102] Accordingly, in some embodiments of the methods described herein, an effective amount of a composition comprising a ROBO2 inhibitor described herein is administered to a subject in order to alleviate a symptom of chronic kidney disease. As used herein, "alleviating a symptom chronic kidney disease" is ameliorating any condition or symptom associated with the chronic kidney disease. Alternatively, alleviating a symptom of a chronic kidney disease can involve reducing one or more symptoms of the chronic kidney disease in the subject relative to an untreated control suffering from chronic kidney disease or relative to the subject prior to the treatment. As compared with an equivalent untreated control, or the subject prior to the treatment with the ROBO2 inhibitor, such reduction or degree of prevention is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or more, as measured by any standard technique. Desirably, the chronic kidney disease is significantly reduced or undetectable, as detected by any standard method known in the art, in which case the chronic kidney disease is considered to have been treated. A patient who is being treated for a chronic kidney disease is one who a medical practitioner has diagnosed as having such a condition. Diagnosis can be by any suitable means known to

one of ordinary skill in the art. Diagnosis and monitoring can involve, for example, detecting the level of specific proteins or molecules in a urine, blood, or serum sample, such as, for example, albumin, calcium, cholesterol, complete blood count (CBC), electrolytes, magnesium, phosphorous, potassium, sodium, or any combination thereof; assays to detect, for example, creatinine clearance; creatinine levels; BUN (blood urea nitrogen); through the use of specific techniques or procedures, such as an abdominal CT scan, abdominal MRI, abdominal ultrasound, kidney biopsy, kidney scan, kidney ultrasound; via detection of changes in results of assays or tests for erythropoietin, PTH; bone density test, or Vitamin D; or any combination of such detection methods and assays.

[00103] The terms "subject" and "individual" as used in regard to any of the methods described herein are used interchangeably herein, and refer to an animal, for example a human, recipient of the ROBO2 inhibitors described herein. For treatment of disease states which are specific for a specific animal such as a human subject, the term "subject" refers to that specific animal. The terms "non-human animals" and "non-human mammals" are used interchangeably herein, and include mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term "subject" also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a human, or other mammals such as a domesticated mammal, *e.g.* dog, cat, horse, and the like.

[00104] In some embodiments of these methods and all such methods described herein, the method further comprises administering to the subject an additional therapeutic agent, in addition to the ROBO2 inhibitor. Such an additional therapeutic agent can be co-administered with the ROBO2 inhibitor. As used herein, the phrase "co-administering" or to "co-administer" means the administration of a ROBO2 inhibitor described herein and another compound, e.g., a therapeutic agent, separately, simultaneously, and/or sequentially over a period of time as determined by a qualified care giver.

[00105] In some such embodiments, the additional therapeutic agent is an angiotensin-converting enzyme (ACE) inhibitor, an angiotensin II receptor blocker (ARB), or a mineralocorticoid receptor (MR) antagonist.

[00106] ACE inhibitors for use with the ROBO2 inhibitors described herein include, but are not limited to, benazepril (marketed in the U.S. as LOTENSIN™), captopril (marketed in the U.S. as CAPOTEN™), enalapril/enalaprilat (marketed in the U.S. as VASOTEC™ oral and injectable), fosinopril (marketed in the U.S. as MONOPRIL™), lisinopril (marketed in the U.S. as ZESTRIL™ and PRINIVIL™), moexipril (marketed in the U.S. as UNIVASC™), perindopril (marketed in the U.S. as ACEON™), quinapril (marketed in the U.S. as ACCUPRIL™), ramipril (marketed in the U.S. as ALTACE™), and trandolapril (marketed in the U.S. as MAVIK™). ARBs for use with the ROBO2 inhibitors described herein include candesartan (marketed in the U.S. as ATACAND™), irbesartan (marketed in the U.S. as AVAPRO™), olmesartan (marketed in the U.S. as BENICAR™), losartan (marketed in the U.S. as COZAAR™), valsartan (marketed in the U.S. as DIOVAN™), telmisartan (marketed in the U.S. as MICARDIS™), and eprosartan (marketed in the U.S. as TEVETEN™).

[00107] In some embodiments of these methods and all such methods described herein, the method further comprises administering to the subject an effective amount of a diuretic, in addition to the ROBO2 inhibitor. Diuretics include, but are not limited to, torsemide (marketed in the U.S. as DEMADEX™), furosemide (marketed in the U.S. as LASIX™), bumetanide (marketed in the U.S. as BUMEX™), ethacrynic acid (marketed in the U.S. as EDECRIN™), torsemide (marketed in the U.S. as DEMADEX™), amiloride, (marketed in the U.S. as MIDAMOR™), acetazolamide (marketed in the U.S. as DIAMOX™), pamabrom (marketed in the U.S. as AQUA-BAN™), mannitol (marketed in the U.S. as ARIDOL™ or OSMITROL™), traimterene (marketed in the U.S. as DYRENIUM™), spironolactone (marketed in the U.S. as ALDACTONE™), amiloride (marketed in the U.S. as MIDAMOR™), indapamide (marketed in the U.S. as LOZOL™), hydrochlorothiazide (marketed in the U.S. as HYDRODIURIL™), metolazone (marketed in the U.S. as ZAROXOLYN™ or MYKROX™), methylclothiazide (marketed in the U.S. as AQUATENSEN™ or ENDURON™), hydrocholorthiazide (marketed in the U.S. as AQUAZIDE H™ or ESIDRIX™ or MICROZIDE™), chlorothiazide (marketed in the U.S. as DIURIL™), bendroflumethiazide (marketed in the U.S. as NATURETIN™), polythiazide (marketed in the U.S. as RENESE™), hydroflumethiazide (marketed in the U.S. as SALURON™), and chlorthalidone (marketed in the U.S. as THALITONE™). For a complete listing also see, e.g., Physician's Desk Reference, 2012 Edition, PDR Network (2011).

[00108] As used herein, in regard to any of the compositions and methods comprising ROBO-2 inhibitors or combination treatments thereof described herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with, a disease or disorder. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a chronic kidney disease, such as, but not limited to, diabetic nephropathy. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of at least slowing of progress or worsening of symptoms that would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00109] The term "effective amount" as used herein refers to the amount of a ROBO-2 inhibitor described herein, needed to alleviate at least one or more symptom of the disease or disorder being treated, and relates to a sufficient amount of pharmacological composition to provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of the ROBO-2 inhibitor described herein, using the methods as disclosed herein, that is sufficient to provide a particular effect

when administered to a typical subject. An effective amount as used herein would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not possible to specify the exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[00110] Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the ROBO-2 inhibitor described herein, which achieves a half-maximal inhibition of measured function or activity) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. Depending on the type and severity of the chronic kidney disease, about 1 μg/kg to 100 mg/kg (e.g., 0.1-20 mg/kg) of a ROBO2 inhibitor described herein is an initial candidate dosage range for administration to the subject, whether, for example, by one or more separate administrations, or by continuous infusion.

Modes of Administration

[00111] The ROBO2 inhibitors or combination treatments thereof described herein can be administered to a subject in need thereof by any appropriate route which results in an effective treatment in the subject. As used herein, the terms "administering," and "introducing" are used interchangeably and refer to the placement of a ROBO-2 inhibitor into a subject by a method or route which results in at least partial localization of such agents at a desired site, such that a desired effect(s) is produced.

[00112] In some embodiments, the ROBO2 inhibitor is administered to a subject having a chronic kidney disease by any mode of administration that delivers the agent systemically or to a desired surface or target, and can include, but is not limited to, injection, infusion, instillation, and inhalation administration. To the extent that polypeptide agents can be protected from inactivation in the gut, oral administration forms are also contemplated. "Injection" includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. In some

embodiments, the ROBO-2 inhibitors for use in the methods described herein are administered by intravenous infusion or injection.

[00113] The phrases "parenteral administration" and "administered parenterally" as used herein, refer to modes of administration other than enteral and topical administration, usually by injection. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein refer to the administration of the ROBO-2 inhibitor, other than directly into a target site, tissue, or organ, such as a tumor site, such that it enters the subject's circulatory system and, thus, is subject to metabolism and other like processes.

[00114] For the clinical use of the methods described herein, administration of the ROBO-2 inhibitors described herein, can include formulation into pharmaceutical compositions or pharmaceutical formulations for parenteral administration, *e.g.*, intravenous; mucosal, *e.g.*, intranasal; ocular, or other mode of administration. In some embodiments, the ROBO-2 inhibitors described herein can be administered along with any pharmaceutically acceptable carrier compound, material, or composition which results in an effective treatment in the subject. Thus, a pharmaceutical formulation for use in the methods described herein can contain a ROBO-2 inhibitor, as described herein, in combination with one or more pharmaceutically acceptable ingredients.

[00115] The phrase "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, media, encapsulating material, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in maintaining the stability, solubility, or activity of, a ROBO-2 inhibitor. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) excipients, such as cocoa butter and suppository waxes; (8) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (9) glycols, such as propylene glycol; (10) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (11) esters, such as ethyl oleate and ethyl laurate; (12) agar; (13) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (14) alginic acid; (15) pyrogen-free water; (16) isotonic saline; (17) Ringer's solution; (19) pH buffered solutions; (20) polyesters, polycarbonates and/or polyanhydrides; (21) bulking agents, such as polypeptides and amino acids (22) serum components, such as serum albumin, HDL and LDL; (23) C2-C12 alcohols, such as

ethanol; and (24) other non-toxic compatible substances employed in pharmaceutical formulations. Release agents, coating agents, preservatives, and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein.

[00116] The ROBO-2 inhibitors described herein can be specially formulated for administration of the compound to a subject in solid, liquid or gel form, including those adapted for the following: (1) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (2) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (3) intravaginally or intrarectally, for example, as a pessary, cream or foam; (4) ocularly; (5) transdermally; (6) transmucosally; or (79) nasally. Additionally, a ROBO-2 inhibitor can be implanted into a patient or injected using a drug delivery system. See, for example, Urquhart, *et al.*, Ann. Rev. Pharmacol. Toxicol. 24: 199-236 (1984); Lewis, ed. "Controlled Release of Pesticides and Pharmaceuticals" (Plenum Press, New York, 1981); U.S. Pat. No. 3,773,919; and U.S. Pat. No. 35 3,270,960.

[00117] Further embodiments of the formulations and modes of administration of the compositions comprising the ROBO-2 inhibitors described herein, that can be used in the methods described herein are described below.

[00118] Parenteral Dosage Forms. Parenteral dosage forms of the ROBO-2 inhibitors can also be administered to a subject with a chronic kidney condition by various routes, including, but not limited to, subcutaneous, intravenous (including bolus injection), intramuscular, and intraarterial. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, controlled-release parenteral dosage forms, and emulsions.

[00119] Suitable vehicles that can be used to provide parenteral dosage forms of the disclosure are well known to those skilled in the art. Examples include, without limitation: sterile water; water for injection USP; saline solution; glucose solution; aqueous vehicles such as but not limited to, sodium chloride injection, Ringer's injection, dextrose Injection, dextrose and sodium chloride injection, and lactated Ringer's injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

[00120] In some embodiments, compositions comprising an effective amount of a ROBO2 inhibitor are formulated to be suitable for oral administration, for example as discrete dosage forms, such as, but not limited to, tablets (including without limitation scored or coated tablets), pills, caplets, capsules,

chewable tablets, powder packets, cachets, troches, wafers, aerosol sprays, or liquids, such as but not limited to, syrups, elixirs, solutions or suspensions in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion, or a water-in-oil emulsion. Such compositions contain a predetermined amount of the pharmaceutically acceptable salt of the disclosed compounds, and may be prepared by methods of pharmacy well known to those skilled in the art. See generally, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing, Easton, Pa. (1990).

[00121] Due to their ease of administration, tablets and capsules represent the most advantageous solid oral dosage unit forms, in which case solid pharmaceutical excipients are used. If desired, tablets can be coated by standard aqueous or nonaqueous techniques. These dosage forms can be prepared by any of the methods of pharmacy. In general, pharmaceutical compositions and dosage forms are prepared by uniformly and intimately admixing the active ingredient(s) with liquid carriers, finely divided solid carriers, or both, and then shaping the product into the desired presentation if necessary. In some embodiments, oral dosage forms are not used for the antibiotic agent.

[00122] Typical oral dosage forms of the compositions comprising an effective amount of a ROBO2 inhibitor are prepared by combining the pharmaceutically acceptable salt of the ROBO2 inhibitor in an intimate admixture with at least one excipient according to conventional pharmaceutical compounding techniques. Excipients can take a wide variety of forms depending on the form of the composition desired for administration. For example, excipients suitable for use in oral liquid or aerosol dosage forms include, but are not limited to, water, glycols, oils, alcohols, flavoring agents, preservatives, and coloring agents. Examples of excipients suitable for use in solid oral dosage forms (*e.g.*, powders, tablets, capsules, and caplets) include, but are not limited to, starches, sugars, microcrystalline cellulose, kaolin, diluents, granulating agents, lubricants, binders, and disintegrating agents.

[00123] Binders suitable for use in the pharmaceutical formulations described herein include, but are not limited to, corn starch, potato starch, or other starches, gelatin, natural and synthetic gums such as acacia, sodium alginate, alginic acid, other alginates, powdered tragacanth, guar gum, cellulose and its derivatives (*e.g.*, ethyl cellulose, cellulose acetate, carboxymethyl cellulose calcium, sodium carboxymethyl cellulose), polyvinyl pyrrolidone, methyl cellulose, pre-gelatinized starch, hydroxypropyl methyl cellulose, (*e.g.*, Nos. 2208, 2906, 2910), microcrystalline cellulose, and mixtures thereof.

[00124] Examples of fillers suitable for use in the pharmaceutical formulations described herein include, but are not limited to, talc, calcium carbonate (*e.g.*, granules or powder), microcrystalline cellulose, powdered cellulose, dextrates, kaolin, mannitol, silicic acid, sorbitol, starch, pre-gelatinized starch, and mixtures thereof. The binder or filler in pharmaceutical compositions described herein is typically present in from about 50 to about 99 weight percent of the pharmaceutical composition.

[00125] Disintegrants are used in the oral pharmaceutical formulations described herein to provide tablets that disintegrate when exposed to an aqueous environment. A sufficient amount of disintegrant

that is neither too little nor too much to detrimentally alter the release of the active ingredient(s) should be used to form solid oral dosage forms of the ROBO2 inhibitors described herein. The amount of disintegrant used varies based upon the type of formulation, and is readily discernible to those of ordinary skill in the art. Disintegrants that can be used to form oral pharmaceutical formulations include, but are not limited to, agar, alginic acid, calcium carbonate, microcrystalline cellulose, croscarmellose sodium, crospovidone, polacrilin potassium, sodium starch glycolate, potato or tapioca starch, other starches, pre-gelatinized starch, clays, other algins, other celluloses, gums, and mixtures thereof.

[00126] Lubricants that can be used to form oral pharmaceutical formulations of the ROBO2 inhibitors described herein, include, but are not limited to, calcium stearate, magnesium stearate, mineral oil, light mineral oil, glycerin, sorbitol, mannitol, polyethylene glycol, other glycols, stearic acid, sodium lauryl sulfate, talc, hydrogenated vegetable oil (*e.g.*, peanut oil, cottonseed oil, sunflower oil, sesame oil, olive oil, corn oil, and soybean oil), zinc stearate, ethyl oleate, ethyl laureate, agar, and mixtures thereof. Additional lubricants include, for example, a syloid silica gel (AEROSIL® 200, manufactured by W. R. Grace Co. of Baltimore, Md.), a coagulated aerosol of synthetic silica (marketed by Degussa Co. of Piano, Tex.), CAB-O-SIL® (a pyrogenic silicon dioxide product sold by Cabot Co. of Boston, Mass.), and mixtures thereof. If used at all, lubricants are typically used in an amount of less than about 1 weight percent of the pharmaceutical compositions or dosage forms into which they are incorporated.

[00127] In other embodiments, lactose-free pharmaceutical formulations and dosage forms are provided, wherein such compositions preferably contain little, if any, lactose or other mono- or di-saccharides. As used herein, the term "lactose- free" means that the amount of lactose present, if any, is insufficient to substantially increase the degradation rate of an active ingredient. Lactose-free compositions of the disclosure can comprise excipients which are well known in the art and are listed in the USP (XXI)/NF (XVI), which is incorporated herein by reference.

[00128] The oral formulations of the ROBO2 inhibitors further encompass, in some embodiments, anhydrous pharmaceutical compositions and dosage forms comprising the ROBO2 inhibitors described herein as active ingredients, since water can facilitate the degradation of some compounds. For example, the addition of water (*e.g.*, 5%) is widely accepted in the pharmaceutical arts as a means of simulating long-term storage in order to determine characteristics such as shelf life or the stability of formulations over time. See, *e.g.*, Jens T. Carstensen, Drug Stability: Principles & Practice, 379-80 (2nd ed., Marcel Dekker, NY, N.Y.: 1995). Anhydrous pharmaceutical compositions and dosage forms described herein can be prepared using anhydrous or low moisture containing ingredients and low moisture or low humidity conditions. Pharmaceutical compositions and dosage forms that comprise lactose and at least one active ingredient that comprises a primary or secondary amine are preferably anhydrous if substantial contact with moisture and/or humidity during manufacturing, packaging, and/or storage is expected. Anhydrous compositions are preferably packaged using materials known to prevent exposure to water such that they can be included in suitable formulary kits. Examples of suitable packaging

include, but are not limited to, hermetically sealed foils, plastics, unit dose containers (*e.g.*, vials) with or without desiccants, blister packs, and strip packs.

[00129] Aerosol formulations. A ROBO-2 inhibitor can be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. A ROBO-2 inhibitor can also be administered in a non-pressurized form such as in a nebulizer or atomizer. A ROBO-2 inhibitor can also be administered directly to the airways in the form of a dry powder, for example, by use of an inhaler.

[00130] Suitable powder compositions include, by way of illustration, powdered preparations of a ROBO-2 inhibitor, thoroughly intermixed with lactose, or other inert powders acceptable for intrabronchial administration. The powder compositions can be administered via an aerosol dispenser or encased in a breakable capsule which can be inserted by the subject into a device that punctures the capsule and blows the powder out in a steady stream suitable for inhalation. The compositions can include propellants, surfactants, and co-solvents and can be filled into conventional aerosol containers that are closed by a suitable metering valve.

[00131] Aerosols for the delivery to the respiratory tract are known in the art. See for example, Adjei, A. and Garren, J. Pharm. Res., 1: 565-569 (1990); Zanen, P. and Lamm, J.-W. J. Int. J. Pharm., 114: 111-115 (1995); Gonda, I. "Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract," in Critical Reviews in Therapeutic Drug Carrier Systems, 6:273-313 (1990); Anderson *et al.*, Am. Rev. Respir. Dis., 140: 1317-1324 (1989)) and have potential for the systemic delivery of peptides and proteins as well (Patton and Platz, Advanced Drug Delivery Reviews, 8:179-196 (1992)); Timsina et. al., Int. J. Pharm., 101: 1-13 (1995); and Tansey, I. P., Spray Technol. Market, 4:26-29 (1994); French, D. L., Edwards, D. A. and Niven, R. W., Aerosol Sci., 27: 769-783 (1996); Visser, J., Powder Technology 58: 1-10 (1989)); Rudt, S. and R. H. Muller, J. Controlled Release, 22: 263-272 (1992); Tabata, Y, and Y. Ikada, Biomed. Mater. Res., 22: 837-858 (1988); Wall, D. A., Drug Delivery, 2: 10 1-20 1995); Patton, J. and Platz, R., Adv. Drug Del. Rev., 8: 179-196 (1992); Bryon, P., Adv. Drug. Del. Rev., 5: 107-132 (1990); Patton, J. S., *et al.*, Controlled Release, 28: 15 79-85 (1994); Damms, B. and Bains, W., Nature Biotechnology (1996); Niven, R. W., *et al.*, Pharm. Res., 12(9); 1343-1349 (1995); and Kobayashi, S., *et al.*, Pharm. Res., 13(1): 80-83 (1996), contents of all of which are herein incorporated by reference in their entirety.

[00132] The formulations of the ROBO-2 inhibitors described herein further encompass anhydrous pharmaceutical compositions and dosage forms comprising the disclosed compounds as active ingredients, since water can facilitate the degradation of some compounds. For example, the addition of water (*e.g.*, 5%) is widely accepted in the pharmaceutical arts as a means of simulating long-term storage in order to determine characteristics such as shelf life or the stability of formulations over time. See, *e.g.*, Jens T. Carstensen, Drug Stability: Principles & Practice, 379-80 (2nd ed., Marcel Dekker, NY, N.Y.: 1995). Anhydrous pharmaceutical compositions and dosage forms of the disclosure can be prepared using anhydrous or low moisture containing ingredients and low moisture or low humidity

conditions. Pharmaceutical compositions and dosage forms that comprise lactose and at least one active ingredient that comprises a primary or secondary amine are preferably anhydrous if substantial contact with moisture and/or humidity during manufacturing, packaging, and/or storage is expected. Anhydrous compositions are preferably packaged using materials known to prevent exposure to water such that they can be included in suitable formulary kits. Examples of suitable packaging include, but are not limited to, hermetically sealed foils, plastics, unit dose containers (*e.g.*, vials) with or without desiccants, blister packs, and strip packs.

[00133] Controlled and Delayed Release Dosage Forms. In some embodiments of the aspects described herein, a ROBO-2 inhibitor can be administered to a subject by controlled- or delayed-release means. Ideally, the use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include: 1) extended activity of the drug; 2) reduced dosage frequency; 3) increased patient compliance; 4) usage of less total drug; 5) reduction in local or systemic side effects; 6) minimization of drug accumulation; 7) reduction in blood level fluctuations; 8) improvement in efficacy of treatment; 9) reduction of potentiation or loss of drug activity; and 10) improvement in speed of control of diseases or conditions. (Kim, Cherng-ju, Controlled Release Dosage Form Design, 2 (Technomic Publishing, Lancaster, Pa.: 2000)). Controlled-release formulations can be used to control a compound of formula (I)'s onset of action, duration of action, plasma levels within the therapeutic window, and peak blood levels. In particular, controlled- or extended-release dosage forms or formulations can be used to ensure that the maximum effectiveness of a compound of formula (I) is achieved while minimizing potential adverse effects and safety concerns, which can occur both from under-dosing a drug (*i.e.*, going below the minimum therapeutic levels) as well as exceeding the toxicity level for the drug.

[00134] A variety of known controlled- or extended-release dosage forms, formulations, and devices can be adapted for use with the ROBO-2 inhibitors described herein. Examples include, but are not limited to, those described in U.S. Pat. Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5674,533; 5,059,595; 5,591 ,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; 5,733,566; and 6,365,185 B1, each of which is incorporated herein by reference in their entireties. These dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydroxypropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems (such as OROS® (Alza Corporation, Mountain View, Calif. USA)), multilayer coatings, microparticles, liposomes, or microspheres or a combination thereof to provide the desired release profile in varying proportions. Additionally, ion exchange materials can be used to prepare immobilized, adsorbed salt forms of the disclosed compounds and thus effect controlled delivery of the drug. Examples of specific anion exchangers include, but are not limited to, DUOLITE® A568 and DUOLITE® AP143 (Rohm&Haas, Spring House, Pa. USA).

[00135] In some embodiments of the methods described herein, a ROBO-2 inhibitor for use in the methods described herein is administered to a subject by sustained release or in pulses. Pulse therapy is not a form of discontinuous administration of the same amount of a composition over time, but comprises administration of the same dose of the composition at a reduced frequency or administration of reduced doses. Sustained release or pulse administrations are particularly preferred when the disorder occurs continuously in the subject, for example where the subject has chronic kidney disease. Each pulse dose can be reduced and the total amount of a ROBO-2 inhibitor described herein administered over the course of treatment to the subject or patient is minimized.

[00136] The interval between pulses, when necessary, can be determined by one of ordinary skill in the art. Often, the interval between pulses can be calculated by administering another dose of the composition when the composition or the active component of the composition is no longer detectable in the subject prior to delivery of the next pulse. Intervals can also be calculated from the *in vivo* half-life of the composition. Intervals can be calculated as greater than the *in vivo* half-life, or 2, 3, 4, 5 and even 10 times greater the composition half-life. Various methods and apparatus for pulsing compositions by infusion or other forms of delivery to the patient are disclosed in U.S. Pat. Nos. 4,747,825; 4,723,958; 4,948,592; 4,965,251 and 5,403,590.

[00137] In some embodiments, sustained-release preparations comprising the ROBO-2 inhibitor can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the inhibitor, in which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

[00138] The formulations comprising the ROBO-2 inhibitors to be used for *in vivo* administration are preferably sterile. This is readily accomplished by filtration through, for example, sterile filtration membranes, and other methods known to one of skill in the art.

[00139] Also provided herein, in some aspects, are assays, methods, and systems for determining whether an individual has a chronic kidney disease or a pre-disposition for a chronic kidney disease or proteinuria based on expression profiles or sequence information of ROBO2 as a biomarker indicative of chronic kidney disease or a pre-disposition for a chronic kidney disease or proteinuria. As demonstrated herein, ROBO2 is useful as a biomarker to identify a subject having chronic kidney disease or at high risk for chronic kidney disease or proteinuria or to monitor the effects of treatment on the progression of chronic kidney disease or proteinuria.

[00140] As used herein, a "biomarker" refers to an organic biomolecule which is differentially present in a sample taken from a subject of one phenotypic status (e.g., having a disease) as compared

with another phenotypic status (e.g., not having the disease). A biomarker is differentially present between different phenotypic statuses if the mean or median expression level of the biomarker in the different groups is calculated to be statistically significant. Common tests for statistical significance include, among others, t-test, ANOVA, Kruskal-Wallis, Wilcoxon, Mann-Whitney and odds ratio. Biomarkers, alone or in combination, provide measures of relative risk that a-subject belongs to one phenotypic status or another. As such, they are useful as markers for disease (diagnostics), therapeutic effectiveness of a drug (theranostics) and of drug toxicity.

[00141] ROBO2 expression for use in the assays described herein can be detected by any suitable method, including detection or protein levels or detection of mRNA expression levels. ROBO2 polypeptide can be detected in any form that may be found in a biological sample obtained from a subject, or in any form that may result from manipulation of the biological sample (e.g., as a result of sample processing). Modified forms of ROBO2 can include modified proteins that are a product of allelic variants, splice variants, post-translational modification (e.g., glycosylation, proteolytic cleavage (e.g., fragments of a parent protein), glycosylation, phosphorylation, lipidation, oxidation, methylation, cysteinylation, sulphonation, acetylation, and the like), oligomerization, de-oligomerization (to separate monomers from a multimeric form of the protein), denaturation, and the like.

[00142] The assays described herein can be designed to detect all forms or particular forms of ROBO2. Where desired, differentiation between different forms of ROBO2, e.g., different isoforms, can be accomplished by use of detection methods dependent upon physical characteristics that differ between the forms, e.g., different molecular weight, different molecular size, presence/absence of different epitopes, and the like.

[00143] Accordingly, provided herein, in some aspects, are assays for the diagnosis of a subject at having chronic kidney disease or at risk for chronic kidney disease or proteinuria, the assay comprising: measuring the level of ROBO2 protein or nucleic acid in a biological sample obtained from the subject, wherein if the level of the ROBO2 in the biological sample from the subject is at the same level or greater than (e.g., greater than by a statistically significant amount) a threshold reference level for ROBO2, the subject likely is at risk for chronic kidney disease or proteinuria or has chronic kidney disease. For example, an increase in the level of ROBO2 by more than about 10%, or more than about 20%, or more than about 30%, or more than about 40%, or more than about 50%, or more than about 60%, or more, as compared to a reference threshold level of ROBO2. In some embodiments, the increase in the level of ROBO2 is at least one standard deviation greater than, or at least two standard deviations, or more, greater than a median or mean ROBO2 reference threshold level. Such median or mean ROBO2 reference levels can be obtained, for example, from five or more samples obtained from subjects not having chronic kidney disease or proteinuria, or from five or more samples obtained from the same subject at different timepoints.

[00144] In some embodiments of these assays, the amount of ROBO2 measured in a biological sample is compared to a reference threshold level, or a reference biological sample, such as biological

sample obtained from an age-matched normal control (e.g., an age-matched subject not having a risk of chronic kidney disease or proteinuria), or a healthy subject, e.g., a healthy individual.

[00145] In some embodiments, the assays, systems and kits as disclosed herein are also useful for monitoring a course of treatment being administered to a subject. For example, one can measure the level of ROBO2 in a biological sample in the subject at a first timepoint (e.g., t1) and compare with the ROBO2 biomarker reference threshold level, and if the measured level for ROBO2 is the same or higher than the reference threshold level, the subject can be administered an appropriate therapeutic treatment or regimen to delay or reduce the occurrence of chronic kidney disease or proteinuria, e.g., for example, increase exercise, reduce heart pressure, adjust diet etc. as disclosed in the methods herein, and then the level of the panel of ROBO2 biomarker protein can be measured at a second (e.g., t2) and subsequent timepoints (e.g., t3, t4, t5, t5 … etc.), and compared to levels of tROBO2 at one or more time points (e.g., at t1 or any subsequent timepoint) or the reference threshold levels of ROBO2 to determine if the therapeutic treatment or medical treatment or regimen for the treatment to reduce the risk of, delay, or reduce the occurrence of chronic kidney disease or proteinuria is effective. In some such embodiments, the assays, systems and kits as disclosed herein can be used to monitor a therapeutic treatment in a symptomatic subject (e.g., a subject known to have chronic kidney disease or proteinuria), where an effective treatment can be a decrease in ROBO2 in the subject, or alternatively the assays, systems and kits as disclosed herein can be used to monitor the effect of prophylactic treatment in an asymptomatic subject (e.g., to prevent chronic kidney disease or proteinuria occurring in a subject), for example, where the subject has been identified to be at risk of chronic kidney disease or proteinuria according to the methods as disclosed herein, or others known in the art, or due to hereditary reasons, for example.

[00146] The term "sample" as used herein generally refers to any material containing nucleic acid, either DNA or RNA, or amino acids. Generally, such material will be in the form of a blood sample, stool sample, tissue sample, cells, bacteria, histology section, or buccal swab. Samples can be prepared, for example samples can be fresh, fixed, frozen, or embedded in paraffin. The term "biological sample" as used herein refers to a cell or population of cells or a quantity of tissue or fluid from a subject. Most often, the sample has been removed from a subject, but the term "biological sample" can also refer to cells or tissue analyzed in vivo, i.e. without removal from the subject. Often, a "biological sample" will contain cells from the animal, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure gene expression levels. Biological samples include, but are not limited to, tissue biopsies, scrapes, whole blood, plasma, serum, urine, saliva, cell culture, or cerebrospinal fluid. Biological samples also include tissue biopsies, cell culture. A biological sample or tissue sample can refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, urine, blood, plasma, serum, kidney biopsy, stool, sputum, spinal fluid, pleural fluid, nipple aspirates, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, cells (including but not limited to

blood cells), tumors, organs, and also samples of an in vitro cell culture constituent. In some embodiments, where a urine sample is obtained, the urine sample is centrifuged to pellet any kidney cells, on which the assays and methods described herein can be performed. In some embodiments, the sample is from a kidney biopsy, such as a core needle biopsy of a kidney or portion thereof, such as a podocyte sample. In addition, fine needle aspirate samples are used. In some embodiments, the biological samples can be prepared, for example biological samples can be fresh, fixed, frozen, or embedded in paraffin. The sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (e.g. isolated by another person), or by performing the methods described herein *in vivo*.

[00147] The term “expression" as used herein refers to interchangeably to the expression of a polypeptide or protein or expression of a polynucleotide or expression of a gene. Expression also refers to the expression of pre-translational modified and post-translationally modified proteins, as well as expression of pre-mRNA molecules, alternatively spliced and mature mRNA molecules. Expression of a polynucleotide can be determined, for example, by measuring the production of RNA transcript molecules, for example messenger RNA (mRNA) transcript levels. Expression of a protein or polypeptide can be determined, for example, by immunoassay using an antibody(ies) that bind with the polypeptide. The term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide or protein if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed to produce the RNA which can be translated into an amino acid sequence to generate the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom. The term “endogenously expressed" or “endogenous expression" refers to the expression of a gene product at normal levels and under normal regulation for that cell type.

[00148] Detection methods that can be used with the assays, methods, and systems described herein to measure levels of ROBO2 protein or nucleic acid in a sample or biological sample include optical methods, electrochemical methods (voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods, e.g., multipolar resonance spectroscopy. Optical methods include microscopy, both confocal and non-confocal, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry).

[00149] In those embodiments of the assays, methods, and systems described herein in which the level of ROBO2 protein is determined, such as, for example, the level of a protein of SEQ ID NO: 1 or SEQ ID NO: 3, one can use any proteomic approach commonly known to persons of ordinary skill in the art to measure the level of biomarker proteins in a biological sample. The measurement can be either quantitative or qualitative, so long as the measurement is capable of determining or indicating whether

the level of ROBO2 protein in the biological sample is the same as, or above or below a reference threshold value for ROBO2 protein.

[00150] The measured level of ROBO2 protein can, in some embodiments, be a primary measurement of the level of ROBO2 protein measuring the quantity of ROBO2 protein itself, such as by detecting the number of ROBO2 protein molecules in the sample) or it can be, in some embodiments, a secondary measurement of ROBO2 protein (a measurement from which the quantity of ROBO2 protein can be but not necessarily deduced, such as a measure of functional activity or a measure of nucleic acid, such as mRNA, encoding ROBO2 protein). Qualitative data can also be derived or obtained from primary measurements.

[00151] In some embodiments of the assays and methods described herein, ROBO2 protein levels can be measured using an affinity-based measurement technology. "Affinity" as relates to an antibody is a term well understood in the art and means the extent, or strength, of binding of antibody to the binding partner, such as a biomarker as described herein (or epitope thereof). Affinity can be measured and/or expressed in a number of ways known in the art, including, but not limited to, equilibrium dissociation constant (K_D or Kd), apparent equilibrium dissociation constant ($K_{D'}$ or $K_{d'}$), and IC_{50} (amount needed to effect 50% inhibition in a competition assay; used interchangeably herein with "I50"). It is understood that, for purposes of this invention, an affinity is an average affinity for a given population of antibodies which bind to an epitope.

[00152] Affinity-based measurement technology utilizes a molecule that specifically binds to the biomarker protein being measured (an "affinity reagent," such as an antibody or aptamer), although other technologies, such as spectroscopy-based technologies (e.g., matrix-assisted laser desorption ionization-time of flight, MALDI-TOF spectroscopy) or assays measuring bioactivity (e.g., assays measuring mitogenicity of growth factors) can also be used. Affinity-based technologies for use with the assays and methods described herein can include antibody-based assays (immunoassays) and assays utilizing aptamers (nucleic acid molecules which specifically bind to other molecules), such as ELONA. Additionally, assays utilizing both antibodies and aptamers are also contemplated (e.g., a sandwich format assay utilizing an antibody for capture and an aptamer for detection). A wide variety of affinity-based assays are also known in the art.

[00153] Affinity-based assays typically utilize at least one epitope derived from the biomarker protein, i.e., ROBO2, and many affinity-based assay formats utilize more than one epitope (e.g., two or more epitopes are involved in "sandwich" format assays; at least one epitope is used to capture the biomarker protein, and at least one different epitope is used to detect the marker).

[00154] Affinity-based assays can be in competition or direct reaction formats, utilize sandwich-type formats, and can further be heterogeneous (e.g., utilize solid supports) or homogenous (e.g., take place in a single phase) and/or utilize immunoprecipitation. Many assays involve the use of labeled affinity reagent (e.g., antibody, polypeptide, or aptamer); the labels can be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the

signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA and ELONA assays. For example, the biomarker concentrations from biological fluid samples may be measured by LUMINEX® assay or ELISA. Either of the biomarker or reagent specific for the biomarker can be attached to a surface and levels can be measured directly or indirectly.

[00155] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels can be measured using an immunoassay affinity-based measurement technology.

[00156] Immunoassay technologies can include any immunoassay technology which can quantitatively or qualitatively measure the level of ROBO2 protein in a biological sample. Suitable immunoassay technologies include, but are not limited to radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blot analysis, immunoprecipitations, immunofluorescence assays, immunoelectrophoresis assays, fluoroimmunoassay (FiA), immunoradiometric assay (IRMA), immunoenzymometric assay (IEMA), immunoluminescence assay and immunofluorescence assay (Madersbacher S, Berger P. Antibodies and immunoassays. Methods 2000;21:41-50), chemiluminescent assay, immuno-PCR, and western blot assay. Likewise, aptamer-based assays which can quantitatively or qualitatively measure the level of a biomarker in a biological sample can be used in the assays, methods, and systems described herein. Generally, aptamers can be substituted for antibodies in nearly all formats of immunoassay, although aptamers allow additional assay formats (such as amplification of bound aptamers using nucleic acid amplification technology such as PCR (U.S. Pat. No. 4,683,202) or isothermal amplification with composite primers (U.S. Pat. Nos. 6,251,639 and 6,692,918).

[00157] In some embodiments of the assays, methods, and systems described herein, where ROBO2 protein levels are measured using an immunoassay affinity-based measurement technology, the immunoassay is performed by measuring the extent of the protein/antibody interaction of the biomarker/antibody interaction. Any known method of immunoassay can be used.

[00158] In some embodiments, a binding partner, e.g., an antibody or a ligand binding to the ROBO2 protein in the binding assay, is preferably a labeled specific binding partner, but not necessarily an antibody. The binding partner is usually labeled itself, but alternatively it can be detected by a secondary reaction in which a signal is generated, e.g. from another labeled substance.

[00159] Thus, the antibody which specifically binds to ROBO2 protein can be used in the assays, methods, and systems described herein to determine the presence and/or amount of ROBO2 protein n a biological sample, which can be used to detect the increased or decreased concentration of ROBO2 protein present in a diagnostic sample. Such antibodies can be raised by any of the methods well known in the immunodiagnostics field. The antibodies can be anti-ROBO2 protein antibodies to any biologically relevant state of the protein. Thus, for example, they could be raised against the

unglycosylated form of ROBO2 protein, which exists in the body in a glycosylated form, or against a peptide carrying a relevant epitope of ROBO2 protein.

[00160] In some embodiments of these assays, method, and systems, an amplified assay form can be used, whereby an enhanced "signal" is produced from a relatively low level of protein to be detected. One particular form of an amplified immunoassay is enhanced chemiluminescent assay. For example, the antibody is labeled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

[00161] In some embodiments of these assays, method, and systems, an amplified immunoassay can be used comprising immuno-PCR. In this technique, the antibody is covalently linked to a molecule of arbitrary DNA comprising PCR primers, whereby the DNA with the antibody attached to it is amplified by the polymerase chain reaction. See E. R. Hendrickson et al., Nucleic Acids Research 23: 522-529 (1995).

[00162] Accordingly, in all embodiments of the assays, method, and systems described herein, the level of ROBO2 protein can be determined using a protein-binding agent, also referred to herein as "protein-binding entity" or an "affinity reagent" can be used, in particular, antibodies. For instance, the affinity reagents, in particular, antibodies such as anti-biomarker antibodies can be used in an immunoassay, particularly in an ELISA (Enzyme Linked Immunosorbent Assay). In embodiments where the level of a biomarker protein can be measured in a biological sample using methods commonly known in the art, and including, for example but not limited to isoform-specific chemical or enzymatic cleavage of isoform proteins, immunobloting, immunohistochemical analysis, ELISA, and mass spectrometry.

[00163] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using "Enzyme-Linked Immunosorbent Assay (ELISA)." ELISA is a technique for detecting and measuring the concentration of an antigen using a labeled (e.g. enzyme linked) form of the antibody. There are different forms of ELISA, which are well known to those skilled in the art. The standard techniques known in the art for ELISA are described in "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W. A. Benjamin, Inc., 1964; and Oellerich, M. 1984, J. Clin. Chem. Clin. Biochem., 22:895-904.

[00164] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using a sandwich assay ELISA. In a "sandwich ELISA", an antibody (e.g. anti-enzyme) is linked to a solid phase (i.e. a microtiter plate) and exposed to a biological sample containing antigen (e.g. enzyme). The solid phase is then washed to remove unbound antigen. A labeled antibody (e.g. enzyme linked) is then bound to the bound-antigen (if present) forming an antibody-antigen-antibody sandwich. Accordingly, using this method, a first antibody to ROBO2 protein is bound to the solid phase such as a well of a plastics microtiter plate, and incubated with the

sample and with a labeled second antibody specific to ROBO2 protein to be assayed. Examples of enzymes that can be linked to the antibody are alkaline phosphatase, horseradish peroxidase, luciferase, urease, and B-galactosidase. The enzyme linked antibody reacts with a substrate to generate a colored reaction product that can be measured.

[00165] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using an antibody capture assay or competitive ELISA. In a "competitive ELISA", antibody is incubated with a sample containing antigen (i.e. enzyme). The antigen-antibody mixture is then contacted with a solid phase (e.g. a microtiter plate) that is coated with antigen (i.e., enzyme). The more antigen present in the sample, the less free antibody that will be available to bind to the solid phase. A labeled (e.g., enzyme linked) secondary antibody is then added to the solid phase to determine the amount of primary antibody bound to the solid phase. Accordingly, in some such embodiments, a biological test sample is allowed to bind to a solid phase, and the anti-ROBO2 protein antibody (e.g., antibodies that specifically bind ROBO2 protein) can be added and allowed to bind. After washing away unbound material, the amount of antibody bound to the solid phase is determined using a labeled second antibody, anti- to the first.

[00166] In some embodiments of these assays, method, and systems, a label is preferably an enzyme. The substrate for the enzyme can be, for example, color-forming, fluorescent or chemiluminescent.

[00167] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using immunohistochemistry. In an "immunohistochemistry assay" a section of tissue is tested for specific proteins by exposing the tissue to antibodies that are specific for the protein that is being assayed. The antibodies are then visualized by any of a number of methods to determine the presence and amount of the protein present. Examples of methods used to visualize antibodies are, for example, through enzymes linked to the antibodies (e.g., luciferase, alkaline phosphatase, horseradish peroxidase, or beta-galactosidase), or chemical methods (e.g., DAB/Substrate chromagen). The sample is then analyzed microscopically, most preferably by light microscopy of a sample stained with a stain that is detected in the visible spectrum, using any of a variety of such staining methods and reagents known to those skilled in the art.

[00168] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using radioimmunoassays. A radioimmunoassay is a technique for detecting and measuring the concentration of an antigen, i.e., ROBO2, using a labeled (e.g.. radioactively or fluorescently labeled) form of the antigen. Examples of radioactive labels for antigens include 3H, 14C, and 125I. The concentration of ROBO2 in a biological sample is measured by having the ROBO2 in the biological sample compete with the labeled (e.g. radioactively) ROBO2 for binding to an antibody to ROBO2. To ensure competitive binding between the labeled ROBO2 and the unlabeled ROBO2, the labeled ROBO2 is present in a concentration sufficient to saturate the binding

sites of the antibody. The higher the concentration of ROBO2 in the sample, the lower the concentration of labeled ROBO2 that will bind to the antibody.

[00169] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using an immunoradiometric assay (IRMA). IRMA is an immunoassay in which the antibody reagent is radioactively labeled. An IRMA requires the production of a multivalent antigen conjugate, by techniques such as conjugation to a protein e.g., rabbit serum albumin (RSA). The multivalent antigen conjugate must have at least 2 antigen residues per molecule and the antigen residues must be of sufficient distance apart to allow binding by at least two antibodies to the antigen. For example, in an IRMA the multivalent antigen conjugate can be attached to a solid surface such as a plastic sphere. Unlabeled "sample" antigen and antibody to antigen which is radioactively labeled are added to a test tube containing the multivalent antigen conjugate coated sphere. The antigen in the sample competes with the multivalent antigen conjugate for antigen antibody binding sites. After an appropriate incubation period, the unbound reactants are removed by washing and the amount of radioactivity on the solid phase is determined. The amount of bound radioactive antibody is inversely proportional to the concentration of antigen in the sample.

[00170] Other techniques can be used to detect the level of ROBO2 protein in a biological sample can be performed according to a practitioner's preference, and based upon the present disclosure and the type of biological sample (i.e. plasma, urine, tissue sample etc.). One such technique is Western blotting (Towbin et at., Proc. Nat. Acad. Sci. 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid support, such as a nitrocellulose filter. Detectably labeled anti-ROBO2 antibodies or protein binding molecules can then be used to assess the level of ROBO2 protein, where the intensity of the signal from the detectable label corresponds to the amount of ROBO2 protein. Levels of the amount of ROBO2 protein present can also be quantified, for example by densitometry.

[00171] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using mass spectrometry such as MALDI/TOF (time-of-flight), SELDI/TOF, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), capillary electrophoresis-mass spectrometry, nuclear magnetic resonance spectrometry, or tandem mass spectrometry (e.g., MS/MS, MS/MS/MS, ESI-MS/MS, etc.). See for example, U.S. Patent Application Nos: 20030199001, 20030134304, 20030077616, which are incorporated herein in their entirety by reference.

[00172] In some such embodiments, these methodologies can be combined with the machines, computer systems and media to produce an automated system for determining the level of ROBO2 protein in a biological sample and analysis to produce a printable report which identifies, for example, the level of ROBO2 protein in a biological sample. In some instances, the measurement of the level of ROBO2 is done remotely from the determination and comparison modules.

[00173] Mass spectrometry methods are well known in the art and have been used to quantify and/or identify biomolecules, such as proteins (see, e.g., Li et al. (2000) Tibtech 18:151-160; Rowley et al. (2000) Methods 20: 383-397; and Kuster and Mann (1998) Curr. Opin. Structural Biol. 8: 393-400). Further, mass spectrometric techniques have been developed that permit at least partial de novo sequencing of isolated proteins. Chait et al., Science 262:89-92 (1993); Keough et al., Proc. Natl. Acad. Sci. USA. 96:7131-6 (1999); reviewed in Bergman, EXS 88:133-44 (2000).

[00174] In certain embodiments, a gas phase ion spectrophotometer is used. In other embodiments, laser-desorption/ionization mass spectrometry is used to analyze the sample. Modern laser desorption/ionization mass spectrometry ("LDI-MS") can be practiced in two main variations: matrix assisted laser desorption/ionization ("MALDI") mass spectrometry and surface-enhanced laser desorption/ionization ("SELDI"). In MALDI, the analyte is mixed with a solution containing a matrix, and a drop of the liquid is placed on the surface of a substrate. The matrix solution then co-crystallizes with the biological molecules. The substrate is inserted into the mass spectrometer. Laser energy is directed to the substrate surface where it desorbs and ionizes the biological molecules without significantly fragmenting them. See, e.g., U.S. Pat. No. 5,118,937 (Hillenkamp et al.), and U.S. Pat. No. 5,045,694 (Beavis & Chait) which are incorporated herein by reference.

[00175] In SELDI, the substrate surface is modified so that it is an active participant in the desorption process. In one variant, the surface is derivatized with adsorbent and/or capture reagents that selectively bind the protein of interest. In another variant, the surface is derivatized with energy absorbing molecules that are not desorbed when struck with the laser. In another variant, the surface is derivatized with molecules that bind the protein of interest and that contain a photolytic bond that is broken upon application of the laser. In each of these methods, the derivatizing agent generally is localized to a specific location on the substrate surface where the sample is applied. See, e.g., U.S. Pat. No. 5,719,060 and WO 98/59361 which are incorporated herein by reference. The two methods can be combined by, for example, using a SELDI affinity surface to capture an analyte and adding matrix-containing liquid to the captured analyte to provide the energy absorbing material.

[00176] For additional information regarding mass spectrometers, see, e.g., Principles of Instrumental Analysis, 3rd edition., Skoog, Saunders College Publishing, Philadelphia, 1985; and Kirk-Othmer Encyclopedia of Chemical Technology, 4th ed. Vol. 15 (John Wiley & Sons, New York 1995), pp. 1071-1094.

[00177] Detection of the level of ROBO2 protein will typically depend on the detection of signal intensity. This, in turn, can reflect the quantity and character of a polypeptide bound to the substrate. For example, in certain embodiments, the signal strength of peak values from spectra of a first sample and a second sample can be compared (e.g., visually, by computer analysis etc.), to determine the relative amounts of particular biomolecules. Software programs such as the Biomarker Wizard program (Ciphergen Biosystems, Inc., Fremont, Calif.) can be used to aid in analyzing mass spectra. The mass spectrometers and their techniques are well known to those of skill in the art.

[00178] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using gel electrophoresis techniques, in particular SDS-PAGE (Sodium Dodecylsulfate Polyacrylamide Gel Elektrophoresis), especially two dimensional PAGE (2D-PAGE), preferably two dimensional SDS-PAGE (2D-SDS-PAGE). According to a particular example, the assay is based on 2D-PAGE, in particular, using immobilized pH gradients (IPGs) with a pH range preferably over pH 4-9.

[00179] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using gel electrophoresis techniques, in particular, the above mentioned techniques combined with other protein separation methods, particularly methods known to those skilled in the art, in particular, chromatography and/or size exclusion.

[00180] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using resonance techniques, in particular, plasma surface resonance.

[00181] In some embodiments of the assays, methods, and systems described herein, ROBO2 protein levels are measured using a protein biochip. A biochip generally comprises a solid substrate having a substantially planar surface, to which a capture reagent (e.g., an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations having bound capture reagent bound. The biochip may also include bound capture reagent that serves as a control. Protein biochips are biochips adapted for the capture of polypeptides. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems, Inc. (Fremont, Calif.), Zyomyx (Hayward, Calif.), Invitrogen (Carlsbad, Calif.), Biacore (Uppsala, Sweden) and Procognia (Berkshire, UK). Examples of such protein biochips are described in the following patents or published patent applications: U.S. Pat. No. 6,225,047 (Hutchens &Yip); U.S. Pat. No. 6,537,749 (Kuimelis and Wagner); U.S. Pat. No. 6,329,209 (Wagner et al.); PCT International Publication No. WO 00156934 (Englert et al.); PCT International Publication No. WO 031048768 (Boutell et al.) and U.S. Pat. No. 5,242,828 (Bergstrom et al.).

[00182] The reference threshold levels or values of ROBO2 protein levels used for comparison with the level of ROBO2 protein from a subject can vary, depending on the aspect or embodiment described herein being practiced, as will be understood throughout this specification, and below. A reference threshold value can be based on an individual sample value, such as for example, a value obtained from a biological sample from the subject being tested, but at an earlier point in time (e.g., at a first timepoint (t1), e.g., a first biomarker level measured, or at a second timepoint (t2), e.g.,). A reference threshold value can also be based on a pool of samples, for example, value(s) obtained from samples from a pool of subjects being tested. For example, in some embodiments, reference threshold values for ROBO2 protein are based on measured the 50% value (e.g., median) of ROBO2 protein measured in subjects known to have chronic kidney disease or proteinuria. For example, subjects in the top 50% (e.g., at or above the median level) for ROBO2 protein can be selected to be at risk of having chronic kidney disease or proteinuria. Reference value(s) can also be based on a pool of samples including or excluding

the sample(s) to be tested. The reference value can be based on a large number of samples, such as from population of healthy subjects of the chronological age-matched group, or from subjects who do not have or do not have a risk of chronic kidney disease or proteinuria. In some embodiments, the reference value can be at least one, more typically at least two, standard deviations above the mean or median of any of these assays or a predetermined mean or median.

[00183] For assessing the risk of a subject likely to experience or have chronic kidney disease or proteinuria by the assays, methods, and systems as disclosed herein, a "reference threshold value" is typically a predetermined reference threshold level, such as the median urine, serum or blood ROBO2 protein obtained from a population of healthy subjects that are in the chronological age group matched with the chronological age of the tested subject. As indicated earlier, in some situations, the reference samples can also be gender matched, and/or matched based on ethnicity. In some embodiments, the reference threshold value for ROBO2 protein is the median level for that biomarker in a type of biological sample, e.g., urine, blood, serum, in a panel subjects for the same ethnicity, e.g., Caucasian, Black, Hispanic, Asian, and Asian-Indian, Pakistani, Middle Eastern and/or Pacific Islander .

[00184] For assessing the risk of a subject likely to experience or have chronic kidney disease or proteinuria by the assays, methods, and systems as disclosed herein, the reference threshold level for ROBO2 protein can be a predetermined level, such as an average or median of levels obtained from a population of healthy subjects that are in the chronological age group matched with the chronological age of the tested subject. Alternately, the reference threshold level for ROBO2 protein can be a historical reference level for the particular subject that was obtained from a sample derived from the same subject, but at an earlier point in time, and/or when the subject did not have a risk of chronic kidney disease or proteinuria. In some instances, the reference threshold level for ROBO2 protein can be a historical reference level of ROBO2 protein for a particular group of subjects whom have all had chronic kidney disease or proteinuria, due to, for example, diabetes.

[00185] In some embodiments, healthy subjects are selected as the control subjects. In some embodiments, controls are age-matched controls. Healthy subject can be used to obtain a reference threshold level ROBO2 protein in, for example, a urine or serum sample. A "healthy" subject or sample from a "healthy" subject or individual as used herein is the same as those commonly understood to one skilled in the art. For example, one may use methods commonly known to evaluate kidney function, as described herein, to select control subjects as healthy subjects for diagnosis and treatment methods described herein. In some embodiments, subjects in good health with no signs or symptom suggesting chronic kidney disease can be recruited as healthy control subjects. The subjects are evaluated based on extensive evaluations consisted of medical history, family history, physical and renal examinations by clinicians, laboratory tests. Examples of analyses of chronic kidney disease and/or proteinuria include, but are not limited to detecting the level of specific proteins or molecules in a urine, blood, or serum sample, such as, for example, albumin, calcium, cholesterol, complete blood count (CBC), electrolytes, magnesium, phosphorous, potassium, sodium, or any combination thereof; assays to detect, for

example, creatinine clearance; creatinine levels; BUN (blood urea nitrogen); through the use of specific techniques or procedures, such as an abdominal CT scan, abdominal MRI, abdominal ultrasound, kidney biopsy, kidney scan, kidney ultrasound; via detection of changes in results of assays or tests for erythropoietin, PTH; bone density test, or Vitamin D; or any combination of such detection methods and assays.

[00186] Age-matched populations (from which reference values can be obtained) are ideally the same chronological age as the subject or individual being tested, but approximately age-matched populations are also acceptable. Approximately age-matched populations may be within 1, 2, 3, 4, or 5 years of the chronological age of the individual tested, or can be groups of different chronological ages which encompass the chronological age of the individual being tested.

[00187] A subject that is compared to its "chronological age matched group" is generally referring to comparing the subject with a chronological age-matched within a range of 5 to 20 years. Approximately age-matched populations can be in 2, 3, 4, 5, 6, 7, 8, 9, 10 or 15, or 20 year increments (e.g. a "5 year increment" group can serve as the source for reference values for a 62 year old subject might include 58-62 year old individuals, 59-63 year old individuals, 60-64 year old individuals, 61-65 year old individuals, or 62-66 year old individuals). In a broader definition, where there are larger gaps between different chronological age groups, for example, when there are few different chronological age groups available for reference values, and the gaps between different chronological age groups exceed the 2, 3, 4, 5, 6, 7, 8, 9, 10 or 15, or 20 year increments described herein, then the "chronological age matched group" can refer to the age group that is in closer match to the chronological age of the subject (e.g. when references values available for an older age group (e.g., 80-90 years) and a younger age group (e.g., 20-30 years), a chronological age matched group for a 51 year old can use the younger age group (20-30 years), which is closer to the chronological age of the test subject, as the reference level.

[00188] Other factors to be considered while selecting control subjects include, but not limited to, species, gender, ethnicity, and so on. Hence, in some embodiments, a reference level can be a predetermined reference level, such as an average or median of levels obtained from a population of healthy control subjects that are gender-matched with the gender of the tested subject. In some embodiments, a reference level can be a predetermined reference level, such as an average or median of levels obtained from a population of healthy control subjects that are ethnicity-matched with the ethnicity of the tested subject (e.g., Caucasian, Black, Hispanic, Asian, and Asian-Indian, Pakistani, Middle Eastern and Pacific Islander). In other embodiments, both chronological age and gender of the population of healthy subjects are matched with the chronological age and gender of the tested subject, respectively. In other embodiments, both chronological age and ethnicity of the population of healthy subjects are matched with the chronological age and ethnicity of the tested subject, respectively. In other embodiments, chronological age, gender, and ethnicity of the population of healthy control

subjects are all matched with the chronological age, gender, and ethnicity of the tested subject, respectively.

[00189] The process of comparing a level of ROBO2 protein in a biological sample from a subject and a reference threshold level for ROBO2 protein can be carried out in any convenient manner appropriate and known to one of skill in the art. Generally, values of ROBO2 protein levels determined using the assays, methods, and systems described herein can be quantitative values (e.g., quantitative values of concentration, such as milligrams of ROBO2 protein per liter (e.g., mg/L) of sample, or an absolute amount). Alternatively, values of ROBO2 protein levels can be qualitative depending on the measurement techniques, and thus the mode of comparing a value from a subject and a reference value can vary depending on the measurement technology employed. For example, the comparison can be made by inspecting the numerical data, by inspecting representations of the data (e.g., inspecting graphical representations such as bar or line graphs), and using standard deviations of, for example, at least one, or at least two standard deviations. In one example, when a qualitative assay is used to measure ROBO2 protein levels, the levels can be compared by visually comparing the intensity of the colored reaction product, or by comparing data from densitometric or spectrometric measurements of the colored reaction product (e.g., comparing numerical data or graphical data, such as bar charts, derived from the measuring device).

[00190] As described herein, ROBO2 protein levels can be measured quantitatively (absolute values) or qualitatively (relative values). In some embodiments, quantitative values of ROBO2 protein levels in the biological samples can indicate a given level (or grade) of risk of chronic kidney disease or proteinuria.

[00191] In some embodiments, the comparison is performed to determine the magnitude of the difference between the values from a subject and reference values (e.g., comparing the "fold" or percentage difference between the measured ROBO2 protein levels obtained from a subject and the reference threshold ROBO2 protein value). A fold difference can be determined by measuring the absolute concentration of the ROBO2 protein levels, and comparing that to the absolute value to the reference threshold ROBO2 protein level, or a fold difference can be measured by the relative difference between a reference value and a sample value, where neither value is a measure of absolute concentration, and/or where both values are measured simultaneously. For example, an ELISA measures the absolute content or concentration of a protein from which a fold change is determined in comparison to the absolute concentration of the same protein in the reference. As another example, an antibody array measures the relative concentration from which a fold change is determined. Accordingly, the magnitude of the difference between the measured value and the reference value that suggests or indicates a particular diagnosis will depend on the method being practiced.

[00192] As will be apparent to those of skill in the art, when replicate measurements are taken for measurement of ROBO2 protein levels, the measured values from subjects can be compared with the reference threshold ROBO2 protein levels, and take into account the replicate measurements. The

replicate measurements can be taken into account by using either the mean or median of the measured values.

[00193] In some embodiments, the process of comparing can be manual or it can, preferably, be automated. For example, an assay device (such as a luminometer for measuring chemiluminescent signals) can include circuitry and software enabling it to compare a value from a subject with a reference value for ROBO2 protein. Alternately, a separate device (e.g., a digital computer) can be used to compare the measured ROBO2 protein levels from subject(s) and the reference threshold levels for ROBO2 protein. Automated devices for comparison can include stored reference values for the ROBO2 protein, or can compare the measured ROBO2 protein levels from subject(s) with reference threshold levels for ROBO2 protein that are derived from contemporaneously measured reference samples

[00194] In some embodiments, a subject tested for ROBO2 protein levels is assigned into one of two or more groups (statuses) based on the results of the assays, methods, and systems described herein. The diagnostic assays, methods, and systems described herein can be used to classify between a number of different states.

[00195] Accordingly, in some embodiments, determining whether a subject has a high risk of having chronic kidney disease or proteinuria (status: low-risk v. high risk) is performed using the diagnostic assays, methods, and systems described herein . Biomarker amounts or patterns of ROBO2 protein determined as being characteristic of various risk states, e.g., high, medium or low, are identified. The risk of developing chronic kidney disease or proteinuria is determined by measuring ROBO2 protein alone or in combination with other known biomarkers, and then either submitting them to a classification algorithm or comparing them with a reference amount (e.g., a cut off reference amount as disclosed herein) that is associated with the particular risk level.

[00196] In some embodiments, provided herein are diagnostic assays, methods, and systems for determining the severity or stage or risk of having a chronic kidney disease or proteinuria in a subject. Each stage of chronic kidney disease, for example, has a characteristic amount of ROBO2 protein or relative amounts of ROBO2 protein. The stage of a disease is determined by measuring ROBO2 protein, alone or in combination with other biomarkers, and then either submitting them to a classification algorithm or comparing them with a reference amount and/or pattern of biomarkers that is associated with the particular stage, e.g., how soon the subject will likely develop chronic kidney disease or proteinuria. For example, one can classify between likely to have chronic kidney disease or proteinuria within a year (e.g., a poor prognosis) or a subject likely to have chronic kidney disease or proteinuria in the next 5 years.

[00197] Additional embodiments of the diagnostic assays, methods, and systems relate to the communication of results or diagnoses or both to technicians, physicians or patients, for example. In certain embodiments, computers are used to communicate assay results or diagnoses or both to interested parties, e.g., physicians and their patients. In some embodiments, the assays are performed or the assay results analyzed in a country or jurisdiction which differs from the country or jurisdiction to

which the results or diagnoses are communicated, for example. In some embodiments, a risk of having chronic kidney disease or proteinuria based on levels of ROBO2 protein in a biological sample from the subject is communicated to the subject after the levels or prognosis are obtained. The prognosis or diagnosis can be communicated to the subject by the subject's treating physician. Alternatively, the prognosis or diagnosis can be sent to the subject by email or communicated to the subject by phone. A computer can be used to communicate the prognosis or diagnosis by email or phone, or via the internet using a secure gateway patient log-in service. In certain embodiments, the message containing results of the prognosis or diagnostic test can be generated and delivered automatically to the subject using a combination of computer hardware and software which will be familiar to artisans skilled in telecommunications. In certain embodiments of the assays, methods, and systems described herein, all or some of the method steps, including the assaying of samples, diagnosing of diseases, and communicating of assay results or diagnoses, can be carried out in diverse (e.g., foreign) jurisdictions.

[00198] In some embodiments of the diagnostic assays, methods, and systems of qualifying or assessing a risk of chronic kidney disease or proteinuria described herein, the assays, methods, or systems further comprise managing subject treatment based on the determination of the risk of having a chronic kidney disease or proteinuria. Such management includes the actions of the physician or clinician subsequent to determining the subjects risk of having chronic kidney disease or proteinuria. For example, if a physician makes a diagnosis of the subject at risk of chronic kidney disease or proteinuria, then a certain regimen of treatment can follow. A suitable regimen of treatment can include, without limitation, a supervised exercise program; control of blood pressure, sugar intake, and/or lipid levels; and drug therapies. In some embodiments, a diagnosis of a risk of having chronic kidney disease or proteinuria can be followed by further testing to determine whether a patient is suffering from a chronic kidney disease, or whether the patient is suffering from a related disease. Also, if the diagnostic test gives an inconclusive result on the risk of a major adverse event status, further tests may be called for. In some embodiments of the diagnostic assays, methods, and systems of qualifying or assessing a risk of chronic kidney disease or proteinuria described herein, if a physician makes a diagnosis of the subject not being at risk of chronic kidney disease or proteinuria, then no treatment is provided.

[00199] The assay and ROBO2 detection methods described herein can be automated using robotics and computer directed systems. A biological sample, such as a urine, plasma, or blood sample, can be injected into a system, such as a microfluidic device entirely run by a robotic station from sample input to output of the result.

[00200] Accordingly, also provided herein, in some aspects are systems (and computer readable medium for causing computer systems) to perform a method for determining whether an individual has a chronic kidney disease or proteinuria or a pre-disposition for a chronic kidney disease or proteinuria based on expression profiles or sequence information.

[00201] In some aspects, provided herein are systems for assessing if a subject has or is at increased risk for chronic kidney disease or proteinuria, the systems comprising: (a) a determination module

configured to receive a at least one biological sample and perform at least one analysis on said biological sample to measure a level of ROBO2 in the biological sample or determine the expression ratio of ROBO2 relative to a pre-determined or threshold reference level and to output said measured level or expression ratio; (b) a storage device configured to store data output information from the determination module; (c) a comparison module adapted to receive input from the storage device and compare the data stored on the storage device with at least one reference threshold ROBO2 level, wherein if the measured ROBO2 protein level is at least the same or higher than the reference threshold level, the comparison module provides information to an output module that the biological sample is associated with a subject that deviates from the reference threshold biomarker level; and (d) an output module for displaying the information to the user.

[00202] In all aspects of the invention, methods to determine the levels of ROBO2 protein can be performed using an automated machine or system. Such machines and systems generate a report, such as displaying a report on a visible screen or a printable report which indicates the levels of ROBO2 protein and/or report an increase or the same as a reference threshold level for ROBO2 protein, and/or if the subject from which the sample was obtained is at risk of chronic kidney disease or proteinuria.

[00203] Accordingly, some embodiments described herein also provide for a machine, computer systems and computer readable media for performing the steps of (i) determining the levels of ROBO2 protein, and (ii) indicating or reporting whether a subject is at risk of having chronic kidney disease or proteinuria.

[00204] Embodiments of these aspects are described through functional modules, which are defined by computer executable instructions recorded on computer readable media and which cause a computer to perform method steps when executed. The modules have been segregated by function for the sake of clarity. However, it should be understood that the modules need not correspond to discreet blocks of code and the described functions can be carried out by the execution of various code portions stored on various media and executed at various times. Furthermore, it should be appreciated that the modules can perform other functions, thus the modules are not limited to having any particular functions or set of functions.

[00205] The computer readable media can be any available tangible media that can be accessed by a computer. Computer readable media includes volatile and nonvolatile, removable and non-removable tangible media implemented in any method or technology for storage of information such as computer readable instructions, data structures, program modules or other data. Computer readable media includes, but is not limited to, RAM (random access memory), ROM (read only memory), EPROM (eraseable programmable read only memory), EEPROM (electrically eraseable programmable read only memory), flash memory or other memory technology, CD-ROM (compact disc read only memory), DVDs (digital versatile disks) or other optical storage media, magnetic cassettes, magnetic tape, magnetic disk storage or other magnetic storage media, other types of volatile and non-volatile memory,

and any other tangible medium which can be used to store the desired information and which can accessed by a computer including and any suitable combination of the foregoing.

[00206] Computer-readable data embodied on one or more computer-readable media, or computer readable medium, can define instructions, for example, as part of one or more programs, that, as a result of being executed by a computer, instruct the computer to perform one or more of the functions described herein (e.g., in relation to a system, or computer readable medium), and/or various embodiments, variations and combinations thereof. Such instructions can be written in any of a plurality of programming languages, for example, Java, J#, Visual Basic, C, C#, C++, Fortran, Pascal, Eiffel, Basic, COBOL assembly language, and the like, or any of a variety of combinations thereof. The computer-readable media on which such instructions are embodied can reside on one or more of the components of either of the system, or computer readable medium described herein, can be distributed across one or more of such components, and can be in transition there between.

[00207] The computer-readable media can be transportable such that the instructions stored thereon can be loaded onto any computer resource to implement the aspects of the present invention discussed herein. In addition, it should be appreciated that the instructions stored on the computer readable media, or the computer-readable medium, described above, are not limited to instructions embodied as part of an application program running on a host computer. Rather, the instructions can be embodied as any type of computer code (e.g., software or microcode) that can be employed to program a computer to implement aspects of the present invention. The computer executable instructions can be written in a suitable computer language or combination of several languages. Basic computational biology methods are known to those of ordinary skill in the art and are described in, for example, Setubal and Meidanis et al., *Introduction to Computational Biology Methods* (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), *Computational Methods in Molecular Biology*, (Elsevier, Amsterdam, 1998); Rashidi and Buehler, *Bioinformatics Basics: Application in Biological Science and Medicine* (CRC Press, London, 2000) and Ouelette and Bzevanis *Bioinformatics: A Practical Guide for Analysis of Gene and Proteins* (Wiley & Sons, Inc., 2nd ed., 2001).

[00208] The functional modules of certain embodiments of the aspects described herein include a determination module, a storage device, a comparison module and a display module. The functional modules can be executed on one, or multiple, computers, or by using one, or multiple, computer networks or computer systems.

[00209] In some aspects, provided herein are computer systems that can be used to determine if a subject is likely to have or be at risk of chronic kidney disease or proteinuria. In some embodiments, a computer system is connected to a determination module and is configured to obtain output data from a determination module regarding a biological sample, where the determination module is configured to detect the levels of ROBO2 protein in a biological sample obtained from the subject; and where the computer system comprises (a) a storage device configured to store data output from the determination module as well as reference data; where the storage device is connected to (b) a comparison module

which in some embodiments, is adapted to compare the output data stored on the storage device with stored reference data, and in alternative embodiments, adapted to compare the output data with itself, where the comparison module produces report data and is connected to (c) a display module for displaying a page of retrieved content (i.e. report data from the comparison module) for the user on a client computer, wherein the retrieved content can indicate the levels of ROBO2, and/or likelihood of the subject experiencing chronic kidney disease or proteinuria in the future.

[00210] In some embodiments, the determination module has computer executable instructions to provide expression data, sequence information, information related to sequence information in computer readable form. As used herein, "sequence information" refers to any nucleotide and/or amino acid sequence, including but not limited to full-length nucleotide and/or amino acid sequences, partial nucleotide and/or amino acid sequences, or mutated sequences. Moreover, information "related to" the sequence information includes detection of the presence or absence of a sequence (e.g., detection of a mutation or deletion), determination of the concentration of a sequence in the sample (e.g., amino acid sequence expression levels, or nucleotide (RNA or DNA) expression levels), and the like. The term "sequence information" is intended to include the presence or absence of post-translational modifications (e.g. phosphorylation, glycosylation, summylation, farnesylation, and the like).

[00211] As an example, determination modules for determining ROBO2 sequence or nucleic acid expression information can include known systems for automated sequence analysis including but not limited to Hitachi FMBIO® and Hitachi FMBIO® II Fluorescent Scanners (available from Hitachi Genetic Systems, Alameda, California); SPECTRUMEDIX® SCE 9610 Fully Automated 96-Capillary Electrophoresis Genetic Analysis Systems (available from SpectruMedix LLC, State College, Pennsylvania); ABI PRISM® 377 DNA Sequencer, ABI® 373 DNA Sequencer, ABI PRISM® 310 Genetic Analyzer, ABI PRISM® 3100 Genetic Analyzer, and ABI PRISM® 3700 DNA Analyzer (available from Applied Biosystems, Foster City, California); Molecular Dynamics FLUORIMAGER™ 575, SI Fluorescent Scanners, and Molecular Dynamics FLUORIMAGER™ 595 Fluorescent Scanners (available from Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England); GENOMYXSC™ DNA Sequencing System (available from Genomyx Corporation (Foster City, California); and PHARMACIA ALF™ DNA Sequencer and Pharmacia ALFEXPRESS™ (available from Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England).

[00212] In some embodiments for determining sequence or protein information, determination modules include systems for protein and DNA analysis. For example, mass spectrometry systems including Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) systems; SELDI-TOF-MS ProteinChip array profiling systems, e.g. Machines with CIPHERGEN PROTEIN BIOLOGY SYSTEM II™ software; systems for analyzing gene expression data (see for example U.S. 2003/0194711); systems for array based expression analysis, for example HT array systems and cartridge array systems available from Affymetrix (Santa Clara, CA 95051) AutoLoader, COMPLETE

GENECHIP® Instrument System, Fluidics Station 450, Hybridization Oven 645, QC Toolbox Software Kit , Scanner 3000 7G, Scanner 3000 7G plus Targeted Genotyping System, Scanner 3000 7G Whole-Genome Association System, GENETITAN™ Instrument , GeneChip® Array Station, HT Array; an automated ELISA system (e.g. DSX® or DS2® form Dynax, Chantilly, VA or the ENEASYSTEM III®, TRITURUS®, THE MAGO® Plus); Densitometers (e.g. X-Rite-508-Spectro Densitometer®, The HYRYS™ 2 densitometer); automated Fluorescence *in situ* hybridization systems (see for example, United States Patent 6,136,540); 2D gel imaging systems coupled with 2-D imaging software; microplate readers; Fluorescence activated cell sorters (FACS) (e.g. Flow Cytometer FACSVantage SE, Becton Dickinson); radio isotope analyzers (e.g. scintillation counters), or a combination thereof.

[00213] In some embodiments of this aspect and all other aspects of the present invention a variety of software programs and formats can be used to store the biomarker protein level information on the storage device. Any number of data processor structuring formats (e.g., text file or database) can be employed to obtain or create a medium having recorded thereon the sequence information or expression level information.

[00214] The ROBO2 expression information or information related to ROBO2 expression information determined in the determination module can be read by the storage device. As used herein the "storage device" is intended to include any suitable computing or processing apparatus or other device configured or adapted for storing data or information. Examples of electronic apparatus suitable for use with the present invention include stand-alone computing apparatus, data telecommunications networks, including local area networks (LAN), wide area networks (WAN), cloud storage systems, Internet, Intranet, and Extranet, and local and distributed computer processing systems. Storage devices also include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage media, magnetic tape, optical storage media such as CD-ROM, DVD, electronic storage media such as RAM, ROM, EPROM, EEPROM and the like, cloud storage systems, general hard disks and hybrids of these categories such as magnetic/optical storage media. The storage device is adapted or configured for having recorded thereon sequence information or expression level information. Such information may be provided in digital form that can be transmitted and read electronically, e.g., via the Internet, via a cloud system, on diskette, via USB (universal serial bus), or via any other suitable mode of communication.

[00215] As used herein, "expression level information" refers to any nucleotide and/or amino acid expression level information, including but not limited to full-length nucleotide and/or amino acid sequences, partial nucleotide and/or amino acid sequences, or mutated sequences. Moreover, information "related to" the expression level information includes detection of the presence or absence of a sequence (e.g., presence or absence of an amino acid sequence, nucleotide sequence, or post translational modification), determination of the concentration of a sequence in the sample (e.g., amino

acid sequence levels, or nucleotide (RNA or DNA) expression levels, or level of post translational modification), and the like.

[00216] As used herein, "stored" refers to a process for encoding information on the storage device. Those skilled in the art can readily adopt any of the presently known methods for recording information on known media to generate manufactures comprising the sequence information or expression level information.

[00217] A variety of software programs and formats can be used to store the sequence information or expression level information on the storage device. Any number of data processor structuring formats (e.g., text file or database) can be employed to obtain or create a medium having recorded thereon the sequence information or expression level information.

[00218] By providing sequence information or expression level information in computer-readable form, one can use the sequence information or expression level information in readable form in the comparison module to compare a specific sequence or expression profile with the reference data within the storage device. For example, search programs can be used to identify fragments or regions of the sequences that match a particular sequence (reference data, e.g., sequence information obtained from a control sample) or direct comparison of the determined expression level can be compared to the reference data expression level (e.g., expression level information obtained from a control sample). The comparison made in computer-readable form provides a computer readable comparison result which can be processed by a variety of means. Content based on the comparison result can be retrieved from the comparison module to indicate a specific disease or disorder, such as chronic kidney disease or proteinuria.

[00219] In some embodiments, the reference data stored in the storage device to be read by the comparison module is ROBO2 sequence or expression information data obtained from a control biological sample of the same type as the biological sample to be tested. Alternatively, the reference data are a database, e.g., a part of the entire genome sequence of an organism, or a protein family of sequences, or an expression level profile (RNA, protein or peptide). In some embodiments, the reference data are sequence information or expression level profiles that are indicative of a specific disease or disorder, such as chronic kidney disease or proteinuria.

[00220] In some embodiments, the reference data are electronically or digitally recorded and annotated from databases including, but not limited to GenBank (NCBI) protein and DNA databases such as genome, ESTs, SNPS, Traces, Celara, Ventor Reads, Watson reads, HGTS, and the like; Swiss Institute of Bioinformatics databases, such as ENZYME, PROSITE, SWISS-2DPAGE, Swiss-Prot and TrEMBL databases; the Melanie software package or the ExPASy WWW server, and the like; the SWISS-MODEL, Swiss-Shop and other network-based computational tools; the Comprehensive Microbial Resource database (available from The Institute of Genomic Research). The resulting information can be stored in a relational data base that may be employed to determine homologies between the reference data or genes or proteins within and among genomes.

[00221] By providing the levels of ROBO2 in readable form in the comparison module, it can be used to compare with the reference threshold levels of ROBO2 within the storage device. The comparison made in computer-readable form provides computer readable content which can be processed by a variety of means.

[00222] The "comparison module" can use a variety of available software programs and formats for the comparison operative to compare ROBO2 sequence or expression level information determined in the determination module to reference ROBO2 sequence or expression level data. In some embodiments, the comparison module is configured to use pattern recognition techniques to compare ROBO2 sequence or expression level data from one or more entries to one or more reference data patterns. The comparison module can be configured using existing commercially-available or freely-available software for comparing patterns, and can be optimized for particular data comparisons that are conducted. The comparison module provides computer readable information related to the sequence information that can include, for example, detection of the presence or absence of a sequence (e.g., detection of a mutation or deletion (protein or DNA), information regarding distinct alleles, detection of post-translational modification, or omission or repetition of sequences); determination of the concentration of a sequence in the sample (e.g., amino acid sequence/protein expression levels, or nucleotide (RNA or DNA) expression levels, or levels of post-translational modification), or determination of an expression profile.

[00223] In some embodiments, the comparison module permits the comparison of levels of ROBO2 from the output data of the determination module with reference threshold level data for each ROBO2.

[00224] In some embodiments, the comparison module performs comparisons with mass-spectometry spectra, for example comparisons of peptide fragment sequence information can be carried out using spectra processed in MATLB with script called "Qcealign" (see for example WO2007/022248, herein incorporated by reference) and "Qpeaks" (Spectrum Square Associates, Ithaca, NY), or Ciphergen Peaks 2.1™ software. The processed spectra can then be aligned using alignment algorithms that align sample data to the control data using minimum entropy algorithm by taking baseline corrected data (see for example WIPO Publication WO2007/022248, herein incorporated by reference). The comparison result can be further processed by calculating ratios. Protein expression profiles can be discerned.

[00225] Any available comparison software can be used, including but not limited to, the Ciphergen Express (CE) and Biomarker Patterns Software (BPS) package, Ciphergen Biosystems, Inc., CA, USA. Comparative analysis can be done with protein chip system software (e.g. The Proteinchip suite for Bio-Rad Laboratories).

[00226] The comparison module, or any other module described herein, can include an operating system (e.g., UNIX) on which runs a relational database management system, a World Wide Web application, and a World Wide Web server. World Wide Web application includes the executable code necessary for generation of database language statements (e.g., Structured Query Language (SQL)

statements). Generally, the executables will include embedded SQL statements. In addition, the World Wide Web application can include a configuration file which contains pointers and addresses to the various software entities that comprise the server as well as the various external and internal databases which must be accessed to service user requests. The Configuration file also directs requests for server resources to the appropriate hardware--as can be necessary should the server be distributed over two or more separate computers. In some embodiments, the World Wide Web server supports a TCP/IP protocol. Local networks such as this are sometimes referred to as "Intranets." An advantage of such Intranets is that they allow easy communication with public domain databases residing on the World Wide Web (e.g., the GenBank or Swiss Pro World Wide Web site). Thus, in some preferred embodiments, users can directly access data (via Hypertext links for example) residing on Internet databases using a HTML interface provided by Web browsers and Web servers.

[00227] In some embodiments, the comparison module compares gene expression profiles. For example, detection of gene expression profiles can be determined using Affymetrix Microarray Suite software version 5.0 (MAS 5.0) (available from Affymetrix, Santa Clara, California) to analyze the relative abundance of a gene or genes on the basis of the intensity of the signal from probe sets, and the MAS 5.0 data files can be transferred into a database and analyzed with Microsoft Excel and GeneSpring 6.0 software (available from Agilent Technologies, Santa Clara, California). The detection algorithm of MAS 5.0 software can be used to obtain a comprehensive overview of how many transcripts are detected in given samples and allows a comparative analysis of 2 or more microarray data sets.

[00228] In some embodiments, the comparison module compares protein expression profiles. Any available comparison software can be used, including but not limited to, the Ciphergen Express (CE) and Biomarker Patterns Software (BPS) package (available from Ciphergen Biosystems, Inc., Freemont, California). Comparative analysis can be done with protein chip system software (e.g., The Proteinchip Suite (available from Bio-Rad Laboratories, Hercules, California). Algorithms for identifying expression profiles can include the use of optimization algorithms such as the mean variance algorithm (e.g. JMP Genomics algorithm available from JMP Software Cary, North Carolina).

[00229] The comparison module provides computer readable comparison result that can be processed in computer readable form by predefined criteria, or criteria defined by a user, to provide a content based in part on the comparison result that can be stored and output as requested by a user using a display module. The display module enables display of a content based in part on the comparison result for the user, wherein the content is a signal indicative of a chronic kidney disease or proteinuria. Such signal, can be for example, a display of content indicative of the presence or absence of a chronic kidney disease or proteinuria on a computer monitor, a printed page of content indicating the presence or absence of a chronic kidney disease or proteinuria from a printer, or a light or sound indicative of the presence or absence of a chronic kidney disease or proteinuria.

[00230] The content based on the comparison result can include an expression profile of one or more proteins, or an expression profile of one or more genes. In some embodiments, the content based on the comparison result is merely a signal indicative of the presence or absence of a chronic kidney disease or proteinuria based on ROBO2 protein levels.

[00231] In some embodiments, the content based on the comparison result is displayed a on a computer monitor. In one embodiment of the invention, the content based on the comparison result is displayed through printable media. In one embodiment of the invention, the content based on the comparison result is displayed as an indicator light or sound. The display module can be any suitable device configured to receive from a computer and display computer readable information to a user. Non-limiting examples include, for example, general-purpose computers such as those based on Intel PENTIUM-type processor, Apple computer and tablet devices, Motorola PowerPC, Sun UltraSPARC, Hewlett-Packard PA-RISC processors, any of a variety of processors available from Advanced Micro Devices (AMD) of Sunnyvale, California, or any other type of processor, visual display devices such as tablet devices, smartphone mobile devices, flat panel displays, cathode ray tubes and the like, as well as computer printers of various types.

[00232] In some embodiments, a World Wide Web browser is used for providing a user interface for display of the content based on the comparison result. It should be understood that other modules of the invention can be adapted to have a web browser interface. Through the Web browser, a user may construct requests for retrieving data from the comparison module. Thus, the user will typically point and click to user interface elements such as buttons, pull down menus, scroll bars and the like conventionally employed in graphical user interfaces. The requests so formulated with the user's Web browser are transmitted to a Web application which formats them to produce a query that can be employed to extract the pertinent information related to the sequence information, e.g., display of an indication of the presence or absence of mutation or deletion (DNA or protein); display of expression levels of an amino acid sequence (protein); display of nucleotide (RNA or DNA) expression levels; display of expression, SNP, or mutation profiles, or haplotypes, or display of information based thereon. In one embodiment, the sequence information of the reference sample data is also displayed.

[00233] In some embodiments, the display module displays the comparison result and whether the comparison result is indicative of a disease, e.g., whether the expression profile of ROBO2 is indicative of chronic kidney disease or proteinuria.

[00234] In some embodiments, the content based on the comparison result that is displayed is a signal (e.g. positive or negative signal) indicative of the presence or absence of a chronic kidney disease or proteinuria, thus only a positive or negative indication can be displayed.

[00235] Thus, provided herein are systems (and computer readable medium for causing computer systems) to perform assays and methods for determining whether an individual has a chronic kidney disease or proteinuria or a pre-disposition, for a chronic kidney disease or proteinuria based on expression profiles or sequence information.

[00236] Systems and computer readable medium, are merely an illustrative embodiments of the invention for performing assays and methods of determining whether an individual has a specific disease or disorder or a pre-disposition, for a specific disease or disorder based on expression profiles or sequence information, and is not intended to limit the scope of the invention. Variations of systems, and computer readable medium, are possible and are intended to fall within the scope of the invention.

[00237] The modules of the system or used in the computer readable medium, can assume numerous configurations. For example, function can be provided on a single machine or distributed over multiple machines.

Robo2 is a Podocyte Protein Localized to the Basal Cell Surface of Mouse Podocyte

[00238] During kidney development, *Robo2* mRNA is expressed in the metanephric mesenchyme surrounding the arborizing ureteric bud and later in the proximal end of the S-shaped body (Piper et al., 2000), the location of primordial podocytes. To investigate whether Robo2 is also involved in podocyte maturation, in addition to its role in early kidney induction, we performed *in situ* hybridization and found *Robo2* mRNA was expressed in the capillary loop stage of developing glomeruli of mouse embryos at embryonic day 16.5 (E16.5) (**FIGS. 5A** and **5B**). Robo2 protein became detectable by immunofluorescence staining in the developing glomerulus around E14.5 and reached peak expression at E16.5 (**FIGS. 5C-5E**). Although the expression decreased after E17.5 during development (**FIG. 5F**), specific Robo2 expression was maintained in glomeruli after birth and was detectable in adult mice at 5 weeks of age (**FIGS. 5G**, **5H**, **5L-5M**).

[00239] To determine the cellular localization of Robo2 in the developing glomerulus, we performed dual-label immunohistochemistry with glomerular cell type specific markers. We found that Robo2 protein was co-localized with nephrin (**FIGS. 1A-1C**) and podocin (**FIGS. 1D-1F**), two podocyte slit-diaphragm associated proteins. Robo2 was also co-expressed in the glomeruli with the nephrin-interacting adaptor protein Nck (**FIGS. 1G, 1I**) and with WT1, a constituent of podocyte nuclei (FIGS. 5H-5K). Dual-labeling with antibodies against nidogen, a basement membrane marker (**FIGS. 1J-1L** and **1P**) and Pecam1, an endothelial cell marker (**FIGS. 1M—1O, 5M**) showed that Robo2 was localized adjacent to the external surface of the glomerular basement membrane and absent from endothelial cells. High-resolution confocal microscopy further demonstrated that subcellular Robo2 was most abundant on the basal surface of podocytes (**FIG. 1Q**). Immunogold electron microscopy of postnatal mouse kidneys with an antibody against the cytoplasmic domain established that Robo2 was localized to podocyte foot processes close to the cytoplasmic face of the slit-diaphragms (**FIG. 1R**). These results demonstrate that Robo2 is a podocyte protein and its basal subcellular localization in the foot processes indicates that it plays a role in regulating podocyte foot process structure.

Robo2 Intracellular Domain Interacts Directly with SH3 Domains of Adaptor Protein Nck

[00240] Nephrin extracellular domain engagement leads to tyrosine phosphorylation of its intracellular domain by Src kinases and recruitment of the SH2 domain of the adaptor protein Nck, which in turn induces actin polymerization (Jones et al., 2006; Verma et al., 2006). Nck bears one SH2

domain in the C-terminus and three SH3 domains near the N-terminus. Actin polymerization is mediated by the SH3 domains of Nck (Rivera et al., 2004), which can recruit various cytoskeleton regulators including N-WASP and Pak (Jones et al., 2006). Previous studies have shown that the SH3 domains of the *Drosophila* Nck homolog Dreadlock (Dock) also directly interact with the intracellular domain of Robo to inhibit actin polymerization (Fan et al., 2003; Yang and Bashaw, 2006).

[00241] We tested whether mammalian Nck can also interact directly with Robo2 in the podocyte to regulate the F-actin cytoskeleton. To answer this question, we used a yeast two-hybrid assay to examine if Robo2 interacted with Nck. Since two mammalian Ncks (*i.e.* Nck1, Nck2) share similar structure and function in kidney development (Jones et al., 2006), we used Nck1 in this study and observed that the intracellular domain of Robo2 directly interacted with Nck1 (**FIGS. 2A-2C**). Binding site mapping in Robo2 for Nck1 showed that the sequence from amino acid 1085 to 1301, which contains 4 proline-rich motifs, was crucial for the interaction (**FIGS. 2A** and **2C**). Absence of this proline-rich region prevented its interaction with Nck1 (**FIG. 2A**). Binding site mapping in Nck1 for Robo2 showed that the first two SH3 domains were required for its interaction with Robo2 because deleting either or both of them abrogated the interaction (**FIG. 2B**). Thus Robo2 and Nck1 interaction was mediated by two well-characterized protein domains, the SH3 domains and proline-rich motifs (**FIG. 2C**). CD2AP, another podocyte adaptor protein, also bears three SH3 domains in its N-terminus (Shih et al., 2001), but we did not detect any interaction between CD2AP and Robo2 in either the yeast two-hybrid or co-precipitation assays. These observations indicate that the binding between Robo2 and Nck1 in the podocyte is a specific interaction.

Full-Length Robo2 Forms a Complex with Nephrin through Nck

[00242] We confirmed the interaction between Robo2 and Nck by pull down and co-precipitation assays. His- and myc-tagged human full-length Robo2 (His-myc-Robo2) or his- and myc-tagged human Robo2 with a deletion of the Nck1 binding domain (His-myc-Robo2-ΔNBD) were expressed in HEK cells. Transfected HEK cells were stimulated with Slit2 conditioned medium (prepared from Slit2 stably transfected cells) to activate Robo2 and increase Nck binding (Fan et al., 2003). Nck was pulled down with His-mycRobo2 from the HEK cell lysates using Ni-NTA beads but not with His-myc-Robo2-ΔNBD (**FIG. 2D**). Since the SH2 domain of Nck interacts with phosphotyrosines in the nephrin cytoplasmic domain (NCD) (Jones et al., 2006; Verma et al., 2006), we examined whether Robo2 formed a complex with nephrin through Nck using a co-precipitation assay. To establish proof of principle, we co-expressed Robo2 and nephrin in HEK cells with Fyn kinase to increase nephrin phosphorylation (Verma et al., 2006). Pull-down of His-myc-Robo2 from the HEK cell lysates with Ni-NTA beads co-precipitated Nck and nephrin when Fyn was expressed (**FIG. 2E**). In the reverse order, pulling down His-myc-nephrin co-precipitated Nck and Robo2 when Fyn kinase was expressed (**FIG. 2F**). Furthermore, the precipitates prepared with the anti-Nck antibody contained both Robo2 and nephrin when Fyn was over-expressed (**FIG. 6A**). These data indicate that nephrin, Nck, and Robo2 form a complex in vitro. To validate these findings in vivo, we immunoprecipitated Robo2 from

newborn mouse kidney lysates and found that Nck and nephrin were co-precipitated (**FIG. 2G**). Conversely, the precipitates prepared with the anti-nephrin antibody also contained Nck and Robo2 (**FIG. 2H**). Since nephrin is uniquely expressed in podocytes, and Nck and Robo2 are also localized in these cells in the kidney, these results indicate that nephrin, Nck, and Robo2 are able to form a complex in podocytes.

[00243] To determine the role of Slit2 in the formation of the Robo2-Nck-nephrin protein complex, His-myc-Robo2, nephrin, and Fyn were co-expressed in HEK cells that were stimulated with Slit2 conditioned medium or control conditioned medium without Slit2 prior to co-precipitation (**FIG. 2I**). We observed that Slit2 stimulation increased Robo2 binding to Nck and complex formation with nephrin. Both ratios of Nck1 versus Robo2 and nephrin versus Robo2 were increased after Slit2 stimulation (**FIG. 2J**). Consistent with this finding, we observed that Slit2 was strongly expressed in newborn mouse glomeruli (**FIGS. 6B, 6C**).

Slit2-Robo2 Signaling Inhibits Nephrin-Induced Actin Polymerization

[00244] Since Slit binds Robo to recruit Dock and srGAPs to inhibit actin polymerization (Fan et al., 2003), we wished to test whether Robo2 also recruits Nck to inhibit actin polymerization in mammalian cells, an opposite role to nephrin that promotes actin polymerization. To address this question, we studied actin polymerization by analyzing F-actin tails in cells expressing the CD16/7-NCD chimeric protein as previously described (Jones et al., 2006; Verma et al., 2006). This model utilizes the extracellular and transmembrane domains of the human immunoglobulin Fc receptors CD16 and CD7 fused to the nephrin cytoplasmic domain (NCD). CD16/7-HA, in which NCD was replaced by an HA tag, was used as a negative control. These chimeric proteins were co-expressed with Robo2 in HEK cells and clustered by treatment with anti-CD16 antibody and a secondary antibody conjugated to rhodamine. We first examined if clustering of the nephrin cytoplasmic domain could recruit Robo2. We observed that engagement of CD16/7-NCD brought Robo2 into the clusters since most of the Robo2 co-localized with the CD16/7-NCD clusters (**FIGS. 6D-6F**). However, no colocalization of the Robo2 was observed either with the CD16/7-HA control (**FIG. 6D'**) or with the Robo2-ΔNBD construct (**FIG. 6E'**), in which the Robo2 Nck binding domain (NBD) was deleted. Interestingly, in the absence of Slit2, colocalization of CD16/7-NCD and Robo2 was significantly reduced (**FIG. 6F'**). These data provide further evidence that the nephrin cytoplasmic domain is able to complex with the Robo2 intracellular domain in the presence of Slit2 and validates the model to determine if the formation of a Robo2-Nck-nephrin complex affects actin polymerization.

[00245] HEK cells expressing CD16/7-NCD and Robo2 were stimulated with Slit2 or control conditioned medium without Slit2 while clustered by the anti-CD16 antibody. Actin polymerization was evaluated by quantifying the number of HEK cells with visible F-actin tails (Rivera et al., 2004). We observed that ~80% of the CD16/7-NCD clustered cells formed F-actin tails that could be revealed by phalloidin staining as previously reported (Jones et al., 2006; Verma et al., 2006). Upon Slit2 stimulation, however, the number of cells with F-actin tails was significantly reduced to approximately

40% (**FIGS. 3A** and **3C**). Only a few cells were observed to contain shorter F-actin tails when the control CD16/7-HA proteins were clustered (**FIGS. 3B** and **3C**). To further investigate whether this inhibition of actin polymerization required Nck, we repeated this assay using Robo2 without Nck binding domain (Robo2-ΔNBD) to determine if blocking of Nck binding to Robo2 would prevent Slit2-Robo2 inhibition on nephrin-induced actin polymerization. CD16/7-NCD was co-expressed with either full-length Robo2 (**FIG. 7A**) or Robo2-ΔNBD (**FIG. 7B**) in HEK cells. We observed that deletion of Nck binding domain in Robo2 significantly compromised Slit2- Robo2 inhibition on nephrin-induced actin polymerization (**FIG. 7C**).

[00246] Previous study has shown that nephrin is linked to the F-actin cytoskeleton (Yuan et al., 2002). To determine if Slit2-Robo2 signaling could inhibit F-actin associated to nephrin, we immunoprecipitated CD16/7-NCD and CD16/7-HA with anti-CD16 antibody and examined the amount of F-actin in the precipitates by Western blot. We observed that the abundance of F-actin associated with nephrin was significantly reduced upon Slit2 stimulation (**FIGS. 3D** and **3E**). Conversely, in vivo immunoprecipitation assay showed that F-actin associated with nephrin immunoprecipitated by an anti-nephrin antibody from *Robo2* newborn null mouse kidneys was significantly increased compared with that from wild type or *Robo2* heterozygous mouse kidneys (**FIGS. 3F** and **3G**). Taken together, these results indicate that Slit2-Robo2 signaling inhibits nephrin-induced actin polymerization.

Loss of Robo2 in Podocytes Causes Altered Foot Process Structure in Mice

[00247] We and others have previously shown that almost all *Robo2* homozygous null mice in mixed genetic background die shortly after birth due to a severe CAKUT phenotype (Grieshammer et al., 2004; Lu et al., 2007; Wang et al., 2011). After breeding mice with a $Robo2^{del5}$ mutant allele for five generations onto C57BL/6 genetic background, mating of $Robo2^{del5/+}$ heterozygous parents revealed three $Robo2^{del5/del5}$ homozygous null mice that survived to 3 weeks (among a total of 160 mice analyzed at weaning). To determine if *Robo2* was required for podocyte foot process formation during development, we examined the ultrastructure of glomeruli in *Robo2* null mice at birth and 3 weeks of age. Although the podocyte body, foot processes and slit-diaphragm were formed at birth, transmission electron microscopy showed focal foot process effacement in newborn $Robo2^{del5/del5}$ homozygous null mice (**FIGS. 8A-8F**). By scanning electron microscopy, we observed irregular interdigitating foot processes in $Robo2^{del5/del5}$ homozygous null mice at birth and 3 weeks of age (**FIGS. 4A-4H**). These findings indicate that *Robo2* is required for normal podocyte foot process patterning during kidney development.

[00248] To examine the role of *Robo2* in the maintenance of foot process structure in mature glomeruli, we generated podocyte specific *Robo2* knockout mice by crossing conditional $Robo2^{flox/flox}$ mice with $Robo2^{del5/+}$; $Tg^{Nphs2\text{-}Cre/+}$ heterozygous mice carrying a podocin-Cre transgene. Twenty podocyte specific *Robo2* mutant mice with $Robo2^{del5/flox}$; $Tg^{Nphs2\text{-}Cre/+}$ genotype and 20 littermate control mice were analyzed up to one year of age. Podocyte specific *Robo2* knockout mice were viable and

fertile. However, they displayed unusually broad podocyte foot processes and focal segmental foot process effacement at one month (**FIGS. 4I-4M**). At 6 weeks of age the mutant mice developed significant microalbuminuria, which was detected by both ELISA and Western blot analyses (**FIGS. 4N** and **4O**). In addition, scanning electron microscopy revealed foot process patterning defects in *Robo2* podocyte specific knockout mice. Instead of orderly zipper-like interdigitating secondary foot processes in the wild-type, *Robo2* podocyte specific knockout mice displayed irregular and disorganized foot process interdigitation patterning at one month (**FIGS. 8G-8J**). These defects became more obvious over time. At seven months of age, overtly disorganized, shorter, and meandering foot processes were observed in *Robo2* podocyte specific knockout mice (**FIGS. 8K-8N**), which were similar to the phenotype of three-week old *Robo2* null mice. Although *Robo2* podocyte specific knockout mice displayed normal podocyte number, matrix deposition was significantly increased in glomeruli (**FIGS. 8O-8T**, **Tables 1** and **2**). These morphological changes indicate that Robo2 plays a role in regulating and maintain glomerular podocyte foot process structure.

Loss of Robo2 Alleviates the Podocyte Structural Defect in Nephrin Null Mice

[00249] Nephrin homozygous mice develop a characteristic phenotype with dilation of the Bowman's space, abnormally broad foot processes, absence of glomerular slit-diaphragms, and significant proteinuria (Done et al., 2008; Hamano et al., 2002). Since Robo2 formed a complex with nephrin, and Slit2-Robo2 signaling inhibited nephrin-induced actin polymerization, we wondered if loss of Robo2 would modify the podocyte phenotype in nephrin null mice. To test this hypothesis of a possible genetic interaction between Robo2 and nephrin, we generated both germline $Robo2^{-/-}$;$Nphs1^{-/-}$ and podocyte-specific $Robo2^{flox/flox}$;$Tg^{Nphs2\text{-}Cre/+}$;$Nphs1^{-/-}$ double Robo2-nephrin knockout mice. Like $Nphs1^{-/-}$ single homozygote, both $Robo2^{-/-}$;$Nphs1^{-/-}$ (4/4, 100%) and $Robo2^{flox/flox}$;$Tg^{Nphs2\text{-}Cre/+}$;$Nphs1^{-/-}$ (3/3, 100%) double knockout mice died within 10 hours after birth. Histological analysis, however, revealed that the morphology of glomeruli in the $Robo2^{-/-}$;$Nphs1^{-/-}$ double homozygous mice appeared relatively normal compared with the phenotype in $Nphs1^{-/-}$ single nephrin homozygous mice, which had a dilated Bowman's space (**FIGS. 8U-8X**). The number of glomeruli with dilated Bowman's space was significantly reduced in $Robo2^{-/-}$;$Nphs1^{-/-}$ double homozygous mice (2/55, 3.6%) compared with nephrin single null mice (31/122, 25.4%) (**FIGS. 8Y** and **Table 3**). In addition, analysis of glomerular ultrastructure by scanning electron microscopy showed that the interdigitating podocyte foot process structure was observed in only 1 (6.67%) of 15 glomeruli from nephrin single homozygous mice (**FIGS. 4P** and **4Q**) compared with 100% in $Robo2^{-/-}$ single homozygotes (**FIGS. 4T** and **4U**) and wild-type controls (**FIG. 4V** and **4W**). Remarkably, the interdigitating pattern of the podocyte foot processes was restored in 12 (75%) of 16 glomeruli from $Robo2^{-/-}$;$Nphs1^{-/-}$ double homozygous newborn mouse kidneys (**FIGS. 4R** and **4S**, **Table 4**), indicating that a concomitant loss of Robo2 and nephrin alleviated the podocyte foot process structural phenotype in these mice. These findings indicate that the Robo2-Nck-nephrin physical interactions described above have a substantial effect on podocyte foot process morphology in vivo when the levels of expression of Robo2 and nephrin are genetically altered.

[00250] Podocytes exhibit a remarkable degree of plasticity. During development they differentiate from simple cuboidal epithelial cells into the elaborate process-bearing cells that we recognize as mature podocytes (Reeves et al., 1978). This plasticity is retained after maturation. It is seen most graphically as reversible foot process effacement following experimental surface charge neutralization with protamine sulfate and restoration with heparin (Seiler et al., 1975) and during relapse and remission of proteinuria in children with minimal change disease (Nachman et al., 2008). More subtle changes in foot processes probably occur under physiological conditions in response to positive and negative signals in the form of hemodynamic, hormonal or paracrine stimuli. Given the abundance of F-actin in the foot processes, it is likely, without wishing to be bound or limited by theory, that such stimuli bring about those subtle changes in response to positive and negative signals transduced to the F-actin cytoskeleton. Too much and unbalanced positive signals may lead to disease phenotype. Indeed, although a physiological ligand has yet to be identified, it is clear that clustering and phosphorylation of nephrin induces actin polymerization by recruiting Nck, a mechanism that can be involved in the proteinuria induced in rats by a nephritogenic monoclonal antibody to the extracellular domain of nephrin (Topham et al., 1999) and in cases of congenital nephrotic syndrome that develop anti-nephrin alloantibodies after renal transplantation (Patrakka et al., 2002).

[00251] Our studies described herein reveal another level of negative regulation of podocyte actin polymerization in which Robo2, when bound by Slit, inhibits nephrin-induced actin polymerization. We propose, without wishing to be bound or limited by theory, that Slit-Robo2 signaling can inhibit nephrin-induced actin polymerization to maintain normal podocyte foot process structure as follows: Under physiological conditions (e.g. during foot process development), nephrin engagement leads to phosphorylation of the intracellular Y1191/1208/1232 to which the Nck SH2 domain binds (Jones et al., 2006; Verma et al., 2006). Nck in turn recruits cytoskeleton regulators such as N-WASP through its SH3 domains to promote actin polymerization for podocyte foot-process extension or spreading (**FIG. 8Z**). Local secretion and binding of Slit increases the interaction of Robo2 with Nck through its proline rich region and the first two SH3 domains of Nck. Sequestering the first two SH3 domains of Nck by Robo2 would inhibit nephrin-Nck mediated actin polymerization and decrease F-actin associated with nephrin to maintain a dynamic and balanced F-actin cytoskeleton and normal podocyte foot process structure (**FIG. 8Z**). In addition to direct inhibition of nephrin induced actin polymerization through Nck, Slit-Robo2 signaling can inactivate actin polymerization through other pathways, such as recruiting Ena, Abl, srGAPs to negatively regulate F-actin cytoskeleton as previously reported (Bashaw et al., 2000; Wong et al., 2001). In the absence of Slit2-Robo2 signaling (e.g., when Robo2 is knocked out), the inhibitory effects of Robo2 on nephrin induced polymerization is lost. The SH3 domains of Nck are able to interact with downstream cytoskeletal regulators to increase actin polymerization (**FIG. 8Z**), which can explain the altered podocyte foot process structure identified in *Robo2* mutant mice. Our results described herein thus support a mechanism whereby Slit-Robo signaling can regulate podocyte plasticity by negatively regulating F-actin cytoskeleton, which is similar to the role of Slit-Robo

signaling in axon growth cone pathfinding (Guan and Rao, 2003). The pathological finding of increased matrix deposition in the *Robo2* mutant mouse glomeruli likely represents a secondary response.

[00252] Although it is clear from our studies described herein that Robo2 localizes to the basal surface of podocytes and forms a complex with other established foot process slit-diaphragm proteins through its intracellular domain, it remains uncertain if it actually forms part of the slit-diaphragm itself. Interestingly, the extracellular domain of Robo2 resembles that of nephrin, which has eight immunoglobulin (Ig)-like motifs and one fibronectin domain whereas Robo2 has five Ig-like motifs and three fibronectin domains (**FIG. 8Z**) (Tryggvason et al., 2006). We have tested the interaction between the intracellular domain of Robo2 and the cytoplasmic domain of nephrin in the yeast two-hybrid assay. Our biochemical data (**FIGS. 2E** and **2F**) also did not support a direct interaction between these two receptors in vitro. However, it is possible that the extracellular domain of Robo2 can associate with the extracellular domain of nephrin in vivo on the cell membranes of adjacent foot processes through a trans-interaction in the slit-diaphragm.

[00253] We found that *Robo2* homozygous null and podocyte specific knockout mice developed an altered foot process interdigitating pattern, a phenotype that is different from that of the nephrin null mice (Hamano et al., 2002; Done, 2008). This is not surprising since nephrin and Robo2 play opposite roles in regulating podocyte F-actin cytoskeleton. While nephrin signaling induces localized actin polymerization, Slit2-Robo2 signaling acts as a negative regulator on actin polymerization to maintain podocyte foot process plasticity and dynamics. It is noteworthy that a similar foot process organization defect is observed in mice in which the actin-depolymerizing factor Cofilin-1, another negative regulator of the F-actin cytoskeleton in podocytes, is knocked out (Garg et al., 2010). This indicates that the absence of either an actin polymerization promoting factor such as nephrin signaling or an inhibitory factor such as Robo2 signaling will affect the normal structure of podocytes. Thus the balance between positive and negative F-actin cytoskeleton regulators in podocytes is important to maintain normal foot process structure. Regaining this balance by knocking out both positive (nephrin) and negative (Robo2) signals can explain the restoration of podocyte foot process interdigitation and milder glomerular phenotype in the Robo2-nephrin double knockout mice. Our studies described herein demonstrate the dual roles of nephrin as an essential component of the slit-diaphragm to control glomerular permselectivity on the one hand (Tryggvason et al., 2006) and as a regulator of foot process morphology through its interaction with the actin cytoskeleton (Jones et al., 2006; Verma et al., 2006) on the other. While Robo2 signaling clearly counters the positive signaling effects of nephrin on the foot processes, it remains to be determined if it also influences slit-diaphragm integrity.

[00254] Accordingly, as described herein, we have identified Robo2 as a new component of the podocyte intercellular junction in the kidney. We have demonstrated interactions between Robo2 and nephrin using biochemical, functional, and genetic techniques and have shown that Slit2-Robo2 signaling inhibits nephrin-induced actin dynamics. Our results indicate that Robo2 signaling acts as a negative regulator on nephrin to modulate podocyte foot process architecture. This study extends our

understanding of the role of Slit-Robo signaling and identifies a novel crosstalk mechanism by which guidance cue receptor Robo might influence F-actin cytoskeleton dynamics.

[00255] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. 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 is defined solely by the claims. Definitions of common terms in immunology, and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 18th Edition, published by Merck Research Laboratories, 2006 (ISBN 0-911910-18-2); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006. Definitions of common terms in molecular biology are found in Benjamin Lewin, Genes IX, published by Jones & Bartlett Publishing, 2007 (ISBN-13: 9780763740634); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1982); Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987); Current Protocols in Molecular Biology (CPMB) (Fred M. Ausubel, et al. ed., John Wiley and Sons, Inc.), Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.) and Current Protocols in Immunology (CPI) (John E. Coligan, et. al., ed. John Wiley and Sons, Inc.), which are all incorporated by reference herein in their entireties.

[00256] As used herein, the term “comprising” means that other elements can also be present in addition to the defined elements presented. The use of “comprising” indicates inclusion rather than limitation.

[00257] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00258] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00259] Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. As used in this specification and the appended claims, the singular forms “a,” "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[00260] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used in connection with percentages can mean ±1%.

[00261] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. 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 is defined solely by the claims.

[00262] All patents and other publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that could be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00263] Embodiments of the various aspects described herein can be illustrated by the following numbered paragraphs:

2. A method for the treatment of chronic kidney disease in a subject in need thereof, comprising administering to a subject having or at risk for a chronic kidney disease a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.
3. A method for the reduction of proteinuria in a subject in need thereof, comprising administering to a subject having or at risk for proteinuria a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.
4. The method of any one of paragraphs 1or 2, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.
5. The method of any one of paragraphs 1-3, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.
6. The method of any one of paragraphs 1-4, wherein the ROBO2 inhibitor is specific for the Ig1

SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

7. The method of paragraph 3, wherein the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.
8. The method of any one of paragraphs 1-6, wherein the subject having or at risk for a chronic kidney disease has diabetic nephropathy or high blood pressure.
9. The method of any one of paragraphs 1-7, further comprising administering to the subject an additional therapeutic agent.
10. The method of paragraph 8, wherein the additional therapeutic agent is an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin II receptor blocker (ARB).
11. A method comprising:
    a. assaying a biological test sample from a subject to determine an expression level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide;
    b. determining whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in the biological test sample is above a reference threshold level; and
    c. diagnosing the subject as in need of treatment or therapy for chronic kidney disease.
12. The method of paragraph 10, wherein assaying the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.
13. The method of paragraph 10, wherein assaying the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.
14. The method of any one of paragraphs 10-12, wherein the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.
15. The method of any one of paragraphs 10-13, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.
16. The method of any one of paragraphs 10-13, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.
17. An assay comprising:
    a. contacting a biological test sample isolated from a subject with a reagent that detects ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide; and
    b. measuring the level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide,

c. wherein an increased level of said ROBO2 polypeptide or said RNA encoding a ROBO2 polypeptide, relative to a normal biological sample, identifies a subject having chronic kidney disease and/or progression of chronic kidney disease or proteinuria.

18. The assay of paragraph 16, wherein detecting the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.
19. The assay of paragraph 16, wherein detecting the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.
20. The assay of any one of paragraphs 16-18, wherein the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.
21. The assay of any one of paragraphs 16-19, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.
22. The assay of any one of paragraphs 16-19, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.
23. The assay of any one of paragraphs 16-21, wherein the subject has been diagnosed with diabetes or high blood pressure.
24. A system for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:
    a. a measuring module configured to determine the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in a biological sample obtained from a subject;
    b. a comparison module configured to receive said expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide determined by the measuring module and perform at least one analysis to determine whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is greater than a pre-determined reference level or ratio, and to provide a retrieved content; and
    c. a display module for displaying a content based the data output from said comparison module, wherein the content comprises a signal indicative that the expression level or ratio of ROBO2 polypeptide or RNA is greater than the pre-determined reference level or ratio, or a signal indicative that the level or expression ratio of ROBO2 is not greater than the reference level or pre-determined ratio.
25. The system of paragraph 23, wherein the content displayed on said display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.

26. A system for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:
    a. a determination module configured to receive at least one test sample obtained from a subject and perform at least one analysis on said at least one test sample to determine the presence or absence of either of the following conditions:
        i. an expression ratio of ROBO2 greater than a pre-determined ratio, or
        ii. an expression level of ROBO2 greater than a pre-determined level
    b. a storage device configured to store data output from said determination module; and
    c. a display module for displaying a content based on the data output from said determination module, wherein the content comprises a signal indicative that the expression ratio of ROBO2 is greater than the pre-determined ratio or level of ROBO2 greater than a pre-determined level, or a signal indicative that the expression ratio of ROBO2 is not greater than the pre-determined ratio or not greater than a pre-determined level.
27. The system of paragraph 25, wherein the content displayed on said display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.
28. A method for treating a human subject with a risk of chronic kidney disease or proteinuria, comprising administering a treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria to a human subject who is determined to have a level of ROBO2 protein above a reference threshold level.
29. The method of paragraph 27, wherein the level of ROBO2 protein is at least 20% above the reference level.
30. The method of paragraph 27, wherein the level of ROBO2 protein is at least two standard deviations above the reference level.
31. The method of any one of paragraphs 27-29, wherein the treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria comprises a ROBO2 inhibitor.
32. The method of paragraph 30, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.
33. The method of any one of paragraphs 30-31, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.
34. The method of any one of paragraphs 30-32, wherein the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.
35. The method of paragraph 31, wherein the ROBO2 inhibitory polypeptide is a dominant

negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

36. A ROBO2 inhibitor for use in treating a chronic kidney disease.
37. A ROBO2 inhibitor for use in treating proteinuria.
38. The use of any one of paragraphs 35 or 36, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.
39. The use of any one of paragraphs 35-37, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.
40. The use of any one of paragraphs 35-38, wherein the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.
41. The use of paragraph 37, wherein the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.
42. The use of any one of paragraphs 35-40, wherein the chronic kidney disease or proteinuria is caused by diabetic nephropathy or high blood pressure.

[00264] This invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLES

Tissue in Situ Hybridization, Immunohistochemistry, and Immunogold Electron Microscopy

[00265] In situ hybridization analysis was performed with digoxigenin-labeled *Robo2* riboprobes as previously described (Grieshammer et al., 2004). Immunohistochemistry was performed on mouse embryonic kidney tissues fixed in 4% paraformaldehyde and in adult mouse kidney tissues fixed in methanol. For immunogold electron microscopy, wild-type mouse kidneys were dissected and fixed in paraformaldehyde-lysine-periodate (PLP). Ultrathin sections of the mouse kidney were prepared and incubated with goat anti-Robo2 antibody (DAKO Corporation) and a secondary antibody coupled to 10 nm colloidal gold (Ted Pella).

Yeast Two-Hybrid, Co-Precipitation, and Actin Polymerization Assays

[00266] The DUPLEX-A™ yeast two-hybrid system (OriGene Tech) was used to characterize Robo2 and Nck1 interaction according to manufacturer's instructions. Cell culture, His-tagged protein co-precipitation, and immunoprecipitaion were performed as previously reported (Fan et al., 2003). For endogenous immunoprecipitation, mouse newborn kidneys were utilized. CD16 antibody-mediated

crosslinking of CD16/7 fusion proteins and the actin polymerization assay were performed as previously described (Jones et al., 2006; Rivera et al., 2004; Verma et al., 2006).

Knockout Mouse study, Transmission and Scanning Electron Microscopy, and Kidney Glomerular Analysis

[00267] Mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University Medical Center (#14388). The generation and genotyping of *Robo2*^{flox} conditional allele, *Robo2*^{del5} (also called *Robo2*$^{-}$ interchangeably in this paper) germline mutant allele, and *Robo2*$^{+}$ wild-type allele were described previously (Lu et al., 2007; Wang et al., 2011). To generate *Robo2*-nephrin double knockout mice, *Robo2*$^{+/-}$ mice were crossed with *Nphs1*$^{+/-}$ mice that were generated previously (Hamano et al., 2002). For transmission electron microscopy, kidneys were fixed, incubated in 2% glutaraldehyde in 0.15 M sodium cacodylate, dehydrated in graded ethanol, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate. Ultrathin kidney sections were examined using a JEM-1011 electron microscope. For scanning electron microscopy, kidneys were prepared following the standard protocol. For kidney pathological studies, kidneys were fixed in 4% paraformaldehyde, paraffin embedded, sectioned, and stained using standard Periodic acid-Schiff (PAS) or eosin hematoxylin (H&E) methods. For quantification of podocyte number, WT1 was used as a podocyte nuclear marker and immunoperoxidase staining was performed on kidney sections following the standard protocol. WT1 positive podocyte nuclei in each glomerular cross section were counted. For proteinuria analysis, "spot" urine specimens from 6 weeks old mice were examined using a murine albuminuria ELISA quantitation kit (Exocell) according to manufacturer's instruction and urine dipstick (Multistix, Bayer, IN) as a screening method.

Tissue In Situ Hybridization and Immunohistochemistry

[00268] *In situ* hybridization analysis was performed with digoxigenin-labeled *Robo2* riboprobes as previously described (Grieshammer et al., 2004). The *Robo2* cDNA was linearized with *Not*I and probes were generated using the DIG DNA labeling and detection kit (Roche Applied Science). Hybridization was performed on 4% paraformaldehyde fixed OCT embedded mouse embryonic kidney frozen sections. Immunohistochemistry was performed on mouse embryonic kidney tissues fixed in 4% paraformaldehyde followed by 30% sucrose cryoprotection (Mugford et al., 2008) and in adult mouse kidney tissues fixed in methanol. Mouse kidneys embedded in OCT compound were frozen and sectioned using Cryostat at 8-10 µm. Sections were stained with primary antibodies and followed by an appropriate FITC or Cy3 conjugated secondary antibodies. The primary antibodies used in this study include the ones against ROBO2 (R&D System, Abnova, Santa Cruz Biotechnology), nephrin (custom synthesized) (Topham et al., 1999), Nck (Upstate/Millipore), podocin (Sigma), nidogen (Santa Cruz Biotechnology), Pecam1 (BD Biosciences), WT1 (Santa Cruz Biotechnology), SLIT2 (Santa Cruz Biotechnology), PDGFR beta (Cell Signaling), Synaptopodin (Santa Cruz Biotechnology). Images were obtained using a Perkin Elmer UltraView LCI multi-point spinning disc laser-scanning confocal

microscope and a Zeiss LSM 510 confocal laser scanning microscope with a 60x oil immersion objective.

Immunogold Electron Microscopy

[00269] Wild-type mouse kidneys were dissected and fixed in paraformaldehyde-lysine-periodate (PLP) at 4°C overnight. The tissue was washed in 1x PBS and dehydrated in graded ethanol and embedded in LR White resin (Electron Microscopy Sciences). Ultrathin sections of the mouse kidney were prepared and transferred to Formvar-coated gold grids, and blocked with 1% bovine serum albumin and 5% normal goat serum in 1x PBS. The sections were then incubated with goat anti-Robo2 antibody with a 1:50 dilution in DAKO (DAKO Corporation) at 4°C overnight. Non-immune serum was used as a control. After three washes with 1x PBS, sections were incubated with a IgG secondary antibody coupled to 10 nm colloidal gold (Ted Pella) for 2 hours at room temperature. Sections were finally post-fixed with 1% glutaraldehyde and contrasted with uranyl acetate. Sections were examined with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) at 80kV, and images were acquired using an AMT digital imaging system (Advanced Microscopy Techniques, Danvers, MA.) and imported into Adobe Photoshop. Subcellular localization of Robo2 stained with gold particles in glomeruli was recognized on digital electron micrographs in comparison with control micrographs stained with non-immune serum.

Yeast Two-Hybrid Assay

[00270] The DUPLEX-A™ yeast two-hybrid system (OriGene Tech, Rockville, MD) was used to characterize Robo2 and Nck1 interaction. The cDNAs encoding the intracellular domain of human Robo2 and its truncated forms were cloned into the pJG4-5 vector at *Eco*RI/*Xho*I sites, fusing them to the transcription activation domain of B42. The cDNAs of human Nck1 and its truncated forms were cloned into the pEG202 vector at *Eco*RI/*Xho*I to fuse them to the DNA binding domain of LexA. The *lacZ* gene in the construct pSH18-34 and the *LEU2* gene in the EGY48 strain yeast genome were used as reporter genes. The pEG202, pSH18-34, and pJG4-5 constructs were co-transformed into yeast EGY48 cells. The interaction was considered positive if the yeast cells turned blue in the presence of X-gal and grew in the absence of leucine.

Cell Culture, DNA Constructs, Transfection, Co-Precipitation, and Western Blot Analyses

[00271] HEK (293T) cells were transfected at 60% confluency using calcium phosphate transfection. To make C-terminal his- and myc- tagged fusion proteins, full-length human nephrin and Robo2 were cloned into pSecTag B vector (Invitrogen) at *Hin*d III/*Eco*R1 and *Eco*R1/*Xho*1 restriction sites respectively. Robo2-ΔNBD was obtained by deleting the Nck binding domain (FIG. 2C) using QUIKCHANGE site-directed mutagenesis kit (Strategene) according to manufacturer's instructions. Non-tagged Robo2 and Nck1 were cloned into pCS2 vector (Addgene) at *Eco*R1/*Xho*1 sites, nephrin at *Hin*dIII/*Eco*R1 sites. Human Fyn and myc- tagged Slit2 constructs have been reported previously (Li et al., 2008; Wong et al., 2001). CD16/7-NCD and CD16/7-HA constructs were also reported previously (Verma et al., 2006). To detect Robo2 and Nck1 interaction, C-terminal His- and myc-tagged human

Robo2 or Robo2-ΔNBD was expressed in HEK cells. Forty-eight hour post-transfection, cells were lysed in the lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 0.5% TX100, 1x protease inhibitor [pH 8.0]). Cell lysates were centrifuged for 10 min at 4°C; supernatants were incubated with Ni-NTA resin (Qiagen) at 4°C for 2 hours to precipitate His-Robo2, NTA resin without Ni was used as a control. The resin was washed three times with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.5% TX100 [pH 8.0]) and heated at 95°C for 10 min. The precipitates were resolved on SDS-PAGE gels and blotted with rabbit anti-myc, rabbit monoclonal anti-Nck1 (Cell Signaling) antibodies at a 1:1000 dilution. To examine the triple interaction among Robo2, Nck1, and nephrin, His-myc-Robo2 or His-myc-Robo2-ΔNBD were co-expressed in HEK cells with human nephrin and human Fyn. His-myc-Robo2 was precipitated with Ni-NTA beads as described above. To confirm the triple interaction, His-myc-nephrin was co-expressed with Robo2, and Fyn in HEK cells and His-myc-nephrin was pulled-down by Ni-NTA beads. Precipitates were blotted with rabbit polyclonal anti-myc, rabbit monoclonal anti-Nck1, rabbit polyclonal anti-nephrin, mouse monoclonal anti-Robo2 (R&D systems), and rabbit polyclonal anti-Fyn (Santa Cruz Biotechnology) antibodies at a 1:1000 dilution. For co-immunoprecipitation of endogenous proteins, kidneys from newborn mice were homogenized in the RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1x protease inhibitor) on ice. Samples were centrifuged for 10 min at 4°C and the supernatant was incubated with 1 µg mouse monoclonal anti-Robo2 antibody (R&D Systems) for 1 hour at 4°C. The control goat IgG (Santa Cruz Biotechnology) was used as a control. Samples were then mixed with 30 µl of protein A/G Plus agarose bead slurry (Santa Cruz Biotechnology) and further incubated for 12 hours at 4°C. Beads were then washed three times in the RIPA buffer and proteins were eluted in 1x protein loading buffer by heating at 95°C for 10 min. Precipitates were resolved on SDS-PAGE gels and blotted with mouse anti-Robo2, rabbit anti-nephrin, and rabbit anti-Nck1 antibodies as described above. Actin was blotted with anti-beta-actin mouse antibody from Sigma. Intensity of the bands was measured using ImageJ. For proteinuria detection, mice spot urines were collected and diluted with 1x protein loading buffer at 1:100 dilution. Urine proteins were then resolved on SDS-PAGE gels and purified albumin was used as a control (MP Biomedicals). Gels were blotted with rabbit anti-albumin polyclonal antibody (MP Biomedicals).

CD16/7-NCD Crosslinking and Actin Polymerization Assay

[00272] CD16 antibody-mediated crosslinking of CD16/7 fusion proteins has been described previously (Jones et al., 2006; Rivera et al., 2004; Verma et al., 2006). Briefly, CD16/7-NCD or CD16/7-HA was co-expressed in HEK cells with Robo2. After 24 hours, cells were transferred and seeded on glass coverslips coated with polylysine for another 24 hours. Cells were then incubated with 1 µg/ml mouse monoclonal anti-CD16 (Beckman Coulter) for 30 min at 37°C, washed once with DMEM, incubated with rhodamine-conjugated secondary antibody (Thermo Scientific) diluted in Slit2 conditioned medium (Wong et al., 2001) or control conditioned medium for 30 min and fixed in 4%

paraformaldehyde in 1x PBS. F-actin was stained using FITC-conjugated phalloidin (Invitrogen) according to manufacturer's instruction. The newly formed F-actin bundles stick to the clustered nephrin (CD16/7-NCD) and look like comet tails (i.e. actin tails in the main text) under fluorescence microscope. In this experiment, we only analyzed the F-actin bundles formed by clustering of CD16/7-NCD and attached to the clusters. The cells with F-actin tails were counted and compared to the total CD16/7-NCD transfected cells. The quantification formula is: Percentage % = (number of transfected cells with F-actin tails / total number of cells transfected) × 100. Images were obtained using a LSM510 confocal microscope with a 60x oil immersion objective.

Generation and Characterization of Robo2 Podocyte Specific Knockout Mice and Robo2-Nephrin Double Knockout Mice

[00273] The generation and genotyping of *Robo2*^{flox} conditional allele, *Robo2*^{del5} (also called *Robo2*$^{-}$ interchangeably in this paper) germline mutant allele, and *Robo2*$^{+}$ wild-type allele were described previously (Lu et al., 2007; Wang et al., 2011). Standard breeding scheme was followed to generate *Robo2* podocyte specific *Robo2*$^{del5/flox}$;Tg$^{Nphs2\text{-}Cre/+}$ knockout mice, which carry one *Robo2*^{del5} allele and one *Robo2*^{flox} allele. In this compound mutant, podocyte specific Cre recombinase driven by podocin promoter deletes only the *Robo2*^{flox} allele to facilitate the penetrance of a phenotype because the other allele, *Robo2*^{del5}, has been deleted ubiquitously from germline expression. The authenticity of *Robo2*$^{del5/flox}$;Tg$^{Nphs2\text{-}Cre/+}$ mice was determined by tail DNA genotyping for the presence of *Robo2*^{del5} and *Robo2*^{flox} alleles as well as Tg$^{Nphs2\text{-}Cre}$ transgene. F2 littermates *Robo2*$^{flox/+}$ mice without *Robo2*^{del5} allele and Tg$^{Nphs2\text{-}Cre}$ transgene were used as controls. To generate *Robo2*-nephrin double knockout mice, *Robo2*$^{+/-}$ heterozygous mice were crossed with *Nphs1*$^{+/-}$ heterozygous mice that were generated previously (Hamano et al., 2002). After the generation of *Robo2*$^{+/-}$;*Nphs1*$^{+/-}$ double heterozygous mice, the cross of double heterozygous mice was performed to generate *Robo2*$^{-/-}$;*Nphs1*$^{-/-}$ double homozygous mice as well as *Nphs1*$^{-/-}$ single homozygous, *Robo2*$^{-/-}$ single homozygous, and *Robo2*$^{+/+}$;*Nphs1*$^{+/+}$ wild-type controls. Mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University Medical Center (#14388).

[00274] **Transmission and Scanning Electron Microscopy**

[00275] For transmission electron microscopy, kidneys were dissected from *Robo2* homozygous null mice and podocyte specific knockout mice, fixed in PLP at 4°C overnight, and then incubated in 2% glutaraldehyde in 0.15 M sodium cacodylate for 6 hours. After washing in 1x PBS, fixed kidneys were dehydrated in graded ethanol, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate. Ultrathin kidney sections were prepared and examined using a JEM-1011 electron microscope. Wild-type littermates were used as controls. For scanning electron microscopy, kidney samples from *Robo2* homozygous null mice, podocyte specific knockout mice, nephrin homozygous null mice, and *Robo2*-nephrin double homozygous mice were prepared following the protocol described previously (Friedman and Ellisman, 1981) with minor modifications. Briefly, the kidney was perfused with 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1M cacodylate buffer

(Karnovsky's fixative, Electron Microscopy Sciences), and subsequently fixed in the Karnovsky's fixative for 24 hrs followed by postfixation in 2% osmium tetraoxide solution (Electron Microscopy Sciences). Kidney samples were cryofractured, dehydrated and dried using hexamethyldisilazane (Electron Microscopy Sciences). Kidney samples were imaged using an Amray 1000A and Jeol 6340F scanning electron microscopes. Three glomeruli from each animal were examined to provide representative images.

Mice Kidney Pathology Studies, Quantification of Podocyte Number, and Proteinuria Analysis

[00276] For kidney pathological studies, kidneys were dissected and fixed in 4% paraformaldehyde overnight, and then treated with a graded ethanol series for paraffin embedding. The kidney paraffin blocks were sectioned at 4 μm using a MT-920 microtome (MICROM) and stained using standard Periodic acid-Schiff (PAS) or eosin hematoxylin (H&E) methods. The glomeruli were examined and assessed for matrix deposition, segmental glomerulosclerosis, and dilatations of the Bowman's space using an Olympus BHT light microscope equipped with a SPOT digital camera system. For quantification of podocyte number, WT1 was used as a podocyte nuclear marker and immunoperoxidase staining was performed on kidney sections following the protocol described previously (Sanden et al., 2003). Briefly, paraffin embedded kidney sections from 4 one-year old $Robo2^{del5/flox};Tg^{Nphs2\text{-}Cre+}$ podocyte-specific knockout mice and 4 age-matched wild-type control mice were sectioned at 4 μm and stained with WT1 antibody (Santa Cruz Biotechnology) after microwave antigen retrieval. Biotinylated secondary antibody and Vectastain ABC kit (Vector Laboratories) were used to detect WT1 signal. WT1 positive podocyte nuclei in each glomerular cross section were counted in total 165 glomeruli from four mutant mice and 166 glomeruli from four control mice. For proteinuria analysis, "spot" urine specimens from 6 weeks old mice were examined using a sensitive murine albuminuria ELISA quantitation kit (Exocell) according to manufacturer's instruction and urine dipstick (Multistix from Bayer, IN) as a screening method. Urine albumin was normalized with creatinine to provide an albumin/creatinine ratio. Creatinine in urine was determined using the creatinine detection kit (Sigma) according to manufacturer's instruction. Urine albumin was also examined by 12% SDS-PAGE and blotted with anti-albumin antibody (MP Biomedicals). The data from mutants and controls were analyzed using one-way ANOVA, Student *t*-test, and Chi-square test.

Table 1. Quantitative Analysis of Glomeruli with Increased Matrix Expansion in 2 to 9 Months Old Robo2 Podocyte Specific Knockout Mice (Mutant) Compared to Controls (Wild type)

Genotype	Mouse ID#	Age (months)	Total glomeruli counted	Glomeruli with mesangial matrix expansion	% of Glomeruli with mesangial matrix expansion*
Mutant	4048	2	96	13	12.35%
Mutant	1721	3	107	18	16.82%
Mutant	4005	6	103	17	16.51%

Mutant	1190	7	102	20	19.61%
Mutant	2396	9	80	14	17.50%
Mutant total			**488**	**82**	**16.80%**
Wild type	4058	2	90	4	4.44%
Wild type	4052	3	105	6	5.71%
Wild type	3919	6	107	4	3.74%
Wild type	1191	7	103	5	4.85%
Wild type	2385	9	106	5	4.72%
Wild type total			**511**	**24**	**4.70%**

$p < 0.01$, n=5, t-test.

Table 2. Quantitative Analysis of Glomeruli with Increased Matrix Expansion in 12-months-old Robo2 Podocyte-Specific Knockout Mice (Mutant) Compared to Controls (Wild type)

Genotype	Mouse ID#	Age (months)	Total glomeruli counted	Glomeruli with mesangial matrix expansion	% of Glomeruli with mesangial matrix expansion*
Mutant	1844	12	136	17	12.50%
Mutant	1847	12	125	18	14.40%
Mutant	1877	12	127	11	8.66%
Mutant	1878	12	132	20	15.15%
Mutant	1948	12	142	28	19.72%
Mutant total			**662**	**94**	**14.20%**
Wild type	1901	12	125	5	4.00%
Wild type	2429	12	179	8	4.47%
Wild type	2834	12	154	9	5.84%
Wild type	2836	12	159	7	4.40%
Wild type	2837	12	124	5	4.03%
Wild type total			**741**	**34**	**4.59%**

$p < 0.01$, n=5, t-test.

Table 3. Morphology Analysis of Glomeruli with Dilated Bowman's Space in *Nphs1*$^{-/-}$ Single-Homozygous (*Robo2*$^{+/-}$;*Nphs1*$^{-/-}$) Compared to *Robo2*$^{-/-}$;*Nphs1*$^{-/-}$ Double- Homozygous Newborn Mice by Histology

Genotype	Total glomeruli counted	Glomeruli with dilated Bowman's space	% of Glomeruli with dilated Bowman's space
Robo2$^{+/-}$;*Nphs1*$^{-/-}$	122	31	25.4%
Robo2$^{-/-}$;*Nphs1*$^{-/-}$	55	2	3.6%
Robo2$^{-/-}$;*Nphs1*$^{+/-}$	158	3	1.9%
Robo2$^{+/+}$;*Nphs1*$^{+/+}$	271	1	0.4%

Note: The glomerulus was scored as positive with dilated Bowman's space if the glomerulus displayed similar phenotype as shown in Fig S4U was observed, and was scored as negative if similar glomerulus as shown in FIG. 8V-8X was observed. Three mice from each genotype were analyzed. *Robo2*$^{-/-}$ single homozygous (*Robo2*$^{-/-}$;*Nphs1*$^{+/-}$) and wild-type (*Robo2*$^{+/+}$;*Nphs1*$^{+/+}$) were used as controls.

Table 4. Morphology Analysis of Glomerular Podocyte Interdigitating Foot Process (FP) Phenotype in *Nphs1*$^{-/-}$ Single-Homozygous (*Robo2*$^{+/-}$;*Nphs1*$^{-/-}$) Compared to *Robo2*$^{-/-}$;*Nphs1*$^{-/-}$ Double-Homozygous Newborn Mice by Scanning Electron Microscopy

Genotype	Total glomeruli counted	Glomeruli with interdigitating FP structure	% of Glomeruli with interdigitating FP structure
Robo2$^{+/-}$;*Nphs1*$^{-/-}$	15	1	6.67%
Robo2$^{-/-}$;*Nphs1*$^{-/-}$	16	12	75%
Robo2$^{-/-}$;*Nphs1*$^{+/-}$	13	13	100%
Robo2$^{+/+}$;*Nphs1*$^{+/+}$	13	13	100%

REFERENCES

Bashaw, G. J., Kidd, T., Murray, D., Pawson, T., and Goodman, C. S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell *101*, 703-715.

Dickson, B. J., and Gilestro, G. F. (2006). Regulation of commissural axon pathfinding by slit and its Robo receptors. Annu Rev Cell Dev Biol *22*, 651-675.

Done, S. C., Takemoto, M., He, L., Sun, Y., Hultenby, K., Betsholtz, C., and Tryggvason, K. (2008). Nephrin is involved in podocyte maturation but not survival during glomerular development. Kidney Int *73*, 697-704.

Fan, X., Labrador, J. P., Hing, H., and Bashaw, G. J. (2003). Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline. Neuron *40*, 113-127.

Faul, C., Asanuma, K., Yanagida-Asanuma, E., Kim, K., and Mundel, P. (2007). Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. Trends Cell Biol *17*, 428-437.

Furness, P. N., Hall, L. L., Shaw, J. A., and Pringle, J. H. (1999). Glomerular expression of nephrin is decreased in acquired human nephrotic syndrome. Nephrol Dial Transplant *14*, 1234-1237.

Garg, P., Verma, R., Cook, L., Soofi, A., Venkatareddy, M., George, B., Mizuno, K., Gurniak, C.,

Witke, W., and Holzman, L. B. (2010). Actin-depolymerizing factor cofilin-1 is necessary in maintaining mature podocyte architecture. J Biol Chem *285*, 22676-22688.

Grieshammer, U., Le, M., Plump, A. S., Wang, F., Tessier-Lavigne, M., and Martin, G. R. (2004). SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. Dev Cell *6*, 709-717.

Guan, K. L., and Rao, Y. (2003). Signalling mechanisms mediating neuronal responses to guidance cues. Nat Rev Neurosci *4*, 941-956.

Hamano, Y., Grunkemeyer, J. A., Sudhakar, A., Zeisberg, M., Cosgrove, D., Morello, R., Lee, B., Sugimoto, H., and Kalluri, R. (2002). Determinants of vascular permeability in the kidney glomerulus. J Biol Chem *277*, 31154-31162.

Jones, N., Blasutig, I. M., Eremina, V., Ruston, J. M., Bladt, F., Li, H., Huang, H., Larose, L., Li, S. S., Takano, T., *et al.* (2006). Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. Nature *440*, 818-823.

Lu, W., van Eerde, A. M., Fan, X., Quintero-Rivera, F., Kulkarni, S., Ferguson, H. L., Kim, H., Fan, Y., Xi, Q., Li, Q. G., *et al.* (2007). Disruption of ROBO2 is associated with urinary tract anomalies and confers risk of vesicoureteral reflux. Am J Hum Genet *80*, 616-632.

Nachman, P. H., Jennette, J. C., and Falk, R. J. (2008). Primary glomerular disease. In Brenner & Rector's The Kidney, B. M. Brenner, ed. (Pheladelphia, Saunders), pp. 987-1066.

Patrakka, J., Ruotsalainen, V., Reponen, P., Qvist, E., Laine, J., Holmberg, C., Tryggvason, K., and Jalanko, H. (2002). Recurrence of nephrotic syndrome in kidney grafts of patients with congenital nephrotic syndrome of the Finnish type: role of nephrin. Transplantation *73*, 394-403.

Piper, M., Anderson, R., Dwivedy, A., Weinl, C., van Horck, F., Leung, K. M., Cogill, E., and Holt, C. (2006). Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. Neuron *49*, 215-228.

Piper, M., Georgas, K., Yamada, T., and Little, M. (2000). Expression of the vertebrate Slit gene family and their putative receptors, the Robo genes, in the developing murine kidney. Mech Dev *94*, 213-217.

Reeves, W., Caulfield, J. P., and Farquhar, M. G. (1978). Differentiation of epithelial foot processes and filtration slits: sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. Lab Invest *39*, 90-100.

Rivera, G. M., Briceno, C. A., Takeshima, F., Snapper, S. B., and Mayer, B. J. (2004). Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. Curr Biol *14*, 11-22.

Seiler, M. W., Venkatachalam, M. A., and Cotran, R. S. (1975). Glomerular epithelium: structural alterations induced by polycations. Science *189*, 390-393.

Shih, N. Y., Li, J., Cotran, R., Mundel, P., Miner, J. H., and Shaw, A. S. (2001). CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am J Pathol *159*, 2303-2308.

Topham, P. S., Kawachi, H., Haydar, S. A., Chugh, S., Addona, T. A., Charron, K. B., Holzman, L. B., Shia, M., Shimizu, F., and Salant, D. J. (1999). Nephritogenic mAb 5-1-6 is directed at the extracellular

domain of rat nephrin. J Clin Invest *104*, 1559-1566.

Tryggvason, K., Patrakka, J., and Wartiovaara, J. (2006). Hereditary proteinuria syndromes and mechanisms of proteinuria. N Engl J Med *354*, 1387-1401.

Verma, R., Kovari, I., Soofi, A., Nihalani, D., Patrie, K., and Holzman, L. B. (2006). Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. J Clin Invest *116*, 1346-1359.

Wang, H., Li, Q., Liu, J., Mendelsohn, C., Salant, D. J., and Lu, W. (2011). Noninvasive assessment of antenatal hydronephrosis in mice reveals a critical role for Robo2 in maintaining anti-reflux mechanism. PLoS One *6*, e24763.

Wong, K., Ren, X. R., Huang, Y. Z., Xie, Y., Liu, G., Saito, H., Tang, H., Wen, L., Brady-Kalnay, S. M., Mei, L., *et al.* (2001). Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell *107*, 209-221.

Yuan, H., Takeuchi, E., and Salant, D. J. (2002). Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. Am J Physiol Renal Physiol *282*, F585-591.

Friedman, P. L., and Ellisman, M. H. (1981). Enhanced visualization of peripheral nerve and sensory receptors in the scanning electron microscope using cryofracture and osmium-thiocarbohydrazide-osmium impregnation. J Neurocytol 10, 111-131.

Li, X., Gao, X., Liu, G., Xiong, W., Wu, J., and Rao, Y. (2008). Netrin signal transduction and the guanine nucleotide exchange factor DOCK180 in attractive signaling. Nat Neurosci 11, 28-35.

Mugford, J. W., Sipila, P., Kobayashi, A., Behringer, R. R., and McMahon, A. P. (2008). Hoxd11 specifies a program of metanephric kidney development within the intermediate mesoderm of the mouse embryo. Dev Biol 319, 396-405.

Sanden, S. K., Wiggins, J. E., Goyal, M., Riggs, L. K., and Wiggins, R. C. (2003). Evaluation of a thick and thin section method for estimation of podocyte number, glomerular volume, and glomerular volume per podocyte in rat kidney with Wilms' tumor-1 protein used as a podocyte nuclear marker. J Am Soc Nephrol 14, 2484-2493.

CLAIMS

We claim:

1. A method for the treatment of chronic kidney disease in a subject in need thereof, comprising administering to a subject having or at risk for a chronic kidney disease a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.

2. A method for the reduction of proteinuria in a subject in need thereof, comprising administering to a subject having or at risk for proteinuria a therapeutically effective amount of a composition comprising a ROBO2 inhibitor.

3. The method of any one of claims 1or 2, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

4. The method of any one of claims 1-3, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

5. The method of any one of claims 1-4, wherein the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

6. The method of claim 3, wherein the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

7. The method of any one of claims 1-6, wherein the subject having or at risk for a chronic kidney disease has diabetic nephropathy or high blood pressure.

8. The method of any one of claims 1-7, further comprising administering to the subject an additional therapeutic agent.

9. The method of claim 8, wherein the additional therapeutic agent is an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin II receptor blocker (ARB).

10. A method comprising:
    a. assaying a biological test sample from a subject to determine an expression level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide;
    b. determining whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in the biological test sample is above a reference threshold level; and

c. diagnosing the subject as in need of treatment or therapy for chronic kidney disease.

11. The method of claim 10, wherein assaying the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.

12. The method of claim 10, wherein assaying the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.

13. The method of any one of claims 10-12, wherein the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.

14. The method of any one of claims 10-13, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.

15. The method of any one of claims 10-13, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.

16. An assay comprising:

a. contacting a biological test sample isolated from a subject with a reagent that detects ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide; and

b. measuring the level of ROBO2 polypeptide or an RNA encoding a ROBO2 polypeptide,

wherein an increased level of said ROBO2 polypeptide or said RNA encoding a ROBO2 polypeptide, relative to a normal biological sample, identifies a subject having chronic kidney disease and/or progression of chronic kidney disease or proteinuria.

17. The assay of claim 16, wherein detecting the expression level of ROBO2 polypeptide is performed using an antibody or antigen-binding fragment thereof specific for the ROBO2 polypeptide.

18. The assay of claim 16, wherein detecting the expression level of the RNA encoding a ROBO2 polypeptide is performed using PCR or a hybridization assay.

19. The assay of any one of claims 16-18, wherein the biological test sample is a kidney biopsy, urine, blood, serum sample, or cells pelleted from a urine sample.

20. The assay of any one of claims 16-19, wherein the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is at least 20% above the reference threshold level.

21. The assay of any one of claims 16-19, wherein the expression level of ROBO2 polypeptide or

the expression level of the RNA encoding a ROBO2 polypeptide is at least two standard deviations above the reference threshold level.

22. The assay of any one of claims 16-21, wherein the subject has been diagnosed with diabetes or high blood pressure.

23. A system for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:

a. a measuring module configured to determine the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide in a biological sample obtained from a subject;

b. a comparison module configured to receive said expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide determined by the measuring module and perform at least one analysis to determine whether the expression level of ROBO2 polypeptide or the expression level of the RNA encoding a ROBO2 polypeptide is greater than a pre-determined reference level or ratio, and to provide a retrieved content; and

c. a display module for displaying a content based the data output from said comparison module, wherein the content comprises a signal indicative that the expression level or ratio of ROBO2 polypeptide or RNA is greater than the pre-determined reference level or ratio, or a signal indicative that the level or expression ratio of ROBO2 is not greater than the reference level or pre-determined ratio.

24. The system of claim 23, wherein the content displayed on said display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.

25. A system for determining if a subject is at risk for chronic kidney disease or proteinuria, or has chronic kidney disease comprising:

a. a determination module configured to receive at least one test sample obtained from a subject and perform at least one analysis on said at least one test sample to determine the presence or absence of either of the following conditions:

i. an expression ratio of ROBO2 greater than a pre-determined ratio, or

ii. an expression level of ROBO2 greater than a pre-determined level

b. a storage device configured to store data output from said determination module; and

c. a display module for displaying a content based on the data output from said determination module, wherein the content comprises a signal indicative that the expression ratio of ROBO2 is greater than the pre-determined ratio or level of ROBO2 greater than a pre-determined level, or a signal indicative that the expression ratio of

ROBO2 is not greater than the pre-determined ratio or not greater than a pre-determined level.

26. The system of claim 25, wherein the content displayed on said display module further comprises a signal indicative of the subject being recommended to receive a particular treatment regimen.

27. A method for treating a human subject with a risk of chronic kidney disease or proteinuria, comprising administering a treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria to a human subject who is determined to have a level of ROBO2 protein above a reference threshold level.

28. The method of claim 27, wherein the level of ROBO2 protein is at least 20% above the reference level.

29. The method of claim 27, wherein the level of ROBO2 protein is at least two standard deviations above the reference level.

30. The method of any one of claims 27-29, wherein the treatment or therapy to prevent the occurrence of chronic kidney disease or proteinuria comprises a ROBO2 inhibitor.

31. The method of claim 30, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

32. The method of any one of claims 30-31, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

33. The method of any one of claims 30-32, wherein the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

34. The method of claim 31, wherein the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

35. A ROBO2 inhibitor for use in treating a chronic kidney disease.

36. A ROBO2 inhibitor for use in treating proteinuria.

37. The use of any one of claims 35 or 36, wherein the ROBO2 inhibitor is a blocking antibody or antigen-binding fragment thereof specific for ROBO2, an antisense molecule specific for ROBO2, a short interfering RNA (siRNA) specific for ROBO2, a small molecule inhibitor of

ROBO2, a ROBO2 inhibitory polypeptide, or a ROBO2 structural analog.

38. The use of any one of claims 35-37, wherein the ROBO2 inhibitor blocks or reduces binding of ROBO2 to SLIT, to Nck, or to both.

39. The use of any one of claims 35-38, wherein the ROBO2 inhibitor is specific for the Ig1 SLIT binding domain, the Ig1 and Ig2 SLIT binding domains, the Nck intracellular binding domain, or any combination thereof.

40. The use of claim 37, wherein the ROBO2 inhibitory polypeptide is a dominant negative ROBO2 fusion protein, a polypeptide comprising a ROBO2 extracellular domain without the intracellular domain, or a polypeptide comprising a ROBO2 intracellular domain without the extracellular domain.

41. The use of any one of claims 35-40, wherein the chronic kidney disease or proteinuria is caused by diabetic nephropathy or high blood pressure.