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Inhibitory Effects of Robo2 on Nephrin: A Crosstalk between Positive and Negative Signals Regulating Podocyte Structure

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SUMMARY

Robo2 is the cell surface receptor for the repulsive guidance cue Slit and is involved in axon guidance and neuronal migration. Nephrin is a podocyte slit-diaphragm protein that functions in the kidney glomerular filtration barrier. Here, we report that Robo2 is expressed at the basal surface of mouse podocytes and colocalizes 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, Slit2-Robo2 signaling inhibits nephrin-induced actin polymerization. In addition, the amount of F-actin associated with nephrin is increased in Robo2 knockout mice that develop an altered podocyte foot process structure. Genetic interaction study further reveals that loss of Robo2 alleviates the abnormal podocyte structural phenotype in nephrin null mice. These results suggest that Robo2 signaling acts as a negative regulator on nephrin to influence podocyte foot process architecture.

INTRODUCTION

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 semipermeable 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 may 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).

Roundabout (Robo) family proteins are cell surface receptors for the secreted ligand Slits (Dickson and Gilestro, 2006), which were originally found as repulsive guidance cues for axon path-finding and migrating neurons during nervous system development (Guan and Rao, 2003). The transmembrane protein Robo2 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 Slit2, the first Ig1 motif of Robo2 is the primary binding site for Slit2 (Dickson and Gilestro, 2006). The intracellular domain of Robo2 has four cytoplasmic conserved (CC) sequences named CC0 to CC3 (Dickson and Gilestro, 2006). While both immunoglobulin (Ig) motifs 1 and 2 interact with Slit2, the first Ig1 motif of Robo2 is the primary binding site for Slit2 (Dickson and Gilestro, 2006). The intracellular domain of Robo2 has four cytoplasmic conserved (CC) sequences named CC0 to CC3 (Dickson and Gilestro, 2006). CC0 and CC1 contain conserved tyrosine residues, while CC2 and CC3 are proline-rich stretches. The repulsive Slit2-Robo2 signaling inhibits actin polymerization (Guan and Rao, 2003) or induces F-actin depolymerization (Piper et al., 2006).

Slit2-Robo2 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 leads to a broad spectrum of urinary tract anomalies (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). We recently showed that Robo2 is crucial for the formation of a normal ureteral orifice and for the maintenance of an effective antireflux mechanism (Wang et al., 2011). However, it is not known if Robo2 also plays a role in the kidney after ureteric bud outgrowth.

Here, we report that Robo2 is a podocyte protein expressed at the basal surface of kidney podocytes and colocalizes with...
nephrin and podocin. Robo2 interacts directly with adaptor protein Nck SH3 domains and forms a complex with nephrin. In addition, Robo2 signaling inhibits actin polymerization induced by 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 suggest that Robo2 signaling acts as a negative regulator on nephrin signaling to influence podocyte foot process architecture.

RESULTS

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

During kidney development, Robo2 mRNA is expressed in the metanephric mesenchyme surrounding the ureteric bud and later in the proximal end of the S-shaped body (Piper et al., 2000), the location of primordial podocytes. This raises the possibility that Robo2 may also be involved in podocyte biology in addition to its role in early kidney induction. To investigate this, 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) (Figures S1A and S1B). Robo2 protein became detectable by immunofluorescence staining in the developing glomerulus around E14.5 and reached peak expression at E16.5 (Figures S1C–S1E). Although the expression decreased after E17.5 (Figure S1F), Robo2 expression was maintained in glomeruli after birth and was detectable in adult mice at 5 weeks of age (Figures S1G, S1H, and S1L–S1M).

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 colocalized with nephrin (Figures 1A–1C) and podocin (Figures 1D–1F), two podocyte slit-diaphragm-associated proteins. Robo2 was also coexpressed in the glomeruli with the nephrin-interacting adaptor protein Nck (Figures 1G and 1I) and with WT1, a constituent of podocyte nuclei (Figures S1H–S1K). Dual labeling with antibodies against nidogen, a basement membrane marker (Figures 1J–1L and 1P) and Pecam1, an endothelial cell marker (Figures 1M–1O; Figure S1M) 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 (Figure 1Q). Immunogold electron microscopy of postnatal mouse kidneys with an antibody against the cytoplasmic domain established that Robo2 was localized to podocyte foot processes near to the cytoplasmic face of the slit diaphragms (Figure 1R). These results demonstrate that Robo2 is a podocyte protein and its basal subcellular localization in the foot processes suggests that it may play a role in regulating podocyte foot process structure.

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

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 harbors 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 may recruit various cytoskeleton regulators including N-WASP and Pak (Jones et al., 2006). Interestingly, 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). This prompted us to question whether mammalian Nck can also interact directly with Robo2 in the podocyte to regulate the F-actin cytoskeleton.

To address this possibility, 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 (Figures 2A–2C). Binding site mapping in Robo2 for Nck1 showed that the sequence from amino acid 1085–1301, which contain 4 proline-rich motifs, was crucial for the interaction (Figures 2A and 2C). Absence of this proline-rich region prevented its interaction with Nck1 (Figure 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 (Figure 2B). Thus, Robo2 and Nck1 interaction was mediated by two well-characterized protein domains: the SH3 domains and proline-rich motifs (Figure 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 coprecipitation assays (data not shown). These observations suggest that the binding between Robo2 and Nck1 in the podocyte is a specific interaction.

Full-Length Robo2 Forms a Complex with Nephlin through Nck

We confirmed the interaction between Robo2 and Nck by pull-down and coprecipitation 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 (293T) 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-myc-Robo2 from the HEK cell lysates using Ni-NTA beads but not with His-myc-Robo2-ΔNBD (Figure 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 coprecipitation assay. To establish proof of principle, we coexpressed Robo2 and nephrin in HEK cells with Fyn kinase to increase nephrin phosphorylation (Verma et al., 2006). Pull-down of His-myc-Robo2 (but not of His-myc-Robo2-ΔNBD) from the HEK cell lysates with Ni-NTA beads coprecipitated Nck and nephrin when Fyn was expressed (Figure 2E). In the reverse order, pulling
His-myc-nephrin coprecipitated Nck and Robo2 when Fyn kinase was expressed (Figure 2F). Furthermore, the precipitates prepared with the anti-Nck antibody contained both Robo2 and nephrin when Fyn was overexpressed (Figure S2A). These data suggest 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 coprecipitated (Figure 2G). Conversely, the precipitates prepared with the antinephrin antibody also contained Nck and Robo2 (Figure 2H). Since nephrin is uniquely...
expressed in podocytes of kidney lysates, 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.

To determine the role of Slit2 in the formation of the Robo2-Nck-nephrin protein complex, His-myc-Robo2, nephrin, and Fyn were coexpressed in HEK cells that were stimulated with Slit2 conditioned medium or control conditioned medium without Slit2 prior to coprecipitation (Figure 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.

Figure 2. Robo2 Interacts with the Adaptor Protein Nck and Forms a Complex with Nephrin

(A) Yeast two-hybrid assays show a positive interaction between Robo2 intracellular domain (Robo2-ICD) and Nck1. LacZ reporter (X-gal): +++, yeast turned dark blue; ++, light blue; −, white in 24 hr. Leucine reporter (−Leu): +, yeast grew; −, yeast did not grow. CC, cytoplasmic conserved region. Numbers indicate residue positions in the full-length protein.

(B) Yeast two-hybrid assays show the first two SH3 domains of Nck1 are required for its interaction with Robo2-ICD.

(C) Yeast two-hybrid assays show the 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 in red.

(D) Coprecipitation 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-△NBD transfected cells (used in lanes 3 and 4).

(E) Coprecipitation of Robo2 and Nck, and nephrin.

(F) A similar coprecipitation as (E) except that His-myc-nephrin is pulled down instead of His-myc-Robo2.

(G) Coimmunoprecipitation of kidney endogenous Robo2, Nck, and nephrin.

(H) A similar assay as (G) except that precipitates are prepared using mouse antinephrin 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 (lanes 2, 4).

(J) Intensity quantification of (I). 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 Figure S2.
stabilization (Figure 2J). Consistent with this finding, we observed that Slit2 was also expressed in newborn and adult mouse glomeruli (Figures S2B and S2C).

**Slit2-Robo2 Signaling Inhibits Nephrin-Induced Actin Polymerization**

Since Slit binds Robo to recruit Dock and srGAPs, thereby inhibiting actin polymerization (Fan et al., 2003; Wong et al., 2001), we wondered if Robo2 might also recruit 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 coexpressed 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 in the presence of Slit2. We observed that engagement of CD16/7-NCD moved nephrin into the Robo2 complexes since most of the Robo2 colocalized with the CD16/7-NCD clusters (Figures S2D–S2F). However, no colocalization of the Robo2 was observed either with the CD16/7-HA control (Figure S2D') or with the Robo2-ΔNBD construct (Figure S2E'), 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 (Figure S2F'). 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.

HEK cells expressing CD16/7-NCD and Robo2 were stimulated with Slit2 or control conditioned medium 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% (Figures 3A and 3C). Only a few cells were observed to contain F-actin tails when the control CD16/7-HA proteins were clustered (Figures 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 coexpressed with either full-length Robo2 (Figure S3A) or Robo2-ΔNBD (Figure S3B) in HEK cells. We observed that deletion of the Nck binding domain in Robo2 significantly compromised Slit2-Robo2 inhibition on nephrin-induced actin polymerization (Figure S3C).

A previous study shows that nephrin is linked to the F-actin cytoskeleton (Yuan et al., 2002). To determine if Slit2-Robo2 signaling could inhibit the association of F-actin and 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 (Figures 3D and 3E). Conversely, in vivo immunoprecipitation assay showed that F-actin associated with nephrin immunoprecipitated by an antinephrin antibody from Robo2 newborn null mouse kidneys was significantly increased compared with that from wild-type or Robo2 heterozygous mouse kidneys (Figures 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**

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<sup>ΔNBD</sup> (also called Robo2<sup>−/−</sup>) mutant allele for five generations onto C57BL/6 genetic background, mating of Robo2<sup>ΔNBD/+</sup> heterozygous parents revealed three Robo2<sup>ΔNBD/ΔNBD</sup> homozygous mice that survived to 3 weeks (among a total of 160 mice analyzed at weaning). To determine if Robo2 is required for podocyte foot process formation during development, we examined the ultrastructure of glomeruli in Robo2<sup>−/−</sup> 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<sup>ΔNBD/ΔNBD</sup> homozygous mice (Figures S4A–S4F). By scanning electron microscopy, we observed irregular interdigitating foot processes in Robo2<sup>ΔNBD/ΔNBD</sup> homozygous null mice at birth and 3 weeks of age (Figures 4A–4H). These findings suggest that Robo2 plays a role in normal podocyte foot process patterning during kidney development.

To examine the role of Robo2 in the maintenance of foot process structure in mature glomeruli, we generated podocyte specific Robo2<sup>−/−</sup> knockout mice by crossing conditional Robo2<sup>flox/flox</sup> mice with Robo2<sup>ΔNBD/+ , Tg<sup>Nphs2-Cre/+</sup> heterozygous</sup> mice carrying a podocin-Cre transgene. Twenty podocyte specific Robo2<sup>−/−</sup> mutant mice with Robo2<sup>flox/flox , Tg<sup>Nphs2-Cre/+</sup> genotype and 20 littermate control mice were analyzed up to 1 year of age. Podocyte specific Robo2<sup>−/−</sup> knockout mice were viable and fertile. However, they displayed unusually broad podocyte foot processes and focal segmental foot process effacement at 1 month (Figures 4I–4M). At 6 weeks of age, the mutant mice developed microalbuminuria, which was detected by both ELISA and western blot analyses (Figures 4N and 4O). In addition, scanning electron microscopy revealed foot process patterning changes 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 patterns at 1 month (Figures S4G–S4J). These changes became more obvious over time. At 7 months of age, overly

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disorganized, shorter, and meandering foot processes were observed (Figures S4K–S4N), which were similar to the phenotype of 3-week-old Robo2 null mice. Although Robo2 podocyte specific knockout mice had normal podocyte number, matrix deposition was significantly increased in glomeruli (Figures S4O–S4T; Tables S1 and S2). These morphological and functional changes suggest that Robo2 plays a role in regulating and maintaining glomerular podocyte foot process structure.

Figure 3. Slit2-Robo2 Signaling Inhibits Nephrin-Mediated Actin Polymerization

A) CD16/7-NCD is coexpressed 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 (Slit2) 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.

B) A similar assay as (A) except that CD16/7-NCD is replaced by CD16/7-HA and is used as a control assay.

C) 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.

D) CD16/7-NCD in (A) is immunoprecipitated by anti-CD16 antibody after Slit2 conditioned medium stimulation (lanes 1 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.

E) Intensity quantification of (D). Data are represented as mean ± SEM; n = 4, *p < 0.05 compared with the control, paired Student’s t test.

F) Immunoprecipitation of nephrin from Robo2 knockout homozygous (Robo2−/−), heterozygous (Robo2+/−), and wild-type (Robo2+/+) mouse kidneys using the antinephrin antibody. Note increased F-actin in lane 3.

G) Intensity quantification of (F). Data are represented as mean ± SEM; n = 4, *p < 0.05 compared with the wild-type and heterozygous, ANOVA analysis. See also Figure S3.
Figure 4. Podocyte Structural Phenotypes in the Robo2 Homozygous Null, Robo2 Podocyte Specific Knockout, and Robo2 and Nphs1 Double-Knockout Mice

(A and B) Representative images of newborn kidneys show podocyte bodies (arrowheads) and Bowman’s capsule (arrows) in wild-type (A) and Robo2 homozygous null mice (B).

(E and F) Representative images of 3-week-old kidneys at low magnification show podocyte cell body (arrowheads) in a Robo2 homozygous null mouse (F) compared to an age-matched control (E).

(G and H) Higher magnification images of (E and F) show disorganized shorter meandering foot processes (arrow) in a 3-week-old Robo2 homozygous null mouse (H) compared to well-organized zip-like foot processes in the age-matched control (G). Scale bars: 2 μm.

(I and J) Representative transmission electron microscopy images (magnification at 5,000x) depict the focal segmental podocyte foot process effacement in a 1-month-old Robo2 podocyte-specific knockout mouse and the normal phenotype in the control (I). Abbreviations: gc, glomerular capillary; us, urinary space.

(K and L) Higher magnification transmission electron microscopy images (40,000x) show broader podocyte foot processes (arrow in L) in a 2-month-old Robo2 podocyte-specific mutant mouse compared with the control (K). Abbreviations: fp, podocyte foot process; GBM, glomerular basement membrane.

Ab/creatinine in WT and Robo2 KO mice shown in bar graph (N). Scale bars: 2 μm.
**DISCUSSION**

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., 1999). 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 that such stimuli bring about those subtle changes in response to positive and negative signals transduced to the F-actin cytoskeleton. Too much or unbalanced positive signals may lead to disease phenotype. Indeed, although a physiological ligand has yet to be identified, it is now clear that clustering and phosphorylation of nephrin induces actin polymerization by recruiting Nck. This mechanism may 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).

Our studies reveal another level of negative regulation of podocyte actin polymerization in which Robo2, when bound by Slt, inhibits nephrin-induced actin polymerization. While the precise mechanisms have yet to be defined, we propose that Slt-Robo2 signaling might 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 (Figure S4Z). Local secretion and binding of Slt increases the interaction of Robo2 with Nck thereby recruiting Nck-actin to the podocyte foot processes and inducing actin polymerization. These findings indicate that the Robo2-Nck-neph-rin 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.

(M) Quantification of podocyte foot process width in 1-month-old Robo2del5/flox;Nphs1−/−:Nphs1−/− double-knockout mice. Data are represented as mean ± SEM, n = 33, *p < 0.01.
(N) ELISA assay of spot urine shows an elevated albumin/creatinine ratio in Robo2del5/flox;Nphs1−/−:Nphs1−/− double-knockout mice compared with control adult wild-type (WT). Data are represented as mean ± SEM, n = 20, *p < 0.01.
(O) Western blot analysis shows the presence of albumin in urine; 1 μl urine was loaded on each well, 0.2 μg albumin was used as a positive control. WT, three wild-type littermates; Robo2 KO, three individual Robo2del5/flox;Nphs1−/−:Nphs1−/− mouse. (P and Q) Representative scanning electron microscopy images show disrupted interdigitating podocyte foot processes that resemble disorganized cellular protrusions (arrows) in the Nphs1−/−:Nphs1−/− single homozygous newborn mouse kidney. Scale bars: 1 μm. (R and S) Glomeruli from Nphs1−/−:Robo2−/− double-homozygous newborn mice exhibit restored interdigitating foot processes (arrows), indicating alleviation of nephrin null phenotype by knocking out Robo2. (T and U) Glomeruli from Robo2−/− single homozygous newborn mice display irregular and broader foot processes but extensive interdigitating pattern formation (arrows). See also Figure S4 and Tables S1, S2, S3, and S4.
F-actin associated with nephrin to maintain a dynamic and balanced F-actin cytoskeleton and normal podocyte foot process structure (Figure S4Z). In addition to direct inhibition of nephrin-induced actin polymerization through Nck, Slit-Robo2 signaling may inactivate actin polymerization through other pathways such as recruiting Ena, Abl, srGAPs to negatively regulate F-actin cytoskeleton (Bashaw et al., 2000; Wong et al., 2001). In the absence of Slit2-Robo2 signaling (e.g., when Robo2 is knocked out), the inhibitory effect 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 (Figure S4Z), which may explain the altered podocyte foot process structure identified in Robo2 mutant mice. Our results thus support the concept that Slit-Robo signaling may 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 although the mechanisms and consequences have yet to be defined.

Although it is clear from our studies 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 (Figure S4Z) (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 but did not observe any positive results. Our biochemical data (Figures 2E and 2F) also did not support a direct interaction between these two receptors in vitro. However, we cannot exclude the possibility that the extracellular domain of Robo2 may 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.

We found that Robo2 knockout mice developed an altered foot process interdigitating pattern, a phenotype that is different from that of the nephrin null mice (Doné et al., 2008; Hamano et al., 2002). This is not surprising since nephrin and Robo2 play opposite roles in regulating the podocyte F-actin cytoskeleton. While nephrin signaling induces localized actin polymerization, Slit2-Robo2 signaling acts as a negative regulator on nephrin-induced 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 suggests that the absence of either an actin polymerization promoting factor such as nephrin or an inhibitory factor such as Robo2 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 and negative signals may explain the restoration of podocyte foot process interdigitation in the Robo2-nephrin double-knockout mice. Our studies highlight 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.

In summary, we have identified Robo2 as a 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 suggest that Robo2 signaling acts as a negative regulator on nephrin signaling to modulate podocyte foot process architecture. This study extends our understanding of the role of Slit-Robo signaling and identifies a crosstalk mechanism by which guidance cue receptor Robo might influence F-actin cytoskeleton dynamics. Further studies are needed to determine if eliminating or blocking Slit2-Robo2 signaling can be used therapeutically to restore podocyte foot process structure in those diseases in which nephrin expression is reduced (Furness et al., 1999).

**EXPERIMENTAL PROCEDURES**

Detailed information can be found in the Extended Experimental Procedures in Supplemental Information.

**Tissue in Situ Hybridization, Immunohistochemistry, and Immunogold Electron Microscopy**

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-peroxidase (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, Coprecipitation, and Actin Polymerization Assays**

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 coprecipitation, and immunoprecipitation 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**

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<sup>flx</sup> conditional allele, Robo2<sup>3H9</sup> (also called Robo2<sup>interchangeably in this paper</sup>) germline mutant allele, and Robo2<sup>+</sup> wild-type allele were described previously (Lu et al., 2007; Wang et al., 2011). To generate Robo2-nephrin double-knockout mice, Robo2<sup>−/−</sup> mice were crossed with Nphs1<sup>−/−</sup> mice that were generated previously (Hamano et al., 2002). Other mice were generated and genotyped in-house.
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. Ultra-thin 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 hematoxylin and eosin (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-week-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.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.06.002.

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REFERENCES
Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

Tissue In Situ Hybridization and Immunohistochemistry
In situ hybridization analysis was performed with digoxigenin-labeled Robo2 riboprobes as previously described (Grieshammer et al., 2004). The Robo2 cDNA was linearized with NotI 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), PDGF 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
Wild-type mouse kidneys were dissected and fixed in paraformaldehyde-lysine-periodate (PLP) at 4 ºC 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 ºC 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 hr 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
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 EcoRI/Xhol 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 EcoRI/Xhol 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 cotransformed 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, Coprecipitation, and Western Blot Analyses
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 HindIII/EcoR1 and EcoR1/Xho1 restriction sites respectively. Robo2-ΔNBD was obtained by deleting the Nck binding domain (Figure 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 EcoR1/Xho1 sites, nephrin at HindIII/EcoR1 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 NaH2PO4, 300 mM NaCl, 10 mM Imidazole, 0.5% TX100, 1x protease inhibitor [pH 8.0]). Cell lysates were centrifuged for 10 min at 4 ºC; supernatants were incubated with Ni-NTA resin (Qiagen) at 4 ºC for 2 hr to precipitate His-Robo2, Ni-NTA resin without Ni was used as a control. The resin was washed three times with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM Imidazole, 0.5% TX100 [pH 8.0]) and heated at 95 ºC 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 coexpressed in HEK cells with human nephrin and human Fyn. His-myc-Robo2 was precipitated with Ni-NTA beads as described previously. To confirm the triple interaction, His-myc-nephrin was coexpressed 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 coimmunoprecipitation 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 Na2VO4, 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 hr 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 hr 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 antinephrin, 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 antialbumin polyclonal antibody (MP Biomedicals).

**CD16/7-NCD Crosslinking and Actin Polymerization Assay**

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 coexpressed in HEK cells with Robo2. After 24 hr, cells were transferred and seeded on glass coverslips coated with polylysine for another 24 hr. 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 Sili2 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**

The generation of Robo2 podocyte specific double-knockout mice and Robo2-Nephrin double-knockout mice was conducted as follows. 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-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-Cre/+ mice was determined by tail DNA genotyping for the presence of Robo2 ∆del5 and Robo2 flox alleles as well as Tg Nphs2-Cre transgene. F2 littermates Robo2−/- mice without Robo2 ∆del5 allele and Tg Nphs2-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).

**Transmission and Scanning Electron Microscopy**

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 hr. 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 each animal were examined to provide representative images.

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

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 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**

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-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-Cre/+ mice was determined by tail DNA genotyping for the presence of Robo2 ∆del5 and Robo2 flox alleles as well as Tg Nphs2-Cre transgene. F2 littermates Robo2−/- mice without Robo2 ∆del5 allele and Tg Nphs2-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).

Transmission and Scanning Electron Microscopy

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 hr. 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 hr followed by postfixation in 2% osmium tetroxide 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.
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<sup>del5/flox;Nphs2-Cre+</sup> 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-week-old mice were examined using a sensitive murine albuminuria ELISA quantitation kit (Exocell) according to manufacturer’s instruction and urine dipstick (Multi-stix 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 antialbumin antibody (MP Biomedicals). The data from mutants and controls were analyzed using one-way ANOVA, Student t test, and Chi-square test.

**SUPPLEMENTAL REFERENCES**


Figure S1. Robo2 Is Expressed in the Developing and Adult Glomeruli, Related to Figure 1
(A and B) In situ hybridization analysis shows that Robo2 transcripts are expressed in developing glomeruli (arrows) at E16.5. Magnification: 60X (A) and 200X (B).
(C–F) Immunohistochemistry (IHC) studies reveal that Robo2 is expressed during developing glomeruli from E14.5 to E17.5. Magnification: 600X.
(G) IHC shows that Robo2 (Red) is specifically expressed in adult mouse glomeruli at 5 weeks of age. DAPI (blue) marks cell nuclei in the kidney. Magnification: 400X.
(H) IHC colocalization stainings of 5w kidney show Robo2 is coexpressed in the glomerulus with podocyte marker Wt1. Magnification: 600X.
(I–K) Robo2 and WT1 are coexpressed in the mouse glomerulus at E16.5. Magnification: 600X.
(L and M) IHC colocalization stainings of 5w kidney show Robo2 is coexpressed in the glomerulus with mesangial cell marker Pdgfrb (L), and endothelial cell marker Pecam1 (M). Magnification: 600X.
Figure S2. Robo2 Interacts with Nck and Forms a Complex with Nephrin, which Is Enhanced by Slit2 Stimulation, Related to Figure 2

(A) 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. (B and C) Slit2 is expressed in the newborn mouse glomeruli by Immunoperoxidase staining (B) and is coexpressed in the glomerulus with the podocyte marker Synaptopodin (C). Magnification: 600X.

(D and D*) 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 (D) but no colocalization is observed in control CD16/7-HA clusters (D*). Scale bar: 5 μm. NCD: nephrin cytoplasmic domain.

(E and E*) 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 (E) but no colocalization is observed in Robo2-DNBD clusters (E*). Scale bar: 5 μm.

(F and F*) 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 (F) but not with control conditioned medium (F*). Scale bar: 5 μm.
Figure S3. Deletion of Nck Binding Domain in Robo2 Compromises Slit2-Robo2 Inhibition on Nephrin-Induced Actin Polymerization, Related to Figure 3

(A) 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, 5 μm. NCD, nephrin cytoplasmic domain.

(B) CD16/7-NCD and Robo2-ΔNBD were co-expressed in HEK cells. Scale bar, 5 μm. NBD, Nck binding domain.

(C) 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 = 1.436x10⁻⁵, **p = 6.32x10⁻⁵, n = 5, ANOVA.
Figure S4. 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, Related to Figure 4

(A–F) Transmission electron microscopy analysis of glomerular ultrastructure in newborn (NB) Robo2<sup>del5/del5</sup> mutant mice kidney. (A, C, E) Glomerular ultrastructure from a newborn heterozygous Robo2 control mouse at low (A, 2200X), medium (C, 15500X) and high (E, 52000X) magnifications. (B, D, F) Glomerular ultrastructure from a newborn homozygous Robo2<sup>/C0/C0</sup> (i.e., Robo2<sup>del5/del5</sup>) mutant mouse at low (B), medium (D) and high (F) magnifications. Arrows indicate focal foot process effacement. Abbreviations: gc: glomerular capillary; us: urinary space; GBM: glomerular basement membrane.

(G–N) Abnormal podocyte foot process patterns in Robo2 podocyte-specific knockout mice. (G–J) Representative scanning electron microscopy images of glomeruli from 1-month-old Robo2<sup>del5/flox;Nphs2-Cre<sup>+</sup></sup> podocyte-specific knockout mice and aged matched Robo2<sup>flox/+</sup> control mice. Mild irregularities of the interdigitating podocyte foot processes were found in a 1-month-old Robo2<sup>del5/flox;Nphs2-Cre<sup>+</sup></sup> podocyte-specific knockout mouse (K and N). At 7 months old, Robo2

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podocyte-specific knockout mice developed markedly irregular foot processes (L and N). Scale bars: 10 μm (G, H, K, L at 2000x magnification) and 2 μm (I, J, M, N at 13000x magnification).

(O–T) Glomerular morphology in Robo2 podocyte-specific knockout mice. (O–R) Periodic acid-Schiff (PAS) staining showed mesangial matrix expansion in the glomeruli from 2- and 6-month-old Robo2 podocyte-specific knockout mice (P, R) compared to age-matched controls (O, Q). (S) 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); TgNphs2-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.

(U–Y) Glomerular phenotype in Robo2 and Nphs1 double-knockout mice. (U) H&E staining shows glomeruli with characteristic dilatations of the Bowman’s space (asterisks) in a Nphs1/−/− single homozygous newborn mouse, 400x. (V) Glomeruli from a Robo2/−/− single homozygous newborn mouse show absence of Bowman’s space dilatations; 400x. (W) 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. (X) H&E staining of normal kidney and glomeruli from an age-matched wild-type newborn mouse control; 400x. (Y) 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.

(Z) A proposed 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 podocyte 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.