CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Nephrin—Ephrin-B1—Na⁺/H⁺ Exchanger Regulatory Factor 2—Ezrin—Actin Axis Is Critical in Podocyte Injury

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Ephrin-B1 is one of the critical components of the slit diaphragm of kidney glomerular podocyte. However, the precise function of ephrin-B1 is unclear. To clarify the function of ephrin-B1, ephrin-B1-associated molecules were studied. RNA-sequencing analysis suggested that Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2), a scaffolding protein, is associated with ephrin-B1. NHERF2 was expressed at the apical area and the slit diaphragm, and interacted with the nephrin—ephrin-B1 complex at the slit diaphragm. The nephrin—ephrin-B1—NHERF2 complex interacted with ezrin bound to F-actin. NHERF2 bound ephrin-B1 via its first postsynaptic density protein-95/disk large/zonula occludens-1 domain, and podocalyxin via its second postsynaptic density protein-95/disk large/zonula occludens-1 domain. Both *in vitro* analyses with human embryonic kidney 293 cells and *in vivo* study with rat nephrotic model showed that stimulation of the slit diaphragm, phosphorylation of nephrin and ephrin-B1, and dephosphorylation of NHERF2 and ezrin, disrupted the linkages of ephrin-B1—NHERF2 and NHERF2—ezrin. It is conceivable that the linkage of nephrin—ephrin-B1—NHERF2—ezrin—actin is a novel critical axis in the podocytes. Ephrin-B1 phosphorylation also disrupted the linkage of an apical transmembrane protein, podocalyxin, with NHERF2—ezrin—actin. The phosphorylation of ephrin-B1 and the consequent dephosphorylation of NHERF2 are critical initiation events leading to podocyte injury. (*Am J Pathol* 2021, 191: 1209–1226; <https://doi.org/10.1016/j.ajpath.2021.04.004>)

Kidney glomerular visceral epithelial cells (podocytes) are highly differentiated unique epithelial cells that have lost their ability to proliferate like neurons. Podocytes have primary and secondary processes.^{1–3} The unique secondary processes, where filamentous actin (F-actin) is densely located, are called foot processes that are interdigitated and cover the outer side of the glomerular basement membrane. The neighboring foot processes are bridged by a slit diaphragm observed as a zipper-like structure by electron microscopy.⁴ Because neighboring foot processes are derived from different cell bodies, a slit diaphragm connecting them is a cell-cell junction and is considered a variant of tight junction.^{5,6} The slit diaphragm functions as a final barrier preventing the leak of plasma proteins into primary urine, and proteinuria in major clinical cases of kidney diseases is caused by dysfunction of the slit diaphragm.⁷ However, the precise mechanisms of formation and maintenance of slit diaphragm and pathogenesis of slit diaphragm dysfunction remain unclear.

In the past two decades, molecules such as nephrins, podocin, and NPH-1 (IRRE-like protein 1) have been identified as critical components of the slit diaphragm.^{8–10} Nephrin is the gene mutated in Finnish-type nephrotic syndrome⁸ and is a key molecule of the slit diaphragm in preventing proteinuria. Ephrin-B1 is expressed at the slit diaphragm¹¹ and binds to nephrin.¹² It is expressed in several tissues and is estimated to be involved in maintaining tissue-specific functions.^{11,13,14} Although the role of ephrin-B is well investigated in the neuronal field, the function of ephrin-B in epithelial cells is not well understood. Podocyte-specific conditional knockout (CKO) mouse of ephrin-B1 has altered expression of the slit

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diaphragm components, proteinuria, and foot process effacement, which indicates that ephrin-B1 plays a critical role in maintaining the structure and barrier function of slit diaphragm.¹² Ephrin and Eph are membrane-bound proteins that function as receptor-ligand pairs.¹⁵ Although ephrin-B is generally stimulated by EphB, the expression of EphB has not been detected in the podocytes. Ephrin-B1 is phosphorylated by the stimulation to nephrin in the podocytes,¹² and phosphorylation of ephrin-B1 activates c-Jun N-terminal kinase¹² and alters the molecular composition of the Par complex.¹⁶ These observations imply that ephrin-B1 plays an important role as a signaling molecule in podocytes. However, the precise function of ephrin-B1 in podocytes is not fully understood. To better understand the role of ephrin-B1 in podocytes, molecules associated with ephrin-B1 were explored. Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2) was down-regulated in the glomerular sample of podocyte-specific ephrin-B1 CKO mice, as indicated by the RNA-sequencing assay, suggesting that NHERF2 is related to ephrin-B1. Thus, we focused on NHERF2 in this study.

NHERF2, an isoform of NHERF proteins, was initially identified by two-hybrid screening using the cytoplasmic tail of Na⁺/H⁺-exchanger 3.¹⁷ NHERF proteins are scaffold proteins that connect plasma membrane proteins with the members of the ezrin/moesin/radixin family, thereby linking them to the actin cytoskeleton.¹⁸ NHERF2 functions to regulate the surface expression of its binding membrane proteins.¹⁸ It is reported to interact with podocalyxin, an apical transmembrane protein of podocytes, form a complex with ezrin, and link the complex to the actin cytoskeleton.¹⁹ It also interacts with Schip1 in the foot processes.²⁰ However, the precise localization and function of NHERF2 in podocytes remain unclear.

The present study indicates that NHERF2 is expressed at not only apical membrane but also the slit diaphragm. NHERF2 interacted with the nephrin–ephrin-B1 complex and the nephrin–ephrin-B1–NHERF2 complex interacted with ezrin, as indicated by the interaction assay. Stimulation of the slit diaphragm and phosphorylation of nephrin and ephrin-B1 led to the dephosphorylation of NHERF2 and ezrin and disruption of the nephrin–ephrin-B1–NHERF2–ezrin–actin complex, as indicated by *in vitro* and *in vivo* studies in the nephrotic model. Additionally, the phosphorylation of ephrin-B1 and consequent dephosphorylation of NHERF2 also disrupted the interaction of NHERF2 with podocalyxin, an apical transmembrane protein.

Materials and Methods

Animal Experiments

All animal experiments conformed to the NIH *Guide for the Care and Use of Laboratory Animals*.²¹ All procedures in this study were approved by the Animal Committee at Niigata University (Niigata, Japan; permit numbers SA00710 and SA00712). Podocyte-specific conditional ephrin-B1

knockout mice were prepared, as previously described.¹² For the experiments with rats, specific pathogen-free, 6-week-old Wistar rats were used. For the analyses of developmental expression, kidneys of neonatal Wistar rats were used. Rat nephrotic model was induced by the injection of mouse anti-nephrin antibody, as previously described.^{12,16,22}

Immunohistochemical Analysis

Immunofluorescence and immunoelectron microscopy were performed according to prior methods.^{11,12,23–25} The primary antibodies used for immunohistochemical analyses were as follows: goat anti-NHERF2 antibody (Santa Cruz Biotechnology, Dallas, TX), goat anti-ephrin-B1 antibody (R&D Systems, Minneapolis, MN), rabbit anti-ephrin-B1 antibody (Santa Cruz Biotechnology), mouse anti-podocalyxin antibody (prepared in our laboratory),²⁶ rabbit anti-podocalyxin antibody,²³ rabbit anti-nephrin antibody (prepared in our laboratory),²² mouse monoclonal anti-rat Thy1.1 antibody (OX-7),²⁷ mouse anti-rat endothelial cell antigen-1 antibody (Serotec, Oxford, UK), mouse anti-ezrin antibody (Invitrogen, Carlsbad, CA), rabbit anti-ezrin antibody (Cell Signaling, Danvers, MA), rabbit anti-ezrin (pThr567)/radixin (pThr564)/moesin (pThr558) antibody (Abcam, Cambridge, UK), and EphB2-Fc probe (R&D Systems). Fluorescein isothiocyanate–conjugated rabbit anti-goat IgG (Dako, Glostrup, Denmark), fluorescein isothiocyanate–conjugated donkey anti-goat IgG (Protos Immunoresearch, Burlingame, CA), fluorescein isothiocyanate–conjugated swine anti-rabbit IgG (Dako), tetramethylrhodamine–conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL), tetramethylrhodamine–conjugated rabbit anti-mouse IgG (Dako), tetramethylrhodamine–conjugated goat anti-mouse IgG2a (Southern Biotech), or DyLight 594–conjugated rabbit anti-human IgG (Bethyl Laboratories, Montgomery, TX) were used as secondary antibodies. The immunofluorescence staining in glomeruli was semiquantified, as previously reported.²⁸ More than 30 glomeruli of each rat or each mouse were analyzed in a blinded manner.

HEK293 Cell Transfection

Assays with human embryonic kidney (HEK) 293 cell transfection were performed, as previously described.^{12,16} HEK293 cells were transfected with TwinStrepII-tagged SLC9A3R2 (NHERF2; Addgene, Cambridge, MA; plasmid number 83,200), myc-DDK-tagged podocalyxin (OriGene Technologies, Rockville, MD; catalog number RR213127), HA-tagged ephrin-B1,¹² or FLAG-tagged nephrin¹² by calcium phosphate method.

Immunoprecipitation Assays with Glomerular Lysates and Transfected HEK293 Cell Lysates

Immunoprecipitation assays with rat glomerular lysates and transfected HEK293 cell lysates were performed as

described previously,^{11,12,16} with anti-NHERF2 antibody (Santa Cruz Biotechnology), anti-ephrin-B1 antibody (Santa Cruz Biotechnology), anti-nephin antibody,²² anti-ezrin antibody (Invitrogen), rabbit anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-HA antibody (Bethyl Laboratories), mouse anti-HA antibody (Santa Cruz Biotechnology), rabbit anti-myc antibody (MBL, Woburn, MA), mouse anti-myc antibody (Santa Cruz Biotechnology), mouse anti-streptavidin antibody (MBL), rabbit anti-phosphorylated ephrin-B (Cell Signaling), rabbit anti-phosphorylated nephrin (Abcam), mouse anti-phosphorylated serine antibody (Sigma-Aldrich), mouse anti-phosphorylated threonine antibody (Cell Signaling), or mouse anti-phosphorylated tyrosine antibody (Merk, Darmstadt, Germany). Alkaline phosphatase–conjugated anti-rabbit IgG (Invitrogen), alkaline phosphatase–conjugated anti-mouse IgG (Rockland Immunochemicals, Limerick, PA), and alkaline phosphatase–conjugated anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies. The reaction was developed with an alkaline phosphatase chromogen kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Real-Time RT-PCR

Real-time RT-PCR analyses were performed according to the method described previously.^{12,23,29} PCR was performed with the following primers: mouse *NHERF2*: sense, 5'-TGACCTGCACTGAGGAGATG-3'; antisense, 5'-AGAGCCAGGGTCCACAGAG-3'; human *NHERF2*: sense, 5'-TGACCTGCACTGAGGAGATG-3'; antisense, 5'-TGAGCCCGGGTCCACAGAG-3'; mouse and human *ephrin-B1*: sense, 5'-GGCAAGCATGAGACTGTGAA-3'; antisense, 5'-CCGTAAGGGGATGATGATGT-3'; mouse *GAPDH*: sense, 5'-CTCCACTCACGGCAAATTCAA-3'; antisense, 5'-GGATGACCTTGCCACAGC-3'; and human *GAPDH*: sense, 5'-TTCCACCCATGGCAAATTCCA-3'; antisense, 5'-GGATGACCTTGCCACAGC-3'. The data are shown as ratios relative to control findings and expressed as means $\pm$ SD of four independent experiments.

RNA Silencing

The siRNA targeting NHERF2, ephrin-B1, and negative control siRNA were purchased from Qiagen Inc. (Valencia, CA). A conditionally immortalized human podocyte cell line kindly donated by Dr. Moin Saleem (University of Bristol, Bristol, UK) was used for the siRNA analysis. Cells were cultivated and treated with siRNA for 48 hours, as previously described.^{16,30} Fluorescence intensity of NHERF2, ephrin-B1, and phosphorylated ezrin/moesin/radixin in the cells treated was evaluated with ImageJ software version 1.53e (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>), as previously described.¹⁶ Actin staining was

evaluated as score 1 to 4, as previously described.^{31,32} (Score 1, >90% of cell area filled with thick cables; score 2, at least two thick cables running under nucleus and rest of cell area filled with fine cables; score 3, no thick cables, but some cables present; and score 4, no cables visible in the central area of the cell.) The immunofluorescence intensity was assigned to each cell, and 30 cells were analyzed from each image. To detect actin fiber, rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO) was used.

Transcript Profiling (RNA Sequencing)

Total RNA was isolated from isolated glomeruli of ephrin-B1 CKO mice, according to the manufacturer's protocol. cDNA library was synthesized by Ion total RNA-sequencing kit (ThermoFisher Scientific, Waltham, MA) and the sequence was analyzed by Ion proton system, according to the manufacturer's protocol. The RNA-sequencing data were aligned to the University of California, Santa Cruz transcriptome (build mm10) using the Torrent Browser. The reads were normalized on the basis of reads per kilobase per million. Fold change was computed as (sign of log fold change) $\times$ 2 |log fold change| by StrandNGS software version 2.6 (Tomy Digital Biology, Tokyo, Japan).

Duolink *in Situ* Proximity Ligation Assay

A Duolink *in situ* proximity ligation assay (PLA), which can detect protein-protein interactions,³³ was performed with Duolink *in situ* PLA probes purchased from Sigma-Aldrich. The assay involves two primary antibodies raised in different species and species-specific secondary antibodies, called PLA probes, with a unique, short DNA strand, that bind to the primary antibodies. The DNA strands interact when the PLA probes are in proximity (<40 nm). After amplification, the DNA circle is detected with a fluorescence microscope. The analysis was performed according to the method previously reported.^{23,34} To analyze the interaction of NHERF2 with podocalyxin, ephrin-B1, and ezrin, kidney sections were incubated with goat anti-NHERF2 antibody and rabbit anti-podocalyxin antibody, rabbit anti-ephrin-B1 antibody, or rabbit anti-ezrin antibody. After the incubations of the antibodies, the interaction was analyzed with Duolink *in situ* PLA probe anti-goat MINUS and Duolink *in situ* PLA probe anti-rabbit PLUS. More than 30 glomeruli were analyzed from each sample in a blinded manner.

Statistical Analysis

Statistical significance was evaluated using the unpaired *t*-test or the *U*-test. Data were analyzed using GraphPad Prism 5.0 software version 5.04 (GraphPad Software, San Diego, CA).

Results

NHERF2 Is Down-Regulated in Glomeruli of the Podocyte-Specific Ephrin-B1 CKO Mice

The expression of NHERF2 was decreased in the glomeruli of the podocyte-specific ephrin-B1 CKO mice (Figure 1 and Supplemental Table S1). mRNA expression of other post-synaptic density protein-95/disk large/zonula occludens-1 (PDZ) molecules reported to be expressed in podocyte was not altered (Figure 1 and Supplemental Table S1). The observations suggest that NHERF2 is an ephrin-B1-associated PDZ molecule. Thus, the next few studies focused on NHERF2.

NHERF2 Is Expressed Not Only at Apical Membrane Area but also at Slit Diaphragm, Where It Is Colocalized with Ephrin-B1

Localization of NHERF2 in glomeruli indicated NHERF2 expression along the capillary loop (Figure 2A). The NHERF2 staining did not overlap with the rat endothelial cell antigen-1 (an endothelial cell marker) and Thy1.1 (a mesangial cell marker) staining. Some NHERF2 costained with nephrin, some costained with ephrin-B1, and almost all ephrin-B1 costained with NHERF2. Some NHERF2

staining colocalized with podocalyxin, while other did not. Ephrin-B1 and podocalyxin did not colocalize (Figure 2B). Immunoelectron microscopic analysis showed that NHERF2 was expressed at the apical membrane and the slit diaphragm (Figure 2C).

The developmental expression of NHERF2 in glomeruli was analyzed with neonatal rat kidney sections. At this stage, NHERF2 was already present in presumptive podocytes of the early S-shaped body stage, and expressed along the whole cell surface, including the basal side. At the late S-shaped body stage, NHERF2 was detected along the whole cell surface, and costained with ephrin-B1 at the basolateral end. At the capillary loop stage, NHERF2 was restricted at the lateral and the apical areas, and ephrin-B1 was restricted at the lateral area. Some NHERF2 costained with ephrin-B1 (Figure 2D).

NHERF2 Interacts with Nephrin via Ephrin-B1

Next, the interaction of NHERF2 with ephrin-B1 and nephrin was analyzed. In the immunoprecipitation assay with normal rat glomerular lysate, a specific band of NHERF2 was detected in the precipitate with anti-ephrin-B1 antibody (Figure 3A) and that with anti-nephrin antibody (Figure 3A). Immunoprecipitation with the HEK293 cell lysates cotransfected with ephrin-B1 and NHERF2 showed

Figure 1 NHERF2 expression is down-regulated in glomeruli of the ephrin-B1 conditional knockout (CKO) mice. **A:** Real-time PCR analysis of the isolated glomeruli of ephrin-B1 CKO mice. The glomerular mRNA expression of NHERF2 was decreased in the ephrin-B1 CKO mice ($17.4\% \pm 5.7\%$; two-tailed *t*-test). Glomerular mRNA expression of ephrin-B1 was clearly decreased ($0.86\% \pm 0.06\%$; two-tailed *t*-test). Glomerular mRNA expressions of zonula occludens protein 1 (ZO-1), membrane associated guanylate kinase, WW and PDZ domain containing 1, and calcium/calmodulin-dependent serine protein kinase were not altered in the ephrin-B1 CKO mice. **B:** Representative immunofluorescence findings of NHERF2 and ephrin-B1 in kidney sections. The staining of NHERF2 was decreased in ephrin-B1 CKO mice (2.67 ± 0.18 versus 3.59 ± 0.16 ; *U*-test). No ephrin-B1 staining in glomeruli was detected. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Scale bar = 20 μ m (**B**). IF, immunofluorescence; WT, wild type.

Figure 2 Localization of NHERF2 in glomeruli. **A:** Representative immunofluorescence images of NHERF2 in rat kidney section. NHERF2 staining was detected in glomeruli in the kidney section, and NHERF2 staining in glomeruli was detected along the capillary loop as a podocyte pattern. **B:** Dual-labeling finding of NHERF2 (green) with glomerular cell markers (red). The staining of NHERF2 did not overlap with Thy1.1, a mesangial cell marker, or rat endothelial cell antigen-1 (RECA1), an endothelial cell marker. NHERF2 costained with nephrin (arrow), with ephrin-B1 (arrow), and ephrin-B1 costained with NHERF2. Some NHERF2 costained with podocalyxin (arrow), while other did not (arrowhead). Ephrin-B1 did not costain with podocalyxin. **C:** Immunoelectron microscopic finding of NHERF2. Gold particles for NHERF2 were detected not only at the apical area (arrowhead) but also at the slit diaphragm (arrows). **D:** Developmental expression of NHERF2 in glomeruli. NHERF2 staining was detected in presumptive podocytes of the early S-shaped body stage along with ephrin-B1 staining. At this stage, NHERF2 staining was detected along the whole cell surface, including the basal side. At the late S-shaped body stage, NHERF2 was detected along the whole cell surface, and NHERF2 at the basolateral side costained with ephrin-B1. At the capillary and maturing stages, NHERF2 staining was detected at the lateral and apical areas, and the NHERF2 at the lateral area costained with ephrin-B1 (arrows). Scale bars: 100 μ m (**A**); 20 μ m (**B** and **D**); 1 μ m (**C**).

an interaction between NHERF2 and ephrin-B1 (Figure 3B), which was not detected in the HEK293 cells cotransfected with NHERF2 and nephrin (Figure 3B). In contrast, the interaction was detected in the HEK293 cells transfected with NHERF2, ephrin-B1, and nephrin (Figure 3B). These results indicate that NHERF2 interacts with nephrin via ephrin-B1.

Ephrin-B1 Binds to the First PDZ Domain of NHERF2, and Podocalyxin Binds to Its Second PDZ Domain

Next, the interaction of NHERF2 with the PDZ binding motif of ephrin-B1 was analyzed. NHERF2 did not interact with a truncated ephrin-B1 in which C-terminus, including the PDZ binding motif, was deleted, indicating that NHERF2 interacted with the PDZ binding motif of ephrin-B1 (Figure 3C). Because NHERF2 has two PDZ domains, their interaction with ephrin-B1 was analyzed. Immunoprecipitation with the truncated NHERF2 showed that NHERF2 interacted with ephrin-B1 via its first PDZ domain (Figure 3D), and with podocalyxin via its second PDZ domain (Figure 3D). Next, the triadic relationship between NHERF2, ephrin-B1, and podocalyxin was analyzed. The interaction between ephrin-B1 and podocalyxin was not detected in the HEK293 cell lysates transfected with NHERF2, ephrin-B1, and podocalyxin. This indicated that ephrin-B1 bound to NHERF2 did not interact with podocalyxin, and podocalyxin bound to NHERF2 did not interact with ephrin-B1. On the other hand, the interaction between ephrin-B1 and NHERF2 was diminished by the co-expression of podocalyxin (Figure 3E), and the interaction between podocalyxin and NHERF2 was diminished by the co-expression of ephrin-B1 (Figure 3E). The results suggest that ephrin-B1 and podocalyxin are compete for NHERF2 binding.

NHERF2 Links the Nephrin–Ephrin-B1 Complex to Ezrin

Next, the interaction of nephrin–ephrin-B1–NHERF2 complex with ezrin was analyzed. In the immunoprecipitation assay with normal rat glomerular lysate, an ezrin band was detected in the precipitates with an anti-nephrin antibody and an anti-ephrin-B1 antibody (Figure 4A). In the precipitation assay with the HEK293 cells transfected with ephrin-B1 and NHERF2, an endogenous ezrin band was detected in the precipitate with the anti-ephrin-B1 antibody, which was not detected in the precipitation assay with the HEK293 cells transfected with ephrin-B1 only (Figure 4B). The results indicate that ephrin-B1 interacts with ezrin via NHERF2. The interaction of NHERF2 with endogenous ezrin was not detected in the lysates of HEK293 cells transfected with NHERF2 alone (Figure 4B). The results indicate that NHERF2, in the absence of the transmembrane proteins as binding partners, does not interact with ezrin.

In HEK293 cells transfected with nephrin, ephrin-B1, and NHERF2, an endogenous ezrin band was detected in the precipitate with the anti-nephrin antibody. However, an ezrin band was not detected in the precipitate with the anti-nephrin antibody in the lysates of the HEK293 cells transfected with nephrin and NHERF2 but not ephrin-B1 (Figure 4C).

Ezrin Is Dephosphorylated, and the F-Actin Structure Is Deranged, in NHERF2 Knockdown Podocytes

Next, the expression and the phosphorylation of ezrin were analyzed in podocytes treated with NHERF2 siRNA. Although total ezrin staining was not altered, phosphorylated ezrin staining was decreased, and F-actin staining detected with rhodamine-phalloidin was altered in the cells treated with NHERF2 siRNA (Figure 5). Ephrin-B1 was down-regulated in the NHERF2 knockdown cells, indicating that NHERF2 is highly associated with ephrin-B1 and that NHERF2 is necessary for the expression of ephrin-B1.

Ephrin-B1 Is Crucial for the Proper Expression of NHERF2 and Ezrin

NHERF2 was down-regulated in podocytes treated with ephrin-B1 siRNA. Although total ezrin staining was not clearly altered, phosphorylated ezrin staining was decreased in the cells treated with ephrin-B1 siRNA. F-actin staining was altered in the ephrin-B1–knockdown podocytes at the same level as that in the NHERF2 knockdown podocytes (Figure 6). Similar to NHERF2, the expression of ezrin was reduced in the ephrin B1 CKO mice, and the remaining ezrin was dephosphorylated and was dissociated from NHERF2 (Figure 7, A and B). Next, the interaction between NHERF2 and podocalyxin was analyzed. Although the expression of podocalyxin was not altered in the CKO mice, almost no costaining of podocalyxin and NHERF2 was detected (Figure 7, A and B). The dissociation of NHERF2 from podocalyxin in the CKO mice was confirmed by the PLA assay (Figure 7C). These results indicate that the remaining NHERF2 does not interact with podocalyxin in the ephrin-B1 CKO mice.

The Linkage of Nephrin–Ephrin-B1–NHERF2–Ezrin Is Disrupted in the Rat Nephrotic Model Induced by the Anti-Nephrin Antibody

NHERF2 was decreased at 1 hour in the rat nephrotic model induced by the anti-nephrin antibody injection, and the decrease was evident on day 5 when severe proteinuria was observed (Figure 8). Ezrin was down-regulated, and the phosphorylated ezrin staining was decreased in the nephrotic model (Figure 8). Dual-labeling analyses showed that the remaining NHERF2 did not colocalize with ephrin-B1 and ezrin (Figure 9A). The dissociation of NHERF2 from ephrin-B1 and ezrin was confirmed by the PLA assay (Figure 9B).

Figure 3 Interaction of NHERF2 with ephrin-B1, nephrin, and podocalyxin. **A:** Interaction assays with rat glomerular lysates. A specific NHERF2 band of approximately 45 kDa was detected in the precipitate with anti-ephrin-B1 antibody (**left panel**), and with anti-nephrin antibody (**right panel**). **B: Left panel:** Human embryonic kidney (HEK) 293 cells cotransfected with NHERF2-streptavidin and ephrin-B1–HA. NHERF2 band was detected in the precipitate with anti-HA antibody. Ephrin-B1 band was detected in the precipitate with anti-streptavidin antibody. **Middle panel:** HEK293 cells transfected with NHERF2-strep and nephrin-FLAG. Nephrin band was not detected in the precipitate with anti-streptavidin antibody. NHERF2 band was not detected in the precipitate with anti-FLAG antibody. **Right panel:** HEK293 cells transfected with NHERF2-streptavidin, ephrin-B1–HA, and nephrin-FLAG. NHERF2 band was detected in the precipitate with anti-FLAG antibody. **C: Left panel:** NHERF2 band was detected in the precipitate with anti-FLAG antibody. **Right panel:** NHERF2 band was not detected in the precipitate with anti-HA antibody in the HEK293 cells cotransfected with NHERF2-streptavidin and the truncated ephrin-B1–HA in which PDZ binding motif was deleted. **D: Left panel:** NHERF2 band was not detected in the precipitate with anti-HA antibody in the HEK cells transfected with ephrin-B1–HA and the truncated NHERF2-streptavidin in which PDZ domain 1 was deleted. **Right panel:** NHERF2 band was not detected in the precipitate with anti-myc antibody from the lysate of the HEK293 cells transfected with podocalyxin-myc and the truncated NHERF2-streptavidin in which PDZ domain 2 was deleted. **E: Left panel:** The NHERF2 band in the precipitate with the antibody recognizing ephrin-B1 was diminished by the co-expression of podocalyxin. No interaction between ephrin-B1 and podocalyxin was detected. **Right panel:** The NHERF2 band in the precipitate with the antibody recognizing podocalyxin was diminished by the co-expression of ephrin-B1. IP, immunoprecipitation; NMS, normal mouse serum; NRS, normal rabbit serum; WB, Western blot analysis.

Figure 4 Nephrin interacts with ezrin via ephrin-B1 and NHERF2. **A:** Interaction assays with rat glomerular lysates. A specific ezrin band of approximately 80 kDa was detected in the precipitate with the anti-ephrin-B1 antibody (**left panel**), and in the precipitate with the anti-nephrin antibody (**right panel**). **B:** **Left panel:** An endogenous ezrin band was detected in the precipitate with the antibody recognizing ephrin-B1 in the HEK293 cells cotransfected with ephrin-B1 and NHERF2. The ezrin band was not detected in the precipitate with the antibody recognizing ephrin-B1 in the human embryonic kidney (HEK) 293 cells transfected with ephrin-B1 only. **Right panel:** The endogenous ezrin band was detected in the precipitate with the antibody recognizing NHERF2 in the HEK293 cells cotransfected with ephrin-B1 and NHERF2. The ezrin band was not detected in the precipitate with the antibody recognizing NHERF2 in the HEK293 cells transfected with NHERF2 only. Co-expression of ephrin-B1 was required for the interaction of NHERF2 with ezrin. **C:** Assay with the HEK293 cells transfected with nephrin-FLAG, ephrin-B1-HA, and NHERF2-streptavidin. The NHERF2 and ezrin bands were detected in the precipitate with the antibody recognizing nephrin from the lysate of the HEK293 cells transfected with nephrin, ephrin-B1, and NHERF2. Neither NHERF2 nor ezrin band was detected in the precipitates with the antibody detecting nephrin from the lysate of the HEK cells without transfection of ephrin-B1. IP, immunoprecipitation; NRS, normal rabbit serum; WB, Western blot analysis.

Phosphorylation of Nephrin and Consequent Ephrin-B1 Phosphorylation Lead to Dephosphorylation of NHERF2 and Ezrin and Disrupt the Linkage of Nephrin–Ephrin-B1–NHERF2–Ezrin

Next, the effect of nephrin phosphorylation on nephrin–ephrin-B1–NHERF2–ezrin linkage was analyzed. In HEK293 cells transfected with nephrin, ephrin-B1, and NHERF2, tyrosine phosphorylation of nephrin and consequent tyrosine phosphorylation of ephrin B1 disrupted the interactions among nephrin–ephrin-B1, ephrin-B1–NHERF2, and NHERF2–ezrin (**Figure 10A**). Next,

more precise analyses were performed with HEK293 cells transfected with ephrin-B1 and NHERF2. Ephrin-B1 phosphorylation by the stimulation with Eph-Fc, dephosphorylation of NHERF2 and endogenous ezrin, and dissociation of ephrin-B1 from NHERF2, led to a disruption in the interaction between NHERF2 and ezrin (**Figure 10B**).

Ephrin-B1 Phosphorylation Disrupts the Linkage of Podocalyxin and NHERF2

Because the observations in the ephrin-B1 CKO mice (**Figure 7**) and in the nephrotic model (**Figures 8 and 9**)

Figure 5 Ezrin is dephosphorylated, and the F-actin structure is deranged, in NHERF2 knockdown podocytes. mRNA expression of NHERF2 decreased to 21.5% (two-tailed *t*-test), and the staining intensity of NHERF2 was reduced to 16.5% (two-tailed *t*-test) in the cells treated with NHERF2 siRNA for 48 hours. Ephrin B1 staining was also decreased ($37.2\% \pm 2.46\%$; two-tailed *t*-test), and phosphorylated ezrin was decreased in the cells treated with NHERF2 siRNA. Phosphorylation of ezrin was evaluated by staining with the anti-phosphorylated ezrin/moesin/radixin (ERM) antibody. The staining intensity of the phosphorylated ERM was decreased to 16.5% (two-tailed *t*-test), although the staining with the antibody detecting total ezrin was not altered. The data are shown as the ratio (percentage) relative to the control siRNA-treated cells. F-actin was detected by rhodamine-phalloidin, and the phalloidin staining was evaluated as score 1 to 4. In the NHERF2 knockdown podocytes, cells with score 1 and 2 were fewer than, and cells with score 3 and 4 were more than, in the control podocytes (3.41 ± 0.29 versus 2.06 ± 0.54 ; NHERF2 siRNA versus control; *U*-test). **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001. Scale bar = 100 μ m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

suggested that ephrin-B1 affects not only ephrin-B1-binding NHERF2 but also podocalyxin-binding NHERF2, the interaction between NHERF2 and podocalyxin was analyzed in HEK293 cell transfected with NHERF2, podocalyxin, and ephrin-B1. Although in the basal state, an NHERF2 band was detected in the precipitate with podocalyxin antibody, NHERF2 band was not detected in case of ephrin B1 phosphorylation (Figure 11). Additionally, ezrin band was not

detected in the precipitate with the NHERF2 antibody if ephrin-B1 was phosphorylated. Unexpectedly, the ezrin band was still detected in the precipitate with the podocalyxin antibody, even if ephrin-B1 was phosphorylated. It is estimated that ezrin dissociated from NHERF2 directly interacted with podocalyxin. NHERF2 and endogenous ezrin were almost completely dephosphorylated in the presence of phosphorylated ephrin B1 (Figure 11).

Figure 6 NHERF2 is down-regulated and ezrin is dephosphorylated in ephrin-B1 knockdown cultured podocyte. mRNA expression of ephrin-B1 decreased to 6.6% (two-tailed *t*-test), and the staining intensity of ephrin-B1 was reduced to 14.3% (two-tailed *t*-test) in the cells treated with ephrin-B1 siRNA for 48 hours. NHERF2 staining was decreased (two-tailed *t*-test), and phosphorylated ezrin was decreased in the cells treated with the ephrin-B1 siRNA. Phosphorylation of ezrin was evaluated by the staining with the anti-phosphorylated ezrin/moesin/radixin (ERM) antibody. The staining intensity of the phosphorylated ERM was decreased to 14.1% (two-tailed *t*-test), although the staining with the antibody detecting total ezrin was not clearly altered. The data are shown at the ratio (percentage) relative to the control siRNA-treated cell. The structure of F-actin detected by rhodamine-phalloidin is altered in the ephrin-B1 knockdown podocytes (3.42 ± 0.08 versus 1.86 ± 0.18 ; *U*-test). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. Scale bar = 100 μ m.

Discussion

Immunohistochemical analyses in this study demonstrated that NHERF2 is expressed not only at the apical membrane but also at the slit diaphragm (Figure 2). Immunoprecipitation assay indicated that NHERF2 interacts with ephrin-B1 and with nephrin, a key functional molecule of slit diaphragm via ephrin-B1. These findings point to NHERF2 being a member of the slit diaphragm complex. The study also showed that ephrin-B1 bound to the first PDZ domain of NHERF2, and podocalyxin bound to the second PDZ

domain (Figure 3). However, unbound NHERF2 did not interact with ephrin-B1 or podocalyxin.

This study also revealed that nephrin interacts with ezrin via ephrin-B1 and NHERF2 (Figure 4). NHERF2 was a linker connecting transmembrane proteins of the slit diaphragm to ezrin. This is the first report demonstrating the linkage of nephrin-ephrin-B1–NHERF2–ezrin. Ezrin, a member of the ezrin-radixin-moesin family of proteins, connects membrane proteins to the actin cytoskeleton and regulates actin polymerization and the surface expression of the membrane proteins.^{35,36} Physiologically active ezrin is

Figure 7 NHERF2 is dissociated from ezrin in the ephrin-B1 conditional knockout (CKO) mice. **A:** The expression and the phosphorylation of ezrin were decreased in the ephrin-B1 CKO mice (*U*-test). Stainings of nephrin and NHERF2 were decreased, whereas the staining of podocalyxin was not altered in the CKO mice. **B:** Dual-labeling immunofluorescence (IF) findings of NHERF2 with ezrin and podocalyxin. NHERF2 almost completely colocalized with ezrin in wild-type (WT) mice. In the CKO mice, NHERF2 was down-regulated and the remaining NHERF2 was dissociated from ezrin (arrow). Some NHERF2 costained with podocalyxin in WT mice. The remaining NHERF2 did not colocalize with podocalyxin in the CKO mice (arrow). **C:** Findings of the proximity ligation assay (PLA). Almost no positive PLA signals showing proximity of NHERF2 and podocalyxin were detected in the CKO mice (WT versus CKO, 28.54 ± 1.40 versus 0.26 ± 0.07 ; two-tailed *t*-test). ***P* < 0.01, *****P* < 0.0001. Scale bar = 20 μ m (**A–C**). ERM, ezrin/moesin/radixin.

serine/threonine phosphorylated and interacts with actin.^{37,38} Several protein kinases, including Rho-associated kinases,^{38,39} protein kinase C,⁴⁰ and Ste20-like kinase,⁴¹ can phosphorylate ezrin. In podocytes, ezrin plays an important role in maintaining foot processes.^{19,42–44} Podocyte-specific CKO mice of ephrin-B1 show effacement of foot processes and proteinuria.¹² Herein, the data show that ezrin was dephosphorylated and down-regulated in the CKO mice (Figure 7A). F-actin structure was deranged in the NHERF2-deleted cultured podocytes (Figure 5) and in the

ephrin-B1–deleted cultured podocytes (Figure 6). These observations imply that ephrin-B1, NHERF2, and ezrin play an important role in maintaining the structure of the actin cytoskeleton. The slit diaphragm is reported to be linked to the actin cytoskeleton via CD2AP^{45,46} and via klin of IRRE-like protein 1–zonula occludens protein 1.⁴⁷ However, the linkages of the slit diaphragm to the actin cytoskeleton are not fully elucidated yet. It is conceivable that the linkage of nephrin–ephrin-B1–NHERF2–ezrin–actin is one of the critical linkages connecting the slit diaphragm to the actin

Figure 8 NHERF2 and phosphorylated ezrin are decreased in the rat nephrotic model induced by the anti-nephrin antibody injection. Down-regulation and tyrosine phosphorylation of ephrin-B1 were already detected at 1 hour after the disease induction. Stainings of NHERF2 and ezrin were decreased at 1 hour. Podocalyxin staining was not altered through the period examined. Ezrin was threonine phosphorylated in normal rat glomeruli, and phosphorylated ezrin detected by the anti-phosphorylated ezrin/moesin/radixin (ERM) decreased at 1 hour (2.28 ± 0.09 versus 3.83 ± 0.10 ; *U*-test). **P* < 0.05. Scale bar = 20 μ m. IF, immunofluorescence.

cytoskeleton, and that this linkage plays an important role in maintaining the interdigitating foot processes and the slit diaphragm.

The nephrin–ephrin-B1–NHERF2–ezrin–actin axis was analyzed in the pathologic state. Rats intravenously injected with the anti-nephrin antibody show transient but severe proteinuria.^{22,24,48} Proteinuria in this model is induced in the absence of inflammatory factors, and is caused by disarrangement of the slit diaphragm components.^{22,49–51} Not only nephrin but also ephrin-B1 is down-regulated and tyrosine phosphorylated in glomeruli of this nephrotic model.¹² The significance of nephrin phosphorylation in physiological and pathologic states is still controversial, and there are site-specific differences of tyrosine residues of nephrin.³ Phosphorylation of Y1204 of nephrin and consequent ephrin-B1 phosphorylation are critical initiation events leading to podocyte injury in this nephrotic model.¹² The present study showed that NHERF2

was also down-regulated in this model, and the remaining NHERF2 was dissociated from ephrin-B1 (Figures 8 and 9). In the HEK293 cell expression system, serine/threonine of NHERF2 and ezrin were phosphorylated in the basal state, and in case of nephrin and consequently ephrin-B1 phosphorylation, NHERF2 and endogenous ezrin were almost completely dephosphorylated (Figure 10B). We propose the following mechanisms to explain these results: first, the phosphorylated ephrin-B1 is dissociated from NHERF2. Next, NHERF2 gets dephosphorylated, becomes inactive, and dissociates from ezrin.⁵² Then, ezrin, which is dissociated from NHERF2, gets dephosphorylated, becomes inactive, and is unable to bind F-actin.^{36,53} Effacement of foot process with derangement of F-actin is usually observed in kidney biopsy samples of patients with massive proteinuria.⁵⁴ However, the causal relationship between the alterations of foot processes and proteinuria is not clear. This study presents a novel pathogenic mechanism of how

Figure 9 The linkage of nephrin–ephrin-B1–NHERF2–ezrin is disrupted in the anti-nephrin antibody-induced nephropathy. **A:** Dual-labeling immunofluorescence findings of NHERF2 with ephrin-B1, ezrin, and podocalyxin. Ephrin-B1 and nephrin were dissociated on day 5 at the peak of proteinuria. NHERF2 almost completely colocalized with ezrin, some NHERF2 colocalized with ephrin-B1, while some NHERF2 colocalized with podocalyxin in normal rat kidney sections. NHERF2 staining was decreased on day 5, and the remaining NHERF2 was isolated from ephrin-B1, ezrin, and podocalyxin (arrow). **B:** The findings of proximity ligation assay (PLA). Almost no positive PLA signal showing close proximity of NHERF2 with ephrin-B1 or ezrin was detected on day 5 in this nephropathy (NHERF2/ephrin-B1: normal versus day 5: 89.22 ± 4.75 versus 0.53 ± 0.10 , two-tailed *t*-test; NHERF2/ezrin: normal versus day 5: 142.23 ± 8.96 versus 1.23 ± 0.23 , two-tailed *t*-test). *****P* < 0.0001. Scale bar = 20 μ m (**A** and **B**).

phosphorylation of nephrin and ephrin-B1 disrupts the slit diaphragm and F-actin (Figure 12). It is conceivable that the phosphorylation of ephrin-B1 and the consequent dephosphorylation of NHERF2 are critical initiation events leading to proteinuria and foot process effacement. Although the mechanisms of phosphorylation of NHERF2 are not well elucidated, it is estimated that several kinases and phosphatase, including those acting on ezrin, are involved in the regulation of NHERF2 phosphorylation.^{55,56} We propose that inhibition of dephosphorylation of NHERF2 is a rational strategy for developing a novel therapy for a nephrotic syndrome.

Although the role of ephrin-B is well investigated in the neuronal field,⁵⁷ the function of ephrin-B in epithelial cells is not well understood. Herein, NHERF2 was shown to interact with ephrin-B1 in podocytes. This is the first report identifying the PDZ protein interacting with the PDZ-binding motif of ephrin-B1 in epithelial cells. Both ephrin-B1 and NHERF2 are highly expressed in epithelial cells in several major organs, including lung and small

intestine.^{17,58–60} NHERF2 plays a critical role in ezrin/radixin/moesin phosphorylation of pulmonary endothelial cells.⁶¹ The ephrin-B1–NHERF2 complex participates in maintaining the specific tissue functions in several major organs.

Another important finding that should be noted is that studies with the CKO mice (Figure 7) and the nephrotic model (Figures 8 and 9) suggest that defect or phosphorylation of ephrin-B1 affects not only the ephrin-B1–NHERF2–ezrin axis but also the podocalyxin–NHERF2–ezrin axis. The association between these axes was investigated in HEK293 cells transfected with podocalyxin, ephrin-B1, and NHERF2. Ephrin-B1 phosphorylation led to the complete dephosphorylation of NHERF2, disrupting not only the linkage of ephrin-B1–NHERF2 but also that of podocalyxin–NHERF2 (Figure 11). Endogenous ezrin was completely dephosphorylated in the event of ephrin-B1 phosphorylation. Because the structural linkage of the ephrin-B1–NHERF2 complex with the podocalyxin–NHERF2 complex was not detected by the

Figure 10 Phosphorylation of ephrin-B1 disrupts the linkage of nephrin–ephrin-B1–NHERF2–ezrin. **A:** Human embryonic kidney (HEK) 293 cells transfected with NHERF2-streptavidin, ephrin-B1–HA, and nephrin-FLAG. Nephrin and ephrin-B1 were tyrosine phosphorylated by the anti-nephrin antibody treatment. The interaction of nephrin–endogenous ezrin was detected in the HEK293 cells without the anti-nephrin antibody treatment. All interactions were disrupted in the HEK293 cells treated with the anti-nephrin antibody. **B:** HEK293 cells transfected with NHERF2-streptavidin and ephrin-B1–HA. Ephrin-B1 was phosphorylated by the EphB2-Fc treatment. The interactions of ephrin-B1–NHERF2, NHERF2–endogenous ezrin, and ephrin-B1–endogenous ezrin were detected in the HEK293 cells without Eph-Fc treatment. All interactions were disrupted in the HEK293 cells treated with the EphB2-Fc. NHERF2 and ezrin were serine/threonine phosphorylated in the HEK293 cells without the EphB2-Fc treatment. NHERF2 and ezrin were dephosphorylated by the EphB2-Fc treatment. IP, immunoprecipitation; Tx, treatment; WB, Western blot analysis.

immunoprecipitation assay with HEK293 cell lysates (Figure 3), the mechanism of ephrin-B1 affecting the podocalyxin-NHERF2 complex cannot be explained. However, multiple mechanisms are proposed (Figure 12). Because NHERF proteins form dimers or oligomers,^{62,63} the ephrin-B1–NHERF2 complex may interact with the podocalyxin-NHERF2 complex via the dimerized NHERF2. Ephrin-B1 phosphorylation led to the dissociation of NHERF2 from ephrin-B1 and its subsequent dephosphorylation. Some phosphatase may act not only on NHERF2 from the ephrin-B1–NHERF2 complex but also on the neighboring NHERF2 from the podocalyxin-NHERF2 complex. Dephosphorylated NHERF2 was dissociated from podocalyxin and ezrin. The present study implies that stimulation of the slit diaphragm and consequent phosphorylation of nephrin and ephrin-B1 disrupts not only the nephrin–ephrin-B1–NHERF2–ezrin–actin

linkage but also the podocalyxin-NHERF2-ezrin-actin linkage. This is the first report demonstrating that the stimulation of the slit diaphragm alters the interaction of apical transmembrane protein with its scaffolding protein.

The slit diaphragm of podocytes is considered a variant of tight junction because of its fence function.^{5,6} One of the most important functions of tight junction of epithelial cells is acting as a fence that prevents the intermixing of apical and basolateral plasma membrane proteins and lipids, which helps to maintain cell surface polarity. Although the precise molecular mechanisms of how tight junctions act as a fence are not clear, tight junctions presumably play a role in regulating the expression and the property of apical and basolateral membrane proteins. The current study demonstrated that phosphorylation of ephrin-B1 at the slit diaphragm and the consequent dephosphorylation of NHERF2 altered the binding property of the apical membrane protein.

Figure 11 Phosphorylation of ephrin-B1 disrupts the linkage of podocalyxin-NHERF2-ezrin. Human embryonic kidney (HEK) 293 cells transfected with NHERF2-streptavidin, ephrin-B1–HA, and podocalyxin-myc. Ephrin-B1 was phosphorylated by the EphB2-Fc treatment. **Left panel:** The interaction of podocalyxin-NHERF2, NHERF2–endogenous ezrin, and podocalyxin–endogenous ezrin was detected in the HEK293 cells without the Eph-Fc treatment. The interaction of podocalyxin-NHERF2 and NHERF2–endogenous ezrin was disrupted in the HEK293 cells treated with EphB2-Fc. The interaction of podocalyxin–endogenous ezrin was still detected in the HEK293 cells treated with the EphB2-Fc. **Right panel:** NHERF2 and ezrin were serine/threonine phosphorylated in the HEK293 cells without the EphB2-Fc treatment. NHERF2 and ezrin were dephosphorylated by the EphB2-Fc treatment. IP, immunoprecipitation; Tx, treatment; WB, Western blot analysis.

Ephrin-B1 and NHERF2 are expressed at the tight junction in several types of epithelial cells. Observations in this study suggest that the ephrin-B1–NHERF2 complex at the tight junction acts as a fence by regulating the property of apical membrane proteins.

In conclusion, NHERF2 is expressed at not only the apical membrane but also at the slit diaphragm. NHERF2 interacts with the nephrin–ephrin-B1 complex, and the nephrin–ephrin-B1–NHERF2 complex interacts with ezrin bound to F-actin, which indicates that the linkage of nephrin–ephrin-B1–NHERF2–ezrin–actin is a novel critical axis in the podocytes. Stimulation of slit diaphragm leads to the phosphorylation of nephrin and ephrin-B1,

dephosphorylation of NHERF2 and ezrin, and subsequent disruption of not only the nephrin–ephrin-B1–NHERF2–ezrin–actin axis, but also the podocalyxin–NHERF2–ezrin–actin axis. It is conceivable that phosphorylation of ephrin-B1 and the consequent dephosphorylation of NHERF2 are critical initiation events leading to podocyte injury.

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A Normal

B Stimulation to nephrin

Figure 12 Schematic diagram of the proposed pathogenic mechanism of disruption of the linkages of nephrin–ephrin-B1–NHERF2–ezrin and podocalyxin–ephrin-B1–NHERF2–ezrin. **A:** The nephrin–ephrin-B1 complex at the slit diaphragm is linked to F-actin via NHERF2 and ezrin. Podocalyxin is an apical membrane protein linked to F-actin via NHERF2 and ezrin. Podocalyxin is an apical membrane protein linked to F-actin via NHERF2 and ezrin. Podocalyxin binds to the second PDZ domain of NHERF2, and ephrin-B1 binds to the first PDZ domain of NHERF2. But they do not share a molecule of NHERF2. It is estimated NHERF2 forms dimer. In the physiological state, nephrin and ephrin-B1 are not phosphorylated, and NHERF2 and ezrin are serine/threonine phosphorylated. **B:** If the slit diaphragm is stimulated and nephrin and ephrin-B1 are tyrosine phosphorylated, nephrin is dissociated from ephrin-B1, and ephrin-B1 is dissociated from NHERF2. Then, NHERF2 changed to be an inactive dephosphorylated form and then dissociated from ezrin. Ezrin, which is dissociated from NHERF2, changed to be an inactive dephosphorylated form unable to bind F-actin. The phosphorylation of ephrin-B1 also dephosphorylated a neighboring NHERF2 of the linkage of podocalyxin-NHERF2-ezrin and disrupted the linkage. GBM, glomerular basement membrane.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2021.04.004>.

Author Contributions

Y.F. and H.K. designed the experiments; Y.F. performed *in vivo* experiments and wrote the manuscript; Y.F. performed the studies with conditional knockout mice as well as *in vitro* experiments; H.Y. performed the human cultured podocyte study; Y.Z. performed the developmental study; H.K. conceived and directed the project and wrote the manuscript.

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