Vasohibin-1 has α-tubulin detyrosinating activity in glomerular podocytes

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Abstract

Podocytes are highly specialized epithelial cells in glomeruli, with a complex morphology composed of a cell body, primary processes, and foot processes, which maintain barrier function in glomerular filtration. The microtubule-based cytoskeleton is necessary for podocyte morphology. Microtubule structure and function can be affected by post-translational modification of tubulin, including detyrosination. Recent studies have shown that vasohibin-1 (VASH1), an antiangiogenic factor, has tubulin carboxypeptidase activity that causes detyrosination of α-tubulin. We aimed to examine the role of VASH1 in regulating α-tubulin detyrosination in podocytes and the potential involvement of VASH1 deficiency in renal morphology. In normal mouse kidneys, detyrosinated α-tubulin was mainly identified in glomeruli, especially in podocytes; meanwhile, in cultured immortalized podocytes, α-tubulin detyrosination was promoted with cell differentiation. Notably, α-tubulin detyrosination in glomeruli was diminished in *Vash1* homozygous knockout (*Vash1*$^{-/-}$) mice, and knockdown of *VASH1* in cultured podocytes prevented α-tubulin detyrosination. Although VASH1 deficiency-induced downregulation of detyrosination caused no remarkable glomerular lesions, urinary albuminuria excretion and glomerular volume were significantly higher in *Vash1*$^{-/-}$ mice than in wild-type mice. Furthermore, decreased glomerular nephrin expression and narrower slit diaphragms width were observed in *Vash1*$^{-/-}$ mice. Taken together, we demonstrated that α-tubulin detyrosination in podocytes was mainly regulated by VASH1 and that VASH1 deficiency-mediated decreases in α-tubulin detyrosination led to minor alterations in podocyte morphology and predisposition to albuminuria. VASH1 expression and α-tubulin detyrosination may be novel targets for maintaining glomerular filtration barrier integrity.

1. Introduction

Proteinuria reflects the disruption of the glomerular filtration barrier and represents a major risk factor for the progression of chronic kidney disease[1]. The barrier function is ensured by the structure of three layers: capillary endothelial cells, the glomerular basement membrane (GBM), and podocytes. Podocytes are highly specialized epithelial cells, with long cytoplasmic projections termed primary processes that extend to form smaller foot processes[2]. These processes cover the urinary lumen side of glomerular capillaries and foot processes interdigitate with those of neighboring podocytes to form slit diaphragms (SDs). The normal structure of foot processes and SDs is the most important aspect of the barrier function against proteinuria. In addition, podocytes produce and secrete angiogenic factors, including vascular endothelial growth factor (VEGF), to maintain the glomerular endothelium, contributing to the integrity of the filtration barrier[3].

Vasohibin-1 (VASH1) was originally identified as an endothelium-derived antiangiogenic factor that is upregulated by VEGF stimulation[4]. However, recent studies have reported VASH1 expression in different cell types, including neurons[5]. Although insufficient VASH1 expression in *Vash1* heterozygous knockout mice does not cause any detectable abnormalities in the kidney, it can accelerate various renal pathological processes such as diabetic nephropathy[6], renal fibrosis[7], and acute kidney injury[8], suggesting that VASH1 deficiency may enhance susceptibility to renal diseases. However, the significance of VASH1 expression in the kidney has not been fully elucidated.

In 2017, a novel biological function of VASH1 was reported. Two studies independently demonstrated that VASH1 has detyrosinating enzymatic activity, the representative post-transcriptional modification (PTM) that occurs on α-tubulin[5, 9]. When α- and β-tubulin heterodimers polymerize to form stabilized microtubules, the C-terminal

tyrosine residue of α-tubulin is removed, and this process can be reversed in depolymerized α-tubulin monomers; these processes are termed detyrosination and tyrosination, respectively. The detyrosination/tyrosination cycle in microtubule dynamics is mediated by tubulin carboxypeptidase (TCP) in the former and tubulin tyrosine ligase in the latter[10]. Although the putative TCP had not been identified for over 40 years, VASH1 was first determined to possess TCP activity. Vasohibin-2 (VASH2), which is a homolog of VASH1 but serves as a proangiogenic factor, has also been reported to catalyze α-tubulin detyrosination[5, 9]. Detyrosination of α-tubulin has been implicated in various microtubule functions, such as mitosis and the cytoskeleton[10]. However, there is little evidence regarding the roles of detyrosination of α-tubulin mediated by VASH1 in the kidney. Here, we examined VASH1-mediated detyrosination of α-tubulin in the kidney and determined the renal phenotype of *Vash1* homozygous knockout mice.

2. Materials and Methods

2.1 Animals

C57BL/6J background *Vash1*^{tm1Ysat} (*Vash1* knockout) and *Vash2*^{tm1Ysat} (*Vash2* knockout) mice were obtained from the Institute of Development, Aging, and Cancer at Tohoku University (Sendai, Japan) and were bred at the Department of Animal Resources in the Advanced Science Research Center at Okayama University (Okayama, Japan). All animal experiments complied with ARRIVE guidelines and the protocol were approved by the Animal Care and Use Committee at Okayama University (approval no. OKU-2018716 and OKU-2021373). All male wild-type and knockout mice used in this study were fed standard pellet laboratory chow and provided with water ad libitum. At eight-week-old, twenty-four hours urine samples were collected using metabolic cages, and arterial blood pressure was

measured by a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan). Then, under isoflurane anesthesia, blood samples were collected from the inferior vena cava, and the kidneys were harvested. Urinary creatinine, serum creatinine, and blood urea nitrogen (BUN) concentrations were measured by Oriental Yeast Co., Ltd. (Tokyo, Japan). Urinary albumin concentration was determined using a Mouse Albumin ELISA Kit (Bethyl Laboratories, Montgomery, TX, USA) and normalized to urinary creatinine concentration.

2.2 Cell culture

Conditionally immortalized human podocytes were kindly provided by Prof. Moin A. Saleem and Prof. Peter W. Mathieson, and were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, penicillin-streptomycin (Thermo Fisher), and Insulin-Transferrin-Selenium A (Thermo Fisher), as previously described[11]. Cells were cultured at 33 °C to express large T antigen, allowing cell proliferation, followed by incubation at 37 °C to induce cell differentiation[12]. For the *VASH1* silencing experiment, cells were transfected with siRNA (Stealth RNAi; Thermo Fisher), as previously described[13]. Briefly, the day after transfer to 37 °C, the cells were incubated with culture medium without penicillin-streptomycin for twenty-four hours. Then, human *VASH1* siRNA or control siRNA were transfected using Lipofectamine RNAiMAX (Thermo Fisher). The nucleotide sequences of human *VASH1* siRNA were 5′-CAA GGA CCG GAA GGA UGU UUC U-3′, and the control siRNA was 5′-CGA CCU GCC CAA GAU UCC CAU ACC A-3′. Twelve hours later, the reagents were replaced with RPMI 1640 containing 1% Non-Essential Amino Acid Solution (Thermo Fisher). After the following day, the cells were incubated in complete medium as described above at 37 °C for cell differentiation.

2.3 Histology and morphometrics

Formalin-fixed, paraffin-embedded kidney sections (4 μm thickness) were stained with periodic acid-Schiff. More than twenty-five glomerular images at 200× magnification from each kidney section were obtained and the areas surrounded by glomerular capillary tufts were measured using cellSens imaging software (Olympus, Tokyo, Japan) to determine the mean glomerular cross-sectional tuft area (G_A). The mean glomerular volume (G_V) was calculated using the following equation: $G_V = \beta/k \times (G_A)^{3/2}$. Here, β = 1.38 is the shape coefficient for spheres, k = 1.1 is the size distribution coefficient[13, 14].

2.4 Immunofluorescence

Frozen kidney sections (4 μm thickness) were used for immunofluorescence, as previously described[13, 15]. Briefly, sections were incubated with the following primary antibodies overnight at 4 °C; 1) rabbit anti-detyrosinated α-tubulin (Sigma-Aldrich, St. Louis, MO, USA), 2) rabbit anti-tyrosinated α-tubulin (Sigma-Aldrich), 3) mouse anti-synaptopodin (Progen Biotechnik, Heidelberg, Germany), and 4) guinea pig anti-nephrin (Progen Biotechnik). Then, the sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit, goat anti-mouse, or donley anti-guinea pig secondary antibodies (Thermo Fisher) for one hour at room temperature. For quantitative analysis, more than twenty glomerular images at 200× magnification were obtained from each kidney sample, and fluorescent positive areas in each glomerulus were measured using cellSens imaging software (Olympus). The positive area was expressed as a percentage of the glomerular tuft area.

For double immunofluorescence, frozen kidney sections were first incubated with a rat anti-podocalyxin antibody (R&D Systems, Minneapolis, MN, USA) overnight at 4 °C and then incubated with a rabbit anti-detyrosinated α-tubulin antibody (Sigma-Aldrich) overnight

at 4 °C. Alexa Fluor 488-conjugated donkey anti-rabbit and Alexa Fluor 546-conjugated goat anti-rat antibodies (Thermo Fisher) were used as secondary antibodies. Podocytes were seeded on Lab-Tek 8-well Chamber Slides (Thermo Fisher) and were fixed with 4% paraformaldehyde after differentiation. Detyrosinated α-tubulin was first stained using rabbit anti-detyrosinated α-tubulin (Sigma-Aldrich) and Alexa Fluor 488-conjugated donkey anti-rabbit (Thermo Fisher), and then α-tubulin was stained by Alexa Fluor 594-conjugated anti-α-tubulin antibody (Abcam, Cambridge, UK). 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for nuclear staining. Images were obtained using All-in-one Fluorescence Microscopy (BZ-X700; Keyence, Osaka, Japan).

2.5 Electron microscopy

Transmission electron microscopy was performed to observe the ultrastructure of podocytes and their processes, as previously described[13, 16]. Each kidney cortex tissue was fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and then embedded in EPON epoxy resins. Ultra-thin sections were photographed using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) in the Central Research Laboratory, Okayama University Medical School. In the magnified images, (1) widths of SDs, defined as the distances between the neighboring foot processes, and (2) widths of the foot processes were measured using ImageJ.

2.6 Immunoblotting

Immunoblot was performed as previously described[8, 16]. Briefly, 10 μg of extracted protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes using an iBlot2 Dry Blotting System (Thermo Fisher). The membranes were

incubated with a rabbit anti-detyrosinated α-tubulin antibody (Sigma-Aldrich) or mouse anti-α-tubulin antibody (Cell Signaling) overnight at 4 °C. Then, peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Cell Signaling) were used as secondary antibodies. Enzyme activity was detected using the ECL Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK), and images were obtained using ImageQuant LAS 4000 (GE Healthcare). The density of each band was determined using ImageJ.

2.7 Statistical analysis

All values are expressed as the mean ± standard deviation. The unpaired t-test was used for comparisons of multiple variables. Statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Expression of detyrosinated α-tubulin in the kidney

Detyrosinated α-tubulin generally accumulates in a subset of non-centrosomal, stable microtubules. In the wild-type mouse kidney, strong immunoreactivity for detyrosinated α-tubulin was detected in glomeruli (Fig. 1*A*), whereas tyrosinated α-tubulin was mainly located in tubular epithelial cells but not in glomeruli (Fig. 1*B*). Double immunofluorescence analysis revealed that the detyrosinated α-tubulin-positive area mainly overlapped with podocalyxin-positive podocytes (Fig. 1C). Although detyrosination of α-tubulin can occur in any cell type, these results suggest that microtubules in podocytes consist of highly detyrosinated α-tubulin.

3.2 Detyrosination of α-tubulin in differentiated podocytes in vitro

Immortalized human podocyte can proliferate at 33 °C and differentiate at 37 °C[12]. Immunofluorescence showed that detyrosinated α-tubulin was barely observed in proliferating undifferentiated podocytes, whereas α-tubulin in differentiated podocytes was highly detyrosinated (Fig. 1D). Immunoblotting also demonstrated that the detyrosination of α-tubulin was markedly increased with podocyte differentiation (Fig. 1E). These results suggest that the detyrosination of α-tubulin in microtubules was promoted by the establishment of the podocyte cytoskeleton.

3.3 VASH1-mediated regulation of α-tubulin detyrosination in podocytes

Since VASH1, encoded by *Vash1* in mice and *VASH1* in humans, has been reported to have α-tubulin detyrosinating activity, the expression of detyrosinated α-tubulin in the kidneys of wild-type and VASH1-deficient mice was examined. In contrast to glomeruli in wild-type mouse kidneys, immunoreactivity for detyrosinated α-tubulin was markedly reduced in glomeruli from *Vash1* heterozygous knockout (*Vash1*$^{+/-}$) mice (Fig. 2A and B). Furthermore, detyrosinated α-tubulin in the *Vash1* homozygous knockout (*Vash1*$^{-/-}$) mice was nearly undetectable (Fig. 2C). In the lower magnification images of the transverse section of the kidney, the dot-staining pattern of detyrosinated α-tubulin-positive glomeruli observed in wild-type mice disappeared in *Vash1*$^{-/-}$ mice (Fig. 2D and E). Although VASH2 has been shown to be another α-tubulin detyrosinating enzyme[5, 9], immunoreactivity for detyrosinated α-tubulin in glomeruli was not attenuated in *Vash2*$^{-/-}$ mice (Fig. 2F). Therefore, it is conceivable that the detyrosination of α-tubulin in podocytes can be exclusively regulated by VASH1. Consistent with these results, *VASH*1 knockdown in cultured human podocytes led to a reduction in α-tubulin detyrosination (Fig. 2G and H).

3.4 Renal phenotype of Vash1 homozygous knockout mice

The effect of detyrosinated α-tubulin deficiency on glomerular structure in *Vash1*$^{-/-}$ mice was examined. No significant differences in body weight (24.6 ± 0.54 g in wild-type and 23.7 ± 1.19 g in *Vash1*$^{-/-}$) and systolic blood pressure (116.1 ± 4.7 mmHg in wild-type and 109.5 ± 6.1 mmHg in *Vash1*$^{-/-}$) were observed. There were no significant differences in BUN and serum creatinine levels between wild-type and *Vash1*$^{-/-}$ mice (Fig. 3A and B), whereas creatinine clearance, a glomerular filtration marker, tended to be higher in *Vash1*$^{-/-}$ mice (Fig. 3C). In contrast, urinary albumin excretion was significantly higher in *Vash1*$^{-/-}$ mice than in wild-type mice (Fig. 3D). Light microscopy revealed no apparent alterations in the glomerular structure (Fig. 3E). However, glomerular size was significantly larger in *Vash1*$^{-/-}$ mice than in wild-type mice (Fig. 3F).

3.5 Effects of VASH1 deficiency on filtration barrier in podocytes

The expression of podocyte-specific proteins in *Vash1*$^{-/-}$ mice was evaluated by immunofluorescence. Synaptopodin is an actin-binding protein that is distributed along the podocyte cytoskeleton. There were no differences in the synaptopodin-positive area between wild-type and *Vash1*$^{-/-}$ mice (Fig. 4A and B). However, expression of nephrin, which is specifically localized in podocyte SDs, was significantly lower in *Vash1*$^{-/-}$ mice than in wild-type mice (Fig. 4C and D). Therefore, VASH1 deficiency affected the foot process structure rather than the whole cytoskeleton in podocytes.

Next, electron microscopy was performed to evaluate ultrastructural alterations in podocyte foot processes. No apparent differences in podocyte structure, including primary processes and foot processes, were observed in *Vash1*$^{-/-}$ and wild-type mice (Fig. 4E).

However, morphometric analysis demonstrated that the width of SDs was significantly narrower in *Vash1*$^{-/-}$ mice than in wild-type mice (Fig. 4F), which could be consistent with the lower nephrin expression as mentioned above, whereas there was no significant difference in the width of foot processes (Fig. 4G).

4. Discussion

In the present study, we demonstrated that VASH1 acts as an α-tubulin detyrosinating enzyme in glomerular podocytes, and that defective α-tubulin detyrosination in glomeruli from *Vash1* knockout mice resulted in mild albuminuria and minor morphological changes in podocyte foot processes.

VASH1 was originally identified as an endothelium-derived antiangiogenic factor[4]. In the kidney, the antiangiogenic effects of VASH1 could prevent glomerular injury in murine models of early-stage diabetic nephropathy[14, 17], in which increased VEGF expression is involved in this process. In addition to its antiangiogenic ability, VASH1 has been shown to enhance stress tolerance in cultured endothelial cells[18]. Indeed, heterozygous *Vash1* deficiency exacerbates acute kidney injury with accelerated peritubular capillary loss[8]. Nevertheless, *Vash1* deficiency does not lead to any obvious organ malformation[19], including the kidney as shown in the present study. Therefore, the protective effects of VASH1 on the vascular endothelium may be essential only under stressed conditions.

The tyrosination/detyrosination cycle of α-tubulin in microtubules was identified more than forty years ago[10], and the function of VASH1 as the detyrosinating enzyme for α-tubulin was first reported in 2017[5, 9]. The C-terminal tyrosine residue of α-tubulin is generally removed as α- and β-tubulin heterodimers polymerize to form microtubules. Therefore, detyrosination is a marker for microtubule stabilization. In neurons, stable

microtubules are important for maintaining the structure of axons[20], and can also provide tracks for long-distance transport of cargo between the cell body and axon terminal[21]. Neurons and podocytes are known to share several characteristics: In addition to the expression of common proteins, including synaptopodin and nephrin[22], both cell types have specialized microtubule-based processes[23]. A previous *in vitro* study reported that knockout of *VASH1* and *VASH2* genes using the CRISPR/Cas9 system led to decreased α-tubulin detyrosination and differentiation defects in neural processes[5]; in contrast, in the present study, *Vash1* but not *Vash2* knockout mice showed decreased α-tubulin detyrosination in glomeruli. Although there are obvious differences between these studies in the experimental protocols, podocytes may have a unique VASH1-mediated regulation of α-tubulin detyrosination which is different from that in neurons.

In our previous study, VASH1 heterozygous deficiency was shown to enhance hyperglycemia-induced albuminuria[6]. This finding raises the possibility that glomeruli with less α-tubulin detyrosination in podocytes are prone to albuminuria. Indeed, *Vash1* homozygous knockout mice showed modest but significant albuminuria and glomerular hypertrophy. Recently, detyrosinated α-tubulin has been shown to increase cardiomyocyte stiffness in heart failure[24, 25]. Therefore, decreased detyrosination of α-tubulin in *Vash1*-deficient podocytes may impair the tolerance to mechanical stress produced by intraglomerular pressure, possibly leading to ultrastructural changes in foot processes. In addition, since VASH1 deficiency decreased nephrin expression and shortened the width of SDs, decreased detyrosination of α-tubulin might affect the protein transport between podocyte cell bodies and foot processes along with microtubules. Unfortunately, cultured podocytes cannot reproduce foot processes and SDs *in vitro*; thus, it is difficult to determine whether ultrastructural alterations of foot processes in *Vash1* homozygous knockout mice

were ascribed to impaired tolerance to intraglomerular pressure or altered protein transport.

VASH1-mediated α-tubulin detyrosination may also have important roles in glomerular endothelial and mesangial cells. A recent study demonstrated that the antiangiogenic effects of VASH1 are regulated by α-tubulin detyrosinationin in endothelial cells[26]. Considering the crosstalk between podocytes and glomerular endothelial cells[3], decreased detyrosination of α-tubulin in endothelial cells may affect the structure of podocyte foot processes. However, based on the observation that α-tubulin detyrosination was markedly reduced in cultured podocytes with *VASH1* silencing, it can be speculated that ultrastructural changes in foot processes in *Vash1* homozygous knockout mice were mainly caused by decreased detyrosination of α-tubulin in podocytes.

In conclusion, VASH1 is likely to be the main α-tubulin detyrosinating enzyme in glomerular podocytes. Defective α-tubulin detyrosination can lead to mild albuminuria and glomerular hypertrophy, but not overt kidney disease, at least under unstressed conditions. However, since VASH1 deficiency has been shown to exacerbate diabetes-induced albuminuria and glomerular injury, VASH1-mediated α-tubulin detyrosination may be a novel therapeutic target in some glomerular diseases.

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Conflict of interest

Jun Wada receives speaker honoraria from Astra Zeneca, Daiichi Sankyo, Novartis, Novo Nordisk Pharma, Tanabe Mitsubishi and receives grant support from Astellas, Baxter, Bayer, Chugai, Dainippon Sumitomo, Kyowa Kirin, Novo Nordisk Pharma, Ono, Otsuka, Tanabe Mitsubishi, and Teijin.

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Figure legends

Fig. 1 Detyrosinated α-tubulin in normal mouse kidney and cultured podocytes

Immunofluorescence for detyrosinated **(A)** and tyrosinated **(B)** α-tubulin in wild-type mouse kidneys (original magnification: ×100). **C)** Double immunofluorescence for glomerular detyrosinated α-tubulin (green) and podocalyxin (red) in the kidney (original magnification: ×200). In the merged image, the nuclei were stained with DAPI (blue). **D)** Double immunofluorescence for detyrosinated α-tubulin (green) and α-tubulin (red) in immortalized

human podocytes in undifferentiated and differentiated condition (original magnification: ×400). In the merged image, the nuclei were stained with DAPI (blue). **E)** Immunoblots for detyrosinated α-tubulin and α-tubulin. Each lane was loaded with 10 μg of protein obtained from the cultured podocytes.

Fig. 2 Detyrosinated α-tubulin in *Vash1* deficient mouse kidney and *VASH1* knockdown podocytes

Immunofluorescence for detyrosinated α-tubulin in glomeruli from wild-type (WT; **A**), *Vash1* heterozygous knockout (*Vash1*$^{+/-}$; **B**), and *Vash1* homozygous knockout (*Vash1*$^{-/-}$; **C**) mice (original magnification: ×400). In the lower magnification images of the kidney (original magnification: ×40), immunoreactivity for glomerular detyrosinated α-tubulin was observed as many small dots in WT mouse kidney **(D)**, whereas these dots nearly disappeared in *Vash1*$^{-/-}$ mouse kidneys **(E)**. **F)** Glomerular detyrosinated α-tubulin in *Vash2* homozygous knockout (*Vash2*$^{-/-}$) mouse kidneys (original magnification: ×400). **G)** Immunoblots for detyrosinated α-tubulin and α-tubulin in cultured podocytes treated with control siRNA (siCont) or *VASH1* siRNA (siVASH1). Each lane was loaded with 10 μg of protein obtained from cultured podocytes.

Fig. 3 Renal function and morphology in *Vash1* homozygous knockout mice

Blood urea nitrogen (BUN; **A**) and serum creatinine (**B**) in wild-type and *Vash1* homozygous knockout (*Vash1*$^{-/-}$) mice. **C)** Creatinine clearance was higher in *Vash1*$^{-/-}$ mice, but the difference was not statistically significant. **D)** Urinary albumin excretion was significantly higher in *Vash1*$^{-/-}$ mice than in wild-type mice. **E)** Representative light microscopic images of glomeruli from wild-type and *Vash1*$^{-/-}$ mice (periodic acid-Schiff

staining, original magnification: ×400). **F)** Glomerular volume was significantly higher in *Vash1*$^{-/-}$ mice than in wild-type mice. n = 5 for each group. Each column represents the mean ± standard deviation.

Fig. 4 Expression of podocyte proteins and electron microscopic alterations of podocytes in *Vash1* homozygous knockout mice

A, B) Immunofluorescence of synaptopodin in glomeruli from wild-type and *Vash1* homozygous knockout (*Vash1*$^{-/-}$) mice (original magnification: ×400). **C, D)** Immunofluorescence of nephrin in glomeruli from wild-type and *Vash1*$^{-/-}$ mice (original magnification: ×400). **E)** Representative transmission electron microscopic images of glomeruli from wild-type and *Vash1*$^{-/-}$ mice (original magnification: ×15,000). The morphometric analysis showed that the width of slit diaphragms **(F)** was significantly smaller in *Vash1*$^{-/-}$ mice, but there was no difference in the width of the foot processes **(G)**. n = 5 for each group. Each column represents the mean ± standard deviation.