Vps34 Deficiency Reveals the Importance of Endocytosis for Podocyte Homeostasis

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ABSTRACT

The molecular mechanisms that maintain podocytes and consequently, the integrity of the glomerular filtration barrier are incompletely understood. Here, we show that the class III phosphoinositide 3-kinase vacuolar protein sorting 34 (Vps34) plays a central role in modulating endocytic pathways, maintaining podocyte homeostasis. In mice, podocyte-specific conditional knockout of Vps34 led to early proteinuria, glomerular scarring, and death within 3–9 weeks of age. Vps34-deficient podocytes exhibited substantial vacuolization and foot process effacement. Although the formation of autophagosomes and autophagic flux were impaired, comparisons between podocyte-specific Vps34-deficient mice, autophagy-deficient mice, and doubly deficient mice suggested that defective autophagy was not primarily responsible for the severe phenotype caused by the loss of Vps34. In fact, Rab5-positive endosomal compartments, endocytosis, and fluid-phase uptake were severely disrupted in Vps34-deficient podocytes. Vps34 deficiency in nephrocytes, the podocyte-like cells of Drosophila melanogaster, resulted in a block between Rab5- and Rab7-positive endosomal compartments. In summary, these data identify Vps34 as a major regulator of endolysosomal pathways in podocytes and underline the fundamental roles of endocytosis and fluid-phase uptake for the maintenance of the glomerular filtration barrier.


Podocytes are specialized cells of the renal glomerulus that maintain a complex cytoarchitecture to form the glomerular filtration barrier. The concept that podocytes internalize and remove proteins was proposed over 50 years ago.1–3 Multiple electron microscopy studies have shown vesicles in podocytes under normal conditions and further pronounced under pathologic conditions.2–7 However, the physiologic relevance of podocyte uptake mechanisms remained elusive so far. Intriguingly, recent studies indicate a possible role of endocytosis for podocyte homeostasis. A fragment of the (Pro)Renin Receptor (PRR) also known as ATPase H(+)-transporting lysosomal accessory protein 2 (ATP6AP2) is functionally associated with the vacuolar (V-type) adenosine triphosphatase (v-ATPase). Conditional knockout of ATP6AP2 in podocytes leads to impaired lysosomal processing and defective autophagy accompanied by endoplasmic reticulum (ER) stress but also indicates disturbed processing of multivesicular bodies.8 Uptake or endocytosis of extracellular material in membrane-bound vesicles is crucial for a wide range of cellular functions, including antigen presentation, nutrient acquisition, clearance of apoptotic cells, pathogen entry, receptor regulation, hypertension, and synaptic transmission.9,10 Although some studies pinpointed to the requirement of endocytic processes for podocytes,11,12 the signaling crosstalk between endocytosis and signal transduction in this specialized cell population is not yet fully elucidated.
Phosphatidylinositol phosphates (PIPs) and Rab family guanosine triphosphatases coordinate cellular functions like growth, proliferation, migration, differentiation, survival, cell adhesion, or degranulation. They are key regulators of vesicle identity, formation, and trafficking. Phosphoinositide kinases (PIKs) produce second messenger molecules through phosphorylation of inositolphospholipids at the 3'-OH position of the inositol ring. Three classes of PIK are known, with vacuolar protein sorting 34 (Vps34; also called Pik3c3) representing the only known class III PI 3-kinase. Vps34 catalyzes phosphate transfer from ATP to lipid (PtdIns) and protein substrates (phosphorylation of PtdIns to PtdIns3P). It is evolutionarily conserved and was described first in yeast. In mammalian cells, Vps34 is part of a large multiprotein complex consisting of either Beclin1 and UV radiation resistance-associated gene (complex I) or early endosome antigen 1 (EEA1; complex II). As part of these distinct multiprotein complexes, Vps34 specifically produces PtdIns3(3)P to initiate autophagosome formation and regulate endocytic processes. PtdIns3P binding proteins contain a FYVE domain, a specific zinc finger domain (named after the four cysteine-rich proteins: Fab1 [yeast orthologue of PIKfyve], YOTB [uncharacterized protein YobT], Vac1 [vesicle transport protein], and EEA1 [Early Endosome Antigen 1]) that allows them to elaborate their function in cellular protein trafficking at specific localizations in cells. Concisely, Vps34 controls several vesicular trafficking processes and is required as an early regulator of vesicle docking/fusion at the endosome through the recruitment/activation of components in the PI 3-kinase signaling cascade.

Inhibition of Vps34 has been shown to result in dysfunctional autophagy, vesicular trafficking, and endocytic sorting. Conditional knockout of Vps34 in mammals, however, displays organ-specific phenotypes. Conditional knockout in neurons leads to a severe endocytic defect, whereas in liver and heart, conditional knockout of Vps34 results in dysfunctional autophagy. In humans, Vps34 (encoded by PIK3C3) has been shown to play a role in metabolic disorders, neurodegenerative diseases, and cancer. The differences between various species and cell lines in regard to Vps34 deficiency indicate a cell-specific importance of autophagic versus endosomal processes. Here, we show that Vps34 is crucial for the regulation of endocytosis-maintaining podocyte homeostasis.

RESULTS

Conditional Vps34 Depletion in Mouse Podocytes Induces Massive Proteinuria and Early Lethality

To study the podocyte-specific function of Vps34 in vivo, Vps34null mice were bred with Nphs2-cre;Vps34fl/fl mice to generate podocyte-specific Nphs2-cre;Vps34fl/fl mice and Vps34null litters of controls (Figure 1A). Efficient depletion of Vps34 was confirmed by Western blot analysis of lysates of freshly isolated podocytes (Figure 1B) and immunofluorescence staining of Nphs2-cre;Vps34fl/fl:tomato+/−EGFP primary podocytes and wild-type controls (Figure 1C). Co-labeling immunofluorescence with the early endosomal marker Rab5 and Vps34 on kidney sections of conditionally Vps34-deficient mice and wild-type controls indicated efficient deletion of Vps34 and a massive accumulation of Rab5 in Vps34-deficient podocytes at 3 weeks of age (Figure 1D, white arrowheads). Tubular Rab5 expression and colocalization with Vps34 are shown in Supplemental Figure 1. Colocalization of Rab5 and Vps34 were quantified using ImageJ software. although Nphs2-cre;Vps34fl/fl mice seemed normal at birth, they developed early-onset proteinuria at 3 weeks of age (Figure 1E) and significant growth retardation at week 4 (Figure 1F and G). Nphs2-cre;Vps34fl/fl mice all died within 3–9 weeks after birth (Figure 1H).

Podocyte-Specific Vps34 Deficiency Causes Rapid Podocyte Degeneration and Early-Onset Glomerulosclerosis

On postnatal day 7, glomeruli of Nphs2-cre;Vps34fl/fl mice were structurally indifferent from glomeruli of littermate controls (Figure 2A and Supplemental Figure 2A). They showed equal distribution of the slit diaphragm proteins nephrin and podocin (Supplemental Figure 3, A and B) and equal numbers of primary podocytes and wild-type controls (Figure 1C). Co-localization of Rab5 and Vps34 were quantified at birth, they developed early-onset proteinuria at 3 weeks of age (Figure 1E) and significant growth retardation at week 4 (Figure 1F and G). Nphs2-cre;Vps34fl/fl mice all died within 3–9 weeks after birth (Figure 1H).

Impairment of Autophagy in Nphs2-cre;Vps34fl/fl Mice

One of the reported functions of Vps34 in intracellular vesicle transport is the induction of autophagy on nutrient deprivation. Immunofluorescence staining and Western blot analyses showed increased levels of Lamp1 and -2 in Nphs2-cre;Vps34fl/fl mice, indicating insufficient lysosomal degradation (Figure 3, B and E).
During induction of autophagy, microtubule-associated protein 1 light chain 3A (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II, which is subsequently recruited to form autophagosomes. Immunofluorescence staining and Western blot analysis identified increased LC3-I and -II levels in Nphs2-cre;Vps34fl/fl conditional knockout mice (Figure 3, D–F), indicating that LC3 conjugation can still occur in the absence of Vps34. In agreement with insufficient autophagosome formation, we observed substantial increase of polyubiquitin binding protein p62/SQSTM1 (referred to as p62) in podocytes of Nphs2-cre;Vps34fl/fl mice (Figure 3, A and F). p62
identifies toxic cellular waste that is usually consumed through autophagy. Accumulation of p62 induces a cellular stress response, confirmed by an accumulation of the ER marker Calnexin (Figure 3C). Confocal imaging analysis showed that LC3 did not colocalize with Lamp2 in Vps34-deficient podocytes (Figure 3E). This finding indicates a defect in autolysosomal formation. To further define the localization of LC3 in the podocytes, we performed immunogold–electron microscopy analyses (Supplemental Figure 4). Anti-LC3 gold particles were present on autophagosomal membranes in tubular cells in Nphs2-cre;Vps34fl/fl mice and littermate controls (Supplemental Figure 4A, i and B, i). However, Vps34-deficient podocytes exhibited a diffuse cytosolic distribution of LC3 lacking any association with membrane structures, which is indicative of a defect in early autophagosome formation (Supplemental Figure 4B). Consistently, the large-sized cargo-containing vacuoles of Vps34-deficient podocytes were not labeled with anti-LC3 gold particles and thus, do not represent autophagosomes.
Figure 3. Autophagy is impaired in Nphs2-cre;Vps34flo/flo mice. Immunofluorescence staining of kidney sections of 3-week-old Nphs2-cre;Vps34flo/flo and littermate control mice. (A–E) Quantification of confocal immunofluorescence microscopy and Western blot analyses of glomerular or primary podocyte protein lysates displayed significant accumulation of the autophagy marker p62, the lysosomal markers Lamp1 and -2, the ER stress marker Calnexin, and the autophagy marker LC3 in podocytes of Nphs2-cre;Vps34flo/flo mice. (E) Confocal microscopy showed no significant colocalization of LC3 and the lysosomal marker Lamp2 in Vps34-deficient podocytes. (F) Colocalization of LC3 and p62 in Vps34-deficient podocytes in confocal microscopy. *P<0.05, **P<0.01, ***P<0.001. Scale bars, 20 μm; 5 μm in detail.
Abrogated Autophagic Flux Is Not Causative of the Severe Podocyte Phenotype of Vps34-Deficient Podocytes

To study the dynamic cellular mechanisms of Vps34, we generated primary podocyte cell cultures (Figure 4, A and B and Supplemental Figure 6). Vps34\(^{-/-}\) mice were bred to Nphs2-Cre;tomato\(^{fl+}\)–EGFP mice to generate podocyte-specific GFP-positive Vps34-deficient mice (Figure 4A and Supplemental Figure 5A). \(^{36}\) Nphs2-cre;Vps34\(^{fl-}\);tomato\(^{fl+}\)–EGFP primary podocytes displayed substantial perinuclear vacuolization (Figure 4B) and a significant reduction in the total cell count after 9 days (Supplemental Figure 6A) compared with GFP-positive control podocytes. To further differentiate and quantify diminished proliferation versus increased cell death in Vps34-deficient podocytes, we isolated primary podocytes of conditionally Vps34-deficient mice and littermate controls and stained cells with Ki-67 as well as cleaved caspase 3 (Supplemental Figure 6, B–E). Isolated primary podocytes from young mice proliferated for one to two cycles and thus, behaved differently from a podocyte in vivo. In vitro, primary wild-type podocytes displayed enhanced Ki-67 staining, whereas Vps34-deficient podocytes did not show signs of proliferation. Active caspase 3 staining, however, suggested increased apoptosis in Vps34-deficient cultured podocytes. To investigate diminished proliferation and increased apoptosis in vivo, we stained sections from 1-, 3-, and 9-week-old mice for Ki-67 and cleaved caspase 3 (Supplemental Figure 6, F–I). Podocytes were costained with wild type 1.

In vivo, Ki-67 staining indicated that proliferation activity of neither wild-type nor Vps34-deficient podocytes was present at different time points. Active caspase 3 staining, however, was increased in all glomerular cell types of 9-week-old Nphs2-cre;Vps34\(^{fl-}\) mice. At early stages of beginning glomerulosclerosis, however, active caspase 3 staining was indifferent from wild type.

To determine if the extensive perinuclear vacuoles seen in Vps34-deficient podocytes represented lysosomal accumulation, vesicles were counterstained with Lysotracker Red (Figure 4B). Equal to our observations in kidney sections of Nphs2-cre;Vps34\(^{fl-}\) mice and littermate controls (Figure 3, A and D–F), LC3 and p62 accumulated in Nphs2-cre;Vps34\(^{fl-}\);tomato\(^{fl+}\)–EGFP podocytes (Figure 4C), which is characteristic for a block in autophagy. To verify impaired autophagic flux, Nphs2-cre;Vps34\(^{fl-}\);tomato\(^{fl+}\)–EGFP primary podocytes and wild-type controls were serum-starved in the presence or absence of 10 \(\mu\)M chloroquine (Figure 4, E and F). Chloroquine increases the lysosomal pH, which leads to inhibition of autophagosome–lysosomal fusion and impaired lysosomal protein degradation. Chloroquine treatment of wild-type control cells led to an accumulation of LC3-II over time on starvation representative for autophagic flux (Figure 4, E and F). Nphs2-cre;Vps34\(^{fl-}\);tomato\(^{fl+}\)–EGFP primary podocytes, however, already showed high LC3-II levels under normal conditions (Figure 4, E and F) and showed only a little additional increment on treatment with chloroquine (Figure 4, E and F), confirming that autophagic flux is largely abrogated in Vps34-deficient podocytes.

To answer the question of whether autophagy is the main underlying reason for the severe phenotype observed in Nphs2-cre;Vps34\(^{fl-}\) mice, we generated podocyte-specific Nphs2-cre;Atg5\(^{fl-}\) mice, Nphs2-cre;Vps34\(^{fl-}\) mice, and Nphs2-cre;Atg5\(^{fl-}\);Vps34\(^{fl-}\) double conditional knockout mice (Figure 5A) and compared functional data (Figure 5B). Atg5 is essential for the extension and completion of autophagosomes. Our earlier studies showed that specific disruption of autophagy in podocytes by deletion of Atg5 leads to late-onset proteinuria and glomerulosclerosis. \(^{37}\) Nphs2-cre;Atg5\(^{fl-}\) mice showed no signs of proteinuria, weight loss, or histologic aberration within the first 5 weeks of observation (Figure 5, B–D). Double deficient Nphs2-cre;Atg5\(^{fl-}\);Vps34\(^{fl-}\) mice exhibited an analogous phenotype to Nphs2-cre;Vps34\(^{fl-}\) single conditional knockout mice (Figure 5B). Impaired autophagy, shown here by increased LC3 deposits and p62 accumulation (Figure 5, C and D), was similar in Nphs2-cre;Vps34\(^{fl-}\) single conditional knockout mice and Nphs2-cre;Atg5\(^{fl-}\);Vps34\(^{fl-}\) conditional double knockout mice (Figure 5, C and D). The late-onset generation of podocytes because of single Atg5 deficiency is, thus, both histologically and phenotypically entirely distinct from the degeneration caused by Vps34 deficiency (Figure 5, B–D).

Ablation of Vps34 in Mouse Podocytes Leads to Impaired Endocytosis

Endocytosis of extracellular material in membrane-bound vesicles is crucial for a wide range of cellular functions. Although there is evidence that endocytosis is important for podocytes, \(^{8,11,12}\) the signaling crosstalk between endocytosis and signal transduction in this specialized cell population is still largely unacquainted. Vps34 is implicated in the regulation of intracellular vesicle transport at numerous key positions. \(^{17}\) Because the late-onset degeneration of podocytes caused by Atg5 deficiency is histologically and phenotypically utterly distinct from the degeneration caused by Vps34 deficiency, we hypothesized that, in podocytes, Vps34 deficiency leads to defects in endolysosomal pathways. To study endolysosomal trafficking in Vps34-deficient podocytes, we examined early and late endosomal compartments and PI(3)P-dependent endolysosomal fusion (Figure 6). Rab5 recruits Vps34 to the early endosome to secure the local production of PI(3)P. The presence of PI(3)P is required for the fusion between endocytic vesicles and early endosomes by the recruitment of a subset of proteins (e.g., EEA1). \(^{38,39}\) Similar to the large vacuoles observed in Vps34-deficient mouse podocytes (Figure 2D and Supplemental Figures 2B and 4), Rab5 deficiency causes giant endosomes in cells. \(^{40}\) Vps34-deficient podocytes showed substantial accumulation of Rab5 (Figure 6A). In contrast, Vps34-deficient podocytes displayed no significant differences of the late endosomal marker Rab7 compared with littermate controls (Figure 6B), indicative of a block in endosomal maturation upstream of the late endosomal compartment. To functionally address the question of impaired fluid-phase endocytosis in Vps34-deficient podocytes, we performed an FITC-dextran uptake assay (Figure 6, C and D). Serum-starved Nphs2-cre;Vps34\(^{fl-}\);tomato\(^{fl+}\)–EGFP primary podocytes and wild-type controls were stimulated with...
EGF in the presence of fluorescent-labeled dextran to monitor endosome/macropinosome formation (Figure 6, C and D). In the absence of EGF, dextran was internalized into a few endosomes of variable sizes (Figure 6, C and D). The addition of EGF induced a marked stimulation of dextran uptake at 5 minutes after EGF addition (Figure 6, C and D).

Figure 4. Autophagic flux is abrogated in primary Vps34-deficient podocytes. (A) Nphs2-cre;tomato<sup>/+</sup>/EGFP mice were crossed with Vps34<sup>fl/fl</sup> and Vps34<sup>+/+</sup> mice to obtain Vps34-deficient, GFP-positive podocytes and GFP-positive control podocytes. (B) Primary podocytes at passage 1 were observed by phase/contrast microscopy for 9 days. Vps34-deficient podocytes showed substantial perinuclear vacuolization. Counterstaining with Lysotracker Red was performed to determine if the vacuoles seen in Vps34-deficient podocytes represent accumulation of lysosomal acidic vesicles. Scale bars, 20 µm. (C) Immunofluorescence staining showed massive accumulation of LC3 and p62 in Nphs2-cre;Vps34<sup>fl/fl</sup>;tomato<sup>+/−</sup>/EGFP podocytes. Scale bars, 20 µm. (D-F) Autophagic flux was impaired in Nphs2-cre;Vps34<sup>fl/fl</sup>;tomato<sup>+/−</sup>/EGFP primary podocytes. Serum deprivation induced LC3 accumulation in wild-type GFP-positive primary podocytes that was further enhanced by blockade of autophagosomal–lysosomal fusion with the lysosomal inhibitor chloroquine (10 µM). In contrast, LC3 was already accumulated in Vps34-deficient primary GFP-positive podocytes with no further increase on serum starvation or disruption of autophagosomal–lysosomal fusion by chloroquin. **P<0.01. Scale bars, 50 µm.

EGF in the presence of fluorescent-labeled dextran to monitor endosome/macropinosome formation (Figure 6, C and D). In the absence of EGF, dextran was internalized into a few endosomes of variable sizes (Figure 6, C and D). The addition of EGF induced a marked stimulation of dextran uptake at 5 minutes after EGF addition (Figure 6, C and D). Nphs2-cre;Vps34<sup>fl/fl</sup>;tomato<sup>+/−</sup>/EGFP primary podocytes, however, showed impaired uptake of dextran in the presence of EGF, indicating disturbed fluid-phase uptake (Figure 6, C and D). Coseconding with the early endosomal marker Rab5 revealed significant overlap with dextran in wild-type podocytes, whereas in Vps34-deficient primary podocytes, accumulated Rab5 did not colocalize with fluorescent-labeled dextran. To functionally address other forms of endocytosis, we evaluated receptor-mediated streptavidin uptake in Vps34-deficient and wild-type primary podocytes (Figure 6E). Serum-starved Nphs2-cre;Vps34<sup>fl/fl</sup>;tomato<sup>+/−</sup>/EGFP primary podocytes and wild-type controls were stimulated with biotinylated EGF in the presence of fluorescent-labeled streptavidin (Figure 6E). In the absence of EGF, streptavidin was internalized into a few endosomes of variable sizes. The addition of biotinylated EGF induced a marked stimulation of streptavidin uptake at 30 minutes after the addition of biotinylated EGF. Nphs2-cre;Vps34<sup>fl/fl</sup>;tomato<sup>+/−</sup>/EGFP primary podocytes, however, showed impaired uptake of streptavidin in the presence of EGF.
Figure 5. Abrogated autophagic flux is not causative for the severe podocyte phenotype of Vps34-deficient podocytes. (A) Vps34<sup>fl/fl</sup> mice were crossed to Nphs2-cre;Atg5<sup>fl/fl</sup> mice to obtain Nphs2-cre;Atg5<sup>fl/fl</sup>;Vps34<sup>fl/fl</sup> conditional double knockout mice. (B) Albumine/creatinine ratios were significantly increased from 3 weeks of age in Vps34<sup>fl/fl</sup>;Nphs2-cre and Nphs2-cre;Atg5<sup>fl/fl</sup>;Vps34<sup>fl/fl</sup> conditional double knockout mice compared with Nphs2-cre;Atg5<sup>fl/fl</sup> mice and littermate controls (n=10 per group, *P<0.05, **P<0.01, two-tailed t test, mean values ± SEM are shown). (C) Confocal microscopy showed accumulation of LC3 and Lamp2 in podocytes of Nphs2-cre;Vps34<sup>fl/fl</sup> single mutant mice and Nphs2-cre;Atg5<sup>fl/fl</sup>;Vps34<sup>fl/fl</sup> conditional double knockout mice. No significant colocalization of


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biotinylated EGF, indicating disturbed receptor-mediated endocytosis. Costaining with the early endosomal marker Rab5 indicated a significant overlap with streptavidin in wild-type podocytes, whereas Rab5 did not colocalize with fluorescent-labeled streptavidin in Vps34-deficient primary podocytes.

**Vps34 Is an Important Regulator of the Early Endosomal Compartment in the Podocyte-Like Drosophila Cell—the Nephrocyte**

To test whether these fundamental Vps34-mediated endocytosis mechanisms are a general theme of filtering cells, we analyzed podocyte-like cells in *Drosophila melanogaster*. Garland cell nephrocytes (GCNs) are highly endocytic active so-called storage kidneys of *D. melanogaster* (Figure 7A). Immunofluorescence staining with Rabenosyn5, a marker for the early endosome, shows the characteristic ring-like pattern of early endosomes in wild-type controls (Figure 7). Underneath this ring, late endosomes can be visualized by immunofluorescence staining with anti-Rab7 antibodies (Figure 7, A and B). On knockdown of Vps34, this pattern was utterly abrogated, and the Rabenosyn5-positive early endosome compartment was significantly extended (Figure 7, B and C), characteristic for a complete early endosomal block in nephrocytes comparable with the observations in Vps34-deficient podocytes (Figure 6).

**DISCUSSION**

The podocyte may be the most complicated and intricate cell in the kidney, responsible for maintaining the glomerular filtration barrier and the glomerular basement membrane. The presence of endocytic vesicles within the podocyte is one of the earliest observations in electron microscopy of the glomerulus.1 Marylin Farquhar and other investigators have hypothesized that these bodies represent transcytotic vesicles of primary urine or protein reabsorption droplets.41–43 Large foot process phagosomes have been identified removing subepithelial immune deposits and giant protamine heparin aggregates.44,45 More recently, it was shown that defects in multivesicular body formation were associated with increased susceptibility to glomerular disease, suggesting the importance of an intact endocytosis machinery for the integrity of the glomerular filter.46 However, the mechanisms and the intracellular pathways of endocytosis in podocytes had remained unclear. Recently, the type 2 phosphoinositide 3-kinase PI3KC2-α has been shown to play a role in glomerular structure, suggesting a function for podocyte maintenance.47 Here, we used a podocyte-specific Vps34-deficient mouse model to identify the fundamental role of endocytosis for podocyte homeostasis. Through the localized phosphorylation of phosphatidylinositol to phosphatidylinositol-3-phosphate (PI[3]P), Vps34 has a unique function in several vesicular trafficking processes and early vesicle docking/fusion at the endosome.17 It recruits downstream effectors that contain PI[3]P binding motif, such as a FYVE domain (a specific zinc finger domain named after the four cysteine-rich proteins: Fab 1, YOTB, Vac 1 and EEA1) or a PX domain (a phosphoinositide-binding structural domain involved in targeting of proteins to cell membranes), components in the PI 3-kinase signaling cascade to regulate membrane trafficking processes, such as endocytosis and autophagy.20–22,48 Furthermore, Vps34 plays a crucial role in endosome tethering and membrane fusion through EAA1 and other Rab5 effectors, vesicle invagination and cargo selection within multivesicular bodies, and fusion of autophagosomes with lysosomes.23–26

The first study of conditional Vps34 knockout mice described a disruption of endolysosomal pathways in mammalian neurons. Zhou et al.30 observed that, similar to Vps34-deficient podocytes, large-diameter Vps34-deficient sensory neurons showed dramatic defects in endolysosomal pathways leading to rapid degeneration within 5–9 days because of an accumulation of atypical vacuoles and endosomes. However, in mouse liver and heart, depletion of Vps34 shows rather mild phenotypes, similar to the phenotypes seen in autophagy-deficient mice.31,37 Similar to these mice, autophagy is impaired in Vps34-deficient podocytes, and autophagic flux is abrogated. Interference with Vps34 was found to inhibit mammalian target of rapamycin (mTOR) activation. Vps34 can function in a nutrient-sensing pathway upstream of mTOR in several mammalian cell lines, indicating a role for Vps34 in mTOR signaling.49,50,51 Interestingly, Cinà et al.52 have recently shown that deletion of mTOR in podocytes resulted in glomerulosclerosis as a result of decreased autophagic flux. Intriguingly, however, podocyte-specific ablation of Vps34 in mice leads to severe proteinuria and early-onset glomerulosclerosis, showing an entirely distinct phenotype from podocytes that are unable to execute autophagy. We showed that specific disruption of macroautophagy in podocytes by deletion of Atg5 leads to accelerated aging, late-onset proteinuria, and glomerulosclerosis.52 Conditionally, Vps34-deficient mice, however, developed large-sized vacuoles and unprocessed endosomes within the podocyte cytoplasm, resulting in rapid podocyte degeneration and death within 3–9 weeks. The late-onset degeneration of podocytes caused by Atg5 deficiency is, thus, both histologically and phenotypically entirely distinct from the degeneration caused by Vps34 deficiency, indicating that the lack of autophagy is not the main reason underlying the rapid degeneration of Vps34-deficient podocytes (Figure 8). Because of the disparities in Vps34- and autophagy-deficient mice, we hypothesized that Vps34-mediated endocytosis...
Figure 6. Ablation of Vps34 in mouse podocytes leads to impaired endocytosis. (A and B) Confocal immunofluorescence microscopy of kidney sections of 3-week-old Nphs2-cre;Vps34fl/fl and littermate control mice. (A) Rab5, a marker for early endosomes and activator of Vps34, was strongly increased in Nphs2-cre;Vps34fl/fl podocytes at 3 weeks of age. Scale bars, 5 μm. (B) Rab7, a marker for the late endosome, showed no significant differences in podocytes of Nphs2-cre;Vps34fl/fl and Vps34fl/fl mice. Scale bars, 5 μm. (A and B)
might be of particular importance for podocyte maintenance. In fact, the observed disruption of early endosomal sorting in Vps34-deficient podocytes as well as Vps34-deficient D. melanogaster nephrocytes suggests a block of endocytosis at the level of early endocytosis (Figure 8). A large number of distinct endocytic pathways exist to allow the internalization of nutrients, solutes, and growth factors. The disruption at the early steps of these endocytosis mechanisms is likely to abrogate podocyte-specific functions. To this end, it is not clear which exact subset of endocytic mechanisms is mostly affected in podocyte physiology. Several studies have previously shown that growth factor pathways are significantly regulated by endocytosis of their respective receptors. Hence, essential growth factor signaling axes, such as vascular EGF and EGF pathways, might be affected by the disruption of Vps34-controlled endocytosis in podocytes. Dysregulation of vascular EGF expression within the glomerulus has been shown in a wide range of primary and acquired renal diseases.

In our study, we observed an almost complete abrogation of receptor-mediated endocytosis in Vps34-deficient podocytes, emphasizing the importance of receptor-mediated endocytosis for growth factor signaling. Interestingly, we also detected fluid-phase uptake to be seriously compromised in Vps34-deficient podocytes. Larger dextrans are known to be endocytosed through fluid-phase uptake rather than receptor-mediated endocytosis, suggesting that Vps34 deficiency likewise leads to a failure of fluid-phase uptake in podocytes. Because some of the vesicles that can be observed in wild-type podocytes in vivo seem to be nonclathrin-coated, it can be speculated that these vesicles might represent micro- or macropinosomes representing an active podocytic fluid-phase uptake. We speculate that our data point to the importance of receptor-mediated endocytosis and fluid-phase uptake for the maintenance of the filtration barrier. Endocytotic mechanisms are highly conserved features of filtering cells. This result is highlighted by our observation that nephrocytes, podocyte-like cells of D. melanogaster, show an equally severe disruption of the early endosomal compartment on Vps34 knockdown as Vps34-deficient podocytes.

Vps34 deficiency in podocytes as well as nephrocytes disrupts active vesicle transport at early stages of endocytosis, causing an increase in unprocessed early endosomes, lysosomes, and impaired endolysosomal fusion, which ultimately results in mislocalization of proteins and ER stress. It is important to note that the alterations in vesicular trafficking pathways in Vps34-deficient podocytes were already detectable at week 1 after birth (accumulation of Rab5, Lamp1/2, and LC3) before a significant phenotype. At this early time point, no functional or histologic changes were yet evident, and secondary effects on cell homeostasis caused by proteinuria can, therefore, be excluded.

Here, we provide first evidence that endocytosis is a key mechanism in podocyte homeostasis. Lack of autophagy is not the major reason underlying the rapid degeneration of Vps34-deficient podocytes. Instead, the massive vacuolization and rapid degeneration of Vps34-deficient podocytes that is entirely distinct from autophagy-deficient podocytes point to an early block in endocytic pathways in podocytes caused by impaired vesicle fusion and maturation. Additional investigation is needed to unravel which extracellular and intracellular signals trigger endolysosomal trafficking for podocyte homeostasis.

**CONCISE METHODS**

**Mice**

Vps34fl/fl mice (129/B6 mixed background) were a gift from F. Wang, Duke University. Nphs2-cre mice (C56BL/6 background) and Atgs3fl/fl mice (C56BL/6 background) were previously described. STOCK Gt (ROSA)26Sor14STv1/ACTB-Atdomino-EGFP/LacZ129/SvJ mice were purchased from JAX (strain of origin: 129X1/SvJ×129S1/Sv). Vps34fl/fl mice were bred with Nphs2-cre/+ mice to generate podocyte-specific Vps34 knockout mice (referred to as Nphs2-cre;Vps34fl/fl). The podocyte-specific expression of Cre recombinase excises the ATP binding domain of the kinase (encoded by exons 17 and 18) in Vps34fl/fl mice to create a conditional mutant allele resulting in a truncated functionally inactive protein if expressed. Vps34fl/fl littermates served as controls. Nphs2-cre;tomato>EGFPfl/+ mice were crossed with Vps34fl/fl mice to obtain Nphs2-cre;Vps34fl/fl;tomato-EGFPfl/+ mice for the isolation of primary podocytes. Vps34fl/fl mice were bred with Nphs2-cre+;Atg5fl/fl mice to generate podocyte-specific Vps34fl/fl;Atg5fl/fl conditional double knockout mice. All animal studies were approved by the Committee on Research Animal Care, Regierungspräsidium Freiburg.

**Functional Analysis of Podocyte-Specific Vps34-Deficient Mice**

Urinary albumin and creatinine were measured at postnatal day 1 and then, one time per week from week 1 to week 9 using mouse albumin-specific Western blot analysis of glomerular protein lysates of 3-week-old mice shows that the early endosomal marker Rab5 is accumulated in glomerular lysates of Nphs2-cre;Vps34fl/fl mice compared with littermate controls. No changes in the late endosomal marker Rab7 can be observed. β-actin was used as loading control. (C and D) Immunofluorescence and quantification of fluid-phase uptake in primary podocytes. Stimulation with 20 nM EGF for 5 minutes induced uptake of dextran in GFP-positive primary control podocytes. In contrast, Vps34-deficient primary GFP-positive podocytes showed impaired uptake of dextran, indicating a blockade in endocytosis. Scale bars, 50 μm. (D) Costaining with Rab5 revealed significant overlap in wild-type podocytes, whereas in Vps34-deficient primary podocytes, Rab5 accumulation did not colocalize with dextran. (E) Immunofluorescence and quantification of streptavidin uptake in primary podocytes. Stimulation of GFP-positive primary wild-type podocytes with 20 nM biotinylated EGF induced uptake of streptavidin coupled to Alexa Fluor 555. In contrast, Vps34-deficient primary GFP-positive podocytes showed impaired uptake of streptavidin, indicating a blockade in receptor-mediated endocytosis. Scale bars, 50 μm. Costaining with Rab5 revealed significant overlap in wild-type podocytes, whereas in Vps34-deficient primary podocytes, Rab5 accumulation did not colocalize with streptavidin. **P<0.01, ***P<0.001, ****P<0.0001.
Mikrofloral Mikroalbumin Test; Progen) and creatinine kits (Creatinine PAP LT-SYS, Labor&Technik; Eberhard Lehmann GmbH) according to the manufacturer’s instructions. Albumin/creatinine ratio was calculated and expressed as milligrams albumin/milligrams creatinine.

Histologic Analysis
Kidneys from 1-, 3-, and 9-week-old Nphs2-cre;Vps34fl/fl and littermate control mice were dissected, fixed in 4% paraformaldehyde, and embedded in paraffin or Lowicryl K4M resin (Electron Microscopy Sciences). Tissues were further processed for periodic acid–Schiff (PAS) staining or electron microscopy, respectively. For PAS staining, 3-μm sections were cut on a Leica microtome and analyzed and photographed with an Axioplan 2 microscope (Zeiss) and an AxioCam camera (Zeiss).

Semiquantitative Histological Analyses
Severity of glomerulosclerosis was evaluated using an index score that includes the percent of glomeruli showing sclerosis and the extension of the glomerulosclerosis within the glomeruli. Glomeruli were graded from 0 to +4: grade 0, normal; grade 1, <25% involvement of the glomerular tuft; grade 2, 25%–50% involvement of the glomerular tuft; grade 3, 50%–75%; grade 4, sclerosis occupying >75% of the glomerular tuft. The glomerulosclerosis score was obtained as follows: (1×number of

Figure 7. Vps34 is an important regulator of the early endosomal compartment in the podocyte-like Drosophila cell—the nephrocyte. (A) Combined schematic of electron microscopy and confocal immunofluorescence microscopy of GCNs. (a–d) Structural comparison of (a) GCN with (b) podocytes and (c) nephrocyte diaphragm with (d) slit diaphragm. The rough surface of the GCN underneath the (a) basement membrane is formed by the (c) nephrocyte diaphragm. In contrast to the mammalian slit diaphragm, which is formed between (b) neighboring podocytes, (a) the Drosophila nephrocyte diaphragm is formed within one GCN. The illustration shows the endocytosis process by formation of early endosomes from invaginations of the nephrocyte diaphragm and their maturation to late endosomes. (B) Confocal immunofluorescence microscopy of Vps34-deficient and wild-type control GCNs. The control shows the thin outer ring of Rabenosyn5-positive early endosomes (red) and the inner ring of Rab7-positive late endosomes (green). On expression of Vps34RNAi, this pattern is disrupted, and the whole cytoplasm is filled with Rabenosyn5-positive endosomes. Control: prospero GAL4/+; Vps34RNAi: prospero-GAL4/upstream activating sequence-Vps34-RNAi(2). Scale bars, 5 μm. (C) Distribution profile of the early endosomal marker Rabenosyn5.
Figure 8. Schematic illustration of Vps34 deficiency in podocytes. (A) Vps34 deficiency leads to incomplete formation of the autophagosomal membrane, resulting in deficient autophagy and autophagosomal fusion. LC3 can still be converted from LC3-I to -II, but no functional autophagosome is formed. It leads to an accumulation of nondegraded LC3 and p62 as well as an accumulation of vacant lysosomes and accumulation of Lamp1/2. The absence of PI(3)P causes a blockade in autophagosomal formation and autophagosomal–lysosomal fusion. (B) Endosomal trafficking is blocked in Vps34-deficient podocytes. Lack of PI(3)P production inhibits fluid-phase uptake, receptor-mediated endocytosis, and maturation of the early endosome to the late endosome, resulting in an accumulation of Rab5. Rab7, a marker for the late endosome, is not affected. Lamp1/2, markers for the lysosome, are upregulated, indicating unused lysosomes because of insufficient endolysosomal fusion.
glomeruli with +1)+2×number of glomeruli with +2)+3×number of glomeruli with +3)+4×number of glomeruli with +4)/total number of glomeruli examined.59

Quantification of Foot Process Effacement
We performed quantification of foot process effacement. Data are means ± SEM. **P<0.01. Random electron microscopy images were used for morphometric analysis of foot process effacement. Three glomeruli of n=3 mice each were evaluated. Electron images were analyzed using ImageJ software. The quantification of foot process effacement was adapted from van den Berg et al.60 Briefly, from each picture, the mean width of the foot processes (FPWs) was calculated according to the following formula: 

\[ FPW = \pi/4 \times (\Sigma \text{glomerular basement membrane length} / \Sigma \text{foot process}) \]

A foot process was defined as any connected epithelial segment butting on the basement membrane between two neighboring filtration slits.

Immunogold Electron Microscopy
Kidneys of 3-week-old Nphs2-cre;Vps34fl/fl mice and control littermates were perfused transaortically with 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 phosphate buffer. Kidneys were removed and postfixed in the same fixative (overnight at 4°C). Tissues were washed in PBS, and then, sections (50 μm) were cut on a vibratome and cryoprotected in a solution containing 25% sucrose and 10% glycerol in 50 mM PBS. The sections were freeze-thawed and incubated in blocking solution containing 2% normal goat serum in 50 mM Tris-buffered saline for 1 hour followed by incubation with an anti-LC3 antibody (24 hours at 4°C; Cell Signaling). After the sections were washed, the sections were incubated with 1.4-nm gold-coupled goat anti-mouse secondary antibody (1:100, Nanogold; Nanoprobes, Stony Brook, NY) for immunogold reaction. Immunogold labeling was then enhanced with the HQ Silver Kit (Nanoprobes). After the sections were treated with OsO4, the sections were stained with uranyl acetate, dehydrated, and flat-embedded in epoxy resin (Durcupan ACM, Fluka; Sigma-Aldrich, Gillingham, United Kingdom). Ultrathin sections were cut and analyzed using a Philips CM 100 electron microscope.

Immunofluorescence Staining of Kidney Sections
Kidneys were snap-frozen in cryogenic Tissue-Tek O.C.T. compound (Electron Microscopy Science). The embedded tissue was sectioned at 6 μm with a Leica cryostat (Leica). The sections were fixed with 4% paraformaldehyde, blocked with PBS containing 5% BSA, and incubated for 1 hour with primary antibodies. After three PBS rinses, fluorophore-conjugated Alexa secondary antibodies (Invitrogen) were applied for 30 minutes. Confocal microscopy and acquisition of images were performed using a Zeiss laser scan confocal microscope. To determine the number of podocytes per glomerulus, kidney sections were stained with the podocyte nuclear marker WT1. WT1-positive cells were counted in 100 glomeruli per mouse (n=3 for Nphs2-cre;Vps34fl/fl and littermate controls).

Quantification of Immunofluorescence and Colocalization
Integrated density and corrected total cell fluorescence was measured using ImageJ software. Ten eight-bits images each were analyzed. Pearson’s correlation coefficient was calculated using ImageJ software (1=perfect colocalization, 0=no colocalization). Three independent experiments were conducted, and the percent inhibition of streptavidin endocytosis was calculated. The cells were classified as cells internalizing avidin or cell not internalizing avidin. The percent inhibition represents the ratio of noninternalizing cells to the total number of cells.

Antibodies
Antibodies were obtained from Abcam (anti-WT1 rabbit pAb, ab15249; anti-Lamp1 rabbit mAb, ab24170; anti-Lamp2 rabbit mAb, ab37024; anti-Rab5 rabbit pAb, ab13253; anti-Rab7 mouse mAb, ab50533), Cell Signaling Biotechnology (anti-LC3B rabbit pAb, 2775; anti-Calnexin rabbit pAb, 2433), Epitomics (anti-Vps34 rabbit mAb, 3838–1), MBL (anti-LC3 mouse mAb, M152–3), Progen (anti-nephrin guinea pig pAb, GP-N2; anti-p62 guinea pig pAb, GP62-C), Santa Cruz Biotechnology (anti-Rab5 mouse mAb, sc-46692), Sigma-Aldrich (antipodocin rabbit pAb, P0372; anti-β-actin mouse mAb, A5441), and Enzo (anticalnexin rabbit pAb, ADI-SPA-860). Secondary antibodies and nuclear staining reagents were obtained from Invitrogen. For Western blotting, goat anti-rabbit or antimouse IgG–horseradish peroxidase (HRP) secondary antibodies were used for the above primary antibodies (anti-rabbit IgG, HRP-linked antibody, 7074; Cell Signaling; anti-mouse IgG, HRP-linked antibody, P0447; Dako). Antibodies for D. melanogaster experiments, rabbit anti-Rab7 and rat anti-Rabenosyn5, were a gift from A. Nakamura, RIKEN Center for Developmental Biology, Japan.

Isolation of Mouse Glomeruli and Primary Podocytes
Glomeruli were isolated from 10-day-old triple transgenic Nphs2-cre;Vps34fl/fl;tomatofts->EGFP and littermate control mice and sieved through sieves with decreasing pore sizes (100, 70, and 40 μm). For the isolation of primary podocytes, glomeruli were plated on Collagen IV-coated cell culture dishes. After 4 days, glomerular cells were FACs-sorted for green fluorescent protein (GFP) positive cells. For Western blot analyses, glomeruli were glass–glass homogenized in lysis buffer (containing 20 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate and 1% Triton X-100)61 and centrifuged at 15,000×g for 15 minutes at 4°C.

Western Blotting
Mouse glomeruli were isolated by either graded sieving or magnetic bead isolation. The proteins were extracted from the isolated glomeruli, lysed in lysis buffer containing EDTA, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, sodium orthovana-date, and protease inhibitors, and quantified by Lowry protein assay (Bio-Rad). Equal amounts of protein samples (25 μg/lane) were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA and incubated overnight at 4°C with primary antibodies as indicated (Concise Methods, Antibodies). The membranes were then incubated with the appropriate secondary antibodies (Concise Methods, Antibodies). After extensive washing in Tris-buffered saline with Tween20, the proteins were visualized using chemiluminescence reagents and exposed to film. For the quantitative analysis, the blots were scanned,
and the relative density of each band was calculated and normalized to the density of β-actin using LabImage software.

**Primary Podocyte Cell Culture**
Primary isolated podocytes from Nphs2–cre;Vps34fl/fl;tomato<sup>Wts</sup>–EGFP and littermate control mice were cultured in collagen IV-coated tissue culture flasks (Nunc) in RPMI supplemented with penicillin/streptomycin, FCS (Life Technologies), and insulin-transferrin-selenium (Roche Applied Science) at 37°C in 95% O<sub>2</sub> and 5% CO<sub>2</sub>. For immunofluorescence staining, cells were cultured on collagen IV-coated glass coverslips.

**Autophagy Flux Assay**
For autophagy flux assays, medium was removed and replaced with serum-free RPMI for 6 hours before the cells were incubated with chloroquine (Sigma Aldrich) dissolved in PBS (25 μM final concentration) for 0, 2, and 4 hours. Western blot quantification was performed using ImageJ software.

**Dextran Fluid-Phase Uptake**
For dextran uptake assays, medium was removed and replaced with serum-free RPMI for 6 hours before the cells were activated with EGF (20 nM final concentration) and incubated with dextran and Alexa Fluor 555 (Invitrogen) or streptavidin alone. To visualize dextran uptake, cells were fixed with 4% paraformaldehyde, costained with the early endosomal marker Rab5, and embedded in ProLong Gold Antifade Reagent (Invitrogen) before performing confocal microscopy. Quantification and colocalization analyses were performed using ImageJ software.

**Streptavidin Uptake**
For avidin uptake assays, medium was removed and replaced with serum-free RPMI for 6 hours before the cells were activated with biotinylated EGF (20 nM final concentration) and incubated with streptavidin coupled with Alexa Fluor 555 (Invitrogen) or streptavidin alone. To visualize avidin uptake, cells were fixed with 4% paraformaldehyde, costained with the early endosomal marker Rab5, and embedded in ProLong Gold Antifade Reagent (Invitrogen) before performing confocal microscopy. Quantification and colocalization analyses were performed using ImageJ software.

**D. melanogaster Experiments**
*D. melanogaster* stocks were cultured on standard cornmeal molasses agar food and maintained at 29°C. Virgins of upstream activating sequence–VPS34RNAi (VDRC TID100296) were crossed to prospero<sup>c knockdown of VPS34. GCNs (isolated from wandering third-instar larvae) were dissected in PBS, fixed in 4% paraformaldehyde, washed in PBS and 0.2% Triton X-100, incubated with primary antibodies (in PBS, 0.2% Triton X-100, and 0.05% sodium azide), and mounted in Vectashield (Vector Labs). The mounting medium for GCNs contained 4’,6-diamidino-2-phenylindole for nuclear staining. Samples were imaged using NIKON A1 CLEM with inverted microscope Eclipse TI. Rabbit anti-Rab7 and rat anti-Rabenosyn5 were gifts from A. Nakamura, RIKEN Center for Developmental Biology, Japan.

**Statistical Analyses**
Data are presented as mean ± SEM throughout the text unless otherwise specified. All experiments were performed at least three times. Statistical comparisons were performed using two-tailed t test where applicable. A value of P<0.05 was considered to represent statistically significant differences.

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**DISCLOSURES**
None.

**REFERENCES**


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Figure S1. Tubular Rab5 expression and co-localisation with Vps34. Co-labelling immunofluorescence stainings with Rab5 and Vps34 on mouse kidney sections.
**Supplementary Figure 2**

**Figure S2. Podocyte specific Vps34 deficiency causes massive vacuolization and rapid podocyte degeneration.** (A) Electron microscopy of kidney sections of 1 week old mice. Glomerular overview of Nphs2-cre;Vps34fl/fl and littermate controls (left upper panels) displayed no significant differences in podocyte morphology. Podocytes (right panels) and slit diaphragm (lower panels) show only marginal early signs of foot process effacement in 1 week old Nphs2-cre;Vps34fl/fl kidney sections compared to littermate control sections. Scale bars 5μm (upper left panels), 2μm (upper middle panels), 1μm (right panels) and 500nm lower panels. (B) Electron microscopy of kidney sections of 3 weeks old mice showed single-membrane, large-sized vacuoles in Vps34 deficient podocytes (arrows). Scale bars 1μm for upper left panel, 500nm for upper and lower right panel, 200nm for lower left panel. (C) Electron microscopy of kidney sections of 9 weeks old Nphs2-cre;Vps34fl/fl mice showed substantial vacuolization of podocytes (white arrows), complete loss of foot processes (black arrow heads) and severe glomerulosclerosis compared to littermate control mice. Scale bars 5μm (upper left panels), 2μm (upper middle panels), 1μm (right panels) and 500nm lower panels.
Supplementary Figure 3

(A) Immunofluorescence stainings of kidney sections of Nphs2-cre;Vps34fl/fl and littermate controls for the slit diaphragm proteins Nephrin and Podocin and the podocyte nucleus marker WT1 demonstrated equal distribution of slit diaphragm proteins and no difference in podocyte cell numbers in Nphs2;Vps34fl/fl mice and littermate controls 1 week after birth. Scale bars 10 μm; 5 μm for details. (C) Podocyte cell quantification at 1 week of age. Podocyte numbers were estimated by counting the number of WT1-positive cells per glomerular cross-section in 100 randomly selected glomeruli/kidney. No differences in the number of podocytes/glomerulus were detected in kidneys of Nphs2-cre;Vps34fl/fl mice or littermate controls (P = 0.84, 2-tailed Student’s t test, n = 3 mice for each condition).

Figure S3. Staining for slit diaphragm proteins and the podocyte marker WT1 displayed no significant difference in Nphs2-cre;Vps34fl/fl mice compared to littermate control mice at 1 week of age. (A, B) Immunofluorescence stainings of kidney sections of Nphs2-cre;Vps34fl/fl and littermate controls for the slit diaphragm proteins Nephrin and Podocin and the podocyte nucleus marker WT1 demonstrated equal distribution of slit diaphragm proteins and no difference in podocyte cell numbers in Nphs2;Vps34fl/fl mice and littermate controls 1 week after birth. Scale bars 10 μm; 5 μm for details. (C) Podocyte cell quantification at 1 week of age. Podocyte numbers were estimated by counting the number of WT1-positive cells per glomerular cross-section in 100 randomly selected glomeruli/kidney. No differences in the number of podocytes/glomerulus were detected in kidneys of Nphs2-cre;Vps34fl/fl mice or littermate controls (P = 0.84, 2-tailed Student’s t test, n = 3 mice for each condition).
Figure S4. Immunogold electron microscopy analysis. Fixed kidney sections of 3-weeks-old Nphs2-cre;Vps34fl/fl mice and control littermates were incubated overnight with anti-LC3 and with a 1.4-nm gold-coupled goat anti-mouse secondary antibody for immunogold reaction and further processed for electron microscopy. (A) Wildtype podocytes display only very few LC3 gold particles. Representative sections of tubular cells (i), rich in autophagosomes confirm the specific localization of LC3-gold particles to autophagosomes. Scale bars: 500nm. (B) In Vps34-deficient podocytes, gold particles aggregate diffusely in the cytoplasm but do not co-localize with autophagosomal membranes. Gold particle deposition to the basal membrane is equal in wildtype and Nphs2-cre;Vps34fl/fl renal sections and most likely unspecific due to immunocomplex deposits and/or negative electric charge of the basal membrane. Representative sections of tubular cells (i), rich in autophagosomes confirm the specific localization of LC3-gold particles to autophagosomes. Scale bars: 500nm.
Supplementary Figure 5

(A) Significant reduction in the total cell count of Vps34-deficient primary podocytes after 9 days compared to GFP-positive control podocytes. (B-E) Quantification and immunofluorescence stainings of primary podocytes isolated from conditionally Vps34-deficient mice and littermate controls. (B, D) Primary wildtype podocytes show enhanced proliferation (illustrated by Ki-67 staining) whereas Vps34-deficient podocytes did not. (C, E) Active caspase 3 staining revealed that, in vitro, primary Vps34-deficient podocytes die, at least in part, from apoptosis. (F, G) Immunofluorescence stainings and quantification of kidney sections of conditionally Vps34-deficient mice and littermate controls. (F) In vivo, Ki-67 staining showed no significant proliferation activity of neither WT nor Vps34-deficient podocytes at different time points. Podocytes were co-stained with WT1. (G) Active caspase 3 staining of kidney sections showed increased apoptosis in 9 weeks old mice in parietal cells, mesangial cells and podocytes. At early stages of beginning glomerulosclerosis (week 3), active caspase 3 staining was indifferent from WT podocytes.

Figure S5. Primary podocytes isolated from Nphs2-cre;Vps34fl/fl;tomatofl/+>EGFP mice exhibited strongly reduced proliferation compared to wildtype controls. (A) Significant reduction in the total cell count of Vps34-deficient primary podocytes after 9 days compared to GFP-positive control podocytes. (B-E) Quantification and immunofluorescence stainings of primary podocytes isolated from conditionally Vps34-deficient mice and littermate controls. (B, D) Primary wildtype podocytes show enhanced proliferation (illustrated by Ki-67 staining) whereas Vps34-deficient podocytes did not. (C, E) Active caspase 3 staining revealed that, in vitro, primary Vps34-deficient podocytes die, at least in part, from apoptosis. (F, G) Immunofluorescence stainings and quantification of kidney sections of conditionally Vps34-deficient mice and littermate controls. (F) In vivo, Ki-67 staining showed no significant proliferation activity of neither WT nor Vps34-deficient podocytes at different time points. Podocytes were co-stained with WT1. (G) Active caspase 3 staining of kidney sections showed increased apoptosis in 9 weeks old mice in parietal cells, mesangial cells and podocytes. At early stages of beginning glomerulosclerosis (week 3), active caspase 3 staining was indifferent from WT podocytes.
Supplementary Figure 6

**A**

NPHS2 Promoter \[\xrightarrow{\text{Cre}}\] Tomato \(\xrightarrow{\text{loxP}}\) EGFP \(\xrightarrow{\text{loxP}}\) Vps34

Supplementary Figure 6

**B**

Single cells \hfill Living cells \hfill GFP+-cells

**Figure S6. FACS sorting of GFP-positive primary podocytes.** (A) On postnatal day 10, glomeruli from Nphs2-cre;Vps34fl/fl;tomatofl/+EGFP mice and littermate controls were isolated by a sieving protocol, established in our lab and cultured for several days. (B) After podocytes had grown out of the glomeruli, cells were trypsinated and GFP-positive cells were FACS sorted and maintained in primary cell culture medium. Representative flow cytometric data, regions and sort gate.