Role of Ragulator in the Regulation of Mechanistic Target of Rapamycin Signaling in Podocytes and Glomerular Function

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ABSTRACT
Aberrant activation of mechanistic target of rapamycin complex 1 (mTORC1) in glomerular podocytes leads to glomerular insufficiency and may contribute to the development of glomerular diseases, including diabetic nephropathy. Thus, an approach for preventing mTORC1 activation may allow circumvention of the onset and progression of mTORC1-dependent podocyte injury and glomerular diseases. mTORC1 activation requires inputs from both growth factors and nutrients that inactivate the tuberous sclerosis complex (TSC), a key suppressor of mTORC1, on the lysosome. Previous studies in mice revealed that the growth factor-phosphatidylinositol 3-kinase pathway and mTORC1 are essential for maintaining normal podocyte function, suggesting that direct inhibition of the phosphatidylinositol 3-kinase pathway or mTORC1 may not be an ideal approach to sustaining physiologic podocyte functions under certain disease conditions. Here, we report the role of the Ragulator complex, which recruits mTORC1 to lysosomes in response to nutrient availability in podocytes. Notably, podocytes lacking Ragulator maintain basal mTORC1 activity. Unlike podocyte-specific mTORC1-knockout mice, mice lacking functional Ragulator in podocytes did not show abnormalities in podocyte or glomerular function. However, aberrant mTORC1 activation induced by active Rheb in podocyte-specific TSC1-knockout (podo-TSC1 KO) mice did require Ragulator. Moreover, ablation of Ragulator in the podocytes of podo-TSC1 KO mice or streptozotocin-induced diabetic mice significantly blocked the development of pathologic renal phenotypes. These observations suggest that the blockade of mTORC1 recruitment to lysosomes may be a useful clinical approach to attenuate aberrant mTORC1 activation under certain disease conditions.

Mechanistic target of rapamycin (mTOR) kinase exists in two distinct multiprotein complexes, termed mTORC1 and mTORC2.1 mTORC1, a rapamycin-sensitive complex, stimulates essential cellular anabolic processes, such as protein synthesis, and induces cell growth in response to nutrients and growth factors.2 mTORC2 is a rapamycin-insensitive complex and regulates cytoskeleton reorganization and cell survival in response to growth factors. However, prolonged treatment with rapamycin also inhibits mTORC2 in a variety of cells, including podocytes.3,4 Importantly, aberrant mTORC1 activation also leads to a negative feedback to growth factor-dependent PI3K activation, thereby inhibiting mTORC2 activity.5–7

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We have recently demonstrated that mTORC1 is hyperactivated in podocytes of diabetic mice and patients, and activation of mTORC1 is responsible for podocyte injury and plays a critical role in the development of diabetic nephropathy (DN) in mouse models.\(^6\)\(^9\) Conditional activation of mTORC1 in podocytes by specific deletion of the upstream negative regulator tuberous sclerosis complex 1 (TSC1) leads to severe podocyte injury and recapitulates many features of DN in nondiabetic animals. Conversely, reduction of mTORC1 activity in podocytes prevents the development of DN in diabetic animals.\(^6\)\(^8\)\(^9\) Although these observations suggested a possible application of rapamycin-analogs to prevent podocyte injury and DN, we have also found that podocytes require physiologic levels of mTORC1 activity for their normal functions. Genetic ablation of mTORC1 in podocytes causes podocyte dysfunction and proteinuria.\(^9\)\(^10\) In addition, currently available inhibitors of mTORC1 result in progressive proteinuria in certain patients with renal problems.\(^11\)\(^13\) Possibly due to their inhibitory effects on basal mTORC1 and/or on the mTORC2-Akt pathway, which is essential for the integrity of podocytes,\(^14\)\(^15\) Thus, an approach that specifically attenuates but does not completely block mTORC1 activity represents a promising alternative therapeutic strategy.

Activation of mTORC1 requires inputs from both growth factors and nutrients that stimulates two distinct small GTPases, Rheb and Rag, respectively. In response to growth factor stimulation, active Akt phosphorylates and inhibits the TSC complex, an essential GTPase activating protein complex, which suppresses the activity of Rheb small GTPase, a direct activator of mTORC1, on the lysosomal membrane.\(^16\)\(^18\) Akt-dependent TSC2 phosphorylation leads to the dissociation of the TSC complex from lysosomal membranes, thereby preventing its access to Rheb.\(^19\) Moreover, multiple cellular cues such as glucose and oxidative stress have been reported to inhibit the activity of the TSC complex.\(^20\) Thus, a wide array of growth and nutrient signals stimulate the Rheb by inhibiting the activity of the TSC complex on the lysosome membrane.

In parallel to Rheb activation, amino acids stimulate the Ragulator complex, which localizes to lysosomal membranes.\(^21\)\(^22\) Ragulator consists of five subunits, p18/LAMTOR1, p14, MP1, HBXIP, and C7orf59.\(^22\) Importantly, Ragulator functions as both a scaffold and a guanine nucleotide exchange factor for RagA/B small GTPases,\(^22\)\(^23\) which directly recruit mTORC1 to the lysosomal membrane.\(^24\) The mammalian Rag family consists of four small GTPase proteins (A–D). Rag proteins form heterodimers between RagA/B and RagC/D. Ragulator-dependent RagA/B activation triggers mTORC1 lysosomal localization,\(^24\) where mTORC1 can be activated by active Rheb. These observations explain why mTORC1 activation requires both inputs from nutrients and growth factors for its activation.

In this study, we characterized the function of Ragulator in cultured podocytes and its role in podocyte and glomerular function in a mouse model in which p18, an essential component of Ragulator, was genetically ablated in glomerular podocytes (podocyte-specific p18-knockout [podo-p18 KO]). Surprisingly, podocytes lacking p18 maintain basal mTORC1 activity. Accordingly, podo-p18 KO mice do not show the obvious pathologic phenotypes seen in podo-Raptor or podocyte-specific mTOR-knockout (podo-mTOR KO) animals.\(^9\)\(^10\) However, p18 ablation significantly prevented the pathologic renal phenotypes, including podocyte injury and glomerulosclerosis, in podocyte-specific TSC1-knockout (podo-TSC1 KO) mice or diabetic mice. To specifically attenuate mTORC1 activity and maintain mTORC2 activity, the reduction of the lysosomal localization of mTORC1 may be a suitable alternative approach to mitigate aberrant mTORC1 activity with fewer side effects.

RESULTS

p18 Plays a Critical Role in mTOR Localization on Lysosomes in Podocytes

Podocytes express p18 and RagA/RagC but not RagB/RagD heterodimer (Figure 1A), suggesting that mTORC1 recruitment to the lysosomes is mainly operated by a Ragulator-RagA/RagC complex. To examine the functional importance of p18 in podocytes, we ablated the p18 gene in isolated primary podocytes, which express WT1 (Figure 1B), from the \(p18^{\text{fl}ox}/\text{fl}ox\) mice by expressing Cre recombinase \textit{in vitro}. Complete ablation of the p18 gene was confirmed by PCR analysis of genomic DNAs from the \(p18^{\text{fl}ox}/\text{fl}ox\) and \(p18^{\text{fl}ox}\) knockout (KO) podocytes (Figure 1C). Accordingly, the expression of p18 protein was abolished in \(p18^{\text{KO}}\) podocytes (Figure 1D). While ablation of p18 did not affect the expression of mTOR, Raptor, or Rheb, the expression of Rictor, an essential component of mTORC2, was increased in \(p18^{\text{KO}}\) podocytes compared with wild-type (WT) podocytes (Figure 1D). As seen in other cell lines,\(^22\)\(^23\) p18 is largely colocalized with LAMP2, a lysosomal transmembrane protein, in WT podocytes, indicating that p18 is exclusively expressed on the surface of lysosomes (Figure 2A). In WT podocytes, RagC was also found to be colocalized with LAMP2. In contrast, RagC was diffusively expressed in the cytoplasm without obvious colocalization with LAMP2 in \(p18^{\text{KO}}\) podocytes (Figure 2B). While mTOR is mainly localized in the cytoplasm of podocytes under amino acid starvation conditions, it translocated to lysosomes after amino acid replenishment in WT primary podocytes and MPC5 podocytes (Figure 2C, Supplemental Figure 1). However, amino acid-induced mTOR localization on lysosomes was largely blocked in \(p18^{\text{KO}}\) primary podocytes and \(p18^{\text{KO}}\) MPC5 cells (Figure 2C, Supplemental Figures 1 and 2A). These observations indicate that p18 plays a critical role in recruiting mTORC1 to the lysosomal membrane in response to nutrients in podocytes.
in response to serum or insulin occurred 30 minutes after stimulation in p18 KO podocytes (Figure 3, B and C). The inability of growth factors in p18 KO podocytes to quickly activate mTORC1 is not due to a decrease in sensitivity to growth factors because higher concentrations of serum or insulin are unable to accelerate these phosphorylations events in p18 KO podocytes (Supplemental Figure 3). Importantly, growth factors more effectively stimulated Akt phosphorylation, an mTORC2 substrate, in p18 KO podocytes with the same kinetics observed in WT cells. These data indicate that sensitivity of Akt activation to growth factors was enhanced in p18 KO podocytes possibly, in part, due to the increase in the amount of mTORC2 (Figure 1D). Furthermore, mTORC1 activation in response to amino acids was also delayed in p18 KO podocytes (Figure 3D). Taken together, these results indicate that although the p18-Ragulator-Rag system plays a critical role in acute mTORC1 activation in response to growth factor or amino acids, the system is dispensable, at least for maintaining basal mTORC1 activity in podocytes, and even in cell lines such as MEFs (Supplemental Figure 4). Our observations also suggest that there are other yet to be identified mechanisms that growth factors and amino acids utilize to activate cellular mTORC1 activity independent of the Ragulator-Rag system.

Characterization of podo-p18 KO Mice
To determine the roles of p18 in podocytes and glomerular function, we created podo-p18 KO mice. While the germline p18 KO showed an embryonic lethal phenotype, the podo-p18 KO mice were born at the normal Mendelian ratio and developed without any discernable abnormalities (data not shown). Unlike podo-Raptor or podo-mTOR KO mice, in which mTORC1 activity in podocytes is abolished and podocyte injury with proteinuria occurs,9,10 p18KO podocytes maintain normal glomerular structures without obvious abnormality of intracellular organelles (Figure 4A), slit diaphragm protein expression and cellular localization (Figure 4B), and glomerular function (Figure 4C) even at an old age (2 years). These observations indicate that loss of functional Ragulator does not cause podocyte dysfunction, which is likely due to the preservation of basal mTORC1 activity in the podocytes of podo-p18 KO mice.

Ragulator Plays a Key Role in Aberrant mTORC1 Activation and Podocyte Injury Caused by Loss of TSC Function
Recent studies have revealed that multiple growth-associated cellular cues, including growth factors, oxidative stress, glucose, and even amino acids, exert inhibitory effects on the TSC complex, a functional Rheb GAP protein complex, thereby activating mTORC1 on lysosomes.20 To investigate the functional role of Ragulator for active Rheb-induced aberrant mTORC1 activation in podocytes, we knocked down TSC1 in p18 KO primary podocytes and MPC5 cells and

**Figure 1.** p18 KO podocytes show unique characteristics in mTOR-related protein expression. (A) Tissue distribution of mTOR-Rag-Ragulator related proteins. Equal amount of proteins from different mouse tissues were applied to SDS-PAGE and different target proteins were examined by Western blotting. GAPDH is used as a loading control. (B) p18KO and p18 KO podocytes were examined for their expression of WT1. Immortalized mouse podocytes (MPC5)41 were used as a positive control and mouse embryonic fibroblasts (MEF) as a negative control. (C) Genomic DNA was extracted from p18fl/fl and p18 KO podocytes under basal growing conditions. Equal amounts of protein lysates were used for Western blotting.

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examined levels of mTORC1 activity and cell viability. As expected, in TSC1 knockdown podocytes, mTORC1 activity was constitutively active as S6K1 phosphorylation stayed at higher levels, both in the absence and presence of growth factors in cultured media (Figure 5A, Supplemental Figure 5A). However, active Rheb-induced S6K1 phosphorylation by TSC1 knockdown was largely attenuated in p18 KO podocytes. In contrast, the sensitivity of Akt phosphorylation to serum stimulation was decreased in TSC1 knockdown podocytes. Furthermore, the increased cleaved caspase-3 observed in TSC1 knockdown podocytes in culture, we demonstrated that induction of endoplasmic reticulum (ER) stress in podocytes causes podocyte injury and decreases its viability.25–28 In addition, phenotypic alterations such as epithelial-mesenchymal transition (EMT) changes have been observed in podocytes treated with various injurious stimuli and in several proteinuric disease conditions.29,30 We have previously demonstrated that hyper-activation of mTORC1 in glomerular podocytes caused ER stress (enhancement of Bip expression), EMT-like phenotypic change (desmin expression), and the mislocalization of slit diaphragm proteins such as nephrin.8 Importantly, these pathologic phenotypes seen in podo-TSC1 KO mice were largely attenuated in podo-TSC1/p18 DKO mice (Figure 6, C and D). Glomerular hypertrophy and mesangial expansion with pathologic accumulation of extracellular matrix proteins seen in podo-TSC1 KO mice were also blocked by ablation of p18 (Figure 6E).

Transmission electron microscopy (TEM) analyses demonstrated that disruption of normal foot process formation in podo-TSC1 KO mice was attenuated in podo-TSC1/p18 DKO mice (Figure 7A). In TSC1 KO podocytes, rough ERs with abundant ribosomes were unstructurally developed (Figure 7A). However, these pathologic alterations were not observed in TSC1/p18 DKO podocytes. As previously reported,8 the number of podocytes per glomerulus was significantly reduced in podo-TSC1 KO mice. In contrast, podo-TSC1/p18 DKO mice maintained a similar number of podocytes to WT mice (Figure 7B). Despite the increase of apoptosis in TSC1 knockdown podocytes in culture, we did not observe apoptotic podocytes in podo-TSC1 KO glomeruli by terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling staining (data not shown) or immunoblot analysis, monitoring cleaved caspase-3 (Supplemental Figure 6A). Instead, we observed cells expressing podocyte-specific mRNAs, such as nephrin, in the urine from podo-TSC1 KO mice but not those from WT or podo-TSC1/p18 DKO mice (Supplemental Figure 6B), suggesting that the ablation of p18 in podocytes attenuates podocyte loss due to their detachment from the glomerular basement membrane in podo-TSC1 KO mice. Consistent with the above observations, massive proteinuria observed in podo-TSC1 KO mice was prevented in podo-TSC1/p18 DKO mice (Figure 7C).

To investigate the effects of p18 ablation in the podocytes on the development of DN, we induced type 1 diabetes in WT and podo-p18 KO mice. mTORC1 activity in some
glomerular cells was slightly enhanced in WT but not podo-p18 KO mice under diabetic conditions (Figure 8A). The accumulation of periodic acid–Schiff-positive components and extracellular matrix proteins within the mesangial area observed in WT diabetic mice was slightly but significantly reduced in podo-p18 KO diabetic mice (Figure 8, A–D). TEM study revealed that foot process effacement observed in WT diabetic mice was prevented in podo-p18 KO mice (Figure 9A), although the levels of blood glucose and urine volume were similarly increased in both WT and podo-p18 KO diabetic mice (Figure 9, B and C). Furthermore, the number of podocytes per glomerulus was slightly reduced in WT diabetic mice compared with nondiabetic mice. In contrast, podo-p18 KO diabetic mice maintained a similar number of podocytes to that of control podo-p18 KO mice (Figure 9D). Accordingly, podo-p18 KO mice showed a slower development of albuminuria than WT diabetic mice (Figure 9E).

DISCUSSION

It has been well documented that growth factor and amino acids are two essential inputs for mTORC1 activation, as lack of either input largely diminishes cellular mTORC1 activity in vitro. A series of recent studies have identified the molecular mechanisms and provide a model whereby amino acids stimulate Ragulator, which is required for Rag-dependent mTORC1 recruitment to lysosomal membranes (Supplemental Figure 7).22–24 On the lysosome membrane, mTORC1 is directly activated by Rheb, which is stimulated by PI3K-Akt-TSC signaling. Interestingly, more recent studies show that the TSC complex is also localized on lysosomes. Importantly, both amino acids and growth factors induce the dissociation of the TSC complex from the lysosomal membrane, thereby stimulating Rheb-dependent mTORC1 activation.19,31 In this study, we show that lysosomal mTORC1 localization is indeed a key event for active Rheb-induced mTORC1 activation in vivo.

Consistent with previous observations in HEK293Tor MEF cells,22,23 ablation of p18, an essential component of Ragulator, abolished not only lysosomal Rag localization but also the recruitment of mTORC1 to lysosomes in cultured podocytes. However, we also found that mTORC1 can still be activated by growth factor or amino acids with different kinetics in the p18 KO podocytes. In fact, we also found that slower activation of mTORC1 in response to growth factor or amino acids can also be seen in p18 KO MEF cells (Supplemental Figure 4). These observations suggest that while Ragulator is required for lysosome-dependent, fast, and robust activation of mTORC1, lower levels of active mTORC1 can be stimulated by amino acids or growth factors independent of the Ragulator-Rag system. Although further studies will be required to reveal the mechanism underlying Ragulator-independent mTORC1 activation in p18 KO cells, this low activity of mTORC1 seems to play an important role in maintaining podocyte integrity both in vitro and in vivo.

Recent studies have demonstrated that inhibition of mTORC1 activity with rapamycin prevents the onset/progression of glomerular disorders including DN in animal models.32–38 Our previous studies demonstrated that mTORC1 activity is enhanced in podocytes of both diabetic animals and humans. Pathologic roles of aberrant mTORC1 activation in podocytes were confirmed in the podo-TSC1 KO nondiabetic mice.8,9 Conversely, reduction, but not ablation of mTORC1 activity in podocytes prevents the development of DN. Furthermore, mTORC1 inhibitors such as rapamycin often cause podocyte injury and proteinuria in individuals with kidney problems, likely due to its potent inhibition of mTORC1 and/or the indirect inhibitory effect on the mTORC2-Akt pathway. The latter possibility was supported by recent genetic studies demonstrating that conditional inactivation of the insulin receptor or Akt2 in podocytes leads to severe podocyte dysfunctions.14,39 Thus, effective treatments for mTORC1-associated podocyte dysfunction
may require the attenuation of mTORC1 activity in the setting of both an intact mTORC2-Akt pathway and normal basal mTORC1 activities in order to preserve podocyte function. The results of this study propose that attenuation of lysosomal localization of mTORC1, which is required for maximal activation of mTORC1, may be a promising alternative approach to reduce aberrant activation of mTORC1 in podocytes with fewer side effects.

**CONCISE METHODS**

**Animals and Animal Treatment**
Animal experiments were conducted following a protocol approved by the University of Michigan Committee on the Use and Care of Animals. To
generate mice with p18 deletion specifically in podocytes (podo-p18 KO), we crossed p18<sup>fl/fl</sup> mice (129S4/SvJae) with Tg ([Nphs2 [podocin]-Cre]) (C57BL/6J) mice.8 To generate podo-Tsc1 KO and podo-Tsc1/p18 DKO mice, [Tg ([Nphs2-Cre]), Tsc1<sup>fl/+</sup>, p18<sup>fl/+</sup>] mating was used for this study. Mice were maintained on the segregating (129S4/SvJae; C57BL/6J) genetic background. Diabetes was induced in 7-week-old mice by intraperitoneal injections of low dose streptozotocin (50 mg/kg<sup>2</sup> per day<sup>2</sup> for 5 consecutive days) dissolved in 10 mM citrate buffer, pH 4.5. Only mice that developed hyperglycemia (nonfasting blood glucose: approximately 300–500 mg/dl) during the following 4 weeks were selected for the study.

**Isolation of Glomeruli and Mouse Primary Podocytes**

Glomeruli of p18<sup>fl/fl</sup> mice were collected by magnetic bead–based isolation. Briefly, transcardiac perfusion was performed with PBS containing precleaned beads (Dynabeads; Invitrogen, Carlsbad, CA). The perfused renal cortex was briefly digested with collagenase and filtered, and the glomeruli choked with beads were collected with magnets, followed by further isolation with a pipet under a microscope. Isolated pure glomeruli were briefly treated with collagenase and passed through a sieve. Cells that passed through the sieve were cultured in plates coated with type I collagen. p18<sup>fl/fl</sup> podocytes were isolated and cultured in DMEM/F12 medium (Gibco, Carlsbad, CA), supplemented with 10% FBS.
(HyClone Laboratories), penicillin/streptomycin (Invitrogen), 1× Insulin-Transferin-Selenium-X (Invitrogen) and (10⁻⁷ M) hydrocortisone (Sigma-Aldrich, St. Louis, MO). To generate the p18 KO podocytes, Ade-CRE virus was added to the p18⁻/⁻ podocytes for the deletion of p18 exon 1. Both normal and p18 KO podocytes were maintained in a 37°C incubator with 5% carbon dioxide. For culturing podocytes, cell culture dishes were precoated with collagen type IV (Sigma-Aldrich). Cells between passage three and ten were used for the experiments.

**Immunofluorescence and Immunohistochemistry**

Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. After rinsing twice with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, and blocked with 2% BSA in PBS for 1 hour. Cells on coverglass were incubated with primary antibody diluted in PBS with 2% BSA at 4°C overnight. Antibodies were used at the following dilutions: p18 (1:300; Cell Signaling Technology, Danvers, MA); LAMP2 (1:500; Abcam, Inc., Cambridge, MA); RagC (1:300; Cell Signaling Technology); mTOR (1:500; Cell Signaling Technology). Cells were rinsed three times with PBS containing 0.02% Tween 20. Secondary antibodies were diluted in PBS with 2% BSA, and cells were incubated at room temperature for 1 hour. The secondary antibodies used were Alexa Fluor 594 goat anti-rabbit IgG and 488 goat anti-rat IgG (1:500; Invitrogen). After rinsing three times with PBS containing 0.02% Tween 20, cells on the coverglass were mounted on microscope slides with Prolong Gold antifade reagent (Invitrogen). Images were taken using a Leica TCS SP5 confocal microscope with a 63X oil immersion objective and processed using Photoshop software (Adobe Systems, Inc., San Jose, CA). For immunohistochemistry, we used paraffin-embedded tissue sections in mouse models. Briefly, the renal tissues were fixed by transcardiac perfusion with PBS containing 4% PFA. Antibodies were used at the following dilutions: pS6 (1:300; Cell Signaling Technology); pAkt S473 (1:400; Cell Signaling Technology); synaptopodin (1:100; Genway); collagen type IV (1:150; EMD Millipore, Billerica, MA); fibronectin (1:400; Sigma-Aldrich), Desmin (1:50; Dako), Bip (1:100; Abcam, Inc.). Staining was visualized by indirect/direct immunofluorescence or 3,3′-Diaminobenzidine.

**Figure 7.** Ablation of p18 in the podocytes prevents mTORC1-induced podocyte loss and glomerular dysfunction in podo-TSC1 KO mice. (A) The formation of the podocyte foot process in the indicated 8-week-old male mice was analyzed by TEM. The arrow and arrowhead indicate developed rough ERs and increased ribosomes, respectively, in podo-TSC1 KO podocytes. (B) Ratios (a number of WT1-positive cells/glomerular tuft area) were determined in about 25–35 glomeruli from the indicated animals. The data were expressed as the mean fold change. **P<0.001 versus other groups; mean±SEM (n= approximately 3–5 mice). (C) Urine was collected from the indicated animals at 4 and 8 weeks of age and subjected to SDS-PAGE. Coomassie blue staining was performed to visualize urinary proteins (upper panels). Urinary albumin concentrations in 24-hour urine from the indicated animals were measured. **P<0.001; mean±SEM (n= approximately 5–8 mice).
Figure 8. Ablation of p18 in the podocytes prevents mesangial expansion in diabetic mice. (A) Kidney sections from the indicated 20-week-old mice were stained with pS6, synaptopodin, periodic acid–Schiff (PAS), type IV collagen, and fibronectin. (B–D) Quantifications of PAS-, type IV collagen-, and fibronectin-positive area within a glomerulus were shown. Ratios (positive area/ glomerular tuft area) were determined in 40 glomeruli from the indicated mice and expressed as the mean fold change. **P<0.001; mean±SEM (n=4 mice).
Figure 9. The effects of p18 ablation in the podocytes on their foot process formation, number, and glomerular function in diabetic mice. (A) Representative WT1 staining and TEM images (foot process and cytoplasm) of the indicated 20-week-old mice. The arrow and arrowhead indicates rough ERs and ribosomes, respectively. (B) 24-hour urine volume of the indicated diabetic mice. Data are expressed as an average of the amount of urine over 3 days. Mean±SEM (n=5 mice). (C) Blood glucose levels in the indicated diabetic mice at
Immunoblot Analysis
Cells or purified glomeruli were lysed in NP-40 lysis buffer (40 mM HEPES, pH 7.5, 120 mM sodium chloride, 1% NP-40, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM EDTA, 1× Protease Inhibitor Cocktail [Roche, Basel, Switzerland]). Lysates were then boiled in SDS sample buffer (20 mM Tris, pH 6.8, 2% SDS, 0.01% bromphenol blue, 10% glycerol, 5% 2-mercaptoethanol) and subjected to SDS-PAGE and immunoblotting according to standard techniques.

Cell Treatment
For amino acid stimulation, podocytes were rinsed twice with prewarmed HBSS (Invitrogen), and incubated with amino acid-free DMEM/F12 (US Biologic) containing 2% dialyzed FBS (Life Technologies, Carlsbad, CA), for 2 hours. Amino acids (Sigma-Aldrich) were added to the medium for the indicated times in Figure 3D and Supplemental Figure 4A. For growth factor stimulation, podocytes were rinsed twice with serum-free DMEM/F12 and incubated in serum-free DMEM/F12 for 10 hours. Serum-starved cells were stimulated with the indicated concentration of FBS (HyClone Laboratories) or Insulin (Sigma-Aldrich) for the time as indicated in Figure 3, B and C and Supplemental Figure 2B and 4B.

Lentivirus Production and Infection
For lentivirus packaging, the pLKO vectors encoding shRNA specific to the Tsc1 gene were transfected into HEK293T cells with lentiviral packaging plasmid pSIN2G and envelope plasmid pMD2.G. The condition media containing the lentivirus were collected every 24 hours until 72 hours post-transfection. The media containing the lentivirus were concentrated by ultracentrifugation at 23,000 rpm for 90 minutes in a Beckman SW32 rotor. Viral pellets were resuspended in 1/300th of the original volume. For the lentivirus were concentrated every 24 hours until 72 hours post-transfection. The media containing the lentivirus were collected and concentrated, and used to infect MPC5 cells. At 24 hours after infection, cells were selected with puromycin at 3 μg/ml for another 48 hours.

At 7 days postinfection, the expression of LAMTOR1/p18 was examined and used for other experiments.

EM Analysis
The renal tissues were fixed by transcardiac perfusion with Sorensen phosphate buffer containing 4% PFA and 2.5% glutaraldehyde. The processed samples were analyzed by Philips CM100 TEM or AMRAY 1910 field emission scanning electron microscope.

In vivo Physiologic Studies
Urinary albumin and creatinine concentrations were determined using mouse-specific Albuwell M and Creatinine Companion kit (Exocell). Glomerular tuft area, periodic acid–Schiff-positive mesangial area, and other pixel densities obtained by immunohistochemical experiments were measured by ImageJ software.

Statistical Analyses
All data were analyzed by ANOVA with Scheffe post hoc tests. Asterisks represent statistically significant differences. P values less than 0.05 were considered significant. *P<0.01; **P<0.001.

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DISCLOSURES
None.

4 weeks after STZ treatment. Mean ± SEM, (n=5 mice). (D) Ratios (the number of WT1-positive cells/glomerular tuft area) were determined in 40 glomeruli from the indicated animals. Data is expressed as the mean fold change. *P<0.01 versus WT diabetic mice (WT STZ); mean±SEM (n=5 mice). (E) Urinary albumin and creatinine concentrations were measured in 24-hour urine samples from the indicated animals. Albumin-to-creatinine ratios were shown (n=5 mice). STZ, streptozotocin.
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