Inhibition of MTOR Disrupts Autophagic Flux in Podocytes

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ABSTRACT
Inhibitors of the mammalian target of rapamycin (MTOR) belong to a family of drugs with potent immunosuppressive, antiangiogenic, and antiproliferative properties. De novo or worsening proteinuria can occur during treatment with these agents, but the mechanism by which this occurs is unknown. We generated and characterized mice carrying a podocyte-selective knockout of the Mtor gene. Although Mtor was dispensable in developing podocytes, these mice developed proteinuria at 3 weeks and end stage renal failure by 5 weeks after birth. Podocytes from these mice exhibited an accumulation of the autophagosome marker LC3 (rat microtubule-associated protein 1 light chain 3), autophagosomes, autophagolysosomal vesicles, and damaged mitochondria. Similarly, human podocytes treated with the MTOR inhibitor rapamycin accumulated autophagosomes and autophagolysosomes. Taken together, these results suggest that disruption of the autophagic pathway may play a role in the pathogenesis of proteinuria in patients treated with MTOR inhibitors.


The mammalian target of rapamycin (MTOR) is an evolutionarily conserved serine-threonine kinase that interacts with regulatory associated protein of MTOR (Rptor) or Rptor independent companion of MTOR (Rictor) to form mTORC1 and mTORC2 complexes, respectively. In turn, mTORC1 and mTORC2 regulate different aspects of MTOR function. mTORC1 is a key regulator of cellular metabolism, including protein translation, ribosomal biogenesis, cell growth and proliferation, and suppression of autophagy in response to amino acids, growth factors, and elevated cellular ATP levels.¹ mTORC2 is regulated primarily by growth factors to promote actin cytoskeletal rearrangement, cell survival, and cell cycle progression.² In mammalian cells, rapamycin and other MTOR inhibitors associate with the FKBP12 protein, and together they directly bind MTOR to prevent the RPTOR-MTOR interaction and thus inhibit mTORC1 function.³ In certain cell types, including the podocyte, chronic inhibition of MTOR by rapamycin also results in downregulation of mTORC2 functions.⁴–⁶ Although this mechanism of action has not been completely elucidated, data in podocytes suggest that prolonged rapamycin treatment directly downregulates MTOR and Rictor, both of which are required for mTORC2 function.⁶

Sirolimus (rapamycin) was originally proposed as an immunosuppressant to prevent rejection of solid organ transplants. There were expectations that MTOR inhibitors would replace nephrotoxic calcineurin inhibitors (CNIs).

In one prospective trial, patients treated with sirolimus or switched to sirolimus from CNIs had comparable rates of biopsy-confirmed acute allograft rejection and 2-year graft survival to those treated with CNIs.⁷ In addition, sirolimus-treated patients had fewer malignancies and a better estimated GFR (eGFR) at 24 months if their baseline eGFR was >40 ml/min. Because of their antiproliferative and antiangiogenic effects, indications for MTOR inhibitors have expanded to include treatment of various cancers such as renal cell carcinoma, nonmalignant conditions such as autosomal dominant polycystic kidney disease (AD-PKD), and primary glomerulopathies.⁸–¹²

Despite its potential advantages in the transplant setting, evidence that sirolimus causes de novo or worsening proteinuria is unequivocal. In one randomized clinical trial in which patients with AD-PKD received sirolimus or placebo, the group receiving an MTOR inhibitor had a significantly higher median
Given the well recognized proteinuric effect of MTOR inhibitors, we were interested in understanding its mechanism. To explore the role of MTOR in vivo, we developed a mouse model with a podocyte-selective deletion of the Mtor gene (Mtor pod-KO).

A conditional Mtor allele was generated using BAC recombinering to place loxP sites around the first three exons of the Mtor gene (Figure 1A). The successful generation of a floxed Mtor allele was confirmed by Southern blot (Figure 1B). In this mouse, Cre-mediated excision results in a null Mtor allele, abolishing function of both mTORC1 and mTORC2 complexes. Because chronic use of mTOR inhibitors downregulates both mTORC1 and mTORC2 functions, we chose to delete the Mtor gene—as opposed to its partners Rictor or Rictor—to obtain the most complete knockdown and simulate the clinical effects of MTOR inhibitors.

To confirm functional deletion of the Mtor gene, we bred the Mtor^fl/fl mouse to a pCagsCre-driver strain that deletes the gene at the one cell embryo stage, giving a germline deletion of Mtor (Mtor^del) (Figure 1C). Heterozygous Mtor^wt/del mice appear healthy but have reduced Mtor protein levels (Supplemental Figure 1). Homozygous Mtor^del/del mice die in utero, similar to the conventional knockout (Figure 1D). In all subsequent breeding, one Mtor^del allele was utilized to enhance the degree of Cre-mediated excision in the podocyte.

To generate an Mtor pod-KO, Podocin-Cre mice were bred to mice carrying one conditional allele (Mtor^fl) and one deleted (Mtor^del) allele (Figure 1C). Glomerular deletion of Mtor was verified by analysis of genomic DNA isolated from the renal cortex and identification of the deleted band (Figure 1E). Further confirmation was obtained by Western blot analysis of glomerular protein showing downregulation of Mtor (Figure 1F). Mice of all genotypes were born in expected Mendelian frequency (Supplemental Table 1) and mutants appeared as healthy as wild-type or mixed genotype controls at birth. Interestingly, no overt phenotype was observed until 3 weeks of age, suggesting that Mtor is dispensable in developing podocytes.

Several in vitro and patient biopsy studies have addressed a role for MTOR in the glomerulus. One group described thrombotic microangiopathic glomerular lesions in renal biopsies from five patients who developed proteinuria when treated with sirolimus. These lesions were associated with downregulation of vascular endothelial growth factor A (VEGFA) expression in podocytes, a molecular mechanism that has been associated with thrombotic microangiopathy in patients with pre-eclampsia. Although not all patients with proteinuria who take sirolimus have a distinct glomerular lesion, Stallone et al. performed a biopsy study showing that sirolimus treatment was associated with decreased expression of synaptopodin, podocin, CD2AP, and nephrin in podocytes. Cell culture studies support the results of these biopsy studies and further suggest a role for MTOR in regulating actin and slit-diaphragm–associated proteins in the podocyte. Finally, genetic deletion of Riptor alone or both Rictor and Riptor from podocytes results in glomerular injury in mice by an unknown mechanism. These data suggest that inhibition of MTOR signaling within the podocyte may play a complex role to promote proteinuria in patients.

Despite the presence of foot process effacement, previous studies indicate that this is unlikely to be the primary mechanism of glomerular injury because other transgenic mouse models with even more marked foot process defects do not exhibit such a dramatic disease course or the vacuolated podocyte phenotype. For example, mice lacking the adaptor proteins, Nck1/2, in their podocytes do not form any foot processes.
Although they are born with severe proteinuria, their renal injury progresses more slowly than that seen in Mtor pod-KO mice.22 In keeping with structural changes observed by EM, podocin expression was decreased at 3 weeks in glomeruli of Mtor pod-KO mice (Supplemental Figure 2A). However, Wt1 and Nphs1 expression were unaffected at this time point (Supplemental Figure 2B).

Given the observations of decreased VEGFA levels in renal biopsies from patients treated with MTOR inhibitors and regulation of VEGFA by mTORC1 through upregulation of hypoxia-inducible...
Figure 2. Disease course and glomerular histology in Mtor pod-KO mice. (A) Mtor pod-KO mice appear smaller than their control littermates at 3 weeks of age. (B) Mtor pod-KO mice are significantly growth restricted by 4 weeks of age (*P<0.01; **P<0.05). (C) Protein/creatinine ratios are significantly increased by 3 weeks of age in Mtor pod-KO mice compared with wild-type (*P<0.01; **P<0.05). (D) At 3 weeks of age, glomeruli look similar in mutants and controls although proteinaceous casts are visible in tubules (periodic acid–Schiff stain, magnification ×40). By 4 weeks of age, glomerular scarring and vacuolization of podocytes are evident. (E) By 5 weeks of age, glomeruli are end stage with widespread vacuolization of podocytes. (F) Electron micrographs from control and mutant glomeruli at 3 weeks of age show focal foot process effacement in mutants compared with controls. The endothelium and glomerular basement membrane appear largely intact. Scale bars represent 1 μm. (G) Electron micrographs of glomeruli from 2-week-old and 3-week-old Mtor pod-KO mice show frequent double-membraned cytoplasmic vesicles characteristic of autophagosomes. Scale bar in the top left panel represents 500 nm. All other scale bars represent 1 μm. (H) Isolated glomeruli from Mtor pod-KO mice show increased levels of LC3-II compared with controls. AV, autophagosomal vesicles; ALV, autophagolysosomal vesicles.
We next examined whether reduced Vegfa in podocytes might be driving the disease in Mtor pod-KO mice. In our model, Vegfa levels were similar in mutants and controls at the onset of disease (between 2 and 3 weeks of age). However, by 3 weeks of age, Vegfa levels were visibly reduced but not absent (Supplemental Figure 1C). Despite this finding, a primary reduction of Vegfa alone in podocytes results in a very different phenotype and disease course in mice, including marked endothelial swelling with mesangiolysis, and, in the case of developmental deletion, complete loss of the glomerular endothelium. Similarly, reduction of Vegfa by approximately 75% of normal levels results in dramatic mesangiolysis by 3 weeks of age. None of these pathologic findings were observed in Mtor pod-KO mice.

If primary cytoskeletal defects and Vegfa deficiency are unlikely to be the initiating events of the glomerular injury in Mtor pod-KO mice, what explains the dramatic phenotype? MTOR is known to regulate and inhibit many cellular processes, including autophagy (reviewed by Zoncu et al.1). Autophagy is a lysosomal-dependent cellular survival response to starvation or lack of growth factors in which cells degrade cellular constituents from proteins to entire organelles, such as mitochondria, to provide a supply of nutrients under conditions of stress. A basal level of autophagy is, however, necessary to remove damaged organelles, excessive lipids, and long-lived or misfolded proteins. Terminally differentiated, nondividing cells such as neurons have an elevated basal level of autophagy. Similarly to neurons, the basal level of autophagy also seems to be increased in podocytes. In the podocyte, it has been suggested that autophagy may be required to protect the cell from injury. In this regard, deletion of Atg5, a key component of the autophagic pathway, results in late onset of glomerular disease in mice at 20–24 months of age, presumably due to the accumulation of damaged organelles and ubiquitinated protein complexes. Active autophagy is characterized by the presence of double-membraned cytoplasmic vesicles known as autophagosomal vesicles (AV), which can be seen on electron micrographs. Importantly, AVs were readily observed in podocytes from Mtor pod-KO mice (Figure 2G) beginning at 2 weeks of age but were not seen in controls. To quantitate the number of AVs, we isolated protein from the glomeruli of wild-type or Mtor pod-KO mice. LC3 (rat microtubule-associated protein 1 light chain 3) is the mammalian homolog of the essential yeast autophagy protein Atg8 and is the most commonly used molecular marker of the autophagosome. There are two LC3 isoforms generated by post-translational modification: LC3-I and LC3-II. LC3-I localizes to the cytoplasmic compartment, whereas LC3-II localizes to the autophagosome membrane as well as the cytoplasm. LC3-II is increased in glomeruli isolated from mutant animals (Figure 2H) consistent with an increase in AVs.

After engulfment of the intracellular targets, the AV fuses with lysosomes to generate the autophagolysosomal vesicle (ALV), permitting degradation of intracellular organelles. The process is successfully terminated after reformation of the autophagosome and lysosomes from the ALV (Figure 3A). Failure to regenerate these components will disrupt the autophagic cycle, with consequent accumulation of acidophilic ALVs and damaged organelles such as mitochondria, ultimately leading to cell death. A recent cell culture study showed that MTOR plays a role in both phases of the autophagic cycle and that inhibition of MTOR therefore has two distinct effects on the process. First, MTOR inhibition causes enhanced autophagosome formation, followed by failure of MTOR-dependent lysosomal reformation, which is accompanied by decreased clearance of damaged organelles due to accumulation of autophagolysosomes. In this sense, autophagy stimulated by naturally occurring endogenous factors or events such as starvation or growth factor withdrawal has a very different effect on the cell than autophagy that is initiated through pharmacologic or genetic inhibition of MTOR. Although starvation or loss of growth factor signals initiate autophagy through inhibition of MTOR, continued starvation, paradoxically, leads to upregulation of MTOR activity, establishing a negative feedback loop that is important for autolysosomal vesicle recycling and cell survival (Figure 3A). In the setting of rapamycin or a knockout of the Mtor gene, this negative feedback loop is blocked and autophagic vesicles will accumulate, will fail to recycle, and the process will arrest, resulting in toxicity to the cell. On the basis of these data, we posit that disruption of both steps of the autophagic cycle contribute to the development of dramatic glomerular injury observed in Mtor pod-KO mice.

We tested this model in human podocytes treated with rapamycin. Human podocytes were stably transfected with an LC3-GFP construct. After exposure to rapamycin, accumulation of fluorescent ringed structures was observed (Supplemental Movie 1 and Figure 4A) consistent with an increased number of AVs and enhanced level of autophagy. Western blot analysis confirmed an upregulation of the AV-associated isoform LC3-II in the rapamycin-treated human podocytes. These findings were associated with coincident inhibition of phosphorylated S6K, a marker of mTORC1 activity (Figure 4B).

To determine whether ALVs accumulate after MTOR inhibition (i.e., arrest of the autophagic cycle), we studied distribution of LC3-GFP puncta and Lysotracker, a lysosomal marker. After 5 days of exposure to rapamycin, there was almost complete co-localization of LC3-GFP and acidophilic Lysotracker Red (Figure 4A). In addition, there was evidence of enhanced Mitosox staining indicative of superoxide activity derived from mitochondria in podocytes treated with rapamycin. This suggests an accumulation of dysfunctional mitochondria that would normally be cleared by autophagy (Figure 4A). Consistent with the model in Figure 3A, ALV accumulation in podocytes is transient under starved conditions, coinciding with a transient downregulation of MTOR activity (Figure 4, C and D). At 4 hours of starvation, the autophagosome and
lysosomes often co-localized. After 10 hours of starvation, there was almost complete clearance of LC3-GFP–labeled vesicles and restoration of lysosomal size. Although phosphorylated S6K is downregulated after 4 hours of starvation alone, it recovers by 6 hours, thereby demonstrating reactivation of mTORC1.

By contrast, administration of rapamycin results in massive accumulation of ALVs in podocytes as demonstrated by LC3 and Lysotracker markers (Figure 4C) and robust suppression of phosphorylated S6K that does not recover even by 6 hours (Figure 4D). These findings were recapitulated in vivo in the Mtor pod-KO mouse model (Figure 2). Specifically, LC3-II is increased in glomeruli isolated from mutant mice (Figure 2H) and EM studies revealed large intravesicular structures characteristic of AVs and ALVs together with accumulation of damaged mitochondria in podocytes of mutant mice (Figure 2G) (data not shown) consistent with a substantive defect in lysosomal reformation.

Our data support a model whereby MTOR inhibition in podocytes results in enhanced initiation of autophagy followed by failure to regenerate key components of this process (Figure 3B). Why might this be important? Although reduced autophagy can lead to cell damage in podocytes, activation and dysregulation of autophagy are also likely to result in susceptibility to glomerular disease in patients. This is notably apparent in patients with lysosomal storage diseases, such as Fabry’s disease, aspartyl-glucosaminuria, or Scheie’s disease, in which failure of lysosomes to acidify causes failure of lysosomal reformation and these patients are also prone to developing proteinuria. In addition to the kidney, there are a number of other pathologic states, including cystic fibrosis, certain myopathies, and neurodegenerative diseases, characterized by dysfunctional or incomplete autophagy causing tissue damage.

Our results suggest that dysregulation of autophagy may be an important component in the pathogenesis of proteinuria and glomerular injury in patients treated with MTOR inhibitors and perhaps other types of glomerular disease. Although actin cytoskeletal defects and reduction of Vegfa may contribute to this damage, the Mtor pod-KO phenotype does not resemble the phenotypes observed in mouse models with primary defects in either of these pathways. In turn, these data suggest that strategies to protect the cell from enhanced and/or dysregulated autophagy may be useful in protecting the kidney during treatment with MTOR inhibitors.

Finally, it is important to consider these results in light of recent data demonstrating a critical role for Mtor activation in the development and progression of diabetic nephropathy. Haploinsufficiency for Rptor slowed the development of glomerular changes in diabetic mice, suggesting that rapalogs...
may be useful agents to treat diabetic nephropathy. Our findings highlight the importance of tight MTOR regulation in podocytes and suggest that the use of rapalogs to treat renal disease must be considered with caution.

### Concise Methods

#### Generation of Mtor Pod-KO
Podocin-Cre recombinase transgenic mice were used as previously described. A detailed targeting strategy was designed for the Mtor gene using BAC recombineering to generate a floxed Mtor gene. The first loxP site was introduced into the unconserved 5' flanking region of the Mtor promoter and the second loxP site into an unconserved region in intron 3. Correctly targeted clones analyzed by Southern blot were used for aggregation to produce chimeras. After identification of F1 offspring carrying the targeted allele, the neomycin selection cassette was removed by breeding Mtor<sup>wt/lox</sup>neo-in mice to a mouse carrying the FLPe recombinase transgene. Heterozygous Mtor<sup>wt/fllox</sup> mice were bred to mice carrying a pCaggs-Cre transgene to generate Mtor<sup>wt/del</sup> and then bred to the Podocin-Cre driver mouse strain. Mtor<sup>wt/del</sup> Podocin-Cre mice were bred to Mtor<sup>flfl/Del</sup> mice to obtain Mtor<sup>fl/del</sup> Podocin-Cre double-transgenic mice.

#### Phenotypic Analysis
Spot urine was collected from mice 1, 2, 3, and 4 weeks of age. We used a urine dipstick (Chemstrip7; Roche Diagnostics Corp) to detect the presence or absence of protein in the urine. A standard colorimetric assay for urine protein and urine creatinine was performed according to the manufacturer's instructions (Sigma) to estimate urine protein/creatinine ratios.

#### Histologic Analysis and Immunofluorescence
Kidneys from mice 1, 2, 3, and 4 weeks of age were dissected and fixed in 10% formalin in PBS and then embedded in paraffin for histologic analysis. Sections 4 μm in thickness were cut and stained with periodic acid–Schiff or hematoxylin and eosin and then examined and photographed with a DC200 Leica camera and Leica DMLB microscope (Leica Microsystems Inc).

Immunofluorescent tissue staining for podocin was performed using a rabbit anti-podocin antibody (P0372; Sigma).

#### In Situ Hybridization
Kidneys from mice at 2, 3, and 4 weeks of age were dissected and washed briefly in RNAase-free PBS and then embedded in paraffin for histologic analysis. Sections 4 μm in thickness were cut and stained with periodic acid–Schiff or hematoxylin and eosin and then examined and photographed with a DC200 Leica camera and Leica DMLB microscope (Leica Microsystems Inc).

In situ hybridization was performed using a rabbit anti-podocin antibody (P0372; Sigma).

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**Figure 4.** Chronic MTOR inhibition disrupts the autophagic pathway in podocytes in vitro. (A) Human podocytes stably transfected with LC3-GFP and treated with rapamycin exhibit increased fluorescent-tagged ringed structures characteristic of autophagosomes. The AVs colocalize with the lysosomal marker Lysotracker Red. Podocytes exposed to rapamycin also demonstrate increased superoxide activity as shown by Mitosox Red, suggestive of mitochondrial damage. (B) Western blot analysis confirms downregulation of phospho-S6K (pS6K) and inhibition of mTOR activity in podocytes exposed to rapamycin along with upregulation of the autophagosome-associated LC3 isoform. (C) Podocytes exposed to starvation show only a transient increase in ALVs. In contrast, rapamycin-treated cells exhibit prolonged and marked accumulation of ALVs. ALVs are marked by colocalization of the lysosomal dye (Lysotracker Red) and GFP-LC3 (green). (D) Western blot analysis shows recovery of mTOR activity after 6 hours of starvation as evidenced by increasing levels of phospho-S6K. In contrast, rapamycin-treated cells show continued suppression of phospho-S6K.
frozen. Sections 10 μm in thickness were cut on a Leica Jung cryostat (model CM3050; Leica Microsystems Inc) and transferred to Superfrost microscope slides (Fischer Scientific Co.). Digoxigenin-labeled probes were prepared according to the Roche Molecular Biochemicals protocol (Roche Molecular Biochemicals). Probes used for in situ analysis were Nphs1, Vegfa, and Wt1. Further details regarding the in situ protocol can be obtained on request.

Cell Culture
The immortalized human podocyte cell line was a kind gift from Moin Saleem (University of Toronto, University of Toronto). It was previously characterized.38 Cells were maintained in RPMI 1640 medium supplemented with 10% FBS and insulin-transferrin-selenium at 33°C.

Human podocytes were co-transfected with a pCMV-GFP-LC3 expression vector (Cell Biolabs Inc) and a marker plasmid expressing a puromycin-resistant gene. Two days after transfection, cells were selected in 2 mg/L of puromycin (Sigma) until resistant colonies of cells appeared. GFP positive clones were selected. Cells were treated with 50–1000 ng/ml of rapamycin (R878; Sigma) in RPMI.

lysotracker Red (Molecular Probes) or Mitosox Red (Invitrogen) was incubated with selected. Cells were treated with 50 μM, respectively, for 20 minutes. After 20 minutes, cells were washed with PBS and visualized by confocal microscopy.

Un transfected human podocytes were used for Western blotting. Cells were treated with vehicle, 10, 100, 500, and 1000 ng/ml rapamycin for 5 days and were then lysed using RIPA buffer (5 ml 1M tris-HCl pH 7.4, 30 ml 5M NaCl, 5 ml 20% Triton-X, 5 ml 10% sodium deoxycholate, 0.5 ml 20% SDS, 50 ml ddH2O). Protein was isolated and concentration was determined by Bradford assay (BioRad).

Isolation of Mouse Glomeruli
Glomeruli were isolated from 3-week-old mice using Dynabead (Invitrogen) magnetic bead perfusion as previously described.39,40 Glomeruli from four mice were pooled in each sample and homogenized in radiimmunoprecipitation assay buffer. Protein concentration was determined by Bradford assay (BioRad).

Western Blotting
Protein electrophoresis was performed on protein isolated from mouse glomeruli or cultured human podocytes. Antibodies against MTOR (#2972; Cell Signaling), LC3 (PM046; MBL), phospho-P70S6K (#9205; Cell Signaling), total P70S6K (#9202; Cell Signaling), and β-actin (ab8227; Abcam) were used. A goat anti-rabbit IgG-horseradish peroxidase secondary antibody was used for all of the above primary antibodies (sc-2054; Santa Cruz).

ACKNOWLEDGMENTS
We thank Doug Holmyard and Ken Harpal for electron microscopy and histologic stains and Anne-Claude Gingras, John Brummell, and the entire Quaggin laboratory for helpful discussions. We also thank Jeffrey Zaltzman whose thoughtful question stimulated this study.

This work was supported by the Kidney Foundation of Canada (KFOC110014), Canadian Institutes of Health Research (CIHR MOP29351 and MOP77756), and CIHR Team Grant/Grants (CIHR MOP62931 and MOP77756), and CIHR Canadian Institutes of Health Research D.P. C. is supported by an American Society for Clinical Investigation Merit Award, an American Society of Nephrology Team Grant/Terry Fox TFF105268 to S.E.Q.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2011070690/-/DCSupplemental.
Supplementary Table 1: Normal Mendelian distribution of pups born from breeding $Mtor^{wt/del}$ Podocin-Cre mice to $Mtor^{flox/flox}$ mice.

Supplementary Figure 1: $Mtor$ protein is reduced in glomeruli from $Mtor$ haploinsufficient mice ($Mtor^{wt/del}$)

Supplementary Figure 2: Molecular markers of podocyte differentiation.

A. Immunostaining for podocin shows similar levels at 2 weeks of age in mutants and controls, but downregulation at 3 weeks of age in mutants, although staining pattern is similar.

B. At 3 weeks of age, Wilms tumor protein 1 ($Wt1$) and $Nphs1$ are not different between mutants and control mice.

C. Vascular endothelial growth factor A ($Vegfa$) expression in glomeruli. In situ analysis shows that $Vegfa$ expression in podocytes is similar in mutants and controls at 2 weeks of age but is downregulated in mutants at 3 weeks of age.

Supplementary Movie:

Human podocyte transfected with LC3-GFP shows accumulation of ringed structures characteristic of autophagosomes upon exposure to rapamycin.
Supplementary table 1: Normal mendelian distribution of pups born from breeding $M_{\text{tor}}^{\text{wt/del}}$ Podocin-Cre mice to $M_{\text{tor}}^{\text{flox/flox}}$ mice.

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mTOR flox/del

mTOR

b-actin