Supplemental information

Primer information

	Forward (5'-3')	Reverse (5'-3')
nephrin	CCAGAGGGGCAGAGCATCC	TCACCGGGCGCGAATCCTTA
podocin	GCTGCCACCGCCACCGTAGT	GATCAGGGAGGCGAGGACAAGAAG
cadherin3	GAGGGAGTGTTCTGGAGGGAGTA	CCGTGCTTTTATGGATGGTGAAC
actinin4	CTGAGACCGCTGCCAACC	TGCGCCGAATCCACTCTA
WT-1	GCATAGCCGGAAGCACA	GTCGGACCGGGAAAACT
ILK1	CCGCTGGCAGGGCAATGATA	GGGGAGCCTGGCAAGCACCTA
TGF-BETA1	GCGGACTACTATGCTAAAGAGGT	AGCAATGGGGGTTCGGGCACTG
ATIIRtype2	GATGGAGGGAGCTCGGAACT	TTGAACTGCAGCAACTCCAAATT
Fibronectin	GGGCTTTGGCAGTGGTCATTTCA	TCATCCGCTGGCCATTTTCTCC
COL4alppha3	CCAGGTGACCAAGGGCATCCAG	GGTCCCCTTTCCTTCCACTCAG
ACTIN BETA	ACCGTGAAAAGATGACCCAG	AGCCTGGATGGCTACGTACA
HGPRT	TGTTGTTGGATATGCCCTTG	TTGCGCTCATCTTAGGCTTT

Supplemental Figure Legends

Supplemental Figure 1.

Rapamycin prevents the development of DN in db/db mice.

(A) Rapamycin treatment prevents mesangial expansion. db/+ and db/db mice were treated with or without rapamycin (1mg/kg BW, ip, 3 times/week) from 8 to 25 week. Representative Hematoxylin-Eosin (H&E) staining of glomeruli from the indicated mice at 25 weeks of age is shown (male, n=8 each group). Original magnification: x 400.

(B) Rapamycin prevented albuminuria in db/db mice. The ratio of urinary albumin and creatinine concentrations of 25 weeks of age animals is shown (male, *p<0.001 vs other groups, mean±SD, n=8).

Supplemental Figure 2.

Characterization of podocyte-specific *Tsc1* knockout (PcKOTsc1) mice.

(A) Birth ratio of PcKOTsc1 mice. [*Tsc1*$^{flox/+}$, Tg (*NPHS2*-cre)$^{+/-}$] x [*Tsc1*$^{flox/flox}$, Tg (*NPHS2*-cre)$^{-/-}$] mating was used for this study. All genotypes were born at the expected Mendelian ratio.

(B) Time course of the mTORC1 activation in podocytes in PcKOTsc1 mice. The renal tissues from the indicated old animals were stained with phospho-specific S6 antibody. Enhanced S6 phosphorylation was observed in the PcKOTsc1 mice podocytes starting 2 weeks of age.

(C) A maximal diameter of the circular podocytes located around the center of glomerulus in SEM images was directly measured (*p<0.001 vs other groups, mean±SEM, no statistical significance between wild type and PcKOTsc1 treated with rapamycin, n=30~50 podocytes from 3 mice in each group).

(D) Eosin positive area in glomerulus was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3). Data were expressed as a percentage of Eosin positive area / glomerular area.

(E) PAS positive area in glomerulus was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3). Data were expressed as (D).

(F) Total cell number (hematoxylin positive nuclei) was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3).

(G) qRT-PCR analysis of the indicated mRNA in isolated glomeruli from wild type (Wt) and PcKOTsc1 mice.

Data were normalized to HPRT1 (*p<0.05 vs wild type, mean±SEM, n=7).

(H) The expression of TGF-β1 in the indicated genotypes was shown. Quantification of positive signals / glomerular area from 30 glomeruli in the indicated genotypes was shown. Data were expressed as a fold induction (*p<0.01 vs wild type, mean±SEM, n=3)

(I) The expression of angiotensin type2 receptor (AT-2R) was examined by immunohistochemistory and western blotting using glomerular proteins purified from the indicated genotypes. HSP90 as a loading control. The relative protein levels (AT-2R / HSP90) were quantified and the data were expressed as a fold reduction (*p<0.05 vs wild type, mean±SEM, n=3). Original magnification: x 200 (B), x 400 (H and I).

Supplemental Figure 3.

Podocyte-specific TSC1 KO mice show proteinuria and ascites.

(A) Time course of the proteinuria in PcKOTsc1 mice. 1 μl of urine from the indicated mice was subjected to SDS-PAGE. Urinary proteins were visualized by Coomassie blue staining. The data demonstrate a correlation between onset of proteinuria and mTORC1 activation.

(B) Pictures of representative 8 week-old Wt and PcKOTsc1 mice. Right image demonstrates massive ascites in a 6 week old PcKOTsc1 mouse.

Supplemental Figure 4.

Podocyte-specific TSC1 KO mice show renal failure.

(A) Serum creatinine concentrations in 8 week-old wild type and PcKOTsc1 mice were determined by ELISA.

The serum creatinine concentrations were significantly elevated in PcKOTsc1 mice (*$p<0.001$ vs wild type, mean±SEM, n=7). Note upper normal range is around 0.5 mg/dl.

(B) Serum ALT activity (sGPT) was measured in 8 week-old wild type and PcKOTsc1 mice. The serum ALT activities of PcKOTsc1 mice were higher than those of wild type, but within normal range (*$p<0.01$ vs wild type, mean±SEM, n=7). Note upper normal range of ALT activity in mice is around 50 (IU/L).

Supplemental Figure 5.

B6 background podocyte-specific TSC1 KO mice show proteinuria and glomerulosclerosis.

(A) C57BL/6J background TSC1 KO mice show proteinuria. Urinary proteins from the indicated animals were visualized by Coomassie blue staining. 1 µl of spontaneous urine from the indicated mice was subjected to SDS-PAGE.

(B) Urinary albumin concentrations were determined by ELISA (*$p<0.001$ vs Wt, mean±SEM, n=8).

(C) C57BL/6J background TSC1 KO mice develop glomerulosclerosis. Representative H&E stains of the renal cortex in mice of age 12 weeks are shown. Original magnification: x 200 (left panels), x 400 (right panels).

Supplemental Figure 6.

mTORC1 activation in podocyte does not affect Neph1 localization.

(A) NEPH1 expression in the indicated glomerulus. NEPH1 expression in PcKOTsc1 mice and rapamycin-treated PcKOTsc1 mice were weaker than those in Wt. However, the pattern of membrane localization of Neph1 was still conserved in PcKOTsc1 mice. Original magnification: x 400.

Supplemental Figure 7.

mTORC1-induced ER stress causes fibroblastic phenotypic change in podocytes.

(A) PBA treatment prevents desmin expression in the podocytes of PcKOTsc1 mice. As rapamycin prevents the expression of desmin in PcKOTsc1 podocytes (Figure 7A, Supplemental Figure 7B, 7C, and 7D), PBA treatment largely inhibited desmin expression in PcKOTsc1 podocytes.

(B) PBA treatment has little effect on S6 phosphorylation in PcKOTsc1 podocytes. In contrast to rapamycin treatment, PBA treatment only suppressed the expression of desmin but not phosphorylation of S6, suggesting that PBA inhibits mTORC1-induced desmin expression at the downstream of mTORC1.

(C) Higher magnification of the double staining using desmin and phospho-S6 antibodies.

(D) The ratio of desmin positive podocyte (desmin positive podocyte/total podocyte) was measured in the indicated animals (10 images per mouse, 3 mice per group, *$p<0.001$ vs other groups, mean±SEM).

(E) Quantification of glomerular pS6 (red) pixel densities divided by glomerular area was shown. Data were expressed as fold induction (10 images per mouse, 3 mice per group,

*p<0.001 vs wild type and KO treated with rapamycin, no statistical significance between KO and KO treated with PBA, mean±SEM).

(F) PBA treatment failed to prevent nephrin mis-localization in PcKOTsc1 mice.

PBA treatment was performed from 2 to 4 week as Figure 7D. Original magnification: x 200 (A and B), x 400 (C and F).

Supplemental Figure 8.

Reduction of mTORC1 complex in podocytes prevents the development of DN.

(A) Relative mesangial area was shown. PAS staining was performed in the indicated renal tissues and PAS-positive mesangial area and glomerular area of 10-20 glomeruli cut at their vascular pole were measured. The data were expressed as mesangial/glomerular area (*p<0.001 vs other groups, mean±SEM, n=6~8).

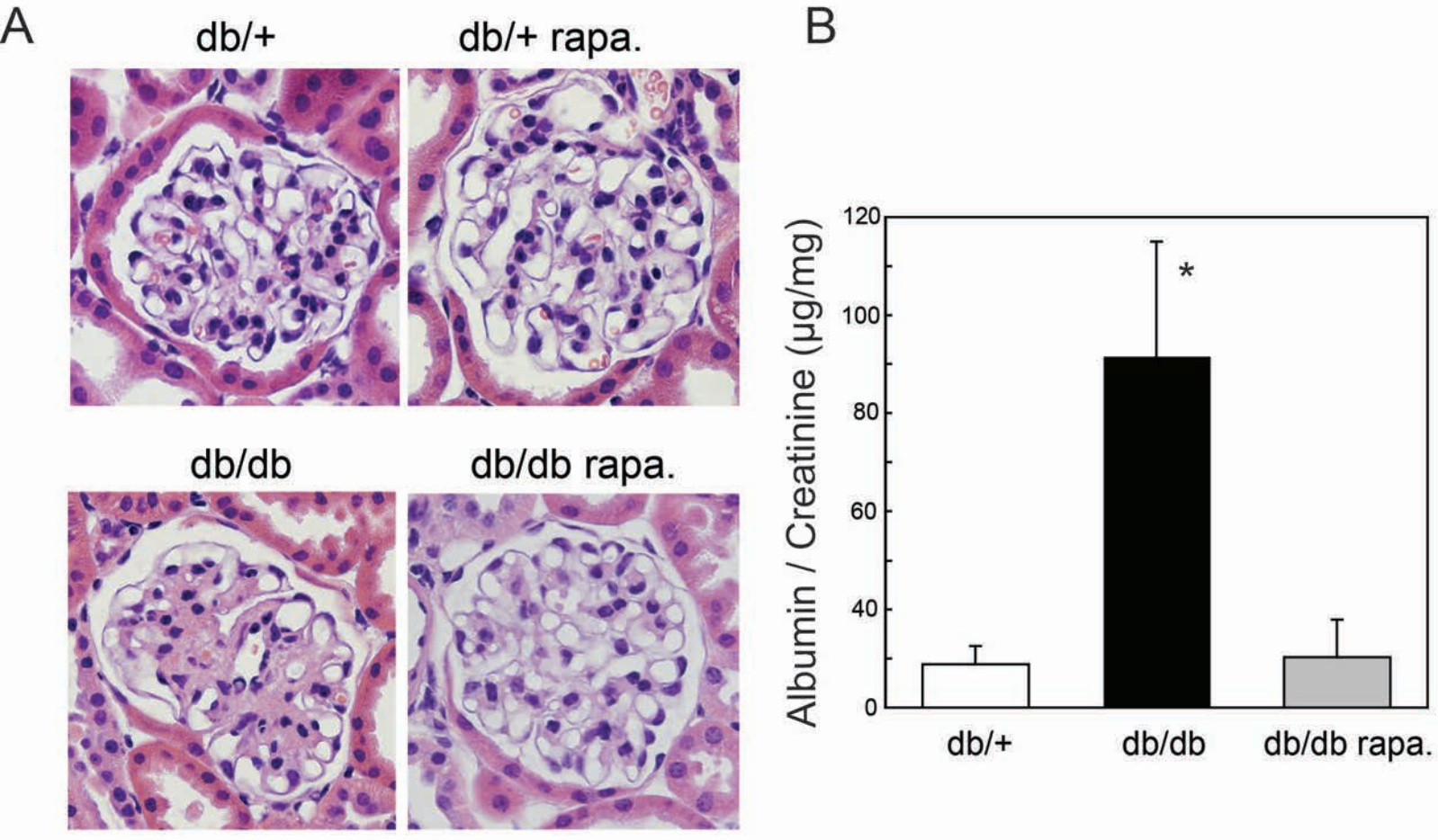
(B) DM *Raptor* +/- mice display a thinner GBM and maintain foot process formation. Representative TEM images from the indicated 40 week- and 53 week-old mice were shown. Original magnification: x 7,900.

(C) Urinary albumin concentration in the indicated animals. Urinary albumin concentrations were measured in 24 hours urine from the indicated animals. (*p<0.05 vs DM and Control, mean±SEM, n=6~8).

Supplemental Figure 9.

A model of mTORC1-dependent podocyte injury in diabetes.

mTORC1 activation in podocytes plays a crucial role in the development of DN. mTORC1 activation in podocytes causes not only podocyte injury including slit diaphragm protein mis-localization and fibroblast-like phenotypic change but also GBM thickening and mesangial expansion.



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Rapamycin prevents the development of DN in db/db mice.
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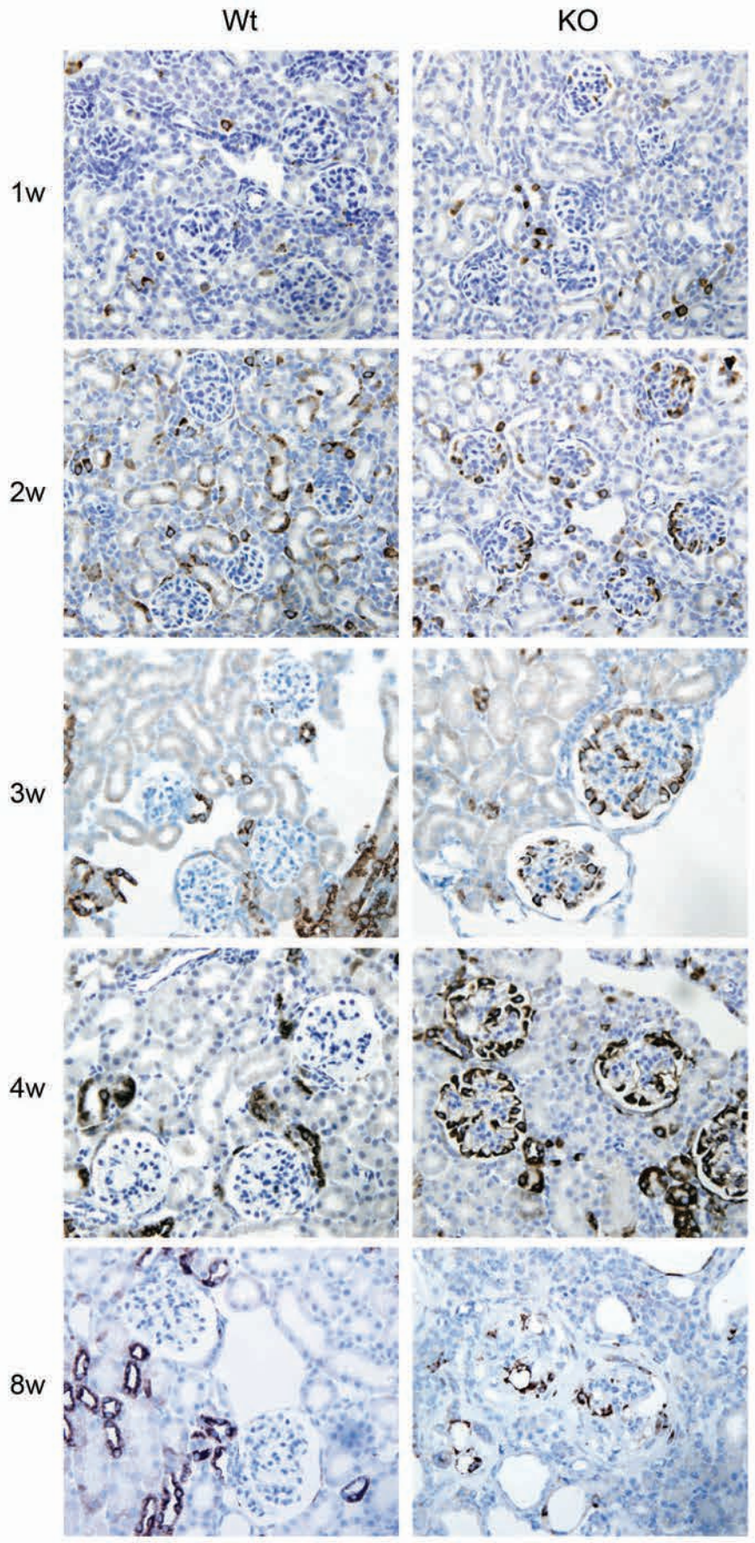
(B) Rapamycin prevented albuminuria in db/db mice. The ratio of urinary albumin and creatinine concentrations of 25 weeks of age animals is shown (male, *$p<0.001$ vs other groups, mean±SD, n=8).

A

FF, +/+ 67/268 Wt	F+, +/+ 61/268 F+	FF, cre/+ 75/268 KO	F+, cre/+ 65/268 Het
25%	23%	28%	24%

B



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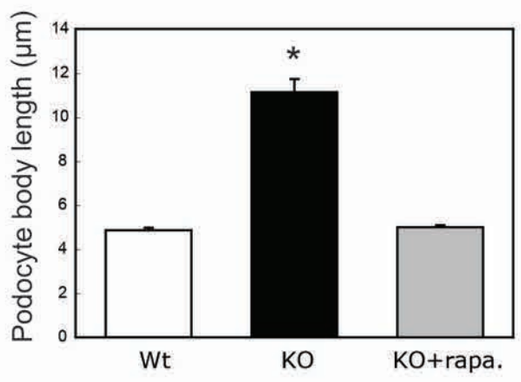

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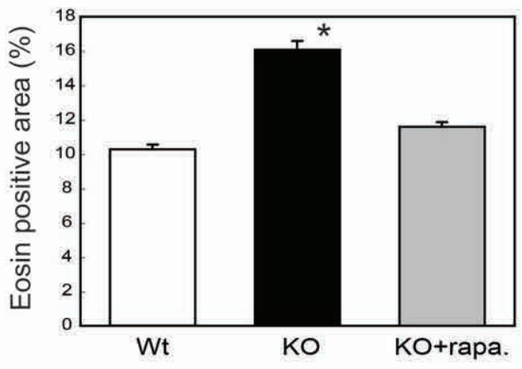

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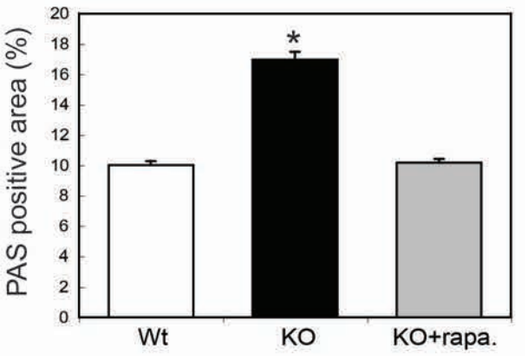

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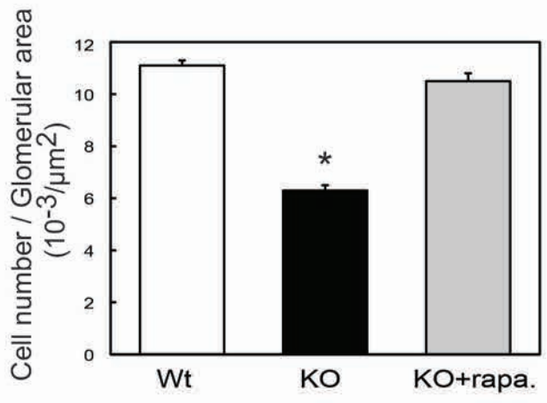

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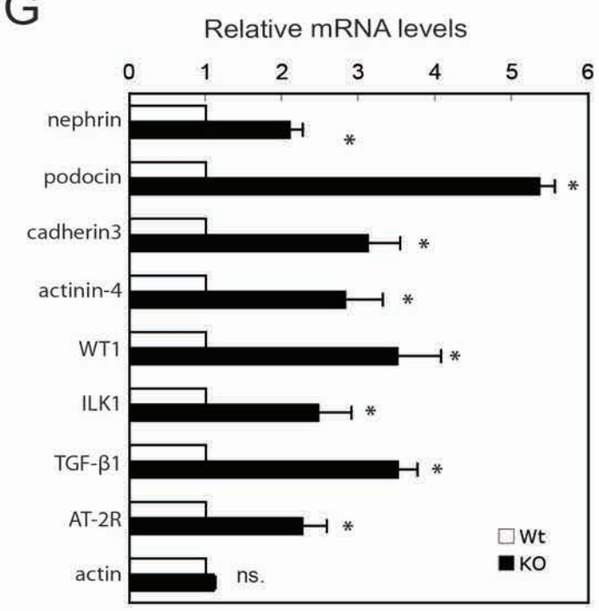

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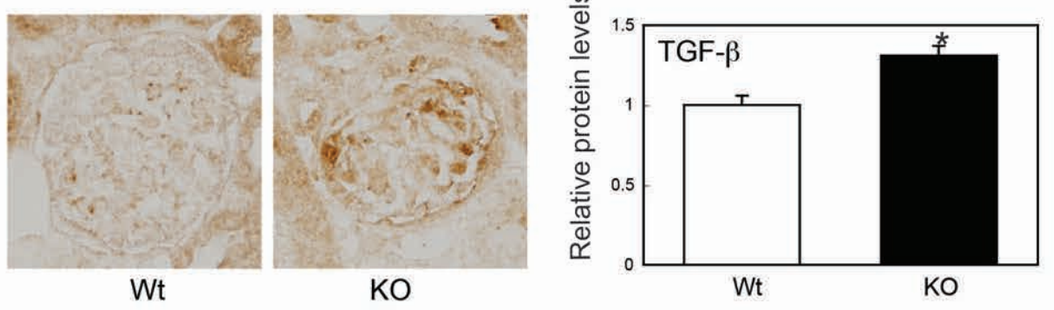

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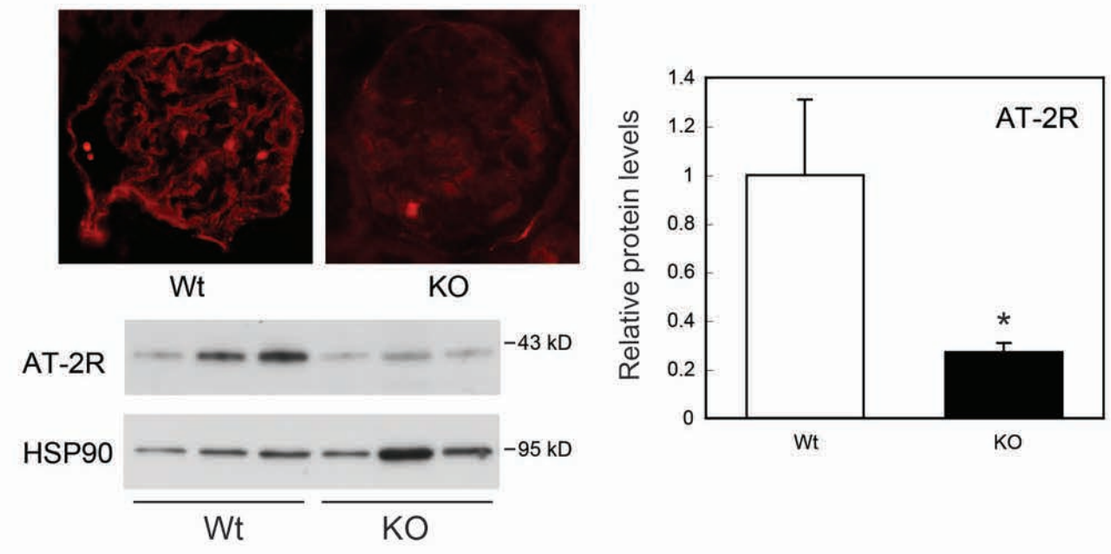

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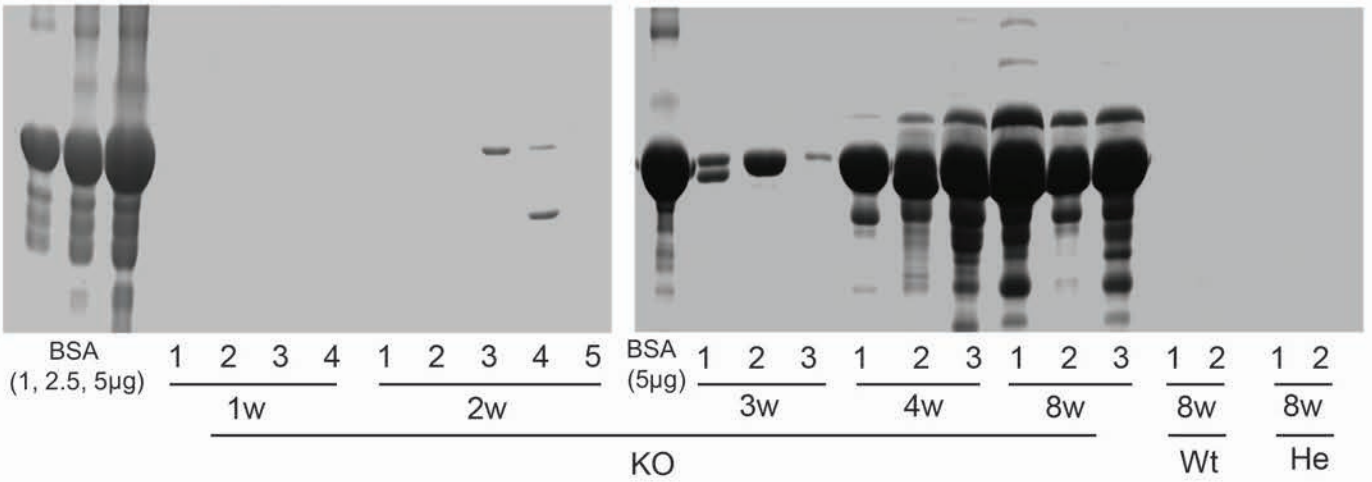
Characterization of podocyte-specific Tsc1 knockout (PcKOTsc1) mice.
(A) Birth ratio of PcKOTsc1 mice. [Tsc1flox/+, Tg (NPHS2-cre)+/-] x [Tsc1flox/flox , Tg (NPHS2-cre)-/-] mating was used for this study. All genotypes were born at the expected Mendelian ratio.
(B) Time course of the mTORC1 activation in podocytes in PcKOTsc1 mice. The renal tissues from the indicated old animals were stained with phospho-specific S6 antibody. Enhanced S6 phosphorylation was observed in the PcKOTsc1 mice podocytes starting 2 weeks of age.
(C) A maximal diameter of the circular podocytes located around the center of glomerulus in SEM images was directly measured (*p<0.001 vs other groups, mean±SEM, no statistical significance between wild type and PcKOTsc1 treated with rapamycin, n=30~50 podocytes from 3 mice in each group).
(D) Eosin positive area in glomerulus was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3). Data were expressed as a percentage of Eosin positive area / glomerular area.
(E) PAS positive area in glomerulus was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3). Data were expressed as (D).
(F) Total cell number (hematoxylin positive nuclei) was measured in 30 glomerular sections from the indicated animals (*p<0.001 vs other groups, n=3).
(G) qRT-PCR analysis of the indicated mRNA in isolated glomeruli from wild type (Wt) and PcKOTsc1 mice.
Data were normalized to HPRT1 (*p<0.05 vs wild type, mean±SEM, n=7).
(H) The expression of TGF-β1 in the indicated genotypes was shown. Quantification of positive signals / glomerular area from 30 glomeruli in the indicated genotypes was shown.
Data were expressed as a fold induction (*p<0.01 vs wild type, mean±SEM, n=3)
(I) The expression of angiotensin type2 receptor (AT-2R) was examined by immunohistochemistory and western blotting using glomerular proteins purified from the indicated genotypes.
HSP90 as a loading control. The relative protein levels (AT-2R / HSP90) were quantified and the data were expressed as a fold reduction (*p<0.05 vs wild type, mean±SEM, n=3). Original magnification: x 200 (B), x 400 (H and I).

A



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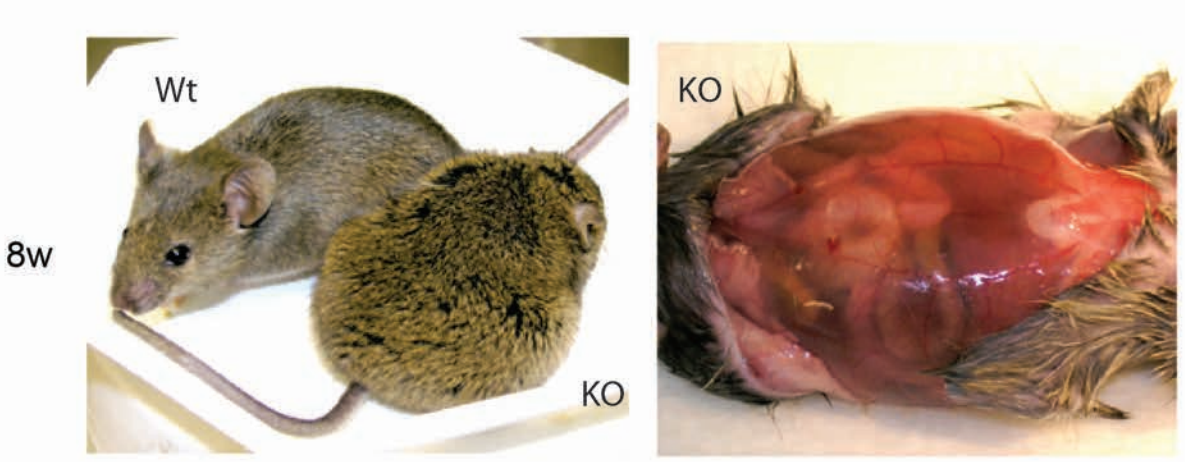
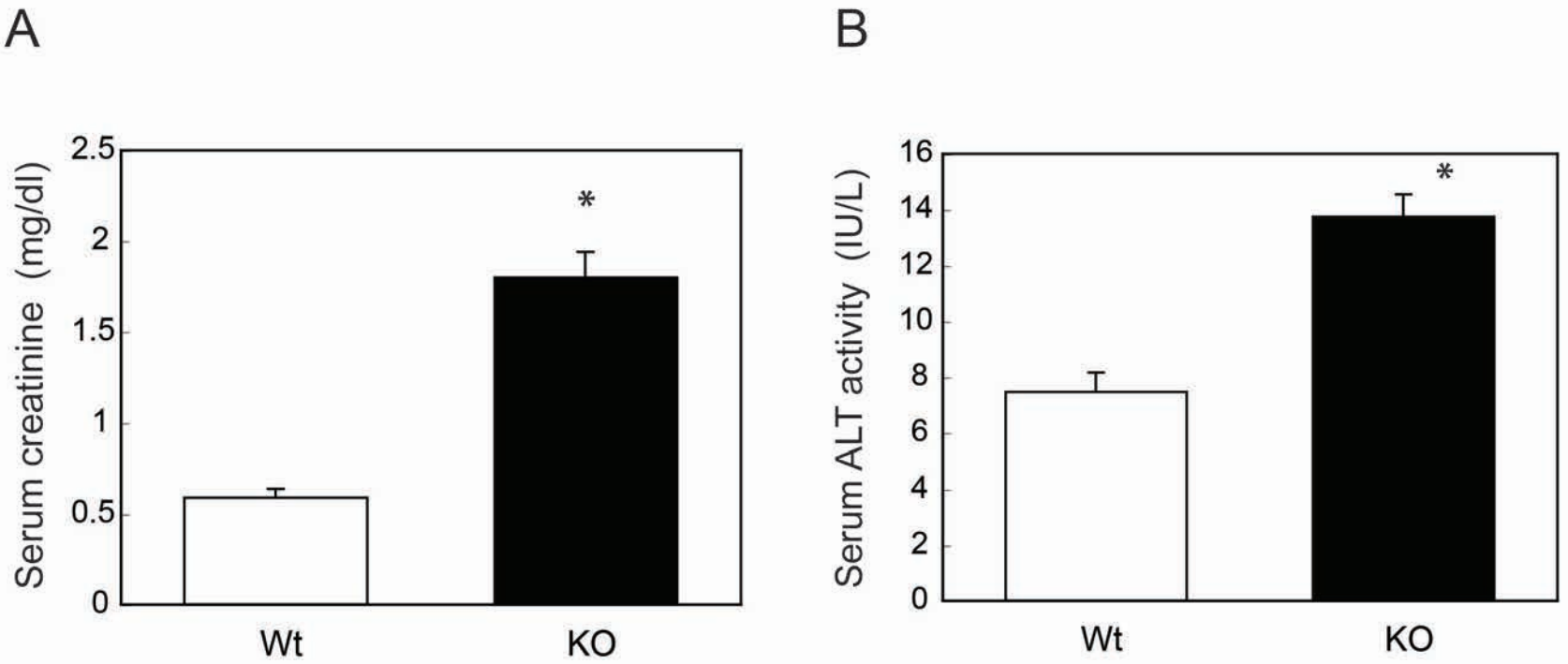
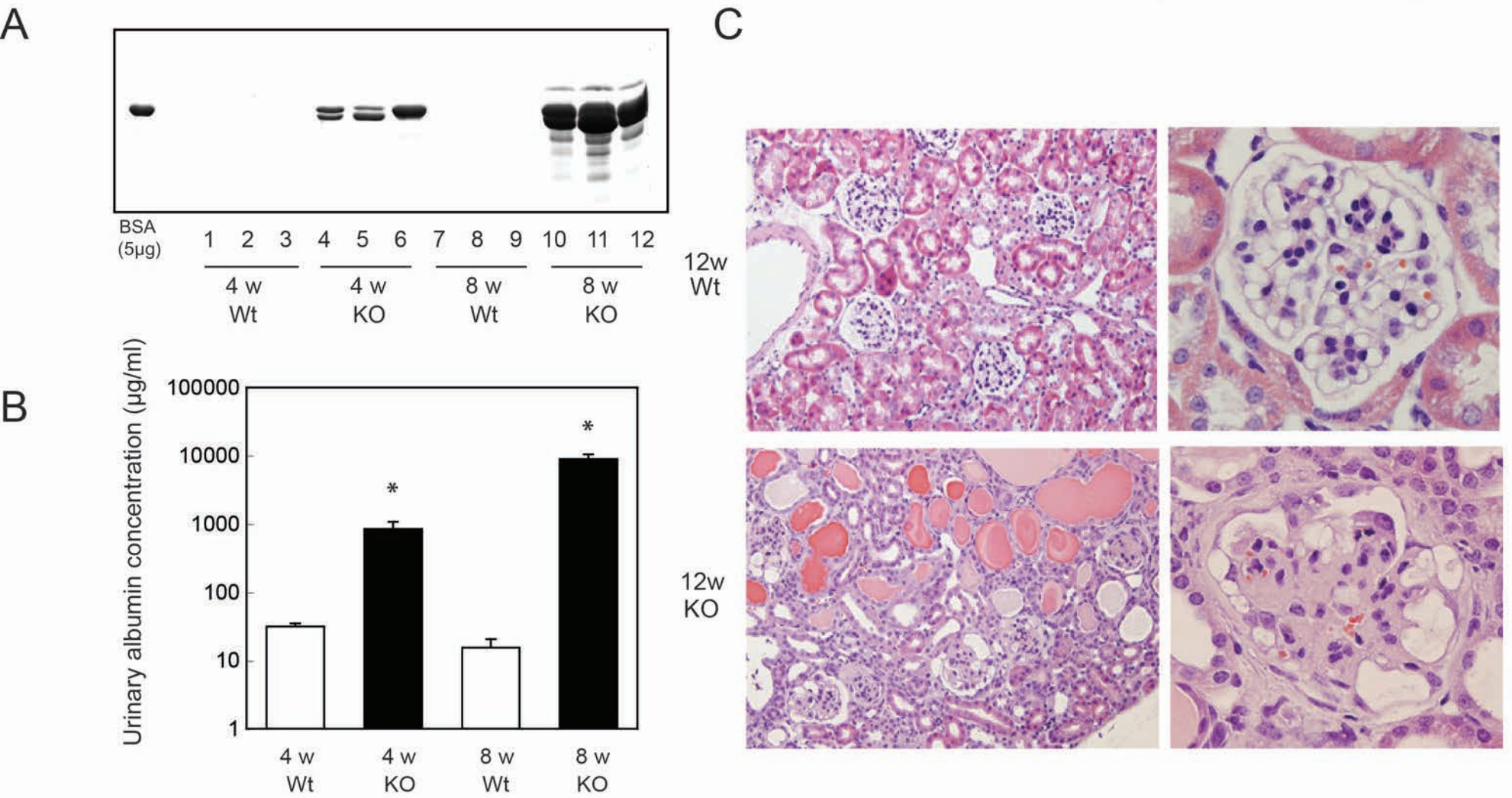


Supplemental Figure 3.
Podocyte-specific TSC1 KO mice show proteinuria and ascites.
(A) Time course of the proteinuria in PcKOTsc1 mice. 1 µl of urine from the indicated mice was subjected to SDS-PAGE. Urinary proteins were visualized by Coomassie blue staining. The data demonstrate a correlation between onset of proteinuria and mTORC1 activation.
(B) Pictures of representative 8 week-old Wt and PcKOTsc1 mice. Right image demonstrates massive ascites in a 6 week old PcKOTsc1 mouse.



Supplemental Figure 4.
Podocyte-specific TSC1 KO mice show renal failure.
(A) Serum creatinine concentrations in 8 week-old wild type and PcKOTsc1 mice were determined by ELISA.
The serum creatinine concentrations were significantly elevated in PcKOTsc1 mice (*$p<0.001$ vs wild type, mean±SEM, n=7).
Note upper normal range is around 0.5 mg/dl.
(B) Serum ALT activity (sGPT) was measured in 8 week-old wild type and PcKOTsc1 mice. The serum ALT activities of PcKOTsc1 mice were higher than those of wild type, but within normal range (*$p<0.01$ vs wild type, mean±SEM, n=7). Note upper normal range of ALT activity in mice is around 50 (IU/L).



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B6 background podocyte-specific TSC1 KO mice show proteinuria and glomerulosclerosis.
(A) C57BL/6J background TSC1 KO mice show proteinuria. Urinary proteins from the indicated animals were visualized by Coomassie blue staining. 1 μl of spontaneous urine from the indicated mice was subjected to SDS-PAGE.
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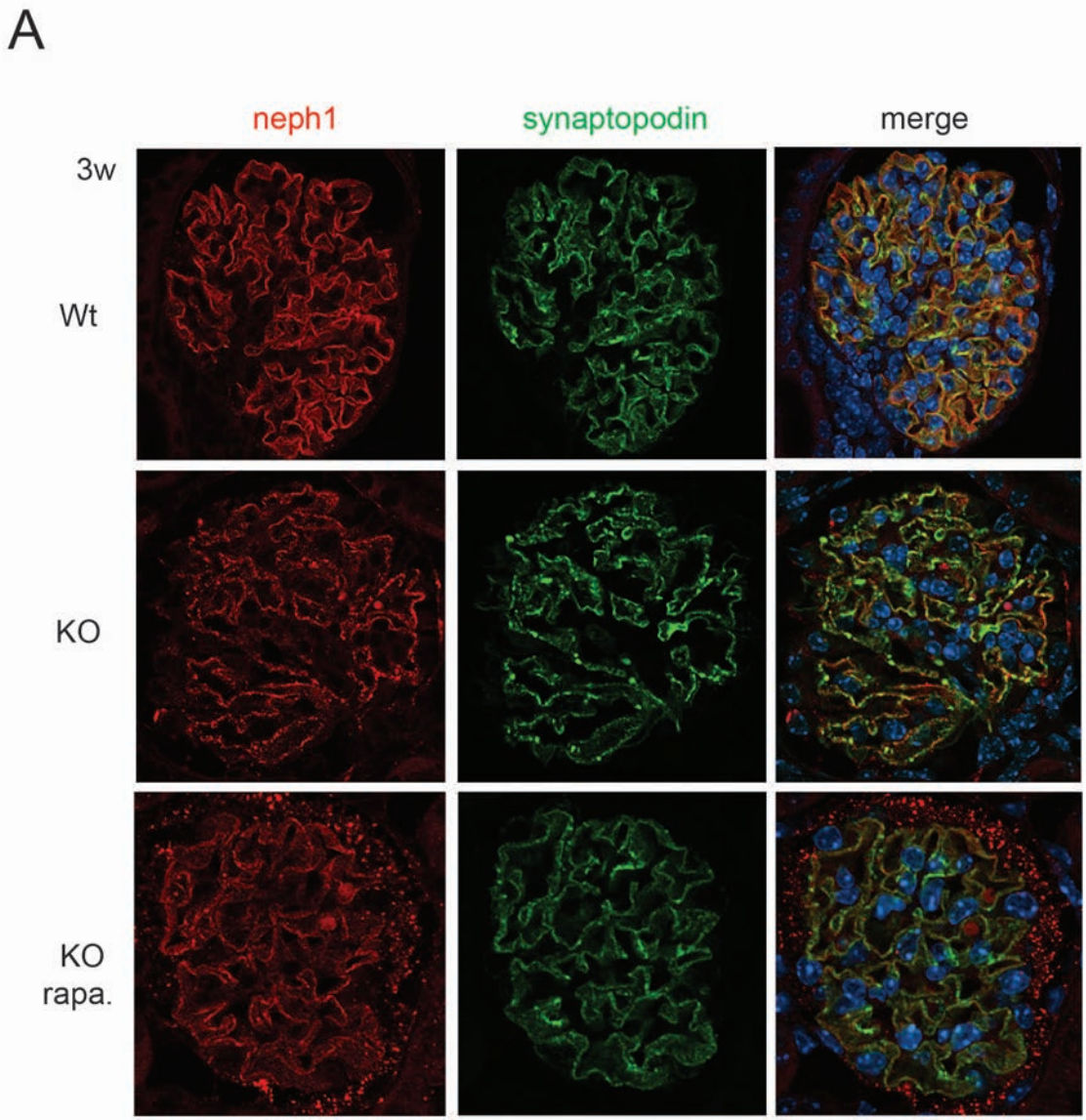
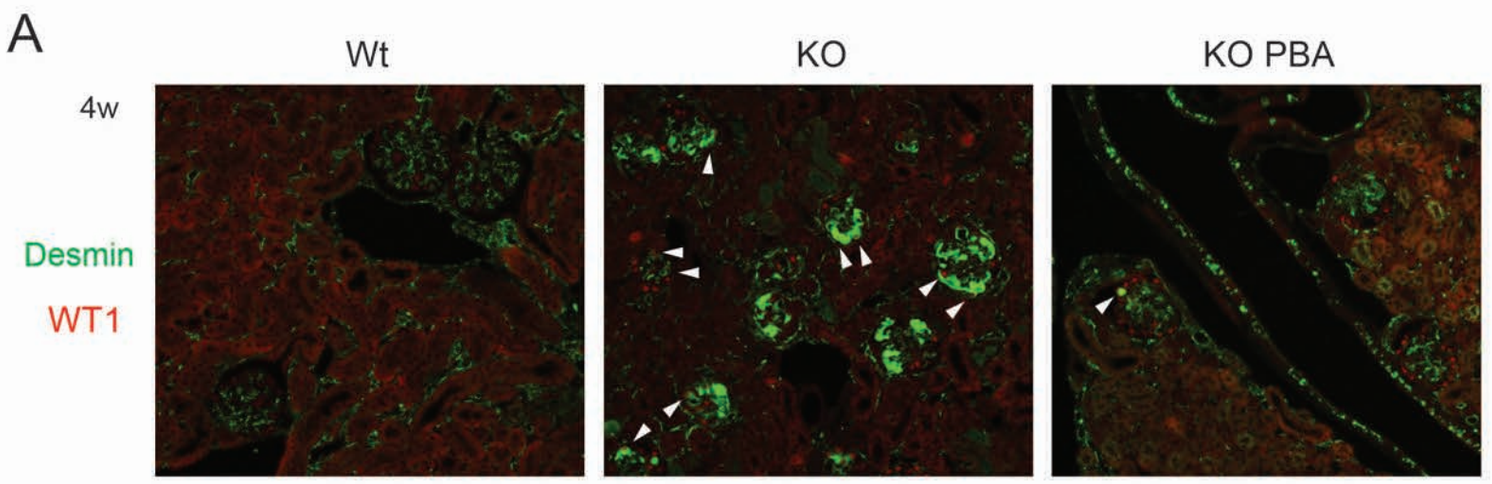
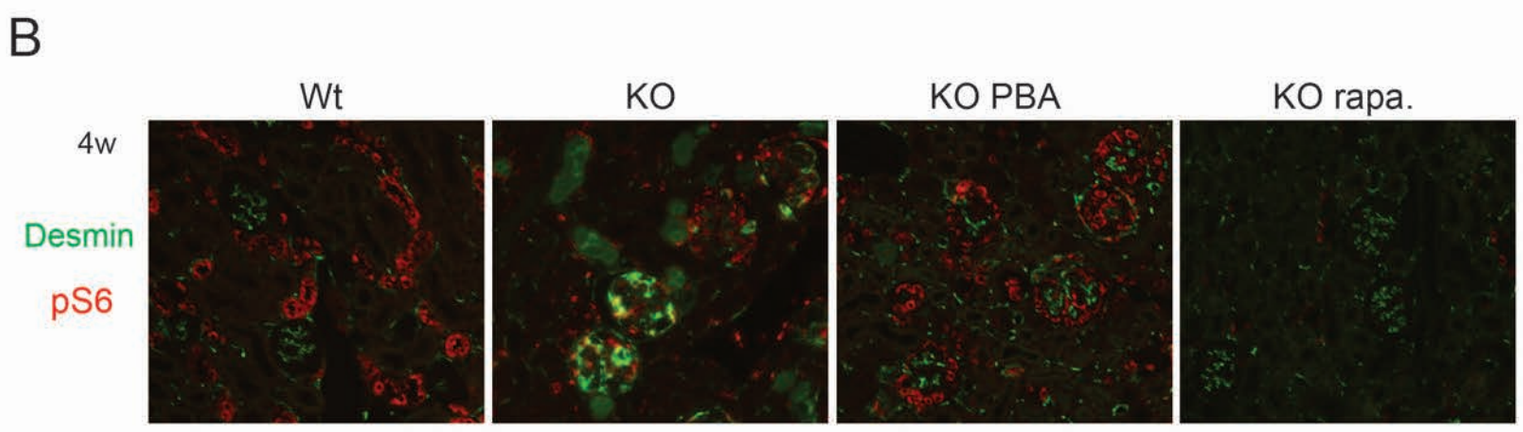
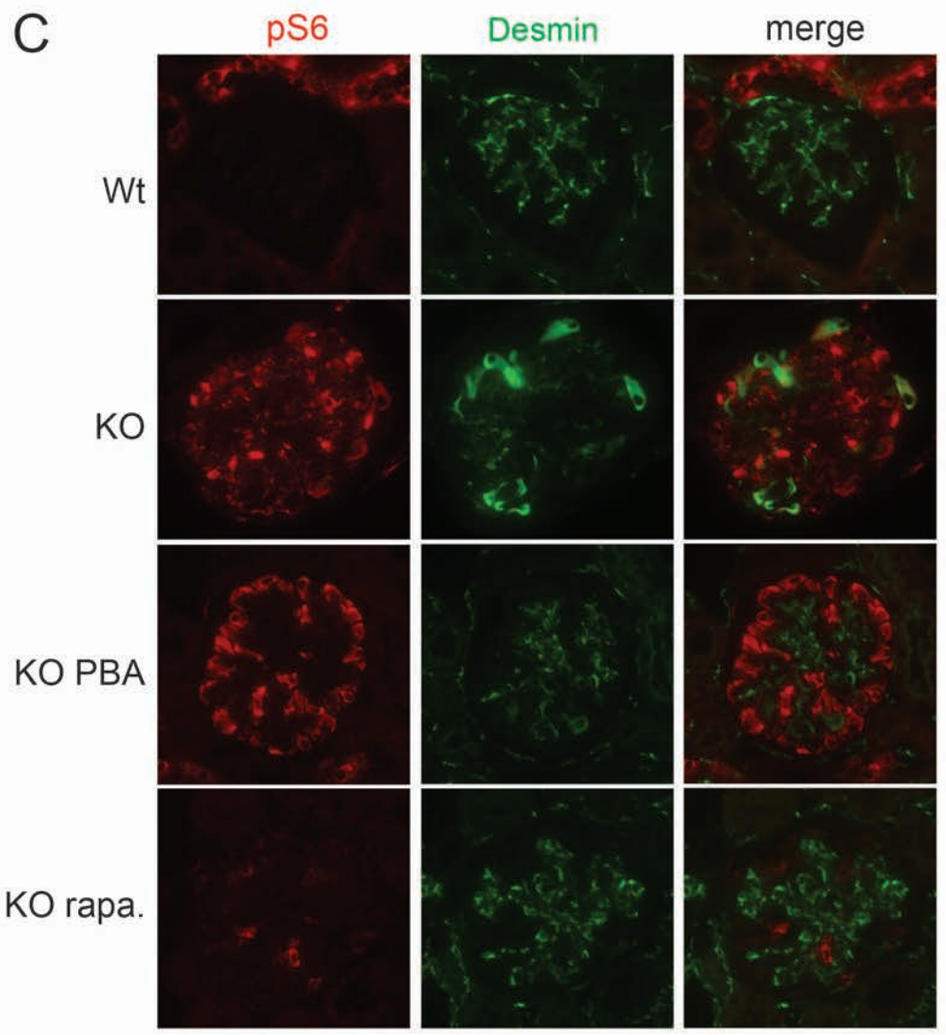
(C) C57BL/6J background TSC1 KO mice develop glomerulosclerosis. Representative H&E stains of the renal cortex in mice of age 12 weeks are shown. Original magnification: x 200 (left panels), x 400 (right panels).

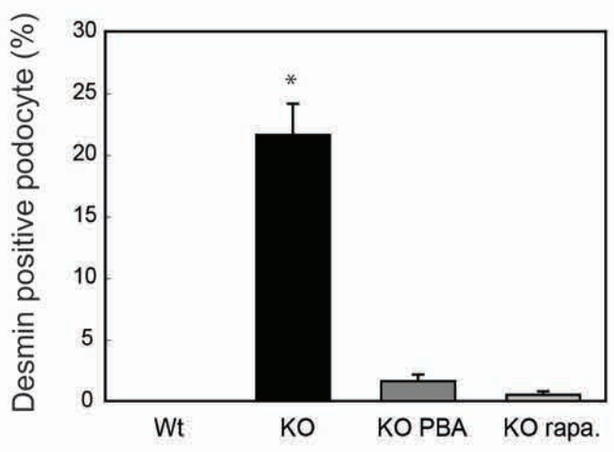


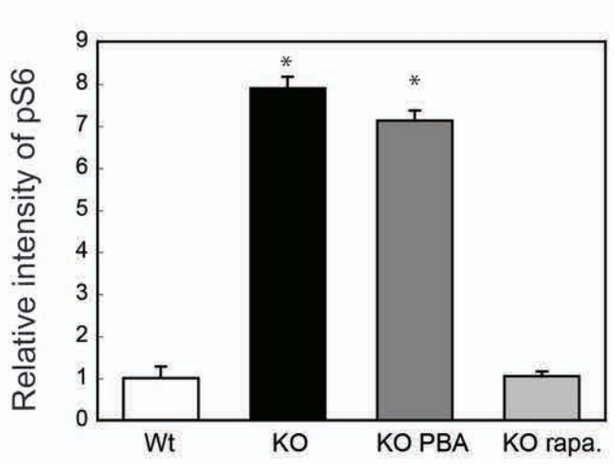
Supplemental Figure 6.
mTORC1 activation in podocyte does not affect Neph1 localization.
(A) NEPH1 expression in the indicated glomerulus. NEPH1 expression in PcKOTsc1 mice and rapamycin-treated PcKOTsc1 mice were weaker than those in Wt. However, the pattern of membrane localization of Neph1 was still conserved in PcKOTsc1 mice. Original magnification: x 400.

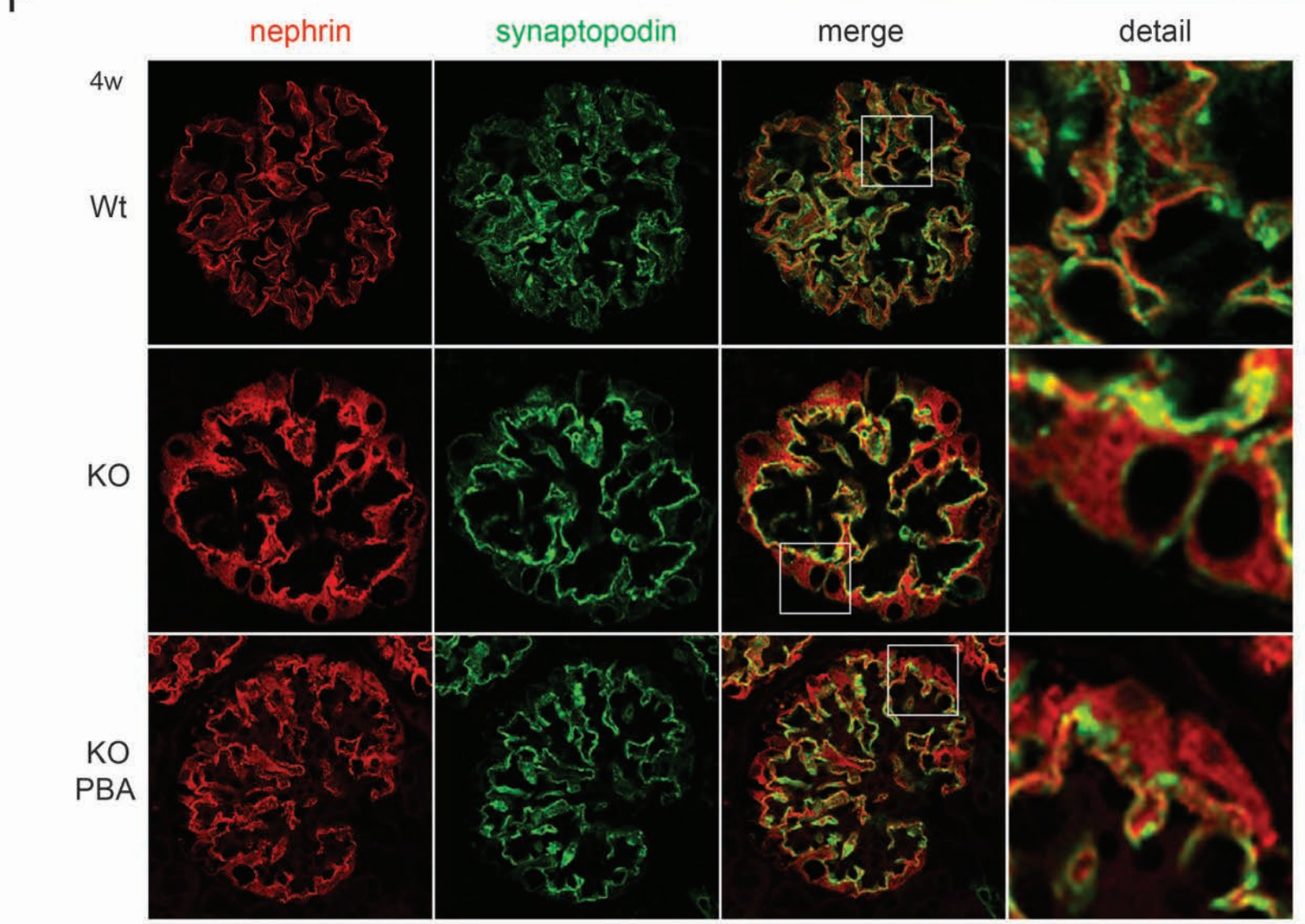






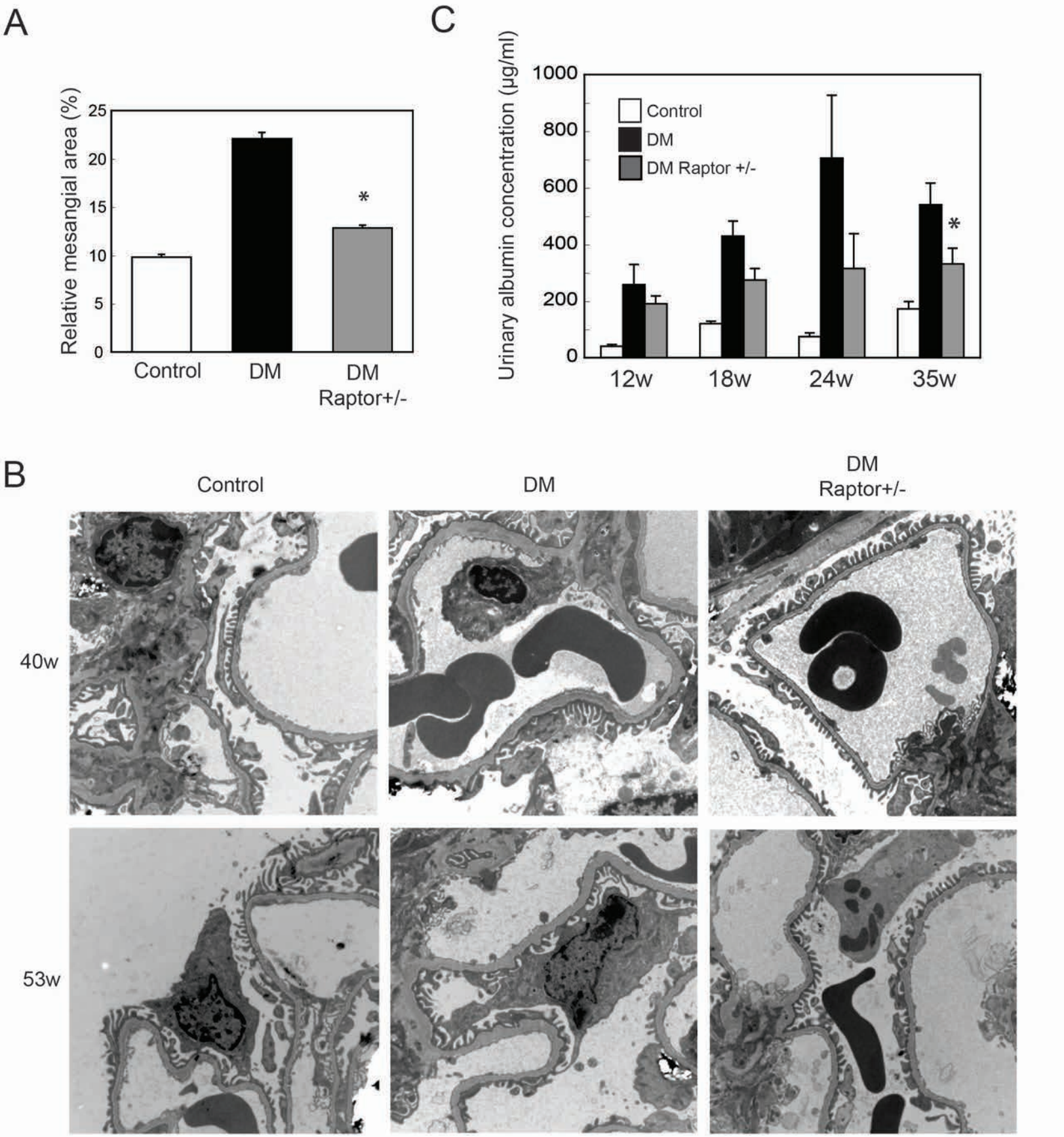






Supplemental Figure 7.
mTORC1-induced ER stress causes fibroblastic phenotypic change in podocytes.
(A) PBA treatment prevents desmin expression in the podocytes of PcKOTsc1 mice.
As rapamycin prevents the expression of desmin in PcKOTsc1 podocytes (Figure 7A, Supplemental Figure 7B, 7C, and 7D), PBA treatment largely inhibited desmin expression in PcKOTsc1 podocytes.
(B) PBA treatment has little effect on S6 phosphorylation in PcKOTsc1 podocytes.
In contrast to rapamycin treatment, PBA treatment only suppressed the expression of desmin but not phosphorylation of S6, suggesting that PBA inhibits mTORC1-induced desmin expression at the downstream of mTORC1.
(C) Higher magnification of the double staining using desmin and phospho-S6 antibodies.
(D) The ratio of desmin positive podocyte (desmin positive podocyte/total podocyte) was measured in the indicated animals (10 images per mouse, 3 mice per group, *$p<0.001$ vs other groups, mean±SEM).
(E) Quantification of glomerular pS6 (red) pixel densities divided by glomerular area was shown. Data were expressed as fold induction (10 images per mouse, 3 mice per group, *$p<0.001$ vs wild type and KO treated with rapamycin, no statistical significance between KO and KO treated with PBA, mean±SEM).
(F) PBA treatment failed to prevent nephrin mis-localization in PcKOTsc1 mice. PBA treatment was performed from 2 to 4 week as Figure 7D. Original magnification: x 200 (A and B), x 400 (C and F).

Supplemental Figure 8



Supplemental Figure 8.
Reduction of mTORC1 complex in podocytes prevents the development of DN.
(A) Relative mesangial area was shown. PAS staining was performed in the indicated renal tissues and PAS-positive mesangial area and glomerular area of 10-20 glomeruli cut at their vascular pole were measured. The data were expressed as mesangial/glomerular area (*$p<0.001$ vs other groups, mean±SEM, n=6~8).
(B) DM Raptor +/- mice display a thinner GBM and maintain foot process formation. Representative TEM images from the indicated 40 week- and 53 week-old mice were shown. Original magnification: x 7,900.
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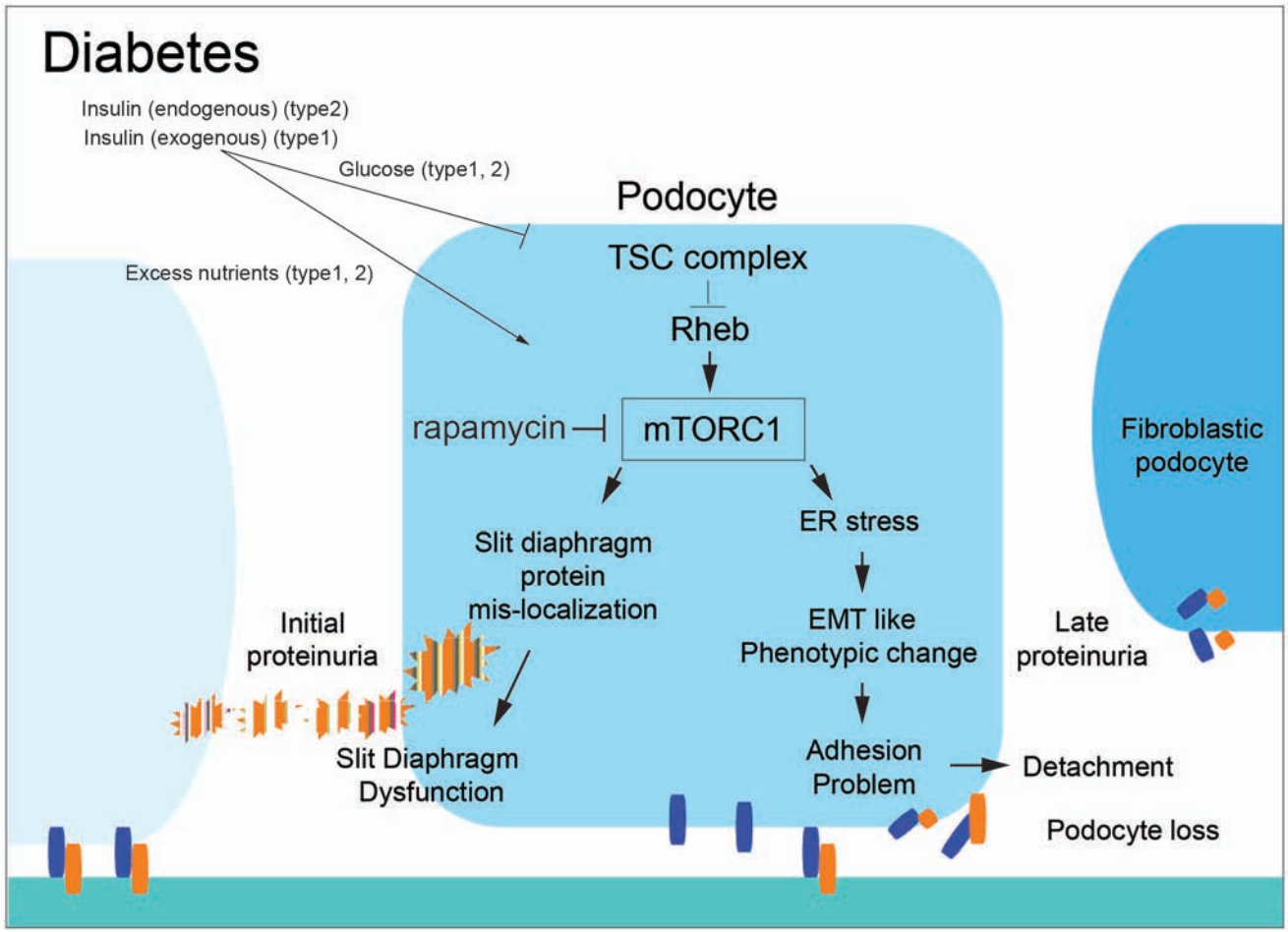

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