

SUPPLEMENTARY METHODS

Mice and Cell lines

A plasmid containing cDNA encoding a ligand-independent Tbrl mutant Tbrl(AAD) was obtained from Dr. Joan Massague¹. Tbrl(AAD) cDNA contains mutations to replace Leu193 and Pro194 with Alanine residues to eliminate binding of the Tbrl inhibitor FKBP12, and substitution of Thr204 with charged aspartic acid residue to mimic the GS domain phosphorylation enabling binding of receptor-regulated Smad proteins (Supplementary Fig. 1). We placed Tbrl(AAD) cDNA under control of tet-On promoter and confirmed inducible activation of Tbrl/Smad signaling in vitro. To generate double-transgenic mice for podocyte-specific, inducible Tbrl(AAD) expression (called PodTbrl mice), we intercrossed the generated doxycycline-inducible tet-O-Tbrl(AAD) transgenic mice on FVB/N background with NPHS2-rtTA transgenic mice provided by Dr. Jeffrey Kopp².

PodTbrl mice were crossed with Immortomouse® transgenic mice (Charles River, Wilmington, MA) to generate conditionally immortalized podocytes as previously described³. We established clones from Immorto® transgenic mice carrying either tet-O-Tbrl(AAD) or NPHS2-rtTA.tet-O-Tbrl(AAD). The conditionally immortalized mouse podocytes were maintained in RPMI-1640 containing 10% FBS on collagen I coated flasks under either permissive conditions (33°C with 10 units/ml of INF- γ), or non-permissive condition (37°C without INF- γ) under humidified conditions (95% air, 5% CO₂).

Ki-67 localization.

Paraffin fixed kidney section were washed and permeabilized with 0.2% Triton X-100. Slides were blocked followed by incubation with rabbit anti Ki-67 antibody (Invitrogen) and FITC-labeled anti-mouse. Fluorescence was detected using a Zeiss Axioplan 2 microscope and to quantify the Ki-67 positive nuclei per glomerular section, we used MetaMorph 6.3r3 software (Molecular Devices, Downingtown, PA), provided by the MSSM microscopy core facility.

Telemetry probe implantation for blood pressure (BP) measurements

Implantable mouse blood pressure (BP) transmitters (TA11PA-C10; Data Sciences International; DSI, St. Paul, MN) were used to directly measure arterial blood pressure in individual animals. The catheter tip was placed in the aortic arch via the carotid artery, and the transmitter was placed under the skin at the flank. Mice were treated with buprenorphine 0.03 mg/kg and ciprofloxacin 4 mg/kg post-surgery. Mice were caged individually and unrestricted. After allowing 7 days to recover, Dox diet was introduced and BP recordings were collected in each group over a 14 day period by a receiver (model RPC-1; DSI) passed to an analogue converter (model RP11A; DSI) and average daily systolic BP, diastolic BP tracings generated as well as mean arterial pressure (MAP), heart rate and mouse activity were recorded and analyzed using Dataquest ART 4.31 Gold software.

Microarray study

mRNA from isolated glomeruli was used for microarray studies. mRNAs were reverse transcribed into cDNA, then cRNA and hybridized onto Affymetrix genechip mouse gene 1.0 ST array. Affymetrix standard protocols were followed for these steps. Chips were scanned and intensity files (in DTT format) were transferred with Affymetrix data transfer tool version 1.1.1, and quality control and normalization was performed with Affymetrix expression console version 1.1. Probe sets intensity values were analyzed with Multiple Array viewer version 4.5. Network and pathway analysis were performed with ingenuity system software (See Supplementary Table 1).

Western blotting

Cultured podocytes lysates were made using RIPA buffer containing protease inhibitor cocktail (Roche) and Halt phosphatase inhibitor (Thermo Scientific). After SDS-PAGE gel fractionation and membrane transfer, the blots were incubated with primary antibodies for HA and phosphorylated Smad2 (Cell Signaling, Beverly, MA).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Inducible expression of constitutively-active Tbrl(AAD) in transgenic glomeruli.

a) Illustration of Tbrl amino acid substitutions L193A, P194A, and T203D in Tbrl(AAD) mutant. b) Tbrl(AAD) mRNA expression in isolated glomeruli of single transgenic Tet-O-Tbrl(AAD) control mice (STG), and double-transgenic PodTbrl mice on regular chow or Dox chow for 1-14 days. c) Ki67 staining for glomerular cell proliferation rate of PodTbrl mice without Dox and with Dox for 1 - 14 days.

Supplementary Figure 2. Survival analysis, longterm blood pressure monitoring and glomerular (mesangial) cell proliferation

a) Survival percentage of PodTbr1 mice controls (n=20) and PodTbr1 in Dox diet (n=20) was recorded and plotted according to time (0 to 30 weeks) maintained on Dox chow. b) Average of sequential traces of blood pressure and heart rate of conscious PodTbrl mice by telemetry; 2 days before Dox at day 0 (arrow) and up to 14 days with Dox chow (n = 7 ± s.e.m.).

Supplementary Figure 3. Glomerular DNA oxidation injury assessed by 8-oxoG immunolabelling in PodTbrl glomeruli

8-oxoG immunodetection in PodTbrl kidneys. Top panels left to right: untreated control, 1 day and 2 days of Dox. Bottom panels left to right: 4 days, 7 days and 14 days of Dox showing increased glomerular staining starting with d1 (arrows) and late tubular staining starting at d7 (arrowheads) of Dox chow.

Supplementary Figure 4. Glomerular endothelial mtDNA damage in kidneys of mice with podocyte-specific knockout of dicer (PodDicKo) and Adriamycin-induced segmental glomerulosclerosis in Balb/c mice

a) Top left to right: representative glomerular triple-immunofluorescence staining in PodDicKo mice showing Smad2/3 (red), WT1 (green), and Smad2/3, WT1 and DAPI localization. Arrows depict co-localization of nuclear Smad2/3, WT1 and DAPI. Bottom panels left to right: immunoperoxidase detecting 3-nitrotyrosine (3-NT), and immunofluorescence detecting 8-oxoG (green), Cd31 (red) and merged image of PodDicKo mice. b) Histopathology stain (PAS) of Balb/c mouse treated with Adriamycin (AD) (6 daily AD (10mg/Kg) injections) showing evidence of glomerular sclerosis and tubular casts (20x magnification). c) Immunoperoxidase detecting 3-NT in Balb/c saline control, and AD kidneys, arrows depict positive 3-NT staining. d) Triple-immunofluorescence staining of kidney sections from Adriamycin (AD) treated Balb/c mice showing Smad2/3 (red), WT1 (green), and Smad2/3, WT1 and DAPI localization (top panels). Arrows depict co-localization of nuclear Smad2/3, WT1 and DAPI in podocytes. Immunofluorescence detecting: Synaptopodin (red) and 8-oxoG (green), Cd31 (red) and 8-oxoG (green), and Ednra (green) and Cd31 (red) in kidneys of AD treated Balb/c mice.

Supplementary Figure 5. Phenotypic and molecular validation of conditionally-immortalized PodTbri-derived podocyte (POD) cell lines and Edn1 synthesis.

a) Western blot analysis for hemagglutinin tagged-Tbri(AAD) (anti-HA antibody), phosphorylated Smad2 (pSmad2), total Smad2 in conditionally-immortalized podocyte cell line (POD) established from double-transgenic PodTbri(AAD) mice: podocytes without or with Dox (1ug/ml) for 24hr. b) Representative immunofluorescence staining for pSmad2 and DAPI by control untreated (left), 1hr TGF β (5ng/ml; middle) and 1hr Dox (1ug/ml; right) treated POD (100x Magnification). c) Oxygen consumption rate (OCR) and d) extracellular acidification rate (ECAR) in POD untreated or Dox (1ug/ml) treated as indicated. Histograms show mean \pm s.e.m of three independent experiments. These POD cells were used for in vitro co-culture system to study paracrine signaling crosstalk with murine glomerular endothelial cell lines (mGEC). e) mRNA expression of Edn1 in WT, Smad2KO and Smad2/3KO (DKO) podocytes incubated with TGF β , or TGF β +LY374947 for 48hr. f) Amount of Edn1 protein released in supernatant as detected by ELISA of WT, Smad2KO and DKO podocytes treated as indicated. Mean \pm s.e.m. in three independent experiments; * P < 0.05 versus scramble controls, +P < 0.05 versus TGF β).

Supplementary Figure 6. Inhibition of Edn-1 signaling and mitochondrial oxidation prevented albuminuria and oxidative DNA damage in Dox treated PodTbri or Adriamycin-induced Balb/c mice

a) Urinary albumin-creatinine ratio (ACR), b) urinary 8oxodG-creatinine ratio in PodTbri control and Dox-treated mice in the absence or presence of BQ-123 (0.1nM/kg/day) or mitoTEMPO (1mg/kg/day), as indicated. c) Urinary ACR, d)

urine 8oxodG-creatinine ratio in control Balb/c mice (after 6 days of saline injections), or Adriamycin (AD)-induced Balb/c mice (6 daily AD (10mg/Kg) injections), in the absence or presence of BQ-123 or mitoTEMPO, as indicated. Representative glomerular triple-immunofluorescence staining showing Smad2/3 (red) with DAPI, WT1 (green) with DAPI, and Smad2/3, WT1 and DAPI localization in 14d Dox-fed PodTbr1 mice co-treated with e) BQ-123 (0.1nM/kg/day) or f) mitoTEMPO (1mg/kg/day). Arrows depict co-localization of nuclear Smad2/3, WT1 and DAPI. For histograms in a) and b), each bar represents mean \pm s.e.m of n = 6 mice/group for c) and d) each bar represents mean \pm s.e.m or n = 4-7 mice/group. *P < 0.05, ** P<0.01, *** P<0.001 versus control mice; +P < 0.05, ++ P<0.01 versus Dox or AD only treated mice.

Supplementary Figure 7. Supernatant from DOX-treated POD upregulated Ednra to induce mitochondrial oxidative stress in mGEC.

a) mRNA expression of Edn1 and Ednra in mGEC incubated with control supernatant from untreated POD (control PSN), or with supernatant from POD treated for 24hr with Dox (Dox PSN) or TGF β (TGF β PSN) as indicated. Bars represent mean \pm s.e.m. (n = 3) *P < 0.05, **P < 0.01 versus controls. b) Histograms of cell surface expression of Ednra by mGEC controls, co-cultured with control PSN for 24hr or DOX PSN for 6hr or 24hr (n = 2). c) Quantification of lesion frequencies in mtDNA and nDNA by QPCR in mGEC co-cultured with DOX stimulated POD SN as indicated. Values are relative amplification ratio and represent mean \pm s.e.m. of 3 independent experiments normalized to the

amplification of untreated controls (*P < 0.05). d) mGEC mRNA expression of nuclear genes encoding mitochondrial proteins including encoded complex I subunits NDUFA4A and NDUFb7, and mitochondrial DNA-encoded complex I subunit ND1 and ND4 genes, after 24hr co-culture with TGFβ (5ng/ml) or DOX- (1ug/ml) -treated PSN. Values represent mean ± s.d. relative to no DOX controls (n = 2 independent experiments).

Supplementary Figure 8. siRNA knockdown of Ednra in mGEC, and of Edn1 in POD.

a) Edn1 mRNA expression in POD treated with Dox (1ug/ml) for 48hr, following transfection with scramble control or Edn1-specific small interference (siRNA) for 2 days prior to Dox treatment. Mean ± s.e.m. relative to Dox-treated control in three independent experiments; *P < 0.05). b) Amount of Edn1 protein released in supernatant as detected by ELISA of POD treated as indicated. Mean ± s.e.m. in four independent experiments; * P < 0.05 versus scramble controls, ++P < 0.01 versus Dox. c) Ednra mRNA expression after 48hr transfection with scramble siRNA or siRNA for Ednra in mGEC, followed by 24hr Edn1 or Dox PSN co-incubation ± s.e.m. relative to Edn1 ++P < 0.01, or to Dox PSN +P < 0.05). d) Ednra protein expression by western blot after 48hr transfection as indicated (n = 2 ± s.d. relative to scramble controls).

Supplementary Figure 9. Edn1 treatment of mGEC induces mitochondrial oxidative stress and release of podocyte apoptosis inducing factors after co-incubation.

a) Percentage of MitoSOX bright fluorescent mGECs after 24h Edn1 treatment at doses indicated. b) Percentage apoptotic POD +/- Dox after 48hr co-culture with control mGEC SN (Ctrl ESN) or with Edn1 ESN at the doses indicated. For a) and b) mean \pm s.e.m of 3 independent experiments; *P < 0.05.

Supplementary Table 1. Summary of molecular pathway progression in PodTbr1

Glomerular fractions were separated to high purity (>98%) and subjected to genome- wide expression profiling using Affymetrix GeneChips (M430), followed by gene enrichment analysis and pathway analysis (Ingenuity System). Genes encoding protein complexes of the mitochondrial electron transport chain were strikingly and broadly downregulated between day 1-4, suggesting profound decrease in glomerular oxidative phosphorylation (OXPHOS) and possibly mitochondrial ATP production. Also nuclear genes encoding mitochondrial antioxidant proteins were downregulated on day 4, suggesting a potential increase in reactive oxygen species (ROS) generation. The early phase was also characterized by: upregulation of N-glycan and chondroitin/keratan/heparan sulfate biosynthesis, concomitant with glycosaminoglycan degradation, suggesting turnover of extracellular reservoir proteins for mitogenic cytokine pathways, such as Wnt, FGF, and others, as well as possibly basement

membrane; upregulation of Wnt/ β -catenin and PI3K/AKT networks, suggesting mitogenic and prosurvival signaling; G1/S cell cycle regulators, suggesting cellular proliferative response; as well as upregulation of fatty acid metabolism, suggesting possibly release of free fatty acid and lipotoxicity. By day 7, we observed a striking switch to activation of pathways involved in damage regulation, apoptosis and wound healing/coagulation with upregulation of: coagulation pathways; p53 and p38 MAPK signaling; TGF β signaling; and fibrogenesis. With advanced glomerulosclerosis typically observed by day 14, the predominant pathways included: TGF β signaling; broadening of fibrogenesis programs; and G2/M cell cycle and DNA damage regulation.

Supplementary Table 2. Human biopsy characterization

Patient characteristics at the time of biopsy.

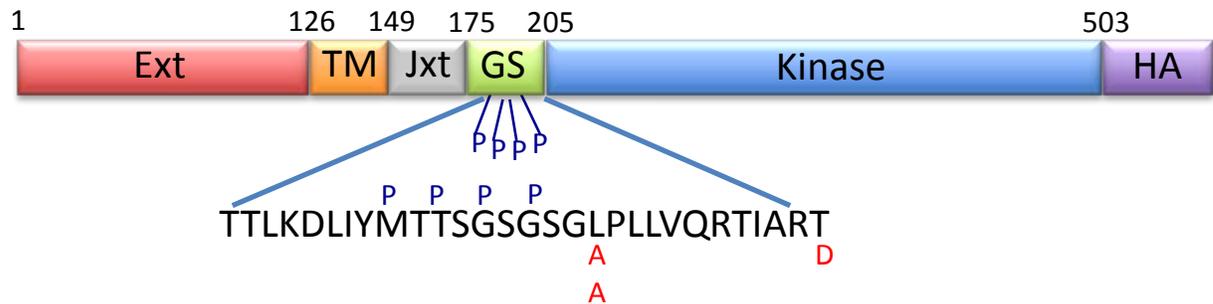
Supplementary Table 3. RT-PCR primer sequence

Supplementary References

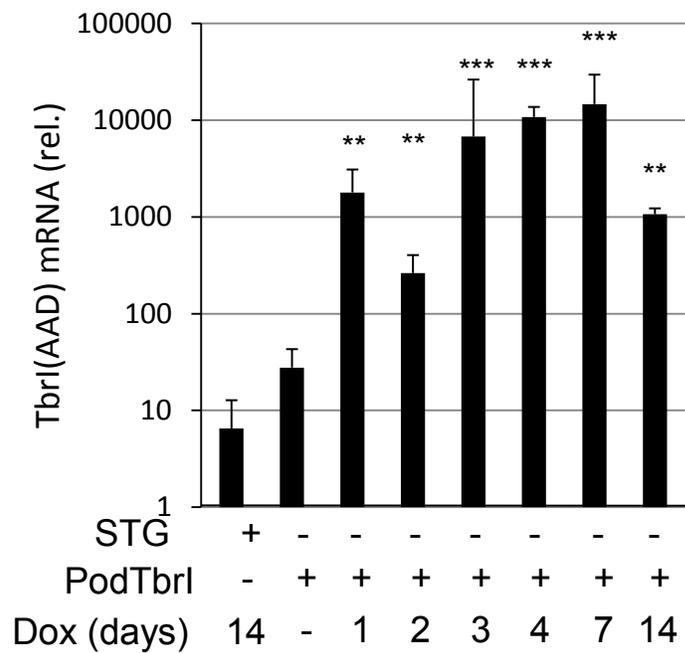
1. Wieser, R., Wrana, J.L. & Massague, J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* **14**, 2199-2208 (1995).
2. Shigehara, T., *et al.* Inducible podocyte-specific gene expression in transgenic mice. *J.Am.Soc.Nephrol.* **14**, 1998-2003 (2003).
3. Mundel, P., *et al.* Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp.Cell Res.* **236**, 248-258 (1997).
4. Ma LJ, Fogo AB. Model of robust induction of glomerulosclerosis in mice: importance of genetic background. *Kidney Int.* **64**, 350-5 (2003).

Supplementary Figure. 1

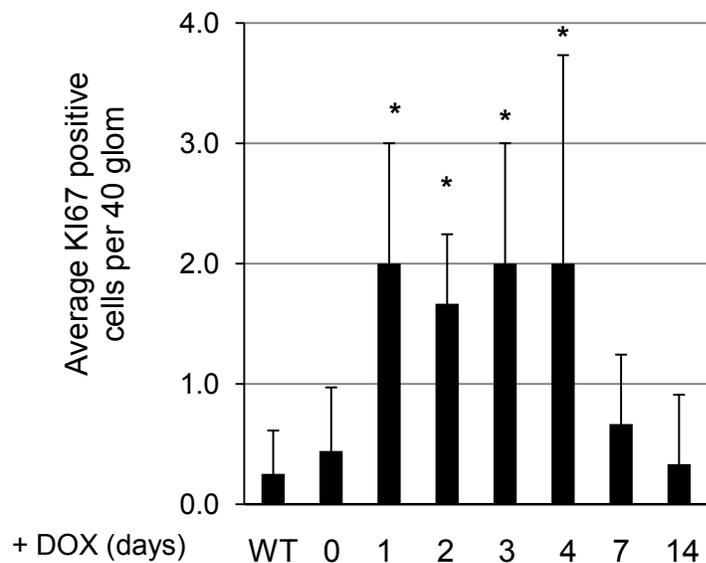
a)



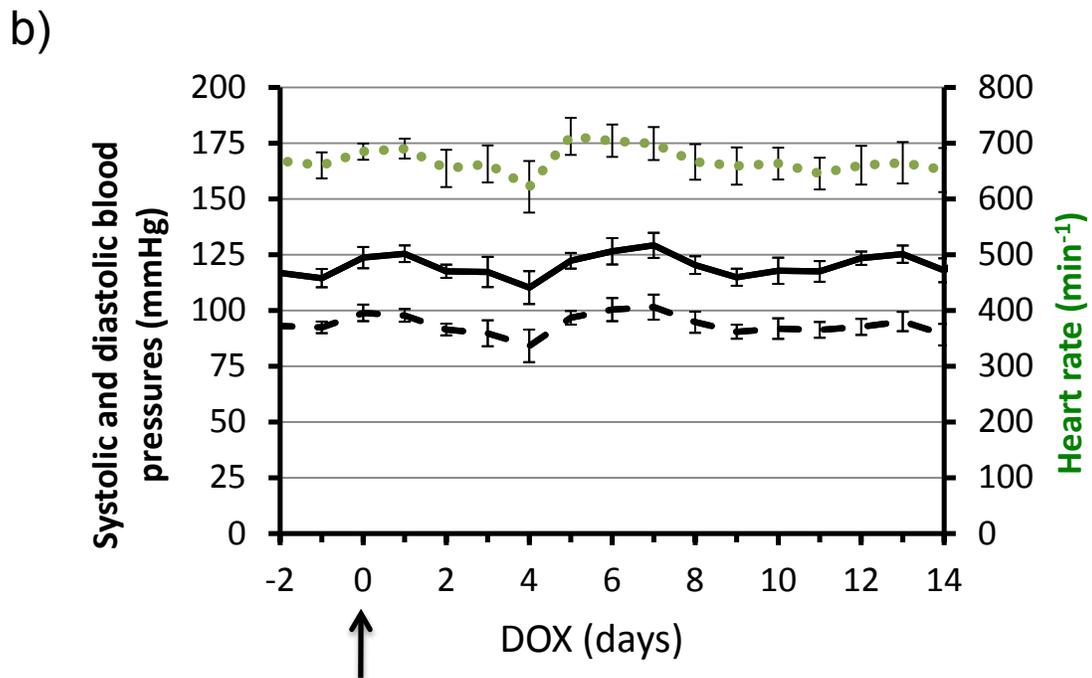
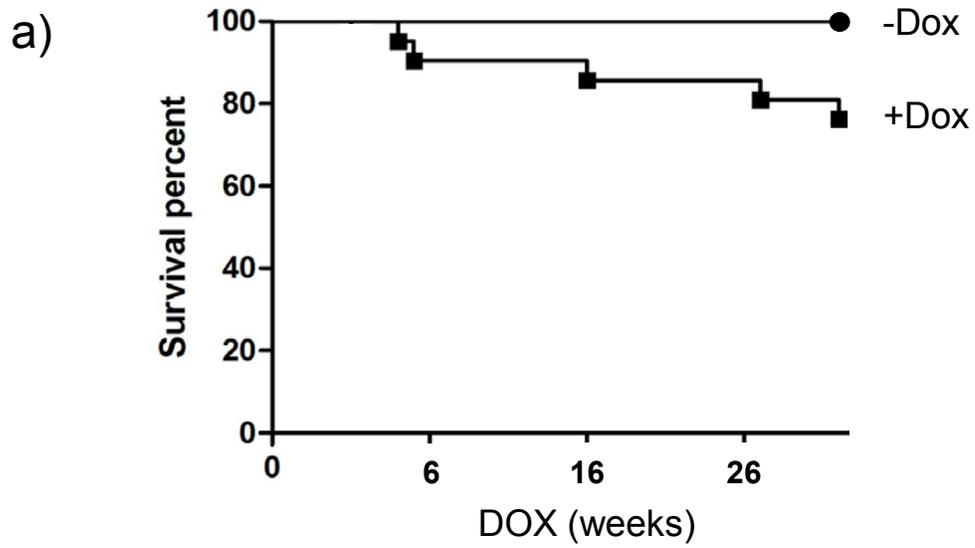
b)



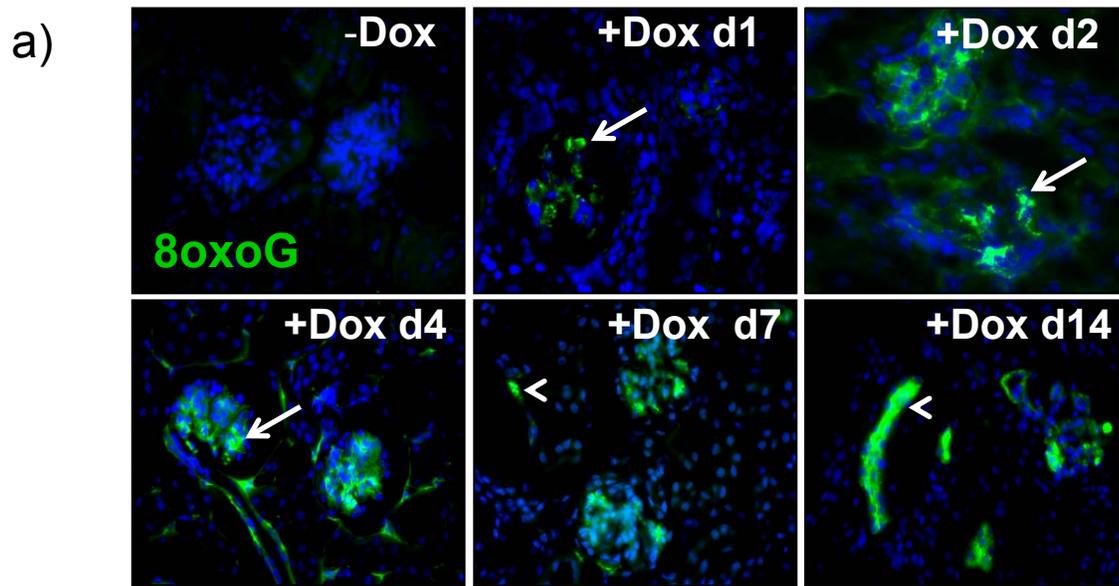
c)



Supplementary Figure. 2

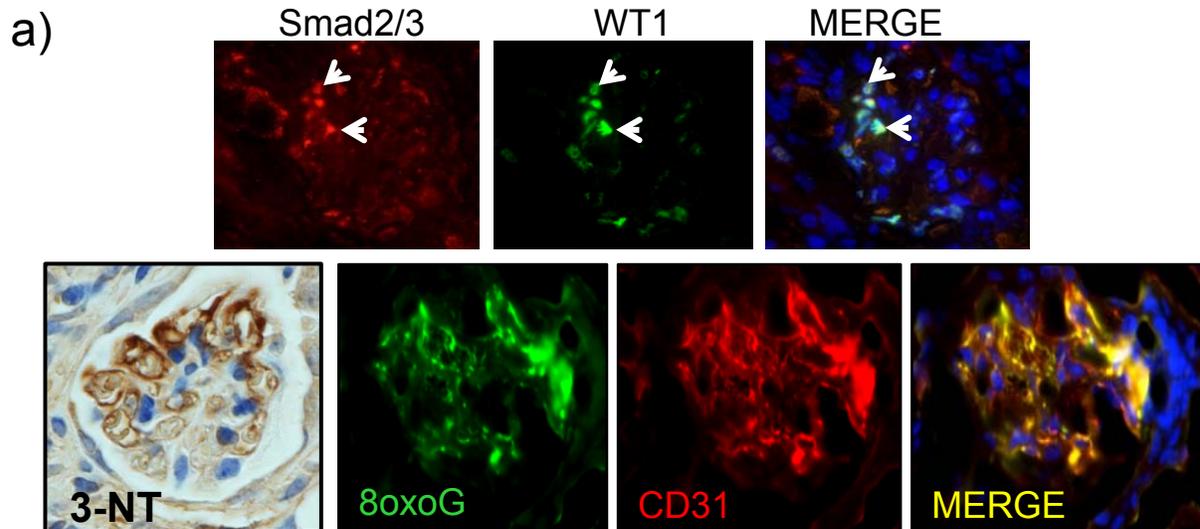


Supplementary Figure. 3

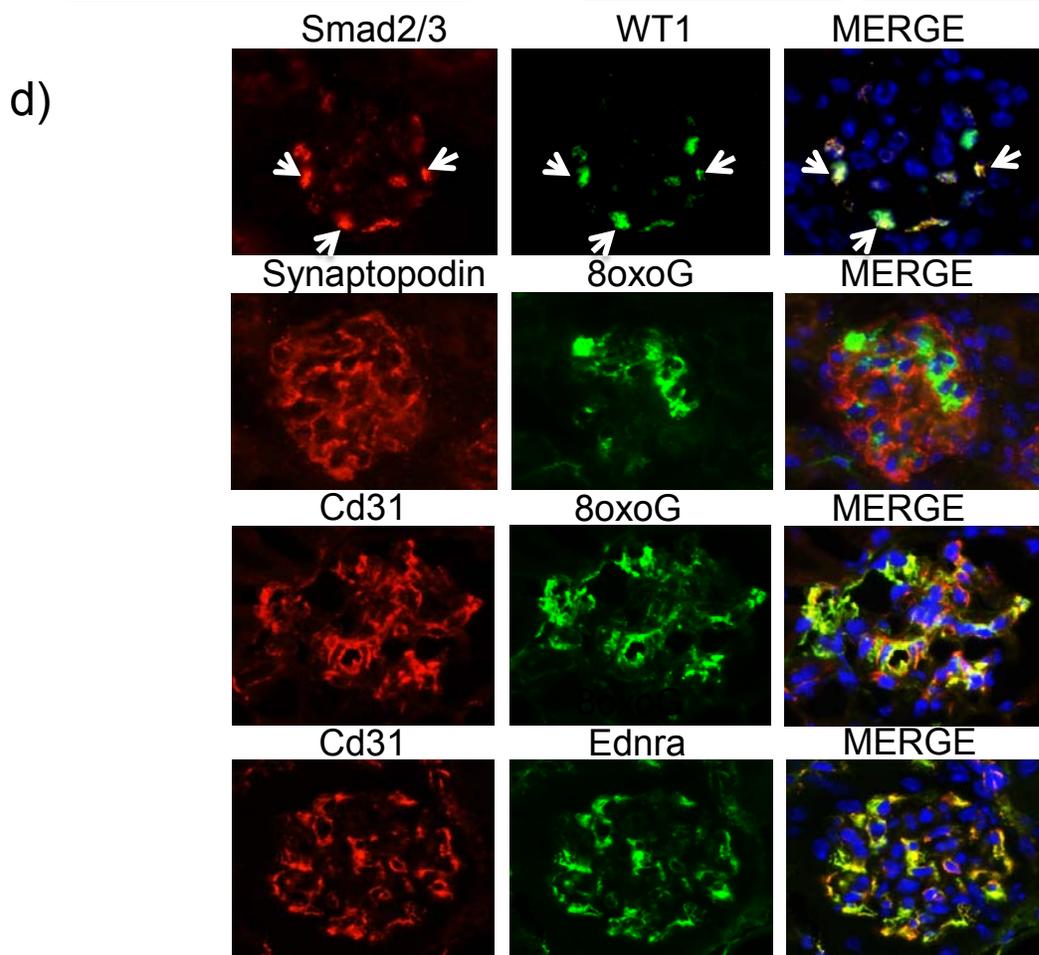
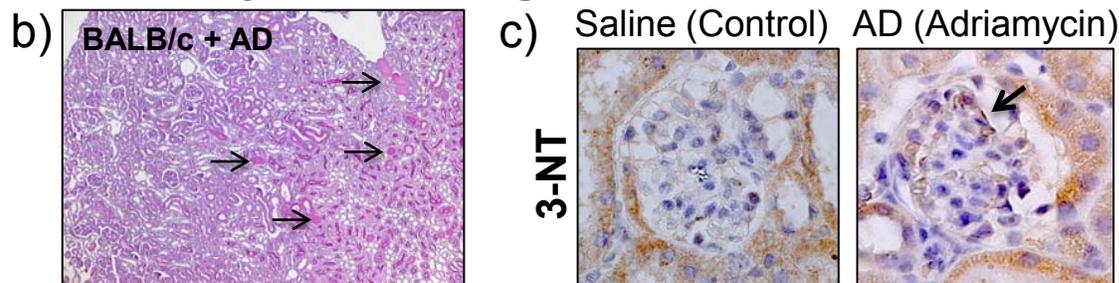


Supplementary Figure. 4

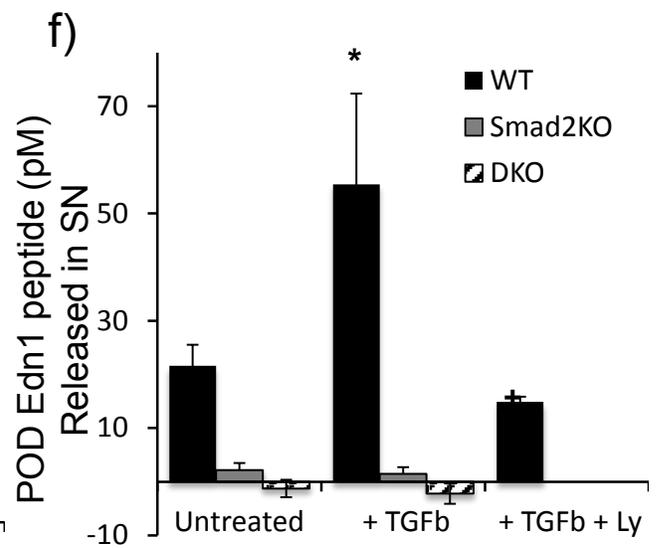
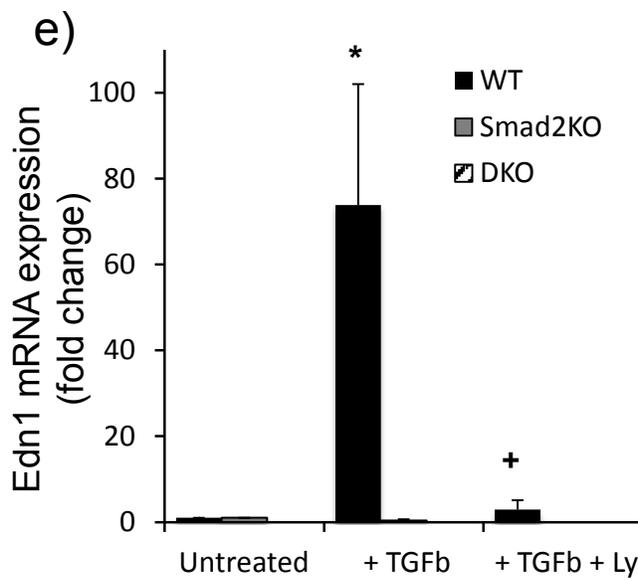
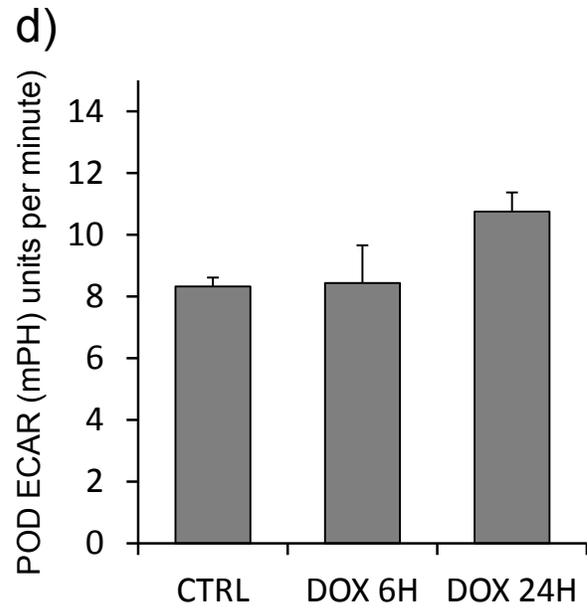
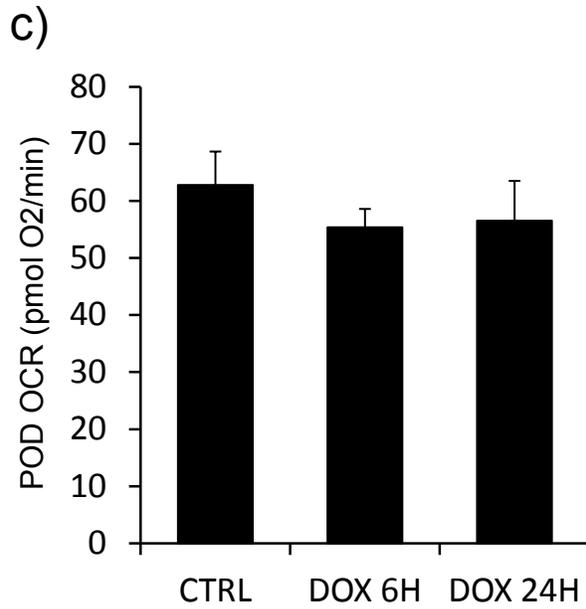
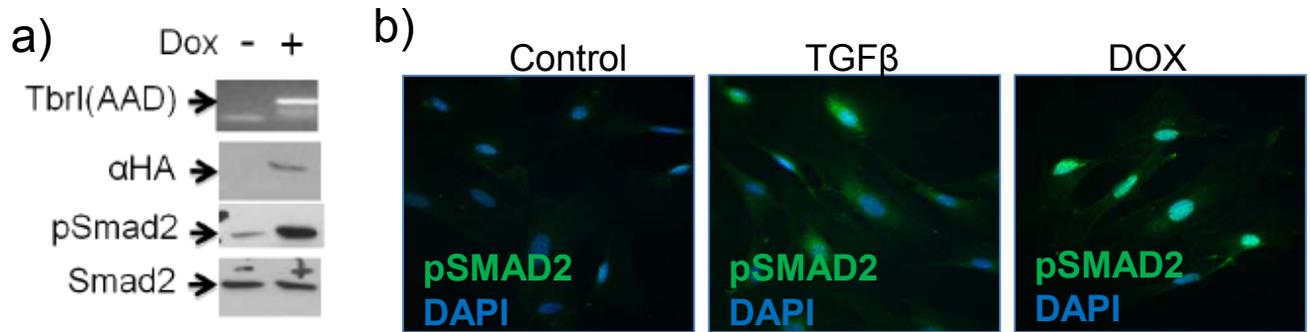
Podocyte-specific knockout dicer (PodDicker)



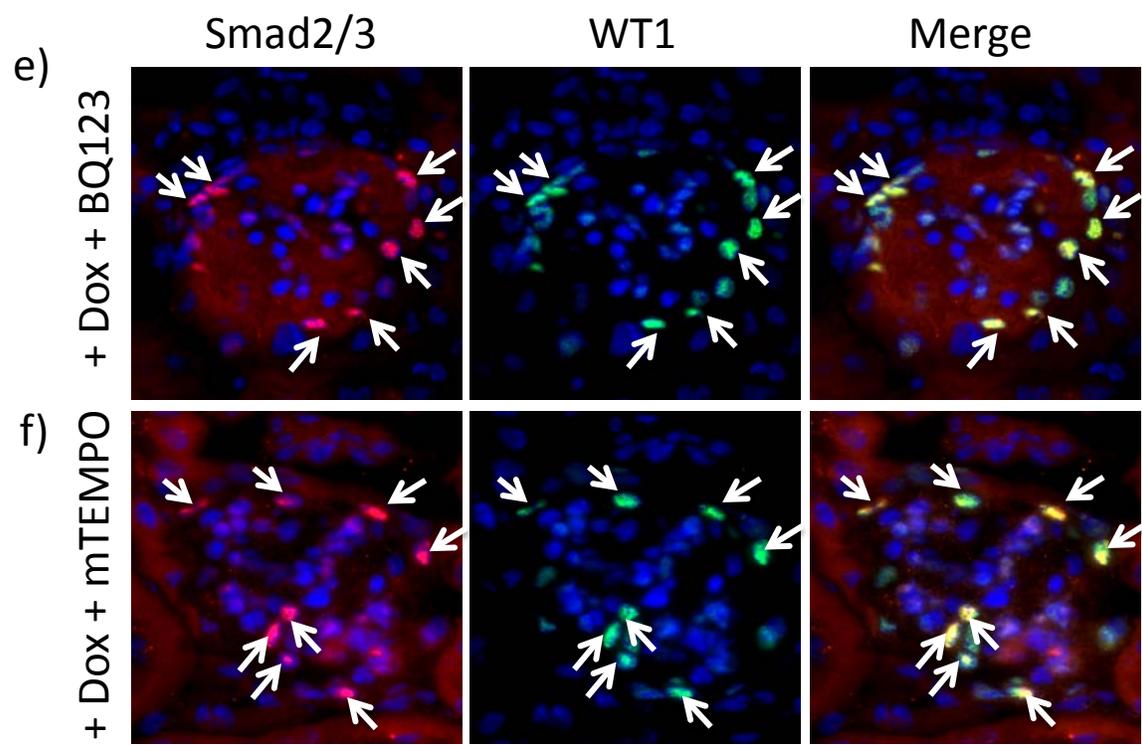
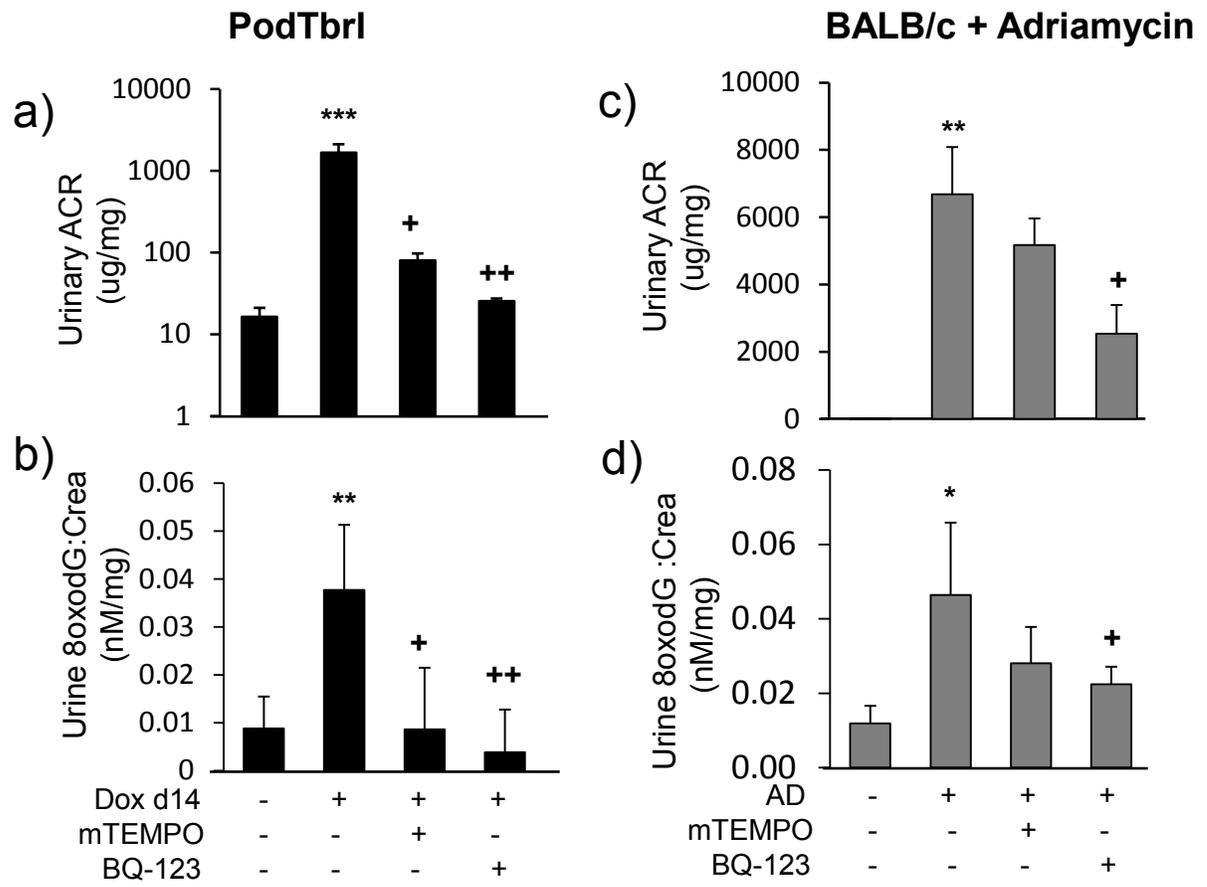
Adriamycin-induced glomerulosclerosis in BALB/c mice



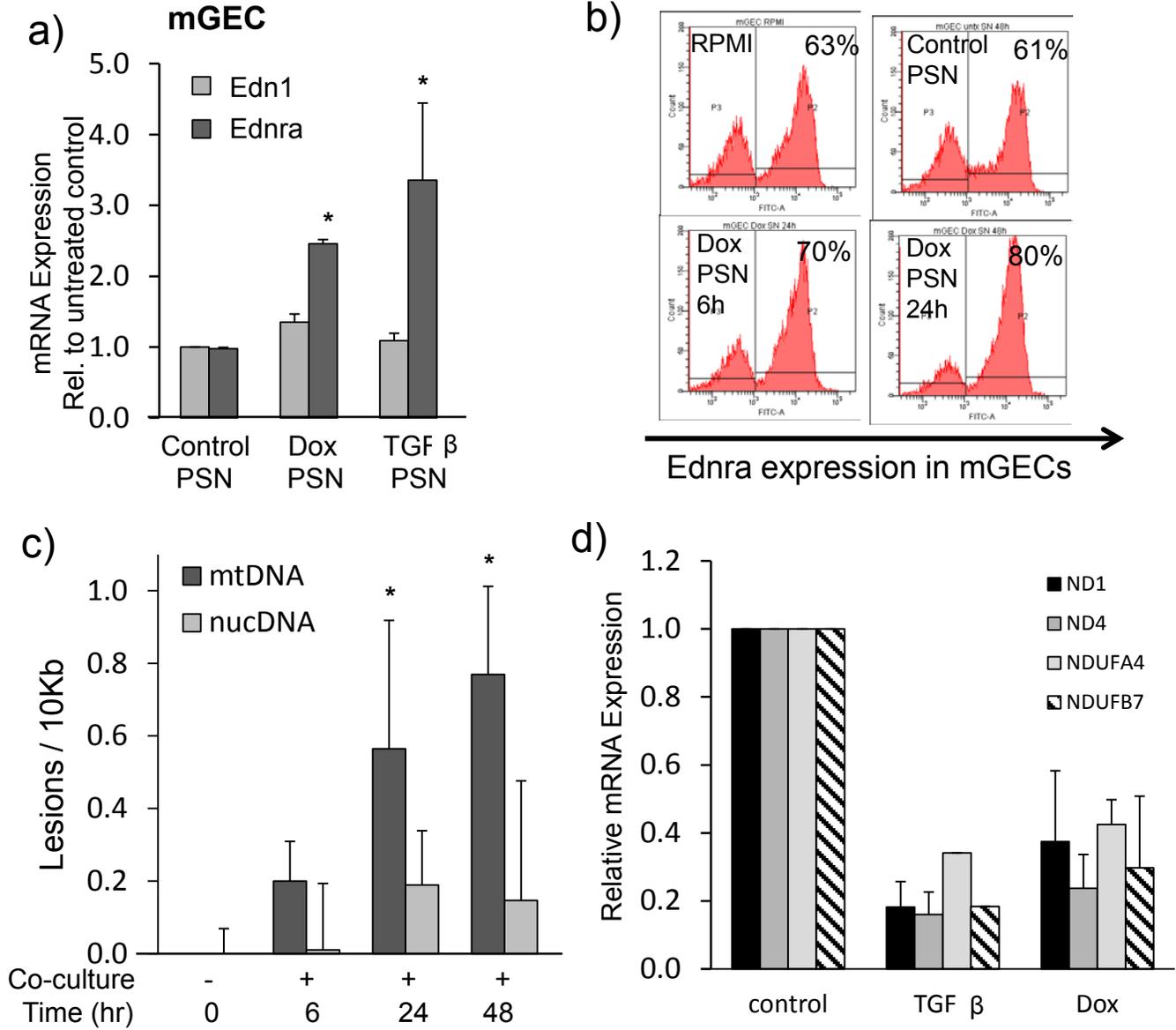
Supplementary Figure. 5



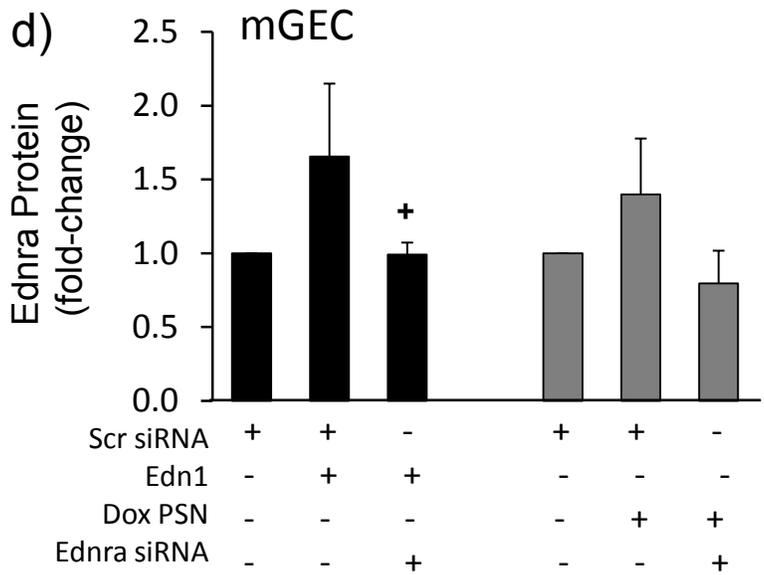
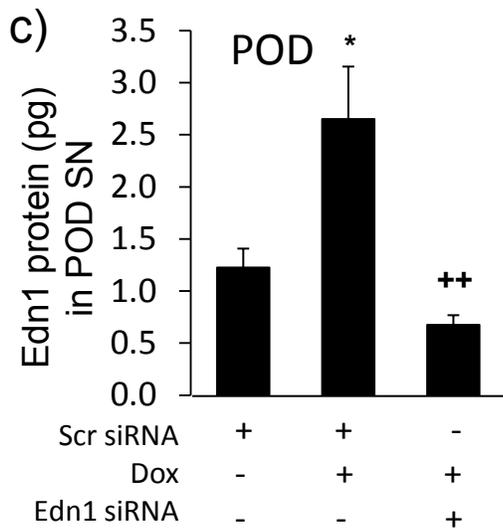
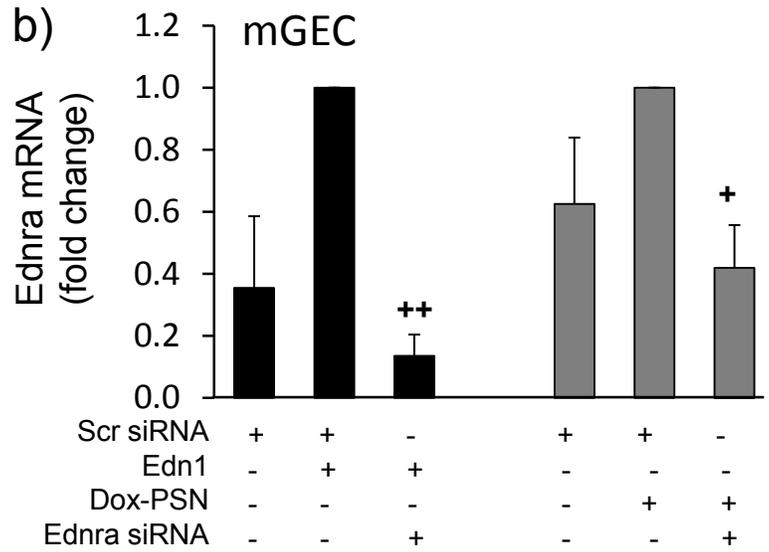
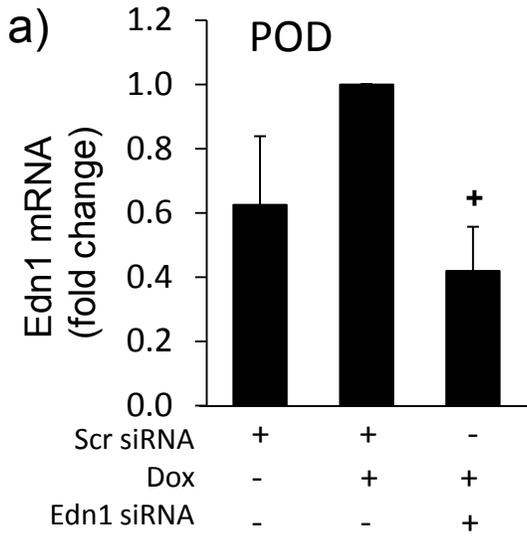
Supplementary Figure. 6



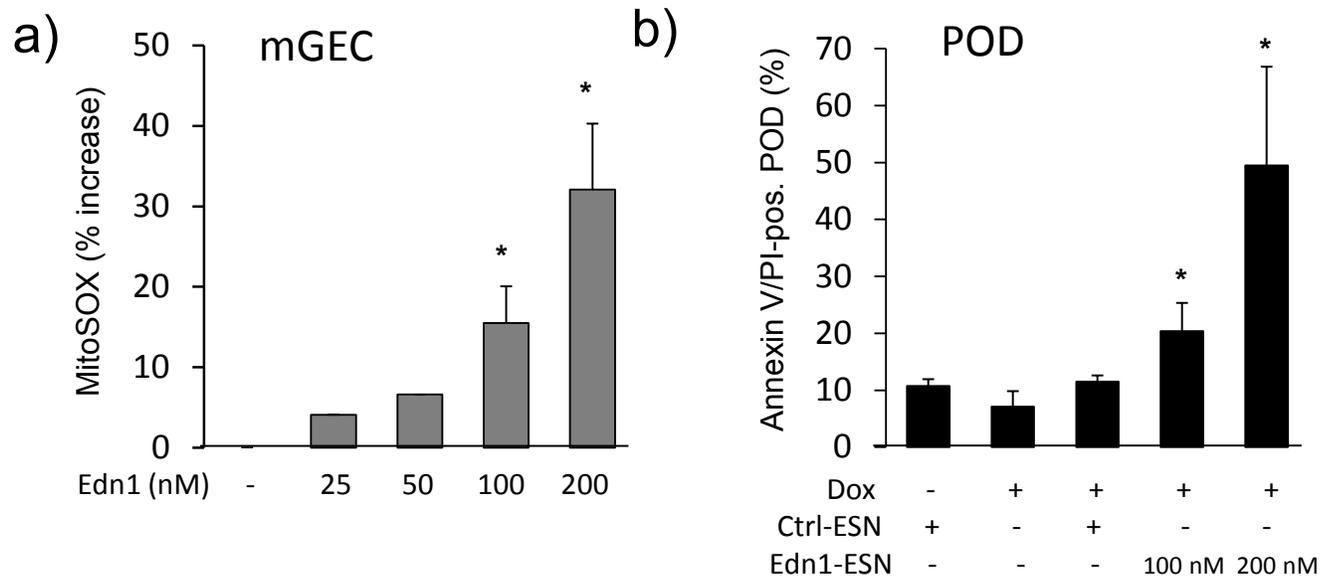
Supplementary Figure. 7



Supplementary Figure. 8



Supplementary Figure. 9



Supplementary Table. 1
 Summary of molecular pathway progression in PodTbr1

+ DOX (days)	1	4	7	14
Oxidative Phosphorylation	Green	Green	White	White
N-GAG Biosynthesis	Red	Red	Red	White
Wnt/ β -catenin	Red	White	White	White
PI3K/AKT	Red	White	White	White
Cell cycle G1/S regulation	Green/Red	White	Green/Red	White
Mitochondrial (dys)function	White	Green	White	White
C-/K-/Heparan sulfate biosynthesis	White	Red	White	White
GAG Degradation	White	Red	White	White
Fatty acid metabolism	White	Red	White	White
Coagulation system	White	White	Red	White
p53 signaling	White	White	Red	White
p38 MAPK signaling	White	White	Red	White
Fibrosis	White	White	Red	Red
TGF β signaling	White	White	Red	Red
Cell cycle G2/M DNA damage regulation	White	White	White	Red

Supplementary Table. 2

Pathology of human samples

	patient					Serum	
	code	age	sex	race/ethnicity	Protenuria	Creatinine	ACEi/ARB
FSGS	KC11 960	53	M	W	13.0 g	1.83 mg/dL	no
FSGS	KC11 836	61	M	B	8.0 g	1.3	yes
FSGS	KC11 830	39	M	B	16.0 g	2.48	yes
FSGS	KC11 806	44	M	Hispanic	5.0 g	1.3	yes
FSGS	KC11 687	23	M	B	2.9 g	3.6	yes
FSGS	KC11 436	44	M	W	1.0 g	1.9	yes
FSGS	KC11 937	22	M	B	2.0 g	2.2	yes
FSGS	KC11 518	34	M	W	3.6 g	4.4	yes
FSGS	KC11 492	23	M	W	19.0 g	1.7	yes
FSGS	KC11 295	57	F	W	2.0 g	2.6	yes
NORMAL	KC11 835	55	M	W	0.9 g	1.51	no
NORMAL	KC11 854	19	F	W	trace	0.6	no
NORMAL	KC11 532	65	F	Hispanic	trace	1.26	no
NORMAL	KC11 493	36	M	Black/Asian	0	1.41	no
NORMAL	KC11 382	7	F	W	0.21 g	0.4	no
NORMAL	KC11 333	10	F	W	0.527 g	0.7	no

Supplementary Table. 3

RT-PCR primers

Gene	Forward	Reverse
Tgbrl(ADD)	AAAGTCATCACCTGGCCTTA	TTCTCAAATCGACCTTTGC
ND1	TGCACCTACCCTATCACTC	ATTGTTTGGGCTACGGCT
ND4	ATAATTATAACTAGCTCAATCTGC	TCGTAGTTGGAGTTTGCTAG
NDUFA4	AGCCAGGTTGCAGAAGCGGC	GCTGCTCCAGTACCCCCTGC
NDUFB7	AGAGTAGCCATGGGGCGCA	AGCAGTCGCCGCTCACGTTC
Cox8A	TCCGGCTGGTTCGGCCATCT	CCAGCCCGCAGGCAGAAGAC
ATP50	GGGAGTGAGCTGGCCTGC	GCATGCAGCATCCCCTGCCA
UQCR	CGTAGTGCTCCAGGGCAGCG	AGGATCAGCCGCCAGTCCGT
SOD2	AATGTTGTGTCTCGGGCGGCGT	AAGCCAGCCCCAGCCTGAAC
ET1	TTA GCAAGACCATCTGTGTG	GAGTTTCTCCCTGAAATGTG
ETA	GTCTTGAACCTCTGTGCTCTC	GATCCCGATTCTTGAACCTCG