

SUPPLEMENTARY MATERIALS

ADDITIONAL METHODS

Histology and tissue immunostainings. Dissected kidneys fixed overnight in 2% formaldehyde in PBS buffer were sectioned for histology or immunostainings from either paraffin-embedded or cryo-mounted tissue blocks at 5- μ m or 10- μ m thickness, respectively. Alternatively, fixed kidneys were sliced into 50-100 μ m free-floating vibratome sections. Histology, immunofluorescence and immunohistochemical stainings were performed according to standard methods using antibodies listed in Table S1. Relative vascular densities were obtained by quantifying areas of positive Emcn immunofluorescence normalized to total tissue area in serially-sectioned kidneys from P5 animals using TissueGnostics slide imaging system and HistoFAXS imaging analysis software (TissueGnostics GmbH, Vienna, Austria).

Ultrastructure analysis. Kidney specimens were immersion-fixed in 4% formaldehyde/2% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2). Transmission electron microscopy (TEM) specimens were postfixed in 1% OsO₄. Ultrathin resin sections were stained with uranyl acetate and lead citrate and were visualized in an FEI CM100 transmission electron microscope (FEI, Hillsboro, OR). For scanning electron microscopy, kidneys specimens were hemisected before dehydration and subsequently sputter-coated with gold. Gold-plated specimens were observed using an FEI XL30 scanning electron microscope.

Western blot analysis. Tissue samples were homogenized in RIPA buffer and used for denaturing polyacrylamide gel electrophoresis followed by blotting to PVDF membranes. PVDF immunoblots were incubated with antibodies against Tie2 and tubulin (Table S1) and imaged by peroxidase-based enhanced chemiluminescence method (ThermoFisher Scientific, Waltham, MA). Tie2 protein expression were normalized against tubulin levels.

Quantitative Real-Time PCR analysis. RNA was extracted using Trizol (ThermoFisher Scientific,) and reverse-transcribed using iSCRIPT synthesis kit (Bio-Rad, Des Plaines, IL).

Quantitative PCR using SYBR green fluorescent reporter was done on Applied Biosystems 7500 Fast Real-Time PCR system (ThermoFisher Scientific). Primers used are listed on Table S2.

Urinalysis and GFR measurements. Urinary albumin was measured using Albuwell M kit (Exocell, Philadelphia, PA) while urinary creatinine was analyzed through a microtiter-format colorimetric Jaffe reaction assay using alkaline picrate. Qualitative albuminuria was assessed on colloidal Coomassie dye-stained protein gels of urine samples alongside bovine serum albumin standards. Glomerular filtration rates (GFR) were obtained by measuring plasma clearance of FITC-labeled sinistrin delivered by retro-orbital injection⁵⁸.

Urine output and osmolality measurements. 24-h urinary outputs were measured from mice kept in individual metabolic cage with free access to food and water. Urine osmolality measurements were obtained using a Micro Osmette 5004 automatic osmometer (Precision Systems, Inc., Natick, MA).

Statistics. Quantitative data were expressed as means with their corresponding standard errors. Means were compared for statistical significance by 2-tailed Student's t-test and ANOVA. $P < 0.05$ were considered statistically significant. Statistical analyses and graphical representation of numeric data were done using Prism software version 5.0a (GraphPad Software, Inc.).

TABLE S1. List of Antibodies Used

Antibody	Source/Company	Catalog Number	Application (Dilution)*
Acta2 (α SMA)	Sigma-Aldrich	F3777	IF (1:300)
AQP2 (Aquaporin 2)	Santa Cruz Biotechnology	sc-9882	IF (1:100)
Atp1b1 (Na ⁺ /K ⁺ ATPase)	Developmental Studies Hybridoma Bank	a5	IF (1:500)
CD34	Abcam	ab8158	IF (1:100)
Cdh16 (Kidney specific cadherin)	Generous gift from Peter S. Aronson, Yale University		IF (1:1000)
Cnn1 (calponin)	Abcam	ab46794	IF (1:100)
Desmin	Novocastra	NCL-c-DES-DERII	IF (1:20)
Emcn (endomucin)	Abcam	Ab106100	IF/IHC (1:200)
GFP (chicken polyclonal)	Abcam	ab13970	IF (1:500)
GFP (rabbit polyclonal)	Life Technologies	A11122	IF (1:500)
LTL (<i>Lotus tetraglobulus</i> lectin)	Vector Laboratories	FL-1321	IF (1:100)
Lyve1	R&D	AF2125	IF (1:100)
PCK (pan-cytokeratin)	Cell Signaling Technology	4545	IF (1:100)
PDGFR β	Cell Signaling Technology	3169	IF (1:100)
Pecam (CD31)	BD Biosciences	55337	IF (1:100)
Pdpn (Podoplanin)	Biologend	127401	IF (1:500)
Plvap	Developmental Studies Hybridoma Bank	MECA-32	IF (1:50)
Plvap	Radu Stan, Darmouth Medical School		IF (1:500)
Podxl (Podocalyxin)	R&D	AF1556	IF (1:100)
Tagln (SM22 α)	Abcam	ab14106	IF (1:40)
Tie2	Santa Cruz	SC-324	WB (1:1000)
Tubulin	Genscript	A01410	WB (1:2000)
Umod (Uromodulin)	Biomed Technologies, Inc.	BT-590	IF (1:100)
UTB (Urea Transporter B)	Jeff Sands and Janet Klein, Emory University School of Medicine		IF (1:1000)
Vim (Vimentin)	Cell Signaling	5741	IF (1:100)

*IF, immunofluorescence; IHC, immunohistochemistry; and WB, western blot

TABLE S2**Real-time PCR Primers**

Primer Name	Sequence
Angpt1-F	5'-GGGGGAGGTTGGACAGTAA-3'
Angpt1-R	5'-CATCAGCTCAATCCTCAGC-3'
Angpt2-F	5'-GATCTTCCTCCAGCCCCTAC-3'
Angpt2-R	5'-TTTGTGCTGCTGTCTGGTTC-3'
Gapdh-F	5'- AAGGTCATCCCAGAGCTGAA-3'
Gapdh-R	5'-CTGCTTCACCACCTTCTTGA-3'
Pdgfb-F	5'- GAGCTTTCCAACCTCGACTCC-3'
Pdgfb-R	5'-ATCCGCTCCTTTGATGATCT-3'
Pecam1-F	5'- CGGTGTTTCAGCGAGATCC -3'
Pecam1-R	5'-ACTCGACAGGATGGAAATCAC-3'

SUPPLEMENTARY FIGURES

FIGURE S1. Emcn co-localizes with Tie2-expressing cells in the kidney. Most EGFP-labeled Tie2-Cre expressing vessels show strong co-expression of Emcn in **(A)** the cortex and **(B)** the medulla as shown by confocal immunofluorescence. Scale bars: 100 μm .

FIGURE S2. Gene expression analysis. **(A,B)** qRT-PCR analysis on whole kidneys from A1/A2 $\Delta\text{E}^{16.5}$ and control littermates 3-5 wk of age showing 92% reduction in Angpt1 and 84.5% reduction in Angpt2 mRNA transcript levels. **(C,D)** Western blot analysis of Tie2 total kidney expression in Tie2 $\Delta\text{E}^{16.5}$ and control littermates 2-3 weeks of age showing 66.8% reduction of Tie2 protein levels in mutants compared to controls.

FIGURE S3. Unremarkable glomerular phenotype in A1/A2 $\Delta\text{E}^{16.5}$ and Tie2 $\Delta\text{E}^{16.5}$ mutants. **(A)** No significant differences in albumin to creatinine ratios measured in 10-wk old A1/A2 $\Delta\text{E}^{16.5}$ and Tie2 $\Delta\text{E}^{16.5}$ mutants and control littermates. **(B)** Coomassie blue-stained protein gels of urine samples from A1/A2 $\Delta\text{E}^{16.5}$ and Tie2 $\Delta\text{E}^{16.5}$ mutants and control littermates showing lack of albumin in mutant animals. **(C)** Periodic acid-Schiff staining of kidneys from adult animals showing normal glomerular histology of A1/A2 $\Delta\text{E}^{16.5}$ and Tie2 $\Delta\text{E}^{16.5}$ mutants. **(D)** Scanning electron micrographs of adult glomeruli showing normal podocyte ultrastructure in Tie2 $\Delta\text{E}^{16.5}$ mutants and control animals. Scale bars: (C) 10 μm ; and, (D), 5 μm .

FIGURE S4. Prox1⁺ vessels do not express classic lymphatic markers. **(A)** Cortical co-expression of Lyve1 and Prox1-GFP in small arcuate lymphatics (arrowheads). **(B)** Lack of Lyve1 expression in clustered Prox1-GFP⁺ vessels in the outer medulla. **(C)** Co-expression of Pdpn and Prox1-GFP in cortical lymphatic vessels (arrowheads). Pdpn is also notably

expressed in podocytes within the glomerulus (g). **(D)** Medullary Prox1-GFP^{+ve} vessels do not express Pdpn. Scale bars: 100 μ m.

FIGURE S5. Expression of myofibroblast markers in wild-type kidneys.

Immunofluorescence staining of myofibroblast markers in wild type adult kidneys showing strong expression of **(A)** Acta2, **(B)** Cnn1, **(C)** Tagln, and **(D)** Vim around larger caliber blood vessels. Vim is also found strongly expressed in podocytes within glomeruli (g). Scale bars: 100 μ m.

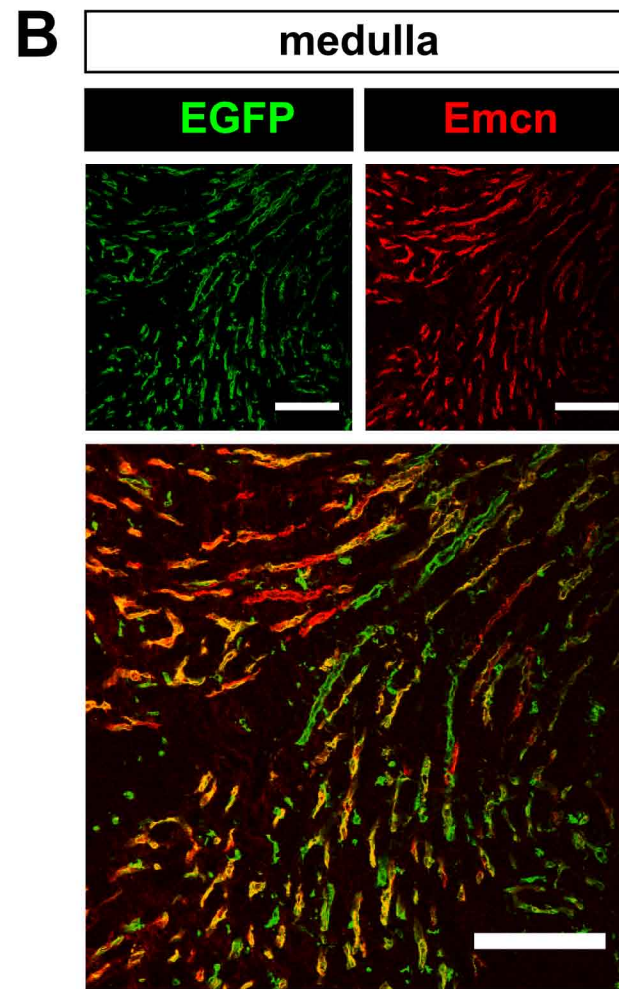
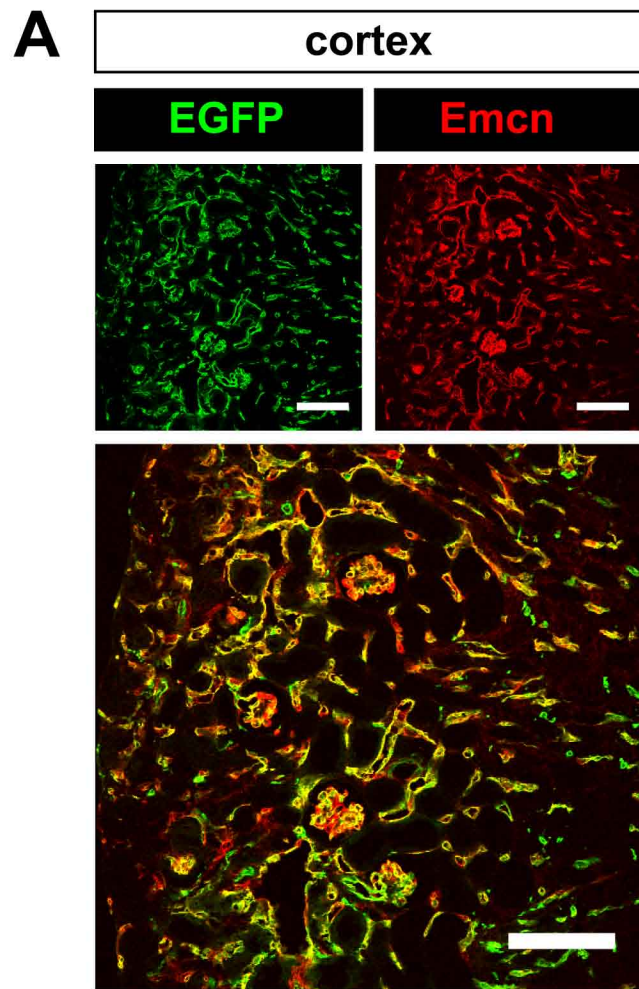
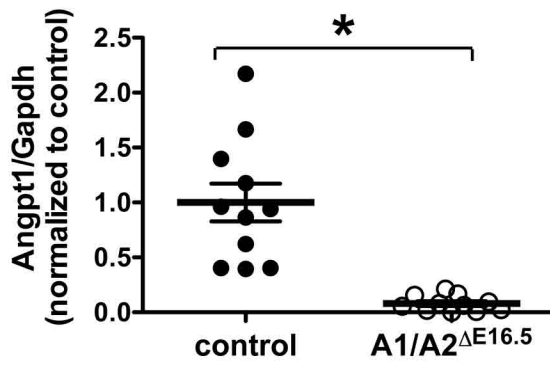
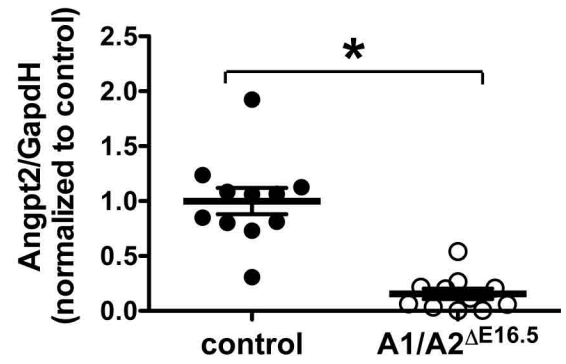
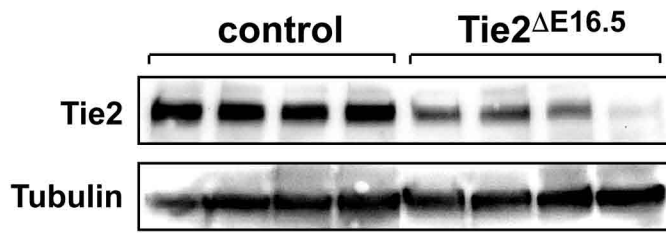
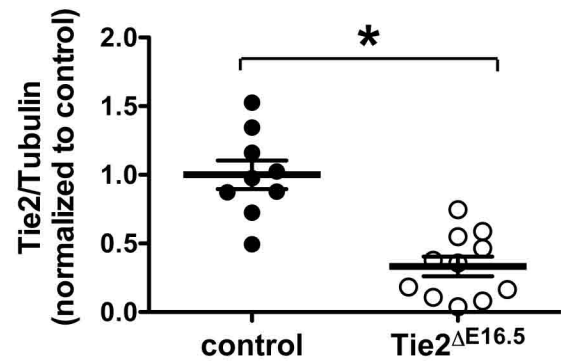
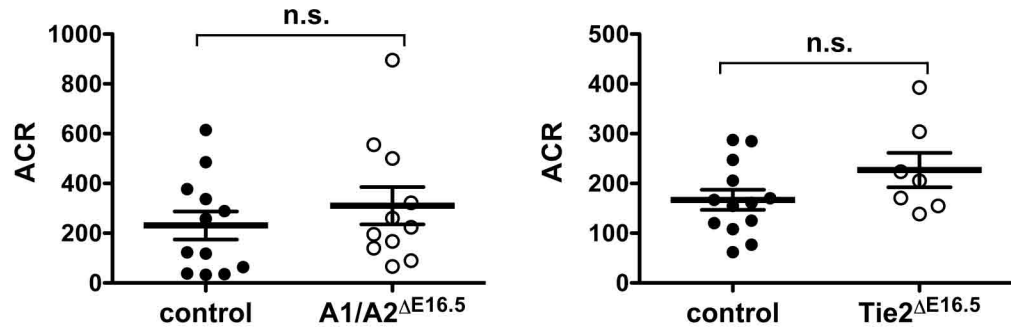
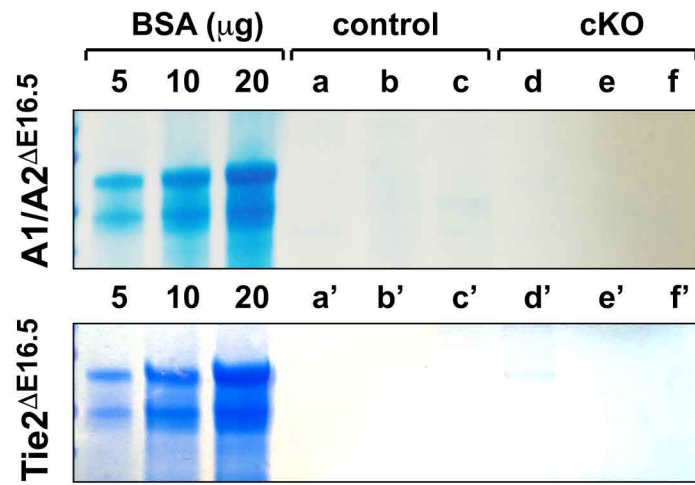
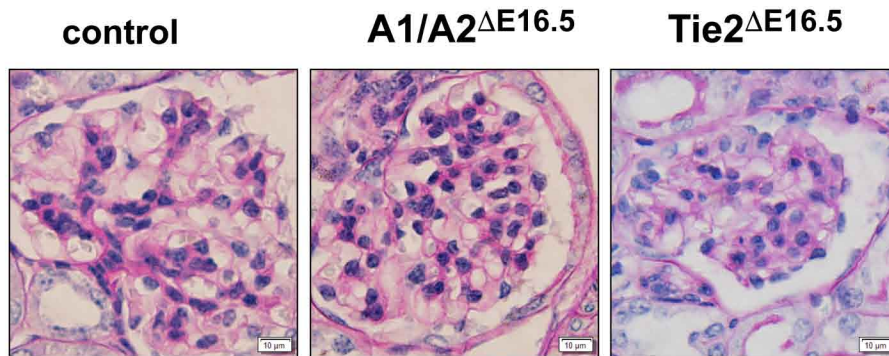
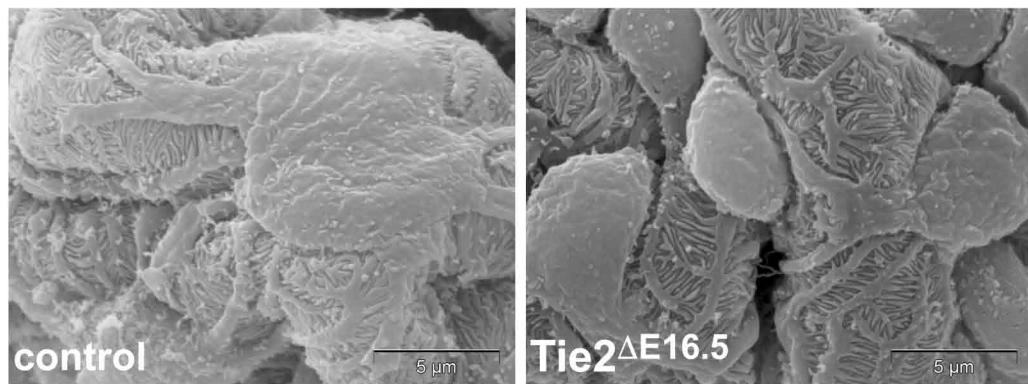


FIGURE S1

A**B****C****D****FIGURE S2**

A**B****C****D****FIGURE S3**

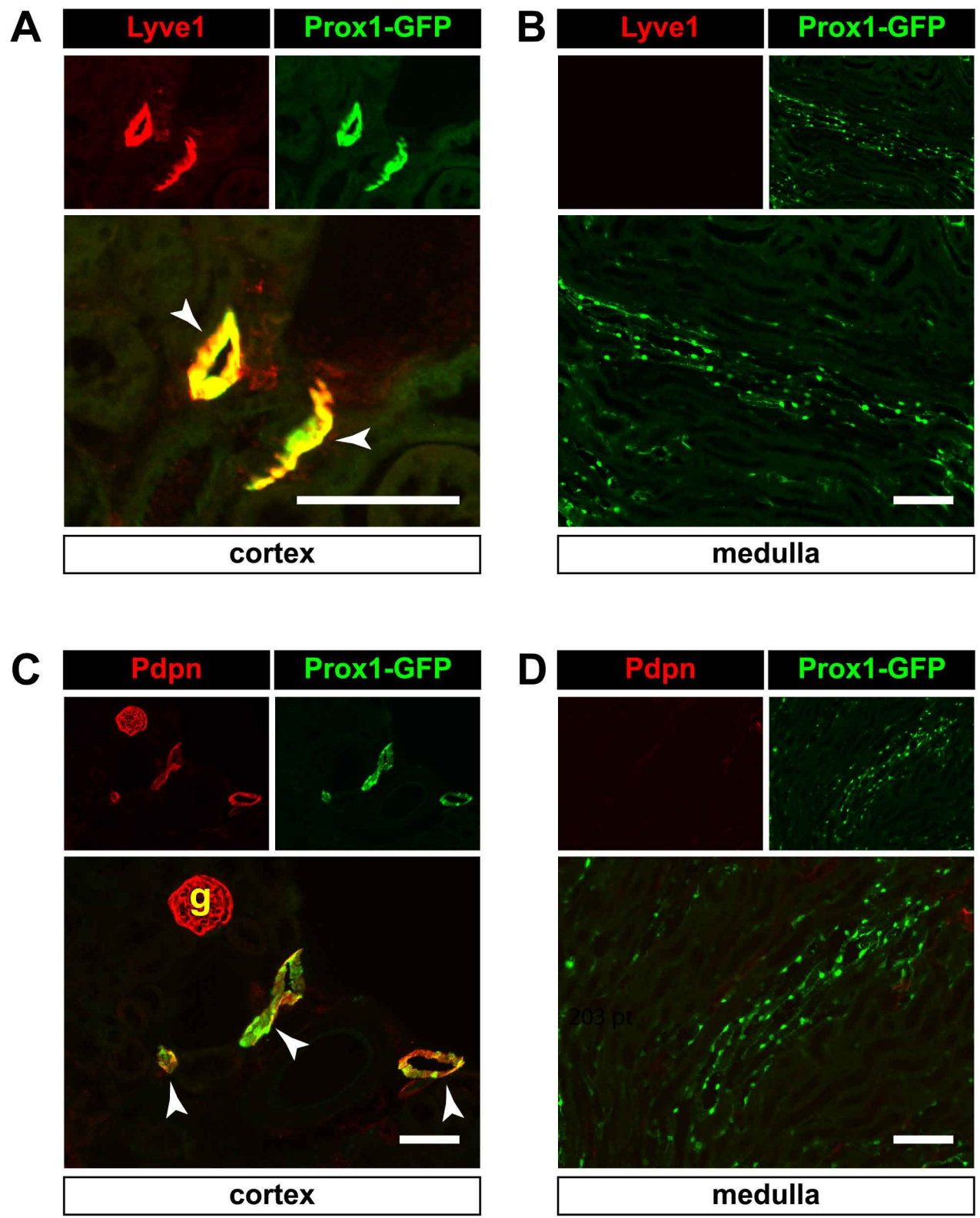


Figure S4

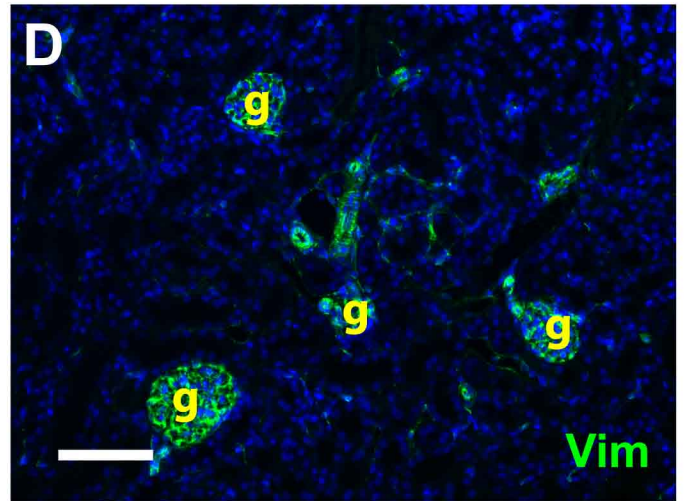
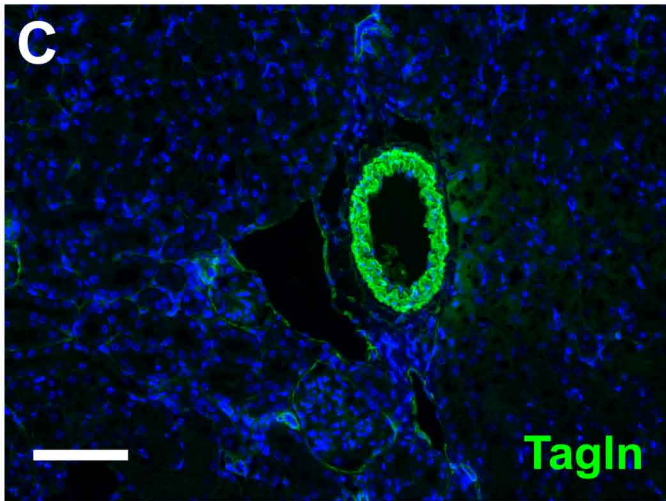
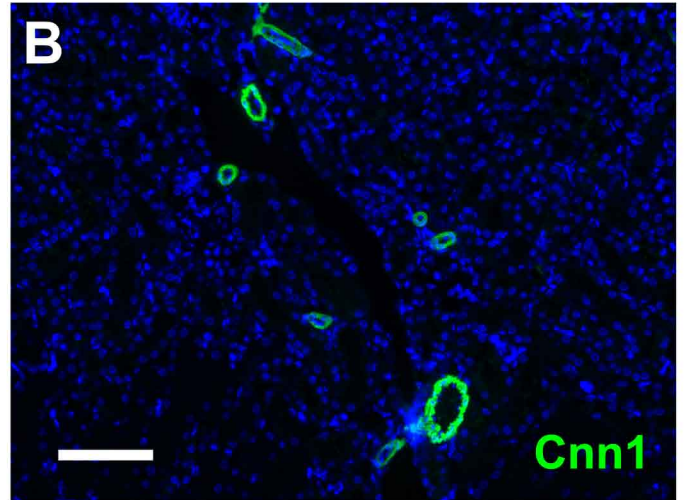
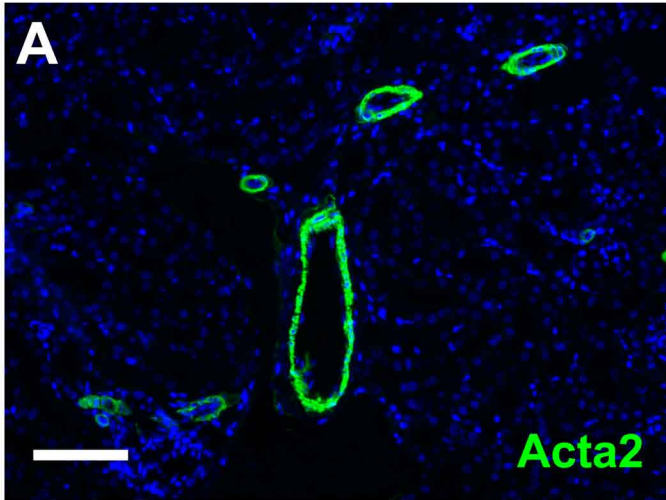


FIGURE S5