Glomerular function and structural integrity depend upon hyaluronan synthesis by glomerular endothelium.

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Running headline: Endothelial hyaluronan loss results in glomerular injury

SUPPLEMENTAL MATERIAL

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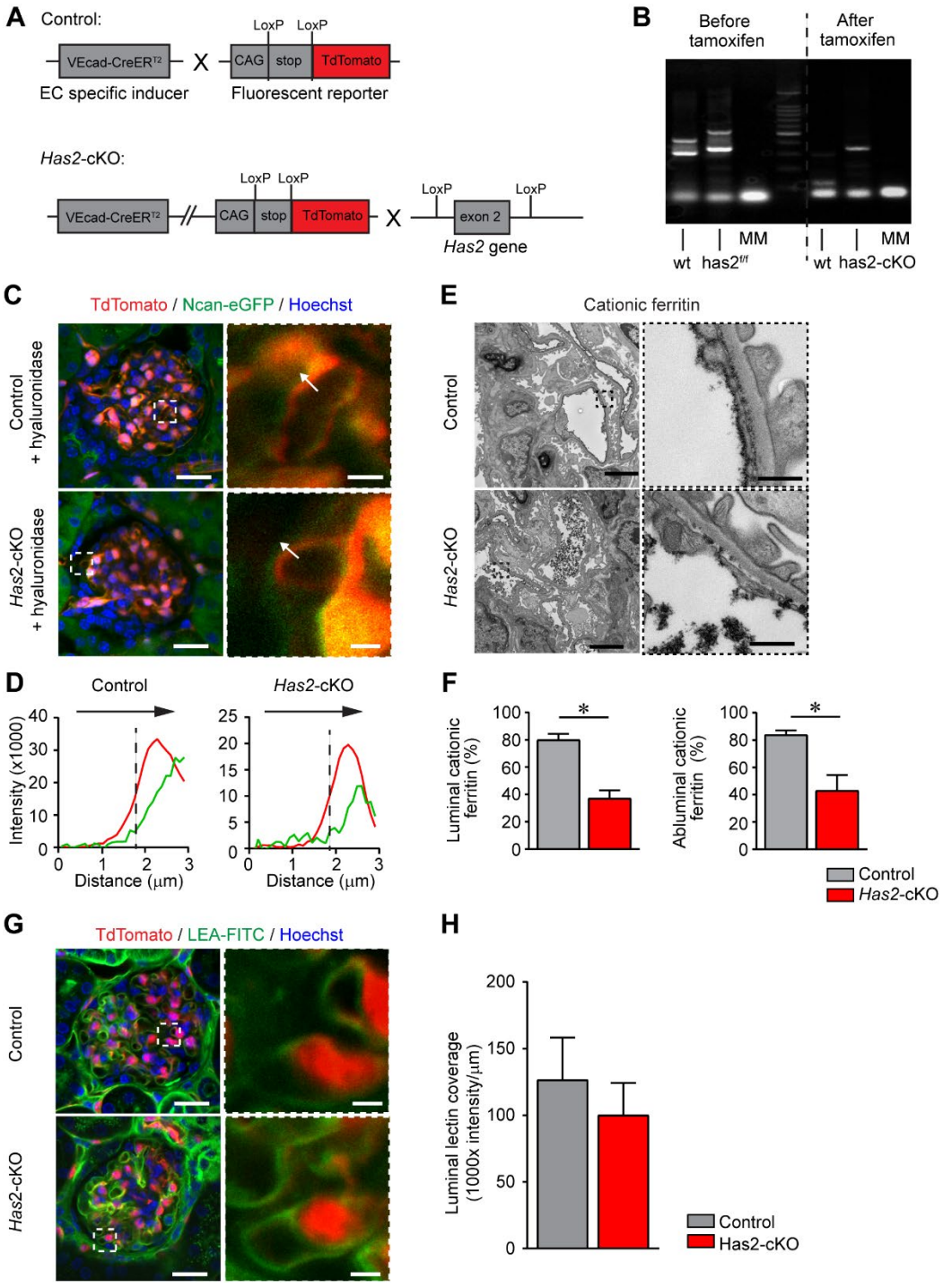
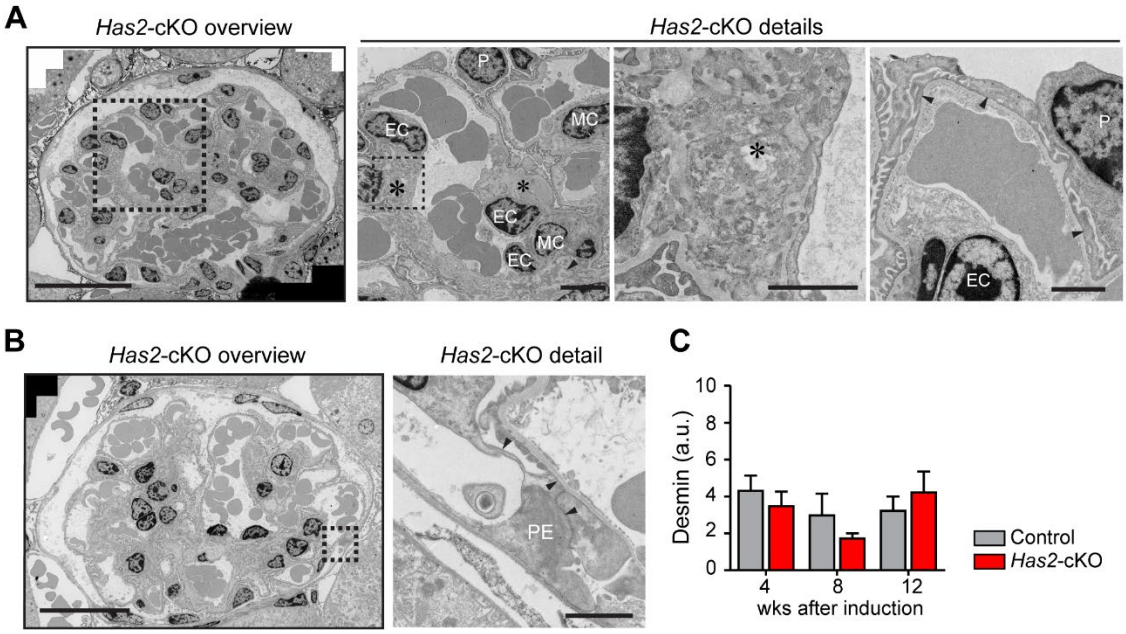


Supplemental Figure 1. Generation of conditional endothelial specific hyaluronan synthase 2 knock-out mice.

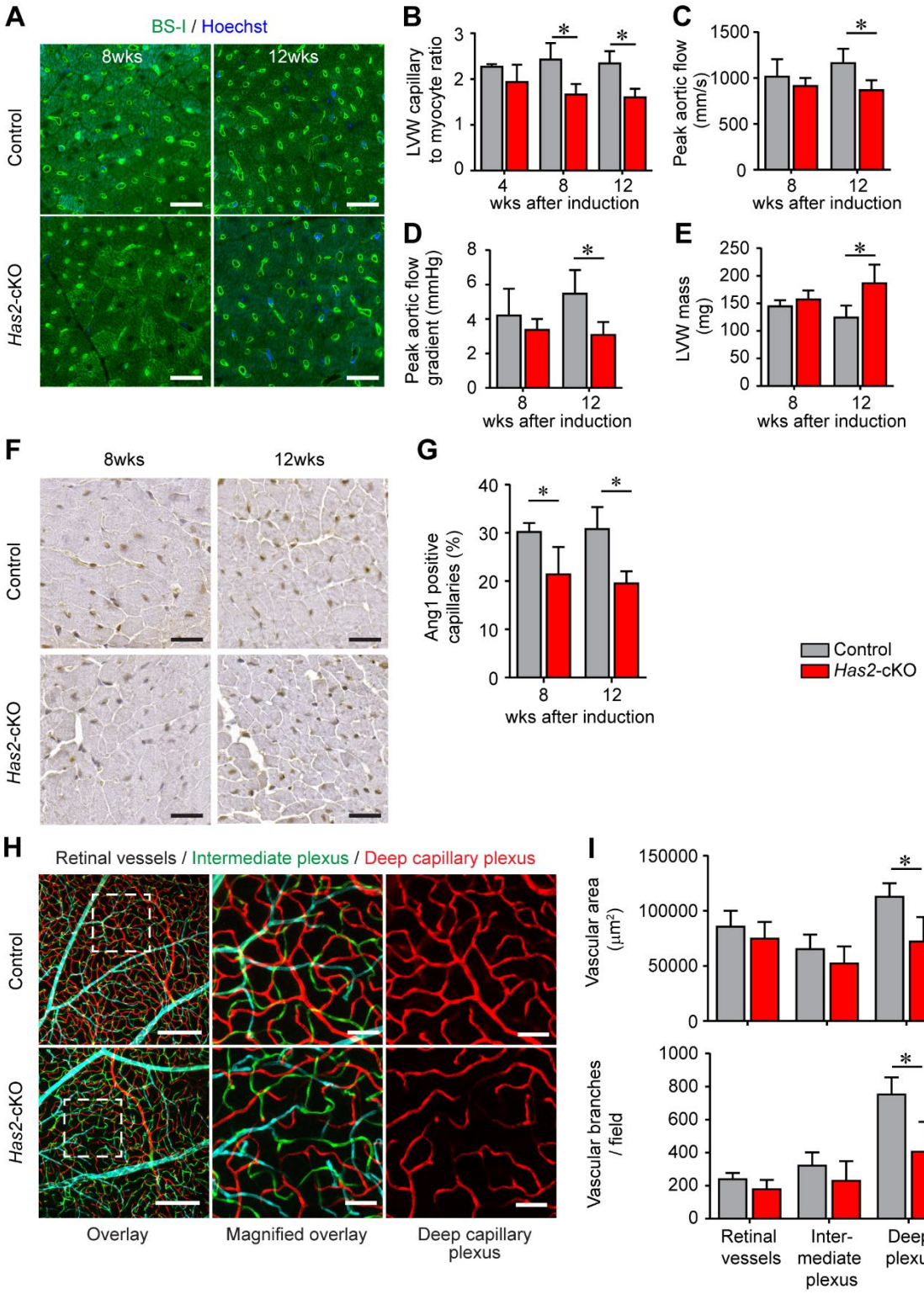
(A) Schematic representation of specific genes introduced in control and hyaluronan synthase 2 knock-out (*Has2*-cKO) mice to allow for conditional endothelial changes. CAG = synthetic CAG promoter; stop = stop sequence; LoxP = Cre-Lox specific recombination site. **(B)** Example of genotyping of mice

homozygous for the floxed has2 gene (*has2*$^{f/f}$) before tamoxifen induction (left lanes) and confirmation of mutation of *has2* gene (exon 2 deletion) after tamoxifen induction (right panel). MM = PCR master mix only. **(C)** Representative cross-sectional average intensity projections of control (upper panel) and *Has2*-cKO (lower panel) glomerular tissue sections incubated with the hyaluronan degrading enzyme hyaluronidase before staining with the hyaluronan binding probe Ncan-eGFP. Endothelial cells are colored red (TdTomato label) upon tamoxifen induction (scale bar 30µm). Insets (right panels) show measurement lines producing the **(D)** line-graphs demonstrating the absence of luminal hyaluronan. **(E)** Representative transmission electron micrographs of cationic ferritin stained glomerular endothelial surfaces in control and *Has2*-cKO mice, 4 weeks after tamoxifen induction, with magnifications on the right (scale bars 5µm, left, and 0.5µm, right). **(F)** Quantification of luminal- (left graph) and subendothelial (right graph) cationic ferritin coverage (*n = 3/group*). **(G)** Representative cross-sectional confocal images of glomerular tissue sections, 4 weeks after tamoxifen induction. Sections are stained with the lectin *Lycopersicon esculentum* (LEA-FITC), endothelial cells are labelled with TdTomato (red) upon induction with tamoxifen (scale bar 30µm). **(H)** Luminal hyaluronan surface coverage quantification (*n = 3/group*). Values are given as mean ± s.e.m. and difference was assessed by non-paired 2-tailed Student's *t* test; $^{*}P < 0.05$.



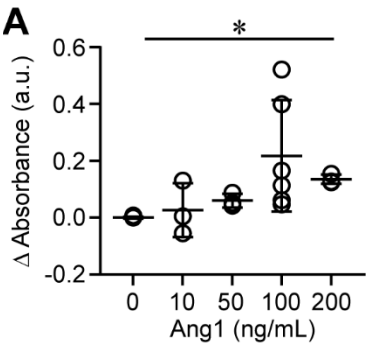
Supplemental Figure 2. Changes in renal morphology and inflammation markers.

(A) TEM overview of a *Has2*-cKO glomerular stitch for reference with indications of matrix expansion (scale bar 25µm). Detailed TEM images from week 8 up to 12 after *Has2* deletion shows mesangial sclerosis (left- and middle panel, asterisk) and foot process effacement (right panel, arrowheads) (scale bars 5µm and 0.5µm). **(B)** TEM overview of a *Has2*-cKO glomerular stitch for reference with indications of occasional parietal epithelial cells bound to the capillary loop basement membranes and podocytes (right panel detail, arrowhead) (scale bar 25µm and 0.5µm). During the 12-week period glomerular changes were without changes in **(C)** cytoskeletal protein desmin levels. Values at 4- (*n = 5-, 4/group*), 8- (*n = 4/group*) and 12wks (*n = 4/group*) are given as mean ± s.e.m and differences between control and *Has2*-cKO mice were determined by 1-tailed Mann-Whitney U-test. ***P < 0.05.***



Supplemental Figure 3. Loss of endothelial hyaluronan results in cardiac morphologic- and functional changes and retinal capillary rarefaction.

As endothelial loss of hyaluronan was not confined to kidneys, we also studied the microvasculature in myocardium and retina. **(A)** Concurrent with endothelial loss of hyaluronan, capillary rarefaction developed in the myocardium as exemplified by *Griffonia simplicifolia* (BS-1) lectin LVW staining for determination of capillary density (scale bar 50µm) and **(B)** quantification of LVW capillary to myocyte ratio density at 4- (*n = 6-, 3/group*), 8- (*n = 5/group*) and 12wks (*n = 5/group*) after tamoxifen induction (*n = 5-6*/group). Further M- mode Doppler echocardiography, and ascending aortic pulse wave-Doppler flow measurements demonstrate reduced myocardial contractility as measured by a significant lower **(C)** peak aortic flow and **(D)** peak aortic flow gradient and accompanied by increased **(E)** left ventricular mass 12 weeks after tamoxifen induction (n = 5-6/group). **(F)** Representative myocardial angiopoietin-1 (Ang1) presence at 8- and 12 weeks after induction in control (upper panels) and *Has2*-cKO (lower panels) mice. **(G)** In concordance with observations in the glomerular tufts, Ang1 was significantly reduced. **(H)** Representative images depicting endothelial cell-TdTomato reporter fluorescence of isolated retinal vascular plexus of control (upper panels) and *Has2*-cKO (lower panels) mice at 6 weeks after induction (*n = 3-5/group*). Left panels show a pseudo-colored overview of the retinal vascular network with central feeding artery and vein plus branches in cyan, the superficial capillary plexus in green and the deep capillary plexus in red (scale bar 200µm). Middle panels show a magnified overlay of left panels, and right panels show the TdTomato stained endothelial cells of the deep capillary plexus (scale bar 50µm). Compared to control plexus loss of endothelial cells in the has2-cKO retina can be observed, which is quantified in **(I)** vascular area and number of vascular branches per field.
Values are given as mean ± s.e.m. (**B-E,F-I**) or mean ± s.d. (**I**) and differences between control and *Has2*-cKO mice were assessed by non-paired 2-tailed Student's *t* test; ***P < 0.05***.



B

	Free binding energy ΔG (kcal/mol)
Fibrinogen	- 11.99
CD44	- 11.69
Ang1	- 12.49

Supplemental Fig. 4. Hyaluronan binds angiopoietin 1.

(A) Direct binding of recombinant human Ang1 to hyaluronan is shown as the difference between absorbance of Ang1 bound to surface coated HA and absorbance of Ang1 binding to the surface in presence of buffer alone (Δ Absorbance) of 6 separate experiments. **(B)** Comparison of free binding energies obtained from binding predictions of hyaluronan to fibrinogen, CD44 and Ang1, respectively. No binding was found for HA to Ang2.

Values are given as mean ± s.d. and difference in added angiopoietin 1 concentrations was assessed using ANOVA; $^{*}P < 0.05$.

Supplemental table 1 Detailed glomerular morphology quantification at 12wks after tamoxifen induction

	Control	*Has2-cKO*
Endothelial layer thickness (nm)	89.8 ± 14.4	*136.5 ± 21.8*[#]
% Endothelial fenestration	38.6 ± 6.5	*19.1 ± 4.8*[#]
GBM thickness (nm)	191.7 ± 12.4	*234.7 ± 10.2*[#]
Podocyte foot process base width (nm)	491.0 ± 19.5	*621.5 ± 46.9*[#]

Mean ± sd

[#] $P < 0.05$ versus control

Supplemental table 2 Primers used for genotyping

	Forward (5'-3')	*Reverse (5'-3')*	*Product (bp)*
VE cadherin	gcaggcagctcacaaaggaacaat	tgtccttgctgagtgacagtggaa	*550*
VE cad-CreER^{T2}	gcaggcagctcacaaaggaacaat	atcactcgttgcatcgaccggtaa	*950*
R26R wt	aagggagctgcagtggagta	ccgaaaatctgtgggaagtc	*297*
R26R-TdTomato	ctgttcctgtacggcatgg	ggcattaaagcagcgtatcc	*196*
Has2 wt	tgcagaatttaggggcgaattgggagctaa	atgaggttagagattagcaagactgagttc	*441*
	cttgaaccttgagtgtgccattttgtagtc	cattcttgttttgaagtttgtttccttgac	*322*
Has2 floxed	tgcagaatttaggggcgaattgggagctaa	atgaggttagagattagcaagactgagttc	*550*
	cttgaaccttgagtgtgccattttgtagtc	cattcttgttttgaagtttgtttccttgac	*355*
Has2 mutant	tcacatgcatgcacaatcag	acagatggtcacagccttcc	*660*

Supplemental complete methods

Reagents. All materials are from Sigma-Aldrich (St. Louis, MO, USA) or stated otherwise.

Mice. Endothelial specific conditional homozygous has2 knockout (has2-cKO) mice were generated by breeding B6.*Cdh5*-Cre-ER^{T2} with B6.*Has2*$^{fl/fl}$ [1-5]. For reporter analysis, *Cdh5-Cre-ER*^{T2}.*Has2*$^{fl/fl}$ and *Cdh5-Cre-ER*^{T2} (ctrl) mice were crossed to *Rosa-TdTomato*$^{fl/fl}$ reporter mice, in which Cre-mediated excision resulted in endothelial Tomato expression, as illustrated in Supplementary Figure S1a. Animal experiments were approved by the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences (permit no. HI 11.2504) or by the ethical committee on animal care and experimentation of the Leiden University Medical Center (permit no. 14-033). All animal work was performed in compliance with the Dutch government guidelines. Presence of introduced genes from ear pinched DNA samples is performed on all animals during breeding and for confirmation of has2 gene mutation (exon 2 deletion) after tamoxifen on *Has2*-cKO mice at the end of the experiment using PCR and primers (Supplementary Fig. 1B & Supplementary Table 2). Only *Has2*-cKO mice with a confirmed mutation within their has2 gene were examined further. Eight-week-old male *Has2*-cKO and control mice (*n = 5-6*/time-point) received intra-peritoneal 2mg/0.2mL tamoxifen for 5 consecutive days. Two days before tamoxifen treatment and at indicated time intervals (2-, 4-, 8- and 12 weeks after induction), body weight and blood pressure measurements were performed. One week before the endpoints at 8- and 12 weeks, echocardiography was performed. Additional animals, 4wks after tamoxifen, were used for renal cationic ferritin perfusion (*n = 3/group*), renal tissue slice collection (*n = 3/group*) and at 6wks after tamoxifen for collection of retinas (n = 3-5/group).

Urine collection and analysis. Mice (Has2-ckO and control) were weighted before and after residing in the metabolic cage (Tecniplast S.p.a, Buguggiate, Italy) and water- and food intake, urine and fecal excretion were collected. After acclimatization, 24hrs-urine was collected (maximal 4 times per mouse). Urine was centrifuged and subsequently stored at -20°C. Albumin levels were quantified using Rocket immunoelectrophoresis [6] and creatinine levels were determined by the Jaffé method using 0.13% picric acid, and quantified using a creatinine standard set. Optical densities were measured with an Ascent Multiscan (Thermo electron corp., Vantaa, Finland).

Non-invasive tail-cuff blood pressure measurement. Blood pressure (BP) was measured in conscious animals using the tail-cuff method (CODA-2; Kent Scientific, Torrington, CT). Briefly, each session consisted of five acclimatization cycles, followed by 15 BP measurement cycles. The average from the automatic approved sessions (>50% valid) was used for mean BP, and heart rate in each individual mouse.

Echocardiographic assessment. Transthoracic echocardiography was performed on temperature controlled and lightly, isoflurane, anesthetized mice using a Vevo 2100 high-resolution system (Visual Sonics, Toronto, Canada) equipped with an 18-38-MHz MS400 scan head. Two-dimensional guided M-mode tracings were recorded in both parasternal long and short axis views at the level of the papillary muscles8. Diastolic left ventricular posterior wall thickness, ventricular parameters including diastolic interventricular septal thickness and systolic left ventricular internal diameters were measured (data not shown) and left ventricle wall mass, ejection fraction and stroke volume were calculated with the established standard equation. For measuring systolic flow parameters pulse wave (PW) Doppler was used in the aortic arch for obtaining peak flow velocity and peak aortic velocity gradient9. All measurements were made from more than three beats and averaged.

Isolation of retinal tissue. Six weeks after tamoxifen treatment of eyes of has2-cKO and control mice were excised and fixed in 4% paraformaldehyde in PBS at 20°C for 10 min. Retinas were dissected and post-fixed in 4% paraformaldehyde in PBS at 4°C overnight and embedded in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) for whole-mount examination of TdT-Tomato positive retinal vessels. Tissues were examined using a LEICA TCS SP8 X WLL (Leica, Rijswijk, The Netherlands) and a 20x objective (HC PL APO 20x/0.70 DRY, Leica). Sequential 16-bit confocal images (xyz dimensions, 0.758×0.758×1.0µm) were recorded using LAS-X Image software (Leica) and analyzed with ImageJ.

Tissue preparation and histology. Mice were anaesthetized by isoflurane and after abdominal exposure the right kidney was ligated and fixed immediately by injecting 2% paraformaldehyde (PFA) in PBS under its capsule. Next, the beating heart was excised and directly placed in 4% PFA, followed by excision of both kidneys, liver, spleen and lungs. Kidney capsules were removed, one half of the right kidney was fixed overnight at 4°C in 2% PFA in PBS for detection of endogenous albumin[7], data not shown, or detailed glomerular morphometric analysis, as described elsewhere. [8] Both halves of the left kidney

were placed in 2% PFA in PBS overnight at 4°C, followed by paraffin embedding for periodic acid-Schiff (PAS) or immunofluorescence staining. Some human biopsy samples were stained with a methenamine silver-Periodic acid-Schiff (MPAS) stain.

Creation of Neurocan-dsRed construct. The N-terminus rat neurocan construct of the hyaluronan specific neurocan-eGFP (Ncan-eGFP, gift from J. Kappler) construct [9], linked to the BM 40 signal peptide with APLGRGSHHHHHHGGLA and a GSSGA linker at the C-terminus, was excised and fused into a pDsRedMonoN1 (ClonTech, Mountain View, CA, USA) plasmid. The modified neurocan-dsRed (Ncan-dsRed) construct was incorporated into a self-inactivating (SIN) lentiviral vector (pLV-CMV-IE.Ncan-dsRed) and transduced into Hek293 cells for production. Ncan-eGFP and Ncan-dsRed were isolated from supernatant and purified on a Ni-NTA resin column using the incorporated 6xHistidine tag within the protein.

Tissue slice imaging. Slices were glued to the bottom of a Petri dish with Permabond 910 (Electron Microscopy Sciences), submerged in HBSS and examined using a Zeiss 710-NLO CLSM microscope (CarlZeiss, Göttingen, Germany) with a 20x water dipping objective (W-Plan Apochromat, NA 1.0 DIC M27 70mm; Carl Zeiss). Sequential 16-bit confocal images (xy dimensions, 0.05×0.05µm) were recorded using ZEN-2009 Image software (Carl Zeiss) and analyzed with ImageJ, as described earlier[7]. For each glomerular image, the amount and location of intensity profiles were quantified by estimating the distance from the luminal half-width of the endothelial TdTomato signal to the half-width of the intraluminal Ncan-eGFP (ctrl, *n = 7*; ctrl + hyal *n = 5*; *Has2*-cKO, *n = 12*; *Has2*-cKO + hyal, *n= 10*) or LEA-FITC (ctrl, *n = 5*; has2cKO, *n = 9*) fluorescence along the lines (5 per image) of interest as shown in Figure 2A,B. Total luminal endothelial fluorescence was calculated from fluorescence within this section.

Immunohistochemistry. Deparaffinized kidney sections (4 µm thick) were washed in PBS and antigen retrieval was performed in a citrate (pH 6.0) buffer in an autoclave. Slides were incubated with 3% H_2O_2 for 15 minutes at rT and after washing with 0.01% Triton-X100 in PBS, blocked in Serum-Free Protein Block buffer (Dako, Agilent Technologies Netherlands B.V., Amstelveen, the Netherlands) for 1hr at rT. Primary Goat anti-mouse Ang1 (AF923, R&D Systems) was incubated overnight (at 4°C), followed by Rabbit Anti-Goat-IgG-HRP (Dako) for 1 hour, both in blocking buffer. Kidney tissue samples were developed with DAB solution (1:100, Dako) for about 1-2 minutes and counterstained for 1-2 sec with Hematoxylin. Mouse heart tissue was processed for paraffin embedding and 4µm tangential sections of

the entire heart were incubated with fluorescent linked *Griffonia simplicifolia I* isolectin B4 (isolectin BS-I-FITC) for determination of capillary density. Renal and heart tissue slides were recorded using a 3D Histech Pannoramic MIDI Scanner (Sysmex, Etten-Leur, the Netherlands) and a 40x objective (Plan Apochromat, NA 0.95; Carl Zeiss). Quantification was performed using the public domain NIH ImageJ software (FIJI version 1.49m; http://rsb.info.nih.gov/ij); for renal morphology 20-25 sub-cortical glomeruli per kidney slice (*n = 5-6*/time-point), cardio-myocyte area determination of approximately 40-50 individual cells per cardiac segment (*n = 5*/time-point), and number capillaries and cardiomyocytes (to calculate capillary to myocyte ratio) of 3 separate fields per cardiac segment (*n = 5*/time-point) were measured.

Deparaffinized sections (4 µm thick) of post-mortem *human diabetic nephropathy* (DN) samples or DN kidney biopsies were washed in PBS and antigen retrieval was performed in antigen retrieval buffer (Dako) in an autoclave. After washing with PBS, blocked in Serum-Free Protein Block buffer (Dako) for 1hr at rT, slides were incubated overnight with Ncan-dsRed (200µg/mL), 10µg/mL LEA-FITC (L0401) and 20µg/mL Rabbit anti-laminin (L9393) at 4°C, followed by Goat Anti-Rabbit-AF-647 (Molecular probes) for 1 hour in blocking buffer, embedded in Prolong™ gold antifade mountant with DAPI (ThermoFisher, P36931). Tissue slides were recorded using a 3D Histech Pannoramic MIDI Scanner (Sysmex, Etten-Leur, the Netherlands) and a 40x objective (Plan Apochromat, NA 0.95; Carl Zeiss). Quantification was performed using the public domain NIH ImageJ software (FIJI version 1.49m; http://rsb.info.nih.gov/ij).

Cationic ferritin labeling. Detection of glomerular endothelial surface layer with cationic ferritin. Four weeks after tamoxifen induction of control and Has2-cKO mice (n = 3/group) were anesthetized, the abdominal aorta was exposed and cannulated adjacent to the left renal artery. The right renal artery was ligated at the renal stalk after which the left kidney was perfused with 5mL Hanks-buffered salt solution (HBSS, Gibco) containing 0.5% BSA (Sigma, A7030, essentially globulin free) and 5U/mL heparin at 2mL/minute to remove blood, followed by 2mL of cationic ferritin (horse spleen, 2.5mg/mL, Electron Microscopy Sciences, Fort Washington, PA) in HBSS at 2mL/minute. The left kidney was excised, its capsule removed, and stored in fixative (1.5% glutaraldehyde and 1% paraformaldehyde (both from Electron Microscopy Sciences, Hatfield, PA) in 0.1M sodium-cacodylate buffered solution, pH 7.4) overnight at 4˚C. The tissue was subsequently sectioned in 180µm thick slices with a Leica VT1000S vibratome, rinsed 2x with 0.1M sodium cacodylate-buffered solution, and post-fixated for 1hr with 1% osmium tetroxide (Electron Microscopy Sciences) and 1.5% potassium ferrocyanide in demineralized

water. Samples were further washed, dehydrated in ethanol, infiltrated with a mixture of epon LX-112 and propylene oxide (1:1) for 1 hr, followed by pure epon for 2hrs, embedded in epon mounted in BEEM capsules (Agar Scientific, Essex, United Kingdom) and polymerized for 48hrs at 60°C. 100nm Thick sections were cut using a diamond knife (Diatome, Biel, Switzerland), collected on single slot copper grids covered with formvar film and carbon layer, and then stained with 7% uranyl acetate in demineralized water for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Data was collected at an acceleration voltage of 120kV on a Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM), equipped with an FEI 4k Eagle CCD camera, in a similar fashion as described above. Virtual slides were acquired with 18,500x magnification at the detector plane, corresponding to a 1.2nm pixel size at the specimen level. Each recorded glomerulus from representative virtual slides of control (n = 5) and Has2-cKO (n = 7) mice were divided into 4 quadrants from which 2 capillary loops per quadrant were selected for high resolution imaging. Quantification was performed using an automatic grid overlay (ImageJ). Cross-hairs at the intersection of endothelium (>80) were counted and the percentage of coverage with cationic ferritin was determined.

Ultrastructural Large-Scale Virtual Slides. For analysis of detailed morphologic changes in glomeruli of mice at 4-, 8- and 12 weeks after tamoxifen induction virtual slides were recorded using the Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Eindhoven, The Netherlands), equipped with an FEI 4k Eagle CCD camera, at an acceleration voltage of 120 kV. To create an overview of the glomerulus, a large amount of 6,800x magnification images, corresponding to a 3.2nm pixel size at the specimen level, collected automatically using stitching software13. Quantification of morphologic changes was performed for control and has2-cKO mice (n = 3 each) at 12 weeks after tamoxifen induction in 8 capillary loops per glomerulus, using an automatic grid overlay (in ImageJ). Cross-hairs were introduced in each image and at the intersection with the endothelium (>15) the endothelial cell thickness, basement membrane thickness and podocyte foot process width were measured, essentially as shown previously11. Percentage fenestrations were calculated from counting fenestrated or non-fenestrated sites at the cross-hairs intersection.

In silico Angiopoietin / hyaluronan prediction model. Angiopoietin 1 (GenBank: AAM92271.1) and Angiopoietin 2 (GenBank: AAI43903.1) amino acid sequences were analyzed using online *PredictProtein* software (www.predictprotein.org/home) and the predicted glycosaminoglycan binding domain within the secondary structure was evaluated against fibrinogen (GenBank: CAA50740.1) and a functional

region map was drawn (Figure 3B). 3D structures of Ang1 (target; PDB code 4JYO) and HA (2BVK) from the protein databank (www.rcsb.org/pdb/home) were downloaded and the HA code was transformed to a mol2 format using UCSF *Chimera* software (www.cgl.ucsf.edu/chimera/) for use as ligand in the online *Swissdock* software (www.swissdock.ch/docking), to obtain 3D docking models of the Ang1-HA binding domain, as predicted above. Using the UCSF *Chimera* software, the predicted model with lowest binding energy is shown (Figure 3C).

Angiopoietin / hyaluronan binding assay. First, addition of 50 µL of Coating buffer (0.2 M sodium carbonate/bicarbonate pH 9.4) / well (Nunc-Covalink, Thermo-Fisher Scientific) was followed by 40 ng/ml hyaluronan standard (HA Quantikine ELISA kit, DHYALO, R&D Systems) or RD5-18 buffer (DHYALO, R&D Systems) to each well and incubated at room temperature for 2hrs on a horizontal orbital microplate shaker. Wells are washed 5x in between consecutive steps with wash buffer (DHYALO, R&D Systems). Next, recombinant human Ang1 protein (923-AN, R&D Systems) was added and incubated at room temperature for 2hrs on shaker, washed and blocked with Protein Block Serum-Free buffer (X0909, Dako) for 1hr at rT on shaker. Wells are aspirated and 100 µL of 0.2 µg/mL Goat Anti-Human angiopoietin-1 antibody (AF923, R&D Systems) was added, incubated for 1hr at rT on shaker, and washed. Secondary Rabbit Anti-Goat IgG HRP antibody (P0160, Dako) was added and incubated for 1hr at rT on shaker, washed and followed by 100 µL Substrate Solution (color reagent A and color reagent B; DHYALO, R&D Systems) for 30 minutes at rT. Reaction was stopped with Stop Solution (DHYALO, R&D Systems) and absorbance was read at 450 nm within 30 minutes with wavelength correction to 540 nm or 570 nm. Values are given as Δ Absorbance [A450(40 ng/mL HA)- A450(RD5-18 buffer)], of 3-6 separate experiments.

To differentiate Ang1 / Ang2 binding to HA, 250µg/mL of 10-20kDa HA (Lifecore Biomedical LCC, Chaska, MN) is mixed with either 500ng/mL Ang1 or Ang2 (R&D Systems) and incubated overnight at rT (control HA alone). To separate bound from unbound HA, samples diluted with PBS are centrifuged through a Amicon ultra-15 centrifugal filter unit (30kDa cut-off, Merck) at 4000g for 15 minutes, excess unbound is removed with 4 washes of PBS. For HA detection, 20µg/mL (100µL) Neurocan HA-binding peptide in carbonate buffer (0.2M, pH 9.4) was added to 96-well plate and incubated overnight at 4°C. Wells were washed, followed by protein block serum-free buffer (Dako) at rT for 1 hr. Next, angiopoietin bound-HA (residual Amicon samples) were added and incubated at rT for 2hrs on a shaker, washed and followed by 100 µL HABP-biotin conjugate (DHYALO, R&D Systems) and incubated for 2hrs at rT on a shaker. Wells were washed and 100 µL Substrate Solution (color reagent A and color reagent B; DHYALO, R&D

Systems) for 30 minutes at rT. Reaction was stopped with Stop Solution (DHYALO, R&D Systems) and absorbance was read at 450 nm within 30 minutes with wavelength correction to 540 nm or 570 nm. Values are given as bound HA of 3 separate experiments.

Viral constructions.

The hyaluronan synthase 2-short-hairpin RNA lentivirus (pLV-*CMV-IE.HAS2shRNA*) was created through transfecting Hek293 cells (60-80% confluence) with a mixture of combined plasmid DNA of *HAS2shRNA* (Sigma-Aldrich, Mission shRNA library #9868), H1/VSVG and H23/pspax2 with polyethylenimine (PEI, 0.05 mg/mL final concentration) in DMEM (Gibco) to obtain the self-inactivating (SIN) lentiviral construct from supernatant, as described. [10]

In vitro culture experiments. Primary human glomerular-derived microvascular endothelial cells (hgMVECs) were purchased from Cell Systems (ACBRI-128, Kirkland, WA) and cultured in serum-free endothelial cell medium (EC-SFM, Gibco, Thermo-Fisher Scientific, Waltham, MA) supplemented with 1% bovine platelet poor plasma-derived serum (Biomedical Technologies Inc., Stoughton, MA), 50 µg/mL VEGF-165 (R&D Systems) and 100 µg/mL hFGF (Miltenyi Biotech) (EC-SFM full medium) at 37°C and 5% CO_2.

Flow experiments. Flow experiments were performed using an ibidi flow system (Ibidi, Martinsried, Germany) and cells were cultured for 4 days at a constant laminar shear stress of 5 dyne/cm^2 in EC-SFM full medium in the presence of 100 ng/mL recombinant human ANG1 (R&D Systems). Control hgMVEC (pLV-CMV-IE) or pLV-CMV-IE.HAS2shRNA silenced cells were seeded into closed perfusion chambers (ibiTreat, 0.4 µ-Slide VI Luer) at a concentration of 1.0 x 10^6 cells per mL. Hereafter, the chamber is connected to a computer-controlled air pressure pump and a fluidic unit with a two-ways switching valve which allows pumping of 16 mL cell culture medium from two reservoirs in a unidirectional way through the flow channel over the endothelial cell monolayer. Medium was refreshed after 1 day, non-adhered cells removed, and cultured under laminar flow for another three days at 37°C and 5% CO_2.

Next, cells were fixed with 4% PFA and 0.2% Triton-X100 in HBSS or 4%PFA in HBSS (for HA staining) for 10 minutes at rT, washed in HBSS containing 1% BSA (A7030), and blocked for 30 minutes at room temperature in 5% BSA in HBSS. Primary monoclonal Mouse Anti-Human VE cadherin (CD144, 55-7H1 BD Biosciences), monoclonal Mouse Anti-Human active β-catenin (anti-ABC, 8E7, Merck Millipore), Goat

Anti-Human ANG1 (R&D Systems), or Ncan-dsRed were incubated overnight (at 4°C), followed by an appropriate secondary antibody and phalloidin-TRITC (VEcadherin, β-catenin samples) for 1 hour, all in blocking buffer. Cells were counterstained with 10µg/mL Hoechst 33528, embedded in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Cells were examined using a LEICA TCS SP8 X WLL (Leica, Rijswijk, The Netherlands) and a 40x objective (HC PL APO CS2 40x/1.30 OIL, Leica). Sequential 16-bit confocal images (xyz dimensions, 0.142×0.142×0.3µm (Ang1/Ncan-dsRed) or 0.142×0.142×1µm (VEcadherin/β-catenin) were recorded using LAS-X Image software (Leica) and analyzed with ImageJ, as described earlier[7]. Angiopoietin 1 binding was quantified as total fluorescence intensity per cell surface area. Surface hyaluronan expression (Ncan-dsRed) was quantified on 5 cells per field of view (5 views) in essence as described earlier[11]. From a side-view, resliced from a line (10pixels wide) over the nucleus 3 additional lines were drawn at the nuclear position and the mean fluorescence was calculated between the distance from half-maximum signal of the nuclear staining to half-maximum signal at the luminal end. Membrane localization of β-catenin was quantified as percentage cell-perimeter coverage. Difference between stable adherence junctions (AJ) and focal adherence junctions (FAJ) upon junction remodeling[12] was quantified as ratio FAJ length over total junction length[13]. From each independent experiment (*n = 5*) 25 cells are analyzed. (mean ± s.e.m.).

Mouse genotyping analysis. DNA was isolated from mouse ear samples using Extracta™ DNA Prep for PCR-Tissue (Quanta bio, 023131) following the manufacturer's instructions. PCR was performed using AccuStart™ II PCR SuperMix (Quanta bio, 015729) and respective primers (Supplementary Table 2). PCR products were put on 2% agarose gel, and mouse genotyping was confirmed by band analysis.

Western blot analysis. kidney samples from both HAS2-cKO and control mice or cultured hgMVECs described above were washed with PBS and lysed in 50mM TRIS HCL buffer (pH 7.5) containing 150mM NaCl, 1% SDS, 0.5% deoxycholate and 0.5% triton X-100. Protein samples were diluted 5x in SDS sample buffer (pH 6.8; 10% SDS, 25% 2-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue, 0.3125M Tris-HCl, 0.5M DTT), incubated at 95°C for 10 minutes and subjected to SDS-PAGE and western blotting. Proteins, transferred to PVDF membrane (1704156, Bio-Rad Laboratories BV, Veenendaal, The Netherlands) were detected with antibodies against HAS2 (MABC712, Merck Millipore), Phospho-Tie2 (Y992; AF2720-SP, R&D Systems), Pospho-Tie2 (Y1102/Y1100; AF3909, R&D Systems), Tie2 (33, 557039, BD Biosciences), phospho-Akt (S473; P31749, Cell Signaling Technologies, Danvers, MA), Akt (C67E7;

4691, Cell Signaling) and GAPDH (NB300-221SS, Novus Biologicals) after blocking the membrane with 5% fat-free milk in PBS and 0.1% Tween-20. Followed by incubation with a secondary HRP-conjugated antibody and Pierce ECL Western Blotting substrate (32106, Thermo Scientific). Band intensity was analyzed using ImageJ software.

Statistical analysis. Results are presented as mean ± s.d. or mean ± s.e.m., n defines the number of biological replicates. Differences between groups were assessed by non-paired 2-tailed Student's t test, paired 2-tailed Student's t test or, when not normally distributed, by two-tailed F-test. P values < 0.05 were considered statistically significant.

Data and materials availability. Plasmid DNA of *HAS2shRNA* is available from Sigma-Aldrich (Mission shRNA library #9868). The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files. Extra data are available from the corresponding author (T.J.R.) on request.

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