SUPPLEMENTARY MATERIAL

Collagen-specific molecular imaging of renal fibrosis

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Supplementary figures

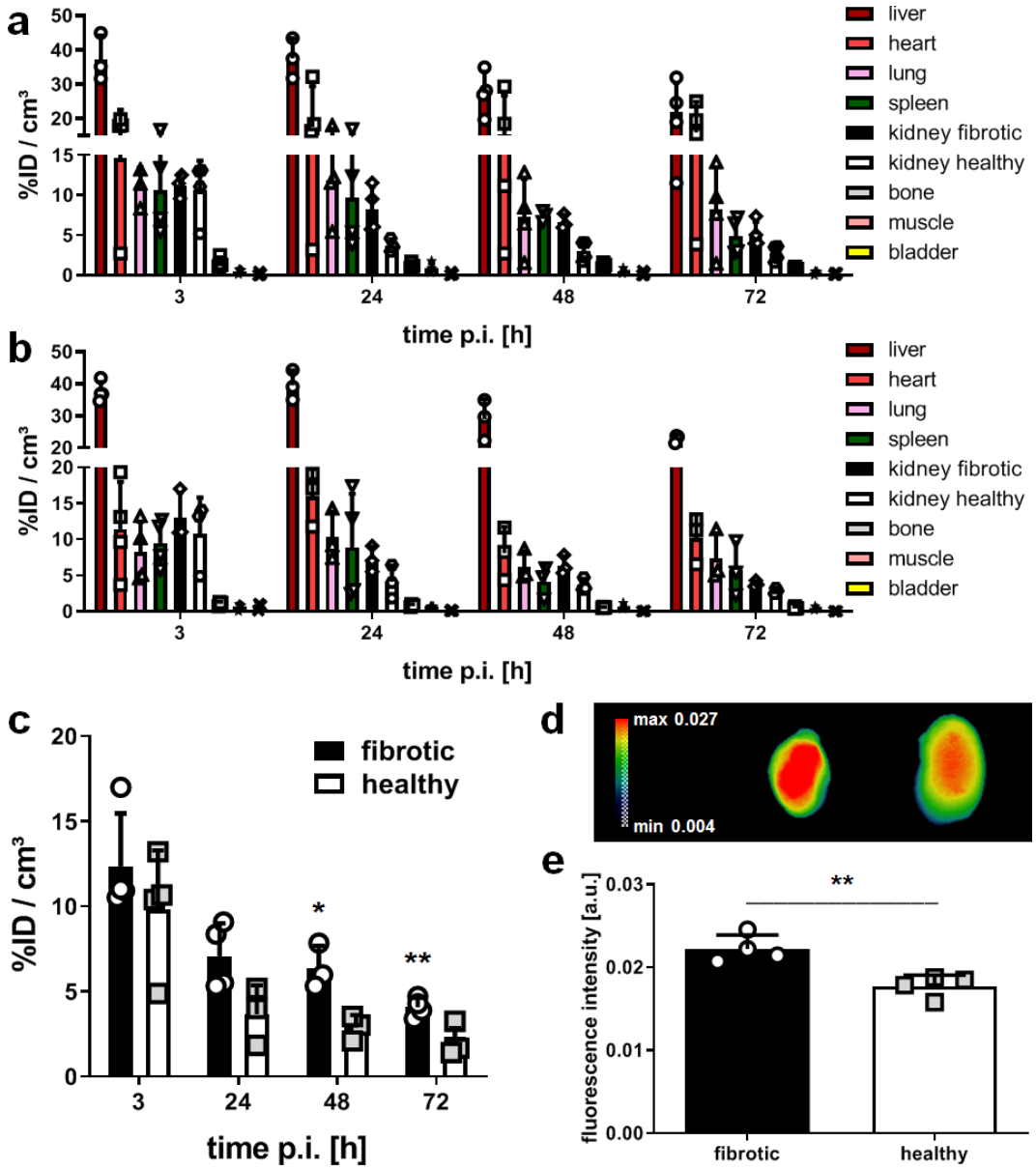

Figure S1| Optical imaging of collagen deposition in renal fibrosis using Cy7 fluorophore-labeled CNA35. (a-c) Biodistribution of CNA35 over time in mice after i.v. injection of 1 nmol **(a)** or 5 nmol **(b)** on day 14 after unilateral I/R. Hybrid CT-FMT imaging indicated a significantly higher accumulation of CNA35 in fibrotic kidneys compared to the healthy kidneys, when 5 nmol are administered **(c)**. **(d, e)** *Ex vivo* FRI analysis 72 h post injection confirmed the *in vivo* findings for the 5 nmol injection. Values represent mean ± SD based on quantifying n=4 mice. *p<0.05, **p<0.01.

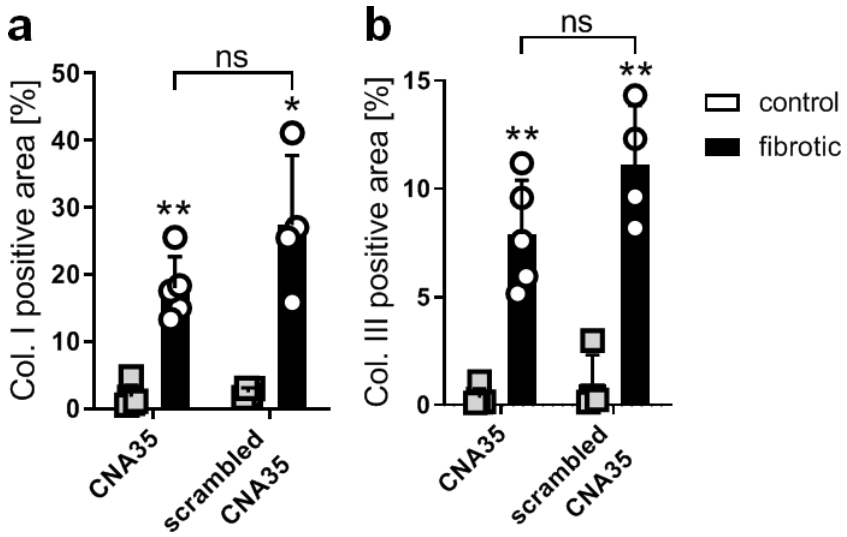

Figure S2| Characterization of collagen deposition in fibrotic kidneys (a, b) Quantification of the collagen type I **(a)** and collagen type III **(b)** positive area fraction of I/R and healthy kidneys. Tissues were obtained from mice used in the *in vivo* experiments with CNA35 and scrambled CNA35. Collagen deposition was significantly elevated in fibrotic kidneys in both experimental setups. Values represent mean ± SD based on quantifying n = 5 mice per group. *p<0.05, **p<0.01.

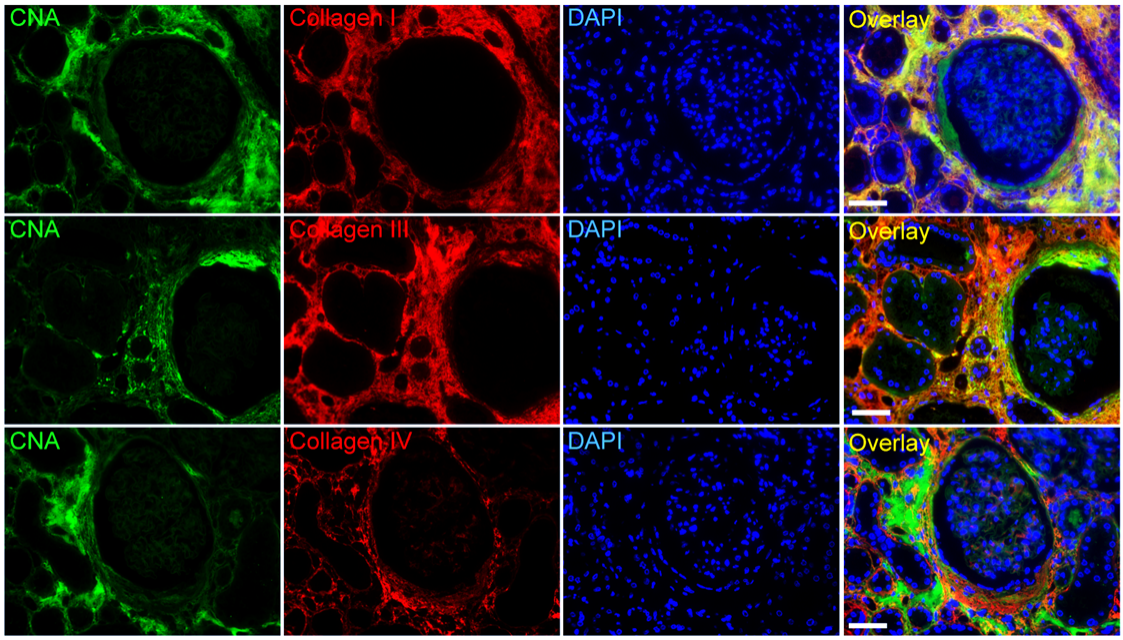

Figure S3| CNA35 co-localizes with collagen fibers in human kidney biopsies. Fluorescence microscopy images of CNA35 (green), collagen fibers (red) and cell nuclei (DAPI: blue) of fibrotic kidney biopsies. CNA35 shows high co-localization with collagen type I and III, but only partly overlaps with collagen type IV. Scale bar: 50 µm.

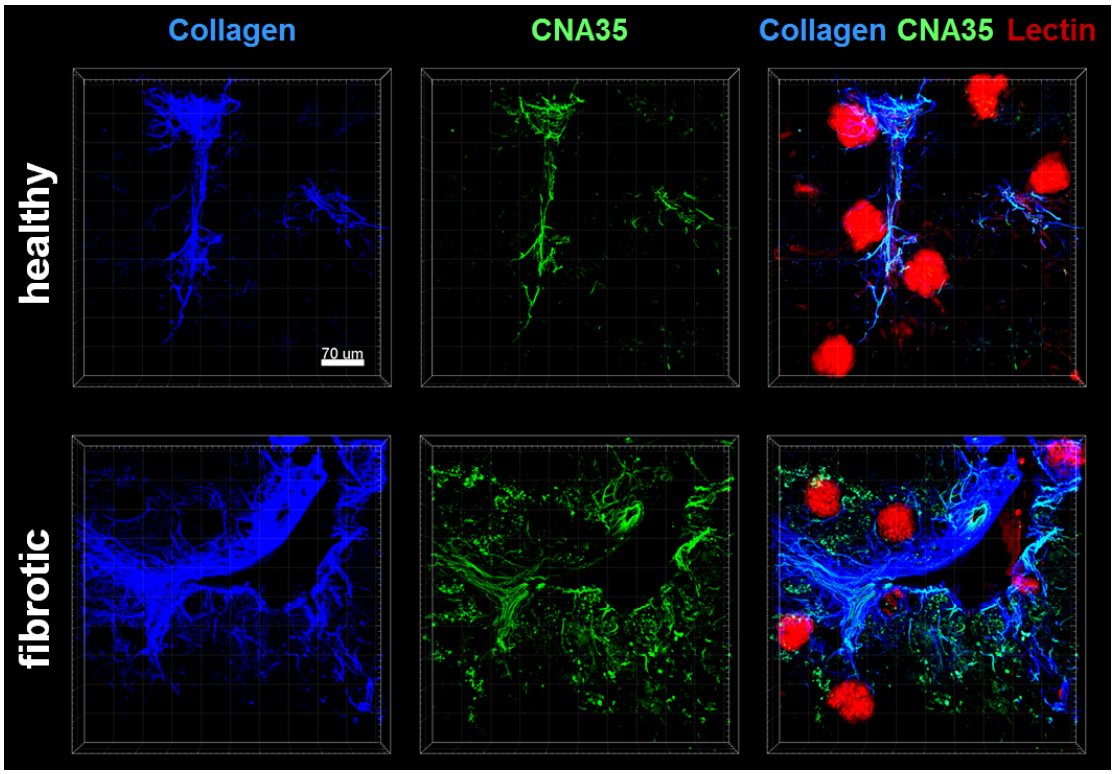

Figure S4| TPLSM-based characterization of CNA35 distribution in kidneys. CNA35 is able to bind specifically to collagen I and III fibers, visualized by means of second harmonic generation (SHG), independent of the blood vessel (red) localization. FITC labeled CNA35 signal (green) overlaps with collagen (blue), except for spot-like green signal that is likely due to low abundant collagen and extremely small vessels with impaired extravasation. Scale bar: 70 µm.

Supplementary methods

Kidney fibrosis animal models

Animal experiments were performed according to the regulations of the local and national ethical committee for animal welfare (LANUV NRW). Mice were housed under SPF-free conditions with constant temperature and humidity under a 12-hour phase light-dark cycle with free access to drinking water and food. All surgeries were performed under anesthesia by ketamine (14 g/kg body weight) and xylazine (8 g/kg body weight) following analgesia with Temgesic (0.05 mg/kg) until 72 h post OP. Every effort was made to minimize fear and pain of animals. Two established unilateral models of renal fibrosis with abundant interstitial collagen accumulation were used, i.e. ischemia-reperfusion (I/R) for 14 days and unilateral ureteral

obstruction (UUO) for 5 days, as previously described [S1] in male 10-12 week old Balb/C mice (Charles River, Germany). Prior to the imaging experiments, mice were fed chlorophyll-free food (ssniff Spezialdiäten GmbH, Germany). All *in vivo* experiments were conducted under inhalation anesthesia (1.5%-v/v isoflurane). After imaging, a ~2 mL blood sample was taken directly from the heart under 5%-v/v isoflurane anesthesia. Kidneys and other organs were harvested and the *ex vivo* FMT image was acquired (approximately within 5 minutes after death). Afterwards, kidneys were cut, fixed and embedded in paraffin or embedded in O.C.T. and snap frozen. All other organs (liver, heart, aorta, lung, spleen, stomach, gut, pancreas, muscle, skin, bladder) were embedded in O.C.T. and snap frozen.

Human kidney samples, histology and microscopy analysis

Kidney tissues from the Institutes of Pathology in Aachen and Erlangen were used for immunohistochemical and immunofluorescent collagen staining. The study was approved by the local review boards of Aachen and Erlangen and is in line with the Declaration of Helsinki. Eleven fibrotic kidneys were used, resulting from IgA nephropathy in different CKD stages (n=5), chronic pyelonephritis (n=3), obstructive nephropathy with hydronephrosis (n=2) and nephrolithiasis (n=1). Non-fibrotic/healthy kidney tissue was obtained from nephrectomy specimens due to traumatic injury (n=2), discarded transplant kidney (n=1) and tumor-distant areas of tumor nephrectomies (n=3).

Renal histology, immunohistochemistry and immunofluorescence

Mouse kidneys were harvested and fixed in methyl Carnoy's solution and embedded in paraffin. Human archive samples were formalin fixed and paraffin embedded. 1 µm sections were cut and stained as previously described.[S2] Collagen type I and III antibodies (Southern Biotechnology Associates, USA) were both diluted 1:100 in phosphate-buffer saline containing 1% bovine serum albumin (Sigma-Aldrich, Germany). Secondary anti-goat antibody was purchased from Vector Laboratories (USA). DAB (3, 3'-diaminobenzidine) (Sigma-Aldrich, Germany) was used as HRP substrate to produce a dark reaction. Methyl green (Sigma-Aldrich, Germany) was used for nuclear counterstaining.

Stained tissue slides were scanned using NanoZoomer digital slide scanner (Hamamatsu Photonics, Japan). For collagen quantification murine renal cortices were photographed (magnification x400). The positively stained area fraction was evaluated by computer-based morphometry using ImageJ (National institutes of Health, USA). For analyses of perivascular fibrosis, the thickness of the adventitia as well as the diameter of the vessel lumen were measured in all a. arcuatae, a. interlobulares and arterioles per cross section using NDPview software (Hamamatsu Photonics, Japan).

Double stainings using CNA35-FITC and antibodies against collagen type I, III and IV (Southern Biotechnology Associates, USA) were performed on methyl Carnoy's fixed 1 µm thick paraffin sections of mouse and human renal tissues. Sections were first incubated with CNA35-FITC overnight at 4°C followed by the incubation with collagen type I (1:100), III (1:100) or IV (1:200) antibodies for 1 h (all Southern Biotechnology Associates, USA). Secondary antibodies (Alexa 647, anti-goat, 1:100) were applied for 30 minutes at room temperature. DAPI (4'-6-diamidine-2'-phenylindole dihydrochloride) (Roche diagnostics, Germany) was used as nuclear counter stain. Stained slides were viewed and photographed with a digital fluorescence microscope (BZ-9000, Keyence, Japan).

Two-photon laser scanning microscopy and fluorescence microscopy

To demonstrate the *in vivo* specificity of CNA35 binding to collagen, 1 nmol of the CNA35-FITC probe was injected intravenously into UUO mice on day 5. To better visualize the renal vasculature, rhodamine labeled Ricinus Communis Agglutinin I (Lectin, Vector Laboratories, USA) was injected 10 min before the sacrifice. Mice were sacrificed after 0.5, 4 and 24 h and the excised kidneys were snap frozen. Both the fibrotic kidney and the contralateral healthy kidney were cut into 5 µm and 100 µm thick sections.

For the thicker sections image stacks of 50 images with a step size of 1 µm were acquired using a 25× water-immersed objective mounted on the Olympus FV1000MPE multiphoton microscopy system. TPLSM images were analyzed using the Imaris Software, Version 9.2.0 (Bitplane AG, Switzerland). We assessed n=3 sections of six kidneys and acquired six images per specimen.

A mix of DAPI (1:50, Sigma-Aldrich, Germany) in Mowiol® 4-88 (Roth, Germany) was used for nuclear counterstaining on the 5 µm thick sections. Images were acquired using the Axio Imager M2 fluorescence microscopy system (Carl Zeiss AG, Germany).

Supplementary references

S1. Sun Q, Baues M, Klinkhammer BM*, et al.* Elastin imaging enables noninvasive staging and treatment monitoring of kidney fibrosis. *Sci Transl Med* 2019; **11**.
S2. Boor P, Babickova J, Steegh F*, et al.* Role of platelet-derived growth factor-CC in capillary rarefaction in renal fibrosis. *Am J Pathol* 2015; **185:** 2132-2142.