Supplementary Information

Materials

The following antibodies were used in the current study: goat polyclonal anti-DDAH1 and rabbit polyclonal anti-SOCS3 were purchased from Santa Cruz biotechnology. Rabbit polyclonal anti-TIM-1 (T-cell transmembrane, immunoglobulin, and mucin; also known as KIM-1) was obtained from Abcam, mouse monoclonal anti-ATF6 and rabbit polyclonal anti-GADD153/CHOP were purchased from Novusbio. Rabbit polyclonal anti-GAPDH was obtained from Sigma-Aldrich, TEXAS RED anti-rabbit/mouse/goat were purchased from Vector laboratories.

Tauroursodeoxycholic acid (TUDCA) sodium salt was purchased from Merck Millipore, Z-guggulsterone was purchased from Santa Cruz biotechnology. Methyl cellulose, Angiotensin II ELISA, Trichrome staining kit (HT15) and enalapril maleate salt were purchased from Sigma-Aldrich.

Other reagents used in the current study were as follows: mouse albumin ELISA quantification kit (Bethyl Laboratories); DMEM/F12, RPMI1640, Trypsin-EDTA, penicillin, streptomycin, DPBS and fetal bovine serum (FBS) (Sigma-Aldrich). ITS supplement and HEPES (PAA Laboratories); IFN-γ (Cell Sciences); Accu-Chek test strips, Accu-Chek glucometer, and protease inhibitor cocktail (Roche Diagnostics); BCA reagent (Thermoscientific); Vectashield mounting medium with DAPI, PVE and M.O.M kit (Vector Laboratories); ammonium persulphate (APS) (Merck); PVDF membrane immobilon^{TM} and western chemiluminescent HRP substrate (Millipore); Periodic acid-Schiff (PAS) reagent, Hematoxylin and DMSO (ROTH).

Determination of albuminuria

Mice were individually placed in metabolic cages to collect 12 h urine samples before (16 weeks of age or 10 weeks of hyperglycemia) and at the end (22 or 26 weeks of age or 16 weeks of hyperglycemia) of the intervention period. Urine albumin was determined using a mouse albumin ELISA (Mouse albumin ELISA quantification set, Bethyl Laboratories) according to the manufacturer's instructions. Urine creatinine was determined using a commercially available assay of a modified Jaffé method (X-Pand automated platform, Siemens, Eschborn, Germany).[1-4] The ΔUACR represents the fold-change, determined by dividing the absolute UACR (µg/mg) following 6 / 10 weeks of treatment in 22 / 26 weeks old mice by the absolute UACR (µg/mg) measured at 16 weeks (just before starting the treatment).

Histological analyses of the kidney

We perfused animals with ice-cold PBS and then with 4% buffered paraformaldehyde. Tissues were further fixed in 4% buffered paraformaldehyde for 2 days, embedded in paraffin, and processed for sectioning. Extracellular matrix deposition in glomeruli was assessed by Periodic acid–Schiff (PAS) staining. The fractional mesangial area (FMA) was calculated following the current DCC (Diabetes Complications Consortium) protocol. Briefly, 5 µm thick sections were stained with PAS reagent. For every investigated glomerulus, total glomerular area and glomerular tuft area were determined by tracing the outline of the Bowman's capsule and the tuft, respectively, using ImageJ. The FMA was calculated as the percentage of the glomerular area relative to the tuft area.[1] For determination of the tubular and glomerular diameter adjacent sections were compared to identify the maximal glomerular diameter and to ensure that a sagittal tubular cross-section was analyzed. At least 30 randomly chosen glomeruli and tubules were analyzed per group using ImageJ software. Images were taken with Olympus BX43 microscope, Olympus XC30 Camera and Olympus cellSens Dimension 1.5 Image software. All images were taken with the same settings.

Human renal biopsy

Human renal biopsy samples from renal malignant individuals (without other kind of chronic diseases) were provided by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the ethics committee of the University of Heidelberg and after obtaining informed consent.

Immunofluorescence

Paraffin-embedded sections were de-paraffinized and rehydrated (30 min, at 65°C followed by Xylene for 10 min two times; rehydration in a descending ethanol series). Antigen-retrieval was conducted using an antigen unmasking solution (Vector # H-3300; 1:100) and boiling samples for 30 min. ATF6 staining was conducted following incubation with Mouse-on-Mouse (M.O.M.) blocking solution (Vector) for 1 h followed by M.O.M. protein concentrate solution for 10 min as per manufacturer's instructions. Sections were incubated with the primary antibody (ATF6, Novus Biologicals, 1:50 in protein concentrate solution) overnight at 4°C, washed twice in PBS, and then with the secondary antibody (TEXAS RED anti-mouse IgG 1:100 in protein concentrate solution) for 90 min at room temperature. CHOP staining was conducted following incubation with 1% donkey serum in PBST (blocking solution) for 1 h. Sections were incubated

with the primary antibody (CHOP Novus Biologicals, 1:50 in blocking solution) overnight at 4°C, washed twice in PBS, and then with the secondary antibody (TEXAS RED anti-rabbit IgG (H+L) TI-1000, Vector; 1:100 in blocking solution) for 90 min at room temperature. Then sections were incubated for 10 min. in 10 mM copper-II-sulfate solution and 25 min in 0.5% Sudan black B solution. Images were taken with Olympus BX43 microscope, Olympus XC30 Camera and Olympus cellSens Dimension 1.5 Image software. The exposure setting and gain of laser light were kept the same for each analysis.

Immunofluorescence staining for SOCS3 and DDAH1 was performed on mouse kidney sections and control human biopsies. In brief, sections were fixed in ice-cold acetone for 1 min, incubated in PBST (0.1% Tween20 + 0.1% sodium citrate) for 10 min, blocked in 2.5% donkey serum for 1 h and incubated overnight at 4°C with the primary antibodies against human SOCS3 (1:25) or DDAH1 (1:25). Corresponding fluorescently labelled secondary antibody (anti-rabbit IgG-TEXAS RED 1:300) was added for 60 min at room temperature and sections were rinsed 3 times in PBS. Slides were covered with Vectashield mounting medium containing nuclear stain DAPI. Images were taken with Olympus BX43 microscope, Olympus XC30 Camera and Olympus CellSens Dimension 1.5 Image software. The exposure setting and gain of laser light were kept the same for each analysis.

Masson's Trichrome staining (MTS)

5 µm thick kidney sections were stained using the trichrome staining kit (Sigma HT15) according to the manufacturer's instructions and as previously described.[5] At a magnification of 200x ten visual fields per slide were randomly selected, covering nearly the whole renal cortex. The assessment and the evaluation were performed by a blinded investigator.

Protein isolation

Proteins were isolated as previously described.[2, 4, 6, 7] In brief, cell lysates were prepared in RIPA buffer (50 mM Tris at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF supplemented with protease inhibitor cocktail). Lysates were centrifuged (10.000 g for 10 min at 4°C) and insoluble debris was discarded. The protein concentration in supernatants was quantified using BCA reagent.

Immunoblot

Cell lysates were prepared using RIPA buffer containing 50 mM Tris (pH 7.4), 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF supplemented with protease inhibitor cocktail. Lysates were centrifuged (13.000 g for 10 min at 4°C) and insoluble debris was discarded. Protein concentration in supernatants was quantified using BCA reagent. Equal amounts of protein were electrophoretically separated on 10% or 12.5% SDS polyacrylamide gel, transferred to PVDF membranes and probed with desired primary antibodies at a concentration of KIM-1 (1:1000), ATF6 (1:400), CHOP (1:1000), FXR (1:500), SOCS3 (1:500), and DDAH1 (1:500). After overnight incubation with primary antibodies at 4°C membranes were washed with TBST and incubated with anti-mouse IgG (1:5000), anti-rabbit IgG (1:2000), or anti-Goat IgG (1:2000) horseradish peroxidase-conjugated antibodies for 1 h at room temperature. Blots were developed with the enhanced chemiluminescence system. To compare and quantify levels of proteins, the density of each band was measured using ImageJ software. Equal loading for total cell or tissue lysates was determined by GAPDH immunoblot using the same blot.[3]

Nephroseq

Nephroseq (Life Technologies, Ann Arbor, MI; previously known as Nephromine; nephroseq.org) was used for analysis and visualization of human glomerular expression data. Nephroseq is an open-access web-based platform for integrative data analysis of microarray gene expression data sets specifically for renal diseases.[8] FXR, SOCS3 and DDAH1 mRNA expression were analyzed in microdissected glomeruli of healthy controls within the Woroniecka dataset, which is a collection of gene expression profiling of 13 healthy diabetic nephropathies using Affymetrix expression arrays. Array type: Human Genome U133A 2.0 Array. The detailed clinical characteristics are described.[9] *P*-values and significances shown reflect results if interrogating the Nephromine database for overexpression.

References for Supplementary Information

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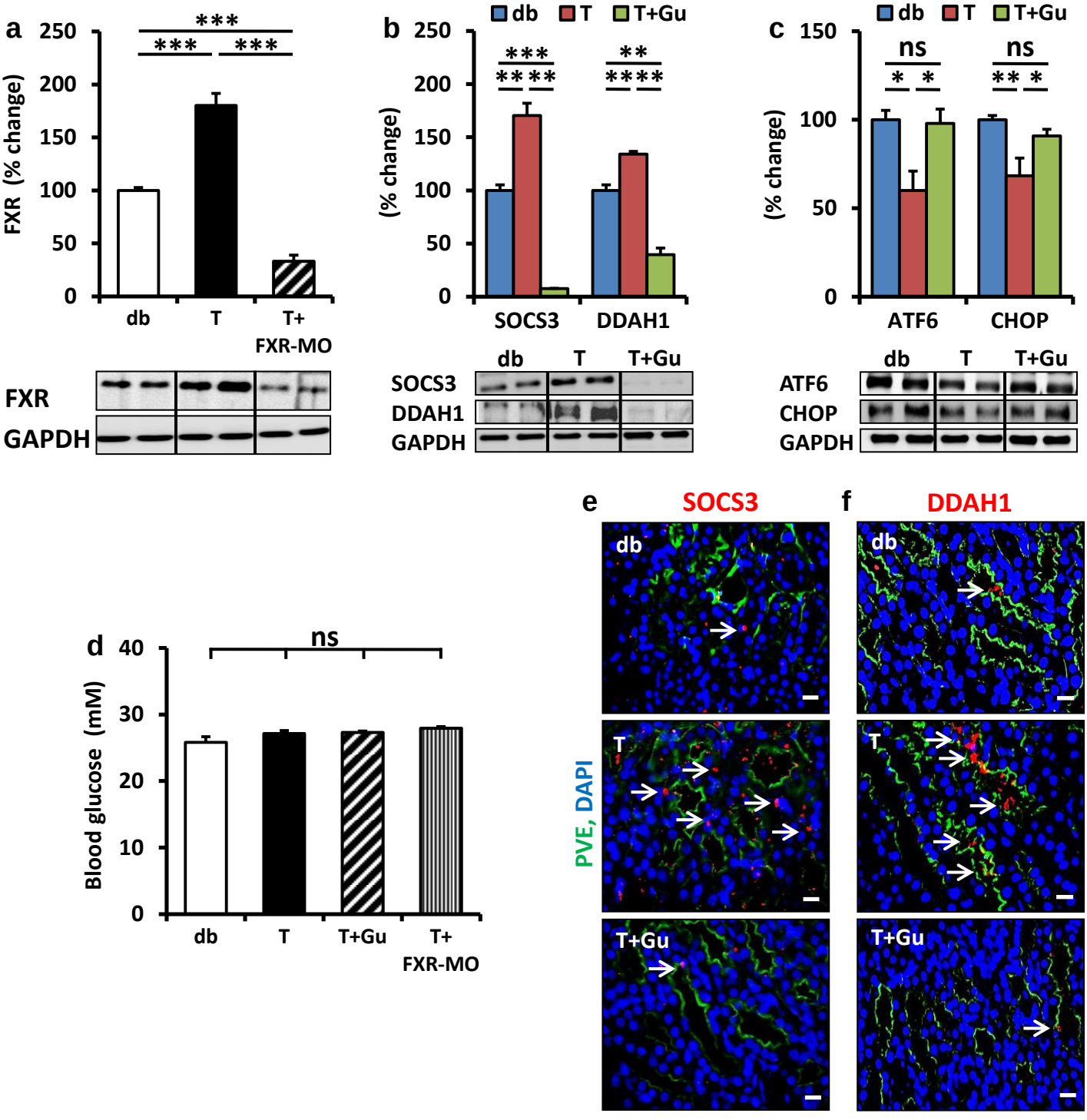
Supp. 1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Blood glucose (mM)
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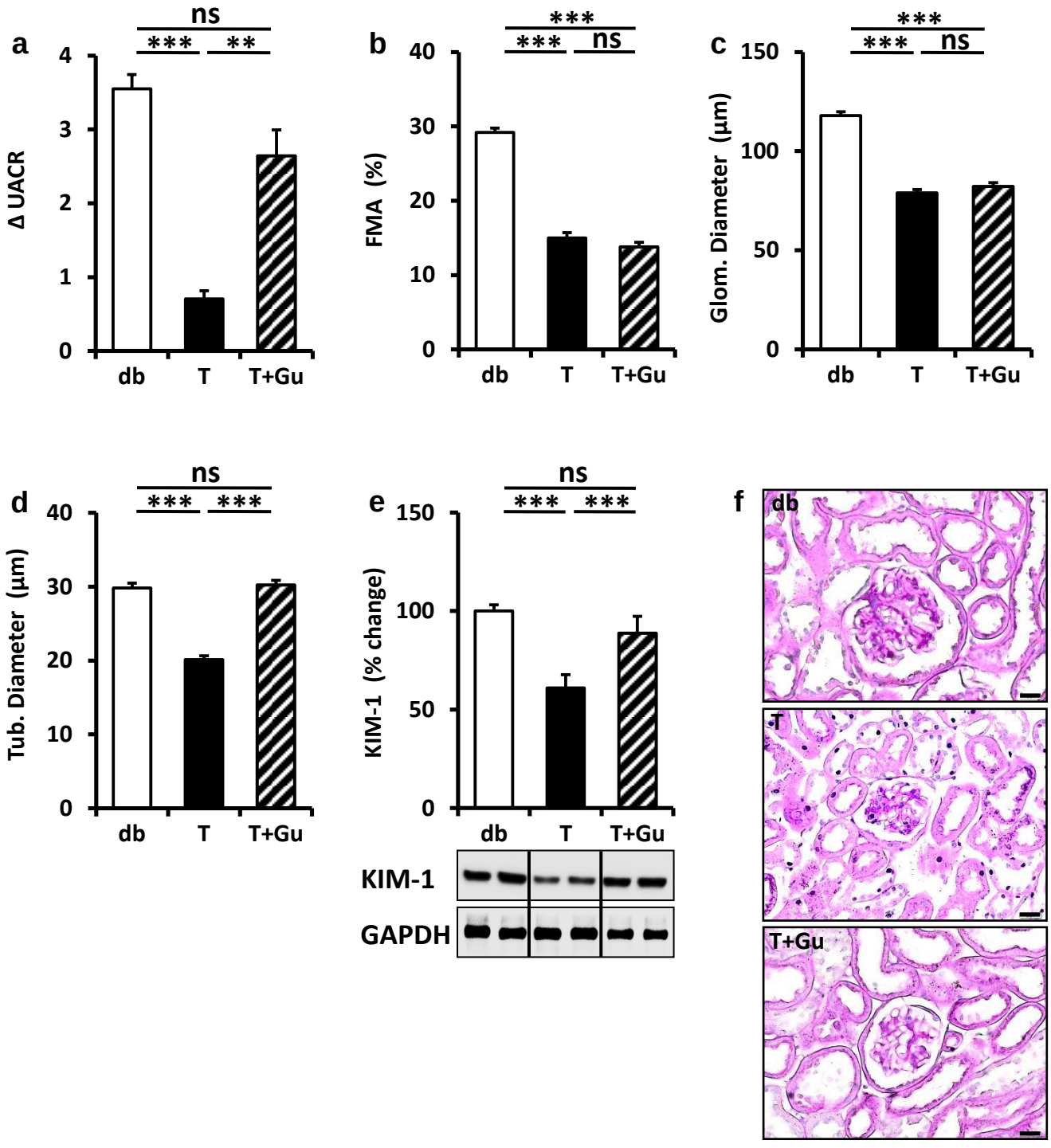
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Glom. Diameter (µm)
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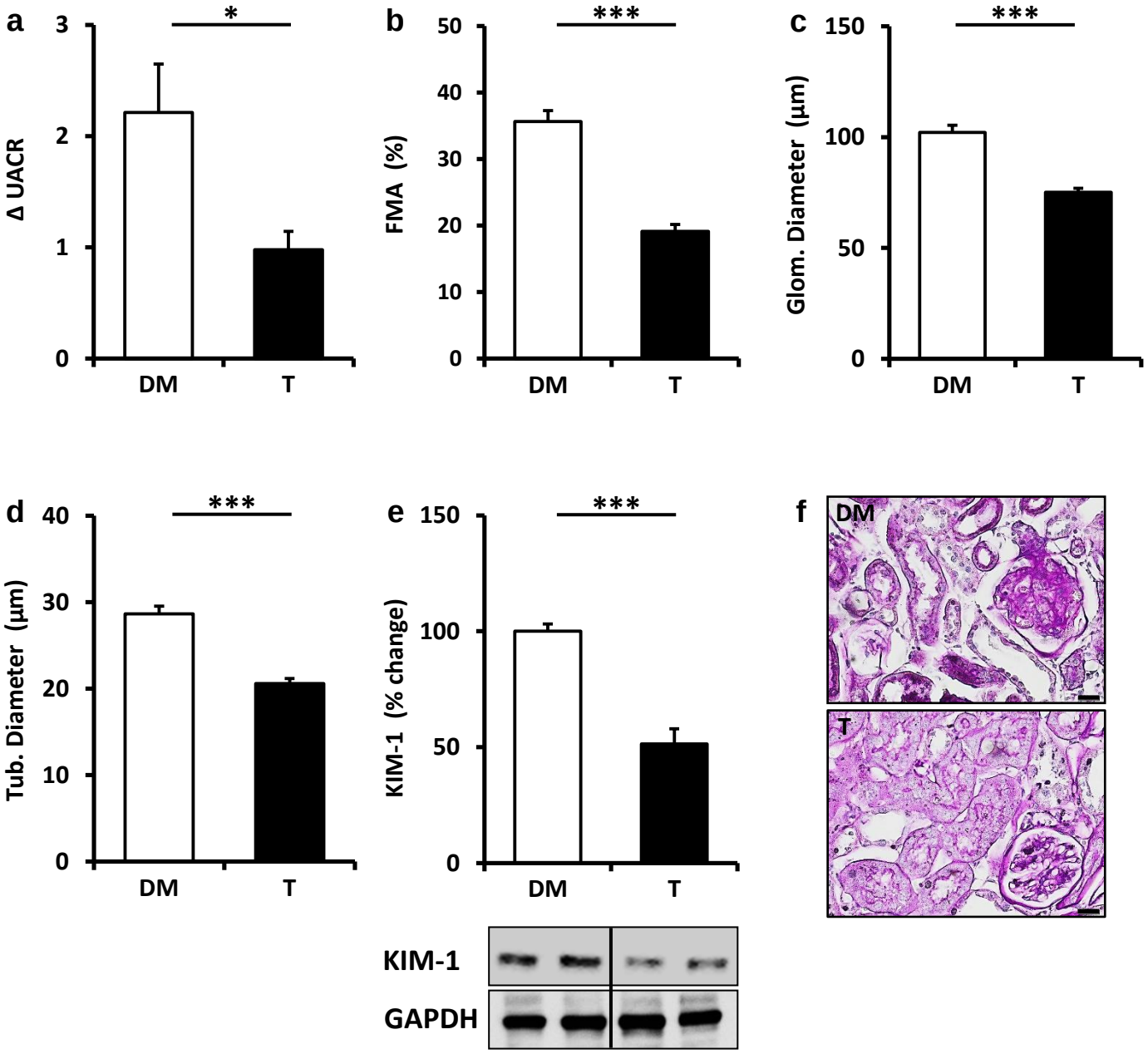
Supp. 3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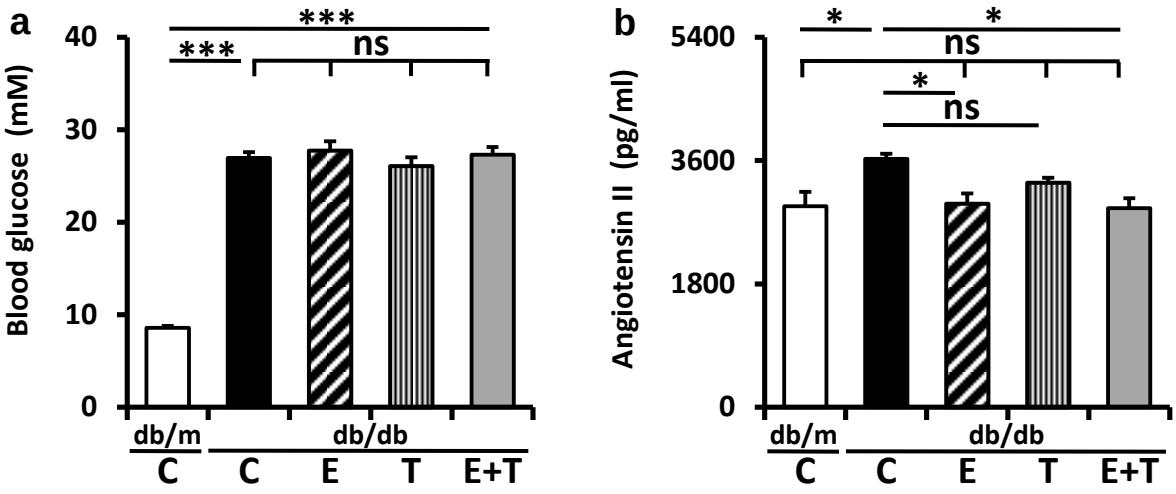
Supp. 4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b
Angiotensin II (pg/ml)
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3600
1800
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*
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ns
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ns
db/m
db/db
C
C
E
T
E+T