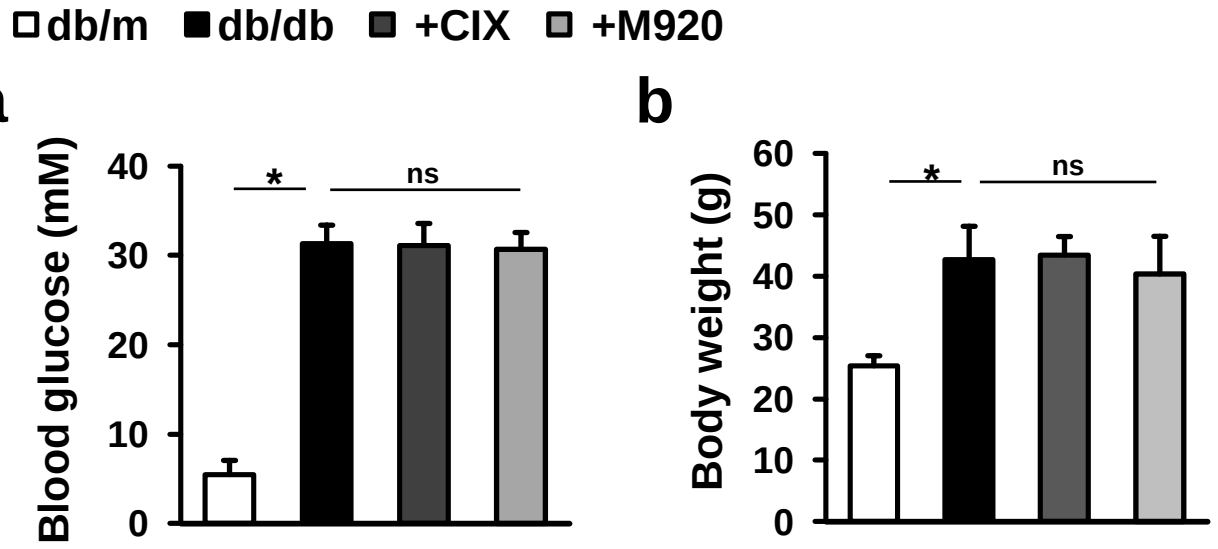
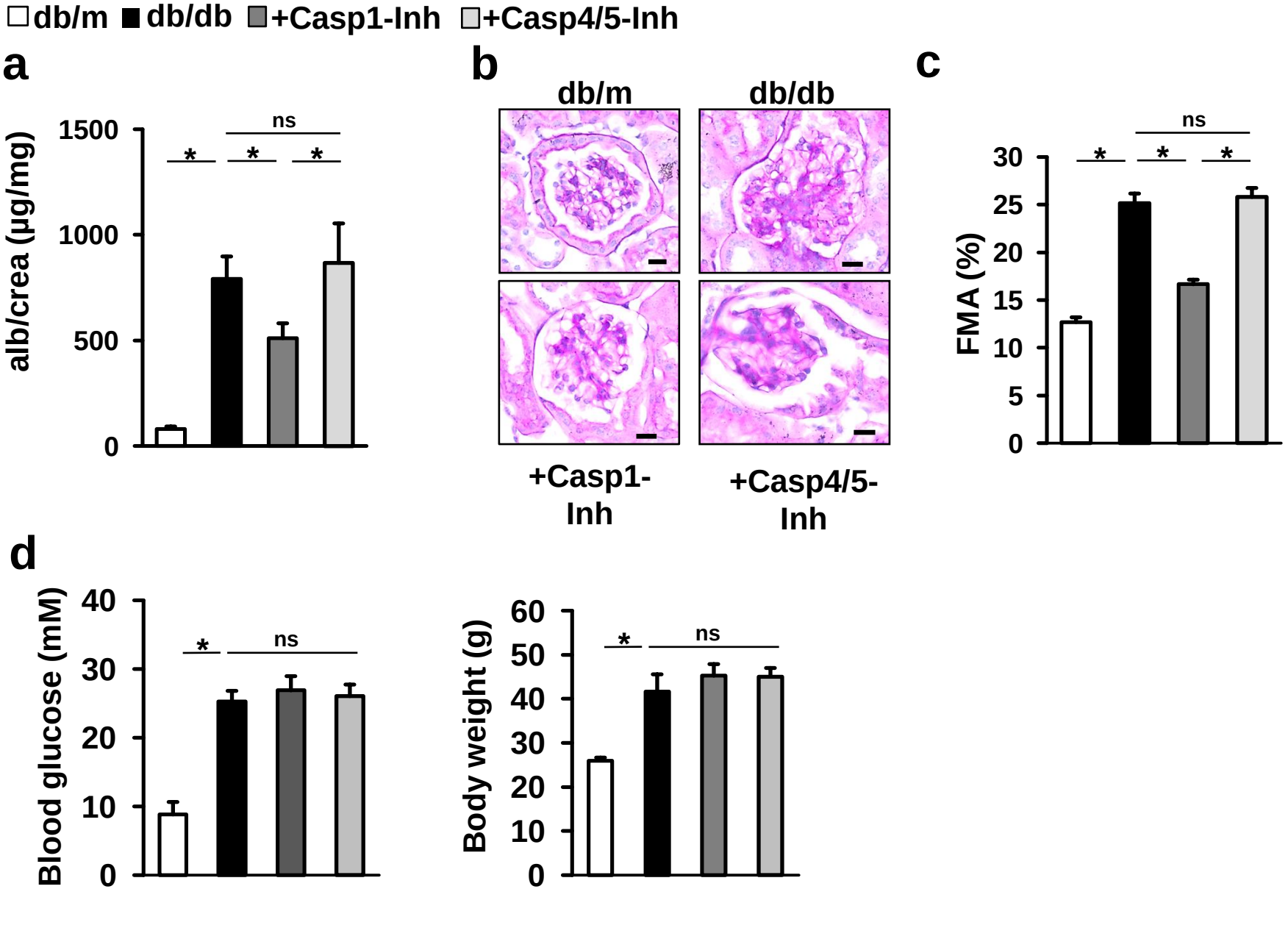
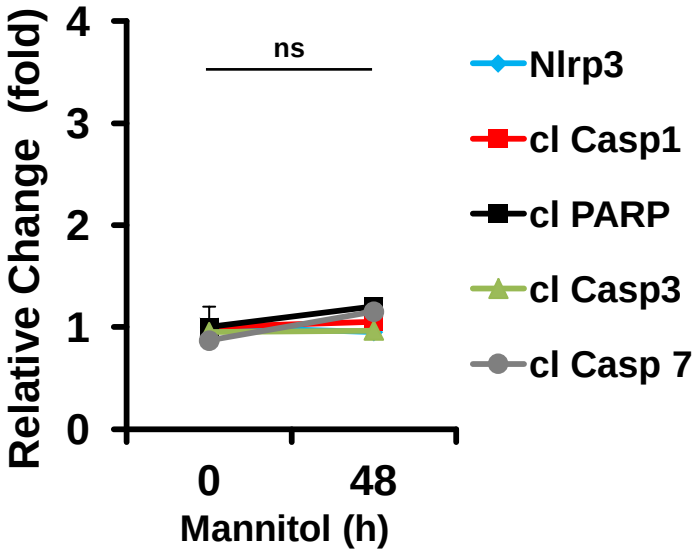
Supp Fig 1


db/m
db/db
+CIX
+M920
a
Blood glucose (mM)
0
10
20
30
40
*
ns
b
Body weight (g)
0
10
20
30
40
50
60
*
ns

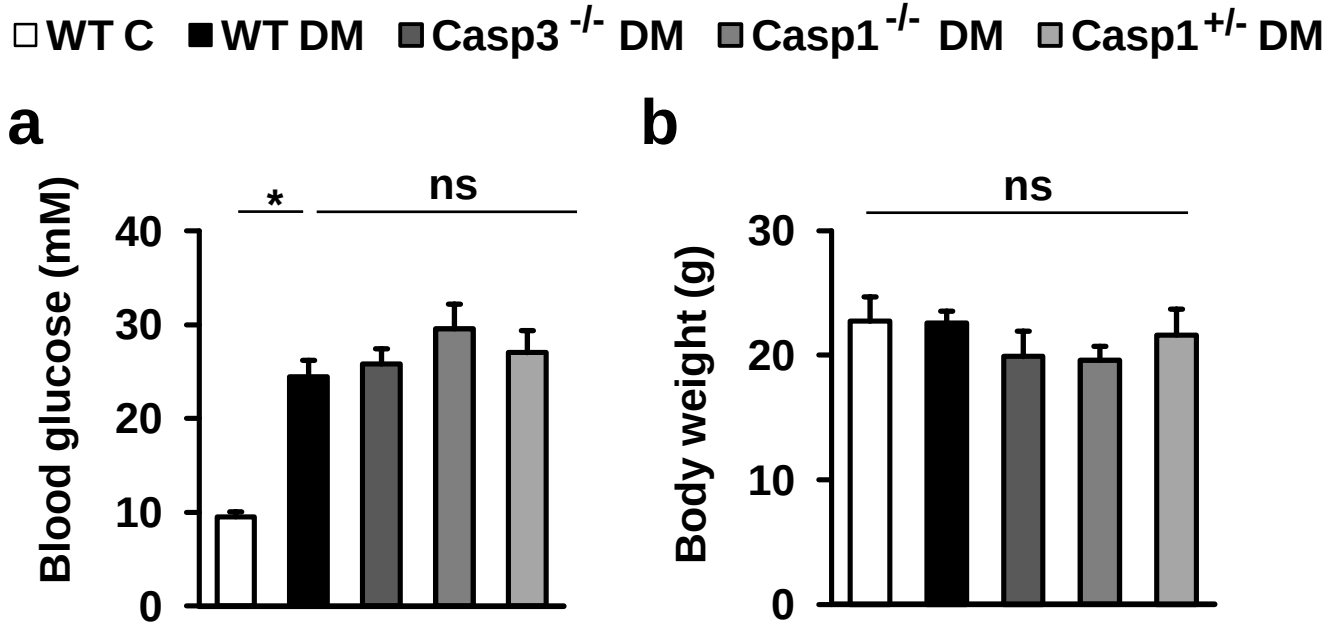
Supp Fig 2


db/m
db/db
+Casp1-Inh
+Casp4/5-Inh
a
alb/crea (µg/mg)
1500
1000
500
0
ns
*
*
*
b
db/m
db/db
+Casp1-Inh
+Casp4/5-Inh
c
FMA (%)
30
25
20
15
10
5
0
ns
*
*
*
d
Blood glucose (mM)
40
30
20
10
0
*
ns
Body weight (g)
60
50
40
30
20
10
0
*
ns

Supp Fig 3


Relative Change (fold)
4
3
2
1
0
ns
0
48
Mannitol (h)
Nlrp3
cl Casp1
cl PARP
cl Casp3
cl Casp 7

Supp Fig 4


WT C
WT DM
Casp3 -/- DM
Casp1 -/- DM
Casp1 +/- DM
a
Blood glucose (mM)
40
30
20
10
0
*
ns
b
Body weight (g)
30
20
10
0
ns

Additional online Supplementary material and methods

Reagents

The following antibodies were used in the current study: rabbit anti-caspase-1 (pro- and cleaved caspase-1), Millipore; rabbit anti-Nlrp3, biorbyt; polyclonal anti-mouse IL-1β, Boster Immunoleader; rabbit anti-cleaved caspase-3, rabbit anti-PARP1, rabbit anti-β-actin, rabbit anti caspase-7, from Cell Signaling Technology; rat anti-IL-18, from MBL international corporation; polycaspase inhibitor M-920 (which inhibits caspase-1, -3, -4, -5, -6, -7 and -8) and polycaspase inhibitor CIX (which inhibits caspase-3, -6, -7, -8 and -10) from Merck Bioscience; FLICA™ caspase-1 and caspase-3,-7 assay kit from Immunochemistry Technologies LLC. The following reagents were obtained from Sigma-Aldrich: rabbit polyclonal anti-GAPDH, streptozotocin, 1% collagen, HOECHST 33258, and pentobarbital. Other reagents used in the current study were as follows: mouse albumin ELISA quantification kit, Bethyl Laboratories; mouse IL-1β ELISA kit, R&D system; DMEM, Trypsin-EDTA, penicillin, streptomycin, FCS, FBS, ITS supplement, and Hepes, PAA Laboratories; IFN-γ, Cell Sciences; Accu-Chek test strips, Accu-Check glucometer, and protease inhibitor cocktail and TUNEL detection kit, Roche Diagnostics; BCA reagent, Perbio Science; Vectashield mounting medium with DAPI and DAB reagent, Vector Laboratories; ammonium persulphate (APS) and D-glucose from Merck; PVDF membrane immobilon™, western chemiluminescent HRP substrate, periodic acid-Schiff reagent, Haemotoxylin, and powdered milk from ROTH, Trizol reagent, life technologies. RevertAid™ H Minus First Strand cDNA Synthesis kit, Fermentas.

Diabetic nephropathy models

We used two different mouse models of diabetic nephropathy within the current study. First, we use the db/db mice, in which treatment was initiated at age 8-weeks and mice were sacrificed after 12 weeks of treatment at the age of 20 weeks. In non-treated db/db mice indices of dNP, e.g. albuminuria and glomerular extracellular matrix accumulation (FMA, fractional mesangial area), are markedly increased in 20 weeks old db/db mice.[1]

In addition we used the streptozotocin (STZ) plus unilateral nephrectomy model of diabetic nephropathy. [2-5] In this model diabetes was induced by injections of STZ (i.p., 40 mg/kg body weight, freshly dissolved in 0.05 M sterile sodium citrate, pH 4.5) for five consecutive days two weeks after unilateral nephrectomy. Age-matched control mice received 100 µL PBS i.p. for five consecutive days. Mice were considered diabetic if blood glucose levels were above 300 mg/dL 16 d after the last STZ injection. Blood and tissue samples were obtained after 10 weeks of persistent hyperglycemia in uni-nephrectomised diabetic mice. Age-matched littermates served as controls.

Immunoblotting

Proteins were isolated and immunoblotting was performed as described.[2, 3, 6-8] In brief, whole 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, and 1 mM Na_3VO_4, supplemented with protease inhibitor cocktail). Lysates were centrifuged (10,000 × g for 20 min at 4 °C) and the supernatant was kept, while the pellet containing debris was discarded. The protein concentration in supernatants was quantified using BCA reagent. Equal amounts of protein were electrophoretically separated on 10% (vol/vol) or 12.5% (vol/vol) SDS polyacrylamide gels, transferred to PVDF membranes, and probed with the desired primary antibodies overnight at 4 °C. Membranes were then washed with PBS-T and incubated with anti-mouse (1:2,000), anti-goat IgG (1:2000), anti-rat IgG (1:2,000) or anti-rabbit IgG (1:2,000) horseradish peroxidase-conjugated antibodies, as indicated. Blots were developed with the immobilon

western chemiluminiscent HRP substrate. To compare and quantify levels of proteins, the density of each band was measured by using ImageJ software. Equal protein loading was confirmed by immuno-blotting with β-actin or GAPDH antibody.

Cell culture

Conditionally immortalized mouse wild-type podocytes were cultured as described previously. [2, 3, 6-8] In brief, podocytes were grown on 10 cm^2 cell culture plates coated with 0.2% collagen type 1 at 33°C in the presence of interferon γ (10 U/ml) to enhance expression of the thermosensitive T antigen. Under these conditions, cells proliferate and remain undifferentiated. To induce differentiation, podocytes were grown at 37°C in the absence of interferon γ for 14 days. Experiments were performed after 14 days of differentiation. Differentiation was confirmed by determining expression of synaptopodin and Wilms tumor-1 protein.

RT-PCR

RNA was isolated using Trizol reagent according to manufacturer's instructions. cDNA was generated using 1 µg total RNA following treatment with DNAse (5U/5µg RNA) followed by reverse transcription using RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Heidelberg, Germany). PCR primers used for expression of IL-1β analyses are as follows: forward 5'- GCAGGCAGTATCACTCATTG -3', reverse 5'- CGTTGCTTGGTTCTCCTTGT -3'. PCR conditions were optimized (95°c for 2 min, then 35 cycles of 94°C, for 20 sec; 60°C for 20 sec; 72 °c for 30 sec; final extension at 72°C for 12 min) to detect the logarithmic increase of the amplimer, which was separated on a 1.8% agarose gel and visualized by ethidium bromide staining. Expression was normalized to β-actin. Reactions lacking reverse transcriptase served as negative controls.

Determination of albuminuria

The day before tissue preparation mice were placed individually in metabolic cages and 12 hour urine samples were collected.[2, 3, 6-8] Urine albumin was determined using an ELISA for mouse albumin according to the manufacturer's instructions, and urine creatinine was determined using a commercially available assay of a modified version of the Jaffe method (X-Pand automated platform; Siemens).[2, 3, 6-9]

Histology and immuohistochemical analysis

Freshly sacrificed mice were perfused first with ice cold PBS-T and then with 4% buffered paraformaldehyde. Tissues were further fixed with 4% buffered paraformaldehyde for 2 days, embedded in paraffin and processed for sectioning. Extracellular matrix deposition in glomeruli was assessed by Periodic acid–Schiff staining. The fractional mesangial area (FMA) was calculated following the current DCC (Diabetes Complications Consortium) protocol. Briefly, 5 µm thick sections were stained with Periodic acid-Schiff reagent. At least 30 different superficial glomeruli per tissue section were randomly chosen for analysis. 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. The FMA was calculated as the percentage of the glomerular area relative to the tuft area.[5] The Image Pro Plus software (version 6.0) and Image J software were used for image analysis. All histological analyses were performed by two independent blinded investigators. Immunohistochemistry and immunofluorescence images were captured with an Olympus Bx43-Microscope (Olympus, Hamburg, Gemany).

Nephromine

Nephromine (Life Technologies, Ann Arbor, MI) was used for analysis and visualization of human glomerular expression data. Nephromine (nephromine.org) is a web-based platform for integrative data analysis of microarray gene expression data sets specifically for renal diseases.[10] CASP1, IL-18, Nlrp3, CASP3, CASP7 and PARP1 mRNA expression were analyzed in microdissected glomeruli of healthy controls and patients with diabetic nephropathy within the Woroniecka dataset, which is a collection of gene expression profiling of 13 healthy (defined as control samples from healthy, living transplant donors or biopsy samples of the unaffected portion of tumor nephrectomies. and having an eGFR >60 (mL/min), absence of proteinuria, normal serum creatinine and BUN, and <10% glomerular and tubulointerstitial fibrosis) and 9 samples from patients with diabetic nephropathy using Affymetrix expression arrays. Array type: Human Genome U133A 2.0 Array. The detailed clinical characteristics are described elsewhere.[11] Furthermore two different mouse models for diabetic nephropathy within the Hodgin cohort in nephromine, BKS db/db and $eNOS^{-/-}$ BKS db/db were analyzed for overexpression of the aforementioned markers. The diabetic mice were compared to db/m or $eNOS^{+/+}$ m/m mice, respectively, with 5-9 mice in each group. The phenotypic characterization of these mice is described elsewhere.[12] Folds, P values and significances shown reflect results if interrogating the Nephromine database for overexpression.

IL-1β/IL-18 immunoassay

Mouse blood samples were obtained from the inferior vena cava of anticoagulated mice (500 U unfractionated heparin intraperitoneally intraperitoneal 20 mins before blood sampling).[2, 3, 6-8] Plasma was obtained by centrifugation of blood samples for 10 min at 2000 g at RT. Plasma samples were stored at -80°C until analyses. We measured the concentrations of mouse cleaved IL-1β and IL-18 by ELISA (R & D system) according to manufacturer's instructions.

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