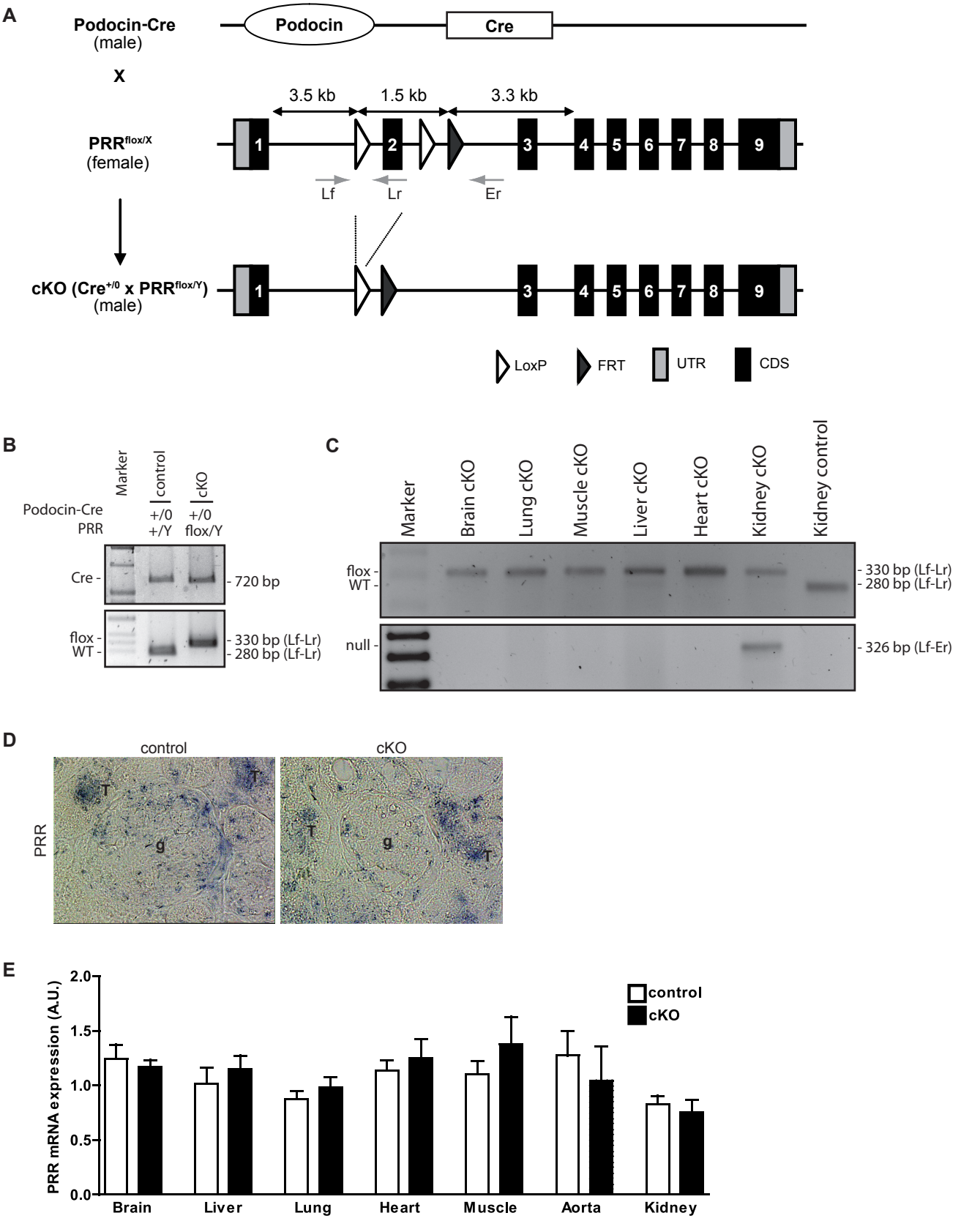
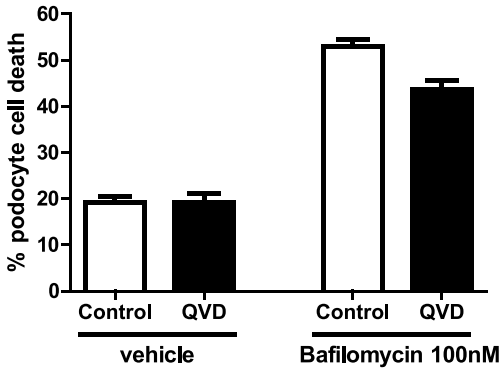
SUPPLEMENTAL DATA

Supplemental Figure 1



Supplemental Figure 1. Conditional inactivation of the prorenin receptor/ATP6ap2 (PRR) gene. (A) To generate floxed PRR mice, the PRR gene was targeted by homologous recombination using a construct in which exon 2 was flanked by *loxP* sites. Podocyte-specific excision of exon 2 was achieved by Cre recombinase under the control of the podocin promoter. (B) Wild type control (WT) male animals were Cre-positive and flox-negative analysing genomic DNA, whereas male conditional knockout animals (cKO) were genotyped Cre-positive and flox-positive. (C) After the exon 2 excision, a 326bp PCR product specific for excised PRR gene was only detected in cKO kidneys. All other organs of cKO were flox-positive except kidneys from control mice and did not show an excision of exon 2; neither did kidneys of control animals. (D) *In situ* analysis revealed podocyte specific loss of PRR in cKO mice, while tubular cell PRR expression was similar in control and cKO animals (g=glomerulus, T=tubule). (E) RT-PCR mRNA analyses in different organs did not show any significant difference in PRR expression between WT and cKO. Please note that we created a pododyte-specific knockout; therefore it is not expected to detect any difference in renal PRR expression.

Supplemental Figure 2



Supplemental Figure 2. Inhibition of v-ATPase activity leads to cell death, which is mostly caspase-independent. FACS analysis of cultured murine podocytes showed an enhanced cell death indicated by measuring propidium iodide (PI) uptake. Cells were treated with bafilomycin A1 or vehicle (DMSO) for 96 hours with or without the pan-caspase inhibitor QVD (q-VD-OPh) (n=3 independent experiments).

SUPPLEMENTAL MATERIAL AND METHODS

Genotyping

Standard PCR methods were used to differentiate between unaltered, floxed or excised exon 2 of PRR/ATP6AP2 genomic DNA, which were either extracted from mouse tails (Supplemental Figure 1B) or indicated organs (Supplemental Figure 1C) using commercial available lysis buffer (Peqlab, Erlangen, Germany) according to manufacturer's instructions. Floxed exon 2 of genomic DNA was detected using primers Lf and Lr (Supplemental Figure 1A) resulting in a 330 bp product (floxed exon 2) or a 280 bp product (not floxed), respectively. Excision of exon 2 resulted in a 326 bp product by using primers Lf and Er. Primer sequences were: Lf: 5'-AGCACTCTCTTCCAGGTATGTTGTG-3'. Lr: 5'-CTGGATCCCGGAGCATGGGT AAAGG-'3. Er: 5'-GCCCCTCTCTTACAGTTCTATCAGT-3'. Cycling conditions were: 94°C for 3 minutes; 2 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute; 30 cycles of 94°C for 30 seconds, 62°C, for 30 seconds, 72°C for 30 seconds, and 1 cycle of 72°C for 1 minute. Cre-positivity was confirmed using these primers which result in a 720 bp product: fw (5'- GCTGCCACGACCAAGTG-3'), rev (5'- TCGCCATCTTCCAGCAG-3').

Serum measurements

Blood was obtained via cardiac puncture, centrifuged at 3000 rpm for 10 min to obtain serum, and blood urea nitrogen, albumin, creatinine, and cholesterol were measured using an Olympus AU-400 multiparametric analyzer.

Quantitative TaqMan RT-PCR

RNA isolation and TaqMan RT-PCR were performed as described.[1] We analyzed total kidney tissue for PRR, NGAL, and Kim1 expression and normalized the data to

the house keeping gene 18S. Each sample was performed in triplicate. For TaqMan RT-PCR analyses the following set of primers (BioTez, Germany) were used: PRR mRNA: fw (5'-CCAGTTTGTTGTCTCGTCATAAGC-3'), rev (5'-ACCTGCCAGCTCCAATGAAT-3'), probe (5'-FAM-TCTAGCCAAGGACCATTCACC CGACTT-TAMRA 3'). NGAL mRNA: fw (5'-TGATCCCTGCCCCATCTCT-3'), rev (5'-GGAACTGATCGCTCCGGAA-3'), probe (5'-FAM-TCACTGTCCCCCTGCAGCCAGA-TAMRA-3'). Kim1 mRNA: fw (5'-CTGGAGTAATCACACTGAAGCAATC-'3), rev (5'-GATGCCAACATAGAAGCCCTTAGT-3'), probe (5'-FAM-CTCCAGGGAAGCCGCAGAAAAACC-TAMRA-3'). As housekeeper we used 18S: fw (5´-ACATCCAAGGAAGGCAGCAG-3´), rev (5´-TTTTCGTCACTACCTCCCCG-3´), probe (5'-FAM-CGCGCAAATTACCCACTCCCGAC-TAMRA-3').

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling)-Diaminobenzidine Assay

ApopTag® Fluorescein In Situ Apoptosis Detection Kit S7110 (CHEMICON, Millipore corp., Billerica, MA, USA) was used to detect DNA fragmentation by labeling the terminal end of nucleic acids. The kit was used according to manufacture's instructions.

FACS analysis

Cell death of podocytes was assessed by uptake of the DNA-intercalating dye propidium iodide (PI). In brief, cells were harvested via trypsinization together with the detached cells in the supernatant and subsequently incubated for 15 minutes in PBS containing 50 µg/ml PI at 4°C in the dark. After two washes with PBS the cells were resuspended in 200 µl PBS and analysed via flow cytometry in a FACSCalibur

(BD Bioscience, San Jose, CA, USA). The viable cell population was determined using an FL3/FSC dot plot.

In situ hybridisation

Digoxigenin in situ hybridization was performed in mouse kidney tissue using a riboprobe for mouse prorenin receptor with primer sequences as follows: forward primer 5´-GTGCTTTAGCGAATTTAG-3´ and reverse primer 5´-TTCCTTCACAGAGAAGCCC-3´ (accession no. XM_217592.5) The cDNAs were inserted into pGEM-T easy, and sequencing was performed to confirm identity of the sequences and the orientation of the sequences in the vector. Templates for the preparation of riboprobes were made by restriction enzyme digestion with Spe-I and runoff transcripts incorporating DIG-UTP were made using T7 RNA polymerase.

Briefly, 5-μm-thick sections of formalin-fixed, paraffin-embedded mouse kidney was dewaxed, rehydrated, and postfixed in formalin. Slides were incubated with 20 μg/ml proteinase K (Roche) in PBS for 30 min at 37°C before inactivation of proteinase K in 0.2% glycine (W/V) for 2 min at room temperature. Sections were prehybridized at 37°C for 1 h in hybridization buffer containing 50% formamide, 5× SSC, 5% blocking reagent (Roche), 10% dextran sulfate, and 0.1% Tween20. Hybridization was performed overnight at 65°C in hybridization buffer containing the digoxigenin-labeled anti-sense and sense probes in 50% formamide-humidified chambers. Slides were washed in 2× SSC at 60°C for 15 min, treated with 150 μg/ml ribonuclease (RNase) A (Sigma), and washed again in graded SSC. The hybridized digoxigenin riboprobe was detected with alkaline phosphatase–coupled anti-digoxigenin antibody (Roche). The final signal was developed using NBT-BCIP detection system (Roche).

Immunofluorescence staining and histological analyses of kidney sections

Kidneys were frozen in OCT compound and sectioned at 6 µm (Leica Kryostat). The sections were fixed with 4% paraformaldehyde, blocked in PBS containing 5% BSA, and incubated for 1 h with primary antibodies. After several PBS rinses, fluorophore-conjugated secondary antibodies (Invitrogen) were applied for 30 minutes. Confocal images were taken using a Zeiss laser scan microscope equipped with a ×63 water immersion objective. For histological analyses, sagital sections of the kidney were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 2 µm. For morphological analysis was stained with trichrome stain after Masson-Goldner or Periodic Acid-Schiff (PAS) using a standard procedure.

Antibodies

Antibodies were obtained from Abcam (anti-GRP78 rabbit pAb, ab21685), Acris (anti-nephrin guinea pig pAb, BP5030), Sigma-Aldrich (anti-ATP6ap2 rabbit pAb, HPA003156; anti-podocin rabbit pAb, P0372), Biomol (anti-calnexin rabbit pAb, SPA-860), Cell Signaling (anti-beta-actin rabbit mAb, 4970; anti-LC3B rabbit pAb, 2775; anti-alpha-actinin rabbit pAb, 3134), Chemicon (anti-ubiquitin rabbit pAb, AB1690), Santa Cruz (anti-phospho-cofilin rabbit pAb, sc-12912; anti-Lamp2 pAb, sc-81729; anti-WT1 rabbit pAb, sc-192), Cytoskeleton (anti-cofilin rabbit pAb, ACFL02), Lifespan Biosciences (anti-Cre recombinase rabbit pAb, LS-C79887), Progen (anti-p62 guinea pig pAb, GP62-C; anti-synaptopodin mouse mAb, G1D4).

Viral transduction of primary podocytes

For viral transduction primary podocytes were cultured in a T75 flask at 37 °C. Approximately 3 x 10^6 cells were infected with adenovirus vectors containing either

LacZ gene (control) or Cre recombinase gene at a viral titer of 1.2×10^8 plaque forming units (pfu) in serum free DMEM medium for 12 hours. Then medium was changed to primary podocyte culture medium and the podocytes were the plated at a density of $3.0 \times 10^3/cm^2$ on cover slips or 6 wells. The efficiency of transfection was determined by X-galactosidase (X-gal) staining.

Immunofluorescence of cultured podocytes

Podocytes were plated on glass slides and differentiated for 10 days. Cells were then either simultaneously incubated with bafilomycin A1 (100 nM) or vehicle (DMSO) or adenoviral Cre recombinase or adenoviral LacZ for the indicated time. Thereafter, the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde. For Lysotracker stainings, Lysotracker Red Lysosomal Probe (Lonza Walkersville, MD, USA) was incubated in a concentration of 50nM 30 min before fixation. Incubation with the primary antibody was done in 4% BSA and 0.01% Triton overnight at 4^oC. After washing with PBS 3 times, cells were incubated with the secondary antibody for 1 h at room temperature. Finally, cells were washed 3 times with PBS and the slides were mounted with Prolong Antifade Gold (Invitrogen, Paisley, UK). Photomicrographs were taken with a Zeiss Axio Observer Z1 microscope using Axio Vision Software.

Western Blot

Cells were washed twice with PBS and lyzed in a 1% Triton X-100 lysis buffer. After centrifugation (15,000 × g for 15 min at 4°C), cell lysates containing equal amounts of total protein were dissolved in 2 x Laemmli buffer and separated by 12% SDS/PAGE. Proteins were then blotted on PVDF membrane and visualized by a standard enhanced chemiluminescence (ECL) procedure.

REFERENCES

1. Gratze P, Dechend R, Stocker C, Park JK, Feldt S, Shagdarsuren E, Wellner M, Gueler F, Rong S, Gross V, Obst M, Plehm R, Alenina N, Zenclussen A, Titze J, Small K, Yokota Y, Zenke M, Luft FC, Muller DN. Novel role for inhibitor of differentiation 2 in the genesis of angiotensin II-induced hypertension. *Circulation.* 2008;117(20):2645-2656.