Supplemenatal Materials

- Supplemental Methods and References
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Supplemental Methods

Blood urea nitrogen (BUN) and aldosterone measurement

BUN was measured by the Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Serum aldosterone concentration was measured by SRL Inc. (Tokyo, Japan).

Nitrate/Nitrite, 8-OHdG, cGMP, SOD, and ACE measurement

Urine nitrate/nitrite concentrations were determined using a Griess reagent kit (Nitrate/Nitrite Colorimetric Assay Kit; Cayman Chemical). Urine 8-hydroxy-2'-deoxyguanosine (8-OHdG) (JaICA, Shizuoka, Japan), serum cGMP (Cayman Chemical), and serum superoxide dismutase (SOD) (Cayman Chemical) were measured by using commercial kits according to the protocol provided by the manufacturer. Lung ACE activity was measured by a fluorescence assay using an ACE activity assay kit as described previously (Life Laboratory Company, Yamagata, Japan)[1].

Western blot analysis

The details of our Western blot analysis method have been described previously [1, 2]. The blots were incubated with mouse anti-ACE monoclonal antibody (dilution, 1:1000;

Chemicon International, Millipore) and monoclonal anti-β-actin antibody (dilution, 1:1000; Sigma-Aldrich), followed by incubation with mouse IgG (dilution, 1:5000; Jackson Immunoresearch Laboratories Inc.) Proteins were visualized using an enhanced chemiluminescence detection system (GE Healthcare).

ACE-inhibitor model

Enalapril (120mg/L, 16mg/kg/day; Sigma) were dissolved in water and added directly to the drinking water of the animals (which was changed at twice a week). After 2weeks of enarapril treatment, 14,15-EEZE (140ug/kgBW i.v.) was injected under monitored BP as described above.

Real-time PCR

RNA extraction and real-time PCR were performed as described previously[1]. To validate changes in gene expression, we performed real-time PCR analysis with an Applied Biosystems Prism 7500HT Sequence Detection System using TaqMan Gene Expression Assays according to the manufacturer's specifications (Applied Biosystems). The TaqMan probes and primers were as follows. For endothelin-1 (Mm00438656_m1), Ptgis (Mm00447271_m1), gp22phox (Mm01287743_m1), Ephx2 (Mm00514706_m1),

cyp2c44(Mm01197188_m1), cyp2c29 (Mm00725580_s1), cyp2c38 (Mm00658527_m1), cyp2c39 (Mm04207909_m1), cyp2j5 (Mm00487292_m1), cyp2j6 (Mm01268197_m1), cyp2j9 (Mm01264620_m1), cyp2j13 (Mm01262154_m1), tnfα (Mm00443260_g1), GAPDH (Mm99999915_g1) (Applied Biosystems), Renin(5'-TTGTTGCTCTGGAGTCCTTGC-3′, 5′-CAGGATTTCCCGGACAGAAGG-3′), Nox1 (5′-TTGGCACAGTCAGTGAGGATG-3′, 5′-AGATTTCAAGATGGAAG CAAAGGG-3′), Nox2 (5′-ACTTTCCATAAGATGGTAGCTTGG-3′, 5′-GCATTCACACACCACTCAACG-3′), and Nox4 (5′-ACCAGAATGAGGATCCCAGAAAG-3′, 5′-GTAGAAGCTGTAACCATGAGGAAC-3′).

F4/80 counting

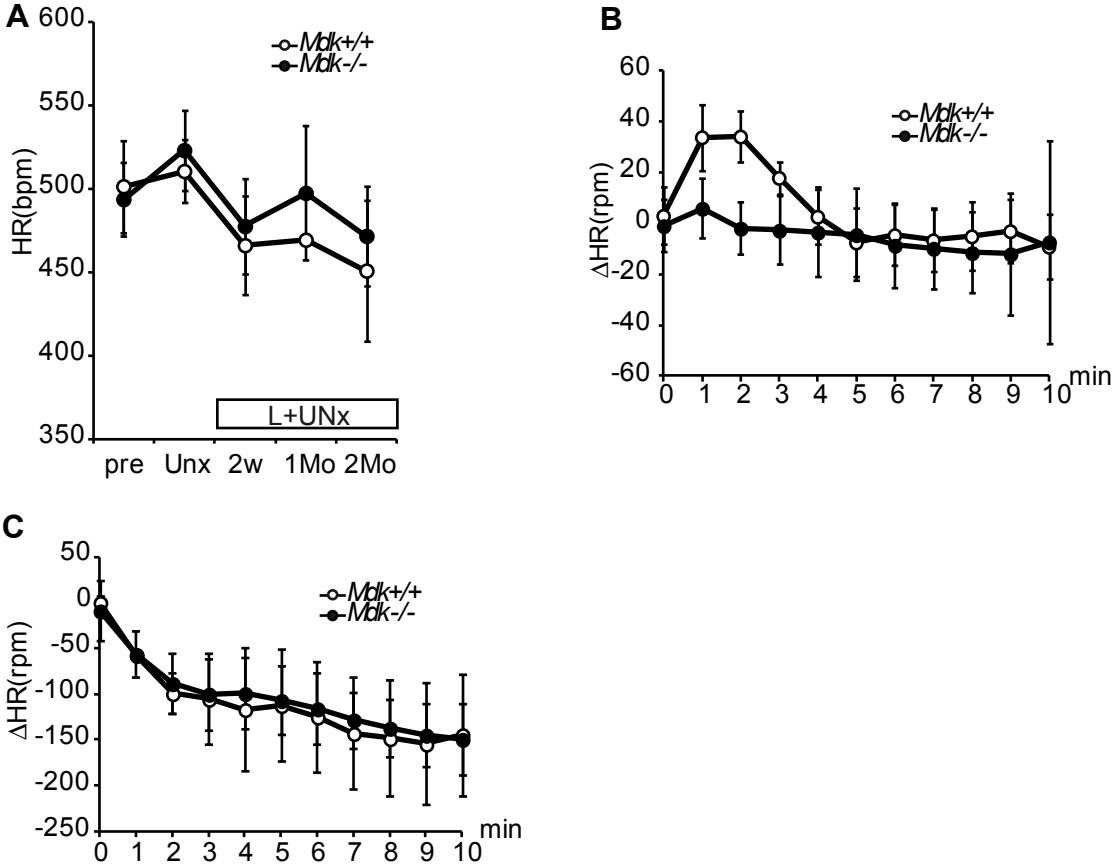
Immunohistochemical staining was performed described previously[3]. Quantitative analysis of macrophage by counted from 10 fields of each kidney (n=4, each).

Supplemental References

1. Hobo, A, Yuzawa, Y, Kosugi, T, Kato, N, Asai, N, Sato, W, Maruyama, S, Ito, Y, Kobori, H, Ikematsu, S, Nishiyama, A, Matsuo, S, Kadomatsu, K: The growth factor midkine regulates the renin-angiotensin system in mice. *J Clin Invest* 119**:** 1616-1625, 2009
2. Kadomatsu, K, Hagihara, M, Akhter, S, Fan, QW, Muramatsu, H, Muramatsu, T: Midkine induces the transformation of NIH3T3 cells. *Br J Cancer* 75**:** 354-359, 1997

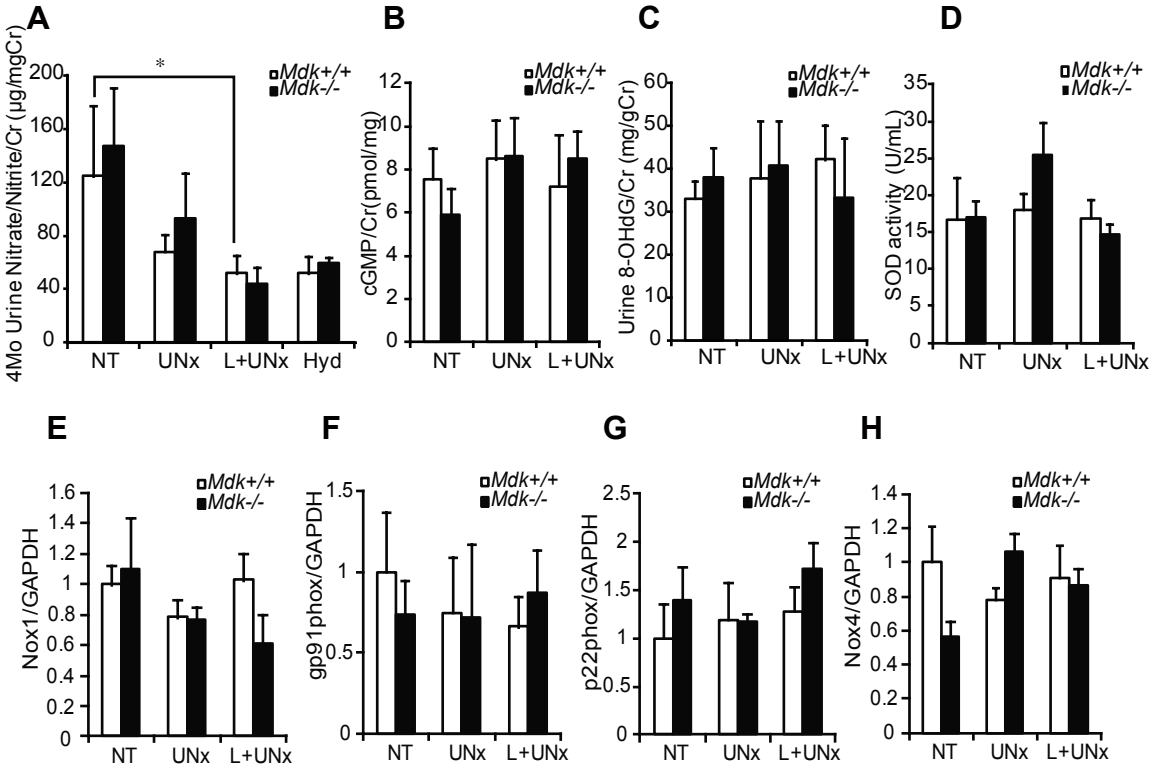
3. Sato, W, Kadomatsu, K, Yuzawa, Y, Muramatsu, H, Hotta, N, Matsuo, S, Muramatsu, T: Midkine is involved in neutrophil infiltration into the tubulointerstitium in ischemic renal injury. *J Immunol* 167**:** 3463-3469, 2001

Supplemental Figure 1



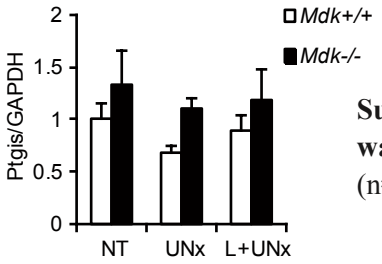
Supplemental Figure 1. Heart rate was not different between *Mdk*+/+ and *Mdk*-/- mice. (A) Heart rate was measured in conscious mice by the radiotelemetry system before uni-nephrectomy (pre), at 2 weeks after uninephrectomy (UNx), and at 2 weeks, 1 month, and 2 months after L-NAME administration. (pre: *Mdk*+/+, n= 4; pre: *Mdk*-/-, n=4; UNx: *Mdk*+/+, n=4, UNx: *Mdk*-/-, n=4; 2w: *Mdk*+/+, n=4; 2w: *Mdk*-/-, n=3; 1Mo: *Mdk*+/+, n=4; 1Mo: *Mdk*-/-, n=3; 2Mo:*Mdk*+/+, n=3; 2Mo: *Mdk*-/-, n=3). **(B,C)** The heart rate change (ΔHR) after intravenous injection of 14, 15-EEZE (a 14, 15-EET antagonist) (**B**) and hexametonium (a nictinic acetylcholine receptor antagonist) (**C**) was not different between *Mdk*+/+ and *Mdk*-/- mice (n=4). Data are presented as the mean ± SEM.

Supplemental Figure 2



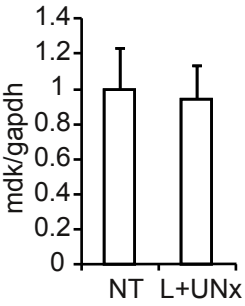
Supplemental Figure 2. The NO axis shows the lack of difference between *Mdk+/+* and *Mdk-/-* mice. (A) Urine nitrate/nitrite were significantly decreased by L+UNx to a similar extent in *Mdk+/+* and *Mdk-/-* mice. (NT: *Mdk+/+*, n=7; NT: *Mdk-/-*, n=5; UNx: *Mdk+/+*, n=9; UNx: *Mdk-/-*, n=9; L+UNx: *Mdk+/+*, n=8; L+UNx: *Mdk-/-*, n=8; Hyd: *Mdk+/+*, n=10; Hyd: *Mdk-/-*, n=8). *p<0.05. Data are presented as the mean ± SEM. (**B**) Urinary excretion of cGMP corrected by urine creatinine was not significantly different between *Mdk+/+* and *Mdk-/-* (NT: *Mdk+/+*, n=7; NT: *Mdk-/-*, n=4; UNx: *Mdk+/+*, n=7; UNx: *Mdk-/-*, n=7; L+UNx: *Mdk+/+*, n=8; L+UNx: *Mdk-/-*, n=8). (**C-F**) The urine 8-OHdG concentration corrected by urine creatinine (**C**), the superoxide dismutase (SOD) activity in serum (**D**), and the renal mRNA expression of Nox and p22phox normalized by GAPDH (**E-H**) were not significantly different between *Mdk+/+* and *Mdk-/-* mice (urine 8-OHdG: NT: *Mdk+/+*, n=5; NT: *Mdk-/-*, n=5; UNx: *Mdk+/+*, n=9; UNx: *Mdk-/-*, n=9; L+UNx: *Mdk+/+*, n=7; L+UNx: *Mdk-/-*, n=9), (SOD: NT: *Mdk+/+*, n=4; NT: *Mdk-/-*, n=3; UNx: *Mdk+/+*, n=5; UNx: *Mdk-/-*, n=4; L+UNx: *Mdk+/+*, n=4; L+UNx: *Mdk-/-,* n=5), (Nox, p22phox: n=4, each). Data are presented as the mean ± SEM.

Supplemental Figure 3



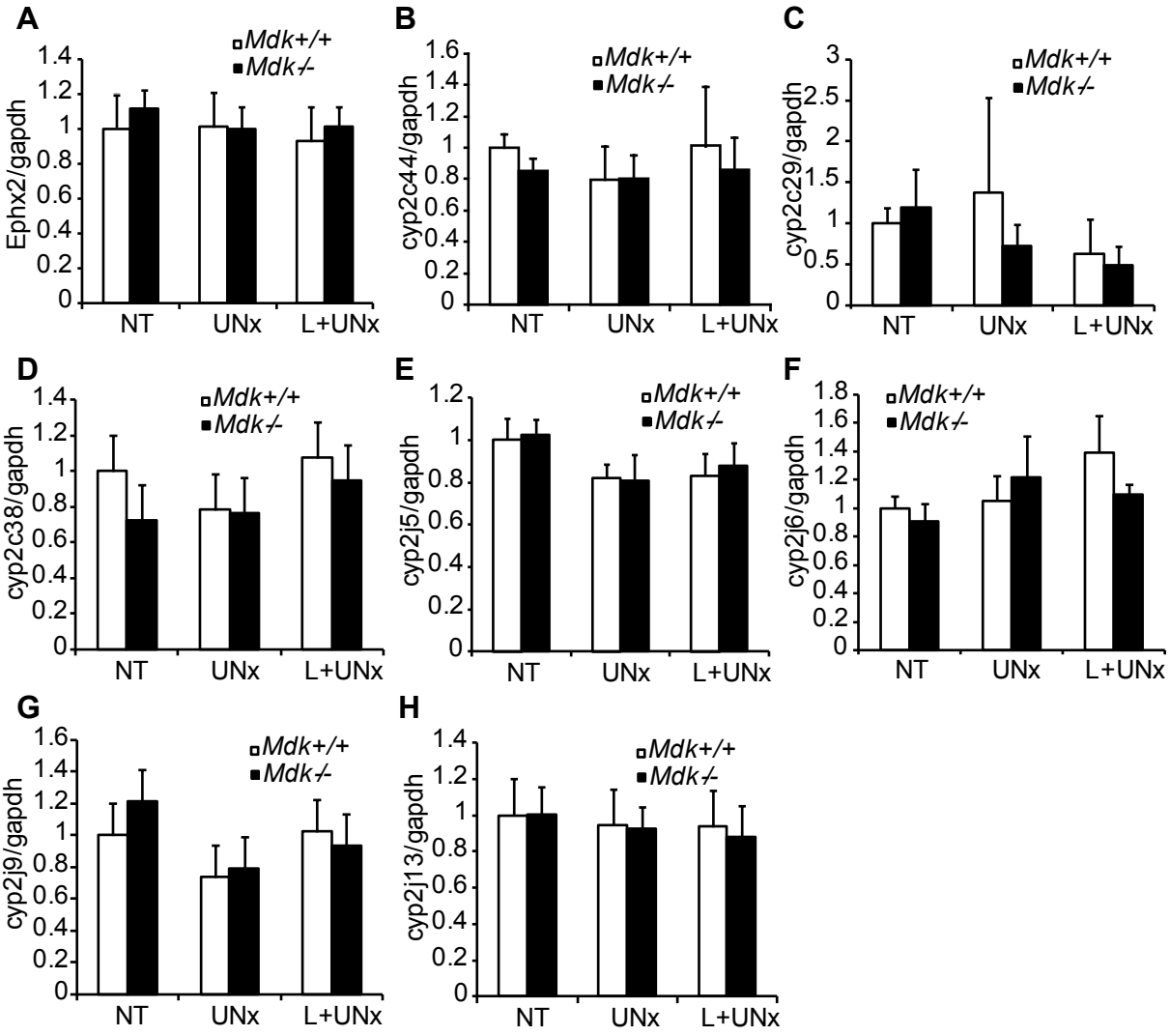
Supplemental Figure 3. Renal mRNA expression of PGI_2 synthase was not significantly different between *Mdk+/+* and *Mdk-/-* mice (n=4, each).

Supplemental Figure 4



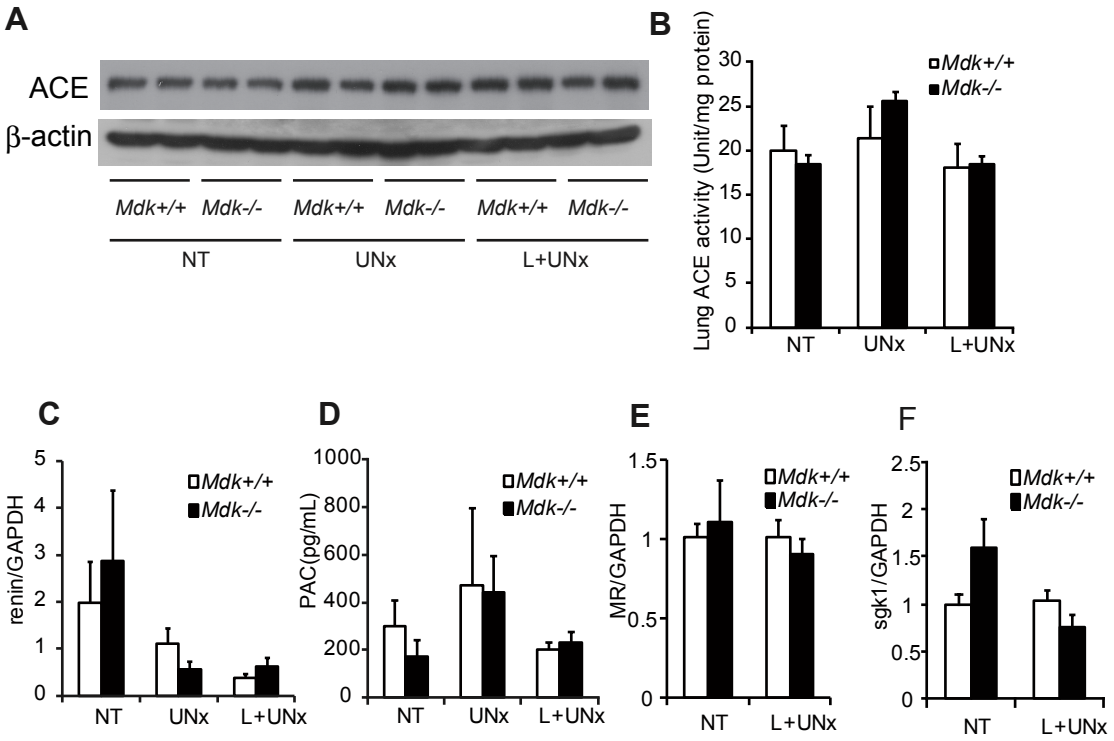
Supplemental Figure 4. MK expression in the kidney after L-NAME+UNx treatment. The renal mRNA expression of MK in L+UNx was not significantly decreased compared to NT (n=4, each). Data are presented as the mean ± SEM.

Supplemental Figure 5



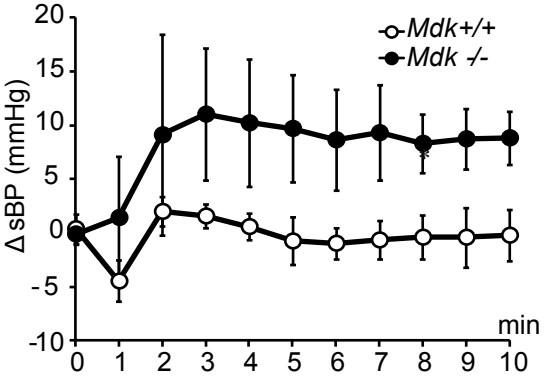
Supplemental Figure 5. Expression of soluble epoxide hydrolase and CYP epoxygenases. **(A-H)** The renal mRNA expression of soluble epoxide hydrolase, cyp2c44, 2c29, 2c38, cyp2j5, 2j6, 2j9 and 2j13 normalized by GAPDH were not significantly different between *Mdk*+/+ and *Mdk*-/- (NT: *Mdk*+/+, n=8; NT: *Mdk*-/-,n=6; UNx: *Mdk*+/+, n=8; UNx: *Mdk*-/-, n=8; L+UNx: *Mdk*+/+, n=7; L+UNx: *Mdk*-/-, n=8). Data are presented as the mean ± SEM.

Supplemental Figure 6



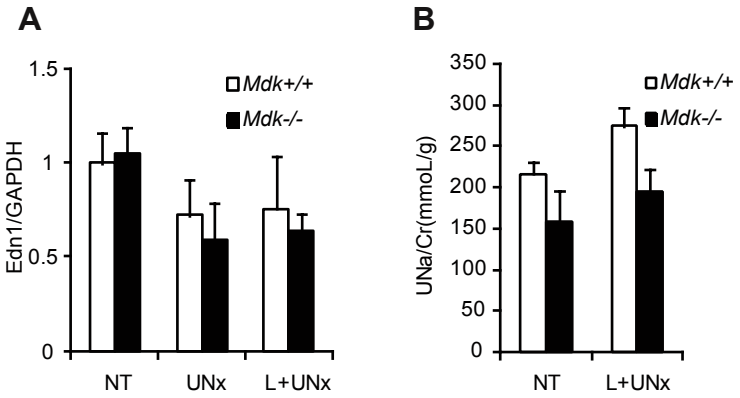
Supplemental Figure 6. RAS at 4 months after L+UNx in *Mdk*+/+ and *Mdk*-/- mice.
(A, B) Angiotensin converting enzyme (ACE) protein expression (**A**) and ACE activity (**B**) in the lung exhibited no difference between *Mdk*+/+ and *Mdk*-/- (NT: *Mdk*+/+, n=6; NT: *Mdk*-/-, n=6; UNx: *Mdk*+/+, n=7; UNx: *Mdk*-/-, n=8; L+UNx: *Mdk*+/+, n=7; L+UNx: *Mdk*-/-, n=7). **(C, D)** The renin mRNA normalized to GAPDH in the kidney (**C**) and the plasma aldosterone concentration (PAC) (**D**) were not significantly different between *Mdk*-/- and *Mdk*+/+ (renin: NT, n=4; UNx, n=5; L+UNx, n=4, PAC: NT: *Mdk*+/+, n=4; NT: *Mdk*-/-, n=3; UNx: *Mdk*+/+, n=4; UNx: *Mdk*-/-, n=4; L+UNx: *Mdk*+/+, n=4; L+UNx: *Mdk*-/-, n=4). **(E, F)** The levels of mineralcorticoid receptor (MR) mRNA (**E**) and sgk1 mRNA (**F**) normalized to GAPDH determined by real-time PCR showed no difference between *Mdk*+/+ and *Mdk*-/- (n=4, each). Data are presented as the mean ± SEM.

Supplemental Figure 7



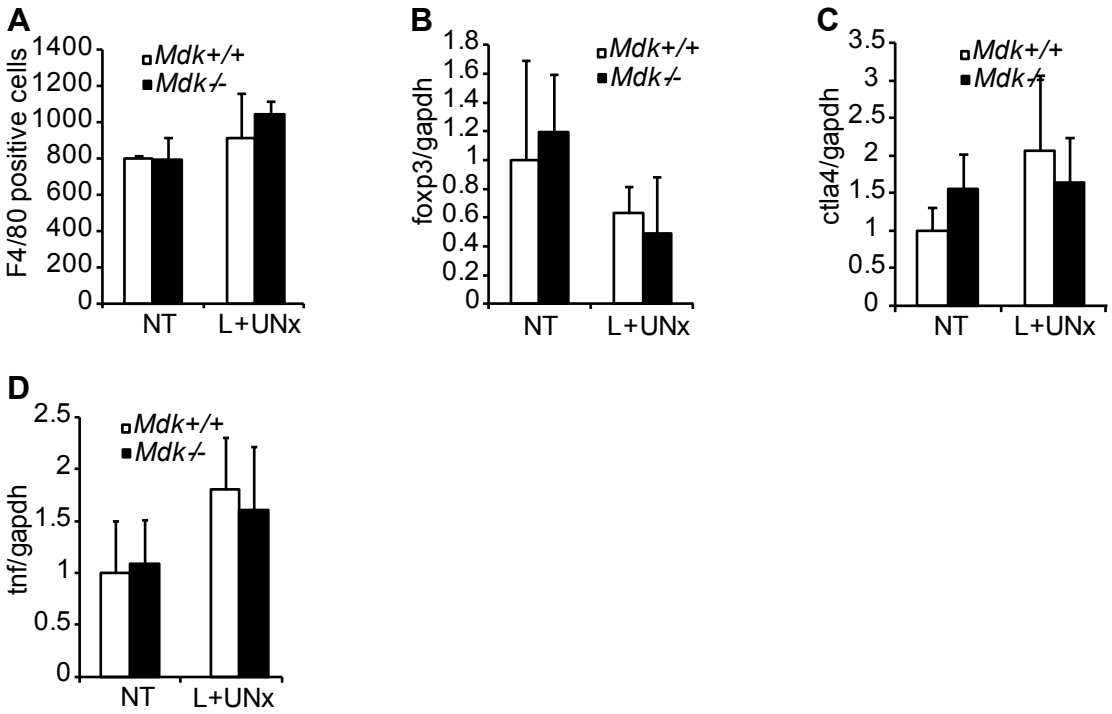
Supplemental Figure 7. RAS-independent models. After 2weeks of pretreatment of enarapril, mice were injected with 14,15-EEZE, and BP was measured (n=3, each). p=0.088 (*Mdk*+/+ vs. *Mdk*-/-). Data are presented as the mean ± SEM.

Supplemental Figure 8



Supplemental Figure 8. Endothelin-1 expression and Urinary sodium exretion.
(A) Endothelin-1 mRNA in the kidney normalized to GPADH was not affected in *Mdk-/-* mice (NT, n=4; UNx, n=5; L+UNx, n=4). (**B**) Sodium excretion into the urine was also not affected. Data are presented as the mean ± SEM.

Supplemental Figure 9



Supplemental Figure 9. (A) Quantitative analysis of macrophage by counting in 10 fields of kidney section with immunochemical staining of F4/80 (n=4, each) (n=4, each). **(B-D)** The renal mRNA expression of TNFα, Foxp3 and CTLA4 normalized by GAPDH were not significantly different between *Mdk*+/+ and *Mdk*-/- in L+UNx (NT: *Mdk*+/+, n=8; NT: *Mdk*-/-,n=6; UNx: *Mdk*+/+, n=8; UNx: *Mdk*-/-, n=8; L+UNx: *Mdk*+/+, n=7; L+UNx: *Mdk*-/-, n=8). Data are presented as the mean ± SEM.