

## Online Supplement

Original research article:

**Enhanced angiotensin receptor-associated protein in renal tubule suppresses angiotensin dependent hypertension (HYPE201200572.R2)**

Running title:

Enhanced renal tubular ATRAP inhibits hypertension

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## **Supplemental Materials and Methods**

### **Generation of ATRAP Transgenic (Tg) Mice**

A speed congenic method was used to generate the ATRAP Tg mice. C3H/He and C57BL/6J were selected as the parental inbred lines and backcrossed to a pure C57BL/6J background. Hemagglutinin-tagged mouse ATRAP (HA-ATRAP) cDNA was subcloned into a Tg vector between the 1.2-kb fragment of the cytomegalovirus enhancer-promoter and the bovine growth hormone polyadenylation sequence, as illustrated in **Supplemental Figure S1A**. This transgene was microinjected into the pronucleus of fertilized mouse embryos. The resulting pups were screened for the presence of the transgene by PCR, using forward (5'-TGCTTGGGGCAACTTCACTATC-3') and reverse (5'-ACGGTGCATGTGGTAGACGAG-3') primers. PCR was performed as follows: 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min for 35 cycles, with a final extension step at 72°C for 10 min.

### **BP Measurements and Metabolic Cage Analysis**

All of the mice were fed a normal (0.3%) sodium diet throughout the present study. BP and Heart rate (HR) were measured in the conscious state using a radiotelemetry system, as described previously.<sup>1</sup> Briefly, under anesthesia with isoflurane, an incision was made from the chin to the superior sternum and the left common carotid artery was surgically exposed. A small incision was made in the artery adjacent to the bifurcation, and the tip of a BP transducer (PA-C10, Data Science International [DSI]) was placed in the artery. The catheter was then tied and the transducer secured in place under the skin of the right flank with tissue adhesive. All skin wounds were closed with 5-0 nylon (Sigma Rex). Fourteen days after transplantation, when the circadian rhythm had been restored, mice were acclimated for the following week to metabolic cages (Techniplast). After an additional 3 days of baseline, Ang II (1000 or 2000 ng/kg/min) was continuously infused subcutaneously into the mice via an osmotic minipump (ALZA) for 11 days and hemodynamic measurements were recorded every 5 min using the software Dataquest A.R.T. 4.1 (DSI). The BP values at baseline were the average of data obtained on three consecutive days. Daily BW, food intake and water intake were measured, and urine was collected. The mice were given free access to tap water and fed the indicated diet. The urinary excretion of angiotensinogen was measured using ELISA, as described previously.<sup>2</sup>

### **Analysis of ATRAP and HA-ATRAP Protein Expression**

The characterization and specificity of the anti-mouse ATRAP antibody was described previously in detail.<sup>1, 3, 4</sup> The anti-HA polyclonal antibody was obtained from Bethyl Laboratories (A190-107A). Western blot analysis was performed as described previously.<sup>1, 3, 4</sup> Briefly, the total protein was extracted from the tissues with SDS-containing sample buffer, and the protein concentration of each sample was measured with a DC protein assay kit (Bio-Rad) using bovine serum albumin as the standard. Equal amounts of protein extract from the tissue samples were fractionated on a 5-20% polyacrylamide gel (ATTO), then transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked for 1 h at room temperature with phosphate-buffered saline containing 5 % skim milk powder, and probed overnight at 4°C with specific primary antibodies. Then the membranes were washed and incubated with secondary antibodies for 40 min at room temperature. After they were washed, the sites of the antibody-antigen reaction were visualized by enhanced chemiluminescence substrate (GE healthcare). The images were quantitated using a FUJI LAS3000 Image Analyzer (FUJI Film).

### **Membranous Protein Extraction and Immunoblot Analysis for Sodium Channels**

Membranous proteins were extracted from kidney tissues using the Plasma Membrane Extraction Kit (Biovision; K268-50) according to the manufacturer's protocol and then used for SDS-PAGE. Membranes were incubated with affinity-purified primary antibodies to NHE3, phosphorylated NKCC2, NKCC2, phosphorylated NCC, NCC, and the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits of the ENaC. The antibody against NHE3 was obtained from Alpha Diagnostic Intl. Inc. (NHE31-A); The antibody against phospho-NKCC2 on Thr96 was kindly provided by Shih-Hua Lin (Tri-Service General Hospital, Taipei, Taiwan);<sup>5</sup> The antibody against NKCC2 was obtained from Abcam (ab60301); The antibody against phospho-NCC on Ser71 was characterized previously.<sup>6</sup> The antibody against NCC was obtained from Chemicon (AB3553); the antibody against  $\alpha$ ENaC was obtained from Affinity Bioreagents (PA1-920A); the antibody against  $\beta$ ENaC was obtained from Santa Cruz Biotechnology (sc-48428); the antibody against  $\gamma$ ENaC was obtained from Abcam (ab3468).

### **Real-Time Quantitative RT-PCR Analysis**

Total RNA was extracted from the kidney with ISOGEN (Nippon Gene) and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed by incubating the RT product with the TaqMan Universal PCR Master Mix and designed TaqMan probe (NHE3: Mm01352473\_m1, NKCC2: Mm01275821\_m1, NCC: Mm00490213\_m1,  $\alpha$ ENaC: Mm00803386\_m1,  $\beta$ ENaC: Mm00441215\_m1,  $\gamma$ ENaC: Mm00441228\_m1) (Applied Biosystems), as described previously.<sup>1</sup> the RNA quantity was expressed relative to the 18S rRNA endogenous control.

### **Immunohistochemical Analysis**

Immunohistochemistry was performed as described previously.<sup>3, 4</sup> Mice kidneys were perfusion-fixed with 4% paraformaldehyde and subsequently embedded in paraffin. The 4  $\mu$ m-thick sections were dewaxed and rehydrated, and antigen retrieval was performed by microwave heating. The sections were blocked for endogenous biotin activity using Peroxidase Blocking Reagent (DAKO) and treated for 60 min with 10% normal goat serum in phosphate-buffered saline. The sections were then incubated with one of the following: 1) anti-HA antibody diluted at 1:100, 2) anti-ATRAP antibody diluted at 1:100, 3) anti-aquaporin 2 antibody (254-271, CALBIOCHEM) diluted at 1:200, 4) anti-calbindin D-28K antibody (C9848, Sigma-Aldrich) diluted at 1:3000, or 5) anti-megalin antibody (NB110-96417, Novus Biologicals) diluted at 1:1000.

### **Confocal Microscopy Analysis**

Cryostat sections (5  $\mu$ m) of snap-frozen kidneys from Wt and Tg mice were fixed with acetone for 5 minutes at room temperature and subsequent blocking with 2% BSA in PBST. Then, the sections were subjected to staining with anti- $\alpha$ ENaC antibody, which was characterized previously,<sup>7</sup> diluted at 1:50 overnight at 4°C, followed by an incubation with the Alexa Fluor-conjugated secondary antibody. Phalloidin was added for F-actin staining. DAPI was added for nuclear staining. The images were observed with a FV300 confocal laser microscopy (Olympus) immediately after immunostaining.

### **Laser Capture Microdissection (LMD) and Subsequent qRT-PCR Analysis**

LMD was performed on a Leica LMD System (LMD6000) according to the

manufacturer's standard manual. Briefly, formalin-fixed paraffin-embedded tissues were cut into 10  $\mu\text{m}$ -thick sections and mounted on polyethylene terephthalate (PET) membrane slides and stained with hematoxylin/eosin. Then, proximal or distal tubules in the renal cortex were microdissected under LMD 6000 laser microdissection microscopy. A hundred areas (approximately  $700,000 \mu\text{m}^2$ ) of the proximal or distal tubules were microdissected from the renal cortex per mouse. The total microdissected area was approximately  $2,800,000 \mu\text{m}^2$  in each group ( $N=4$  in each group). Total RNA was extracted from the microdissected tissues using the RNeasy FFPE Kit (Qiagen) and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen), and applied to Taqman qRT-PCR analysis.

### **Distal Convoluted Tubule Cell Analysis**

Mouse distal convoluted tubule (mDCT) cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized.<sup>8-10</sup> Cells were grown on 100-mm dishes (Corning) in DMEM/HAM F-12 media (1:1, Sigma-Aldrich) supplemented with 5% heat-inactivated fetal calf serum (MBL), 2 mM L-glutamine (GIBCO), 0.5 mg/ml streptomycin, 0.5 mg/ml penicillin, and 1 mg/ml neomycin (GIBCO), in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. Adenoviral vectors were prepared using cDNAs coding for the NH<sub>2</sub>-terminal HA epitope-tagged ATRAP (Ad.HA-ATRAP) and bacterial  $\beta$ -galactosidase (Ad.LacZ) using a commercially available system (Adeno X Expression System, Clontech), and the virus titer was determined with a plaque assay.<sup>11</sup> For the adenoviral gene transfer experiments, mDCT cells were subcultured in 6 cm-diameter dishes ( $5 \times 10^4$ /ml), incubated overnight, infected with recombinant adenovirus (Ad.HA-ATRAP or Ad.LacZ) at 50 multiplicity of infection for 24 h, and further incubated in a serum-free medium for an additional 24 h. The cells were then treated with Ang II ( $10^{-6}$  M) for the indicated time and subsequently harvested for analysis, as described previously.<sup>10, 12, 13</sup>

### **Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software. All the quantitative data are expressed as the means  $\pm$  SE. Differences were analyzed by Student's unpaired *t*-test or analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. Values of  $P < 0.05$  were considered

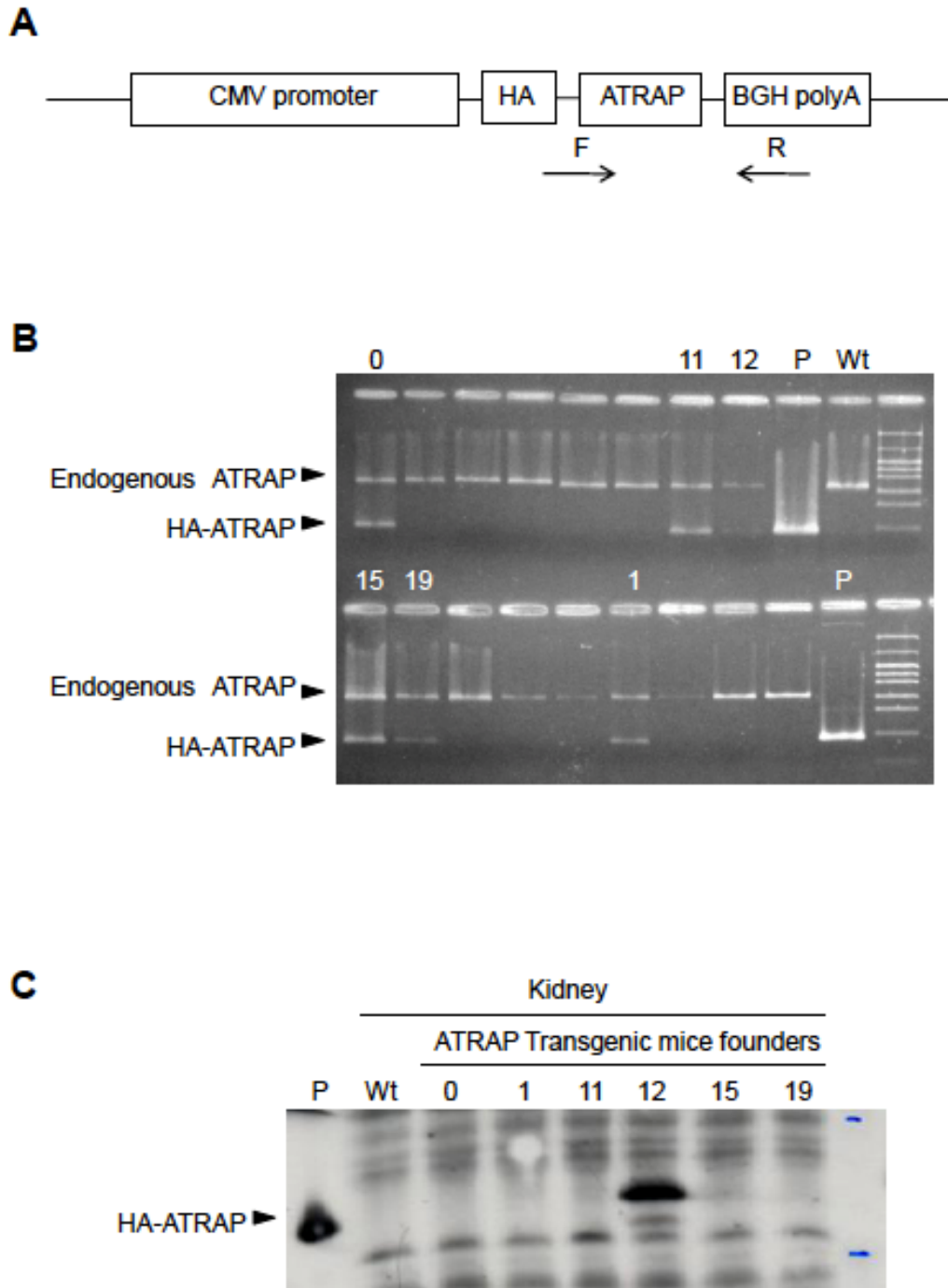
statistically significant.

### Supplemental References

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Supplemental Figure S1



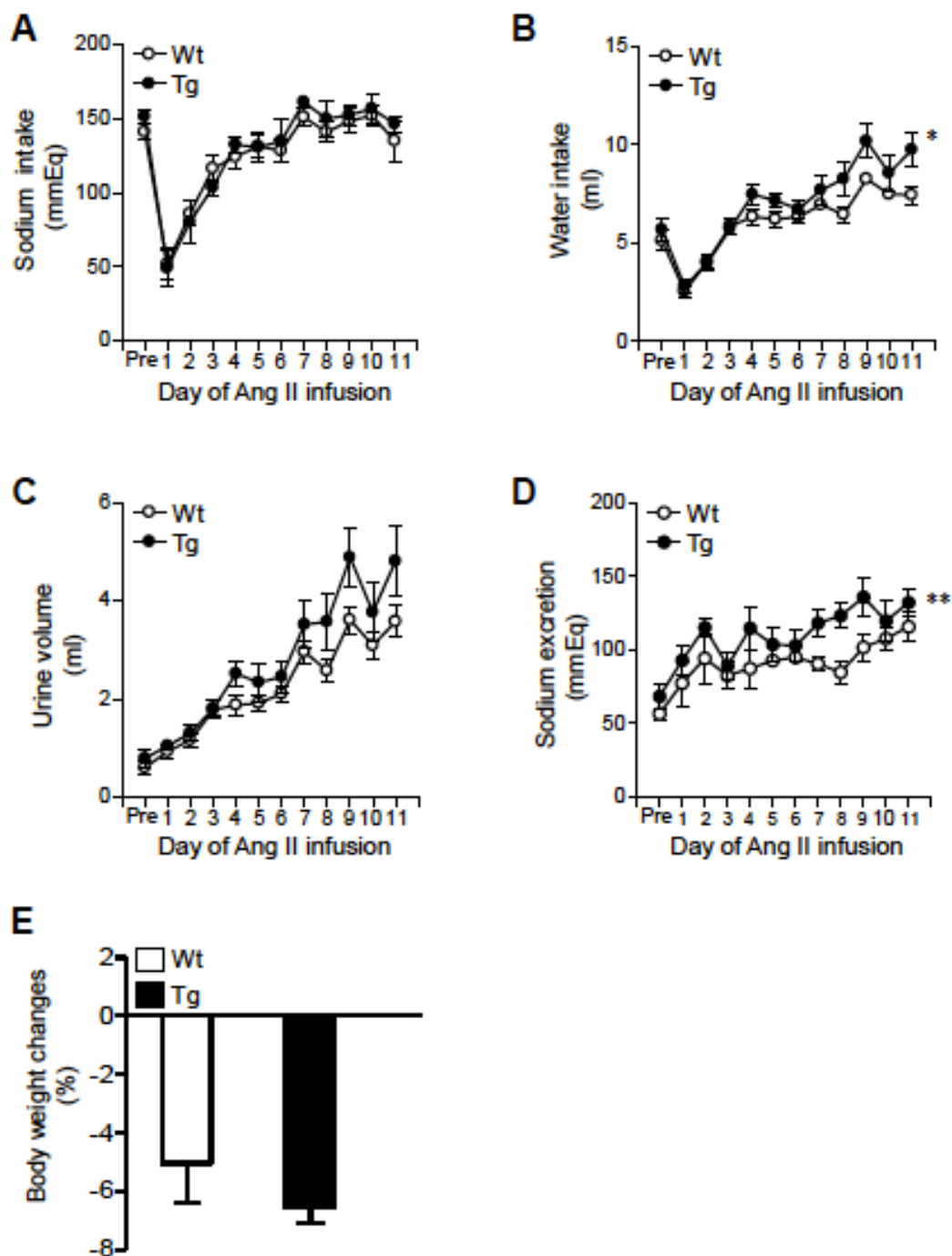


## **Supplemental Figure S1**

### **Generation of Renal ATRAP Tg Mice**

**A**, Schematic representation of the Tg vector. F and R indicate the locations of the forward and reverse primers used for the genotyping by PCR. CMV indicates cytomegalovirus, HA; hemagglutinin-tag, BGH polyA; bovine growth hormone polyadenylation. **B**, Agarose gel electrophoresis of PCR products after DNA amplification. The 755-bp endogenous ATRAP band appeared in both the Wt and ATRAP Tg mice founders. The 243-bp HA-ATRAP transgene band appeared only in the ATRAP transgenic mice founders. P indicates the positive control. **C**, Representative Western blot analysis of HA-ATRAP with the polyclonal anti-HA antibody in the kidney of Wt and ATRAP Tg mice founders.

Supplemental Figure S2

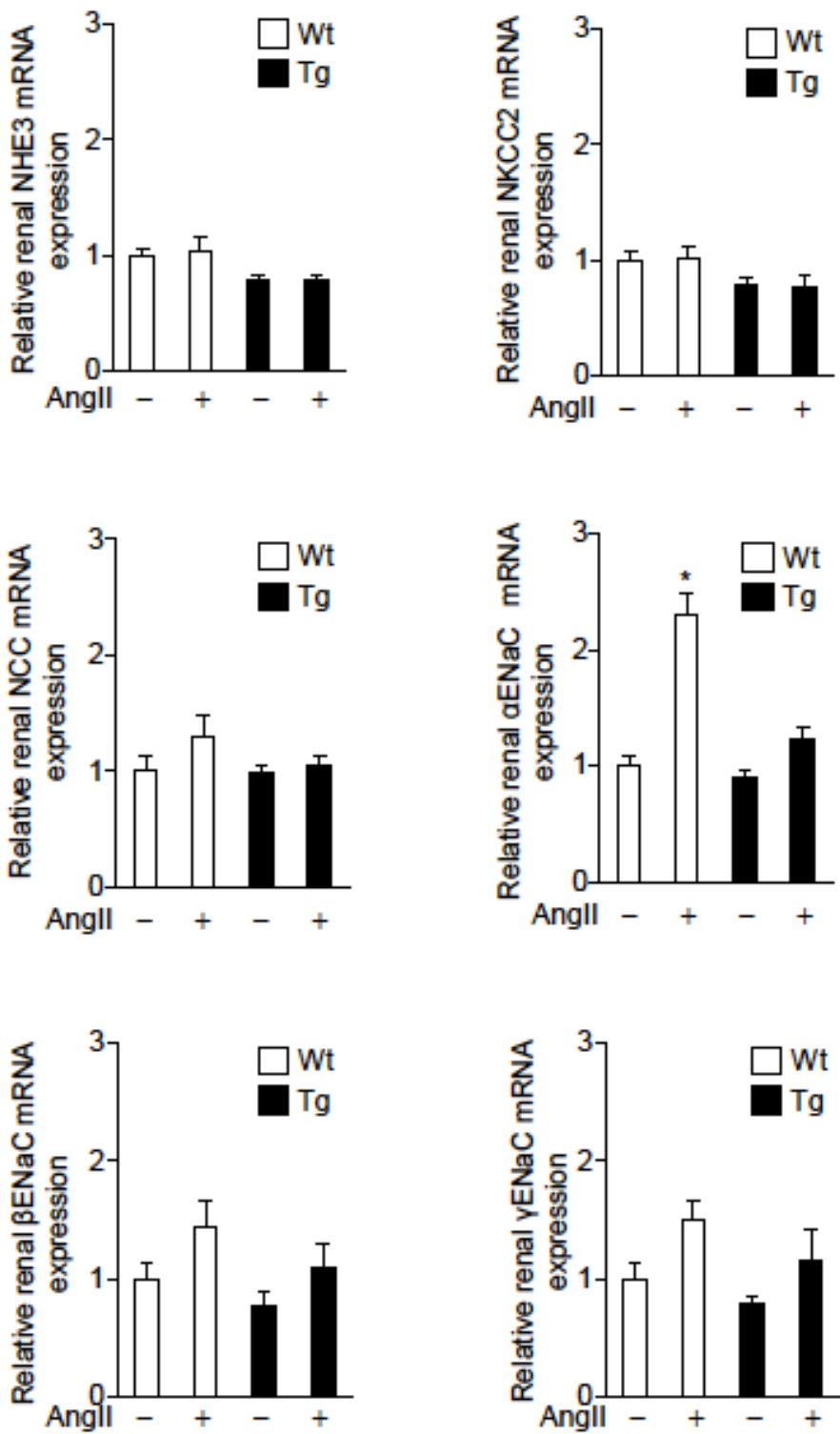


Supplemental Figure S2

### **Effects of Ang II Infusion on Metabolic Parameters and Urinary Patterns of Angiotensinogen in Renal ATRAP Tg Mice**

**A**, Daily sodium intake; **B**, water intake; **C**, urine volume; and **D**, urinary sodium excretion of the Wt and Tg mice during the Ang II (2000 ng/kg/min) infusion period. Sodium intake was comparable in the Wt and Tg mice during the infusion period (2-way repeated measures ANOVA  $F=0.1047$ ,  $P=0.75$ ). Water intake was significantly increased in the Tg mice compared with the Wt mice (2-way repeated measures ANOVA  $F=5.288$ ,  $P=0.037$ ). Urine volume tended to be increased in the Tg mice compared with the Wt mice (2-way repeated measures ANOVA  $F=3.585$ ,  $P=0.079$ ). Urine sodium excretion was significantly increased in the Tg mice compared with the Wt mice during the infusion period (2-way repeated measures ANOVA  $F=12.91$ ,  $P=0.0029$ ). Values are expressed as the mean $\pm$ SE ( $N=6$  in each group), \* $P<0.05$ , versus Wt mice; \*\* $P<0.01$ , versus Wt mice. **E**, BW changes calculated as follows: BW change= [(BW at day 11) - (BW at baseline)] / (BW at baseline)  $\times$  100. Values are expressed as the mean $\pm$ SE ( $N=6$  in each group).

### **Supplemental Figure S3**



Supplemental Figure S3

### **Suppression of Ang II-mediated Up-regulation of Renal $\alpha$ ENaC mRNA in Renal ATRAP Tg Mice**

Effects of Ang II infusion on the mRNA expression of major sodium transporters (NHE3, NKCC2, NCC and ENaC subunits) in the kidney of Wt and ATRAP Tg mice. Values are expressed as the mean $\pm$ SE ( $N=6$  in each group). \* $P<0.05$ , versus vehicle.