**ONLINE SUPPLEMENTAL MATERIAL**

# Loss of *Arhgef11* in the Dahl salt-sensitive rat protects against hypertension-induced renal injury

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**Running Title:** Arhgef11 loss protects against renal injury

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## SUPPLEMENTAL MATERIAL AND METHODS

The authors declare that all supporting data are available within the article and its online supplementary files.

### Animals

All experimental procedures were approved by the University of Mississippi Medical Center (UMMC) Institutional Animal Care and Use Committee (IACUC). The Dahl salt-sensitive (SS/Jr) and SS-*Arhgef11*$^{-/-}$ are maintained at our institutional animal facility. All rats were housed in a 12-hour light-dark cycle, maintained on low-salt rodent chow (0.3% NaCl Teklad 7034), and provided water ad libitum. All animals studied were male. The SS-*Arhgef11*$^{-/-}$ (SS/JrHsdMcw) model was developed by Medical College of Wisconsin's "Gene Editing Rat Resource Center" R24 resource grant (R24 HL114474) via gene nomination process. The knockout model was made using CRISPR/Cas9 system, which resulted in a 17 bp deletion in exon 2 of the *Arthgef11* gene, resulting in premature stop codon and protein truncation (**Fig. S1**). The SS-*Arhgef11*$^{-/-}$ (SS/JrHsdMcw) was backcrossed to the SS/Jr at UMMC five times before being fixed in the homozygous state. Once the model was received at UMMC, there were two options to study the animals: (1) import the SS/JrHsdMcwi model and directly compare it with the SS-Arhgef11-/- (SS/JrHsdMcwi); or (2) backcross the SS-*Arhgef11*$^{-/-}$ model to the SS/Jr model, essentially diluting out any genetic differences between the two sub-strains while retaining the 17 bp deletion in Arhgef11. It was decided that the SS-*Arhgef11*$^{-/-}$ to the SS/Jr for several reasons, including: (1) phenotypically, the SS/Jr strain develops age-related hypertension/kidney on low-salt (which is greatly attenuated or variable in the SS/JrHsdMcwi) as well as the model is more salt-sensitive than the SS/JrHsdMcw[1]. In our opinion the SS/Jr strain was a better genetic background to test the impact of *Arhgef11*; and more importantly (2) successive backcrosses would have the benefit to select against any possible off target effects of the CRISPR/Cas9 that could have occurred elsewhere in the genome as these would be selected against.

### Phenotyping

<u>Study 1: Temporal study of renal injury and impact of salt-loading</u>. At 4 weeks of age, age matched male SS and SS-*Arhgef11*$^{-/-}$ were weaned onto a low-salt diet (0.3% NaCl; TD7034; Harlan Teklad). 24-hr urine collections were done at 4 and 6 weeks of age on low-salt diet for the determination of proteinuria [2]. After urine collections were performed at week 6, each group was randomly divided and either continued on low-salt diet (0.3% NaCl) or placed on elevated salt diet (2% NaCl, TD 94217, Harlan Teklad). The following groups were studied: low-salt, SS-WT (n= 9) and SS-*Arhgef11*$^{-/-}$ (n=9); and high-salt, SS-WT (n=9), S, SS-*Arhgef11*$^{-/-}$ (n=9). To ensure rigor and reproducibility of the phenotypic differences, Study 1 was performed a second time (n=7-9 animals per group/conditions). Both studies gave similar results and the data were combined for final analysis using low-salt, SS-WT (n=16) and SS-*Arhgef11*$^{-/-}$ (n=17); and high salt, SS-WT (n=18) and SS-*Arhgef11*$^{-/-}$ (n=18). Urine collections were performed at week 8, 10, and 12 for the determination of proteinuria. After week 12 urine collections, a terminal blood pressure measurement was performed as done previously under isoflurane anesthesia [2]. Serum samples were obtained from cardiac puncture to measure blood parameters of renal function (creatinine and blood urine nitrogen), lipid metabolism (total cholesterol, HDL, LDL, and triglycerides), and

electrolytes (sodium, potassium, calcium, and chloride) as previously described [3]. Upon euthanasia, kidney and heart weights were taken, followed by snap freezing half kidney in liquid nitrogen for downstream -omics analyses and formalin fixing the other half kidney for histological examination.

Study 2: Temporal study of renal injury and telemetric blood pressure measurements. At 4 weeks of age, age matched male SS-WT (n=9) and SS-*Arhgef11*$^{-/-}$ (n=10) were weaned onto a low-salt diet (0.3% NaCl). 24-hr urine collections were done at 6 weeks of age on low-salt and then each group was placed on high-salt (2.0% NaCl) for the remainder of the study. Albuminuria was measured using a rat albumin ELISA kit (Abcam, ab108789). At week 7 a subset of animals from each group (n=8) were implanted with telemetry transmitters (model HD-S10, Data Sciences International) as previously described [2,4]. After one week of recovery, blood pressure was measured for 24 hours one day each week from weeks 8-15, followed by 24-hour urine collections were made for the determination of proteinuria and albuminuria. Euthanasia was performed after week 15 blood pressure and urine collections were complete. Organ weights, serum, and kidney samples for histological examination were collected.

Study 3: Measurement of renal function, survival, and assessment of renal injury at an advanced age. An additional group of age matched male SS-WT (n=16) and SS-*Arhgef11*$^{-/-}$ (n=17) were weaned onto a low-salt diet (0.3% NaCl) at 4 weeks of age. Similar to Study 2, 24-hr urine collections were done at 6 weeks of age on low-salt and then each group was placed on high-salt (2.0% NaCl) for the remainder of the study. Tail vein blood was collected immediately after the urine collection for the measurement of serum creatinine and calculation of creatinine clearance at weeks 6, 9, 12, and 15. Subsequently, this study was continued as a survival study. Animals were evaluated daily and if they were found dead the date was recorded. Any animal that displayed any significant signs of distress (e.g. shaking, lethargy, or tachypnea) were euthanized [5]. To provide some indication of renal injury and function between groups in older animals, measurements were collected at weeks 25 and 35. Given the small sample size of SS-WT at these later time points, the data from both time points were averaged representing ~30 weeks of age.

**RNA Sequencing and Real-Time PCR**. RNA was extracted using the Pure Link RNA Mini Kit from archived Study 1 kidney samples (Invitrogen) according to manufacturer instructions and assessed for quality control parameters of minimum concentration and fidelity (i.e. 18S and 28S bands, RQI >8). cDNA libraries were prepared using the TruSeq mRNA Stranded Library Prep Kit (Set-A-indexes), quantified with the Qubit Fluorometer (Invitrogen), and assessed for quality and size using Bio-Rad Experion System[6]. Samples were pooled into single library (n=8-12 pooled samples per library) and sequenced using the NextSeq 500 High Output Kit (300 cycles, paired end 100bp) on the Illumina NextSeq 500 platform. For RNA sequencing performed on week 4 samples an n=4 per group (WT and KO) were used. The run generated 109 Gb at QC30=89% with 590 million or 74 million reads passing filter per sample. For RNA sequencing performed on week 12 samples an n=6 per group (WT and KO) were used. The run generated 115 Gb at QC30=83.6% with 664 million or 28.8 million reads passing filter per sample. Sequenced reads were assessed for quality using the Illumina BaseSpace Cloud Computing Platform and FASTQ sequence files were used to align reads to the rat reference genome (RGSC 5.0/rn5) using RNA-Seq Alignment Application (using STAR aligner). On average, >96% reads per sample mapped to the reference genome for each run. Differential expression was determined using Cufflinks Assembly & DE

workflow (v2.1.0). Gene expression differences are denoted as $Log_2$ (ratio) and q>0.05. Gene Set Enrichment Analysis (GSEA) was performed using Enrichr (http://amp.pharm.mssm.edu/Enrichr/)[7,8], which provides multiple tools for gene list enrichment analysis. Raw reads can be accessed under BioProject PRJNA600744 and Short Read Archive (SRA) accession numbers SRR10874291-SRR10874310.

Validation of RNAseq data was performed using SYBR-green dye chemistry on Bio-Rad Real Time PCR Platform. RNA was reverse-transcribed to cDNA using the iScript™ cDNA Synthesis Kit and real-time PCR (Bio-Rad CFX96/384) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad). All primers were pre-designed, validated primers from Bio-Rad PrimePCR assays. Expression levels were normalized to beta-actin (Actb) and Glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Statistical analysis of Real-Time PCR data was performed by Bio-Rad Maestro Software. The data are presented as mean + SEM.

**Mass Spectrometry Proteomics.** Total protein was isolated from kidney using Protein Extraction Reagent Type 4. Proteins were reduced, alkylated, and trypsin-digested into peptides Peptides were cleaned using Sep-Pak Vac C18 cartridge (Waters, Milford, MA) and analyzed label-free by liquid chromatography-tandem mass spectrometry using a Q Exactive (ThermoFisher, Waltham, MA) coupled with a 15 cm × 75 µm C18 column (5 µm particles with 100 Å pore size)[6]. Samples were run in duplicate and combined for analysis. Target values in MS were 1e6 ions at a resolution setting of 70,000 and in MS2 1e5 ions at a resolution setting of 17,500.MS/MS spectra were searched with SEQUEST using Proteome Discoverer (version 1.4; ThermoFisher) against the RefSeq database (November 3, 2013) containing 53,918 sequences. The false discovery rate (FDR) was set at 0.01 to eliminate low-probability protein identifications. Peptides were quantified using spectral counting and total spectral counts of each sample were used for normalization. Missing values were replaced with a small value (0.01) in order to calculate the ratio and p-value. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011758.

**Flow Cytometry.** Single cell suspensions from the kidney cortex were prepared in 5 mL RPMI (Roswell Park Memorial Institute) media containing 200 U/mL DNase and 10 mg/mL collagenase IV using the Gentle MACS dissociator (Milltenyi Biotec, Bergisch Gladbach, Germany) using a user-defined protocol for rodent kidney[9]. The resulting suspension was filtered through a 70 µm cell strainer and washed with 1× PBS containing 2% FCS and 2 mmol/L EDTA. The single cell suspension was centrifuged at 300g for 10 minutes. The resulting cell pellet was resuspended in PBS. One million cells were aliquoted into a flow cytometry tube and incubated with Zonbie Green viability dye (BioLegend, San Diego, CA) for 20 min at room temperature protected from light. The cells were subsequently washed with 1X PBS containing 2% FCS and 0.09% sodium azide (stain buffer). The cells were resuspended in 50 µL of stain buffer and incubated with 0.5 µg of anti-rat CD32 (FcR block, BD Biosciences) and then stained with either isotype control antibodies or ant-rat CD3 (clone 1F4), anti-rat CD4 (clone OX-35), anti-rat CD8a (clone OX-8), and anti-rat CD11b/c (clone OX-42) diluted 1:100 in stain buffer (BD Biosciences). The samples were incubated on ice for 30 min and protected from light. Samples were analyzed on a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN), and a total of 25,000 events were acquired for each sample. Data were analyzed using FCS express software (DeNovo Software, Glendale, CA).

**Histological Analysis.** Kidneys were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-µm sections and stained with Periodic acid Schiff (PAS) and/or Masson's trichrome. For glomeruli, morphometric analysis [diameter (um) and area ($um^2$)] was performed on 20 randomly selected images (PAS stained at 40X) per section. Tubulointerstitial injury was determined by evaluation of slides stained with Masson's Trichrome to quantify the percent fibrosis (blue staining) compared to background in 20 randomly selected images from renal cortex as previously done[3,5]. Tubulointerstitial injury was evaluated separately on a semi-quantitative scale from 0 (normal) to 4 (severe) using a minimum of 20 randomly selected images (Masson's Trichrome at 20 X) as follows: grade 0, no changes; grade 1, mild tubule atrophy/fibrosis involving less than 25%; grade 2, lesions affecting 25-50%; grade 3, lesions affecting 50- 75%; and grade 4, lesions affecting >75%[10,11]. All histology analyses were performed with the analyst blinded to the animal/ID/category. Images were captured using SeBaP4-PH1 Brightfield/Phase contrast microscope (Laxco, Mill Creek, WA) and analyzed using Nikon Elements image analysis software.

**Statistical Analysis.** All physiological data are presented as mean + standard error (SE). Time course experimental data (e.g., proteinuria, and telemetry blood pressure) was evaluated by one-way or two-way ANOVA followed by either Dunnett or Tukey post-test (GraphPad Prism 6, La Jolla, CA) [12]. Data for single time point measures (e.g. organ weight, terminal blood pressure, etc.) were analyzed using student t-test or ANOVA when more than 2 groups. Survival was evaluated by the Kaplan-Meier method (GraphPad Prism 5, La Jolla, CA). A $p< 0.05$ was considered to be statistically significant.

## SUPPLEMENTAL REFERENCES

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**SUPPLEMENTAL TABLES FOR ANIMAL STUDIES**

**Table S1: Organ Weights and Clinical Chemistries at Week 12 on 0.3% and 2.0% NaCl Diet (Study 1)**

| | 0.3% NaCl | | 2.0% NaCl | |
|---|---|---|---|---|
| | SS-WT | SS-*Arhgef11*$^{-/-}$ | SS-WT | SS-*Arhgef11*-/- |
| BW(g) | 338 ± 6.2 | 330 ± 5.8 | 339 ± 5.8 | 336 ± 4.9 |
| HW (mg/gBW) | 3.0 ± 0.05 | 3.0 ± 0.02 | 3.4 ± 0.08$^{*}$ | 3.1 ± 0.03$^{††}$ |
| TKW(mg/gBW) | 7.7 ± 0.14 | 7.4 ± 0.11 | 9.7 ± 0.34$^{*}$ | 8.3 ± 0.10$^{††}$ |
| CrCl (ml/min/gTKW) | 0.84 ± 0.07 | 1.1± 0.11 | 0.80 ± 0.05 | 1.1 ± 0.05$^{††}$ |
| BUN (mg/dl) | 26 ± 0.9 | 22 ± 0.9† | 32 ± 1.5$^{*}$ | 27 ± 1.0$^{*,††}$ |
| CHOL (mg/dL) | 97 ± 5.1 | 77 ± 2.0† | 99 ± 4.1 | 81 ± 2.3$^{††}$ |
| HDL (mg/ml) | 52 ± 3.7 | 45 ± 1.4 | 60 ± 2.8 | 51 ± 2.5 |

One-way ANOVA adjustment for multiple comparisons (Tukey) * p<0.05, 2.0% vs 0.3 either group, † p<0.05, KO vs SS-WT for 0.3% NaCl, †† p<0.05, KO vs SS-WT for 2.0% NaCl

**Table S2:Organ Weights and Clinical Chemistries at Week 15 on 2.0% NaCl Diet (Study 2)**

| | 2.0% NaCl | |
|---|---|---|
| | SS-WT | SS-*Arhgef11*-/- |
| BW(g) | 380 ± 8.7 | 389 ± 5.6 |
| HW (mg/gBW) | 3.8 ± 0.08 | 3.4 ± 0.04$^{*}$ |
| TKW(mg/gBW) | 9.1± 0.31 | 8.3 ± 0.13$^{*}$ |
| PROT (g/dl) | 6.3 ± 0.13 | 6.7 ± 0.08$^{*}$ |
| ALB (g/dl) | 3.4 ± 0.13$^{*}$ | 3.8 ± 0.08$^{*}$ |
| CHOL (mg/dL) | 135 ± 5.4 | 102 ± 3.9$^{*}$ |
| HDL (mg/ml) | 72 ± 3.0 | 51 ± 2.1$^{*}$ |

Unpaired t-test, *, p<0.05 KO vs SS-WT

**SUPPLEMENTAL TABLES FOR –OMICS DATASET**

**Tables S3-S10 in separate Excel File**

**SUPPLEMENTAL FIGUREs AND LEGENDS**

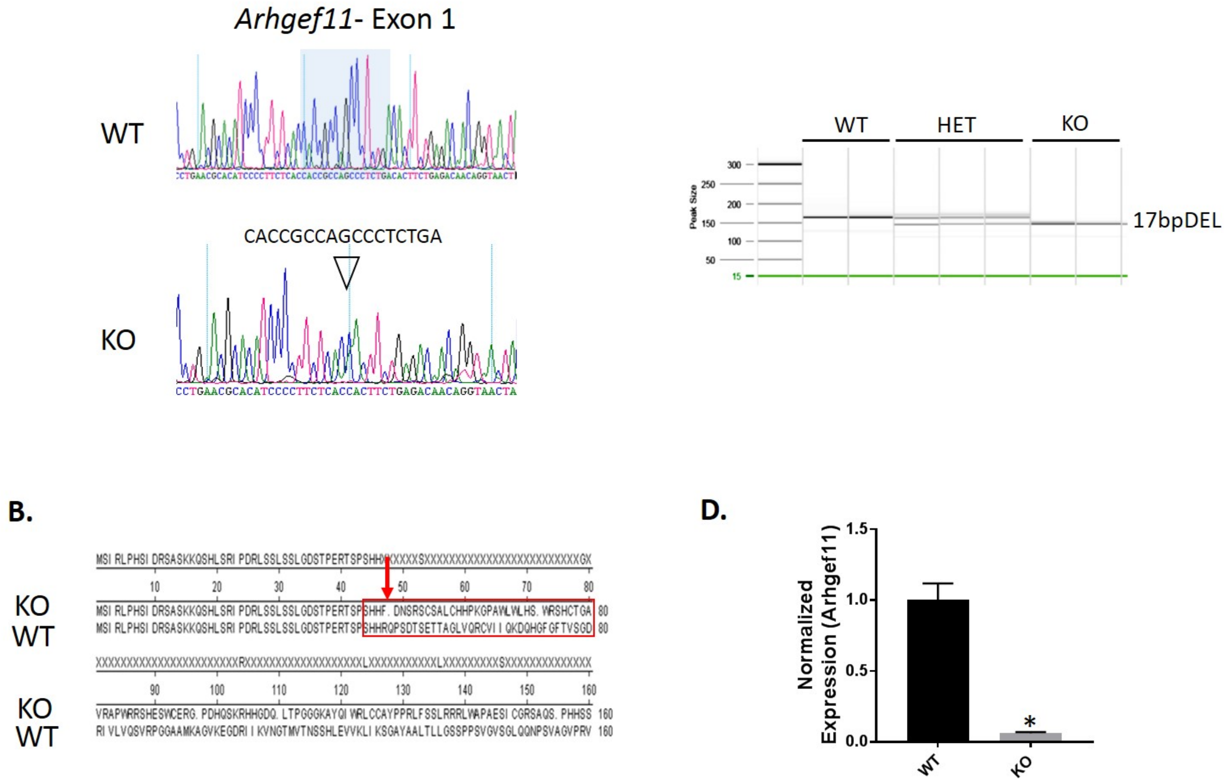


**Figure S1.** Validation of the CRISPR/Cas9 induced deletion in *Arhgef11* gene. **(A)** Sanger sequencing chromatograph demonstrating a 17bp deletion in Exon 1. **(B)** Protein translation of CRISPR/Cas9 deletion of 17 bps in *Arhgef11* resulting in predicted stop codon in exon 1. **(C)** Qiagen QIAxcel fragment analysis of exon 1 PCR products from SS-WT, heterozygous animals (SS-WT/KO), and KO animals. **(D)** Real-time PCR of *Arhgef11* expression in WT (n=6) and KO (n=6). Unpaired t-test, * p<0.05 KO vs WT.

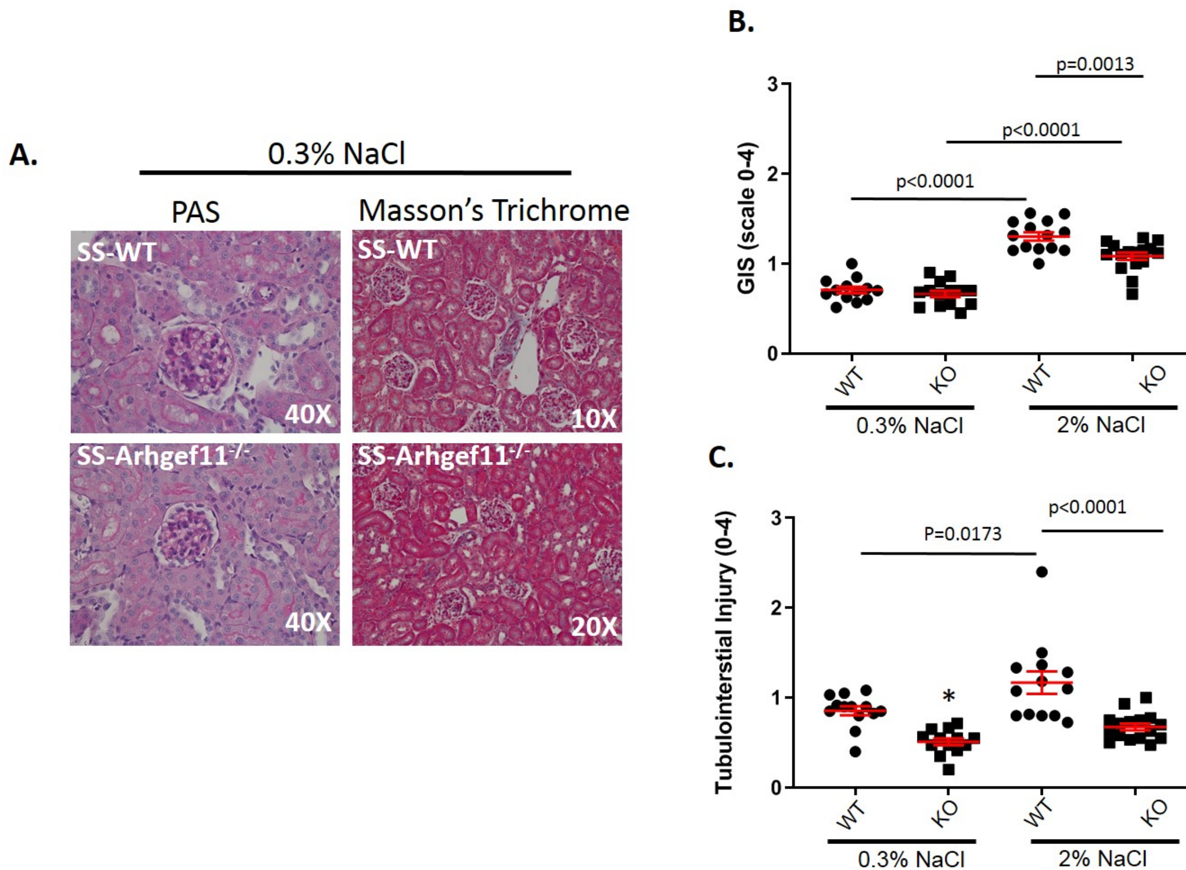


**Figure S2.** Histology and semi-quantitative scoring of renal injury in SS-WT and SS-Arhgef11$^{-/-}$ (KO) rats on low-salt (LS, 0.3% NaCl) and/or elevated-salt diet (ES, 2% NaCl). **(A)** Representative image of glomeruli and tubulointerstitial region for SS-WT and KO on LS at week 4. **(B)** Glomerular injury score at week 12. Each glomerulus was scored on a scale from 0 (normal) to 4 (global sclerosis). A minimum of 20 glomeruli were scored for each kidney section (n= 9 per group/diet). **(C)** Tubular injury at week 12 was analyzed for degree of tubular atrophy, vacuolization, dilation, and proteinaceous casts on a scale from 0 (normal) to 4 (severe with > 75% tubules demonstrating injury). One-way ANOVA with Tukey multiple comparisons. *, t-test KO vs SS-WT. Mean values ± SE of diff are presented.

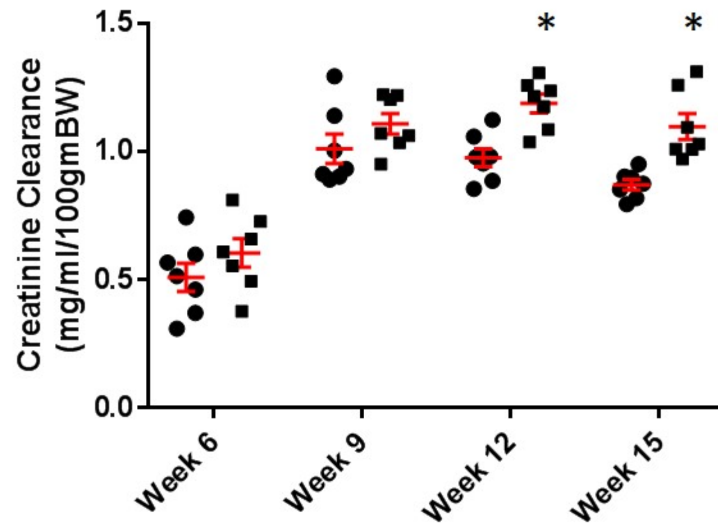


**Figure S3.** Time course measurement of renal function in SS-WT and SS-*Arhgef11*$^{-/-}$ (KO) rats on elevated-salt diet (ES, 2% NaCl). Creatinine clearance (CrCl, ml/min) normalized to 100 gm body weight. WT (n=7) and KO (n=7). Two way ANOVA- Sidak's multiple comparison (strain and time, $p<0.0001$, strain x time, NS). * $p<0.05$ KO vs WT. Mean values ± SE of diff are presented.

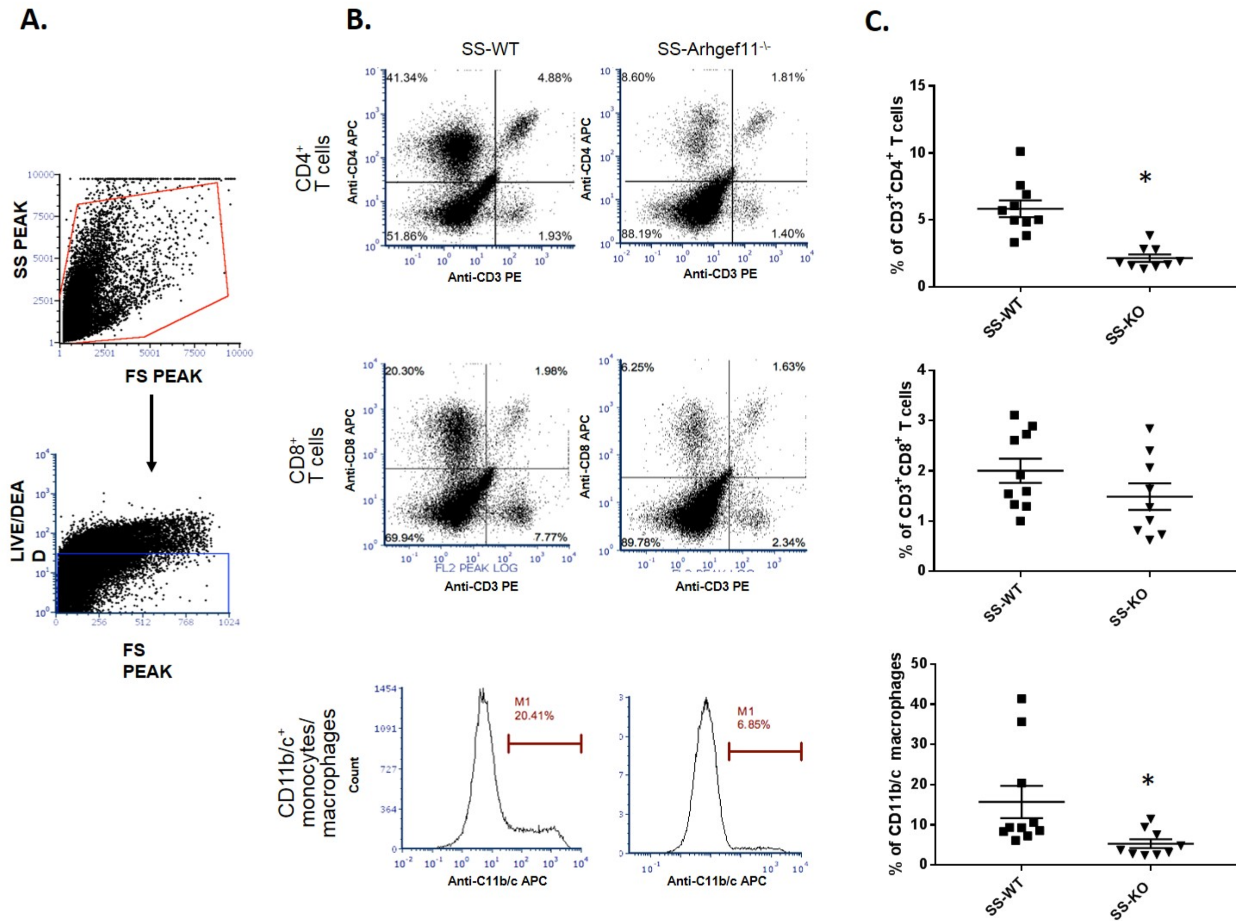


**Figure S4.** Flow cytometric immune cell profiling in the kidney in SS-WT and SS-*Arhgef11*$^{-/-}$ (KO) rats on elevated-salt diet (ES, 2% NaCl). **(A)** Representative scatter profile and gating strategy to identify live cells. **(B)** Representative dot plots to identify CD3$^+$CD4$^+$ T cells and CD3$^+$CD8$^+$ T cells, and histograms for CD11b/c$^+$ monocytes and macrophages in WT and KO rats. **(C)** Percentages of CD3$^+$CD4$^+$ T cells, CD4$^+$CD8$^+$ T cells, and CD11b/c$^+$ monocytes and macrophages in WT (n=11) and KO (n=9). Unpaired t-test, *, $p<0.05$ KO vs WT. Mean values ± SE.

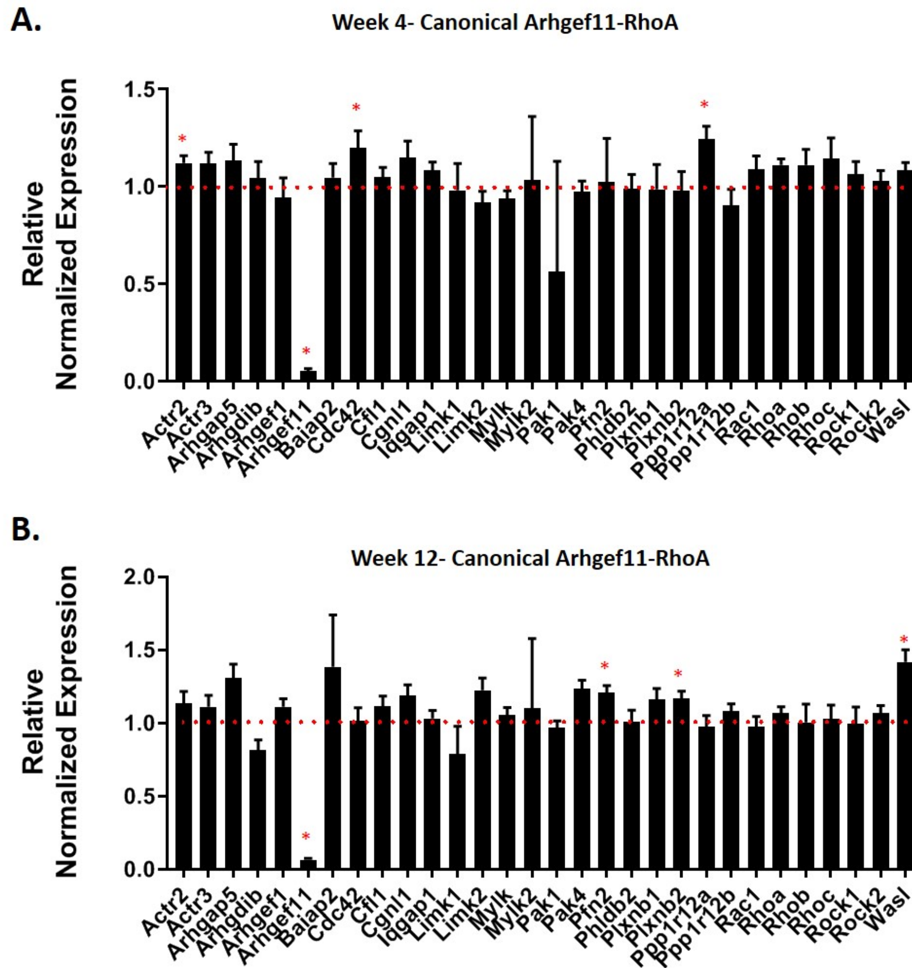


**Figure S5.** Quantitative real-time PCR of genes known to be associated with *Arhgef11* and associated *RhoA* signaling pathway on low-salt (LS, 0.3% NaCl). WT genes were normalized to 1 and expression level is represented by the red-dotted line. **(A)** Expression of Arhgef11-RhoA related genes at week 4. **(B)** Expression of Arhgef11-RhoA related genes at week 12. SS-WT (n=6) and KO (n=6). Unpaired t-test, *, $p<0.05$ KO vs WT. Mean values ± SE.

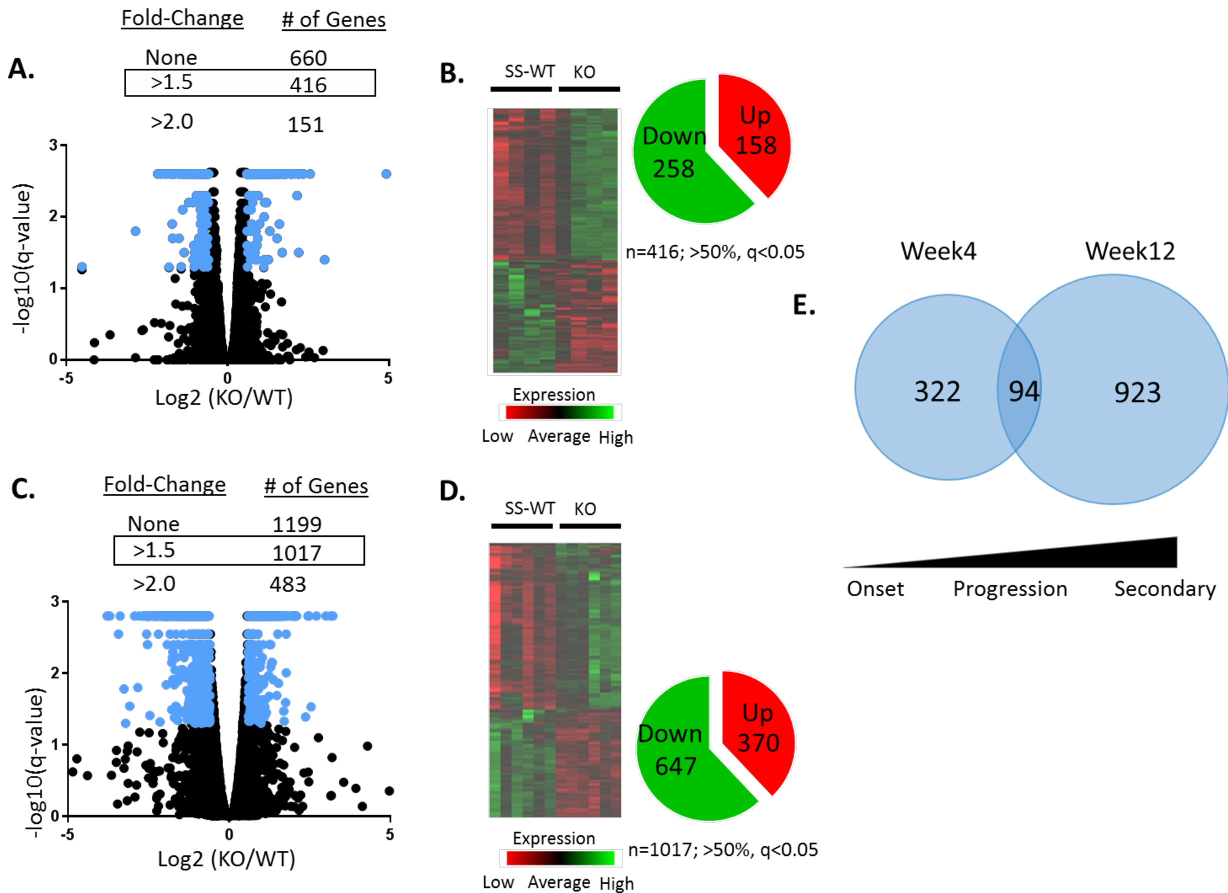


**Figure S6.** Transcriptome analysis of kidney from SS-WT and SS-*Arhgef11*$^{-/-}$ (KO) rats on low-salt (0.3% NaCl) at week 4 and week 12. **(A)** Volcano plot differentially expressed genes at week 4 (blue denoted genes expressed at >50% change and q<0.05). **(B)** Hierarchical clustering and pie chart of upregulated/downregulated genes in KO vs WT at week 4. **(C)** Volcano plot differentially expressed genes at week 12 (blue denoted genes expressed at >50% change and q<0.05). **(D)** Hierarchical clustering and pie chart of upregulated/downregulated genes in KO vs WT at week 12. **(E)** Venn diagram of significantly differentially genes at week 4 and 12.Overlap (n=94) and unique genes observed at week 4 (n=322) or week 12 (n=923). Genes unique to week 4 are likely involved in early molecular changes that contribute to injury in the WT, whereas those unique to week 12 are likely associated with progression of injury and downstream factors.

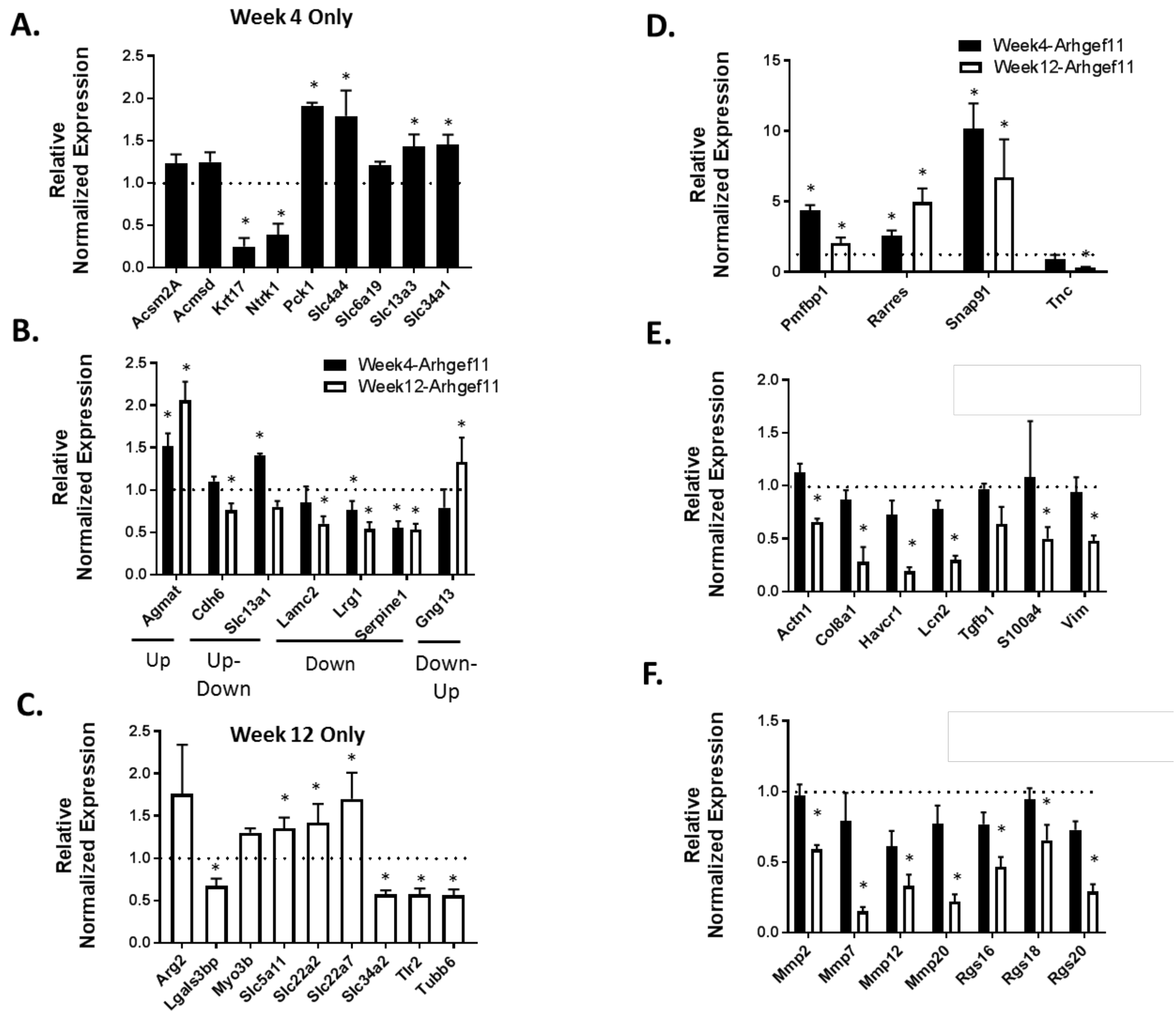


**Figure S7.** Validation of select genes/ enriched pathways via quantitative real-time PCR. SS-WT genes were normalized to 1 and expression level is represented by the red-dotted line. **(A)** Expression of genes associated with enriched pathways at week 4. **(B)** Expression of genes expressed at both week 4 and week 12. **(C)** Expression of genes associated with enriched pathways at week 12. **(D)** Genes that demonstrated the largest differences at either week 4 and/or 12. **(E)** Known gene markers associated with renal injury. **(F)** Two gene families, matrix metalloproteinases (MMP) and regulator of G protein signaling (RGS) with several dysregulated genes. SS-WT (n=6) and KO (n=6). Unpaired t-test, *, $p<0.05$ KO vs WT. Mean values ± SE.

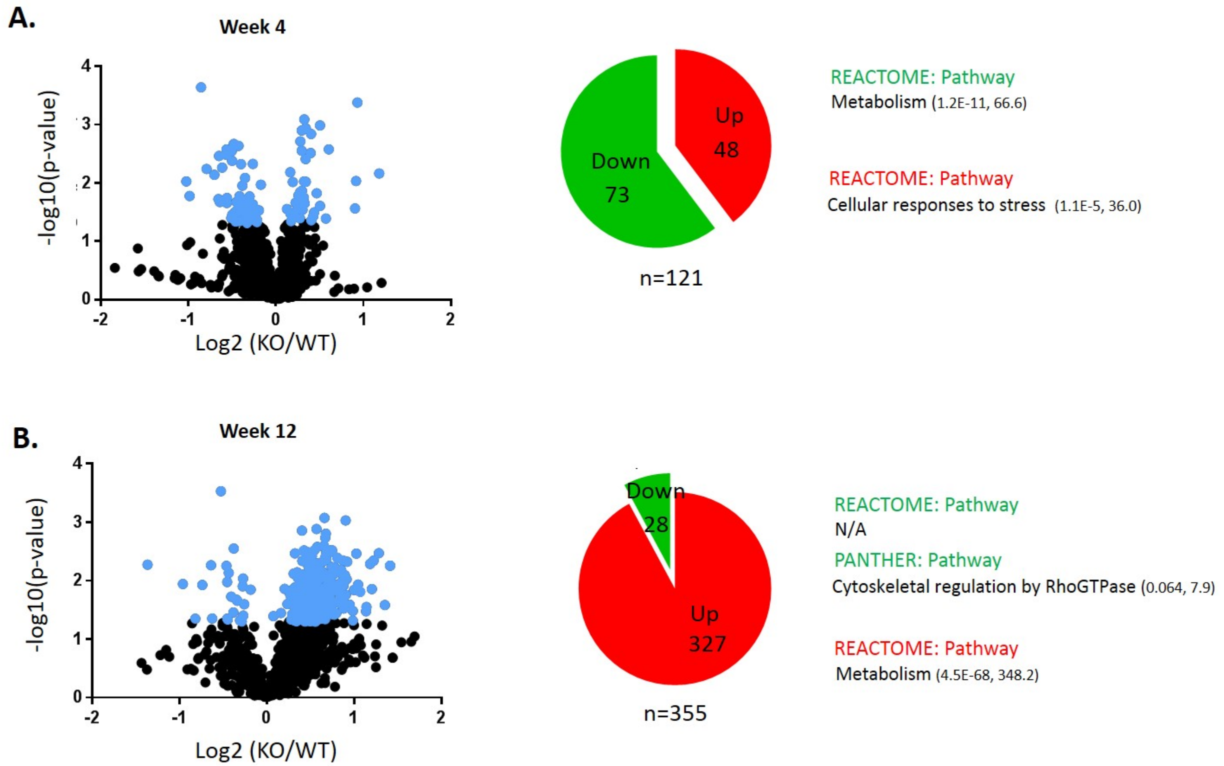


**Figure S8.** Proteomic analysis of kidney from SS-WT and SS-*Arhgef11*$^{-/-}$ (KO) rats on low-salt (0.3% NaCl) at week 4 and week 12. **(A)** Volcano plot of protein abundance differences between KO and WT at week 4 and 12 (blue dotes denoted protein level differences). **(B)** Chart of upregulated/downregulated proteins and significantly enriched pathways (GO terms and Reactome pathways) at week 4 and 12. For GO terms and Reactome pathways, the numbers within the parenthesis note the q-value (adjusted p-value using the Benjamini-Hochberg method for correction for multiple hypotheses testing) and combined score (combination of the p-value and z-score), both measures of significance for enrichment.

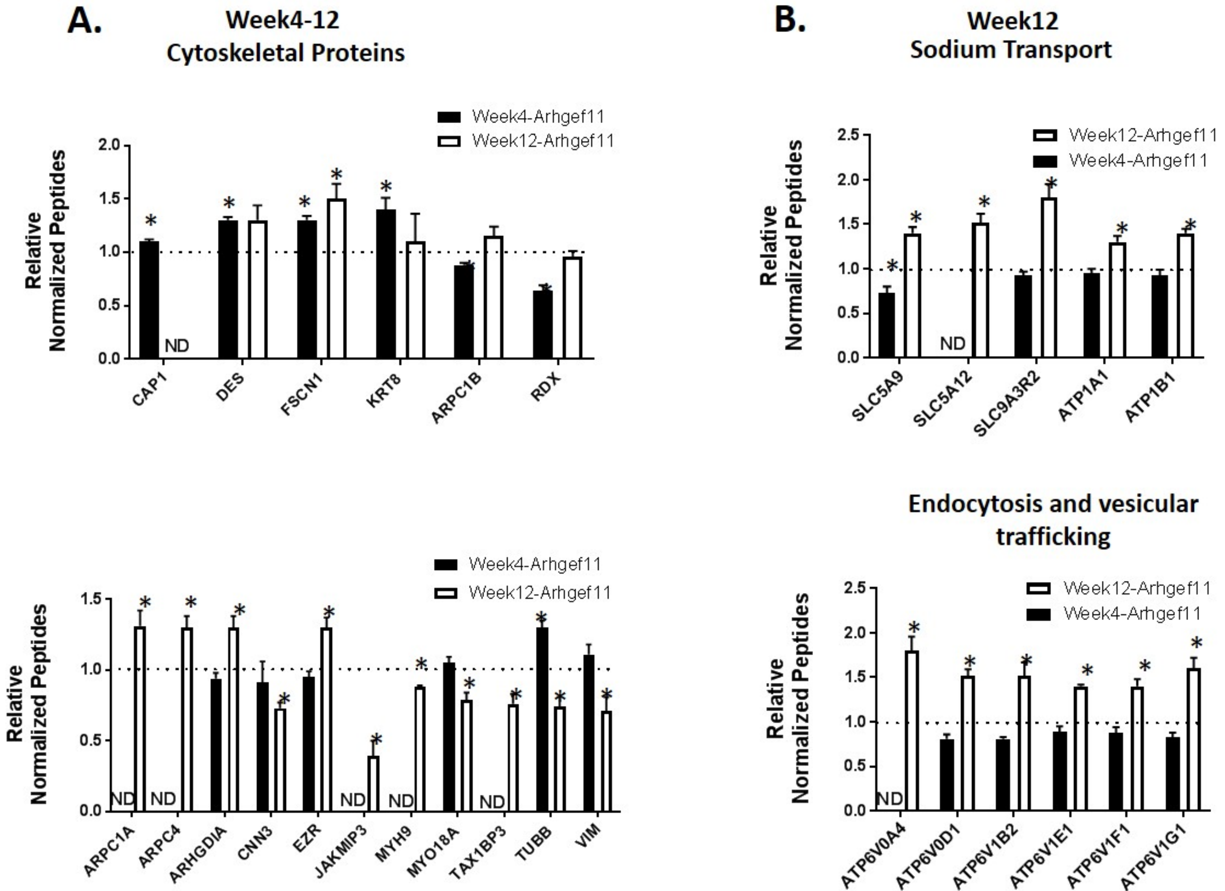


**Figure S9.** Highlight of key proteins identified via proteomic analysis of kidney from SS-WT and SS-*Arhgef11*$^{-/-}$ (KO) rats on low-salt (0.3% NaCl) at week 4 and week 12. These proteins were queried from the mass spectrometry data based on being enriched in the either or both dataset and/or involved in cytoskeleton regulation. The number of peptide hits for WT were normalized to 1 which is denoted by the dashed line. The peptide hits for KO were normalized to WT so the data represents an increase (above the line) or decreased (below the line) compared to the number of protein peptide identified for WT. **(A)** Proteins differences between KO and SS-WT involved in cytoskeleton regulation at week 4 and 12. **(B)** Proteins differences between KO and SS-WT involved in sodium transport and endocytosis and vesicular trafficking. * $p<0.05$ KO vs WT. Mean values ± SE of diff are presented.

| | SS-WT | SS-*Arhgef11*$^{-/-}$ | SS-*Arhgef11*$^{SHR}$ |
|---|---|---|---|
| **Hypertension** | +++ | + | ++ |
| **CKD** | +++ | + | ++ |
| Renal Injury | | | |
| Glomerulosclerosis | +++ | + | ++ |
| Tubulointerstitial fibrosis | +++ | + | + |
| Immune cell infiltration | ++ | + | + |
| Vascular injury | +++ | + | ++ |
| Renal Function | | | |
| Gradual decline in GFR | +++ | + | ++ |
| **Mortality** | +++ | - | ++ |

**Figure S10.** Comparison of cardiovascular and renal phenotypes between SS-WT, SS-*Arhgef11*$^{-/-}$ (KO) rats, and SS-*Arhgef11*$^{SHR}$.