RENAL FKBP12 DELETION ATTENUATES TACROLIMUS-INDUCED HYPERTENSION

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Supplementary methods:

Nephron Segment qRT-PCR Primers:

FKBP12	F:5'ACTACACGGGGATGCTTGAA 3'
	R:5'GCTCTCTGACCCACACTCAT 3'
Calcineurin-α	F:5'CCAACACTCGCTACCTCTTC 3'
	R:5' GTGCCTACATTCATGGTTTCC 3'
Calcineurin-β	F:5' GCAACCATGAATGCAGACACC 3'
	R:5'CAAGGGGCAAGCTGTCAAAAG 3'

Animals: Control and FKBP12$^{-/-}$ mice were generated by crossing FKBP12$^{fl/fl}$ mice with Pax8-rtTA/TRE-LC1 mice. Dr. Susan Hamilton graciously supplied the FKBP12$^{-/-}$ mice, which were generated using AB2.1 ES cells[1] and were crossed with C57bl/6 mice (personal correspondence) to produce chimeric offspring. Dr. Jim McCormick generously supplied the CRE Pax8-rtTA/TRE-LC mice, which are on a C57bl/6 background (personal correspondence).

Tacrolimus: Tacrolimus stock solutions of 30 mg/mL were prepared by dissolving powdered tacrolimus in a 3:1 solution of DMSO:tween-20. Working solutions were prepared fresh, daily, by diluting with PBS to 15 ug/mL. Mice were injected subcutaneously with 3 mg/Kg tacrolimus daily, for 18 days at 9 AM and tissues were collected at 4 PM.

Use of Male Mice: We restricted the analysis to male mice, as this is a follow on paper, extending prior results obtained in males. Further, there are gender differences in tacrolimus metabolism that would have made the feasibility of these difficult studies difficult.[2]

Power Calculations: We did power calculations to determine the number of mice needed. To detect a 5 mmHg difference in blood pressure, using an alpha value of 0.05 power of 0.8, and a delta of 3 mmHg (reported by the manufacturer, DSI international) n=6 is required. Previously, we found that tacrolimus induces a 40% increase in NCC phosphorylation.[3] To detect this, assuming a standard deviation of 27 (previous work[3]) and using an alpha of 0.05 and a power of 0.8, an n=7 is needed to detect such differences by Western Blot.

Genotyping

Genomic DNA from mouse tail clippings were heated in 75 uL NaOH (pH 12.0) at 95 C for 45 min and neutralized with 75 uL Tris-HCl (pH 5.0). Genotypes were determined by PCR using 4 uL of crude genomic lysate, 21 uL of Invitrogen TaqDNA Polymerase native master mix and the following primers:

FKBP12	F:5' AGAACTTGCCCTTCAGTATT 3'
	R:5'AGGCTTGTACCACTATTTTCT3'
PAX8-rtTA	F:5'CCATGTCTAGACTGGACAAGA 3'
	R:5'CAGAAAGTCTTGCCATGACT 3'
TRE-LC1 (CRE)	F:5'TTTCCCGCAGAACCTGAAGATG 3'
	R:5'TCACCGGCATCAACGTTTTCTT 3'

Genotypes were confirmed by immunoblotting for FKBP12 (abcam 2918, 1:5000) at the conclusion of experiments.

Genomic DNA semi-quantitative PCR Primers:

FKBP12	F 5' GTCCTCTTTTCTCACGGT 3'
	R:5'AGGCTTGTACCACTATTTTCT 3'

mRNA PCR

Tissue was preserved at the time of collection in RNALater, snap frozen and stored at -80C. mRNA was extracted using oligotex direct mRNA mini kit. cDNA was transcribed using Finnzymes Phusion Kit and amplified by PCR using the following exon spanning primers:

FKBP12	F: 5'GAGTGCAGGTGGAGACCATCTCTC 3'
	R: 5'CATGGCAGATCCACGTGCAGAG 3'

Immunoblotting: Tissue lysates were prepared by dounce homogenizing snap frozen samples, on ice, in 1 mL chilled lysis buffer as previously described by McCormick et al.[4] Samples were spun down at 6000 RPM for 15 min at 4 C, proteins were separated on 4-12% Bis-Tris gels (Invitrogen) transferred overnight at 4 C and immunoblotted.

Antibodies: The following specific antibodies were used:

pNCC (1:4000) (1:6000 anti-rabbit)[5]

tNCC (1:10,000) (1:5000 anti-rabbit)[6]

tNKCC2 (1:3000)(1:5000)[7]

β-actin abcam ab8227 (1:10,000) (1:10,000 anti-rabbit)

FKBP12 abcam ab2918 (1:5000) (1:5000 anti-rabbit)

WNK4 (1:4000) (1:5000 anti-rabbit)[8]

SPAK (1:5000 overnight) (1:5000 anti-rabbit)-generous gift from Eric Delpire

OxSR1 (1:5000 overnight) (1:5000 anti-rabbit)-generous gift from Eric Delpire

pNKCC2 (1:7000) (1:75000)-Generous gift from Sebastian Bachmann

Blood sample Analysis: Blood was obtained by cardiac puncture (under anesthesia) and put into heparinized tubes. 100 ul of blood was immediately pipetted into an I-STAT chem 8+ cartridge (Abbott Pointe of Care) for analysis. The remaining sample was spun down at 2000 RPM for 5 min and the plasma was used to determine Mg^{2+} concentrations (Pointe Scientific, Xylidyl Blue assay).

Blood Pressure: Mice were given 0.3mg/kg Buprenorphine (analgesic) and 10 mg/Kg Ciprofloxacin (antibiotic) prior to surgery. Under anesthesia (isoflurane in O_2; 5%-loading 1.5-2% maintenance) TA11PA-C20 radiotelemetry probes (Data Sciences International) were implanted into the left carotid artery of mice. Data collection began after 6-10 days of recovery and was collected for 20 seconds every 10 minutes for the length of experiments.

Metabolic Cage gel diets: Gel diets consisted of 5 g of powdered NaCl deficient diet (Harlan) which was reconstituted to a standard 0.49% with NaCl, 8 mL of water and 0.0225 g of bacterial agar per serving.

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3. Hoorn EJ, Walsh SB, McCormick JA, et al. The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension. *Nature medicine.* 2011;17(10):1304-1309.

4. McCormick JA, Mutig K, Nelson JH, et al. A SPAK isoform switch modulates renal salt transport and blood pressure. *Cell metabolism.* 2011;14(3):352-364.
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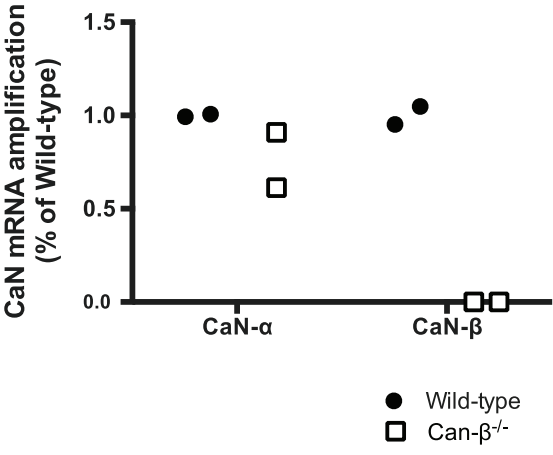
Supplementary Figures and Table

SUPPLEMENTARY TABLE 1

Plasma values	units	Control	KS-FKBP12$^{-/-}$	*p*-value	n
Na^+	mmol/L	142.50	142.00	0.67	4
K^+	mmol/L	4.58	4.35	0.31	4
Cl^-	mmol/L	102.25	102.50	1	4
Ca^{2+}	mmol/L	1.27	1.27	1.00	4
TCO_2	mmol/L	27.25	28.25	0.65	4
BUN	mg/dL	24.25	21.25	0.09	4
Creatinine	mg/dL	0.50	0.48	0.75	4
Hematocrit	%PCV	38.00	36.00	0.11	4
Hemaglobin	g/dL	12.93	12.23	0.10	4
Anion Gap	mmol/L	18.50	17.00	0.47	4

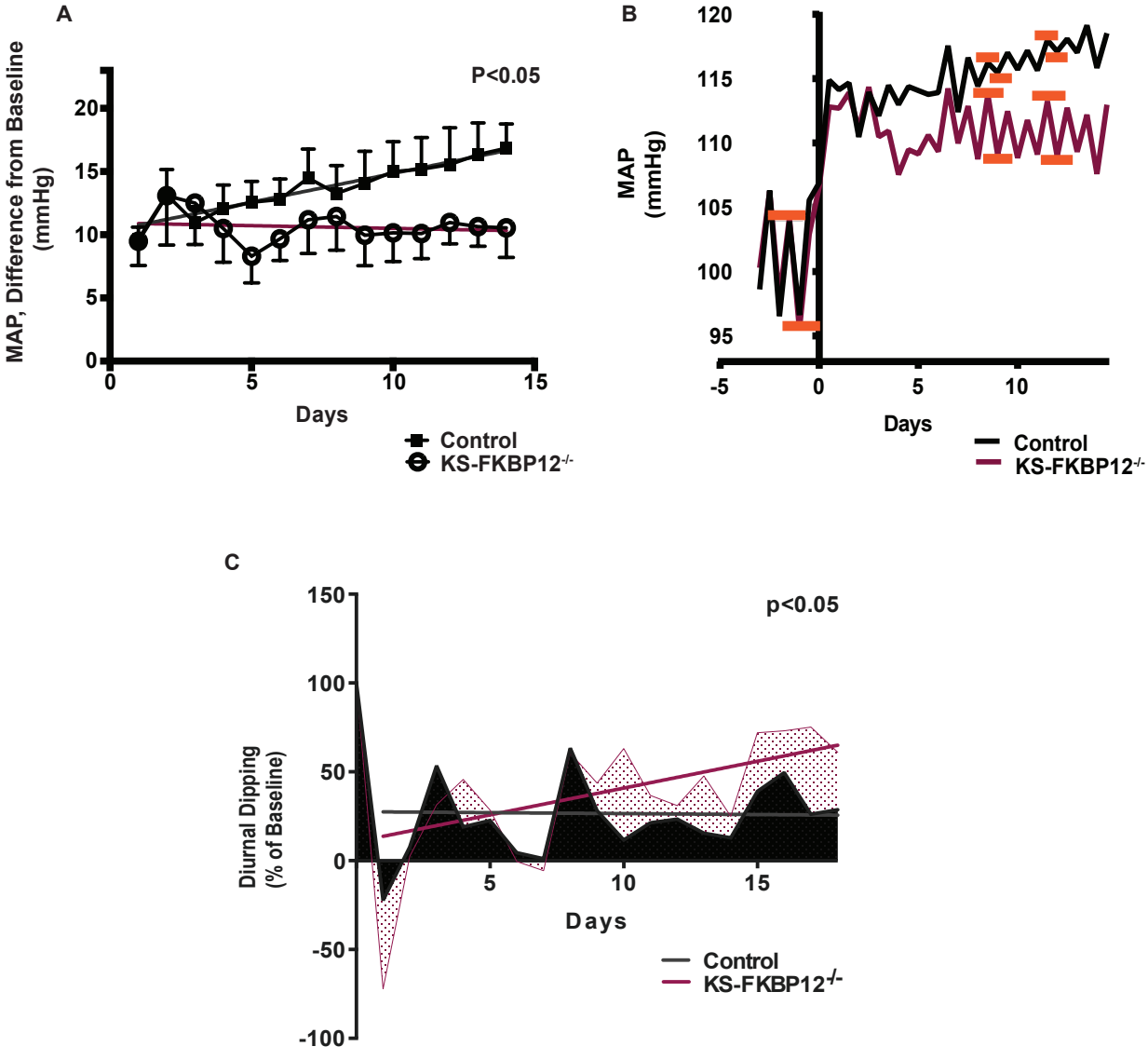
Supplementary Table 1-Baseline electrolytes of Aged Mice. Electrolytes of mice 6 months after doxycycline-induced FKBP12 recombination (T-test, $p<0.05$).

SUPPLEMENTAL FIGURE 1



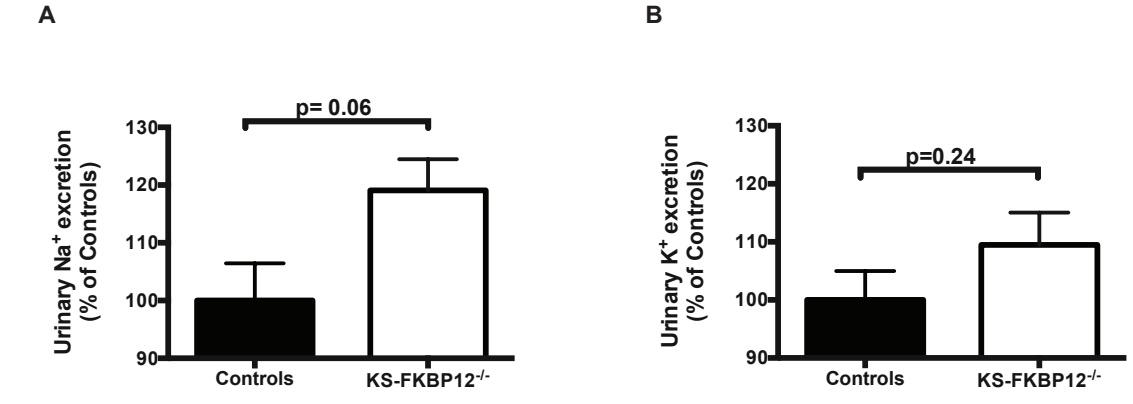
Supp #1: Calcineurin qRT-PCR primers are specific. Primers specific to Calcineurin-α (CaN-α) and Calcineurin-β (CaN-β) were used to amplify Wild type and CaN-$\beta^{-/-}$ kidney samples. A) CaN-α primers were able to generate a signal in CaN-$\beta^{-/-}$ tissues while CaN-β primers did not, confirming primer specificity (n=2).

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2: Quantification of KS-FKBP12$^{-/-}$ protection from tacrolimus-induced blood pressure abnormalities. A) Differences from baseline in average 24 hour MAP in control (-Dox) and KS-FKBP12$^{-/-}$ (+Dox) mice treated with 3 mg/Kg subcutaneously for 18 days (difference in slopes of linear regression, $p<0.05$, $n=6$). B) Illustration of maximal and minimal values used to calculate diurnal dipping patterns. C) The average diurnal-dipping pattern (difference between average MAP during 12 hour dark cycle and 12 hour light cycle) at baseline (average of values from pretreatment days) is set at 100%. Daily diurnal dipping patterns with tacrolimus treatment are represented as a percentage of baseline dipping. Linear regression illustrates that though there is initial disruption in both groups KS-FKBP12$^{-/-}$ mice begin to recover their diurnal dipping patterns more effectively than control mice (difference in slopes of linear regression, $p<0.05$, $n=6$).

SUPPLEMENTAL FIGURE 3



Supp #3) Tacrolimus-treated KS-FKBP12$^{-/-}$ mice have a tendency to excrete more Na^+ and K^+ than tacrolimus-treated controls. Ion excretion was normalized to the tacrolimus-treated controls in each of the 2 experimental replicates. A) Na^+ excretion (mmol/24 hours/g bodyweight, % of controls) B) K^+ excretion (mmol/24 hours/g bodyweight, %of controls) (*t*-test, P>0.05, n=5-7, +/- SEM)