Renal Deletion of 12 kDa FK506-Binding Protein Attenuates Tacrolimus-Induced Hypertension

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ABSTRACT

Tacrolimus is a widely used immunosuppressive drug that inhibits the phosphatase calcineurin when bound to the 12 kDa FK506-binding protein (FKBP12). When this binding occurs in T cells, it leads to immunosuppression. Tacrolimus also causes side effects, however, such as hypertension and hyperkalemia. Previously, we reported that tacrolimus stimulates the renal thiazide-sensitive sodium chloride cotransporter (NCC), which is necessary for the development of hypertension. However, it was unclear if tacrolimus-induced hypertension resulted from tacrolimus effects in renal epithelial cells directly or in extrarenal tissues, and whether inhibition of calcineurin was required. To address these questions, we developed a mouse model in which FKBP12 could be deleted along the nephron. FKBP12 disruption alone did not cause phenotypic effects. When treated with tacrolimus, however, BP and the renal abundance of phosphorylated NCC were lower in mice lacking FKBP12 along the nephron than in control mice. Mice lacking FKBP12 along the nephron also maintained a normal relationship between plasma potassium levels and the abundance of phosphorylated NCC with tacrolimus treatment. In cultured cells, tacrolimus inhibited dephosphorylation of NCC. Together, these results suggest that tacrolimus causes hypertension predominantly by inhibiting calcineurin directly in cells expressing NCC, indicating thiazide diuretics may be particularly effective for lowering BP in tacrolimus-treated patients with hypertension.

To inhibit calcineurin, a serine/threonine phosphatase, tacrolimus must bind to an endogenous protein, the 12 kDa FK506-binding protein (FKBP12). When this occurs in T cells, cytokine production is inhibited and it results in immunosuppression.

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Calcineurin and FKBP12, however, are not expressed exclusively in T cells. Both proteins are widely distributed, so it is possible that calcineurin inhibition in the vasculature, the nervous system, or even T cells themselves, contributes to tacrolimus-induced hypertension. Alternatively, calcineurin inhibition in the distal convoluted tubule (DCT), where NCC is expressed, may activate NCC directly, leading to hypertension. Finally, tacrolimus-induced hypertension may be unrelated to calcineurin inhibition. FKBP12 has many effects that are independent of calcineurin; FKBP12 is a peptidyl prolyl isomerase involved in protein folding, TGF-β signaling, and calcium receptor stabilization. Systemic deletion of FKBP12 in mice is embryonic-lethal, and disruption of FKBP12 in the vasculature and hematopoietic cells causes hypertension. Thus, tacrolimus may disrupt FKBP12 functions that are independent of calcineurin. Here, we tested the hypothesis that tacrolimus activates NCC and causes systemic hypertension by inhibiting calcineurin in the DCT. We generated a mouse model in which FKBP12 can be deleted along the nephron to test this hypothesis in vivo, and corroborated the findings in cultured cells. The results confirm that FKBP12-dependent effects of tacrolimus along the nephron, such as calcineurin inhibition, are largely responsible for its actions on BP.

RESULTS

Renal Distribution of FKBP12 and Calcineurin
To determine whether tacrolimus could exert direct effects in the DCT, we used nephron-segment quantitative RT-PCR to identify sites along the nephron where FKBP12 and calcineurin are expressed. FKBP12 was expressed by all nephron segments and within glomeruli, as was calcineurin (Figure 1).

Generating KS-FKBP12−/− Mice
We next generated mice in which FKBP12 could be deleted along the nephron of adult mice, in response to doxycycline. We bred FKBP12fl/fl mice with mice carrying Pax8-rtTA and TRE-LC1. The resulting offspring were screened and selected for FKBP12fl/fl homozygosity and copies of both Pax8-rtTA and TRE-LC1. These mice were born at the expected rate, and appeared normal at birth and throughout development. At 4–7 weeks of age, the mice were given doxycycline (see Concise Methods section). Doxycycline treatment was tolerated well and resulted in FKBP12 gene recombination that was detected in the kidney, but not the heart, brain, or muscle (Figure 2A). Recombination was also detected in the liver, consistent with prior reports using this system. RT-PCR of mRNA from mice treated with doxycycline showed that the expected full-length FKBP12 transcript was absent in the kidney. Instead, there was a smaller band, consistent with a transcript missing the excised exon 3 (Figure 2B). Western blotting showed >90% reduction in FKBP12 abundance in the kidneys of KS-FKBP12−/− mice (Figure 2C). A reduction in FKBP12 abundance was also observed in the liver (Figure 2D).

KS-FKBP12−/− Baseline Phenotype
The body weight, plasma creatinine, plasma electrolytes (Table 1), arterial pressure, abundance of pNCC, total NCC (tNCC), and their ratio (Figure 3) were similar in KS-FKBP12−/− and control mice. Although plasma [Cl−] and anion gap were significantly lower in KS-FKBP12−/− mice (Table 1), these differences were small and of questionable physiologic significance. Further, when electrolyte values were determined 6 months after deletion of FKBP12, the phenotype remained normal relative to controls, including plasma [Cl−] and anion gap (Supplemental Table 1). The kidney structure of KS-FKBP12−/− mice also appeared normal relative to controls (data not shown).

Effects of Tacrolimus on Arterial Pressure are Attenuated in KS-FKBP12−/− Mice
To inhibit calcineurin, tacrolimus must bind to FKBP12. Thus, KS-FKBP12−/− mice are vulnerable to the effects of tacrolimus outside of the kidney, but protected from FKBP12-dependent events along the nephron. Thus, we tested if tacrolimus had different effects in control and KS-FKBP12−/− mice. Tacrolimus treatment caused an initial increase in mean arterial pressure in both groups, but BP continued to rise in the control mice, whereas it remained stable in KS-FKBP12−/− mice (Figure 4). The difference between the slopes of the BP curves was significant (Supplemental Figure 2A). After 14 days of tacrolimus treatment, although mean arterial pressure remained significantly higher in control mice relative to KS-FKBP12−/− mice, BP in both groups began to decline, suggesting some adaptation (data not shown).

Figure 1. FKBP12 and calcineurin mRNA are present along the nephron. qRT-PCR of mRNA, extracted from microdissected nephron segments. (A) FKBP12 mRNA is distributed evenly across nephron segments (one-way ANOVA, P<0.05, n=3, ±SEM). (B) Confirming prior results, calcineurin is found in the DCT. Both the calcineurin-α and -β isoform are present along the nephron (n=2). Segmental controls published in reference 25, Primer controls in Supplemental Figure 1. Glom, glomerulus; PT, proximal tubule; TAL, thick ascending limb; DCT, distal convoluted tubule; CT/CD, cortical collecting duct.
In rodents and humans, BP typically exhibits a diurnal rhythm, with a decline (called ‘dipping’) during the inactive period of the day. Calcineurin inhibitors have been reported to attenuate diurnal BP dipping in humans. We found that dipping was initially disrupted by tacrolimus injections in both groups; however, it appeared to recover better in KS-FKBP12 2/− mice than in controls (Supplemental Figure 2, B and C).

### Effects of Tacrolimus on Plasma [K+] Homeostasis

Plasma [K+] was not significantly higher in tacrolimus-treated control mice than in tacrolimus-treated KS-FKBP12 2/−/− mice (Figure 6A), although plasma [K+] tended to be higher. As our prior report documented only subtle potassium dysregulation in tacrolimus-treated wild-type mice,3 we explored this issue further. In wild-type mice, pNCC abundance exhibits a strong curvilinear relationship with plasma [K+] under many different conditions.11 This suggests that pNCC abundance is driven strongly by plasma [K+], an effect that impacts downstream K+ excretion and homeostasis. Here, we confirmed this relationship in wild-type mice that were not treated with tacrolimus (reference mice). pNCC values from tacrolimus-treated control mice were similar to values from control mice, although these differences did not reach statistical significance (Supplemental Figure 3).

### Table 1. Baseline phenotype of KS-FKBP12 2/−/− mice.

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*p values are from nonpaired t tests.

*P<0.05.

In rodents and humans, BP typically exhibits a diurnal rhythm, with a decline (called ‘dipping’) during the inactive period of the day. Calcineurin inhibitors have been reported to attenuate diurnal BP dipping in humans. We found that dipping was initially disrupted by tacrolimus injections in both groups; however, it appeared to recover better in KS-FKBP12 2/−/− mice than in controls (Supplemental Figure 2, B and C).

### Effects of Tacrolimus on NCC in Control and KS-FKBP12 2/−/− Mice

Our previous work illustrated the importance of NCC in tacrolimus-induced hypertension and showed that tacrolimus treatment increased pNCC abundance without affecting tNCC abundance. Here, the abundance of pNCC was significantly higher in the kidneys of tacrolimus-treated control mice than in the kidneys of tacrolimus-treated KS-FKBP12 2/−/− mice (Figure 5), suggesting that tacrolimus increases pNCC by inhibiting calcineurin along the nephron. tNCC was slightly lower in tacrolimus-treated control mice than in tacrolimus-treated KS-FKBP12 2/−/− mice (Figure 5). Tacrolimus-treated KS-FKBP12 2/−/− mice also tended to excrete more Na⁺ and K⁺ than tacrolimus-treated control mice, although these differences did not reach statistical significance (Supplemental Figure 3).

### Figure 2. Generating KS-FKBP12 2/−/− mice.

(A) Semiquantitative PCR of genomic DNA collected from control (+doxycycline) and KS-FKBP12 2/−/− mice (+doxycycline), demonstrating genoic recombination of FKBP12. (B) Semiquantitative PCR products derived from control and KS-FKBP12 2/−/− mouse renal mRNA. A stable FKBP12 transcript is detected in KS-FKBP12 2/−/− mice at the smaller, predicted size for FKBP12 missing floxed exon 3. (C) Western blot of homogenized control and KS-FKBP12 2/−/− kidneys. (D) Quantification of panel C, showing significant reduction in FKBP12 renal protein expression (t test, n=7, ****p<0.001, ±SEM). (E) Western blot of FKBP12 across a panel of tissues showing recombination specificity at the protein level. BP here indicates base pairs. NT indicates nucleotides. Kit indicates the positive control sample.
As FKBP12 deletion along the nephron alone does not have phenotypic effects, and FKBP12 deletion along the nephron largely prevents tacrolimus effects on BP and pNCC, this suggests that calcineurin is involved in NCC regulation. Although calcineurin is a phosphatase and might be expected to regulate NCC by dephosphorylating it, previous work suggested that tacrolimus might also affect the abundance of NCC-activating kinases. We determined the abundance of two previously implicated kinases, WNK4 and SPAK.3 The abundance of the two kinases was similar in control and KS-FKBP12−/− mice after tacrolimus treatment (Figure 7, A and B). Although SPAK is believed to be the predominant kinase that activates NCC, a homologous kinase, OxSR1 (also called OSR1) is also able to activate NCC and appears to play an especially important role in some circumstances.11,12 The abundance of OxSR1 was lower in KS-FKBP12−/− mice treated with tacrolimus than in control mice treated with tacrolimus. However, a smaller molecular weight band observed using the same antibody was similar in density between the two groups (Figure 7, C and D). Though NKCC2, a NCC homolog, is regulated by OxSR1, neither total NKCC2 nor pNKCC2 was different between tacrolimus-treated control and KS-FKBP12−/− mice (Figure 7, C and D).

Tacrolimus Inhibits pNCC Dephosphorylation in Cells

The results described above suggested that tacrolimus inhibits calcineurin along the nephron to activate NCC, but these effects could still be indirect. To provide additional support for a direct mechanism, we tested whether tacrolimus could inhibit NCC dephosphorylation in cells. We developed a dephosphorylation assay system in HEK-293 cells using the K+ channel blocker BaCl2 to depolarize them.13 Before inducing dephosphorylation, NCC phosphorylation was stimulated by transfecting constitutively active SPAK (T243E/S383D). Acute treatment with BaCl2 led to a significant reduction in pNCC, whereas tNCC levels remained unchanged, suggesting dephosphorylation (Figure 8, A and B). Short-term (25 minute) pretreatment with tacrolimus significantly attenuated this response to BaCl2, abrogating NCC dephosphorylation (Figure 8, C and D).

DISCUSSION

Tacrolimus is a widely used immunosuppressive drug that inhibits calcineurin when bound to FKBP12. Our previous work showed that tacrolimus increased pNCC, and that NCC is necessary for tacrolimus-induced hypertension to develop.
While those results suggested that tacrolimus might be stimulating NCC through direct effects on calcineurin in DCT cells, this remained speculative. Here, we tested the hypothesis that tacrolimus acts directly on kidney tubule cells to cause hypertension, and that inhibition of calcineurin is required for this. To do this, we generated a new mouse model, Ks-FKB12−/−, in which FKB12 can be deleted along the nephron.

The structurally dissimilar calcineurin inhibitors cyclosporine and tacrolimus both cause hypertension, suggesting that calcineurin inhibition contributes importantly to this pathology. Yet, calcineurin-independent effects of tacrolimus may also contribute. FKB12 is a peptidyl prolyl isomerase involved in many reactions, and its systemic deletion is lethal. Further, Chiasson et al. showed that deleting FKB12 in endothelial and hematopoietic cells leads to hypertension in vivo.7 We found that FKB12 is expressed along the nephron, but its role there is unknown. However, our results show that FKB12 disruption along the nephron does not increase pNCC or cause hypertension; this provides strong evidence that FKB12 disruption along the nephron is not involved in the hypertensive response.

A second possibility is that the effects of tacrolimus in tissues other than the kidney are involved in hypertension and NCC activation. Tacrolimus causes vasoconstriction,14 increases aldosterone,3 and activates the sympathetic nervous system,15 all of which can contribute to hypertension. These systems also ultimately lead to an increase in NCC activity.11,16 As arterial pressure rose during tacrolimus treatment of both control and Ks-FKB12−/− mice, extrarenal effects likely contributed to tacrolimus-induced hypertension as well. Nevertheless, deletion of FKB12 along the nephron significantly attenuated the hypertensive response to tacrolimus, indicating that the effects caused in kidney cells are essential to the development of hypertension.

The current results corroborate results from our previous study showing an essential role for NCC in mediating tacrolimus-induced hypertension. However, the finding in this study that Ks-FKB12−/− mice are not completely protected from the tacrolimus-induced increase in BP does contrast with the complete protection in NCC−/− mice observed previously.3 Both mechanistic and technical factors may account for this difference. In this study, tacrolimus effects on regulatory pathways outside of the kidney, such as the nervous system and the renin/angiotensin/aldosterone axis, may have stimulated NCC despite deletion of FKB12 from kidney cells. As such upstream effects would be retained in Ks-FKB12−/− mice, BP could still rise through secondary activation of NCC. However, in NCC−/− mice the final common pathway, NCC activation, is absent, and the sympathetic nervous system and aldosterone cannot enhance NCC activity.

In our previous study,3 volume pressure recording tail-cuff monitoring, which is less sensitive than radiotelemetry, was used to assess systolic BP. Additionally, the dose of tacrolimus used was lower than that used here. In pilot studies (not shown), we observed both an increase in BP and a statistically significant difference in pNCC using the lower tacrolimus dose in control and Ks-FKB12−/− mice. Yet, in order to ensure that we could detect partial protection in the current experiments, we increased the dose, which magnified both the BP and pNCC phenotype. The higher dose of tacrolimus may also have increased extrarenal effects, contributing to an increase in BP that is both dependent on NCC and independent of it. Nevertheless, the qualitative effects of tacrolimus on both arterial pressure and on pNCC abundance were similar in the two studies.

One advantage of telemetric BP monitoring is that pressures can be monitored throughout the day and night. Surprisingly, despite being woken up for daily tacrolimus injections, nocturnal Ks-FKB12−/− mice largely recovered their diurnal BP dipping patterns. Nondipping patterns, which are associated with several adverse outcomes,17 are also correlated with salt-sensitive hypertension, such as that which results from tacrolimus administration.18 Phosphorylation of NCC has recently been shown to oscillate in a diurnal rhythm,19 with lower levels of pNCC preceding the dip in BP associated with the inactive period. This suggests that disruption of diurnal NCC regulation by tacrolimus may contribute to the attenuation in diurnal BP dipping.

The abundance of pNCC in tacrolimus-treated control mice was higher than in tacrolimus-treated Ks-FKB12−/− mice. This is in line with our previous work3 and suggests a role for calcineurin in NCC regulation. Earlier work described an increase in the abundance of kinases that activate NCC, WNK4, and SPAK,3 as well an electric mobility shift in the latter, when control mice were treated with tacrolimus. An increased abundance of WNK4 was also implicated in an
increase in pNCC during administration of the calcineurin inhibitor cyclosporine. As calcineurin is involved in regulating transcription factors, such as NFAT, differences in protein abundance are in line with calcineurin activity. While differences between tacrolimus-treated control and KS-FKBP12−/− mice were not observed in this study, both groups in this study were treated with tacrolimus. Only FKBP12-dependent effects along the nephron would result in detectable differences in this study. Thus, WNK4 and SPAK abundance or activation may have changed in response to extrarenal signals or FKBP12/calcineurin-independent events, but we would not be able to detect such differences here. However, this does suggest that if WNK4 and SPAK dysregulation are playing a role in transport relative to controls; however, calcineurin is not active at baseline. Activation by depolarization, a physiologically relevant stimulus, may explain the differences between our studies. Nonetheless, this study does not provide proof of a direct effect of calcineurin to dephosphorylate pNCC, as we utilized transfected HEK-293 cells, which may contain important intermediary proteins.

Figure 6. Tacrolimus-treated KS-FKBP12−/− mice maintain their pNCC:plasma [K+] relationship. (A) Plasma [K+] levels are not statistically different between tacrolimus (Tac)-treated control (doxycycline+Tac) and KS-FKBP12−/− mice (doxycycline+Tac) (t test, P<0.05, n=14–16, ±SEM). (B) The relationship between pNCC and plasma [K+] levels has been established by others and is shown as a line graph. Reference mice (control mice from separate experiments used to validate this curve) are shown to adhere to this relationship well (C), whereas Tac-treated control mice do not (D). (E) Tac-treated KS-FKBP12−/− mice do maintain this relationship. (E) Quantification of B-D. The absolute difference between the values predicted by the pNCC: plasma [K+] curve and actual values were taken and averaged for each group (n=4–8, t test **P<0.01, ±SEM).

The most straightforward interpretation of the current results is that calcineurin dephosphorylates NCC, and that by inhibiting this process in the DCT, tacrolimus leads to an increase in pNCC and hypertension. To test this directly we developed an NCC dephosphorylation assay in HEK-293 cells. Sorensen et al. showed in vivo that K+ loading acutely decreases the abundance of pNCC; they suggested that this results from NCC dephosphorylation. This effect is likely triggered by cell depolarization, brought on by the high level of plasma [K+]. Interestingly, depolarization has also been shown to activate calcineurin in other tissues. Here, we mimicked the depolarization triggered by high plasma [K+] levels with BaCl2, which depolarizes cells and increases pNCC in transfected HEK-293 cells exposed for 24 hours. However, instead of overnight treatment, we used a short-term BaCl2 exposure and included constitutively active SPAK (T243E/S383D) to ensure that NCC was fully activated. As tacrolimus inhibited NCC dephosphorylation in this model, it provides support for the hypothesis that calcineurin plays a direct role in NCC dephosphorylation. As these effects were observed in the presence of constitutive activation of the proximate NCC-phosphorylating kinase (SPAK), they strongly suggest that dephosphorylation is involved. These results contrast with those of Glover et al. in which Xenopus oocytes co-injected with NCC and the catalytic subunit of calcineurin showed no difference in 22Na+ transport. However, calcineurin is not active at baseline. Activation by depolarization, a physiologically relevant stimulus, may explain the differences between our studies. Nonetheless, this study does not provide proof of a direct effect of calcineurin to dephosphorylate pNCC, as we utilized transfected HEK-293 cells, which may contain important intermediary proteins.

Deletion of FKBP12 did not significantly affect the plasma [K+] during tacrolimus treatment. Even control mice treated with tacrolimus had physiologic plasma [K+] levels. This is in line with our previous findings that mice are relatively resistant to tacrolimus-induced hyperkalemia. Yet there was a trend for plasma [K+] to be lower in the KS-FKBP12−/− mice during administration of the calcineurin inhibitor cyclosporine. As calcineurin is involved in regulating transcription factors, such as NFAT, differences in protein abundance are in line with calcineurin activity. While differences between tacrolimus-treated control and KS-FKBP12−/− mice were not observed in this study, both groups in this study were treated with tacrolimus. Only FKBP12-dependent effects along the nephron would result in detectable differences in this study. Thus, WNK4 and SPAK abundance or activation may have changed in response to extrarenal signals or FKBP12/calcineurin-independent events, but we would not be able to detect such differences here. However, this does suggest that if WNK4 and SPAK dysregulation are playing a role in transport relative to controls; however, calcineurin is not active at baseline. Activation by depolarization, a physiologically relevant stimulus, may explain the differences between our studies. Nonetheless, this study does not provide proof of a direct effect of calcineurin to dephosphorylate pNCC, as we utilized transfected HEK-293 cells, which may contain important intermediary proteins.

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mice in this study. As the abundance of pNCC has recently been shown to be strongly dependent on plasma [K\(^+\)] across a wide range of conditions,\(^{11}\) and as this relationship appears crucial for normal K\(^+\) homeostasis,\(^{13}\) we tested whether the relationship between pNCC and plasma [K\(^+\)] was altered by tacrolimus treatment. The results clearly show that NCC is less susceptible to suppression by K\(^+\) during tacrolimus treatment, and that this effect is dependent on renal FKBP12. This suggests that, although tacrolimus-treated mice do not become frankly hyperkalemic, a subtle K\(^+\) regulatory defect is present. However, power calculations indicate that a very large number of mice would need to be studied to detect a significant difference in K\(^+\) excretion. Our data suggest that calcineurin is involved in pNCC dephosphorylation, although as noted, additional work will be required to show that these effects are direct. Tacrolimus, which inhibits calcineurin, would therefore increase pNCC by inhibiting its dephosphorylation, contributing to hypertension and likely hyperkalemia. While the present results confirm an important role of the kidney in tacrolimus-induced hypertension, they also suggest a role for external effects, as the KS-FKBP12\(^{-/-}\) mice did experience a rise in arterial pressure during tacrolimus treatment. The results also suggest that the therapeutic and toxic effects of tacrolimus both arise from calcineurin inhibition; thus, to reduce calcineurin inhibitor toxicity, novel agents will need to be targeted directly toward immune cells or specific calcineurin isoforms. Very recent work has found that pNCC abundance is higher in human transplant recipients with hypertension than in controls,\(^{25}\) providing additional support for a role of NCC in patients. Based on this work, thiazide diuretics may prove especially useful in patients who develop tacrolimus-related hypertension.

**CONCISE METHODS**

**Nephron Segment Quantitative RT-PCR**

mRNA was extracted from microdissected nephron segments using RNA extraction kit (Invitek Inc.). cDNA was synthesized by reverse transcription (Tetro Reverse transcription, Promega). Quantitative PCR (qRT-PCR) was performed using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). Gene expression analysis was performed applying the $\Delta\Delta Ct$ method and normalized against $\beta$-actin.

**Animals**

This study was approved by Oregon Health and Science University’s (OHSU) animal care and use committee (protocol IS918) and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. KS-FKBP12\(^{-/-}\) were generated by breeding FKBP12\(^{fl/fl}\) mice with Pax8-rtTA/TRE-LC1 mice. Mice homozygous for flanked FKBP12 and possessing at least one copy of Pax8-rtTA and TRE-LC1 were identified by PCR genotyping of tail-clipping DNA. Genetically identical mice were treated with either doxycycline (2 g/l, 50 g sucrose, 1 L water) or vehicle (50 g sucrose, 1 L water) at 4–7 weeks of age for 2 weeks. Male mice from 10- to 20-weeks-old were used for experiments. For details please see the Supplemental Material.

**Genomic DNA Semiquantitative PCR**

Genomic DNA was extracted from snap-frozen tissues using Qiagen DNAeasy kit and amplified by semiquantitative PCR.

**mRNA PCR**

Tissue was preserved in RNA. Later, mRNA was extracted with Oligotex Direct mRNA Mini Kit. cDNA was transcribed using Finnzymes Phusion Kit and amplified by PCR using exon-spanning primers.
Immunoblotting
Tissue lysates were prepared by dounce homogenizing snap-frozen samples, in chilled lysis buffer as described.24 Samples were spun and separated on 4%–12% Bis-Tris gels (Invitrogen) and immunoblotted with antibodies described in the Supplemental Material. Blots were quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

Blood Sample Analysis
Blood was obtained by cardiac puncture in heparinized tubes. 100 μl was used for analysis by I-STAT (chem 8+ cartridge, Abbott Point of Care). The rest was spun and the plasma used for a colorimetric Mg2+ assay (Pointe Scientific Inc., Xylidyl Blue assay).

BP
Mice were given 0.3 mg/kg buprenorphine (analgesic) and 10 mg/kg ciprofloxacin (antibiotic) prior to surgery. Under isoflurane anesthesia, 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 for 20 seconds every 10 minutes for the length of experiments.

Tacrolimus
Powdered tacrolimus was dissolved in a 3:1 solution of DMSO:Tween 20–30 mg/ml. This was diluted with PBS to 15 μg/ml, and mice were injected subcutaneously with 3 mg/kg for 18 days.

Urinary Na+ and K+ Values
Mice were housed individually in metabolic cages on a standard gel diet (Supplemental Materials). After 18 days of tacrolimus treatment, including 2 days of acclimation, 24-hour urine samples were collected under a layer of water-saturated mineral oil. Urine was analyzed for Na+ and K+ by dual flame photometry using a Li+ internal standard.

pNCC:Plasma [K+] Ratio
The absolute difference between predicted pNCC values, using the best fit line for previous data11 y=6.593(-0.5383*x) -0.01761, and actual pNCC values (pNCC densitometry was normalized to untreated control mice on a normal diet run on the same Western blot) were averaged.

In vitro NCC Dephosphorylation Assay
HEK-293 cells expressing NCC (described in Hoorn et al.)3 and transiently transfected with SPAK (T243E/S383D) were treated for 25 minutes with 10 mM BaCl2. Cells were pre-treated with 30 μg/ml of tacrolimus for 25 minutes before treating with BaCl2.

Statistical Analyses
FKBP12 nephron tubule segment-specific qRT-PCR and deviations from the predicted pNCC; plasma [K+] relationship were analyzed by one-way ANOVA with Tukey post hoc test, BP changes were analyzed by linear regression. t tests were used for all other analyses. For all analyses, P<0.05 was considered to be significant.

ACKNOWLEDGMENTS
R.A.L. designed this study, performed the experiments except for nephron segment PCR, collected and analyzed the data, performed the figures, and wrote the manuscript. B.H.M. taught R.A.L. how to perform telemetry surgeries. A.S.T. generated the pNCC:plasma [K+] curve used to analyze data in Figure 6. N.H. and M.B. provided the dissected mouse nephron segments for qRT-PCR. K.B., K.M., and S.B. performed qRT-PCR for FKBP12 and calcineurin in these dissected segments. C.L.Y. and D.H.E. conceived of this study, supervised the work, and edited the manuscript.

This work was performed by R.A.L. in partial fulfillment of the requirements for a PhD in Pharmacology and Physiology from Oregon Health and Science University.
We thank N. Desmarais for technical assistance and Dr. Susan Hamilton and Dr. Jim McCormick for the mice used to generate the KS-FKBP12^−/− line. We also thank Eric Delpiere and Sebastian Bachman for the use of their antibodies.

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Portions of this work have been presented at scientific meetings (Terker et al., FASEB J 29: S967.6, 2015; Lazelle et al., 2013; Lazelle et al., FASEB J 28: 1136.6, 2014; Lazelle et al., FASEB J 29: 811.1, 2015)

DISCLOSURES
None.

REFERENCES

RENAL FKBP12 DELETION ATTENUATES TACROLIMUS-INDUCED HYPERTENSION

Rebecca A. Lazelle, Belinda H. McCully, Andrew S. Terker, Nina Himmerkus, Katharina Blankenstein, Kerim Mutig, Markus Bleich, Sebastian Bachman, Chao-Ling Yang, and David H. Ellison

Supplementary methods:

Nephron Segment qRT-PCR Primers:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'→3')</th>
<th>Reverse Primer (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP12</td>
<td>F:5'ACTACACGGGGATGCTTGAA 3'</td>
<td>R:5'GCTCTCTGACCCACACTCAT 3'</td>
</tr>
<tr>
<td>Calcineurin-α</td>
<td>F:5'CCAACACTCGCTACCTCTTC 3'</td>
<td>R:5'GTGCCTACATTCTGTTTCC 3'</td>
</tr>
<tr>
<td>Calcineurin-β</td>
<td>F:5'GCAACCATGAATGCAGACACC 3'</td>
<td>R:5'CAAGGGGCAAGCTGTCAAAAG 3'</td>
</tr>
</tbody>
</table>

Animals: Control and FKBP12−/− mice were generated by crossing FKBP12−/− mice with Pax8-rtTA/TRE-LC1 mice. Dr. Susan Hamilton graciously supplied the FKBP12−/− mice, which were generated using AB2.1 ES cells1 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.¹

**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.² To detect this, assuming a standard deviation of 27 (previous work³) 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:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP12</td>
<td>F:5’ AGAAGGTTGCCCCCTTCAGTATT 3’</td>
<td>R:5’AGGCTTGTACCACATTTCTT3’</td>
</tr>
<tr>
<td>PAX8-rtTA</td>
<td>F:5’CCATGTCTAGACTGGACAAGA 3’</td>
<td>R:5’CAGAAAGTCTTGCCATGACT 3’</td>
</tr>
<tr>
<td>TRE-LC1 (CRE)</td>
<td>F:5’TCTCCCCGCAGAACCTGAAGATG 3’</td>
<td>R:5’TCACCGGCATCAACGTTTCTT3’</td>
</tr>
</tbody>
</table>

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

**Genomic DNA semi-quantitative PCR Primers:**
mRNA PCR

Tissue was preserved at the time of collection in RNAlater, snap frozen and stored at -80°C. 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:

<table>
<thead>
<tr>
<th>F</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP12 F: 5’</td>
<td>5’GTCCTTTTCTCACGGT 3’</td>
</tr>
<tr>
<td>R: 5’AGGCTTGTACCACTATTTTCT 3’</td>
<td></td>
</tr>
</tbody>
</table>

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. 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)
- tNCC (1:10,000) (1:5000 anti-rabbit)
- tNKCC2 (1:3000)(1:5000)
- 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)
- 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, Xyliyl 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.


Supplementary Figures and Table
**SUPPLEMENTARY TABLE 1**

<table>
<thead>
<tr>
<th>Plasma values</th>
<th>units</th>
<th>Control</th>
<th>KS-FKBP12/-/-</th>
<th>p-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>mmol/L</td>
<td>142.50</td>
<td>142.00</td>
<td>0.67</td>
<td>4</td>
</tr>
<tr>
<td>K⁺</td>
<td>mmol/L</td>
<td>4.58</td>
<td>4.35</td>
<td>0.31</td>
<td>4</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mmol/L</td>
<td>102.25</td>
<td>102.50</td>
<td>1</td>
<td>4</td>
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<tr>
<td>Ca²⁺</td>
<td>mmol/L</td>
<td>1.27</td>
<td>1.27</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td>TCO₂</td>
<td>mmol/L</td>
<td>27.25</td>
<td>28.25</td>
<td>0.65</td>
<td>4</td>
</tr>
<tr>
<td>BUN</td>
<td>mg/dL</td>
<td>24.25</td>
<td>21.25</td>
<td>0.09</td>
<td>4</td>
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<tr>
<td>Creatinine</td>
<td>mg/dL</td>
<td>0.50</td>
<td>0.48</td>
<td>0.75</td>
<td>4</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>%PCV</td>
<td>38.00</td>
<td>36.00</td>
<td>0.11</td>
<td>4</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>g/dL</td>
<td>12.93</td>
<td>12.23</td>
<td>0.10</td>
<td>4</td>
</tr>
<tr>
<td>Anion Gap</td>
<td>mmol/L</td>
<td>18.50</td>
<td>17.00</td>
<td>0.47</td>
<td>4</td>
</tr>
</tbody>
</table>

**Supplementary Table 1-Baseline electrolytes of Aged Mice.** Electrolytes of mice 6 months after doxycycline-induced FKBP12 recombination (T-test, p<0.05).
**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-β⁻/⁻ kidney samples. A) CaN-α primers were able to generate a signal in CaN-β⁻/⁻ tissues while CaN-β primers did not, confirming primer specificity (n=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).
Supp #3) Tacrolimus-treated KS-FKBP12<sup>−/−</sup> mice have a tendency to excrete more Na<sup>+</sup> and K<sup>+</sup> than tacrolimus-treated controls. Ion excretion was normalized to the tacrolimus-treated controls in each of the 2 experimental replicates. A) Na<sup>+</sup> excretion (mmol/24 hours/g bodyweight, % of controls) B) K<sup>+</sup> excretion (mmol/24 hours/g bodyweight, % of controls) (t-test, P>0.05, n=5-7, +/- SEM)