Title: Mice carrying ubiquitin-specific protease 2 (Usp2) gene inactivation maintain normal sodium balance and blood pressure.

Authors: Pouly D, Debonneville A, Ruffieux-Daidié D, Maillard M, Abriel H, Loffing J, Staub O

Journal: American journal of physiology. Renal physiology

Year: 2013 Jul 1

Issue: 305

Volume: 1

Pages: F21-30

DOI: 10.1152/ajprenal.00012.2013

Mice carrying Ubiquitin Specific Protease 2 (*Usp2*) gene inactivation maintain normal sodium balance and blood pressure

Running title: Normal Na^+ homeostasis in *Usp2*-KO mice

Daniel Pouly[1], Anne Debonneville[1], Dorothée Ruffieux-Daidié[1], Marc Maillard[2], Hugues Abriel[3], Johannes Loffing[4] and Olivier Staub[1]

[1]Department of Pharmacology & Toxicology, University of Lausanne, Lausanne, Switzerland,

[2] Service of Nephrology, Centre Universitaire Hospitalier Vaudois (CHUV), Lausanne, Switzerland

[3]Department of Clinical Research, University of Bern, Bern, Switzerland.

[4]Institute of Anatomy, University of Zurich, Zurich, Switzerland

Correspondence:

Olivier Staub
Department of Pharmacology & Toxicology
University of Lausanne
Rue du Bugnon 27
1005 Lausanne
Switzerland
Tel. +41-21-692-5407
FAX +41-21-692-5355
Olivier.Staub@unil.ch

ABSTRACT

Ubiquitylation plays an important role in the control of Na^+ homeostasis by the kidney. It is well established that the epithelial Na^+ channel ENaC is regulated by the ubiquitin-protein ligase NEDD4-2, limiting ENaC cell surface expression and activity. Ubiquitylation can be reversed by the action of deubiquitylating enzymes (DUBs). One such DUB, USP2-45, was identified previously as an aldosterone-induced protein in the kidney, and is also a circadian output gene. In heterologous expression systems USP2-45 binds to ENaC, deubiquitylates it and enhances channel density and activity at the cell surface. Because the role of USP2-45 in renal Na^+ transport had not been studied *in vivo*, we investigated here the effect of *Usp2* gene inactivation in this process. We demonstrate first that the USP2-45 protein has a rhythmic expression with a peak at ZT12. *Usp2*-KO mice did not show any differences to wild-type littermates with respect to the diurnal control of Na^+ or K^+ urinary excretion and plasma levels neither on standard diet, nor after acute and chronic changes to low and high Na^+ diets, respectively. Moreover, they had similar aldosterone levels either at low or high Na^+ diet. Blood pressure measurements using telemetry did not reveal variations as compared to control mice. *Usp2*-KO did not display alterations in proteins involved in sodium homeostasis or the ubiquitin system, as evidenced by transcriptome analysis in the kidney. Our data suggest that USP2 does not play a primary role in the control of Na^+ balance or blood pressure.

Keywords: sodium transport, ENaC, blood pressure, circadian rhythm, deubiquitylation

INTRODUCTION

The kidneys play a major role in the maintenance of blood plasma composition and volume, achieved by glomerular filtration of large volumes of plasma followed by controlled reabsorption of the liquid and solutes (7). Of particular interest is the homeostasis of Na^+, as this is critical for determining blood volume and arterial blood pressure (50). The fine tuning of the Na^+ balance is achieved in the aldosterone-sensitive distal nephron (ASDN) (29) that includes the late part of the distal convoluted tubule (DCT2), connecting tubule (CNT) and collecting duct (CD). This process is primarily regulated by the mineralocorticoid hormone aldosterone (50), which promotes the reabsorption of Na^+ by stimulation of transporters and channels, including the thiazide-sensitive Na^+,Cl^--cotransporter NCC, the amiloride-sensitive epithelial Na^+ channel ENaC, or the basolateral Na^+,K^+-ATPase, and stimulates secretion of K^+ via ROMK channels (for a review see (4, 50)). In this context, aldosterone binds in the cytosol either to its high affinity receptor, the mineralocorticoid receptor (MR) and/or the low affinity receptor, the glucocorticoid receptor (GR) (2), which then translocate into the nucleus and promote a transcriptional/translational program involving aldosterone-induced and –repressed proteins. During the past 15 years, considerable progress has been made in the characterization of these cellular events, and numerous aldosterone-induced proteins have been identified, including the Serum- and Glucocorticoid-dependent Kinase 1 (SGK1) (10, 33), Glucocorticoid-Induced Leucine Zipper protein GILZ (40), the adaptor protein 14-3-3β (26), Scaffold Protein Connector Enhancer of Kinase Suppressor of RAS Isoform 3 (CNK3) (49) or the deubiquitylating enzyme USP2-45 (14). Importantly, evidence has been provided that these proteins form a complex which may regulate ENaC function via post-translational modifications including ubiquitylation (i.e. the post-translational modification of target proteins with ubiquitin (11, 26, 48)). Ubiquitylation is now recognized as an essential mechanism for regulating cellular and physiological processes (12, 43, 54). It involves the

75 action of an enzymatic cascade, including E1 (ubiquitin-activating), E2 (ubiquitin-
76 conjugating) enzymes, and E3 ubiquitin-protein ligases, the latter being the substrate
77 recognizing enzymes. The process is reversible, implicating deubiquitylating enzymes
78 (DUBs), such as the Ubiquitin Specific Protease (USP) protein family that comprises close to
79 60 members (25, 34). With respect to renal Na^+ transport, it is now well established that the
80 ubiquitin-protein ligase NEDD4-2 ubiquitylates either ENaC or NCC and negatively controls
81 their expression and activity at the plasma membrane (19, 43). Moreover, it has been
82 discovered recently that mutations in the genes encoding KLHL3 and CULLIN3, which form
83 a ubiquitin-protein ligase complex, cause pseudohypoaldosteronism type II (PHAII), a rare
84 form of hypertension involving increased NCC activity (8, 30). As mentioned, the previously
85 cited aldosterone-induced proteins appear to be all related to the action of NEDD4-2. SGK1
86 phosphorylates NEDD4-2, creating binding sites for 14-3-3β, and interfering with the
87 interaction of NEDD4-2 with ENaC (6, 11, 16). Likewise, GILZ and CNK3 are part of the
88 ERC (epithelial sodium channel regulatory) complex that comprises SGK1 and NEDD4-2
89 (47).

90 We had identified previously *Usp2-45* as an aldosterone-induced mRNA in a gene
91 expression screen of microdissected CCD segments from mice that were treated for 1 hour
92 with aldosterone, or untreated controls. The corresponding gene encodes 2 deubiquitylating
93 enzymes, USP2-45 and USP2-69, which are generated by different promoters, and alternative
94 splicing. In *Xenopus laevis* oocytes, USP2-45 but not USP2-69 increased ENaC activity (14).
95 Moreover, it was shown *in vitro* that USP2-45 was able to bind to ENaC, to deubiquitylate the
96 channel and increase ENaC activity, both by increasing its cell surface expression and by
97 promoting proteolytic activation (14, 36, 44, 45). *Usp2* is also a bona fide circadian output
98 gene (31, 37-39, 46, 51). In this context, the importance of the circadian clock in renal Na^+
99 and K^+ handling has been highlighted by several reports (13, 35, 53, 57) (for a review see

100 (18)). The circadian timing system of mammals is critical to allow anticipation of daily
101 changes in physiology. It is controlled by cellular, molecular oscillators that regulate the
102 cyclic expression of output genes (52).

103 As all the data regarding ENaC regulation by USP2-45 were achieved in heterologous
104 expression systems, we were interested to know if USP2-45 plays also a role *in vivo* in ENaC
105 control. We addressed this question by taking advantage of a total constitutive *Usp2* knockout
106 mouse model (referred to as *Usp2*-KO), and tested if Na^+ and K^+ homeostasis are disturbed,
107 and if blood pressure is misregulated. We found that the *Usp2*-KO mice are able to perfectly
108 well handle different Na^+ challenges.

109

MATERIAL AND METHODS

Generation of Usp2 total knockout animals. Usp2-KO mice were generated by Deltagen (San Mateo CA, USA) by targeting the genomic sequence of mouse chromosome 9 encompassing the last nucleotide of exon 3, all intron 3-4 and the 34 first nucleotides of exon 4. The sequence corresponds to bp 44'089'180 – 44'090'064 on mouse chromosome 9 (RefSeq: NT_039472.8) with a neomycin resistance β-galactosidase cassette. This insertion targets a stretch of aminoacids containing the catalytic cysteine (C67 and C290 in USP2-45 and -69, respectively) and induces a frameshift. The animals were backcrossed for 10 generations against a pure C57BL6/N genetic background

Genotyping. Genomic DNA from ear biopsy was extracted in 50 mM NaOH and neutralized in Tris-HCl pH 7.4. 1 µl of this preparation was used for multiplex PCR reaction with oligonucleotides specific for the endogenous (E), targeted (T) or both allele (E,T): AAGTGTTGGGCGAGAACTAGTACAG (E,T, sense), GACGTTGTTTGTCTTCAAGAAGCTTC (T, antisense) and CAGGAGGGGACTCTGTAAAACTATC (E, antisense) using GoTaq polymerase (Promega). The PCR products were analyzed on 2% agarose gels.

Reverse transcription PCR and real-time quantitative PCR. Total RNA was extracted from one half of the left kidney by homogenization in Trizol (Invitrogen) and phenol-chloroform extraction. 2 µg of RNA was reverse-transcribed using Superscript II (Invitrogen) and 1 µg of random hexamer primers (Applied Biosystems). 20 ng of cDNA was used for PCR with the following mUsp2 specific primers GCTTCATGAACTCAATTCTTCAG, GCATGGTTGGTGTTTTCTGAAGC using GoTaq DNA polymerase (Promega). The PCR products were analyzed on 1% agarose gel. Real time quantitative PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) using *Usp2-45* oligonucleotides

and probe described in (14) and Taqman Gene Expression Assays for *Sgk1* (Mm00441380_m1) and *Gapdh* (Mm99999915_g1, Applied-Biosystems). The relative amount of *Usp2-45* and *Sgk1* mRNA was normalized to Gapdh mRNA expression.

Gene expression microarray. Kidneys of 8-week old *Usp2*-KO and wild-type littermates males were harvested at Zeitgeber time ZT12 (where ZT0 is light onset and ZT12 is light shutoff) and quickly frozen in liquid nitrogen. Total RNA from one half of the left kidney was extracted using the phenol-chloroform method described above. The obtained total RNA was then purified using the RNAqueous kit (Ambion). All analysis procedures were performed by the Lausanne Genomic Technologies Facility. RNA quality and concentration was verified with an Agilent Bioanalyzer 2100. Equal amounts of RNA extracted from three mice from the same group were combined as a pool. For all samples, 300 ng of total RNA was used to perform target preparation using the Whole Transcript Sense Target Labeling Protocol procedure (Affymetrix, High Wycombe, UK). Affymetrix Mouse Gene 1.0 ST arrays were hybridized with 5 micrograms of labeled, amplified cDNA from two pools per genotype, washed, stained and scanned on an Affymetrix GeneChip Scanner 7G according to the protocol described in Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol FS450_0007). Gene level normalized expression signals were calculated from Affymetrix CEL files using RMA (23). Data were finally reported as $\log_2$ of the normalized expression signals and analyzed with two-tailed Student t-test and Benjamini-Hochberg correction for multiple testing. The data were deposited in the NCBI Gene Expression Omnibus database (accession number: GSE43517).

Metabolic cages. 8-12 week old *Usp2* WT and KO mice were fed a normal housing chow (Kliba-Nafag) and were habituated to 12h light:12 h dark cycles (LD) for at least 2 weeks. The animals were placed in individual metabolic cages (Techniplast, Buguggiate,

159 Italy) for body weight, water and food consumption measurements and urine and feces
160 collections every 4 hours for 32 hours in LD.

161 For the dietary Na^+ challenge experiment the animals were fed a normal Na^+ control
162 diet (0.17% Na^+, Ssniff Spezialdiäten GmbH, Soest, Germany) for 2 days and switched to
163 either Na^+ deficient (<0.01%, Ssniff Spezialdiäten GmbH, Soest, Germany) or Na^+ rich (3.2%
164 Na^+, Ssniff Spezialdiäten GmbH, Soest, Germany). Body weight, water and food
165 consumption were measured and urine and feces were collected every 12 hours at ZT1 and 13
166 and every 6 hours at ZT13, 19, 1 and 7 for 24 hours after diet change. Mice were irreversibly
167 anesthetized with 0.8 mg Xylazine and 1 mg Ketamine per kg BW in 0.9% NaCl injected
168 intraperitoneally. Blood was collected by retroorbital punction. The animals were then
169 sacrificed by cervical dislocation and tissues were harvested and quickly frozen in liquid
170 nitrogen.

171 *Plasma and urine chemistry.* Urinary and plasma Na^+ and K^+ were measured using a
172 VG Instrumentation Laboratory 943 automatic flame photometer, urine osmolality with an
173 Advanced 2020 osmometer (Advanced Instruments) and plasma aldosterone was measured
174 using a RIA (Coat-a-count Diagnostics Products Inc.). Plasma and urinary creatinine were
175 measured by the Clinical Chemistry Lab of the University Hospital in Lausanne, using
176 standard techniques. Glomerular Filtration Rate was calculated on creatinine clearance.

177 *Blood pressure measurements.* Briefly, the animals were surged and implanted a
178 transponder in the carotid artery (Data Science International) as described previously (41).
179 After a 10-day period of recovery cardiovascular parameters were measured every minute for
180 9 seconds for 1-5 days in LD cycles. Data were separated into day (ZT0-12) and night (ZT12-
181 0) and mean systolic and diastolic blood pressure were calculated for each animal.

182 *Diurnal tissue collection. Usp2*-WT and KO animals were housed for at least 3 weeks in
183 LD and sacrificed every 4 hours around the clock by decapitation. Sacrifices at ZT12, 16 and

20 were done under red dim light. Tissues were collected quickly frozen in liquid nitrogen and stored at -70°C before analysis.

Study approval. All experimental procedures were approved by the Swiss animal welfare authorities and carried out in accordance with the local animal welfare act.

Statistical analyses. All datasets were first screened for the presence of outliers using the 1.5 Interquartile Range as exclusion criteria (i.e. data farther than 1.5 IQR above or under the median of the dataset were considered as outliers and removed of the analysis). Multiple comparisons were performed by two-way ANOVA and post-hoc two-tailed Student T-tests were performed using the Holm-Sidak correction of significance threshold for multiple testing if not stated otherwise.

RESULTS

Characterization of Usp2-KO *mice. Usp2*-KO mice were purchased from Deltagen and backcrossed against a C57BL/6N background for more than 10 generations. In these mice, the last base pair of exon 3, intron 3/4 and exon 4 were targeted with a Lac0-SA-IRES-lacZ-WT Neo/Kan cassette, leading to the deletion of amino acids T**C**FMNSILQCLSN, situated at the beginning of the catalytic domain and including Cys67 in USP2-45 (or Cys290 in USP2-69) that is essential for enzymatic activity. Both *Usp2* splice variants are targeted in these mice (Fig. 1A). PCR analysis of genomic DNA isolated from the skin demonstrates the transmission of the KO allele (Fig. 1B), further confirmed by β-galactosidase stainings of various tissues (data not shown). Moreover, RT-PCR analysis on whole kidney total RNA extracts demonstrates deletion of *Usp2-45* and *Usp2-69* (Fig. 1C). At the protein level, Western blot analysis using either an antibody against the N-terminus of USP2-45 (USP2-45NT), or an antibody against the C-terminal catalytic region shared by both isoforms (USP2-CC), confirmed the deletion of USP2-45 (Fig. 1D,E). Intriguingly, the 2 antibodies did not recognize bands of similar size. Whereas the N-terminal antibody marked a protein at an apparent molecular weight of 45 kDa (Fig. 1D), which disappeared in the KO mice, this 45 kDa band was not observable by the C-terminal antibody, due to a crossreacting protein in this region. In contrast, USP2-CC labeled a protein below 40 kDa (Fig. 1E), which may represent a proteolytic cleavage product missing the N-terminus, and consequently not recognizable by the N-terminal antibody. We were not able to detect USP2-69 in the kidney, consistent with previous findings showing that USP2-69 is not or only very weakly expressed in this tissue (20). According to the detailed phenotypic analysis provided by Deltagen and our own observations, homozygote KO mice are viable, and were born at Mendelian ratio, indicating that *Usp2*-KO animals do not die prematurely during development. Similarly to what was described previously with another *Usp2*-KO model, the fertility of male KO mice

221 was strongly reduced (5). The mice did not show any growth defect or any other obvious
222 morphological changes, as assessed by the mouse phenotyping platform of Deltagen.
223
224 *Diurnal expression of USP2-45 in the kidney.* As outlined in the introduction, *Usp2* is
225 controlled by the circadian timing system in numerous tissues, including the kidney (24, 38,
226 39). Thereby the peak of expression of mRNA in mice in constant darkness (DD) is at
227 Circadian Time 8 to 12 and between ZT12 and 16 in liver and kidney in LD (32, 57)
228 corresponding to the end of the subjective resting period (39, 46). At the protein level, the
229 diurnal expression of USP2-45 had been followed previously in the suprachiasmatic nucleus
230 (SCN) and found to peak at ZT16 (46). We followed USP2-45 every 4 hours in total kidney
231 lysates, using the anti USP2-45NT antibody and observed that USP2-45 displays a rhythmic
232 expression in the kidney that peaks at ZT12 in control mice (Fig. 2). No USP2-45 was
233 detectable at any time point in the KO mice. Our data nicely demonstrate that the protein is
234 closely following mRNA expression, suggesting that USP2-45 has a short turnover.
235
236 *Maintained diurnal rhythm of Na^+ and K^+ excretion in Usp2 KO mice.* As it was
237 previously proposed that USP2-45 is an aldosterone-induced protein in the kidney, able to
238 stimulate ENaC expression and activity in heterologous expression systems, including
239 *Xenopus laevis* oocytes, renal epithelial cells ($mpkCCD_{cl4}$ cells) and Hek293 cells (14, 44,
240 45), we were interested to know if *Usp2* KO mice are defective in the regulation of Na^+
241 homeostasis and other related renal parameters. Because USP2-45 protein is highly rhythmic,
242 we housed mice in metabolic cages during 24 hours, and recorded water and food intake,
243 urine volume and creatinine excretion every 4 hours around the clock (Fig. 3 A-D). In both
244 *Usp2* KO mice and control animals, these parameters followed an expected diurnal rhythm
245 and were maximal at ZT12 to 16, hence at the beginning of the activity period of mice. There

246 was no observable difference between the 2 groups of animals (Fig. 3). We looked then at
247 diurnal rate of Na^+ and K^+ excretion, which peaked between ZT12-16, and were not different
248 throughout the day (Fig. 3E-F).
249
250 *Usp2-KO mice are able to adapt to dietary salt changes.* After having found that there
251 are no alterations in excretory rates of Na^+ or K^+, we wondered if there might be a difference
252 in how mice handle dietary Na^+ changes. Animals were switched from a control diet
253 (containing 0.17 % of Na^+) either to a low (< 0.01% Na^+) or high Na^+ diet (3.2 % Na^+) before
254 an activity period to ensure an immediate as possible experimental food intake. Na^+ or K^+
255 excretion were measured in metabolic cages over 6 days and half (Fig. 4), by collecting the
256 urine every 12 hours. As USP2-45 was reported to be induced in the early phase of
257 aldosterone response, we increased the resolution of the measures on the first 24 hours of
258 challenge by collecting the urine every 6 hours. As can be seen in Fig. 4, wild-type and *Usp2*
259 KO animals were able to handle the switch to either diet by rapidly (6 - 12 hours) adapting
260 their Na^+ excretion rate to the altered Na^+ intake. In both groups, K^+ excretion was not
261 affected by the diet change. No difference in either Na^+ (Fig. 4A) or K^+ excretion (Fig. 4B)
262 was observed during the entire period and plasma levels of Na^+, K^+ and were not altered in
263 *Usp2*-KO mice (Fig. 5A and B). In addition, the plasma levels of aldosterone were in the
264 expected ranges reached under low and high Na^+ dietary conditions (Fig. 5C). The analysis of
265 a number of other metabolic parameters did not show any change as summarized in Table I.
266 Hence, our data suggest that USP2 does not play a crucial role in the regulation of Na^+ or K^+
267 homeostasis by the kidney (Fig. 5, Table I).
268
269

The blood pressure is not changed in Usp2-KO *mice.* It is well established that disturbances in Na^+ reabsorption cause either hyper- or hypotension (27). We therefore wondered if suppressing USP2 may influence blood pressure and measured systolic and diastolic blood pressure by telemetry in mice under low, normal and high Na^+ diet (Fig. 6). Average day or night systolic or diastolic pressures are displayed of control and KO mice. We did not observe any difference at any period of the day between control and *Usp2*-KO mice. Taken together, our data do not reveal any disturbances in Na^+ or K^+ handling by the kidney, nor is there any evidence for disturbances in blood pressure regulation. As it is possible that there are compensatory mechanisms, affecting the ubiquitin system or the Na^+ transport regulation, if such regulatory pathways are affected.

Suppression of USP2 is not compensated by proteins involved in the ubiquitin system or regulation of Na^+ homeostasis. Because we had previously shown that heterologous expression of USP2-45 regulates ENaC (14, 44, 45), we were wondering if there is come compensation by proteins involved in ubiquitylation/deubiquitylation or in the control of Na^+ homeostasis. We therefore carried out gene expression profiling of total kidneys of control or KO mice, using Affymetrix oligonucleotide arrays. The kidneys were isolated at ZT12, when USP2-45 is the strongest expressed. Our analysis did not allow us to detect transcriptomal differences between kidneys of control and *Usp2*-KO mice.

Usp2 is not regulated by changes in dietary Na^+ intake. We wondered if varying Na^+ diet may change the expression of *Usp2-45*. We carried out real-time PCR on RNA extracted from isolated CCD of mice were kept under low, normal of high Na^+ diet for 2 weeks, as described in (28). As one can see in Fig. 9, low Na^+ diet increased the expression of *Sgk1* mRNA, and high Na^+ diet decreased it, whereas these dietary changes had no effect on *Usp2-*

295 *45* mRNA, indicating that *Usp2-45* is not regulated by Na^+ intake, at least not under chronic
296 conditions.
297

DISCUSSION

It is well accepted that regulation of Na^+ homeostasis involves the ubiquitin system. Indeed, both ENaC and NCC are regulated by post-translational modification comprising ubiquitin and this ubiquitylation negatively controls the functional expression of these proteins at the cell surface (19, 42, 43). The importance of this mechanism in the regulation of ENaC and NCC is documented by the Liddle's syndrome, in which mutations in the genes encoding the β and γENaC subunit lead to the inactivation of binding motifs for the ubiquitin-protein ligase NEDD4-2 (1), or in PHAII, where mutations in either CUL3 or in KLHL3, both subunits of a ubiquitin-protein ligase complex, cause the disease, likely by increasing NCC activity (8, 30). Ubiquitylation is a reversible process, catalyzed by DUBS. As mentioned in the introduction, USP2-45 had been identified previously as an aldosterone-induced protein and shown to regulate ENaC by binding to and deubiquitylating the channel in heterologous expression systems (14). Therefore, we were interested to know if *Usp2*-KO mice have impaired regulation of Na^+ homeostasis and blood pressure control. Surprisingly, however, these mice did not show any sign of disturbed salt or water regulation, nor did they display troubles in diastolic or systolic blood pressure.

In mice deficient of USP2, the intron 3 to 4, and exon 4 were targeted, leading to the frame-shifted replacement of a short stretch of amino acids by a neomycin resistance and β-galactosidase cassette at the beginning of the USP2 catalytic domain, including the catalytically critical cysteine 67 in USP2-45. PCR analysis on genomic DNA, RT-PCR on kidney mRNA, and Western blot analysis on kidney lysates (using 2 different antibodies) confirmed the deletion of USP2.

323 The conclusion that *in vivo* inactivation of *Usp2*, and specifically *Usp2-45* do not
324 obviously alter Na^+ and K^+ homeostasis, nor blood pressure, is based on a number of
325 experimental evidences: First, KO mice do not show any differences with respect to food and
326 water intake, nor urine production. Second, we did not observe changes in plasma Na^+ or K^+
327 concentration, or defect in the excretion of these cations, and the mice were perfectly able to
328 handle either low Na^+ or high Na^+ diets. This is different for example from *Sgk1*-KO mice,
329 which, when kept under low Na^+ diet, are wasting salt (16, 17, 55). Moreover, there was no
330 change in circulating aldosterone levels between control and KO animals, as would be
331 expected if these animals had a defect in Na^+ reabsorption, and were for example
332 hypovolemic. Again, this is different from *Sgk1*-KO mice, which have elevated aldosterone
333 concentrations or from inducible renal tubule-specific *Nedd4-2*-KO mice, which display salt-
334 sensitive hypertension, increased NCC activity and low aldosterone levels (3, 41). In contrast
335 to *Sgk1* or *Nedd4-2*-KO mice, *Usp2*-KO mice are perfectly able to maintain the same diastolic
336 and systolic blood pressure as WT mice, independent of the time of the day, or the diet (low,
337 normal or high Na^+ diet).
338
339 The finding that *Usp2*-KO mice do not show any apparent defect in the handling of Na^+
340 and K^+ is unexpected, in the view of previous findings that USP2-45 is an early aldosterone-
341 induced protein. However, in the original screen, the intraperitoneal injection of aldosterone,
342 achieving 10 nM aldosterone after 30 minutes, induced only a relatively weak increase of
343 USP2-45 (1.6-fold for mRNA, or 2.4-fold at the protein level), as compared for example to
344 *Sgk1* mRNA, which was increased more than 8-fold (14). Indeed, when looking at the effect
345 of various Na^+ diets (low, normal or high Na^+ diets for 10 days), with 0.1 nM aldosterone at
346 high Na^+ and 1 nM at low Na^+ diet, we see no effect on the expression of *Usp2* mRNA in
347 microdissected CNT/CCD tubules, whereas *Sgk1* mRNA is regulated (Fig. 9). Previously, we

had also shown that USP2-45 is able to regulate ENaC *in vitro*. It has to be taken into account, however, that most of these data were established in heterologous expression systems, such as *Xenopus laevis* oocytes, or Hek293 cells. In these situations, overexpression of USP2-45 may non-specifically deubiquitylate ENaC, and consequently lead to increase of ENaC cell surface expression and activity. On the other hand, in the kidney, where endogenous USP2-45 is much more weakly expressed (as judged from immunoblotting), the DUB does not seem to be involved in sodium transport regulation or its function is so redundant that its absence is compensated without any overt phenotype.

It might have been possible that compensatory effects develop in *Usp2*-KO mice within regulatory pathways controlling ENaC and/or NCC. Gene expression analysis on total kidney RNA, using Affymetrix microarrays, did not reveal significant abnormalities in the expression of any transcript. Particularly, no enzyme involved in ubiquitylation or deubiquitylation was changed, even not UCH-L3, encoding a DUB previously proposed to regulated ENaC (9), nor any of the proteins playing directly or indirectly a role in Na^+ homeostasis. The one exception is the mineralocorticoid receptor (MR), whose expression appears to be regulated by USP2-45 at the protein level, suggesting a negative feedback loop for limiting the aldosterone response (15) As we do not observe any effect on Na^+ handling, the physiological role of this feedback regulation remains to be determined. However, a limitation of our study relates to the fact that the analysis was carried out in whole kidney. Therefore, we cannot exclude that transcriptional compensatory effects taking place in the ASDN may be masked due to dilution in the whole tissue. Moreover, we do not know about other mechanisms of gene expression regulation, such as miRNAs that may be play a compensating role.

To date we can only speculate on the function of USP2-45 in the kidney. As mentioned above, and also shown in Fig. 2, USP2-45 has a strongly rhythmic expression pattern, with a peak of expression around ZT12, which is at the end of the resting period. Such a pattern of expression has been found at the mRNA level in any tissue analyzed, suggesting that USP2-45 plays a role in the rhythmic regulation of physiological processes everywhere in the organism (24, 38, 39). However, *Usp2*-KO mice show only relatively minor defects in the regulation of the circadian clock in general, as evidenced for example by the rhythmic control of behavior in running wheels (which we also carried out; data not shown) (46, 56). It is therefore likely that the rhythmic levels of USP2-45 control diurnal expression of target proteins, by stabilizing such proteins. It is clear now that the circadian clockwork controls renal functions, for example the Na^+ handling (21, 22, 35), but our data indicate that *Usp2* is not crucial in this process.

In conclusion, we show here that inactivation of *Usp2* in mice does not impair the ability of the animals to handle Na^+ balance, or to control blood pressure. These mice do not display any sign of compensatory mechanism, neither at the functional level, nor at the transcriptional level, suggesting that USP2-45 is not playing a key role in these processes.

ACKNOWLEDGEMENTS

We thank Drs. Caroline Ronzaud and Renuga Devi Rajaram for critically reading the manuscript.

GRANTS

This work was supported by the Leducq Foundation Transatlantic Network on Hypertension, the Swiss National Science Foundation 310030_141013/1 (to OS), 310030-143929/1 (to JL),

398 the Novartis Foundation of Medical-Biological Research (to HA), and the Swiss NCCR
399 Kidney.CH.

400

401

402

REFERENCES

1. **Abriel H, Loffing J, Rebhun JF, Pratt JH, Horisberger J-D, Rotin D, and Staub O.** Defective regulation of the epithelial Na^+ channel (ENaC) by Nedd4 in Liddle's syndrome. *J Clin Invest* 103: 667-673, 1999.

2. **Ackermann D, Gresko N, Carrel M, Loffing-Cueni D, Habermehl D, Gomez-Sanchez C, Rossier BC, and Loffing J.** In vivo nuclear translocation of mineralocorticoid and glucocorticoid receptors in rat kidney: differential effect of corticosteroids along the distal tubule. *Am J Physiol Renal Physiol* 299: F1473-1485, 2010.

3. **Arroyo JP, Lagnaz D, Ronzaud C, Vazquez N, Ko BS, Moddes L, Ruffieux-Daidie D, Hausel P, Koesters R, Yang B, Stokes JB, Hoover RS, Gamba G, and Staub O.** Nedd4-2 Modulates Renal Na+-Cl- Cotransporter via the Aldosterone-SGK1-Nedd4-2 Pathway. *J Am Soc Nephrol* 22: 1707-1719, 2011.

4. **Arroyo JP, Ronzaud C, Lagnaz D, Staub O, and Gamba G.** Aldosterone paradox: differential regulation of ion transport in distal nephron. *Physiology (Bethesda)* 26: 115-123, 2011.

5. **Bedard N, Yang Y, Gregory M, Cyr DG, Suzuki J, Yu X, Chian RC, Hermo L, O'Flaherty C, Smith CE, Clarke HJ, and Wing SS.** Mice lacking the USP2 deubiquitinating enzyme have severe male subfertility associated with defects in fertilization and sperm motility. *Biol Reprod* 85: 594-604, 2011.

6. **Bhalla V, Daidie D, Li H, Pao AC, Lagrange LP, Wang J, Vandewalle A, Stockand JD, Staub O, and Pearce D.** Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally down-regulated protein 4-2 by inducing interaction with 14-3-3. *Mol Endocrinol* 19: 3073-3084, 2005.

7. **Boron WF and Boulpaep EL.** *Medical Physiology*. Philadelphia: Saunders Elsevier, 2009.

8. **Boyden LM, Choi M, Choate KA, Nelson-Williams CJ, Farhi A, Toka HR, Tikhonova IR, Bjornson R, Mane SM, Colussi G, Lebel M, Gordon RD, Semmekrot BA, Poujol A, Valimaki MJ, De Ferrari ME, Sanjad SA, Gutkin M, Karet FE, Tucci JR, Stockigt JR, Keppler-Noreuil KM, Porter CC, Anand SK, Whiteford ML, Davis ID, Dewar SB, Bettinelli A, Fadrowski JJ, Belsha CW, Hunley TE, Nelson RD, Trachtman H, Cole TR, Pinsk M, Bockenhauer D, Shenoy M, Vaidyanathan P, Foreman JW, Rasoulpour M, Thameem F, Al-Shahrouri HZ, Radhakrishnan J, Gharavi AG, Goilav B, and Lifton RP.** Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities. *Nature* 482: 98-102, 2012.

9. **Butterworth MB, Edinger RS, Ovaa H, Burg D, Johnson JP, and Frizzell RA.** The deubiquitinating enzyme UCH-L3 regulates the apical membrane recycling of the epithelial sodium channel. *J Biol Chem* 282: 37885-37893, 2007.

10. **Chen S, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, and Pearce D.** Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A* 96: 2514-2519, 1999.

11. **Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, and Staub O.** Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. *EMBO J* 20: 7052-7059, 2001.

12. **Dikic I, Wakatsuki S, and Walters KJ.** Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol* 10: 659-671, 2009.

13. **Doi M, Takahashi Y, Komatsu R, Yamazaki F, Yamada H, Haraguchi S, Emoto N, Okuno Y, Tsujimoto G, Kanematsu A, Ogawa O, Todo T, Tsutsui K, van der Horst GT, and Okamura H.** Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6. *Nat Med* 16: 67-74, 2010.

14. **Fakitsas P, Adam G, Daidie D, van Bemmelen MX, Fouladkou F, Patrignani A, Wagner U, Warth R, Camargo SM, Staub O, and Verrey F.** Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation. *J Am Soc Nephrol* 18: 1084-1092, 2007.

15. **Faresse N, Debonneville A, and Staub O.** USP2-45 Represses Aldosterone mediated Responses by Decreasing Mineralocorticoid Receptor Availability. *Cell Biochem Biophys* in press, 2013.

16. **Faresse N, Lagnaz D, Debonneville A, Ismailji A, Maillard M, Fejes-Toth G, Naray-Fejes-Toth A, and Staub O.** Inducible kidney-specific Sgk1 knockout mice show a salt-losing phenotype. *Am J Physiol Renal Physiol* 302: F977-985, 2012.

17. **Fejes-Toth G, Frindt G, Naray-Fejes-Toth A, and Palmer LG.** Epithelial Na+ channel activation and processing in mice lacking SGK1. *Am J Physiol Renal Physiol* 294: F1298-1305, 2008.

18. **Firsov D, Tokonami N, and Bonny O.** Role of the renal circadian timing system in maintaining water and electrolytes homeostasis. *Mol Cell Endocrinol* 349: 51-55, 2012.

19. **Gamba G.** Regulation of the Renal Na+:Cl- Cotransporter by Phosphorylation and Ubiquitylation. *Am J Physiol Renal Physiol*, 2012.

20. **Gousseva N and Baker RT.** Gene structure, alternate splicing, tissue distribution, cellular localization, and developmental expression pattern of mouse deubiquitinating enzyme isoforms Usp2-45 and Usp2-69. *Gene Expr* 11: 163-179, 2003.

21. **Gumz ML, Cheng KY, Lynch IJ, Stow LR, Greenlee MM, Cain BD, and Wingo CS.** Regulation of alphaENaC expression by the circadian clock protein Period 1 in mpkCCD(c14) cells. *Biochim Biophys Acta* 1799: 622-629, 2010.

22. **Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD, Weaver DR, and Wingo CS.** The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest* 119: 2423-2434, 2009.

23. **Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, and Speed TP.** Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4: 249-264, 2003.

24. **Kita Y, Shiozawa M, Jin W, Majewski RR, Besharse JC, Greene AS, and Jacob HJ.** Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. *Pharmacogenetics* 12: 55-65, 2002.

25. **Komander D, Clague MJ, and Urbe S.** Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol* 10: 550-563, 2009.

26. **Liang X, Peters KW, Butterworth MB, and Frizzell RA.** 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. *J Biol Chem* 281: 16323-16332, 2006.

27. **Lifton RP, Gharavi AG, and Geller DS.** Molecular mechanisms of human hypertension. *Cell* 104: 545-556, 2001.

28. **Loffing-Cueni D, Flores SY, Sauter D, Daidie D, Siegrist N, Meneton P, Staub O, and Loffing J.** Dietary sodium intake regulates the ubiquitin-protein ligase nedd4-2 in the renal collecting system. *J Am Soc Nephrol* 17: 1264-1274, 2006.

29. **Loffing J, Zecevic M, Feraille E, B. K, Asher C, Rossier BC, Firestone GL, Pearce D, and Verrey F.** Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol* 280: F675-F682, 2001.

30. **Louis-Dit-Picard H, Barc J, Trujillano D, Miserey-Lenkei S, Bouatia-Naji N, Pylypenko O, Beaurain G, Bonnefond A, Sand O, Simian C, Vidal-Petiot E, Soukaseum C, Mandet C, Broux F, Chabre O, Delahousse M, Esnault V, Fiquet B, Houillier P, Bagnis CI, Koenig J, Konrad M, Landais P, Mourani C, Niaudet P, Probst V, Thauvin C, Unwin RJ, Soroka SD, Ehret G, Ossowski S, Caulfield M, International Consortium for Blood P, Bruneval P, Estivill X, Froguel P, Hadchouel J, Schott JJ, and Jeunemaitre X.** KLHL3 mutations cause familial hyperkalemic hypertension by impairing ion transport in the distal nephron. *Nat Genet* 44: 456-460, 2012.

31. **Molusky MM, Li S, Ma D, Yu L, and Lin JD.** Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11beta-hydroxysteroid dehydrogenase 1. *Diabetes* 61: 1025-1035, 2012.

32. **Molusky MM, Ma D, Buelow K, Yin L, and Lin JD.** Peroxisomal localization and circadian regulation of ubiquitin-specific protease 2. *PLoS One* 7: e47970, 2012.

33. **Naray-Fejes-Toth A, Canessa CM, Cleaveland ES, Aldrich G, and Fejes-Toth G.** sgk is an aldosterone-induced kinase in the renal collecting duct. *J Biol Chem* 274: 16973-16978, 1999.

34. **Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, and Bernards R.** A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123: 773-786, 2005.

35. **Nikolaeva S, Pradervand S, Centeno G, Zavadova V, Tokonami N, Maillard M, Bonny O, and Firsov D.** The circadian clock modulates renal sodium handling. *J Am Soc Nephrol* 23: 1019-1026, 2012.

36. **Oberfeld B, Ruffieux-Daidie D, Vitagliano JJ, Pos KM, Verrey F, and Staub O.** Ubiquitin-specific protease 2-45 (Usp2-45) binds to epithelial Na+ channel (ENaC)-ubiquitylating enzyme Nedd4-2. *Am J Physiol Renal Physiol* 301: F189-196, 2011.

37. **Oishi K, Amagai N, Shirai H, Kadota K, Ohkura N, and Ishida N.** Genome-wide Expression Analysis Reveals 100 Adrenal Gland-dependent Circadian Genes in the Mouse Liver. *DNA Res* 12: 191-202, 2005.

38. **Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T, and Ishida N.** Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* 278: 41519-41527, 2003.

39. **Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, and Hogenesch JB.** Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109: 307-320, 2002.

40. **Robert-Nicoud M, Flahaut M, Elalouf JM, Nicod M, Salinas M, Bens M, Doucet A, Wincker P, Artiguenave F, Horisberger JD, Vandewalle A, Rossier BC, and Firsov D.** Transcriptome of a mouse kidney cortical collecting duct cell line: Effects of aldosterone and vasopressin. *Proc Natl Acad Sci U S A* 98: 2712-2716, 2001.

41. **Ronzaud C, Loffing-Cueni D, Hausel P, Debonneville A, Malsure SR, Fowler-Jaeger N, Boase NA, Perrier R, Maillard M, Yang B, Stokes JB, Koesters R, Kumar S, Hummler E, Loffing J, and Staub O.** Renal tubular NEDD4-2 deficiency causes NCC-mediated salt-dependent hypertension. *J Clin Invest* 123: 657-666, 2013.

42. **Rotin D and Staub O.** Nedd4-2 and the regulation of epithelial sodium transport. *Front Physiol* 3: 212, 2012.

43. **Rotin D and Staub O.** Role of the ubiquitin system in regulating ion transport. *Pflugers Arch* 461: 1-21, 2010.

44. **Ruffieux-Daidie D, Poirot O, Boulkroun S, Verrey F, Kellenberger S, and Staub O.** Deubiquitylation regulates activation and proteolytic cleavage of ENaC. *J Am Soc Nephrol* 19: 2170-2180, 2008.

45. **Ruffieux-Daidie D and Staub O.** Intracellular Ubiquitylation of the Epithelial Na+ Channel Controls Extracellular Proteolytic Channel Activation via Conformational Change. *J Biol Chem* 286: 2416-2424, 2011.

46. **Scoma HD, Humby M, Yadav G, Zhang Q, Fogerty J, and Besharse JC.** The De-Ubiquitinylating Enzyme, USP2, Is Associated with the Circadian Clockwork and Regulates Its Sensitivity to Light. *PLoS One* 6: e25382, 2011.

47. **Soundararajan R, Melters D, Shih IC, Wang J, and Pearce D.** Epithelial sodium channel regulated by differential composition of a signaling complex. *Proc Natl Acad Sci U S A* 106: 7804-7809, 2009.

48. **Soundararajan R, Pearce D, and Ziera T.** The role of the ENaC-regulatory complex in aldosterone-mediated sodium transport. *Mol Cell Endocrinol* 350: 242-247, 2012.

49. **Soundararajan R, Ziera T, Koo E, Ling K, Wang J, Borden SA, and Pearce D.** Scaffold Protein Connector Enhancer of Kinase Suppressor of Ras Isoform 3 (CNK3) Coordinates Assembly of a Multiprotein Epithelial Sodium Channel (ENaC)-regulatory Complex. *J Biol Chem* 287: 33014-33025, 2012.

50. **Staub O and Loffing J.** Mineralocorticoid action in the aldosterone sensitive distal nephron. In: *The Kidney: Physiology and Pathophysiology* (5 ed.), edited by Alpern RJ, Caplan MJ and Moe OW. London, Waltham, San Diego: Adademic Press, 2013, p. 1181-1211.

51. **Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, and Weitz CJ.** Extensive and divergent circadian gene expression in liver and heart. *Nature* 417: 78-83, 2002.

52. **Takahashi JS, Hong HK, Ko CH, and McDearmon EL.** The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet* 9: 764-775, 2008.

53. **Wang Q, Maillard M, Schibler U, Burnier M, and Gachon F.** Cardiac hypertrophy, low blood pressure, and low aldosterone levels in mice devoid of the three circadian PAR bZip transcription factors DBP, HLF, and TEF. *Am J Physiol Regul Integr Comp Physiol* 299: R1013-1019, 2010.

54. **Weissman AM, Shabek N, and Ciechanover A.** The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat Rev Mol Cell Biol* 12: 605-620, 2011.

55. **Wulff P, Vallon V, Huang DY, Volkl H, Yu F, Richter K, Jansen M, Schlunz M, Klingel K, Loffing J, Kauselmann G, Bosl MR, Lang F, and Kuhl D.** Impaired renal Na(+) retention in the sgk1-knockout mouse. *J Clin Invest* 110: 1263-1268, 2002.

56. **Yang Y, Duguay D, Bedard N, Rachalski A, Baquiran G, Na CH, Fahrenkrug J, Storch KF, Peng J, Wing SS, and Cermakian N.** Regulation of behavioral circadian rhythms and clock protein PER1 by the deubiquitinating enzyme USP2. *Biol Open* 1: 789-801, 2012.

57. **Zuber AM, Centeno G, Pradervand S, Nikolaeva S, Maquelin L, Cardinaux L, Bonny O, and Firsov D.** Molecular clock is involved in predictive circadian adjustment of renal function. *Proc Natl Acad Sci U S A* 106: 16523-16528, 2009.

FIGURE LEGENDS

Table I. Summary of the metabolic parameters measured on Usp2-WT and KO mice during the last 24 hours of each dietary Na^+ condition (NSD, LSD, HSD). Data are means ± SD of 12 (NSD) or 5-6 (LSD, HSD) animals per genotype. LSD : Low Sodium Diet <0.01% Na^+ ; NSD : Normal Sodium Diet 0.17% Na^+ ; HSD : High Sodium Diet, 3.2% Na^+

Fig. 1. Generation of *Usp2* knockout animals. A: The genomic region of the *Usp2* gene coding for the catalytic C67 / C290 of USP2-45 and USP2-69 was replaced by a neomycin resistance (Neo-R)/β-Galactosidase (LacZ) cassette resulting in a total constitutive knockout model. B: Genotyping by multiplex PCR yields to DNA fragments of 204 and 531 bp corresponding to the WT and KO alleles respectively. C: RT-PCR analysis of total RNA extracted from the kidneys of *Usp2* WT and KO animals. 20 ng of retro-transcribed RNA was assayed for PCR and the DNA products were analyzed on a 1% agarose gel. (n=4). D,E: The presence of USP2 protein products in total kidneys was assayed by SDS-PAGE/immunoblotting using two different anti-USP2 antibodies. Anti USP2-45NT (D) was raised against the specific N-terminal extension of mUSP2-45 whereas anti-USP2-CC (E) was raised against the common C-terminal catalytic core of both USP2 isoforms. As expected no USP2 immunoreactive signal was detected in the kidney of the KO animals.

Fig. 2. Renal expression of USP2-45 expression is rhythmic. Kidneys were obtained from 4 WT and 4 KO animals at the indicated Zeitgeber time points (where ZT0 is light onset and ZT12 is light shutoff). Total kidney protein extracts were assayed for anti-USP2-45 immunoblotting. Shown is a representative western blot out of 4 series.

Fig. 3. *Usp2*-KO mice show normal diurnal renal Na^+ and K^+ handling. Water (A), food intake (B), urinary volume (C), creatinine (D), Na^+ (E) and K^+ (F) excretion rates were

618 measured on the indicated Zeitgeber time periods. Data are means ± SD of 12-18 samples
619 obtained from 12 animals. WT: black bars, KO : gray bars.

620 Fig. 4. *Usp2*-KO mice adapt to Na^+ dietary challenges. Mice were kept in metabolic cages for
621 3 days under normal Na^+ diet and switched to either low Na^+ (LSD, upper panels) or high Na^+
622 (HSD; lower panels) diet at time 0. Na^+ (A) and K^+ (B) excretion rates were calculated at the
623 indicated time points. Data are means ± SD of 5-6 animals per genotype. WT: black bars, KO:
624 gray bars; Normal Na^+: 0.17% Na^+; LSD: Low Na^+ diet: < 0.01% Na^+; HSD: High Na^+ diet:
625 3.2% Na^+.

626 Fig. 5. *Usp2*-KO mice maintain normal plasma Na^+, K^+ and aldosterone. Plasma Na^+ (A), K^+
627 (B) and aldosterone (C) were measured on plasma collected at ZT12 after 7 days of LSD (left
628 columns) or HSD (right columns) dietary challenge. Data are presented as means ± SD of 6
629 animals per genotype. WT: black bars, KO: gray bars.

630 Fig. 6. *Usp2*-KO mice have normal blood pressure. Telemetry measurements were performed
631 on 5 KO and 5 control animals under LSD (A), NSD (B) and HSD (C) dietary conditions.
632 Data are presented as means ± SD of day and night periods. WT: black bars, KO: gray bars;
633 SBP : Systolic blood pressure, DBP: Diastolic blood pressure.

634 Fig. 7. Dietary Na^+ intake does not influence *Usp2-45* expression in the WT mouse CCD.
635 *Usp2-45* (A) and *Sgk1* (B) mRNA expression was assayed by real-time semi-quantitative
636 PCR in microdissected CCD of animals fed Low (LSD), Normal (NSD) or High Sodium
637 (HSD) diets. Data are means ± SD of 6-7 animals per condition. Statistical significance was
638 tested by One-Way ANOVA and post-hoc two-tailed T-test using the Holm-Sidak correction
639 for multiple testing *: $p<0.05$; **: $p<0.01$.

640
641
642

643

			NSD 3 days		LSD 7 days		HSD 7 days	
			WT	KO	WT	KO	WT	KO
	- Metabolism							
Metabolism	Body Weight	% of initial	106.12 ± 2.88	104.35 ± 2.93	117.0 ± 3.2	113.8 ± 2.9	99.9 ± 2.6	96.6 ± 3.4
	Water consuption	ml / gBW / 24h	0.211 ± 0.102	0.181 ± 0.063	0.294 ± 0.175	0.194 ± 0.077	0.544 ± 0.062	0.433 ± 0.036
	Food consumption	g / gBW / 24h	0.198 ± 0.033	0.192 ± 0.023	0.173 ± 0.016	0.158 ± 0.028	0.156 ± 0.009	0.157 ± 0.007
	Urine volume	ml / gBW / 24h	0.081 ± 0.047	0.074 ± 0.016	0.089 ± 0.016	0.065 ± 0.023	0.249 ± 0.054	0.209 ± 0.019
		ml / ml drunk / 24h	0.847 ± 0.345	0.966 ± 0.205	0.881 ± 0.283	0.905 ± 0.398	0.961 ± 0.181	1.011 ± 0.110
	Feces mass	g / gBW / 24h	0.026 ± 0.004	0.025 ± 0.003	0.022 ± 0.003	0.018 ± 0.002	0.023 ± 0.002	0.024 ± 0.004
		g / g eaten / 24h	0.293 ± 0.054	0.293 ± 0.047	0.270 ± 0.024	0.239 ± 0.044	0.320 ± 0.018	0.331 ± 0.039
	- Plasma							
Plasma	plasma Na^+	mM			146.3 ± 3.2	146.8 ± 1.7	146.1 ± 5.7	145.2 ± 6.7
	plasma K^+	mM			5.9 ± 0.6	5.4 ± 0.2	6.1 ± 1.0	5.9 ± 0.4
	plasma Aldosterone	pg / ml			567.9 ± 372	573.7 ± 369.3	101.3 ± 83.1	144.1 ± 89.5
	- Urine							
Urine	Osmolality	mOsm / kg			2203 ± 597	2542 ± 902	1723 ± 247	1760 ± 255
	Creatinine	µmol / gBW / 24h	0.166 ± 0.043	0.183± 0.036	0.164 ± 0.009	0.146 ± 0.025	0.178 ± 0.023	0.181 ± 0.012
	Na^+ excretion rate	µmol / gBW / 24h	11.6 ± 2.6	12.7± 2.3	1.1 ± 0.3	1.1 ± 0.5	124 ± 25.2	109 ± 3.1
	K^+ excretion rate	µmol / gBW / 24h	31.9 ± 7.1	32.6± 5.9	29.6 ± 1.7	22.9 ± 4.8	26.1 ± 3.2	25.5 ± 1.2

Table 1
Pouly et al.

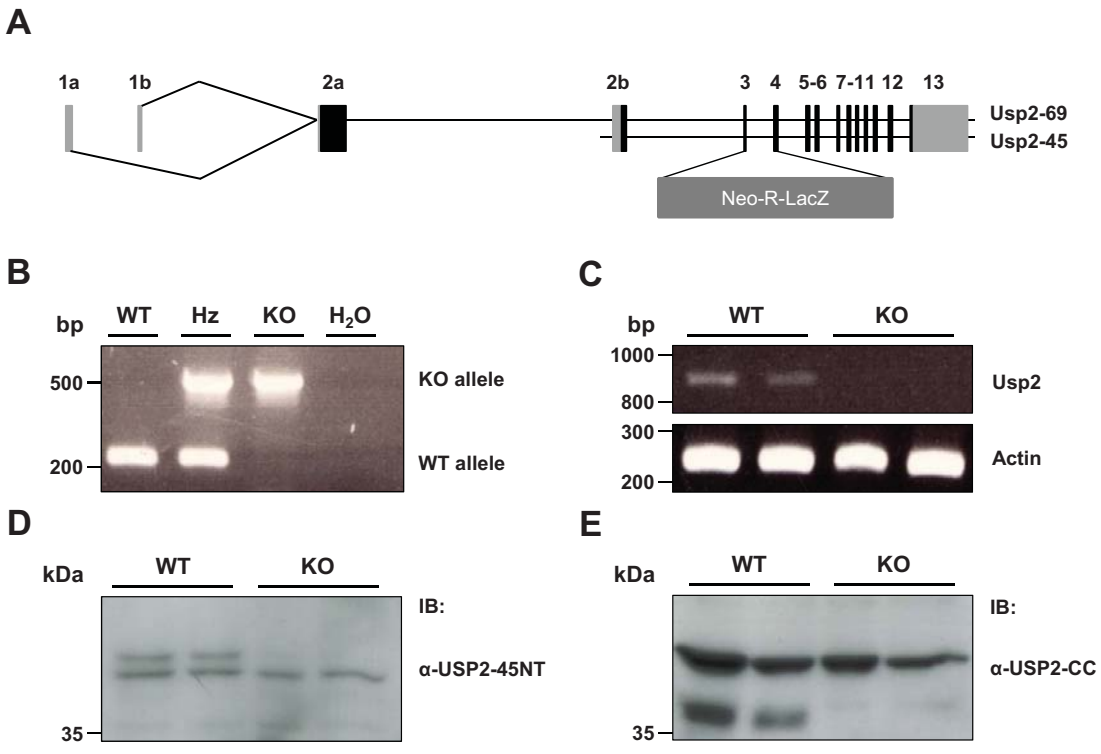

Figure 1
Pouly et al.

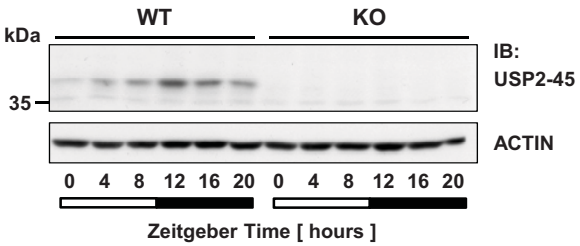

Figure 2
Pouly et al.

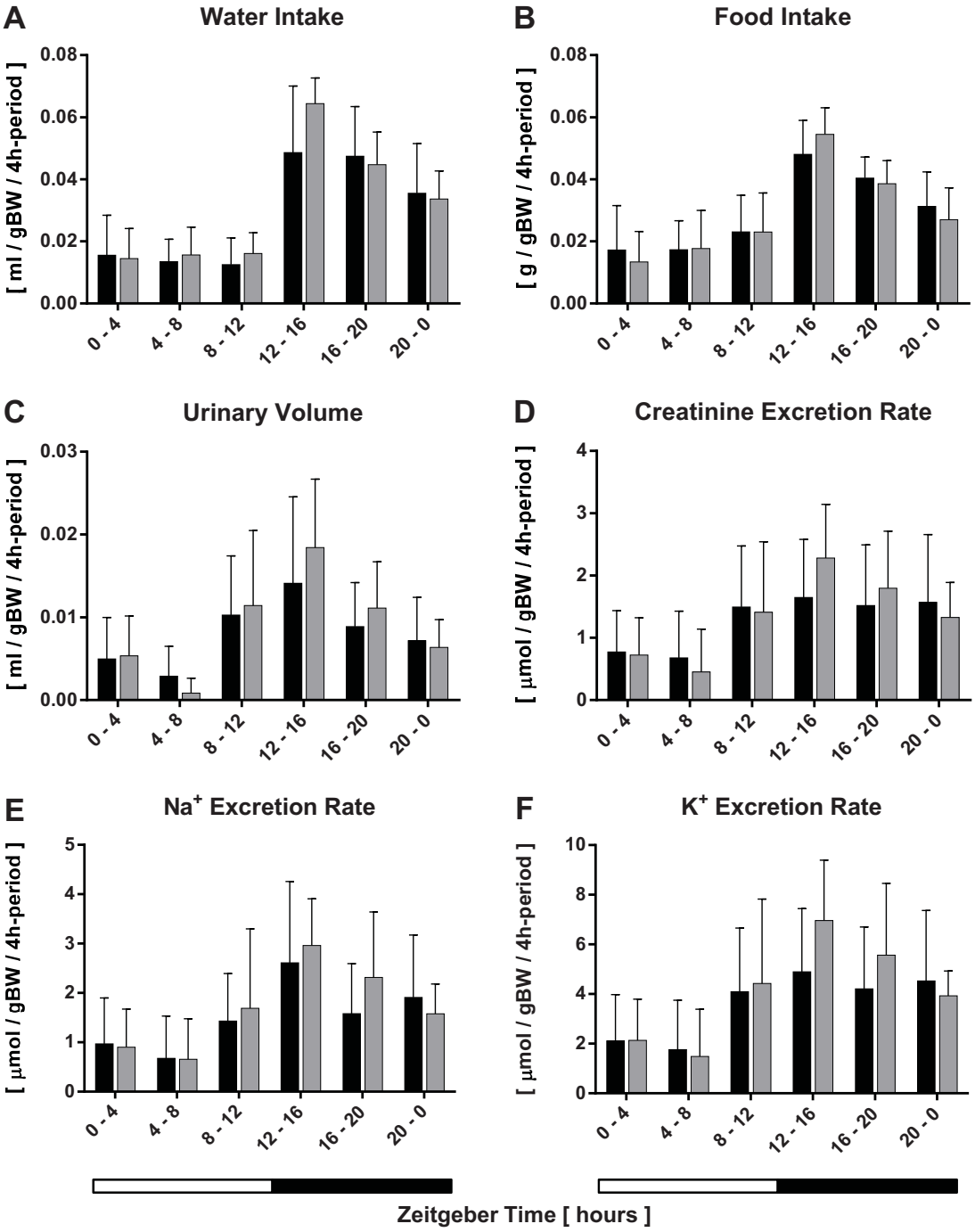

Figure 3
Pouly et al.

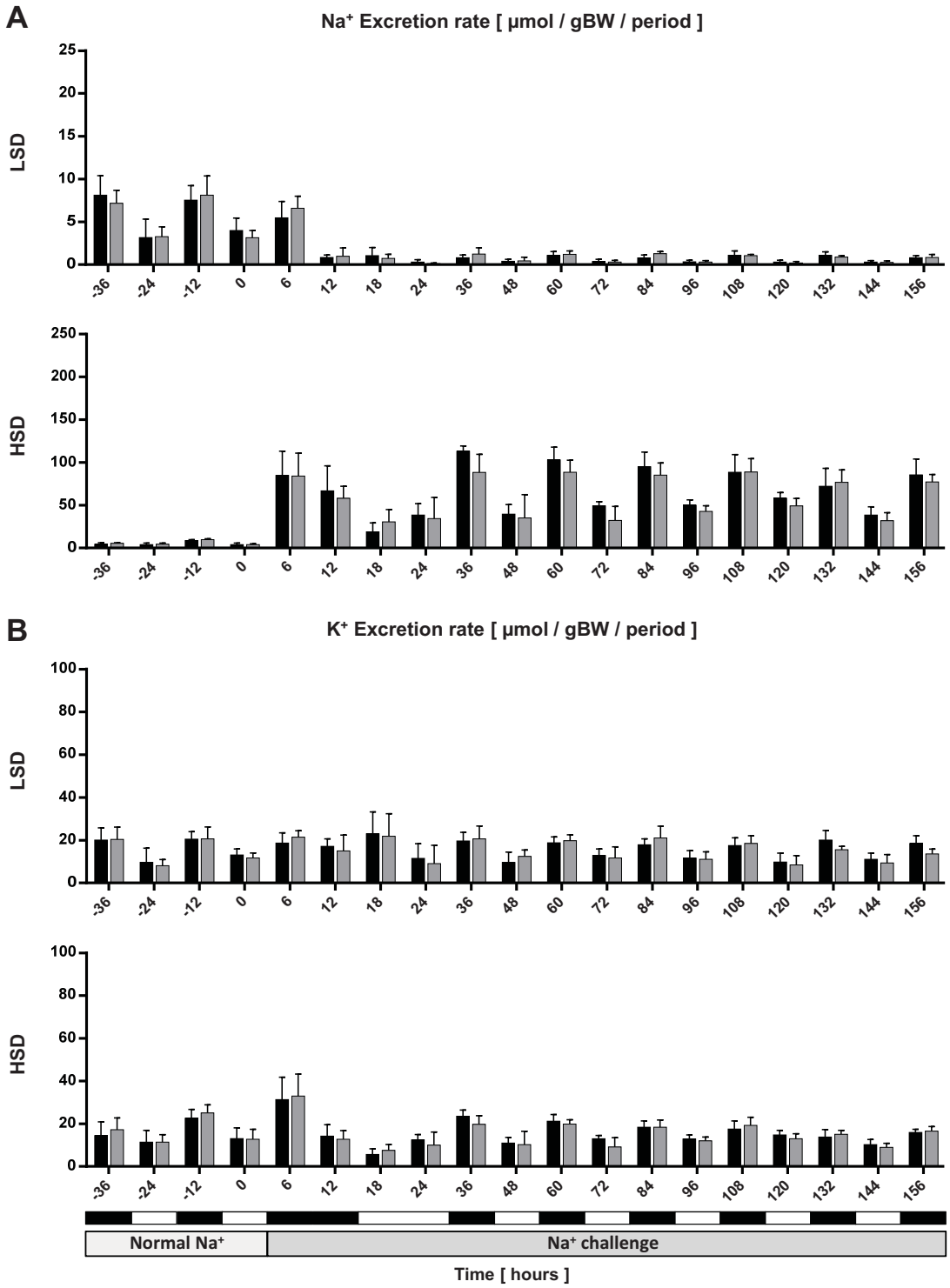

Figure 4
Pouly et al.

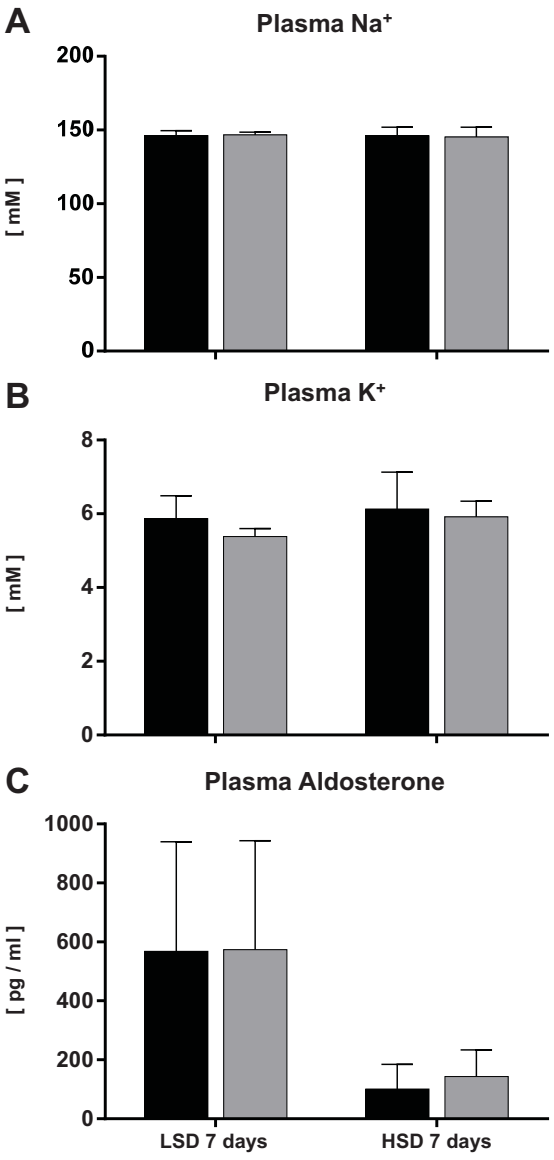

Figure 5
Pouly et al.

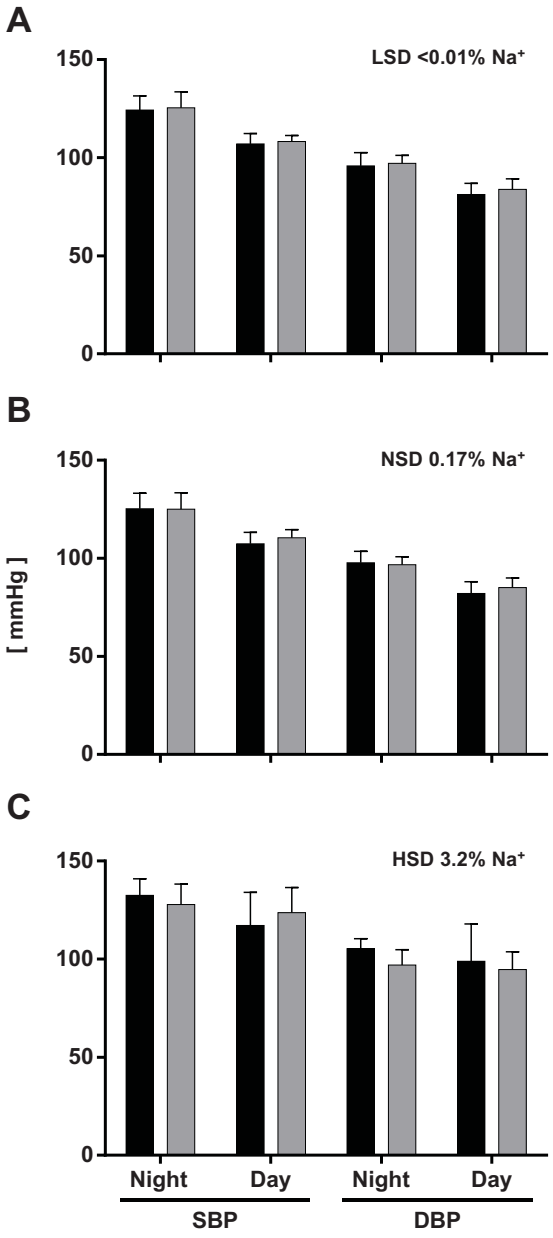

Figure 6
Pouly et al.

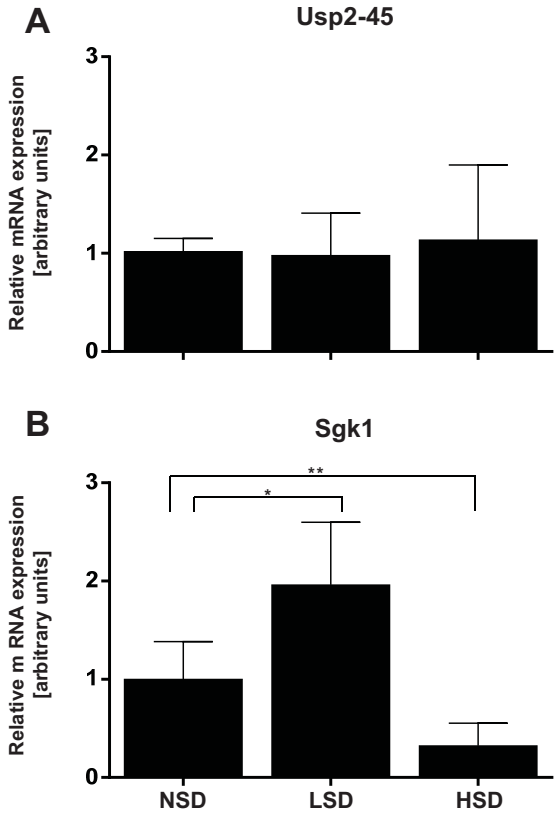

Figure 7
Pouly et al.