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# Transmembrane Serine Protease 2 and Proteolytic Activation of the Epithelial Sodium Channel in Mouse Kidney

--Manuscript Draft--





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# Transmembrane Serine Protease 2 and Proteolytic Activation of the Epithelial Sodium Channel in Mouse Kidney

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***Background***

The renal epithelial sodium channel (ENaC) is essential for sodium balance and blood pressure control. ENaC undergoes complex proteolytic activation by not yet clearly identified tubular proteases. Here, we examined a potential role of transmembrane serine protease 2 (TMPRSS2).

***Methods***

Murine ENaC and TMPRSS2 were (co-)expressed in *Xenopus laevis* oocytes. ENaC cleavage and function were studied in TMPRSS2-deficient murine cortical collecting duct ($mCCD_{cl1}$) cells and TMPRSS2-knockout (*Tmprss2*$^{-/-}$) mice. Short-circuit currents ($I_{SC}$) were measured to assess ENaC-mediated transepithelial sodium transport of $mCCD_{cl1}$ cells. The $mCCD_{cl1}$ cell transcriptome was studied using RNA sequencing. The effect of low-sodium diet with or without high potassium were compared in *Tmprss2*$^{-/-}$ and wildtype mice using metabolic cages. ENaC-mediated whole-cell currents were recorded from microdissected tubules of *Tmprss2*$^{-/-}$ and wildtype mice.

***Results***

In oocytes, co-expression of murine TMPRSS2 and ENaC resulted in fully cleaved γ-ENaC and ~2-fold stimulation of ENaC currents. High baseline expression of TMPRSS2 was detected in $mCCD_{cl1}$ cells without a stimulatory effect of aldosterone on its function or transcription. TMPRSS2 knockout in $mCCD_{cl1}$ cells compromised γ-ENaC cleavage and reduced baseline and aldosterone-stimulated $I_{SC}$ which could be rescued by chymotrypsin. A compensatory transcriptional upregulation of other proteases was not observed. *Tmprss2*$^{-/-}$ mice kept on standard diet exhibited no apparent phenotype, but renal γ-ENaC cleavage was altered. In response to a low-salt diet, particularly with high potassium intake, *Tmprss2*$^{-/-}$ mice increased plasma aldosterone significantly more than wildtype mice to achieve a similar reduction of renal sodium excretion. Importantly, the stimulatory effect of trypsin on renal tubular ENaC currents was much more pronounced in *Tmprss2*$^{-/-}$ mice than that in wildtype mice. This indicated the presence of incompletely cleaved and less active channels at the cell surface of TMPRSS2-deficient tubular epithelial cells.

***Conclusions***

TMPRSS2 contributes to proteolytic ENaC activation in mouse kidney *in vivo*.



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## Introduction

The epithelial sodium channel (ENaC) is a heterotrimeric ion channel that consists of an α, β, and γ subunit and belongs to the ENaC/degenerin family of ion channels[1-3]. ENaC provides the rate-limiting step for transepithelial sodium absorption in several epithelia. Among these is the distal nephron comprising the late distal convoluted tubule (DCT2) the connecting tubule (CNT) and the cortical collecting duct (CCD). Precise ENaC regulation in the distal nephron is essential for adjusting renal sodium excretion to oral intake and, hence, for maintaining sodium homeostasis, extracellular volume, and blood pressure[4,5]. Aldosterone plays a key role in hormonal ENaC stimulation, in particular in the CNT/CCD transition zone, where ENaC activity is strictly aldosterone-dependent. In contrast, ENaC activity is largely aldosterone-independent in the DCT2/CNT region[6-9], albeit dependent on the mineralocorticoid receptor[10,11].

It is well established that ENaC requires specific proteolytic processing to become an active channel[12-15]. Proteolytic cleavage removes autoinhibitory peptide fragments from the extracellular loops of α- and γ-ENaC[16-18]. According to the currently accepted paradigm, the serine protease furin and/or related furin-like proprotein convertases target three cleavage sites (two in α- and one in γ-ENaC) during channel maturation in the intracellular biosynthetic pathway[13,19]. Importantly, a final cleavage event in γ-ENaC is required to achieve full channel activation[20,21]. Relevant proteases involved in this pivotal last cleavage event in γ-ENaC in the kidney remain elusive[22,23]. These may include membrane-anchored proteases expressed by tubular cells or soluble plasma proteases aberrantly filtered in disease states (e.g., nephrotic syndrome)[24-27].

Recently, we have demonstrated that transmembrane serine protease 2 (TMPRSS2), a membrane-anchored serine protease with trypsin-like substrate specificity, proteolytically activates human ENaC in a heterologous expression system and H441 airway epithelial

cells[28]. TMPRSS2 is highly expressed in the renal distal nephron, where it may functionally interact with ENaC[29-34]. In addition, TMPRSS2 could interact with ENaC through the secretion of its catalytic domain or *via* urinary microvesicles[35-37]. Therefore, we hypothesized that TMPRSS2 may contribute to proteolytic ENaC activation in the kidney. To test this hypothesis, we explored the effect of TMPRSS2 deficiency on renal ENaC cleavage and function in murine model systems.

## Methods

More methodological details can be found in the Supplemental Material.

### *Two-electrode voltage-clamp experiments in Xenopus laevis oocytes*

Isolation of oocytes and two-electrode voltage-clamp experiments were performed essentially as described previously[28,38,39].

### *TMPRSS2 knockout in mCCD$_{cl1}$ cells, Ussing chamber measurements, and RNA sequencing (RNA-seq)*

The mCCD$_{cl1}$ cell line[40] was kindly provided by Bernard C. Rossier and Edith Hummler (Université de Lausanne, Switzerland). TMPRSS2-knockout and nontargeting control mCCD$_{cl1}$ cells were generated using CRISPR/Cas9[41,42]. Equivalent short circuit current ($I_{SC}$) measurements were performed on mCCD$_{cl1}$ cells grown on permeable supports[43-45]. mRNA was isolated using the NucleoSpin RNA kit (Machery-Nagel) and RNA-seq was performed at the Next Generation Sequencing Core Unit (Institute of Human Genetics, FAU Erlangen-Nürnberg).

***Mouse studies***

An established TMPRSS2-knockout mouse model (global constitutive TMPRSS2-knockout; background: C57BL/6J)[46] was obtained from Jackson Laboratories (B6.129-*Tmprss2*$^{tm1Psn}$/J, JAX stock #026196). The effects of different diets were studied in metabolic cages. Plasma aldosterone was measured using an ELISA kit (IBL, Hamburg, Germany).

***Immunoblotting***

γ-ENaC was detected using an established antibody (Stressmarq; catalog no.: SPC-405) following deglycosylation (PNGase F, New England BioLabs)[47]. For TMPRSS2 detection a commercially available antibody[37] was used (EMD Millipore Corp.; clone P5H9-A3; catalog no.: MABF2158) and validated (Supplemental Figure 1).

***RNAscope stainings and immunohistochemistry***

*Tmprss2* mRNA was detected in mouse kidney using the RNAscope® Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics ACD, Cat. No 323100) according to the manufacturer's protocol[48,49]. To detect β- or γ-ENaC, a polyclonal rabbit antibody directed against mouse β-ENaC[44] or rat γ-ENaC (see above) was used, respectively.

***Preparation of Renal Tubules and Electrophysiology***

Tubules were prepared and whole-cell patch-clamp recordings were performed essentially as described previously[6,7,10,50]. Two tubular regions were distinguished according to morphological criteria[6]: i) DCT2 and initial CNT (DCT2/CNT), and ii) late CNT and initial CCD (CNT/CCD).

### *Statistical methods*

Data are presented as mean ± SEM. Normal distribution of data was assessed using the D'Agostino-Pearson omnibus or Shapiro-Wilk test. Statistical significance was assessed using appropriate tests as indicated in figure legends.

## Results

### *Co-expression of mouse TMPRSS2 increased mouse ENaC currents in X. laevis oocytes*

To test whether murine TMPRSS2 proteolytically activates murine ENaC, we expressed murine αβγ-ENaC in *X. laevis* oocytes with or without co-expression of murine TMPRSS2. In oocytes co-injected with ENaC cRNA (0.1 ng/subunit) and TMPRSS2 cRNA (0.2 ng), baseline amiloride-sensitive currents were about twice as high as in oocytes expressing ENaC alone (Supplemental Figure 3A-C). Importantly, ENaC currents were not further stimulated by chymotrypsin application, unlike in oocytes expressing ENaC alone (Supplemental Figure 3A-D). Consistent with these functional results, we demonstrated that TMPRSS2 co-expression converted partially cleaved γ-ENaC (~60 kDa) at the cell surface into its fully cleaved active form (~55 kDa) (Supplemental Figure 3E). Thus, we confirmed proteolytic ENaC activation by TMPRSS2 due to γ-ENaC cleavage in murine orthologs.

### *TMPRSS2 contributed to proteolytic ENaC activation in $mCCD_{cl1}$ cells*

To investigate whether TMPRSS2 is involved in proteolytic ENaC activation in $mCCD_{cl1}$ cells, we generated TMPRSS2-knockout cells. Successful TMPRSS2 knockout was confirmed using western blot analysis (Figure 1A, Supplemental Figure 2). TMPRSS2-

knockout $mCCD_{cl1}$ cells formed tight epithelial monolayers but had a significantly lower transepithelial resistance compared to control $mCCD_{cl1}$ cells (Supplemental Figure 4A). However, overall epithelial monolayer integrity was preserved, as evidenced by immunofluorescence staining for the tight junction marker zona occludens protein 1 (Supplemental Figure 4B).

Using a fluorogenic substrate assay, we assessed trypsin-like proteolytic activity in the apical medium collected from $mCCD_{cl1}$ cells (Figure 1B). A high degree of proteolytic activity was detected in the medium collected from control $mCCD_{cl1}$ cells. Importantly, this proteolytic activity was significantly reduced in the medium collected from TMPRSS2-knockout cells. In line with this, using protein precipitation we detected the catalytic domain of TMPRSS2 in the apical medium of control but not of TMPRSS2-deficient cells (Supplemental Figure 5).

Reduced endogenous protease activity may impair ENaC cleavage and activation in TMPRSS2-deficient $mCCD_{cl1}$ cells. To investigate this, we assessed ENaC-mediated transepithelial transport by $I_{SC}$ measurements. Average baseline $I_{SC}$ values were higher in control than in TMPRSS2-deficient cells (Figure 1C, D). Applying chymotrypsin to the apical bath solution did not significantly alter $I_{SC}$ in control cells. This indicated that endogenous proteases were sufficient for full proteolytic ENaC activation at the cell surface. In contrast, in TMPRSS2-knockout $mCCD_{cl1}$ cells, apical application of chymotrypsin substantially stimulated $I_{SC}$ by 2.3 ± 0.2 µA/cm², to a level similar to baseline $I_{SC}$ of control cells (Figure 1C, E). Thus, in TMPRSS2-deficient cells, proteolytic ENaC activation at the cell surface was incomplete. In the presence of amiloride, chymotrypsin failed to stimulate $I_{SC}$ in $mCCD_{cl1}$ cells with TMPRSS2 deficiency (Supplemental Figure 6). Furthermore, we demonstrated that apical application of aprotinin, a broad-spectrum serine protease inhibitor, reduced $I_{SC}$ in control but not in TMPRSS2-deficient $mCCD_{cl1}$ cells. The inhibitory effect of

aprotinin on $I_{SC}$ in control $mCCD_{cl1}$ cells could be rescued by chymotrypsin (Supplemental Figure 7). These results demonstrated a substantial contribution of apical TMPRSS2 activity to proteolytic ENaC activation in $mCCD_{cl1}$ cells, whereas the role of other proteases seemed to be negligible. The finding that proteolytic ENaC activation was incomplete in TMPRSS2-deficient $mCCD_{cl1}$ cells was confirmed by western blot detection of γ-ENaC cleavage fragments in the apical membrane of these cells. Indeed, compared to control cells, the fraction of fully cleaved cell surface γ-ENaC was decreased in TMPRSS2-deficient $mCCD_{cl1}$ cells, whereas the partially cleaved γ-ENaC fraction was increased (Figure 1F, Supplemental Figure 8).

Taken together, these results indicated that endogenously expressed TMPRSS2 contributed to proteolytic γ-ENaC processing and channel activation in $mCCD_{cl1}$ cells.

***TMPRSS2 knockout reduced the stimulatory effect of aldosterone in $mCCD_{cl1}$ cells***

Next, we studied whether TMPRSS2-deficiency altered the stimulatory effect of aldosterone on ENaC in this cell line. In control cells, aldosterone (3 nM) increased $I_{SC}$ over two hours from 4.3 ± 0.9 to 21.0 ± 2.4 μA/cm$^2$. Subsequent addition of chymotrypsin to the apical compartment had no significant additional stimulatory effect on $I_{SC}$ (Figure 2A, C). Thus, like under basal conditions, ENaC was fully proteolytically activated at the cell surface of aldosterone-treated cells. Exposure of TMPRSS2-deficient $mCCD_{cl1}$ cells to aldosterone also resulted in an $I_{SC}$ increase. However, the degree of stimulation (from 3.9 ± 0.8 to 14.5 ± 2.3 μA/cm$^2$) appeared to be reduced compared to control cells (Figure 2B, C). Importantly, in aldosterone-treated TMPRSS2-deficient cells subsequent apical application of chymotrypsin caused a substantial additional rise in $I_{SC}$ of 5.0 ± 0.7 μA/cm² (Figure 2C), essentially rescuing the full stimulatory effect of aldosterone. Indeed, in aldosterone and chymotrypsin treated TMPRSS2-deficient cells, application of amiloride at the end of the experiments

caused an $I_{SC}$ decrease similar to that observed in control $mCCD_{cl1}$ cells having received the same treatment. Thus, in TMPRSS2-deficient cells the total amount of ENaC present at the cell surface after aldosterone treatment was similar to that in control cells (Figure 2C). It is noteworthy that in TMPRSS2-deficient $mCCD_{cl1}$ cells the stimulatory effect of chymotrypsin on $I_{SC}$ after aldosterone treatment was enhanced by ~2.2-fold compared with that observed in the absence of aldosterone (5.0 ± 0.7 µA/cm² in the presence *vs.* 2.3 ± 0.2 µA/cm² in the absence of aldosterone; $p$=0.008, two-sided Student's *t*-test; Figures 1C, 1E, 2B, 2C). This indicated that aldosterone increased ENaC at the cell surface also in TMPRSS2 deficient cells, but that proteolytic activation of newly inserted channels remained incomplete due to TMPRSS2-deficiency.

To summarize, TMPRSS2-deficiency impaired the stimulatory effect of aldosterone by partially preventing proteolytic channel activation.

***Aldosterone did not upregulate TMPRSS2 transcription, and TMPRSS2 deficiency was not associated with a substantial transcriptional upregulation of other serine proteases.***

Using RNA-seq analysis of $mCCD_{cl1}$ cells, we demonstrated that TMPRSS2 had the highest level of mRNA expression among transmembrane serine proteases detected (Table 1, Supplemental Figure 9E, F; Supplemental Figure 12C). To investigate whether aldosterone regulates TMRPSS2 expression, $mCCD_{cl1}$ cells were treated for 2 h or 24 h with 3 nM aldosterone (Supplemental Figure 10) before RNA-isolation. After a 2-hour aldosterone exposure, we observed transcriptional upregulation of known aldosterone target genes[51], but not of *Tmprss2* or any other serine protease (Supplemental Figure 9C; Table 1, Supplemental Table 1). With a 24-hour aldosterone treatment, only a minor 1.09-fold transcriptional upregulation of *Tmprss2* could be detected (Supplemental Figure 9D; Table 1, Supplemental Table 2). In line with the RNA-seq data, TMPRSS2 protein expression at the cell surface

(Figure 2D, Supplemental Figure 11A), trypsin-like proteolytic activity, and the amount of TMPRSS2 in the apical medium were not significantly affected by aldosterone exposure (Figure 2E, Supplemental Figure 11B). To conclude, aldosterone had no substantial effect on expression or activity of TMPRSS2 in $mCCD_{cl1}$ cells.

Finally, RNA-seq analysis of TMPRSS2-deficient cells provided no evidence for marked compensatory upregulation of mRNA expression of other highly expressed transmembrane serine proteases in these cells, except for a modest (~20%) increase of *Prss23* transcription (Table 1, Supplemental Table 3; Supplemental Figure 12).

***In Tmprss2$^{-/-}$ mice, proteolytic processing of renal γ- and α-ENaC was altered***

To study the role of TMPRSS2 in ENaC cleavage *in vivo*, we used constitutive TMPRSS2-knockout mice (*Tmprss2*$^{-/-}$)[46]. The successful knockout of *Tmprss2* in kidneys was confirmed using RNAscope technology (Supplemental Figure 13, 3). Moreover, combining this approach with an immunofluorescence staining for β-ENaC, we demonstrated that *Tmprss2* mRNA was present in renal tubular cells with β-ENaC protein expression (Figure 3). *Tmprss2* mRNA was not restricted to ENaC-positive cells but demonstrated a ubiquitous expression pattern along the renal tubule.

Next, we detected γ- and α-ENaC cleavage fragments in membrane-enriched fractions obtained from kidney cortex of *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice. Similar to our observations in $mCCD_{cl1}$ cells (Figure 1F), knockout of TMPRSS2 in mice resulted in a significant increase of the partially cleaved γ-ENaC fraction (Figures 4, Supplemental Figure 14). In contrast, the signal of uncleaved or fully cleaved γ-ENaC in *Tmprss2*$^{-/-}$ mice was similar to that in wildtype (*Tmprss2*$^{+/+}$) mice. We also observed altered proteolytic processing of α-ENaC in kidneys from *Tmprss2*$^{-/-}$ mice with a significantly increased portion of (furin-)cleaved α-ENaC at the expense of uncleaved α-ENaC (Supplemental Figure 15).

In summary, TMPRSS2 deficiency altered proteolytic processing of renal γ- and α-ENaC in vivo.

***Reduction of renal sodium excretion in response to dietary sodium restriction required higher plasma aldosterone levels in Tmprss2$^{-/-}$ mice than in wildtype controls***

Using immunohistochemistry, we found a similar tubular expression pattern of γ-ENaC in both genotypes (Figure 5A *top panels*). Moreover, in *Tmprss2*$^{-/-}$ mice, the acute natriuretic response to amiloride (Supplemental Figure 16), as well as baseline plasma $Na^+$ and $K^+$ concentrations (Supplemental Figure 17A, B), were not different from those in wildtype controls. Taken together, this indicated that ENaC-mediated sodium transport was not substantially altered in *Tmprss2*$^{-/-}$ mice under baseline conditions.

Intriguingly, both *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice responded in a similar manner to dietary sodium restriction with trafficking of ENaC to the apical membrane (Figure 5A *lower panels*) and an appropriate reduction of urinary sodium excretion (Figure 5B *left panel*) to maintain sodium balance. Consistent with this finding, the acute natriuretic response to amiloride was higher in mice kept on a low sodium diet compared to that on a control diet, but was similar in both genotypes (Supplemental Figure 16). With similar food intake and fecal $Na^+$ excretion, *Tmprss2*$^{-/-}$ and *Tmprss2*$^{+/+}$ mice maintained similar body weight under low salt diet (Supplemental Figures 17D, 18A, B). In addition, in both genotypes, plasma $Na^+$ and $K^+$ concentrations, as well as renal $K^+$ excretion, were unaffected by low sodium diet (Supplemental Figure 17A-C). Water intake and urine volume were slightly higher in *Tmprss2*$^{-/-}$ mice (Supplemental Figure 18C, D). *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice also responded in a similar manner to dietary sodium restriction in combination with an increased potassium intake (Figures 5B *right panel*, Supplemental Figures 19 and 20). Consistent with previously reported evidence[47,52-55], we detected a trend towards an increase in the cleaved γ-

ENaC fragments in kidneys from sodium-restricted wildtype mice (Supplemental Figure 21). Interestingly, under sodium restriction no obvious differences in proteolytic processing and overall expression of γ- and α-ENaC were observed between *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice (Supplemental Figures 21 and 22). Collectively these data demonstrated that the ability of *Tmprss2*$^{-/-}$ mice to adjust renal sodium excretion to low sodium diet was fully preserved.

When animals were maintained on standard diet, there was a non-significant trend for slightly higher plasma aldosterone values in *Tmprss2*$^{-/-}$ mice compared to *Tmprss2*$^{+/+}$ mice (Figure 5C), averaging 187 ± 24 pg/ml and 138 ± 19 pg/ml, respectively. After four days of dietary sodium restriction, there was a trend towards elevated plasma aldosterone levels (210 ± 41 pg/ml) in *Tmprss2*$^{+/+}$ mice (Figure 5C). This was consistent with previously reported data[38] and appeared sufficient to downregulate renal sodium excretions adequately. Importantly, in *Tmprss2*$^{-/-}$ mice plasma aldosterone increased in response to dietary sodium restriction to 482 ± 77 pg/ml, a value 2.3-fold higher than in *Tmprss2*$^{+/+}$ mice (Figure 5C). Plasma aldosterone values reached even higher values in *Tmprss2*$^{-/-}$ mice after four days of dietary sodium restriction when combined with an increased potassium intake (565 ± 99 pg/ml, Figure 5C). This clearly indicated that in response to dietary sodium restriction, in particular in combination with increased potassium intake, *Tmprss2*$^{-/-}$ mice needed to upregulate aldosterone to much higher levels probably to compensate for incomplete proteolytic ENaC activation due to TMPRSS2 deficiency.

***The stimulatory effect of trypsin on ENaC activity in microdissected tubules was more pronounced in Tmprss2$^{-/-}$ than in wildtype mice***

To explore the relevance of TMPRSS2 for ENaC function in native renal tubules, we performed whole-cell patch-clamp recordings in DCT2/CNT and CNT/CCD from *Tmprss2*$^{-/-}$ and wildtype control mice (Figure 6, Supplemental Figure 23). Repeated amiloride

applications were used to monitor ENaC-mediated currents ($\Delta I_{ami}$). To reveal the presence of incompletely cleaved channels at the cell surface, the current response to apical trypsin application was investigated using a previously established protocol[56].

In both genotypes average initial $\Delta I_{ami}$ values in CNT/CCD were significantly lower than corresponding values in DCT2/CNT (Figure 6A, C). In DCT2/CNT we observed a trend towards reduced baseline $\Delta I_{ami}$ in *Tmprss2*$^{-/-}$ mice (372 ± 81 pA) compared to control mice (449 ± 75 pA; Figure 6C). Apical application of trypsin to DCT2/CNT from *Tmprss2*$^{-/-}$ mice increased $\Delta I_{ami}$ on average by ~75% within about three minutes (Figure 6B, D). In contrast, no trypsin response was observed in DCT2/CNT from wildtype animals (Figures 6A, B, D; Supplemental Figure 23B). In CNT/CCD, baseline ENaC currents were significantly lower in *Tmprss2*$^{-/-}$ mice (45 ± 13 pA) than those in wildtype mice (78 ± 15 pA; Figure 6C). The latter currents were slightly stimulated by application of trypsin (~40%). Importantly, in CNT/CCD from *Tmprss2*$^{-/-}$ mice, the stimulatory effect of trypsin was much larger with an average increase of ENaC currents by ~280% within three minutes (Figure 6B, D).

## Discussion

Key findings of the present study were the following: i) Co-expression of murine TMPRSS2 proteolytically activated murine ENaC by cleaving its γ-subunit consistent with our previous findings with the human orthologues[28]; ii) TMPRSS2 deficiency reduced baseline ENaC activity in $mCCD_{cl1}$ cells and the stimulatory effect of aldosterone due to incomplete γ-ENaC cleavage; iii) in *Tmprss2*$^{-/-}$ mice renal ENaC cleavage was compromised and animals required a much larger increase in plasma aldosterone to reduce renal sodium excretion adequately in response to dietary sodium restriction, in particular when combined with increased potassium intake; iv) renal ENaC whole-cell currents could be stimulated by trypsin to a larger extent in *Tmprss2*$^{-/-}$ mice than in wildtype mice, particularly in the CNT/CCD. This indicated that TMPRSS2 deficiency reduced average open probability of ENaC probably due to impaired proteolytic channel activation. Collectively, our results support the conclusion that TMPRSS2 is a functionally important protease co-expressed with ENaC in distal tubular epithelial cells.

The additional stimulatory effect of chymotrypsin on baseline $I_{SC}$ provided functional evidence for incomplete proteolytic ENaC activation in TMPRSS2-deficient $mCCD_{cl1}$ cells. The finding that the fraction of fully cleaved γ-ENaC at the cell surface of TMPRSS2-deficient cells was lower than in control cells further confirmed the concept that TMPRSS2 is essential for complete γ-ENaC cleavage. Importantly, the stimulatory effect of aldosterone on $I_{SC}$ was reduced in TMPRSS2-deficient $mCCD_{cl1}$ cells. ENaC regulation by aldosterone is highly complex[3,9] and involves the stimulation of channel trafficking to the apical membrane and enhanced proteolytic ENaC cleavage[47,52-55]. Our findings indicated that TMPRSS2 deficiency did not impede the stimulatory effect of aldosterone on channel insertion into the apical membrane, but prevented full proteolytic activation of the newly inserted channels. This was evidenced by the finding that in TMPRSS2-knockout $mCCD_{cl1}$ cells the stimulatory

effect of chymotrypsin was enhanced after a two-hour exposure to aldosterone. RNA-seq analysis did not reveal a prominent regulatory effect of aldosterone on the transcriptional expression of TMPRSS2 or any other serine protease detected, in line with previous research[51,57,58]. Moreover, aldosterone treatment did not enhance TMPRSS2 expression or proteolytic activity detected in the apical medium from $mCCD_{cl1}$ cells. To conclude, increased TMPRSS2 expression or function was not required to achieve full proteolytic ENaC activation following aldosterone stimulation. Thus, in $mCCD_{cl1}$ cells, proteolytic activity of constitutively expressed TMPRSS2 appears to be sufficient to process all channels trafficking to the cell surface under baseline and aldosterone-stimulated conditions. This supports the concept that ENaC cleavage depends on the regulation of channel trafficking[53,54]. However, this does not rule out the possibility that long-term stimulation of ENaC activity *in vivo* also involves upregulation of protease activity.

Importantly, we demonstrated in microdissected tubules that ENaC could be stimulated by trypsin to a larger extent in *Tmprss2*$^{-/-}$ mice than in wildtype mice. Indeed, the ~280% stimulation of ENaC currents by trypsin in CNT/CCD of *Tmprss2*$^{-/-}$ mice was much more pronounced than in wildtype mice, where trypsin had only a modest (~40%) stimulatory effect on ENaC consistent with previously reported findings[55,56]. Interestingly, in DCT2/CNT of wildtype mice trypsin had no apparent effect but significantly increased ENaC currents in DCT2/CNT of *Tmprss2*$^{-/-}$ mice by ~75%. The different responsiveness of ENaC currents to trypsin in DCT2/CNT *versus* CNT/CCD in the presence and absence of TMPRSS2 suggests a site-specific role of TMPRSS2 in proteolytic ENaC processing. This implies that in addition to TMPRSS2, other proteases contribute to proteolytic ENaC activation, possibly in a site-specific manner. Site-specific differences in renal ENaC regulation are increasingly being recognised. In this context, it is noteworthy that in *Tmprss2*$^{-/-}$ mice, like in wildtype mice, baseline ENaC currents in DCT2/CNT were significantly larger than those in CNT/CCD.

This is in good agreement with the emerging concept that, unlike in CNT/CCD, baseline ENaC currents in DCT2/CNT are aldosterone-independent, albeit not independent of the mineralocorticoid receptor[6-11,59]. In conclusion, our patch-clamp studies in microdissected tubules indicated that in TMPRSS2 deficient mice, proteolytic ENaC activation at the apical surface of tubular cells was incomplete mainly in CNT/CCD but also in DCT2/CNT.

In kidneytissue from *Tmprss2*$^{-/-}$ mice maintained on standard diet, the fraction of partially cleaved γ-ENaC was increased compared to wildtype controls. It is tempting to speculate that a pool of incompletely cleaved γ-ENaC builds up to compensate for the insufficient generation of fully cleaved γ-ENaC due to TMPRSS2 deficiency. Indeed, TMPRSS2 deficient mice had no overt phenotype and adequately reduced their renal sodium excretion when challenged with a low sodium diet. Moreover, under baseline conditions as well as under sodium restriction, their natriuretic response to acute administration of amiloride was similar to that of wildtype mice. Thus, overall ENaC activity in TMPRSS2 deficient mice was similar to that in wildtype mice. This indicated that animals were able to compensate for impaired γ-ENaC cleavage due to TMPRSS2 deficiency.

Compensation for TMPRSS2 deficiency was probably achieved by an increased stimulation of the renin-angiotensin-aldosterone axis. This was evidenced by the significantly higher aldosterone levels reached in TMPRSS2 deficient mice compared to control mice when animals were challenged with a sodium-deficient diet, in particular in combination with a high potassium intake. The latter combination is a particularly strong stimulus for aldosterone secretion and ENaC activation. In this context, it should be noted that a local increase in basolateral potassium concentration may also directly stimulate renal ENaC activity[60]. Moreover, angiotensin II can stimulate ENaC by aldosterone-independent mechanisms in particular in DCT2/CNT[59], and additional factors independent of γ-ENaC cleavage may modulate ENaC open probability[61,62].

Interestingly, western blot analysis of kidney tissue from sodium-restricted mice did not reveal differences in overall ENaC expression and γ-ENaC cleavage pattern between wildtype versus TMPRSS2-deficient mice. However, with our experimental approach, we cannot distinguish between ENaC in the cytosol and at the apical membrane. Moreover, unlike our patch clamp experiments in microdissected tubules, our western blot analysis could not distinguish between ENaC expression in DCT2/CNT versus CNT/CCD. Thus, we may have missed subtle differences between wildtype and *Tmprss2*$^{-/-}$ mice regarding the expression level and cleavage state of γ-ENaC, in particular at the apical cell surface in CNT/CCD. Moreover, additional endogenous proteases may contribute to ENaC activation in sodium-restricted mice and may at least in part compensate for the loss of TMPRSS2 activity, in particular in the DCT2/CNT.

During preparation of this manuscript, a study was published on TMPRSS2-knockout $mCCD_{cl1}$ cell lines generated by clonal selection[34]. TMPRSS2 deficiency in these cell clones was associated with a strong downregulation of α-ENaC mRNA expression that essentially abrogated ENaC currents[34]. In contrast, in our study we generated polyclonal TMPRSS2-knockout cells to avoid any clonal off-target effects. In our TMPRSS2-knockout $mCCD_{cl1}$ cells, we could reliably detect amiloride-sensitive $I_{SC}$ and observed no transcriptional downregulation of α-ENaC. Thus, the different methods of cell line generation might explain the different results obtained in the two studies. Importantly, our western blot analysis (Figure 4, Supplemental Figures 14, 15, 21, 22) and immunofluorescence experiments in mouse kidney (Figure 5A), as well as the observation that the natriuretic response to amiloride was similar in *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice (Supplemental Figure 16), argue against a substantial downregulation of ENaC expression by *Tmprss2*-knockout *in vivo*.

Increased ENaC activity is likely to contribute to the pathophysiology of essential hypertension, in particular in a subset of patients with salt-sensitive hypertension[63].

TMPRSS2 may emerge as novel pharmacological target to reduce ENaC activity in the context of hypertension. Indeed, previous studies have demonstrated a blood pressure-lowering effect of the protease inhibitor camostat mesylate in Dahl salt-sensitive rats[64,65]. This was attributed to an attenuation of proteolytic ENaC activation.

In summary, our data show that TMPRSS2 contributes to proteolytic ENaC activation in the kidney, particularly in CNT/CCD where ENaC activity is aldosterone-dependent. In the future, the development of specific TMPRSS2 inhibitors[66,67] may open new therapeutic perspectives to limit proteolytic ENaC activation in the kidney in disease states with inappropriately high ENaC activity.

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## Supplemental Material Table of Contents

Supplemental Figure 18: Like *Tmprss2*+/+ control mice, *Tmprss2*−/− mice maintained body weight under low sodium diet with similar food intake but higher water intake and urinary output

Supplemental Figure 19: In response to low sodium diet in combination with increased potassium intake, *Tmprss2*−/− mice maintained sodium and potassium balance

Supplemental Figure 20: Under low sodium diet in combination with increased potassium intake body weight, food intake, water intake and urine output were similar in *Tmprss2*+/+ and *Tmprss2*−/− mice

Supplemental Figure 21: Analysis of proteolytic processing of renal γ-ENaC under low salt diet

Supplemental Figure 22: Analysis of proteolytic processing of renal α-ENaC under low salt diet

Supplemental Figure 23: Representative time-matched control recordings from microdissected tubules and summary of $\Delta I_{ami}$ time-courses in absolute values from individual experiments

Supplemental Table 1. Significantly upregulated gene transcripts in $mCCD_{cl1}$ cells treated for 2 h with 3 nM aldosterone

Supplemental Table 2. Significantly up- and downregulated transcripts in $mCCD_{cl1}$ cells treated for 24 h with 3 nM aldosterone

Supplemental Table 3. Differentially expressed transcripts in TMRPSS2-ko *vs.* control $mCCD_{cl1}$ cells

## References


1. Canessa CM, Schild L, Buell G, et al. Amiloride-sensitive epithelial $Na^+$ channel is made of three homologous subunits. *Nature*. 1994; 367(6462):463-467. doi:10.1038/367463a0

2. Noreng S, Bharadwaj A, Posert R, Yoshioka C, Baconguis I. Structure of the human epithelial sodium channel by cryo-electron microscopy. *Elife*. 2018; 7. doi:10.7554/eLife.39340

3. Rotin D, Staub O. Function and regulation of the epithelial $Na^+$ channel ENaC. *Compr Physiol*. 2021; 11(3):2017-2045. doi:10.1002/cphy.c200012

4. Shimkets RA, Warnock DG, Bositis CM, et al. Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell*. 1994; 79(3):407-414. doi:10.1016/0092-8674(94)90250-x

5. Rossier BC. Epithelial sodium channel (ENaC) and the control of blood pressure. *Curr Opin Pharmacol*. 2014; 15:33-46. doi:10.1016/j.coph.2013.11.010

6. Nesterov V, Dahlmann A, Krueger B, Bertog M, Loffing J, Korbmacher C. Aldosterone-dependent and -independent regulation of the epithelial sodium channel (ENaC) in mouse distal nephron. *Am J Physiol Renal Physiol*. 2012; 303(9):F1289-1299. doi:10.1152/ajprenal.00247.2012

7. Nesterov V, Krueger B, Bertog M, Dahlmann A, Palmisano R, Korbmacher C. In Liddle syndrome, epithelial sodium channel is hyperactive mainly in the early part of the aldosterone-sensitive distal nephron. *Hypertension*. 2016; 67(6):1256-1262. doi:10.1161/HYPERTENSIONAHA.115.07061

8. Yang L, Frindt G, Xu Y, Uchida S, Palmer LG. Aldosterone-dependent and -independent regulation of $Na^+$ and $K^+$ excretion and ENaC in mouse kidneys. *Am J Physiol Renal Physiol*. 2020; 319(2):F323-F334. doi:10.1152/ajprenal.00204.2020

9. Pearce D, Manis AD, Nesterov V, Korbmacher C. Regulation of distal tubule sodium transport: mechanisms and roles in homeostasis and pathophysiology. *Pflügers Arch*. 2022; 474(8):869-884. doi:10.1007/s00424-022-02732-5

10. Nesterov V, Bertog M, Canonica J, et al. Critical role of the mineralocorticoid receptor in aldosterone-dependent and aldosterone-independent regulation of ENaC in the distal nephron. *Am J Physiol Renal Physiol*. 2021; 321(3):F257-F268. doi:10.1152/ajprenal.00139.2021

11. Maeoka Y, Su XT, Wang WH, et al. Mineralocorticoid receptor antagonists cause natriuresis in the absence of aldosterone. *Hypertension*. 2022; 79(7):1423-1434. doi:10.1161/HYPERTENSIONAHA.122.19159

12. Vallet V, Chraibi A, Gaeggeler HP, Horisberger JD, Rossier BC. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature*. 1997; 389(6651):607-610. doi:10.1038/39329

13. Hughey RP, Mueller GM, Bruns JB, et al. Maturation of the epithelial $Na^+$ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem*. 2003; 278(39):37073-37082. doi:10.1074/jbc.M307003200

14. Rossier BC, Stutts MJ. Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol*. 2009; 71:361-379. doi:10.1146/annurev.physiol.010908.163108

15. Kleyman TR, Eaton DC. Regulating ENaC's gate. *Am J Physiol Cell Physiol*. 2020; 318(1):C150-C162. doi:10.1152/ajpcell.00418.2019

16. Carattino MD, Passero CJ, Steren CA, et al. Defining an inhibitory domain in the alpha-subunit of the epithelial sodium channel. *Am J Physiol Renal Physiol*. 2008; 294(1):F47-52. doi:10.1152/ajprenal.00399.2007

17. Bruns JB, Carattino MD, Sheng S, et al. Epithelial $Na^+$ channels are fully activated by furin- and prostasin-dependent release of an inhibitory peptide from the gamma-subunit. *J Biol Chem*. 2007; 282(9):6153-6160. doi:10.1074/jbc.M610636200

18. Passero CJ, Carattino MD, Kashlan OB, Myerburg MM, Hughey RP, Kleyman TR. Defining an inhibitory domain in the gamma subunit of the epithelial sodium channel. *Am J Physiol Renal Physiol*. 2010; 299(4):F854-861. doi:10.1152/ajprenal.00316.2010

19. Hughey RP, Bruns JB, Kinlough CL, et al. Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem*. 2004; 279(18):18111-18114. doi:10.1074/jbc.C400080200

20. Diakov A, Bera K, Mokrushina M, Krueger B, Korbmacher C. Cleavage in the gamma-subunit of the epithelial sodium channel (ENaC) plays an important role in the proteolytic activation of near-silent channels. *J Physiol*. 2008; 586(19):4587-4608. doi:10.1113/jphysiol.2008.154435

21. Haerteis S, Krappitz M, Diakov A, Krappitz A, Rauh R, Korbmacher C. Plasmin and chymotrypsin have distinct preferences for channel activating cleavage sites in the gamma subunit of the human epithelial sodium channel. *J Gen Physiol*. 2012; 140(4):375-389. doi:10.1085/jgp.201110763

22. Anand D, Hummler E, Rickman OJ. ENaC activation by proteases. *Acta Physiol (Oxf).* 2022; 235(1):e13811. doi:10.1111/apha.13811

23. Althaus M, Lawong RY. Proteolytic ENaC activation in health and disease-a complicated puzzle. *Pflügers Arch*. 2022; 474(2):177-179. doi:10.1007/s00424-021-02644-w

24. Svenningsen P, Bistrup C, Friis UG, et al. Plasmin in nephrotic urine activates the epithelial sodium channel. *J Am Soc Nephrol*. 2009; 20(2):299-310. doi:10.1681/ASN.2008040364

25. Bohnert BN, Menacher M, Janessa A, et al. Aprotinin prevents proteolytic epithelial sodium channel (ENaC) activation and volume retention in nephrotic syndrome. *Kidney Int*. 2018; 93(1):159-172. doi:10.1016/j.kint.2017.07.023

26. Worn M, Bohnert BN, Alenazi F, et al. Proteasuria in nephrotic syndrome-quantification and proteomic profiling. *J Proteomics*. 2021; 230:103981. doi:10.1016/j.jprot.2020.103981

27. Picard N, Eladari D, El Moghrabi S, et al. Defective ENaC processing and function in tissue kallikrein-deficient mice. *J Biol Chem*. 2008; 283(8):4602-4611. doi:10.1074/jbc.M705664200

28. Sure F, Bertog M, Afonso S, et al. Transmembrane serine protease 2 (TMPRSS2) proteolytically activates the epithelial sodium channel (ENaC) by cleaving the channel's gamma-subunit. *J Biol Chem*. 2022; 298(6):102004. doi:10.1016/j.jbc.2022.102004

29. Jacquinet E, Rao NV, Rao GV, Hoidal JR. Cloning, genomic organization, chromosomal assignment and expression of a novel mosaic serine proteinase: epitheliasin. *FEBS Lett*. 2000; 468(1):93-100. doi:10.1016/s0014-5793(00)01196-0

30. Jacquinet E, Rao NV, Rao GV, Zhengming W, Albertine KH, Hoidal JR. Cloning and characterization of the cDNA and gene for human epitheliasin. *Eur J Biochem*. 2001; 268(9):2687-2699. doi:10.1046/j.1432-1327.2001.02165.x

31. Vaarala MH, Porvari KS, Kellokumpu S, Kyllonen AP, Vihko PT. Expression of transmembrane serine protease TMPRSS2 in mouse and human tissues. *J Pathol*. 2001; 193(1):134-140. doi:10.1002/1096-9896(2000)9999:9999<::AID-PATH743>3.0.CO;2-T

32. Lee JW, Chou CL, Knepper MA. Deep sequencing in microdissected renal tubules identifies nephron segment-specific transcriptomes. *J Am Soc Nephrol*. 2015; 26(11):2669-2677. doi:10.1681/ASN.2014111067

33. Chen L, Lee JW, Chou CL, et al. Transcriptomes of major renal collecting duct cell types in mouse identified by single-cell RNA-seq. *Proc Natl Acad Sci U S A*. 2017; 114(46):E9989-E9998. doi:10.1073/pnas.1710964114

34. Rickman OJ, Guignard E, Chabanon T, Bertoldi G, Auberson M, Hummler E. Tmprss2 maintains epithelial barrier integrity and transepithelial sodium transport. *Life Sci Alliance*. 2024; 7(3). doi:10.26508/lsa.202302304

35. Gonzales PA, Pisitkun T, Hoffert JD, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol*. 2009; 20(2):363-379. doi:10.1681/ASN.2008040406

36. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 2004; 101(36):13368-13373. doi:10.1073/pnas.0403453101

37. Lucas JM, True L, Hawley S, et al. The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *J Pathol*. 2008; 215(2):118-25. doi: 10.1002/path.2330. PMID: 18338334.

38. Essigke D, Ilyaskin AV, Worn M, et al. Zymogen-locked mutant prostasin (Prss8) leads to incomplete proteolytic activation of the epithelial sodium channel (ENaC) and severely compromises triamterene tolerance in mice. *Acta Physiol (Oxf)*. 2021; 232(1):e13640. doi:10.1111/apha.13640

39. Ilyaskin AV, Korbmacher C, Diakov A. Inhibition of the epithelial sodium channel (ENaC) by connexin 30 involves stimulation of clathrin-mediated endocytosis. *J Biol Chem*. 2021; 296:100404. doi:10.1016/j.jbc.2021.100404

40. Gaeggeler HP, Gonzalez-Rodriguez E, Jaeger NF, et al. Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. *J Am Soc Nephrol*. 2005; 16(4):878-891. doi:10.1681/ASN.2004121110

41. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014; 11(8):783-784. doi:10.1038/nmeth.3047

42. Shalem O, Sanjana NE, Hartenian E, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014; 343(6166):84-87. doi:10.1126/science.1247005

43. Bertog M, Letz B, Kong W, et al. Basolateral proteinase-activated receptor (PAR-2) induces chloride secretion in M-1 mouse renal cortical collecting duct cells. *J Physiol*. 1999; 521 Pt 1(Pt 1):3-17. doi:10.1111/j.1469-7793.1999.00003.x

44. Mansley MK, Neuhuber W, Korbmacher C, Bertog M. Norepinephrine stimulates the epithelial $Na^+$ channel in cortical collecting duct cells via alpha2-adrenoceptors. *Am J Physiol Renal Physiol*. 2015; 308(5):F450-458. doi:10.1152/ajprenal.00548.2014

45. Mansley MK, Niklas C, Nacken R, et al. Prostaglandin $E_2$ stimulates the epithelial sodium channel (ENaC) in cultured mouse cortical collecting duct cells in an autocrine manner. *J Gen Physiol*. 2020; 152(8):e201912525. doi:10.1085/jgp.201912525

46. Kim TS, Heinlein C, Hackman RC, Nelson PS. Phenotypic analysis of mice lacking the Tmprss2-encoded protease. *Mol Cell Biol*. 2006; 26(3):965-975. doi:10.1128/MCB.26.3.965-975.2006

47. Bohnert BN, Essigke D, Janessa A, et al. Experimental nephrotic syndrome leads to proteolytic activation of the epithelial $Na^+$ channel in the mouse kidney. *Am J Physiol Renal Physiol*. 2021; 321(4):F480-F493. doi:10.1152/ajprenal.00199.2021

48. Wang F, Flanagan J, Su N, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn*. 2012; 14(1):22-29. doi:10.1016/j.jmoldx.2011.08.002

49. Broeker KAE, Fuchs MAA, Schrankl J, et al. Prolyl-4-hydroxylases 2 and 3 control erythropoietin production in renin-expressing cells of mouse kidneys. *J Physiol*. 2022; 600(3):671-694. doi:10.1113/JP282615

50. Nesterov V, Bertog M, Korbmacher C. High baseline ROMK activity in the mouse late distal convoluted and early connecting tubule probably contributes to aldosterone-independent $K^+$ secretion. *Am J Physiol Renal Physiol*. 2022; 322(1):F42-F54. doi:10.1152/ajprenal.00252.2021

51. Loughlin S, Costello HM, Roe AJ, et al. Mapping the transcriptome underpinning acute corticosteroid action within the cortical collecting duct. *Kidney360*. 2023; 4(2):226-240. doi:10.34067/KID.0003582022

52. Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest*. 1999; 104(7):R19-23. doi:10.1172/JCI7840

53. Ergonul Z, Frindt G, Palmer LG. Regulation of maturation and processing of ENaC subunits in the rat kidney. *Am J Physiol Renal Physiol*. 2006; 291(3):F683-693. doi:10.1152/ajprenal.00422.2005

54. Frindt G, Gravotta D, Palmer LG. Regulation of ENaC trafficking in rat kidney. *J Gen Physiol*. 2016; 147(3):217-227. doi:10.1085/jgp.201511533

55. Frindt G, Ergonul Z, Palmer LG. Surface expression of epithelial $Na^+$ channel protein in rat kidney. *J Gen Physiol*. 2008; 131(6):617-627. doi:10.1085/jgp.200809989

56. Nesterov V, Dahlmann A, Bertog M, Korbmacher C. Trypsin can activate the epithelial sodium channel (ENaC) in microdissected mouse distal nephron. *Am J Physiol Renal Physiol*. 2008; 295(4):F1052-1062. doi:10.1152/ajprenal.00031.2008

57. Swanson EA, Nelson JW, Jeng S, et al. Salt-sensitive transcriptome of isolated kidney distal tubule cells. *Physiol Genomics*. 2019; 51(4):125-135. doi:10.1152/physiolgenomics.00119.2018

58. Poulsen SB, Limbutara K, Fenton RA, Pisitkun T, Christensen BM. RNA sequencing of kidney distal tubule cells reveals multiple mediators of chronic aldosterone action. *Physiol Genomics*. 2018; 50(5):343-354. doi:10.1152/physiolgenomics.00084.2017

59. Wu P, Gao ZX, Zhang DD, et al. Effect of angiotensin II on ENaC in the distal convoluted tubule and in the cortical collecting duct of mineralocorticoid receptor deficient mice. *J Am Heart Assoc*. 2020; 9(7):e014996. doi:10.1161/JAHA.119.014996

60. Saha B, Shabbir W, Takagi E, et al. Potassium Activates mTORC2-dependent SGK1 phosphorylation to stimulate epithelial sodium channel: role in rapid renal responses to dietary potassium. *J Am Soc Nephrol*. 2023; 34(6):1019-1038. doi:10.1681/ASN.0000000000000109

61. Kashlan OB, Wang XP, Sheng S, Kleyman TR. Epithelial $Na^+$ channels function as extracellular sensors. *Compr Physiol*. 2024; 14(2):1-41. doi:10.1002/cphy.c230015

62. Ray EC, Nickerson A, Sheng S, et al. Influence of proteolytic cleavage of ENaC's γ subunit upon $Na^+$ and $K^+$ handling. *Am J Physiol Renal Physiol*. 2024; 326(6):F1066-F1077. doi: 10.1152/ajprenal.00027.2024.

63. Mutchler SM, Kirabo A, Kleyman TR. Epithelial sodium channel and salt-sensitive hypertension. *Hypertension*. 2021; 77(3):759-767. doi:10.1161/HYPERTENSIONAHA.120.14481

64. Maekawa A, Kakizoe Y, Miyoshi T, et al. Camostat mesilate inhibits prostasin activity and reduces blood pressure and renal injury in salt-sensitive hypertension. *J Hypertens*. 2009; 27(1):181-189. doi:10.1097/hjh.0b013e328317a762

65. Kitamura K, Tomita K. Proteolytic activation of the epithelial sodium channel and therapeutic application of a serine protease inhibitor for the treatment of salt-sensitive hypertension. *Clin Exp Nephrol*. 2012; 16(1):44-48. doi:10.1007/s10157-011-0506-1

66. Mahoney M, Damalanka VC, Tartell MA, et al. A novel class of TMPRSS2 inhibitors potently block SARS-CoV-2 and MERS-CoV viral entry and protect human epithelial lung cells. *Proc Natl Acad Sci U S A*. 2021; 118(43). doi:10.1073/pnas.2108728118

67. Shapira T, Monreal IA, Dion SP, et al. A TMPRSS2 inhibitor acts as a pan-SARS-CoV-2 prophylactic and therapeutic. *Nature*. 2022; 605(7909):340-348. doi:10.1038/s41586-022-04661-w

68. Lucas JM, Heinlein C, Kim T, et al. The androgen-regulated protease TMPRSS2 activates a proteolytic cascade involving components of the tumor microenvironment and promotes prostate cancer metastasis. *Cancer Discov*. 2014; 4(11):1310-25. doi: 10.1158/2159-8290.CD-13-1010.

69. Thomas W, Harvey BJ. Estrogen-induced signalling and the renal contribution to salt and water homeostasis. *Steroids*. 2023; 199:109299. doi:10.1016/j.steroids.2023.109299

**Table 1**

**Effects of aldosterone treatment and TMPRSS2 deficiency on the expression of serine proteases in $mCCD_{cl1}$ cells.**

Listed are five genes encoding serine proteases with the highest expression in control cells from the RNA sequencing analyses shown in Supplemental Figures 9, 12, and Supplemental Tables 1-3. Fold change in RNA levels after 2 h (n=6) or 24 h exposure to 3 nM of aldosterone (n=7), or in TMPRSS2-ko cells (n=6) compared to respective control cells are given with adjusted *p*-values. Please note that despite residual expression of altered TMPRSS2 transcripts, on protein and functional level TMPRSS2-knockout was successful as shown in Figures 1A, 1B, 2D, 2E, Supplemental Figure 5, Supplemental Figure 11B. Significant changes (adj. p-value < 0.05) are highlighted in bold. TPM, transcripts per million.

| **Gene name** | **Gene description** | **average expression in control** *(TPM)* | **2 h aldo** | | **24 h aldo** | | **TMPRSS2-ko** | |
|---|---|---|---|---|---|---|---|---|
| | | | fold change | adj. p-value | fold change | adj. p-value | fold change | adj. p-value |
| ***Tmprss2*** | Transmembrane protease, serine 2 | 266 ± 25 | 1.03 | 0.95 | **1.09** | **7E−3** | **0.37** | **<9E−99** |
| ***St14*** | Suppression of tumorigenicity 14 (matriptase) | 110 ± 2 | 1.00 | 1.00 | 1.00 | 1.00 | **0.91** | **4E−3** |
| ***Prss23*** | Protease, serine 23 | 102 ± 5 | 0.99 | 0.75 | 0.99 | 0.46 | **1.20** | **6E−3** |
| ***Furin*** | Furin (subtilisin-like proprotein convertase 1) | 92 ± 2 | 1.00 | 0.98 | 1.00 | 0.92 | 1.00 | 0.90 |
| ***Prss8*** | Protease, serine 8 (prostasin) | 86 ± 4 | 1.02 | 1.00 | 1.02 | 0.85 | 0.99 | 0.36 |

# Figure Legends

**Figure 1: TMPRSS2 contributed to proteolytic ENaC activation in $mCCD_{cl1}$ cells**

**(A)** *Left panel:* Representative western blot analysis of whole-cell $mCCD_{cl1}$ lysates to assess endogenous expression of TMPRSS2 in wildtype, non-targeting control (Control), and TMPRSS2-knockout (TMPRSS2-ko) cells. Grey and black arrowheads indicate TMPRSS2 in its activated (catalytic chain, ~26 kDa) or zymogen form (~60 kDa), respectively. Similar protein loading in all lanes was confirmed using Ponceau S total protein staining (Supplemental Figure 2). *Right panel:* Densitometric evaluation of TMPRSS2 expression from similar blots as shown in the left panel. In each blot, the density value of the ~26 kDa band was normalized to the corresponding density value from wildtype cells. The dotted line indicates a normalized density value of one (no effect). Mean ± SEM and data points for individual samples are shown; n=6-10, Two-sided one sample Student's *t*-test of log-transformed values with Bonferroni correction for multiple testing.

**(B)** Progress curves of trypsin-like proteolytic activity in medium taken from the apical compartment of non-targeting control (blue, n=42), or TMPRSS2-knockout $mCCD_{cl1}$ cells (red, n=60) are shown (Mean ± SEM). Freshly prepared medium served as control (grey, n=54). In each sample, the recorded RFU (relative fluorescent unit) values were normalized to the RFU value at the beginning of the measurement. A dotted line indicates a relative effect of one (no change). Kruskal-Wallis test (*p*-value <0.001) with Dunn's Multiple Comparisons Test of log-transformed values was used to calculate *p*-values for comparisons with the RFU at time point 180 min obtained in control cells.

**(C)** Representative equivalent short-circuit current ($I_{SC}$) traces recorded from non-targeting control (*left panels*, n=21), or TMPRSS2-knockout $mCCD_{cl1}$ cells (*right panels*, n=20) are shown. Chymotrypsin (chymo, 20 µg/ml) and amiloride (ami, 10 µM) were present in the apical bath solution as indicated by grey and black bars, respectively. The dotted lines indicate zero current levels. Summary data from similar experiments are shown to the right of the representative traces. $I_{SC}$ values were obtained immediately before application of chymotrypsin or amiloride and at the end of the experiment. Values obtained in the same measurement are connected with a line. Mean ± SEM and data points for individual measurement are shown. ANOVA (*p*-values: <0.001 for control; <0.001 for TMPRSS2-ko) with Bonferroni post-hoc test.

**(D)** Summary data from the same experiments as shown in (C) and in Supplemental Figure 6. Baseline $I_{SC}$ at the beginning of the experiment. Mean ± SEM and data points for individual measurements are shown. Two-sided unpaired Wilcoxon Signed Rank test.

**(E)** Summary data from the same experiments as shown in (C). The effect of chymotrypsin (chymo) on $I_{SC}$ ($\Delta I_{SC}$) was calculated by subtracting the $I_{SC}$ value measured immediately before chymotrypsin application from the current level reached in the presence of chymotrypsin immediately before amiloride application. The effect of amiloride (ami) on $I_{SC}$ ($\Delta I_{SC}$) was calculated by subtracting the $I_{SC}$ value measured immediately before amiloride application from the current level reached at the end of the recording. Mean ± SEM and data points for individual measurements are shown. Two-sided unpaired Student's *t*-test with Bonferroni correction for multiple testing.

**(F)** *Left panel:* Representative western blots showing cell surface expression of γ-ENaC in $mCCD_{cl1}$ cells. Uncleaved (~70 kDa), partially cleaved (~60 kDa), and fully cleaved (~55 kDa) γ-ENaC are indicated by black, dark grey, and light grey arrowheads, respectively. *Right panel:* Densitometric evaluation of the western blot shown in the *left panel* and additional blots shown in Supplemental Figure 8. The densitometric signal of uncleaved, partially cleaved and fully cleaved γ-ENaC was normalized to the total signal of all three bands. Mean ± SEM and data points for individual western blots are shown (n=6). Two-sided unpaired Student's *t*-test with Bonferroni correction for multiple testing.

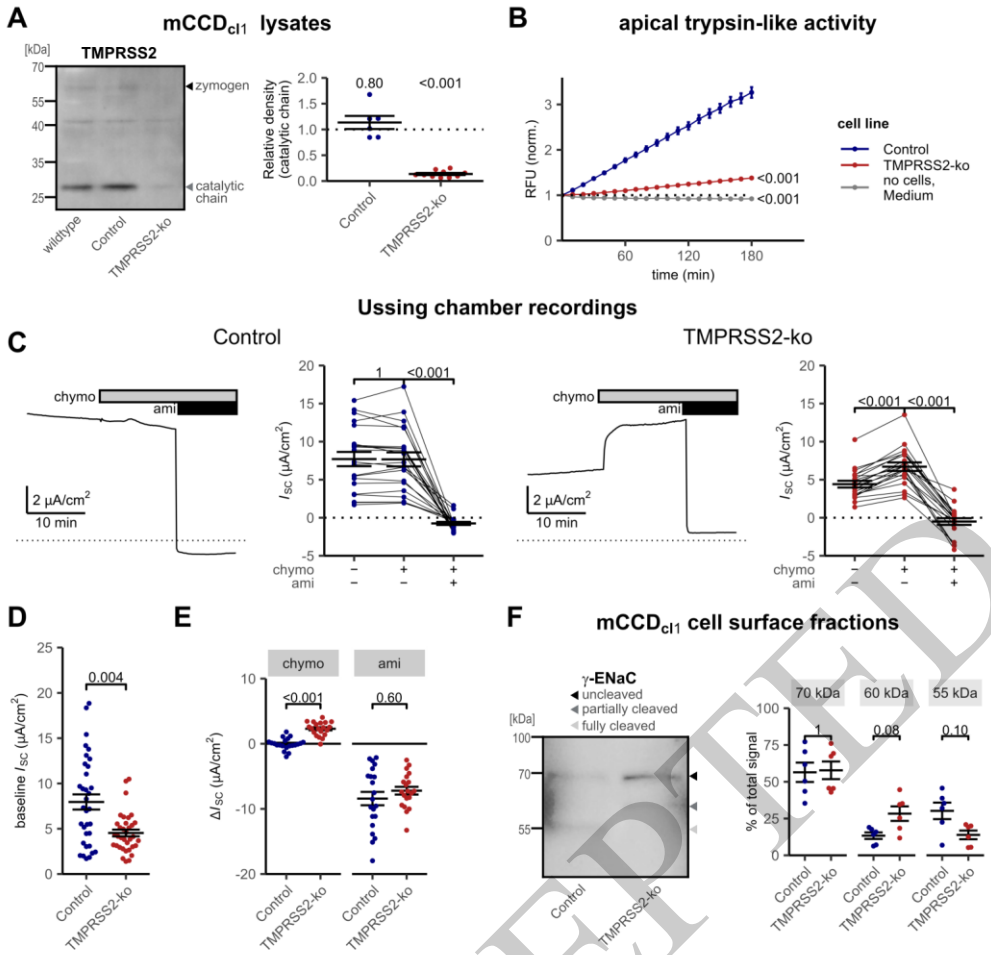


**Figure 2: TMPRSS2 knockout reduced the stimulatory effect of aldosterone in $mCCD_{cl1}$ cells**

**(A,B)** *Left panels:* Representative equivalent short circuit current ($I_{SC}$) recordings from non-targeting control (A), or TMPRSS2-knockout $mCCD_{cl1}$ cells (B) are shown. Aldosterone (aldo, 3 nM, apical and basolateral), chymotrypsin (chymo, 20 µg/ml, apical) and amiloride (ami, 10 µM, apical) were present in the bath solution as indicated by white, grey, and black bars, respectively. The dotted lines indicate zero current levels. *Right panels:* Summary data from similar experiments as shown in the corresponding left panels. Values were obtained immediately before application of aldosterone, chymotrypsin or amiloride and at the end of the experiment. Values obtained in the same measurement are connected with a line. Mean ± SEM and data points for individual measurements are shown. A: n=10, B: n=8. ANOVA (*p*-values: <0.001 (A), <0.001 (B)) with Bonferroni post-hoc test.

**(C)** Summary data from the same experiments as shown in (A, B). $\Delta I_{SC}$ was calculated essentially as described in Figure 1E. Mean ± SEM and data points for individual measurements are shown. Two-sided unpaired Wilcoxon Signed Rank test with Bonferroni correction for multiple testing.

**(D)** *Left panel:* Western blot analysis of the apical cell-surface fraction of $mCCD_{cl1}$ cells to assess endogenous expression of TMPRSS2 in non-targeting control (control, blue bars), and TMPRSS2 knockout (TMPRSS2-ko, red bars) cells. A black arrowhead indicates TMPRSS2 in its activated (catalytic chain, ~26 kDa) form. The absence of the ~26 kDa TMPRSS2 band in TMPRSS2-ko cells confirmed the specificity of TMPRSS2 detection. Prior to harvesting, cells were maintained for 3 hours in the presence (+) or absence (−) of 3 nM aldosterone (aldo) as indicated. *Right panel:* Densitometric evaluation of TMPRSS2 expression in control cells from western blots shown in the left panel and an additional blot shown in Supplemental Figure 11A. In each blot, the density value of the ~26 kDa TMPRSS2 band obtained from aldosterone-treated $mCCD_{cl1}$ cells was normalized to the corresponding TMPRSS2 signal obtained from vehicle-treated $mCCD_{cl1}$ cells. The dotted line indicates a normalized density value of one (no effect). Mean ± SEM and data points for individual samples are shown (n=5). Two-sided one-sample Student's *t*-test with log-transformed values.

**(E)** Progress curves of trypsin-like proteolytic activity in medium taken from the apical compartment of non-targeting control (blue, n=42), or TMPRSS2-knockout $mCCD_{cl1}$ cells (red, n=60) are shown (Mean ± SEM). Freshly prepared medium (with or without aldosterone) served as control (grey, n=54). In each sample, the recorded RFU (relative fluorescent unit) values were normalized to the RFU value at the beginning of the measurement. Cells received either standard medium (Ø aldo, open symbols) or medium supplemented with 3 nM aldosterone (closed symbols) on apical and basolateral sides. Samples were taken 3 h after medium exchange which probably explains lower normalized RFU values compared to Figure 1B where samples were taken after 12 h. A dotted line indicates the relative effect of one (no change). 2-way ANOVA (*p*-values: <0.001 (effect of cell type), 1 (effect of aldosterone)) of log-transformed values.

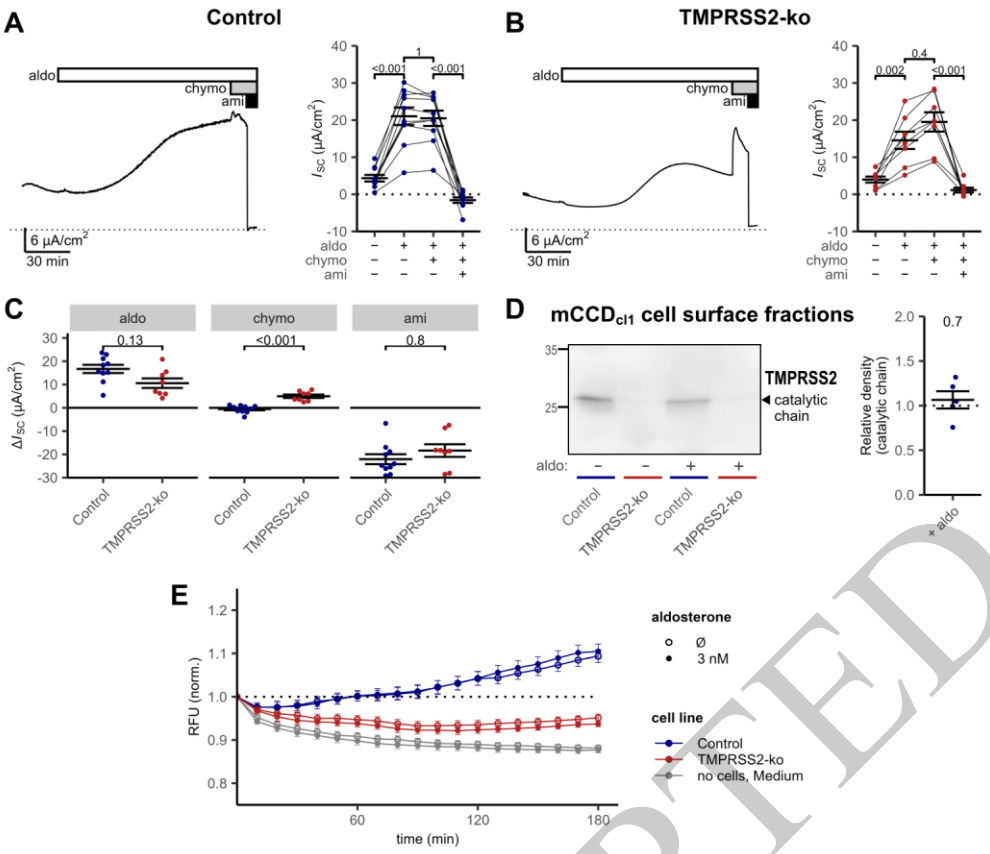


**Figure 3: TMPRSS2 mRNA was expressed in cells positive for β-ENaC protein**
Representative microscopic images from mouse kidney cortex of *Tmprss2*$^{+/+}$ *(top row)* and *Tmprss2*$^{-/-}$ *(bottom row)* mice are shown. RNAscope staining for *Tmprss2* mRNA (*left panels, red*) and immunofluorescence staining for β-ENaC protein (*middle panels, green*) are merged with nuclear DAPI-staining (*blue*) in *right panels.*

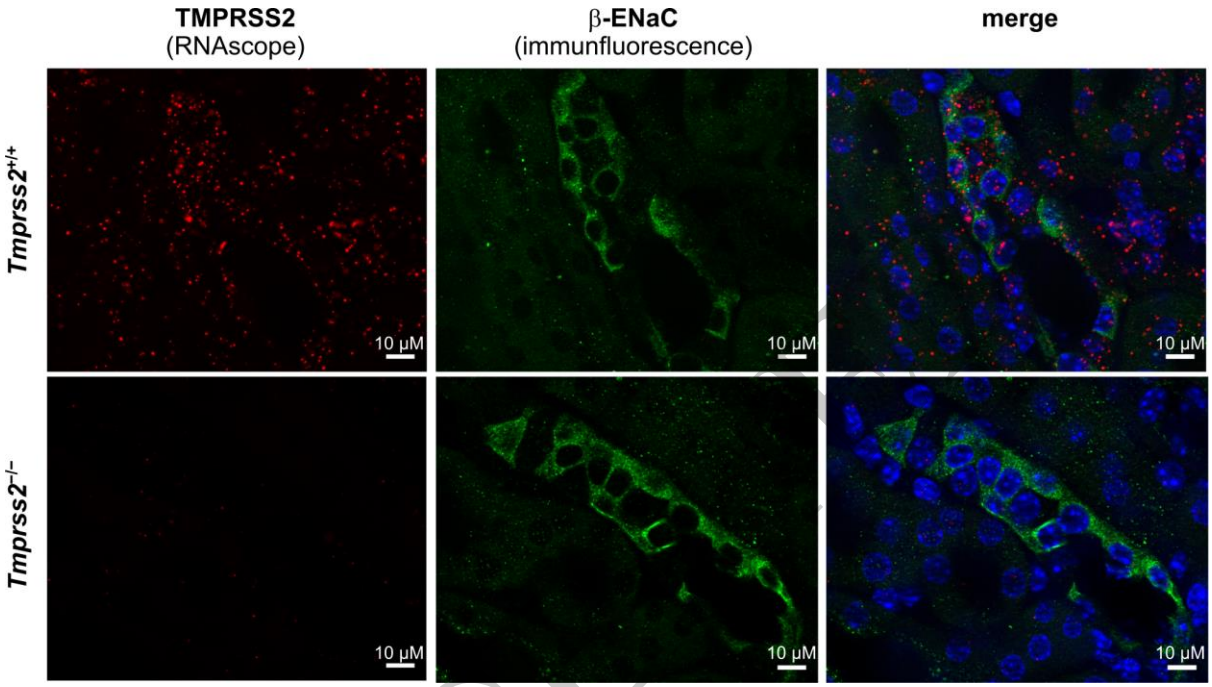


**Figure 4: In *Tmprss2*$^{-/-}$ mice, proteolytic processing of γ-ENaC was altered.**

**(A)** Western blot analysis shows expression of endogenous PNGase treated γ-ENaC in membrane enriched fractions obtained from mouse kidney cortex lysates. Uncleaved (~70 kDa), partially cleaved (~60 kDa), and fully cleaved (~55 kDa) γ-ENaC are indicated by black, dark grey, and light grey arrowheads, respectively. Vertical lines represent positions at which the original blot images were cut to re-order the lanes for clarity. Original blots are shown in Supplemental Figure 14.

**(B)** Densitometric evaluation of similar western blots as in (A). The densitometric signal of uncleaved, partially cleaved and fully cleaved γ-ENaC in each lane was normalized to the Ponceau S total protein staining from the same lane. Mean ± SEM and data points for individual western blots are shown. Data points from female and male mice are represented with open and closed symbols, respectively. TMPRSS2 and ENaC has been reported to be modulated by sex hormones including testosterone[68] and estrogens[69], respectively. However, we observed no sex-specific differences in all our analyses and therefore the data from male and female mice were pooled together. Two-sided Wilcoxon Signed Rank test with Bonferroni correction for multiple testing.

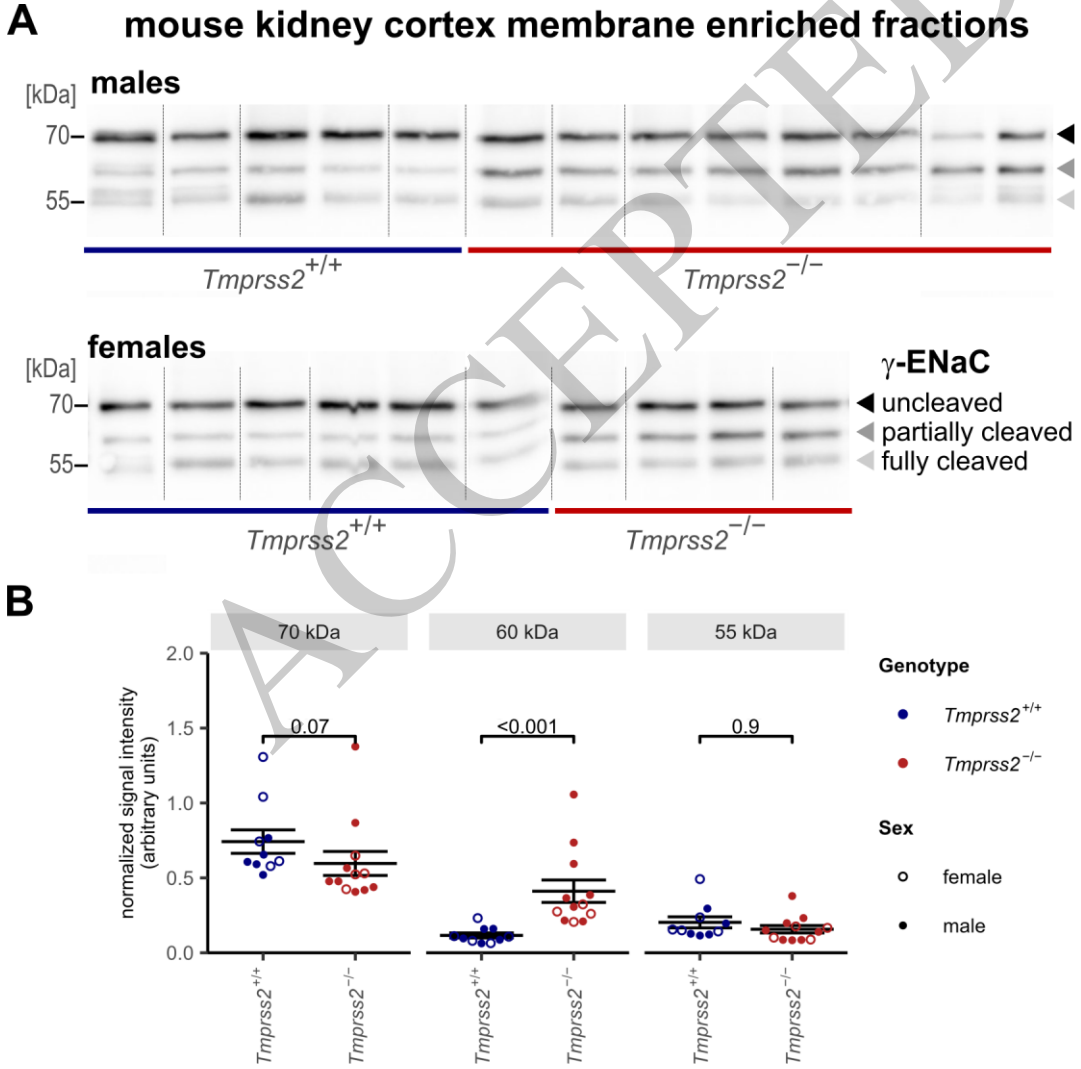


**Figure 5: *Tmprss2*$^{-/-}$ mice required significantly higher plasma aldosterone levels than wildtype mice to maintain sodium balance under dietary sodium restriction with or without increased potassium intake**

**(A)** Representative immunohistochemical staining of γ-ENaC in fixed kidney tissue from *Tmprss2*$^{+/+}$ versus *Tmprss2*$^{-/-}$ mice, shown in 20x magnification (scale 20µm) and 63x magnification (scale 10µm). In both genotypes there is an increased apical staining under low sodium diet (n=3 each).

**(B)** Time course of urinary sodium excretion, normalized to food intake, during dietary sodium restriction without (*left panel*) or with increased potassium intake (*right panel*). Mean ± SEM (n=14-36) are shown. Data are pooled from both sexes. Red and blue *p*-values indicate comparisons to day 0 (Friedman test with Dunn's Multiple Comparison Test). Black *p*-values indicate comparisons between genotypes (Mann-Whitney Test).

**(C)** Plasma aldosterone concentration under control diet and after 4 days of a low sodium diet with or without increased potassium intake. Data points from female and male mice are represented with open and closed symbols, respectively. Mean ± SEM and individual datapoints (n=10-22) are shown. Kruskal-Wallis with Dunn's Multiple Comparison Test.

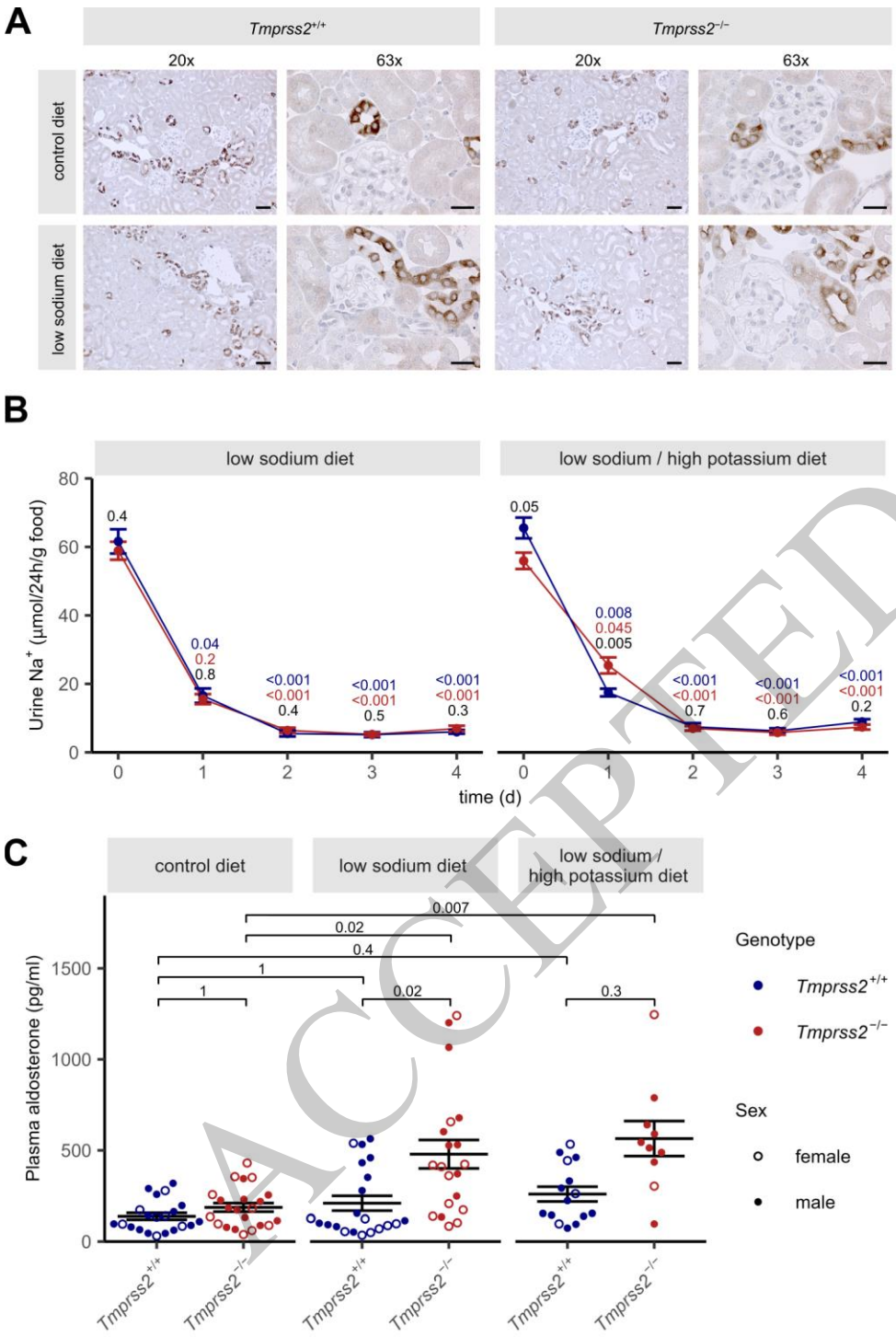


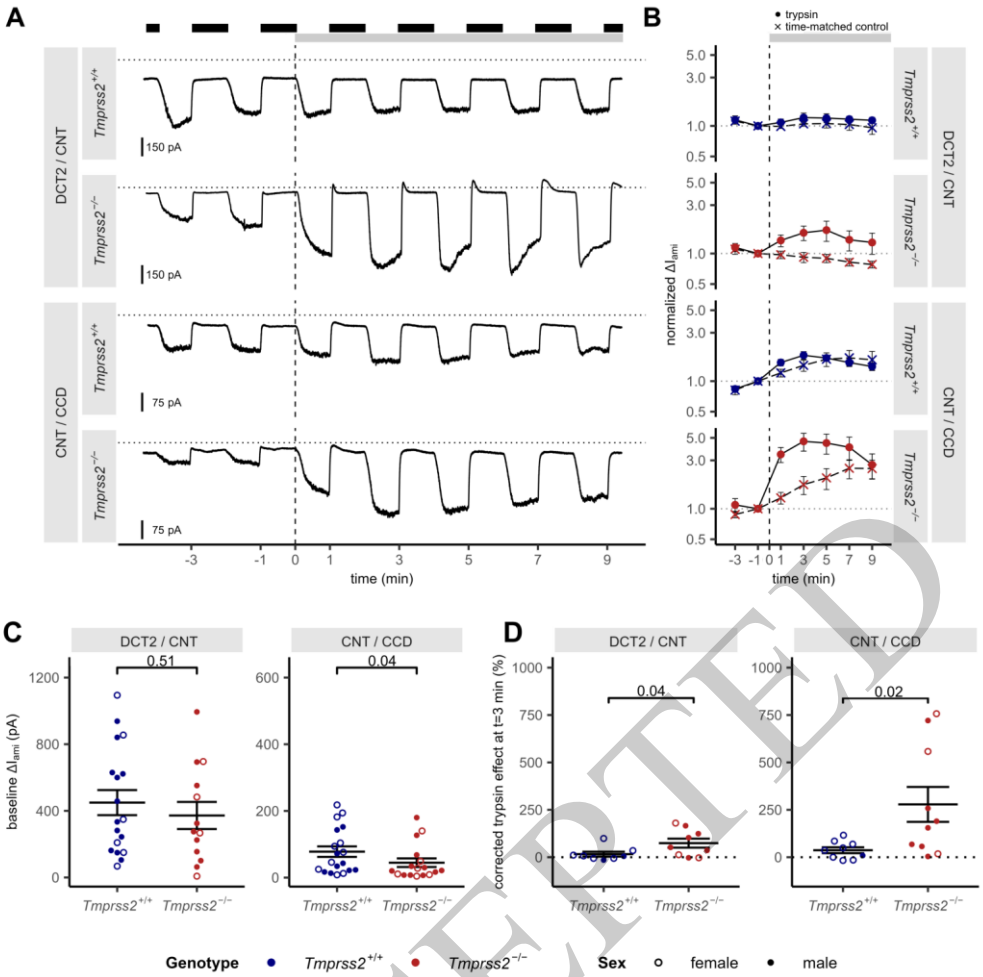
**Figure 6: Whole-cell current recordings in microdissected tubules revealed impaired proteolytic ENaC activation in *Tmprss2*$^{-/-}$ mice.**

**(A)** Representative whole cell current traces are shown from DCT2/CNT and CNT/CCD isolated from *Tmprss2*$^{+/+}$ and *Tmprss2*$^{-/-}$ mice, as indicated. Presence of amiloride (4 µM) and trypsin (20 µg/ml) in the bath solution is indicated by black and grey bars, respectively. Unless trypsin was added, all bath solutions contained 2 µg/ml soybean trypsin inhibitor (SBTI) to reduce the risk of a contamination with trypsin. A dotted line indicates zero current level. Timepoint of trypsin application or of mock solution exchange in time-matched control experiments is referred to as 0 min and marked with a dashed vertical line.

**(B)** Summary of normalized amiloride-sensitive current values ($\Delta I_{ami}$) from similar experiments as shown in A with trypsin application (filled circles) and from time-matched control recordings shown in Supplemental Figure 23 (crosses) displayed on a logarithmic scale. For each cycle of amiloride washout and reapplication $\Delta I_{ami}$ was calculated by subtracting the current value measured immediately before amiloride application from that reached in the presence of amiloride. $\Delta I_{ami}$ values determined at different time points in each individual recording were normalized to the $\Delta I_{ami}$ obtained in the second cycle of the same experiment (at t= −1), i.e. from the amiloride application just prior to trypsin application or mock solution exchange. Absolute $\Delta I_{ami}$ values are shown in Supplemental Figure 23B. It is noteworthy, that the slow time-course of spontaneous current runup observed in time-matched control recordings in CNT/CCD was clearly different from the rapid stimulatory response to trypsin which usually reached a maximum within three to five minutes followed by a gradual current decline. The latter was probably due to ENaC degradation during prolonged trypsin exposure. Mean ± SEM are shown with symbols and error bars. *p*-values from Kruskal-Wallis test of log-transformed values comparing trypsin application and time-matched control: 0.1 (first panel), <0.001 (second panel), 0.6 (third panel), 0.02 (fourth panel).

**(C)** Summary of baseline $\Delta I_{ami}$ values obtained in the second cycle of amiloride washout/reapplication (t=−1) from the same experiments shown in (A) and (B) and in Supplemental Figure 23. Mean ± SEM and individual datapoints are shown. Sex is indicated with open (female) and closed (male) symbols. One-sided Wilcoxon Signed Rank Test with Bonferroni correction for multiple testing.

**(D)** Summary of corrected normalised effects of trypsin on $\Delta I_{ami}$ three minutes after its application from experiments shown in (A) and (B). To account for spontaneous changes in $\Delta I_{ami}$, each normalized $\Delta I_{ami}$ value at t=3 min obtained from trypsin-treated cells was corrected by subtracting the average normalized $\Delta I_{ami}$ at t=3 min from corresponding time-matched control recordings (see Supplemental Figure 23A). Mean ± SEM and individual datapoints are shown. One-sided Wilcoxon Signed Rank Test of log-transformed values with Bonferroni correction for multiple testing.



# Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney

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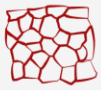
Basic Research

## Experimental models

Co-expression of murine ENaC and TMPRSS2 in *Xenopus laevis* oocytes



Murine cortical collecting duct cells ($mCCD_{cl1}$) with and without TMPRSS2-deficiency

Wild-type and TMPRSS2-knockout (*Tmprss2*$^{-/-}$) mice

## Key findings

In oocytes, co-expression of murine TMPRSS2 and ENaC resulted in fully cleaved γ-ENaC and ~2-fold stimulation of ENaC currents

High baseline expression of TMPRSS2 was detected in $mCCD_{cl1}$ cells without aldosterone having a stimulatory effect on its transcription or function

In $mCCD_{cl1}$ cells, TMPRSS2-deficiency compromised γ-ENaC cleavage and reduced baseline and aldosterone-stimulated ENaC currents

In response to dietary sodium restriction, *Tmprss2*$^{-/-}$ mice upregulated aldosterone to much higher levels than wild-type controls to achieve a similar reduction of renal sodium excretion

Whole-cell patch-clamp recordings in microdissected renal tubules from *Tmprss2*$^{-/-}$ mice revealed incomplete proteolytical ENaC activation, particularly in CNT/CCD

ENaC = epithelial sodium channel, TMPRSS2 = transmembrane serine protease 2, CNT = connecting tubule, CCD = cortical collecting duct

**Conclusions:** TMPRSS2 contributes to proteolytic ENaC activation in mouse kidney *in vivo*.

Florian Sure, Sara Afonso, Daniel Essigke, et al. ***Transmembrane Serine Protease 2 and Proteolytic Activation of the Epithelial Sodium Channel in Mouse Kidney***. JASN. doi: 10.1681/ASN.0000000521.
**Visual Abstract by Brian S. Rifkin, MD**

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Date of Completion: September 5, 2024
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Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
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Name: Daniel Essigke
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: September 6, 2024
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As per ASN journal policy, I have disclosed any financial relationships or commitments I have held in the past 36 months as included below. I have listed my Current Employer below to indicate there is a relationship requiring disclosure. If no relationship exists, my Current Employer is not listed.

T. Gramberg has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Thomas Gramberg
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: August 20, 2024
Disclosure Updated Date: August 20, 2024

ASN American Society of Nephrology
JASN Journal of the American Society of Nephrology
KIDNEY360 Accessing Our World From Every Angle
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A. Ilyaskin has nothing to disclose.

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Name: Alexandr V. Ilyaskin
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: August 16, 2024
Disclosure Updated Date: August 16, 2024

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M. Kalo reports the following:
Employer: Universitätsklinikum Tübingen

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: M. Zaher Kalo
Manuscript ID: JASN-2024-000415R3
Manuscript Title: ransmembrane Serine Protease 2 and Proteolytic Activation of the Epithelial Sodium Channel in Mouse Kidney.
Date of Completion: September 27, 2024
Disclosure Updated Date: September 27, 2024

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A. Kißler has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Alicia Kißler
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: September 9, 2024
Disclosure Updated Date: September 9, 2024

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C. Korbmacher has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Christoph Korbmacher
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: August 16, 2024
Disclosure Updated Date: May 22, 2024

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V. Nesterov reports the following:
Employer: Institute of molecular and cellular physiology

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Viacheslav Nesterov
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: September 14, 2024
Disclosure Updated Date: September 14, 2024

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R. Rinke reports the following:
Employer: FAU Erlangen-Nuernberg

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Ralf G. Rinke
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney,
Date of Completion: August 16, 2024
Disclosure Updated Date: August 16, 2024

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P. Schmidt reports the following:
Employer: Universität Tübingen

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Paul Torsten Walter Schmidt
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: August 18, 2024
Disclosure Updated Date: August 18, 2024

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JASN Journal of the American Society of Nephrology
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F. Sure has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Florian Sure
Manuscript ID: JASN-2024-000415R1
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: August 20, 2024
Disclosure Updated Date: August 20, 2024

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S. Wittmann has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Sabine Wittmann
Manuscript ID: JASN-2024-000415R2
Manuscript Title: Transmembrane serine protease 2 and proteolytic activation of the epithelial sodium channel in mouse kidney
Date of Completion: September 17, 2024
Disclosure Updated Date: September 17, 2024