**Supplemental material**

**to the manuscript**

**Urokinase-type plasminogen activator (uPA) is not required for epithelial sodium channel (ENaC)-mediated sodium retention in experimental nephrotic syndrome**

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**Supplemental methods**

*Protease activity fluorescence assay*

Proteolytic activity was quantified using the fluorogenic substrate Boc-Gln-Ala-Arg-AMC (Boc-QAR-AMC) (Boc: t-Butyloxycarbonyl; AMC: 7-Amino-4-methylcoumarin; R&D systems, Abingdon, UK). This substrate detects the activity of a wide range of trypsin-like proteases. The experimental protocol was similar to that described by Reihill et al. 2016. The substrate was used in a concentration of 20 µM using a sample volume of 100 µl. The fluorescence signal resulting from substrate hydrolysis was continuously recorded over a period up to 150 min using a 96-well TECAN plate reader (360 nm excitation/465 nm emission wavelength).

**Reference:**

Reihill JA, Walker B, Hamilton RA, Ferguson TE, Elborn JS, Stutts MJ, Harvey BJ, Saint-Criq V, Hendrick SM, Martin SL. Inhibition of Protease-Epithelial Sodium Channel Signaling Improves Mucociliary Function in Cystic Fibrosis Airways. Am J Respir Crit Care Med. 194:701-10, 2016

**Supplemental figure 1.** **A high concentration of urokinase-type plasminogen activator (uPA) (2000 IU/ml) does not significantly stimulate murine or human ENaC.**

(a, c) *Xenopus laevis* oocytes expressing murine (a, mENaC) or human (c, hENaC) ENaC were pre-incubated for 12 h in protease-free vehicle solution (ND96) or in a solution containing uPA (150 IU mL-1), uPA (2000 IU mL-1) or uPA (150 IU mL-1) + plasminogen (Plg;1 mg mL-1). Subsequently amiloride-sensitive whole-cell currents (∆Iami) were determined. The filled circles shown in (a) and (c) represent ∆Iami values measured in individual oocytes. n indicates the number of individual oocytes measured. \*\*\*, p<0.001; \*, p<0.05; ns, not significant; one-way ANOVA with Bonferroni post-hoc test

(b, d) Using the fluorogenic substrate Boc-QAR-AMC trypsin-like proteolytic activity was assessed in the incubation solution of each oocyte at the end of the pre-incubation period. The fluorescence data (RFU=relative fluorescence unit; n=7) in (b) and (d) correspond to the current data shown in a and c, respectively. Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. The data shown in (b) and (c) demonstrate that the proteolytic activity of uPA (2000 IU mL-1) was similar to that of uPA (150 IU mL-1) + Plg (1 mg mL-1) but failed to stimulate ENaC currents in the oocytes. Error bars, SEM



**Supplemental figure 2.** **The ENaC stimulating effect and proteolytic activity of a combination of urokinase-type plasminogen activator (uPA) and plasminogen (plg) are inhibited by 500 µM amiloride (ami).**

*Xenopus laevis* oocytes expressing murine ENaC were pre-incubated for 30 min in protease-free control solution (ND96) or in solutions containing uPA (150 IU mL-1) + Plg (1 mg mL-1), uPA (150 IU mL-1) + Plg (1 mg mL-1) + amiloride (Ami; 500µM) or amiloride alone (ND96 + Ami).

(a)Amiloride-sensitive currents (∆Iami) were determined before (-) and after (+) pre-incubation. Measurements performed in the same oocyte are connected by a line.

(b)Summary of the individual data shown in (a) normalized as relative stimulatory effect on ∆Iami. N indicates the number of different batches of oocytes, n the numbers of individual oocytes measured. \*\*\*, p<0.001; paired *t*-test (a) or one-way ANOVA with Bonferroni post-hoc test (b).

(c)Trypsin-like proteolytic activity in the incubation solutions was detected using the fluorogenic substrate Boc-QAR-AMC (RFU=relative fluorescence unit; n=5-6). Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. Error bars, SEM

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**Supplemental figure 3. Proteolytic activity of plasmin (pl) and its stimulatory effect on ENaC are inhibited by 500 µM amiloride (ami).**

*Xenopus laevis* oocytes expressing murine ENaC were pre-incubated for 30 min in protease-free control solution (ND96) or in ND96 solution supplemented with 500 µM amiloride (+Ami), with 25µg mL-1 of plasmin (+Pl) or with 25µg mL-1 of plasmin and 500 µM amiloride (+ Pl, +Ami). (a) Amiloride-sensitive currents (∆Iami) were determined before (-) and after (+) preincubation. Measurements performed in the same oocyte are connected by a line.   
(b) Summary of individual data shown in (a) normalized as relative stimulatory effect on ∆Iami. n indicates the numbers of individual oocytes measured. \*\*\*, p<0.001; paired t-test (a) or one-way ANOVA with Bonferroni posthoc test (b). (c) Trypsin-like proteolytic activity in the incubation solutions was detected using the fluorogenic substrate Boc-QAR-AMC (RFU=relative fluorescence unit; n=7). Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. Error bars, SEM. (d) BOC-QAR-AMC substrate was used to detect trypsin-like proteolytic activity in ND96 solution containing plasmin (50µg mL-1) without amiloride (control) or with different concentrations (1µM, 10µM, 100µM, 500µM, 1mM) of amiloride (RFU=relative fluorescence unit; n=2). (e) Concentration-response relationship of the inhibitory effect of amiloride on proteolytic activity of plasmin. RFU values at 95 min (RFU95; mean ± SEM) from the same experiments as shown in (d) were normalized to RFU95 of control.



**Supplemental figure 4. Food and fluid intake during the course of nephrotic syndrome**

Daily food (a) and fluid (b) intake in wild-type mice treated with vehicle or amiloride (5 µg g bw-1) as well as *uPA+/+* or *uPA-/-* mice before and after induction of nephrotic syndrome by doxorubicin. Due to inappetence mice food and fluid intake transiently decreased on day 3 after injection of doxorubicin

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between treatments/genotypes

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**Supplemental figure 5. Sodium balance in wild-type mice before and after induction of doxorubicin-induced nephrotic syndrome.**

Calculated Na+ intake from food and drink, urinary and fecal Na+ excretion and calculated Na+ balance in wild-type mice before and after induction of nephrotic syndrome.

Arithmetic means ± SEM.

# significant difference between healthy and nephrotic mice

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**Supplemental figure 6. Renal ENaC activity is not different in healthy *uPA-/-* mice compared to *uPA+/+* mice.**

(a, b) Time course of urinary amiloride excretion after injection of vehicle or amiloride   
(5 / 10 µg g bw-1). Urinary amiloride concentration reaches its peak after 4 h and disappears after 24 h. Natriuresis parallels urinary amiloride concentration and is lower 24 h after injection indicating counterregulation. There is no difference between healthy *uPA+/+-* mice *uPA-/-* mice.

(c) Urinary sodium excretion and body weight change (inset) on day 5 of a low salt diet.

(d) Plasma aldosterone concentration under control conditions and after amiloride injection and a low salt diet

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between genotypes

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**Supplemental figure 7. Plasma and urinary plasmin(ogen) concentration in *uPA+/+* and *uPA-/-* mice before and after induction of nephrotic syndrome**

The used ELISA recognizes both plasminogen as a zymogen and heavy chain after proteolytic activation by uPA.

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between genotypes

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**Supplemental figure 8. Amiloride-sensitive natriuresis and urinary amiloride concentration in healthy and nephrotic *uPA+/+* and *uPA-/-* mice.**

(a) Natriuretic effect of mice after a single injection of vehicle and amiloride. Spot urinary samples were collected 4 h after injection of vehicle and amiloride (5 µg g-1) in healthy and nephrotic wild-type *uPA+/+* mice (day 8 after induction). The injections were done one day apart in the same mouse before and after induction of nephrotic syndrome.

(b) Amiloride-sensitive natriuresis calculated from the difference of the urinary sodium excretion between vehicle and amiloride treatment was enhanced in *uPA+/+* mice in the nephrotic state compared to the healthy state. This was not the case in *uPA-/-* mice which had already high amiloride-sensitive natriuresis in the healthy state.

(c) In the urine samples, urinary amiloride was measured. Compared to healthy mice, nephrotic *uPA+/+* show diminished urinary amiloride concentration that can be explained by binding of amiloride to uPA that is aberrantly filtered into the tubular lumen. The lack of this effect explains a higher urinary amiloride concentration in *uPA-/-* mice.

Arithmetic means ± SEM.

# significant difference vs. healthy, § significant difference between vehicle and amiloride injection

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**Supplemental figure 9. Expression of ENaC subunits and their cleavage products in kidney cortex from healthy mice**

(a) Administration of the blocking peptide for α-ENaC attenuated all bands at 22, 26 and 87 kDa.

(b) Probing with an anti-β-ENaC antibody revealed a single band at 85 kDa.

(c) Administration of the blocking peptide for γ-ENaC attenuated bands at 45, 71 and 82 kDa while bands at 50 and 62 kDa were not blocked.

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**Supplemental figure 10: Expression of γENaC and its cleavage products in kidney cortex from a healthy and nephrotic mouse after treatment with the deglycosylating enzyme PNGase F.**

Western blot from renal cortex showing several bands between 45 and 82 kDa in a healthy and nephrotic wild-type mouse (lane 1 and 3). Administration of the blocking peptide attenuated bands at 45, 71 and 82 kDa while bands at 50 and 62 kDa were not blocked (lane 5 and 7). After treatment with PNGase F, only two bands at 71 and 45 kDa were visible (lane 2 and 4) both of which disappeared after application of the blocking peptide (lane 6 and 8). There was no specific band that could correspond to fully-cleaved γ-ENaC in mouse tissue with an expected size between 53-65 kDa.

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