Supplemental Material: Supplemental Figures1–3and Complete Methods

(Second revision – revisions highlighted in red font)

A Missense Mutation in the Extracellular Domain of Alpha ENaC Causes Liddle Syndrome

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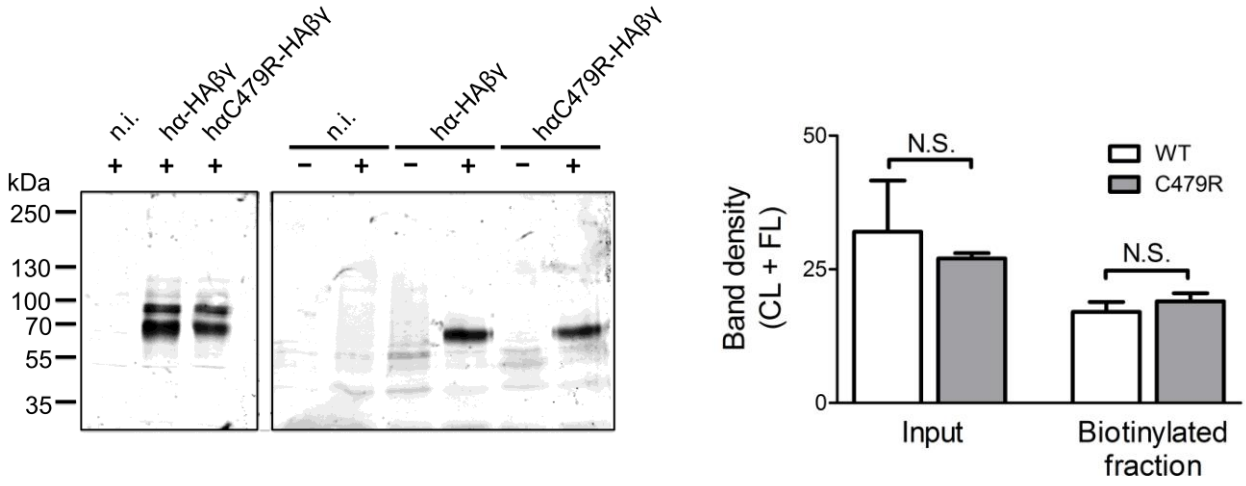
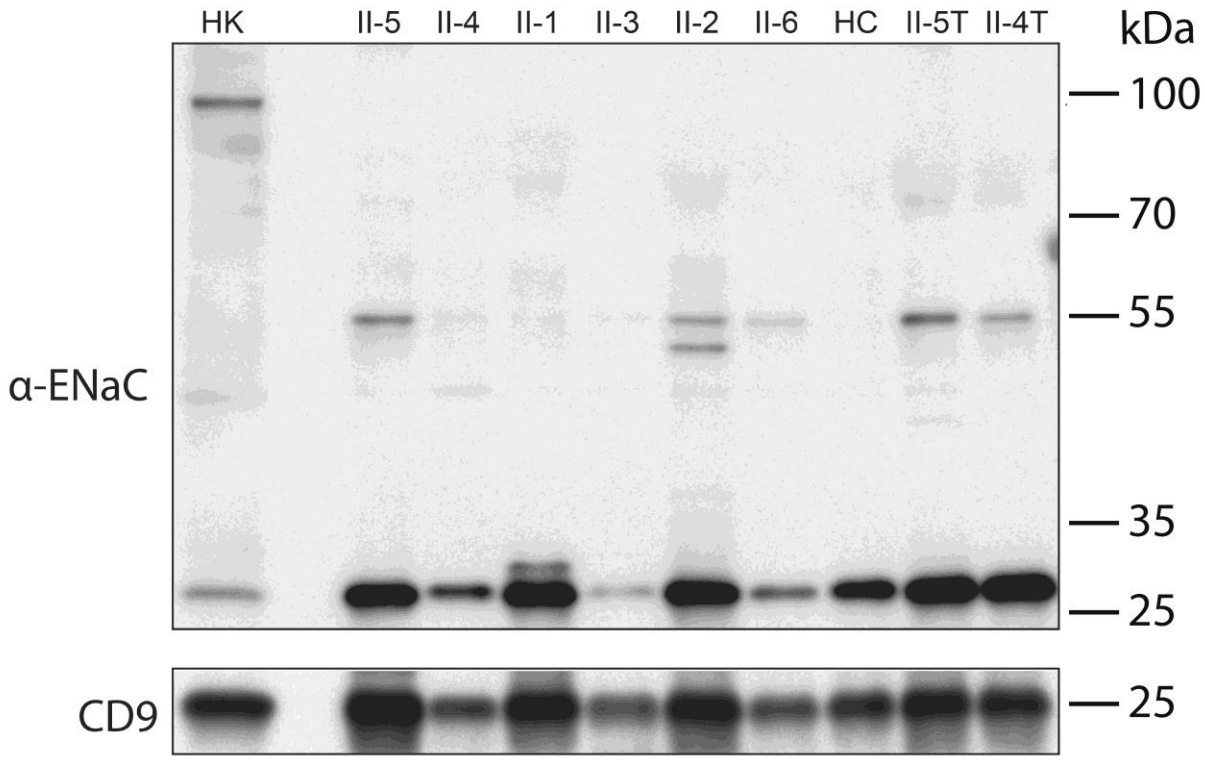
Supplemental Figure 1. αENaC C479R mutation does not result in increased γENaC levels at the cell surface of injected oocytes.

A **B**



Legend: After probing with anti-HA antibody (see **Figure 4**), blots were re-blocked and analyzed with anti-γENaC antibody as described for lysates in the Materials and Methods section. **(A)** Right panel, immunoblot analysis of Neutravidin-bound fractions isolated from control (-) or cell-surface biotinylated (+) Xenopus oocytes, non-injected (n.i.), or injected with either α_{wt}-HA/β/γ or α_{C479R}-HA/β/γ cRNAs. Left panel. Inputs corresponding to 1% of the Triton-soluble preparations from biotinylated oocytes used in the pull-down experiments. (**B**) Values (mean ± SEM) of CL+FL band intensities of inputs or of Neutravidin-bound fractions corresponding to experiments shown in A. Results from three blots with samples of three independent experiments. Differences are non-significant (N.S.).

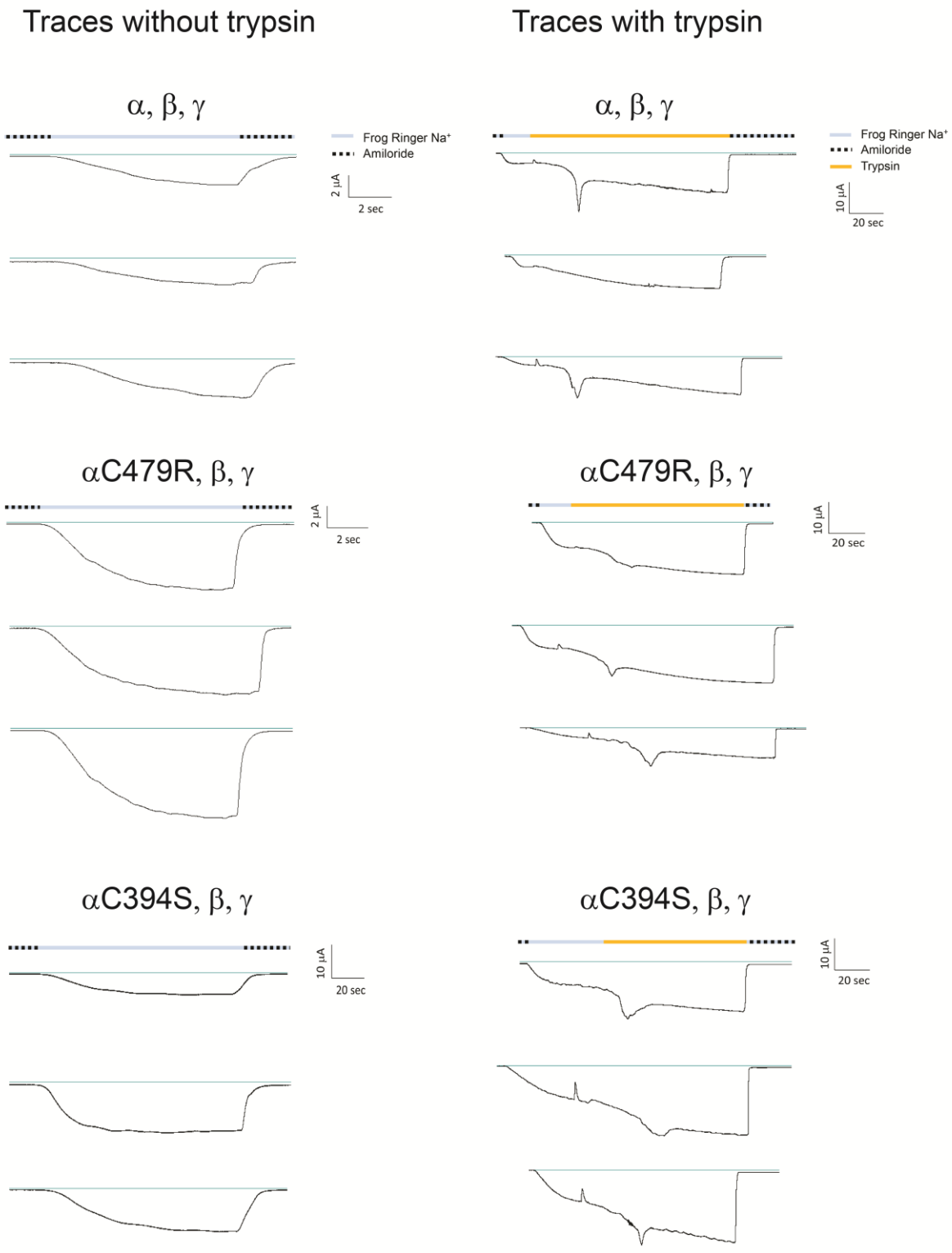
Supplemental Figure 2.αENaC in human urinary extracellular vesicles.



Legend: αENaC in urinary extracellular vesicles isolated from the reported family. Urinary extracellular vesicles were collected in the absence of interfering medication (no renin-angiotensin inhibitors or diuretics). A human kidney (HK) sample was included as positive control and showed the expected bands at 100 kDa (full-length αENaC) and ~30 kDa (cleaved αENaC). It is unclear what the low intensity band at ~45 kDa represents. Of these bands, only the 30 kDa band was detected in human urinary extracellular vesicles. Although no firm conclusions can be drawn based on the small number of individuals,no clear difference in the abundance of this 30 kDa band was visible between the two subjects carrying the C479R mutation (II-5 and II-4) compared to those without the mutation (II-1, II-2, II-3, and II-6) and a healthy control (HC). II-5T and II-4T are urinary extracellular vesicles isolated after the standardized diuretic test with triamterene (T).Of note, an additional band at ~55 kDa was visible in some of the subjects.

Although this band could represent partially or atypically cleaved αENaC, it could also be non-specific, as it was also vaguely visible in kidneys of αENaC -/- mice.[1]

Supplemental Figure 3. Original current traces for the different ENaC types in the presence or absence of trypsin.



Legend Supplemental Figure 3: Three original traces from hENaCwt, C479R, and C394S in the absence (left) or presence (right) of trypsin. Results from independent experiments; please note the current and time scales.

Complete Methods

Studies in patients

All subjects provided written informed consent for diagnostic exome sequencing or genotyping. Our medical ethics committee approved the isolation of urinary extracellular vesicles (MEC-2015-204, see below for details). Plasma renin concentration and plasma aldosterone were measured by enzyme-kinetic assay and LC/MS, respectively. These measurements were performed in the absence of interfering drugs (renin-angiotensin system inhibitors or diuretics). All routine serum and urinary measurements were determined using the Cobas 8000 modular analyzer series (Roche, Basel, Switzerland). Ambulatory blood pressure measurements were performed with the ultralite 90217A (Spacelabs Healthcare, Snoqualmie, USA). Salt-sensitivity was tested by providing sodium chloride tablets (6 g/day). The triamterene test was based on the thiazide test.[2] Briefly, after an overnight fast, patients were invited to drink tap water (10 ml/kg body wt) to facilitate spontaneous voiding. After four basal clearances, 100 mg of triamterene was administered orally and hourly clearances were performed for six hours. Results were compared to a historic cohort of healthy subjects receiving 100 mg triamterene in a similar setting.[3]

Exome sequencing

The analysis of the exome data was divided into two steps: the renal gene panel analysis and the exome analysis. In the renal gene panel analysis, an in silico enrichment of genes associated with genetic renal disorders was performed (version: DGD141114). After the patient consented for the second step, exome analysis, likely pathogenic variants in all coding genes were analyzed. Exome sequencing was performed using a Illumina HiSeq2000TM sequencer at BGI-Europe (Copenhagen, Denmark). Read alignment to the human reference genome (GrCH37/hg19) and

variant calling was performed at BGI using BWA and GATK software, respectively. Variant annotation was performed using a custom designed in-house annotation and variant prioritization pipeline.

Urinary extracellular vesicles

Previously we showed that increased or reduced abundances of renal epithelial transport proteins (e.g., in Gitelman or Gordon syndrome) are reflected in urinary extracellular vesicles.[4, 5] To address whether the novel mutation reported in our family is characterized by increased αENaC abundance, we isolated and analyzed urinary extracellular vesicles, as reported previously.[5] Briefly, all urine samples were treated with a protease inhibitor (Complete Protease Inhibitor Tablet, Roche Diagnostics, Mannheim, Germany) before storage at -80°C. Urinary exosomes were isolated using a 2-step centrifugation process. First, urine was centrifuged at 17,000 x g for 15 minutes at 37°C to remove whole cell membranes and other high density particles. Dithiothreitol was used to disrupt the Tamm-Horsfall polymeric network. Subsequently, the samples were subjected to ultracentrifugation at 200,000 x g for 105 minutes at 25°C (Beckman L8-70M ultracentrifuge, Rotor 45 Ti). The pellet that formed during ultracentrifugation was suspended in isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6). Finally, the suspended pellets were solubilized in Laemmli buffer for Western blot analysis. Western blotting of the urinary exosomes was performed as described previously.[4, 5] The antibody against αENaC was a kind gift by Dr. J. Löffing (Institute of Anatomy, University of Zürich, Switzerland) and binds the N-terminal tail. For the patient study, the amount of sample loaded during Western blotting was normalized by the urinary creatinine concentration. CD9 was also blotted as urinary extracellular vesicle marker (R&D systems, Minneapolis, USA).

Site-directed mutagenesis, RNA in vitro transcription, and expression in Xenopus laevis oocytes

Mutant forms of the wild-type forms of human α-, β-, and γENaC subunits had been cloned in the pBSK(+)_Xglob vector.[6] In these vectors, the ENaC cDNAs are flanked by sequences corresponding to the 5' and 3' non-coding stretches of Xenopus β-globin, which boosts protein expression when injected into Xenopus oocytes.[7] Plasmids suitable for *in vitro* transcription of wild-type and mutant forms of the human αENaC subunit (hαENaC) were generated as follows. Using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA), we replaced the SacII-SacII fragment of pBSK(+)_hαENaC by a synthetic DNA strand (Eurofins Genomics GmbH, Ebersberg, Germany) including the 15-20 base pairs overlapping the termini of the digested plasmid, the 3' Xglobin UTR, but lacking the polyA-encoding stretch. A similar synthetic stand, containing the required T>C change was used to generate hαENaC_C479R mutant. The thus generated pBSK(+)_hαENaC_Δ(pA) plasmids were subsequently used to construct vectors containing the PolyA-encoding stretch by subcloning their EcoRI-SpeI inserts (comprising both 5' and 3' UTRs, and the αENaC ORFs) into pSDEasy(SB)Δ(SmaI-EcoRV) (a version of pSDEasy(SB) containing one single EcoRI recognition site in the multiple cloning stretch), leading to pSD(Xglob)_hαENaCwt and pSD(Xglob)_hαENaC(C479R). To generate vectors for wildtype and C479R mutant hαENaC containing a C-terminal triple-HA-tag (3x(YPYDVPDYA)), we used synthetic DNA strands corresponding to the Bpu10I-BamHI inserts of the previously generated vectors, flanked by 15-18 bp stretches overlapping the ends of the digested vectors, and with the sequence encoding the extended HA-tag in-frame with and preceding the stop codon. A novel HpaI/HincII site (GTT/AAC) was generated as well by introducing an additional Adenosine base shortly after the stop codon to facilitate the

identification of positive clones. Cys394Ser and Cys479Ser mutants of hαENaC were generated by replacing the SmaI-MluNI insert of pSD(Xglob)_hαENaCwt by the corresponding synthetic DNA fragments with the required mutated sequences to introduce either a Cys394Ser or a Cys479Ser conversion. The synthetic DNA fragments contain as well a silent mutation resulting in a novel, unique XbaI site, and comprise flanking sequences for annealing with the ends of the digested plasmid. Plasmids with wildtype and mutant forms of rat ENaC subunits have been described elsewhere.[8] All constructs were verified by sequencing.

In vitro transcription of cRNAs

pSDEasy vectors containing hαENaC cDNAs were linearized with BglII. pBSK(+)_Xglob vectors containing hβ-, and hγENaC cDNAs were linearized with NotI. Capped cRNAs were *in vitro* synthesized using either the SP6 (pSDEasy) or T7 RNA polymerase (pBSK(+)). Healthy stage V and VI Xenopus laevis oocytes were pressure-injected with mixes containing equal amounts of cRNAs encoding α-, β-, and γ-subunits for a total of (unless stated otherwise) 1 or 3 ng cRNA for human and rat ENaC, respectively. Oocytes were kept at 19°C in a solution containing (in mM) 85 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 10 HEPES, and 4.08 NaOH.

Electrophysiology

Electrophysiological measurements were made 24-32 h after injection with the standard two-electrode voltage clamp technique, using a TEV-200A voltage clamp amplifier (Dagan, Minneapolis, MN), an ITC-16 digitizer interface (Instrutech Corp. Elmont, NY), and the PatchMaster data acquisition and analysis package (HEKA Elektronik Dr. Schulze GmbH,

Ludwigshafen/Rhein, Germany). The two electrodes contained a 1 M KCl solution. All electrophysiological experiments were performed at room temperature (22°C). The holding potential was −80 mV. The composition of the perfusion solution was (in mM) 120 NaCl, 2.5 KCl, 1.8 CaCl2, and 10 HEPES. The amiloride-sensitive currents were measured in the presence of 10 μM of this blocker adjusted in a separate solution. Inward Na^+ currents were generated by switching from the amiloride-containing perfusion solution to that without amiloride. In the experiment with proteases, the oocytes were perfused for 2 min with the amiloride-free solution supplemented with 2 μg/ml trypsin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). All INa values were normalized in all experiments to the mean of the amiloride-sensitive currents measured for wildtype ENaC with the same oocyte batch.

Isolation of ENaC-enriched fractions

To isolate membrane fractions, 15-30 oocytes were disrupted by pipetting in 1.5 ml of membrane isolation buffer (in mM): 50 Tris/HCl (pH 7.0 at room temperature), 150 NaCl, 5 MgCl2, 1 DTT, followed by centrifugation through cell shredders (Macherey and Nagel, Oensingen, Switzerland). The membrane pellets obtained after 30 min centrifugation at 20,000 g (4°C) were resuspended in membrane solubilization solution (25 μl per oocyte) containing (in mM): 50 Tris/HCl (pH 7.0 at RT), 150 NaCl, 1 DTT and 1% (v/v) Triton X100. Membrane proteins were solubilized by 45-60 min incubation on an orbital shaker at 4°C, and centrifuged for 12 min as before. The Triton-soluble fractions thus obtained were mixed with 5x Sample buffer (30 mM DTT final concentration) and heated for 20-30 min at 37°C.

Western blot analysis of Triton-soluble fractions

Proteins in samples were resolved, along with pre-stained molecular weight markers (peqGold Protein Marker V, Peqlab Biotechnologie GmbH, Erlangen, Germany #27-2210), by SDS-PAGE on 5–15% acrylamide gradient minigels supplemented with 0.5% (v/v) of 2,2,2-Trichloroethanol (Acros-Organics, Geel, Belgium) for subsequent, in-gel, fluorescently labelling of proteins.[9] After electro-transfer onto nitrocellulose (Membrane Solutions, Bellevue, WA, #MSNC020230301), the blotted, fluorescently-labelled proteins were imagined on an UV-transilluminator using an 8-bit digitizing system (Vilber Lourmat GmbH, Eberhardzell, Germany) and data stored as non-compressed TIF images. Total protein per lane was assessed densitometrically from these images using ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016). A rabbit antibody against γENaC was a generous gift from Dr. J. Löffing's lab (Institute of Anatomy, University of Zürich, Switzerland). Anti-HA tag mouse monoclonal antibody (clone 16B12, #HA.11) was purchased from Covance, Dedham, MA. After 1 h of blocking at room temperature in 0.1% (w/v) casein solution, the blots were incubated overnight with the primary antibody in blocking solution supplemented with 0.1% (v/v) Tween-20 (CBB-T). After three rounds of washing over 20–30 minutes in TBS, 0.1% Tween-20 (TBS-T), the blot was incubated for 1 h in the presence of IRDye-conjugated secondary antibodies (LI-COR Biosciences GmbH, Bad Homburg, Germany): Polyclonal goat antibody anti-rabbit IRDye 800CW (#926–32211) and goat anti-mouse IRDye 680CW (#926–68070), all diluted 1/12,000 in CBB-T. After washing in TBS-T, the blots were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensities were assessed with the Odyssey v2.1 software and normalized with the amount of total, TCE-labelled, protein in the corresponding lanes.

Analysis of cell-surface biotinylated fractions

Control or injected oocytes (~25 per condition) were incubated for 15 min on ice in 1 ml Biotinylation buffer (in mM, Triethanolamine 10, NaCl 150mM, $CaCl_2$ 2, pH 9.5, supplemented with 1 mg/ml NHS-Sulfo-S-S-Biotin; Thermo-Scientific #21331). To have controls for non-specific binding to Neutravidin beads, equal amounts of oocytes were incubated in the absence of biotinylation reagent. The residual reagent was quenched by replacing the biotinylation solution with 1 ml of MBS supplemented with (mM) Glycine 192, Tris/HCl 25, pH 7.5 and incubating for 5 min at 22°C. After one rinsing step in MBS, drained oocytes were stored at -20°C. Biotinylated fractions were isolated from ENaC-enriched fractions which had been purified as described before, except for the substitution of 1 M DTT with 10 mM N-Ethylmaleimide. 25 µl samples of each Triton-X100-soluble fraction were withdrawn and mixed with equal volumes of 2xSample buffer/DTT (50 mM final) for further analysis. The rest of the isolated fractions were adjusted to 150 mM NaCl (from a 5 M stock solution) and incubated on an orbital shaker for 5-6 h at 4°C in the presence of 25 µl (bed volume) of Neutravidin-agarose beads (Thermo Scientific #29202). Non-bound fractions were discarded, the beads were washed twice with high salt solution (in mM, Tris/HCl pH 7.5, 50; NaCl, 500; EDTA, 5; PMSF 1, and 1% (v/v) TritonX100) by incubating each time for 5 min on an orbital shaker at 22°C. Beads were rinsed once in low salt solution (in mM, Tris/HCl pH 7.5, 50; NaCl, 150; PMSF 0.5, and 0.5% (v/v) TritonX100), and resuspended in 50 µl of 2xSample buffer/DTT (50 mM final). Bound fractions were eluted by heating for 10 min at 72°C. Triton-soluble fractions (1% of total) and Neutravidin-bound fractions were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blocked as described before. Nitrocellulose membranes were subsequently incubated overnight at 4°C in the presence of rat monoclonal anti-HA Antibody (Clone 3F10,

Roche #11 867 423 001) diluted 1/1,000 in 0.1% (w/v) casein solution and, after washing, 2 hours in the presence of HRP-conjugated Goat Anti-Rat immunoglobulins (Jackson ImmunoResearch Europe #112-035-03) diluted 1/5,000 in 1% (w/v) skimmed milk, in PBS. After washing, HRP signal was revealed using Western Bright Sirius detection reagent (Advansta, Menlo Park, CA, #K12043-D20) and detected using a Fusion Solo imaging system (Vilber Lourmat, Marne-la-Vallée, France). Band intensities were measured from 16-bit gray-scale images using ImageJ software. To account for non-specific binding to Neutravidin beads, band intensity values from control, non-biotinylated samples were subtracted from the values of the corresponding, biotinylated samples.

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