Supporting Information

Garcia-Caballero et al. 10.1073/pnas.0903609106

SI Materials and Methods

Tissue Procurement and Cell Culture. Human excess donor lungs and excised recipient lungs were obtained at the time of lung transplantation from portions of main stem or lumbar bronchi, and cells were harvested by enzymatic digestion as previously described under a protocol approved by the University of North Carolina School of Medicine Institutional Review Board (1). All preparations were maintained at an air-liquid interface in a modified bronchial epithelial medium and used 2–5 weeks after seeding on 12 mm T-Clear inserts (Corning Costar) coated with human placental type VI collagen (Sigma). PBS was used for washing human bronchial epithelial culture mucosal surfaces.

Sampling of Short Palate, Lung, and Nasal Epithelial Clone (SPLUNC)1 and Western Blotting. Airway surface liquid was collected by lavaging human bronchial epithelial cultures with 100 μL PBS at 37 °C for 15 min. The lavage was then centrifuged for 5 min at 4,000 $\times$ *g* to remove dead cells, and the supernatants were incubated overnight on an end-over-end rotator at 4 °C with trypsin-agarose beads (Sigma) ± aprotinin (Sigma). The beads were eluted using 30 μL Laemmli buffer, boiled at 95 °C for 5 min, separated on a 15% SDS PAGE gel per the University of North Carolina–Duke Michael Hooker Proteomics Center standard procedures (http://proteomics.unc.edu/protocol.shtml). Visible bands were excised and prepared for MS analysis by MALDI-MS/MS as described previously (2).

Airway surface liquid collected as described above was also placed in protease-inhibitor mixture (Roche) for Western blotting. The protein concentration was determined using the BCA Assay (Pierce). To obtain SPLUNC1 from *Xenopus* oocytes, oocytes were lysed in laemlli buffer or oocyte media was directly sampled and placed in laemlli buffer. Proteins were resolved using SDS/PAGE and transferred to a PVDF membrane. The membrane was then probed using αSplunc1 or αV5 antibodies and a Donkey anti-Mouse HRP antibody (R & D Systems). The blots were then incubated with ECL reagents (Pierce).

Transepithelial Voltage and Resistance Studies. A single-barreled PD-sensing electrode was placed in the airway surface liquid by micromanipulator and used in conjunction with a macroelectrode in the serosal solution to measure transepithelial voltage using a voltmeter (World Precision Instruments). Trypsin (2 U/mL; Sigma) was added mucosally as a dry powder in perfluorocarbon to test for changes in regulation of epithelial Na^+ channel (ENaC) as previously described (1). Transepithelial resistance was routinely measured using the EVOM system (WPI) as previously described (1).

Flp-in HEK293 Cell Culture and SPLUNC1 Protein Purification. Flp-In HEK293 cells (Invitrogen) were transfected with pcDNA5/FRT/V5-his-TOPO/hSplunc1 vector. SPLUNC1-expressing clones were selected using hygromycin, isolated, and analyzed for expression. The clones that stably express SPLUNC1 were cultured in T75 flasks in DMEMH media containing 5% FBS and at 37 °C in 5% CO_2. His-tagged SPLUNC1 was purified from cultured media by dialyzing the media into the His-Select Binding Buffer (50 mM sodium phosphate, pH 8.0/300 mM sodium chloride/10 mM imidazole) overnight at 4 °C, incubating the dialyzed media with His-Select Nickel Affinity Matrix (Sigma) for 4 h at 4 °C on an end-over-end rotator in the presence of protease inhibitors (Roche), applied to a column, and washed with 40 mL of His-Select Binding Buffer. SPLUNC1 was then eluted from the Cobalt affinity matrix in 0.5 mL fractions with 600 mM imidazole in His-Select Binding Buffer. Purified SPLUNC1 was then exchanged into Ringer. Cultured media from FlpIn HEK293 cells lacking SPLUNC1 was processed in the same way as media from FlpIn HEK293-SPLUNC1 cells and used as a control for experiments where purified SPLUNC1 was added.

Fluorogenic Assay. To determine whether SPLUNC1 inhibited trypsin activity, we assayed cleavage of the Di-tert-butyl dicarbonate-Gln-Ala-Arg-7-methoxycoumarin-4-yl)acetyl (BGAR-MCA) fluorogenic substrate in Ringer (Peptides Int.) excited at 350 nm and emission collected at 460 nm in a 96-well plate reader format (1420 VICTOR^2; Wallac). For cell-free assays, reactions were carried out in 50 μL Ringer in a 96-well plate format with 100 μM BGAR-MCA. To measure endogenous protease activity in human bronchial epithelial cultures, 30 μL Ringer with 100 μM BGAR-MCA were placed directly onto the mucosal surfaces of human bronchial epithelial cultures grown on 12 mm T-clear inserts, and the cultures were assayed in 12-well plates.

Oocyte Studies. *Xenopus laevis* oocytes were harvested and injected as described (3). Defolliculated healthy stage V–VI oocytes were injected with 0.3 ng of cRNA of each ENaC subunit. Injected oocytes were kept in modified Barth's saline [in millimolars, 88 NaCl/1 KCl/2.4 $NaHCO_3$/0.3 $Ca(NO_3)_2$/0.41 $CaCl_2$/0.82 $MgSO_4$/15 Hepes, adjusted to pH 7.35 with Tris]. Oocytes were studied 24 h after injection using the 2 electrode voltage clamp technique as previously described (3). Oocytes were clamped at a holding potential of −60 mV. The change in amiloride-sensitive whole cell current as an indicator of ENaC activity was determined by subtracting the corresponding current value measured in the presence of 10 μM amiloride from that measured before the application of amiloride.

Coimmunoprecipitation. *Xenopus* oocytes were injected with either HA-N-Terminus or V5-C-Terminus (HA-NT/V5-CT) tagged subunits in combination with WT untagged rat $\alpha\beta\gamma$ENaC subunits (0.3 ng cRNA each) with or without V5-tagged SPLUNC1 and CAP2 (1 ng cRNA each). After 24 h, 40 eggs per experimental condition were lysed with buffer containing (in millimolars): 20 Tris, 50 NaCl, 50 NaF, 10 β-glycerophosphate, 5 $Na4P_2O_7$ pyrophosphate, 1 EDTA, pH 7.5, and protease inhibitors (complete, Roche), and aprotinin (Sigma). Cell lysates were prepared by passing the eggs through a 27G1/2 needle twice followed by centrifugation at 3,600 $\times$ *g* for 10 min at 4 °C. Supernatants were transferred to new tubes and samples were spun at 14,000 $\times$ *g* for 20 min at 4 °C. Supernatants were discarded and pellets were solubilized in (millimolars) 50 Tris, 100 NaCl, 0.1% triton X-100, 0.1% Nonidet P-40, 20 NaF, 10 $Na_4P_2O_7$, 10 EDTA + protease inhibitor mixture (Sigma), pH 7.5. Total inputs were taken from whole-cell samples representing 4% of total protein. Solubilized proteins were incubated with 50 μL of protein A and 5 μL of anti-HA antibody (Covance) overnight while tumbling at 4 °C. Samples were washed three times with (millimolars) 150 NaCl 50 Tris pH 7.5 buffer. Laemli buffer was added and samples were loaded on a 15% gradient Tris-glycine gel after incubation for 10 min at 96 °C. Samples were transferred to PDVF membranes, and Western blot analysis was performed using an anti-V5 (Invitrogen) monoclonal antibody. SPLUNC1 bound to ENaC only when ENaC and

SPLUNC1 lysates were used. Uninjected and SPLUNC1 lysates lacking ENaC were both negative for coimmunoprecipitation.

Short Hairpin RNA-Induced Knockdown of SPLUNC1. Our strategy was to select shRNA sequences from Dharmacon that targeted SPLUNC1 effectively by using transient siRNA in an immortalized human airway epithelial cell line (denoted AALEB) (4). We then generated viruses encoding the most effective siRNA. Passage-1 airway cells surviving 1 week of selection were then trypsinized, plated down on 12-mm T-clear inserts, and differentiated under air liquid interface conditions. At the time of the functional assays, we measured airway surface liquid SPLUNC1 protein levels by Western blotting to verify stable knockdown. An anti-luciferase shRNA-expressing adenovirus was infected separately as a control.

Generation of yfp-αENaC Expressing Cell Line and SPLUNC1 Binding Assay. The yfp-αENaC construct has previously been shown to function normally (5), and was subcloned into a lentiviral vector (pQCXIN). JME nasal epithelial cells express functional ENaCs (6). However, this attribute is lost after several passages. Thus, passaged JME cells that no longer expressed ENaC were stably infected with a lentivirus containing yfp-αENaC or an empty vector as a control, and the presence or absence of αENaC was confirmed using an antibody that was constructed "in-house" that was directed against αENaC (Fig. S5).

Recombinant SPLUNC1 was labeled with Texas Red according to the manufacturer's instructions (Pierce), and was freshly labeled on the day of each binding experiment. JME cells were plated on 12-mm T-Clear culture inserts (Corning Costar), and were cultured until confluent. Cultures were then washed 3× with PBS to remove cellular debris and incubated with varying concentrations of Texas Red-SPLUNC1 for 30 min in PBS^{++} (with Ca^{2+} and Mg^{2+}; 10 μL total volume) followed by a 5× wash with PBS. After this time yfp (514 nm excitation) and Texas Red fluorescence (590 nm excitation) were imaged a 60× water objective on a Nikon Ti-S inverted microscope equipped with an Orca CCD camera (Hamamatsu) switchable filter wheels (Ludl). Background fluorescence was subtracted from all images, and the mean thresholded intensity was quantified to obtain specific and nonspecific binding using Image J.

Confocal Microscopy. To label airway surface liquid, Ringer containing Texas Red-dextran (2 mg/mL; Invitrogen) was added to human bronchial epithelial culture mucosal surfaces. Perfluorocarbon was added mucosally to prevent evaporation of the airway surface liquid and the culture placed in a chamber containing 100 μL Ringer on the stage of a Leica SP5 confocal microscope with a 63× glycerol immersion objective. Five points per culture were scanned, and an average airway surface liquid height determined. For detailed methods, see ref. 1. For confocal microscopy, human bronchial epithelial cultures were bathed serosally in a modified Ringer solution containing (in millimolars): 116 NaCl/10 $NaHCO_3$/5.1 KCl/1.2 $CaCl_2$/1.2 $MgCl_2$/20 TES/10 glucose, pH 7.4. At all other times, human bronchial epithelial cultures were maintained in a modified BEGM growth medium which contained 24 mM $NaHCO_3$ gassed with 5% CO_2 (1). Perfluorocarbon (FC-77) was obtained from 3M and had no effect on apical surface liquid (ASL) height as previously reported (1).

PCR and Primer Sequences. PCR was performed using Amplitaq Gold Mastermix (ABI) and primers specific for SPLUNC1 at a final concentration of 200 nM. The primers used were: forward 5′- ctgatggccaccgtcctat-3′ and reverse 5′- aggtggatcctctcctgctt-3′. The reaction was performed according to the manufacturer's instructions with an extension time of 30 s for an Eppendorf MasterCycler. Water was used as a negative control, and SPLUNC1 cDNA as a positive control. Human cDNA was prepared from 200 ng of RNA using SuperScript II (Invitrogen), and 1 μL was used for each reaction. A product of the appropriate size ≈150 bp was detected by gel electrophoresis.

Statistical Analyses. All data are presented as the mean ± SE for n experiments. Airway cultures derived from 3 or more separate donors were used for each study, and each oocyte study was repeated on 3 separate occasions. Differences between means were tested for statistical significance using paired or unpaired t tests or their nonparametric equivalent as appropriate to the experiment. From such comparisons, differences yielding $P \leq 0.05$ were judged to be significant. All binding assays were fitted to the Hill equation.

1. Tarran R, Trout L, Donaldson SH, Boucher RC (2006) Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia. *J Gen Physiol* 127:591–604.
2. Loiselle DR, et al. (2005) Improved protein identification through the use of unstained gels. *J Proteome Res* 4:992–997.
3. Donaldson SH, et al. (2002) Regulation of the epithelial sodium channel by serine proteases in human airways. *J Biol Chem* 277:8338–8345.
4. Lundberg AS, et al. (2002) Immortalization and transformation of primary human airway epithelial cells by gene transfer. *Oncogene* 21:4577–4586.
5. Berdiev BK, et al. (2007) Molecular proximity of cystic fibrosis transmembrane conductance regulator and epithelial sodium channel assessed by fluorescence resonance energy transfer. *J Biol Chem* 282:36481–36488.
6. Tong Z, et al. (2004) Prostasin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* 287:L928–935.

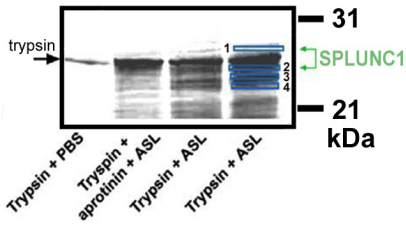

Fig. S1. SPLUNC1 is present in the airway surface liquid of human bronchial cultures. Airway surface liquid was incubated with trypsin-agarose beads ± aprotinin, and proteins were separated on 15% SDS gel and visualized with a silver stain. The outlined bands were then cut out and analyzed by MALDI-MS/MS, and the proteins identified are shown in Table S1. SPLUNC1 was detected in bands 1 and 2, and its binding to trypsin was attenuated in the presence of aprotinin.

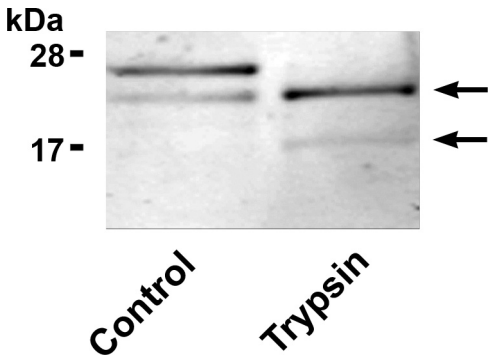

Fig. S2. SPLUNC1 is cleaved by trypsin. A 60-min treatment with 1 U/mL trypsin caused both a ≈1- and a 10-kDa shift in SPLUNC1 size. SPLUNC1 was labeled C-terminally with a V5 tag and detected with an anti-V5 antibody. SPLUNC1 cleavage products are shown with arrows.

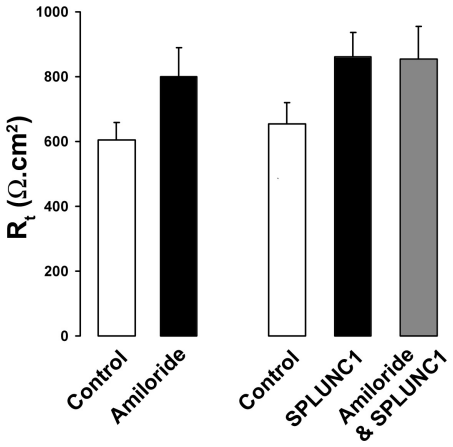

Fig. S3. SPLUNC1 affects the transepithelial resistance (Rt) in a similar fashion to amiloride. Human bronchial epithelial cultures were washed 5× with PBS over 1 h to remove native SPLUNC1, and then exposed to either 50 ng/mL recombinant SPLUNC1 for 30 min or 100 μM amiloride for 10 min or SPLUNC1 followed by amiloride. All $n = 12$. *, $P < 0.05$ different to control.

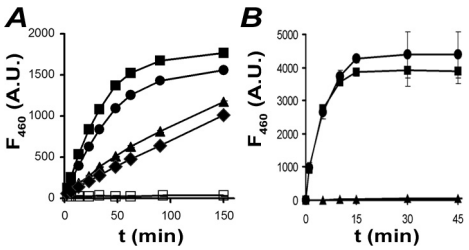

Fig. S4. SPLUNC1 moderately inhibits serine protease activity. (*A*) Cleavage of a serine protease-specific fluorogenic substrate (BGAR-MCA) in 50 μL Ringer solution measured as a change in >460 nm fluorescence (AU, arbitrary units); 1 U/mL trypsin (■); 1 U/mL trypsin and rSPLUNC1 (·); 0.3 U/mL trypsin (▲); 0.3 U/mL trypsin and rSPLUNC1 (♦); 1 U/mL trypsin and 2 U/mL aprotinin (□). All $n = 4$. (*B*) Spontaneous BGAR-MCA cleavage on human bronchial epithelial mucosal surfaces. Cultures were exposed to 30 μL Ringer containing BGAR-MCA and no additional proteases. Ringer alone (■); Ringer and rSPLUNC1 (·); Ringer and 2 U/mL aprotinin (▲). All $n = 8$. SPLUNC1 was used at 50 ng/mL. *, $P < 0.01$ different ± rSPLUNC1. Note: in some cases, error bars were obscured by the graph symbols.

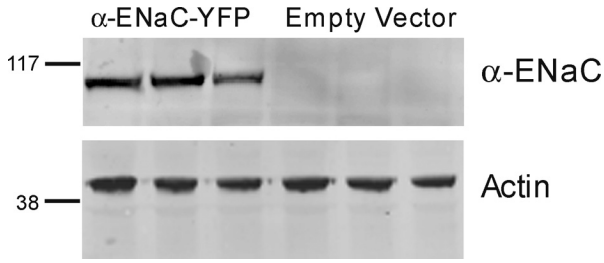

Fig. S5. Western blotting showing αENaC expression in JME cells stably transfected with pQCXIN-α-ENaC-YFP, but not in cells from the same passage infected with the empty pQCXIN vector.

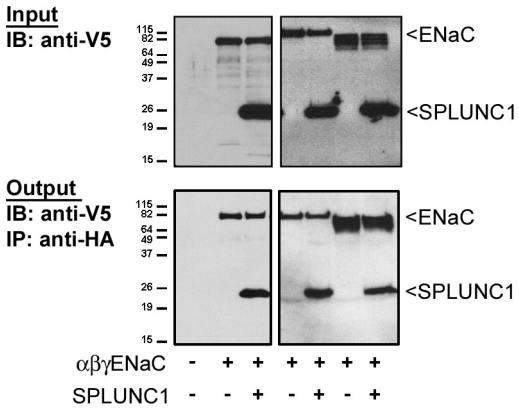

Fig. S6. SPLUNC1 binds to α, β, and γ-ENaC subunits. Oocytes were coinjected with 0.3 ng $\alpha\beta\gamma$ ENaC subunits ± SPLUNC1 (1 ng). Gels show representative coimmunoprecipitation of V5-tagged SPLUNC1 and HA-tagged ENaC subunits. Arrowheads, ENaC or SPLUNC1 bands; UI, uninjected control oocytes.

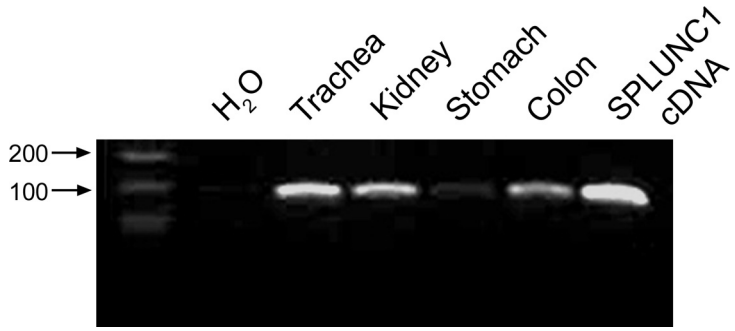

Fig. S7. SPLUNC1 is highly expressed in the trachea, colon, and kidney; cDNA was obtained from whole trachea, kidney, stomach, and colon vs. specific SPLUNC1 cDNA.

Table S1. Proteins in ASL identified by MS that bind to trypsin-agarose beads

Band no.	Protein name	Accession no.
1	SPLUNC1	AAF70860
2	SPLUNC1	AAF70860
	AY513239	AAR89906
3	Complement C3 Precursor	C3HU
4	Hypothetical protein	Q8WVW5_HUMAN

For corresponding blot, see Fig. S1.