Supporting Information

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SI Materials and Methods.

Genotyping. DNA was extracted from tails of mice by using REDExtract-N-Amp Tissue PCR Kit (Sigma) by a standard protocol. For genotyping, to detect Nedd4L, the following primers were used: Forward: CTCCCCACTGCAGTTCCTACC; Reverse: AGCTGCTCAGGCTGAATCACC, with the expected WT allele of 250 bp and the floxed allele of 300 bp. To detect SPC-rtTA, forward primer: GAC ACA TAT AAG ACC CTG GTC; Reverse primer: AAA ATC TTG CCA GCT TTC CCC, with an expected 350-bp fragment. To detect Cre: Forward primer: GTT CGC AAG AAC CTG ATG GAC A; and Reverse primer: CTA GAG CCT GTT TTG CAC GTT C, with an expected 290-bp fragment.

Single Cell RT-PCR Analysis. To verify knockout of Nedd4L (i.e., the excision of exon 15), type II cells were isolated as below. Single type II cells were then obtained under the microscope to avoid contamination of other cells. RT-PCR was performed using Qiagen Single Cell RT-PCR kit with primers spanning exon 11 to 18, followed by nested PCR with primers from exon 12 to a proximal portion of exon 18, by HotStar Taq plus polymerase (Qiagen). PCR products from wild type and knockout type II cells were then sequenced to further verify the deletion of exon 15.

Histology. Ten-day-old pups were sacrificed by cervical dislocation, the abdomen opened, and an incision made in the diaphragm to collapse the lungs. A 30-gauge blunted needle was inserted into the trachea and lungs were inflated with 10% neutral-buffered formalin under 20-cm H_2O pressure. Lungs were then excised and fixed with 10% neutral-buffered formalin for 12 h. They were subsequently dehydrated, embedded in paraffin, sectioned (5 μm), and stained with H&E, periodic acid/Schiff, or collagen (Trichrome) staining.

For cryo sections, lungs were fixed in 4% formaldehyde in PBS plus 0.02% NP-40 and 2 mM $MgCl_2$ overnight at 4 °C and then washed with PBS plus 0.02% NP-40 and 2 mM $MgCl_2$ (15 min, ×3). The lungs were subsequently soaked in 30% sucrose in PBS overnight at 4 °C and then embedded in optimal cutting temperature (OCT) for 30 min at room temperature and 30 min on dry ice. The OCT blocks were then sectioned for subsequent staining or stored at −80 °C.

Lung Wet/Dry Ratio Measurements. Newborn mice were sacrificed 4 h after birth, both lungs isolated immediately, blotted on filter paper, and weighed to obtain lung wet weight. The lungs were then incubated in a 50 °C oven and weighed every 24 h until lung weight stabilized (>48 h).

Lung Explant Growth in Culture. Lung explant growth assay was performed as described (1). Briefly, 16-d gestational age pregnant female were sacrificed by cervical dislocation. The abdomen was opened and fetuses transferred to a cold F-12 medium (GIBCO) on ice. The left lungs of the fetuses were placed in DMEM/F12 medium (GIBCO) on ice. Under the microscope, the peripheral-most ribbon of the left lung was dissected and cut into small pieces (lung explants). Three pieces of lung explants per fetus were placed in 24-mm Corning Transwell-Clear permeable supports with DMEM-F12 medium containing bovine serum albumin (1 μg/mL), in the absence/presence of amiloride (10 μM, 24 h, Sigma), and incubated at 37 °C in a 95% air-5% CO_2 environment. Fresh medium was changed after 24 h and kept throughout the experiment. Photographs of the explants were then taken at 2, 24, 48, and 72 h. The size of lung explants was measured by pixel count using Photoshop (Adobe). The change in size of the explants was then calculated.

Isolation of Alveolar Type II Cells. Alveolar type II cells were isolated from lungs of ~2-wk-old mice as described (2), with minor modifications. Briefly, mice were sacrificed by cervical dislocation and the abdominal artery severed to exsanguinate the animals. The thoracic cavity was quickly opened to expose the heart, and the right ventricle was perfused with PBS to remove blood from the lungs. Dispase (0.8 mL, BD Biosciences) was injected into lungs via a blunted 20-gauge needle installed into the trachea, followed by 0.4 mL of 1% low-melting-point agarose (Sigma). Lungs were then excised and incubated in 2 mL dispase for 45 min at room temperature. They were then dissected into a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DME/F-12; Invitrogen) with 0.01% DNase, 1 mM L-glutamine and antibiotics, and chopped into fine pieces, followed by 10-min incubation at room temperature. This crude preparation was filtered through a series of Nitex filters (100, 41, and 10 μm; Millipore) and then centrifuged at 300 × *g* for 8 min at 4 °C. The cell pellet was resuspended in 1 mL medium, and 1 mL RBC lysis buffer was added to lyse residual RBC. After centrifugation, cells were incubated with biotinylated antimacrophage antibodies (anti-CD45, anti-Ter 119, and anti-CD16/32; BD Biosciences) for 15 min and then selected by streptavidin-conjugated magnetic beads (Promega) in a magnetic block (Stemcell). Cells were then incubated in tissue culture dishes precoated with mouse IgG for 2 h at 37 °C. Nonadherent type II cells were removed and resuspended in tissue culture medium consisting of DME/F-12, 1 mM L-glutamine, 0.25% bovine serum albumin (Sigma), 5% rat serum, 10 mM Hepes (Wisent), 0.1 mM nonessential amino acids (Invitrogen), 0.05% insulin-transferrin-sodium selenite (Roche), and 100 μg/mL Primocin (Invitrogen). The cells were then plated onto 35-mm dishes (Nunc) or coverslip glass that was precoated with 1 μg laminin 5 (Millipore) for 2 h at 37 °C and maintained at 37 °C with 10% CO_2.

Double Immunofluorescence Staining. For double staining of αENaC and surfactant protein C (SPC) on lung tissue cryo sections, first, an antigen retrieval was performed in citrate buffer (pH 6.0) in a steamer for 20 min. Sections were then blocked with 10% normal donkey serum for 1 h at room temperature followed by overnight incubation with rabbit anti-αENaC antibodies (1:100) under nonpermeabilizing conditions. Subsequently, Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) was used to detect αENaC staining. After PBS washes, cells were permeabilized in 0.1% Triton X-100 and then incubated with goat anti-SPC antibodies (1:200) for 3 h. The sections were then washed in PBS and incubated with FITC-conjugated anti-goat secondary antibody (Jackson ImmunoResearch Laboratories). Cytospin slides prepared for immunocytochemistry were stained using the same procedure as for tissues but antigen retrieval step was omitted. Controls had primary antibodies omitted to ensure antibody staining specificity. The immunostained lung tissues and cells were assessed with confocal microscopy (Carl Zeiss) using the Zeiss LSM program. For EM analysis, lungs were isolated and placed in fixative (4% formaldehyde and 1% glutaraldehyde in phosphate buffer). Pieces containing the trachea and some bronchi were cut out, and processed exactly as described (1).

Patch-Clamp Recording in Primary Cultured Alveolar Type II Cells. After two days in culture, the above isolated type II cells were used for patch-clamp recording. Conventional whole-cell recordings were performed using an Axopatch-1D amplifier (Axon Instruments) at room temperature (22 °C). The extracellular solution used for recordings contained (in mM) 140 NaCl, 2 $CaCl_2$, 5.4 KCl, 15 Hepes, 1 $MgCl_2$, and 10 glucose (pH 7.35). Recording electrodes were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments) using a P-87 horizontal pipette puller (Sutter Instrument Company). The intracellular solution (ICS) consisted of (in mM) 154 K-gluconate, 10 Hepes, 2 MgATP, 1 $MgCl_2$, and 2 EGTA. The pH of the ICS was adjusted to 7.25 with KOH and the osmolarity was corrected to a range of 310–315 mOsM. After whole-cell configuration the recorded cell was held at −60 mV and under such conditions the access resistance ranged from 25 to 40 MΩ. Three to five minutes after whole-cell configuration, transmembrane currents were elicited by a voltage-ramp protocol (a steady voltage change from −100 to +60 mV within 1.0 s). To examine ENaC conductance, the ENaC inhibitors amiloride (0.3–10 μM, Sigma) or Benzamil (0.1–1.0 μM, Sigma) were focally applied to the cell that was under recording, via a computer-controlled multibarrelled fast-step perfusion system (SF-77B, Warner Instruments). All electrical signals were digitized, filtered (2 kHz), and acquired online using Clampex software and analyzed off-line using Clampfit software (Axon Instruments). Traces of the V-ramp elicited transmembrane currents were plotted using Sigma Plot software. The amiloride-sensitive or benzamil-sensitive component of transmembrane current was obtained by subtracting the total transmembrane current recorded in the presence of drug from that recorded in its absence. The amiloride-sensitive or benzamil-sensitive conductance was calculated for each cell studied. The Mann–Whitney U test was used for statistical analysis and $P < 0.05$ was considered significant.

1. Elias N, et al. (2007) The role of alpha-, beta-, and gamma-ENaC subunits in distal lung epithelial fluid absorption induced by pulmonary edema fluid. *Am J Physiol-Lung C* 293:L537–545.
2. Demaio L, et al. (2009) Characterization of mouse alveolar epithelial cell monolayers. *Am J Physiol-Lung C* 296:L1051–1058.

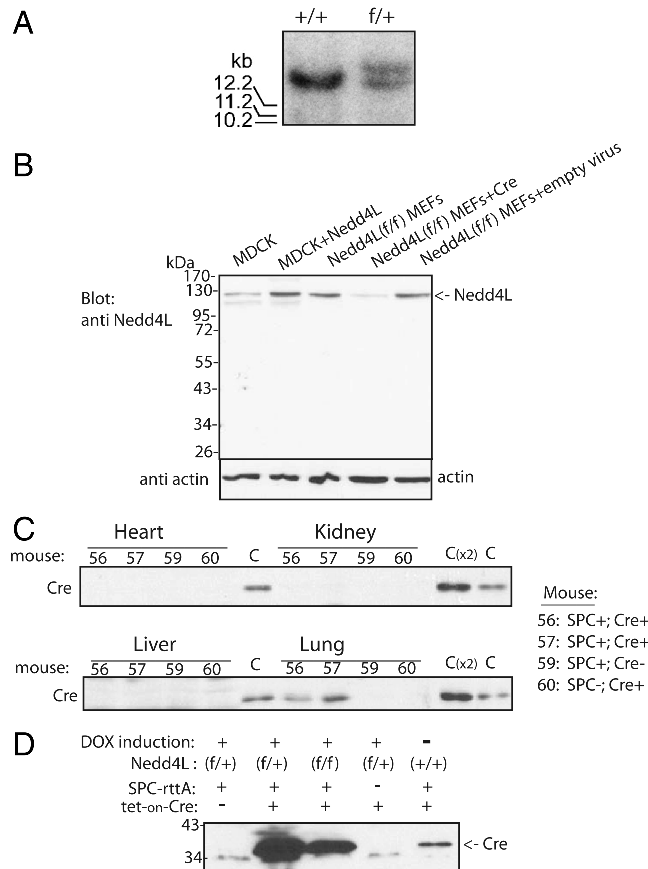

Fig. S1. Characterization of targeted ES cells, mouse embryonic fibroblast (MEF) cells, and Cre expression in Nedd4L floxed mice. (*A*) Southern blot analysis of genomic DNA of targeted embryonic stem cells. Genomic DNA purified from cells was digested with BamHI for Southern blotting. The 15-kb and 17-kb bands represent WT (+/+) and heterozygote $Nedd4L^{flox/+}$ (floxed, $f/+$) alleles, respectively. (*B*) Knockout of Nedd4L in $Nedd4L^{f/f}$ MEFs infected with a retrovirus expressing Cre-IRES-GFP leads to ablation of the whole protein in these cells, not just the HECT domain. The anti-Nedd4L antibodies used for immunoblotting were directed against an N-terminal region of the protein. The presence of trace amounts of Nedd4L in the MEFs infected with Cre-IRES-GFP most likely represents incomplete infection by the virus. Controls include $Nedd4L^{f/f}$ MEFs infected with empty virus (IRES-GFP alone), MDCK cells (which express endogenous Nedd4L), and MDCK cells transfected with cDNA for Nedd4L. (*C*) Expression of Cre (driven by the SPC promoter) only in the lung, but not in kidney, heart, or liver of mice. Each lane was loaded with 60 μg protein and blots immunoblotted with anti-Cre antibodies. The genotype of each mouse (numbered 56, 57, 59, and 60) is provided. C and C(×2) are positive controls loaded at 1× and 2× amounts, respectively. (*D*) Level of expression of Cre analyzed by immunoblotting in lungs of the indicated lines, in the absence or presence of doxycycline (Dox) induction (200 mg/kg in food plus 2 mg/mL in 5% sucrose solution in water) given to pregnant mothers at E8-E14. Note the presence of Cre expression in the absence of Dox induction. All lanes are loaded with equal amounts (100 μg) of proteins.

A Nedd4L$^{+/+}$;SPC-rtTA; tet^{o}-Cre

1x

20x

B Nedd4L$^{+/f}$;SPC-rtTA; tet^{o}-Cre

2x

20x

C E18.5, Control

2x

20x

Fig. S2. Additional controls showing normal lungs in Nedd4L WT or heterozygote mice. Additional controls for analysis of lung phenotype in (*A*) Nedd4L$^{+/+}$;SPC-rtTA;tet^{o}-Cre and (*B*) Nedd4L$^{+/f}$;SPC-rtTA;tet^{o}-Cre mice. (*C*) Control lung from E18.5 embryo harvested from mothers treated with Dox, as described in Fig. 2*D*. (*Left*) Whole (or section) of a lung (1× or 2× original magnification) and (*Right*) enlargement of the lung segment (20× original magnification).

A KO

B Control

50µm

Fig. S3. Trichrome (collagen) staining of Nedd4L knockout lungs. Lungs from 10-day-old (*A*) Nedd4L KO (Nedd4L$^{f/f}$;SPC-rtTA;tet°-Cre) mice or (*B*) controls (Nedd4L$^{f/f}$; SPC-rtTA) mice were stained with trichrome (collagen, blue color). Original magnification: 20×.

Fig. S4. Enhanced amiloride-sensitive dehydration of Nedd4L knockout lung explants. Lung explants harvested at E16 from Nedd4L KO (Nedd4L$^{f/f}$;SPC-rtTA;tet^{o}-Cre) mice and controls were grown in culture for 4 d and their size measured daily. (*A*) An example of lung explants grown in culture. (*B*) Summary of explant size (relative to day 1) from control and KO mice, in the presence of absence of 10 μM amiloride (Amil). Amiloride was added to the medium bathing the explants upon seeding (before the explant is enclosed in a sac, to ensure lumenal access to the drug). Note the restoration of explant expansion upon addition of amiloride to the KO explants. Values are mean ± SD of 39 (control), 27 (KO), and 12 (KO + amiloride) explants. $P < 0.005$ for both control vs. KO and KO + amiloride vs. KO alone (Student's *t* test).

Fig. S5. Decreased periciliary liquid (PCL) and increased proportion of secretory cells in lung tracheal epithelium of Nedd4L conditional KO mice. (*A*) An example of ciliated cells showing reduced cilia height in the Nedd4L KO (Nedd4L$^{f/f}$;SPC-rtTA;tet^{o}-Cre) trachea vs. controls (Nedd4L$^{f/f}$;SPC-rtTA). (*B*) A summary of PCL height from 42–52 cells, exhibiting a reduction in height of the KO cilia ($p = 0.004$, Student's *t* test). (*C*) Abnormal architecture of tracheal epithelium exhibiting increased proportion of secretory (most likely goblet) cells relative to ciliated cells in the Nedd4L KO mice (Nedd4L$^{f/f}$;SPC-rtTA;tet^{o}-Cre) vs. controls (Nedd4L$^{f/f}$;SPC-rtTA). (*D*) Morphometric analysis revealed 56% secretory cells in the knockout trachea ($n = 288/517$ cells) relative to 42% (51/120 cells) in the controls ($p < 0.005$, Student's *t* test).