Knockout mice reveal a major role for alveolar epithelial type I cells in alveolar fluid clearance

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ONLINE DATA SUPPLEMENT

Online Supplement

Materials and Methods

Generation of knockout mice

Mice with a floxed allele of the Na pump β1 subunit gene (*Atp1b1*) were generated. For this purpose, a conditional (floxed) *Atp1b1* gene targeting vector was constructed by bacterial recombineering using methods and reagents provided by the National Cancer Institute, Fredrick, MD (http://ncifrederick.cancer.gov/research/brb/recombineeringInformation.aspx) (1, 2). A Bacterial Artificial Chromosome (BAC) clone containing the *Atp1b1* gene was obtained after screening a mouse BAC library (RPCI-22, derived from mouse strain 129S6/SvEvTac) from Children's Hospital Oakland Research Institute, Oakland, CA. An 8,387 bp genomic *Atp1b1* fragment, representing the sequence starting 1,471 bp upstream of exon 3, was retrieved from the BAC clone into PL253, a pBluescript II SK vector containing Herpes Simplex Thymidine Kinase (HSV-*tk*) for negative selection. LoxP sites were subsequently inserted by bacterial homologous recombination to flank exon 4 of the *Atp1b1* gene, together with an FRT-flanked phosphoglycerate kinase promoter-Neomycin resistance (PGK-Neo^R) marker for positive selection. The construct was introduced into W4 ES cells (derived from mouse strain 129S6/SvEvTac), and positive clones that had gone through homologous recombination were identified by Southern blot hybridization with a flanking probe and verified by PCR. Positive clones with correct karyotype were injected into C57BL/6J host blastocysts and chimeric males were generated. Germline-transmitting chimeras were bred to FLPeR mice (3), strain 129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J, stock #003946, Jackson Laboratories, Bar Harbor, MA, to remove the FRT-flanked PGK-Neo^R selection marker. Resultant

floxed *Atp1b1* mice (*Atp1b1*$^{F/F}$) were backcrossed to 129S6/SvEvTac mice for 10 generations and then crossed to *Aqp5-cre* mice (also on 129S6/SvEvTac background), a line recently established in our laboratory (4), to generate mice deficient in the β1 subunit in AT1 cells (called *Atp1b1*$^{Aqp5\text{-}cre}$). In order to knock out the β1 subunit in the entire alveolar epithelium (i.e., both AT1 and AT2 cells), *Atp1b1*$^{F/F}$ mice were crossed to *Sftpc-cre* mice (on C57BL/6 genetic background) to generate *Atp1b1*$^{Sftpc\text{-}cre}$ mice. The *Sftpc-cre line* has been described previously (5) and was generously provided by Brigid Hogan, Duke University, Durham, NC. Therefore, *Atp1b1*$^{Aqp5\text{-}cre}$ and the corresponding *Atp1b1*$^{F/F}$ control mice were on a pure 129S6/SvEvTac background, while *Atp1b1*$^{Sftpc\text{-}cre}$ and *Atp1b1*$^{F/F}$ controls were on a mixed 129S6/SvEvTac:C57BL/6J background. Specificity and efficiency of the Cre transgenes in both lines was verified by crosses to the Cre-dependent reporter line *ROSA*$^{mT/mG}$ (6), purchased from Jackson Laboratories (strain Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J). For GFP reporter expression analysis, lungs were fixed/frozen and cryosectioned as described (6) and analyzed by confocal microscopy. Initially, we used *Nkx2.1-cre* mice (7) to generate an alveolar epithelium-specific β1 knockout line. The *Nkx2.1-cre* line was kindly provided by Stewart A. Anderson (Cornell University, New York, NY). Since *Atp1b1*$^{Nkx2.1\text{-}cre}$ mice died postnatally at 2-3 weeks of age, *Atp1b1*$^{Sftpc\text{-}cre}$ mice were used in this study. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California.

Verification of Atp1b1 gene deletion

Verification of correct Cre/loxP-mediated deletion of exon 4 in the floxed *Atp1b1* allele (*Atp1b1*$^{F/F}$) was performed by PCR amplification. Genomic DNA isolated from lungs of *Atp1b1*$^{Aqp5\text{-}cre}$ mice was used as template to amplify a predicted 343 bp PCR product from the deleted allele (*Atp1b1*$^{\Delta exon4}$), along with a 1226 bp product amplified from the intact *Atp1b1*$^{F/F}$ allele. The following PCR primers flanking exon 4 were used: forward (P1): 5'-GGGTCACCACAACATGAGGAACTA-3', reverse (P2): 5'-TGGTTATCAAAGGGCAGAGACCGT-3').

Alveolar fluid clearance (AFC)

AFC was measured according to previously published methods (8, 9). Mice 3-5 months old and 20-30 grams were used. Animals were sedated with diazepam, anesthetized with pentobarbital and placed supine on a warming pad. An incision was made in the neck area, trachea was exposed and transected, and a 20-gauge plastic catheter (Angiocath #381702, BD Infusion Therapy Systems, Sandy, UT) was inserted into the trachea for administration of an instillate comprised of 5% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO) plus 0.025% Alexa Fluor 594-conjugated BSA (Life Technologies, Carlsbad, CA) in phosphate-buffered saline (PBS). Before instillation, mice were paralyzed with pancuronium bromide (1 mg/kg) and mechanically ventilated with an Inspira ASV ventilator (Harvard Apparatus, Holliston, MA) with 100% O_2 at tidal volume (V_t) = 10 ml/kg, 150 breaths per minute (bpm), peak inspiratory pressure (PIP) = 12 cmH_2O and positive-end expiratory pressure (PEEP) = 3 cmH_2O. Arterial O_2 saturation and heart rate were measured continuously using a MouseOx (Starr Life Sciences, Oakmont, PA). Using the tracheal catheter, 12 ml instillate/kg of body weight

was administered and, after 30 minutes, fluid was aspirated. Fluorescence (excitation at 590 nm) in the instillate (F_i) and aspirate (F_a) was measured at an emission wavelength of 622 nm and used for AFC (%/hour) calculation as $(1 - F_i/F_a) \times 100 \times 2$.

In vivo lung permeability

Measurement of alveolar epithelial permeability *in vivo* was performed as described previously (10) using fluorescein-labeled BSA (F-BSA, Life Technologies). Animals were anesthetized with Ketamine/Xylazine (100 mg/kg and 20 mg/kg, respectively) and 10 mg/kg body weight of F-BSA (1 mg/ml) was injected into the jugular vein. Mice were then kept anesthetized during the following 2 hour period by re-administration of Ketamine (50 mg/kg). Two hours after injection of F-BSA, bronchoalveolar lavage fluid (BALF) and blood was collected. Blood was obtained by heart puncture and after coagulation for 30 min at room temperature and subsequent centrifugation at 1,300 x *g* for 10 minutes, serum was collected. BALF was obtained by lavaging lungs three times with PBS (30 µl per kg body weight). The three BALF samples were pooled and centrifuged for 10 minutes at 600 x *g* at 4°C to pellet cells and the supernatant was collected. Fluorescence in BALF supernatant and serum (diluted 100-fold in PBS) was measured using excitation/emission wavelengths of 494/520 nm. Lung permeability index was defined as the ratio between the fluorescence observed in undiluted BALF supernatant and that in 1:100 diluted serum.

Wet-to-dry lung weight ratios

Lungs were surgically removed, weighed and then dried at 65°C for 48 hours. Dry weight was recorded and wet-to-dry lung weight ratios were calculated.

Isolation of AT2 cells and primary culture of mouse alveolar epithelial cell monolayers (MAECM)

AT2 cells were isolated from Na-K-ATPase β1 subunit knockout (*Atp1b1*$^{Sftpc\text{-}cre}$) and floxed control (*Atp1b1*$^{F/F}$) mice (20-30 grams) according to our previously established laboratory protocols (11). Briefly, after mice were anesthetized with a lethal dose of Euthasol, lungs were cleared of blood by cardiac perfusion with PBS. Dispase (BD Biosciences, Bedford, MA) was instilled into lungs via the trachea, followed by 0.5 ml of 1% low-melting-point agarose (Sigma, St. Louis, MO). Lungs were excised and incubated in dispase for 45 minutes at room temperature. Lungs were dissected into wash medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12; Sigma) supplemented with 0.01% DNase, 10 mM HEPES (Sigma-Aldrich), 0.1 mM nonessential amino acids (Sigma-Aldrich), 1 mM L-glutamine (Sigma-Aldrich) and 0.2% Primocin (InvivoGen, San Diego, CA)). Lung pieces were chopped and the resulting crude cell mixture was passed through a series of Nitex filters (100, 40, 20 and 10 μm; Tetko, Elmsford, NY), followed by centrifugation at 300 x *g* for 10 minutes at 4°C. Erythrocytes and macrophages were removed from this crude cell mixture by incubation with biotinylated antibodies (anti-CD45, anti-Ter 119, and anti-CD16/32; BD Biosciences), followed by selection with streptavidin-conjugated magnetic beads (Promega, Madison, WI). Partially purified cell mixtures were then incubated on Petri dishes precoated with mouse IgG (Sigma-Aldrich) for 2 hours at 37°C.

Nonadherent AEC were removed from IgG plates. Purified AT2 cells were resuspended in complete mouse medium (CMM): DMEM/F-12 supplemented with 1 mM L-glutamine, 0.25% BSA (BD Biosciences), 10 mM HEPES, 0.1 mM nonessential amino acids, 0.05% insulin-transferrin-sodium selenite (ITS; Roche, Basel, Switzerland) and 0.2% Primocin. Purified AT2 cells in CMM supplemented with 2% newborn bovine serum (Omega Scientific, Tarzana, CA) were plated onto tissue culture-treated polycarbonate filters (0.4 μm pores, 1.13 cm^2; Corning Costar, Cambridge, MA) precoated with laminin-5 (1 μg/ml; Millipore, Billerica, MA) at $7.5x10^5$ cells/cm^2. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 + 95% air. Confluent monolayers formed by day 3 in primary culture. MAECM exhibit alveolar epithelial type I cell-like morphology and phenotype (11). Cells were fed with serum-free CMM every other day starting on day 3.

Bioelectric properties of MAECM

Transepithelial electrical resistance (R_T; $k\Omega \cdot cm^2$) and spontaneous potential difference (*PD*; mV) were measured using a Millicell-ERS (Millipore) device. Background R_T and *PD* were measured using blank filters and used to correct for measured R_T and *PD*. Equivalent short-circuit current (I_{EQ}; $\mu A/cm^2$) was calculated as PD/R_T using background-corrected values. R_T and *PD* were measured until day 10 after plating, starting from day 3 in culture. For measurements of short-circuit current (I_{SC}; $\mu A/cm^2$) in response to amiloride (10 μM; Sigma-Aldrich) or pimozide (10 μM; Sigma-Aldrich), monolayers were mounted in modified Ussing chambers and bathed on both sides with ITS-free CMM at 37°C. A stream of humidified 5% CO_2 in air was continuously blown

across surfaces of the bathing fluids (10 ml) to maintain constant pH and agitate bathing fluids. Monolayers were continuously short-circuited throughout the experimental period, except for brief interruptions (at 5 minute intervals) to allow measurements of *PD*. R_T of short-circuited monolayers was estimated intermittently as PD/I_{SC}.

Unidirectional flux of Na^+

Unidirectional flux of Na^+ was determined in the apical-to-basolateral (A→B) and basolateral-to-apical (B→A) directions across MAECM at 37°C using ^{22}NaCl (American Radiolabeled Chemicals, St. Louis, MO). Briefly, ^{22}NaCl (with a final specific activity of 0.5 μCi/ml) was added to the upstream (either apical or basolateral) fluid of monolayers mounted in modified Ussing chambers. During measurements of unidirectional Na^+ fluxes, monolayers were short-circuited to eliminate electrical gradients across the alveolar epithelial barrier. Downstream samples were taken at 30 minute intervals for up to 120 minutes. Aliquots of upstream fluid were taken at 10 minutes after ^{22}NaCl addition and at the end of each experiment. Samples were mixed with 10 ml of Ecoscint (National Diagnostics, Manville, NJ) and assayed for radioactivity in a liquid scintillation counter (LS 6000TA, Beckman, Fullerton, CA).

Western analysis

Whole lung lysates for Western analysis were prepared as follows: lungs were cleared of blood by transcardiac perfusion with PBS and homogenized on ice with a Polytron homogenizer in lysis buffer (2% SDS/10% glycerol/62.5 mM Tris, pH 6.8) including protease inhibitors (Protease Inhibitor Cocktail Set III, Millipore). After further lysis for 1

hour on ice and 15 minutes at 37°C, DNA was sheared by passing samples 5 times through a 25-gauge needle. Debris was removed by centrifugation for 15 minutes at 20,000 x *g* at 4°C. Lysates (supernatants) were stored at -80°C in aliquots. Protein from freshly isolated AT2 cells was prepared as above with the exception that the Polytron homogenizer was replaced by vortexing. Protein concentrations in all samples were measured with the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA). Protein samples to be subjected to Western analysis of Na-K-ATPase β subunit expression were deglycosylated with PNGase F (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. For Western analysis, equal amounts of protein in sample buffer (20-60 µg) were resolved by 10% SDS-PAGE under reducing conditions using the buffer system of Laemmli (12) and transferred to Immobilon-P membranes (Millipore). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 for 60 minutes and incubated overnight at 4°C with primary antibody. The following primary antibodies were used: rabbit anti-α1 subunit of Na-K-ATPase (#06-520, Millipore), rabbit anti-β1 subunit of Na-K-ATPase (#GTX113390, GeneTex Irvine, CA), goat anti-β3 subunit of Na-K-ATPase (sc-66343, Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-β_2 adrenergic receptor (#ab36956, Abcam, Cambridge, MA). The following antibodies were used for sample normalization purposes: rabbit anti-lamin A/C (#sc20681, Santa Cruz Biotechnology), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #AM4300, Life Technologies) and mouse anti-actin (#ab6276, Abcam). After washing, membranes were incubated with species-specific secondary antibodies (anti-rabbit, -goat or -mouse IgG) conjugated to horseradish peroxidase for 1 hour at room temperature, and antigen-antibody complexes were visualized by

enhanced chemiluminescence (Pierce, Rockford, IL) using the FluorChem Imaging System (Model 8900, Alpha Innotech, San Leandro, CA), which was also used for quantitation of specific protein bands and loading controls for normalization.

Lung histology

Lungs were cleared of blood by cardiac perfusion with PBS and then inflated with 4% paraformaldehyde (PFA) in PBS at 20 cmH_2O pressure. When pressure was stable, the trachea was tied off with surgical sutures. Lungs were then immersed in 4% PFA in PBS and fixed at 4°C for 16-20 hours. After embedding in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin (H&E).

Antibody staining

Na pump β3 subunit protein in MAECM was identified by immunofluorescence microscopy using a rabbit polyclonal β3 antibody (generously provided by Alicia McDonough, USC, CA). Briefly, MAECM on day 6 were rinsed with ice-cold PBS, fixed with 4% paraformaldehyde for 15 minutes and rinsed in PBS. Antigen retrieval was performed in Antigen Unmasking Solution, High pH (#H-3301, Vector Laboratories, Burlingame, CA). After permeabilization with 0.2% Triton X-100 in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 15 minutes at room temperature and rinses in TBS-T, MAECM were incubated in CAS Block (Life Technologies) plus 5% donkey serum for 1 hour at room temperature. Next, MAECM were incubated with the β3 antibody (diluted 1:1,000 in CAS Block) on a rocker overnight at 4°C. After washes in TBS-T, MAECM were incubated with secondary antibody (Alexa Fluor 488-conjugated donkey anti-rabbit

IgG (H+L), Life Technologies, diluted 1:500 in CAS Block) for 1 hour at room temperature on a rocker. After washes in TBS-T, MAECM were placed on glass slides, one drop of propidium iodide (PI) mounting media was added and coverslips placed on top. Stained specimens were viewed with an epifluorescence microscope (BX60, Olympus, Tokyo, Japan).

RNA isolation, reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from whole lungs, freshly isolated AT2 cells or MAECM using TRIzol (Life Technologies). RNA was quantified spectrophotometrically by determining absorption at 260 nm (A_{260}) and calculating concentration (µg/ml) from A_{260} x 40 x sample dilution factor. RNA purity was calculated from A_{260}/A_{280}. Integrity of RNA was checked by agarose gel electrophoresis prior to RT. RNA was reverse transcribed into complementary DNA (cDNA) using ThermoScript RT-PCR System (Life Technologies) with random hexamer primers as follows: incubation of 2 µg RNA at 65°C for 5 minutes, at 50°C for 50 minutes and at 85°C for 5 minutes. In parallel, RT reactions without reverse transcriptase were performed as control for contaminating genomic DNA. cDNA was analyzed by SYBR-Green qPCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Primers were designed using Primer Express 3.0 software (Applied Biosystems) and tested for specificity by dissociation analysis. PCR mixtures consisted of 0.5 µl cDNA, 10 µl SYBRGreen (Applied Biosystems), 5.5 µl PCR-water and 4 µl primer mix (2 µM forward and reverse primer). The qPCR procedure was comprised of three components; pre-amplification denaturation (one cycle at 50°C for 2

minutes and at 95°C for 10 minutes), amplification (40 cycles at 95°C for 15 seconds and at 60°C for 1 minute) and post-amplification (one cycle at 95°C for 15 seconds, at 60°C for 15 seconds, and at 95°C for 15 seconds). Sequences of all primers used, along with the expected product size and accession numbers, are shown in Table 1. *Polr2a* (RNA polymerase II α subunit), a housekeeping gene, was used as internal control.

Hyperoxia exposure

Mice were housed in cages with free access to food and water inside a hyperoxia chamber (Terra Universal, Fullerton, CA) for 65 hours. Sodium bicarbonate (Sigma-Aldrich) was used to absorb exhaled carbon dioxide. Oxygen concentration inside the chamber was continuously monitored with a MiniOX I Oxygen Analyzer (Ohio Medical, Gurnee, IL) and maintained at >95% at a flow of 15 l/hour (Visi-Float Flowmeter VFA, Dwyer Instruments, Michigan City, IN).

Ventilator-induced lung injury (VILI)

VILI was performed as previously described (13, 14). Mice of the same age and weight ranges as those used for AFC experiments (see above) were anesthetized with Ketamine/Xylazine (100/20 mg/kg, i.p.). An incision was made in the neck area, trachea was exposed and transected, and a 20-gauge plastic catheter (BD Infusion Therapy Systems) was inserted. The catheter was secured inside the trachea with surgical suture, after which the incision was closed. Mice were then placed in supine position on a warming pad (37°C) on a flat platform and connected to an Inspira ASV ventilator

(Harvard Apparatus). Non-injurious ventilation (room air) was performed for 3 hours with PIP = 20 cmH_2O, PEEP = 0 cmH_2O and respiratory rate = 70 bpm. Injurious ventilation (VILI) was performed for 3 hours with PIP = 40 cmH_2O, PEEP = 0 cmH_2O and respiratory rate = 25 bpm. Animals were monitored throughout the experiment by measuring arterial O_2 saturation and heart rate with a MouseOx device (Starr Life Sciences). As needed, mice were re-anesthetized with 50 mg Ketamine/kg body weight, based on increases in heart rate (approximately every 30 minutes).

Data analysis

Data are shown as mean ± SEM (standard error of the mean). Unpaired, two-tailed Student's *t*-test was used for comparisons of two group means. Multiple (≥3) group means were analyzed by one-way analysis of variance (ANOVA) with post-hoc tests based on Student-Newman-Keuls approaches. P <0.05 is considered statistically significant.

Results

Figure Legends

Figure E1. Verification of Na pump β1 subunit deletion. **A.** Verification of knockout in $Atp1b1^{Aqp5\text{-}cre}$ lung. Deletion of exon 4 in the floxed allele ($Atp1b1^{F/F}$) by Cre generates the deleted allele $Atp1b1^{\Delta exon4}$. PCR amplification of genomic DNA from $Atp1b1^{F/F}$ lung with primers P1 and P2 results in a 1226 bp product (lane "1"), while the deleted allele present in $Atp1b1^{Aqp5\text{-}cre}$ lung is amplified as a 343-bp product (lane "2") using the same primers. The $Atp1b1^{F/F}$ (1226 bp) product in lane 2 is contributed by cells not expressing

Cre in the lung. M = 100 bp ladder. **B**. Verification of knockout by Western analysis of Na pump β1 subunit protein in AT2 cells isolated from *Atp1b1*$^{Sftpc\text{-}cre}$ (KO) and *Atp1b1*$^{F/F}$ (F/F) mice. Results from three separate cell isolations (#1-3) are shown. In each cell isolation experiment, AT2 cells from two mice of each genotype were pooled. **C.** Activity of Cre lines used to generate knockouts of *Atp1b1*. Left panel: In *Aqp5*^{cre};*ROSA*$^{mT/mG}$ double transgenic mice, *Aqp5*^{cre} activates GFP (*mG*) expression from the *ROSA*$^{mT/mG}$ reporter transgene specifically in AT1 cells in the alveolar epithelium. Red fluorescence represents Tomato (*mT*) reporter in cells not expressing Cre (i.e., AT2, endothelial and other cells). Right panel: In *Sftpc*^{cre};*ROSA*$^{mT/mG}$ double transgenic mice, *Sftpc*^{cre} activates GFP expression from the *ROSA*$^{mT/mG}$ reporter transgene in both AT1 and AT2 cells, although the GFP signal is considerably stronger in AT2 cells. Tomato reporter is observed in cells not expressing Cre (i.e., endothelial and other cells). Blue is DAPI (nuclear).

Figure E2. Lung histology. No apparent differences were observed in distal lung histology between *Atp1b1*$^{F/F}$ and *Atp1b1*$^{Aqp5\text{-}cre}$ lungs (upper panels) or between *Atp1b1*$^{F/F}$ and *Atp1b1*$^{Sftpc\text{-}cre}$ lungs (lower panels).

References

1. Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* 2001; 2: 769-779.

2. Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* 2003; 13: 476-484.

3. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* 2000; 28: 106-110.

4. Flodby P, Borok Z, Banfalvi A, Zhou B, Gao D, Minoo P, Ann DK, Morrisey EE, Crandall ED. Directed expression of Cre in alveolar epithelial type 1 cells. *Am J Respir Cell Mol Biol* 2010; 43: 173-178.

5. Okubo T, Knoepfler PS, Eisenman RN, Hogan BL. Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation. *Development* 2005; 132: 1363-1374.

6. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis* 2007; 45: 593-605.

7. Xu Q, Tam M, Anderson SA. Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. *J Comp Neurol* 2008; 506: 16-29.

8. Mutlu GM, Dumasius V, Burhop J, McShane PJ, Meng FJ, Welch L, Dumasius A, Mohebahmadi N, Thakuria G, Hardiman K, Matalon S, Hollenberg S, Factor P. Upregulation of alveolar epithelial active Na^+ transport is dependent on β2-adrenergic receptor signaling. *Circ Res* 2004; 94: 1091-1100.

9. Hardiman KM, Lindsey JR, Matalon S. Lack of amiloride-sensitive transport across alveolar and respiratory epithelium of iNOS$^{-/-}$ mice in vivo. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L722-L731.

10. Han X, Fink MP, Uchiyama T, Yang R, Delude RL. Increased iNOS activity is essential for pulmonary epithelial tight junction dysfunction in endotoxemic mice. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L259-L267.

11. DeMaio L, Tseng W, Balverde Z, Alvarez JR, Kim KJ, Kelley DG, Senior RM, Crandall ED, Borok Z. Characterization of mouse alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2009; 296: L1051-L1058.

12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.

13. Kage H, Flodby P, Gao D, Kim YH, Marconett CN, DeMaio L, Kim KJ, Crandall ED, Borok Z. Claudin 4 knockout mice: normal physiological phenotype with increased susceptibility to lung injury. *Am J Physiol Lung Cell Mol Physiol* 2014; 307: L524-536.

14. Li G, Flodby P, Luo J, Kage H, Sipos A, Gao D, Ji Y, Beard LL, Marconett CN, DeMaio L, Kim YH, Kim KJ, Laird-Offringa IA, Minoo P, Liebler JM, Zhou B, Crandall ED, Borok Z. Knockout mice reveal key roles for claudin 18 in alveolar barrier properties and fluid homeostasis. *Am J Respir Cell Mol Biol* 2014; 51: 210-222.

Figure E1 (online supplement)

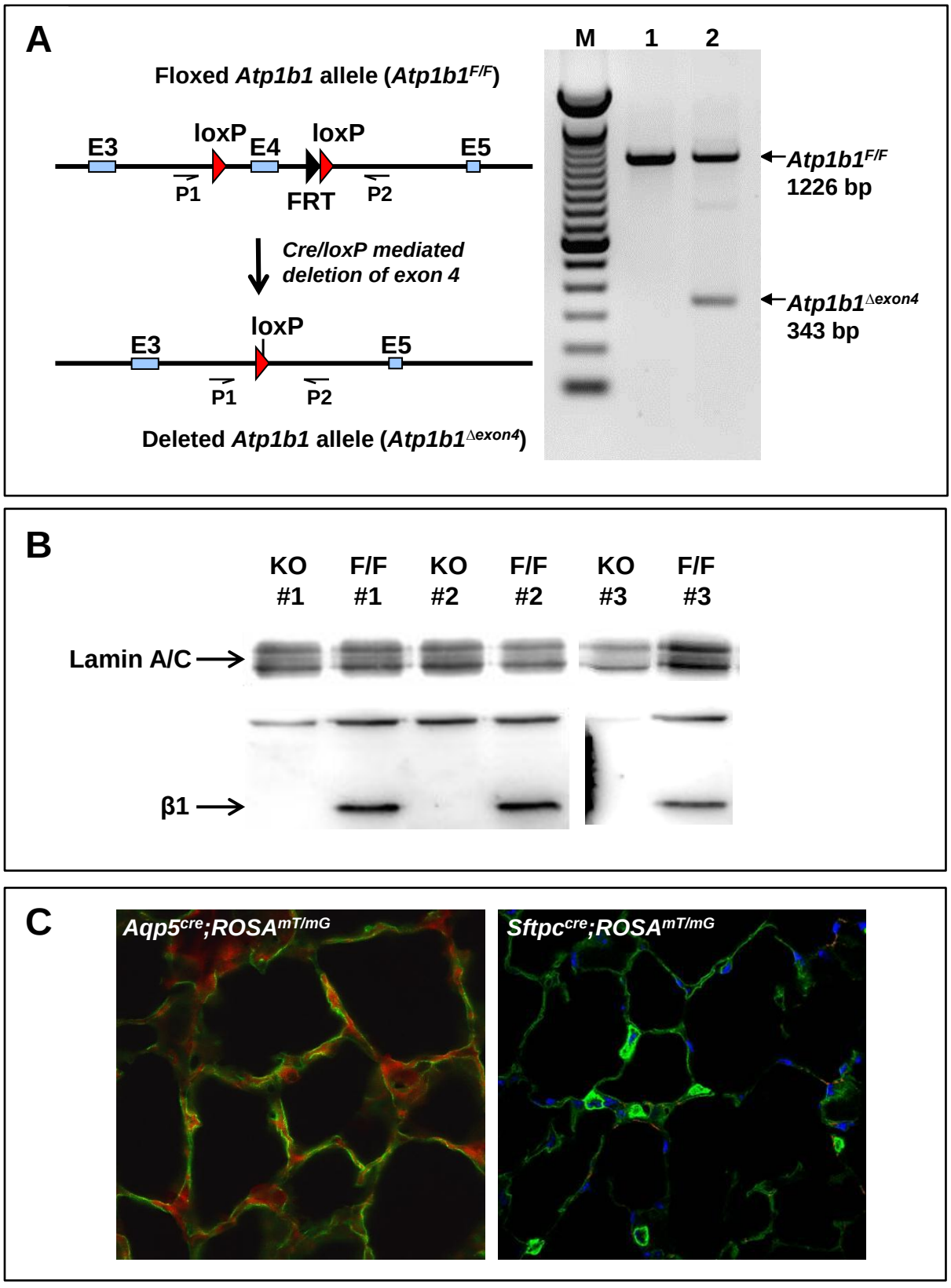
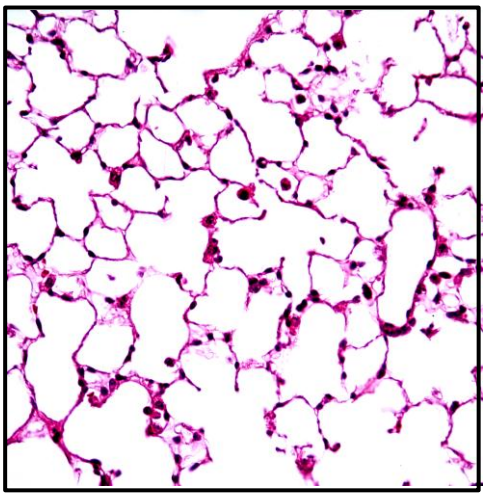
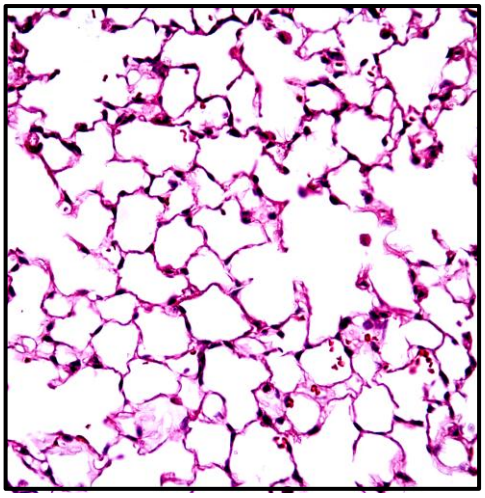


Figure E2 (online supplement)



***Atp1b1*$^{F/F}$**



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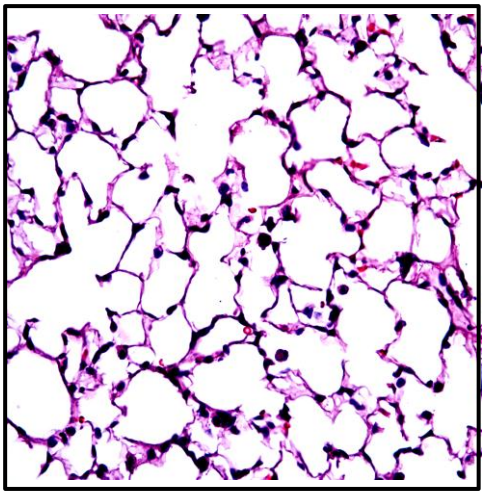

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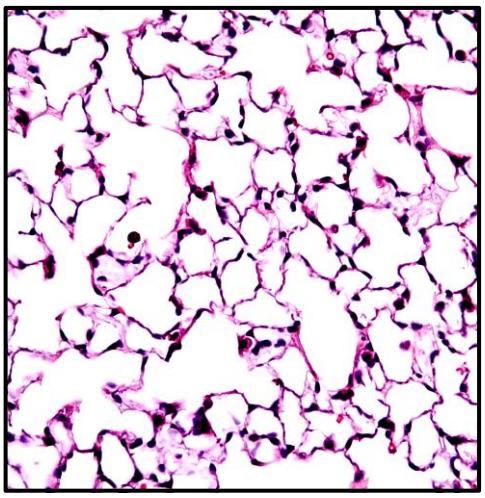

***Atp1b1*$^{Sftpc\text{-}cre}$**