Extended Experimental Procedures

Gpr116 Gene Targeting and Animal Studies

The second exon of *Gpr116* was chosen for targeting because it contains the start codon and signal peptide. A *LoxP* site was introduced upstream and an *Frt-loxP-Neo-Frt-loxP* cassette was inserted downstream of exon 2 by sequential recombineering (Liu et al., 2003; Southon and Tessarollo, 2009; Tessarollo et al., 2009). The *Gpr116* conditional KO vector was electroporated into V6.4 129/SvJae X C57BL/6J hybrid F1 embryonic stem (ES) cells, and ES cell clones with one targeted allele were identified by Southern blot analysis by probing BglII or SacI digested DNA with PCR-generated probes. Targeted ES clones were microinjected into C57BL/6 blastocysts to generate chimeric mice and transfer the $Gpr116^{neo}$ allele to the germline. Mice with a $Gpr116^{+/neo}$ allele were identified by Southern blotting and crossed with either a transgenic *β-actin-flp* deleter strain to generate the conditional "floxed" allele or a transgenic *β-actin-Cre* deleter strain to generate the *Gpr116* null allele. The mice with *Gpr116* null or floxed alleles described in this study have been backcrossed to C57BL6 at least 6 generations and only WT and KO littermates were used for comparisons. Offspring from heterozygous intercrosses were born at the expected Mendelian frequency. To delete *Gpr116* in endothelial cells, heterozygous $Gpr116^{+/-}$ mice were crossed with transgenic *Tg(Tie2-Cre)* mice that carry the *cre*-recombinase gene driven by the Tie2 promoter [B6.Cg-Tg(Tek-cre)12Flv/J; The Jackson Laboratory] to generate $Gpr116^{+/-}$*; Tg(Tie-2-Cre)* mice with a single copy of the *Tie-2-Cre* transgene. Male $Gpr116^{+/-}$*; Tg(Tie-2-Cre)* mice were crossed with

female *Gpr116*$^{flox/flox}$ mice to generate *Gpr116*$^{-/flox}$*; Tg (Tie-2-Cre)* offspring that harbor one globally deleted allele and one endothelial deleted allele. To delete *Gpr116* in Vav-positive hematopoietic cells or SP-C positive ATII cells, the same strategy was applied using transgenic mice that carry iCre-recombinase driven by the *Vav1* promoter [B6.Cg-Tg(Vav1-cre)A2Kio/J; The Jackson Laboratory] or Cre driven by the *SFTPC* promoter (Okubo et al., 2005). Others working with *SFTPC-Cre* on a different strain background (i.e. 129/Sv) have noted enlarged airspaces (but not PAP) in mice with the *SFTPC-Cre* transgene alone (Jeannotte et al., 2011) raising concerns about this model. However, in control experiments we have performed extensive histopathological analysis of lungs from mice with the *SFTPC-Cre* transgene alone, and have not observed enlarged airspaces or any other lung abnormalities in our inbred C57BL6 mice. Furthermore, the transgene is present in both groups from our comparison of *Gpr116*$^{+/flox}$; *SFTPC-Cre* versus *Gpr116*$^{-/flox}$; *SFTPC-Cre*, yet we only observed a PAP phenotype in the latter group.

To create an inducible model for *Gpr116* deletion in ATII cells, conditional *Gpr116* KO mice were crossed with B6.Cg-Tg(tetO-cre)1Jaw/J mice (The Jackson Laboratory) that express Cre recombinase under the control of a tetracycline-responsive promoter element (*TRE*; *tetO*) and with B6.Cg-Tg(SFTPC-rtTA)5Jaw/J mice (The Jackson Laboratory) that express the reverse tetracycline-controlled transactivator (rtTA) protein under the control of the human *SFTPC* promoter. To induce deletion, doxycycline was added to the

drinking water and chow from post-natal day 1 (P1) to P10. QPCR performed on ATII cells isolated from adult lungs revealed that we were able to induce deletion in 90.3% of ATII cells. Although the tet system has been reported to have off-target affects on lung (Perl et al., 2009), in our studies we employed the C57BL6 strain that is evidently resistant (Morimoto and Kopan, 2009; Perl et al., 2009). Furthermore, histopathological analysis of doxycycline-treated *Gpr116* WT control lungs that also contain *SFTPC-rtTA* and/or *tetO-Cre* failed to reveal any lung abnormalities.

Endothelial Cell Isolations. Endothelial cell isolations from each of the organs indicated in Figure S1C were previously described (Seaman et al., 2007).

Type II Cell Isolation. *Gpr116*$^{+/-}$ mice were crossed with *SFTPC-GFP* mice (Lo et al., 2008), a kind gift from Drs. Joe Rae Wright and John Heath, to create *Gpr116*$^{-/-}$ or *Gpr116*$^{+/+}$ mice with the *SFTPC-GFP* transgene. In some experiments further crosses were made with transgenic SFTPC-Cre mice (Okubo et al., 2005), a kind gift from Dr. Brigid L.M. Hogan, and *Gpr116*$^{flox/flox}$ mice to create *Gpr116*$^{+/flox}$*; SFTPC-Cre; SFTPC-GFP* or *Gpr116*$^{-/flox}$*; SFTPC-Cre; SFTPC-GFP* mice. ATII cells were isolated using a procedure modified from Corti *et. al.* (Corti et al., 1996) and Lo et al (Lo et al., 2008). In brief, lungs were perfused by intracardial injection of heparin in PBS (200 U/ml). Perfused lungs were lavaged once with 1 ml of collagenase A solution [hepatocyte wash buffer (Invitrogen) containing 2 mg/ml collagenase A (Roche)] then filled with fresh

collagenase A solution through a tracheal catheter, and allowed to collapse naturally. Low melting agarose (1%, 1 ml, 50ºC) was instilled, and lungs were immediately covered with crushed ice for 2-3 min. The lungs were removed and digested in 15 mL of collagenase A solution for 30 min at 37ºC. Next, the lungs were teased apart, incubated a further 10 min in collagenase A solution, then filtered through a 40 µm strainer and a 20 µm nylon mesh. Leucocytes and endothelial cells were depleted using streptavidin-280 magnetic Dynabeads (Invitrogen) that had been pre-armed with biotinylated anti-CD45 (eBioscience), CD11b (eBioscience) and CD31 antibodies (BD Pharmingen). The cells were pelleted, resuspended in PBS/0.5% BSA, and GFP-positive ATII cells were isolated by Fluorescence Activated Cell Sorting (FACS). All ATII cell samples were >90% pure based on flow cytometry post-sort analysis.

SPA uptake into ATII cells

Surfactant was cleared of macrophages by low speed centrifugation (250xG, 10 min), concentrated by pelleting at high speed (165,000xG for 1h), and resuspended in DMEM. Samples were normalized for PC content, aliquoted and frozen. 5ug of human SP-A purified from individuals with pulmonary alveolar proteinosis (a gift from Drs. Todd M. Umstead and Joanna Floros) was mixed with 100ug of *Gpr116* WT or KO surfactant at room temperature for 20 min. The surfactant/SP-A mixture was then mixed with partially purified ATII cells that had been enriched from the lungs of *SFTPC-GFP* mice by negative selection with magnetic beads as described above (see Type II Cell Isolation). After rotating for

10 min, 37^{o}C, cells were rinsed 1x with DMEM/5% fatty acid-free BSA (FAFB), pelleted, rinsed 2X with DMEM/0.1% FAFB/10 mM EGTA to remove non-specifically bound proteins, pelleted, and then rinsed with PBS/0.1% BSA to remove traces of EGTA. GFP-positive ATII cells were then isolated by flow cytometry and lysed in denaturing SDS sample buffer. Uptake of human SPA was evaluated by Immunoblotting with mouse anti-human SP-A monoclonal antibody (clone 6F10; Santa Cruz, cat # SC-80621) using ECL2 Western Blotting Substrate and the SuperSignal Western Blot Enhancer kit (Thermo Scientific).

Genotyping

Mice were genotyped for *Gpr116* by mixing the following three primers in a single PCR: F: 5'-GGAGGCTCTGTGCGTTTC-3', R1: 5'-CTGTGGACATGATGAAGGGTG-3', and R2, 5'-CTCCCTGAATCATAGTCTAGTCTCC-3'. The expected amplicon sizes are 263 bp, 486bp and 312bp for *Gpr116* wild-type, null, and conditional floxed alleles, respectively.

Cell Lines and Culture Conditions

The A549 cell line was obtained from the DCTD Tumor Repository at NCI Frederick National Laboratory for Cancer Research and was maintained in DMEM supplemented with 10% fetal bovine serum.

Quantitative RT-PCR

RNA was isolated from whole tissues or purified cells (ATII cells, endothelial cells, or macrophages) immediately following purification. Total RNA or mRNA was isolated from cells using the RNeasy mini kit (Qiagen) or the QuickPre Micro mRNA Kit (GE Healthcare) respectively, and cDNA was synthesized using the Superscript III 1st strand synthesis system (Invitrogen). The *Gpr116* primers used to primers used to evaluate deletion efficiency in *Gpr116* conditional KO mice were For: AAGTTGAGCAAAGGAGTGGC and Rev: TGAAGGTTGAACAATGGGCTC. The remaining primer pairs used for QPCR are listed on Table S1. Primers were designed to span large introns thus preventing any potential amplification of genomic DNA.

Vectors

The full-length human *GPR116* cDNA was subcloned from the IMAGE clone 5298732 (GenBank accession number BC066121) into pcDNA4/TO (Invitrogen). Using PCR-based tag insertion, a pcDNA4/*GPR116-pyo* plasmid was generated, which encodes two tandem copies of the pyo sequence “MEYMPME” immediately following the C-terminal asparagine residue. The GPR116-GFP vector, which produces a full-length GPR116 protein fused to GFP at the C terminus, was generated by cloning the *GPR116* open reading frame (ORF) into the pAcGFP1-N1 vector (Clontech). The pcDNA3/ABCA3-3xHA plasmid was generated by subcloning the full ABCA3 ORF from IMAGE clone 9021763

(GenBank accession number BC140895) into pcDNA3.1 (Invitrogen) and then using PCR-based tag insertion to clone the 3xHA tag "YPYDVPDYAYPYDVPDYAYPYDVPDYA" immediately following the C-terminal arginine residue. Each of the vectors was sequenced and found to be mutation free. Vectors were stably transfected into A549 cells using an Amaxa nucleofector kit (Lonza) according to the manufacturer's recommendations.

Histological Studies

Tissues were fixed in formalin, routinely processed, paraffin embedded, sectioned at 5μm, stained with hematoxylin and eosin (H&E), and evaluated by a boarded veterinary pathologist (D.C.H). The initial comprehensive evaluation included an analysis of 44 organs or tissues taken from 6-month-old mice *Gpr116* WT and KO mice (n=6 per group). For immunohistological studies of human lung, paraffin sections were deparaffinized, incubated with proteinase K, heated at 95^{o}C for 20 min in citrate buffer (pH 6) (Invitrogen), and then treated with biotin block (Dako) and 0.1% Sudan Black (Sigma) to minimize background. Human lung sections were incubated with polyclonal rabbit antibodies against GPR116 (MBL, cat # LS-A1513) or CC10 (Bioscience, cat # LS-B6822) followed by biotin-conjugated anti-rabbit and streptavidin-AP (Jackson Immunoresearch), and detected with the AP substrate Fast Red TR/Napthol AS-MX (Sigma). For co-immunofluorescence, GPR116 was labeled with Fast Red and combined with mouse anti-SP-B antibodies (Thermo Pierce, cat # MA5-13975) followed by FITC-goat anti-mouse, Alexa 488-goat anti-FITC and Alexa 488 donkey anti-

goat. Fast Red fluorescence was detected using a Texas Red filter. Mouse lung sections were stained with rabbit anti-SP-A (Lifespan, cat # LS-C17949) followed by biotinylated goat anti-rabbit secondary antibodies (Vector Labs) and streptavidin-HRP and detected with 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin or DAPI. The anonymized human lung tissue samples were obtained from the Cooperative Human Tissue Network with approval from the NIH Office of Human Subject Research. Non-malignant lung tissues were derived from excess lung material removed at the time of surgical tumor removal. *In situ* mRNA hybridization was performed on murine tissues as previously described (Seaman et al., 2007).

Immunofluorescence

Cells grown on poly-D-lysine treated chamber slides were chilled on ice and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were blocked and permeabilized with the TNT block [1% blocking reagent (Roche) in 100 mM Tris HCl (pH 7.5), 150mM NaCl, containing 0.1% Triton-X 100] and labeled with anti-pyo (Covance) to detect GPR116-Pyo or mouse anti-HA antibodies (Sigma) to detect ABCA3-HA. Surfactant labeling with rhodamine-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids) and uptake was performed as previously described (Ikegami et al., 2005). Sections were counterstained with DAPI and immunofluorescent images captured using a Zeiss LSM510 confocal microscope.

Electron Microscopy

Lungs were removed from 1-, 3- and 13-month-old mice, diced into 1 to 2 mm cubes, fixed in 4% formaldehyde, 2% glutaraldehyde, and 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and 0.5% uranyl acetate in 0.1 M acetate buffer. The tissues were dehydrated stepwise with ethanol and embedded in Embed-812 epoxy resin and cured at 55oC for 48 hr. The blocks were then trimmed and thin sectioned at 80-100nm using an ultramicrotome and were transferred onto copper mesh grids. Sections were stained with uranyl acetate and lead citrate. Following staining, sections were carbon coated and imaged in a Hitachi H7600 and H7650 microscopes running at 80 kV. Images were captured with an AMT digital camera. ATII cells were identified based on their unique intracytoplasmic lamellar bodies.

Metabolic Labeling of SP-A

ATII cells were isolated from lung and seeded onto 6-well plates coated with fibronectin (10 μg/ml) and incubated overnight at 37^{o}C. Cells were washed twice with pulse-labeling media (Met/Cys-free DMEM containing 2% dialyzed FBS), incubated with the same medium for 30 min, then pulsed for 4 hours at 37^{o}C with fresh pulse-labeling medium containing 1 ml of [^{35}S]Met/Cys (100 uCi/mL) (Perkin Elmer). After rinsing with pulse-labeling media cells were immediately lysed with 1 volume of denaturing lysis buffer 1 [1% (w/v) SDS, 50 mM TrisCl, (pH 7.4), 5 mM EDTA, 10 mM DTT, 15 U/ml DNase I, plus protease inhibitors]

and boiled at 95^{o}C for 5 min. After rapid cooling, 9 volumes of non-denaturing lysis buffer were added [1% (w/v) Triton X-100, 50 mM TrisCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, protease inhibitors, 10 mM iodoacetamide], the sample was clarified, and SPA was immunoprecipitated using goat anti-SP-A antibodies (Santa Cruz cat # sc-7700). Labeled SP-A was separated by SDS-PAGE. The gel was dried using a Large Gel Drying kit (Life Technologies) and the [^{35}S]-SP-A detected using a Typhoon 9200 imager.

Phosphatidylcholine Measurement

Phosphatidylcholine (PC) from BALF of mice was measured using phosphatidylcholine assay kit from BioVision (Mountain View, CA) according to the manufacturer's protocol.

Cholesterol Measurement

Cholesterol was measured using total cholesterol assay kit from Cell Biolabs, Inc (San Diego, CA) according to the manufacturer's protocol.

SP-A Immunoprecipitation. To remove lipids samples were extracted with 1-butanol prior to immunoprecipitation as previously described (Haagsman et al., 1987). Briefly, surfactant was enriched from the BALF by pelleting at 165,000g and resuspending in 0.9% NaCl. Surfactant was added to butanol at a ratio of 20:1 (mL of butanol : mg of total protein), mixed at RT for 30 minutes, and pelleted at 5000g. After drying the pellet with a flux of N_2 gas, pellets were

dissolved in OGP buffer [20mM OGP/10mM HEPES/150mM NaCl (pH 7.4)]. The SP-A was immunoprecipitated from either the OGP samples (butanol extracted) or BALF using rabbit and anti-SP-A antibodies (US Biologicals, Cat # S8400-02) and Protein A agarose (Roche) according to the manufactures instructions.

Phagocytosis Assay

AM were collected by lavage from WT and KO mice. Uptake of pHrodo *E. Coli* BioParticles Conjugates (Invitrogen) were measured according to the manufacturer's protocol.

Glycerophospholipid Analysis by Electrospray Ionization Mass Spectrometry (ESI-MS)

BALF (2mL) in PBS was collected from *Gpr116* WT or KO mice that were 6-weeks (n=5/group) or 6-months (n=3/group) old. After pelleting alveolar macrophages by low speed centrifugation (250xG, 10min), supernatants were transferred to new microfuge tubes, frozen and stored at -80^{o}C until analyzed.

Glycerophospholipids from BALF were extracted by a modified Bligh and Dyer procedure (Bligh and Dyer, 1959) using acidified methanol. Briefly, 1 mL of ice-cold 0.1 N methanolic HCl and 1 mL of ice-cold $CHCl_3$ were added to 1 mL of BALF. Following 1 minute of vortexing at 4°C, layers were separated by centrifugation (5 min, 4^{o}C, 18,000 x g) and the lower organic layer was collected and solvent evaporated. The resulting lipid film was dissolved in 100 µL of isopropanol:hexane:100 mM NH_4COOH(aq) 58:40:2 (mobile phase A).

Quantification of glycerophospholipids was achieved by the use of an LC-MS technique employing synthetic odd-carbon diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per sample. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-µm particle size) using a gradient elution as previously described (Ivanova et al., 2007; Myers et al., 2011). The identification and confirmation of the individual species, achieved by LC-MS/MS, was based on their chromatographic and mass spectral characteristics. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn-1* versus *sn-2*).

Bone Marrow Transplantation

To monitor transplantation efficiency, a transgenic line that expresses EGFP in hematopoietic cells [C57BL6-Tg(CAG-EGFP)1Osb/J mice from The Jackson Laboratory] was crossed with Gpr116 KO mice. Gpr116 WT or KO mice containing the EGFP transgene were euthanized, and bone marrow was sterilely extracted from the excised femurs and tibia. Bone marrow cells ($10 \text{ X } 10^6$) were injected into 2-month-old recipient mice that had been irradiated with 1000 cGy. Recipient mice received sulfamethoxazole (SMZ) antibiotic (60 mg per 250 ml of acid water) 7 days before and 7 days after irradiation. Post irradiation mice were

placed under Laminar flow 10 days and provided a Nutra-Gel Diet (Bio-Serv) for at least two weeks. Transplantation efficiency was ~95% at 6-months post-transplant based on flow cytometry comparisons of BM from recipients versus the parent EGFP transgenic line. Images were taken at 8 months of age, 6 months post-BM transplantation.

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Figure S1. *Gpr116* mRNA expression analysis and gene targeting in mice.
(A) *Gpr116* mRNA is expressed in endothelial cells derived from Brain (Br), Heart (H), Kidney (Kid), Liver (Liv), Lung (Lg) and Muscle (Mus), but not in a CD45+ hematopoietic cell fraction (HCF) isolated as previously described (Seaman et al., 2007). The Serial Analysis of Gene Expression (SAGE) tag numbers shown represent mRNA transcript copies per 100,000 transcripts.
(B) GPR116 is a seven-pass transmembrane receptor that contains a long N-terminal extracellular domain with a SEA domain and two immunoglobulin-like domains. A G-proteolysis site (GPS) resides next to the transmembrane domain.
(C) QPCR analysis was used to evaluate *Gpr116* mRNA levels in ECs isolated from the indicated organs compared to unfractionated whole tissues.
(D-H) mRNA *in situ* hybridization validated *Gpr116* mRNA expression (red) predominantly in vasculature from normal liver (D) and various tumors including murine Lewis lung carcinoma (E) grown subcutaneously in immunocompetent mice, or human SW620 (F), HCT116 (G) and KM12SM (H) grown in immunodeficient mice.
(I) The targeting vector used to disrupt the *Gpr116* locus was designed to incorporate lox-p sites (green triangles) on either side of exon 2 (E2) that contains the start codon and signal peptide (forward arrow). Frt sites (red triangles) were incorporated on either side of the neomycin (*neo*) gene to facilitate removal of the neo cassette by crossing the $Gpr116^{neo}$ mice with transgenic *β-actin-flp* mice. Mice with *Gpr116* 'floxed' alleles ($Gpr116^{flox}$) were crossed with either *β -actin-cre* transgenic mice to generate offspring with a *Gpr116* null allele or various cre-drivers to disrupt the conditional *Gpr116* allele in particular cell types. B: BglII, S: ScaI.
(J-K) Southern blots were used to identify the correctly targeted neo allele in ES cells (J) or the null allele from tail DNA following cre-mediated recombination in vivo (K). The 5' and 3' probes used for the Southern analysis are shown in *A*.
(L) PCR screen used for routine genotyping. The PCR reaction contained the three primers F, R1 and R2 indicated in *A* (black arrowheads).

Figure S2. Phenotypic characterization of *Gpr116* knockout mice.

(A) Weights were monitored from 3 to 14 months of age in female and male *Gpr116* wildtype (WT) or knockout (KO) mice. The difference in size between *Gpr116* KO versus WT mice was significant (p= 0.0001, 2-way ANOVA). The reduced body mass in the KO may be secondary to the labored breathing and increased energy expenditure. Values represent mean ±SEM.

(B) PAS staining was performed on paraffin sections derived from 6-month-old *Gpr116* WT or KO lungs. Note the red staining of the material in the alveolar space of the KO (yellow arrowheads). Scale bar is 20μm.

(C) H&E staining and SP-A staining was performed on lungs from *Gpr116* WT and KO mice. Note that the accumulation of SP-A-positive eosinophilic material in the alveolar space was detectable by 2 weeks but more pronounced by 6 weeks of age. Scale bar is 50μm.

(D) Immunohistochemistry was performed on normal human lung tissues in order to evaluate expression of GPR116 in Clara cells. Antibodies against the Clara cell marker CC10 were used on a serial section as a positive control. Scale bar is 100μm.

(E) Both free and total cholesterol (free cholesterol plus cholesterol ester) were evaluated in bronchoalveolar lavage (BAL) fluid of *Gpr116* WT and KO at 3 and 16 months of age. Cholesterol levels were also evaluated in blood samples derived from *Gpr116* KO versus WT mice, but in this case the differences were not statistically significant. n=3 mice per group. Error bars represent SD.

Figure S3. Disruption of *Gpr116* in ECs and macrophages does not result in altered surfactant homeostasis in the lung.

(A-B) QPCR for the endothelial markers *Pecam1* (A) and *Cdh5* (*VE-cadherin)* (B) was used to evaluate the purity of endothelial cells isolated from the lungs of *Gpr116*$^{-/flox}$ mice with or without the Tie2-cre transgene. Lung endothelial fractions were compared to unfractionated (UF) lung tissue.

(C) *Gpr116* mRNA levels in lung ECs were reduced by 97% in Tie-2-cre positive mice.

(D) SP-A staining was used to determine if SP-A could be detected in the alveolar space of *Gpr116*$^{-/flox}$ mice containing the endothelial *Tie2-cre* transgene. Scale bar is 50μm.
(E) QPCR was used to evaluate *Csf2* (*GM-CSF*) mRNA levels in whole lung tissues from *Gpr116* WT or KO mice.
(F) QPCR was used to assess the levels of *Emr1*, the gene encoding the macrophage marker F4/80, in alveolar macrophages (M∅) isolated from BAL fluid compared to unfractionated lung tissue.
(G) To assess macrophage responsiveness to GM-CSF signaling, QPCR was used to evaluate *Sfpi1* (*PU.1*), *pparg* (PPARγ) and *Abcg1* mRNA levels in alveolar macrophages isolated from *Gpr116* WT and KO lungs. N.S.: non-significant ($p>0.05$).
(H) Macrophages were isolated from the BAL fluid of *Gpr116*$^{-/flox}$ mice with or without the *Vav-iCre* transgene and the deletion of the conditional allele was verified by PCR analysis of genomic DNA. Tail DNA, which is comprised of mixture of different cell types, was used for comparison.
(I) SP-A staining was used to determine if SP-A could be detected in the alveolar space of *Gpr116*$^{-/flox}$ mice containing the *Vav-iCre* transgene. Scale bar is 50μm.
(J) SP-A staining was used to determine if SP-A could be detected in the alveolar space of *Gpr116* WT mice that had been previously reconstituted with *Gpr116* KO bone marrow (BM). Images of *Gpr116* WT and KO lungs were included as controls for comparison. Scale bar is 50μm.
(K) A phagocytosis assay was used to evaluate functional activity of macrophage in *Gpr116* KO mice. Alveolar macrophages were collected by lavage from *Gpr116* WT and KO mice and mixed with the pH-sensitive pHrodo E. Coli bioparticles that are non-fluorescent outside the cell, but fluoresce red inside phagosomes. Note that the macrophages from the BAL fluid of both WT and KO mice readily phagocytose the bioparticles at both 6 weeks and 6 months of age. Nuclei were stained with DAPI (blue). Scale bar is 50μm.
In (A-C, E-G) error bars represent SD.

Figure S4. Ultrastructural analysis reveals evidence of ATII cell hyperplasia, defective lamellar body packaging by ATII cells, and increased lamellar body (LB) number and size in *Gpr116* KO mice.

(A-C) *Gpr116* WT ATII cells were typically found in the corners of the alveoli (*A*), but in the KO ATII cells were often found next to one another (*B* and *C*). Tubular myelin, often found in the alveolar space of the WT (arrowheads in *A*), was rarely observed in the KO, although onion-like structures were widespread (for example, *E*; also see Figure *2D* of main text).

(D-F) Surfactant secretion by ATII cells in the KO appeared normal at 1 month (D) and 3 months (E) of age (asterisks), but by 1 year severe accumulation of surfactant was occasionally observed in some ATII cells (F).

(G) The number of lamellar bodies (LBs) was quantified in ATII cells from WT and KO mice by counting the average number of LBs per ATII cell section. Cell images were only included in the analysis if they spanned the main body of the ATII cell including part of the nucleus, such as those depicted from the 1-year-old WT or KO lungs in A. n = 10 sections/group.

(H) LBs were grouped according to size in KO versus WT mice. Scale bar is 2μm.

In (G-H) error bars represent SD.

Table S1. Primers used for QPCR analysis, related to Figures 1G and 4C and S3E-G.

Gene	Forward Primer	Reverse Primer
Normalizer		
Actb	CACCCGCCACCAGTTCG	AAGGTCTCAAACATGATCTGGG
Surfactant proteins		
Sftpa	GCAATGGGACAGAAGTTTGTG	CACTGACAGCATGGATCCTTG
Sftpb	GCCTCACACTCAGGACTTCTCT	CCCAGCAGTGCGTCTAGC
Sftpc	ATGAGTAGCAAAGAGGTCCTG	TGGTGTCTGCTCGCTCACTC
Sftpd	AGAGCCTCTCGCAGAGATCAG	GAGGTCCACTTAGTCCACGTTC
Other genes		
Abca3	CAGTAAAGCCAACATAGCAGCAG	GAGTCCAGCAGCAGCATCC
Abcg1	CCTGAAGAAGGTGGACAACAAC	GCCAGTCTCCCTGTATCCTG
Pcyt1a	GGCTCTGATGCAAGCAAAGA	CTGCGTCCTTGATGTGCTTA
Pecam1	CGATTGTAGCCACCTCCAAG	TGATACTGCGACAAGACCGTC
Fasn	AAGCAGGCACACACAATGG	GCTGTGTCCAGGGCAATG
Emr1	GAATGACTGCCACAGTACGATG	CAGACACTCATCAACATCTGCG
Csf2	CCAGGAGATTCCACAACTCAGG	CTCTTCATTCAACGTGACAGGC
Gpam	GTGAGGACTGGGTTGACTGTG	GACAGCCGTCTCGCCAG
Gpr116	TGCTGCGTAGAAGAGGATGC	GAGGTAAGTTCGCTGGGAGAC
Hmgcr	CTAGAGCGAGTGCATTAGCAAAG	GAGCTGCCAAATTGGACGA
Pparg	GATAGGTGTGATCTTAACTGCCG	AGGGCTCGCAGATCAGCA
Sfpi1	GGATCTGACCAACCTGGAGC	CGAGGACGTGCATCTGTTC
Srebf2	GGACATCGACGAGATGCTACAG	GGCTGGAGAACAGGGGTTG
Cdh5	GCTACCTGCCCACCATCG	CATCCACTGCTGTCACACGG

Table S2. Phospholipid composition in BALF from GPR116 WT and KO mice, related to Figure 4.

	6 Weeks (n=5)			6 months (n=3)		
	WT*	KO	*P*-value[†]	WT	KO	*P*-value
Phosphatidylcholine						
PC 28:0	0.5 ± 0.0	0.9 ± 0.0	<0.001	0.7 ± 0	1.0 ± 0.1	<0.05
PC 30:1	0.7 ± 0.0	0.8 ± 0.0	NS	1.4 ± 0.1	1.9 ± 0.1	<0.01
PC 30:0	10.6 ± 0.3	8.4 ± 0.2	<0.001	11.8 ± 0.1	14.3 ± 0.7	<0.05
PC 32:1	17.9 ± 0.6	11.0 ± 0.1	<0.001	24.9 ± 0.5	21.6 ± 0.7	<0.05
PC 32:0	21.6 ± 0.5	17.8 ± 0.3	<0.001	26.9 ± 0.3	27.7 ± 1.5	NS
PC 34:2	5.7 ± 0.2	4.6 ± 0.1	<0.001	7.1 ± 0.1	5.7 ± 0.3	<0.01
PC 34:1	8.8 ± 0.2	8.0 ± 0.1	<0.01	8.4 ± 0.2	6.1 ± 0.6	<0.05
PC 34:0	2.1 ± 0.1	2.9 ± 0.1	<0.001	0 ± 0	0 ± 0	NS
PC 36:4	10.8 ± 0.3	16.3 ± 0.2	<0.001	6.6 ± 0.2	7.5 ± 0.8	NS
PC 36:2	4.1 ± 0.3	3.4 ± 0.1	NS	0 ± 0	0 ± 0	NS
PC 36:1	0 ± 0	0 ± 0	NS	0.9 ± 0	0.7 ± 0	<0.05
PC 38:6	7.4 ± 0.3	9.1 ± 0.2	<0.01	5.9 ± 0.3	6.3 ± 0.2	NS
PC 38:5	2.7 ± 0.1	3.7 ± 0.1	<0.001	1.6 ± 0.1	1.9 ± 0.1	NS
PC 38:4	4.4 ± 0.3	8.3 ± 0.3	<0.001	1.9 ± 0.1	2.7 ± 0.1	<0.01
PC 40:6	2.0 ± 0.1	3.6 ± 0.1	<0.001	1.8 ± 0.2	2.6 ± 0.1	<0.05
Phosphatidylethanolamine						
PE 32:1	3.5 ± 0.1	2.3 ± 0.2	<0.001	2.7 ± 0.3	2.2 ± 0.2	NS
PE 34:2p[‡]	0.7 ± 0.1	0.5 ± 0.1	NS	1.5 ± 0.1	1.3 ± 0.1	NS
PE 34:1p	0.9 ± 0.1	0.8 ± 0.1	NS	1.6 ± 0.1	1.7 ± 0.1	NS
PE 34:2	7.3 ± 0.5	5.1 ± 0.5	<0.05	7.0 ± 0.7	5.3 ± 0.3	NS
PE 34:1	6.4 ± 0.3	5.2 ± 0.5	NS	6.4 ± 0.4	4.7 ± 0.2	<0.05
PE 36:4p	5.7 ± 1.3	14.5 ± 2.3	<0.01	10.6 ± 1.0	17.6 ± 1.1	<0.01
PE 36:4	23.3 ± 1.1	19.4 ± 2.2	NS	17.2 ± 0.7	15.8 ± 0.7	NS
PE 36:2	3.0 ± 0.2	2.4 ± 0.3	NS	2.4 ± 0.1	2.2 ± 0.1	NS
PE 36:1	1.4 ± 0.1	1.0 ± 0.1	<0.05	1.1 ± 0.1	1.0 ± 0	NS
PE 38:6p	4.5 ± 0.8	9.7 ± 1.5	<0.05	9.6 ± 1.6	9.6 ± 0.4	NS
PE 38:5p	5.7 ± 0.6	8.3 ± 0.6	<0.05	7.2 ± 0.5	7.4 ± 0.2	NS
PE 38:4p	2.4 ± 0.4	4.1 ± 0.8	NS	3.5 ± 0	5.5 ± 0.5	NS
PE 38:6	16.4 ± 0.6	11.7 ± 0.6	<0.001	15.4 ± 1.0	11.8 ± 0.7	<0.05
PE 38:5	9.7 ± 0.2	8.0 ± 0.3	<0.01	8.2 ± 0.2	7.1 ± 0.4	<0.05
PE 38:4	9.1 ± 0.4	6.9 ± 0.6	<0.05	5.7 ± 0.1	6.9 ± 0.3	<0.05
Phosphatidylglycerol						
PG 30:0	1.6 ± 0.1	1.9 ± 0.1	<0.01	0 ± 0	0 ± 0	NS
PG 32:1	6.6 ± 0.1	5.3 ± 0.3	<0.01	6.5 ± 0.6	9.4 ± 0.1	<0.01
PG 32:0	14.2 ± 0.5	18.3 ± 0.6	<0.001	12.6 ± 0.8	20.9 ± 0.8	<0.01
PG 34:2	16.1 ± 0.5	14.7 ± 0.5	NS	17.5 ± 0.8	16.7 ± 0.3	NS
PG 34:1	19.9 ± 0.4	21.9 ± 0.4	<0.01	19.5 ± 0.3	20.4 ± 0.4	NS
PG 34:0	0 ± 0	0 ± 0	NS	1.7 ± 0.4	0.8 ± 0.2	NS
PG 36:4	16.1 ± 0.3	13.8 ± 0.2	<0.001	15.8 ± 0.3	13.1 ± 0.4	<0.01
PG 36:3	2.2 ± 0	2.5 ± 0.1	<0.05	2.5 ± 0.3	2.2 ± 0.2	NS
PG 36:2	2.3 ± 0.1	2.7 ± 0.2	NS	3.5 ± 0.4	3.2 ± 0.1	NS
PG 36:1	1.0 ± 0	1.2 ± 0.1	NS	2.3 ± 0.2	2.1 ± 0.1	NS
PG 38:6	9.7 ± 0.3	8.7 ± 0.2	<0.05	12.1 ± 0.7	7.9 ± 0.2	<0.01
PG 38:5	4.9 ± 0.1	4.3 ± 0.2	<0.05	6.1 ± 0.5	3.4 ± 0.2	<0.01
PG 38:4	5.3 ± 0.1	4.7 ± 0.2	<0.05	0 ± 0	0 ± 0	NS
Phosphatidylinositol						
PI 32:1	1.5 ± 0	1.0 ± 0	<0.001	0 ± 0	0 ± 0	NS
PI 34:2	11.5 ± 0.3	11.4 ± 0.2	NS	11.2 ± 0.4	8.5 ± 0.4	<0.01
PI 34:1	9.0 ± 0.2	8.1 ± 0.1	<0.01	9.4 ± 0.6	6.4 ± 0.5	<0.05
PI 36:4	15.2 ± 0.4	17.2 ± 0.3	<0.01	12.6 ± 0.3	16.2 ± 0.2	<0.001
PI 36:3	3.9 ± 0.1	4.7 ± 0.2	<0.05	5.2 ± 0.2	5.1 ± 0.4	NS
PI 36:2	3.6 ± 0.1	3.1 ± 0	<0.01	3.4 ± 0.1	3.4 ± 0.1	NS
PI 38:6	18.3 ± 0.4	18.3 ± 0.4	NS	21.2 ± 0.2	20.0 ± 0.7	NS
PI 38:5	10.3 ± 0.3	9.9 ± 0.1	NS	12.3 ± 0.3	10.9 ± 0.1	<0.05
PI 38:4	22.3 ± 0.9	22.2 ± 0.9	NS	19.6 ± 0.7	25.0 ± 1.6	<0.05
PI 40:6	4.3 ± 0.1	4.1 ± 0.2	NS	5.0 ± 0.1	4.5 ± 0.2	NS
Phosphatidylserine						
PS 32:1	2.4 ± 0.1	2.3 ± 0.1	NS	1.9 ± 0.1	3.0 ± 0.3	<0.05
PS 34:2	2.6 ± 0.1	2.3 ± 0.1	<0.05	3.0 ± 0.1	3.2 ± 0.2	NS
PS 34:1	5.9 ± 0.1	7.2 ± 0.1	<0.001	7.4 ± 0.3	9.5 ± 0.4	<0.05
PS 36:4	4.1 ± 0.2	7.3 ± 0.2	<0.001	5.6 ± 0.3	9.7 ± 0.2	<0.001
PS 36:2	5.3 ± 0.1	4.2 ± 0.1	<0.001	6.7 ± 0.2	7.1 ± 0.3	NS
PS 36:1	11.4 ± 0.4	9.6 ± 0.2	<0.01	12.2 ± 0.3	12.1 ± 0.4	NS
PS 38:4	24.4 ± 1.3	28.5 ± 0.5	<0.05	28.4 ± 1.3	28.0 ± 0.4	NS
PS 38:3	6.9 ± 0.2	6.4 ± 0.2	NS	5.4 ± 0.2	4.6 ± 0.2	NS
PS 38:2	1.9 ± 0.1	0.7 ± 0	<0.001	0.8 ± 0	0.9 ± 0.2	NS
PS 40:7	3.2 ± 0.2	3.4 ± 0.1	NS	2.8 ± 0.1	3.7 ± 0.5	NS
PS 40:6	14.9 ± 0.3	15.1 ± 0.1	NS	16.2 ± 0.5	10.5 ± 0.5	<0.001
PS 40:5	6.9 ± 0.3	4.7 ± 0.1	<0.001	5.8 ± 0.2	3.6 ± 0.1	<0.001
PS 40:4	7.6 ± 0.3	5.8 ± 0.1	<0.001	0 ± 0	0 ± 0	NS
PS 42:1	2.5 ± 0.1	2.3 ± 0	NS	3.8 ± 0.3	4.1 ± 0.5	NS

*mol% within GPL class; [‡](p): Plasmalogen; [†]values indicate mean ± SEM, NS; not significant..

Table S3. Lysophospholipid composition in BALF from 6-week old GPR116 WT and KO mice, related to Figure 4.

	WT*	KO	*P*-value†
Lysophosphatidylcholine			
LPC 14:0	5.7 ± 0.2	8.4 ± 1.0	<0.05
LPC 16:1	19.8 ± 1.3	14.6 ± 1.4	<0.05
LPC 16:0§	**40.4 ± 2.2**	**52.6 ± 2.5**	**<0.01**
LPC 18:2	11.6 ± 0.7	4.6 ± 0.2	<0.001
LPC 18:1	7.6 ± 0.4	5.8 ± 0.5	<0.05
LPC 18:0	**7.1 ± 0.6**	**9.8 ± 1.0**	**<0.05**
LPC 20:4	5.4 ± 0.3	2.6 ± 0.3	<0.001
LPC 24:0	2.4 ± 0.2	1.6 ± 0.2	<0.05
Lysophosphatidylethanolamine			
LPE 14:0	1.6 ± 0.2	1.1 ± 0.1	<0.05
LPE 15:1	3.2 ± 0.5	8.4 ± 2.0	<0.05
LPE 16:1	5.3 ± 0.2	3.9 ± 0.1	<0.001
LPE 16:0	**7.6 ± 0.7**	**38.7 ± 4.3**	**<0.001**
LPE 18:2	8.7 ± 0.3	2.7 ± 0.5	<0.001
LPE 18:1	8.2 ± 0.3	12.3 ± 1.1	<0.01
LPE 18:0	**5.4 ± 0.7**	**12.1 ± 1.7**	**<0.01**
LPE 20:4	36.6 ± 1.8	13.0 ± 5.5	<0.01
LPE 24:0	23.3 ± 0.8	7.7 ± 2.9	<0.001
Lysophosphatidylglycerol			
LPG 16:0	**20.0 ± 0.7**	**61.4 ± 0.9**	**<0.001**
LPG 18:2	28.2 ± 0.5	6.0 ± 0.6	<0.001
LPG 18:1	27.1 ± 0.5	14.6 ± 0.2	<0.001
LPG 18:0	**4.2 ± 0.2**	**13.1 ± 0.4**	**<0.001**
LPG 20:4	10.2 ± 0.2	2.3 ± 0.2	<0.001
LPG 22:6	10.4 ± 0.2	2.7 ± 0.3	<0.001
Lysophosphatidylinositol			
LPI 16:0	**13.9 ± 0.4**	**28.7 ± 1.1**	**<0.001**
LPI 18:2	10.6 ± 0.3	5.8 ± 0.4	<0.001
LPI 18:1	9.4 ± 0.3	8.7 ± 0.3	NS
LPI 18:0	**11.8 ± 0.6**	**22.4 ± 1.2**	**<0.001**
LPI 20:4	27.1 ± 1.2	19.5 ± 1.3	<0.01
LPI 22:6	18.9 ± 0.4	10.5 ± 0.6	<0.001
LPI 22:5	8.4 ± 0.3	4.4 ± 0.2	<0.001
*Lysophosphatidylserin*e			
LPS 16:0	25.6 ± 0.9	16.7 ± 0.4	<0.001
LPS 18:0	**23.2 ± 1.5**	**57.0 ± 0.5**	**<0.001**
LPS 20:3	15.3 ± 0.3	9.0 ± 0.2	<0.001
LPS 22:0	12.7 ± 0.5	3.1 ± 0.1	<0.001
LPS 24:1	11.2 ± 0.4	2.7 ± 0	<0.001
LPS 24:0	11.9 ± 0.5	11.5 ± 0.2	NS

*mol% within GPL class, †values indicate mean ± SEM, NS; not significant

§Saturated lysophospholipids that are increased in Gpr116 KO are indicated in bold.