ABCA3 inactivation in mice causes respiratory failure, loss of pulmonary surfactant and depletion of lung phosphatidylglycerol

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Running Title: Abca3 null mice die of neonatal respiratory failure

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Abbreviations footnote: apolipoprotein AI (apoA-I); ATP cassette binding transporter (ABC transporter); bacterial artificial chromosome (BAC); Electrospray ionization-tandem mass spectrometry (ESI-MS); Phosphatidylglycerol (PG); Phosphatidylcholine (PC); Phosphatidylethanolamine (PE); Phosphatidylserine (PS); Phosphatidylinositol (PI); Sphingomyelin (SM).
ABSTRACT

The highly branched mammalian lung relies on surfactant, a mixture of phospholipids, cholesterol and hydrophobic proteins, to reduce intra-alveolar surface tension and prevent lung collapse. Human mutations in the ABCA3 transporter have been associated with childhood respiratory disease of variable severity and onset. Here we report the generation of Abca3 null mice, which became lethargic, cyanotic and died within an hour of birth. Tissue blots found ABCA3 expression was highest in lung but was also detectable in other tissues including the kidney. Gross development of kidney and lung was normal in neonatal Abca3−/− pups, but the mice failed to inflate their lungs, leading to death from atelectatic respiratory failure. Ultrastructural analysis of the Abca3−/− lungs revealed an absence of surfactant from the alveolar space and a profound loss of mature lamellar bodies, the intracellular storage organelle for surfactant. Mass spectrometry measurement of over 300 phospholipids in lung tissue taken from Abca3−/− mice showed a dramatic reduction of phosphatidylglycerol levels, as well as selective reductions in phosphatidylcholine species containing short acyl chains. These results establish a requirement of ABCA3 for lamellar body formation and pulmonary surfactant secretion and suggest a unique and critical role for the transporter in the metabolism of pulmonary phosphatidylglycerol. They also demonstrate the utility of the Abca3 null mouse as a model for a devastating human disease.
INTRODUCTION

ABC transporters are large polytopic membrane proteins that move molecules across bilayer membranes by hydrolyzing ATP. The A class of this gene family has at least 11 members and has evolved rapidly during the vertebrate radiation (1). The functional importance of the A class transporters is clear, as mutations in members of the class cause Tangier disease (ABCA1), Stargardt’s macular degeneration (ABCA4), and Harlequin icthyosis (ABCA12), disorders in which defects in transporter activity lead to major disruptions in human physiology (2-5). Recently, mutations in the gene encoding the ABCA3 transporter were associated with human respiratory diseases with either a neonatal or later childhood onset (6-8).

A role for ABCA3 in lung function was first indicated when antibodies against the transporter were found to stain type II alveolar cells at the plasma membrane, as well as at the limiting membrane of lamellar bodies (9, 10). The lamellar body is a unique lysosomal derived storage organelle characterized by internal lamellae enriched in phospholipids, cholesterol and hydrophobic proteins that comprise pulmonary surfactant. Through a process of regulated exocytosis the type II cells secrete stored pulmonary surfactant into the alveolar space where it functions to reduce surface tension at low lung volumes and thus prevents alveolar collapse. Considering the close homology between ABCA3 and ABCA1, it is reasonable to suspect that like ABCA1, ABCA3 may be involved in a lipid trafficking step, possibly at the limiting membrane of the lamellar body. Indeed, Cheong et al. have presented data analyzing cells transfected with an ABCA3 cDNA, or with an siRNA targeting the endogenous ABCA3 message, and concluded that ABCA3 can stimulate the uptake of fluorescent labeled analogs of phosphatidylcholine, sphingomyelin and cholesterol, suggesting the transporter may have broad lipid transport activity, not unlike ABCA1, but opposite in direction (11).
Human mutations in ABCA3 have been associated with respiratory disease of variable onset and severity but the precise role of ABCA3 in pulmonary function is unknown. To explore the physiologic transport function of ABCA3, we engineered mice that lack ABCA3 expression. Null embryos were generated in Mendelian frequencies and had grossly normal development in utero. In contrast, at birth, despite attempts to clear their lungs of fluid and initiate breathing, the Abca3<sup>−/−</sup> mice rapidly become cyanotic and perished within the hour. Histologic and ultrastructural analysis of Abca3<sup>−/−</sup> lung tissue indicated an invariable collapse of the airspaces at birth and a profound lack of secreted surfactant. These findings were associated with a failure to develop mature lamellar bodies in the alveolar type II cells. This phenotype was also associated with a dramatic reduction in lung phosphatidylglycerol, and lesser reductions in phosphatidylcholine species with short acyl chains, suggesting a lipid transport activity that to date appears unique among the members of the A class of the ABC transporter superfamily.
METHODS

Reagents: A rabbit anti-ABCA3 antibody was generated against the last 100 amino acids of mouse ABCA3 as previously described (12). To generate a mouse ABCA3 cDNA an Open Biosystems clone (ID# 5044171, Huntsville, AL) was sequenced on both strands confirming it represented nucleotides 960 to 5779 of the mouse cDNA and contained no mutations. However this cloned lacked nucleotides 1 to 954 that code for the first 167 amino acids of the open reading frame. Reverse transcription PCR amplification of a mouse lung mRNA pool generated a 1.4 kp product containing the missing sequences that was inserted back into the cDNA as a Kpn-I/AfeI restriction fragment. Sequence analysis of the resulting clone showed that it represented an intact error free open reading frame.

Statement of animal care: All animal procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and were conducted in accordance with the United States Department of Agriculture Animal Welfare Act for the Humane Care and Use of Laboratory Animals.

Generation of Abca3 knockout mice: The Abca3 locus was disrupted in mouse 129/SvEv embryonic stem cells using an Abca3 targeted BAC as previously described (13). In brief, using lambda red-mediated recombination in bacteria, a BAC from a 129 genomic library containing the Abca3 locus had exons 4 and 5 replaced with a zeo'/neo' dual selection cassette. Insertion of the cassette at exon 4 produces a targeted locus capable of generating only truncated ABCA3 peptides encoding the first 17 amino acids of the transporter. The structure of the targeted BAC, verified by PCR and Southern blotting was linearized and electroporated into 129/SvEv cells. Two G418 resistant colonies screened for the lack of BAC vector sequences and showing only two fluorescent in-situ hybridization signals were selected for injection into C57BL/6 blastocysts. This screening insures the injected clones had no illegitimate copies of the BAC and that the
targeted Abca3 allele had replaced one of the wild-type Abca3 alleles. Two chimeric lines were produced one of which transmitted the targeted allele to F1 progeny as analyzed by multiplex PCR reactions (P1, 5’-TCCTCTAAGGGCATGTTCAGG-3’; P2, 5’-ATGGCCACCCTTCCTTGGGTC-3’; P3, 5’-GGCCAGGGTGTTGTCCGGACC-3’) and Southern blotting of genomic BamHI digests with a probe against nucleotides 8555 to 9075 located in the 3rd intron of the Abca3 locus.

**ABCA3 tissue and macrophage immunoblots:** Specificity of the anti-ABCA3 antibody was tested using cell lysates from 293-EBNA-T cells transfected with empty vector or with cDNAs for ABCA1, ABCA2, ABCA7 and ABCA3 (20 ug of total cell protein in a lysis buffer composed of 20 mM Tris-HCl, pH 7.5, 150 mm NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1 % SDS and 0.001% Sigma protease cocktail). The lysates were separated by 6% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked overnight at 4°C in blocking buffer (1% bovine serum albumin, 5% dried milk protein, 0.1% Tween-20 in 1X PBS) and then incubated with either pre-immune or immune sera at a 1:1000 dilution. Antibody binding was detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, enhanced chemiluminescence (Pierce) and x-ray film. Only the immune sera detected two bands of approximately 180 kDa and 150 kDa molecular weight in the lysates from the ABCA3 transfected cells (Fig. 1C). The 150 kDa isoform co-migrated with the most prominent isoform detected in lung. The expression of ABCA3 protein in tissues from wild type adult C57/BL6 mice was assessed in lysates (50 ug total cell protein) prepared and analyzed as above (Fig. 1C). For the analysis of ABCA3 protein in tissues of Abca3+/− mice either 15 or 20 ug of total cell protein was used as indicated (Fig. 1E). Bone marrow macrophages were isolated by flushing femurs of C57/BL6 mice with cold Dulbecco’s modified Eagle’s medium (DMEM) and collecting the marrow by spinning at 2200 rpm. Red blood cells were lysed with 0.17 M NH₄Cl, and bone marrow was suspended in DMEM supplemented with 10 % fetal bovine serum, 100 units/ml
penicillin, 100 ug/ml streptomycin, and 15% L929-conditioned medium. After 24 hours of culture, the nonadherant cells were collected and cultured for an additional 6 days to obtain mature macrophages.

To obtain alveolar macrophages bronchial alveolar lavages were performed on adult mice by inserting a 22G Abbocath-T catheter (Abbot) into the trachea of euthanized mice and flushing the lungs with five 1 ml aliquots of Hank’s balanced salt solution. The combined aliquots were spun at 250 X g for 5 min to pellet the alveolar macrophages. The cell pellet was washed once with 1X PBS and lysed in lysis buffer as described above.

Histological analysis: Lung architecture was assessed in E18.5 embryos that had not respired, or in postnatal day 0 pups that had respired as previously described (14). To assess airspace morphometry the mean chord length of the of saccule airspaces in five randomly chosen microscopic fields of each sample was measured by a blinded viewer using a Leica DMLB microscope interfaced with Leica Q Win 550 image analysis software (Leica Microsystems Inc). The development of the pulmonary airways and vasculature in the lungs of E18.5 embryos was assessed by smooth muscle actin staining using a commercially available antibody coupled to alkaline phosphatase (Sigma) and counterstaining with haematoxylin. The amount of SP-B secreted into the air spaces of E18.5 lungs was measured by staining E18.5 embryo lung sections for mature SP-B using a mouse anti-SP-B antibody (Abcam) and horseradish peroxidase detection. This antibody preferentially detects mature secreted SP-B. The amount of airspace SP-B staining was assessed by a blinded viewer using a Leica DMLB microscope interfaced with Leica Q Win 550 image analysis software (Leica Microsystems Inc.). A saccule airspace was considered positive for secreted SP-B if the airspace staining was clearly associated with acellular material that was not counterstained for nuclei with methyl green. Approximately 600 hundred saccules were scored from three littermate matched Abca3^{+/+} and Abca3^{-/-} samples. The percent of
SP-B positive saccules was calculated and the significance in the difference of positive saccules between genotypes was determined by a two-tailed Student’s t-test.

**Electron Microscopy:** Lungs were fixed overnight in 4% paraformaldehyde, 1% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C, rinsed in 0.1 M cacodylate buffer, and postfixed for 1 hour in 1% OsO₄ in cacodylate buffer at room temperature. The samples were rinsed in buffer, then in distilled water, and stained en bloc in 2% aqueous uranyl acetate for 1 hour. Samples were then rinsed in distilled water and dehydrated through a graded series of ethanol to 100%. Samples were infiltrated overnight on shaker in a 1:1 solution of Epon-812 resin: 100% ethanol at room temperature. Following further infiltration in 100% Epon-812 (Electron Microscopy Sciences), samples were embedded in fresh Epon-812 overnight at 60°C. Thin sections were cut on a Reichert Ultracut E ultramicrotome, collected onto formvar-coated slot grids, poststained with uranyl acetate and lead citrate, and imaged using a JEOL 1011 transmission electron microscope with AMT digital camera at 80 kV (JEOL USA). An extensive survey of lamellar body formation and surfactant secretion in lung samples from littermate paired E18.5 pups (Abca3+/+ and Abca3−/−, n=5) was carried out on 182 micrographs at magnifications of 10,000 to 60,000.

**Phospholipidomics:** After homogenization by 20 strokes in a dounce in 0.4 ml 1X PBS, total lipids were isolated from littermate paired E18.5 embryo (Abca3+/+ and Abca3−/−, n=5) lungs by one 2 ml extraction with chloroform/methanol (1:1, v:v) and two 0.5 ml chloroform extractions. The combined organic phases were washed once with 0.5ml KCL (1M), twice with 0.5 ml of H₂O, dried under a stream of N₂ gas and stored at −80°C till analysis. For phospholipid profiling an automated electrospray ionization-tandem mass spectrometry approach was used and data acquisition and analysis and acyl group identification were carried out as described previously with minor modifications (15, 16). The dried extracts were resuspended in chloroform and an aliquot of extract (30 µL out of 1 mL) was taken for mass spectrometry analysis. The lipid
extract was combined with solvents and internal standards, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.23 mL. Internal standards, obtained and quantified as previously described (16), were 0.66 nmol di14:0-PC, 0.66 nmol di24:1-PC, 0.66 nmol 13:0-lyso PC, 0.66 nmol 19:0-lyso PC, 0.36 nmol di14:0-PE, 0.36 nmol di24:1-PE, 0.36 nmol 14:0-lyso PE, 0.36 nmol 18:0-lyso PE, 0.36 nmol di14:0-PG, 0.36 nmol di24:1-PG, 0.36 nmol 14:0-lyso PG, 0.36 nmol 18:0-lyso PG, 0.24 nmol di14:0-PS, 0.24 nmol di20:0(phytanoyl)-PS, 0.20 nmol 16:0-18:0-PI, and 0.16 nmol di18:0-PI. Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with a 1 mL injection loop and presented to the ESI needle at 30 μl/min. The collision gas pressure was set at 2 (arbitrary units) for phospholipids. The collision energies, with nitrogen in the collision cell, were 28 V for PE, 40 V for PC and SM, -58 V for PI, -57 V for PG, and -34 V for PS. Declustering potentials were 100 V for PE, SM, and PC and -100 V PG, and PI. Entrance potentials were 15 V for PE, 14 V for PC and SM, and -10 V for PI, PG, and PS. Exit potentials were 11 V for PE, 14 V for PC, -15 V for PI, -14 V for PG, and -13 V for PS. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer mode. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

Lipid species were detected, using the scans previously described, including neutral loss of 87 in the negative mode for PS (16, 17). Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a
common head group fragment. Sphingomyelin was determined from the same mass spectrum as PC (precursors of m/z 184 in positive mode) (17, 18) and by comparison with PC internal standards using a molar response factor for sphingomyelin (in comparison to PC) determined experimentally to be 0.37. The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. Isotopic overlap corrections were applied, and the lipids in each class were quantified in comparison to the two internal standards of that class using standard curve shapes determined for the API 4000 mass spectrometer.

**Individual acyl group identification:** The acyl groups of PC and PG species found to be significantly affected by loss of ABCA3 expression were identified as acyl anions from the appropriate negative ion precursors. The collision energies were 20-55V. The solvent was chloroform/methanol/300 mM ammonium acetate in water (300/665/35). PG was analyzed as [M - H]-, and PC was analyzed as [M + OAc]-.

**Cholesterol and triglyceride analysis:** Oil-red-O staining of 4% paraformaldehyde fixed frozen lung sections was use to assess the distribution and levels of cholesterol and triglycerides in littermate E18.5 Abca3+/+ and Abca3-/- embryos as previously described (19). Total cholesterol, triglyceride and free glycerol levels were determined on lung lipid extracts by enzymatic assays using commercially available reagents (Sigma Aldrich) using the method of Carr et al (20). Cholesterol efflux assays were carried out as previously described (12). In brief, 293-EBNA-T cells were seeded into 24 well poly-D-lysine coated tissue culture plates at 100,000 cells/well and 72 h later were transfected in triplicate with empty vector or the indicated cDNAs using Lipofectamine 2000 (Invitrogen). Twenty-four hours post transfection the cells were incubated with 0.5 uCi/ml [1H]-cholesterol in complete media (10% FBS/DMEM) for 24 hours. Non-cell associated cholesterol was removed by two washes with 1X PBS, a 2 hour incubation in media at
37°C and two additional washes in 1X PBS. The cells were further incubated in media alone (1mg/ml fatty acid free bovine serum albumin/DMEM) or in media with 10 ug/ml delipidated apoA-I for 12 hours. Media was collected from the cells and cleared of debris by a 800 X g spin for 10 min. To calculate total cholesterol uptake and efflux, the cell layers were dissolved in 0.1 N NaOH, and the amount of radioactivity in the media and cell lysates was measured by scintillation counting. Total cell associated cholesterol was expressed as counts per minute/ 24 well. ApoA-I dependent cholesterol efflux was expressed as the difference in the percentage of efflux (media cpm/(media + cell cpm) X 100) for the apoA-I treated cells minus the percentage of efflux from the cells treated with media alone.

Statistical analysis: Data sets were tested for equal variance and when found to have equal variance were further compared by a two-tailed Student’s T test using the SigmaStat software package. The lung weights of the adult wild type and ABCA3+/- mice were found to have unequal variance and thus the lung weights were first transformed to their natural logarithm before being compared by a two-tailed Student’s T test. Statistical significance was defined a P < 0.05.
RESULTS

Homozygous null Abca3 mutations in mice result in neonatal lethality

Recombination of the Abca3 locus was accomplished by electroporating a bacterial artificial chromosome (BAC) lacking exons 4 and 5 into 129/SvEv embryonal stem cells (Fig. 1A) (13). Injection of targeted ES cells generated two chimeric lines, one of which transmitted the targeted allele to F1 progeny. Southern analysis of DNA from late term embryos (E15.5-18.5) derived from Abca3<sup>+/−</sup> intercrosses demonstrated transmission of the 11 kb targeted allele at the expected Mendelian frequencies (Fig.1B, wt:het:null embryo ratios of 51:102:52, respectively), however, no homozygous null animals survived the immediate post-natal period. Thus, deletion of ABCA3 in the mouse results in neonatal lethality, consistent with a hypothesis that ABCA3 transport function is essential for respiratory function.

To characterize the defect in these mice further, we generated an anti-ABCA3 antibody that lacked cross-reactivity to other ABCA class members, including A1, A2 and A7 (Fig.1C). In 293 cells transfected with an ABCA3 cDNA, the antibody detected two bands, the lower of which co-migrated with the predominant ABCA3 isoform expressed in murine lung. We then analyzed expression of ABCA3 in adult mouse tissues by immunoblot (Fig.1C, lower panel). As has been reported for ABCA3 mRNA in the rat, ABCA3 protein expression was highest in mouse lung, with moderate expression in the kidney (9). Other tissues that demonstrated substantial expression included brain, white adipose tissue and bone marrow-derived macrophages. Immunoblotting whole embryo lysates of Abca3<sup>−/−</sup> mice confirmed the loss of ABCA3 protein in these animals, whereas heterozygous embryos exhibited ABCA3 protein levels approximately half those of wild type mice (Fig. 1D). Evaluation of other ABCA transporters in these mice revealed that ABCA1 was modestly up-regulated in Abca3<sup>−/−</sup> mice and that expression of ABCA7 was unchanged. Compared to age-matched wild type animals, twelve-week old heterozygote mice continued to demonstrate reduced expression of ABCA3 protein in lung and kidney tissues, as well as in alveolar macrophages (Fig. 1E). The body weights, as well as lung and kidney
weights in the adult Abca3\textsuperscript{+/−} mice were statistically indistinguishable from their wild type littermates (Table I). These data indicate that mice whose expression of ABCA3 is reduced by approximately half develop and grow normally, and are able to survive into adulthood.

**Abca3\textsuperscript{−/−} mice display normal embryonic development but fail to inflate their lungs upon birth**

To assess the cause of death of Abca3\textsuperscript{−/−} pups, studies were performed on late stage embryos, as well as neonates. Gross examinations of day 18.5 embryos obtained by Caesarian section showed the null embryos to be normally developed, with total body, lung and kidney weights statistically indistinguishable from those of the wild type or heterozygous embryos (Table II). Histologic analysis of E18.5 lung tissue also showed grossly normal architecture (Fig. 2A), although a slight trend towards reduced alveolar chord length in the null tissues was noted (Abca3\textsuperscript{+/+}, 9.96\text{um} ± 2.15\text{um} (n=6), Abca3\textsuperscript{−/−}, 7.34\text{um} ± 2.12\text{um} (n=5), p=0.7). However, staining of the lung tissue for alpha smooth muscle actin indicated the airways and pulmonary vasculature had developed normally in the absence of ABCA3 (Supplemental figure 1). Finally, loss of ABCA3 expression in the E18.5 kidneys also resulted in no grossly discernible abnormalities (Fig. 2A). Thus complete loss of ABCA3 in mice does not appear to affect in utero development as detected by light microscopy.

Next, litters resulting from crosses of heterozygous animals were allowed to develop to term and newborn pups were observed for signs of distress immediately following birth. Abca3\textsuperscript{−/−} pups were born with initially normal color and exhibited typical early motor activity, including concerted efforts to breathe (see supplemental video). However, shortly after birth, the null pups became lethargic, cyanotic, and failed to nurse. Inflation of the lungs and oxygenation of the blood, evident in the development of a pulsating white patch in the thoracic region of wild type and heterozygous mice, never occurred in Abca3\textsuperscript{−/−} pups (Fig. 2B). All Abca3\textsuperscript{−/−} pups ceased activity.
and died within 10-60 minutes following birth. Analysis of the lungs of Abca3<sup>−/−</sup> mice indicated that the primary cause of death was atelectasis, or collapse of the alveolar space. This was grossly apparent in that Abca3<sup>−/−</sup> lungs contained little or no air and sank when placed in phosphate-buffered saline (Fig. 2C), a finding consistent with the histologic evidence of no inflated airspaces in the Abca3<sup>−/−</sup> lungs (Fig 2D).

**Abca3<sup>−/−</sup> lungs lack secreted surfactant and mature lamellar bodies**

As pulmonary surfactant is critical for lung inflation and the maintenance of the alveolar space, electron microscopy was used to test whether loss of ABCA3 activity disrupted surfactant production. Micrographs of lungs from an Abca3<sup>−/−</sup> P0 mouse exhibited little or no secreted surfactant, compared to the lungs of a littermate Abca3<sup>+/+</sup> mouse (Fig. 3A and supplementary Fig. 2, arrows point to secreted surfactant in the airspaces of the wild type lung). Along with the lack of surfactant, the null lung exhibited tissue damage and leakage of red blood cells into the collapsed air spaces (Fig. 3A, arrowhead). To avoid the potential of post-mortem tissue damage artifacts, the lungs of E18.5 embryos delivered by Caesarian section were further examined. Additionally, since ABCA3 mutations in humans have been reported to have a variable impact on lamellar body structure, a more extensive analysis of 128 micrographs of 5 littermate paired lung samples was undertaken. In wild type mice, secreted surfactant and normal surfactant storage organelles, with their characteristic internal lamellae (lamellar bodies), were consistently observed throughout the lung tissue (Fig. 3B, arrows point to lamellar bodies). In contrast, the lungs of Abca3<sup>−/−</sup> embryos again showed little or no secreted surfactant and lacked mature lamellar bodies, although the lamellae precursor multivesicular bodies appeared to be normal (Fig 3B). These results suggested loss of ABCA3 produced a strong block in the secretion of surfactant. Because the hydrophobic surfactant protein B is also stored in lamellar bodies and is co-secreted with surfactant lipids we analyzed whether loss of ABCA3 activity was also associated with a block in the secretion of SP-B. This was found to be the case in that
immunostaining of wild type and Abca3−/− lungs from E18.5 embryos revealed dramatically less mature SP-B staining in the airspaces of the Abca3−/− lungs while intracellular staining was largely unchanged (supplementary Fig 3A, arrows point to airspace staining that is not cell associated as assessed by the lack of counterstaining with methyl green). Blinded counts of three samples from littermate paired animals showed a significant 95% reduction in the number of airspaces stained positive for SP-B in the Abca3−/− lungs (supplementary Fig 3B). In composite these results indicate ABCA3 activity is critical for the formation of lamellar bodies and the secretion of surfactant.

**Phospholipid profiling indicates ABCA3 activity maintains phosphatidylglycerol levels in the lung**

To date mutations in transporters of the ABCA class have been associated with prominent disruptions in lipid homeostasis suggesting that all members of this class may play a role in lipid transport. Profiling of lipid classes by mass spectrometry is a recently developed technique that permits a more global analysis of tissues for changes in their lipid composition. As surfactant is principally composed of phospholipids, and loss of ABCA3 in our mice appeared to have strongly disrupted the storage and secretion of surfactant, we used mass spectrometry to profile the phospholipid content of lung tissue derived from littermate paired P0 pups (n=5). We analyzed 333 individual species covering the major phospholipids, as well as their lyso and ether derivatives (see supplementary table I). The total phospholipid mass per gram of lung tissue did not differ significantly between the genotypes although a trend for the null lungs to have less phospholipid was evident (Table III). In contrast, the null lungs showed an 85% reduction in the mass of phosphatidylglycerol while the other major phospholipid classes did not differ significantly (Table III). However, when analyzing individual PG and PC sub-species, further differences were observed. The reduction in phosphatidylglycerol mass was accompanied by decreases across all the major sub-species of PG detected in this assay, including PG 34:1 (Fig.
4A). In contrast, for phosphatidylcholine, shorter acyl chain species were reduced in the Abca3<sup>-/-</sup> lungs, but PC 34:1 along with other longer chain species were not significantly different in the varying mouse genotypes (Fig. 4B & C). The acyl groups of the PC and PG species found to be significantly reduced by the loss of ABCA3 expression were identified as acyl anions from the appropriate negative ion precursors. As expected, the PC and PG 32:0 species contained only fully saturated palmitoyl chains (16:0), the 32:1 species contained palmitoyl and palmitoleoyl chains (16:0 and 16:1, respectively) while the 34:1 species contained palmitoyl and oleoyl chains (16:0 and 18:1, respectively).

For phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and sphingomyelin, no significant differences were found among the major species, although a few minor species were also found to be lower in the Abca3<sup>-/-</sup> samples, including PE 36:6, PI 36:3 and PI 40:7 (Fig. 5A-D). In composite, these data indicate ABCA3 activity is most important for maintaining lung levels of phosphatidylglycerol, and to a lesser degree the level of phosphatidylcholine species with short acyl chains.

Lost of ABCA3 activity does not influence lung cholesterol levels

As lamellar bodies and surfactant also contain cholesterol, and in vitro assays by Cheong et al have suggested ABCA3 can stimulate the cellular uptake of cholesterol, we sought to assess whether cholesterol homeostasis had been disrupted in the Abca3<sup>-/-</sup> lung (11). Staining frozen lung sections with Oil Red O, which detects cholesterol as well as other neutral lipids, did not indicate loss of ABCA3 had a major effect on the levels of these lipids, although staining in the null tissue was slightly more diffuse, consistent with a disruption of lamellar bodies (data not shown). Cholesterol levels were measured in Abca3<sup>+/+</sup> and Abca3<sup>-/-</sup> lungs by enzymatic assays further confirming loss of ABCA3 activity did not significantly alter the amount of this neutral sterol (Fig. 6A). Although total lung cholesterol levels were not prominently altered by loss of ABCA3 expression the disruption of lamellar body structure may have altered the cellular
distribution of cholesterol and could have been responsible for the slight up-regulation of ABCA1 expression that was seen in whole embryo lysates of the Abca3<sup>−/−</sup> mice (Fig. 1D). However, immunoblots showed levels of ABCA1 protein in the lungs of the Abca3<sup>−/−</sup> mice to be similar to levels in the lungs of the wild type mice, whereas a slight increase in ABCA1 protein was again detected in the brain of the Abca3<sup>−/−</sup> mice (Supplementary Fig. 4). Finally, although 293-EBNA-T cells transfected with the mouse ABCA3 cDNA led to robust expression of transporter (Fig. 6B), these cells were not found to accumulate greater amounts of tritiated cholesterol than did cells that were mock-transfected (Fig. 6C). The ability of the ABCA3 transfected cells to efflux cholesterol to apolipoprotein A-I was additionally measured in these assays and compared to efflux activity of ABCA1 transfected cells. ABCA3 expression did not significantly alter this cholesterol trafficking process (Fig. 6D). In composite, our experiments do not indicate a prominent role for ABCA3 in maintaining lung cholesterol homeostasis, although do not rule out more subtle effects on sterol trafficking in the lung and other tissues such as the brain.

**DISCUSSION**

This work demonstrates that Abca3<sup>−/−</sup> mice die of respiratory failure due to an inability to secrete pulmonary surfactant into the alveolar space. The phenotype is completely penetrant in that all of the Abca3<sup>−/−</sup> pups died within an hour of birth having failed to inflate their lungs. These results indicate ABCA3 transport function is essential for mammals to transition to air respiration. In contrast to our mouse model, children with ABCA3 genetic mutations who developed neonatal respiratory distress showed a more variable disease course and time of death (6, 8), perhaps because their mutant transporters retained some functional activity. Indeed, a more recent report suggests compound missense ABCA3 mutations are associated with chronic lung disease that is compatible with survival into adulthood (7). Our results make clear that some ABCA3 activity is required for respiratory function and survival. The insertion of hypomorphic ABCA3 alleles into the null animals may provide additional useful models to study the pathogenesis of more...
commonly occurring respiratory disorders that have been suggested by the recent human genetic association data.

Why is ABCA3 function critical for lung function and the generation surfactant? The alveolus of the mammalian lung dynamically expands and contracts during the respiratory cycle. During expiration, as the alveoli contract, the surface tension generated by the aqueous hypophase lining the airspaces can cause their collapse. To reduce surface tension and prevent collapse, type II alveolar cells secrete surfactant, a mixture composed primarily of phospholipid with lesser amounts of cholesterol and hydrophobic proteins. Here we show that the formation of the lamellar body, which stores surfactant before its release into the alveolus, is severely disrupted in mice lacking ABCA3 activity. In contrast to this near complete disruption of lamellar body structure, various human ABCA3 mutations have been associated with a more variable impact on lamellar body structure (6, 8). This again may reflect differences in residual ABCA3 transport function among the various mutations identified, and possibly on the ability of the mutant transporters to localize to the lamellar body (11, 21). Our result shows that in mice completely lacking ABCA3 protein, the transporter’s function is essential for the formation of the lamellar body and surfactant release into the airspaces.

How the absent lamellar body structure and surfactant secretion relates to ABCA3 transport activity is less clear. Because other close homologues of ABCA3 are know to stimulate the movement of lipids across membrane bilayers it is reasonable to suspect ABCA3 may also possess such activity. Indeed, Cheong et. al. have suggested ABCA3 has a broad transport capacity that stimulates the cellular uptake of phosphatidylcholine, sphingomyelin and cholesterol as determined by in vitro assays using microscopy and uptake of fluorescent lipid analogues (11). This suggests an ABCA3 transport activity similar in nature, but opposite in direction, to that of ABCA1, a homologue of ABCA3. However, our analysis of the cholesterol levels in lungs of the
Abca3\textsuperscript{-/-} mice did not indicate a major change in the levels of this lipid, and we found that forced expression of ABCA3 in 293 cells did not stimulate the uptake or inhibit the release of radiolabeled cholesterol. These experiments make it less likely that ABCA3 plays a major role in cholesterol homeostasis, but they do not exclude a more subtle role in sterol trafficking as suggested by the results of Cheong et. al. As with cholesterol, our results indicate lung triglyceride and free glycerol were also not strongly dependent on ABCA3 activity (data not shown). In contrast, our mass spectrometry profiling of the null lungs did reveal a more specialized role for ABCA3 in the metabolism of phosphatidylglycerol and short acyl chained phosphatidylcholine species. To our knowledge, this is first description of an ABC transporter that has such a restricted and dramatic effect on tissue phosphatidylglycerol levels (22-24). This finding highlights the utility of profiling lipid levels by mass spectrometry, and suggests the method may help identify the transport function of other poorly characterized ABCA transporters. The result also provides a mechanistic rationale for the use of clinical assays that measure phosphatidylglycerol levels in amniotic fluids as a metabolic marker of lung maturity.

Phosphatidylglycerol is uniquely enriched in the lung and comprises approximately 10% of the phospholipid content in secreted surfactant. Phosphatidylcholine comprises up to 80% of the phospholipid content in surfactant and of this nearly 40% is dipalmitoyl PC (PC 32:0), while palmitoyloleyl PC (PC 32:1) at 25% is the next most abundant surfactant PC species in the mouse (25) (Unpublished observations, ML Fitzgerald and MW Freeman). The loss of ABCA3 activity prominently affected the level of these PC species as well as levels of PC 32:2, PC 30:1 and PC 30:0. However, PC 34:1 and the other major PC species with longer acyl chains were not significantly reduced. In contrast, PG 34:1, as well as all other major PG species, were all reduced, thus significantly lowering total PG levels in the lung. Surfactant PG stored in lamellar bodies contains a broad range of acyl chain species and this pool encompasses the majority of the lung PG (26). In contrast, surfactant PC is restricted at birth to short acyl chain species and this
pool of PC comprises a much smaller fraction of total lung PC (27). Our results indicate the loss of ABCA3 activity selectively affected the metabolism of those phospholipids preferentially stored in lamellar bodies. It is these phospholipids, especially the unsaturated species that play a critical role in reducing surface tension within the alveolus. That these phospholipids are specific and direct transport substrates for ABCA3, dependent on the transporter for accumulation in the lamellar body, is suggested by our results. Alternatively, the loss of ABCA3 activity could disrupt lamellar body formation by another mechanism, thus leading to a decrease in specific stored phospholipids by a secondary feedback mechanism.

In conclusion, the generation of Abca3 null mice has established an essential role for this transporter in the formation of pulmonary lamellar bodies and the secretion of surfactant from alveolar type II cells. The loss of transporter function results in neonatal respiratory failure and death, as is seen in humans with some mutations in the ABCA3 gene. The transporter plays a critical role in the accumulation of phosphatidylglycerol in murine lung tissue at birth, suggesting that it plays a key role in the transport of this phospholipid into the lamellar body. This work also provides additional evidence that strengthens the hypothesis that all members of the ABCA class of transporters will be involved in cellular lipid transport. Interestingly, mutation of the amphipathic helical surfactant protein B in humans and mice is also associated with neonatal respiratory distress, depletion of lung phosphatidylglycerol and disruption of lamellar body structure, a phenotype remarkably similar to what we have observed in the Abca3 null mice (28, 29). Thus, while we do not provide direct evidence for the mechanism of lipid transport, our results suggest the intriguing possibility that the general process of ABCA-mediated cellular lipid export may share the common feature of loading intracellular lipid onto a specific amphipathic helical carrier protein, such as surfactant protein B (ABCA3) or apolipoprotein A-1 (ABCA1).
ACKNOWLEDGEMENTS

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References


**Abbreviations footnote:** apolipoprotein AI (apoA-I); ATP cassette binding transporter (ABC transporter); bacterial artificial chromosome (BAC); Electrospray ionization-tandem mass spectrometry (ESI-MS); Phosphatidylglycerol (PG); Phosphatidylcholine (PC); Phosphatidylethanolamine (PE); Phosphatidylserine (PS); Phosphatidylinositol (PI); Sphingomyelin (SM).
Figure legends

Fig. 1. Targeted deletion of Abca3 results in neonatal lethality. A: Exons 4 and 5 of the Abca3 wild-type locus (WT) were disrupted by homologous recombination using a targeted BAC (TG BAC). B: Southern analysis of DNA from day 18.5 embryos derived from Abca3+/− intercrosses shows transmission of the 11 kb targeted allele and generation of the null state. C: A rabbit anti-mouse ABCA3 antiserum was generated that detects a 180 kDa protein in 293 cells transfected with ABCA3 cDNA and does not cross-react with other A class transporters (top panels, 20 ug total cellular protein). Immunoblotting of mouse tissues using this antibody demonstrated that ABCA3 protein is most highly expressed in the lung and moderately expressed in the kidney, adipose, macrophage and spleen (lower panel, 50 ug total cellular protein). D: Embryonic Abca3+/− mice do not express ABCA3 protein. Immunoblotting of whole body lysates from wild type, Abca3+/− and Abca3+/− day E18.5 embryos confirmed the loss of ABCA3 protein and revealed a mild up-regulation of ABCA1, but not ABCA7, in these mice (40 ug total protein). E: Diminished levels of ABCA3 protein are maintained in the adult heterozygous state as determined by immunoblotting of lung, kidney and alveolar macrophage lysates from 12 week old Abca3+/− and Abca3+/− mice (lung sample: 15 ug total protein and 10 sec exposure; kidney samples: 20 ug and 30 sec exposure; alveolar macrophage sample: 15 ug total protein and 5 min exposure).

Fig. 2. Abca3−/− mice suffer fatal neonatal respiratory distress. A: Hematoxylin/eosin stains of tissues from day E18.5 wild type and Abca3−/− mice reveal no gross abnormalities in lung or kidney structure (20X). B: At birth Abca3−/+ pups attempt breathing movements, but fail to inflate their lungs, rapidly becoming cyanotic and then inactive (arrows indicate the inflated lungs of the of the Abca3+/− pup that are lacking in the Abca3−/+ pup). C: The lung from Abca3−/+ pups sink in
phosphate-buffered saline confirming a lack of inflation. D: Hematoxylin/eosin stained lung sections show collapsed air spaces in the lungs of the P0 Abca3−/− pups (40x).

**Fig. 3.** Abca3−/− lungs lack secreted surfactant and mature lamellar storage bodies. A: Electron micrographs of lungs from P0 pups demonstrate secreted surfactant (arrows) in the alveolus of an Abca3+/+ mouse that is lacking in the Abca3−/− sample (bars represent 3.7 um, arrowhead points to a red blood cell in the Abca3−/− alveolus). B: E18.5 Abca3+/+ lungs show copious secreted surfactant and mature lamellar bodies that are lacking in the Abca3−/− lung (bars represent 500nM, arrows point to lamellar bodies in the Abca3+/+ lung and a multivesicular body in the Abca3−/− lung, arrowheads point to microvilli on type II cells).

**Fig. 4.** Phosphatidylglycerol levels are depleted in Abca3−/− lungs. Organic lipid extracts from littermate Abca3+/+ and Abca3−/− lungs were profiled for phospholipid content by ESI-MS/MS and expressed as nmol of the indicated phospholipid species per gram of lung tissue (n=5, ±SEM, *p<0.05). The total acyl carbon: total double bond content of each phospholipid species is indicated on the x-axis. A: Phosphatidylglycerol levels including PG 34:1 and PG 34:2 are significantly depleted in Abca3−/− lungs. B&C: Phosphatidylcholine species with short acyl chains are selectively depleted in the Abca3−/− lungs.

**Fig. 5.** Loss of ABCA3 activity does not disrupt lung levels of the other major phospholipids. Organic lipid extracts from E18.5 littermate Abca3+/+ & Abca3−/− lungs were profiled for phospholipid content by ESI-MS/MS and expressed as nmol of the indicated phospholipid species per gram of lung tissue (n=5, ±SEM, *p<0.05). The total acyl carbon: total double bond content of each phospholipid species is indicated on the x-axis. A: Phosphatidylserine levels. B: Phosphatidylethanolamine levels. C: Phosphatidylinositol levels. D: Sphingomyelin levels.
Fig. 6. Loss of ABCA3 does not significantly disrupt lung cholesterol homeostasis.

A: Enzymatic quantification of total lung cholesterol showed no significant differences between E18.5 littermate matched Abca3+/+ & Abca3−/− lungs (n=5, ±SD, p=0.53). Forced expression of ABCA3 in 293-EBNA-T cells does not stimulate uptake of radiolabelled cholesterol or modulate efflux to apoA-I. B: Immunoblots demonstrate significant ABCA1 and ABCA3 expression in 293-EBNA-T cells after transfection with the respective cDNAs. C: Uptake of 3H-cholesterol by these ABCA3 transfected cells was not significantly different (n=3, ±SD, p=0.78), and only the ABCA1 transfected cells significantly stimulated efflux to apoA-I (D) (n=3, ±SD, p=0.005).
### Table I: Body, lung and kidney weights of adult wild type and Abca3+/− mice at 4 months of age

<table>
<thead>
<tr>
<th></th>
<th>Abca3+/+ SD</th>
<th>Abca3+/− SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>38.2 (18) ± 5.2</td>
<td>39.8 (27) ± 5.1</td>
<td>0.31</td>
</tr>
<tr>
<td>Lung</td>
<td>0.39 (16) ± 0.04</td>
<td>0.40 (24) ± 0.08</td>
<td>0.54</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.50 (15) ± 0.07</td>
<td>0.53 (24) ± 0.09</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Body and tissue weights are in grams ± S.D.
Sample number is in parentheses
P values are derived from a two-tailed Student’s T test.

### Table II: Body, lung and kidney weights of embryonic day 18.5 wild type, Abca3+/− and Abca3−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Abca3+/+ SD</th>
<th>Abca3+/− SD</th>
<th>Abca3−/− SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body</td>
<td>1.307 (8) ± 0.17</td>
<td>1.23 (18) ± 0.14</td>
<td>1.29 (11) ± 0.11</td>
<td>0.26, 0.81</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.037 (14) ± 0.005</td>
<td>0.039 (19) ± 0.005</td>
<td>0.043 (17) ± 0.005</td>
<td>0.4, 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.009 (8) ± 0.0014</td>
<td>0.009 (11) ± 0.0019</td>
<td>0.009 (6) ± 0.002</td>
<td>0.44, 0.46</td>
</tr>
</tbody>
</table>

Body and tissue weights are in grams ± S.D.
Sample number is in parentheses
P values are derived from a two-tailed Student’s T test comparing the Abca3+/+ values to either the Abca3+/− or Abca3−/− values, respectively.

### Table III: Lung phospholipid content of embryonic day 18.5 wild type, and Abca3−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Abca3+/+ SE</th>
<th>Abca3−/− SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>191 ± 50</td>
<td>30 ± 9</td>
<td>0.01</td>
</tr>
<tr>
<td>PC</td>
<td>2265 ± 192</td>
<td>1636 ± 356</td>
<td>0.16</td>
</tr>
<tr>
<td>PS</td>
<td>386 ± 44</td>
<td>351 ± 78</td>
<td>0.71</td>
</tr>
<tr>
<td>PE</td>
<td>1027 ± 132</td>
<td>801 ± 176</td>
<td>0.33</td>
</tr>
<tr>
<td>PI</td>
<td>225 ± 31</td>
<td>194 ± 56</td>
<td>0.64</td>
</tr>
<tr>
<td>SM</td>
<td>430 ± 40</td>
<td>372 ± 83</td>
<td>0.55</td>
</tr>
<tr>
<td>Total PL</td>
<td>4526 ± 456</td>
<td>3384 ± 751</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The phospholipid masses are in nmol/g lung tissues ± S.E., n=5
P values are derived from a two-tailed Student’s T test.
Figure 1
Figure 3
Figure 4

A

phosphatidylcholine (nmol/g lung)

WT
Abca3^-/-

Figure 4

B

phosphatidylglycerol (nmol/g lung)

WT
Abca3^-/-

Figure 4

C

phosphatidylcholine (nmol/g lung)

WT
Abca3^-/-
Running Title: Abca3 null mice die of neonatal respiratory failure
Figure 6