Loss of Rab27 function results in abnormal lung epithelium structure in mice

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While a subset of “housekeeping” Rabs are expressed ubiquitously, Rab27 GTPases (Rab27a and Rab27b) are expressed in cell types with specialized secretory functions (6, 29). In these cells, Rab27 localizes to, and regulates, the motility and exocytosis of lysosome-related organelles (LROs) and secretory granules; e.g., melanosomes in melanocytes, lytic granules in cytotoxic T cells (CTL), and dense granules in platelets (1, 8, 11, 25, 28, 32). To facilitate these diverse functions Rab27 interacts with eleven known downstream effectors (5, 13). For instance, in melanocytes, we and others (9, 21) have shown that Rab27a-GTP sequentially recruits effector Melaphenil/Slac2-a and molecular motor myosin 5a to melanosomes thereby allowing their retention in actin-rich peripheral dendrites. Meanwhile, in CTLs, Rab27a mediates granule docking and exocytosis via effector Munc13–4 (17). Differential engagement of effectors is in part due to their cell type-specific pattern of expression (10). In some cell types, Rab27a and Rab27b isoforms are expressed together, and recent data suggest that they may perform sequential roles in granule docking and exocytosis via engagement of different effectors (14, 20).

Previous analysis revealed that Rab27 proteins are expressed in the murine lung, suggesting that they play an important role in lung cell types with specialized secretory functions (6, 29). Within the conducting airways (bronchioles), Clara cells perform an important role in production and release of lung lining fluid components; e.g., surfactant protein A, Clara cell-specific protein (CCSP). While in the gas exchange, (alveolar) epithelial cells perform a similar function producing surfactant protein and phospholipid components (3, 15). Lining fluid proteins and lipids perform an essential role in the innate immune response to invading pathogens; e.g., defensins and collectins, and reduction of surface tension that maintains the alveolar structure; e.g., surfactant proteins and lipids. Interestingly, within AEII cells a subset of surfactant components are stored in lysosome-related organelles (LROs), known as lamellar bodies (LB), that release their cargo into the airspace upon stimulation (4).

Several studies provide evidence for an important role for Rab GTCPases in the physiological function of the lung. For example, Rab3D has been found to localize to a population of LBs in AEII cells, although its precise function in this context remains unknown (30). Rab14, meanwhile, was found partially localized to LBs, and knockdown resulted in partial inhibition of their evoked release suggesting a role for Rab14 in this process (7). Most recently, analysis of the chocolate mutant mouse and the ruby mutant rat that contain Rab38 gene mutations revealed a role for this protein in surfactant exocytosis and LB structure (18, 19). In this study, we investigated the cell-type specific and intracellular localization of Rab27 proteins; intracellular transport; alveolar epithelium type II cell; Clara cell

SMALL GTPASES of the Rab family regulate intracellular transport in eukaryotic cells (5, 22, 24). Analyses of mammalian genomes indicate that this family comprises ~60 members that have intracellular compartment-specific localization and function. Rabs act as molecular switches cycling between an active GTP and inactive GDP bound states. Active Rabs recruit effectors to the membrane upon which they reside, and these transduce functions in vesicle formation, transport, docking, or fusion. After nucleotide hydrolysis, inactive Rab is then retrieved to the cytosol in complex with Rab GDP dissociation inhibitor (GDI) in readiness for reutilization in further rounds of transport. Nascent Rabs are posttranslationally modified by covalent attachment of geranylgeranyl groups to carboxyl-terminus cysteine residues, which is essential for their targeting to specific intracellular membranes.
proteins in the murine lung as well as the effects of loss of Rab27 upon morphology of the pulmonary epithelium.

MATERIALS AND METHODS

Chemicals and antibodies. Unless otherwise stated all chemicals were obtained from Sigma-Aldrich (Poole, UK). The following antibodies were used for immunoblotting: rabbit polyclonal anti-Rab27a and anti-Rab27b raised against the COOH-terminus hypervariable region of each protein at 1:1,000 (Davids Biotechnologie, Regensburg, Germany) and rabbit anti-Calnexin Stressgen (SPA-860D, BioWhittaker; Wokingham, UK) at 1:10,000. For immunohistochemistry, rabbit anti-pro-surfactant protein C (SPC) antibody (Abcam ab28744, Cambridge, UK) was used at 1:1,000, rabbit anti-Clara cell-specific protein (CCSP) (UK cat no. 07-623, Upstate, Dundee) was at 1:1,000, goat anti-SPC (Research Diagnostics RTSURFCCabG) was at 1:100, and rabbit polyclonal anti-Rab27a and anti-Rab27b antibodies (as above) were each at 1:100.

Mice strains. All mice were treated humanely and in accordance with the UK Home Office regulations under project license PPL 70/7078 at the Central Biomedical Services of Imperial College, London, UK. C57BL/6J wild-type mice were purchased from B&K Universal Limited (Hull, UK). Rab27a-deficient mice, ashen (Rab27aash/ash) mice were described previously (31) and the generation of Rab27b KO mice and double Rab27KO was described elsewhere (27). All strains were maintained on a C57/B1/6 background.

Immunoblotting. Immunoblotting was performed as described previously (12). Perfused lung lysates were prepared using a Polytron homogenizer and an appropriate volume (10× vol/wt) of lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1% CHAPS, and 1× PI cocktail) followed by incubation on ice for 15 min. Nuclei and debris were then harvested by centrifugation (3,000 g at 4°C for 10 min), and the protein content of the PNS was quantified using BCA protein assay kit (Pierce, UK).

Real-time PCR analysis. Total RNA from resected human lung tissue obtained from 19 transplant donors was reverse transcribed using a reaction mix of 1× RT buffer (500 µM each dNTP, 3 mM MgCl2, 75 mM KCl, 50 mM Tris-HCl, pH 8.3), 20 units of RNasin Rnase inhibitor (Promega, Madison, WI), 10 mM dichloro-diphenyl-trichloroethane (DDT), 100 units of Superscript II RNase H-reverse transcriptase (Invitrogen, Uppsala, Sweden), and 250 ng of random hexamers (Promega). First-strand cDNA synthesis was carried out in a final volume of 20 µL, incubating at 20°C for 10 min and 42°C for 30 min, and inactivating reverse transcriptase by heating at 99°C for 5 min and cooling at 5°C for 5 min.

Real-time PCR were performed using the 7000 Abi Prism (Applied Biosystems, Foster City, CA) with optimized PCR conditions. The reaction was carried out in a 96-well plate adding 3 µl of diluted template cDNA to a final reaction volume of 25 µl. The PCR master mix was assembled with TaqMan Universal Master Mix Reagents (Applied Biosystems) and each Taqman Gene Expression Assay, Hs00608302 (Applied Biosystems) for human Rab27a, Hs01072206 (Applied Biosystems) for Rab27b. Each target assay was replicated three times and performed in multiplex reaction with the 18S rRNA endogenous control gene (4310893E, Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, and 50 cycles at 95°C for 15 s and 65°C for 1 min. Real-time quantitative values were obtained from the Ct number at the end of exponential growth of Ct value of the target gene from the average Ct value of the 18s rRNA gene.

Lung histology and morphometry. Male mice were terminally anesthetized by intraperitoneal injection of ketamine-xylazine (100 and 12 mg/kg, respectively) and heparin (300 U/ml). Animals were perfused with PBS through the right ventricle of the heart until the lungs were visually free of blood. The trachea was then exposed, and a Luer cannula (BD Insyte; 20 gauge 1.1 × 30 mm) was inserted and secured with surgical thread. The lungs and heart were then removed and fixed by careful inflation with 10% formalin neutral buffer solution via the trachea at a constant hydrostatic pressure of 30 cmH2O at the height of the carina in the upright position for 15 min. The lungs were further incubated overnight in fixative, and the right lung was embedded in paraffin. After deparaffinization and rehydration, 4-µm sections were stained using hematoxylin and eosin. Stained sections were then observed using a Zeiss Axiosvert 200 inverted microscope and images captured using a Hamamatsu Orca ER CCD. For mean linear intercept analysis of the integrity of alveolar epithelium, 10 randomly acquired low-magnification (20×) images for each of 3 age-matched animals were overlaid with a grid comprising 10 equally spaced horizontal lines, and the number of times that the epithelium intersected the grid was recorded for each image. The mean was determined and plotted as a percentage of age-matched control (wild-type, WT) samples. For measurement of bronchiolar thickness, bronchiolar epithelium areas were identified on the basis of intensity; the area was then calculated by subtraction of the luminal-airway area from the total bronchiolar area. The bronchiolar perimeter was then determined by taking the average of the inner and outer perimeter measurements (determined with area measurements). The area was then divided by the perimeter to give a thickness measurement (area/length of perimeter). This measurement was performed on 10 randomly selected bronchioles.
from each of 3 age-matched animals. P values were derived using the Student’s t-test.

Immunohistochemistry. Immunofluorescence was performed on either deparaffinized, formalin-fixed paraffin sections or frozen sections. Sections were deparaffinized with Histoclear and then taken through reducing alcohols to water, and rinsed in 1× PBS. For antigen retrieval, samples were boiled for 9 min in 10 mM sodium citrate buffer (pH 6.0) and cooled for 5–10 min in water. Slides were washed in 1× PBS and incubated with blocking buffer (1× PBS, 0.025% Triton X-100, 1% BSA, 10% donkey serum) for 1 h and then incubated overnight at 4°C with primary antibody diluted in blocking buffer. The following day slides were washed three times with washing buffer (1× PBS, 0.1% Triton X-100), incubated with Alexa488- or Alexa568-conjugated secondary antibodies, and finally washed as before and mounted using Immunofluor mountant (MP Biomedicals, Cambridge, UK). Nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI) stain. For immunofluorescence of frozen sections, perfused lungs were manually inflated via the trachea with a 1:1 mixture of OCT (Cellpath, Powys, UK) and PBS, and frozen sections, perfused lungs were manually inflated via the trachea. Inverted confocal microscope (Zeiss, Welwyn Garden City, UK). Images were edited using Adobe Photoshop CS software. For AEII cell counting 10 randomly collected images of alveolar areas of the lungs of 3 age-matched, sex-matched mice stained with pro-SPC antibody were imported into Volocity 5.4 image analysis software, and AEII cells were identified on the basis of staining intensity above background as well as area measurements. P values were derived using the Student’s t-test.

Conventional electron microscopy. Lungs of C57BL/6 mice were infused and fixed with a mixture of 2% (wt/vol) PFA, 2% (wt/vol) glutaraldehyde (TAAB) in 0.1 M sodium cacodylate buffer (Agar), pH 7.4, postfixed with 1% (wt/vol) OsO4 supplemented with 1.5% (wt/vol) potassium ferrocyanide, dehydrated in ethanol, and infiltrated with propylene oxide (Agar)/Epon (Agar) (1:1) followed by Epon embedding. Ultrathin sections were cut with an Ultracut S microtome (Leica), counter-stained with lead citrate, and observed with a transmission electron microscope (TEM) Jeol 1010. Images were obtained using a Gatan ORIUS CCD camera. For measurement of lamellar body area, electron microscope images were imported into Image J software, the perimeter of each organelle was defined manually, and the area was measured using the measure tool within the software.

Ultracytotomy and immunogold labeling. Lungs of C57BL/6 mice were infused and fixed with 2% (wt/vol) PFA and 0.1% (wt/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.1). Samples were cut in 0.5-mm3 blocks, embedded in 12% gelatin, and infused in 2.3 M sucrose (26). Mounted gelatin blocks were frozen in N2, and ultrathin (50 nm) cryosections were cut at −80°C with a cryostat (Bright Instruments, Huntingdon, UK) and fixed with 4% paraformaldehyde (PFA) (wt/vol), 1× PBS for 20 min at ambient temperature and then processed for blocking and immunolabeling as described for the deparaffinized sections. Images of fluorescent antibody-stained sections were obtained using either wide-field Zeiss Axiovert 200 controlled by Simple PCI acquisition software with images recorded using a Hamamatsu EM-CCD or a Zeiss LSM-510 inverted confocal microscope (Zeiss, Welwyn Garden City, UK). Images were edited using Adobe Photoshop CS software. For AEII cell counting 10 randomly collected images of alveolar areas of the lungs of 3 age-matched, sex-matched mice stained with pro-SPC antibody were imported into Volocity 5.4 image analysis software, and AEII cells were identified on the basis of staining intensity above background as well as area measurements. P values were derived using the Student’s t-test.

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Fig. 2. Immunohistochemistry of frozen sections of murine lung reveals that Rab27 proteins are expressed in the bronchiolar and alveolar epithelium. Frozen sections of C57BL/6 murine lungs were fixed and stained with antibodies specific for Rab27a (A and B) or Rab27b (C) (green in overlay images). In A and C, arrows indicate localization of Rab27a and Rab27b to alveolar epithelial type II (AEII) cells, whereas arrowhead indicates localization of Rab27a and Rab27b to the bronchiolar epithelium. In B, arrow shows localization of Rab27a to granules in the apical projections of Clara cells, whereas arrowhead highlights the expression of Rab27a in ciliated cells. Scale bars A and C = 100 μm and B = 5 μm.
RESULTS

Rab27A and Rab27B are expressed in the murine lung. As a first step to investigate the role of Rab27 proteins in the function of the pulmonary epithelium, we used isoform-specific Rab27 antibodies to test the expression of Rab27a and Rab27b proteins in murine lung lysates. In control (WT) lung lysate we observed that both isoforms are expressed (Fig. 1A). The specificity of Rab27a and Rab27b signal was confirmed by blotting of lysate derived from mutant mice lacking individually Rab27a (Rab27aash/ash) or Rab27b (Rab27bKO), respectively. Moreover, quantitative real-time PCR analysis of Rab27a and Rab27b mRNA levels in resected human lung tissue samples showed that both genes are significantly expressed in the lung (Fig. 1B).

Rab27 proteins are expressed in Clara cells and AEII cells. We then used immunofluorescence microscopy to reveal the distribution of Rab27 isoforms in frozen sections of C57Bl/6 mouse lung. This showed that Rab27a is highly expressed in the bronchiolar epithelium (Fig. 2A, arrow) where it is present in both ciliated and Clara cells (Fig. 2B, arrowhead and arrow, respectively). In high-magnification images of bronchiolar epithelium, Rab27a was found to be particularly apparent in apical vesicles in Clara cells that may represent secretory vesicles (Fig. 2B, arrow). Within the alveolar epithelium, Rab27a was found to be highly expressed within a subset of cells (Fig. 2A, arrow). Double immunolabeling of surfactant protein C (SPC), which is specifically expressed in AEII cells, revealed that Rab27a-positive cells are AEII cells (Fig. 3A). High-magnification images of AEII cells showed that while SPC is present in punctate structures (presumably LBs), Rab27a is present on small vesicles throughout the cytoplasm that do not significantly colocalize with LBs (Fig. 3B). Similar analysis using specific antibodies indicated that Rab27b is also expressed in ciliated and Clara cells of the bronchiolar epithelium (Fig. 2C, arrow), as well as a subset of cells in the alveolar epithelium (Fig. 2C, arrow). Double immunolabeling of frozen sections with SPC-specific antibodies confirmed that a subset of these Rab27b-positive cells are AEII cells (Fig. 3C, AJP-Cell Physiol • VOL 300 • MARCH 2011 • www.ajpcell.org

Fig. 3. Rab27 proteins are localized to cytoplasmic vesicles in AEII cells. Frozen sections of C57Bl/6 murine lungs were fixed and stained with antibodies specific for Rab27a (A, B) or Rab27b (C, D) (green in overlay images), surfactant protein C (SPC, red in overlay images), and DAPI (blue in overlay images) and analyzed using confocal immunofluorescence microscopy. Low-magnification images show Rab27a (A) and Rab27b (C) expression in AEII cells (arrows). High-magnification images show the intracellular distribution of Rab27a (B) and Rab27b (D) in AEII. Scale bars A and C = 25 μm, B and D = 5 μm.
Fig. 4. Electron microscopy of ultrathin cryosections of murine lungs reveals Rab27 proteins localization to cytoplasmic vesicles, lamellar bodies, and multivesicular bodies in AEII cells. Ultrathin cryosections of C57Bl/6 murine lungs were labeled with antibodies specific for surfactant protein C (SPC; A and B), Rab27a (C and D) or Rab27b (E and F) and detected using second antibodies conjugated to 10-nm protein A gold (PAG). Electron microscopy analysis of labeled sections revealed strong expression of SPC (A and B) in the internal vesicular membranes of MVBs as well as LBs and cytoplasmic vesicles in AEII cells. Similarly Rab27a (C and D) and Rab27b (E and F) were localized to the membranes of cytoplasmic vesicles, LBs, and multivesicular bodies (MVBs) in AEII cells. Scale bars = 100 nm.
arrows). High-magnification images of double-labeled sections reveal little colocalization of Rab27b with SPC-positive LBs (Fig. 3D). This analysis also showed that Rab27b is strongly expressed on cytoplasmic granules in an additional cell type present throughout the alveolar epithelium (Fig. 3C, arrowhead and see online supplementary Fig. S1A at AJP-Cell Physiol website). The specificity of antibody staining was confirmed by staining frozen sections of lung from double knockout (DKO) mice (see online supplementary Fig. S2).

Rab27 proteins associate with cytoplasmic vesicles, LBs, and multivesicular bodies in AETII cells and apical secretory granules in Clara cells. We then used cryoimmunoelectron microscopy to investigate further the identity of Rab27-positive structures within AEII cells in ultrathin cryosections of the lungs of C57BL/6 mice. Staining with SPC-specific antibodies (Fig. 4, A and B) showed that this protein is present in the internal membranes of multivesicular bodies (MVBs), LB precursor vesicles, and mature LBs. Similar analysis showed that Rab27a is associated with MVBs and vesicles present throughout the cytoplasm (Fig. 4, C and D). Furthermore, Rab27a did not show strong colocalization with LBs. Meanwhile, Rab27b was localized to cytoplasmic vesicles and in some cases to the limiting membrane of LBs (Fig. 4, E and F). Consistent with immunofluorescence, immunoelectron microscopy analysis confirmed the existence of a second class of Rab27b-expressing cell type within the capillary network of the alveolar wall. High-magnification images revealed that these cells are eosinophils, based on the presence of cytoplasmic crystalloid granules that were labeled with anti-Rab27b antibodies (see online supplementary Fig. S1, B–E). Meanwhile, in the bronchiolar epithelium, examination of immunogold-labeled cryosections revealed that Rab27a is present on the limiting membrane of secretory CCSP granules in Clara cells (Fig. 5, A and B). We also observed gold particles in Clara cells in cryosections labeled with Rab27b-specific antibodies (data not shown).

Loss of Rab27 protein results in atrophy of the pulmonary epithelium. Based on the previous observation that Rab27 proteins are expressed in the murine lung, we next aimed to investigate the function of Rab27 in this context. As a first step we examined the morphology of lungs from mice lacking both Rab27 isoforms (DKO). As shown in Fig. 6A, we observed thinning of the bronchiolar epithelium as well as marked enlargement of the alveolar air spaces in lungs from the DKO compared with control (WT) samples. These morphological alterations were clearly observed in high-magnification images of bronchioles (Fig. 6B), which show disorganization and shortening of bronchiolar cilia, while alveolar areas clearly show enlargement of alveolar spaces and thinning of the alveolar epithelium (Fig. 6C). Quantification using mean linear intercept measurement (Fig. 6D) indicated that airspace enlargement in the DKO reaches significance in the lungs of 12 wk- and 18-mo-old mice. This also indicated that airspace enlargement becomes more evident with increasing age. Meanwhile, measurement of the thickness of the epithelium in multiple bronchioles confirmed that there is significant thinning of the epithelium in DKO compared with age-matched, sex-matched control (WT) samples (Fig. 6E). In addition to these changes, we also observed increased incidence of activated foamy macrophages (see online supplementary Fig. S3A, arrows) and cellular infiltrates containing granulocytes (supplementary Fig. S3, B and C, arrows) in 12-mo-old DKO mice compared with those of age-matched, sex-matched controls (WT) (4/5 DKO vs. 2/9 control samples). Morphological analyses of the lungs of mice lacking individual Rab27 isoforms indicate that Rab27b plays a more significant role in maintenance of the integrity of the pulmonary epithelium than does Rab27a (data not shown).

The numbers of cells expressing AEII and Clara marker proteins are reduced in the lungs of DKO mice. Given that loss of Rab27 proteins resulted in generalized atrophy of the pulmonary epithelium, we investigated the effect of loss of Rab27
specifically in Clara and AEII cells. To do this we stained deparaffinized sections of mutant and control lungs with pro-SPC and CCSP-specific antibodies that detect AEII and Clara cells, respectively, and then used immunofluorescence microscopy to investigate the effects of loss of Rab27 on the number and distribution of these cell types. By this approach we observed a significant reduction in the number of SP-C immune-positive cells in DKO compared with control samples from age-matched, sex-matched WT mice (mean AEII cells/20× field 32 vs. 49.3, respectively, Fig. 7A). Similar analysis of the number of Clara cells in both large and small bronchioles of DKO mice revealed a similar reduction in the number of CCSP immune-positive cells compared with control (WT) bronchioles (Fig. 7B). In addition, we frequently observed that, in contrast to the control, the coverage of Clara cells in DKO bronchioles was patchy with large areas entirely devoid of Clara cells. These observations suggest that the number of AEII and Clara cells is reduced in the lungs of DKO versus WT mice.

**DKO AEII cells accumulate large, mature LBs and contain few LB precursors.** Finally, we used electron microscopy to investigate the ultrastructural alterations in Clara and AEII cells resulting from loss of Rab27 protein. Consistent with immunofluorescence data, this indicated that the bronchiolar epithelium in DKO mice is thinner than that of controls (Fig. 8A). While in Clara cells we observed a dramatic accumulation of cytoplasmic vesicles that most likely represent CCSP-containing secretory granules, together with a reduction in the abundance of early secretory pathway organelles, such as endoplasmic reticulum, that was commonly observed in control samples (Fig. 8A, high magnification image). Similarly, using electron microscopy to study AEII cells, we observed a marked increase in both the number and size of mature LBs and a concomitant reduction in the number of LB precursor structures and mitochondria in DKO compared with control samples (Fig. 8B). This was confirmed by quantification (Fig. 8, C and D). This analysis also confirms that the changes in morphology are progressive and more apparent with aging (Fig. 8C). Parallel electron microscopy analysis of AEII cells in mice deficient in individual Rab27 isoforms revealed similar accumulation of mature LB in AEII cells of mice lacking Rab27b alone, indicating that Rab27b, and not Rab27a, plays a significant role in the regulation of LB homeostasis and AEII cell function.
Ultrastructural analysis of AEII cells confirmed that there is marked thinning of the alveolar epithelium in DKO compared with control lungs (Fig. 8B).

DISCUSSION

In this study we used light and electron microscopy to investigate the localization and function of Rab27 proteins in the lung. Our main findings are that Rab27a and Rab27b are expressed in Clara and ciliated cells in bronchioles as well as AEII cells in the alveolar region. Rab27b is additionally strongly expressed in cells in the parenchyma that we have defined as eosinophils based on our finding that they contain eosinophil-specific crystalloid granules. In particular, we find that Rab27b is associated with cytoplasmic face of these granules. These observations are largely consistent with previous analysis of expression of GFP-Rab27a transgenic protein and Rab27b-driven lacZ expression in the lungs of mice (6, 29). However, our finding of high Rab27b expression in eosinophils is surprising given that previous studies indicated that Rab27a, not Rab27b, is expressed in circulating eosinophils in humans (2). Meanwhile, our analysis of the structure of the lungs of DKO mice revealed a generalized atrophy of both the alveolar and bronchiolar epithelium together with mild airspace enlargement that was particularly apparent in mice.
Fig. 8. Electron microscopy reveals an accumulation of mature secretory organelles in AEII and Clara cells in DKO mice. Ultrathin sections of lungs from control (WT) and DKO mice at 18 mo of age were observed by transmission electron microscopy. A and B: typical appearance of Clara cells in the bronchiolar epithelium (A) and alveolar epithelium type II and type I cells (B) in DKO and control mice. A, right: high magnifications of regions of the left image. B, insets: high magnification images of typical lamellar bodies from each sample. This analysis reveals a marked accumulation of enlarged secretory granules in Clara cells and lamellar bodies in AEII cells as well as thinning of the AEI cells. Mlb, mitochondria-like bodies; prec, precursors; cv, caveolae; s, surfactant; LB, lamellar body; nu, nucleus. Scale bars = 0.5 μm. C: quantification of the differences in organelle content (lamellar body, lamellar body precursor, and mitochondria) in AEII cells in control (WT) and DKO lung samples for mice of indicated ages. For WT and DKO 5-wk-old samples, WT 12-wk-old, and 18-mo-old samples: n = 18, whereas for DKO 12-wk-old and 18-mo-old samples: n = 11 and 15, respectively. *P < 0.05; **P < 0.005. D: quantification of the differences in lamellar body size in AEII of 18-mo-old WT (n = 231 LBs in 18 different AEII cells) and DKO (n = 121 LBs in 14 different AEII cells) mice (ns, observed differences did not reach significance).

over 12 mo old. Moreover, we observed a reduction in the abundance of SP-C/CCSP-positive cells in DKO mice that was more apparent after 12 mo of age. This suggests that Rab27-expressing AEII and Clara cell numbers may be reduced in the DKO mutant compared with WT control. At the subcellular level, we observed that Rab27 was localized to apical granules in Clara cells that may represent secretory granules, whereas in AEII cells, Rab27 was located on cytoplasmic vesicles and MVBs. In DKO samples, we observed a striking accumulation of mature secretory organelles; i.e., Clara cell granules and LBs in AEII cells, and a reciprocal reduction in the numbers of mitochondria, granule precursors, and ER membranes.

Based on these findings, we hypothesize that the function of Rab27 proteins in the lung is to regulate the release of exocytic
granules in specialized secretory cell types such as Clara and AEII cells. This suggestion is consistent with the localization data, as well as the observed accumulation of end-stage secretory organelles in these cell types in mice lacking Rab27 proteins. Further support for this idea comes from the large number of other studies that have linked Rab27 function to regulated exocytosis in a variety of cell types, including CTLs, platelets, and neutrophils (5, 13). Leading from this, we hypothesize that chronic disruption of exocytosis in Clara and AEII cells is the cause of pulmonary epithelium atrophy in DKO. Consistent with this, the reduction in the number of granule precursor structures, early secretory pathway organelles, and mitochondria point to a mechanism whereby accumulation of end-stage organelles feeds back into a reduction in metabolic activity in these cells. Given that other cell types; e.g., bronchiolar ciliated cells and AEII cells, are thought to be derived from these Clara cells and AEII cells, reduced metabolic, and possibly proliferative, activity in these cells may go some way to explaining the widespread atrophy of the lung tissue observed in the DKO mice (16). Interestingly, the changes in lung architecture observed in DKO are similar to some way to explaining the widespread atrophy of the lung tissue observed in the DKO mice (16). Interestingly, the changes in lung architecture observed in DKO are similar to those that occur during natural aging, suggesting that the DKO may represent a useful model for the study of accelerated aging of the lung (23). The relatively mild phenotype observed in the DKO mutant lungs suggests that the function of Rab27 proteins is partially redundant. In line with this idea it has been reported that the closely protein Rab3d, whose function is also linked to exocytosis, is expressed in the pulmonary epithelium (30). Also consistent with the relatively mild nature of the DKO mutant phenotype, we did not observe significant differences in the levels of secreted SP-A, SP-D, SP-C, and CCSP in the bronchoalveolar lavage fluid of DKO and WT animals (data not shown).

Future studies should address the functional consequences of these changes in lung structure in the DKO mice; they should also identify the cargo whose exocytosis is regulated by Rab27 and the molecular mechanisms by which this occurs.

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REFERENCES


