Mitofusins regulate lipid metabolism to mediate the development of lung fibrosis

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Accumulating evidence illustrates a fundamental role for mitochondria in lung alveolar type 2 epithelial cell (AEC2) dysfunction in the pathogenesis of idiopathic pulmonary fibrosis. However, the role of mitochondrial fusion in AEC2 function and lung fibrosis development remains unknown. Here we report that the absence of the mitochondrial fusion proteins mitofusin1 (MFN1) and mitofusin2 (MFN2) in murine AEC2 cells leads to morbidity and mortality associated with spontaneous lung fibrosis. We uncover a crucial role for MFN1 and MFN2 in the production of surfactant lipids with MFN1 and MFN2 regulating the synthesis of phospholipids and cholesterol in AEC2 cells. Loss of MFN1, MFN2 or inhibiting lipid synthesis via fatty acid synthase deficiency in AEC2 cells exacerbates bleomycin-induced lung fibrosis. We propose a tenet that mitochondrial fusion and lipid metabolism are tightly linked to regulate AEC2 cell injury and subsequent fibrotic remodeling in the lung.
Alveoli, the basic units for gaseous exchange in the lung, are composed of alveolar type 1 (AEC1) and alveolar type 2 (AEC2) epithelial cells, capillary networks, and interstitial matrix. Although AEC2 cells cover only 5% of the alveolar surface area, they are highly specialized, metabolically active cells that have a high density of sub-cellular organelles. Importantly, AEC2 cells are the main progenitor cells in the alveoli, differentiating into AEC1 cells for alveolar repair, and proliferating for self-renewal. AEC2 cells primarily function to secrete lung surfactant, a surface-active lipoprotein complex containing ~90% lipid, predominantly composed of phospholipids, particularly dipalmitoylphosphatidylcholine, pamitoyl-myristoyl-phosphati-
dylcholine, and phosphatidylglycerol, along with cholesterol. Lung surfactant reduces the surface tension, preventing the alveoli from collapsing, as well as playing a critical role in lipid metabolism, both of which share common upstream sub-
controls 20, suggesting that mitochondrial fusion may be per-
duced in AEC2 cells after bleomycin treatment. AEC2 cells were isolated from mice treated with bleomycin and AEC2 cells from controls identified several significantly altered mitochondrial and metabolic cellular pathways, including mitochondrial organization, apoptotic signaling, nucleotide metabolic process, regulation of protein transport, RNA transport, and autophagy (Fig. 1b and Supplementary Fig. 2e). Examination of genes included in the mitochondrial organization annotation revealed the upregulation of genes involved in mitochondrial dynamic regulation (such as Mfn1, Mfn2, Dnm1l, and March5) mitochondrial apoptotic control (such as Bcl2l1, Mcl1, Bax, Bid, and Bak1), and mitochondrial oxidative phosphorylation (such as Ndufs6, Ndufs6 and Ndufa12 for complex I, Sdhd for complex II, Cyc1, Cys, Ucerb, and Ucraq for complex III, and Cox5a, Cox5b, Cox6a1 and Cox7c for complex IV) (Fig. 1c, d and Supplementary Data 1), while there was downregulation of genes involved in mitophagy (Pink1, Bnip3, and Atg13) (Fig. 1e). This data highlighted similar transcriptomic responses between murine AEC2 cells after bleomycin treatment and human AEC2 cells from IPF lungs. We next examined mitochondrial ultrastructural changes in AEC2 cells in the murine model of bleomycin-induced lung fibrosis through transmission electron microscopy (TEM). AEC2 cells of mice exposed to bleomycin (8 days post treatment) showed swollen mitochondria with disrupted cristae (Fig. 1f and Supplementary Fig. 2a), which, when compared to the controls, had significantly decreased mitochondrial number (Fig. 1h) and area (Fig. 1g, i and Supplementary Fig. 2b). Immunoblotting showed decreased OPA1 (for optic atrophy 1) protein levels with no change in DRP1 (for dynamin-1-like protein), Mfn1 or Mfn2 expression in AEC2 cells 8 days post bleomycin exposure (Supplementary Fig. 2c, d). Collectively, the data suggested that bleomycin alters mitochondrial dynamics, leading to mitochondrial fragmentation, and also alters the expression of several genes related to mitochondrial regulation in AEC2 cells.

Loss of Mfn1 or Mfn2 in AEC2 cells promotes lung fibrosis. To elucidate the precise function of Mfn1 and Mfn2 in AEC2 cells, we conditionally deleted Mfn1 or Mfn2 in murine AEC2 cells. Specifically, genetically modified mice harboring Mfn1 or Mfn2 flanked by two loxp sites were crossed with Sftp7CreERT2+/+ mice (Fig. 2a). AEC2 cells were isolated from murine lungs, through CD45-negative selection and subsequent EpCAM-positive selection (Supplementary Fig. 1b-e). Tamoxifen
treatment resulted in the selective deletion of Mfn1 and Mfn2 genes in AEC2 cells (Mfn1loxP/loxPSftpcCreERT2+/− (Mfn1ΔAEC2) and Mfn2loxP/loxPSftpcCreERT2+/− (Mfn2ΔAEC2)) as confirmed by genotyping (Fig. 2b) and immunoblotting (Fig. 2c). To access for potential off-target toxicity by CreERT2, heterozygous SftpcCreERT2+/− transgenic mice were used as controls.

Given the pivotal role for MFN1 and MFN2 in regulating mitochondrial fusion, we first evaluated mitochondrial ultrastructural changes in Mfn1−/− and Mfn2−/− AEC2 cells. TEM analysis confirmed that at baseline Mfn1−/− AEC2 cells displayed fragmented mitochondria with decreased mitochondrial area, increased mitochondrial number, but normal cristae (Fig. 2d-f and Supplementary Fig. 3b). In contrast, abnormally enlarged...
mitochondria with irregular and disrupted cristae were seen in the Mfn2−/− AEC2 cells (Supplementary Fig. 3a). These mitochondrial morphological changes were restricted to AEC2 cells and were not observed in other lung cells, such as bronchial epithelial cells (Fig. 2g). To confirm the above findings, we depleted MFN1 or MFN2 in the murine AEC2 cell line MLE 12 through shRNA lentiviral transduction (Supplementary Fig. 4a). Loss of Mfn1 induced more mitochondrial fragmentation than loss of Mfn2 in MLE 12 cells (Supplementary Fig. 4b). Depletion of MFN1 or MFN2 in the human AEC2 cell line A549 also altered mitochondrial morphology (Supplementary Fig. 4c, d). Ultrastructural examination further revealed the increased presence of abnormal mitochondria (swollen, irregular cristae) in Mfn2−/− MLE 12 cells (Supplementary Fig. 4e). We surmised that such morphological changes might be indicative of a failure of Mfn2−/− MLE 12 cells to activate mitophagy for mitochondrial quality control. To verify the importance of MFN2 in mitophagy regulation, we generated a mitophagy reporter system using mtKeima fluorescent protein. We found mitophagy induced by oligomycin and antimycin A was only mildly suppressed by Mfn1 deficiency, but markedly suppressed by Mfn2 deficiency in MLE 12 cells (Supplementary Fig. 4f). Collectively, these data show that loss of either Mfn1 or Mfn2 alters mitochondrial morphology and turnover in AEC2 cells.

Despite evidence of mitochondrial dysfunction, Mfn1ΔAEC2 and Mfn2ΔAEC2 mice continued to thrive at 28–32 weeks post tamoxifen treatment, without remarkable lung pathologies (Fig. 2h). To investigate whether deficiency of Mfn1 or Mfn2 in AEC2 cells altered the development of lung fibrosis after bleomycin treatment, Mfn1ΔAEC2 and Mfn2ΔAEC2 mice were instilled with bleomycin. TEM analysis showed that Mfn1 or Mfn2 deletion enhanced bleomycin-induced mitochondrial damage in AEC2 cells (Fig. 3a, b). After bleomycin treatment, compared to control AEC2 cells, Mfn1−/− AEC2 cells showed decreased mitochondrial area and increased mitochondrial number, while Mfn2−/− AEC2 cells showed increased mitochondrial area and decreased mitochondrial number (Fig. 3c–e and Supplementary Fig. 5a–f). The data suggested that Mfn1 deletion leads to excessive mitochondrial fragmentation, while Mfn2 deletion leads to swollen mitochondria in AEC2 cells after bleomycin treatment. We also found that bleomycin treatment and deletion of Mfn1 or Mfn2 did not alter the amount of mtDNA present in AEC2 cells (Supplementary Fig. 5g)

The bleomycin model, weight loss occurs with disease progression and correlates with the severity of lung fibrosis. We found, compared to control mice, both Mfn1ΔAEC2 and Mfn2ΔAEC2 mice demonstrated persistent weight loss (Fig. 4a) and increased mortality (Fig. 4b) after bleomycin exposure. Lungs from Mfn1ΔAEC2 and Mfn2ΔAEC2 mice also showed more intense Masson’s trichrome staining of fibrotic regions, along with increased immunohistochemical (IHC) staining for collagen III (Fig. 4c). Quantification of acid-soluble collagen showed that Mfn1ΔAEC2 and Mfn2ΔAEC2 mice, compared to the control, had more lung collagen deposition after bleomycin treatment (Fig. 4d). Compared to the control, Mfn1ΔAEC2 or Mfn2ΔAEC2 mice had similar protein levels in bronchoalveolar lavage fluid (BALF), and did not have increased inflammatory cell infiltrates after bleomycin treatment (Supplementary Fig. 6a–c).

Loss of AEC2 cell Mfn1/2 induces spontaneous lung fibrosis. Single-gene deletion of Mfn1 or Mfn2 in AEC2 cells exacerbated bleomycin-induced lung fibrosis, but at baseline did not cause any obvious lung pathology. We hypothesized that Mfn1 and Mfn2 may compensate for the loss of each other to maintain the basal function of AEC2 cells. We therefore generated mice in which both Mfn1 and Mfn2 were simultaneously deleted in AEC2 cells (Mfn1loxP/loxP;Mfn2loxP/loxP;SftpcCreERT2+/− (Mfn1/2ΔAEC2)) and confirmed by genotyping and immunoblotting (Fig. 5a–c). TEM analysis of mitochondrial ultrastructure showed loss of both Mfn1/2 led to increased mitochondrial area (Supplementary Fig. 7a–c), decreased mtDNA copy number (Supplementary Fig. 7d) and considerable accumulation of abnormal mitochondria with disrupted cristae in AEC2 cells (Fig. 5d and Supplementary Fig. 7e). Strikingly, 36.4% of mice deficient in both Mfn1 and Mfn2 in AEC2 cells died by 16 weeks post tamoxifen treatment, with equal penetrance in both sexes (Fig. 5e). Morphological and pathological assessment of lung sections from surviving (~17 weeks post tamoxifen treatment) Mfn1/2ΔAEC2 mice revealed significant increases in Masson trichrome positive staining for collagen deposition, indicative of lung fibrosis (Fig. 5f). All the remaining surviving mice which displayed signs of respiratory distress (i.e. gasping) developed severe and widespread fibrosis involving both lungs. Such trichrome positive regions principally extended from the sub-pleural parenchyma, with no predilection toward right or left lungs, and the pattern of progression resembled those observed in human IPF. IHC staining of the fibrotic zone showed strong positivity for several fibrotic markers, including vimentin, a-smooth muscle actin, and collagen III (Fig. 5g). Immunofluorescent staining of Mfn1/2ΔAEC2 murine lungs also demonstrated increased localization of ER-TR7 positive fibroblastic aggregates (Fig. 5h), which were surrounded by AEC2 cells (Supplementary Fig. 8), possibly indicative of more fibrosis. Morphological features of fibrosis or distinct fibroblastic aggregations were not observed in the lungs of Mfn1ΔAEC2, Mfn2ΔAEC2, SftpcCreERT2+/− or SftpcCreERT2+/− mice (Fig. 5i). Collectively, the above results show that Mfn1/2ΔAEC2 mice develop spontaneous lung fibrosis, which is associated with extensive mitochondrial damage in AEC2 cells.
MFN1/2 regulate lipid metabolism in AEC2 cells. Deletion of either Mfn1 or Mfn2 in murine AEC2 cells aggravated bleomycin-induced lung fibrosis, while deletion of both induced spontaneous lung fibrosis. Given that both bleomycin and depletion of MFN1/2 can impair mitochondrial respiration through mtDNA damage13,21, we next examined whether mtDNA damage-associated mitochondrial bioenergetic failure28 in AEC2 cells can directly induce lung fibrosis. Mice with a mutation in mtDNA polymerase γ (PolgA257A/D257A), the polymerase responsible for proofreading during mtDNA replication, display accumulation of mtDNA mutations and failure of mitochondrial bioenergetics, leading to premature aging and shortened lifespan28,29. We found that PolgA257A/D257A mice had increased swollen mitochondria in AEC2 cells (Supplementary Fig. 9), but did not demonstrate...
Fig. 2 Mice with AEC2 cell-specific deletion of Mfn1 or Mfn2. a Schema demonstrating the generation of AEC2 cell-specific mice deficient in Mfn1 or Mfn2 using a tamoxifen-inducible Sftpc-promoter driven CreERT2. SftpcCreERT2+/− mice were used as controls. b Genotyping of DNA extracted from CD45(−) EpCAM(+) cells and CD45(−)EpCAM(−) cells isolated from control and Mfn1−/−AEC2 mice 6 weeks after tamoxifen injection (n = 3 mice per group). c Representative immunoblots of AEC2 cell lysates obtained 3 weeks after tamoxifen-induced deletion, showing decreased protein levels of MFN1 or MFN2 in the respective knockout cells (n = 3 mice per group). d Representative TEM images (×12,000) of murine left lung at 28-32 weeks post tamoxifen-induced deletion (control mice, n = 20; Mfn1ΔAEC2 mice, n = 10; Mfn2ΔAEC2 mice, n = 9; scale bar 3 mm). Source data (c, e, f) are provided as a Source Data file.

Fig. 3 Mitofusin-deficient AEC2 cells susceptible to bleomycin-induced mitochondrial damage. a, b Representative TEM images (12,000X; scale bar 2 μm) in Mfn1−/− (a) or Mfn2−/− (b) AEC2 cells before (n = 3 mice) and after bleomycin treatment (n = 2 mice). c-e Quantification of mitochondria area of each mitochondrion (control n = 521 mitochondria, Mfn1−/− n = 464 mitochondria, Mfn2−/− n = 119 mitochondria; data presented as the median [interquartile range], and the comparison performed by Mann-Whitney U test) (c), and mitochondrial number per μm² of cytosolic area (d) and mitochondrial area (μm²) (e) in each AEC2 cell (d, e, each dot represents one AEC2 cell; *p < 0.05, **p < 0.001, ***p < 0.001, vs. control by unpaired Student’s t test), using TEM images (12,000X) of control, Mfn1−/− or Mfn2−/− AEC2 cells 8 days after bleomycin treatment (control AEC2 cells n = 26, Mfn1−/− AEC2 cells n = 21, Mfn2−/− AEC2 cells n = 16, from 2 mice per group). Source data (c-e) are provided as a Source Data file.

any pathological changes indicative of the development of spontaneous lung fibrosis, up to the age of 36–40 weeks. These findings indicate that the phenotype of lung fibrosis may be specific to Mfn1/2ΔAEC2 mice and independent to bioenergetic failure alone.

To assess AEC2 cell injury and proliferation in the bleomycin-induced lung fibrosis mouse model and to explore the biological processes affected in AEC2 cells after deletion of Mfn1 or Mfn2, we generated Mfn1loxP/loxP SftpcCreERT2+/− ROSA26tdTomato+/− (Mfn1ΔAEC2/tdTmato−AEC2) and Mfn2loxP/loxP SftpcCreERT2+/− ROSA26tdTomato+/− (Mfn2ΔAEC2/tdTmato−AEC2) mice (Supplementary Fig. 10a), and performed transcriptomic profiling in Mfn1−/− and Mfn2-deficient AEC2 cells. The generation of these mice allowed for tamoxifen-inducible tdTomato fluorescent labeling in Mfn1−/− and Mfn2-deficient AEC2 cells, and was confirmed by demonstrating excision of the floxed allele after...
tamoxifen injection (Supplementary Fig. 10b, c). In the bleomycin-induced lung fibrosis model, we did not observe that Mfn1Δ−/− or Mfn2Δ−/− AEC2 cells had significantly increased cell death 5 days after bleomycin administration (Supplementary Fig. 11a–d). The expression levels of the proliferative marker Mki67 (RNA-Seq data) in AEC2 cells did not significantly increase (Supplementary Fig. 11e), and was not significantly different between controls and Mfn1ΔAEC2 and Mfn2ΔAEC2 mice 5 days after bleomycin exposure (Supplementary Data 3). We further observed minimal Ki67 positive nuclear staining in tdTomato-positive AEC2 cells, 10 days after bleomycin treatment (Supplementary Fig. 11f).

Transcriptomic profiling at baseline showed Mfn2 deletion, compared to Mfn1 deletion, led to more robust changes in gene expressions in AEC2 cells (Supplementary Fig. 10d and Supplementary Data 2). Specifically, Mfn2Δ−/− AEC2 cells, but not Mfn1Δ−/− AEC2 cells, activated genes involved in ATF5-mediated mitochondrial unfolded protein responses (UPRMT) (Atf5, Lonp1, Clpp, and Hspa9), ATF4-mediated stress pathways (Atf4, Ddit3, Asns, Chac1, Pck2, and Trib3), along with genes involved in de novo serine/glycine synthesis pathways (Phgdh, Psat1, Smit2) (Supplementary Fig. 10e)30,31. Neither Mfn1Δ−/− nor Mfn2Δ−/− AEC2 cells activated genes related to UPRER, such as Hspa5, Atf6, Pdia2, Ero1l, Xbp1, Hsp90b1, and Calr. Furthermore, in addition to organelle fusion, the common metabolic biological processes revealed by functional enrichment analyses included lipid localization, nucleotide phosphate metabolic process, and alcohol metabolic process (Supplementary Fig. 10f).

In the bleomycin-induced lung fibrosis model, we found increased common genes which were different between both Mfn1Δ−/− and Mfn2Δ−/− AEC2 cells (Fig. 6a and Supplementary Data 3). Functional enrichment analyses of these common genes identified fatty acid and acylglycerol metabolic process, carbohydrate derivative biosynthetic and nucleoside triphosphate metabolic process and cofactor metabolic process as the major metabolic processes affected in both Mfn1Δ−/− and Mfn2Δ−/− AEC2 cells after bleomycin treatment (Fig. 6b). Examination of genes included in the carbohydrate derivative biosynthetic and nucleoside triphosphate metabolic process, we found upregulation of genes involving oxidative respiratory complexes, and genes involving purine metabolism, particularly nucleoside diphosphate kinase, adenylyl kinase, polyribonucleotide nucleotidytransferase, and adenosine monophosphate deaminase (Fig. 6c). Moreover, a number of genes involved in lipid metabolism were differentially regulated between control and Mfn1Δ−/− or Mfn2Δ−/− AEC2 cells (Fig. 6d), with the downregulation of genes related to fatty acid synthesis, long-chain fatty acid transport, fatty acid activation, elongation and modification (Supplementary Fig. 11g).

The above transcriptomic results suggest upregulated purine metabolism and downregulated lipid metabolism in Mfn1Δ−/− or Mfn2Δ−/− AEC2 cells after bleomycin. Considering Mfn1Δ/2ΔAEC2 mice develop spontaneous lung fibrosis, we evaluated whether the transcriptomic response in Mfn1Δ−/− AEC2 cells at baseline resembled those in the Mfn1Δ−/− or Mfn2Δ−/− AEC2 cells after bleomycin treatment, and whether Mfn1Δ−/− AEC2 cells had functional annotations in common with control AEC2 cells after bleomycin treatment. Functional enrichment analyses comparing isolated AEC2 cell transcripts from Mfn1Δ/2ΔAEC2 and SftpccERTR2+/+/ (control) mice showed that Mfn1/2-deletion markedly affected nucleoside phosphate metabolic process in AEC2 cells (Fig. 6e and Supplementary Data 4), of which purine metabolism is the major biological pathway included in this annotation. Compared to control AEC2 cells, Mfn1/2-deletion in AEC2 cells activated profound ATF5-mediated UPRMT, and
ATF4-mediated stress response pathways (Supplementary Fig. 12a), along with activation of de novo glycolytic serine/glycine synthesis pathways and mitochondrial one-carbon metabolism (Fig. 6f)30,31. Together, the results from transcriptomic analyses suggested upregulation of purine metabolism in Mfn1/2−/− AEC2 cells.

We next compared the overlapping genes identified in Mfn1/2−/− AEC2 cells with those identified AEC2 cells after bleomycin treatment (Supplementary Fig. 12b). Functional enrichment analysis of these overlapping genes confirmed that altered purine metabolism and oxidative phosphorylation are common biological processes altered in Mfn1/2−/− AEC2 cells and in AEC2 cells after bleomycin treatment (Supplementary Fig. 12c). Gene-set enrichment analysis (GSEA) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database further revealed that Mfn1/2−/− AEC2 cells, compared with AEC2 cells after bleomycin treatment, markedly enhance the upregulation of purine metabolism (Supplementary Fig. 12d). Collectively,
transcriptomic upregulation of purine metabolism was a common prominent feature in both Mfn1/2−/− AEC2 cells and Mfn1−/− and Mfn2−/− AEC2 cells after bleomycin treatment.

Loss of MFN1/2 alters lamellar body structure in AEC2 cells. Purine synthesis and phospholipid synthesis share common upstream substrates, including serine and derivatives from glycolysis. Changes in the preferential flux of these substrates towards purine metabolism (as observed in Mfn1−/− and Mfn2−/− AEC2 cells after bleomycin treatment, and in Mfn1/2−/− AEC2 cells) and away from lipid synthesis (as observed in Mfn1−/− and Mfn2−/− AEC2 cells after bleomycin treatment) may alter the innate function of the AEC2 cells to generate phospholipids for surfactant production32. As previously mentioned, AEC2 cells require proper lipid metabolism to continuously produce and store (in lamellar bodies) lung surfactant, a lipoprotein complex primarily composed of lipids (90%) (particularly phosphatidylcholines and phosphatidylglycerol, and cholesterol)43. In this study we observed disrupted and disorganized lipid membranes in the lamellar bodies of Mfn1−/− and Mfn2−/− AEC2 cells treated with bleomycin. This was in stark contrast to control AEC2 cells treated with bleomycin or to control, Mfn1−/− and Mfn2−/− AEC2 cells, which all showed relatively normal lamellar body structure with organized and densely-packed lipid membranes (Fig. 7a). Unlike Mfn1−/− or Mfn2−/− AEC2 cells, that showed no disrupted lamellar body structure at baseline, Mfn1/2−/− AEC2 cell lamellar bodies displayed severe disorganization of lipid lamellae (Fig. 7b), suggestive of disrupted lipid homeostasis in Mfn1/2−/− AEC2 cells.

Mfn1/2 deletion impairs lipid metabolism in AEC2 cells. To evaluate if lipid metabolism was altered in AEC2 cells upon bleomycin treatment, we performed high throughput targeted lipidomic profiling in AEC2 cells of control, Mfn1ΔAEC2 and Mfn2ΔAEC2 mice at baseline and upon bleomycin treatment (8 days). At baseline, Mfn1−/− and Mfn2−/− AEC2 cells did not show any significant alterations in lipid species, except for a modest increase in phosphatidic acid species (Supplementary Fig. 13a-c). However, cholesterol, ceramides, phosphatidic acids, phosphatidylethanolamine, phosphatidyserine and plasmalogen phosphatidylethanolamine were all increased in AEC2 cells 8 days after bleomycin exposure (Fig. 7c, d). In contrast, these lipids were significantly decreased in Mfn1−/− or Mfn2−/− AEC2 cells treated with bleomycin. Specifically, acylceramines and phosphatidylcholines with long unsaturated aliphatic chains increased in control AEC2 cells treated with bleomycin, but not in the Mfn1−/− or Mfn2−/− AEC2 cells treated with bleomycin. Not in the control AEC2 cells treated with bleomycin (Supplementary Fig. 14c). Surfactant protein gene (Sftpfb, Sftpcc) expression was significantly downregulated in control, Mfn1−/− and Mfn2−/− AEC2 cells treated with bleomycin (Supplementary Fig. 14d). These lipid profiling results confirm that deletion of either Mfn1 or Mfn2 perturbs lipid metabolism in murine AEC2 cells after bleomycin treatment.

We next evaluated the lipodome of Mfn1/2−/− AEC2 cells. Strikingly, lipodomic changes in cholesterol, acylceramine, monacrylglycerol, diacylglycerol, phosphatidylserine, and phosphatidylglycerol were distinctively apparent in the Mfn1/2−/− AEC2 cells, when compared to controls (Fig. 7e). Specifically, we found that long-chain acylceramides significantly decreased in Mfn1/2−/− AEC2 cells, when compared to control AEC2 cells (Supplementary Fig. 15a). Phosphatidylglycerol synthesized in mitochondria were the major phospholipid species affected in Mfn1/2−/− AEC2 cells (Supplementary Fig. 15b). Furthermore, diacylglycerol, which is derived from phosphatidic acid, is required for the synthesis of glycerophospholipids in the ER. Several diacylglycerol species (Supplementary Fig. 15c) and certain glycerophospholipids and sphingolipids (Supplementary Fig. 15d) were all markedly decreased in the Mfn1/2−/− AEC2 cells. Surfactant protein gene (Sftpfb, Sftpcc) expression was not altered between control and Mfn1/2−/− AEC2 cells (Supplementary Fig. 15e). The above results together strongly implicate perturbed lipid metabolism in Mfn1/2−/− AEC2 cells.

Impaired AEC2 cell lipid synthesis promotes lung fibrosis. To test the hypothesis that loss of surfactant associated lipid metabolism in AEC2 cells contributes to the development of lung fibrosis (Supplementary Fig. 16), we compromised lipid synthesis in AEC2 cells. FASN encodes the principal enzyme that catalyzes the synthesis of palmitoyl-CoA, the substrate required for glycerophospholipid and sphingolipid synthesis (Supplementary Fig. 11g)33. We generated mice with tamoxifen-inducible Fasn deletion in AEC2 cells (FasnloxP/loxP SftpccCreERT2+/−, referred to as FasnΔAEC2), by crossing SftpccCreERT2+/+ with FasnloxP/loxP mice (Fig. 8a). Fasn is mainly expressed in AEC2 cells34, and immunoblot of AEC2 cell lysates showed FASN depletion after tamoxifen injection (Fig. 8b). Notably, Fasn-deletion did not alter the expression levels of MFN1 and MFN2. Exposure of FasnΔAEC2 mice to bleomycin resulted in higher mortality (Fig. 8c), more weight loss (Fig. 8d), and increased collagen deposition and lung fibrosis (Fig. 8e, f), when compared to SftpccCreERT2+/− controls. These findings confirm that defective lipid metabolism in AEC2 cells promotes bleomycin-induced lung fibrosis, and supports our hypothesis that impaired regulation of lipid metabolism in AEC2 cells of Mfn1ΔAEC2, Mfn2ΔAEC2, and Mfn1/2ΔAEC2 mice contributes to development of lung fibrosis (Supplementary Fig. 16).
Discussion

Here we uncover a critical function for MFN1 and MFN2 in AEC2 lipid metabolism and development of lung fibrosis. In the absence of mitofusins in AEC2 cells, we observe significant morbidity and mortality associated with disordered mitochondrial dynamics, including impairment of surfactant lipid metabolism and the development of spontaneous lung fibrosis.

We herein confirm that mitochondrial damage is a key pathogenic event in AEC2 cell injury and the development of lung fibrosis. Using the AEC2 cell-specific Sftpc-promoter, we are capable of introducing mitochondrial damage exclusively in
AEC2 cells, supporting prior evidence that persistent AEC2 cell mitochondrial damage is pathogenic in the lung fibrotic process in IPF lungs and murine models\textsuperscript{35,36}. Our findings also echo prior studies suggesting that IPF may be a single-cell disease affecting AEC2 cells\textsuperscript{35,36}, which may in turn promote the activation of highly activated fibroblasts and myofibroblasts. Although the interaction between AEC2 cells and fibroblasts in the lung fibrotic process is not completely understood, our current work supports the present theory that injury to AEC2 cells hampers the maintenance of the epithelial cell barrier integrity. This in turn may encourage the aberrant alveolar repair process eventually leading to the extensive lung remodeling observed in IPF\textsuperscript{35,36}.

Although it is technically difficult to access the role of mito-chondrial fusion proteins MFN1 and MFN2 in regulating lipid metabolism in human AEC2 cells from healthy and IPF lungs, in this study we provide evidence that MFN1 and MFN2 regulate lipid metabolism in murine AEC2 cells, which has important ramifications for surfactant lipid production in these cells and importantly for the development of lung fibrosis. Notably, this study confirms mitochondrial fragmentation and increased synthesis of cholesterol, ceramides, and specific glycerophospholipids in response to bleomycin-induced mitochondrial damage. We believe that, in response to mitochondrial damage, AEC2 cells upregulate these lipids as an attempt to maintain surfactant lipid production under conditions of AEC2 cell injury. Loss of surfactant integrity leads to loss of normal lung physiology and may promote the development of lung fibrosis\textsuperscript{37–39}. Most interestingly, we herein find that AEC2 cells require mitofusins to engage such lipid metabolic rewiring programs in order to respond to mitochondrial damage. We reveal that MFN1 or MFN2 deficiency abolishes the lipogenic metabolic response in AEC2 cells under bleomycin-induced mitochondrial damage and demonstrate that impaired regulation of lipid metabolism in the Mfn1\textsuperscript{+/-} Mfn2\textsuperscript{+/-} mice drives lung fibrosis. These findings are consistent with prior findings that both human MFN1 and MFN2 are required to restore cholesterol synthesis in Drosophila larvae incapable of mitochondrial fusion\textsuperscript{40} and that both Fzo1 (the Caenorhabditis elegans homolog of mitofusins in mammalian cells), and lipid synthesis are required for mitochondrial stress adaptation\textsuperscript{41}. Our findings are also consistent with mitofusins, particularly MFN2, directly regulating the interaction between the mitochondrial outer membrane and the ER\textsuperscript{8,42}. Consistently, such distinct functions of MFN1 and MFN2 are highlighted by the observation that Mfn2\textsuperscript{−/−} mice develop more severe lung fibrosis than the Mfn1\textsuperscript{−/−} mice. In addition, the mechanisms by which Mfn1\textsuperscript{−/−} or Mfn2\textsuperscript{−/−} mice are more susceptible to bleomycin may not fully explain the development of spontaneous fibrosis in the Mfn1\textsuperscript{−/−} mice, with SftpC expression reduced in Mfn1- or Mfn2-deficient AEC2 cells in bleomycin-induced lung fibrosis, but remaining unchanged in Mfn1/2-deficient AEC2 cells. The differential roles of MFN1 and MFN2 in AEC2 function regulation and lung fibrosis development require further exploration.

Intra-tracheal bleomycin administration induces acute lung inflammation and epithelial cell injury, followed by epithelial cell repair and fibrotic reactions\textsuperscript{3,22,43}. In this study, we did not observe any difference in inflammation or altered AEC2 cell death or proliferation in Mfn1\textsuperscript{−/−} Mfn2\textsuperscript{−/−} AEC2 cells, suggesting AEC2 cell dysfunction may promote lung fibrosis independent of inflammation and cell injury. Mechanistically, data generated using Polg\textsuperscript{−/−}/Polg\textsuperscript{−/−} mice indicates that failure of mitochondrial bioenergetics alone may not account for the phenotypes observed in the Mfn1\textsuperscript{−/−} Mfn2\textsuperscript{−/−} AEC2 cells after bleomycin treatment. Instead, we put forward a hypothesis whereby transcriptional alterations in key enzymes important for lipid metabolism including the fatty acid synthesis enzyme, FASN regulate lipid synthesis in AEC2 cells after bleomycin treatment. Through AEC2 cell-specific deletion of Fasn, we confirm that loss of lipid synthesis in AEC2 cells, upon mitochondrial damage exacerbates lung fibrosis in murine models. Similar to our findings, others have shown that perturbed long-chain fatty acid synthesis in AEC2 cells aggravates bleomycin-induced lung fibrosis\textsuperscript{44}. Our results are also supported by findings of abnormal lipid profiles in the BALF\textsuperscript{45} and altered lipid synthesis in the lungs\textsuperscript{46} and AEC2 cells from IPF patients\textsuperscript{20}. These findings also re-emphasize the critical role for and the proper composition of alveolar surfactant in maintaining the function and the intactness of the lung during lung injury\textsuperscript{37–38}. Although the mechanistic link between impaired lipid synthesis in AEC2 cells and fibroblast activation requires further investigation, our data suggest that alterations in the lipidome of the lung microenvironment may promote the activation of fibroblasts and myofibroblasts. Interestingly, FASN has been identified as a TGF-β-regulated target in fibroblasts in vitro and in response to bleomycin in vivo and pharmacologically inhibiting this pathway reverses the profibrotic response in the lung\textsuperscript{47}. These findings suggest that lipid synthesis may play a distinct role in AEC2 cells and fibroblasts of the lung.

In addition to altered lipid metabolism we observed another metabolic feature of cells with mitochondrial damage\textsuperscript{48}, namely, the upregulation of de novo purine synthesis, a common feature between Mfn1\textsuperscript{−/−} AEC2 cells at baseline and Mfn1\textsuperscript{−/−} AEC2 cells after bleomycin treatment. We hypothesize that the robust upregulation of purine metabolism in AEC2 cells may promote lung fibrosis indirectly by impairing lipid metabolism in AEC2 cells. The correct regulation of purine and lipid metabolism during mitochondrial damage is important for the diversion and utilization of common upstream substrates shared by both of these pathways, and our data indicate that mitofusins and mitochondrial fusion are essential in balancing such metabolic reprogramming. The biological significance of increased purine synthesis upregulation is not yet clear, but may occur as a
Fig. 7 MFN1 and MFN2 regulates surfactant lipid synthesis in AEC2 cells. a, b Representative TEM images of lamellar bodies (LB) in control, Mfn1- and Mfn2-deficient AEC2 cells at baseline (×25,000; n = 3 mice per group) and 8 days after bleomycin treatment (×50,000; n = 2 mice per group) (scale bar 500 nm) (a) and in SftpcCreERT2+/− and Mfn1/2−/− AEC2 cells (×25,000; n = 3 mice per group; scale bar 1 μm) (b, left panel). Quantification of the percentage of LB with disorganized lipid membranes in AEC2 cells by TEM image analysis (×12,000) (b, right panel). Each dot represents one AEC2 cell (SftpcCreERT2+/− AEC2 cells n = 15 from 3 mice, Mfn1/2−/− AEC2 cells n = 17 from 2 mice; ***p < 0.001, vs. control by unpaired Student’s t-test). c, d Heat map (c) and bar graph (d) of differential changes of specific lipid contents in control, Mfn1−/− and Mfn2−/− AEC2 cells (n = 4 biologically independent samples per group) 8 days after bleomycin treatment. The fold-changes of specific lipids in AEC2 cells after bleomycin treatment relative to those after PBS treatment (n = 3 biologically independent samples per group) were calculated and log-transformed (base 2) (d, *p < 0.05, **p < 0.01, calculated fold change vs. 1 by unpaired Student’s t-test). e Lipidomic analysis in SftpcCreERT2+/− and Mfn1/2−/− AEC2 cells (n = 4 mice per group; *p < 0.05, **p < 0.01, ***p < 0.001, vs. SftpcCreERT2+/− AEC2 cells by unpaired Student’s t test). Data are presented as mean±s.e.m. (d, e). Source data (b–e) are provided as a Source Data file.
27 cells are consistent with previous studies demonstrating that the role of AEC2-specific Mfn1 or Mfn2 deficiency in AEC2 cells may play a direct role in promoting lung fibrosis, and the pathogenic role of AEC2-specific purine metabolism warrants future investigation.

Our findings of impaired mitophagy in Mfn2-deficient MLE12 cells are consistent with previous studies demonstrating that Mfn2 regulates mitophagy and autophagy. However, despite the key role for Mfn2 and possibly Mfn1 in mitophagy regulation in AEC2 cells, Mfn1ΔAEC2 and Mfn2ΔAEC2 mice did not develop lung fibrosis, suggesting that mitofusin-related mitophagy regulation might not be the most critical mechanism accounting for lung fibrosis in the Mfn1/2ΔAEC2 mice. Currently, the cell-specific role of Pink1 and other mitophagy related pathways in the development of lung fibrosis is controversial; with some showing that global Pink1 deficiency promotes pulmonary fibrosis and others demonstrating that Pink1 deficiency in lung macrophages is anti-fibrotic. Further studies into the AEC2 cell-specific role of Pink1 and mitophagy in the development of pulmonary fibrosis are required.

In conclusion, we reveal a function for Mfn1 and Mfn2 in mediating lung fibrosis by regulating lipid metabolism in response to mitochondrial injury in AEC2 cells of the lung, supporting the mitochondrion as a critical signaling and compensatory mechanism to ATP synthesis when mitochondrial bioenergetic function is impaired. Altered purine synthesis has been shown to promote lung inflammation and collagen deposition in murine models and inhibiting purine synthesis may offer therapeutic potential for IPF.

Collectively, the marked upregulation of purine metabolism in Mfn1/2−/− AEC2 cells and in bleomycin-treated Mfn1- or Mfn2-deficient AEC2 cells may play a direct role in promoting lung fibrosis, and the pathogenic role of AEC2-specific purine metabolism warrants future investigation.

Methods

Mice. Mfn1loxP/loxP (stock 029901-UCD) and Mfn2loxP/loxP (stock 029902-UCD) mice were both generated by David C Chan, and were purchased from the Jackson Laboratory. Mfn1loxP/loxP mice were kindly provided by Dr. Clay F Senesnikovich, Washington University School of Medicine. To generate mice with tamoxifen-inducible Mfn1, Mfn2, Mfn1/2 or FASN deletion specifically in AEC2 cells, Mfn1loxP/loxP, Mfn2loxP/loxP and FasnloxP/loxP mice were crossed to SftpC CreERT2/+/−. Mice were used as control for experiments.

Additionally, ROSA26tdTomato+ mice (stock 007914) were purchased from the Jackson Laboratory and were bred with SftpC CreERT2/− to express tamoxifen-inducible tdTomato fluorescence in AEC2 cells. To sort AEC2 cells with loss of Mfn1 or Mfn2 through tamoxifen-inducible tdTomato fluorescence, ROSA26tdTomato+ mice were crossed to Mfn1loxP/loxP or Mfn2loxP/loxP mice to
respectively generate Mfn1\textsuperscript{fl}\textsubscript{ox}/\textsuperscript{lox}/ROSA26\textsuperscript{tdTomato/+} or Mfn2\textsuperscript{fl}\textsubscript{ox}/\textsuperscript{lox}/ROSA26\textsuperscript{tdTomato/+} mice, which were subsequently crossed to Mfn1\textsuperscript{fl}\textsubscript{ox}/\textsuperscript{lox}/ROSA26\textsuperscript{CreERT2/+} or Mfn2\textsuperscript{fl}\textsubscript{ox}/\textsuperscript{lox}/ROSA26\textsuperscript{CreERT2/+} mice. ROSA26\textsuperscript{CreERT2/+} mice were used as the control. All animal experiments and procedures in this study were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine, and were performed in compliance with all relevant ethical regulations.

**Bleomycin model of lung fibrosis.** 12-week-old sex and weight matched mice were used for bleomycin instillations. Induction of anesthesia was performed in the induction chamber by 3.5% Isoflurane, and 0.5–0.75 mg/kg bleomycin (catalog 13877, Cayman Chemical Company) in 50 μL phosphate-buffered saline (PBS) was then given by intratracheal instillation, through gel-loading tips under the assis-
tance of direct laryngoscopy using the otoscope\textsuperscript{65,66}. Control mice received intra-
tracheal instillation of 50 μL PBS only. The weight of mice was recorded before and every 2 days after bleomycin treatment. Mice were euthanized at different time points after bleomycin instillation for sample harvest as outlined in the manuscript and figure legends.

**Sircol assay.** Murine lungs were harvested 14 days after bleomycin or PBS instillation for quantification of the acid soluble collagen, using the Sircol assay (catalog S1000, Biocolor). Murine lungs were first perfused using PBS, and the right lungs were obtained for the measurements, according to the manufacturer’s instructions.

**Bronchoalveolar lavage.** After the mouse was euthanized, the trachea was intub-
bated with a 20-gauge catheter (Terumo). Murine lungs were lavaged with 0.7-ml Bronchoalveolar lavage fluid solution (BD Biosciences) for 1 h at room temperature. Hoechst 33342 (1:1000 dilution in TBS) was incubated with the Alexa Fluor-488-conjugated secondary antibody (Thermo Fisher) for 1 h at room temperature. Hoechst 33342 (1:1000 dilution in TBS) was used to stain the nucleus. The slides were mounted using Prolong Gold antifade solution (Invitrogen), and the images of the slides were obtained by confocal microscopy (Zeiss LSM 880 laser scanning microscope).

**Flow cytometry analysis.** Flow cytometric analyses of EpCAM or SP-C positivity were performed using a LSRII Fortessa cell analyzer (BD Biosciences). For the staining of EpCAM, cells were fixed by 1% PFA for 15 min at room temperature, followed by EpCAM binding with biotin-conjugated anti-EpCAM (1:50; eBioscience) and anti-mouse Fc receptor blocking reagent (1:10; catalog 130–092–575, Miltenyi Biotec) for 1 h on ice. After washing, a FITC-conjugated anti-biotin antibody (1:10; catalog 130–098–796, Miltenyi Biotec) was added for 10 min on ice in the dark. After further washing, the samples were used for flow cytometric analyses. SP-C intracellular staining for flow cytometric analysis was the same as the protocol for immunofluorescent SP-C staining using cytoplasmic slides. MknEa mice were carried out in Flowjo. Induction of mitophagy. To induce mitophagy, cells with mKeima expression were treated with the combination of oligomycin and antimycin A (4 μM/μL; Sigma-Aldrich) for 24 h. Mitophagy measurement was performed by the pH-sensitive mKeima fluorescence by the excitation using 405 nm (for detecting mKeima at pH 7.0) and 561 nm (for detecting acidic mKeima at pH 4.0) lasers\textsuperscript{55,56}. The intensity of mitophagy was calculated by the ratio of cell percentage with acidic mKeima (upper gate) to cell percentage with neutral mKeima (lower gate) (see also Supplementary Fig. 3h). The flow cytometric data were analyzed with Flowjo analytical software (version 10) (https://www.
flowjo.com/). (BD Biosciences).

**AEC2 cell isolation by tdTomato fluorescence.** To isolate AEC2 cells through tdTomato fluorescence, whole lung cell suspension was obtained after digestion and homogenization of mouse lungs, as described for AEC2 cell isolation by MACS separation. DAPI (0.1 μg/mL) was added to assist cell viability. Flow cytometric cell sorting was then performed by an InCyte cell sorter (BD Biosciences) (see also Supplementary Fig. 1a).

**Genotyping for Mfn1 deletion in AEC2 cells.** DNA samples were extracted from AEC2 cells obtained from Spc\textsuperscript{CreERT2/+}; Mfn1\textsuperscript{fllox/flox}/Mfn2\textsuperscript{fllox/flox}/tdTomato-/- AEC2 cells, and Mfn1\textsuperscript{fllox/flox}/tdTomato-/+ AEC2 mice using DNeasy blood and tissue kit (Qiagen), and were used for genotyping through PCR reactions and the subsequent resolution by agarose gel electrophoresis, based on the protocol by MMRC (forward 5′-TGGTTAAATCTTGGGCGGCTGTC-3′ reverse 5′-TTTAAGACAGCGGCTAAGAGCAG-3′).

**Real-time qPCR for quantification of mtDNA copy number.** DNA samples were extracted from isolated AEC2 cells, and were used for the real-time qPCR, using primers for Ndr2 gene of mitochondrial genome (forward 5′-CTATGACCCCTCG CATCATT-3′, reverse 5′-GAGGCT-GTGCTGGTGTCAGC-3′) and Pecan gene of nuclear genome (forward 5′-ATGGAAGCCTGCGCATCATC-3′, reverse 5′-CTTC GGTGCTAGCAGTACAT-3′) or SYBR green PCR master mix (Applied Biosystems), in the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). mtDNA copy number was calculated relative to genomic DNA (gDNA) copy number, through the 2\textsuperscript{ΔΔCt} method.

**Cell lines.** The murine AEC2 cell line MLE 12 and human AEC2 cell line A549 were purchased from ATCC (CRL-2100 and CCL-185, respectively), and were maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin (Gibco). For stable knockdown of Mfn1 mRNA using Lipofectamine 2000 (Invitrogen), shRNA lentiviral particles, followed by pur-
ymycin (2 μg/mL; catalog A11138–03, Gibco) positive selection for 10–14 days, and were then maintained in RPMI 1640 medium containing 2 μg/mL puromycin and 0.5% penicillin-streptomycin. Retroviral packaging plasmids were gifts from David C Chan. The retroviral construct pCHAC-mt-mKeima was a gift from Richard Youle (Addgene plasmid #72342)\textsuperscript{25,56}, and was used to express mtKeima in MLE 12 cells through retroviral transduction. Cell sorting by InCyte sorter (BD Biosciences) was performed to obtain mKeima-positive cells. Based on manufacturer’s instructions, A549 cells were transfected with non-targeting control siRNA (Dharmacon, D-001206-14-05) or siRNA targeting at human Mfn1 (Dharmacon SMARTpool, M-012961-00-0005) or Mfn2 (Dharmacon SMARTpool, M-012961-00-0005) mRNA using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). The above cell lines were free of mycoplasma infection, assessed using EZ-PCR\textsuperscript{TM} Mycoplasma detection kit (Biological Industries).

**Immunoblots.** Immunoblotting was performed using lysis of MLE 12 or Mfn1\textsuperscript{fllox/flox} AEC2 cells. RIPA buffer with protease inhibitor cocktail (Cell Signaling Technology) was used to prepare the lyses, and the protein concentra-
tions were measured using BCA protein assay (Thermo Fisher). Proteins were resolved by NuPAGE 4–12% Bis-Tris gel or 3–8% Tris-Acetate gel
Immuno-sequential incubation with an endogenous peroxidase block, primary antibody, and secondary antibodies against goat IgG (linked to Alexa Fluor-488 or Alexa Fluor-568) (1:500, Thermo Fisher), were used to detect murine MFN1 (1:1000, Antibodies Incorporated 75-162), human MFN1 (1:1000, Proteintech 13798-1-AP), MFN2 (1:1000, Cell Signaling Technology 5741), a-smooth muscle actin (1:640, Cell Signaling Technology, 29245), and collagen III (1:1000, Abcam, ab7778) were used for IHC staining. For IHC staining, the paraffin-embedded lung sections were dewaxed and rehydrated with the RNA-Seq set (Qiagen). These slides were then heated on the Bond III Autostainer at 99–100 °C, and the sections subjected to sequential incubation with an endogenous peroxidase block, primary antibody, secondary antibody, polymer, diamino benzidine, and hematoxylin. Finally, the sections were dehydrated in 100% ethanol, and mounted in Cytoxyl XYL (Richard Allan Scientific). Appropriate positive and negative controls were included.

**Immunofluorescent staining of mouse lung cryosections.** To prepare lung cryosections for immunofluorescent staining, murine lungs were infiltrated using 1.2 ml 4% paraformaldehyde (PFA) (Electron Microscopy Sciences), and then transferred to a 50 ml polypropylene tube containing 10 ml 4% PFA. After 24 h of fixation at 4 °C, the lobes of the mouse lungs were isolated and transferred to a cryomold (Tissue-Tek), and the tissue cassettes were then immersed in 70% ethanol at 4 °C. Primary antibodies against vimentin (1:100, Cell Signaling Technology, 5741), a-smooth muscle actin (1:640, Cell Signaling Technology, 29245), and collagen III (1:1000, Abcam, ab7778) were used for IHC staining. For IHC staining, the paraffin-embedded lung sections were dewaxed and rehydrated with the RNA-Seq set (Qiagen) followed by microwave treatment and heating on the Bond III Autostainer at 99–100 °C, and the sections subjected to sequential incubation with an endogenous peroxidase block, primary antibody, secondary antibody, polymer, diamino benzidine, and hematoxylin. Finally, the sections were dehydrated in 100% ethanol, and mounted in Cytoxyl XYL (Richard Allan Scientific). Appropriate positive and negative controls were included.

**Transmission Electron Microscopy (TEM).** Fixatives for TEM sample preparation were composed of 4% paraformaldehyde, 2.5% glutaraldehyde, 0.02% picric acid in 0.1 M sodium cacodylate buffer (pH 7.3). Murine lungs were infiltrated with 1.2 ml TEM fixative, and were then excised and transferred to a 50 ml polypropylene tube containing 10 ml TEM fixative, and then submitted to the WCMC Microscopy and Image Analysis Core Facility for sample processing and image acquisition. A Zeol electron microscope (EM-1400) was used to obtain images with an accelerating voltage of 100 kV. AEC2 cells were identified according to the appearance of lamellar bodies and the microvilli at the apical cell membrane. The quantification of the number (μm²) of antimycin-treated area and the area (μm²) of mitochondria or lamellar bodies was performed using Fiji running Imagej software.

**RNA-Seq analysis in AEC2 cells.** RNA samples were obtained from MACS-isolated AEC2 cells from SfpCERET2+/+ and Mfn2ΔAEC2 mice, or from AEC2 cells isolated from tdTomato-ΔCreERT2/tdTomato-AEC2 mice. RNA-Seq analysis was performed using Trizol reagent (Invitrogen), and was purified by the RNAasy Plus Mini Kit (Qiagen), according to the manufacturer’s instructions. The cDNA library was sequenced on the HiSeq4000 sequencer (Illumina). The raw sequencing reads in binary base call (BCL) format were processed through bcl2fastq 2.19 (Illumina) for FASTQ format conversion and demultiplexing, RNA reads were aligned and mapped to the mm9 mouse reference genome by TopHat2 (version 2.0.11) (https://ccb.jhu.edu/software/tophat/index.shtml) and the pheatmap package (https://github.com/raikovolke/pheatmap), based on the z scores calculated using the Limma package (https://bioconductor.org/packages/release/bioc/html/limma.html) [65]. Differentially expressed genes were identified using the Limma package (http://bioconductor.org/packages/release/bioc/html/limma.html) [65]. To assess the differential expression, p-values were derived from linear modelling and empirical Bayes moderation and adjusted for multiple testing by the Benjamini-Hochberg method. Gene ontology (GO) over-representation analysis was performed using the clusterProfiler package (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) [65]. GO terms related to biological process were used, and adjusted p-values for multiple testing were calculated based on a false discovery rate (FDR) of 0.1. The most significant GO terms were extracted using the enrichmentMap function of the “networkD3” package (version 1.3). The GO terms were plotted using the R package “visNetwork” and the pheatmap package (https://github.com/raikovolke/pheatmap), based on the z scores calculated using the gene expressions by FPKM.

**Lipidomic profiling in AEC2 cells.** MACS-isolated AEC2 cells from 1 to 2 mice formed one sample for lipidomic analyses. AEC2 cells were snap frozen by liquid nitrogen immediately after isolation, and were stored at −80 °C before lipidomic analysis. The lipidomic samples were submitted to the Columbia University Core Laboratory [66,67]. Lipids were extracted from equal amounts of material (50 μg protein/sample). Lipid extracts were prepared using a modified Bligh and Dyer procedure [68], spiked with appropriate internal standards, and analyzed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies). Glycerophospholipids, sphingolipids and sphingolipids were separated with normal-phase HPLC as described in the manufacturer’s instructions. The cDNA library was sequenced on the HiSeq4000 sequencer (Illumina). The raw sequencing reads in binary base call (BCL) format were processed through bcl2fastq 2.19 (Illumina) for FASTQ format conversion and demultiplexing, RNA reads were aligned and mapped to the mm9 mouse reference genome by TopHat2 (version 2.0.11) (https://ccb.jhu.edu/software/tophat/index.shtml) and the pheatmap package (https://github.com/raikovolke/pheatmap), based on the z scores calculated using the Limma package (http://bioconductor.org/packages/release/bioc/html/limma.html) [65]. Differentially expressed genes were identified using the Limma package (http://bioconductor.org/packages/release/bioc/html/limma.html) [65]. To assess the differential expression, p-values were derived from linear modelling and empirical Bayes moderation and adjusted for multiple testing by the Benjamini-Hochberg method. Gene ontology (GO) over-representation analysis was performed using the clusterProfiler package (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) [65]. GO terms related to biological process were used, and adjusted p-values for multiple testing were calculated based on a false discovery rate (FDR) of 0.1. The most significant GO terms were extracted using the enrichmentMap function of the “networkD3” package (version 1.3). The GO terms were plotted using the R package “visNetwork” and the pheatmap package (https://github.com/raikovolke/pheatmap), based on the z scores calculated using the gene expressions by FPKM.
Statistical analysis. Data are represented as outlined in the figure legends. Unpaired Student’s t-test was used for the comparisons between two groups, and one-way ANOVA with post-hoc Bonferroni test was used for multi-group comparisons. The log-rank test was used to compare the differences of the survival between two groups. The detailed statistical analyses for RNA-seq data were described in the method details of RNA-seq. A two-sided p value <0.05 was statistically significant. All analyses were performed using SPSS version 17.0 (IBM Corporation) or GraphPad Prism version 5.0 (GraphPad Software).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

K.P.C., S.M.C. and A.M.K.C. conceived and designed the study. K.P.C., L.C.F., S.H., S.J.C., K.N., M.I., Z.H., D.B. and Y.J.C. performed the experiments. S.J.C. and M.I. provided technical support for the blomycin model. K.P.C. and C.L.H. performed the data analyses, and C.L.Y., S.M.C., M.E.C. and A.M.K.C. provided critical discussions for data interpretation. K.P.C., S.M.C. and A.M.K.C. wrote the manuscript and all coauthors reviewed and approved the final manuscript.

Additional information

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Competing interests: The spouse of M.E.C. is a co-founder, shareholder and serves on the Scientific Advisory Board of Proterris, Inc. A.M.K.C. is a co-founder, stock holder and serves on the Scientific Advisory Board for Proterris, which develops therapeutic uses for carbon monoxide. A.M.K.C. also has a use patent on CO. He served on the TEVA Advisory Board meeting as a consultant on July 12–13, 2018. The remaining authors declare no competing interests.

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