Acquisition of cellular properties during alveolar formation requires differential activity and distribution of mitochondria

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Running Title: Mitochondria and alveologenesis
Abstract

Alveolar formation requires coordinated movement and interaction between alveolar epithelial cells, mesenchymal myofibroblasts and endothelial cells/pericytes to produce secondary septa. These processes rely on the acquisition of distinct cellular properties to enable ligand secretion for cell-cell signaling and initiate morphogenesis through cellular contraction, cell migration and cell shape change. In this study, we showed that mitochondrial activity and distribution play a key role in bestowing cellular functions on both alveolar epithelial cells and mesenchymal myofibroblasts for generating secondary septa to form alveoli in mice. These results suggest that mitochondrial function is tightly regulated to empower cellular machineries in a spatially-specific manner. Indeed, such regulation via mitochondria is required for secretion of ligands, such as platelet-derived growth factor, from alveolar epithelial cells to influence myofibroblast proliferation and contraction/migration. Moreover, mitochondrial function enables myofibroblast contraction/migration during alveolar formation. Together, these findings yield novel mechanistic insights into how mitochondria regulate pivotal steps of alveologenesis. They highlight selective utilization of energy in cells and diverse energy demands in different cellular processes during development. Our work serves as a paradigm for studying how mitochondria control tissue patterning.
Introduction

Production of alveoli during development and following lung injury is essential for lung function (1-5). Defective alveologenesis underlies bronchopulmonary dysplasia (BPD) (6) and ongoing destruction of alveoli is characteristic of chronic obstructive lung disease (COPD) (7). COPD is a major cause of morbidity and mortality globally (8, 9). During alveolar formation, alveolar epithelial cells (type I [AT1] and type II [AT2] cells), myofibroblasts and endothelial cells/pericytes undergo coordinated morphogenetic movement to generate secondary septa within saccules. As a result, secondary septa are comprised of a layer of alveolar epithelial cells that ensheathes a core of myofibroblasts and endothelial cells/pericytes. Secondary septa formation (or secondary septation) is the most important step during alveolar formation. Platelet-derived growth factor (PDGF) produced by alveolar epithelial cells is a key player in controlling myofibroblast proliferation and contraction/migration during alveologenesis (10, 11). In response to PDGF signaling, the traditional model posits that myofibroblasts proliferate and migrate to the prospective site of secondary septation and secrete elastin. Myofibroblasts and endothelial cells/pericytes are subsequently incorporated with alveolar epithelial cells to form secondary septa. All of these principal components play a key role in driving secondary septa formation (2).

Generation of alveoli increases the surface area and efficiency of gas exchange, enabling high activity in terrestrial environments. Despite the progress that has been made, our mechanistic understanding of alveologenesis remains incomplete.

Mitochondrial activity is essential for every biological process and mitochondria provide a major source of ATP production through oxidative phosphorylation (OXPHOS) (12, 13). Unexpectedly, we have limited mechanistic insight into how mitochondria control cellular processes in vivo. In particular, little is known about if certain cellular processes have a higher
energy demand during alveolar formation. Many genetic and molecular tools have been
developed in mice to study mitochondrial function. They offer a unique opportunity to address
the central question of how mitochondria control alveologenesis at the molecular level.

Mitochondria exhibit dynamic distribution within individual cells. This process is mediated
by the cytoskeletal elements that include microtubules, F-actin and intermediate filaments. For
instance, Rhot1 (ras homolog family member 1), which is also called Miro1 (Mitochondrial Rho
GTPase 1), encodes an atypical Ras GTPase and plays an essential role in mitochondrial
transport (14). RHOT1 associates with the Milton adaptor (TRAK1/2) and motor proteins
(kinesin and dynein), and tethers the adaptor/motor complex to mitochondria. This machinery
facilitates transport of mitochondria via microtubules within mammalian cells. Whether
regulated mitochondrial distribution is essential for lung cell function during alveologenesis is
unknown.

In this study, we have demonstrated a central role of mitochondrial activity and distribution
in conferring cellular properties to alveolar epithelial cells and myofibroblasts during alveolar
formation. In particular, PDGF ligand secretion from alveolar epithelial cells and motility of
myofibroblasts depend on regulated activity and distribution of mitochondria. Moreover, loss of
mitochondrial function does not have a uniform effect on cellular processes, indicating diverse
energy demands in vivo. We also reveal regulation of mitochondrial function by mTOR complex
1 (mTORC1) (15, 16) during alveolar formation and establish a connection between
mitochondria and COPD/emphysema. Taken together, these findings provide new insight into
how different cell types channel unique energy demands for cellular machinery into distinct
cellular properties during alveolar formation.
Results

Mitochondria display dynamic subcellular distribution in alveolar epithelial cells and mesenchymal myofibroblasts during alveolar formation

To uncover the functional role of mitochondria during alveologenesis, we first examined the distribution of mitochondria in murine lung cells involved in alveolar formation. We used antibodies against mitochondrial components to visualize the distribution of mitochondria in lung epithelial cells and myofibroblasts. For instance, we performed immunostaining on lung sections derived from Sox9Cre/++; ROSA26mTmG/+ mice with anti-MPC1 (mitochondrial pyruvate carrier 1) and anti-MTCO1 (mitochondrially encoded cytochrome C oxidase I) (17). In particular, anti-MPC1 serves as a general marker for mitochondria. Lung epithelial cells were labeled by GFP produced from the ROSA26mTmG reporter (18) due to selective Cre expression in SOX9+ epithelial cells (19). We found that mitochondria were widely distributed in alveolar epithelial cells (distinguished by T1α and SPC for AT1 and AT2 cells, respectively) and myofibroblasts (marked by PDGFRA, PDGF receptor α, and smooth muscle actin, SMA) (20, 21) (Figure 1A). This is consistent with an essential role of mitochondrial activity in proper functioning of lung cells. In addition, we observed an uneven subcellular distribution of mitochondria (Figure 1A, 1B). For instance, mitochondria were concentrated in areas adjacent to the trans-Golgi network (TGN38+) in alveolar epithelial cells (especially AT1 cells) where proteins were sorted to reach their destinations through vesicles and in areas that surrounded SMA in myofibroblasts (Figure 1A, 1B). This finding suggests that localized mitochondrial distribution is required for cellular function in mammalian lungs.
Compromised mitochondrial activity in the postnatal murine lung leads to defective alveologenesis

We first tested if mitochondrial activity is required for alveologenesis by inactivating Tfam (transcription factor A, mitochondria), which encodes a master regulator of mitochondrial transcription (22), in the mouse lung after birth. We produced CAGG\textsuperscript{CreER/+}; ROSA26\textsuperscript{mTmG/+} (control) and Tfam\textsuperscript{f/f}; CAGG\textsuperscript{CreER/+}; ROSA26\textsuperscript{mTmG/+} mice. Tamoxifen was administered to neonatal mice to activate CreER and lungs were collected at postnatal (P) day 10 (Figure 2A). CreER expression under the CAGG promoter/enhancer (23) was ubiquitous in lung cells, including NKX2.1\textsuperscript{+} epithelial cells and PDGFRA\textsuperscript{+} fibroblasts/myofibroblasts, and converted a floxed allele of Tfam (Tfam\textsuperscript{f}) (24) into a null allele (Figure 2B). We noticed that multiple regions in the lungs of mutant mice displayed alveolar defects concomitant with an increased mean linear intercept (MLI), a measure of air space size (25, 26) (Figure 2C, 2D). Alveolar defects were associated with disorganized SMA (Figure 2E). We anticipated that Tfam removal led to shutdown of mitochondrial transcription and reduction of mitochondrial activity. Indeed, the relative ratio of mitochondrial DNA (mtDNA) (27), 16S rRNA and mtND1 (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), was reduced in Tfam-deficient lungs compared to controls (Figure 2F). Loss of Tfam was accompanied by diminished immunoreactivity of MTCO1, the expression of which is controlled by Tfam (Figure 2G). Together, these results indicate that mitochondrial activity is required for alveolar formation.

Selective reduction of mitochondrial activity in the lung epithelium disrupts alveologenesis
To investigate the function of mitochondrial activity in distinct compartments, we selectively reduce mitochondrial activity in either the lung epithelium or mesenchyme. We produced control and \( Tfm^{lf} \); \( Sox9^{Cre/+} \) mice to establish a platform for mechanistic studies on mitochondrial activity in lung epithelial cells during alveolar formation. The \( Sox9^{Cre} \) mouse line (19) is highly efficient in removing sequences flanked by loxP sites in the distal lung epithelium. \( Sox9^{Cre} \) is active at or later than 11.5 days post coitus (dpc) and converted \( Tfm^{lf} \) into a null allele and compromised mitochondrial activity (Figure 3–figure supplement 1A). \( Tfm^{lf} \); \( Sox9^{Cre/+} \) mice were born at the expected Mendelian frequency and could not be distinguished from their wild-type littermates by their outer appearance at birth. Moreover, histological analysis revealed no difference between control and mutant lungs prior to P5, confirming that branching morphogenesis and saccule formation were unaffected by inactivating \( Tfm \) in SOX9+ cells (Figure 3, figure supplement 2A). In addition, differentiation of alveolar type I and type II cells proceeded normally in \( Tfm^{lf} \); \( Sox9^{Cre/+} \) lungs (Figure 3–figure supplement 2B). These results highlight a difference in dependence on mitochondrial activity in distinct cellular processes during development. To uncover the cellular processes that are highly dependent on mitochondrial activity, we investigated alveolar formation in control and \( Tfm^{lf} \); \( Sox9^{Cre/+} \) lungs.

After P5, \( Tfm^{lf} \); \( Sox9^{Cre/+} \) mice could be discerned by their slightly reduced body size in comparison with the littermate controls. Histological analysis of \( Tfm^{lf} \); \( Sox9^{Cre/+} \) lungs at various postnatal stages revealed defects in secondary septa formation with an increased MLI (Figure 3A, 3B) and reduced primary septal thickness (Figure 3C). In this setting, primary septal thickness (P2–P7) appears to be an earlier and more sensitive indicator than MLI in detecting defects in secondary septation. No apparent difference in cell death was noted between control
and Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs (Figure 3–figure supplement 3A), suggesting that mitochondria-mediated apoptosis was not activated. Moreover, lysates from Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs displayed a reduction in mtDNA/nDNA ratio (Figure 3D), mitochondrial complex I activity (Figure 3E) and ATP production (Figure 3F). By contrast, loss of epithelial Tfam did not affect the major regulators of mitochondrial fusion and fission such as OPA1 processing and DRP1 phosphorylation (13) (Figure 3–figure supplement 4). Together, these findings are consistent with reduced mitochondrial activity in Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs and reveal a critical role of mitochondrial activity in lung epithelial cells during alveologenesis. We noted that removal of Tfam in T cells by Foxp3-Cre (28) or Cd4-Cre (29) and in macrophages by activated Cx3cr1-CreER (30) did not exhibit alveolar defects (Figure 3–figure supplement 5) (31, 32). Histological analysis revealed no difference between control and Tfam\textsuperscript{ff}; Foxp3\textsuperscript{Cre/+} and Tfam\textsuperscript{ff}; Cd4\textsuperscript{Cre/+} lungs, and between control and Tfam\textsuperscript{ff}; Cx3cr1\textsuperscript{CreER/+} lungs that had received tamoxifen (Figure 3–figure supplement 5). This suggests that structural components of the secondary septa are more susceptible to reduced mitochondrial function.

**Disruption of mitochondrial distribution in the lung epithelium disturbs alveologenesis**

As described above, mitochondria display dynamic distribution in lung cells, raising the possibility that proper subcellular distribution of mitochondria is vital for cellular function during alveolar formation. To test this hypothesis, we generated control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} mice. Sox9\textsuperscript{Cre} converted a floxed allele of Rho1 (Rho1\textsuperscript{f}) (33) to a null allele in SOX9\textsuperscript{+} alveolar epithelial cells. Loss of Rho1 is expected to perturb normal subcellular distribution of mitochondria. Rho1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} mice were born at the expected Mendelian frequency and cannot be distinguished from their wild-type littermates by their outer appearance or activity at
birth. Similarly, no difference between control and mutant lungs prior to P5 was detected by histological analysis (Figure 3–figure supplement 2C, 2D). After P5, Rhot1^{f/f}; Sox9^{Cre/+} mice displayed defects in secondary septation (Figure 3G) with an increased MLI (Figure 3H) and reduced primary septal thickness (Figure 3I). Loss of epithelial Rhot1 did not induce cell death (Figure 3–figure supplement 3B). The alveolar phenotypes could first appear anywhere between P5 and P12 (Figure 3G-I). As expected, mitochondrial activity was unperturbed by disrupting epithelial Rhot1. No changes in mtDNA/nDNA ratio, mitochondrial complex I activity or ATP production were observed in lysates from Rhot1^{f/f}; Sox9^{Cre/+} lungs (Figure 3J-L). These results support the notion that localized mitochondrial distribution plays a functional role in alveolar formation. We noticed that the alveolar defects in Rhot1^{f/f}; Sox9^{Cre/+} lungs were less severe than those in Tfam^{f/f}; Sox9^{Cre/+} lungs. This is likely due to the fact that only the distribution and not the activity of mitochondria was perturbed in Rhot1^{f/f}; Sox9^{Cre/+} lungs.

**PDGF signal reception is perturbed and the number of mesenchymal myofibroblasts is reduced in the absence of proper mitochondrial activity or distribution in the lung epithelium**

We examined various lung cell types in Tfam^{f/f}; Sox9^{Cre/+} lungs to explore the molecular basis of their alveolar phenotypes. Interestingly, the number of fibroblasts/myofibroblasts marked by PDGFRA was reduced in the absence of epithelial Tfam (Figure 4A, 4D). Likewise, we found that the number of fibroblasts/myofibroblasts was reduced in Rhot1^{f/f}; Sox9^{Cre/+} lungs where epithelial Rhot1 was lost (Figure 4G, 4I). A diminished population of fibroblasts/myofibroblasts in Tfam^{f/f}; Sox9^{Cre/+} and Rhot1^{f/f}; Sox9^{Cre/+} lungs prompted us to investigate whether PDGF signaling was disrupted.
Phosphorylation of PDGFRα (p-PDGFRα), indicative of PDGF signaling, was reduced in Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} or Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs (Figure 4A, 4G). This observation suggests that PDGF signal reception by fibroblasts/myofibroblasts was impaired in Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} or Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs. Defective PDGF signal reception in fibroblasts/myofibroblasts could be due to lack of PDGF production, trafficking or release.

We found that production of the PDGF ligand (PDGFA) in alveolar epithelial cells was unaffected in Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} or Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs by qPCR analysis (Figure 4E, 4J). To substantiate this model, we utilized a PDGF reporter mouse line (Pdgfa\textsuperscript{ex4COIN}) (34) that faithfully recapitulates the spatial and temporal expression of Pdgfa. Of note, no reliable PDGF antibody is available to detect PDGF in lungs or other tissues (21, 34). We generated Pdgfa\textsuperscript{ex4COIN/+}; Sox9\textsuperscript{Cre/+} (control) and Tfam\textsuperscript{ff}; Pdgfa\textsuperscript{ex4COIN/+}; Sox9\textsuperscript{Cre/+} mice. Cre recombinase activated β-galactosidase (lacZ) expression in Pdgfa-expressing cells from the Pdgfa\textsuperscript{ex4COIN} allele. We found that LacZ expression in Pdgfa-expressing cells (i.e., alveolar epithelial cells) displayed a similar pattern and intensity between control and Tfam-deficient lungs (Figure 4C, Figure 4–figure supplement 1A). Together, these results pointed to disrupted PDGF trafficking or release. This defect would subsequently disturb signal reception in mesenchymal fibroblasts/myofibroblasts of Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs.

PDGF secretion from lung cells is diminished without proper mitochondrial activity or distribution

Our model posits that secretion of PDGF ligand from Tfam- and Rhot1-deficient alveolar epithelial cells is compromised. To test this idea, we derived Tfam- and Rhot1-deficient cells from Tfam\textsuperscript{ff}; Pdgfra\textsuperscript{Cre/+} and Rhot1\textsuperscript{ff}; Pdgfra\textsuperscript{Cre/+} lungs (see below), respectively. We
transduced control and Tfam- and Rhot1-deficient cells with lentiviruses that produced epitope-tagged PDGF (Figure 4–figure supplement 1B). Using this assay, we determined the amount of PDGF released from control and Tfam- and Rhot1-deficient cells (Figure 4F, 4K). PDGF levels in the conditioned media derived from Tfam- or Rhot1-deficient cells were reduced compared to controls (Figure 4F, 4K). These findings support a model in which loss of mitochondrial activity or distribution results in a failure of vesicular transport and PDGF release from alveolar epithelial cells. We surmise that these defects are in part due to an incapacitated actomyosin cytoskeleton caused by reduced mitochondrial function.

Selective reduction of mitochondrial activity or distribution in fibroblasts/myofibroblasts compromises alveogenesis

We then investigated the functional requirement of mitochondria in the lung mesenchyme. To this end, we produced control and Tfam\(^{fl/fl}\); Pdgfra\(^{Cre/+}\) (35) mice for Tfam inactivation in lung fibroblasts/myofibroblasts. It was reported that ~95% of lineaged cells (PDGFR\(^{A}\)) are myofibroblasts (36). Nevertheless, we have adopted PDGFR\(^{A}\) fibroblasts/myofibroblasts throughout this study for accuracy. Cre expression in PDGFR\(^{A}\) fibroblasts/myofibroblasts eliminated Tfam and reduced mitochondrial activity. Indeed, the expression of MTCO1, a transcriptional target of Tfam, was decreased in lung fibroblasts/myofibroblasts derived from Tfam\(^{fl/fl}\); Pdgfra\(^{Cre/+}\) mice (Figure 3–figure supplement 1B). Prior to P3, Tfam\(^{fl/fl}\); Pdgfra\(^{Cre/+}\) mice could not be distinguished from their wild-type littermates by appearance, activity, morphological and immunohistochemical analysis (Figure 5–figure supplemental 1A, 1B). This suggests that loss of Tfam in the lung mesenchyme did not affect branching morphogenesis or saccule formation (37). This permitted us to assess the contribution of mesenchymal
mitochondria to alveolar formation. Histological analysis of Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> lungs at various postnatal stages prior to P5 revealed defective secondary septa formation with an increased MLI (Figure 5A, 5B) and reduced primary septal thickness (Figure 5C). Loss of mesenchymal Tfam did not induce cell death (Figure 5, figure supplement 2A). Moreover, lysates from Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> lungs displayed a reduction in mtDNA/nDNA ratio (Figure 5D), mitochondrial complex I/IV activity (Figure 5E) and ATP production (Figure 5F). All of them are consistent with reduced mitochondrial activity in Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> lungs. Most Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> mice died before P30.

We also generated control and Tfam<sup>fl/fl</sup>; Twist2 (Dermo1)<sup>Cre/+</sup> mice, in which Tfam was eliminated by mesenchymal Twist2<sup>Cre</sup> (38). Tfam<sup>fl/fl</sup>; Twist2<sup>Cre/+</sup> mice exhibited alveolar defects (Figure 5–figure supplemental 3A, 3B) similar to those in Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> mice, further supporting a central role of mitochondrial activity in the lung mesenchyme during alveologenesis.

Of note, we bred Lrpprc<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> mice as an alternative means to disrupt mitochondrial activity. Lrpprc (<i>Leucine-rich PPR motif-containing</i>) (39) is required for mitochondrial translation. Similarly, Pdgfra<sup>Cre</sup> converted a floxed allele of Lrpprc (Lrpprc<sup>f</sup>) into a null allele. Lrpprc affects different aspects of mitochondrial activity and serves the purpose of confirming our findings using Tfam. We found that Lrpprc<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> mice developed alveolar defects (Figure 5–figure supplemental 3C, 3D), albeit the phenotypes were less severe than those in Tfam<sup>fl/fl</sup>; Pdgfra<sup>Cre/+</sup> mice. This was likely due to the presence of residual proteins after removal of Lrpprc. Together, these studies establish a crucial role of mitochondrial activity in fibroblasts/myofibroblasts for alveologenesis.
We went on to determine whether proper subcellular distribution of mitochondria in fibroblasts/myofibroblasts is necessary for their function during alveolar formation. We generated control and \( Rhot1^{ff}; Pdgfra^{Cre/+} \) mice (Figure 5, figure supplemental 1C, 1D). In this case, the subcellular distribution of mitochondria is expected to be perturbed in mesenchymal fibroblasts/myofibroblasts as indicated by loss of proper MTCO1 distribution in fibroblasts/myofibroblasts derived from lungs of \( Rhot1^{ff}; Pdgfra^{Cre/+} \) mice (Figure 3–figure supplement 1C). \( Rhot1^{ff}; Pdgfra^{Cre/+} \) mice exhibited alveolar defects (Figure 5G–I, Figure 5–figure supplement 2B), milder than those in \( Tfat1^{ff}; Pdgfra^{Cre/+} \) lungs. Mitochondrial activity was unperturbed by disrupting mesenchymal \( Rhot1 \) (Figure 5J–L).

We discovered that fibroblasts/myofibroblasts proliferation was reduced in \( Tfat1^{ff}; Pdgfra^{Cre/+} \) or \( Rhot1^{ff}; Pdgfra^{Cre/+} \) lungs compared to controls (Figure 6A, 6B, 6E, 6F), suggesting defective PDGF signal reception. This may be related to a failure in PDGFR trafficking when mitochondrial function is impaired. As a result, the pool of PDGFR+ myofibroblasts was decreased with a concomitant reduction in SMA (encoded by Acta2) and/or elastin (Figure 6C, 6G), contributing to alveolar defects. In summary, these findings suggest that proper activity and distribution of mitochondria in alveolar fibroblasts/myofibroblasts are critical to generating a sufficient number of fibroblasts/myofibroblasts for secondary septation.

Contraction/migration of fibroblasts/myofibroblasts is reduced without proper mitochondrial activity or distribution, which is associated with a disrupted cytoskeleton

We speculate that myofibroblasts deficient in \( Tfat1 \) are defective in their ability to migrate to the prospective site of secondary septation. To test this idea, we isolated fibroblasts/myofibroblasts from control and \( Tfat1^{ff}; Pdgfra^{Cre/+} \) lungs. \( Tfat1 \)-deficient fibroblasts/myofibroblasts displayed
short tubular and fragmented mitochondria (Figure 6–figure supplement 1A, 1B).

Fibroblasts/myofibroblasts were seeded onto the migration chamber for wound healing assays (40), in which the rate of fibroblasts/myofibroblasts migration into the cell-free area was measured. While control fibroblasts/myofibroblasts occupied the cell-free area after 36–48 hr, only scant Tfam-deficient fibroblasts/myofibroblasts were detected in the cell-free area (Figure 6D). Introduction of TFAM into Tfam-deficient fibroblasts/myofibroblasts rescued their migratory defects (Figure 6–figure supplement 2A, 2B, 2E). This result indicates that mobility of fibroblasts/myofibroblasts was compromised due to loss of mitochondrial activity in these cells.

We conjecture that the migration defect was in part due to an incapacitated actomyosin cytoskeleton without mitochondrial activity. Consistent with this idea, organization of the cytoskeleton (labeled by phalloidin) was perturbed in Tfam<sup>f/f</sup>; Pdgfra<sup>Cre/+</sup> lungs in comparison with controls (Figure 6A).

Similarly, fibroblasts/myofibroblasts derived from Rhot1<sup>f/f</sup>; Pdgfra<sup>Cre/+</sup> lungs displayed a compromised response in wound healing assays compared to controls (Figure 6H, Figure 6–figure supplement 1C, 1D), which was restored by RHOT1 expression (Figure 6–figure supplement 2C-E). To sum up, these results affirm the role of mitochondrial activity and distribution in regulating fibroblasts/myofibroblasts contraction and/or migration during alveologenesis.

**mTOR complex 1 regulates mitochondrial function and alveologenesis**

We have discovered an essential role of mitochondria in controlling alveolar formation. To dissect the signaling cascade that regulates mitochondrial function during alveologenesis, we first investigated mTOR complex 1 (mTORC1), a known regulator of mitochondrial activity and
biogenesis. We generated control, Rptor<sup>flo</sup>; Sox9<sup>Cre/+</sup> and Rptor<sup>flo</sup>; Pdgfra<sup>Cre/+</sup> mice. Rptor<sup>314</sup> (Raptor, rapamycin-sensitive regulatory associated protein of mTOR) (41) encodes an essential component of mTORC1, which includes RPTOR, mTOR and several other proteins. Rptor<sup>flo</sup>; Sox9<sup>Cre/+</sup> and Rptor<sup>flo</sup>; Pdgfra<sup>Cre/+</sup> mice failed to survive to term, precluding the analysis of potential alveolar phenotypes.

To circumvent this problem, we produced control and Rptor<sup>flo</sup>; CAG<sup>GCreER/+</sup> mice and administered tamoxifen postnatally (Figure 7A). Analysis of Rptor<sup>flo</sup>; CAG<sup>GCreER/+</sup> mice at P10 revealed alveolar defects with an increased MLI (Figure 7B, 7C). Defective secondary septation in Rptor<sup>flo</sup>; CAG<sup>GCreER/+</sup> lungs was associated with reduced SMA (Figure 7D). As expected, the protein levels of phosphorylated ribosomal protein S6 (p-RPS6), a downstream target of mTORC1, was decreased in lysates from Rptor-deficient lungs compared to controls (Figure 7E, Figure 7–figure supplement 1). The relative ratio of mtDNA to nDNA was reduced in Rptor-deficient lungs (Figure 7F). Moreover, immunoreactivity of both MPC1 and MTCO1 was diminished in Rptor<sup>flo</sup>; CAG<sup>GCreER/+</sup> lungs compared to controls (Figure 7G). These findings show that elimination of Rptor in the lung resulted in loss of mitochondria. Impaired mitochondrial function then contributed to alveolar defects. Collectively, our results suggest that mTORC1 controls alveolar formation partly through its effects on mitochondrial function.

Mitochondrial copy number and TFAM protein levels are decreased in lungs from COPD/emphysema patients

To explore whether studies of mitochondrial function in alveolar formation in mice can shed new light on human diseases, we assessed the status of mitochondria in lung tissues of normal subjects and COPD/emphysema patients (Figure 8A, Figure 8–figure supplement 1). We found
that the copy number of mitochondria (27) relative to nuclear DNA was significantly reduced in COPD/emphysema patients (Figure 8B). In addition, TFAM protein levels were reduced in lysates from emphysema lungs compared to normal lungs (Figure 8C). These results established a connection between mitochondrial function and pathogenesis of COPD/emphysema. Interestingly, a disorganized cytoskeleton was noted in COPD/emphysema lungs, in which actin bundles seen in normal alveoli were sparse (Figure 8D). We did not detect a difference in the protein levels of either RPS6 or p-RPS6 in lysates from emphysema and normal lungs (Figure 8E, Figure 8–figure supplement 2). However, we observed heterogeneous expression of p-RPS6 in emphysema lungs. Whether regional reduction of p-RPS6 is correlated with disease progression needs future studies.

To further explore the connection between mitochondrial function and cellular properties, we conducted gain- and loss-of-function studies on TFAM and RHOT1 in both human lung epithelial cells and human lung fibroblasts. TFAM or RHOT1 knockdown in human lung epithelial cells impaired PDGF secretion from epithelial cells (Figure 8F, Figure 8–figure supplement 3A). This defect was rescued by introduction of mouse TFAM or RHOT1 (Figure 8F, Figure 8–figure supplement 3A). In addition, TFAM or RHOT1 knockdown in human lung fibroblasts compromised cell migration of fibroblasts (Figure 8G Figure 8–figure supplement 3B). Likewise, migration defects were restored upon expression of mouse TFAM or RHOT1 (Figure 8G, Figure 8–figure supplement 3B). Results from the experiments using human lung cells affirmed the observations obtained in mouse cells and mouse lungs. Taken together, these findings using human lungs and cells complement our mouse work and lay the foundation for further investigation into the disease mechanisms of COPD/emphysema.
Discussion

Our studies have provided new insight into how mitochondrial function controls alveolar formation. We discovered a major role of mitochondria in conferring requisite cellular properties to both alveolar epithelial cells and myofibroblasts during alveologenesis. These findings define the molecular basis of the functional requirement of mitochondria in distinct compartments and reveal the energy demand in a given process. They also add a new layer of complexity to the interactions between the major players of secondary septa. Moreover, our work establishes the foundation for investigating the interplay between mitochondria and signaling pathways in endowing cellular properties in alveologenesis during development and following injury. We expect that this framework will be applicable to other developmental systems (Figure 9).

A reduction in mitochondrial function either globally or in distinct compartments in the lung results in alveolar defects. We found that not all cellular processes are perturbed to the same extent when mitochondrial activity or distribution is perturbed. For instance, while alveolar development is disrupted, saccule formation and cell type specification are unaffected. These observations highlight the differential requirement of mitochondrial function in a given cellular process. Events that necessitate a higher demand of energy will be the first to exhibit phenotypes once mitochondrial function is compromised. It implies that the severity of phenotypes resulting from a reduction in mitochondrial function could be used to characterize the energy demand of a particular cellular process in development. This information cannot be obtained from cell-based studies. It is unclear why alveolar formation has a higher energy demand than saccule formation. Perhaps, construction of a more complex structure such as the alveolus requires additional energy consumption.
A limitation of our approach lies in the broad expression of Cre lines in multiple cell types over an extended developmental time. We propose that distinct cellular processes exhibit differential dependence on mitochondrial activity. While there is no evidence that a new epithelial cell type emerges from saccular to alveolar stages, the transcriptome of any cell type changes as development proceeds. The simplest model is that the same cell types with an altered transcriptome (dubbed cell subtypes) display different sensitivity to mitochondrial perturbation (and energy expenditure) as lung development proceeds from saccular to alveolar stages. This is based on the assumption that the subcellular events (e.g., ligand secretion and cellular contraction) that drive a cellular process (e.g., sacculation and secondary septation) are the main consumer of energy for a given cell subtype. However, it is also possible that the subcellular events that execute a given cellular process may not be the main consumer of energy. In this scenario, one would conclude that the phenotypic defects of a given cellular process due to compromised mitochondrial activity are indicative of the sensitivity of the subcellular events to mitochondrial activity. Finally, we cannot rule out the possibility that functions mediated by mitochondria other than energy production play an important role in a given cellular process (42). However, lack of apoptosis in the mutant lungs in our study suggests that mitochondria-controlled cell death does not contribute to the lung phenotypes.

Our work has focused on PDGF ligand secretion. Alveolar epithelial cells, especially AT1 cells, are the reservoir for multiple ligands, including PDGFA, VEGFA and SHH (40, 43, 44). While other pathways have not been interrogated, different pathways may exhibit a varying degree of dependence on mitochondrial function. Similarly, this could provide a new way to functionally categorize signaling pathways on the basis of their energy demand in vivo. Such insight will provide a new blueprint of how cells dispense their energy source in tissues.
Secretion of the PDGF ligand from alveolar epithelial cells is impaired due to mitochondrial dysfunction in either activity or distribution. We surmise that the actomyosin cytoskeleton likely underlies this defect. However, it is also possible that the energy produced by mitochondria is required in many key steps of vesicular transport and membrane fusion. Additional insight would come from a careful assessment of the dependence of each process on mitochondrial function. Similarly, failure to power the actomyosin cytoskeleton in myofibroblasts due to disruption of mitochondrial activity or distribution could cause the contraction and/or migratory defect. ATP produced by mitochondria is known for the assembly of the actomyosin cytoskeleton. By contrast, how regulation of mitochondrial distribution is superimposed upon the formation of the actomyosin cytoskeleton is less understood at the molecular level. This scenario is further complicated by the observation that F-actin and intermediate filaments also play a key role in mitochondrial dynamics and functions (45).

Technical advances that enable live imaging (46) of mitochondria (47) and the cytoskeleton would provide new tools to address this important issue.

We have uncovered the role of mitochondria in alveolar epithelial cells and fibroblasts/myofibroblasts during alveolar formation. Whether the function of endothelial cells/pericytes or other cell types not yet tested also relies on mitochondrial activity and/or distribution in this process is unknown (48, 49). This would require future studies using an approach similar to that employed in this work. Again, the dependence of cell types, cellular processes and signaling pathways on mitochondrial function can only be revealed through studies in vivo.

We envision that a complex regulatory network must be in place to regulate mitochondrial number and distribution in distinct cellular processes. Our work shows that mTOR complex 1 is
a key player in controlling mitochondrial function during alveolar formation. This is consistent
with the role of mTORC1 in regulating mitochondrial function in cell-based assays. Identifying
additional components in the signaling network would reveal the key hubs in the signaling
network that control mitochondrial function during alveologenesis.

Mitochondrial copy number and TFAM expression levels are reduced in the lungs of
COPD/emphysema patients. Moreover, knockdown of TFAM or RHOT1 in human lung
epithelial cells or fibroblasts impairs their cellular properties, including PDGF secretion and
myofibroblast migration. We speculate that changes in cellular properties due to disturbed
mitochondrial activity and distribution contribute to the pathogenesis of COPD/emphysema (50-53). Disturbance of mitochondrial function and cellular properties could be related to
inflammatory responses in COPD/emphysema. To further test this idea would rely on the
analysis of lungs at different stages of disease progression with a focus on identifying cellular
changes that are directly related to mitochondrial dysfunction. It also necessitates new
approaches that enable a mechanistic correlation between mitochondrial function and disease
pathogenesis and progression. For instance, analysis of the transcriptome and proteome of
various lung cell types at single cell levels could reveal alterations in a subset of cells that herald
the process of emphysematous changes. Such analysis could also uncover changes in the
signaling network that connects mitochondria to cellular processes.

Taken together, our work has yielded new molecular insight into the old question of energy
utilization in vivo. In particular, energy production by mitochondria is channeled in a spatially-
specific manner to power cellular machinery and drive cellular processes in distinct cell types.
Diverse cell types and cellular processes have a unique energy demand, which is likely executed
by a signaling network. These investigations form the basis of additional studies to explore this
new concept in alveolar formation and repair and in other physiological and pathological processes *in vivo*.
Materials and methods

Animal husbandry

Mouse strains used in this study are listed in the Key Resources table. All mouse experiments described in this study were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco (UCSF).

Tamoxifen administration

Tamoxifen was prepared by dissolving in corn oil to a concentration of 50 mg/ml (40). For postnatal (P) injection, the tamoxifen stock was diluted 1/10 in corn oil to make a final concentration of 5 mg/ml. 50 μL of tamoxifen was delivered through oral gavage or direct injection into the stomach of neonatal mice.

Measurement of mean linear intercept (MLI)

Measurement of MLI was performed as previously reported (40). Briefly, 15 fields without visible blood vessels or airways from three different histological sections per animal were captured at 20x magnification using a Nikon Eclipse E1000 microscope. A grid with horizontal and vertical lines (10 each) was superimposed on the images using ImageJ. The mean linear intercept (Lm) was calculated as: Lm = L/N, where L is the total length of horizontal plus vertical lines, and N is the total number of the intercepts.
Measurement of alveolar wall thickness

To measure the thickness of the primary septal wall (54), 15 representative fields from histological sections of lungs at the indicated age were imaged at 100x magnification using a Nikon Eclipse E1000 microscope. Three vertical lines were drawn on each image such that they can cross the primary septal wall. The thickness of the primary septal wall was evaluated by Image J as the vertical distance of the line that traversed the primary septal wall.

Whole lung imaging, histology and immunohistochemistry

To image the whole lungs that carried GFP or RFP, dissected mouse lungs at the indicated stages were placed under a Nikon Eclipse E1000 microscope equipped with a SPOT 2.3 CCD camera. Mouse lungs at indicated time points were collected and fixed with 4% paraformaldehyde (PFA) in PBS on ice for 1 hr. The tissues were embedded in paraffin wax or OCT, and sectioned at 7 μm. For histological analysis of lung sections, hematoxylin and eosin (H&E) staining was performed as previously described (40, 55). Images were taken using a SPOT 2.3 CCD camera connected to a Nikon Eclipse E1000 microscope.

To detect PDGFA in lung cells, lungs from Sox9^{Cre/+}; Pdgfa^{ex4COIN/+} (control) and T{fam}^{ff}; Sox9^{Cre/+}; Pdgfa^{ex4COIN/+} mice at P3 were dissected and fixed in 4% PFA on ice for 1 hr. Lungs were washed in 0.02% NP40 in PBS for 2 hr, then placed in X-gal staining solution (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, 1 mg/ml X-gal) for 72 hr at 37°C. The stained lungs were paraffin embedded and sectioned. Images were taken using a SPOT 2.3 CCD camera connected to a Nikon Eclipse E1000 microscope.
Immunofluorescence was performed as previously described (55). Antibodies used in this study are listed in the Key Resources table. The primary antibodies used for wax sections were: chicken anti-GFP (1:200, abcam, Cat# ab13970), rabbit anti-NKX2.1 (1:100, Epitomics, Cat# 2044–1), goat anti-CC10 (1:200, Santa Cruz Biotechnology, Cat# sc-9773), mouse anti-acetylated tubulin (1:200, Sigma-Aldrich, Cat# T6793), rabbit anti-prosurfactant protein C (proSP-C) (1:200, MilliporeSigma, Cat# AB3786), hamster anti-T1α (1:200, Developmental Studies Hybridoma Bank, Cat# 8.1.1), mouse anti-HOPX (1:100, Santa Cruz Biotechnology, Cat# sc-398703). The primary antibodies used for frozen sections were: rabbit anti-MPC1 (1:100, Millipore/Sigma, Cat# HPA045119), rat anti-E-cadherin (1:200, Invitrogen, Cat# 13–1900), mouse anti-MTCO1 (1:100, abcam, Cat# ab14705), chicken anti-GFP (1:300, abcam, Cat# ab13970), mouse anti-ACTA2 (1:200, Thermo Scientific Lab Vision, Cat# MS-113-P0), rat anti-PECAM-1 (CD31) (1:150, Santa Cruz Biotechnology, Cat# sc-18916), rabbit anti-PDGFRA (1:150, Cell Signaling Technology, Cat# 3164), rabbit anti-phospho-PDGFRa (Tyr754) (1:100, Cell Signaling Technology, Cat# 2992), mouse anti-S6 Ribosomal Protein (1:100, Cell Signaling Technology, Cat# 2317), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (1:100, Cell Signaling Technology, Cat# 4856). Secondary antibodies and conjugates used were: donkey anti-rabbit Alexa Fluor 488 or 594 (1:1000, Life Technologies), donkey anti-chicken Alexa Fluor 488 or 647 (1:1000, Life Technologies), donkey anti-mouse Alexa Fluor 488 or 594 (1:1000, Life Technologies), and donkey anti-rat Alexa Fluor 594 (1:1000, Life Technologies). The biotinylated secondary antibodies used were: goat anti-hamster (1:1000, Jackson ImmunoResearch Laboratories), donkey anti-rabbit (1:1000, Jackson ImmunoResearch Laboratories), donkey anti-rat (1:1000, Jackson ImmunoResearch Laboratories) and horse anti-mouse (1:1000, Jackson ImmunoResearch Laboratories). The signal was detected using
streptavidin-conjugated Alexa Fluor 488, 594, or 647 (1:1000, Life Technologies) or HRP-conjugated streptavidin (1:1000, Perkin-Elmer) coupled with fluorogenic substrate Alexa Fluor 594 tyramide for 30 s (1:200, TSA kit; Perkin Elmer). F-actin was stained with rhodamine-conjugated phalloidin (1:200, Sigma) in PBS for 2 hr. Since the filamentous actin is sensitive to methanol, ethanol and high temperature, we only used OCT-embedded frozen sections for F-actin staining.

Confocal images were captured on a Leica SPE laser-scanning confocal microscope. Adjustment of red/green/blue/grey histograms and channel merges were performed using LAS AF Lite (Leica Microsystems).

**Fibroblast/myofibroblast proliferation assays**

The rate of cell proliferation was determined through EdU incorporation as previously described (40). Mouse pups at the indicated time points were intraperitoneally injected with EdU/PBS solution for 1 hr before dissection. The Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies) was used to quantify EdU incorporation. The sections were co-stained with antibody against PDGFRA. Cell proliferation rate was calculated as the ratio of (EdU+PDGFRA+ cells)/(PDGFRA+ cells).

**Fibroblast/myofibroblast migration assay in vitro**

The migratory capacity of lung fibroblasts/myofibroblasts was assessed by the Culture-Insert 2 Well system (ibidi) (40). Briefly, at postnatal (P) day 3, dissected lungs from control, Tfat/l; PdgfraCre/+ and Rhot1f/f; PdgfraCre/+ mice were minced into small pieces and digested in solution (1.2 U/ml dispase, 0.5 mg/ml collagenase B and 50 U/ml DNase I), rocking at 37°C for 2
hr to release single cells. After adding an equal volume of culture medium (DMEM with 10% FBS, 2x penicillin/streptomycin and 1x L-glutamine), the samples were filtered through 40 μm cell strainers and centrifuged at 600 g for 10 min. The dissociated cells were resuspended in 200 μl of culture medium and plated into wells (100 μl per well). The lung fibroblasts/myofibroblasts were allowed to attach to the fibronectin–coated plates for 2-3 hr. Fresh culture medium was added and the attached fibroblasts/myofibroblasts were cultured 2 or 3 days to reach 100% confluence. The confluent fibroblasts/myofibroblasts were switched to starvation medium (DMEM with 0.5% FBS and 1x penicillin/streptomycin) for 16 hr before removal of the insert. Fibroblasts/myofibroblasts migration was assessed 36-48 hr afterwards.

**Lentiviral production and transduction**

Lentiviruses were produced in HEK293T cells maintained in DMEM containing 10% FBS, 1x penicillin/streptomycin and 1x L-glutamine (40). HEK293T cells were plated and transfected when they reached 70% confluence on the following day. 2 μg of pMD2.G, 2 μg of psPAX2, 4 μg of PDGFA-3xFLAG (in the modified pSECC lentiviral vector) and 50 μl of polyethylenimine (PEI) (1 μg/μl) were mixed in 1000 μl OPTI-MEM and added to a 10 cm dish. Incubation medium was replaced one day after transfection. 48 hr post-transfection, the viral supernatant was collected, filtered through 0.45 μm PVDF filter, then added to primary lung cells together with 8 μg/ml polybrene. 12 hr post-transduction, the medium was replaced with fresh culture medium.

**shRNA-mediated gene silencing**
shRNAs against the 3'UTR of human TFAM and RHOT1 were designed by pSicOligomaker (56). Paired oligonucleotides were annealed and inserted into the pLentiLox3.7 lentiviral vector. The packaging vectors used were either the pLP1/pLP2/pLP/VSV-G or pMD2.G/psPAX2 combinations. Primers used for shRNAs were: shTFAM-1 forward, 5'-

TGGTGCTGAGGAGTGTAAATTCAAGAGATTATCCTCAGCACACCTTTTTTTC-3'; reverse, 5'-

TCGAGAAAAAGGTGCTGAGGAGTGTAAATCTCTTGAAATTTAACACTCCTCAGCACACCA-3', shTFAM-2 forward, 5'-

TGACTTCTGCCAGCATAATATTCAAGAGATATTATGCTGGCAGAAGTCTTTTTTTC-3'; reverse, 5'-

TCGAGAAAAAGACTTCTGCCAGCATAATATTCTCTTGAAATTTAATTGCTGGCAGAAGTCA-3', shTFAM-3 forward, 5'-

TGTACTCTTTTCTTTATATTCAAGAGATATAAGGAAACAAGAGTACTTTTTTTC-3'; reverse, 5'-

TCGAGAAAAAGTACTCTTGGTCTTTATATTCTCTTGAAATTTAAGGAAACAAAGAAGTCA-3', shRHOT1-1 forward, 5'-

TGAAACAGCGATGATATAAATTCAAGAGATTTATATCATCGCTTTTCTTTTTTTC-3'; reverse, 5'-

TCGAGAAAAAGAAACAGCGATGATATAAAATCTCTTGAAATTTTATATCATCGCTTTTTTTC-3', shRHOT1-2 forward, 5'-

TGTACATTCTGAAATGCTTTTATTTCAAGAGATAAAGCATTACAGAATGTACTTTTTTTC-3'; reverse, 5'-

TCGAGAAAAAGTACATTCTGAAATGCTTTTATCTCTTGAAATTTAAGGACATTACAGAATGTA
CA-3', shRHO1-3 forward, 5'-
TGAAATGATGTTTCTAGACATTCAAGAGATGTCTAGAAACATCATTTCTTTTTTC-3';
reverse, 5'-
TCGAGAAAAAGAAATGATGTTTCTAGACATCTCTTTGAATGTCTAGAAACATCATTT
CA-3'.

**PDGFA secretion assay**

PDGFA secretion assay was performed as previously described (40). In brief, PDGFA-3xFLAG was stably expressed in control and Tfam- and Rhot1-deficient primary lung cells (derived from the lungs of control, Tfam^{f/f}; Pdgfra^{Cre/+} and Rhot1^{f/f}; Pdgfra^{Cre/+} mice, respectively) that were plated onto 10 cm dishes. The culture media were replaced with OPTI-MEM supplemented with insulin, transferrin and selenium (ITS) once the cells reached 100% confluence. 24 hr post-incubation, the supernatants were mixed with 1x protein inhibitor cocktail, filtered through 0.45 μm filters, and centrifuged at high speed (>12000 rpm) for 15 min at 4°C. The filtrates were then concentrated in protein concentration columns (Millipore CENTRICON YM-10 Centrifugal Filter Unit 2 mL 10 kDa) through centrifugation at 2000 g for 1 hr at 4°C. Concentrated supernatants were mixed with immunoprecipitation (IP) buffer (50 mM Tris pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 1x protein inhibitor cocktail) in a total volume of 500 μl. Meanwhile, cells seeded on the plate were harvested and lysed in IP buffer. Immunoprecipitation was performed using FLAG-M2 beads following the standard procedure. Western blotting was performed to detect PDGFA in the supernatants and lysates derived from control, Tfam-deficient, and Rhot1-deficient primary lung cells.
qPCR analysis

qPCR was performed as previously described (40). Briefly, the right cranial lobe was dissected from the mouse lungs of the indicated genotypes and time points, and homogenized in 1 ml TRIzol (Life Technologies). The homogenates were added to 200 μl chloroform, and then centrifuged for 15 min at 12000 rpm. The upper aqueous layer was collected and mixed with an equal volume of 70% ethanol. RNAs were extracted with the RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. The extracted RNAs were reverse-transcribed with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR (qPCR) was performed on the Applied Biosystems QuantStudio™ 5 Real-Time PCR System. Primers used for qPCR were: mouse Pdgfa forward, 5’-CTGGCTCGAAGTCAGATCCACA-3’; reverse, 5’-GACTTGTCCTCAAGGGCATCCTC-3’, mouse Pdgfra forward, 5’-

TGCAGTTGCCTTACGACTCCAG-3’; reverse, 5’-

AGCCACCTTCATTACAGGTTGGGA-3’, mouse Acta2 forward, 5’-

ATGCAGAAGGAGATCACAGC-3’; reverse, 5’-GAAGGTAGACAGCGAAGCC-3’, mouse Eln forward, 5’-GCCAAAGCTGCCAAATACG-3’; reverse, 5’-

CTCCAGCTCAACACCATAG-3’, mouse Gapdh forward, 5’-

AGGTTGTCTCTGCGACTTCA-3’; reverse, 5’-CCAGGAATGAGCTTGACAAAGTT-3’.

Analysis of mtDNA/nDNA ratio

Lung samples from Tfamfl/fl; CAGGCreER/+, Rptorf/f; CAGGCreER/+ mice and human patients were lysed in lysis buffer (100 mM Tris pH 7.5, 5 mM EDTA, 0.4% SDS, 200 mM NaCl, 50 μg/ml proteinase K), and incubated in a 55°C chamber overnight. The tissues were digested with 100 μg/ml RNase A at 37°C for 30 min to degrade the RNAs. An equal volume of phenol/chloroform
was added to the lysed samples, which were centrifuged at 12,000 rpm for 10 min. Lung DNAs were concentrated by ethanol precipitation. For mouse lungs, the mitochondrial 16S rRNA or ND1 gene and the nuclear Hk2 gene were used to calculate the relative ratio of mitochondrial (mt) to nuclear (n) DNA copy number. For human lungs, the mitochondrial tRNA^{Leu(UUR)} or 16S rRNA gene and the nuclear Hk2 gene were employed to determine the relative ratio of mtDNA to nDNA. Quantitative PCR (qPCR) was performed on the Applied Biosystems QuantStudio™ 5 Real-Time PCR System. The primer pairs used for the indicated genes were: mouse 16S rRNA forward, 5’-CCGCAAGGGAAGATGAAAGAC-3’; reverse, 5’-TCGTTTGGTTTCCGGGTTTC-3’, mouse mt-ND1 forward, 5’-
CTAGCAGAAACAAACCGGGC-3’; reverse, 5’-CCGGCTGCCTATTCTACGT-3’, mouse Hk2 forward, 5’-GCCAGCCTCTCTGATTTTAGTGT-3’; reverse, 5’-
GGGAACACAAAGACCTCTTGGG-3’, human tRNA^{Leu(UUR)} forward, 5’-
CACCCAGAACACCGTTTTG-3’; reverse, 5’-TGCCATGATGGTGTTGA-3’, human 16S rRNA forward, 5’-GCCTTCCCCGTAAATGATA-3’; reverse, 5’-
TTATGCGATTACCUGGCTCT-3’, human β-2-microglobulin (β2M) forward, 5’-
TGCTGTCTCCATGTTTGATGT-3’; reverse, 5’-TCTCTGCTCCCCACCTCTAAGT-3’.

**Enzymatic activities of the mitochondrial electron transport chain (ETC) complex**

Measurement of the enzymatic activity of mitochondrial ETC complex was performed as previously described (57). In brief, frozen lung tissues from control, Tfam^{-/}; Pdgfra^{Cre/+}, Tfam^{-/}; Sox9^{Cre/+}; Rhot1^{-/}; Pdgfra^{Cre/+} and Rhot1^{-/}; Sox9^{Cre/+} mice at P5 were homogenized in homogenization buffer (120 mM KCl, 20mM HEPES, 1mM EGTA, pH 7.4) using a Dounce homogenizer. The Pierce BCA Protein Assay Kit was used to determine protein concentrations.
before spectrophotometric kinetic assays. Complex I (NADH:ubiquinone oxidoreductase) activity was determined by measuring the oxidation of NADH at 340nm in a reaction mixture (50 mM potassium phosphate [pH 7.5], 1.7 mM potassium ferricyanide, 0.2 mM NADH and homogenate of interest). Potassium ferricyanide was used as the electron acceptor for Complex I. Complex IV (Cytochrome c oxidase) activity was determined by measuring the oxidation of cytochrome c at 550 nm in a reaction solution (50 mM potassium phosphate [pH 7.0], 100 μM reduced cytochrome c and homogenate of interest).

Mitochondrial network
Fibroblasts were derived from control, Tjfatf; PdgfrafCre/+ and Rhot1ff; PdgfrafCre/+ lungs and transduced with lentiviral constructs expressing Mito-7-mEmerald to label mitochondria. After 48 hrs, cells were fixed and co-stained with MTCO1. Tubular mitochondria are the dominant form in control fibroblasts, while short tubular and fragmented mitochondria could be found in Tjfat-deficient fibroblasts. Fragmented mitochondria are characterized as dot-like structures; the morphology of short tubular mitochondria is intermediate between tubular and fragmented mitochondria.

Human lung tissues
Human lung samples were processed as previously described (40). Briefly, lung tissues were obtained from severe Emphysema (Global Initiative for Chronic Obstructive Lung Disease Criteria, stages III or IV) at the time of lung transplantation. The donor control lung samples were indicated physiologically and pathologically normal (58). Written informed consent was
obtained from all subjects and the study was approved by the University of California, San Francisco Institutional Review Board (IRB approval # 13-10738).

**Human lung cells**

The human lung epithelial cell line (1310 cell line) was derived from human alveolar type II cells. Cells were immortalized by lentiviral transduction with hTERT and CDK4 to create a stable cell line (59).

Human airway fibroblasts were derived from donor lungs not utilized for lung transplantation. It was approved by the Committee on Human Research at the University of California, San Francisco in full accordance with the declaration of Helsinki principles. Airway fibroblasts were cultured from the lung parenchyma by the explant technique and used passages 1 to 4, as previously described (60). The identity of the cell lines has been confirmed by STR profiling and no mycoplasma contamination was found.

**Western blotting analysis of human lung tissues**

Human lung tissues from normal subjects and emphysema patients were homogenized in RIPA buffer with 1x Protease Inhibitor Cocktail and 1x PMSF. The lysates were centrifuged at full speed for 15 min at 4°C and analyzed by Western blotting as previously described (55). The primary antibodies used were: rabbit anti-TFAM (1: 2000, Proteintech, Cat# 22586-1-AP), mouse anti-S6 Ribosomal Protein (1:2000, Cell Signaling Technology, Cat# 2317), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (1:2000, Cell Signaling Technology, Cat# 4856), mouse anti-OPA1 (clone 18) (1: 2000, BD Transduction Laboratories, Cat# 612606), mouse anti-DLP1 (1: 2000, BD Transduction Laboratories, Cat# 611113), rabbit anti-Phospho-DRP1
(Ser616) (1: 1000, Cell Signaling Technology, Cat# 3455S), mouse anti-alpha-tubulin (1:3000, Developmental Studies Hybridoma Bank, Cat# 12G10).

**Statistical analysis**

All the statistical comparisons between different groups were shown as mean value ± SEM. The P values were calculated by two-tailed Student’s t-tests and statistical significance was evaluated as (*) p<0.05; (**) p<0.01; (***) p<0.001. More than or equal to three biological repeats were performed, and the detailed biological replicates (n numbers) were indicated in the figure legends.
Acknowledgments

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Figure legends

Figure 1. Mitochondria display subcellular concentration in alveolar epithelial cells and mesenchymal myofibroblasts of mouse lungs

(A) Immunostaining of lung sections collected from Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mGm/+} mice at 18.5 days post coitus (dpc) and different postnatal (P) stages as indicated. The GFP signal identified alveolar epithelial cells (alveolar type I [AT1] and alveolar type II [AT2] cells) while myofibroblasts were characterized by SMA expression. Moreover, mitochondria were labeled by MPC1; the trans-Golgi network was visualized by TGN38. Enhanced MPC1 signal was distributed non-uniformly in both alveolar epithelial cells and myofibroblasts. (B) Transmission electron micrographs of lungs collected from wild-type mice at P3. Prominent features in a given lung cell type include lamellar bodies in AT2 cells, elongated cell membrane in AT1 cells, and actin bundles and collagen fibers in myofibroblasts. RBC, red blood cell.

Figure 2. Global inactivation of Tfam in postnatal mice results in alveolar defects

(A) Schematic diagram of the time course of postnatal (P) administration of tamoxifen and harvest of mouse lungs. (B) Immunostaining of lungs collected from CAG\textsuperscript{CreER/+}; ROSA26\textsuperscript{mGm/+} (control) and Tfam\textsuperscript{ff}; CAG\textsuperscript{CreER/+}; ROSA26\textsuperscript{mGm/+} mice at P10 that had received tamoxifen at P0. The GFP signal represents sites of induced CreER activity. Nuclear NKX2.1 staining marked all lung epithelial cells while PDGFRA immunoreactivity labeled mesenchymal fibroblasts/myofibroblasts. (C) Hematoxylin and eosin-stained lung sections of control and Tfam\textsuperscript{ff}; CAG\textsuperscript{CreER/+}; ROSA26\textsuperscript{mGm/+} mice at P10. Histological analysis revealed the presence of enlarged saccules and retarded development of secondary septa in the mutant lungs. (D) Measurement of the mean linear intercept (MLI) in control and Tfam\textsuperscript{ff}; CAG\textsuperscript{CreER/+};
ROSA26^mTmG/+ lungs at P10 (n = 4 for each group). The MLI was increased in Tfam-deficient lungs. (E) Immunostaining of lung sections collected from control and Tfam^{ff}; CAGG^{CreER/+}; ROSA26^mTmG/+ mice at P10. SMA expression was characteristic of myofibroblasts and phalloidin stained the actin filaments. (F) Quantification of the relative ratio of mitochondrial DNA (mtDNA), 16S rRNA and mtND1 (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), in lysates derived from control and Tfam^{ff}; CAGG^{CreER/+}; ROSA26^mTmG/+ lungs (n = 4 for each group). (G) Immunostaining of lung sections collected from control and Tfam^{ff}; CAGG^{CreER/+}; ROSA26^mTmG/+ mice at P10. MPC1 antibodies marked mitochondria; MTCO1 antibodies detected cytochrome c oxidase, the expression of which was controlled by Tfam. All values are mean ± SEM. (*) p<0.05; (**) p<0.01 (unpaired Student’s t-test).

Figure 3. Elimination of Tfam or Rhot1 in the epithelium of mouse lungs disrupts alveolar formation

(A) Hematoxylin and eosin-stained lung sections of control and Tfam^{ff}; Sox9^{Cre/+} mice at different postnatal (P) stages as indicated. Histological analysis revealed the presence of enlarged saccules and failure in secondary septation in the mutant lungs. (B) Measurement of the MLI in control and Tfam^{ff}; Sox9^{Cre/+} lungs at P3–P10 (n = 3 for each group). The MLI was increased in Tfam-deficient lungs. (C) Measurement of the primary septal thickness in control and Tfam^{ff}; Sox9^{Cre/+} lungs at P3–P7 (n = 3 for each group). (D) Quantification of the relative ratio of mitochondrial DNA (mtDNA), 16S rRNA and mtND1 (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), in lysates derived from control and Tfam^{ff}; Sox9^{Cre/+} lungs at P5 (n = 5 for each group). (E) Quantification of the relative
enzymatic activity of mitochondrial complex I and complex IV in control and \( T\text{fam}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5 (\( n = 5 \) for each group). (F) Measurement of relative ATP production in control and \( T\text{fam}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5 (\( n = 5 \) for each group). (G) Hematoxylin and eosin-stained lung sections of control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) mice at different postnatal stages as indicated. Histological analysis detected enlarged saccules and lack of secondary septa in the mutant lungs. (H) Measurement of the MLI in control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P3–P10 (\( n = 3 \) for each group). The MLI was increased in \( R\text{hot1} \)-deficient lungs. (I) Measurement of the primary septal thickness in control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5–P7 (\( n = 3 \) for each group). (J) Quantification of the relative ratio of mitochondrial DNA (mtDNA), 16S rRNA and \( mt\text{ND1} \) (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), in lysates derived from control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5 (\( n = 5 \) for each group). (K) Quantification of the relative enzymatic activity of mitochondrial complex I and complex IV in control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5 (\( n = 5 \) for each group). (L) Measurement of relative ATP production in control and \( R\text{hot1}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) lungs at P5 (\( n = 5 \) for each group). All values are mean ± SEM. (**) \( p<0.01 \); (***) \( p<0.001 \); ns, not significant (unpaired Student’s \( t \)-test).

**Figure 4. Loss of epithelial Tfam or Rhot1 compromises PDGF release**

(A) Immunostaining of lungs collected from control and \( T\text{fam}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) mice at postnatal (P) day 5 or 10, some which were injected with EdU as indicated. (B) Immunostaining of lungs collected from control and \( T\text{fam}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}} \) mice at P5. (C) LacZ-staining (blue) of lung sections collected from \( \text{Sox9}^{\text{Cre/+}}; P\text{dgfa}^{\text{ex4COIN/+}} \) (control) and \( T\text{fam}^{\text{ff}}; \text{Sox9}^{\text{Cre/+}}; P\text{dgfa}^{\text{ex4COIN/+}} \) mice. The slides were counterstained with eosin (red). No difference in the intensity of LacZ staining in the lung was noted between these two mouse lines. (D)
Quantification of fibroblast/myofibroblast proliferation in control and Tjam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs at P5, P7 and P10 (n = 3 for each group). The rate of fibroblast/myofibroblast proliferation was calculated as the ratio of the number of EdU\textsuperscript{+} fibroblasts/myofibroblasts (EdU\textsuperscript{+}PDGFRA\textsuperscript{+}) to the number of fibroblasts/myofibroblasts (PDGFRA\textsuperscript{+}). The percentage of proliferating fibroblasts/myofibroblasts was reduced in Tjam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} compared to controls at P7 and P10.

(E) qPCR analysis of gene expression in control and Tjam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs at P5 (n = 3 for each group). While no difference in expression levels was noted for Pdgfa and Pdgfra between control and Tjam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs, the expression levels of Acta2 (SMA) and Eln (elastin) were significantly reduced in the absence of Tjam. (F) Western blot analysis of cell lysates and supernatants from control and Tjam-deficient cells (n = 4 for each group) lentivirally transduced with PDGFA-expressing constructs. The amount of PDGFA released into the media was reduced in Tjam-deficient cells compared to controls. α-Tubulin served as a loading control. (G) Immunostaining of lungs collected from control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} mice at P5, P7 or P10, some which were injected with EdU as indicated. (H) Immunostaining of lungs collected from control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} mice at P5. (I) Quantification of fibroblast/myofibroblast proliferation in control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs at P7 and P10 (n = 3 for each group). The percentage of proliferating fibroblasts/myofibroblasts was reduced in Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} compared to controls at P7 and P10. (J) qPCR analysis of gene expression in control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs at P5 (n = 3 for each group). The expression levels of Pdgfa and Pdgfra were unaltered between control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+} lungs; the expression levels of Acta2 and Eln were significantly reduced in the absence of Rhot1. (K) Western blot analysis of cell lysates and supernatants from control and Rhot1-deficient cells (n = 4 for each group) lentivirally transduced with PDGFA-expressing constructs. The amount of PDGFA released into the media...
was reduced in *Rhot1*-deficient cells compared to controls. α-Tubulin served as a loading control. All values are mean ± SEM. (*) p<0.05; ns, not significant (unpaired Student’s *t*-test).

**Figure 5. Elimination of *Tfam* or *Rhot1* in mouse lung fibroblasts/myofibroblasts impairs alveolar formation**

(A) Hematoxylin and eosin-stained lung sections of control and *Tfam*/*Pdgfra*Cre/+ mice at different postnatal (P) stages as indicated. Histological analysis revealed the presence of enlarged saccules and defective development of secondary septa in the mutant lungs. (B) Measurement of the MLI in control and *Tfam*/*Pdgfra*Cre/+ lungs at P0–P5 (n = 3 for each group). The MLI was increased in *Tfam*-deficient lungs. (C) Measurement of the primary septal thickness in control and *Tfam*/*Pdgfra*Cre/+ lungs at P2–P3 (n = 3 for each group). (D) Quantification of the relative ratio of mitochondrial DNA (mtDNA), 16S rRNA and mtND1 (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), in lysates derived from control and *Tfam*/*Pdgfra*Cre/+ lungs at P5 (n = 5 for each group). (E) Quantification of the relative enzymatic activity of mitochondrial complex I and complex IV in control and *Tfam*/*Pdgfra*Cre/+ lungs at P5 (n = 5 for each group). (F) Measurement of relative ATP production in control and *Tfam*/*Pdgfra*Cre/+ lungs at P5 (n = 5 for each group). (G) Hematoxylin and eosin-stained lung sections of control and *Rhot1*/*Pdgfra*Cre/+ mice at different postnatal stages as indicated. Histological analysis detected enlarged saccules and lack of secondary septa in the mutant lungs. (H) Measurement of the MLI in control and *Rhot1*/*Pdgfra*Cre/+ lungs at P3–P10 (n = 3 for each group). The MLI was increased in *Rhot1*-deficient lungs. (I) Measurement of the primary septal thickness in control and *Rhot1*/*Pdgfra*Cre/+ lungs at P5–P7 (n = 3 for each group). (J) Quantification of the relative ratio of mitochondrial DNA (mtDNA), 16S rRNA and
mtND1 (mitochondrially encoded NADH dehydrogenase 1), to nuclear DNA (nDNA), Hk2
(hexokinase 2), in lysates derived from control and Rhot1ff; PdgfraCre/+ lungs at P5 (n = 5 for
each group). (K) Quantification of the relative enzymatic activity of mitochondrial complex I and
complex IV in control and Rhot1ff; PdgfraCre/+ lungs at P5 (n = 5 for each group). (L)
Measurement of relative ATP production in control and Rhot1ff; PdgfraCre/+ lungs at P5 (n = 5
for each group). All values are mean ± SEM. (*) p<0.05; (**) p<0.01; ns, not significant
(unpaired Student’s t-test).

Figure 6. Loss of mesenchymal Tfam or Rhot1 compromises fibroblast/myofibroblast
migration

(A) Immunostaining of lungs collected from control and Tfamff; PdgfraCre/+ mice at postnatal
(P) day 2 and 5, some of which injected with EdU as indicated. (B) Quantification of
fibroblast/myofibroblast proliferation in control and Tfamff; PdgfraCre/+ lungs at P2, P3 and P5
(n = 3 for each group). The rate of fibroblast/myofibroblast proliferation was calculated as the
ratio of the number of EdU+ fibroblasts/myofibroblasts (EdU+PDGFRA+) to the number of
fibroblast/myofibroblasts (PDGFRA+). The percentage of proliferating
fibroblasts/myofibroblasts was reduced in Tfamff; PdgfraCre/+ compared to controls at P3 and
P5. (C) qPCR analysis of gene expression in control and Tfamff; PdgfraCre/+ lungs at P3 (n = 3
for each group). The expression levels of Pdgfra, Acta2 and Eln were significantly reduced in
Tfamff; PdgfraCre/+ lungs compared to controls. (D) Wound recovery assays to assess the
migratory ability of fibroblasts/myofibroblasts derived from control and Tfamff; PdgfraCre/+
lungs (n = 3 for each group). Within 36–48 hr, the wound area has been populated by migrating
fibroblasts/myofibroblasts derived from control lungs. By contrast, fewer
fibroblasts/myofibroblasts from Tfam\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs reached the wound area within the same time frame. Wound recovery by fibroblasts/myofibroblasts from control and Tfam\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs was quantified. (E) Immunostaining of lungs collected from control and Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} mice at P5 and P10, some of which were injected with EdU as indicated. (F) Quantification of fibroblasts/myofibroblasts proliferation in control and Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs at P5 and P10 (n = 3 for each group). The percentage of proliferating fibroblasts/myofibroblasts was decreased in Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} compared to controls at P10. (G) qPCR analysis of gene expression in control and Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs at P7 (n = 3 for each group). The expression levels of Eln were significantly reduced in Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs in comparison with controls. (H) Wound recovery assays to assess the migratory ability of fibroblasts/myofibroblasts derived from control and Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs (n = 3 for each group). Fewer fibroblasts/myofibroblasts from Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs reached the wound area within the same time frame compared to controls. Wound recovery by fibroblasts/myofibroblasts from control and Rhot1\textsuperscript{f/f}; Pdgfra\textsuperscript{Cre/+} lungs was quantified. All values are mean ± SEM. (*) p<0.05; ns, not significant (unpaired Student’s t-test).

**Figure 7. Postnatal inactivation of Rptor in mice results in alveolar defects**

(A) Schematic diagram of the time course of postnatal (P) administration of tamoxifen and harvest of mouse lungs. (B) Hematoxylin and eosin-stained lung sections of control and Rptor\textsuperscript{f/f}; CAGG\textsuperscript{CreER/+} mice at postnatal (P) day 10. Histological analysis revealed the presence of enlarged saccules and retarded development of secondary septa in the mutant lungs. (C) Measurement of the mean linear intercept (MLI) in control and Rptor\textsuperscript{f/f}; CAGG\textsuperscript{CreER/+} lungs at P10 (n = 5 for each group). The MLI was increased in Rptor-deficient lungs. (D)
Immuno
staining of lung sections collected from control and Rptor^{ff}; CAGG^{CreER/+};

ROSA26^{mTmG/+} mice at P10. SMA expression was characteristic of myofibroblasts and
phalloidin stained the actin filaments. (E). Quantification of the protein levels of RPS6 and
phosphorylated RPS6 (p-RPS6) in lung lysates derived from control and Rptor^{ff}; CAGG^{CreER/+};
ROSA26^{mTmG/+} lungs (n = 5 for each group). (F) Quantification of the relative ratio of
mitochondrial DNA (mtDNA), 16S rRNA and mtND1 (mitochondrially encoded NADH
dehydrogenase 1), to nuclear DNA (nDNA), Hk2 (hexokinase 2), in lysates derived from control
and Rptor^{ff}; CAGG^{CreER/+}; ROSA26^{mTmG/+} lungs (n = 5 for each group). (G) Immunostaining of
lung sections collected from control and Rptor^{ff}; CAGG^{CreER/+}; ROSA26^{mTmG/+} mice at P10.
MPC1 antibodies marked mitochondria; MTCO1 antibodies detected cytochrome c oxidase, the
expression of which was controlled by Tfam. All values are mean ± SEM. (*) p<0.05; (**) p<0.01; (***) p<0.001 (unpaired Student’s t-test).

Figure 8. Lungs from emphysema patients exhibit a reduction in mitochondrial DNA and
TFAM expression

(A) Hematoxylin and eosin-stained lung sections of normal and emphysema patients. Disruption
do of alveoli in emphysema patients resulted in enlarged airspace with thin primary septa. (B) qPCR
analysis of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) in lung lysates derived from
normal and emphysema patients (n = 22 for each group). mtDNA-encoded tRNA^{Leu} and 16S
rRNA and nDNA-encoded β2M (β2 microglobulin) were used in this study. The relative ratio of
mtDNA to nDNA was calculated. (C) Quantification of TFAM protein levels in lung lysates
derived from normal and emphysema patients (n = 21 for each group). (D) Immunostaining of
lungs collected from control and emphysema patients. Phalloidin detected actin filaments. (E).
Quantification of the protein levels of RPS6 and phosphorylated RPS6 (p-RPS6) in lung lysates derived from normal and emphysema patients (n = 21 for each group). (F) Western blot analysis of cell lysates and supernatants from human lung epithelial cells transduced with control constructs, constructs expressing shRNAs against human TFAM and constructs expressing mouse TFAM\textsuperscript{FLAG} (mTFAM\textsuperscript{FLAG}) as indicated. qPCR analysis revealed efficient knockdown of human TFAM transcripts. The amount of PDGFA released into the media was reduced in TFAM-knockdown cells compared to controls and was rescued by expression of mTFAM\textsuperscript{FLAG} (n = 3 for each group). α-Tubulin served as a loading control. (G) Western blot analysis of cell lysates from human lung fibroblasts transduced with control constructs, constructs expressing shRNAs against human TFAM and constructs expressing mouse TFAM\textsuperscript{FLAG} (mTFAM\textsuperscript{FLAG}) as indicated. qPCR analysis revealed efficient knockdown of human TFAM transcripts. Wound recovery assays using control, TFAM-knockdown fibroblasts and TFAM-knockdown fibroblasts expressing mTFAM\textsuperscript{FLAG} were quantified. The migratory defect of fibroblasts was rescued by mTFAM\textsuperscript{FLAG} expression (n = 3 for each group). α-Tubulin served as a loading control. All values are mean ± SEM. (*) p<0.05; (**) p<0.01; ns, not significant (unpaired Student’s t-test).

Figure 9. A model of regulating alveolar formation through mitochondrial activity and distribution

The main players during alveolar formation are shown in the schematic diagram. Mitochondria display dynamic subcellular distribution in alveolar epithelial cells and mesenchymal fibroblasts/myofibroblasts. Tfam controls mitochondrial activity while Rhot1 regulates mitochondrial distribution. Mitochondrial activity and distribution in alveolar epithelial cells (type I [AT1] and type II [AT2]) contribute to secretion of the PDGF-A ligand. Reception of
PDGF-A by mesenchymal fibroblasts/myofibroblasts is critical to fibroblast/myofibroblast proliferation and migration, a key step in secondary septation. Similarly, mitochondrial activity and distribution in fibroblasts/myofibroblasts are also required for fibroblast/myofibroblast contraction and migration, likely through powering the cytoskeleton. The mTORC1 pathway plays a central role in controlling mitochondrial function during alveolar formation. Specification of alveolar epithelial cells and fibroblasts/myofibroblasts was unaffected in mutant mouse lungs in which mitochondrial activity and distribution were perturbed. We propose that essential cellular processes have differential requirements of mitochondrial activity and distribution. Investigating how mitochondria control signaling pathways and cellular processes in vivo provide a new way to functionally define signaling pathways and cellular processes. We surmise that the regulatory circuitry mediated by mitochondria activity and distribution is also deployed during alveolar repair following lung injury and could contribute to the pathogenesis of COPD/emphysema.
Figure legends for figure supplements

Figure 3–figure supplement 1. Loss Tfam or Rhot1 disrupts mitochondrial activity and distribution, respectively

(A) Immunostaining of lung sections collected from Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} (control) and Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} mice at 16.5 days post coitus (dpc). MPC1 antibodies marked mitochondria while MTCO1 antibodies detected cytochrome c oxidase, the expression of which was controlled by Tfam. (B) Immunostaining of fibroblasts derived from control or Tfam\textsuperscript{ff}; Pdgfra\textsuperscript{Cre/+} lungs at postnatal (P) day 3. (C) Immunostaining of fibroblasts derived from control or Rhot1\textsuperscript{ff}; Pdgfra\textsuperscript{Cre/+} lungs at P5.

Figure 3–figure supplement 2. Elimination of Tfam or Rhot1 in the epithelium of mouse lungs does not perturb saccule formation or cell type specification

(A) Surface view of dissected lungs from Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} (control) and Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (B) Immunostaining of lung sections from control and Tfam\textsuperscript{ff}; Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} mice at P5. Specification of lung cell types (e.g., club cell [CC10\textsuperscript{+}], ciliated cell [Ac-tub\textsuperscript{+}], AT1 cell [T1\textalpha\textsuperscript{+}], AT2 cell [SPC\textsuperscript{+}]) was unaffected. (C) Surface view of dissected lungs from Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} (control) and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (D) Immunostaining of lung sections from control and Rhot1\textsuperscript{ff}; Sox9\textsuperscript{Cre/+}; ROSA26\textsuperscript{mTmG/+} mice at P5. Specification of lung cell types was unaffected.
Figure 3–figure supplement 3. Loss of epithelial Tfam or Rhot1 does not lead to cell death in the lungs

(A) Immunostaining of lung sections collected from control and Tfam$f/f$; Sox9Cre/+ mice at postnatal (P) day 5. NKX2.1 antibodies marked lung epithelial cells. The rate of cell death by TUNEL assay was unaltered in the absence of epithelial Tfam. (B) Immunostaining of lung sections collected from control and Rhot1$f/f$; Sox9Cre/+ mice at P5. The rate of cell death by TUNEL assay was similar between control and Rhot1-deficient lungs.

Figure 3–figure supplement 4. Loss of epithelial Tfam does not affect the regulators of mitochondrial fusion and fission

Western blotting of lysates derived from control and Tfam$f/f$; Sox9Cre/+ mouse lungs at postnatal (P) day 5. The expression levels of OPA1 (dynamin-like 120 KDa protein), DRP1 (dynamin-related protein 1), pDRP1 (phosphorylated DRP1) showed no apparent difference between control and Tfam-deficient lungs. OPA1 controls mitochondrial fusion while DRP1 regulates mitochondrial fission. Note that multiple isoforms of OPA1 were present. The numbers indicate the positions of protein size standards.

Figure 3–figure supplement 5. Loss of Tfam in T cells by Foxp3–Cre or Cd4-Cre and in macrophages by activated Cx3cr1-CreER does not perturb alveolar formation

Hematoxylin and eosin-stained lung sections of control, Tfam$f/f$; Foxp3Cre/+ and Tfam$f/f$; Cd4Cre/+ mice and of control and Tfam$f/f$; Cx3cr1CreER/+ mice at postnatal (P) day 12. Control and Tfam$f/f$; Cx3cr1CreER/+ mice were injected with tamoxifen (TM) at P2 and P5 and lungs were
harvested at P12. Histological analysis revealed no difference between control, \(Tfam^{ff}\); \(Foxp3^{Cre/+}\) and \(Tfam^{ff}\); \(Cd4^{Cre/+}\) lungs and between control and \(Tfam^{ff}\); \(Cx3cr1^{CreER/+}\) lungs.

**Figure 4—figure supplement 1. Controls for LacZ-staining and lentiviral transduction**

(A) LacZ-staining (blue) of lung sections collected from \(Pdgfa^{ex4COIN/+}\) and \(Tfam^{ff}\); \(Sox9^{Cre/+}\) mice at postnatal (P) day 5. The slides were counterstained with eosin (red). No LacZ staining in the lung was detected in these two mouse lines. (B) Immunostaining of control, \(Tfam\)-deficient (n = 4 for each group) and \(Rhot1\)-deficient cells (n = 3 for each group) lentivirally transduced with PDGFA\(^{\text{FLAG}}\)-expressing constructs. (C) Quantification of PDGFA\(^{\text{FLAG}}\)-expressing cells revealed no difference in transduction efficiency among these three cell lines.

**Figure 5—figure supplement 1. Removal of \(Tfam\) or \(Rhot1\) in mouse lung fibroblasts/myofibroblasts does not perturb saccule formation or cell type specification**

(A) Surface view of dissected lungs from \(Pdgfra^{Cre/+}\); \(ROSA26^{mTmG/+}\) (control) and \(Tfam^{ff}\); \(Pdgfra^{Cre/+}\); \(ROSA26^{mTmG/+}\) mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (B) Immunostaining of lung sections from control and \(Tfam^{ff}\); \(Pdgfra^{Cre/+}\); \(ROSA26^{mTmG/+}\) mice at P5. Specification of lung cell types was unaffected. (C) Surface view of dissected lungs from control and \(Rhot1^{ff}\); \(Pdgfra^{Cre/+}\); \(ROSA26^{mTmG/+}\) mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (D) Immunostaining of lung sections from control and \(Rhot1^{ff}\); \(Pdgfra^{Cre/+}\); \(ROSA26^{mTmG/+}\) mice at P5. Specification of lung cell types was unaffected.

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Figure 5–figure supplement 2. Loss of mesenchymal Tfam or Rhot1 does not lead to cell death in the lungs

(A) Immunostaining of lung sections collected from control and Tfam$^{f/f}$; Pdgfra$^{Cre/+}$ mice at postnatal (P) day 5. NKX2.1 antibodies marked lung epithelial cells. The rate of cell death by TUNEL assay was unaltered in the absence of mesenchymal Tfam. (B) Immunostaining of lung sections collected from control and Rhot1$^{f/f}$; Pdgfra$^{Cre/+}$ mice at P5. The rate of cell death by TUNEL assay was similar between control and Rhot1-deficient lungs.

Figure 5–figure supplement 3. Inactivation of Tfam or Lrpprc in mouse lung fibroblasts/myofibroblasts leads to alveolar defects

(A) Hematoxylin and eosin-stained lung sections of control and Tfam$^{f/f}$; Twist2$^{Cre/+}$ mice at different postnatal (P) stages as indicated. Histological analysis revealed the presence of enlarged saccules and defective development of secondary septa in the mutant lungs. (B) Measurement of the MLI in control and Tfam$^{f/f}$; Twist2$^{Cre/+}$ lungs at P5 and P28 (n = 3 for each group). The MLI was increased in Tfam-deficient lungs. (C) Hematoxylin and eosin-stained lung sections of control and Lrpprc$^{f/f}$; Pdgfra$^{Cre/+}$ mice at P21 and P30. Saccules were enlarged with loss of secondary septation in the mutant lungs. (D) The MLI was increased in Lrpprc-deficient lungs at P21 and P30 (n = 3 for each group). All values are mean ± SEM. (*) p<0.05; (**) p<0.01; (***) p<0.001; ns, not significant (unpaired Student’s t-test).

Figure 6–figure supplement 1. Tfam- but not Rhot1-deficient fibroblasts display short tubular and fragmented mitochondria
(A) Immunostaining of fibroblasts derived from control and \textit{Tfam}^{f/f}; \textit{Pdgfra}^{Cre/+} lungs at postnatal (P) day 5. Cells were lentivirally transduced with Mito-7-mEmerald-expressing constructs to visualize mitochondria and stained with MTCO1 antibodies to detect cytochrome c oxidase. (B) Quantification of mitochondrial network and morphology. Tubular mitochondria were the dominant form in control fibroblasts, while short tubular and fragmented mitochondria were found in \textit{Tfam}-deficient fibroblasts. (C) Immunostaining of fibroblasts derived from control and \textit{Rhot1}^{f/f}; \textit{Pdgfra}^{Cre/+} lungs at postnatal (P) day 5. (D) Quantification of mitochondrial network and morphology. No apparent difference in mitochondrial morphology was found between control and \textit{Rhot1}-deficient fibroblasts.

Figure 6—figure supplement 2. The migratory defect in \textit{Tfam}- and \textit{Rhot1}-deficient fibroblasts/myofibroblasts is rescued by expression of TFAM and RHOT1, respectively

(A) Wound recovery assays to assess the migratory ability of fibroblasts/myofibroblasts derived from \textit{Tfam}^{f/f}; \textit{Pdgfra}^{Cre/+} mouse lungs (n = 3 for each group). Cells were transduced with control or mouse TFAM\textsuperscript{FLAG} (mTFAM\textsuperscript{FLAG})-expressing constructs. Within 36–48 hr, the wound area has been populated by migrating \textit{Tfam}-deficient fibroblasts/myofibroblasts expressing mTFAM\textsuperscript{FLAG}. By contrast, fewer \textit{Tfam}-deficient fibroblasts/myofibroblasts reached the wound area within the same time frame. (B) Wound recovery by \textit{Tfam}-deficient fibroblasts/myofibroblasts and \textit{Tfam}-deficient fibroblasts/myofibroblasts expressing mTFAM\textsuperscript{FLAG} was quantified. (C) Wound recovery assays to assess the migratory ability of fibroblasts/myofibroblasts derived from \textit{Rhot1}^{f/f}; \textit{Pdgfra}^{Cre/+} mouse lungs (n = 3 for each group). Cells were transduced with control or mouse RHOT1\textsuperscript{FLAG} (mRHOT1\textsuperscript{FLAG})-expressing constructs. Within 36–48 hr, the wound area has been populated by migrating \textit{Rhot1}-deficient...
fibroblasts/myofibroblasts expressing mRHOT1\textsuperscript{FLAG}. By contrast, fewer \textit{Rhot1}-deficient fibroblasts/myofibroblasts reached the wound area within the same time frame. (D) Wound recovery by \textit{Rhot1}-deficient fibroblasts and \textit{Rhot1}-deficient fibroblasts/myofibroblasts expressing mRHOT1\textsuperscript{FLAG} was quantified. (E) Western blotting of cell lysates from control fibroblasts/myofibroblasts, \textit{Tfam}-deficient and \textit{Rhot1}-deficient fibroblasts/myofibroblasts expressing mTFAM\textsuperscript{FLAG} or mRHOT1\textsuperscript{FLAG}, respectively as indicated. The numbers indicate the positions of protein size standards.

**Figure 7–figure supplement 1. The ratio of p-RPS6 to RPS6 is reduced in the absence of \textit{Rptor}**

Western blotting of lung lysates from control (C1–C8) and \textit{Rptor}-deficient (M1–M5) mice at postnatal (P) day 10. Quantification of the ratio of p-RPS6 to RPS6 protein levels is shown in Figure 7E. The numbers indicate the positions of protein size standards.

**Figure 8–figure supplement 1. Mitochondria display inhomogeneous distribution in human lungs**

Immunostaining of human lungs from normal subjects and emphysema patients. Mitochondria labeled by MTCO1 showed inhomogeneous distribution in different cell types of normal lungs. Likewise, immunoreactivity of phosphorylated RPS6 (pRPS6), an indicator of mTORC1 activity, also displayed non-uniform distribution. Regional but not global reduction of MTCO1 was observed in lungs of emphysema patients.

**Figure 8–figure supplement 2. TFAM protein levels are decreased in emphysema patients**
Western blotting of lung lysates from normal (C1–C21) and emphysema (M1–M21) patients.

Quantification of protein levels of TFAM, RPS6 and p-RPS6 is shown in Figure 8C, 8E.

**Figure 8–figure supplement 3. RHOT1 controls PDGF secretion in human lung epithelial cells and migration of human lung fibroblasts**

(A) Western blot analysis of cell lysates and supernatants from human lung epithelial cells transduced with control constructs, shRNA constructs against human RHOT1 and constructs expressing mouse RHOT1^{FLAG} (mRHOT1^{FLAG}) as indicated. qPCR analysis revealed efficient knockdown of human RHOT1 transcripts. The amount of PDGFA released into the media was reduced in RHOT1-knockdown cells compared to controls and was rescued by expression of mouse RHOT1^{FLAG} (mRHOT1^{FLAG}) (n = 3 for each group). α-Tubulin served as a loading control.

(B) Western blot analysis of cell lysates and supernatants from human lung fibroblasts transduced with control constructs, shRNA constructs against human RHOT1 and constructs expressing mRHOT1^{FLAG} as indicated. qPCR analysis revealed efficient knockdown of human RHOT1 transcripts. Wound recovery by control fibroblasts, RHOT1-knockdown fibroblasts and RHOT1-knockdown fibroblasts expressing mRHOT1^{FLAG} was quantified. The migratory defect was rescued by mRHOT1^{FLAG}. α-Tubulin served as a loading control.

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Figure 2 source data 1. Mean linear intercept and relative mtDNA/nDNA ratio

Figure 3 source data 1. Mean linear intercept, primary septal thickness, relative mtDNA/nDNA ratio, relative enzymatic activity and relative ATP production

Figure 4-figure supplement 1 source data 1. Efficiency of lentiviral transduction

Figure 4 source data 1. EdU quantification, relative transcript levels and quantification of PDGFA secretion

Figure 5-figure supplement 3 source data 1. Mean linear intercept

Figure 5 source data 1. Mean linear intercept, primary septal thickness, relative mtDNA/nDNA ratio, relative enzymatic activity and relative ATP production

Figure 6-figure supplement 1 source data 1. Quantification of mitochondrial morphology

Figure 6-figure supplement 2 source data 1. Quantification of wound recovery

Figure 6 source data 1. EdU quantification, relative transcript levels and quantification of wound recovery
Figure 7 source data 1. Mean linear intercept, relative protein levels and relative mtDNA/nDNA ratio

Figure 8-figure supplement 3 source data 1. Relative transcript levels, quantification of PDGFA secretion and quantification of wound recovery

Figure 8 source data 1. Relative mtDNA/nDNA ratio, protein levels, relative transcript levels, quantification of PDGFA secretion and quantification of wound recovery
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Figure 3—figure supplement 1. Loss Tfn or Rhot 1 disrupts mitochondrial activity and distribution, respectively

(A) Immunostaining of lung sections collected from SoxgCre/+; ROSA26mTmG/+ (control) and Tfn−/−; SoxgCre/+; ROSA26mTmG/+ mice at 16.5 days post coitus (dpc). MPC1 antibodies marked mitochondria while MTCO1 antibodies detected cytochrome c oxidase, the expression of which was controlled by Tfn. (B) Immunostaining of fibroblasts derived from control or Tfn−/−; PdgfraCre/+ lungs at postnatal (P) day 3. (C) Immunostaining of fibroblasts derived from control or Rhot 1−/−; PdgfraCre/+ lungs at P5.
Figure 3–figure supplement 2. Elimination of Tfam or Rhot1 in the epithelium of mouse lungs does not perturb saccule formation or cell type specification

(A) Surface view of dissected lungs from Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> (control) and Tfam<sup>flk</sup>; Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (B) Immunostaining of lung sections from control and Tfam<sup>flk</sup>; Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> mice at P5. Specification of lung cell types (e.g., club cell [CC10<sup>+</sup>], ciliated cell [Ac-tub<sup>+</sup>], AT1 cell [T1α<sup>+</sup>], AT2 cell [SPC<sup>+</sup>]) was unaffected. (C) Surface view of dissected lungs from Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> (control) and Rhot1<sup>flk</sup>; Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (D) Immunostaining of lung sections from control and Rhot1<sup>flk</sup>; Sox9<sup>Cre/+</sup>; ROSA26<sup>mTmG/+</sup> mice at P5. Specification of lung cell types was unaffected.
Figure 3–figure supplement 3. Loss of epithelial Tfam or Rhot1 does not lead to cell death in the lungs

(A) Immunostaining of lung sections collected from control and Tfam<sup>fl/fl</sup>; Sox9<sup>Cre/+</sup> mice at postnatal (P) day 5. NKX2.1 antibodies marked lung epithelial cells. The rate of cell death by TUNEL assay was unaltered in the absence of epithelial Tfam. (B) Immunostaining of lung sections collected from control and Rhot1<sup>fl/fl</sup>; Sox9<sup>Cre/+</sup> mice at P5. The rate of cell death by TUNEL assay was similar between control and Rhot1-deficient lungs.
Figure 3—figure supplement 4. Loss of epithelial *Tfam* does not affect the regulators of mitochondrial fusion and fission

Western blotting of lysates derived from control and *Tfam*<sup>fl/fl</sup>; *Sox9<sup>Cre/+</sup>* mouse lungs at postnatal (P) day 5. The expression levels of OPA1 (dynamin-like 120 KDa protein), DRP1 (dynamin-related protein 1), pDRP1 (phosphorylated DRP1) showed no apparent difference between control and *Tfam*-deficient lungs. OPA1 controls mitochondrial fusion while DRP1 regulates mitochondrial fission. Note that multiple isoforms of OPA1 were present. The numbers indicate the positions of protein size standards.
Figure 3—figure supplement 5. Loss of Tfat in T cells by Foxp3-Cre or Cd4-Cre and in macrophages by activated Cx3cr1-CreER does not perturb alveolar formation

Hematoxylin and eosin-stained lung sections of control, Tfat<sup>ff</sup>, Foxp<sup>3Cre/+</sup> and Tfat<sup>ff</sup>; Cd4<sup>Cre/+</sup> mice and of control and Tfat<sup>ff</sup>; Cx3cr1<sup>CreER/+</sup> mice at postnatal (P) day 12. Control and Tfat<sup>ff</sup>; Cx3cr1<sup>CreER/+</sup> mice were injected with tamoxifen (TM) at P2 and P5 and lungs were harvested at P12. Histological analysis revealed no difference between control, Tfat<sup>ff</sup>; Foxp<sup>3Cre/+</sup> and Tfat<sup>ff</sup>; Cd4<sup>Cre/+</sup> lungs and between control and Tfat<sup>ff</sup>; Cx3cr1<sup>CreER/+</sup> lungs.
Figure 4—figure supplement 1. Controls for LacZ-staining and lentiviral transduction

(A) LacZ-staining (blue) of lung sections collected from $Pdgfa^{ex4COIN/+}$ and $Tiam^{fl}; Sox9^{Cre/+}$ mice at postnatal (P) day 5. The slides were counterstained with eosin (red). No LacZ staining in the lung was detected in these two mouse lines. (B) Immunostaining of control, $Tiam$-deficient ($n = 4$ for each group) and $Rhot1$-deficient cells ($n = 3$ for each group) lentivirally transduced with PDGFA$^{FLAG}$-expressing constructs. (C) Quantification of PDGFA$^{FLAG}$-expressing cells revealed no difference in transduction efficiency among these three cell lines.
Figure 5—figure supplement 1. Removal of Tfacm or Rhot1 in mouse lung fibroblasts/myofibroblasts does not perturb saccule formation or cell type specification (A) Surface view of dissected lungs from Pdgfra^{Cre/+}; ROSA26^{mTmG/+} (control) and Tfacm^{ff}; Pdgfra^{Cre/+}; ROSA26^{mTmG/+} mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (B) Immunostaining of lung sections from control and Tfacm^{ff}; Pdgfra^{Cre/+}; ROSA26^{mTmG/+} mice at P5. Specification of lung cell types was unaffected. (C) Surface view of dissected lungs from control and Rhot1^{ff}; Pdgfra^{Cre/+}; ROSA26^{mTmG/+} mice at postnatal (P) day 0. No difference in saccule formation was noted between control and mutant lungs. (D) Immunostaining of lung sections from control and Rhot1^{ff}; Pdgfra^{Cre/+}; ROSA26^{mTmG/+} mice at P5. Specification of lung cell types was unaffected.
Figure 5—figure supplement 2. Loss of mesenchymal *Tfat* or *Rhot1* does not lead to cell death in the lungs
(A) Immunostaining of lung sections collected from control and *Tfat*; *Pdgfra*Cre/+ mice at postnatal (P) day 5. NKX2.1 antibodies marked lung epithelial cells. The rate of cell death by TUNEL assay was unaltered in the absence of mesenchymal *Tfat*. (B) Immunostaining of lung sections collected from control and *Rhot1*; *Pdgfra*Cre/+ mice at P5. The rate of cell death by TUNEL assay was similar between control and *Rhot1*-deficient lungs.
Figure 5—figure supplement 3. Inactivation of Tfail or Lrpprc in mouse lung fibroblasts/myofibroblasts leads to alveolar defects
(A) Hematoxylin and eosin-stained lung sections of control and Tfail<sup>−/−</sup>; Twist2<sup>Cre/+</sup> mice at different postnatal (P) stages as indicated. Histological analysis revealed the presence of enlarged saccules and defective development of secondary septa in the mutant lungs. (B) Measurement of the MLI in control and Tfail<sup>−/−</sup>; Twist2<sup>Cre/+</sup> lungs at P5 and P28 (n = 3 for each group). The MLI was increased in Tfail-deficient lungs. (C) Hematoxylin and eosin-stained lung sections of control and Lrpprc<sup>−/−</sup>; Pdgfra<sup>Cre/+</sup> mice at P21 and P30. Saccules were enlarged with loss of secondary septation in the mutant lungs. (D) The MLI was increased in Lrpprc-deficient lungs at P21 and P30 (n = 3 for each group). All values are mean ± SEM. (*) p<0.05; (**) p<0.01; (***) p<0.001; ns, not significant (unpaired Student’s t-test).
Figure 6—figure supplement 1. Tfam- but not Rhot1-deficient fibroblasts display short tubular and fragmented mitochondria

(A) Immunostaining of fibroblasts derived from control and Tfam^fl/fl; Pdgfra^{Cre/+} lungs at postnatal (P) day 5. Cells were lentivirally transduced with Mito-7-mEmerald-expressing constructs to visualize mitochondria and stained with MTCO1 antibodies to detect cytochrome c oxidase. (B) Quantification of mitochondrial network and morphology. Tubular mitochondria were the dominant form in control fibroblasts, while short tubular and fragmented mitochondria were found in Tfam-deficient fibroblasts. (C) Immunostaining of fibroblasts derived from control and Rhot1^fl/fl; Pdgfra^{Cre/+} lungs at postnatal (P) day 5. (D) Quantification of mitochondrial network and morphology. No apparent difference in mitochondrial morphology was found between control and Rhot1-deficient fibroblasts.
Figure 6—figure supplement 2. The migratory defect in Tfam- and Rhot1-deficient fibroblasts/myofibroblasts is rescued by expression of TFAM and RHOT1, respectively

(A) Wound recovery assays to assess the migratory ability of fibroblasts/myofibroblasts derived from Tfam−/−; PdgfraCre/+ mouse lungs (n = 3 for each group). Cells were transduced with control or mouse TFAMFLAG (mTFAMFLAG)-expressing constructs. Within 36–48 hr, the wound area has been populated by migrating Tfam-deficient fibroblasts/myofibroblasts expressing mTFAMFLAG. By contrast, fewer Tfam-deficient fibroblasts/myofibroblasts reached the wound area within the same time frame. (B) Wound recovery by Tfam-deficient fibroblasts/myofibroblasts and Tfam-deficient fibroblasts/myofibroblasts expressing mTFAMFLAG was quantified. (C) Wound recovery assays to assess the migratory ability of fibroblasts/myofibroblasts derived from Rhot1−/−; PdgfraCre/+ mouse lungs (n = 3 for each group). Cells were transduced with control or mouse RHOT1FLAG (mRHOT1FLAG)-expressing constructs. Within 36–48 hr, the wound area has been populated by migrating Rhot1-deficient fibroblasts/myofibroblasts expressing mRHOT1FLAG. By contrast, fewer Rhot1-deficient fibroblasts/myofibroblasts reached the wound area within the same time frame. (D) Wound recovery by Rhot1-deficient fibroblasts/myofibroblasts and Rhot1-deficient fibroblasts/myofibroblasts expressing mRHOT1FLAG was quantified. (E) Western blotting of cell lysates from control fibroblasts/myofibroblasts, Tfam-deficient and Rhot1-deficient fibroblasts/myofibroblasts expressing mTFAMFLAG or mRHOT1FLAG, respectively as indicated. The numbers indicate the positions of protein size standards.
Figure 7—figure supplement 1. The ratio of p-RPS6 to RPS6 is reduced in the absence of Rptor

Western blotting of lung lysates from control (C1–C8) and Rptor-deficient (M1–M5) mice at postnatal (P) day 10. Quantification of the ratio of p-RPS6 to RPS6 protein levels is shown in Figure 7E. The numbers indicate the positions of protein size standards.
Figure 8—figure supplement 1. Mitochondria display inhomogeneous distribution in human lungs

Immunostaining of human lungs from normal subjects and emphysema patients. Mitochondria labeled by MTCO1 showed inhomogeneous distribution in different cell types of normal lungs. Likewise, immunoreactivity of phosphorylated RPS6 (pRPS6), an indicator of mTORC1 activity, also displayed non-uniform distribution. Regional but not global reduction of MTCO1 was observed in lungs of emphysema patients.
Figure 8–figure supplement 2. TFAM protein levels are decreased in emphysema patients. Western blotting of lung lysates from normal (C1–C21) and emphysema (M1–M21) patients. Quantification of protein levels of TFAM, RPS6 and p-RPS6 is shown in Figure 8C, 8E.
Figure 8—figure supplement 3. RHOT1 controls PDGF secretion in human lung epithelial cells and migration of human lung fibroblasts
(A) Western blot analysis of cell lysates and supernatants from human lung epithelial cells transduced with control constructs, shRNA constructs against human RHOT1 and constructs expressing mouse RHOT1\( ^{\text{FLAG}} \) (mRHOT1\( ^{\text{FLAG}} \)) as indicated. qPCR analysis revealed efficient knockdown of human RHOT1 transcripts. The amount of PDGFA released into the media was reduced in RHOT1-knockdown cells compared to controls and was rescued by expression of mouse RHOT1\( ^{\text{FLAG}} \) (mRHOT1\( ^{\text{FLAG}} \)) (n = 3 for each group). α-Tubulin served as a loading control.
(B) Western blot analysis of cell lysates and supernatants from human lung fibroblasts transduced with control constructs, shRNA constructs against human RHOT1 and constructs expressing mRHOT1\( ^{\text{FLAG}} \) as indicated. qPCR analysis revealed efficient knockdown of human RHOT1 transcripts. Wound recovery by control fibroblasts, RHOT1-knockdown fibroblasts and RHOT1-knockdown fibroblasts expressing mRHOT1\( ^{\text{FLAG}} \) was quantified. The migratory defect was rescued by mRHOT1\( ^{\text{FLAG}} \). α-Tubulin served as a loading control.
Figure 9

Wild-type AT2 cell

AT1 cell

Golgi

Mitochondria

mTORC1

Tfam

Mitochondrial activity

Mitochondrial distribution

PDGF-A

PDGFRA

Alveoli

Secondary septa

Shape change

Proliferation
Migration
Contracton

Fibroblast/myofibroblast

Wild-type

Primary septa

Primary septa

Myofibroblast

Blood vessel

PDGFRA+ fibroblast

PDGFRA+ fibroblast

AT2

AT1