Differential regulation of autophagy and mitophagy in pulmonary diseases

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Autophagy is a lysosomal-mediated intracellular degradation pathway that maintains cellular homeostasis by turning over cellular components (19). During autophagy, cellular components, such as proteins, lipids, and organelles, are sequestered into double-membrane vesicles, termed autophagosomes, and transferred to endosomes or lysosomes. Lysosomal hydrolases within the lysosomes digest these autophagic cargoes to their basic components (i.e., amino acids and fatty acids) to be reutilized for anabolic pathways and ATP generation. By disposing of dysfunctional or damaged organelles such as ribosomes, peroxisomes, and mitochondria through ribophagy, pexophagy, and mitophagy, respectively, the autophagy pathway therefore plays a vital role in organelle quality control (46).

Mitochondria are constantly exposed to reactive intermediates and become damaged, and hence constant turnover is needed to maintain functional networks (24, 58, 121). This oxidative stress can impair mitochondrial protein homeostasis (proteostasis) and promote cell death (87). To maintain mitochondrial proteostasis, dysfunctional mitochondria are removed through selective degradation via autophagy, a process known as mitophagy (62, 62, 91, 120, 120). Under pathological conditions, mitophagy can become dysregulated (23, 121, 122). Perturbation of autophagy in HEK cells due to exogenous expression of human surfactant protein C mutation is associated with decrease mitochondrial membrane potential and decrease the ability of the cells to degrade aggregation prone mutant huntingtin protein (34). Emerging evidence suggests that autophagy and mitophagy have diverse functions in human diseases that may include both protective and potentially deleterious processes (17, 75, 76, 95).

The regulation of autophagy and mitophagy involves complex interaction and signal transduction networks (Fig. 1). Autophagy is upregulated during starvation or energy depletion by the activation of 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) and inhibition of the mechanistic target of rapamycin (mTOR) pathway (93). The formation of the autophagosomes is mediated by two ubiquitin-like conjugation systems: microtubule-associated protein 1, light chain 3 (LC3) or its homologs, and autophagy protein, autophagy-related gene 5–12 (ATG5-12) (25, 48, 106). In mammals, the conversion of LC3-I (unconjugated cytosolic form) to LC3-II (autophagosome-associated phosphatidylethanolamine-conjugated form) is a hallmark of autophagosome formation (25, 48, 106). In addition, the SQSTM1/p62 protein, also known as sequestosome-1, has a ubiquitin association domain (UBA) and a LC3-interacting region (LIR), which play an important role in recognizing cargos and targeting them for autophagy (42). Autophagy deficiencies lead to accumulation of p62 and induction of cellular stress and disease (44, 54).

Autophagosome formation is also upregulated by the autophagy protein, BECN1, which associates with a macromolecular complex that activates autophagy, including VPS34, a class III phosphatidylinositol-3-kinase (PI3KC3) (35, 49). BECN1 interaction with BCL2 family of proteins may also impact on cell death pathways (49), since association of B-cell lymphoma-1 (BCL2)-associated X protein (BAX) and BCL2-antagonistic/killer (BAK) and the opening of the inner membrane mitochondrial permeability transition pores induce apoptosis and necrotic cell death (50, 111). The permeabilization of the outer membrane releases proapoptotic proteins, such as cytochrome c, while the rapid influx of solutes and water through the inner membrane into the mitochondrial matrix collapses the proton gradient and impairs ATP synthesis, culminating in cell death (110, 127). Damaged mitochondria undergo dynamin-related protein 1 (DRP1)-mediated fission, membrane depolarization, accumulation of phosphatase and tensin homolog-induced putative kinase 1 (PINK1), and recruitment of the E3 ubiquitin ligase PARKIN, which promotes the ubiquitination of mitochondrial proteins and targets the damaged mitochondria for elimination by autophagosomes (120). This review will focus on the role of autophagy and mitophagy on mitochondrial function in pulmonary diseases.
Autophagy and Mitophagy in Acute Lung Injury

Acute lung injury (ALI) is a life-threatening syndrome associated with high morbidity and mortality. ALI is characterized by the increased permeability of the alveolar-capillary membrane, edema, uncontrolled neutrophil migration to the lung, and diffuse alveolar damage, leading to acute hypoxemic respiratory failure. Many studies have suggested the important role of mitochondrial dysfunction in the underlying pathology in the development of ALI (72, 96). Dysfunctional mitochondria generate excessive reactive species and regulate apoptosis, cytokine production, and innate immunity (32, 72, 96, 110). Mitophagy in ALI has been hypothesized either to enhance lung cell survival by turning over dysfunctional mitochondria (59, 78, 95, 124, 125) or to contribute to lung pathology by disrupting mitochondrial homeostasis and promoting cell death (66, 123). As the causes of ALI are diverse, whether mitophagy is upregulated or downregulated and whether mitophagy plays a protective or harmful role seems to depend on the underlying cause of the lung injury (Table 1, Fig. 2). Further complicating the interpretation of whether mitophagy is elevated or inhibited during any pathological or therapeutic process is the divergence of methods used, some of them only assessed whether LC3-II protein or LC3 immunoreactive puncta are up or down. As LC3-II can accumulate in response to both activation of autophagy initiation or inhibition of autophagy clearance, a careful evaluation of existing observations regarding autophagy activities is essential (53).

In a mouse model of ALI caused by *Staphylococcus aureus* infection, BECN protein and LC3-II are increased, p62 is decreased, and GFP-LC3 fluorescence signals are increased in type 2 epithelium and alveolar macrophages in the mouse lungs and colocalized to mitochondria (12). Nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) knockout mice exhibited decreased LC3-II and increased p62; however, whether lung injury is also exacerbated by *Nrf2* knockout has not been assessed in this study (12). In alveolar macrophages, infection with *Klebsiella pneumoniae* increases the ATG7 protein and LC3-II. *Atg7* knockout mice exhibit decreased bacterial clear-
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<td>Staphylococcus aureus-induced model of sepsis</td>
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<td>Infection with Klebsiella pneumoniae increased ATG7 and LC3-II</td>
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<td>Transgenic mice overexpressing the Le3 gene exhibited increased clearance of autophagosomes and improved survival after CLP.</td>
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<td>Hemorrhagic shock/resuscitation plus intratracheal injection of muramyl dipeptide (two-hit model)</td>
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<td>Hyperoxia decreased LC3B-II/I, increased p62, PINK1, and PARKIN, and increased caspase-3 activation.</td>
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<td>Carbon monoxide increases LC3BII/I and protects against hyperoxia-induced cell death.</td>
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<td>Sea water aspiration-induced ALI</td>
<td>Aspiration of sea water increased LC3B-II protein in rat lungs. In IR-induced ALI, the upregulation of complement, C5a, increased ratio of LC3-II/I and decreased Bcl-2 in alveolar macrophages.</td>
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<td>Intestinal IR-induced ALI</td>
<td>Mice with macrophage specific Atg5 knockout exhibited attenuated IR-induced ALI.</td>
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and more severe lung injury (116). Additional consequences of Atg7 deficiency in macrophages also include increased reactive oxygen species (ROS) as assessed by 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) probe and Europium-tetracycline (EuTc) assay, decreased JAK2/STAT1/H9251, decreased Nos2 mRNA, decreased nitric oxide release (63), and increased binding of p-I\(\beta\)/H9260 to ubiquitin (117). In response to chlorine exposure, lung epithelial cells exhibit mitochondrial dysfunction, oxidative stress, and increased LC3B-II. Autophagy activator trehalose protects against, and autophagy

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**Table 1.—Continued**

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<td>VILI</td>
<td>In a mouse model of VILI, mechanical ventilation with a high tidal volume (28 ml/kg) caused rapid increase in LC3B-II levels and ratio of LC3B-II/I in macrophages, causing lung inflammation via NLRC inflammasome signaling. Mechanical ventilation increased LC3II/I, p-JNK, and p-mTOR.</td>
<td>Pharmacological inhibition of autophagy by 3-MA or trichostatin A, or silencing lung macrophage Atg5 significantly attenuated the inflammatory injury.</td>
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<td>Influenza A virus-induced lung injury</td>
<td>H5N1 suppress mTOR signaling in MEFs Electron microscopy of lung tissue from a H5N1-infected patient and mice, and from H5N1-infected human lung adenocarcinoma cells contain autophagosomes, LC3-II is upregulated by H5N1</td>
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<td>H5N1 HA protein is responsible for and NF-(\kappa)B promotes autophagosome formation. Not assessed</td>
<td>Atg5 lung epithelial knockout attenuate H3N2 viral replication and mortality. Bafilomycin at low concentration suppresses H1N1-induced LC3-II accumulation and decreased viral replication in A549 cells. Atg5, Atg14, Fip200, and Atg7, but not Atg4b or Atg16l1, myeloid cell knockout protected mice from influenza H1N1-induced lethality.</td>
<td>Hahn et al. 2014</td>
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<td></td>
<td>H5N1 HA protein is responsible for and NF-(\kappa)B promotes autophagosome formation. Not assessed</td>
<td>Not assessed</td>
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3-MA, 3-methyladenine.

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**Fig. 2. Acute lung injury and autophagy.** Autophagy may play a protective or a detrimental role in acute lung injury, as the blue shaded boxes summarize examples of inhibition of autophagy by genetic or pharmacological approaches exacerbated injury. Brown shaded boxes summarize examples that activation of autophagy decreased lung injury, and converse examples of inhibition of autophagy by genetic or pharmacological approaches decreased lung injury.
inhibitor 3-methyladenine (3-MA) exacerbates, chlorine-induced mitochondrial dysfunction. In vivo, trehalose decreases pulmonary inflammation, consistent with a protective role of autophagy enhancement (47).

In a cecal ligation and puncture (CLP) model of sepsis-induced ALI, it has been reported that in C57BL/6J mice CLP increased LC3-II. *Beecn*+/− mice exhibited more CLP-induced mortality in this model, and carbon monoxide protects. Furthermore, the protective effects of carbon monoxide is dependent on BECN1, as carbon monoxide induces BECN1, ATG7, and LC3 and the protection is attenuated in *Beecn*+/− mice (59). Similarly, in C3H/HeN mice in response to CLP, autophagosome-lysosome fusion was impaired and LC3-II accumulates. Overexpression of *Lc3* enhanced autophagosome-lysosome fusion; decreased lung injury, edema, and inflammation; and enhanced animal survival (67). The same group also reported however, that CLP decreased LC3-II and ATG5, and the treatment of mice with rapamycin, an inhibitor of the mTOR signaling pathway, attenuated the CLP-induced suppression of LC3-II and ATG5 and improved mouse survival after CLP (119). This is one example of discrepancies observed sometimes with the same mouse strain and the same injury model yet different LC3-II responses.

In ALI caused by lipopolysaccharide (LPS)-induced endotoxemia, LC3-II was increased in the lungs of LPS-treated mice and in human bronchial cells exposed to serum from septic patients (1). Starvation ameliorated lung injury, and autophagy-deficient mice (*Atg4b-null*) had increased mortality after endotoxemia and exacerbated lung injury (1). One study found that in septic patients MAP kinase kinase 3 (MKK3) activities are higher in isolated peripheral blood mononuclear cells compared with controls, suggesting a correlation between MKK3 activity and sepsis (72). LPS increased mitochondria, and LC3B colocalization was further elevated in MKK3−/− lung endothelial cells, associated with higher levels of PINK1 and PARKIN, higher mitochondrial mass and membrane potential, and decreased mitochondrial-derived ROS as assessed by MitoSOX fluorescence. MKK3−/− mice released less mitochondrial DNA to the serum associated with enhanced survival of LPS exposure (72). In contrast, *Pink1*+/− mice exhibited worsened survival during LPS-induced sepsis (72). *Lc3b−/−* or *Beecn−/−* macrophages exhibited enhanced LPS+ATP-induced caspase-1 activation, the accumulation of swollen or depolarized mitochondria, increased mitochondrial ROS as indicated by MitoSOX intensity, the translocation of mitochondrial DNA into the cytosol, and the secretion of IL-1β and IL-18 (78).

In a mouse two-hit model (hemorrhagic shock followed by intratracheal injection of muramyl dipeptide, HS-MDP) of ALI, HS-MDP induced high mobility group box 1 (HMG B1) signaling and activation of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in alveolar macrophages, induced inflammation, and elevated Lc3-II. *Lc3* knockout alveolar macrophages exhibited persistent receptor-interacting protein 2 (RIP2) and NOD2 association and enhanced inflammation, indicating that autophagy plays an inhibitory role in the inflammation process. Polymorphonuclear leukocytes also promote lung inflammation, albeit through an autophagy-independent mechanism via upregulation of Toll-like receptor 2 (TLR2) signaling and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation (112).

ALI induced by hyperoxia is associated with increased LC3B-II and p62 in the lungs of mice and bronchial epithelial cells (64, 105). *Lc3b* and *p62* siRNAs promoted hyperoxia-induced epithelial cell death, consistent with autophagy playing a protective role in ALI pathology (64, 105). The protective role of autophagy has been demonstrated by additional evidence in the context of ALI. Nlrp3 knockout mice exhibited increased LC3B-II/I at normal or hyperoxia condition, increased PINK1, resistance to hyperoxia-induced bronchoalveolar lavage (BAL) fluid protein exudation, oxidative stress, and apoptosis (124). Conversely, mice with *Pink1* knockout or lung endothelial-targeted silencing exhibited increased oxidative stress, BAL, apoptosis, and mortality in hyperoxia-exposed mice (124). Carbon monoxide increases LC3B-II/I in cultured human epithelial cells, decreases mitochondrial ROS in A549 cells, and is protective against hyperoxia-induced human epithelial cell death (60).

In contrast, several studies have shown that autophagy may have a detrimental role in lung pathology in certain circumstances. For instance, studies have shown that in response to seawater-exposure, both the LC3 mRNA and the LC3-II proteins are increased in the lung. Inhibition of autophagy by intraperitoneal injection of 3-MA attenuated seawater-induced lung edema and improved lung function (66). Similarly, in response to intestinal injection of 3-MA or lung endothelial-targeted silencing exhibited increased oxidative stress, BAL, apoptosis, and mortality in hyperoxia-exposed mice (124). Carbon monoxide increases LC3B-II/I in cultured human epithelial cells, decreases mitochondrial ROS in A549 cells, and is protective against hyperoxia-induced human epithelial cell death (60).

Pathogenic influenza A (H5N1) has been shown to suppress mTOR signaling in embryonic fibroblasts and inhibition of autophagy by 3-MA or *Beecn1* knock down decreased H5N1-induced cell death (71). Autophagosomal accumulation has been observed from H5N1 infected human patient lung tissue. Mouse lung and A549 cells infected with H1N1 or H5N1 viruses exhibited higher LC3-II levels, which are even higher in the presence of E64d and peptatin A, the lysosomal protease inhibitors. 3-MA and *Atg 5* siRNA decrease H5N1-induced cell death in A549 cells in a process that is dependent on the mTOR pathway, tuberous sclerosis complex 2 (TSC2). In vivo, 3-MA or *Atg 5* siRNA attenuated H5N1-induced inflammation in mice (102). Further studies indicated that the hemagglutinin protein of H5N1 may be responsible for stimulating autophagy and that NF-κB activation promotes autophagosome formation (81). In addition to H5N1, H3N2 viral replication and mortality were also attenuated by *Atg5* knockout in the bronchoalveolar epithelium (30). In A549 cells, bafilomycin A1 (an inhibitor of vacuolar ATPase and autophagy completion in the lysosomes), at concentrations below IC50 of inhibiting vacuolar ATPase, suppressed H1N1 virus-induced LC3-II accumulation, and diminished viral replication (118). The mechanisms of how low concentrations of bafilomycin suppress LC3-II accumulation still need to be further investigated. Interestingly, *Atg5* whole body knockout and *Atg 14* myeloid cell knockout elevated basal level of lung inflammation-
tion; Atg5, Atg14, focal adhesion kinase family interacting protein of 200 kD (Fip200), and Atg7, but not Atg4b or Atg16l1 myeloid cell knockout, protected mice from influenza H1N1-induced lethality (70).

Together, although the exact mechanisms are unclear regarding how autophagy is regulated in ALI pathologies, modulating autophagy through pharmacological and molecular genetic approaches has significant impact on ALI. Whether autophagy and mitophagy play protective or detrimental role in ALI depends on the type of injury and at which step autophagy is inhibited. In summary, important highlights of autophagy/mitophagy in ALI are

- In *Staphylococcus aureus* infection, LC3 are increased in type 2 epithelium and alveolar macrophages in the mouse lungs. Role of autophagy and mitophagy is not known.
- In infection with *Klebsiella pneumoniae*, ATG7 protein and LC3-II are increased in alveolar macrophages. Autophagy/mitophagy is protective.
- After chlorine exposure, lung epithelial cells exhibit increased LC3B-II levels. Autophagy/mitophagy is protective.
- In CLP model of sepsis-induced ALI, LC3-II is increased in the lungs of C57BL/6J mice. However, in C3H/HeN mice whether LC3-II is increased or decreased is controversial. Autophagy/mitophagy is protective.
- In LPS-treated mice, LC3-II is increased in the lungs and also in bronchial epithelial cells exposed to serum from septic patients. Autophagy/mitophagy is protective.
- In H5-MDP model of ALI, LC3-II levels are elevated in alveolar macrophages. Autophagy/mitophagy is protective.
- In hyperoxia, ALI is associated with increased LC3B-II and p62 in the lungs of mice and bronchial epithelial cells. Autophagy/mitophagy is protective.
- Seawater aspiration increases LC3-II in the lung. Autophagy/mitophagy is deleterious.
- Intestinal ischemia-reperfusion increases LC3-II in alveolar macrophages. Autophagy/mitophagy is deleterious.
- In VILI, LC3-II/I ratio is increased in lung macrophages. Autophagy/mitophagy is deleterious.
- H1N1 or H5N1 viral infection increases LC3-II in mouse lung and A549 cells. Autophagy/mitophagy is deleterious.

**Autophagy and Mitophagy in Chronic Obstructive Pulmonary Disease**

Chronic obstructive pulmonary disease (COPD) is a leading cause of death worldwide and is the third leading cause of death in the United States. COPD includes the clinical phenotypes of emphysema characterized by the enlargement of distal air spaces and chronic bronchitis associated with mucus hypersecretion and obstruction of the bronchioles. Cigarette smoke (CS) is the most common risk factor for COPD, while the main inherited risk factor is α1-antitrypsin deficiency (α1AT). In COPD, disruptions in mitochondrial function have been well documented (7, 31, 37). The mitochondria in small airway epithelial cells of COPD patients appear fragmented compared with smokers without COPD (31). Similarly, the mitochondria from bronchial epithelial cells treated with cigarette smoke extract (CSE) are depolarized and fragmented in a DRP1-dependent manner (31). Interestingly, these aberrations in mitochondrial function are also linked to bronchial epithelial cell senescence and are ultimately prevented by using a mitochondrial-targeted antioxidant (31). In addition to mitochondrial fragmentation, decreased numbers of cristae, increased mtDNA damage, increased Opal mRNA and MnSOD protein, and increased expression of IL-1β, IL-6, and IL-8 are evident in bronchial epithelial cells exposed to CSE for 6 mo (37). Mitochondria in bronchial epithelial cells isolated from COPD patients exhibit excessive branching, elongation, and swelling and fewer cristae and have higher complex V F1a and MnSOD proteins and lower Pink1 and Pgc1α mRNA, compared with mitochondria in bronchial epithelial cells from individuals who never smoked (37). In human lung microvascular endothelial cells, CSE has been shown to induce the expression of mitochondrial E3 ubiquitin protein ligase 1 (MUL1), which causes Akt ubiquitination and endothelial cell dysfunction (52). In murine lung epithelial (MLE)12 cells, nontoxic concentrations of CSE induce mitochondrial elongation and increased levels of the mitochondrial fusion protein, MFN2, without changing PINK1. This is associated with increased mitocondrial membrane potential; increased basal, ATP-linked, and maximal mitochondrial oxygen consumption rate; and total ATP, therefore representing a beneficial adaptive stress (8). Decreasing mitochondrial iron loading either by knockout the iron-responsive element-binding protein 2 (IRP2) gene or by an iron chelator alleviated CS-induced impairment of mucociliary clearance, pulmonary inflammation, and lung injury (18).

Like in ALI, the role of autophagy and mitophagy in COPD is still controversial regarding whether autophagy and mitophagy in COPD are beneficial processes that are hampered in COPD or are maladaptive processes exacerbating the pathology. The presence of elevated autophagic flux remains a contentious issue (95). Many studies report an increase in the levels of autophagic markers (4, 14, 40, 126), but whether autophagic flux is actually elevated or autophagosome clearance is impaired or insufficient is still under investigation and seems to be cell type and context dependent (Table 2, Fig. 3).

Increased LC3-II/I, ATG4, ATG5-12 conjugate, ATG7, and autophagic vacuoles have been found in the lungs of COPD patients, associated with increased caspase-3 activation (14). *Lc3b* knockdown in bronchial epithelial cells exposed to CSE prevented caspase-3 activation and apoptosis and improved cell viability, consistent with the notion that autophagy is detrimental (14). In these COPD patients and CSE-exposed bronchial epithelial cells, increased expression of the transcription factor EGR-1 (early growth response protein 1) was required for enhancing the levels of LC3B and ATG4B (14). Egr-1-deficient mice displayed decreased LC3B-II and ATG4B and decreased emphysema after CS exposure (14). This is important considering that genetic polymorphisms in Egr-1, as well as autophagy gene *Atg16l1*, are associated with an increased risk of developing COPD (13).

LC3B function is regulated by multiple membrane associated and cytosolic factors. LC3B association with extrinsic apoptotic factor Fas in lipid rafts is caveolin-1 dependent and is disrupted after CSE exposure. Cave-1 knockdown sensitized CS-induced apoptosis in epithelial cells, and Cave-1 knockout mice exhibited enhanced chronic CS exposure-induced apoptosis in vivo (15). Enhanced expression of lipid raft proteins was also observed in CFTR-deficient mice, concurrent with increased p62 and LC3 puncta and worsened CS-induced inflam-
Table 2. Autophagy and mitophagy in COPD

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<td>Primary bronchial epithelial cells exposed to CSE</td>
<td>While PINK1 levels and LC3B colocalization with the mitochondria were elevated, PARKIN levels were significantly decreased in the lungs and small airway epithelial cells of COPD patients. Prolonged exposure-induced accumulation of ubiquitinated proteins and p62, cell senescence and IL-8 secretion.</td>
<td>Silencing of Pink1 or Parkin in human bronchial epithelial cells enhanced cellular senescence, suggesting a beneficial role of PARKIN and PINK1 in airway epithelial cells in attenuating cell senescence. Lc3 silencing exacerbated the accumulation of ubiquitinated proteins and IL-8 release, and autophagy activation by Torin1, an mTOR inhibitor, suppressed accumulation of ubiquitinated proteins and senescence. These observations are consistent with a beneficial role of autophagy.</td>
<td>Ito et al. 2015; Fujii et al. 2012</td>
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<td>Exposure of human lung fibroblasts to 0.5% CSE for 10–15 days</td>
<td>CS impaired mitophagy as evident by impaired mitochondrial localization to autophagosome, increased perinuclear localization of mitochondria, and increased mitochondrial DNA damage. Macrophages had defective autophagy, as evidenced by decreased protein degradation, increased autophagic vesicles on electron microscopy, impaired trafficking of autophagosomes to the lysosome (autophagic flux), blocked delivery of ubiquitin binding chaperone protein, p62, to the lysosome, and accumulated aggregates of ubiquitin and SUMO-modified proteins.</td>
<td>Mitochondrial fission inhibitor, Mdivi-1, increased cellular senescence. PARKIN overexpression or Mito-TEMPO decreased cellular senescence. In this case, it was implied that autophagy is beneficial.</td>
<td>Ahmad et al. 2015; Monick et al. 2010</td>
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<td>Alveolar macrophages from smokers with &gt;10 pack a year of smoking history</td>
<td>Decreased SIRT1 level in macrophages and lungs of COPD patients and in MonoMac6 cells exposed to CSE. H292 cells, SIRT1 activation by resveratrol decreases CSE-induced LC3-II, and SIRT1 inhibition by sirtinol enhances CSE-induced LC3-II.</td>
<td>Sirt1 knockout mice exhibited more increase of LC3-II in response to CS.</td>
<td>Hwang et al. 2010</td>
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<td>Human lung homogenates from COPD patients</td>
<td>SIRT6 levels are decreased. Sirt6 overexpression increased LC3-II while siRNA of Sirt6 decreased LC3-II, correlating with suppressed and enhanced senescence, respectively suggesting a protective role of SIRT6.</td>
<td>Not assessed</td>
<td>Takasaka et al. 2014</td>
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<td>Human bronchial epithelial cells exposed to CSE</td>
<td>Not assessed</td>
<td>Carbamazepine decreases CSE-induced perinuclear accumulation of ubiquitinated proteins and p62 accumulation in response to CSE.</td>
<td>Lin et al. 2013</td>
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<td>Beas-2b cells exposed to CSE</td>
<td>LacCer-synthase inhibitor decreased p62 accumulation in response to CSE.</td>
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<td>Guo et al. 2013</td>
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<td>Whole lungs from smoking patients; Epithelial cells or fibroblasts exposed to CSE; Mice exposed to CS</td>
<td>Increased LC3B-II ratio, ATG4, ATG5-ATG12, and ATG7 proteins, increased autophagosome vacuoles in COPD patients. Egr-1+/− mice are resistant to cigarette smoke-induced apoptosis and emphysema. Ho-1 overexpression inhibited the increase of LC3B-II or BECN1, the activation of DISC and the activation of caspase 3, 8, and 9. Ho-1 siRNA augmented DISC activation. Nrfl siRNA increases, while N-acetylcysteine, Keap1 siRNA, Nrfl2 overexpression, or p62 overexpression decreases LC3B-II. LC3B dissociation from Fas might be responsible for induction of apoptosis in response to CS or CSE. Increased LC3B in COPD patients, associated with higher TLR4.</td>
<td>In human pulmonary epithelial cells, knockdown of Egr-1 inhibited CSE-induced LC3B and ATG4B expression. Inhibition of autophagy by Lc3b knockdown protected epithelial cells from CSE-induced apoptosis. Both Lc3b and Becn1 siRNA decreased activation of caspase 3 and 8 in Beas-2b cells. Knockout Cav-1 which mediates LC3B-Fas association exacerbates CS exposure-induced apoptosis.</td>
<td>Chen et al. 2008; Kim et al. 2008; Zha et al. 2013; Chen et al. 2010; An et al. 2012</td>
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The observation of increased LC3B-II in the lungs of COPD patients has been confirmed in additional studies. One such study also correlated increased LC3B-II with higher TLR4 expression. Tlr4-deficient mice exhibited higher levels of LC3B both at basal conditions and after CS, with higher cleaved caspase-3 and augmented emphysema (4). An increase of placental growth factor (PGF) and LC3B-II/I and a decrease of p-mTOR and p62 have also been found in response to excessive elastase leading to apoptosis lung epithelial cells, suggesting involvement of protease/growth factors in regulation of autophagy in COPD (38). In primary human airway epithelial cells, IL13 induces MUC5AC hypersecretion and autophagy. Atg5 or Atg14 silencing attenuated mucin MUC5AC secretion, with mechanisms speculated to involve modulating exocytosis. These observations suggest the potential of inhibition of autophagy as a treatment of mucin secretion and chronic lung disease (22).

The involvement of antioxidant response in epithelial cells exposed to CSE has been demonstrated by the observation that Nrf2 siRNA increases and N-acetylcysteine, Keap1 siRNA, Nrf2 overexpression, or p62 overexpression decreases LC3B-II (126). In Beas-2b cells, overexpression of heme oxygenase (HO-1) inhibited the CSE-induced increase of LC3B-II or BECN1, the activation of death-inducing signaling complex (DISC), and the activation of caspase 3, 8, and 9. Ho-1 siRNA augmented the DISC activation and DCF-DA fluorescence induced by CSE. Becn1−/− fibroblasts exhibited decreased DISC activation and both Lc3b and Becn1 siRNA decreased activation of caspase 3 and 8 in Beas-2b cells (51). HO-1 overexpression has also been found to be protective against cadmium-induced emphysema, while this is associated with further increased LC3B-II/I (103).

An important hallmark of bronchial epithelial damage in COPD is cilia shortening and disruption of mucociliary clearance. In addition, a selective autophagic process termed ciliophagy may underlie CS-induced mucociliary clearance disruption in COPD (56). CS exposure causes the shortening and the loss of epithelial cilia, primarily by inducing oxidative stress and ciliary protein misfolding, aggregation, and ubiquitination (56). Ciliary protein IFT88, ARL13, centrin1, and pericentrin

<table>
<thead>
<tr>
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<tr>
<td>Human LMVEC from smokers and nonsmokers</td>
<td>LMVEC from smokers were resistant to ceramide-induced apoptosis. Treatment of cells with ceramide increased LC3B-II/I and increased autophagosomes, regardless of the smoking status of the donor.</td>
<td>Cells from smokers are resistant to 3-MA-induced caspase-3 activation.</td>
<td>Petrusca et al. 2014</td>
</tr>
<tr>
<td>Mice exposed to CS</td>
<td>Not assessed</td>
<td>Deficits in mucociliary clearance and cilia shortening are exacerbated by Nrf2 knockout and attenuated by Becn, Lc3b, and Hdac6 disruption, or chemical chaperone 4-phenylbutyric acid.</td>
<td>Lam et al. 2013</td>
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<tr>
<td>Exposure of human bronchial epithelial cells to 20% aqueous CSE for 4 h</td>
<td>In CSE-exposed cells there was an increased transportation of mt-mKeima to the lysosomes, indicating increased mitophagy.</td>
<td>Pharmacological inhibition of mitochondrial fission by Mdivi-1 protected against CS-induced cell death and mitochondrial dysfunction in vitro. Genetic disruption of PINK1 increased mucociliary clearance and protected against mitochondrial depolarization.</td>
<td>Mizumura et al. 2014</td>
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</table>

Fig. 3. COPD and autophagy/mitophagy. COPD may be associated with either increased LC3B-II and autophagosome numbers and increased mitophagy, or decreased mitophagy protein and decreased clearance of autophagy/mitophagy substrates. Examples that downregulation of autophagy may be detrimental are shown in the blue shaded boxes, and examples that both upregulation and downregulation of autophagy may be protective are shown in the brown shaded box.
are targeted for LC3B- and Beclin-1-mediated autophagy degradation, as Beclin1, LC3b, and Hdac6 impairments protected against cilia shortening (56). Interestingly, PINK1, which is increased in bronchial epithelial cells from COPD patients compared with nonsmoking controls, seems to regulate mitophagy. It has been found that CSE exposure in bronchial epithelial cells decreased the oxygen consumption rate, the mitochondria membrane potential, and increased mitochondrial-derived reactive species (76). This is associated with increased PINK1 levels and DRP1 phosphorylation at Ser616. Mitophagy has been shown to be increased as assessed by translocation of a mitochondria-targeted mKeima probe (mt-mKeima) to the lysosomes, and cells die by necroptosis (76). An inhibitor of mitochondrial fission, Mdivi-1, prevented CSE-induced mitophagy, loss of the mitochondria membrane potential, and cell death (76). Pink1-deficient mice exposed to CS exhibited increased mucociliary clearance and were protected against air space enlargement and loss of mitochondrial membrane potential, consistent with the notion that PINK1 plays a detrimental role (76).

However, there were also studies that challenge the detrimental role of mitophagy in the context of COPD. For example, it was shown that while PINK1 levels and LC3B colocalization with the mitochondria were elevated, PARKIN levels were significantly decreased in the lungs and small airway epithelial cells of COPD patients (43). Low levels of PARKIN correlated with low forced expiratory volume in 1 s/forced vital capacity (FEV1/FVC) percentages, an indicator of lung obstructive disease (43). The knockdown of Pink1 or Parkin in human bronchial epithelial cells (HBECs) enhanced cellular senescence, suggesting a beneficial role of PARKIN and PINK1 in airway epithelial cells in attenuating cell senescence associated with COPD (43).

Also supporting a beneficial role of autophagy in epithelial cells, it has been shown that although autophagic flux was transiently increased (12–24 h) in primary bronchial epithelial cells (HBECS) isolated from COPD patients, accumulation of ubiquitinated proteins and p62, cell senescence as assessed by β-galactosidase staining, and senescence-associated secretory phenotype with IL-8 release still occurred beginning at 24 h and further increased after prolonged CSE exposure. Lc3 silencing exacerbated the accumulation of ubiquitinated proteins and IL-8 release, and autophagy activation by Torin1, an mTOR inhibitor, suppressed accumulation of ubiquitinated proteins and senescence (26). This study suggested a beneficial role of autophagy in attenuating epithelial cell senescence in COPD.

By measurement of translocation of LC3B to mitochondria, mitophagy has been shown to be impaired in lung fibroblasts in response to CSE exposure, despite the increased PINK1 levels in lung fibroblasts (2). Increased cell senescence, mitochondrial DNA damage, and decreased mitochondrial membrane potential and ATP levels in lung fibroblasts were also evident (2). There was also an increase of p53, and p53 interaction with PARKIN may be responsible for inhibition of PARKIN translocation to the mitochondria (2). PARKIN overexpression and Mito-TEMPO, a mitochondria-targeted superoxide dismutase mimetic, decreased DNA damage and cellular senescence in CSE-exposed HFL1 cells, consistent with a beneficial effect of enhancement of mitophagy (2).

Using a combined approach of assessing the accumulation of p62, the rate of protein degradation assay, and LC3-II turnover in the absence and presence of lysosomal clearance inhibitor bafilomycin, it has been demonstrated that, in alveolar macrophages, CS or CSE exposures blocked autophagic flux, associated with increased accumulation of LC3-II (77). The decreased autophagic flux in CS- or CSE-exposed macrophages was correlated with decreased bacterial clearance and increased mitochondrial dysfunction (77).

Decreased nuclear sirtuin 1 (SIRT1) has been found in macrophages and lungs of COPD patients and in MonoMac6 cells exposed to CSE (88). SIRT1 has been shown to impact autophagy, as demonstrated in H922 cells that SIRT1 activation by resveratrol decreases CSE-induced LC3-II, and SIRT1 inhibition by sirtinol enhances CSE-induced LC3-II. Since SIRT1 activity is also regulated by NAD+ depletion induced by activation of poly(ADP-ribose) polymerase 1 (PARP-1), the impact of PARP-1 inhibition on CSE-induced LC3-II has been investigated. It was found that PARP-1 inhibition by an inhibitor 3-AB in HFL1 fibroblasts decreased CSE-induced LC3-II, consistent with PARP-1 inhibition activating SIRT1. Sirt1 knockout mice exhibited more increase of LC3-II in response to CS, although whether SIRT1 inhibition exacerbates lung pathology has not been examined in this particular study (40). Resveratrol (40) and Vam3 (97), a resveratrol dimer, have been shown to attenuate CSE-induced LC3-II increase in bronchial epithelial cells. The protective effects of resveratrol and Vam3 in CS-exposed mice and bronchial epithelial cells were also associated with suppressed apoptosis, preserved mitochondrial membrane potential, attenuated cytochrome c release, and decreased caspase-9 activation (115). Similar to SIRT1, SIRT6 levels were also found decreased in lung homogenates from COPD patients and in HBECS exposed to CSE. Sirt6 overexpression increased LC3-II while siRNA of Sirt6 decreased LC3-II (104). As Sirt1 activation attenuated cell death, Sirt6 overexpression suppressed while siRNA of Sirt6 enhanced CSE-induced HBECS senescence (104). In addition, low levels of SIRT6 expression levels correlated with low FEV1/FVC percentages (104). The mechanism of protective effects of SIRT6 against CSE-induced bronchial epithelial cell senescence has been found to involve the inhibition of mTOR (104).

Beas-2b cells exposed to CSE exhibited increased perinuclear accumulation of ubiquitinated proteins colocalizing with LC3B. Carbamazepine, which targets to voltage-gated sodium channel and GABA receptor along with previously noted autophagy-activating functions (65), decreased these perinuclear ubiquitinated protein aggregates both in Beas-2b cells exposed to CSE and in CS-exposed mice, although further confirmation is needed regarding whether carbamazepine enhances autophagosomal turnover in this context (109).

LC3B-II/I increase was also associated with exposure of human lung microvascular endothelial cells (LMVECs) to ceramide regardless of the smoking status of the donor, although cells from the smokers are resistant to 3-MA or ceramide-induced apoptosis (85). This study suggests that endothelial cell remodeling in response to CS may change cellular autophagic activities, which may contribute to COPD pathogenesis. Because increased ceramides have been found in patients with smoke-induced emphysema (84), a recent study further demonstrated that in Beas-2b cells CSE-induced p62 accumulation can be rescued by pharmacological inhibitor of
LacCer-synthase. Muscle in COPD patients also exhibited increased autophagosomes; increased LC3B-II, BECN1, BNIP3, p62, VPS34, ATG7, PARKIN, and AMPK proteins; increased p62, Gabarapl1, Uvrag, Ambra1 and Ctsl mRNA; as well as decreased p-AKT, p-S6, and p-ULK1 proteins. These observations provide evidence of alterations of autophagy pathway that may contribute to the muscle weaknesses associated with COPD (28).

In total, it has been found that LC3B is detrimental for CS-or CSE-induced apoptosis in pulmonary epithelial cells and that BECN, LC3B, and PINK1 are detrimental in mucociliary clearance deficits. However, PINK1 and PARKIN are protective against epithelial cell senescence and clearance of ubiquitinated proteins. In addition to this evidence, many autophagy regulators exhibited changes of levels in different cells and in different models of COPD. Pharmacological and genetic control of autophagy and mitophagy in COPD is still far from being clear. The major highlights of autophagy/mitophagy in COPD are:

- In the lungs and small airway epithelial cells of COPD patients, PINK1 and LC3B colocalization is elevated. Autophagy/mitophagy is protective.
- Human lung fibroblasts exposed to CSE have impaired mitophagy as evident by impaired mitochondrial localization to autophagosome, increased perinuclear localization of mitochondria, and increased mitochondrial DNA damage. Autophagy/mitophagy is protective.
- Alveolar macrophages from smokers have defective autophagy, as evidenced by decreased protein degradation, increased autophagic vesicles on electron microscopy, impaired trafficking of autophagosomes to the lysosome, blocked delivery of the ubiquitin binding chaperone protein p62 to the lysosome, and accumulated aggregates of ubiquitin- and SUMO-modified proteins. Autophagy/mitophagy is protective.
- In human lung homogenates from COPD patients and HBECs exposed to CSE, SIRT6 levels are decreased. Sirt6 overexpression increased LC3-II suggesting a protective role of SIRT6. Autophagy/mitophagy is protective.
- In human pulmonary epithelial cells exposed to CSE, the inhibition of autophagy by Lc3b knockdown protected epithelial cells from CSE-induced apoptosis. Autophagy/mitophagy is detrimental.
- In mice exposed to CS, the deficits in mucociliary clearance and cilia shortening are attenuated by Becn, Lc3b, and Hdac6 disruption. Autophagy/mitophagy is detrimental.

### Autophagy and Mitophagy in Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrosis process that typically affects patients over 40 years of age. The median time of survival from the time of diagnosis is about 3 years. Lung transplantation used to be the only therapeutic approach that improved survival, whereas recent studies have also led to the FDA approval of two drugs, nintedanib and pirfenidone, for the treatment of IPF. Although the etiology of IPF was unclear recently, research into the pathology has identified several key pathways and mechanisms of disease. It is now recognized that IPF is a result of repeated injuries of the alveolar epithelium with dysregulation of cellular homeostasis and aberrant wound repair. Repeated microinjuries to the alveolar epithelial cells lead to a fibrotic environment with recruitment of fibroblasts that differentiate into myofibroblasts, cells that have features of both fibroblasts and smooth muscle cells. The myofibroblasts secrete collagen, which is deposited in a disorganized manner within the extracellular matrix. The fibroblastic foci are a characteristic feature of IPF and are composed of clusters of fibroblasts and myofibroblasts (113).

Lung tissues from IPF patients showed evidence of increased p62 and increased number of autophagosomes (5, 82). It has been suggested that autophagy is involved in the development of pulmonary fibrosis by altering the stress and injury responses of epithelial cells, fibroblasts, and myofibroblasts. During pulmonary fibrosis tissue injury occurs and fibroblasts are activated to produce extracellular matrix to facilitate wound injury. It is known that the extracellular matrix such as type 1 collagen induces stress responses in activated fibroblasts and subsequently causes apoptosis. This is a mechanism to limit the fibrogenic potential of the activated fibroblasts. However, IPF fibroblasts are resistant to this process and are apoptosis resistant and highly proliferative.

Cultured fibroblasts from lungs of IPF patients on polymerized collagen exhibited decreased LC3-II and increased p-AKT and p-mTOR (79). Inhibition of mTOR using rapamycin or AKT inhibition using a dominant negative mutant plasmid increased LC3-II and sensitized the IPF fibroblasts to polymerized collagen-induced cell death (79). Moreover, mTOR activity was high and LC3-II level was low in cells within the fibroblastic foci of patients with IPF (79). Fibroblasts from IPF lungs showed a downregulated expression of BECN1 in fibroblasts while the antiapoptotic protein Bcl-2 expression was high (92). Fibroblasts from IPF lung exhibited decreased FoxO3A and LC3B levels and resistance to type I collagen matrix-induced cell death (41). Restoration of FoxO3A activity or LC3B in IPF fibroblasts with expression vectors sensitized the fibroblasts to collagen matrix-induced cell death.

IPF had also been linked to mitophagy deficits, with marked accumulation of dysfunctional mitochondria in alveolar type II epithelial cells (AECIIIs) from lungs of IPF patients (11). Ultrastructural and biochemical studies demonstrated that mitochondria in IPF lungs are enlarged and dysmorphic with impaired Complex I and IV activity (11). Furthermore, IPF lung had higher levels of LC3 that colocalize to the mitochondrial marker ATP synthase, higher levels of p62, and lower levels of LAMP1 (a lysosome marker), indicating an impairment in the autophagy flux leading to accumulation of dysfunctional mitochondria (11).

Alveolar macrophages also play an important role in the pathogenesis of pulmonary fibrosis by generating ROS and are a source of TGFβ, a major mediator of fibrosis (114). Akt1 has been found to be one of the important factors regulating mitophagy and macrophage production of TGFβ, as macrophage-specific Akt1 knockout mice exhibit decreased mitophagy and increased macrophage apoptosis, decreased TGFβ, and protection from bleomycin-induced pulmonary fibrosis (57).

Microinjuries including endoplasmic reticulum (ER) stress, TGFβ, bleomycin, aging, and surfactant genetic defects have all been shown to perturb autophagy. Here we summarize the evidence for the role of autophagy in each of these aspects of pulmonary fibrosis (Table 3, Fig. 4). ER stress is a potential...
TGFβ/H9252

ER stress

- LC3 and ubiquitinated protein immunoreactive staining and P62 protein levels are increased in IPF lung tissues.
- ER stress (tunicamycin) increases LC3-II
- Tunicamycin intratracheal injection combined with infection with MHV68 (a murine gammaherpesvirus homologous to EBV) (an in vivo ER stress model) led to lung fibrosis.
- PINK1 is downregulated in AECIIs of IPF patient lung, and in mice exposed to ER stress.

TGFβ/H9252

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Table 3. Autophagy and mitophagy in pulmonary fibrosis

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<tbody>
<tr>
<td>ER stress</td>
<td>LC3 and ubiquitinated protein immunoreactive staining and P62 protein levels are increased in IPF lung tissues.</td>
<td>Autophagy induction by mTOR inhibitor Torin1 suppresses ER stress-induced cell senescence in HBEC. Le3 and Atg5 silencing exacerbate ER stress-induced senescence.</td>
<td>Araya et al. 2013</td>
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<td>TGFβ</td>
<td>TGFβ decreased senescence and increased differentiation of fibroblasts from explanted human lungs.</td>
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<td>TGFβ increased LC3-II and decreased p62 in lung fibroblasts.</td>
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<td>TGFβ increases LC3-II and p62 in Beas-2b cells.</td>
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<td>In normal human lung fibroblasts, TGFβ suppresses autophagic flux as assessed by measurement of LC3-II levels with and without chloroquine. Furthermore, many autophagy genes are regulated by TGFβ.</td>
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<td>Bleomycin</td>
<td>Bleomycin (a single intratracheal instillation of 2.5 U/kg of bleomycin)-treated rats (28th day) exhibited higher fibrotic markers including α-SMA, fibronectin, collagens I and III, together with higher p-mTOR, lower BECN1 and lower LC3-II.</td>
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<td>In mice, bleomycin (3.0 U/kg, intratrachea instillation) on 28th day increases LC3-II, BECN1, p-mTOR, and p62; decreased VPS34 and p-Bcl-2. IL-17A neutralizing antibodies attenuate bleomycin-induced lung fibrosis, p-mTOR, and p62 in mice and further increase BECN1, VPS34, and p-Bcl-2.</td>
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<td>Bleomycin-induced fibrosis is exaggerated by Mmp19 knockout in mice. Mmp19+/− fibroblasts exhibited diminished ATG4C proteins.</td>
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<td>Not assessed</td>
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<td>Aging</td>
<td>There is an age effect in bleomycin induction of LC3 puncta, LC3-TOM20 colocalization, lipofuscin, and collagen deposition in mice.</td>
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<td>There is an age effect on mitochondrial dynamics, ER stress, susceptibility to MHV68-induced mitochondrial dysfunction, Pink1 expression, and fibrosis in AECIIs.</td>
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<tr>
<td>Familial</td>
<td>Lung epithelia from patient with SFTPC I73T heterozygous mutation showed increased autophagic vacuoles. Exogenous expression of I73T in HEK cells increased autophagosomes, increased PARKIN and mitochondrial MTCO2, and increased LC3-II response to bafilomycin, while attenuating degradation of aggregate-prone protein substrates. HPS is an autosomal recessive disorder and some patients develop lung fibrosis, with surfactant accumulation, lysosomal stress, and AECII apoptosis. LC3B and p62 are increased in HPS1/2 mice and human patients’ lungs. Transgenic mice carrying misfolded variant α1-antitrypsin Z exhibited increased LC3-II in the lung, collagen deposition, and leukocyte infiltration.</td>
<td>Not assessed</td>
<td>Hawkins et al. 2015</td>
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<td>TGFβ overexpression decreased HPS1 knockdown-induced p62 accumulation in A549 cells. CBZ and Flu, or overexpression of transcription factor TFEB, decreased lung collagen deposition and leukocyte infiltration in α1-antitrypsin Z mice.</td>
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<td>Ahuja et al. 2015</td>
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<tr>
<td>TGFβ</td>
<td>Nintedanib, an inhibitor of multiple tyrosine kinases, increased autophagic flux in primary human lung fibroblast from explants of patient lungs, downregulated fibronectin and collagen 1a1 production and inhibited TGFβ signaling.</td>
<td>Becn1 silencing did not change nintedanib effects on fibronectin and collagen 1a1. Lc3 silencing further decreased fibronectin and collagen 1a1, implying that even though nintedanib enhances autophagic flux and attenuates fibrosis, the endogenous LC3 promotes fibrosis when nintedanib is present.</td>
<td>Rangarajan et al. 2015</td>
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<td>Bleomycin</td>
<td>Mice with bleomycin-induced injury had increased Pink1, Parkin, and LC3-II expression in alveolar macrophages.</td>
<td>Parkin knockout mice were protected against collagen deposition and destruction of lung architecture after administration of bleomycin.</td>
<td>Larson-Casey et al. 2016</td>
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Inducer of autophagy, through mechanisms involving PERK-eIF2α-ATF4-CHOP cascade to activate transcription of autophagy genes, as well as IRE1-JNK-mediated Bcl-2 phosphorylation to regulate dissociation of BECN1 from Bcl-2 and activation of VPS34 (6, 9, 20, 21, 29, 80). Autophagy induction by mTOR inhibitor Torin1 suppressed and silencing autophagy gene Lc3 or Atg5 exacerbated ER stress-induced senescence in HBECs (5). Stimulation of ER stress in A549 lung epithelial cells with tunicamycin caused depolarization of mitochondria and apoptosis. This change was exacerbated when autophagy was inhibited with bafilomycin A1, indicating that autophagy ameliorates the deleterious effect of ER stress on mitochondrial homeostasis (11). In vivo ER stress by tunicamycin intratracheal injection and injection with MHV68 (a murine gammaherpesvirus homologous to EBV) led to lung fibrosis and downregulation of PINK1. The essential role of PINK1 has been demonstrated in Pink1 knockout mice where increased p62, enlarged swollen mitochondria, and decreased complex I and IV activities are evident in the lung, with fibrosis in response to MHV68 (11).

TGFβ decreases senescence and increases differentiation of lung fibroblasts from explanted human lungs; this is associated with increased LC3-II levels and decreased p62 levels. Silencing Lc3b and Atg5 enhanced, and mTOR inhibitor Torin1 suppressed, TGFβ-induced expression of α-smooth muscle actin (α-SMA) and type I collagen in fibroblasts, consistent with an inhibitory role of autophagy against fibroblast differentiation (5). TGFβ also induced human lung fibroblast MRC-5 cells differentiation with increased levels of fibronectin, collagen-1 and α-SMA (82). In response to TGFβ, mTOR is activated, LC3-II is decreased, and p62 is increased. Silencing of Becn1 or Lc3 resulted in enhanced TGFβ-induced differentiation with even higher fibronectin and α-SMA (82). Conversely mTOR inhibitor rapamycin attenuated TGFβ-induced differentiation (82). Although PINK1 was shown to be lower in AECII and IPF patients (11), it was found to be higher in IPF lung mitochondria (83). In normal human lung fibroblasts (NHLFs), TGFβ suppresses autophagic flux as assessed by measurement of LC3-II levels with and without chloroquine. Furthermore, many autophagy genes, including Atg4c, Atg5, Atg7, Atg16l1, Atg16l2, Gabarap, Parkin, Pink1, p62, and Ulk2 are regulated by TGFβ (99). In Beas-2b cells, TGFβ increased PINK1, mitochondrial fragmentation, and colocalization of PINK1 with LC3 (83). Silencing Pink1 enhanced cell death in response to TGFβ (83). Nintedanib, an FDA-approved drug for treatment of IPD patients and an inhibitor of multiple tyrosine kinases, has been shown to induce autophagy, downregulate fibronectin and collagen 1a1 production, and inhibit TGFβ signaling in primary human fibroblasts. Becn1 silencing did not change nintedanib effects on fibronectin and collagen 1a1, indicating nintedanib effects are BECN independent. Paradoxically, Lc3 silencing further decreased fibronectin and collagen 1a1, indicating nintedanib effects are BECN independent. Bleomycin-treated rats and mice both exhibited higher p-mTOR, while apparently opposite changes were seen in BECN1 and LC3-II (16, 74). Inhibition of mTOR protected against bleomycin-induced fibrosis in mice (82). Bleomycin has also been reported to elevate IL-17A in animals (74). Blocking IL-17A with neutralizing antibodies decreased fibro-
sis, p62, and p-mTOR, increased BECN1, VPS34, and p-Bcl-2 (74). Matrix metalloproteinase 19 (MMP19) deficiency exacerbated bleomycin-induced lung fibrosis in mice, this is associated with a diminished ATG4C protein, together with dysregulation of several profibrotic pathways including ECM formation, migration, and proliferation in \textit{Mmp19}\textsuperscript{-/-} mice (45). Future studies will need to investigate the mechanisms and consequences of ATG4C decrease in lung fibrosis. Interestingly, as in ER stress and TGF\textbeta\textsuperscript{-mediated fibrosis studies, \textit{Pink1}-deficient mice are also more susceptible to developing pulmonary fibrosis in a bleomycin model, consistent with a protective role of mitophagy (11, 83).

Aging is a risk factor for IPF. Autophagy appears to decrease and mitochondrial dysfunction increases with age, prompting the research to determine whether autophagy and/or mitochondrial dysfunction may contribute to age-related IPF. In mice, mitochondrial function is impaired in old compared with young mice, as demonstrated by enlarged mitochondria in AECII\textsuperscript{s} (11). Infection with MHV68 (a murine gamma herpes-virus) induced ER stress in AECII\textsuperscript{s} and lung fibrosis in aging mice, whereas young mice are protected (11, 108). Age-dependent susceptibility to bleomycin-induced lung fibrosis has also been demonstrated and associated with age-dependent bleomycin induction of LC3 puncta, LC3-TOM20 colocalization, lipofuscin, and collagen deposition in mice (99).

In addition to aging and environmental stress, genetic mutations were associated with familial forms of IPF. For example, mutations of surfactant protein C-induced familial IPF has been found to be associated with autophagy and mitophagy deficits. The mutation of threonine for isoleucine at codon 73 (I73T) in the human surfactant protein C (hSP-C) gene (SFTPC) accounts for a significant portion of SFTPC mutations associated with interstitial lung disease (ILD). Biopsy from a patient with heterozygous I73T mutation showed enlarged double membrane vesicles containing amorphous and organellar debris. HEK cells expressing exogenous I73T mutant protein exhibited increased autophagosomes, increased LC3-II in response to bafilomycin, increased PARKIN and mitochondrial MTCO2, but an attenuated degradation of aggregate-prone protein substrates (34). Similarly, lung biopsies from patients with Hermansky-Pudlak syndrome (HPS)-associated pulmonary fibrosis and \textit{Hps1/2}\textsuperscript{-/-} mutant mice exhibited increased LC3B-II, p62, ATG5, ATG7, TFE3, and LAMP2 levels. \textit{Hps1} knockout in A549 cells increased LC3B-II and p62, decreased LC3B colocalization with LAMP1, and induced apoptosis. Overexpression of \textit{Lc3} decreased \textit{Hps1} knockdown-induced p62 accumulation (3). Similarly, the pharmacological agents carbamazepine (CBZ) and fluphenazine (Flu), which are autophagy inducers, or nasal instillation of HDAd-TFE3, which regulates the expression of autophagy and lysosomal genes, decreased lung collagen deposition and leukocyte infiltration in transgenic mice carrying misfolded variant a1-antitrypsin Z (36).
Together, there was clear evidence of autophagy perturbation in human IPF patients and rodent and fibroblast models of IPF induced by ER stress, TGFβ, bleomycin, aging, and genetic factors. Pharmacological agents and knockout or overexpression of autophagy genes strongly impacted fibrosis development. In the studies discussed above, there was generally a protective role of autophagy and mitophagy in structural cells such as epithelial cells, fibroblasts, and myofibroblasts against ER stress or TGFβ-induced senescence and mitochondrial dysfunction, bleomycin-induced fibrosis, and lung collagen deposition in mice expressing misfolded proteins. However, there appears to be a cell-specific effect of autophagy and mitophagy in the development of pulmonary fibrosis. For example in macrophages, induction of mitophagy in profibrotic conditions promotes apoptosis resistance, production of TGFβ, and development of fibrosis. The major highlights of autophagy/mitophagy in pulmonary fibrosis are:

- In IPF patient lungs with ER stress, LC3 and P62 protein levels are increased, while PINK1 levels are decreased. Autophagy/mitophagy is protective.
- In fibroblasts from explanted human lungs, TGFβ increased LC3-II and decreased P62. TGFβ also induces fibronectin, collagen-1, and α-SMA in human lung fibroblast. Autophagy/mitophagy is protective.
- In human IPF lung, mitochondria exhibit higher PINK1. In Beas-2b cells, TGFβ increased PINK1 and colocalization of PINK1 with LC3. Autophagy/mitophagy is protective.
- In mice exposed to bleomycin, LC3-II, BECN1, p-mTOR, and p62 levels are increased, while VPS34 and p-Bcl-2 levels are decreased. However, in rats exposed to bleomycin, BECN1 and LC3-II levels are decreased. Autophagy/mitophagy is protective.

**Autophagy and Mitophagy in Pulmonary Hypertension**

Pulmonary hypertension (PH) is a disease characterized by elevations of the pulmonary arterial pressures and right ventricular failure. The pathogenesis of PH is complex and involves inflammation and dysfunction of the endothelial cells and excessive proliferation of pulmonary artery smooth muscles cells (PASMCs) leading to obliteration of the vascular lumen. Lungs from human patients with PH showed increased LC3B levels and autophagosomes prompting study of the role of autophagy in PH (61). In addition, mitochondrial dysfunction is also known to be involved in the pathogenesis of PH as mitochondrial fragmentation is increased in the PASMCs of patients with pulmonary arterial hypertension (PAH), suggesting insufficient clearance of dysfunctional mitochondria by mitophagy (73) (Table 4, Fig. 5).

As with other pulmonary diseases discussed above, autophagy also appears to play a dual role depending on the model of PH used in animal models. The most commonly used animal models of PH are the chronic hypoxia and the monocrotaline models (100). In rats, although hypobaric hypoxia for 2 wk did not change LC3B-II, 17β-estradiol increased LC3-II in the lungs exposed to hypobaric hypoxia and attenuates hypoxic PH via estrogen receptor-mediated inhibition of pulmonary vascular remodeling (55). LC3B is increased in the lung in mice exposed to hypoxia for 3 wk and increased in pulmonary artery endothelial cells (PAECs) and PASMCs exposed to hypoxia for 24 h. Lc3b-deficient mice had exacerbated PH in response to hypoxia, suggesting that autophagy is protective against chronic hypoxia-induced PH (61). In contrast, in a monocrotaline rat model of PH there was an increase in LC3B-II and a decreased in p62 in the lung. Inhibition of autophagy by chloroquine further increased LC3B-II and restored p62, concurrent with an attenuation of PH, right ventricular hypertrophy, and vascular remodeling (68). In vitro chloroquine or siRNA of Atg5 inhibits proliferation in rat PASMCs (68).

In addition to the chronic hypoxia and the monocrotaline models, persistent PH (PPHN) in newborn lambs with intrauterine ductus arteriosus constriction induced PH. PAECs isolated from PPHN lambs exhibited increased LC3-II but no further increase in response to serum starvation induction. Pharmacological agents chloroquine or 3-MA or Beclin-1 knockdown decreased dihydroethidium (DHE) fluorescence after ATP simulation and improved angiogenesis and scratch recovery, indicating that autophagy impairs angiogenesis and recovery in these PAECs (107). In the rat PAH model induced by a single subcutaneous injection of Sugen-5416 on day 1 and exposed to 3 wk of normobaric hypoxia, followed by reexposure to normoxia for 5 additional weeks, there was an increase of autophagic vacuoles and LC3A/B-II in the right ventricle (RV), but not in the left ventricle. Treatment of the animals with dehydroepiandrosterone (DHEA), a 17-ketosteroid, for 5 wk (from weeks 3–8) decreased LC3A/B-II and diastolic heart failure phenotypes (90). In a mouse PAH model induced by pulmonary artery constriction, increased LC3 and p62 were associated with RV failure, and folic acid decreased these increases and attenuated RV failure (86). Although increase of LC3-II correlated with PAH, neither the experiments with rat PAH model induced by Sugen hypoxia-normoxia nor the experiments with mouse PAH model induced by pulmonary artery constriction examined whether autophagy is protective or detrimental in these models.

Mitophagy and mitochondrial dynamics are interrelated processes that maintain mitochondrial homeostasis. Fission protein DRP1 contributes to quality control by enabling removal of damaged mitochondria by mitophagy, while fusion protein MFN1 and MFN2 help mitigate mitochondrial stress by complementation of damaged DNA, proteins, and lipids between mitochondria (121). As discussed before, inhibition of mitochondrial fission by Mdivi-1 increased cellular senescence in human lung fibroblasts exposed to CSE (2). In idiopathic human PAH lungs, the Ser616-phosphorylated active form of DRP1 is elevated in blood vessels and HIF1α is activated in lungs and PASMCs (73). In CoCl2 and chronic hypoxia-induced PH in rats, inhibition of DRP1 by Mdivi-1 decreased PH (73). Opposite to DRP1, human PAH PASMC and PASMC of rat exposed to chronic hypoxia+Sugen-5416 or monocrotaline have less MFN2 (94). Inhibition of fission by Mdivi-1 or overexpression of MFN2 by intravenous Ad-Mfn2 attenuated PAH in rats exposed to chronic hypoxia+Sugen-5416 and appear to have additive effects (94). In the above studies, fission and fusion have been clearly linked to PH, but whether mitophagy plays a role in mediating some of the protective or detrimental process was unclear. The evidence suggesting that excessive mitophagy may contribute to PH came from a
study that showed that both loss of Ucp2 and intermittent hypoxia (IH) led to increased PINK1, PARKIN, and LC3B-II in the lung, and even higher in Ucp2 knockout mice exposed to IH. Silencing PINK1 increased RV systolic pressure after IH with wild-type UCP2 but decreased the increase of RV systolic pressure and RV hypertrophy in Ucp2 knockout after IH, thus indicating a protective role of PINK1 in response to IH but a detrimental role in response to IH when Ucp2 is impaired (33).

There is conflicting evidence on the role of autophagy and mitophagy in development of PH. This may partially be due to the fact that clinical diagnosis of PH is determined by hemo-

Table 4. Autophagy and mitophagy in pulmonary hypertension

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<tr>
<th>Experimental Models</th>
<th>Evidence of Altered Autophagy</th>
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<tr>
<td>Human PH</td>
<td>Lungs from human patients with pulmonary hypertension showed increased LC3B levels and autophagosomes prompting study of the role of autophagy in PH. Mitochondrial dysfunction is also known to be involved in the pathogenesis of pulmonary hypertension as mitochondrial fragmentation is increased in the PASMCs of patients with PAH.</td>
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<td>Rat chronic hypoxia</td>
<td>Hypobaric hypoxia for 2 wk did not change LC3B-II. 17β-Estradiol increased LC3-II in the lungs exposed to hypobaric hypoxia, and attenuates hypoxic pulmonary hypertension via estrogen receptor-mediated inhibition of pulmonary vascular remodeling.</td>
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<td>Mouse chronic hypoxia</td>
<td>LC3B is increased in the lung in mice exposed to hypoxia for 3 wk, and increased in PAECs and PASMC exposed to hypoxia for 24 h. Le3b-deficient mice had exaggerated PH in response to hypoxia, suggesting that autophagy is protective against chronic hypoxia-induced PH.</td>
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<tr>
<td>Rat monocrotaline</td>
<td>LC3B-II is increased and p62 is decreased and a decreased in p62 in the rat lung in response to monocrotaline. Inhibition of autophagy by chloroquine further increased LC3B-II and restored p62, concurrent with an attenuation of pulmonary hypertension, right ventricular hypertrophy, and vascular remodeling. In vitro chloroquine or siRNA of Atg5 inhibits proliferation in rat PASMCs.</td>
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<tr>
<td>Lamb</td>
<td>In a model of PPHN in newborn lambs with intraterine ductus arteriosus constriction, PAEC isolated from PPHN lambs exhibited increased LC3-II but no longer responded to serum starvation induction. Chloroquine, 3-MA, or Beclin1 knockdown decreased DHE fluorescence after ATP simulation, improved angiogenesis and scratch recovery, indicating that autophagy impairs angiogenesis and recovery in these PAEC.</td>
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<td>Rat PAH induced by Sugen hypoxia-normoxia</td>
<td>A single subcutaneous injection of Sugen-5416 on day 1 and exposed to 3 wk of normobaric hypoxia, followed by reexposure to normoxia for 5 additional weeks elevated LC3-II in the RV. DHEA decreased LC3-II and improved diastolic heart failure phenotypes.</td>
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<td>Mouse PAH induced by pulmonary artery constriction</td>
<td>Increased LC3 and p62 were associated with RV failure. Folic acid decreased the raise in LC3 and p62 and attenuated RV failure.</td>
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<td>Human PH, CoCl2 and chronic hypoxia-induced PH in rat</td>
<td>In idiopathic human PH lungs, Ser616 phosphorylated active form of DRP1 is elevated in blood vessels, HIF1a is activated in lungs and PASMCs. In CoCl2 and chronic hypoxia-induced pulmonary hypertension in rats, inhibition of DRP1 by Mdivi-1 decreased pulmonary hypertension.</td>
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<tr>
<td>Human PAH, female rats exposed to chronic hypoxia+Sugen-5416 or monocrotaline</td>
<td>PASMCs from human PAH and rat exposed to chronic hypoxia+Sugen-5416 or monocrotaline have less Mfn2 and more fragmented mitochondria. Ad-Mfn2 and Mdivi-1 attenuated PAH phenotypes in rats exposed to hypoxia+Sugen-5416, and their effects appear to be additive.</td>
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<tr>
<td>human PAH, mice exposed to IH</td>
<td>PINK1 is elevated in human PAH and in mice in response to either Ucp2 knockout or IH. Silencing PINK1 increased right ventricle systolic pressure after IH, but decreased the increase of right ventricle systolic pressure and right ventricle hypertrophy induced by Ucp2 knockout after IH, thus indicating a protective role of PINK1 in response to IH but a detrimental role in response to IH when Ucp2 is impaired.</td>
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Detrimental effect of autophagy on pulmonary hypertension

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<td>Haslip et al. 2015</td>
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dynamic characteristics and it encompasses a wide variety of groups with diverse pathological mechanisms (98). Further complicating the scenario is the fact that so far divergent methods have been used to assess autophagy and mitophagy. Most of the studies only touch on part of the autophagy process; due to technical limitations, there are limitations in understanding of the participation of more than 40 proteins in the process and of the even more intricate regulation of the process. Based on current studies, it does not appear likely that a universal theory on the role of autophagy and mitophagy in PH would exist. The major highlights of autophagy/mitophagy in PH are

- In human patients with PH, LC3B levels and autophagosomes are increased. The role of autophagy/mitophagy is not known.
- In rats with chronic hypoxia-induced PH, LC3B levels are unchanged. Upregulation of LC3-II by estradiol is protective.
- In mice with chronic hypoxia-induced PH, LC3B is increased in the lung. Autophagy/mitophagy is protective.
- In rats with monocrotaline-induced PH, LC3-II is increased and p62 is decreased in lung. Autophagy/mitophagy is detrimental.
- In newborn lambs with PPHN with intrauterine ductus arteriosus constriction, LC3-II levels are increased in PAECs. Autophagy/mitophagy is detrimental.
- In rats with PH induced by a single subcutaneous injection of Sugen-5416 followed by hypoxia and normoxia, LC3-II levels are elevated in the RV. Autophagy/mitophagy is detrimental.
- In mice with PAH due to pulmonary artery constriction, LC3 and p62 levels are elevated in RV, associated with RV failure.
- In mice exposed to IH, PINK1 is elevated. Autophagy/mitophagy is either protective or detrimental depending on the Ucp2 status in the animals.

**Conclusion**

Significant studies in the past 20 years have clearly demonstrated the involvement of autophagy and mitophagy in maintaining cellular homeostasis and clearance of toxic proteins and organelles. Both increases and decreases of proteins involved in autophagy and mitophagy have been observed in pulmonary diseases in human biopsies and in animal models. In the cases of ALI, COPD, and PH, autophagy and mitophagy have been shown to play either a protective or a detrimental role, as assessed by using both pharmacological and genetic manipulations that up- or downregulate autophagy activities or protein levels. In most of the IPF studies, autophagy and mitophagy appear to be protective against IPF. But the diversity in autophagy and mitophagy effects in pulmonary fibrosis may be cell type specific. It is evident from the literature that the etiology of the stressors (acute vs. chronic, endogenous vs. exogenous), the mechanisms of disease progression (cell death, cell differentiation, or cell senescence), and the cell types involved all play significant roles in determining whether autophagy and mitophagy will be upregulated or downregulated and whether autophagy and mitophagy will be protective or detrimental to cellular health.

Furthermore, more standardized assessment methods are needed to compare experimental observations, especially with regard to autophagy activities (53). Currently, the ability to monitor and quantify mitophagy in the tissues is limited. LC3-based fluorescent probes may have a high rate of false positive signals due to the transient nature of the interaction between autophagosome and mitochondria. Newly developed pH-dependent fluorescent probes such as Keima may provide additional means to monitor mitophagy. Keima allowed accurate assessment of protein location in the mitochondria (pH ~8.0) or the lysosome (pH ~4.5). Transgenic mice that express mt-Keima provide robust assessment of in vivo mitophagy under a wide range of experimental conditions (101).
Last but not least, cautions need to be exercised even when interpreting pharmacological and genetic manipulations of autophagy activities, since nonautophagy targets may be perturbed and gene redundancy or compensatory changes may be in place when these manipulations are conducted. Despite these caveats, it is clear some of these pharmacological and genetic manipulations attenuate disease development in the lung and present therapeutic potentials that warrant further investigations.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
S.A. and I.Z. prepared figures; S.A. and P.M. drafted manuscript; S.A., P.M., and I.Z. approved final version of manuscript.

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