Endoplasmic reticulum stress enhances fibrotic remodeling in the lungs

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Evidence of endoplasmic reticulum (ER) stress has been found in lungs of patients with familial and sporadic idiopathic pulmonary fibrosis. We tested whether ER stress causes or exacerbates lung fibrosis by (i) conditional expression of a mutant form of surfactant protein C (L188Q SFTPC) found in familial interstitial pneumonia and (ii) intratracheal treatment with the protein misfolding agent tunicamycin. We developed transgenic mice expressing L188Q SFTPC exclusively in type II alveolar epithelium by using the Tet-On system. Expression of L188Q SFTPC induced ER stress, as determined by increased expression of heavy-chain Ig binding protein (BiP) and splicing of X-box binding protein 1 (XBP1) mRNA, but no lung fibrosis was identified in the absence of a second profibrotic stimulus. After intratracheal bleomycin, L188Q SFTPC-expressing mice developed exaggerated lung fibrosis and reduced static lung compliance compared with controls. Bleomycin-treated L188Q SFTPC mice also demonstrated increased apoptosis of alveolar epithelial cells and greater numbers of fibroblasts in the lung. With a complementary model, intratracheal tunicamycin treatment failed to induce lung remodeling yet resulted in augmentation of bleomycin-induced fibrosis. These data support the concept that ER stress produces a dysfunctional epithelial cell phenotype that facilitates fibrotic remodeling. ER stress pathways may serve as important therapeutic targets in idiopathic pulmonary fibrosis.

S100A4 | unfolded protein response

Idiopathic pulmonary fibrosis (IPF) is the most common and severe form of idiopathic interstitial pneumonia (IIP). IPF is characterized by dyspnea, decreased exercise tolerance, and progression to respiratory failure as a result of ongoing fibrotic remodeling of the distal lung parenchyma (1). Although the cause of IPF remains unknown, recent cases of familial interstitial pneumonia (FIP) have begun to shed light on potential pathogenic mechanisms. FIP, which represents a small proportion of IIP, is defined as two or more biologically related family members with a diagnosis of IIP (2, 3). In FIP, 85% of biopsy-proven cases have pathology consistent with usual interstitial pneumonia, the pathological equivalent of IPF (2). In 2002, we reported a large FIP family with a heterozygous mutation in the carboxyl-terminal region of surfactant protein C (SFTPC) (4). This exon 5 +128 T→A transversion results in substitution of glutamine for leucine at amino acid 188 (L188Q) in the carboxyl-terminal region of the pro-SFTPC precursor protein (pro-SP-C). This region of pro-SP-C is known as the BRICHO5 domain, which is essential for protein folding and processing. Mutations in proteins containing BRICHO5 domains are linked to several degenerative and proliferative diseases through mechanisms related to altered posttranslational protein processing (5).

In cultured alveolar epithelial cells (AECs), expression of L188Q SFTPC results in a precursor protein that cannot be folded properly in the endoplasmic reticulum (ER), leading to ER stress and activation of the unfolded protein response (UPR) (4, 6, 7). We evaluated lung tissue from individuals with FIP who carried the L188Q mutation and found up-regulation of ER stress markers in the alveolar epithelium (6). Subsequently, we studied lung tissue from individuals with FIP and sporadic IPF without mutations in SFTPC and noted that ER stress markers were also present in the alveolar epithelium in the same pattern (6), a finding confirmed by other investigators (8). Therefore, it appears that ER stress and UPR activation are common features of the alveolar epithelium in IPF. These findings raise a number of important and unresolved issues, including whether ER stress causes or exacerbates fibrosis, and, if so, how ER stress in the epithelium regulates fibrotic remodeling. To address these issues, we developed a transgenic mouse model by using the Tet-On system in which mutant L188Q SFTPC can be inducibly expressed in type II AECs in the adult mouse. In this model, expression of L188Q SFTPC in type II AECs resulted in ER stress and UPR activation; however, no fibrosis was seen in the absence of a second profibrotic stimulus. In contrast, enhanced bleomycin-induced lung fibrosis was found in mice expressing L188Q SFTPC, as well as in mice treated with the ER stress-inducing agent tunicamycin, in association with increased epithelial cell death and increased fibroblast accumulation. Our data support the idea that dysfunctional type II AECs facilitate lung fibrosis through increased susceptibility to injury, leading to excessive and disregulated remodeling.

Results

Expression of Mutant L188Q SFTPC Induces ER Stress in AECs. We generated an expression construct under control of the (tet-O)7 promoter carrying human SFTPC (six exons and introns) with an exon 5 +128 T→A substitution and insertion of an 11-aa myc tag in exon 2 that is expressed in the mature peptide. We also created a construct in which the murine SFTPC promoter drives the reverse tetracycline transactivator (rtTA), msFTPC::rtTA. Coexpression of msFTPC::rtTA and (tet-O)→L188Q SFTPC-myc led to doxycycline (Dox)-inducible expression of mutant pro-SP-C in A549 cells (Fig. S14). Next, we purified msFTPC::rtTA and (tet-O)→L188Q SFTPC-myc constructs, as well as a construct expressing a tetracycline-controlled transcriptional silencer (iTS) under the murine SFTPC promoter (msFTPC::iTS) to prevent


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basal leakiness of transgene expression (9) (Fig. S1B) and performed simultaneous pronuclear microinjection of the three DNA constructs into fertilized oocytes from C57BL/6j mice. This strategy leads to tandem integration of constructs into a single integration site in the majority of cases (10). We identified 4 founders (of 52 potential founders) that possessed all three constructs (Fig. S2A). Transgenic mice developed normally, appeared healthy, and bred well, transmitting all three transgenes to their progeny. Adult mice were given Dox in drinking water, and RNA was isolated from whole-lung tissue. Evaluation by real-time RT-PCR revealed that induction of L188Q SFTPC expression did not affect native pro-SP-C mRNA levels and was ~10-fold less than expression of the endogenous gene in the highest expressing founder line (Fig. S2B). Furthermore, mutant L188Q SFTPC was expressed for up to 6 mo with colocalized exposure to Dox (Fig. S2C). Immunohistochemistry (IHC) for the myc-tagged transgene demonstrated that mutant L188Q pro-SP-C expression localized exclusively to cells in corners of alveoli, consistent with the site and appearance of type II AECs (Fig. S2D–F). No transgene expression was identified in the absence of Dox treatment.

Formalin-fixed, paraffin-embedded lung tissue sections were immunostained for the ER stress markers heavy-chain Ig binding protein (BiP) and X-box binding protein 1 (XBP1). BiP is a protein chaperone that assists with protein folding and increases during ER stress, and protein accumulation and ER stress. XBP1 is a potent transactivator of UPR gene expression, regulating a variety of cellular functions (11, 12). After administration of Dox for 1 wk, both BiP and XBP1 were found by IHC to be up-regulated in type II AECs in L188Q SFTPC mice (Fig. 1A–D). In addition, BiP protein and mRNA expression was increased in whole-lung tissue samples after Dox treatment (Fig. 1E and F). XBP1 is normally expressed as a full-length unspliced mRNA. When protein accumulation induces ER stress, unspliced XBP1 mRNA undergoes inositol requiring enzyme 1 (IRE1)-dependent splicing, yielding a spliced XBP1 isoform that permits translation of the biologically active XBP1 protein. The ratio of the spliced isoform to total XBP1 mRNA can be used as a marker of the IRE1-mediated ER stress response (13). Dox-treated L188Q SFTPC mice had evidence of increased XBP1 splicing in lung tissue, indicative of ER stress (Fig. 1G and H). Together, these studies show that transgene expression in L188Q SFTPC mice causes ER stress localized to type II AECs.

After Dox treatment for 1 wk, we isolated primary type II AECs to determine trafficking of the myc-tagged mutant pro-SP-C. In type II AECs from L188Q SFTPC mice, immunofluorescence for the myc tag colocalized with BiP, which is expressed exclusively in the ER (Fig. 2A–C). In contrast, myc expression did not colocalize with gialtin, which is found in the Golgi apparatus (Fig. 2D–F). Thus, it appears that mutant L188Q SFTPC is principally localized to the ER with little presence in the Golgi, suggesting that processing of mutant pro-SP-C is impaired. Consistent with this idea, we have been unable to detect mature myc-tagged SP-C in supernatant of primary type II AECs or in the airway of L188Q SFTPC-expressing mice. Consistent with studies using whole-lung tissue, type II AECs from mice with L188Q SFTPC expression exhibited greater BiP protein and mRNA expression and increased XBP1 splicing compared with type II AECs from WT mice (Fig. 2G–J). In the absence of Dox treatment, expression of BiP and XBP1 splicing in type II AECs from L188Q SFTPC mice were similar to WT cells, confirming the inducibility of ER stress in our model.

Expression of Mutant L188Q SFTPC Exacerbates Lung Fibrosis. We treated L188Q SFTPC mice for up to 6 mo with Dox to determine whether induction of ER stress was sufficient to cause lung remodeling; however, lungs from these mice appeared histologically normal despite persistent transgene expression (Fig. S3). Although L188Q SFTPC expression alone did not result in lung fibrosis, we reasoned that a second stimulus might induce greater fibrosis in L188Q SFTPC mice as a result of vulnerable type II AECs. Thus, we turned to intratracheal (i.t.) bleomycin, the most commonly used model of experimental lung fibrosis. Mutant L188Q SFTPC mice and WT controls were started on Dox and, 1 wk later, received low-dose i.t. bleomycin (0.04 unit); 3 wk later, lung fibrosis was increased in L188Q SFTPC mice, as determined by evaluation of trichrome-stained sections (Fig. 3 A–C). Furthermore, total lung collagen was greater in L188Q SFTPC mice compared with WT mice after bleomycin treatment (Fig. 3D). In previous studies, we have used S100A4 as a fibroblast marker in the lungs (14–17). Therefore, we evaluated lung sections by IHC for S100A4 expression and noted greater numbers of S100A4-positive fibroblasts in L188Q SFTPC mice compared with WT controls after bleomycin treatment (Fig. S4A and B and Fig. 3E). Similarly, L188Q SFTPC mice had greater numbers of myofibroblasts positive for α-smooth muscle actin (αSMA) in lung parenchyma after bleomycin treatment than WT controls did (Fig. S4C and D and Fig. 3F).
Additionally, we wanted to determine whether lung mechanics were different at 3 wk after bleomycin treatment in L188Q SFTPC mice. We found that static lung compliance was decreased in both WT mice and L188Q SFTPC mice treated with bleomycin compared with their respective saline-treated controls. In the bleomycin-treated group, static lung compliance was further reduced in L188Q SFTPC mice compared with WT controls (Fig. 3G). No differences were noted in static lung compliance between the two groups in the absence of bleomycin.

Although our findings indicated a probiotic effect of L188Q SFTPC expression, we wondered whether the impact of transgene expression was limited by the relatively low ratio of mutant SFTPC expression compared with the endogenous gene. Thus, we crossed L188Q SFTPC mice with mice that use the human SFTPC promoter (bSFTPC::rtTA) (18), in hopes of increasing mutant L188Q SFTPC expression. Dox-inducible mRNA expression of mutant pro-SP-C expression was limited by the relatively low ratio of mutant SFTPC expression; however, that level of transgene expression is not a limiting factor in determining the phenotype of L188Q SFTPC-expressing mice.

Expression of L188Q SFTPC Increases AEC Apoptosis After Bleomycin Treatment. Because high levels of ER stress have been linked to increased epithelial cell apoptosis (6, 7, 19), we wanted to determine whether ER stress in L188Q SFTPC mice was associated with increased AEC apoptosis. In the absence of bleomycin, TUNEL+ AECs were rare in Dox-treated L188Q SFTPC mice and were present in similar numbers compared with WT littersmates. We have previously shown that the number of TUNEL+ epithelial cells peaks in the lungs at 1 wk after i.t. bleomycin injection (17). At this time point, lung sections from L188Q SFTPC mice showed greater numbers of TUNEL+ AECs than lungs from WT controls did (Fig. 4A–C), indicative of greater AEC apoptosis. This finding indicates that AECs in L188Q SFTPC
mice are more susceptible to bleomycin-induced injury. Furthermore, active caspase-3 expression was increased in the lungs of L188Q SFTP C mice compared with WT controls (Fig. 4 D and E), suggesting that caspase-dependent cell-death pathways are selectively activated in the presence of ER stress in this model. ER stress has been linked to apoptosis through activation of ER-bound caspase-12 in mice (caspase-4 in humans) (20, 21). By Western blot analysis from whole-lung lysates, we noted that caspase-12 expression was increased in L188Q SFTP C mice at 1 wk after bleomycin treatment compared with WT controls (Fig. 4 F and G). Apoptosis mediated through expression of CCAAT/enhancer-binding protein homologous transcription factor (CHOP) has also been linked to ER stress (22), but we did not identify increased CHOP expression in our model.

Interestingly, we found that BIP mRNA expression and XBPI splicing were increased in lungs of WT mice at 1 wk after bleomycin treatment (Fig. S6). In addition, BIP expression was further increased in the lungs of L188Q SFTP C mice treated with bleomycin in addition to Dox, although XBPI splicing was unchanged. Together, these findings indicate that bleomycin itself induces ER stress in the lungs, potentially contributing to the pathological lung remodeling in this model.

Next, we asked whether L188Q SFTP C mice had altered lung inflammation after bleomycin. At 1 wk after bleomycin treatment, L188Q SFTP C and WT mice had similar total cells, neutrophils, macrophages, and lymphocytes in bronchoalveolar lavage (Fig. 5 A–D). To further evaluate inflammatory signaling in the lungs, we crossed L188Q SFTP C mice to NF-κB GFP/luciferase (NGL) reporter mice. The NGL reporter mouse expresses luciferase as a function of NF-κB activation, providing a surrogate readout of inflammatory pathway activation (23). With Dox alone, neither WT/NGL nor L188Q SFTP C/NGL mice had increased luciferase expression above baseline, and values were similar between the two groups. After bleomycin treatment, luciferase expression increased similarly in both groups (Fig. 5 B and Fig. S7). Furthermore, whole-lung luciferase levels were similar between WT/NGL and L188Q SFTP C/NGL mice after 14 d of Dox alone (23.4 ± 22.5 vs. 22.5 ± 11.7 relative light units per μg) and after 14 d of Dox + bleomycin (285.7 ± 53.4 vs. 297.3 ± 58.7 relative light units per μg). Thus, we found no evidence for differences in bleomycin-induced inflammation in L188Q SFTP C-expressing mice.

**Induction of ER Stress via Tunicamycin Administration Enhances Bleomycin-induced Lung Fibrosis.** Although our data suggest that ER stress is the underlying process by which L188Q SFTP C expression contributes to lung fibrosis, we sought to determine whether ER stress induced by other means could lead to a similar phenotype. Among ER stress-inducing agents, tunicamycin, an antibiotic that induces ER stress by blocking N-linked protein glycosylation, is the most commonly used (24). We administered i.t. tunicamycin (20 μg/mL in 100 μL of 20% DMSO diluted in PBS) to WT (C57BL/6J background) mice. IHC on sections of lungs harvested 2 d later showed that BiP and XBPI were induced most prominently in AECs after tunicamycin treatment (Fig. S8 A–D). Tunicamycin also induced BiP protein and mRNA expression and XBPI mRNA splicing in whole-lung samples (Fig. S8 E–H).

As with L188Q SFTP C expression, there was no evidence of lung fibrosis or architectural change 3 wk after a single dose of tunicamycin or after twice-weekly tunicamycin administration for 3 wk. We then tested the combination of tunicamycin followed by bleomycin treatment. WT mice received i.t. tunicamycin (or vehicle control) followed 48 h later by i.t. bleomycin (0.04 unit). At 2 wk after bleomycin injection, the tunicamycin + bleomycin group had much greater lung fibrosis than the vehicle + bleomycin group (Fig. 6 A–D). Thus, tunicamycin-induced ER stress resulted in a similar profibrotic phenotype as in the L188Q SFTP C model, strongly supporting the idea that ER stress facilitates lung fibrosis.

**Discussion**

Evaluation of IFP lung biopsies reveals evidence of ER stress in AECs lining the areas of fibrosis (6, 8), but the degree to which ER stress contributes to disease pathogenesis remains undefined. Although SFTP C mutation-associated interstitial lung disease is rare in the broad scope of IPF (25), modeling such mutations may serve as a paradigm to better understand IFP and the role of ER stress. With these studies, we have shown that expression of mutant L188Q SFTP C results in ER stress in type II AECs in vivo, leading to a vulnerable type II AEC population that is highly susceptible to the effects of bleomycin. With expression of L188Q SFTP C and resultant ER stress, AECs were more prone
to apoptosis by induction of CHOP/GADD153, via activation of caspase-12/4, and through phosphorylation of eIF2α. In our studies with L188Q SFTPC mice and in humans with IPF (6), we have not identified increased p-eIF2α. In addition, we did not note increased up-regulation of CHOP. We did, however, note increased caspase-12 levels in the lung after bleomycin treatment in mutant L188Q SFTPC mice compared with littermate controls. ER-bound caspase-12 (and its human homolog, caspase-4) has been linked to ER stress-induced apoptosis (20, 21). Previous studies by Mullageta et al. detailed that mutant forms of SFTPC, including L188Q, lead to caspase-4–mediated caspase-3 activation and cell apoptosis in vitro (7), a finding complementary to results from our in vivo model. AEC apoptosis has been linked to fibrosis in both human IPF lung biopsy samples (34) and in animal models (35), including in a recent study by Sisson et al. in which directed expression of diphtheria toxin by means of the SFTPC promoter led to type II AEC apoptosis and lung fibrosis (36). Thus, increased apoptosis may be one of the mechanisms contributing to exuberant fibrosis in L188Q SFTPC mice.

In our models, expression of L188Q SFTPC and treatment with tunicamycin induce ER stress but do not result in fibrotic remodeling, suggesting that murine type II AECs are able to manage the UPR response without obvious pathology. Rather, ER stress likely places the type II AEC population in a vulnerable state in which a second stimulus, in this case bleomycin, exerts a prominent effect, leading to enhanced lung fibrosis. Given the fact that patients with FIP, including many with SFTPC mutations, frequently present with interstitial lung disease in adulthood, one could infer that a “second hit” might be required to induce clinical disease in at-risk individuals. In FIP (and sporadic IPF), the relevant injurious stimulus likely results from an environmental exposure, but the nature of this agent(s) is currently unknown. To date, only a history of cigarette smoking and the presence of herpesvirus antigens in the lungs have been associated with clinical disease in FIP. Hopefully, future studies with L188Q SFTPC–expressing mice or other genetic models treated with relevant environmental stimuli will help clarify the potential second hits that contribute to lung fibrosis.

In summary, our studies demonstrate that ER stress in AECs predisposes the lung to greater injury and fibrosis in experimental models. These findings provide mechanistic information supporting an important role for ER stress, which is prominent in the lungs of individuals with IPF, in disease pathogenesis. ER stress and downstream UPR pathways may serve as therapeutic targets for future interventions in this devastating lung disease.

Materials and Methods

Transgenic Mice. To generate transgenic mutant L188Q SFTPC mice, three constructs were cojected at the Vanderbilt Transgenic/Eis Shared Resource facility (Fig. 5I): (i) (tet-O2)–L188Q SFTPC-myct, (ii) mSFTPC.rTAT, and (iii) mSFTPC.rTS (37). Transgenic mice expressing rTAT under the human SFTPC promoter (nSFTPC.rTAT) were obtained from Jackson Laboratories. NGL mice, which have consensus NF-κB binding sites upstream of the HSV minimal thymidine kinase promoter driving the GPP/Phucerase construct, have been described previously (23). All mice were C57Bl/6j background and entered experiments at 8–10 wk of age. All mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Animal Model and Drug Administration. Bleomycin (0.04 unit) (Bedford Laboratories) was injected i.t. in mice by intubation as previously described (17). Lungs were harvested for histology, frozen tissue, bronchoalveolar lavage,
or cell isolation as previously described (14–17). L188Q SFTP C mice and controls were maintained on normal water ad libitum until transgene activation was desired. Then, mice were given Dox (Sigma-Aldrich) in sterile water (2 g/dL with additional 2% sucrose).

Tunicamycin (Sigma) was dissolved in DMSO and diluted to 20 μg/mL in 20% DMSO diluted in PBS, and then 100 μL of solution was delivered to mice by i.t. intubation. A similar volume of 20% DMSO diluted in PBS was used as vehicle control.

**Lung Sample Processing and Analysis.** Formalin-fixed lung sections were prepared as previously described (14–16). IHG (14, 15), immunofluorescence (16, 17), TUNEL (15, 17), and bronchoalveolar lavage with cell counts (15, 17) were performed as previously described. RNA isolation, real-time RT-PCR, and Western blot analysis were performed by using standard techniques (detailed in *SI Materials and Methods*).

**Cell Culture/Isolation.** Plasmids were transfected into A549 cells with an Effectene transfection kit (Qiagen) per the manufacturer's instructions. Cells were incubated with the transfection complexes under normal growth conditions for 4 h, and then 0.5–1.0 μg/mL Dox was added to the medium. After 24 h, cells were harvested for Western blot analysis. Type II AECs were isolated as previously described (14, 16).

**In Vivo Bioluminescence and Luciferase Measurements.** Live bioluminescence imaging was performed after i.p. injection of luciferin (1 mg in 100 μL of saline), and whole-lung tissue luciferase levels were determined as previously described (23).

**Measurement of Airway Resistance and Compliance.** For airway resistance and compliance measurements, mice were anesthetized with i.p. pentobarbital, and tracheas were cannulated with a 20-gauge metal stub adapter. Each mouse was placed on a small-animal ventilator, flexiVent (SCIREQ), with 150 breaths per min and a tidal volume of 10 mL/kg of body weight. Airway resistance (cm of H2O/mL per s) and static lung compliance (using a 2-s breath pause) (mL/cm H2O) were determined with the manufacturer’s software.

**Semi quantitative Scoring and Collagen Content.** Scoring of lung fibrosis (15, 17), fibroblasts (15), and TUNEL staining (9) and determination of collagen content by hydroxyproline assay (38) were performed as previously described.

**Statistics.** Statistical analyses were performed with InStat (GraphPad Software). Differences among groups were assessed with one-way ANOVA and between pairs with Student's t test. Results are presented as mean ± SEM. P values <0.05 were considered significant.

Detailed methods are available in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Development of Mutant L188Q Surfactant Protein C (SFTPC) Mice. The plasmid containing mutant L188Q SFTPC was generated as previously described (1, 2). The L188Q SFTPC construct was cloned into the EcoRV site of a modified pBluescript II SK expression vector (pBSII KS/Asc). This vector contains a (tet-O)–CMV promoter together with bovine growth hormone polyadenylation sequences. The final plasmid, (tet-O)–L188Q SFTPC–myc–BGH;polyA, was verified by sequencing. A plasmid containing murine SFTPC–rtTA (mSFTPC–rtTA) was provided by E.E.M. To prevent basal leakiness, we used a third construct expressing a tetracycline-controlled transcriptional silencer (tTS) under control of the murine SFTPC promoter (3). We purified these three constructs with a GELase Agarose Gel-Digesting Preparation Kit (Epicentre) following the manufacturer’s instructions, and these constructs were cojected at the Vanderbilt Transgenic/ES Shared Resource facility to generate transgenic mice. All transgenic and WT mice for these experiments were in a C57BL/6 background with additional 2% sucrose. The bottles containing Dox were delivered to experimental mice by i.t. intubation. A similar volume of 20% DMSO diluted in PBS was used as a vehicle control.

Histology and Microscopy. Formalin-fixed, paraffin-embedded lung tissue was sectioned and stained with H&E or Mason’s trichrome as previously described (7, 8). Light and fluorescent microscopy was performed with an Olympus IX81 Inverted Research Microscope configured with an Olympus IX2-DSU Biological Disk Scanning Unit.

Immunostaining. Immunohistochemistry (IHC) on paraffin-embedded lung tissue sections and immunocytochemistry on cell preparations were performed with primary antibodies and standard immunoperoxidase techniques as previously described (7, 8). Immunofluorescence staining was performed on cell preparations using primary antibodies followed by appropriate fluorescent secondary antibodies (Jackson ImmunoResearch) with nuclear staining performed with DAPI using Vectashield mounting medium (Vector Laboratories) as previously described (5, 6).

Cell Culture/Isolation. Plasmids were transfected into A549 cells with an Effectene transfection kit (Qiagen) per the manufacturer’s instructions. Cells were incubated with the transfection complexes under normal growth conditions for 4 h, and then 0.5–1.0 μg/mL Dox was added to the medium for induction of transgene expression. After transient transfection for 24 h, cells were harvested for determination of gene expression by Western blot analysis. Type II alveolar epithelial cells (AECs) were isolated from adult mice using techniques as previously described (6, 7).

Lung Lavage and Cell Counts. Bronchoalveolar lavage was performed as detailed previously (5, 8). After euthanasia, three 800-μL lavages of sterile saline were performed with a 20-gauge blunt-tipped needle inserted into the trachea. Samples were centrifuged at 400 × g for 10 min, and cells were resuspended and counted manually under light microscopy with a hemocytometer. Approximately 30,000 cells from each specimen were loaded onto slides using a Cytospin 2 centrifuge (Shandon Southern Products). These slide preparations were then stained with a modified Wright stain.

RNA Isolation, Real-Time RT-PCR, and Densitometry. Total RNA from type II AECs and lung tissue was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s specifications. To remove contaminating genomic DNA, samples were incubated with DNase (Ambion) and then converted to cDNA using SuperScript II reverse transcriptase (Invitrogen). For BiP evaluation, PCR amplification and quantification were performed with SYBR Green PCR Master Mix (Ambion). Primer sequences were as follows: BiP (forward, 5′-CCT GCG TCG GTG TGT TCA AG-3′; reverse, 5′-AAG GGT CAT TCC AAG TGC G-3′) and RPL19 wrapped with foil to prevent light-induced Dox degradation, and Dox bottles were replaced twice per week.

Tunicamycin Administration. Tunicamycin (Sigma) was dissolved in DMSO and diluted to a concentration of 20 μg/mL in 20% DMSO diluted in PBS, and then 100 μL of solution was delivered to experimental mice by i.t. intubation. A similar volume of 20% DMSO diluted in PBS was used as a vehicle control.

Antibodies. The following primary antibodies were used in these investigations: pro-SFTPC precursor protein (pro-SP-C) goat polyclonal antibody (Santa Cruz Biotechnology); S100A4 rabbit polyclonal antibody (obtained from Eric Neilson, Vanderbilt University); α-smooth muscle actin (αSMA) rabbit polyclonal antibody (Abcam); heavy-chain Ig binding protein (BiP) goat polyclonal antibody (Santa Cruz Biotechnology); X-box binding protein 1 (XBP1) rabbit polyclonal antibody (Santa Cruz); giantin rabbit polyclonal antibody (Abcam); myc mouse monoclonal antibody (Invitrogen Life Technologies); myc goat polyclonal antibody (Santa Cruz Biotechnology); active caspase-3 rabbit polyclonal antibody (Millipore); β-actin rabbit polyclonal antibody (Sigma); caspase-12 rabbit polyclonal antibody (Cell Signaling Technology); and CCAT enhancer-binding protein homologous transcription factor (CHOP) rabbit polyclonal antibody (Cell Signaling Technology).

 Bleomycin Model. Bleomycin (0.04 unit; Bedford Laboratories) was injected intratracheally (i.t.) in WT and transgenic mice by using an intubation technique as previously described (5). At baseline and at designated time points after bleomycin injection, lungs were harvested for histology, frozen tissue, bronchoalveolar lavage, or cell isolation as previously described (5–8).

 Doxycycline (Dox) Administration. All L188Q SFTPC mice and controls were maintained on normal water ad libitum until transgene activation was desired. At that time, Dox (Sigma-Aldrich) was mixed in sterile water at a concentration of 2 g/dL with additional 2% sucrose. The bottles containing Dox were
with i.p. pentobarbital, and tracheas were cannulated with a 20-gauge metal stub adapter. Each mouse was placed on a small-animal ventilator, flexiVent (SCIREQ), with 150 breaths per min and a tidal volume of 10 mL/kg of body weight. Airway resistance and static lung compliance (using a 2-s breath pause) were assessed with SCIREQ manufacturer-provided software, which calculates the resistance by dividing the change in pressure by the change in flow (cm of H2O/mL per s) and compliance by dividing change in volume by change in pressure (mL/cm of H2O).

**Semi-quantitative Scoring.** Quantification of lung fibrosis on histological specimens was performed by an investigator blinded to the group using a semi-quantitative score on 10 sequential, non-overlapping fields (magnification: ×300) as previously described (5, 8). For scoring of S100A4+ and αSMA+ lung fibroblasts, slides immunostained for S100A4 or αSMA were evaluated on a 0- to 4-point scale as previously described (8): 0, no positive cells; 1, few (<3) positive cells; 2, multiple (>3) individual positive cells; 3, multiple positive cells in isolated clumps; and 4, multiple clumps of positive cells. The mean score for the 10 sequential fields represented the score for each individual specimen. For evaluation of TUNEL staining, slides were evaluated on 10 sequential, non-overlapping fields (magnification: ×600) of lung parenchyma for each specimen and were scored using a 0- to 4-point semi-qualitative scale as previously described: 0, no positive cells; 1, ≤1% of cells in field positive; 2, 1–5% of cells in field positive; 3, 5–10% of cells in field positive; and 4, 10–25% of cells in field positive (10).

**Collagen Content.** Frozen lung tissue samples were hydrolyzed in 6 M HCl and hydroxyproline content was quantitated by using a microplate assay based on Ehrlich’s reaction as previously described (11). Lung collagen content was calculated from these results as hydroxyproline accounts for ~13.3% of collagen by weight.

**Statistics.** Statistical analyses were performed with GraphPad InStat (GraphPad Software). Differences among groups were assessed with one-way ANOVA. Differences between pairs were assessed with Student’s t test. Results are presented as mean ± SEM. P values <0.05 were considered significant.

Constructs were developed to express myc-tagged mutant L188Q SFTPC in a tetracycline-dependent fashion. (A) Western blot analysis for the myc-tagged transgene in A549 cells transfected with both the mSFTPC.rtTA and tetO.L188Q SFTPC-myc constructs. β-Actin is shown as a loading control. (B) Schematic of rtTA, tTS, and L188Q myc-tagged SFTPC constructs that were designed for the purpose of generating the transgenic L188Q SFTPC mouse.

Fig. S2. Expression of mutant pro-SP-C is detected in vivo in L188Q SFTPC mice. (A) Southern blot analysis for the three transgenic constructs demonstrating identification of four founder lines: 1, 2, 45, and 52. (B) pro-SP-C-myc mRNA levels for native versus mutant pro-SP-C for each founder line exposed to Dox for 1 wk. (n = 3 per group.) (C) pro-SP-C-myc mRNA levels in whole lung from founder line 1 mice exposed to Dox for up to 6 mo. (n = 3 per group.) Graphical data are presented as mean ± SEM. (D–F) IHC for myc tag in WT mice with Dox (D) and L188Q SFTPC mice without Dox (E) and with Dox (F). (Magnification: ×400.)
Fig. S3. Expression of mutant L188Q SFTPC in vivo does not lead to aberrant lung histology. H&E-stained lung tissue sections from WT (A) and L188Q SFTPC (B) mice exposed to Dox for 6 mo. (Magnification: ×200.)

Fig. S4. Mice expressing mutant L188Q SFTPC had more lung fibroblasts after i.t. bleomycin (Bleo) than littermate controls did. (A and B) IHC for the fibroblast marker S100A4 in lung sections from WT (A) and L188Q SFTPC (B) mice at 3 wk after bleomycin treatment. (C and D) IHC for the myofibroblast marker αSMA in lung sections from WT (C) and L188Q SFTPC (D) mice at 3 wk after bleomycin treatment. (Magnification: ×200.)

Fig. S5. Crossing L188Q SFTPC mice to the human SFTPC promoter-driven rtTA (hSFTPC:rtTA) did not alter lung fibrosis. (A) L188Q pro-SP-C–myc mRNA levels in whole-lung tissue from WT, L188Q SFTPC, and hSFTPC:rtTA × L188Q SFTPC mice exposed to Dox for 1 wk. (n = 3 per group.) (B) Semiquantitative fibrosis scoring of trichrome-stained lung sections from WT, L188Q SFTPC, and hSFTPC:rtTA × L188Q SFTPC mice at 3 wk after 0.04 unit of i.t. bleomycin (Bleo). (n = 5–7 per group; *P < 0.05 compared with WT.) Graphical data are presented as mean ± SEM.
Markers of endoplasmic reticulum (ER) stress are increased by bleomycin (Bleo) treatment in WT mice and mutant L188Q SFTPC mice. (A) Real-time RT-PCR for expression of BiP mRNA normalized to expression of the housekeeping gene RPL19 at 1 wk after bleomycin treatment. (n = 4–5 per column; *P < 0.001 for WT versus L188Q SFTPC in both Dox and Dox + Bleo groups; #P < 0.05 for Dox versus Dox + Bleo for both WT and L188Q SFTPC.) (B) RT-PCR gel demonstrating splice variants for XBP1 mRNA in lungs of WT and L188Q SFTPC mice treated with Dox and harvested 1 wk after bleomycin injection. (C) XBP1 splicing analysis by densitometry. (n = 5 per column; *P < 0.01 for WT versus L188Q SFTPC in Dox group; #P < 0.001 for Dox versus Dox + Bleo for WT.) Graphical data are presented as mean ± SEM.

Representative in vivo photon-capture images from WT/NGL (A) and L188Q SFTPC/NGL (B) mice after 14 d of Dox treatment. (C and D) Representative in vivo photon-capture images from WT/NGL (C) and L188Q SFTPC/NGL (D) mice at 3 d after i.t. bleomycin (Bleo) with continued Dox.
Fig. S8. Tunicamycin (TM) leads to ER stress in the lungs. (A and B) IHC for the ER stress marker BiP in lung sections from WT mice at 48 h after i.t. administration of vehicle (DMSO; A) and tunicamycin (B). (C and D) IHC for the ER stress marker XBP1. (Magnification: ×600.) (E) Western blot analysis for BiP using whole-lung lysates from WT mice at 48 h after i.t. administration of vehicle or tunicamycin. β-Actin is shown as loading control. (F) Real-time RT-PCR for expression of BiP mRNA normalized to expression of the housekeeping gene RPL19. (n = 3 per column; *P < 0.05 between columns.) (G) RT-PCR gel demonstrating splice variants for XBP1 mRNA in lungs of WT mice treated with either vehicle or tunicamycin. (H) XBP1 splicing analysis by densitometry. (n = 3 per column; *P < 0.0001 between columns.) Graphical data are presented as mean ± SEM.