

The 78-kD Glucose-Regulated Protein Regulates Endoplasmic Reticulum Homeostasis and Distal Epithelial Cell Survival during Lung Development

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Abstract

Bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity, has been linked to endoplasmic reticulum (ER) stress. To investigate a causal role for ER stress in BPD pathogenesis, we generated conditional knockout (KO) mice (*cGrp78^{fl/fl}*) with lung epithelial cell-specific KO of *Grp78*, a gene encoding the ER chaperone 78-kD glucose-regulated protein (GRP78), a master regulator of ER homeostasis and the unfolded protein response (UPR). Lung epithelial-specific *Grp78* KO disrupted lung morphogenesis, causing developmental arrest, increased alveolar epithelial type II cell apoptosis, and decreased surfactant protein and type I cell marker expression in perinatal lungs. *cGrp78^{fl/fl}* pups died immediately after birth, likely owing to respiratory distress. Importantly, *Grp78* KO triggered UPR activation with marked induction of the proapoptotic transcription factor CCAAT/enhancer-binding proteins (C/EBP) homologous protein (CHOP). Increased expression of genes involved in oxidative stress and cell

death and decreased expression of genes encoding antioxidant enzymes suggest a role for oxidative stress in alveolar epithelial cell (AEC) apoptosis. Increased Smad3 phosphorylation and expression of transforming growth factor- β /Smad3 targets *Cdkn1a* (encoding p21) and *Gadd45a* suggest that interactions among the apoptotic arm of the UPR, oxidative stress, and transforming growth factor- β /Smad signaling pathways contribute to *Grp78* KO-induced AEC apoptosis and developmental arrest. Chemical chaperone Tauroursodeoxycholic acid reduced UPR activation and apoptosis in *cGrp78^{fl/fl}* lungs cultured *ex vivo*, confirming a role for ER stress in observed AEC abnormalities. These results demonstrate a key role for GRP78 in AEC survival and gene expression during lung development through modulation of ER stress, and suggest the UPR as a potential therapeutic target in BPD.

Keywords: 78-kD glucose-regulated protein knockout; endoplasmic reticulum stress/unfolded protein response signaling; alveolar epithelial cells; apoptosis

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Clinical Relevance

Bronchopulmonary dysplasia (BPD) is a major cause of mortality and morbidity in preterm neonates. Recent studies in mouse models as well as patients with BPD have suggested a role for endoplasmic reticulum (ER) stress in the pathogenesis of BPD. The chaperone 78-kD glucose-regulated protein (GRP78) is a master regulator of ER homeostasis and the unfolded protein response (UPR). In this study, analysis of mice with lung epithelial-specific knockout of *Grp78* revealed developmental arrest, impaired alveolar epithelial cell (AEC) differentiation, and increased alveolar epithelial type II cell apoptosis in perinatal lungs. These results demonstrate a key role for GRP78 in AEC survival and differentiation during lung development through modulation of ER stress/UPR signaling. Pharmaceutical targeting of the ER stress/UPR pathway might be a potential treatment strategy in BPD.

Endoplasmic reticulum (ER) is the intracellular site of maturation and folding of secretory and membrane proteins. Disturbances in the ER luminal environment, genetic mutations, viral infection, and other insults cause misfolded proteins to accumulate in the ER (1–3). ER stress results when the capacity of the ER to process misfolded proteins is exceeded. Eukaryotic cells have developed a defense mechanism, known as the unfolded protein response (UPR), which alleviates ER stress to promote cell survival, but can trigger apoptosis if ER stress is severe or prolonged. Activation of all three arms of UPR signaling via protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 α (IRE1 α), and activating transcription factor 6 (ATF6) initiates adaptive responses to increase protein folding capacity and degradation of misfolded proteins and inhibit protein translation (4). Severe or prolonged ER stress leads to apoptosis via activation of c-Jun amino terminal kinase (JNK) signaling and CCAAT/enhancer-binding proteins (C/EBP) homologous protein

(CHOP) by IRE1 α and PERK, respectively (4–6).

The chaperone protein 78 kD glucose-regulated protein (GRP78; also known as BiP/HSPA5) is an important modulator of ER stress/UPR signaling that binds to PERK, IRE1 α , and ATF6 in nonstressed cells. In response to stress, sequestration of GRP78 by misfolded proteins leads to its release from UPR effector molecules, resulting in UPR activation (4, 7). GRP78 itself plays a crucial role in protein folding, translocation, and degradation, and its up-regulation upon UPR activation protects against apoptosis (8, 9). Loss of GRP78 leads to ER stress and UPR activation in a number of contexts. For example, GRP78 cleavage by subtilase cytotoxin in eukaryotic cells induces severe ER stress and UPR signaling (10). Homozygous deletion of *Grp78* is embryonically lethal, due to ER stress and apoptosis of the inner cell mass (11). In tissue-specific *Grp78* knockout (KO) animal models, resulting ER stress and activation of the UPR causes apoptosis of hematopoietic stem cells (12), while inducing differentiation of progenitor cells in intestine and esophagus (13, 14), suggesting context-dependent roles for GRP78-regulated UPR signaling/ER homeostasis in stem/progenitor cell survival versus differentiation during development and in the adult.

Lung development is initiated at Embryonic Day (E) 9.5 in the mouse (15) with budding of an epithelial tube from anterior foregut endoderm. Late lung maturation (E16.5–Postnatal Day 5) is characterized by reduction in cell proliferation and emergence of more differentiated cell types, including alveolar epithelial type (AT) 1 and AT2 cells in distal lung (15). AT2 cells are cuboidal, surfactant-producing cells containing lamellar bodies that serve as progenitors for AT1 cells (which provide the major surface area for gas exchange) during development and after injury (16–18). Although signaling pathways that regulate lung morphogenesis and maturation have been extensively evaluated, effects of GRP78-modulated ER stress/UPR signaling on lung development and cellular differentiation have not been fully explored. Newborn mice expressing mutant GRP78 lacking the Lys-Asp-Glu-Leu (KDEL) retrieval

sequence needed for recycling of GRP78 protein show atelectasis and respiratory failure due to impaired surfactant protein biosynthesis, although lung development before birth is normal (19). This mutant GRP78 retains sufficient chaperone function to circumvent prenatal defects, and the effect of complete GRP78 deficiency during lung maturation and distal epithelial cell differentiation has not been investigated.

Bronchopulmonary dysplasia (BPD) is a major complication of premature birth, characterized by alveolar simplification, surfactant deficiency, and respiratory distress (20). A recent study showed elevated expression of UPR components and increased apoptosis in a mouse model of hyperoxia-induced BPD and in patients with BPD, suggesting a role for ER stress/UPR signaling in BPD pathogenesis (21). In transgenic mice overexpressing a disease-linked mutation in the C-terminal peptide of surfactant protein C (SFTPC) in AT2 cells, Bridges and colleagues (22) reported that accumulation of mutant SFTPC protein (SFTPC ^{Δ exon4}) perturbed lung development due to epithelial cell cytotoxicity, although epithelial cell apoptosis, as the result of ER stress/UPR activation, was not directly addressed. Although these studies are suggestive, a causal role for ER stress in disruption of lung maturation/distal lung epithelial cell differentiation has not been established.

To more directly evaluate effects of ER stress/UPR signaling on lung development and epithelial cell maturation, we generated lung epithelial cell-specific *Grp78* KO mice (*cGrp78^{fl/fl}*), with the genotype *SFTPC-cre^{+/-};Grp78^{fl/fl}*, by breeding *Grp78^{fl/fl}* with *SFTPC-cre* mice. *cGrp78^{fl/fl}* mice died shortly after birth, and fetal KO lungs displayed defects in morphogenesis and alveolar epithelial cell (AEC) phenotype associated with AT2 cell apoptosis, likely resulting from ER stress/UPR-mediated cross-talk with oxidative stress and transforming growth factor (TGF)- β /Smad signaling. These results indicate that GRP78 is required for pulmonary epithelial survival/differentiation, likely through modulation of UPR signaling/ER homeostasis during lung development, and suggest that interventions to modulate UPR signaling may offer

effective strategies to treat neonatal lung diseases, such as BPD.

Materials and Methods

Generation of *cGrp78^{ff}* Mice and Animal Husbandry

Heterozygous *cGrp78^{ff}* mice (genotype *SFTPC-cre^{+/-};Grp78^{ff}*) were generated by breeding *Grp78^{ff}* mice (11) and *SFTPC-cre^{+/-}* mice (23). Heterozygotes were normal and fertile, and further crossing with *Grp78^{ff}* was performed to generate homozygous *cGrp78^{ff}* mice (genotype *SFTPC-cre^{+/-};Grp78^{ff}*).

Genotyping details are available in the online supplement. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Tissue Collection

Embryos were dissected free from the uterus and lungs were collected at E14 and E18 for RNA and protein extraction and histology. Brains and hearts were collected at E18 for RNA extraction.

Quantitative RT-PCR

cDNA was synthesized using SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA) and quantitative RT-PCR (qRT-PCR) was performed with primers shown in Table E1 in the online supplement, using Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the 7900-HT Fast Real-Time PCR System (Applied Biosystems). Further methods are available in the online supplement.

Western Analysis

Proteins were resolved by SDS-PAGE and electrophoretically blotted onto Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA). Western blots (WBs) were performed using antibodies detailed in the online supplement.

Electron Microscopy

Lung samples were fixed in 0.5× Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde [PFA] in 0.1 M cacodylate buffer), postfixed in 2% osmium tetroxide, stained *en bloc* with 1% uranyl acetate overnight, dehydrated, and embedded in Epon for transmission electron microscopy, according to standard

procedures. Ultrathin sections were photographed on a JEOL JEM-2100 Electron Microscope operated at 100 kV (JEOL, Tokyo, Japan).

Immunostaining

Lung samples were fixed in 4% PFA overnight, except E18 lungs used for Pro-surfactant protein C (pro-SFTPC) staining that were fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid), and processed into paraffin sections using routine procedures. Antibody staining protocols are available in the online supplement.

Terminal Deoxynucleotidyl

Transferase dUTP Nick End Labeling Assay

Detection of apoptosis based on labeling of DNA strand breaks was performed using the *In Situ* Cell Death Detection Kit (no. 11684795910; Roche Diagnostics USA, Indianapolis, IN) according to the manufacturer's instructions.

Microarray Analysis of Gene Expression

RNA (1 μg) from four *Grp78^{ff}* and four *cGrp78^{ff}* lungs was converted into cRNA and used for Illumina mouseRef-8 v2 whole-genome expression analysis (Illumina, San Diego, CA). Data are available in Tables E2 and E3 in the online supplement and under Gene Expression Omnibus record GSE70208 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gvuviigpnollypandacc=GSE70208>). Details regarding analysis of microarray data are available in the online supplement.

Explant Culture

Lung explants were dissected from E12 *Grp78^{ff}* and *cGrp78^{ff}* mice and cultured on Whatman polycarbonate membranes (13 mm in diameter and 8 μm in pore size [VWR 28158-089]; Visalia, CA) for 6 days in Dulbecco's modified Eagles medium/F12 containing 2% FBS (no. AXE40802; Hyclone, Logan, Utah). To inhibit ER stress, Tauroursodeoxycholic acid (TUDCA; no. 580549; EMD Millipore, Temecula, CA) was added at 250 μM on the second day of culture and media changed every 48 hours. PBS was used as vehicle control. After culture, explants were photographed using a Spot FLEX digital camera (SPOT Imaging, Sterling Heights, MI) and then fixed in 4% PFA for histology.

Results

Lung Epithelial Cell-Specific KO of *Grp78* Causes Early Perinatal Lethality

Floxed *Grp78* (*Grp78^{ff}*) mice (11) were crossed to *SFTPC-cre^{+/-}* mice (23) to generate mice with lung epithelial cell-specific KO of *Grp78* with genotype *SFTPC-cre^{+/-};Grp78^{ff}* (referred to as *cGrp78^{ff}*). In these mice, Cre is under control of the human *SFTPC* promoter, which is active in early embryonic lung development (24) and should result in deletion of floxed exons 5–7 of *Grp78* throughout the lung epithelium (Figure 1A; see also Figure E1). WB using whole-lung lysates showed an approximately 70% decrease in GRP78 expression in *cGrp78^{ff}* mice compared with controls (*Grp78^{ff}*; Figures 1B and 1C). Heterozygous mice (*SFTPC-cre^{+/-};Grp78^{ff}*) were alive and healthy with a normal lifespan, and did not show any abnormalities, similar to previously reported heterozygous conventional *Grp78* KO mice (11). There was a slight increase in embryonic lethality in KOs, as the number of *cGrp78^{ff}* embryos was less than the expected Mendelian ratio of 25% throughout development, beginning as early as E14 (Figure 1D). Embryonic lethality is likely due to KO of *Grp78* in fetal membranes (amnion and chorion) and placenta, because *SFTPC* expression was detected in these tissues (25). *cGrp78^{ff}* mice did not survive after birth. The very few KO pups that were born died within roughly 12 hours with respiratory distress, including cyanosis (Figure 1E), indicating that GRP78 is required for postnatal survival. Cre expression in brain and heart was detected by PCR at E18, suggesting that brain and heart defects due to *Grp78* deletion may also contribute to early postnatal mortality of *cGrp78^{ff}* mice (Figure E2).

GRP78 Is Necessary for Normal Distal Lung Maturation

cGrp78^{ff} embryos appear normal at E14 and E18 (Figure 2A). At E12, no significant differences were observed between *Grp78^{ff}* and *cGrp78^{ff}* lungs. At E14, *cGrp78^{ff}* lungs appeared slightly smaller in size, with dilated distal airways compared with control lungs, although the severity of dilation varied among individual lungs. These phenotypes persisted until E18

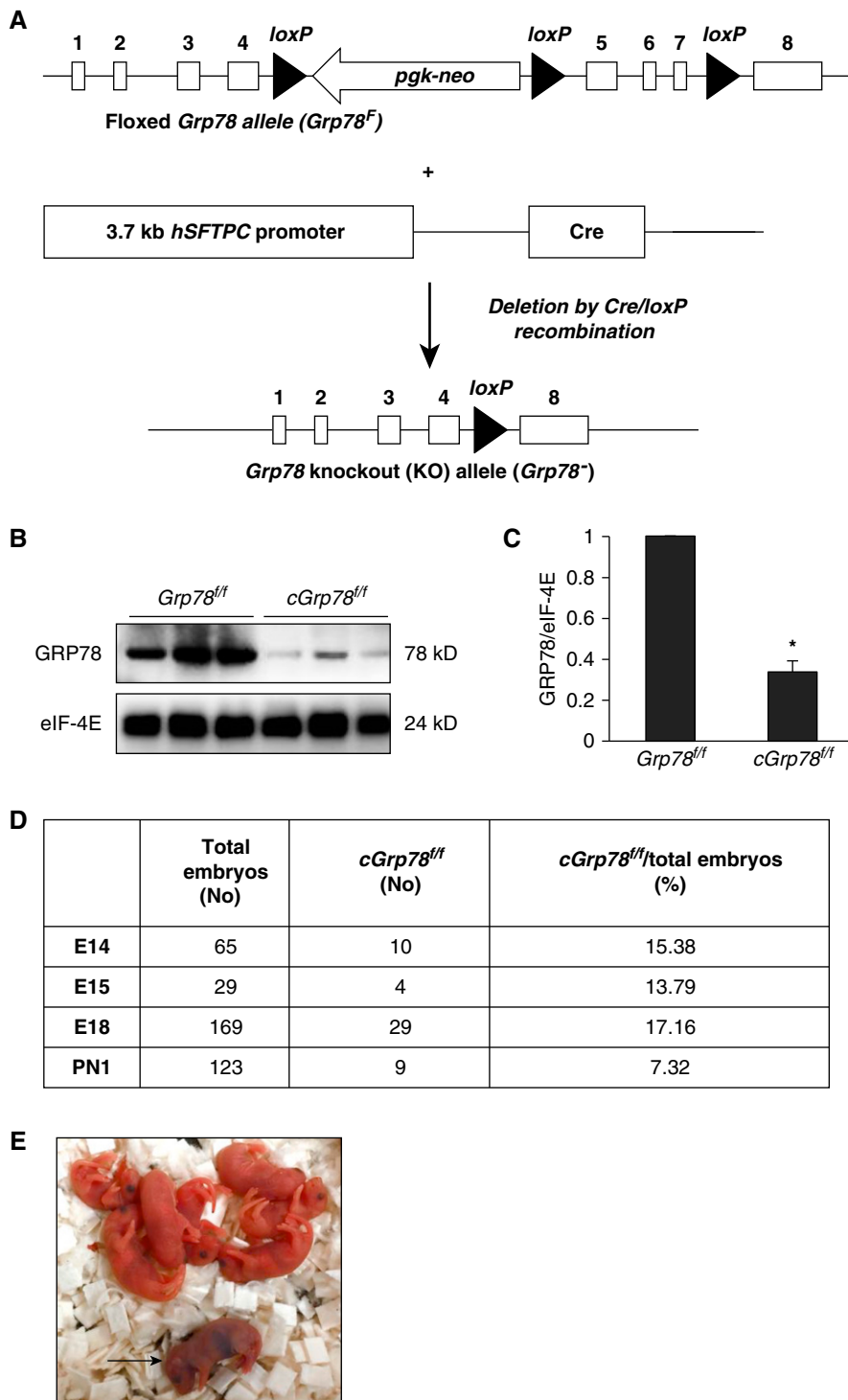


Figure 1. Embryonic and early perinatal lethality in chaperone 78-kD glucose-regulated protein (*cGrp78^{ff}*) mice. (A) Schematic diagram of floxed *Grp78* (*Grp78^F*) and knockout (KO) alleles (*Grp78⁻*). Western blotting (WB) (B) and corresponding quantitative analysis (C) show significantly decreased GRP78 expression in *cGrp78^{ff}* whole-lung lysates at Embryonic Day 18 (E18). Eukaryotic translation Initiation factor 4E (eIF-4E) was used as loading control. * $P < 0.05$, $n = 3$. (D) The number of *cGrp78^{ff}* embryos and live births at Postnatal Day 1 (PN1) was less than the expected Mendelian ratio of 25%. (E) *cGrp78^{ff}* mice (arrow) died within a few hours after birth. hSFTPC, human surfactant protein C.

(Figure 2B). As shown in Figure 2C, hematoxylin and eosin staining revealed that the distal airways were indeed dilated in E14 *cGrp78^{ff}* lungs. At E18, enlarged saccules with irregular size, thickened septal walls, and large amounts of cellular debris were observed in *cGrp78^{ff}* lungs. At Postnatal Day 1, KO lungs appeared simplified, with thin saccular septa (Figure 2C). Furthermore, consistent with previous findings that heterozygous conventional *Grp78* KO mice are normal, morphology of lung epithelial-specific heterozygous *Grp78* KO mice was normal (Figure E3).

Expression of AEC Differentiation Markers Is Reduced in Lungs of *cGrp78^{ff}* Mice

To examine if GRP78 has a function in AEC differentiation, WB was performed to evaluate expression of AT2 and AT1 cell markers in *cGrp78^{ff}* mice at E18 using whole-lung lysates. Although AT2 cell marker NK2 Homeobox 1 (NKX2-1), expression was unchanged, expression of other AT2 cell markers, surfactant protein A (SFTPA), surfactant protein B (SFTPB), and surfactant protein C (SFTPC), and AT1 cell markers, Aquaporin5 (AQP5), T1 α , receptor for advanced glycation endproducts (RAGE), and caveolin-1, was significantly reduced in *cGrp78^{ff}* mice (Figures 3A and 3B). Consistent with these findings, immunostaining demonstrated reduced expression of pro-SFTPC and AQP5 in lungs of *cGrp78^{ff}* mice at E18 (Figures 3C and 3D). Expression levels of the transcripts encoding SFTPC, SFTPA, NKX2-1, T1 α , and RAGE (*Sftpc*, *Sftpa*, *Nkx2-1*, *Pdpln* (*Pdpln*) and Advanced Glycosylation End Product-Specific Receptor [*Ager*]) were also significantly reduced in *cGrp78^{ff}* mice (Figure E4).

Activation of ER Stress/UPR Signaling in Lungs of *cGrp78^{ff}* Mice

To investigate effects of *Grp78* deficiency on UPR signaling in AECs, lungs were harvested from embryos at E14 and E18. WB showed increased GRP94 and p-eIF2 α expression at E14 in *cGrp78^{ff}* lungs (Figures 4A and 4B). Immunostaining and colocalization with NKX2-1 at E14 showed increased GRP94 specifically in respiratory epithelial cells (Figure 4C). At E18, GRP94, p-eIF2 α , CHOP, and protein disulfide isomerase (PDI) were all significantly increased (Figures 4D and 4E). Furthermore, qRT-PCR analysis showed

increased expression of transcripts encoding GRP94 (*Hsp90b1*), CHOP (*Ddit3*), PDI (*P4 hb*), and other major UPR components in *cGrp78^{ff}* mice (Figure 4F). Immunostaining showed that cells strongly expressing GRP94 and PDI are epithelial cells lining the saccules (Figures 4G and 4H) in distal lung. We evaluated whether *Grp78* KO also induces ER stress in bronchiolar epithelial cells at E18. Although ER stress markers, GRP94 and PDI, were increased in AECs, they were not induced in bronchiolar epithelial cells at E18 (Figures E5A and E5B). Moreover, club cell marker CCSP (SCGB1A1) immunostaining appeared similar in *cGrp78^{ff}* and *Grp78^{ff}* mice (Figure E5C), together indicating selective effects of *Grp78* KO on AT2 cells.

Altered ER Structure in Lungs of *cGrp78^{ff}* Mice

ER expansion has been reported in cells undergoing ER stress (26, 27), leading us to examine ER structure using electron microscopy. As shown in Figure 5, epithelial cells are tightly packed in *Grp78^{ff}* mice (Figure 5, *i*), but are more loosely arranged and appear to be detaching from the epithelial layer in *cGrp78^{ff}* mice (Figure 5, *iii*) at E18. Abnormal ER expansion (*white arrows*) was observed in *cGrp78^{ff}* mice (Figures 5, *iv–vi*) when compared with *Grp78^{ff}* mice (Figure 5, *ii*) at E18. Although secreted lamellar bodies and normally developed intracellular lamellar bodies were observed in *cGrp78^{ff}* mice (*black arrows*), lamellar bodies were seldom observed in cells with greater ER expansion (Figures 5, *iv, v, vii, and viii*). In addition, whereas there was no increase in the number of autophagosomes, more mitochondria (Figures 5, *arrowheads, iv* and *vi*), and features of apoptosis (28) (e.g., condensed chromatin at the nuclear membrane [Figures 5, *iv, v, vii, and viii*], cell surface blebbing [Figure 5, *black arrowheads, vii*], and possible apoptotic bodies [Figure 5, *viii*]) were observed in *cGrp78^{ff}* mice.

Increased Apoptosis in Lung Epithelial Cells in *cGrp78^{ff}* Mice

To further investigate effects of UPR activation on AEC apoptosis in *cGrp78^{ff}* mice, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed in E14 and E18 lungs. Very

few apoptotic epithelial cells (*green, arrow*) were detected in *cGrp78^{ff}* lungs at E14 (Figure 6A), whereas increased numbers of apoptotic cells located within saccules were detected in E18 lungs. Detection of cleaved (CL)-caspase 3 by immunohistochemistry confirmed that cells located in airspaces, but not bronchiolar epithelial cells (Figure E6), were undergoing apoptosis (Figure 6B). Double staining for CL-caspase 3 and NKX2-1 showed some NKX2-1-positive epithelial cells expressing CL-caspase 3 (Figure 6C, *arrow*), indicating that AECs undergo apoptosis in *cGrp78^{ff}* mice. Ki67 staining showed no apparent differences in cell proliferation between *cGrp78^{ff}* and *Grp78^{ff}* mice (Figure E7). Together with up-regulation of CHOP (Figure 4), these data suggest that ER stress due to *Grp78* KO induces AEC apoptosis at E18. We also determined if *Grp78* KO affects pulmonary vascular development by staining lung sections for endothelial marker CD31 (platelet/endothelial cell adhesion molecule 1 [PECAM-1]). No obvious differences in CD31 expression were found between *Grp78^{ff}* and *cGrp78^{ff}* embryos (Figure E8).

Activation of Oxidative Stress and TGF- β Signaling in *cGrp78^{ff}* Mice

To determine if ER stress caused by absence of GRP78 activates oxidative stress in *cGrp78^{ff}* mice, qRT-PCR using whole-lung RNA harvested from embryos at E18 was performed. Loss of *Grp78* reduced expression of genes encoding antioxidant enzymes, *Sod1*, *Sod2*, *Usp2*, *Gpx1*, *Cat* (Catalase), and *Nrf2* (Figure 6D), whereas expression of *Nox1*, *P4 hb* (PDI), *Ppp1r15a* (growth arrest and DNA damage-inducible protein [GADD34]), and *Ero1lb* (endoplasmic reticulum oxidoreductase beta [ERO1 β]), genes involved in generation of reactive oxygen species (ROS), was increased (Figures 4F and 6E). In addition, p-Smad3 (but not p-Smad2) (Figure 6F), and known targets of TGF- β -mediated apoptosis, *Cdkn1a* (encoding p21; Figure 6G) (29) and *Gadd45a* (Figure 6H) (30), were increased in *cGrp78^{ff}* lungs at E18. We also examined if ER stress due to loss of GRP78 stimulated autophagy, as the autophagy pathway has been reported to be activated in response to ER stress (31). WB for microtubule-associated protein 1A/1B-light chain (LC) 3 showed no change in conversion of LC3-I (cytoplasmic form) to LC3-II

(autophagosome membrane-bound form) in *cGrp78^{ff}* lungs at E18 (Figure E9).

Microarray Analysis of Messenger RNA from *cGrp78^{ff}* and *Grp78^{ff}* Lung

Microarray analysis using whole-lung RNA from E18 *cGrp78^{ff}* and *Grp78^{ff}* embryos (Figures E10A and E10B) revealed 83 up-regulated genes with greater than twofold change, with CHOP (*Ddit3*) at the top of the list, and 7 down-regulated genes (Figure E10B and Tables E2 and E3). Genes labeled with an asterisk (*) in Tables E2 and E3 were confirmed to be up-regulated by qRT-PCR (Figure 4). Ingenuity pathway analysis revealed that the most significantly altered pathway in *cGrp78^{ff}* lungs was ER stress/UPR signaling (Figure E10C). Analysis of gene networks and pathways affected in *cGrp78^{ff}* lungs demonstrated UPR activation, as evidenced by up-regulation of several genes involved in ER stress-mediated apoptosis and death (e.g., *Ddit3*, *Trib3*, *Atf3*, *Lcn2*, and *Gadd45a*), ER-associated protein degradation (e.g., *Ayvn1*, *Herpud1*, and *Sel1l*), and protein folding and trafficking (e.g., *Mdfd2*, *Creld2*, and *Srm*) (Figure E10D). Ingenuity pathway analysis also showed that genes involved in calcium, cytoskeletal, and GADD45A signaling, which are highly associated with ER homeostasis and apoptosis, were affected in *cGrp78^{ff}* E18 embryos (Figure E10C). Genes involved in protein synthesis, which lead to increased ROS generation, ATP depletion, and apoptosis in cells with ER stress (32) (*Acns*, *Slc7a5*, *Slc7a11*, *Slc6a9*, and *Wars*) were also increased in *cGrp78^{ff}* mice (Table E2). In microarray analysis (Table E2), *Grp78* expression appears increased in *cGrp78^{ff}* lungs, presumably due to the fact that the Illumina probe used was located upstream of the portion of the gene deleted by Cre (exon 5–7). Although the remaining truncated *Grp78* mRNA is up-regulated, probably due to compensatory mechanisms induced by ER stress as result of *Grp78* KO, it is not translated into a detectable protein (11). All microarray data used in this study are publicly available in Gene Expression Omnibus record GSE70208.

TUDCA Inhibits AEC Apoptosis in *cGrp78^{ff}* Lungs in Ex Vivo Culture

To examine whether inhibition of ER stress blocks AEC apoptosis, we cultured E12 lung explants (33) for 6 days in the presence or absence of the chemical

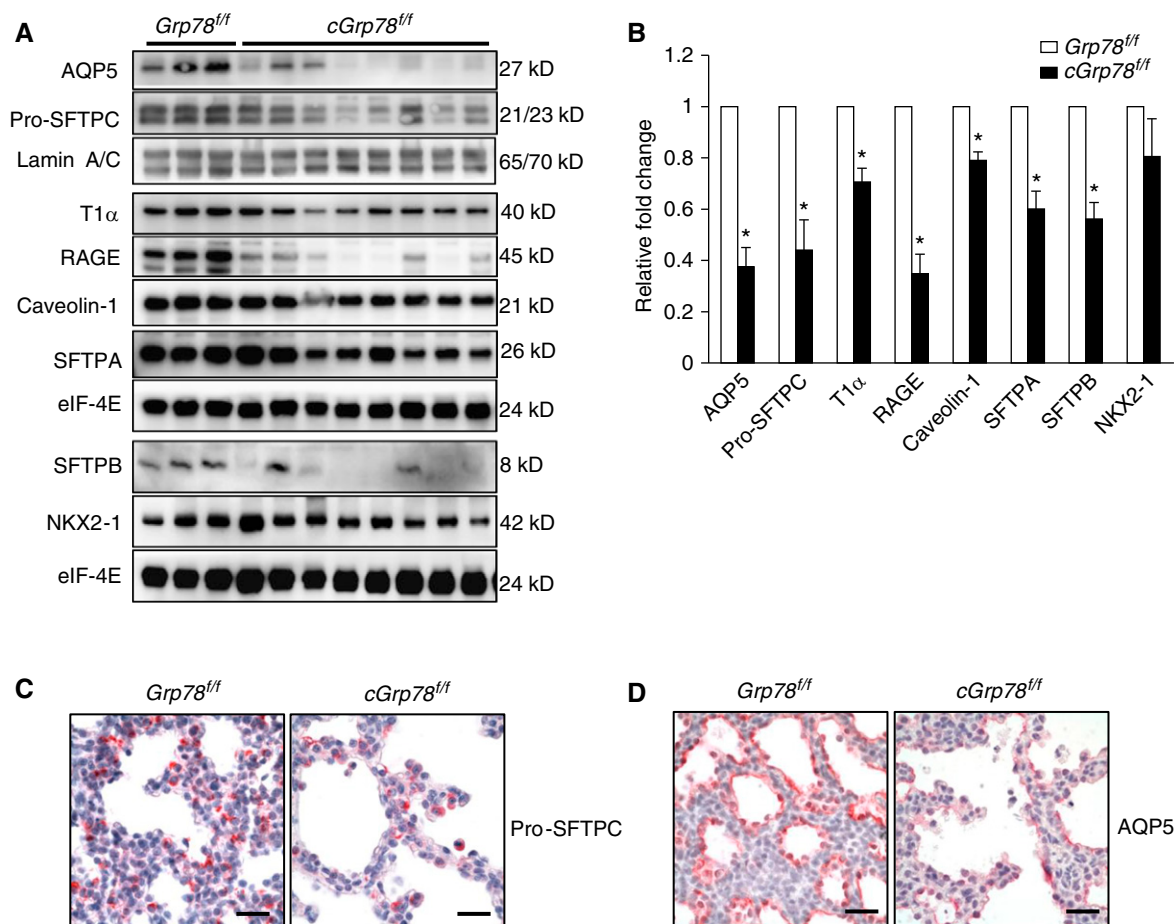


Figure 3. KO of *Grp78* reduces expression of alveolar epithelial type (AT) 2 and AT1 cell markers. Representative WB using whole-lung lysates (A) and corresponding quantitative analysis (B) show decreased expression of AT2 and AT1 cell markers in *cGrp78^{fl/fl}* mice at E18 ($n \geq 3$, $*P < 0.05$). Immunohistochemistry confirmed decreased expression of pro-SFTPC (red) (C) and AQP5 (red) (D) in *cGrp78^{fl/fl}* mice at E18. Scale bars: 50 μ m. AQP5, Aquaporin5; NKX2-1, NK2 homeobox 1; RAGE, receptor for advanced glycation endproducts; SFTPA, surfactant protein A; SFTPB, surfactant protein B.

chaperone and ER stress inhibitor, TUDCA (34, 35). Lungs of *cGrp78^{fl/fl}* mice were normal at E12 and largely appeared to grow similarly to control lungs during 6 days in culture (Figure E11). However, TUNEL assay and immunostaining showed increased apoptosis and expression of GRP94 and CHOP in *cGrp78^{fl/fl}* lungs cultured without TUDCA, whereas TUDCA treatment decreased TUNEL staining and expression of GRP94 and CHOP in *cGrp78^{fl/fl}* epithelial cells (Figures 7A–7C). These data indicate that ER stress/UPR signaling mediates AEC apoptosis in *cGrp78^{fl/fl}* mice.

Discussion

GRP78 modulates UPR activation and plays a crucial role in early embryogenesis.

To examine the role of GRP78/ER stress in lung development and maturation/differentiation of distal lung epithelial cells, we generated *cGrp78^{fl/fl}* mice with lung epithelial-specific *Grp78* KO. *cGrp78^{fl/fl}* mice died soon after birth from respiratory failure. Histological analysis revealed dilated airways in *cGrp78^{fl/fl}* lungs at E14, whereas airspaces at E18 were enlarged and irregularly shaped, and contained considerable amounts of cell debris. Electron microscopy showed irregularly arranged and detaching AT2 cells with abnormal ER expansion, fewer lamellar bodies, and features of apoptosis. In addition, *Grp78* KO resulted in reduced expression of AT2 and AT1 cell markers. Loss of *Grp78* induced UPR activation at E14, which shifted to an apoptotic response at E18, as evidenced by greater induction of apoptotic ER stress marker,

CHOP. Consistent with a role for GRP78 as a master regulator of ER stress/UPR signaling, these results suggest that GRP78 is required for maintenance of ER homeostasis and AEC survival during lung development. Together with activated oxidative stress signaling and TGF- β /Smad-mediated apoptotic signaling, these data suggest that interactions among UPR apoptotic response, oxidative stress, and TGF- β /Smad signaling pathways contribute to *Grp78* KO-induced AEC apoptosis and resultant disruption of lung development in *cGrp78^{fl/fl}* mice. AEC apoptosis, impaired AEC differentiation, and AT1 function, decreased surfactant protein expression, as well as oxidative stress, likely all contributed to postnatal respiratory failure.

The *SFTPC-cre* transgenic line used in this study has not been extensively

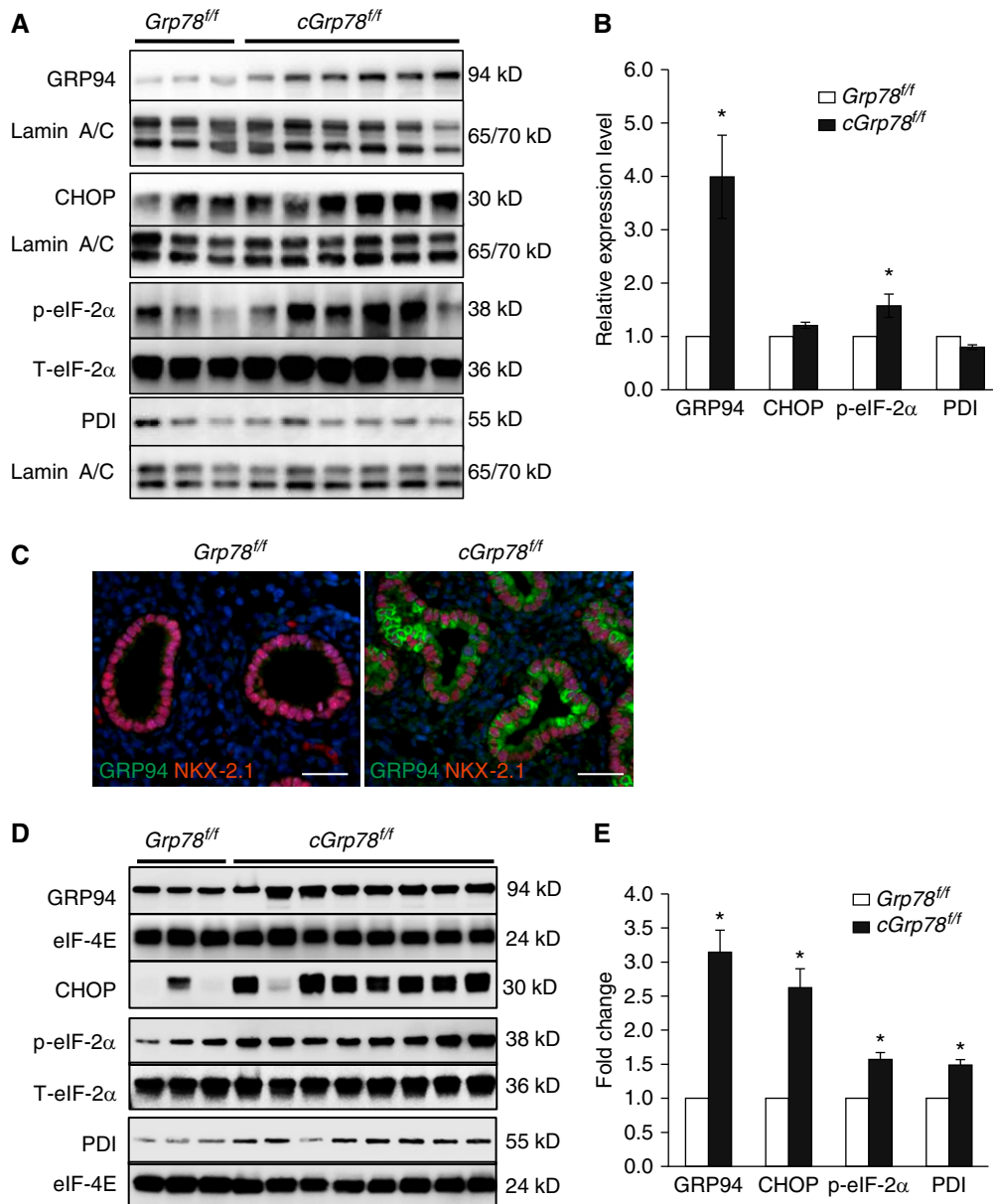


Figure 4. Unfolded protein response activation in lungs of *cGrp78^{fl/fl}* mice. Representative WB using whole-lung lysates (A) and corresponding quantitative analysis (B) show significant increases of GRP94 and p-eIF2α protein in E14 lungs of *cGrp78^{fl/fl}* mice ($n \geq 3$, $*P < 0.05$). (C) Immunostaining demonstrates increased GRP94 expression in NKX2-1⁺ epithelial cells of *Grp78* KO *cGrp78^{fl/fl}* mice at E14. Scale bars: 50 μm. Representative WB (D) and corresponding quantitative analysis (E) using whole-lung lysates shows significant increases of GRP94, CCAAT/enhancer-binding proteins (C/EBP) homologous protein (CHOP), p-eIF2α, and protein disulfide isomerase (PDI) in E18 lung of *cGrp78^{fl/fl}* mice ($n \geq 3$, $*P < 0.05$). (F) Quantitative RT-PCR (qRT-PCR) shows increased mRNA expression of *Hsp90b1*, *Ddit3*, and *P4 hb* (encoding GRP94, CHOP, and PDI), and other endoplasmic reticulum (ER) stress markers in E18 lungs of *cGrp78^{fl/fl}* mice ($n = 3$, $*P < 0.05$). Immunostaining demonstrates increased GRP94 (green) (G) and PDI (red) (H) expression in epithelial cells lining the airspaces in *cGrp78^{fl/fl}* mice at E18. Scale bars: 50 μm.

characterized with regard to tissue specificity of Cre expression and activity. For this reason, we cannot exclude the possibility that deletion of *Grp78* in other tissues might also contribute to perinatal lethality. Newborn KO pups featured clear signs of respiratory distress, including cyanosis, likely due to lung defects observed

in these animals at E18. However, because our RT-PCR data showed Cre expression in brain and heart, defects in the brain and heart could also conceivably contribute to postnatal lethality in *cGrp78^{fl/fl}* mice.

In this study, we found phenotypic effects of *Grp78* gene deletion in the

alveolar epithelium to be more severe compared with previously described GRP78 KDEL mutant mice (19). In this mutant, deletion of the KDEL domain makes the GRP78 protein unable to recycle back into the ER after performing protein chaperone and ER export functions. Despite decreased availability of functional

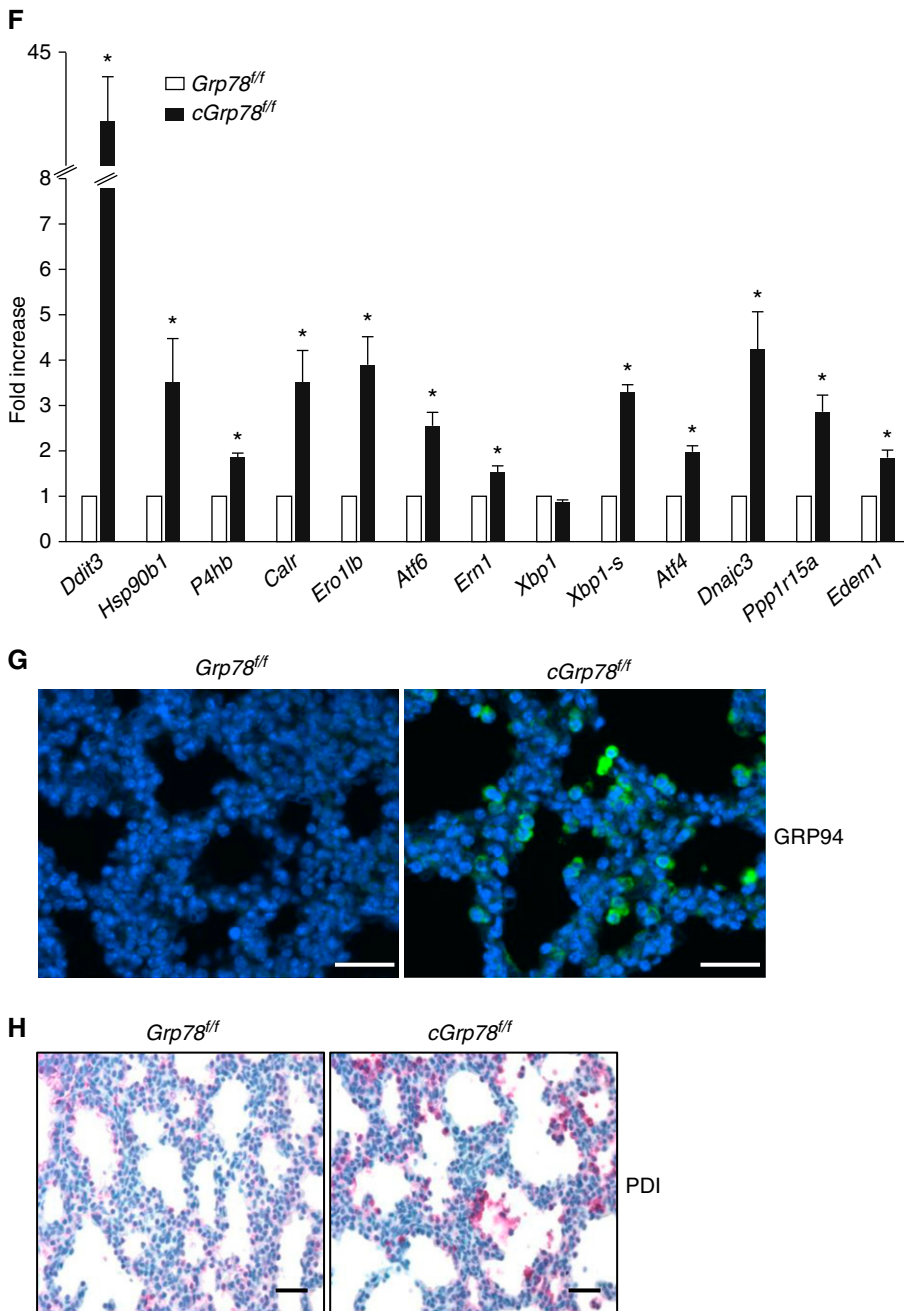


Figure 4. (Continued).

GRP78 in the ER, GRP78 function is apparently sufficient to support normal development, and resultant ER stress is not severe enough to impair normal development in KDEL mutant embryos. However, postnatally, when production of surfactant protein increases, KDEL mutant GRP78 is unable to maintain ER stress homeostasis, leading to impaired SFTPC processing/production and resultant respiratory dysfunction. In the

current study, chaperone function of GRP78 was completely abolished, inducing sustained ER stress in *cGrp78^{fl/fl}* mice. This is likely the reason that we observed defects in *cGrp78^{fl/fl}* mice already during embryonic development, whereas severe abnormalities did not appear until after birth in KDEL mutant mice.

Although the UPR is initially protective against ER stress, it promotes cell

death when ER stress is severe and persistent. We observed apoptotic epithelial cells (Figures 5 and 6) in *cGrp78^{fl/fl}* mice and strong expression of CHOP in E18, but not E14, lungs (Figure 4), despite UPR activation at E14. This suggests that a switch from an adaptive to an apoptotic UPR takes place in *cGrp78^{fl/fl}* AT2 cells with sustained ER stress as lung development progresses. AT2 cells are specialized secretory cells for synthesis and secretion of surfactant proteins. During the saccular stage, production of surfactant proteins increases rapidly to prepare the lung for postnatal respiration. Thus, like other secretory cells (e.g., pancreatic β -cells and plasma cells [36, 37]), AT2 cells are highly dependent on ER homeostasis. Loss of GRP78 likely led to persistent ER stress (7) in AT2 cells of *cGrp78^{fl/fl}* mice at E18, resulting in apoptosis and decreased surfactant protein production.

Grp78 loss has different effects in various tissues, including stem/progenitor cells. Tissue-specific *Grp78* KO induced ER stress/apoptosis in Purkinje and liver cells (38, 39), but no abnormalities in prostate epithelial cells (40, 41). *Grp78* KO induced apoptosis in inner cell mass and hematopoietic stem cells (11, 12), whereas, in intestine and esophagus, *Grp78* KO forced stem/progenitor cell differentiation with no/low levels of apoptosis (13, 42). These data suggest that GRP78 regulates cell survival and function in a tissue-specific manner. In this study, unlike in stem/progenitor cells in intestine and esophagus, lung-specific *Grp78* KO induced AEC apoptosis and prevented AEC differentiation, as evidenced by reduced expression of both AT2 and AT1 markers. AT2 cells are believed to be progenitors that can both self-renew and differentiate into AT1 cells. Thus, a decrease in both AT1 and AT2 cell marker expression suggests that loss of *Grp78* induces apoptosis of AT2 cells, as well as arrested AT2 cell maturation and transdifferentiation into AT1 cells. Because both AT2 and AT1 cells have the *Grp78* KO, AT1 cell function/survival might also be affected by ER stress caused by GRP78 deficiency, which could contribute to respiratory dysfunction after birth. Recent studies also reported the presence of bipotential progenitors at the tips of distal tubules that express both AT2 (SFTPC) and

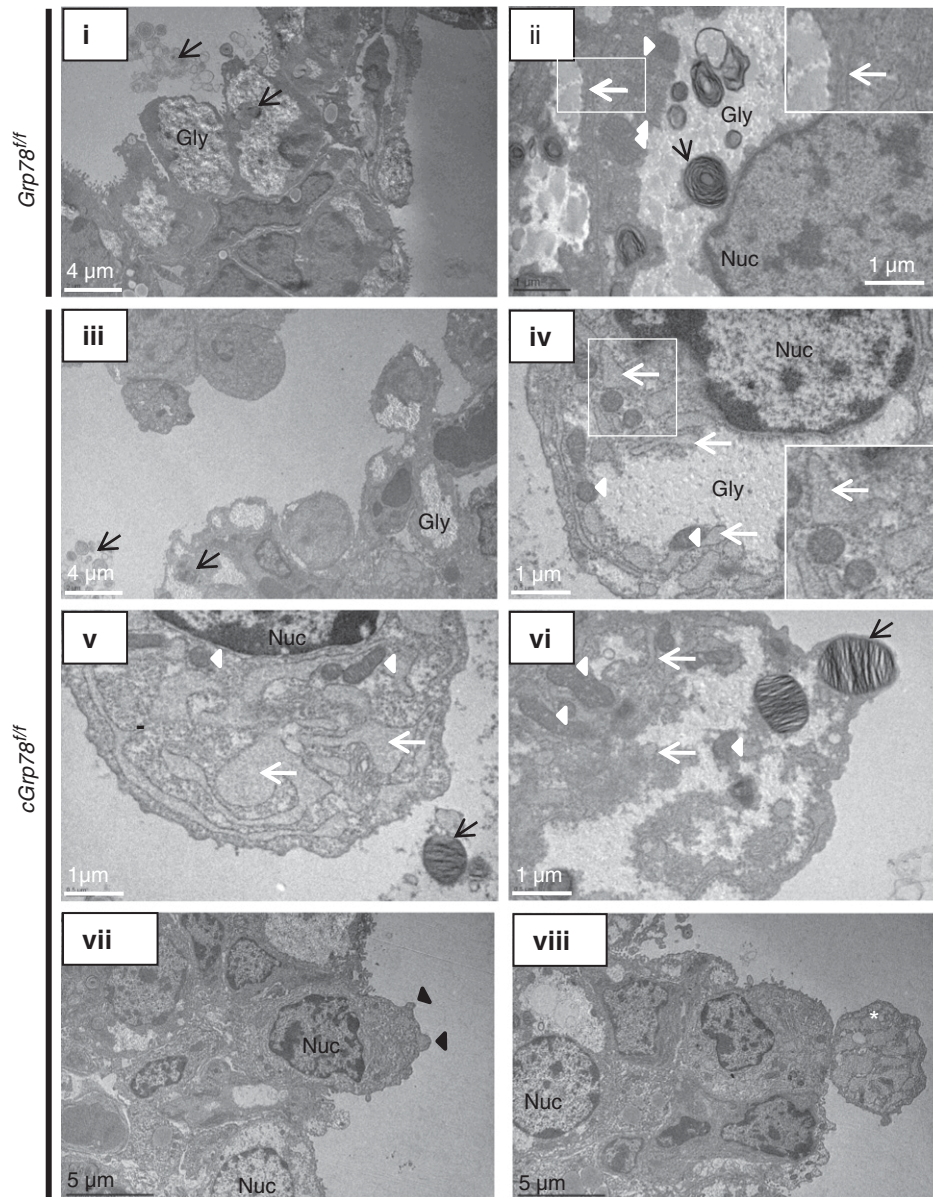


Figure 5. *Grp78* KO alters ER structure. Representative electron micrographs of lung at E18 show that epithelial cells are not tightly connected, and some are detaching from the epithelium (*iii*) and ER (*white arrows*) is abnormally expanded (*iv*, *v*, and *vi*) in *cGrp78*^{+/+} compared with control *Grp78*^{+/+} mice (*i* and *ii*). Lamellar bodies (*black arrows*) are observed within cells and in airspaces in *cGrp78*^{+/+} mice (*iii* and *vi*) and *Grp78*^{+/+} mice, but they are seldom observed in cells with greater ER expansion (*iv* and *v*). More mitochondria (*white arrowheads*) and no increase in autophagosomes are observed in *cGrp78*^{+/+} mice. Marginalization and condensation of chromatin in the nucleus (*iv*, *vii*, and *viii*), cell surface blebbing (*black arrowheads*; *vii*) and possible apoptotic bodies (*white star*; *viii*) are observed in *cGrp78*^{+/+} mice. Gly, Glycogen; Nuc, nucleus.

AT1 (T1 α) cell markers, and have the capability to give rise to both AT1 and AT2 cell populations (43). Although not examined, we cannot exclude the possibility that bipotential epithelial progenitors also undergo ER stress-mediated apoptosis in *cGrp78*^{+/+} mice. Interestingly, we did not observe UPR activation or apoptosis in bronchiolar epithelial cells, suggesting that loss of GRP78 in *cGrp78*^{+/+} mice induced ER

stress specifically in AECs. This difference is likely due to the fact that AT2 cells produce large amounts of surfactant protein, especially at the time of birth, making this cell type more sensitive to GRP78 deficiency from higher levels of ER stress.

Several studies have shown that PERK and IREs regulate the switch between adaptive and apoptotic UPRs. Activation of PERK upon ER stress regulates cell

survival by attenuation of protein translation via Eukaryotic translation Initiation factor 2 α (eIF-2 α)-2 α phosphorylation and promotes apoptosis via the PERK-eIF-2 α -ATF4-CHOP pathway (5). CHOP up-regulates expression of genes encoding proapoptotic proteins (e.g., GADD34, death receptor 5 [DR5], ERO1 β , and Tribbles 3 [TRB3]) and inhibits antiapoptotic proteins (e.g., B-cell

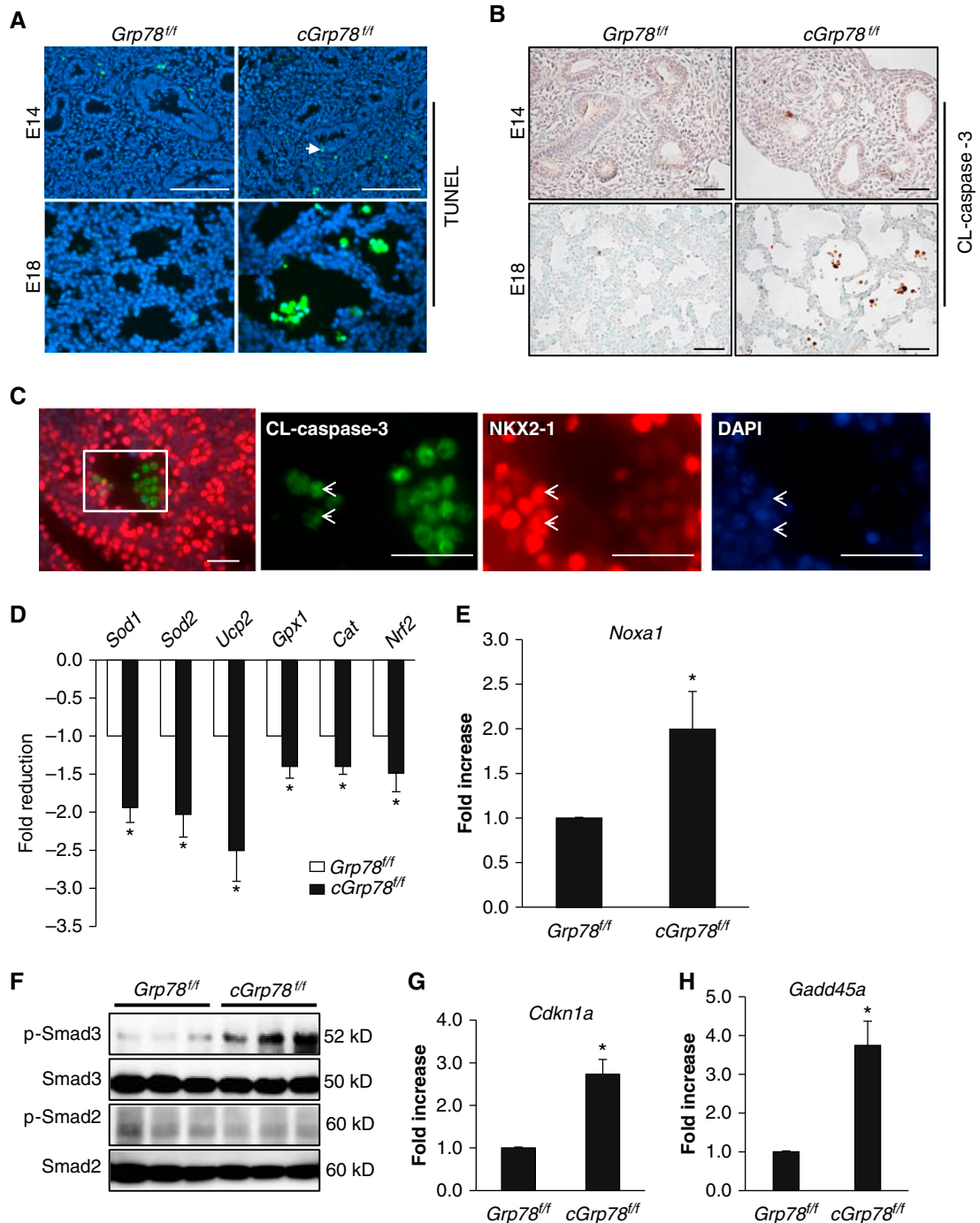


Figure 6. *Grp78* KO results in apoptosis, activation of oxidative stress and transforming growth factor (TGF)- β signaling in alveolar epithelial cell (AEC). (A) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and (B) immunohistochemistry for cleaved (CL)-caspase 3 demonstrate increased numbers of TUNEL⁺ cells (green) and CL-caspase 3⁺ cells (brown) located in the airspaces in E18, but not E14, *cGrp78^{fl/fl}* embryos. 4',6-diamidino-2-phenylindole (DAPI) or methyl green is the nuclear counterstain. Scale bars: 50 μ m. (C) Double immunofluorescence staining shows colocalization of CL-caspase 3 and NKX2-1 in cells detaching from the epithelial layer in *cGrp78^{fl/fl}* lung. The three panels to the right are individual channels showing higher magnification of the rectangular area in the left image. Arrowheads shows CL-caspase 3⁺/NKX2-1⁺ cells. Scale bars: 50 μ m. qRT-PCR using whole-lung RNA harvested from *cGrp78^{fl/fl}* and *Grp78^{fl/fl}* mice at E18 shows that *Grp78* KO reduces expression of genes encoding antioxidant enzymes (D) and increases expression of *Noxa1*, a gene involved in generation of reactive oxidant species (E) ($n = 3$, $*P < 0.05$). (F) WB demonstrates that *Grp78* KO increases p-Smad3, but not p-Smad2, expression. qRT-PCR shows increased expression of *Cdkn1a* (encoding p21) (G) and *Gadd45a* (H) in *cGrp78^{fl/fl}* mice ($n = 3-4$, $*P < 0.05$).

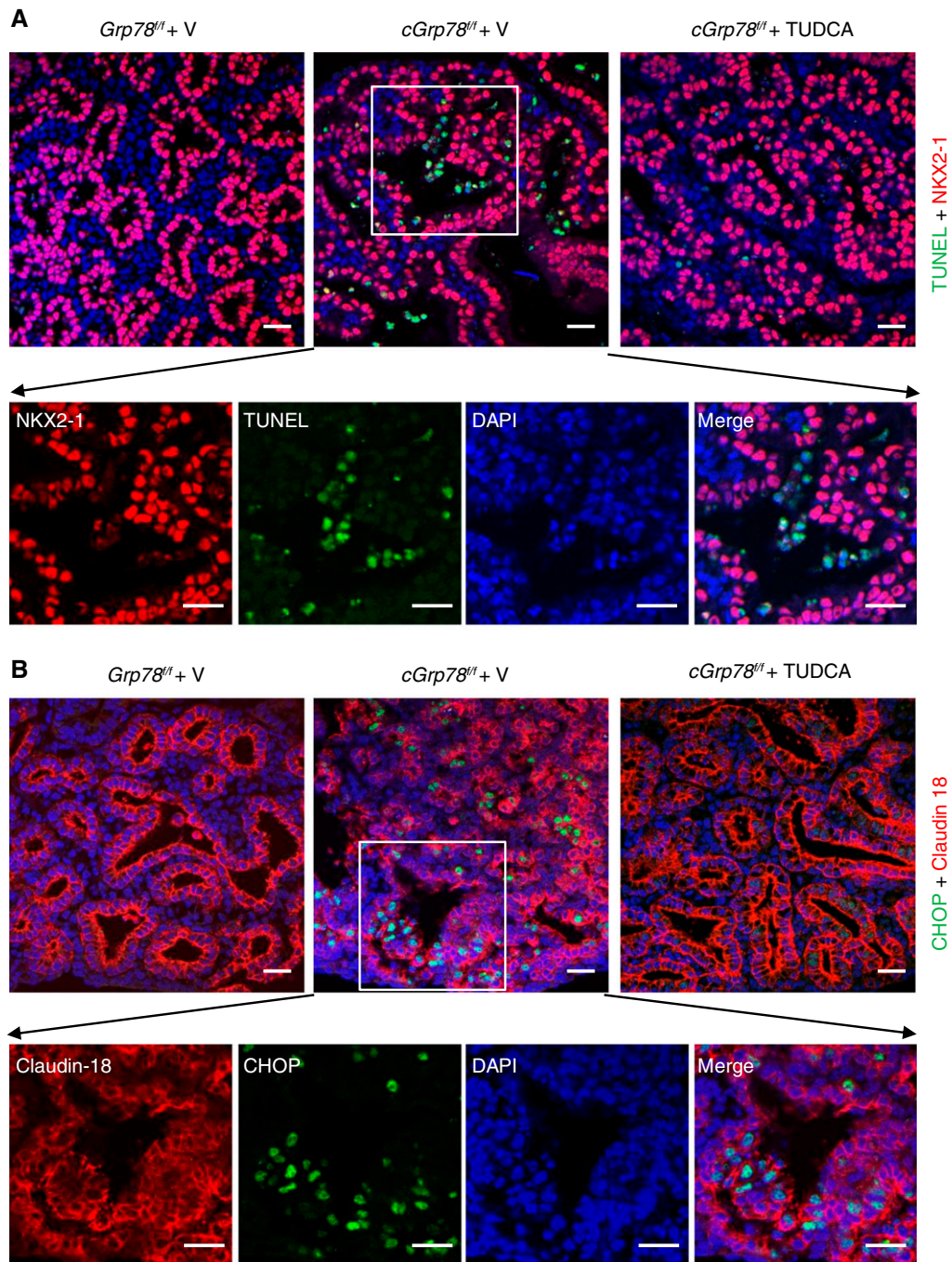


Figure 7. Tauroursodeoxycholic acid (TUDCA) inhibits AEC apoptosis in *cGrp78^{fl/fl}* lung. (A) Staining for NKX2-1 (red) and TUNEL (green) shows increased TUNEL⁺ cells in *cGrp78^{fl/fl}* (middle panel) compared with *Grp78^{fl/fl}* lung (left panel). TUNEL⁺ cells in *cGrp78^{fl/fl}* lung were decreased by TUDCA treatment (right panel). Scale bar: 20 μ m. The four lower panels are individual and merged channels showing higher magnification of the rectangular area in the upper middle image. Scale bars: 20 μ m. (B) Staining for tight junction protein claudin 18 (red) and CHOP (green) show increased CHOP⁺ cells in *cGrp78^{fl/fl}* (middle panel) compared with *Grp78^{fl/fl}* lung (left panel). CHOP⁺ cells in *cGrp78^{fl/fl}* lung were decreased by TUDCA treatment (right panel). Scale bar: 20 μ m. The four lower panels are individual and merged channels showing higher magnifications of the rectangular area in the upper middle image. Scale bars: 20 μ m. (C) Staining for NKX2-1 (red) and GRP94⁺ (green) shows increased GRP94⁺ cells in *cGrp78^{fl/fl}* lung (middle panel) compared with *Grp78^{fl/fl}* lung (left panel). GRP94⁺ cells in *cGrp78^{fl/fl}* lung were decreased by TUDCA treatment (right panel). Scale bars: 20 μ m. The four lower panels are individual and merged channels showing higher magnifications of the rectangular area in the upper middle image. Scale bars: 20 μ m.

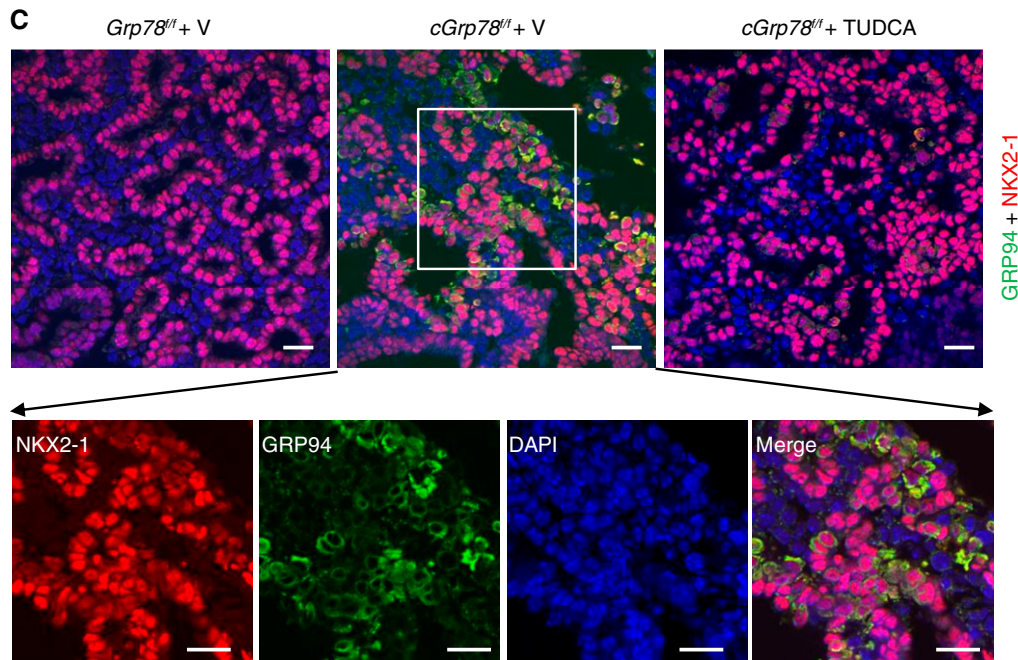


Figure 7. (Continued).

lymphoma 2 [BCL2]) (4, 44). CHOP is also involved in regulation of oxidative stress. Up-regulation of GADD34, ERO1 β , and PDI by CHOP promotes ROS generation, which, in turn, promotes apoptosis (6, 45). In addition, CHOP deletion in islet cells was associated with increased expression of genes encoding proteins with antioxidative functions (5). Recently, CHOP was found to interact with ATF4 to induce genes involved in protein synthesis, which resulted in increased ROS and cell death in stressed cells (32). In *cGrp78^{fl/fl}* mice, CHOP was the most up-regulated gene, whereas both ATF4 and phospho (p)-eIF-2 α were also up-regulated. Expression of genes involved in ER stress-mediated oxidative stress and cell death (e.g., *Ero1 β* , *Gadd34*, *Edem1*, and *Nox1*) was up-regulated, whereas expression of genes encoding antioxidant enzymes (e.g., *Sod1*, *Sod2*, *Ucp2*, *Gpx1*, *Cat*, and *Nrf2*) (Figures 4 and 6) was decreased at E18. These data suggest that ER stress-mediated AEC apoptosis in *cGrp78^{fl/fl}* mice involves cross-talk between oxidative stress and the PERK-eIF-2 α -ATF4-CHOP arm of UPR signaling, although other pathways, such as IRE1 α -mediated activation of JNK signaling through apoptosis signal-regulating kinase 1/JNK may also be involved (4, 6). These data also suggest that

activation of oxidative stress may increase sensitivity of *cGrp78^{fl/fl}* mice to oxygen exposure at birth, contributing to early postnatal death. We did not find activation of autophagy in lungs of *cGrp78^{fl/fl}* mice, as evidenced by unchanged number of autophagosomes by electron microscopy and absence of conversion of LC-3I to LC-3II. This is similar to observations in human embryonic kidney (HEK) 293 and HeLa cells after GRP78 knockdown, supporting the idea that GRP78 is required for stress-induced autophagy (9). Interestingly, in *cGrp78^{fl/fl}* mice, *Grp78* loss also increased p-Smad3, a key transducer of TGF- β signaling, and the downstream apoptosis regulators, p21 and GADD45A (46). TGF- β signaling is involved in the regulation of apoptosis in lung epithelial cells in addition to its role in branching and septation during lung development (47). Our results suggest that UPR and TGF- β /Smad signaling pathways may be tightly interconnected, contributing to AEC apoptosis, although the precise link between UPR and TGF- β /Smad signaling pathways requires further investigation.

Up-regulation of ER stress pathway components has been reported in neonatal human lungs with BPD (21). Although risk factors (e.g., hyperoxia, ventilator-induced lung injury, and antenatal infection) for

BPD are well described, mechanisms underlying disrupted alveolar growth remain unclear. To date, no therapy has been identified that produces consistently efficient results (48). Our lung epithelial cell-specific *cGrp78^{fl/fl}* mice feature arrest of alveolar development, suggesting that mice lacking *Grp78* display a BPD phenocopy that is histologically consistent with what is observed in human neonates with BPD (49). Analysis of *cGrp78^{fl/fl}* mice shows that perturbation of ER homeostasis induced AEC apoptosis via interaction of the UPR with oxidative stress and TGF- β /Smad pathways and impaired prenatal lung development/AEC differentiation, suggesting that imbalanced UPR signaling in the developing lung may contribute to BPD in neonates. Importantly, our data show that treatment with TUDCA alleviated ER stress/UPR activation, as evidenced by decreased expression of CHOP and GRP94 and reversal of ER stress-induced AEC apoptosis in *cGrp78^{fl/fl}* lungs in *ex vivo* culture. TUDCA is a U.S. Food and Drug Administration-approved chemical chaperone, and has the ability to decrease ER stress and enhance the adaptive capacity of the ER by targeting PERK/CHOP and IRE/JNK pathways (35, 50). Recent evidence suggests that TUDCA protects the liver against injury and regeneration failure (34) and improves

symptoms of obesity in a mouse model of severe obesity and type 2 diabetes associated with ER stress/UPR activation (35). Therefore, our data open a new perspective on the use of TUDCA as a potential therapeutic approach to modulation of ER stress/UPR signaling for treatment of BPD.

In summary, GRP78 is required in lung epithelium for neonatal survival. Loss of GRP78 causes an ER stress

response and dilation of the ER compartment, and alveolar simplification accompanied by AEC apoptosis and decreased expression of differentiation markers (including surfactant proteins) in distal lung epithelial cells. These observations highlight the general importance of a properly functioning UPR regulated by GRP78 in different tissues, including AEC. In addition, because AT2 cells function as

progenitor cells for both AT2 and AT1 cells, decreased survival of AT2 cells will affect the entire alveolar epithelium in the developing lung, consistent with studies showing effects of GRP78 KO on stem cell survival/differentiation in other tissues (11–14). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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