EMC3 coordinates surfactant protein and lipid homeostasis required for respiration

Xiaofang Tang, …, Xinhua Lin, Jeffrey A. Whitsett


Adaptation to respiration at birth depends upon the synthesis of pulmonary surfactant, a lipid-protein complex that reduces surface tension at the air-liquid interface in the alveoli and prevents lung collapse during the ventilatory cycle. Herein, we demonstrated that the gene encoding a subunit of the endoplasmic reticulum membrane complex, EMC3, also known as TMEM111 (Emc3/Tmem111), was required for murine pulmonary surfactant synthesis and lung function at birth. Conditional deletion of Emc3 in murine embryonic lung epithelial cells disrupted the synthesis and packaging of surfactant lipids and proteins, impaired the formation of lamellar bodies, and induced the unfolded protein response in alveolar type 2 (AT2) cells. EMC3 was essential for the processing and routing of surfactant proteins, SP-B and SP-C, and the biogenesis of the phospholipid transport protein ABCA3. Transcriptomic, lipidomic, and proteomic analyses demonstrated that EMC3 coordinates the assembly of lipids and proteins in AT2 cells that is necessary for surfactant synthesis and function at birth.

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EMC3 coordinates surfactant protein and lipid homeostasis required for respiration

Xiaofang Tang, John M. Snowball, Yan Xu, Cheng-Lun Na, Timothy E. Weaver, Geremy Clair, Jennifer E. Kyle, Erika M. Zink, Charles Ansong, Wei Wei, Meina Huang, Xinhua Lin, and Jeffrey A. Whitsett

Introduction

Pulmonary surfactant is a complex mixture of lipids and associated proteins synthesized by alveolar type 2 (AT2) cells in the peripheral lung. Surfactant is required at birth and throughout postnatal life to reduce surface tension at the air-liquid interface in the alveoli. Lack of surfactant causes atelectasis and respiratory distress syndromes in premature infants (RDS) and adults (ARDS) (1). Surfactant proteins, and a phospholipid transport protein, ABCA3, are required for the packaging of phospholipids into specialized organelles termed lamellar bodies (LBs) by AT2 cells and for the function of surfactant in the alveoli. Mutations in the ABCA3, SFTPB, and SFTP C genes cause severe lung disease in newborn infants and children, usually presenting with respiratory failure soon after birth (ABCA3 and SFTPB) or in infancy (SFTP C) (1–4). During transit from the ER to LBs, surfactant proteins B and C (SP-B and SP-C) are folded, proteolytically processed, and trafficked from the Golgi to multivesicular bodies, and ultimately stored with surfactant phospholipids in LBs. The fully processed SP-B and SP-C are secreted together with phospholipids onto alveolar surfaces to reduce surface tension and prevent lung collapse during the ventilatory cycle (5). ABCA3, a multipass transmembrane protein, is routed to the limiting membrane of the LB where it is required for transporting phosphatidylcholine (PC) and phosphatidylglycerol (PG) into the organelle (6, 7). Cellular mechanisms coordinating synthesis and assembly of phospholipids, surfactant proteins, and ABCA3 required for normal surfactant homeostasis remain incompletely understood.

EMC3, encoded by the mouse Tmem111 gene, is a subunit in the highly conserved ER membrane protein complex termed the EMC. The EMC was first identified in Saccharomyces cerevisiae as a 6-subunit transmembrane membrane protein complex required for protein folding in the ER (8). Loss of EMC subunits in yeast causes accumulation of misfolded membrane proteins and induction of the unfolded protein response (UPR). Emc1, Emc2, and Emc3 form a complex with ER-associated degradation (ERAD) pathway components Ubac2 and Derlin-2, indicating a close link between the EMC and ERAD (9). Moreover, EMCs are essential for the assembly of several multipass membrane proteins including nicotinic acetylcholine receptors (AChRs) in Caenorhabditis elegans (10, 11) and rhodopsin in Drosophila (12) and Danio (13). Despite its involvement in important cellular processes, the functions of Emc3 in vertebrate development and organ function are unclear.

Acute respiratory failure and chronic interstitial lung disease are caused by mutations in ABCA3, SFTPB, and SFTP C that produce misfolded proteins, inducing UPR and causing AT2 cell injury (1–4, 14–16). The folding, proteolytic processing, and intracellular transport of ABCA3 and surfactant proteins through the ER are critical for the production of pulmonary surfactant. In this report, we tested whether EMC3 plays a role in the assembly of surfactant lipids and proteins in AT2 cells in the neonatal mouse lung. We show that, in spite of normal lung morphogenesis, deletion of Emc3 in embryonic lung epithelial cells led to respiratory distress and neonatal lethality related to surfactant deficiency. Furthermore, we demonstrate that EMC3 controls the processing and routing of surfactant proteins SP-B and SP-C as well as the biogenesis of the phospholipid transporter protein ABCA3. Therefore, we have identified EMC3 as a critical component of an ER complex that mediates the processing and trafficking of surfactant.
associated proteins and lipids by AT2 cells and is required for lung function and respiration at birth.

**Results**

Conditional deletion of Emc3 in respiratory epithelium causes surfactant deficiency and neonatal respiratory failure. To assess the role(s) of EMC3 in lung epithelial cells, an Emc3fl/fl allele was produced and mated to Shh-Cre–transgenic mice to delete Emc3 in respiratory epithelial cells prior to birth (Figure 1, A and B). While Shh-Cre;Emcfl/+ mice survived normally, all Shh-Cre;Emcfl/fl mice died of respiratory failure immediately after birth (Table 1). Cre-mediated excision in Shh-Cre;Emcfl/fl (referred to as Emc3 cKO hereafter) mice reduced Emc3 mRNA levels in whole-lung homogenates and isolated EpCAM+ (CD326+) epithelial cells at E18.5, the day before birth (Figure 1C and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI94152DS1). Western blot analysis demonstrated decreased EMC3 protein levels (Figure 1D). Deletion of Emc3 decreased the other EMC proteins we examined including EMC1, 4, 7, and 10 but did not influence their RNAs, indicating the essential role of EMC3 in stabilizing the entire EMC complex (Figure 1D, and Supplemental Figure 1, A and C). Without any observable malformations, Emc3-deficient pups remained cyanotic and failed to inflate their lungs in spite of vigorous respiratory efforts, while control (Emc3+/+) littermates were well oxygenated and survived (Figure 2, A–D). Histological analysis of lung sections at E18.5 showed no abnormalities in lung morphogenesis (Figure 2, E and F). Differentiation of AT1 cells, indicated by podoplanin (PDPN), AGER, and HOPX staining, was undisturbed (Supplemental Figure 2), supporting the concept that defective surfactant homeostasis rather than a developmental delay may underlie the respiratory failure seen in the Emc3-cKO mice. Ultrastructure of AT2 cells in Emc3-mutant lungs lacked normal LBs and instead contained small, poorly lamellated vesicles, while the ultrastructure of other intracellular organelles was unchanged (Figure 2, G and H, and Table 2). Given the essential function of LBs in surfactant packaging, secretion, and function, the abnormal LB morphology associated with Emc3 deletion demonstrated that EMC3 played an important role in surfactant biosynthesis before birth.

**Figure 1. Conditional deletion of Emc3 with Shh-Cre.** (A) Design of the Emc3 gene targeted allele is shown. The critical exons 4 and 5 are flanked by loxP sites. (B) The breeding strategy to generate Emc3 conditional knockout mice is shown. The Emc3 conditional allele was created after flippase recombination. Subsequently, the Shh-Cre promoter–driven targeting cassette deletes exons 4 and 5. (C) qPCR of Emc3 RNA isolated from E18.5 control and Emc3-cKO whole-lung tissue or EpCAM+ sorted lung epithelial cells. Emc3 RNA was significantly decreased in homozygous Emc3-cKO mice. Data are the mean ± SEM. **P < 0.01, ***P < 0.001 using unpaired, 2-tailed Student’s t test. n = 4/group. (D) Whole-lung lysates were prepared from control and mutant mice at E18.5. Western blots were performed using indicated EMC and β-actin antibodies. Levels of all EMC subunits tested were decreased by deletion of Emc3.

<table>
<thead>
<tr>
<th>Table 1. Postnatal death in Emc3-cKO mice</th>
<th>Male Shh-Cre;Emc3fl/+ × Female Emc3fl/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny genotype</td>
<td>Emc3fl/+</td>
</tr>
<tr>
<td>Expected</td>
<td>25%</td>
</tr>
<tr>
<td>Progeny at weaning (n = 80)</td>
<td>32.5% (26)</td>
</tr>
<tr>
<td>Progeny at E18.5 (n = 149)</td>
<td>21.5% (32)</td>
</tr>
</tbody>
</table>

The genotypes and numbers of progeny generated are listed. Of 80 pups genotyped at weaning, no Shh-Cre;Emc3fl/+ mice were observed. For progeny analyzed at E18.5, the percentage of each genotype was consistent with the expected Mendelian ratio.
Emc3 deficiency leads to decreased ABCA3 and abnormal processing and trafficking of surfactant-associated proteins. Deleterious mutations in SFTPβ or ABCA3 impair LB formation and surfactant production in both humans and mice (1, 17, 18). We therefore assessed components of the surfactant system required for normal postnatal respiration. While the expression of assessed components of the surfactant system required for normal production in both humans and mice (1, 17, 18). We therefore impaired LB formation and surfactant-associated proteins (SFTPB, ABCA3, or SFTPC) mutations in Emc3. Deleterious processing and trafficking of surfactant-associated proteins (SP-B and SP-C) was nearly absent while the active 8-kDa SP-B peptide (mSP-B) was abnormally secreted, and was abnormally secreted, while the active 8-kDa SP-B peptide (mSP-B) was nearly absent (Figure 3, A, B, and H). The precursor of SP-B protein (proSP-B) accumulated and was abnormally secreted, while the active 8-kDa SP-B peptide (mSP-B) was nearly absent (Figure 3, A, B, and H), demonstrating a lack of proteolytic processing and misroutting of proSP-B. ProSP-C staining was increased in the cytoplasm of AT2 cells, while the mature 3-kDa SP-C peptide (mSP-C) was abnormally colocalized with proSP-C and was not secreted into the alveolar spaces (Figure 3, C, D, and H). To determine intracellular distributions of proSP-B and proSP-C, we performed coimmunofluorescence staining with LAMP-1, which stains lysosome-related organelles, including LBs, multivesicular bodies (MVBs), and lysosomes. ProSP-B and proSP-C were colocalized with LAMP-1 in AT2 cells of Emc3-deficient AT2 cells (Figure 3, E and F), indicating retention of unprocessed precursors. Consistent with these findings, immunogold-labeled proSP-B and proSP-C were increased in the MVBs of Emc3-deficient AT2 cells (Supplemental Figure 4, C–F). Altogether, our data demonstrate that Emc3 is required for stabilization of the ABCA3 protein and for the normal processing and trafficking of SP-B and SP-C, processes that are necessary for surfactant function.

**EMC3 interacts with ABCA3 and controls ABCA3 stability.** Since Emc3 was previously shown to influence folding of multi-pass transmembrane proteins, including AChRs and rhodopsin (10–13), we sought to test whether EMC interacts with ABCA3 to influence its stability. To model AT2 cells in vivo, we utilized MLE-15 cells (mouse lung epithelial cell line transformed with SV40 large T-antigen) that express both ABCA3 and the surfactant proteins SP-B and SP-C (19). Consistent with its function as a subunit of the EMC complex, endogenous Emc3 was localized in the ER, as revealed by its colocalization with the ER marker, protein disulfide isomerase (PDI) (Supplemental Figure 4A). Expression of Flag-tagged ABCA3 recruited endogenous Emc3 to intracellular compartments positive for LAMP-1, indicating the interaction and cotransport of ABCA3 and Emc3 from the ER to MVBs/LBs (Supplemental Figure 4, B and C). To further characterize the interaction between Emc3 and ABCA3, a construct encod-

Table 2. Quantification of the size of lamellar body–like inclusions in E18.5 AT2 cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of surveyed LBs</th>
<th>Area (mean ± SEM; μm²)</th>
<th>Maximal diameter (mean ± SEM; μm)</th>
<th>Minimal diameter (mean ± SEM; μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>377</td>
<td>0.67 ± 0.03</td>
<td>1.04 ± 0.02</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>Emc3-cKO</td>
<td>388</td>
<td>0.28 ± 0.01</td>
<td>0.79 ± 0.02</td>
<td>0.53 ± 0.01</td>
</tr>
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</table>

Lamellar body (LB) sizes were quantified from random fields of electron micrographs of AT2 cells from control and Emc3-cKO lungs as shown in Figure 2, G and H (n = 2 each). Morphometric analysis was used to determine morphology of the lamellar body–like inclusions in control and Emc3-deleted AT2 cells at E18.5. Compared with age-matched control mice, Emc3-cKO mice had significantly smaller lamellar body–like inclusions. *P < 0.001 by Kruskal-Wallis test.
Emc3 deletion induces the UPR in AT2 cells. AT2 cell function is intimately related to the synthesis and processing of surfactant proteins and lipids. Mutations in SFTPC, SFTPB, ABCA3, and genes encoding EMC3 was produced with an N-terminal c-Myc tag. When transfected into MLE-15 cells, Myc-EMC3 acted the same way as endogenous EMC3; it was readily detected in the ER, where it was colocalized with calnexin (Supplemental Figure 5A). In MLE-15 cells coexpressing Myc-EMC3 and ABCA3-Flag, the 2 proteins were primarily colocalized with LAMP-1, but not with calnexin, in intracellular compartments (Figure 4, A and B). Furthermore, Myc-EMC3 and ABCA3-Flag coimmunoprecipitated (co-IP) with other endogenous EMC subunits, including EMC1 and EMC4 (Figure 4C and Supplemental Figure 4D), indicating that ABCA3 and EMC proteins formed a complex in MLE-15 cells. RNAi-mediated knockdown of Emc1, Emc3, or Emc4 destabilized the complex (Figure 4D), demonstrating that they function together. Consistent with Emc3-cKO defects, efficient knockdown of the EMC complex by Emc3 siRNA and Emc4 siRNA decreased the stability of ABCA3-Flag in MLE-15 cells (Figure 4D). Taken together, these data support the concept that EMC3 interacts with ABCA3 to regulate its biogenesis. With similar assays, we were not able to detect direct interactions between proSP-C and Myc-EMC3 (Supplemental Figure 5, B and C), implying a distinct mechanism underlying the control of processing and trafficking of this surfactant protein by EMC3. Consistent with this finding, changes in the profiles from whole-lung mRNA in Emc3-cKO mice were distinct from those previously shown in Abca3-deficient lung (18) (Supplemental Table 1), supporting an independent role of EMC3 in surfactant biogenesis. 

Figure 3. EMC3 is required for normal processing and trafficking of surfactant-associated proteins. (A and B) E18.5 control and Emc3-cKO lung sections were stained for ABCA3 and proSP-B. ABCA3 staining was reduced and proSP-B increased in AT2 cells from Emc3-cKO mice. ProSP-B was abnormally present in peripheral lung saccules in Emc3-cKO mice, indicating its abnormal trafficking. (C and D) E18.5 control and Emc3-cKO lung sections were stained for proSP-C and mature SP-C (mSP-C). Both proSP-C and mSP-C accumulated in the cytoplasm and mSP-C was not secreted from Emc3-cKO AT2 cells. (E and F) E18.5 control and Emc3-cKO lung sections were stained for proSP-B, proSP-C, and LAMP-1, a marker for lysosome-related organelles. Intracellularly retained proSP-B and proSP-C colocalized with LAMP-1 in Emc3-cKO AT2 cells. (G) Western blotting of whole-lung lysates demonstrated decreased ABCA3 in Emc3-cKO lung. (H) Western blotting of whole-lung lysates showing the accumulation of proSP-B and proSP-C, and lack of mature SP-B (mSP-B) in Emc3-cKO lung. Scale bars: 50 μm.
critical for PC synthesis cause severe and acute lung diseases mediated by AT2 cell injury or surfactant deficiency (1). Misfolding and misprocessing of surfactant proteins cause AT2 cell injury, related in part to the activation of UPR due to the misfolding and accumulation of the abnormal proteins within AT2 cells (1, 2, 14–16). Expression of disease-associated mutant ABCA3 or SP-C proteins in an AT2-like cell line modestly induced expression of EMC3 and EMC4, concomitantly with ER stress (Supplemental Figure 6).

To further interrogate the function of EMC3 in the regulation of pulmonary surfactant protein and lipid homeostasis, we isolated epithelial cells (EpCAM+ cells) from E18.5 control and Emc3-cKO lungs by magnetic cell sorting (Supplemental Figure 7). EpCAM+ cells were then subjected to RNA sequencing, proteomic, and lipidomic analyses. Data from these experiments were integrated and correlated to enable a comprehensive view and quantitative measurements of lipid and protein in AT2 cells, seeking to identify mechanisms by which EMC3 regulates AT2 cell functions. Transcriptional and/or posttranscriptional changes in Emc3-deficient epithelial cells were revealed by integration of transcriptomic and proteomic analyses. Consistent with the presence of misfolded/misprocessed ABCA3 and surfactant proteins SP-B and SP-C, both proteomic and transcriptomic analyses of Emc3-cKO AT2 cells predicted activation of the UPR signaling pathway (Figure 5, A and B, and Supplemental Table 2). The UPR comprises 3 parallel sensing pathways: IRE1α/XBP1, ATF6, and PERK/eIF2α. The PERK branch of the UPR was activated in Emc3-cKO AT2 cells, as indicated by increased expression of ATF4 (Figure 5, E, F, and I), the major effector of the integrated stress response (ISR) (20), and its downstream target, GADD34 (Figure 5I). In response to PERK-mediated phosphorylation of eIF2α, ATF4 dimerizes with other transcription factors, including ATF3 (Figure 5, C and D) and DDIT3/CHOP, to restore cellular homeostasis or, in the face of prolonged stress, to induce cell death. Likewise, the IRE1α/XBP1 branch of the UPR was activated by Emc3 deficiency. As a surrogate of IRE1α activity, splicing of Xbp1 mRNA was increased, as indicated by the increased ratio of spliced Xbp1 (S) to full-length Xbp1 (U) (Figure 5J). The functionally active transcription factor Xbp1(S) upregulates a series of chaperones, including HSPA5/BiP and DNAJB9/Erdj4 (Figure 5A and Supplemental Table 2), which are involved in folding of surfactant proteins (21). In contrast to the above 2 branches, ATF6 was not activated, as both the full-length ATF6(F90) and the cleaved transcription factor ATF6(F50) were reduced in Emc3-cKO AT2 cells (Figure 5, G–I). Taken together, our data demonstrated that Emc3 deletion selectively induced UPR pathways in AT2 cells of the murine lung (Figure 5K).

Emc3 deletion disrupts surfactant phospholipid and protein synthesis in AT2 cells. Consistent with accumulation of surfactant proproteins (Figure 3), proteomic analysis of Emc3-cKO AT2
Figure 5. Loss of Emc3 induced the unfolded protein response in EpCAM+ sorted epithelial cells. (A and B) Heatmap of the mRNAs (A, blue/red) and proteins (B, blue/yellow) involved in the UPR pathway are shown. Proteomic and RNA sequencing data were obtained from EpCAM+ sorted epithelial cells from control and Emc3-cKO mice at E18.5. Genes and proteins were categorized by ToppGene. P values and fold changes for each mRNA and protein are listed in Supplemental Table 2. (C–H) Immunohistochemical staining for ATF3 (C and D), ATF4 (E and F), and ATF6 (G and H) was performed on lung sections from E18.5 control and Emc3-cKO embryos. ATF3 and ATF4 staining was increased and ATF6 staining was unaltered in the mutant lungs. Scale bars: 100 μm. (I) Western blots using EpCAM+ cell lysates from control and mutant lungs at E18.5 were performed using the indicated antibodies. (J) Increased Xbp1 splicing in Emc3-cKO mice. Levels of the spliced Xbp1 transcript [Xbp1(S)] were normalized to that of the full-length Xbp1 by qPCR. mRNAs were isolated from E18.5 control and Emc3-cKO EpCAM+ cells. Data are the mean ± SEM. *P < 0.05 using unpaired, 2-tailed Student’s t test. n = 4/group. (K) Model for the induction of UPR in Emc3-cKO AT2 cells. The model was built based on the integration of RNA sequencing and proteomic data. Relationships between differentially expressed genes and proteins were determined by Genomatrix Pathway System (GePS) and Ingenuity Pathway Analysis (IPA) suites. System models were created using IPA’s Path Designer.
cells demonstrated a reduction of a number of proteases critical for the processing of surfactant proteins, including cathepsin H (CTSH) and napsin (NAPSA) that occurred without changes in their encoding RNAs (Figure 6, A and B). Similarly, levels of other cathepsins, CTSB, CTSC, and CTSZ, were significantly decreased (Figure 6B), which together may reflect altered sorting/trafficking of surfactant proteins to the lysosome and LB compartments.

Besides ABCA3 and UPR components, major groups of proteins were affected at RNA and/or protein levels, including enzymes and transporters involved in lipid homeostasis (Figure 6, A and B, and Supplemental Table 2). Similar to ABCA3 and other EMC subunits, some ER proteins (e.g., ACAT1/2 and ABCD3) were decreased in spite of normal RNA concentrations, indicating that these proteins may be directly or indirectly regulated by EMC3 at the level of protein folding or stabilization. RNAs encoding a number of key enzymes (e.g., PON1, SCD1/2, and ACAT1/2) and lipid transport proteins (e.g., FABPS, SCP2, and ABCD3), some with known roles in surfactant phospholipid synthesis, were decreased (Figure 6A and Supplemental Table 2). Expression of PON1, SCD1/2, FABPS, and SCP2 changed coordinately at both RNA and protein levels, perhaps mediated by loss of SREBP signaling in the Emc3-cKO AT2 cells. This change could be due to the transcriptional regulation of active UPR in response to the accumulation of unfolded/misfolded proteins in Emc3-cKO AT2 cells. As a master transcription regulator of cellular lipogenesis, suppression of SREBP1/2 may mediate alterations in surfactant lipid homeostasis (22, 23). In Emc3-cKO AT2 cells, lipid concentrations were markedly decreased (Supplemental Table 3). While loss of PG moieties is consistent with defects in Abca3-mutant lungs (18, 24), differential effects among various PC species were observed beyond those changed by ABCA3 deficiency. In contrast to findings in Abca3 gene–targeted mice (18, 24), triglycerides accumulated to high levels in Emc3-deficient AT2 cells (Supplemental Table 3). Since triglycerides serve as a source of substrate for surfactant synthesis, their accumulation may indicate the disruption of normal lipid biogenesis in the absence of EMC3.

Discussion
Since the identification of the EMC complex almost 2 decades ago, our study provides the first evidence to our knowledge that the EM3/EMC complex is essential in mammalian development. The 3 major defects in Emc3-cKO lung include disrupted surfactant

Figure 6. Heatmap of the mRNAs and proteins involved in surfactant protein and lipid metabolism. (A and B) RNA (A, blue/red) and proteomic (B, blue/yellow) sequencing data were obtained from EpCAM* sorted epithelial cells from control and Emc3-cKO mice at E18.5. Genes and proteins were categorized by ToppGene. P values and fold changes for individual mRNAs and proteins are listed in Supplemental Table 2.
sis. Transcriptional profiling demonstrated that Abca3 and Emc3 gene–deleted mice shared few changes in their lung mRNA profile (Supplemental Table 1). While loss of ABCA3 compromised the expression of genes related to the biosynthesis and transport of fatty acids and phospholipids, enzymes and transporters primarily involved in lipogenesis were changed at RNA and/or protein levels after deletion of Emc3 (18, 24) (Figure 6 and Supplemental Table 2). Moreover, decreased SREBP signaling, perhaps mediated in part by activation of the UPR, may contribute to the inhibition of lipid synthesis in the Emc3-deleted mice. While deletion of Abca3 decreased lung PC and PG content (18, 24), the marked increase in triglyceride in Emc3-cKO lungs indicates neutral lipid accumulation and a more general disruption of cellular lipid homeostasis.

EMC3 directly bound ABCA3 and was required for its stabilization in AT2 cells, but also disrupted proteolytic processing and intracellular transport of proSP-B and proSP-C. While EMC3 did not bind to the surfactant proteins, napsin and several cathepsins known to mediate their processing were reduced in the Emc3-deleted mice. Misprocessed surfactant proteins are usually misrouted as has been shown in cultured cells (25), isolated AT2 cell injury. The UPR itself may disrupt lipid homeostasis through suppression of SREBP1/2 signaling. Thus, the respiratory failure caused by deletion of Emc3 may reflect combined effects of EMC3 on surfactant production via direct and indirect processes (Figure 7).

While Emc3 deficiency dramatically decreased ABCA3 protein levels, Abca3-cKO and Emc3-cKO lungs have distinct transcriptional profiles and defects in cellular functions, which is likely due to the more pleiotropic effects of EMC3 on surfactant homeostasis. Lipid synthesis (Supplemental Table 3), abnormal processing and trafficking of surfactant proteins (Figure 3), and induction of the UPRs (Figure 5), all of which contribute to surfactant deficiency at birth. The production of surfactant by AT2 cells is a highly orchestrated process in which disruption of individual genes causes secondary defects in surfactant lipid and protein homeostasis (1); for example, SFTPB deficiency inhibits packaging of phospholipids into LBs and causes misprocessing of proSP-C. ABCA3 mutations result in failure to transport surfactant lipids into LBs and abnormal routing of surfactant proteins. ABCA3, SFTPB, and SFTPC mutations produce misfolded proteins and induce the UPR, some of which cause AT2 cell injury. The UPR itself may disrupt lipid homeostasis through suppression of SREBP1/2 signaling. Thus, the respiratory failure caused by deletion of Emc3 may reflect combined effects of EMC3 on surfactant production via direct and indirect processes (Figure 7).

Figure 7. Role of EMC3 in surfactant biogenesis modeled by integration of RNA sequencing and proteomic data. Model for disrupted surfactant production in Emc3-cKO AT2 cells. The model is based on the integration of RNA sequencing and proteomic data. Relationships between differentially expressed genes and proteins were determined by Genomatix Pathway System (GePS) and Ingenuity Pathway Analysis (IPA) suites. System models were created using IPA’s Path Designer.
cells (26), and in vivo (27), consistent with the abnormal accumulation of surfactant proteins in Emc3-deleted AT2 cells.

Perinatal lung maturation is associated with increasing synthesis of surfactant proteins and lipids required for adaptation to air breathing. AT2 cells are highly metabolic and express high levels of the surfactant-associated proteins and lipids, rendering them sensitive to alterations in surfactant homeostasis that cause ER stress. In the present study, deletion of Emc3 caused misprocessing and accumulation of surfactant proteins and other AT2 cell proteins, as well as accumulation of triglyceride and alterations in lipid concentrations, both impairing AT2 cell functions and probably inducing ER stress. In response to ER stress, UPR pathways are induced to increase ER folding capacity and to maintain cellular homeostasis. The induction of the UPR by the loss of EMC3 and other EMC subunits has been reported in previous findings. In Saccharomyces cerevisiae, deletion of Emc1–6 activated the sensor of misfolded proteins, Ire1p, and induced Hac1p splicing and the UPR (8). In Drosophila, loss of Emc3 in the retina activated Ire1/Xbp1 and eIF2α phosphorylation (12). Consistent with those findings, we found that deletion of Emc3 in AT2 cells activated 2 of the 3 branches of the UPR, including Ire1/XBP1 and PERK/eIF2α, as indicated by XBP1 and ATF4 activities, respectively. While the transcription factor XBP1 induces expression of a number of chaperones involved in protein folding and ERAD, PERK inhibits translation initiation and protein synthesis, thereby reducing protein cargoes in the ER.

We did not detect the activation of ATF6, a transcription factor that induces UPR target genes, including a number of ER chaperones. During ER stress, activated UPR can interfere with SREBP signaling and thus disrupt lipid metabolism. PPARγ signaling pathways mediated by numerous multipass transmembrane proteins, for example, DISP1 and WLS. Preservation of normal lung growth and structure in the Emc3-cKO mice supports a relatively restricted substrate specificity of proteins regulated by the EMC3/EMC complex in AT2 cells.

In summary, our findings support the critical role of EMC3 and the EMC complex that coordinates (a) folding and stability of proteins critical for surfactant function at birth, (b) the UPR in the ER, and (c) regulation of lipogenesis by AT2 cells. EMC3 is a critical component of an ER membrane complex that integrates surfactant protein and lipid synthesis required for air breathing after birth.

Methods

Mice. To generate the Emc30 mice, an embryonic stem cell line containing the knockout-first Emc3 allele (Emc30/Emc30) was purchased from The European Conditional Mouse Mutagenesis Program (EUCOMM) (Figure 1A). After obtaining germ-line transmission, the conditional allele Emc30/1 was generated and bred with Shh-Cre (The Jackson Laboratory, stock number 005622) for endoderm-specific deletion of Emc3 (Figure 1B). Emc30 and Emc30 alleles were genotyped using primers F (GAGAAGCTTGCAGCACACTCC) and R (GTGAGTGTCCTTAACTGCTTCT). For timed matings, E0.5 was determined by the presence of a copulation plug in the morning. Pregnant dams were sacrificed by CO2 inhalation and embryos were harvested at E18.5. P0 newborn pups were sacrificed by anesthesia using a mixture of ketamine, acepromazine, and xylazine and exsanguinated by severing the inferior vena cava and descending aorta. All experiments were performed using at least 4 animals of each genotype per group.

Lung immunohistochemistry, immunofluorescence, and biochemical analysis. Lungs from E18.5 embryos and P0 newborns were fixed by 4% paraformaldehyde followed by paraffin embedding. Histological staining, immunohistochemistry, and immunofluorescence were performed on 5-μm paraffin sections. Bright-field images were obtained using a Zeiss Axios ImagerA2 microscope equipped with AxioVision Software. Fluorescence images were obtained using a Nikon A1Rsi inverted laser confocal microscope. For the detection of surfactant proteins in embryonic lungs by Western blots, frozen lung lobes were homogenized in phosphate-buffered saline (PBS) supplemented with Protease Inhibitor Cocktail. The following antibodies were used in this work: rabbit anti–proSP-C (WRAB-9337, Seven Hills Bioreagents); guinea pig anti–proSP-C (generated in the Whitsett lab); rabbit anti–mSP-B (CREAM-B, Developmental Studies Hybridoma Bank); rabbit anti-GAPDH (A-19, Santa Cruz Biotechnology); goat anti–Flag (MA1-91878, Thermo Fisher Scientific); rabbit anti-Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma-Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit anti-ABCA3 (generated in the Whitsett lab); mouse anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–mSP-C (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rat anti–mouse LAMP-1 (1D4B, Developmental Studies Hybridoma Bank); rabbit anti–GAPDH (G5455, Sigma–Aldrich); mouse anti–β-actin (A5441, Sigma–Aldrich); rabbit anti–Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma-Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit anti-ABCA3 (generated in the Whitsett lab); mouse anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rat anti–mouse LAMP-1 (1D4B, Developmental Studies Hybridoma Bank); rabbit anti–GAPDH (G5455, Sigma–Aldrich); mouse anti–β-actin (A5441, Sigma–Aldrich); rabbit anti–Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma–Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit anti-ABCA3 (generated in the Whitsett lab); mouse anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rabbit anti–proSP-C (generated in the Whitsett laboratory, raised against the N terminus of proSP-C); rabbit anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–proSP-B (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rat anti–mouse LAMP-1 (1D4B, Developmental Studies Hybridoma Bank); rabbit anti–GAPDH (G5455, Sigma–Aldrich); mouse anti–β-actin (A5441, Sigma–Aldrich); rabbit anti–Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma–Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit anti-ABCA3 (generated in the Whitsett lab); mouse anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rat anti–mouse LAMP-1 (1D4B, Developmental Studies Hybridoma Bank); rabbit anti–GAPDH (G5455, Sigma–Aldrich); mouse anti–β-actin (A5441, Sigma–Aldrich); rabbit anti–Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma–Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit anti-ABCA3 (generated in the Whitsett lab); mouse anti–mSP-C (WRAB-76694, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-55522, Seven Hills Bioreagents); rabbit anti–mSP-B (WRAB-48604, Seven Hills Bioreagents); guinea pig anti–ABC-A3 (generated in the Whitsett lab); rat anti–mouse LAMP-1 (1D4B, Developmental Studies Hybridoma Bank); rabbit anti–GAPDH (G5455, Sigma–Aldrich); mouse anti–β-actin (A5441, Sigma–Aldrich); rabbit anti–Hopx (sc-30216, Santa Cruz Biotechnology); goat anti–AGER (AF1I45, R&D Systems); rabbit anti–Flag (F7425, Sigma–Aldrich); mouse anti–Flag (MA1-91878, Thermo Fisher Scientific); mouse anti–c-Myc (sc-40, Santa Cruz Biotechnology); chicken anti–c-Myc (A-21281, Molecular Probes); rabbit anti-calnexin (SPA-860D, StressGen); mouse anti-PDI (MA3-019, Thermo Fisher Scientific); rabbit
anti-EMC1 (PA5-23732, Thermo Fisher Scientific); mouse anti-EMC3/TMEM111 (sc-365903, Santa Cruz Biotechnology); rabbit anti-EMC3/TMEM111 (HPA042372, Sigma-Aldrich); rabbit anti-EMC4/TMEM85 (ab184162, Abcam); mouse anti-EMC7 (sc-514440, Santa Cruz Biotechnology); rabbit anti-EMC10 (ab181209, Abcam); hamster anti-podoplanin (MA5-16113, Developmental Studies Hybridoma Bank); rabbit anti-GRP78/Bip (G9043, Sigma-Aldrich); rabbit anti-NXX2.1 (also known as TTF-1; WRAB-1231, Seven Hills Bioreagents); mouse anti-ATF3 (ab191513, Abcam); rabbit anti-ATF4 (ab184909, Abcam); mouse anti-ATF6 (70B1413.1, Novus); mouse anti-CHOP (2895, Cell Signaling Technology); rabbit anti-GADD34 (PAI-139, Thermo Fisher Scientific); and mouse anti-GFP (sc-9996, Santa Cruz Biotechnology).

**Electroscopy and immunogold labeling.** Embryonic mouse lung tissues at E18.5 were fixed and processed using a protocol modified from a previous publication (36). E18.5 mouse lungs were fixed in situ with 2% paraformaldehyde (Electron Microscopy Sciences, EMS), 2% glutaraldehyde (EMS), 0.1% calcium chloride (Sigma-Aldrich), and 0.1 M sodium cacodylate buffer, pH 7.3 (SCB; EMS) overnight at 4°C. Fixed lungs were cut into 1- to 2-mm blocks, postfixed with 1% osmium tetroxide (EMS) and 1.5% potassium ferrocyanide (Sigma-Aldrich) in 0.1 M SCB, pH 7.3, dehydrated in a graded series of alcohol, and infiltrated and embedded with EMBed 812 (EMS). Immunogold labeling was performed as previously described (37). Electron micrographs of lung sections were acquired using a Hitachi TEM H-7650 (Hitachi High Technologies America) with an AMT CCD camera (Advanced Microscopy Techniques).

**Morphometric analysis of LBs.** To determine whether loss of EMC3 influences LB formation, 40 AT2 cells obtained per animal at E18.5 were randomly selected (n = 2 for control and Emc3-cKO mice) for ultrastructural morphometric analysis. Electron micrographs of AT2 cells were acquired at magnification of ×5,000 using a Hitachi H-7650 electron microscope and an AMP TEM camera, followed by manual tracings of LBs in surveyed cells using Fiji, a variant of ImageJ image analysis software. Surface areas and maximal and minimal diameters of traced LBs were estimated. Differences in surface area and diameter between control and Emc3-cKO animals were compared using the Kruskal-Wallis test, where a P value less than 0.05 was considered statistically significant.

**Constructs.** The C-terminal Myc-DDK-tagged EMC3 construct was purchased from ORIGENE (catalog MR203415). To make the N-terminal Myc-tagged EMC3, Emc3 cDNA was amplified by PCR using primers F (ttgctacCGCCatggaacaaaaacttatttctgaagaagatctgGCAG- GACCAGAGCTGCTGCTTGACTC) and R (ttgctacTTAGAATATG - CCACAGAGCTGCTGCTTGACTC) and then subcloned into the pShuttle vector using Sall restriction sites. The construct encoding Flag-tagged mouse ABCA3 was generated by the Whitsett laboratory. Those encoding human SFTPC variants were generated by the Weaver laboratory. The constructs encoding hABCA3-GFP, hABCA3(E292V)-GFP, and hABCA3(L101P)-GFP were provided by Jennifer A. Wambach and Francis S. Cole (Washington University, St. Louis, Missouri, USA).

**Cell culture, transfection, and Western blots.** MLE-15 cells were cultured as previously described (19). Cell transfection with plasmid and/or siRNA was done using the Lipofectamine 3000 reagent (Life Technologies) following the manufacturer’s protocol. Upon harvest, cells were lysed in celllyt Lysis buffer supplemented with Protease Inhibitor Cocktail. Cell debris was removed by centrifugation. For co-IP assays, cells were harvested 48 hours after transfection in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with Protease Inhibitor Cocktail). Cell lysates were incubated with control beads (the Ezview Red Protein G Affinity Gel, Sigma-Aldrich) or anti-Myc beads (Anti-c-Myc Agarose Affinity Gel, Sigma-Aldrich) or anti-Flag beads (ANTi-FLAG M2 Affinity Gel, Sigma-Aldrich) overnight at 4°C. After removal of the supernatant, immunoprecipitates were recovered from the gel by incubation with Laemmli SDS reducing sample buffer (Bio-Rad) for 20 minutes at 42°C.

Proteins were resolved by SDS-PAGE using 4%–12% gels (NuPAGE Novex gel, Invitrogen) and the MES SDS running buffer (NuPAGE, Invitrogen). Chemiluminescence was observed using the Luminata Western HRP substrate (Millipore) and images were generated using the ChemiDoc Touch imaging system (Bio-Rad) and quantified using Image Lab software (Bio-Rad). For each experiment presented, at least 3 independent experiments were performed and representative results are shown.

The following Silencer Select siRNAs (Thermo Fisher Scientific) were used for transfection: control siRNA (catalog 4457289), Emc1 siRNA 1 (catalog s106663), Emc1 siRNA 2 (s106664), Emc3 siRNA 1 (catalog s82552), Emc3 siRNA 2 (catalog s2553), Emc4 siRNA 1 (catalog s8611), and Emc4 siRNA 2 (catalog s8612).

**Isolation of epithelial cells by magnetic cell sorting.** To isolate pulmonary epithelial cells, E18.5 lung lobes were harvested from control and Emc3-cKO embryos and digested with Dispase (Corning) at 37°C for 15 minutes. The lobes were transferred to 5 ml of DMEM containing 25 mM HEPES (Gibco) and 120 U of DNase I (Sigma-Aldrich) and dissociated with a gentleMACS Dissociator (Miltenyi Biotec). The cell suspensions were passed through a 40-μm nylon mesh strainer and then pelleted by centrifugation at 300–400 g for 10 minutes. Cell pellets were resuspended in 90 μl of autoMACS Running Buffer (Miltenyi Biotec) and 10 μl CD326 (EpCAM) MicroBeads (Miltenyi Biotec). After incubation for 15 minutes at 4°C, 900 μl of autoMACS Running Buffer was added and cells pelleted. After washing twice with autoMACS Running Buffer, cells were resuspended in 500 μl of Running Buffer and passed through a 40-μm nylon filter cap into a round-bottom tube prior to sorting using an autoMACS Pro Separator (Miltenyi Biotec). EpCAM-positive (epithelial) and -negative (non-epithelial) cells were isolated. Cells were counted and approximately 5 × 10⁴ EpCAM⁺ cells were centrifuged and stained with NKX2.1/ΔΔ (Gibco) and DAPI. Sorting efficiency was determined by the ratio of NKX2.1⁺ cells (epithelial) and proSP-C⁺ cells (AT2) in all DAPI-stained cells. Results from 4 control and 3 Emc3-cKO mice were quantified using at least 3 independent images per sample.

**RNA analyses.** Total RNA was isolated from EpCAM⁺ cells or frozen lung lobes using an RNeasy Micro Kit (Qiagen), and reverse transcription reactions were performed using an iScript cDNA synthesis kit (Bio-Rad) following the manufacturers’ instructions. Quantitative PCR (qPCR) was performed using a StepOnePlus Real-Time PCR System and TaqMan gene expression assays (Applied Biosystems). Emc3 RNA was detected using Taqman probe Mm01184718_g1 and normalized to the levels of 18S RNA (TaqMan probe 4352930, Applied Biosystems). The levels of the spliced form of XBP1 (Taqman probe Mm00464496_m1; Applied Biosystems) was normalized to that of the unspliced XBP1 (Taqman probe Mm00464497_s1; Applied Biosystems). Relative expression was calculated using the ΔΔCt method and statistical significance was determined by 2-tailed unpaired Student’s t test.

**RNA, protein, and lipid analyses.** RNA sequencing was performed on whole-lung RNAs or RNAs extracted from E18.5 lung EpCAM⁺.
cells. Sequencing and alignment were done by Cincinnati Children’s Hospital Medical Center’s Gene Expression Core. Emc3-cKO and control samples (n = 2 each) were analyzed. Raw reads were aligned using Bioconductor’s GenomicAlignment (38). DeSeq was used to analyze the raw gene counts and to calculate differentially expressed genes (39). AltAnalyzer was also used to determine significance (40). Genes that passed only 1 differentially expressed analysis were manually reviewed to determine relevance. All differentially expressed genes had a fold change greater than 1.2, an nbinomTest P value less than 0.05, and reads per kilobase of transcript per million mapped reads (RPKMs) greater than 1 for both samples in at least one condition (39). The complete data set has been submitted to the NCBI’s Gene Expression Omnibus (GEO) database with the accession number GSE90969.

Mass spectrometry–based proteomic and lipidomic analyses were performed at Pacific Northwest National Laboratory on EpCAM+ cells isolated from control and Emc3-cKO fetal lungs (n = 4 each) at E18.5. Proteomic analyses were performed using tandem mass spectrometry (LC-MS/MS) to profile protein changes employing a label-free relative quantification approach (41). Lipid analyses were performed using LC-MS/MS employing a label-free relative quantification approach (42–44). Data were log2 transformed and median normalized within each sample. Statistically significant changes were determined using 2-tailed, homoscedastic t tests. All significantly changed proteins and lipid species had a P value less than 0.05 and a fold change greater than 1.2. The lipidomic and proteomic data were deposited and are freely available at the MassIVE data repository with the MassIVE ID of MSV000080389 (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp).

RNAs, proteins, and lipids that were significantly altered were further analyzed using functional genomics tools. ToppGene (https://toppgene.cchmc.org/) was used to detect functional enrichment of significantly altered RNAs and proteins (45). Genomatix Pathway System (GePS) (www.genomatix.de) and Ingenuity Pathway Analysis (IPA) suites (www.ingenuity.com) were used to determine relationships between differentially expressed genes and proteins based on literature mining. Predicted relationships were then manually reviewed to ensure relevance before being represented in the figures. BRB-ArrayTools was used to generate heatmaps of significant gene and protein changes (46). System models were created using IPA’s Path Designer.

**Statistics.** For qPCR results and quantification of Western blots, values are expressed as the mean ± SEM. Data were analyzed using a 2-tailed, unpaired Student’s t test. P values of 0.05 or less were considered statistically significant. Statistical analyses of RNA, protein, and lipid are described in corresponding sections in the Methods.

**Study approval.** Mice were housed in pathogen-free conditions according to the protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Research Foundation.

**Author contributions**

XT and MH generated the conditional allele of Emc3. XT, XL, and JAW designed experiments and analyzed the data. XT characterized the defects and performed all cell culture experiments. XT and WW examined the unfolded protein response in EpCAM+ lung cells by Western blots. JMS and YX analyzed the RNA sequencing, lipidomic, and proteomic data. CLN performed the electron microscopy and immuno-electron microscopy experiments. GC, JEK, EMZ, and CA performed the lipidomic and proteomic analyses. XT, TEW, XL, and JAW wrote the manuscript.

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