Pulmonary surfactant proteins and lipids are required for lung function after birth. Lung immaturity and resultant surfactant deficiency cause respiratory distress syndrome, a common disorder contributing to morbidity and mortality in preterm infants. Surfactant synthesis increases prior to birth in association with formation of the alveoli that mediate efficient gas exchange. To identify mechanisms controlling perinatal lung maturation, the Calcineurin b1 (Cnb1) gene was deleted in the respiratory epithelium of the fetal mouse. Deletion of Cnb1 caused respiratory failure after birth and inhibited the structural maturation of the peripheral lung. Synthesis of surfactant and a lamellar body–associated protein, ABC transporter A3 (ABCA3), was decreased prior to birth. Nuclear factor of activated T cells (Nfat) calcineurin-dependent 3 (Nfatc3), a transcription factor modulated by calcineurin, was identified as a direct activator of Sfpq, Sftpβ, Sftpδ, Abca3, Foxa1, and Foxa2 genes. The calcineurin/Nfat pathway controls the morphologic maturation of lungs prior to birth and regulates expression of genes involved in surfactant homeostasis that are critical for adaptation to air breathing.

Introduction
In late gestation, the fetal lung undergoes the structural and functional maturation required for adaptation to air breathing at birth. The respiratory sacculles dilate, the mesenchyme thins, and the vascular bed grows into close apposition to squamous type II respiratory epithelial cells to form an efficient gas exchange surface required for respiration after birth (1). Cuboidal type II cells undergo marked ultrastructural and biochemical changes, including depletion of glycogen, increased surfactant protein and lipid synthesis, increased numbers of lamellar bodies, and secretion of surfactant into the air spaces. Surfactant reduces surface tension at the air-liquid interface and is required for pulmonary homeostasis at birth (2). Preterm birth or dysregulation of these processes result in pulmonary immaturity and surfactant deficiency, causing respiratory distress syndrome (RDS), a common disorder contributing to morbidity and mortality in preterm infants. Mechanisms controlling perinatal lung maturation and surfactant homeostasis are poorly understood and complex, mediated by autocrine-paracrine and endocrine signaling, including cell-cell and matrix-cell interactions (3).

A number of signaling and transcriptional programs are required in early lung morphogenesis and respiratory epithelial differentiation (4, 5). The important role of prenatal glucocorticoids that are used for prevention of RDS emphasizes the importance and utility of regulating gene transcription to improve lung maturation (6, 7). Knowledge of molecular pathways coordinating perinatal lung maturation and surfactant homeostasis is limited to the identification of mutations in Sftpc, Sftpβ, and ABC transporter A3 (ABCA3) transporter genes that cause respiratory failure at birth in humans (5, 8). The identification of molecular pathways that enhance epithelial maturation in late gestation to facilitate perinatal lung function represents an important strategy for development of new therapies for prevention or treatment of RDS in preterm infants.

Calcineurin (Cn) is a Ca2+/calmodulin-dependent serine-threonine protein phosphatase involved in many physiological processes (9). Cn exists as a heterodimer containing a 61-kDa catalytic A subunit (CnA) and a 19-kDa subunit (CnB) that binds calcium. In the cytosol, Cn is bound to calmodulin (CaM), forming a trimer. A rise in cytosolic Ca2+ concentration following a signaling event leads to a conformational change in CaM and CnB that is essential for the regulatory subunit (CnB1 and CnB2) (10). The CnAα, CnAβ, and CnB1 genes are expressed ubiquitously, while CnAγ and CnB2 are expressed in the brain and testis (10). Cn directly dephosphorylates a family of Rel-like homology–containing transcription factors called nuclear factors of activated T cells (Nfats) within the cytoplasm and participates in the transcriptional control of diverse genes (9). In addition to established functions of Nfats in the immune and cardiovascular system, Nfats have recently been identified to play critical roles in vertebrate organogenesis and cellular processes in several tissues (9). The 4 Nfat genes (Nfat1–Nfat4) arose at the origin of vertebrates, implying that they have evolved for the development of vertebrate-specific functions such as complex nervous, immune, and cardiovascular systems (e.g., the 4-chambered heart) (11). The lung evolved relatively late in higher vertebrates; therefore, it is not surprising that the Nfat pathway might play important roles in lung formation and function. In a recent study, we found that Nfatc3 directly interacted with thyroid transcription factor 1 (TTF-1), a homeodomain-containing transcription factor critical for expression of surfactant proteins in the lung (12). Consistent
with a potential role for the Cn/Nfat signaling pathway in the lung, Nfatc3 and CnB1 were identified in the respiratory epithelium of the fetal mouse lung (12). To determine the potential role of Cn/Nfat signaling in perinatal lung maturation and function, here we selectively deleted the mouse Cnb1 gene in the developing respiratory epithelium. Deletion of the Cnb1 gene in the respiratory epithelium caused respiratory distress and death associated with morphological and biochemical immaturity of the lung.

Results
Expression of components of the Cn/Nfat pathway during lung development. Expression of Cn/Nfat signaling components was assessed by immunostaining in the mouse lung from E11.5 to birth. Both CnA and CnB1 were detected in the apical and basal regions of respiratory epithelial cells at E11.5 and persisted until the day of birth at E18.5 (Figure 1). Nfatc3 was coexpressed with CnA and CnB1 in respiratory epithelial cells. In early lung buds (E11.5–E14.5), CnA was detected in the apical membrane of the respiratory epithelium and the mesenchyme. In late gestation, Nfatc3 staining was predominantly nuclear, suggesting its activation by Cn and nuclear translocation. Expression of Nfatc4 was not detected in the lung. Nfatc1 was detected only in the pulmonary mesenchyme (data not shown), and Nfatc2 was not expressed in type II epithelial cells (12), supporting the concept that Nfatc3 is the potential transcriptional effector of Cn in the respiratory epithelium.

Lung epithelial cell–specific deletion of Cnb1. Triple-transgenic mice harboring loxp-flanked exons III–V of the regulatory subunit of the Cn gene Cnb1 (Cnb1floxed/Δm), SP-C–rtTA+Δm, and Teto-Cre+Δm were produced in which Cnb1 was selectively deleted in the respiratory epithelium following administration of doxycycline to the dam (Figure 2A). Since Cnb1 is expressed early in embryonic lung epithelium (E11.5), we used the human SFTPC gene promoter that is active in the early embryonic lung epithelial cells (13). In the presence of doxycycline, reverse tetracycline transactivator (rtTA) bound to the TetO promoter and activated expression of Cre recombinase, deleting exons III, IV, and V of the Cnb1 gene (Figure 2A) and producing Cnb1Δm/Δm mice. At birth, genotype analysis (Figure 2B) demonstrated the transmission of the genes as predicted by Mendelian inheritance. In Cnb1Δm mice, while whole body weight was unaltered, the lung weight–to–body weight ratio was decreased by 10% (Figure 2D). Mice expressing rtTA or Cre genes without the Cnb1lox/+ allele from the time of conception to birth had a normal phenotype. No lung abnormalities were detected in mice expressing rtTA alone; therefore, these mice were used for further breeding.

Cn/Nfat signaling components after Cnb1 deletion in the respiratory epithelium. Absence of Cnb1 staining was observed at E16.5 and E18.5 in lungs from Cnb1Δm/Δm mice (Figure 2, C and F). Loss of Cnb1 correlated with sites of Cre expression (Figure 2F). Cnb1 staining was not altered in control Cnb1lox/lox littermates (Figure 2F). During normal lung development, CnA and CnB1 were coordinately expressed in the respiratory epithelium (Figure 1). Deletion of Cnb1 did not affect the sites or intensity of staining of CnA or Nfatc3 at E18.5 (Figure 2F), suggesting that Cn does not regulate CnA or Nfatc3 in the respiratory epithelium.

Function of the Cn/Nfat pathway is required for lung maturation and perinatal survival. Deletion of Cnb1 caused respiratory failure within 6 hours to 2 days after birth. While branching morphogenesis of the lung was apparently normal in Cnb1Δm/Δm mice at E16.5, decreased sacculation was observed at E17.5 and persisted at E18.5 (Figure 2E). The pulmonary mesenchyme failed to thin, and peripheral saccules were not septated, findings consistent with pulmonary immaturity. Squamous type I cells were decreased or absent (Figure 3F), and cuboidal type II epithelial cells remained glycogen rich, also consistent with pulmonary immaturity (Figure 3G). Lungs of control littermates consisted of dilated peripheral lung saccules with thinning of mesenchyme and differentiation of squamous type I and cuboidal type II epithelium (Figure 2E and Figure 3F), typical of normal lung maturation in late gestation. Thus, deletion of the Cnb1 gene impaired morphological maturation of the lung. Nfatc1 staining was detected in the developing pulmonary vasculature of...
control lungs, demonstrating an extensive vascular bed with capillaries that were observed in close proximity to epithelial cells. In contrast, pulmonary vessels in Cnb1Δ/Δ mice were embedded in the thick mesenchyme that surrounded the immature saccules at E18.5, consistent with a delay in lung maturation. Formation of pulmonary arteries and bronchiolar smooth muscle was unaltered as assessed by NFATc1 and α-SMA staining (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI27331DS1).

Synthesis of pulmonary surfactant is dependent on the Cn/Nfat pathway. RDS, a common cause of perinatal death in premature infants, is induced by the lack of pulmonary surfactant lipids and proteins (14). To determine whether respiratory failure in Cnb1Δ/Δ mice was associated with decreased surfactant production, surfactant proteins and lipids were assessed at E18.5. While pro–surfactant protein B (proSP-B) and proSP-C were readily detected in control mice expressing Cnb1, both proteins were markedly decreased in the respiratory cells of Cnb1Δ/Δ mice (Figure 3A). Sfpa, Sfph, and Sfpc mRNAs were significantly decreased (Figure 3, B and C). Likewise, surfactant protein levels — including those of SP-D — were significantly decreased in lung homogenates from Cnb1Δ/Δ mice (Figure 3D). Saturated phosphatidylcholine (Sat PC), a critical surfactant lipid, was decreased by approximately 40% (Figure 3E). Thus, Cnb1 is required for normal perinatal surfactant synthesis. Staining of T1α and aquaporin-5 were decreased after deletion of Cnb1 (Figure 3F), consistent with the paucity of type I cell differentiation observed in histology. In contrast, staining for Foxj1 and CCSP, differentiation markers of conducting airway epithelial cells, was not altered (Figure 3F).

Surfactant protein genes Sfpa, Sfph, and Sfpc are direct transcriptional targets of the Cn/Nfat pathway. Luciferase reporters containing
promoter regions of the Sftpa, SftpB, and SftpC genes (Supplemental Table 1) were activated when cotransfected with constitutively active Nfatc3 (CA-Nfatc3) in mouse lung epithelial cells (MLE-15 cells; Figure 4A). VIVIT, a potent Nfat-inhibitory peptide (15), inhibited Sftpa, SftpB, and SftpC promoter activity (Figure 4B), suggesting direct transcription activation by Nfats. Cotransfection of Nfatc3 with TTF-1, an Nkx-2 homeodomain protein that is required for lung formation and gene expression in the respiratory epithelium,
synergistically activated transcription from Sftpa, Sftpβ, and Sftpγ gene promoters in HeLa cells (Figure 4C). Direct binding of Nfatc3 to Sftpβ promoter elements in chromatin was tested in MLE-15 cells, which express high levels of Nfatc3 (Figure 4D). Chromatin immunoprecipitation (ChIP) assay using DNA primers spanning the Sftpβ 5′-regulatory region containing 3 adjacent consensus Nfat sites readily detected Nfatc3 binding in the chromatin of MLE-15 cells (Figure 4D). In contrast, Nfatc3 failed to bind Gapdh proximal promoter, implicating Nfatc3 as a direct transcriptional activator of Sftpβ. Immunostaining of Nfatc3 and TTF-1 in adult mouse lungs demonstrated their colocalization in nuclei of alveolar and bronchiolar epithelial cells (Figure 4E). Thus Nfatc3 activated by Cn regulates surfactant protein gene transcription.

Abca3 is a direct transcriptional target of the Cn/Nfat pathway. In humans, mutations in ABCA3 cause respiratory failure at birth associated with surfactant deficiency (8). ABCA3 is present in the limiting membranes of lamellar bodies in type II epithelial cells (16) and plays a critical role in surfactant homeostasis. ABCA3 staining was markedly decreased in respiratory epithelial tubules of the peripheral lungs from Cnb1Δ/Δ mice at E18.5 (Figure 5A). The mechanism for transcriptional regulation of the Abca3 gene in lung epithelial cells is unknown. To test the role of the Cn/Nfat pathway in transcriptional regulation of the Abca3 gene, a 2.6-kb 5′ promoter region of the Abca3 gene cloned into a luciferase reporter plasmid (Figure 5B and Supplemental Table 1) was cotransfected with CA-Nfatc3 (0, 0.5, 1, and 2 μg). The Abca3 promoter was activated by Nfatc3 in MLE-15 cells, and Nfatc3 and TTF-1 synergistically activated transcription from the Abca3 promoter in HeLa cells, while VIVIT inhibited Abca3 gene promoter activity in MLE-15 cells (Figure 5C). EMSAs were performed on 5 con-
sensus Nfat sites identified in the Abca3 promoter. Sites II and III (Supplemental Table 2) bound proteins in MLE-15 cell nuclear extracts that were competed by 100-fold excess of unlabeled self DNA (Figure 5D) but not by 100-fold excess of a strong TTF-1 site (17), indicating that the Cn/Nfat pathway was required for normal Foxa1/Foxa2 expression in late gestation. To determine whether Nfatc3 regulates the Foxa1 and Foxa2 genes, luciferase reporter plasmids containing 5′ promoter regions of Foxa1 and Foxa2 genes (Figure 6, A and F) were transiently transfected into HeLa and MLE-15 cells. Nfatc3 activated Foxa1 and Foxa2 promoters in both cell lines (Figure 6, C and G). Since Foxa2 and Foxa1 regulate genes required for lung function at birth, the effects of Cnb1 deletion may be mediated in part by its effects on Foxa activity. Since the –3.4-kb Abca3 promoter contains 15 consensus Nfat binding sites as identified by MatInspector (version 7.0), EMSAs were performed on DNA elements with high matrix similarity (Supplemental Table 2). Sites IV, V, and VI (Figure 6D) bound Nfatc3 from MLE-15 nuclear extracts. An Nfat site competitor and unlabeled self DNA disrupted the DNA-protein complex, while a 100-fold molar excess of the TTF-1 probe did not affect the complex. Antibodies to Nfatc3 interfered with specific DNA-protein complex formation.

CnB1 participates with TTF-1 in a transcriptional network during lung formation. TTF-1, a homeodomain-containing member of the Nkx family of transcription factors, is expressed in the respiratory epithelial cells. TTF-1 regulates expression of surfactant protein genes, and mutations in Titf1 leads to defects in lung formation and function (21). Since Cnb1 is expressed in early embryonic lung buds at E11, we sought to determine whether lack of Cnb1 influences Titf1 expression and to explain, in part, the maturation defects seen in Cnb1−/− mice. The sites and intensity of TTF-1 staining were not altered in the lungs of Cnb1−/− mice, suggesting that Cnb1 does not regulate TTF-1 expression (Figure 7A). Conversely, we tested whether TTF-1 regulates Cnb1 and Nfatc3 expression. Cnb1 staining was lacking in the respiratory epithelial cells in the lungs of TTF-1 null (Titf1−/−) mice, while staining was observed in the surrounding mesenchyme (Figure 7B). In contrast,
intense Nfatc3 staining was evident in epithelial cells lining the lung saccules in Titf1–/– mice at E16.5 and E18.5, suggesting that while TTF-1 regulated expression of Cnb1, Nfatc3 expression was independent of TTF-1. Indeed, deletion of the Cnb1 gene inhibited the nuclear localization of Nfatc3, while Nfatc3 and TTF-1 staining were colocalized in the nuclei of airway epithelial cells in lungs from normal Cnb1fl/fl mice (Figure 7C).

RNA microarray analysis of Cnb1-regulated genes. Microarray analysis of lung RNA from Cnb1Δ/Δ and control mice identified 437 genes that were significantly altered (≥1.5-fold change): 243 mRNAs were upregulated and 194 were downregulated in Cnb1Δ/Δ mice. The most significantly upregulated genes included Ttf1 and Ttf2, which are known to regulate Cnb1 expression.

**Figure 6**
Cnf influences Foxa1 and Foxa2 gene regulation. (A) Consensus Nfat site GGAAA in the Foxa1 promoter. (B) Foxa1 staining was decreased in lungs from Cnb1Δ/Δ mice at E17.5 and E18.5. Scale bars: 200 μm. (C) CA-Nfatc3 expression (0, 1, 2, and 4 μg) enhanced activity of Foxa1 promoter-luciferase construct (0.165 μg) in MLE-15 cells. (D) Sites IV, V, and VI of Foxa1 promoter bound Nfatc3 from nuclear extracts of MLE-15 cells. Complexes were competed by 100-fold molar excess of Nfat site from the IL-2 promoter (lanes 3, 10, and 17) and by unlabeled self DNA (lanes 5, 12, and 19) but not by TTF-1 site DNA (lanes 4, 11, and 18). Antibodies to Nfatc3 interfered with complex formation (lanes 6, 13, and 20). Free DNA probes containing Nfat site (lanes 1, 8, and 15). MLE-NE bound to DNA probes containing Nfat sites (lanes 2, 9, and 16). BSA incubated with MLE-NE and DNA probes containing Nfat sites (lanes 7, 14, and 21). NS, nonspecific protein binding to DNA probes containing Nfat site. (E) Decreased Foxa2 immunostaining in lungs from Cnb1Δ/Δ mice at E17.5 and E18.5. Scale bars: 200 μm. (F) Consensus Nfat sites (solid box: GGAAA; and open box: AGAAA) were identified in Foxa2 promoter. (G) Nfatc3 activated the Foxa2 promoter in MLE-15 cells. Expression of VIVIT inhibited transcription from the Foxa2 promoter in MLE-15 cells.
increased, and 194 mRNAs were decreased (Supplemental Table 3). An additional filter was used (see Methods) to identify genes classified as involved in regulation of surface tension, respiratory air and gas exchange, lipid metabolism, and ion transport (See Supplemental Table 3 for gene ontology classification). Genes regulating lung function, including regulation of surface tension, respiratory air and gas exchange, lipid metabolism, and ion transport, were significantly decreased in the lungs of Cnb1Δ/Δ mice (Table 1). Pearson correlation analysis identified lung mRNAs that were similarly influenced after deletion of Cnb1 and Foxa2 and mutation of Ttf1 in respiratory epithelial cells (Figure 9A and Supplemental Table 4). The list of genes differentially expressed in response to Cnb1 deletion correlated closely with those differentially expressed in Foxa2Δ/Δ mice (r = 0.71) and those in Ttf1 phosphorylation mutant mice (Ttf1PMP/PM mice; r = 0.58).

Foxa2 and Ttf1-1 regulate expression of surfactant proteins and respiratory epithelial cell differentiation (21, 22). Comparison of lung mRNAs from Cnb1Δ/Δ, Foxa2Δ/Δ (22), and Ttf1PMP/PM mice (21) demonstrated that a number of mRNAs were similarly influenced in each model, supporting the concept that their biological processes were shared (Figure 9A and Supplemental Table 4). Genes involved in the regulation of lung lipid homeostasis (Lrp2, SftpB, Abca3, Dlk1, Scd1, and Pon1), fluid and electrolyte transport (Aqp5, Clic5, Scnn1g, and Slc34a2), and inflammation (Sftpα1, Hc, Lyz3, and Lzp-s) were decreased in response to the lung-selective deletion of Cnb1 or Foxa2 or mutation of phosphorylation sites in the Ttf1 gene (see Supplemental Table 4 for a complete list of overlapping genes).

Deletion of Cnb1 altered expression of a number of genes regulating lipid synthesis. Pla2g1b mRNA, which encodes a calcium-depen-

dent phospholipase referred to as phosphatidylcholine 2-acylhy-
drolase (23), was increased approximately 3.7-fold in Cnb1Δ/Δ mice over that of Cnb1floxflox mice. mRNA levels for Aytl2, a putative acetyltransferase that may regulate synthesis of Sat PC by addition of acyl groups, was decreased approximately 3.9-fold in Cnb1Δ/Δ mice compared with that of Cnb1floxflox mice as assessed by real-time PCR, consistent with the RNA microarray data (Figure 9B).

Discussion

Failed lung maturation at birth results in surfactant deficiency, causing respiratory distress in the perinatal period. The molecular mechanisms controlling maturation of the lung are poorly understood. The present study demonstrates that Cn/Nfat signaling was required for the structural, biochemical, and functional maturation of the lung prior to birth. Since Cnb1-null embryos die before lung formation (24), we used a conditional deletion strategy to ascertain the function of Cn in the developing respiratory epithelium. While the growth and branching of the lungs appeared to be normal in Cnb1Δ/Δ mice, structural abnormalities associated with delayed lung maturation were apparent in late gestation. Deletion of Cnb1 impaired sacculation and inhibited lung epithelial cell differentiation, consistent with a generalized arrest in lung maturation.

Expression of SftpB and Abca3 mRNA and protein as well as that of the surfactant lipids were markedly decreased in the lungs of Cnb1Δ/Δ mice. Since deletion of either SftpB or ABCA3 causes lethal respiratory failure at birth (8, 25), the lack of their expression is sufficient to explain respiratory failure in Cnb1Δ/Δ mice. The abnormalities in lung structure and decreased surfactant lipid synthesis are also likely factors contributing to poor respiratory function at birth.
The temporal and spatial expression of CnA, CnB1, and Nfatc3 in the developing lung epithelial cells is consistent with the concept that the Cn/Nfat pathway regulates critical genes involved in lung formation and function at birth. Nfatc3, a transcription factor and downstream effector of the Cn signaling pathway, enhanced transcription via direct binding and activation of target genes, including genes encoding surfactant proteins, providing evidence that disruption of the Cn/Nfat-mediated pathway is responsible, at least in part, for the observed phenotype after deletion of Cnb1 in the lung.

RNA microarray analysis demonstrated that Cnb1 regulated genes mediating innate host defense, electrolyte and fluid transport, and surfactant protein and lipid homeostasis, thus identifying a group of genes likely to play important roles in perinatal lung homeostasis. Since TTF-1 and Foxa2 regulate expression of surfactant proteins, and either mutations in Cnb1 or deletion of Cnb1 delays lung maturation causing respiratory failure at birth, it seems likely that they share common transcriptional targets. This assumption is supported by the following findings: First, a number of common transcriptional target genes were identified that were influenced by both Cnb1 and Foxa2 deletion (22) with a high Pearson correlation, implying that the Cn/Nfat pathway appears to be upstream of Foxa2 activity, consequently sharing an overlapping set of transcriptional targets with Cnb1. Thus, the 3 transcription factors Nfatc3, Foxa1, and Foxa2, and TTF-1 are known to activate a number of shared target genes critical for lung function (blue arrows). Cn may also function via Nfat-independent pathways (dashed arrow).

**Figure 8**

Regulation of Cnb1 and its participation with TTF-1 in a transcriptional network during lung formation. (A) Consensus TTF-1 and Nfat sites were detected in the Cnb1 proximal promoter. Black and white boxes represent Nfat sites (GGAAA and AGAAA, respectively); black circles represent TTF-1 sites. (B) The Cnb1 gene promoter was activated by TTF-1 and Nfatc3. Cotransfection of TTF-1 expression vector (0, 0.25, 0.5, and 1 µg) with a fixed amount of Cnb1-755-luc (0.25 µg; a promoter-reporter construct containing −755 bps of 5′-upstream regulatory sequence of mouse Cnb1 gene cloned into Xhol and HindIII site of pGL3 basic plasmid) increased luciferase activity in HeLa and MLE-15 cells. TTF-1 (0.5 µg) and Nfatc3 (0.5 µg) synergistically activated the Cnb1 gene promoter in HeLa cells. (C) Proposed network of transcription factors — regulated by or interacting with the Cn/Nfat pathway — driving perinatal lung maturation. This maturation process begins in the sacchular stage from E17 to postnatal day 5 (PND-5) and is completed at the end of the alveolar stage by PND-21 in mice. TTF-1 regulates Cnb1 expression. Cnb1 dephosphorylates and activates Nfatc3 (red dashed arrow). In turn, Nfatc3 activates Foxa1 and Foxa2; (b) synergistically interacts with TTF-1 to activate surfactant protein genes and Abca3; and (c) increases transcription of CnA required for its own activity (61). Foxa1, Foxa2, and TTF-1 are known to activate a number of shared target genes critical for lung function (blue arrows). Cn may also function via Nfat-independent pathways (dashed arrow).
CnB1 interact in a complex transcriptional network that orches-
trates the expression of groups of genes critical for the structural
adaptation that may provide a basis for the prevention or treat-
ment of respiratory disease before and after birth.

Methods

Transgenic mouse lines. CnA and CnB1 are both indispensable for the phosphatase activity of Cn. Thus deletion of Cnb1, the only isoform found in all tissues except the testis, is sufficient to eliminate Cn activity in the lung. Therefore, mice bearing floxP-flanked exons III-V of Cnb1 (Figure 1A) were produced (45) and maintained as homozygotes in a mixed C57BL/6– SV129–FVB/N background. To achieve lung epithelium–specific deletion of the Cnb1 gene, these mice were first mated to SP-C–rtTA Δ-Δ mice and TetO-Cre Δ-Δ mice. Administration of doxycycline to dams from E0.5 to birth activated rtTA from the SP-C promoter in the lung epithelium and induced Cre (Cre recombinase), which deleted exons III-V of Cnb1 in the lung epithelium and produced Cnb1 f/f mice. Cnb1 f/f littermates lacking either rtTA or Cn genes served as controls, herein termed Cnb1 f/f mice. Further controls included mice expressing rtTA or Cre genes under the control of the SP-C promoter from the time of conception. In the absence of loxP alleles (Cnb1 f/f), rtTA- and Cre-expressing mice survived normally and without pulmonary abnormality. Genotypes were identified by PCR as previously described (13, 45). Titf1 Δ-Δ mice (46), kindly provided by S. Kimura (NIH, Bethesda, Maryland, USA), were bred to obtain Titf1 Δ-Δ lungs at E16.5 and E18.5. Lungs from Titf1 Δ-Δ mice served as controls.

Animal husbandry and doxycycline administration. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Research Foundation. Mice were housed in humidity- and temperature-controlled rooms on a 12-hour light/12-hour dark cycle with food and water ad libitum. There was no serologic or histologic evidence

and biochemical maturation of the lung required for respiration at birth (Figure 8C). Because TTF-1 phosphorylation is critical in regulating expression of genes involved in lung morphogenesis and surfactant protein production (21), it is plausible that distinct signaling events influence TTF-1 and Nfatc3 function at transcriptional and posttranslational levels, enabling them to synergize in the temporal, spatial, and cell-specific control of gene expression during perinatal lung maturation.

Taken together, our results suggest that under the influence of Cn signaling, Nfatc3 binds and activates the promoters of transcriptional targets mediating surfactant homeostasis, including Abca3, SftpA, SftpB, SftpC, and SftpD. Nfatc3 may directly interact with TTF-1 at transcriptional target sites. Cn activation is dependent on receptors present on the cell membrane (9, 32), providing a potential mechanism by which Nfatc3 function is influenced by external signals. The identification of an Nfat signaling axis in the respiratory epithelial cells presents opportunities to modulate this transcriptional program during perinatal

Table 1
Gene ontology analysis after Cnb1 deletion in the lung epithelial cells

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Expression of genes involved in lung maturation, lung function, and homeostasis — including regulation of surfactant, respiratory gas exchange, ion transport, fluid balance, lipid metabolism, and transport — were decreased. Note decrease in expression of Abca3, SftpB, Scd1, and Aqp5 mRNAs.

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of either pulmonary pathogens or infections in sentinel mouse colonies. Gestation was dated E0.5 by vaginal plug. Dams were maintained on dosycycline in food (25 mg/g; Harlan Teklad) from E0.5 were and killed by injection of anesthetic to obtain embryos.

Histology and immunohistochemistry. Fetal and adult lungs were prepared as previously described (47). Antibodies used were as follows: CnA (diluted 1:200, anti-mouse rabbit polyclonal; Chemicon International AB-1695); CnB1 (diluted 1:700, anti-mouse rabbit polyclonal, PA-3025; Affinity BioReagents); Cre (diluted 1:20, K, Novagen, EMD Biosciences); Nfatc3 (diluted 1:100, mouse monoclonal, F-1, SC-8405X; Santa Cruz Biotechnology Inc.); Nfatc1 (diluted 1:200, mouse monoclonal, SC-7294X; Santa Cruz Biotechnology Inc.); proSP-B (diluted 1:1,500, rabbit polyclonal, AB-1695); CCSP (diluted 1:1,000, rabbit polyclonal, kindly provided by B. Stripp, University of Pittsburgh, Pittsburgh, Pennsylvania, USA); TTF-1 (diluted 1:3,000, rabbit polyclonal, kindly provided by A. Menon, University of Cincinnati Medical Center, Cincinnati, Ohio, USA); TTF-1 (diluted 1:20, rabbit monoclonal; Chemicon International); proSP-B (diluted 1:1,500, rabbit polyclonal, AB-1695); CCSP (diluted 1:1,000, rabbit polyclonal, kindly provided by B. Stripp, University of Pittsburgh, Pittsburgh, Pennsylvania, USA); proSP-B (diluted 1:1,500, rabbit polyclonal, generated in the lab of J.A. Whitsett); FoxJ1 (diluted 1:5,000, anti-mouse guinea pig polyclonal, generated in this lab); T1-α (diluted 1:1,000 anti-mouse hamster polyclonal 8.1.1, University of Iowa Hybridoma bank); aquaporin-5 (diluted 1:20, kindly provided by A. Menon, University of Cincinnati Medical Center, Cincinnati, Ohio, USA); TTF-1 (diluted 1:3,000, rabbit polyclonal, kindly provided by R. DiLauro, Laboratory of Biochemistry and Molecular Biology, Stazione Zoologica, Naples, Italy); FoxA1 and FoxA2 (both diluted 1:16,000, generated in this lab; ref. 48); ABCA3 (diluted 1:500, anti-mouse rabbit polyclonal, kindly provided by T. Weaver, University of Cincinnati, Cincinnati, Ohio, USA); and α-SMA (diluted 1:10,000, Sigma-Aldrich 1A4). Immunostaining and electron microscopy procedures were as previously described (47, 49). For immunofluorescence studies a 10-fold higher titer of antibodies was used. All results shown are representative of at least 3–4 triple-transgenic offspring that were compared with control littermates.

Cell culture, transfection, and transcriptional reporter assays. HeLa cells were cultured in DMEM with 10% FBS. MLE-15 cells were cultured as previously described (50). Transfection and reporter assays were carried out as previously described (12).

EMSA. Nuclear extracts from MLE-15 cells were made as previously described (12). EMSA probes (Supplemental Table 2) were derived from the 5′ promoter region of Abca3 and Foxa1 genes containing consensus Nfat sites (core similarity, 1.0; matrix similarity, ≥0.85; MatInspector 7.0 Software; Genomatix). EMSAs and antibody supershift assays were performed as previously described (12). Unlabeled double-stranded DNA competitors with a strong TTF-1 binding site from the rat thyroglobulin gene (rTg; ref. 17) and a strong Nfat binding site from the IL-2 promoter (18) were added at 100-fold molar excess. The gels were dried and autoradiographed.

Surfactant lipid and protein analysis. Lungs were harvested at E18.5. The left lobe was homogenized in 0.9% NaCl. Sat PC was extracted using the osmium tetroxide (OsO4) method (51) followed by phosphorous measurement (52). For SP-B and SP-C immunoblots, equal aliquots of the left lung homogenates were loaded on a 10%–20% Tricine SDS-PAGE (Invitrogen) and electrophobted to nitrocellulose membranes (0.1 μm; MidSci). Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 8.5, 150 mM NaCl, 0.1% Tween 20) and incubated with antibodies against human SP-B and SP-C (Chemicon International). To estimate SP-A and SP-D content, proteins from lung homogenates were resolved on 10%–20% Tris Glycine SDS-PAGE and electrophobted to nitrocellulose membranes (0.45 μm; Bio-Rad). For immunoblot analysis, guinea pig anti-rat SP-A or rabbit anti-mouse SP-D antibodies (53) were used. Secondary antibodies used were as follows: SP-A (goat anti-guinea pig IgG-peroxidase conjugate; Sigma-Aldrich); SP-B, SP-C, and SP-D (goat anti-rabbit heavy- and light-chain-peroxidase conjugate; Calbiochem, EMD Biosciences). MLE-15 whole-cell lysates run on 10%–20% Tris Glycine SDS-PAGE detected Nfatc3 by immunoblotting using mouse monoclonal antibody (F-1, SC-8405X; diluted 1:5,000, Santa Cruz Biotechnology Inc.), followed by anti-mouse goat IgG (Calbiochem, EMD Biosciences). Blots were developed by chemiluminescence (Pierce Biotechnology) and autoradiographed.

RNA isolation and S1 nuclease analysis. Lung RNA prepared by TRIZOL method (Invitrogen) was measured by UV absorbance and used for microarray and S1 analyses as previously described (21). Relative changes in SP-A, SP-B, SP-C, and SP-D mRNAs were assessed by S1 nuclease assays with ribosomal protein L32 as an internal control (54) using a phosphorimager.

RNA microarray analysis. Lung cRNA was hybridized to the murine genome MOE430 chips (Affymetrix) according to the manufacturer’s protocol. Affymetrix Microarray Suite 5.0 was used to scan and quantitate the gene chips under default settings. Normalization was performed using the robust multichip average model (54, 56). Data were analyzed using Genespring 7.2 (Silicon Genetics). A volcano plot was used to identify significance (negative log of P values from Welch’s approximate t test on y axis) and magnitude of change (log2 of fold change on the x axis) in the expression of a set of genes between Cnb1 Δ/Δ mice and control littermates. The selection criteria included a P value of 0.05 or less by 2-tailed Student’s t test, false discovery rate (FDR) of no more than 10% (57), and fold change of at least 1.5. Differentially expressed genes were subjected to an additional filter and classified according to Gene Ontology classification on Biological Process using the publicly available web-based tool David (58). The Fisher exact test was used to calculate the probability of each gene ontology category that was overrepresented in the selected list, using the entire MOE430 mouse genome as a reference data set. Pearson correlation identified lung mRNAs that were...
similarly influenced after deletion of Foxa2 and Cnb1 and mutation of Tgfβ1 in respiratory epithelial cells. Differentially expressed genes (P < 0.05, 2-tailed Student’s t test; fold change, >1.5) were compared, and correlations of transcript changes among 3 microarray experiments were measured.

**ChIP assays.** ChIP assays were performed as described previously by Li et al. (59), with modifications. Nfatac3 expression vector was transfected into about 80% confluent MLE-15 cells on 2 150-mm dishes using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were treated with 1% formaldehyde for 10 minutes at 24°C, crosslinking was terminated by 0.125 M glycine, and cells were then washed in cold PBS and centrifuged to pellet cells that were resuspended in 10 ml lysis buffer 1 (50 mM HEPES-KOH, pH 7.9; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% IGE 0.125 M glycine, and cells were then washed in cold PBS and centrifuged to remove the lysis buffer 1 (50 mM HEPES-KOH, pH 7.9; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% IGE 0.125 M glycine, and cells were then washed in cold PBS and centrifuged to remove the lysis buffer.

The lysates were collected by centrifugation, resuspended in 10 ml lysis buffer 2 (10 mM Tris-HCl, pH 7.8; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 1% SDS; and protease inhibitors from Roche Diagnostics), and once with 1 ml TE buffer and were resuspended in 1 ml elution buffer (50 mM Tris-HCl, pH 7.8; 10 mM EDTA; and 1% SDS).

Precipitated chromatin was eluted at 65°C for 10 minutes and incubated with 5 times 1 ml RIPA buffer (50 mM HEPES-KOH, pH 7.9; 1 mM EDTA; 0.5 mM EGTA; and protease inhibitors from Roche Diagnostics) and incubated overnight at 4°C on a neutron. After centrifugation, the pellet was resuspended in 2 ml lysis buffer 3 (10 mM Tris-HCl, pH 7.8; 1 mM EDTA; 0.5 mM EGTA; and protease inhibitors from Roche Diagnostics) and once with 1 ml TE buffer and were resuspended in 50 μl elution buffer (50 mM Tris-HCl, pH 7.8; 10 mM EDTA; and 1% SDS). Precipitated chromatin was eluted at 65°C for 10 minutes and incubated at 65°C overnight to reverse the crosslinks. The next day, the DNA was ethanol precipitated, with ethanol, and dissolved in 50 μl of 10 mM Tris-HCl (pH 8.0). PCRs were performed using primers spanning the 5′ regulatory regions of the Sftp8, Abca3, and Gapdh genes (Supplemental Table 1). Reaction conditions were as follows: 95°C for 5 minutes; 35 cycles of amplification at 95°C for 30 seconds, then 55°C (Sftp8 and Abca3) or 53°C (Gapdh) for 30 seconds; followed by 72°C for 30 seconds as an extension reaction. The products were analyzed by agarose gel electrophoresis.

**Real-time RT-PCR assays.** Lung RNA was prepared as described above. cDNA was synthesized from 5 μg total RNA as previously described (12) and analyzed by real-time PCR for Plazgβ and Aytl2 mRNA on a SmartCycler (Cepheid) as previously described (60). The values were normalized to β-actin values in each sample. Reaction conditions were as follows: 95°C for 150 seconds; 40 cycles of amplification at 95°C for 10 seconds, then 60°C (Aytl2) or 57.5°C (Plazgβ and β-actin) for 10 seconds; followed by 72°C for 20–25 seconds (see primers in Supplemental Table 2).

**Statistics.** Quantitative results for gene activation analysis represent the average of at least 3 transfection experiments performed in duplicate (n = 6) and depicted as mean ± SD for data shown in Figures 4–6 and 8. For quantitative RNA and Sat PC analysis represented in Figure 3, statistical differences were determined using unpaired 2-tailed Student’s t tests. Mean ± SEM values are shown (n = 7–9 mice per genotype). A P value of less than 0.05 was considered to be significant throughout this article.

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