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C/EBPα is required for lung maturation at birth

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Epithelial cells lining the peripheral lung synthesize pulmonary surfactant that reduces surface tension at the air-liquid interface. Lack of surfactant lipids and proteins in the lungs causes respiratory distress syndrome, a common cause of morbidity and mortality in preterm infants. We show that C/EBPα plays a crucial role in the maturation of the respiratory epithelium in late gestation, being required for the production of surfactant lipids and proteins necessary for lung function. Deletion of the *Cebpa* gene in respiratory epithelial cells in fetal mice caused respiratory failure at birth. Structural and biochemical maturation of the lung was delayed. Normal synthesis of surfactant lipids and proteins, including SP-A, SP-B, SP-C, SP-D, ABCA3 (a lamellar body associated protein) and FAS (precursor of fatty acid synthesis) were dependent upon expression of the C/EBPα in respiratory epithelial cells. Deletion of the *Cebpa* gene caused increased expression of *Tgfb2*, a growth factor that inhibits lung epithelial cell proliferation and differentiation. Normal expression of C/EBPα required *Titf1* and *Foxa2*, transcription factors that also play an important role in perinatal lung differentiation. C/EBPα participates in a transcriptional network that is required for the regulation of genes mediating perinatal lung maturation and surfactant homeostasis that is necessary for adaptation to air breathing at birth.

KEY WORDS: CCAAT/enhancer-binding protein α, Respiratory epithelial cell differentiation, Lung maturation, Pulmonary surfactant, ABCA3, FOXA2, TTF1, Mouse

INTRODUCTION

In mammals, transition of the fetus from an aqueous to an air breathing environment at birth is dependent upon the formation and function of the lung. Lung morphogenesis and differentiation are mediated by complex intracellular, autocrine, paracrine and endocrine signaling that influences transcriptional programs to regulate cell behavior. A number of transcription factors have been identified that play important roles in lung formation and regulation of genes and processes required for the function of the lung at birth, including TTF1 (Kimura et al., 1996; Minoo et al., 1999; Cardoso, 2001), NFI (Bachurski et al., 2003) GATA6 (Morrisey et al., 1996), β-catenin (Mucenski et al., 2003), FOXA1 (Wan et al., 2005) and FOXA2 (Wan et al., 2004; Wan et al., 2005). These transcription factors influence lung morphogenesis, epithelial cell differentiation, and the synthesis of surfactant lipids and proteins required for respiration after birth.

C/EBPα (CCAAT enhancer binding protein α) is a member of a family of basic leucine zipper (bZIP) transcription factors (Alam et al., 1992) that serves an important role in normal tissue development, regulation of cell proliferation and differentiation, lipid metabolism, and lipid biosynthesis (Cao et al., 1991; De Simone and Cortese, 1992; Sugahara et al., 1999; Sugahara et al., 2001). Increased levels of C/EBPα mRNA were observed in tissues that have high rates of synthesis of lipids and cholesterol-linked compounds, including liver, fat, intestine, lung, adrenal gland and placenta (Birkenmeier et al., 1989; Lekstrom-Himes and Xanthopoulos, 1998; Takiguchi, 1998). In fetal lung, C/EBPα expression was detected in subsets of respiratory epithelial cells prior to birth and was abundantly expressed in alveolar type II cells in the peripheral lung (Li et al., 1995). However, elucidation of the roles of C/EBPα in postnatal lung development and function was complicated by the precocious death of mice with a homozygous null mutation in the *Cebpa* gene. Newborn $Cebpa^{-/-}$ pups die primarily from hypoglycemia caused by liver dysfunction (Wang et al., 1995; Burgess-Beusse and Darlington, 1998); however, a subset exhibit clinical symptoms of respiratory distress, displaying a primitive lung (Flodby et al., 1996; Sugahara et al., 2001). In cultured type II epithelial cells, marked increases in C/EBPα mRNA and protein were observed following exposure to FGF7, a growth factor that induces proliferation and surfactant synthesis in the lung (Cardoso et al., 1997; Yano et al., 2000; Mason et al., 2003; Portnoy et al., 2004; Zhang et al., 2004). The temporal-spatial expression of C/EBPα and its known function in lipid metabolism provided a rationale supporting its role in respiratory epithelial cell differentiation and surfactant homeostasis in the perinatal period. To identify the potential roles of C/EBPα in lung morphogenesis and function, we deleted the mouse *Cebpa* gene in respiratory epithelial cells of the fetal lung using a conditional Cre/LoxP recombination system. Deletion of *Cebpa* inhibited differentiation of the fetal lung, causing death from respiratory failure at birth.

MATERIALS AND METHODS

Animals and transgene genotype

$Cebpa^{flox/flox}$ mice were kindly provided by Dr P. Johnson (National Cancer Institute – Frederick, Frederick, MD) (Lee et al., 1997) and maintained as homozygotes in a mixed C57/BL6/FVB/N background. Homologous recombination between loxP sites was accomplished by expression of Cre recombinase using $(tetO)_7CMV\text{-}Cre^{tg/tg}$ mice. The SP-C-$rtTA^{-/tg}$ transgenic mouse line (Perl et al., 2002; Perl et al., 2005) was used for respiratory epithelium specific expression of rtTA (reverse tetracycline transactivation) to cause recombination of the floxed allele after exposure of the dam to doxycycline (Perl et al., 2002; Perl et al., 2005). Triple transgenic mice, herein termed $Cebpa^{\Delta/\Delta}$ mice, were generated by mating $(tetO)_7CMV\text{-}Cre^{-/tg}/Cebpa^{flox/flox}$ to SP-C-$rtTA^{-/tg}/Cebpa^{flox/flox}$. $Cebpa^{flox/flox}$ littermates lacking either rtTA or Cre genes served as controls. Genotypes were identified by PCR with genomic DNA from the tails of fetal and postnatal mice using the forward primer 5′-CCA CTC ACC GCC TTG GAA AGT CAC A-3′, the reverse primer 5′-CCG CGG CTC CAC CTC GTA GAA GTC G-3′ and the knockout primer 5′-AGG GAC CTA ATA ACT TCG TAT AGC A-3′ for $Cebpa^{flox/flox}$. Genotyping for SP-C-rtTA and $(tetO)_7CMV$-Cre DNA was performed by PCR as described previously

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(Perl et al., 2002). *Foxa2*$^{\Delta/\Delta}$ mice in which *Foxa2* was selectively deleted from the respiratory epithelium were generated as previously reported (Wan et al., 2004). Lungs were obtained at E18.5 from *Titf1*-null mice, kindly provided by Dr S. Kimura from the National Institutes of Health (Kimura et al., 1996).

Animal husbandry and doxycycline administration

Animals were maintained in a pathogen-free environment in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation. All animals were housed in humidity- and temperature-controlled rooms on a 12 hour-12 hour light-dark cycle. Mice were allowed food and water ad libitum. There was no serological evidence of pulmonary pathogens or bacterial infections in sentinel mice maintained within the colony. Gestation was dated by detection of the vaginal plug (as E0.5) and correlated with weight of each pup at the time of sacrifice. Dams bearing control and *Cebpa*$^{\Delta/\Delta}$ mice were maintained on doxycycline in food (625 mg/kg; Harlan Teklad, Madison, WI) from E0. At E18.5, dams were killed by exsanguination and lungs were dissected for analysis.

Blood glucose analysis

Glucose concentrations in neonatal blood were measured immediately upon collection using Ascensia Elite Blood Glucose Meter (Model 9662A, Bayer) with Ascensia Elite *XL* Blood Glucose Test Strips that have a 1.1-33.3 mmol/l (20-600 mg/dl) sensitivity.

Morphological analysis

Tissues from fetal lungs were fixed in situ after opening the chest and immersion in 4% paraformaldehyde in PBS and processed into paraffin blocks. Antibodies used for immunohistochemistry were: C/EBPα (1:5000, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA), FOXA1 (1:2000, guinea pig polyclonal antibody) (Wan et al., 2005), TTF1 (1:5000, rabbit polyclonal) (Wan et al., 2005), mature SP-B (1:1000, rabbit polyclonal) (Perl et al., 2002), pro-SP-C (1:2000, rabbit polyclonal AB3428, Chemicon, Temecula, CA), AQP5 (aquaporin 5) (1:10, kindly provided by Dr Anil Menon), vWF (von Willebrand Factor) (1:800, DAKO, Carpinteria, CA), PAS (Periodic Acid Schiff) (K047, Poly Scientific R&D, Bay Shore, NY), TGFβ2 (1:300, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) and α-SMA (α-smooth muscle actin) (1:10,000, mouse monoclonal, clone IA4, Sigma-Aldrich, St Louis, MO). Electron microscopy was performed on lung tissue obtained from E18.5 *Cebpa*$^{\Delta/\Delta}$ mice and littermate controls after fixation in glutaraldehyde (Clark et al., 2001). All experiments shown are representative of findings from at least two independent dams, generating at least four triple-transgenic offspring that were compared with littermates.

RNA isolation and analysis

RNA was prepared by using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Total RNA was isolated by phenol-chloroform extraction and precipitation with isopropanol. A spectrophotometer was used to measure total RNA concentration. Quantification of surfactant protein (SP)-A, SP-B, SP-C and SP-D mRNAs was performed by S1 nuclease assays. C/EBPα, C/EBPβ, C/EBPδ and FOXA2 mRNAs were quantified by RNase protection assays with ribosomal protein L32 as an internal control (Dranoff et al., 1994). Rat FOXA2 cDNA probe was kindly provided by Dr R. Costa (University of Illinois).

RNA microarray analysis

Methods for RNA isolation, amplification and data analysis are essentially as described previously (Xu et al., 2003). Lung cRNA was hybridized to the murine genome MOE430 (consists of ≈45,000 gene entries) chips (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Affymetrix MICROARRAY SUITE 5.0 was used to scan and quantitate the gene chips under default scan settings. Normalization was performed using the three step Robust Multichip Average Model (Irizarry et al., 2003a; Irizarry et al., 2003b): background adjustment, quartile normalization and summarization. Data were further analyzed using Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) and Genespring 7.2 (Silicon Genetics, Redwood City, CA). Multiple criteria were used to select differentially expressed genes conservatively. Detection of differential expression was performed using random permutation and Welch's approximate *t*-test for mutant and control groups at $P \leq 0.01$, False Discovery Rate (FDR) ⩽10%, coefficient variation <50%. Additional filters for positive candidate selection, including a minimal twofold change in absolute ratio and a minimum 2-Present call by Affymetrix algorithm in three samples with a relative higher expression, were used. Gene Ontology (GO) analysis was performed using the publicly available web-based tool, DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Dennis et al., 2003).

Surfactant lipid and protein analysis

The left lobe was homogenized in 0.9% NaCl and protease inhibitor (1:100) (P 8340) (Sigma Chemical, St Louis, MO) was added to the lung homogenate (LH). Saturated phosphatidylcholine (Sat PC) was isolated from lipid extracts of lung homogenate using osmium tetroxide (OsO_4) (Mason et al., 1976), followed by measurement of phosphorous (Bartlett, 1959). Phospholipid composition was determined after 2D thin-layer chromatography (Ikegami et al., 2003). For SP-B and SP-C immunoblot analysis, extracted lipids of lung homogenate (5% for SP-B, 10% for SP-C) were loaded on a SDS/PAGE gel and transferred to nitrocellulose (0.10 μm) with antiserum against mature human SP-B peptide (Chemicon, Temecula, CA) or high-titer anti-SP-C antibody, raised against a modified human recombinant 34 amino acid SP-C peptide (Glasser et al., 2001). Supernatant of lung homogenate (1500 ***g*** for 15 minutes) was subjected to a SDS/PAGE gel to estimate the content of SP-A and SP-D. Resolved proteins were transferred to a nitrocellulose membrane (0.45 μm) and immunoblot analysis was performed with guinea pig anti-rat SP-A (Ikegami et al., 2003) or rabbit anti-mouse SP-D (Stahlman et al., 2002) antiserum diluted in Tris-buffered saline with 0.1% Tween (TBS-T). Goat anti-guinea pig IgG peroxidase-conjugate (Sigma, St Louis, MO) was used for SP-A. Goat anti-rabbit (heavy and light chain) peroxidase-conjugate (Calbiochem, La Jolla, CA) was used as the secondary antibody for SP-B, SP-C and SP-D.

For identification of ABCA3 (ATP-binding cassette transporter 3), homogenized lung tissue was sonicated in buffer containing 5% 1 M Tris-HCl, pH 7.5; 1% 100 mM EGTA; 0.2% 500 mM EDTA with Complete Mini – protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), and 1% PMSF with a Fisher Sonic Dismembrator Model 300 using a micro-tip operated at 35%. The homogenate was centrifuged at 20,000 ***g*** for 15 minutes and 50 μg of protein from the supernatant was electrophoresed on a SDS/PAGE gel under non-reducing conditions. Following electrophoresis, proteins were transferred to nitrocellulose paper and immunoblot analysis was performed with antisera against rabbit polyclonal ABCA3 (kind gift from Dr T. Weaver, Cincinnati Children's Hospital Medical Center). Incubation in goat anti-rabbit (heavy and light chain) peroxidase-conjugate (Calbiochem, La Jolla, CA) was used to detect the antigen-antibody complexes.

For detection of FAS, RNA was treated with DNase inactivation reagent (Ambion, Austin, TX) before cDNA synthesis. DNase-treated total-lung RNA (10 μg) was reverse transcribed into cDNA using oligo (dT) and analyzed by real-time PCR with the Smart Cycler System (Cepheid, Sunnyvale, CA). The relative concentration of FAS mRNA was standardized to the internal control β-actin. Primers used to quantify β-actin mRNA were 5′-TGG AAT CCT GTG GCA TCC ATG AAC-3′ and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′, and to quantify FAS mRNA were 5′-GGA CAT GGT CAC AGA CGA TGA C-3′ and 5′-GTC GAA CTT GGA CAG ATC CTT CA-3′.

Transient transfection of promoter constructs

Promoter-luciferase constructs (see Table S1 in the supplementary material) were co-transfected with increasing amounts of pCMV5-C/EBPα (0, 0.05, 0.1 and 0.4 pmol) into HeLa and H441 cells. Mouse FOXA2 promoter luciferase construct was kindly provided by Dr K. Kaestner, University of Pennsylvania School of Medicine. FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) was used for transfection in accordance with the manufacturer's specifications. Forty-eight hours following

transfection, luciferase activity was assessed and normalized for co-transfection by β-galactosidase activity. All transfections were performed in triplicate.

Quantitative analysis

Immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham, Chicago, IL) and band intensities were quantified by densitometry (ImageQuant v5.2, GE Healthcare, Piscataway, NJ). Statistical differences were determined using unpaired Student's *t*-tests. Comparisons among groups were assessed by Analysis of Variance (ANOVA) with Tukey's tests used for post-hoc analyses. Results were expressed as mean±s.e.m. Differences were considered significant at the 5% level.

RESULTS

Deletion of *Cebpa*

Cebpa was deleted by expression of Cre recombinase under conditional control of the reverse tetracycline transactivator (rtTA) expressed in respiratory epithelial cells of the fetal lung directed by the *Sftpc* gene promoter, producing $Cebpa^{\Delta/\Delta}$ mice. Analysis of genotyped litters at E18.5 demonstrated Mendelian inheritance, indicating no fetal loss related to the $Cebpa^{\Delta/\Delta}$ alleles. SP-C-rtTA$^{-/tg}$, (tetO)$_7$Cre$^{-/tg}$, $Cebpa^{flox/flox}$ dams (n=24 litters) were maintained on doxycycline from E0. Pups were genotyped between E16.5 and P1. At birth, body weights and lung weights of control and $Cebpa^{\Delta/\Delta}$ mice were similar: 1.73±0.06 g versus 1.71±0.06 g (P>0.05) and 53.1±3.2 mg versus 49.9±3.9 mg (P>0.05), respectively. Blood glucose concentrations in $Cebpa^{\Delta/\Delta}$ mice were similar to control littermates at birth: 127±6 mg/dl versus 132±7 mg/dl (P>0.05).

The extent of *Cebpa* gene deletion in respiratory epithelial cells in $Cebpa^{\Delta/\Delta}$ mice was assessed the day before birth (E18.5), demonstrating extensive reduction of *Cebpa* by immunohistochemistry. C/EBPα was detected in alveolar type II cells in littermate controls throughout the respiratory epithelium at E16.5 (data not shown), E18.5 (Fig. 1A) and P1 (data not shown), consistent with previous findings (Alam et al., 1992; Sugahara et al., 2001). By contrast, nuclear staining of C/EBPα was generally absent in terminal lung saccules of $Cebpa^{\Delta/\Delta}$ mice (Fig. 1B).

C/EBPα is required for pulmonary maturation in late gestation

When observed during normal delivery (E19.5-E21), control mice became oxygenated and survived normally. $Cebpa^{\Delta/\Delta}$ mice developed severe respiratory distress and generally died within 2-3 hours of birth. Histological examination of $Cebpa^{\Delta/\Delta}$ pups revealed extensive atelectasis and pulmonary congestion (data not shown), findings consistent with impaired maturation of the respiratory epithelium. During the saccular period (E17.5–E18.5) of fetal lung

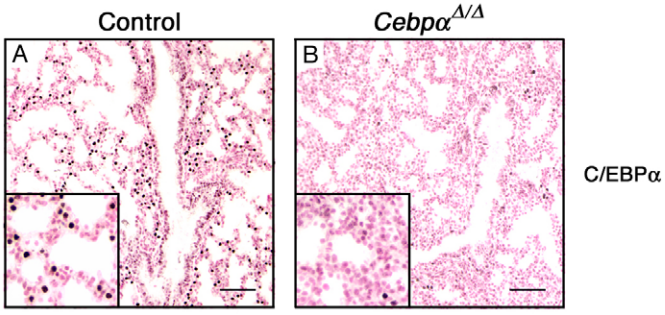

Fig. 1. Deletion of *Cebpa*. Lung sections from control (**A**) and $Cebpa^{\Delta/\Delta}$ (**B**) mice were prepared at E18.5 and stained using a C/EBPα polyclonal antibody. Nuclear C/EBPα staining was observed in alveolar epithelial type II cells of control littermates and was absent or decreased in $Cebpa^{\Delta/\Delta}$ mice. Scale bar: 100 μm.

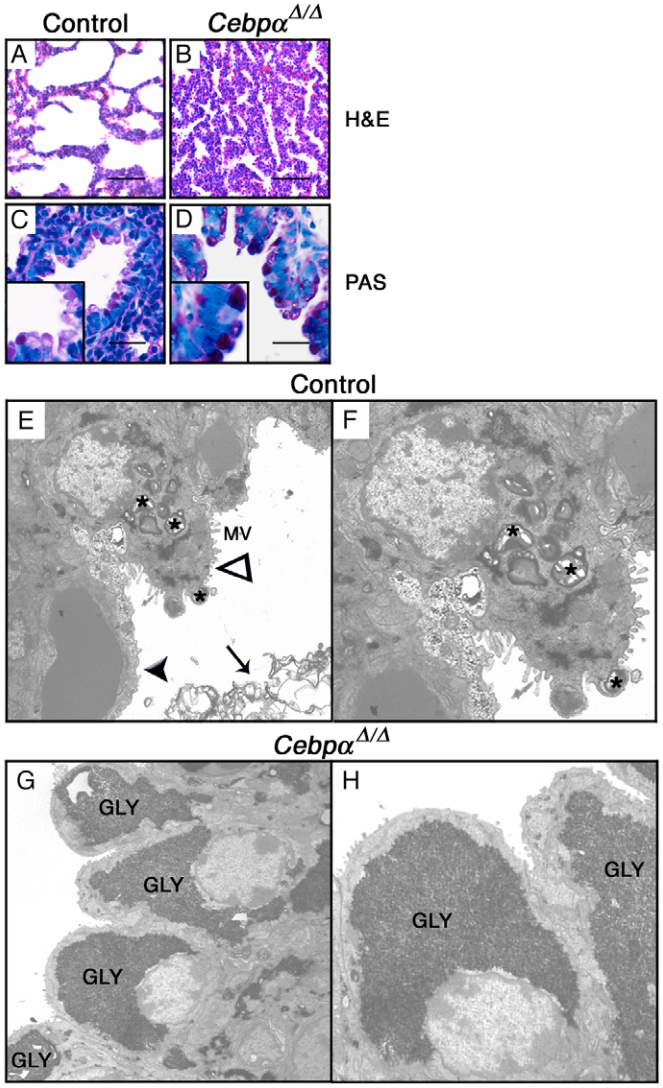

Fig. 2. Pulmonary immaturity in $Cebpa^{\Delta/\Delta}$ mice. (**A**) Lung sections prepared at E18.5 and stained with Hematoxylin and Eosin revealed normal lung inflation and histology, indicated by saccular structures lined by squamous type I cells and cuboidal type II cells, in control littermates. (**B**) Severe atelectasis, pulmonary congestion, reduced septation, thickened mesenchyme and columnar epithelium lining the peripheral saccules were observed in the $Cebpa^{\Delta/\Delta}$ lung. Scale bar: 50 μm. (**C**,**D**) Consistent with the observed immaturity in the $Cebpa^{\Delta/\Delta}$ lung, PAS staining demonstrated increased glycogen content in the bronchiolar epithelium of $Cebpa^{\Delta/\Delta}$ mice (D) compared with control littermates (C). Scale bar: 1 μm. Electron microscopy was performed on lungs from control (**E**,**F**) and $Cebpa^{\Delta/\Delta}$ (**G**,**H**) mice at E18.5. Squamous type I cells (black, arrowhead) and cuboidal type II cells (white triangle) containing numerous lamellar bodies (*), apical microvilli (MV) and highly organized rosette glycogen were observed in the lungs of control mice. Lamellar bodies were observed in the lumen of peripheral airspaces and secreted surfactant (thin arrow) was identified in the airspaces (E). By contrast, lamellar bodies, secreted surfactant, and type I cells were not observed in the lungs of $Cebpa^{\Delta/\Delta}$ mice (G). Cytoplasmic glycogen (GLY) was dispersed and apical microvilli were smaller in epithelial cells lining the abnormal lung tubules (H). Micrographs are representative of three $Cebpa^{\Delta/\Delta}$ mice and littermate controls.

morphogenesis, the terminal lung buds dilate, the mesenchyme and epithelium thin, and extensive ingrowth of pulmonary capillaries invades in close proximity to squamous respiratory epithelial cells (type I cells) as the gas exchange surface of the lung forms. At E18.5, the peripheral lung of control mice consisted of saccules lined by both squamous type I cells and cuboidal type II cells, indicating structural maturation typical at this stage of gestation (Fig. 2A). By contrast, dilation of peripheral saccules was reduced, the mesenchyme remained thickened, and the peripheral saccules were lined by a cuboidal epithelium that lacked squamous type I cells in the lungs of *Cebpa*$^{\Delta/\Delta}$ mice (Fig. 2B), findings consistent with pulmonary immaturity.

Immature type II cells are glycogen-rich and as they differentiate, glycogen is converted into phospholipids and mobilized to lamellar bodies (Ridsdale and Post, 2004). Sections from *Cebpa*$^{\Delta/\Delta}$ mice showed increased levels of PAS staining in bronchiolar epithelium, indicated by the red deposit (Fig. 2D), compared with their control littermates (Fig. 2C).

At the ultrastructural level, lungs from E18.5 *Cebpa*$^{\Delta/\Delta}$ mice were immature compared with their littermates. In controls, cuboidal type II cells contained numerous lamellar bodies, apical microvilli and highly organized rosette glycogen (Fig. 2E,F). Lamellar bodies and secreted surfactant were observed in the lumen of peripheral airspaces at E18.5. By contrast, cytoplasmic glycogen was dispersed and apical microvilli were smaller in type II epithelial cells lining the immature lung tubules of *Cebpa*$^{\Delta/\Delta}$ mice (Fig. 2G,H). Squamous type I cells were lacking and the extent of capillary invasion was decreased. Lamellar bodies, the intracellular storage form of pulmonary surfactant, were absent in type II cells, and secreted surfactant was not detected in the airspaces of *Cebpa*$^{\Delta/\Delta}$ mice at E18.5.

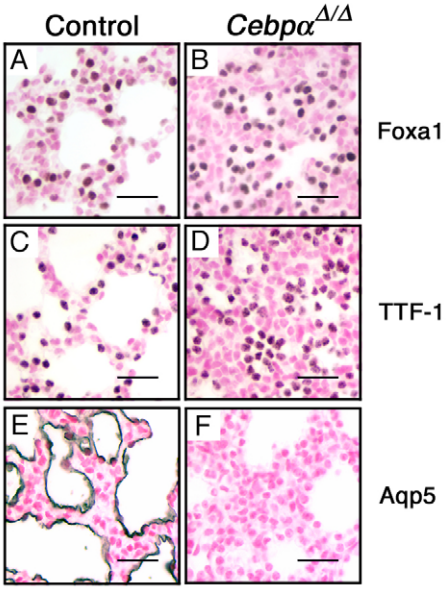

Fig. 3. Alveolar type I and type II cell maturation is disrupted in *Cebpa*$^{\Delta/\Delta}$ mice. Serial sections of lung from E18.5 control and *Cebpa*$^{\Delta/\Delta}$ mice were stained for FOXA1 (**A**,**B**), TTF1 (**C**,**D**) and Aqp5 (**E**,**F**). In *Cebpa*$^{\Delta/\Delta}$ mice, the cuboidal epithelium of the peripheral lung saccules homogeneously expressed FOXA1 and TTF1, indicative of structural immaturity. Consistent with pulmonary immaturity, Aqp5 staining, a marker of squamous type I cells, was absent in lungs of *Cebpa*$^{\Delta/\Delta}$ mice. Scale bar: 25 μm.

Alveolar type I and type II cell maturation is disrupted in *Cebpa*$^{\Delta/\Delta}$ mice

FOXA1 and TTF1, transcription factors expressed in respiratory epithelial cells lining both the conducting and peripheral airways of the lung, each play distinct roles in lung formation and function. At E18.5, expression of FOXA1 and TTF1 was restricted to type II cells in peripheral lung saccules of control mice, as previously reported (Stahlman et al., 1996; Besnard et al., 2004; Wan et al., 2004; Wan et al., 2005) (Fig. 3A,C). In *Cebpa*$^{\Delta/\Delta}$ mice, the cuboidal epithelium of the peripheral lung saccules expressed FOXA1 and TTF1, in a relatively homogenous pattern, indicating a lack of squamous type I cells and the predominance of immature cuboidal epithelial cells that is characteristic of earlier stages of lung morphogenesis (Fig. 3B,D). Aquaporin 5 staining, a selective marker of squamous type I cells, was decreased in the peripheral lung saccules of *Cebpa*$^{\Delta/\Delta}$ mice (Fig. 3F), consistent with pulmonary immaturity.

Expression of surfactant proteins, SP-A, SP-B, SP-C and SP-D in type II epithelial cells in the peripheral lung normally increases prior to birth (Randell and Young, 2004). Of these, SP-B and SP-C play crucial roles in surfactant function and homeostasis (Clark et al., 1995; Clark et al., 2001; Ikegami et al., 2003; Shulenin et al., 2004). Mature SP-B and proSP-C staining was decreased in conducting airways and peripheral lung saccules in the lungs of *Cebpa*$^{\Delta/\Delta}$ mice (Fig. 4B,D), demonstrating that normal pulmonary epithelial cell differentiation and expression of surfactant proteins are dependent upon C/EBPα.

In contrast to the inhibitory effect of *Cebpa* deletion on differentiation of the peripheral lung, no abnormalities in formation or differentiation of epithelial cells in conducting airways was observed. Morphology and expression of CCSP, a non-ciliated secretory cell bronchial marker, and Foxj1, a ciliated cell marker, were not altered in conducting airways at sites of *Cebpa* deletion (data not shown).

C/EBPα is required in synthesis of surfactant lipids and proteins

Pulmonary surfactant is required in lung function. Lung Sat PC, a crucial component of pulmonary surfactant, and FAS, a major precursor of fatty acid synthesis in the lung, normally increases before birth (Das, 1980; Pope and Rooney, 1987; Sa et al., 1990;

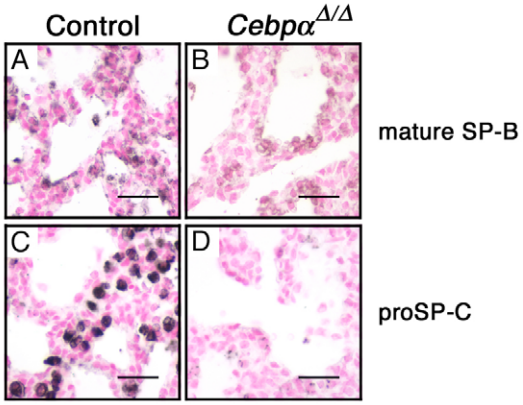

Fig. 4. C/EBPα regulates expression of surfactant proteins in vivo. Serial sections of lung from control and *Cebpa*$^{\Delta/\Delta}$ mice at E18.5 were stained for mature SP-B (**A**,**B**) and proSP-C (**C**,**D**). Staining for mature SP-B and proSP-C was markedly reduced in conducting airways and peripheral lung saccules in *Cebpa*$^{\Delta/\Delta}$ mice. Scale bar: 25 μm.

Table 1. Phospholipid composition after deletion of *Cebpα*

	Control (*n*=6)	*Cebpα*$^{\Delta/\Delta}$ (*n*=6)
SatPC μmol/kg of body weight	89.7±6.9	38.8±2.8*
FAS mRNA	2.35±0.11	1.09±0.07*
Phospholipid composition† (%)		
Phosphatidylcholine	57.6±0.2	57.8±1.4
Phosphatidylethanolamine / Phosphatidylserine	6.0±0.6	6.1±1.2
Phosphatidylglycerol	15.4±1.0	16.8±0.3
Phosphatidylinositol	5.7±0.3	5.9±0.4
Sphingomyelin	9.9±1.5	10.4±0.7
Lyso-phosphatidylcholine	1.5±0.3	1.5±0.2
Lysobisphosphatidic acid	2.7±0.7	1.5±0.2

Values are mean±s.e.m.
*P<0.05 compared with control littermates.
†Three samples of pooled lung homogenate from two mice were evaluated for the relative abundance of phospholipids.

Stahlman et al., 1996). In *Cebpa*$^{\Delta/\Delta}$ mice at E18.5, Sat PC and FAS expression were significantly decreased, whereas the fractional content of PC and other minor phospholipid components were unaltered in the lungs of *Cebpa*$^{\Delta/\Delta}$ mice (Table 1). Likewise, levels of SP-A, SP-B, SP-C and SP-D mRNAs (Fig. 5A) and proteins (Fig. 5B) were significantly decreased in *Cebpa*$^{\Delta/\Delta}$ mice, consistent with immunohistochemical findings.

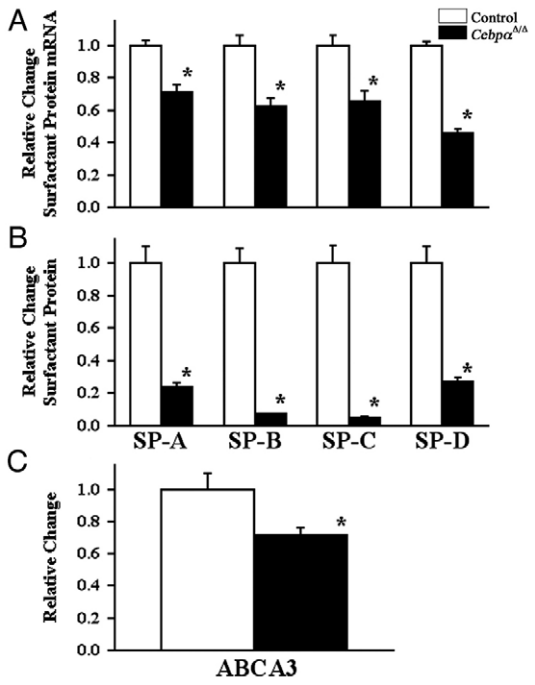

Fig. 5. C/EBPα regulates expression of surfactant proteins and ABCA3. Surfactant protein mRNAs and surfactant proteins were decreased in *Cebpa*$^{\Delta/\Delta}$ mice. Total lung RNA was prepared from fetal mouse lung at E18.5. S1 nuclease assay was performed with 3 μg of total RNA for estimation of SP-A, SP-B, SP-C, SP-D and ribosomal protein L32 mRNA. (**A**) Protected fragments were standardized to the internal control L32 and normalized to control littermates. (**B**) Decreased surfactant proteins (SP-A, SP-B, SP-C and SP-D) were detected in lung homogenates from *Cebpa*$^{\Delta/\Delta}$ mice. (**C**) Western blot analysis demonstrated that ABCA3 protein was reduced after deletion of *Cebpa*. Lung homogenates were prepared from *Cebpa*$^{\Delta/\Delta}$ and littermate controls at E18.5. Surfactant proteins and ABCA3 were identified by western blot analysis, quantified by densitometry and normalized to controls. n=6 animals per group; *P<0.05 versus control.

Mutations in *ABCA3* cause respiratory failure in newborn infants (Shulenin et al., 2004). ABCA3 is present in the limiting membranes of lamellar bodies in type II epithelial cells. The structure of the ABCA3 transporter and its localization in the cell suggests an intrinsic role in intracellular surfactant lipid homeostasis (Whitsett et al., 2004). In *Cebpa*$^{\Delta/\Delta}$ mice at E18.5, ABCA3 protein levels were significantly decreased (Fig. 5C). As lack of either SP-B or ABCA3 causes respiratory failure at birth (Clark et al., 1995; Perl et al., 2002; Shulenin et al., 2004; Whitsett et al., 2004), deficiency of these proteins and decreased surfactant lipids are likely to contribute to respiratory failure in *Cebpa*$^{\Delta/\Delta}$ mice.

Vascular and smooth muscle development in lungs of *Cebpa*$^{\Delta/\Delta}$ mice

vWF staining was used to identify the pulmonary capillary bed at E18.5. In control lungs at E18.5, an extensive vascular network associated with thinning of the walls of the peripheral saccules was observed (Fig. 6A). By contrast, vWF staining revealed a relatively undeveloped capillary bed, consistent with the generalized immaturity of the lung in *Cebpa*$^{\Delta/\Delta}$ mice. vWF stained vessels were embedded within the thickened mesenchyme of the immature lung saccules (Fig. 6B). There was no evidence of hemorrhage. α-SMA staining, a marker for pulmonary arteries and bronchial smooth muscle was similar in control and *Cebpa*$^{\Delta/\Delta}$ lungs (Fig. 6C,D), suggesting that the deletion of *Cebpa* did not inhibit pulmonary smooth muscle cell differentiation.

Expression of *Cebpb* and *Cebpd* in *Cebpa*$^{\Delta/\Delta}$ mice

Because C/EBPα, C/EBPβ and C/EBPδ are closely related bZIP transcription factors that are co-expressed in respiratory epithelial cells, they may serve agonistic or antagonistic roles at specific transcriptional targets in the lung. Expression of C/EBPα was significantly reduced; however, levels of C/EBPβ and C/EBPδ mRNA in *Cebpa*$^{\Delta/\Delta}$ mice were comparable with control littermates (Fig. 7).

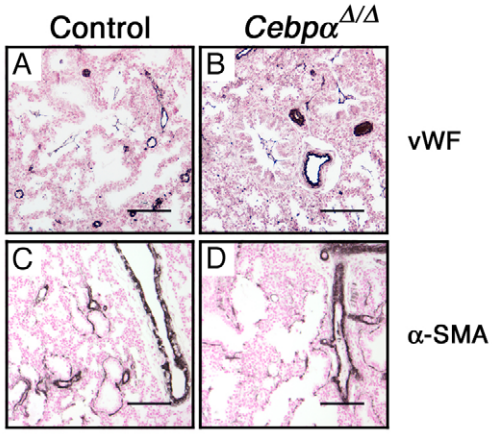

Fig. 6. C/EBPα influences maturation of the capillary-epithelial interface. Lung sections from *Cebpa*$^{\Delta/\Delta}$ mice and control littermates were obtained at E18.5 and immunostained for vWF and α-SMA. In controls (**A**), an extensive vascular network was observed in which vessels are located in close apposition to squamous type I cells. In *Cebpa*$^{\Delta/\Delta}$ mice (**B**), a relatively undeveloped capillary bed, with vessels embedded within the thickened mesenchyme of the immature lung saccules, was observed. Expression of α-SMA was detected in smooth muscle cells surrounding the bronchioles in both control (**C**) and *Cebpa*$^{\Delta/\Delta}$ (**D**) mice. Scale bar: 50 μm.

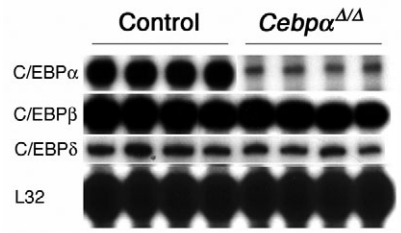

Fig. 7. C/EBPα does not regulate C/EBPβ and C/EBPδ in vivo. C/EBPα mRNA was decreased but expression of C/EBPβ and C/EBPδ mRNA was maintained in lungs from *Cebpa*$^{\Delta/\Delta}$ mice. Total lung RNA was prepared from fetal mouse lung at E18.5. RNAse protection assay was performed with 7 μg of total RNA for estimation of C/EBPα, C/EBPβ, C/EBPδ, and ribosomal protein L32 mRNA. Protected fragments were standardized to the internal control L32 and normalized to control littermates.

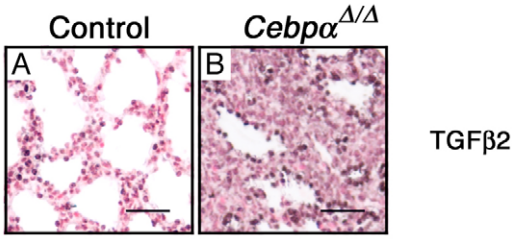

Fig. 8. C/EBPα regulates expression of TGFβ2. (**A**) Control section. (**B**) Increased TGFβ2 immunostaining was detected in lungs of *Cebpa*$^{\Delta/\Delta}$ mice at E18.5. TGFβ2 expression was detected in mesenchymal tissue, type II cells and in the epithelial lining of the developing airway along a proximodistal gradient. Scale bar: 25 μm.

C/EBPα regulates gene expression: RNA microarray analysis

To identify the RNAs influenced by the conditional deletion of *Cebpa*, lung RNA from control and *Cebpa*$^{\Delta/\Delta}$ mice were compared at E18.5 using Affymetrix murine genome MOE430 gene chips. One hundred and twelve genes were identified that were significantly altered after deletion of *Cebpa*. Among them, 64 mRNAs were increased and 48 mRNAs were decreased in response to the deletion of *Cebpa* in the lung (see Table S2 in the supplementary material). Selected genes are shown in a heat map (see Fig. S1 in the supplementary material). Differentially expressed genes were classified according to Gene Ontology (GO) classification on Biological Process. The Fisher Exact Test was used to calculate the probability of each category that was over-represented in the selected list using the entire MOE430 mouse genome as a reference dataset. Genes involved in lipid metabolism ($P=1\times10^{-7}$) and fatty acid metabolism ($P=4\times10^{-5}$) were markedly decreased in lungs of *Cebpa*$^{\Delta/\Delta}$ mice. Genes regulating related biological processes, including lipid biosynthesis and lipid catabolism, were also decreased in lungs of *Cebpa*$^{\Delta/\Delta}$ mice ($P<0.05$), demonstrating that *Cebpa* plays a crucial role in the regulation of genes influencing lipid homeostasis in the lung. By contrast, expression of genes involved in muscle contraction ($P=8\times10^{-7}$), morphogenesis ($P=2\times10^{-6}$), and growth and development ($P=1\times10^{-5}$) was significantly increased in response to the deletion of *Cebpa* (see Table S3 in the supplementary material).

As pulmonary maturation is delayed in *Cebpa*$^{\Delta/\Delta}$ mice, we queried the microarray to retrieve factors involved in lung epithelial cell differentiation. TGFβ2 mRNA was increased in the lungs of *Cebpa*$^{\Delta/\Delta}$ mice. Consistently, intensity and numbers of cells immunostained for TGFβ2 was increased in the lungs of *Cebpa*$^{\Delta/\Delta}$ mice at E18.5 (Fig. 8). Growth factor signaling is an essential component of the regulatory network controlling proliferation, differentiation and pattern formation of the lung (Cardoso et al., 1997; Yano et al., 2000; Mason et al., 2003; Portnoy et al., 2004). As TGFβ2 signaling is known to inhibit lung maturation and type II cell differentiation (Whitsett et al., 1992; Lebeche et al., 1999), effects of *Cebpa* deletion may be mediated in part by increased expression of TGFβ2.

As FOXA2 and TTF1 regulate expression of surfactant proteins and deletion of either *Foxa2* or mutations in *Titf1* delays lung maturation and causes respiratory failure at birth (Minoo et al., 1999; Cassel and Nord, 2003; Wan et al., 2004), we sought to determine whether these genes share transcriptional targets or participate in regulatory programs crucial for lung differentiation at birth. Comparison of mRNA profiles in lung tissue from *Cebpa*$^{\Delta/\Delta}$, *Foxa2*$^{\Delta/\Delta}$ (Wan et al., 2004), *Titf1*$^{PM/PM}$ (deFelice et al., 2003) (phosphorylation mutant) mice at E18.5 demonstrated that a number of mRNAs and their biological processes were shared, compared with age-matched control littermates (data not shown). Genes involved in the regulation of lung lipid homeostasis (*Lrp2*, *Sftpb*, *Ldlr*, *Abca3*, *Dlk1*, *Scd1* and *Pon1*) and inflammation (*Sftpa1*, *Hc* and *Lyzs*) were decreased in response to the selective deletion of either *Cebpa*, *Foxa2* or mutation of *Titf1* in the respiratory epithelium. To test whether *Foxa2* or *Titf1* were required for expression of C/EBPα in respiratory epithelial cells, immunohistochemistry for C/EBPα was assessed in the lungs from *Titf1*$^{-/-}$ (Kimura et al., 1996) and *Foxa2*$^{\Delta/\Delta}$ (Wan et al., 2004) transgenic mice at E18.5. C/EBPα immunostaining was markedly decreased or absent in the respiratory epithelium of mutant *Titf1*$^{-/-}$ and was decreased in *Foxa2*$^{\Delta/\Delta}$ mice (Fig. 9), providing evidence that normal expression of C/EBPα is dependent upon both *Foxa2* and *Titf1*.

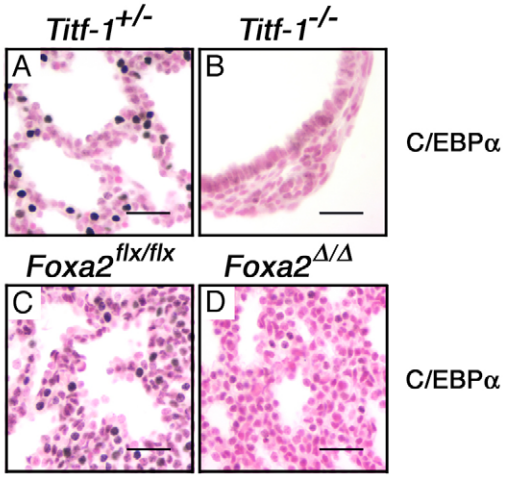

Fig. 9. Decreased expression of C/EBPα in *Titf1*$^{-/-}$ mice and *Foxa2*$^{\Delta/\Delta}$ mice. Lung sections from *Titf1*$^{+/-}$, *Titf1*$^{-/-}$, *Foxa2*$^{flx/flx}$ and *Foxa2*$^{\Delta/\Delta}$ mice were prepared on E18.5 and immunostained for C/EBPα. C/EBPα was not altered in lungs from *Titf1*$^{+/-}$ (**A**) and *Foxa2*$^{flx/flx}$ (**C**) mice. *Titf1*$^{-/-}$ mice (**B**) have a rudimentary bronchial tree associated with absent peripheral lung formation in which C/EBPα staining was markedly decreased or absent. Expression of C/EBPα was decreased in respiratory epithelial cells in the lungs of *Foxa2*$^{\Delta/\Delta}$ (deficient) mice (**D**). Scale bar: 25 μm.

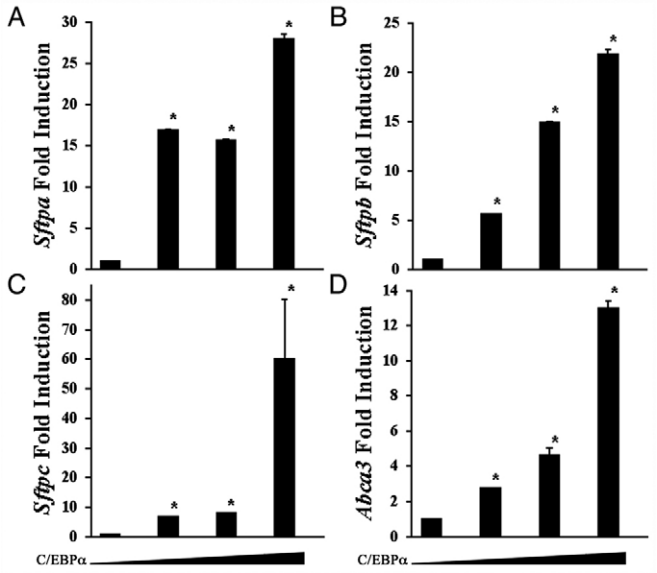

Fig. 10. C/EBPα activates gene transcription in vitro. C/EBPα enhanced luciferase activity in HeLa cells after co-transfection with *Sftpa* (**A**), *Sftpb* (**B**), *Sftpc* (**C**) and *Abca3* (**D**) promoter constructs in a dose-dependent manner. **P*<0.05 versus control.

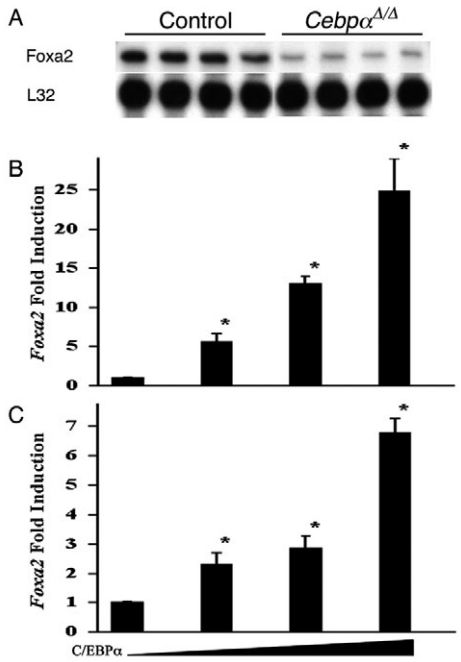

Fig. 11. C/EBPα regulates *Foxa2* in vitro and in vivo. FOXA2 mRNA levels were decreased in lungs from *Cebpa*$^{\Delta/\Delta}$ mice (**A**). Total lung RNA was prepared from fetal mouse lung at E18.5 from dams that were treated with doxycycline from E0 to E18.5. RNase protection assay was used to estimate FOXA2 and ribosomal protein L32 mRNA. Protected fragments were standardized to the internal control L32 and normalized to control littermates. (**B**,**C**) HeLa (B) and H441 (C) cells were transfected with reporter plasmid pGL3-*Foxa2*p1.6 with increasing amounts of expression plasmid pCMV5-C/EBPα. FOXA2 promoter activity was determined by relative luciferase activity and normalized for co-transfection using β-galactosidase. Plasmid pcDNA was used as an empty control. Activation of FOXA2-luciferase construct by C/EBPα was dose dependent in both HeLa and H441 cells. **P*<0.05 versus control.

Transcription of selected C/EBPα target genes in vitro

To test whether the effects of C/EBPα on target gene expression was induced by transcriptional activation, co-transfection experiments were performed using promoter constructs expressing luciferase under control of several genes regulating surfactant homeostasis in vitro. C/EBPα enhanced activities of *Sftpa*, *Sftpb*, *Sftpc* and *Abca3* promoters in HeLa cells in vitro (Fig. 10).

mRNA analysis demonstrated that FOXA2 expression was decreased in lungs from *Cebpa*$^{\Delta/\Delta}$ mice in vivo (Fig. 11A). Therefore, the potential role of C/EBPα on *Foxa2* promoter activity was assessed in vitro. C/EBPα increased the activity of a reporter construct containing 1.6 kb of the promoter region of *Foxa2* in a dose-dependent manner in both HeLa (Fig. 11B) and H441 pulmonary adenocarcinoma cells (Fig. 11C). Thus, C/EBPα may influence perinatal lung maturation by activating surfactant protein gene expression or by regulating FOXA2 expression, the latter also required for normal lung function (Wan et al., 2004). Conversely, C/EBPα staining was decreased in the lungs of mice in which *Foxa2* was selectively deleted (Fig. 9D), supporting the concept that each gene influences expression of the other, indicating that *Cebpa* and *Foxa2* participate in a network regulating perinatal lung maturation.

DISCUSSION

Deletion of the *Cebpa* gene in the respiratory epithelium

Normal formation and function of the lung is essential for the transition of the fetus to an air-breathing environment at birth. Although branching morphogenesis was unaltered, morphological maturation, epithelial cell differentiation and surfactant synthesis were inhibited after deletion of the *Cebpa* gene in epithelial cells of the fetal mouse lung. Previous studies of *Cebpa*$^{-/-}$ mice have reported high incidence of early perinatal loss. Studied survivors demonstrated glucose abnormalities and died from hypoglycemia in the newborn period (Wang et al., 1995; Flodby et al., 1996; Sugahara et al., 2001). In the present study, the *Cebpa* gene was selectively deleted in the respiratory epithelium. Thus, *Cebpa*$^{\Delta/\Delta}$ mice were euglycemic but, nevertheless, died from respiratory failure after birth, demonstrating its crucial role in perinatal lung function. Normal expression of C/EBPα was dependent upon TTF1 and FOXA2, transcription factors that play a crucial role in perinatal lung differentiation. C/EBPα regulated a number of genes influencing surfactant synthesis, some of which were co-regulated by TTF1 and FOXA2. Thus, *Cebpa* participates in a transcriptional network orchestrating perinatal lung maturation and function at birth.

Deletion of *Cebpa* disrupts lung maturation and epithelial cell differentiation

C/EBPα has previously been shown to regulate cell differentiation in lung cancer cell lines (Halmos et al., 2002) and other cell culture models (MacDougald and Lane, 1995; Nord et al., 1998). C/EBPα also plays a crucial role in adipocyte differentiation (Rosen and Spigelman, 2000) and may be involved in the synthesis of lipid precursors of surfactant phospholipids through lipogenesis. Targeted disruption of the *Cebpa* gene in the mouse lung inhibited maturation of type II cells, suppressed lipid precursors and surfactant synthesis, and delayed thinning of lung saccules associated with normal pulmonary maturation in late gestation. Structural and biochemical maturation of the developing lung involves coordinate biosynthesis and regulation of surfactant components. Surfactant phospholipids and surfactant proteins increase prior to birth (Hallman and Gluck,

1976; Stahlman et al., 1996), correlating temporally with C/EBPα expression (Li et al., 1995; Rosenberg et al., 2002; Barlier-Mur et al., 2003). Analysis of whole lung lipid indicated decreased surfactant lipid content without alteration of phospholipid composition in the lungs of *Cepaba*$^{\Delta/\Delta}$ mice. It is unclear, however, whether reduced lipid synthesis in *Cebpa*$^{\Delta/\Delta}$ lungs is mediated by a direct inhibitory effect on transcription or by a generalized delay in lung maturation. Activity of the surfactant protein promoters was stimulated by C/EBPα in vitro and a marked decrease in surfactant protein expression was observed in type II cells of *Cebpa*$^{\Delta/\Delta}$ mice in vivo, supporting a cell-autonomous effect of C/EBPα on surfactant protein expression. Consistent with the anatomic immaturity seen in the *Cebpa*$^{\Delta/\Delta}$ mice, alveolar type I cells were lacking in association with decreased expression of aquaporin 5 (an alveolar type I cell marker). Delayed vascular invasion suggests that C/EBPα also influences paracrine or paracellular signaling from the epithelium to the endothelium.

Branching morphogenesis is unaltered in *Cebpa*$^{\Delta/\Delta}$ mice

The present findings demonstrate that C/EBPα plays a regulatory role in maturation of the lung in late gestation but is not required for branching morphogenesis. TTF1, a transcription factor required for branching morphogenesis of the embryonic lung (Stahlman et al., 1996; Minoo et al., 1999; Cassel and Nord, 2003), was not altered in *Cebpa*$^{\Delta/\Delta}$ mice. This is consistent with the developmental pattern of C/EBPα which increases in late gestation in temporal proximity to alveolar type II cell differentiation (Li et al., 1995; Rosenberg et al., 2002).

C/EBPα is required for surfactant synthesis at birth

SP-B and ABCA3 are required for transition to air breathing at birth. Mutations in either gene cause respiratory failure in human infants (Clark et al., 1995; Shulenin et al., 2004; Whitsett et al., 2004). Targeted disruption of *Sftpb* in mice and mutations in *ABCA3* in humans impaired formation of lamellar bodies and caused respiratory distress because of the lack of pulmonary surfactant (Nogee et al., 2000; Randell and Young, 2004; Shulenin et al., 2004). In the lungs of *Cebpa*$^{\Delta/\Delta}$ mice, lamellar bodies were absent, and levels of surfactant proteins and ABCA3 were significantly decreased. Lack of surfactant phospholipid, SP-B and ABCA3, as well as the structural immaturity of the lung, are probably sufficient to account for perinatal respiratory failure seen after deletion of *Cebpa*.

Distinct roles of *Cebpa*, *Cebpb* and *Cebpd* in regulating gene expression

C/EBPα, C/EBPβ and C/EBPδ transcription factors exhibit highly conserved regions of amino acid sequence identity to the bZIP DNA-binding domain and demonstrate overlapping patterns of spatial expression (He and Crouch, 2002; Ramji and Foka, 2002; Rosenberg et al., 2002). Furthermore, these transcription factors can homo- or heterodimerize to regulate transcription (Lekstrom-Himes and Xanthopoulos, 1998). Therefore, C/EBPα, C/EBPβ and C/EBPδ may compete or serve complementary roles at specific translational targets. C/EBPα, C/EBPβ and C/EBPδ were shown to transcriptionally activate expression of SP-A (Matlapudi et al., 2002; Rosenberg et al., 2002) and SP-D (He and Crouch, 2002) in vitro. However, expression of C/EBPβ and C/EBPδ was unchanged in the *Cebpa*$^{\Delta/\Delta}$ mice; therefore, it is unclear whether maintenance of C/EBPβ and C/EBPδ plays a role in the phenotypic changes observed in the lung.

Role of *Tgfb2* in *Cebpa*$^{\Delta/\Delta}$ mice

Growth factors play important roles in mammalian development, including lung epithelial cell proliferation and differentiation. Deletion of *Cebpa* enhanced expression of TGFβ2 in the respiratory epithelium. Increased expression of TGFβ inhibited lung maturation in late gestation (Zhou et al., 1996; Bartram and Speer, 2004) and inhibited expression of surfactant synthesis (Whitsett et al., 1992; Jaskoll et al., 1996). SMAD3 is a transcription factor mediating TGFβ signaling (Massagué, 1998; Attisano and Wrana, 2000; Shi and Massagué, 2003). The expression of both TGFβ2 and SMAD3 was increased in *Cebpa*$^{\Delta/\Delta}$ mice, providing a potential mechanism by which *Cebpa* influences pulmonary maturation and surfactant synthesis.

Overlapping roles of the *Cebpa*, *Foxa2* and *Titf1* genes

Deletion of *Cebpa* inhibited expression of a number of genes involved in host defense and lipid metabolism, including several genes identified as *Cebpa* targets during acute phase responses (Burgess-Beusse and Darlington, 1998) and adipogenesis (Tong et al., 2005). *Sftpa*, *Sftpb*, *Lys*, *Scd1* and *Slc34a2* were significantly decreased in *Cebpa*$^{\Delta/\Delta}$ mice and are selectively expressed in respiratory epithelial cells (Li et al., 1995; Rosenberg et al., 2002; Barlier-Mur et al., 2003; Cassell and Nord, 2003; Zhang et al., 2004). The increased expression of these genes correlates temporally with increased C/EBPα expression that occurs prior to birth (Li et al., 1995; Rosenberg et al., 2002). Surfactant proteins, lysozyme, Scd1 (Stearoyl-coenzyme A desaturase 1) and Slc34a2 [solute carrier family 34 (sodium phosphate, member 2)] were decreased in lungs of mice bearing mutations in phosphorylation sites in *Titf1*, and in *Cebpa*$^{\Delta/\Delta}$ and *Foxa2*$^{\Delta/\Delta}$ mice (deFelice et al., 2003), suggesting that these transcription factors influence a group of genes associated with perinatal lung maturation and function.

This study demonstrated that C/EBPα regulates a group of genes that mediate lipid synthesis, surfactant homeostasis and host defense required for postnatal adaptation to air breathing. Targeted deletion of the *Cebpa* gene inhibited the differentiation of respiratory epithelial cells, resulting in decreased surfactant synthesis causing respiratory failure at birth. C/EBPα, FOXA2, and TTF1 share transcriptional targets crucial for surfactant synthesis and host defense. The finding that FOXA2 and TTF1 are required for normal

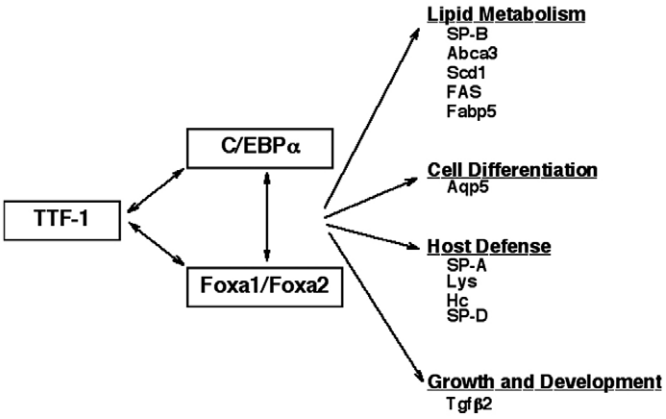

Fig. 12. Interactive roles of *Cebpa*, *Foxa2* and *Titf1*. C/EBPα, FOXA2 and TTF1 reciprocally or synergistically interact in a transcriptional network to regulate genes required for maturation and function of the lung at birth.

Cebpa gene expression and that C/EBPα regulates *Foxa2* gene expression supports the concept that these factors function in a transcriptional network that regulates genes required for maturation and function of the lung at birth (Fig. 12).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/6/1155/DC1

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