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Mesenchymal Nuclear factor I B regulates cell proliferation and epithelial differentiation during lung maturation

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ABSTRACT

The Nuclear factor I (NFI) transcription factor family consists of four genes (*Nfia*, *Nfib*, *Nfic* and *Nfix*) that regulate the development of multiple organ systems in mice and humans. *Nfib* is expressed in both lung mesenchyme and epithelium and mice lacking *Nfib* have severe lung maturation defects and die at birth. Here we continue our analysis of the phenotype of $Nfib^{-/-}$ lungs and show that *Nfib* specifically in lung mesenchyme controls late epithelial and mesenchymal cell proliferation and differentiation. There are more PCNA, BrdU, PHH3 and Ki67 positive cells in $Nfib^{-/-}$ lungs than in wild type lungs at E18.5 and this increase in proliferation marker expression is seen in both epithelial and mesenchymal cells. The loss of *Nfib* in all lung cells decreases the expression of markers for alveolar epithelial cells (*Aqp5* and *Sftpc*), Clara cells (*Scgb1a1*) and ciliated cells (*Foxj1*) in E18.5 lungs. To test for a specific role of *Nfib* in lung mesenchyme we generated and analyzed $Nfib^{flox/flox}$, *Dermo1-Cre* mice. Loss of *Nfib* only in mesenchyme results in decreased *Aqp5*, *Sftpc* and *Foxj1* expression, increased cell proliferation, and a defect in sacculation similar to that seen in $Nfib^{-/-}$ mice. In contrast, mesenchyme specific loss of *Nfib* had no effect on the expression of *Scgb1a1* in the airway. Microarray and QPCR analyses indicate that the loss of *Nfib* in lung mesenchyme affects the expression of genes associated with extracellular matrix, cell adhesion and FGF signaling which could affect distal lung maturation. Our data indicate that mesenchymal *Nfib* regulates both mesenchymal and epithelial cell proliferation through multiple pathways and that mesenchymal NFI-B-mediated signals are essential for the maturation of distal lung epithelium.

Introduction

The Nuclear factor I (NFI) family of transcription factors functions both in mammalian development and in adenoviral DNA replication (de Jong and van der Vliet, 1999; Nagata et al., 1982, 1983). In vertebrates, the NFI family consists of four genes, *Nfia*, *Nfib*, *Nfic* and *Nfix*, that encode proteins which bind as homo- and heterodimers to the consensus sequence TTGGC(N5)GCCAA on duplex DNA (Gronostajski, 1986; Meisterernst et al., 1988). NFI-binding sites have been identified in the promoter, enhancer and silencer regions of more than 100 genes expressed in multiple organs, including brain, lung, liver and intestine (Gronostajski, 2000). Here we address the role of *Nfib* in lung development.

Lung immaturity is a major problem in premature infants. It is associated with respiratory distress syndrome, an acute lung problem that presents shortly after birth, and bronchopulmonary dysplasia, a chronic lung disease of premature infants (Coalson et al., 1999; Jobe, 2005). Previous studies of mice lacking *Nfib* revealed defects in lung development and $Nfib^{-/-}$ mice die at birth with immature lungs (Grunder et al., 2002; Steele-Perkins et al., 2005). At E18.5 $Nfib^{-/-}$ lungs lack saccules, have an increased DNA content, and decreased levels of surfactant protein transcripts. This phenotype indicates that *Nfib* plays an important role in lung maturation. Previous studies also showed that NFI-B can directly regulate the expression of genes in lung epithelium (Bachurski et al., 2003). However, the specific cell types in which *Nfib* is required for normal lung development, and the critical target genes regulated by *Nfib* during lung maturation remain unknown.

Lung development is regulated by mesenchymal–epithelial interactions (Deimling et al., 2007; Demayo et al., 2002; Morrisey and Hogan, 2010). Some signals from epithelial and/or mesenchymal cells

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that control smooth muscle cell differentiation and vasculogenesis have been well characterized, including the VEGF-A (Akeson et al., 2003; White et al., 2007; Zeng et al., 1998), PDGF (Hellstrom et al., 1999; Li and Hoyle, 2001), and WNT (Cohen et al., 2009; Li et al., 2002; Shu et al., 2002) signaling pathways. In contrast, the signaling pathways from mesenchymal cells that influence epithelial cell proliferation and differentiation are less well understood. One mesenchymal-expressed factor that is known to affect lung epithelial cell proliferation and differentiation is FGF-10. *Fgf10* is expressed in mesenchyme and *Fgf10*$^{-/-}$ mice were characterized by the absence of lungs (Min et al., 1998). Overexpression of FGF-10 in late lung development results in the impairment of lung branching and attenuation of distal epithelial cell differentiation (Clark et al., 2001; Nyeng et al., 2008), suggesting that FGF-10 must be tightly regulated for normal lung development. However, much more information is needed regarding how mesenchyme regulates lung maturation.

Here we demonstrate an essential role of *Nfib* in lung maturation using *Nfib*$^{-/-}$ mice and mice in which *Nfib* is deleted specifically in mesenchyme using *Dermo1-Cre*. We show that while *Nfib* is expressed in both mesenchyme and epithelium during lung development, loss of *Nfib* specifically in mesenchyme affects both mesenchymal and epithelial cell proliferation, and distal epithelial cell differentiation. These data demonstrate a heretofore unrecognized pathway of mesenchymal regulation of late epithelial maturation. In addition, microarray and QPCR analyses were used to identify biochemical pathways regulated by NFI-B that appear important for lung maturation.

Materials and methods

Histology and immunohistochemistry

Fetal lungs were dissected and fixed in 4% paraformaldehyde overnight at 4 °C. After paraffin embedding, 4 μm sections were cut and stained with hematoxylin and eosin. For immunohistochemistry, antibodies against the following proteins were used: CC10 (Santa Cruz T-18, 1:200), pro-SPC (Chemicon AB3786, 1:2000), PCNA (Santa Cruz FL-261, 1:200), phospho-histone H3 (Sigma HTA28, 1:200), cleaved Caspase-3 (Cell Signaling #9661, 1:200), Ki67 (Abcam ab15580, 1:400), TTF-1 (Dako 8G7G3/1, 1:200), Vimentin (Sigma LN-6, 1:400), Caveolin-1 (Santa Cruz N-20, 1:200), smooth muscle actin (SMA) (Sigma 1A4, 1:5000), AQP5 (Alomone labs AQP-005, 1:200) and NFI-B (Active Motif 1:1000). In general, primary antibodies were incubated with slides overnight at 4 °C. After a PBS wash, slides were incubated with secondary antibodies (Alexa 488 or Alexa 568, Invitrogen) for 1 h at room temperature and auto-fluorescence was eliminated with 0.5% Sudan black B. TOPRO3 (Invitrogen, 1:5000) was used as a nuclear counterstain on some sections. For BrdU staining, pregnant mice were injected with 0.1 mg/g BrdU 2 h before sacrifice, lungs were fixed and processed, and sections were stained using a BrdU staining kit (Zymed).

Statistics

Comparisons were made between at least three KO and littermate control mice. Statistical significance was assessed using the two-tailed student t-test in Microsoft Excel. P values of less than 0.05 were considered statistically significant. Values for all experiments are expressed as the mean +/− SD. In all QPCR analyses duplicate or triplicate PCR reactions from each of at least 3 biological replicates were analyzed. For statistical quantification of cell number using immunohistochemistry, 15 random fields were imaged from 3 mice of each genotype. A minimum of 100 positive cells were counted for each condition. Intra-litter comparisons of each genotype were used whenever possible and multiple litters were assessed for consistency of phenotype. The numbers of fluorescent and total cells were quantified using Image J software.

RNA extraction and RT-QPCR

RNA was extracted with TRIzol reagent (Invitrogen) and 2–5 μg was used for random hexamer primed cDNA synthesis with Superscript II (Invitrogen). Transcript levels were quantified by quantitative PCR (QPCR) with a Bio-Rad iCycler real-time PCR machine using SYBR Green as described previously (Steele-Perkins et al., 2005). All results were normalized to β2-microglobulin levels. Sequences of the primers are available upon request.

Chromatin immunoprecipitation (ChIP) assay

E16.5 lungs were isolated from wild type embryos, minced and fixed with 1% formaldehyde. Chromatin was sheared to ~200–500 bp using a Branson Sonifier 250 sonicator. Immunoprecipitation was performed with a ChIP assay kit (Upstate Biotechnology) and αNFI-B antibody (Geneka Biotechnology). After immunoprecipitation and reversal of cross-links the chromatin was subjected to PCR and QPCR. ChIP analysis was performed on E16.5 lung in an effort to identify potential early mesenchymal targets of *Nfib* that could contribute to the later morphological phenotype seen at E18.5. The large number of biochemical and morphological differences between E18.5 wild type and mutant lungs and the increase in epithelial NFIB expression could complicate the identification of direct mesenchymal NFIB targets at this stage.

Transient transfection assays

Mouse embryo fibroblasts (MEFs) were isolated from WT E13 embryos as described previously (Plasari et al., 2009). Cells were transfected using Lipofectamine as recommended by the manufacturer with vectors expressing GFP or NFIB2 from the CMV promoter (Chaudhry et al., 1999). After 48 h cells were harvested using Trizol, RNA was isolated, cDNA was prepared using random primers and QPCR was performed with primers specific for Elastin as described earlier.

Gene targeting and mouse strains

A targeting vector was constructed with a 4.4 kb 5′ homology arm containing all of exon 2 of *Nfib* and 300 bp of intron 2, with a loxP site inserted 363 bp 5′ to the start of exon 2. The 5′ arm was followed by an FRT-flanked PGK-neo expression cassette in the opposite transcriptional orientation, a 3′ loxP site, the contiguous 3.6 kb 3′ homology arm and a PGK-diphtheria toxin A chain cassette in the opposite transcriptional orientation (Fig. S1A) (Campbell et al., 2008). Cre-mediated recombination deletes all of exon 2, 363 bp of intron 1 and 300 bp of intron 2. This vector was linearized with *Asc*I, electroporated into J1 ES cells and G418-resistant colonies were picked, expanded and banked until PCR screening was completed. Five of six correctly targeted clones (6 targeted/256 total) were thawed, targeting was confirmed, and two were expanded and injected into C57Bl/6 blastocysts to generate chimeric animals. Chimeras were bred with C57Bl/6 females and agouti progeny were screened for the presence of the targeted conditional KO allele by PCR. *Nfib*$^{flox/flox}$ mice survive birth with no overt lung phenotype, grow to adulthood and at E18.5 express ~70–80% of wild type levels of *Nfib*.

Dermo1-Cre mice were kindly provided by David Ornitz (Yu et al., 2003). *Nfib*$^{flox/flox}$ mice were crossed with *Dermo1-Cre* mice to generate *Nfib*$^{flox/+}$, *Dermo1-Cre* offspring. *Nfib*$^{flox/+}$, *Dermo1-Cre* mice were then crossed to *Nfib*$^{flox/flox}$ mice to generate mesenchymal-specific *Nfib* null mice. All mice were genotyped using PCR and sequences of the primers are available upon request. All protocols were approved by the IACUC at Roswell Park Cancer Institute.

Microarray analysis

Total RNA was isolated from three different *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *Dermo1-Cre* lungs at E18.5 using TRIzol reagent (Invitrogen) and then purified using an RNeasy Mini Kit (Qiagen). The purified RNA was examined using an Agilent Bioanalyzer 2100 (Agilent Technologies) and then labeled and hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Arrays using the manufacturer's protocol. Three independent sets of biological replicates (*Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *Dermo1-Cre* lungs) were used. Scanned microarray images were imported into GeneChip Operating Software (GCOS, Affymetrix) to generate raw signal values for each probe. The MAS5.0 algorithm in the 'Affy' package of Bioconductor in the R statistical computing environment was used to generate expression summary values, followed by trimmed mean global normalization to bring the mean expression values of all six GeneChips to the same scale (Gentleman et al., 2004). For data quality control, MAS5.0 present/absent calls were used to filter out probe sets whose expression intensities were close to background noise across the majority of samples. Specifically, filtering of three 'present calls' was applied to either the *Nfib*$^{flox/flox}$ or *Nfib*$^{flox/flox}$, *Dermo1-Cre* group, with 25,086 unique transcripts passing the quality control. We used the linear model implemented in *Limma* program to calculate the level of gene differential expression (Smyth, 2004).Transcripts that were altered at a *P*-value less than 0.05 and with at least 1.5-fold expression change between *Nfib*$^{flox/flox}$, *Dermo1-Cre* and *Nfib*$^{flox/flox}$ mice were considered significant and used for further analysis. These differentially expressed genes were analyzed for statistically enriched gene ontology terms using the NCBI DAVID package with the default setting (Huang da et al., 2007). The mRNA expression profiling datasets have been deposited in the NCBI Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nlm.nih.gov/geo/) under Accession number GSE24465.

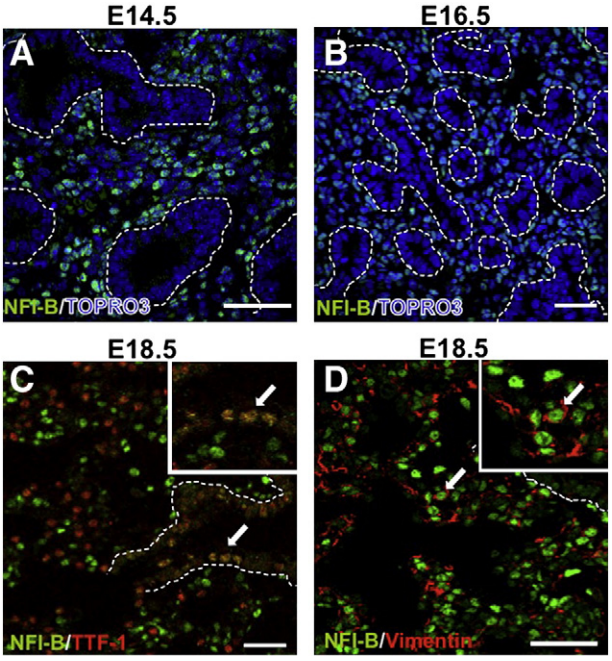

Fig. 1. Expression of NFI-B in mesenchyme and epithelium during lung development. Paraffin sections of E14.5 (A), E16.5 (B), and E18.5 (C, D) lungs were immunostained for NFI-B expression. At E14.5 and E16.5, NFI-B was expressed predominantly in mesenchyme. At E18.5, NFI-B was expressed in both epithelial cells (TTF-1 positive cells) (C) and mesenchymal (Vimentin positive cells) (D). The dashed lines denote the border between epithelial and mesenchymal cells in bronchioles. Arrows denote the colocalization of NFI-B and TTF-1 (C) or NFI-B and Vimentin (D). Insets in C and D show a 5× magnification. TOPRO-3 shows nuclear staining. Scale bars, 50 μm.

Results

NFI-B is expressed predominantly in mesenchyme at E14.5 and E16.5, and in both epithelial cells and mesenchyme at E18.5

We showed previously by lacZ staining in heterozygous *Nfib*$^{lacZ/+}$ mice that *Nfib* is expressed almost exclusively in mesenchyme at E14.5–E16.5 but is expressed in both mesenchyme and epithelium by E18.5 (Steele-Perkins et al., 2005). To assess the expression pattern of NFI-B in WT mice, αNFI-B antibodies were used for immunohistochemistry on sections of E14.5, E16.5 and E18.5 lungs. As expected, from E14.5 to E16.5, NFI-B was expressed almost exclusively in the nuclei of mesenchymal cells surrounding bronchial tubules (dashed lines, Figs. 1A, B). At E18.5, dual immunostaining showed that NFI-B was colocalized with both Thyroid Transcription Factor 1 (TTF-1) (arrows in Fig. 1C, magnified 5× in inset), a bronchiolar and alveolar type II epithelial cell marker, and Vimentin (arrows in Fig. 1D, magnified 5× in inset), a mesenchymal cell marker. These data suggest that NFI-B likely function primarily in mesenchyme until E14.5 and in both mesenchyme and epithelium after E16.5.

Loss of Nfib increases cell proliferation but doesn't affect apoptosis

We showed previously that the DNA content/total body weight of E18.5 *Nfib*$^{-/-}$ lungs was about double that of WT lungs, suggesting changes in either cell proliferation or apoptosis late in lung development (Steele-Perkins et al., 2005). To assess cell proliferation, antibodies against proliferating cell nuclear antigen (PCNA), Ki67 or phosphohistone H3 (PHH3) were used to stain E18.5 lung sections from *Nfib*$^{+/+}$ and *Nfib*$^{-/-}$ embryos. Multiple markers were used to ensure that any change in proliferation detected was not marker-dependent. The number of PCNA positive cells was increased in *Nfib*$^{-/-}$ lungs compared with *Nfib*$^{+/+}$ lungs (Fig. S2A, B) and quantification of these data (PCNA positive nuclei/ total nuclei) supported this conclusion (Fig. S2C). In addition, there were more Ki67 positive cells and PHH3 positive cells in *Nfib*$^{-/-}$ lungs than in *Nfib*$^{+/+}$ lungs (Fig. S2G, H, J, K) and quantification showed an increase in the number of cells expressing multiple proliferation markers in *Nfib*$^{-/-}$ lungs (Fig. S2I, L). To further assess cell proliferation, bromodeoxyuridine (BrdU) was injected into mice 2 h before sacrifice and then detected with αBrdU antibodies. BrdU staining was increased significantly in *Nfib*$^{-/-}$ lungs compared to *Nfib*$^{+/+}$ lungs (Fig. S2D, E, F). These data indicate that loss of *Nfib* results in dramatically increased cell proliferation at E18.5.

Apoptosis in E18.5 lungs was examined by immunostaining of cleaved Caspase 3. The number of cleaved Caspase 3 positive cells is similar in *Nfib*$^{+/+}$ and *Nfib*$^{-/-}$ lungs (Fig. S3A, B, C). Together, these data indicate that loss of *Nfib* increases lung DNA content through increasing cell proliferation with no affect on apoptosis.

Loss of Nfib increases both TTF-1 positive and TTF-1 negative cell proliferation

To identify the hyperproliferative cells in *Nfib*$^{-/-}$ lungs at E18.5, αTTF-1 antibody was used to label epithelial cells. Dual immunostaining for PCNA and TTF-1 revealed more double positive cells (PCNA^{+}TTF-1^{+} cells) (arrows in Figs. 2A, B, magnified 5× in insets) in *Nfib*$^{-/-}$ lungs than in *Nfib*$^{+/+}$ lungs. Quantification of these data (PCNA^{+}TTF-1^{+} cells/ total TTF-1^{+} cells) (Fig. 2C) supported this conclusion. We also examined TTF-1^{-} cell proliferation. There was a substantial increase in TTF1^{-}PCNA^{+} cells, indicating an increase in TTF-1^{-} cell proliferation (Fig. 2C). Dual immunostaining for Ki67 and TTF-1 was used to further assess TTF-1^{+} and TTF-1^{-} cell proliferation. These data indicate that more of both Ki67^{+}TTF-1^{+} and Ki67^{+}TTF-1^{-} cells are present in *Nfib*$^{-/-}$ lungs than in *Nfib*$^{+/+}$ lungs (Figs. 2D–F, magnified 5× in insets). These data are consistent with our previous PCNA analysis and show that loss of *Nfib* leads to an increase in proliferation of both epithelial and mesenchymal cells during lung development.

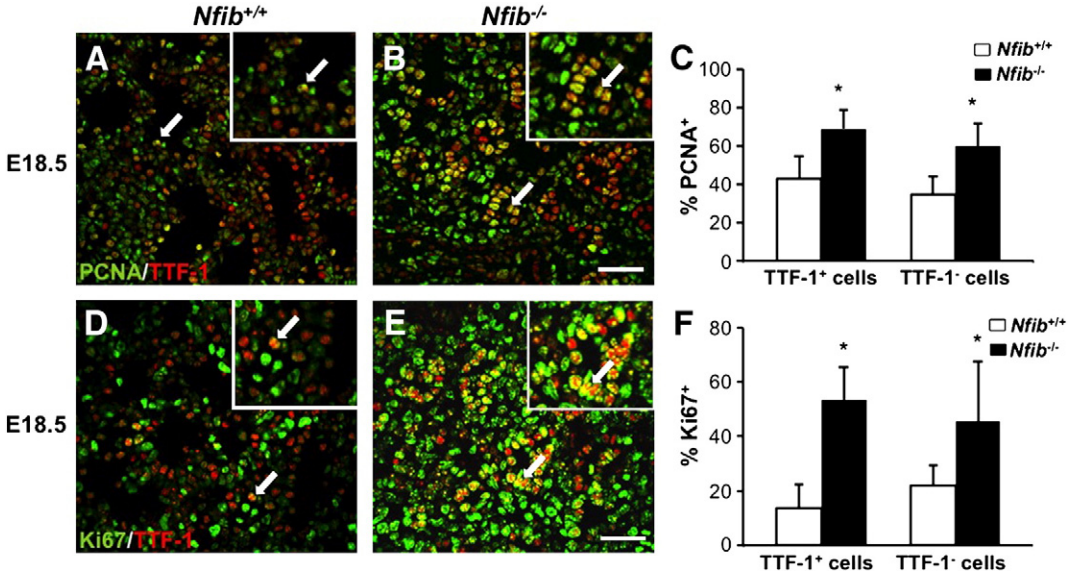

Fig. 2. Loss of *Nfib* increases both epithelial (TTF-1$^+$) and mesenchymal (TTF-1$^-$) cell proliferation. Paraffin sections of E18.5 lungs from *Nfib*$^{+/+}$ and *Nfib*$^{-/-}$ embryos were stained for both PCNA and TTF-1 (A, B) or for both Ki67 and TTF-1 (D, E). Immunostaining reveals there are more PCNA$^+$TTF-1$^+$ cells (arrows) and Ki67$^+$TTF-1$^+$ cells (arrows) in *Nfib*$^{-/-}$ lungs compared with *Nfib*$^{+/+}$ lungs. Insets in panels A, B, D and E show a 5× magnification. Quantification (C, F) indicates there are more PCNA$^+$TTF-1$^-$ cells and Ki67$^+$TTF-1$^-$ cells in *Nfib*$^{-/-}$ lungs compared with *Nfib*$^{+/+}$ lungs. Arrows denote the colocalization of PCNA and TTF-1 or Ki67 and TTF-1 and the region of magnification; *P<0.05; Scale bars, 50 μm.

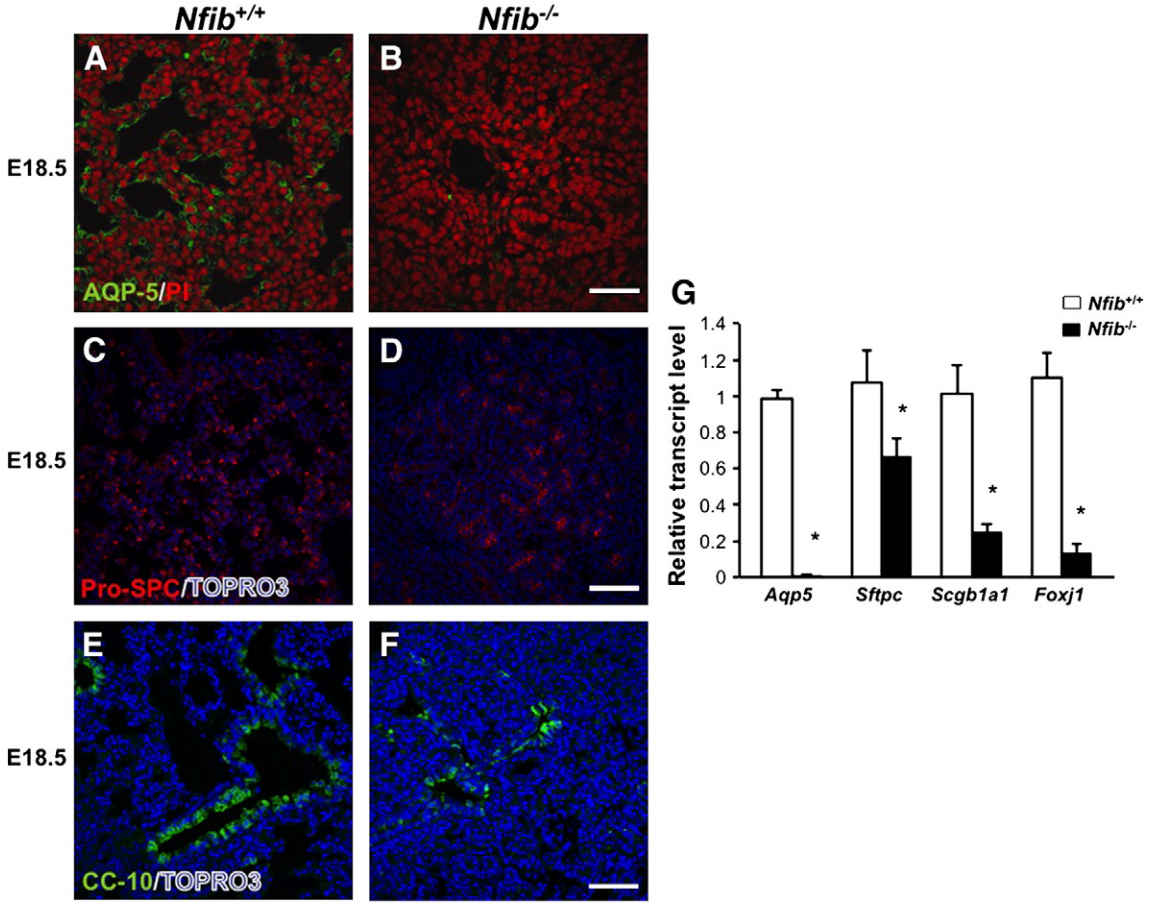

Fig. 3. *Nfib*$^{-/-}$ lungs exhibit decreased epithelial cell differentiation at E18.5. E18.5 lung sections from *Nfib*$^{+/+}$ and *Nfib*$^{-/-}$ embryos were stained for AQP5 (type I epithelial cell marker) (A, B), pro-SPC (type II epithelial cell marker) (C, D) and CC10 (Clara cell marker) (E, F). Loss of *Nfib* at E18.5 lungs led to decreased expression of all three epithelial cell markers. RT-QPCR analysis (G) reveals *Aqp5*, *Sftpc* and *Scgb1a1* and *Foxj1* expression is significantly decreased in *Nfib*$^{-/-}$ lungs at E18.5. PI and TOPRO3 staining show nuclei; *P<0.05; Scale bars, 50 μm.

Loss of Nfib results in decreased epithelial type I, type II, ciliated cell and Clara cell differentiation

To confirm and extend our previous findings that *Nfib* is essential for epithelial cell differentiation, we performed both RT-QPCR and immunohistochemistry for several cell-type-specific markers. Aquaporin 5 (*Aqp5*), an alveolar type I cell marker, was essentially absent from $Nfib^{-/-}$ lungs compared with $Nfib^{+/+}$ lungs (Figs. 3A, B), consistent with the result of RT-QPCR (Fig. 3G). Expression of pro-surfactant protein C (*Sftpc*), an alveolar type II cell marker, was also decreased in $Nfib^{-/-}$ lungs (Figs. 3C, D) and confirmed by RT-QPCR (Fig. 3G). Since alveolar type II cells potentially differentiate into type I alveolar cells during lung injury and lung development (Sugahara et al., 2006), loss of *Nfib* could affect alveolar type II cell differentiation directly and indirectly reduce alveolar type I cell number. Both Immunostaining and RT-QPCR for CC10 (*Scgb1a1*), a Clara cell marker, showed a dramatic decrease in CC10 expression in $Nfib^{-/-}$ lungs versus $Nfib^{+/+}$ lungs (Figs. 3E–G). In addition, the levels of *Foxj1* transcripts (a ciliated cell marker) were dramatically reduced in the $Nfib^{-/-}$ lungs (Fig. 3G). These data indicate that loss of *Nfib* results in defects in both distal and proximal epithelial cell differentiation.

Dermo1-Cre deletes a floxed Nfib allele specifically in lung mesenchyme

Because *Nfib* is required for lung maturation and is expressed almost exclusively in mesenchyme at E14.5, we proposed that *Nfib* in lung mesenchyme may regulate the phenotype seen in $Nfib^{-/-}$ lungs. To test this hypothesis, we created a conditional *Nfib* allele ($Nfib^{flox}$) such that exposure to Cre deletes exon 2 and ~660 bp of adjacent intronic sequence (Fig. S1A). $Nfib^{flox/flox}$ mice have no overt lung phenotype, survive to adulthood, are fertile, and at E18.5 express at least ~70–80% of wild type levels of *Nfib* (data not shown). To determine the effect of deleting *Nfib* exclusively in the mesenchyme, *Dermo1-Cre* mice (here termed *D1-Cre*) were crossed with $Nfib^{flox/flox}$ mice to generate $Nfib^{flox/+}$, *D1-Cre* offspring. $Nfib^{flox/+}$, *D1-Cre* mice were then crossed to $Nfib^{flox/flox}$ mice to generate mesenchymal-specific *Nfib* null mice (here termed $Nfib^{flox/flox}$, *D1-Cre*). By E16.5, *D1-Cre* had efficiently eliminated NFI-B expression in lung mesenchyme (Fig. 4A, B arrows, magnified 5× in insets). At E18.5, *D1-Cre* had no obvious affect on NFI-B expression in epithelium (arrows in Figs. 4C, D, magnified 5× in insets), indicating that *Nfib* was deleted specifically in the mesenchyme of $Nfib^{flox/flox}$, *D1-Cre* mice. These data are consistent with previous studies showing that *D1-Cre* specifically deletes floxed alleles only in lung mesenchyme, but not lung epithelium (De Langhe et al., 2008; White et al., 2006; Yu et al., 2003).

Perinatal lethality and sacculation defects in $Nfib^{flox/flox}$, D1-Cre mice

No live $Nfib^{flox/flox}$, *D1-Cre* mice were recovered at P0 from 4 litters (Table 1), while all other genotypes were found in appropriate numbers. This lethality occurs perinatally since the number of animals of each genotype recovered at E16.5 and E18.5 was near the predicted Mendelian frequency of 1:1:1:1 (12 $Nfib^{flox/+}$ mice, 11 $Nfib^{flox/+}$, *D1-Cre* mice, 8 $Nfib^{flox/flox}$ mice and 12 $Nfib^{flox/flox}$, *D1-Cre* mice, 5 litters). Perinatal lethality is consistent with our previous results with $Nfib^{-/-}$ mice (Steele-Perkins et al., 2005).

Our and other studies previously found a defect in sacculation in $Nfib^{-/-}$ mice (Grunder et al., 2002; Steele-Perkins et al., 2005). Therefore, we examined the morphology and histology of $Nfib^{flox/flox}$, *D1-Cre* lungs at E18.5. Like $Nfib^{-/-}$ lungs, $Nfib^{flox/flox}$, *D1-Cre* lungs were larger than $Nfib^{flox/flox}$ lungs (Fig. 5A). Histological analysis of E18.5 lungs showed that lungs from $Nfib^{flox/flox}$, *D1-Cre* mice had a severe defect in sacculation, while those from control $Nfib^{flox/flox}$ mice were well sacculated (Figs. 5D, E). In addition to the defect in sacculation, we found aberrant clefts in $Nfib^{flox/flox}$, *D1-Cre* lungs at E16.5 and E18.5 (arrows in Figs. 5C, F). Similar clefts were seen previously in $Nfib^{-/-}$ lungs (Steele-Perkins et al., 2005). These data show that the $Nfib^{flox/flox}$, *D1-Cre* lungs share multiple aberrant morphological changes with $Nfib^{-/-}$ lungs.

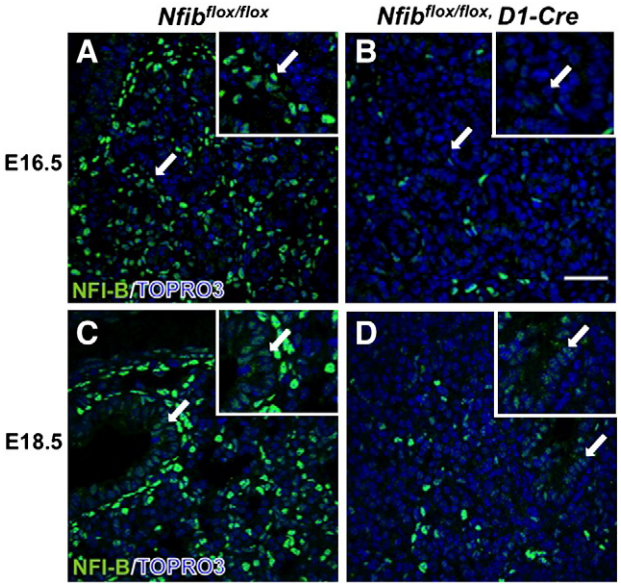

Fig. 4. *Dermo1-Cre* reduces NFI-B expression in $Nfib^{flox/flox}$ lungs in mesenchyme, but not in epithelium. Lung sections from E16.5 $Nfib^{flox/flox}$ (A) and $Nfib^{flox/flox}$, *D1-Cre* (B) embryos stained for NFI-B showed *Dermo1-Cre* eliminated almost all NFI-B in mesenchyme at E16.5. Immunostaining for NFI-B (C, D) in E18.5 $Nfib^{flox/flox}$ and $Nfib^{flox/flox}$, *D1-Cre* lungs reveals *Dermo1-Cre* eliminated NFI-B expression throughout the lung mesenchyme at E18.5 but didn't affect NFI-B expression in the epithelial cells (arrows). Insets show a 5× magnification. Arrows denote NFI-B in bronchiolar epithelial cells and the region of magnification; Scale bar, 50 μm.

Loss of Nfib in mesenchyme increases cell proliferation

To determine whether *Nfib* in lung mesenchyme is required for the control of cell proliferation during lung maturation, we performed PHH3 immunostaining on E18.5 $Nfib^{flox/flox}$ and $Nfib^{flox/flox}$, *D1-Cre* lung sections. There were more PHH3 positive cells in $Nfib^{flox/flox}$, *D1-Cre* lungs than in $Nfib^{flox/flox}$ lungs (Fig. S4A, B), indicating a higher rate of proliferation at E18.5 in the former. Ki67 staining was also performed on E16 and E18.5 lung samples. These data show more Ki67 positive cells in $Nfib^{flox/flox}$, *D1-Cre* lung at both E16.5 and E18.5 (Fig. S4D–G). Quantification of the immunostaining (Fig. S4C, H) was consistent with the data from $Nfib^{-/-}$ lungs (Fig. S2C–L) in showing an increase in cell proliferation.

Loss of Nfib in mesenchyme increases both TTF-1 positive and TTF-1 negative cell proliferation

To determine whether the increased proliferation in $Nfib^{flox/flox}$, *D1-Cre* lungs was predominantly in the epithelial or mesenchymal compartments, we performed dual staining for TTF-1 and Ki67 on sections of E16 and E18.5 lungs. Notably, more $Ki67^{+}TTF\text{-}1^{+}$ cells

Table 1
Loss of *Nfib* in mesenchyme results in perinatal lethality.

No. of live animals of each genotype from $Nfib^{flox/+}$, *D1-Cre* X $Nfib^{flox/flox}$ matings				
Genotype	$Nfib^{flox/+}$	$Nfib^{flox/flox}$	$Nfib^{flox/+}$, *D1-Cre*	$Nfib^{flox/flox}$, *D1-Cre*
Observed	10	14	9	0
Expected	10	10	10	10

4 litters were examined in this experiment. The expected numbers are based on the number of $Nfib^{flox/+}$ progeny obtained.

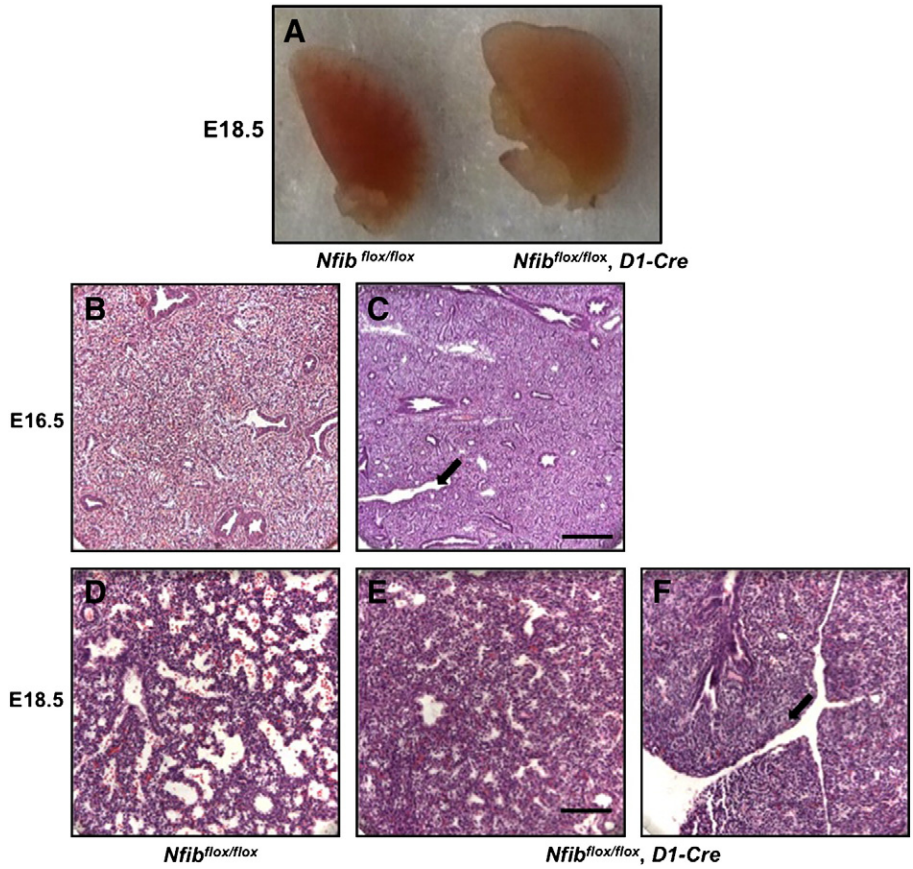

Fig. 5. Sacculation and lung morphology is affected by loss of *Nfib* in mesenchyme. Dissected left lobes of *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs are shown unstained at E18.5 (A), and H&E stained at E16.5 (B, C) and E18.5 (D, E, F). *Nfib*$^{flox/flox}$, *D1-Cre* lungs were larger than *Nfib*$^{flox/flox}$ lungs (A). H&E staining shows abnormal clefts (arrows in C, F) in *Nfib*$^{flox/flox}$, *D1-Cre* lungs at E16.5 (C) and E18.5 (F). At E18.5, there is increased mesenchyme thickness and reduced sacculation in *Nfib*$^{flox/flox}$, *D1-Cre* lungs. Scale bar, 50 μm.

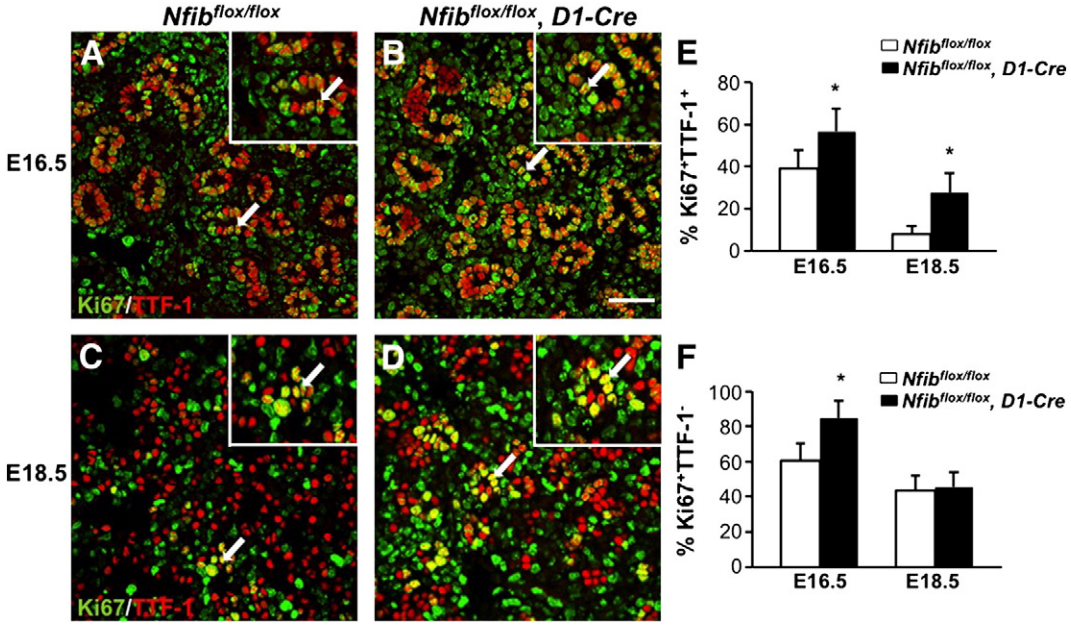

Fig. 6. Loss of *Nfib* in mesenchyme increases TTF-1 positive and TTF-1 negative cell proliferation. Paraffin sections of E18.5 and E16.5 lungs from *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* embryos were stained for both Ki67 and TTF-1 (A, B, C, D). Insets show a 5× magnification. Immunofluorescence staining reveals there were more Ki67^{+}TTF-1^{+} cells (arrows) in *Nfib*$^{flox/flox}$, *D1-Cre* lungs compared with *Nfib*$^{flox/flox}$ lungs at E16.5 and E18.5 (E). The percentage of Ki67^{+}TTF-1^{-} cells was also higher in *Nfib*$^{flox/flox}$, *D1-Cre* lungs at E16.5 (F). In contrast, the percentage of Ki67^{+}TTF-1^{-} cells was similar between *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs at E18.5 (F). Arrows denote the colocalization of Ki67 and TTF-1 and the region of magnification; *P<0.05; Scale bar, 50 μm.

were present in *Nfib*$^{flox/flox}$, *D1-Cre* lungs relative to *Nfib*$^{flox/flox}$ lungs indicating increased proliferation of epithelial cells (arrows in Figs. 6A–D, magnified 5× in insets). Quantification confirmed an increase in the percentage of Ki67^{+}TTF-1^{+} cells (Fig. 6E). Furthermore, we determined the percentage of Ki67^{+}TTF-1^{-} cells at E16.5 and E18.5. The percentage of Ki67^{+}TTF-1^{-} mesenchymal cells in *Nfib*$^{flox/flox}$, *D1-Cre* lungs was higher at E16.5 and then decreased to a level similar to that seen in *Nfib*$^{flox/flox}$ lungs by E18.5 (Fig. 6F). These data indicate that loss of *Nfib* in mesenchyme results in an increase in both TTF-1^{+}and TTF-1^{-} cell proliferation during lung development. However, at E18.5 the apparent rate of TTF-1^{-} cell proliferation appears similar in *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs, which is different from that seen in *Nfib*$^{+/+}$ vs. *Nfib*$^{-/-}$ lungs (Figs. 2D–F). We discuss later possible reasons for these relatively minor differences in apparent TTF-1^{-} cell proliferation.

Loss of Nfib in mesenchyme affects epithelial cell differentiation and surfactant gene expression

As done with *Nfib*$^{-/-}$ lungs, we assessed multiple epithelial cell differentiation markers in *Nfib*$^{flox/flox}$, *D1-Cre* lungs by immunostaining. AQP5 and pro-SPC staining was significantly reduced in *Nfib*$^{flox/flox}$, *D1-Cre* lungs relative to *Nfib*$^{flox/flox}$ lungs (Figs. 7A–D). RT-QPCR analysis showed that *Aqp5* and *Sftpc* transcript levels were also significantly decreased with loss of *Nfib* in the mesenchyme (Fig. 7G). In addition, as was seen in germline *Nfib* KO lungs (Fig. 3G) the expression of the ciliated cell marker *Foxj1* was substantially decreased in the *Nfib*$^{flox/flox}$, *D1-Cre* lungs (Fig. 7G). In contrast, *Scgb1a1* (CC10) expression was similar in *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs as assessed by both immunostaining (Figs. 7E–F) and RT-QPCR (Fig. 7G). These data show that mesenchymal *Nfib* is clearly required for Type I, Type II and ciliated cell differentiation, but fail to support a role of mesenchymal expression of *Nfib* in the regulation of Clara cell differentiation.

RT-QPCR analysis showed that mRNA levels of the other surfactant proteins, including surfactant protein A (*Sftpa1*), surfactant protein B (*Sftpb*) and surfactant protein D (*Sftpd*), were dramatically decreased in *Nfib*$^{flox/flox}$, *D1-Cre* lungs compared with *Nfib*$^{flox/flox}$ lungs at E18.5 (Fig. 7H). Thus, loss of *Nfib* in mesenchyme results in defects in distal epithelial cell differentiation as assessed by surfactant protein gene expression.

Loss of Nfib in mesenchyme affects the expression of genes related to lipid production, extracellular matrix, cell adhesion and the FGF signaling pathway

To assess potential target genes of NFI-B that could affect cell proliferation and distal epithelial cell differentiation during lung maturation, we performed microarray analysis on RNA from E18.5 *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs. Genes with expression changes of 1.5 fold or greater and p-values of <0.05 were selected for further analysis (see Table S1).

The expression of genes involved in a number of biological processes, including extracellular matrix and cell adhesion appear affected by loss of *Nfib* in lung mesenchyme (Fig. 8A). To confirm these expression data, the transcript levels of several genes, including

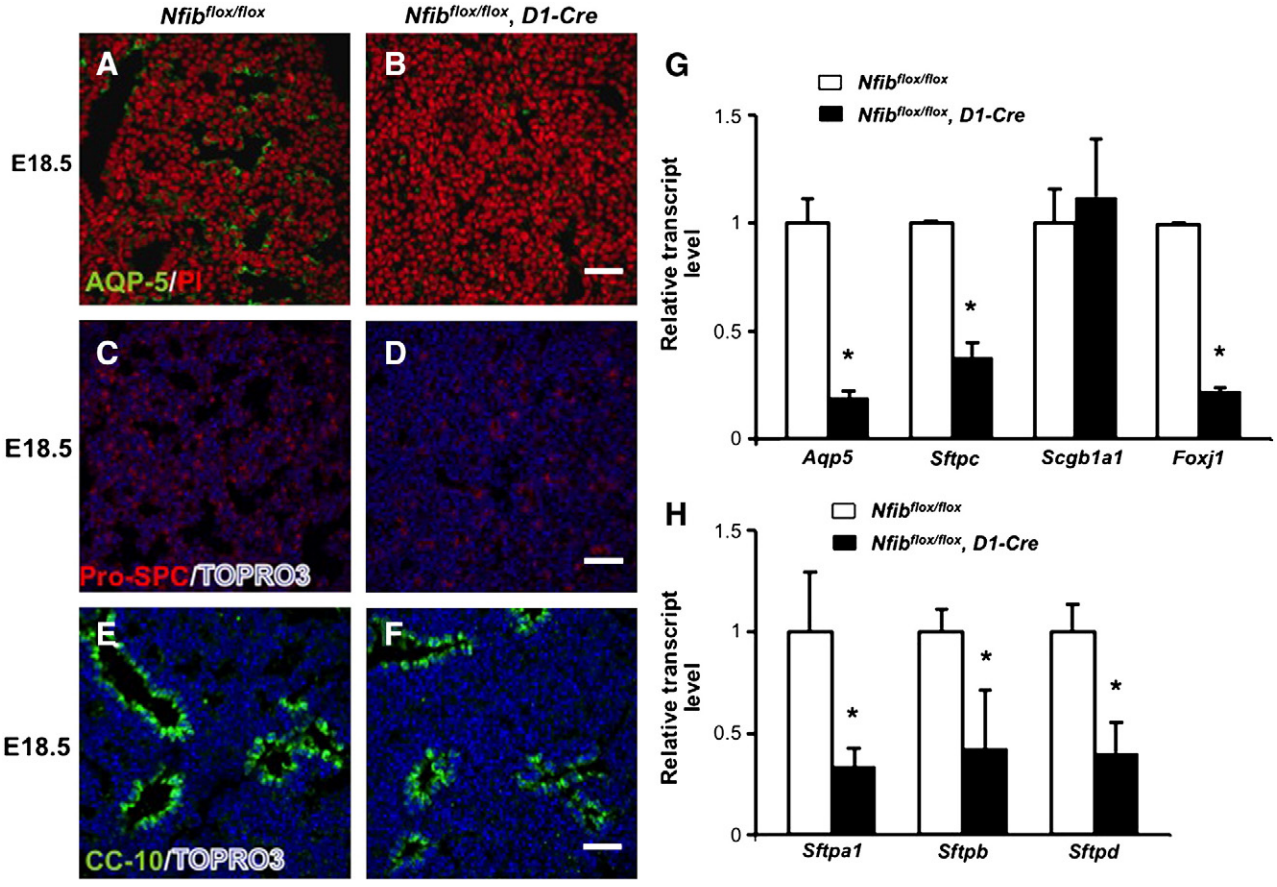

Fig. 7. Loss of *Nfib* in mesenchyme affects type I and type II epithelial cell differentiation. E18.5 lung sections from *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* embryos were stained for AQP5 (A, B), pro-SPC (C, D) and CC10 (E, F). AQP5, pro-SPC and *Foxj1* transcript expression was also reduced in *Nfib*$^{flox/flox}$, *D1-Cre* lungs, as shown by RT-QPCR (I). In contrast, immunostaining and RT-QPCR revealed CC10 (*Scgb1a1*) protein and transcript levels were similar in *Nfib*$^{flox/flox}$ and *Nfib*$^{flox/flox}$, *D1-Cre* lungs. Along with reduced *Sftpc* expression, *Sftpa1*, *Sftpb* and *Sftpd* expression was significantly reduced in *Nfib*$^{flox/flox}$, *D1-Cre* lungs (H). *P<0.05; Scale bars, 50 μm.

Elastin (*Eln*), Fibronectin (*Fn1*), collagen, type IV, alpha 3 (*Col4a3*) and Ephrin A3 (*Efna3*), were examined using RT-QPCR. The RT-QPCR data are consistent with our microarray data (Figs. 8B–E), indicating an effect on genes involved in extracellular matrix production and cell adhesion in $Nfib^{flox/flox}$, *D1-Cre* mice. The transcripts levels of Tenascin C (*Tnc*), an extracellular matrix protein involved in lung vasculogenesis and smooth muscle cell differentiation (Cohen et al., 2009) were also examined. There was no significant change of *Tnc* transcript levels between $Nfib^{flox/flox}$ and $Nfib^{flox/flox}$, *D1-Cre* lungs (fold change = 1, P = 0.5) (Fig. S5).

In addition, we examined the expression of *Fgf7* and *Fgf10* transcripts because both of these FGFs are expressed in lung mesenchyme and have been implicated in epithelial cell differentiation as part of a paracrine pathway (Min et al., 1998; Nyeng et al., 2008; Shannon et al., 1999). Interestingly, *Fgf7* expression is significantly decreased in $Nfib^{-/-}$ lungs (fold change = −1.6, P = 0.03) but essentially unchanged in $Nfib^{flox/flox}$, *D1-Cre* lungs (fold change = −1.2, p = 0.2) (Fig. 8F). Increased levels of *Fgf10* transcripts were detected in both $Nfib^{-/-}$ and $Nfib^{flox/flox}$, *D1-Cre* lungs (Fig. 8G), suggesting an excess of FGF-10 might attenuate epithelial cell differentiation and trigger cell proliferation, as was seen previously in *Fgf10* overexpressing mice (Nyeng et al., 2008).

Finally, lipid and surfactant production are critical for lung function. Our microarray analysis also found decreased expression of several genes related to lipid and surfactant protein production (Table S2), suggesting that multiple pathways influencing epithelial cell function and differentiation are altered in $Nfib^{flox/flox}$, *D1-Cre* lungs.

Elastin is an NFI-B target gene during lung development

We next asked whether any of the genes whose expression was affected by loss of *Nfib* might be direct NFI-B target genes. A NFI binding site between −401 and −415 in the human *Elastin* gene was characterized previously using *in vitro* DNA-binding assays and transient transfection analysis (Degterev and Foster, 1999). We examined the corresponding sequence of the mouse *Eln* promoter and identified 3 putative NFI binding sites located between −467 bp and −344 bp in a region highly homologous to the human *ELN* promoter (Fig. 9A). To test whether these sites are bound by NFI-B *in vivo* in E16.5 mouse lungs we performed ChIP analysis. Both standard PCR and QPCR indicate that NFI-B binding occurs in the region containing these three predicted NFI-B binding sites (Figs. 9B–C). In contrast, there is no enrichment in the region of the first intron of β2-microglobulin, the negative control region used in our ChIP assay (Figs. 9B–C). These data indicate that NFI-B binds to this region of the mouse *Eln* promoter at E16.5. To determine whether NFI-B could activate endogenous *Eln* expression we transfected MEFs with either a control GFP vector or a vector expressing NFI-B2 from the CMV promoter (Fig. 9D). The NFI-B2 isoform was used because it is the major spliced isoform of NFIB in lung (data not shown). Transient expression of NFI-B2 increased Eln expression by ~4 fold in MEFs when compared to the control GFP vector. Taken together these data indicate that *Nfib* can regulate *Eln* expression, most likely through the conserved NFI binding sites in the promoter region. Since *Eln* is expressed exclusively in mesenchyme (Mariani et al., 1997; Pierce et

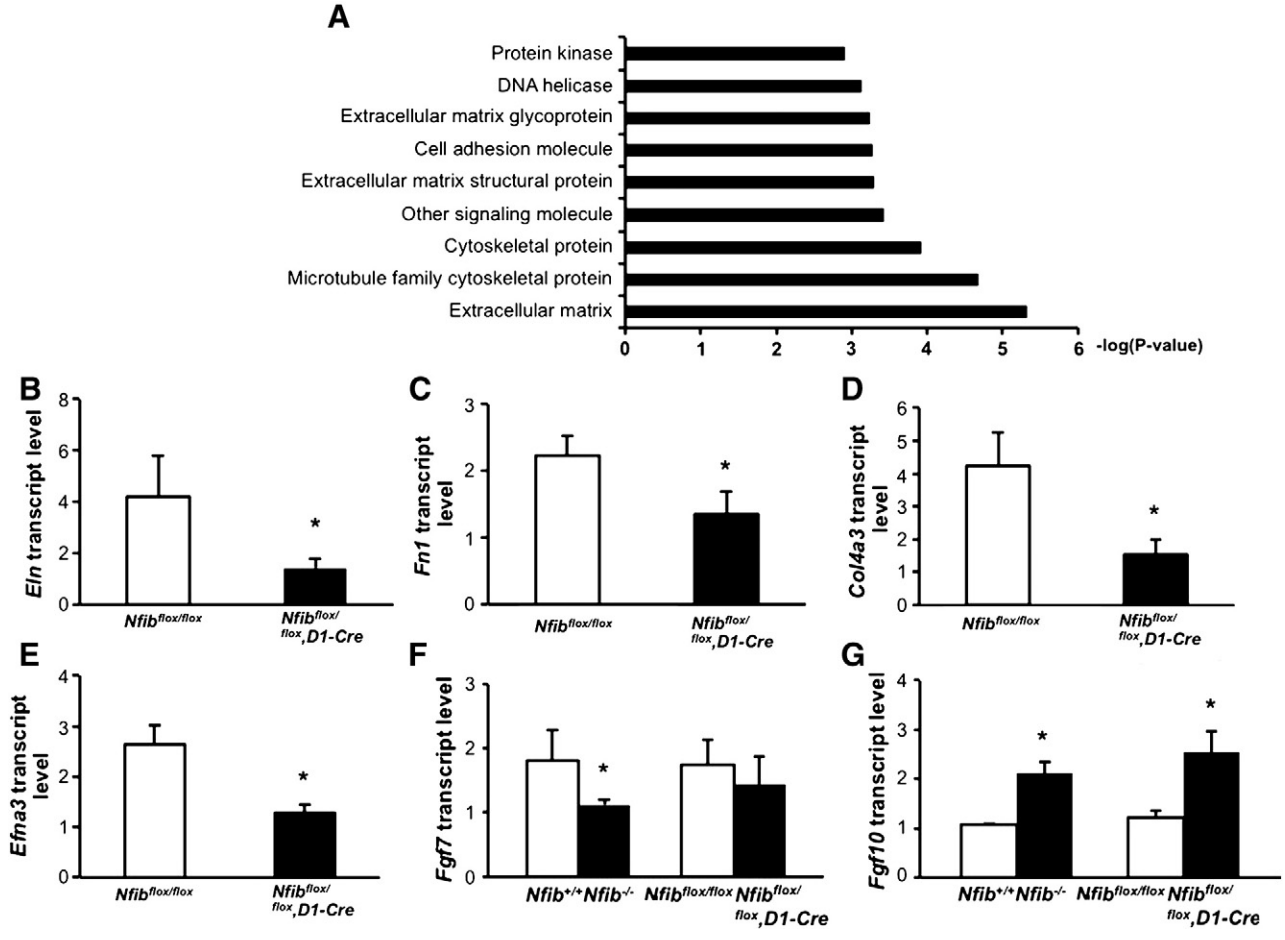

Fig. 8. Microarray and RT-QPCR analysis show changes in the expression of genes involved in extracellular matrix, cell adhesion and FGF signaling pathway in $Nfib^{-/-}$ E18.5 lungs. (A) Microarray studies show that loss of *Nfib* in mesenchyme influences the expression of genes associated with extracellular matrix and cell adhesion. Decreased expression of several genes from the microarray data were confirmed by RT-QPCR from lung RNA isolated from E18.5 *Nfib* mesenchyme-specific knockout and WT mice (B–E). In addition, the expression of *Fgf7* and *Fgf10* were measured using RT-QPCR from lung RNA isolated from E18.5 *Nfib* knockout, mesenchyme-specific knockout and WT mice (F–G). *P < 0.05.

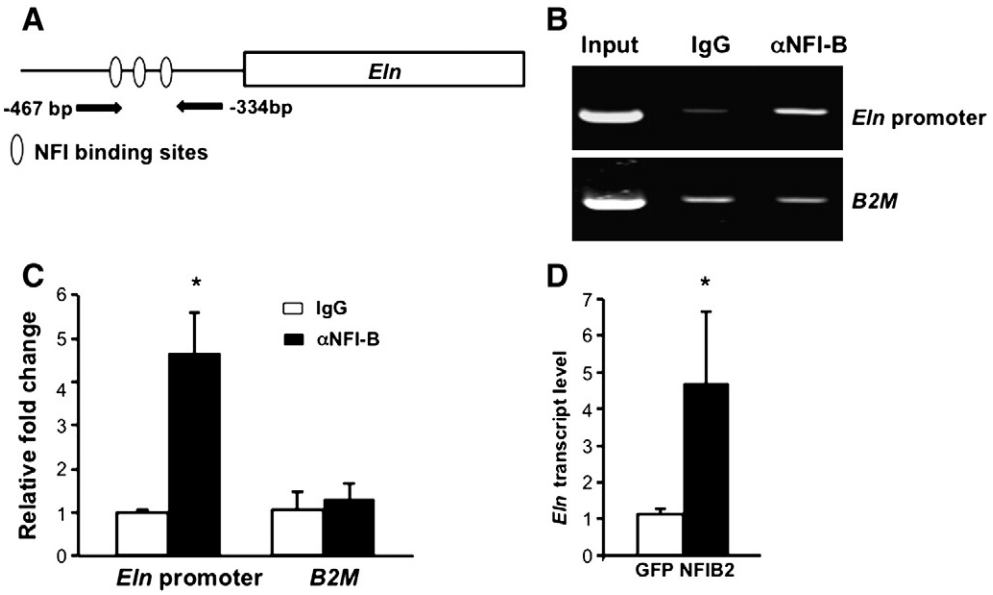

Fig. 9. The promoter region of *Eln* is bound by NFI-B during lung development and NFI-B can activate endogenous *Eln* expression. Three putative NFI binding sites were found in the mouse *Eln* promoter (A). ChIP-PCR (B) and ChIP-QPCR (C) were performed as described in methods and indicate that NFI-B in binds to the mouse *Eln* promoter in E16.5 mouse lungs. Transient expression of NFI-B in MEFs increases the expression of endogenous *Eln* expression (D). *P<0.05.

al., 2006), we propose *Eln* as a mesenchyme-specific target gene of *Nfib*. We are currently investigating which other genes whose expression is affected by loss of *Nfib* may be direct targets of NFI-B.

Discussion

Here we show a critical role for mesenchymal expression of NFI-B in regulating fetal lung maturation. We confirmed that NFI-B is expressed predominantly in lung mesenchyme at E14.5 and in both epithelial cells and mesenchyme at E18.5 (Fig. 1). Loss of *Nfib* results in lung hyperplasia and inhibition of Clara cell, ciliated cell, and distal epithelial cell differentiation (Figs. 2 and 3, S2). Further, the data from our conditional KO mice show that the loss of *Nfib* in only mesenchyme appears responsible for the majority of defects seen in $Nfib^{-/-}$ lungs, with the exception of the inhibition of Clara cell differentiation (Figs. 4–7, S4). Finally, the microarray analysis reveals that *Nfib* in mesenchyme may directly regulate the expression of genes related to the synthesis of extracellular matrix, cell adhesion and the FGF signaling pathway (Figs. 8 and 9). Taken together, these data provide new insights into the role of mesenchymal *Nfib* in the regulation of cell proliferation and maturation in the lung.

NFI-B regulates lung maturation and cell proliferation

Previous studies showed that loss of *Nfib* in all cells in the lung prevents normal lung maturation (Grunder et al., 2002; Steele-Perkins et al., 2005). Here, similar though not identical defects in lung maturation were found in $Nfib^{flox/flox}$, *D1-Cre* lungs, indicating that *Nfib* expression specifically in mesenchyme is required for normal lung maturation. In contrast, CC10 expression was decreased in $Nfib^{-/-}$ lungs but unchanged in $Nfib^{flox/flox}$, *D1-Cre* lungs, suggesting a possible direct effect of NFI-B on Clara cell differentiation. Further loss-of function and gain-of-function studies on *Nfib* in lung epithelium will be needed to address this possibility.

Cell proliferation and relatively low levels of apoptosis occur throughout embryonic lung maturation (Stiles et al., 2001). A fraction of lung mesenchymal cells undergo apoptosis during the pseudo-glandular and saccular stages of lung development, and this process is more obvious around birth (Kresch et al., 1998; Scavo et al., 1998). In our *Nfib*-deficient lungs, there is no change in the number of cleaved Caspase 3 positive cells at E18.5 (Fig. S2A-C). In contrast, an increase in cell proliferation marker expression was observed in both germline *Nfib*-deficient and mesenchyme-specific *Nfib* deficient lungs (Figs. 2 and 6, S2 and 4). These data strongly indicate that an increase in cell proliferation rather than a decrease of apoptosis is the cause of the increase in DNA content and lung size in *Nfib*-deficient mice. We observed increases in proliferation marker expression in both TTF^{+} and TTF^{-} cells in germline *Nfib*-deficient lungs at E18.5 (Fig. 2). Interestingly, in the mesenchyme-specific *Nfib*-deficient lungs there was an increase in proliferation marker expression in both cell populations at E16.5, but no apparent increase in expression in mesenchymal cells at E18.5 (Fig. 6). The lack of increased proliferation marker expression in the TTF^{-} cells at E18.5, despite the clear loss of *Nfib* in the TTF^{-} cells, may indicate that *Nfib* in TTF^{+} lung epithelium can influence the proliferation of TTF^{-} cells during lung maturation. Further studies where *Nfib* is deleted specifically in epithelial cells are needed to address this issue.

Prenatal glucocorticoid treatment has been a standard therapy to stimulate lung maturation in premature infants (Crowley, 1995; Liggins, 1968, 1969; Liggins and Howie, 1972). Glucocorticoid receptor $(GR)^{-/-}$ (Cole et al., 1995) and corticotrophin-releasing hormone $(Crh)^{-/-}$ (Muglia et al., 1999) mice display defective lungs with few or no saccules and an increase in cell proliferation, similar in many respects to the phenotype of *Nfib*-deficient lungs. While the expression levels of these two genes is not reduced in *Nfib*-deficient lungs (data not shown), it is possible that NFI-B may function downstream of GR signaling and/or may cooperate with GR to promote lung maturation. Indeed the transcription of several GR-regulated genes has been shown to be co-regulated both positively and negatively by NFI proteins (Chaudhry et al., 1999; Hebbar and Archer, 2007). Since microarray data from E18.5, $GR^{-/-}$ lungs has been published (Bird et al., 2007), it will be of interest to identify common lung maturation-related genes in the $Nfib^{-/-}$ and $GR^{-/-}$ microarray data which could relate to the common morphological changes seen in these mutant lungs.

Mesenchymal NFI-B regulates epithelial cell differentiation

Defects of distal epithelial cell differentiation were found in both $Nfib^{-/-}$ and $Nfib^{flox/flox}$, *D1-Cre* lungs, indicating NFI-B may regulate

this process through paracrine signaling. Among the genes whose transcript levels are reduced in *Nfib*$^{flox/flox}$, *D1-Cre* lungs is Fibronectin 1 (*Fn1*) *Fn1* is necessary for epithelial cell differentiation in primary cultures of lung epithelial cells (Isakson et al., 2001; Olsen et al., 2005; Roman, 1997). It is possible that decreased mesenchymal expression of *Fn1* could affect distal epithelial cell differentiation in our *Nfib*-deficient mice. *Eln* transcript expression was decreased ~3 fold in *Nfib*-deficient lungs (fold change = −3.0, P = 0.007). Elastin synthesis is required for breath and lung extension (Shifren and Mecham, 2006; Starcher, 2000) and *Eln*$^{-/-}$ mice die by P3.5 with lung alveolar defects (Wendel et al., 2000). In addition, mechanical stretch has been demonstrated to promote type II epithelial cell differentiation (Sanchez-Esteban et al., 2001; Wang et al., 2009). While *Eln*$^{-/-}$ mice show no lung maturation defects at E18.5 (Wendel et al., 2000), it is possible that the reduction in *Eln* expression, in conjunction with the decreased expression of *Fn1* and other genes, could inhibit lung extension in *Nfib*-deficient mice and thus inhibit epithelial cell differentiation. Previous studies showed that the human *ELN* promoter contained an NFI binding site that was required for NFI-induced expression of the promoter in transient reporter assays (Degterev and Foster, 1999). Here we have demonstrated that NFI-B binds to the *Eln* promoter in mouse lungs *in vivo* at E16.5 and can activate endogenous *Eln* expression (Fig. 9). It will be important in future studies to determine how direct binding of NFI-B to the *Eln* promoter *in vivo* regulates *Eln* expression and whether decreased *Eln* expression in *Nfib*$^{-/-}$ lungs directly or indirectly influences lung maturation. In addition, it will be important to assess gene expression profiles at multiple stages of lung development to determine how many of the changes in gene expression seen in the E18.5 *Nfib*$^{flox/flox}$, *D1-Cre* lungs reflect direct targets of *Nfib* that could mediate the lung-maturation phenotype, versus secondary changes due to the altered differentiation state of the mutant lungs.

While the initial microarray data showed no significant difference in the levels of *Fgf10* transcripts (fold change = 1.2, P = 0.13), subsequent QPCR analysis showed that *Fgf10* expression was consistently increased in both *Nfib*$^{-/-}$ (fold change = 2.0, P = 0.0007) and *Nfib*$^{flox/flox}$, *D1-Cre* lungs (fold change = 2.1, P = 0.003). Such discrepancies indicate the importance of careful testing of individual candidate genes from microarray analysis by QPCR. The overexpression of *Fgf10* during lung development results in some changes similar to those seen in *Nfib*-deficient lungs, including lung hyperplasia and inhibition of distal epithelial cell differentiation (Clark et al., 2001; Nyeng et al., 2008). However, the defects in branching morphogenesis, increase in pro-SPC and TTF-1 expression, and the formation of a layer of smooth muscle cells around the lung perimeter seen in lungs in which *Fgf10* is highly overexpressed were not observed in *Nfib*-deficient lungs. This may be due to differences in the timing, degree and spatial distribution of *Fgf10* overexpression in the two systems. In future studies, it will be useful to determine whether reduction of *Fgf10* expression can partially rescue the defects of *Nfib*-deficient lungs by crossing *Fgf10*$^{-/+}$ mice with *Nfib*$^{-/+}$ mice.

Previous studies on lung maturation focused primarily on genes expressed in lung epithelial cells, such as *Klf5* (Wan et al., 2008), *Tgfb1* (Zhou et al., 1996), *Cebpa* (Berg et al., 2006; Martis et al., 2006), *Vegf* (Zeng et al., 1998), *T1a* (Millien et al., 2006), *GR* (Manwani et al., 2010), *Carm1* (O'Brien et al., 2010), *Pparγ* (Simon et al., 2006), *Alk5* (Xing et al., 2010) and *Pdgfa* (Li and Hoyle, 2001). There is far less data on how gene expression in mesenchyme regulates lung maturation. Here we found that *Nfib* in lung mesenchyme is necessary for lung maturation and epithelial cell differentiation. These studies suggest that *Nfib* in lung mesenchyme regulates cell proliferation and distal epithelial cell differentiation through regulation of the expression of genes related to extracellular matrix deposition, cell adhesion and/or FGF signaling. Future studies will focus on defining in more detail the molecular mechanisms by which NFI-B in mesenchyme affects epithelial cell differentiation and both epithelial and mesenchymal cell proliferation during lung maturation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.04.002.

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References

Akeson, A.L., Greenberg, J.M., Cameron, J.E., Thompson, F.Y., Brooks, S.K., Wiginton, D., Whitsett, J.A., 2003. Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. Dev. Biol. 264, 443–455.
Bachurski, C.J., Yang, G.H., Currier, T.A., Gronostajski, R.M., Hong, D., 2003. Nuclear factor I/thyroid transcription factor 1 interactions modulate surfactant protein C transcription. Mol. Cell. Biol. 23, 9014–9024.
Berg, T., Didon, L., Nord, M., 2006. Ectopic expression of C/EBPalpha in the lung epithelium disrupts late lung development. Am. J. Physiol. Lung Cell. Mol. Physiol. 291, L683–L693.
Bird, A.D., Tan, K.H., Olsson, P.F., Zieba, M., Flecknoe, S.J., Liddicoat, D.R., Mollard, R., Hooper, S.B., Cole, T.J., 2007. Identification of glucocorticoid-regulated genes that control cell proliferation during murine respiratory development. J. Physiol. 585, 187–201.
Campbell, C.E., Piper, M., Plachez, C., Yeh, Y.T., Baizer, J.S., Osinski, J.M., Litwack, E.D., Richards, L.J., Gronostajski, R.M., 2008. The transcription factor Nfix is essential for normal brain development. BMC Dev. Biol. 8, 52.
Chaudhry, A.Z., Vitullo, A.D., Gronostajski, R.M., 1999. Nuclear factor I-mediated repression of the mouse mammary tumor virus promoter is abrogated by the coactivators p300/CBP and SRC-1. J. Biol. Chem. 274, 7072–7081.
Clark, J.C., Tichelaar, J.W., Wert, S.E., Itoh, N., Perl, A.K., Stahlman, M.T., Whitsett, J.A., 2001. FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. Am. J. Physiol. Lung Cell. Mol. Physiol. 280, L705–L715.
Coalson, J.J., Winter, V.T., Siler-Khodr, T., Yoder, B.A., 1999. Neonatal chronic lung disease in extremely immature baboons. Am. J. Respir. Crit. Care Med. 160, 1333–1346.
Cohen, E.D., Ihida-Stansbury, K., Lu, M.M., Panettieri, R.A., Jones, P.L., Morrisey, E.E., 2009. Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway. J. Clin. Invest. 119, 2538–2549.
Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., Schutz, G., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608–1621.
Crowley, P.A., 1995. Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994. Am. J. Obstet. Gynecol. 173, 322–335.
de Jong, R.N., van der Vliet, P.C., 1999. Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication. Gene 236, 1–12.
De Langhe, S.P., Carraro, G., Tefft, D., Li, C., Xu, X., Chai, Y., Minoo, P., Hajihosseini, M.K., Drouin, J., Kaartinen, V., Bellusci, S., 2008. Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signaling. PLoS One 3, e1516.
Degterev, A., Foster, J.A., 1999. The role of NF-1 factors in regulation of elastin gene transcription. Matrix Biol. 18, 295–307.
Deimling, J., Thompson, K., Tseu, I., Wang, J., Keijzer, R., Tanswell, A.K., Post, M., 2007. Mesenchymal maintenance of distal epithelial cell phenotype during late fetal lung development. Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L725–L741.
Demayo, F., Minoo, P., Plopper, C.G., Schuger, L., Shannon, J., Torday, J.S., 2002. Mesenchymal-epithelial interactions in lung development and repair: are modeling and remodeling the same process? Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L510–L517.
Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.
Gronostajski, R.M., 1986. Analysis of nuclear factor I binding to DNA using degenerate oligonucleotides. Nucleic Acids Res. 14, 9117–9132.
Gronostajski, R.M., 2000. Roles of the NFI/CTF gene family in transcription and development. Gene 249, 31–45.
Grunder, A., Ebel, T.T., Mallo, M., Schwarzkopf, G., Shimizu, T., Sippel, A.E., Schrewe, H., 2002. Nuclear factor I-B (Nfib) deficient mice have severe lung hypoplasia. Mech. Dev. 112, 69–77.
Hebbar, P.B., Archer, T.K., 2007. Chromatin-dependent cooperativity between site-specific transcription factors in vivo. J. Biol. Chem. 282, 8284–8291.
Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., Betsholtz, C., 1999. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126, 3047–3055.
Huang da, W., Sherman, B.T., Tan, Q., Kir, J., Liu, D., Bryant, D., Guo, Y., Stephens, R., Baseler, M.W., Lane, H.C., Lempicki, R.A., 2007. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. 35, W169–W175.
Isakson, B.E., Lubman, R.L., Seedorf, G.J., Boitano, S., 2001. Modulation of pulmonary alveolar type II cell phenotype and communication by extracellular matrix and KGF. Am. J. Physiol. Cell Physiol. 281, C1291–C1299.

Jobe, A.H., 2005. Antenatal associations with lung maturation and infection. J. Perinatol. 25 (Suppl 2), S31–S35.
Kresch, M.J., Christian, C., Wu, F., Hussain, N., 1998. Ontogeny of apoptosis during lung development. Pediatr. Res. 43, 426–431.
Li, J., Hoyle, G.W., 2001. Overexpression of PDGF-A in the lung epithelium of transgenic mice produces a lethal phenotype associated with hyperplasia of mesenchymal cells. Dev. Biol. 239, 338–349.
Li, C., Xiao, J., Hormi, K., Borok, Z., Minoo, P., 2002. Wnt5a participates in distal lung morphogenesis. Dev. Biol. 248, 68–81.
Liggins, G.C., 1968. Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. J. Endocrinol. 42, 323–329.
Liggins, G.C., 1969. Premature delivery of foetal lambs infused with glucocorticoids. J. Endocrinol. 45, 515–523.
Liggins, G.C., Howie, R.N., 1972. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 50, 515–525.
Manwani, N., Gagnon, S., Post, M., Joza, S., Muglia, L., Cornejo, S., Kaplan, F., Sweezey, N.B., 2010. Reduced viability of mice with lung epithelial-specific knockout of glucocorticoid receptor. Am. J. Respir. Cell Mol. Biol. 43, 599–606.
Mariani, T.J., Sandefur, S., Pierce, R.A., 1997. Elastin in lung development. Exp. Lung Res. 23, 131–145.
Martis, P.C., Whitsett, J.A., Xu, Y., Perl, A.K., Wan, H., Ikegami, M., 2006. C/EBPalpha is required for lung maturation at birth. Development 133, 1155–1164.
Meisterernst, M., Gander, I., Rogge, L., Winnacker, E.L., 1988. A quantitative analysis of nuclear factor I/DNA interactions. Nucleic Acids Res. 16, 4419–4435.
Millien, G., Spira, A., Hinds, A., Wang, J., Williams, M.C., Ramirez, M.I., 2006. Alterations in gene expression in T1 alpha null lung: a model of deficient alveolar sac development. BMC Dev. Biol. 6, 35.
Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Genes Dev. 12, 3156–3161.
Morrisey, E.E., Hogan, B.L., 2010. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev. Cell 18, 8–23.
Muglia, L.J., Bae, D.S., Brown, T.T., Vogt, S.K., Alvarez, J.G., Sunday, M.E., Majzoub, J.A., 1999. Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. Am. J. Respir. Cell Mol. Biol. 20, 181–188.
Nagata, K., Guggenheimer, R.A., Enomoto, T., Lichy, J.H., Hurwitz, J., 1982. Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA 79, 6438–6442.
Nagata, K., Guggenheimer, R.A., Hurwitz, J., 1983. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80, 6177–6181.
Nyeng, P., Norgaard, G.A., Kobberup, S., Jensen, J., 2008. FGF10 maintains distal lung bud epithelium and excessive signaling leads to progenitor state arrest, distalization, and goblet cell metaplasia. BMC Dev. Biol. 8, 2.
O'Brien, K.B., Alberich-Jorda, M., Yadav, N., Kocher, O., Diruscio, A., Ebralidze, A., Levantini, E., Sng, N.J., Bhasin, M., Caron, T., Kim, D., Steidl, U., Huang, G., Halmos, B., Rodig, S.J., Bedford, M.T., Tenen, D.G., Kobayashi, S., 2010. CARM1 is required for proper control of proliferation and differentiation of pulmonary epithelial cells. Development 137, 2147–2156.
Olsen, C.O., Isakson, B.E., Seedorf, G.J., Lubman, R.L., Boitano, S., 2005. Extracellular matrix-driven alveolar epithelial cell differentiation in vitro. Exp. Lung Res. 31, 461–482.
Pierce, R.A., Moore, C.H., Arikan, M.C., 2006. Positive transcriptional regulatory element located within exon 1 of elastin gene. Am. J. Physiol. Lung Cell. Mol. Physiol. 291, L391–L399.
Plasari, G., Calabrese, A., Dusserre, Y., Gronostajski, R.M., McNair, A., Michalik, L., Mermod, N., 2009. Nuclear factor I-C links platelet-derived growth factor and transforming growth factor beta1 signaling to skin wound healing progression. Mol. Cell. Biol. 29, 6006–6017.
Roman, J., 1997. Fibronectin and fibronectin receptors in lung development. Exp. Lung Res. 23, 147–159.
Sanchez-Esteban, J., Cicchiello, L.A., Wang, Y., Tsai, S.W., Williams, L.K., Torday, J.S., Rubin, L.P., 2001. Mechanical stretch promotes alveolar epithelial type II cell differentiation. J. Appl. Physiol. 91, 589–595.
Scavo, L.M., Ertsey, R., Chapin, C.J., Allen, L., Kitterman, J.A., 1998. Apoptosis in the development of rat and human fetal lungs. Am. J. Respir. Cell Mol. Biol. 18, 21–31.
Shannon, J.M., Gebb, S.A., Nielsen, L.D., 1999. Induction of alveolar type II cell differentiation in embryonic tracheal epithelium in mesenchyme-free culture. Development 126, 1675–1688.
Shifren, A., Mecham, R.P., 2006. The stumbling block in lung repair of emphysema: elastic fiber assembly. Proc. Am. Thorac. Soc. 3, 428–433.
Shu, W., Jiang, Y.Q., Lu, M.M., Morrisey, E.E., 2002. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. Development 129, 4831–4842.
Simon, D.M., Arikan, M.C., Srisuma, S., Bhattacharya, S., Tsai, L.W., Ingenito, E.P., Gonzalez, F., Shapiro, S.D., Mariani, T.J., 2006. Epithelial cell PPAR [gamma] contributes to normal lung maturation. FASEB J. 20, 1507–1509.
Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 Article3.
Starcher, B.C., 2000. Lung elastin and matrix. Chest 117, 229S–234S.
Steele-Perkins, G., Plachez, C., Butz, K.G., Yang, G., Bachurski, C.J., Kinsman, S.L., Litwack, E.D., Richards, L.J., Gronostajski, R.M., 2005. The transcription factor gene Nfib is essential for both lung maturation and brain development. Mol. Cell. Biol. 25, 685–698.
Stiles, A.D., Chrysis, D., Jarvis, H.W., Brighton, B., Moats-Staats, B.M., 2001. Programmed cell death in normal fetal rat lung development. Exp. Lung Res. 27, 569–587.
Sugahara, K., Tokumine, J., Teruya, K., Oshiro, T., 2006. Alveolar epithelial cells: differentiation and lung injury. Respirology 11 (Suppl), S28–S31.
Wan, H., Luo, F., Wert, S.E., Zhang, L., Xu, Y., Ikegami, M., Maeda, Y., Bell, S.M., Whitsett, J.A., 2008. Kruppel-like factor 5 is required for perinatal lung morphogenesis and function. Development 135, 2563–2572.
Wang, Y., Maciejewski, B.S., Soto-Reyes, D., Lee, H.S., Warburton, D., Sanchez-Esteban, J., 2009. Mechanical stretch promotes fetal type II epithelial cell differentiation via shedding of HB-EGF and TGF-alpha. J. Physiol. 587, 1739–1753.
Wendel, D.P., Taylor, D.G., Albertine, K.H., Keating, M.T., Li, D.Y., 2000. Impaired distal airway development in mice lacking elastin. Am. J. Respir. Cell Mol. Biol. 23, 320–326.
White, A.C., Xu, J., Yin, Y., Smith, C., Schmid, G., Ornitz, D.M., 2006. FGF9 and SHH signaling coordinate lung growth and development through regulation of distinct mesenchymal domains. Development 133, 1507–1517.
White, A.C., Lavine, K.J., Ornitz, D.M., 2007. FGF9 and SHH regulate mesenchymal Vegfa expression and development of the pulmonary capillary network. Development 134, 3743–3752.
Xing, Y., Li, C., Li, A., Sridurongrit, S., Tiozzo, C., Bellusci, S., Borok, Z., Kaartinen, V., Minoo, P., 2010. Signaling via Alk5 controls the ontogeny of lung Clara cells. Development 137, 825–833.
Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., et al., 2003. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development 130, 3063–3074.
Zeng, X., Wert, S.E., Federici, R., Peters, K.G., Whitsett, J.A., 1998. VEGF enhances pulmonary vasculogenesis and disrupts lung morphogenesis in vivo. Dev. Dyn. 211, 215–227.
Zhou, L., Dey, C.R., Wert, S.E., Whitsett, J.A., 1996. Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-beta 1 chimeric gene. Dev. Biol. 175, 227–238.