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# GATA and Nkx factors synergistically regulate tissue-specific gene expression and development in vivo

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In vitro studies have suggested that members of the GATA and Nkx transcription factor families physically interact, and synergistically activate pulmonary epithelial- and cardiac-gene promoters. However, the relevance of this synergy has not been demonstrated in vivo. We show that *Gata6-Titf1* (*Gata6-Nkx2.1*) double heterozygous (*G6-Nkx* DH) embryos and mice have severe defects in pulmonary epithelial differentiation and distal airway development, as well as reduced phospholipid production. The defects in *G6-Nkx* DH embryos and mice are similar to those observed in human neonates with respiratory distress syndromes, including bronchopulmonary dysplasia, and differential gene expression analysis reveals essential developmental pathways requiring synergistic regulation by both Gata6 and Titf1 (Nkx2.1). These studies indicate that Gata6 and Nkx2.1 act in a synergistic manner to direct pulmonary epithelial differentiation and development in vivo, providing direct evidence that interactions between these two transcription factor families are crucial for the development of the tissues in which they are co-expressed.

**KEY WORDS: GATA, Nkx, Transcription factor, Synergy, Lung, Mouse**

## INTRODUCTION

Mounting evidence suggests that the combinatorial action of multiple transcription factor families is required for cell specification, differentiation and tissue development. Evidence for this comes from multiple in vitro studies in which the physical interaction between transcription factors results in the synergistic regulation of tissue-restricted target-gene promoters. Two such families of transcription factors that have been shown to interact in vitro are the GATA family of zinc-finger proteins and the Nkx family of homeodomain transcription factors.

The six known GATA factors in vertebrates can be divided into two subfamilies; Gata1-3 and Gata4-6. All six GATA family members share a highly conserved double zinc-finger DNA-binding domain. Gata1-3 are primarily expressed in hematopoietic tissues, and are required for erythrocyte differentiation (Gata1 and Gata2), T cell development (Gata3) and hematopoietic stem cell development (Gata2) (reviewed in Weiss and Orkin, 1995). Gata4-6 are expressed in heart and endodermal-derived tissues, such as the lung and intestine, and all three members have been shown to play crucial roles in the development of these tissues (reviewed in Molkentin, 2000). In particular, Gata6 can activate several lung epithelial-restricted genes, including surfactant protein A (*SP-A*, also known as *Sftpa1* – Mouse Genome Informatics), surfactant protein C (*SP-C*, also known as *Sftpc* – Mouse Genome Informatics) and *Wnt7b*, and is required for proper lung epithelial differentiation in vivo (Bruno et al., 2000; Liu et al., 2002a; Weidenfeld et al., 2002).

Members of the Nkx family of homeodomain transcription factors are expressed in pulmonary epithelium and cardiomyocytes. Titf1 (also known as Nkx2.1, and hereafter referred to as Nkx2.1) is expressed throughout the conducting airway epithelium and both loss- and gain-of-function experiments have demonstrated an essential role for this factor in lung development (Kimura et al., 1996). Loss of Nkx2.1 results in an early and severe block in branching morphogenesis of the lung, as well as a severe loss of epithelial cell differentiation (Kimura et al., 1996; Minoo et al., 1999; Yuan et al., 2000). In the heart, Nkx2.5 is expressed in the developing bilateral precardiac mesoderm early in development, starting at E7.5 (Lyons et al., 1995). Loss of Nkx2.5 in mice results in embryonic demise at E9.5 resulting from aberrant cardiac development with defects in looping morphogenesis and ventricular specification (Lyons et al., 1995). Dominant mutations in *NKX2.1* and *NKX2.5* in humans lead to congenital pulmonary and cardiac defects, respectively, demonstrating their importance in adult tissue homeostasis (Krude et al., 2002; Schott et al., 1998).

The lung arises from an outpouching of the ventral foregut at approximately E9.5 of mouse development. The primitive airways grow quickly in an arborized fashion through branching morphogenesis. The lung is patterned in a distinct proximal-distal manner and expression patterns of lung-restricted genes define epithelial cell types within the developing lung. For example, SP-C is expressed first at E10.5 in the distal tips of the growing airway epithelium and later its expression is restricted to alveolar type-2 cells (AEC-2) in the alveolus. By contrast, Clara cell 10 kd protein (CC10, also known as Scgb1a1 – Mouse Genome Informatics) is expressed exclusively by Clara cells lining the bronchioles and upper airways, beginning at approximately E16.5. Transcription factors such as Gata6 and Nkx2.1 are also expressed in a proximal-distal manner, with Gata6 being expressed at high levels in the distal airway epithelium and later in AEC-2 cells while Nkx2.1 is expressed throughout the developing airway epithelium, with highest levels in the distal airway epithelium (Morrisey et al., 1996; Yuan et al., 2000).

Recent evidence demonstrates that GATA- and Nkx-family members physically interact to synergistically activate target genes in lung, heart and vascular smooth muscle in vitro (Liu et al., 2002a; Nishida et al., 2002; Sepulveda et al., 2002; Weidenfeld et al., 2002). Whether these physical interactions are required for proper

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development of any of these tissues in vivo is unknown. To determine whether GATA-Nkx interactions are required for the regulation of tissue-specific gene expression and development in vivo, we generated *Gata6-Nkx2.1* double heterozygous (*G6-Nkx* DH) mice to assess the result of haploinsufficiency of both genes on lung epithelial differentiation and development. Gata6 and Nkx2.1 are the only known GATA and Nkx family members expressed in lung epithelia, and both are essential for the proper development of this tissue (Kimura et al., 1996; Liu et al., 2002b; Yang et al., 2002). *G6-Nkx* DH embryos and mice exhibit specific defects in lung epithelial differentiation, which cannot be accounted for by a classic genetic epistatic relationship between the two genes, indicating that the protein-protein interaction between GATA- and Nkx-family members is crucial for tissue-specific gene regulation and cell differentiation in vivo.

## MATERIALS AND METHODS

### Mouse lines

Generation of the *Gata6*$^{+/-}$ and *Nkx2.1*$^{+/-}$ mouse lines has been previously described (Kimura et al., 1996; Morrisey et al., 1998). These lines were maintained on a C57BL/6:CD-1 mixed background, as described previously (Kimura et al., 1996; Morrisey et al., 1998) and were intercrossed to generate *G6-Nkx* DH embryos and adult mice. Embryonic age was established by considering noon of the day that the vaginal plug was observed as E0.5. Embryos were harvested by Cesarean section. Genotyping was performed by PCR from genomic DNA isolated from either yolk sac or tails of the fetuses and adult mice. The PCR conditions and oligos for genotyping the *Nkx2.1*$^{+/-}$ mice have been previously described (Niimi et al., 2001). Genotyping for the *Gata6*$^{+/-}$ allele was performed using the following oligonucleotides: forward 5′-CATT-CCTCCCACTCATGATCTATAG-3′, reverse 5′-GGTCACATTACAAT-TAAGAGCAGC-3′.

### Histology

Dissected lungs (E18.5 and adult) were fixed in 4% paraformaldehyde for 48 hours. Tissues were then dehydrated in ethanol and embedded in paraffin. Paraffin sections were cut at 5 μm and used for histochemical staining, in situ hybridization and immunohistochemistry. Immunohistochemistry using CC10 (Santa Cruz T-18, 1:500), SP-C (Chemicon AB3786, 1:1000) and Bmp4 (Santa Cruz N-16, 1:100) was performed as previously described (Shu et al., 2005). In situ probes for Gata6, Nkx2.1 and aquaporin-5 (Aqp5) have been previously described (Shu et al., 2005; Yang et al., 2002). Oligonucleotides to generate the in situ probes for RBP-L (also known as Rbpsuhl – Mouse Genome Informatics), stearoly-CoA desaturase 1 (Scd1), dipeptidyl peptidase 4 (Dpp4) and napsin (also known as Napsa – Mouse Genome Informatics) are: Scd1 forward 5′-GGTGCCAAACACTCAGTT-CACTTG-3′, reverse 5′-TGTAATACGACTCACTATAGGGAGCCTC-TTGACTATTCCCACTCG-3′; Dpp4 forward 5′-TCCAGAAGACAA-CCTTGACCATTAC-3′, reverse 5′-TGTAATACGACTCACTATAGGG-GGGGACAGGCATCCTTAGTTAGG-3′; RBP-L forward 5′-AAAG-AGCAGGAAGGAGAGAGATAGC-3′, reverse 5′-TGTAATACGACT-CACTATAGGGTCACCACAGGCAGATAGACGC-3′; napsin forward 5′-ATCGCTTTAATCCCAAGGCCTTCC-3′, reverse 5′-TGTAATACG-ACTCACTATAGGGGCCAACATCGCTCTGAAGAATC-3′. All reverse oligonucleotides contain a T7 RNA polymerase recognition sequence used to generate labeled cRNA probes. Periodic acid-Schiff (PAS) staining was performed as previously described (Yang et al., 2002). Additional details on histological methods can be found at the University of Pennsylvania Molecular Cardiology Research Center web site http://www.uphs.upenn.edu/mcrc/. All data are representative of at least five embryos of each genotype.

### Lung morphometry

Mesenchymal thickness was calculated by capturing digital images at 200× magnification; overlaying grid lines in a vertical, horizontal and diagonal fashion; and measuring the mesenchymal thickness on at least five inter-alveolar regions per field of view. This was performed on ten fields of view per sample and on four samples of each indicated genotype. The Student's *t*-test was used to calculate the significance of the differences between each group.

### Electron microscopy

Lung tissue from the indicated mouse embryos (three samples for each genotype) were fixed in 2% gluteraldehyde with 0.1 M sodium cacodylate (pH 7.4) for 72 hours at 4°C. Samples were further incubated with 2% osmium tetroxide and 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4°C. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed on a JEM 1010 microscope.

### Microarray and quantitative PCR studies

RNA was isolated from E18.5 lungs from wild-type and *G6-Nkx* DH littermates (three samples from each genotype). Total RNA was transcribed to generate biotinylated cRNA to use as a probe for Affymetrix mouse 230A GeneChips. Three chips each were hybridized for both wild-type and *G6-Nkx* DH samples. Data from these arrays was normalized using Microarray Suite 5.0 (MAS5, Affymetrix) and Significance Analysis of Microarrays (SAM). Changes in gene expression of 2.0-fold and greater were considered significant. Treeview software was used to generate the heatmap (http://rana.lbl.gov). Total RNA was isolated with Trizol and quantitative-PCR was performed using the oligonucleotides listed in Table 3 with an Applied Biosystems 7900HT system and Syber green reaction mixture, as previously described (Lepore et al., 2005).

### Identification of transcription factor-binding sites

For each murine gene, 1 kb of genomic sequence immediately upstream from the transcription start site was extracted using the RefSeq genome sequence database (www.genome.ucsc.edu). These promoter regions were searched using the 546 transcription factor-binding-site motifs obtained from the TRANFAC database v8.4. A binding-site motif is represented as a Positional Weight Matrix (PWM), which is a $4\times k$ matrix, where $k$ denotes the number of bases in the binding site. For each of the $k$ positions, this matrix provides the preferences for the four nucleotide bases at that position. Matches between TRANFAC PWMs and promoter regions of cardiac genes were selected using the tool PWMSCAN (Levy and Hannenhalli, 2002). The criterion for a match was $P$-value cutoff of $2\times10^{-4}$, corresponding to a probability of less than one random match in every 5 kb of genomic sequence. These matches were further filtered using human-mouse genome-sequence alignments to focus our analyses on promoter regions that showed evolutionary conservation. These criteria for matching have been evaluated previously and were shown to accurately detect experimentally verified binding sites with a low false-positive rate of one random match in every 50 kb of genomic sequence searched (Levy and Hannenhalli, 2002). Because of similar DNA-binding sites, this methodology cannot distinguish between the enrichment of different members of transcription factor families. For example, 'Gata-2' denotes enrichment of conserved GATA DNA-binding sites.

### Cell transfection studies

The mouse versus human comparisons of the Dpp4 and Scd1 genomic regions were performed using the mVista genomic analysis software (http://genome.lbl.gov/vista/index.shtml). The indicated regions of the mouse *Scd1* and *Dpp4* genomic regions were cloned into the pGL3promoter vector using the following oligonucleotides: Dpp4 1250 bp region A enhancer forward 5′-TGGTACCGTGGTAACAGGTTACGGCAAAG-TTAGC-3′, reverse 5′-ATCTCGAGCCTTTCCCTCTAAACAATTGC-AGTAAC-3′; Scd1 732 bp region A enhancer forward 5′-TGGT-ACCAACAGTGTGGTCCCCAAGAAGCAG-3′, reverse 5′-ATCTCGA-GCACCACCCAGCCTGGCTTGGCAAC-3′; Scd1 466 bp region B enhancer forward 5′-ATGGTACCTGACGCTGGACACCCAGACAT-3′, reverse 5′-ATCTCGAGTGTTGGTTCCCAGGACAATCC-3′. The Gata6 and Nkx2.1 expression plasmids that were used have been previously described (Weidenfeld et al., 2002). NIH-3T3 cells were transfected with the indicated plasmids using Fugene 6 as previously described (Weidenfeld et al., 2002). All transfections were assayed after 48 hours. Luciferase activity was determined using a commercially available kit (Promega). Reported

DEVELOPMENT

**Table 1. Survival of *G6-Nkx* DH embryos-E18.5**

| | Genotype | | | |
|---|---|---|---|---|
| | Wild type | GATA6$^{+/-}$ | Nkx2.1$^{+/-}$ | *G6-Nkx* DH |
| Numbers recovered at E18.5 (percent) | 23 (28%) | 21 (26%) | 18 (22%) | 20 (24%) |
| Numbers recovered at 2 weeks (percent) | 55 (38%) | 49 (33%) | 32 (22%) | 10 (7%) |

**Table 2. ChIP PCR oligonucleotide sequences**

| Region | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| *Dpp4* – region A | CAATCCCTCAAGACTTGCCATAC | GAAGGCACCAGCAAGGAAAG |
| *Dpp4* – region B | CCCCATGCCACCAGATACTAA | CAACTCGGAATGAACCAAAACA |
| *Dpp4* – region C | TGGGCTACATGCTGAGAAGAAA | TTCCAGGAGCACCAGATGAAT |
| *Dpp4* – region D | CTTCAGTGCCTTCCTGGTGAGT | CACCAGTGCTGCACTGGAGTA |
| *Dpp4* – region E | CTTTCTTTGCCAGCCAGATAACA | TGCAGAAACTAGCTGCCAGTTC |
| *Scd1* – region A | TGTTCTAGTCAAGAGGCATCTCATG | TCTGTTTACCCGTCTAACAGGAAAA |
| *Scd1* – region B | GTGGCCTAAAAAGGTGGGAAT | GCCAATCAGAAAGCAAGCCTAGT |
| *Scd1* – region C | TTGGGAAGAAAGAGGAAAATAATCC | ATGTAACTGTGGTGCGTTTCCA |

values are normalized to cells lacking Gata6- or Nkx2.1-expression-plasmids and represent the average of three assays performed in triplicate ± standard error of the mean (s.e.m.).

#### Phospholipid analysis

Lungs from wild-type and *G6-Nkx* DH-mutant mice (age 3-5 months) were lavaged, and phospholipids levels were measured as previously described (Atochina et al., 2000). Three mice from each genotype were used in these studies. Briefly, bronchoalveolar lavage (BAL) fluid was subfractionated into two surfactant fractions: the biophysically active large-aggregate (LA) form and the biophysically inactive small-aggregate (SA) form. Lavage fluid was centrifuged at 1000 ***g*** for 10 minutes at 4°C to remove cells. The cell-free supernatant was recentrifuged at 20,000 ***g*** for 40 minutes at 4°C for separation of LA surfactant in the pellet and SA surfactant in the supernatant fraction. The resulting LA pellets were resuspended in saline for biophysical and biochemical characterization. LA- and SA-surfactant fractions were analyzed for total phospholipid content by extraction of total phospholipid and determination of inorganic phosphorus content with a modified method of Bartlett (Itoh et al., 1986).

#### Chromatin immunoprecipitation assays

Chromatin was made from E18.5 mouse lung tissue using a commercially available kit (Upstate Biotechnology). Lung tissue was minced, fixed with 1% formaldehyde and chromatin was sheared by sonication to an average length of 500-600 bp. The antibodies used for immunoprecipitation were as follows: Gata6 (Santa Cruz Biotech, C-20) and Nkx2.1 (Santa Cruz Biotech, H-190). Reverse cross-linked immunoprecipitated chromatin was subjected to both standard PCR and quantitative PCR on an ABI 7900 using Syber green and the oligonucleotides listed in Table 2.

## RESULTS

### Both alleles of Gata6 and Nkx2.1 are required for lung development

If the synergism between Gata6 and Nkx2.1 observed in vitro is crucial for lung epithelial differentiation and development, these factors should regulate lung development in a synergistic manner. Therefore, to determine whether mice haploinsufficient for both Gata6 and Nkx2.1 exhibited defects in lung epithelial differentiation, *Gata6*$^{+/-}$ and *Nkx2.1*$^{+/-}$ mice were crossed to each other to generate *G6-Nkx* DH embryos and mice, and the resulting offspring were genotyped. This cross should yield approximately 25% *G6-Nkx* DH mice. Remarkably, of 142 mice genotyped at 2 weeks of age, only 7% of the surviving mice were *G6-Nkx* DH, whereas the expected number of *G6-Nkx* DH embryos was recovered prior to birth at E18.5 (Table 1). The only tissue in which Gata6 and Nkx2.1 are co-expressed is lung airway epithelia (Fig. 1A-E) (Minoo et al., 1999; Morrisey et al., 1996). Both Gata6 and Nkx2.1 expression is observed in distal airway epithelium, as well as in more-proximal bronchiolar epithelium that expresses the Clara-cell-marker gene product CC10 (Fig. 1C-H). This suggests that postnatal survival requires the combined expression of Gata6 and Nkx2.1, probably through the regulation of lung airway epithelial development.

Lungs from E18.5 embryos were analyzed histologically for lung-specific defects in *G6-Nkx* DH embryos. Hematoxylin and Eosin staining (H+E) showed an increased thickness in the mesenchyme of *G6-Nkx* DH lungs, with a concomitant loss of airway sacculation (Fig. 2A,J). *Gata6*$^{+/-}$ and *Nkx2.1*$^{+/-}$ lungs appeared histologically normal (Fig. 2D,G). The difference in mesenchymal thickness was quantitated, revealing a more than twofold increase in inter-alveolar mesenchymal thickness in *G6-Nkx* DH lungs versus wild-type littermates (Fig. 2Q). Airway epithelial differentiation was assessed by SP-C and CC10 immunohistochemistry. SP-C is expressed exclusively in distal lung epithelium and later in alveolar type 2 cells, whereas CC10 expression is confined to Clara epithelial cells lining the bronchiolar and tracheal airways. Although *G6-Nkx* DH embryos exhibited SP-C staining in distal airway epithelium, fewer cells stained, and the ones that did were organized in a very focal pattern, probably due to defective sacculation in the distal airways (Fig. 2K,M,N). Similar focal expression was observed for aquaporin-5, a late maturation marker of type 1 cells in the distal airways whose expression is initiated at E17.5 (Fig. 2O,P) (Funaki et al., 1998; Lee et al., 1997; Yang et al., 2002). CC10 expression was dramatically reduced in *G6-Nkx* DH lungs (Fig. 2L). These changes in SP-C and CC10 expression were not observed in either *Gata6*$^{+/-}$ or *Nkx2.1*$^{+/-}$ lungs (Fig. 2B,C,E,F,H,I). Quantitative RT-PCR (Q-PCR) confirmed the decrease in CC10 expression observed by immunohistochemistry, whereas expression of SP-B, another crucial surfactant component that is expressed after E15.5 in alveolar type 2 cells, was not significantly altered (Fig. 3A). Expression of other putative target genes, such as SP-C and Wnt7b, was decreased by approximately 50% (Fig. 3A). In contrast to these findings, SP-C, SP-B and CC10 expression are all severely attenuated or lost upon loss of Gata6- or Nkx2.1-function in vivo (Liu et al., 2002b; Minoo et al., 1999; Yang et al., 2002). Thus, although *G6-Nkx* DH lungs exhibited defects associated with epithelial immaturity, expression of aquaporin-5 and SP-B argues against a general inhibition of lung maturation in *G6-Nkx* DH embryos due to the late gestational onset of their expression.

The defects observed in *G6-Nkx* DH mice could be caused by a genetic epistatic relationship between these two factors, or it could be due to their reported physical interaction (Liu et al., 2002a;

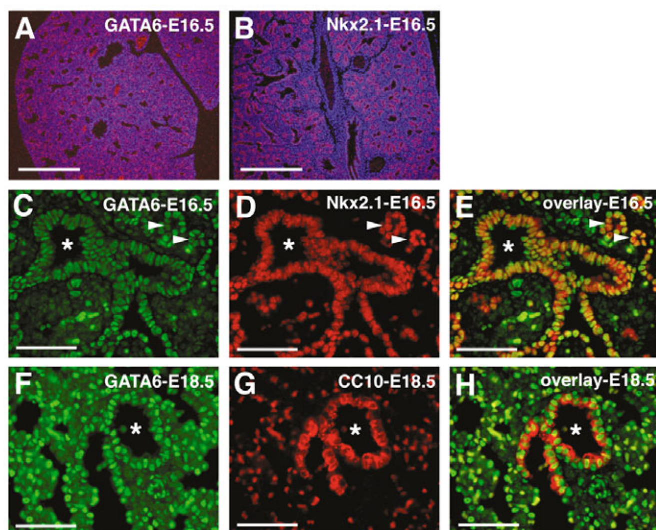



**Fig. 1. Expression of Gata6 and Nkx2.1 during lung development.** In situ hybridization for Gata6 (**A**) and Nkx2.1 (**B**) expression was performed at E16.5. Immunofluorescent staining for Gata6 and Nkx2.1 expression reveals extensive overlap between the expression of both of these proteins at E16.5, including in the distal airway epithelium (**C-E**, arrowheads) and more proximal bronchiolar airways (C-E, asterisks). Expression of Gata6 at E18.5 in proximal bronchiolar airways is confirmed by co-staining with the Clara cell marker gene CC10 (**F-H**, asterisks). Scale bars: 500 μm in A,B; and 100 μm in C-H.

Weidenfeld et al., 2002). To determine whether Gata6 and Nkx2.1 regulate the expression of each other, Q-PCR was performed to determine the expression of Gata6 and Nkx2.1 in wild-type, *Gata6*$^{+/-}$, *Nkx2.1*$^{+/-}$ and *G6-Nkx* DH lungs. Loss of either Gata6 or Nkx2.1 expression did not alter the expression of the other gene (Fig. 3B). This is supported by our previous finding that loss of Gata6 activity does not lead to changes in Nkx2.1 expression (Yang et al., 2002). Thus, the cooperative relationship between Gata6 and Nkx2.1 is unlikely to be caused by classic genetic epistasis, but rather by the physical interaction of the two factors.

### Differentiation of alveolar epithelium and phospholipid production are compromised in *G6-Nkx* DH mutants

Transmission electron microscopy (TEM) was performed to further assess distal airway epithelial differentiation. As expected, at E18.5, distal airway cells in wild-type, *Gata6*$^{+/-}$ and *Nkx2.1*$^{+/-}$ were lined with type-2 AECs containing lamellar bodies (Fig. 4A-D). However, *G6-Nkx* DH lungs were lined with large cuboidal cells that contained large glycogen-filled vacuoles but lacked lamellar bodies (Fig. 4E,F). Glycogen is a precursor of the phospholipid component of pulmonary surfactant, and levels decrease during gestation as it is processed by type-2 AECs into phospholipids for surfactant production and lamellar-body formation. Increased periodic acid-Schiff (PAS) staining in *G6-Nkx* DH lungs confirms the dramatic increase in glycogen content (Fig. 4G,H). These data indicate defective differentiation of distal lung epithelium in *G6-Nkx* DH mutants.

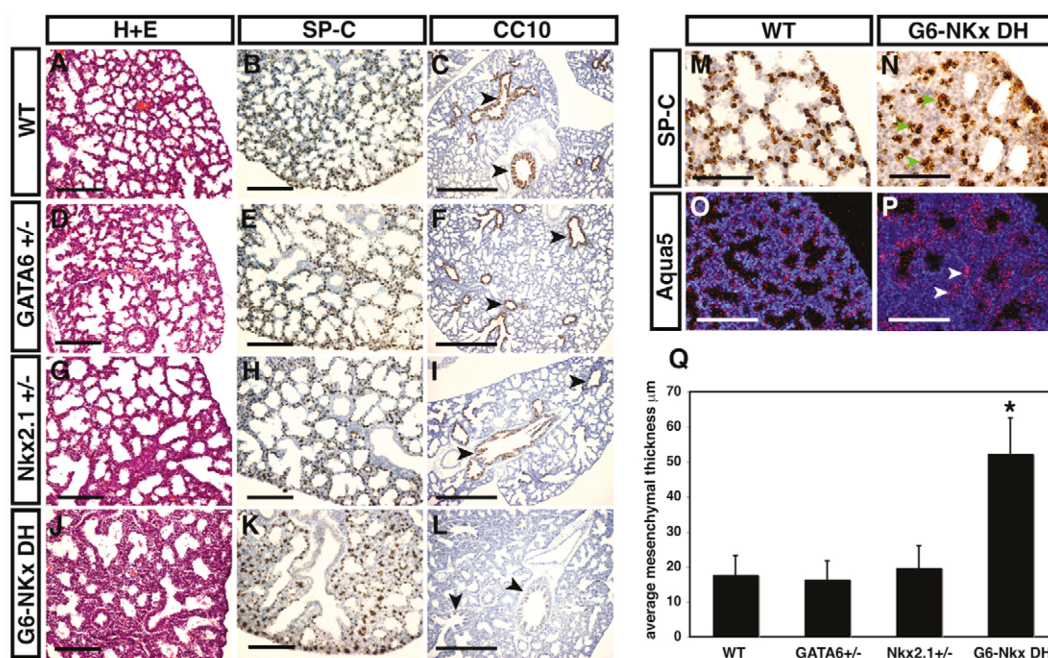



**Fig. 2. Defects in lung epithelial differentiation in *G6-Nkx* DH embryos.** (**A-P**) *G6-Nkx* DH lungs exhibit defects in airway development and differentiation. (A,D,G,J) Hematoxylin and Eosin (H+E) staining of lung sections. Notice the increased mesenchymal thickness and reduced sacculation in *G6-Nkx* DH lungs (J). Wild-type (A) and single heterozygous (D,G) lungs appear normal. SP-C protein expression is more focal in *G6-Nkx* DH lungs (B,E,H,K). Both aquaporin-5 and SP-C are expressed in a tight, focal pattern that is less evenly distributed throughout the distal alveolar airspaces in *G6-Nkx* DH lungs compared with wild-type lungs (M-P, arrowheads). CC10 protein expression is reduced in *G6-Nkx* DH lungs compared with wild-type or single heterozygous lungs (C,F,I,L, arrowheads). Data are from either the left lung or the cranial lobe of the right lung. All histological data are representative of at least five embryos of each genotype. (**Q**) Microscopic measurements were made to determine the mesenchymal thickness in *G6-Nkx* DH lungs, as described in the Materials and methods section. These data show a significant thickening in the inter-alveolar mesenchyme of *G6-Nkx* DH lungs at E18.5. *P* values *<0.001 versus wild-type, *Gata6*$^{+/-}$ or *Nkx2.1*$^{+/-}$ lungs. Scale bars: 500 μm in C,F,I,L; and 100 μm in A,B,D,E,G,H,J,K,M-P.

DEVELOPMENT

**Table 3. RT-PCR oligonucleotide sequences**

| Gene | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| *Scd1* | CACTGAATGCGAGGGTTGGT | CCAAAGCCACATTACCTTGCA |
| *Dpp4* | GGTGGATGCTGGTGTGGATT | GGCTCATGTGGGAATAGATGTG |
| *RBP-L* | GGCGTGCTTCCTGTTTTTGT | TCTGGAGAGCAAATCCCCAAT |
| *CC10* | TCCTAACAAGTCCTCTGTGTAAGCA | AGGAGACACAGGGCAGTGACA |
| *SP-B* | ACGTCCTCTGGAAGCCTTCA | TGTCTTCTTGGAGCCACAACAG |
| *SP-C* | ACCCTGTGTGGAGAGCTACCA | TTTGCGGAGGGTCTTTCCT |
| *napsin* | ABI catalog # Mm00492829 | ABI catalog # Mm00492829 |
| *β-actin* | CAGAAGGAGATTACTGCTCT | GGACCACCGATCCACACA |
| *Gata6* | TGTATTGCTCCAAATCATGTGCTT | CTCTCCACGAACGCTTGTGA |
| *Nkx2.1* | TCCAGCCTATCCCATCTGAACT | CAAGCGCATCTCACGTCTCA |
| *Bmp4* | CGAGCCAACACTGTGAGGAG | TATACGGTGGAAGCCCTGTTC |
| *Wnt7b* | GCATCCAAGGTCAACGCAAT | CTCAGAGTCTCATGGTCCCTTTG |

Given the increased glycogen content in *G6-Nkx* DH lungs, phospholipid levels were determined on surviving *G6-Nkx* DH as well as wild-type, $Gata6^{+/-}$ and $Nkx2.1^{+/-}$ mice to determine whether these mice had altered surfactant levels. A significant and reproducible decrease of approximately 60% in phospholipid content in bronchioalveolar lavage fluid was observed in *G6-Nkx* DH adult mice (Fig. 5A). A small but significant decrease in phospholipid content was also observed in the $Nkx2.1^{+/-}$ mice (Fig. 5A). By contrast, lung-to-body weight ratios and tidal volumes were

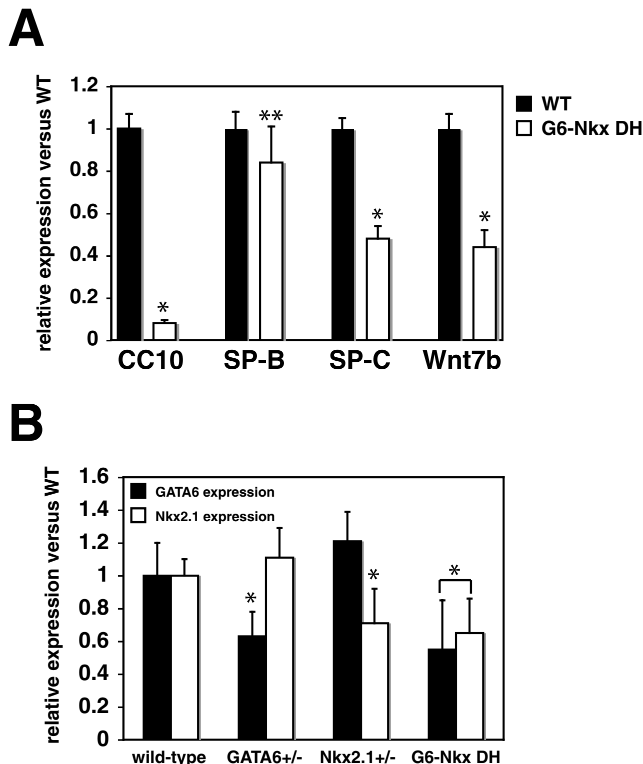



**Fig. 3. Quantitative measurements of gene expression changes in *G6-Nkx* DH lungs.** (**A**) Q-PCR expression levels of CC10, SP-B, SP-C and Wnt7b in wild-type and *G6-Nkx* DH lungs. (**B**) Q-PCR expression of Gata6 and Nkx2.1 in wild-type, $Gata6^{+/-}$, $Nkx2.1^{+/-}$ and *G6-Nkx* DH lungs. Q-PCR was performed on RNA extracted from whole lungs of the indicated genotypes at E18.5. All values were compared with expression in wild-type lungs, which was arbitrarily set at 1. Values are the average of four lung samples from each genotype performed in triplicate ±s.e.m. *$P<0.001$ versus wild-type, $Gata6^{+/-}$ or $Nkx2.1^{+/-}$ lungs. **$P<0.05$, non-significant changes compared to wild type.

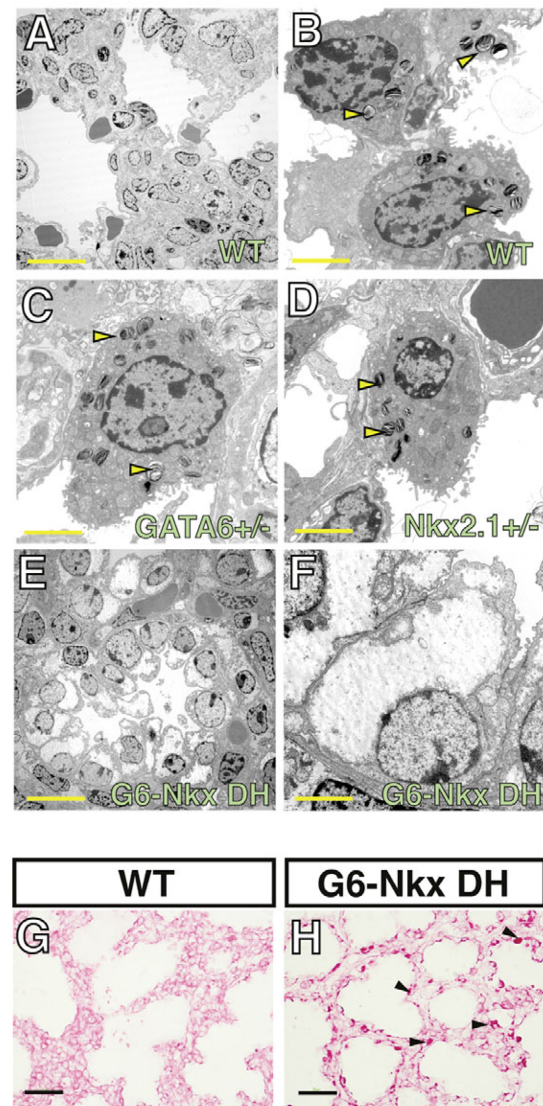



**Fig. 4. Increased glycogen content in *G6-Nkx* DH lung epithelium.** *G6-Nkx* DH lungs have increased glycogen deposits, as measured by transmission electron microscopy (**A-F**), and increased PAS staining (**G**,**H**, red staining and arrowheads). Lamellar bodies were observed in wild-type and single heterozygous lung epithelium (B-D, arrowheads) but were absent or only rarely found in *G6-Nkx2.1* DH lung epithelium (E,F). Scale bars: 10 μm in A,E; 2 μm IN B-D,F; and 50 μm in G,H.

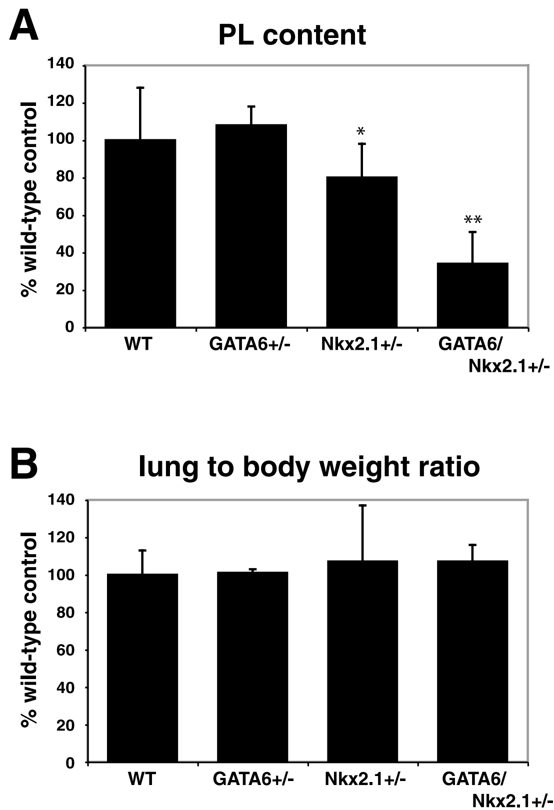
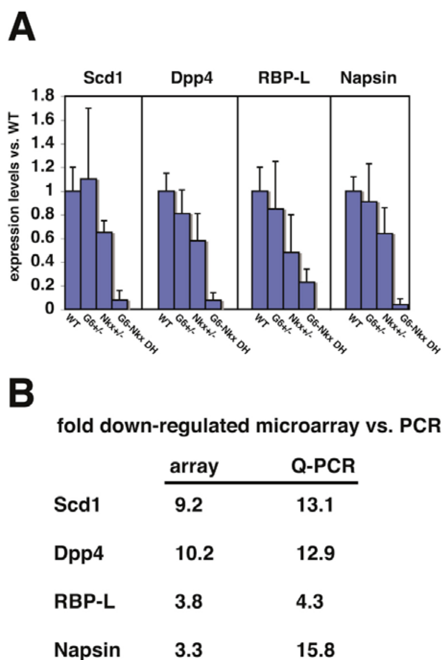



**Fig. 5. Decreased phospholipid levels in the lungs of adult *G6-Nkx* DH mutants.** Phospholipids levels, measured as described in Materials and methods, were decreased by approximately 60% in *G6-Nkx* DH mice (**A**). Phospholipid levels were decreased by a small but significant amount in *Nkx2.1*$^{+/-}$ mice. Lung-to-body weight ratios were not significantly different among all genotypes tested (**B**). **P*<0.05; ***P*<0.005.

not appreciably changed in any of the mice (Fig. 5B, and data not shown). The decreased level of phospholipids correlates with the increased glycogen content observed at E18.5 in *G6-Nkx* DH embryos and supports the hypothesis that Gata6 and Nkx2.1 act synergistically to regulate surfactant production and processing. Assuming that the most severely affected animals died in the neonatal period, these data demonstrate that epithelial differentiation and surfactant production is exquisitely sensitive to regulation by Gata6 and Nkx2.1.

### Identification of a subset of lung epithelial genes that require the synergistic activity of Gata6 and Nkx2.1

The increased glycogen levels coupled with relatively normal aquaporin-5 and SP-B levels suggests that Gata6 and Nkx2.1 are required in a synergistic manner for a specific gene-expression program that directs lung epithelial differentiation and surfactant production. To identify putative target genes regulated by heterozygous loss of both Gata6 and Nkx2.1, RNA from lungs of E18.5 wild-type and *G6-Nkx* DH embryos were compared using microarray analysis. In total, 292 genes were downregulated by more than twofold (see Table S1 in the supplementary material). Supporting our immunohistochemical data, expression of the *CC10* gene was reduced by tenfold in *G6-Nkx* DH lungs. Interestingly, a large percentage of downregulated genes are involved in phospholipid metabolism and surfactant protein processing (see Fig. S1 and Table S1 in the supplementary material). Several key transcriptional regulators were also downregulated in *G6-Nkx* DH lungs, including regulators of Notch signaling (RBP-L, also known as Rbpsuhl – Mouse Genome Informatics) and endodermal differentiation (Sox11 and Sox17) (see Table S1 in the supplementary material). Using a computational genomics approach that exploits the known transcription-factor-binding motifs and evolutionary conservation in the promoter sequence (Levy and Hannenhalli, 2002), we found that GATA DNA-binding sites were




| | array | Q-PCR |
|---|---|---|
| Scd1 | 9.2 | 13.1 |
| Dpp4 | 10.2 | 12.9 |
| RBP-L | 3.8 | 4.3 |
| Napsin | 3.3 | 15.8 |

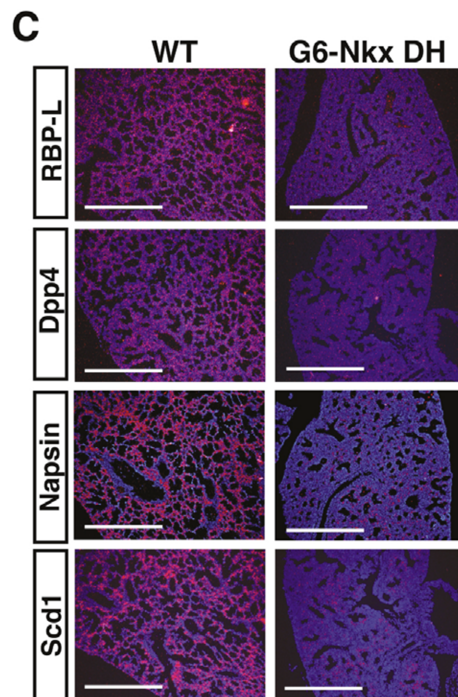



**Fig. 6. Genes involved in phospholipid production and processing are downregulated in the lungs of *G6-Nkx* DH embryos.** Q-PCR was performed on E18.5 lung tissue from wild-type, *Gata6*$^{+/-}$, *Nkx2.1*$^{+/-}$ and *G6-Nkx* DH embryos using the oligonucleotides listed in Table 3. (**A**) Scd1, Dpp4, RBP-L and napsin are all downregulated to a small extent in *Nkx2.1*$^{+/-}$, but not *Gata6*$^{+/-}$, lungs. However, all four genes are severely downregulated in *G6-Nkx* DH lungs. (**B**) Comparison of changes in gene expression of Scd1, Dpp4, RBP-L and napsin from microarray and Q-PCR experiments. (**C**) In situ hybridization performed on wild-type and *G6-Nkx* DH lungs using riboprobes for RBP-L, Dpp4, napsin and Scd1 shows reduced expression of all four genes in *G6-Nkx* DH lungs at E18.5. Scale bars: 500 μm in C.

enriched in the upstream regulatory regions of the downregulated genes (see Fig. S1C in the supplementary material). The overall lack of enrichment of Nkx-binding sites may reflect the restricted region (1 kb) that was analyzed. However, Nkx-binding sites are enriched in the subset of genes that are enriched in GATA DNA-binding sites (see Fig. S1C in the supplementary material).

Quantitative RT-PCR and in situ hybridizations were performed for a subset of these genes to compare their expression in *G6-Nkx* DH versus wild-type and single heterozygous lungs. These genes were chosen based on their importance for a variety of processes that are crucial for lung development and function. Dipeptidyl peptidase 4 (Dpp4) is a serine protease with known tumor-suppressor function that is expressed in type 2 cells in the lung (Hildebrandt et al., 1991; Wesley et al., 2005). RBP-L is a transcription factor related to RBP-J (also known as Rbpsuh – Mouse Genome Informatics), the downstream nuclear effector of the Notch signal-transduction pathway (Minoguchi et al., 1997). RBP-L expression is restricted to the lung and brain during development (Minoguchi et al., 1999). Stearoly-CoA desaturase 1 (Scd1) is an enzyme that converts saturated fatty acids into monounsaturated fatty acids and is crucial for the conversion of glycogen into phospholipids in the pulmonary surfactant (Zhang et al., 2004). Napsin (also known as Napsa – Mouse Genome Informatics) is an aspartyl protease known to process SP-B and SP-C into their mature forms (Brasch et al., 2003; Ueno et al., 2004). Remarkably, the expression of Dpp4, Scd1, RBP-L and napsin were all dramatically downregulated in *G6-Nkx* DH mutants, as measured by Q-PCR and in situ hybridization (Fig. 6A-C), indicating that synergism between Gata6 and Nkx2.1 is required for their expression. Comparison of wild-type, *Gata6*$^{+/–}$, *Nkx2.1*$^{+/–}$ and *G6-Nkx* DH samples showed that loss of Nkx2.1, but not of Gata6, leads to a small but detectable decrease in expression of these target genes, whereas *G6-Nkx* DH lungs have much more dramatic decreases in expression of these four genes (Fig. 6A). This suggests that Gata6 acts as a modifier of Nkx2.1 function, and that Nkx2.1 DNA-binding sites may be required for synergistic interaction between the two factors. Thus, the microarray data from the present studies allowed us to identify a global network of genes in the lung whose expression is finely controlled in a synergistic manner in vivo by Gata6 and Nkx2.1. The large percentage of genes involved in surfactant production and processing identified in these analyses suggests that the synergism between Gata6 and Nkx2.1 is crucial to this overall gene-expression program in the lung.

## Bmp4 expression is compromised in *G6-Nkx* DH lungs

Because the microarray experiment was performed on lungs from E18.5 embryos, the genes identified represent the later stages of lung development. Given that Nkx2.1 and Gata6 are both expressed earlier in lung development, we sought to determine whether expression of putative target genes known to be regulated by both these factors were altered. Bmp4 is a crucial morphogen expressed in the distal tips of the growing airways during early lung development. Bmp4 and its receptor Bmpr1a are both important in the early stages of lung epithelial differentiation and branching morphogenesis (Eblaghie et al., 2006). Moreover, Bmp4 has been shown to be a direct target of Gata6 and Nkx2.1 (Nemer and Nemer, 2003; Zhu et al., 2004). To determine whether Bmp4 expression was specifically altered in *G6-Nkx* DH mutants, immunohistochemistry was performed on E12.5 embryos to assess Bmp4 expression. No difference was observed between wild-type, *Gata6*$^{+/–}$ and *Nkx2.1*$^{+/–}$ embryos (Fig. 7A-C). However, a significant decrease in Bmp4 expression was observed in the airways *G6-Nkx* DH mutants (Fig. 7D). Q-PCR using cDNA derived from E12.5 lungs confirmed a

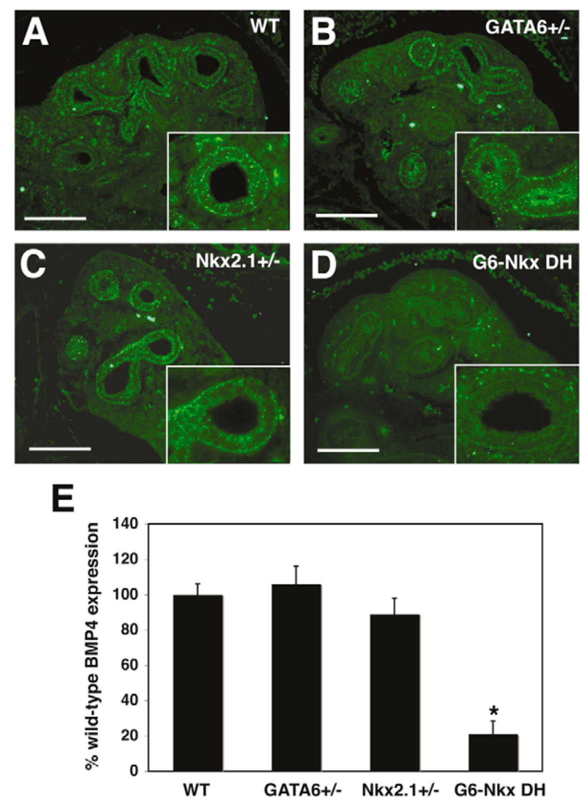
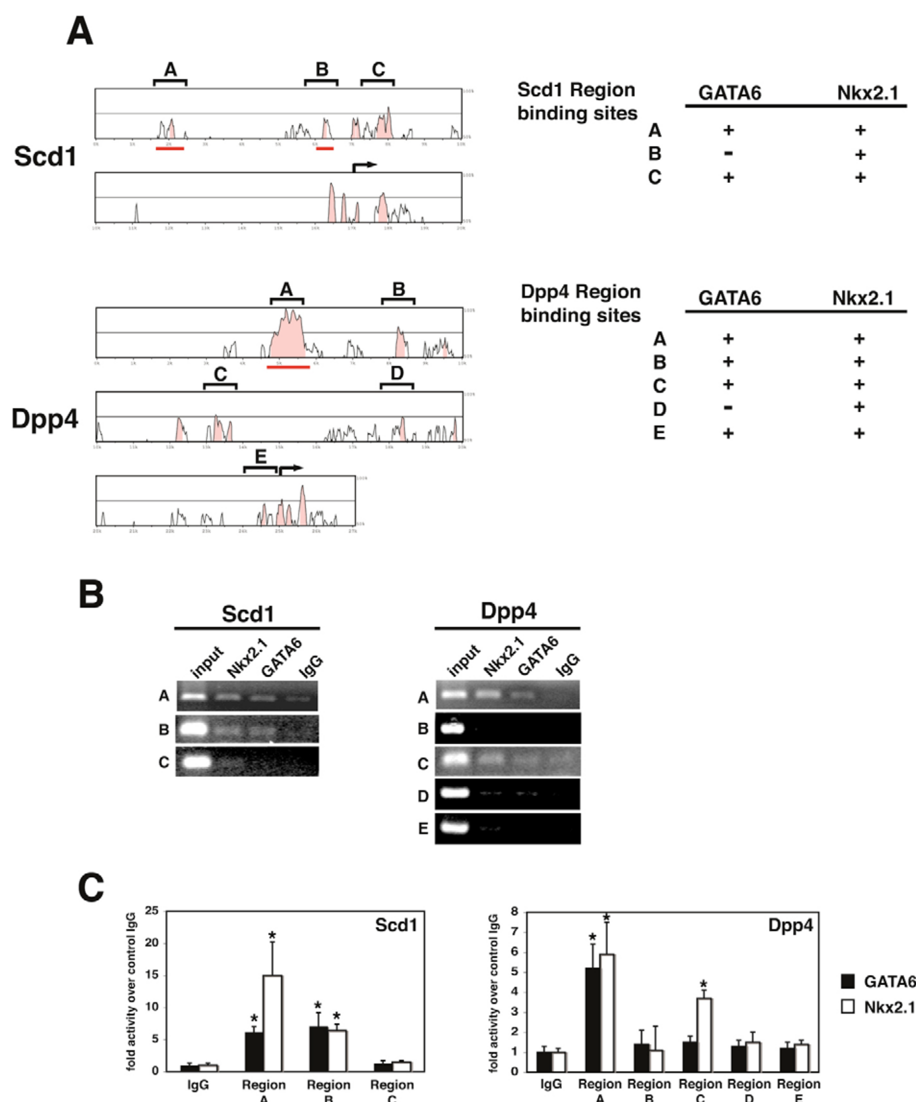



**Fig. 7. Bmp4 expression is downregulated in *G6-Nkx* DH lungs.** Immunohistochemistry using a Bmp4 antibody was performed to determine the expression of Bmp4 in the developing lungs of E12.5 wild-type (**A**), *Gata6*$^{+/–}$ (**B**), *Nkx2.1*$^{+/–}$ (**C**) and *G6-Nkx* DH (**D**) embryos. Bmp4 expression was significantly reduced in *G6-Nkx* DH lungs, but was unchanged in *Gata6*$^{+/–}$ and *Nkx2.1*$^{+/–}$ lungs. Inset for each panel is a higher magnification of a single distal airway. Q-PCR was performed using oligonucleotides listed in Table 3 to quantitatively determine the decrease in expression of Bmp4 in *G6-Nkx* DH lungs at E12.5 (**E**). Three samples from each indicated genotype were evaluated in these assays. *$P$<0.001 wild-type versus *G6-Nkx* DH samples. Scale bars: 100 μm in A-D.

decrease of almost 80% (Fig. 7E). These data suggest that the synergistic role of Gata6 and Nkx2.1 is required for the expression of crucial target genes, such as Bmp4, supporting a role for this synergy in the early stages of lung development.

## Regulation of Scd1 and Dpp4 by Gata6/Nkx2.1 synergy

To understand better how Gata6 and Nkx2.1 regulate specific target genes identified in late lung development, we characterized the regulatory regions of two of the genes identified in our analysis – *Scd1* and *Dpp4*. These two genes were chosen because of their crucial importance in phospholipid production, tumor suppression and glycogen metabolism in the lung, and because of their previously described promoter/transcription initiation sequences (Bernard et al., 1994; Ntambi et al., 1988). Comparison of mouse and human genomic regions for these two genes shows that there are several conserved regions both proximal and distal to the start of transcription (Fig. 8A). Within some of these regions, multiple conserved GATA- and Nkx2.1-DNA-binding sites are found clustered together. By contrast, other regions contained only GATA- or Nkx2.1-DNA-binding sites. To determine whether Gata6 and




| Scd1 Region binding sites | GATA6 | Nkx2.1 |
|---|---|---|
| A | + | + |
| B | - | + |
| C | + | + |

| Dpp4 Region binding sites | GATA6 | Nkx2.1 |
|---|---|---|
| A | + | + |
| B | + | + |
| C | + | + |
| D | - | + |
| E | + | + |

**Fig. 8. Gata6 and Nkx2.1 interact at specific regions within the upstream regulatory regions of *Scd1* and *Dpp4*.** (**A**) mVista analysis was used to determine conserved regions in the upstream regulatory sequences of *Scd1* and *Dpp4*. Regions probed using ChIP analysis are highlighted in brackets. Red underlined regions are genomic enhancers used in transactivation reporter assays. (**B**) ChIP analysis demonstrates an interaction of Gata6 with regions A and B in *Scd1* and region A in *Dpp4*, whereas Nkx2.1 interacts with regions A and B in *Scd1* and regions A and C in *Dpp4*. (**C**) Q-PCR of ChIP reactions on the same regions shown in A and B demonstrating quantitative differences in binding of Gata6 and Nkx2.1 to the identified conserved regions in the *Scd1* and *Dpp4* regulatory regions. *$P<0.01$ Gata6 and Nkx2.1 antibodies versus normal IgG.

Nkx2.1 bound to these conserved sites in vivo, chromatin immunoprecipitation (ChIP) assays were performed using chromatin from E18.5 mouse lungs. ChIP showed that Gata6 and Nkx2.1 associate with specific regions within both of these genes (Fig. 8B,C). In particular, regions A and B in the *Scd1* gene and region A in the *Dpp4* gene showed high-level association with both Gata6 and Nkx2.1. Gata6 and Nkx2.1 were not found associated with region C in *Scd1* or regions B, D, and E in *Dpp4*, despite all these regions containing highly conserved Gata6- and/or Nkx2.1-DNA-binding sites. Nkx2.1, but not Gata6, was found to associate with region C in *Dpp4*, even though this region contains conserved Gata6 DNA-binding sites. Remarkably, Gata6 was found to associate in region B of the *Scd1* regulatory locus even though GATA DNA-binding sites were not found in this region, indicating that Gata6 can interact through other DNA-binding factors to associate with specific regions of chromatin. Given the presence of Nkx2.1-binding sites and the ability of Gata6 to bind to Nkx2.1 (Liu et al., 2002a; Weidenfeld et al., 2002), this interaction probably explains this association, as well as the sensitivity of these genes to combined Gata6 and Nkx2.1 haploinsufficiency.

Region A in *Scd1* and *Dpp4*, and region B in *Scd1*, were further analyzed for their ability to be directly regulated by Gata6 and Nkx2.1 using luciferase reporter assays. In agreement with the ChIP assays, Gata6 and Nkx2.1 individually and robustly activated region A in *Dpp4* and *Scd1* but did not do so in a synergistic manner (Fig. 9A,B). By contrast, Gata6 and Nkx2.1 did not individually activate region B in *Scd1* but together could synergistically activate this enhancer region (Fig. 9C). This is similar to what has been previously reported for the *Wnt7b* promoter and indicates that Gata6 can act through Nkx2.1 to activate gene transcription independently of direct DNA binding (Weidenfeld et al., 2002). These data also indicate that this synergy occurs preferentially in enhancer elements containing a single type of DNA-binding site, in this case Nkx2.1 sites, and that Nkx2.1 acts as a DNA-binding ‘platform’ for further interaction with Gata6, which acts as a modifier of Nkx2.1 function. Together, these data demonstrate that Gata6 and Nkx2.1 synergistically regulate a distinct subset of genes in vivo, including *Scd1*, *Dpp4* and *Bmp4*, that are crucial for lung epithelial differentiation, surfactant expression and airway homeostasis.

## DISCUSSION

Our studies demonstrate that the in vitro biochemical interactions described for GATA and Nkx have important in vivo consequences on gene expression and tissue-specific development. Most lung epithelial-restricted genes contain both GATA- and Nkx-DNA-binding sites within their promoter regions, providing evidence of

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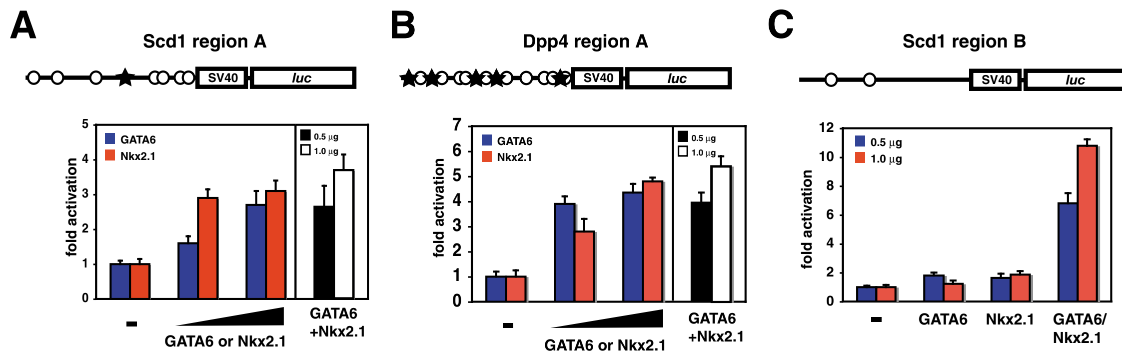


**Fig. 9. Activation of *Scd1* and *Dpp4* enhancers by Gata6 and Nkx2.1.** Region A in *Scd1* and *Dpp4*, and region B in *Scd1* were all cloned into the pGL3promoter plasmid. These regions contain both Gata6 (stars) and Nkx2.1 (circles) DNA-binding sites. (**A**,**B**) In NIH-3T3 cells, expression of either Gata6 or Nkx2.1 transactivated region A in *Scd1* and *Dpp4* in a dose-dependent manner. However, co-expression of Gata6 and Nkx2.1 did not synergistically activate either of these enhancer regions. (**C**) Gata6 and Nkx2.1 synergistically activated *Scd1* region B, even though this region lacks Gata6 DNA-binding sites. Data represent the average of three assays ±s.e.m.

the importance of these factors in their regulation. Additionally, several reports have demonstrated that Gata6 and Nkx2.1 physically interact with each other (Liu et al., 2002a; Weidenfeld et al., 2002). Despite these important observations, no data on the in vivo relevance of these interactions has been reported. Although Gata6 and Nkx2.1 could directly regulate the expression of one another, the data presented here and in previous reports on the lack of an effect on the expression of Nkx2.1 in dominant-negative Gata6-expressing lungs, as well as the high level of expression of Gata6 throughout the developing foregut prior to Nkx2.1 expression, argues against this mechanism (Liu et al., 2002b; Yang et al., 2002). Thus, the in vivo synergism between Gata6 and Nkx2.1 is probably caused by the physical interaction between these two transcription factors, which leads to the regulation of a subset of lung epithelial genes required for lung epithelial development and homeostasis. Given the changes in expression of target genes to Nkx2.1, but not to Gata6, haploinsufficiency, and that Gata6 and Nkx2.1 are both associated with regions of chromatin that contain only Nkx2.1 DNA-binding sites, our data suggest that Gata6 acts as modifier of Nkx2.1 function.

GATA and Nkx factors are co-expressed in several other tissues besides lung epithelium, including in myocardium and smooth muscle (Durocher et al., 1997; Lyons et al., 1995; Sepulveda et al., 1998; Sepulveda et al., 2002). Thus, our findings may have important implications for the synergy between these transcription factor families in other developmental systems. However, tissues such as myocardium express multiple GATA- and Nkx-factors that probably act redundantly, making it difficult to analyze a synergistic effect using genetic experiments (Evans, 1999; Molkentin, 2000). Given that lung epithelium expresses a single Nkx- and GATA-factors, lung morphogenesis is an ideal developmental process in which to study such synergistic interactions. In addition to redundancy, there may be specific differences in how GATA- and Nkx-factors work in other tissues. GATA factors have been shown to act directly upstream of Nkx2.5 in myocardium and Nkx2.5 has been shown to be crucial for Gata6 expression in the heart, identifying a self-enforced regulatory loop for these factors (Lien et al., 1999; Molkentin et al., 2000). Based on the data in this report, as well as on that from previous studies, Gata6 and Nkx2.1 do not appear to act in such a manner in the lung (Liu et al., 2002b; Yang et al., 2002).

Recent data has demonstrated that a host of factors are required for proper lung epithelial differentiation, including Foxa1, Foxa2 and C/EBPα (Cebpa) (Martis et al., 2006; Wan et al., 2005; Wan et al., 2004). We show that haploinsufficiency in both Gata6 and Nkx2.1 leads to a disruption in a genetic program required for Bmp4 expression in early lung development, as well as a disruption in surfactant production later in lung development. Bmp4 has been shown to be a direct target of Gata6 and Nkx2.1, and its downregulation in *G6-Nkx* DH lungs suggests that the synergistic interaction between Gata6 and Nkx2.1 is required for its full expression (Nemer and Nemer, 2003; Zhu et al., 2004). These data indicate that Gata6-Nkx2.1 interactions are crucial from the earliest stages of lung development. Interestingly, the combined activity of Gata6 and Nkx2.1 is required for the expression of a set of genes important for surfactant production and homeostasis, including *Scd1* and *Dpp4*. Both Gata6 and Nkx2.1 directly associate with conserved genomic regulatory regions in *Scd1* and *Dpp4* in vivo, and directly activate, in some cases synergistically, conserved upstream regulatory regions in each gene. This synergy appears to occur only in enhancer elements containing Nkx2.1 DNA-binding sites, suggesting the differential regulation and usage of such crucial protein-protein interactions. Such interactions could increase the affinity of Gata6 and Nkx2.1 for additional co-factors or could increase the affinity of the binding of Nkx2.1 to DNA, as has been suggested in Gata4-Nkx2.5 interactions (Sepulveda et al., 1998). The identification of *Scd1* and *Dpp4* as direct targets of Gata6- and Nkx2.1-regulation provides novel information on how these transcription factors regulate both phospholipid production and airway-epithelial homeostasis.

Defects in saccular development and surfactant production are common in human neonates with bronchopulmonary dysplasia. Our finding that Gata6 can act as a modifier of Nkx2.1 may help to explain why some human patients with *NKX2.1* mutations have respiratory defects whereas others appear normal (Krude et al., 2002; Pohlenz et al., 2002). The gene-expression defects uncovered in *G6-Nkx* DH embryos and mice represent targets that are specifically regulated by the synergistic activity of these two transcription factors. Further studies into this and other combinatorial interactions between GATA- and Nkx-factors in vivo will undoubtedly reveal novel insights into the gene-gene and protein-protein interactions described in other tissues.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02720/DC1

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