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Mutations in the thyroid transcription factor gene *NKX2-1* result in decreased expression of *SFTPB* and *SFTPC*

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Abstract

BACKGROUND: Mutations in the NK2 homeobox 1 (*NKX2-1*) gene are associated with lung disease in infants and children. We hypothesize that disruption of normal surfactant gene expression with these mutations contributes to the respiratory phenotypes observed.

METHODS: To assess transactivational activity, cotransfection of luciferase reporter vectors containing surfactant protein B or C (*SFTPB* or *SFTPC*) promoters with *NKX2-1* plasmids was performed and luciferase activity was measured. To assess the binding of mutated proteins to target DNA, electrophoretic mobility shift assays (EMSA) were performed using nuclear protein labeled with oligonucleotide probes representing *NKX2-1* consensus binding sequences followed by gel electrophoresis. The effect of overexpression of wild-type (WT) and mutant *NKX2-1* on *SFTPB* and *SFTPC* was evaluated with quantitative real-time PCR.

RESULTS: Decreased transactivation of the *SFTPB* promoter by both mutants and decreased transactivation of the *SFTPC* promoter by the L197P mutation was observed. EMSA demonstrated decreased DNA binding of both mutations to *NKX2-1* consensus binding sequences. Transfection of A549 cells with *NKX2-1* expression vectors demonstrated decreased stimulation of *SFTPB* and *SFTPC* expression by mutant proteins compared with that of WT.

CONCLUSION: Disruption of transcriptional activation of surfactant protein genes by these DNA-binding domain mutations is a plausible biological mechanism for disruption of surfactant function and subsequent respiratory distress.

The NK2 homeobox 1 (*NKX2-1*) gene encodes thyroid transcription factor-1 (TTF-1), a critical regulator of gene transcription in the brain, thyroid gland, and lung (1,2). The 4 kb *NKX2-1* is located on human chromosome 14 (3,4) and includes a DNA-binding

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homeodomain (HD) that encodes amino acids 189–253 (Figure 1). Mutations in this region of the gene disrupt transcriptional activity of TTF-1 target genes by altering the binding of TTF-1 to elements within target genes (5–8). *NKX2-1* expression is essential for early lung morphogenesis, as well as later regulation of the expression of genes necessary for lung function, including the surfactant genes *SFTPB* or *SFTPC* (5,9–12). The molecular mechanisms by which *NKX2-1* mutations result in lung disease phenotypes remain poorly understood.

The incidence of respiratory distress associated with mutations in the *NKX2-1* gene is unknown. Pulmonary phenotypes observed in infants and children with brain–thyroid–lung syndrome and *NKX2-1* mutations are diverse, including respiratory distress in the newborn period, interstitial lung disease in infancy or childhood, and recurrent respiratory infections (3,10,13–20). Affected infants and children may also demonstrate neurological abnormalities such as newborn hypotonia, developmental delay in infancy, and/or movement disorders in childhood, as well as hypothyroidism (21–27).

We previously described 21 individuals with brain–thyroid–lung syndrome, 16 of whom presented with neonatal respiratory distress (10). Two of these individuals presented with respiratory distress syndrome and progressed to severe interstitial lung disease that ultimately required lung transplantation. These individuals were found to have heterozygous, *de novo*, missense mutations in the *NKX2-1* HD (R195W and L197P). Neither child had mutations identified in *SFTPB* or *SFTPC* (10). The HD location of these mutations prompted us to investigate the encoded transcriptional mechanisms that disrupt surfactant gene expression in these patients.

Previous studies, including ours with these same patients, have noted the presence of surfactant proteins in lung samples and in lung lavage (3,10,19,28). In some cases, there appears to be decreased expression of surfactant proteins in the presence of *NKX2-1* mutations, and in other cases, surfactant protein expression appears to be unchanged or possibly increased. These results, however, are difficult to interpret in the context of various treatments, including mechanical ventilation, oxygen, and medications to which these patients have been exposed. It becomes difficult to separate the changes in gene expression directly due to the mutations from the effects of these treatments. Therefore, we turned to an *in vitro* system to demonstrate the biological plausibility that these mutations are disease causing.

METHODS

Patient Selection

Subjects were identified retrospectively through protocols approved by the Washington University School of Medicine Institutional Review Board by sequencing *SFTPB*, *SFTPC*, and *NKX2-1* in a cohort of infants who had undergone lung transplantation for respiratory failure (10). Neither patient had an identified etiology or pathogenic mutations in *SFTPB* or *SFTPC* (10). Written informed consent was obtained from the parents of each patient.

Cell Culture

The human epithelial cell line, A549, was cultured in F12K medium with 10% fetal bovine serum and penicillin/streptomycin (standard medium) at 37 °C. In some experiments, *SFTPC* expression was induced with 33 nM dexamethasone (29).

Site-Directed Mutagenesis

The mutations c.583C > T (R195W) and c.590T > C (L197P) were introduced by site-directed mutagenesis (Stratagene, La Jolla, CA) into the pRC/CMV/NKX2-1 expression vector that contained the full-length, wild-type, human *NKX2-1* cDNA. Construct sequences were confirmed by Sanger sequencing. Forward primer 5'-GCGCAGGAAGCGCTGG GTGCTCTTCTC-3' and reverse primer 5'-GAGAAGAGCACCC AGCGCTTCTGCGC-3' were used to introduce the R195W mutation. Forward primer 5'-GCGCCGGGTGCCCTTCTCGCAGG-3' and reverse primer 5'-CCTGCGAGAAGGGCACCCGGCGC-3' were used for the L197P mutation. *NKX2-1* encodes two isoforms that differ by 30 amino acids (11). For this study, numbering of residues based on the longer, 401-amino-acid isoform was used to be consistent with our previous report and Human Genome Variation Society nomenclature (10). Other authors have used numbering based on the shorter isoform (3). The wild-type *NKX2-1* plasmid was a kind gift from Parviz Minoov (University of Southern California, Los Angeles, CA).

Transient Transfection

The *SFTPB* promoter or *SFTPC* promoter was cloned into a firefly luciferase vector lacking a promoter (pGL4.10[luc2]; Promega, Madison, WI) to create reporter plasmids (*SFTPB_luc* and *SFTPC_luc*). The *SFTPB* promoter was a 1.0 kb human promoter and the *SFTPC* promoter was a 3.7 kb human promoter (kind gift from Dr J. Whitsett, Cincinnati Children's Hospital, Cincinnati, OH). A549 cells (1.5×10^5 cells/well in 12-well plates 24 h before transfection) were cotransfected using Fugene 6 (Roche Applied Science, Indianapolis, IN) and 0.1 µg of either the *SFTPB_luc* or *SFTPC_luc* reporter plasmid together with 0.1 µg of pRC/CMV expressing wild-type or mutant *NKX2-1*. Cotransfection with a Renilla-luciferase expression vector (pRL-TK) was used to normalize for cell transfection efficiency. Transfections with empty pRC/CMV served as a control. After 48 h, cells were lysed, and firefly luciferase and then Renilla luciferase were measured in a sequential manner with a dual luciferase assay (Promega). After normalization of firefly luciferase activity with Renilla luciferase activity, normalized luciferase activities were compared between the wild-type *NKX2-1* plasmids and the mutations using Student's paired *t* tests.

Electrophoretic Mobility Shift Assay

To assess the ability of the mutated TTF-1 proteins to bind to target DNA sequences in the promoter region of *SFTPB* or *SFTPC*, electrophoretic mobility shift assays were performed. A549 cells (4×10^6 /well) were transfected with 8 µg of wild-type or mutant *NKX2-1* expression vectors by nucleofection (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Nuclear protein was isolated 48 h after transfection and extracted using nuclear and cytoplasmic extraction reagents (NE-PER; ThermoScientific,

Rockford, IL). Next, 6 µg of nuclear protein was incubated with IRDye 700-labeled DNA probes (Integrated DNA Technologies, Coralville, IA) that included *SFTPB*- or *SFTPC*-specific TTF-1 binding sequences. Forward and reverse polyacrylamide gel electrophoresis-purified, IRDye 700-labeled probes for *SFTPB* were 5'-AGCACC TGGAGGGCTCTTCAGAGC-3' and 5'-GCTCTGAAGAGCCCTCCAGGTGCT-3', respectively, and for *SFTPC*, 5'-TAGGCCAAGGGCCTTGGGGCTCT-3' and 5'-AGAGCCCCCAAGGCCCTTGGCCTA-3', respectively. Electrophoretic mobility shift assay was performed using the Lightshift Chemiluminescent EMSA Kit and protocol (Thermoscientific). In some reactions, an excess of unlabeled probe (10 pmol) was added to demonstrate reaction specificity. To confirm the presence of TTF-1 protein in the reaction products, 0.8 µg of mouse anti-TTF-1 antibody (Seven Hills Bioreagents, Cincinnati, OH) was added to some reactions. The reactions were then resolved on 6% DNA acrylamide gels (Life Technologies, Grand Island, NY) and imaged with Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Quantitative PCR

To assess the ability of wild-type and mutant *NKX2-1* to regulate the expression of endogenously expressed *SFTPB* and *SFTPC*, quantitative real-time PCR was used in A549 cells transfected with vectors expressing wild-type or mutant *NKX2-1*. A549 cells (3×10^6 /well) were transfected with 2 µg of wild-type or mutant *NKX2-1* pRC/CMV expression vectors using Fugene 6 (Promega) according to the manufacturer's instructions and cultured in standard media for 72 h. Because endogenous expression of *SFTPC* was low in A549 cells, expression was stimulated by culturing A549 cells with 33 nM dexamethasone beginning 24 h after transfection. RNA was isolated 72 h after transfection and extracted using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Taqman Gene Expression Assays were performed in triplicate to assess gene expression of *SFTPB* and *SFTPC* in transfected vs. untransfected A549 cells.

RESULTS

Clinical Description

Patient 1 (R195W mutation) was a male of Hispanic descent born at 38 weeks of gestation with respiratory failure at birth and treated with mechanical ventilation. He received multiple doses of surfactant replacement therapy and was discharged home on supplemental oxygen at 80 days of life. During the neonatal period, he was also diagnosed with hypothyroidism that was treated with levothyroxine. He underwent gastrostomy tube placement and Nissen fundoplication for poor feeding and concerns for aspiration. For the first 4 months of life, he remained on supplemental oxygen with multiple hospital admissions for respiratory decompensations. Computed tomography scan of his lungs showed a diffusely reticular nodular pattern. Histology from a lung biopsy showed diffuse interstitial lung disease with prominent pneumocyte hyperplasia and alveolar septal widening. Electron microscopy revealed hyperplastic type II pneumocytes with lamellar bodies of variable size that appeared crinkled and densely packed forming irregular concentric whorls. Immunohistochemistry demonstrated the presence of surfactant protein B and surfactant protein C in the tissue from his lungs, which was removed at the time of transplantation (10).

Due to progressive respiratory failure, he required mechanical ventilation at 5 months of age until he underwent lung transplantation at 8 months of age. He expired at 3 years of age of pulmonary vein stenosis and pulmonary hypertension. No mutations were identified in *SFTPB* or *SFTPC*. Sequence analysis revealed a c.583C > T missense mutation in the *NKX2-1* gene.

Patient 2 (L197P mutation) was a male of European descent born at 37 weeks who had respiratory distress syndrome at birth for which he was intubated, received surfactant replacement therapy, and was supported with mechanical ventilation for 12 days. His chest radiographs demonstrated a ground glass appearance. He was discharged on supplemental oxygen. At 3 months of age, he underwent fundoplication, pyloroplasty, and gastrostomy tube placement for poor oral feeding and possible aspiration. At 5 months of age, he was hospitalized for respiratory syncytial virus and received systemic steroids. Computed tomography scan of his lungs showed generalized ground glass opacities and lung hyperinflation. Histology from lung biopsy showed nonspecific parenchymal changes, including diffuse pneumocyte hyperplasia, mild to focally moderate interstitial widening, and increased numbers of histiocytic-like cells in the alveolar septae. Histology also demonstrated mild inflammatory infiltrates, including lymphocytes, scattered plasma cells, eosinophils, and intra-alveolar foamy macrophages. Electron micrographs revealed numerous lamellar bodies of various sizes and some with a “fried egg” appearance (10). Immunohistochemistry demonstrated the presence of surfactant protein B and surfactant protein C in the tissue from his lungs, which was removed at the time of transplantation (10). Owing to progressive respiratory failure and interstitial lung disease, he underwent lung transplantation at 10 months of age. He developed attention-deficit/hyperactivity disorder at 10 years of age and a choreiform movement disorder at 12 years of age. Thyroid studies were normal. He is now 17 years old and has maintained his first set of donor lungs, although his course is now complicated by bronchiolitis obliterans. No mutations were identified in *SFTPB* or *SFTPC*. Sequence analysis revealed a c.590T > C missense mutation in the *NKX2-1* gene.

Transient Transfection

To investigate disruption of transcriptional activity encoded by the R195W and L197P mutants, transient cotransfection was performed with *NKX2-1* wild-type or mutant expression vectors and *SFTPB* or *SFTPC* luciferase reporter vectors. Cotransfection of a wild-type *NKX2-1* expression vector with an *SFTPB*-luciferase reporter increased luciferase activity sevenfold compared with that of the vector alone (luc2) ($P < 0.05$) (Figure 2a). Cotransfection with the R195W or L197P mutation expression vectors decreased *SFTPB* promoter luciferase activity by 34% and 50%, respectively, compared with that of wild-type *NKX2-1* ($P < 0.05$ for each) (Figure 2a).

Cotransfection of the wild-type *NKX2-1* expression vector with the *SFTPC* reporter construct increased luciferase activity 15-fold compared with that of the vector alone (luc2) ($P < 0.05$) (Figure 2b). Cotransfection with the R195W mutation expression vector did not change luciferase activity, whereas cotransfection with the L197P mutation expression

vector decreased luciferase activity of the *SFTPC* promoter by 25% compared with that of the wild-type vector ($P < 0.05$) (Figure 2b).

Electrophoretic Mobility Shift Assay

To evaluate the binding of mutant TTF-1 proteins to *SFTPB*- or *SFTPC*-specific *NKX2-1* target promoter sequences, electrophoretic mobility shift assays were performed with nuclear protein extracted from transiently transfected A549 cells. Nuclear protein from A549 cells transfected with wild-type TTF-1 was bound to the *SFTPB* target promoter sequence (Figure 3a; lane 2), and binding was inhibited by excess unlabeled probes (Figure 3a; lane 3). When quantified with infrared imaging and compared with wild-type TTF-1 protein, nuclear protein from A549 cells transfected with the R195W or L197P mutant decreased binding to *SFTPB* target promoter sequence by 33% (Figure 3a; lane 4) and 15%, respectively (Figure 3a; lane 6). The binding reactions were inhibited by excess unlabeled probes (Figure 3a; lanes 5 and 7). Supershift (arrow) with anti-TTF-1 antibody indicated the presence of TTF-1 protein in the protein–DNA complex (Figure 3a; lane 8). Each experiment was repeated a minimum of three times with consistent changes observed in binding.

Wild-type TTF-1 was also bound to the *SFTPC*-specific target promoter sequence (Figure 3b; lane 2), and binding was inhibited by excess unlabeled probe (Figure 3b; lane 3). Nuclear protein from A549 cells transfected with each mutation decreased binding by 30% compared with that of wild-type TTF-1 (Figure 3b; lanes 4 and 6). Binding of both mutant proteins was inhibited by the addition of excess unlabeled probe (Figure 3b; lanes 5 and 7). Supershift (arrow) with anti-TTF-1 demonstrates the presence of TTF-1 protein in the protein–DNA complex (Figure 3b, lane 8). Each experiment was repeated a minimum of three times with consistent changes in binding observed.

Endogenous *SFTPB* and *SFTPC* Expression after Transfection with Wild-Type or Mutant *NKX2-1* Vectors

To examine the effect of wild-type and mutant TTF-1 on the expression of surfactant protein genes, vectors expressing wild-type or one of the mutant *NKX2-1* genes were transfected into A549 cells. *SFTPB* and *SFTPC* transcript expression was then measured by quantitative PCR. Transfection of A549 cells with a wild-type *NKX2-1* expression vector increased endogenous *SFTPB* expression 64-fold ($P < 0.05$) (Figure 4a). Transfection of the R195W or L197P *NKX2-1* mutants decreased endogenous *SFTPB* expression by 63% and 95%, respectively, compared with that of wild-type ($P < 0.05$) (Figure 4a).

Because endogenous expression of *SFTPC* by A549 cells is low, A549 cells were cultured in the presence of dexamethasone to increase *SFTPC* expression. Transfection of A549 cells cultured in the presence of dexamethasone with wild-type *NKX2-1* increased the endogenous *SFTPC* expression 9.0-fold ($P < 0.05$) (Figure 4b). Transfection of the R195W or L197P *NKX2-1* mutants decreased endogenous *SFTPC* expression by 73% and 76%, respectively ($P < 0.05$) (Figure 4b).

DISCUSSION

Advances in computational prediction of the functional impact of DNA sequence changes on gene function have enabled the identification of genotype–phenotype associations. However, confirmation of pathogenicity requires model systems to demonstrate functional disruption of computationally discovered genomic variants. The *NKX2-1* HD mutations described here were identified in patients with extreme respiratory phenotypes who required lung transplantation. Both patients had brain–thyroid–lung syndrome, although some of the neurologic symptoms in patient 1 may have been confounded by his illness severity (10). The phenotypes of patient 1 and a previously reported patient with the same mutation are similar (3). The mutation in patient 2 (L197P) is novel.

Functional analysis of these mutations suggests that both of them encode TTF-1 mutant proteins with decreased binding to NKX-2-specific target sites in the *SFTPB* and *SFTPC* promoters. Both mutants also demonstrated reduced upregulation of endogenous *SFTPB* and *SFTPC* expression compared with that of wild-type mutants. Both mutants resulted in reduced *SFTPB* promoter activation, but only the L197P mutant reduced *SFTPC* promoter activation with no significant effect of the R195W mutant. The absence of an effect of the R195W mutation on *SFTPC* promoter transactivation, despite decreased binding of the mutant protein, may be the result of the unique characteristics of the truncated promoter used for transient transfection vs. the endogenous promoter. These results are also in contrast to the results of Guillot *et al.* (3) where increased activation of the *SFTPC* promoter was reported following cotransfection with the R195W mutant (R165W in their manuscript). This difference may be attributable to the different *SFTPC* promoters tested. We used a 3.7 kb human *SFTPC* promoter, whereas Guillot *et al.* (3) used a 4.8 kb mouse *SFTPC* promoter in their studies.

The genotype–phenotype association of the *NKX2-1* mutations in our patients and those of other patients with *NKX2-1* disease-associated mutations is consistent (10,18,27,30,31) and suggests loss-of-function haploinsufficiency or gain-of-function dominant-negative mechanisms. In addition to disruption of the TTF-1 DNA-binding HD encoded by the mutations in our two patients, *NKX2-1* mutations in two transactivation domains, a nuclear localization signal domain and functionally important phosphorylation sites (32–35), have also been described (3,10,14,18,22,26,27,31,36–39).

Disruption of the transactivating interaction of TTF-1 with the surfactant-associated genes suggests disrupted surfactant composition or function as a mechanism by which mutations in *NKX2-1* result in a respiratory phenotype. Although surfactant proteins are present in the lungs of the patients reported here at the time of transplantation in what appears to be normal or even increased amounts, it is difficult to interpret these data in the context of the multiple treatments these patients have received over a period of months. The use of animal models would be helpful in distinguishing between the direct effects of the mutations and the effect of treatments on surfactant protein expression *in vivo*. In addition to its role in surfactant metabolism, *NKX2-1* also has a critical role in early lung development, as well as interacting with a large number of other genes in addition to the surfactant protein genes. The targeted mutation of the mouse *nkx2-1* gene resulted in a disruption of distal lung

development (40). In addition, genome-wide analysis in mice revealed that TTF-1 binds to more than 1,300 genes. This included genes involved in early and late lung development, cell cycle regulation, ion transport, *RAS* signal transduction, and response to injury (7). Thus, these other fundamental disturbances in lung development and function could contribute to the respiratory phenotype seen in these patients. There is suggested disruption of normal lung growth in these patients but, once again, it is difficult to separate the effects of the mutation from the effects of treatments (10). This again underscores the importance of *in vitro* surrogate cell systems and animal models in unraveling the pathogenic mechanisms involved. Careful phenotyping of patients who are identified with *NKX2-1* mutations will provide opportunities for further characterization of the mechanisms of respiratory dysfunction.

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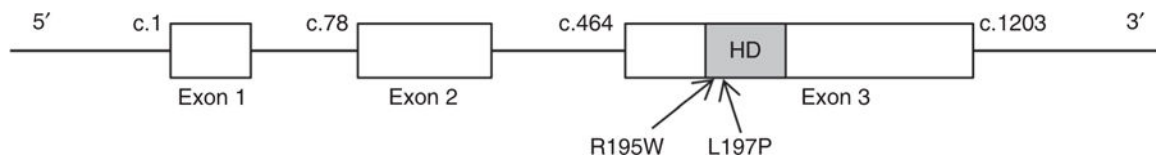


Figure 1. NK2 homeobox 1 (*NKX2-1*) gene showing three exons and the region encoding the homeodomain (HD) located in exon 3. The two patients described in the manuscript had mutations in the homeodomain at nucleotide 583 (c.583C > T) and nucleotide 590 (c.590T > C), resulting in the R195W and L197P amino-acid substitutions, respectively.

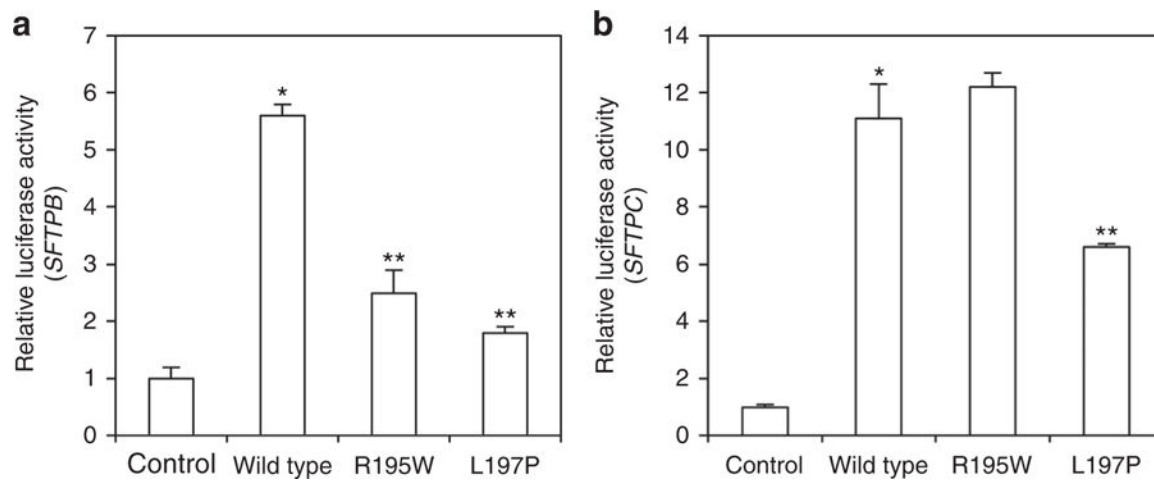


Figure 2. Transactivation of surfactant protein B (*SFTP B*) (a) and surfactant protein C (*SFTP C*) (b) promoters by wild-type and mutant NK2 homeobox 1 (*NKX2-1*). Relative luciferase activity is indicated following correction for transfection efficiency. Error bars indicate standard error of the mean. * $P < 0.05$ vs. vector alone. ** $P < 0.05$ vs. wild type.

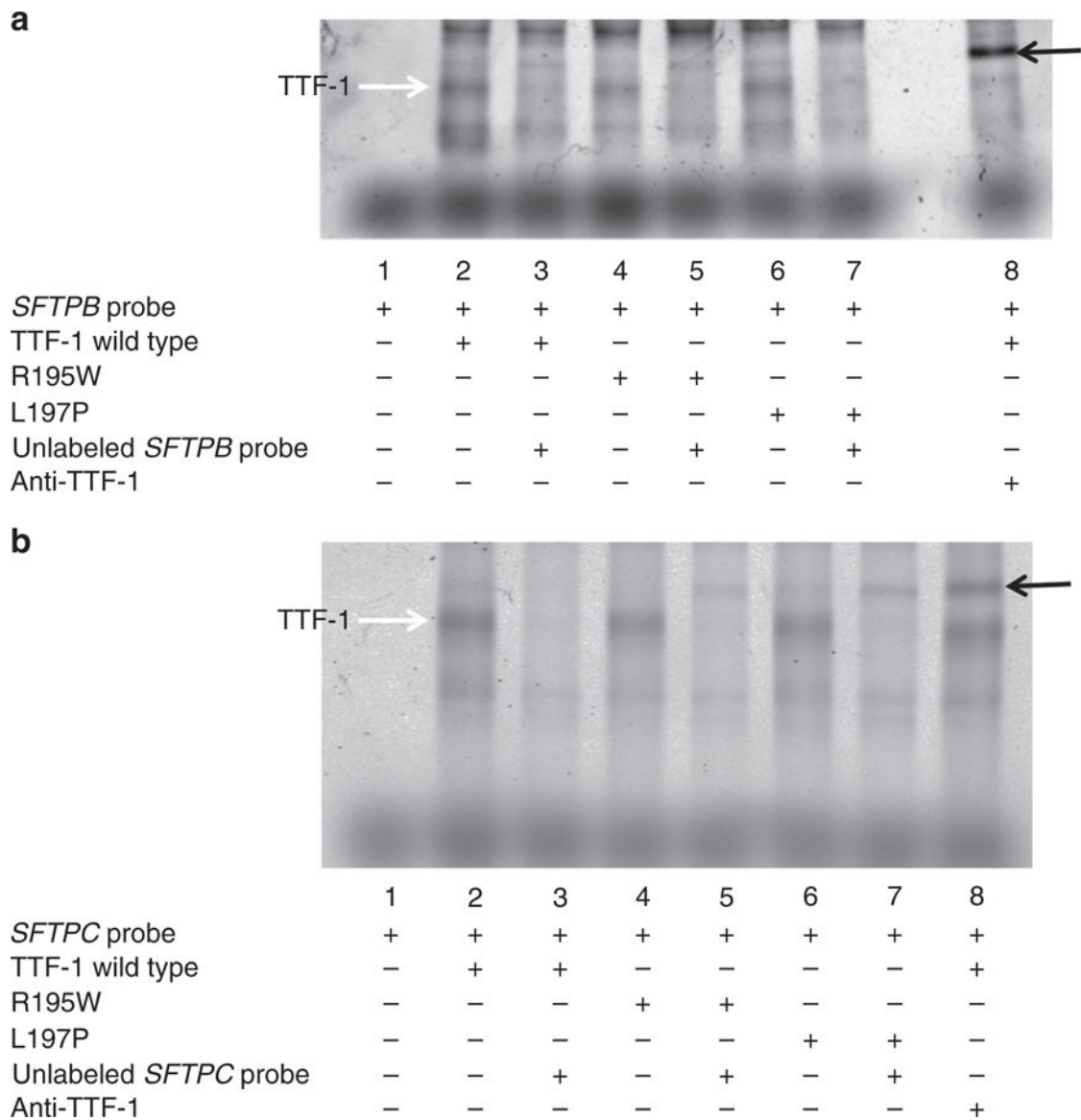


Figure 3.

Electrophoretic mobility shift assays were performed with nuclear protein, including wild-type or mutant thyroid transcription factor-1 (TTF-1). Reactions were performed with labeled surfactant protein B (*SFTP B*) (**a**) and surfactant protein C (*SFTP C*) (**b**) TTF-1 binding sites. In some reactions, unlabeled probe or anti-TTF-1 antibody was included. The white arrow indicates the TTF-1 binding complex; the dark arrow indicates the supershifted binding complex.

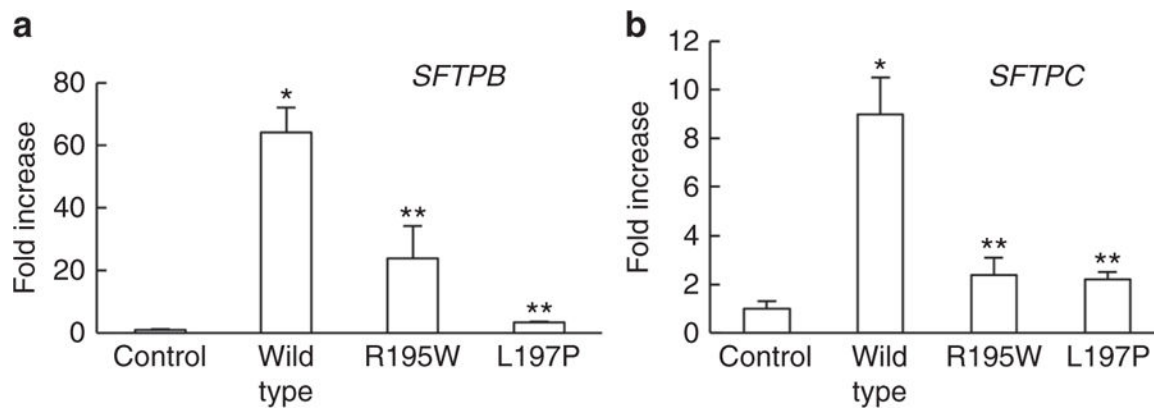


Figure 4. Expression of surfactant protein B (*SFTPB*) (a) and surfactant protein C (*SFTPC*) (b) by A549 cells in response to wild-type or mutant NK2 homeobox 1 (*NKX2-1*) expression. Relative transcript abundance is shown compared with that of untransfected cells. Error bars indicate standard error of the mean. * $P < 0.05$ vs. untransfected cells. ** $P < 0.05$ vs. cells transfected with wild-type vector.