TTF-1 Phosphorylation Is Required for Peripheral Lung Morphogenesis, Perinatal Survival, and Tissue-specific Gene Expression*

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Mario deFelice‡, Daniel Silberschmidt‡, Roberto DiLauro‡, Yan Xu§, Susan E. Wertz§, Timothy E. Weaver§, Cindy J. Bachurski§, Jean C. Clark§, and Jeffrey A. Whitsett§

From the ‡Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039 and §Stazione Zoologica “A. Dohrn,” Villa Comunale, 80121 Naples, Italy

Thyroid transcription factor-1 (TTF-1) is a 43-kDa, phosphorylated member of the Nkx2 family of homeodomain-containing proteins expressed selectively in lung, thyroid, and the central nervous system. To assess the role of TTF-1 and its phosphorylation during lung morphogenesis, mice bearing a mutant allele, in which seven serine phosphorylation sites were mutated, Titf1PM/PM, were generated by homologous recombination. Although heterozygous Titf1PM/+ mice were unaffected, homozygous Titf1PM/PM mice died immediately following birth. In contrast to Titf1 null mutant mice, which lack peripheral lung tissues, bronchiolar and peripheral acinar components of the lung were present in the Titf1PM/PM mice. Although lobulation and early branching morphogenesis were maintained in the mutant mice, abnormalities in acinar tubules and pulmonary hypoplasia indicated defects in lung morphogenesis later in development. Although TTF-1PM protein was readily detected within the nuclei of pancreatic epithelial cells at sites and abundance consistent with that of endogenous TTF-1, expression of a number of known TTF-1 target genes, including surfactant proteins and secretoglobin 1A, was variably decreased in the mutant mice. Vascular endothelial growth factor mRNA was readily detected within the nuclei of pulmonary epithelial cells at sites and abundance consistent with that of endogenous TTF-1, expression of a number of known TTF-1 target genes, including surfactant proteins and secretoglobin 1A, was variably decreased in the mutant mice. Vascular endothelial growth factor mRNA was decreased in association with decreased formation of peripheral pulmonary blood vessels. Genes mediates surfactant homeostasis, vasculogenesis, host defense, fluid homeostasis, and inflammation were highly represented among those regulated by TTF-1. Thus, in contrast to the null Titf1 mutation, the Titf1PM/PM mutant substantially restored lung morphogenesis. Direct and indirect transcriptional targets of TTF-1 were identified that are likely to play important roles in lung formation and function.

Lung formation begins with the outpouching of endodermal tissues from the laryngeal-tracheal-esophageal groove at embryonic day (E)9–9.5 in the mouse embryo. Epithelial lined tubules invade the splanchnic mesenchyme and undergo branching morphogenesis to form bronchi, bronchioles, and alveolar regions of the adult lung. Thyroid transcription factor-1 (genomic designation Titf1; also termed TEBP, or Nkx2.1) is a phosphorylated, homeodomain-containing, nuclear transcription factor expressed in respiratory epithelial cells of the developing lung, thyroid, and central nervous system (1). Although the trachea and main stem bronchi were formed in Titf1 null mutant mice, peripheral components of the lung, including bronchioles, acinar ducts, and respiratory sacculles were lacking in these mice, causing death at the time of birth (2). Likewise, expression of surfactant proteins was lacking in the Titf1 null mice (3, 4). TTF-1 is critical for formation of the lung and thyroid, regulating distinct subsets of genes expressed in both organs (2, 5, 6). TTF-1 binds to regulatory elements located in the promoters of a number of transcriptional targets in the lung (e.g. secretoglobin 1A, and the surfactant proteins Sftpa, Sftpb, and Sftpc) (5).

In thyroid and pulmonary epithelial cells, TTF-1 is phosphorylated at serine and/or threonine residues (7–9). Although the protein kinases and sites of phosphorylation mediating the interactions of TTF-1 with its various protein partners or DNA at cis-acting sites are not known with certainty, activation of protein kinase A enhanced transcriptional activation of Sftpb (8). However, direct effects of phosphorylation of TTF-1 on activity or DNA binding to thyroid-specific transcriptional target genes were not found in FRTL5 or HeLa cells in vitro (7, 9, 10). Hypophosphorylation of TTF-1 was observed in transformed thyroid cells in which TTF-1 target genes were not expressed; however, TTF-1 phosphorylation did not alter its binding to the thyroglobulin promoter (11). cAMP-dependent protein kinase stimulated phosphorylation of TTF-1 in several cell types; however, the effects of cAMP-dependent protein kinase on TTF-1-dependent transcription were not directly mediated by its phosphorylation (12). Taken together, TTF-1 is highly phosphorylated in many cell types, but the role of phosphorylation on transcriptional activation of target genes or on cell differentiation in target tissues remains unclear.

TTF-1 interacts directly or indirectly with other transcription factors and co-factors, including Foxa2, NF-1, GATA-6, AP-1, retinoic acid receptors, and associated co-factors, at or near TTF-1 binding, cis-acting elements located in regulatory regions of its target genes (5, 13–16). Furthermore, TTF-1 expression is spatially regulated during lung morphogenesis, being more highly expressed in peripheral regions of the growing lung buds with advancing development (1, 17). In the

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1 The abbreviations used are: E, embryonic day; VEGF, vascular endothelial cell growth factor; eSMA, α-smooth muscle actin; CCSP, Clara cell secretory protein; PECAM, platelet endothelial cell adhesion molecule; EST, expressed sequence tag; MMTV, mouse mammary tumor virus; SP, surfactant protein.
postnatal lung, TTF-1 is most abundant in type II epithelial cells in the alveolus, where it regulates surfactant protein synthesis. Thus, the activity of TTF-1 may be regulated by stochastic mechanisms, by interactions of TTF-1 with various protein partners, and by phosphorylation, oxidation-reduction, and cytoplasmic-nuclear trafficking (7, 8, 17–19).

Because TTF-1 phosphorylation mutants retained transcriptional activities in vitro, the ability of TTF-1PM to replace TTF-1 during lung morphogenesis was assessed in vivo. TTF-1PM substantially, but not completely, corrected the defects in lung morphogenesis characteristic of Titf1 null mice. Microarray analysis was used to identify genes for which expression was influenced by the Titf1PM/PM gene.

EXPERIMENTAL PROCEDURES

Generation of Titf1PM/PM Mice—Mouse Titf1 gene was isolated from a strain 129/SV mouse genomic library (Stratagene) using a probe corresponding to the 3’-untranslated region of rat Titf1 (Fig. 1). To prepare the targeting vector, a fragment extending from bp 4656 to bp 10443 of the reported mouse genomic sequence (GenBankTM accession no. U19755), containing the entire coding sequence for Titf1, was cloned in pBlueScript. A fragment, spanning from the translation start site of Titf1 (bp 7957) to the end of homeobox (bp 9480) was removed and replaced by the sequence encoding S80, a phosphorylation mutant allele of rat Titf1 in which seven serine phosphorylation sites were replaced by alanine codons as described (10). The probe used for genotyping ES cell clones and mice is indicated by a black bar B in Fig. 1. Southern blot analysis of genomic DNA from mouse tails digested with BamHI and probed with the probe indicated in panel A. The lower band corresponds to the mutated allele (4.5 kb), the upper band to the wild type allele (12 kb).

To genotype mice, DNA was obtained from a piece of tail from the mouse fetuses. The tissue was incubated overnight at 60 °C with lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K), and genomic DNA was extracted by adding 0.3 volumes of 6 M NaCl and then precipitated with isopropanol alcohol. The genomic DNA was digested with BamHI and analyzed by Southern blotting.

Lung Histology, Immunohistochemistry, and in Situ Hybridization—Lungs were collected at E18.5 by Cesarean section. Lung tissue was processed according to standard methods and embedded in paraffin. Paraffin sections of lung tissue were cut at 5 μm for histochemical analysis. Staining for the surfactant proteins SP-B and proSP-C, TTF-1, the Clara cell secretory protein (CCSP), PECAM (CD31), and α-smooth muscle actin (αSMA) was performed as described previously (17). In situ hybridization for SP-A, SP-B, SP-C, and VEGF-A mRNAs were performed using 32P-labeled riboprobes as previously described (21). Slides were coated in NTB-2, emulsion exposed for 2–5 days, and developed with Kodak D19.

RNA Protection and Western Blot Analysis—RNA protection assays for SP-A, SP-B, SP-C, and CCSP mRNAs were performed on lung RNA using 32P-end labeled DNA probes as previously described (22); L32 mRNA was used to normalize loading. Blots were scanned and differences compared by Student’s t test. Proteins from lung homogenates from wild type and Titf1PM/PM mice (E18) were separated by SDS-PAGE and blotted using antisera against proSP-B, SP-B, proSP-C, (Chemicon AB3430, AB3436, and AB3428, respectively), and napsin (kidney-derived aspartyl proteinase). To generate napsin antibody, the mouse napsin A cDNA was amplified from type II epithelial cell cDNA, sequenced, and the region encoding pronapsin cloned into the bacterial expression vector pET-21 (Novagen). Recombinant napsin protein was purified from bacterial lysates by chromatography on nickel-nitrilotriacetic acid resin and injected into rabbits. The napsin antibody detected a single protein band (Mr, 38,000) in immunoblots of mouse kidney.

RNA Microarray and Promoter Analysis—Total RNA from lungs at E18 Titf1PM/PM and wild type littermates was subjected to reverse transcription using oligo(dT) with T7 promoter sequences attached, followed by second strand cDNA synthesis. Antiense cRNA was amplified and biotinylated using T7 RNA polymerase, prior to hybridization to the version 2 of murine genome U74 set, which consists of three GeneChips and ~36,000 full-length mouse genes/ESTs (Affymetrix Inc.), using the Affymetrix recommended protocol (23, 24). Affymetrix MicroArray Suite version 5.0 was used to scan and quantitate the GeneChips using default scan settings. Intensity data was collected from each chip and scaled to a target intensity of 1500. The results were
analyzed using GeneSpring 5.0 (Silicon Genetics, Inc.), JMP4 (SAS Institute, Inc.), and Spotfire 7.12 (Spotfire, Inc.) software. A total of 18 chips were used in this experiment. Hybridization data (~216,000 data points) were sequentially subjected to normalization, transformation, filtering, clustering, and function classification as previously described (25). Data were normalized to enable the direct comparisons across chips and across genes. Statistical differences between Titf1PM/PM and control littermates were identified by distribution analysis and Welch’s t test at p value ≤ 0.05. Variations related to processing and biological replicates were calculated and separated from the candidate genes to identify primary genotype response. Fold changes were calculated for each gene against its specific control to determine relative gene expression. Additional filters included minimal absolute intensity ≥ 30, a minimum of 4 detectable judgments for A-set (12 chips) and 2 detectable judgments for B and C-sets (6 chips), and coefficient of variation among replicates ≤ 50%. Genes with average fold changes ≥ 2 and genes that were cross-validated via different probes on the same chip, or the same gene on different chips (A, B, and C sets), were prioritized. Differentially expressed genes were classified into functional categories based on gene ontology definitions. To determine representation of functional categories in the selected gene list, the binomial probability was calculated for each category using corresponding U74A2v2 genome as the reference dataset. Hierarchical clustering was applied to visualize and further group the selected genes based on their expression similarity. Pearson correlation was used for similarity measure. Clusters were constructed by the unweighted pair cluster analysis using average linkage and intensity of surfactant protein staining for SP-B and SP-A, proSP-C and CCSP (Fig. 4). Immunostaining for CCSP was decreased in the conducting airways of PM/PM mice, the peripheral respiratory parenchyma was composed of 2-3 generations of abnormally branched, dilated, acinar tubules (*), ending in smaller acinar buds (arrowheads) (D and F), which were lined by TTF-1-positive epithelial cells (arrow) and surrounded by abundant mesenchyme. Br, bronchiole; t, terminal bronchiole; *, acinar tubules/alveolar duct. Illustrations are representative of n = 8–9 for each genotype. Bars equal 1 mm (A and B), 100 μm (C and D), and 50 μm (E and F).
Decreased Surfactant Protein and Processing—Decreased but variable expression of known TTF-1 target genes suggested that the Titf1<sup>PM/PM</sup> may function, at least in part, as a hypo-morphic allele. Reduction of SP-B to less than 50% of normal levels causes lung dysfunction in mice (26, 27); therefore, reduction in SP-B expression may contribute to lack of postnatal...
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creased in lung homogenates of the 
type II epithelial cell-selective aspartyl protease, was de-
deficient (Fig. 6). RNA microarray data indicated a 21-fold 
increased, indicating that proteolytic processing of SP-B was 
mice, whereas abundance of the 42-kDa proSP-B precursor was 
and actin.

ally reprobed with antibodies directed against proSP-B, SP-B, SP-C, 
and actin. 

survival of Titf1PM/PM mice. Because processing of proSP-B and 
proSP-C is known to be cell-specific and proteolytically pro-
cessed SP-B peptide is required for surfactant function, proc-
essing of proSP-B was assessed in lung homogenates from Titf1PM/PM mice. The active SP-B peptide (18-kDa dimer) was 
markedly decreased in lung homogenates from the mutant mice, whereas abundance of the 42-kDa proSP-B precursor was 
increased, indicating that proteolytic processing of SP-B was 
deficient (Fig. 6). RNA microarray data indicated a 21-fold 
decrease in expression of napsin (Kdap) (see below). Napsin, a 
type II epithelial cell-selective aspartyl protease, was de-
creased in lung homogenates of the Titf1PM/PM mice (Fig. 6). 
Together with the previous report that an aspartyl protease is 
required for maturation of proSP-B, these findings support the 
likelihood that napsin plays a role in the processing of SP-B. 
Deficient processing of SP-B may contribute to the respiratory 
failure in the Titf1PM/PM mice, although the observed alterations 
in lung morphogenesis likely contribute to respiratory failure after birth.

Identification of Genes Influenced by the TTF-1 Phosphorylation Mutation—To identify genes responsive to Titf1PM/PM, 
lung RNAs from Titf1PM/PM mice (E18) were subjected to SDS-
PAGE, electrophoretically transferred to nitrocellulose and probed with 
napsin antibody. The membrane was subsequently stripped and seri-
ally reprobed with antibodies directed against proSP-B, SP-B, SP-C, 
and actin. 

Data are shown in two-dimensional matrix, and remarkably 
ordered gene expression profiles were displayed on genes se-
lected from A chip (Fig. 7A) and B and C chips (Fig. 7B). At the 
chip level (top dendrogram), RNAs influenced by Titf1PM/PM 
formed two distinct groups. Within the mutant group, samples 
collected from the same littermates were more closely related 
than those from different litters, whereas no differences among 
litters were observed in wild type controls. At the RNA level 
(see the dendrogram at the left of Fig. 7), genes were clearly 
separated into those mRNAs increased or decreased corre-
sponding to the Titf1 genotypes.

Because TTF-1 plays critical roles in lung development and 
morphogenesis, we specifically inspected the effects of the 
Titf1PM/PM mutant on genes that are considered important for 
lung formation and/or function, including some known TTF-1 
transcriptional targets (Fig. 8). Among them Kdap (napsin), 
Calb3, Sftpalpha, Vegfa, Sgcblalpha1 (also known as CCSP), Aap5, 
Sox17, Lzap-s, Fgf1, and Pdgfra were decreased in Titf1PM/PM 
mice; Sftpbeta, Sftpalpha, and Bmp4 were significantly, but moder-
ately, decreased. The decrease in Sftpalpha observed in the array 
was not great as seen by S1 nuclease assay, but was statisti-
cally significant. Other genes including Sftpd, Mdk, Evi1, Clu, 
Znflalpha1, and FoxM1 were increased in the mutant mice. To 
further test whether the Titf1PM/PM influenced expression of 
these genes directly or indirectly, we searched for potential 
TTF-1 binding sites within 1 kb upstream of the start of tran-
scription. Genes expressed selectively in the respiratory epite-
thelium versus lung mesenchyme were identified. Genes 1) 
fluenced by Titf1PM/PM, 2) selectively expressed in respira-
tory epithelium, and 3) containing TTF-1 binding site(s) within 
1 kb of the start of transcription were considered as possible 
direct transcriptional targets of TTF-1. A number of genes fit 
these criteria, including Sftpalpha, Sftpbeta, Sftpalpha, Sgcblalpha1, Clu, Tcf7, 
Sox17, β-catenin, Aap5, Bmp4, Lzap-s, Zfp386 (Kruppel-like), H3b-Qa, 
Calb3, Gsta4, Mdk, and Evi1.

Differentially expressed genes were further classified according 
to their known or predicted functions. Each gene was anno-
tated and assigned to a functional category. To simplify the 
calculation, we assumed that genes in each category could be fit 
to a binomial distribution. The binomial probability was calcul-
dated for each category using the entire U74Av2 as the reference 
dataset. The “defense response,” which includes immune, inflam-
matory, and stress responses, was the most represented category 
of those RNAs increased in the Titf1PM/PM mice. Among RNAs for 
which abundance was decreased, those involved in lipid metab-
olism, signal transduction, and defense response were most 
highly represented (Tables V, parts a and b).

DISCUSSION

Titf1PM/PM Supports Early Branching Morphogenesis but Not Maturation of Acinar Saccules—lobulation and early 
branching morphogenesis were maintained, whereas formation 
and differentiation of peripheral bronchioles and acini were de-
cicient in the Titf1PM/PM mice. Peripheral pulmonary vessels, 
as indicated by regional differences in PECAM staining, were 
perturbed in the abnormal lung saccules and the numbers of 
cells expressing VEGF mRNA decreased. Deficient formation of 
acinar buds and terminal saccules, as well as of the alveolar 
capillary bed, in the mutant mice indicates a critical role for 
TTF-1 in the regulation of genes required for reciprocal inter-
actions between the epithelium and mesenchyme during for-
mation of the peripheral lung. These findings are consistent 
with recent studies supporting the concept that early vascular-
ization is required for normal morphogenesis of the developing 
pancreas (28).

Lack of Terminal Differentiation in Peripheral Lung Struc-
tures—Peripheral lung tubules in the Titf1PM/PM mice were 
poorly developed, and squamous type I cell differentiation, 
typical of the normal E18 lung, was not observed. Likewise,
there was a paucity of small blood vessels that normally come into close apposition with the respiratory epithelium at this time. Fewer terminal sacculles were observed in the mutant mice, indicating arrest of late branching morphogenesis, resulting in fewer acinar tubules. Taken together, lung morphology in the Titf1<sup>PM/PM</sup> mice is consistent with abnormalities in branching morphogenesis and/or delay in cytodifferentiation that normally occur in the late pseudoglandular, canalicular, branching morphogenesis and/or delay in cytodifferentiation resulting in fewer acinar tubules. Taken together, lung morphology in the mutant mice at levels similar to that expressed by individual cells in control mice. However, SP-B- and SP-C-positive cells were not observed in many of the larger, dilated peripheral tubules of the Titf1<sup>PM/PM</sup> mice. Changes in relative numbers of cells expressing these RNAs rather than transcriptional activity of the genes were influenced by Titf1<sup>PM</sup>. Expression of CCSP and SP-A mRNA was more markedly decreased in all cells in the mutant mice, likely indicating an effect of Titf1<sup>PM</sup> on their transcription. Thus, proximal/peripheral patterning of the epithelial cell differentiation was generally maintained, but the level of expression of these TTF-1 target genes was variably decreased. Because the numbers of peripheral tubules were decreased, reduction in peripheral lung markers may indicate reduction in numbers of specific cell types, decreased transcription of target genes, or both. Although type II epithelial cell differentiation was observed, as indicated by the expression of SP-C mRNA and proSP-C staining, squamous alveolar type I cells were not present in the abnormal lung tubules. SP-B mRNA, normally expressed in both conducting and peripheral airways at E18–18.5, was present throughout the pulmonary lining of the lungs of the wild-type and heterozygous mice, but was restricted to the distal airways in the Titf1<sup>PM/PM</sup> mice. The expression of SP-C mRNA was also decreased in the Titf1<sup>PM/PM</sup> mice, consistent with the decrease in SP-B expression. The decrease in SP-C expression may be due to a decrease in the expression of the SP-C gene itself, or to a decrease in the expression of the SP-C promoter. The decrease in SP-C expression may also be due to a decrease in the expression of the SP-C protein, or to a decrease in the expression of the SP-C mRNA. The decrease in SP-C expression may also be due to a decrease in the expression of the SP-C promoter, or to a decrease in the expression of the SP-C gene itself. The decrease in SP-C expression may also be due to a decrease in the expression of the SP-C protein, or to a decrease in the expression of the SP-C gene itself.
epithelium in the Titf1PM/PM mice, its level of expression being similar to controls on a per cell basis.

Expression of genes known to be direct targets of TTF-1, including surfactant proteins and CCSP, was significantly, but variably, decreased in the Titf1PM/PM mice, supporting the concept that the Titf1PM/PM mutant represents, in part, a hypomorphic TTF-1 allele. Because lung structure was perturbed in the mutant mice, differences in the proportions of specific subcategories of cells may be influenced by changes in RNA concentration related to differences in cell types rather than by transcriptional mechanisms. For example, peripheral tubules were decreased in number, and squamous cells (type I cells) failed to form in the mutant lung. Thus, decreased aquaporin-1 mRNA (a marker of type I epithelial cells) may also reflect changes related to the absence of cell type and/or regulation by TTF-1.

**Decreased Processing of ProSP-B**—Immunostaining indicated the lack of the active SP-B protein in the airways and increased intracellular staining for SP-B in the mutant mice (data not shown), indicating lack of secretion or proteolytic processing of SP-B that normally occurs in the perinatal period.

Consistent with this observation, SP-B processing was decreased and the abundance of proSP-B increased in the Titf1PM/PM mice. Napsin mRNA and protein were also decreased, indicating a potential role for this protease in cell-specific processing of SP-B.

**mRNAs Relevant to Perinatal Lung Function**—Expression of a number of known and potential TTF-1 target genes was reduced in the Titf1PM/PM mice. Decreased Processing of ProSP-B—Immunostaining indicated the lack of the active SP-B protein in the airways and increased intracellular staining for SP-B in the mutant mice (data not shown), indicating lack of secretion or proteolytic processing of SP-B that normally occurs in the perinatal period.

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homeostasis that is required for surfactant production prior to birth. The expression of β-adrenergic receptor 2, known to regulate surfactant secretion and ion transport in the neonatal lung, was decreased ~3-fold. The numbers and activity of β2-adrenergic receptors increase dramatically in the perinatal and postnatal period (30), a process induced by perinatal exposure to glucocorticoid, consistent with its role in surfactant secretion at birth.

**Genes Regulating Fluid and Electrolyte Transport**—Aquaporin-1 and -5 mRNAs were decreased (4- and 3-fold, respectively) in the Titf1PM/PM mice. Although the functional significance of these findings is unclear, TTF-1 may influence airway reactivity; aquaporin-5-deficient mice develop airway hyperactivity in response to cholinergic challenge (31). Expression of a number of solute carriers (neurotransmitter transporter, sodium/sulfate supporter, and organic anion transporter), ion channels (sodium channel non-voltage-gated 1γ, and voltage-gated type IV α polypeptide) were significantly decreased (2- and 9-fold), indicating that TTF-1 may regulate these genes to maintain fluid and electrolyte balance in the lung.

**Regulation of Genes Modulating Host Defense Functions**—Genes involved in host defense and inflammation were most influenced by the Titf1PM/PM. Calbindin D9K (a neutrophil chemoattractant molecule), hemolytic complement (Hc), Sgb1a1, Sftpa, Kit ligand (stem cell factor), and lysozyme were decreased. Some of these genes are known to be expressed in respiratory epithelial cells and are direct transcriptional targets of TTF-1, e.g. Sftpa and Sgb1a1. Scavenger receptor class A, IL-7, leukocyte cell-derived chemotaxin-1, clusterin, peptoglycan recognition protein, chitinase (acidic), glutathione S-transferase, trefoil factor 2, small proline-rich polypeptide, serum amyloid, CD14, and others were increased. TTF-1 staining was decreased in human lung tissues following lung injury and infection (32); thus, decreased activity of TTF-1 may influence transcription of host defense genes involved in protection from lung injury and in repair. The observed increase in the expression of mRNAs selectively expressed in lymphocytes may represent the presence or absence of thymic tissue that may be

### Table IV

| Gene Description | GenBank™ | Change | p value | Category |
|------------------|----------|--------|---------|----------|
| Sodium channel, non-voltage-gated 1γ | NM_011326 | −8.96 | 1.29E−04 | Transporter/channel |
| Phosphatidylinositol glycan, class M | NM_026234 | −6.83 | 1.34E−04 | Transporter/channel |
| Surfactant-associated protein A | NM_023134 | −6.54 | 5.75E−06 | Defense response |
| Low density lipoprotein receptor-related protein 2 | NM_029630 | −4.84 | 9.64E−04 | Metabolism |
| Similar to JC7286 liver-specific organic anion transporter-1 | AA903320 | −4.06 | 1.21E−04 | Transporter/channel |
| Cytooglobin | NM_030206 | −3.97 | 1.50E−04 | Transporter/channel |
| Zinc finger protein 386 (Kruppel-like) | NM_019965 | −3.55 | 4.27E−02 | Regulation of transcription |
| Wingless-related MMTV integration site 5B | NM_099525 | −3.41 | 8.39E−04 | Signal transduction |
| Par-6 (partitioning defective 6) homolog β (Caenorhabditis elegans) | NM_021409 | −3.40 | 3.15E−04 | Transporter/channel |
| Aquaporin 5 | NM_099670 | −3.12 | 6.96E−06 | Transporter/channel |
| Megalencephalic leukoencephalopathy with subcortical cysts 1 homolog (human) | NM_133241 | −3.02 | 3.86E−04 | Development |
| Solute carrier family 21 (organic anion transporter), member 10 | NM_020495 | −2.72 | 2.61E−03 | Transporter/channel |
| Serine protease inhibitor 12 | NM_011454 | −2.71 | 6.70E−04 | Protein modification |
| Similar to JC5629 nullerian-inhibiting substance type II receptor | AV253399 | −2.69 | 3.95E−03 | Signal transduction |
| Naked cuticle homolog 1 (Drosophila) | NM_027280 | −2.66 | 2.53E−06 | Transporter/channel |
| G protein-coupled heptahelical receptor Ig-Hepta (rat) | AI835456 | −2.58 | 5.32E−04 | Signal transduction |
| Solute carrier family 6 (neurotransmitter transporter), member 14 | NM_020049 | −2.53 | 9.05E−03 | Transporter/channel |
| Similar to solute carrier family 13 (sodium/sulfate symporters), member 1 | AA021940 | −2.28 | 9.74E−04 | Transporter/channel |
| Poliovirus receptor-related 3 | NM_021409 | −2.26 | 2.00E−02 | Cell adhesion |
| Similar to UDP-N-acetyl-D-glucosamine | AW124105 | −2.17 | 3.28E−05 | Carbohydrate metabolism |
| Heparan sulfate 2-O-sulfotransferase 2 | NM_011828 | −2.17 | 1.33E−03 | Carbohydrate metabolism |
| Tetraspan 2 (human) | NM_027533 | −2.08 | 1.93E−03 | Signal transduction |
| Endothelial cell-selective adhesion molecule | NM_027102 | −2.03 | 1.06E−03 | Cell adhesion |
| Glucocorticoid-induced transcript 1 | NM_133236 | 1.64 | 4.36E−03 | Other |
| Catenin β | NM_007614 | 1.77 | 2.36E−03 | Regulation of transcription |
| N-Acetylgalactosamine-phosphate mutase (human) | NM_028352 | 2.02 | 3.40E−02 | Carbohydrate metabolism |
| RAD51 homolog (S. cerevisiae) | NM_011249 | 2.06 | 1.10E−02 | DNA recombination |
| Similar to hydroxysteroid (17-β)-dehydrogenase 11 (human) | AI914343 | 2.16 | 5.45E−03 | Metabolism |
| Ectodermal-nervous cortex 1 | NM_007930 | 2.20 | 1.57E−02 | Development |
| Ras GTase-activating-like protein (human) | AA717658 | 2.29 | 1.46E−03 | Signal transduction |
| Androgen-induced basic leucine zipper | NM_009080 | 2.41 | 2.73E−03 | Regulation of transcription |
| UDP-glucuronosyltransferase 8 | NM_011674 | 2.43 | 1.62E−02 | Metabolism |
| Scavenger receptor class A, member 3 (human) | AI851432 | 2.48 | 1.09E−02 | Transporter/channel |
| Metal response element-binding transcription factor 1 | NM_008636 | 2.51 | 6.50E−03 | Regulation of transcription |
| Phenyllalnine-tRNA synthetase-like (human) | AV305194 | 2.54 | 2.42E−02 | Protein biosynthesis |
| Interleukin 7 | NM_008371 | 2.54 | 1.95E−03 | Defense response |
| 5-Hydroxybutyrate dehydrogenase | AW045975 | 2.58 | 1.01E−02 | Metabolism |
| Paired related homeobox 2 | NM_009116 | 2.59 | 2.19E−03 | Regulation of transcription |
| Procollagen, type IX, Paired related homeobox 2 | NM_007740 | 2.74 | 3.37E−03 | Cell adhesion |
| Phospholipase A2, group Ib, pancreas | NM_011107 | 2.74 | 2.79E−03 | Metabolism |
| Forkhead box Q1 | NM_008239 | 2.79 | 1.52E−02 | Regulation of transcription |
| Ribonucleotide reductase M2 | NM_009104 | 3.93 | 1.65E−02 | DNA replication |
| Whn-dependent transcript 2 | AA606601 | 4.08 | 6.31E−03 | Regulation of transcription |
| Thymocyte selection-associated HMG box gene | NM_145711 | 4.49 | 5.61E−03 | Transporter/channel |
| HSCARG protein (human) | AW046381 | 6.06 | 5.99E−04 | Metabolism |
| Leukocyte cell-derived chemotaxin 1 | NM_010701 | 7.78 | 8.82E−03 | Defense response |
adherent to lung tissues from which the RNAs were prepared. Because these changes were found in each of the individual lungs assayed, it is unclear whether changes in lymphocytes or the relative proportion of thymic tissues contributing to the RNA pools were influenced by TTF-1.

The increased representation of genes involved in host defense in the Titf1PM/PM mice supports the concept that TTF-1 phosphorylation plays a role in host defense responses in the lung. It is of interest that increased expression of TTF-1 in the postnatal lung in transgenic mice caused marked inflammation, emphysema, and eosinophilic infiltration (21). It is also possible that increased expression of some of these genes represent cell injury responses related to the TTF-1 mutant protein (e.g., glutathione S-transferase and serum amyloid, etc.), a possibility that cannot be excluded. However, there was no observable histologic evidence of cell necrosis or inflammation in wild type or Titf1PM/PM mice.

Alterations in Transcriptional Pathways Modulating Respiratory Epithelial Cell Differentiation—Significant differences were observed in the abundance of mRNAs encoding a number of transcriptional proteins known to be expressed in the developing lung. Such changes may indicate that these proteins are direct or indirect targets of TTF-1 phosphorylation or TTF-1 per se. The increase in myb may represent a compensatory response to decreased TTF-1 activity, because myb is known to act synergistically with TTF-1 and binds to elements in the Sftpa gene (33). Expression of several transcription factors were decreased, perhaps representing potential transcriptional targets of TTF-1 phosphorylation. Forkhead F2 (known to be expressed in lung mesenchyme), naked cuticle homologue, androgen-induced basic leucine zipper, metal response element binding transcription factor-1, homeobox2, forkhead boxQ, paired related, thymocyte-selective HMG box, EVI-1, and FoxM1 (a Fox family member regulatory cell cycle and expressed in the lung mesenchyme) mRNAs were increased. Although these changes may represent reciprocal or compensatory responses to the lack of TTF-1 activity or phosphorylation, changes in their abundance may reflect changes in cell populations in which they are expressed.

Several mRNAs in the Wnt signaling cascade were altered in the Titf1PM/PM mice, including Wnt-4 and -11, β-catenin, Tcf-7 (Tcf-1), and Lef-1, which were increased 2–3-fold. In contrast, expression of Wnt-3a and Wnt-5b were decreased in the mutant lungs. Nuclear β-catenin is present in epithelial cells of the developing lung during the embryonic period, at sites overlapping with TTF-1 (34). The present findings support the concept that
TTF-1- and β-catenin-dependent pathways interact in the peripheral lung, directly or indirectly, during lung morphogenesis. 

Genes Modulating Lung Vasculogenesis—Regional decreases in vascularity of the abnormal peripheral lung tubules, as detected by PECAM staining, were associated with decreased VEGF-A mRNA, indicating that TTF-1 phosphorylation is required for normal levels of expression of VEGF in the developing respiratory epithelium. Surprisingly, PECAM RNA was increased in the mutant mice, perhaps related to the extensive tissue remodeling. BMP-4, ECAM, carbonic anhydrase, VEGFR1, and ephrin A2, proteins known or considered to be markers or regulators of pulmonary mesenchyme differentiation and vasculature formation, were perturbed in the Titf1PM/PM mice.

Identification and Mapping of Known and Predicted TTF-1 Response Elements—Genes known to be expressed in a respiratory epithelial cell-specific manner under direct transcriptional control of TTF-1 were subjected to a computer-assisted analysis of their regulatory regions. Consensus elements for TTF-1 binding were readily detected within the regulatory regions of Sftpa, Sftpb, Sftpc, and Scgb1a genes. A number of these elements were previously validated by direct site-specific mutagenesis, transfection assays, and gel retardation analyses. A consensus for a TTF-1 binding sequence was utilized to identify potential TTF-1 regulatory elements in the subset of genes for which expression was influenced in the Titf1PM/PM mice. A distinct subset of genes expressed in the lung mesenchyme did not contain the element, but were consistently influenced by the Titf1PM/PM, supporting the likelihood that TTF-1 influences their expression indirectly, via reciprocal tissue interactions between the epithelium and the mesenchyme or by changing the proportions of cells expressing the gene.

Conclusion—The lung developed relatively late during vertebrate evolution, representing a singular solution to the problem of air-breathing. TTF-1 is required for normal formation of the peripheral lung at birth. Perhaps it is not surprising that Titf1PM/PM influences lung structure and the expression of subsets of genes regulating biological functions uniquely required for adaptation following birth, including host defense, fluid balance, surfactant homeostasis, and the formation of an extensive interface between the peripheral-vascular bed and the alveolar surfaces upon which gas exchange depends.

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