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Foxp2 and Foxp1 cooperatively regulate lung and esophagus development

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The airways of the lung develop through a reiterative process of branching morphogenesis that gives rise to the intricate and extensive surface area required for postnatal respiration. The forkhead transcription factors Foxp2 and Foxp1 are expressed in multiple foregut-derived tissues including the lung and intestine. In this report, we show that loss of Foxp2 in mouse leads to defective postnatal lung alveolarization, contributing to postnatal lethality. Using in vitro and in vivo assays, we show that T1alpha, a lung alveolar epithelial type 1 cell-restricted gene crucial for lung development and function, is a direct target of Foxp2 and Foxp1. Remarkably, loss of a single Foxp1 allele in addition to complete loss of Foxp2 results in increased severity of morphological defects in mutant lungs and leads to perinatal loss of all *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mice. Expression of N-myc and Hop, crucial regulators of lung development, is compromised in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants. In addition to the defects in lung development, esophageal muscle development is disrupted in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ embryos, a tissue where Foxp2 and Foxp1 are co-expressed. These data identify Foxp2 and Foxp1 as crucial regulators of lung and esophageal development, underscoring the necessity of these transcription factors in the development of anterior foregut-derived tissues and demonstrating functional cooperativity between members of the Foxp1/2/4 family in tissues where they are co-expressed.

KEY WORDS: Foxp1, Foxp2, Lung development, Esophagus, T1alpha (podoplanin), Mouse

INTRODUCTION

The foregut endoderm is derived from the early definitive endoderm of the vertebrate embryo. Multiple tissues derive from the foregut endoderm including cell lineages within the liver, thyroid, pancreas and the lung. Development of the mammalian lung, which is initiated at E9.5 in the mouse, begins as a simple epithelial tube budding from the ventral side of the anterior region of the foregut. This tube bifurcates and quickly grows through a process termed branching morphogenesis. Early in lung development, distinct proximal-distal patterning of both the endoderm and mesoderm occurs and is driven by specific molecular pathways required for the proper differentiation of epithelial cells needed for gas exchange, surfactant production, detoxification and particle clearance. Disruption of this intricate process can lead to respiratory distress syndromes, including bronchopulmonary dysplasia and pulmonary hypoplasia in humans.

In the lung, the surfactant protein genes have acted as surrogate readouts for the important transcriptional mechanisms underlying lung development. These studies have lead to the identification of several transcription factors including Nkx2.1 (Titf1 – Mouse Genome Informatics), Gata6, Foxa1/2 and C/EBPα as important regulators of lung endoderm differentiation and development (reviewed by Cardoso and Lu, 2006). Many of these factors are part of large families of proteins, which act both individually and cooperatively to control gene transcription in tissues where multiple family members are expressed.

Among the transcription factor families known to be crucial for lung development, the Fox family is of particular importance. Fox proteins are characterized by their highly homologous DNA-binding domain, which forms a 'winged-helix' motif (Kaestner et al., 2000). Fox genes are important regulators of foregut development, including tissues such as the liver, pancreas, intestinal epithelium and lung. Fox factors are crucial activators of lung-specific gene expression and both Foxa1 and Foxa2 are important for airway morphogenesis and epithelial differentiation in the lung. Loss of Foxa2 expression results in distinct defects in alveolarization, whereas *Foxa1*-null mice exhibit transient defects in lung epithelial differentiation (Besnard et al., 2005; Wan et al., 2004). Loss of both genes results in a severe disruption in branching morphogenesis with a concurrent loss of epithelial differentiation (Wan et al., 2005). Such redundancy is likely to be important for large transcription factor families such as the Fox family that are crucial for tissue-specific development.

We have previously identified a subfamily of Fox factors, Foxp1/2/4, that are highly expressed in distinct patterns in the developing airway epithelium (Lu et al., 2002). Loss-of-function of each gene resulted in distinct and severe defects in cardiovascular, neural and hematopoietic development (Hu et al., 2006; Li et al., 2004b; Shu et al., 2005a; Wang et al., 2004). However, it remains unclear what role these factors play in lung development, as *Foxp1* and *Foxp4* mutants exhibit normal lung specification but do not survive past E13.5. We show that *Foxp2*-null mice exhibit defects in postnatal alveolarization. Given their overlapping patterns of expression, redundancy of Foxp2 and Foxp1 was addressed by generating *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants. In contrast to *Foxp2*-null animals, these compound mutants die at birth due to increased severity in airway morphogenesis and differentiation defects leading to respiratory failure. Furthermore, *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants have severe defects in esophageal development, indicating a broader role in regulation of anterior foregut development. These data identify Foxp2 and Foxp1 as crucial regulators of lung airway morphogenesis and differentiation as well as esophageal muscle development, pointing to a complex interplay amongst Foxp factors in the regulation of anterior foregut development.

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Table 1. PCR oligonucleotides

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Foxp1	CAGGCAGATCCCCTATGCAA	GGACAGAGGGCCTTCAGCTT
Foxp4	CAGCCCGCCTCGTCTTT	CCGGCCGTCGGTCTTC
Sp-A (*Sftpa1*)	CTCCAGACCTGTGCCCATATG	ACCTCCAGTCATGGCACAGTAA
SP-B (*Sftpb*)	ACGTCCTCTGGAAGCCTTCA	TGTCTTCTTGGAGCCACAACAG
SP-C (*Sftpc*)	ACCCTGTGTGGAGAGCTACCA	TTTGCGGAGGGTCTTTCCT
CC10 (*Scgb1a1*)	TCCTAACAAGTCCTCTGTGTAAGA	AGGAGACACAGGGCAGTGACA
aquaporin 5	ATGAACCCAGCCCGATCTTT	ACGATCGGTCCTACCCAGAAG
T1alpha (*Pdpn*)	AGGTACAGGAGACGGCATGGT	CCAGAGGTGCCTTGCCAGTA
N-myc (*Mycn*)	ACAAGGCGGTAACCACTTTCA	AACACAGCGCTTGAGGATCA
Hop (*Hod*)	GGAGTACAACTTCAACAAGGTCACA	GCGCTGCTTAAACCATTTCTG
Nkx2.1 (*Titf1*)	TCCAGCCTATCCCATCTGAACT	CAAGCGCATCTCACGTCTCA
T1alpha*	ACGTGCCTTGCATGAAGGTTC	CACGTGCAGTAAACAGTTCTG

*Used for ChIP (all other primer sets were used for Q-PCR).

MATERIALS AND METHODS

Animals

Generation and genotyping of *Foxp1* and *Foxp2* mutants has been described previously (Shu et al., 2005a; Wang et al., 2004). Mice were kept on a C57BL/6×129SVj mixed background. $Foxp1^{+/-}$ and $Foxp2^{+/-}$ were intercrossed to generate $Foxp2^{+/-};Foxp1^{+/-}$, which were further crossed with $Foxp2^{+/-}$ to generate $Foxp2^{-/-};Foxp1^{+/-}$ animals. For timed matings, noon of the day that the vaginal plug was observed was considered E0.5.

Histology

Embryos were dissected free from the uterus and fixed in 4% paraformaldehyde (PFA) for 48 hours. Lungs from postnatal day (P) 8 and 20 animals were inflation fixed (20 cm of water pressure) in 4% PFA then submerged in 4% PFA for 48 hours. Tissues were dehydrated through a series of ethanol washes and embedded in paraffin. Sections (5 μm) were used for subsequent in situ hybridizations and immunohistochemical analysis. In situ hybridization probes for genes encoding N-myc, Hop, Gata6, SP-B (Sftpb), Shh, Foxa2 and Nkx2.1 have been described previously (Shu et al., 2005b; Yin et al., 2006). The following antibodies and concentrations were used in the histological studies: SP-C (Chemicon #07-647, 1:500), CC10 (Santa Cruz T-18, 1:50), SP-B (Chemicon #AB3436, 1:250), T1alpha (Developmental Studies Hybridoma Bank, University of Iowa, mAb 8.1.1, 1:100), phospho-histone H3 (Cell Signaling Technologies #9701, 1:500), smooth muscle α-actin (Sigma clone 1A4, 1:200), Foxp1 antisera (1:400), Foxp2 antisera (1:400), MyoD (Novacastra NCL-MyoD1, 1:20). Complete details on all histological procedures can be found at the University of Pennsylvania Cardiovascular Institute Histology Core web site http://www.uphs.upenn.edu/mcrc/histology/histologyhome.html.

Lung morphometry and proliferation index

The mean linear intercept (MLI) on E18.5 and postnatal lung samples was calculated as follows, based on previously published protocols (Neptune et al., 2003; Thurlbeck, 1967). Digital images were captured at both 200× and 400× magnification. Horizontal, vertical and diagonal grid lines were overlaid and used to count the number of alveolar septa intersections. MLI was calculated as follows: length of grid lines divided by the number of intersections with alveolar septa. Data are from three samples of each indicated genotype. Data were analyzed using Student's *t*-test and values considered significant if $P<0.05$.

Lung-to-body weight ratios were based on four embryos of each genotype at each age tested. To determine changes in E14.5 and E18.5 airspace luminal area, ImageJ software was used to compare distal airspace area in arbitrary pixel units. This was performed on four sections from each of three different lung samples of the indicated genotypes. Data were analyzed using Student's *t*-test and values considered significant if $P<0.05$.

A proliferation index for both the epithelial and mesenchymal cells in the indicated wild-type and mutant lungs was generated by counting the percentage of Ki-67-positive cells in five fields of view from three different embryos. Epithelial cells were selected on the basis of their position lining distal airways, whereas mesenchymal cells were selected on the basis of not lining airways. Data were analyzed using Student's *t*-test.

Quantitative RT-PCR (Q-PCR) and chromatin immunoprecipitation (ChIP) assays

Total RNA was isolated using Trizol (Invitrogen) and Q-PCR was performed using the oligonucleotides listed in Table 1 and an Applied Biosystems 7900HT system with Syber Green reaction mixture as previously described (Lepore et al., 2005). Chromatin was prepared from E18.5 mouse lung tissue using the ChIP Kit (Upstate Biotechnology). Lung tissue was minced, fixed with 1% formaldehyde and chromatin sheared by sonication to an average length of 500-600 bp. The Foxp1 and Foxp2 antibodies used for

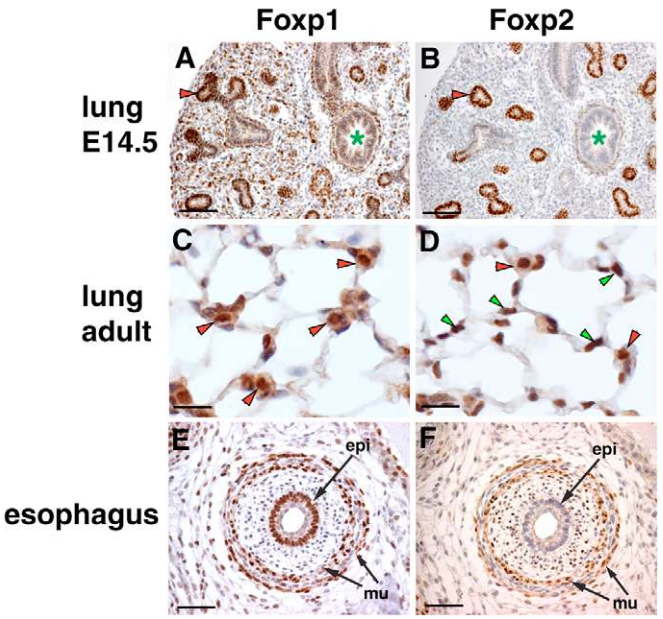

Fig. 1. Expression of Foxp1 and Foxp2 during lung and esophagus development. Previously characterized rabbit polyclonal antibodies were used to perform immunohistochemistry on E14.5 embryonic and adult mouse lung tissue sections to determine the expression pattern of Foxp1 and Foxp2. (**A**,**B**) Expression is observed for both proteins in the distal tips of the developing airway epithelium at E14.5 (arrowheads). Foxp1 is expressed at low levels in developing bronchial airways, whereas Foxp2 is not (asterisk). (**C**,**D**) In adult lungs, Foxp1 is expressed primarily in AEC-2 cells (C, red arrowheads), whereas Foxp2 is expressed in both AEC-2 (D, red arrowheads) and AEC-1 (D, green arrowheads) cells. (**E**,**F**) Foxp1 and Foxp2 are both expressed in the muscular component of the developing esophagus at E14.5, whereas only Foxp1 is expressed in the epithelium. epi, epithelium; mu, muscular layers. Scale bars: 100 μm in A,B; 50 μm in C,D; 10 μm in E,F.

immunoprecipitation have been described previously (Lu et al., 2002). Reverse cross-linked immunoprecipitated chromatin was subjected to PCR using the oligonucleotides listed in Table 1.

Cell transfection studies

NIH-3T3 cells were transfected with Fugene 6 as previously described (Shu et al., 2001). Expression plasmids for Foxp1 and Foxp2 and the 1.3 kb rat T1alpha luciferase reporters have been described previously (Ramirez et al., 1997; Shu et al., 2001). Luciferase assays were performed 48 hours after transfection using the Dual Luciferase Kit (Promega). All data are the average of three assays performed in triplicate±s.e.m.

RESULTS

Loss of Foxp2 leads to lung alveolarization defects

Foxp1/2/4 are highly expressed in foregut-derived tissues during development (Lu et al., 2002). To directly compare Foxp1 and Foxp2 expression in the lung and esophagus, immunohistochemistry was performed on E14.5 embryos. Foxp1 was found to be expressed at high levels in distal lung epithelium and at lower levels in more proximal bronchial epithelium (Fig. 1A). Foxp1 expression was also observed in the developing mesenchyme, most likely in endothelial precursors as Foxp1 is expressed in the developing vascular endothelium later in development (Fig. 1A) (Lu et al., 2002). In contrast to Foxp1, Foxp2 expression was restricted to distal lung epithelia and was not expressed in the developing mesenchyme (Fig. 1B). In adult lungs, Foxp1 was found to be expressed primarily in alveolar epithelial type 2 (AEC-2) cells located in the corners of alveoli, whereas Foxp2 expression was observed in AEC-2 as well as alveolar epithelial type 1 (AEC-1) cells, which contain a more flattened nucleus in the walls of the alveolus (Fig. 1C,D). Both Foxp1 and Foxp2 were expressed in the muscular component of the developing esophagus, whereas only Foxp1 was expressed in the developing esophageal epithelium (Fig. 1E,F). These data demonstrate both extensive overlap and distinct differences in Foxp1 and Foxp2 expression during foregut development.

Given the high-level expression of Foxp2 in distal lung epithelium, we sought to determine whether there were defects in lung morphogenesis or maturation in *Foxp2*-null mice. We have previously demonstrated that *Foxp2*-null mice die approximately three weeks postnatally (Shu et al., 2005a), a time when active lung alveolarization occurs. Histological analysis revealed that at E18.5, *Foxp2*-null lungs are morphologically similar to wild-type littermates (Fig. 2A,D). However, by P8, airways in *Foxp2*-null lungs appeared severely dilated (Fig. 2B,E). This airway dilation was also evident at P20 (Fig. 2C,F). Mean linear intercept analysis revealed significant dilation of distal airspaces at P8 and P20, indicating postnatal alveolarization defects in *Foxp2*-null mice (Fig. 2G). Transmission electron microscopy (TEM) was performed to visualize the alveolar airspaces at high magnification. Little difference was observed in the number or morphology of AEC-1 and AEC-2 cells (Fig. 2H,I).

Immunohistochemistry was performed on wild-type and *Foxp2*-null mice to determine the effect that loss of Foxp2 expression had on lung epithelial cell lineage differentiation. Expression of the AEC-2 cell markers SP-B and SP-C and the Clara cell marker CC10 (also known as Sftpb, Sftpc and Scgb1a1, respectively – Mouse Genome Informatics) were unaffected by loss of Foxp2 (Fig. 3A,B,E-H). However, expression of the AEC-1 cell marker T1alpha (also known as podoplanin – Mouse Genome Informatics) was markedly increased in *Foxp2*-null animals (Fig. 3C,D). Q-PCR also revealed an increase in T1alpha expression, whereas expression of aquaporin 5, another AEC-1-restricted marker gene, as well as other AEC-2 marker genes, were unaffected (Fig. 3N). Foxp2 and T1alpha proteins are co-expressed in the same cells in the distal airways at E18.5, indicating that Foxp2 could have a direct affect on T1alpha gene expression (Fig. 3I-K). In addition to AEC-1 cells, T1alpha is also expressed in the lymphatic endothelium of the lung (Schacht et al., 2003). However, T1alpha expression in this cell type did not appear to be affected by loss of Foxp2 (Fig. 3L,M). Given the expanded nature of the airways, these data suggest either disruption in alveolar epithelial differentiation or increased numbers of AEC-1 cells in the distal airways of *Foxp2*-null mice. Since TEM studies did not indicate an increased number of AEC-1 cells in *Foxp2*$^{-/-}$ mutants, these data suggest that the increase in T1alpha is due to Foxp2 acting directly on T1alpha gene expression. Thus, Foxp2 is required for postnatal lung alveolarization and regulation of the AEC-1 cell-restricted gene T1alpha.

T1alpha is a direct in vivo target of Foxp2 and Foxp1

The above data suggest that Foxp2 acts as a transcriptional repressor of T1alpha gene expression in vivo. This concurs with previous studies by our laboratory that have demonstrated that all members of the Foxp1/2/4 family are transcriptional repressors (Li et al., 2004a; Shu et al., 2001). Three consensus Fox DNA-binding sites

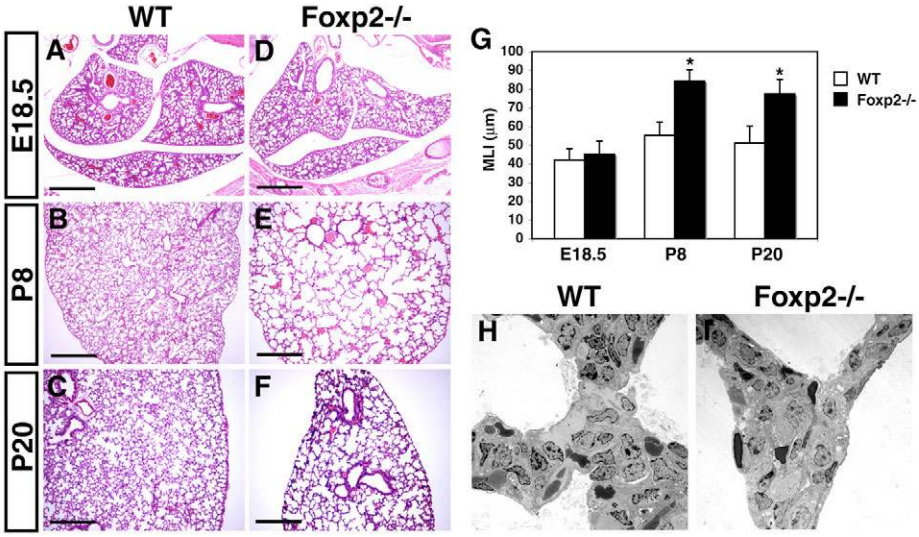

Fig. 2. Loss of Foxp2 leads to postnatal lung alveolarization defects.
(**A-F**) Hematoxylin and Eosin staining of wild-type and *Foxp2*$^{-/-}$ mutant mice revealing dilated airspaces in the *Foxp2* mutant lungs at E18.5, P8 and P20. (**G**) Mean linear intercept (MLI) calculation of wild-type and *Foxp2* mutant lungs at the indicated times showing airspace enlargement in *Foxp2* mutant mice. (**H**,**I**) TEM of wild-type (H) and *Foxp2* mutant lungs (I) does not reveal a significant change in alveolar epithelial morphology at P8. Scale bars: 500 μm in A-F.

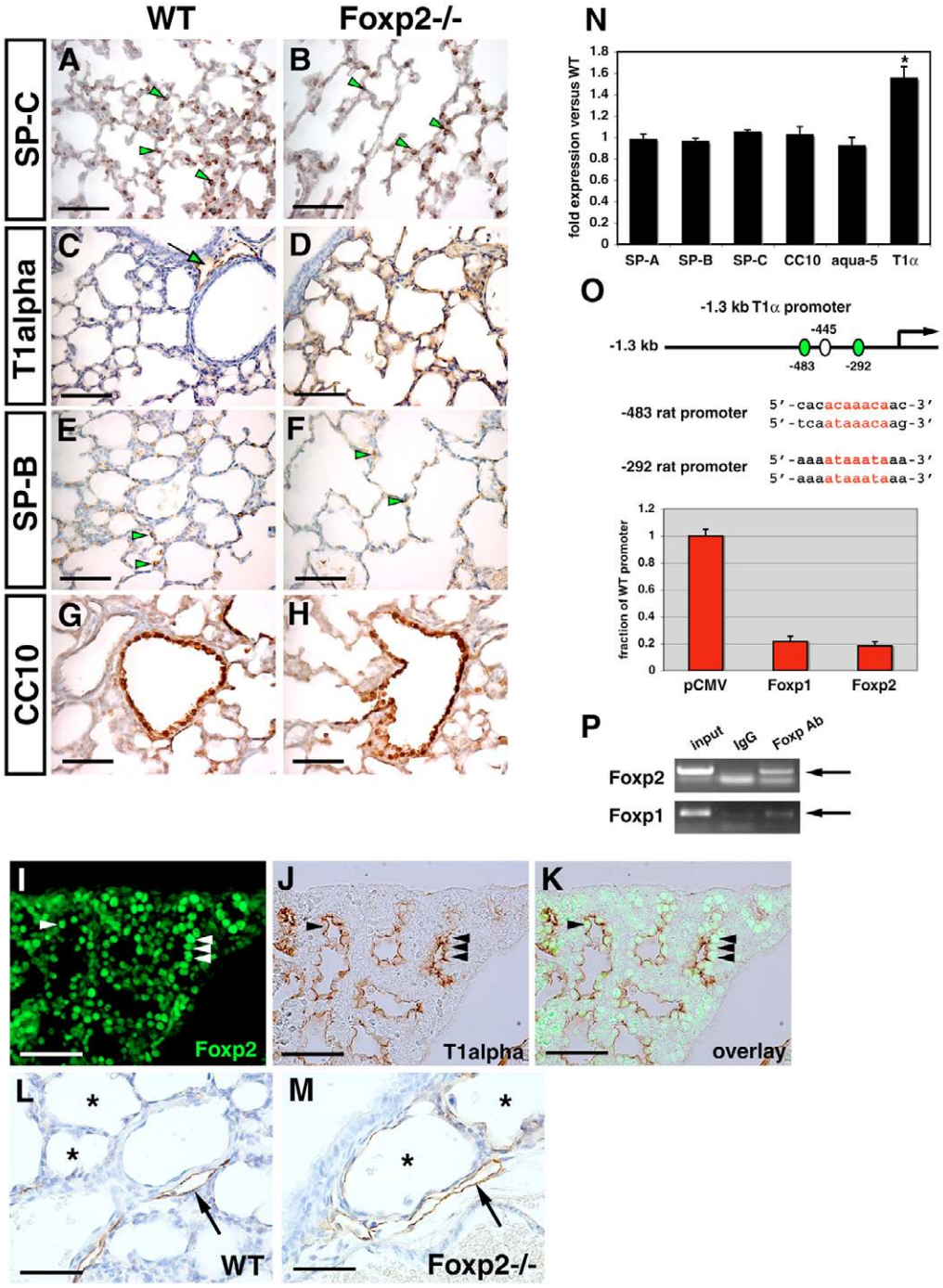

Fig. 3. T1alpha is a direct in vivo target of Foxp2 and Foxp1 in the lung. (**A-H**) Immunohistochemical detection of the expression of SP-C (A,B), T1alpha (C,D), SP-B (E,F) and CC10 (G,H) in wild-type (A,C,E,G) and *Foxp2* mutant (B,D,F,H) mouse lungs at E18.5, showing elevated T1alpha expression in the *Foxp2* mutant. Arrows, AEC-2 cells; arrowheads, lymphatic vessel. (**I-K**) Co-staining for expression of Foxp2 (I) and T1alpha (J) reveals that both proteins are co-expressed in the same cells within the distal lung at E18.5 (K). Note that the dim fluorescence displayed by cells located between the airways in I is non-specific background. (**L**,**M**) Expression of T1alpha in lymphatic endothelium at E18.5 (arrows) does not differ between wild-type and *Foxp2*$^{-/-}$ lungs in contrast to the upregulation of T1alpha expression in alveoli (asterisk) of the mutant. (**N**) Q-PCR to assess expression of the genes encoding the indicated lung cell markers at E18.5 confirming specific upregulation of T1alpha (T1α) expression in *Foxp2*$^{-/-}$ lungs. Values are relative to wild-type expression levels, which are set at 1.0 for each gene. aqua-5, aquaporin 5. Q-PCR results are the average of three lung samples performed in triplicate±s.e.m.; *, P<0.001. (**O**) Luciferase reporter assays were performed in NIH-3T3 cells using the rat 1.3 kb T1alpha (T1α) promoter. Fox DNA-binding sites located in the T1alpha promoter are indicated by either green ovals (conserved between mouse and rat) or white ovals (present in the rat promoter but not conserved across species). Comparison between the rat sequence (upper sequence) and the mouse sequence (lower sequence) in the two conserved sites in the T1alpha promoter. Bar chart showing that co-expression of either Foxp1 or Foxp2 leads to a fivefold repression of the T1alpha promoter relative to pCMV. (**P**) ChIP assays performed using mouse lung chromatin and the previously characterized Foxp1 and Foxp2 polyclonal antibodies show that Foxp1 and Foxp2 are both found associated with the region containing the conserved Fox DNA-binding sites in the mouse T1alpha promoter in vivo. Scale bars: 50 μm in A-M.

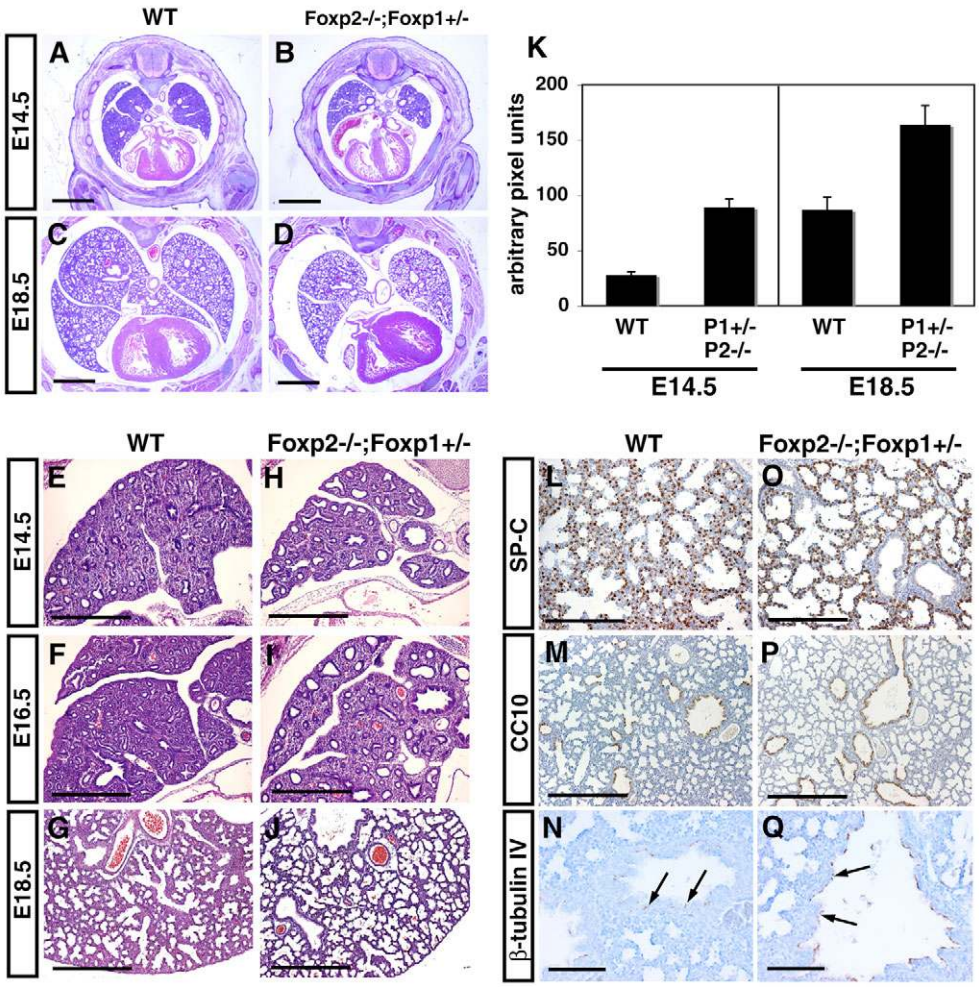

Fig. 4. *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutants exhibit dramatic defects in lung airway morphogenesis. *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutants were generated by crossing *Foxp1*$^{+/-}$;*Foxp2*$^{+/-}$ double heterozygous mice to *Foxp1*$^{+/-}$ mice. (**A-D**) Overall lung size was reduced at both E14.5 and E18.5 in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants. (**E-J**) At E14.5, E16.5 and E18.5, significant defects were observed in airway development including decreased branching morphogenesis as demonstrated by the dilated nature of the developing airways. (**K**) Distal airspace area, as measured using ImageJ software, was significantly increased in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs at both E14.5 and E18.5. (**L-Q**) Despite these defects, proximal-distal epithelial patterning was maintained in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutants as determined by normal expression patterns of SP-C (L,O), CC10 (M,P) and β-tubulin IV (N,Q) proteins. Scale bars: 500 μm in A-J,L,M,O,P; 100 μm in N,Q.

are located in the 1.3 kb rat T1alpha promoter, two of which are conserved in the mouse gene (Fig. 3O) (Ramirez et al., 1997). This promoter has been used to direct lung epithelial gene expression in transgenic mice in previous studies (Ramirez et al., 1999). To determine whether Foxp2 could repress the T1alpha promoter, a luciferase reporter plasmid with the 1.3 kb rat T1alpha promoter was co-transfected into NIH-3T3 cells along with expression plasmids for Foxp2 and Foxp1. Expression of either Foxp2 or Foxp1 repressed the T1alpha promoter approximately fivefold (Fig. 3O).

To determine whether Foxp2 and Foxp1 directly interact with the T1alpha promoter in vivo, ChIP assays were performed on chromatin isolated from lungs of E18.5 embryos. Using specific antibodies, ChIP assays revealed that Foxp2 and Foxp1 associated with the conserved Fox DNA-binding sites in the T1alpha promoter in vivo (Fig. 3P). Together, these data indicate that T1alpha is a direct target of Foxp1 and Foxp2 transcriptional repression in vivo and suggest that the alveolarization defects observed in *Foxp2*-null mice might at least in part be due to increased expression of T1alpha.

Loss of a single *Foxp1* allele in a *Foxp2*-null background leads to dramatic embryonic lung defects

The above studies suggested that Foxp1 acts cooperatively with Foxp2 to regulate lung epithelial gene expression. First, Foxp1 and Foxp2 are co-expressed at high levels in distal lung epithelium during development. Second, both Foxp1 and Foxp2 are found associated with conserved Fox DNA-binding sites on the T1alpha promoter in vivo. Third, the T1alpha promoter was repressed by both Foxp1 and Foxp2. Finally, there is no compensatory upregulation of Foxp1 or Foxp4 in *Foxp2*-null lungs (see Fig. S1 in the supplementary material). We generated *Foxp1*$^{-/-}$;*Foxp2*$^{-/-}$ mutants, but they die prior to E11.5 and are severely runted, not allowing an accurate assessment of lung development (data not shown). Thus, we generated *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants to determine whether loss of a single *Foxp1* allele in addition to complete loss of Foxp2 expression would lead to increased severity in lung defects.

In contrast to *Foxp2*-null mice, *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mice did not survive beyond the neonatal stage (Table 2). However, at least some *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ animals did survive gestation, although the exact percentage is difficult to determine owing to the fact that *Foxp1* and *Foxp2* are linked on mouse chromosome 6 (Table 2). This suggested that loss of a single *Foxp1* allele in a *Foxp2*-null background increased the severity of defects in specific tissues where they are co-expressed during development, such as the lung. Histological analysis revealed severe lung airway defects as early as E14.5 (Fig. 4A-J). These included dilated airways and reduced lung size, indicating defects in branching morphogenesis and cellular proliferation in the lung. Lung-to-body weight ratios were significantly reduced for *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants at both E17.5 and P0 (Table 3). Measurement of distal airspace luminal area reveal

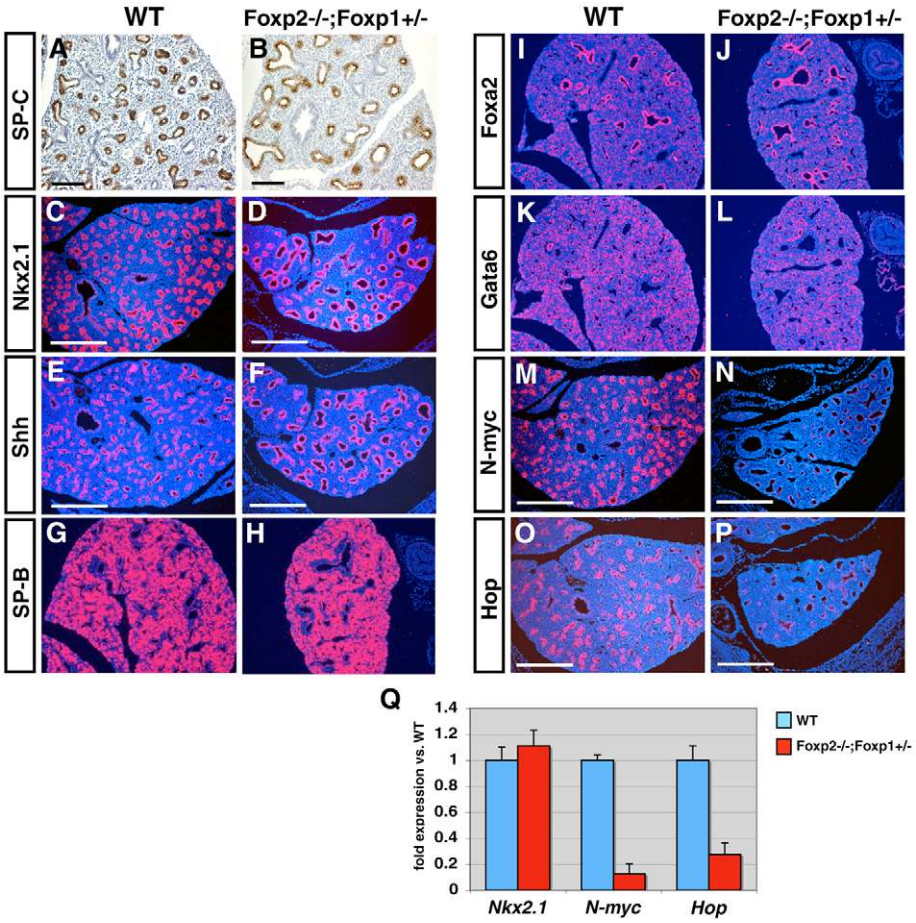

Fig. 5. Decreased expression of *N-myc* and *Hop* in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutant mice. In situ hybridization was performed on wild-type (A,C,E,G,I,K,M,O) and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutants (B,D,F,H,J,L,N,P) at E16.5 to determine the lung epithelial expression of genes encoding SP-C (**A**,**B**), Nkx2.1 (**C**,**D**), Shh (**E**,**F**), SP-B (**G**,**H**), Foxa2 (**I**,**J**), Gata6 (**K**,**L**), N-myc (**M**,**N**), and Hop (**O**,**P**). (**Q**) Expression of *N-myc* and *Hop* was significantly decreased in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutants, whereas expression of *Nkx2.1* was not significantly affected as measured by Q-PCR. Scale bars: 100 μm in A,B; 500 μm in C-P.

an almost threefold increase at E14.5 and an almost twofold increase at E18.5, further indicating defects in lung airway morphogenesis (Fig. 4K).

To determine whether proximal-distal patterning in the lung was disrupted, expression of SP-C, CC10 and β-tubulin IV was determined at E18.5 in wild-type and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs. The expression patterns for all three marker genes were unchanged in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs (Fig. 4L-Q). These data indicate that Foxp1 works cooperatively with Foxp2 to regulate lung airway morphogenesis, but that proximal-distal patterning of the lung airways is not dramatically affected in these mutants.

Given the defects observed in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs, we performed in situ hybridization and immunohistochemistry for expression of genes that function in airway epithelial development and alveolarization to determine the extent of airway epithelial differentiation in these embryos. As observed at E18.5, expression of the gene encoding SP-C was not disrupted at E16.5, although airway dilation was evident (Fig. 5A,B). Expression of the genes encoding Nkx2.1, sonic hedgehog (Shh), SP-B, Foxa2 and Gata6 were unchanged in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs (Fig. 5C-L). However, expression of the genes encoding N-myc (Mycn – Mouse Genome Informatics) and Hop (Hod – Mouse Genome Informatics) were significantly reduced as assessed by in situ hybridization in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs (Fig. 5M-P). This reduction was confirmed by Q-PCR in wild-type and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs at E16.5 (Fig. 5Q). N-myc is a crucial regulator of distal lung development and its loss leads to dramatic defects in airway morphogenesis, including defects in airway epithelial proliferation (Okubo et al., 2005). Hop is a homeodomain protein expressed in the developing airway epithelium in a unique temporal expression pattern and loss of Hop leads to lung alveolarization defects and partial perinatal lethality (Yin et al., 2006). Thus, the reduction in N-myc and Hop expression, but unchanged expression of other lung epithelial markers, suggests that Foxp2 and Foxp1 cooperatively regulate a specific transcriptional program required post-specification to regulate lung epithelial differentiation and airway morphogenesis. The significant loss in N-myc expression could be responsible for the observed defects in airway morphogenesis as a complete loss in N-myc results in dramatic loss in branching morphogenesis (Okubo et al., 2005).

Given the relatively small size of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs and the loss in N-myc expression, cell proliferation and apoptosis was assessed in wild-type and mutant lungs. Immunohistochemistry

Table 2. Genotype and viability of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants (*Foxp1*$^{+/-}$;*Foxp2*$^{+/-}$ × *Foxp2*$^{+/-}$)

Age	Total offspring	Number of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$
E12.5-E18.5	122	10
P0-P14 (live)	114	0

Table 3. Lung-to-body weight ratios of wild type versus *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants

Age	Wild type	*Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$
E17.5	0.0436±0.0021	0.0221±0.0045
P0	0.02647±0.0010	0.0195±0.0018

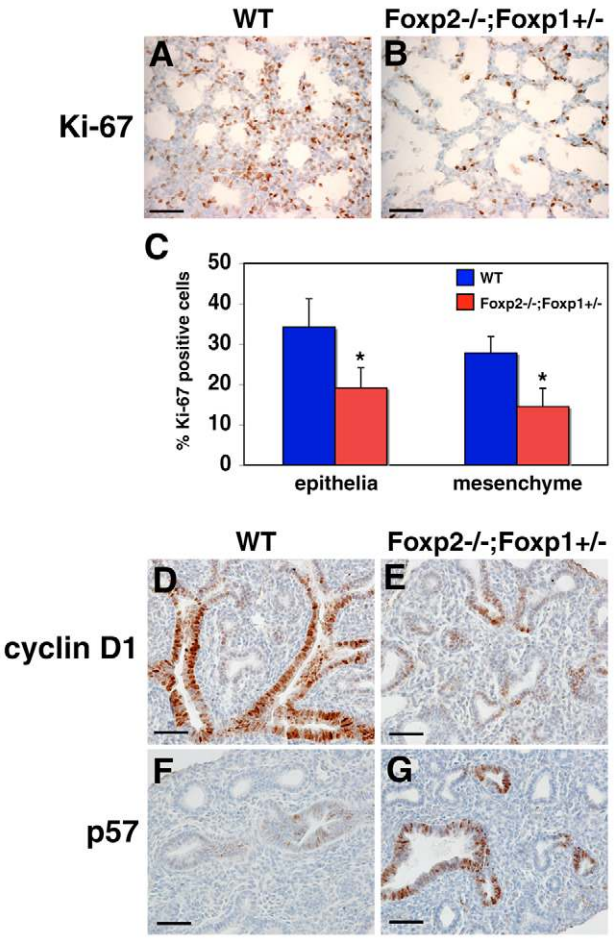

Fig. 6. Decreased epithelial and mesenchymal cell proliferation in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutant lungs. Immunohistochemistry was performed using a Ki-67 antibody to detect proliferating cells in the lungs of wild-type (**A**) or *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutant (**B**) mice. Quantitation (**C**) shows that there is a more than 40% reduction in cell proliferation in both the epithelia and mesenchyme of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ compound mutant lungs. Expression of cyclin D1 (**D**,**E**) is decreased in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs, whereas expression of the CDKI p57 (**F**,**G**) is increased. Scale bars: 50 μm.

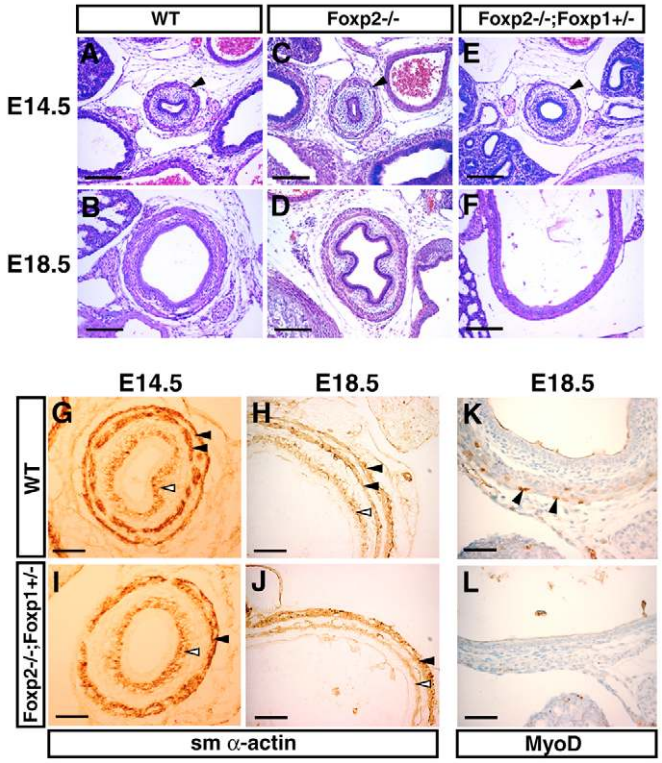

Fig. 7. *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants have defects in esophageal muscle development. (**A-F**) Esophageal development was examined by Hematoxylin and Eosin staining in wild-type (A,B), *Foxp2*$^{-/-}$ mutant (C,D) and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutant (E,F) mice at E18.5. As early as E14.5, the smooth muscle surrounding the esophagus was thinner in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants than in either *Foxp2*$^{-/-}$ or wild-type littermates (A,C,E). By E18.5, *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants had severely dilated esophagi with a very thin muscular layer (B,D,F). (**G-J**) Smooth muscle actin (sm α-actin) staining revealed a single muscular layer surrounding *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ esophagi as compared with wild-type animals (black arrowheads). This single muscular layer also appeared thicker in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants (I,J). The sm-actin-positive submucosal layer was unchanged in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants (white arrowheads). (**K**,**L**) MyoD immunohistochemistry demonstrated the presence of skeletal muscle in the esophagi of wild-type embryos (K, arrowheads) and revealed a lack of skeletal muscle contribution to *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ esophagi (L). Scale bars: 100 μm in A-F; 50 μm in G-L.

using the cell proliferative marker Ki-67 (Mki67 – Mouse Genome Informatics) revealed a significant reduction in cell proliferation in the epithelia and mesenchyme of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs (Fig. 6A-C). However, TUNEL staining did not reveal any significant changes in apoptosis in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ lungs (data not shown). These data suggest that the reduced cell proliferation may account for the reduced lung size in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants. To determine the underlying mechanism by which Foxp1 and Foxp2 regulate cell proliferation in the lung, expression of cyclin D1 and the cyclin-dependent kinase inhibitors (CDKI) p21, p27 and p57 (Cdkn1a, Cdkn1b and Cdkn1c, respectively – Mouse Genome Informatics) were assessed. Remarkably, we observed reduced levels of cyclin D1 and increased levels of p57 in the airways of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants (Fig. 6D-G). Changes in expression of p21 and p27 were not observed (data not shown). These data, along with previous studies showing that loss of Foxp1 in the heart leads to increased p21 and decreased p27 (Wang et al., 2004), indicate that Foxp1 and Foxp2 regulate cell cycle regulators in a cell type-specific manner. Together with the dramatic loss in N-myc and Hop expression, these data suggest that Foxp1 and Foxp2 cooperatively regulate cell proliferation programs in lung epithelia required for proper growth of the airways.

Foxp2 and Foxp1 regulate esophageal muscle development

As shown in Fig. 1, Foxp2 and Foxp1 are both expressed in esophageal muscle. Careful analysis of esophageal development in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ embryos revealed significant defects in the muscle surrounding the esophagus. Esophageal muscle is composed of both smooth and skeletal muscle in the mid-thoracic region and above. In *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants, esophageal muscle appeared underdeveloped at E14.5, and by E18.5 the entire esophagus was highly dilated with a very thin muscular layer (Fig. 7A-F). Anatomically, esophageal muscle is composed of two layers: an

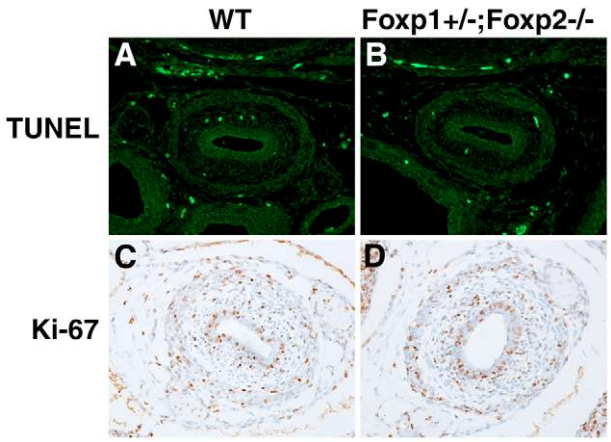

Fig. 8. Apoptosis and cell proliferation in the esophagus of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutant mice. TUNEL staining (**A**,**B**) and Ki-67 immunostaining (**C**,**D**) were performed on wild-type (A,C) and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ (B,D) esophagi at E14.5. No difference in either apoptosis or cell proliferation was detectable.

outer longitudinal and inner circumferential layer. Immunostaining with an antibody for smooth muscle α-actin (sm-actin) was used to delineate the muscular layers within the developing esophagus. In wild-type mice, sm-actin expression was observed in three layers of the developing esophagus: an inner submucosal layer directly adjacent to the epithelium and two outer layers representing the circumferential and longitudinal layers (Fig. 7G,H). In contrast to wild-type embryos, *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants contained only a single outer layer of muscle in addition to the submucosal layer adjacent to the esophageal endoderm (Fig. 7I,J).

Although there is contribution of skeletal muscle to the esophagus, its origins and function are somewhat controversial. Whether skeletal muscle in the developing esophagus arises from distinct skeletal muscle precursors or from a smooth muscle to skeletal muscle trans-differentiation event is unclear (Kablar et al., 2000; Rishniw et al., 2003). To determine whether there was a normal contribution of skeletal muscle to *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ esophagus, tissues were stained for the presence of skeletal muscle development using an antibody to the skeletal muscle-specific transcription factor MyoD (MyoD1 – Mouse Genome Informatics). As expected, wild-type esophagus at E18.5 contained numerous MyoD-positive cells in the outer muscular layer (Fig. 7K). By contrast, *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants had no detectable MyoD-positive cells in the muscle surrounding the esophagus (Fig. 7L).

The loss of skeletal and smooth muscle development in the esophagus of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants could be due to a loss of proliferation of these cells, their apoptosis during development, or to defects in differentiation of these cell types. Therefore, we assessed cell proliferation and apoptosis in wild-type and *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutant esophagi. No changes in either proliferation as assessed by Ki-67 immunostaining, or in apoptosis as assessed by TUNEL staining, were observed (Fig. 8). These data suggest a loss of esophageal muscle differentiation in *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants. However, given that a complete loss of skeletal muscle in the esophagus did not result in a phenotype as dramatic as that exhibited by *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants (Kablar et al., 2000), the loss of skeletal muscle in these mutants does not completely account for the severe reduction in muscle mass or dilated appearance of the esophagus. Together, these results indicate that both smooth and skeletal muscle differentiation is disrupted in the esophagi of *Foxp2*$^{-/-}$;*Foxp1*$^{+/-}$ mutants.

DISCUSSION

The Foxp1/2/4 family of forkhead transcription factors regulate diverse developmental processes including cardiomyocyte growth, cardiac outflow tract development, cerebellar development and B lymphocyte development (Hu et al., 2006; Li et al., 2004b; Shu et al., 2005a; Wang et al., 2004). In this report, we show that Foxp2 and Foxp1 work cooperatively to regulate lung gene expression, airway morphogenesis and esophageal muscle development. Given that both factors are highly expressed in the developing airway epithelia and esophageal muscle, these data demonstrate that Foxp1 and Foxp2 act in an allelic dose-dependent manner to regulate gene expression in these cell types.

Since Foxp1/2 are known transcriptional repressors (Li et al., 2004a), the increased expression of T1alpha in Foxp mutants is provocative and is likely to reflect these factors acting directly on the T1alpha promoter to restrict its expression in alveolar epithelial cells. Our data showing in vivo occupancy of the T1alpha promoter by Foxp1 and Foxp2 support this concept. T1alpha expression is restricted to AEC-1 cells in late gestation, but it is expressed throughout distal airway epithelium during early lung development (Ramirez et al., 1999; Ramirez et al., 1997). T1alpha is also expressed in lymphatic endothelium in the lung and elsewhere (Schacht et al., 2003). Interestingly, we did not observe an increase in T1alpha expression in the lymphatic endothelium in the lung or elsewhere in the developing embryo. This may be explained by the fact that Foxp2 is not expressed in lymphatic endothelium (data not shown).

The function of T1alpha is largely unknown, but T1alpha-null mice exhibit both airway epithelial defects and lymphatic endothelial defects (Ramirez et al., 2003; Schacht et al., 2003). Based on the protein structure, T1alpha is a mucin-type glycoprotein with extensive *O*-glycosylation (Kato et al., 2003). T1alpha-null mice have defects in lymphatic vascular patterning leading to large dilated lymphatics (Schacht et al., 2003). T1alpha also appears to regulate lymphatic endothelial cell migration and adhesion. Overexpression of T1alpha in microvascular endothelial cells leads to increased cell migration and adhesion (Schacht et al., 2003). AEC-1 cells cover the vast majority of alveolar airspace in the late gestational and postnatal lung. Undoubtedly, cell migration and adhesion play a key role in the ability of AEC-1 cells to form the thin gas-permeable interface by spreading their cytoplasmic processes to cover such a vast surface area. Given its potential affects in regulating both lung epithelial and lymphatic endothelial differentiation, increased expression of T1alpha could lead to defects in late-stage AEC-1 cell differentiation, which in turn leads to defective lung alveolarization, a developmental process whereby AEC-1 cells help to remodel the distal airspaces to produce the well established alveolus required for efficient gas exchange in the lung. In support of this concept, gain-of-function experiments in which T1alpha is overexpressed in distal lung epithelium using the human *SP-C* (*SFTPC* – Human Gene Nomenclature Database) promoter, showed that increased T1alpha expression leads to increased postnatal mortality after hyperoxic injury (Girod et al., 1999). Although the data presented in this study are preliminary and do not suggest a distinct mechanism to explain these results, they do suggest that increased levels of T1alpha might predispose the lung to additional defects or injury leading to alveolar dysfunction. Gain-of-function experiments of crucial signaling and transcriptional

regulators in the lung, including Shh, Bmp4 and Gata6, revealed severe defects in late airway epithelial development, including alveolarization defects (Bellusci et al., 1997; Liu et al., 2003; Weaver et al., 1999). These studies support the concept that transcriptional repression to control temporal and spatial gene expression is an important mechanism for regulating lung epithelial morphogenesis and differentiation. Thus, increased expression of T1alpha caused by the loss of Foxp2-mediated repression could contribute to the lung defects in *Foxp2*-null animals, but is unlikely to be the sole cause. Given the dearth of information regarding the direct targets of Foxp1/2 and the function of T1alpha in vivo, these data add critical insight into how these factors regulate lung epithelial differentiation.

Previous studies have implicated *Foxp1* as a tumor suppressor gene, with reduced expression observed in cancers from several tissues including the lung. *Foxp1* maps to chromosome 3p14.1, a region commonly associated with loss of heterozygosity in several forms of cancer (Banham et al., 2001). In the lung, Foxp1 expression is reduced in lung tumors induced with the carcinogenic reagent N-nitrosobis(2-hydroxypropyl)amine (Shimizu et al., 2006). Moreover, loss of Foxp1 leads to an aberrant increase in cardiomyocyte proliferation and defective differentiation (Wang et al., 2004). Less is known about the potential oncogenic role of Foxp2 and Foxp4. In contrast to the lung and heart, esophageal smooth muscle proliferation does not appear to be affected in $Foxp2^{-/-};Foxp1^{+/-}$ mutants. This could be explained by an inability to detect a short temporal window of decreased proliferation with the techniques used, or to intrinsic differences in the roles of Foxp1 and Foxp2 in esophageal smooth muscle. The decreased proliferation in both the epithelial and mesenchymal compartments of $Foxp2^{-/-};Foxp1^{+/-}$ lungs is somewhat counterintuitive to the result observed in lung tumors and cardiomyocytes. However, the significant decrease in N-myc, which plays a crucial role in lung epithelial cell proliferation, may override any increase in proliferation from loss of Foxp1 and Foxp2 expression. In the adult lung after injury, N-myc expression could be reactivated to help in re-epithelialization of the airways, and in this instance Foxp1 (or Foxp2) may be required for its proper expression. Loss or gain of Foxp1/2 expression could lead to an aberrant injury response in the lung, leading to epithelial hyperplasia and eventually tumorigenesis. Future studies to specifically delete *Foxp1* in the postnatal lung will be required to determine whether this gene acts as a tumor suppressor in the lung.

Most Fox factors exist in small subfamilies of highly related factors that have overlapping patterns of expression. Foxp1/2/4 are all highly expressed in lung airway epithelium in addition to other tissues such as the developing endocardial cushions in the heart (Lu et al., 2002; Wang et al., 2004). However, there are important differences in the expression patterns of Foxp1/2/4 in the lung and these differences may indicate specific roles for each of these family members. Foxp1 is expressed in a polarized fashion, with the highest level of expression in developing distal airway epithelium and lower levels in more proximal airways. By contrast, Foxp2 expression is restricted to the distal airways, with little expression detected in proximal airway epithelium by immunohistochemistry or in situ hybridization. Foxp4 is expressed evenly throughout the airway epithelium with no noticeable polarization along the proximal-distal axis. Foxp1 and Foxp4 cannot compensate for the loss of Foxp2 in the lung, as demonstrated by the distinct alveolarization defects in *Foxp2* mutant lungs and by the lack of compensatory upregulation of Foxp1 or Foxp4. The extensive overlap in expression of Foxp1/2/4 in tissues such as the lung, as well as their ability to heterodimerize, suggest that a significant degree of redundancy might exist and all three factors may regulate the same set of target genes in a dose-dependent manner. Moreover, because AEC-1 cells are thought to derive from AEC-2 cells, expression of Foxp2 in either cell type could contribute to the alveolarization phenotype in $Foxp2^{-/-}$ animals.

The increased severity in lung-related defects in $Foxp2^{-/-};Foxp1^{+/-}$ mutants supports the hypothesis that Foxp1 and Foxp2 act in a cooperative and dose-dependent manner to regulate tissue-specific gene expression and development where these factors are co-expressed. The complete loss of both Foxp1 and Foxp2 in the lung will have to await the generation of a conditional *Foxp1* allele, as $Foxp1^{-/-};Foxp2^{-/-}$ animals die prior to E11.5 (data not shown). Similar studies determining the role of Foxp1 and Foxp4 will also require conditional alleles owing to the early embryonic demise of compound mutants. These studies are the focus of future work and should reveal important dose-dependent functions in the developing lung as well as in other tissues, such as the cardiovascular system, where Foxp1/2/4 are co-expressed.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/10/1991/DC1

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