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Foxp transcription factors suppress a non-pulmonary gene expression program to permit proper lung development

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Abstract

The inhibitory mechanisms that prevent gene expression programs from one tissue to be expressed in another are poorly understood. Foxp1/2/4 are forkhead transcription factors that repress gene expression and are individually important for endoderm development. We show that combined loss of all three Foxp1/2/4 family members in the developing anterior foregut endoderm leads to a loss of lung endoderm lineage commitment and subsequent development. Foxp1/2/4 deficient lungs express high levels of transcriptional regulators not normally expressed in the developing lung, including Pax2, Pax8, Pax9 and the Hoxa9-13 cluster. Ectopic expression of these transcriptional regulators is accompanied by decreased expression of lung restricted transcription factors including Nkx2-1, Sox2, and Sox9. Foxp1 binds to conserved forkhead DNA binding sites within the Hoxa9-13 cluster, indicating a direct repression mechanism. Thus, Foxp1/2/4 are essential for promoting lung endoderm development by repressing expression of non-pulmonary transcription factors.

INTRODUCTION

The foregut endoderm is a multipotent tissue that generates multiple organs in the adult mammal including the lung, thyroid, liver, stomach and pancreas. The molecular programs that promote development of each of these organs have been studied extensively, and

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multiple transcriptional regulators essential for organ specific endoderm commitment and differentiation have been identified. Despite this progress, important questions remain including what transcriptional repressive mechanisms are critical for promoting and maintaining organ specific endoderm identity and differentiation.

The forkhead box transcription factor (Fox) family is a large group of transcriptional regulators that are defined in part through their common forkhead DNA binding domain. Fox family members can be further divided into subfamilies based on protein similarities outside the forkhead DNA binding domain. The Foxp subfamily consists of four members, Foxp1/2/3/4, which are highly related through multiple conserved domains required for transcriptional repression (Li et al., 2004). Foxp1/2/4 are expressed in multiple endoderm cell lineages including the lung, pancreas, and thyroid (Lu et al., 2002; Spaeth et al., 2015), while Foxp3 is restricted to the T cell lineage within the hematopoietic system (Hori et al., 2003). Previous work has demonstrated a cooperative role of Foxp1/2 and Foxp1/4 in regulating lung endoderm differentiation (Li et al., 2012; Shu et al., 2007), and all three family members are expressed in an overlapping pattern in the lung endoderm during development (Lu et al., 2002). Recently, the combined role for Foxp1/2/4 in pancreatic alpha cell proliferation was demonstrated (Spaeth et al., 2015). In all of these studies, deletion of more than one member of the Foxp family was required to elicit a notable phenotype during development, suggesting significant redundancy and cooperativity.

To assess the total contribution of Foxp1/2/4 mediated repression to lung endoderm development, we inactivated all three genes using the Shh[cre] line (Harfe et al., 2004). In contrast to the relatively normal structure of the lung in previous studies where Foxp1/2 or Foxp1/4 were inactivated, complete loss of Foxp1/2/4 in early lung endoderm resulted in a dramatic inhibition of normal lung branching and development. This is accompanied by a significant decrease in early lung endoderm progenitor gene expression programs. In turn, ectopic expression of non-pulmonary transcriptional regulators including Pax2, Pax8, Pax9, and Hoxa9-13 indicate that Foxp1/2/4 are required to silence transcriptional programs active in other cell lineages including those found in the kidney and thyroid. Our studies highlight the importance of transcriptional repression in promoting proper organ identity and reveal that Foxp1/2/4 are critical to this repressive mechanism during development of anterior foregut endoderm derivatives.

RESULTS

Foxp1/2/4 are cooperatively required for lung endoderm development

Previous work has suggested that members of the Foxp1/2/4 transcription factor family function in a cooperative manner to promote lung endoderm development (Li et al., 2012; Shu et al., 2007). However, the overall role that this transcription factor family plays during early lung endoderm development is unknown. To inactivate Foxp1/2/4 during early lung endoderm development, we generated Shh^{cre}:$Foxp1^{flox/flox}$:$Foxp2^{flox/flox}$:$Foxp4^{flox/flox}$ mutants, which we will hereafter refer to as Foxp[TKO] mutants. The Shh^{cre} line drives high level of cre recombinase in the early ventral anterior foregut endoderm (Goss et al., 2009; Harris et al., 2006). Foxp[TKO] mutants died within minutes after birth due to respiratory failure. Examination of E18.5 lungs from Foxp[TKO] mutants showed that they consisted of

large unbranched sacs (Fig. 1A–C). Histological analysis showed that while the endodermal lining of these malformed sacs expressed the lung restricted transcription factor Nkx2-1 (Fig. 1D and E), little to no expression of differentiated lung epithelial cell marker genes was observed (Fig. 1F–I). Quantitative PCR (Q-PCR) analysis confirmed these changes in gene expression (Fig. 1J). This phenotype is much more severe than loss of either Foxp1/2 or Foxp1/4 (Li et al., 2012; Shu et al., 2007). These data show that complete loss of Foxp1/2/4 leads to a dramatic arrest in lung endoderm development.

Loss of Foxp1/2/4 leads to decreased expression of early lung endoderm transcription regulators

Given the phenotype observed at E18.5 in Foxp^{TKO} mutants, we examined lung development at an earlier stage in these mutants to assess whether the defects were the result of loss of lung endoderm progenitor development. Histological sections from E14.5 Foxp^{TKO} mutants reveal a severe block in branching morphogenesis (Fig. 2A and B). While expression of the early lung endoderm commitment transcription factor Nkx2-1 was noted in Foxp^{TKO} mutants at E18.5, at E14.5 expression of Nkx2-1 was patchy and quantitatively reduced by more than five-fold (Fig. 2C–F, O). Immunostaining and Q-PCR also revealed a dramatic reduction in expression of the early proximal lung endoderm transcription factor Sox2 and the distal lung endoderm transcription factor Sox9 (Fig. 2G–N, O). In addition to these transcriptional regulators, early markers of lung epithelial differentiation including Sftpc, an early marker of distal alveolar epithelium, and Scgb3a2, an early marker of proximal secretory epithelium, were not expressed at detectable levels (Fig. 2O). Proliferation and apoptosis was not significantly altered in E14.5 Foxp^{TKO} mutants (Fig. 2, Supplement 1). Interestingly, tracheoesophageal fistulas were found in a subset of Foxp^{TKO} mutants at E11.5–E12.5 suggesting that the severe disruption in lung endoderm development caused an overall defect in anterior foregut development (Fig. 2, Supplement 2). These data indicate that loss of Foxp1/2/4 leads to a severe inhibition of lung endodermal commitment and differentiation.

Loss of Foxp1/2/4 leads to a loss of airway smooth muscle differentiation

Given the severe block in endodermal commitment and differentiation in Foxp^{TKO} mutants, we next sought to determine whether there were commensurate defects in lung mesenchymal development due to defective epithelial-mesenchymal interactions. Immunostaining for expression of the smooth muscle specific protein SM22α at E14.5, reveals a complete absence of airway smooth muscle development in Foxp^{TKO} mutants (Fig. 3A–D). This absence of airway smooth muscle development persists through E18.5 (Fig. 3E and F). However, smooth muscle is still observed surrounding the vasculature in the developing lung at both stages of development (Fig. 3C–F). Immunostaining for CD31 expression to detect vascular endothelial development shows that there is evidence for initial endothelial plexus formation in Foxp^{TKO} mutants at E14.5 and E18.5 (Fig. 3G–L). Thus, while vascular endothelial and vascular smooth muscle cell commitment is relatively unperturbed, airway smooth muscle development is blocked in Foxp^{TKO} mutants. This is consistent with the extra-pulmonary origins of this component of the pulmonary vasculature (Peng et al., 2013).

Ectopic activation of alternative cell lineage commitment in Foxp^TKO mutants

To better understand the underlying cause of the severe defects in lung endoderm development in Foxp^TKO mutants, we assessed transcriptome changes at E14.5 due to loss of Foxp1/2/4 during lung development. As predicted by the preceding data, there was a severe loss of lung endoderm specific gene expression signatures including decreased expression of multiple markers of lung epithelium including Sftpc, Cyp2f2, and Cbr2 (Supplemental Table 1). Importantly, we observed decreased expression of critical transcriptional regulators of lung endodermal development including Nkx2-1, Foxa2, Sox2, and Sox9 (Fig. 4 and Supplement 1). Using gene set enrichment analysis (GSEA), the transcriptome data show that lung endoderm commitment and development is severely impeded in Foxp^TKO mutants while gene signatures representing kidney and thyroid development are significantly up-regulated (Fig. 4, Supplements 1 and 2). Importantly, key transcriptional regulators for non-lung cell lineages including members of the Hox and Pax gene families were dramatically up-regulated (Fig. 4 and Supplement 1).

To confirm these ectopic gene expression signatures and where in the lung they are located, we performed in situ hybridization (ISH) for a subset of these genes. ISH for Hoxa9-13 revealed ectopic expression of all of these genes in Foxp^TKO mutants at E14.5 (Fig. 5A–K). Q-PCR confirmed that these genes were significantly up-regulated in Foxp^TKO mutants (Fig. 5C,F,I,L). ISH and Q-PCR for Pax2, Pax3, Pax9, and Nkx3-1 also revealed up-regulated expression in Foxp^TKO mutants (Fig. 6A–L). The ectopic expression of Pax, Hox and Nkx3-1 genes was restricted to the mutant lung endoderm suggesting a cell autonomous affect from loss of Foxp1/2/4 expression (Figs. 5A–K and 6A–K). Moreover, ectopic expression of these genes was not uniform in the Foxp^TKO mutant endoderm suggesting stochastic alterations in endoderm cell fate commitment and differentiation.

Foxp1/2/4 repress Hoxa9-13 through two conserved and distinct DNA binding sites

To assess whether Foxp1/2/4 directly bind to genomic regions of some of these putative target genes, we identified conserved Fox DNA binding sites within Hoxa9-13 cluster genomic locus. We identified four sites that are conserved in mammals (Fig. 7A and B). Assessment of these four conserved Fox DNA binding sites showed that sites B and D efficiently bound Foxp1 at high levels using ChIP-QPCR assays (Fig. 7C). These data suggest that Foxp1/2/4 can directly repress expression of the Hoxa9-13 through distinct conserved Fox DNA binding sites located in this region of the genome.

DISCUSSION

The regulatory mechanisms by which different tissue specific endodermal cell lineages are specified and directed into lineage specific programs remain poorly understood. We show that the forkhead transcription factors Foxp1/2/4 are co-required for proper lung endodermal lineage commitment and differentiation. Foxp1/2/4 act in part through their combined repression of important transcriptional regulators not normally expressed in the lung endoderm including Hoxa9-13 and Pax2/8/9. Our data point towards a broad role for Foxp1/2/4 in restricting ectopic activation of cell lineage specific gene expression programs to allow for proper tissue development.

Previous work has demonstrated that Foxp1/2 are co-required for development of alveolar type 1 epithelial cells during late lung maturation (Shu et al., 2007). In contrast, Foxp1/4 are co-required for both development of airway secretory cells as well as their regeneration after napathalene based injury (Li et al., 2012). In the case of Foxp1/4, loss of these two transcription factors results in the ectopic activation of the goblet cell gene expression program (Li et al., 2012). This abnormal differentiation into goblet cells in the airways of Foxp1/4 deficient mice is mediated in part through the de-repression of anterior gradient 2. Interestingly, Foxp family members are potent transcriptional repressors that inhibit gene expression through their interaction with repressive chromatin remodeling complexes such as NuRD (Chokas et al., 2010). Thus, the ability of Foxp transcription factors to restrict cell lineage differentiation programs likely resides in their inherent ability to repress lineage specific gene expression.

The significant similarity in the protein sequence and expression pattern of Foxp1/2/4 in certain tissues including the lung indicates a large degree of redundancy or cooperativity in regulation of gene expression. Indeed, recent evidence has shown that loss of Foxp1/2/4 in pancreatic alpha cells resulted in reduced proliferation and mass of these cells in adult mice leading to hypoglycemia (Spaeth et al., 2015). The loss of all three of these factors was required to induce this phenotype in the pancreas suggesting that only a low level of Foxp activity was required for alpha cell proliferation. This is in contrast to the lung where complete loss of two genes, Foxp1 and Foxp4, results in a significant airway epithelial developmental and regenerative phenotype (Li et al., 2012).

Little is known about the molecular mechanisms of initial specification of tissue specific endoderm progenitors in the anterior foregut. Wnt signaling has been shown to play a critical role in specifying Nkx2-1+ lung endoderm progenitors and is one of the few genetic models that results in complete respiratory agenesis (Goss et al., 2009; Harris-Johnson et al., 2009). Interestingly, Foxp2 expression also demarcates the same region within the anterior foregut endoderm marked by Nkx2-1 expression and which is known to generate both the lung and thyroid (Sherwood et al., 2009). While our work shows the critical importance of cooperative Foxp1/2/4 function in regulating early lung development, the morphological presence of a respiratory organ suggests that these three transcription factors may not be absolutely necessary for specification of lung endoderm progenitors. Our studies do reveal for the first time that Foxp1/2/4 are necessary for promoting early lung endoderm progenitor development, in part, through inhibition of non-lung gene expression programs.

MATERIALS AND METHODS

Animals

Shh-cre, Foxp1^flox/flox, Foxp2^flox/flox and *Foxp4^flox/flox* mice were previously generated and genotyped as describe (Feng et al., 2010; French et al., 2007; Harfe et al., 2004; Li et al., 2012). All animal procedures were performed in accordance with the Institute for Animal Care and Use Committee at the University of Pennsylvania.

Histology

Tissues were fixed in 10% formalin and processed using standard procedures. In situ hybridization was performed as previously described (Herriges et al., 2014; Morrisey et al., 1996; Wang et al., 2013). Immunohistochemistry was performed using the following antibodies: goat anti-Scgb1a1 (Santa Cruz, 1:20), rabbit anti-SP-C (Chemicon, 1:500), rabbit anti-Nkx2.1 (Santa Cruz, 1:50), rabbit anti-Sox9 (Santa Cruz, 1:100), rabbit anti-Sox2 (Seven Hills Bioreagents, 1:500), mouse anti-β-tubulin IV (BioGenex, USA; 1:20), rabbit anti-e-cadherin (Cell Signaling, 1:100), Anti-Mouse CD31 (PECAM-1) (HistoBioTec, 1:20). The Foxp1 (1:200), Foxp2 (1:200) and Foxp4 (1:200) polyclonal antibodies have been previously described (Lu et al., 2002).

Microarray studies

RNA was isolated from E14.5 lungs of *Foxp1/4^TKO* and *Shh^cre* control mice and used to determine changes in the transcriptome of airway epithelial cells due to genetic inactivation of *Foxp1/2/4*. Microarray assay and data analysis were performed as described previously (Li et al., 2012). Heatmaps were generated using the R library gplots. Genes with a 2-fold (or 1.2-fold for transcript factor analysis) change over that of the control samples and with an experimental mean with a Student's T-test of $P<0.01$ (ANOVA), were considered to be statistically significant. Microarray data are deposited in Gene Expression Omnibus under accession number GSE64777.

Quantitative PCR

Total RNA was isolated from lung tissue at the indicated time points using Trizol reagent or RNeasy Micro Kit (QIAGEN), reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real-time PCR analysis with the primers listed in Supplemental Table 2.

ChIP analysis

To determine if the up-regulated Hox9a-13 cluster is direct target of Foxp1/2/4, we used reported Foxp1 binding motifs to blast against mouse genome mm9 and obtain binding sequences conserved in mammals using the FIMO program (Grant et al., 2011). We restricted our analysis to conserved DNA binding sequences found outside of exonic sequences. The ChIP-QPCR analysis was performed as previously described (Li et al., 2012), and the primers used are listed in Supplemental Table 2.

Statistics

Data are reported as means ± SEM of at least three independent assays. Paired or unpaired Student's T test was used for single comparisons and one-way ANOVA for multiple comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Foxp1/2/4 are essential for lung development

2. Combined loss of Foxp1/2/4 leads to defective lung progenitor commitment

3. Foxp1/2/4 restrict expression of non-lung genes including Hox and Pax factors
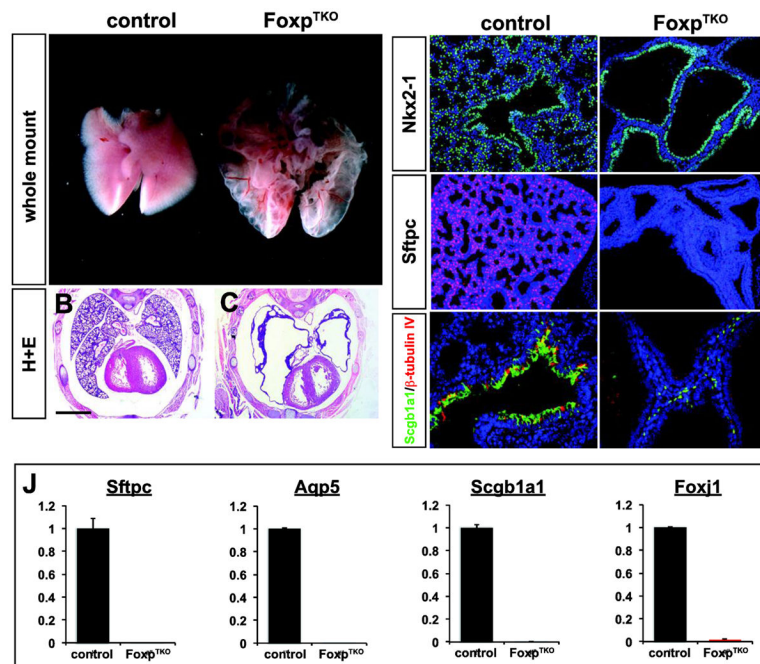
Figure 1. Foxp^{TKO} mutants display a severe disruption in lung development

Whole mount (A) and H+E staining of histological sections (B and C) at E18.5 of Foxp^{TKO} mutants reveals a lack of branching morphogenesis with replacement of normal lung structures with large dilated sacs. Immunostaining for the pan-lung epithelial marker Nkx2-1 (D and E) and markers of differentiated epithelial lineages including Sftpc (F and G), Scgb1a1 (H and I), and β-tubulin IV (H and I) reveal that while Nkx2-1 expression is still detectable, expression of differentiated epithelial lineages is absent or dramatically reduced in Foxp^{TKO} mutants. Q-PCR for markers of differentiated lung epithelial lineages (J). Scale bars: B and C=200μm, D–I=100μm.
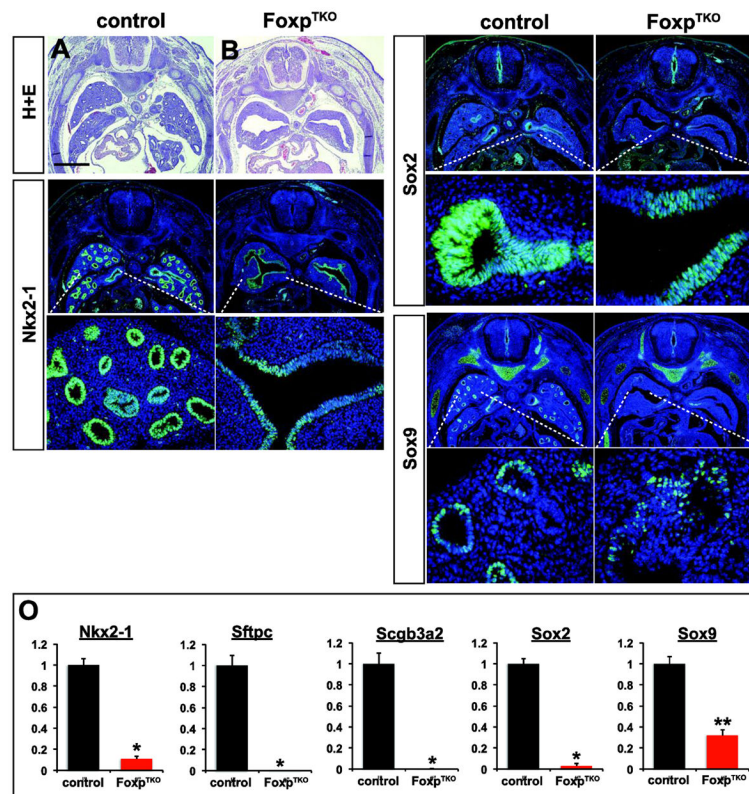
Figure 2. Decreased expression of lung endoderm progenitor markers in Foxp^{TKO} mutants
H+E staining at E14.5 (A and B) reveal lack of branching morphogenesis. Expression of the early pan-lung endoderm markers Nkx2-1 is reduced in Foxp^{TKO} mutants (C–F). Immunostaining reveals reduced expression of the early proximal endoderm progenitor marker Sox2 and the distal endoderm progenitor marker Sox9 in Foxp^{TKO} mutants at E14.5 (G–N). Q-PCR for early lung endoderm progenitor markers showing almost complete absence or dramatic reduction in expression in Foxp^{TKO} mutants (O). Scale bars: C,D,G,H,K,L=150μm; E,F,I,J,M,N=100μm.
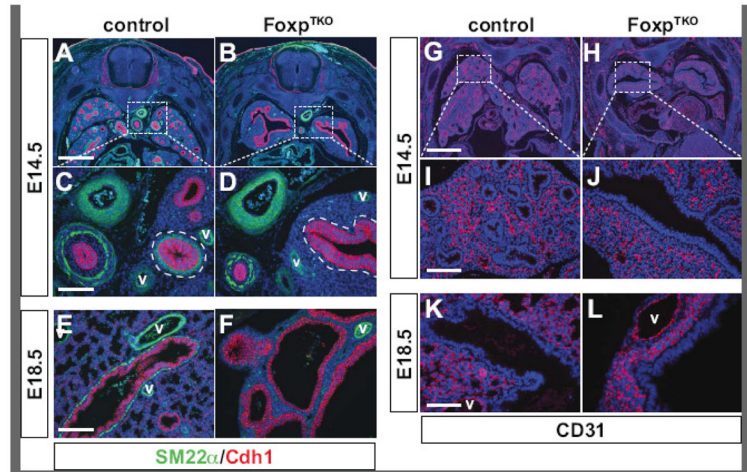
Figure 3. Endoderm specific loss of Foxp1/2/4 leads to absence of airway smooth muscle development

Immunostaining for SM22α reveals lack of airway smooth muscle development in Foxp^{TKO} mutants at E14.5 (A–D) and E18.5 (E and F). However, Foxp^{TKO} mutants still develop inter-pulmonary blood vessels (C–F). Cdh1 immunostaining is used to reveal the airway epithelium in A–F, which is surrounded by dotted lines in C and D. CD31 immunostaining reveals development of a capillary plexus in Foxp^{TKO} mutants. C–D and I–J are insets of A–B and G–H, respectively. V=blood vessel. Scale bars: A,B,E,F,G,H=150μm; C,D,I,J,K,L=100μm.
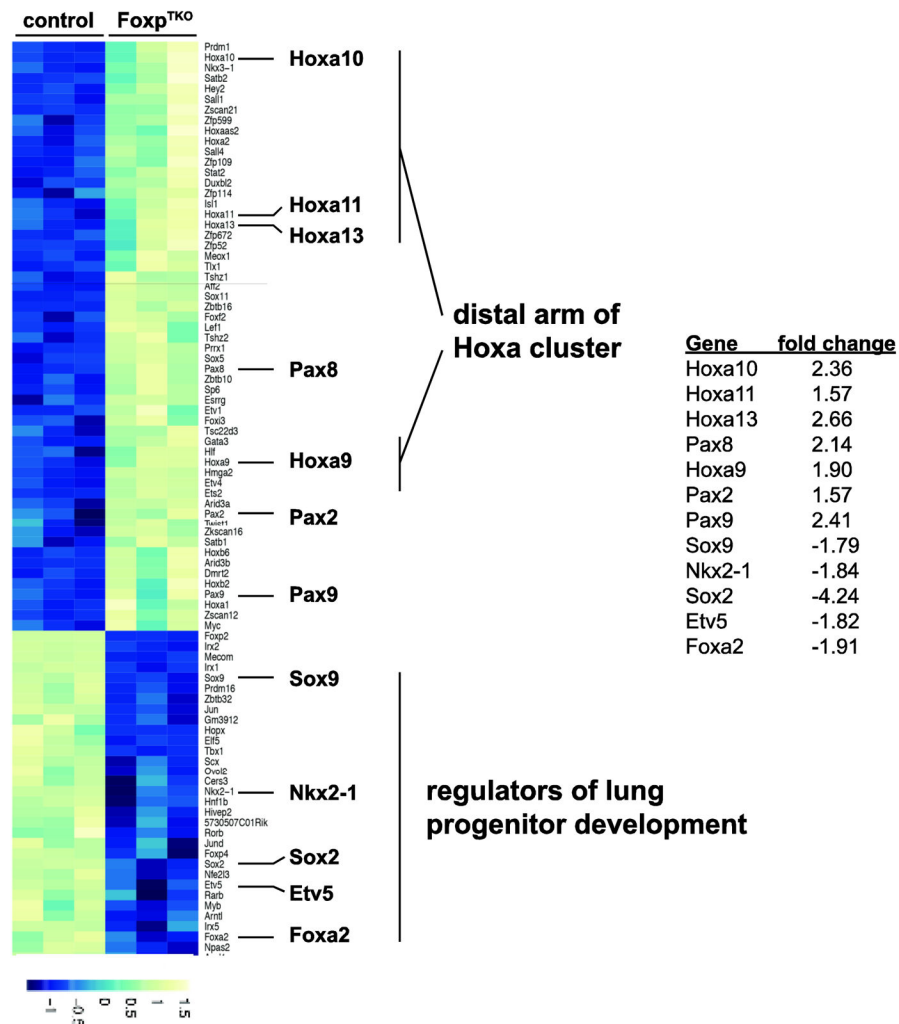
Figure 4. Transcriptome analysis reveals decreased expression of several lung transcriptional regulators and ectopic expression of Hox and Pax factors

A heatmap of transcriptional regulators whose expression is deregulated in Foxp^{TKO} mutants at E14.5. The table on the right lists fold-changes of the various transcription factors as detected in the microarray experiments.
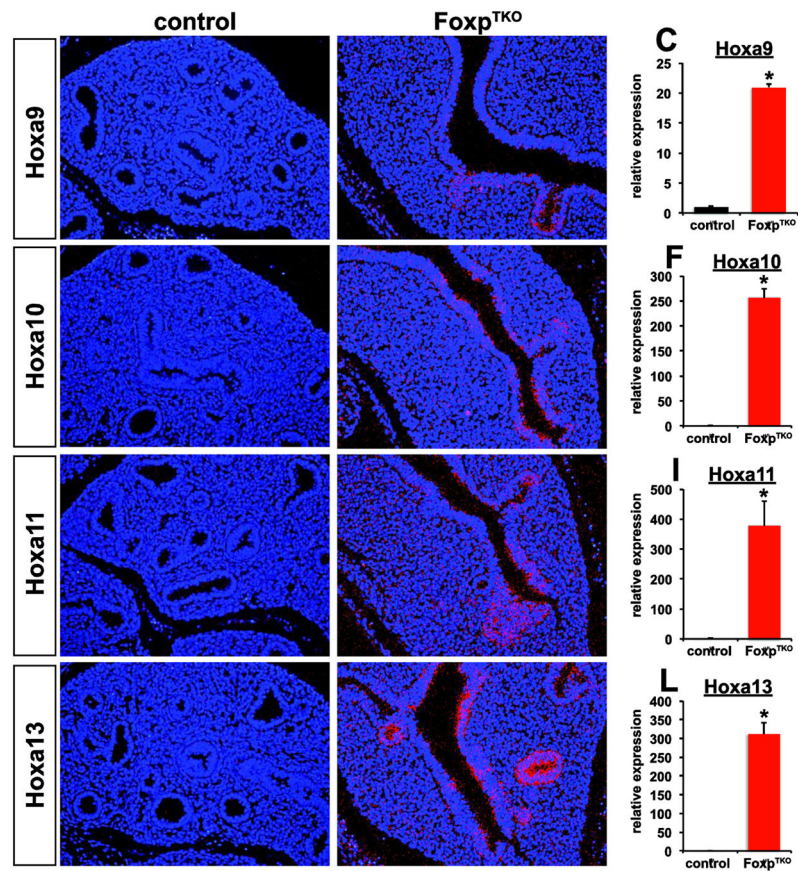
Figure 5. Expression of Hoxa9-13 is ectopically activated in the lungs of Foxp^{TKO} mutants
In situ hybridization was performed at E14.5 for Hoxa9 (A–B), Hoxa10 (D–E), Hoxa11 (G–H), and Hoxa13 (J–K). Dotted lines outline the areas of airway epithelium that ectopically express the various Hoxa genes. Q-PCR for Hoxa9-13 in Shh^{cre} control and Foxp^{TKO} mutants (C,F,I,L). Scale bars: A–K=150μm.
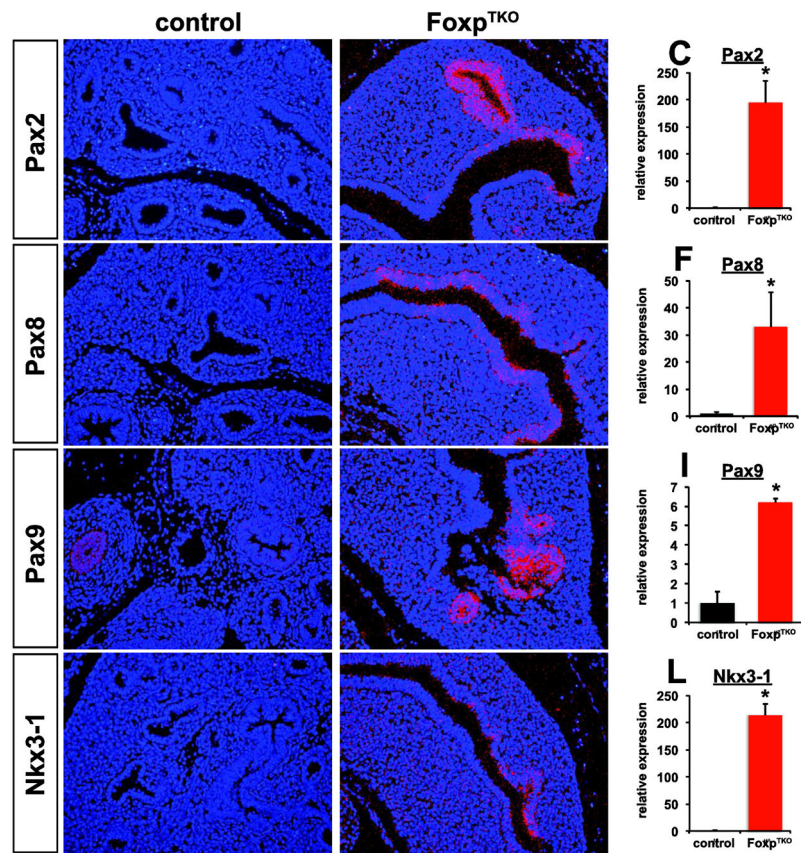
Figure 6. Expression of Pax2/8/9 and Nkx3-1 is ectopically activated in the lungs of Foxp^TKO mutants

In situ hybridization was performed at E14.5 for Pax2 (A–B), Pax8 (D–E), Pax9 (G–H), and Nkx3-1 (J–K). Dotted lines outline the areas of airway epithelium that ectopically express the various Pax or Nkx3-1 genes. Of note, Pax9 is expressed in the esophageal epithelium (G). Q-PCR for Pax2, Pax8, Pax9, and Nkx3-1 in Shh^cre control and Foxp^TKO mutants (C,F,I,L). eso=esophagus. Scale bars: A–H=150μm.
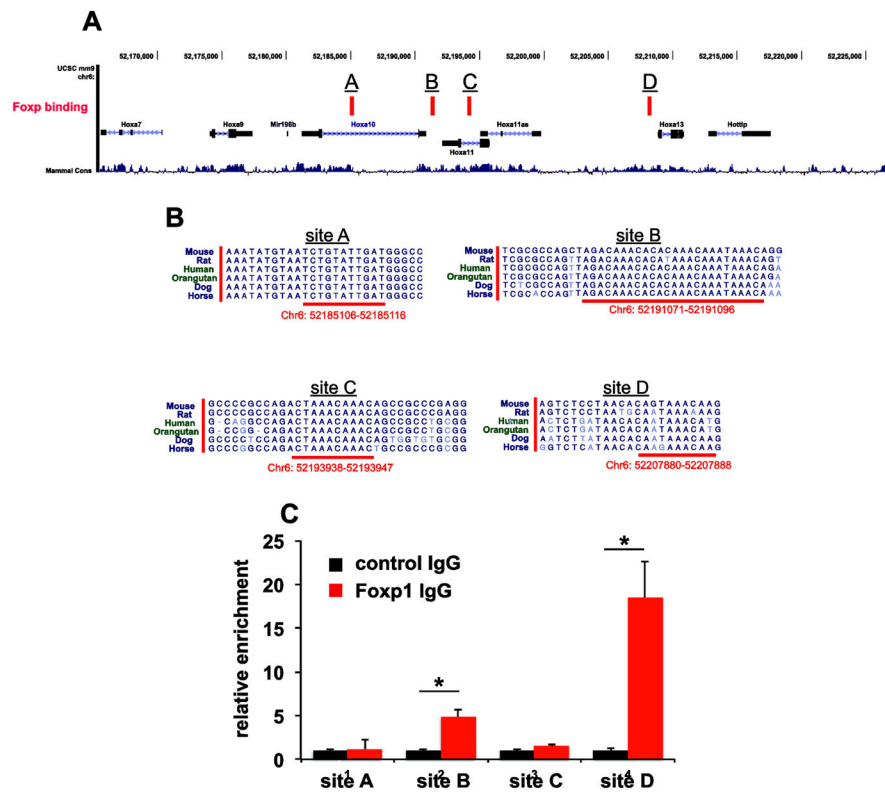
Figure 7. Foxp1 binds to two sites in the Hoxa9-13 genomic region
We used the mouse genome mm9 build to identify conserved Fox DNA binding sites within the Hoxa9-13 genomic region. Four sites were identified and labeled A–D (A). All of these sites are conserved across mammals with site B containing a duplex Fox DNA binding sequence (B). ChIP-QPCR was performed on these four sites using a Foxp1 antibody and this revealed binding to sites B and D but not A and C (C).