Multiple Roles and Interactions of \( Tbx4 \) and \( Tbx5 \) in Development of the Respiratory System

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Abstract

Normal development of the respiratory system is essential for survival and is regulated by multiple genes and signaling pathways. Both \( Tbx4 \) and \( Tbx5 \) are expressed throughout the mesenchyme of the developing lung and trachea; and, although multiple genes are known to be required in the epithelium, only Fgfs have been well studied in the mesenchyme. In this study, we investigated the roles of \( Tbx4 \) and \( Tbx5 \) in lung and trachea development using conditional mutant alleles and two different Cre recombinase transgenic lines. Loss of \( Tbx5 \) leads to a unilateral loss of lung bud specification and absence of tracheal specification in organ culture. Mutants deficient in \( Tbx4 \) and \( Tbx5 \) show severely reduced lung branching at mid-gestation. Concordant with this defect, the expression of mesenchymal markers \( Wnt2 \) and \( Fgf10 \), as well as \( Fgf10 \) target genes \( Bmp4 \) and \( Spry2 \), in the epithelium is downregulated. Lung branching undergoes arrest \textit{ex vivo} when \( Tbx4 \) and \( Tbx5 \) are both completely lacking. Lung-specific \( Tbx4 \) heterozygous; \( Tbx5 \) conditional null mice die soon after birth due to respiratory distress. These pups have small lungs and show severe disruptions in tracheal/bronchial cartilage rings. \( Sox9 \), a master regulator of cartilage formation, is expressed in the trachea; but mesenchymal cells fail to condense and consequently do not develop cartilage normally at birth. \( Tbx4;Tbx5 \) double heterozygous mutants show decreased lung branching and fewer tracheal cartilage rings, suggesting a genetic interaction. Finally, we show that \( Tbx4 \) and \( Tbx5 \) interact with \( Fgf10 \) during the process of lung growth and branching but not during tracheal/bronchial cartilage development.

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Introduction

The development of the respiratory system represents an evolutionary hallmark that allowed vertebrates to survive on land utilizing air as a source of oxygen. Because the respiratory system is dispensable for embryonic survival in mammals, defects in development of the respiratory system manifest at or after birth. Indeed, abnormal development of the respiratory system in humans is associated with multiple disorders such as tracheal/bronchial atresia, tracheoesophageal fistula, bronchogenic cysts, pulmonary/lobar atresia and pulmonary hypoplasia [1]. Thus, it is important to understand the genetic basis of development of the respiratory system.

In the mouse embryo, the endodermal foregut tube is patterned by signals from the lateral plate mesoderm leading to specification of the lung and trachea at embryonic day (E) 9.0 (19–24 somites). \(\text{Nkx}2.1\) has been identified as the earliest marker of lung endoderm specification. At E9.25 (25–28 somites), the primary lung buds appear as ventro-lateral outpouchings of the foregut connected with the anterior foregut or absence of pulmonary/lobar atresia and pulmonary hypoplasia [1]. Thus, it is important to understand the genetic basis of development of the respiratory system.

In the mouse embryo, the endodermal foregut tube is patterned by signals from the lateral plate mesoderm leading to specification of the lung and trachea at embryonic day (E) 9.0 (19–24 somites). \(\text{Nkx}2.1\) has been identified as the earliest marker of lung endoderm specification. In the mouse, the left lung bud remains a single lobe and the right lung bud forms 4 lobes - cranial, medial, caudal and accessory [2]. The airways undergo a stereotypic pattern of branching beginning at E11.5 [4]; development and maturation of the alveoli occurs later.

Genes involved in different signaling pathways, including \(\text{Wnt}2\), \(\text{Fgf10}\), \(\text{Bmp4}\), \(\text{Shh}\) and retinoic acid synthesis genes, have been shown to play important roles in lung specification and branch formation. Complete absence of both \(\text{Wnt}2\) and \(\text{Wnt}2\) in mesenchyme surrounding the anterior foregut or absence of \(\beta\)-catenin in the foregut epithelium leads to a loss of specification of lung primordia as seen by the absence of \(\text{Nkx}2.1\) expression [5–7]. Embryos lacking \(\text{Fgf10}\), which is normally expressed in mesenchyme surrounding the epithelial branching tips, form a short trachea but have no lungs [8,9]. Inhibition of epithelial \(\text{Bmp4}\) signaling by overexpression of \(\text{Xrog}\) leads to a decrease in lung size and irregularly shaped lung lobes [10]. \(\text{Shh}\) null mutant mice have only a rudimentary lung sac due to branching severe branching defect [11]. Additionally, conditional inactivation of \(\text{Shh}\) in lung epithelial cells leads to the formation of hypoplastic lungs with reduced branching of the peripheral tubules [12]. Retinoic acid receptor (RAR) \(\alpha\) and RAR \(\beta2\) double null mutants show left lung agenesis and a hypoplastic right lung at E18.5 [13]. Thus, genes expressed in both the mesenchyme and the epithelium are essential for correct lung bud specification and branching.

After E11.5, mesenchyme surrounding the dorsal aspect of the trachea differentiates into the trachealis smooth muscle. Mesen-
Author Summary

Defective development of the mammalian respiratory system can lead to tracheal, bronchial, or pulmonary malformations causing severe consequences at birth or during postnatal life. Studies using mouse genetics have begun to reveal complex regulatory mechanisms that guide the development of the respiratory system, but understanding how disruption of these mechanisms leads to malformations is far from complete. In this study, we analyze the role of two T-box transcription factors, *Tbx4* and *Tbx5*, in three processes essential to the development of the respiratory system: the specification of the lung and trachea primordia, the growth and branching of the airways to form the lung, and formation of cartilage rings of the trachea and bronchi. In the absence of *Tbx5*, only one lung is specified, and no trachea. Both *Tbx4* and *Tbx5* regulate the process of lung branching by controlling the expression of the secreted growth factor Fgf10 and activation of Fgf10 signaling. In the trachea, both *Tbx4* and *Tbx5* are important for condensation of cells to form cartilage rings, although this is regulated by a distinct pathway that does not involve Fgf10.

The T-box transcription factor genes are important during embryonic development. All members of this gene family contain a conserved DNA-binding T-box domain, which binds to a conserved sequence, the T-box binding element, to activate or repress transcription of specific target genes [23]. All Tbx2 subfamily genes, *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5* are expressed in the developing chick lung buds and trachea between stages 15–21 [24]. In the mouse, *Tbx1* is expressed in lung epithelium at E12.5, *Tbx2* and *Tbx3* are expressed in lung mesenchyme at E11.5, and *Tbx4* and *Tbx5* are expressed in both lung and trachea mesenchyme at E12.5 and later [25]. *Tbx1* homozygous null mutants die at birth due to severe heart defects; the lungs are never fully inflated [26] but lung development has not been further investigated. In *Tbx4* homozygous mutants, lung buds form but the embryos die at E10.5 due to failure of allantois development and the subsequent lack of chorio-allantoic fusion leading to placental insufficiency [27]. *Tbx5* mutants die around E10 due to defects in heart development [28]; lung development has not been previously investigated. Antisense oligonucleotide depletion of both *Tbx4* and *Tbx5*, but not *Tbx2* and *Tbx3*, in lung organ cultures results in inhibition of branching and loss of Fgf10 expression [29] suggesting a role for these factors in lung branching. In the chick embryo, interference with *Tbx4* function leads to a reduction in *Fgf10* expression in lung mesenchyme and inhibits lung bud formation. Ectopic expression of *Tbx4* leads to ectopic expression of *Fgf10* and *Nkx2.1* and lung bud formation in the esophagus. Additionally, ectopic expression of *Tbx4* at the boundary between the trachea and the esophagus can lead to lack of separation of these two structures, resulting in a tracheoesophageal fistula [30]. In humans, *Tbx5* mutations cause Holt-Oram syndrome characterized by heart and forelimb abnormalities. A single de-novo mutation in *TBX5* has been linked to right lung agenesis [31].

To study the roles of *Tbx4* and *Tbx5* in lung and trachea development in the mouse, we made use of conditional alleles to bypass early embryonic lethality. We studied three distinct processes, namely 1) lung bud and trachea specification, 2) lung branching morphogenesis, and 3) tracheal/bronchial cartilage formation. We show that during early stages of development, *Tbx5* is important for specification of the lung buds and the trachea. After specification, *Tbx4* and *Tbx5* interact during lung growth and branching and the regulation of branching is dependent on Fgf10 signaling. Additionally, *Tbx4* and *Tbx5* interact in the formation of mesenchymal condensations, which ultimately form the tracheal/bronchial cartilage rings independent of Fgf10 signaling.

Results

Expression of *Tbx4* and *Tbx5* in the developing lung and trachea

*Tbx5* expression is first detected using in situ hybridization (ISH) at E9.0 (24 somites) in the mesenchyme of the lung and trachea primordia, concurrent with *Nkx2.1* expression in the ventral foregut epithelium (Figure 1A–1D). The anterior extent of expression of both genes coincides with the posterior extent of the third pharyngeal pouch (red arrow in Figure 1A, 1B). *Tbx4* expression is detected in the lung buds when they first appear a few hours later at E9.25 (28 somites) in a pattern similar to *Tbx5* (Figure 1E, 1F). *Tbx4* and *Tbx5* are expressed at E11.5, E13.5 and E15.5 throughout the lung mesenchyme but not in the epithelium (Figure 1G–1L) [25].

Both *Tbx4* and *Tbx5* show a dynamic expression pattern in the developing trachea. At E11.5 and E13.5 both genes are expressed throughout the tracheal mesenchyme surrounding the epithelium but are excluded from the epithelium and the outermost layer, the mesothelium (Figure 1G, 1H, 1I, 1M, 1N, 1O). At these stages, *Tbx4* and *Tbx5* are expressed in ventral tracheal cells that also express *Sox9* (Figure 1O, 1O’). *Tbx4* and *Tbx5* are expressed at E11.5, 13.5 and 15.5 in the mesenchyme but not in the epithelium (Figure 1G–1L) [25].

Efficiency of recombination of conditional alleles

The genotypes of embryos used in this study and the corresponding descriptive shorthand nomenclature are shown in Table 1. PCR genotyping was used to determine the efficiency of recombination of the conditional alleles. For embryos carrying the tamoxifen-inducible *CreER* transgene, a dose of 8 mg tamoxifen was injected into pregnant females at E9.0 and embryos were dissected at E12.5. Yolk sacs were analyzed as an estimate of recombination in the whole embryo. Both alleles of *Tbx4*/*5* embryos were completely recombined at E12.5 to produce the mutant allele at this dose of tamoxifen (Figure S1A), but the single floxed allele of *Tbx4*/*5* embryos was only partially recombined to
the mutant form (Figure S1E). Doses of tamoxifen higher than 8 mg at E9.0 lead to a loss of pregnancy. When 7 mg tamoxifen was injected at E8.5, complete recombination of the Tbx4fl alleles (Figure S1B) and the single Tbx5fl allele (Figure S1F) was obtained at E13.5.

In lung bud cultures a concentration of 1 μM 4-OH tamoxifen produced near-complete recombination of the Tbx4fl allele after 24 hours (Figure S1C) whereas the Tbx5fl allele was only partially recombined (Figure S1G). Virtually complete recombination of all floxed alleles was achieved after 4 days of culture (Figure S1D, S1H). These data suggest that the Tbx5fl allele has a lower efficiency of Cre-mediated recombination than the Tbx4fl allele. Thus we assume that there may be some residual Tbx5 activity from the Tbx5fl allele in the in vivo experiments, even in the presence of the CreER and Tbx4fl alleles.

Early loss of Tbx5, but not Tbx4, leads to a unilateral loss of lung bud specification and absence of tracheal specification

To explore the role of Tbx4 and Tbx5 in the earliest stages of lung and trachea specification, foregut culture [34] was used with Nkx2.1 as a marker of specification. This ex vivo technique allows for analysis of mutants in culture, circumventing early embryonic lethality of the Tbx4 homozygous mutants due to allantois defects and Tbx5 homozygous mutants due to heart defects. When

Figure 1. Expression of Tbx4 and Tbx5 in the developing lung and trachea. (A–L) Tbx4 and Tbx5 expression analyzed using ISH on lungs. Tbx5 is first expressed at E9.0 (B,D) when the specification of lung primordia occurs, as seen by Nkx2.1 expression (A,C). Red arrows point to the posterior extent of the third pharyngeal pouch which marks the anterior of the expression domain of both Nkx2.1 and Tbx5. Right views (A,B), ventral views (C,D). Tbx4 is first expressed at E9.5 along with Tbx5 in the newly formed lung buds (E,F). Expression is seen in lung whole mounts at E11.5 (G,H), E13.5 (I,J) and in lung mesenchyme in cryosections at E15.5 (K,L). (M–S) Tbx4 and Tbx5 expression analyzed using ISH on tracheas. Tbx4 and Tbx5 are expressed throughout tracheal mesenchyme (m) at E13.5 (M,M’,N,N’) but not in the epithelium (e) or the mesothelium (arrowheads). Caudal view of cut tracheas after whole mount ISH (M–P). ISH on cryosections (M’–P’). At E13.5, Sox9 is expressed in the mesenchyme on the ventral side (O,O’) and SM22a is expressed on the dorsal side (P,P’) of the trachea. D-dorsal; V-ventral; R-right; L-left. At E15.5, Tbx4 and Tbx5 are expressed around the condensing cartilage mesenchyme and in the intercartilage mesenchyme (Q,R). Sox9 is expressed in the condensing cartilage rings (S). Asterisks indicate areas of cartilage condensation. Insets in (Q) and (S) show ISH on E15.5 sagittal cryosections with Tbx4 and Sox9 probe, respectively. Scale bars represent 50 μm.

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foreguts and surrounding tissue are isolated at E8.75 (3–16 somites), the foregut tube is devoid of Nkx2.1 expression and the lung buds are not present [6]. At the end of 3 or 4 days of culture lung buds and trachea have formed as seen by Nkx2.1 expression (Figure 2A, 2D, 2G). Nkx2.1 is also expressed in the thyroid primordia at this stage (Figure 2D–2I and [35]). Expression of Tbx4 and Tbx5 was confirmed in control foreguts that were cultured for 4 days (Figure 2B, 2C). Foreguts from E8.5 embryos were cultured in the presence of 4-hydroxy (OH) tamoxifen and analyzed for Nkx2.1 expression. Reduction of Tbx4 alone did not affect Nkx2.1 expression after 3 days of culture (data not shown) and removal of Tbx4 in addition to Tbx5 did not exacerbate the Tbx5 phenotype (Figure 2F, 2I). Therefore, Tbx5 but not Tbx4 is important for the bilateral specification of lung buds ex vivo. Wnt2 and Wnt2b, genes essential for specification of respiratory primordia [5], were analyzed in the conditional Tbx5 null foreguts. Wnt2 expression was reduced (Figure 2J, 2K) and Wnt2b expression was absent (Figure 2L, 2M) suggesting that Tbx5 lies upstream of these genes in regulating the process of specification.

With respect to tracheal specification, Nkx2.1 expression was not observed in the foregut tube after 3 or 4 days of culture in embryos lacking Tbx5 (arrowheads in Figure 2E, 2H) suggesting a lack of tracheal specification in the absence of Tbx5. Additional loss of Tbx4 alleles did not alter the phenotype (Figure 2F, 2I), supporting a role for Tbx5 in the specification of the trachea, independent of Tbx4.

Table 1. Different allelic combinations and the descriptive nomenclature.

| Genotype                  | Nomenclature                      |
|---------------------------|----------------------------------|
| Tbx4fl/fl; Tbx5fl/+; CreER | Conditional Tbx4 null            |
| Tbx4+/-; Tbx5fl/+; CreER  | Conditional Tbx5 null            |
| Tbx4fl/+; Tbx5fl/fl; CreER | Conditional Tbx4, Tbx5 double     |
| Tbx4+/-; Tbx5fl/fl; CreER  | heterozygous                     |
| Tbx4fl/fl; Tbx5fl/fl; CreER | Conditional Tbx4 null, Tbx5       |
| Tbx4+/-; Tbx5fl/fl; CreER  | null heterozygous                |
| Tbx4fl/fl; Tbx5fl/fl; CreER | Lung-specific Tbx5 null          |
| Tbx4fl(fl/+); Tbx5fl(fl/+)|                         |
| Tbx4+/-; Tbx5fl/fl; CreER  |                         |

1Tbx4 and Tbx5 alleles are generated by recombination of the floxed alleles in the germ line due to germ line expression of the Tbx4 allele [38,63]. doi:10.1371/journal.pgen.1002866.t001

Figure 2. Early loss of Tbx5 leads to aberrant lung bud and trachea specification. (A–I) Foreguts were isolated between 8–16 somite stages and analyzed by ISH following culture. Nkx2.1 (A), Tbx4 (B) and Tbx5 (C) expression in lung buds (arrows) and tracheal primordia (yellow arrowhead) was confirmed in control cultures. Excision of Tbx5 using 4-OH tamoxifen in conditional null foreguts with CreER leads to the loss of Nkx2.1 expression in one of the lung buds at 3 (E) or 4 days (H). Conditional double null (F,J) carrying CreER show a phenotype similar to the conditional Tbx5 nulls, suggesting additional removal of Tbx4 does not exacerbate the phenotype. Nkx2.1 expression was absent in the foregut tube of conditional Tbx5 null and conditional double null foreguts after 3 or 4 days of culture (yellow arrowheads in E,F,H,J) compared to controls (D,G). Conditional Tbx5 nulls show reduced Wnt2 expression (K) and absence of Wnt2b expression (M) in the developing lung buds as compared to controls (L,J). Black arrowheads (J) point to Wnt2 expression in the heart in the controls. ht, heart; th, thyroid primordia. doi:10.1371/journal.pgen.1002866.g002

Lung branching is severely affected by the loss of Tbx4 and Tbx5

To analyze the effect of loss of Tbx4 and Tbx5 on lung branching in vivo we made use of the tamoxifen-inducible CreER transgene. Tamoxifen was injected at E8.75 (8–16 somites), late enough to bypass lethality but well before lung branching begins. Embryos with CreER and different combinations of the Tbx4fl and Tbx5fl alleles were examined at E12.5 and E13.5. Conditional Tbx4 null lungs were similar to controls at E12.5 but had fewer branching tips at E13.5 (Figure 3A, 3B, 3E, 3F). Conditional Tbx4; Tbx5 double heterozygous lungs (Figure 3C) were smaller in size and had fewer branching tips than conditional Tbx4 null lungs at E13.5 (Figure 3B) but were more advanced developmentally (Figure 3E, 3F) than conditional Tbx4 null; Tbx5 heterozygous lungs (Figure 3D), which were severely retarded. Lociation in the right lung was disrupted in conditional Tbx4 null; Tbx5 heterozygous lungs; the accessory lobe was missing and only rudimentary cranial and medial lobes were present (Figure 3H). The lobes had a fused appearance (Figure 3H’) suggesting a failure of separation. Histologically, there were no obvious structural defects other than an overall reduction in size (Figure 3G, 3G’, 3H, 3H’).
Conditional Tbx4 null;Tbx5 heterozygous mutants could not be analyzed later than E13.5, due to hematopoietic defects caused by Cre-induced apoptosis [36] and it was not possible to analyze conditional Tbx5 null embryos using the inducible CreER in vivo due to lethal heart defects. To circumvent these limitations, we used the Tbx4cre allele, which is expressed in the lung and trachea [37]. From this allele, Cre was expressed in the majority of cells of the developing lung and trachea mesenchyme [38]. Lung-specific Tbx4 heterozygous;Tbx5 null lungs show hypoplasia at E13.5 (K,L) and at birth (N,O). The number of branching tips in control lungs and the lung-specific Tbx4 heterozygous;Tbx5 null lungs, labeled as experimental in the box plot (M), are significantly different at E13.5. The green boxes contain 50% of the values; the median is indicated by a horizontal line in the box; bars represent the 5th and 95th percentiles. H & E staining on sections shows lung morphology for control (P) and mutants (Q) at birth. Scale bars represent 100 µm.

Figure 3. Loss of Tbx4 and Tbx5 causes reduced lung branching and lethality at birth. (A–H) Tbx4fl and Tbx5fl alleles were excised using CreER by injecting tamoxifen at E8.75 and lungs were dissected at different time points. Loss of Tbx4 alone or both Tbx4 and Tbx5 leads to lung hypoplasia at E13.5 (A–D). The number of branching tips was quantitated following E-cadherin staining for different genotypes at E12.5 (E) and E13.5 (F). H & E on histological sections shows a decrease in lung size at E12.5 in the conditional Tbx4 null;Tbx5 heterozygous lungs (G,H). (G’) and (H’) are magnified views of the boxed regions in (G) and (H), respectively. Black arrowheads indicate separation between the lobes of the right lung in the control (G’) and lack of separation in the mutants (H’). (I–Q) Tbx4cre was utilized for lung and trachea-specific excision of Tbx4fl and Tbx5fl alleles. Tbx4cre was expressed in most of the trachea mesenchyme (I) and lung mesenchymal cells (J) as seen by a R26RlacZ reporter expression at E13.5. doi:10.1371/journal.pgen.1002866.g003

Tbx4, Tbx5 in Development of the Respiratory System

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hypothesize that the variability in phenotype is due to variable extent of recombination of the Tbx5 allele (Figure S1). All lung-specific Tbx4 heterozygous;Tbx5 null pups (n = 13/13 from 5 litters) became cyanotic at birth and died shortly thereafter due to respiratory distress. Unlike the variable lung size in the lung-specific Tbx5 null mutants, the lungs of these mutants were consistently smaller than controls at E13.5 (Figure 3K, 3L) and at birth (Figure 3N, 3O) and had significantly fewer branching tips at E13.5 (Figure 3M). At P0 histology of the lung-specific Tbx4 heterozygous;Tbx5 null lungs is comparable to controls although the mutant tracheas show accumulation of a mucus like substance (Figure 3P, 3Q). Epithelia of mutant lungs show expression of T1α and Pro-surfactant protein C (Pro-SPC) (Figure S2A, S2D), markers for alveolar cell differentiation [10,19], suggesting that appropriate differentiation of the lung epithelium occurs in the lung-specific Tbx4 heterozygous;Tbx5 null lungs. These lung show lobation defects very similar to the conditional Tbx4 null;Tbx5 heterozygous mutant lungs. The accessory lobe was absent in most embryos but when present showed less branching; the cranial and caudal lobes also showed decreased branching. The cranial, medial and caudal lobes were not separated (Figure S3A, S3B). In addition, although tertiary dorsal branches were present in these lungs, they were crowded together and the secondary lateral branches had outgrown a shorter distance compared to controls (Figure S3C, S3D).

Using Tbx4F<sup>Cre</sup>, it is not possible to study conditional double nulls as Tbx4 is also expressed from this allele. Thus, to further explore branching morphogenesis in conditions where both alleles of Tbx4 and Tbx5 could be deleted, a lung bud culture system was used in which lung buds from embryos with or without the CreER transgene were cultured in the presence of 4-OH tamoxifen. Conditional Tbx4 null;Tbx5 heterozygous, or conditional Tbx4 heterozygous;Tbx5 null lung buds showed reduced branching (Figure 4A, 4B, 4C) consistent with the reduced number of branching tips observed in vivo. The conditional double null lungs showed a complete branching arrest by 3 days of culture (Figure 4D, 4E). The existing branches continued to elongate as seen at 4 days of culture (arrow in Figure 4E). Therefore, Tbx4 and Tbx5 are essential for continuing branching morphogenesis ex vivo.

Loss of Tbx4 and Tbx5 affects the Fgf10 signaling pathway and Wnt2 expression

Expression of the lung mesenchymal marker Fgf10 as well as epithelial targets of the Fgf10 signaling pathway, Bmp4, Spdy2 and Etn5 [39,40,41], was analyzed in lungs with reduced Tbx4 and Tbx5 expression. Fgf10 is expressed in mesenchyme surrounding the distal epithelial tips that mark the site of future bud formation (Figure 5A) [42]. Consistent with the smaller overall lung size, there were fewer foci of Fgf10 expression in the conditional Tbx4 null;Tbx5 heterozygous lungs (Figure 5B) and in the lung-specific Tbx4 heterozygous;Tbx5 nulls (Figure 5C). The primary receptor for this pathway, Fgfr2 [43], is expressed normally in the epithelium of lung-specific Tbx4 heterozygous;Tbx5 null lungs (Figure 5D, 5E). Bmp4 (Figure 5F, 5G, 5H) and Spdy2 (Figure 5I, 5J) were downregulated in Tbx4 and Tbx5-deficient lungs but Etn5 (Figure 5N, 5O) expression was not affected, although it was drastically reduced in conditional double nulls cultured ex vivo (see Figure 6J, 6K).

Wnt2, which is normally expressed in the developing lung mesenchyme (Figure 5K) [5], was greatly reduced at E13.5 in conditional Tbx4 null;Tbx5 heterozygous (Figure 5L) and lung-specific Tbx4 heterozygous;Tbx5 null lungs (Figure 5M). In addition to Fgf10 and Wnt2 signaling pathways, Shh signaling has also been implicated in branching morphogenesis in the lung [11]. Epithelial Shh (Figure 5S, 5T) and its mesenchymal receptor Ptce (Figure 5U, 5V) showed normal expression in lung-specific Tbx4 heterozygous;Tbx5 null lungs. The epithelial marker Nkx2.1, which is necessary for lung branching [44] and showed unilateral expression in the conditional Tbx5 null foreguts at the time of specification (Figure 2I), showed expression similar to controls at E13.5 in the lung-specific Tbx4 heterozygous;Tbx5 null lungs (Figure 5W, 5X). At E13.5, expression of the vascular marker Pecam indicated normal development of vessels around individual bronchioles of Tbx4 and Tbx5-deficient mutants (Figure 5P, 5Q, 5R).

Tbx4 and Tbx5 interact with Fgf10 during lung development

Since mesenchymal Fgf10 expression and expression of epithelial targets was affected in mutants with reduced Tbx4 and Tbx5, we investigated the interactions between Tbx4, Tbx5 and Fgf10 during branching morphogenesis using double and triple heterozygous mutants. Tbx4;Tbx5 double heterozygous lungs were smaller than control lungs at E18.5 (Figure 6A, 6B). Tbx4;Tbx5;Fgf10 triple heterozygous lungs were smaller than Tbx4;Tbx5 double heterozygous lungs (Figure 6B, 6C), suggesting that Tbx4 and Tbx5 genetically interact with the Fgf10 signaling pathway during lung development. Removing one copy of Fgf10 from lung-specific Tbx4 heterozygous;Tbx5 nulls did not reduce lung size further (Figure 6D, 6E).

Despite the genetic interaction, the addition of exogenous Fgf10 to lung bud cultures of conditional double nulls did not rescue branching (Figure 6F–6I). The lack of change in Etn5 expression, an Fgf10 target, indicated that the Fgf10 signaling pathway was not activated in the presence of exogenous Fgf10 in the conditional double null lungs (Figure 6J–6M). To ensure that the Fgf10 used for the rescue experiments was active, Fgf10 coated heparin beads were placed near the branching tips of lung explants. The tips of both control and conditional double null lungs swelled up in response to Fgf10 beads but not BSA-coated heparin beads (Figure 6N, 6O) [45]. Since Fgfr2 is expressed normally in the Tbx4 and Tbx5-deficient lungs (Figure 5E) and the conditional double null lungs (data not shown), it is not surprising that the conditional double null lung tips can respond to Fgf10. However the conditional double null lungs fail to undergo branching in the presence of Fgf10. Thus, in addition to Fgf10 there must be other factors under the control of Tbx4 and Tbx5 important for activation of the Fgf10 signaling pathway leading to branching morphogenesis.

Loss of Tbx4 and Tbx5 causes tracheal/bronchial ring defects

To analyze the development of the tracheal/bronchial cartilage in embryos with reduced Tbx4 and Tbx5, cartilage rings were visualized at birth using alcian blue staining. Lung-specific Tbx5 nulls and lung-specific Tbx4 heterozygous;Tbx5 null embryos had defective cartilage ring development (Figure 7A–7C) with some normal rings (arrows in Figure 7B, 7C) and isolated foci of cartilage (black arrowheads in Figure 7B, 7C). The tracheal and bronchial lumen of newborn pups was expanded in the controls (Figure 7D, 7E) but collapsed in lung-specific Tbx4 heterozygous;Tbx5 nulls, and contained a mucus-like substance (Figure 7F, 7G). The tracheal epithelium of these mutants showed an increase in the number of mucus-producing cells, as seen by alcian blue staining (Figure 7D*, 7D’, 7F*, 6P) [19]. To assess the development of cartilage rings at earlier stages, Sox9 expression was analyzed at E12.5 and E13.5 in lung-specific Tbx4 heterozygous;Tbx5 nulls. At E12.5, these mutant tracheas have Sox9 expression on the ventral aspect of the trachea similar to controls (Figure 7H, 7I) but fail to form mesenchymal condensations at E13.5 (Figure 7J, 7K). Expression of two genes genetically
downstream of Sox9, Sox6 (Figure 7L, 7M) and Sox5 (Figure 7N, 7O), was downregulated at E13.5 whereas Col2a1, a Sox9 target, was expressed at apparently normal levels in those rings that were present (Figure 7P, 7Q). The smooth muscle marker SM22α was analyzed to assess the development of the dorsal trachealis muscle. SM22α was expressed in an expanded domain and there was a loss of the characteristic banding pattern in the mutant tracheas (Figure 7R, 7S) indicating a disruption in smooth muscle formation due to loss of Tbx4 and Tbx5.

**Tbx4 and Tbx5 do not interact with Fgf10 during trachea development**

Tracheas of controls (Figure 8A), Tbx4;Fgf10 double heterozygotes (Figure 8B) and Tbx5;Fgf10 double heterozygotes (Figure 8C) showed a normal pattern of 10–11 cartilage rings, suggesting a lack of genetic interactions between Tbx4 or Tbx5 and Fgf10 in trachea formation. Fgf10 null mutants do not form lungs but have a truncated trachea with 6–8 cartilage rings, some of which are aberrantly formed [22] (Figure 8D). Tbx4;Tbx5 double heterozygous mice also have tracheas with 6–8 cartilage rings but in addition have main stem bronchial cartilage rings (Figure 8E). Removing a copy of Fgf10 in these double heterozygotes did not alter the phenotype (Figure 8F) showing an Fgf10-independent role for Tbx4 and Tbx5 in the formation of tracheal/bronchial cartilage rings. Also, removing a copy of Fgf10 in the lung-specific Tbx4 heterozygous;Tbx5 nulls did not alter the phenotype of the tracheal/bronchial cartilage rings (Figure 8G, 8H). Thus, Tbx4 and Tbx5 affect lung development via control of Fgf10 expression but affect tracheal/bronchial cartilage development independently of the Fgf10 signaling pathway.
Discussion

Loss of Tbx5 affects specification of the lung buds and trachea

Tbx5 is expressed around the lung/trachea primordia at the same time that Nkx2.1, a marker of specification, is first expressed in the primordia of the foregut endoderm. Tbx4 is expressed slightly later at the time of lung bud formation. In our study, loss of Tbx5, but not Tbx4, leads to unilateral loss of lung bud specification, indicating that Tbx5 has a distinct function in lung bud formation. In contrast, in the chick, although ectopic expression of Tbx4 in the esophagus can specify lung fate, expression of a dominant negative form of Tbx4 leads to a lack of primary budding in only a third of the mutants analyzed [30]. However, the dominant negative Tbx4 could also be affecting the expression of Tbx5 targets as the repressor construct utilized the complete T-box domain and Tbx4 and Tbx5 have 94% amino acid identity in their T-box domains [46]. This interpretation is compatible with our hypothesis that Tbx5 plays a distinct role in vertebrate lung primordia specification [Figure 9A]. Further our results suggest that Tbx5 regulates specification by regulating the activity of Wnt2 and Wnt2b.

Loss of Tbx5 alone leads to a lack of tracheal specification ex vivo, a phenotype similar to that of mice mutant for Bmpr1 and Bmpr2. In these mutants, although lung bud specification occurs, the ventral foregut fails to acquire tracheal identity [47]. Therefore, Tbx5 either acts in parallel with or in the BMP signaling pathway in specification of the foregut into a trachea (Figure 9A).

Tbx4 and Tbx5 interactions in lung development

In lung branching morphogenesis, Tbx4 and Tbx5 genetically interact with one another (Figure 9B). Although, neither Tbx4 (data not shown) nor Tbx4 single heterozygous lungs show a branching defect, double heterozygous lungs are smaller at E13.5 and E18.5 with a reduced number of branching tips at E13.5. Because these genes are closely related [46], they could potentially regulate the same target genes by binding to a similar T-box binding element, independently of one another. Alternatively, Tbx4 and Tbx5 could physically interact with each other as heterodimers to activate or repress transcription of downstream targets, as has been suggested for other T-box genes [48]. Even though Tbx4 and Tbx5 have a shared role in regulating lung branching, their relative contributions may not be equal, as Tbx4;Tbx5 double heterozygous lungs are
smaller than conditional Tbx4 nulls. Unequal and distinct functions could be explained by domains outside of the T-box. For instance, the C terminal domain of both Tbx4 and Tbx5 has transcription activating capability which has been correlated to the shared limb outgrowth promoting activity of these genes [49] but Tbx4 also has a C terminal repressor domain which is proposed to be responsible for its distinct hind limb specific patterning activity [50]. In another example Tbx3 and Tbx4 both bind to a distinct LIM domain repeat of the LMP4 protein in the chick [51], illustrating distinct protein-protein interactions which could have functional implications.

In conditional Tbx4 nulls; Tbx5 heterozygous and lung-specific Tbx4 heterozygous; Tbx5 null lungs, reduction of Tbx4 or Tbx5 leads to a severe decrease in branching, a defect in formation of the lobes and failure of lobe separation, while absence of Tbx4 and Tbx5 leads to branching arrest. These results are in line with the published observations that antisense RNAs used against both Tbx4 and Tbx5 inhibit lung branching in culture and that this affect is explained by loss of Fgf10 expression [29]. Tbx5 binds and activates the Fgf10 promoter in vitro suggesting that Fgf10 is a direct downstream target [52]. The drastic effect on lung branching in our study is explained by loss of expression of the important regulatory genes Fgf10 and Wnt2 in the lung mesenchyme. The Fgf10 targets Bmp4 and Spy2 show very low levels of expression in the epithelium of Tbx4 and Tbx5-deficient mutants and E15.5, another Fgf10 target, shows greatly reduced expression in the conditional double null lungs, leading to the hypothesis that the Fgf10 signaling pathway is activated downstream of Tbx4 and Tbx5 in the developing lung and that Fgf10 genetically interacts with Tbx4 and Tbx5. Lungs that are triple heterozygous for Tbx4, Tbx5 and Fgf10 are reduced in size compared to Tbx4; Tbx5 double heterozygous lungs, which supports this hypothesis. Lung-specific Tbx4 heterozygote; Tbx5 null lungs are severely retarded but lung size is not affected by further loss of Fgf10, demonstrating epistasis and supporting the hypothesis that Fgf10 lies downstream of Tbx4 and Tbx5. Lack of rescue of branch formation and lack of activation of the Fgf10 signaling pathway by exogenously supplied Fgf10 in culture suggests that there are other factors downstream of Tbx4 and Tbx5 that affect Fgf10 signaling. Although, lungs deficient in Tbx4 and Tbx5 retain the ability to respond to Fgf10 coated beads (our study and [29]), they fail to activate additional factors necessary for branching morphogenesis (X in Figure 9B).

One possibility for such a factor is a mesenchymal signaling molecule that communicates with the epithelium and activates downstream target(s) required for the activation of the Fgf10 pathway. We examined expression of Ptc and Shh to determine whether the Shh pathway was involved, but Shh signaling is not affected in the lung-specific Tbx4 heterozygous; Tbx5 nulls or in the conditional double null cultures (data not shown). The extracellular matrix (ECM) molecules, heparan sulfate (HS) proteoglycans aid in Fgf10-Fgf2 interactions during lung development [45] and hence are good candidates for factors missing in the Tbx4 and Tbx5-deficient mutants. Inhibition of heparanase decreases submandibular gland morphogenesis in culture due to deficiency of Fgf signaling [53]. Additionally, HS-deficient null mouse embryos fail to respond to Fgf signaling and the spatiotemporal expression of cell surface-tethered HS chains regulates the local reception of Fgf-signaling activity during embryonic development [54]. Tbx4 regulates ECM molecules in the developing allantois, specifically the chondroitin sulfate proteoglycan versican (R. Arora and V. E. Papaioannou unpublished observations), suggesting that ECM might be one of the targets for T-box genes in regulating development of other organs as well.

Figure 6. Tbx4 and Tbx5 interact with Fgf10, but Fgf10 fails to rescue Tbx4- and Tbx5-deficient lungs. (A–E) Lungs of different genotypes at E18.5. Tbx4;Tbx5 double heterozygous lungs (B) are smaller than control lungs (A). Tbx4;Tbx5;Fgf10 triple heterozygous lungs (C) are smaller than the double heterozygous lungs (B) but comparable in size to the lung-specific Tbx4 heterozygous; Tbx5 null lungs (D). Removing an additional copy of Fgf10 from the lung-specific Tbx4 heterozygous; Tbx5 null lungs does not further affect lung size (E). ht, heart. (F–I) E-cad expression in epithelium of the control lungs and conditional double null lungs in the absence (F,G) and presence (H,I) of exogenous Fgf10 showing a lack of rescue of branching morphogenesis. (J–M) Etv5 expression in control lungs and conditional double null lungs in the absence (J,K) and presence (L,M) of exogenous Fgf10. Etv5 expression remains unchanged after addition of Fgf10. (N,O) Both control and conditional double null lungs respond to Fgf10 coated beads (f) but not to BSA coated beads (b) as seen by swelling of tips (arrows) in proximity to the FGF10 bead. doi:10.1371/journal.pgen.1002866.g006
Appropriate dorsal smooth muscle development and ventral tracheal/bronchial cartilage development is important for the normal functioning of the trachea. Smooth muscle provides tracheal flexibility and the cartilaginous rings prevent tracheal collapse. Defects in trachea formation but there were fewer rings with apparently normal expression in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (Q). SM22α expression was analyzed on the dorsal trachea at E13.5 in the control (R) and in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (S). Arrowheads in (S) point to ectopic expression in an uncharacteristic pattern in the mutants. Scale bars represent 100 μm.

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**Figure 7. Loss of** Tbx4 **and** Tbx5 **disrupts tracheal/bronchial cartilage and smooth muscle formation.** (A–G) Alcian blue staining was used to visualize tracheal/bronchial cartilage. Ten-eleven cartilage rings were seen in control tracheas (E18.5, A); normal (arrow) and incomplete rings were seen in tracheas and bronchi (black and red arrowheads respectively) of lung-specific Tbx5 null (E18.5, B) and lung-specific Tbx4 heterozygous; Tbx5 nulls (postnatal day P0, C). Asterisks in (A–C) indicate the point of bronchial separation from the trachea. Alcian blue on P0 sections showed that the control trachea (D) and main stem bronchial lumens (E) were expanded and the chondrocytes were present as C shaped rings (arrowheads). The lung-specific Tbx4 heterozygous; Tbx5 null tracheal lumen (F) and main stem bronchial lumen (G) were collapsed and filled with a mucus like substance (arrows) and alcian blue positive foci were seen (black arrowheads). Higher magnification views of control (D’, D”) and lung-specific Tbx4 heterozygous; Tbx5 null (F’, F’’) tracheas. Yellow arrowheads indicate alcian blue positive mucus-producing cells in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas. TL, Tracheal lumen; RBL, Right bronchial lumen; LBL, Left bronchial lumen. (H–S) Genes important for chondrogenesis and smooth muscle development. Sox9 expression at E12.5 showed a comparable ventral expression pattern in control (H) and lung-specific Tbx4 heterozygous; Tbx5 null tracheas (I). At E13.5, Sox9 positive cells begin to condense and appear in a ring like pattern in controls (J) but not in the mutant tracheas (K). Sox6 (L,M) and Sox5 (N,O) expression was downregulated in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas at E13.5. Col2α1 was expressed in its characteristic ring like pattern (P) similar to Sox9 in the control tracheas but there were fewer rings with apparently normal expression in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (Q). SM22α expression was analyzed on the dorsal trachea at E13.5 in the control (R) and in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (S). Arrowheads in (S) point to ectopic expression in an uncharacteristic pattern in the mutants. Scale bars represent 100 μm.

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**Tbx4 and Tbx5 interactions in tracheal/bronchial cartilage development**

Appropriate dorsal smooth muscle development and ventral tracheal/bronchial cartilage development is important for the normal functioning of the trachea. Smooth muscle provides tracheal flexibility and the cartilaginous rings prevent tracheal collapse. Defects in trachea formation but there were fewer rings with apparently normal expression in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (Q). SM22α expression was analyzed on the dorsal trachea at E13.5 in the control (R) and in the lung-specific Tbx4 heterozygous; Tbx5 null tracheas (S). Arrowheads in (S) point to ectopic expression in an uncharacteristic pattern in the mutants. Scale bars represent 100 μm.

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**Tbx4 and Tbx5** heterozygous tracheae have 10–11 cartilage rings but Tbx4; Tbx5 double heterozygous tracheae have 6–8 cartilage rings, some of which are incomplete. Additional reduction of Tbx4 and Tbx5 in the lung-specific Tbx4 heterozygous; Tbx5 null lungs leads to a complete disruption of cartilage ring formation supporting a genetic interaction between Tbx4 and Tbx5 in trachea formation.

Sox9, a master regulator of the process of chondrogenesis, is expressed normally at E12.5 throughout the ventral mesenchyme in lung-specific Tbx4 heterozygous; Tbx5 null tracheae but at E13.5 these tracheae show a lack of characteristic mesenchymal condensations.
and reduced Sox9 expression. Either Tbx4 and Tbx5 control of Sox9 expression becomes more sensitive to dosage at E13.5 or Tbx4 and Tbx5 control another factor, possibly an ECM molecule, which is important for formation of mesenchymal condensations and, in the absence of these condensations, there is a down regulation of Sox9 expression (Figure 9C). Expression of Sox5 and Sox6, genes genetically downstream of Sox9 and important for condensation and cartilage formation, is downregulated in lung-specific Tbx4 heterozygous; Tbx5 null tracheas, concordant with an aberration in the process of chondrogenesis.

While Tbx4 and Tbx5 regulate lung branching by controlling Fgfl0 signaling, their control of tracheal/bronchial cartilage formation is independent of Fgfl0 signaling. Fgfl0 homozygous mutants have 6–8 tracheal cartilage rings, although the spacing between them is reduced and the rings do not always form the characteristic C shape [8,9,22]. Neither Tbx4; Fgfl0 double heterozygous tracheas nor Tbx5; Fgfl0 double heterozygous tracheas show cartilage condensation defects or a reduction in the number of cartilage rings. In contrast, Tbx4; Tbx5 double heterozygotes show a shorter trachea with 6–8 tracheal cartilage rings suggesting Tbx4 and Tbx5 interact with each other during tracheal formation but do not interact with Fgfl0. The triple heterozygous tracheas do not show an exacerbation of the Tbx4; Tbx5 double heterozygous trachea phenotype. Additionally, aberrant Fgfl0 signaling has been shown to affect tracheal cartilage formation and loss of Fgfl0 expression but not Sox9 expression [22]. In contrast, in the Tbx4 and Tbx5-deficient mutants Shh expression appears to be unaffected whereas Sox9 expression is reduced at E13.5. Hence, Tbx4 and Tbx5 control tracheal/bronchial cartilage formation via Sox9, independent of the Fgfl0 signaling pathway.

**Materials and Methods**

**Mouse strains, crosses, and embryo collection**

Mice carrying the following alleles were genotyped as previously described: a Tbx4 conditional ‘floxed’ allele, Tbx4tm1.2Pa [55], hereafter referred to as Tbx4f; a Tbx5 conditional floxed allele, Tbx5tm1.2Jse [56], hereafter referred to as Tbx5f; an Fgfl0 null allele [8]; ROSA26CreER;ERT2, a ubiquitous tamoxifen-inducible cre transgene [57], hereafter referred to as CreER, Tbx4;cre, an insertion into the endogenous Tbx4 allele resulting in a bicistronic allele that expresses both cre and Tbx4 in all of Tbx4 expression including lung and trachea [37,38], hereafter referred to as Tbx4fcre; and a R26RlacZ reporter [58]. All lines of mice were kept on mixed genetic backgrounds. Embryos were dissected from timed matings and yolk sacs were removed for PCR genotyping. The dark period was 19:00 to 05:00 hours and the day of a mating plug was identified as E0.5. All mouse work was carried out under Columbia University Medical Center Institutional Animal Care and Use Committee guidelines.

**Tamoxifen injections**

Tamoxifen (Sigma) at a concentration of 20 mg/ml in sunflower oil (Sigma) was administered to pregnant females by intraperitoneal injection between 15.30 and 19.30 hours on E9.0 or between 23.00 and 24.00 hours on E9.0.

**In situ hybridization, immunohistochemistry, and histology**

Whole-mount ISH, immunohistochemistry (IHC), immunofluorescence (IF) and ISH on cryosections was performed as described previously [59,60,61]. Primary antibodies used were anti-PECAM (Pharmingen, catalog number 01951D), anti-E-cadherin (Takara clone ECCD-2), anti T1a (Developmental Studies Hybridoma Bank antibody 8.1.1) and anti Prosurfactant Protein C (Millipore catalog number AB3786). All secondary antibodies were either peroxidase-conjugated donkey IgG from Jackson Immunochemicals or Alexa Fluor 488 from Invitrogen. For histology embryos were removed from the uterine horns, dissected out of the decidua and fixed in Bouin’s fixative (Sigma). After dehydration in ethanol, embryos were embedded in paraffin wax, sectioned at 8 μm thickness and stained with hematoxylin and cosin (H & E).

**Alcian blue staining**

Alcian blue staining was performed according to standard protocols [62]. Lungs and trachea were dissected out at different stages, fixed in Bouin’s fixative and washed with 70% ethanol. The tissue was then equilibrated in 5% acetic acid and stained with 0.05% alcian blue in 5% acetic acid for 2 hours. The tissue was washed in 5% acetic acid to remove excess stain and dehydrated in 100% methanol, cleared in BABB (benzyl alcohol, benzyl benzoate) and photographed. For alcian blue staining on sections,
8 µm paraffin sections were rehydrated, treated with 0.05% alcian blue in 5% acetic acid and then counterstained with nuclear fast red.

Foregut and lung bud culture

Foregut culture was carried out as described previously [34]. Foreguts were isolated from 8–16 somite stage embryos using tungsten needles and cultured at 37°C in the presence of 95% air and 5% CO2 on Transwell-Col filters (Fisher Scientific) containing 1.5 ml BGJb media (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.2 mg/ml vitamin C (Sigma) and 2 µM 4-OH tamoxifen (Sigma). For lung bud culture, lung buds were dissected at E11.5 in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (Sigma) and cultured in media containing DMEM (Invitrogen) with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen) and 1 µM 4-OH tamoxifen on 3.0 µm filters (Millipore) or 0.4 µm Transwell filters (Fisher Scientific). Similar results were obtained using both types of filter; results reported are for experiments with Millipore filters. Where specified, Fgf10 (R&D) was added after 1 day of culture at a concentration of 500 ng/ml. In some experiments heparin beads coated with Fgf10 (100 µg/ml) or BSA (100 µg/ml) were placed near the branching tips of the explants after 1 day of culture. Transwell filters were used for the bead experiments.

Lung branching analysis

Lungs were stained with either E-cadherin antibody using IHC or E-cadherin RNA probe using ISH. In case of whole mount lungs, lobes were separated and photographed to count the number of branching tips. The cultured lungs were photographed and the branching tips were counted. For some experiments, after E-cadherin ISH, lungs were post fixed in 4% PFA, washed with PBT, dehydrated in 100% methanol, cleared in BABB and then photographed.

Supporting Information

Figure S1  Analysis of excision efficiency of Tbx4fl and Tbx5fl. Females carrying embryos with Tbx4fl, Tbx5fl and CreER alleles were injected with either 8 mg tamoxifen at E9.0 and dissected at E12.5 (A,E) or 7 mg tamoxifen at E8.5 and dissected at E13.5 (B,F). Tbx4fl/fl was completely excised to mutant Tbx42/2 at both doses and times (A,B) whereas the single conditional allele of Tbx5fl/+ was incompletely excised by injection at E9.0 (E) but completely excised by injection at E8.5 (F). In addition when lungs with the genotype Tbx4fl/fl; Tbx5fl/fl; CreER were treated with 1 µm tamoxifen in culture, the Tbx4 locus was nearly completely excised at the end of 1 day of culture (C) but the Tbx5 locus was only

Figure 9. Model for the role of Tbx4 and Tbx5 in lung and trachea development. (A) Lung and trachea specification begins at E9.0 in the ventral foregut and at this time Tbx5 expression (light purple) is adjacent to the presumptive endoderm. Later, Tbx4 and Tbx5 expression (dark purple) is in mesenchyme associated with the lung and trachea. Tbx5 but not Tbx4 is important for specification of bilateral lung buds and the trachea. (B) Magnification of box shown in (A) representing the events in the growing tip during branching morphogenesis. Grey denotes epithelium and purple denotes mesenchyme. Tbx4 and Tbx5 interact with each other and act upstream of the Fgf10 signaling pathway. Decrease in Tbx4 and Tbx5 affects mesenchymal Fgf10 expression and expression of its targets in the epithelium – Bmp4, Spry2 and Etv5 – but not the expression of the epithelial Fgf10 receptor Fgfr2. In addition to Fgf10 expression in the mesenchyme, Tbx4 and Tbx5 also control the expression of an unknown factor(s) (X) that is essential for activation of the Fgf10 signaling pathway. Furthermore, Tbx4 and Tbx5 act upstream of Wnt2 in the mesenchyme. (C) In the trachea and the main stem bronchi Tbx4 and Tbx5 either control Sox9 expression, which in turn regulates cartilage condensation, or Tbx4 and Tbx5 regulate another factor (X) essential for chondrogenesis secondarily affecting Sox9 expression.

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partially excised (G). At the end of a 4 day culture both Tbx4 (D) and Tbx5 (H) loci achieved virtually complete excision, II, floxed conditional PCR band; WT, wild type PCR band; Mut, excised PCR band. (TIF)

**Figure S2** Loss of Tbx4 and Tbx5 does not affect alveolar differentiation. (A,B) T1α IHC on cryosections of control and lung-specific Tbx4 heterozygous; Tbx5 null lungs at E18.5 shows comparable staining in the lung epithelium. Nuclear fast red was used as a counter stain. A' and B' are higher magnification views of boxed regions in A and B. (C,D) Prosurfactant protein C (ProSPC) IF on cryosections of control and lung-specific Tbx4 heterozygous; Tbx5 null lungs at E18.5 shows comparable staining in the lung epithelium. DAPI was used to stain the nuclei. Scale bars represent 100 μm. (TIF)

**Figure S3** Loss of Tbx4 and Tbx5 has multiple affects on branching morphogenesis. (A,B) H&E sections of lung-specific Tbx5 null lungs at E18.5 shows lack of separation of the cranial (cr), medial (m) and caudal (cd) lobes in the right lung as compared to control lungs (A). Black arrowheads point to the space created between the lobes in the control lungs (A) and to the corresponding regions of the mutant lungs (B). (C,D) Control lungs stained for E-cadherin at E13 show greater outgrowth (yellow parentheses) of lateral branches (C) than the lung-specific Tbx4 heterozygous; Tbx5 null lungs (D). Scale bars represent 100 μm. (TIF)

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**Author Contributions**

Conceived and designed the experiments: RA VEP. Performed the experiments: RA VEP. Analyzed the data: RA VEP RJM. Contributed reagents/materials/analysis tools: RA VEP. Wrote the paper: RA VEP RJM.

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