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A genome-scale study of transcription factor expression in the branching mouse lung

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Abstract

Background—Mammalian lung development consists of a series of precisely choreographed events that drive the progression from simple lung buds to the elaborately branched organ that fulfills the vital function of gas exchange. Strict transcriptional control is essential for lung development. Among the large number of transcription factors encoded in the mouse genome, only a small portion of them are known to be expressed and function in the developing lung. Thus a systematic investigation of transcription factors expressed in the lung is warranted.

Results—To enrich for genes that may be responsible for regional growth and patterning, we performed a screen using RNA *in situ* hybridization to identify genes that show restricted expression patterns in the embryonic lung. We focused on the pseudoglandular stage during which the lung undergoes branching morphogenesis, a cardinal event of lung development. Using a genome-scale probe set that represents over 90% of the transcription factors encoded in the mouse genome, we identified sixty-two transcription factor genes with localized expression in the epithelium, mesenchyme or both. Many of these genes have not been previously implicated in lung development.

Conclusions—Our findings provide new starting points for the elucidation of the transcriptional circuitry that controls lung development.

Keywords

lung; mouse; transcription factors; expression patterns; branching

Introduction

The mammalian lung is essential for survival starting at birth. It is a prominent branching organ, with an endoderm-derived epithelium supported by a mesoderm-derived mesenchyme. In the mature lung, the epithelial tubules assume the shape of a tree-like structure, where the proximal portion forms the conducting airways and the distal portion forms the gas-exchanging alveoli. The mesenchyme consists primarily of the vasculature, smooth muscles, and tracheal/bronchial cartilage that forms intimate contacts with the

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epithelial tree and are also critical for respiration (Cardoso and Whitsett, 2008; Shi et al. 2009; Warburton et al. 2010).

In mice, lung development and maturation can be divided into five stages: embryonic bud initiation (Embryonic Day, or E9.0–E11.5), pseudoglandular (E11.5–E16.5), canalicular (E16.5–E17.5), saccular (E17.5–P5), and alveolar (P5–P28) stages (Maeda et al. 2007). The first sign of respiratory specification is marked by the expression of a transcription factor encoding gene, *Nkx2-1* in a small group of ventral foregut endoderm cells at E9.0 (Lu et al. 2004). Signals from the surrounding cardiac and prospective lung mesenchyme are critical for this specification (Domyan and Sun 2011). The first morphologically distinct respiratory structure, the primary lung buds, are observed at E9.5. From E9.5–11.5, these buds elongate and undergo secondary branching to establish the basis of one left lung lobe and four right lung lobes. The bulk of branching morphogenesis occurs during the pseudoglandular stage, and follows a strict program (Metzger et al. 2008). Towards the end of this stage and continuing into the canalicular stage, the conducting airway epithelium differentiates into specialized cell types, including Clara and ciliated cells (Rawlins et al. 2006). Drastic changes occur in the saccular stage as the lung transitions from being fluid filled to air breathing. The distal epithelium undergoes saccular branching and subdivides into primary alveolar sacs that allow for gas exchange. Meanwhile, the vascular network surrounding the alveolar sacs remodels to facilitate oxygen uptake. Within the distal epithelium, type II pneumocytes mature and start to produce surfactants, a protein and lipid mixture that lowers surface tension within the lung and allows lung expansion at birth (Whitsett and Weaver 2002). The expression of channel proteins also increases at birth, leading to rapid clearing of the fluid from the lung. While the lung can carry out gas-exchange at the end of saccular stage, it only contains approximately 5% of the final alveolar surface area. During alveologensis, thousands of secondary septae rise from the walls of the primary alveolar sac into the lumen; each serving as a new surface for gas-exchange (Mund et al. 2008).

Transcription factors have been shown to play critical roles in lung development (Warburton et al. 2000; Whitsett and Matsuzaki 2006; Maeda et al. 2007). They control diverse processes, including epithelial branching, epithelial and mesenchymal cell differentiation, surfactant and channel gene expression, and septae formation. Thus studies of transcription factor function in lung development have been a fruitful direction of research in the field. There are over 1,400 transcription factors encoded in the mouse genome. To date only a small fraction of these are known to be expressed and play a role in the lung. A systematic investigation of transcription factor expression in the lung is necessary, as it will help to elucidate the genetic control of lung development.

We performed an RNA *in situ* hybridization screen to identify transcription factor genes expressed within the branching mouse lung. There are several previously published expression screens in the branching lung (Liu and Hogan 2002, Lu et al. 2004, Lu et al. 2005, Metzger et al. 2007). Some of these focus on genes differentially expressed in particular regions of the wildtype lung (Liu and Hogan 2002, Lu et al. 2004), while others focus on genes that are regulated by specific signals essential for branching, such as FGF (Lu et al. 2005, Metzger et al. 2007). In addition to these studies, there are public databases such as GenePaint (www.genepaint.org) where gene expression patterns in tissue sections are available on a genome scale (Visel et al. 2004). However, these large-scale databases are not focused on any particular tissue. Therefore, annotation of the expression pattern is not always precise. For example, in GenePaint, *Sox17* is listed as not expressed in E14.5 lung, while it is clearly expressed at this stage and plays a critical role in lung development (Park et al. 2006).

To enrich the existing datasets, we utilized a mouse transcription factor probe collection that represents approximately 90% of all transcription factors encoded in the mouse genome (Gray et al. 2004). We conducted wholemount *in situ* hybridization using over 1,100 probes on E13.5 mouse lungs. Using a stringent standard, we identified sixty-two genes that are expressed in a subset of the cells within the lung epithelium, mesenchyme, or both. It should be noted that not all transcription factor genes are represented in the cDNA collection. Furthermore, since one probe per gene was screened, it is likely that a fraction of the probes do not produce the optimal hybridization signal with the specific transcript. Due to these limitations, our data only account for a subset of the transcription factor genes expressed in the branching lung. However, as many of the genes identified in our screen have not been previously implicated in lung development, our findings make an important contribution to existing gene expression datasets in the branching lung.

Results/Discussions

We chose to carry out our screen in E13.5 lungs for several reasons. First, a meticulous mapping of the lung epithelial ductal system during development shows that epithelial branching follows a stereotypical program, suggestive of strict genetic control including transcriptional regulation (Metzger et al. 2008). E13.5 is a stage in the middle of pseudoglandular branching, and is thus representative of the process. Second, several key signaling pathways that are essential for lung branching, including the fibroblast growth factor (FGF), sonic hedgehog (SHH) and bone morphogenetic protein (BMP) signaling pathways, are all active in the E13.5 lung. Members of these pathways are known to influence each other's transcription through feedback loops, yet few transcription factors that mediate these regulations have been identified. Third, E13.5 is a stage when active patterning of the epithelium and mesenchyme occurs. These broad patterns then serve as blueprints for fine-scale cellular differentiation that occurs at later stages of lung development. Finally, we chose to carry out an *in situ* screen because we are particularly interested in genes expressed in subsets of cells in the lung as they may direct regional growth and patterning events. We determined that E13.5 is the latest, and therefore most advanced stage when distinct patterns can still be consistently discerned from wholemount *in situ* hybridization.

As we are interested in genes that may drive regional patterning and morphogenesis in the lung, we focused on genes that show restricted expression patterns. Overall, we identified sixty-two genes with differential expression in distinct sub-regions of the lung (Table 1, Figs. 1–4). Some of these factors have previously been shown to play a role in lung development (Maeda et al. 2007). However, the majority of these genes do not have a well-characterized association with the branching lung. We grouped these genes into four categories to present their expression patterns and discuss known or potential functions. The first three categories are distinguished based on their expression site: genes that are expressed solely in the epithelium (Category 1) or mesenchyme (Category 2), and genes that are expressed in subregions of both tissue layers (Category 3). Hox genes are presented as a separate group (Category 4) to facilitate a direct comparison of their overlapping expression patterns (Fig. 4). For each gene, expression patterns in the wholemount lung were confirmed by the patterns in vibratome sections.

Category 1-genes expressed in the lung epithelium

Twenty-three transcription factor genes were identified as having epithelial specific expression patterns in the lung (Fig. 1A–W). We found more than one member in each of the following families containing specific domains: homeobox, zinc finger, forkhead box (FOX), SRY box (SOX), and E-twenty six (ETS).

Homeobox containing: *Irx3*, *Nkx2-1*, *Nkx2-9*, *Nkx1-2*, *Hnf1b*, and *Mnx1*—

Homeobox containing transcription factors are essential for patterning throughout the developing embryo. In the E13.5 lung, we identified five genes belonging to this family that are expressed solely in the epithelium (Fig. 1A–F). Of these, only *Irx3* and *Nkx2-1* have been previously shown to play roles in embryonic lung development.

The Iroquois homeobox (*Irx*) transcription factor family consists of six members that have extensive overlaps in expression in the developing mouse embryo (Bosse et al. 1997; Christoffels et al. 2000; Cohen et al. 2000; Houweling et al. 2001). At E13.5, *Irx3* expression is detected at a high level in the distal portion of the lung epithelium (Fig. 1A). Three other *Irx* transcription factors (*Irx1,2,5*) are also expressed in the lung epithelium in a similar pattern as *Irx3* (Becker et al. 2001; Van Tuyl et al. 2006; Diez-Roux et al. 2011). No lung abnormalities have been reported in any of the existing *Irx* single gene mutant mice (Lebel et al. 2003). However, knockdown of all *Irx* transcription factors using antisense oligonucleotides in lung explants causes branching defects (Van Tuyl et al. 2006), suggesting that *Irx* factors may act redundantly to regulate lung branching.

Three *Nkx* transcription factors were identified in our screen, *Nkx2-1*, *Nkx2-9*, and *Nkx1-2* (Fig. 1B–D). *Nkx2-1* (also termed *TTF-1*) is one of the earliest genes expressed in the nascent respiratory lineage (Lazzaro et al. 1991). Knockdown or knockout of *Nkx2-1* leads to early lung branching arrest (Minoo et al. 1995; Minoo et al. 1999). However, lung buds form in each case, suggesting that *Nkx2-1* is dispensable for lung budding, but essential for secondary branching morphogenesis. Furthermore, *Nkx2-1* mutants lack the trachea, suggesting that this gene is essential for trachea specification (Minoo et al. 1999; Que et al. 2007). *Nkx2-9* knockout mice form functional lungs and survive to adulthood, but show tracheobronchial epithelial hyperplasia (Tian et al. 2006). Further analysis of the *Nkx2-9* mutants demonstrates that *Nkx2-9* maintains the cellular architecture of the upper airway by restricting cell proliferation in the epithelium. Little is known regarding the role of *Nkx1-2* in development, including in the lung. No knockout mouse line has been reported. In the *Foxa2* mutant mouse gastrula, *Nkx1-2* expression is downregulated (Tamplin et al. 2008). Since *Foxa2* is critical for lung epithelial differentiation and alveologenesis (Wan et al. 2005), these results raise the possibility that *Nkx1-2* may function downstream of *Foxa2* in the lung.

The roles of either *Hnf1b* or *Mnx1* in lung development have not been closely interrogated. Within the branching lung, *Hnf1b* shows higher expression in the distal region of the lung epithelium, while *Mnx1* expression appears present in both the proximal and distal regions (Fig. 1E,F). Homozygous null mutants of *Hnf1b* (also known as *vHnf1* and *Tcf2*) are lethal at the early post-implantation stage due to defects in extra-embryonic endoderm differentiation (Barbacci et al. 1999; Coffinier et al. 1999). Using a conditional allele, it has been shown that inactivation of *Hnf1b* in the bile duct led to a change from cuboidal to pseudostratified squamous epithelium (Coffinier et al. 2002). Conditional inactivation of *Hnf1b* within the kidney results in a polycystic kidney phenotype, suggesting that *Hnf1b* is essential in controlling planar cell polarity (Fischer et al. 2006; Verdeguer et al. 2010). A recent study has demonstrated the importance of planar cell polarity in lung development (Yates et al. 2010), raising the possibility that *Hnf1b* may play a role in this process in lung. *Mnx1* (also known as *Hb9*) knockout mice die at birth due to an inability to inflate the lung (Arber et al. 1999). However, it remains unresolved whether this is the result of a lung-specific defect or a neuronal defect in diaphragm innervation. In the epithelium of other tissues, such as the pancreas, *Mnx1* is known to regulate cell differentiation, raising the possibility that *Mnx1* may regulate a similar process within the lung (Harrison et al. 1999; Li et al. 2001).

Zinc finger containing: *Gata6*, *Lmo1*, *Zfp746*, and *Tcea2*—Four zinc finger containing transcription factors were found to be expressed within the E13.5 lung epithelium: *Gata6*, *Lmo1*, *Zfp746*, and *Tcea2* (Figure 1G–J). Of these, only *Gata6* has been shown to be essential for lung development (Keijzer et al. 2001; Yang et al. 2002; Liu et al. 2002; Zhang et al. 2008). Lung epithelium-specific inactivation of *Gata6* leads to defects in branching morphogenesis and cell differentiation.

Homozygous *Lmo1* null mutants are viable and show no obvious defects including in the lung (Tse et al. 2004). This lack of a phenotype within the lung may reflect redundancy with other *Lmo* transcription factors, as several other *Lmo* members are also expressed in the lung, including *Lmo4* and *Lmo7* (Sum et al. 2005; Ott et al. 2008). In neural tissues such as the hindbrain, LMO factors regulate proximal-distal patterning (Matis et al. 2007). This raises the possibility that LMO factors may function collectively to control lung epithelium patterning.

Zfp746 (also termed *PARIS*) has not been shown to be expressed or function within the developing lung. A recent study using overexpression and knockdown techniques show that *Zfp746* promotes neural degeneration in Parkinson's disease models (Shin et al. 2011). No *Zfp746* mutant mouse has been reported.

Tcea2 is expressed within the distal region of the lung epithelium and is one of two transcriptional elongation factors identified within this screen (together with *Tcea1*, see below). Existing evidence suggests that *Tcea2* promotes transcription by preventing the arrest of RNA polymerase (Reines et al. 1996). It remains unclear how this molecular role impacts tissue development, as no *Tcea2* mutant mice have been reported. It is interesting to note that *Tcea2* is not expressed in the adult lung (Umehara et al. 1997), suggesting that it may act specifically in cells and tissues that are undergoing active proliferation and expansion.

FOX domain containing: *Foxa1*, *Foxd4*—A number of FOX domain transcription factors are expressed in the developing lung (Maeda et al. 2007). Our screen highlights two that are detected within the lung epithelium at E13.5, *Foxa1* and *Foxd4* (Fig. 1K,L). *Foxa1*, along with the closely related *Foxa2*, has been shown to be critical for branching and epithelial differentiation in the lung (Wan et al. 2004a; Wan et al. 2004b; Wan et al. 2005). Inactivation of *Foxa1* and *Foxa2* in the mouse lung epithelium leads to fewer, and more dilated epithelial branches than the control. Furthermore, markers of differentiated cell types in both the proximal airway (CC10 or SCGB1A1, and FOXJ1) and distal alveoli (SFTPC and SFTPB) are no longer expressed in the absence of these FOX factors, suggesting that *Foxa1*; *Foxa2* are required broadly in the lung epithelium for cell differentiation. On the protein level, it has been shown that FOXA1 interacts with NKX2-1 to regulate the expression of *Sftpc* in the lung epithelium (Minoo et al. 2007). Moreover, examination of major signaling pathways essential for lung development suggests that *Foxa1* and *Foxa2* act to control the expression of *Shh* in the lung epithelium (Wan et al. 2005).

While *Foxd4* has been shown to be expressed at a low level in the adult lung (Kaestner et al. 1993), its expression has not been reported in the embryonic lung (Fig. 1L). In yeast, *Foxd4* controls the cell cycle by regulating the G2/M transition (Kumar 2000). Whether it plays a similar role in mice has not been tested, and no knockout mouse has been reported. Outside of the lung, in the gastrula stage embryo, the expression of *Foxd4* is positively regulated by FOXA2 (Tamplin et al. 2008). Thus it is possible that *Foxd4* may function downstream of *Foxa2* in the lung.

Although not identified by probes used in our screen, several other FOX domain transcription factors are expressed within the developing lung epithelium, including the Foxp sub family (Lu et al. 2002). *Foxp1*, *Foxp2* and *Foxp4* exhibit overlapping expression patterns within the lung epithelium and mesenchyme (Shu et al. 2001; Lu et al. 2002). Inactivation of *Foxp1* and one allele of *Foxp2* (*Foxp1*^{-/-};*Foxp2*^{+/-}) produces a smaller lung due to a decrease in cell proliferation (Shu et al. 2007). At later stages these mice also develop a simplified alveolar network. FOX factors act mainly as transcriptional repressors and this effect is mediated through interaction with HDAC proteins (Chokas et al 2010). In the adult lung, double heterozygous *Foxp1*^{+/-};*HDAC2*^{+/-} mice are resistant to hyperoxia-induced lung injury compared to control. These data together suggest that Foxp genes play critical roles in both lung development and homeostasis.

ETS domain containing: *Etv5* and *Elf5*—Two genes encoding ETS domain proteins, *Etv5* and *Elf5*, were identified as being expressed solely in the distal lung epithelium (Fig. 1M,N). Both have been shown to be positively regulated by FGF signaling, which could explain their expression within the distal epithelium, as *Fgf10* is strongly expressed within the distal mesenchyme of the branching lung and signals to the adjacent epithelium (Liu et al. 2003; Metzger et al. 2007). ETV5 belongs to the three-member Pea3 subgroup of ETS domain containing proteins (ETV1 also known as ER81, ETV4 also known as PEA3, and ETV5 also known as ERM), which share homology not only in the ETS domain, but also throughout the rest of the protein. Expression of a dominant-negative form of *Etv5* in the epithelium of late branching stage lung causes proximal-distal patterning defects (Liu et al. 2003). No overt lung phenotype was found in *Etv5* loss-of-function mutants (Chen et al. 2005), likely due to redundancy with *Etv1* and *Etv4*.

Overexpression of full-length form of *Elf5* in the distal epithelium of the lung causes dilated branching tips, and disrupted type II cell differentiation (Metzger et al. 2008a). On the molecular level, *Elf5* overexpression leads to decreased expression of *Etv5* and increased expression of *Spdef*, another ETS family gene. This result suggests intricate feedback regulation among ETS family members. Homozygous null mutants of *Elf5* die by E7.5, prior to lung initiation (Zhou et al. 2005). Lung epithelium-specific inactivation of *Elf5* produces no overt lung development defects (X. Sun unpublished results), possibly due to redundancy with other ETS containing genes expressed in the lung epithelium.

SRY box containing: *Sox2* and *Sox9*—Two genes encoding SOX domain containing transcription factors, *Sox2* and *Sox9*, were identified as being expressed in the epithelium (Fig. 1O,P). They are known to exhibit complementary expression patterns in the epithelium, with *Sox2* expression localized to the proximal epithelium and *Sox9* expression localized to the distal epithelium. Conditional inactivation of *Sox2* within the respiratory epithelium leads to a decrease in ciliated and Clara cells in both the trachea and bronchiole, but an increase in goblet cells in the trachea and a decrease in goblet cells in the bronchiole (Que et al. 2009; Tompkins et al. 2009). Furthermore in the adult, loss of *Sox2* in the trachea disrupts its ability to repair after sulfur dioxide (SO₂)-induced epithelial damage, possibly due to a reduced number of basal stem cells in the mutant epithelium (Que et al. 2009). Similarly, loss of *Sox2* in the adult bronchiole disrupts the increase in goblet cell differentiation and mucus production in response to allergen challenge (Tompkins et al. 2009). Compared to these loss-of-function phenotypes, overexpression of *Sox2* in the lung epithelium causes altered branching morphogenesis, proximalization of epithelial cell fate, and a slight decrease in cell proliferation (Gontan et al. 2008).

Inactivation of *Sox9* in the lung epithelium does not result in any reported defects (Perl et al. 2005), suggesting that by itself, *Sox9* is not required in the epithelium for lung development. However, *Sox9* is also expressed in the proximal mesenchyme, specifically in the precursor

and differentiated cartilages of both the trachea and main bronchi (Elluru et al. 2004). As *Sox9* is a key gene essential for cartilage development (Bi et al. 2001), it likely plays a role in airway cartilage ring formation.

Miscellaneous epithelial genes: *Kat2b*, *Ash2l*, *Tfap2c*, *Nab1*, *Id2*, *Irf6*, and *Grhl2*—Seven of the epithelium-expressed transcription factors, *Kat2b*, *Ash2l*, *Tfap2c*, *Nab1*, *Id2*, *Irf6*, and *Grhl2*, were identified in our screen as the sole member of their respective families (Fig. 1Q–W). The roles of these genes in lung development are largely unknown.

Kat2b—*Kat2b* (also known as *Pcaf*) is a bromodomain-containing acetyl transferase. *Kat2b* knockout mice survive postnatally, and no lung defect has been reported (Yamauchi et al. 2000). The absence of an apparent lung phenotype may be due to redundancy with a close family member *Kat2a*. In support of this possibility, while *Kat2a* is expressed at a relatively low level in the wild-type lung, its level increases dramatically in the *Kat2b* mutant lung (Yamauchi et al. 2000). As *Kat2a* global knockout mice die prior to E11.5, identification of a possible combined role of *Kat2a* and *Kat2b* in lung awaits the generation of tissue-specific double mutants. A recent study identifies *Kat2b* as a potential target of miR302/367, two GATA6-regulated miRNAs that control saccular branch patterning and the balance between proliferation and differentiation in the lung (Tian et al. 2011). Whether misexpression of *Kat2b* contributes to the defects in the miR302/367 overexpression or knockdown lungs has yet to be established.

Ash2l—*Ash2l* is a SPRY domain containing methyl-transferase that promotes the trimethylation of lysine 4 of histone 3 (H3K4), an epigenetic signature of actively transcribed regions (Steward et al. 2006). RNAi knockdown of *Ash2l* in HeLa cells causes decreased trimethylation of H3K4, and decreased transcription of target genes (Steward et al. 2006). *Ash2l* knockout mice die at a stage when lung buds first initiate (Stoller et al. 2010), and its role in lung development has not been studied. Despite this, *Ash2l* has been shown to regulate the expression of several genes implicated in lung development. For example, in muscle differentiation, *Ash2l* regulates the expression of *MyoG* (Rampalli et al. 2007). Previous studies show that *MyoG* is required in the lung for transition from pseudoglandular to saccular stages (Tseng et al. 2000). *Ash2l* has also been shown to regulate the expression of *Fgfr3* in a neuroblastoma cell line (Tan et al. 2009). *Fgfr3*, together with *Fgfr4*, are required for lung alveologenesis (Weinstein et al. 1998; Srisuma et al. 2010). These data raise the possibility that *Ash2l* may act upstream of these factors during lung development.

Tfap2c—*Tfap2c* (also known as *Ap-2γ*) is an Ap2 family gene. The role of *Tfap2c* in the developing lung remains unknown. Knockout mice are lethal between E7 and E9, prior to lung budding (Werling et al. 2002). Conditional ablation of *Tfap2c* in the skin shows that *Tfap2c* is important for terminal differentiation of epidermal cells (Wang et al. 2008). This role is executed through an interaction with members of the NOTCH signaling pathway. In the lung, NOTCH signaling is essential for epithelial patterning and differentiation (Tsao et al. 2008; Guseh et al. 2009; Tsao et al. 2009; Morimoto et al. 2010). Furthermore, recent data show that CEBPα, a critical factor for the maturation of the distal epithelium, is a downstream target of TFAP2C (Martis et al. 2006; Xu et al. 2008). Thus, it is plausible that TFAP2C may interact with these genes to control lung development. It should be noted that another *Tfap2* transcription factor, *Tfap2* (also known as *Ap-2α*), is expressed within the lung epithelium (Visel et al. 2004). Double knockout mice for these two TFAP2 factors die prior to the lethality of either single mutant, suggesting that they function redundantly during development, possibly also in the lung (Winger et al. 2006).

Nab1—*Nab1* single mutant mice are viable, fertile, and no lung defect has been reported (Le et al. 2005). In contrast, double mutants of both *Nab1* and the related *Nab2* develop labored breathing. It is not clear if the respiratory defect results from the loss of *Nab1* and *Nab2* within the lung or in other tissues required for breathing, as histology of the double mutant lungs has not been reported (Le et al. 2005). On the molecular level, *Nab1* is a transcriptional repressor of early growth response proteins 1 and 2 (EGR1 and EGR2) (Swirnoff et al. 1998; Le et al. 2005). Loss of *Egr1* protects the lung from bleomycin-induced fibrosis, a mouse model for idiopathic pulmonary fibrosis (IPF) (Lee et al. 2004). Furthermore, *Egr1* has been implicated in mediating cell death in smoking-induced chronic obstructive pulmonary disease (COPD) (Chen et al. 2008). *Egr2* knockout mice develop increased breathing rates (Desmazieres et al. 2008). These findings suggest that *Nab1* and *Nab2* may control lung maturation through their ability to regulate the activity of *Egr1* and *Egr2*.

Id2—*Id2* is a member of the inhibitor of differentiation (Id) family of bHLH domain transcription factors. There are four Id transcription factor genes and all are expressed in the lung: *Id2* and *Id4* are expressed in the distal and proximal epithelium, respectively, while *Id1* and *Id3* are expressed in the mesenchyme (Fig. 1U, Fig. 2Q; Jen et al. 1996). Lineage tracing using an *Id2*-creER^{T2} has shown that *Id2*-expressing cells represent a multipotent progenitor population that contributes to both the proximal airway epithelial and the distal alveolar epithelial cell populations (Rawlins 2009). The expression of Id genes is regulated by BMP signaling (Miyazono and Miyazawa 2002; ten Dijke et al. 2003). Previous work has demonstrated that genetic inactivation of BMP signaling leads to trachea specification, proximal-distal lung patterning as well as lung cell differentiation defects (Bellusci 1996; Weaver et al. 1999; Eblaghie et al. 2006; Que et al. 2006; Li et al. 2008; Sun et al. 2008; Domyan et al. 2011). These results suggest that the Id genes may mediate the critical roles of BMP signaling in lung. However, there are no reported lung developmental defects associated with single Id gene mutants, including *Id2* mutants. These results suggest that either these Id genes function redundantly amongst themselves, or with other bHLH factors during lung development (Yokota et al. 1999).

Irf6—*Irf6* is an interferon regulatory factor whose human homolog has been associated with Van der Woude syndrome, a craniofacial disease characterized by the formation of a cleft lip (Kondo et al. 2002). *Irf6* is expressed throughout both the proximal and distal portions of the lung epithelium (Fig. 1V). *Irf6* knockout mice develop skin defects as a result of increased proliferation, and decreased terminal differentiation of epidermal progenitor cells (Ingraham et al. 2006). *Irf6* knockout mice also display esophageal closure and limb-tail fusion as a result of increased adhesion between epidermal layers. At the molecular level, *Irf6* has been shown to act as a downstream mediator of NOTCH signaling (Restivo et al. 2011). Since NOTCH signaling is critical for lung epithelial patterning and differentiation (Tsao et al. 2008; Guseh et al. 2009; Tsao et al. 2009; Morimoto et al. 2010), it is plausible that *Irf6* may also function downstream of NOTCH in the lung epithelium.

Grhl2—*Grhl2* is one of three Grainyhead homologs (*Grhl1-3*), and all are expressed within the developing lung epithelium (Fig. 1W; Auden et al. 2006). While *Grhl2* targeted knockout mutants die at the stage of development that precludes a study of their requirement in lung (Rifat et al. 2010), mice carrying a *Grhl2* point mutant hypomorphic allele exhibit reduced lung size and inability to inflate the lung at birth (Pyrgaki et al. 2011). In *Grhl2* point mutant lungs, cell adhesion proteins including E-Cadherin, β -Catenin, and Ezrin are detected at lower levels and/or mislocalized. Regulation of cellular adhesion molecules appears to be a general function of *Grhl2* during development, as a similar role was also found in the epidermis and the neural tube (Werth et al. 2010; Boglev et al. 2011). These

data together suggest that *Grhl2* is essential for lung development via controlling the adhesion properties of the lung epithelium.

Category 2-genes expressed in the lung mesenchyme

From our screen, twenty-three genes were identified as showing mesenchyme-specific expression patterns in the lung (Fig. 2A–W). Most of these factors have not been characterized for their role in early lung development. We found multiple transcription factors that contained the following domains: zinc finger, homeobox, SOX, ETS, bHLH, and FOX. Six of these factors, *Nr2f2*, *Hhex*, *Sox7*, *Sox18*, *Elk3*, and *Ets1*, are expressed in a pattern consistent with their general known roles in vasculature formation (Fig. 2E, I–M). Several of the rest of the factors are detected in the subepithelial mesenchyme portion of the lung (White et al 2006).

Zinc finger containing: *Gli2*, *Snai1*, *Baz1b*, *Nr2f1*, and *Nr2f2*—Five zinc finger containing transcription factors were identified within the mesenchyme (Fig. 2A–E). Of these, only *Gli2* is known to be required for early lung development, and is detected in the subepithelial mesenchyme (Fig. 2A). *Gli2*, together with *Gli1* and *Gli3*, are core components of the Hedgehog signaling pathway. Loss of *Gli2* leads to reduced lung branching (Motoyama et al. 1998). Further inactivation of *Gli3* in a *Gli2* knockout background (generating *Gli2*^{−/−};*Gli3*^{−/−} embryos) results in trachea and lung agenesis, suggestive of shared function (Motoyama et al. 1998).

Snai1 is abundantly expressed in the subepithelial mesenchyme of the lung (Fig. 2B). Its function in lung has not been directly assessed, as *Snai1* knockout mutants are lethal prior to E8.5 due to aberrant conversion of epiblast cells into mesoderm cells during gastrulation (Carver et al. 2001). As exemplified by its role during gastrulation, SNAI1 is part of the core machinery that promotes epithelial to mesenchymal transitions (EMT) throughout development as well as in postnatal events such as metastasis in tumorigenesis (Vincent et al. 2009). In normal lung development, a known EMT event is the transition of mesothelium into cells in the mesenchyme (Que et al. 2008). Whether *Snai1* plays a role in this event remains to be tested using conditional gene inactivation approaches. In pathological conditions of the lung, it has been shown that *Snai1* promotes EMT in lung epithelium-derived cell lines (Jayachandran et al. 2009). This suggests that *Snai1* may contribute to lung fibrosis in diseases such as Idiopathic Pulmonary Fibrosis (IPF) (Kim et al. 2006; Coward et al. 2010). However, a recent study throws into question the involvement of EMT in lung fibrosis (Rock et al 2011).

Baz1b has been shown to be expressed within the adult lung through Northern blot analysis (Peoples et al. 1998). Here, we localize its expression to the E13.5 lung mesenchyme (Fig. 2C). On the molecular level, BAZ1B controls transcription by interacting with a number of chromatin remodeling proteins including the SWI/SNF-type WINAC and ISWI-type WICH complexes (Oya et al. 2009). This interaction is regulated by MAPK signaling. In humans, *BAZ1B* deletions have been associated with the congenital disorder Williams Syndrome (WS), where patients develop supravalvular aortic stenosis along with neurological abnormalities (Williams et al. 1961; Lu et al. 1998; Riby and Porter 2010; Mohan et al. 2011). Consistent with disease symptoms, *Baz1b* knockout mice develop heart abnormalities, including atrial/ventricular septation defects and abnormal trabeculation (Yoshimura et al. 2009). However no lung phenotype has been reported in these mice.

Nr2f1 and *Nr2f2* (also known as *Coup-TfI* and *Coup-TfII*) encode zinc finger-containing steroid/thyroid hormone nuclear receptor proteins. In the lung, they are expressed in overlapping patterns with *Nr2f1* being more restricted to the subepithelial mesenchyme while *Nr2f2* being more widely expressed in the mesenchyme (Fig. 2D,E). *Nr2f1* has

primarily been shown to control neural development, where it regulates both the patterning of neural tissues as well as the differentiation of progenitor cells into mature neurons (Qiu et al. 1997; Faedo et al. 2008). Despite neural differentiation phenotypes, *Nr2f1* knockout mice survive postnatally, with no report of cyanosis or labored breathing. *Nr2f2* knockout mice show abnormal angiogenesis and heart patterning, and are lethal by E10.0 (Pereira et al. 1999). Conditional knockout of *Nr2f2* in the foregut mesentery using *Nkx3-2-cre* produces defects that mimic congenital diaphragmatic hernia (CDH), a human birth defect characterized by failed closure of the diaphragm, herniation of abdominal content into the chest cavity, and hypoplastic lungs (You et al. 2005). It remains to be determined whether this lung defect is due to a primary requirement for *Nr2f2* in lung, or is secondary to the mechanical pressure exerted by the herniating tissues on the lung.

Homeobox domain containing: Meis1, Pou3f1, Lhx6 and Hhex—We identified four homeobox containing genes that are expressed within the mesenchyme of the E13.5 lung: *Meis1*, *Pou3f1*, *Lhx6* and *Hhex* (Fig. 2F–I). While there are no reported role for the latter three factors in the developing lung, a previous study indicates that *Meis1* null mutants show hypoplastic lungs (Hisa 2004).

The *Meis* family of transcription factors is primarily recognized as Hox cofactors in their regulation of gene expression (Moens and Selleri 2006). In mice, all three *Meis* genes are expressed in the developing lung mesenchyme, with *Meis1* RNA being detected throughout the mesenchyme (Fig. 2F and data not shown). Multiple Hox genes are expressed in the lung mesenchyme and all are possible partners of *Meis* (Fig. 4). *Meis1* knockout mice die around E14.5 due to hematopoietic and microvascular defects, and exhibit hypoplastic lungs (Hisa et al. 2004). However the primary role of *Meis1* in lung development has not been determined.

Pou3f1 (also known as *Oct6*) is primarily known for its function in neuronal differentiation (Bermingham et al. 1996; Bermingham et al. 2002; Ghazvini et al. 2002). *Pou3f1* mutants are unable to form myelinating Schwann cells within the embryonic peripheral nervous system. These mutants die at birth due to respiratory distress but examination of the lungs revealed no gross defect. Instead, it was proposed that the respiratory deficiency is caused by a possible decrease in number and function of phrenic motor neurons, which may lead to failed diaphragm function during respiration (Bermingham et al. 1996). However, given its expression in the lung, a more thorough analysis of the lung defects in these mutants and a tissue-specific inactivation of *Pou3f1* in lung may be worthwhile to rule out a direct requirement for *Pou3f1* in lung development.

Lhx6 belongs to a large family of LIM homeodomain containing transcription factors. *Lhx6* knockout mice die shortly after two weeks of age due to unknown causes (Liodis et al. 2007). Whether these mice develop lung defects has not been shown. Functional redundancy among *Lhx* factors is observed during development. For example, during dentition, *Lhx6*;*Lhx7* double mutants display molar agenesis due to failed mesenchyme differentiation (Denaxa et al. 2009). Following the detection of *Lhx6* expression in lung (Fig. 2H), a search of existing databases revealed that *Lhx4*, *Lhx5*, and *Lhx9* are also expressed in the lung mesenchyme (Visel et al. 2004). These results suggest that *Lhx6* may function redundantly with other *Lhx* members to control lung mesenchyme development.

Hhex (also known as *Hex*) is expressed in the lung in a pattern that resembles the vascular network (Fig. 2I, Lazarus et al. 2011). *Hhex* knockout mice die prior to E14.5 due to abnormal development of the heart and vasculature (Keng et al. 2000; Hallaq et al. 2004). In the heart, *Hhex* mutants show abnormal outflow tract and valve formation as a result of persistent EMT of endocardial cells. This persistent EMT is thought to be mediated, at least

in part, by increased VEGFA protein levels. In the vascular network of *Hhex* knockout mice, blood vessels initiate normally. However, the vascular lumens become dilated, possibly due to a decrease in vascular smooth muscle. Whether *Hhex* mutants development any lung defects has not been reported.

SOX domain containing: Sox7 and Sox18—From our screen, we identified two genes encoding F subgroup SOX domain containing transcription factors, *Sox7* and *Sox18*. These genes, together with the remaining F subgroup member *Sox17*, are expressed in overlapping, but non-identical patterns in the vasculature (Fig. 2J,K) (Park et al. 2006; Sakamoto et al. 2007; François et al. 2010). For example, in a normal embryo, *Sox18*, but not *Sox7* or *Sox17*, is expressed in the lymphatic endothelial cells. Interestingly, in the absence of *Sox18*, *Sox7* becomes ectopically expressed in the lymphatics (Hosking et al. 2009). Consistent with this, these genes play redundant roles in endothelial cell differentiation in blood and lymphatic vessels in both mice and zebrafish (Cermenati et al. 2008; Sakamoto et al. 2007). In humans, a dominant-negative mutation in human *SOX18*, which likely interferes with the function of multiple SOX genes, is known to cause Hypotrichosis-Lymphedema-Telangiectasia, a congenital lymphatic disorder (Irrthum et al. 2003). The specific requirement for these genes in the lung vasculature has not been directly addressed.

ETS domain containing: Elk3, Ets1, and Ets2—We identified three ETS domain containing transcription factors that are expressed within the lung mesenchyme: *Elk3*, *Ets1*, and *Ets2* (Fig. 2L–N). While their function in the lung remains unclear, all three of these genes have been implicated in endothelial development in other tissues.

Elk3 (also known as *Net*) knockout mice survive past birth and show no lung defects (Ayadi et al. 2001). While these mutant mice do eventually die due to respiratory distress, the cause is not believed to be intrinsic to the lung, but rather due to a build up of chyle in the thoracic body cavity from a defective lymphatic system (Zheng et al. 2003; McGrath et al. 2010). In addition to its role in lymphatic development, *Elk3* has also been shown to be necessary for endothelial response to pro-angiogenic growth factors such as FGF2 during wound healing (Ayadi et al. 2001).

While *Ets1* knockout mice are viable, *Ets2* knockout embryos die around E8.5 due to a defect in extra-embryonic tissue development (Muthusamy et al. 1995; Yamamoto et al. 1998). Double mutant mice of *Ets1* and *Ets2* that utilize a null allele of *Ets1*, and either a partial loss of function allele or a conditional deletion of *Ets2* within the epiblast, are lethal at approximately E14.5 and display severe hemorrhaging due to a drastic decrease of embryonic vasculature (Wei et al. 2009). While no lung phenotypes have been reported in these mice, it is possible that these *Ets* genes play redundant roles in the formation of the pulmonary vasculature.

bHLH containing: Tcf21, Hif3a, Id3, and Heyl—We identified four bHLH family transcription factors that were expressed within the lung mesenchyme: *Tcf21*, *Hif3a*, *Id3* and *Heyl* (Fig. 2O–R). Of these, *Tcf21* and *Hif3a* have known roles in lung development.

Previous work has shown that *Tcf21* (also known as *Pod1*) is essential for lung branching morphogenesis (Quaggin et al. 1999). *Tcf21* knockout mice develop fewer epithelial branches compared to control lungs and these mutant branches do not go on to form terminal sacs. The lack of terminal sacs is likely due to proximalization of the lung epithelium, as evidenced by the expansion of the proximal marker *Scgb1a1* and reduction of the distal marker *Sftpc*. Additionally, *Bmp4* expression is decreased within the *Tcf21* mutant lung. Previous work has demonstrated that inhibition of BMP signaling results in a similar phenotype as the *Tcf21* knockout (Bellusci 1996; Weaver et al. 1999; Sun et al. 2008). Thus,

Tcf21 may control lung branching and proximal-distal patterning through promoting *Bmp4* expression.

Hif3a is a well-known hypoxia induced factor (HIF) that is expressed within the distal region of the lung mesenchyme (Fig. 2P). *Hif3a* knockout mutants show impaired alveologenesi s with an increase in the amount of vasculature lining the alveolar walls (Yamashita et al. 2008). Whether the vasculature defect is the mechanism underlying the alveolar phenotype remains to be determined.

Id3, similar to *Id2* mentioned above, is a member of the Id family of genes (Fig. 2Q). *Id3* knockout mice are viable and no lung defect has been reported (Pan et al. 1999). The lack of an apparent developmental phenotype is likely due to its redundancy with *Id1*, which shows overlapping expression patterns with *Id3* throughout the embryo, including in the mesenchyme of the lung (Jen et al. 1996). In support of this, *Id1;Id3* double knock out mice die by E13.5 and develop severe vascular and neural defects (Lyden et al. 1999). It is not clear whether these mutants exhibit lung defects. A recent study within the postnatal lung shows that *Id3* expression is increased in *Id1* knockout lungs, further suggesting that these two genes may share redundant function (Lowery et al. 2010). Given that their expression is regulated by BMP signaling (Miyazono and Miyazawa et al. 2002; Ten Dijke et al. 2003), it is possible that together they may mediate the essential role of BMP signaling in lung mesenchyme development.

Hey1 is a downstream component of the NOTCH signaling pathway (Nakagawa et al. 2000; Leimeister et al. 2000). As mentioned above, extensive work has demonstrated that the NOTCH signaling pathway regulates lung proximal-distal patterning and cell differentiation (Tsao et al. 2008; Guseh et al. 2009; Tsao et al. 2009; Morimoto et al. 2010). In the lung, *Hey1* expression is restricted to the vascular smooth muscle (Fig. 2R), likely overlapping with the expression of *Notch3*, which plays an essential role in vascular smooth muscle differentiation and maturation (Domenga et al. 2004). While knockout mice for *Hey1* have no reported lung phenotype, *Hey1* may function together with other components of the Notch signaling pathway during lung vascular smooth muscle development.

FOX domain containing: Foxf1a and Foxf2—*Foxf1a* (also known as *Foxf1*) and *Foxf2* encode closely related FOX domain containing factors and have very similar patterns preferentially in the subepithelial mesenchyme of the lung (Fig. 2S,T). This expression is positively regulated by SHH signaling and negatively regulated by BMP signaling (Mahlapuu et al. 2001; Lim et al. 2002), both critical signals for lung development. *Foxf1a* has previously been shown to be essential for lung development in a haploinsufficient manner, since mice carrying one mutant allele (*Foxf1a*^{+/-}) display lobe fusion, altered epithelial branching, and high incidents of postnatal lethality due to pulmonary hemorrhaging (Mahlapuu et al. 2001; Lim et al. 2002). Tissue-specific inactivation of both copies of *Foxf1a* in the lung has not been reported. *Foxf2* knockout mice have no known lung phenotype and die postnatally due to inability to feed (Wang et al. 2003). *Foxf1a* and *Foxf2* have been shown to act redundantly in the intestine, where they regulate extracellular matrix production through their ability to mediate WNT signaling (Ormestad et al. 2006). Whether *Foxf1a* and *Foxf2* have redundant roles in lung development has not been reported.

Miscellaneous: Lef1, Creb3l1, and Tbx3—Three of the mesenchyme-expressed transcription factors, *Lef1*, *Creb3l1* and *Tbx3*, were identified in our screen as the sole member of their respective families (Fig. 2U–W). Of these, *Lef1* is the only gene implicated in lung development (Okubo et al. 2004).

Lef1—*Lef1* is an HMG domain-containing transcription factor, and a downstream component of the WNT signaling pathway. Within the lung, *Lef1* expression is higher within the distal mesenchyme, which corresponds to the distal expression of *Wnt2* and *Wnt7b* within the mesenchyme and epithelium respectively (Fig. 2U and Visel et al. 2004). Both canonical (e.g. *Wnt2*, *Wnt2b* and *Wnt7b*) and non-canonical (e.g. *Wnt5a*) WNT signaling has been shown to control branching morphogenesis, patterning, and differentiation in the developing lung (Li et al. 2002; Li et al. 2005; Cohen et al. 2009; Goss et al. 2009; Goss et al. 2011). Consistent with this, expression of a β -Catenin-Lef1 fusion protein within the lung epithelium leads to constitutive activation of WNT signaling and a change of cell fate of the lung epithelium into the intestinal epithelium (Okubo et al. 2004). *Lef1* knockout mice die after birth and before weaning (van Genderen et al. 1994), however no lung defect has been reported. The lack of a lung phenotype in *Lef1* knockout mice may result from its overlapping expression with the highly related *Tcf1*, which is also expressed within the lung mesenchyme (Oosterwegel et al. 1993).

Creb3l1—*Creb3l1* (also known as *Oasis*) is a bZIP (basic leucine zipper) domain containing transcription factor that is tethered to the endoplasmic reticulum (ER) and cleaved in response to ER stress (Murakami et al. 2006). Upon cleavage, the DNA binding domain-containing portion of the protein can be transported to the nucleus where it regulates downstream gene expression. *Creb3l1* cleavage can also be regulated by signaling ligands such as BMPs (Murakami et al. 2009). Consistent with this regulation, *Creb3l1* knockout mice show decreased bone formation due to reduced secretion of bone matrix proteins. *Creb3l1* has also been shown to regulate extracellular matrix production within the pancreas (Vellanki et al. 2010). *Creb3l1* mutant mice are viable and no lung defect has been reported (Murakami et al. 2006; Murakami et al. 2009).

Tbx3—*Tbx3* is one of the four members of the T-box transcription factor family genes, *Tbx2-5* that have been shown to be expressed within the lung mesenchyme (Fig. 2W, Chapman et al. 1996; Li et al. 2004). Their expression is promoted by SHH signaling, which is critical for lung epithelial branching and mesenchymal differentiation (Pepicelli et al. 1998; Li et al. 2004). However, no lung phenotype has been reported in *Tbx3* knockout mice (Davenport et al. 2003), potentially due to overlapping expression with *Tbx2,4,5*.

Category 3—genes expressed in both the epithelium and mesenchyme

We identified ten transcription factors as being expressed in both the epithelium and the surrounding mesenchyme (Fig. 3A–J). Within this group, there are multiple members of the homeobox and FOX domain containing transcription factors.

Homeobox domain-containing: Pitx2, Zfhx3, and Pou2f1—Of the three homeobox domain-containing transcription factors found to be expressed in both the epithelium and the mesenchyme (Fig. 3A–C), *Pitx2* is the only gene known to be required for normal lung development. *Pitx2* knockout mice exhibit a left-right asymmetry defect, which manifests in the transformation of the normally single-lobed left lung into the multi-lobed right lung (Lu et al. 1999; Liu et al. 2001). It remains to be determined whether this phenotype is due to a primary role of *Pitx2* in the lung, or an earlier requirement in the node or lateral plate mesenchyme for overall determination of left-right asymmetry. Of note, *Pitx2* is expressed in only the left lobe of the E9.5 lung, but it becomes bilaterally expressed throughout the lung epithelium and mesenchyme by E13.5 (Kitamura et al. 1999 and data not shown). Two other Pitx transcription factor genes, *Pitx1* and *Pitx3*, are also expressed in regions of the lung mesenchyme that overlap with the *Pitx2* expression domain, suggesting possible redundancy (Fig. 3A; De Langhe et al. 2008). In addition, it has been shown that the expression of both *Pitx2* and *Pitx3* are positively regulated by WNT signaling in the lung

(De Langhe et al. 2008), raising the possibility that *Pitx* genes may mediate the essential functions of WNT signaling during lung development.

Zfhx3 (also known as *Atbf1*) shows strong expression in the epithelium and subepithelial mesenchyme of the developing lung (Fig. 3B) (Ido et al. 1996). It has been shown that *Zfhx3* promotes *Pdgfrsβ* expression in P19 mouse embryonic carcinoma cells, protecting these cells from oxidative stress (Kim et al. 2010). *Pdgfrsβ* is expressed within the subepithelial mesenchyme of the lung, and a recent study suggests that signaling through PDGFRB promotes smooth muscle development in the lung (Cohen et al. 2009). Mice carrying a gene-trap allele of *Zfhx3* have been produced, but no lung phenotype has been described (Stryke et al. 2003; Qi et al. 2008).

Pou2f1 (also known as *Oct1*) is strongly expressed in the lung epithelium, and weakly expressed in the adjacent mesenchyme (Fig. 3C). *Pou2f1* knockout embryos are smaller in size, underrepresented after E13.5, and none survive after birth (Wang et al. 2004). No lung phenotype has been reported. However, in lung derived NCI-H441 cells, *Pou2f1* has also been shown to directly bind to the promoter of *SCGB1A1* (also known as *CC10* or *CCSP*), a key marker for Clara cells (Sawaya et al. 1993). Furthermore, in the lens and nasal placode, *Pou2f1* genetically interacts with *Sox2* to promote the induction of these tissues (Donner et al. 2007). *Sox2* is essential for the differentiation of airway epithelial cells (Gontan et al. 2008; Que et al. 2009; Tompkins et al. 2009). Whether similar interactions occur between *Pou2f1* and *Sox2* in the lung remains to be determined.

FOX domain containing: *Foxm1* and *Foxh1*—Two FOX domain containing transcription factor genes, *Foxm1* and *Foxh1* are identified as being expressed in both the epithelium and mesenchyme of the lung (Fig. 3D,E). The expression level of *Foxm1* in lung diminishes from E11.0–E18.5, but peaks again during the initiation of alveologenesis in the postnatal lung (Wang et al. 2010). During lung development, *Foxm1* promotes blood vessel formation, epithelial cell differentiation, and inhibits arterial smooth muscle formation. Conditional inactivation of *Foxm1* in the lung mesenchyme leads to an increase of smooth muscle around the proximal airways and a decrease of pulmonary microvasculature (Kim et al. 2005). Conditional inactivation of *Foxm1* in the lung epithelium leads to a decrease in sacculization and a delay in the differentiation of alveolar type I cells (Kalin et al. 2008). Expression of a constitutively active form of *Foxm1* within the embryonic lung epithelium results in impaired sacculization and epithelial hyperplasia in the major airways (Wang et al. 2010). These results demonstrate that precise control of *Foxm1* level is crucial to multiple processes of lung development.

Similar to the loss of *Pitx2*, inactivation of *Foxh1* in the lateral plate mesoderm leads to left-right asymmetry defects, which includes the transformation of the one-lobe left lung into the four-lobe right lung (Hoodless et al. 2001; Yamamoto et al. 2003; Kofron et al. 2004; Von Both et al. 2004). And like in *Pitx2* mutants, it is unclear whether this transformation of lung lobes is due to the requirement for *Foxh1* in the lung, or at earlier stages in cells essential for left-right determination. In other tissues, *Foxh1* has been shown to interact with several signaling pathways that are critical for lung development. For example, in the brain, *Foxh1* acts downstream of TGFβ and upstream of retinoic acid signaling (Silvestri et al. 2008). However, whether similar relationships operate within the lung has not been shown.

Miscellaneous: *E2f1*, *Hif1a*, *Smarca5*, *Brd7*, and *Tcea1*—Of the five single family member transcription factors expressed in both the epithelium and mesenchyme (Fig. 3F–J), *E2f1* and *Hif1a* have been previously shown to play a role in lung development.

E2f1—*E2f1* is one of eight E2f transcription factor genes present in the mouse genome. E2F transcription factors play very diverse roles including the control of cell proliferation, cell death, and cell differentiation (Wang et al. 1998; Fajas et al. 2002; Sharma et al. 2006; Chong et al. 2009). In the lung, *E2f1* is more strongly expressed within the distal epithelium and mesenchyme (Fig. 3F). In multiple settings especially that of cancer, E2F transcription factors interact closely with retinoblastoma (RB) and p53 proteins (Udayakumar et al. 2010). Recent studies have identified *Rb* as an important regulator of cell proliferation and differentiation in lung development and repair, raising the possibility that E2f1 may play a role in these processes through its association with RB (Wikenheiser-Brokamp 2004; Mason-Richie et al. 2008; Simpson et al. 2009). While *E2f1* single knockout mice show no lung phenotype, *E2f1*;*E2f3* double knockout mice have simplified alveoli (Tsai et al. 2008). The precise role of these E2f factors in lung development has not been determined.

Hif1a—Like *Hif3a* described above, *Hif1a* is a member of the HIF group. It is known to promote vascular growth in response to low oxygen levels (Tian et al. 1997; Semenza et al. 2001a; Semenza et al. 2001b). Knockout mice are lethal by E11.0, due to defective vascular development and cardiac patterning (Iyer et al. 1998; Compennolle et al. 2003). As for its role in the lung, an early report shows that conditional inactivation of *Hif1a* in the lung epithelium results in lethality shortly after birth due to loss of surfactant production and increased septal wall thickness (Saini et al. 2008). However, this result is challenged by a more recent study, which shows that a similar lung epithelium-specific inactivation of *Hif1a* permits survival of the mutant mice (Shannon and Young 2011). While the absolute requirement for *Hif1a* in the lung for perinatal survival awaits further clarification, the importance of a close regulation of *Hif1a* level in lung is further demonstrated by the findings that overexpression of *Hif1a* in lung epithelium leads to defects in epithelial branching, epithelium differentiation and vasculature maturation, which resulted in lethality at birth (Bridges et al. 2012).

Smarca5 and Brd7—Two of the transcription factors expressed in the epithelium and mesenchyme, *Smarca5* and *Brd7* are known chromatin regulators (Fig. 3H,I). SMARCA5 is a SWI/SNF related protein that is highly expressed within the distal epithelium and mesenchyme, while BRD7 is a bromodomain containing protein that acts as a component of the SWI/SNF complex and is expressed more uniformly in the lung (Fig. 3H, I and Stopka et al. 2003; Kaeser et al. 2008). *Smarca5* global knockout mice are lethal during the pre-implantation stage of development (Stopka et al. 2003). *Smarca5*^{+/-} embryonic stem cells or spermatocytes exhibit a global decrease in H3K9Ac and H3K79Me2 levels, indicating an inactive gene expression state (Vargova et al. 2009). These results suggest that the general role of *Smarca5* within the embryo is to promote an active chromatin state. No *Brd7* knockout mutant has been reported, and the function of either of these genes during lung development awaits to be determined.

Tcea1—Similar to *Tcea2* described above, *Tcea1* is a transcriptional elongation factor that assists RNA polymerases read through transcriptional blocks (Wind et al. 2000). *Tcea1* is strongly expressed in the distal tips of the branches in both the epithelium and mesenchyme (Fig. 3J). Recent work has suggested that *Tcea1* boosts RNA polymerase function through regulation of histone modifications (Nagata et al. 2009). *Tcea1* knockout mice die prior to E16.5, with defects in the maintenance and differentiation of hematopoietic progenitor cells (Ito 2006). No lung phenotype has been reported, possibly due to redundancy with *Tcea2*, which is also expressed in the lung epithelium (Fig. 1J).

Category 4-Hox genes

Hox genes encode an evolutionarily conserved family of transcription factors that control pattern formation in animals. Thirty-nine *Hox* genes are encoded in the mouse genome. Previous results obtained using multiple approaches including Northern blotting, RT-PCR/qRT-PCR and *in situ* hybridization suggest that as many as twenty *Hox* genes are expressed in the lung during development (Cardoso 1995; Mollard and Dziadek 1997; Grier et al. 2009). Despite the clear presence of expression, the function of *Hox* genes during lung development is largely unknown as only a single *Hox* mutant, *Hoxa5*, displays primary lung defects (Aubin et al. 1997; Kinkead et al. 2004; Mandeville et al. 2006). *Hoxa5* mutant mice exhibit respiratory distress and partial lethality at birth, likely due to reduced surfactant production and/or reduced and disorganized tracheal cartilage, which may lead to tracheal occlusion. *Hoxa5* mutants that survive postnatally develop impaired alveologenesis. The lack of a lung phenotype in other single *Hox* mutants suggests that *Hox* factors may act redundantly. Furthermore, the range of defects observed in *Hoxa5* mutants demonstrates that *Hox* factors regulate a variety of processes over the course of lung development. To elucidate the function of *Hox* in lung branching morphogenesis, we assessed the expression of fourteen *Hox* genes (*Hoxa1*, *Hoxa2*, *Hoxa5*, *Hoxa9*, *Hoxb2*, *Hoxb3*, *Hoxb5*, *Hoxb6*, *Hoxb8*, *Hoxb9*, *Hoxc5*, *Hoxc6*, *Hoxd4* and *Hoxd9*) previously noted for their expression in the lung. We identified six genes, *Hoxa1*, *Hoxa2*, *Hoxa5*, *Hoxa9*, *Hoxb5*, and *Hoxb8* that showed restricted expression patterns in E11.5, and either E12.5 or E13.5 lung and trachea (Fig. 4A–L).

While most of the *Hox* genes we assayed show similar expression between E11.5 and E13.5, *Hoxa1* expression changes over time. At E11.5, similar to at E10.5, *Hoxa1* expression is readily detected, particularly in the distal mesenchyme of both the medial and accessory buds (Fig. 4A and data not shown). However, this expression tapers off by E13.5 (Fig. 4B). Progressive loss of *Hoxa1* expression is also supported by data from northern blot analysis (Mollard and Dziadek 1997). Northern blot data further indicate that other genes, *Hoxa7*, *Hoxb3*, and *Hoxb4* also exhibit temporally dynamic expression (Mollard and Dziadek 1997). It should be noted that *Hoxa1* mutants develop breathing abnormalities at birth (Lufkin et al. 1991). However, existing evidence has attributed the defect to a requirement for *Hoxa1* in patterning the respiratory neuronal circuits in the brain (Chatonnet et al. 2002; Gray 2008).

Different *Hox* genes are often expressed in distinct cell lineages. For example, *Hoxa2*, *Hoxa5*, and *Hoxa9* are expressed in the trachea/bronchi region, while *Hoxb5* and *Hoxb8* are not. At both E11.5 and E13.5, *Hoxa2* and *Hoxa5* are observed in the trachea and main bronchi in a pattern consistent with cartilage precursors (Fig. 4C–F). Interestingly, while no cartilage defects have been reported in *Hoxa2* mutants, *Hoxa5* mutants do exhibit tracheal cartilage malformations, suggesting that *Hoxa5* may be the primary *Hox* factor that controls airway cartilage patterning (Aubin et al. 1997). Although *Hoxa9* is also expressed in the trachea/bronchi region, its expression is primarily in the epithelium (Fig. 4G,H). The epithelial expression of *Hoxa9* continues into the lung, where it is also detected in the lung mesenchyme with a proximal bias. In contrast to this bias, *Hoxa2*, *Hoxa5*, and *Hoxb5* expression in the lung is more widespread throughout the mesenchyme at E12.5/E13.5 (Fig. 4D,F,J).

A vast majority of the genes that have been studied in the mouse lung show similar expression in the five lung lobes. Hence, it is interesting that several of the *Hox* genes that we have assayed exhibit biased expression in the different lobes. For example, at E11.5, both *Hoxa1* and *Hoxa5* show concentrated expression in the distal mesenchyme of the medial and accessory lobes (Fig. 4A,E). At both E11.5 and E12.5, *HoxB8* shows concentrated expression in the distal mesenchyme of the left lobe and in the cranial and

caudal lobes of the right lung, but is not detected in the right, medial, and accessory lobes (Fig. 4K,L). Whether these transcription factors coordinate the shape and size of the lung lobes remains to be determined. It is important to note that the Hox cluster was first identified as genes that provide segmental identity in *Drosophila*. In mouse, *Hox* gene expression often marks particular sub-regions within a tissue lineage such as in the anterior-posterior axial skeleton, the brain and the limb bud. Our results suggest that *Hoxa1*, *Hoxa5* and *Hoxb8* can be used as markers of early lobe compartments within the developing lung.

Conclusions

Using a probe collection that represents a majority of the transcription factors encoded in the mouse genome, we identified sixty-two transcription factors that show patterned expression in the pseudoglandular stage mouse lung. Many of the transcription factors that we identified have not been previously implicated in lung development. Furthermore, characterization of the expression pattern of some of these genes, such as *Grhl2* and *Tbx3*, has led to consideration of other members of the gene families and their expression in lung. We envision that our findings will promote future studies by: providing additional genes to be investigated for their roles in lung development; increasing nodes of the transcriptional circuits that act either upstream or downstream of known factors; and suggesting putative markers for specialized cell populations in the lung at later stages of development and maturation.

Going beyond development, our dataset may be of value to lung disease research such as the studies of lung cancer. Recent investigations of lung cancer have revealed strong correlations between patient survival rates and misexpression of a number of the transcription factors highlighted in our screen. For example, increased nuclear localization of *ID2* or increased expression of *FOXM1*, *HIF1A*, *SNAIL* or *HOXA9* is associated with higher mortality rates (Choi et al. 2006; Rauch et al. 2007; Gialmanidis et al. 2009; Hung et al. 2009; Rollin et al. 2009). These findings raise the possibility that while the expression of many of these factors is downregulated at the completion of development, they are reactivated in tumor cells. Thus, our dataset may suggest candidate biomarkers that can be used in early lung cancer diagnosis.

Experimental Procedures

Tissue Collection

The embryos used in these experiments were harvested from time-mated females, with noon the day of the vaginal plug counted as E0.5. Dissected lung tissues were fixed in 4% paraformaldehyde for 4 hours to overnight at 4°C. After fixation the right lungs were embedded in 4% low melt agarose and sectioned at 100µm thickness using a vibratome. Intact left lung lobes and the right lung sections were dehydrated through a methanol series and stored at -20 °C in 100% methanol until RNA in situ hybridization.

RNA in situ hybridization

RNA in situ probes were generated from a published plasmid collection (Gray 2004). RNA in situ hybridization was performed following a protocol that was adapted from previously published protocols (Wall and Hogan 1995; Abler et al. 2011). Briefly, either wholemount or vibratome-sectioned tissues stored in methanol were rehydrated using PBS with 0.1% Tween (PBT). Tissues were then permeabilized using 10µg/ml proteinase K in PBT for 10 minutes. Tissues were fixed with 4% paraformaldehyde, 0.2% glutaraldehyde in PBT for 20 minutes. They were then washed and allowed to hybridize with probes at 70°C overnight. After the hybridization, tissues were washed three times, twenty minutes each in 2x SSC,

0.1% CHAPS, and then in 0.2x SSC, 0.1% CHAPS at 70°C. Tissues were then washed in KTBT and blocked for 2 hours in 2% blocking solution (Roche) and 20% heat inactivated sheep serum (Sigma) in KTBT at room temperature. The tissues were then incubated overnight at 4°C in the primary antibody, AP conjugated α -DIG, Fab fragment (Roche) at a dilution of 1:2000 using 2% blocking solution and 20% heat inactivated sheep serum in KTBT. The tissues were then washed 5 times, 1 hour each in KTBT at room temperature. Following this, the tissues were washed twice, 5 minutes each with 0.1% Tween, 1mM Levamisole in ddH₂O. Finally the tissues were stained in BM purple (Roche) containing 0.1% Tween and 1mM levamisole. The staining was carried out for varying amount of time depending on the probes until desired intensity, from 30 minutes to 8 hours.

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Figure 1. Patterns of transcription factor genes with primarily epithelial expression in the developing lung

(A–W) Twenty-three genes were identified by RNA *in situ* hybridization as having epithelium-specific expression patterns in the E13.5 lung. For each gene, the left panel is a wholemount *in situ* hybridization of a left lung lobe, and the right panel is an enlarged vibratome section of a right lung lobe. The tissue in each panel is oriented so that the upper left corner and the lower right corner represent the proximal and distal regions of the lung, respectively.

Figure 2. Patterns of transcription factor genes with primarily mesenchymal expression in the developing lung

(A–W) Twenty-four genes were identified by RNA *in situ* hybridization as having mesenchyme-specific expression patterns in the E13.5 lung. For each gene, the left panel is a wholemount *in situ* hybridization of a left lung lobe, and the right panel is an enlarged vibratome section of a right lung lobe. The tissue in each panel is oriented so that the upper left corner and the lower right corner represent the proximal and distal regions of the lung, respectively.

Figure 3. Patterns of transcription factor genes with both epithelial and mesenchymal expressions in the developing lung

(A–J) Ten transcription factor genes were identified by RNA *in situ* hybridization to be expressed in both the epithelium and the mesenchyme of the E13.5 lung. For each gene, the left panel is a wholemount *in situ* hybridization of a left lung lobe, and the right panel is an enlarged vibratome section of a right lung lobe. The tissue in each panel is oriented so that the upper left corner and the lower right corner represent the proximal and distal regions of the lung, respectively.

Figure 4. Patterns of Hox transcription within the developing lung

(A–L) Six Hox genes were identified by RNA *in situ* hybridization to be expressed within the E11.5 (A,C,E,G,I,K) and either E12.5 (J,L) or E13.5 (B,D,F,H) lung and trachea. Each gene is a wholemount *in situ* hybridization of both the trachea and lung.

Table 1

A summary of transcription factors identified in the screen

Gene Name	Sites of expression in lung	Function during lung development	References	Figure
<i>Ash2l</i>	Epithelium	KO: dies at lung bud initiation stage.	Stoller et al. 2010	1R
<i>Baz1b</i>	Mesenchyme	KO: die at birth with heart defects, no reported lung defect.	Yoshimura et al. 2009	2C
<i>Brd7</i>	Epithelium and Mesenchyme	No knockout line available.	Kaeser et al. 2008	3I
<i>Creb3l1</i>	Mesenchyme	KO: viable and no reported lung defect.	Murakami et al. 2009	2V
<i>E2f1</i>	Epithelium and Mesenchyme	KO: no reported lung defect. Double KO of <i>E2f1;E2f3</i> develops simplified alveoli.	Tsai et al. 2008	3F
<i>Elf5</i>	Epithelium	OE: dilated branching tips and disrupted epithelial differentiation. KO: no reported lung defect.	Metzger et al. 2008 and Xin Sun unpublished	1N
<i>Elk3</i>	Mesenchyme	KO: survive birth, but eventually die due to respiratory distress secondary to chyle built up in thorax.	Ayadi et al. 2001	2L
<i>Ets1</i>	Mesenchyme	KO: viable and no reported lung defect.	Muthusamy et al. 1995	2M
<i>Ets2</i>	Mesenchyme	KO: embryonic lethal at E8.5.	Yamamoto et al. 1998	2N
<i>Etv5</i>	Epithelium	DN: proximalization of the lung epithelium in late branching stage. KO: no reported lung defect.	Liu et al. 2003; Chen et al. 2005	1M
<i>Foxa1</i>	Epithelium	KO: crucial for epithelial branching and differentiation.	Wan et al. 2005	1K
<i>Foxd4</i>	Epithelium	No knockout line available.	Kaestner et al. 1993	1L
<i>Foxf1a</i>	Mesenchyme	Heterozygous KO: lung lobe fusion and altered branch pattern.	Mahlapuu et al. 2001	2S
<i>Foxf2</i>	Mesenchyme	KO: dies postnatally with no reported lung defect.	Wang et al. 2003	2T
<i>Foxh1</i>	Epithelium and Mesenchyme	KO: left-right asymmetry lung defect.	Yamamoto et al. 2003; Hoodless et al. 2001	3E
<i>Foxm1</i>	Epithelium and Mesenchyme	KO: defects in vascular and epithelial cell differentiation and inhibit arterial smooth muscle formation in lung.	Kim et al 2005; Kalin et al. 2008	3D
<i>Gata6</i>	Epithelium	KO: lung branching and epithelial differentiation defects.	Keijzer et al 2001; Yang et al. 2002	1G
<i>Gli2</i>	Mesenchyme	KO: reduced lung branching.	Motoyama et al. 1998	2A
<i>Grhl2</i>	Epithelium	Point mutant: reduced lung size and lung collapse at birth. KO: dies at beginning of lung development.	Pyrgaki et al. 2011	1W
<i>Heyl</i>	Mesenchyme	KO: viable and no reported lung defect.	Fischer et al. 2007	2R
<i>Hhex</i>	Mesenchyme	KO: dies at E14.5, no reported lung defect.	Hallaq et al. 2004	2I
<i>Hif1a</i>	Epithelium and Mesenchyme	CKO in epithelium: lethal at birth due to low surfactant gene expression.	Saini et al 2008	3G
<i>Hif3a</i>	Mesenchyme	KO: impaired alveologenesis with an increase of the vasculature lining the alveolar walls.	Yamashita et al. 2008	2P
<i>Hnf1b</i>	Epithelium	KO: embryonic lethal at post implantation stage.	Coffinier et al. 2002	1E
<i>Id2</i>	Epithelium	KO: dies postnatally, no reported lung defect.	Yokota et al. 1999	1U
<i>Id3</i>	Mesenchyme	KO: viable and no reported lung defect.	Pan et al. 1999	2Q
<i>Irf6</i>	Epithelium	Unknown. KO: no reported lung defect.	Ingraham et al. 2006	1V

Gene Name	Sites of expression in lung	Function during lung development	References	Figure
<i>Irx3</i>	Epithelium	KD: abnormal branching.	Van Tuyl et al. 2006	1A
<i>Kat2b</i>	Epithelium	KO: no reported lung defect.	Yamauchi et al. 2000	1Q
<i>Lef1</i>	Mesenchyme	KO: dies postnatally, no reported lung defect.	van Genderen et al. 1994	2U
<i>Lhx6</i>	Mesenchyme	KO: dies around 2 weeks postnatally, no reported lung defect.	Liodis et al. 2007	2H
<i>Lmo1</i>	Epithelium	KO: viable and no reported lung abnormalities.	Tse et al. 2004	1H
<i>Meis1</i>	Mesenchyme	KD: dies at E14.5 and exhibit lung hypoplasia.	Hisa et al. 2004	2F
<i>Mnx1</i>	Epithelium	KO: postnatal lethal due to failure to inflate lungs.	Arber et al. 1999	1F
<i>Nab1</i>	Epithelium	KO: viable and no reported lung defect.	Le et al. 2005	1T
<i>Nkx1-2</i>	Epithelium	No knockout line available.	Tamplin et al. 2008	1D
<i>Nkx2-1</i>	Epithelium	KO or KD: failed trachea specification and lung branching arrest.	Minoo et al. 1995; Minoo et al. 1999	1B
<i>Nkx2-9</i>	Epithelium	KO: tracheobronchial epithelium hyperplasia in adult lung.	Tian et al. 2006	1C
<i>Nr2f1</i>	Mesenchyme	KO: postnatal lethal with no reported lung defect.	Qiu et al. 1997	2D
<i>Nr2f2</i>	Mesenchyme	KO: dies at E10.0. CKO: lung hypoplasia.	Pereira et al 1999; You et al. 2005	2E
<i>Pitx2</i>	Epithelium and Mesenchyme	KO: left-right asymmetry lung defect.	Lu et al. 1999; Liu et al. 2001	3A
<i>Pou2f1</i>	Epithelium and Mesenchyme	KO: prenatal lethal with erythropoiesis defect, no reported lung defect.	Wang et al. 2004	3C
<i>Pou3f1</i>	Mesenchyme	KO: dies at birth due to respiratory distress possibly secondary to neuronal defects.	Bermingham et al. 1996	2G
<i>Smarca5</i>	Epithelium and Mesenchyme	KO: lethal at pre-implantation stage.	Stopka et al. 2003	3H
<i>Snai1</i>	Mesenchyme	KO: lethal at E8.5.	Carver et al. 2001	2B
<i>Sox18</i>	Mesenchyme	KO: variable prenatal lethality, cardiovascular defects, no reported lung defect.	Dunn et al. 1995	2K
<i>Sox2</i>	Epithelium	CKO: disruption of cell differentiation. OE: altered branching morphogenesis.	Gontan et al. 2008; Tompkins et al. 2009; Que et al. 2009	1O
<i>Sox7</i>	Mesenchyme	No knockout line available.	Cermenati et al 2008	2J
<i>Sox9</i>	Epithelium	CKO: no reported lung defect.	Perl et al. 2005	1P
<i>Tbx3</i>	Mesenchyme	KO: dies before birth, no reported lung defect.	Davenport et al. 2003	2W
<i>Tcea1</i>	Epithelium and Mesenchyme	KO: dies before E16.5 and no reported lung defect.	Ito et al. 2006	3J
<i>Tcea2</i>	Epithelium	No knockout line available.	Reines et al. 1996	1J
<i>Tcf21</i>	Mesenchyme	KO: branching defects with proximalization of the lung epithelium.	Quaggin et al. 1999	2O
<i>Tfap2c</i>	Epithelium	KO: dies at E7–9.	Werling et al. 2002	1S
<i>Zfmx3</i>	Epithelium and Mesenchyme	Gene-trap mutant: no reported lung defect.	Stryke et al. 2003	3B
<i>Zfp746</i>	Epithelium	No knockout line available.	Shin et al 2011	1I

Abbreviations:

KO Knock Out

KD Knock Down

CKO Conditional Knock Out

DN Dominant Negative

OE Over Expression

*
Hox gene expression patterns are presented separately in Figure 4.