Epithelial inactivation of \( Yy1 \) abrogates lung branching morphogenesis

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**ABSTRACT**

Yin Yang 1 (YY1) is a multifunctional zinc-finger-containing transcription factor that plays crucial roles in numerous biological processes by selectively activating or repressing transcription, depending upon promoter contextual differences and specific protein interactions. In mice, \( Yy1 \) null mutants die early in gestation whereas \( Yy1 \) hypomorphs die at birth from lung defects. We studied how the epithelial-specific inactivation of \( Yy1 \) impacts on lung development. The \( Yy1 \) mutation in lung epithelium resulted in neonatal death due to respiratory failure. It impaired tracheal cartilage formation, altered cell differentiation, abrogated lung branching and caused airway dilation similar to that seen in human congenital cystic lung diseases. The cystic lung phenotype in \( Yy1 \) mutants can be partly explained by the reduced expression of \( Shh \), a transcriptional target of YY1, in lung endoderm, and the subsequent derepression of mesenchymal Fgf10 expression. Accordingly, SHH supplementation partially rescued the lung phenotype in vitro. Analysis of human lung tissues revealed decreased YY1 expression in children with pleuropulmonary blastoma (PPB), a rare pediatric lung tumor arising during fetal development and associated with DICER1 mutations. No evidence for a potential genetic interplay between murine \( Dicer \) and \( Yy1 \) genes during lung morphogenesis was observed. However, the cystic lung phenotype resulting from the epithelial inactivation of \( Dicer \) function mimics the \( Yy1 \) lung malformations with similar changes in \( Shh \) and \( Fgf10 \) expression. Together, our data demonstrate the crucial requirement for YY1 in lung morphogenesis and identify \( Yy1 \) mutant mice as a potential model for studying the genetic basis of PPB.

**INTRODUCTION**

YY1 is a ubiquitous zinc finger transcription factor that contains diverse domains, enabling a plethora of protein-protein interactions. YY1 can recruit co-activators or co-repressors, which determine whether YY1 will execute inhibitory or activating functions on transcriptional targets (Deng et al., 2010). In regulating a multitude of genes, YY1 plays crucial functions in numerous biological processes, including cell proliferation and differentiation, X-chromosome inactivation, and embryogenesis (Donohoe et al., 2007; Nicholson et al., 2011). The \( Yy1 \) null mutation in mice results in peri-implantation lethality, a phenotype precluding investigation of YY1 requirement at later developmental stages (Donohoe et al., 1999). Using mouse lines carrying a \( Yy1 \) conditional allele and tissue-specific \( Cre \)-expressing transgenes, \( Yy1 \) was found to widely participate in developmental processes. However, despite the fact that mice expressing 25% of normal YY1 levels die at birth from respiratory failure due to collapsed lungs, little is known about the role of YY1 in lung morphogenesis (Affar et al., 2006).

Lung development is subdivided into five overlapping periods (Morrissey and Hogan, 2010). The embryonic phase is characterized by the formation of the ventral diverticulum that arises from the laryngotracheal groove of the foregut endoderm. Subsequently, this diverticulum, destined to become the trachea, divides into the left and right lung buds. At the pseudoglandular stage, the two primary buds elongate and undergo stereotypical branching via complex epithelium-mesenchyme interactions to form the respiratory tree. This is followed by the canalicular and saccular stages, during which the progressive differentiation of the pulmonary epithelium, the expansion of the vasculature with thinning of the mesenchyme, and the formation of functional air-blood barriers happen. Alveologenesis occurs after birth and is characterized by the formation of alveoli, the final gas-exchange units. Recent data indicate that the transition from branching morphogenesis to epithelial cell differentiation depends on control mechanisms involving key players of lung development such as Fgf10 and SOX9. These latter act by regulating the delicate balance between distal and proximal endodermal progenitors, promoting branching and preventing precocious alveolar differentiation (Chang et al., 2013; Volkaert et al., 2013; Yang and Chen, 2014).

We showed that the specific ablation of YY1 function in lung mesenchyme causes neonatal death of mutant pups due to collapsed lungs, a phenotype similar to that of \( Yy1 \) hypomorph mutants (Bérubé-Simard et al., 2014). In the present study, we investigated the impact of the epithelial-specific inactivation of YY1 in the developing lung. The loss of YY1 function in lung epithelium also resulted in death at birth. It affected tracheal cartilage formation, cell differentiation and lung branching, leading to the formation of large cysts. The reduced expression of \( Shh \) and the subsequent upregulation of \( Fgf10 \) expression likely contributed to the branching defects in \( Yy1 \) mutants. Accordingly, in vitro SHH supplementation partially rescued the lung phenotype of \( Yy1 \) mutant explants. The cysts in \( Yy1 \) mutants mimicked the lung phenotype of \( Dicer \) mutants with similar \( Shh \) and \( Fgf10 \) expression variations (Harris et al., 2006). DICER is a ribonuclease III essential for the biogenesis of mature microRNAs (Bartel, 2004). Cystic lesions characterize several pulmonary diseases such as congenital cystic adenomatoid malformation (CCAM) and pleuropulmonary blastoma (PPB), the latter being a rare dysontogenetic neoplasm.
RESULTS

Loss of epithelial Yy1 function causes defective lung morphogenesis

To circumvent the early embryonic lethality of the Yy1 null mutants and address Yy1 function in lung morphogenesis, we specifically deleted the Yy1 gene in lung epithelium using the Shh \(^{+/Cre}\) deleter mouse line (supplementary material Fig. S1; Harfe et al., 2004). All Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) newborns died at birth due to respiratory failure, whereas Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mice were viable and normal (Table 1). Analysis of lungs from embryonic (E) day 18.5 Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) embryos revealed a disorganized architecture with the presence of dilated fluid-filled sacs (Fig. 1). At E12.5, lungs from Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) embryos presented two hypoplastic lobes in contrast to the expected asymmetric pattern of four right lobes and one left lobe. Moreover, lung epithelium of mutant specimens had an abnormal stratified structure.

To identify the causes of the lung phenotype, we looked at cell proliferation. At E12.5, reduced immunostaining for cyclin D1, a marker for S-phase, and for pHH3, a marker for cells in late G2 and mitosis, was specifically observed in lung epithelium of Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants (Fig. 2A,B; supplementary material Fig. S2). Apoptosis, as indicated by cleaved caspase-3 immunostaining, was also more important in the multilayered lung epithelium of Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) specimens (Fig. 2C,D). At E14.5, apoptosis was increased in lung mesenchyme from mutants (supplementary material Fig. S2). Together, reduced epithelial proliferation and augmented apoptosis contribute to the abnormal lung structure in Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants.

Altered lung patterning and cell differentiation in Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants

To investigate whether the proximal-distal patterning of airway epithelium was disrupted in Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants, we examined the expression of the transcription factors sex-determining region Y (SRY)-box 2 and 9 (SOX2 and SOX9), two lineage commitment markers of proximal and distal endodermal lung progenitor cells, respectively (Tian et al., 2011). In controls, SOX2 was expressed in the epithelium of trachea and proximal lung progenitor cells, whereas SOX9 was present in the epithelium of distal airways, whereas SOX9 was expressed in the epithelium of distal tubular tips and in the mesenchyme lining proximal airways. In mutants, SOX2 and SOX9 epithelial expression also displayed a reciprocal pattern, but SOX2 expression was downregulated and restricted to a more proximal region (Fig 2E-H). We further characterized epithelial cell specification by examining the expression of NKX2-1 and FOXA2, two transcription factors essential for lung branching and epithelial cell differentiation (Kimura et al., 1996; Minoo et al., 1999; Wan et al., 2004). No major difference was observed (Fig. 2I-L).

In agreement with the lack of SOX2 expression along the cysts, no secretory club (Clara) cells or ciliated cells, normally found along the proximal airway epithelium, were detected in the cystic epithelium (Fig. 2M-P). In contrast, Types I and II pneumocytes were present along the cyst-lining epithelium (Fig. 2Q-X). Microvascular development, as detected by PECAM immunostaining, also occurred within the mesenchyme surrounding the cysts (Fig. 2Y,Z).

In E12.5 controls, expression of alpha smooth muscle actin (\(\alpha\)SMA), a marker of airway smooth muscle differentiation, was detected in myofibroblasts surrounding blood vessels and conducting airways, but excluded from the tip of growing buds. In mutants, \(\alpha\)SMA-positive cells were lacking around cysts, but still present around pulmonary vasculature (Fig. 2AA,BB). Thus, Yy1 epithelial ablation prevents branching morphogenesis with no major consequences on the specification of the distal epithelium. However, it interferes with the differentiation or the survival of airway myofibroblasts.

Abnormal formation of cartilage rings and impaired cell differentiation in trachea from Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants

The trachea from Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) mutants appeared thinner with disorganized cartilage rings (Fig. 1A-B). Alcian Blue staining verified the abnormal banding pattern (Fig. 3A,B). Tracheal stenosis was confirmed by the measurement of the external diameter (625±26 µm versus 339±25 µm; \(P<0.001\)) and the luminal surface area (61±16 µm² versus 4.6±2 µm²; \(P<0.01\)), both of which were significantly smaller in mutants. The trachea was also longer in mutants (2029±130 µm versus 2487±47 µm; \(P<0.01\)) (Fig. 3A-E).

We analyzed the expression of SOX9, a master regulator of chondrogenesis essential for tracheal cartilage patterning (Park et al., 2010). In E14.5 controls, the punctuated SOX9 expression pattern reflected future cartilage rings. Conversely, mutants displayed continuous SOX9 expression along the upper airways (Fig. 3H-G). Thus, epithelial YY1 controls the condensation of SOX9-positive mesenchymal cells into cartilage nodules.

We assessed the expression of p63, a marker of basal cells, which are known to generate ciliated, club and goblet cells (Rock et al., 2009). Basal cells were distributed irregularly along the cyst-lining epithelium (Fig. 2Q-X). Microvascular development, as detected by PECAM immunostaining, also occurred within the mesenchyme surrounding the cysts (Fig. 2Y,Z).

Although the number of p63-positive basal cells was not noticeably altered, ciliated, club and goblet cells were scarce in mutants (Fig. 3H-M). SOX2 is crucial for tracheal cartilage

Table 1. Ratios of genotypes in litters from crosses between Yy1\(^{flox/+}\); Shh\(^{+/Cre}\) and Yy1\(^{flox/+}\) mice

<table>
<thead>
<tr>
<th>Age</th>
<th># litters</th>
<th># pups</th>
<th>Yy1(^{flox/+}); Shh(^{+/Cre})</th>
<th>Yy1(^{flox/+}); Shh(^{+/Cre})</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>E12.5</td>
<td>21</td>
<td>144</td>
<td>(25)</td>
<td>(25)</td>
</tr>
<tr>
<td>E14.5</td>
<td>7</td>
<td>54</td>
<td>31 (21.5)</td>
<td>41 (28.5)</td>
</tr>
<tr>
<td>E18.5</td>
<td>9</td>
<td>59</td>
<td>19 (35.2)</td>
<td>6 (11.1)</td>
</tr>
<tr>
<td>P21</td>
<td>13</td>
<td>74</td>
<td>26 (35.1)</td>
<td>21 (28.4)</td>
</tr>
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</table>

The percentage obtained is indicated in parentheses.
YY1 in tracheal cartilage patterning and its cell-autonomous function in airway epithelial cell differentiation.

**YY1 epithelial inactivation affects the expression of key players of lung development**

We assessed by qRT-PCR if the YY1 mutation caused the misregulation of molecules with established roles in lung morphogenesis. During branching, FGF10 is dynamically expressed in mesenchymal clustered cells, inducing the activation of the ERK/MAPK pathway in the adjacent epithelium to control the directional outgrowth of lung buds (Bellusci et al., 1997). At E14.5, Fgf10 expression was 2.7-fold higher in YY1flox/flox;Shh+/Cre lungs compared with YY1flox/flox;Shh−/Cre controls. Consequently, expression of Bmp4, Spry2 and Etv4 genes, known targets of lung FGF10 signaling, was upregulated in mutants (Fig. 4A; Weaver et al., 2000; Mailleux et al., 2001; Liu et al., 2003). Reduced expression of Etv5, another target of FGF10 signaling, was also observed. In situ hybridization assays revealed an expanded spatial distribution of Fgf10 mRNA in lungs from E12.5 mutants (Fig. 4B,C). Accordingly, increased expression of phospho-ERK (pERK), the activated form of ERK, was observed in the cystic epithelium of YY1flox/flox;Shh+/Cre embryos (Fig. 4D,E). Expression of Fg9, Hoxa5, Hoxb5, Foxp1, Foxp2, Foxp4, Hdac1, and Hdac2 genes, all involved in lung branching, was also monitored (Aubin et al., 1997; White et al., 2006; Shu et al., 2007; Wang et al., 2011, 2013). With the exception of a reduction in expression of Fg9 and Foxp2, no change in gene expression was detected (Fig. 4A).

**YY1 positively regulates Shh expression**

Shh null mutants present rudimentary lung sacs and tracheobronchial cartilage defects that mirror the YY1flox/flox;Shh+/Cre phenotype. Moreover, increased Fgf10 and Bmp4 lung expression levels are common denominators in YY1 and Shh mutants. Finally, αSMA-positive cells are absent around the bronchi in Shh+/− mutants (Litingtung et al., 1998; Pepicelli et al., 1998; van Tuyl et al., 2007). We examined Shh expression in YY1 mutants. The ShhCre allele used to generate the YY1 epithelial deletion is a Cre knock-in producing a Shh null allele (Harfe et al., 2004). To eliminate any bias that might result from Shh haploinsufficiency, Shh expression levels were measured by qRT-PCR in lungs from E14.5 YY1flox/flox;Shh+/Cre mutants and compared to YY1flox/flox;Shh−/Cre specimens. Shh expression, as well as that of Ptc1, Hip1 and Foxf1, targets of the SHH pathway, was significantly decreased in YY1flox/flox;Shh−/Cre lungs (Fig. 4F; Pepicelli et al., 1998; Chuang and McMahon, 1999; Mahlapuu et al., 2001).

Shh expression is confined to lung epithelium, suggesting that Shh may be a direct target of YY1. Sequence comparison with TFSEARCH and TESS databases revealed putative YY1 binding sites clustered into five domains spread along the 4.5 kb upstream sequences relative to the transcription start site (TSS) of the Shh murine gene. To establish whether these sites were effective in vivo, we performed ChIP assay on cross-linked chromatin from lungs of E14.5 wild-type embryos. We observed high occupancy in vivo of YY1flox/flox;Shh−/Cre specimens. Shh expression, as well as that of Ptc1, Hip1 and Foxf1, targets of the SHH pathway, was significantly decreased in YY1flox/flox;Shh−/Cre lungs (Fig. 4F; Pepicelli et al., 1998; Chuang and McMahon, 1999; Mahlapuu et al., 2001).
Taken together, these data support the notion that YY1 is a transcriptional activator of Shh expression in lung epithelium. Yy1^flox/flox; Tg^Shh+/Cre and Yy1^flox/flox; Shh+/Cre mice share lung phenotype characteristics. To inactivate Yy1 in the developing lung endoderm in a Shh haploinsufficiency-independent context, we used the BAC-Nkx2-1-Cre transgenic deleter line (Xu et al., 2008). Yy1^flox/flox; Tg^Nkx2-1Cre embryos exhibited cysts in the proximal region of the lobes, a phenotype consistent with the gradient of activity of the recombinase (Fig. 5A-I). As observed in Yy1^flox/flox; Shh+/Cre mutants, Types I and II pneumocytes, but not club and ciliated cells, were present along the cystic epithelium (supplementary material Fig. S3). Decreased Shh and Hip1 and increased Fgf10 expression levels were detected in lungs from Yy1^flox/flox; Shh+/Cre mutants, providing additional evidence that epithelial YY1 regulates Shh expression (Fig. 5J).

SHH partially rescues the lung phenotype of Yy1^flox/flox; Shh+/Cre mutants

We tested whether addition of recombinant mouse SHH (rmSHH) could rescue the Yy1 lung phenotype in vitro. Lungs from E12.5 Yy1^flox/flox; Tg^Nkx2-1Cre embryos cultured in control media failed with branch. They showed a loss of αSMA immunoreactivity around the cysts and an increased number of apoptotic cells when compared with controls (Fig. 6). Addition...
of rmSHH to $Yy^{\text{fox/fox;Shh}^{+/Cre}}$ explants caused an increased overall size, probably due to the thickened mesenchymal layer (Fig. 6C,D). The rmSHH treatment of $Yy^{\text{fox/fox;Shh}^{+/Cre}}$ explants did not rescue the branching defect (Fig. 6G,H). However, apoptosis in rmSHH-supplemented $Yy^{\text{fox/fox;Shh}^{+/Cre}}$ lungs returned to control levels (Fig. 6M-P). Moreover, αSMA expression at the periphery of cysts was partially recovered, corroborating the importance of SHH in airway smooth muscle cell specification (Fig. 6I-L).

**Decreased YY1 expression in PPB lung specimens**

The presence of lung cysts in $Yy^{\text{fox/fox;Shh}^{+/Cre}}$ and $Yy^{\text{fox/fox;Tg^{+}\text{Nkx2-1Cre}}}$ mutants mimics features of CCAM and PPB, two human pediatric cystic lung diseases (Stocker, 2009). To define the clinical relevance of our findings, YY1 immunostaining was performed on lung sections from children suffering from types I and II CCAM, and types I, II and III PPB and compared with lung specimens from age-matched patients who died from a non-pulmonary cause (supplementary material Table S1). PPB evolves...
through sequential pathological changes; the early stage (Type I) is characterized by a pure cystic architecture that progresses over time into a mixed cystic and solid lesion (Type II), which further evolves into a purely solid aggressive tumor with anaplastic undifferentiated sarcomatous components (Type III; Messinger et al., 2015). In controls and CCAM specimens, YY1 protein was strongly expressed in lung epithelium and mesenchyme. In specimens from all three types of PPB, YY1 staining was reduced, as assessed by IHC semiquantitative scoring (Fig. 7). These findings suggest a pathogenic role for YY1 in PPB.

No genetic interplay between YY1 and Dicer genes in lung morphogenesis

PPB is an extremely rare lung sarcoma that arises during fetal lung development and occurs in young children. PPB is associated with germ line and somatic mutations in the DICER1 gene (Hill et al., 2009; Pugh et al., 2014). In mice, the conditional loss of Dicer function in lung epithelium causes neonatal death due to defective lung branching and airway dilation, similar to the phenotype of Yy1flkox/lykox;Shhcre/+ mutants (Fig. 8A-D; Harris et al., 2006). These resemblances raised questions about a potential interplay between YY1 and Dicer genes in lung morphogenesis. We assessed Dicer expression levels between lungs from E14.5 Yy1flkox/+;Shhcre/+ and control embryos when compared with Yy1flkox/+;Shhcre/+ embryos. No significant difference was found, indicating that YY1 did not control Dicer expression during lung formation (Fig. 8E). Likewise, a Dicer epithelial mutation did not perturb YY1 lung expression in Dicerflkox/lykox;Shhcre/+ mutants at the RNA and protein levels (Fig. 8F; not shown).

As reported, the Dicer mutation caused increased Fgf10 expression (P<0.001) (Fig. 8F; Harris et al., 2006). We also found...
**Fig. 5.** Yy1 inactivation in the developing lung endoderm with the Nkx2-1Cre transgenic mouse line causes cyst formation. (A-B) Lungs from E18.5 Yy1<sup>floxed/floxed</sup>, Tg<sup>Nkx2-1Cre</sup> embryos exhibited cysts in the proximal region of the lobes (arrows). (C) A robust Cre activity was detected in the proximal respiratory epithelium from E12.5 R26<sup>tm1Tim</sup>, Tg<sup>Nkx2-1Cre</sup> embryos. (D-F) H&E-stained lung sections revealed that E18.5 Yy1<sup>floxed/floxed</sup>, Tg<sup>Nkx2-1Cre</sup> mutants presented defective airway branching as shown by the formation of cysts (asterisks). Panels E and F correspond to the proximal and distal parts of the lung, respectively. (G-I) YY1 immunostaining demonstrated a near-complete loss of YY1 expression in the epithelium lining cysts in the proximal portion of the lungs from E18.5 Yy1<sup>floxed/floxed</sup>, Tg<sup>Nkx2-1Cre</sup> mutants (H). The deletion was incomplete in distal lung as shown by YY1-positive cells (arrows; I). (J) qRT-PCR analysis revealed decreased Shh and Hip1 expression and increased Fgf10 levels in lungs from E14.5 Yy1<sup>floxed/floxed</sup>, Tg<sup>Nkx2-1Cre</sup> embryos. Values are expressed as mean±s.e.m.; **P<0.01, ***P<0.001. Asterisks indicate cysts. Scale bars: 2 mm in A, B; 100 µm in C-F, 50 µm in G-I.

Reduced expression of Shh (P<0.01) and its targets Ptc1 (P<0.01) and Hip1 (P<0.05) in Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> specimens compared with Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> controls (Fig. 8F). Therefore, both Yy1 and Dicer genes regulate Shh expression and subsequent lung branching morphogenesis.

To address if genetic interactions occur between the Yy1 and Dicer genes in vivo, we generated Yy1<sup>flox/flox</sup>/Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> double heterozygous animals. Like single heterozygous mutants, Yy1<sup>flox/flox</sup>/Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> embryos did not present lung defects, suggesting no genetic interplay (not shown). Accordingly, Shh expression levels were similar in lungs from single and double heterozygous mutants and comparable to that detected in Shh<sup>Cre</sup> specimens (Fig. 8G). Thus, the homozygous mutation of either Yy1 or Dicer in lung epithelium is required to significantly reduce Shh expression.

Microarray analysis of RNA from lungs of E14.5 Shh<sup>Cre</sup>, Yy1<sup>flox/flox</sup>/Shh<sup>Cre</sup> and Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> embryos was performed to assess the molecular consequences of the Yy1 and Dicer mutations in lung epithelium (supplementary material Fig. S5). An analysis of the total number of differentially expressed genes with a fold change ≥1.5 and P<0.05 revealed an overlap of 210 genes of which only seven were regulated in opposite direction. This suggested that a core pattern of altered gene expression is associated with airway dilation common to the two models. A heat map was established. In addition to confirming the modified expression of Shh, Hip1 and Fgf10 genes, it revealed the upregulation of Cdkn1a, known to be repressed by YY1 (Affar et al., 2006). Cdkn1a encodes the cyclin-dependent kinase inhibitor p21, and its upregulation concurred with the reduced proliferation observed in mutants. The detected decrease in expression of Elf5, Irx2, Irx3 and Irx5 genes, encoding transcriptional regulators of lung morphogenesis, also corroborates the observed lung phenotype. Inhibition of Irx expression has been shown to cause reduced lung branching (van Tuyl et al., 2006). Elf5 misexpression in lung epithelium also disrupts branching, suggesting that precise levels of Elf5 are required for lung morphogenesis (Metzger et al., 2008).

**Yy1<sup>flox/flox</sup>/Nkx2-1Cre mice present a PPB-like phenotype**

The neonatal lethality of Yy1<sup>flox/flox</sup>/Shh<sup>Cre</sup> mice precluded the study of lung tumor formation and progression. However, proliferation assays in lungs from E18.5 Yy1<sup>flox/flox</sup>, Shh<sup>Cre</sup> and Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> embryos revealed a dramatic increase in cell proliferation of the cystic walls, suggesting tissue overgrowth (Fig. 9A-D).

The lung phenotype of Yy1<sup>flox/flox</sup>, Tg<sup>Nkx2-1Cre</sup> embryos was less severe than the Yy1<sup>flox/flox</sup>/Nkx2-1Cre phenotype. However, increased proliferation was also detected in lungs from E18.5 Yy1<sup>flox/flox</sup>, Shh<sup>Cre</sup> and Dicer<sup>flox/flox</sup>, Shh<sup>Cre</sup> embryos (supplementary material Fig. S4K,L). Most Yy1<sup>flox/flox</sup>, Tg<sup>Nkx2-1Cre</sup> mutants died at birth but some survived up to weaning age (Table 2). Out of 86 pups, seven Yy1<sup>flox/flox</sup>, Tg<sup>Nkx2-1Cre</sup> mice born alive survived until weaning or were sacrificed due to health problems. The lungs of these seven mice exhibited cysts in the proximal region and histological analyses showed a multiloculated structure with septa of variable thicknesses around cysts (Fig. 9E-G). Elevated proliferation was observed in the disorganized mass (Fig. 9I,J). Vimentin immunostaining confirmed the mesenchymal nature of the tissue (Fig. 9K,L). Putative primitive small mesenchymal cells, a hallmark of type I PPB, were also detected (Fig. 9H). Finally, Nkx2-1 and Tαα positive cells revealed the alveolar nature of the cyst epithelium (Fig. 9O,P). Altogether, these characteristics are reminiscent of the histologic features of an evolving type I PPB (Hill et al., 2008).
DISCUSSION

YY1 is a multifunctional transcription factor member of the Polycomb group protein family, and its actions are controlled by protein-protein interactions (Gordon et al., 2006). YY1 plays pivotal roles throughout development. In the lung, there is a dosage-dependent requirement for YY1 with a lower limit of expression for survival as hypomorph mutants die at birth from respiratory distress due to collapsed lungs (Affar et al., 2006). This phenotype was reproduced when we specifically ablated \textit{Yy1} function in the mesenchyme. Moreover, \textit{Yy1} lung mesenchymal deletion affects epithelial cell differentiation indicating an YY1 non-cell autonomous role (Bérubé-Simard et al., 2014). Here, we demonstrated that the \textit{Yy1} mutation in lung epithelium also impacts on lung formation. \textit{Yy1} function is thus required in both mesenchymal and epithelial cell layers for the correct development of the respiratory tract.

Epithelial inactivation of the \textit{Yy1} gene inhibited lung branching but maintained distal epithelial cell differentiation, which agrees with the notion of negative correlation between branching and alveolar differentiation (Chang et al., 2013). The \textit{Yy1} mutation also caused the formation of large cysts that mimicked the \textit{Shh} lung phenotype (Litingtung et al., 1998; Pepicelli et al., 1998). \textit{Shh} expression was decreased in \textit{Yy1} mutant lungs independently of the \textit{Cre} allele used. SHH, a diffusible factor secreted by the lung epithelium, inhibits \textit{Fgf10} expression in lung mesoderm (Bellusci et al., 1997). Consequently, \textit{Fgf10} expression was upregulated in \textit{Yy1} mutants, resulting in the loss of \textit{Fgf10}-expressing foci in lung mesenchyme that normally trigger branching events. Gain in \textit{Fgf10} expression may generate a driving force favoring the cystic dilation of airways in \textit{Yy1} mutants (Fig. 10). This mechanistic explanation is further supported by the observations that trans-uterine intraparenchymal microinjections of a vector carrying an \textit{Fgf10} transgene induce cystic lung malformations in rat fetuses that recapitulate the phenotype of human congenital cystic lung diseases (Gonzaga et al., 2008). Therefore, our work uncovers a functional cascade sequentially implicating \textit{Yy1}, \textit{Shh} and \textit{Fgf10} genes in lung branching coordination.

One \textit{Shh} wild-type allele remains in \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} specimens, raising concerns about the impact of \textit{Shh} haploinsufficiency on the lung phenotype. We have shown that: (1) \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} specimens did not present lung anomalies; (2) \textit{Shh} expression was reduced in \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} specimens compared with \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} samples; and (3) \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} and \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} mutants share a similar lung phenotype. Moreover, \textit{Shh}\textsuperscript{+/−} mutants do not present lung anomalies and variations in \textit{Fgf10} expression (van Tuyl et al., 2007). These data provide concordant evidence for a role of YY1 in the regulation of \textit{Shh} expression in the lung.

![Fig. 6. Addition of rmSHH partially rescues the airway smooth muscle cells and the apoptotic defects in \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} lung explants. Lung explants from E12.5 \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} (A-D,I-N) and \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} (E-H,K-P) embryos were cultured for 3 days with (C,D,G,H,J,L,N,P) or without (A,B,E,F,K,M,O) rmSHH. BSA-treated \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} explants exhibited cysts and failed to branch (E,F). They showed a loss of αSMA immunoreactivity around the cysts (I,K) and an increased number of apoptotic cells (M,O). Addition of rmSHH caused an increased size of the mesenchymal layer in both \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} and \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} explants but did not improve lung branching in mutants (C,D,G,H). rmSHH partially restored αSMA expression in \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} explants (I-L). The number of apoptotic cells in \textit{Yy1}\textsuperscript{floxed}\textsuperscript{−};\textit{Shh}\textsuperscript{+/Cre} lungs supplemented with rmSHH returned to control levels (M-P). Arrows indicate cleaved caspase 3-positive cells. Asterisks indicate cysts, which are delineated by a dashed line. (\textit{n}=3-5 per condition). Scale bars: 1 mm in A-H; 100 µm in I-P.](image-url)
transcriptional regulator of Shh expression in lung endoderm and alleviate the potential effect of the Shh heterozygous status on the phenotype observed.

The left pulmonary isomerism, the peribronchial smooth muscle differentiation defect and the tracheal–bronchial ring anomalies encountered in Yy1flox/flox,Shh+;Cre specimens further support a role for YY1 in the positive regulation of Shh expression in the respiratory tract epithelium as these anomalies were also reported in Shh null mutants (Pepicelli et al., 1998; Tsukui et al., 1999). The tracheoesophageal fistula observed in Shh mutants was not reproduced in Yy1flox/flox,Shh−;Cre mutants, probably due to the remaining low levels of Shh expression, suggesting that different SHH thresholds define specific SHH functions during respiratory tract development.

In the trachea, epithelial YY1 plays a non-cell autonomous role in the formation of the cartilage rings, whereas it acts directly on epithelial cell differentiation. In the distal lung, the Yy1 epithelial mutation does not affect the specification of the epithelial cell types but it interferes with the specification of the airway smooth muscle cells. Addition of rmSHH to Yy1 mutant lung explants can rescue the defective specification of αSMA-positive myofibroblasts and control apoptosis levels, strengthening the importance of SHH in mediating the non-cell autonomous role of epithelial YY1 in lung mesenchyme. However, the lack of branching of Yy1flox/flox,Shh−;Cre explants in presence of rmSHH indicated that SHH is not the sole player in the Yy1 lung phenotype. This is further supported by the microarray data revealing that several transcriptional regulators of lung development were differentially expressed in Yy1flox/flox,Shh−;Cre specimens.

Formation of cystic-like structures is a characteristic of several mutations targeted to lung epithelium in mice. The epithelial inactivation of Dicer function with the ShhCre allele caused similar lung defects to those seen in Yy1flox/flox,Shh−;Cre mice (Harris et al., 2006). Moreover, reduced Shh expression and augmented Fgf10 expression occurred in Dicerflox/flox,Shh−;Cre lung specimens, reinforcing the idea that dysregulated SHH-FGF10 signaling acts as a convergent point that induces events leading to cystic lung malformations.

Increased Fgf10 expression appears to be a common denominator in the formation of lung cysts (Gonzaga et al., 2008). However, the reduced expression of En5 and Elf5 genes, encoding two transcription factors of the lung epithelium known to be under the positive control of FGF10, indicates that Yy1 acts also on gene expression independently of FGF10 signaling (Liu et al., 2003; Metzger et al., 2007).

Addition of Fgf9, another FGF important for lung development, to lung explant cultures also causes cyst-like structures (del Moral et al., 2006). Fgf9 overexpression in lung epithelium induces epithelial branching arrest and luminal dilatation in combination with decreased and increased Shh and Fgf10 expression, respectively (White et al., 2006). Recently, augmented Fgf9 expression was reported in the lung epithelium of E12.5 Dicerflox/flox,Shh−;Cre embryos and type I PPB patients, whereas the Dicerflox/flox,Shh−;Cre lung phenotype was attenuated by reduced Fgf9 epithelial gene dosage (Yin et al., 2015). Our results indicated that Fgf9 expression was not affected in lungs from E14.5 Dicer mutants, whereas it was reduced in Yy1 mutant specimens. As Fgf9 is expressed in both lung mesothelium and epithelium, a possibility remains that local variations in distinct cell populations, not detectable by a qRT-PCR global approach, may occur (Yin et al., 2011). Alternatively, variations in temporal Fgf9 expression in Dicer mutant lungs might explain the differences.
Despite the resemblances between \( Yy^1 \) and \( Dicer \) mutant lung phenotypes and the important overlap in differentially expressed lung genes, production of \( Yy^1 \) and \( Dicer \) double heterozygous mice and expression analyses did not provide evidence for a genetic interaction between \( Yy^1 \) and \( Dicer \) genes during lung morphogenesis. Therefore, \( Dicer \) may act via distinct pathways to control lung development.

![Fig. 8. Loss of Dicer function in the developing lung epithelium phenocopies the lung defects of Yy1 mutants.](image)

Although our data pointed toward a direct transcriptional regulation of \( Shh \) expression by \( Yy1 \), the mechanisms of \( Dicer \) action in lung epithelium remain elusive (Harris et al., 2006).

\( Yy1 \) acts on target genes via the recruitment of co-factors, and elucidating \( Yy1 \) partners may unveil the molecular mechanisms underlying the lung phenotype. For instance, \( Yy1 \) recruits protein modifiers, such as \( Ezh2 \) and histone deacetylases (HDAC), that mediate posttranslational modifications involved in chromatin remodeling (Deng et al., 2010). The epithelial deletion of \( Ezh2 \) function does not cause cyst formation (Snitow et al., 2015). Conversely, the combined epithelial mutations of \( Hdac1 \) and \( Hdac2 \) genes led to airway dilation and to upregulated \( Cdkn1a \) expression, raising the possibility that \( Yy1 \) and HDAC act together to some extent during lung morphogenesis (Wang et al., 2013).

Aberations in lung developmental processes may give rise to structural abnormalities, such as congenital cystic diseases that encompass a spectrum of rare but clinically significant conditions like CCAM and PPB. PPB is characterized in its earliest manifestation by cyst formation (Snitow et al., 2015). Conversely, the combined epithelial mutations of \( Hdac1 \) and \( Hdac2 \) genes led to airway dilation and to upregulated \( Cdkn1a \) expression, raising the possibility that \( Yy1 \) and HDAC act together to some extent during lung morphogenesis (Wang et al., 2013).

A whole exome sequencing analysis of PPB patients revealed somatic \( DICER1 \) missense mutations, but no \( Yy1 \) mutations were found (Pugh et al., 2014). The somatic \( DICER1 \) mutations were mainly localized in the RNaseIIIb domain responsible for the cleavage of the 3' end from the -5p strand of microRNA precursors. Mutations of the RNaseIIIb domain were shown to cause a -3p mature microRNA strand bias due to the loss of -5p strand cleavage of the pre-microRNAs (Anglesio et al., 2013).
Serum microRNA profiling from a PPB patient carrying a somatic mutation in the DICER1 RNaseIIIb domain showed the overexpression of microRNAs largely derived from the -3p strand (Murray et al., 2014). Among the microRNAs presenting the higher fold change were let-7a-3p and let-7b-3p, which are predicted to target the human YY1 gene (http://mirdb.org). It is tempting to speculate that abnormal levels of particular miRNAs may alter YY1 expression providing a mechanistic explanation for the reduced YY1 levels seen in lungs from PPB patients (Fig. 10).

Because YY1 expression and function are closely associated with cell-cycle progression and apoptosis, the potential role of YY1 in cancer was extensively explored. Both increased and decreased YY1 expression levels were linked to a wide range of cancers (Wang et al., 2014).
2008; Nicholson et al., 2011). Analysis of $Yy1^{flox/flox}; Shh^{+/Cre}$ lungs at late gestation revealed a burst in cell proliferation in both epithelium and mesenchyme that may reflect a potential subsequent malignant transformation. The reduced expressivity of the lung phenotype of $Yy1^{flox/flox}; Tg^{+/Nkx2-1Cre}$ mutants allowed us to overcome the neonatal death of $Yy1^{flox/flox}; Shh^{+/Cre}$ pups. The cystic architectural and histological features observed in mutant mice were reminiscent of an evolving type I PPB, further supporting the notion that reduced expression of YY1 may participate in PPB pathogenesis. It must be underscored that the unrepressed $Fgf10$ expression seen in $Yy1$ mutants should also be considered to play a role in cancer development as $Fgf10$ overexpression in the lung during the postnatal period can cause multifocal pulmonary tumors (Clark et al., 2001).

In conclusion, we present evidence of the crucial role played by the transcription factor YY1 in the developing epithelium of the respiratory tract for lung branching morphogenesis. Although further works are needed to fully elucidate the underlying mechanisms, SHH appears to be a key player in mediating YY1 function in the lung. Our $Yy1$ mouse models also reproduced characteristics of the rare early childhood cancer PPB and indicated that a more precise gene dosage mouse model operating within the context of a developmental window should provide a strong model for the exploration of PPB molecular pathogenesis.

### MATERIALS AND METHODS

#### Mice, genotyping and tissue collection

$Yy1^{flox/+}; Tg^{+/Nkx2-1Cre}$ mice were obtained from Dr Shi (Affar et al., 2006). The Rosa26 reporter line ($Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$), the $Dicer1^{flox/flox}$ conditional line ($Dicer1^{tm1Bdh/J}$), and the $Shh^{Cre}$ [$Shh^{tm1(EGFP/cre)Cjt}$] and $Tg^{+/Nkx2-1Cre}$ deleter strains were purchased from The Jackson Laboratory (Harfe et al., 2004, 2005; Muzumdar et al., 2007; Xu et al., 2008). As only individuals carrying the $Yy1^{flox/flox}; Shh^{+/Cre}$, $Yy1^{flox/flox}; Nkx2-1-Cre$ and $Dicer^{flox/flox}; Shh^{+/Cre}$ genotypes presented defects, all the other genotypes were referred hereafter as controls except when specified. Age of the embryos was estimated by considering the morning of the day of the vaginal plug as E0.5. Experimental specimens were genotyped by PCR analyses. Lungs were collected at E12.5, E14.5, E18.5 or postnatal day (P) 21 as described (Boucherat et al., 2014). For RNA extraction, lungs were snap-frozen in N2. Experiments were performed according to the guidelines of the Canadian Council on Animal Care and approved by the institutional animal care committee.

#### Human tissues

This study was conducted with anonymised specimens of CCAM and PPB patients from the Department of Pathology of Hôpital Necker-Enfants Malades, Paris, France and from McGill University, Montréal, Canada and

### Table 2. Ratios of genotypes in litters from crosses between $Yy1^{flox/+}; Tg^{+/Nkx2-1Cre}$ and $Yy1^{flox/flox}$ mice

<table>
<thead>
<tr>
<th>Age</th>
<th># litters</th>
<th># pups</th>
<th>$Yy1^{flox/+}$</th>
<th>$Yy1^{flox/flox}$</th>
<th>$Tg^{+/Nkx2-1Cre}$</th>
<th>$Yy1^{flox/+}$</th>
<th>$Yy1^{flox/flox}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
<td>3</td>
<td>23</td>
<td>9 (39.1)</td>
<td>7 (30.4)</td>
<td>2 (8.8)</td>
<td>5 (21.7)</td>
<td></td>
</tr>
<tr>
<td>E18.5</td>
<td>2</td>
<td>14</td>
<td>4 (28.6)</td>
<td>4 (28.6)</td>
<td>3 (21.4)</td>
<td>3 (21.4)</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>14</td>
<td>86</td>
<td>17 (19.8)</td>
<td>37 (43)</td>
<td>25 (29.1)</td>
<td>7 (8.1)</td>
<td></td>
</tr>
</tbody>
</table>

The percentage obtained is indicated in parentheses.
approved by the respective Ethics Committee. Controls corresponded to normal lung specimens from the Department of Pathology of Hôpital Raymond Poincaré, Garches, France. They were collected at autopsy from age-matched children who died from a non-pulmonary cause (sudden infant death syndrome). Characteristics of patients are summarized in supplementary material Table S1.

**Histology, immunohistochemistry (IHC) and immunofluorescence (IF) analyses**

Experiments were performed as described (Boucherat et al., 2014). The Cryo 3 Tyramide Signal Amplification Kit (PerkinElmer) was used for pERK detection. Antibodies are listed in supplementary material Table S2.

**Proliferation and apoptosis**

Experiments were performed as described (Boucherat et al., 2014). Three to four random fields were taken, for an average number of 650 cells per field, from four to five embryos per genotype.

**In situ hybridization**

RNA in situ hybridization was performed on 15 µm cryosections of E12.5 embryos (Schaefer-Wienkem and Gerras-Moser, 1993). A 584-bp mouse Fgf10 CDNA fragment was used for the digoxigenin-labeled riboprobe (provided by Dr B. Hogan, Duke University Medical Center, USA). Experiments were performed on four specimens per genotype.

**Alcian Blue cartilage staining**

Dissected respiratory tracts from E18.5 embryos were stained in a solution of 0.03% Alcian Blue and 20% acetic acid prepared in 95% ethanol. The external tracheal diameter was measured at five rostrocaudal locations along the most linear portion of the trachea and quantified using NIH ImageJ software. Tracheal luminal surface was measured using Leica SCN 400 F SlideScanner and SlidePath Gateway Software.

**Quantitative RT-PCR (qRT-PCR)**

Lung total RNA was isolated from individual E14.5 embryos. qRT-PCR experiments were performed as described (Boucherat et al., 2012). Three to eight specimens were used per genotype tested. Primer sequences are listed in supplementary material Table S3A.

**Chromatin immunoprecipitation (ChIP) assays**

Lungs from E14.5 wild-type embryos were collected and ChIP assay was performed with rabbit anti-YY1 antibody, rabbit anti-histone H3, or control rabbit IgG as described (Bérubé-Simard et al., 2014). qPCR-ChIP analyses were performed with primers specific to each domain containing YY1 binding sites (supplementary material Table S3B). The values for the samples immunoprecipitated by anti-YY1, anti-histone H3, or control IgG were recorded as the percentage relative to input. ChIP results were confirmed by two independent experiments. qPCR was performed in triplicate for each sample. ChIP efficiency was calculated by dividing the amount of PCR product obtained with the immunoprecipitated DNA by the amount obtained with the input DNA (Aparicio et al., 2005).

**Transactivation assays**

Human pCMV-YY1 and control pCMV-GFP-Lpa expression vectors were obtained from Drs G. Blanck (University of South Florida, USA) and R. Aasland (University of Bergen, Norway), respectively. A 5 kb BgrII-Xhol genomic fragment located between positions 5016 bp and +60 bp of the mouse Shh gene (relative to TSS) was cloned into the pGL3 basic luciferase reporter expression plasmid (Promega). HEK293 cells were transiently co-transfected in 24-well plates (40,000 cells/well) with 0.3 µg/well of luciferase reporter construct and 0.2 µg/well of YY1 or the RL-SV40 control expression vectors using the FuGENE 6 transfection reagent (Promega). The RL-SV40 Renilla reniformis luciferase expression vector (Promega) was used as an internal control for transfection efficiency (0.001 µg/well). Luciferase activity was measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Transfections were performed in triplicate in at least two independent experiments. Data from a representative experiment are presented as the fold induction±s.d. of normalized relative luciferase activity.

**Lung explant cultures**

Experiments were performed as described (Boucherat et al., 2014). Lungs were kept for 72 h in serum-free DMEM/F12 medium (Gibco) in presence or not of rmSHH protein, N-Terminus (R&D Systems) at a concentration of 3 µg/ml. BSA was used as control.

**Microarray analysis**

Total RNA was isolated from lungs of E14.5 Shh+/Cre, Yy1flacoxo/Shh+/Cre and Dicerflacoxo/Shh+/Cre embryos (n=4/genotype). RNA quality and quantity assessment, cDNA probe preparation, hybridization to the Affymetrix Mouse Gene 2.0ST Array and image scan were performed at the Genome Quebec Innovation Centre at McGill University (Montreal, Canada). Data were pre-processed and normalized using Affymetrix Power Tools with the rma-sketch method. Raw and normalized data were uploaded to the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE66171 according to MIAME standards (Edgar et al., 2002). Significantly modulated probes were identified using the empirical Bayes statistics available in limma (Smyth, 2004). Probes were considered to be significantly modulated when the Benjamin–Hochberg-adjusted was significant to P<0.05.

**Statistical analyses**

Student’s t-test was performed for comparative studies. A significance level inferior to 5% (P<0.05) was considered statistically significant.

**Author contributions**

The authors declare no competing or financial interests.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.120469/-/DC1

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