

RESEARCH ARTICLE

Cyp26b1 is an essential regulator of distal airway epithelial differentiation during lung development

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ABSTRACT

Proper organ development depends on coordinated communication between multiple cell types. Retinoic acid (RA) is an autocrine and paracrine signaling molecule essential for the development of most organs, including the lung. Despite extensive work detailing effects of RA deficiency in early lung morphogenesis, little is known about how RA regulates late gestational lung maturation. Here, we investigate the role of the RA catabolizing protein Cyp26b1 in the lung. Cyp26b1 is highly enriched in lung endothelial cells (ECs) throughout development. We find that loss of Cyp26b1 leads to reduction of alveolar type 1 cells, failure of alveolar inflation and early postnatal lethality in mouse. Furthermore, we observe expansion of distal epithelial progenitors, but no appreciable changes in proximal airways, ECs or stromal populations. Exogenous administration of RA during late gestation partially mimics these defects; however, transcriptional analyses comparing Cyp26b1^{-/-} with RA-treated lungs reveal overlapping, but distinct, responses. These data suggest that defects observed in Cyp26b1^{-/-} lungs are caused by both RA-dependent and RA-independent mechanisms. This work reports crucial cellular crosstalk during lung development involving Cyp26b1-expressing endothelium and identifies a novel RA modulator in lung development.

KEY WORDS: Lung, Cyp26b1, Epithelium, Retinoic acid, Endothelial cell, Epithelial cell, Mouse

INTRODUCTION

Organogenesis requires tightly orchestrated crosstalk between endothelial cells (ECs), epithelial cells and stromal cells to form a mature and functional organ. These cell types communicate with one another using a multitude of distinct signaling pathways that must be activated at the right place and time to promote proper development (Kraus and Grapin-Botton, 2012; Rankin et al., 2018). Coordination is central to development, and aberrations in any one step of this multistep process can have catastrophic developmental consequences. Despite this, there is still much to learn concerning the mechanisms that underlie spatiotemporal control of developmental signaling cues over the course of organogenesis.

Lung development begins at embryonic day (E)9.0 with specification of lung progenitors along the ventral side of the

anterior foregut endoderm (Herriges and Morrisey, 2014; Shi et al., 2009; Warburton et al., 2010). These progenitors undergo initial bud formation, followed by highly stereotyped and hierarchical branching to form the lung airway tree (Metzger et al., 2008). During this process, the lung epithelium segregates into two separate groups: the distal airways, which give rise to alveoli where gas exchange occurs, and proximal airways, which form the bronchi and bronchioles. By E16.5, the distal airways begin to differentiate into pre-alveolar structures called canaliculi and saccules, consisting of alveolar type 2 (AT2) surfactant-producing cells and alveolar type 1 (AT1) gas-exchanging cells. These structures mediate gas exchange in neonates until full alveolarization occurs postnatally (Herriges and Morrisey, 2014; Morrisey and Hogan, 2010). Understanding the drivers of this process has the potential to impact human health, as premature infants are at an increased risk for developing respiratory distress syndrome (RDS) because of immaturity of distal airways, with a mortality rate as high as 50% (Gallacher et al., 2016).

Retinoic acid (RA) is an essential signaling molecule that exhibits highly regulated spatiotemporal control during embryogenesis. RA directs cell fate by binding to the RAR and RXR family of nuclear receptors to direct changes in gene transcription. RA is derived from vitamin A (retinol) consumed in our diet. Vitamin A is first dehydrogenated to form retinaldehyde, then retinaldehyde is further dehydrogenated by Raldh1-Raldh3 (also known as Aldh1a1-Aldh1a3) to form the active forms of RA, with all-trans retinoic acid (atRA) being the most abundant and potent form. Once synthesized, RA can act as an autocrine signal or can diffuse to nearby cells as a paracrine signal, forming a local gradient (Duester, 2008). Antagonizing RA signaling is the Cyp26 family of P450 enzymes, consisting of Cyp26a1, Cyp26b1 and Cyp26c1. These enzymes metabolize RA into inactive forms. The expression of these genes requires precise control, as genetic or pharmacologic manipulations resulting in RA excess or deficiency drastically affect nearly every developing organ (Duester, 2008; Rhinn and Dolle, 2012).

Lung development and maturation are known to require proper spatiotemporal regulation of RA signaling. Mice lacking Raldh2 or fed on a vitamin A-deficient diet fail to initiate lung formation, resulting in lung agenesis (Wang et al., 2006; Wilson et al., 1953). In the initial lung bud, RA activates the Wnt cascade via Shh, and inhibits TGF- β signaling (Chen et al., 2010, 2007; Rankin et al., 2016, 2018). Together, this leads to upregulation of Fgf10, a crucial factor necessary for lung bud formation and branching (Desai et al., 2004; Park et al., 1998; Wang et al., 2006). Once lung branching is initiated, RA activity decreases until birth, allowing for proper epithelial branching and distal airway differentiation. Culturing E12.5 lung explants with RA leads to a reduction in branching due to decreased Fgf10 (Malpel et al., 2000). Likewise, forcing increased RA signaling in distal lung epithelial cells through a

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constitutively active RAR *in vivo* prevents distal sacculle differentiation (Wongtrakool et al., 2003). Lastly, a third, separate role for RA occurs postnatally during alveolar maturation. At this stage, RA promotes increased alveolar septation, alveolar number and alveolar surface area (Massaro and Massaro, 1996, 2000; Massaro et al., 2003; McGowan et al., 2000). Taken together, these studies indicate that RA is essential for the regulation of several distinct steps during lung development in a highly temporally defined manner. However, the role of RA signaling in lung development during late gestation (later than E15.5) is not fully understood.

Although RA signaling has been shown to regulate specification and differentiation of lung cell types, the role of RA-catabolizing Cyp26 enzymes during lung development remains unknown. Cyp26a1 is expressed in the epithelium during early stages of lung branching, but its expression diminishes and is absent by E16.5 when distal epithelial differentiation begins (Malpel et al., 2000). On the other hand, Cyp26b1 is expressed in non-epithelial cells at E18.5 (Abu-Abed et al., 2002). Deletion of Cyp26b1 leads to neonatal lethality, which was suggested to occur from pulmonary dysfunction, although this phenotype was not characterized further (Yashiro et al., 2004). These data suggest that Cyp26b1 may be required in the lung during late gestation to reduce RA signaling and promote proper distal epithelial differentiation in a paracrine manner.

Here, we identify a crucial role for Cyp26b1 during lung organogenesis. Cyp26b1 is highly enriched in lung ECs throughout development, and loss of Cyp26b1 results in a delay

in the formation of distal airways in late gestation. E18.5 Cyp26b1^{-/-} lungs exhibit increased cellular density and contain an expansion of a distal tip progenitor population at the expense of mature gas-exchanging AT1 cells. Exogenous administration of atRA during late gestation phenocopies loss of Cyp26b1, suggesting that the phenotype is due, at least in part, to excess RA. However, transcriptional analyses of atRA-treated and Cyp26b1^{-/-} lungs reveal only partially overlapping responses, suggesting RA-independent signaling pathways downstream of Cyp26b1 in the lung. These findings identify Cyp26b1 as a novel endothelial modulator of RA activity in the developing lung and reveal RA-independent functions during lung development.

RESULTS

Cyp26b1 is highly enriched in lung and kidney ECs

We have previously identified *Cyp26b1* as an endothelial-enriched gene in embryonic organs, including kidney and lung, using RNA-seq (Daniel et al., 2018). *Cyp26b1* was expressed in the mesenchyme of these embryonic organs, suggesting possible lung EC enrichment (Abu-Abed et al., 2002) (Fig. 1). To differentiate between the two major cell types resident in the lung mesenchyme – lung ECs and stromal cells – we assessed *Cyp26b1* expression in publicly available single cell (sc)RNA-seq datasets of fetal, early postnatal and adult tissues. These studies confirmed elevated expression of *Cyp26b1* in ECs compared with non-ECs (Fig. S1; Du et al., 2015, 2017; Guo et al., 2019; Lindstrom et al., 2018; Sabbagh et al., 2018; Tabula Muris et al., 2018).

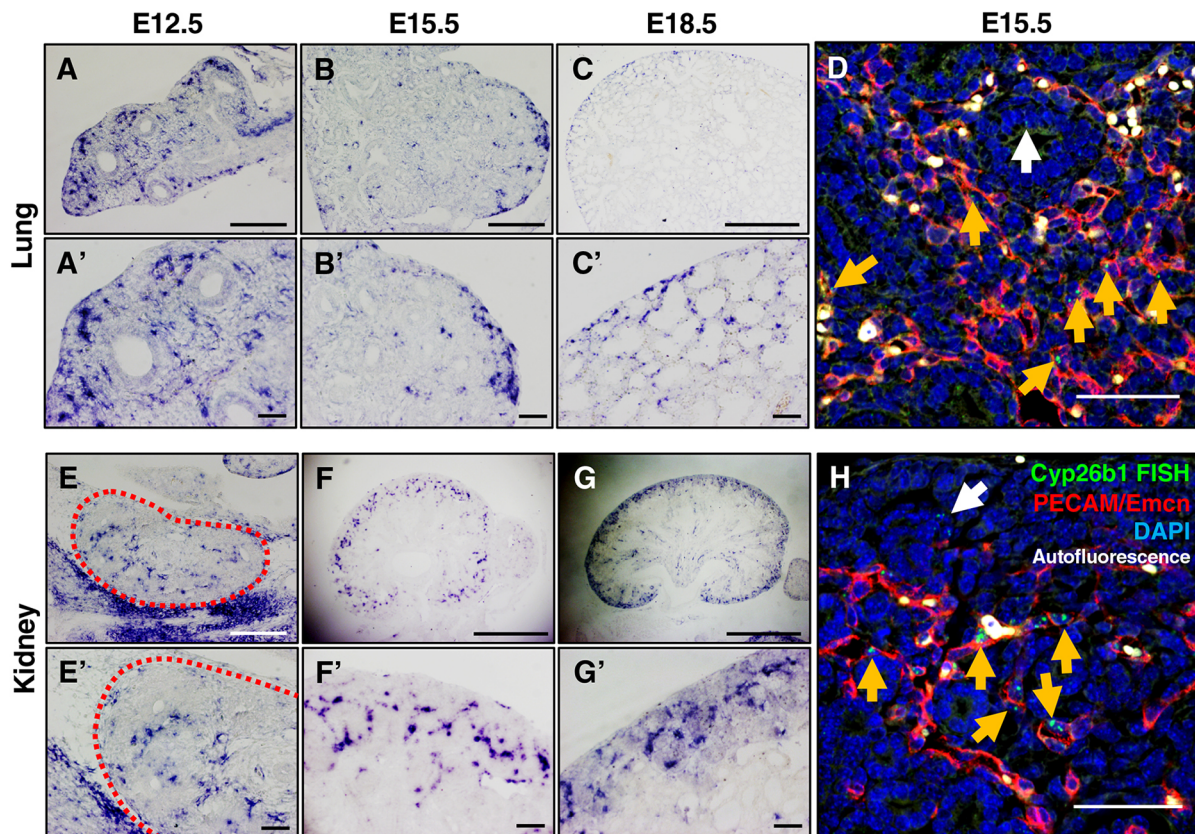


Fig. 1. Cyp26b1 is highly enriched in lung and kidney endothelial cells during development. (A-H) ISH of *Cyp26b1* in the lung (A-D) and kidney (E-H) at E12.5 (A,A',E,E'), E15.5 (B,B',D,F,F',H) and E18.5 (C,C',G,G'). Magnifications for chromogenic assays are shown. Red dotted outline (E,E') delineates the E12.5 kidney. D and H show FISH for *Cyp26b1* (green) co-stained with PECAM and Emcn (red) to mark ECs. Orange arrows, *Cyp26b1*⁺ puncta in PECAM⁺/Emcn⁺ ECs; white arrow, *Cyp26b1*⁺ puncta in non-EC cells. Scale bars: 200 μm (A,B,E); 100 μm (A',B',C',E',F',G'); 500 μm (C,F,G); 50 μm (D,H).

To validate these data, we performed *in situ* hybridization (ISH) for *Cyp26b1* in E12.5, E15.5 and E18.5 embryonic organs. These experiments revealed an endothelial-like expression pattern in both lung and kidney (Fig. 1A-C',E-G'). Consistent with previously published data, *Cyp26b1* was also expressed in the developing limbs, the face and palate, the tongue, the hindbrain, intersomitic regions along the back, in endocardial cushions extending into the great vessels and the epicardium (Fig. S2A-E') (Abu-Abed et al., 2002; Spoorendonk et al., 2008). By E15.5, *Cyp26b1* expression in lungs was restricted to the ECs in the distal periphery (Fig. 1B-C'). Likewise, *Cyp26b1* expression in the kidney became restricted to ECs in the outer cortex (Fig. 1F-G'). We validated EC specificity of *Cyp26b1* using fluorescent *in situ* hybridization (FISH) analysis and co-staining with the endothelial markers PECAM and endomucin (Emcn) (Fig. 1D,H). Further, *Cyp26b1* FISH co-stained with Lyve1 revealed that *Cyp26b1* is also expressed in lymphatic endothelium in the lung (Fig. S2F-F'). Of note, *Cyp26b1*⁺ punctae were observed in some epithelial and stromal regions, but at much lower levels compared with ECs (white arrows). Thus, *Cyp26b1* expression is highly enriched in lung and kidney ECs throughout development.

Generation of *Cyp26b1* null mice using CRISPR/Cas9

Previous work using a *Cyp26b1* germline deficient mouse model showed that *Cyp26b1* null mutants die shortly after birth because of respiratory distress (Yashiro et al., 2004). We generated two independently derived *Cyp26b1*-null mouse models using CRISPR/Cas9 to further explore these defects. Our first model contained an in-frame 2.6 kb deletion from exon 3 to exon 6 (referred to as *Cyp26b1*⁻) and the second contained a 10 bp deletion in exon 3 leading to a frame-shift mutation (referred to as *Cyp26b1*^{Δ10}). Both *Cyp26b1*⁻ and *Cyp26b1*^{Δ10/Δ10} embryos exhibited many of the same developmental defects as previously described, including limb defects, craniofacial abnormalities, micrognathia, cleft palate, skin abnormalities and spleen hypoplasia, although edema and hemorrhages were not observed at this stage as previously described (Fig. 2A,B) (Bowles et al., 2014; Dranse et al., 2011; Lenti et al., 2016; Okano et al., 2012a,b; Yashiro et al., 2004). In addition, *Cyp26b1*⁻ mice died shortly after birth and exhibited signs of respiratory distress, including air hunger, mirroring the previously generated null model. These observations and previously published data both support a role for *Cyp26b1* in regulating lung development.

Cyp26b1 null mice exhibit lung defects

To further explore how loss of *Cyp26b1* affects lung development in late gestation, we analyzed *Cyp26b1*^{+/+} and *Cyp26b1*^{-/-} lungs at E18.5, immediately before birth. E18.5 *Cyp26b1*^{-/-} lungs were smaller but with grossly normal lobation (Fig. 2C,D). Interestingly, *Cyp26b1*^{-/-} lungs had decreased distal airspaces compared with control littermates (Fig. 2C',D'). In tissue sections, *Cyp26b1*^{-/-} lungs appeared to be hypercellular, with increased septal wall thickness and smaller airspaces, although the total number of airspaces was not different (Fig. 2E-K). To determine whether this phenotype was due to an increase in total cell number or due to a partial failure of alveologenesis, we measured the wet and dry weights of these lungs. Measurements of the wet and dry weight standardized to total body weight confirmed that *Cyp26b1*^{-/-} lungs are smaller without an increase in total mass (Fig. 2L-N). These data suggest that the phenotype in *Cyp26b1*^{-/-} lungs is due to an increase in the density of cells within the lung and not due to increased total number of cells.

Cyp26b1 is necessary for distal epithelial maturation

Based on these gross changes in epithelial morphology, we asked whether distal epithelial specification was affected in E18.5 *Cyp26b1*^{-/-} lungs. We first assessed changes in the frequency of AT1 and AT2 cells. Immunofluorescent (IF) stains for the AT1 cell marker HOPX revealed a significant decrease in AT1 cells upon loss of *Cyp26b1* (Fig. 3A-C). Of note, *Cyp26b1*^{-/-} lungs contain a lower proportion of AT1 cells after standardizing for the increased number of DAPI⁺ cells per given area. Staining for the additional AT1 markers podoplanin (Pdpn) and aquaporin 5 (Aqp5) confirmed the reduction in distal airway cells in the mutants (Fig. S3A-D). IF stains for the AT2 marker pro-surfactant protein C (proSP-C; *Sftpc*) at E18.5 revealed that *Cyp26b1*^{-/-} lungs contain an increase in AT2 cells compared with control littermates (Fig. 3D-F). Because proSP-C is not exclusively expressed in AT2 cells during development (Frank et al., 2019), we repeated the analysis using the AT2 cell-specific marker lysosomal-associated membrane protein 3 (Lamp3 or DC-Lamp). Quantification of AT2 cells using Lamp3 demonstrated a similar increase in Lamp3⁺ AT2 cells compared with proSP-C⁺ AT2 cells in *Cyp26b1*^{-/-} lungs, with the vast majority of AT2 cells expressing both proteins (Fig. S3E-J). To determine whether this increase in AT2 cells could be the result of a general increase in the total number of epithelial cells, we stained E18.5 lungs for pan-epithelial nuclear marker Nkx2.1. Quantification revealed no change in the total number of distal epithelial cells (Fig. S3K-M). These data show that there is no change in total distal epithelial cell number, but rather a change in the proportion of AT1 and AT2 cells.

These apparent changes in epithelial differentiation led us to ask whether there were changes in epithelial AT1 or AT2 progenitor populations. Sox9⁺ cells located at the tips of the distal epithelium are primarily fated to become AT2 cells (Frank et al., 2019; Rockich et al., 2013). These cells first express both Sox9 and proSP-C/Lamp3, then become exclusively proSP-C/Lamp3-positive as they differentiate into mature AT2 cells (Rockich et al., 2013). We observed an increase in Sox9⁺ distal epithelial progenitor cells in *Cyp26b1*^{-/-} lungs (Fig. 3G-I). To determine whether the increase in Sox9⁺ and proSP-C⁺/Lamp3⁺ cells in *Cyp26b1*^{-/-} lungs represents an increase in two distinct populations or in the same distal tip progenitor population, we co-stained E18.5 lungs with both Sox9 and Lamp3. Quantification of these populations revealed an expansion in only Sox9⁺/Lamp3⁺ cells, with no change in Sox9⁺/Lamp3⁻ or Sox9⁻/Lamp3⁺ cells (Fig. 3J and Fig. S3N,O). We sought to further validate the IF stains with western blot and qRT-PCR analyses on E18.5 tissues. Western blot analyses demonstrated a decrease in the AT1 cell marker Aqp5 and an increase in proSP-C protein levels, validating the IF data (Fig. S3P). Likewise, qRT-PCR for the AT1 cell markers *Aqp5*, *Pdpn*, *HOPX* and *Ager* (*RAGE*) revealed ~40% decrease in mRNA abundance in *Cyp26b1*^{-/-} lungs; however, in contrast to the IF analysis, *Sox9*, *Sftpc* and *Lamp3* mRNA levels were not significantly altered (Fig. S3Q).

Defects in lung development are not due to off-target CRISPR lesions

Because our *Cyp26b1* mutant mice were generated using CRISPR/Cas9-mediated mutagenesis, we considered the possibility that there may be off-target effects contributing to these phenotypes. These off-target effects can be eliminated by crossing two independently derived null alleles to create compound heterozygotes. Any phenotypes observed in compound heterozygotes can then be fully

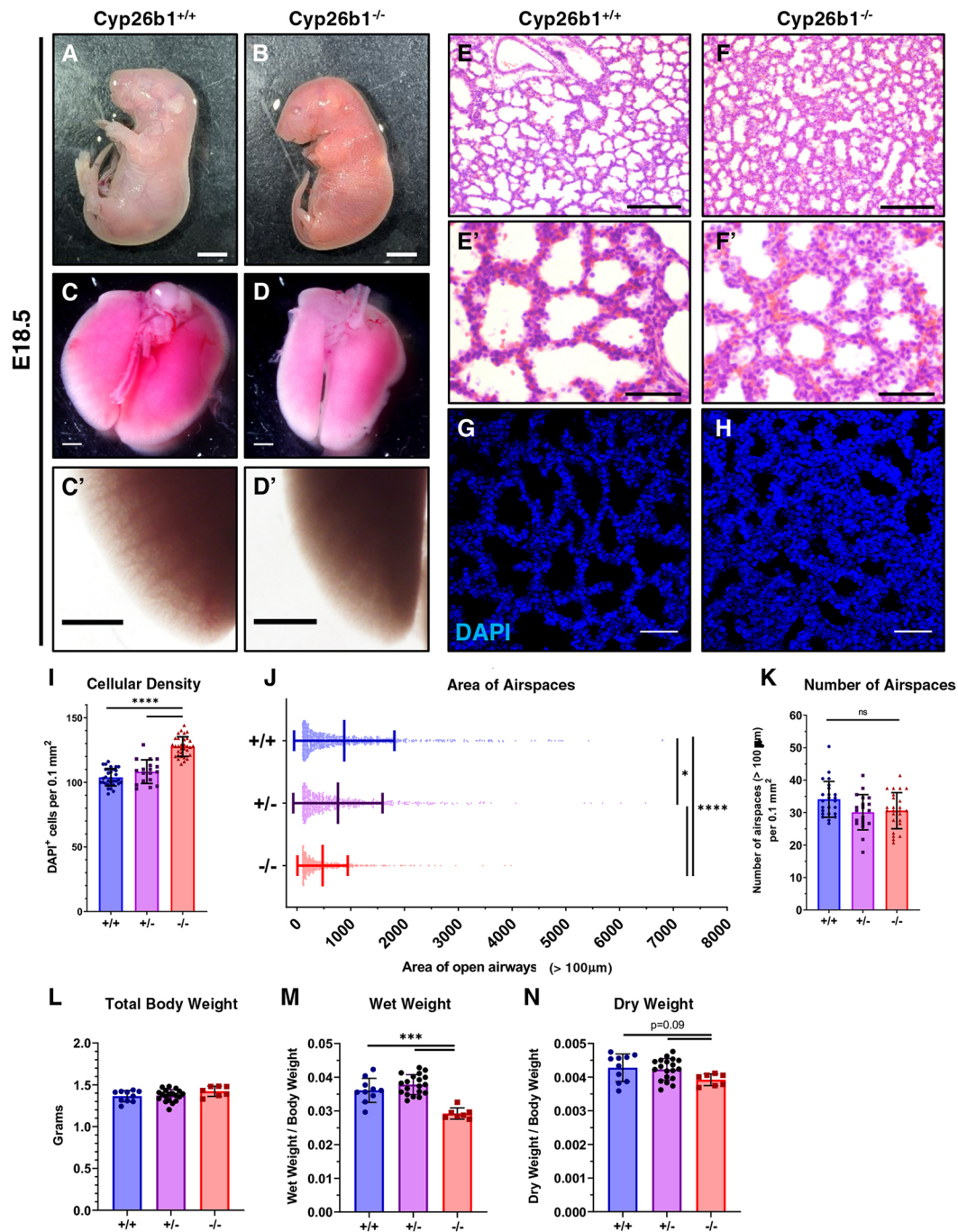


Fig. 2. Cyp26b1 mutant lungs exhibit increased cellular density and decreased airspaces at late gestation. (A,B) E18.5 Cyp26b1^{+/+} and Cyp26b1^{-/-} embryos at dissection. (C,D') Lungs from Cyp26b1^{+/+} and Cyp26b1^{-/-} E18.5 embryos. C' and D' show magnified areas of lungs in C and D, respectively, with backlight to highlight loss of distal airspaces in Cyp26b1^{-/-} lungs. (E-F') H&E stain of E18.5 Cyp26b1^{+/+} and Cyp26b1^{-/-} lungs at 10× (E,F) and 40× (E',F') magnification. (G,H) Representative images of DAPI stains used in quantifications for I-K. (I) Number of DAPI⁺ cells per 0.1 mm². *n*=5 (+/+, -/-) and 3 (+/-) with six random views per sample. (J) Scatter plots depicting area of open airspaces (>100 μm²) in Cyp26b1^{+/+}, Cyp26b1^{+/-} and Cyp26b1^{-/-} lungs. *n*=4 (+/+, -/-) and 3 (+/-) with six random views per sample. (K) The total number of open airspaces (>100 μm²) per 0.1 mm². *n*=4 (+/+, -/-) and 3 (+/-) with six random views per sample. (L) Total body weight of E18.5 embryos. (M) Weight of lungs immediately after dissection (wet) relative to total body weight in E18.5 embryos. (N) Weight of lungs after drying relative to total body weight in E18.5 embryos. *n*=10 (+/+), 19 (+/-) and 7 (-/-). Data are mean±s.d. **P*<0.05, ****P*<0.001, *****P*<0.0001. Significance for all experiments was determined using one-way ANOVA with Tukey multiple comparison test. ns, not significant. Scale bars: 5 mm (A,B); 1 mm (C-D'); 500 μm (E,F); 50 μm (E',F',H).

attributed to loss of the target gene. We generated compound heterozygotes of Cyp26b1 by crossing the Cyp26b1⁻ allele with the Cyp26b1^{Δ10} allele. E18.5 Cyp26b1^{-Δ10} embryos completely phenocopied Cyp26b1^{-/-} embryos. When analyzed similarly to the

Cyp26b1^{-/-} embryos, E18.5 Cyp26b1^{-Δ10} embryos displayed the same gross developmental defects, decreased distal airspaces, increased cellularity and increased relative numbers of distal tip epithelial progenitors (Sox9⁺) and AT2 cells (Fig. S4).

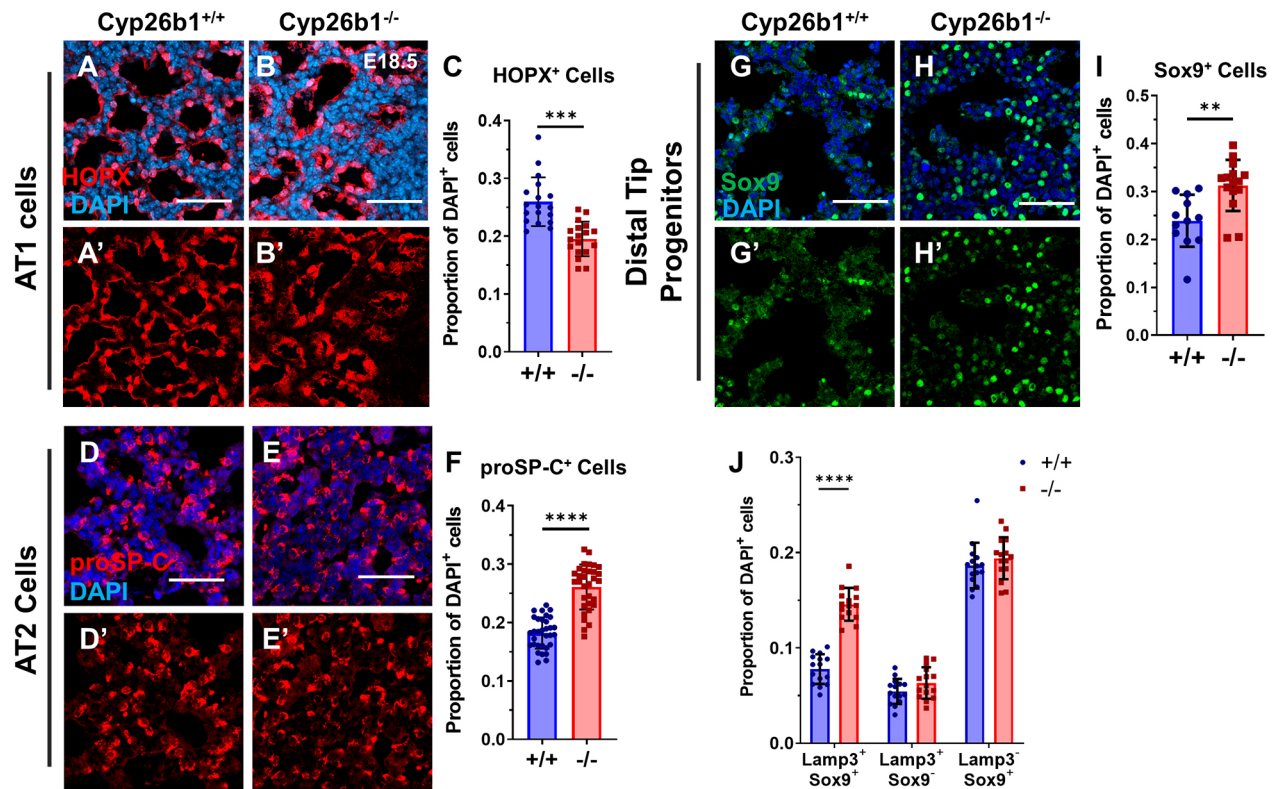


Fig. 3. Distal tip epithelial progenitors are expanded at the expense of AT1 cells in *Cyp26b1*^{-/-} lungs. (A-C) IF stain for the AT1 marker HOPX (A-B') and quantification of cell counts (C). *n*=3 biological replicates with six random views per sample. (D-F) IF stain for the AT2 marker proSP-C (D-E') and quantification of cell counts (F). *n*=5 biological replicates with six random views per sample. (G-I) IF stain for the distal progenitor cell marker Sox9 (G-H') and quantification of cell counts (I). *n*=3 biological replicates with six random views per sample. (J) Quantification of Lamp3/Sox9 double positive cells (first set), Lamp3 single positive cells (second set) and Sox9 single positive cells (third set) as a proportion of DAPI⁺ cells. *n*=3 biological replicates with five random views per sample. Note that the quantifications in C, F, I, and J are standardized to the total number of DAPI⁺ cells. Data are mean±s.d. ***P*<0.01, ****P*<0.001, *****P*<0.0001. Significance determined with unpaired Student's *t*-test (C,F,I) and two-way ANOVA with Sidak multiple comparison test (J). Scale bars: 50 μm.

Proximal airways, stroma, endothelia and lymphatics of *Cyp26b1*^{-/-} lungs are grossly normal

After confirming that observed changes in distal epithelial development were not due to off-target effects of CRISPR/Cas9, we assayed for changes in other populations in *Cyp26b1*^{-/-} lungs. Proximal airways in *Cyp26b1*^{-/-} lungs appeared to be unaffected as there were no appreciable differences in gross morphology or in the localization of CCSP⁺ (Scgb1a1) secretory cells (Clara cells), Foxj1⁺ ciliated cells (bronchial in embryonic lungs) or Sox2⁺ proximal epithelial cells (Fig. S5A-D'). CCSP⁺/Sca-1⁺ (Ly6a) bronchioalveolar stem cells (BASCs) are a stem cell population that reside at the transition between proximal and distal airways and can give rise to distal epithelium in the adult during lung regeneration (Kim et al., 2005; Lee et al., 2014). Analysis of BASCs by flow cytometry revealed no differences in this population at any stage in late gestation (Fig. S5E,F).

As ECs are closely associated with many different cell types in the lung, we set out to determine whether there were defects in cell types other than the distal epithelium. We analyzed Pdgfr-α and Pdgfr-β stromal populations using IF at E18.5 and did not observe any striking differences. Flow cytometry analysis of Pdgfr-α cells confirmed that there was no significant difference in the stromal makeup of *Cyp26b1*^{-/-} lungs at E18.5 (Fig. S6A-C). Several groups have established a link between RA signaling and proper vascular formation in the heart through directing proper vascular smooth muscle cell differentiation and association of the vasculature with these smooth muscle cells (Braitsch et al., 2012; Wang et al., 2018;

Xiao et al., 2018). Co-stains for VE-Cad (Cdh5) and Pdgfr-β to mark the ECs and pericytes, respectively, did not reveal any differences in EC-pericyte coupling (Fig. S6D-E'). Despite the fact that *Cyp26b1* is highly enriched in ECs and also observed in lymphatic ECs, we did not observe gross defects in vascular organization (Fig. S6F-G'). We also observed no obvious differences in arterial, venous or lymphatic development (Fig. S6H-K'). Lastly, RA has been shown to direct the differentiation of stromal-derived smooth muscle cells during lung organogenesis (Chen et al., 2014). Analysis using the marker Sm22a (protein product of *Tagln*) revealed that smooth muscle cells around both airways and vessels were not appreciably altered in *Cyp26b1*^{-/-} lungs (Fig. S6L-M'). These data demonstrate that formation of the vasculature, stroma and associated lineages is not grossly affected in *Cyp26b1*^{-/-} lungs.

Cyp26b1 is required for distal airway morphogenesis in late gestation

Our results show that AT1 and AT2 cell differentiation is altered in *Cyp26b1*^{-/-} lungs at the end of gestation. To determine when this phenotype arises, we analyzed earlier stages of lung development. Distal airway terminal differentiation begins at E16.5 when the columnar epithelium undergoes dramatic cell-shape changes that lead to lumen expansion and formation of distal saccules for gas exchange (Herriges and Morrissey, 2014; Shi et al., 2009; Warburton et al., 2010). Consistent with this timeline, *Cyp26b1*^{-/-} lungs begin to show gross morphological defects beginning at E16.5,

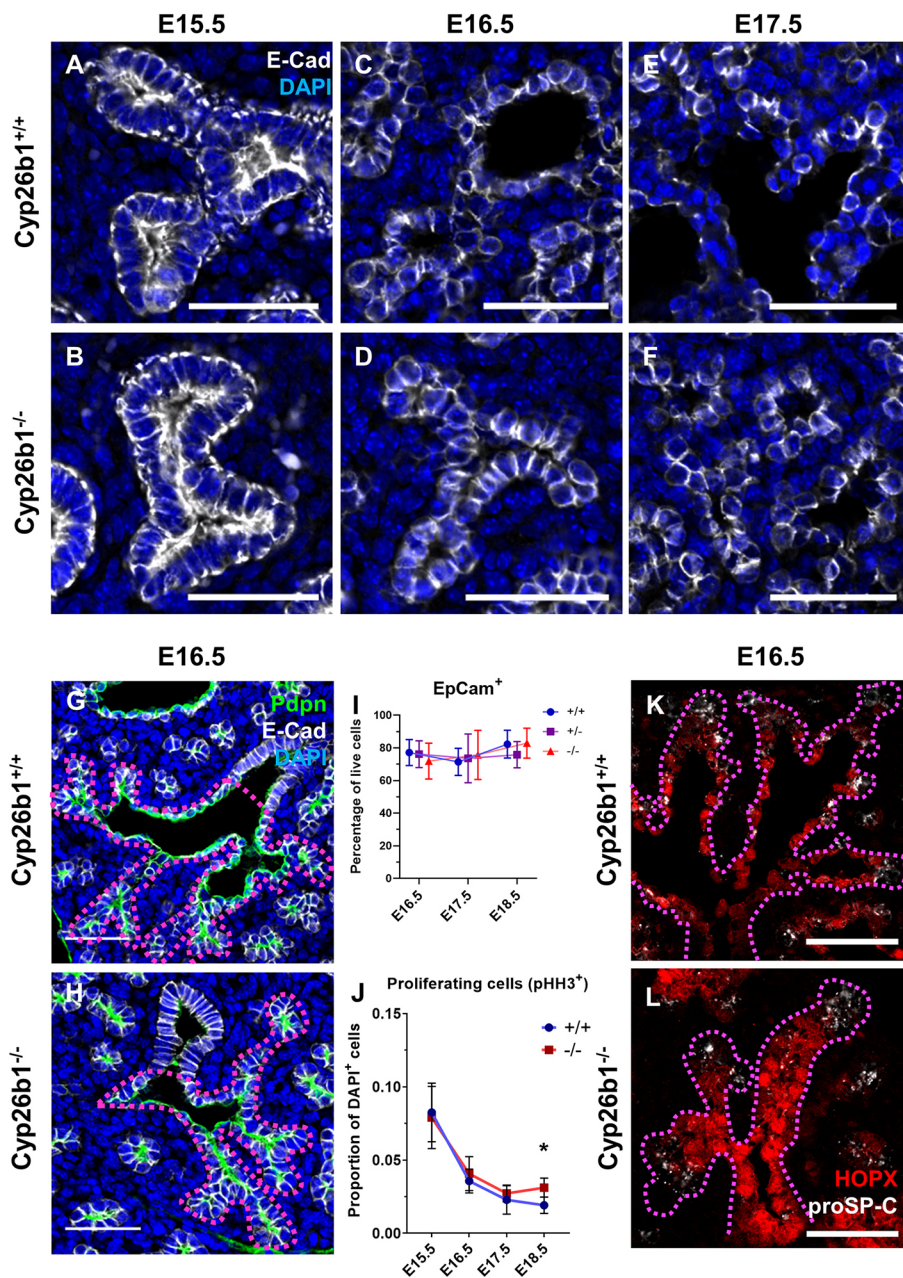


Fig. 4. Defects in epithelial morphogenesis in *Cyp26b1*^{-/-} lungs arise at E16.5. (A-F) IF stains for E-Cad (white) to mark the epithelial structure at E15.5 (A,B), E16.5 (C,D) and E17.5 (E,F). (G,H) Proximal to distal epithelial transition in E16.5 lungs stained with Pdpn (green) and E-Cad (white). Magenta dotted outlines mark distal epithelial tree extending from the terminal bronchiole. (I) Flow cytometry analysis of EpCam⁺/CD31⁻/CD45⁻ cells in E16.5-E18.5 lungs. *n*=9 (E16.5 +/+), 4 (E16.5 ±, E18.5 -/-), 3 (E16.5 -/-), 10 (E17.5 +/+), 10 (E17.5 ±), 6 (E17.5 -/-, E18.5 ±) and 5 (E18.5 +/+). (J) Quantification of pHH3⁺ cells as a proportion of DAPI⁺ cells from E15.5-E18.5 based on IF sections. *n*=3 with six random views per sample. (K-L) Distal epithelial tree in E16.5 lungs stained with E-Cad (green), HOPX (red) and proSP-C (white). Magenta dotted outlines mark distal epithelial tree. Data are mean±s.d. **P*<0.05. Significance was determined using two-way ANOVA with Sidak multiple comparison test. Scale bars: 50 μm.

characterized by increased cellular density and decreased distal airspace formation (Fig. S7). During distal epithelial differentiation, subsets of epithelial cells marked by E-cadherin (E-Cad; Cdh1) undergo morphogenetic changes from a columnar, glandular-like structure (E15.5) to a flattened, or squamous, morphology for AT1 cells and a rounded morphology for AT2 cells (E17.5) (Fig. 4A,C,E). In *Cyp26b1*^{-/-} lungs, the epithelium undergoes relatively normal cell rounding; however, the transition to flattened epithelium is delayed (Fig. 4B,D,F). Although distal epithelial lumens in the mutant lungs can be observed at E16.5, these lumens are appreciably narrower and are open primarily in regions adjacent to the proximal epithelium (Fig. 4G,H, magenta dotted outlines).

As morphological defects arise in *Cyp26b1*^{-/-} lungs as early as E16.5, we aimed to confirm our previous observation that the total epithelial volume was unchanged at E18.5. We performed flow cytometry on E16.5-E18.5 lungs with the epithelial marker EpCam, which indicated that the total number of epithelial cells did not differ

between *Cyp26b1*^{+/+} and *Cyp26b1*^{-/-} lungs at any of the three time points examined (Fig. 4I). We next sought to determine whether proliferation rates were increased at earlier stages of lung development at a time when *Cyp26b1* mutant lungs begin to display an increase in cellular density. Phospho-Histone H3 (pHH3) staining for proliferating cells revealed a small but significant increase in proliferation only at E18.5, indicating that altered proliferation alone cannot account for the observed changes in cellular density seen as early as E16.5 (Fig. 4J).

Recent data have led to the development of new models concerning the origin of mature distal epithelial cells. These data indicate that specification of distal epithelial progenitors to either AT1 or AT2 cells begins as early as E13.5, with AT1 cells arising primarily from HOPX⁺ cells in the stalk region, whereas AT2 cells derive primarily from proSP-C⁺/Sox9⁺ cells at the most distal tips (Frank et al., 2019; Jain et al., 2015). Based on these studies, we sought to determine whether the skewing of AT1 and AT2

differentiation at E18.5 could be accounted for by changes in early progenitor specification. Staining for HOPX (AT1 progenitors) and proSP-C (AT2 progenitors) at E16.5 revealed no overt qualitative differences in AT1 or AT2 progenitor patterning (Fig. 4K,L, magenta dotted outlines). This suggests the observed skewing of AT1 and AT2 cell populations at E18.5 is the result of defects in differentiation and not progenitor specification.

Cyp26b1 mutant lungs exhibit a partial RA transcriptional response

Cyp26b1 is known to dampen RA signaling by catabolizing RA into an inactive metabolite; therefore, we assessed levels of RA signaling in lungs lacking Cyp26b1. As RA primarily effects cellular change by modulating gene transcription, we assayed for genes involved in RA metabolism by qRT-PCR, several of which are direct targets of RA signaling (*Rarb*, *Stra6*, *Crabp2*, *Rbp1*) (Balmer and Blomhoff, 2002; Rhinn and Dolle, 2012; Ross and Zolfaghari, 2011; Wu and Ross, 2010). Transcriptional analyses of these genes in E18.5 whole-lung lysates revealed significant upregulation of *Rarb* and *Stra6*, significant downregulation of the RA-synthesizing enzymes *Raldh1*

and *Raldh3*, and a trend towards increased and decreased expression of *Crabp2* and *Raldh2*, respectively (Fig. 5A). We validated these results by performing ISH for *Raldh1*. We found that *Raldh1* is expressed specifically in the proximal epithelium, consistent with previous data, and that loss of Cyp26b1 led to a decrease in its expression in those cells (Fig. S8A,B) (Chazaud et al., 2003).

As Cyp26 family genes can also be regulated by RA (Rhinn and Dolle, 2012; Ross and Zolfaghari, 2011; Wu and Ross, 2010), we investigated their expression in Cyp26b1^{-/-} lungs. We first verified the gene deletion by assessing *Cyp26b1* expression using primers within the deleted region (Fig. 5B, Cyp26b1-E4-5). Repeating the qRT-PCR using primers outside of the deleted region demonstrated that remaining *Cyp26b1* transcript is strongly upregulated in Cyp26b1^{-/-} lungs (Fig. 5B, Cyp26b1). Interestingly, expression levels of *Cyp26a1* and *Cyp26c1* were unaffected (Fig. 5B). ISH and FISH for *Cyp26b1* in mutant lungs co-stained with *Emcn* validated the qRT-PCR data and revealed that the upregulation of *Cyp26b1* came specifically from ECs (Fig. 5C-H).

We next asked whether other targets of RA signaling are altered in Cyp26b1^{-/-} lungs. We first analyzed expression levels of *Ret*,

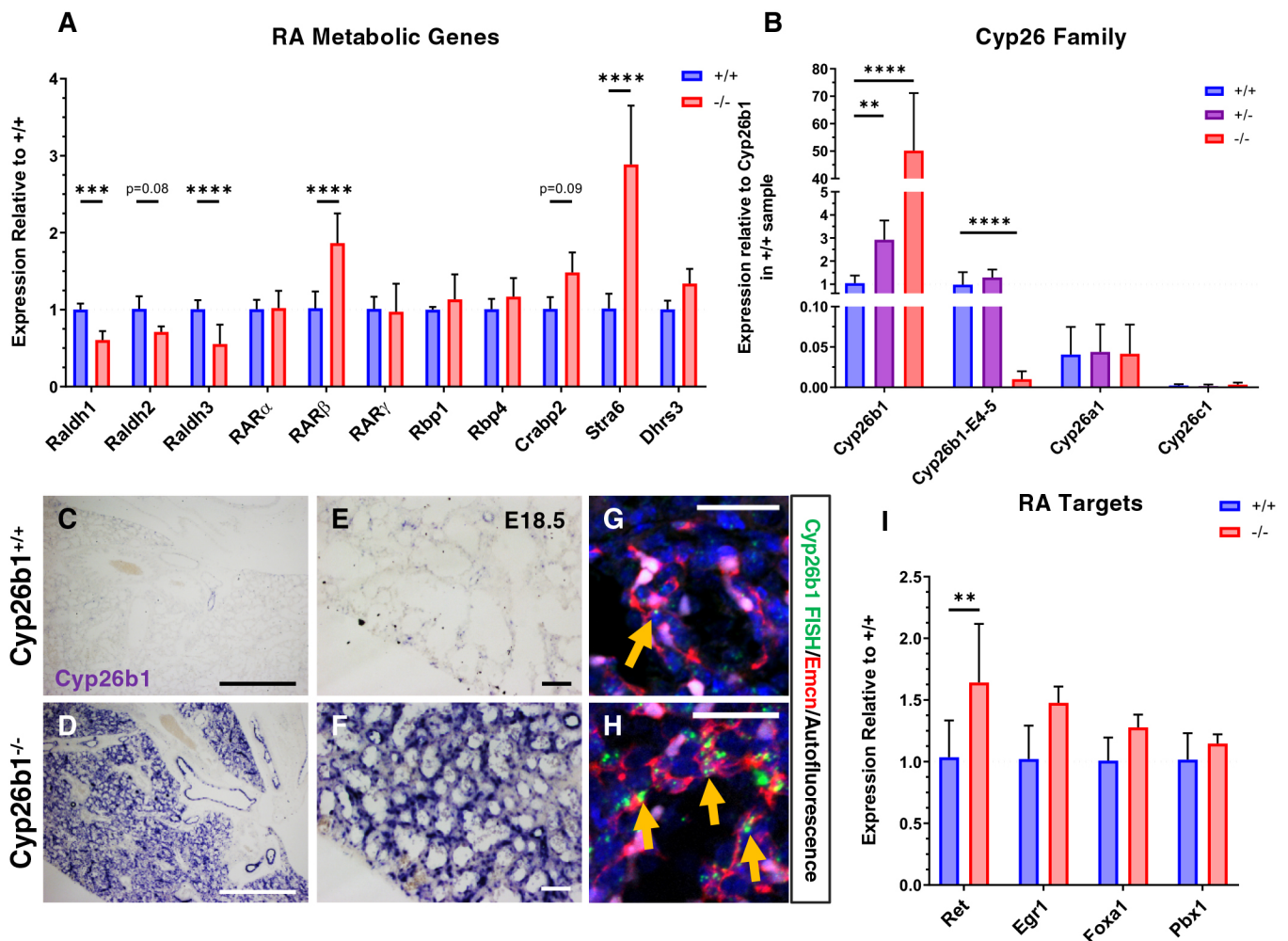


Fig. 5. Cyp26b1^{-/-} lungs exhibit a mixed RA response. (A) qRT-PCR for several RA metabolic genes in whole E18.5 lungs standardized to the expression of +/+ samples for each gene. $n=6$. (B) qRT-PCR for the three Cyp26 family members all standardized to the expression of Cyp26b1 in Cyp26b1^{+/+} samples. 'Cyp26b1' uses primers outside of the deleted region in the Cyp26b1⁻ line, whereas 'Cyp26b1-E4-5' uses primers designed within the deleted region. $n=4$. (C-F) ISH for Cyp26b1 in E18.5 Cyp26b1^{+/+} (C,E) and Cyp26b1^{-/-} (D,F) lungs at 5 \times (C,D) and 20 \times (E,F) zoom. (G,H) FISH for Cyp26b1 (green) co-stained with *Emcn* (red). Orange arrows, Cyp26b1⁺ punctae in ECs. (I) qRT-PCR for *Ret*, *Egr1*, *Foxa1* and *Pbx1* in whole E18.5 lungs. $n=4$. Data are mean \pm s.d. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Significance was determined using two-way ANOVA with Sidak multiple comparison test on the $\Delta\Delta$ Ct values. Scale bars: 500 μ m (C,D); 100 μ m (E,F); 25 μ m (G,H).

Egr1, *Foxa1* and *Pbx1*, which have all been shown to be direct targets of RA signaling in other contexts (Balmer and Blomhoff, 2002; Probst et al., 2011; Rhinn and Dolle, 2012; Wong et al., 2012). qRT-PCR analyses of whole-lung lysates for these genes revealed significant upregulation of *Ret* alone (Fig. 5I). We similarly asked whether other genes in other signaling pathways may also be differentially regulated. During early lung morphogenesis, RA regulates multiple signaling pathways, including Fgfs, Tgf- β /Bmps, Wnts and Shh (Chen et al., 2007; Desai et al., 2004; Malpel et al., 2000; Rankin et al., 2016, 2018). Surprisingly, qRT-PCR analysis for all of these pathways showed no change, except for downregulation of the Tgf- β target *Tgfb1* (Fig. S8C-F). Taken together, these data indicate that loss of *Cyp26b1* can lead to a partial RA response in the lung, in which genes involved in RA metabolism are transcriptionally altered but other established downstream targets are unaffected.

RA partially contributes to the *Cyp26b1*^{-/-} phenotype in the lung

We sought to more clearly determine whether increased RA is sufficient to induce the changes seen in the *Cyp26b1*^{-/-} lungs. Previous reports have shown that exogenous administration of atRA can partially phenocopy the defects in limb development, palate fusion and skin barrier formation that are seen in *Cyp26b1*^{-/-} embryos (Cadot et al., 2012; Okano et al., 2012b). Following a similar dosing regimen, we gavaged atRA to *Cyp26b1*^{+/-} pregnant dams at E15.5, E16.5 and E17.5, dissected out the lungs at E18.5, and assessed for changes in lung development (Fig. 6A). Of embryos receiving exogenous atRA, 100% of *Cyp26b1*^{-/-} and ~30% of *Cyp26b1*^{+/-} and *Cyp26b1*^{+/+} died *in utero*, and the remainder died shortly after birth (Fig. 6B). As all atRA-treated *Cyp26b1*^{-/-} embryos were dead by E18.5, we focused our analysis on the lungs of atRA-treated *Cyp26b1*^{+/+} and *Cyp26b1*^{+/-} embryos. atRA-treated *Cyp26b1*^{+/+} and *Cyp26b1*^{+/-} lungs exhibited a loss of distal airspaces and increased cellular density that were indistinguishable from control *Cyp26b1*^{-/-} lungs (Fig. 6C-L,W, Fig. S9A-E). Sox9⁺ distal tip progenitor cells were relatively more abundant in atRA-treated lungs per given area compared with their matched controls, but not to the same degree as in *Cyp26b1*^{-/-} lungs (Fig. 6M-Q,X). Interestingly, proSP-C⁺ AT2 cells were only proportionally increased in atRA-treated *Cyp26b1*^{+/-} lungs, but not in atRA-treated *Cyp26b1*^{+/+} lungs (Fig. 6R-V,Y). These data show that excess RA is sufficient to induce morphologic changes in *Cyp26b1*^{-/-} lungs but does not fully recapitulate loss of *Cyp26b1*.

Exogenous RA and loss of *Cyp26b1* exhibit distinct transcriptional responses

To further validate these data, we performed qRT-PCR to assess whether RA treatment induces the same changes in transcription as loss of *Cyp26b1* in the lung. Consistent with previous results, exogenous atRA treatment induced expression of *Rarb*, *Stra6* and *Cyp26b1* (Fig. S9F,G). We also observed a small, but significant, increase in *Cyp26a1* (Fig. S9G). Next, we assayed for changes in markers of each distal epithelial population. Although expression levels of *Sfpic*, *Aqp5*, *Pdpn*, *Ager* and *HOPX* in atRA-treated lungs mirrored that of *Cyp26b1*^{-/-} lungs, *Sox9* expression was increased and *CCSP* expression was decreased in atRA-treated lungs only (Fig. 6Z, Fig. S9H). These data raise the possibility that exogenous RA may induce additional transcriptional changes not observed in *Cyp26b1*^{-/-} lungs, suggesting that *Cyp26b1* may play novel roles beyond reduction of RA levels. Indeed, qRT-PCR analysis of potential signaling pathways downstream of RA reveal differential

expression of several Shh, Wnt and Tgf- β members in atRA-treated lungs that are not differentially expressed in control *Cyp26b1*^{-/-} lungs (Fig. 7A-D). Specifically, we found upregulation of *Gli2*, *Gli3*, *Wnt4*, *Tgfb2* and *Tgfb3* in atRA-treated lungs compared with *Cyp26b1*^{-/-} lungs. In addition, several other genes – namely *Wnt2*, *Tgfb2*, *Tgfb3*, *Id1*, *Shh* and *Ptch1* – were not differentially expressed between atRA-treated and *Cyp26b1*^{-/-} lungs but showed significant differences when the atRA-treated lungs were compared with their genotyped-matched controls (Fig. 7). These data show that exogenous administration of atRA and loss of *Cyp26b1* both lead to similar morphogenetic effects on lung development, but do so via partially overlapping but distinct transcriptional mechanisms.

DISCUSSION

Here, we identify a novel role for *Cyp26b1* in lung development. *Cyp26b1* is enriched in lung ECs throughout development and loss of *Cyp26b1* leads to profound changes in distal epithelial differentiation and morphogenesis, ultimately resulting in neonatal lethality. *Cyp26b1*^{-/-} lungs exhibit an expansion of distal tip progenitors by E18.5 marked by both Sox9 and Lamp3/proSP-C at the expense of mature gas-exchanging AT1 cells. Currently, the only known function of *Cyp26b1* is as a negative regulator of RA; however, our data indicate RA signaling is only partially increased in *Cyp26b1*^{-/-} lungs. Likewise, administration of exogenous atRA during late lung formation does not fully phenocopy loss of *Cyp26b1*. Instead, we make the unexpected observation that *Cyp26b1*^{-/-} and atRA-treated lungs undergo overlapping, but distinct, cellular and transcriptional responses. Therefore, our study both identifies an essential role for *Cyp26b1* during lung development and suggests that *Cyp26b1* has important roles in lung development beyond RA regulation.

Cyp26b1 enrichment in lung ECs throughout development raises interesting questions and implicates ECs in driving the *Cyp26b1*^{-/-} phenotype. ECs have been shown to regulate lung development during branching morphogenesis and alveolarization (Del Moral et al., 2006; Jakkula et al., 2000; Lazarus et al., 2011). In addition, ECs are crucial in directing distal epithelial development in models of adult lung regeneration (Ding et al., 2011; Lee et al., 2014; Rafii et al., 2015). Our data suggest a new role for ECs during normal lung development in which ECs express *Cyp26b1* to locally decrease RA signaling and promote proper distal epithelial differentiation. Although EC organization and specification appear to be unaltered in the mutants, we observed strong self-upregulation of *Cyp26b1* specifically in ECs of *Cyp26b1*^{-/-} lungs. Interestingly, there was no compensatory change in *Cyp26a1* or *Cyp26c1* expression. Both *Cyp26b1* and *Cyp26a1* are known targets of RA (Rhinn and Dolle, 2012; Ross and Zolfaghari, 2011; Wu and Ross, 2010) but it is unclear why both loss of *Cyp26b1* and exogenous atRA strongly upregulate only *Cyp26b1* and not *Cyp26a1* in the lung. Based on these expression patterns, there may be unidentified EC-specific mechanisms that lead to upregulation of *Cyp26b1* over the other *Cyp26* enzymes. It must be stated that we cannot rule out possible roles played by other cells that express low levels of *Cyp26b1*. However, both the enrichment of *Cyp26b1* in wild-type ECs and the specific upregulation of *Cyp26b1* expression in *Cyp26b1* null ECs are highly suggestive of an EC-driven phenotype. We hypothesize that ECs expressing *Cyp26b1* act as a local sink for RA, thereby reducing local RA signaling and creating an RA gradient within the developing lung.

Recent models of distal epithelial development have demonstrated that specification of AT1 and AT2 cells occurs as early as E13.5 and can be identified by location along the distal epithelial tree and by gene

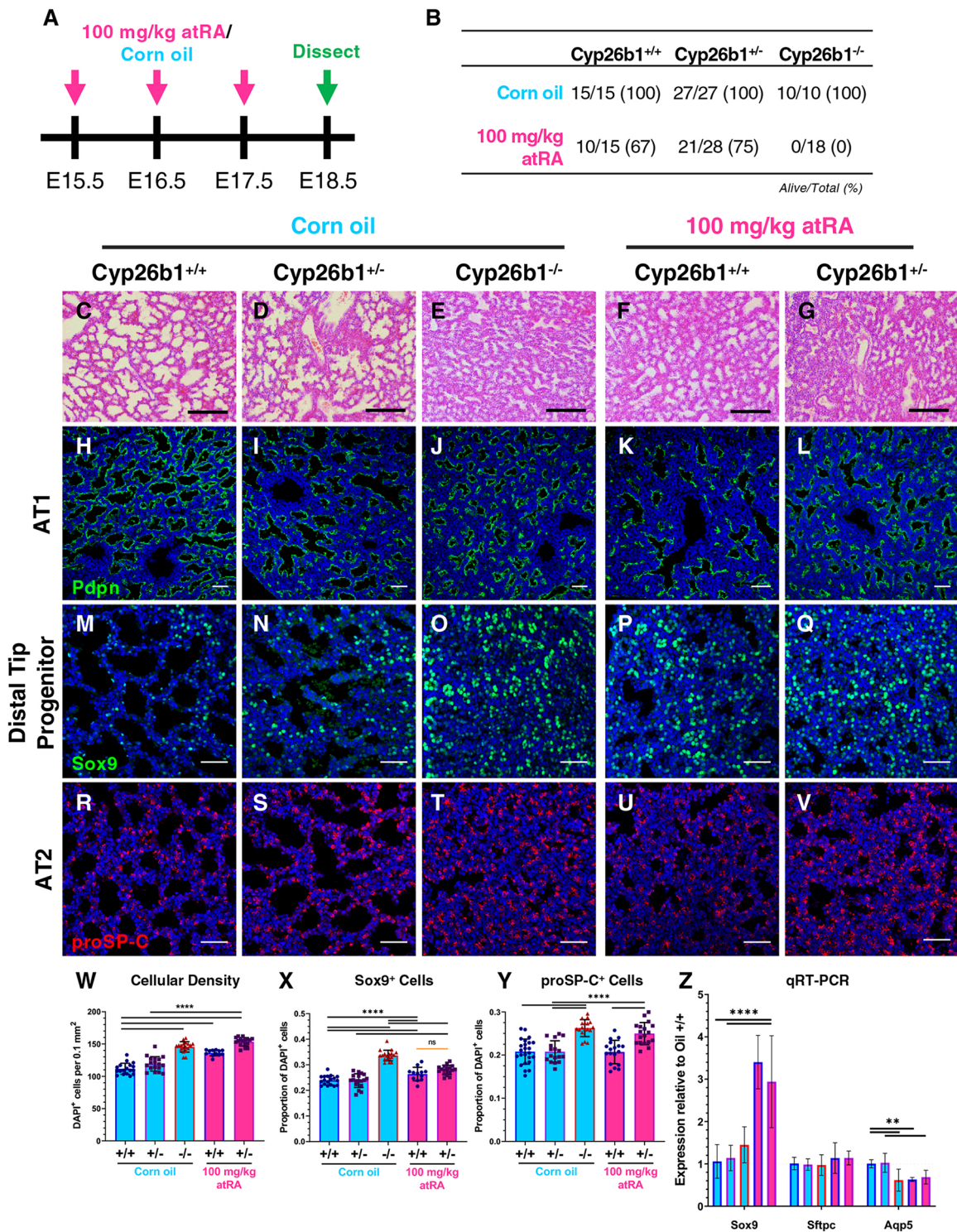


Fig. 6. Exogenous atRA partially phenocopies loss of Cyp26b1. (A) Experimental design for experiments with exogenous atRA: 100 mg/kg atRA (magenta) or equivalent dose of corn oil (control, cyan) was gavaged to pregnant dams at E15.5, E16.5 and E17.5, and dissected at E18.5. (B) Survival chart for E18.5 embryos at the time of dissection for each genotype and treatment. (C-G) H&E stains of control (C-E), and atRA-treated (F-G) lungs at 10 \times magnification. (H-L) IF stain for the AT1 cell marker Pdpn in control (H-J) and atRA-treated (K-L) lungs. (M-Q) IF stain for Sox9 in control (M-O) and atRA-treated (P-Q) lungs. (R-V) IF stain for the AT2 cell marker proSP-C in control (R-S) and atRA-treated (U-V) lungs. (W) Number of DAPI⁺ cells per 0.1 mm². Outline color corresponds to genotype (blue, Cyp26b1^{+/+}; purple, Cyp26b1^{+/-}; red, Cyp26b1^{-/-}) and fill color corresponds to treatment group (cyan, control; magenta, atRA). $n=3$ with six random views per sample. (X) Quantification of Sox9⁺ cells in M-Q following the same coloring scheme as in W. $n=3$ with six random views per sample. (Y) Quantification of proSP-C⁺ cells in R-V following the same coloring scheme as in W. $n=3$ with six random views per sample. (Z) qRT-PCR for Sox9, Sftpc and Aqp5 in control and atRA-treated lungs following the same coloring scheme as in W, standardized to the expression of +/+ samples for each gene. $n=3$ (oil -/-), 4 (oil +/-), 5 (atRA +/-), 6 (oil +/+, atRA +/+). Data are mean \pm s.d. ** $P<0.01$, **** $P<0.0001$. Significance was determined using one-way ANOVA with Tukey multiple comparison test (W-Y) and two-way ANOVA with Sidak multiple comparison test on the $\Delta\Delta$ Ct values (Z). Scale bars: 500 μ m (C-G); 50 μ m (H-V).

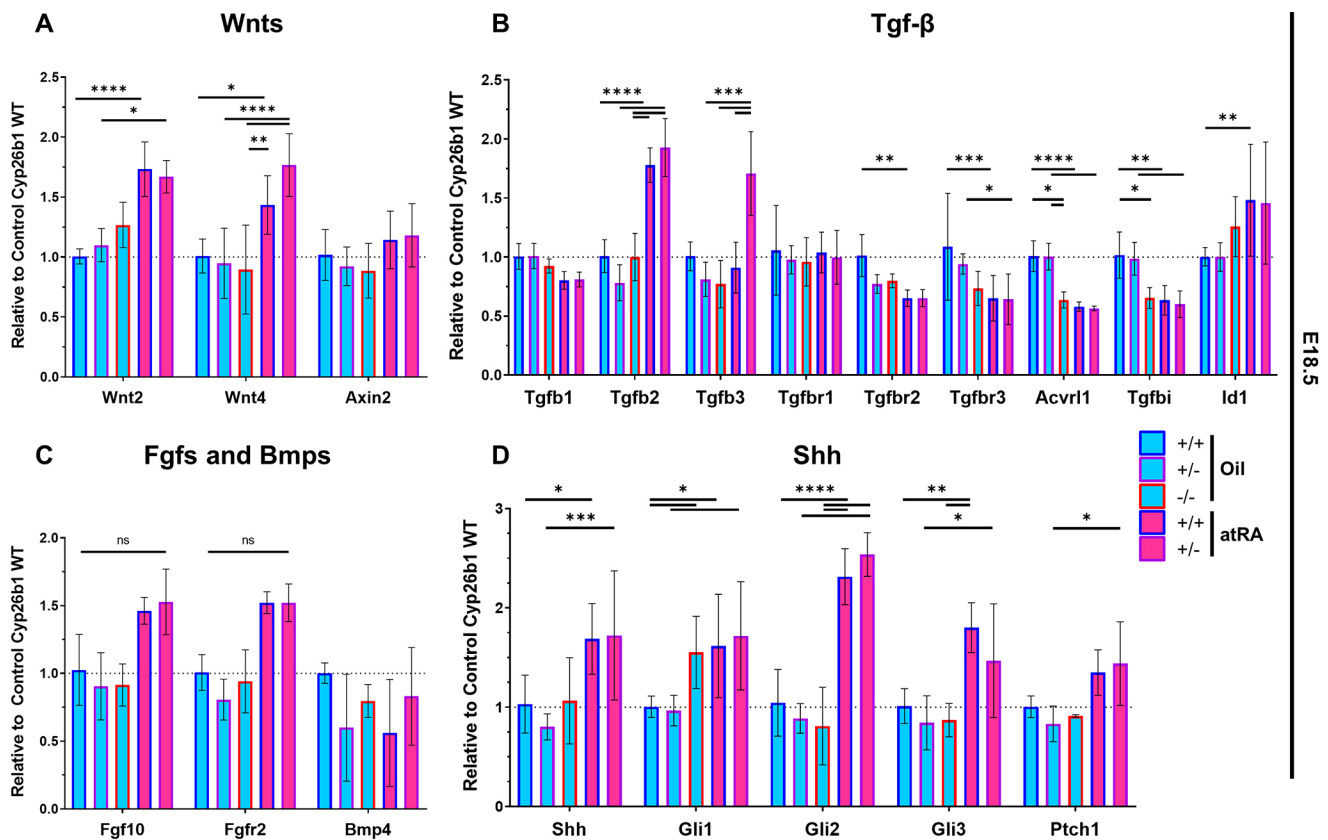


Fig. 7. Exogenous atRA induces distinct transcriptional changes compared with *Cyp26b1*^{-/-} lungs. (A) qRT-PCR for the Wnt pathway genes *Wnt2*, *Wnt4* and *Axin2* standardized to the expression of *+/+* samples for each gene. Outline color corresponds to genotype (blue, *Cyp26b1*^{+/+}; purple, *Cyp26b1*^{+/-}; red, *Cyp26b1*^{-/-}) and fill color corresponds to treatment group (cyan, control; magenta, atRA). (B) qRT-PCR for members of the TGF- β signaling pathway following the coloring scheme in A, standardized to the expression of *+/+* samples for each gene. (C) qRT-PCR for established regulators of lung branching following the coloring scheme in A, standardized to the expression of *+/+* samples for each gene. (D) qRT-PCR for members of the Shh pathway following the coloring scheme in A, standardized to the expression of *+/+* samples for each gene. $n=3$ (oil *-/-*), 4 (oil *+/-*), 5 (atRA *+/-*) and 6 (oil *+/+*, atRA *+/+*). Data are mean \pm s.d. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Significance was determined using two-way ANOVA with Sidak multiple comparison test on the $\Delta\Delta$ Ct values. ns, not significant.

expression pattern (Frank et al., 2019). Similarly, our previous work in the pancreas has shown that disruption of early morphogenesis drives shifts in progenitor cell differentiation (Azizoglu et al., 2017). Our analysis here, by contrast, did not reveal overt changes in early patterning of the distal progenitor epithelial tree. Instead, we did observe late defects in cellular morphology, as the transition from columnar to squamous epithelium during terminal differentiation is impaired in the absence of *Cyp26b1*. This suggests that *Cyp26b1* is not required for progenitor specification, but rather it regulates AT1 and AT2 differentiation.

Of note, we observed expansion of distal tip progenitors marked by both *Sox9* and *Lamp3* at E18.5 possibly at the expense of AT1 cells, whereas the formation of mature *Lamp3*⁺/*Sox9*⁻ AT2 cells was unaffected. Although death in *Cyp26b1* mutants was previously characterized as ‘respiratory distress’ (Yashiro et al., 2004), RDS in humans is characterized by decreased surfactant production due to decreased formation of AT2 cells. Instead, we observe an increase in surfactant production upon loss of *Cyp26b1*. In that regard, our work stands in contrast to other studies that have also identified similar defects in distal epithelial differentiation but found a decrease in surfactant production or proSP-C⁺ AT2 cells (Comperolle et al., 2002; Hogmalm et al., 2014; Kersbergen et al., 2018; Rockich et al., 2013; Woik et al., 2014; Yang et al., 2002). The defects seen in these studies most likely arise from an inability

of distal progenitor cells to first differentiate into AT2 cells. By contrast, loss of *Cyp26b1* appears to prevent the maturation of AT1, but not AT2, cells which culminates in neonatal lethality.

An important issue arising from our work concerns the role of RA signaling in the phenotype of *Cyp26b1* mutants. RA has previously been shown to block distal epithelial differentiation in other contexts. Human pluripotent stem cell-derived lung bud tip organoids containing *Sox9*⁺ distal progenitors can be maintained in a progenitor state when cultured with *Fgf7*, CHIR-99021 and RA (Miller et al., 2018). Removal of CHIR-99021 and RA lead to differentiation of these progenitors into the mature airway lineages. Although the effect of removing RA individually was not tested directly, this study is consistent with the model that RA inhibits differentiation of the distal airways. Similarly, hyperactive RA signaling through constitutively active *RAR α* or *RAR β* leads to defects in distal airway formation (Wongtrakool et al., 2003). Interestingly, lungs with the constitutively active *RAR α* transgene had a complete loss of AT2 and AT1 cells, whereas lungs with the constitutively active *RAR β* transgene did contain some AT2 and AT1 cells, more closely mirroring *Cyp26b1*^{-/-} lungs. These data raise the question of the extent to which the effects observed in *Cyp26b1*^{-/-} lungs are due to elevated RA signaling and whether they are primarily mediated through *RAR β* or other RARs and nuclear receptors. Whereas these previous studies relied on *in vitro*

systems or transgenic overexpression systems, we identify *Cyp26b1* as an endogenous physiologic mechanism that decreases RA signaling during normal lung development.

To address the role of RA in the *Cyp26b1* phenotype, we carried out exogenous administration of atRA beginning at E15.5 and found that it partially phenocopied loss of *Cyp26b1*. Although morphologic defects in atRA-treated and *Cyp26b1*^{-/-} lungs were indistinguishable from one another, cell fate changes in atRA-treated lungs did not fully recapitulate that of *Cyp26b1*^{-/-} lungs. One possible explanation for this is that distal epithelial progenitors are specified as early as E13.5 and may no longer be receptive to RA-driven changes in fate by the first dose of RA at E15.5 (Frank et al., 2019). *Cyp26b1*^{-/-} lungs are consistently exposed to higher levels of RA throughout lung development, potentially leading to a more severe difference in epithelial differentiation. We confirm the role of RA in driving these morphological defects, as exogenous RA mirrors morphological defects seen upon loss of *Cyp26b1*. In addition, RA pathway gene expression is similarly altered in RA-treated lungs. However, RA treatment is unable to fully recapitulate cell differentiation defects, but it does lead to additional changes in transcriptional regulation not observed in *Cyp26b1*^{-/-} lungs, suggesting an additional RA-independent mechanism. First, we saw little change in the expression of multiple signaling pathways known to be regulated by RA during lung development in *Cyp26b1*^{-/-} lungs. Second, several genes in these pathways were differentially expressed in atRA-treated lungs but not in *Cyp26b1*^{-/-} lungs. Additional studies need to be performed to identify RA-independent mechanisms driving defects in *Cyp26b1*^{-/-} lungs.

From our studies, it is clear that *Cyp26b1* is essential for the development of a functional lung through its impact on differentiation of distal lung epithelium. As *Cyp26b1* is enriched in ECs, we propose that our study identifies *Cyp26b1* as a novel endothelial modulator for RA activity in embryonic organs. Given that *Cyp26b1* null lungs display increased cellular density and contain an expansion of distal tip progenitors at the expense of mature gas-exchanging ATI cells, it will be of great interest to see whether its loss causes progenitor differentiation defects in other organs which have not yet been studied. Together, our data provide new perspectives on the complex mechanisms by which RA regulates lung development and suggest novel mechanisms downstream of *Cyp26b1*. Studies of cell-type specific signaling events such as this one will contribute to better understanding of neonatal lung maturation and to the development therapies for newborns with respiratory disease.

MATERIALS AND METHODS

Mice and embryo handling

Experiments were performed in accordance with protocols approved by the University of Texas Southwestern Medical Center (UTSW) Institutional Animal Care and Use Committee. *Cyp26b1* mutant alleles were generated using CRISPR/Cas9 as previously described (Yang et al., 2013). In brief, Cas9 mRNA and *in vitro* transcribed single guide (sg)RNAs were injected directly into C57BL/6J oocytes (Transgenic Core Facility, UTSW). sgRNA sequences used are in Table S1. Mice were crossed to generate a mixed CD1/C57BL/6J background, up to five backcrosses. Deletions were validated and characterized by sequencing (Sanger Sequencing Core, UTSW) using the primers indicated in Table S1. Images of whole embryos and organs were taken using an iPhone XS (Apple) and NeoLumar stereomicroscope (Zeiss) using a DP-70 camera (Olympus), respectively.

RA administration to pregnant dams was performed as previously described (Cadot et al., 2012; Okano et al., 2012b). atRA (Sigma-Aldrich) was reconstituted in corn oil at 50 mg/ml. Pregnant dams were gavaged either 100 mg/kg atRA or equivalent dose of corn oil at E15.5, E16.5 and E17.5.

Measurements of embryo and lung weights

Wet and dry weights for the E18.5 embryo lungs were determined as previously described (Murata et al., 2007). Briefly, lungs were dissected out of embryos, blotted dry and weighed to determine the wet weight. Lungs were then dried overnight at 55°C and weighed again to determine the dry weight. Wet and dry weights were standardized to embryonic weight to determine relative wet and dry weights.

Histologic and IF analysis on sections

E12.5-E18.5 embryos, lungs and kidneys were dissected and fixed in 4% paraformaldehyde/PBS overnight at 4°C and embedded in paraffin as previously described (Azizoglu et al., 2017). For Hematoxylin and Eosin (H&E) stains, sections were deparaffinized in xylene, 100% ethanol and 95% ethanol and washed under running water. Slides were then incubated with Hematoxylin (Gill's Method, Fisher Chemical), destained in acid alcohol (99 ml 70% ethanol+1 ml 12N HCl) and blued in 0.1% sodium bicarbonate. Eosin staining was performed by incubating slides with Eosin Y (Acros Organics). Slides were washed under running water after each staining step. Lastly, slides were dehydrated to 100% ethanol, washed in xylene and mounted using Permount. Images were taken using a Zeiss Axiovert 200 M scope and a DP-70 camera (Olympus).

IF stains were performed as previously described (Daniel et al., 2018). Primary antibody incubations were carried out at 4°C overnight (for antibody information and dilutions, see Table S2). Images were obtained using an A1R Nikon confocal microscope.

Digoxigenin-labeled RNA probes and *in situ* hybridizations

The *Cyp26b1* full coding sequence clone was acquired from Dharmaco (BC059246) and linearized using EcoRI. The *Raldh1* full coding sequence clone was acquired from Dharmaco (BC044729) and linearized using KpnI. Probe synthesis was performed as previously described (Daniel et al., 2018). Briefly, probes were synthesized at 37°C for 2-4 h in digoxigenin-synthesis reaction mixture with T3 or T7 RNA polymerase (Roche). After synthesis, DNA was eliminated by adding RQ1 DNase I (Promega) and RNA probes were purified using Micro Bio-spin columns (Bio-Rad). Then 10× hybridization stock was prepared at 10 µg/ml by adding the appropriate volume of pre-hybridization buffer.

In situ hybridizations were performed as previously described (Daniel et al., 2018). E12.5-E18.5 embryos, lungs and kidneys were fixed and embedded in paraffin as described above. Images were taken using a Zeiss Axiovert 200 M scope and a DP-70 camera (Olympus).

For fluorescent *in situ* hybridizations, the protocol is the same as the chromogenic assay up to the 0.2× SSC wash on the second day. After that wash, slides were transferred to 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20 (TNT) and treated with 0.3% H₂O₂ for 30 min before washing in TNT 3× for 5 min. Slides were then blocked in 1% blocking reagent (Perkin Elmer) for at least 1 h at room temperature. Slides were then incubated with anti-Dig-peroxidase (Roche, 1:500), and either rabbit anti-Lyve1 (1:100) or rat anti-Endomucin (1:200) and rat anti-PECAM (1:200) overnight at 4°C (see Table S2 for full antibody information). After primary antibody incubation, slides were washed in TNT and treated with Fluorescein Amplification Reagent (Perkin Elmer, 1:50 in Plus Amplification Diluent) for 15 min at room temperature. Slides were then washed with TNT and incubated with donkey anti-rabbit 555 (Invitrogen, A-31572) or donkey anti-rat 555 (Abcam, ab150154) for 1 h at room temperature. Lastly, slides were incubated with DAPI, washed in TNT and mounted using Prolong Gold Mounting Medium (Thermo Fisher Scientific). Images were obtained using an A1R Nikon confocal microscope.

RNA isolation and qRT-PCR

E18.5 lungs and kidneys were dissected and placed in RNase-free Eppendorf tubes (Ambion) in which they were manually dissociated using disposable plastic pestles. RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. mRNA concentrations were quantified on a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). RNA was standardized to the sample with the lowest concentration and was reverse transcribed using SuperScript III (Invitrogen) kits following the manufacturer's instructions using oligo-dTs.

Transcripts were quantified using Power SYBR Green PCR Master Mix (Applied Biosciences) on a QuantStudio 3 Real-Time PCR System (Applied Biosciences). Primers used for qRT-PCR are listed in Table S1. Relative levels of transcripts were determined using the $\Delta\Delta C_t$ method by first standardizing mean C_t for a given gene to the housekeeping gene cyclophilin (*Ppib*) in the same sample and then calculating changes between samples. Data and statistical analyses were plotted and performed in GraphPad Prism 8.

Flow cytometry

Primary lung cells were isolated from E16.5-E18.5 lungs as previously described (Kim et al., 2005) using pan CD45-FITC, CD31-APC, Sca1 (Ly-6A)-APC-Cy7 (BD Pharmingen), EpCAM-PECy7 (BioLegend) or CD140a-FITC (Thermo Fisher Scientific), with DAPI (Sigma-Aldrich) staining to eliminate dead cells. Whole E16.5-E18.5 lungs were manually dissociated with a pestle in separate microcentrifuge tubes or with forceps in a sterilized petri dish and then incubated with 2.5 mg/ml Collagenase A (Roche) and 20 μ g/ml DNase 1 (Sigma-Aldrich) for 45 min at 37°C on a nutator. The reaction was stopped by adding wash media (PBS+0.5% bovine serum albumin (BSA)+2 mM EDTA+1 mM CaCl₂) to each reaction. Cells were pelleted and incubated with ACK Lysis buffer for 10 min on ice to lyse red blood cells. Next, cells were washed and filtered through 100 μ m and 40 μ m cell filters before incubating with the antibodies listed above. Cells were then washed again and resuspended with wash media+DAPI for analysis. Samples were analyzed on an LSR II (BD Biosciences) in the UTSW Flow Cytometry Core Facility.

Western blot

Protein extraction and western blot analyses were performed as previously described (Azizoglu et al., 2017). Briefly, E18.5 lungs were mechanically dissociated with a pestle and homogenized in PBS with 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin. Triton X-100 was added to each tube to a final concentration of 1%. Samples were frozen at -80°C overnight, thawed and centrifuged at 10,000 g for 5 min. Protein samples were quantified using Pierce BSA protein assay (Thermo Fisher Scientific) and standardized in Laemmli's SDS-Sample Buffer (Boston BioProducts). Then 40 μ g of total protein from each lung lysate were run on a western blot. Antibodies and concentrations used are listed in Table S2.

Quantification and statistical analysis

Cell counting for DAPI⁺, pHH3⁺, proSP-C⁺, Lamp3⁺, HOPX⁺, Nkx2.1⁺ and Sox9⁺ cells was performed using Bitplane Imaris v.9.0.2. Positive cells were first marked using the spots function to generate an initial count, followed by manual editing to ensure proper counts. Lamp3⁺/proSP-C⁺ cells or Lamp3⁺/Sox9⁺ cells were quantified using the 'colocalize spots' function in the Imaris XT analysis package. Images used in these quantifications encompass at least five random views of distal epithelium from at least three embryos per genotype and treatment condition. Images were captured to avoid all proximal epithelial structures that may improperly skew the results. Airspace area and number were calculated using FIJI. First, a threshold was applied to images of distal airways using the DAPI stain such that the areas occupied by cells were mostly filled. Next, the number and sizes of all blank spaces greater than 100 μ m² were calculated using the Analyze Particles function. The 100 μ m² threshold was chosen to eliminate false positive blank spaces that are due to gaps in between adjacent DAPI⁺ cells and not from actual open airspaces.

Data and statistical analyses were plotted and performed in GraphPad Prism 8 for all experiments described. Significance for cell quantifications, qRT-PCR analysis and flow cytometry was determined using unpaired Student's *t*-test or two-way ANOVA with Sidak multiple comparison test. Significance for embryo and lung weights was determined using one-way ANOVA with Tukey multiple comparison test (as specified in figure legends).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.D., O.C.; Methodology: E.D.; Validation: E.D., H.R.B., X.G.; Formal analysis: E.D., H.R.B., M.A.C., O.C.; Investigation: E.D., H.R.B., G.I.S., X.G., Y.H.; Resources: E.D., G.I.S., Y.H.; Data curation: E.D., H.R.B.; Writing - original draft: E.D., O.C.; Writing - review & editing: E.D., H.R.B., G.I.S., Y.H., O.C.; Visualization: E.D., H.R.B., O.C.; Supervision: O.C.; Project administration: O.C.; Funding acquisition: O.C.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.181560.supplemental>

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