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Supplemental information

Patterning the embryonic pulmonary mesenchyme

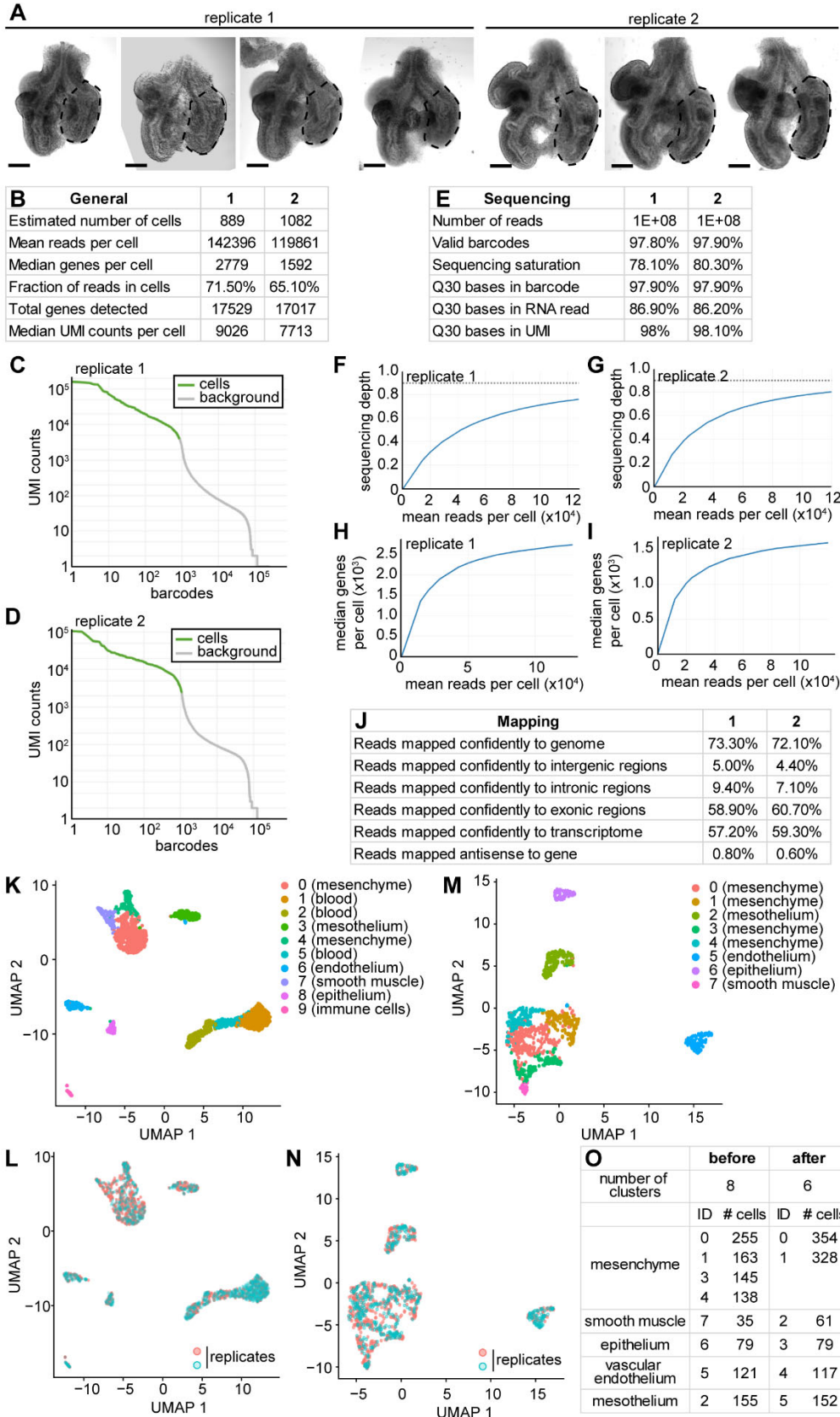
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Supplementary Information

Supplementary Figures

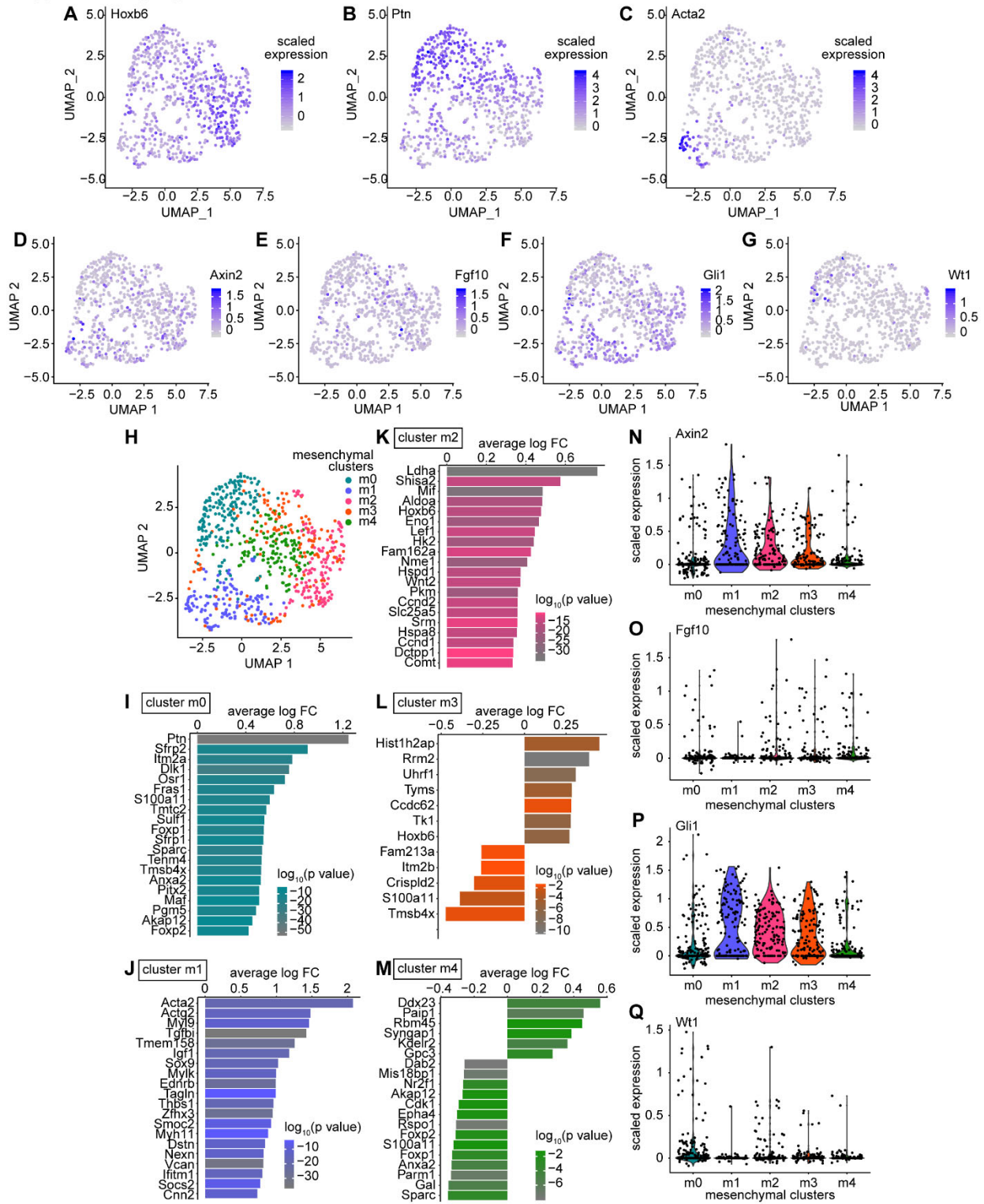
Supplementary Figure 1, Related to Figure 1. scRNA-seq experimental design, summary statistics, and initial clustering. (A) Images of all *E11.5* lungs used for the two replicates of the scRNA-seq experiment. Each lung was micro-dissected to isolate the left lobe (indicated by dotted outlines). Scale bars show 100 μm . (B) Table of general statistics for each sample. UMI (unique molecular identifiers) reflects the number of transcripts captured per cell. (C-D) UMI counts as a function of number of barcodes for replicates 1 (C) and 2 (D). Portion of curve above threshold includes all cells (green) and portion below is considered background (grey). (E) Table of sequencing statistics for each replicate. Q30 statistics give the percentage of bases in the indicated sequence type with a quality score greater than 30. (F-G) Sequencing depth as a function of mean reads per cell for each replicate. (H-I) Median genes per cell as a function of mean reads per cell. (J) Table of mapping statistics for each replicate. Mapping was performed using STAR. (K-L) UMAP plot of all cells isolated by scRNA-seq of *E11.5* CD1 mouse lungs, color-coded by cluster (K) or by replicate (L). (M-N) UMAP plot after all blood and immune cells have been removed, color-coded by cluster (M) or by replicate (N). (O) Numbers of clusters by cell type and number of cells in each cluster before and after cell cycle regression.

Supplementary Figure 1



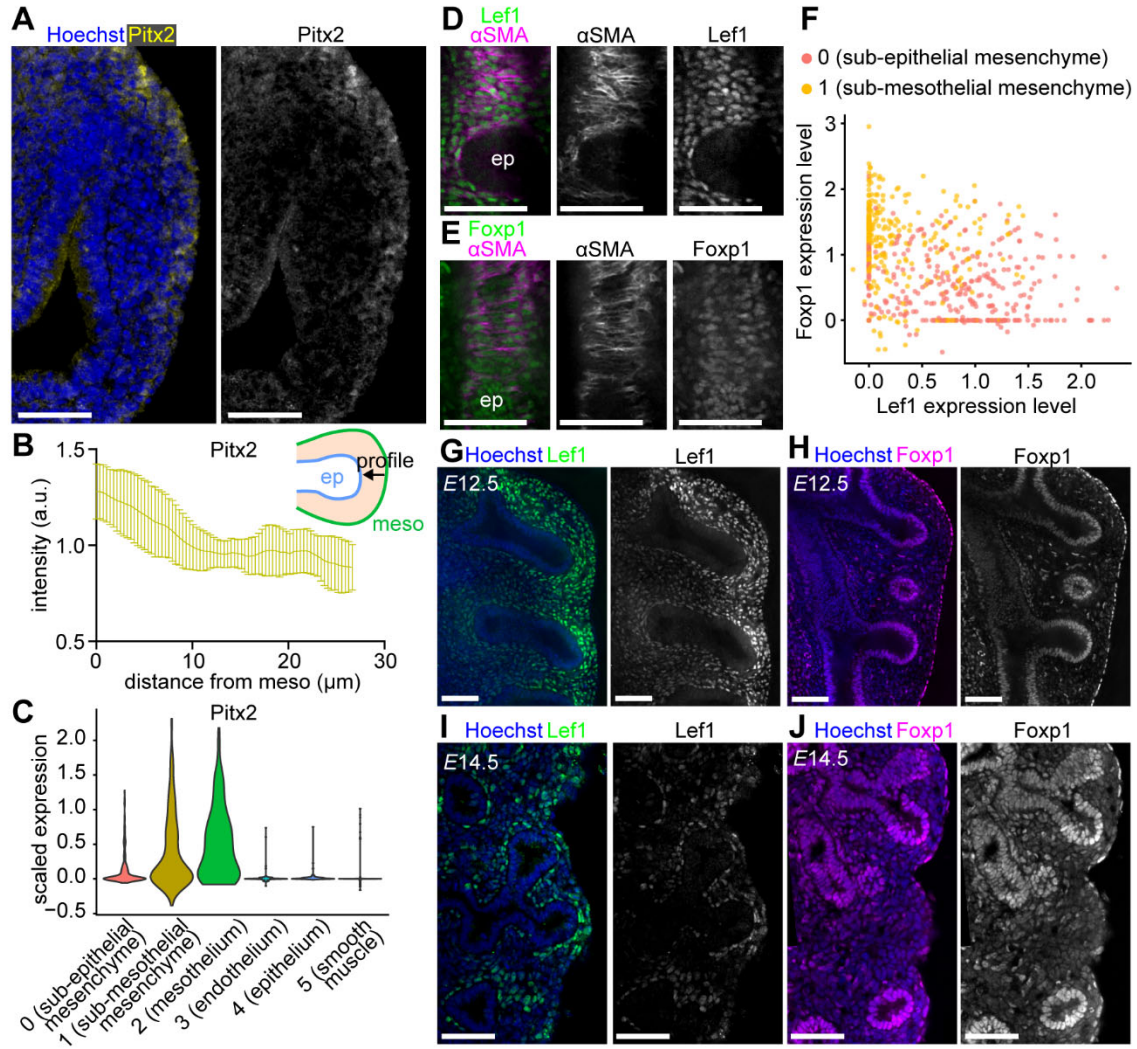
Supplementary Figure 2, Related to Figure 1. Pulmonary mesenchymal cells are heterogeneous but ultimately closely related. (A-C) UMAP plots of isolated mesenchymal cells color-coded to show the expression of key markers from Fig. 1: *Hoxb6*, *Ptn*, and *Acta2*. (D-G) UMAP from (A) color-coded to show the expression of putative smooth muscle progenitor markers *Axin2*, *Fgf10*, *Gli1*, and *Wt1*. (H) UMAP of mesenchymal and smooth muscle cells corrected for cell-cycle stage and color-coded based on cluster identified by Seurat. (I-M) Top 20 marker genes for each cluster based on average log fold-change (FC) and color-coded by adjusted p-value. (N-Q) Violin plots showing the expression of putative smooth muscle progenitor markers *Axin2*, *Fgf10*, *Gli1*, and *Wt1* in each of the mesenchymal clusters represented in (H).

Supplementary Figure 2



Supplementary Figure 3, Related to Figure 2. Pitx2, Lef1, and Foxp1 expression patterns throughout the lung and across developmental stages. (A) *E11.5* lungs immunostained for cluster 1 marker *Pitx2* and counterstained with Hoechst. (B) Quantification of *Pitx2* intensity profiles emanating from the mesenchyme. Schematic shows lines and direction along which intensity profiles were measured. Mean and s.d. are plotted (n=7). (C) Violin plots showing the expression of *Pitx2* in each cluster. (D-E) Zoomed-in view of a confocal slice of the left primary bronchus of lungs isolated at *E12.5* and immunostained for α SMA and either *Lef1* or *Foxp1*. Epithelium indicated by ep. (F) Expression levels of *Foxp1* plotted against those of *Lef1* in mesenchymal cell clusters 0 and 1. (G-J) Confocal slices of the left lobe of lungs isolated at *E12.5* or *E14.5* and immunostained for either *Lef1* or *Foxp1* and counterstained with Hoechst. Scale bars show 50 μ m.

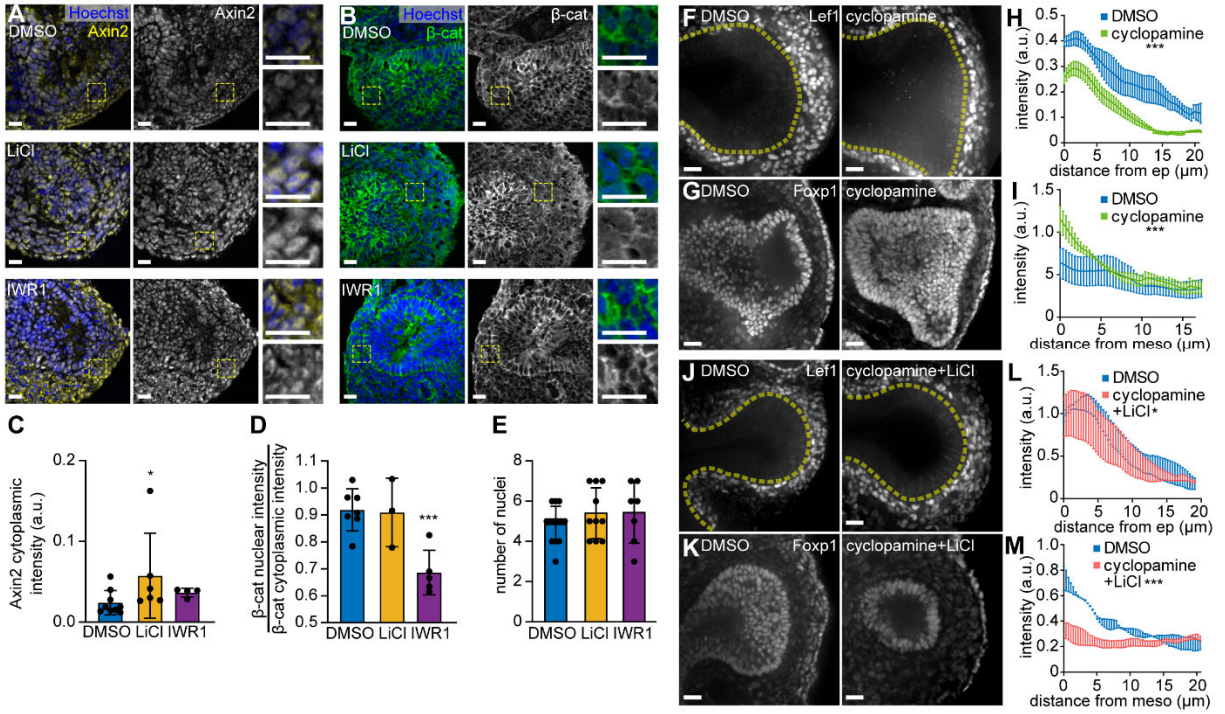
Supplementary Figure 3



Supplementary Figure 4, Related to Figure 3. Manipulating Wnt and Shh signaling ex vivo.

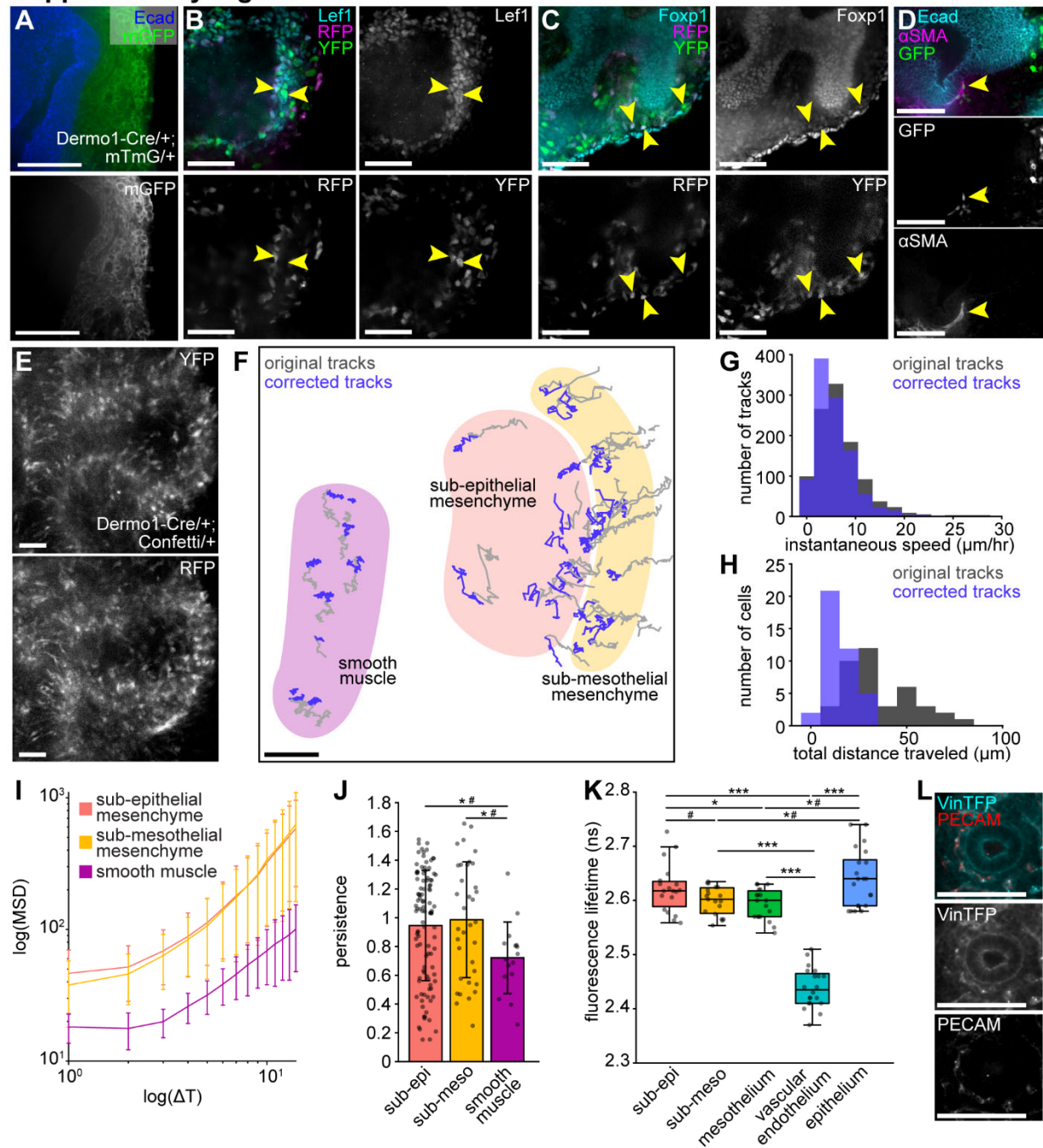
(A-B) Sections of lungs isolated at *E11.5* from CD1 embryos, immunostained for Axin2 or β -catenin, and counterstained with Hoechst after treatment with either DMSO, LiCl (10 mM), or IWR1 (100 μ M) for 24 hrs. Yellow dashed boxes indicate zoomed-in regions displayed to the right. **I** Quantification of mean cytoplasmic Axin2 immunofluorescence intensity in the mesenchyme for each treatment (n=4-11 sections, n=2-3 lungs). **(D)** Quantification of the ratio of nuclear to cytoplasmic β -catenin immunofluorescence intensity in the mesenchyme for each treatment (n=3-7 sections, n=2-3 lungs). **(E)** Quantification of the number of nuclei in the mesenchyme between the epithelium and mesothelium near branch L.L2 for each treatment (n=7-13 lungs). **(F-I)** Confocal sections and quantification of Lef1 and Foxp1 intensity profiles around branch L.L2 in lungs isolated at *E11.5* and treated with DMSO or cyclopamine (1 μ M) for 24 hr (n=4-5). **(J-M)** Confocal sections and quantification of Lef1 and Foxp1 intensity profiles around branch L.L2 in lungs isolated at *E11.5* and treated with DMSO or a combination of cyclopamine and LiCl for 24 hr (n=2-3). Scale bars show 25 μ m. Treatment conditions were compared using one-way ANOVA. * indicates $p < 0.05$ and ** indicates $p < 0.001$.

Supplementary Figure 4



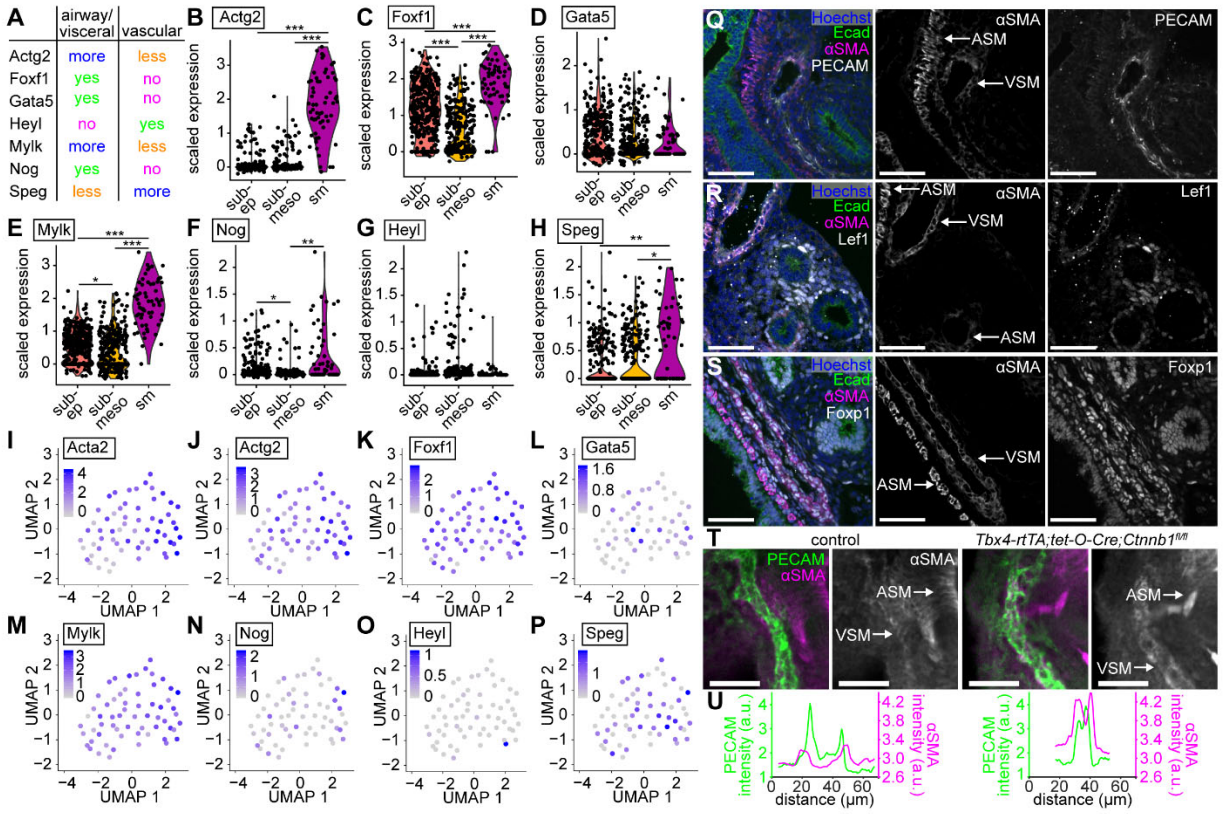
Supplementary Figure 5, Related to Figure 4. Confetti fluorescent reporters driven by Dermo1-Cre can be used to track single-cell movements in the embryonic pulmonary mesenchyme. (A) Confocal slice of the left lobe of lungs isolated at *E11.5* from a *Dermo1-Cre;mTmG* embryo and immunostained for E-cadherin and GFP. GFP expression throughout the mesenchyme indicates that Cre is active throughout this tissue. (B-D) Confocal sections of *Dermo1-Cre;Confetti^{fl/+}* lungs isolated at *E11.5*, cultured for 48 hrs under time-lapse conditions, and immunostained for Lef1, Foxp1, or α SMA and GFP. Arrowheads indicate examples of cells that exhibit colocalization of Confetti label and mesenchymal or smooth muscle cell markers. (E-F) Snapshots from a time-lapse of *Dermo1-Cre;Confetti^{fl/+}* lung isolated at *E11.5* I and plot of cell tracks (F). Tracks colored in gray are uncorrected and tracks colored in blue are corrected for local tissue movement. Cell-type assignments are indicated by the shaded regions. (G-H) Distribution of instantaneous speeds from each cell track and of total cell displacements before and after correcting for local tissue movement. (I) Log-log plot of mean MSD curves of each mesenchymal cell type. Error bars show s.d. (J) Persistence of cell tracks measured as slope of MSD curves from (G). (K) Fluorescence lifetime of each lung tissue. Data points represent average per sample. Boxes show 25th and 75th percentiles, center lines show median. (L) VinTS lung stained for PECAM. Cells with high VinTS expression dispersed throughout the mesenchyme are vascular endothelial cells. Scale bars show 50 μ m. Groups were compared using two-sided t-test and Bartlett's test for unequal variances. *** indicates $p < 0.0001$ and * indicates $p < 0.05$ for t-test. #### indicates $p < 0.0001$ and # indicates $p < 0.05$ for Bartlett's test.

Supplementary Figure 5



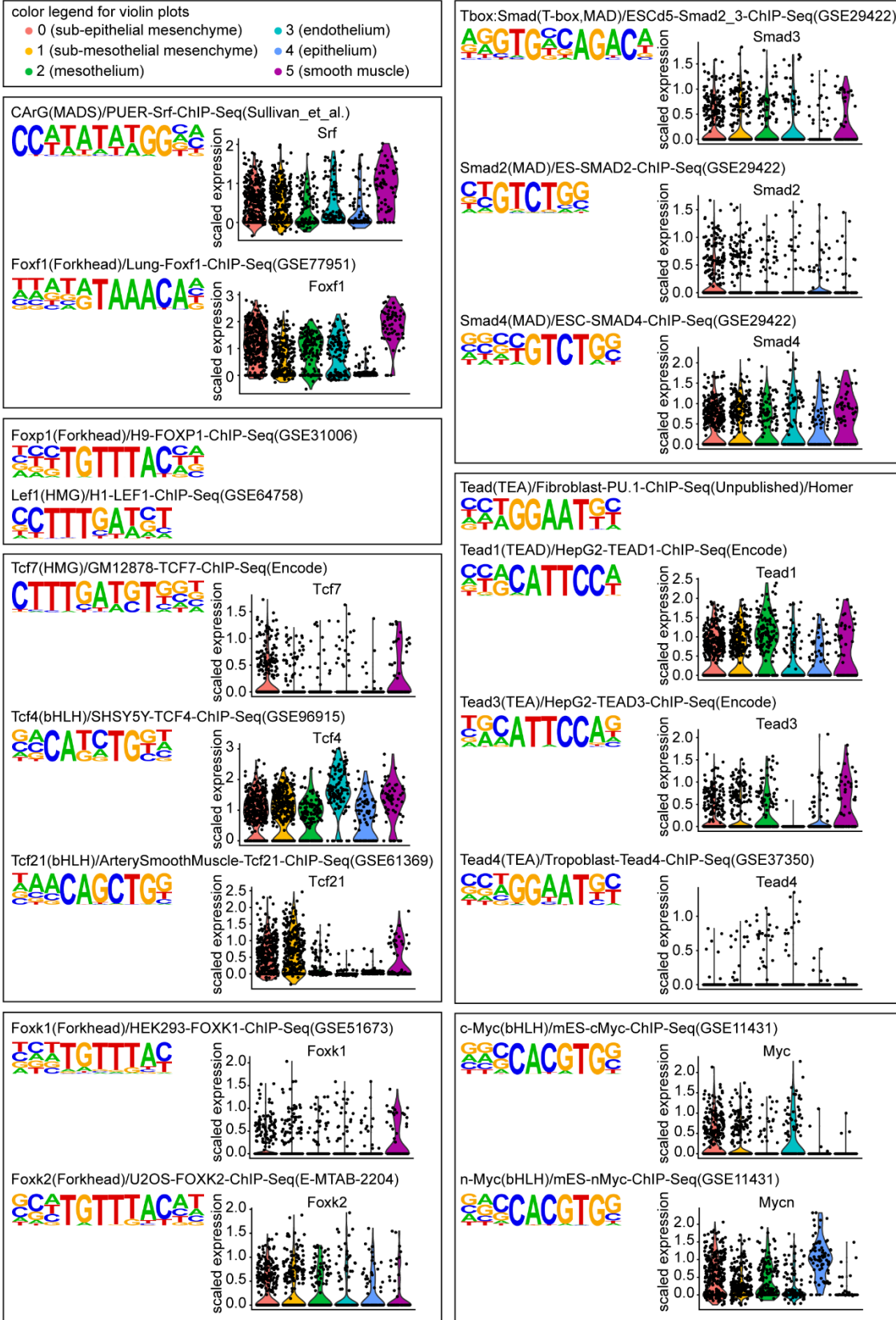
Supplementary Figure 6, related to Figure 5. Sub-mesothelial cells may preferentially give rise to vascular smooth muscle. (A) Table showing the expected expression of each of marker in airway/visceral smooth muscle versus vascular smooth muscle³. (B-H) Violin plots showing the scaled expression of *Actg2*, *Foxf1*, *Gata5*, *Heyl*, *Mylk*, *Nog*, and *Speg* in each of the mesenchymal and smooth-muscle clusters from **Fig. 1B**. Gene expression per cell cluster compared by Wilcoxon Rank Sum Test using the FindMarkers function. (I-P) UMAP of smooth muscle cells computationally isolated from the dataset in **Fig. 1B** color-coded based on scaled expression of marker genes for airway and vascular smooth muscle. (Q-S) Sections of E14.5 lungs immunostained for E-cadherin, α SMA, and either PECAM, Lef1, or Foxp1 and counterstained with Hoechst. Airway smooth muscle (ASM) and vascular smooth muscle (VSM) are indicated. (T) E12.5 control and *Tbx4-rtTA; tet-O-Cre; Ctnnb1^{fl/fl}* lungs immunostained for α SMA and PECAM. Airway smooth muscle (ASM) and vascular smooth muscle (VSM) are indicated. (U) Intensity profiles along lines traced across blood vessels showing PECAM and α SMA staining intensity in controls and mutants. Scale bars show 50 μ m. * indicates $p < 0.05$, ** indicates $p < 0.001$, and *** indicates $p < 0.0001$.

Supplementary Figure 6



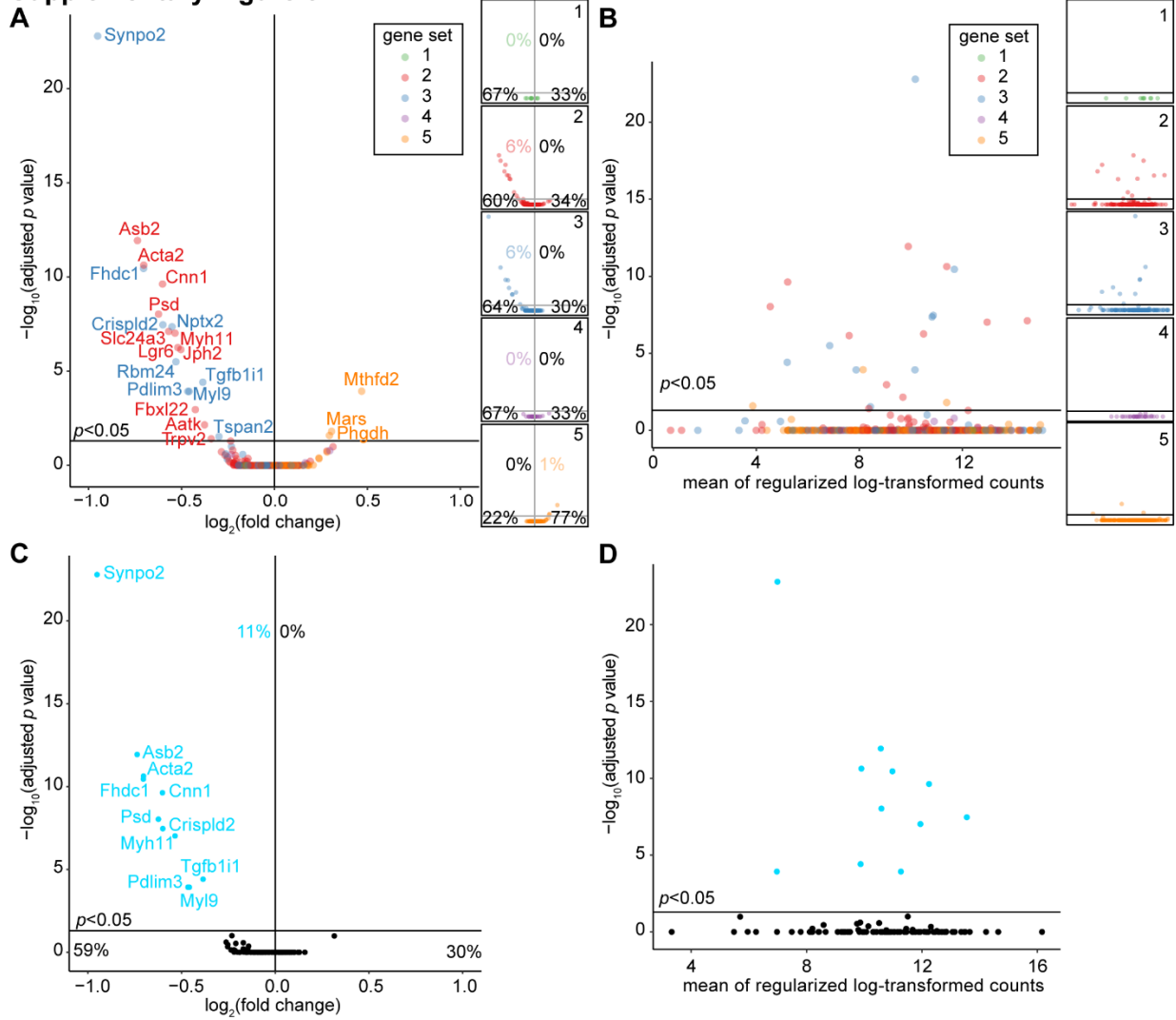
Supplementary Figure 7, Related to Figure 5. Transcription factors identified in motif discovery analysis are expressed in the embryonic lung. Motif logos and tissue-specific expression of corresponding transcription factors for results presented in **Fig. 5F**. For expression of Lef1 and Foxp1, see **Fig. 2C**.

Supplementary Figure 7



Supplementary Figure 8, Related to Figure 6. The majority of smooth muscle marker genes are Myocd-independent. (A) Volcano plot showing the adjusted p-values and log fold changes of gene-set members (**Fig. 5D**) that were detected in bulk RNA-seq of *E13.5* Myocd-KO lungs compared to controls. Insets to show each gene set separately with the percentage of genes in each quadrant of the volcano plot indicated. (B) Adjusted p-values plotted against the mean of regularized log-transformed counts across all samples showing that genes that were not significantly different were detected at similar levels as genes that were significantly different. Points are color-coded according to gene set and insets to the right show each gene set separately. (C) Volcano plot showing the adjusted p-values and log fold changes of smooth-muscle cluster markers (**Fig. 1B, E**) that were detected in bulk RNA-seq of *E13.5* Myocd-KO lungs compared to controls. The percentages of genes in each quadrant of the volcano plot are indicated. (D) Adjusted p-values plotted against the mean of regularized log-transformed counts across all samples.

Supplementary Figure 8



Supplementary Figure 9, Related to Figure 7. (A-B) Confocal sections of the left lobe of lungs isolated from *E11.5* embryos and immunostained for Tbx3 after treatment with DMSO or LiCl for 24 hr and quantification of Tbx3 intensity profiles emanating from the epithelium (n=3). **(C)** *E12.5* control and *Tbx4-rtTA; tet-O-Cre; Ctnnb1^{fl/fl}* lungs immunostained for Ecad and Tbx3. **(D-E)** Original and segmented images of lungs immunostained for α SMA used to quantify % coverage of smooth muscle. First, images are processed and thresholded to generate a mask of regions with α SMA staining. Morphological closing (a dilation followed by an erosion) is then performed on the resultant binary images to generate a closed mask that represents the total area of the airways in contact with smooth muscle. % coverage is calculated as the area of the mask (A_{mask}) divided by the area of the closed mask ($A_{\text{closed mask}}$) and reflects the extent to which the airways are wrapped by smooth muscle.

Supplementary Figure 9

