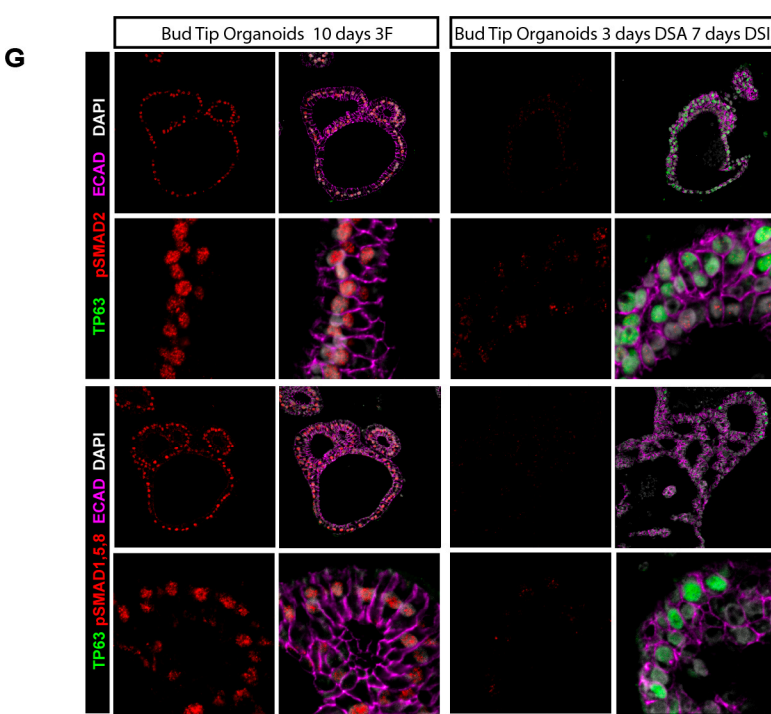
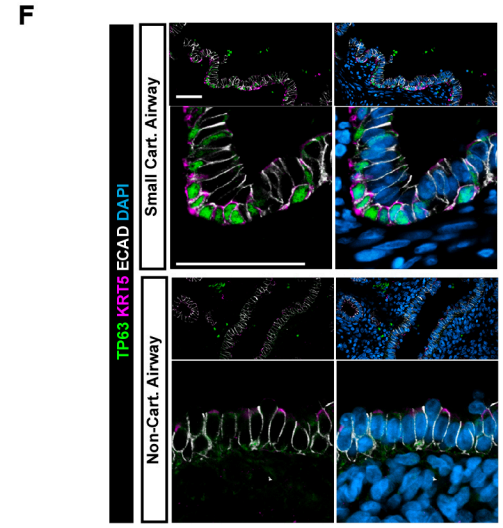
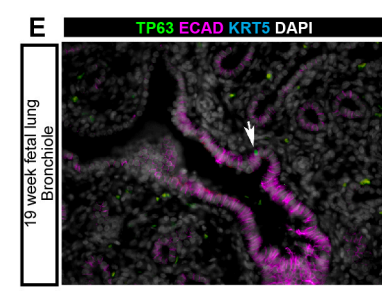
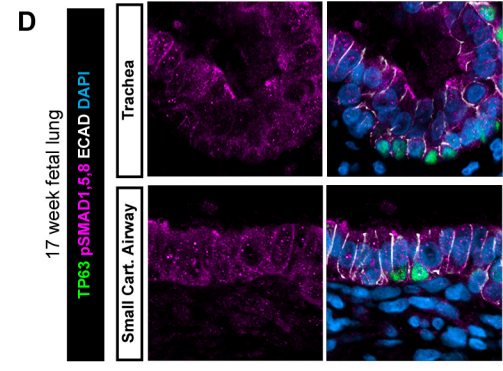
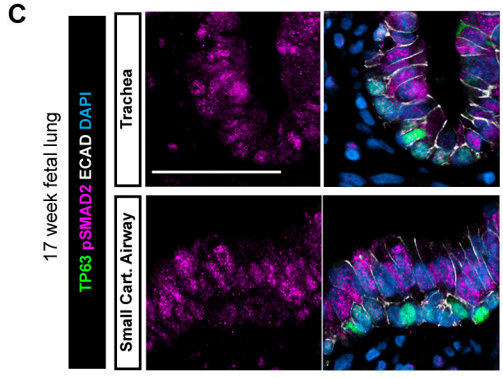
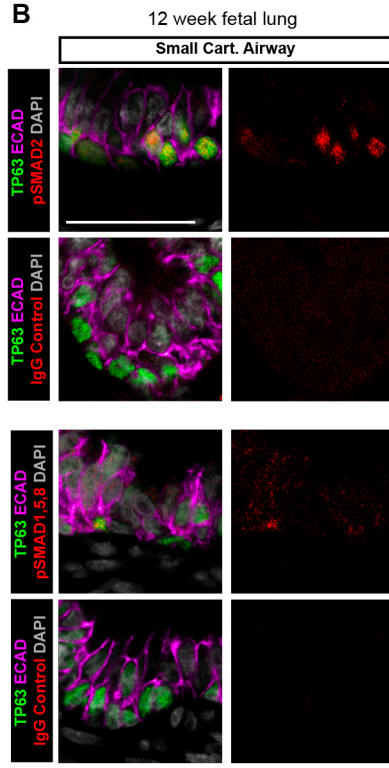
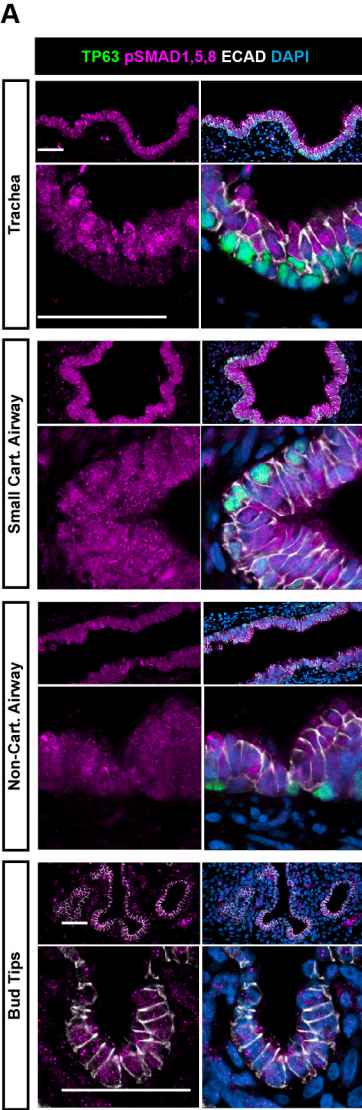
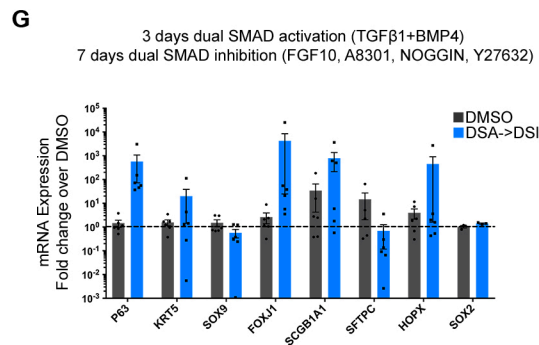
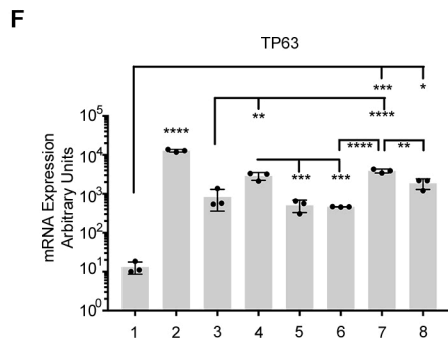
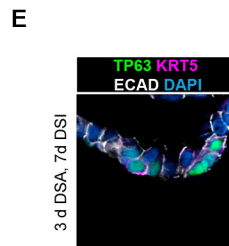
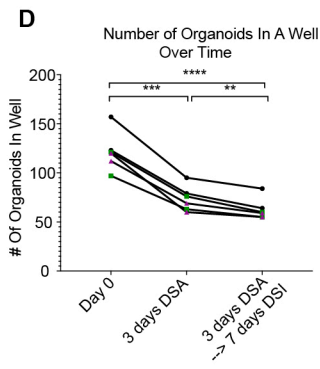
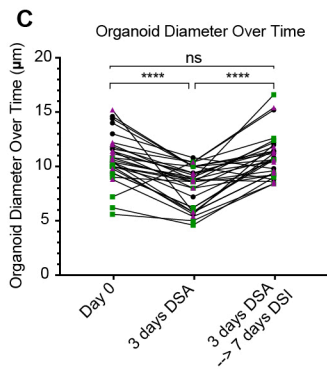
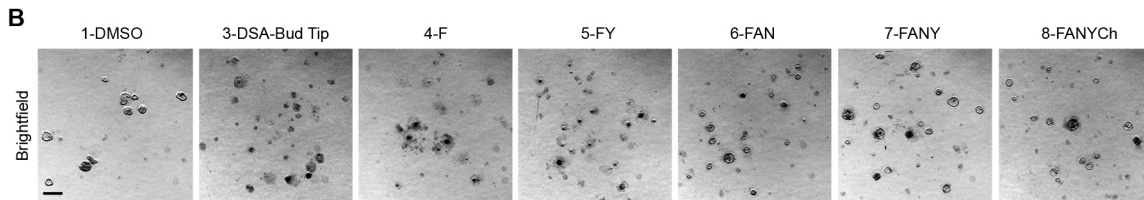
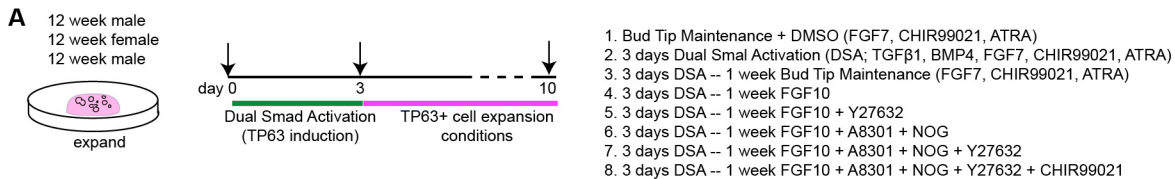


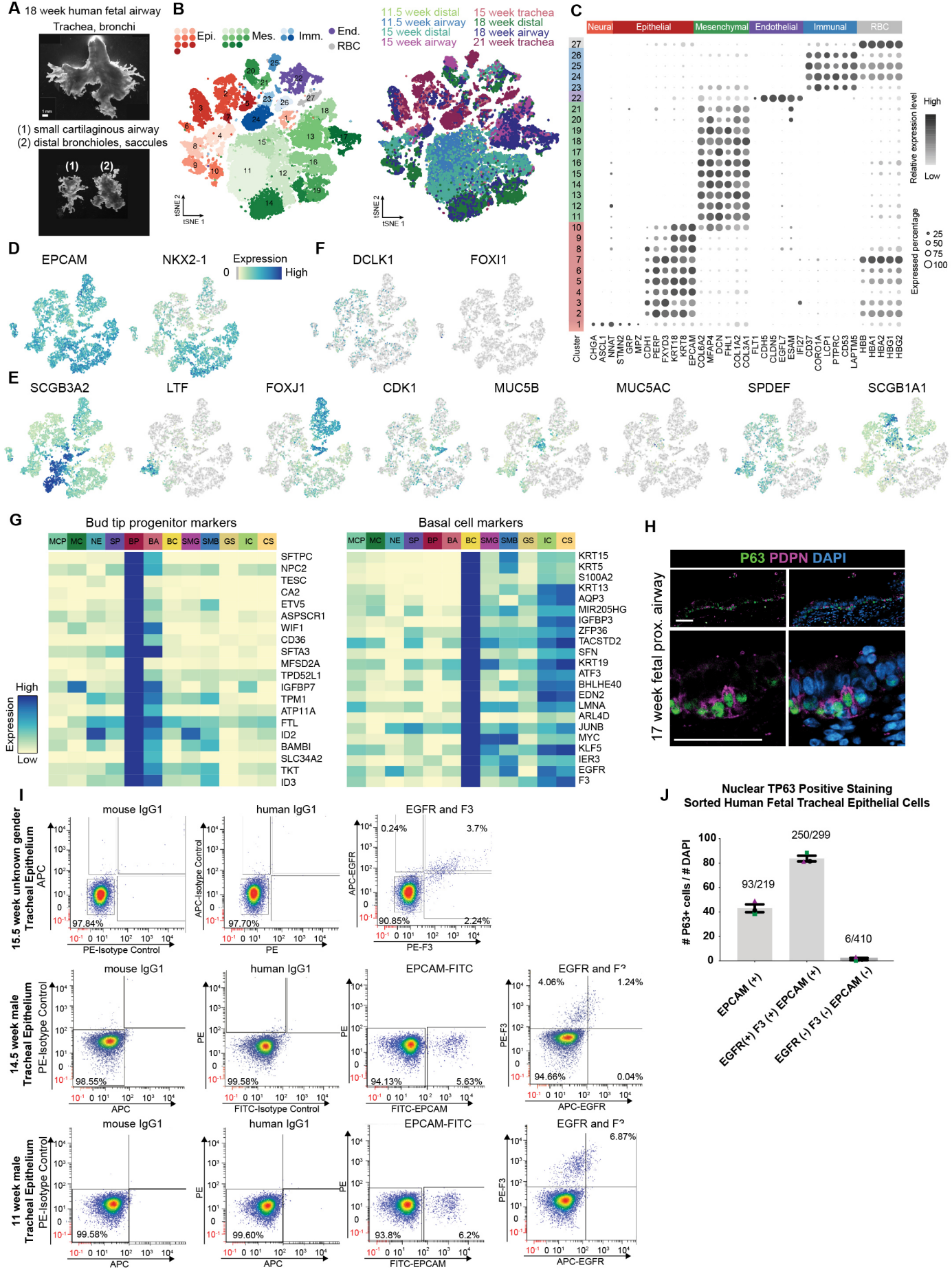
**Figure Supplement 1. Characterization of SMAD manipulation on bud tip progenitor organoids and fetal lung explants. Related to Figure 1.** **A)** Schematic of experimental design. **B)** QRT-PCR for expression of basal cell marker KRT5 in bud tip progenitor organoids treated serum-free progenitor maintenance medium (3 Factor or '3F'; FGF7, CHIR99021 and ATRA), supplemented with combinations of activators and/or inhibitors of TGF $\beta$  and BMP signaling. Data is presented as mean +/- the standard error of the mean. N=3 biological replicates. Significant variation between the means of each group was analyzed by a one-way ANOVA, alpha=0.05. **C)** Brightfield images of a single well of organoids for each treatment group at day 0 and after 3 days of treatment. Scale bar represents 400  $\mu$ m. **D)** Protein staining of proliferation marker KI67 (pink) and TP63 (green) in organoids treated with DMSO or with 3 days of DSA. Scale bars represent 50  $\mu$ m. **E)** Quantification of (D). Total number of KI67+ cells were counted for 4-5 individual organoids across 3 biological replicates. A two-sided Mann-Whitney test, P<0.0001 was used to evaluate the difference in the mean of each group. **F)** Protein staining of apoptosis marker Cleaved Caspase 3 (CC3; pink) and TP63 (green) in organoids treated with DMSO or with 3 days of DSA. Scale bar represent 50  $\mu$ m. **G)** Quantification of (F). Total number of CC3+ cells were counted for 5 individual organoids across 3 biological replicates. DSA treated organoids exhibited a statistically significant increase in the number of CC3+ cells (Two-sided Mann-Whitney test, p<0.0001). **H)** QRT-PCR for basal cell marker *TP63* in organoids treated with serum free progenitor maintenance medium with or without the addition of CHIR99021 in the presence of SMAD signaling pathway activists and inhibitors. No statistical differences were observed between groups treated in the presence or absence of CHIR99021, though we note that variability within groups is high (one-way ANOVA, p>0.05). Data is reported as arbitrary units and error bars represent the mean +/- the standard error of the mean. **I)** Schematic of experimental design. **J)** Bright field images of lung explants on day 0 (2 days post plating) and day 3 of treatment. Scale bar represents 1 mm. **K)** Protein staining for TP63 (green), KRT5 (pink), E-Cadherin (ECAD; white) and DAPI (blue) in lung explants after 3 days in culture with DMSO control, Dual SMAD Activation (DSA) or Dual SMAD Inhibition (DSI). Scale bars represent 100  $\mu$ m. **L)** QRT-PCR of *TP63* from 2 explants from the 10 week lung (technical replicates) and 3 explants from the 11 week lung (technical replicates). Significant variation in means was evaluated by one-way ANOVA, alpha=0.05, p<0.001, followed by Tukey's multiple comparisons comparing the mean of each group to the mean of every other group. Error bars represent the mean +/- the standard error of the mean. Estimated p values are reported on the graph as follows: \* p<0.05; \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001.



**Figure Supplement 2. Characterization of fetal TP63+ cells and SMAD signaling. Related to Figure 2.** **A)** Protein staining for phospho-SMAD1,5,8 (pSMAD1,5,8; pink; middle panels) with TP63 (green), E-cadherin (ECAD; white) and DAPI (blue) along the proximal-distal axis in a fetal 12 week lung. Scale bars represent 50  $\mu$ m. **B)** Adjacent tissue sections of 12 week fetal lung were stained with pSMAD2 or pSMAD1,5,8 and the rabbit IgG isotype control. Slides were imaged with identical laser power and microscope settings as those in A. Scale bar represents 50  $\mu$ m. **C-D)** Staining for pSMAD2 (**C**, pink; left panels), and **D)** pSMAD1,5,8 (pink; middle panels) with TP63 (green) at 17 weeks gestation along the proximal-distal axis. Scale bars represent 50  $\mu$ m. **E)** Despite low abundance, rare TP63+/KRT5- cells were observed in the distal airways (arrow) in 19-20 fetal lungs. Image is representative of n=3 biological replicates between 19 and 20 weeks gestation. Scale bar represents 50  $\mu$ m. **F)** Protein staining for TP63 (green), KRT5 (pink), E-Cadherin (white) and DAPI (white) in a 17 week fetal lung specimen. Image is representative of n=3 biological replicates. Scale bar represents 50  $\mu$ m. **G)** Protein staining for phospho-SMAD2 (pSMAD2; red, top rows) or phospho-SMAD 1,5,8 (pSMAD1,5,8, red, bottom rows) with TP63 (green), E-cadherin (ECAD; pink) and DAPI (white) in bud tip organoids treated for 10 days with progenitor maintenance medium 3F, or treated for 3 days with dual SMAD activation (DSA) followed by 7 days dual SMAD inhibition (DSI). Scale bars represent 50  $\mu$ m.

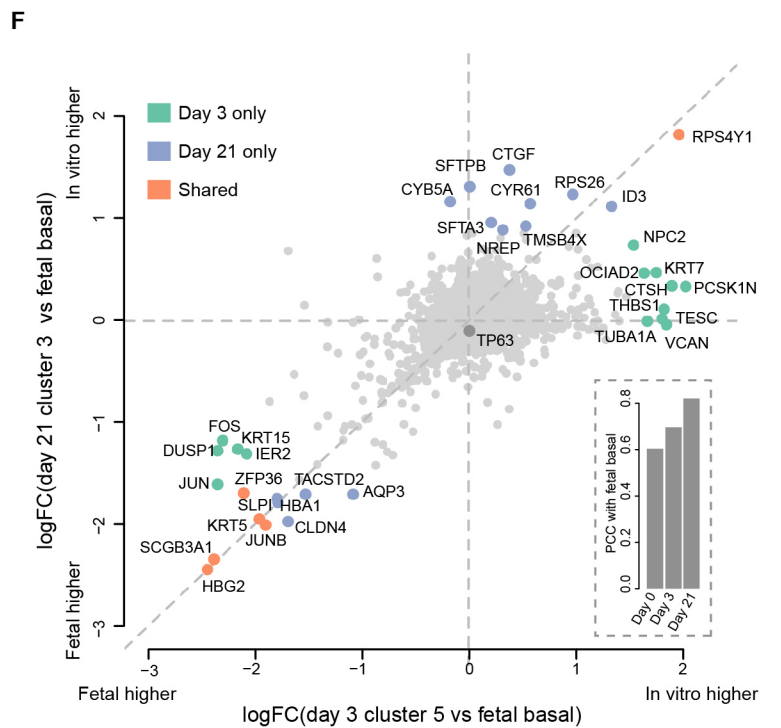
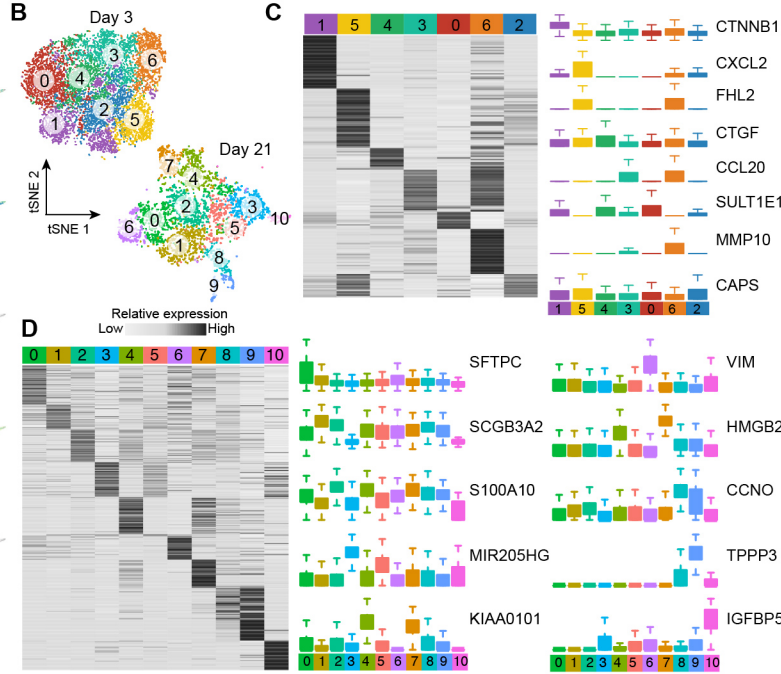
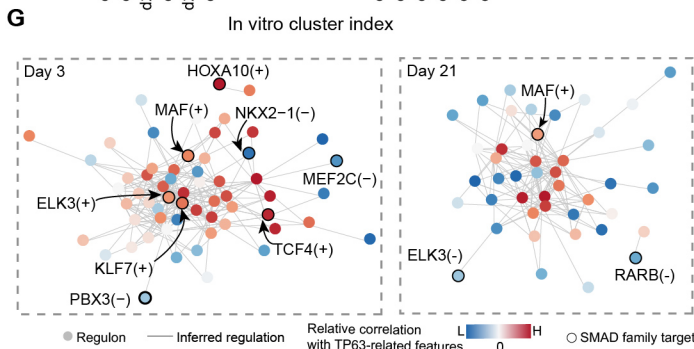
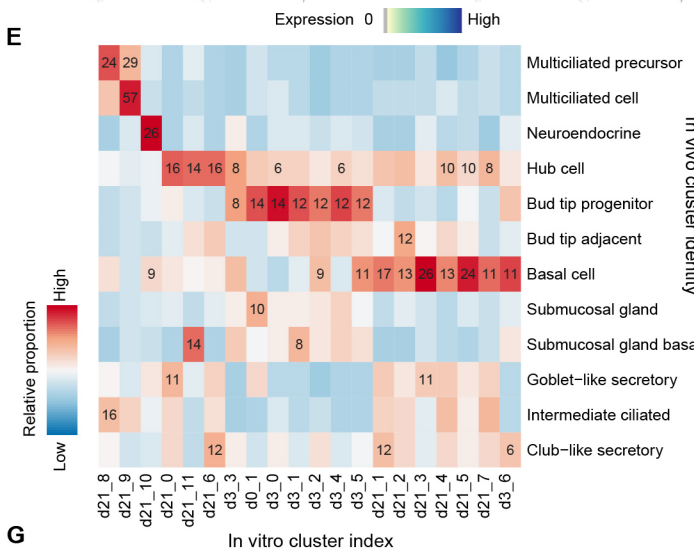
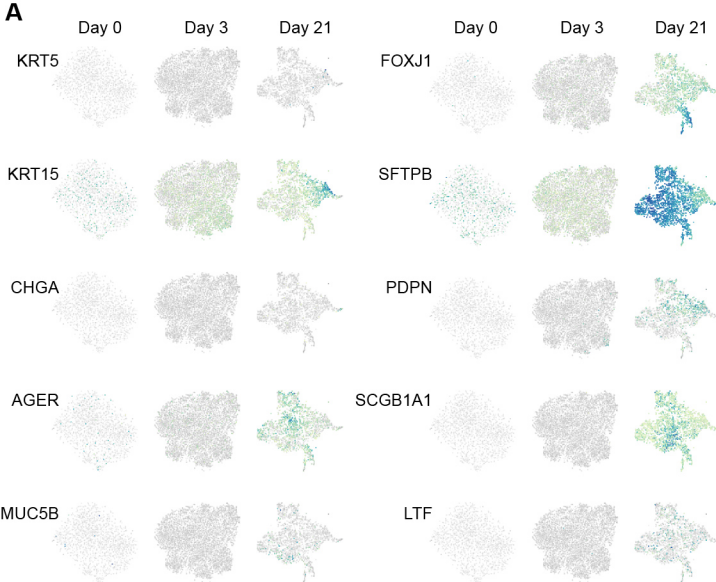


**Supplemental Figure 3. Screen for factors that maintain growth and expansion of TP63+ cells in culture. Related to Figure 2.** **A)** Experimental schematic. Experimental conditions are listed as groups 1-8. **B)** Brightfield images of organoids 10 days after treatment. Scale bar represents 500  $\mu\text{m}$ . **C)** Quantification of organoid diameter over time prior to culture with DSA (day 0), after 3 days of DSA treatment (day 3) and after 3 days of DSA treatment followed by 7 days of DSI treatment. Each data point represents a single organoid connected by a line to measurements of that organoid on a different day. Statistical variation in means was determined by a one-way paired ANOVA,  $\alpha=0.05$ ,  $p<0.0001$ ,  $f=25.3$ , followed by Tukey's multiple comparison's test to compare the mean of each group to the mean of every other group. Error bars represent the mean  $\pm$  the standard error of the mean.  $N=3$  biological replicates denoted by color. Estimated p values are reported on the graph:  $p<0.05= *$ ;  $p<0.01 = **$ ,  $p<0.001= ***$ ,  $p<0.0001= ****$ . **D)** Quantification of the number of organoids in a well over time prior to culture with DSA (day 0), after 3 days of DSA treatment (day 3) and after 3 days of DSA treatment followed by 7 days of DSI treatment. Each data point represents a single well, connected by lines to measurements from that same well on a different day. Statistical variation in means was determined by a one-way paired ANOVA,  $\alpha=0.05$ ,  $p<0.0001$ , followed by Tukey's multiple comparison's test to compare the mean of each group to the mean of every other group. Error bars represent mean  $\pm$  the standard error of the mean.  $N=3$  biological replicates denoted by color. Estimated p values are reported on the graph:  $p<0.05= *$ ;  $p<0.01 = **$ ,  $p<0.001= ***$ ,  $p<0.0001= ****$ . **E)** Protein staining for TP63 (green), Keratin 5 (KRT5; pink), E-Cadherin (ECAD; white), and DAPI (blue) of bud tip organoids treated for 3 days with Dual SMAD Activation (DSA) followed by 7 days treatment with Dual SMAD Inhibition (DSI). Scale bar represents 50  $\mu\text{m}$ . **F)** QRT-PCR for expression of *TP63* for all treatment groups. Significant variation in means between groups was determined by one-way ANOVA,  $\alpha=0.05$ ,  $p<0.0001$ ; Tukey's multiple comparisons of the mean of each group versus the mean in all other groups, estimated p values are reported on the graph.  $p<0.05= *$ ;  $p<0.01 = **$ ,  $p<0.001= ***$ ,  $p<0.0001= ****$ ).  $N=3$  biological replicates. Data is plotted as arbitrary units. Error bars are plotted to show mean  $\pm$  the standard error of the mean. Data is from a single experiment and is representative of  $n=3$  experiments. **G)** QRT-PCR for markers of canonical differentiated lung epithelial cell types showing DMSO (gray bars) and DSA--DSI treated (blue bars) organoids after 3 days DSI and 7 days DSI treatment. Data is plotted as fold change over DMSO controls. DSA--DSI treated organoids exhibited a 394-fold increase over DMSO controls in mean *TP63* expression. Significant differences in expression between controls and experimental treatment groups was determined by Wilcox Rank-Sum tests,  $\alpha=0.05$ . No statistically significant differences between groups were found. Error bars represent the mean  $\pm$  the standard error of the mean.

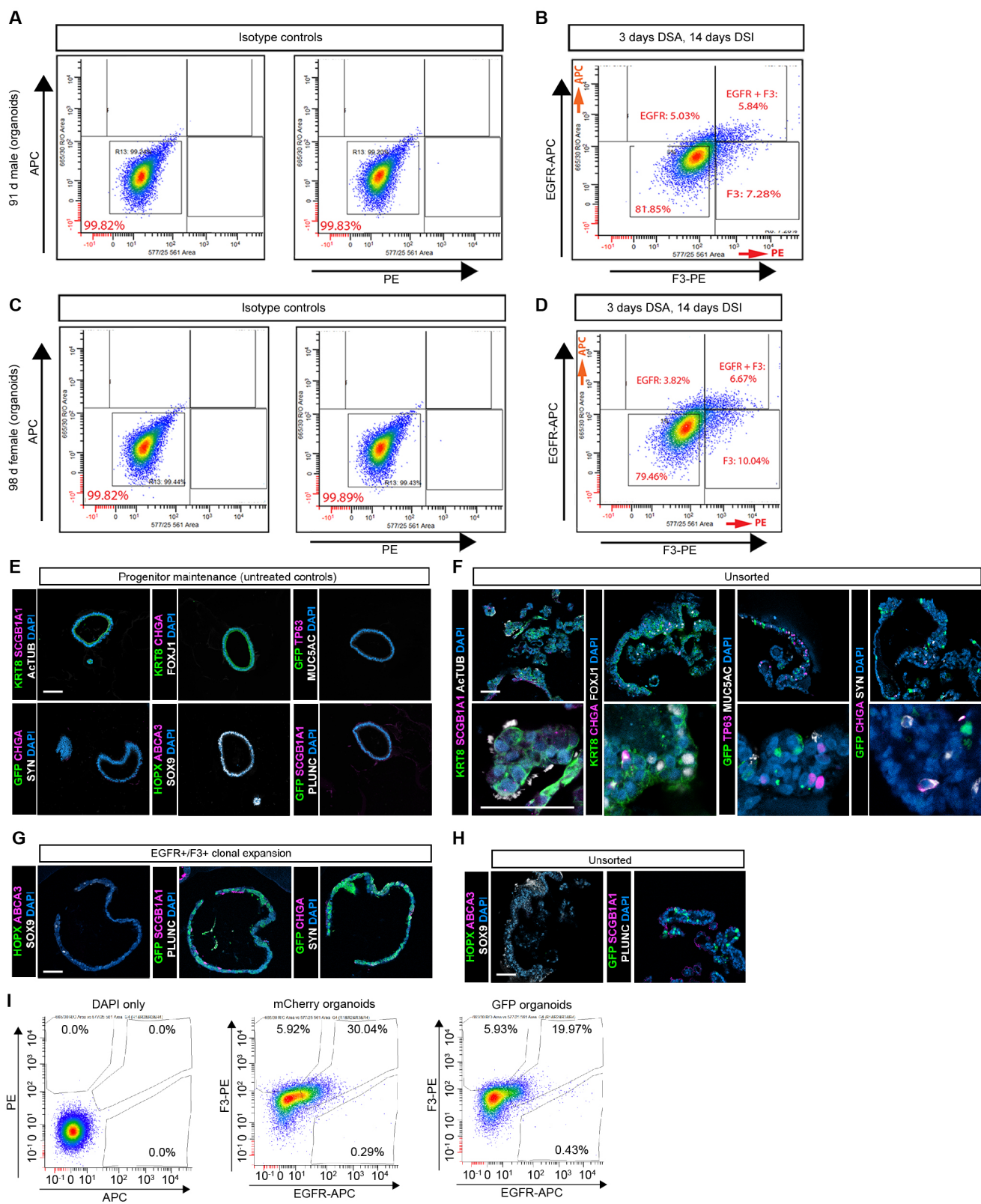


**Figure Supplement 4. Fetal lung characterization at single cell resolution. Related to Figure 3.** **A)** Brightfield images of 18 week dissected human fetal lung tissue. Top panel shows the trachea and main bronchi. To collect cells for scRNA-seq from the trachea, the tracheal tube was cut lengthwise, and epithelial cells were scraped with a scalpel and processed for scRNA-seq. Bottom panel shows the small cartilaginous airways (1) and the distal lung (2). Scale bar represents 1mm. **B)** Cellular transcriptome heterogeneity visualized by tSNE, with cells colored by identity of cell lineages (top; Epi: epithelial clusters; Mes: mesenchymal clusters; Imm: immune clusters; End: endothelial cluster; RBC: red blood cell cluster) and location of specimen (bottom), respectively. Numbers on the top panel tSNE indicate identity of *de novo* identified clusters. **C)** Dot plot showing z-transformed cluster average expression levels and proportion of cells with detectable expression levels in each cluster for canonical cell lineage markers. Colors of cluster index indicate inferred cell lineages. Red: epithelial lineage. Green: mesenchymal lineage. Purple: endothelial cells. Blue: immune lineage. Gray: red blood cells. Cell lineage identity of marker genes are represented by side bar on top, following the same color scheme as that for clusters. Orange: neuronal lineage. **D)** Feature plots show the expression patterns of lung epithelial marker NKX2.1 and pan-epithelial marker EPCAM. **E)** Feature plots on tSNE embeddings of canonical human lung epithelial cell types, including goblet cell markers *MUC5B* and *MUC5AC*, secretory marker *SPDEF*, club cell marker *SCGB1A1*, fetal secretory progenitor marker *SCGB3A2*, submucosal gland cell marker *LTF*, multiciliated cell marker *FOXJ1* and proliferation marker *CDK1* **F)** Feature plots showing expression patterns of canonical markers for rare lung epithelial cell types, Tuft cells (*DCLK1*) and Ionocytes (*FOXI1*). **G)** Heatmaps show quantiles of cluster average expression levels normalized to expression range of each gene in the scRNA-seq data of 11.5-21 week gestation human fetal lung specimens. Only genes with top fold change in bud tip progenitors (BT, left) or basal cell (BC, right) are presented. **H)** TP63+ basal cells (green) in the trachea of 17 week fetal lungs (n=3 biological replicates) stain positive for surface marker PDPN (pink). Scale bar represents 50  $\mu$ m. **G-H)** MCP=multiciliated precursor, MC=multiciliated, NE=neuroendocrine, SP=secretory progenitor, BP=bud tip progenitor, BA=bud tip adjacent, BC=basal cell, SMG=submucosal gland, SMB=submucosal basal, GS=goblet-like secretory, IC=intermediate ciliated, CS=club-like secretory. **I)** Fluorescence Activated Cell Sorting (FACS) data for epithelial cells scraped from fetal tracheas of N=3 biological replicates and stained with EGFR and F3 to isolate native basal cells. Gates were set using IgG negative controls. **J)** Quantification of the number of cells from F3+/EPCAM+ and F3-/EGFR- populations that stained positive for nuclear TP63 after cytospin.





**Figure Supplement 5. scRNA-seq analysis of *in vitro*-derived TP63+ cells and airway organoids. Related to Figure 4.** **A)** Feature plots of gene expression in day 0, day 3 and day 21 *in vitro* organoids from scRNA-seq. These data are derived from the same experiment as those shown in Figure 1 and Figure 4. **B)** tSNE projection of scRNA-seq from bud tip organoids treated for 3 days with Dual SMAD Activation (Day 3) or for 3 days with Dual SMAD Activation followed by 18 days of Dual SMAD Inhibition (Day 21). **C)** Left panel: heatmap showing z-transformed cluster average expression levels of top marker genes ranked by log-transformed fold change of cluster average expression levels compared to other clusters for Day 3 *in vitro* cells. Right panel: boxplots (interquartile range with minimum and maximum, outliers removed from plot) showing expression level distributions of selected cluster markers in each cluster. **D)** Left panel: heatmap showing z-transformed cluster average expression levels of top marker genes ranked by log-transformed fold change of cluster average expression levels compared to other clusters for Day 21 *in vitro* cells. Right panel: boxplots (interquartile range with minimum and maximum, outliers removed from plot) showing expression level distributions of selected cluster markers in each cluster. **E)** Percentage of top markers of each fetal epithelial cluster that are also top cluster markers of each *in vitro* cell cluster from day 3 and day 21. Numbers listed within boxes are percentages, and are only listed for results within the top two highest percentage groups. **F)** Differential expression (DE) analysis between *in vitro* basal like cells (day 3 cluster 5, and day 21 cluster 3) and fetal basal cells. Scatter plot shows the Log-transformed fold changes of gene expression between day 3 cluster 5 and fetal basal cells (X-axis), and between day 21 cluster 3 and fetal basal cells (Y-axis). DE genes with top 20 fold changes only at day 3, or day 21, or both are highlighted with different colors. Bottom right islet: Transcriptome similarity between day 0, best of the correlation between *in vitro* clusters and fetal basal cells in day 3 and day 21. **G)** Representation of reconstructed regulons from the *in vitro* scRNA-seq data. Only regulons with significant overrepresentation of top 50 TP63 best correlated genes (TP63+ cell gene features) in each time point are presented (One-sided Fisher's exact test, Benjamini & Hochberg corrected  $P < 0.05$ ). Each dot represents one regulon. Regulons are highlighted if the master TF is annotated to be bound by SMAD or SMAD3 or SMAD4 and shows significant correlation at indicated time point (Two-sided correlation test, nominal  $P < 0.05$ ). Each line indicates that a target gene of a regulon is the master regulon of another regulon. Regulons are colored according to correlation between sum of Z-transformed expression levels of TP63+ cell gene features and master TF of each regulon. Blue colors indicate negative correlations and red colors indicate positive correlations.



**Figure Supplement 6. Sorting and clonal expansion of fetal bud tip progenitor organoids. Related to Figure 5. A)** Fluorescence Activated Cell Sorting (FACS) plots of organoids treated with DSA-DSI and stained with isotype controls for biological replicate #2. **B)** Fluorescence Activated Cell Sorting (FACS) plots of organoids treated with DSA-DSI and stained with EGFR-APC and F3-PE for biological replicate #2. **C)** Fluorescence Activated Cell Sorting (FACS) plots of organoids treated with DSA-DSI and stained with isotype controls for biological replicate #3. **D)** Fluorescence Activated Cell Sorting (FACS) plots of organoids treated with DSA-DSI and stained with EGFR-APC and F3-PE for biological replicate #3. **E)** Bud tip progenitor organoids from n=3 biological replicates from 12 week fetal lungs were maintained in serum-free progenitor maintenance medium (FGF7, CHIR99021, ATRA) for 56 days and stained for markers of differentiated lung epithelial cell types: club cell marker SCGB1A1 (pink), neuroendocrine markers Chromagranin A (CHGA; pink) and synaptophysin (SYN; white), ciliated cell marker FOXJ1 (white), basal cell marker TP63 (pink), goblet cell marker mucin 5AC (MUC5AC; white), AECII marker ABCA3 (pink), AECI and secretory progenitor cell marker HOPX (green), secretory lineage marker PLUNC (white) and SOX9 (white). Scale bar represents 50  $\mu$ m. **F)** Organoids that had been infected with GFP lentivirus but not sorted and left to expand in basal cell expansion medium were collected after 56 days in culture and stained for differentiated epithelial cell markers: secretory marker SCGB1A1 (pink), multiciliated markers Acetylated Tubulin (white) and FOXJ1 (white), Neuroendocrine markers (CHGA, pink; SYN, white), TP63 (pink) and goblet cell marker MUC5AC (green). Scale bar represents 50  $\mu$ m. **G)** Organoids treated for 3 days DSA and 18 days DSI-expansion, FACS sorted for EGFR/F3 and clonally expanded in DSI expansion medium were stained for: AECI marker HOPX (green), AECII marker ABCA3 (pink), secretory markers SCGB1A1 (pink) and PLUNC (white), Neuroendocrine markers Chromagranin A (CHGA) and Synaptophysin (white), or GFP (green). Nuclei are stained by DAPI (blue). Scale bar represents 50  $\mu$ m. **H)** Protein staining for AECI marker HOPX (green), AECII marker ABCA3 (pink), bud tip progenitor marker SOX9 (white), secretory markers SCGB1A1 (pink) and PLUNC (white), and GFP (green). Nuclei are stained with DAPI (blue). Scale bar represents 50  $\mu$ m. **I)** FACS isolation of EGFR+/F3+ cells from organoids that had been treated with DSA-DSI, expanded in DSI, and then infected with a lentivirus driving expression of either GFP or mCherry and expanded again.

**Supplementary Table 2. Primary and Secondary Antibodies Used. Related to Figures 1, 2, 3 and 5.**

<b>Primary Antibody</b>	<b>Source</b>	<b>Catalog #</b>	<b>Dilution</b>	<b>Clone</b>
*Biotin-Mouse anti-MUC5AC	Abcam	ab79082	1:500	Monoclonal
*Biotin-Goat anti-P63	R&D systems	BAF1916	1:500	
Goat anti-CC10 (SCGB1A1)	Santa Cruz Biotechnology	sc-9770	1:200	C-20
Goat anti-Chromogranin A (CHGA)	Santa Cruz Biotechnology	sc-1488	1:100	C-20
Goat anti-SOX2	Santa Cruz Biotechnology	Sc-17320	1:200	polyclonal
Mouse anti-ABCA3	Seven Hills Bioreagents	WMAB-17G524	1:500	17-H5-24
Mouse anti-Acetylated Tubulin (ACTTUB)	Sigma-Aldrich	T7451	1:1000	6-11B-1
Mouse anti-E-Cadherin (ECAD)	BD Transduction Laboratories	610181	1:500	36/E-Cadherin
Mouse anti-F3	Sigma-Aldrich Atlas Antibodies	AMAb91236	1:200	monoclonal
Mouse anti-MUC5B	Abcam	AB77995	1:250	monoclonal
Mouse anti-PLUNC	R&D systems	MAP1897	1:500	monoclonal
Mouse anti-Surfactant Protein B (SFTPB)	Seven Hills Bioreagents	Wmab-1B9	1:250	monoclonal
Rabbit anti-Clara Cell Secretory Protein (CCSP; SCGB1A1)	Seven Hills Bioreagents	Wrab-3950	1:250	polyclonal
Rabbit anti-Cleaved Caspase 3	Cell Signaling	9664	1:500	monoclonal
Rabbit anti-EGFR	Sigma-Aldrich Atlas Antibodies	HPA018530	1:250	polyclonal
Rabbit anti-HOPX	Santa Cruz Biotechnology	Sc-30216	1:250	polyclonal
Rabbit anti-IL33	Sigma-Aldrich Atlas Antibodies	HPA024426	1:250	polyclonal
Rabbit anti-KRT15	Sigma-Aldrich Atlas Antibodies	HPA024554	1:500	polyclonal
Rabbit anti-KRT5	Sigma-Aldrich Atlas Antibodies	HPA059479	1:500	polyclonal
Rabbit anti-NKX2.1	Abcam	ab76013	1:200	EP1584Y
Rabbit anti-PDPN	Santa Cruz Biotechnology	Sc-134482	1:500	polyclonal
Rabbit anti-phospho-SMAD1,5,8	Millipore	AB3848	1:100	polyclonal
Rabbit anti-phospho-SMAD2	Abcam	AB188334	1:250	polyclonal
Rabbit anti-Pro-Surfactant protein C (Pro-SFTPC)	Seven Hills Bioreagents	Wrab-9337	1:500	polyclonal
Rabbit anti-SOX9	Millipore	AB5535	1:500	polyclonal

Rabbit anti-Synaptophysin	Abcam	AB32127	1:500	monoclonal
Rat anti-KI67	Biolegend	652402	1:100	16A8
<b>Secondary Antibody</b>	<b>Source</b>	<b>Catalog #</b>	<b>Dilution</b>	
Donkey anti-goat 488	Jackson Immuno	705-545-147	1:500	
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Donkey anti-goat 647	Jackson Immuno	705-605-147	1:500	
Donkey anti-goat 647	Jackson Immuno	705-605-147	1:500	
Donkey anti-goat Cy3	Jackson Immuno	705-165-147	1:500	
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Donkey anti-mouse 488	Jackson Immuno	715-545-150	1:500	
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Donkey anti-mouse 647	Jackson Immuno	415-605-350	1:500	
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Donkey anti-mouse Cy3	Jackson Immuno	715-165-150	1:500	
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Donkey anti-rabbit 488	Jackson Immuno	711-545-152	1:500	
Donkey anti-rabbit 647	Jackson Immuno	711-605-152	1:500	
Donkey anti-rabbit 647	Jackson Immuno	711-605-152	1:500	
Donkey anti-rabbit Cy3	Jackson Immuno	711-165-102	1:500	
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Streptavidin 488	Jackson Immuno	016-540-084	1:500	

**Supplementary Table 3. QRT-PCR Primers Used. Related to Figures 1, 2, 3 and 5.**

<b>Species</b>	<b>Gene Target</b>	<b>Forward Primer Sequence</b>	<b>Reverse Primer Sequence</b>
Human	FOXJ1	CAACTTCTGCTACTCCGCC	CGAGGCACTTTGATGAAGC
Human	GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA
Human	HOPX	GCCTTCCGAGGAGGAGAC	TCTGTGACGGATCTGCACTC
Human	KRT5	CTGGTCCAACCTCTCTCCA	GGAGCTCATGAACACCAAGC
Human	KRT14	TCTGCAGAAGGACATTGGC	GGCCTGCTGAGATCAAAGAC
Human	MUC5AC*	GCACCAACGACAGGAAGGATGAG	CACGTTCCAGAGCCGGACAT
Human	TP63	CCACAGTACACGAACCTGGG	CCGTTCTGAATCTGCTGGTCC
Human	SCGB1A1	ATGAAACTCGCTGTCACCCT	GTTTCGATGACACGCTGAAA
Human	SOX2	GCTTAGCCTCGTCGATGAAC	AACCCCAAGATGCACAACCTC
Human	SOX9	GTACCCGCACCTTGACAAC	ATTCCACTTTGCGTTCAAGG
Human	SFTPC	AGCAAAGAGGTCCTGATGGA	CGATAAGAAGGCGTTTCAGG