nature cell biology

DOI: 10.1038/ncb3580

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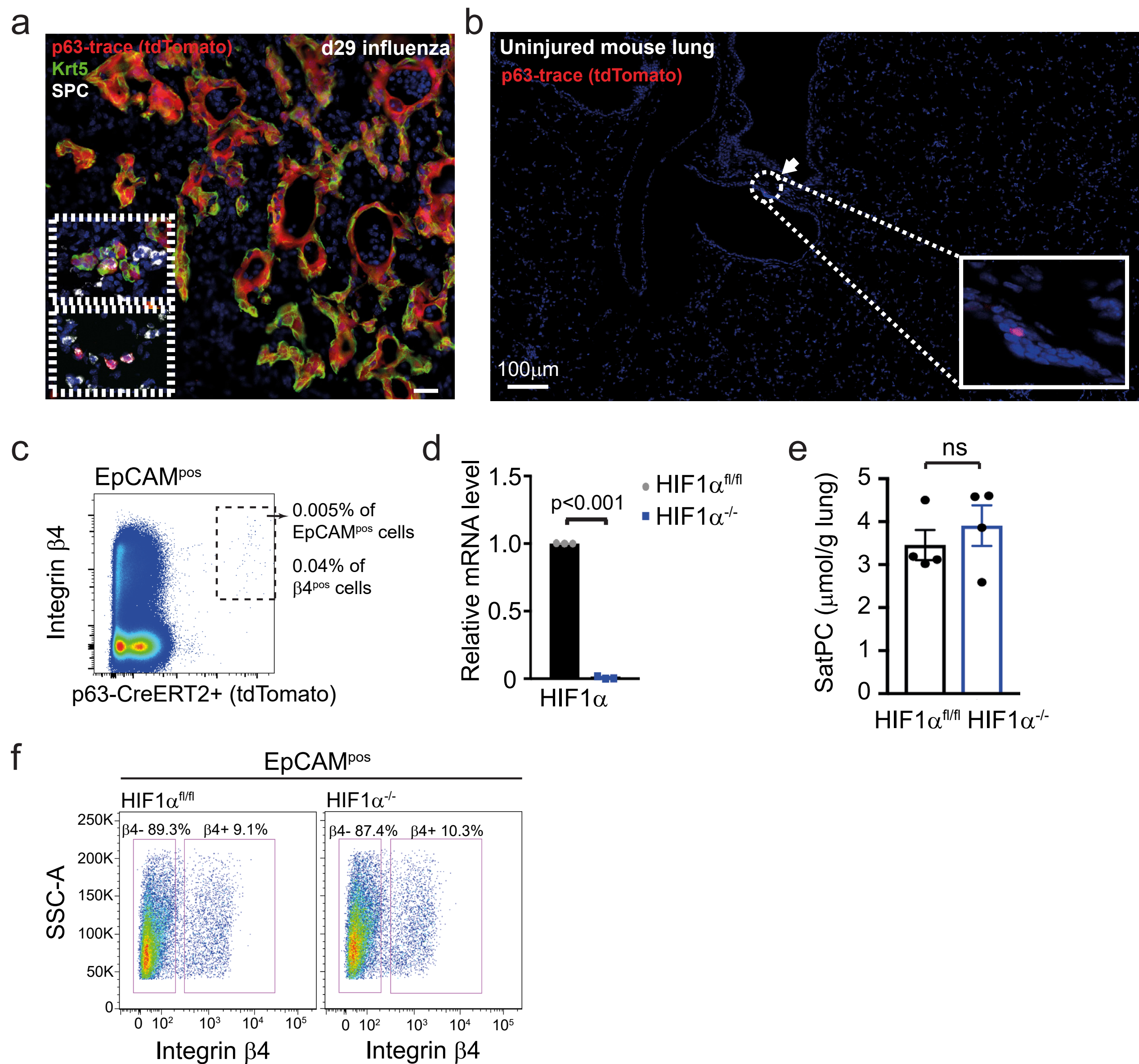

Supplementary Figure 1 p63^{pos} LNEPs are very rare and do not efficiently generate AEC2s. Lung epithelial ablation of HIF1α with Shh-Cre causes no defects in uninjured mice. (a) While p63^{pos} LNEPs generate Krt5 metaplasia, they make only a minor contribution to AEC2 regeneration after influenza injury (insets). (b) Rare p63-CreERT2 traced (tdTomato+) cells are scattered throughout airways in uninjured mice, representing 0.005% of the total epithelium as judged by FACS analysis (c). Data represent n=3 mice for histology (b), n=2 independent experiments with pooled live cells from 3 mice for FACS (c). (d) HIF1α is deleted in all the epithelial cells sorted from Shh-Cre; HIF1α$^{fl/fl}$ mouse lungs. Data are mean ± SEM from n=3 independent experiments, in which each group is a pool of 3 mice. (e) Lung SatPC content and (f) LNEP-enriched population (integrin β4^{pos} EpCAM^{pos}) remain unchanged after HIF1α deletion in epithelial cells. (e) Data are represented as mean ± SEM from n=4 mice per group from two independent experiments. (f) Data are represented as a percentage in EpCAM^{pos} live cells from a pool of 3 mice in each group. p values derived by unpaired two-tailed Student's t test.

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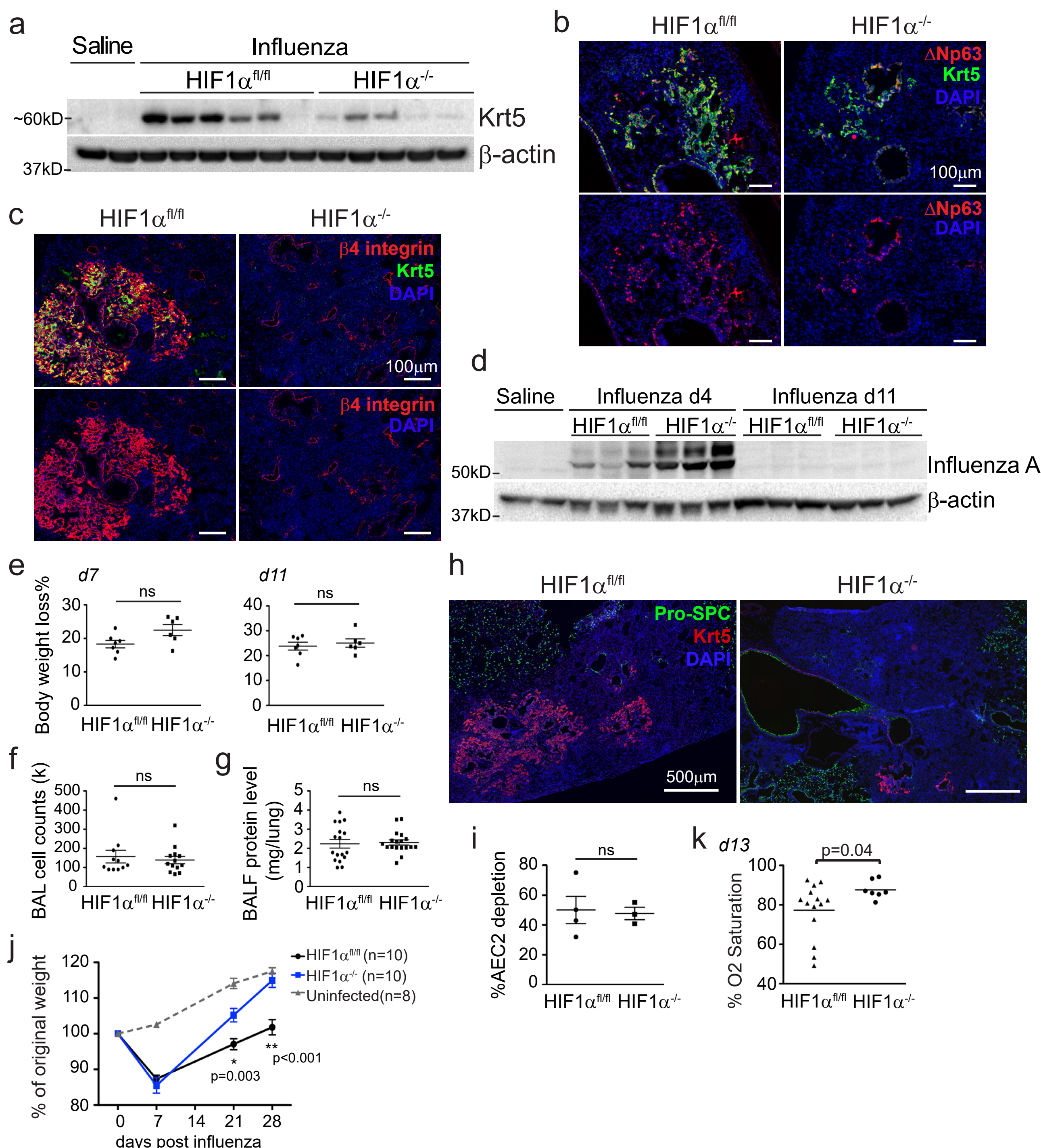

Supplementary Figure 2 HIF1α$^{-/-}$ mice are injured at similar levels as wild-type mice, but lack alveolar Krt5^{pos} cell expansion. (a) Representative blot showing Krt5 induction is inhibited by epithelial HIF1α deletion. (b-c) No large expansion of DNp63 (b) or integrin b4 (c) positive cells in the alveoli of HIF1α$^{-/-}$ mice, indicating alveolar Krt5^{pos} cell expansion is inhibited by HIF1α deletion. No difference in virus infection (d), weight loss (e), immune cell numbers in BAL (f) and BALF protein level (g) between wild-type and HIF1α$^{-/-}$ mice. (e-g) Data are represented as mean ± SEM, (e) n=7 wild-type, n=6 HIF1α$^{-/-}$; (f) n=11 wild-type, n=13 HIF1α$^{-/-}$; (g) n=17 wild-type, n=18 HIF1α$^{-/-}$ mice from three independent experiments. Each data point represents one mouse. (h) Large areas of AEC2 depletion are present in both wild-type and HIF1α$^{-/-}$ lungs, quantified in (i). SPC staining in airways (h, right) is an artifact of the goat anti-pro-SPC antibody. (i) Data are represented as mean ± SEM, n=4 wild-type, n=3 HIF1α$^{-/-}$ mice from 2 independent experiments. (j) HIF1α$^{-/-}$ mice recover weight more rapidly than their wild-type counterparts after influenza injury. Data are represented as mean ± SEM, n=10 wild-type, n=10 HIF1α$^{-/-}$, n=8 uninfected wild-type mice from three independent experiments. (k) Significant difference in average arterial oxygen saturation at 13 days post-infection between HIF1α$^{-/-}$ and wild-type mice. Each data point represents the average % O2 saturation reading for a single mouse at this time point (see Fig. 1h). Data are mean ± SEM, n= 7 HIF1α $^{-/-}$, n = 14 wild-type (2 Shh-Cre^{neg}, 12 C57BL6) mice from two independent experiments. Analysis is 11 days post-infection unless otherwise indicated. p values derived by unpaired two-tailed Student's t test, except in (k) derived by Mann Whitney.

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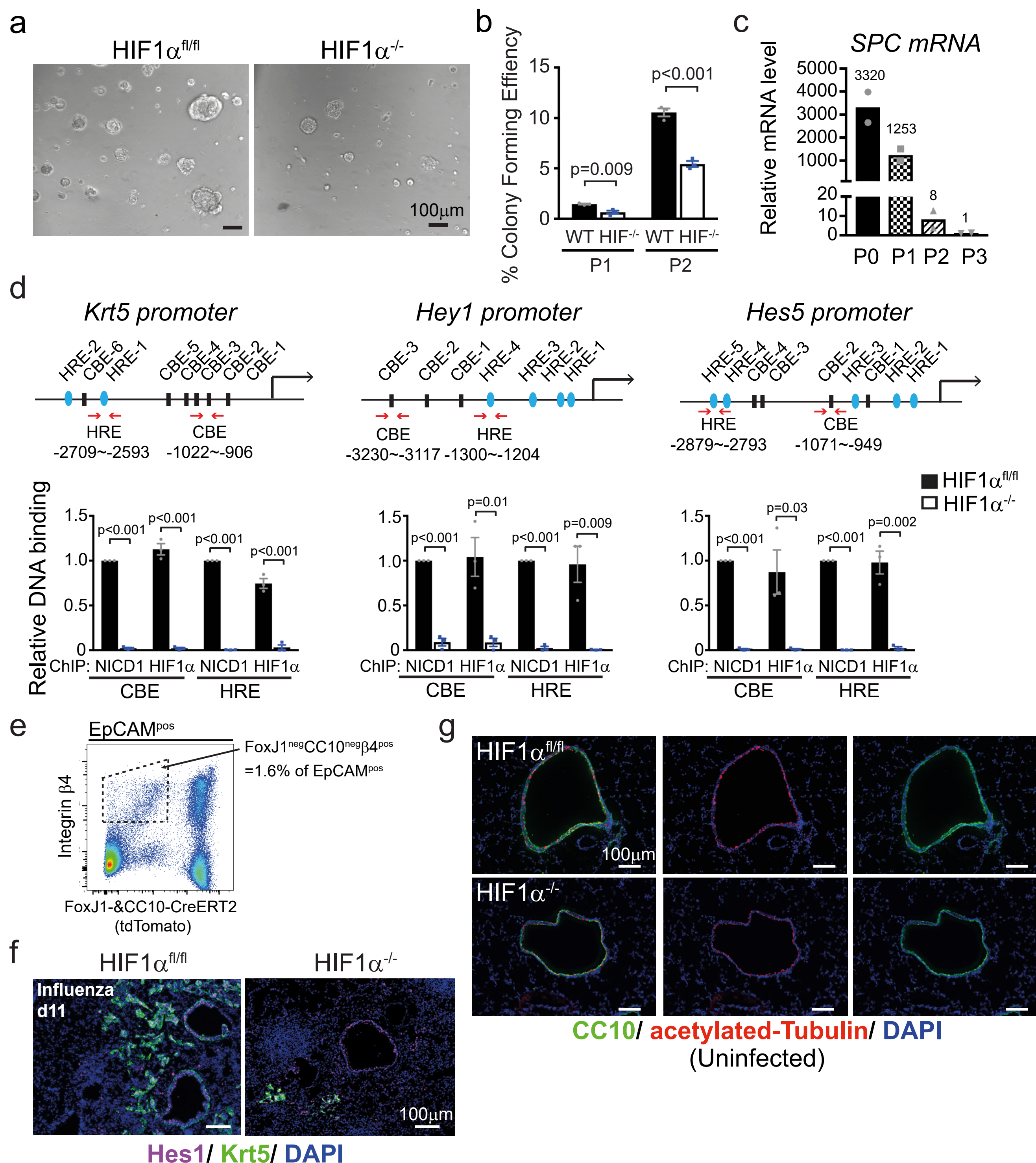

Supplementary Figure 3 HIF1α promotes Notch activity in LNEPs but has no effect on airway Notch activity. (a-b) Reduced colony size and number of HIF1α$^{-/-}$ LNEPs in culture. WT, HIF1α$^{fl/fl}$. (c) qPCR analysis of SPC in freshly sorted (P0) and cultured (P1-P3) LNEPs showing SPC mRNA dramatically decreases upon culture, n=2 independent experiments. (d) Top, mouse Krt5, Hey1 and Hes5 promoters contain HRE and CBE. The primers used in bottom are highlighted in red. Bottom, qPCR analysis of ChIP demonstrating HIF1α deletion blocks NICD1 DNA binding on Krt5, Hey1 and Hes5 promoters in cultured LNEPs. Ct value of pulled down DNA was normalized by Ct of input DNA and the abundance was calculated relative to NICD1 association of each site. (b,d) Data are represented as mean ± SEM from n=3 independent experiments. p values derived by unpaired two-tailed Student's t test. (e) FACS isolation of highly purified LNEPS (FoxJ1^{neg} CC10^{neg} integrin β4+) from uninjured mice used for RNA-Seq analysis. (f) HIF1α deletion inhibits Hes1 staining in the alveoli but not airways. (g) HIF1α deletion has no effect on airway Notch activity in uninfected mice, as judged by the ratio between club cells (CC10^{pos}) and multi-ciliated cells (acetylated-Tubulin^{pos}) remaining unchanged.

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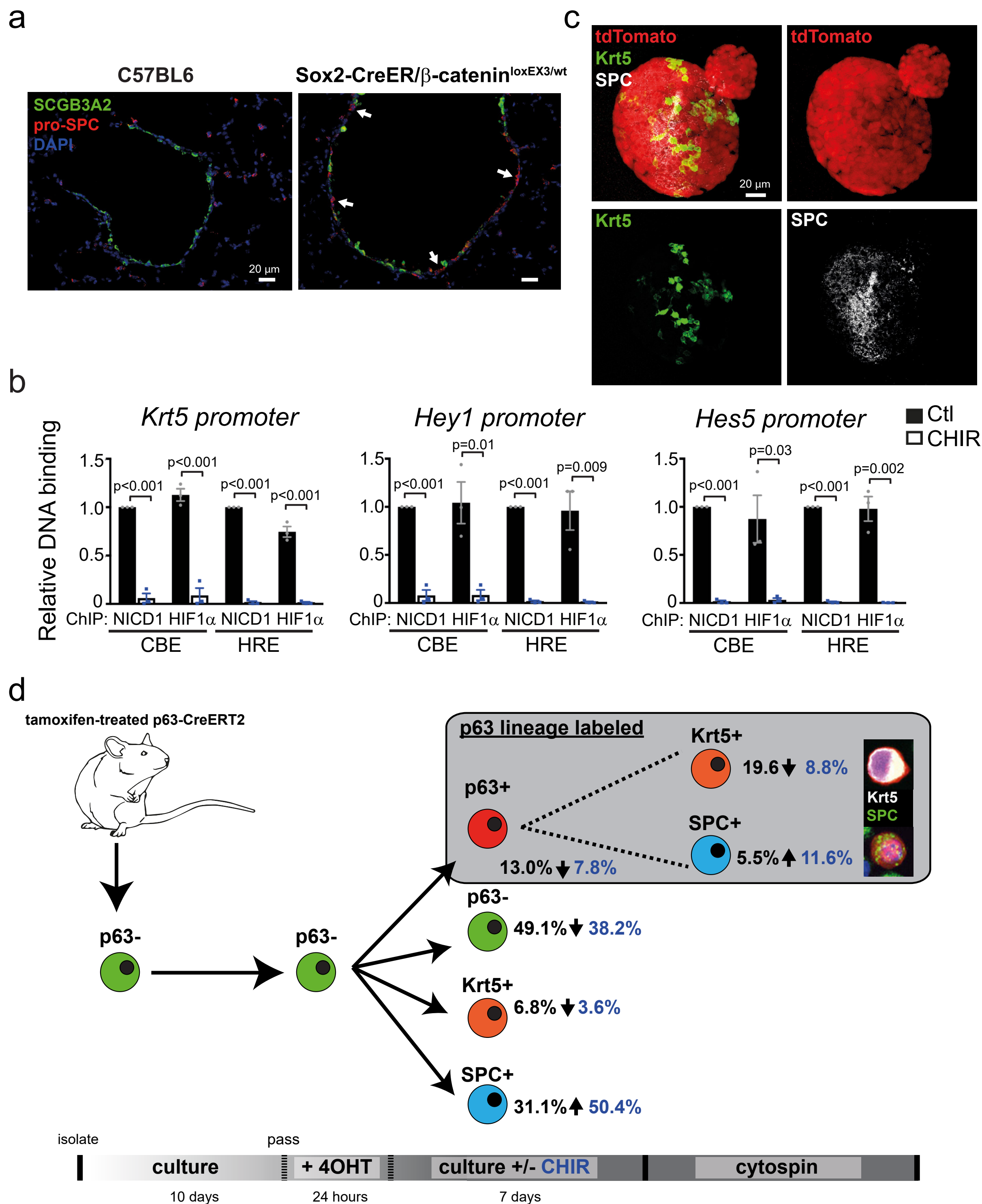

Supplementary Figure 4 Stabilization of b-catenin inhibits Notch and HIF1α activity by blocking their DNA association. (a) β-catenin stabilization increases ectopic SPC expression in the airways largely independent of club cells expressing Scgb3a2. About 27% (97 cells out of 362) Sox2-traced airway cells express SPC 7 days after tamoxifen induced β-catenin stabilization, n = 3 mice examined. (b) qPCR analysis of ChIP demonstrating NICD1 and HIF1a DNA binding on Krt5, Hey1 and Hes5 promoters are blocked by CHIR. The same control sample (LNEPs from HIF1α$^{fl/fl}$ mice) was used as Supplementary Fig. 3d. Data are represented as mean ± SEM from n=3 independent experiments. p values derived by unpaired two-tailed Student's t test. (c) Individual fluorescent channels of the colony from Fig. 3g demonstrating Krt5 and SPC expression in a single clone. (d) p63^{neg} LNEPs *in vitro* either remain undifferentiated, are activated into p63^{pos} cells (visualized by tdTomato expression after a brief 4OHT treatment), or differentiate into Krt5+ or SPC+ cells. Wnt agonism (blue) results in more SPC+ cells and fewer Krt5+ cells as described in Fig. 4d. Grey inset quantifies these outcomes specifically within those cells that become p63 traced. Quantification is via immunostaining of cytospins, n=2 experiments.

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a

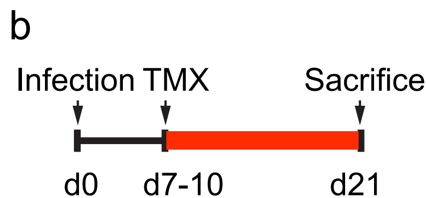
Krt5-CreER | Krt5-CreER/ $HIF1\alpha^{fl/fl}$ | Krt5-CreER/ β-catenin$^{loxEx3/wt}$

200μm

Krt5-trace / Krt5 / DAPI

Krt5-trace / Pro-SPC / DAPI

b



c Relative mRNA level

	Krt5-trace	Krt5-trace; $HIF1\alpha^{fl/fl}$
Hey1	1.0	1.9
Hey2	1.0	1.2
Hes5	1.0	4.0
HIF1α	1.0	0.1

d Relative mRNA level

	Krt5-trace	Krt5-trace; loxEx3
Hey1	1.0	0.9
Hey2	1.0	0.4
Hes5	1.0	0.9
Axin2	1.0	32280.3

Supplementary Figure 5 Deleting HIF1α or stabilizing b-catenin does not alter LNEP differentiation after full Notch/Krt5 activation. (a) HIF1a deletion or β-catenin stabilization subsequent to Krt5 activation as described in (b) has no effect on Krt5 (green, upper panel) and SPC (green, lower panel) expression. (c) Relative mRNA levels in sorted Krt5-CreERT2-traced cells 21 days post infection with (n=8) or without (n=2) HIF1α deletion. Notch activity is not downregulated by HIF1α deletion in Krt5 expressing cells. (d) Relative mRNA levels in sorted Krt5 traced cells 21 days post infection with (n=2) or without (n=5) b-catenin stabilization. Notch activity is not inhibited by Wnt signaling in Krt5 expressing cells. (c-d) Sorted cells from two independent experiments were pooled together for RNA isolation.

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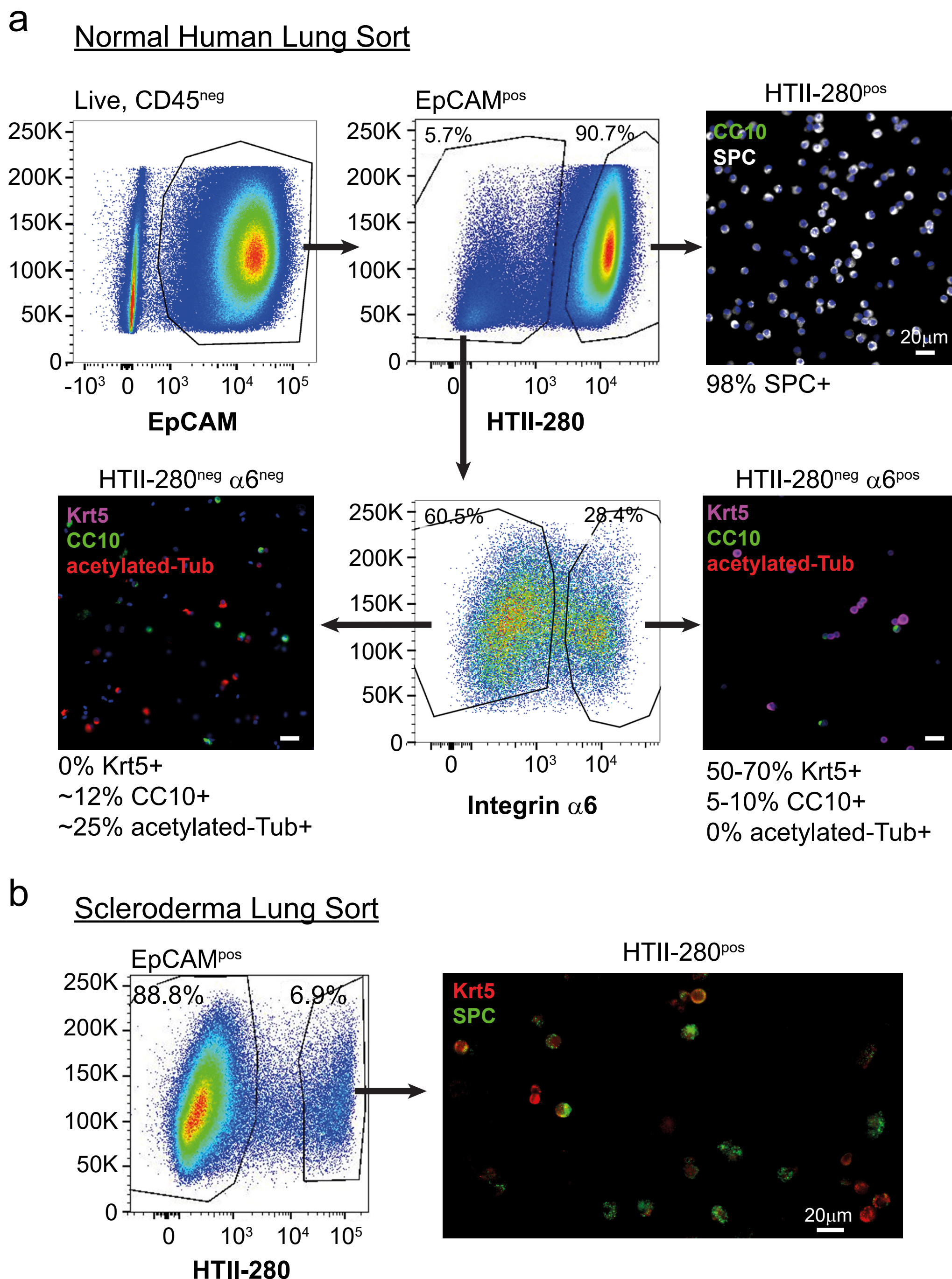

Supplementary Figure 6 Flow plots and cytospin of human lung epithelial cell sort. (a) As judged by cytospin, the HTII-280^{pos} population captures all the SPC^{pos} AEC2s, and the HTII-280^{neg}α6^{pos} population is enriched for basal cells in normal human lungs. (b) In scleroderma lung, the percentage of HTII-280^{pos} cells as a function of total EpCAM^{pos} cells decreases and both Krt5^{pos} and Krt5/SPC double positive cells are observed in HTII-280^{pos} cells.

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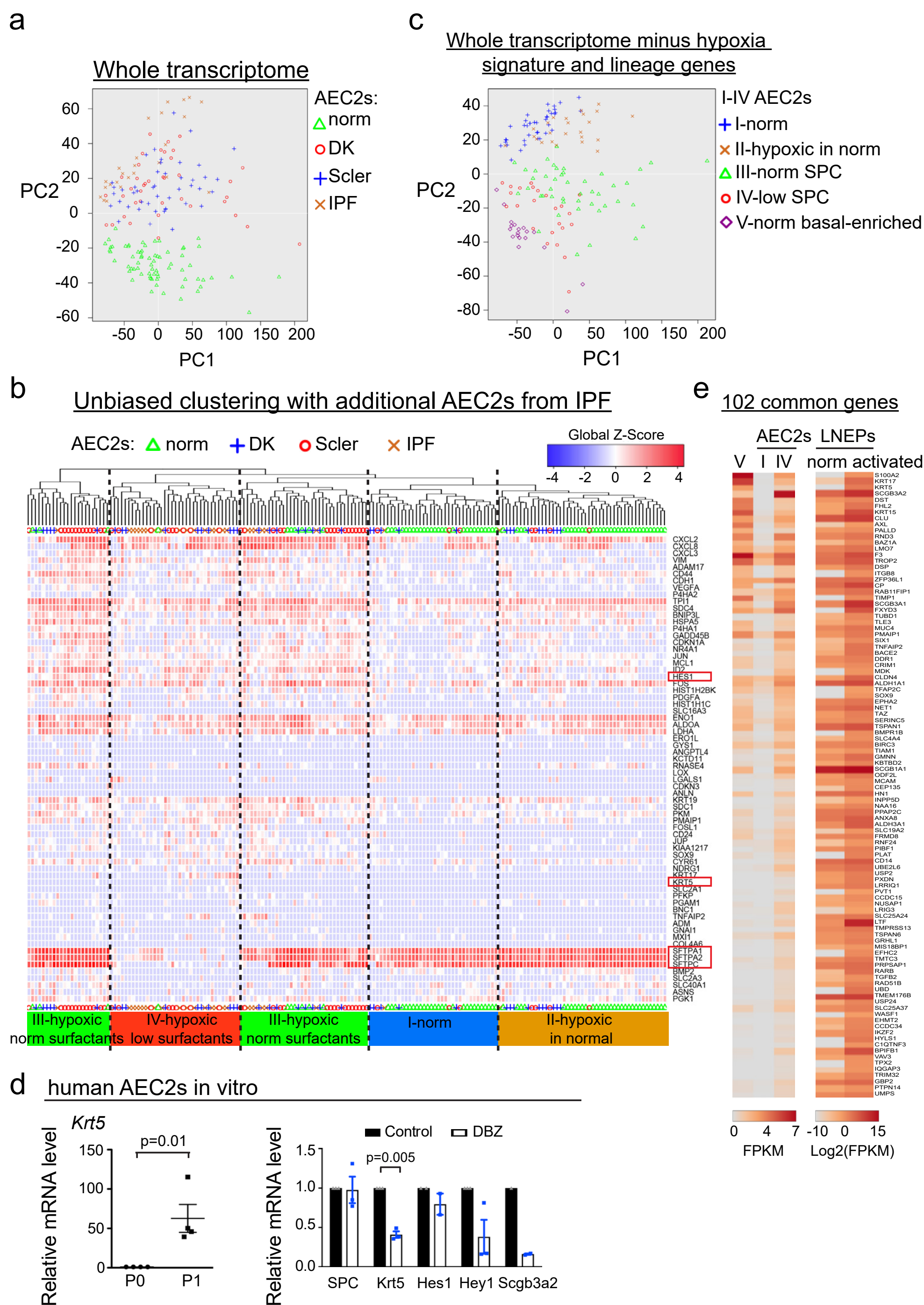

Supplementary Figure 7 Transdifferentiation of human AEC2s to basal-like cells and single cell RNA-Seq analysis of primary human lung epithelial cells show distinct hypoxia signature in fibrotic lungs. (a) Whole-genome wide PCA analysis of HTII-280^{pos} cells from normal, DK, Scleroderma and IPF lungs, showing AEC2s from fibrotic lungs are distinct from that of normal lungs. (b) Hierarchical clustering of single cell transcriptomes of HTII-280^{pos} cells isolated from normal, DK, scleroderma and IPF lungs. Listed genes (rows) are hypoxia signature (listed in Figure 5c) plus *STFPA1, STFPA2, SFTPC, KRT5, HES1* (highlighted with red rectangles). Four distinct groups (I-IV) are highlighted. Cells from IPF lungs are mostly in Group IV. (c) PCA analysis of all human cells using the entire genome except for the signature genes from Fig. 6a, demonstrating that the hypoxia & lineage gene set is predictive of meaningful differences in cell identity at the whole-transcriptome level. (d) Primary human AEC2s (HTII-280^{pos}) upregulated Krt5 mRNA after one passage in culture by qPCR analysis. Notch inhibition (DBZ) during this initial culture attenuated Krt5 upregulation (right). Data are represented as mean ± SEM, where each point represents one independent cell isolation and experiment (n=4 left, n=3 right). p values derived by unpaired two-tailed Student's t test (d, left) or one-sample t-test (d, right). (e) Average FPKM values of human cells (Group I, IV and V), and mouse quiescent and activated LNEPs from RNA-seq are indicated in the heatmap (right) for the shared upregulated 102 gene set (see Fig. 7).

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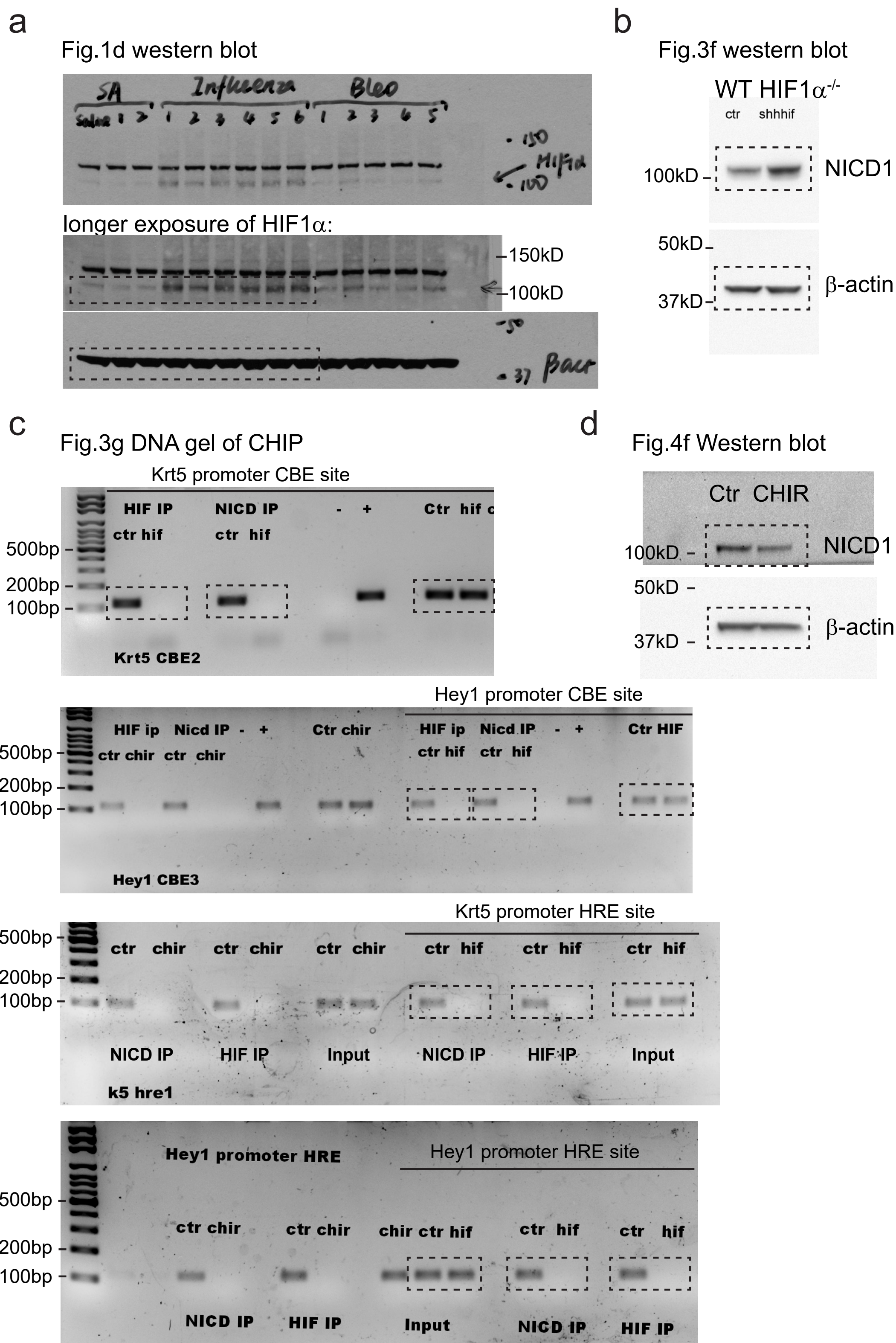

Supplementary Figure 8 Unprocessed original scans of immunoblots and agarose gel electrophoresis images. (a,b,d,f,g,h) Unprocessed western blot scans for Figures 1d, 3f, 4f, 5d, S2a and S2d. (c, e) Unprocessed DNA gel electrophoresis of ChIP for Figures 3g and 4e.

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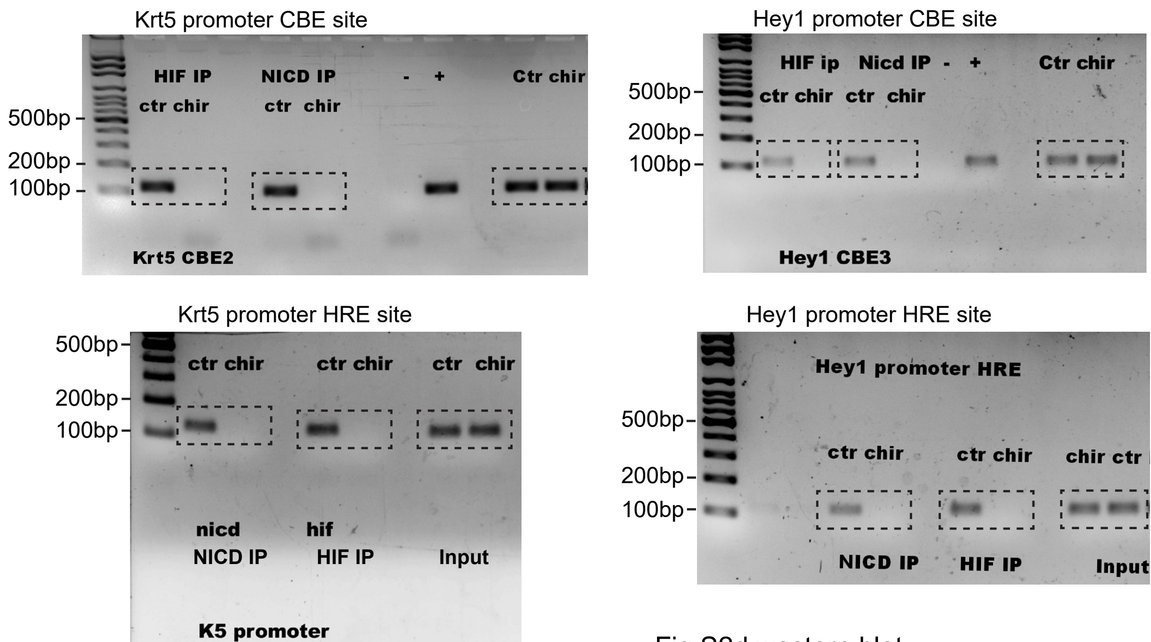

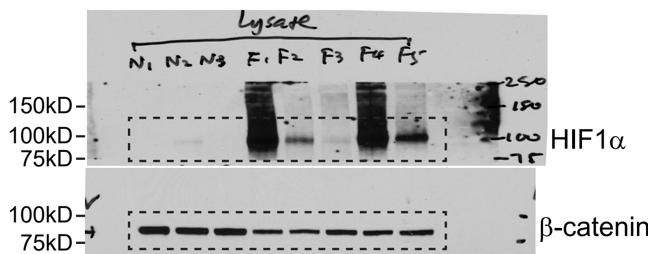

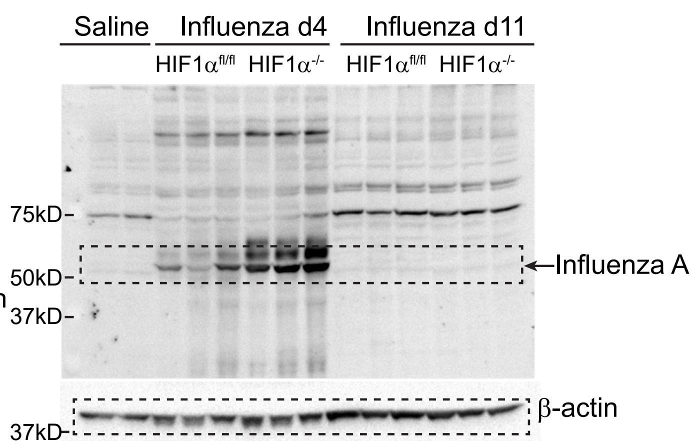

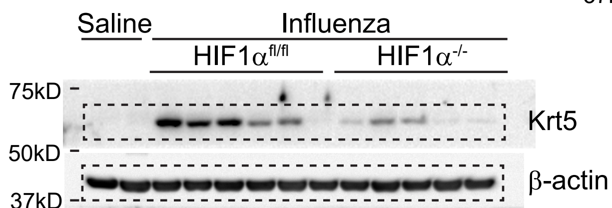

Supplementary Figure 8 Continued

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Supplementary Tables Legends

Supplementary Table 1 Top 100 differentially expressed genes between normal and diseased human AEC2s (HTII-280^{pos}).

Supplementary Table 2 Top upstream regulators of human diseased versus normal AEC2s (HTII-280^{pos}) from IPA® analysis.

Supplementary Table 3 Expression of hypoxia signature plus *HES1*, makers of basal cells and AEC2s in normal and diseased human AEC2s (HTII-280^{pos}).

Supplementary Table 4 Top 100 differentially expressed genes in Group I-V cells.

Supplementary Table 5 Expression of top differentially expressed genes and motility genes in Group I, III, IV and V cells.

Supplementary Table 6 Upregulated and downregulated genes shared by activated LNEPs and Group IV hypoxic AEC2s.

Supplementary Table 7 Top upstream regulators of the 102 common genes from IPA® analysis (see excel).

Supplementary Table 8 Pathway analysis of the 102 common genes from IPA® analysis (see excel).

Supplementary Table 9 Diseases and functions affected by the 102 common genes from IPA® analysis (see excel).

Supplementary Table 10 Primers.

Supplementary Table 11 Statistics Source Data.

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nature research

Corresponding Author: Harold A. Chapman

Date: Jun 5, 2017

Life Sciences Reporting Summary

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▸ Experimental design

1. Sample size

Describe how sample size was determined.	No statistical method was used to predetermine sample size. The sample size was determined on the basis of our prior knowledge of the variability of experimental output and on initial results or pilot experiments for each line of in vitro or in vivo experiments reported.

2. Data exclusions

Describe any data exclusions.	No data were excluded from analyses.

3. Replication

Describe whether the experimental findings were reliably reproduced.	All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.	The experiment were not randomized.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.	The investigators were not blinded to group allocation during data collection and/or analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
☐	☒	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Only commercially available or free software was used for data analysis. This includes Graphpad Prism, Fluidigm Singular, FlowJo, and Microsoft Excel.

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	Goat anti-CC10 antibody was a gift from Dr. Barry Stripp and HTII-280 antibody was a gift from Dr. Leland Dobbs.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	IF: rabbit anti-pro-SPC (1:3000; Millipore, #AB3786), goat anti-pro-SPC (1:2000; Santa Cruz, #SC-7706), rabbit anti-Krt5 (1:1000; Covance, #PRB-160P), chicken anti-Krt5 (1:1000; Covance, #SIG-3475), rabbit anti-ΔNp63 (1:100; Cell Signaling, #13109), rat anti-mouse integrin β4 (1:200; BD, #555721), goat anti-CC10 (1:10,000, a gift from Dr. B. Stripp), mouse anti-acetylated tubulin (1:500, Sigma, 6-11B-1), rat anti-E-cadherin (1:500, Invitrogen, #13-1900), rabbit anti-Hes1 (1:1000; Cell Signaling, #11988), goat anti-Scgb3a2 (1:100, R&D, AF3465). Western blots: HIF1α (1:500, R&D systems, #AF1935), pro-SPC (1:500, Millipore, #AB3786), Krt5 (1:1000, Covance, #PRB-160P), Cleaved Notch1 (1:1000, Cell signaling, #4147), E-cadherin (1:2000, BD, #610181), ?-actin (1:10000, Sigma-Aldrich, #A5441). To detect influenza A virus, unboiled lysates in 1mM DTT and 2% SDS were blotted with anti-Influenza A antibody (1:1000, Millipore, #AB1074). FACS: rat anti-mouse CD45 (1:200, BD, #553078), rat anti-mouse CD16/CD32 (1:200, BD, #553143), rat anti-mouse CD31 (1:200, BD, #553371), then incubated for 1 hr at 4°C with the following primary antibodies or viability dye diluted in DMEM (without phenol red) plus 2% FBS (Gibco): phycoerythrin (PE) or BV421-conjugated rat anti-mouse EpCAM (1:500; Biolegend, #563477, #563214), Alexa Fluor® 647 or PE-conjugated rat anti-mouse integrin β4 (1:75; BD, #553745), fixable viability dye eFluor® 780 (1:2000, eBioscience), Goat anti-pro-SPC (1:500; Santa Cruz, #SC-7706), BV421 rat anti-mouse EpCAM, Alexa Fluor® 488 donkey anti-goat IgG (1:2000, ThermoFisher, #A-11055). Validation for species and application was from manufacturer website.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	No eukaryotic cell line was used.
b. Describe the method of cell line authentication used.	No eukaryotic cell line was used.
c. Report whether the cell lines were tested for mycoplasma contamination.	No eukaryotic cell line was used.
d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

HIF1αfl/fl, Shh-Cre, Krt5-CreERT2, Sox2-CreERT2, β-cateninloxEx3, CC10-CreERT, FoxJ1-CreERT2, p63-CreERT2, Ub-GFP, SPC-CreERT2, and Ai14-tdTomato mice were used in the study and they were all previously described. For all experiments, 6–8 week old animals of both sexes were used in equal proportions.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involved human research participants. All human samples are non-identified, otherwise discarded tissues.

nature research

Corresponding Author: Hal Chapman

Date: Jun 6, 2017

Flow Cytometry Reporting Summary

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Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	Lung epithelial cells were obtained by Dispase digestion of primary tissue, exactly as described in Methods.
6. Identify the instrument used for data collection.	All data was collected on either FACSAria II or LSR II.
7. Describe the software used to collect and analyze the flow cytometry data.	Analysis was performed using FlowJo.
8. Describe the abundance of the relevant cell populations within post-sort fractions.	Purity checks were routinely performed by re-running ~100-500 sorted cells back through the sorter and ensuring they fell within appropriate gates.
9. Describe the gating strategy used.	As a general rule cells were gated on forward and side scatter corresponding to the known scatter profile of lung epithelial cells. Next they were gated as singlets, and then gated as live (viability dye negative) and CD45 negative. All positive gates were based off of the background fluorescence signal obtained by staining with an isotype antibody conjugated to the same fluorophore as the primary antibody.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒