Local lung hypoxia determines epithelial fate decisions during alveolar regeneration

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After influenza infection, lineage-negative epithelial progenitors (LNEPs) exhibit a binary response to reconstitute epithelial barriers: activating a Notch-dependent ΔNp63/cytokeratin 5 (Krt5) remodelling program or differentiating into alveolar type II cells (AEC2s). Here we show that local lung hypoxia, through hypoxia-inducible factor (HIF1α), drives Notch signalling and Krt5pos basal-like cell expansion. Single-cell transcriptional profiling of human AEC2s from fibrotic lungs revealed a hypoxic subpopulation with activated Notch, suppressed surfactant protein C (SPC), and transdifferentiation toward a Krt5pos basal-like state. Activated murine Krt5pos LNEPs and diseased human AEC2s upregulate strikingly similar core pathways underlying migration and squamous metaplasia. While robust, HIF1α-driven metaplasia is ultimately inferior to AEC2 reconstitution in restoring normal lung function. HIF1α deletion or enhanced Wnt/β-catenin activity in Sox2pos LNEPs blocks Notch and Krt5 activation, instead promoting rapid AEC2 differentiation and migration and improving the quality of alveolar repair.

The adult lung is a largely quiescent tissue but responds effectively to injury by activating stem/progenitor populations and promoting proliferation of surviving, mature lineages. Depending on the type and severity of cellular injury, different cell types are involved in repair1–3. A remarkable expansion of potentially regenerative ΔNp63pos/Krt5pos cells was observed in mouse lungs infected by murine-adapted H1N1 (PR8) influenza virus6. Lineage-tracing experiments showed that >80% of the expanded Krt5pos cells arise from LNEP populations in distal airways and probably alveoli2. These LNEPs proliferate and migrate dramatically to damaged sites to reconstitute the alveolar epithelium and promote recovery. Specific ablation of the newly expanded Krt5pos cells by diphtheria toxin resulted in prolonged hypoxemia, confirming involvement of these cells in the repair process2. Notch signalling is critical for activating this ΔNp63/Krt5 remodelling program; however, persistent Notch signalling prevents alveolar epithelial differentiation8, leading to cystic structures indicative of incomplete regeneration. Similar Krt5pos honeycombing cysts also exist in the lungs from patients with pulmonary fibrosis, suggesting that common mechanisms apply to injury responses in both mice and humans2.

In this study we explored the basis for activation and expansion of lung epithelial stem/progenitor cells observed in mice infected with H1N1 (PR8) influenza. Mice and humans infected with H1N1 influenza develop large areas virtually devoid of alveolar epithelial cells, requiring both expansion and migration of surviving AEC2s and more undifferentiated progenitors to restore alveolar epithelial barriers2,9,10. Studies here interrogate the underlying mechanisms and sources of epithelial progenitors that determine the alternative pathways of alveolar repair.

RESULTS

Influenza-activated Krt5pos cells are derived from p63pos lineage-negative progenitors

Our previous studies indicated that LNEPs are a Sox2pos heterogeneous population comprised of both cells expressing the stem cell transcription factor ΔNp63 and ΔNp63pos cells, although p63 expression was restricted to a minor LNEP fraction2,11. Transplantation studies suggested the possibility that these subtypes are biased toward either basal-like or AEC2 differentiation, respectively2. To clarify this, we infected tamoxifen-treated
Figure 1 Epithelial HIF1α deletion blocks alveolar Krt5 activation post H1N1 infection. (a) Krt5<sup>pos</sup> cells are completely traced (tdTomato<sup>pos</sup>) after influenza injury in p63-CreERT2/tdTomato mice. (b) Quantification of lineage tracing by manual cell counts in tissue sections. Data are mean ± s.e.m., n = 3 mice. (c) Krt5<sup>pos</sup> cells (red) invariably appear in hypoxic alveolar regions (hypoxprobe, green) after influenza injury in mice, although some Krt5<sup>pos</sup> hypoxic regions were also observed (n = 7 mice). (d) HIF1α protein accumulates in influenza-infected mouse lungs (n = 6 influenza, n = 3 saline, β-actin as loading control). (e) Alveolar Krt5<sup>pos</sup> expansion (green) (left) is largely blocked by epithelial HIF1α deletion (right). (f) Quantification of e. Data are mean ± s.e.m., n = 5 wild-type, n = 4 HIF1α<sup>−/−</sup> mice from two independent experiments. (g) Krt5 mRNA levels are reduced in HIF1α<sup>−/−</sup> mice. Data are mean ± s.e.m., n = 8 wild-type, n = 9 HIF1α<sup>−/−</sup> mice from 3 independent experiments. (h) Arterial oxygen saturation values obtained by pulse oximetry are greater in HIF1α<sup>−/−</sup> at the indicated times. (i) HIF1α<sup>−/−</sup> mice exhibit less excess lung water after influenza, indicative of improved barrier function. Data for h and i are mean ± s.e.m., n = 7 HIF1α<sup>−/−</sup>, n = 14 wild-type (2 Shh-Cre<sup>−/−</sup>, 12 C57BL6) or n = 13 wild-type for i (2 Shh-Cre<sup>−/−</sup>, 11 C57BL6) from two independent experiments. (j) AEC2 recovery/regeneration is improved in HIF1α<sup>−/−</sup> as judged by intracellular FACS for SPC<sup>pos</sup> cells as a fraction of total EpCAM<sup>pos</sup> cells. The percentage of EpCAM<sup>pos</sup> live cells is unchanged in HIF1α<sup>−/−</sup> and wild-type mice. Data are mean ± s.e.m., n = 3 HIF1α<sup>−/−</sup>, n = 4 wild-type mice from 2 independent experiments. Analysis is 11 days post-infection unless otherwise indicated. All P values were derived by unpaired two-tailed Student’s t-test, except in h, derived by Mann–Whitney. NS, not significant. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

p63-CreERT2 mice and determined that essentially all arising Krt5<sup>pos</sup> cells were lineage labelled (Fig. 1a,b and Supplementary Fig. 1a). Uninjured mice possessed p63-lineage-labelled cells scattered throughout airways, comprising <0.01% of all lung epithelial cells (Supplementary Fig. 1b,c). These rare cells must possess remarkable resistance to infection as well as migratory and proliferative capacity. However, the scarcity of p63<sup>pos</sup> LNEPs and their infrequent contribution to AEC2 regeneration (Supplementary Fig. 1a) suggest that additional p63<sup>pos</sup> Sox2<sup>pos</sup> LNEPs are a likely source of previously observed AEC2 differentiation following LNEP transplantation post...
infection, we examined the role of hypoxia, a likely consequence of extensive epithelial necrosis. Krt5pos areas were found exclusively in hypoxic regions identified by immunostaining of pimonidazole (hypoxyprobe) (Fig. 1c). HIF1α, a key mediator of cell responses to hypoxia, accumulated in the whole lung lysates 11 days post infection (Fig. 1d), confirming that regions of the lung are hypoxic.

We then explored the functional relationship between hypoxia and Krt5pos cell expansion/activation. Pan epithelial deletion of HIF1α (Shh-Cre; HIF1αfl/fl, designated as HIF1α−/−) results in viable mice, born at expected Mendelian ratios, and displaying no overt defects, while epithelial HIF1α messenger RNA was decreased by 99% (Supplementary Fig. 1d–f). Eleven days after influenza injury, the HIF1α−/− mice showed a dramatic decrease in alveolar Krt5pos cell expansion (Fig. 1e–j), as well as Krt5 protein (Supplementary Fig. 2a) and mRNA (Fig. 1g) levels, with no inhibition of airway Krt5pos expansion. Expansion of LNEPs was attenuated, demonstrated by a lack of integrin β4pos and p63pos expanding cells (Supplementary Fig. 2b,c). We next addressed the possibility that HIF1α−/− mice were less injured. Regardless of HIF1α deletion, viral protein was detected on day 4 and cleared by day 11 (Supplementary Fig. 2d). Both groups lost 20–30% weight (Supplementary Fig. 2e) and no difference was observed in the inflammatory cell counts or protein levels in bronchoalveolar lavage (Supplementary Fig. 2f,g). Comparable large regions depleted of alveolar epithelial cells developed in all mice (Supplementary Fig. 2h,i). HIF1α−/− mice are therefore injured at similar levels as wild-type mice, but lack alveolar Krt5pos cell expansion.

**HIF1α deletion promotes functional regeneration**

While injury levels were equivalent, epithelial HIF1α−/− mice regained weight more rapidly after the acute injury phase (Supplementary Fig. 2j). Remarkably, arterial oxygen saturation in HIF1α−/− mice also recovered more quickly, and barrier function was likewise improved as judged by less interstitial and/or alveolar edema (Fig. 1h,i and Supplementary Fig. 2k). Furthermore, AEC2s constituted a higher fraction of the total epithelium in HIF1α−/− mice after recovery (Fig. 1j). To address the source of the newly generated AEC2s, we deleted HIF1α specifically in Sox2pos cells, including both p63pos and p63pos LNEPs but not AEC2s, with Sox2-CreERT2/tomato mice (Fig. 2a). HIF1α deletion from the Sox2pos lineage resulted in strong attenuation of alveolar Krt5pos cell expansion after infection (Fig. 2b), similar to global epithelial HIF1α deletion (Fig. 1e). Instead, the majority of Sox2-traced alveolar cells exhibited AEC2 differentiation, as judged by location and SPC expression (Fig. 2a,c). Sox2-labelled AEC2s were much more proliferative than either endogenous AEC2s in the same injured regions or AEC2s in influenza-injured lungs of mice with HIF1α deletion specifically in pre-existing AEC2s (Fig. 2d,e). While HIF1α-driven Krt5pos expansion supports some degree of functional recovery, HIF1α deletion substantially improves lung function by re-directing airway progenitor responses toward rapid AEC2 expansion and migration, bypassing basal-like metaplasia entirely. Given the impact of HIF1α on the quality of epithelial recovery, we explored the mechanistic link between hypoxia and Krt5pos cell expansion.

**Hypoxia promotes Notch signalling and Krt5 expression in a HIF1α-dependent manner**

We validated that submersion cultures are hypoxic, as previously reported, in comparison with air/liquid interface demonstrated by hypoxyprobe staining and elevation of HIF1α targets (Fig. 3a). Hypoxia also promoted Notch target and Krt5 mRNA expression (Fig. 3a), suggesting that HIF1α regulates Notch activity. Primary LNEP-enriched cells (EpCAMpos integrin β4pos) expressed negligible Krt5 but its expression dramatically increased during the first 7–10 days in vitro, and continued over several passages alongside other basal cell genes (Fig. 3b). In contrast, LNEPs isolated from HIF1α−/− mice expressed lower levels of basal cytokeratins and Notch target genes, indicating that HIF1α is required for Notch activity. HIF1α−/− LNEPs formed fewer and smaller colonies (Fig. 3e and Supplementary Fig. 3a,b) but demonstrate increased SPC expression (Fig. 3c,d and Supplementary Fig. 3c) consistent with the suppressive effects of Notch signalling on alveolar differentiation.

Because HIF1α loss had no effect on total NICD1 level (Fig. 3f), we tested whether HIF1α could promote NICD DNA binding via chromatin immunoprecipitation (ChIP) experiments. Both HIF1α and NICD1 associated with CSL-binding sites (CBE) and HIF1α-binding sites (HRE) on Krt5, Hey1 and Hex5 promoters (Fig. 3g, and Supplementary Fig. 3d). NICD1 binding on DNA was abolished by HIF1α deletion. Multiple binding sites on the promoters were tested and all showed similar results.

To confirm Notch signalling as a driver of LNEP expansion, we analysed the transcriptomes of highly purified quiescent LNEPs (EpCAMpos β4pos CC10pos FoxJ1pos) (Supplementary Fig. 3e) and activated LNEPs (Krt5-CreERT2 traced cells 17 days post infection). Activated LNEPs showed high induction of Notch and HIF1α target genes and Krt5 expression (Fig. 3h). The loss of Notch activity in HIF1α-deficient cells correlates directly with the failure of Krt5pos cell expansion in vivo in HIF1α−/− mice. Thus, injury-induced hypoxia, through HIF1α, drives Notch activity by empowering NICD binding on Notch target gene promoters, and promoting LNEP differentiation towards Krt5pos basal-like cells. Of note, HIF1α deletion had little apparent effect on Notch regulation of airway epithelium.

**Stabilization of β-catenin promotes LNEP differentiation towards AEC2s**

Because Wnt signalling is important for the development of alveolar epithelium, we tested whether activating Wnt could promote LNEP differentiation into AEC2s in vivo, similar to HIF1α deletion. We crossed Sox2-CreERT2/tomato mice with β-catenin gain-of-function mice (β-cateninlox/lox) to stabilize β-catenin in Sox2pos LNEPs. β-catenin stabilization in Sox2-traced cells (tdTomato) led to ectopic SPC expression in the airways even without challenge, demonstrating that Wnt activity alone could induce a fate change even in quiescent adult progenitors (Supplementary Fig. 4a). Expression of SPC and expression of Scgb3a2 (a highly specific club cell marker) were largely mutually exclusive, and previous reports activating...
β-catenin in mature club cells resulted in no increase in SPC expression\textsuperscript{24}, indicating that most newly arising SPC\textsuperscript{pos} cells were derived from Sox2\textsuperscript{pos} LNEPs and not mature club cells. After infection, Sox2-traced cells expanded and differentiated primarily into Krt5\textsuperscript{pos} cells, whereas β-catenin stabilization dramatically switched differentiation from Krt5\textsuperscript{pos} to SPC\textsuperscript{pos}, leading to AEC2 expansion (Fig. 4a,b).

We further explored the impact of Notch and Wnt signalling on LNEP differentiation in vitro. Treatment with the GSK3β inhibitor CHIR99021 inhibited Notch and hypoxia target gene expression while activating Wnt target gene Axin2 expression (Fig. 4c), indicating that Wnt activity antagonizes Notch and hypoxia signalling, favouring AEC2 differentiation of LNEPs (Fig. 4d). ChIP experiments indicated that the inhibition occurs by prevention of NICD1 and HIF1α association on target promoters, without affecting NICD1 protein level (Fig. 4e,f and Supplementary Fig. 4b). We established clonal cultures of fluorescently tagged LNEPs and observed multiple instances of Krt5\textsuperscript{pos}
and SPCpos cells in a single-colour clone, indicating that both cell types could arise from a single cell (Fig. 4g and Supplementary Fig. 4c), although some colonies were homogeneously Krt5pos or SPCpos. Likewise, p63neg LNEPs isolated from tamoxifen-treated p63-CreERT2 mice demonstrated ~10% recombination after one culture passage following addition of 4OHT (Supplementary Fig. 4d), confirming differentiation of p63neg to p63pos cells. Most p63neg LNEPs in culture expand as either undifferentiated cells or as SPCpos cells, the latter significantly enhanced by Wnt activation (Fig. 4d and Supplementary Fig. 4d). Therefore, stabilization of β-catenin blocks Notch and hypoxia signalling, attenuates Krt5 activation, and promotes AEC2 expansion after influenza infection. Interestingly, while Krt5pos cells in vitro arise from p63pos LNEPs (Supplementary Fig. 4d), following influenza infection, activated Krt5pos cells in vivo are derived virtually entirely from rare LNEPs already expressing p63 (Fig. 1a), suggesting that p63neg LNEPs in vivo are intrinsically slower to differentiate to Krt5pos cells or rendered dysfunctional by H1N1 infection.

Deleting HIF1α or stabilizing β-catenin does not alter LNEP differentiation after full Notch/Krt5 activation.

To investigate the possibility of LNEP transdifferentiation to AEC2s after Krt5 activation, we crossed Krt5-CreERT2; tdTomato mice with either HIF1α-/- or β-catenin(ΔN) mice and treated with tamoxifen 7–10 days after injection to delete HIF1α or stabilize β-catenin specifically in activated LNEPs. Neither blocking HIF1α nor activating Wnt signalling altered Krt5pos cell fate: Krt5 traced cells did not give

Figure 3 HIF1α drives Notch signalling in vitro and in vivo. (a) LNEPs in subculture after infection with HIF1α (green) in comparison with LNEPs in air/liquid interface culture. Subculture results in upregulation of hypoxia and Notch target genes as well as Krt5. Data are mean ± s.e.m., n = 4 independent experiments. (b) Krt5 and Vegfa mRNA increase with time (passage) in cultured LNEPs. P0, primary cells, P1–P3, passages. n = 2 independent experiments. (c) HIF1α-deficient LNEPs exhibit reduced Krt5 and increased SPC expression as measured by the percentage of positive cells in culture. WT, wild-type; HIF, HIF1α−/−. (d) Expression levels of basal cytokeratins and Notch target genes are similarly reduced in HIF1α−/− LNEPs, in contrast to elevated SPC expression. In c, d, data are mean ± s.e.m. from n = 3 independent experiments. (e) Representative images of P1 colonies (primary cells cultured for 1 week) showing smaller colony size, reduced Krt5 and increased SPC expression at 15 days post infection. (f) Representative blots of WT colonies (primary cells cultured for 1 week) showing expression of HIF1α-deficient LNEPs culture. (f) Representative blots of three independent experiments showing that the NICD1 protein level in LNEPs is not altered by HIF1α deletion in cultured LNEPs. (g) HIF1α and NICD1 associate with both CSL-binding element (CBE) and HIF-responsive element (HRE) on the Krt5 and Hey1 promoters by ChIP. HIF1α deletion completely prevents NICD1 association on promoter DNA. See qPCR quantification as well as Hes5 promoter analysis from three independent experiments in Supplementary Fig. 3d. (h) RNA-Seq results showing that activated LNEPs (Krt5-CreERT2 traced cells 17 days post infection) have higher Krt5, Notch and hypoxia target gene expression as compared with LNEPs from uninfected mice (n = 5 mice per group). P values derived by unpaired two-tailed Student’s t-test, except in a, derived by one-sample t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Figure 4 Stabilization of β-catenin promotes LNEP differentiation towards an alveolar fate. (a) Krt5pos cells and to a lesser degree SPCpos cells are traced by Sox2-CreERT2 in response to influenza injury (left). Stabilization of β-catenin in Sox2-expressing cells prior to injury by tamoxifen administration results in a dramatic increase in traced SPCpos cells and concurrent decrease in Krt5pos cells (right). (b) Quantification of the percentage of the Sox2-CreERT2 traced cells expressing Krt5 (bottom) or SPC (top) in injured alveolar areas. Data are mean ± s.e.m., n = 4 with β-catenin stabilization, n = 6 without (same mice from Fig. 2c) from two independent experiments. (c) CHIR99021 treatment phenocopies HIF1α deletion (see Fig. 3d), reducing expression of Notch and HIF1α target genes and basal cytokeratins while increasing SPC and Axin2 by qPCR analysis. (d) Wnt agonism of LNEPs in vitro decreases the frequency (%) of Krt5pos cells with a concurrent increase of SPCpos cells in cytospin analysis. In (d), data are mean ± s.e.m. from n = 3 independent experiments. (e) CHIR99021 treatment further mirrors HIF1α deletion in preventing association of NICD1 and HIF1α with CBE and HRE sites on the Krt5 and Hey1 promoters as determined by ChIP. See qPCR quantification as well as Hes5 promoter analysis from three independent experiments in Supplementary Fig. 4b. (f) Representative blots of three independent experiments showing that the NICD1 protein level is not affected by β-catenin stabilization. (g) LNEPs isolated from ubGFP (GFP pos) and mTmG (tdTomato pos) were mixed in equal ratios and cultured resulting in predominantly single-colour colonies (clones). Representative images of tdTomato pos colonies demonstrate SPCpos and Krt5pos cells in single clones whereas most colonies exhibit uniform SPC expression when treated with GSK3β inhibitor CHIR99021 (2 μM). Krt5 is pseudocoloured green for visualization since these colonies were uniformly tdTomato pos. (h) Schematic model of Sox2pos LNEP activation in mouse lungs in response to severe injury. HIF1α/Notch promotes Krt5pos basal-like cell expansion from p63pos LNEPs. Wnt/β-catenin signalling antagonizes hypoxia and Notch signalling and promotes AEC2 expansion from p63pos LNEPs. LNEPs that upregulate p63 can still respond to Wnt signals to generate AEC2s (see Supplementary Fig. 4d). P values were derived by unpaired two-tailed Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Fig. B.

rise to AEC2s two weeks post tamoxifen, retaining Krt5 expression and Notch activity (Supplementary Fig. 5). In the context of severe influenza infection, it appears that once the Notch/Krt5 program is activated, signals that redirect initial LNEP differentiation become ineffective in inducing transdifferentiation of Krt5-committed cells.

Krt5pos expansion represents a common response to major lung injury in both mice and humans

As hyperactive Notch/Krt5pos cysts exist in lungs from patients with pulmonary fibrosis, we examined whether the expansion of Krt5pos cells is a common response to major injury. In lung tissue obtained from six patients who succumbed to H1N1 influenza A virus there was widespread destruction of parenchymal cells and three patient samples evidenced an extensive reparative response (Fig. 5a,b). Large areas were devoid of SPCpos AEC2s and comprised instead of Krt5pos cells that appear flattened and cover much of the alveolar surfaces, reminiscent of LNEP expansion in influenza-infected mouse lungs. In contrast to mice, some areas of affected lung contained numerous Krt5pos cells that were also clearly positive for SPC, suggesting either differentiation of Krt5pos cells toward AEC2s or transdifferentiation of AEC2s toward basal-like cells. This was paralleled in sections from ARDS (acute respiratory distress syndrome) lungs (Fig. 5c) and by prior observations in scleroderma in which SPCpos/Krt5pos alveolar cells were observed. We conclude that as in murine influenza injury, human stem/progenitor cells demonstrate robust attempts at re-establishing alveolar epithelial barriers with Krt5pos cells. Although variable, HIF1α protein was also increased in all idiopathic pulmonary fibrosis (IPF) samples relative to normal lungs, consistent with reports of hypoxia signalling in IPF25,26 (Fig. 5d). To further investigate the relationship between hypoxia and Krt5pos cell expansion in tissue...
Evidence that Notch and hypoxia regulate human lung epithelial repair processes

We performed single-cell RNA-Seq using the Fluidigm C1 to analyse the transcriptomes of AEC2s (HTII-280pos, of which 98% express SPC) and basal-enriched cells (integrin α6pos HTII-280neg, of which 50–70% express Krt5) (Supplementary Fig. 6). We sequenced between 25 and 72 single cells from normal lungs as well as lungs from patients diagnosed with scleroderma, dyskeratosis congenita and IPF, all fibrotic diseases despite differing etiologies. Principal component analysis (PCA) and whole-genome unsupervised hierarchical clustering clearly separated AEC2s from normal and fibrotic lungs (Supplementary Fig. 7a). The Notch targets HES1 and SCGB3A2 were among the most differentially expressed genes in diseased versus normal populations, corroborating our findings in fibrotic human lungs (Supplementary Table 1). Furthermore, HIF1α stands out as a top upstream regulator in the diseased AEC2s based on ingenuity pathway analysis (IPA, QIAGEN, www.qiagen.com/ingenuity) (Supplementary Table 2).

We generated a hypoxia-induced gene expression signature by combining three published hypoxia signatures (IPA HIF1α pathway). Hierarchical clustering based on the hypoxia signature plus Notch target HES1, KRT5 and AEC2 markers showed three distinct populations: normal AEC2s (Groups I and II), pathologic AEC2s with a hypoxia signature and high HES1 (Group III), and pathologic AEC2s with hypoxia targets and HES1, but decreased surfactants and upregulated basal cytokeratins (Group IV) (Fig. 6a–c and Supplementary Table 3). Adding normal basal-enriched cells (Group V) demonstrated that Group IV was transcriptionally more similar to basal-enriched cells than normal AEC2s. Groups I and II contained nearly all cells isolated from normal lungs that do not clearly segregate based on unbiased clustering, suggesting that the mild hypoxia target expression in Group II may be a technical artefact (Fig. 6d and Supplementary Table 4). Adding 27 AEC2s from IPF lungs placed most IPF cells in Group IV (Supplementary Fig. 7b). Clustering based on the entire transcriptome except the hypoxia signature and lineage markers resulted in nearly identical clustering, validating the hypoxia signature in predictive categorization and demonstrating that meaningful whole-transcriptome (phenotypic) changes occur coordinately with hypoxia signalling (Supplementary Fig. 7c).

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A subset of human alveolar epithelial cells, distal airway cells, and murine LNEPs share a migratory gene expression profile

Activated murine LNEPs and Group IV hypoxic AEC2s shared 102 upregulated genes and 25 downregulated genes (Fig. 7a and Supplementary Fig. 7c and Supplementary Table 6). IPA analysis of the common upregulated genes indicates that HIF1α is a top upstream regulator (Supplementary Table 7) and cell movement/invasion and proliferation are the top two processes affected (Supplementary Tables 8, 9). Importantly, normal human distal airway basal-enriched cells (Group V) appear inherently primed by expression of the motility-linked genes induced by hypoxia/Notch in AEC2s (Supplementary Fig. 7c).

We therefore compared motility of human basal-enriched cells with normal human AEC2s. Basal-enriched cells and activated LNEPs were highly motile in vitro in comparison with AEC2s (Fig. 7b). We further analysed the function of two of these motility genes, (c) Representative cells and genes of the four groups. (d) Whole-genome PCA analysis of the four groups plus normal basal-enriched cells (HTII-280™, Group V) demonstrates progressive evolution to basal cell-like expression profiles in diseased AEC2s. (e) The expression pattern of the top 20 up- and downregulated genes derived from ANOVA analysis of Group IV versus I, showing the progressive transition from Group I to Group IV, which is very similar to Group V basal-enriched cells. The average FPKM values of the indicated genes averaged within each group (I–V) are displayed in the heatmap. A subset of human alveolar epithelial cells, distal airway cells, and murine LNEPs share a migratory gene expression profile

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Activated murine LNEPs and Group IV hypoxic AEC2s shared 102 upregulated genes and 25 downregulated genes (Fig. 7a and Supplementary Fig. 7c and Supplementary Table 6). IPA analysis of the common upregulated genes indicates that HIF1α is a top upstream regulator (Supplementary Table 7) and cell movement/invasion and proliferation are the top two processes affected (Supplementary Tables 8, 9). Importantly, normal human distal airway basal-enriched cells (Group V) appear inherently primed by expression of the motility-linked genes induced by hypoxia/Notch in AEC2s (Supplementary Fig. 7c).

We therefore compared motility of human basal-enriched cells with normal human AEC2s. Basal-enriched cells and activated LNEPs were highly motile in vitro in comparison with AEC2s (Fig. 7b). We further analysed the function of two of these motility genes, (c) Representative cells and genes of the four groups. (d) Whole-genome PCA analysis of the four groups plus normal basal-enriched cells (HTII-280™, Group V) demonstrates progressive evolution to basal cell-like expression profiles in diseased AEC2s. (e) The expression pattern of the top 20 up- and downregulated genes derived from ANOVA analysis of Group IV versus I, showing the progressive transition from Group I to Group IV, which is very similar to Group V basal-enriched cells. The average FPKM values of the indicated genes averaged within each group (I–V) are displayed in the heatmap.
hypoxia/Notch-activated progenitors (Krt5<sup>pos</sup> cells) activate hypoxia/Notch signalling and a motile phenotype in response to major injury. (a) Overlap between mouse activated LNEPs (Krt5<sup>pos</sup> cells) versus quiescent LNEPs and human hypoxic AECs (Group IV versus I) identifies pathways strongly implicated in cell movement. Average FPKM values of human cells (Group I, IV and V), and mouse quiescent and activated LNEPs from RNA-Seq are indicated in the heat map (right) for 30 of these 102 genes directly implicated in migration. IPA analysis of these 102 genes implicates cell movement and cell proliferation as major cellular processes affected, with at least 30 reported to promote cell migration. (b) Human Group V basal-enriched cells are strikingly motile in a transwell assay, similar to activated LNEPs. Cells that migrated through the pores to the bottom of the insert were stained and quantified. Data are mean ± s.e.m. from n = 3 independent experiments. (c) Two tyrosine kinases identified from the common 102 genes, AXL and EPiHA2, are functionally important for LNEP migration. Activated LNEPs treated with AXL- and EPiHA2-specific inhibitors (3 μM R428 and 1 μM ALW-II-247) show compromised motility in wound closure assays. Relative wound area at 24 h as compared with 0 h is graphed as mean ± s.e.m. from n = 3 independent experiments. P values were derived by unpaired two-tailed Student’s t-test. (d) Schematic summary of a common, hypoxia-mediated epithelial progenitor response. Following major lung injury, local hypoxia promotes Notch signalling and a Krt5<sup>pos</sup>/SPC<sup>pos</sup>remodelling program in quiescent p63<sup>pos</sup>LNEPs via HIF1α, leading to robust migration, squamous metaplasia, and the ultimate formation of dysplastic alveolar barriers. However, Wnt activity prior to Krt5 activation favours p63<sup>pos</sup>LNEP expansion and differentiation toward AEC2s, leading to normal alveolar epithelial repair. The appearance of Krt5<sup>pos</sup>/SPC<sup>pos</sup>cells in several human disease/injury settings further suggests an intermediate response that may result in concurrent dysplastic and appropriate alveolar repair.

**Figure 7** Both human and mouse lung epithelial progenitor cells activate hypoxia/Notch signalling and a motile phenotype in response to major injury. (a) Overlap between mouse activated LNEPs (Krt5<sup>pos</sup> cells) versus quiescent LNEPs and human hypoxic AECs (Group IV versus I) identifies pathways strongly implicated in cell movement. 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 30 of these 102 genes directly implicated in migration. IPA analysis of these 102 genes implicates cell movement and cell proliferation as major cellular processes affected, with at least 30 reported to promote cell migration. (b) Human Group V basal-enriched cells are strikingly motile in a transwell assay, similar to activated LNEPs. Cells that migrated through the pores to the bottom of the insert were stained and quantified. Data are mean ± s.e.m. from n = 3 independent experiments. (c) Two tyrosine kinases identified from the common 102 genes, AXL and EPiHA2, are functionally important for LNEP migration. Activated LNEPs treated with AXL- and EPiHA2-specific inhibitors (3 μM R428 and 1 μM ALW-II-247) show compromised motility in wound closure assays. Relative wound area at 24 h as compared with 0 h is graphed as mean ± s.e.m. from n = 3 independent experiments. P values were derived by unpaired two-tailed Student’s t-test. (d) Schematic summary of a common, hypoxia-mediated epithelial progenitor response. Following major lung injury, local hypoxia promotes Notch signalling and a Krt5<sup>pos</sup>/SPC<sup>pos</sup>remodelling program in quiescent p63<sup>pos</sup>LNEPs via HIF1α, leading to robust migration, squamous metaplasia, and the ultimate formation of dysplastic alveolar barriers. However, Wnt activity prior to Krt5 activation favours p63<sup>pos</sup>LNEP expansion and differentiation toward AEC2s, leading to normal alveolar epithelial repair. The appearance of Krt5<sup>pos</sup>/SPC<sup>pos</sup>cells in several human disease/injury settings further suggests an intermediate response that may result in concurrent dysplastic and appropriate alveolar repair.
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS
This work was supported by NIH grants R01 HL128484, U01 HL110154, U01 134766, and a sponsored research agreement with Biogen Idec (H.A.C.). A.E.V. was supported by T32 HL007185-36, F32 HL117600-01, and K99 HL131817. M.A.M. and J.E.G. were supported by NHLBI grant R37 HL51856 and R37HL57156. J.X. was supported by NIH grants R01 CA112403 and R01 CA193455 and CRPIT grants R0120732-P5 and RP150197. J.M.S. was supported by NIH grant R01 HL04376. We thank M.A.M. and P. Wolfers at the UCSF Interstitial Lung Disease Blood and Tissue Repository for procuring non-donor and diseased lungs, respectively. We also thank J.E.G. for providing influenza PR8 virus and technical assistance, B. Sennino for hypoxyprobe and technical advice, M. Zhang for CHIR99021 and technical advice, R. Lao of the Institute for Human Genetics core facility, UCSC, for assistance with RNA-sequencing, and D. A. Denison and S. Zaki of the CDC for lung tissue slides obtained from patients with H1N1 influenza.

AUTHOR CONTRIBUTIONS
Conceptualization: Y.X., H.A.C., A.E.V.; investigation: Y.X., T.K., A.N.B., J.X., D.-K.L., Y.W., J.R.J., J.M.S., H.A.C., J.E.G., M.A.M., A.E.V.; formal analysis: Y.X., I.H.D., A.E.V.; writing: Y.X., H.A.C., A.E.V.; supervision: H.A.C.; funding acquisition: H.A.C., A.E.V.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3580
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METHODS

Animals and treatment All animal procedures were approved by the Institutional Animal Care and Use Committee of UCSF and all animal experiments were done in compliance with ethical guidelines and the approved protocols. HIIFiuCreERT2 (ref. 39), Shh-CreERT2 (ref. 40), Krt5-CreERT2 (ref. 41), Sox2-CreERT2 (ref. 42), β-catenin-loxEx3 (ref. 43), CC10-CreERT (ref. 44), FoxJ1-CreERT2 (ref. 45), p63-CreERT2 (ref. 46), Ub-GFP (ref. 47), SPC-CreERT2 (ref. 48), and Ai14-tDTomato (ref. 49) mice were previously described. For all experiments, 6–8-week-old animals of both sexes were used in equal proportions. All animal studies utilized a minimum of 3 mice per group.

For influenza infection, mice were administered 280 FFU of Influenza H1N1 (PB8) intranasally (survival rate >90%). Briefly, PB8 virus dissolved in 30 μl of PBS was pipetted onto the nostrils of heavily anesthetized mice (agonal breathing), whereupon mice aspirated the fluid directly into their lungs.

For all animal studies, no statistical method was used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Lineage tracing For analysing the lineage fate of Sox2-expressing cells, a single dose of 0.25 mg g⁻¹ body weight tamoxifen dissolved in 50 μl corn oil was administered i.p. one week before PB8 infection. For HIIFiu deletion in Sox2⁺ cells, three doses of tamoxifen were utilized to ensure complete depletion. To trace Krt5⁺ cells, a single dose of 0.125 mg g⁻¹ tamoxifen was administered i.p. at day 7 to 10 post-PB8 infection when Krt5⁺ cells were abundant. To purify LNEPs by excluding club cells and multi-ciliated cells, five doses of 0.25 mg g⁻¹ body weight tamoxifen were administered to CC10-CreERT; FOXJ1-CreERT2; Ai14-tDTomato mice i.p. two weeks before euthanizing the mice to maximize the recombination. To determine the cell of origin for post-influenza Krt5⁺ cells, p63-CreERT2/tdTomato mice were administered five doses of 0.25 mg g⁻¹ body weight tamoxifen. We waited 6 weeks for tamoxifen clearance and then infected as described above. Background (tamoxifen-independent) recombination in p63-CreERT2 mice is <1%.

Tissue hypoxia detection To detect hypoxic regions in tissue, mice received pimonidazole hydrochloride (60 mg kg⁻¹ body weight i.p., Hypoxprobe Plus kit) 1 h before euthanization. The lungs were infected with 1% paraformaldehyde (PFA), incubated with 30% sucrose overnight and embedded with OCT. Sections (80 μm thick) were cut and air-dried overnight on Superfrost plus slides (Fisher Scientific).

The sections were stained with FITC-conjugated anti-pimonidazole mouse IgG (1:100, 4.3.11.3 mouse FITC-MAH, Hypoxprobe Plus kit) together with rabbit anti-Krt5 (1:1,000; Covance, no. PR-160P) overnight in PBS plus 1% BSA, 5% sodium azide (Sigma), followed by secondary antibody and DAPI staining. The slides were mounted with Prolong (Invitrogen) and imaged with a Yokogawa spinning-disc confocal (UCSF Biological Imaging Development Center).

Saturated phosphatidylcholine (SatPC) measurement SatPC was isolated from lungs of newborn animals. Lungs were first weighed, then placed in ice-cold 0.9% saline and pulse sonicated. Total lipids were extracted as previously described (ref. 50). SatPC was isolated as previously described (ref. 51) and normalized to lung weight.

Pulse oximetry Arterial oxygen saturation was measured using the MOUSEOX Pulse Oximeter system (Starr Life Science). Mice were shaven prior to infection and the CollarClip utilized so that measurements could be taken without anesthesia. Measurements were taken for at least 3 min per mouse after establishing the first successful reading. Error-free measurements were then averaged for each time point for each mouse.

Excess lung water analysis This analysis was performed as previously described52. Briefly, the lungs were removed, weighed and homogenized (after addition of 1 ml distilled water). The blood was collected through right-ventricle puncture. The homogenate was weighed and a fraction was centrifuged (10,000 g, 8 min) for assay of haemoglobin concentration in the supernatant. Another fraction of homogenate, supernatant, and blood were weighed and then desiccated in an oven (60 °C for 24 h). Calculations of water fraction, blood volume, water volume, whole lung dry weight, and ultimately excess lung water calculations were performed using equations previously described52. The mice used for this analysis were the same cohort used for pulse oximetry.

Tissue preparation and immunofluorescence (IF) Freshly dissected mouse lungs were inflated with 4% PFA plus 50% OCT dissolved in PBS for 1 h at room temperature. After fixation, the lungs were washed with PBS and incubated with 30% sucrose plus 50% OCT overnight, then embedded in OCT and frozen the following day. Cryosections (7 μm thick) were cut and fixed for an additional 5 min in 4% PFA at room temperature, then incubated with 0.1% sodium borohydride (Sigma-Aldrich) in PBS to reduce aldehyde-induced background fluorescence for 3 × 10⁻⁵ min intervals, and subsequently blocked and stained in PBS plus 0.1% BSA (Albemarle), 5% nonimmune horse serum, 0.1% Triton X-100, and 0.02% sodium azide.

Cryosections were fixed with 4% PFA for 5 min before cytospin and then stained as above. Cell grown on Matrigel were fixed in IHC Zinc Fixative (BD) for 5 min and subsequently blocked and stained in TBS-based blocking buffer, as the zinc fixative reacts with phosphatase.

The following antibodies were used for IF: rabbit anti-pro-SPC (1:3,000; Millipore, no. A8376), goat anti-pro-SPC (1:2,000; Santa Cruz, no. SC-7706), rabbit anti-Krt5 (1:1,000; Covance, no. PR-160P), chicken anti-Krt5 (1:1,000; Covance, no. SIG-13475), rabbit anti-Np63 (1:100; Cell Signaling, no. 13109), rat anti-mouse integrin β4 (1:200; BD, no. 555721), goat anti-CC10 (1:10,000, a gift from B. Stripp, Cedars-Sinai Medical Center, USA), mouse anti-acetylated tubulin (1,500, Sigma, 6-11B-1), rat anti-E-cadherin (1,500;Invitrogen, no. 13-1900), rabbit anti-Hes1 (1:1000; Cell Signaling, no. 11988), goat anti-Scl6a2 (1:140, R&D, AF3465).

Quantification of immunofluorescence. To quantify Krt5⁺ or AECA-depleted area, mosaic images covering the whole lobes were generated from multiple ×10 fields captured on a Zeiss Axiosimager upright fluorescent microscope and tiled using 10% image overlap by Axiovision 4.7 software. The Krt5⁺ or SPC⁺ areas and total areas were measured using outline spline in the measure menu of Axiovision 4.7. At least 4 sections, each section containing 2–3 individual lobes and separated by over 300-μm depth were quantified for each mouse (n ≥ 5 mice).

To line trace 6-11B-1-expressing cells into alveolar cell fate, all the traced cells in injured alveolar area were captured at ×20 magnification and counted using events in the measure menu of Axiovision 4.7. On average, ≥3,000 cells were counted per section. For each mouse, we quantified at least 2 sections, each containing 2–3 individual lobes and separated by over 300-μm depth (n ≥ 4 mice).

To quantify p63-CreERT2 trace of Krt5⁺ cells post-influenza, images were captured as above and cells were counted from >4 sections per mouse and included at least 3 individual lobes. In total, ≥3,200 cells were scored from n = 5 mice.

To quantify Krt5⁺ or SPC⁺ cell percentage in cultured LNEPs accurately, the colonies were dissociated from Matrigel and digested into single-cell suspension as described below, followed by cytospin and staining. Stained cells were captured at ×20 and at least 600 cells were counted per condition.

Mouse lung epithelial cell isolation and flow cytometry. Mouse lung epithelial cells were isolated as previously described6. For FACS analysis, single-cell preparations were negatively selected with biotinylated antibodies: rat anti-mouse CD45 (1:200, BD, no. 553078), rat anti-mouse CD16/CD32 (1:200, BD, no. 553314), rat anti-mouse CD31 (1:200, BD, no. 553317), and then incubated for 1 h 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 CD3 (1:500; Biologend, no. 563547),rat anti-acetylated tubulin (1,500, Sigma, 6-11B-1), rat anti-E-cadherin (1,500, Invitrogen, no. 13-1900), rabbit anti-Hes1 (1:100; Cell Signaling, no. 11988), goat anti-Scl6a2 (1:140, R&D, AF3465).

Cell line identity. No cell lines were used in this study.

Mouse LNEPs culture. Mouse lung LNEPs were flow sorted by EpCAM⁺/CD45⁻ cells and cultured on Matrigel (BD) as follows. A 48-well plate or 8-well chamber slide was coated with 150 μl Matrigel per well, allowed to solidify at 37 °C, and then equilibrated with SABM (Lonza) for at least 30 min prior to cell plating. A total of 15,000 to 25,000 cells were plated in each well and maintained in SAGM (Lonza) supplemented with 5% charcoal-stripped FBS (ThermoFisher), 10 ng ml⁻¹ KGF (PGF-7, Peprotech), 10 μM Y 27632 (Sigma) and 50 ng ml⁻¹ murine noggin (Peprotech) for the first 2 days, and then replaced with SAGM supplemented with 5% charcoal-stripped FBS and 10 ng ml⁻¹ KGF with or without CHIR99021 (2 mM dissolved in dimethylsulfoxide (DMSO)) for an additional 5–7 days. Cells were passed once a week by incubation with 25 μl ml⁻¹ Dispase II (Roche) at 37 °C for
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DOI: 10.1038/ncb3580

in PBS at 37 °C to dissociate the colonies and obtain a single-cell suspension. The cells were then plated on Matrigel and maintained in SAGM supplemented with 5% charcoal-stripped FBS and 10 ng/ml KGF with or without CHIR99021 through additional passages. After that, they were gradually activated (expressed Krt5) over time in culture, as described above.

For air/liquid interface experiments, mouse LNEPs were isolated as described above and grown for a single passage in submersion conditions to establish the culture. Cells were then liberated and replated into continued Matrigel submersion culture or on Matrigel layered on top of a Transwell membrane exposed to air (COSTAR Transwell Permeable Support, no. 3413), cultured for 7 days, and then isolated as above for RNA. For hypoxia probe staining, 2 μM pimonidazole was added to culture media in each condition for 1 h on the seventh day after seeing. Cells were then washed thoroughly with TBS before fixation and stained as described above.

For LNEP isolation from p63-CreERT2/tdTomato mice, mice were treated with 5 doses of tamoxifen, and EpCAM+Krt5+ cells were flow sorted and cultured as normal. Wells were visually inspected to confirm that cultures only rarely contained endogenous p63-labelled (tdTomato+) cells. Labelling was induced by a 24 h pulse of 100 μM hydroxytamoxifen (4OH)T 24 h after replating for the second passage (~10 days after isolation), demonstrating induction of p63 expression in cells that were p63+/in situ (see Supplementary Fig. 4d).

Migration assay. Transwell assays were performed with Corning BioCoat Invasion Chambers (inserts coated with Matrigel, 8.0 μm pore size). Primary human basal- and AEC2s (80,000 and 200,000 respectively) were seeded on top of the inserts in SAGM with KGF, Noggin, and Y-27632 for 23 days for recovery, then washed with PBS and starved with SABM for 6 h before migration through Matrigel toward 1% FBS plus 50 ng ml−1 EGF in SAGM at 37 °C for ~66 h (with SABM on top of the inserts). Non-invaded cells and Matrigel were removed by swabbing. Invaded cells were fixed in methanol, stained with 1% crystal violet (Sigma-Aldrich), and imaged with a Zeiss AxioImager at ×10 magnification. Invaded cells were counted in random fields avoiding the edge of the insert.

Wound closure assays were performed using specific culture inserts (Ibidi, no. 80209). A culture insert was transferred to an individual well of a 24-well plate and the surface in the insert was coated with 1:20 diluted Matrigel for 2 h before cell seeding. Activated LNEPs were disaggregated as described above and resuspended in SAGM with KGF, Noggin and Y-27632 at a density of 0.7–1 million ml−1. Seventy microliter of cell suspension was seeded into each well of the insert. After cell attachment for about 24 h forming a confluent layer, the cells were pretreated with inhibitors for 1 h before removing the culture inserts, and then washed with PBS twice and incubated with fresh SAGM medium in the presence/absence of the AXL inhibitor R428 (Apexbio) or the Eph2A inhibitor ALW-II–41–27 (Apexbio) dissolved in DMSO. The cell migration into the defined cell-free gap (500 μm) was observed for 24 h under an inverted microscope (Nikon). Images of the cell-free gap were captured at 0 h and 24 h, and total cell-free area was quantified with Axiovision 4.7.

Chromatin immunoprecipitation (ChIP). ChIP experiments were performed following the Imprint Chromatin Immuno-precipitation protocol (Sigma). Expanded LNEP colonies were digested as described above, seeded on 1:20 diluted Matrigel-coated 10 cm dishes and harvested on reaching 80–90% confluence. Cells were crosslinked with 4% formaldehyde for 10 min followed by nuclei isolation, sonication to shear DNA into 100–500 bp fragments, protein/DNA complex pulldown and crosslink recoil reversal. One microgram of rabbit anti-Cleaved Notch1 (Cell Signaling, no. 4147) and goat anti-HIF1α (R&D Systems, no. AF1935) antibody were used for each IP. The precipitated DNA and input DNA was quantified by qPCR using specific primers on Krt5, Hey1 and Hey5 promoters.

Human tissues. All human normal and fibrotic tissue samples were obtained from UCSF Intrstitial Lung Disease Blood and Tissue Repository and are classified as Non-identifiable Otherwise Discarded Human Tissues, for which no consent/IRB approval is required. Slides from lung tissue blocks of non-identified human subjects with documented H1N1 influenza A infection 10–21 days after diagnosis and without secondary bacterial infection, as judged by PCR screening, were obtained from A. Denison at the Center for Disease Control (Atlanta).

Human lung epithelial cell isolation. Distal lung tissue was obtained and dissected into roughly 5 cm3 pieces. Tissue was washed in 500 ml sterile PBS for 10 min at 4°C at least two times, or until PBS no longer appeared obviously bloody. An additional 10 min wash was then performed with Hank's buffered saline solution (HBSS). Using autoclaved Kim Wipes, tissue was compressed to remove as much liquid as possible and further disected into 1 cm3 pieces. Sterile HBSS buffer containing 5 μM DII, 1 μg/ml penicillin/streptomycin was added to the small tissue pieces. Tissue rapidly takes up the digest solution at this point, becoming visibly engorged. Tissue was digested for 2 h at 37 °C and Fungizone (1:400) was added for the final 30 min of the digest. The digest solution was then stored overnight at 4 °C without further degradation of cells due to lack of Dispase activity at this temperature. The following day, the tissue in digestion solution was warmed to 37 °C and liquefied using an Osterizer 12 Speed Blender as follows: 5 s pulse, 5 s grate, and 5 s pulse again. The suspension was further glassed for 4 min 4 s 4 gauze, applying some compression to recover as much of the solution as possible. The cell suspension was sequentially filtered through 100 μm, 70 μm and 40 μm strainers. Finally, red blood cells were removed using Red Blood Cell Lysis Buffer (Sigma-Aldrich).

Single-cell RNA-Seq of human lungs. The cell preparations were stained as described for mouse cells with corresponding anti-human antibodies: HTII-280 (1:100, a generous gift from L. Dobbs, University of California - San Francisco, USA), anti-human/mouse CD49F (1:100, Biolegend, no. 313602), PE anti-human EpCAM (1:400, Biolegend, no. 324206), and anti-human CD45 APC-Cy7 (1:200, Biolegend, no. 304014). As judged by cytospin, all the SPC+ cells were captured by HTII-280 sorted. Single cells were captured on a small-sized (7–10 μm cell diameter) microfluidic mRNA seq chip (Fluidigm) using the Fluidigm C1 Single-Cell Auto Prep System. The downstream steps (lysis, cDNA synthesis/amplification, library preparation) were carried out following the Fluidigm C1 protocol, incorporating the Illumina Nextera XT DNA Sample Preparation Kit. Nextera libraries consisting of barcoded single-cell samples were pooled and sequenced on an Illumina HiSeq 2500 sequencer using high-output mode with 100 bp paired-end reads. This results in a sequencing depth of ~2–3 million reads per single-cell sample. Paired-end fastq files were aligned to the genome using the Tophat/Bowtie2 software28 and annotated using the Cufflinks package24 and the UCSC hg19 index (Illumina), followed by normalization using Cuffnorm. Cells with a fragment mapping rate below 50% and less than 1,000 genes sequenced (1 FPKM threshold) were filtered out. Genes that were not expressed at 1 FPKM in at least 3 cells were also removed prior to subsequent Fluidigm Singular analysis running in R, leading to ~13,000 genes. We successfully sequenced a total of 72 AEC2s and 23 basal-enriched cells from a normal lung, 49 AEC2s from scleroderma, 48 AEC2s from dyskeratosis congenita, and 27 AEC2s from IPF lung. Differential expression analysis from Singular is included in Supplementary Tables 1.3–6. Ingenuity pathway analysis (IPA, QIAGEN, www.qiagen.com/ingenuity) was performed on this differential expression data and is found in Supplementary Tables 2, 7–9.

RNA-Seq of mouse normal and activated LNEPs. Highly purified quiescent LNEPs (EpCAM+Krt5+CC10+FosI+) and activated LNEPs (Krt5-CreERT2 traced cells 17 days post infection) were flow sorted and RNA extracted using ReliaPrep RNA Tissue Miniprep kit (Promega). cDNA synthesis/amplification, library preparation and sequencing followed the same protocol used in Single Cell RNA-Seq.

Western blot analysis. Snap-frozen mouse lungs were ground into tissue powder and then lysed in the RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with Protease Inhibitor Cocktail, 1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium vanadate, 10 mM sodium fluoride, and Phosphatase Inhibitor Cocktail. The lysates were quantified using Pierce BCA protein assay kit (Thermo, no. 23225), normalized and blotted to HIF1α (1:500, R&D Systems, no. AF1935), pro-SPC (1:500, Millipore, no. AB3786), Krt5 (1:1000, Covance, no. PRB160P), Cleaved Notch1 (1:100, Cell Signaling, no. 4147), E-cadherin (1:2,000, BD, no. 610181), β-actin (1:10,000, Sigma-Aldrich, no. A5441). To detect influenza A virus, unboiled lysates in 1 mM dithiothreitol were electrophoresed on a 10% SDS-PAGE gel and blotted with anti-Influenza A antibody (1:1,000, Millipore, no. AB1074).

Bronchoalveolar lavage. After the trachea was exposed, a 20-G-catheter was inserted into the trachea for lavage. Cold PBS (1 ml) was instilled into the mouse lungs followed by gentle aspiration repeated three times. All of the bronchoalveolar lavage fluid was centrifuged and the supernatant was collected to measure total protein content using BCA assay. The cell pellet was resuspended in Red Blood Lysis Buffer and counted using a haemocytometer.

 Colony-formation assay. Ten thousand freshly sorted or cultured single LNEPs were plated onto a Matrigel-coated 8-well chamber slide and cultured for one week. Colonies were fixed with IHC Zinc Fixative and stained. Only colonies containing more than 5 nuclei were counted.

qRT-PCR analysis. Snap-frozen lungs were ground into tissue powder and RNA was isolated using the ReliaPrep RNA Tissue Miniprep kit (Promega). ReliaPrep RNA Cell Miniprep kit (Promega) was used to isolate RNA from freshly sorted cells or cultured cells. To isolate RNA from cultured LNEPs on Matrigel, colonies were dissociated from Matrigel using Dispase II first, and then the cell pellets were lysed following the Promega protocol. cDNA was synthesized using Superscript III (Invitrogen)
and assayed for gene expression using Faststart Universal SYBR green Master Mix (Roche). PCR reaction and analysis was run on Eppendorf Mastercycler ep realplex 2. HPRT, L19 and GAPDH were used as internal controls and all of the data were normalized by L19. All primer sequences are as listed in Supplementary Table 10.

Statistics and reproducibility. For single-cell RNA-Seq analysis, Fluidigm Singular software was used. All other statistical calculations were performed using Graphpad Prism. Variance for all group data is expressed as ±s.e.m. The statistical test used to determine significance for each experiment is stated in the corresponding figure legend. A P value less than 0.05 was accepted as significant. The investigators were not blinded to allocation during experiments and outcome assessment. All images are representative of at least three independent experiments or mice of the same genotype. Western blots are representative of multiple independent experiments, and unprocessed original scans of blots are displayed in Supplementary Fig. 8. No statistical method was used to predetermine sample size and experiments were not randomized. All experiments presented were reproducible and n for each experiment is presented in each figure legend.

Data availability. RNA-Seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession codes GSE83467 and GSE83501. Source data for the main and supplementary figures have been provided as Supplementary Table 11. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Supplementary Figure 1  p63<sup>pos</sup> 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<sup>pos</sup> LNEPs generate Krt5 metaplasia, they make only a minor contribution to AEC2 regeneration after influenza injury (insets). (b) Rare p63-CreERT2 traced (tdTomato<sup>+</sup>) 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/flfl 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<sup>pos</sup> EpCAM<sup>pos</sup>) 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<sup>pos</sup> live cells from a pool of 3 mice in each group. p values derived by unpaired two-tailed Student's t test.
Supplementary Figure 2 HIF1α−/− mice are injured at similar levels as wild-type mice, but lack alveolar Krt5pos cell expansion. (a) Representative blot showing Krt5 induction is inhibited by epithelial HIF1α deletion. (b-c) No large expansion of Dnpp63 (b) or integrin b4 (c) positive cells in the alveoli of HIF1α−/− mice, indicating alveolar Krt5pos 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=8 uninfect control 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−/−, 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.
**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 HIF1αfl/fl LNEPs in culture. WT, HIF1αfl/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 (FoxJ1neg CC10neg integrin $\beta_4$+) 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 (CC10pos) and multi-ciliated cells (acetylated-Tubulinpos) remaining unchanged.
Supplementary Figure 4 Stabilization of b-catenin inhibits Notch and HIF1α activity by blocking their DNA association. (a) b-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 HIF1α DNA binding on Krt5, Hey1 and Hes5 promoters are blocked by CHIR. The same control sample (LNEPs from HIF1αfl/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) p63neg LNEPs in vitro either remain undifferentiated, are activated into p63pos 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.
**Supplementary Figure 5** Deleting HIF1α or stabilizing β-catenin does not alter LNEP differentiation after full Notch/Krt5 activation. (a) HIF1α 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.
a Normal Human Lung Sort

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^{neg} 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.

Supplementary Figure 6
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<sup>pos</sup> 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<sup>pos</sup> 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<sup>pos</sup>) 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).
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.
**Supplementary Figure 8 Continued**

**Fig. 4e** DNA gel of CHIP

- **Krt5 promoter CBE site**
  - HIF IP
  - NICD IP
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir

- **Krt5 promoter HRE site**
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir

- **Hey1 promoter CBE site**
  - HIF IP
  - Nicd IP
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir

- **Hey1 promoter HRE site**
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir
  - Ctrl chir

**Fig. S2d western blot**

- **Saline**
- **Influenza d4**
- **Influenza d11**
  - HIF1α
  - HIF1α-/-
  - HIF1α-/- HIF1α-/-

**Fig. S2a western blot**

- **Saline**
- **Influenza**
  - HIF1α
  - HIF1α-/-
  - Krt5
  - β-actin

**Supplementary Figure 8 Continued**
Supplementary Tables Legends

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

Supplementary Table 2 Top upstream regulators of human diseased versus normal AEC2s (HTII-280pos) 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-280pos).

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.
# 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 (1:1000; 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-Sgcβ3a2 (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), β-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.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

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.
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 involve human research participants. All human samples are non-identified, otherwise discarded tissues.
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. ☑