

Peer Review Information

Journal: Nature Cell Biology

Manuscript Title: Release of Notch activity coordinated by IL-1 β signalling confers differentiation plasticity of airway progenitors via Fosl2 during alveolar regeneration

Corresponding author name(s): Joo-Hyeon Lee

Reviewer Comments & Decisions:

Decision Letter, initial version:

Date: 1st December 20 14:11:01

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Triggered By: Christine Weber

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Subject: Decision on Nature Cell Biology submission NCB-L44082

Message: *Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Joo-Hyeon,

Your manuscript, "Release of Notch activity coordinated by IL-1 β signalling confers cross-compartment differentiation plasticity during alveolar regeneration", has now been seen by 3 referees, who are experts in lung architecture, niches (referee 1); lung organoids (referee 2); and Notch signalling, lung development/disease (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to address the following key points:

(A) Additional experimental work should be included to strengthen the conclusion that Notch inhibition induces the conversion of secretory into AT2 cells, clarify the effects of Notch signalling on other cell types and resolve concerns noted by all three referees about cell identity and/or origins, unspecific markers and pharmacological approaches and a possible direct role of IL-1 β signalling.

Referee 1 notes:

"One major technical caveat that needs to be addressed is the fact that the Scgb1a1creERT used in this study also labels AEC2s during homeostasis (Rawlins et al., Cell Stem Cell, 2009), which the authors actually demonstrated in Figure 1i (~7-8% of SPC+ cells are Scgb1a1 Lin+ at homeostasis). Do the authors consider these alveolar SPC+/Scgb1a1 Lin+ cells as secretory AEC2s? They do not display any features of club cells other than scgb1a1 transcript, nor are they located at the bronchioalveolar ductal junction to be considered BASCs. Labeling endogenous AEC2s with Scgb1a1creERT confounds the interpretation of some results, as some phenotypes could be ascribed to the behavior of endogenous AEC2s rather than airway club cells differentiating into AEC2s. However, I do not believe this to be a fatal flaw, and perhaps the authors can address this with certain experiments to bolster their claims."

"2. For Figure 2 and 3 on lineage tracing of Scgb1a1 Lin+ cells with Notch manipulation, see comment about scgb1a1creERT tool above. The authors should demonstrate whether endogenous SPC+ cells expansion is potentially affected by Notch manipulation (e.g. does Notch inactivation also expand endogenous AEC2 pool), or consider additional experiments where AEC2s can be excluded for genetic lineage tracing or transplant studies. Furthermore, it has been reported that KRT5+ alveolar basal cells found in bleomycin injury are also derived from scgb1a1+ club cells in the airway (and not the scgb1a1+/spc+ cells in the alveoli), does Notch alteration affect Scgb1a1Lin+ cells that differentiate into KRT5+ alveolar basal cells, if any? Finally, does Notch manipulation also alter club cell differentiation into ciliated cell fate at the airway with bleomycin, or do you have evidence that AEC2 differentiation is already occurring at the airway? Clarifying the last two points would improve the resolution of how the secretory cell differentiation is altered in the setting of alveolar injury."

"4. Figure 4 presents very intriguing data that Notch ligand expression in ciliated cells are regulated by inflammation and modulates Club cell transdifferentiation. However, this hypothesis is still largely based on circumstantial evidence as presented. The data that IL1R1 deletion reduces Jag1/2 in ciliated cells, and the data that club cells transdifferentiates into AEC2s with ciliated IL1R1 deletion could be true-true but unrelated. It would be important to assess whether Notch activation in the club cells at the airway is altered by IL1R1 deletion in ciliated cells during injury. Additionally, in figure 4b, what is the cellular composition of Il1r1+ CD24+ cells (?% ciliated cells vs other cell types) vs. Il1r1+/CD24- cells? In figure 4E, what is missing is the effect of IL1R1 deletion in ciliated cells during PBS, which is important to determine whether there is tonic IL1 stimulation in the airways during homeostasis. Finally, does IL1b have an effect on club cell organoids that

might be independent of notch signaling?

5. Same point regarding the Scgb1a1creERT for the ATAC-seq analysis. To provide more assurance that the secretory AT2s are mostly derived from airway Scgb1a1+ cells (and not endogenous Scgb1a1+/SPC+ cells in the alveoli) in the ATAC-seq, authors should demonstrate whether there is an increase in GFP+/DsRed+ population (Figure 5b) after injury compared to homeostasis, to assess whether there is an increased proportion of GFP+ cells (secretory) that acquire AT2 fate (DSRed+). Furthermore, the demonstration that Fosl1 KD suppresses Sftpc expression alone does not prove Fosl1 is necessary for AEC2 fate. An alternative explanation is that Fosl1 might be repressing the fate of an alternative lineage (e.g. basal, ciliated, and etc.), and KD of Fosl1 results in increase in a non-AEC2 lineage derived from club cells. This should be interrogated by immunophenotyping organoids derived from DAPT treated and Fosl1 KD conditions. Do these organoids express no mature markers of differentiation? Or do they express alternative lineage markers that are neither secretory nor AEC2."

Referee 2 notes:

"1. Does interfering with the mechanism (be it at the IL1r, Notch or Fosl2 level) interfere with regeneration after bleomycin injury? In other words, this paper shows what can happen, not how important it is physiologically."

"2. If SCO were isolated from more proximal airway, would they still generate AT2 after Notch inhibition, or might there be heterogeneity in the capacity of secretory cells to do this? Would the cells capable of doing this be variant secretory cells?"

"4. In ED 4e, what are the dTom+CD24- cells? And why do they express so little IL1r if they are dTom+? What are those cells?"

"7. The finding that sAT2 and rAT2 appear different is interesting. How long are these sAT2 detectable after injury?"

Referee 3 notes:

"Specifically, the authors often refer to the repair-driving progenitor cell type as a club cell or secretory cell. In many cases, this cellular identity (and the language used to describe it, "club cell" or "secretory" cell) is based on expression of one (or no more than a few markers), such as CC10. While club cells certainly express CC10 and other markers, these markers are shared with other cells, most notably stem/progenitor cells that reside at the bronchial/alveolar junctions (BAJs). Numerous studies have clearly established that cells at this location express markers of both club cells and AE2 cells, thus displaying markers indicative of a dual lineage. An equally plausible, or more plausible model, compared to the authors' model, is that these dual lineage progenitor cells are the precursors that provide the repaired AE2 cells, and that the fate decision to AE2 is driven by loss of Notch signaling. Put another way, perhaps the "airway" organoids are really "BASC" organoids and Notch controls the fate between airway, alveolar and mixed.

One other general comment is that the authors do not consider that in some of their experiments, treatment with a gamma-secretase inhibitor before damage

would likely have converted nearly all of the club cells to ciliated cells, since both genetic and pharmacological inhibition of Notch induces a club-to-ciliated cell transdifferentiation (for example, see Pardo-Saganta et al., Nature 2015 and Lafkas et al., Nature 2015). Given the expected absence of the majority of club cells under such conditions (but not the loss of CC10+ cells at the BAJs), how do the authors explain the improved AE2 regeneration? This omission seems like a major issue that must be addressed."

"2. Figure 1 and related:

- a. Cells are described as "secretory" or "alveolar" when often the observation is simply CC10+ or SPC+. This is an oversimplification. Indeed, CC10 or SPC would also mark the dual-lineage progenitors noted above.
- b. The organoid system seems like an important technical advance. I would like to see more characterization, particularly of the state of Notch signaling. For example, in the three types of organoids (e.g., see panel f), where are Notch ligands and receptors expressed? Which cells show not just Notch receptor expression but, more importantly, Notch receptor activity, as evidenced by nuclear localization of the Notch ICD? One thing I am confused by...in the first (left-most) panel of Fig. 1f, almost all (if not all) of the cells in the airway organoids appear to be CC10 positive and therefore are described as secretory airway cells. In vivo, airway secretory cells (club cells) require Notch signaling for their fate to be maintained, and as the authors note later in their paper, the Notch signal is induced by Jag ligands expressed on neighboring ciliated cells. There are no ciliated cells in these organoids, so what is inducing the Notch signal?
- c. Additional markers of other lung cell types should be examined in the organoids (ciliated cell markers, AE1 markers, for example).

3. Fig 2 and related

- a. The dnMAML exposure in Ext Data Fig 2 occurs 4-10 days prior to the isolation of Tom+ cells. Since club cells require persistent Notch signaling to maintain their fate, and because loss of Notch signaling causes their rapid conversion (4-6 days) to ciliated cells, the observation that dnMAML generates increased CFE does not easily fit with the proposed model. One could argue that these results in fact argue that normal airway club cells are likely not the progenitors for the AE2 (CFE) regeneration activity. As noted in my general comments above, could cells that are CC10+ SPC+ at the BAJ be responsible instead? What happens to the airway organoids after DAPT treatment? I realize that such a treatment is included in studies in Fig 2, but only CC10 and SPC markers are examined (do ciliated cell markers get induced?). [...]"

"Fig 2c. Panel 2g: how has dnMAML impacted club-ciliated cell ratios in the airways? The ratio should be greatly reduced. What is the control evidence that the dnMAML mice are working as intended, that is, how do the authors know that dnMAML is inhibiting Notch in the intended target cells? Have the authors tried DAPT (or other GSI) treatment in vivo, as an alternative to the genetic method of dnMAML, or is the GSI too toxic, especially when used in combination with bleomycin?"

"4. Fig 3 and related: I am surprised that ciliated and secretory airway cells don't appear more closely related as "airway" cells, as in previous lung single-cell sequencing experiments. Are these results inconsistent with such previously

published results (Krasnow, Whitsett labs, for example)?

5. Fig 4 and related:

- a. The authors do not note that club cell maintenance requires Notch signaling in the adult (not just during development), as noted above, which is an important consideration that should be addressed.
- b. The authors should assess Notch signaling in the secretory cells. For example, they could stain for Notch1 or Notch2 ICD in the nuclei as a transcript-independent measure of Notch activity. Also, they could examine expression in situ of Notch transcriptional targets. The change in ligand expression is nicely shown but it is important to show that this decrease in ligands impacts the signal.
- c. Ext data Fig 4: What does bleomycin treatment do the airways? They certainly don't look the same by H & E, comparing PBS and bleomycin (the obvious club-ciliated cell architecture seems lost after treatment). Are "normal" ciliated cells present?
- d. An important missing piece of data is an assessment of IL-1beta levels. Is there a technical reason why this cannot be measured? An increase in IL-1beta after damage is a key piece of the model and is not established.
- e. The authors could further test their model by treating mice with a pulse of GSI to convert the bulk of club cells to ciliated cells, then treating with bleomycin to induce damage. If airway secretory cells are key to repair, then the GSI treatment should inhibit or slow repair. The dnMAML results, as discussed above, suggest that loss of club cells does not slow repair, which presents a challenge to the model. The GSI pre-pulse experiment would be another simple way to address this question."

(B) The technical advance of this work should be further enhanced by clarifying methodological details and the feasibility of the organoid model, confirming the efficiency of the methods used and adding more evidence for KDR as an ideal marker to isolate secretory cells.

Referee 1 notes:

"6. The data in Figure 6 that secretory-derived AEC2s have more clonogenic capacity is really interesting, but perhaps more details could be given in terms of the exact culture condition and what is the cell composition of the organoids at late passage."

"7. The discovery of KDR as a potential tool to isolate secretory cells in human could prove to be very useful for the field, but more is needed to validate the strategy aside from qpcr shown in Figure 7c. It would be important to include more precise information about what fraction of different epithelial lineages are in the KDR+/HTII280+, KDR+/HTII280-, KDR-/HTII280+ populations (with a cytospin). Furthermore, for the data showing that DAPT is increasing alveolar lineage differentiation, it would be important to quantify it at the protein level (e.g. IHC, % cell or organoid that are positive for specific marker) to show certain canonical AEC2 markers are present (e.g. % cell or organoid with SPC, LAMP3, ABCA3, and etc), because SP-B is not specific to AEC2s in humans and it is not really clear what anti-HTII-280 is actually marking."

Referee 2 notes:

"5. Very complex mouse models are used. Do you have any idea of deletion efficiency and leakiness of these lineage tracing reporters?"

6. How efficient was the knockdown of the genes shown in Fig. 5 and ED 5? (only shown for *Etv5*). How come that knockdown of *RBPJ*, which is involved in repressing *Fosl2*, still allows an effect of DAPT on the expression of AT2 markers? And did this knockdown induce any AT2 markers?"

"8. The human data focus on KDR as a marker for secretory cells. This part is, admittedly likely by necessity, less strong. The data are shown as MFI (Fig. ED7d). What fraction is really positive? Is there heterogeneity? According to ref. 38, KDR is expressed in basal cells. scRNAseq also shows KDR to be expressed in only a fraction of secretory cells, and in other cell types as well (ED 7f). In addition, there seems to be a population of KDR+HT2-280+ cells (Fig 7c). What are these? The AT2 cells arising after Notch inhibition also seem to be located at the interior of the organoids. What are the other cells?"

Referee 3 notes:

"[as above] Fig 1b. The organoid system seems like an important technical advance. I would like to see more characterization, particularly of the state of Notch signaling. For example, in the three types of organoids (e.g., see panel f), where are Notch ligands and receptors expressed? Which cells show not just Notch receptor expression but, more importantly, Notch receptor activity, as evidenced by nuclear localization of the Notch ICD? One thing I am confused by...in the first (left-most) panel of Fig. 1f, almost all (if not all) of the cells in the airway organoids appear to be CC10 positive and therefore are described as secretory airway cells. In vivo, airway secretory cells (club cells) require Notch signaling for their fate to be maintained, and as the authors note later in their paper, the Notch signal is induced by Jag ligands expressed on neighboring ciliated cells. There are no ciliated cells in these organoids, so what is inducing the Notch signal? c. Additional markers of other lung cell types should be examined in the organoids (ciliated cell markers, AE1 markers, for example)."

(C) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes should also be addressed.

(D) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with

data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).
- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.
- provide the completed Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-research/editorial-policies/image-integrity"](https://www.nature.com/nature-research/editorial-policies/image-integrity)>Digital Image Integrity Guidelines. and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We are aware that many researchers are currently facing disruptions because of the COVID-19 pandemic. If you anticipate significant delays for these revisions, please do let us know as we are happy to extend deadlines as necessary.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Christine.

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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

Choi, Jang, and colleagues are attempting to address how epithelial stem cells migrate and transdifferentiate into another stem cell population in the setting of injury and repair. Primarily using Scgb1a1creERT as a tool to mark secretory club cells in the airway, this paper demonstrates that genetic manipulation of Notch signaling can alter the ability of secretory cells to undergo transdifferentiation into SFTPC+alveolar type 2 cells (AEC2s) in the alveoli after injury. More interestingly, inflammation-mediated signals such as IL1b might be regulating the expression of Notch ligands in the secretory cell niche to modulate Notch-mediated effects on AEC2 transdifferentiation. The paper then transitions to perform chromatin accessibility studies to identify potential binding motifs for transcription factors that modulates AEC2 transdifferentiation, and demonstrates intrinsic differences between AEC2 derived from secretory club cells vs. resident AEC2s. Finally, the authors demonstrate a potential new marker to isolate human secretory cells for further studies.

Overall, this is a very comprehensive and nicely written study on secretory club cells and how they adopt fate decision to transdifferentiate into AEC2s during alveolar repair, unified by a focus on Notch regulation. The role of Notch in promoting secretory cell fate is very well characterized, as acknowledged by the authors, and the expression of Notch ligands and components in the airway has been described as referenced. The authors argue that the main novelty here is the

demonstration that notch can regulate club to AEC2 transdifferentiation, which is debatable. There was prior evidence that Notch inhibition can induce AEC2 differentiation from secretory cell subset in vitro (previously described as Lineage-negative epithelial progenitors), and persistent Notch signaling might serve to maintain airway differentiation in the alveoli in vivo after influenza (Vaughan et al, Nature, 2015, Fig. 3), although the current manuscript goes much further in formally testing Notch in vivo using numerous genetic models. What I think is more novel is the demonstration that inflammatory signals might play a role in modulating notch in the niche, as well as uncovering techniques to isolate secretory cells in the human lung. Focusing on these latter aspects of the manuscript would potentially do more to move the field forward in learning about how stem cells integrate inflammation to fate switch and whether human correlates exist.

One major technical caveat that needs to be addressed is the fact that the Scgb1a1creERT used in this study also labels AEC2s during homeostasis (Rawlins et al., Cell Stem Cell, 2009), which the authors actually demonstrated in Figure 1i (~7-8% of SPC+ cells are Scgb1a1 Lin+ at homeostasis). Do the authors consider these alveolar SPC+/Scgb1a1 Lin+ cells as secretory AEC2s? They do not display any features of club cells other than scgb1a1 transcript, nor are they located at the bronchioalveolar ductal junction to be considered BASCs. Labeling endogenous AEC2s with Scgb1a1creERT confounds the interpretation of some results, as some phenotypes could be ascribed to the behavior of endogenous AEC2s rather than airway club cells differentiating into AEC2s. However, I do not believe this to be a fatal flaw, and perhaps the authors can address this with certain experiments to bolster their claims.

Major Comments:

1. Mesenchymal free culturing conditions have been published for murine AEC2s (Shiraishi et al, iScience, 2019, and Weiner et al, NPJ Regen Med, 2019), which should be acknowledged for the data generated in Figure 1. It would be nice to determine what percentage of Scgb1a1 Lin+ cells are SPC+ prior to culture.
2. For Figure 2 and 3 on lineage tracing of Scgb1a1 Lin+ cells with Notch manipulation, see comment about scgb1a1creERT tool above. The authors should demonstrate whether endogenous SPC+ cells expansion is potentially affected by Notch manipulation (e.g. does Notch inactivation also expand endogenous AEC2 pool), or consider additional experiments where AEC2s can be excluded for genetic lineage tracing or transplant studies. Furthermore, it has been reported that KRT5+ alveolar basal cells found in bleomycin injury are also derived from scgb1a1+ club cells in the airway (and not the scgb1a1+/spc+ cells in the alveoli), does Notch alteration affect Scgb1a1Lin+ cells that differentiate into KRT5+ alveolar basal cells, if any? Finally, does Notch manipulation also alter club cell differentiation into ciliated cell fate at the airway with bleomycin, or do you have evidence that AEC2 differentiation is already occurring at the airway? Clarifying the last two points would improve the resolution of how the secretory cell differentiation is altered in the setting of alveolar injury.
3. For Figure 3, it is unclear how YFP- AEC2s are isolated. Please clarify as to how the RFP-/YFP- AEC2 population is derived on single cell seq analysis in Fig 3l.
4. Figure 4 presents very intriguing data that Notch ligand expression in ciliated cells are regulated by inflammation and modulates Club cell transdifferentiation. However, this hypothesis is still largely based on circumstantial evidence as

presented. The data that IL1R1 deletion reduces Jag1/2 in ciliated cells, and the data that club cells transdifferentiate into AEC2s with ciliated IL1R1 deletion could be true-true but unrelated. It would be important to assess whether Notch activation in the club cells at the airway is altered by IL1R1 deletion in ciliated cells during injury. Additionally, in figure 4b, what is the cellular composition of IL1r1+ CD24+ cells (% ciliated cells vs other cell types) vs. IL1r1+/CD24- cells? In figure 4E, what is missing is the effect of IL1R1 deletion in ciliated cells during PBS, which is important to determine whether there is tonic IL1 stimulation in the airways during homeostasis. Finally, does IL1b have an effect on club cell organoids that might be independent of notch signaling?

5. Same point regarding the Scgb1a1creERT for the ATAC-seq analysis. To provide more assurance that the secretory AT2s are mostly derived from airway Scgb1a1+ cells (and not endogenous Scgb1a1+/SPC+ cells in the alveoli) in the ATAC-seq, authors should demonstrate whether there is an increase in GFP+/DsRed+ population (Figure 5b) after injury compared to homeostasis, to assess whether there is an increased proportion of GFP+ cells (secretory) that acquire AT2 fate (DsRed+). Furthermore, the demonstration that Fosl1 KD suppresses Sftpc expression alone does not prove Fosl1 is necessary for AEC2 fate. An alternative explanation is that Fosl1 might be repressing the fate of an alternative lineage (e.g. basal, ciliated, and etc.), and KD of Fosl1 results in increase in a non-AEC2 lineage derived from club cells. This should be interrogated by immunophenotyping organoids derived from DAPT treated and Fosl1 KD conditions. Do these organoids express no mature markers of differentiation? Or do they express alternative lineage markers that are neither secretory nor AEC2.

6. The data in Figure 6 that secretory-derived AEC2s have more clonogenic capacity is really interesting, but perhaps more details could be given in terms of the exact culture condition and what is the cell composition of the organoids at late passage.

7. The discovery of KDR as a potential tool to isolate secretory cells in human could prove to be very useful for the field, but more is needed to validate the strategy aside from qPCR shown in Figure 7c. It would be important to include more precise information about what fraction of different epithelial lineages are in the KDR+/HTII280+, KDR+/HTII280-, KDR-/HTII280+ populations (with a cytospin). Furthermore, for the data showing that DAPT is increasing alveolar lineage differentiation, it would be important to quantify it at the protein level (e.g. IHC, % cell or organoid that are positive for specific marker) to show certain canonical AEC2 markers are present (e.g. % cell or organoid with SPC, LAMP3, ABCA3, and etc), because SP-B is not specific to AEC2s in humans and it is not really clear what anti-HTII-280 is actually marking.

Minor Comments:

1. When graphs with individual data points are presented throughout the manuscript, it is not always clear in the legends what each dot represents in terms of biological vs. technical replicates.

2. The abstract is nicely written but perhaps a bit overstated. It is not clear that the authors produced enough evidence that there is a "2-stage process" for Notch-mediated club-AEC2 differentiation, if so, it is not well delineated as to what is stage 1 and 2. Furthermore, the term "transcriptional licensing" would be more accurately described as a factor that is capable of initiating origin of replication at the DNA level, or acting as a pioneer transcription factors, which is not really demonstrated in the manuscript. I would probably remove these terms and save

them for speculation in Discussion.

3. Finally, a big question from this manuscript is why does downregulation of Notch leads to different secretory cell differentiation outcomes in different contexts (differentiation to ciliated cell in homeostasis, AEC2s in injury). Addressing this experimentally is beyond the scope of a revision, but some speculation as to possible mechanisms and acknowledging the knowledge gap in the Discussion would help the field move forward.

Reviewer #2:

Remarks to the Author:

This is a very elegant paper describing how alveolar injury induces IL1b from ciliated cells, which inhibit Notch signaling between ciliated and neighboring secretory cells, which in turn, through derepression of Fosl2, permits differentiation into AT2 cells. In addition, and very interestingly, the authors show that AT2 derived from secretory cells differ from other AT2 cells. This paper reads very well, offers in depth mechanistic insight into lung regeneration, and will be important for the field. I have a few comments, the most important of which is on physiological relevance.

1. Does interfering with the mechanism (be it at the IL1r, Notch or Fosl2 level) interfere with regeneration after bleomycin injury? In other words, this paper shows what can happen, not how important it is physiologically.

2. If SCO were isolated from more proximal airway, would they still generate AT2 after Notch inhibition, or might there be heterogeneity in the capacity of secretory cells to do this? Would the cells capable of doing this be variant secretory cells?

3. Several data require ANOVA rather than a t-test for statistical analysis, as multiple groups are compared. These include Fig. 2i, 4b, 4e.

4. In ED 4e, what are the dTom+CD24- cells? And why do they express so little IL1r if they are dTom+? What are those cells?

5. Very complex mouse models are used. Do you have any idea of deletion efficiency and leakiness of these lineage tracing reporters?

6. How efficient was the knockdown of the genes shown in Fig. 5 and ED 5? (only shown for Etv5). How come that knockdown of RBPJ, which is involved in repressing Fosl2, still allows an effect of DAPT on the expression of AT2 markers? And did this knockdown induce any AT2 markers?

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8. The human data focus on KDR as a marker for secretory cells. This part is, admittedly likely by necessity, less strong. The data are shown as MFI (Fig. ED7d). What fraction is really positive? Is there heterogeneity? According to ref. 38, KDR is expressed in basal cells. scRNAseq also shows KDR to be expressed in only a fraction of secretory cells, and in other cell types as well (ED 7f). In addition, there

seems to be a population of KDR+HT2-280+ cells (Fig 7c). What are these? The AT2 cells arising after Notch inhibition also seem to be located at the interior of the organoids. What are the other cells?

Minor

1. Line 166: please mention for clarity that the dnMAML are in fact dnDMAMf/+.

Reviewer #3:

Remarks to the Author:

Choi et al. describe a series of experiments aimed at understanding how alveoli regenerate after damage (bleomycin) and induction of latent stem cell programs. Their model systems rely on in vitro analysis of club cell derived organoids and in vivo studies using a variety of sophisticated mouse genetic tools, both combined with molecular studies of chromatin states, single-cell sequencing and pharmacological manipulations. The authors assert that their data are best described by a model in which alveolar damage generates an IL-1 β signal that in turn downregulates JAG1/2 expression on ciliated cells, further downregulating Notch signaling in club cells to release a Fosl2/Fra2 transcriptional program that licenses the secretory cells, as a progenitor, to differentiate to alveolar type 2 cells (AE2 cells).

In general, the experiments are performed thoughtfully and are well presented. The systems employed are technically advanced and directed to answering relevant and important questions in lung biology and damage/repair. My main criticism, however, is that there seem to be other at-least-as-plausible models to explain the data, and I suggest that these alternative interpretations need to be addressed.

Specifically, the authors often refer to the repair-driving progenitor cell type as a club cell or secretory cell. In many cases, this cellular identity (and the language used to describe it, "club cell" or "secretory" cell) is based on expression of one (or no more than a few markers), such as CC10. While club cells certainly express CC10 and other markers, these markers are shared with other cells, most notably stem/progenitor cells that reside at the bronchial/alveolar junctions (BAJs). Numerous studies have clearly established that cells at this location express markers of both club cells and AE2 cells, thus displaying markers indicative of a dual lineage. An equally plausible, or more plausible model, compared to the authors' model, is that these dual lineage progenitor cells are the precursors that provide the repaired AE2 cells, and that the fate decision to AE2 is driven by loss of Notch signaling. Put another way, perhaps the "airway" organoids are really "BASC" organoids and Notch controls the fate between airway, alveolar and mixed.

One other general comment is that the authors do not consider that in some of their experiments, treatment with a gamma-secretase inhibitor before damage would likely have converted nearly all of the club cells to ciliated cells, since both genetic and pharmacological inhibition of Notch induces a club-to-ciliated cell transdifferentiation (for example, see Pardo-Saganta et al., Nature 2015 and Lafkas et al., Nature 2015). Given the expected absence of the majority of club cells under such conditions (but not the loss of CC10+ cells at the BAJs), how do

the authors explain the improved AE2 regeneration? This omission seems like a major issue that must be addressed.

Specific comments:

1. Writing: The paper is generally well-written and fairly easy to follow. However, the beginning sections (Title and abstract in particular) were very hard for me to understand. For example, the title is much too obtuse and relies on jargon. I don't think most readers will understand, until finishing the paper, what is meant by "cross-compartment differentiation plasticity"). This kind of language persists in the Introduction and could be rewritten using a more direct and straightforward approach.

2. Figure 1 and related:

a. Cells are described as "secretory" or "alveolar" when often the observation is simply CC10+ or SPC+. This is an oversimplification. Indeed, CC10 or SPC would also mark the dual-lineage progenitors noted above.

b. The organoid system seems like an important technical advance. I would like to see more characterization, particularly of the state of Notch signaling. For example, in the three types of organoids (e.g., see panel f), where are Notch ligands and receptors expressed? Which cells show not just Notch receptor expression but, more importantly, Notch receptor activity, as evidenced by nuclear localization of the Notch ICD? One thing I am confused by...in the first (left-most) panel of Fig. 1f, almost all (if not all) of the cells in the airway organoids appear to be CC10 positive and therefore are described as secretory airway cells. In vivo, airway secretory cells (club cells) require Notch signaling for their fate to be maintained, and as the authors note later in their paper, the Notch signal is induced by Jag ligands expressed on neighboring ciliated cells. There are no ciliated cells in these organoids, so what is inducing the Notch signal?

c. Additional markers of other lung cell types should be examined in the organoids (ciliated cell markers, AE1 markers, for example).

3. Fig 2 and related

a. The dnMAML exposure in Ext Data Fig 2 occurs 4-10 days prior to the isolation of Tom+ cells. Since club cells require persistent Notch signaling to maintain their fate, and because loss of Notch signaling causes their rapid conversion (4-6 days) to ciliated cells, the observation that dnMAML generates increased CFE does not easily fit with the proposed model. One could argue that these results in fact argue that normal airway club cells are likely not the progenitors for the AE2 (CFE) regeneration activity. As noted in my general comments above, could cells that are CC10+ SPC+ at the BAJ be responsible instead? What happens to the airway organoids after DAPT treatment? I realize that such a treatment is included in studies in Fig 2, but only CC10 and SPC markers are examined (do ciliated cell markers get induced?).

b. Panels a and d: Are these heat maps showing the top differentially expressed genes? The panels need to be better described in the figure legends. In general, a bit more experimental detail should be included in many of the legends as it was often difficult to understand exactly what was done or what was being shown (e.g., length of treatments).

c. Panel 2g: how has dnMAML impacted club-ciliated cell ratios in the airways? The ratio should be greatly reduced. What is the control evidence that the dnMAML mice are working as intended, that is, how do the authors know that dnMAML is inhibiting Notch in the intended target cells? Have the authors tried DAPT (or other GSI) treatment in vivo, as an alternative to the genetic method of dnMAML, or is

the GSI too toxic, especially when used in combination with bleomycin?

4. Fig 3 and related: I am surprised that ciliated and secretory airway cells don't appear more closely related as "airway" cells, as in previous lung single-cell sequencing experiments. Are these results inconsistent with such previously published results (Krasnow, Whitsett labs, for example)?

5. Fig 4 and related:

a. The authors do not note that club cell maintenance requires Notch signaling in the adult (not just during development), as noted above, which is an important consideration that should be addressed.

b. The authors should assess Notch signaling in the secretory cells. For example, they could stain for Notch1 or Notch2 ICD in the nuclei as a transcript-independent measure of Notch activity. Also, they could examine expression in situ of Notch transcriptional targets. The change in ligand expression is nicely shown but it is important to show that this decrease in ligands impacts the signal.

c. Ext data Fig 4: What does bleomycin treatment do the airways? They certainly don't look the same by H & E, comparing PBS and bleomycin (the obvious club-ciliated cell architecture seems lost after treatment). Are "normal" ciliated cells present?

d. An important missing piece of data is an assessment of IL-1beta levels. Is there a technical reason why this cannot be measured? An increase in IL-1beta after damage is a key piece of the model and is not established.

e. The authors could further test their model by treating mice with a pulse of GSI to convert the bulk of club cells to ciliated cells, then treating with bleomycin to induce damage. If airway secretory cells are key to repair, then the GSI treatment should inhibit or slow repair. The dnMAML results, as discussed above, suggest that loss of club cells does not slow repair, which presents a challenge to the model. The GSI pre-pulse experiment would be another simple way to address this question.

6. Fig 6 panel e: The top and bottom labels are switched relative to what is referenced in the legend.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

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REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

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- Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.
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- At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.
- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can be found at www.nature.com/protocolexchange/about.

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FIGURES – Colour figure publication costs \$600 for the first, and \$300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend.

Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

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- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

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Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

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Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1,

Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>; <http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

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STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

Author Rebuttal to Initial comments

Summary of additional data and modified text in response to the reviewers' comments

Reviewer	Comment	Response: additional/modified data/text
Reviewer 1	1	Rev1_Fig. 1
	2-1	Rev1_Fig. 2 (Revised Extended Data Fig. 7) Rev1_Fig. 3 (Revised Extended Data Fig. 6a-i)
	2-2	Rev1_Fig. 4
	2-3	Rev1_Fig. 5 (Revised Extended Data Fig. 4), Rev1_Fig. 6
	3	Rev1_Fig. 7 (Revised Extended Data Fig. 8a)
	4-1	Rev1_Fig. 8 (Revised Extended Data Fig. 10)
	4-2	Rev1_Fig. 9 (Revised Extended Data Fig. 9e)
	4-3	Rev1_Fig. 10 (Revised Extended Data Fig. 11a)
	4-4	Rev1_Fig. 11
	5-1	Rev1_Fig. 12
	5-2	Rev1_Fig. 13 (Revised Fig. 5g)
	6	Methods Rev1_Fig. 14 (Revised Extended Data Fig. 15)
	7-1	Rev1_Fig. 15 (Revised Fig. 7c-f)
	7-2	Rev1_Fig. 16 (Revised Fig. 7i,j)
	Minor-1	Detailed description provided
	Minor-2	Text changed as suggested
	Minor-3	Rev1_Fig. 17 (Revised Extended Data Fig. 17) in the Discussion as suggested
Reviewer 2	1	See point-to-point response
	2	Rev2_Fig. 1
	3	See point-to-point response
	4	Rev2_Fig. 2 (Revised Extended Data Fig. 9e)
	5	Rev2_Fig. 3 (Revised Extended Data Fig. 3)
	6	Rev2_Fig. 4 (Revised Extended Data Figs. 13h and 2b)
	7	Rev2_Fig. 5
	8-1	Rev2_Fig. 6
	8-2	Rev2_Fig. 7
	8-3	Rev2_Fig. 8 (Revised Fig. 7f,i,j; Revised Extended Data Fig. 16h,i)
	Minor-1	Text changed as suggested
Reviewer 3	1	Text changed as suggested
	2-1	See our response for comment 3
	2-2	Rev3_Fig. 1 (Revised Fig. 1f,h; Revised Extended Data Fig. 2a)
	2-3	Rev3_Fig. 2 (Revised Fig. 1f)
	3-1	Rev3_Fig. 3, Rev3_Fig. 4 (Revised Extended Data Fig. 6)
	3-2	Rev3_Fig. 5 (Revised Fig. 2c)
	3-3	Detailed description provided
	3-4	Rev3_Fig. 6 (Revised Extended Data Fig. 3) Rev3_Fig. 7 (Revised Extended Data Fig. 4)
	3-5	Rev3_Fig. 8
	4	Rev3_Fig. 9 (Revised Extended Data Fig. 8b,c)
	5-1	Addressed in the Discussion as suggested
	5-2	Rev3_Fig. 10 (Revised Extended Data Fig. 10)
	5-3	Rev3_Fig. 11
	5-4	Rev3_Fig. 12
	5-5	Rev3_Fig. 13
	6	Error corrected

Response to Reviewers' comments

We are grateful for the reviewers' valuable comments, which helped us strengthen our paper. We have comprehensively addressed all concerns with additional experiments which we think to provide stronger and clearer support for our conclusions. We hope that the new data will solidify the reviewer's enthusiasms for our manuscript.

Reviewer #1:

Choi, Jang, and colleagues are attempting to address how epithelial stem cells migrate and transdifferentiate into another stem cell population in the setting of injury and repair. Primarily using *Scgb1a1creERT* as a tool to mark secretory club cells in the airway, this paper demonstrates that genetic manipulation of Notch signaling can alter the ability of secretory cells to undergo transdifferentiation into SFTPC+alveolar type 2 cells (AEC2s) in the alveoli after injury. More interestingly, inflammation-mediated signals such as IL1b might be regulating the expression of Notch ligands in the secretory cell niche to modulate Notch-mediated effects on AEC2 transdifferentiation. The paper then transitions to perform chromatin accessibility studies to identify potential binding motifs for transcription factors that modulates AEC2 transdifferentiation, and demonstrates intrinsic differences between AEC2 derived from secretory club cells vs. resident AEC2s. Finally, the authors demonstrate a potential new marker to isolate human secretory cells for further studies.

Overall, this is a very comprehensive and nicely written study on secretory club cells and how they adopt fate decision to transdifferentiate into AEC2s during alveolar repair, unified by a focus on Notch regulation. The role of Notch in promoting secretory cell fate is very well characterized, as acknowledged by the authors, and the expression of Notch ligands and components in the airway has been described as referenced. The authors argue that the main novelty here is the demonstration that notch can regulate club to AEC2 transdifferentiation, which is debatable. There was prior evidence that Notch inhibition can induce AEC2 differentiation from secretory cell subset in vitro (previously described as Lineage-negative epithelial progenitors), and persistent Notch signaling might serve to maintain airway differentiation in the alveoli in vivo after influenza (Vaughan et al, Nature, 2015, Fig. 3), although the current manuscript goes much further in formally testing Notch in vivo using numerous genetic models. What I think is more novel is the demonstration that inflammatory signals might play a role in modulating notch in the niche, as well as uncovering techniques to isolate secretory cells in the human lung. Focusing on these latter aspects of the manuscript would potentially do more to move the field forward in learning about how stem cells integrate inflammation to fate switch and whether human correlates exist.

Response: We thank the Reviewer for highlighting the novelty of our findings that advance the fields. We hope that the new data added in the revised manuscript provide the important insight into how airway stem/progenitor cells integrate inflammatory signals to differentiate into alveolar lineages via Il1r1-Notch-Fosl2 axis in mouse and, potentially, human lungs.

One major technical caveat that needs to be addressed is the fact that the *Scgb1a1creERT* used in this study also labels AEC2s during homeostasis (Rawlins et al., Cell Stem Cell, 2009), which the authors actually demonstrated in Figure 1i (~7-8% of SPC+ cells are *Scgb1a1* lin+ at homeostasis). Do the authors consider these alveolar SPC+/Scgb1a1 Lin+ cells as secretory AEC2s? They do not display any features of club cells other than *scgb1a1* transcript, nor are they located at the bronchioalveolar ductal junction to be considered BASCs. Labeling endogenous AEC2s with *Scgb1a1creERT* confounds the interpretation of some results, as some phenotypes could be ascribed to the behavior of endogenous AEC2s rather than airway club cells differentiating into AEC2s. However, I do not believe this to be a fatal flaw, and perhaps the authors can address this with certain experiments to bolster their claims.

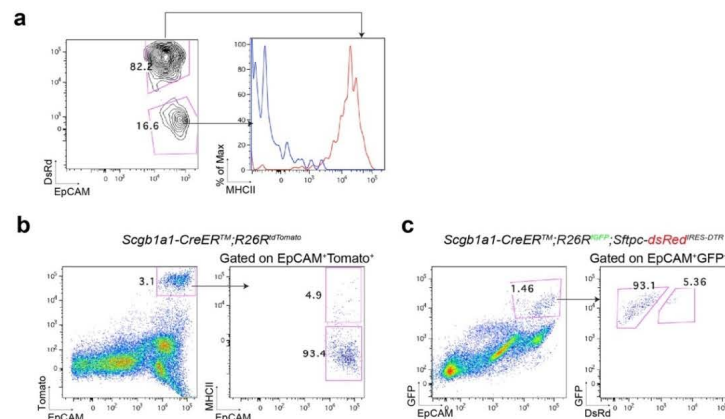
Response: We agree with the Reviewer's concern. We have thoroughly addressed this point with additional experiments. Please see our response to **Specific Comment 2** below.

Major Comments:

1. Mesenchymal free culturing conditions have been published for murine AEC2s (Shiraishi et al, iScience, 2019, and Weiner et al, NPJ Regen Med, 2019), which should be acknowledged for the data generated in Figure 1. It would be nice to determine what percentage of *Scgb1a1* Lin⁺ cells are SPC⁺ prior to culture.

Response: As suggested, we have cited those previous studies in our revised manuscript.

To verify the percentage of *Scgb1a1*⁺ lineage-labelled AT2 cells of *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs, we performed flow cytometry analysis for MHCII which is known to specifically mark AT2 cells (Hasegawa et al., 2017). We first confirmed the specificity of MHCII in SPC⁺ AT2 cells using our *Sftpc-dsRed* reporter mice (**Rev1_Fig. 1a**). All *dsRed*⁺ AT2 cells were specifically marked by MHCII. We then assessed the expression of MHCII in lineage-labelled cells isolated from *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs. Approximately 5% of lineage-labelled cells expressed MHCII, similar to our immunofluorescent (IF) quantification data (**Revised Fig. 2g,i**) in lung tissue sections (**Rev1_Fig. 1b**). In addition, we further analysed *Scgb1a1*⁺ lineage-labelled AT2 cells using our *Scgb1a1-CreER*^{TM/+}; *R26R*^{GFP/+}; *Sftpc-dsRed* mouse line (**Rev1_Fig. 1c**). The similar percentage of lineage-labelled AT2 cells (fGFP⁺dsRed⁺, ~5-6%) were detected in the dissociated lung cells.



Rev1_Figure 1. *Scgb1a1*⁺ lineage-labelled AT2 cells in *Scgb1a1-CreER*TM mouse line.

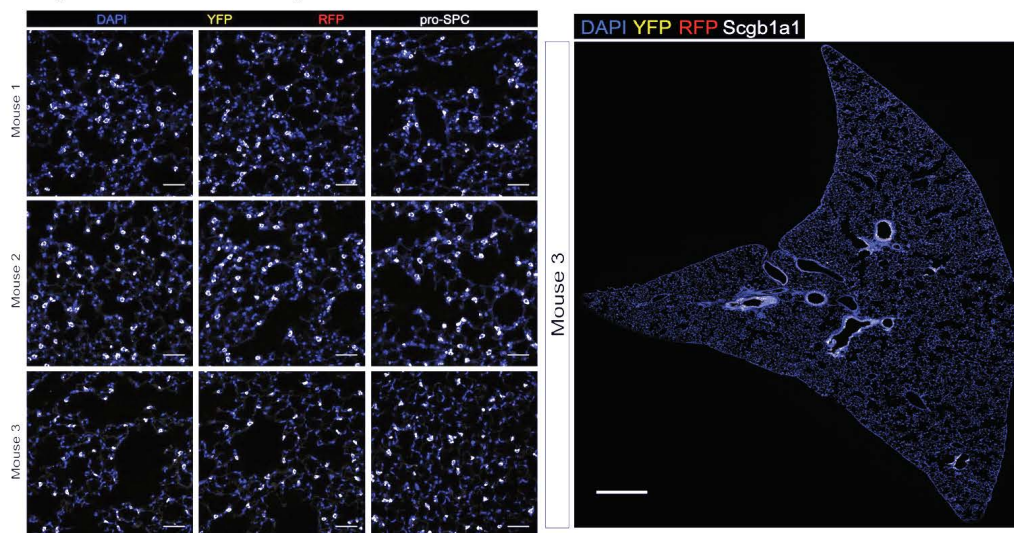
(a) MHCII expression marks AT2 cells. Flow cytometry analysis showing the expression of MHCII in SPC⁺ AT2 (EpCAM⁺dsRed⁺) or non-AT2 (EpCAM⁺dsRed⁻) cells of *Sftpc-dsRed* reporter mouse lungs. Numbers adjacent to the outlined area indicate the percentage of populations.

(b) Flow cytometry analysis showing the percentage of lineage-labelled AT2 cells (EpCAM⁺Tomato⁺MHCII⁺) in *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs. Numbers adjacent to the outlined area indicate the percentage of populations.

(c) Flow cytometry analysis showing the percentage of lineage-labelled AT2 cells (EpCAM⁺GFP⁺dsRed⁺) in *Scgb1a1-CreER*^{TM/+}; *R26R*^{GFP/+}; *Sftpc-dsRed* mouse lungs. Numbers adjacent to the outlined area indicate the percentage of populations.

2-1. For Figure 2 and 3 on lineage tracing of *Scgb1a1* Lin⁺ cells with Notch manipulation, see comment about *scgb1a1creERT* tool above. The authors should demonstrate whether endogenous SPC⁺ cells expansion is potentially affected by Notch manipulation (e.g. does Notch inactivation also expand endogenous AEC2 pool), or consider additional experiments where AEC2s can be excluded for genetic lineage tracing or transplant studies.

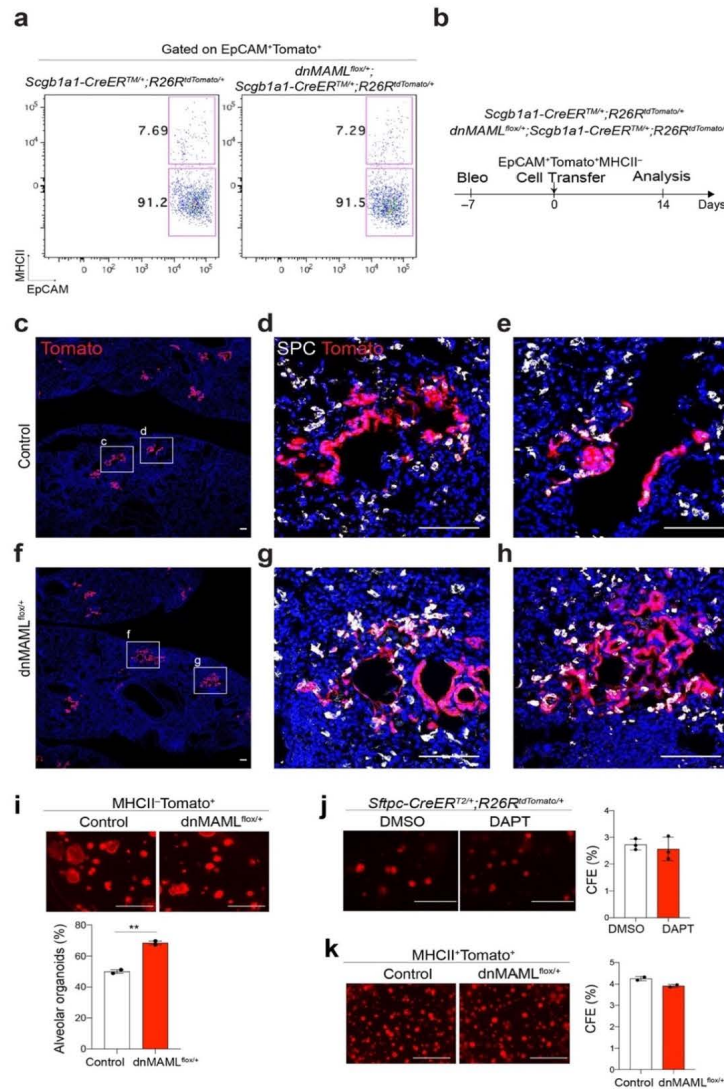
Response: We thank the Reviewer for raising this issue. We agree with the Reviewer that 7-8% of SPC⁺ AT2 cells were labelled in *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs and could be affected by Notch signalling. However, a recent study clearly showed that Notch activity is dispensable for the proliferation and survival of AT2 cells, although Notch inactivation is required for AT1 cell differentiation (Finn et al., 2019). In addition, we confirmed that there is no lineage-labelled AT2 cells in our *Scgb1a1-CreER*^{TM/+}; *Red2-Notch*^{NIICD/+} tamoxifen-treated mouse lungs at homeostasis (PBS control) (**Rev1_Fig. 2, Revised Extended Data Fig. 7**). These data support our conclusion that expanded lineage-labelled AT2 cells post injury are derived from airway secretory cells, not from endogenous AT2 cells, via regulation of Notch activity in our mouse models.



Rev1_Figure 2. No AT2 cells were labelled in *Scgb1a1-CreER*^{TM/+}; *Red2-Notch*^{NIICD/+} mouse lungs. Representative immunofluorescent (IF) images showing *Scgb1a1-CreER*TM lineage-labelled cells in alveolar regions (left) and a whole lobe (right) post tamoxifen treatment: YFP (yellow), RFP (red), pro-SPC (white, left), *Scgb1a1* (white, right) and DAPI (blue). Scale bar, 50µm (left) and 500µm (right).

Furthermore, following the Reviewer's comment, we also performed a cell transplantation study to prove the contribution of secretory cells to alveolar regeneration via Notch inhibition, by excluding lineage-labelled AT2 cells. We isolated lineage-labelled cells from *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} or *dnMAML*^{flox/+}; *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs, and excluded AT2 cells based on MHCII expression (**Rev1_Fig. 3a; Revised Extended Data Fig. 6a**). Lineage-labelled secretory cells (EpCAM⁺Tomato⁺MHCII⁻) were transplanted into B6 WT mice at day 7 after bleomycin injury (**Rev1_Fig. 3b; Revised Extended Data Fig. 6b**). At 14 days post transplantation, we found that secretory cells of *dnMAML*^{flox/+}; *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs showed a greater ability to contribute to AT2 cell differentiation compared to that of *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato} mouse lungs (**Rev1_Fig. 3c-h; Revised Extended Data Fig. 6c-h**). Additionally, we also confirmed that secretory cells from *dnMAML*^{flox/+}; *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs revealed the greater

frequency of alveolar organoids retaining AT2 cells compared to secretory cells from *Scgb1a1-CreER^{TM/+}*; *R26R^{tdTomato/+}* mouse lungs (**Rev1_Fig. 3i**; Revised Extended Data Fig. 6i).



Rev1_Figure 3. Secretory cells, but not AT2 cells, contribute to AT2 cell differentiation via inhibition of Notch activity.

(a) Flow cytometry analysis showing the exclusion of lineage-labelled AT2 cells from *Scgb1a1-CreER^{TM/+}*; *R26R^{tdTomato/+}* or *dnMAML^{lox/+}*; *Scgb1a1-CreER^{TM/+}*; *R26R^{tdTomato/+}* mouse lungs.

(b) Experimental scheme for transplantation of lineage-labelled secretory cells (EpCAM⁺Tomato⁺MHCII⁻) after excluding lineage-labelled AT2 cells in (a).

(c-h) Representative IF images of engrafted lineage-labelled secretory cells in (a,b): *Scgb1a1-CreER^{TM/+}*; *R26R^{tdTomato/+}* (control, c-e) or *dnMAML^{lox/+}*; *Scgb1a1-CreER^{TM/+}*; *R26R^{tdTomato/+}* (*dnMAML^{lox/+}*, f-h). Tomato (for *Scgb1a1* lineage, red), SPC (white), and DAPI (blue). Scale bars, 100µm.

(i) Representative fluorescent images of organoids (top) and statistical quantification of colony forming efficiency (CFE) of alveolar organoids (bottom) derived from lineage-labelled secretory cells in (a). 5,000 cells of cells were co-cultured with stromal cells with 1:5 ratio for 14 days. Scale bar, 2,000µm.

(j) Representative fluorescent images of organoids (left) and statistical quantification of CFE (right) derived from lineage-labelled AT2 cells isolated from *Sftpc-CreER*^{T2}; *R26R*^{tdTomato/+} mouse lungs with DMSO or DAPT treatment for 14 days. DAPT (20µM) were treated every other day during organoid culture. Scale bar, 1,000µm.

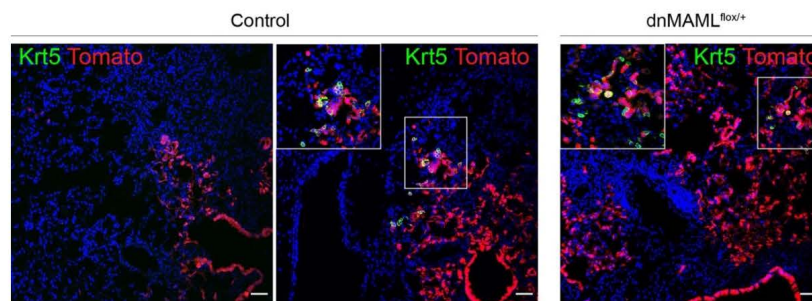
(k) Representative fluorescent images (left) and statistical quantification of CFE (right) derived from lineage-labelled AT2 cells (EpCAM⁺Tomato⁺MHCII⁺) isolated from *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (control) or *dnMAML*^{lox/+}; *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (*dnMAML*^{lox/+}) mouse lungs. Scale bar, 2,000µm. (i-k) Each individual dot represents one experiment from one mouse and data are presented as mean and s.e.m.

Lastly, to investigate the functional role of Notch inhibition on the behaviour of AT2 cells, we isolated AT2 cells from *Sftpc-CreER*^{T2/+}; *R26R*^{tdTomato/+} mouse lungs and cultured them in organoids with or without DAPT treatment. Consistent with a previous study (Finn et al., 2019), there was no discernible change in the growth of organoids with Notch inactivation (**Rev1_Fig. 3j**). Furthermore, we also isolated lineage-labelled AT2 cells (EpCAM⁺Tomato⁺MHCII⁺) from *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} or *dnMAML*^{lox/+}; *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs and cultured them in organoids. We observed no significant difference in the proliferation ability based on the colony forming efficiency and size of organoids (**Rev1_Fig. 3k**).

Taken together, our data strongly suggest that enhanced expansion of *Scgbl1a1*⁺ lineage-labelled AT2 cells via Notch inhibition is due to the differentiation of secretory cells but not due to the altered behaviour of lineage-labelled SPC⁺ AT2 cells.

2-2. Furthermore, it has been reported that KRT5⁺ alveolar basal cells found in bleomycin injury are also derived from *scgbl1a1*⁺ club cells in the airway (and not the *scgbl1a1*⁺/spc⁺ cells in the alveoli), does Notch alteration affect *Scgbl1a1*Lin⁺ cells that differentiate into KRT5⁺ alveolar basal cells, if any?

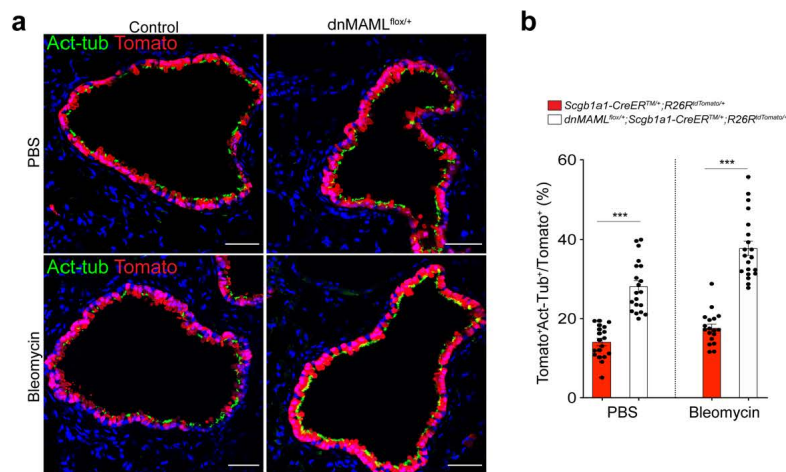
Response: We analysed the generation of basal cell-like Krt5⁺ pods in *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} or *dnMAML*^{lox/+}; *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs at 28 days after bleomycin injury. As shown in **Rev1_Fig. 4**, we barely observed lineage-labelled Krt5⁺ pods in our injury models, possibly due to the different level of injury severity. Even though we observed a few lineage-labelled Krt5⁺ cells in parenchyma regions, there was no discernible difference between *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (control) and *dnMAML*^{lox/+}; *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs.



Rev1_Figure 4. *Scgbl1a1*⁺ lineage-labelled Krt5⁺ cells were barely detected in bleomycin injured lungs regardless of Notch activity. Representative IF images showing the derivation of *Scgbl1a1*⁺ lineage-labelled Krt5⁺ cells in bleomycin-treated lungs of *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (control) and *dnMAML*^{lox/+}; *Scgbl1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mice. Tomato (for *Scgbl1a1* lineage, red), Krt5 (green), and DAPI (blue). Scale bar, 50µm.

2-3. Finally, does Notch manipulation also alter club cell differentiation into ciliated cell fate at the airway with bleomycin, or do you have evidence that AEC2 differentiation is already occurring at the airway? Clarifying the last two points would improve the resolution of how the secretory cell differentiation is altered in the setting of alveolar injury.

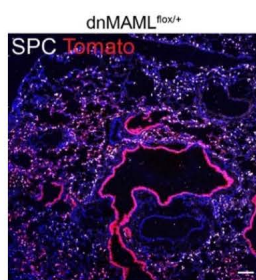
Response: We thank the Reviewer for this important question. We investigated whether the modulation of Notch activity induces the fate conversion of secretory cells into ciliated cells. Consistent with previous studies showing that Notch inhibition promotes the differentiation of secretory cells into ciliated cells (Lafkas et al., 2015; Morimoto et al., 2010; Pardo-Saganta et al., 2015), we observed a 2-fold increase of lineage-labelled ciliated cells in *dnMAML^{flox/+};Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs compared to *Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* (control) mouse lungs at homeostasis (PBS control) (**Rev1_Fig. 5; Revised Extended Data Fig. 4**). A 2-fold difference in lineage-labelled ciliated cells between *dnMAML^{flox/+}* and control mouse lungs remained at the similar level, although there were slight increases in lineage-labelled ciliated cells both in control and *dnMAML^{flox/+};Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs after bleomycin injury.



Rev1_Figure 5. Inhibition of Notch activity induces the differentiation of secretory cells into ciliated cells in distal airways.

(a) Representative IF images showing the derivation of *Scgbl1a1*⁺ lineage-labelled ciliated cells at day 28 in PBS- or bleomycin-treated lungs of *Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* (control) or *dnMAML^{flox/+};Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* (*dnMAML^{flox/+}*) mice. Tomato (for *Scgbl1a1* lineage, red), Acetylated Tubulin (Act-Tub, green), and DAPI (blue). Scale bar, 50µm.

(b) Statistical quantification of *Scgbl1a1*⁺ lineage-labelled ciliated cells in (a). Each individual dot represents one section and data are presented as mean ± s.e.m. (n=2 PBS control, n=2 for bleomycin). ***p<0.001.



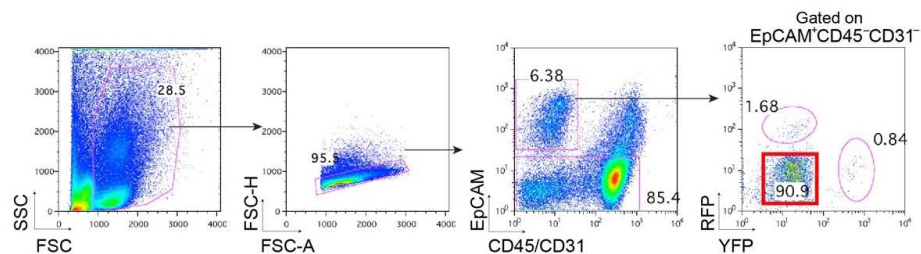
We further examined the potential cell conversion of secretory cells into AT2 cells in airways by assessing the expression of SPC in *dnMAML^{flox/+};Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs after bleomycin injury. Lineage-labelled AT2 cells were observed only in alveoli but not in airways (**Rev1_Fig. 6**).

Rev1_Figure 6. Lineage-labelled AT2 cells in the alveoli of *dnMAML^{flox/+};Scgbl1a1-CreER^{TM/+};R26R^{tdTomato/+}* mice after bleomycin injury. Representative IF image showing the derivation of *Scgbl1a1*⁺ lineage-labelled

AT2 cells expressing SPC in the alveoli of bleomycin-treated mice. Of note, there are no SPC-expressing AT2 cells in airways by inhibition of Notch activity. Tomato (for *Scgb1a1* lineage, red), SPC (white), and DAPI (blue). Scale bar, 100µm.

3. For Figure 3, it is unclear how YFP⁺ AEC2s are isolated. Please clarify as to how the RFP⁺/YFP⁺ AEC2 population is derived on single cell seq analysis in Fig 3I.

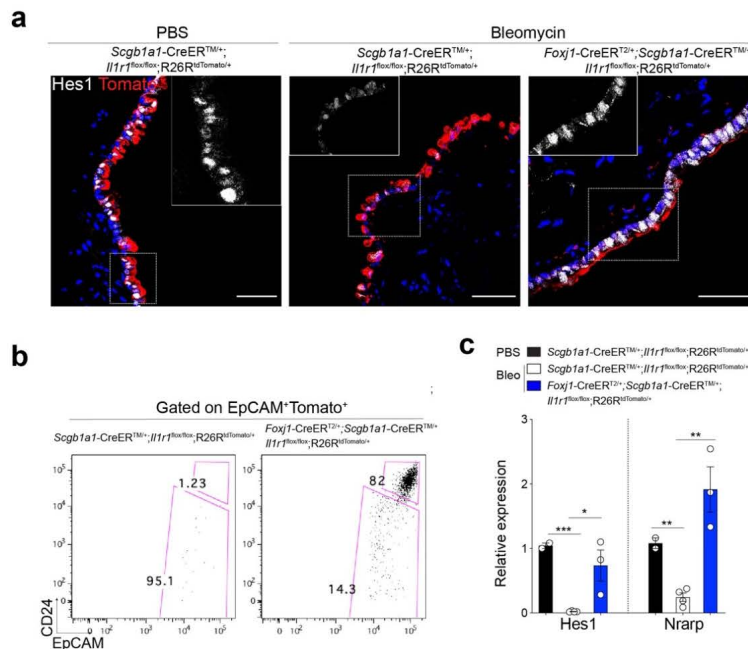
Response: We obtained non-lineage-labelled epithelial cells by sorting for CD31⁺CD45⁺EpCAM⁺RFP⁺YFP⁺ populations, which include endogenous AT2 cells that are not derived from labelled secretory cells (**Rev1_Fig. 7**). We have added a detailed description of how to isolate each population for the generation of libraries for scRNA-seq in the revised manuscript (**Revised Extended Data Fig. 8a**).



Rev1_Figure 7. Sorting strategy for unlabelled (RFP⁺YFP⁺) epithelial cells after bleomycin injury.

4-1. Figure 4 presents very intriguing data that Notch ligand expression in ciliated cells are regulated by inflammation and modulates Club cell transdifferentiation. However, this hypothesis is still largely based on circumstantial evidence as presented. The data that IL1R1 deletion reduces Jag1/2 in ciliated cells, and the data that club cells transdifferentiates into AEC2s with ciliated IL1R1 deletion could be true-true but unrelated. It would be important to assess whether Notch activation in the club cells at the airway is altered by IL1R1 deletion in ciliated cells during injury.

Response: We are pleased to see that the Reviewer finds these data interesting. As the Reviewer pointed out, we carefully examined the expression of Hes1 to determine Notch activity in secretory cells. The expression level of Hes1 was highly maintained in homeostatic secretory cells, but dramatically reduced after bleomycin injury, which is consistent with the downregulation of Notch ligands in ciliated cells after injury (**Rev1_Fig. 8a**; **Revised Extended Data Fig. 10a**). Significantly, we found that the reduction of Hes1 expression in secretory cells was impaired by *Il1r1* deletion in ciliated cells after injury. Furthermore, we evaluated the transcriptional activity of Notch signalling by measuring downstream target genes, such as *Hes1* and *Nrarp*, in isolated lineage-labelled secretory cells (EpCAM⁺CD24⁺Tomato⁺) (**Rev1_Fig. 8b,c**; **Revised Extended Data Fig. 10b,c**). Consistent with IF data, deletion of *Il1r1* in ciliated cells altered Notch signalling in secretory cells after bleomycin injury.



Rev1_Figure 8. The activity of Notch signalling in secretory cells is altered by *Il1r1* deletion in ciliated cells.

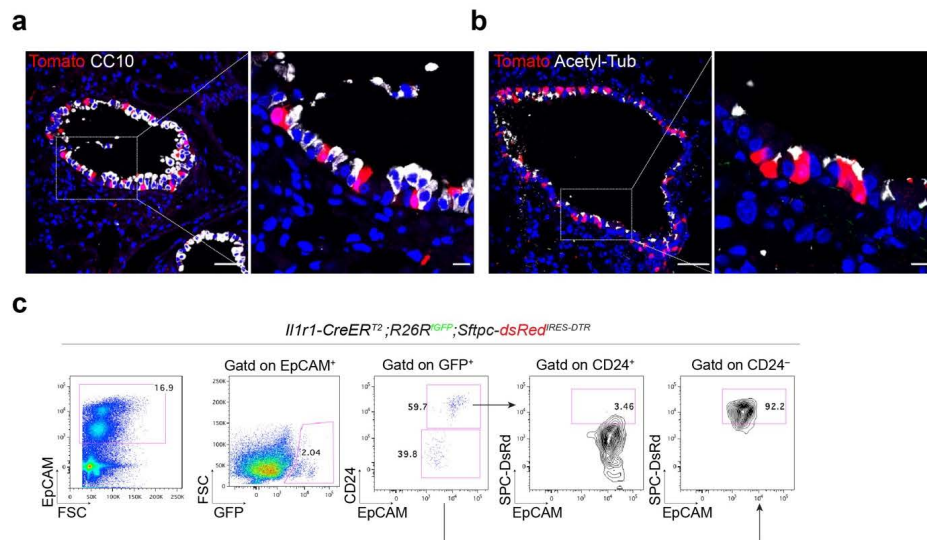
(a) Representative IF images showing Hes1 expression in lineage-labelled secretory cells at day 14 post bleomycin injury in the indicated genotype: Tomato (for *Scgb1a1* lineage, red), Hes1 (white), and DAPI (blue). Insets show high-power view. Scale bars, 50µm.

(b) Sorting strategy for lineage-labelled secretory cells isolated from PBS- or bleomycin-treated lungs in (a). EpCAM⁺CD24⁻ cells gated from EpCAM⁺Tomato⁺ were used for isolation of lineage-labelled secretory cells and qPCR analysis.

(c) qPCR analysis of downstream target genes of Notch signalling, including *Hes1* and *Nrarp*, in isolated lineage-labelled secretory cells in (b). *p<0.05, **p<0.01, ***p<0.001.

4-2. Additionally, in figure 4b, what is the cellular composition of *Il1r1*⁺ CD24⁺ cells (% ciliated cells vs other cell types) vs. *Il1r1*⁺/CD24⁻ cells?

Response: In the distal lungs, we found that *Il1r1* is specifically expressed in ciliated cells in airways (Rev1_Fig. 9a,b), and in the subsets of AT2 cells in alveoli (Choi et al., 2020). IF images of *Il1r1*⁺ lineage labelled cells revealed the specific expression of *Il1r1* only in ciliated cells, not in secretory cells, in airways (Rev1_Fig. 9a,b). Further, qPCR analysis for the ciliated cell marker *Foxj1* confirmed that EpCAM⁺*Il1r1*⁺CD24^{high} cells are composed of ciliated cells (Revised Extended Data Fig. 9e). In contrast, as we previously showed (Choi et al., 2020), EpCAM⁺*Il1r1*⁺CD24⁻ cells consisted of AT2 cells. Flow cytometry analysis of isolated lung cells from *Il1r1-CreERTTM+/+*; *R26R^{GFP}/+*; *Sftpc-dsRed* mice revealed that EpCAM⁺*Il1r1*⁺CD24⁻ cells are SPC⁺ AT2 cells (Rev1_Fig. 9c).



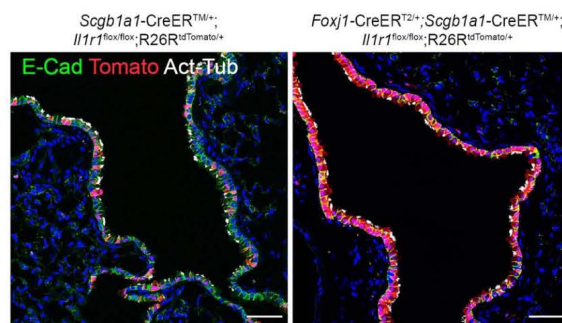
Rev1_Figure 9. *IIR1* expression marks ciliated cells in airways and subsets of AT2 cells in alveoli.

(a-b) Representative IF images showing that *IIR1* expression labels ciliated cells, not secretory cells in homeostatic airways at day 14 post two doses of tamoxifen injection: Tomato (for *IIR1* lineage, red), CC10 (white, a), Act-Tub (white, b), and DAPI (blue). Scale bars, 50µm.

(c) *IIR1*⁺CD24⁻ cells are composed of *SPC*⁺ AT2 cells. Flow cytometry analysis of *IIR1*⁺ lineage-labelled AT2 cells from *IIR1-CreERT2*^{+/+}; *R26R*^{tdTomato}^{+/+}; *Sftpc-dsRed*^{RES-DTR}^{+/+} reporter mouse lungs.

4-3. In figure 4E, what is missing is the effect of IL1R1 deletion in ciliated cells during PBS, which is important to determine whether there is tonic IL1 stimulation in the airways during homeostasis.

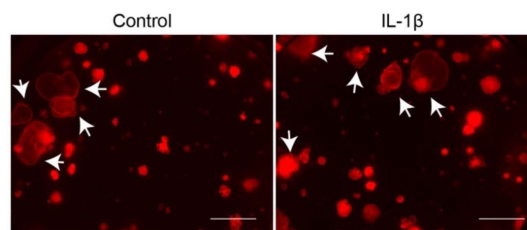
Response: Following the Reviewer's comment, we carefully checked the morphology and percentage of ciliated cells in homeostatic airways upon *IIR1* deletion in ciliated cells. There was no discernible alteration in the morphology and frequency of ciliated cells based on the expression of E-cadherin and Acetylated-Tubulin (**Rev1_Fig 10; Revised Extended Data Fig. 11a**).



Rev1_Figure 10. *IIR1* deletion in ciliated cells does not induce significant phenotypic alteration in homeostatic airways. Representative IF images showing ciliated cells in the airways of *IIR1*^{lox/lox}; *Scgb1a1-CreERTM*^{+/+}; *R26R*^{tdTomato}^{+/+} (control) or *IIR1*^{lox/lox}; *Foxj1-CreERT2*^{+/+}; *Scgb1a1-CreERTM*^{+/+}; *R26R*^{tdTomato}^{+/+} mice at 28 days after PBS treatment. Tomato (for *Scgb1a1* or *Foxj1* lineage, red), Acetylated-Tubulin (Act-Tub, white), E-Cadherin (E-Cad, green), and DAPI (blue). Scale bars, 50µm.

4-4. Finally, does IL1b have an effect on club cell organoids that might be independent of notch signalling?

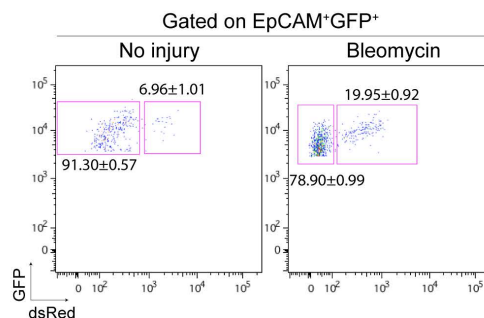
Response: As shown in **Rev1_Fig. 11**, IL-1 β treatment does not significantly affect the overall size and efficiency of secretory cell-derived organoid formation compared to control. As *Il1r1* is not expressed in the secretory cells, it is likely that IL-1 β signalling does not directly modulate the behaviour of secretory cells.



Rev1_Figure 11. Effect of IL-1 β treatment in secretory cell-derived organoids. Representative fluorescent images of organoids derived from lineage-labelled secretory cells from *Scgb1a1-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs. Organoids were cultured with PBS or IL-1 β for 14 days. Arrows point to cystic airway-like organoids. Scale bar, 2,000 μ m.

5-1. Same point regarding the *Scgb1a1creERT* for the ATAC-seq analysis. To provide more assurance that the secretory AT2s are mostly derived from airway *Scgb1a1*⁺ cells (and not endogenous *Scgb1a1*⁺/SPC⁺ cells in the alveoli) in the ATAC-seq, authors should demonstrate whether there is an increase in GFP⁺/DsRed⁺ population (Figure 5b) after injury compared to homeostasis, to assess whether there is an increased proportion of GFP⁺ cells (secretory) that acquire AT2 fate (DsRed⁺).

Response: As suggested, we confirmed the increase of secretory cell-derived AT2 cells (GFP⁺dsRed⁺) in *Scgb1a1-CreER^{TM/+}; R26R^{GFP/+}; Sftpc-dsRed^{ires-DTR/+}* mouse lungs after bleomycin injury compared to PBS control by flow cytometry analysis (**Rev1_Fig. 12**; **Revised Extended Data Fig. 12**).

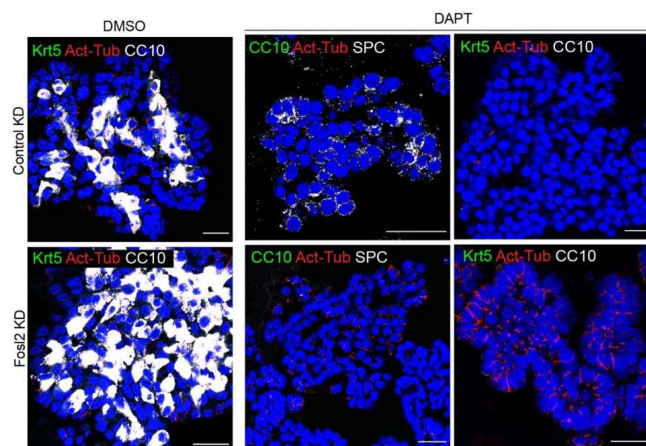


Rev1_Figure 12. Enhanced differentiation of SPC⁺ AT2 cells derived from secretory cells after bleomycin injury. Flow cytometry analysis of secretory cell-derived AT2 cells isolated from *Scgb1a1-CreER^{TM/+};R26R^{GFP/+};Sftpc-dsRed^{ires-DTR/+}* mouse lungs after PBS (left) or bleomycin (right) treatment. Data are presented as mean $\pm$ s.e.m (n=2 individual biological replicate).

5-2. Furthermore, the demonstration that *Fos1* KD suppresses *Sftpc* expression alone does not prove *Fos1* is necessary for AEC2 fate. An alternative explanation is that *Fos1* might be repressing the fate of

an alternative lineage (e.g. basal, ciliated, and etc.), and KD of *Fosl1* results in increase in a non-AEC2 lineage derived from club cells. This should be interrogated by immunophenotyping organoids derived from DAPT treated and *Fosl1* KD conditions. Do these organoids express no mature markers of differentiation? Or do they express alternative lineage markers that are neither secretory nor AEC2.

Response: We further investigated if *Fosl2* KD alters the differentiation capacity of secretory cells into non-AT2 lineages with or without DAPT treatment. Similar to control secretory organoids (control KD), *Fosl2* KD-secretory organoids consisted of mainly CC10⁺ secretory cells and some Act-Tub⁺ ciliated cells in our culture condition, suggesting that, in the presence of Notch activity, *Fosl2* KD itself does not alter the lineage differentiation capacity of secretory cells into other lineages, including Krt5⁺ basal or Act-Tub⁺ ciliated cells (**Rev1_Fig. 13**, DMSO control; **Revised Fig. 5g**). We then examined whether *Fosl2* KD modulates the secretory differentiation when Notch activity is inhibited. As expected, DAPT treatment dramatically facilitated SPC⁺ AT2 cell differentiation at the expense of CC10⁺ secretory cells in control organoids (**Rev1_Fig. 13**, DAPT treatment in control KD organoids; **Revised Fig. 5g**). There were no discernible effects in other lineages, such as basal or ciliated cells, in DAPT-treated control organoids. However, Significantly, *Fosl2*-deficient secretory cells failed to generate SPC⁺ AT2 cells in secretory organoids even with DAPT treatment, suggesting that *Fosl2* is required for AT2 cell conversion of secretory cells by Notch inhibition (**Rev1_Fig. 13**, DAPT treatment in *Fosl2* KD organoids; **Revised Fig. 5g**). Interestingly, we observed that *Fosl2* KD did not alter the reduction of secretory cells, but revealed increased ciliated cells in DAPT-treated organoids. These results suggest that *Fosl2* is a critical regulator for switching the fate conversion of secretory cells into AT2 cells by Notch inhibition during alveolar regeneration after injury.

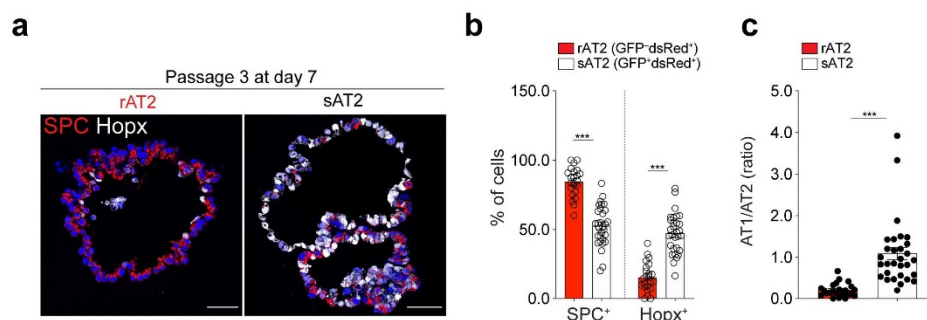


Rev1_Figure 13. *Fosl2* is required for the fate conversion of secretory cells into AT2 cells by Notch inhibition. Representative IF images of secretory cell-derived control (top) or *Fosl2* KD (bottom) organoids treated with DMSO or DAPT (20μM). CC10 (white, left and right panels; green, middle panels), SPC (white, middle panels), Krt5 (green, left and middle panels), Act-Tub (red), and DAPI (blue). Scale bars, 50μm.

6. The data in Figure 6 that secretory-derived AEC2s have more clonogenic capacity is really interesting, but perhaps more details could be given in terms of the exact culture condition and what is the cell composition of the organoids at late passage.

Response: We thank the Reviewer for appreciating our new findings. We co-cultured resident AT2 (rAT2) or secretory cell-derived AT2 (sAT2) cells with lung stromal cells as described in the **Methods**. In

this culture condition, sAT2 cells maintained organoids without loss of colony formation efficiency (CFE) over multiple passages, whereas rAT2 cells lost CFE (**Revised Fig. 6e,f**). Interestingly, we found that sAT2-derived organoids retained greater number of AT1 cells compared to rAT2-derived organoids (**Rev1_Fig. 14a; Revised Extended Data Fig. 15a**). The ratio of AT1 versus AT2 cells in sAT2-organoids was significantly increased compared to that of rAT2-organoids (**Rev1_Fig. 14b,c; Revised Extended Data Fig. 15b,c**). These data suggest that sAT2 cells have an enhanced capacity to give rise to AT1 lineage cells compared to rAT2 cells, which may result in a prolonged contribution to alveolar regeneration after chronic damage.



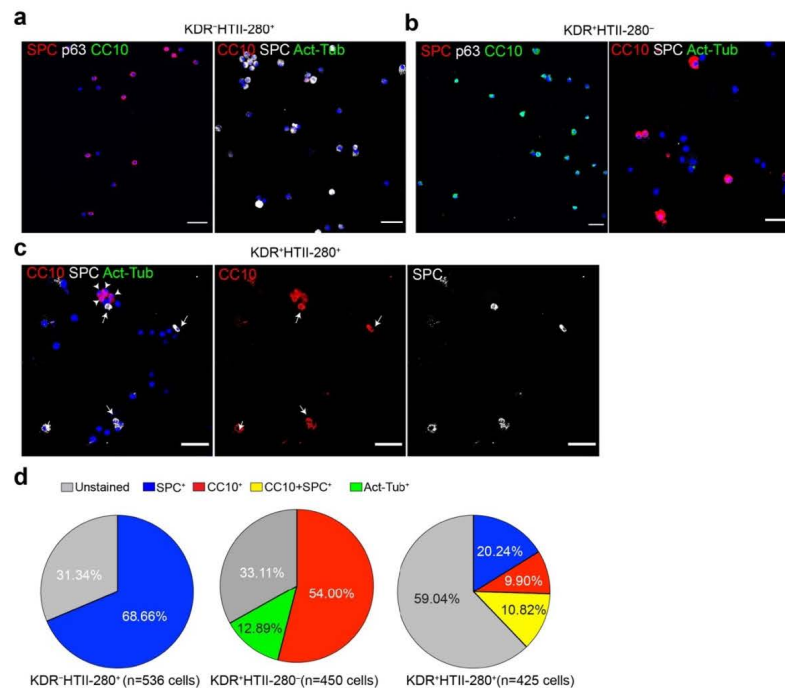
Rev1_Figure 14. Enhanced differentiation capacity of secretory-derived AT2 cells into AT1 lineage.

(a) Representative IF images of organoids derived from rAT2 or sAT2 cells. SPC (for AT2 cells, red), Hopx (for AT1 cells, white), and DAPI (blue). Scale bars, 50 μm.

(b, c) Quantification of the frequency of AT2 (SPC⁺) or AT1 (Hopx⁺) cells (b) and the ratio of AT1/AT2 cells (c) in rAT2- or sAT2-derived organoids. Each individual dot represents one organoid and data are presented as mean ± s.e.m (n=2 independent experiments). ***p<0.001.

7-1. The discovery of KDR as a potential tool to isolate secretory cells in human could prove to be very useful for the field, but more is needed to validate the strategy aside from qPCR shown in Figure 7c. It would be important to include more precise information about what fraction of different epithelial lineages are in the KDR⁺/HTII280⁺, KDR⁺/HTII280⁻, KDR⁻/HTII280⁺ populations (with a cytospin).

Response: As suggested, we performed cytospin staining to verify the cellular composition in the population of KDR⁺HTII-280⁺, KDR⁺HTII-280⁻, and KDR⁻HTII-280⁺ human cells. As expected from our qPCR analysis (**Revised Fig 7e**), the KDR⁺HTII-280⁺ cell population was enriched for SPC⁺ AT2 cells (**Rev1_Fig. 15a,d; Revised Fig. 7c,d**). We barely observed other lineages such as p63⁺ basal cells, CC10⁺ secretory cells, or Act-Tub⁺ ciliated cells in this population. The population of KDR⁺HTII-280⁻ cells revealed enriched CC10⁺ secretory cells (**Rev1_Fig. 15b,d; Revised Fig. 7c,d**). Consistent with scRNA-seq analysis (**Revised Extended Data Fig. 16f**), this population also included small proportion of Act-Tub⁺ ciliated cells that may represent differentiated ciliated cells from secretory cells. The population of KDR⁺HTII-280⁺ cells consisted of heterogeneous cells including CC10⁺ secretory cells and SPC⁺ AT2 cells (**Rev1_Fig. 15c,d**). Interestingly, we observed a small number of CC10⁺SPC⁺ BASC-like population in KDR⁺HTII-280⁺ population. However, the percentage of KDR⁺HTII-280⁺ cells varied from sample to sample. In addition, BASC-like CC10⁺SPC⁺ dual positive population has not been reported yet in the human lung tissues. Thus, it is likely that further validation of the KDR⁺HTII-280⁺ population, in addition to cytospin staining, would be needed.



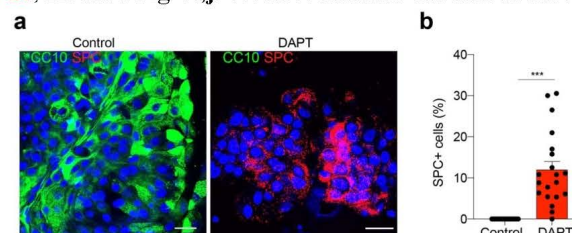
Rev1_Figure 15. KDR expression marks secretory cells in the human lung tissue.

(a-c) Representative IF images of cytospin staining from sorted KDR⁺HTII-280⁺ (a), KDR⁺HTII-280⁻ (b), and KDR⁺HTII-280⁺ (c) populations. SPC (for AT2 cells), p63 (for basal cells), Acetylated Tubulin (Act-Tub, for ciliated cells), and DAPI (blue). Arrowheads point to CC10⁺SPC⁻ and arrows point to CC10⁺SPC⁺ cells in (c). Scale bars, 100µm.

(d) Quantification of SPC⁺, CC10⁺, Act-Tub⁺, and CC10⁺SPC⁺ cells in freshly sorted KDR⁺HTII-280⁺, KDR⁺HTII-280⁻, and KDR⁺HTII-280⁺ cells. Of note, there were no P63⁺ cells in these populations.

7-2. Furthermore, for the data showing that DAPT is increasing alveolar lineage differentiation, it would be important to quantify it at the protein level (e.g. IHC, % cell or organoid that are positive for specific marker) to show certain canonical AEC2 markers are present (e.g. % cell or organoid with SPC, LAMP3, ABCA3, and etc), because SP-B is not specific to AEC2s in humans and it is not really clear what anti-HTII-280 is actually marking.

Response: As suggested, we performed IF staining for SPC in DAPT-treated organoids to specifically quantify AT2 cells, in addition to staining for SP-B and LPCAT1. Consistent with other AT2 markers, SPC⁺ AT2 cells were significantly increased in DAPT-treated organoids compared to control secretory organoids (Rev1_Fig 16; Revised Fig. 7i,j. We have included this data in our revised manuscript.



Rev1_Figure 16. Notch inhibition facilitated the differentiation of secretory cells into AT2 cells in human secretory organoids.

(a) Representative IF images of secretory cell-derived organoids treated with DMSO or DAPT (20 μ M). CC10 (for secretory cells, green), SPC (for AT2 cells, red), and DAPI (blue). Scale bar, 50 μ m.

(b) Quantification of the frequency of SPC⁺ cells in DMSO control or DAPT treated organoids. Each individual dot represents one organoid and data are presented as mean $\pm$ s.e.m (n=3 independent experiments). ***p<0.001.

Minor Comments:

Minor-1. When graphs with individual data points are presented throughout the manuscript, it is not always clear in the legends what each dot represents in terms of biological vs. technical replicates.

Response: We have now included the detailed description for biological or technical replicates in the figure legends in our revised manuscript. For biological individual mouse or experiment, we added biological replicates as number “n”.

Minor-2. The abstract is nicely written but perhaps a bit overstated. It is not clear that the authors produced enough evidence that there is a “2-stage process” for Notch-mediated club-AEC2 differentiation, if so, it is not well delineated as to what is stage 1 and 2. Furthermore, the term “transcriptional licensing” would be more accurately described as a factor that is capable of initiating origin of replication at the DNA level, or acting as a pioneer transcription factors, which is not really demonstrated in the manuscript. I would probably remove these terms and save them for speculation in Discussion.

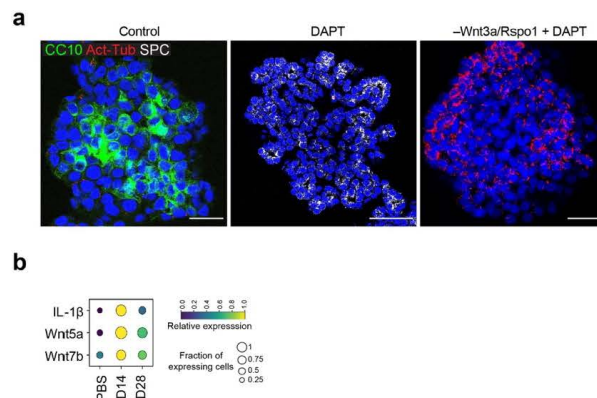
Response: We thank the Reviewer for this valuable comment. We have removed this sentence in the abstract, but further discussed our hypothesis of 2-stage process in the discussion section based on our new findings in the revised manuscript.

Minor-3. Finally, a big question from this manuscript is why does downregulation of Notch leads to different secretory cell differentiation outcomes in different contexts (differentiation to ciliated cell in homeostasis, AEC2s in injury). Addressing this experimentally is beyond the scope of a revision, but some speculation as to possible mechanisms and acknowledging the knowledge gap in the Discussion would help the field move forward.

Response: We thank the Reviewer for raising this important question, but appreciating that it is beyond the scope of our study. Previous studies and our work (**Rev1_Fig. 5**) showed that inhibition of Notch activity promotes the differentiation of secretory cells into ciliated cells in lung development and homeostatic adult lungs, although there are some differences in the incidence of ciliated cell differentiation, potentially due to different model systems (e.g. genetic models vs. pharmacological models) (Lafkas et al., 2015; Morimoto et al., 2010; Pardo-Saganta et al., 2015). In this study, we demonstrated that, during alveolar regeneration after injury, the downregulation of Notch signalling facilitated the differentiation of secretory cells into AT2 cells via a Il1r1-Notch-Fosl2 axis. By developing a feeder-free organoid culture conditions for secretory and AT2 cells, we confirmed that DAPT treatment markedly increased AT2 cells at the expense of secretory cells in secretory organoids, suggesting that Notch inhibition leads to the differentiation of secretory cells into AT2 lineages (**Revised Fig. 2c-e**). There was no noticeable change in ciliated cells in these organoids. Furthermore, we found that Fosl2-deficient secretory cells failed to generate AT2 cells in DAPT-treated organoids, suggesting that Fosl2 is required for AT2 cell differentiation of secretory cells upon Notch inhibition (**Rev1_Fig. 13; Revised Fig. 5g**). Interestingly, we observed that Fosl2 KD failed to recover reduced secretory cells, but induced the production of ciliated cells in DAPT-treated organoids (**Rev1_Fig. 13; Revised Fig. 5g**).

These results suggest that *Fosl2* is a critical regulator for switching the fate conversion of secretory cells into AT2 cells by Notch inhibition during alveolar regeneration after injury.

How does *Fosl2* regulate this process? Interestingly, when we removed WNT signalling-inducing factors, including *Wnt3a* and *R-spondin-1*, in our culture conditions, DAPT treatment induced *Act-Tub*⁺ ciliated cells at the expense of secretory cells in secretory organoids (**Rev1_Fig. 17a; Revised Extended Data Fig.17a**). AT2 cells were barely observed in these organoids. This result suggests that Notch and WNT signalling are likely to cooperatively function for AT2 cell differentiation from secretory cells. Our, and others, previous studies reported the increase of Wnt ligand expressions in mesenchyme cells during alveolar regeneration after injury (Lee et al., 2017; Zepp et al., 2017). We showed that Wnt ligands secreted from *Lgr6*⁺ airway mesenchyme cells are important for secretory cell differentiation into AT2 lineages (Lee et al., 2017). Further, analysis of scRNA-seq in our previous study (Choi et al., 2020) showed dynamic expressions of Wnt ligands during alveolar regeneration. Wnt ligands, such as *Wnt5a* and *Wnt7b*, were highly upregulated at day 14 and returned back to homeostatic levels at day 28 post bleomycin injury (**Rev1_Fig. 17b; Revised Extended Data Fig.17b**). Given these results, it is likely that increased Wnt ligands from mesenchyme cells, in combination with Notch-mediated *Fosl2* activity in secretory cells, may cooperatively confer the differentiation potential of secretory cells to AT2 lineages during alveolar regeneration after injury. While *Fosl2* was identified as a key factor in the axis of Notch signalling, further identification of interacting partners regulated by Wnt signalling will uncover the molecular networks governing the fate decision of secretory cells into ciliated or AT2 cells in homeostasis and regeneration. We have included these data and discussed the potential regulation of secretory cells at different context in our revised manuscript.



Rev1_Figure 17. AT2 lineage conversion of secretory cells by Notch inhibition requires WNT activity.

(a) IF images of secretory organoids treated with DMSO (control) or DAPT (20µM) in the presence (middle) or withdrawals of WNT signalling (right). CC10 (for secretory cells, green), Acetylated Tubulin (for ciliated cells, Act-Tub, red), SPC (for AT2 cells, white), and DAPI (blue). Scale bars, 50µm.

(b) Gene expression of *IL-1β*, *Wnt5a*, and *Wnt7b* at indicated time points after bleomycin injury. Data were from our previous study (Choi et al., 2020).

Reviewer #2:

This is a very elegant paper describing how alveolar injury induces IL1b from ciliated cells, which inhibit Notch signaling between ciliated and neighboring secretory cells, which in turn, through derepression of Fosl2, permits differentiation into AT2 cells. In addition, and very interestingly, the authors show that AT2 derived from secretory cells differ from other AT2 cells. This paper reads very well, offers in depth mechanistic insight into lung regeneration, and will be important for the field. I have a few comments, the most important of which is on physiological relevance.

1. Does interfering with the mechanism (be it at the IL1r, Notch or Fosl2 level) interfere with regeneration after bleomycin injury? In other words, this paper shows what can happen, not how important it is physiologically.

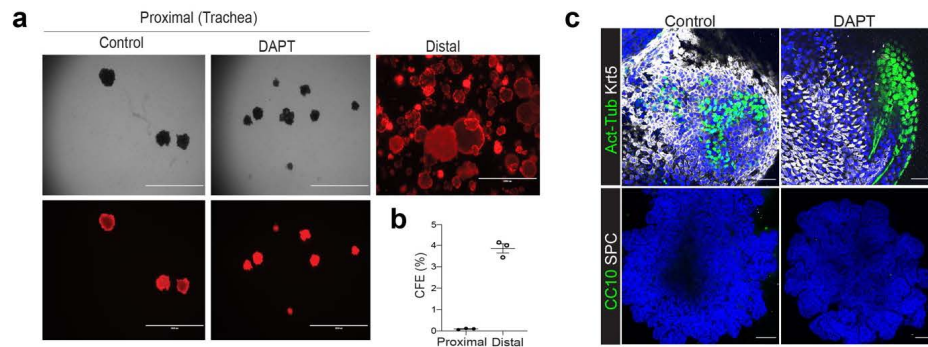
Response: We appreciate the Reviewer's valuable comment. Although resident AT2 cells serve as stem cells to maintain homeostatic and regenerative lungs, airway secretory cells are known to contribute to alveolar regeneration upon severe injury, including bleomycin and Influenza infection. How inflammatory microenvironments caused by tissue injury affect their regenerative contribution during tissue repair is less clear. Our study shows the molecular and cellular links between inflammation and cell fate conversion of airway stem/progenitor cells during alveolar regeneration after injury. We demonstrated that the deletion of *Il1r1* in ciliated cells or persistent Notch activity in secretory cells impaired alveolar regeneration derived from secretory cells with increased fibrotic regions after bleomycin injury. Furthermore, Fosl2-deficient secretory cells also failed to contribute to alveolar differentiation. These results strongly suggest that the contribution of secretory cells to alveolar regeneration via *Il1r1*-Notch-Fosl2 axis upon lung injury. Importantly, we showed the greater regenerative potential of secretory cell-derived AT2 cells based on their long-term maintenance and AT1 differentiation ability in *ex vivo* organoid models, suggesting the potential roles of secretory cells in chronic lung disease.

2. If SCO were isolated from more proximal airway, would they still generate AT2 after Notch inhibition, or might there be heterogeneity in the capacity of secretory cells to do this? Would the cells capable of doing this be variant secretory cells?

Response: We thank the Reviewer for interesting questions. To investigate the potential contribution of proximal secretory cells to alveolar lineages, we isolated lineage-labelled secretory cells from *Scgblal-CreER^{TM/+}; R26R^{tdTomato}* mouse tracheas after tamoxifen treatment. As a control, we also isolated lineage-labelled secretory cells from distal lungs. Isolated cells were placed in our organoid culture conditions described in our manuscript. The organoid forming efficiency of tracheal secretory cells was substantially lower than that of distal secretory cells in this culture condition (**Rev2_Fig. 1a,b**). IF staining revealed that tracheal secretory cell-derived organoids mainly consisted of Krt5⁺ basal cells and Act-Tub⁺ ciliated cells (**Rev2_Fig. 1c**). Surprisingly, CC10⁺ secretory cells were barely observed in these organoids, suggesting de-/trans-differentiation of tracheal secretory cells into basal or ciliated cells in this culture condition. Furthermore, there was no discernible effect by DAPT treatment in these organoids, indicating that secretory cells in proximal airways may differently behave in response to Notch inhibition at least *in vitro* model system.

It has been reported that subsets of secretory cells including Upk3a⁺ variant club cells, H2-K1^{high} secretory cells, and CC10⁺SPC⁺BASCs at the BADJ are capable of differentiating into AT2 cells after bleomycin injury (Guha et al., 2017; Kathiriya et al., 2020; Liu et al., 2019; Salwig et al., 2019). Thus, these subsets of secretory cells localised in the distal lungs, not proximal region, require downregulation

of Notch activity to differentiate into AT2 cells during alveolar regeneration. Further study to address the functional heterogeneity of secretory cells at different context would be important to understand their contribution to regeneration in various lung diseases.



Rev2_Figure 1. Organoid assays derived from secretory cells in proximal and distal airways.

(a) Representative bright-field or fluorescent images of organoids derived from lineage-labelled *Tomato*⁺*Scgb1a1*⁺ secretory cells in the trachea and distal lung. Cells were cultured with WNT3A, RSPO1 (R-spondin 1), EGF, FGF7, FGF10, and NOG (Noggin), with or without DAPT (20μM). Organoids derived from distal secretory cells were used for positive control experiment. Scale bar, 2,000μm.

(b) Statistical quantification of colony forming efficiency.

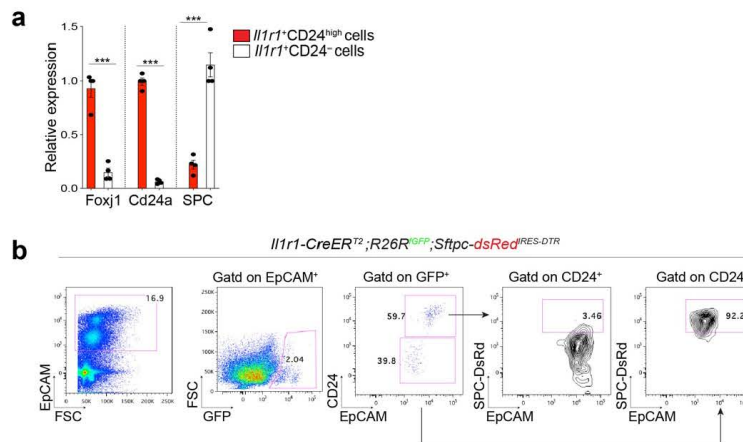
(c) Representative IF images of tracheal secretory cell-derived organoids. Acetylated Tubulin (Act-Tub, green), Krt5 (white), CC10 (green, bottom), SPC (white, bottom), and DAPI (blue). Scale bars, 50μm.

3. Several data require ANOVA rather than a t-test for statistical analysis, as multiple groups are compared. These include Fig. 2i, 4b, 4e.

Response: As suggested, we applied 2-way ANOVA test for statistical analysis of multiple groups in our revised manuscript.

4. In ED 4e, what are the dTom⁺CD24⁻ cells? And why do they express so little IL1r if they are dTom⁺? What are those cells?

Response: We apologize for typos in this graph. Given that *Il1r1* expression marks ciliated cells in airways and subsets of AT2 cells in alveoli (Choi et al., 2020), we isolated ciliated cells (EpCAM⁺*Il1r1*⁺CD24^{high}) and AT2 cells (EpCAM⁺*Il1r1*⁺CD24⁻) for further validation for qPCR analysis. We amended “*Il1r1*” to “*Cd24a*” in the qPCR panel and added the expression level of *SPC* (**Rev2_Fig. 2a**; **Revised Extended Data Fig. 9e**) in our revised manuscript. Additionally, we further verified that EpCAM⁺*Il1r1*⁺CD24⁻ cells are *SPC*⁺ AT2 cells by flow cytometry analysis of isolated cells from *Il1r1*-CreER^{T2/+};R26R^{GFP/+};Sfipc-dsRed reporter mouse lungs (**Rev2_Fig. 2b**).



Rev2_Figure 2. *Il1r1* expression marks ciliated cells in airways and subsets of AT2 cells in alveoli.

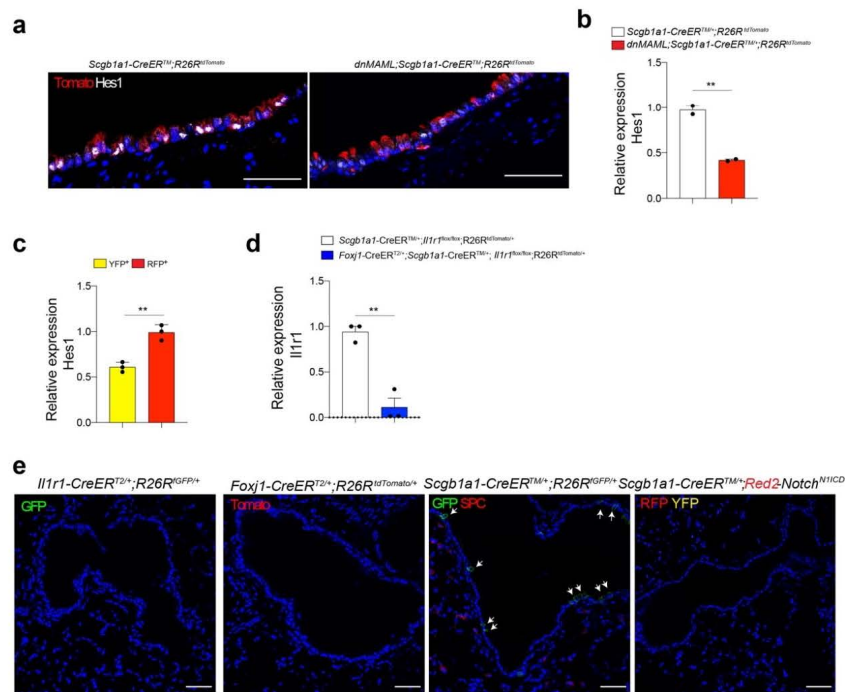
(a) qPCR analysis of *Foxj1*, *Cd24a*, and *SPC* expression in isolated cells from *Il1r1-CreER^{T2/+};R26R^{tdTomato/+}* mouse lungs. Each individual dot represents one individual biological experiment and data are presented as mean $\pm$ s.e.m. *** $p < 0.001$.

(b) *Il1r1*⁺CD24⁻ cells are *SPC*⁺ AT2 cells. Flow cytometry analysis of *Il1r1*⁺ lineage-labelled cells from *Il1r1-CreER^{T2/+};R26R^{GFP/+};Sftpc-dsRed* reporter mouse lungs.

5. Very complex mouse models are used. Do you have any idea of deletion efficiency and leakiness of these lineage tracing reporters?

Response: We carefully validated our mouse models. First, we examined Notch activity in *Scgblal-CreER^{TM/+};R26R^{tdTomato/+}* and *dnMAML^{flox/+};Scgblal-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs by assessing *Hes1* expression in lineage-labelled secretory cells. Induction of *dnMAML* expression revealed the reduction of Notch activity compared to control based on the expression level of *Hes1* by IF staining and qPCR analysis for *Hes1* (Rev2_Fig. 3a,b; Revised Extended Data Fig. 3). We also checked Notch activity in the mouse model of *Scgblal-CreER^{TM/+};Red2-Notch^{NIICD}*. qPCR analysis revealed that RFP⁺ cells expressing *NIICD* showed 2-fold higher expression of *Hes1* compared to YFP⁺ control cells (Rev2_Fig. 3c). Second, we validated the deletion efficiency of *Il1r1* in ciliated cells of *Foxj1-CreER^{T2/+};Scgblal-CreER^{TM/+};Il1r1^{flox/flox};R26R^{tdTomato/+}* mouse lungs. As there is no available antibody to detect the expression level of *Il1r1* at protein level, we performed qPCR analysis in EpCAM⁺CD24^{high} ciliated cells isolated from *Scgblal-CreER^{TM/+};Il1r1^{flox/flox};R26R^{tdTomato/+}* (control) and *Foxj1-CreER^{T2/+};Scgblal-CreER^{TM/+};Il1r1^{flox/flox};R26R^{tdTomato/+}* mouse lungs. The expression level of *Il1r1* was significantly reduced in ciliated cells of *Foxj1-CreER^{T2/+};Scgblal-CreER^{TM/+};Il1r1^{flox/flox};R26R^{tdTomato/+}* mouse lungs compared to control lungs, suggesting the efficient deletion of *Il1r1* in these cells (Rev2_Fig. 3d).

Next, we checked the leakiness of the mouse models used for lineage-tracing analysis. There were no lineage-labelled cells without tamoxifen injection both in *Il1r1-CreER^{T2/+};R26R^{GFP/+}* and *Foxj1-CreER^{T2/+};R26R^{tdTomato/+}* mouse lungs (Rev2 fig. 3e). Whereas we detected low incidence of labelled secretory cells in corn oil treated *Scgblal-CreER^{TM/+};R26R^{GFP/+}* mouse lungs, consistent with an original paper generating this mouse model (Rawlins et al., 2009), our new mouse models of *Red2-Notch^{NIICD};Scgblal-CreER^{TM/+}* didn't show any labelled secretory cells with corn oil treatment (Rev2 fig. 3e).



Rev2_Figure 3. Validation of the efficiency and leakiness of reporter mouse models.

(a) Representative IF images showing Hes1 expression in lineage-labelled secretory cells after tamoxifen treatment in the indicated genotype: Tomato (for *Scgb1a1* lineage, red), Hes1 (white), and DAPI (blue). Scale bars, 50µm.

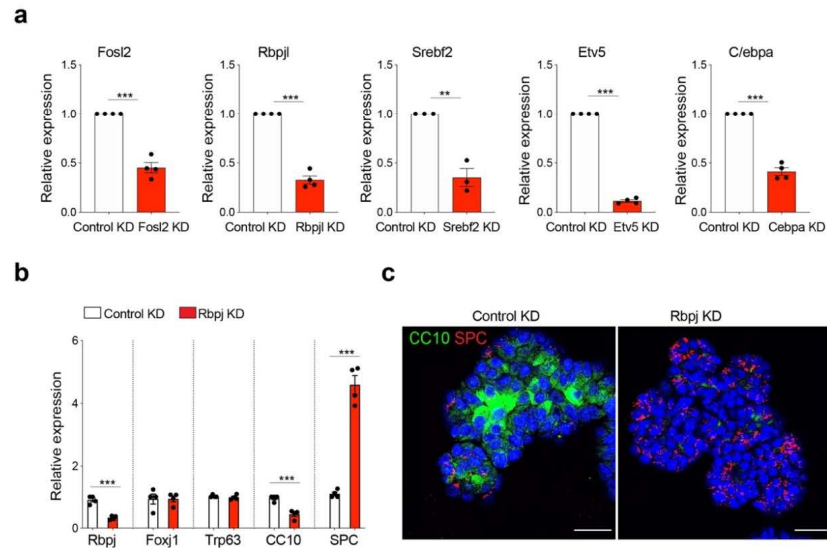
(b-c) qPCR analysis of *Hes1* expression in isolated EpCAM⁺Tomato⁺ (a), EpCAM⁺RFP⁺, or EpCAM⁺YFP⁺ (c) cells. Each individual dot represents one individual biological experiment (n=2 mice for b, n=3 mice for c) and data are presented as mean ± s.e.m. **p<0.01.

(d) qPCR analysis of *Il1r1* expression in isolated Tomato⁺EpCAM⁺CD24^{high} cells from indicated genotypes. Each individual dot represents one individual biological experiment (n=3 mice) and data are presented as mean ± s.e.m. **p<0.01.

(e) Representative IF images showing reporter expression after corn oil treatment in the indicated genotype: GFP (green), Tomato (red), SPC (red), RFP (red), YFP (yellow), and DAPI (blue). Arrows point to report-positive cells. Scale bars, 50µm.

6. How efficient was the knockdown of the genes shown in Fig. 5 and ED 5? (only shown for *Etv5*). How come that knockdown of *Rbpj*, which is involved in repressing *Fosl2*, still allows an effect of DAPT on the expression of AT2 markers? And did this knockdown induce any AT2 markers?

Response: We validated the successful knockdown (KD) of the genes we used in organoid experiments by qPCR analysis (Rev2_Fig. 4a; Revised Extended Data Fig. 13h). Further, we investigated the functional role of *Rbpj* in secretory cells by establishing *Rbpj* KD organoids. The expression level of *Rbpj* was significantly downregulated in *Rbpj* KD organoids compared to control (Rev2_Fig. 4b; Revised Extended Data Fig 2b). Notably, *Rbpj* KD in secretory cells markedly reduced *CC10* expression but increased *SPC* expression (Rev2_Fig. 4b; Revised Extended Data Fig 2b). IF staining confirmed that *Rbpj* KD increased SPC⁺ AT2 cells at the expense of CC10⁺ secretory cells even in the absence of DAPT in organoids, suggesting enhanced AT2 cell differentiation of secretory cells (Rev2_Fig. 4c; Revised Extended Data Fig 2c).



Rev2_Figure 4. Knockdown of Rbpj gene enhances the differentiation of secretory cells into AT2 lineages.

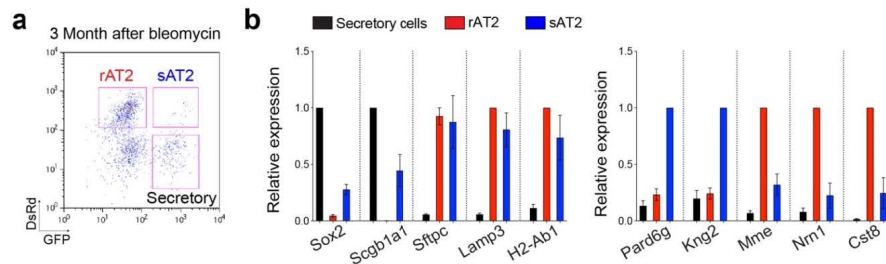
(a) qPCR analysis to confirm the knockdown (KD) efficiency of the genes that are used for indicated constructs in organoids. Each individual dot represents one individual biological experiment (n=4 for KD of *Fosl2*, *Rbpj*, *Etv5*, and *C/ebpa*, n=3 for KD of *Srebf2*) and data are presented as mean $\pm$ s.e.m. **p<0.01, ***p<0.001.

(b) qPCR analysis of gene expressions in Rbpj KD organoids. Each individual dot represents one individual experiment (n=4) and data are presented as mean $\pm$ s.e.m. ***p<0.001.

(c) Representative IF images of control KD or Rbpj KD organoids: CC10 (green), SPC (red), and DAPI (blue). Scale bars, 50 μ m.

7. The finding that sAT2 and rAT2 appear different is interesting. How long are these sAT2 detectable after injury?

Response: We were able to detect sAT2 cells (GFP⁺dsRed⁺) in the lung tissues of *Scgb1a1-CreER^{TM/+};R26R^{flGFP/+};Sftpc-dsRed* mice for least at 3 months post bleomycin injury (**Rev2 Fig. 5a**). Notably, they still retained distinctive features showing higher expression of secretory cell markers such as *Sox2* and *Scgb1a1* compared to rAT2 cells whereas the expression levels of canonical AT2 markers such as *SPC* and *Lamp3* were comparable (**Rev2 Fig. 5b**). In addition, sAT2-specific genes, including *Pard6g* and *Kn2*, were also still highly enriched in sAT2 at this time point, while rAT2 cells showed specific expression of rAT2-specific genes such as *Nrn1* and *Mme*.



Rev2_Figure 5. Long-term sustained distinct transcriptional signatures of sAT2 cells after injury repair.

(a) Flow cytometry analysis of *Scrgb1a1*⁺ lineage-labelled AT2 cells isolated from *Scrgb1a1-CreER*^{TM/+}; *R26R*^{fGFP/+}; *Sftpc-dsRed*^{RES-DTR/+} mouse lungs at 3 months after bleomycin injury.

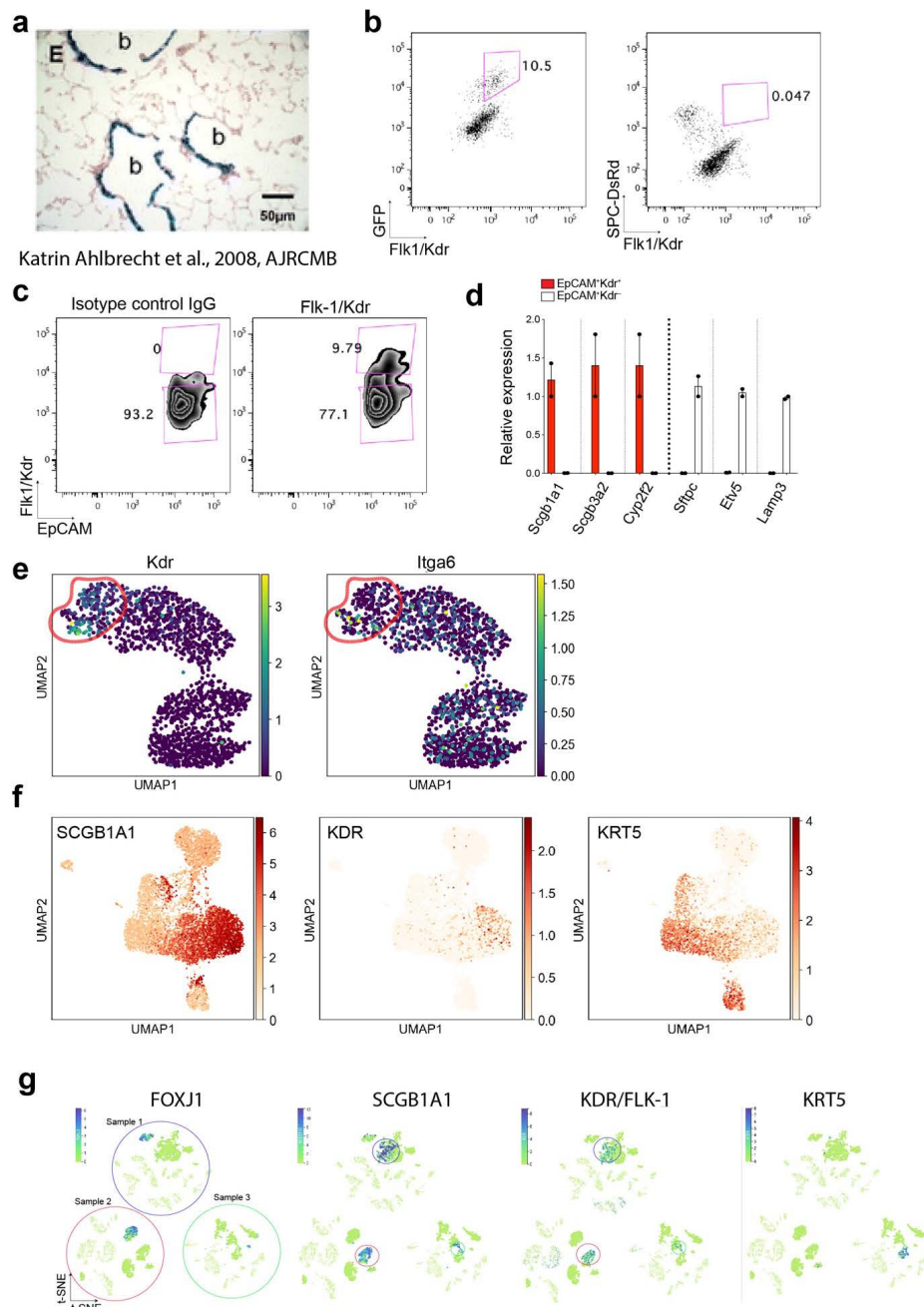
(a) qPCR analysis of the genes in isolated secretory, rAT2, or sAT2 cells. Data are presented as mean ± s.e.m (n=4 individual mice).

8-1. The human data focus on KDR as a marker for secretory cells. This part is, admittedly likely by necessity, less strong. The data are shown as MFI (Fig. ED7d). What fraction is really positive? Is there heterogeneity? According to ref. 38, KDR is expressed in basal cells. scRNAseq also shows KDR to be expressed in only a fraction of secretory cells, and in other cell types as well (ED 7f).

Response: Consistent with our scRNA-seq data showing the restricted expression of *Kdr/Flk-1* in mouse distal airway secretory cells (**Revised Extended Data Fig.16a**), a previous study using Flk-1 knock-in reporter mice identified the specific expression of *Kdr* in bronchial epithelial cells (Ahlfbrecht et al., 2008) (**Rev2_Fig. 6a**). We further analysed flow cytometry of cells from *Scrgb1a1-CreER*^{TM/+}; *R26R*^{fGFP/+}; *Sftpc-dsRed* mouse lungs. As shown in **Rev2_Fig. 6b**, *Kdr*⁺ cells are lineage-labelled GFP⁺ secretory cells. In addition, we also isolated EpCAM⁺*Kdr*⁺ and EpCAM⁺*Kdr*⁻ cells from WT mice using *Kdr* antibody for qPCR analysis (**Rev2_Fig. 6c**). EpCAM⁺*Kdr*⁺ cells revealed the specific expression of secretory cell markers while EpCAM⁺*Kdr*⁻ cells showed the enriched expression of AT2 marker genes (**Rev2_Fig. 6d**).

As the Reviewer pointed out, Chernaya et al. colleagues showed that Itga6⁺ airway progenitor cells express high levels of *Kdr* (Chernaya et al., 2014). Although Itga6⁺ populations were suggested as basal-like progenitors in the distal airway based on their capacity to produce cells expressing various cell markers in 3D cultures, the level of basal cell marker expression, such as *p63* and *Krt5*, was much lower than those in the proximal airways. Moreover, our scRNA-seq analysis showed that *Itga6* is also highly expressed in the subset of secretory cells in parallel with *Kdr* expression (**Rev2_Fig. 6e**). Thus, it is likely that Itgb6⁺ population is a subset of secretory cells in the distal airways. Indeed, Chernaya et al. colleagues also discussed the potential contribution of Itgb6⁺ cells to alveolar regeneration after bleomycin injury.

In the human lung tissue, based on the dataset published by Habermann et al., *KDR* is specifically expressed in secretory cells, differentiating ciliated cells, and *KRT17*⁺ basaloid cells (Habermann et al., 2020). We further analysed scRNA-seq dataset from other recent studies (Salahudeen et al., 2020; Travaglini et al., 2020) and confirmed the specific expression of *KDR* in secretory cells both in *in vitro* airway organoids and *in vivo* primary human lung tissues (**Rev2_Fig. 6f-g**).



Rev2_Figure 6. Specific expression of KDR/FLK-1 in secretory cells of mouse and human lungs.

(a) Kdr expression from the adult lungs of *Kdr/Flk-1 lacZ* knock-in mice.

(b) Flow cytometry analysis for Kdr expression in secretory cells (GFP⁺) or AT2 (dsRed⁺) cells isolated from *Scgb1a1-CreER^{TM/+};R26R^{GFP/+};Sftpc-dsRed* mouse lungs.

(c) Flow cytometry analysis for sorting EpCAM⁺Kdr⁺ and EpCAM⁺Kdr⁻ cells isolated from WT B6 mouse lungs. Isotype IgG was used for negative control.

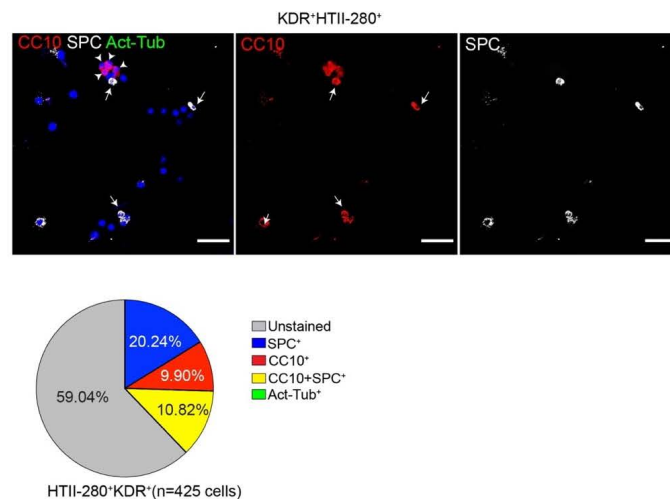
(d) qPCR analysis of the genes in isolated EpCAM⁺Kdr⁺ and EpCAM⁺Kdr⁻ cells. Data are presented as mean $\pm$ s.e.m (n=2 individual mice).

(e) UMAP visualisation of the log-transformed ($\log_{10}(\text{TPM}+1)$), normalised expression of *Kdr/Flk-1* and *Itga6* in distinctive clusters.

(f,g) UMAP visualisation of the log-transformed ($\log_{10}(\text{TPM}+1)$), normalised expression of KDR/FLK-1 in distinctive clusters from the dataset of Salahudeen et al., (f) or Travaglini et al., (g).

8-2. In addition, there seems to be a population of KDR⁺HTII-280⁺ cells (Fig 7c). What are these?

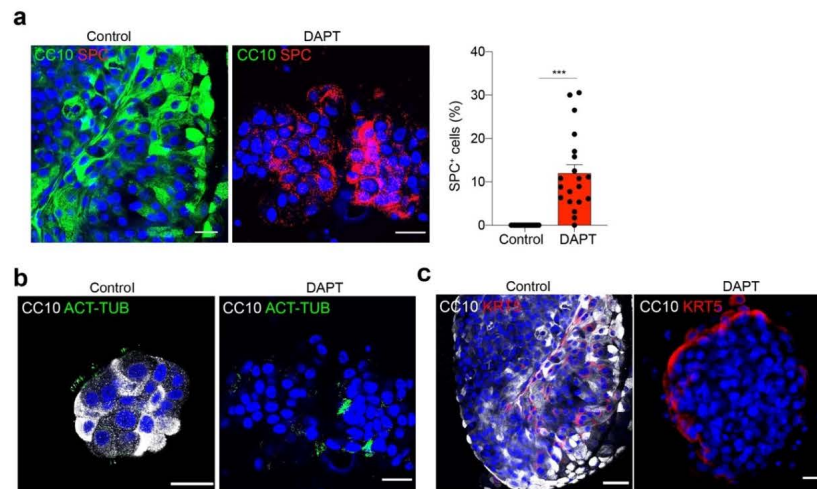
Response: To better understand the cellular composition of KDR⁺HTII-280⁺ cells, we performed cytospin staining for various lineage markers on the isolated KDR⁺HTII-280⁺ cells. We found that the KDR⁺HTII-280⁺ population consisted of heterogenous cells including CC10⁺ secretory cells and SPC⁺ AT2 cells (**Rev2_Fig. 7**). Interestingly, we observed a small number of CC10⁺SPC⁺ BASC-like cells in this population, although CC10⁺SPC⁺ dual-positive cells have not been reported yet in the human lung tissues. In addition, the percentage of KDR⁺HTII-280⁺ cells varied from sample to sample. Thus, it is likely that further validation of the KDR⁺HTII-280⁺ population, in addition to cytospin staining, would be needed.



Rev2_Figure 7. Characterisation of KDR⁺HTII-280⁺ populations isolated from human lung parenchyma tissues. Representative IF images of cytospin staining (top) of sorted KDR⁺HTII-280⁺ populations. CC10 (red), SPC (white), Acetylated Tubulin (Act-Tub, green), and DAPI (blue). Arrowheads point to CC10⁺SPC⁻ and arrows point to CC10⁺SPC⁺ cells. Scale bars, 100μm. Quantification (bottom) of SPC⁺, CC10⁺, Act-Tub⁺, and CC10⁺SPC⁺ cells from cytospin staining of KDR⁺HTII-280⁺ cells (n=3 individual samples).

8-3. The AT2 cells arising after Notch inhibition also seem to be located at the interior of the organoids. What are the other cells?

Response: We further analysed our secretory organoids by IF staining for other lineage markers, in addition to secretory and AT2 markers. Control organoids were composed of mainly CC10⁺ secretory cells and some Act-Tub⁺ ciliated cells and KRT5⁺ basal cells (**Rev2_Fig. 8; Revised Fig.7f**). Notch inhibition dramatically induced SPC⁺ AT2 cells at the expense of secretory cells in DAPT-treated organoids. The proportion of ciliated cells and basal cells remained similar to control organoids (**Revised Fig. 7i,j** and **Extended Data Fig 16h,i**)



Rev2_Figure 8. Enhanced differentiation of secretory cells into AT2 cells by inhibition of Notch activity in human secretory organoids.

(a) Representative IF images (left) of secretory cell-derived organoids treated with DMSO (control) or DAPT (20 μM). CC10 (green), SPC (red), and DAPI (blue). Scale bar, 50 μm. Quantification of the frequency of SPC⁺ cells in control or DAPT-treated organoids (right). Each individual dot represents one organoid and data are presented as mean ± s.e.m (n=3 independent experiments). ***p<0.001.

(b,c) Representative IF images of secretory cell-derived organoids treated with DMSO (control) or DAPT (20 μM). CC10 (white), Acetylated Tubulin (Act-Tub, green, b), KRT5 (red, c), and DAPI (blue). Scale bar, 50 μm.

Minor

Minor-1. Line 166: please mention for clarity that the dnMAML are in fact dnDMAMfl/+.

Response: We have amended it accordingly in the revised manuscript.

Reviewer #3:

Choi et al. describe a series of experiments aimed at understanding how alveoli regenerate after damage (bleomycin) and induction of latent stem cell programs. Their model systems rely on in vitro analysis of club cell derived organoids and in vivo studies using a variety of sophisticated mouse genetic tools, both combined with molecular studies of chromatin states, single-cell sequencing and pharmacological manipulations. The authors assert that their data are best described by a model in which alveolar damage generates an IL-1 β signal that in turn downregulates JAG1/2 expression on ciliated cells, further downregulating Notch signalling in club cells to release a Fos12/Fra2 transcriptional program that licenses the secretory cells, as a progenitor, to differentiate to alveolar type 2 cells (AE2 cells).

In general, the experiments are performed thoughtfully and are well presented. The systems employed are technically advanced and directed to answering relevant and important questions in lung biology and damage/repair. My main criticism, however, is that there seem to be other at-least-as-plausible models to explain the data, and I suggest that these alternative interpretations need to be addressed.

Specifically, the authors often refer to the repair-driving progenitor cell type as a club cell or secretory cell. In many cases, this cellular identity (and the language used to describe it, “club cell” or “secretory” cell) is based on expression of one (or no more than a few markers), such as CC10. While club cells certainly express CC10 and other markers, these markers are shared with other cells, most notably stem/progenitor cells that reside at the bronchial/alveolar junctions (BAJs). Numerous studies have clearly established that cells at this location express markers of both club cells and AE2 cells, thus displaying markers indicative of a dual lineage. An equally plausible, or more plausible model, compared to the authors’ model, is that these dual lineage progenitor cells are the precursors that provide the repaired AE2 cells, and that the fate decision to AE2 is driven by loss of Notch signaling. Put another way, perhaps the “airway” organoids are really “BASC” organoids and Notch controls the fate between airway, alveolar and mixed.

Response: We understand the Reviewer’s point of view, and agree that it would be important to clarify whether secretory cells or CC10⁺SPC⁺ dual-positive cells, including BASCs, are capable of differentiating into AT2 cells via Notch inhibition after injury. Please see our response below to thoroughly address the Reviewer’s concerns and suggestions, in the **Specific Comment 3**.

One other general comment is that the authors do not consider that in some of their experiments, treatment with a gamma-secretase inhibitor before damage would likely have converted nearly all of the club cells to ciliated cells, since both genetic and pharmacological inhibition of Notch induces a club-to-ciliated cell transdifferentiation (for example, see Pardo-Saganta et al., Nature 2015 and Lafkas et al., Nature 2015). Given the expected absence of the majority of club cells under such conditions (but not the loss of CC10⁺ cells at the BAJs), how do the authors explain the improved AE2 regeneration? This omission seems like a major issue that must be addressed.

Response: We thank the Reviewer for this important question. Please see our response below to thoroughly address the Reviewer’s concerns and suggestions, in the **Specific Comment 5**.

Specific comments:

1. Writing: The paper is generally well-written and fairly easy to follow. However, the beginning sections (Title and abstract in particular) were very hard for me to understand. For example, the title is much too obtuse and relies on jargon. I don’t think most readers will understand, until finishing the paper, what is

meant by “cross-compartment differentiation plasticity”). This kind of language persists in the Introduction and could be rewritten using a more direct and straightforward approach.

Response: As suggested, we have rewritten our manuscript in a more straightforward manner.

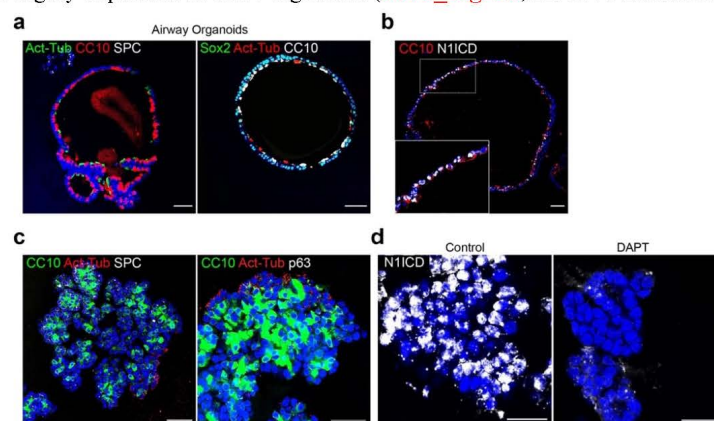
2. Figure 1 and related:

2-1. Cells are described as “secretory” or “alveolar” when often the observation is simply CC10⁺ or SPC⁺. This is an oversimplification. Indeed, CC10 or SPC would also mark the dual-lineage progenitors noted above.

Response: In the field, secretory or AT2 cells are generally defined based on the expression of secretory protein (CC10) and surfactant protein C (SPC), respectively, although we appreciate the heterogeneity of populations within these cells. Given the Reviewers’ valuable comments, we now further determined the functional contribution of CC10⁺ secretory cells, not CC10⁺SPC⁺ dual-positive cells, to SPC⁺ AT2 cells in the revised manuscript. Please see our response below to **Specific Comment 3**.

2-2. The organoid system seems like an important technical advance. I would like to see more characterization, particularly of the state of Notch signaling. For example, in the three types of organoids (e.g., see panel f), where are Notch ligands and receptors expressed? Which cells show not just Notch receptor expression but, more importantly, Notch receptor activity, as evidenced by nuclear localization of the Notch ICD? One thing I am confused by...in the first (left-most) panel of Fig. 1f, almost all (if not all) of the cells in the airway organoids appear to be CC10 positive and therefore are described as secretory airway cells. *In vivo*, airway secretory cells (club cells) require Notch signaling for their fate to be maintained, and as the authors note later in their paper, the Notch signal is induced by Jag ligands expressed on neighboring ciliated cells. There are no ciliated cells in these organoids, so what is inducing the Notch signal?

Response: To further verify our organoid models, we performed IF staining for other lineage marker such as Acetylated Tubulin (Act-Tub) for ciliated cells, in addition to AT2 and secretory cell markers. Airway organoids at P0 contained Act-Tub⁺ ciliated cells in addition to CC10⁺ secretory cells (**Rev3_Fig. 1a; Revised Fig.1f**). Similar to *in vivo*, ciliated and secretory cells were closely localised. Furthermore, we observed the specific expression of N1ICD in the nucleus of secretory cells in our airway organoids (**Rev3_Fig. 1b**). At the later passages, organoids became mixed organoids which are composed of mainly CC10⁺ secretory cells and some SPC⁺ AT2 and Act-Tub⁺ ciliated cells (**Rev3_Fig. 1c; Revised Fig.1h**). N1ICD was also highly expressed in these organoids (**Rev3_Fig. 1d; Revised Extended Data Fig.2a**).

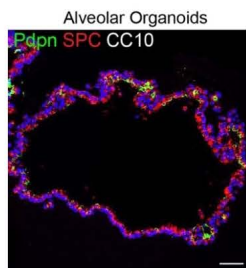


Rev3_Figure 1. Validation of secretory organoids.

- (a) IF images of airway organoids derived from secretory cells at the establishment (P0) under feeder-free culture condition with defined medium; Airway organoids retained CC10⁺ secretory and Act-Tub⁺ ciliated cells. CC10 (red or white), Sox2 (green), Acetylated Tubulin (Act-Tub, green), SPC (white), and DAPI (blue). Scale bars, 50µm.
- (b) IF images showing nuclear localisation of N1ICD in CC10⁺ secretory cells of P0 airway organoids. CC10 (red), N1ICD (white), and DAPI (blue). Scale bars, 50µm.
- (c) IF images for various lung lineage markers, including CC10, Act-Tub, SPC, and p63 in mixed organoids derived from secretory cells at passage 5 (P5). CC10 (green), Acetylated Tubulin (Act-Tub, red), SPC (white), p63 (white), and DAPI (blue). Scale bars, 50µm.
- (d) IF images showing nuclear localisation of N1ICD in mixed organoids derived from secretory cells at P5 with or without DAPT (20µM). CC10 (red), N1ICD (white), and DAPI (blue). Scale bars, 50µm.

2-3. Additional markers of other lung cell types should be examined in the organoids (ciliated cell markers, AE1 markers, for example).

Response: As shown in **Rev3_Fig. 1**, we further investigated the cellular composition of our secretory organoids by IF staining for various lineage markers. We also checked that alveolar organoids derived from secretory cells at P0 retained Pdpn⁺ AT1 cells, in addition to SPC⁺ AT2 cells (**Rev3_Fig. 2**; **Revised Fig. 1f**).

**Rev3_Figure 2. Alveolar organoids contained SPC⁺ AT2 and Pdpn⁺ AT1 cells.**

IF images of alveolar organoids derived from *Tomato⁺Scgbl1a1⁺* cells at the establishment (P0) under stroma-free culture condition with defined medium; Alveolar organoids retained SPC⁺ AT2 cells and Pdpn⁺ AT1 cells, not CC10⁺ secretory cells. CC10 (white), Pdpn (green), SPC (red), and DAPI (blue). Scale bars, 50µm.

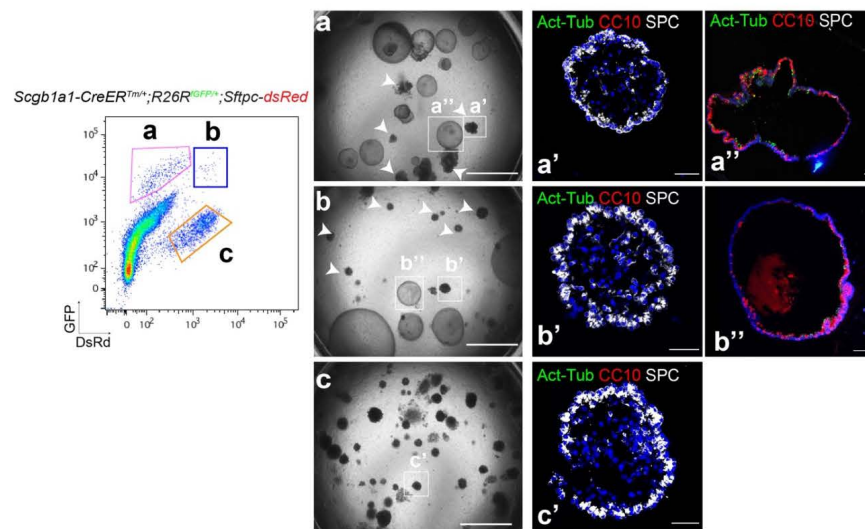
3. Fig 2 and related:

3-1. The dnMAML exposure in Ext Data Fig 2 occurs 4-10 days prior to the isolation of Tom⁺ cells. Since club cells require persistent Notch signaling to maintain their fate, and because loss of Notch signaling causes their rapid conversion (4-6 days) to ciliated cells, the observation that dnMAML generates increased CFE does not easily fit with the proposed model. One could argue that these results in fact argue that normal airway club cells are likely not the progenitors for the AE2 (CFE) regeneration activity. As noted in my general comments above, could cells that are CC10⁺ SPC⁺ at the BAJ be responsible instead?

Response: We appreciate Reviewer's valuable comment. To answer the question, we validated the differentiation capacity of secretory cells into AT2 lineages, after excluding CC10⁺SPC⁺ dual-positive cells, *in vitro* (**Rev3_Fig. 3**) and *in vivo* (**Rev3_Fig. 4**).

Given that *Scgbl1a1⁺* lineage-labelled cells contained CC10⁺SPC⁺ dual-positive cells, including BASCs, we further investigated the differentiation capacity of CC10⁺SPC⁻, CC10⁺SPC⁺, and CC10⁻SPC⁺ cells utilising *Scgbl1a1-CreER^{T2/+};R26R^{GFP/+};Sftpc-dsRed^{RES-DTR/+}* reporter mice (**Rev3_Fig. 3**). We isolated *Scgbl1a1⁺* lineage-labelled SPC⁻ cells (**a**), lineage-labelled SPC⁺ cells (**b**), and non-lineage-labelled resident SPC⁺ AT2 cells (**c**) for organoid co-cultures. As expected, resident AT2 cells (CC10⁻SPC⁺) only formed alveolar organoids retaining AT2 cells (**Rev3_Fig. 3c, c'**). However, organoids derived from

CC10⁺SPC⁻ secretory cells (a) and CC10⁺SPC⁺ dual-positive cells (b) generated airway and alveolar organoids at the comparable manner, indicating that CC10⁺SPC⁻ secretory cells, as well as CC10⁺SPC⁺ cells including BASCs, are capable of converting into AT2 lineages (Rev3_Fig. 3a,b).



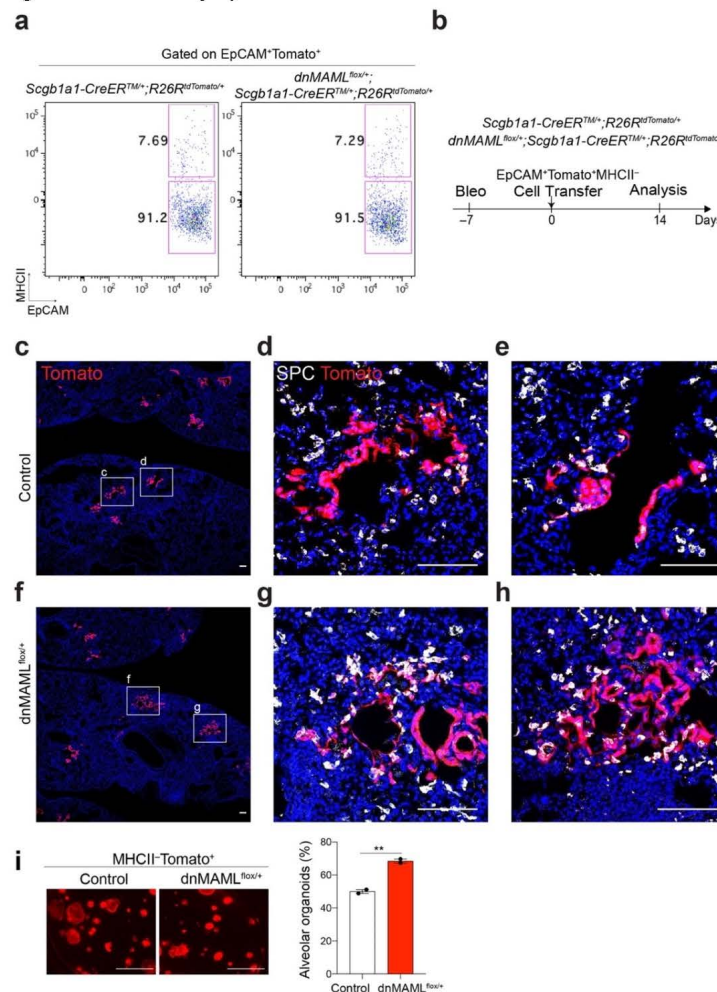
Rev3_Figure 3. Differentiation potentials of CC10⁺SPC⁻, CC10⁺SPC⁺ and CC10⁻SPC⁺ cells to give rise to SPC⁺ AT2 cells in organoids.

Sorting strategy (left) for *Scgb1a1*⁺ lineage-labelled SPC⁻ cells (a), lineage-labelled SPC⁺ cells (b), and non-lineage-labelled resident SPC⁺ AT2 cells (c) by flow cytometry. Bright-field images of organoids (middle). Cells isolated at day 4 post 3 doses of tamoxifen treatment were co-cultured with stromal cells with 1:5 ratio for 14 days. Arrows point to alveolar organoids. IF images (right) showing SPC⁺ AT2-retaining alveolar (a', b', c') or CC10⁺ secretory cell-retaining airway organoids (a'', b''). SPC (white), CC10 (red), Acetylated Tubulin (Act-Tub, green), and DAPI (blue). Scale bars, 2,000µm (a,b,c) and 50µm (a',a'',b',b'',c').

Furthermore, we also evaluated the functional contribution of secretory cells (CC10⁺SPC⁻) to alveolar regeneration by Notch inhibition *in vivo* by transplantation study after excluding CC10⁺SPC⁺ cells (Rev3_Fig. 4; Revised Extended Data Fig.6). We isolated lineage-labelled cells from *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (control) or *dnMAML*^{flox/+}; *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} (dnMAML) mouse lungs, and excluded AT2 cells based on MHCII expression (Hasegawa et al., 2017) (Rev3_Fig. 4a; Revised Extended Data Fig.6a). Lineage-labelled secretory cells (EpCAM⁺Tomato⁺MHCII⁻) were transplanted into B6 WT mice at day 7 after bleomycin injury (Rev3_Fig. 4b; Revised Extended Data Fig.6b). At day 14 post transplantation, we found that *dnMAML*-expressing secretory cells showed a greater ability to contribute to AT2 cell differentiation compared to control secretory cells (Rev3_Fig. 4c-h; Revised Extended Data Fig.6c-h). This result suggests enhanced differentiation capacity of secretory cells into AT2 lineages by Notch inactivation *in vivo*.

Additionally, we isolated lineage-labelled secretory cells (EpCAM⁺Tomato⁺) from control or dnMAML mouse lungs for organoid cultures (Revised Extended Data Fig.6i). The colony forming efficiency of *dnMAML*-expressing secretory cells was comparable to that of control secretory cells. We then further assessed their differentiation capacity after excluding lineage-labelled AT2 cells. Consistent with our *in vivo* transplantation data, *dnMAML*-expressing secretory cells (EpCAM⁺Tomato⁺MHCII⁻) revealed the greater frequency of alveolar organoids retaining AT2 cells compared to control secretory cells

(Rev3_Fig. 4i; Revised Extended Data Fig.6i). Taken together, our new data strongly suggest persistent inhibition of Notch activity facilitates the differentiation potential of secretory cells into AT2 lineages during alveolar regeneration after injury.



Rev3_Figure 4. Secretory cells are capable of contributing to AT2 cell differentiation via inhibition of Notch activity.

(a) Flow cytometry analysis showing the exclusion of lineage-labelled AT2 cells from *Scgb1a1-CreERTM^{+/+}; R26R^{tdTomato}+/+* (Control) or *dnMAML^{flox/+}; Scgb1a1-CreERTM^{+/+}; R26R^{tdTomato}+/+* mouse lungs.

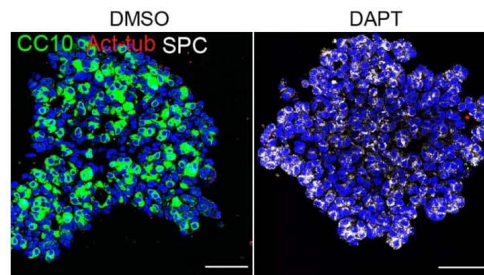
(b) Experimental scheme for transplantation of lineage-labelled secretory cells (EpCAM⁺Tomato⁺MHCII⁻) after excluding lineage-labelled AT2 cells in **(a)**.

(c-h) Representative IF images of engrafted lineage-labelled secretory cells in **(a,b)**: *Scgb1a1-CreERTM^{+/+}; R26R^{tdTomato}+/+* (Control, **c-e**) or *dnMAML^{flox/+}; Scgb1a1-CreERTM^{+/+}; R26R^{tdTomato}+/+* (*dnMAML^{flox/+}*, **f-h**). Tomato (for *Scgb1a1* lineage, red), SPC (white), and DAPI (blue). Scale bars, 100µm.

(i) Representative fluorescent images of organoids (left) and statistical quantification of colony forming efficiency of alveolar organoids (right) derived from lineage-labelled secretory cells in **(a)**. 5,000 cells of cells were co-cultured with stromal cells with 1:5 ratio for 14 days. Each individual dot represents one experiment from one mouse and data are presented as mean and s.e.m. Scale bar, 2,000µm.

3-2. What happens to the airway organoids after DAPT treatment? I realize that such a treatment is included in studies in Fig 2, but only CC10 and SPC markers are examined (do ciliated cell markers get induced?).

Response: As suggested, we further analysed our organoids and included IF staining for other lineages including Act-Tub (ciliated cell marker) in the revised manuscript. As we described the details in the result section, we observed dramatic increases in SPC⁺ AT2 cells at the expense of CC10⁺ ciliated cells with DAPT treatment. However, there was no discernible change in Act-Tub⁺ ciliated cells with or without DAPT treatment in secretory cell-derived organoids (**Rev3_Fig 5; Revised Fig 2c**).



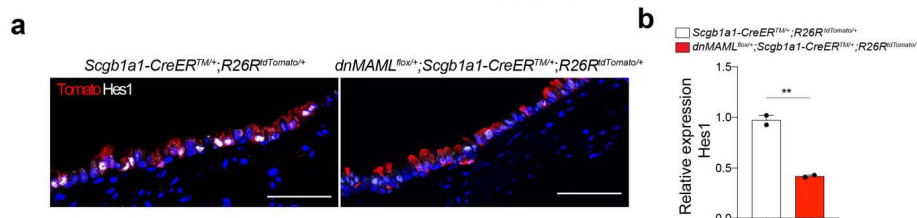
Rev3_Figure 5. Notch inhibition does not facilitate ciliated cell differentiation in secretory cell-derived organoids (SCOs). IF images of secretory organoids treated with DMSO (control) or DAPT (20μM) in SCOs. CC10 (for secretory cells, green), Acetylated Tubulin (for ciliated cells, Act-Tub, red), SPC (for AT2 cells, white), and DAPI (blue). Scale bars, 50μm.

3-3. Panels a and d: Are these heat maps showing the top differentially expressed genes? The panels need to be better described in the figure legends. In general, a bit more experimental detail should be included in many of the legends as it was often difficult to understand exactly what was done or what was being shown (e.g., length of treatments).

Response: We have included the detailed description in the figure legends in our revised manuscript.

3-4. Panel 2g: how has dnMAML impacted club-ciliated cell ratios in the airways? The ratio should be greatly reduced. What is the control evidence that the dnMAML mice are working as intended, that is, how do the authors know that dnMAML is inhibiting Notch in the intended target cells?

Response: We thank the Reviewer for this important comment. To validate the downregulation of Notch activity in *dnMAML*^{flox/+} mouse models, we carefully checked Notch activity by assessing the expression of Hes1, a potent target gene of Notch signalling, in lineage-labelled secretory cells. The overexpression of *dnMAML* showed significant reduction of Notch activity based on the expression level of Hes1 in secretory cells of *dnMAML*^{flox/+}; *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs compared to that of *Scgb1a1-CreER*^{TM/+}; *R26R*^{tdTomato/+} mouse lungs (**Rev3_Fig. 6a,b; Revised Extended Data Fig.3**).

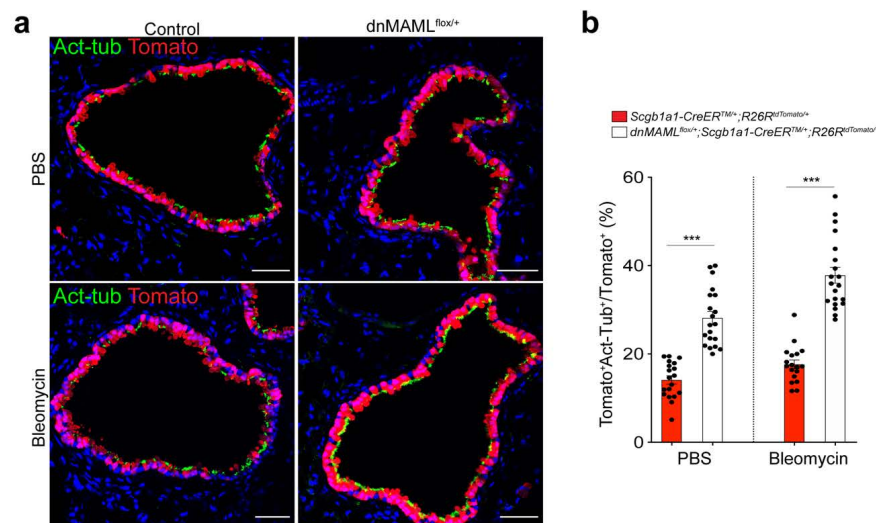


Rev3_Figure 6. Inhibition of Notch activity in the secretory cells expressing *dnMAML*.

(a) Representative IF images showing Hes1 expression in lineage-labelled secretory cells after tamoxifen treatment in the indicated genotype: Tomato (for *Scgbl1a1* lineage, red), Hes1 (white), and DAPI (blue). Scale bars, 50µm.

(b) qPCR analysis of *Hes1* expression in isolated EpCAM⁺Tomato⁺ cells. Data are presented as mean ± s.e.m (n=2 individual mice). **p<0.01.

We then further investigated the fate conversion of secretory cells into ciliated cells by inhibition of Notch activity at homeostatic lungs and after bleomycin injury. Consistent with previous studies showing that Notch inhibition promotes the differentiation of secretory cells into ciliated cells (Lafkas et al., 2015; Morimoto et al., 2010; Pardo-Saganta et al., 2015), we observed a 2-fold increase of lineage-labelled ciliated cells in *dnMAML*^{fllox/+}; *Scgbl1a1*-CreER^{TM/+}; *R26R*^{tdTomato/+} mouse lungs compared to control *Scgbl1a1*-CreER^{TM/+}; *R26R*^{tdTomato/+} mouse lungs both in homeostasis and injury repair (**Rev3_Fig. 7; Revised Extended Data Fig. 4**). Given that the differences in the incidence of ciliated cell differentiation in previous and our studies (Lafkas et al., 2015; Morimoto et al., 2010; Pardo-Saganta et al., 2015), it is likely that the way of Notch inhibition (e.g. genetic models vs. pharmacological models) or the extent of Notch inactivation may affect in the level of this conversion.

**Rev3_Figure 7. Inhibition of Notch activity induces the differentiation of secretory cells into ciliated cells in distal airways.**

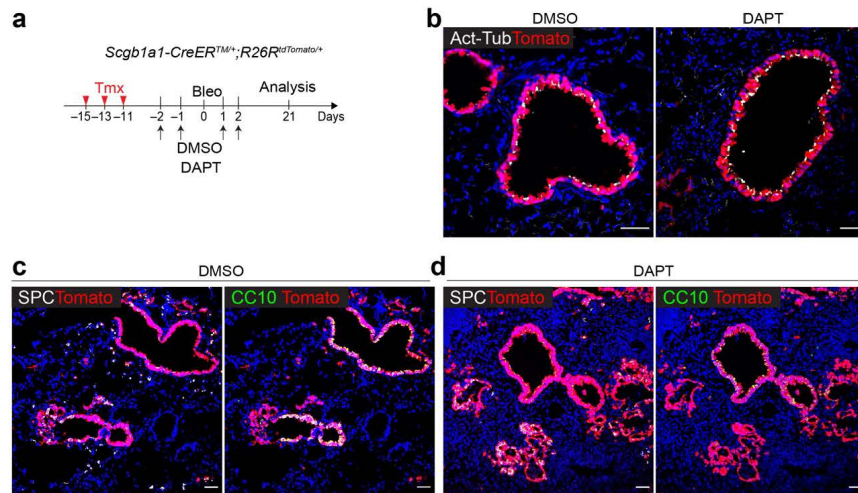
(a) Representative IF images showing the derivation of *Scgbl1a1*⁺ lineage-labelled ciliated cells at day 28 in PBS- or bleomycin-treated lungs of *Scgbl1a1*-CreER^{TM/+}; *R26R*^{tdTomato/+} (control) or *dnMAML*^{fllox/+}; *Scgbl1a1*-CreER^{TM/+}; *R26R*^{tdTomato/+} mice. Tomato (for *Scgbl1a1* lineage, red), Acetylated Tubulin (Act-Tub, green), and DAPI (blue). Scale bar, 50µm.

(b) Statistical quantification of *Scgbl1a1*⁺ lineage-labelled ciliated cells in (a). Each individual dot represents one section and data are presented as mean ± s.e.m. (n=2 PBS control, n=2 for bleomycin). ***p<0.001.

3-5. Have the authors tried DAPT (or other GSI) treatment *in vivo*, as an alternative to the genetic method of *dnMAML*, or is the GSI too toxic, especially when used in combination with bleomycin?

Response: As suggested, we treated DAPT before and after bleomycin injury to pharmacologically inhibit Notch activity during injury repair (**Rev3_Fig. 8a**). Consistent with our results utilising genetic

mouse models of dnMAML^{fllox/+} system, we found enhanced differentiation of lineage-labelled secretory cells into AT2 cells in DAPT-treated mouse lungs compared control lungs (**Rev3_Fig. 8c,d**). Furthermore, we also observed an increase in lineage-labelled ciliated cells in the airways of DAPT-treated lungs compared to control lungs (**Rev3_Fig. 8b**). These results suggest that, despite increased ciliated cell differentiation of secretory cells in airways, persistent Notch inhibition facilitates the differentiation of remaining? secretory cells into AT2 lineages during alveolar regeneration after injury.



Rev3_Figure 8. Pharmacological inhibition of Notch activity by DAPT treatment enhances the differentiation of secretory cells into AT2 cells during injury repair.

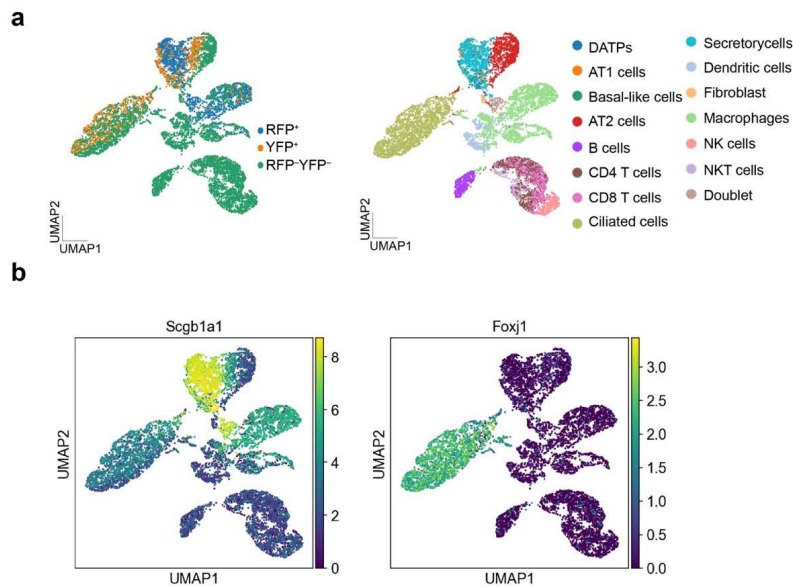
(a) Experimental design of lineage-tracing analysis of *Scgb1a1-CreERTM⁺;R26R^{tdTomato}* mice after bleomycin injury. DMSO or DAPT (50mg/kg body weight) was administrated *via* intraperitoneally as indicated time points.

(b) Representative IF images showing the increased *Scgb1a1*⁺ lineage-labelled Act-Tub⁺ ciliated cells in DAPT-treated mouse lungs at day 21 post bleomycin injury compared to DMSO control mice. Tomato (for *Scgb1a1* lineage, red), Acetylated Tubulin (Act-Tub, white), and DAPI (blue). Scale bar, 50μm.

(c,d) Representative IF images showing the derivation of *Scgb1a1*⁺ lineage-labelled SPC⁺ AT2 (left) or CC10⁺ secretory cells (right) in DMSO control (c) or DAPT-treated (d) mice at day 21 post bleomycin injury. Tomato (for *Scgb1a1* lineage, red), SPC (white), CC10 (green), and DAPI (blue). Scale bar, 100μm.

4. Fig 3 and related: I am surprised that ciliated and secretory airway cells don't appear more closely related as "airway" cells, as in previous lung single-cell sequencing experiments. Are these results inconsistent with such previously published results (Krasnow, Whitsett labs, for example)?

Response: We modified the parameter of "n" _neighbour (the number of approximate nearest neighbours) that is the most important parameter to construct the local versus global structure. To focus on local structure, we generated UMAP using low values by constraining the number of neighbouring points considered (from 30 to 15). This amendment reveals the close proximity between secretory cells and ciliated cells, which is consistent with previous data (**Rev3_Fig. 9; Revised Extended Data Fig.8b,c**).



Rev3_Figure 9. scRNA-seq analysis shows the close proximity of secretory and ciliated cells.

(a) Clusters of cell population after bleomycin injury from 10x Genomics 3' scRNA-seq analysis visualised by UMAP assigned by specific colours.

(b) UMAP visualisation of the log-transformed ($\log_{10}(\text{TPM}+1)$), normalised expression of selected marker genes (*Foxj1* for ciliated cells, *Scgb1a1* for secretory cells).

5. Fig 4 and related:

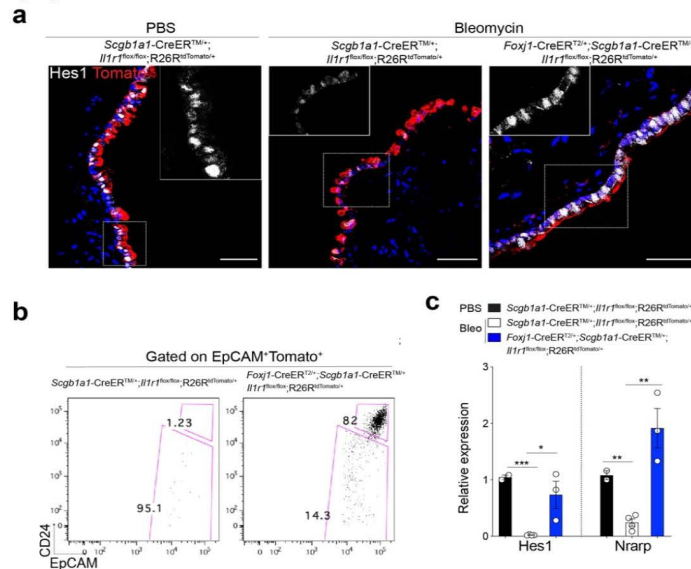
5-1. The authors do not note that club cell maintenance requires Notch signaling in the adult (not just during development), as noted above, which is an important consideration that should be addressed.

Response: We have included additional sentences describing that Notch inhibition promotes the differentiation of secretory cells into ciliated cells in lung development and homeostatic adult lungs in the discussion section of our revised manuscript (Page: 17, line: 514).

5-2. The authors should assess Notch signaling in the secretory cells. For example, they could stain for Notch1 or Notch2 ICD in the nuclei as a transcript-independent measure of Notch activity. Also, they could examine expression in situ of Notch transcriptional targets. The change in ligand expression is nicely shown but it is important to show that this decrease in ligands impacts the signal.

Response: In addition to assessing Notch activity in secretory cells of *Scgb1a1-CreER^{TM/+};R26R^{tdTomato/+}* or *dnMAML^{lox/+};Scgb1a1-CreER^{TM/+};R26R^{tdTomato/+}* mouse lungs (**Rev3_Figure 5**), we also examined *Hes1* expression in secretory cells after *Il1r1* deletion in ciliated cells. As expected, the expression of *Hes1* was highly maintained in homeostatic secretory cells, but dramatically reduced after bleomycin injury, consistent with the downregulation of Notch ligands in ciliated cells after injury (**Rev3_Fig. 10a; Revised Extended Data Fig. 10a**). Significantly, we found that the decrease in *Hes1* expression in secretory cells was impaired by *Il1r1* deletion in ciliated cells after injury. Furthermore, we evaluated the transcriptional activity of Notch signalling by measuring downstream target genes, such as *Hes1* and *Nrarp*, in isolated lineage-labelled secretory cells (EpCAM⁺CD24⁺Tomato⁺) (**Rev3_Fig. 10b,c; Revised Extended Data Fig. 10b,c**).

Consistent with IF data, deletion of *Il1r1* in ciliated cells altered Notch signalling in secretory cells after bleomycin injury.



Rev3_Figure 10. The activity of Notch signalling in secretory cells is altered by *Il1r1* deletion in ciliated cells.

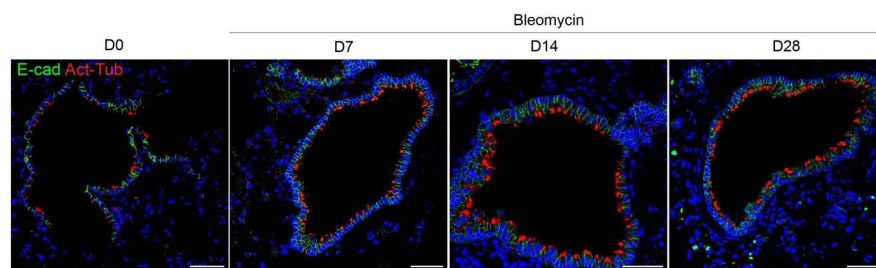
(a) Representative IF images showing Hes1 expression in lineage-labelled secretory cells at day 14 post bleomycin injury in the indicated genotype: Tomato (for *Scgb1a1* lineage, red), Hes1 (white), and DAPI (blue). Insets show high-power view of Hes1 staining. Scale bars, 50µm.

(b) Sorting strategy for lineage-labelled secretory cells isolated from PBS- or bleomycin-treated lungs in **(a)**. EcCAM⁺CD24⁺ cells gated from EpCAM⁺Tomato⁺ were used for isolation of lineage-labelled secretory cells and qPCR analysis.

(c) qPCR analysis for downstream target genes of Notch signalling, including *Hes1* and *Nrarp*, from isolated lineage-labelled secretory cells in **(b)**. **p*<0.05, ***p*<0.01, ****p*<0.001.

5-3. Ext data Fig 4: What does bleomycin treatment do the airways? They certainly don't look the same by H & E, comparing PBS and bleomycin (the obvious club-ciliated cell architecture seems lost after treatment). Are "normal" ciliated cells present?

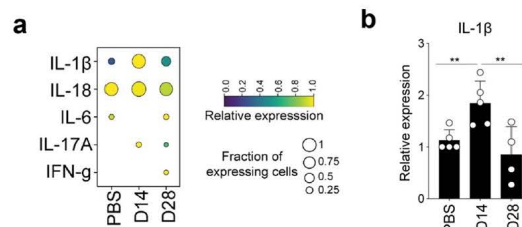
Response: As suggested, we carefully examined ciliated cells during regeneration after bleomycin injury. Although there are some transcriptional programme changes, such as downregulation of Notch ligands, in ciliated cells during injury repair, we observed no significant effects on the morphology and frequency of ciliated cells based on the expression patterns of E-cadherin and Acetylated Tubulin (**Rev3_Fig. 11**).



Rev3_Figure 11. No significant alteration in ciliated cells after bleomycin injury. Representative IF images showing ciliated cells in the lung at indicated time points after bleomycin injury: Acetylated-Tubulin (Act-Tub, red), E-Cadherin (E-Cad, green), and DAPI (blue). Scale bars, 50µm.

5-4. An important missing piece of data is an assessment of IL-1beta levels. Is there a technical reason why this cannot be measured? An increase in IL-1beta after damage is a key piece of the model and is not established.

Response: We thank the Reviewer for this important comment. Our previous study showed that the expression of *IL-1β* is higher at day 14 post bleomycin injury, and returns back to the homeostatic level at day 28 after injury (**Rev3_Fig. 12a**) (Choi et al., 2020). qPCR analysis also confirmed the increased expression of *IL-1β* after bleomycin injury from the total lung tissues (**Rev3_Fig. 12b**).



Rev3_Figure 12. Dynamic expression of *IL-1β* during alveolar regeneration after bleomycin injury.

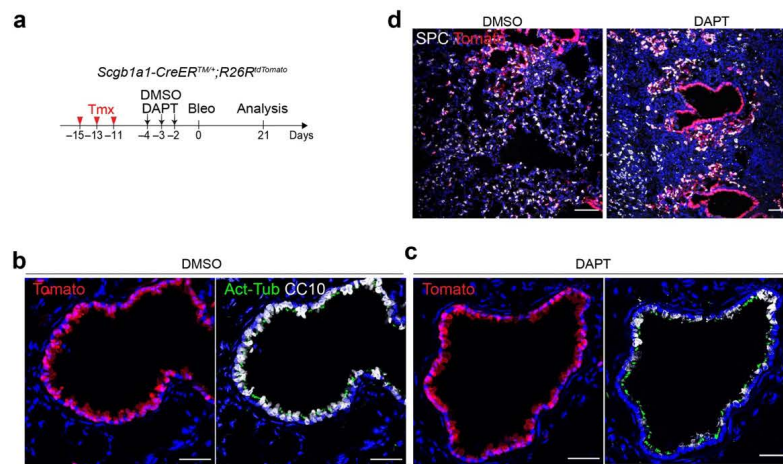
(a) Gene expression of proinflammatory cytokines including *IL-1β* at indicated time points after bleomycin injury. Data were from our previous study (Choi et al., 2020).

(b) qPCR analysis of *IL-1β* in total lung tissue at the indicated time points after bleomycin injury. Data are presented as mean ± s.e.m (n=4 individual mice). **p<0.01.

5-5. The authors could further test their model by treating mice with a pulse of GSI to convert the bulk of club cells to ciliated cells, then treating with bleomycin to induce damage. If airway secretory cells are key to repair, then the GSI treatment should inhibit or slow repair. The dnMAML results, as discussed above, suggest that loss of club cells does not slow repair, which presents a challenge to the model. The GSI pre-pulse experiment would be another simple way to address this question.

Response: We understand the Reviewer's point of view hypothesising that the contribution of secretory cells to AT2 cells can be affected if the absolute number of secretory cells is significantly reduced by Notch inhibition prior to damage. To test this hypothesis, we pre-treated DMSO (control) or DAPT before bleomycin injury, and tracked *Scgblal*⁺ lineage-labelled cells at day 21 post injury (**Rev3_Fig. 13a**). As expected, there were increased lineage-labelled ciliated cells in the airways with DAPT pre-pulse compared to DMSO control (**Rev3_Fig. 13b,c**). However, we were able to observe the differentiation of remaining? secretory cells into AT2 cells in DAPT pre-treated lungs at the comparable level of control lungs (**Rev3_Fig. 13d**). These results lead the potential explanation as followings: 1) Given the presence of lineage-labelled secretory cells even with DAPT treatment, the dosages used in the DAPT pre-pulse experiment may be not sufficient for disrupting the contribution of secretory cells to alveolar regeneration after injury. 2) Two-day wash-out time of DAPT treatment prior to bleomycin injury may be not enough, and still affect the differentiation plasticity of secretory cells upon damage.

Significantly, we demonstrated that *Fosl2*-deficient secretory cells failed to convert into AT2 lineages in DAPT-treated secretory organoids (**Revised Fig. 5g**). Instead, *Fosl2*-KD organoids showed increased ciliated cells at the expense of secretory cells with DAPT treatment. These data strongly suggest that *Fosl2* is a critical regulator that induces the fate conversion of secretory cells into AT2 lineages upon Notch inhibition after lung injury. We also provided the potential mechanisms of how downregulation of Notch leads to different secretory cell differentiation outcomes in different contexts in the response to the comment to Reviewer 1 (please see the response to **Minor Comment 3** from **Reviewer #1**), although we believe that this is beyond the scope of our study. As we discussed, further *in vivo* studies showing the cooperative regulation of Notch inhibition and Wnt signalling in the fate conversion of secretory cell into AT2 lineages during alveolar regeneration would be important to better understand their behaviours at the different contexts.



Rev3_Figure 13. Pre-treatment of DAPT does not affect the efficiency of alveolar regeneration derived by secretory cells.

(a) Experimental design of lineage-tracing analysis of *Scgb1a1-CreER^{TM/+};R26R^{tdTomato}* mice after bleomycin injury. DMSO or DAPT (50mg/kg body weight) was administrated *via* intraperitoneally as indicated time points.

(b, c) Representative IF images showing the increased *Scgb1a1*⁺ lineage-labelled Act-Tub⁺ ciliated cells in DAPT-treated mouse lungs at day 21 post bleomycin injury compared to DMSO control mice. Tomato (for *Scgb1a1* lineage, red), CC10 (white), Acetylated-Tubulin (Act-Tub, green), and DAPI (blue). Scale bar, 50μm.

(d) Representative IF images showing the derivation of *Scgb1a1*⁺ lineage-labelled SPC⁺ AT2 cells in DMSO control (left) or DAPT-treated (right) mice at day 21 post bleomycin injury. Tomato (for *Scgb1a1* lineage, red), SPC (white), and DAPI (blue). Scale bar, 100μm.

6. Fig 6 panel e: The top and bottom labels are switched relative to what is referenced in the legend.

Response: We apologize for mis-cited figure panels. We have amended our manuscript accordingly.

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Decision Letter, first revision:

Date: 28th April 21 11:34:30

Last Sent: 28th April 21 11:34:30

Triggered By: Christine Weber

From: christine.weber@nature.com

To: jhl62@cam.ac.uk

CC: ncb@springernature.com

Subject: Decision on Nature Cell Biology submission NCB-L44082A

Message: *Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Joo-Hyeon,

Thank you for providing a response to the reviewer feedback on your revised manuscript, "Release of Notch activity coordinated by IL-1 β signalling confers differentiation plasticity of airway progenitors via Fosl2 during alveolar regeneration".

As you know, your manuscript has been seen again by all three of the original referees and their comments are attached in full below, for your record.

We have assessed the new information provided and would be willing to reconsider a revision as outlined in your latest rebuttal. Please note that all figures from the latest and previous rebuttals should be included into the revised manuscript. We would also ask that the language in the rebuttal is slightly tempered, focusing on the valid scientific points and clarifications instead of referring to the referee in person.

We therefore invite you to take these points into account when revising the manuscript. In addition, when preparing the revision please:

- ensure that it conforms to our format instructions and publication policies (see below and <https://www.nature.com/nature/for-authors>).
- provide an updated point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.
- provide an updated Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript and will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

When submitting the revised version of your manuscript, please pay close attention

to our [href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-research/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive the revision within four weeks. If submitted within this time period, reconsideration of the revised manuscript will not be affected by related studies published elsewhere, or accepted for publication in Nature Cell Biology in the meantime. We would be happy to consider a revision even after this timeframe, but in that case we will consider the published literature at the time of resubmission when assessing the file.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

With best wishes,

Christine.

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Nature Cell Biology
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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this revised manuscript by Choi, Jang et al., the authors addressed a major technical limitation of the *scgb1a1cre* by performing new transplant assays where AT2s are excluded to demonstrate the effect of notch on airway-derived *Scgb1a1+* club cells, in addition to organoid experiment showing that notch does not alter *sftpc*-derived AT2 organoids. I also appreciate the increased clarity in the organoid compositions reported in the *Fosl2* KD studies, as well as better resolution of *KDR+* cells from the human lung. The revised manuscript has significantly strengthened the authors' thesis, and will add important knowledge to the field. Nice work!

Reviewer #2:

Remarks to the Author:

This paper has significantly improved and is overall impressive, but I remain with some questions. First and foremost, we still don't know how physiological significant this is, and that would be important. Yet, the authors provide a figure in response to reviewer 3 showing the effect of DAPT treatment *in vivo* (Rev3_Fig. 8). This figure does not seem to have made it into the manuscript. DAPT was administered before and after bleomycin treatment, the rationale of which unclear. Did these mice develop less fibrosis or recovered more rapidly? What would be the effect of IL1 antagonism *in vivo*?

Another issue is that other cells in the organoids are still not accounted for (see Rev_2, Fig. 8). This also goes toward comment 3 of reviewer 3, in response to which the authors claim the presence of ATI cells based on *Pdpr1* staining. This is not a specific ATI marker.

Reviewer #3:

Remarks to the Author:

The revised manuscript by Choi et al. represents a significant improvement over the initial submission, which already displayed a number of strong points and important contributions. My main concerns had to do with whether the authors could justify their conclusion that the regenerative cells were truly airway secretory cells versus BASCs or another population. I was concerned that Notch-inhibitor methods, either genetic or pharmacological, would be expected to convert secretory cells to ciliated cells, even in the adult mouse under homeostasis, and that therefore it was unclear why Notch inhibition would enhance AT2 regeneration from secretory cells. I was also underwhelmed by the level of characterization of the organoids.

The revised manuscript, and the rebuttal letter, show a concerted and rigorous

effort to address these general questions and multiple specific questions/concerns. I commend the authors for their progress and thoughtful approach.

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READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT – should not exceed 150 words and should be unreferenced. This paragraph is the most visible part of the paper and should briefly outline the background and rationale for the work, and accurately summarize the main results and conclusions. Key genes, proteins and organisms should be specified to ensure discoverability of the paper in online searches.

TEXT – the main text consists of the Introduction, Results, and Discussion sections and must not exceed 3500 words including the abstract. The Introduction should expand on the background relating to the work. The Results should be divided in subsections with subheadings, and should provide a concise and accurate description of the experimental findings. The Discussion should expand on the findings and their implications. All relevant primary literature should be cited, in particular when discussing the background and specific findings.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements,

detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

REFERENCES – are limited to a total of 70 in the main text and Methods combined,. They must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

All Nature Cell Biology manuscripts submitted on or after March 21 2016, must include a Data availability statement as a separate section after Methods but before references, under the heading "Data Availability". For Springer Nature policies on data availability see <http://www.nature.com/authors/policies/availability.html>; for more information on this particular policy see <http://www.nature.com/authors/policies/data/data-availability-statements-data->

citations.pdf. The Data availability statement should include:

- Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.
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- At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.
- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can be found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables. For Supplementary Information see below.

FIGURES – Colour figure publication costs \$395 per colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend.

Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it has to be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.
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- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.
- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a

single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labeled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>; <http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos must be accompanied by a separate Word document including titles and legends.

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REPORTING REQUIREMENTS – We ask authors to complete a Reporting Summary that collects information on experimental design and reagents. We hope this will aid in your evaluation of the paper. The Reporting Summary can be found here <https://www.nature.com/documents/nr-reporting-summary.pdf>) Please note that these forms are dynamic 'smart pdfs' and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use. If you would like to reference the guidance text as you complete the template, please access these flattened versions at <http://www.nature.com/authors/policies/availability.html>.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

Author Rebuttal, first revision:

Summary of additional data and modified text in response to the reviewers' comments

Reviewer	Comment	Addition in the revised manuscript
Reviewer 1	1	Extended Data Figure 5a,b
	2-1	Extended Data Figure 5c-i
	2-2	Rev1 Fig. 4 (See Cover Letter)
	2-3	Extended Data Figure 2f,g
	3	Extended Data Figure 6a
	4-1	Figure 4f-h
	4-2	Extended Data Figure 7d,e
	4-3	Extended Data Figure 7g,h
	4-4	Extended Data Figure 7i
	5-1	Extended Data Figure 7j
	5-2	Figure 5g
	6	Extended Data Figure 9i-k
	7-1	Figure 7c,d
	7-2	Figure 7i,j
	Minor-1	Detailed description provided in the text
	Minor-2	Text changed as suggested
	Minor-3	Extended Data Figure 10l,m
Reviewer 2_First Round	1	See current version of point-to-point response 1
	2	Rev2 Fig. 1 (See Cover Letter)
	3	Amended the methods for statistical analysis
	4	Extended Data Figure 7e
	5	Extended Data Figure 2d,e
	6	Extended Data Figure 2b,c and 8h
	7	Extended Data Figure 9l,m
	8-1	Extended Data Figure 10d-g
	8-2	Figure 7c,d
	8-3	Extended Data Figure 10h,i
Reviewer 2_Second Round	1	Extended Data Figure 4
	2	Extended Data Figure 10j; Figure 1f
Reviewer 3	1	Text changed as suggested
	2-1	Text changed as suggested
	2-2	Figure 1f-i
	2-3	Figure 1f
	3-1	Extended Data Figure 5a-i
	3-2	Figure 2c
	3-3	Detailed description provided in the text
	3-4	Extended Data Figure 2d-g
	3-5	Extended Data Figure 4a-d
	4	Extended Data Figure 6b
	5-1	Addressed this point in the Discussion
	5-2	Figure 4f-h
	5-3	Extended Data Figure 7g,h
	5-4	Extended Data Figure 10m
	5-5	Rev3 Fig. 13 (See Cover Letter)
	6	Error was corrected

Point-by-point rebuttal to Reviewers' comments**Reviewer #1:**

In this revised manuscript by Choi, Jang et al., the authors addressed a major technical limitation of the *scgblal*CreERT by performing new transplant assays where AT2s are excluded to demonstrate the effect of notch on airway-derived *Scgblal*⁺ club cells, in addition to organoid experiment showing that notch does not alter sftpc-derived AT2 organoids. I also appreciate the increased clarity in the organoid compositions reported in the *Fosl2* KD studies, as well as better resolution of KDR⁺ cells from the human lung. The revised manuscript has significantly strengthened the authors' thesis, and will add important knowledge to the field. Nice work!

Response: We are grateful that the reviewer appreciated our improvements in addressing the technical challenges and the clarity in our revised manuscript. We are thankful for the reviewers' valuable comments, which helped us strengthen our study.

Reviewer #2:

1. This paper has significantly improved and is overall impressive, but I remain with some questions. First and foremost, we still don't know how physiological significant this is, and that would be important. Yet, the authors provide a figure in response to reviewer 3 showing the effect of DAPT treatment in vivo (Rev3_Fig. 8). This figure does not seem to have made it into the manuscript. DAPT was administered before and after bleomycin treatment, the rationale of which unclear. Did these mice develop less fibrosis or recovered more rapidly? What would be the effect of IL1 antagonism in vivo?

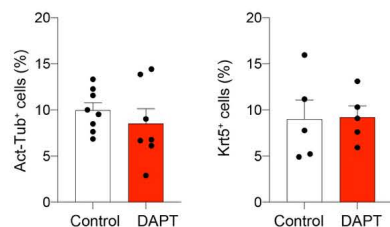
Response: We thank the reviewer for their appreciation that our manuscript has been significantly improved. We believe our study details the physiological significance of inflammatory signals after injury, whereby they direct the fate decision of tissue resident stem cells, which is crucial for tissue maintenance and regeneration. Indeed, as shown in Fig. 4f in the revised manuscript, deletion of *Il1r1* in ciliated cells impaired tissue repair, with defects in AT2 cell differentiation derived from secretory cells. This result clearly suggests that, upon severe alveolar damage, inflammatory signals build regenerative microenvironments which trigger alveolar regeneration. Previously, we also demonstrated that IL-1 β is crucial for alveolar regeneration by enhancing alveolar differentiation of AT2 cells (Choi et al., *Cell Stem Cell*, 2020). Inhibition of IL-1 β signalling in AT2 cells by deletion of *Il1r1* substantially deteriorated tissue regeneration by blocking the generation of AT1 cells. Overall, our studies demonstrate that inhibition of inflammatory niches, mediated by IL-1 β signalling, significantly impairs lung regeneration after tissue injury, which is physiologically significant. Most importantly, we show the conserved functional role of secretory cells and the molecular programme that may contribute to alveolar regeneration in human lungs.

To verify whether pharmacological inhibition of Notch signalling triggers alveolar differentiation of secretory cells, which was originally only shown by genetic ablation of Notch activity using *dnMAML*^{flox/+}; *Scgblal*-*CreER*^{TM/+}; *R26R*^{tdTomato} mice, we then treated *Scgblal*-*CreER*^{TM/+}; *R26R*^{tdTomato} mice with DAPT simultaneously with bleomycin injury (**Rev3_Fig.8; Revised Extended Data Fig. 4**). We confirmed that pharmacological inhibition of Notch signalling also significantly enhanced AT2 cell differentiation of secretory cells, indicating the regulatory role of Notch signalling in modulating the fate behaviours of secretory cells during alveolar regeneration. Given that pharmacological inhibition of Notch signalling induces the fate conversion of secretory cells into ciliated cells in homeostatic lungs, we also asked whether

DAPT treatment prior to bleomycin, which may lead to reduction of absolute numbers of secretory cells, impacts on alveolar regeneration contributed to by secretory cells (**Rev3_Fig. 13**). We confirmed that DAPT treatment prior to injury still showed the enhanced recovery with increased alveolar differentiation of secretory cells compared to control. These results suggest that, despite increased differentiation of secretory cells into ciliated cells in airways, persistent Notch inhibition facilitates the differentiation of remaining secretory cells into AT2 lineages during alveolar regeneration after injury. We have now included these results in our revised manuscript.

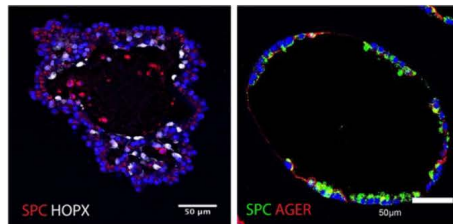
2. Another issue is that other cells in the organoids are still not accounted for (see Rev 2, Fig. 8). This also goes toward comment 3 of reviewer 3, in response to which the authors claim the presence of ATI cells based on Pdpn1 staining. This is not a specific ATI marker.

Response: As shown in **Rev2_Fig.8**, we further analysed our secretory organoids with or without DAPT treatment by immunofluorescence staining for lung epithelial lineage markers. We found that secretory organoids contained Act-Tub⁺ ciliated cells and KRT5⁺ basal cells as well as CC10⁺ secretory cells. Notch inhibition by DAPT treatment decreased CC10⁺ secretory cells with significant induction of SPC⁺ AT2 cells. However, the proportion of ciliated and basal cells remained similar to control organoids (**Revised Fig. 7i,j; Rev2_Fig.1; Revised Extended Data Fig. 10h-j**). We should note that co-staining for all lineages within the section of individual organoids was limited due to the issue of primary antibodies that is raised in the same species.



Rev2_Fig.1 Notch inhibition does not alter the proportion of ciliated and basal cells in organoids derived from secretory cells. Quantification of the frequency of Act-Tub⁺ ciliated cells or Krt5⁺ basal cells in control or DAPT-treated organoids. Each individual dot represents one organoid and data are presented as mean ± s.e.m (n=3 independent experiments).

We also stained the organoids with other canonical AT1 markers such as HOPX or AGER in addition to PDPN (T1α). Immunofluorescence staining clearly showed that alveolar organoids derived from secretory cells at P0 contained AT1 cells (HOPX⁺ or AGER⁺) in addition to SPC⁺ AT2 cells (**Rev2_Fig.2; Revised Fig. 1f**). Therefore, we have used multiple markers to assess the presence of AT1 fate.



Rev2_Fig.2 Alveolar organoids contained AT2 and AT1 cells. IF images of alveolar organoids derived from *Tomato⁺Scgb1a1⁺* cells at the establishment (P0) under stromal-free culture condition with defined medium; Alveolar organoids retained SPC⁺ AT2 cells and HOPX⁺ (left) or AGER⁺ (right) AT1 cells. HOPX (white, left), SPC (red, left), AGER (red, right), SPC (green, right), and DAPI (blue). Scale bars, 50μm.

Reviewer #3:

The revised manuscript by Choi et al. represents a significant improvement over the initial submission, which already displayed a number of strong points and important contributions.

My main concerns had to do with whether the authors could justify their conclusion that the regenerative cells were truly airway secretory cells versus BASCs or another population. I was concerned that Notch-inhibitor methods, either genetic or pharmacological, would be expected to convert secretory cells to ciliated cells, even in the adult mouse under homeostasis, and that therefore it was unclear why Notch inhibition would enhance AT2 regeneration from secretory cells. I was also underwhelmed by the level of characterization of the organoids.

The revised manuscript, and the rebuttal letter, show a concerted and rigorous effort to address these general questions and multiple specific questions/concerns. I commend the authors for their progress and thoughtful approach.

Response: We thank Reviewer #3 for appreciating our rigorous approach to addressing the questions and concerns raised. We are thankful for the reviewers' valuable comments, which helped us strengthen our study.

Decision Letter, second revision:**Date:** 18th June 21 07:41:58**Last Sent:** 18th June 21 07:41:58**Triggered By:** Christine Weber**From:** christine.weber@nature.com**To:** jhl62@cam.ac.uk**CC:** ncb@springernature.com**Subject:** Decision on your manuscript NCB-L44082B**Message:** Dear Joo-Hyeon,

Thank you for submitting your revised manuscript, entitled "Release of Notch activity coordinated by IL-1 β signalling confers differentiation plasticity of airway progenitors via Fosl2 during alveolar regeneration" (NCB-L44082B).

It has now been seen by the original referee and s/he finds that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to comply with our editorial and formatting guidelines.

As the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX) -- we cannot proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about 1-2 weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions.

With best wishes,

Christine.

Christine Weber, PhD
Senior Editor
Nature Cell Biology
E-mail: christine.weber@nature.com
Phone: +44 (0)207 843 4924

Final Decision Letter:

Date: 23rd July 21 09:19:23

Last Sent: 23rd July 21 09:19:23

Triggered By: Christine Weber

From: christine.weber@nature.com

To: jhl62@cam.ac.uk

CC: ncb@springernature.com

BCC: rjsproduction@springernature.com;rjsart@springernature.com

Subject: Decision on Nature Cell Biology submission NCB-L44082C

Message: Dear Joo-Hyeon,

I am pleased to inform you that your manuscript, "Release of Notch activity coordinated by IL-1 β signalling confers differentiation plasticity of airway progenitors via Fosl2 during alveolar regeneration", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With best wishes,

Christine.

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