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Nuclear IL-33/SMAD signaling axis promotes cancer development in chronic inflammation

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1st Editorial Decision 14th Aug 2020

Dear Dr. Demehri,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from their comments, the referees find the analysis interesting. However, they also find that significant revisions are needed in order to consider publication here. In particular they find that some of the mechanistic links need to be better substantiated and that further data is needed to support a causal role of nuclear IL-33 in the described processes.

Should you be able to strengthen the findings along those lines suggested by the referees the we would be interested in considering a revised version. I am happy to discuss the raised points further and maybe it would be most helpful to do so via email or video call. I should add that it is EMBO Journal policy to allow only a single major round of revision and that acceptance of your manuscript will depend on the completeness of your responses in this revised version

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further.

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #1:

The manuscript by Park et al. investigates the role of nuclear IL-33 in the progression of skin and pancreatic cancers. Using global knock out mice for either IL-33 or ST2 (receptor for IL-33), the authors show that both IL-33KO and ST2 KO mice exhibit impaired colon cancer formation in an AOM-DSS model. In contrast, the development of skin tumors following DMBA-DNFB treatment was only negatively impacted by loss of IL-33. This led the authors to focus on a nuclear function of IL-33 (rather than its cytokine function) in skin cancer formation. IL-33 was shown to interact with Runx2, which blocked the ability of Runx2 to induce Smad6 expression. Thus, high IL-33 expression was associated with elevated Smad2/3 and Smad1/5 phosphorylation due to reduced Smad6 levels. The authors revealed that nuclear IL-33 was also important for the induction of pancreatitis associated pancreatic cancer.

The role of IL-33 as a modulator of cancer initiation and progression is quite complex, which is likely due to the fact that IL-33 can be released from cells and function as a cytokine or be retained in the nucleus where it can modulate gene expression. The authors have employed some interesting animal models to dissect the role of nuclear versus secreted IL-33 and have revealed an important

role for nuclear IL-33 in both skin and pancreatic cancer formation. The phenotypes with respect to the carcinogen-induced tumor formation models are very clear. However, some questions remain regarding the precise molecular mechanisms that are engaged by nuclear IL-33, beyond enhanced signaling via Smad2/3 and Smad1/5.

Specific comments:

Figure 2. The authors demonstrate that loss of nuclear IL-33 releases Runx2 so that it can bind the promoter of Smad6 and increase expression. This leads to a suppression of Smad signaling in the absence of nuclear IL-33. If the authors examine the RNA-sequencing data for a TGF-beta regulated gene signature or a Runx-dependent gene signature, do they observe that cells with high nuclear IL-33 would have enhanced expression of TGFbeta target genes and reduced levels of Runx2-regulated genes when compared to cells lacking IL-33.

Figure 3A. The authors use immunoblotting to show that Smad6 levels are reduced and pSmad2/3 and pSmad1/5 levels are elevated in skin tissues treated with DNFB versus acetone. It would strengthen the manuscript if the authors could perform multi-plex IF on these tissues to show that cells with intense nuclear IL-33 are the ones that lack Smad6 and exhibit high pSmad2/3 levels. As seen in Fig. 1G, the degree of nuclear IL-33 is heterogeneous.

Figure 3B. The authors stimulate Pam212 cells with Poly-IC and see an increase of IL-33 (message). Does Poly-IC increase the released for of IL-33 or does it increase the nuclear fraction of IL-33. IF or cellular fractionation would be important to show that the IL-33 that is induced is indeed nuclear.

The authors show that poly-IC or full-length IL-33 increases pSmad2/3 levels (presumably by diminishing Smad6 expression). These blots should be performed using an Li-Cor Odyssey system or similar quantitative platform.

The authors suggest that nuclear IL-33 and elevated TGFbeta signaling promote proliferation of Pam212 cells in vitro and hyperplasia in vivo. How do the authors envision that TGFbeta promotes proliferation (especially in primary keratinocytes, where TGFbeta is typically cytostatic?)? Also, cell growth has been measured; however, proliferation per se has not been examined. Could the skin tissues in Figure 3H be stained for Ki67 and cleaved caspase to look at both proliferation and apoptosis?

Figure 4A-D. Why did the authors not use the original DMBA-DNFB protocol (results in the highest expression of IL-33) to assess the role of TGFbeta signaling?

Fig. 4E-1. Multiplex-IF (rather than immunoblot) could be used to stain the following tissues for nuclear IL-33 and pSmad2/3 for example (K14-IL-33tg, ST2KO + acetone versus K14-IL-33tg, ST2KO + SB431542). I imagine the SB431542 tumors were too small to make lysates from - but the residual tumors could be stained for specific markers.

Ki67 and cleaved caspase staining should also be done on the tissues in described in Fig. 4E-H.

The authors focus on the connection between IL-33/Runx and the impact on Smad6 expression and TGFb signaling. If Runx2 function is impaired by nuclear IL-33, could other Runx2-regulated genes that are induced by IL-33 loss be involved in the observed phenotype? A recent paper published in Science describes an IL-33/TGFbeta axis in the promotion of tumor initiating cells (PMID: 32675345). Could nuclear IL-33 impact TGFbeta signaling and increase TICs in the skin and

pancreatic models. Loss of IL-33, increased Smad6 and reduced TGFbeta signaling that could lead to fewer TICs and reduced skin and pancreatic tumors. It might be interesting to assess stem cell markers in the systems used here.

Referee #2:

Tackling the tumor microenvironment is an important route for future therapies, in particular in tumors with accompanying chronic inflammation. Within this context, Park et al report a tumor-promoting function of the proinflammatory cytokine IL-33 in different mouse models that is independent from its specific receptor ST2 (which has been renamed to IL-1R4). Specifically they show that in skin and pancreatic cancer IL-33 deletion (unlike ST2 deletion) reduces tumor progression. Additionally, transgenic IL-33 expression in keratinocytes in the ST2 knockout background also promotes skin cancer. Based on these observations, the authors conclude that the receptor-independent functions of IL-33 are evidence for a nuclear (cell autonomous) function of this cytokine in cancer. In order to reveal the mechanisms of these effects, the authors have attempted to delineate a signaling pathway by which nuclear IL-33 indirectly augments TGF-beta signaling through downregulation of SMAD6. While the results from the in vivo studies are interesting and the effects are clear, the mechanistic data are less convincing and in my view do not really prove a causal role of nuclear IL-33 in both, tumor development and TGF-beta signaling. There is also a number of technical issues concerning the original data and their further analysis. Specific points:

Fig. 1:
(G/H) a quantification of nuclear IL-33 signals comparing vehicle versus tumor promoting agents is missing. Negative controls demonstrating the specificity of the immunohistochemistry data should be shown.
Fig.2:
(A) The authors used RNA-seq to retrieve candidate genes that might cause the observed IL-33 effects in their mouse models. According to the heatmap pattern, there is substantial variation amongst the upregulated genes in the three IL-33 ko samples. Does this variability impact on the selection process for candidate IL-33 target genes? Was an overrepresentation analysis performed on the 203 DEGs or alternatively, a gene set enrichment analysis taking into accounts the entire lists of expressed genes? If so, did the TGF-beta pathway emerge as enriched and how many components of this pathway were expressed or changed ? Additional point: The scale bar requires labelling (it presumably shows log2 ratio values?).
(B) The integrative analysis of DEGs with ChIP-seq peaks is an important approach to connect putative nuclear functions of IL-33 to changes in gene expression. However, as it is, far too little specific information is provided to judge the data and their analysis. Apparently, the large circle of the Venn diagram shows differentially enriched peaks comparing ectopically expressed full length (fl) IL-33 versus the truncated (non-nuclear) version combined with data from vector transfected cells (why were the latter two groups pooled?). Genome-browser views should be shown to demonstrate: (i) the quality of the ChIP-seq signals, (ii) the differential binding between fl IL-33 and the mutant IL-33 at genomic coordinates, and (iii) the recruitment of fl IL-33 to the promoters/enhancers of genes shown in (C). A further analysis should provide insight into the genomic distribution of all IL-33 peaks and enrichment analyses of biological pathways of the next annotated genes (e.g. using GREAT tools). The method parts needs to be expanded to cover comprehensively the ChIP-seq analysis workflow. A state of the art analysis and presentation of the ChIP-seq data is crucial for this study to reveal more directly the nuclear role of IL-33. ChIP-seq

data of endogenous IL-33 (e.g. after induction by appropriate oncogenic / inflammatory triggers) would also be key to conclude on a direct nuclear function.
(C) On page 7 the authors state that by "cross comparing RNA-seq with ChIP-seq data" they narrowed down the nuclear IL-33 candidates to seven genes whose regulation at the mRNA level is shown in (C). This procedure appears highly selective and the effect sizes (ratios) in changes of gene expression are small (e.g. SMAD6 is downregulated by log2 0.79-fold), although this effect is confirmed by RT-qPCR (shown in (D). Along the issues raised in (A), an unbiased and complete analysis of the genomics data needs to be provided to justify the focus on SMAD6-TGF-beta signaling as key downstream pathway of IL-33.
(E) The effects of ectopically expressed fl IL-33 versus cytosolic IL-33 are minor and may simply result from confounding factors such as differences in transfection efficiency etc. . No immunoblots are provided proving that the constructs were equally well expressed under these conditions. This is of particular concern in the light of the immunoblots presented in (G) which show that IL-33 cyto is stronger expressed. Moreover, the data are presented as fold change; against which sample (non-transfected, vector transfected)?. I doubt that this system is suited to prove a role of nuclear IL-33 in regulation of SMAD6.
(F) This Co-IP lacks crucial negative controls such as IgG pulldowns from lysates of the PyMT cell extracts.
(H) This experiment provides a key link for the mechanism proposed to be regulated by nuclear IL-33. Several improvements are required: (i) ChIP-qPCR enrichment should be shown as percent input and not as relative change. (ii) Since IL-33 is a cofactor which are notoriously difficult to crosslink and to ChIP, a negative control region (chosen from the ChIP-seq data) and IgG IPs need to be added to demonstrate that raw signals were well above background. (iii) The authors need to add endogenous IL-33 ChIP-qPCR data comparing the Runx recruitment to the SMAD6 promoter in wt versus IL-33 ko cells.
Fig.3:
(A) The technical quality of the immunoblots is unconvincing. There is a lot of background and the protein bands are blurry. I appreciate that this may be due to technical issues during PDF conversion or to low abundancies of the proteins. However, the authors should provide all full size blots of all replicates as source data to solve these issues. To conclude that SMAD1/5 phosphorylation is changing (and not just the protein levels), anti SMAD1/5 blots need to be added. All changes need quantification of replicates, graphical presentation and statistical testing of the data. This comment also applies to the other blot panels shown in Fig. 3C/D, Fig. 4I, Fig. 5C, Fig. EV3A/B. All of these experiments serve to proof the counter regulation of P-SMAD2/3, P-SMAD1/5 and SMAD6 and I regard a revision of these data sets as crucial to support the main conclusions of this manuscript.
Fig. 4:
(J) In the line with comments above (Fig.1), data require quantification and addition of negative controls.
Fig. EV2:
(C), (D) Along the comments to Fig. 2:
Why are only the top 20 DEGs and the top 6 enriched pathways are shown? A much more complete and comprehensive analysis and presentation of the genomics data sets is warranted to claim the major role of TGF-beta pathway components for IL-33 signaling.
(E) What is the red signal? IL-33 antibody stainings?

Minor points:
Page 3: In the first paragraph, the authors give the impression that chronic inflammatory microenvironments exclusively lead to resistance to conventional cancer immunotherapies such as checkpoint inhibitors. For two reasons, I find this rather general statement misleading: (i) not all

inflammatory microenvironments will be associated with resistance to therapy; the role of inflammation is highly dependent on the individual tumor type and tumor evolution; (ii) checkpoint inhibitors should not be classified as conventional immunotherapies. It is rather appropriate to refer in this context to the role of inflammation in the response to conventional chemotherapies or to novel immunotherapies, the latter being only suited for tumors expressing checkpoint ligands and containing enough immune cells responding to these signals.
Page 18: Explain the species and tissue type of Pam212 cells.

Referee #3:

General summary
In this study, Jong Ho Park et al. investigate the nuclear function of the cytokine IL-33 in promoting tumorigenesis that develops in the context of chronic inflammation, by revealing its role as a pro-tumorigenic regulator of transcription.
The authors first show that IL-33 supports tumor development independently of the receptor ST2 in a murine model of inflammation-triggered skin cancer, but not during colitis-associated intestinal tumorigenesis. By applying a combination of transcriptomics (RNA-Seq) and protein-DNA interaction (ChIP-Seq) analysis on murine skin and epithelial cell lines, they then identify Smad6 as a key differentially expressed gene found in both IL-33 knockout mice and IL-33 full-length overexpressing murine Pam212 squamous cell carcinoma (SSC) cells, when compared to WT mice and IL-33 (mature) cytokine domain-overexpressing Pam212 cells, respectively.
The authors use several complementary approaches to show that full-length (i.e. nuclear) IL-33 binds to RUNX2, the transcription factor controlling Smad6 transcription, which in turn represses Smad6 expression. Further data show that that Smad6 downregulation leads to increased TGFb-induced Smad signaling, which results in increased epithelial cell proliferation upon TGFb stimulation.
Using a ST2-deficient mouse strain with keratinocyte-specific IL-33 overexpression, the authors determine that nuclear IL-33 is able to promote skin cancer development (independent of DNFB sensitization) through their previously described nuclear IL-33/SMAD6/TGFb axis. Lastly, they show that this nuclear IL-33/SMAD6/TGFb axis also supports the development of pancreatitis-associated pancreatic cancer.
Overall, the authors convincingly detail the mechanisms by which nuclear IL-33 influences TGFb sensitivity, illustrating its role in regulating tumor development during chronic infectious condition. The experiments performed include all necessary controls and the data provided are generally convincing and of good quality. To demonstrate the general relevance of their findings the authors use several different mouse models of inflammation-associated cancer, as well as tissues from human patients. I still have several comments on the current manuscript:

Major comments
While the authors use several different models of inflammation and cancer, and it is not always clear why they switch from one model to another one. The rationale behind it should be better explained in the text, in particular for the models of skin inflammation and cancer. For instance, DFNB/DMBA is used to induce skin cancer in Fig 1, yet after presenting these data, the authors use the skin of DFNB (only)-treated / sensitized mice for their RNA seq analysis in Fig 2, as well as later on for other experiments (Fig. 3). In Fig 4, they then use a model of skin cancer based on TPA/DMBA, in the context of which IL-33 needs to be first genetically overexpressed to see an

effect of TGFb inhibition.

The reason for focusing exclusively on Smad6 as a possible target of nuclear IL-33, rather than any of the other genes identified in their combined RNA-Seq / ChIP-Seq analysis is not convincingly rationalized from the current line of argumentation presented in the manuscript. Contrarily to what the authors claim, several of the other genes identified in Fig 2C have been previously involved in cancer. This is for instance true for BNC2 (PMID: 27899818; PMID: 29750795) and NUP210 (doi: https://doi.org/10.1101/2020.02.05.936518), ...

The representation of some of the histology data needs to be improved to be more convincing. These include Fig 1G (overexposed, somehow unsharp) and Fig 4A (overexposed). In Fig. 4J, the authors should present a magnification (in an inset) of the normal / non-cancerous skin; in addition, the panels showing IL-33 stain is SCC tissue are too bright. Based on the current data it is not clear whether IL-33 is indeed upregulated in SCC versus normal skin.

Lastly, in the discussion the authors could present a possible explanation on why the identified IL-33/SMAD6/TGFb axis is important for inflammation-dependent cancer in the skin and pancreas, but not during colon cancer. Is this due to the expression of IL-33 being mainly detectable in stromal but not in epithelial cells in their model, in the colon of AOM/DSS-treated mice? i.e. IL-33 has a cell-intrinsic role in malignant cells, but not for other cells in the tumor environment? Note that other studies reported IL-33 to be upregulated in the intestinal epithelium in the context of DSS treatment (PMID: 23172891; PMID: 30224451) and AOM/DSS treatment (PMID: 26942077), which should be also mentioned.
They could possibly also discuss about which domains of IL-33 interacts with RUNX2. Is this the N-terminal part that is only present in the full-length IL-33 protein? Or does simply the cellular location (i.e. nuclear versus cytoplasmic/secreted) determines whether an interaction can take place between the 2 proteins? The former seems more likely, based on the overexpression data showed in Fig. 2G and 2H and on a previous report describing an interaction of the same N-terminal domain with NF-kB (PMID: 21734074).

Minor comments
• In the Methods, the chapter on the ChIP assay mentions the use of an anti-HA antibody for RUNX2/DNA immunoprecipitation, while the legend of Fig 2H indicates that an anti-RUNX antibody was employed.
• In the Methods, the authors should indicate how they measure tumor volume in the skin and intestine. Since the data in Fig 1A and D show the pooled volumes of all single tumors from several mice per group, they should also present corresponding data on tumor counts per mouse, as done for instance in Fig 4C and G.
• For all figure legends, the authors should indicate the number of experimental repetitions with similar findings; this information is not provided for each dataset.
• A list of the 203 genes showing differential expressions in IL-33 KO compared with WT skin keratinocytes should be added to this manuscript (e.g. as supplemental data part).
• Fig. EV2B: the current magnification / picture size does not allow an assessment of the lesions on the back of these mice.
• Fig. EV3A: can the resolution / contrast be improved for the WB data indicating SMAD6 expression?
• Fig 3C: Contrarily to what the authors claim, endogenous SMAD2/3 levels seem to change / diminish upon combined TGFb + poly (I:C) treatment of Pam212 cells.
• The authors should precise whether whole (back) skins (more likely) or only skin patches with

tumors were used for preparing the tissue lysates that were analyzed by WB (e.g. for Fig 4I).
• In Fig 5D, the Y axis indicates the percent of tumor-free survival, yet ST2 KO mice apparently only develop severe pancreatitis with fibrosis, without cancer. Therefore, I think the label of the axis shall be accordingly modified.
• The calling of some of the figures should be corrected in the result part (Fig EV5A instead of Fig 5A; Fig EV5D instead of Fig 5D). In addition, Fig 5E but not Fig EV5E shows tissue fibrosis.
• In the discussion, the study by "Gao et al, 2015" employs murine B16 melanoma cells and 4T1 mammary gland cells, but no intestinal epithelial cells.

Response to Referees' Comments:

We thank the referees for their insightful comments that have improved our manuscript. Our replies to the comments are provided in blue below. Manuscript text and figures have also been revised (highlighted in blue) according to the referees' comments.

Referee #1:

The manuscript by Park et al. investigates the role of nuclear IL-33 in the progression of skin and pancreatic cancers. Using global knock out mice for either IL-33 or ST2 (receptor for IL-33), the authors show that both IL-33KO and ST2 KO mice exhibit impaired colon cancer formation in an AOM-DSS model. In contrast, the development of skin tumors following DMBA-DNFB treatment was only negatively impacted by loss of IL-33. This led the authors to focus on a nuclear function of IL-33 (rather than its cytokine function) in skin cancer formation. IL-33 was shown to interact with Runx2, which blocked the ability of Runx2 to induce Smad6 expression. Thus, high IL-33 expression was associated with elevated Smad2/3 and Smad1/5 phosphorylation due to reduced Smad6 levels. The authors revealed that nuclear IL-33 was also important for the induction of pancreatitis associated pancreatic cancer.

The role of IL-33 as a modulator of cancer initiation and progression is quite complex, which is likely due to the fact that IL-33 can be released from cells and function as a cytokine or be retained in the nucleus where it can modulate gene expression. The authors have employed some interesting animal models to dissect the role of nuclear versus secreted IL-33 and have revealed an important role for nuclear IL-33 in both skin and pancreatic cancer formation. The phenotypes with respect to the carcinogen-induced tumor formation models are very clear. However, some questions remain regarding the precise molecular mechanisms that are engaged by nuclear IL-33, beyond enhanced signaling via Smad2/3 and Smad1/5.

We thank the referee for their positive remarks and the critical points raised.

Specific comments:

Figure 2. The authors demonstrate that loss of nuclear IL-33 releases Runx2 so that it can bind the promoter of Smad6 and increase expression. This leads to a suppression of Smad signaling in the absence of nuclear IL-33. If the authors examine the RNA-sequencing data for a TGF-beta regulated gene signature or a Runx-dependent gene signature, do they observe that cells with high nuclear IL-33 would have enhanced expression of TGFbeta target genes and reduced levels of Runx2-regulated genes when compared to cells lacking IL-33.

As suggested, we have performed Gene Set Enrichment Analysis (GSEA) on our RNA-sequencing data, which demonstrates the significant enrichment for negative regulation of TGF-β/BMP signaling pathway in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1A). *Bmper*, *Smad6* and *Smad7* which are negative regulator of BMP and TGF-β signaling are highlighted in the heatmap plot (Fig R1B). In addition, RUNX2 target genes are highly upregulated in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1C).
(Appendix Fig S2E-G, S3D, and lines 125-126 and 150-152 in the revised manuscript)

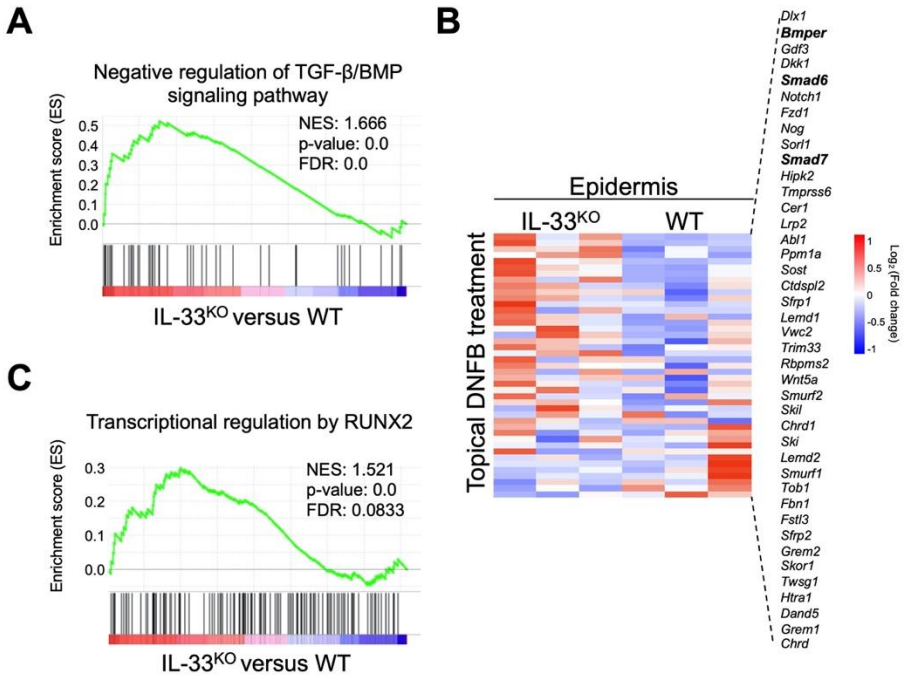

Figure R1. Analysis of RNA-sequencing data comparing IL-33^{KO} and WT epidermis treated with DNFB.

(A) The enrichment plot of negative regulation of BMP signaling pathway gene set in IL-33^{KO} compared with WT epidermis treated with DNFB.

(B) Heatmap of negative regulation of TGF-β/BMP signaling pathway gene set in IL-33^{KO} versus WT epidermis after topical treatment with DNFB. This gene lists contain several genes (highlighted) shared between BMP and TGF-β signaling pathways.

(C) The enrichment plot of transcriptional regulation by RUNX2 gene set in IL-33^{KO} compared with WT epidermis treated with DNFB.

Figure 3A. The authors use immunoblotting to show that Smad6 levels are reduced and pSmad2/3 and pSmad1/5 levels are elevated in skin tissues treated with DNFB versus acetone. It would strengthen the manuscript if the authors could perform multi-plex IF on these tissues to show that cells with intense nuclear IL-33 are the ones that lack Smad6 and exhibit high pSmad2/3 levels. As seen in Fig. 1G, the degree of nuclear IL-33 is heterogeneous.

We appreciate the referee's comment with regard to the co-localization of IL-33 and pSmad2/3 in the keratinocytes' nuclei. However, the available IL-33 and p-SMAD2/3 antibodies do not work on IF platform (Fig R2A). Therefore, we used IHC for both IL-33 and p-SMAD2/3 in the same region of adjacent tissue sections as shown in Fig 4J and 5F. To further substantiate this co-localization, we provide the images of mice skin stained with IL-33, p-SMAD2/3 or SMAD6 antibodies using IHC on adjacent tissue sections from DNFB- and acetone-treated controls (Fig R2B).

(Appendix Fig S4B in the revised manuscript)

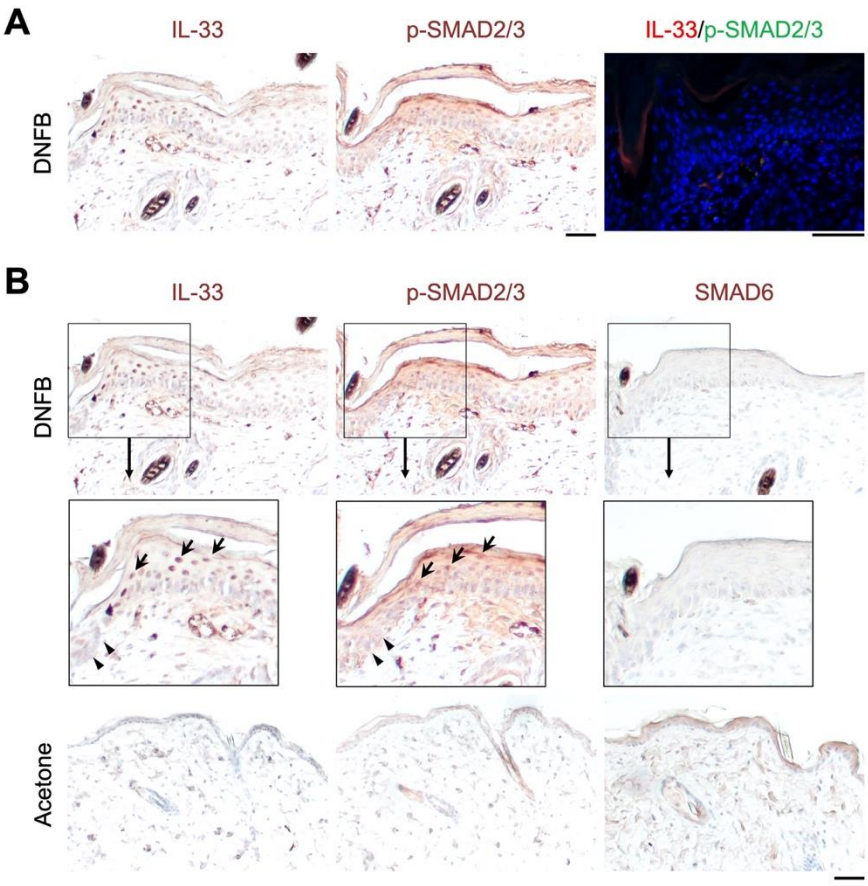

Figure R2. **IL-33, p-SMAD2/3 and SMAD6 localization in DNFB-treated skin.**
(A) Representative images of IL-33 and p-SMAD2/3 immunostaining on the adjacent tissue sections of DNFB-treated mouse skin and negative immunofluorescence staining result for the detection of IL-33 and p-SMAD2/3 in the same tissue.
(B) Representative images of IL-33, p-SMAD2/3 and SMAD6 IHC on the adjacent sections of DNFB versus acetone-treated mouse skin. Arrows in the insets point to nuclear IL-33 and p-SMAD2/3 stains in the epidermal keratinocytes and arrowheads highlight keratinocytes nuclei that are negative for both IL-33 and p-SMAD2/3.
Scale bars: 100 μm

Figure 3B. The authors stimulate Pam212 cells with Poly-IC and see an increase of IL-33 (message). Does Poly-IC increase the released for of IL-33 or does it increase the nuclear fraction of IL-33. IF or cellular fractionation would be important to show that the IL-33 that is induced is indeed nuclear.

This is an important point. As mentioned above, commercially available IL-33 antibody does not work for IF staining. Nonetheless, to address this important question, we have performed nuclear fractionation, which demonstrate that poly (I:C)-induced endogenous IL-33 is localized in the nucleus of Pam212 cells (Fig R3).
(Appendix Fig S5A and lines 172-173 in the revised manuscript)

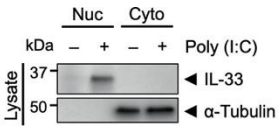

Figure R3. Poly (I:C) induces nuclear IL-33.
IL-33 protein levels in nuclear (Nuc) and cytoplasmic (Cyto) fractions of Pam212 cells treated with poly (I:C). Each fraction's lysate was subjected to immunoblot with IL-33 and α-tubulin antibodies.

The authors show that poly-IC or full-length IL-33 increases pSmad2/3 levels (presumably by diminishing Smad6 expression). These blots should be performed using an Li-Cor Odyssey system or similar quantitative platform.

As requested, we have quantified the western blot data using cSeries Capture Software from azure biosystems (Fig R4).
(Appendix Fig S4A, D, F, S5D-F, S8A, S9D, and lines 433-438 in the revised manuscript)

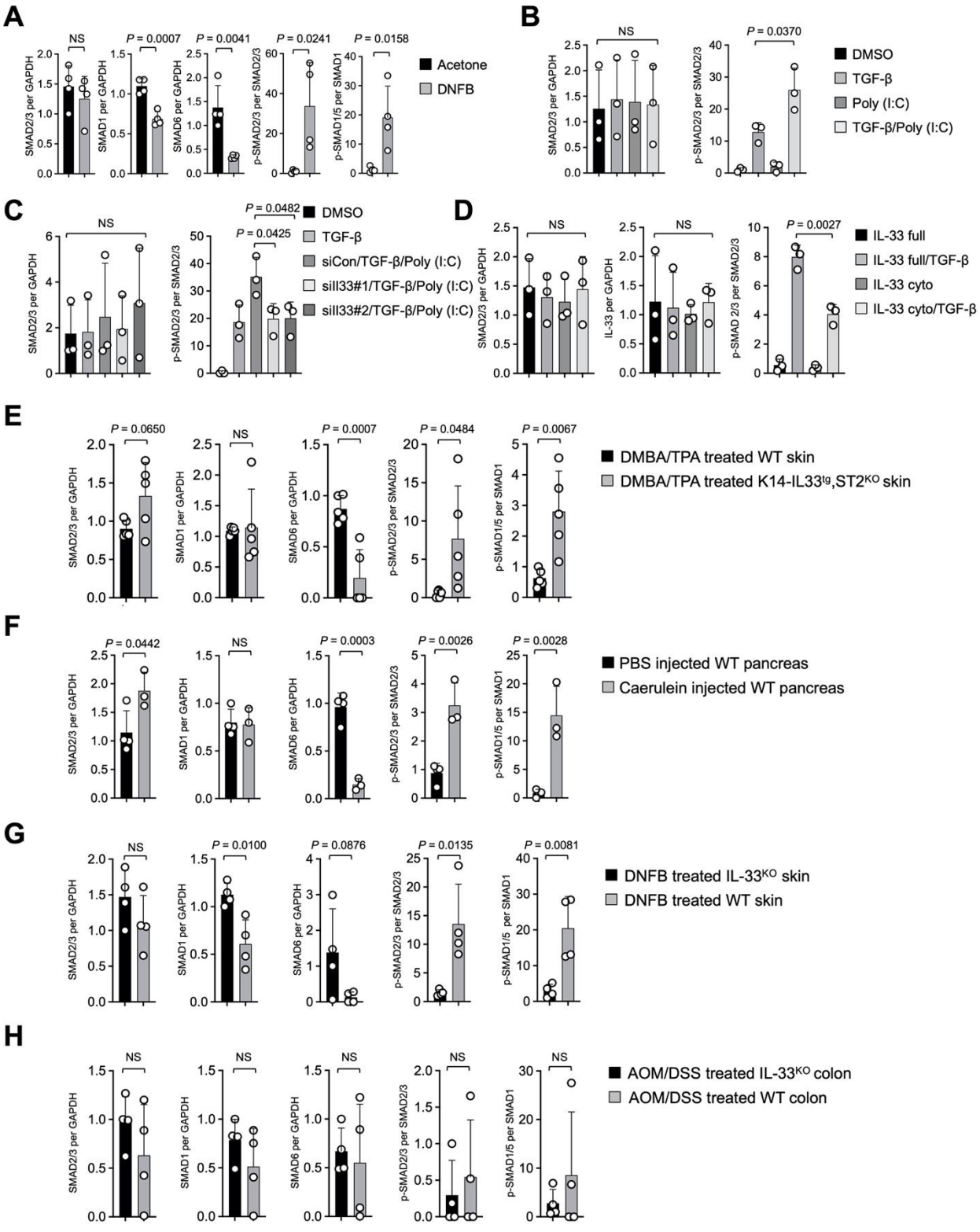

Figure R4. Quantification of western blot results.
(A) Quantification of protein bands shown in Figure 3A.
(B) Quantification of protein bands shown in Figure 3D.

(C) Quantification of protein bands shown in Figure 3E.
(D) Quantification of protein bands shown in Figure 3F.
(E) Quantification of protein bands shown in Figure 4I.
(F) Quantification of protein bands shown in Figure 5C.
(G) Quantification of protein bands shown in Appendix Fig S4C.
(H) Quantification of protein bands shown in Appendix Fig S4E.
NS: not significant, unpaired *t*-test

The authors suggest that nuclear IL-33 and elevated TGFbeta signaling promote proliferation of Pam212 cells in vitro and hyperplasia in vivo. How do the authors envision that TGFbeta promotes proliferation (especially in primary keratinocytes, where TGFbeta is typically cytostatic?)? Also, cell growth has been measured; however, proliferation per se has not been examined. Could the skin tissues in Figure 3H be stained for Ki67 and cleaved caspase to look at both proliferation and apoptosis?

We thank the reviewer for raising this critical point. Although TGF-β has pleiotropic effects on epithelial cells, we have found TGF-β to promote keratinocyte proliferation. To substantiate this finding, we have treated Pam212 cells with SB431542 (TGF-β inhibitor) and found a significant reduction in Ki67$^+$ cells (Fig R5A and B). In addition, we provide Ki67 and cleaved caspase-3 IF staining images for skin samples shown in Fig 3I, which demonstrate the reduction in Ki67$^+$ proliferating cells, but no change in apoptosis, in WT epidermis treated with SB431542 compared with acetone control (Fig R5C).
(Fig 3I, Appendix Fig S6C-E, and lines 193-195 and 201-205 in the revised manuscript)

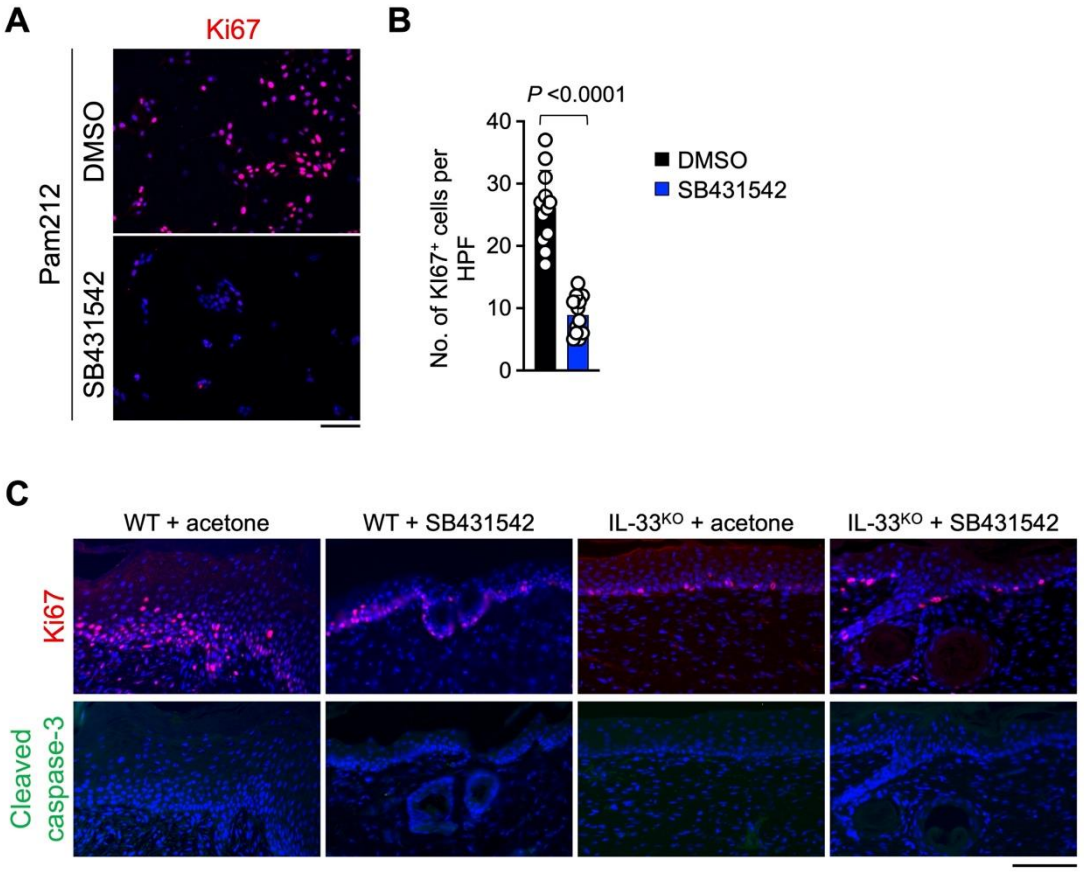

Figure R5. TGF-β inhibitor blocks proliferation of keratinocytes *in vitro* and *in vivo*.
(A) Representative images of Ki67 immunofluorescence staining on Pam212 cells treated with SB431542 or DMSO (carrier control) for 24 hours.
(B) Quantification of $Ki67^{+}$ Pam212 cells treated with SB431542 versus DMSO (unpaired *t*-test).
(C) Representative images of Ki67 and cleaved caspase-3 immunofluorescence stained skin tissues shown in Fig 3I.
Scale bars: 100 μm

Figure 4A-D. Why did the authors not use the original DMBA-DNFB protocol (results in the highest expression of IL-33) to assess the role of TGFbeta signaling?

We appreciate this critical point. IL-33 is highly induced in chronic inflammatory model of skin carcinogenesis (i.e., DMBA/DNFB), and we have shown that blocking TGF-β signaling suppressed epithelial hyperplasia in WT mice treated with DNFB (Fig 3I and J). In Fig 4, we sought to isolate the tumor-promoting mechanism downstream of IL-33 induction. Therefore, in this set of experiments, we used WT versus K14-IL-33 transgenic mice that highly express IL-33 and treated them with standard chemical skin carcinogenesis protocol (DMBA/TPA) to exclude other tumor-promoting effects of chronic DNFB treatment. We have clarified this point in the revised manuscript.
(lines 211-215 in the revised manuscript)

Fig. 4E-1. Multiplex-IF (rather than immunoblot) could be used to stain the following tissues for nuclear IL-33 and pSmad2/3 for example (K14-IL-33tg, ST2KO + acetone versus K14-IL-33tg, ST2KO + SB431542). I imagine the SB431542 tumors were too small to make lysates from - but the residual tumors could be stained for specific markers.
Ki67 and cleaved caspase staining should also be done on the tissues in described in Fig. 4E-H.

To address this point, we performed IL-33 and p-SMAD2/3 IHC staining on adjacent skin sections from K14-IL-33^{tg},$ST2^{KO}$ + acetone and K14-IL-33^{tg},$ST2^{KO}$ + SB431542 mice, which demonstrates the co-localization of p-SMAD2/3 and IL-33 in epidermal keratinocytes' nuclei (Fig R6A). In addition, we performed Ki67 and cleaved caspase-3 IF staining on adjacent skin sections from K14-IL-33^{tg},$ST2^{KO}$ + acetone and K14-IL-33^{tg},$ST2^{KO}$ + SB431542 mice, which highlights the significant reduction in epidermal keratinocyte proliferation, but no change in apoptosis, in K14-IL-33^{tg},$ST2^{KO}$ skin treated with SB431542 (Fig R6B).
(Appendix Fig S7D, E, and lines 227-229 in the revised manuscript)

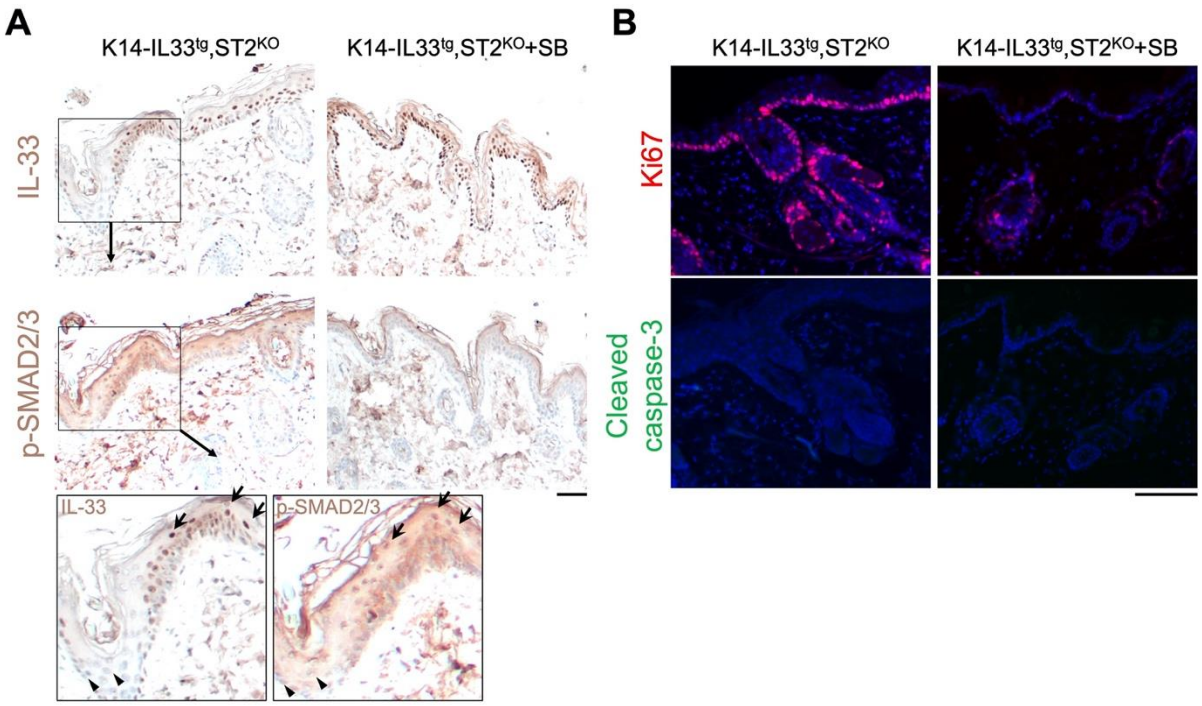

Figure R6. Reduction in epidermal p-SMAD2/3 and proliferation in K14-IL33^{tg},ST2^{KO} skin treated with SB431542.
(A) Representative images of IL-33 and p-SMAD2/3 immunostained adjacent skin sections from SB431542 and acetone-treated K14-IL33^{tg},ST2^{KO} mice that completed DMBA/TPA carcinogenesis protocol.
(B) Representative images of Ki67 and cleaved caspase-3 immunofluorescence staining on the adjacent sections of SB431542 and acetone-treated K14-IL33^{tg},ST2^{KO} skin that underwent DMBA/TPA carcinogenesis protocol.
Scale bars: 100 μm

The authors focus on the connection between IL-33/Runx and the impact on Smad6 expression and TGFb signaling. If Runx2 function is impaired by nuclear IL-33, could other Runx2-regulated genes that are induced by IL-33 loss be involved in the observed phenotype? A recent paper published in Science describes an IL-33/TGFbeta axis in the promotion of tumor initiating cells (PMID: 32675345). Could nuclear IL-33 impact TGFbeta signaling and increase TICs in the skin and pancreatic models. Loss of IL-33, increased Smad6 and reduced TGFbeta signaling that could lead to fewer TICs and reduced skin and pancreatic tumors. It might be interesting to assess stem cell markers in the systems used here.

Thank you for raising this important point. We agree that nuclear IL-33, which is highly and broadly expressed in epidermal keratinocytes and epithelial cells of pancreas in the context of chronic inflammation, can amplify TGF-β signaling within the tumor-initiating cells. We have discussed this critical point in our revised manuscript.
(lines 329-331 in the revised manuscript)

Referee #2:

Tackling the tumor microenvironment is an important route for future therapies, in particular in tumors with accompanying chronic inflammation. Within this context, Park et al report a tumor-promoting function of the proinflammatory cytokine IL-33 in different mouse models that is independent from its specific receptor ST2 (which has been renamed to IL-1R4). Specifically they show that in skin and pancreatic cancer IL-33 deletion (unlike ST2 deletion) reduces tumor progression. Additionally, transgenic IL-33 expression in keratinocytes in the ST2 knockout background also promotes skin cancer. Based on these observations, the authors conclude that the receptor-independent functions of IL-33 are evidence for a nuclear (cell autonomous) function of this cytokine in cancer. In order to reveal the mechanisms of these effects, the authors have attempted to delineate a signaling pathway by which nuclear IL-33 indirectly augments TGF-beta signaling through downregulation of SMAD6. While the results from the in vivo studies are interesting and the effects are clear, the mechanistic data are less convincing and in my view do not really prove a causal role of nuclear IL-33 in both, tumor development and TGF-beta signaling. There is also a number of technical issues concerning the original data and their further analysis.

We thank the referee for the critical points raised.

Specific points:

Fig. 1:
(G/H) a quantification of nuclear IL-33 signals comparing vehicle versus tumor promoting agents is missing. Negative controls demonstrating the specificity of the immunohistochemistry data should be shown.

To address this critique, we quantified nuclear IL-33^{+} epithelial and dermal/stromal cells in the skin and colon impacted by chronic inflammation (Fig R7A and B). In addition, the specificity of IL-33 immunohistochemistry data was confirmed by staining DNFB-treated skin and AOM/DSS-treated colon of IL-33^{KO} animals with IL-33 antibody, which clearly demonstrates lack of staining in IL-33 deficient tissues (Fig R7C). These data have been added to the revised manuscript.
(Fig 1H, J, Appendix Fig S1F, and lines 110-114 in the revised manuscript)

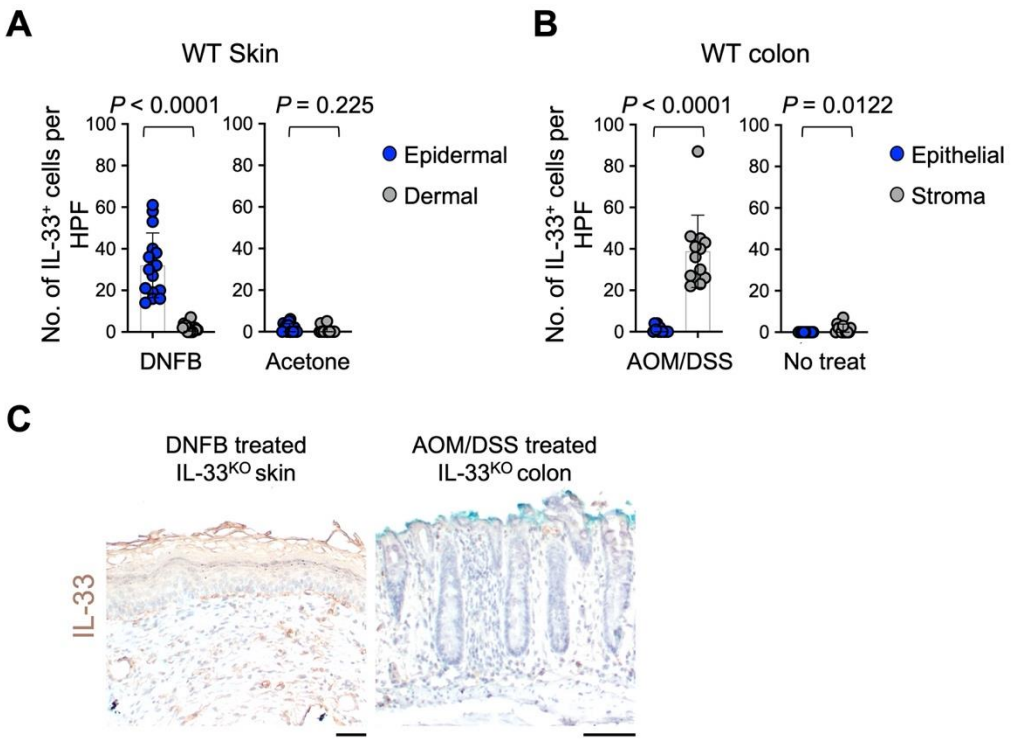

Figure R7. Quantification of nuclear IL-33 in skin and colon tissues.
(A) Quantification of nuclear IL-33^{+} epidermal and dermal cells in the DNFB versus acetone-treated WT skin. Dots represent cell counts from three randomly selected high power field (HPF) images per sample (n=5 per group, unpaired *t*-test).
(B) Quantification of nuclear IL-33^{+} epithelial and stromal cells of WT colon treated with AOM/DSS versus no treatment. Dots represent cell counts from three randomly selected HPF images per sample (n=4 per group, unpaired *t*-test).
(C) Representative images of IL-33 immunostained DNFB-treated skin and AOM/DSS-treated colon tissues from IL-33^{KO} mice. Scale bars: 100 μm

Fig.2:
(A) The authors used RNA-seq to retrieve candidate genes that might cause the observed IL-33 effects in their mouse models. According to the heatmap pattern, there is substantial variation amongst the upregulated genes in the three IL-33 ko samples. Does this variability impact on the selection process for candidate IL-33 target genes? Was an overrepresentation analysis performed on the 203 DEGs or alternatively, a gene set enrichment analysis taking into accounts the entire lists of expressed genes? If so, did the TGF-beta pathway emerge as enriched and how many components of this pathway were expressed or changed? Additional point: The scale bar requires labelling (it presumably shows log2 ratio values?).

As recommended, we have performed gene set enrichment analysis (GSEA) taking into account the entire RNA-Seq results, which demonstrates the significant enrichment for negative regulation of TGFβ/BMP signaling pathway in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1A and B above). In addition, Runx2 target genes are highly upregulated in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1C above). We Thank the referee for pointing out the missing heatmap scale bar label, which is now added to Fig 2A.
(Appendix Fig S2E-G, S3D, and lines 125-126 and 150-152 in the revised manuscript)

(B) The integrative analysis of DEGs with ChIP-seq peaks is an important approach to connect putative nuclear functions of IL-33 to changes in gene expression. However, as it is, far too little specific information is provided to judge the data and their analysis. Apparently, the large circle of the Venn diagram shows differentially enriched peaks comparing ectopically expressed full length (fl) IL-33 versus the truncated (non-nuclear) version combined with data from vector transfected cells (why were the latter two groups pooled?). Genome-browser views should be shown to demonstrate: (i) the quality of the ChIP-seq signals, (ii) the differential binding between fl IL-33 and the mutant IL-33 at genomic coordinates, and (iii) the recruitment of fl IL-33 to the promoters/enhancers of genes shown in (C). A further analysis should provide insight into the genomic distribution of all IL-33 peaks and enrichment analyses of biological pathways of the next annotated genes (e.g. using GREAT tools). The method parts needs to be expanded to cover comprehensively the ChIP-seq analysis workflow. A state of the art analysis and presentation of the ChIP-seq data is crucial for this study to reveal more directly the nuclear role of IL-33. ChIP-seq data of endogenous IL-33 (e.g. after induction by appropriate oncogenic / inflammatory triggers) would also be key to conclude on a direct nuclear function.

We appreciate the importance of the points raised. The integrative analysis of RNA-Seq and ChIP-Seq results enabled us to identify *Smad6* as the nuclear target of IL-33. To further support this analysis:
1) We provide peak calling for the seven DEGs that showed differentially enriched peaks comparing ectopically expressed full length (fl) IL-33 to the truncated (non-nuclear or cyto) version of IL-33 (Fig R8). We did not combine the data from IL-33 cyto and vector only transfected cells. This has been clarified in the revised manuscript.
(Appendix Fig S3C, and lines 130-134 in the revised manuscript)

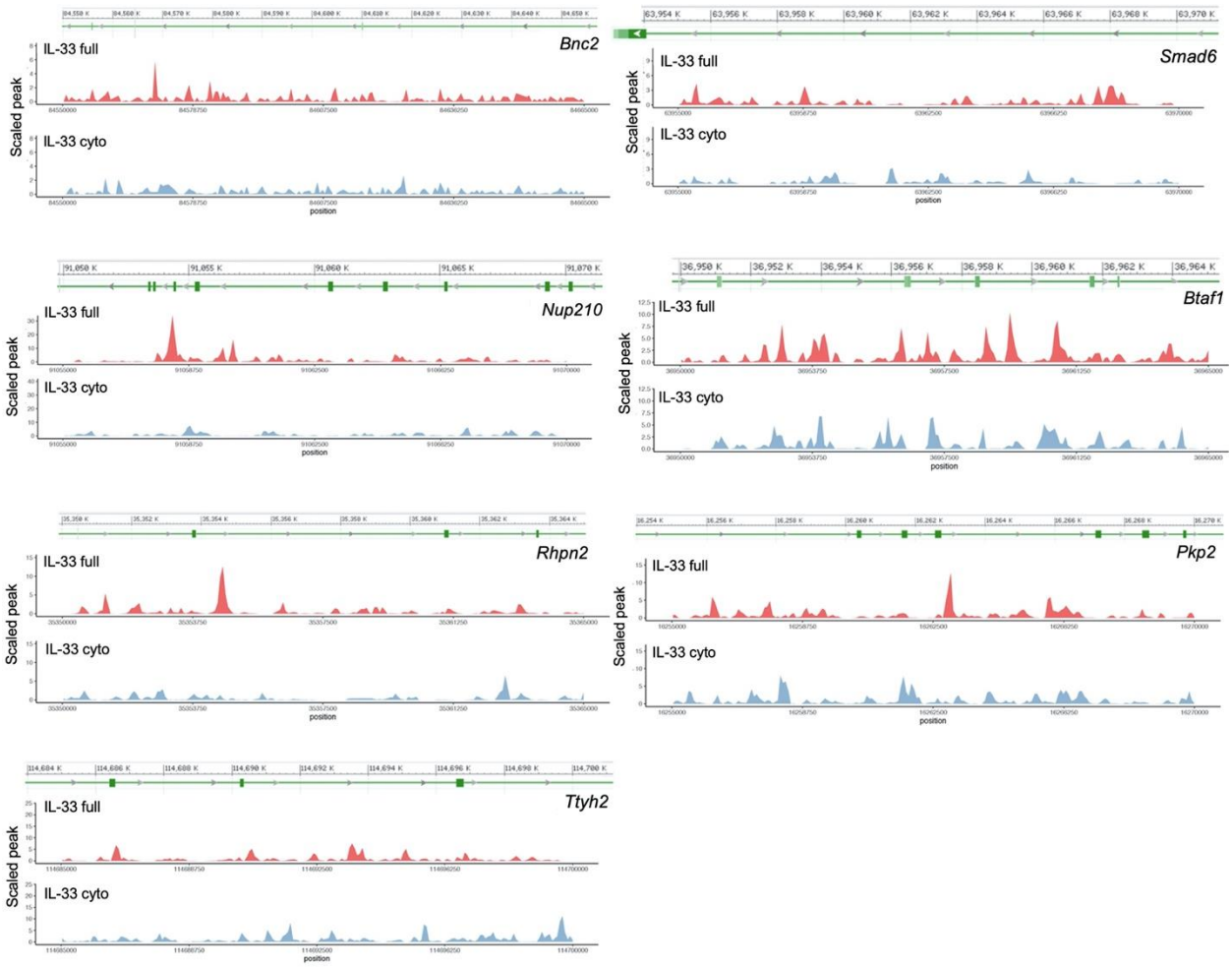

Figure R8. Peak calling analysis of genes identified from the integrative analysis of RNA-Seq and ChIP-Seq results.
Genome-browser views of IL-33 full length (full) and IL-33 cytokine domain (cyto) ChIP-Seq signals for *Bnc2*, *Nup210*, *Rhpn2*, *Ttyh2*, *Smad6*, *Btaf1* and *Pkp2* genes.

2) We have added detailed description of method for ChIP-Seq to revised manuscript.
(lines 481-492 in revised manuscript)

3) In response to referee's comment, we performed ChIP-qPCR assay on Pam212 cells after poly (I:C) mediated induction of endogenous IL-33. Using anti-IL-33 antibody and primer sets for a peak region and a no peak (negative) region of *Smad6* regulatory region from ChIP-Seq data, we confirm the binding of endogenous IL-33 to *Smad6* regulatory region as identified in our ChIP-Seq study (Fig R9).
(Fig 3C, Appendix Fig S5B, and lines 173-174 in the revised manuscript)

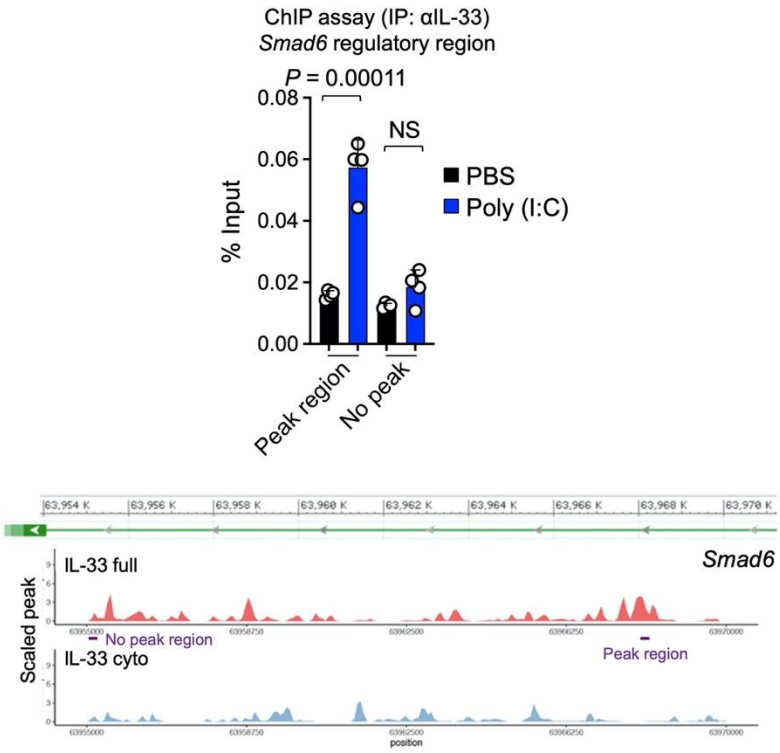

Figure R9. **Endogenous nuclear IL-33 binds to *Smad6* regulatory region.**
ChIP-qPCR assay for *Smad6* in the presence of poly (I:C) or PBS using an anti-IL-33 antibody. After Pam212 cells were transfected with poly (I:C) for 24 hours, cell lysates were subjected to chromatin-immunoprecipitation with anti-IL-33 antibody and eluted IL-33-bound chromatin was used for qPCR with both *Smad6* peak region (63,968k) primers and *Smad6* no peak region (63,955k) primers (NS: not significant, unpaired *t*-test).

(C) On page 7 the authors state that by "cross comparing RNA-seq with ChIP-seq data" they narrowed down the nuclear IL-33 candidates to seven genes whose regulation at the mRNA level is shown in (C). This procedure appears highly selective and the effect sizes (ratios) in changes of gene expression are small (e.g. SMAD6 is downregulated by log2 0.79-fold), although this effect is confirmed by RT-qPCR (shown in (D). Along the issues raised in (A), an unbiased and complete analysis of the genomics data needs to be provided to justify the focus on SMAD6-TGF-beta signaling as key downstream pathway of IL-33.

We agree with referee's comment. To address this critical point, we have performed gene enrichment analysis on our RNA-sequencing data, which demonstrates the significant enrichment for negative regulation of TGFβ/BMP signaling pathway in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1A above). *Bmper*, *Smad6* and *Smad7* which are negative regulator of BMP and TGFβ signaling are highlighted in the heatmap plot (Fig R1B above). In addition, RUNX2 target genes are highly upregulated in IL-33^{KO} compared with WT epidermis treated with DNFB (Fig R1C above). Furthermore, we find IL-33^{KO} group to show down-regulation of SMAD protein phosphorylation compared with WT group by GSEA (Fig R10). We have added these data to the revised manuscript to further substantiate the rationale for the focus on SMAD signaling pathway as key downstream pathway of nuclear IL-33.
(Appendix Fig S2E-G, S3D, and lines 125-126 and 150-152 in the revised manuscript)

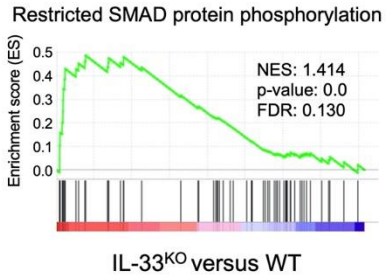

Figure R10. **The enrichment plot of a restricted SMAD protein phosphorylation gene set** in IL-33^{KO} compared with WT epidermis treated with DNFB.

(E) The effects of ectopically expressed fl IL-33 versus cytosolic IL-33 are minor and may simply result from confounding factors such as differences in transfection efficiency etc. No immunoblots are provided proving that the constructs were equally well expressed under these conditions. This is of particular concern in the light of the immunoblots presented in (G) which show that IL-33 cyto is stronger expressed. Moreover, the data are presented as fold change; against which sample (non-transfected, vector transfected)?. I doubt that this system is suited to prove a role of nuclear IL-33 in regulation of SMAD6.

We apologize for the lack of clarity in the presentation of this data. In response to referee's comment, we have revised the graph to clarify *Smad6* gene expression upon IL-33 full length or cytokine domain only (Cyto) expression is compared to empty vector group (as a negative control, Fig R11A). Although we have controlled for expression of IL-33 full length versus IL-33 cytokine domain in this study (Fig R11B), a higher expression of IL-33 cytokine domain compared with IL-33 full length in the cells would provide further support for our finding that IL-33 full length is uniquely capable of binding to RUNX2 and suppressing *Smad6* expression.
(Fig 2E, Appendix Fig S3A, and lines 140-142 in the revised manuscript).

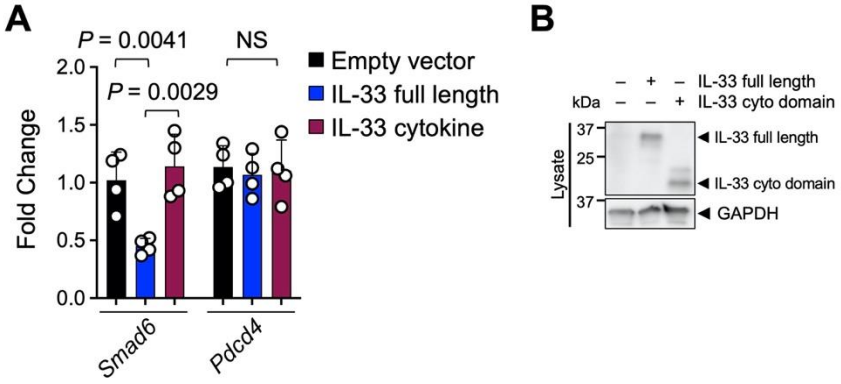

Figure R11. IL-33 nuclear domain is required to block *Smad6* gene expression.
(A) *Smad6* and *Pdcd4* (negative control gene) expression levels upon IL-33 full length or cytokine domain expression compared with HA empty vector expression in Pam212 cells (n=4 in each group, NS: not significant, unpaired *t*-test).
(B) Expression of IL-33 full length and IL-33 cytokine domain in cells assayed in (A).

(F) This Co-IP lacks crucial negative controls such as IgG pulldowns from lysates of the PyMT cell extracts.

Thank you for pointing this out. We have added IgG pull down lane with PyMT cell lysates as a negative control (Figure R12).
(Fig 2F in the revised manuscript)

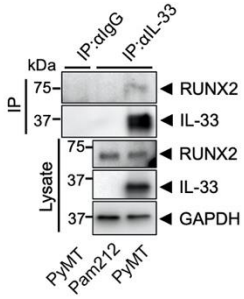

Figure R12. Endogenous IL-33 binds to RUNX2.
Immunoblot for interaction between endogenous IL-33 and RUNX2. Lysates from PyMT and Pam212 cell lines were subjected to immunoprecipitation with an anti-IL-33 antibody followed by immunoblot analysis. Immunoprecipitation with an anti-IgG antibody on equal amount of PyMT lysate is shown as negative control.

(H) This experiment provides a key link for the mechanism proposed to be regulated by nuclear IL-33. Several improvements are required: (i) ChIP-qPCR enrichment should be shown as percent input and not as relative change. (ii) Since IL-33 is a cofactor which are notoriously difficult to crosslink and to ChIP, a negative control region (chosen from the ChIP-seq data) and IgG IPs need to be added to demonstrate that raw signals were well above background. (iii) The authors need to add endogenous IL-33 ChIP-qPCR data comparing the Runx recruitment to the SMAD6 promoter in wt versus IL-33 ko cells.

We appreciate these comments. As referee requested, we have revised the graph to show ChIP-qPCR enrichment as percent input and added anti-IgG IPs as negative controls (Figure R13A). In Fig 2H referenced here, we have used anti-RUNX2 antibody, not anti-IL-33 antibody, for ChIP-qPCR assay. Nonetheless, we agree with referee's point and have performed ChIP-qPCR assay using anti-IL-33 antibody IP and qPCR for positive peak versus no peak (negative control) in regulatory region of *Smad6* shown in Fig R9A above. To determine endogenous IL-33 regulation of RUNX2 recruitment to *Smad6* promoter, we have performed ChIP-qPCR assay on poly (I:C) treated Pam212 cells after transfection with siIl33 or control siRNA, which shows increased RUNX2 binding to *Smad6* promoter upon *Il33* knockdown (Fig R13B).
(Fig 2H, Appendix Fig S5C, and lines 175-179 in the revised manuscript)

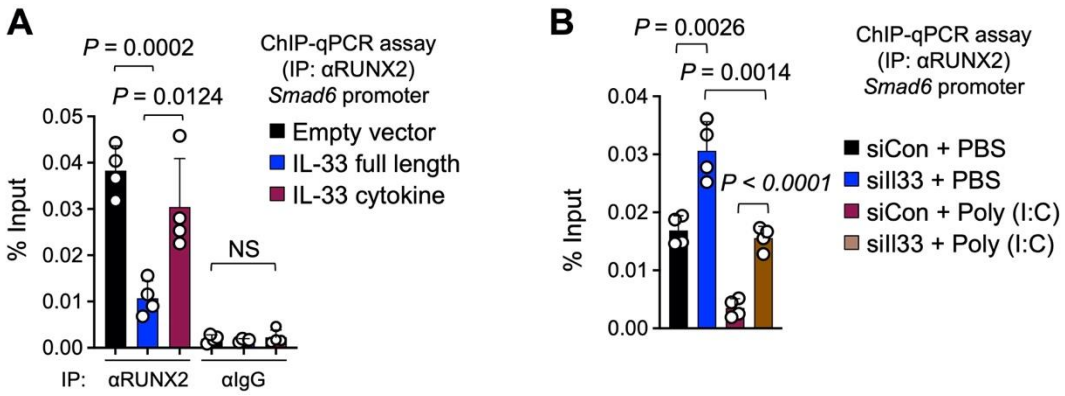

Figure R13. Nuclear IL-33 regulates RUNX2 binding to *Smad6* promoter region.
(A) ChIP-qPCR assay for *Smad6* in the presence of IL-33 full length or cytokine domain using an anti-RUNX2 antibody. After Pam212 cells were transfected with IL-33 full length or cytokine domain for 24 hours, cell lysates were subjected to chromatin-immunoprecipitation with anti-RUNX2 antibody and eluted RUNX2-bound chromatin was used for qPCR with primers against *Smad6* promoter region. Anti-IgG IPs used as negative controls.
(B) ChIP-qPCR assay for *Smad6* in the presence of endogenous IL-33 versus *Il33* knockdown using an anti-RUNX2 antibody. After transfection with siIl33 (#1) or control siRNA (siCon) for 24 hours, Pam212 cells were treated with poly (I:C) or carrier control. Cell lysates were subjected to chromatin-immunoprecipitation with anti-RUNX2 antibody and eluted RUNX2-bound chromatin was used for qPCR with *Smad6* promoter primers.
NS: not significant, unpaired *t*-test

Fig.3:
(A) The technical quality of the immunoblots is unconvincing. There is a lot of background and the protein bands are blurry. I appreciate that this may be due to technical issues during PDF conversion or to low abundancies of the proteins. However, the authors should provide all full size blots of all replicates as source data to solve these issues. To conclude that SMAD1/5 phosphorylation is changing (and not just the protein levels), anti SMAD1/5 blots need to be added. All changes need quantification of replicates, graphical presentation and statistical testing of the data. This comment also applies to the other blot panels shown in Fig. 3C/D, Fig. 4I, Fig. 5C, Fig. EV3A/B. All of these experiments serve to proof the counter regulation of P-SMAD2/3, P-SMAD1/5 and SMAD6 and I regard a revision of these data sets as crucial to support the main conclusions of this manuscript.

We agree with this comment. We have quantified western blots in the revised manuscript by using cSeries Capture Software from azure biosystems with statistical analysis (Fig R4 above). We have also added full blots of all of western blots as a Source Data file. As requested, we have added endogenous SMAD1 to the western blots (Fig R14).
(Fig 3A, 4I, 5C, Appendix Fig S4C, E, and lines 433-438 in the revised manuscript)

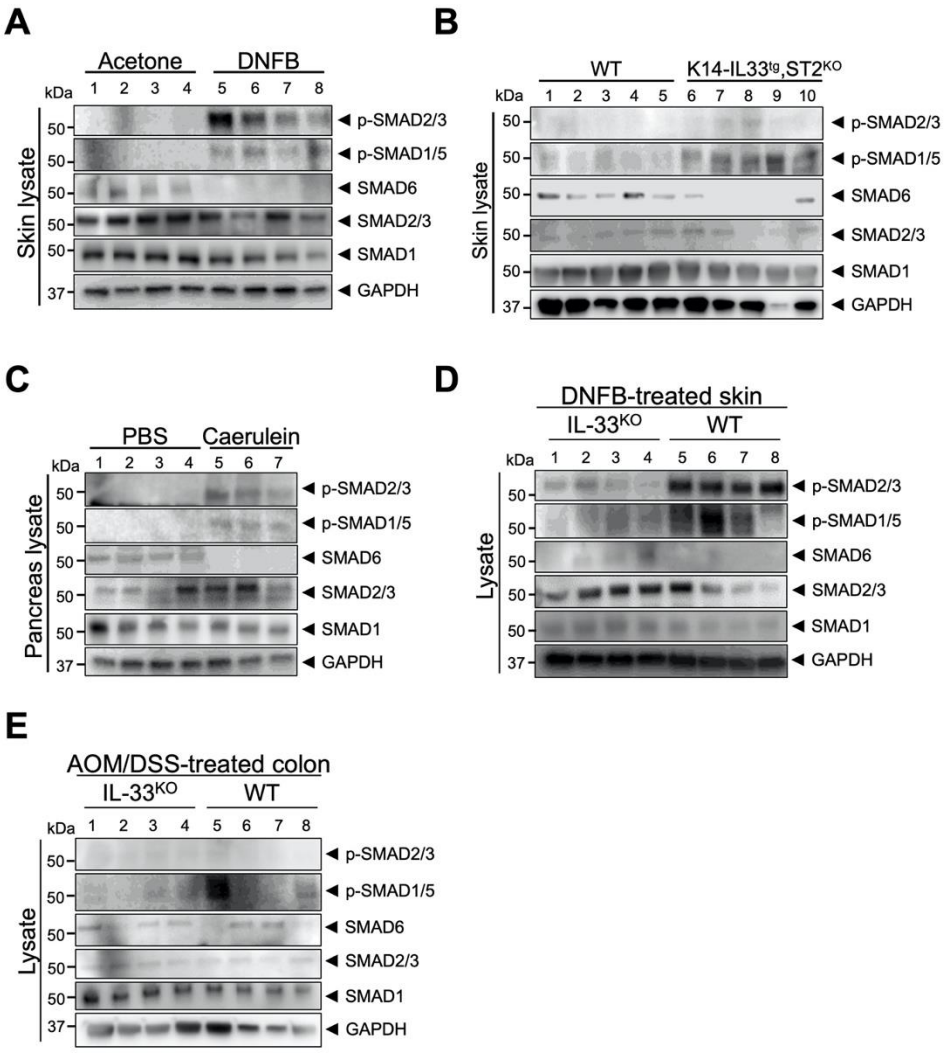

Figure R14. Expression of SMAD1 protein.
(A) Related to Fig 3A.
(B) Related to Fig 4I.
(C) Related to Fig 5C.
(D) Related to Appendix Fig S4C.
(E) Related to Appendix Fig S4E.

Fig. 4:
(J) In the line with comments above (Fig.1), data require quantification and addition of negative controls.

We have added quantification of this data and negative control to the revised manuscript (Fig R15).
(Appendix Fig S8B, C, and lines 236-238 in the revised manuscript)

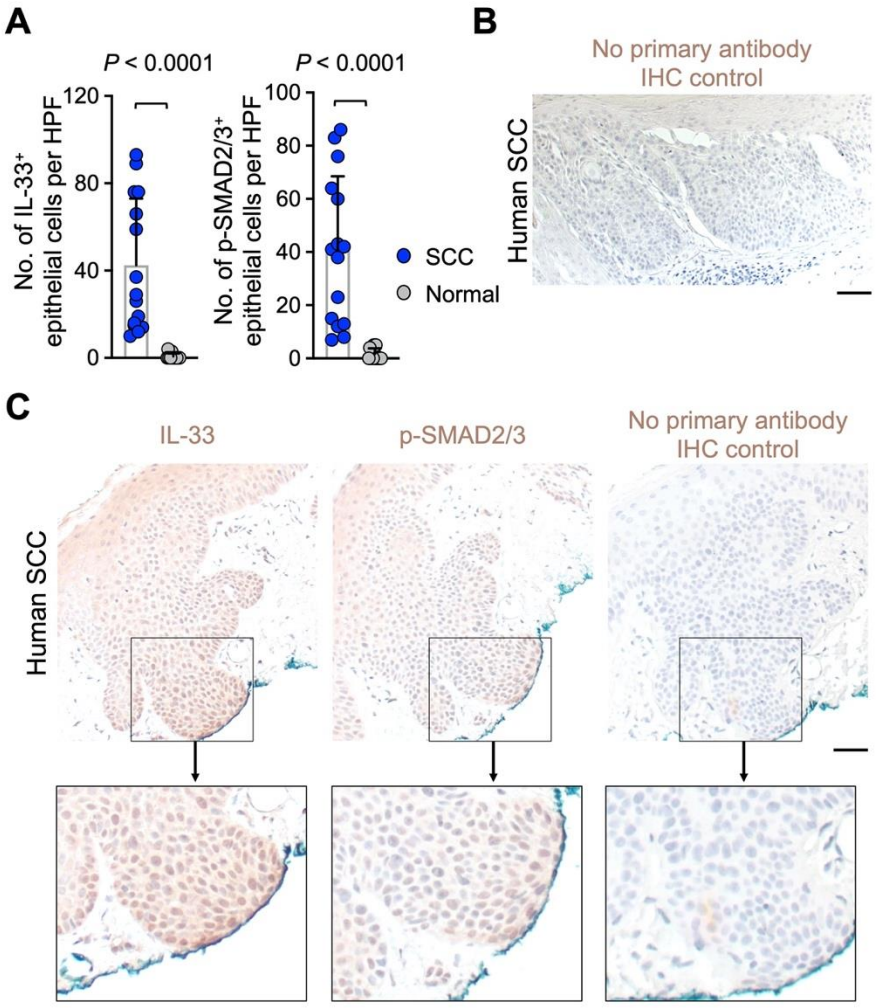

Figure R15. IL-33 and p-SMAD2/3 immunostaining on the adjacent sections of human squamous cell carcinoma (SCC).

(A) Quantification of nuclear IL-33$^+$ and p-SMAD2/3$^+$ epithelial cells per HPF in SCC versus normal human skin. Each dot represents cell counts in three randomly selected HPF images per sample across 5 SCC and 5 normal skin samples (unpaired *t*-test).

(B) Representative image of negative control (no primary antibody) immunostaining on the adjacent tissue section of the SCC sample shown in Fig 4J.

(C) Representative images of IL-33 and p-SMAD2/3 and negative control immunostaining on the adjacent sections of another SCC sample used in this study.

Scale bars: 100 μm

Fig. EV2:

(C), (D) Along the comments to Fig. 2:

Why are only the top 20 DEGs and the top 6 enriched pathways are shown? A much more complete and comprehensive analysis and presentation of the genomics data sets is warranted to claim the major role of TGF-beta pathway components for IL-33 signaling.

We appreciate this point and have revised the manuscript to reflect the full list of DEGs and enriched pathways (Fig R16).
(Appendix Fig S2D and Appendix Table S1 in the revised manuscript)

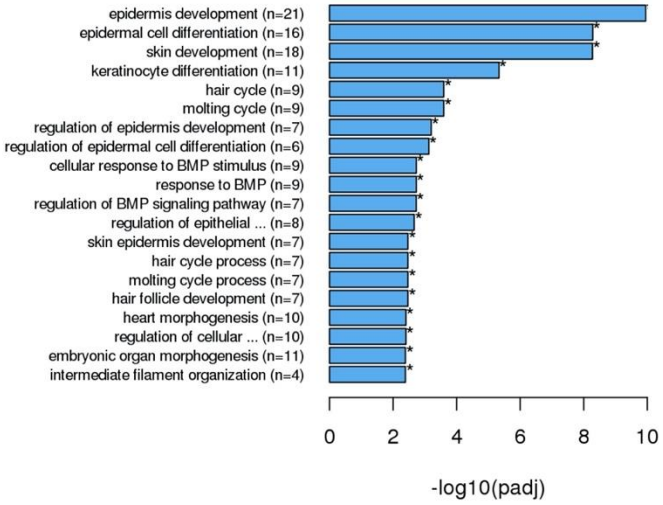

Figure R16. Biological pathways highlighted by DEGs from RNA-Seq analysis of IL-33^{KO} versus WT epidermis treated with DNFB.

(E) What is the red signal? IL-33 antibody stainings?

We apologize for the lack of clarity on this point. Red arrows point to IL-33 positive green signals. This point has been clarified in the Appendix Fig S3B of the revised manuscript.

Minor points:
Page 3: In the first paragraph, the authors give the impression that chronic inflammatory microenvironments exclusively lead to resistance to conventional cancer immunotherapies such as checkpoint inhibitors. For two reasons, I find this rather general statement misleading: (i) not all inflammatory microenvironments will be associated with resistance to therapy; the role of inflammation is highly dependent on the individual tumor type and tumor evolution; (ii) checkpoint inhibitors should not be classified as conventional immunotherapies. It is rather appropriate to refer in this context to the role of inflammation in the response to conventional chemotherapies or to novel immunotherapies, the latter being only suited for tumors expressing checkpoint ligands and containing enough immune cells responding to these signals.

Thank you for raising these points, which we have addressed in the revised manuscript.
(lines 40-43 in the revised manuscript)

Page 18: Explain the species and tissue type of Pam212 cells.

Pam212 is a mouse keratinocytes cell line and this information has been added to the revised manuscript. (line 129 in the revised manuscript)

Referee #3:

General summary

In this study, Jong Ho Park et al. investigate the nuclear function of the cytokine IL-33 in promoting tumorigenesis that develops in the context of chronic inflammation, by revealing its role as a pro-tumorigenic regulator of transcription.

The authors first show that IL-33 supports tumor development independently of the receptor ST2 in a murine model of inflammation-triggered skin cancer, but not during colitis-associated intestinal tumorigenesis. By applying a combination of transcriptomics (RNA-Seq) and protein-DNA interaction (ChIP-Seq) analysis on murine skin and epithelial cell lines, they then identify Smad6 as a key differentially expressed gene found in both IL-33 knockout mice and IL-33 full-length overexpressing murine Pam212 squamous cell carcinoma (SSC) cells, when compared to WT mice and IL-33 (mature) cytokine domain-overexpressing Pam212 cells, respectively.
The authors use several complementary approaches to show that full-length (i.e. nuclear) IL-33 binds to RUNX2, the transcription factor controlling Smad6 transcription, which in turn represses Smad6 expression. Further data show that that Smad6 downregulation leads to increased TGFb-induced Smad signaling, which results in increased epithelial cell proliferation upon TGFb stimulation.
Using a ST2-deficient mouse strain with keratinocyte-specific IL-33 overexpression, the authors determine that nuclear IL-33 is able to promote skin cancer development (independent of DNFB sensitization) through their previously described nuclear IL-33/SMAD6/TGFb axis. Lastly, they show that this nuclear IL-33/SMAD6/TGFb axis also supports the development of pancreatitis-associated pancreatic cancer.

Overall, the authors convincingly detail the mechanisms by which nuclear IL-33 influences TGFb sensitivity, illustrating its role in regulating tumor development during chronic infectious condition. The experiments performed include all necessary controls and the data provided are generally convincing and of good quality. To demonstrate the general relevance of their findings the authors use several different mouse models of inflammation-associated cancer, as well as tissues from human patients. I still have several comments on the current manuscript:

We thank the referee for their positive remarks and important points raised.

Major comments

While the authors use several different models of inflammation and cancer, and it is not always clear why they switch from one model to another one. The rationale behind it should be better explained in the text, in particular for the models of skin inflammation and cancer. For instance, DFNB/DMBA is used to induce skin cancer in Fig 1, yet after presenting these data, the authors use the skin of DFNB (only)-treated / sensitized mice for their RNA seq analysis in Fig 2, as well as later on for other experiments (Fig. 3). In Fig 4, they then use a model of skin cancer based on TPA/DMBA, in the context of which IL-33 needs to be first genetically overexpressed to see an effect of TGFb inhibition.

We appreciate this comment. For chronic inflammation-associated skin carcinogenesis, we have used DMBA/DNFB protocol over a 30 week period; however, to explore the impact of IL-33 on epithelial cells during the onset of cancer-prone chronic skin inflammation (Ameri *et al*, 2019), we treated the mice with DNFB for 22 days. In Fig 4, we sought to isolate the tumor-promoting mechanism downstream of IL-33 induction. Therefore, in this set of experiments, we

used K14-IL-33 transgenic mice that highly express IL-33 and treated them with standard chemical skin carcinogenesis protocol (DMBA/TPA) to exclude other tumor-promoting effects of chronic DNFB treatment. We have clarified these points in the revised manuscript.
(lines 211-215 in the revised manuscript)

The reason for focusing exclusively on Smad6 as a possible target of nuclear IL-33, rather than any of the other genes identified in their combined RNA-Seq / ChIP-Seq analysis is not convincingly rationalized from the current line of argumentation presented in the manuscript. Contrarily to what the authors claim, several of the other genes identified in Fig 2C have been previously involved in cancer. This is for instance true for BNC2 (PMID: 27899818; PMID: 29750795) and NUP210 (doi: https://doi.org/10.1101/2020.02.05.936518), ...

Thank you for pointing this out. We have performed additional analysis on our RNA-Seq results using GSEA, which highlight TGF-β/BMP signaling pathway, RUNX2 targets genes and SMAD phosphorylation as the targets of IL-33 (Fig R1 and R10 above). We have revised the manuscript provide a more accurate rationale for focusing on SMAD signaling pathway in our study.
(lines 134-136 in the revised manuscript)

The representation of some of the histology data needs to be improved to be more convincing. These include Fig 1G (overexposed, somehow unsharp) and Fig 4A (overexposed). In Fig. 4J, the authors should present a magnification (in an inset) of the normal / non-cancerous skin; in addition, the panels showing IL-33 stain is SCC tissue are too bright. Based on the current data it is not clear whether IL-33 is indeed upregulated in SCC versus normal skin.

In response to this point, we have added the high magnification insets to normal skin panel in Fig 4J. In addition, we have added quantification of this data and negative control to the revised manuscript (Fig R15 above).
(Fig 4J, Appendix Fig S8B, C, and lines 236-238 in the revised manuscript)

Lastly, in the discussion the authors could present a possible explanation on why the identified IL-33/SMAD6/TGFb axis is important for inflammation-dependent cancer in the skin and pancreas, but not during colon cancer. Is this due to the expression of IL-33 being mainly detectable in stromal but not in epithelial cells in their model, in the colon of AOM/DSS-treated mice? i.e. IL-33 has a cell-intrinsic role in malignant cells, but not for other cells in the tumor environment? Note that other studies reported IL-33 to be upregulated in the intestinal epithelium in the context of DSS treatment (PMID: 23172891; PMID: 30224451) and AOM/DSS treatment (PMID: 26942077), which should be also mentioned.

We appreciate this comment. IL-33 induced by AOM/DSS mostly localized in stroma of colon tissues compared to epithelial region. We have added quantification of IL-33 expression in AOM/DSS model (Fig R7 above). In addition, we have discussed this point in revised discussion section.
(Fig 1H, J, and lines 326-327 in the revised manuscript)

They could possibly also discuss about which domains of IL-33 interacts with RUNX2. Is this the N-terminal part that is only present in the full-length IL-33 protein? Or does simply the cellular

location (i.e. nuclear versus cytoplasmic/secreted) determines whether an interaction can take place between the 2 proteins? The former seems more likely, based on the overexpression data showed in Fig. 2G and 2H and on a previous report describing an interaction of the same N-terminal domain with NF-kB (PMID: 21734074).

As suggested by the referee, our data (including Fig R13 above) demonstrate that N-terminal nuclear domain of IL-33 is required for its interaction with RUNX2 in the nucleus. we have added this discussion point to our revised manuscript.
(lines 293-294 in the revised manuscript)

Minor comments
• In the Methods, the chapter on the ChIP assay mentions the use of an anti-HA antibody for RUNX2/DNA immunoprecipitation, while the legend of Fig 2H indicates that an anti-RUNX antibody was employed.

Thank you for pointing out this discrepancy. Anti-RUNX2 antibody was used for IP in Fig 2H, which we have clarified in the revised manuscript.

• In the Methods, the authors should indicate how they measure tumor volume in the skin and intestine. Since the data in Fig 1A and D show the pooled volumes of all single tumors from several mice per group, they should also present corresponding data on tumor counts per mouse, as done for instance in Fig 4C and G.

We appreciate this point, which needs clarification. Fig 1A and D show volume of each tumor found in WT, IL-33^{KO} and ST2^{KO} mice. If an animal did not have any tumors, one dot at zero value is used to represent that animal. To address reviewers comment, we have generated a new graph showing skin tumor onset and counts per mouse over time (Fig R17).
(Appendix Fig S1D, E, Fig 1 legend, and lines 105-108 in the revised manuscript).

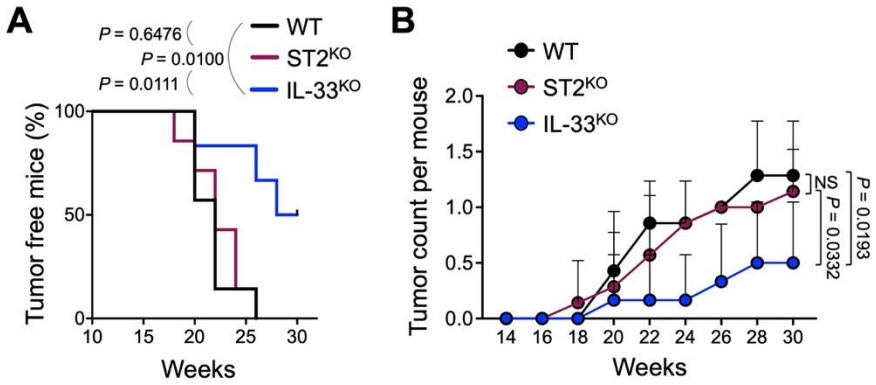

Figure R17. Skin tumorigenesis is suppressed in DMBA/DNFB treated IL-33^{KO} mice.
(A) Skin tumor onset in DMBA/DNFB treated WT, IL-33^{KO} and ST2^{KO} mice over time (log-rank test)
(B) Skin tumor counts in DMBA/DNFB treated WT, IL-33^{KO} and ST2^{KO} mice over time (unpaired *t*-test for tumor counts at the completion of DNFB treatment).

• For all figure legends, the authors should indicate the number of experimental repetitions with similar findings; this information is not provided for each dataset.

We have added experimental repeats to the figure legends.

• A list of the 203 genes showing differential expressions in IL-33 KO compared with WT skin keratinocytes should be added to this manuscript (e.g. as supplemental data part).

We have added 203 DEGs as a new Appendix Table S1.

• Fig. EV2B: the current magnification / picture size does not allow an assessment of the lesions on the back of these mice.

We have added high magnification images of mice back skin.

• Fig. EV3A: can the resolution / contrast be improved for the WB data indicating SMAD6 expression?

We have tried to improve WB resolution as requested by the referee. In addition, we have added western blot quantifications and raw gel files to the revised manuscript, which assists in better assessment of the results.

• Fig 3C: Contrarily to what the authors claim, endogenous SMAD2/3 levels seem to change / diminish upon combined TGFb + poly (I:C) treatment of Pam212 cells.

To address this point, we have performed quantitative analysis of the western blot bands across the experimental repeats. This analysis demonstrates that there is no significant change between endogenous SMAD2/3 across treatment groups (Fig R4B above).

• The authors should precise whether whole (back) skins (more likely) or only skin patches with tumors were used for preparing the tissue lysates that were analyzed by WB (e.g. for Fig 4I).

We used whole back skin to prepare tissue lysates for WB analysis. This point has been clarified in the revised Fig 4I legend.

• In Fig 5D, the Y axis indicates the percent of tumor-free survival, yet ST2 KO mice apparently only develop severe pancreatitis with fibrosis, without cancer. Therefore, I think the label of the axis shall be accordingly modified.

We appreciate this point. $ST2^{KO}$ mice developed highly fibrotic tumors. To better demonstrate this, we have added low power images of H&E-stained section of pancreas/tumor from WT, $IL\text{-}33^{KO}$ and $ST2^{KO}$ mice at the completion of DMBA/caerulein study, which shows of a large tumor in place of pancreas in $ST2^{KO}$ mouse. This data has been added to the revised manuscript. (Appendix Fig S9F, and line 267 in the revised manuscript).

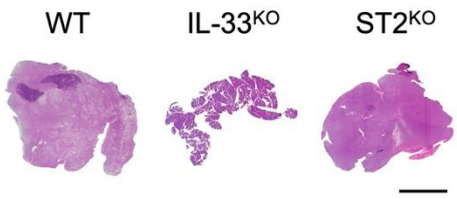

Figure R18. **Representative images** of H&E-stained pancreas/tumor in IL-33^{KO}, WT and ST2^{KO} mice treated with DMBA/caerulein (scale bar: 1 cm).

• The calling of some of the figures should be corrected in the result part (Fig EV5A instead of Fig 5A; Fig EV5D instead of Fig 5D). In addition, Fig 5E but not Fig EV5E shows tissue fibrosis.

The calling of the figures has been revised to conform to EMBO Journal standard ("Appendix Fig S#" to reference supplementary figures).

• In the discussion, the study by "Gao et al, 2015" employs murine B16 melanoma cells and 4T1 mammary gland cells, but no intestinal epithelial cells.

Thanks for the comment. We have revised the discussion section accordingly.

Reference:

Ameri AH, Moradi Tuchayi S, Zaalberg A, Park JH, Ngo KH, Li T, Lopez E, Colonna M, Lee RT, Mino-Kenudson M *et al* (2019) IL-33/regulatory T cell axis triggers the development of a tumor-promoting immune environment in chronic inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 116: 2646-2651

1st Revision - Editorial Decision 8th Dec 2020

Dear Shawn,

Thank you for submitting your revised manuscript to The EMBO Journal. The revised version has now been seen by the three referees and as you can see they appreciate the introduced changes. Referee #3 has a few remaining concerns about the histology but they should be fairly straightforward to address. When you submit the revised version will you also take care of the following points.

- You have at the moment 7 keywords, but can only have 5

- Thanks for uploading individual figure files please remove the figures from the manuscript text file.

- The source data looks good and I also like that you show replicates for some of the gels. Maybe it would be good to indicate in the file which set is for experiments shown in the figure and which ones are replicates. Please upload separate source data files one figure per file.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

That should be all - congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 8th Mar 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have submitted a revised manuscript (EMBO J-2020-106151R) that largely addresses the concerns I raised during the initial review. A considerable amount of new data has been added during revision that clarifies and strengthens the manuscript. In fact, the authors have been highly responsive to all three reviewers. I support publication of the paper in EMBO J.

Referee #2:

The authors have sufficiently adressed all my previous concerns.

Referee #3:

Overall, I think the reviewers answered most of the requests/questions quite well and provided the data needed. The revisions have improved the manuscript and the strength of the data. Yet I still have a couple of minor comments - all about histology - that I believe should be still addressed:

-The data on apparent colocalization of IL-33 / p-SMAD2/3 in the new figure Appendix Fig S4B are not convincing; it does not look that slides have been stained that were directly consecutive. In addition, the p-SMAD2/3 staining is faint.

-The histology in Fig1G has only been moderately improved; can't the authors increase the resolution of their picture with an objective with higher magnification? In addition, they should also show a corresponding magnification for the acetone-treated controls for better direct comparison.

-Figure 1H and J, Fig. 5G and H and other figures in the supplementary data: the authors should mention the methods used to quantify the number of positive cells in these IHC sections. E.g. were these cells counted semi-quantitatively by a pathologist, in a blinded fashion, (or not) etc. ?

Response to Referees' Comments:

We thank the referee #3 for their comments to improve our manuscript. Our replies to the comments are provided in blue below. Manuscript figures have also been revised according to the referee' comments.

Referee #3:

Overall, I think the reviewers answered most of the requests/questions quite well and provided the data needed. The revisions have improved the manuscript and the strength of the data. Yet I still have a couple of minor comments - all about histology - that I believe should be still addressed:

-The data on apparent colocalization of IL-33 / p-SMAD2/3 in the new figure Appendix Fig S4B are not convincing; it does not look that slides have been stained that were directly consecutive. In addition, the p-SMAD2/3 staining is faint.

We appreciate this point. We can confirm that IL-33 and p-SMAD2/3 IHC images shown in Fig S4B are from consecutive skin sections. We have increased the size and resolution of these images in the revised Fig S4B to optimize the visualization of the signals. Here, we provide an additional image of IL-33 and p-SMAD2/3 IHC from the same region of the consecutive tissue sections to further substantiate the co-localization of IL-33 and p-SMAD2/3 nuclear signals in the same region of the DNFB-treated epidermis (Figure R1).

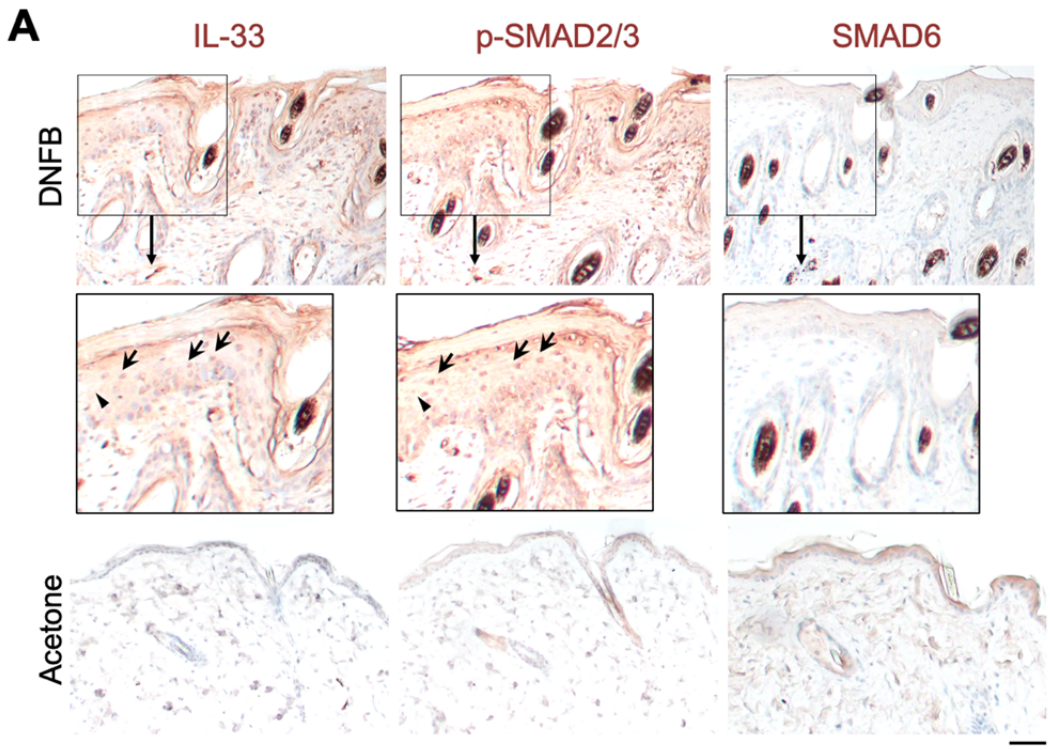

Figure R1. IL-33, p-SMAD2/3 and SMAD6 localization in DNFB-treated skin.

Representative images of IL-33, p-SMAD2/3 and SMAD6 IHC on the adjacent sections of DNFB versus acetone-treated mouse skin. Arrows in the insets point to nuclear IL-33 and p-SMAD2/3 stains in the epidermal keratinocytes and arrowheads highlight keratinocytes nuclei that are negative for both IL-33 and p-SMAD2/3.
Scale bar: 100 μm

-The histology in Fig1G has only been moderately improved; can't the authors increase the resolution of their picture with an objective with higher magnification? In addition, they should also show a corresponding magnification for the acetone-treated controls for better direct comparison.

We have revised Fig. 1G and 1I according to referee's comment.

-Figure 1H and J, Fig. 5G and H and other figures in the supplementary data: the authors should mention the methods used to quantify the number of positive cells in these IHC sections. E.g. were these cells counted semi-quantitatively by a pathologist, in a blinded fashion, (or not) etc. ?

The number of positive cells were counted in randomly selected high power field (HPF, 200X magnification) images in a blinded manner by a trained investigator. Clinical samples were reviewed by a pathologist. This information has been added to the revised manuscript.

Thank you!

2nd Revision - Editorial Decision 11th Jan 2021

Dear Shawn,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at it and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-10615

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was determined based on preliminary studies conducted in our laboratory and power analysis. For experiments in which no preliminary data was available in our laboratory or in similarly published research, the sample size chosen was sufficient to determine significance in all assays, with reproducible statistical significant difference between conditions in all the repeat experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal experiments, we followed the 3 R's of animal reserach and used only the number of animals required to reach conclusive outcomes. The number of mice per group was determined based on preliminary studies conducted in our laboratory and power analysis. For experiments in which no preliminary data was available in our laboratory or in similarly published research, the number of animals per group was sufficient to determine significance in the experiment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from the study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Sample randomization scheme was applied. Mice were randomly allocated to experimental or treatment groups. Test and control mice of the same strain were gender and aged matched.
For animal studies, include a statement about randomization even if no randomization was used.	Same as above.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For outcome measurements in in vitro studies, investigators were aware of the conditions and treatment while relying on unbiased measurements of quantitative parameters.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For outcome measurements in animal studies, investigators were aware of the host genotype and treatments while relying on unbiased measurements of quantitative parameters.
5. For every figure, are statistical tests justified as appropriate?	Yes. Unpaired t-test was used as the test of significance for tumor volume, counts, epidermal thickness, count of nuclear protein staining, RNA and protein expression levels and other quantitative measurements. The log-rank test was used as the test of significance for time to tumor onset outcomes. A paired t-test was used for the comparison of cell counts and t-test for the Pearson correlation coefficient was used to determine significance in correlation for human pancreatic samples.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We considered the outcome significant if P value was lower than 0.05.
Is there an estimate of variation within each group of data?	Yes. Quantitative data are presented with standard diviation.
Is the variance similar between the groups that are being statistically compared?	Yes. We observed simialr variance across the groups being compared.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Western blotting Antibodies Primary antibodies CloneCompany p-SMAD2/3 D27F4 Cell Signaling Technology, Danvers, MA p-SMAD1/5 41D10 Cell Signaling Technology RUNX2 D1L7F Cell Signaling Technology GAPDH D16H11 Cell Signaling Technology Anti-mouse IL-33PolyclonalR&D Systems, Minneapolis, MN SMAD2/3PolyclonalR&D Systems SMAD6PolyclonalNOVUS biologicals, Centennial, CO SMAD1EP565YAbcam, Cambridge, UK HA16B12Biolegend, San Diego, CA Secondary antibodies. Cat # Company Peroxidase Goat Anti-Mouse IgG115-035-003Jackson Immunoresearch, West Grove, PA Peroxidase Goat Anti-Rabbit IgG111-035-003Jackson Immunoresearch Peroxidase Bovine Anti-Goat IgG805-035-180Jackson Immunoresearch
	Immunostanting antibodiesCloneCompany IL-33 (mouse) Nessy-1ENZO, Farmingdale, NY IL-33 (human)PolyclonalSigma, St. Louis, MO p-SMAD2/3 (mouse and human)D27F4Cell Signaling Technology Immunofluorescence staining AntibodiesCloneCompany Ki67PolyclonalAbcam, Cambridge, UK Cleved caspase-3 (Asp 175)5A1ECell Signaling Technology, Danvers, MA Secondary antibodiesCat #Company Goat anti-Mouse IgG, Alexa Fluor 488 conjugateA32723Thermo Fisher Scientific, Waltham, MA Goat anti-Rabbit IgG, Alexa Fluor 647 conjugateA32733Thermo Fisher Scientific, Waltham, MA (This information if provided in Appendix Table S2)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Pam212 and PyMT cell lines are from mouse and negative for mycoplasma.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	WT C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). K14-IL33tg mice were purchased from the Transgenic Inc. (Kobe, Japan). IL-33KO mice were a gift from Dr. Marco Colonna and ST2KO mice were a gift from Dr. Peter Nigrovic. All mouse strains are on the C57BL/6 background. 5-6 weeks age and gender-matched mice were used in all experiments. All mice were housed under specific pathogen free conditions in an animal facility at Massachusetts General Hospital in accordance with animal care regulations.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Massachusetts General Hospital IACUC approved animal studies.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Massachusetts General Hospital IRBs approved the analysis of de-identified clinical samples.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Confirmed.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Original data are available in the NCBI Gene Expression Omnibus (GEO) with accession number GSE149579 (RNA-Seq) and GSE149579 (ChIP-Seq).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Journal's data policy has been reviewed.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A