EMBO reports

Terminal differentiation of villus tip enterocytes is governed by distinct Tgfβ superfamily members

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1st Editorial Decision **23rd Dec 2022**

Dear Dr. Valenta

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and have a number of suggestions how to strengthen your study that should be addressed.

I am also available to discuss the referee points and the revision further via e-mail or a video call, if you wish.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (March 23). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

We recommend to publish your study as a short report. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In case the revision results in a manuscript with more than 5 figures, your study will be considered as article. In this case here are no length limitations and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Data availability: The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) At EMBO Press we ask authors to provide source data for the main figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

[Overall comment]
This article discussed a novel finding that TGF-beta superfamily ligands function differently in the differentiation of intestinal epithelial cells. Given that BMP ligand gradient plays a crucial role in the regulation of intestinal stem cell differentiation, the discovery of this article may offer a conceptual advance in the relevant research field.
However, the experimental evidence from this article relies mostly on mRNA levels rather than proteins and enzymatic function, which are crucial for determining the cellular sub-types in intestinal epithelium. In addition to this worry, I have several recommendations to strengthen and clarify the evidence of author's discovery.
[Major]
In Figure 1, authors described that Pdgfrahigh cells induces differentiation of intestinal epithelium by showing the gene expression changes in intestinal organoids upon initiating co-culture.
- Fig. 1B: Authors cited the concept of terminal differentiation reported by Moor et al. (Ref 5), and proposed that ivMCs promoted the terminal differentiation of enterocytes by showing the increased expression of villus-tip genes. Since Moor provide different clusters of zonated genes expressions throughout villus (form bottom, middle to top of villus), author should check other zonated gene clusters to propose that ivMCs promoted the terminal differentiation. Considering that ivMC secretes not only BMP2 but also BMP4, and BMP inhibitor Grem1, ivMC might influence general differentiation not specific to terminal differentiation. At least, authors should provide differential changes in enterocyte type 1 and type 2 markers upon co-culture with ivMC to propose terminal differentiation.
- Fig. EV 1C,D: Authors proposed that the induction of the villus-tip program could be partially abrogated by treatment with the Bmp type I receptor inhibitor LDN-193189, by showing the mRNA expression changes in villus-tip markers. However, to clarify the reversion of villus-tip differentiation, author should show changes in other zonated gene expressions, especially including stem markers, to exclude the possibility that dead of stem cell by LDN-193189 might randomly reduce all gene expressions.
- Fig. 2: Authors proposed differential effect of BMP2 and BMP4 on enterocyte differentiation by showing gene expression patterns of organoids and by expecting the fraction of cell types based on informatics tools. However, they really did not confirmed different changes in fraction of enterocytes by visualizing the cellular subtypes in organoids, rather solely dependent on mRNA expression of markers. To fully prove that "Bmp4 promoted differentiation into enterocyte type 1 1, characterized by the expression of metabolic genes", authors should provide direct evidence of the increased type 1 population upon Bmp4 as they provided further evidence on BMP2-induced villus-tip program by visualizing Nt5e+ population. And it would be much better if author can show different metabolic activity in BMP2- and BMP4-treated organoids.
-Fig. 3: As mentioned above, authors should show functional evidence on differential effect of BMP ligand on enterocyte differentiation. Since Moor et al defined the villus-tip population with the increase of Nt5e, Ada, and Slc28a2, all of which are included in purine metabolism. It would be better if author can show different purine metabolism ability upon BMP2 or BMP4 treatment.
-Fig. 4: Authors proposed that Tgf-beta seems to activate a program similar to that triggered Bmp2. But authors should state the different changes between Tgf- and Bmp2-treated organoids. The estimated fraction of cell types showed similar patterns in BMP2-treated and Tgf-beta-treated organoids; loss of enterocytes type 1 and increase of tuft cell. But qRT-PCR result showed inconsistent results; BMP2 treatment didn't reduce the expression levels of enterocyte type 1 genes (Fabp1, fabp2, and G6pc, Fig EV2G), while TGF-beta dramatically reduced the expressions of those genes, potentially suggesting further differentiation of type1 into type2.

- Fig. 4: In order to propose that Tgf-beta has similar to BMP2 in activating villus-tip program, authors should provide more evidence like increase in Nt5e+ population and changes in other cell types.
- Fig 4: TGF-beta1 activates signal cascade through type I receptor, such as ALK5. I wonder whether the effect of TGF-beta1 on villus-tip program is dependent on smad2/3, selective mediators of TGF-beta, or Smad1/5/8, common mediators of both BMP and TGF-beta. It might explain the different effect of TGF-beta and BMP2 on reduction of type 1 enterocyte gene expressions.
- Fig4: I wonder whether there would be phenotypic difference upon treatment of TGF-beta1 inhibitor. I wonder if TGF-beta1 inhibitors and BMP inhibitors affect enterocyte differentiation patterns differently or in a similar way
- Fig. 5A,B: Author propose that short-term repression of Bmp receptor complex (4 days) altered villus-tip differentiation in vivo. For that statement, authors should provide more convincing evidence like histological analyses of different subcellular types (including Type 1 and 2 enterocytes) and should confirm whether histological difference is in consistent with gene expression changes. And it would be better if author shows increase of tuft cells as showed in cell fraction changes expected by bioinformatics tools.
- Fig.5C,E: In line with comments above, author should provide direct evidence of changes in cell fraction by visualizing specific cell types in organoids using fluorescence.
- Fig. 5F: There is not big difference in villus-tip gene expression levels between passage 2 and passage 4 organoids. But for me, it makes sense that adding BMP2/4 might help recapitulation of original intestinal structure in long-term culture with preserved metabolic activity. How about showing obvious evidence that there are less villus-tip/and center villus enterocyte population and decreased metabolic activity compared to original intestinal villus? If adding BMPs restores the metabolic activity in intestinal organoids, it would appeal to the researchers who want to study the metabolic function in intestines.
- Fig EV4B: The image shows the elongated crypt upon BMP inhibition. Authors should reconsider whether they can state that the epithelial morphology and proliferation stayed the same after LDN-193189 treatment
- There are several conflicting results in Figures. Please confirm the consistency of results throughout Figures.
1) Fig 1B and Fig EV1D; Ada, one of the strong villus-tip markers, is increased upon co-culture with ivMV in Fig 1B, but not in Fig EV1D.
2) Figure EV2G and Figure 2E; Increased Cdh1 expression upon BMP2 (Fig 2E) but no changes in Fig EV2G in the same condition. And Fig EV2G don't include the result of BMP2+BMP4 treatment as figure legend says. And please describe the result about what happened after BMP2+BMP4 treatment. Reduction of type 1 enterocyte genes upon Bmp2 treatment in Fig 2E is not consistent with Fig EV2G data.
3) Fig2E and Fig EV2F: The increase of Ada, a Villus-tip marker, is much higher in Bmp2-treated organoids than in Bmp4-treated organoids. And authors propose that BMP2 induces terminal differentiation while BMP4 induces central villus differentiation. However, in Figure EV2F, Ada expression is similar in both Bmp2- and Bmp4-treated organoids. Authors should explain the inconsistency in their experiments. Or it would be helpful to directly show the changes in enterocyte population by visualizing specific cell types in organoids.

[Minor]
- Please note the statistical significance in Figures and supplementary Figures (ex. Fig. EV1B, 1D, Fig 1E, 1F....)
- Fig. 1C: Authors provide a complex expression pattern of distinct Tgf superfamily ligands in Pdgfrahigh mesenchymal cells using published scRNAseq dataset. It would be better if authors provide the information of whole Tgf superfamily ligands, not only BMPs and TGF-beta1.
- line 173-178: Authors propose that Bmp4 was expressed in non-epithelial cells from the center to the top of the villus in contrast to the zonated Bmp2 expression at villus tip, supporting the proposed action of Bmp2 on terminal differentiation. But there are conflicting evidence regarding Bmp4 expression patterns. KB Halpern et al. showed the increased Bmp4 expression at villus-tip. And N McCarthy et al. also reported that PDGFRA+ stromal cells which exist at the villus-tip region secrete both Bmp4 and Bmp2. These conflicting evidences suggest that terminal differentiation is not dependent only to zonated Bmp2 secretion from stromal cells rather propose the presence of other complex mechanism on regulation of terminal differentiation. Thus, author should discuss about the conflicting evidences in DISCUSSION section not to overstate on potential relationship between BMP ligand locations and enterocyte differentiation. .
KB Halpern et al., Nat Commun. 2020; 11: 1936.
N McCarthy et al., Cell Stem Cell 2020; 26(3):391-402
- Fig. 5A,B: Author should state how they determined gene expression changes in tissues; was it mixture of diverse cell population including stromal cells or just epithelium using specific enrichment methods?

Referee #2:

In this manuscript Berkova et al. study the differential impact of members of the TGFb superfamily on the differentiation status of enterocytes. Enterocytes exhibit zonated gene expression signatures, that are thought to be controlled by zonated stromal morphogens. Among these are members of the TGFb superfamily - Bmp2, Bmp4, Bmp5, Bmp7 and Tgfb. It is unclear whether these morphogens act in a redundant manner, or rather regulate distinct epithelial programs. The manuscript addresses this important question in a systematic manner, by exposing enteroids to all of the Tgfb ligands and measuring in an unbiased manner, through RNA sequencing, as well as qPCR validations, the resulting expression changes. They find that Bmp4 activates programs associated with the mid-villus zones, such as lipid metabolism genes, whereas Bmp2 induces more villus-tip programs such as cell adhesion. They further find that Wnt5a, another villus tip-restricted morphogen, synergizes with Bmp2 to

up-regulate villus tip enterocyte genes. The authors also introduce an organoid culture supplemented by Bmp2 that better recapitulates the in-vivo villus tip enterocyte programs. The study addresses an important question, is well designed and the results conveyed in a readable and interesting manner. I have a few, generally minor comments:

1)Bmp4 is solely expressed by mesenchymal cells, whereas Bmp2 is expressed both by mesenchymal cells but also by villus tip enterocytes (e.g. Moor et al., 2018). This is actually also seen in the smFISH validation of Figure 2F. This needs to be discussed. Are there any hypotheses to the epithelial expression of Bmp2? Is this a positive feedback loop, whereby enterocyte Bmp2 is induced by mesenchymal Tgfb signals or Wnt5a to enhance the Bmp signal? Does enterocyte Bmp2 mRNA expression change in response to the distinct ligands studied?
2)How do enterocyte anti-microbial programs change upon Bmp2/4 stimulation? It will be interesting to see, based on the RNAseq, how genes like Reg3a, Reg3b, Reg3g, Reg1, Il18, Ccl25, Nlrp6, Lypd8 are expressed in BMP4 treated organoids, as figure 2D suggests BMP4 organoids have some V1-V2 like transcriptional programs.
3)Additional cell types in the small intestine have Bmp receptors (both mesenchymal, endothelial and glia cells), the authors should discuss the potential additional roles of Tgfb ligands beyond the epithelium.
4)Is Chylomicrons assembly and secretion bmp dependent? Beside immunomodulation and adhesion, Villus tip enterocytes are extensively assembling and secreting chylomicrons. It will be interesting to show, based on their RNAseq, the gene expression levels of such genes (e.g., apolipoproteins, MTTP, DGAT2, ABCA1 and more) in the different BMP conditions and conclude whether those functions are governed by Bmp ligands, or rather other factors (e.g. luminal lipids)?
5)Figure 2F - please provide a blow-up. There is a typo in the legend - white should be Bmp4 and not Smad4.
6)Row 140-142 - sentence unclear.
7)Figure 2B caption - what is "top 10 unique genes per each treatment"? Please better explain the genes shown in the heatmap (as done in the Methods section).
8)In general, how were ligand concentrations determined? Are they within physiological ranges? Might there be concentration-dependent behaviors?
9)Related to point 8 above, the results should be discussed in the context of recent work from the Elowitz lab on the distinct behavior of Bmp ligands and their combination on cell lines
(https://www.sciencedirect.com/science/article/pii/S2405471222001296,
https://www.sciencedirect.com/science/article/pii/S0092867417309406).
10)Line 244 - typo, should be "was" instead of "as".

We would like to thank referees for recognizing the scientific potential of our manuscript and for their valuable comments helping us to improve its quality. To address questions of referees we performed additional experiments and re-analyzed our datasets to make our work more solid. Major focus was targeted to accompany the transcriptomic data with protein expression, as one referee criticized the fact our manuscript mainly relies on the expression profiling data. To be honest we were surprised by this type of criticism, as Bmp/Tgfβ are ligands inducing the transcription of specific target genes and thus an unbiased expression profiling may provide sufficient picture about their role(s).
Additionally, we extended our analysis to other important genes (e.g. antimicrobial peptides, constituents of chylomicrons). In spite of all our effort, we could not carry out all the experiments we had planned given the time and financial constraints. We also would like to stress that besides showing new data the part of our manuscript can be seen as the Independent First Confirmation (https://doi.org/10.15252/embr.202255821), the type of the work recently recognized and appreciated by EMBO Reports. Our manuscript not only confirmed the work published by Beumer et al. (2022), but extends it by new observations pointing to different impact of individual members of Bmp/Tgfβ family.
Altogether we believe that the revised version of the manuscript can provide a clear and complex picture how individual members of Tgfβ superfamily differently influence the cellular fate of enterocytes.

Referee #1:

[Overall comment]
This article discussed a novel finding that TGF-beta superfamily ligands function differently in the differentiation of intestinal epithelial cells. Given that BMP ligand gradient plays a crucial role in the regulation of intestinal stem cell differentiation, the discovery of this article may offer a conceptual advance in the relevant research field.
However, the experimental evidence from this article relies mostly* on mRNA levels rather than proteins and enzymatic function, which are crucial for determining the cellular sub-*types in intestinal epithelium. In addition to this worry, I have several recommendations to strengthen and clarify the evidence of author's discovery.

We agree with the referee that the main part of our manuscript is based on transcriptomic data. On the other hand as mentioned above as Bmps/Tgfβ are the ligands activating transcription of target genes, thus the unbiased expression profiling may provide sufficiently interesting information per se. Additionally we already showed some data/results indicating that protein expression follows the transcriptional changes (immunoblot panel showing the induced level of Fabp2 upon Bmp4 stimulation and immunofluorescent image of enhanced Cd73 expression). As the publication (Harnik et al., 2021) from S. Itzkovitz lab showed there is a discordance between induced transcription and translation in the enterocytes (but not in the case of villus tip genes, the main focus of our work) we performed new experiments showing on protein level, that Bmp2 and Bmp4 preferentially activate different enterocytic programs (Fig. 3C,E; Fig.7B,C; Appendix Figure S2B). Similar experiments were done for Tgfβ (Appendix FigureS4B; Fig.EV4H). These figures are in agreement with our RNAseq data.

[Major]
In Figure 1, authors described that Pdgfrahigh cells induces differentiation of intestinal epithelium by showing the gene expression changes in intestinal organoids upon initiating co-culture.
- Fig. 1B: Authors cited the concept of terminal differentiation reported by Moor et al. (Ref 5), and proposed that ivMCs promoted the terminal differentiation of enterocytes by showing the increased

expression of villus-tip genes. Since Moor provide different clusters of zonated genes expressions throughout villus (form bottom, middle to top of villus), author should check other zonated gene clusters to propose that ivMCs promoted the terminal differentiation. Considering that ivMC secretes not only BMP2 but also BMP4, and BMP inhibitor Grem1, ivMC might influence general differentiation not specific to terminal differentiation. At least, authors should provide differential changes in enterocyte type 1 and type 2 markers upon co-culture with ivMC to propose terminal differentiation.

We repeated the co-cultivation experiments (followed by qRT PCR) having the focus also to representative villus center genes (Fabp1, Fabp2, G6pc, Leap2) besides already shown villus tip (Ada, Lama3, Lgals3, Cdh1) marker genes to show the how the ivMCs induce the differentiation of epithelial cells (Fig.1B). The panels show the ivMC ($Pdgfra^{high}$) induced the differentiation to goblet cells, but importantly also to enterocytes (Krt20). The increased expression of some villus tip markers (mainly Lama3, but also others) suggest the potential of ivMCs to induce villus tip fate, whereas villus center programs do not seem to be activated. It is also important to mention that ivMCs ($Pdgfra^{high}$) mesenchymal cells do not express only Bmps, but also other signaling molecules, which might lead to not so straightforward changes in co-co-cultivated organoids in comparison to experiments based on recombinant proteins.

- Fig. EV 1C,D: Authors proposed that the induction of the villus-tip program could be partially abrogated by treatment with the Bmp type I receptor inhibitor LDN-193189, by showing the mRNA expression changes in villus-tip markers. However, to clarify the reversion of villus-tip differentiation, author should show changes in other zonated gene expressions, especially including stem markers, to exclude the possibility that dead of stem cell by LDN-193189 might randomly reduce all gene expressions.

Similarly to previous point we repeated these experiments and expanded the figure Fig. EV1C. Now the figure matches Fig. 1B. We hope that with such a change we made the data more transparent and persuasive. The text related to this figure was changed accordingly. As mentioned in previous point, the influence of ivMCs might be more complex and LDN-193189 may affect also ivMCs. Nevertheless, the expression highly responding villus tip marker Lama3 is reduced upon the treatment. We would like to stress that we used the co-cultivation with ivMCs only as a proxy experiment to suggest the involvement of Bmps produced by ivMCs in the villus tip differentiation. As many labs currently perform analogous types of co-cultivation experiments, we included them as well. We just wanted to directly show that any interaction between ivMCs and intestinal enterocytes really exists and that it is at least partially dependent on Bmp signaling (abrogated by Bmp signaling inhibition). The independent validation of this claim is the main part of our manuscript. It starts with Fig.1D.

- Fig. 2: Authors proposed differential effect of BMP2 and BMP4 on enterocyte differentiation by showing gene expression patterns of organoids and by expecting the fraction of cell types based on informatics tools. However, they really did not confirmed different changes in fraction of enterocytes by visualizing the cellular subtypes in organoids, rather solely dependent on mRNA expression of markers. To fully prove that "Bmp4 promoted differentiation into enterocyte type 1 1, characterized by the expression of metabolic genes", authors should provide direct evidence of the increased type 1 population upon Bmp4 as they provided further evidence on BMP2-induced villus-tip program by visualizing Nt5e+ population. And it would be much better if author can show different metabolic activity in BMP2- and BMP4-treated organoids.

We agree with the statement that increased mRNA level does not directly imply increase in protein level as the discordance between transcription and translation was shown for villus bottom ad villus center genes by Harnik et al., 2021 (but in the case of villus tip genes the translation follows the transcription). On the other hand, Bmps act as inducers of transcription. Hence the unbiased expression profiling may give an sufficient picture concerning their common or distinct roles. To show that Bmp2 and Bmp4 act differently at both transcriptional and translation level, we additionally stained the Bmp2 and Bmp4-treated organoids for villus center marker Fabp2 (Fig. 3C,D). The time point was chosen analogically to previously shown immunoblot picture (now Fig.EV2F) when the organoids showed highest enrichment for the protein. The original Cd73(Nt5e) staining after 24 h treatment (now Fig. EV3) was extended for 48 hour time point (Fig.3C,D), that showed the similar activation upon Bmp2, but not Bmp4. We also stained for general enterocyte differentiation marker AldlB (Appendix Fig.2B). Last but not least, changes in E-cadherin expression are shown at Fig.7. Analogically to Bmp-treatment we added immunostaining for Tgfβ- treated organoids (Appendix FigureS4B).

-Fig. 3: As mentioned above, authors should show functional evidence on differential effect of BMP ligand on enterocyte differentiation. Since Moor et al defined the villus-tip population with the increase of Nt5e, Ada, and Slc28a2, all of which are included in purine metabolism. It would be better if author can show different purine metabolism ability upon BMP2 or BMP4 treatment.

We agree that showing altered metabolic or enzymatic activity upon the Bmps-treatments might be very interesting and important. Unfortunately, due to limited resources and time we could not perform mass-spectroscopy analysis after the treatments. Nevertheless, we invested lot of effort to address this point. Despite many difficulties, we were able to detect changes in purine catabolism after Bmp treatment. We checked the enzymatic activity of Ada using Adenosine Deaminase Activity Assay Kit (Fluorometric, Sigma-Aldrich, EPI020). As shown at Fig.3E the Ada activity increased upon Bmp2 treatment confirming the expression data (Fig.2E).

-Fig. 4: Authors proposed that Tgf-beta seems to activate a program similar to that triggered Bmp2. But authors should state the different changes between Tgf- and Bmp2-treated organoids. The estimated fraction of cell types showed similar patterns in BMP2-treated and Tgf-beta-treated organoids; loss of enterocytes type 1 and increase of tuft cell. But qRT-PCR result showed inconsistent results; BMP2 treatment didn't reduce the expression levels of enterocyte type 1 genes (Fabp1, fabp2, and G6pc, Fig EV2G), while TGF-beta dramatically reduced the expressions of those genes, potentially suggesting further differentiation of type1 into type2.

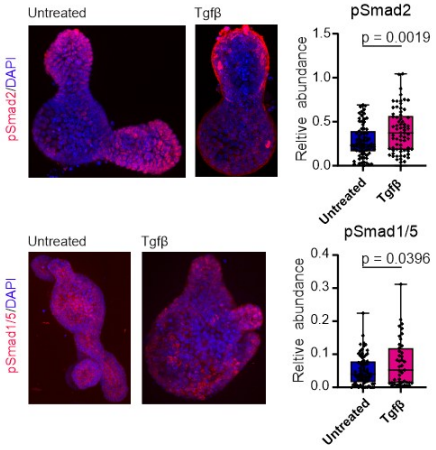
We reanalyzed the overlap in induced gene expression between Bmp2 and Tgfβ (Fig. EV4G). The overlap is really high – almost 80% of genes is similarly induced by Bmp2 and Tgfβ.

- ***Fig. 4: In order to propose that Tgf-beta has similar to BMP2 in activating villus-tip program, authors should provide more evidence like increase in Nt5e+ population and changes in other cell types.***

We checked the changes in expression of Cd73 /Nt5e, Fabp2 and AldlB at protein level after Tgfβ treatment (Appendix Fig.4B). Similarly to Bmp2, also Tgfb treatment upregulated the levels of Cd73, Fabp2 remained unaffected. We also added the analysis of RNAseq data showing stem cell markers, proliferation markers and general differentiation markers (Appendix Fig.4A).

- Fig 4: TGF-beta1 activates signal cascade through type I receptor, such as ALK5. I wonder whether the effect of TGF-beta1 on villus-tip program is dependent on smad2/3, selective mediators of TGF-beta, or Smad1/5/8, common mediators of both BMP and TGF-beta. It might explain the different effect of TGF-beta and BMP2 on reduction of type 1 enterocyte gene expressions.

We tried to address this point by two different approaches. Besides inducible Smad4-KD organoids we worked on generation on inducible Smad5-KD organoids. Despite these organoids survived the puromycin selection they grew very slowly and eventually died before we managed to perform any experiments with them. We could not exclude that their poor fitness was connected to the leakiness of shRNA construct. Thus, we could not perform planed Tgfβ treatment with these organoids. As a second approach we stained Tgfβ- treated organoids with antibodies against p-Smad2 vs. p-Smad1/5. Despite there might be certain trend pointing to increased Smad2 activity (please see the figure below), the result is not clear and to address this question would require further investigation. This is the reason, why we did not incorporate this panel to the manuscript.



-Fig4: I wonder whether there would be phenotypic difference upon treatment of TGF-beta1 inhibitor. I wonder if TGF-beta1 inhibitors and BMP inhibitors affect enterocyte differentiation patterns differently or in a similar way

Unfortunately, we could not perform such type of in vivo experiment we focused our effort to successfully finish other ones. We also could not get an approval to perform this type of in vivo experiment within the provided time. Instead, we improved the in vivo experiment targeted to the block of Bmp receptor type I.

- Fig. 5A,B: Author propose that short-term repression of Bmp receptor complex (4 days) altered villus-tip differentiation in vivo. For that statement, authors should provide more convincing evidence like

histological analyses of different subcellular types (including Type 1 and 2 enterocytes) and should confirm whether histological difference is in consistent with gene expression changes. And it would be better if author shows increase of tuft cells as showed in cell fraction changes expected by bioinformatics tools.

We repeated and extended the experiment and could confirm the decrease in villus tip genes (marked by Ada) upon the inhibitor treatment *in vivo* (Fig.5B,C). Additionally, we confirmed expected changes in gene expression by qRT-PCR (Fig. 5D; Fig. EV5A). Unfortunately, tuft cells are very rare population and we did not have any good and reliable antibody to visualize them. Moreover, the focus of our manuscript is directed towards enterocytes differentiation.

- Fig.5C,E: In line with comments above, author should provide direct evidence of changes in cell fraction by visualizing specific cell types in organoids using fluorescence.

As we showed that Bmp2-induced gene expression is coupled with protein expression on few examples, we believe that to prove the involvement of Smad4 in Bmp2-induced gene expression qRT PCR provides the sufficient and clear evidence. We tested few other antibodies on organoids (Ada, Muc2, Lama3) but those either did not work or suffered with high background.

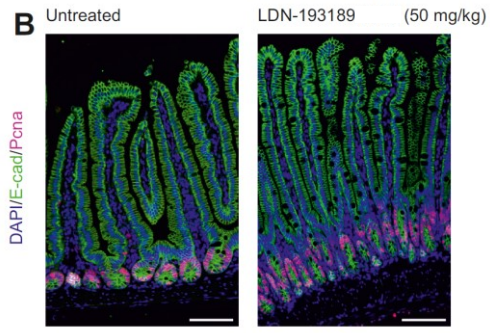
- Fig. 5F: There is not big difference in villus-tip gene expression levels between passage 2 and passage 4 organoids. But for me, it makes sense that adding BMP2/4 might help recapitulation of original intestinal structure in long-term culture with preserved metabolic activity. How about showing obvious evidence that there are less villus-tip/and center villus enterocyte population and decreased metabolic activity compared to original intestinal villus? If adding BMPs restores the metabolic activity in intestinal organoids, it would appeal to the researchers who want to study the metabolic function in intestines.

This is very important point. Now the Fig. EV5D indicates that epithelial cells lost the differentiated identity (differentiated cells) relatively quickly and already after the passage 2 they are enriched for stem cells (as indicated by the increased expression of stem cell and proliferation markers) whereas reduced or missing villus tip genes (with the exception of E-cadherin, but E-cadherin is also in the intestinal epithelium expressed from crypt to tip of the villus, just with varying intensity/level). The addition of low concentration of Bmps can enrich for villus tip gene expression (clearly after passage 2, then decreased again), providing at least 10 days to study differentiation programs (Fig.6E). This experiment should be seen as a starting point for the further adjustment of media composition.

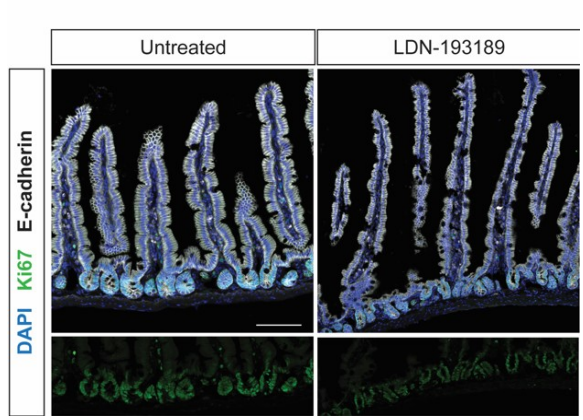
- Fig EV4B: The image shows the elongated crypt upon BMP inhibition. Authors should reconsider whether they can state that the epithelial morphology and proliferation stayed the same after LDN-193189 treatment

The referee is right that crypts seem to be elongated and narrower. However, when we repeated the experiment, but instead of FFPE (left) did frozen sections (right), we could not observe such elongation. Hence, we could not exclude this was an fixation artefact. Importantly in both cases, there are intact crypts containing proliferating cells (either Pcna or Ki67). As our interest goes to villi (these are also intact) we did not follow this issue further. In the text we mentioned that the picture just illustrates the presence of intact(proliferating) crypts and villi.

FFPE (Fig. EV5B)



frozen sections



Treatment and concentration are the same as in the case of FFPE)

- There are several conflicting results in Figures. Please confirm the consistency of results throughout Figures.

1) Fig 1B and Fig EV1D; Ada, one of the strong villus-tip markers, is increased upon co-culture with ivMV in Fig 1B, but not in Fig EV1D.

The co-cultivation experiments presented in Fig. 1A,B and Fig. EV1C,D were performed in series (one independently from the other one, not in parallel) with the ivMCs and TdTomato organoids of the same origin. The Fig. EV1 C,D was performed as the second. The longer cultivation time of both cultures before the experiment might cause lower induction potential of ivMCs and lower responsive potential of the organoids. This might explain the inconsistency, especially ivMCs may slightly differ during the time. What we consider as important is the fact the highly responding villus tip marker Lama3 is sensitive to BMP receptor type I repression. This is reflected in the text now. As we used the co-cultivation experiment only as a proxy and starting point for further experiment we stopped co-cultivation experiments at this stage.

2) Figure EV2G and Figure 2E; Increased Cdh1 expression upon BMP2 (Fig 2E) but no changes in Fig EV2G in the same condition. And Fig EV2G don't include the result of BMP2+BMP4 treatment as figure legend says. And please describe the result about what happened after BMP2+BMP4 treatment. Reduction of type 1 enterocyte genes upon Bmp2 treatment in Fig 2E is not consistent with Fig EV2G data.

Figure Fig. EV2G serves as an additional confirmatory experiment to Fig. 2E. Out of 8 reference genes but only one did not match with bulk RNAseq. This could be explained by primer bias, although we did not test different qRT-PCR primer pairs. As RNAseq provides unbiased evidence and summarized the results from three independently performed experiments we consider this observation as consistent. Moreover,

Fig. 7. B,C shows an increase in Cdh1 expression at protein level upon Bm2 treatment. Even though we performed the experiments using mixed Bmp2+Bmp4 treatment, we decided not to show this data. We realized that we would require many additional controls supported by RNAseq data. Unfortunately, we did not have resources and time to do so. Since we did not want to present insufficiently supported results, we decided to give up on presenting the results of combined Bmp2 and Bmp4 treatment. Nevertheless newly added data (see our previous replies) confirmed the trend – Bmp2 preferentially induced villus tip programs, whereas Bmp4 triggers expression of genes involved in lipid metabolism and uptake.

3) Fig2E and Fig EV2F: The increase of Ada, a Villus-tip marker, is much higher in Bmp2-treated organoids than in Bmp4-treated organoids. And authors propose that BMP2 induces terminal differentiation while BMP4 induces central villus differentiation. However, in Figure EV2F, Ada expression is similar in both Bmp2- and Bmp4-treated organoids. Authors should explain the inconsistency in their experiments. Or it would be helpful to directly show the changes in enterocyte population by visualizing specific cell types in organoids.

The newly added panel (Fig. 3E) reveals the increased enzymatic activity of Ada. This is in the line with transcriptomic data. Concerning the western blot we could not include the sensitivity issue of the antibody or that the chosen timing was not optimal. Despite we tried to use various antibodies for immunofluorescence on treated organoids they did not work. Also western blot required optimalizations. As in previous cases we simply did not want to hide any data we have.

[Minor]

- Please note the statistical significance in Figures and supplementary Figures (ex. Fig. EV1B, 1D, Fig 1E, 1F....)

We adjusted statistical significance according the journal requirements: 1-2 values: values only ; 3-5 values: values with error bars; +5 values: error bars and significance (= exact p-value).

- ***Fig. 1C: Authors provide a complex expression pattern of distinct Tgf superfamily ligands in Pdgfrahigh mesenchymal cells using published scRNAseq dataset. It would be better if authors provide the information of whole Tgf superfamily ligands, not only BMPs and TGF-beta1.***

We additionally provided DotPlots containing information about the expression across all families throughout Tgfβ superfamily by re-analyzing McCarthy 2020 dataset (Appendix Fig.S1). This analysis clearly shows that members of Bmp family are the most abundantly produced by the small intestinal $Pdgfra^{+}$ MCs.

- ***line 173-178: Authors propose that Bmp4 was expressed in non-epithelial cells from the center to the top of the villus in contrast to the zonated Bmp2 expression at villus tip, supporting the proposed action of Bmp2 on terminal differentiation. But there are conflicting evidence regarding Bmp4 expression patterns. KB Halpern et al. showed the increased Bmp4 expression at villus-tip. And N McCarthy et al. also reported that PDGFRA+ stromal cells which exist at the villus-tip region secrete both Bmp4 and Bmp2. These conflicting evidences suggest that terminal differentiation is not dependent only to zonated Bmp2 secretion from stromal cells rather propose the presence of other complex mechanism***

on regulation of terminal differentiation. Thus, author should discuss about the conflicting evidences in DISCUSSION section not to overstate on potential relationship between BMP ligand locations and enterocyte differentiation.
KB Halpern et al., Nat Commun. 2020; 11: 1936.
N McCarthy et al., Cell Stem Cell 2020; 26(3):391-402

We agree that the mechanism of villus tip fate induction might be more complex *in vivo*. As this complexity goes far beyond our work (we only suggest that the individual role of Bmps can contribute, too) we only partially discuss this issue. To dissect this question more comprehensive *in vivo* experiments should be done. Nevertheless, we also mention the aspect of complex regulation of villus tip differentiation. For example, as we mentioned Wnt5a can be also involved.

- Fig. 5A,B: Author should state how they determined gene expression changes in tissues; was it mixture of diverse cell population including stromal cells or just epithelium using specific enrichment methods?

To study changes in gene expression by qRT-PCR after LDN193189-4HCl treatment *in vivo* treatment, we used complete intestinal epithelium, respectively all intestinal epithelial cells released from the proximal small intestine as indicated in the Methods section.

Referee #2:

In this manuscript Berkova et al. study the differential impact of members of the TGFb superfamily on the differentiation status of enterocytes. Enterocytes exhibit zonated gene expression signatures, that are thought to be controlled by zonated stromal morphogens. Among these are members of the TGFb superfamily - Bmp2, Bmp4, Bmp5, Bmp7 and Tgfb. It is unclear whether these morphogens act in a redundant manner, or rather regulate distinct epithelial programs. The manuscript addresses this important question in a systematic manner, by exposing enteroids to all of the Tgfb ligands and measuring in an unbiased manner, through RNA sequencing, as well as qPCR validations, the resulting expression changes. They find that Bmp4 activates programs associated with the mid-villus zones, such as lipid metabolism genes, whereas Bmp2 induces more villus-tip programs such as cell adhesion. They further find that Wnt5a, another villus tip-restricted morphogen, synergizes with Bmp2 to up-regulate villus tip enterocyte genes. The authors also introduce an organoid culture supplemented by Bmp2 that better recapitulates the in-vivo villus tip enterocyte programs. The study addresses an important question, is well designed and the results conveyed in a readable and interesting manner. I have a few, generally minor comments:

We thank the referee for very positive opinion considering our manuscript.

1)Bmp4 is solely expressed by mesenchymal cells, whereas Bmp2 is expressed both by mesenchymal cells but also by villus tip enterocytes (e.g. Moor et al., 2018). This is actually also seen in the smFISH

validation of Figure 2F. This needs to be discussed. Are there any hypotheses to the epithelial expression of Bmp2? Is this a positive feedback loop, whereby enterocyte Bmp2 is induced by mesenchymal Tgfb signals or Wnt5a to enhance the Bmp signal? Does enterocyte Bmp2 mRNA expression change in response to the distinct ligands studied?

This is very interesting question that led us to re-analyze our RNAseq datasets and checked the expression of Bmps after Tgfβ and vice versa (Fig. EV04A, I, J). There is an interesting upregulation of Tgfβ upon Bmp2 treatment a possible feedback loop in Tgfβ activation. Nevertheless, we are not able to distinguish the individual contributions of Bmps originating in mesenchyme and in epithelium. The probed the effect of other molecules such as Wnt5a, Ihh/Shh on the epithelial and/or mesenchymal expression of Bmps might be an interesting question for the future direction of the research. Additionally, one can speculate, whether the Bmp/ Tgfβ positive feedback loop isn't the main reason for the requirements of Bmp signaling inhibition in intestinal organoid culture.

2)How do enterocyte anti-microbial programs change upon Bmp2/4 stimulation? It will be interesting to see, based on the RNAseq, how genes like Reg3a, Reg3b, Reg3g, Reg1, Il18, Ccl25, Nlrp6, Lypd8 are expressed in BMP4 treated organoids, as figure 2D suggests BMP4 organoids have some V1-V2 like transcriptional programs.

We analyzed the expression of anti-microbial program related genes as suggested (Appendix Fig. S3A). The all checked genes are downregulated by Bmp2 treatment, many of them (Reg3a, Reg3b, Reg3g, Reg1 and Nlrp6) are also inhibited by Bmp4 treatment. In general, this observation indicates, that Bmp treatment might help to restrict antimicrobial program to the villus crypt interface as expected.

3)Additional cell types in the small intestine have Bmp receptors (both mesenchymal, endothelial and glia cells), the authors should discuss the potential additional roles of Tgfb ligands beyond the epithelium.

This is very interesting point but we afraid it is far from the focus of our manuscript. As we studied the impact of Bmps/Tgfβ on the intestinal epithelium, we think it might be either distracting or might be not really relevant. We hope, that the referee could agree with us that the addition of few sentences claiming that Tgfβ can affect other tissues beyond the epithelium will not bring much (if we do not show any results, as it is not our focus). Definitely it might be the topic for a prospective review article.

4)Is Chylomicrons assembly and secretion bmp dependent? Beside immunomodulation and adhesion, Villus tip enterocytes are extensively assembling and secreting chylomicrons. It will be interesting to show, based on their RNAseq, the gene expression levels of such genes (e.g., apolipoproteins, MTTP, DGAT2, ABCA1 and more) in the different BMP conditions and conclude whether those functions are governed by Bmp ligands, or rather other factors (e.g. luminal lipids)?

We checked the expression of chylomicrons-related genes in our RNAseq dataset (Appendix Fig. S3B). Majority of them is upregulated by Bmp4 some of them only upon Bmp4-treatment (Mttp, Apoc3, Cd36, Npc1l1, Apoa1, Apoa4). Some are upregulated by both Bmp2 and Bmp4 some are upregulated by both treatments (Apob, Dgat2, Apobec1). Only one gene is upregulated solely after Bmp2 treatment (Abca1). We can conclude, that mainly Bmp4 but partially also Bmp2 ligands are able to trigger the expression of

chylomicrons assembly genes. It could be caused by the fact that chylomicrons might be expressed as "upper center genes". Importantly we could not conclude that Bmp signaling is the only initiator of expression of those genes. The evaluation of the effect of other possible inducers such as luminal lipids is unfortunately beyond the scope of this paper.

5)Figure 2F - please provide a blow-up. There is a typo in the legend - white should be Bmp4 and not Smad4.

We corrected it. The legend and the figure now should provide a clear picture.

6)Row 140-142 - sentence unclear.

The sentence was clarified. Now it is: …. Bmp3 overlapped with untreated samples, did not dramatically affect the expression of stem cell or differentiation markers, indicating that Bmp3 had no effect on the organoids. It could be connected to its specific role acting as a Bmp antagonist rather than the inducer of Bmp activated programs (Bragdon et al., 2011; Wang et al., 2014) (Fig. 2A).

7)Figure 2B caption - what is "top 10 unique genes per each treatment"? Please better explain the genes shown in the heatmap (as done in the Methods section).

The information is now indicated in the caption.

8)In general, how were ligand concentrations determined? Are they within physiological ranges? Might there be concentration-dependent behaviors?

Working concentrations of Bmps were determined based on the dose-dependent activity assays performed with the Bmps by manufacturer (please see the Materials table). We used concentration, which was in the middle of working range for all recombinant proteins used in the study. We also performed experiment with lower concentrations of the recombinant proteins (Fig. 6E). We observed the same kind of effects of Bmps but in dose-dependent decreasing manner. At half dose (250 ng/ml) we could see the same effect of Bmp2 and Bmp4 as in the case of concentration we used (500 ng/ml) We did not use higher concentrations than 500 ng/ml. As it is almost impossible to determine what is the exact concentration of Bmp in vivo, we could not tell, how close is the chosen concentration to physiological range. We also Importantly as the trend of Bmp2-promoting the villus tip differentiation was the same also for 50x lower concentration we believe our data reflect the correct situation.

9)Related to point 8 above, the results should be discussed in the context of recent work from the Elowitz lab on the distinct behavior of Bmp ligands and their combination on cell lines (https://www.sciencedirect.com/science/article/pii/S2405471222001296, https://www.sciencedirect.com/science/article/pii/S0092867417309406).

We thank for this interesting point and idea. We tried to discuss this aspect. However we could not go deeply to the detail, since we do not have sufficient data concerning the intestinal epithelium.
10)Line 244 - typo, should be "was" instead of "as".

Many thanks, the typo is corrected.

Dear Dr. Valenta

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, both referees are very positive about the study and request only minor changes to clarify text and figures.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please add up to 5 keywords

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
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- Figure panels should be called out in an alphabetical order. In this context we notice that Fig 3B is called out before 3A. If possible, please rearrange/swap these panels.

- Please add callouts to Fig EV2D.

- Datasets: Please add their names (Dataset EV#) and legends to the .xls file. The legend should be in a separate tab. Callouts need to be added in the text.

- Appendix: please add a table of content with page numbers.

- Please provide the synopsis image in higher resolution. The text in the current image appears fuzzy/pixelated. The text to the right is also very close to the border of the image. The green text 'Wnt5a' is not so easy to read. Maybe another text color would increase contrast and visibility.

- You mention several datasets in the text, e.g. GSM3747599 in Figure 1 and Appendix Figure S1, GSE92332 and zenodo.3403670 in Figure 2 and several others. Please cite these as data reference. You need to cite the paper that described these datasets and in addition add a data citation. In the text this looks like (Name et al, year, data ref: Name et al, year.) In the reference list you add [DATASET] at the end of the data reference.

See also the paragraph on 'Data citation' in our guide to authors:
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- Key resource table: Please change the name and the callouts to "Reagents and Tools Table". The second part of the table (polynucleotides, qRT-PCR primers should also have three columns to stick to the format. Alternatively, you can move these to another table (Table EV#) and refer to this table in the Reagents and Tools Table. I attach the Word template here for your information.
See also https://www.embopress.org/page/journal/14693178/authorguide

- Please order the manuscript sections according to your style/guidelines.

- Appendix Legends:
- Appendix Figure S1: comma missing. Correct to [...] cells expressing the transcript, color scale indicates [...]
- Appendix Figure S2: (A) Please define whether 'n' stands for technical or biological/independent replicates. (B) Please add a scale bar and define its size in the figure legend. Typo: Left panel: quantification... should be Right panel: quantification; 3th quartile should be 3rd quartile. 'Box blot' might be a better description instead of 'graph'.
- Appendix Figure S3: typo '...mainly Bmp4 influence" should be 'influences'.
Please define the nature of the replicates (technical, biological) in (A) and (B).

- Appendix Figure S4: Suggest changing 'suppresses the stemness' to 'suppresses stemness'.
(A) Please define the nature of the replicates. (B) Please add scale bars and define their size in the legend. The quantifications are shown in the Right panels.

- Source data: All source data should be uploaded as one folder/ZIP per figure. One of the PDF files appears to be mislabelled - sFig 2F is now EV3F and Fig 4F should now be Fig 6B.

- Fig 1B, F, 7A, EV1B contains quantification based on two datapoints with mean and error bars. Please show only the datapoints and not the mean plus error bars.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors completely addressed 11 of 15 major concerns and partially addressed 1 (11.5/15=76.6%). The remaining three concerns are about TGF-beta1 to fully dissect the role of TGF-beta1 in intestinal organoid differentiation.
After revision, the authors pin-pointed on BMP2 and BMP4 not to overstate the conclusion and provided enough data proposing that Bmp2 and Bmp4 preferentially activate different enterocytic programs.
I believe this finding is already novel to be published in EMBO Reports.

Minor: Fig. EV5C (Graph titles are interchanged)

Referee #2:

The authors have addressed most of my comments. This is an important work that should be published in EMBO Reports.

The authors have addressed all editorial requests.

Tomas Valenta
University of Zurich
Department of Molecular Life Sciences
Winterthurer Str. 190
Zurich, 8057
Switzerland

Dear Tomas,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Kind regards,

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Martina Rembold, PhD
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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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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:
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  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures (caption), Material and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Material and methods
Include a statement about **blinding** even if no blinding was done.	Not Applicable	Statement that no specific blinding was applied is included in Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures (caption), Material and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Material and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Material and Methods

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Figures(caption), Material and Methods, References