

Intra-epithelial non-canonical Activin A signalling safeguards prostate progenitor quiescence

Francesco Cambuli, Veronica Foletto, Alessandro Alaimo, Dario De Felice, Francesco Gandolfi, Maria Dilia Palumbieri, Michela Zaffagni, Sacha Genovesi, Marco Lorenzoni, Martina Celotti, Emiliana Bertossio, Giosuè Mazzero, Arianna Bertossi, Alessandra Bisio, Francesco Berardinelli, Antonio Antoccia, Marco Gaspari, Mattia Barbareschi, Michelangelo Fiorentino, Michael Shen, Massimo Loda, Alessandro Romanel, and Andrea Lunardi

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Dear Prof. Lunardi,

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in the detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on $n=2$ (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

- 1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.
- 2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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- 3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: <http://embor.embopress.org/authorguide#datadeposition>

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (DAS - placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets 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. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: <http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (indicating biological or technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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9) Please also note our reference format:
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10) Please provide the abstract (not more than 175 words) written in present tense.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript by Cambuli, Foletto et al is a long due study on the role of intra-epithelial non-canonical Activin A signalling. I would like to compliment Prof Lunardi for the outstanding work. This manuscript sets the basis for the understanding of cancer initiation and possibly targeting of a problematic quiescent cell type.

I have few minor concerns/comments:

1. How do the authors explain the absence of Nkx3.1 in the m ProOs progenitor population in mouse organoids ? Assessing the composition of organoids based on expression of Nkx3.1 along with basal and luminal markers would be very informative and complementary to the androgen sensitivity experiment in Figure EV1, panel B-D.
2. The native expression of Acvr1b seems to be exclusively found in basal KRT5+ cells (Fig.2C). Is this generally the case? Higher magnification images are needed. Also, the authors should show the proliferation status, pSmad2/4 and p38 of the ALK expressing cells in the normal prostate and in the organoids
3. The A8301 specificity and selectivity should be mentioned in the manuscript. Given that A8301 would inhibit both ALK5 and ALK4 it is challenging to extrapolate whether the outcome is due to inhibition of one vs the other receptor. E.g when the canonical ALK5/Smad signaling is inhibited that may also be sufficient for activation of non-canonical signaling. Experiments with knockdown of specific ALK receptors would be more to the point.
4. Given the absence of TGFb ligands in the organoid media and the fact that exogenous TGFb1 (or absence of its inhibitor) canonically leads to p21 activation and cell growth inhibition, the title of the section related Fig.2 is not completely correct. The described ActivinA non canonical signaling is an additional mechanism but not the mediator of the TGFb-induced cytostasis.
5. The mechanisms of the quiescent phenotype found in the prostate epithelium is of great interest, especially with the implications in tumor progression such as in cases with mutations in ACVR2A. For that reason, it would interesting to better characterize the expression of ALK receptors and downstream signaling in the native prostate, and how this dynamically alters in relation to a BPH /aging setting or upon androgen deprivation.

Referee #2:

General comments:

In the present manuscript Cambuli and colleagues generate a valuable collection of prostate organoids with which they describe the association of intra-epithelial non-canonical Activin A signalling to the balance between quiescent and cell proliferation status of prostate epithelia. This finding might be important for future investigations regarding the biological events that occur along the generation of prostate cancer, but for the moment this work is not groundbreaking. The analysis of Activin A signaling (or transcriptional programs associated) in any of the prostate cancer mouse models available (from less aggressive to more) would add preclinical value to the message. Even an attempt to interrogate the multiple PCa databases that are publically available nowadays, to associate their findings to physiopathological scenarios, would be beneficial to the message of the manuscript. In fact, the discussion opens very interesting questions such as the origins of replicative stress, a more than probable precursor of senescence and a biological event relevant to prostate cancer.

Specific and technical comments:

Authors have done a very good technical work establishing this type of culture but there are some issues regarding the statement that Activin A signaling is linked to the proliferation effects:

Authors state that Egf signalling alone is unlikely to explain the excess of proliferation in culture based on receptor and ligand expression, but from the results represented in EV3 (A), Egf is the sole component of the media that blocks organoid formation&maintenance, meaning that it could play an important role. It is not clear the justification to not studying EGF signalling and focus on TGFb inhibition.

Do the different organoid cultures respond equally to EGF depletion and/or A83-01?

Is the presence of secreted Activin A consistent/reproducible in different organoid cultures from different prostate lobes?

In figure 2D, authors rescue the proliferation treating with follistatin. Have authors monitored the efficiency of follistatin to inhibit Activin A (quality control) and no other targets such as BMPs?

As a specificity control of follistatin effect on -A83-01 organoids, authors should treat also ENRAD organoids with the drug. Does follistatin rescue the basal levels (ENRAD condition) of pTAK, p38 and p21? Does Tgfb1 and ActivinA induce the same signalling cascade in the presence of A83-01 (so ENRAD condition) (figure 2E)?

Why authors have used shRNA of Smad4 to study TGFb-cannonical pathway but not Smad2/3, the ones they analysed in fig 2E?

In figure 2G authors should include the vehicle control and the effect of the drugs in ENRAD conditions, to show the specific effect on TGFb inhibition conditions.

In figure 2H blots for total Stat1 blots, total p38 as well as p-Stat2 are missing.

In figure 2, organoids depleted of A83-01 show an enrichment of immune genes, related to cytokine-mediated signalling and

TNF signalling. It would be nice to see qRT-PCR of some of these genes, to validate RNAseq-GSEA. In the enrichment scores plots, the distribution of the genes within the geneset (black bars) do not seem to be specific of one or other experimental group (not such enrichment), but the NES are relatively high and FDR are very low. Can authors comment on that, in first instance, incongruity?

From data on figure 2 authors state that they demonstrated that intra-epithelial non-canonical Activin A signalling induce a cytostatic response in mouse prostate organoids but the data shown is associative not demonstrative because they have not silenced or delete specifically Activin A.

In the orthotopic injection, how authors differentiate between host and organoid-derived epithelial prostate cells? It would be nice to have an H&E picture in which the reader could see the delimitation of host and organoid-derived tissue.

Referee #3:

In this study Cambuli and colleagues performed mouse prostate organoids to model epithelial progenitors. They found that intra-epithelial non-canonical Activin A signalling inhibited cell proliferation in a Smad-independent manner, and could trigger the activity of Tak1 and p38 MAPK, leading to p16 and p21 nuclear import. The results presented in this article are interesting. However, the study was not performed comprehensively, leaving much to be answered. Some of the findings are not convincing enough.

Major points:

1. The authors performed mouse prostate organoids as the key experiment tool to conduct the entire research, however, the organoids culture is under the pressure of in vitro culture systems, the evidence on whether organoids can mimic stem cells in vivo is insufficient, whether the structure of organoids enriched for epithelial progenitor cells can also be clearly observed in vivo. Both basal and luminal progenitor cells have been identified with distinct self-renewal capacity in vivo, however the authors using epithelial progenitors to define the survived cells in the prostate organoids. They need clarify the identity of the "epithelial progenitors", basal progenitors or luminal progenitors.
2. Fig 1B. The authors needs to clarify why the trend of organoid forming efficiency at passage 1-3 is violently fluctuating in different cell lines? Does this come from the unstable culture condition or intrinsic prostate cell difference of different lobes?
3. They claimed the continuous expansion of "epithelial progenitors" in a near-physiological manner. However, both the prostate basal and luminal progenitors are quiescence. They should clearly define the context to precisely describe the morphological similarity.
4. Fig EV1. Androgen is critical for prostate luminal cell survival and lumen formation, and the prostate lumen were clearly reduced after androgen deprivation. They claimed that mouse prostate organoids were reversibly dependent on androgen for lumen formation, but not for their survival. They need to stain the cell apoptosis markers, and count the cell numbers during this treatment to show the evidence of their conclusion.
5. Fig 3. Why only the prostate organoids generated from DLP adapting to the absence of A83-01? They found that the ATR signalling were strongly upregulated upon A83-01 withdrawal adaptation, and indicating potential DNA replication stress. TGF- β signaling pathway has distinct function in the tumor initiation and progression. They need the fully discuss the potential correlation between TGF- β signaling and prostate cancer.

Will the cytokine-mediated and TNF signaling be enriched in mouse prostate organoids cultured in the absence of Activin A receptors inhibition?

6. Fig 4. It is challenge to distinguish the grafted cells and the host cells without any tracing markers. The authors need to describe their methods to distinguish the grafted cells and the host cells.
7. The authors just used one organoid line to identify the evasion from the Tgf- β induced cytostatic response via downregulation of intra-epithelial Activin A signaling, more lines will be better.
8. Which source of Activin A signaling play a role in safeguarding prostate progenitor quiescence, the basal, LumP or PrU cells?
9. Is it possible to inhibit Activin A signaling in vivo to observe the activity of prostate progenitor cells?

Point-by-Point Rebuttal

We thank the reviewers for their constructive criticism. We have addressed all the concerns raised by the reviewers and answered their questions in the point-by-point rebuttal. We sincerely hope that the manuscript is now suitable for publication in EMBO Reports.

Referee #1:

The manuscript by Cambuli, Foletto et al is a long due study on the role of intra-epithelial non-canonical Activin A signalling. I would like to compliment Prof Lunardi for the outstanding work. This manuscript sets the basis for the understanding of cancer initiation and possibly targeting of a problematic quiescent cell type.

I have few minor concerns/comments:

1. How do the authors explain the absence of Nkx3.1 in the m PrOs progenitor population in mouse organoids? Assessing the composition of organoids based on expression of Nkx3.1 along with basal and luminal markers would be very informative and complementary to the androgen sensitivity experiment in Figure EV1, panel B-D.

We thank the Reviewer for the opportunity to clarify this point. Our study found that Nkx3.1 mRNA levels are relatively low in organoids (mPrOs) in comparison to the prostate tissue (Fig. 1D), though Nkx3.1 transcripts remain detectable by RNA-seq (Fig. 1E). Additionally, we have confirmed expression of the Nkx3.1 protein in organoids by western blot (Lorenzoni, De Felice et al., submitted CANLET-D-21-03227), as shown below (Fig. R1)[Figure for referees not shown.] . We have edited the text to better reflect this point.

2. The native expression of Acvr1b seems to be exclusively found in basal KRT5+ cells (Fig.2C). Is this generally the case? Higher magnification images are needed.

We agree with our Reviewer that Acvr1b marks preferentially, but not exclusively, Krt5+ cells in both proximal prostate tissue and organoids, while it is exclusively expressed by Krt5 + cells in the distal part of mouse prostate epithelium. Higher magnification of Acvr1b expression by immunofluorescence is now included in Figure 2C of the revised manuscript, as well as being reported below.

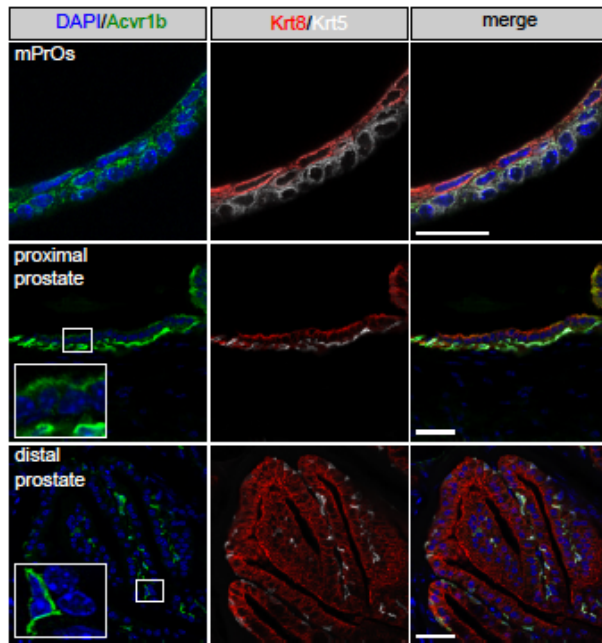


Figure R2. Higher magnification showing immunolocalization of Acvr1b in both basal and luminal cells of the proximal region of mouse prostate while in the distal part of the gland only basal cells express Acvr1b.

Also, the authors should show the proliferation status, pSmad2/4 and p38 of the ALK expressing cells in the normal prostate and in the organoids

We agree with the Reviewer that definition of the proliferative status of ALK (Acvr1b) positive prostate cells in parallel with phospho-Smad2/4 and phospho-p38 would be very valuable. Unfortunately, the phospho-Smad2 and phospho-p38 antibodies tested showed a poor and unreliable immunofluorescence staining on the FFPE biological samples. This is a frequent issue with phospho-specific antibodies, mainly dependent by the loss of phosphate groups from modified proteins during both paraffin inclusion and antigen retrieval. We regret to say that we could not address this point.

3. The A8301 specificity and selectivity should be mentioned in the manuscript. Given that A8301 would inhibit both ALK5 and ALK4 it is challenging to extrapolate whether the outcome is due to inhibition of one vs the other receptor. E.g when the canonical ALK5/Smad signaling is inhibited that may also be sufficient for activation of non-canonical signaling. Experiments with knockdown of specific ALK receptors would be more to the point.

We agree with our Reviewer that lack of specificity of A83-01 for Alk4 (Acvr1b) and Alk5 (TgfbR1) does not allow us to discriminate the relative contribution of the two receptors to Activin A signalling. To address this point, we knocked-down Alk4 in wild-type organoids through stable transduction of a specific shRNA. We found that a reduction in Acvr1b levels was sufficient to restore organoid proliferative capacity. The knockdown experiment is now displayed in Appendix Fig S2C-D of the revised manuscript, as well as below.

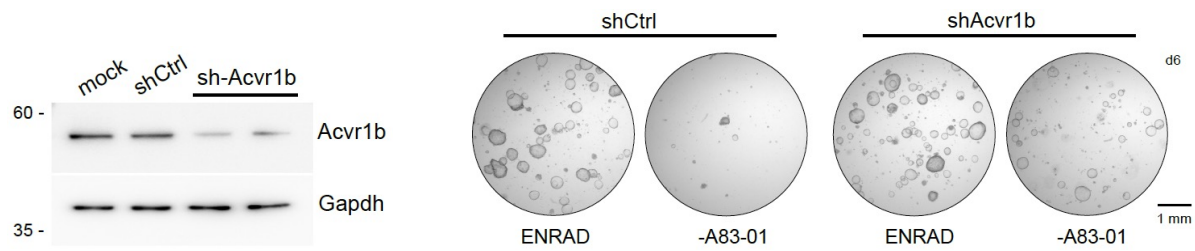


Figure R3. Left, western blot showing reduced amount of Acvr1b protein in mPROs stable transduced with shRNAs against *Acvr1b*. Right, representative images of shAcvr1b mPROs growth in the presence/absence of A83-01.

4. *Given the absence of TGFb ligands in the organoid media and the fact that exogenous TGFb1 (or absence of its inhibitor) canonically leads to p21 activation and cell growth inhibition, the title of the section related Fig.2 is not completely correct. The described ActivinA non canonical signaling is an additional mechanism but not the mediator of the TGFb-induced cytostasis.*

We agree with the Reviewer that Tgfb1 ligand has been described promoting SMAD-dependent p21 transcription and cell cycle inhibition in several context, and especially in the gastrointestinal system. In mouse prostate organoids administration of exogenous Tgfb1 promotes Smad2/3 phosphorylation but it does not change p21 protein levels (Fig 2E, Appendix Figure S6B-C), nor it arrests organoids growth (Figure 3J). In contrast, administration of exogenous Activin A leads to higher p21 protein levels (Figure 2I, Figure 3K, and Appendix Figure S6A), and inhibits organoid proliferation capacity (Figure 3J).

5. *The mechanisms of the quiescent phenotype found in the prostate epithelium is of great interest, especially with the implications in tumor progression such as in cases with mutations in ACVR2A. For that reason, it would be interesting to better characterize the expression of ALK receptors and downstream signaling in the native prostate, and how this dynamically alters in relation to a BPH /aging setting or upon androgen deprivation.*

We thank the Reviewer for this suggestion. Ageing is a well-established risk factor for prostate diseases. Yet, relatively little is known on the impact of age on the prostate, due to the challenge of obtaining tissue specimens in the normal/ageing/BPH setting. Taking advantage of a very recent transcriptomic study by Chris Barbieri, Andrea Sboner, and colleagues (PMID: 32332823), we analysed the expression of key mediators of the non-canonical Activin A signalling pathway, including *INHBA*, *INHBB*, *FST*, *ACVR1B*, *TGFBRI*, *MAP3K7* and *MAPK14*. Despite the limited number of samples in this study (5 normal prostate and 18 BPH), which precludes robust statistical considerations, we observed a trend towards reduced *ACVR1B* expression in BPH compared to normal tissue ($p = 0.07$, unpaired two-samples Wilcoxon test) (Figure R5).

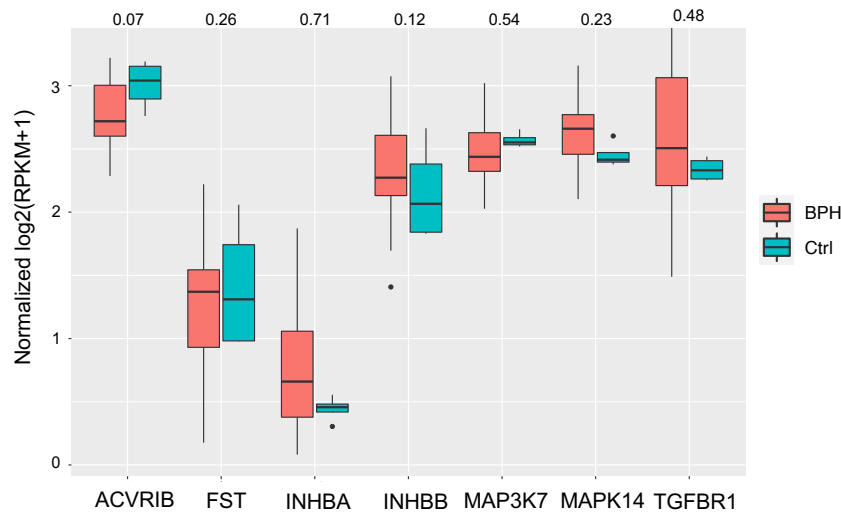


Figure R5. Expression analysis of *ACVR1B*, *FST*, *INHBA*, *INHBB*, *MAP3K7*, *MAPK14*, and *TGFBRI* genes in benign prostatic hyperplasia (BPH, red) and normal (turquoise) human prostate samples.

Referee #2:

General comments:

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Authors state that Egf signalling alone is unlikely to explain the excess of proliferation in culture based on receptor and ligand expression, but from the results represented in EV3 (A), Egf is the sole component of the media that blocks organoid formation&maintenance, meaning that it could play an important role. It is not clear the justification to not studying EGF signalling and focus on TGFb inhibition.

We are sorry if the meaning of this sentence was unclear. Undeniably, Egf signalling is essential for organoid cultures. However, because quiescence is a pervasive condition of adult prostate epithelium, antiproliferative signals must exert a tight control on adult tissue homeostasis. Our understanding of the signalling pathways and molecular mechanisms that promote prostate epithelium quiescence is very limited compared with the amount of literature describing the role of PI3K and MAPK pathways in prostate tumorigenesis. We have edited the text to clarify our focus on the molecular circuits governing quiescence in the adult prostate epithelium.

Do the different organoid cultures respond equally to EGF depletion and/or A83-01?

We agree with the Reviewer that exploring consistency of organoids response to EGF or A83-01 depletion is a very important aspect. *As described in the original articles published by Sawyers and Clevers' groups (Figure R6, Karthaus et al., Cell 2014 and Figure R7), we fully confirmed the essential role of EGF for both lobe-specific and mixed-lobe prostate organoid cultures.*

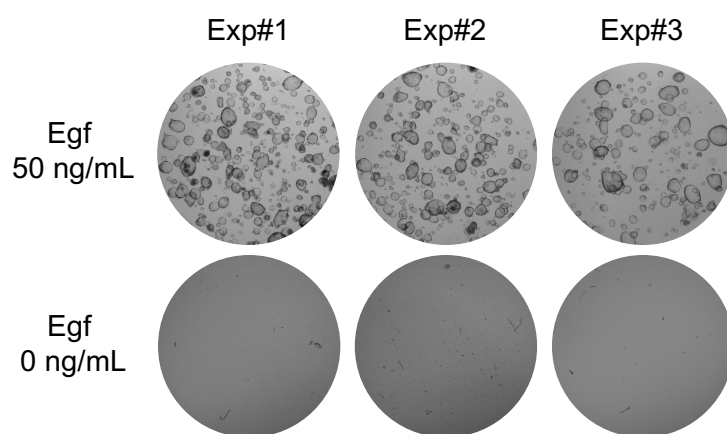
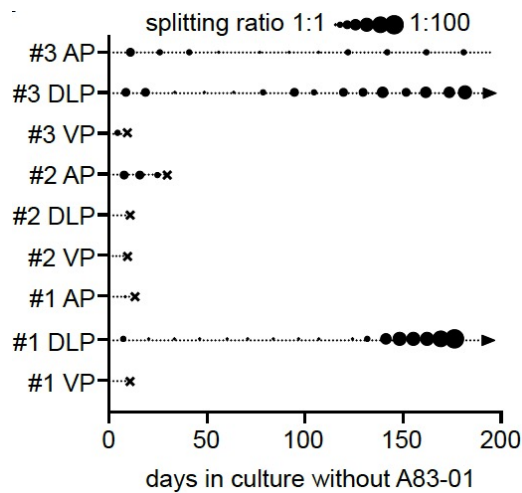


Figure R7. Egf depletion prevents prostate organoids growth (mixed-lobe).

As displayed in Figure 3A of our manuscript (which is also reproduced below), organoid cultures derived from dorsolateral lobes of mouse prostate possess a greater attitude to survive A83-01 depletion.



Is the presence of secreted Activin A consistent/reproducible in different organoid cultures from different prostate lobes?

Activin A was initially identified in the supernatants of organoid cultures via mass spectrometry analyses of four biologically replicas (Figure 2B). Then, we adopted the ELISA approach to compare the amount of Activin A released by normal and adapted organoids of two independent organoids cultures derived from the dorso-lateral lobe of the prostate (Figure 3I). This time, 5 biological replicas were used to evaluate Activin A levels in both normal and adapted DP#1 organoid lines, while 6 were used for both normal and adapted DP#3 organoid lines. Conversely, we agree with our Reviewer that the data supporting Activin A expression in organoids derived from the anterior and ventral lobes of the prostate are limited being these based merely on the analysis of *Inhba* RNA presented in Appendix Figure S2B. To better characterize Activin A expression in all the three lobe-specific prostate organoid cultures, we performed a nonreducing western blot of total cell lysates with a specific antibody against Activin A (MAB3381, R&D System) that recognizes the native form of the protein (Funaba M et al., *Cellular Immunology* 2006). As expected, MAB3381 recognizes two bands in the lysates that are respectively the monomeric (25 kDa) and dimeric (50 kDa) forms of Activin A. Overall, organoids derived from the anterior (AP), ventral (VP), and dorsolateral (DP) lobes of mouse prostate express comparable amounts of Activin A (Figure R8). The results showed below have been integrated in the revised manuscript as Appendix Figure S2E.

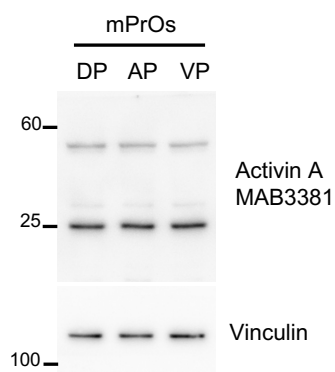


Figure R8. Non-reducing western blot showing comparable amount of monomeric (25 kDa) and dimeric (50 kDa) forms of Activin A in lobe-specific prostate organoids (dorsolateral prostate, DP; anterior prostate, AP; ventral prostate, VP).

In figure 2D, authors rescue the proliferation treating with follistatin. Have authors monitored the efficiency of follistatin to inhibit Activin A (quality control) and no other targets such as BMPs?

We agree with the Reviewer that Follistatin can also bind and inhibit BMP ligands, to a lesser extent. However, the presence in the medium of 100 ng/mL of Noggin should considerably limit the effect of Follistatin on BMP signalling. Moreover, to strengthen the conclusion that inhibition of Activin A rescues organoids formation in the absence of A83-01, we replaced A83-01 with the Activin A neutralizing antibody MAB3381 (R&D System). As shown in Figure R9, neutralization of Activin A rescues organoids growth in the absence of A83-01. These data are described in Figure 2D of the revised manuscript.

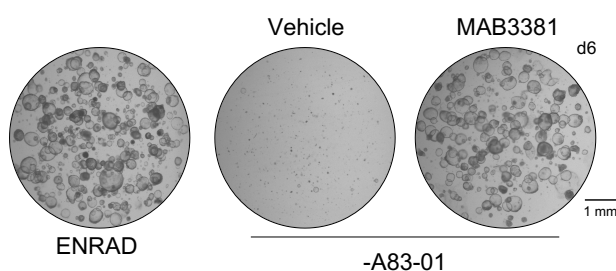


Figure R9. Representative stereoscopic images of mouse prostate organoids cultured in the absence of A83-01 with or without Activin A neutralizing antibody MAB3381 (1 μ g/mL, 6 days).

As a specificity control of follistatin effect on -A83-01 organoids, authors should treat also ENRAD organoids with the drug.

As described in Figure R10, conditioning of ENRAD medium with 500 ng of purified Fst does not alter organoids growth. This result has been included in Figure 2D of the revised manuscript.

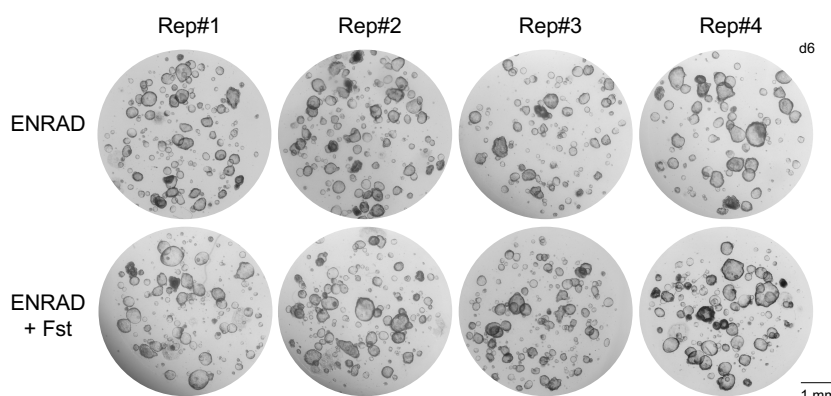


Figure R10. Representative images showing normal organoids growth in ENRAD medium conditioned with Follistatin (Fst).

*Does follistatin rescue the basal levels (ENRAD condition) of pTAK, p38 and p21?
Does Tgfb1 and ActivinA induce the same signalling cascade in the presence of A83-01 (so ENRAD condition) (figure 2E)?*

As suggested by our Reviewer, we analysed the phosphorylation of Tak1 and p38, and the levels of p21 in ENRAD, ENR-D and in ENR-D+Fst. Western blots of Figure R11 show comparable amounts of phospho-Tak1, phospho-p38 and p21 in ENRAD and ENR-D+Fst conditions, thus demonstrating the ability of Fst to compensate for the withdrawal of A83-01. On the other hand, exogenous Activin A or Tgfb1 are equally ineffective in promoting the signalling cascade in presence of A83-01. These results have been integrated into the revised manuscript as Appendix Figure S3A.

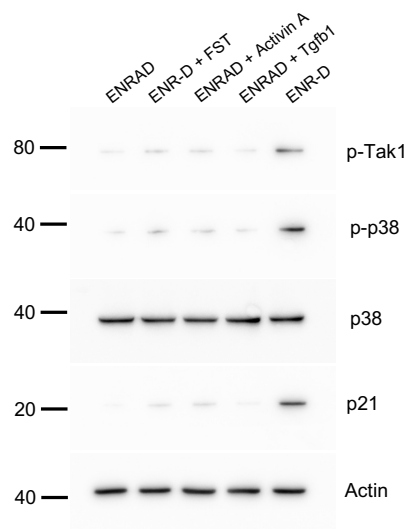


Figure R11. Western blot analysis for key components of the non-canonical Tgf- β pathway in mouse prostate organoids cultured 6 days in ENRAD medium or depleted for the last 48 hours of A83-01, with or without exogenous Activin A (50 ng/mL), Tgfb1 (500 ng/mL), or Follistatin (Fst, 500 ng/mL).

Why authors have used shRNA of Smad4 to study TGFb-cannonical pathway but not Smad2/3, the ones they analysed in fig 2E?

The choice of knocking down Smad4, instead of Smad2 and Smad3, was inspired by the article published in Nature by the De Pinho's group (Ding et al., 2011) entitled '*SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression*'. In addition to the scientific rationale, lowering canonical Tgfb signalling by reducing Smad4 levels is technically easier than the concomitant knock-down of Smad2 and Smad3.

In figure 2G authors should include the vehicle control and the effect of the drugs in ENRAD conditions, to show the specific effect on TGFb inhibition conditions.

As suggested by our Reviewer, Figure R12 showed representative images of organoids cultured in ENRAD medium conditioned with the different molecules used to inhibit Tak1 and p38 function. These data are now included in the revised manuscript as Appendix Figure S3D.

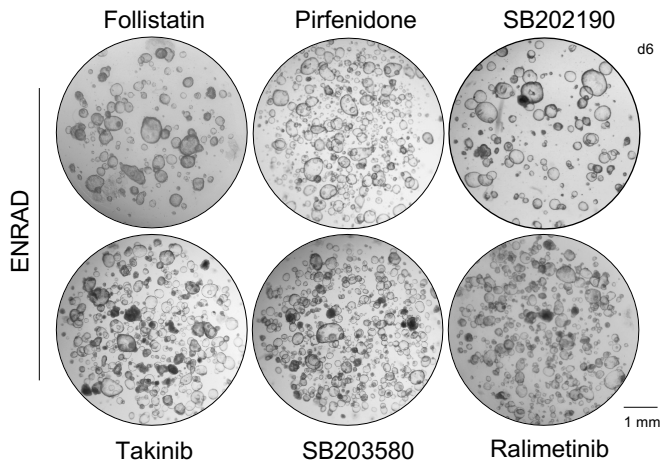


Figure R12. Representative images showing normal organoids growth in ENRAD medium conditioned with Tak1 or p38 inhibitors.

In figure 2H blots for total Stat1 blots, total p38 as well as p-Stat2 are missing.

We thank the Reviewer for this comment. Western blots of total p38 and total Stat1 reported in Figure R13 are now included in Figure 2H and 2I of the revised manuscript. We regret to say that the antibody against phospho-Stat2 did not show any reliable signal.

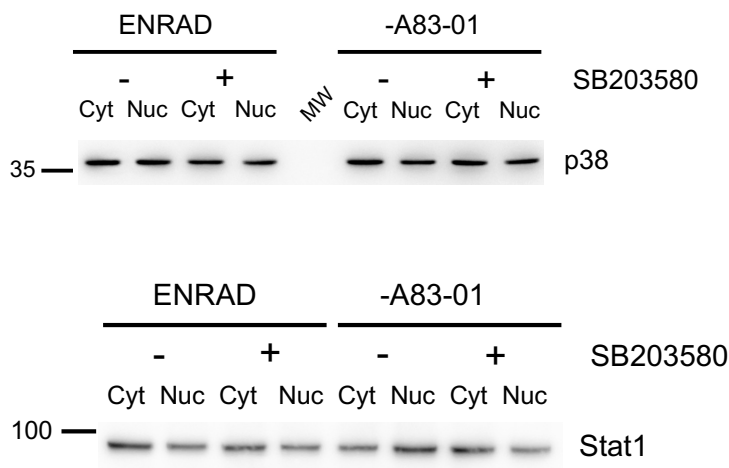


Figure R13. Western blots showing total p38 and total Stat1 proteins in both the nucleus and cytosol fractions described in Figure 2H.

In figure 2, organoids depleted of A83-01 show an enrichment of immune genes, related to cytokine-mediated signalling and TNF signalling. It would be nice to see qRT-PCR of some of these genes, to validate RNAseq-GSEA.

We thank the Reviewer for this comment. qRT-PCR analyses of IFN stimulated genes are shown in Appendix Figure S5.

In the enrichment scores plots, the distribution of the genes within the geneset (black bars) do not seem to be specific of one or other experimental group (not such enrichment), but the NES are relatively high and FDR are very low. Can authors comment on that, in first instance, incongruity?

We thank the Reviewer for the comment. The GO term "Cytokine-mediated signaling pathway" includes >400 genes, therefore the distribution density of the black lines in the NES visual plot is difficult to appreciate, regardless of the highly significant statistics. To better value the strength of the GSEA analysis also in the NES visual plot we have now substituted the first panel of the figure with the related significant GO term "Inflammatory response" (Figure R14), whose graphical trend better reflects the statistical significance of the enrichment. We also corrected the name and statistics reported in the second NES plot of Figure 2J, GO term "Response to Tumor Necrosis Factor", which was erroneously reporting labels and values related to a different significant GO term "Tumor Necrosis Factor Mediated Signaling Pathway".

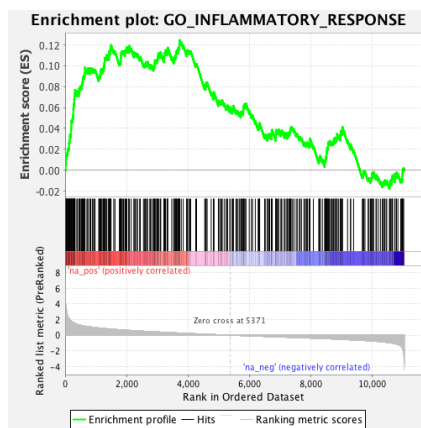


Figure R14. GSEA GO-term Inflammatory Response enriched in A83-01 depleted organoids.

From data on figure 2 authors state that they demonstrated that intra-epithelial non-canonical Activin A signalling induce a cytostatic response in mouse prostate organoids but the data shown is associative not demonstrative because they have not silenced or delete specifically Activin A.

We agree with our Reviewer that silencing/deletion of *Inhba* gene would have strengthened the conclusion that Activin A plays a major role in promoting cell quiescence in mouse prostate progenitors. We consider the administration of exogenous Activin A an acceptable strategy to demonstrate the role of Activin A in the induction of quiescence of mouse prostate progenitors. However, to further strengthen this important aspect, we substituted A83-01 with the Activin A neutralizing antibody MAB3381 from R&D, which is widely used in literature to inhibit Activin A signalling in different context. As shown below (Figure R15), MAB3381 rescues organoids formation in the absence of A83-01 thus confirming the direct role of Activin A in the safeguarding cellular quiescence of mouse prostate progenitors. These data are now described in Figure 2D of the revised manuscript.

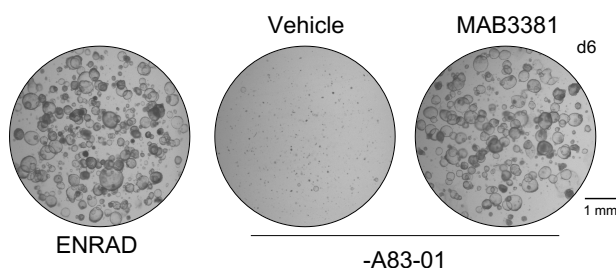


Figure R15. Representative stereoscopic images of mouse prostate organoids cultured in the absence of A83-01 with or without Activin A neutralizing antibody MAB3381 (1 μ g/mL, 6 days).

In the orthotopic injection, how authors differentiate between host and organoid-derived epithelial prostate cells? It would be nice to have an H&E picture in which the reader could see the delimitation of host and organoid-derived tissue.

We agree with our Reviewer that an H&E image of organoid-derived graft within the mouse prostate gland will help the readers appreciate the results of the orthotopic transplantation of the adapted organoids. The H&E picture in Figure R16 represents an anterior lobe of the mouse prostate bearing an organoid-derived tissue. The image is now described in the revised manuscript as Appendix Figure S7.

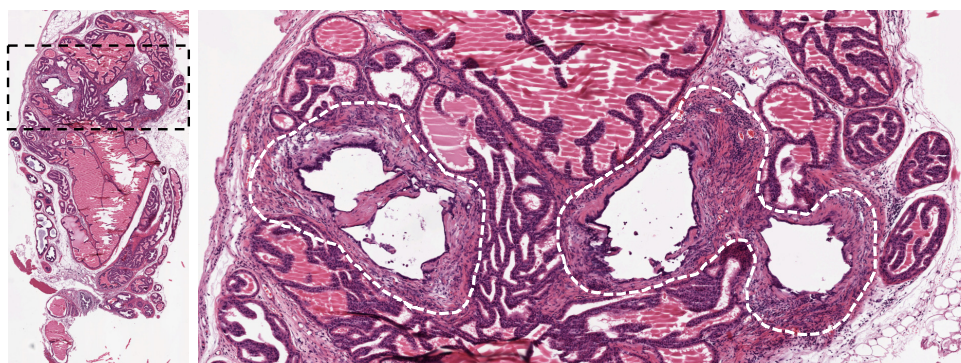


Figure R16. Representative H&E image of grafts (white dashed line) of adapted organoids into the anterior lobe of the prostate of a wild type adult mouse 6 months after transplantation.

Referee #3:

In this study Cambuli and colleagues performed mouse prostate organoids to model epithelial progenitors. They found that intra-epithelial non-canonical Activin A signalling inhibited cell proliferation in a Smad-independent manner, and could trigger the activity of Tak1 and p38 MAPK, leading to p16 and p21 nuclear import. The results presented in this article are interesting. However, the study was not performed comprehensively, leaving much to be answered. Some of the findings are not convincing enough.

Major points:

1. The authors performed mouse prostate organoids as the key experiment tool to conduct the entire research, however, the organoids culture is under the pressure of in vitro culture systems, the evidence on whether organoids can mimic stem cells in vivo is insufficient,

whether the structure of organoids enriched for epithelial progenitor cells can also be clearly observed in vivo. Both basal and luminal progenitor cells have been identified with distinct self-renewal capacity in vivo, however the authors using epithelial progenitors to define the survived cells in the prostate organoids. They need clarify the identity of the "epithelial progenitors", basal progenitors or luminal progenitors.

We thank the Reviewer for this comment. Organoids contain progenitors that need not be strictly luminal or basal, but could have features of both (bipotent), similar to PrU cells in the wild-type mouse prostate. However, the detailed characterization of such progenitors in organoids is beyond the scope of the current work.

2. Fig 1B. The authors needs to clarify why the trend of organoid forming efficiency at passage 1-3 is violently fluctuating in different cell lines? Does this come from the unstable culture condition or intrinsic prostate cell difference of different lobes?

We agree with the Reviewer that organoid forming efficiency at the very early passage can fluctuate significantly regardless the lobe of the prostate. This is a mere technical issue dependent by the fact that organoid culture derived from the peri-urethral/proximal part of the lobe that is very small in size and difficult to extract in its entirety.

3. They claimed the continuous expansion of "epithelial progenitors" in a near-physiological manner. However, both the prostate basal and luminal progenitors are quiescence. They should clearly define the context to precisely describe the morphological similarity.

We regret that this sentence has been misinterpreted. The ‘*near-physiological manner*’ refers to the fact that the sustained proliferation of these progenitor cells *in vitro*, that are normally quiescent in the adult prostate, surprisingly is not accompanied by genomic instability, implying that the mitotic process appears to occur normally.

4. Fig EV1. Androgen is critical for prostate luminal cell survival and lumen formation, and the prostate lumen were clearly reduced after androgen deprivation. They claimed that mouse prostate organoids were reversibly dependent on androgen for lumen formation, but not for their survival. They need to stain the cell apoptosis markers, and count the cell numbers during this treatment to show the evidence of their conclusion.

We agree with our Reviewer that androgen is a pro-survival signal in adult prostate besides its crucial function in the establishment of the luminal compartment of prostate epithelium. We are sorry if the sentence ‘*but not for their survival*’ may appear inaccurate. As expected, western blot analysis on organoid lysates shows clear signs of apoptosis (cleavage of Caspase 3 and Parp) in organoids cultured without DHT. These data have been included in Expanded Figure EV1E of the revised manuscript. Nevertheless, as described by Karthaus and colleagues (Figure R5; Karthaus et al., 2014) and by our work, organoids survive androgen withdrawal and grow indefinitely as compact clusters of cells. We replaced the sentence ‘*but not for their survival*’ with ‘*but minimally for their survival*’.

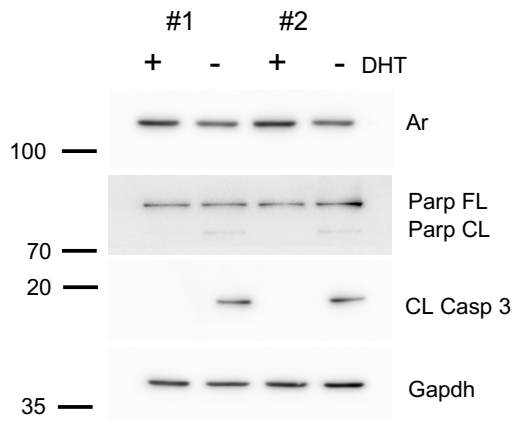


Figure R17. Western blot for apoptotic markers Cleaved Caspase 3 and Parp showing cell death response in organoid cultures depleted of DHT.

5. Fig 3. Why only the prostate organoids generated from DLP adapting to the absence of A83-01?

We thank the Reviewer for raising this interesting question. Based on our RNAseq analysis (Appendix Figure S2B), organoids derived from the dorso-lateral lobe of the mouse prostate express lower amounts of *Inhba* transcripts compared to organoids derived from the anterior and ventral lobes, thus suggesting lower ligand levels. We performed a non-reducing western blot analysis of Activin A to explore such hypothesis, but we found comparable amount of both monomeric and dimeric forms of Activin A protein in the total cell lysates of all the three lobe-specific organoid cultures (Appendix Figure S2F, and included as Figure R18 below). Interestingly, The ability of organoid cultures derived from the dorsolateral lobe of mouse prostate to grow in the absence of A83-01 was also observed by Karthaus and colleagues (PMID: 25201529). Considering that adaptation to non-canonical Activin A signalling (Fig 3A, as well as R18 below) occurs over a period of months – we cannot rule out that small differences in the signalling strength over an extended time can eventually determine the cellular fate.

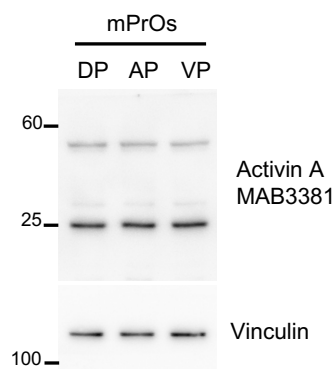


Figure R18. Non-reducing western blot showing comparable amount of monomeric (25 kDa) and dimeric (50 kDa) forms of Activin A protein in lobe-specific prostate organoids (dorsolateral prostate, DP; anterior prostate, AP; ventral prostate, VP).

They found that the ATR signalling were strongly upregulated upon A83-01 withdrawal adaptation, and indicating potential DNA replication stress. TGF- β signaling pathway has distinct function in the tumor initiation and progression. They need the fully discuss the potential correlation between TGF- β signaling and prostate cancer.

We thank the Reviewer for this comment. We have included new parts in the Discussion (lines 241-255 and 273-278) that better explain our view regarding the potential correlation between TGF-b signalling and the very early step of prostate tumorigenesis.

Will the cytokine-mediated and TNF signaling be enriched in mouse prostate organoids cultured in the absence of Activin A receptors inhibition?

We thank the Reviewer for this question. As shown in Appendix Figure S5, classical type-I IFN response genes are significantly over-expressed following acute depletion of A83-01, but they return to normal levels following adaptation.

6. Fig 4. It is challenge to distinguish the grafted cells and the host cells without any tracing markers. The authors need to describe their methods to distinguish the grafted cells and the host cells.

We agree with our Reviewer that organoid-derived graft within the mouse prostate gland may result difficult to distinguish without any tracing markers. However, because we used immune competent wild-type C57BL/6J mice as recipients and tracers such as EGFP are described as immunogenic, we preferred to avoid tracers and rely on blinded pathological studies. H&E images of all the three lobes of the prostate of mice enrolled in the study were analysed by three expert pathologists. Grafts were identified based on the presence of reactive stroma, flat cellular morphology and nuclear atypia on H&E coloured sections, elevated proliferative index via Ki67 IHC, and abnormal Krt5/Krt8 distribution by IF, on serial FFPE sections. As reported in Figure 4, grafts were identified only in the anterior lobe that was injected with adapted organoids. All other lobes were normal except for the presence of a cyst in the anterior lobe of a mouse injected with wild type organoids. As suggested by the Reviewer #2, the revised manuscript includes a representative H&E image of an anterior lobe of the mouse prostate bearing the organoid-derived graft (Appendix Figure S7). This will help the readers appreciate the results obtained with the orthotopic transplantation of adapted organoids.

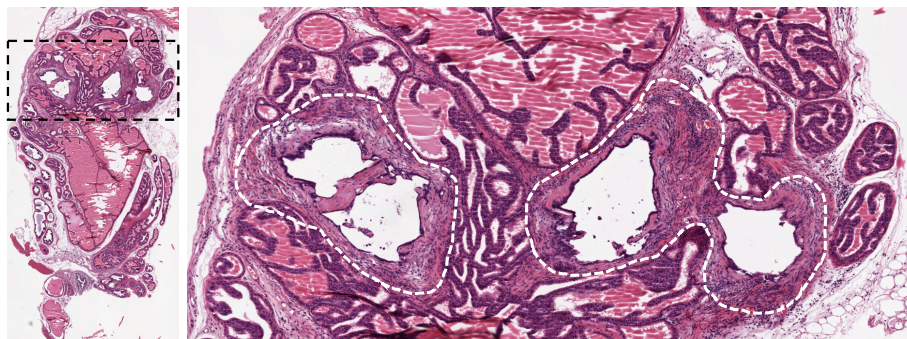


Figure R19. Representative H&E image of a graft (white dashed line) of adapted organoids into the anterior lobe of the prostate of a wild type adult mouse 6 months after transplantation.

7. The authors just used one organoid line to identify the evasion from the Tgf- β induced cytostatic response via downregulation of intra-epithelial Activin A signaling, more lines will be better.

We agree with our Reviewer that recapitulate the same results on different lines is crucial to strength the conclusion of the work. Indeed, all data presented in the manuscript regarding Activin A signalling and the process of adaptation (from Figure 2 to Figure 4, with relative Extended and Appendix Figures) have been obtained in two independent organoid lines, namely C57#1 DLP and C57#3 DLP. To further strengthen the consistency of our results in independent organoid lines, western blots describing Takinib activity in organoids depleted of A83-01 (Appendix Figure S3H) are now showed for both C57#1 and C57#3 DLP lines (Figure R20, right panel) as Appendix Figure S3H-I.

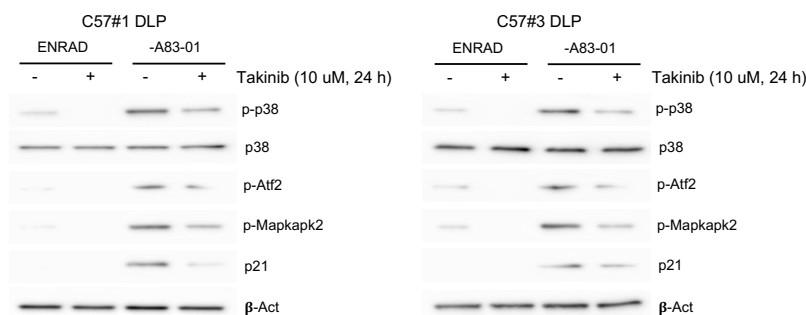


Figure R20. Western blot analysis in mouse prostate organoids (C57DP#1 and C57DP#3) for key components of the non-canonical Tgf- β pathway upon acute A83-01 withdrawal and concomitant inhibition of Tak1 (Takinib; 5 μ M, 24 hours).

8. Which source of Activin A signaling play a role in safeguarding prostate progenitor quiescence, the basal, LumP or PrU cells?

We thank the Reviewer for this interesting question. We analysed the single cell sequencing datasets of adult mouse prostate recently published by Shen's lab (Crowley L., Cambuli F., et al., eLife 2020) for *Inhba* gene expression. As showed by Figure R21, *Inhba* is expressed in a population of basal, lumP, and PrU cells of mouse adult prostate epithelium. This data is now part of the revised manuscript as Appendix Figure S2F.

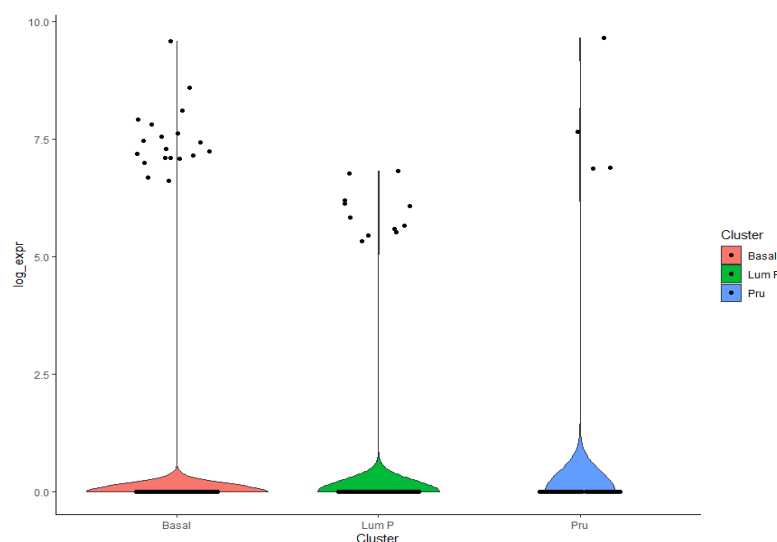


Figure R21. Violin plot showing *Inhba* gene expression in Basal, LumP and PrU epithelial cells of adult mouse prostate based on single cell RNA-seq analysis (Crowley L., Cambuli F., et al., *eLife* 2021).

9. Is it possible to inhibit Activin A signaling in vivo to observe the activity of prostate progenitor cells?

We agree with our Reviewer that it would be very interesting to inhibit Activin A *in vivo*. From a methodological point of view, the injection of neutralizing antibodies (e.g. MAB3381, R&D System) into the adult mouse prostate may represent a valuable alternative to the more classical approach of knocking-out the *Inhba* gene in the prostate epithelium. However, based on my personal experience with mouse models of prostate cancer, the timing of this experiment would far exceed the months allocated for a revision.

Dear Prof. Lunardi,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the report from one of the three referees that were asked to re-evaluate your study, you will find below. As you will see, referees #1 and #2 now fully supports the publication. Referee #3 is still not satisfied with the data shown in Fig. 4 (without tracing markers). However, after cross-commenting with the other referees, I consider this point as adequately addressed and your response as fair.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.
- Please add scale bars of similar style and thickness to the microscopic images (also in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, Figs. 3F/G and EV5C do not have scale bars.
- Please show the Western blots as unmodified as possible and resembling the source data. Presently, several blots shown are overcontrasted (e.g. b-tubulin in Fig. 2H). Please show all blots in one figure panel with same contrast and brightness, resembling closely the original data. Please consider re-scanning some of the blots.
- Please add separate (and sequential) callouts for each panel of Figs. EV1, EV2, EV4 and EV5, and Appendix Figs. S1, S4, S5 and S6 to the manuscript text.
- In the source data file for Fig. 2 data for 2H and 2I are shown (that seem swapped). Moreover, it should be 2H and 2J. In the source data file for Fig. S2 data for 2F are shown, but it should be 2E. The SD for Appendix Fig. S6A misses one box (p-Mapkapk2). Please check and make sure the SD is correctly labelled.
- Please remove the sentence 'Source data are available online for this figure' from the figure legends. For each figure the relevant SD provided will be linked by the typesetter.
- Please add links to directly access the deposited data to the data availability section. Please make sure that the links work and that the datasets are public when the paper is published.
- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy <https://www.embopress.org/competing-interests> and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the acknowledgements section.
- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

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- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (one sentence) bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The manuscript is suitable for publication in EMBO reports without revision.

Referee #2:

I would like to congratulate the authors for the revision work and thank them for answering and clarifying almost all the aspects of my questions and comments.

Referee #3:

It is still a challenge to distinguish the grafted cells and the host cells without any tracing markers (Fig 4). The authors' response is unacceptable and disappointing. EGFP/YFP/tdTomato are valuable tracing reporters and suitable in immune competent wild-type C57BL/6J mice.

The authors have addressed all minor editorial requests.

Prof. Andrea Lunardi
University of Trento, Italy
CIBIO
Via Sommarive 9 (Povo)
Trento, Trento 38123
Italy

Dear Prof. Lunardi,

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Corresponding Author Name: Andrea Lunardi

Journal Submitted to: EMBO Report

Manuscript Number: EMBOR-2021-54049-T

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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 $n \leq 5$, 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 χ^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.

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?	Briefly, all the in vitro experiments involving organoids were repeated at least 3 times. For each replica, all the experimental conditions were performed in triplicates.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Number of mice was established on the basis of published article with similar study design.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not encounter any events that required exclusion of samples or mice 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.	Organoids were seeded in 6-well plates/3 domes per well. 3 wells were randomly allocated to drug treatment, 3 wells allocated to vehicle administration. Experiments were repeated by independent investigators.
For animal studies, include a statement about randomization even if no randomization was used.	Adult wild type mice were randomly assigned to the different groups.
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.	The in vivo transplantation experiments were randomized, and the investigators were blinded to allocation during experiments and outcome assessment. Samples were coded to minimize bias (Material and Methods).
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were performed in blind.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. The statistical details are stated in the Figure legends and Materials and Methods. Differential expression analysis was performed using best practices computational pipelines and tools. Data was normalized using TMM method and edgeR tool was used to compare samples groups. EdgeR tool (PMID:19910308) uses an overdispersed Poisson model to interpret RNA-seq count data accounting for both biological and technical variability.
Is there an estimate of variation within each group of data?	Yes. Variation within each group of data is shown in the figures. EdgeR (PMID:19910308) uses an overdispersion Poisson model to account for both biological and technical variability among groups of data that are compared.

<http://www.antibodypedia.com>

<http://1degreebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-reporting>

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>

<http://ClinicalTrials.gov>

<http://www.consort-statement.org>

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<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-research>

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<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	Yes. EdgeR (PMID:19910308) accounts for data variability using a Poisson model and uses Bayes methods to moderate the degree of overdispersion across transcripts, improving the reliability of inference.
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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).	All antibodies used in this work are reported in Appendix Table S2. Description includes seller, catalog number, RRID, and working dilution.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	Wild-type C57BL/6J (JAX # 000664) mice were purchased from the Jackson Laboratory. Wild-type BALB/c (CRL # 028) and CD-1 (CRL # 022) mice were purchased from the Charles River Laboratories. Mice were housed in groups of maximum 5 animals in IVC cages with food and water ad libitum. Nesting materials and cardboard tunnels were provided as enrichment. The mice were housed in room with 21 °C temperature with 12 hours light/dark cycle with light gradually rising at 7:00 a.m. and gradually decreasing at 7:00 p.m (Material and Methods).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All housing systems were following FELASA guidelines and recommendations, concerning animal welfare, health monitoring and veterinary care, and were in compliance with the Directive 2010/63/UE and its Italian transposition D. L.vo 26/2014. Particular attention was given to prevent animal suffering, by daily check of animals. The CIBIO animal facility is fully accredited and has 7 FTE (veterinarians, technicians, and technologists) charged with the care of research animals, including mice. Mice were monitored daily for general health and well-being and sentinel mice are used for quarterly monitoring for specific pathogens. All animal experiments were performed according to the European Communities Council Directive (2010/63/EU) and approved by the Italian Ministry of Health and the University of Trento Animal Welfare Committee (642/2017-PR) (Material and Methods).
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.	Comply

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	RNA sequencing data have been deposited in the BioProject database under the accession number PRJNA659468. Proteomic data are available via ProteomeXchange with identifier PXD029660 (Material and Methods).
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).	Comply
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).	NA
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 Biocompare (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.	NA

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.	NO
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