2nd Response to Reviewers
TIP5 primes prostate luminal cells for the oncogenic transformation mediated by PTEN-loss
Pietrzak *et al*.

Editor Comments:
Although the authors have done a commendable job in addressing most of the reviewer comments, there are still some aspects that need to be addressed which are conveyed in particular by Reviewer #3.
Reviewer #1:
Comments: The authors have addressed my comments.
Reviewer #2:
Comments: I feel there is enough improvement in the article and potentially publishable. However, the authors failed to answer some of the questions raised by another reviewer.
Author. We want to thank both Reviewers 1&2 for the helpful and constructive comments that clearly improved our work.

Reviewer #3:
Comments:
This is a revised manuscript by Pietrzak et. al. describing an in vitro murine prostate cancer (PCa) organoid model. This model system is important because there are very few PCa models that recapitulate human PCa. The major weakness of the study, noted in the first submission, is the lack of a mechanism. The authors refused to address any mechanistic questions by stating that the mechanism is beyond the scope of the study. While this is reasonable, the study still has several shortcomings as described below.

Major points:
1. In my opinion, the role of TIP5 in priming prostate luminal cells for oncogenic transformation is over-stated. The title of section three of the results part states that "TIP5 is required for the initiation of PCa driven by Pten-loss." This is a big claim. While depletion of TIP5 before induction of Pten-KD altered the translucent phenotype and bilayer structure of the organoids derived from luminal cells, this data is insufficient to make the claim that TIP5 is required for the initiation of PCa driven by Pten-loss. The key experiment is to determine if TIP5 loss can partially or fully rescue the oncogenic/neoplastic properties of the PTEN mouse model.
Author. We are sorry for the confusion raised on this point. We modified the title of section three by adding organoids **"TIP5 is required for the initiation of PCa driven by *Pten*-loss in organoids**".
However, we would like to shortly summarize the main results of this section. The prostate organoid system we developed allowed the control of the order of events, in our specific case TIP5-KO and *Pten*-KD. While TIP5-KO before *Pten-KD* rescued the neoplastic phenotype and gene expression changes mediated by *Pten*-KD in organoids, TIP5-KO after *Pten-KD* does not affect these two features. We would like to clarify that it is extremely difficult to adapt this experimental strategy using PTEN mouse models because it would require a double inducible system to control the different time of TIP5-KO and Pten-KO, a model that is extremely complex to establish and to our knowledge not yet applied in prostate cancer research.

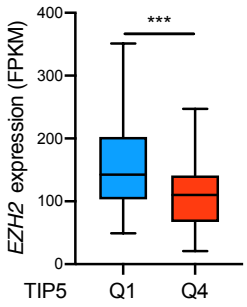
2. The authors have developed the TIP5 whole body knock-out (KO) mouse model but refuse to describe the properties in the manuscript. Some description is provided in the "Response to Reviewers", but these facts are omitted from the manuscript. It does appear that the phenotype of the TIP5 whole-body KO is complex, and the authors do not want to elaborate further. For these reasons, the authors have used the alternate in vitro approach with organoids.
Author. We thank the reviewer to have accepted our reasonable justifications to not include the description of TIP5 whole body knock-out (KO) mouse model in this manuscript. The detailed characterization of the complex TIP5 whole body KO phenotype is not yet complete and we do not feel comfortable to publish these data now and in this manuscript also because this information goes beyond the scope of this work that is focused on prostate. Furthermore, PNAS informed us that it is their policy to not accept statements such as "data not shown".

3. In the "Response to Reviewers," the authors claim that TIP5 is a repressor, but do not state this anywhere in the manuscript.

Author. We are sorry for the confusion raised on this point. The role of TIP5 in epigenetic silencing in metastatic PCa was described in the Introduction section, p.5 lanes 6-8 "TIP5 establishes epigenetic silencing at genes frequently repressed in metastatic PCa and tumors with high TIP5 expression display a CpG island methylator phenotype (CIMP)."

In their earlier publication (Lei Gu et. al. 2015), the authors stated that TIP5 interacts with EZH2 to maintain epigenetic silencing at genes repressed in metastasis. But EZH2 is not over-expressed in the normal prostate or clinically localized PCa. So, how does TIP5 function as a repressor in the absence of EZH2?

Author. We would like to clarify that in this work we did not discuss the role of EZH2 since we did not analyze EZH2 function in PCa organoids. We would also like to clarify that "*not-overexpressed*" does not mean "not-expressed". We would also like to point out that this same topic was already discussed in the previous response to point 4 of Reviewer 2, where we showed data indicating that PCa with high TIP5 expression (TIP5_Q1) display higher EZH2 levels than PCa with low TIP5 expression (TIP5_Q4) (see results below). Although this is a nice correlation that links TIP5 and EZH2 in primary PCa, Reviewer 2 accepted our reasonable request to not include these results in this manuscript. Indeed, the analysis of TIP5 and EZH2 mediated mechanisms in the initiation of PCa requires a complex experimental strategy (i.e. ChIPseq in organoids and primary PCa) that goes beyond the scope of this work. Our future work will clearly take into consideration this interesting result.



The data in Figure 1 indicates that hundreds of genes are both up-regulated and down-regulated upon Tip5 KO. So, the repressor function of Tip5 is insufficient to explain the gene-expression changes.

Author. We are sorry for the confusion raised on this point. It is very well known that RNAseq analyses alone do not allow any conclusions whether changes in gene expression are a direct or indirect effect. There are plenty of examples in the literature where KD/KO of an activator or a repressor gives rise to changes in gene expression in both directions, irrespective of the positive or negative role in transcription of the analyzed factor. Only an intersection of ChIPseq and RNAseq can define the direct role (activator or repressor) of the analyzed factor. Accordingly, we never claim in any part of the manuscript that TIP5 acts as repressor of a defined group of genes in primary PCa since this was not the aim of this work. Instead, we referred to these genes as TIP5-regulated genes. Clearly, the fact that TIP5 was described as the repressor of rRNA genes in healthy cells and tumor suppressor genes in metastatic PCa might favor a model where TIP5 also acts as repressor in prostatic luminal cells and PCa initiation (see also the results of TIP5-EZH2 correlation above), however, this was not the point of our work.

4. Much of the clinical significance has been shown as weak correlation analyses.

Author. We are very sorry but we cannot comment this point because of the lack of specific information or clear requests. However, we think that results obtained using TMA with > 4000 samples with clinical information, validation of *PTEN* gene signature obtained with our cross-species analysis using two different data set, and the Gleason analysis of TCGA samples for

PTEN and TIP5 tumors cannot be considered as weak results. All these analyses were implemented according to the requests of reviewer 1 & 2, which approved our results.

5. It does not appear that the Venn Diagrams in Figures 5 and 6 are drawn to scale. The overlap looks visually impressive, but the real overlap may be very different.

Author. We want to thank the reviewer for the suggestion to use proportional Venn diagram. Accordingly, we modified all the Venn diagrams shown in this study and added this information in the corresponding figure legends and in Material and Methods in the SI document. The visualization of these data is clearly improved and, as expected, the results remained the same since in the previous version of this manuscript the real overlap was already shown by the number of unique and overlapping genes present in each Venn diagram. Furthermore, we would like to clarify that the Venn diagrams represent different experiments with different readouts. While the amount of overlapping of genes regulated by PTEN-KD shown in Figures 5F,H,J (now 5D,F,H) is important (high in organoids of luminal origin, lower in basal organoids and no effect *Pten*-KD→*Tip5*-KO PCa organoids), the overlapping of the cross-species analysis of Fig. 6A plays no role since this image aims to show how the analysis to select the *PTEN* gene signature (validated with two different data sets) was performed.

Minor points:

1. The panels in Figure 5 are mis-labeled. Figure 5C and Figure 5E are missing.

Author. We thank the Reviewer to have spotted this mistake. We corrected the Figure, accordingly.

Statistical or Methodological Comments:

It does not appear that the Venn Diagrams in Figures 5 and 6 are drawn to scale. The overlap looks visually impressive, but the real overlap may be very different.

Author. We modified all the Venn diagrams.