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YAP/BRD4-controlled ROR1 promotes tumor-initiating cells and hyperproliferation in pancreatic cancer.

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript the authors analyzed the role of ROR1 in pancreatic cancer progression and metastasis. They found that ROR1 expression is specifically increased in an partial EMT cell cluster upon scRNA-Seq of tumor cells derived from an orthotopic mouse PDAC model. Moreover, the ROR1 high population in tumors specifies cells with high proliferation and tumor initiation capacities, increased metastatic propensity and chemoresistance, since knockdown of ROR1 shows reduction of these features in vivo. By comparing transcriptomes from several in vivo models the authors identified that ROR1 acts through AURKB and that its expression is regulated by an upstream enhancer that is bound by YAP/TAZ and BRD4 complexes. With this study the authors identified a new targetable pathway that promotes tumor progression and metastasis in PDAC. The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic. However, some of the findings are a bit preliminary and the drawn conclusions are not sufficiently supported by the experimental data. Moreover, some findings seem a bit out of context and do not really help to bring the story forward. At other instances experimental details are missing to mechanistically demonstrate the role of ROR1. In particular it remains elusive how ROR1 is regulated, i.e. which signaling events are crucial to generate ROR1 high vs. low cells. I listed my specific comments below.

1. The authors' initial finding is that in the partial EMT cluster ROR1, but also other RTKs (out of 56) are specifically increased. What about the other RTKs? Why was ROR1 chosen to analyze more thoroughly?
2. The finding of AURKB as crucial target of ROR1 is very weak and needs more in-depth analyses. It is not clear why AURKB was chosen over the other candidates. Is AURKB expression directly regulated by ROR1? Are the two genes directly linked? Can ROR1 deficiency be compensated by AURKB overexpression? Especially the decrease in AURKB protein level in Fig. 4K is not very convincing to account for the different phenotypes in ROR1 high and low cells. Is AURKB and ROR1 expression correlated in TCGA samples (like Fig. 8B)? In Fig. 4L the readout was changed from colony numbers to colony diameter. If AURKB is the crucial player downstream of ROR1, then colony formation efficiency should be affected at first. This needs to be shown. The statement in lines 223,224 that AURKB is a direct downstream target of

ROR1 was not shown!
3. Fig. 4 A-E: The ROR1 KD was induced in vitro but not continued in vitro. The transient KD has a strong impact on tumor forming capacity, even though recovery of expression is likely within the first days in vivo. This is very interesting and underscores the role of ROR1 in tumor initiation and presumably independent of differences in proliferation. Would the results be different, if the DOX treatment would start with injection of the cells and continued in vivo? Is then tumor initiation not affected and maybe only tumor growth?
4. In Fig. 5 the authors show that ROR1 is highly expressed in tumors after gemcitabine treatment and conclude that the ROR1 high cells are a resistant population. However, this statement is too strong, since gemcitabine treatment could also lead to an upregulation of ROR1 in "low" cells during acquisition of chemoresistence. Together with our knowledge on the role of EMT in driving therapy resistance and therapy-mediated induction of EMT, such a scenario is equally likely. Similarly, the statement in lines 370-372 is not supported by experimental evidence.
5. In order to understand how ROR1 is regulated, the authors use ATAC-Seq and cut and run and identified a putative upstream enhancer element (Fig. 7). Although this element increases the activity of the promoter fragment in a reporter construct, the experiments do not help to understand how ROR1 activity is increased specifically in the "high" cells. Are peaks of YAP1 and BRD4 also changed between hi/lo cells? Is YAP OE and KD (BRD4 OE and KD) or the use of the inhibotor JQ1 altering the activity of the reporter constructs (i.e. only of the enhancer-promoter combination but not of the promoter only construct)? This would help to strengthen a direct link between ROR1, YAP and BRD4. Is YAP activity different in ROR1 high vs. low cells?
6. In Fig. 8A the authors identified 202 antigens that match the H3 monomethylation/acetylation pattern. How was YAP etc. chosen?

Minor:

1. Fig. 2D,E: What is actually shown here? Is there an overlap between the genes that define ROR1 high vs. low cells in both approaches? The gene list should be provided.
2. Fig. 3G: I suggest to include the images of the tumors from the ROR1 low cells in the main figure as well

2. Significance:

Significance (Required)

PDAC is a very aggressive desease with very low 5-year survival rates.
Understanding of the pathobiology is of keen interest. The findings of the authors are

of high significance and extremely relevant as they provide a mechanism that can also be targeted by specific drug combinations, i.e. standard care gemcitabine with specific ROR1 inhibition.
The findings are of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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No

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this work Yamazaki and colleagues performed single cell RNA sequencing of one xenograft tumor formed by the S2-VP10 PDAC cell line to explore PDAC intratumor heterogeneity. Using this model they identified ROR1 as heterogeneously expressed in neoplastic cells. Using further in vivo and in vitro models they show that ROR1high cells have higher tumor initiation capacity than ROR1low. By histone and

ATAC-seq analyses, they identify a ROR1 enhancer upstream the promoter and show that YAP and BRD4 bind to this genomic region and that BRD4 inhibition by JQ1 reduces ROR1 expression and organoid formation.
The data, figures and methods are nicely and clearly presented.

Major comments

1. The authors use one xenograft tumor as starting model and all conclusions are derived from the data generated with this model. To support the existence of identifie heterogeneity in the PDAC neoplastic compartment, I would strongly suggest to validate the existence of the partial EMT population and the ROR1 heterogeneity in single cell data bases generated from primary human tumors.
2. In Fig. 3G, it is mentioned that tumors grown from ROR1high cells recapitulate the original PDOx histology thus suggesting that ROR1high cells in the tumor are the actual TICs. ROR1low cells could also grow tumors, just with lower incidence. Are these tumors any different to the ROR1high derived ones? Is it just a lower tumor initiation capacity (TIC) or they can not recapitulate the tumor as the ROR1high cell? Can they also give rise to differentiated progeny cells? This should appear in the main text and not only in the discussion. I would suggest to move panel 3G to supplementary figure.
3. In line 160 you mention that known CSC markers such as CD44, PROM1 and DCLK1 are not differentially expressed between ROR1 high and low populations. Then, in figure 3H,I you analyze the expression of CD44v6 together with ROR1. I would try to put this information together in the text, or at least in fig. 3 start with something like "we had seen that both ROR1high and low express CD44, however...". In any case, I feel that the experiment with CD44 could be obviated (or at least moved to supplementary), as it brings the question of weather this is also true for DCLK1 or CD133.
4. JQ1 has been described to inhibit PDAC growth by downregulation of MYC. To unequivocally link the effect of JQ1 in the downregulation of ROR1 (Fig. 8M) as discussed in the text it would be important to exclude that other mechanisms such as MYC downregulation are taking place. For example, does JQ1 treatment of ROR1low cells also reduce their colony formation capacity (in an experiment such as the one in fig. 3C). Or does ROR1 re-expression in Fig. 8M rescue the JQ1 effect? These or other experiments could help to establish a stronger link between (BRD4/JQ1) and ROR1.

Minor comments

1. The data are nicely presented (text and figures) and the conclusions are clear. My

suggestion to make the story more "catchy" at the beginning would be, if possible, to start from the observation done in primary human data and then move to the PDX model to explore ROR1 as a TIC marker in PDAC. For this, you could use available public single cell data of human PDAC tumors. If this doesn't work (it is of course possible that by unsupervised analysis you don't get the same clusters as in the PDX with the partial EMT cluster popping up), it would be nice if some primary tumor data came early in the story (currently the first figure showing heterogeneity in primary samples is in supplem fig. 4A).
2. It is not clear if the xenografts were subcutaneous or orthotopic. It would be good to include this information in the main text (line 102) and the methods so that the reader knows what is the exact model that has been used.
3. In Fig. 2F and 2G I would highlight the EMT pathway to help the reader.
4. In Supp Fig 4B it would be nice to have an amplified view of the staining as in panel C of the same figure.
5. In the same figure (Fig. 4A-D) ROR1 shows an apical staining pattern that doesn't seem to resemble the staining in patient samples. I am not an expert in pathology evaluation but I would recommend a pathologist to give her/his opinion. Possibly, during the PDX process, few cells from the original patient tumor are selected giving a different staining pattern.
6. In the analyses of TCGA data, be aware that only 150 from the original dataset are actual PDAC tumors. The dataset contains otherwise data from cell lines, PDX, normal tissue, etc that should be removed for a proper analysis (see DOI: 10.3390/cancers11010126)
7. Does ROR1 correlate with RFS? This would nicely fit with the concept of TIC and metastasis.
8. Line 219: ROR1 is not "depleted" in the lines as it is a downregulation model. "ROR1-downregulated" would be more correct.
9. It would be good to have a supplem figure showing that siROR1 cells show reduction organoid formation, to validate that the siRNA model functionally recapitulates the ROR1low vs high phenotype.
10. Some of the supplemental figures are only referred in the discussion although they appear earlier than other in the main text. This is a bit confusing when going through the figures.

Referees cross-commenting

I agree with the importance of addressing points 2 (link to AURKB), 4 (selection vs acquisition), 5 (mechanism in high vs low cells) raised by Reviewer 1, and the comments from Reviewer 3. I think that the study of other RTKs (point 1 from Reviewer 1) is not the focus of the story. It would be nice if the authors can comment on why they chose ROR1 but the fact that are other differentially expressed genes

does not exclude the validity of the current story. I fell that the in vivo sustained KD experiment (point 3 from Reviewer 1) although interesting, it is not mandatory for a revision of this manuscript in case the adaptation of the animal protocol represents a long process. The experiment provided already in the current version is the best approach to address the role of ROR1 at the early initiation phase.

2. Significance:

Significance (Required)

This is a neat and interesting work with potential implications for the clinical field of pancreatic cancer as the authors identified a new subpopulation with enhanced tumor initiating cell capacity. However, the use of JQ1 for pancreatic cancer has been previously discussed mainly linked to MYC inhibition, but also to stromal reprogramming or DNA damage induction. I missed some discussion in this regard in the discussion section. What is adding the work to the field of JQ1 treatment in PDAC? IN a way, how do the authors foresee that the discovery of ROR1high cells and the regulation of ROR1 by BRD4 and YAP will be beneficial when considering JQ1 in the clinics? Maybe by stratifying patients? Or by following ROR1 upregulation upon initial chemotherapy? These questions are just suggestions. In general, some discussion to put the work into the context of previous works using JQ1 in PDAC would be nice.

I believe that this work would be interesting not only to the pancreatic cancer community but also to a more general public working on cancer and/or stemmness as it touches several interesting points in that regard that can be applicable to other systems.

My own work is focused on pancreatic cancer, patient heterogeneity and stromal interactions. I am not an expert on histone or ATACseq analyses.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

Yamazaki et al investigate partial EMT in pancreatic cancer and provide data that ROR1 marks pancreatic tumor cells that are capable of initiating tumors. The authors exploit scRNAseq of pancreatic tumor xenografts to identify a cluster of cells showing a partial EMT phenotype. The found 7 RTKs expressed more highly in this partial EMT cluster and focus their attention on ROR1, an 'orphan' receptor that has been implicated in WNT signaling and EMT previously. Validation experiments using ROR1-high vs low cells support that ROR1 expression correlates with EMT, poor outcome in human PDA patients, tumor forming and colony forming capacity. They also show that ROR1 high cells form tumors that recapitulate parental tumor histology. The authors show that ROR1 expression is associated with EF2 transcription factor activity, elevated expression of multiple targets including AURKB. Pharmacologic inhibition of AURKB reduces colony formation and genetic loss of ROR1 combined with chemotherapy (gemcitabine) has potent anti-tumor activity in vivo. The authors show that ROR1 expression is elevated in metastatic lesions and identify a novel enhancer element that putatively drives ROR1 expression in tumor cells. They provide evidence that this element is engaged by YAP/BRD4 and show that BRD4 inhibition reduces tumor cell colony formation. The manuscript is a solid combination of techniques with adequate controls and statistics.

Major Comments:

The overall conclusion that ROR1 expression marks a subset of pancreatic cancer cells that have the ability to initiate tumors is supported by the data provided. The correlative data are strong and the demonstration that loss of ROR1 reduces colony formation, reduces metastatic lesions and enhances the efficacy of chemotherapy are compelling. Additionally, the demonstration that ROR1 expression is elevated in metastatic lesions is consistent with many other drivers/markers of EMT in pancreatic cancer.

The conclusion that ROR1 expression is driven by YAP/BRD4 is interesting and provides important mechanistic depth to the study. However, this conclusion could be strengthened by use of a suitable rescue experiment. For instance does overexpression of ROR1 rescue the effect of BRD4 inhibition or loss of YAP?

A challenge with the data presented in Figure 1, the scRNA-seq data that lead them to ROR1, is that it is not stated how many tumors are used to generate the scRNA-seq data and the overall number of tumor cells analyzed is relatively low (993). The authors should provide the number of tumors used for the initial scRNA-seq. A general concern with any scRNA-seq data is batch effect, this is mitigated to a degree by the follow on studies that provide functional validation of ROR1 in multiple cell lines.

The data and methods are provided in an adequate manner. Reproduction of the experiments is likely. The authors use multiple cell lines and tools that are generally available.

The authors note a limitation of the study is that only human tumor xenografts were exploited.

Minor comments:

Figure 1E and text page 9. The text identifies MERB3 as a gene that marks the partial EMT cluster, I believe this is a type and the gene should actually be MSRB3.

Please provide the dose of gemcitabine in the legend for figure 5

Referees cross-commenting

I think the comments from Referee #2 are pretty reasonable - have no additions

2. Significance:

Significance (Required)

Intratumor heterogeneity is a major challenge for the treatment of many cancers, including pancreatic cancer. The data provided support that ROR1 marks a subset of cancer cells in pancreatic tumors that have the capacity to drive intratumor heterogeneity. If supported these data have the potential to drive significant impact. Identification of a marker and a targetable pathway that supports tumor initiation in pancreatic cancer has the potential to nominate companion therapies that enhance the efficacy of standard of care approaches. Further, identification of a pathway that drives partial EMT in pancreatic cancer provides a substantial increase in baseline knowledge of intratumor heterogeneity.

These data would be broadly interesting to scientists interested in the tumor microenvironment, metastasis, therapy resistance and tumor progression. In addition, oncologists focused on drug development and combinatorial therapy will find this manuscript of interest.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Revision Plan

Manuscript number: RC-2022-01581
Corresponding author: Masaya Yamazaki

1. General Statements

We are submitting a revision plan and the preliminary revision of our manuscript entitled "ROR1 plays a critical role in pancreatic tumor-initiating cells with a partial EMT signature". We greatly appreciate the helpful comments and suggestions from the reviewers. Below, we include the point-by-point responses with the changes we are planning or have implemented to address the points. All the additions and changes in the manuscript are made in red. We trust that the revision plan will meet with the approval of the editor and reviewers. We would also be glad to respond to any further questions and comments that you may have.

2. Description of the planned revisions

Three main points:

(1) The importance of AURKB as a downstream effector of ROR1
[Reviewer #1: major #2]

Based on these suggestions, we plan to perform a colony formation assay using AURKB-overexpressing cells with ROR1-knockdown. We will clarify this point in the revised manuscript.

(2) The link between *ROR1* expression and YAP/BRD4
[Reviewer #1: major #5 and Reviewer #3: major #1]

Based on the suggestion, we plan to perform the luciferase reporter assay. We will clearly describe this experiment in the revised manuscript.

(3) Single-cell analysis using other models to validate tumor heterogeneity
[Reviewer #2: major #1 and Reviewer #3: major #2]

Based on your suggestion, we plan to analyze primary human tumors (public data: for example, GSE155698, CRA001160) and examine PDO#1 xenografts (in-house data). We will clearly state this information in the revised manuscript.

For the two minor points suggested by Reviewer #2, we plan to

(1) reanalyze TCGA data.

(2) perform the organoid or colony formation assay to validate that the siRNA model functionally recapitulates the ROR1^{low} vs. ROR1^{high} phenotype.

Please see the "Authors' responses to the reviewers' comments" for more details.

3. Description of the revisions that have already been incorporated in the transferred manuscript

As suggested by the reviewer, we have substantially revised our manuscript, and the changes are shown in red.

- Reviewer #1: major comments #2, #3, #4, and #5; minor comments #1 and #2
- Reviewer #2: major comments #2, #3, and #4; minor comments #2, #3, #4, #8, and #10
- Reviewer #3: minor comments #1 and #2

Please see the “Authors’ responses to the reviewers' comments” for more details.

4. Description of analyses that authors prefer not to carry out

Revision Plan

Authors' responses to the reviewers' comments

Reviewer #1

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript the authors analyzed the role of ROR1 in pancreatic cancer progression and metastasis. They found that ROR1 expression is specifically increased in an partial EMT cell cluster upon scRNA-Seq of tumor cells derived from an orthotopic mouse PDAC model. Moreover, the ROR1 high population in tumors specifies cells with high proliferation and tumor initiation capacities, increased metastatic propensity and chemoresistance, since knockdown of ROR1 shows reduction of these features in vivo. By comparing transcriptomes from several in vivo models the authors identified that ROR1 acts through AURKB and that its expression is regulated by an upstream enhancer that is bound by YAP/TAZ and BRD4 complexes. With this study the authors identified a new targetable pathway that promotes tumor progression and metastasis in PDAC. The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic. However, some of the findings are a bit preliminary and the drawn conclusions are not sufficiently supported by the experimental data. Moreover, some findings seem a bit out of context and do not really help to bring the story forward. At other instances experimental details are missing to mechanistically demonstrate the role of ROR1. In particular it remains elusive how ROR1 is regulated, i.e. which signaling events are crucial to generate ROR1 high vs. low cells. I listed my specific comments below.

[Response]
We thank the reviewer for stating that "The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic" and we appreciate the reviewer's constructive suggestions. We have substantially revised our manuscript and plan to perform new experiments based on these valuable comments.

1. The authors' initial finding is that in the partial EMT cluster ROR1, but also other RTKs (out of 56) are specifically increased. What about the other RTKs? Why was ROR1 chosen to analyze more thoroughly?

[Response 1]
We are thankful for the reviewer's suggestion to clarify why ROR1 was selected. (1) Seven candidate genes (*EPHA4, EPHA7, ERBB4*, *FGFR1*, *JAK3*, *LYN*, and *ROR1*) were chosen as surface markers in the partial EMT cluster. (2) The genes were sorted in order of high expression. (3) ROR1 is reported to promote metastasis in breast cancer (Cui et al, 2013). The induction of metastasis is one of the functions of tumor-initiating cells. FGFR1 is already known to enhance the CSC-like phenotype in non-small cell lung cancer (Ji et al, 2016). (4) The antibody against ROR1 was marketed as available for cell sorting using FACS. Therefore, we focused on ROR1 as a potential new marker for tumor-initiating cells with a partial EMT signature.

References
Cui B, Zhang S, Chen L, Yu J, Widhopf GF 2nd, Fecteau JF, Rassenti LZ, Kipps TJ. Targeting ROR1 inhibits epithelial-mesenchymal transition and metastasis. Cancer Res. 2013 Jun 15;73(12):3649-60. doi: 10.1158/0008-5472.CAN-12-3832. PMID: 23771907; PMCID: PMC3832210.
Ji W, Yu Y, Li Z, Wang G, Li F, Xia W, Lu S. FGFR1 promotes the stem cell-like phenotype of FGFR1-amplified non-small cell lung cancer cells through the Hedgehog pathway. Oncotarget. 2016 Mar 22;7(12):15118-34. doi: 10.18632/oncotarget.7701. PMID: 26936993; PMCID: PMC4924774.

2. The finding of AURKB as crucial target of ROR1 is very weak and needs more in-depth analyses. It is not clear why AURKB was chosen over the other candidates. Is AURKB expression directly regulated by ROR1? Are the two genes directly linked? Can ROR1 deficiency be compensated by AURKB overexpression? Especially the decrease in AURKB protein level in Fig. 4K is not very convincing to account for the different phenotypes in ROR1 high and low cells. Is AURKB and ROR1 expression correlated in TCGA samples (like Fig. 8B)? In Fig. 4L the readout was changed from colony numbers to colony diameter. If AURKB is the crucial player downstream of ROR1, then colony formation efficiency should be affected at first. This needs to be shown. The statement in lines 223,224 that AURKB is a direct downstream target of ROR1 was not shown!

[Response 2-1: changed]
We thank the reviewer for noting this issue. We have performed additional experiments to assess the hypothesis that AURKB is a crucial downstream target of ROR1. *ROR1*-knockdown not only suppressed AKT phosphorylation (Supplemental Figure 9A) but also decreased c-Myc protein levels and the expression of c-Myc target genes (*CDK4*, *CCND1*, *CDK2*, and *CCNE1*), leading to a reduction in RB phosphorylation (new Supplemental Figure 9B and 9C). Based on these results, ROR1 regulates c-Myc expression through AKT signaling, leading to the activation of the E2F network (new Supplemental Figure 9D). We added some figures and descriptions to the preliminary revision manuscript (new Supplemental Figure 9B–9D, lines 357–363, lines 649–651).

[Response 2-2: the planned revisions]
We also plan to perform new experiments with a colony formation assay to determine whether ROR1 deficiency is compensated by AURKB overexpression. We agree that this experiment will confirm that AURKB is an important downstream target of ROR1 in PDAC proliferation.

[Response 2-3]
In TCGA-PAAD dataset, *AURKB* expression was not correlated with *ROR1* expression. Since the ROR1^{high} cluster is a minor population in the tumor, a downstream analysis of specific clusters with results from a bulk study such as this TCGA dataset is difficult to perform.

[Response 2-4: changed]
We have added a new graph of organoid formation efficiency (new Figure 4L) and changed some descriptions in the preliminary revision manuscript (line 227).

3. Fig. 4 A-E: The ROR1 KD was induced in vitro but not continued in vitro. The transient KD has a strong impact on tumor forming capacity, even though recovery of expression is likely within the first days in vivo. This is very interesting and underscores the role of ROR1 in tumor initiation and presumably independent of differences in proliferation. Would the results be different, if the DOX treatment would start with injection of the

cells and continued in vivo? Is then tumor initiation not affected and maybe only tumor growth?

[Response 3: changed]
We apologize for the confusing description in the original manuscript. In Fig. 4A–E, we used PDAC cells with stable expression of doxycycline-inducible shROR1. *ROR1*-knockdown was maintained in vivo by adding doxycycline to the drinking water. Continuous *ROR1*-knockdown suppressed tumor growth (Fig. 4C–E). Several statements we made were more ambiguous than intended, and we have adjusted the text and the figures for clarity in the preliminary revision manuscript (new Figure 4A and B, lines 203–204).

4. In Fig. 5 the authors show that ROR1 is highly expressed in tumors after gemcitabine treatment and conclude that the ROR1 high cells are a resistant population. However, this statement is too strong, since gemcitabine treatment could also lead to an upregulation of ROR1 in "low" cells during acquisition of chemoresistence. Together with our knowledge on the role of EMT in driving therapy resistance and therapy-mediated induction of EMT, such a scenario is equally likely. Similarly, the statement in lines 370-372 is not supported by experimental evidence.

[Response 4: changed]
We appreciate the reviewer's critical comments. As suggested, we have not clearly determined whether (1) the ROR1^{high} cells survived gemcitabine treatment and/or (2) the ROR1^{low} cells increased ROR1 expression upon exposure to this treatment. We have carefully changed some descriptions in the preliminary revision manuscript (lines 241–242, 382–383).

5. In order to understand how ROR1 is regulated, the authors use ATAC-Seq and cut and run and identified a putative upstream enhancer element (Fig. 7). Although this element increases the activity of the promoter fragment in a reporter construct, the experiments do not help to understand how ROR1 activity is increased specifically in the "high" cells. Are peaks of YAP1 and BRD4 also changed between hi/lo cells? Is YAP OE and KD (BRD4 OE and KD) or the use of the inhibotor JQ1 altering the activity of the reporter constructs (i.e. only of the enhancer-promoter combination but not of the promoter only construct)? This would help to strengthen a direct link between ROR1, YAP and BRD4. Is YAP activity different in ROR1 high vs. low cells?

[Response 5-1: changed]
We thank the reviewer for this important comment. We have shown differences in chromatin accessibility and histone modification of the ROR1 enhancer between ROR1^{high} and ROR1^{low} cells using ATAC-seq and CUT&RUN assays (Fig. 7B). Very few ROR1$^{high/low}$ cells are present in xenograft. We were not successful in experiments examining the binding of YAP and BRD4 to enhancers in ROR1$^{high/low}$ cells because of the technical limitations in the ChIP and CUT&RUN assays. Instead, we used public data to examine YAP and BRD4 occupancy at the ROR1 enhancer region of cell lines with low ROR1 expression. In T-47D and MCF7 cells (breast cancer cells, low ROR1 expression), YAP and BRD4 did not bind to the ROR1 enhancer region (new Figure 8D and 8I). We have added figures and some descriptions to the preliminary revision manuscript (new Figure 8D and 8I, lines 304–309, line 768).

[Response 5-2: the planned revisions]

We plan to perform new experiments with the reporter assay you suggested. We agree that this experiment will help strengthen the direct link between ROR1, YAP and BRD4.

[Response 5-3]
As shown in Figure 8C, GSEA revealed that ROR1^{high} cells in both S2-VP10 xenografts and PDO#1 xenografts expressed higher levels of YAP-regulated genes than ROR1^{low} cells in these xenografts. We have added a description of this result as follows: “Thus, ROR1^{high} cells have higher YAP activity than ROR1^{low} cells.” (lines 304–305).

6. In Fig. 8A the authors identified 202 antigens that match the H3 monomethylation / acetylation pattern. How was YAP etc. chosen?

[Response 6]
We apologize for the poor description in the original manuscript. We chose YAP and BRD4 based on the following criteria: (1) these antigens are expressed in S2-VP10 cells and PDO#1 and (2) bind to the ROR1 enhancer region (based on an analysis of public data).

Minor:
1. Fig. 2D,E: What is actually shown here? Is there an overlap between the genes that define ROR1 high vs. low cells in both approaches? The gene list should be provided.

[Response: changed]
We apologize for the poor description in the original manuscript. We have added this information to the preliminary revision manuscript (new Supplemental Table 3).

2. Fig. 3G: I suggest to include the images of the tumors from the ROR1 low cells in the main figure as well.

[Response: changed]
We appreciate the reviewer’s suggestion. We have moved this information from the supplementary information to the main figure in the preliminary revision manuscript (new Figure 3G, lines 186–189).

Reviewer #1 (Significance (Required)):

PDAC is a very aggressive desease with very low 5-year survival rates. Understanding of the pathobiology is of keen interest. The findings of the authors are of high significance and extremely relevant as they provide a mechanism that can also be targeted by specific drug combinations, i.e. standard care gemcitabine with specific ROR1 inhibition.
The findings are of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic.

[Response]
We greatly appreciate the reviewer’s comments.

Revision Plan

Reviewer #2

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this work Yamazaki and colleagues performed single cell RNA sequencing of one xenograft tumor formed by the S2-VP10 PDAC cell line to explore PDAC intratumor heterogeneity. Using this model they identified ROR1 as heterogeneously expressed in neoplastic cells. Using further in vivo and in vitro models they show that ROR1high cells have higher tumor initiation capacity than ROR1low. By histone and ATAC-seq analyses, they identify a ROR1 enhancer upstream the promoter and show that YAP and BRD4 bind to this genomic region and that BRD4 inhibition by JQ1 reduces ROR1 expression and organoid formation.
The data, figures and methods are nicely and clearly presented.

[Response]
We thank the reviewer for stating that "The data, figures and methods are nicely and clearly presented", and we appreciate the reviewer's constructive suggestions. We have substantially revised our manuscript and plan to perform new experiments based on these valuable comments.

Major comments

1. The authors use one xenograft tumor as starting model and all conclusions are derived from the data generated with this model. To support the existence of identifie heterogeneity in the PDAC neoplastic compartment, I would strongly suggest to validate the existence of the partial EMT population and the ROR1 heterogeneity in single cell data bases generated from primary human tumors.

[Response 1: the planned revisions]
We thank the reviewer for the positive suggestion. We plan to perform a new analysis of available public single-cell data from human PDAC tumors. In addition, we also launched a single-cell analysis of PDO#1 xenografts.

2. In Fig. 3G, it is mentioned that tumors grown from ROR1high cells recapitulate the original PDOx histology thus suggesting that ROR1high cells in the tumor are the actual TICs. ROR1low cells could also grow tumors, just with lower incidence. Are these tumors any different to the ROR1high derived ones? Is it just a lower tumor initiation capacity (TIC) or they can not recapitulate the tumor as the ROR1high cell? Can they also give rise to differentiated progeny cells? This should appear in the main text and not only in the discussion. I would suggest to move panel 3G to supplementary figure.

[Response 2: changed]
We thank the reviewer for noting this issue and apologize for the confusing description in the original manuscript. ROR1^{low} cells generated tumors at a low frequency, and these tumors showed a hierarchical histology mimicking the original tumor. As suggested, we have added this information to the main text (new Figure 3G, lines 186–189).

3. In line 160 you mention that known CSC markers such as CD44, PROM1 and DCLK1 are not differentially expressed between ROR1 high and low populations. Then, in figure 3H,I you analyze the expression of CD44v6 together with ROR1. I would try to put this information together in the text, or at least in fig. 3 start with something like "we had seen that both ROR1high and low express CD44, however...". In any case, I feel that the experiment with CD44 could be obviated (or at least moved to supplementary), as it brings the question of weather this is also true for DCLK1 or CD133.

[Response 3: changed]
We appreciate and agree with the reviewer's comment on this point. Accordingly, we have moved this figure to the supplementary information and changed the description (new Supplemental Figure 5C and 5D, lines 191–196).

4. JQ1 has been described to inhibit PDAC growth by downregulation of MYC. To unequivocally link the effect of JQ1 in the downregulation of ROR1 (Fig. 8M) as discussed in the text it would be important to exclude that other mechanisms such as MYC downregulation are taking place. For example, does JQ1 treatment of ROR1low cells also reduce their colony formation capacity (in an experiment such as the one in fig. 3C). Or does ROR1 re-expression in Fig. 8M rescue the JQ1 effect? These or other experiments could help to establish a stronger link between (BRD4/JQ1) and ROR1.

[Response 4: changed]
We thank the reviewer for this important comment. As mentioned in the response to Reviewer #1-major comment #2, we newly found that ROR1 regulates c-Myc expression through AKT signaling, leading to the activation of the E2F network (new Supplemental Figures 9B–9D, lines 357–363).

Minor comments

1. The data are nicely presented (text and figures) and the conclusions are clear. My suggestion to make the story more "catchy" at the beginning would be, if possible, to start from the observation done in primary human data and then move to the PDX model to explore ROR1 as a TIC marker in PDAC. For this, you could use available public single cell data of human PDAC tumors. If this doesn't work (it is of course possible that by unsupervised analysis you don't get the same clusters as in the PDX with the partial EMT cluster popping up), it would be nice if some primary tumor data came early in the story (currently the first figure showing heterogeneity in primary samples is in supplem fig. 4A).

[Response: the planned revisions]
We thank the reviewer for these excellent comments. As suggested, we plan to perform several new analyses (please see the previous comment for details: Reviewer #2-major comment #1).

2. It is not clear if the xenografts were subcutaneous or orthotopic. It would be good to include this information in the main text (line 102) and the methods so that the reader knows what is the exact model that has been used.

[Response: changed]

We thank the reviewer for this comment and apologize for the poor description in the original manuscript. As suggested, we have added this information to the preliminary revision manuscript (line 101).

3. In Fig. 2F and 2G I would highlight the EMT pathway to help the reader.

[Response: changed]

We thank the reviewer for this comment. As suggested, we have changed the relevant figures in the preliminary revision manuscript (new Figure 2F and 2G).

4. In Supp Fig 4B it would be nice to have an amplified view of the staining as in panel C of the same figure.

[Response: changed]

We thank the reviewer for this comment. As suggested, we have added high-magnification images of the staining in the preliminary revision manuscript (new Supplemental Figure 4A and 4B).

5. In the same figure (Fig. 4A-D) ROR1 shows an apical staining pattern that doesn't seem to resemble the staining in patient samples. I am not an expert in pathology evaluation but I would recommend a pathologist to give her/his opinion. Possibly, during the PDX process, few cells from the original patient tumor are selected giving a different staining pattern.

[Response]

We appreciate the reviewer's comment on this point. Dr. Ito, a coauthor of this paper, is a pathologist. We have changed some images of staining in patient samples (new Supplemental Figure 4A). We agree that ROR1 shows an apical staining pattern in PDX samples. However, some sites show similar apical staining patterns in patient samples (Patient #2 and Patient #4 in the new Supplemental Figure 4A). We propose that PDX mimics the original patient tissue because it has heterogeneity of ROR1 expression and morphological features indicative of a luminal structure.

6. In the analyses of TCGA data, be aware that only 150 from the original dataset are actual PDAC tumors. The dataset contains otherwise data from cell lines, PDX, normal tissue, etc that should be removed for a proper analysis (see DOI: 10.3390/cancers11010126)

[Response: the planned revisions]

We thank the reviewer for the careful review of this issue. We are currently reconsidering with the pathologist whether the samples are appropriate based on TCGA data (diagnosis and pathology sections) and the paper you presented. The current data (Figures 3A, 4J, and 8B) were analyzed for samples excluding cell lines, PDX, and normal tissue in the TCGA-PAAD dataset.

7. Does ROR1 correlate with RFS? This would nicely fit with the concept of TIC and metastasis.

[Response]
We thank the reviewer for noting this issue. Unfortunately, no correlation was observed between ROR1 expression and RFS.

8. Line 219: ROR1 is not "depleted" in the lines as it is a downregulation model. "ROR1-downregulated" would be more correct.

[Response: changed]
We thank the reviewer for this suggestion and agree with your comment. We have corrected this term accordingly in the preliminary revision manuscript (line 223).

9. It would be good to have a supplem figure showing that siROR1 cells show reduction organoid formation, to validate that the siRNA model functionally recapitulates the ROR1low vs high phenotype.

[Response: the planned revisions]
We thank the reviewer for this suggestion. We plan to perform a colony formation assay.

10. Some of the supplemental figures are only referred in the discussion although they appear earlier than other in the main text. This is a bit confusing when going through the figures.

[Response]
We apologize for the poor description in the original manuscript. We have adjusted the order of the supplemental figures in the preliminary revision manuscript.

CROSS-CONSULTATION COMMENTS
I agree with the importance of addressing points 2 (link to AURKB), 4 (selection vs acquisition), 5 (mechanism in high vs low cells) raised by Reviewer 1, and the comments from Reviewer 3. I think that the study of other RTKs (point 1 from Reviewer 1) is not the focus of the story. It would be nice if the authors can comment on why they chose ROR1 but the fact that are other differentially expressed genes does not exclude the validity of the current story. I fell that the in vivo sustained KD experiment (point 3 from Reviewer 1) although interesting, it is not mandatory for a revision of this manuscript in case the adaptation of the animal protocol represents a long process. The experiment provided already in the current version is the best approach to address the role of ROR1 at the early initiation phase.

[Response]
We thank the reviewer for these positive comments. As suggested, we have substantially revised our manuscript.

Reviewer #2 (Significance (Required)):

Significance:
This is a neat and interesting work with potential implications for the clinical field of pancreatic cancer as the authors identified a new subpopulation with enhanced tumor initiating cell capacity. However, the use of JQ1 for pancreatic cancer has been previously discussed mainly linked to MYC inhibition, but also to stromal reprogramming or DNA damage induction. I missed some discussion in this regard in the discussion section. What is adding the work to the field of JQ1 treatment in PDAC? IN a way, how do the authors foresee that the discovery of ROR1high cells and the regulation of ROR1 by BRD4 and YAP will be beneficial when considering JQ1 in the clinics? Maybe by stratifying patients? Or by following ROR1 upregulation upon initial chemotherapy? These questions are just suggestions. In general, some discussion to put the work into the context of previous works using JQ1 in PDAC would be nice.

[Response: changed]
We thank the reviewer for this comment. As you suggested, we have added a description of the proposed use of JQ1 and BRD4 inhibitors in ROR1^{high} PDAC treatment to the Discussion section (lines 412–416).

I believe that this work would be interesting not only to the pancreatic cancer community but also to a more general public working on cancer and/or stemmness as it touches several interesting points in that regard that can be applicable to other systems.
My own work is focused on pancreatic cancer, patient heterogeneity and stromal interactions. I am not an expert on histone or ATACseq analyses.

[Response]
We greatly appreciate the reviewer's comments.

Reviewer #3

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary
Yamazaki et al investigate partial EMT in pancreatic cancer and provide data that ROR1 marks pancreatic tumor cells that are capable of initiating tumors. The authors exploit scRNAseq of pancreatic tumor xenografts to identify a cluster of cells showing a partial EMT phenotype. The found 7 RTKs expressed more highly in this partial EMT cluster and focus their attention on ROR1, an 'orphan' receptor that has been implicated in WNT signaling and EMT previously. Validation experiments using ROR1-high vs low cells support that ROR1 expression correlates with EMT, poor outcome in human PDA patients, tumor forming and colony forming capacity. They also show that ROR1 high cells form tumors that recapitulate parental tumor histology. The authors show that ROR1 expression is associated with EF2 transcription factor activity, elevated expression of multiple targets including AURKB. Pharmacologic inhibition of AURKB reduces colony formation and genetic loss of ROR1 combined with chemotherapy

(gemcitabine) has potent anti-tumor activity in vivo. The authors show that ROR1 expression is elevated in metastatic lesions and identify a novel enhancer element that putatively drives ROR1 expression in tumor cells. They provide evidence that this element is engaged by YAP/BRD4 and show that BRD4 inhibition reduces tumor cell colony formation. The manuscript is a solid combination of techniques with adequate controls and statistics.

[Response]
We thank the reviewer for stating that "The manuscript is a solid combination of techniques with adequate controls and statistics", and we appreciate the reviewer's constructive suggestions. We have substantially revised our manuscript and plan to perform new experiments based on these valuable comments.

Major Comments:
The overall conclusion that ROR1 expression marks a subset of pancreatic cancer cells that have the ability to initiate tumors is supported by the data provided. The correlative data are strong and the demonstration that loss of ROR1 reduces colony formation, reduces metastatic lesions and enhances the efficacy of chemotherapy are compelling. Additionally, the demonstration that ROR1 expression is elevated in metastatic lesions is consistent with many other drivers/markers of EMT in pancreatic cancer.

The conclusion that ROR1 expression is driven by YAP/BRD4 is interesting and provides important mechanistic depth to the study. However, this conclusion could be strengthened by use of a suitable rescue experiment. For instance does overexpression of ROR1 rescue the effect of BRD4 inhibition or loss of YAP?

[Response 1: the planned revisions]
We thank the reviewer for this comment. We completely agree with the reviewer's suggestion. However, the suggested examination to determine whether overexpression of ROR1 rescues the effect of BRD4 inhibition or loss of YAP may not be suitable because BRD4 and YAP act as transcriptional coregulators of various target genes. Instead, as mentioned in response to Reviewer #1-major comments 5-2, we plan to perform new experiments using a reporter assay.

A challenge with the data presented in Figure 1, the scRNA-seq data that lead them to ROR1, is that it is not stated how many tumors are used to generate the scRNA-seq data and the overall number of tumor cells analyzed is relatively low (993). The authors should provide the number of tumors used for the initial scRNA-seq. A general concern with any scRNA-seq data is batch effect, this is mitigated to a degree by the follow on studies that provide functional validation of ROR1 in multiple cell lines.

[Response 2: changed and the planned revisions]
We appreciate the reviewer's comments. As suggested, we have added this information to the preliminary revision manuscript (line 104). In addition, as mentioned in response to Reviewer #2 major comment #1, we plan to perform a new single-cell analysis of PDO xenografts (in-house data) and human PDAC tumors (available public data).

The data and methods are provided in an adequate manner. Reproduction of the experiments is likely. The authors use multiple cell lines and tools that are generally available.
The authors note a limitation of the study is that only human tumor xenografts were exploited.

[Response]
We thank the reviewer for the positive comment.

Minor comments:
Figure 1E and text page 9. The text identifies MERB3 as a gene that marks the partial EMT cluster, I believe this is a type and the gene should actually be MSRB3.

[Response: changed]
We apologize for the typo. We have corrected this error accordingly (line 114).

Please provide the dose of gemcitabine in the legend for figure 5

[Response: changed]
We apologize for the poor description in the original manuscript. We have added this information.

CROSS-CONSULTATION COMMENTS
I think the comments from Referee #2 are pretty reasonable - have no additions

Reviewer #3 (Significance (Required)):

Intratumor heterogeneity is a major challenge for the treatment of many cancers, including pancreatic cancer. The data provided support that ROR1 marks a subset of cancer cells in pancreatic tumors that have the capacity to drive intratumor heterogeneity. If supported these data have the potential to drive significant impact. Identification of a marker and a targetable pathway that supports tumor initiation in pancreatic cancer has the potential to nominate companion therapies that enhance the efficacy of standard of care approaches. Further, identification of a pathway that drives partial EMT in pancreatic cancer provides a substantial increase in baseline knowledge of intratumor heterogeneity.

These data would be broadly interesting to scientists interested in the tumor microenvironment, metastasis, therapy resistance and tumor progression. In addition, oncologists focused on drug development and combinatorial therapy will find this manuscript of interest.

[Response]
We greatly appreciate the reviewer's comments.

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

Dear Dr Yamazaki,

Thank you for submitting your work for consideration by the EMBO Journal and transferring your manuscript from Review Commons, now listed as EMBOJ-2022-112614. My apologies for getting back to you with unusual protraction due to the high current load of submissions to the journal.

We have now carefully assessed your manuscript together with the referee reports and your point-by-point response to their concerns. I am happy to say that we find the results to be of interest for the EMBO Journal, and thus are positive to have a revised study re-evaluated by the referees.

Given the referees' positive recommendations and based on your detailed response, I would thus like to invite you to submit a revised version of the manuscript, addressing the issues raised. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Please note specifically, that in line with the referees' comments we consider the revision of mechanistic aspects detailing ROR1's function on partial EMT acquisition via AURKB and MYC-E2F as critical for consideration by the EMBO Journal. We realize that you address these points in your rebuttal response; however given the substantial revision required and the open outcome of these experiments we cannot predict the conclusion after re-evaluation and suggest keeping EMBO Reports in mind as an alternative venue for this work.

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

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

Please feel free to approach me any time should you have additional questions related to this.

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response 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.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is

also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

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 .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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 labelled 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.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Further information is available in our Guide to Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (22nd Dec 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

***
Rev_Com_number: RC-2022-01581
New_manu_number: EMBOJ-2022-112614
Corr_author: Yamazaki
Title: ROR1 plays a critical role in pancreatic tumor-initiating cells with a partial EMT signature.

Manuscript number: EMBOJ-2022-112614 (RC-2022-01581)
Corresponding author: Masaya Yamazaki

1. General Statements

We are submitting a revision of our manuscript entitled “ROR1 plays a critical role in pancreatic tumor-initiating cells with a partial EMT signature”. We greatly appreciate the helpful comments and suggestions from the reviewers. Below, we include the point-by-point responses with the changes we have implemented to address the points. All the additions and changes in the manuscript are shown in red. We trust that the revised manuscript will meet with the approval of the editor and reviewers. We would also be glad to respond to any further questions and comments that you may have.

2. Point-by-point description of the revisions

Report of Reviewer #1

(Evidence, reproducibility and clarity (Required)):

In this manuscript the authors analyzed the role of ROR1 in pancreatic cancer progression and metastasis. They found that ROR1 expression is specifically increased in an partial EMT cell cluster upon scRNA-Seq of tumor cells derived from an orthotopic mouse PDAC model. Moreover, the ROR1 high population in tumors specifies cells with high proliferation and tumor initiation capacities, increased metastatic propensity and chemoresistance, since knockdown of ROR1 shows reduction of these features in vivo. By comparing transcriptomes from several in vivo models the authors identified that ROR1 acts through AURKB and that its expression is regulated by an upstream enhancer that is bound by YAP/TAZ and BRD4 complexes. With this study the authors identified a new targetable pathway that promotes tumor progression and metastasis in PDAC. The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic. However, some of the findings are a bit preliminary and the drawn conclusions are not sufficiently supported by the experimental data. Moreover, some findings seem a bit out of context and do not really help to bring the story forward. At other instances experimental details are missing to mechanistically demonstrate the role of ROR1. In particular it remains elusive how ROR1 is regulated, i.e. which signaling events are crucial to generate ROR1 high vs. low cells. I listed my specific comments below.

1. The authors' initial finding is that in the partial EMT cluster ROR1, but also other RTKs (out of 56) are specifically increased. What about the other RTKs? Why was ROR1 chosen to analyze more thoroughly?

2. The finding of AURKB as crucial target of ROR1 is very weak and needs more in-depth

analyses. It is not clear why AURKB was chosen over the other candidates. Is AURKB expression directly regulated by ROR1? Are the two genes directly linked? Can ROR1 deficiency be compensated by AURKB overexpression? Especially the decrease in AURKB protein level in Fig. 4K is not very convincing to account for the different phenotypes in ROR1 high and low cells. Is AURKB and ROR1 expression correlated in TCGA samples (like Fig. 8B)? In Fig. 4L the readout was changed from colony numbers to colony diameter. If AURKB is the crucial player downstream of ROR1, then colony formation efficiency should be affected at first. This needs to be shown. The statement in lines 223,224 that AURKB is a direct downstream target of ROR1 was not shown!

3. Fig. 4 A-E: The ROR1 KD was induced in vitro but not continued in vitro. The transient KD has a strong impact on tumor forming capacity, even though recovery of expression is likely within the first days in vivo. This is very interesting and underscores the role of ROR1 in tumor initiation and presumably independent of differences in proliferation. Would the results be different, if the DOX treatment would start with injection of the cells and continued in vivo? Is then tumor initiation not affected and maybe only tumor growth?

4. In Fig. 5 the authors show that ROR1 is highly expressed in tumors after gemcitabine treatment and conclude that the ROR1 high cells are a resistant population. However, this statement is too strong, since gemcitabine treatment could also lead to an upregulation of ROR1 in "low" cells during acquisition of chemoresistence. Together with our knowledge on the role of EMT in driving therapy resistance and therapy-mediated induction of EMT, such a scenario is equally likely. Similarly, the statement in lines 370-372 is not supported by experimental evidence.

5. In order to understand how ROR1 is regulated, the authors use ATAC-Seq and cut and run and identified a putative upstream enhancer element (Fig. 7). Although this element increases the activity of the promoter fragment in a reporter construct, the experiments do not help to understand how ROR1 activity is increased specifically in the "high" cells. Are peaks of YAP1 and BRD4 also changed between hi/lo cells? Is YAP OE and KD (BRD4 OE and KD) or the use of the inhibotor JQ1 altering the activity of the reporter constructs (i.e. only of the enhancer-promoter combination but not of the promoter only construct)? This would help to strengthen a direct link between ROR1, YAP and BRD4. Is YAP activity different in ROR1 high vs. low cells?

6. In Fig. 8A the authors identified 202 antigens that match the H3 monomethylation/acetylation pattern. How was YAP etc. chosen?

Minor:

1. Fig. 2D,E: What is actually shown here? Is there an overlap between the genes that define ROR1 high vs. low cells in both approaches? The gene list should be provided.

2. Fig. 3G: I suggest to include the images of the tumors from the ROR1 low cells in the main

figure as well

Reviewer #1 (Significance (Required)):

PDAC is a very aggressive desease with very low 5-year survival rates. Understanding of the pathobiology is of keen interest. The findings of the authors are of high significance and extremely relevant as they provide a mechanism that can also be targeted by specific drug combinations, i.e. standard care gemcitabine with specific ROR1 inhibition.

The findings are of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic.

Response to Reviewer #1

(Evidence, reproducibility and clarity (Required)):

In this manuscript the authors analyzed the role of ROR1 in pancreatic cancer progression and metastasis. They found that ROR1 expression is specifically increased in an partial EMT cell cluster upon scRNA-Seq of tumor cells derived from an orthotopic mouse PDAC model. Moreover, the ROR1 high population in tumors specifies cells with high proliferation and tumor initiation capacities, increased metastatic propensity and chemoresistance, since knockdown of ROR1 shows reduction of these features in vivo. By comparing transcriptomes from several in vivo models the authors identified that ROR1 acts through AURKB and that its expression is regulated by an upstream enhancer that is bound by YAP/TAZ and BRD4 complexes. With this study the authors identified a new targetable pathway that promotes tumor progression and metastasis in PDAC. The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic. However, some of the findings are a bit preliminary and the drawn conclusions are not sufficiently supported by the experimental data. Moreover, some findings seem a bit out of context and do not really help to bring the story forward. At other instances experimental details are missing to mechanistically demonstrate the role of ROR1. In particular it remains elusive how ROR1 is regulated, i.e. which signaling events are crucial to generate ROR1 high vs. low cells. I listed my specific comments below.

We thank the reviewer for stating that "The detailed analysis uses many state of the art techniques to address the role of ROR1 and is of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic" and we appreciate the reviewer's constructive suggestions. We have substantially revised our manuscript based on these valuable comments.

#1: The authors' initial finding is that in the partial EMT cluster ROR1, but also other RTKs (out of 56) are specifically increased. What about the other RTKs? Why was ROR1 chosen to analyze more thoroughly?

#1: We are thankful for the reviewer's suggestion to clarify why ROR1 was selected. We described this information in the original manuscript, but we have carefully changed some descriptions in the revised manuscript (new lines 151–160) as follows: "From 56 RTK genes, seven candidate genes were selected with the most enriched expression in the partial EMT cluster compared to other clusters: *EPHA4*, *EPHA7*, *ERBB4*, *FGFR1*, *JAK3*, *LYN*, and *ROR1* (Fig 2A, Fig EV3A and B, and Table EV1). ROR1 is reported as an oncofetal antigen and is widely expressed in multiple

human cancers (Zhang *et al*, 2012b). In addition, high expression of *ROR1* is associated with shorter metastasis-free survival in breast cancer (Cui *et al*, 2013). In addition, we found that *ROR1* is highly expressed in the partial EMT subpopulation of patient PDAC as well as S2-VP10 xenografts (Fig 2B and C). Therefore, we focused on ROR1 as a marker for isolating partial EMT cells."

#2-1: The finding of AURKB as crucial target of ROR1 is very weak and needs more in-depth analyses. It is not clear why AURKB was chosen over the other candidates.

#2-1: We apologize for our confusing explanation. As noted in the original manuscript, AURKB was chosen from four candidates (original Fig 4H / new Fig 4G) because of its functional importance in mitotic chromosome condensation and its overexpression in several cancer tissues. This information has been described in the revised manuscript (new lines 229–235) as follows: "We identified four E2F target genes (*AURKB*, *CDCA8*, *CDK1*, and *E2F8*) commonly upregulated in the three ROR1-enriched datasets (Fig 4G and H). Aurora kinase B (AURKB) plays an important role in mitotic chromosome condensation (Lens *et al*, 2010) and has attracted considerable interest as a potential therapeutic target because of its overexpression in several cancer tissues, such as non-small cell lung carcinoma, glioblastoma, and ovarian cancer (Vischioni *et al*, 2006; Zeng *et al*, 2007; Chen *et al*, 2009)."

#2-2: Is AURKB expression directly regulated by ROR1? Are the two genes directly linked?

#2-2: We thank the reviewer for pointing this out. As noted in the original manuscript, ROR1^{high} cells derived from xenografts had higher expression of *AURKB* than ROR1^{low} cells (original Fig 4H and I / new Fig 4G and H). ROR1-KD also reduced AURKB expression at the protein level original Fig 4K / new Fig 4I). These results indicate that ROR1 controls AURKB expression (original lines 210–220 / new lines 229–236). Moreover, we have performed additional experiments to determine how ROR1 regulates AURKB expression. *ROR1*-knockdown not only suppressed AKT phosphorylation but also decreased c-Myc protein levels and the expression of c-Myc target genes (*CDK4*, *CCND1*, *CDK2*, and *CCNE1*) (new Fig 4L–M). These phenomena led to a reduction in RB phosphorylation (new Fig 4M). These results indicate that ROR1 regulates c-Myc expression through AKT signaling, leading to activation of the E2F network and upregulation of AURKB expression (new Fig 4O). Taken together, these data support the conclusion that AURKB is the downstream target of ROR1. We have added some information to the revised manuscript (new lines 246–255) as follows: "We then sought to link ROR1 activity to E2F-mediated AURKB expression. Previous reports have shown that ROR1 induces AKT

phosphorylation in breast and non-small cell lung cancers (Zhang *et al*, 2012a; Yamaguchi *et al*, 2012). Indeed, we observed a reduction in phospho-AKT levels in ROR1-depleted PDAC cells (Fig 4L). Consistent with the reduced AKT signaling, c-Myc protein levels and the expression of c-Myc target genes, such as *CDK4*, *CCND1*, *CDK2*, and *CCNE1*, were downregulated by *ROR1*-KD (Fig 4M and N). In addition, RB phosphorylation levels were also reduced by *ROR1*-KD (Fig N), suggesting that RB-mediated inhibition of E2F had been promoted. Taken together, these results indicate that the ROR1 enhances AKT/c-Myc signaling, which in turn promotes the E2F-mediated expression of AURKB (Fig 4O).

”

#2-3: Can ROR1 deficiency be compensated by AURKB overexpression? Especially the decrease in AURKB protein level in Fig. 4K is not very convincing to account for the different phenotypes in ROR1 high and low cells.

#2-3: We thank the reviewer for pointing out this issue. In accordance with the reviewer’s comment, we have performed two new experiments using the colony formation assay to determine whether ROR1 deficiency is compensated by AURKB overexpression. (1) In the xenograft model, organoid formation activity was rescued in sorted ROR1^{low} cells by stably expressing AURKB compared to cells expressing of EGFP (control) (new Fig 4K and Fig EV6A and B). (2) In S2-VP10 ishROR1 cells, organoid formation activity was also increased in *ROR1*-KD by stable expression of AURKB (new Fig EV6A–E). These results strengthen the conclusion that AURKB is a critical downstream effector of ROR1. We have added some information to the revised manuscript (new lines 240–242) as follows: “To clarify the importance of AURKB as a downstream effector of ROR1, we examined it in two assays. Stable AURKB expression increased the organoid formation ability of (1) ROR1^{low} cells derived from S2-VP10 AURKB-EGFP xenografts (Fig 4K and Fig EV6A and B) and (2) Dox-inducible *ROR1*-KD cells (Fig EV6A–E).”

#2-4: Is AURKB and ROR1 expression correlated in TCGA samples (like Fig. 8B)?

#2-4: We thank the reviewer for pointing out this issue. To address this point, we analyzed the TCGA-PAAD dataset (accessed the GDC data portal [https://portal.gdc.cancer.gov/], retrieved on March 22, 2021). In the TCGA-PAAD dataset, *AURKB* expression was not positively correlated with *ROR1* expression (Fig 1 for reviewer #1). Since the ROR1^{high} cluster is a minor population in the tumor, we think it would be difficult to perform a downstream analysis of specific clusters using the results of the bulk study.

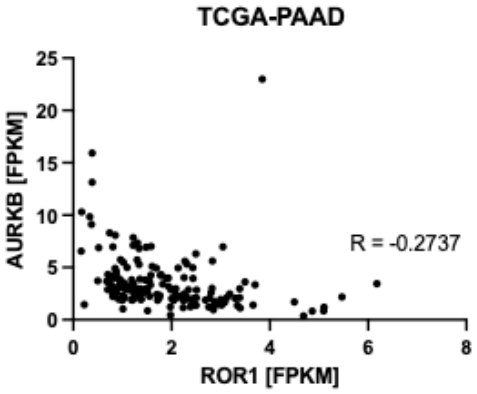

Fig 1 for reviewer #1

#2-5: In Fig. 4L the readout was changed from colony numbers to colony diameter. If AURKB is the crucial player downstream of ROR1, then colony formation efficiency should be affected at first. This needs to be shown.

#2-5: We thank the reviewer for this comment. As suggested, we have added a new graph of organoid formation efficiency (new Fig 4J). Treatment with Aurora kinase inhibitor such as tozesertib and barasertib reduced organoid formation efficiency. We have also added this information to the revised manuscript (new lines 238–240) as follows: “Both tozasertib (a pan-Aurora kinase inhibitor) and barasertib (an Aurora kinase B selective inhibitor) markedly suppressed the formation and growth of organoids (Fig 4J).”

#2-6: The statement in lines 223,224 that AURKB is a direct downstream target of ROR1 was not shown!

#2-6: We apologize for our poor explanation in the original manuscript. As shown in the response to Reviewer #1 [major #2-2], we have demonstrated that (1) ROR1^{high} cells have higher expression of ROR1 than ROR1^{low} cells in xenografts (original Fig 4I / new Fig 4H), (2) ROR1-KD downregulates AURKB at the protein level (original Fig 4K / new Fig 4I), and (3) ROR1 controls AURKB expression through the AKT/c-MYC/E2F axis (new Fig 4L–N). These results clearly indicate that AURKB is a downstream target of ROR1 (new Fig 4O). Consequently, we believe that the manuscript has been greatly improved by these modifications, and we sincerely hope that you approve of our work.

#3. Fig. 4 A-E: The ROR1 KD was induced in vitro but not continued in vitro. The transient KD has a strong impact on tumor forming capacity, even though recovery of expression is likely within

the first days in vivo. This is very interesting and underscores the role of ROR1 in tumor initiation and presumably independent of differences in proliferation. Would the results be different, if the DOX treatment would start with injection of the cells and continued in vivo? Is then tumor initiation not affected and maybe only tumor growth?

#3: We apologize for the confusing description in the original manuscript. In Fig 4A–E, we used PDAC cells with stable expression of doxycycline-inducible shROR1. *ROR1*-KD was maintained in vivo by adding doxycycline to the drinking water. Continuous *ROR1*-KD suppressed tumor growth (original and new Fig 4C–E). Several statements we made were more ambiguous than intended, and we have adjusted the text for clarity in the revised manuscript (new lines 217–218) as follows: "*ROR1-knockdown was maintained in vivo by adding doxycycline to the drinking water (Fig 4B).*"

#4. In Fig. 5 the authors show that ROR1 is highly expressed in tumors after gemcitabine treatment and conclude that the ROR1 high cells are a resistant population. However, this statement is too strong, since gemcitabine treatment could also lead to an upregulation of ROR1 in "low" cells during acquisition of chemoresistence. Together with our knowledge on the role of EMT in driving therapy resistance and therapy-mediated induction of EMT, such a scenario is equally likely. Similarly, the statement in lines 370-372 is not supported by experimental evidence.

#4: We appreciate the reviewer's critical comments and agree. As suggested, we have not clearly determined whether (1) the ROR1^{high} cells survived gemcitabine treatment and/or (2) the ROR1^{low} cells upregulated ROR1 expression upon exposure to this treatment. We have carefully changed some descriptions in the revised manuscript (new lines 268 and 402–403) as follows: "These results suggest that ROR1 supports relapse after chemotherapy.", "The expansion of ROR1high tumor-initiating cells after chemotherapy might be related to efficient tumor growth during relapse."

#5-1. In order to understand how ROR1 is regulated, the authors use ATAC-Seq and cut and run and identified a putative upstream enhancer element (Fig. 7). Although this element increases the activity of the promoter fragment in a reporter construct, the experiments do not help to understand how ROR1 activity is increased specifically in the "high" cells. Are peaks of YAP1 and BRD4 also changed between hi/lo cells?

#5-1: We thank the reviewer for this important comment. As mentioned in the original manuscript, we have shown differences in chromatin accessibility and histone modification of the ROR1 enhancer between ROR1^{high} and ROR1^{low} cells using ATAC-seq and CUT&RUN assays (original

and new Fig 7B). Only a small number of ROR1^{high} and ROR1^{low} cells can be obtained from xenografts. Unfortunately, due to the technical limitations of the ChIP and CUT&RUN assays, we were unsuccessful in experiments examining YAP and BRD4 binding to the ROR1 enhancer in ROR1^{high} and ROR1^{low} cells. Instead, we examined the occupancy of the ROR1 enhancer region by YAP and BRD4 in cell lines with high and low ROR1 expression using public data. In cell lines with high ROR1 expression (MDA-MB-231, PC-9, NCI-H2052), YAP and BRD4 bind to the ROR1 enhancer region, but this is not observed in low ROR1 expression cell lines (MCF7 and T-47D) (new Fig 8D and K). These data support our suggestion that ROR1 expression is dependent on YAP/BRD4 binding to the *ROR1* enhancer region. We have added some information to the revised manuscript (new lines 330–335 and 348–351) as follows: "Analysis of publicly available ChIP-seq datasets revealed that YAP binds to the enhancer region of ROR1 in cancer cell lines with high *ROR1* expression (MDA-MB-231, PC-9, NCI-H2052, and SF268 cells) (Fig 8D and Appendix Fig S4). In contrast, in cancer cell lines with low *ROR1* expression (MCF-7 and T-47D cells), YAP did not bind to its enhancer regions (Fig 8D and Appendix Fig S4)", "Analysis of the ChIP-seq datasets revealed the binding of BRD4 to the *ROR1* enhancer in only ROR1-expressing cancer cell lines (MDA-MB-231 and PC-9 cells) (Fig 8K and Appendix Fig S4)."

#5-2: Is YAP OE and KD (BRD4 OE and KD) or the use of the inhibotor JQ1 altering the activity of the reporter constructs (i.e. only of the enhancer-promoter combination but not of the promoter only construct)? This would help to strengthen a direct link between ROR1, YAP and BRD4.

#5-2: We agree that this experiment will help strengthen the direct link between ROR1, YAP and BRD4. We have performed new experiments using the reporter assay you suggested. YAP/TAZ co-knockdown and treatment with the YAP inhibitor verteporfin reduced ROR1 reporter activity (ROR1 enhancer-promoter-luc) in S2-VP10 cells (new Fig 8H and J). In addition, treatment with a BRD4 inhibitor (JQ1) also decreased reporter activity (new Fig 8M). These results indicate that YAP and BRD4 directly transactivate *ROR1*. We have added some information to the revised manuscript (new lines 337–342 and 352–353) as follows: "YAP/TAZ co-knockdown also reduced luciferase activity, which was under control of *ROR1* enhancer-promoter (Figure 8H). Similarly, treatment of the cells with the YAP inhibitor verteporfin reduced the expression of *ROR1* as well as the known YAP target genes such as *CTGF* and *CYR61* (Fig 8I) and the ROR1 reporter activity (Fig 8J). Together, these results indicate that YAP directly transactivates *ROR1*.", "In addition, treatment with JQ1, one of the most established BET inhibitors, reduced ROR1 reporter activity in S2-VP10 cells (Fig 8M)."

#5-3: Is YAP activity different in ROR1 high vs. low cells?

#5-3: We thank the reviewer for this comment. As noted in the original manuscript, GSEA revealed that ROR1^{high} cells in both S2-VP10 xenografts and PDO#1 xenografts expressed higher levels of YAP-regulated genes than ROR1^{low} cells in these xenografts (original and new Fig 8C). We have added a clear expression of this information to the revised manuscript in the Results section (new lines 328–330) and Methods section (new lines 599–602) as follows: “Moreover, gene set enrichment analysis revealed that intratumor ROR1high cells showed upregulation of YAP-regulated genes compared with ROR1low cells (Fig 8C). Thus, these results suggest that ROR1high cells have higher YAP activity than ROR1low cells.”, “Gene set enrichment analysis was performed using GSEA (version 4.0.3) (Broad Institute) with H hallmark gene sets and CORDENONSI_YAP_CONSERVED_SIGNATURE (M2871) in the Molecular Signatures Database.”

#6. In Fig. 8A the authors identified 202 antigens that match the H3 monomethylation / acetylation pattern. How was YAP etc. chosen?

#6: We thank the reviewer for pointing this out and apologize for the unclear explanation in the original manuscript. We described this information in the original manuscript, but we have carefully changed some descriptions in the revised manuscript (new lines 320–325, 343–348) as follows: “Of the 202 TFs and transcription-associated antigens that showed a similar pattern with our peak data, we focused on Yes-associated protein (YAP). YAP and its close paralog, TAZ, are transcriptional regulators involved in CSC abilities such as tumorigenicity, chemoresistance, and metastasis in breast, esophageal, and hepatocellular cancers as well as in osteosarcoma (Bartucci *et al*, 2015; Basu-Roy *et al*, 2015; Hayashi *et al*, 2015; Song *et al*, 2014).”, “ChIP-Atlas analyses also showed that bromodomain-containing protein 4 (BRD4), an acetylated histone-binding protein, has a highly similar genomic distribution with H3K4me1+/H3K27ac+ shown in our experiment (Tables EV8 and 9). BRD4 is a member of the bromodomain and extraterminal motif (BET) family (Zeng & Zhou, 2002), and BET inhibitors are rapidly being developed for clinical use because of their potent anti-tumor effects (Filippakopoulos et al, 2010; Doroshow et al, 2017).”

Minor:

#1. Fig. 2D,E: What is actually shown here? Is there an overlap between the genes that define ROR1 high vs. low cells in both approaches? The gene list should be provided.

#1: We apologize for the poor description in the original manuscript. We have added this information to the revised manuscript (new Table EV3).

2. Fig. 3G: I suggest to include the images of the tumors from the ROR1 low cells in the main figure as well.

#2: We appreciate the reviewer's suggestion. We have moved this information from the supplementary information to the main figure in the revised manuscript (new Fig 3G).

Reviewer #1 (Significance (Required)):

PDAC is a very aggressive desease with very low 5-year survival rates. Understanding of the pathobiology is of keen interest. The findings of the authors are of high significance and extremely relevant as they provide a mechanism that can also be targeted by specific drug combinations, i.e. standard care gemcitabine with specific ROR1 inhibition.

The findings are of great interest to a large audience including basic researchers in the field of cancer biology and oncologists in the clinic.

We greatly appreciate the reviewer's comments.

Report of Reviewer #2

(Evidence, reproducibility and clarity (Required)):

In this work Yamazaki and colleagues performed single cell RNA sequencing of one xenograft tumor formed by the S2-VP10 PDAC cell line to explore PDAC intratumor heterogeneity. Using this model they identified ROR1 as heterogeneously expressed in neoplastic cells. Using further in vivo and in vitro models they show that ROR1high cells have higher tumor initiation capacity than ROR1low. By histone and ATAC-seq analyses, they identify a ROR1 enhancer upstream the promoter and show that YAP and BRD4 bind to this genomic region and that BRD4 inhibition by JQ1 reduces ROR1 expression and organoid formation.

The data, figures and methods are nicely and clearly presented.

Major comments

1. The authors use one xenograft tumor as starting model and all conclusions are derived from the data generated with this model. To support the existence of identifie heterogeneity in the PDAC neoplastic compartment, I would strongly suggest to validate the existence of the partial EMT population and the ROR1 heterogeneity in single cell data bases generated from primary human tumors.

2. In Fig. 3G, it is mentioned that tumors grown from ROR1high cells recapitulate the original PDOx histology thus suggesting that ROR1high cells in the tumor are the actual TICs. ROR1low cells could also grow tumors, just with lower incidence. Are these tumors any different to the ROR1high derived ones? Is it just a lower tumor initiation capacity (TIC) or they can not recapitulate the tumor as the ROR1high cell? Can they also give rise to differentiated progeny cells? This should appear in the main text and not only in the discussion. I would suggest to move panel 3G to supplementary figure.

3. In line 160 you mention that known CSC markers such as CD44, PROM1 and DCLK1 are not differentially expressed between ROR1 high and low populations. Then, in figure 3H,I you analyze the expression of CD44v6 together with ROR1. I would try to put this information together in the text, or at least in fig. 3 start with something like "we had seen that both ROR1high and low express CD44, however...". In any case, I feel that the experiment with CD44 could be obviated (or at least moved to supplementary), as it brings the question of weather this is also true for DCLK1 or CD133.

4. JQ1 has been described to inhibit PDAC growth by downregulation of MYC. To unequivocally link the effect of JQ1 in the downregulation of ROR1 (Fig. 8M) as discussed in the text it would be important to exclude that other mechanisms such as MYC downregulation are taking place. For example, does JQ1 treatment of ROR1low cells also reduce their colony formation capacity (in an experiment such as the one in fig. 3C). Or does ROR1 re-expression in Fig. 8M rescue the

JQ1 effect? These or other experiments could help to establish a stronger link between (BRD4/JQ1) and ROR1.

Minor comments

1. The data are nicely presented (text and figures) and the conclusions are clear. My suggestion to make the story more "catchy" at the beginning would be, if possible, to start from the observation done in primary human data and then move to the PDX model to explore ROR1 as a TIC marker in PDAC. For this, you could use available public single cell data of human PDAC tumors. If this doesn't work (it is of course possible that by unsupervised analysis you don't get the same clusters as in the PDX with the partial EMT cluster popping up), it would be nice if some primary tumor data came early in the story (currently the first figure showing heterogeneity in primary samples is in supplem fig. 4A).
2. It is not clear if the xenografts were subcutaneous or orthotopic. It would be good to include this information in the main text (line 102) and the methods so that the reader knows what is the exact model that has been used.
3. In Fig. 2F and 2G I would highlight the EMT pathway to help the reader.
4. In Supp Fig 4B it would be nice to have an amplified view of the staining as in panel C of the same figure.
5. In the same figure (Fig. 4A-D) ROR1 shows an apical staining pattern that doesn't seem to resemble the staining in patient samples. I am not an expert in pathology evaluation but I would recommend a pathologist to give her/his opinion. Possibly, during the PDX process, few cells from the original patient tumor are selected giving a different staining pattern.
6. In the analyses of TCGA data, be aware that only 150 from the original dataset are actual PDAC tumors. The dataset contains otherwise data from cell lines, PDX, normal tissue, etc that should be removed for a proper analysis (see DOI: 10.3390/cancers11010126)
7. Does ROR1 correlate with RFS? This would nicely fit with the concept of TIC and metastasis.
8. Line 219: ROR1 is not "depleted" in the lines as it is a downregulation model. "ROR1-downregulated" would be more correct.
9. It would be good to have a supplem figure showing that siROR1 cells show reduction organoid formation, to validate that the siRNA model functionally recapitulates the ROR1low vs high phenotype.
10. Some of the supplemental figures are only referred in the discussion although they appear earlier than other in the main text. This is a bit confusing when going through the figures.

CROSS-CONSULTATION COMMENTS

I agree with the importance of addressing points 2 (link to AURKB), 4 (selection vs acquisition),

5 (mechanism in high vs low cells) raised by Reviewer 1, and the comments from Reviewer 3. I think that the study of other RTKs (point 1 from Reviewer 1) is not the focus of the story. It would be nice if the authors can comment on why they chose ROR1 but the fact that are other differentially expressed genes does not exclude the validity of the current story. I fell that the in vivo sustained KD experiment (point 3 from Reviewer 1) although interesting, it is not mandatory for a revision of this manuscript in case the adaptation of the animal protocol represents a long process. The experiment provided already in the current version is the best approach to address the role of ROR1 at the early initiation phase.

Reviewer #2 (Significance (Required)):

Significance:

This is a neat and interesting work with potential implications for the clinical field of pancreatic cancer as the authors identified a new subpopulation with enhanced tumor initiating cell capacity. However, the use of JQ1 for pancreatic cancer has been previously discussed mainly linked to MYC inhibition, but also to stromal reprogramming or DNA damage induction. I missed some discussion in this regard in the discussion section. What is adding the work to the field of JQ1 treatment in PDAC? IN a way, how do the authors foresee that the discovery of ROR1high cells and the regulation of ROR1 by BRD4 and YAP will be beneficial when considering JQ1 in the clinics? Maybe by stratifying patients? Or by following ROR1 upregulation upon initial chemotherapy? These questions are just suggestions. In general, some discussion to put the work into the context of previous works using JQ1 in PDAC would be nice.

I believe that this work would be interesting not only to the pancreatic cancer community but also to a more general public working on cancer and/or stemmness as it touches several interesting points in that regard that can be applicable to other systems.

My own work is focused on pancreatic cancer, patient heterogeneity and stromal interactions. I am not an expert on histone or ATACseq analyses.

Response to Reviewer #2

(Evidence, reproducibility and clarity (Required)):

In this work Yamazaki and colleagues performed single cell RNA sequencing of one xenograft tumor formed by the S2-VP10 PDAC cell line to explore PDAC intratumor heterogeneity. Using this model they identified ROR1 as heterogeneously expressed in neoplastic cells. Using further in vivo and in vitro models they show that ROR1high cells have higher tumor initiation capacity than ROR1low. By histone and ATAC-seq analyses, they identify a ROR1 enhancer upstream the promoter and show that YAP and BRD4 bind to this genomic region and that BRD4 inhibition by JQ1 reduces ROR1 expression and organoid formation. The data, figures and methods are nicely and clearly presented.

We thank the reviewer for stating that "The data, figures and methods are nicely and clearly presented", and we appreciate the reviewer's constructive suggestions. We have substantially revised our manuscript based on these valuable comments.

Major comments

#1. The authors use one xenograft tumor as starting model and all conclusions are derived from the data generated with this model. To support the existence of identifie heterogeneity in the PDAC neoplastic compartment, I would strongly suggest to validate the existence of the partial EMT population and the ROR1 heterogeneity in single cell data bases generated from primary human tumors.

#1: We thank the reviewer for the positive suggestion. We agree with this comment. As suggested, we analyzed publicly available single-cell RNA sequencing data from human PDAC (Peng et al, 2019). We focus on the T20 sample in Peng's paper, which has a large number of cancer cells. First, we selected the epithelial cell subsets that express *KRT8/KRT18* from all clusters (new Fig 1H and new Fig EV2A). Then, we identified cancer cell cluster from their subsets by checking the expression of *FXYD3* and *MUC1*, which are cancer cell markers shown by Peng et al. (new Fig 1I). The cancer cell cluster was further divided into six clusters based on gene expression (new Fig 1J and new Fig EV2B and C). Within these clusters, there was a malignant subpopulation with high expression of proliferation markers such as *MKI67* and *TOP2A*, suggesting that this population may be a potential origin of tumor growth (new Fig 1K). This subpopulation, cluster 1, also expressed *VIM* but lacked EMT-TFs such as *ZEB1* and *SNA1* (new Fig 1L). Finally, we found that cluster 1 had high expression of ROR1 (new Fig 2C). These data indicate that a similar cluster of ROR1^{high} partial EMT cells existed in patient PDAC as well as in our xenograft model. We have added this information to the revised manuscript (new lines 135–145 and 157–159) as follows:

"To investigate the relevance of our findings in the S2-VP10 xenograft to patient PDAC, we analyzed publicly available scRNA-seq data (Peng *et al*, 2019). This analysis revealed two *KRT8/KRT18* populations (Fig 1H and Fig EV2A), and one of the clusters was a tumor cell population expressing malignant markers such as *FXYD3* and *MUC1* (Fig 1I) (Peng *et al*, 2019). Based on gene expression characteristics, we identified six distinct cell clusters in malignant cells (Fig 1J and Fig EV2B and C). In cluster 1, proliferation-related genes such as *MKI67* and *TOP2A* were highly expressed (Fig 1K), suggesting that this population may be a potential origin of tumor growth. In addition, this cluster exhibited higher expression of *VIM* (Fig 1L) but lacked expression of EMT-TFs (Fig EV2D). These data indicate that similar cell with a partial EMT signature exist not only in our xenograft model but also in patient PDAC.", "In addition, we found that *ROR1* is highly expressed in the partial EMT subpopulation of patient PDAC as well as S2-VP10 xenografts (Fig 2B and C)."

Reference

Peng J, Sun BF, Chen CY, Zhou JY, Chen YS, Chen H, Liu L, Huang D, Jiang J, Cui GS, Yang Y, Wang W, Guo D, Dai M, Guo J, Zhang T, Liao Q, Liu Y, Zhao YL, Han DL, Zhao Y, Yang YG, Wu W. Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. Cell Res 2019 29, 725–738. DOI: 10.1038/s41422-019-0195-y. PMID: 31273297. PMCID: PMC6796938.

#2-1. In Fig. 3G, it is mentioned that tumors grown from ROR1high cells recapitulate the original PDOx histology thus suggesting that ROR1high cells in the tumor are the actual TICs. ROR1low cells could also grow tumors, just with lower incidence. Are these tumors any different to the ROR1high derived ones? Is it just a lower tumor initiation capacity (TIC) or they can not recapitulate the tumor as the ROR1high cell? Can they also give rise to differentiated progeny cells? This should appear in the main text and not only in the discussion.

#2-1: We thank the reviewer for pointing this out and apologize for the poor description in the original manuscript. As mentioned in your comment, ROR1^{low} cells have just a lower tumor initiating capacity. As shown in original or new Fig 3D and G, ROR1^{low} cells generated tumors at a low frequency, and these tumors showed a hierarchical histology mimicking the original tumor containing ROR1^{high} cells and differentiated progeny cells. As noted in the original manuscript, we consider that the expression of ROR1 is flexibly controlled in enhancer activity (original Fig 7 and 8), and some ROR1^{low} cells can revert to ROR1^{high} cells (original lines 354–363 / new lines 386–394). As you suggested, we have added this information clearly to the revised manuscript in Results session (new lines 200–205) and Discussion session (new lines 386–394) as follows:

“Thus, these data demonstrated that intratumor ROR1high cells have a greater ability to initiate tumors than ROR1low cells and produce differentiated progeny. Although present at a low frequency, ROR1low cells also formed tumors with a hierarchical histology similar to the original tumor containing ROR1high cells (Fig 3D, F, and G). These results suggest that ROR1low cells may revert to ROR1high status, contributing to tumor formation.”, “At a low frequency, ROR1^{low} cells also generated tumors that showed hierarchical histology mimicking the original tumor and containing ROR1^{high} cells (Fig 3G). Interestingly, in ROR1^{low} cells, the *ROR1* enhancer employed the H3K4me1+/H3K27ac- poised chromatin state (Fig 7B), suggesting that the expression of *ROR1* is flexibly regulated in PDAC. These data suggest that ROR1^{low} cells may be reversibly converted into ROR1^{high} tumor-initiating cells at a low frequency, resulting in tumor seeding. Our observation is similar to that in a previous report showing that tumor cells expressing the differentiation marker keratin 20 regain their proliferative potential and convert to LGR5^{+} CSCs in colorectal cancer (Shimokawa *et al*, 2017).”

#2-2: I would suggest to move panel 3G to supplementary figure.

#2-2: We appreciate the reviewer for this comment. However, we believe that original Fig 3G should remain in the main figure (new Fig 3G) since it is an important panel showing the heterogeneity of tumors derived from not only ROR1^{high} cells but also ROR1^{low} cells, as mentioned in the response to Reviewer #2 [major #2-1]. We sincerely hope for your approval of this point.

3. In line 160 you mention that known CSC markers such as CD44, PROM1 and DCLK1 are not differentially expressed between ROR1 high and low populations. Then, in figure 3H,I you analyze the expression of CD44v6 together with ROR1. I would try to put this information together in the text, or at least in fig. 3 start with something like "we had seen that both ROR1high and low express CD44, however...". In any case, I feel that the experiment with CD44 could be obviated (or at least moved to supplementary), as it brings the question of weather this is also true for DCLK1 or CD133.

#3: We appreciate and agree with the reviewer's comment on this point. Accordingly, we have moved these figures (colony formation assay of CD44v) to the expanded information (Fig EV5E and F) and changed the description as you mentioned (new lines 207–211) as follows: “We observed that both ROR1^{high} and ROR1^{low} cells express CD44 in scRNA-seq data of S2-VP10 xenograft (Fig EV3D); however, FACS analysis revealed that some of the CD44v9^{high} cells coexpressed ROR1 (Fig EV5E). Only this ROR1^{high}/CD44v9^{high} population exhibited colony-forming capability (Fig EV5F).”

4-1. JQ1 has been described to inhibit PDAC growth by downregulation of MYC. To unequivocally link the effect of JQ1 in the downregulation of ROR1 (Fig. 8M) as discussed in the text it would be important to exclude that other mechanisms such as MYC downregulation are taking place.

#4-1: We thank the reviewer for this important comment. As mentioned in the response to Reviewer #1 [major #2-1 and #5-2], we newly found that ROR1 regulates c-Myc expression through AKT signaling (new Fig 4L–O). We have added this information to the revised manuscript (new lines 246–252) as follows: "We then sought to link ROR1 activity to E2F-mediated AURKB expression. Previous reports have shown that ROR1 induces AKT phosphorylation in breast and non-small cell lung cancers (Zhang *et al*, 2012a; Yamaguchi *et al*, 2012). Indeed, we observed a reduction in phospho-AKT levels in ROR1-depleted PDAC cells (Fig 4L). Consistent with the reduced AKT signaling, c-Myc protein levels and the expression of c-Myc target genes, such as *CDK4*, *CCND1*, *CDK2*, and *CCNE1*, were downregulated by *ROR1*-KD (Fig 4M and N)."

In addition, we demonstrated that JQ1 treatment decreased ROR1 reporter activity in S2-VP10 cells (new Fig 8M). We have added this information to the revised manuscript (new lines 352–353) as follows: "In addition, treatment with JQ1, one of the most established BET inhibitors, reduced ROR1 reporter activity in S2-VP10 cells (Fig 8M)."

Thus, we believe that JQ1 treatment affects c-Myc downregulation not only directly but also through the reduction of ROR1, resulting in the inhibition of PDAC proliferation. This information has been added to the revised manuscript (new lines 436–437) as follows: "We consider that ROR1-mediated suppression of c-Myc function exhibits a useful additive effect of JQ1 or other BRD4 inhibitors during ROR1^{high} PDAC therapy."

4-2. For example, does JQ1 treatment of ROR1low cells also reduce their colony formation capacity (in an experiment such as the one in fig. 3C).

#4-2: We appreciate the reviewer for this suggestion. As noted in the original manuscript, we believe that at low frequency, ROR1^{low} cells can revert to ROR1^{high} cells due to flexible regulation of its expression (original and new Fig 7B; original lines 354–360 and new lines 386–392). Therefore, in the colony/organoid formation assay, some of the ROR1^{low} cells may convert to ROR1^{high} cells and grow (original and new Fig 3C). Considering this, we think that ROR1^{low} cells with JQ1 will also reduce the colony formation capacity.

#4-3. Or does ROR1 re-expression in Fig. 8M rescue the JQ1 effect?

In PDAC, JQ1 treatment leads to a reduction in c-Myc expression (new lines 433–434). Because of this effect, we believe that ROR1 re-expression cannot rescue the JQ1 effect.

4-4. These or other experiments could help to establish a stronger link between (BRD4/JQ1) and ROR1.

#4-4: We appreciate the reviewer in this comment. As mentioned in the response to Reviewer #2 [major #4-1], we found that JQ1 treatment reduced ROR1 reporter activity (new Fig 8M). This result reinforces the link between BRD4/JQ1 and ROR1.

Minor comments

1. The data are nicely presented (text and figures) and the conclusions are clear. My suggestion to make the story more "catchy" at the beginning would be, if possible, to start from the observation done in primary human data and then move to the PDX model to explore ROR1 as a TIC marker in PDAC. For this, you could use available public single cell data of human PDAC tumors. If this doesn't work (it is of course possible that by unsupervised analysis you don't get the same clusters as in the PDX with the partial EMT cluster popping up), it would be nice if some primary tumor data came early in the story (currently the first figure showing heterogeneity in primary samples is in supplem fig. 4A).

#1: We thank the reviewer for these excellent comments. As suggested, we performed scRNA-seq analysis of patient PDAC and found that a similar ROR1^{high} subpopulation with partial EMT signature existed not only in our xenograft model but also in patient PDAC. As mentioned in the response of Reviewer #2 [major #1], we substantially revised our manuscript.

2. It is not clear if the xenografts were subcutaneous or orthotopic. It would be good to include this information in the main text (line 102) and the methods so that the reader knows what is the exact model that has been used.

#2: We thank the reviewer for this comment and apologize for the poor description in the original manuscript. As suggested, we have added this information to the revised manuscript (new lines 102-103) as follow: “...we first performed single-cell RNA sequencing (scRNA-seq) in a subcutaneous xenograft...”

3. In Fig. 2F and 2G I would highlight the EMT pathway to help the reader.

#3: We thank the reviewer for this comment. As suggested, we have changed the relevant figures in the revised manuscript (new Fig 2G and H).

4. In Supp Fig 4B it would be nice to have an amplified view of the staining as in panel C of the same figure.

#4: We thank the reviewer for this comment. As suggested, we added high-magnification images of the staining in the revised manuscript (new Fig EV4A–C).

5. In the same figure (Fig. 4A-D) ROR1 shows an apical staining pattern that doesn't seem to resemble the staining in patient samples. I am not an expert in pathology evaluation but I would recommend a pathologist to give her/his opinion. Possibly, during the PDX process, few cells from the original patient tumor are selected giving a different staining pattern.

#5: We appreciate the reviewer's comment on this point. Dr. Ito, a coauthor of this paper, is a pathologist. We have changed some images of staining in patient samples (new Fig EV4A). We agree that ROR1 shows an apical staining pattern in PDX samples (new Fig EV4B). Similarly, some sites showed similar apical staining patterns in patient samples (Patient #2 and Patient #4 in new Fig EV4A). We propose that PDX mimics the original patient tissue because it has heterogeneity in terms of ROR1 expression and morphological features indicative of a luminal structure.

6. In the analyses of TCGA data, be aware that only 150 from the original dataset are actual PDAC tumors. The dataset contains otherwise data from cell lines, PDX, normal tissue, etc that should be removed for a proper analysis (see DOI: 10.3390/cancers11010126)

#6: We thank the reviewer for pointing this out. We reconsidered with pathologist Dr. Ito (coauthor) the appropriateness of the specimens based on the TCGA-PAAD data (accessed the GDC data portal [https://portal.gdc.cancer.gov/] and referred to the diagnosis and pathology sections) and the paper you provided (DOI: 10.3390/cancers11010126); 169 specimens were diagnosed as PDAC, of which 154 specimens were selected for analysis that had both disease-specific survival and gene expression data (FPKM) available (new Table EV6). Using these 154 data points, we reperformed (1) a survival analysis to determine whether ROR1 expression correlates with disease-specific survival and (2) Pearson correlation analyses of *ROR1* expression with *YAP1* and *WWTR1* expression. In this dataset, (1) high *ROR1* expression was associated with shorter disease-specific survival (new Fig 3A). (2) *ROR1* expression was significantly positively

correlated with *YAP* and *WWTR1* (encoding TAZ) expression (new Fig 8D). We have added this information to the revised manuscript in the Results section (new lines 184–187, 325–327) and Methods section (new lines 799–801 and 852–855) as follows: “In PDAC patients, high expression of *ROR1* was significantly associated with poor clinical outcomes (disease-specific survival) in the TCGA-PAAD dataset from The Cancer Genome Atlas (TCGA) (Fig 3A and Table EV6), suggesting a potential role of ROR1 in PDAC progression.”, “Interestingly, both the *YAP1* and *WWTR1* (encoding TAZ) transcript levels were significantly and positively correlated with *ROR1* transcript levels in the pancreatic adenocarcinoma dataset TCGA-PAAD (Fig 8B and Table EV6).”, “Gene expression data were downloaded from TCGA (TCGA-PAAD, accessed the GDC data portal [https://portal.gdc.cancer.gov/], retrieved on March 22, 2021) (Table EV6).”, “The gene expression data and clinical data were downloaded from TCGA (TCGA-PAAD, accessed the GDC data portal [https://portal.gdc.cancer.gov/], retrieved on March 22, 2021) (Table EV6). The association between the expression of *ROR1* and patient disease-specific survival was examined by the Kaplan–Meier method.”

7. Does ROR1 correlate with RFS? This would nicely fit with the concept of TIC and metastasis.

#7: We thank the reviewer for this suggestion. In TCGA-PAAD dataset (accessed the GDC data portal [https://portal.gdc.cancer.gov/], retrieved on March 22, 2021), there are 58 specimens diagnosed with PDAC that had both RFS (recurrence-free survival) and gene expression data (FPKM) available. Unfortunately, although a trend was noted, no significant association was found between high ROR1 expression and worse RFS (Fig 2 for reviewer #2).

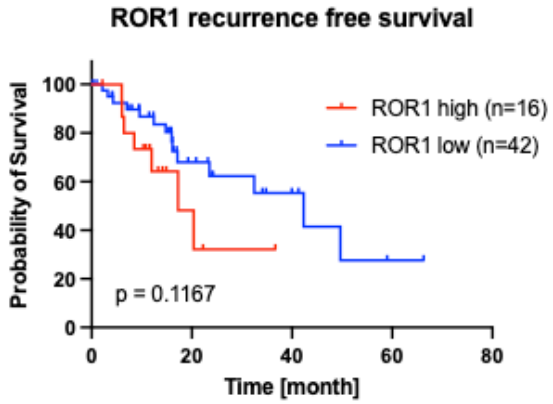

Fig 2 for reviewer #2

8. Line 219: ROR1 is not "depleted" in the lines as it is a downregulation model. "ROR1-downregulated" would be more correct.

#8: We thank the reviewer for this suggestion and agree with your comment. We have corrected this term accordingly in the revised manuscript (new lines 235–236) as follows: “We also confirmed the reduction in AURKB protein in ROR1-downregulated S2-VP10 and S2-013 cells (Fig 4I).”

9. It would be good to have a supplem figure showing that siROR1 cells show reduction organoid formation, to validate that the siRNA model functionally recapitulates the ROR1low vs high phenotype.

#9: We thank the reviewer for this comment. As suggested, we performed an organoid formation assay using S2-VP10 ishROR1 cells. Dox-inducible sustained *ROR1*-knockdown downregulated organoid formation activity (new Fig EV5B–D). This result reinforces our other findings regarding the $ROR1^{high}$ vs. $ROR1^{low}$ phenotype. We have added figures and some descriptions to the revised manuscript (new lines 191–192) as follows: “In addition, stable *ROR1*-knockdown using a doxycycline (Dox)-inducible shRNA system supressed S2-VP10 organoid formation (Fig EV5B–D).”

10. Some of the supplemental figures are only referred in the discussion although they appear earlier than other in the main text. This is a bit confusing when going through the figures.

#10: We apologize for the poor description in the original manuscript. We have adjusted the order of Fig EV (supplemental figures) in the revised manuscript.

CROSS-CONSULTATION COMMENTS

I agree with the importance of addressing points 2 (link to AURKB), 4 (selection vs acquisition), 5 (mechanism in high vs low cells) raised by Reviewer 1, and the comments from Reviewer 3. I think that the study of other RTKs (point 1 from Reviewer 1) is not the focus of the story. It would be nice if the authors can comment on why they chose ROR1 but the fact that are other differentially expressed genes does not exclude the validity of the current story. I fell that the in vivo sustained KD experiment (point 3 from Reviewer 1) although interesting, it is not mandatory for a revision of this manuscript in case the adaptation of the animal protocol represents a long process. The experiment provided already in the current version is the best approach to address the role of ROR1 at the early initiation phase.

We thank the reviewer for these positive comments. As suggested, we have substantially revised our manuscript.

Significance:

This is a neat and interesting work with potential implications for the clinical field of pancreatic cancer as the authors identified a new subpopulation with enhanced tumor initiating cell capacity. However, the use of JQ1 for pancreatic cancer has been previously discussed mainly linked to MYC inhibition, but also to stromal reprogramming or DNA damage induction. I missed some discussion in this regard in the discussion section.

We appreciate the reviewer for this comment. As you pointed out, we have added some description to the discussion session regarding the use of JQ1 on stromal reprogramming and DNA damage induction (new lines 433–437) as follows: “BET inhibitor therapy for PDAC, including JQ1, has previously been discussed with a focus on c-Myc inhibition, stromal remodeling, and accumulation of DNA damage (Mertz *et al*, 2011; Yamamoto *et al*, 2016; Miller *et al*, 2019). We consider that ROR1-mediated suppression of c-Myc function exhibits a useful additive effect of JQ1 or other BRD4 inhibitors during ROR1^{high} PDAC therapy.”

What is adding the work to the field of JQ1 treatment in PDAC? IN a way, how do the authors foresee that the discovery of ROR1high cells and the regulation of ROR1 by BRD4 and YAP will be beneficial when considering JQ1 in the clinics? Maybe by stratifying patients? Or by following ROR1 upregulation upon initial chemotherapy? These questions are just suggestions. In general, some discussion to put the work into the context of previous works using JQ1 in PDAC would be nice.

We also appreciate the reviewer for pointing this out. JQ1 has been previously described to repress MYC expression. Our study shows that ROR1 is an important upstream factor that regulates MYC expression (new Fig 4L–O). Furthermore, we found that ROR1 is regulated by the YAP/BRD axis (Fig 8A–Q). We propose that BRD4 inhibitor therapy has the potential to inhibit MYC not only directly but also by downregulating ROR1, an upstream regulator of MYC (new lines 436–437). In addition, we previously reported an assay that detects cancer-derived ROR1-positive exosomes (Daikuzono, Yamazaki et al.). This new assay may be a useful basic companion diagnostic for ROR1-positive PDAC. Taken together, we believe that ROR1 therapy will be beneficial in a stratified PDAC patient population. We have added this description to the revised manuscript (new lines 436–437 and 438–442) as follows: “We consider that ROR1-mediated suppression of c-Myc function exhibits a useful additive effect of JQ1 or other BRD4 inhibitors during ROR1^{high} PDAC therapy.”, “Our findings will greatly help in developing new therapeutic strategies for ROR1-driven PDAC. In addition, we previously reported an assay that

detects cancer-derived ROR1-positive exosomes, which could be a basic technology for companion diagnostics (Daikuzono *et al*, 2021). ROR1 therapy with patient stratification will be beneficial in PDAC."

Reference

Daikuzono H, Yamazaki M, Sato Y, Takahashi T, Yamagata K. Development of a DELFIA method to detect oncofetal antigen ROR1-positive exosomes. Biochem Biophys Res Commun 2021 Nov 12;578:170-176. DOI: 10.1016/j.bbrc.2021.08.054. PMID: 34597914.

I believe that this work would be interesting not only to the pancreatic cancer community but also to a more general public working on cancer and/or stemmness as it touches several interesting points in that regard that can be applicable to other systems.

My own work is focused on pancreatic cancer, patient heterogeneity and stromal interactions. I am not an expert on histone or ATACseq analyses.

We greatly appreciate the reviewer's comments.

Report of Reviewer #3

(Evidence, reproducibility and clarity (Required)):

Summary

Yamazaki et al investigate partial EMT in pancreatic cancer and provide data that ROR1 marks pancreatic tumor cells that are capable of initiating tumors. The authors exploit scRNAseq of pancreatic tumor xenografts to identify a cluster of cells showing a partial EMT phenotype. The found 7 RTKs expressed more highly in this partial EMT cluster and focus their attention on ROR1, an 'orphan' receptor that has been implicated in WNT signaling and EMT previously. Validation experiments using ROR1-high vs low cells support that ROR1 expression correlates with EMT, poor outcome in human PDA patients, tumor forming and colony forming capacity. They also show that ROR1 high cells form tumors that recapitulate parental tumor histology. The authors show that ROR1 expression is associated with EF2 transcription factor activity, elevated expression of multiple targets including AURKB. Pharmacologic inhibition of AURKB reduces colony formation and genetic loss of ROR1 combined with chemotherapy (gemcitabine) has potent anti-tumor activity in vivo. The authors show that ROR1 expression is elevated in metastatic lesions and identify a novel enhancer element that putatively drives ROR1 expression in tumor cells. They provide evidence that this element is engaged by YAP/BRD4 and show that BRD4 inhibition reduces tumor cell colony formation. The manuscript is a solid combination of techniques with adequate controls and statistics.

Major Comments:

The overall conclusion that ROR1 expression marks a subset of pancreatic cancer cells that have the ability to initiate tumors is supported by the data provided. The correlative data are strong and the demonstration that loss of ROR1 reduces colony formation, reduces metastatic lesions and enhances the efficacy of chemotherapy are compelling. Additionally, the demonstration that ROR1 expression is elevated in metastatic lesions is consistent with many other drivers/markers of EMT in pancreatic cancer.

The conclusion that ROR1 expression is driven by YAP/BRD4 is interesting and provides important mechanistic depth to the study. However, this conclusion could be strengthened by use of a suitable rescue experiment. For instance does overexpression of ROR1 rescue the effect of BRD4 inhibition or loss of YAP?

A challenge with the data presented in Figure 1, the scRNA-seq data that lead them to ROR1, is that it is not stated how many tumors are used to generate the scRNA-seq data and the overall

number of tumor cells analyzed is relatively low (993). The authors should provide the number of tumors used for the initial scRNA-seq. A general concern with any scRNA-seq data is batch effect, this is mitigated to a degree by the follow on studies that provide functional validation of ROR1 in multiple cell lines.

The data and methods are provided in an adequate manner. Reproduction of the experiments is likely. The authors use multiple cell lines and tools that are generally available.

The authors note a limitation of the study is that only human tumor xenografts were exploited.

Minor comments:
Figure 1E and text page 9. The text identifies MERB3 as a gene that marks the partial EMT cluster, I believe this is a type and the gene should actually be MSRB3.

Please provide the dose of gemcitabine in the legend for figure 5

CROSS-CONSULTATION COMMENTS
I think the comments from Referee #2 are pretty reasonable - have no additions

Reviewer #3 (Significance (Required)):
Intratumor heterogeneity is a major challenge for the treatment of many cancers, including pancreatic cancer. The data provided support that ROR1 marks a subset of cancer cells in pancreatic tumors that have the capacity to drive intratumor heterogeneity. If supported these data have the potential to drive significant impact. Identification of a marker and a targetable pathway that supports tumor initiation in pancreatic cancer has the potential to nominate companion therapies that enhance the efficacy of standard of care approaches. Further, identification of a pathway that drives partial EMT in pancreatic cancer provides a substantial increase in baseline knowledge of intratumor heterogeneity.

These data would be broadly interesting to scientists interested in the tumor microenvironment, metastasis, therapy resistance and tumor progression. In addition, oncologists focused on drug development and combinatorial therapy will find this manuscript of interest.

Response to Reviewer #3

(Evidence, reproducibility and clarity (Required)):

Summary

Yamazaki et al investigate partial EMT in pancreatic cancer and provide data that ROR1 marks pancreatic tumor cells that are capable of initiating tumors. The authors exploit scRNAseq of pancreatic tumor xenografts to identify a cluster of cells showing a partial EMT phenotype. The found 7 RTKs expressed more highly in this partial EMT cluster and focus their attention on ROR1, an 'orphan' receptor that has been implicated in WNT signaling and EMT previously. Validation experiments using ROR1-high vs low cells support that ROR1 expression correlates with EMT, poor outcome in human PDA patients, tumor forming and colony forming capacity. They also show that ROR1 high cells form tumors that recapitulate parental tumor histology. The authors show that ROR1 expression is associated with EF2 transcription factor activity, elevated expression of multiple targets including AURKB. Pharmacologic inhibition of AURKB reduces colony formation and genetic loss of ROR1 combined with chemotherapy (gemcitabine) has potent anti-tumor activity in vivo. The authors show that ROR1 expression is elevated in metastatic lesions and identify a novel enhancer element that putatively drives ROR1 expression in tumor cells. They provide evidence that this element is engaged by YAP/BRD4 and show that BRD4 inhibition reduces tumor cell colony formation. The manuscript is a solid combination of techniques with adequate controls and statistics.

We thank the reviewer for stating that “The manuscript is a solid combination of techniques with adequate controls and statistics”, and we appreciate the reviewer’s constructive suggestions. We have performed new experiments and substantially revised our manuscript based on these valuable comments.

Major Comments:

The overall conclusion that ROR1 expression marks a subset of pancreatic cancer cells that have the ability to initiate tumors is supported by the data provided. The correlative data are strong and the demonstration that loss of ROR1 reduces colony formation, reduces metastatic lesions and enhances the efficacy of chemotherapy are compelling. Additionally, the demonstration that ROR1 expression is elevated in metastatic lesions is consistent with many other drivers/markers of EMT in pancreatic cancer.

1. The conclusion that ROR1 expression is driven by YAP/BRD4 is interesting and provides important mechanistic depth to the study. However, this conclusion could be strengthened by use

of a suitable rescue experiment. For instance does overexpression of ROR1 rescue the effect of BRD4 inhibition or loss of YAP?

#1: We thank the reviewer for this comment. We agree with the reviewer's suggestion. However, the suggested examination to determine whether overexpression of ROR1 rescues the effect of BRD4 inhibition or loss of YAP may not be suitable because BRD4 and YAP act as transcriptional coregulators of various target genes. Instead, as mentioned in response to Reviewer #1 [major #5-2] and Reviewer #2 [major #4-1], we have performed new experiments using a reporter assay. YAP/TAZ co-knockdown and treatment with the YAP inhibitor verteporfin or the BRD4 inhibitor JQ1 reduced the luciferase activity of the ROR1 enhancer-promoter-luc reporter in S2-VP10 cells (new Fig 8H, J, and M). These results indicate that YAP and BRD4 directly transactivate *ROR1*. We have added some information to the revised manuscript (new lines 337–342 and 351–352) as follows: "YAP/TAZ co-knockdown also reduced luciferase activity, which was under control of *ROR1* enhancer-promoter (Figure 8H). Similarly, treatment of the cells with the YAP inhibitor verteporfin reduced the expression of *ROR1* as well as the known YAP target genes such as *CTGF* and *CYR61* (Fig 8I) and the ROR1 reporter activity (Fig 8J). Together, these results indicate that YAP directly transactivates *ROR1*.", "In addition, treatment with JQ1, one of the most established BET inhibitors, reduced ROR1 reporter activity in S2-VP10 cells (Fig 8M)."

In addition, as mentioned in response to Reviewer #1 [major #5-1], we examined the occupancy of the ROR1 enhancer region by YAP and BRD4 in cell lines with high and low ROR1 expression using public data. In cell lines with high ROR1 expression (MDA-MB-231, PC-9, and NCI-H2052), YAP and BRD4 were found to occupy the ROR1 enhancer region, but not in cell lines with low ROR1 expression (MCF7 and T-47D) (new Fig 8D and K). These findings support our hypothesis that ROR1 expression is dependent on YAP/BRD4 binding to the *ROR1* enhancer region. This information has been added to the revised manuscript (new lines 330–334, 348–351) as follows: "Analysis of publicly available ChIP-seq datasets revealed that YAP binds to the enhancer region of ROR1 in cancer cell lines with high *ROR1* expression (MDA-MB-231, PC-9, NCI-H2052, and SF268 cells) (Fig 8D and Appendix Fig S4). In contrast, in cancer cell lines with low *ROR1* expression (MCF-7 and T-47D cells), YAP did not bind to its enhancer regions (Fig 8D and Appendix Fig S4).", "Analysis of the ChIP-seq datasets revealed the binding of BRD4 to the *ROR1* enhancer in only ROR1-expressing cancer cell lines (MDA-MB-231 and PC-9 cells) (Fig 8K and Appendix Fig S4)."

2-1. A challenge with the data presented in Figure 1, the scRNA-seq data that lead them to ROR1, is that it is not stated how many tumors are used to generate the scRNA-seq data and the overall number of tumor cells analyzed is relatively low (993). The authors should provide the number of

tumors used for the initial scRNA-seq.

#2-1: We appreciate the reviewer's comments. In the scRNA-seq data of the S2-VP10 xenograft, the initial number of tumor cells, including mouse cells, was 2,655. As suggested, we have added this information to the revised manuscript (new lines 104–106) as follows: "A total of 993 human PDAC cells were carried forward for downstream analysis after filtering low-quality and mitochondria-enriched cells from 2,655 cells (see Methods for details)."

2-2. A general concern with any scRNA-seq data is batch effect, this is mitigated to a degree by the follow on studies that provide functional validation of ROR1 in multiple cell lines.

#2-2: We thank the reviewer for pointing this out. As mentioned in response to Reviewer #2 [major #1], we performed a scRNA-seq analysis using publicly available patient PDAC data (Peng et al, 2019). We focus on the T20 sample in Peng's paper, which has a large number of cancer cells. First, we selected the epithelial cell subsets that express *KRT8/KRT18* from all clusters (new Fig 1H and new Fig EV2A). Then, we identified cancer cell cluster from their subsets by checking the expression of *FXYD3* and *MUC1*, which are cancer cell markers shown by Peng et al. (new Fig 1I). The cancer cell cluster was further divided into six clusters based on gene expression (new Fig 1J and new Fig EV2B and C). Within these clusters, there was a malignant subpopulation with high expression of proliferation markers such as *MKI67* and *TOP2A*, suggesting that this population may be a potential origin of tumor growth (new Fig 1K). This subpopulation, cluster 1, also expressed *VIM* but lacked EMT-TFs such as *ZEB1* and *SNA1* (new Fig 1L). Finally, we found that cluster 1 had high expression of ROR1 (new Fig 2C). These data indicate that a similar cluster of ROR1^{high} partial EMT cells existed in patient PDAC as well as in our xenograft model. We have added this information to the revised manuscript (new line 135–145 and 157–159) as follows: "To investigate the relevance of our findings in the S2-VP10 xenograft to patient PDAC, we analyzed publicly available scRNA-seq data (Peng *et al*, 2019). This analysis revealed two *KRT8/KRT18* populations (Fig 1H and Fig EV2A), and one of the clusters was a tumor cell population expressing malignant markers such as *FXYD3* and *MUC1* (Fig 1I) (Peng *et al*, 2019). Based on gene expression characteristics, we identified six distinct cell clusters in malignant cells (Fig 1J and Fig EV2B and C). In cluster 1, proliferation-related genes such as *MKI67* and *TOP2A* were highly expressed (Fig 1K), suggesting that this population may be a potential origin of tumor growth. In addition, this cluster exhibited higher expression of *VIM* (Fig 1L) but lacked expression of EMT-TFs (Fig EV2D). These data indicate that similar cell with a partial EMT signature exist not only in our xenograft model but also in patient PDAC.", "In addition, we found that *ROR1* is highly expressed in the partial EMT subpopulation of patient PDAC as well as S2-VP10

xenografts (Fig 2B and C)."

Reference

Peng J, Sun BF, Chen CY, Zhou JY, Chen YS, Chen H, Liu L, Huang D, Jiang J, Cui GS, Yang Y, Wang W, Guo D, Dai M, Guo J, Zhang T, Liao Q, Liu Y, Zhao YL, Han DL, Zhao Y, Yang YG, Wu W. Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. Cell Res 2019 29, 725–738. DOI: 10.1038/s41422-019-0195-y. PMID: 31273297. PMCID: PMC6796938.

3. The data and methods are provided in an adequate manner. Reproduction of the experiments is likely. The authors use multiple cell lines and tools that are generally available.
The authors note a limitation of the study is that only human tumor xenografts were exploited.

#3: We thank the reviewer for the positive comment.

Minor comments:

1. Figure 1E and text page 9. The text identifies MERB3 as a gene that marks the partial EMT cluster, I believe this is a type and the gene should actually be MSRB3.

#1: We apologize for the typo. We have corrected this error accordingly (new line 116).

2. Please provide the dose of gemcitabine in the legend for figure 5

#2: We apologize for the poor description in the original manuscript. We have added this information to the revised manuscript.

CROSS-CONSULTATION COMMENTS

I think the comments from Referee #2 are pretty reasonable - have no additions

Reviewer #3 (Significance (Required)):

Intratumor heterogeneity is a major challenge for the treatment of many cancers, including pancreatic cancer. The data provided support that ROR1 marks a subset of cancer cells in pancreatic tumors that have the capacity to drive intratumor heterogeneity. If supported these data have the potential to drive significant impact. Identification of a marker and a targetable pathway that supports tumor initiation in pancreatic cancer has the potential to nominate companion therapies that enhance the efficacy of standard of care approaches. Further,

identification of a pathway that drives partial EMT in pancreatic cancer provides a substantial increase in baseline knowledge of intratumor heterogeneity.

These data would be broadly interesting to scientists interested in the tumor microenvironment, metastasis, therapy resistance and tumor progression. In addition, oncologists focused on drug development and combinatorial therapy will find this manuscript of interest.

We greatly appreciate the reviewer's comments.

Dear Dr Yamazaki,

Thank you for submitting your revised manuscript (EMBOJ-2022-112614R) to The EMBO Journal, as well as for your patience with our response. Your amended study was sent back to the three referees for their re-evaluation, and we have received comments from all of them, which I enclose below. As you will see, the experts stated that the work has been substantially improved by the revisions and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We now need you to take care of a number of minor issues related to formatting and data annotation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Please limit the keywords to maximally five.

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.

>> Please adjust the title of the 'Declaration of competing interests' section to 'Disclosure and Competing Interests Statement'.

>> Data availability section: Add a hyperlink to the database entry and make sure to release data privacy for the GSE dataset.

>> Please remove the synopsis image from the manuscript .doc file.

>> Figure callouts: add callouts for Figure 3B and Table EV7 in the running text.

>> EV Figures: EV figures need to be uploaded separately and limited to maximally five. If you need more space move figure panels to the appendix introducing Appendix Figures S1,S2... . The legends added to the manuscript after the main figure legends.

>> Figure 5A - please add highlight boxes.

>> EV Tables should be uploaded as individual files: Tables EV1, EV2, EV4, EV5, EV7, EV9, EV10 and EV11 should be uploaded as Table file type and renumbered accordingly; Tables EV3, EV6, and EV8 should be renamed to Dataset EV1-EV3 with the legends uploaded as a separate tab of each Excel file, and the corresponding callouts

>> Appendix: a ToC is required on the first page of the appendix.

>> Source data: source data files need to be reorganized to one file/folder per figure and ZIPing for each main figure.

>> Material & Methods: remove the typo: 'the experiments' in line 464,465. Move acknowledgements in lines 662-665 to the 'Acknowledgments' section. Move the current 'study Approval' information on mouse husbandry to the 'Mice' section. Introduce a separate 'Human samples' section, detailing the informed consent.

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (15th Jun 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors have addressed all my points in the new version of the manuscript and included many supporting additional experiments. The manuscript has improved significantly and I do not have further comments. I congratulate the authors for this nice study!

Referee #2:

In this version of the manuscript the authors have addressed most, although not all, of the reviewers' comments. As part of the new results, the authors described the decreased in MYC levels and downstream targets upon downregulation of ROR1. Additionally, they have validated the existence of partial EMT cancer cells in one human PDAC sample. The work is well presented and explained. ROR1 has been already described in other tumor types. In PDAC the findings are new (to the best of knowledge of this reviewer). Thus, the work describes a new gene involved in the increased stem capacity of PDAC cells and has, upon further deeper studies, the potential to impact relapse capacity of tumor cells

Comment:
The authors discuss the role use of ROR1 to classify patients for treatment with ROR1 inhibitory related molecules or BET inhibitors. This is interesting and potentially very relevant from a translationally point of view. One of the points that hasn't been shown by the authors is whether the use of BET inhibitions works on tumors/cells that are ROR1 low (this was previously suggested as a complementary experiment). Since ROR1low cells may give rise to ROR1 high, it is likely that the treatment would also work. Additionally, ROR1 low cells themselves may respond to the treatment. In that case, both patients with high and low expression of ROR1 would benefit from the treatment. This would be, of course, also a nice outcome, but the conclusion of the current study in this regard would need to be revised.

Referee #3:

The authors have done a solid job at addressing concerns of the prior review. The revised manuscript is improved and the impact of the study is elevated.

I have no further experimental suggestions or concerns.

Manuscript number: EMBOJ-2022-112614R (RC-2022-01581)
Corresponding autor: Masaya Yamazaki

We have made the following editorial and formatting changes in our manuscript as instructed. Line numbers were referenced to the following document file: EMBOJ-2022-112614R-Data_edited_MS_file_masaya.docx

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Formatting changes required for the revised version of the manuscript:
>> Please limit the keywords to maximally five.

We have chosen five keywords in the edited manuscript (new line 47) as follows: “AURKB / BRD4 / pancreatic adenocarcinoma / ROR1 / tumor-initiating cells”

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.

We have removed the author contributions information from the manuscript text.

>> Please adjust the title of the 'Declaration of competing interests' section to 'Disclosure and Competing Interests Statement'.

We have changed the description regarding COI to “Disclosure and Competing Interests Statement” (new line 26).

>> Data availability section: Add a hyperlink to the database entry and make sure to release data privacy for the GSE dataset.

We have added some hyperlinks in the ‘data availability’ section (new lines 911–921).

>> Please remove the synopsis image from the manuscript .doc file.

We have removed the synopsis image from the edited manuscript .doc file.

>> Figure callouts: add callouts for Figure 3B and Table EV7 in the running text.

We apologize for forgetting the callout for Figure 3B and have added this description (new line 199). Table EV7 has been renamed and described as Table EV5 (new line 241).

>> EV Figures: EV figures need to be uploaded separately and limited to maximally five. If you need more space move figure panels to the appendix introducing Appendix Figures S1,S2... . The legends added to the manuscript after the main figure legends.

We have been authorized by Dr. Daniel Klimmeck, the editor, to proceed with Figure EV as six. We had described Figure EV legends in the manuscript after the main figure (new lines 1419–1483).

>> Figure 5A - please add highlight boxes.

We have added highlight boxes in Figure 5A and some descriptions in the figure legend (new lines 1328–1329).

>> EV Tables should be uploaded as individual files: Tables EV1, EV2, EV4, EV5, EV7, EV9, EV10 and EV11 should be uploaded as Table file type and renumbered accordingly; Tables EV3, EV6, and EV8 should be renamed to Dataset EV1-EV3 with the legends uploaded as a separate tab of each Excel file, and the corresponding callouts

We have changed Table EV and Dataset EV as instructed.

>> Appendix: a ToC is required on the first page of the appendix.

We have added a ToC to the appendix file.

>> Source data: source data files need to be reorganized to one file/folder per figure and ZIPing for each main figure.

We have changed the folder format of the source data as instructed.

>> Material & Methods: remove the typo: 'the experiments' in line 464,465. Move acknowledgements in lines 662-665 to the 'Acknowledgments' section. Move the current 'study Approval' information on mouse husbandry to the 'Mice' section. Introduce a separate 'Human samples' section, detailing the informed consent.

We have moved the typo in line 464,465 (new line 489).
We have also moved some descriptions from Material & Methods [Lentivirus preparation and establishment of stable cell lines] section to the Acknowledgments section as instructed (new lines 940–943).
We have removed the ‘study approval’ section, and added some description about study approval information to (1) the ‘Mice’ section (new lines 482–484), and (2) the new ‘Human samples’ section (new lines 904–908).

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

We have made the above changes and replies to editorial comments in track mode.

Dear Dr Yamazaki,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We would appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update of the entry status. Thank you in advance!

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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Corresponding Author Name: Masaya Yamazaki
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-112614R [RC-2022-01581]

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- 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
- 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

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods and Table EV11

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
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	Acknowledgement

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.	**Yes**	Materials and Methods

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
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**	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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**	Figure legends, Sourse data

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**	Fiigure
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legend

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.	**Yes**	Material Methods
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 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	

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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**	
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**	Reference