

RhoA^{Y42C} Drives Cell Plasticity and PD-1-Mediated Immune Evasion of Diffuse Gastric Cancer

Corresponding Author: Dr Jae-Il Park

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript titled “RHOA^{Y42C} Drives Cell Plasticity and PD-1-Mediated Immune Evasion of Diffuse Gastric Cancer” by Ko and colleagues investigates the role of RhoA in engineered murine gastric cancer organoids harboring mutant Kras and Trp53 (RKP) to model diffuse gastric cancer independently of Cdh1 loss. Building on their previous work, the authors compare this model with a second diffuse gastric cancer model driven by the combined loss of Cdh1 and mutant Kras and Trp53 (EKP). Using single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics, they examine whether cellular trajectories and the immune landscape differ between these two diffuse gastric cancer organoid models. Furthermore, the authors evaluate the efficacy of checkpoint blockade inhibition (CBI) across different murine models.

Major comments:

(1) The functional consequences of the distinct immune landscapes observed in the EKP and RKP models are not adequately addressed.

The authors show that EKP tumours contain more CD45⁺ immune cells compared to RKP tumours and demonstrate via scRNA seq and cell chat significant differences in immune checkpoint-related molecules, including PD-L1, PD-L2, ICOS, and CD86,. However, these findings are not further validation. For instance, the immune landscape analysis lacks immunohistochemical or flow cytometric validation of CD4⁺ and CD8⁺ T-cell subset populations across both models. Given that CTLA-4 is predominantly expressed on regulatory T cells, the data is important.

Furthermore, the rationale for selecting anti-PD1 as CBI of choice is unclear. The data suggest that RKP are less immune infiltrated than EKP tumours. What is the rational to treat these tumors with CBI and how can the effect be explained in the “cold” RKP model?

Testing anti-PD-L1/or anti-PD-L2 (instead of PD1) or anti-LAG3 may also be (potentially even more) relevant, since these have been identified in their scRNA sequencing and Xenium analyses.

(2) To assess the efficacy of checkpoint blockade inhibition in vivo is important and the authors should be congratulated to address this experimentally. Although the authors see a significant reduction in 2 out of 7 of their mice treated with aPD1, half of the algG treated mice (3/6) did not show proper tumour growth. It is recommended to re-perform the in vivo experiment and start treatment with mice that have a comparable luminescence signal)(instead of starting the treatment on a fixed day after injection with completely and uncomparable tumor loads).

In addition, it is not very well described how anti-PD1 treatment influences the immune landscape? Is there more infiltration or more activation? As diffuse cancers are usually seen to be “cold” tumours, it is important to analyse the therapy induced effect of anti-PD1. It remains unclear whether immune modulation leads to an increased proportion of CD8⁺ T cells in the RKP model and, more importantly, whether such changes translate into functional cytotoxic activity. Markers of CD8⁺ T-cell functionality, such as granzyme B expression, have not been evaluated. Furthermore, it is essential to determine whether CD4⁺ and CD8⁺ T cell subsets are activated or remain anergic following treatment.

(3) The authors describe a murine engineered organoid model, however, the subcutaneous and orthotopic mouse experiments rely on the 2D cultures of RKP and EKP cell lines rather than organoids. This switch from 3D to 2D before

injection might substantially impact the tumour signalling cascade, cellular interactions and the secretome. This might potentially limit the relevance of the in vivo findings. This issue should be discussed more explicitly.

Minor comments:

1. The authors claim that EKP tumours exhibit accelerated growth in Figures 1 and S1. In their spatial transcriptomic analyses however a higher MKI67 expression in RKP tumours observed (Figure 2E), suggesting increased proliferative activity. This apparent discrepancy is not discussed and requires clarification.
2. The figures need to include some more information as it was hard to follow how some information provided in the text was derived from e.g. Fig 1A-C.
3. Please amend the text accordingly for Supplemental Figure 1 A as it is stated in the text (1st paragraph on page 7) "without significant cell death" although the Figure S1A shows that there is indeed a difference.
4. The Supplemental Figure panel 4K-J is missing although it is described in the text.

Reviewer #2

(Remarks to the Author)

The authors leverage single-cell and spatial transcriptomics data from genetically engineered gastric organoids to investigate the oncogenic role of RHOA Y42C, demonstrating its sufficiency for DGC development and proposing PD-1 inhibition as a potential therapeutic strategy for DGC patients harboring this mutation. I have the following concerns:

- 1) The manuscript employs both single-cell and Xenium spatial transcriptomics data; however, it is difficult to determine from the figures and legends which results are derived from single-cell versus spatial data. The authors should clearly specify the data modality used for each analysis in both the main text and figure legends.
- 2) Batch effects between EKP and RKP samples are critical, particularly given the extensive gene expression comparisons and downstream conclusions. The authors should provide a clear description of batch-effect correction strategies and demonstrate the validity and robustness of cross-group comparisons.
- 3) In Fig. 2B–D, the authors conclude that EKP and RKP exhibit clear transcriptional separation. However, in fig2.B, the presented figures appear to primarily reflect differences in cell-type proportions rather than intrinsic transcriptional divergence. Furthermore, the marker genes shown in Fig. 2D do not convincingly support cluster identity and may indicate suboptimal clustering or limited data quality. The authors should refine clustering results and provide more specific and biologically informative marker genes. In addition, cluster annotations introduced in Fig. 3a appear abruptly, and their definition and biological validity should be clarified.
- 4) In Fig. 3, spatial transcriptomics data are mainly used to describe epithelial subtype distribution and infer spatial plasticity. Given the availability of multiple computational approaches for lineage inference from spatial omics data, the authors should provide more direct evidence derived from spatial data and evaluate whether spatial-based lineage inference supports their conclusions.
- 5) In Fig. 4c, it remains unclear how the authors ensured the reliability of cell–cell communication comparisons between groups, particularly in the presence of potential batch effects, sampling bias, and sequencing variability. The authors should clarify normalization strategies and robustness controls.
- 6) The authors attribute AREG expression primarily to cancer cells. However, prior work (Sig Transduct Target Ther 10, 313 (2025)) indicates that myeloid cells can also express AREG. Based on the spatial distribution of AREG alone, the current evidence does not conclusively support cancer-cell-specific expression. The authors should provide additional marker co-localization analyses for cancer and myeloid populations or discuss the possibility of shared or co-expression.

Reviewer #3

(Remarks to the Author)

In this work, the authors analyze the driver function of the RhoY42C mutation in gastric cancer. Using gastric organoids derived from a variety of mouse models, coupled to syngeneic grafts, they show key molecular differences between two gastric cancer genotypes, one comprising combined mutations of ECadherin (CDH)/KRAS/TP53 (EKP) and another comprising RHOA/KRAS/TP53(RKP). The key findings are 1) in human gastric cancer, CDH and RHOA mutations, while not mutually exclusive, are rarely found together; 2) The morphology, growth characteristics, and transcriptomes between these two genotypes have distinct characteristics, many of which are corroborated in human samples; 3) that RKP tumors appear to arise from two stem-like populations, one characterized by Runx1+ and another by Aqp5+; and 4) that the Aqp5+ derived cells activate Hippo/Yap signaling, resulting in increased transcription of Areg, leading to increased PDL1 expression and T-cell exhaustion.

All in all, this is an impressive body of work, with important therapeutic implications. There are, however, opportunities for clarification and improvement.

- 1) I am mystified as to why the EKP tumors grow faster than the RKP tumors as syngeneic grafts (Fig. 1D, 1F). It is strange and seemingly counterintuitive, because the authors show in Fig. 2E that the RKP organoids have more Ki67 staining than EKP. Also, given that the RKP tumors aren't even held in check by T cells, why don't they grow faster than the EKP tumors?
- 2) Verteporfen probably isn't the best choice for blocking Hippo pathway signaling. While I wouldn't insist, it would be better to do these experiments with a more precise and relevant TEAD inhibitor.

3) Whether verteporfin or a better TEAD inhibitor is used, I think the authors should follow-up their experiment in Supp Fig 6E, showing suppression of Areg by verteporfin, with its effects on T cell populations

4) While RhoA Y42C is discussed as being an activated form, this is still controversial and the competing views should be discussed. This is particularly important because the authors discuss strong activators such as RhoA V14 as evoking a different phenotype.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revised manuscript, the authors have substantially revised the mouse work and added additional checkpoint inhibitor data. Taken together, the authors show conclusively the oncogenic role of RhoA by dissecting the molecular pathology, and additional can come up with a potential therapeutic strategy to target these tumors.

All in all, the manuscript is now substantially improved by substantiating the claims with solid data.

Reviewer #2

(Remarks to the Author)

The authors have addressed my questions, and I have no further comments.

Reviewer #3

(Remarks to the Author)

I am satisfied with the author's responses to my critiques and those of the other reviewers

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Responses to Comments

Reviewer 1

“The manuscript titled ‘RHOAY42C Drives Cell Plasticity and PD-1-Mediated Immune Evasion of Diffuse Gastric Cancer’ by Ko and colleagues investigates the role of Rhoa in engineered murine gastric cancer organoids harboring mutant Kras and Trp53 (RKP) to model diffuse gastric cancer independently of Cdh1 loss. Building on their previous work, the authors compare this model with a second diffuse gastric cancer model driven by the combined loss of Cdh1 and mutant Kras and Trp53 (EKP). Using single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics, they examine whether cellular trajectories and the immune landscape differ between these two diffuse gastric cancer organoid models. Furthermore, the authors evaluate the efficacy of checkpoint blockade inhibition (CBI) across different murine models.”

Specific comments

A1. *“The functional consequences of the distinct immune landscapes observed in the EKP and RKP models are not adequately addressed. The authors show that EKP tumours contain more CD45⁺ immune cells compared to RKP tumours and demonstrate via scRNA seq and cell chat significant differences in immune checkpoint-related molecules, including PD-L1, PD-L2, ICOS, and CD86,. However, these findings are not further validated. For instance, the immune landscape analysis lacks immunohistochemical or flow cytometric validation of CD4⁺ and CD8⁺ T-cell subset populations across both models. Given that CTLA-4 is predominantly expressed on regulatory T cells, the data is important. Furthermore, the rationale for selecting anti-PD1 as CBI of choice is unclear. The data suggest that RKP are less immune infiltrated than EKP tumours. What is the rationale to treat these tumors with CBI and how can the effect be explained in the ‘cold’ RKP model? Testing anti-PD-L1/or anti-PD-L2 (instead of PD1) or anti-LAG3 may also be (potentially even more) relevant, since these have been identified in their scRNA sequencing and Xenium analyses.”*

We appreciate the reviewer’s insightful comments and fully agree that the rationale for selecting PD-1 and CTLA4 as key candidates was not sufficiently explained in the original manuscript.

1. To quantitatively characterize the immune landscape, we first compared T-cell populations between EKP and RKP tumors. Consistent with our previous findings showing reduced CD45⁺ immune-cell infiltration in RKP tumors, both CD8⁺ and CD4⁺ T-cell populations were decreased in RKP compared with EKP tumors (**Fig. R1A, B**). However, this observation does not necessarily indicate reduced tumor-immune interactions in RKP tumors. Our Xenium spatial analysis revealed distinct patterns of immune-cell localization between the two models. In EKP tumors, immune cells were broadly distributed throughout the tumor mass, whereas in RKP tumors they were preferentially enriched at the tumor periphery. These findings suggest that tumor-immune interfaces are spatially organized in different ways in the two models and may therefore support distinct modes of cellular communication.
2. To identify therapeutically relevant tumor-immune interactions, we adopted a stepwise validation strategy. First, candidate interactions were predicted using CellChat analysis. Second, the expression of candidate molecules was evaluated using scRNA-seq and spatial transcriptomic analyses to assess consistency across platforms. Finally, selected candidates were validated at the protein level by IHC.

The initial CellChat analysis identified several RKP-enriched signaling pathways, including PD-L1/PD-1, PD-L2/PD-1, CD86/CTLA4, and ICOS/ICOSL-related interactions. We then systematically evaluated these candidates using multimodal approaches, including scRNA-seq, Xenium spatial transcriptomics, and protein-level validation. Among the predicted interactions, PD-1 and CTLA4 consistently showed stronger expression in RKP-associated T cells in both scRNA-seq (**Fig. 4E**) and Xenium analyses (**Fig. 4J**). These findings were further validated at the protein level, where significantly higher numbers of PD-1⁺ and CTLA4⁺ cells were observed in RKP tumors by IHC (**Fig. 4K**). *The concordance across multiple independent platforms led us to prioritize these molecules for further investigation.*

In contrast, other molecules from these CellChat-predicted pathways showed less consistent evidence. Although PD-1 expression was significantly increased in RKP T cells, its ligands PD-L1 and PD-L2 did not

exhibit RKP-specific upregulation (**Fig. R1C, D**), and IHC analysis revealed no significant differences in PD-L1 or PD-L2 protein expression between EKP and RKP tumors (**Fig. R1E, F**). Similarly, although CTLA4 was consistently increased in RKP T cells, its interacting partner CD86 showed higher expression in the myeloid cells of EKP (**Fig. R1G**). However, this was not consistently observed at the protein level by IHC (**Fig. R1H, I**). Another CTLA4-interacting molecule, CD80, at the transcript level, was not significantly different between myeloid cells from EKP and RKP tumors (**Fig. R1G**). These differences across platforms likely reflect the distinct levels of biological information captured by each approach rather than low credibility of the data. CellChat predicts putative cell-cell interactions based on ligand-receptor compatibility, transcriptomic analyses measure gene expression levels, and IHC evaluates the abundance of cells expressing specific genes. Therefore, integrating these multimodal analyses and narrowing down the most consistently supported targets was an important part of our workflow.

ICOS signaling was also identified by CellChat analysis; however, it was not prioritized for further study because robust validation across multiple platforms was not feasible. ICOS and ICOSL were not included in our spatial transcriptomic gene panel, limiting comprehensive evaluation of this pathway. Furthermore, although *ICOS* mRNA expression was elevated in RKP T cells, **no significant difference was observed at the protein level**, and ICOSL expression was not increased in RKP myeloid cells (**Fig. R1G-I**).

Together, PD-1 and CTLA4 were selected not solely based on CellChat predictions but because they were the only candidates that demonstrated consistent evidence across transcriptomic, spatial, and protein-level analyses. To clarify this rationale, we have expanded the description of our candidate-selection workflow and included the additional validation analyses in the revised manuscript.

3. Although our original study focused on anti-PD-1 and anti-CTLA4 therapies based on the candidate-selection workflow described above, we agree with the reviewer that evaluating additional immune checkpoint inhibitors (ICIs) is valuable. As suggested, **in this revision, we tested the therapeutic efficacy of anti-PD-1, anti-PD-L1, anti-PD-L2, and anti-LAG3 antibodies** in the orthotopic RKP transplantation model. Consistent with our previous findings, treatment with anti-PD-1 significantly suppressed RKP tumor growth (**Fig. R2A, B**). Notably, **anti-PD-L1 and anti-LAG3 therapies also produced significant antitumor effects, whereas anti-PD-L2 treatment showed only partial efficacy in tumor suppression**. Anti-PD-L2 treatment displayed tumor suppression until week 5; however, the tumors eventually relapsed, resulting in no significant difference by week 7. Given that PD-L1 expression was substantially higher than PD-L2 expression in RKP tumors (**Fig. R1D**), the limited efficacy of anti-PD-L2 therapy is consistent with the expression patterns observed in our datasets, despite both PD-L1 and PD-L2 serving as ligands for PD-1.

For LAG3, we observed significant therapeutic efficacy despite not initially prioritizing this pathway in our original workflow. Both our scRNA-seq and Xenium spatial transcriptomic analyses demonstrated elevated LAG3 expression in RKP-associated T cells, suggesting that LAG3 contributes to T-cell dysfunction within the RKP tumor microenvironment. However, LAG3-mediated cell-cell interactions were not identified by our initial CellChat analysis and therefore were not included among the candidate pathways selected for further validation in the original study. Although CellChat identified enrichment of MHC-II signaling in RKP tumors, LAG3-mediated interactions are not incorporated into the CellChat MHC-II signaling network and therefore could not be evaluated through this analysis. As a result, LAG3 was not prioritized in our original workflow despite its elevated expression in RKP-associated T cells.

Importantly, the strong anti-tumor activity observed with anti-LAG3 treatment indicates that LAG3 may represent an additional therapeutic vulnerability in RKP tumors. These findings further suggest that complementary approaches beyond CellChat-based interaction analyses may be valuable for identifying biologically relevant immune checkpoint pathways. **We have added these new results and discussion to the revised manuscript and now highlight LAG3** as a promising candidate for future investigation in RHOA-mutant diffuse gastric cancer.

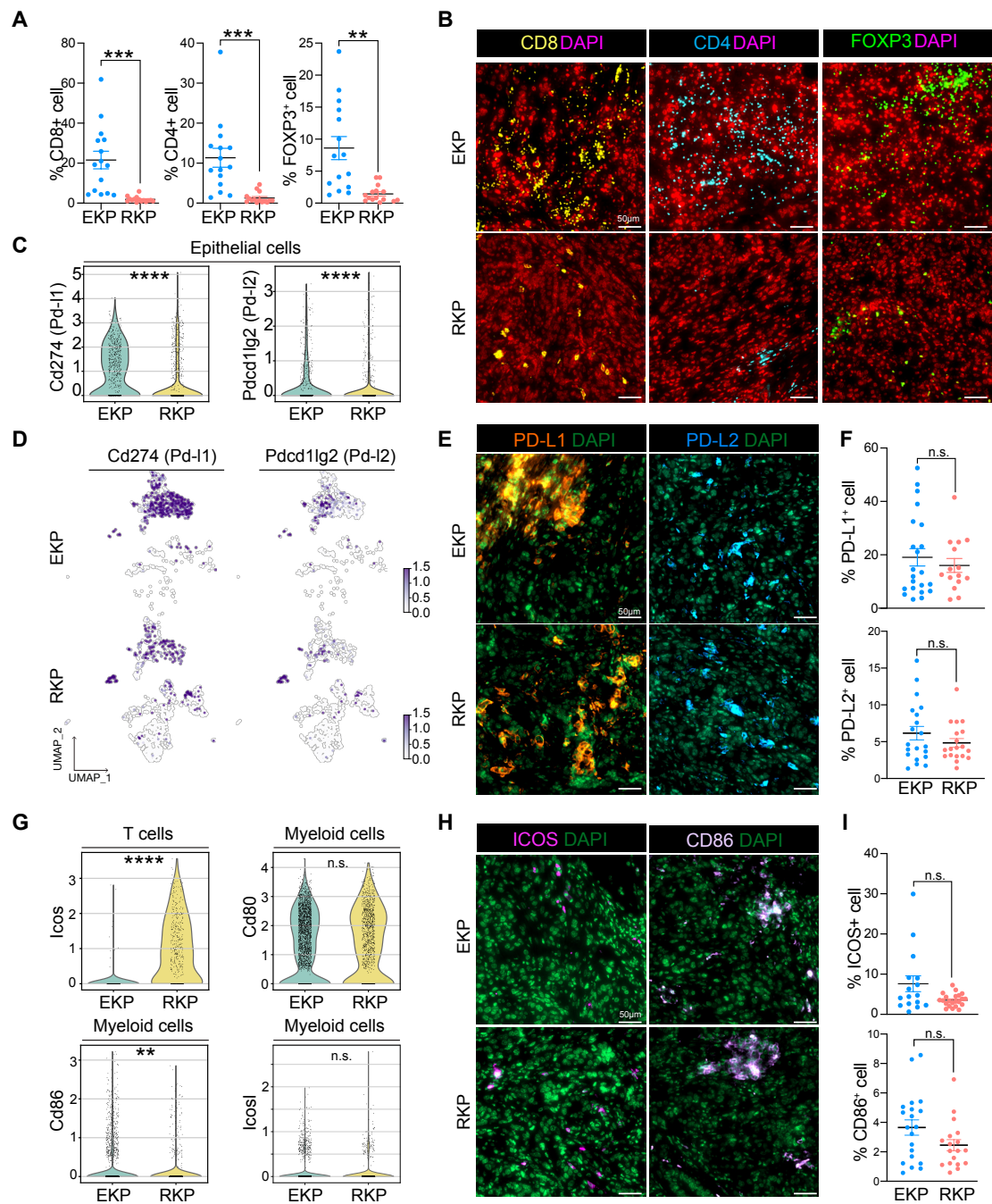


Figure R1. Immune niche of EKP and RKP tumors. **A, B.** Infiltrating CD8⁺, CD4⁺, and FOXP3⁺ T-cell populations were quantified and compared between EKP and RKP tumors. ***P* < 0.01; ****P* < 0.001. **C, D.** *Pd-I1* and *Pd-I2* expression in EKP and RKP tumor scRNA-seq data were compared using violin plots (C) and UMAP visualization (D). *****P* < 0.0001. **E, F.** PD-L1⁺ and PD-L2⁺ cell populations were compared between EKP and RKP tumors using IHC (E) and quantified (F). **G.** Expression levels of *Icos*, *Cd80*, *Cd86*, and *Icosl* were compared using scRNA-seq data. ***P* < 0.01; *****P* < 0.0001; n.s.: Not significant. **H, I.** ICOS- and CD86-expressing cells were detected by IHC (H) and quantitatively compared (I).

A2. *“To assess the efficacy of checkpoint blockade inhibition in vivo is important and the authors should be congratulated to address this experimentally. Although the authors see a significant reduction in 2 out of 7 of their mice treated with aPD1, half of the algG treated mice (3/6) did not show proper tumour growth. It is recommended to re-perform the in vivo experiment and start treatment with mice that have a comparable luminescence signal)(instead of starting the treatment on a fixed day after injection with completely and uncomparable tumor loads). In addition, it is not very well described how anti-PD1 treatment influences the immune landscape? Is there more infiltration or more activation? As diffuse cancers are usually seen to be “cold” tumours, it is important to analyse the therapy induced effect of anti-PD1. It remains unclear whether immune modulation leads to an increased proportion of CD8⁺ T cells in the RKP model and, more importantly, whether such changes translate into functional cytotoxic activity. Markers of CD8⁺ T-cell functionality, such as granzyme B expression, have not been evaluated. Furthermore, it is essential to determine whether CD4⁺ and CD8⁺ T cell subsets are activated or remain anergic following treatment.”*

We deeply appreciate the reviewer's critical evaluation.

As kindly suggested, **we re-performed the orthotopic transplantation** experiment. At this time, after the orthotopic transplantation surgery, we kept mice for 3 weeks to allow the stable establishment of tumor before initiating treatment. Once two consecutive luciferase signals with an ROI > 105, were detected, mice were treated with ICIs (anti-PD-1, anti-PD-L1, anti-PD-L2, and anti-LAG3) or the IgG control. Rather than initiating treatment at a synchronized time point, this signal-dependent strategy allowed treatment to begin at a comparable tumor burden, providing a more reliable assessment of tumor growth and therapeutic response.

In this revised experiment, we obtained clearer results, particularly in the comparison between the IgG control and anti-PD-1 treatment. The mean tumor signal decreased to 0.433 following anti-PD-1 treatment, whereas it increased approximately sixfold (6.039) in the control group during the experimental period (**Fig. R2A** and **Fig. 6N**). Anti-PD-L1 and anti-LAG3 also significantly suppressed tumor growth, with mean values of 0.635 and 0.539, respectively. In contrast, anti-PD-L2 treatment showed only a slight increase in the mean signal (1.903), indicating limited therapeutic efficacy. **Overall, anti-PD-1 showed the strongest antitumor effect.**

Simultaneously, we evaluated the immune landscape following anti-PD-1 treatment by analyzing several T-cell populations. Because the tumor mass was too small in the orthotopic transplantation model, particularly in anti-PD-1-treated mice, we used subcutaneous tumors for this analysis, where the therapeutic effect of anti-PD-1 was relatively modest (**Supplementary Fig. 6K**). Our group has recently compared the tumor microenvironments (TMEs) of subcutaneous and orthotopic gastric tumor transplantation models and found that T cells are more active in the orthotopic TME, whereas T-cell infiltration and exhaustion phenotypes are more readily observed in subcutaneous tumors.¹ Therefore, the stronger tumor suppression observed in the orthotopic model is likely attributable to greater T cell activity. In contrast, the relatively larger residual tumors in the subcutaneous model may reflect a higher degree of exhaustion in the infiltrated T cells, providing a practical advantage for evaluating the function of infiltrating T cells. Based on this rationale, we analyzed changes in T cell function following anti-PD-1 treatment by immunohistochemistry (IHC).

Interestingly, we found that total CD8 T-cell and effector T-cell (Perforin⁺) populations were increased in anti-PD-1-treated RKP tumors, whereas the CD4 T-cell population did not show a significant difference. These findings support our hypothesis that RKP induces T-cell exhaustion by impairing CD8 T-cell activity (**Fig. R2C, D**).

We greatly appreciate the reviewer's constructive comment. The additional experiments and analyses performed in response to this suggestion have significantly enriched the manuscript and were **added** in the **Results** section.

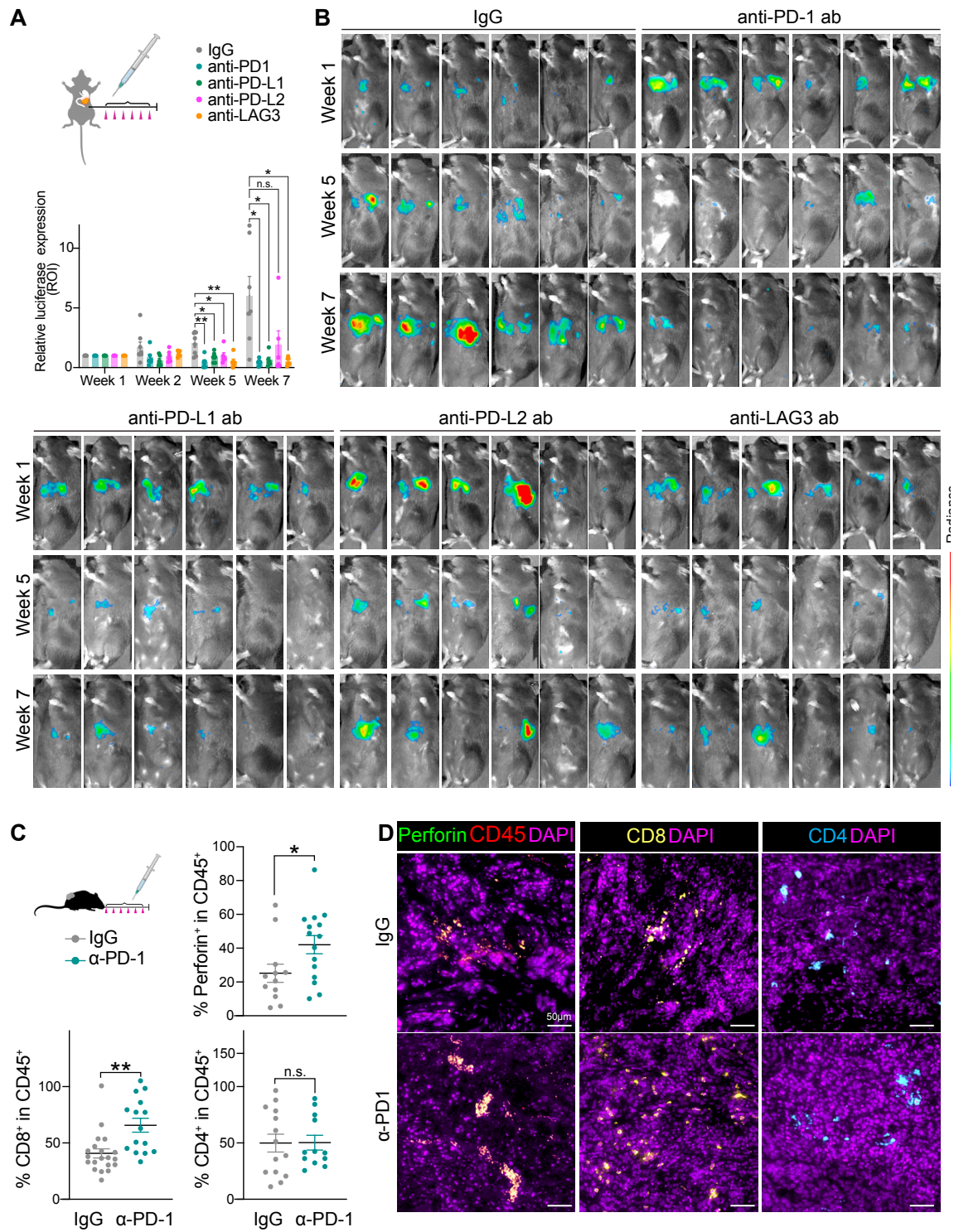


Figure R2. Efficacy of ICLs in RKP tumors. **A.** Orthotopically transplanted RKP tumor-bearing mice were treated with ICLs, including anti-PD-1, anti-PD-L1, anti-PD-L2, and anti-LAG3 antibodies. Tumors were monitored until 7 weeks after treatment initiation. t-test results are shown. * $P < 0.05$; ** $P < 0.01$; n.s.: Not significant. **B.** Luciferase activity of orthotopically transplanted tumors was measured using the S2 LagoX system and visualized. **C, D.** Characterization of infiltrating T cells in RKP tumors. Effector T cell (Perforin⁺), CD8 T cell, and CD4 T cell populations within RKP subcutaneous tumors were detected by IHC (D) and quantitatively analyzed (C). * $P < 0.05$; ** $P < 0.01$.

A3. *"The authors describe a murine engineered organoid model, however, the subcutaneous and orthotopic mouse experiments rely on the 2D cultures of RKP and EKP cell lines rather than organoids. This switch from 3D to 2D before injection might substantially impact the tumour signalling cascade, cellular interactions and the secretome. This might potentially limit the relevance of the in vivo findings. This issue should be discussed more explicitly."*

We appreciate the reviewer's insightful comment regarding the potential impact of using 2D-cultured cells for in vivo transplantation. we agree that the transition from 3D organoid culture to 2D expansion prior to transplantation could potentially influence cellular signaling, drug response, cell-to-cell interactions, or secretory profiles²⁻⁵.

In our study, the engineered organoids were first established and genetically validated in 3D culture. For the in vivo experiments, cells derived from these **organoids were expanded in 2D** culture prior to subcutaneous and orthotopic transplantation **to obtain sufficient numbers of viable cells** for reproducible implantation.

Importantly, the phenotypes observed in the 2D-derived cells were validated in multiple aspects supporting their biological relevance. For example, the RKP model showed high expression of AQP5, MUC1, and MUC4, which are also highly expressed in human DGAC tumors (**Fig. 2T**). In addition, **single-cell transcriptomic analysis of organoid-derived cells demonstrated strong similarity** between RKP-derived tumors and human tumors using TCGA datasets of signet ring cell carcinoma (SRCC) and diffuse gastric adenocarcinoma (DGAC) (**Supplementary Fig. 2A, B**).

We have **added a statement** in the **Discussion** section to explicitly acknowledge this limitation and clarify the context in which the in vivo findings should be interpreted

A4. *"The authors claim that EKP tumours exhibit accelerated growth in Figures 1 and S1. In their spatial transcriptomic analyses however a higher Mki67 expression in RKP tumours observed (Figure 2E), suggesting increased proliferative activity. This apparent discrepancy is not discussed and requires clarification."*

We thank the reviewer for pointing out this important point and for the opportunity to clarify this point.

First, **the spatial transcriptomic and single-cell analyses were performed on EKP and RKP tumors collected at different time points but at comparable tumor sizes**. Therefore, the Mki67 expression shown in **Fig. 2E** reflects proliferative activity at a similar tumor stage, rather than proliferation rates at the same time point during tumor development. As a result, these markers do not directly reflect the different tumor growth kinetics observed in the syngeneic graft experiments shown in **Fig. 1E**.

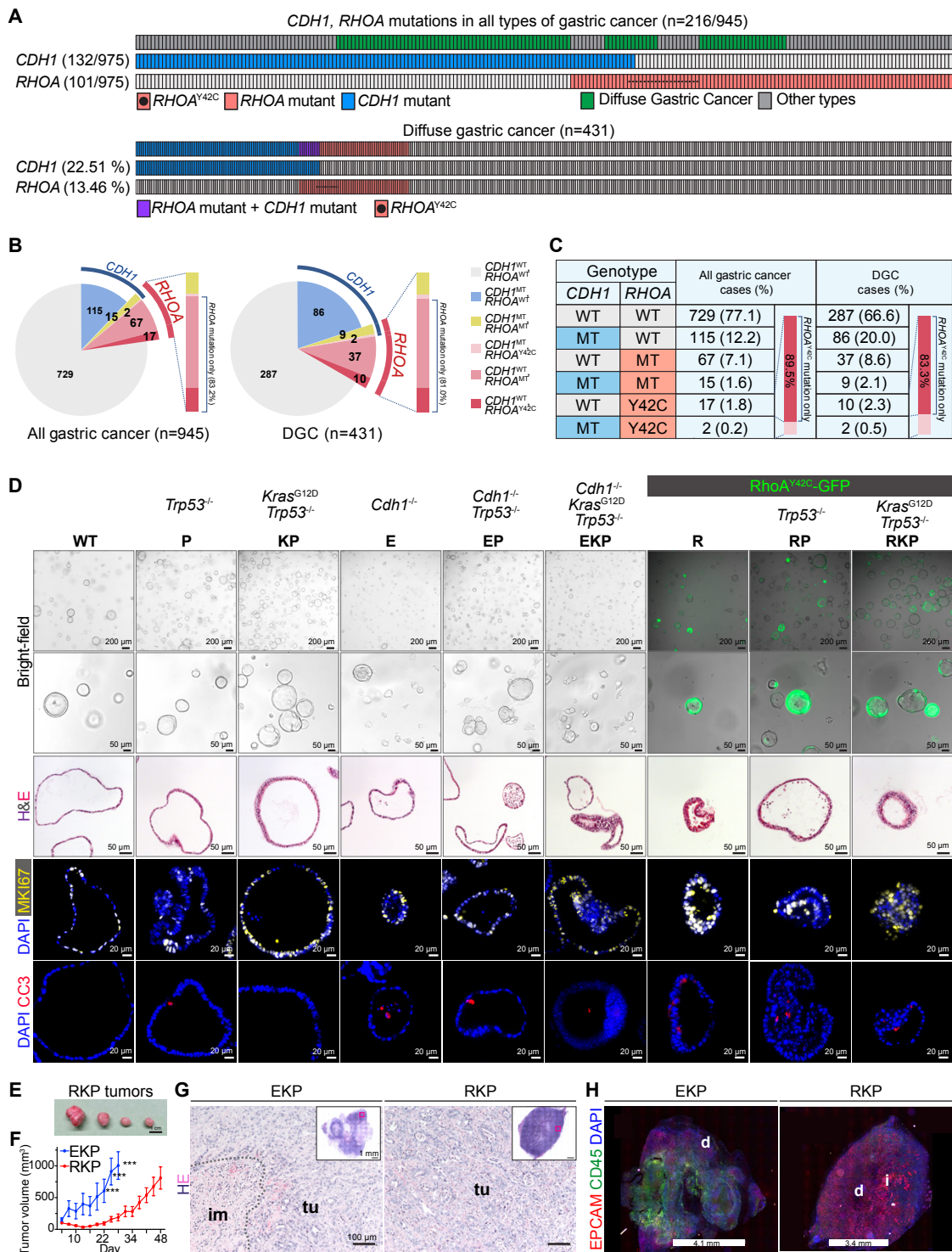
Second, the **cellular composition** of EKP and RKP tumors **differs** substantially. EKP tumors contain extensive immune cell infiltration, meaning that a significant fraction of the tumor volume consists of non-epithelial cells. In contrast, RKP tumors are more densely composed of epithelial tumor cells. Consequently, proliferation markers such as Mki67 could be higher within the epithelial compartment of RKP tumors even though the overall tumor growth rate appears faster in EKP tumors.

Third, our analysis indicates that **Mki67 expression is enriched in an RKP-specific epithelial lineage** (**Supplementary Fig. 3A**), rather than in the lineage shared by both EKP and RKP tumors. Thus, the increased Mki67 signal likely reflects lineage-specific proliferative activity rather than globally increased proliferation across the entire tumor.

Importantly, we do not claim that RKP tumors intrinsically grow faster than EKP tumors. Consistent with this, in vitro growth assays-where immune surveillance is absent-show that **EKP cells exhibit slightly faster cell-autonomous growth than RKP cells** (**Supplementary Fig. 1C**), although the difference is modest compared with the tumor growth differences observed in vivo. We speculate that this reflects differences in cellular heterogeneity and selective pressures between in vitro and in vivo environments. In the absence of immune pressure in vitro, lineages involved in immune evasion may not confer a growth advantage. In contrast, within the immune microenvironment in vivo, selective pressure may favor expansion of RKP-specific cell lineage, which display high proliferative activity in the scRNA-seq and Xenium analyses.

We have clarified these points in the revised manuscript in the **Discussion** section to better explain the relationship between proliferation marker expression and tumor growth kinetics and to avoid potential confusion.

A5. "The figures need to include some more information as it was hard to follow how some information provided in the text was derived from e.g. Fig 1A-C."



Revised Figure 1.

We appreciate the reviewer's important comment and apologize for the unclear description and figure presentation in the previous version. To improve clarity and better link the text with the corresponding figures, we revised **Fig. 1A-C**. In particular, we **added bar plots** in **Fig. 1B** and **1C** to clearly illustrate the cases with *RHOA* mutations without *CDH1* mutations, which are discussed in the Results section. We also **revised** the main text to explicitly reference the corresponding figure panels so that the derivation of the results from **Fig. 1A-C** is easier to follow.

A6. *"Please amend the text accordingly for Supplemental Figure 1 A as it is stated in the text (1st paragraph on page 7) "without significant cell death" although the Figure S1A shows that there is indeed a difference."*

We appreciate the reviewer's important comment. We agree that the previous wording ("without significant cell death") did not accurately reflect the differences observed in **Supplementary Fig. 1A**. We have therefore revised the Results section to more precisely describe the extent of cell death observed among the different organoid genotypes, consistent with the data presented in **Supplementary Fig. 1A**.

A7. *"The Supplemental Figure panel 4K-J is missing although it is described in the text."*

We thank the reviewer for pointing this out. After carefully re-examining the manuscript and supplementary figures, we confirmed that **Supplementary Figure 4** was correctly labeled in the original submission and does not contain panels **J** or **K**. The main text also does not refer to **Supplementary Fig. 4J-K**.

However, during this careful review prompted by the reviewer's comment, we identified a labeling error in **Supplementary Figure 5**. In the original version, panels **E** and **G** were incorrectly labeled. These have now been corrected to **D** and **E** in both the figure and the corresponding text to ensure consistency throughout the manuscript.

We greatly appreciate the insightful and constructive comments.

Reviewer 2

“The authors leverage single-cell and spatial transcriptomics data from genetically engineered gastric organoids to investigate the oncogenic role of RHOA Y42C, demonstrating its sufficiency for DGC development and proposing PD-1 inhibition as a potential therapeutic strategy for DGC patients harboring this mutation. I have the following concerns:”

Specific comments

B1. *“The manuscript employs both single-cell and Xenium spatial transcriptomics data; however, it is difficult to determine from the figures and legends which results are derived from single-cell versus spatial data. The authors should clearly specify the data modality used for each analysis in both the main text and figure legends.”*

We sincerely appreciate the reviewer’s insightful comment. In response, we have revised the figure legends throughout the manuscript to clearly indicate whether each result was generated from scRNA-seq or Xenium in situ spatial transcriptomics data. This clarification has been added to all relevant figure legends in the revised manuscript to ensure that the data modality used for each analysis is clearly specified.

B2. *“Batch effects between EKP and RKP samples are critical, particularly given the extensive gene expression comparisons and downstream conclusions. The authors should provide a clear description of batch-effect correction strategies and demonstrate the validity and robustness of cross-group comparisons.”*

We sincerely thank the reviewer for the valuable comments regarding batch correction strategies. We apologize for not providing sufficient methodological details in the original submission and have now added this information to the Materials and Methods section.

We fully appreciate the concern regarding potential batch effects, particularly because EKP and RKP samples were collected and sequenced at different time points. To address this, both datasets were processed using a consistent preprocessing pipeline in Scanpy. Cells were filtered using `sc.pp.filter_cells` (`min_genes = 200`) and genes were filtered using `sc.pp.filter_genes` (`min_cells = 3`). Gene expression values were then normalized using `sc.pp.normalize_total`. Additional quality control filtering was applied based on gene counts (`adata.obs.n_genes_by_counts < 8000`) and mitochondrial gene proportion (`adata.obs.pct_counts_mt < 20%`).

Following quality control, epithelial cells were isolated based on established marker gene expression. EKP and RKP epithelial datasets were then integrated using the “concatenate” function in Scanpy, followed by batch correction using the Harmony algorithm.

Subsequent analyses, including calculation of neighbors, Leiden clustering, and computing UMAP were performed on the batch-corrected integrated dataset. We have previously evaluated multiple batch correction methods, including BBKNN, Harmony, ComBat, and Scanorama, in large-scale integration analyses of 69 human datasets⁶. Based on those comparative assessments, Harmony demonstrated optimal performance and compatibility with our analytical workflow, and was therefore selected for the current study.

To increase the reliability of our batch correction, we additionally calculated AvgBio and AvgBatch scores using the scIB (single-cell Integration Benchmarking) package.⁷ AvgBio was used to assess how well biological structure, such as cell-type clustering, was preserved after integration, whereas AvgBatch was used to evaluate how effectively technical batch effects were removed. A higher AvgBatch score indicates that cells of the same type from different batches are well mixed, rather than clustering according to their source or experimental batch. In this analysis, EKP and RKP showed the same AvgBio score before and after batch correction (0.566 to 0.566), indicating that the biological signal was preserved without loss of structure. Notably, the AvgBatch score increased from 0.737 to 0.876 after batch correction, demonstrating that batch effects were successfully reduced. These details regarding preprocessing and batch correction have been **added to the Materials and Methods** section of the revised manuscript.

We sincerely apologize for the omission of these details in the initial manuscript and appreciate the reviewer's comment, which has allowed us to clarify this important aspect of our analysis.

B3. *"In Fig. 2B–D, the authors conclude that EKP and RKP exhibit clear transcriptional separation. However, in fig2.B, the presented figures appear to primarily reflect differences in cell-type proportions rather than intrinsic transcriptional divergence. Furthermore, the marker genes shown in Fig. 2D do not convincingly support cluster identity and may indicate suboptimal clustering or limited data quality. The authors should refine clustering results and provide more specific and biologically informative marker genes. In addition, cluster annotations introduced in Fig. 3a appear abruptly, and their definition and biological validity should be clarified."*

We sincerely thank the reviewer for the thoughtful and constructive comments regarding the interpretation of **Fig. 2B-D** and the clarity of cluster annotation in **Fig. 3A**.

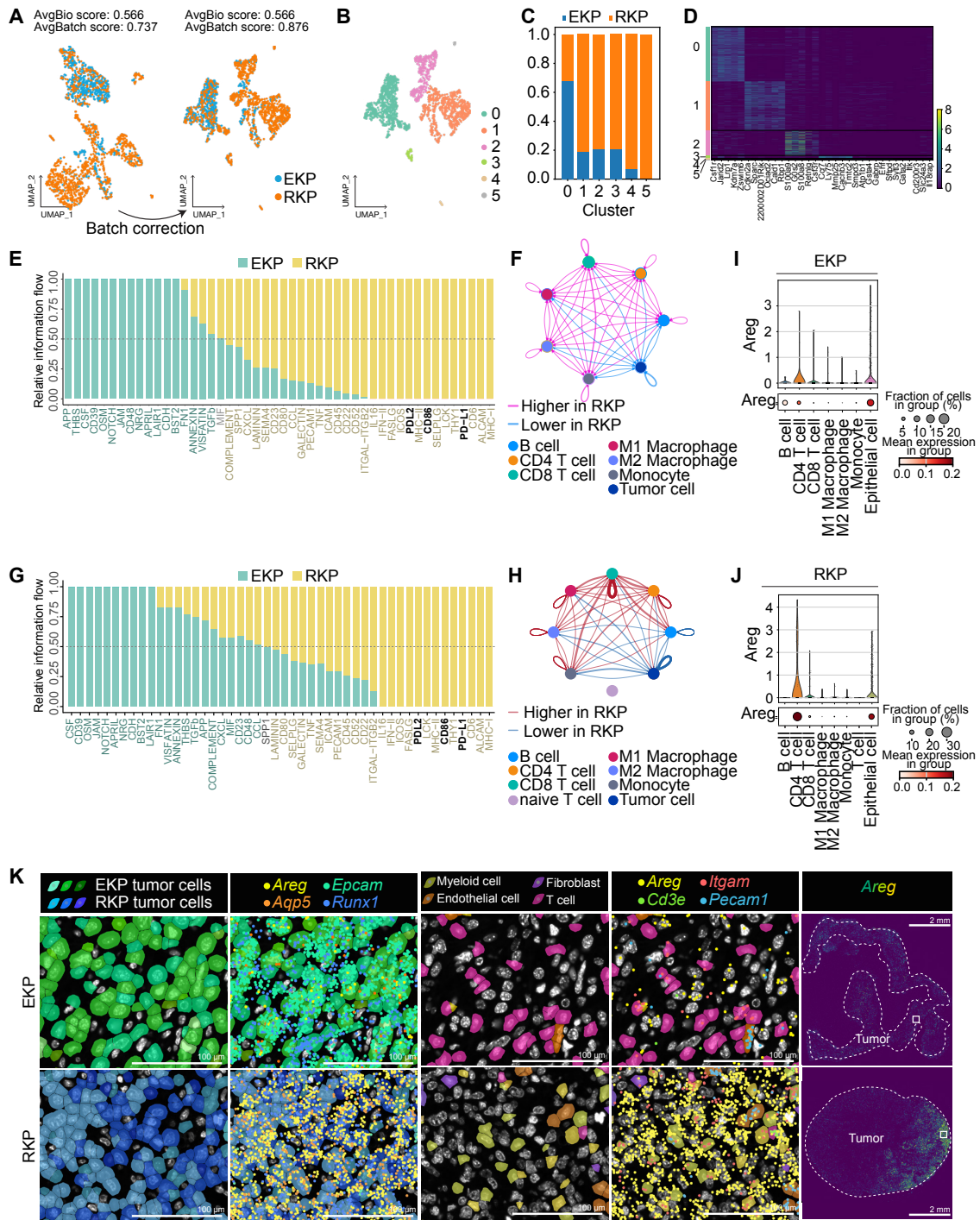
We agree that the original description could have overstated intrinsic transcriptional separation between EKP and RKP tumor cells. As the reviewer correctly pointed out, the primary distinction observed in **Fig. 2B** largely reflects differences in cell-type proportions rather than complete transcriptional segregation. **Accordingly, we have revised** the Results section to state more precisely that "EKP and RKP tumor cells displayed distinct proportional distributions across unbiased clusters, suggesting genotype-associated enrichment of specific cellular states." To better illustrate this point, we added stacked bar plots showing the genotype composition within each cluster, allowing clearer visualization of proportional differences.

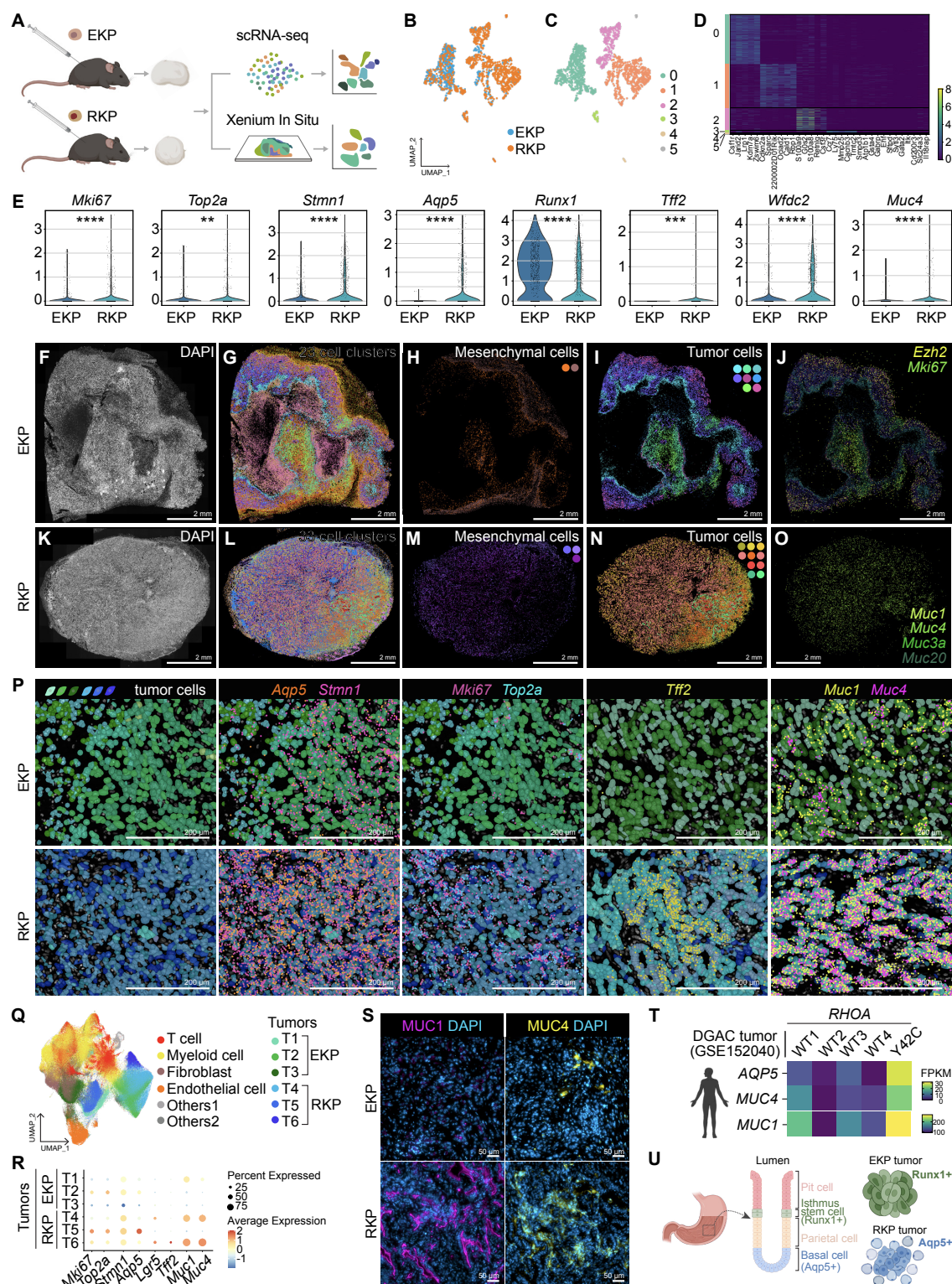
To address concerns regarding clustering robustness and marker gene validity, we refined the clustering strategy. *After Harmony-based batch correction (performed in the original submission), we re-evaluated dimensional reduction and clustering using updated package versions to ensure reproducibility.* Minor differences in UMAP structure resulting from version updates are now shown in **Fig. R3A, B**. We systematically tested multiple Leiden resolution parameters and selected an optimized resolution (0.05), which provided improved separation of clusters and clearer marker gene specificity compared to the previous resolution (0.1). Although this resulted in fewer clusters, marker gene expression patterns are now more distinct and biologically coherent. These results were **added** to the revised **Fig. 2** and **Supplementary Fig. 2**.

Regarding **Fig. 3A**, we apologize for the abrupt introduction of cluster annotations and for insufficient explanation of their biological basis. The clustering shown in **Fig. 3A** was performed independently using the scVelo pipeline, where EKP and RKP datasets were processed separately (filtering, normalization, selection of 2,000 highly variable genes, clustering, and velocity estimation). We have now clarified this workflow in the Methods and Results sections.

For cluster annotation, we used established stem and lineage-associated markers (e.g., *Aqp5*, *Runx1*) to define stem-like populations, *Krt8* to indicate proliferative epithelial states, and *Zeb2* to suggest EMT-associated transitions. *Given that well-defined single-cell references for diffuse-type gastric cancer (DGC) are limited, additional annotations were based on robust cluster-specific DEGs with clear expression enrichment patterns.* We now specifically **describe the criteria used for annotation** and **provide supporting DEG lists** in the revised supplementary materials to strengthen biological validity.

Overall, we have revised the clustering strategy, improved marker specificity, clarified annotation rationale, and refined the interpretation to distinguish proportional differences from intrinsic transcriptional divergence. This detailed information was added in the **Materials and Methods** section. We believe these revisions substantially strengthen the rigor and clarity of the analysis and thank the reviewer again for helping us improve the manuscript.





Revised Figure 2.

B4. “In Fig. 3, spatial transcriptomics data are mainly used to describe epithelial subtype distribution and infer spatial plasticity. Given the availability of multiple computational approaches for lineage inference from spatial omics data, the authors should provide more direct evidence derived from spatial data and evaluate whether spatial-based lineage inference supports their conclusions.”

Thank you for the insightful comments and suggestions.

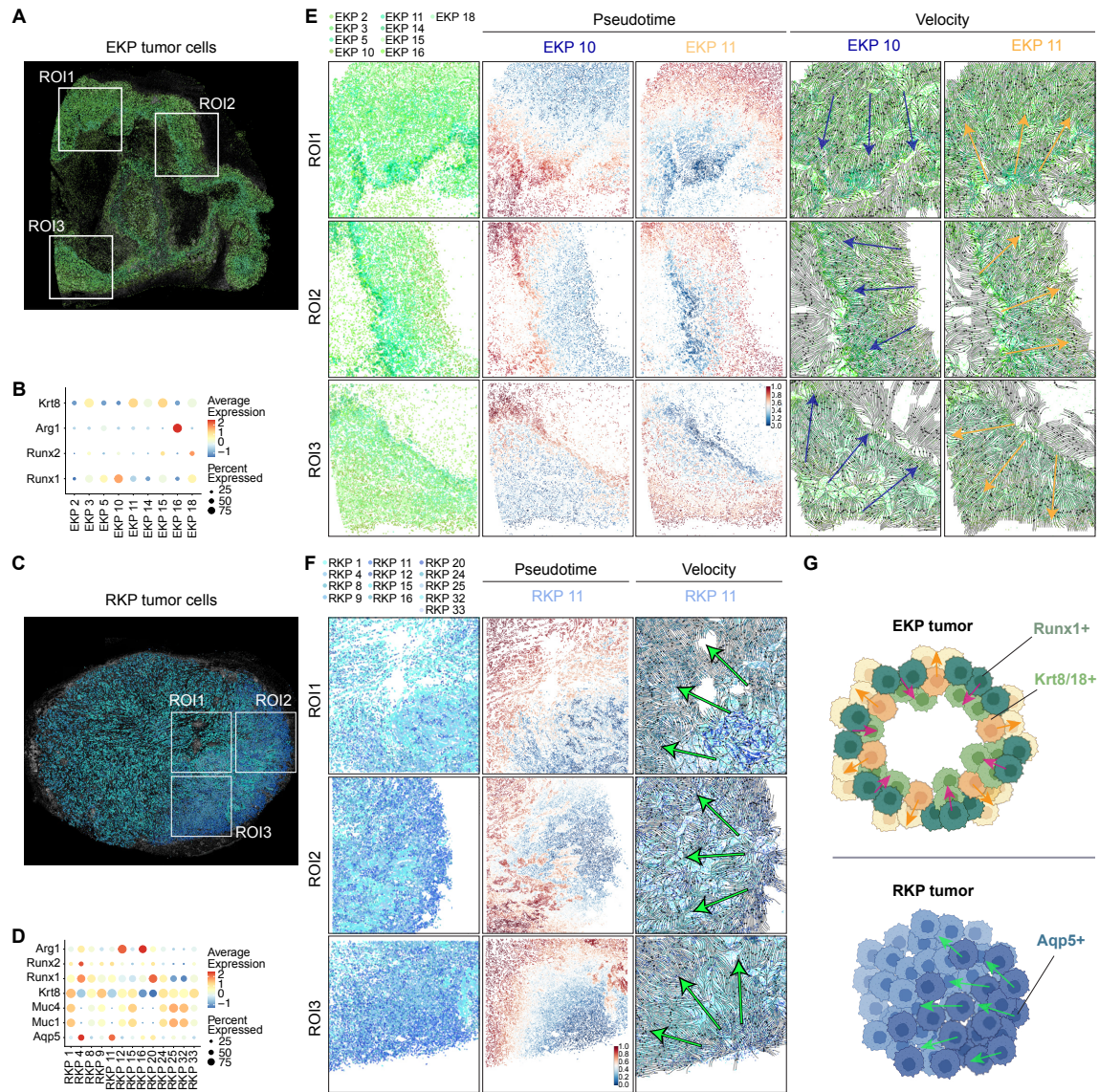
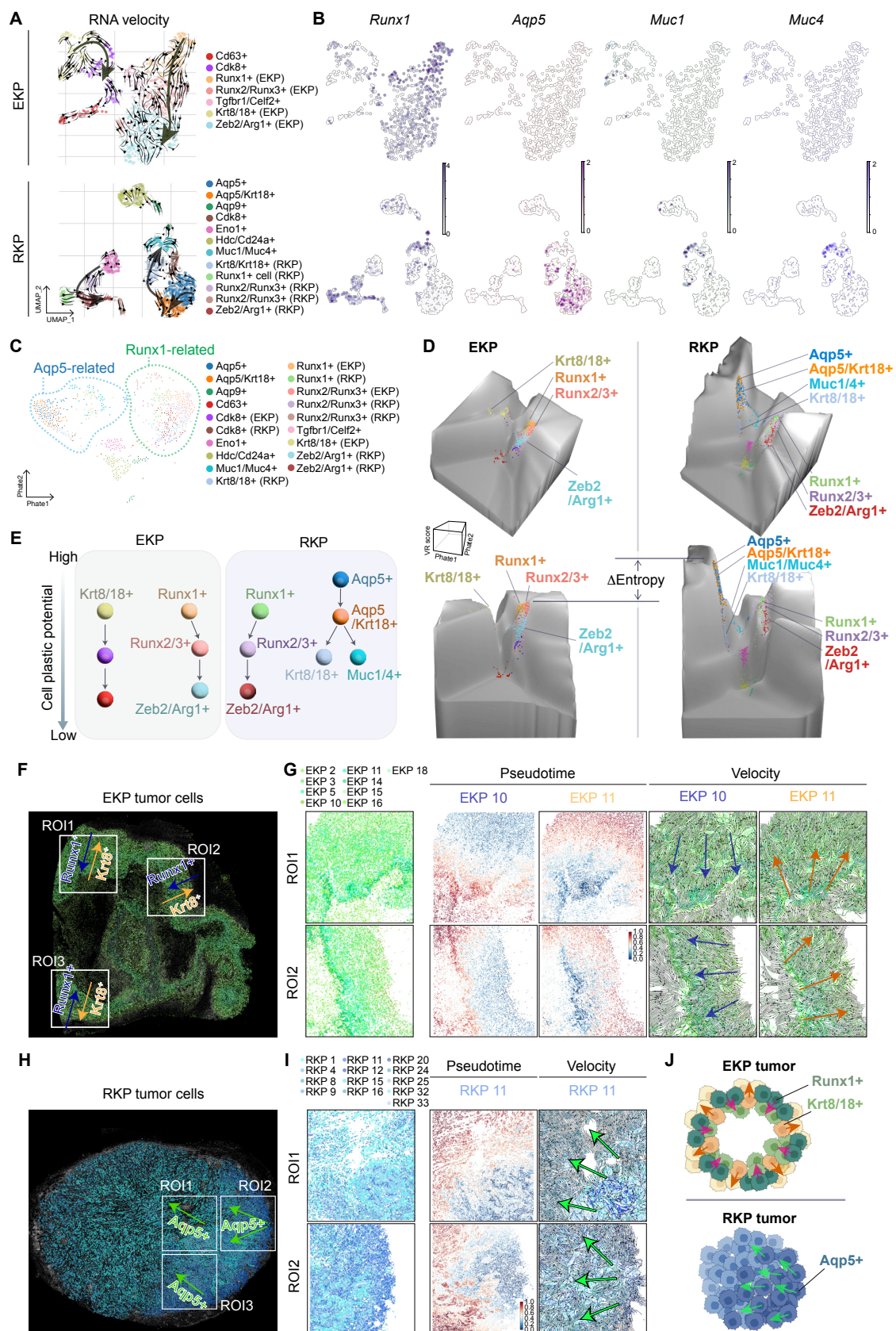


Figure R4. Spatial cell lineage trajectory analysis using SpaTrack in EKP and RKP tumors. **A.** Three ROIs in EKP tumors. **B.** EKP10 and EKP11 clusters were selected as starting cells based on *Runx1* and *Krt8* expression. **C.** Spatial maps of cell clusters, pseudotime, and velocity are shown to illustrate EKP cell lineage trajectories. **D.** Three ROIs in RKP tumors. **E.** The RKP11 cluster was selected as the starting cell population based on its exclusive expression of *Aqp5* and low expression of *Arg1*. **F.** Spatial maps of cell clusters, pseudotime, and velocity are shown to illustrate RKP cell lineage trajectories. **G.** Schematic representation of EKP and RKP tumor cell trajectories.

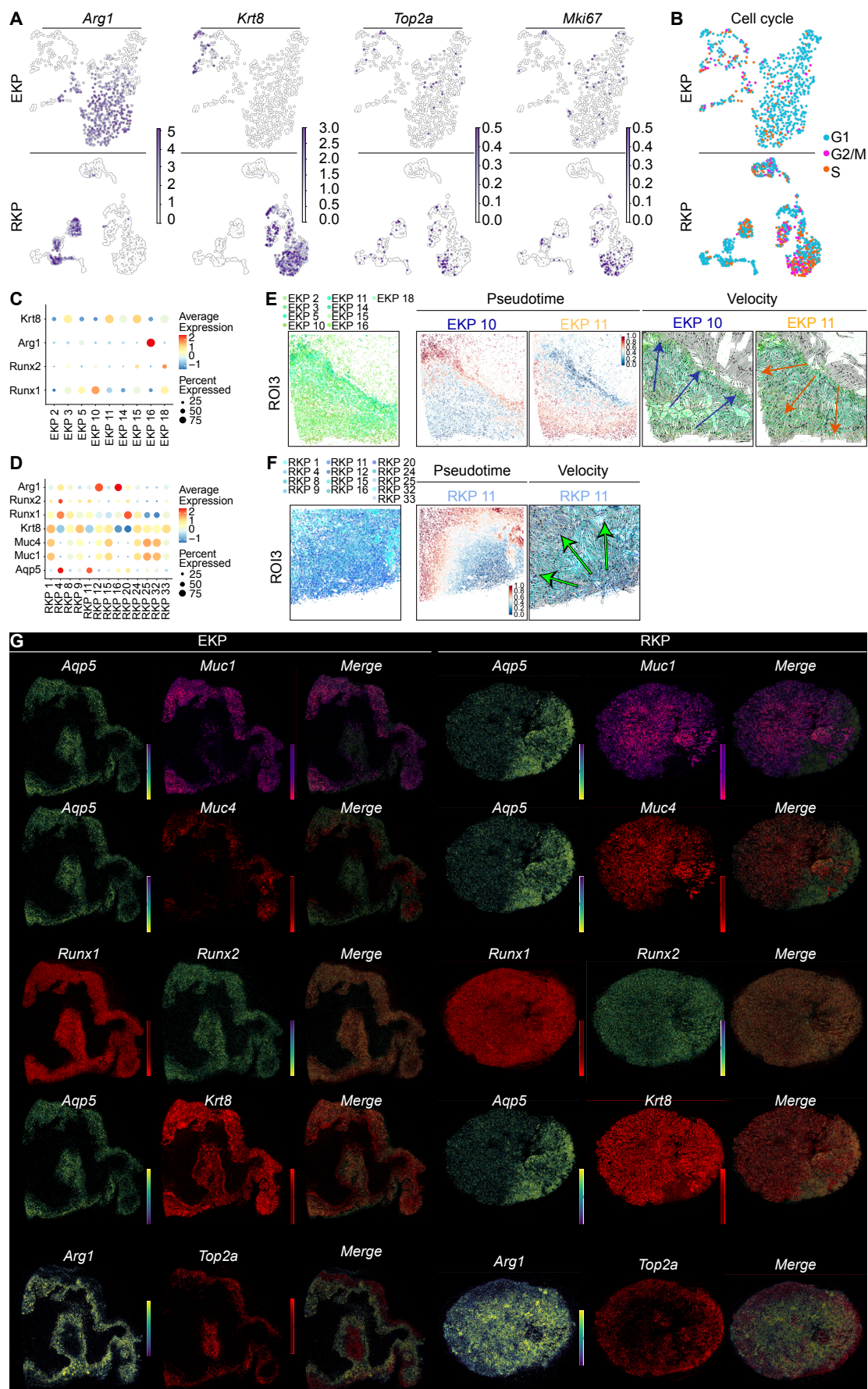
Based on the reviewer’s comment, we **re-performed cell lineage trajectory analysis using spaTrack**.⁸ Consequently, the SpaTrack-based cell lineage showed a perfect match with our previous demonstration. In this analysis, we set three ROIs in both EKP and RKP tumors and assessed the expression of key stem-like cell marker genes, including *Runx1*, *Krt8*, and *Aqp5*, in each tumor cell cluster of EKP and RKP tumors to determine the starting cell populations, (**Fig. R4A-D**). For EKP, EKP10 and EKP11 were selected as starting cell populations because they strongly and exclusively expressed *Runx1* and *Krt8*, respectively. For RKP,

RKP11 was selected because it showed exclusive and strong expression of *Aqp5*. We then analyzed the lineage directions from these cell clusters. Surprisingly, EKP tumors showed opposing lineage directions between Runx1⁺ and Krt8⁺ cells. Runx1⁺ cells exhibited a cortex-to-core lineage direction, whereas Krt8⁺ cells displayed a core-to-cortex lineage direction across three different ROIs (**Fig. R4E**). Unlike EKP tumors, RKP tumors showed a distinct cell lineage trajectory that did not appear to be related to cortex or core localization. In the Aqp5⁺ cell-derived lineage, the trajectory showed a simple spreading pattern from a small region of the tumor mass toward other areas of the tumor mass (**Fig. R4F**). These results indicated that distinct cell lineages driven by different tumor-associated driver genes mutations are subject to specific directional pressures during tumor mass development (**Fig. R4G**).

We added these results in the revised figures (**Fig. R4**, **Fig. 3F-J** and **Supplementary Fig. 3C-F**) and main text.



Revised Figure 3.



Revised Supplementary Figure 3.

B5. *“In Fig. 4c, it remains unclear how the authors ensured the reliability of cell–cell communication comparisons between groups, particularly in the presence of potential batch effects, sampling bias, and sequencing variability. The authors should clarify normalization strategies and robustness controls.”*

We sincerely thank the reviewer for raising this important point regarding the reliability of cell-cell communication comparisons in Fig. 4C. During careful re-examination of our analysis, we identified an error in the normalization procedure used for the CellChat analysis. Specifically, we generated the AnnData object in Python and transferred the gene expression matrix into the R environment to construct the CellChat object. During this transfer, raw count data were used. However, due to the internal handling of the createCellChat function, the raw counts were stored in the @data slot rather than @data.raw, which led us to mistakenly assume that normalization had been applied automatically. Upon recognizing this issue, we re-performed the CellChat analysis after explicitly applying manual library-size normalization followed by log-transformation (log1p), consistent with the normalization strategy implemented internally by CellChat. The raw counts were retained in @data.raw, and the normalized matrix was assigned to the @data slot prior to downstream communication inference.

All CellChat analyses were then recomputed using the properly normalized data. The revised results have replaced the original panels in the main figures (**Fig. 4C, 4D**). Importantly, the key findings remain consistent: RKP tumors continue to show significantly enhanced PD-L1, PD-L2, and CD86-mediated interactions compared with EKP tumors, supporting our original conclusions.

In addition, we acknowledge that insufficient description of preprocessing and batch correction procedures in the initial submission may have contributed to this concern. As detailed in our response to Comment B2, we have now expanded the Materials and Methods section to clearly describe quality control filtering, normalization, integration, and Harmony-based batch correction prior to downstream analyses.

To minimize technical variability, EKP and RKP datasets were processed using an identical preprocessing pipeline in Scanpy, including cell and gene filtering, library-size normalization (sc.pp.normalize_total), mitochondrial filtering, and integration followed by Harmony batch correction. Epithelial cells and immune cells were separately integrated, batch corrected, and annotated with each cell type. Then, epithelial cells and immune cells from each genotype (EKP and RKP) were integrated with cell type label information. Gene count data and annotation information was used for creating CellChat object.

Regarding cell-cell communication inference, we recognize that CellChat is a computational framework that infers potential interactions based on ligand-receptor expression rather than directly measuring physical cell-cell contacts. To improve robustness, we used identical normalization and scaling procedures across groups.

Importantly, to complement the inference-based approach, we performed spatial transcriptomic validation using Xenium in situ analysis. Although the Xenium gene panel does not cover all ligand–receptor pairs included in the CellChat database, key tumor cell-T cell and myeloid cell-T cell interaction signatures, particularly those associated with T cell exhaustion, were validated spatially. These spatial data provide orthogonal support for the communication patterns inferred from single-cell transcriptomics.

We agree that computational inference tools have inherent limitations; therefore, our conclusions rely not solely on CellChat outputs but on concordant evidence spatial validation. We have clarified these normalization strategies, robustness controls, and validation steps in the **Materials and Methods** section of revised manuscript.

We appreciate the reviewer’s insightful comment, which has allowed us to improve the transparency and rigor of our methodological description.

B6. *“The authors attribute AREG expression primarily to cancer cells. However, prior work (Sig Transduct Target Ther 10, 313 (2025)) indicates that myeloid cells can also express AREG. Based on the spatial distribution of AREG alone, the current evidence does not conclusively support cancer-cell-specific expression. The authors should provide additional marker co-localization analyses for cancer and myeloid populations or discuss the possibility of shared or co-expression.”*

We sincerely thank the reviewer for raising this important point regarding the cellular source of AREG and for highlighting prior work (Sig Transduct Target Ther 10, 313 (2025)) demonstrating that myeloid cells can express AREG. We agree that spatial distribution of AREG alone is insufficient to conclude cancer-cell-specific expression and appreciate the opportunity to clarify and strengthen this analysis.

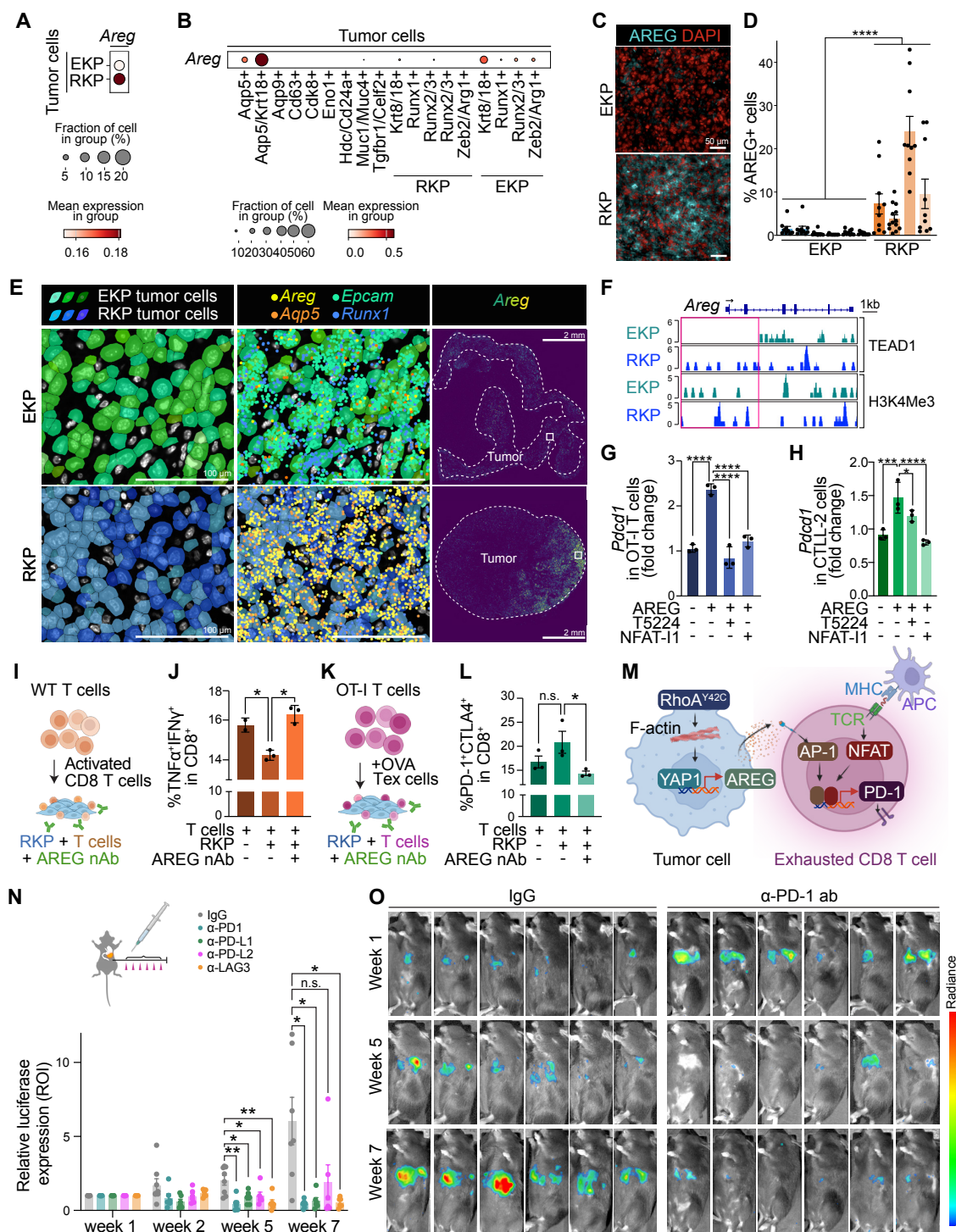
To address this concern, we re-analyzed Areg expression across all annotated cell populations in our scRNA-seq datasets. The updated results are presented (**Fig. R3I-K**) and added in the revised figures (**Fig. 5E** and **Revised Supplementary Fig. 6E**). In both EKP and RKP tumors, epithelial cells exhibit the highest and most consistent Areg expression levels. In contrast, myeloid populations, including macrophage subsets (M1- and M2-like) and monocytes, show minimal to low Areg expression relative to epithelial cells in our dataset.

We did observe relatively high Areg expression in CD4 T cells. However, this enrichment is driven by a very small number of CD4 T cells, which can result in visual overrepresentation. As shown in the violin plots, the absolute number of CD4 T cells is substantially lower than that of epithelial cells. Therefore, although detectable, the overall contribution of CD4 T cells to total Areg expression is limited. We have clarified this point in the revised manuscript to avoid overinterpretation.

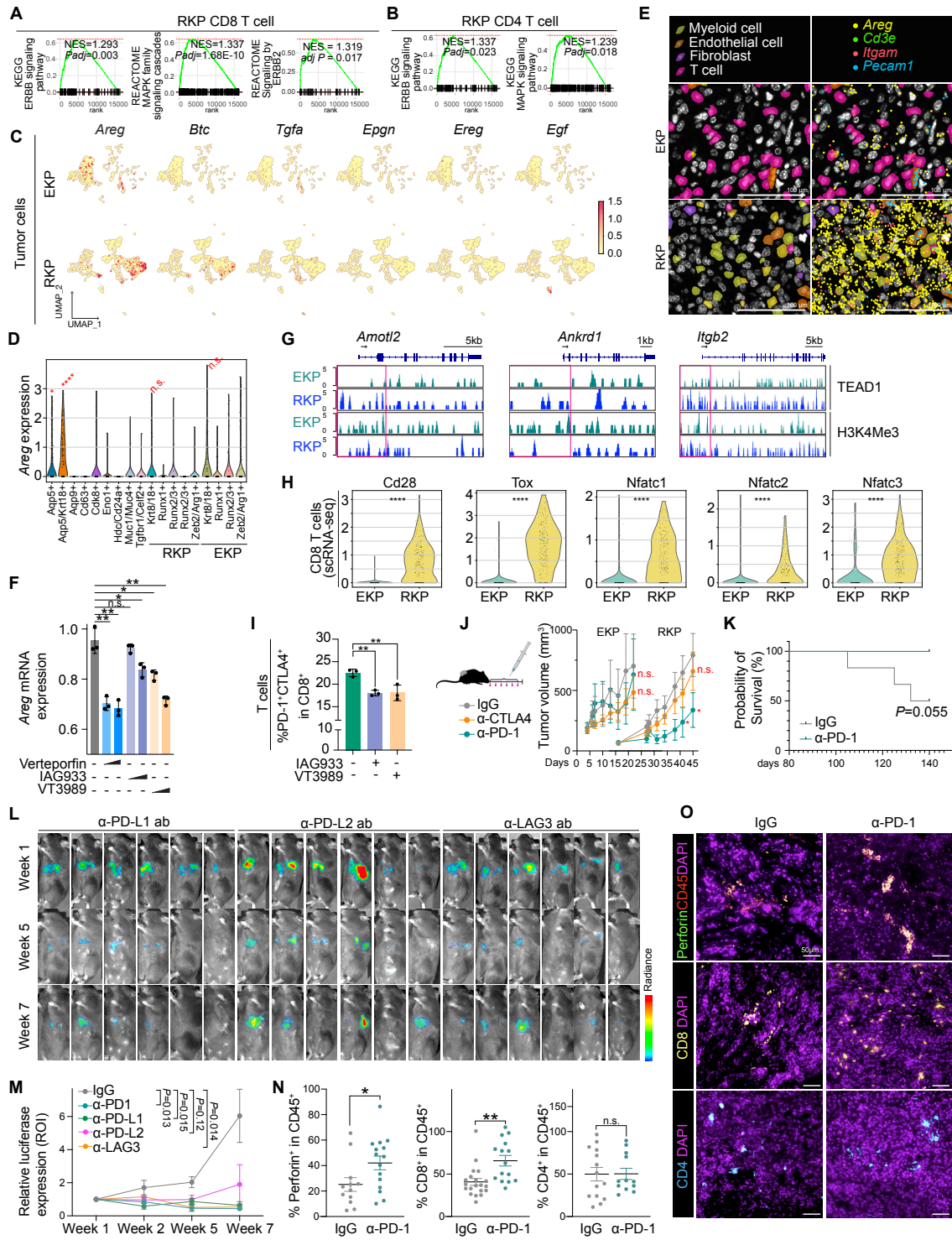
Importantly, we agree that displaying Areg alone in the Xenium spatial data could potentially mislead readers. Therefore, as suggested, we expanded our spatial analysis to include additional epithelial and myeloid cell-type markers and overlaid clear cell boundary segmentation on every cell. This approach enables precise assessment of transcript localization at the single-cell level. The revised Xenium figures demonstrate that Areg transcripts predominantly co-localize with epithelial-marker-positive cells, while overlap with canonical myeloid-marker-positive cells are limited in our model.

Based on these combined single-cell and spatial analyses, we conclude that epithelial tumor cells represent the major source of Areg expression in EKP and RKP tumors in our dataset. At the same time, we have revised the manuscript to avoid absolute statements regarding exclusivity and now acknowledge that myeloid-derived AREG has been reported in other tumor contexts. We have also cited the relevant literature in the **Results** section of the revised manuscript.

We appreciate the reviewer’s insightful comment, which has substantially improved the rigor and clarity of our analysis and interpretation.



Revised Figure 5.



Revised Supplementary Figure 6.

Reviewer 3

"In this work, the authors analyze the driver function of the RhoY42C mutation in gastric cancer. Using gastric organoids derived from a variety of mouse models, coupled to syngeneic grafts, they show key molecular differences between two gastric cancer genotypes, one comprising combined mutations of ECadherin (CDH)/KRAS/TP53 (EKP) and another comprising RHOA/KRAS/TP53(RKP). The key findings are 1) in human gastric cancer, CDH and RHOA mutations, while not mutually exclusive, are rarely found together; 2) The morphology, growth characteristics, and transcriptomes between these two genotypes have distinct characteristics, many of which are corroborated in human samples; 3) that RKP tumors appear to arise from two stem-like populations, one characterized by Runx1+ and another by Aqp5+; and 4) that the Aqp5+ derived cells activate Hippo/Yap signaling, resulting in increased transcription of Areg, leading to increased PDL1 expression and T-cell exhaustion. All in all, this is an impressive body of work, with important therapeutic implications. There are, however, opportunities for clarification and improvement."

Specific comments

C1. *"I am mystified as to why the EKP tumors grow faster than the RKP tumors as syngeneic grafts (Fig. 1D, 1F). It is strange and seemingly counterintuitive, because the authors show in Fig. 2E that the RKP organoids have more Ki67 staining than EKP. Also, given that the RKP tumors aren't even held in check by T cells, why don't they grow faster than the EKP tumors?"*

We sincerely thank the reviewer for raising this important point. We agree that the relationship between the growth kinetics of EKP and RKP tumors and the proliferation marker expression may appear counterintuitive, and we appreciate the opportunity to clarify this.

First, EKP and RKP tumors were collected at different time points, but at comparable tumor sizes, for single-cell RNA-seq analysis. Specifically, EKP tumors were harvested on day 29, whereas RKP tumors were harvested on day 48. Therefore, the proliferation markers shown in [Fig. 2E](#) reflect proliferative activity at comparable tumor sizes, rather than proliferation rates at the same chronological time point during tumor development. Consequently, the proliferation marker expression observed in tumor epithelial cells at the endpoint does not directly represent the early tumor growth kinetics observed in the syngeneic graft experiments. We have clarified this point in the **Materials and Methods** section of the revised manuscript.

Second, the cellular composition of EKP and RKP tumors differs substantially from that of organoid culture conditions. EKP tumors are heavily infiltrated by immune cells, indicating that a substantial fraction of the tumor volume consists of non-epithelial cells ([Fig. 1H](#) and [Supplementary Fig. 5A](#)). In contrast, RKP tumors are more densely composed of epithelial tumor cells. As a result, proliferation markers such as *Mki67* and *Top2a* appear higher in RKP epithelial cells. However, the overall tumor growth rate, which is measured based on tumor length and width, can still appear faster in EKP tumors because a larger proportion of the tumor mass includes stromal and immune components. These non-epithelial components contribute to the total tumor volume, despite the lower relative proportion of epithelial tumor cells.

Third, proliferation markers are enriched in an RKP-specific epithelial lineage ([Supplementary Fig. 3A](#)), rather than in the lineage shared between EKP and RKP tumors (*Runx1-Zeb2/Arg1*). This indicates that the increased MKi67 signal reflects lineage-specific proliferation within RKP tumors, rather than a globally higher proliferative activity across the entire tumor.

Finally, our data suggest that the RKP-specific lineage promotes T-cell exhaustion. Expansion of this lineage likely contributes to accelerated tumor progression at later stages. However, the development of T-cell exhaustion requires sustained antigen exposure, typically over 2-4 weeks,⁹ which may explain why the initial growth of RKP tumors is slower than that of EKP tumors in the syngeneic graft setting. Consistent with this interpretation, a recent study showed that continuous antigen exposure for 28 days induced severe T-cell exhaustion,¹⁰ corresponding to the time point when RKP tumors began to show increased tumor growth in our model ([Fig. 1F](#) and [Supplementary Fig. 6J](#)). Once immune exhaustion is established, RKP tumors may subsequently gain a growth advantage.

We have clarified these points in the **Discussion** section of revised manuscript to better explain the apparent discrepancy between proliferation marker expression and tumor growth kinetics.

C2. “*Verteporfin probably isn't the best choice for blocking Hippo pathway signaling. While I wouldn't insist, it would be better to do these experiments with a more precise and relevant TEAD inhibitor.*”

We sincerely appreciate the insightful and constructive comments.

Although verteporfin has been widely studied and is FDA-approved for the treatment of choroidal neovascularization¹¹, its application in cancer therapy remains limited by concerns regarding adverse effects, including photosensitivity and toxicity.¹² Furthermore, the anti-tumor activity of verteporfin remains controversial. Several studies have reported that verteporfin suppresses tumor growth through inhibition of YAP signaling¹³⁻¹⁶, whereas others have suggested that its tumor-suppressive effects may occur independently of YAP inhibition.^{17,18}

To further support our findings that Areg expression is regulated by the RhoA^{Y42C}-YAP signaling axis, other TEAD-specific inhibitors, IAG933 and VT3989, were tested in this revision. Unlike verteporfin, which has been reported to exert both YAP-dependent and YAP-independent effects, **IAG933** and **VT3989** directly disrupt the YAP–TEAD protein-protein interaction.^{19,20} Both agents have demonstrated promising preclinical activity and are currently being evaluated in clinical trials for cancer treatment.^{19,20} The use of these mechanistically distinct TEAD inhibitors therefore allowed us to more specifically assess the contribution of YAP/TEAD signaling to Areg regulation and the associated immune phenotypes observed in our model. The experimental results were described in the following section.

C3. “*Whether verteporfin or a better TEAD inhibitor is used, I think the authors should follow-up their experiment in Supp Fig 6E, showing suppression of Areg by verteporfin, with its effects on T cell populations*”

We sincerely appreciate the insightful and constructive comments.

To address this question, we first compared the effects of verteporfin, IAG933, and VT3989 on Areg expression in RKP cells. Notably, all three inhibitors significantly suppressed Areg expression, although the strongest suppression was observed with verteporfin (**Fig. R5A**).

Next, we investigated whether inhibition of YAP/TEAD signaling could influence T-cell exhaustion induced by RKP cells. Following the protocol described in the manuscript, CD8 T cells were isolated from OT-I mouse spleens and stimulated with OVA peptide for 7 days to induce exhaustion. Exhausted T cells were subsequently co-cultured with RKP cells for an additional 4 days. IAG933 or VT3989 was added 2 days before the experimental endpoint. At the endpoint, T cells were collected and analyzed by flow cytometry. Importantly, treatment with either IAG933 or VT3989 significantly reduced the proportion of exhausted T cells compared with vehicle-treated controls (**Fig. R5B, C**). These findings support our hypothesis that Areg is transcriptionally regulated by YAP/TEAD signaling and contributes to the promotion of T-cell exhaustion. We have added these results to the revised **Supplementary Fig 6F,I** and to the main text.

As a final validation, we performed an allograft transplantation experiment to determine whether inhibition of YAP/TEAD signaling could suppress T-cell exhaustion within the tumor microenvironment in vivo. For these studies, **we selected verteporfin because it is an FDA-approved drug and therefore provides potential translational relevance as a drug repurposing strategy**, whereas IAG933 and VT3989 remain under clinical investigation.¹⁹⁻²² Moreover, verteporfin showed the strongest suppression of Areg expression in our in vitro experiments (**Fig. R5A**). Verteporfin treatment resulted in a modest reduction in tumor growth; however, this effect did not reach statistical significance (**Fig. R5D**). Despite the limited effect on tumor size, verteporfin markedly altered the immune landscape of RKP tumors. Specifically, we observed increased infiltration of total CD8 T cells and cytotoxic (Perforin⁺) CD8 T cells, whereas CD4 T-cell populations were not significantly affected (**Fig. R5E,F**). These results further support a role for YAP/TEAD signaling in shaping an immunosuppressive tumor microenvironment and promoting T-cell exhaustion in RKP tumors. Although verteporfin showed a favorable effect on the immune landscape, additional studies are needed to determine

whether it can synergize with other therapeutic strategies, such as combination therapy with ICIs, to achieve clinically meaningful tumor suppression. These experiments will be included in our future study.

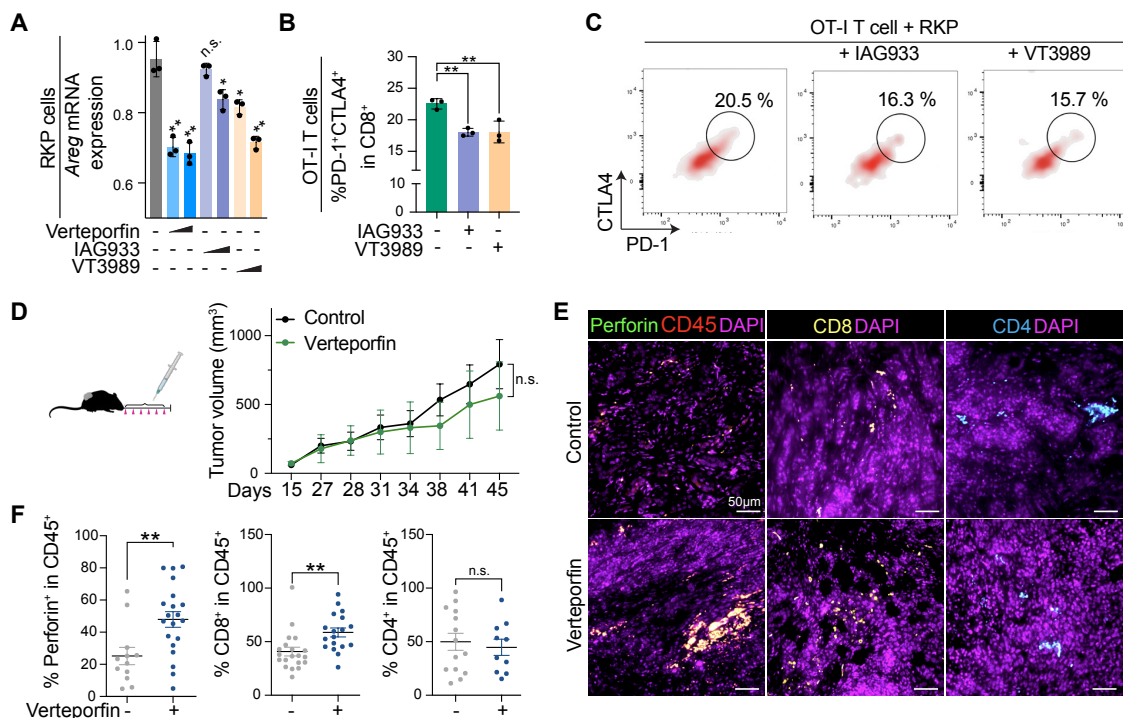


Figure R5. Effects of YAP/TEAD inhibitors. **A.** Verteporfin, IAG933, and VT3989 were tested in RKP cells to assess their effects on Areg expression. * $P < 0.05$; ** $P < 0.01$; n.s.=Not significant. **B, C.** Effects of TEAD inhibitors, IAG933 and VT3989, on T-cell exhaustion. OT-I T cells were cultured with RKP and treated with TEAD inhibitors. Exhausted T cells were evaluated by FACS (C) and quantified (B). **D.** Effect of verteporfin on tumor suppression. **E, F.** Effect of verteporfin on infiltrating T cells. Effector T-cell (Perforin⁺), CD8 T-cell, and CD4 T-cell populations were quantified using IHC images (E) and quantified (F). * $P < 0.05$; ** $P < 0.01$.

C4. “While RhoA Y42C is discussed as being an activated form, this is still controversial and the competing views should be discussed. This is particularly important because the authors discuss strong activators such as RhoA V14 as evoking a different phenotype.”

We thank the reviewer for this important comment. We understand the controversy over the Y42C mutation. Like other small GTPases (Ras, Rac1, and Cdc42), well-characterized activating mutations such as G14V or Q63L impair intrinsic and GAP-mediated GTP hydrolysis, resulting in persistent GTP-bound RhoA and robust activation of downstream signaling pathways, including ROCK-mediated actomyosin contractility.²³⁻²⁵ These mutants typically induce pronounced stress fiber formation, increased contractility, and widespread cytoskeletal remodeling. As such, they represent canonical constitutively active forms of RhoA.

One related study on Rac and Cdc42 showed that such mutations in the effector loop abolish the downstream signaling.²⁶ Similarly, additional studies observed that *RHOA* mutations at Y42 **impair interactions between RhoA and several downstream effector proteins**.²⁷⁻²⁹ Based on this impaired interaction with effector molecule, such as Rhotekin, Y42 mutation was regarded as having loss-of-function on the downstream signaling pathway.²⁹ However, similar to the WT, the Y42C mutant was shown to activate RhoA's downstream pathways, including the actin cytoskeleton and SRF activation.²⁷ Moreover, later study has demonstrated that the **RhoA^{Y42C} mutant alters effector specificity by increasing ROCK-binding affinity while decreasing Rhotekin affinity**.³⁰ **This altered effector specificity impairs GTP hydrolysis activity, resulting in a gain-of-function phenotype** in the canonical RhoA signaling pathway.^{30,31} In addition, multiple independent studies have shown that Y42C promotes phenotypes associated with diffuse gastric cancer progression, including enhanced cell survival, resistance to anoikis, altered cell adhesion, epithelial plasticity, and activation of signaling pathways such as YAP/TAZ.²⁹⁻³⁴ Notably, YAP/TAZ signaling has been

reported to be driven by activated canonical RhoA signaling accompanied by enhanced stress fiber formation, further supporting the possibility that the Y42C mutation enhances RhoA function.^{30,35}

Therefore, although the molecular mechanism of RhoA Y42C within canonical RhoA signaling remains controversial, recent mechanistic studies increasingly support its gain-of-function oncogenic activity in diffuse gastric cancer, with several features overlapping with those of classical constitutively active mutants such as G14V or Q63L.

As kindly suggested, we have revised the **Discussion** to discuss both perspectives.

We sincerely appreciate all insightful and constructive comments.

Reviewer 4

“I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.”

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Responses to Comments

Reviewer 1

“In this revised manuscript, the authors have substantially revised the mouse work and added additional checkpoint inhibitor data. Taken together, the authors show conclusively the oncogenic role of RhoA by dissecting the molecular pathology, and additional can come up with a potential therapeutic strategy to target these tumors. All in all, the manuscript is now substantially improved by substantiating the claims with solid data.”

We sincerely thank the reviewer for the positive assessment of our revised manuscript and for the insightful and constructive comments that helped us substantially improve the rigor of the study.

Reviewer 2

“The authors have addressed my questions, and I have no further comments.”

We sincerely thank the reviewer for carefully evaluating our revised manuscript and for confirming that all concerns have been adequately addressed.

Reviewer 3

“I am satisfied with the author's responses to my critiques and those of the other reviewers.”

We sincerely thank the reviewer for the thoughtful critiques and for confirming that our responses and revisions have addressed the concerns raised during review.

Reviewer 4

“I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.”

We sincerely thank the reviewer and co-reviewer for their careful evaluation of our manuscript and for their contribution to the peer-review process.