nature portfolio

Peer Review File

Differential Activity of MAPK signalling Defines Fibroblast Subtypes in Pancreatic Cancer

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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)
This manuscript from Veghini et. al. documents the role of MAPK signalling in the activation/maintenance of a subset of myofibroblastic (my)CAFs in PDAC, suggesting that this population plays an important role in disease progression and CD8+ T cell infiltration into tumours. This study provides significant insight into the molecular mechanisms that regulate CAF heterogeneity in PDAC, which is an important and poorly understood research question. The study also utilises multiple state-of-the-art techniques to investigate this question, leveraging high-resolution single-cell analysis and bulk transcriptome datasets to provide both detailed evaluation of the biological mechanisms underpinning CAF phenotypes and clinical insight. Therefore, I would anticipate this manuscript to make a significant contribution to progressing this field of research. However, there are some areas of the manuscript that I believe require revision to ensure the validity of the results presented and maximise their potential impact. The main points that require attention prior to publication are: 1) to examine the MAPKhigh CAF phenotype in the wider context of previously identified PDAC subpopulations, iCAF and myCAF are clearly described but how do these cells compare to (for example) the metabolic CAF population previously described by Wang et al?; 2) to determine whether the survival correlations presented are specific to MAPKhigh CAF, as opposed to merely reflecting the presence of myCAFs; 3) to provide further evidence to support a role for the specific involvement of MAPKhigh CAFs in CD8+ T-cell exclusion; 4) to ensure that the conclusions are supported by statistical analyses performed on sample-level data to confirm consistent results are found across biological replicates. Specific comments on the data presented are provided below.
Figure 1:
• Panel 1B) The statistical analysis presented here falsely inflates the sample size (n = 5 cases/subtype) by counting each field of view analysed as a different sample (FOV/sample = 4, creating an n~20). To accurately demonstrate a statistically significant increase in SMA+pERK+ cells between Classical and Squamous, the difference between the 5 biological replicates should be compared after averaging the technical replicates.
Figure 2:
• Panel 2E-F) When examining the increased abundance of CAFs in MEKi treated tumours it is unclear from this analysis whether the difference presented is in in fact an increase in CAFs or merely a reflection of reduced epithelial cells due to treatment response. Performing this analysis to assess CAF abundance as a proportion of the TME (non-epithelial cells) would be more informative and correct for the reduction in malignant cell numbers due to the treatment.
• Line 145, states “eMEK transcriptional signature was significantly downregulated in the epithelial compartment”. However, the analysis presented shows a reduction in the proportion of cells high for this signature (with no statistical significance presented) as opposed to a comparison of the expression level. The text or analysis should be corrected to address this inaccuracy.
Figure 3:
• Do the fibroblasts cluster into iCAF and myCAF populations if analysed in an unsupervised manner or are other subpopulations identified?
• The conclusions drawn from analysis of changes in myCAF vs iCAF ratio on MEKi treatment need to be confirmed by statistical comparison across biological replicates.
o In Figure 3b and 3f a significant association between the treatment and myCAF/iCAF ratio is shown but this analysis is performed by combining cells across biological replicates. To robustly support the conclusions drawn it should be demonstrated that the proportion of iCAF within individual tumours is significantly increased on treatment (and vice versa for

myCAF). Appreciably, this may not be possible for the scRNA-seq data if individual tumours cannot be distinguished but these data should be available for the in-situ hybridisation analysis (Fig. 3f).
• Can the authors explain the increased proportion of iCAFs in C7 vs C2 samples? This difference appears to be similar to the effect induced by 48h MEKi treatment.
• The inference from fig 3c. that the myCAFs showed highest variation in gene expression due to a larger number of differentially expressed genes. Is not sufficiently supported by the data presented. Given the large discrepancy in cell number between iCAFs and myCAFs it is equally likely that the differences in DE genes identified is due to limited power to detect these changes in the smaller iCAF population. As above the authors should try to demonstrate these changes using biological replicates for inferring statistical significance (e.g. using methods such as those described in https://doi.org/10.1038/s41467-020-19894-4).
Figure 4:
• The sMEK high phenotype described in line 215 has many overlapping markers with the metabolic CAF (meCAF) subpopulation previously described by Wang et al (https://doi.org/10.1038/s41421-021-00271-4). Can the authors demonstrate that this MAPK high phenotype is distinct from meCAF? If so what are the key differences?
o If the MAPK high phenotype described here is comparable to the meCAF subpopulation previously described, this does compromise the novelty of the study somewhat. Also, the Wang et al study showed meCAFs to indicate improved response to ICB, which is clearly contradictory to what is shown later in this manuscript and this discrepancy also requires further explanation/investigation.
• The in vitro analysis presented in Fig 4g should be expanded. Given that the myCAF phenotype is known to be induced in cells within close proximity to tumour cells. Have the authors analysed changes in the sMEK signature when PSCs are directly co-cultured with classical or basal PDAC cells? This would likely provide a more physiologically relevant model than pre-treatment with TGF-beta followed by conditioned media and would provide important information regarding the mechanisms of MAPK signalling activation.
• In Figure 4j there is a similar issue to that described above (for Fig. 3b&f) with statistical significance inferred from aggregating samples as opposed to confirming that the difference in sMEKhigh CAFs is consistently increased in basal-like samples vs classical.
Figure 5:
• The results in this figure are potentially of interest and may provide useful clinical insight however there are some issues that need to be addressed.
• Further detail for how these analyses were performed should be provided in the methods section.
• Was the data from the original Moffitt study included in the survival analyses?
o It is surprising that the activated stroma signature performs so poorly in these datasets given the original publication's results.
o In the original study there was a third category of "absent" how was this (and the resulting variation in samples size) handled in the analysis?
• Given that the Moffit stromal subtypes, described as an "established stromal signature", was not significantly associated with patient survival in most datasets analysed. Did the authors compare their signature to more recently described prognostic stromal signatures? For example LRRC15+ CAFs (https://doi.org/10.1158/2159-8290.CD-19-0644 or https://www.nature.com/articles/s41586-022-05272-1), or those described in these studies https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9820557/, https://pubmed.ncbi.nlm.nih.gov/36465388/ .
• It is critical here to determine whether these results are specific to MAPK high myCAF or just a reflection of myCAF in general. Previous studies have already demonstrated that a myCAF signature will indicate poor survival in many of the datasets analysed here. So, it should be demonstrated whether the sMEK signature simply reflects these previously described findings or adds novel independent prognostic value.
• In 5f, the analysis of SMA+pERK+/- goes some way to addressing this point in relation to CD8+ T-cell localisation. However, this staining panel is poorly designed to fully elucidate whether MAPK-high CAFs are specifically associated with CD8+ T-cell exclusion. Further markers (e.g. MCAM) are needed to distinguish SMA+ mural cells from myCAF. With the panel currently used It is possible that the increased abundance of CD8s observed in close proximity to SMA+pERK- cells is explained by higher levels of CD8 T-cells associated with vessels, which is frequently observed in tissue sections.
o Also lower resolution images should be provided to demonstrate the entire region of tissue analysed and a description should be provided for how the regions analysed were selected.

Reviewer #2

(Remarks to the Author)
Veghini et al. present a manuscript entitled "Differential Activity of MAPK signalling defines fibroblast subtypes in pancreatic cancer" for consideration to be published in Nature Communications. The paper begins with a description of MAPK pathway activity in the basal-like and classical cell models, then makes a jump to the fibroblast compartment stemming from an observation of differential MAPK signature activity in basal-like tumors in TCGA, which is known to include low neoplastic cellularity tumors. Then the authors go on to show in mouse models that MEK inhibition drives a reduction in MAPK activity in CAFs, with specific implications for the myCAF populations. They posit that MEK inhibition may cause a myCAF-to-iCAF shift. Human data is presented at the end suggesting that a gene expression signature of MAPK high CAFs may correlate with poor prognosis across tumor types.

The paper is interesting and focuses on an understudied area – MAPK signaling in the CAF compartment and stimuli that cause CAF subtype switching. The authors make several intriguing observations. However, the impact of the paper is limted by several key factors. The paper is almost entirely descriptive with no mechanistic follow-up of any of the observations made, including the association of basal-like cells and the myCAF population (what drives this association), CAF subtype

switching upon MEKi, etc. The conclusions in the paper are based on overreliance and interpretation of results from only one or a few models, mostly mouse models. There is a lack of rigorous quantitation and interpretation of mIF data and an absence of statistical rigor . No multivariable modeling is presented for human correlation data.

Specific comments and suggestions:

The initial description of MAPK pathway activity in basal-like and classical cancer cell models is underpowered and as written does not add much to the paper.

Supp Fig 1a-b: The statement that MAPK activation correlated with higher sensitivity to MEKi is overstated and based on only a few models of each subtype. To state this more conclusively, more models should be investigated and statistical comparisons should be made. Additionally, the GSVA scores for basal-like and classical should be defined more clearly. It has been well described that cell lines have more basal-like character and organoids have more classical character. What are the enrichment scores for the basal-like and classical models, rather than just the binary calls.

Supp Fig 1c: Again conclusion based only on a single model of each subtype. Need to expand to multiple models to be conclusive.

Supp Fig 1d: Would like to see longer term treatment of each model in order to observe what is very likely an epigenetically modified cell state transition. Additionally, please present GSVA scores for basal-like and classical for each model and at each timepoint.

The authors make the argument that the TCGA cohort has low neoplastic cellularity tumors and therefore may be more reflective of fibroblast signaling patterns; however, the neoplastic cellularity is available from TCGA (and other datasets) and the authors could present a much more refined analysis of MAPK activation score and fraction of neoplastic cellularity. Is there a correlation (or anti-correlation) between neoplastic cellularity, basal-like neoplastic signature and MAPK biocarta signature in TCGA, ICGC, etc?

Figure 1b: Show result by tumor, with each tumor indicated as a different color or separate icon. Is this observed across multiple tumors or are one or two tumors driving the difference?

Figure 1d, Supp 1k: only one tumor shown for each class switch and no class switch phenotype? What do the authors not show the aggregate quantitated data for the n = 5 and n = 10 numbers for each of these respective classes? Showing only one tumor without quantification makes it impossible to judge whether this conclusion is valid.

Figure 1e-f: The response to BL_CM and CL-CM must be shown with more than one source cell line of the CMs for each subtype. Is this reproducible across multiple models?

The MEKi treatment of an orthotopic KPC tumor transplant model is interesting but highly descriptive in nature and the differences among cell states are not rigorously demonstrated.

Figure 2 E-F: No legend is provided, although it is assumed the colors represent the same cell populations in the UMAP in Figure 2C. However, what does the gray color in the bar plots represent? This plot is difficult to interpret and unclear what the conclusions are.

Figure 2G-H: Why are there no statistical comparisons performed here? The differences do not appear to be significant in magnitude.

Figure 3C: It is unclear how many cells of each type were evaluated and what the gene capture was for each of these cells.

The observation of MEKi-induced treatment shifts of myCAF and iCAF fractions is one of the more interesting observations presented in the paper. The authors use only one marker and don’t do any co-localization with other known markers of myCAF and iCAFs. The utilization of only a single marker is insufficient. Additionally, using the mIF approach, the authors should be able to more rigorously interrogate co-expression of tumor and fibroblast markers, pERK, etc. The paper would benefit from more refined analysis here.

Does treatment with another therapy (e.g. chemotherapy such as gemcitabine) also induced treatment-related changes in myCAF and iCAF distributions?

The human data in figure 5 are interesting but likely heavily confounded by co-correlations with basal-like phenotype, TGF-B secretion and the activated stromal signatures that have been previously described. No attempt at a multivariable modeling was made.

Reviewer #3

(Remarks to the Author)
In this manuscript the authors define a novel population of myofibroblasts with a high MAPK transcriptional gene expression

signature and link this to prognosis and T cell infiltration.

While the MAPKhigh myCAF population arguably is a novel finding it is not clear how well defined this population is and, more importantly, whether this population is functionally distinct and relevant from already described populations of cancer-associated fibroblasts.

Suggested additional experiments and clarifications:

The authors undertake a number of analyses comparing MAPK activity using gene expression and compare this across transcriptional subtypes of PDAC tumour cells. Ultimately the authors argue that there is a discrepancy in the ability of MAPK gene expression to distinguish between tumour cells of basal and classical transcriptional subtypes, which then lead to the observation that the discrepancy is die to different activation of MAPK in the stromal cells/TME in patient samples.

To strengthen this argument the authors should include an additional analysis of the TCGA data comparing high with high and low with low (tumour cellularity). This should alleviate the observed effect of stromal MAPK.

Supplementary Figure 1. Transcriptional analysis to predict the activity of cell signalling is at best a proxy and should be validated biochemically to ensure specificity within the system presented.

In figure 1b the authors then compare pMAPK in aSMApos CAFs and identify a higher number of these in squamous/basal tumours. There is no general fibroblast marker included and it is therefore difficult to ascertain whether the observed differences are due to different amount of CAFs or difference in pMAPK levels. Moreover, the number of tumour cell samples is a bit low (n=5) to support the conclusion. The similar argument goes for the comparison between MAPK transcriptional signature in TCGA data where the authors observe a positive correlation between MAPK signature and several CAF markers. Have the authors stratified for samples such that the total level of CAFs is comparable between the groups of patient samples compared? The figure/data included in Figure 1c is difficult to interpret and should be presented clearer/better annotated.

The authors then argue that differences in tumour cell subtype is the reason behind the differential pMAPK levels/signature observed and use conditioned medium treated pancreatic stellate cells to illustrate this point. Firstly, the number of exemplar tumour cell lines (basal and classical) is too low to demonstrate consistency in the observation. Secondly, if only PSCs respond to the conditioned medium by elevated pMAPK and not the tumour cells themselves, there must be a receptor which is specifically expressed in the PSCs and not in the tumour cells?

In an effort to improve the understanding of MAPK activity in CAFs the authors then use scRNAseq to annotate the TME and compare control and MAPK inhibitor treated animals. The major challenge with this experiment is that tumour and microenvironment are simultaneously treated with consequential decrease in tumour cell number and CAF subtypes. Thus, delineating causal relationships becomes immensely difficult. Moreover, the distribution of cells doesn't show the variability across experimental conditions and should be followed up with flow for validation.

I am also a little concerned with the FAP staining in Figure S2e. The stain seems a bit high and doesn't seem to align with the relative low abundance of CAFs identified (5-10% as suggested by the scRNA data)?

The shift observed in ration between myCAF and iCAFs (fx Fig 3b) should be followed up and validated. For example, the total number of CAFs observed is between ~5-10% as pr Fig 2e, thus a shift between myCAF and iCAF within a limited number of animals can easily be due to experimental variation and should therefore be confirmed.

The authors propose that MAPK is essential for maintaining of myCAFs, however due to the complexity of the model system the observation could equally be caused by changes in tumour cell state, altered immune infiltration or sensitivity to cell death. Thus, the fundamental observation e.g., that MAPK activity governs myCAF identify should be tested in a simpler in vitro model system. Notably, conditioned medium from basal tumour cells increases pMAPK in PSCs to greater extend, but doesn't seem to increase myCAF signature unless TGFb is added? Doesn't this suggest that pMAPK isn't a driver (and possibly a requirement) for myCAFs unless in the context of TGFb? This should be explored in greater depth.

Finally the authors interrogate other cohorts of scRNA seq data and confirm the MEK transcriptional signature in elevated in myCAF. They then demonstrate that the signature is also associated with outcome, but this is from bulk transcriptional data. The issue here is that the authors haven't validated the specificity of the signature in CAFs and cannot exclude other cells in the tumours e.g., tumour cells, are driving the observation.

Similarly the authors observe an association with low T cell infiltration in areas with high myCAF/pMAPK CAFs. Here the number of samples analysed are low and should be increased for confidence. Moreover, the authors should be able to validate this in their scRNA data from the inhibitor treated animals where T cell infiltrate would be anticipated to increase?

Reviewer #4

(Remarks to the Author)
In this study, Veghini et al investigate MAPK signaling in fibroblasts in pancreatic cancer and its role in promoting the myofibroblast phenotype. They show that basal-like pancreatic tumors, known to be more aggressive than their classical

counterparts, promote MAPK signaling in fibroblasts. They then proceed to test the effects of a MEK inhibitor, trametinib (MEKi), on an orthotopic KPC model that should replicate the human basal-like PDAC phenotype. MEKi treatment transiently reduced p-ERK in fibroblasts and increased the proportion of fibroblasts after 2 and 7 days of treatment. They found that MEKi treatment additionally increased the proportion of iCAFs while reducing the proportion of myCAFs. Single genes were identified that marked myCAFs and iCAFs, respectively, and were used to validate the changes in iCAFs following MEKi. A stromal MEK (sMEK) signature was then defined based on genes that were reduced in fibroblasts following MEKi. This signature was associated primarily with myCAFs and was correlated strongly with basal-like tumors. This sMEK signature predicts worse patient outcome in pancreatic cancer, bladder cancer, lung adenocarcinoma, and uveal melanoma. Additionally, p-ERK signaling in fibroblasts was associated with reduced infiltration of CD8+ T cells, suggesting that MAPK signaling in fibroblasts may be associated with an immunosuppressive microenvironment. Overall, this paper highlights a novel role of MAPK signaling in fibroblasts that may contribute to fibroblast phenotype and drive the immunosuppressive landscape of PDAC. These results could provide new understanding of the molecular drivers of fibroblast heterogeneity within the PDAC TME. However, there are concerns related to the claims that MAPK signaling in CAFs is a driver of the myofibroblast phenotype. Additionally, it is unclear if tumor-dependent effects of MEK inhibition have been fully disentangled from the effects of MEK inhibition on the fibroblast compartment.

Comments:

1. The authors provide evidence to suggest that MAPK signaling in cancer-associated fibroblasts is upregulated when these fibroblasts are close in proximity to basal-like tumor cells, while there is little MAPK signaling in CAFs in classical tumors. MEK inhibition with trametinib seems to increase the proportion of iCAFs, and a stromal MAPK-high signature is associated primarily with myCAFs. The authors thus state that MAPK signaling is a key determinant of the myCAF phenotype. However, classical PDAC tumors are known to have significant myCAF populations even though the results shown in this paper would suggest that there is minimal MAPK signaling in these CAFs. This is also shown in Supplementary Figure 3F-G where myCAFs make up 75-95% of orthotopic tumors from classical tumor cell lines prior to MEKi treatment. It would support your hypothesis if you showed that classical PDAC tumors had a smaller population of myCAFs compared to basal-like PDAC tumors. Otherwise, how would you explain the presence of a large myCAF population in classical PDAC tumors in the context of this study?
2. Treatment with trametinib in your model affects all the cell types found in the tumor microenvironment, including the malignant epithelial cells and the fibroblasts. How can you be sure that effects observed in the fibroblast compartment following trametinib treatment are not due to effects of MEK inhibition on the epithelial cells? This is especially important as dysregulated MAPK signaling is most associated with the malignant epithelium. I realize that the sMEK gene signature is derived from genes downregulated in the stromal compartment following MEKi treatment, but could these transcriptional changes be driven by differential tumor-CAF crosstalk after MEKi?
3. In Supplementary Figure 3F-G, the authors show that orthotopic PDAC tumors derived from cell lines of classical subtype still show an increase in iCAFs following short-term MEKi treatment. They also show that p-ERK activity was limited to the epithelial cells in this model. This result suggests that the increase in iCAFs observed following MEK inhibition is driven by reduction of MAPK signaling in tumor cells as the CAFs show no appreciable MAPK signaling even prior to treatment. Is it likely that the increase in proportion of iCAFs observed in orthotopic models recapitulating the basal-like PDAC subtype also are primarily driven by MEK inhibition in tumor cells rather than the CAFs themselves?
4. In Figure 4C-E and Supplementary Figure 4C, the authors show that the sMEKhigh signature is associated with both myCAFs and a hypoxia-related gene transcriptional signature. This result contrasts with recent studies which have found that hypoxia is a driver of the iCAF phenotype[1,2]. How do you reconcile your results with these other studies?
5. In Figure 4G it seems like TGF-beta alone strongly induces the sMEK gene transcriptional signature in mPSCs. Adding CM from classical or basal-like tumor cell lines does not seem to increase expression of this gene signature, and if anything, the CM from the classical tumor cell line seems to decrease the gene signature. Figure 4F also shows that there is a correlation between TGFB1 expression and the sMEK signature in the TCGA cohort. One way to interpret this data is that TGF-beta derived from tumor cells is a strong driver of the sMEK gene transcriptional signature and is produced more by basal-like tumors. Treatment with MEKi would potentially reduce tumor-derived TGF-beta, reducing expression of this gene signature in the CAFs. Is there a difference in secreted TGF-beta from classical vs basal-like tumor cell lines?
6. In Figure 5E-F you show that p-ERK activity in fibroblasts is inversely correlated with infiltration of CD8+ T cells. However, I think it is a stretch to say that the p-ERK+ CAFs are causing this lack of infiltrating CD8+ T cells. It could also be possible that the basal-like/squamous tumor niches where p-ERK+ CAFs are found are the primary drivers of immunosuppression and reduced CD8+ T cell accumulation. More data would be required to determine whether the MAPK-high CAFs are directly immunomodulatory.

Minor Comments:

• Figure 1B – Are there less aSMA+ cells overall in classical PDAC tissues? Could you quantify the percent of aSMA+ cells that are also pERK+ in these tissues to show that the proportion of aSMA+ pERK+ cells goes up in basal-like tumors rather than just the total number of aSMA+ pERK+ cells? This would support the claim that basal-like tumors are associated with increased MAPK signaling in neighboring CAFs.
• Figure 3A-B - Even though percentage of CAFs that are myCAFs are going down, it looks like the total number of myCAFs are likely increasing. This correlates with the increase in fibroblasts seen following MEKi treatment in Figure 2E-F. This contrasts with the claim that MAPK signaling in CAFs drives the myCAF phenotype, as total number of myCAFs are increasing following MEK inhibition. The increase in the iCAF population is more than that of the myCAF population, which is why the proportion of CAFs which are iCAFs is increasing.
• Figure 1F - Your control mPSCs show no baseline p-ERK. It is only induced with CM from classical or basal-like tumor cell lines. However, in Supplementary Figure 4E, your control mPSCs (both untreated and treated with TGF-B1) show significant p-ERK signaling, which seems to be reduced when treated with CM from either classical or basal-like tumor cell lines. Is the

difference between the two due to serum starvation in the first experiment? If so, can you really say that your basal-like CM is inducing p-ERK activity in your second experiment when the levels of p-ERK are lower than your control?
• Line 224 – Text says that the mPSCs were exposed to CM for 24 hours, while your legend for Supplementary Figure 4E says that they were treated with CM for 1 hour.

References:
1. Schwoerer, S., Cimino, F. v., Ros, M., Tsanov, K. M., Ng, C., Lowe, S. W., Carmona-Fontaine, C., & Thompson, C. B. (2023). Hypoxia potentiates the inflammatory fibroblast phenotype promoted by pancreatic cancer cell-derived cytokines. Cancer Research. https://doi.org/10.1158/0008-5472.can-22-2316
2. Mello, A. M., Ngodup, T., Lee, Y., Donahue, K. L., Li, J., Rao, A., Carpenter, E. S., Crawford, H. C., Pasca di Magliano, M., & Lee, K. E. (2022). Hypoxia promotes an inflammatory phenotype of fibroblasts in pancreatic cancer. Oncogenesis, 11(1). https://doi.org/10.1038/s41389-022-00434-2

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I would like to thank the authors for their clear and thorough reponse to my review. I have no further concerns to raise and support this manuscript progressing to publication.

Reviewer #2

(Remarks to the Author)
The authors have added substantial new data that address most but not all of my comments. Given the breadth of responses to all reviewers critiques and the overall novelty of this body of work, I'm satisfied with their response to reviews. This is an interesting and important study.

Reviewer #3

(Remarks to the Author)
I thank the authors for addressing the queries raised. The resulting manuscript has been much improved. While I think the main question remains to elucidate the function of these mapkCAFs I also appreciate that will be beyond the scope of this manuscript. I would personally encourage follow up on this as the field is booming with new CAF phenotypes and subsets but with limited mechanistic progress.

I have 2 questions/analyses, which shouldn't require any additional experimentation that I would encourage the authors to address:

Firstly, if TGFb paracrine signalling is driving the mapCAF phenotype, and this is regulated by MEKi, it would be expected that TGFb regulated genes are also regulated in mapCAFs following MEKi. This could easily be exemplified using the data in Figure 4C

Secondly, in Figure 2b the number of pERK positive cells seem a bit low. To allow the reader to better interpret the data it would be beneficial to a) show individual channels and b) include additional sections/quantification.

Finally, just because I am puzzled:
In figure 2C Annotated stromal cells express RGS5, which is a pericyte marker. Why not refer to these as pericytes rather than broadly as stromal cells?
If I understand correctly the authors include Rho Kinase inhibitors in their dissociation buffer for the single cell analysis. Would this not affect cell signalling state of cells when included for extended periods of time?

Reviewer #4

(Remarks to the Author)
Veghini et al have resubmitted their previous manuscript with major changes that take into account the initial reviewer comments. The core message of the paper still focuses on MAPK signaling in CAFs in the PDAC microenvironment, but the new manuscript acknowledges that MAPK signaling in malignant epithelium is a key driver of the mapCAF phenotype, predominantly through TGF-beta secretion. The following experiments and data have been added to improve the manuscript:
• Inclusion of additional cell lines and in vivo models
• Additional multiplex immunofluorescence analysis, including podoplanin as a general CAF marker
• IF and flow cytometry to verify changes in epithelial and fibroblast composition
• Measurement of TGF-B1 secretion from PDAC cell lines treated with MEKi
• Refined analysis of existing single-cell sequencing data from human PDAC patients

• Visium spatial transcriptomic analysis of 4 PDAC samples from human patients to show localization of mapCAF gene signature
• Single-cell RNA seq-based analysis of ligand-receptor interactions between cell types in the TME

The authors have responded to most of my initial concerns with a combination of new data and rewriting. I only have a few minor comments listed below:
• Can you clarify in the methods how the mapCAF gene signature, both in mouse and human, was identified? Was this based off differentially expressed genes between sMEKi-high and sMEKi-low CAFs? Could you also include a list of the mapCAF mouse genes in the Supplementary Tables? I only see the mapCAF human genes there. Also, are the mapCAF genes distinct from the sMEKi signature or are they a subset of that signature?
• Line 125: Should be Supplementary Fig. 1k
• Line 375: Should be Supplementary Fig. 7c
• In Supplementary Table 6, the titles for each worksheet reference cluster 0 instead of the respective cluster being analyzed.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
I would like to thank the authors for their detailed responses to my previous critiques. I believe that my concerns have now been adequately addressed, and I recommend acceptance of the manuscript.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in CAFs, transcriptomics

This manuscript from Veghini et. al. documents the role of MAPK signalling in the activation/maintenance of a subset of myofibroblastic (my)CAFs in PDAC, suggesting that this population plays an important role in disease progression and CD8+ T cell infiltration into tumours. This study provides significant insight into the molecular mechanisms that regulate CAF heterogeneity in PDAC, which is an important and poorly understood research question. The study also utilises multiple state-of-the-art techniques to investigate this question, leveraging high-resolution single-cell analysis and bulk transcriptome datasets to provide both detailed evaluation of the biological mechanisms underpinning CAF phenotypes and clinical insight. Therefore, I would anticipate this manuscript to make a significant contribution to progressing this field of research.

We thank the reviewer for his/her positive comment.

However, there are some areas of the manuscript that I believe require revision to ensure the validity of the results presented and maximise their potential impact. The main points that require attention prior to publication are:

1) to examine the MAPKhigh CAF phenotype in the wider context of previously identified PDAC subpopulations, iCAF and myCAF are clearly described but how do these cells compare to (for example) the metabolic CAF population previously described by Wang et al?;

We thank the reviewer for his/her comment which gave us the possibility of clarifying that the $MAPK^{high}$ CAF (now defined as mapCAF) phenotype we describe is profoundly different from the meCAF phenotype reported by Wang and colleagues. The metabolic CAF (meCAF) population has been identified in scRNA-seq data from human PDAC tissues and never explored in the context of mouse PDAC. In their manuscript, Wang and colleagues described meCAFs as a CAF subpopulation (i) displaying high levels of *PLA2G2A* and *CRABP2*; (ii) characterized by marker genes related to translation, mitochondrial translation elongation, proliferation, and glycolysis; (iii) coupled with cancer cells showing elevated OXPHOS activity; and (iv) populating subtumor microenvironments with increased density of $CD8^{+}$ T cells. CREB3L1 was found as main transcription factor driving the meCAF phenotype. Interestingly, another study links CREB3L1 to the iCAF phenotype (please see Hu et al. (Hu et al. 2022).

Collectively, our data show that the mapCAF phenotype is <u>profoundly different</u> from the meCAF phenotype reported by Wang and colleagues. We conclusively demonstrate that the mapCAF is a TGF-β driven phenotype (**Fig. 4e, f** and **Supplementary Fig. 4g,h** of the revised version of the manuscript), mostly restricted to myofibroblastic-like CAFs in both mouse (**Fig. 4a, d** of the revised version of the manuscript) and human PDAC (**Fig. 5b** of the revised version of the manuscript). Furthermore, we integrated spatial with cellular profiles to uncover that mapCAFs are enriched in subtumour microenvironment populated by basal-like cells and scant $CD8^{+}$ T cells (**Fig. 7a** of the revised version of the manuscript). The meCAF phenotype was instead enriched in iCAF clusters (**Supplementary Fig. 5a** of the revised version of the manuscript). Differently from meCAFs (see original publication), the mapCAF phenotype was associated with glycolytic malignant cells (**Supplementary Fig. 5f** of the revised version of the manuscript).

Main pathways enriched in meCAFs are linked to proliferation (e.g., MYC, translational elongation), while pathways enriched in mapCAFs mostly related to hypoxia (**Fig. 4f, Supplementary 4h, Fig. 6b, d** of the revised version of the manuscript) but also extracellular matrix interactions and inflammatory response. This is also evident from our single-cell regulatory network inference that

revealed the activity of transcription factor linked to hypoxia (Hif1a, Epas1) and MAPK (e.g., Fosb, Etv4) as main driver of the mapCAF phenotype (**Supplementary Fig. 4i** of the revised version of the manuscript).

On an additional note, in our scRNA-seq dataset as well as in the dataset from Elyada and colleagues (Elyada et al. 2019), we failed to map the expression of the meCAF main marker (i.e. *Pla2g2a*) as well as of its paralogs.

We hope we have convinced this and the other reviewers that the mapCAF phenotype is a novel CAFs phenotype displaying profound differences with the reported meCAF phenotype.

2) to determine whether the survival correlations presented are specific to MAPKhigh CAF, as opposed to merely reflecting the presence of myCAFs;

We thank the reviewer for the comment. We would like to point out to this reviewer that our mapCAFs signature has been refined to address the concerns about its specificity (see **Supplementary Fig. 5c, d** of the revised version of the manuscript). Spatial transcriptomics on human PDAC tissues (**Fig. 6d** of the revised version of the manuscript) and *in silico* analyses on scRNA-seq data from a pan-cancer study (**Supplementary Fig. 7d)** further supported the specificity of the mapCAFs signature. Stratifying PDAC patients by the new mapCAFs signature showed no difference in overall survival, indicating no prognostic value at least in the evaluated data sets. Nonetheless, the mapCAF signature could still reliably distinguish tumours with elevated expression of the basal-like program in the epithelial compartment (**Fig. 5c, d** and **Fig. 6d**). This was not true for the human myCAFs signature (**Fig. 5c** of the revised version of the manuscript) which has elevated expression in both classical and basal-like PDAC tumours (**Supplementary Fig. 6f**). Based on this result, we have eliminated all data related to survival analysis from the revised version of the manuscript. In line with its association with T cells depleted tumour areas (**Fig. 7a** of the revised version of the manuscript), the mapCAFs still identify primary resistance to immunotherapy in metastatic melanoma (**Fig. 7c** of the revised version of the manuscript). We hypothesize a functional role in tumor-immune interactions in PDAC and other tumor entities based on the results of our study to be further evaluated in future investigations.

3) to provide further evidence to support a role for the specific involvement of MAPKhigh CAFs in CD8+ T-cell exclusion;

We agree with the reviewer that this is a very important point to address. The ability to unveil the specific role of the mapCAFs in T cells exclusion is inevitably linked to the possibility of proper modeling of this phenotype, hence to the identification of its specific molecular driver(s).

At this stage, we were unable to identify reliable candidate targets/pathways for proper modeling of this phenotype. As we have pointed out in the result section (see page 11 of the revised version of the manuscript), similar ligand-receptor expression among CAFs subpopulations (**Supplementary Fig. 4a** of the revised version of the manuscript) and low prevalence of certain phenotypes compromises statistical power in inferring interactions that might be used as candidates for further validation. This is a known limitation of scRNA-seq data and has been evidenced by others (Shiau et al. 2023).

At the same time, we have tried to model this complex phenotype *ex vivo* using both established cell cultures and organoids but failed to identify culture conditions that could reliably replicate the phenotype across a broad range of models. It is likely that, in our reductionistic approach, we are missing some key components of the complex and highly structured ecosystem that can be found *in vivo* and that enables the emergence of this phenotype. We have amended the text to highlight that our observations are restricted to the *in vivo* setting and, accordingly, we have

reduced the prominence of data with less supportive evidence as are those related to mapCAFs driving T cell exclusion. By expanding the number of samples and refining the mIF panel, we have been able to further corroborate our findings related to the association of mapCAFs with T cell depleted areas (**Fig. 7a** of the revised version of the manuscript). Moreover, in our mouse model MAPK inhibition is associated with increased $CD8^{+}$ T cells infiltration (**Fig. 7 b** and **Supplementary Fig. 7c**).

Finally, we now include a paragraph related to the limitation of our study, including the lack of mechanistic insights on the potential immunomodulatory role of mapCAFs. Please, see page 17, Discussion Section, of the revised version of the manuscript.

4) to ensure that the conclusions are supported by statistical analyses performed on sample-level data to confirm consistent results are found across biological replicates.

Based on the reviewer's comment, we have revised our analysis to include statistical tests performed on sample-level data, ensuring that consistent results are found across biological replicates. The data are now presented accordingly, with displays showing sample-level rather than aggregated data.

Specific comments on the data presented are provided below.

5) Figure 1:

• Panel 1B) The statistical analysis presented here falsely inflates the sample size (n = 5 cases/subtype) by counting each field of view analysed as a different sample (FOV/sample = 4, creating an n~20). To accurately demonstrate a statistically significant increase in SMA+pERK+ cells between Classical and Squamous, the difference between the 5 biological replicates should be compared after averaging the technical replicates.

We apologize for the mistake. To adequately respond to this and the other reviewers, we have refined our spatial proteomic analysis. First, to better distinguish between CAFs and mural cells (as requested by this reviewer, see response to comment#9b below) we have included the fibroblast marker Podoplanin (PDPN) to the mIF panel. Second, we have increased the number of cases included in the study (now 12) and annotated neoplastic cells as either basal-like or classical using well-established markers (KRT81 and GATA6). The spatial analysis has highlighted pervasive heterogeneity related to the existence of spatially confined sub-tumour microenvironments and strengthens our previous observation about the existence of p-ERK^{+} CAFs specifically anchored to basal-like cells. Since the data in panel 1b of the original version of the manuscript is not adding to the information that are now included in the **new Fig. 1b** of the revised version of the manuscript, we deemed appropriate to remove it from the manuscript.

6) Figure 2:

• Panel 2E-F) When examining the increased abundance of CAFs in MEKi treated tumours it is unclear from this analysis whether the difference presented is in in fact an increase in CAFs or merely a reflection of reduced epithelial cells due to treatment response. Performing this analysis to assess CAF abundance as a proportion of the TME (non-epithelial cells) would be more informative and correct for the reduction in malignant cell numbers due to the treatment.

We thank the reviewer for the insightful comment. As suggested, we have evaluated CAF abundance as the proportion of the TME cells in our scRNA-seq data and confirmed the increased frequency of fibroblasts in treated tumors (please see **Fig. 2e** of the revised version of the manuscript). Towards a more quantitative description of this, we have evaluated changes in the overall abundance of CAFs and epithelial cells following treatment using cytofluorimetric analysis and multiplex IF. We confirm

the reduction of epithelial cell content, evaluated as pan-cytokeratin^{+} cells, following MEKi (See **Fig. 2g** of the revised version of the manuscript). Conversely, we found that changes in CAFs frequency post-treatment are context (i.e. cell line) dependent (see **Fig. 2h** of the revised version of the manuscript). We have amended the text accordingly (see page 8, Result Section, of the revised version of the manuscript)

6a) • Line 145, states "eMEK transcriptional signature was significantly downregulated in the epithelial compartment". However, the analysis presented shows a reduction in the proportion of cells high for this signature (with no statistical significance presented) as opposed to a comparison of the expression level. The text or analysis should be corrected to address this inaccuracy.

As suggested by the reviewer we have revised our analysis which now includes a statistical analysis of the difference observed between conditions. Please, see **Fig. 2f** of the revised version of the manuscript.

7) Figure 3:

• Do the fibroblasts cluster into iCAF and myCAF populations if analysed in an unsupervised manner or are other subpopulations identified?

We thank the reviewer for his/her comment which gives us the possibility of improving the presentation of our data. Unsupervised clustering of the fibroblast' compartment identifies 10 clusters, of which 7 were reliably annotated as CAFs based on post-hoc marker and gene-program analysis. Indeed, two clusters were defined as "contaminant" (ie, EMT and myeloid cells), while an additional cluster was excluded from the analysis because enriched for "cycling" cells. Two clusters were identified as distinct groups with overlapping signatures of inflammatory CAFs, and accordingly the established iCAF signature was enriched in these two clusters. Clusters 2-5 were identified as mostly composed by myofibroblastic CAFs, while Cluster 9 was characterized by the expression of apCAF markers (e.g., Cd74) and composed by rare cells (n =50). Considering their frequency across stages and conditions, we focused on the most abundant subpopulation of iCAFs and myCAFs. Please, see **Supplementary Fig. 3a-c** and **Fig. 3a** of the revised version of the manuscript.

7a) • *The conclusions drawn from analysis of changes in myCAF vs iCAF ratio on MEKi treatment need to be confirmed by statistical comparison across biological replicates.*

o In Figure 3b and 3f a significant association between the treatment and myCAF/iCAF ratio is shown but this analysis is performed by combining cells across biological replicates. To robustly support the conclusions drawn it should be demonstrated that the proportion of iCAF within individual tumours is significantly increased on treatment (and vice versa for myCAF). Appreciably, this may not be possible for the scRNA-seq data if individual tumours cannot be distinguished but these data should be available for the in-situ hybridisation analysis (Fig. 3f).

As suggested by the reviewer, we present the data at the level of individual tumors rather than aggregating biological replicates. For that, we now present data generated using three different methodologies, namely ISH, multiplex IF and FACS to show that short-term MEKi is associated with a decreased myCAFs/iCAFs ratio across a spectrum of transplantation-based mouse models representative of different neoplastic cell lineages (either basal-like or classical). Please, see **Fig. 3e-g** of the revised version of the manuscript.

7b) • Can the authors explain the increased proportion of iCAFs in C7 vs C2 samples? This difference appears to be similar to the effect induced by 48h MEKi treatment.

We thank the reviewer for the informative critique. Knowing that the myCAFs and iCAFs phenotypes are driven by the antagonistic interaction between TGF-β and IL1, we looked at the proportion of neoplastic cells expressing those two cytokines at different time points and at different condition (ie treated/untreated). We found that the progression is associated with increase in the proportion of *Il1a*, and more importantly, of *Il1b* expressing cells in the neoplastic compartment (see **Supplementary Fig. 3d** of the revised version of the manuscript). This associates with an increased average expression of *Tgfb1*. Conversely, the MEKi treatment is invariably associated with a reduced expression of *Tgfb1 (*at both two and 7 days of treatment) by epithelial cells, which we validated by measuring secreted TGFβ1 in a large panel of human cancer cell lines following MEKi (see **Fig. 3d** of the revised version of the manuscript).

7c) • The inference from fig 3c. that the myCAFs showed highest variation in gene expression due to a larger number of differentially expressed genes. Is not sufficiently supported by the data presented. Given the large discrepancy in cell number between iCAFs and myCAFs it is equally likely that the differences in DE genes identified is due to limited power to detect these changes in the smaller iCAF population. As above the authors should try to demonstrate these changes using biological replicates for inferring statistical significance (e.g. using methods such as those described in https://doi.org/10.1038/s41467-020-19894-4).
We would like to thank the reviewer for the informative critique as we have realized that we lack statistical power to demonstrate a difference in the transcriptional response induced by MEKi in the two CAF populations. Furthermore, novel experiments as well as the re-analysis of available data, which were requested by this and the other reviewers, strongly suggest that the changes in the fibroblast compartment are rather driven by changes in the epithelial compartment. Therefore, we have eliminated this data from the current version of the manuscript.

8) Figure 4:
• The sMEK high phenotype described in line 215 has many overlapping markers with the metabolic CAF (meCAF) subpopulation previously described by Wang et al (https://doi.org/10.1038/s41421-021-00271-4). Can the authors demonstrate that this MAPK high phenotype is distinct from meCAF? If so what are the key differences?
See also response to comment # 1 from this reviewer. We agree with the reviewer that is a critical point of our manuscript. As already discussed, we have refined the analysis of the MAPK^{high} CAF population (now mapCAFs) and derived a novel signature which shares no marker with the meCAF subpopulation. However, our mapCAFs present numerous differences with the meCAFs as outlined above (see response to comment#1 from this reviewer).

8a)o If the MAPK high phenotype described here is comparable to the meCAF subpopulation previously described, this does compromise the novelty of the study somewhat. Also, the Wang et al study showed meCAFs to indicate improved response to ICB, which is clearly contradictory to what is shown later in this manuscript and this discrepancy also requires further explanation/investigation.
As discussed above, we are very confident that the mapCAF phenotype is distinct from meCAFs. In the revised version of the manuscript, we present further evidence to support that mapCAFs are associated to an immunologically cold environment. Please, see **Supplementary Fig. 5a, f** of the revised version of the manuscript.

8b) • The in vitro analysis presented in Fig 4g should be expanded. Given that the myCAF phenotype is known to be induced in cells within close proximity to tumour cells. Have the

authors analysed changes in the sMEK signature when PSCs are directly co-cultured with classical or basal PDAC cells? This would likely provide a more physiologically relevant model than pre-treatment with TGF-beta followed by conditioned media and would provide important information regarding the mechanisms of MAPK signalling activation.

See also response to comment#3 above. We have tried to model the mapCAF phenotype *ex vivo* using both mouse and human cells. However, we have struggled to find culture conditions that could reliably replicate the *in vivo* phenotype in a broad spectrum of models. The reasons for that might be multiple and ranging from the inability to reconstruct a complex and highly structured environment to the fact that *in vivo* basal-like and classical phenotypes are difficult to replicate unless culture medium is complemented with specific TME cues. For example, Raghavan et al.(Raghavan et al. 2021) demonstrated that to enable *ex vivo* a basal-like cell state it is necessary to add TGF-β to culture medium. Given our inability to replicate *ex vivo* the transcriptomic profiles of mapCAFs cells, we have reduced the prominence of the *in vitro* modeling and eliminated some data, included those in **former fig. 4g**, from the new version of the manuscript. To compensate for that, we have now provided a comprehensive and multidimensional (single-cell RNA-seq, spatial proteomics, and spatial transcriptomics) characterization of this CAF phenotype.

8c) • In Figure 4j there is a similar issue to that described above (for Fig. 3b&f) with statistical significance inferred from aggregating samples as opposed to confirming that the difference in sMEKhigh CAFs is consistently increased in basal-like samples vs classical.

We thank the reviewer for the informative critique. Accordingly, we now provide sample-level data to confirm the enrichment of mapCAFs in tumours where basal-like cells represent the bulk of the neoplastic compartment. We would like to point out that this analysis has been conducted on an expanded scRNA-seq dataset that includes four different cohorts(Chan-Seng-Yue et al. 2020; Lin et al. 2020; Peng et al. 2019; Steele et al. 2020) for a total of 126,530 cells and 63 patients. Please, see **Fig. 5d** and **Supplementary Fig. 5e** of the revised version of the manuscript.

9) Figure 5:

• The results in this figure are potentially of interest and may provide useful clinical insight however there are some issues that need to be addressed.

• Further detail for how these analyses were performed should be provided in the methods section.

• Was the data from the original Moffitt study included in the survival analyses?

o It is surprising that the activated stroma signature performs so poorly in these datasets given the original publication's results.

o In the original study there was a third category of "absent" how was this (and the resulting variation in samples size) handled in the analysis?

• Given that the Moffit stromal subtypes, described as an "established stromal signature", was not significantly associated with patient survival in most datasets analysed. Did the authors compare their signature to more recently described prognostic stromal signatures? For example LRRC15+ CAFs (https://doi.org/10.1158/2159-8290.CD-19-0644 or https://www.nature.com/articles/s41586-022-05272-1), or those described in these studies https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9820557/, https://pubmed.ncbi.nlm.nih.gov/36465388/ .

Thanks to this reviewer comment, we have realized that we failed to provide a detail description of the subtyping methods employed in the original version of the manuscript. We apologize for this issue and have now amended the text to provide more details. For subtyping, we have used GSVA and pre-defined gene sets including the "activated" and "normal" stromal signatures from Moffitt and

colleagues(Moffitt et al. 2015). In their original manuscript, Moffitt et al. used consensus clustering for classifying patients into activated or normal. A third category was identified that was defined as absent, not displaying features of activated or normal. To be consistent throughout the manuscript, we have used a GSVA-based subtyping approach throughout our study. When working with the Moffitt dataset, that has translated into the dichotomization of cases into either activated or stromal signature. I hope this will clarify reviewer's concern about this.
As already discussed in response to comment#2, the mapCAF signature has been refined to address the question about its specificity from this and the other reviewers (see **Supplementary Fig. 5c, d; Fig. 6d; Supplementary Fig. 7d** of the revised version of the manuscript). Stratifying PDAC patients of the TCGA cohort by the new mapCAFs signature showed no difference in overall survival. Based on this result, we have eliminated all data related to survival analysis from the revised version of the manuscript. Based on a hypothesized role in immune regulation, we focused on tumour entities treated by immune perturbation. In line with its association with T cell depleted tumour areas, the mapCAFs identified primary resistance to immunotherapy in metastatic melanoma (**Fig. 7c**) and this potential role warrants further investigation in subsequent studies.

9a)• ***It is critical here to determine whether these results are specific to MAPK high myCAF or just a reflection of myCAF in general. Previous studies have already demonstrated that a myCAF signature will indicate poor survival in many of the datasets analysed here. So, it should be demonstrated whether the sMEK signature simply reflects these previously described findings or adds novel independent prognostic value.***
See also response to comment#2. Our integration of spatial and cellular profiles conclusively demonstrates that the mapCAFs phenotype identifies a subset of myCAFs which are specifically paired with basal-like cells. To reflect that, the human myCAFs signature does not distinguish classical from basal-like in bulk RNA-seq data (**Fig. 5c** of the revised version of the manuscript). Spatial transcriptomics further showed that myCAFs have a less variable spatial distribution than mapCAFs in human PDAC tissues displaying different quality of the neoplastic epithelium (Fig. 6c, d of the revised version of the manuscript). We hope we have convinced this and the other reviewers about the peculiarity of this CAF phenotype.

9b) • In 5f, the analysis of SMA+pERK+/- goes some way to addressing this point in relation to CD8+ T-cell localisation. However, this staining panel is poorly designed to fully elucidate whether MAPK-high CAFs are specifically associated with CD8+ T-cell exclusion. Further markers (e.g. MCAM) are needed to distinguish SMA+ mural cells from myCAF. With the panel currently used It is possible that the increased abundance of CD8s observed in close proximity to SMA+pERK- cells is explained by higher levels of CD8 T-cells associated with vessels, which is frequently observed in tissue sections.
See also response to comment#5. Following the reviewer´s suggestion, we have looked at the expression of markers that could reliably differentiate fibroblasts from mural cells. As it can be seen in **Fig R1** below, *ACTA2* (αSMA) and *MCAM* have indeed partially overlapping pattern of expression in scRNA-seq from Peng et al. (Peng et al. 2019). Conversely, *PDPN*, which is widely used in our field, marked exclusively cancer-associated fibroblasts in our analysis (Fig R1). Therefore, we have performed multiplex IF including PDPN in our panel to confirm the increased abundance of $CD8^+$ T cells in close proximity of $PDPN^+$p-ERK^+ CAFs (see **Fig. 7a** and **Supplementary Fig. 7a** of the revised version of the manuscript). Moreover, we have also looked at the difference in $CD34^+$ (endothelial

marker) density between p-ERK$^+$ CAFs vs p-ERK$^-$ CAFs area and found no statistically significant differences (**Supplementary Fig. 7b** of the revised version of the manuscript).

[figure redacted]

Figure R1. Right: UMAP embedding of the scRNA-seq data from Peng et al. (Peng et al. 2019). Left: UMAP plots from Peng et al colored by the expression of the indicated genes.

9c) o Also lower resolution images should be provided to demonstrate the entire region of tissue analysed and a description should be provided for how the regions analysed were selected.

We now provide lower resolution images, and a better annotation of the regions selected. Please see **Fig. 1b** of the revised version of the manuscript.

Reviewer #2 (Remarks to the Author): with expertise in PDAC biology and therapy

Veghini et al. present a manuscript entitled "Differential Activity of MAPK signalling defines fibroblast subtypes in pancreatic cancer" for consideration to be published in Nature Communications. The paper begins with a description of MAPK pathway activity in the basal-like and classical cell models, then makes a jump to the fibroblast compartment stemming from an observation of differential MAPK signature activity in basal-like tumors in TCGA, which is known to include low neoplastic cellularity tumors. Then the authors go on to show in mouse models that MEK inhibition drives a reduction in MAPK activity in CAFs, with specific implications for the myCAF populations. They posit that MEK inhibition may cause a myCAF-to-iCAF shift. Human data is presented at the end suggesting that a gene expression signature of MAPK high CAFs may correlate with poor prognosis across tumor types.
The paper is interesting and focuses on an understudied area – MAPK signaling in the CAF compartment and stimuli that cause CAF subtype switching. The authors make several intriguing observations.
We would like to thank the reviewer for his/her positive comment.

However, the impact of the paper is limted by several key factors. The paper is almost entirely descriptive with no mechanistic follow-up of any of the observations made, including the association of basal-like cells and the myCAF population (what drives this association), CAF subtype switching upon MEKi, etc. The conclusions in the paper are based on overreliance and interpretation of results from only one or a few models, mostly mouse models. There is a lack of rigorous quantitation and interpretation of mIF data and an absence of statistical rigor. No multivariable modeling is presented for human correlation data.
We thank the reviewer for the informative critique. We have tried to address all reviewer' concerns by providing integrative and more quantitative data on an expanded set of models as well as of human PDAC tissues. Accordingly, most of our resources were devoted to generating multidimensional data (FACS, mIF, spatial transcriptomic, bulk RNA-seq). We believe that this is the best approach for a more holistic view of cell states and their response to perturbation.

That said, we did attempt a more reductionistic approach to identify the specific molecular drivers of the described phenotypes for further mechanistic studies but were unable to establish experimental conditions that reliably replicated the observed phenotypes. This is not necessarily surprising given that the emergence of malignant and stromal cell states is dictated by a complex and highly structured *in vivo* ecosystem which is difficult to replicate *ex vivo*. Our effort to identify and characterize mapCAFs and their specific interactions in multiple and complex *in vivo* settings shows the complexity of potentially important TME contributors and provides the framework for further investigations. Thus, while we continue to focus on identifying candidate regulators as targets for specific modulation, this will be a focus of subsequent studies.

To specifically address this reviewer's concerns, we have refined our data analysis to adopt a more quantitative and rigorous approach.

Specific comments and suggestions:

1) The initial description of MAPK pathway activity in basal-like and classical cancer cell models is underpowered and as written does not add much to the paper.
We would like to thank the reviewer for his/her insightful comment. Accordingly, we have expanded the set of human cancer cell lines in our study (n = 12). We have chosen to remove PDOs to avoid that the analyses were confounded by the growth-factor rich media, or any modification needed (i.e.,

addition of TGF-β). Each cell line has been subjected to short-term treatment with trametinib to determine changes in cell fitness. The cells were then profiled before and after treatment with the purpose of identifying molecular correlates of short-term sensitivity. As described in detail below, we have correlated baseline and perturbed biochemical and transcriptomic cell states to pathway activity and drug sensitivity.

2) Supp Fig 1a-b: The statement that MAPK activation correlated with higher sensitivity to MEKi is overstated and based on only a few models of each subtype. To state this more conclusively, more models should be investigated, and statistical comparisons should be made. Additionally, the GSVA scores for basal-like and classical should be defined more clearly. It has been well described that cell lines have more basal-like character and organoids have more classical character. What are the enrichment scores for the basal-like and classical models, rather than just the binary calls.

Following the reviewer' suggestion, we have increased the number of models investigated. As the reviewer correctly pointed out, epithelial cell states (i.e., classical vs basal) cannot be properly replicated *in vitro* as they are also shaped by microenvironmental components (biochemical and/or physical cues). Recent evidence supports the role of sub-tumour microenvironments with distinct signaling cues and co-existence of different epithelial cell states in individual tumours. We are also aware that cancer cell lines and organoids tend to assume more basal-like and classical state, respectively. Therefore, we used models considering their limitation, which is acknowledging that they can be more representative of one cell state or the other. In particular, we decided to focus on human cancer cell lines to avoid the confounding effect of the organoid medium of phenotypic readouts. Accordingly, we have eliminated data related to PDOs from the current version of the manuscript.

As requested by the reviewer, we provide now the GSVA scores for basal-like and classical for each line and further computed a "basalness" score, defined as the difference between the basal-like and the classical scores. This score better captures the dominance of one signature over the other, thereby helping the identification of models that are more representative of one or the other cell state. Please see**, Supplementary Fig. 1b** of the revised version of the manuscript.

Using the extended panel of cell lines (n = 12), we looked at the biochemical and transcriptomic perturbations of the pathway following two days of treatment. The reduction of cell fitness following short-term MEKi was not predicted by differential p-ERK phosphorylation, a proxy for pathway inhibition, nor by the amplitude of drug-induced transcriptomic changes. MAPK transcriptional signatures did not correlate with drug sensitivity, biochemical pathway activation (i.e., p-ERK1/2 levels), or the "basalness" score. MAPK_Biocarta transcriptional signature showed a good correlation with pathway activation as assessed by levels of p-ERK in unperturbed condition. To confirm this result, we used an established cell line (PaTu 8988S) where the basal-like program is induced by RNAi silencing of the classical driver GATA6 (Kloesch et al. 2022). In keeping with our observations, downregulation of GATA6 did not result into significant changes of the MAPK fluxes nor in changes of sensitivity to MEKi. Therefore, we concluded that in cell lines MAPK activity does not discriminate basal-like vs classical PDAC cells nor identifies cell lines with differential sensitivity to pathway inhibition. Please, see **Supplementary Fig. 1a-i** of the revised version of the manuscript.

3) Supp Fig 1c: Again conclusion based only on a single model of each subtype. Need to expand to multiple models to be conclusive.

We now provide data on 12 different cancer cell lines to show that pathway rewiring follows different kinetics in different cell lines. Please, see **Supplementary Fig. 1a** of the revised version of the manuscript.

4) Supp Fig 1d: Would like to see longer term treatment of each model in order to observe what is very likely an epigenetically modified cell state transition. Additionally, please present GSVA scores for basal-like and classical for each model and at each timepoint.

We would like to thank the reviewer for the insightful comment, which gives us the opportunity of clarifying on our strategy. We are perfectly aware that a longer treatment with MEKi might have different outcome on the lineage identity of cancer cells. Indeed, we have data on patient-derived xenografts (PDXs, n = 15) treated long-term (> 4 weeks) with MEKi showing transcriptomic gene expression dynamics associated with a class switch for classical tumours (5 out of 7). These data are the subject of another submission from our group to Nature Communications (manuscript # NCOMMS-24-33807A). Therefore, we cannot show here the data relative to the reduction of GATA6 expressing cells following treatment for classical model. In this model system, only 1 out of 8 basal-like tumours switched upon long-term MEKi.

That said, the effect of long-term treatment on epithelial cell states is beyond the scope of our work. Short-term treatment is a commonly used approach to capture the primary response to a perturbation and accordingly infer pathway activity (Schubert et al. 2018). Given that an anti-tumor response in PDAC is often very early combated by tumour cells, not only are short-term perturbation data informative regarding early tumour-adaptive alterations but also of potential value regarding immune regulatory dynamics. Thus, we believe that our data are of interest and benefit to the field.

As also indicated above (response to comment 2), we now report GSVA score for the treated and untreated cell lines. As you can see from **Supplementary Fig. 1b** of the revised version of the manuscript, the treatment does not significantly affect the identity for almost all cell lines, exception being PANC-1, a cell line known to be poorly representative of *in vivo* cell states. Since we are not making use of the 7-days treatment, we deemed appropriate to remove those data from the manuscript to make room for the new experiments.

5) The authors make the argument that the TCGA cohort has low neoplastic cellularity tumors and therefore may be more reflective of fibroblast signaling patterns; however, the neoplastic cellularity is available from TCGA (and other datasets) and the authors could present a much more refined analysis of MAPK activation score and fraction of neoplastic cellularity. Is there a correlation (or anti-correlation) between neoplastic cellularity, basal-like neoplastic signature and MAPK biocarta signature in TCGA, ICGC, etc?

We thank the reviewer for the insightful comment. We are aware of the intrinsic limitation of this type of analysis (i.e. bulk) considering that our mapCAF phenotype is strongly influenced by spatial "constraints". The interrogation of bulk transcriptomic data was originally intended to confirm/confute the observations made with the models, i.e. no difference between basal-like and classical with the regard to MAPK transcriptional activity. Moreover, we have re-analyzed the data to find that MAPK biocarta does discriminate basal-like vs classical tissues in the ICGC cohort. Indeed, we have realized that we had retained non PDAC tissues (acinar cell carcinomas, IPMN) into the original dataset, which were therefore excluded from downstream analysis. In the light of this result, we have re-arranged the figure to separate transcriptomic data generated on pure epithelium (either cell lines or microdissected samples) from those obtained on tissues (ie, IGCG and TCGA). Please, see **Fig. 1a** and the result section (page 6) of the revised version of the manuscript. See also the response to comment#1 from reviewer#3.

6) Figure 1b: Show result by tumor, with each tumor indicated as a different color or separate icon. Is this observed across multiple tumors or are one or two tumors driving the difference?

We have significantly improved the spatial proteomic analysis in the revised version of the manuscript (see also response to comment# 5 from reviewever#1). We now include a total of 12 cases and data are present at sample-level without aggregating biological replicates. To better distinguish between CAFs and mural cells (as requested by reviewer#1), we have included the fibroblast marker Podoplanin (PDPN) to the mIF panel. Neoplastic cells are annotated as either basal-like or classical using well-established markers (KRT81 and GATA6). The spatial analysis has highlighted pervasive heterogeneity related to the existence of spatially confined sub-tumour microenvironments and strengthen our previous observation about the existence of p-ERK+ CAFs specifically anchored to basal-like cells. Since the data in panel 1b of the original version of the manuscript is not adding to the information that are now included in the new **Fig. 1b** of the revised version of the manuscript, we deemed appropriate to remove it from the manuscript.

7) Figure 1d, Supp 1k: only one tumor shown for each class switch and no class switch phenotype? What do the authors not show the aggregate quantitated data for the n = 5 and n = 10 numbers for each of these respective classes? Showing only one tumor without quantification makes it impossible to judge whether this conclusion is valid.

We thank the reviewer for his/her comment. As discussed in response to comment#4, the data relative to the class switch occurring after long term MEKi and based on evaluation of GATA6 staining are included in another submission to Nature Communications. Of note, staining was performed on tissues from longitudinal biopsies from each PDXs, i.e. before and after treatment. For tumours classified as classical, we observed decreasing GATA6 expression in 5 out of 7 cases (**Fig. R2**).
Therefore, we do not have statistical power to draw any conclusion even if we clearly see a trend for increasing p-ERK in the stroma (**Fig. R3**). Conversely, the switch from basal-like to classical occurred only for one case out of 8 preventing any potential analysis. Based on these results, we retained the PDX data only to make the point that stromal p-ERK is higher in basal-like vs classical models (Fig 1e of the revised version of the manuscript). We have additional data now to show that the MAPK^{high} phenotype is associated with basal-like tumour cells *in vivo*.

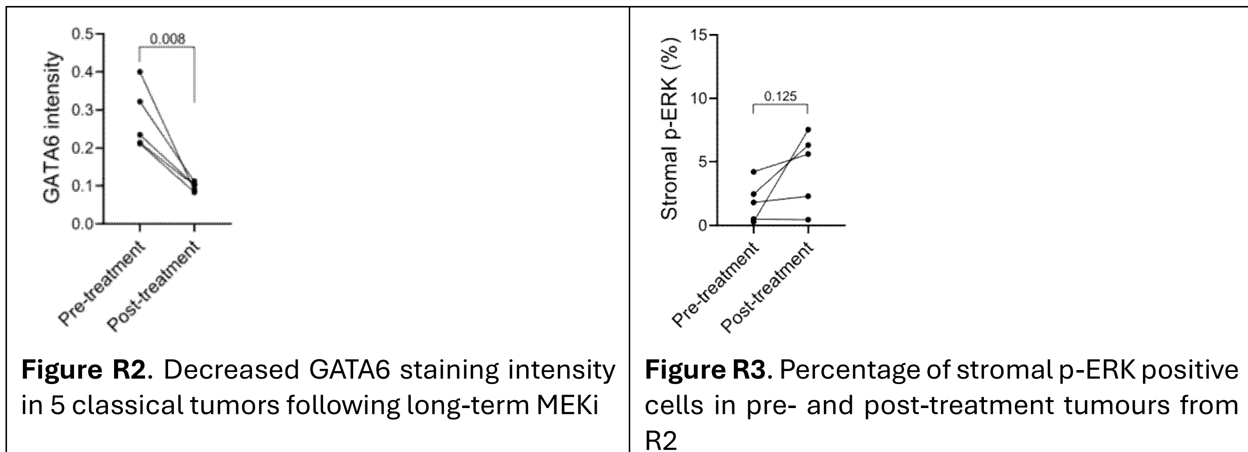

Figure R2. Decreased GATA6 staining intensity in 5 classical tumors following long-term MEKi

Figure R3. Percentage of stromal p-ERK positive cells in pre- and post-treatment tumours from R2

8) Figure 1e-f: The response to BL_CM and CL-CM must be shown with more than one source cell line of the CMs for each subtype. Is this reproducible across multiple models?

Following reviewer' suggestion, we have expanded our analysis using an extended set of cell lines which are representative of the different PDAC cell states. There was no clear trend for a differential enrichment of nuclear p-ERK when mPSCs were exposed to conditioned media from more basal-like cultures (**Supplementary Fig. 1l** of the revised version of the manuscript). We could see statistically significant differences only when mPSCs were treated with conditioned media from isogenic cell lines proficient or deficient for GATA6 expression (**Supplementary Fig. 1m** of the revised version of the manuscript). It is likely that, in our reductionistic approach, we are missing some key components of the complex and highly structured ecosystem that can be found *in vivo* and that enables the emergence of this phenotype. Therefore, we have amended the text to highlight that our observations are restricted to the *in vivo* setting.

9) The MEKi treatment of an orthotopic KPC tumor transplant model is interesting but highly descriptive in nature and the differences among cell states are not rigorously demonstrated.

We acknowledge that our analysis is descriptive in nature. However, since cell states are contributed by both intrinsic and extrinsic (microenvironmental) factors, they are best described through multidimensional analysis of tumour tissues. In the current version of the manuscript, we have refined our data analysis approach and further enriched spatial data to provide a more comprehensive and quantitative overview of cell states and their relationships. Furthermore, we have generated novel data (i.e., FACS, mIF, ISH) on a broader spectrum of models which substantiate our conclusions about changes into stromal compartment of PDAC following perturbation.

10) Figure 2 E-F: No legend is provided, although it is assumed the colors represent the same cell populations in the UMAP in Figure 2C. However, what does the gray color in the bar plots represent? This plot is difficult to interpret and unclear what the conclusions are.

We improved quality and clarity of the displays in Figure 2 as requested by the reviewer. Please see **Fig. 2e** of the revised version of the manuscript. Related to this critical point, we now provide additional data generated through orthogonal methodologies on an extended set of models to evaluate MEKi induced changes in malignant and stromal compartments. Please see **Fig. 2g, i** of the revised version of the manuscript.

11) Figure 2G-H: Why are there no statistical comparisons performed here? The differences do not appear to be significant in magnitude.

We apologize for the oversight. We have added the results of the statistical comparison for both panel 2g and 2h (now **Fig. 2f, h**) as requested by this reviewer. As you can see there is indeed statistical significance with the treatment associated with reduced MAPK transcriptional activity and increased frequency of classical cells.

12) Figure 3C: It is unclear how many cells of each type were evaluated and what the gene capture was for each of these cells.

Please, see also response to comment# 7c from reviewer # 1. We agree with the reviewer that we are underpowered to distinguish the magnitude of transcriptional changes induced by MEKi between myCAFs and iCAFs. Furthermore, the experiments as well as the re-analysis of data that were inspired by this and the other reviewer strongly suggest that the changes in the fibroblast compartment are rather driven by changes in the epithelial compartment. Therefore, we have eliminated this data from the current version of the manuscript also make room to the new findings. We thank the reviewer for this comment.

13) The observation of MEKi-induced treatment shifts of myCAF and iCAF fractions is one of the more interesting observations presented in the paper. The authors use only one marker and don't do any co-localization with other known markers of myCAF and iCAFs. The utilization of only a single marker is insufficient. Additionally, using the mIF approach, the authors should be able to more rigorously interrogate co-expression of tumor and fibroblast markers, pERK, etc. The paper would benefit from more refined analysis here.
We agree with the reviewer that this is a critical point of our study. Following the reviewer's suggestion, we have performed a more thorough analysis of the changes occurring in myCAFs to iCAFs frequencies after treatment. We have generated an ensemble of vertical data (ISH, Flow cytometry, mIF) that conclusively shows that MEKi is associated with a reduction of myCAFs and increase of iCAFs, which we linked to the reduced expression and secretion of TGF-β after MEKi. Please see **Fig. 2d-g** and **Supplementary Fig. 2j** of the revised version of the manuscript.

14) ***Does treatment with another therapy (e.g. chemotherapy such as gemcitabine) also induced treatment-related changes in myCAF and iCAF distributions?***
This is a very interesting question, but we hope that this reviewer will understand that given the questions/comments from all the reviewers we had to prioritize experimentations that would support/confute our major claims. Therefore, we have not conducted this analysis, although it will be very interesting to do so in subsequent studies.

15) ***The human data in figure 5 are interesting but likely heavily confounded by co-correlations with basal-like phenotype, TGF-B secretion and the activated stromal signatures that have been previously described. No attempt at a multivariable modeling was made.***
We would like to thank the reviewer for the insightful comment. In the current version of the manuscript, we present a refined version of the mapCAF signature which was identified to address concerns about its specificity raised by several reviewers. Our integrative analysis conclusively demonstrates that the mapCAF signature is a stromal specific signature that enables the identification of myofibroblastic CAFs paired with basal-like cells. Furthermore, we show that only the mapCAF but not the myCAF signature discriminate basal-like from classical in bulk RNA-seq dataset (**Fig. 5c** of the revised version of the manuscript). However, stratifying PDAC patients by the new mapCAF signature showed no difference in overall survival, indicating no prognostic value. Based on this result, we have eliminated all data related to survival analysis from the revised version of the manuscript. In line with its association with T cell depleted tumour areas, the mapCAFs still identify primary resistance to immunotherapy in metastatic melanoma (**Fig. 7c**). See also responses to comment#2,9 from reviewer#1.

Reviewer #3 (Remarks to the Author): with expertise in PDAC, CAFs

In this manuscript the authors define a novel population of myofibroblasts with a high MAPK transcriptional gene expression signature and link this to prognosis and T cell infiltration. While the MAPKhigh myCAF population arguably is a novel finding it is not clear how well defined this population is and, more importantly, whether this population is functionally distinct and relevant from already described populations of cancer-associated fibroblasts.

We thank the reviewer for his/her comment, and we are confident that this revision has given us the opportunity to further prove that $MAPK^{high}$ CAFs (now defined as mapCAFs) is a phenotype distinct from those already described in the literature.

Suggested additional experiments and clarifications:

1) The authors undertake a number of analyses comparing MAPK activity using gene expression and compare this across transcriptional subtypes of PDAC tumour cells. Ultimately the authors argue that there is a discrepancy in the ability of MAPK gene expression to distinguish between tumour cells of basal and classical transcriptional subtypes, which then lead to the observation that the discrepancy is die to different activation of MAPK in the stromal cells/TME in patient samples. To strengthen this argument the authors should include an additional analysis of the TCGA data comparing high with high and low with low (tumour cellularity). This should alleviate the observed effect of stromal MAPK.

Please, see also response to comment #5 from reviewer#2. Related to this point, we revised our analysis to show that a MAPK transcriptional signature discriminates basal-like from classical tumors also in the ICGC dataset. Indeed, we realized that we had retained non PDAC tissues (acinar cell carcinomas, IPMN) into the original dataset, which were now exclude from further analysis. In the light of this result, we have re-arranged the figure to separate transcriptomic data generated on pure epithelium (either cell lines or microdissected samples) from those obtained on tissues (ie, IGCG and TCGA). Please, see **Fig. 1a** and the result section (page 6) of the revised version of the manuscript.

2) Supplementary Figure 1. Transcriptional analysis to predict the activity of cell signalling is at best a proxy and should be validated biochemically to ensure specificity within the system presented.

We totally agree with this reviewer that context-dependent pathway response signatures can only serve as a proxy for the biochemical activity of a signaling pathway. Nevertheless, this approach has been successfully used to infer pathway activity (Schubert et al. 2018). To respond to this reviewer concerns, we have expanded the number of models subjected to pharmacological perturbation and measured the associated changes in pathway fluxes (evaluated as p-ERK levels) as well as the activity of MAPK transcriptional signatures. Overall, reduction of cell fitness following short term MEKi could not be predicted by baseline MAPK transcriptional activity, differential p-ERK phosphorylation (as a proxy of pathway response), nor by classical or basal-like identity. However, the levels of MAPK Biocarta transcriptional signature positively correlated with p-ERK levels at baseline. This new data supports the MAPK Biocarta as a possible proxy for pathway activity at least in our models/data sets. Please see **Supplementary Fig. 1a-g** of the revised version of the manuscript. Furthermore, spatial transcriptomics data confirm that a $MAPK^{high}$ CAF transcriptional signature (mapCAF) maps onto area with increased density of $p\text{-}ERK^{+}$ signals. Please, see **Fig. 6d** of the revised version of the manuscript.

3) In figure 1b the authors then compare pMAPK in aSMApos CAFs and identify a higher number of these in squamous/basal tumours. There is no general fibroblast marker included and it is therefore difficult to ascertain whether the observed differences are due to different amount of CAFs or difference in pMAPK levels. Moreover, the number of tumour cell samples is a bit low (n=5) to support the conclusion.

We would like to thank the reviewer for his/her comment. We have refined our analysis by incorporating the fibroblast marker PDPN in addition to α–SMA and expanded the number of cases to 12. The incorporation of PDPN gives us the possibility, as requested by the reviewer, of accounting for differences in the density of CAFs between basal-like and classical sub-tumour microenvironments. Overall, we see increased abundance of α–SMA$^+$ CAFs in basal-like tumour subdomain. Nevertheless, even after normalizing for the number of α–SMA$^+$ CAFs, there is still a statistically significant difference in the density of p-ERK$^+$ CAFs between basal-like and classical subdomains (Fig. R4). Please see **Fig. 1b-d** of the revised version of the manuscript. See also responses to comment#5 from reviewer#1 and comment#6 from reviewer#2.

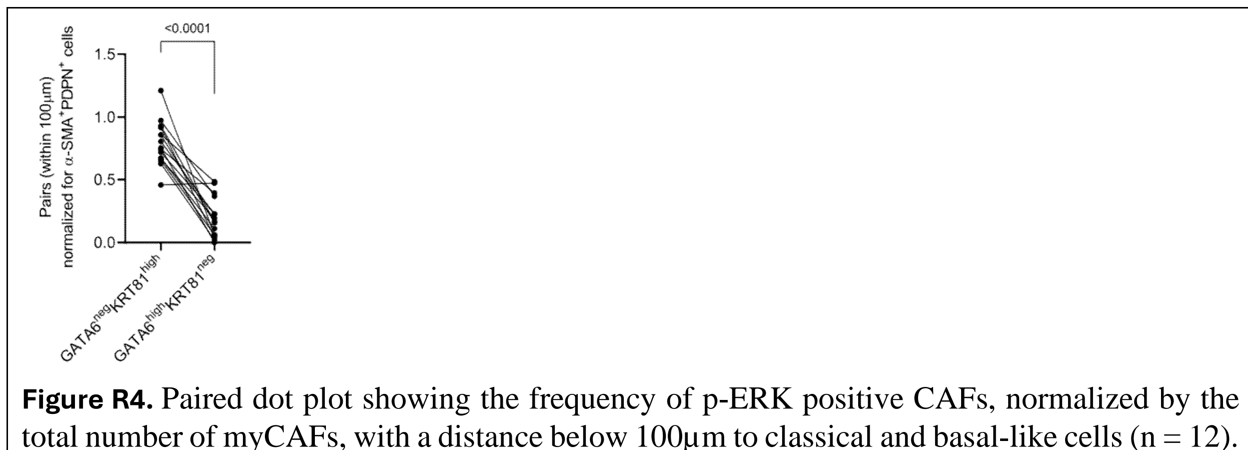

Figure R4. Paired dot plot showing the frequency of p-ERK positive CAFs, normalized by the total number of myCAFs, with a distance below 100μm to classical and basal-like cells (n = 12).

Our result is consistent with a recent report from Moffitt and colleagues, who showed that basal-like patients had increased myCAF proportion compared to classical one from scRNA-seq analysis (Oh et al. Nature Communications). In this manuscript, the authors suggest an increased proportion of myCAFs in basal-like tumours can be explained with the increased production of TGF-β by the epithelial cells with corresponding increased TGF-β response signature in the myCAFS of the classical one.

4) The similar argument goes for the comparison between MAPK transcriptional signature in TCGA data where the authors observe a positive correlation between MAPK signature and several CAF markers. Have the authors stratified for samples such that the total level of CAFs is comparable between the groups of patient samples compared?

See also response to comment 1. We believe that is very difficult to make this type of comparison from bulk transcriptomic data. Nevertheless, we have divided TCGA samples as either high or low "neoplastic cellularity" and then computed the correlation between MAPK signature scores and CAFs gene expression. As shown in **Supplementary Fig. 1i**, there positive correlation between MAPK signature and expression of CAFs markers regardless of the neoplastic cellularity of the tissue.

5) The figure/data included in Figure 1c is difficult to interpret and should be presented clearer/better annotated.

Following reviewers' suggestion, we have expanded and refined the mIF analysis. Please, see the new **Fig 1b** of the revised version of the manuscript.

6) The authors then argue that differences in tumour cell subtype is the reason behind the differential pMAPK levels/signature observed and use conditioned medium treated pancreatic stellate cells to illustrate this point. Firstly, the number of exemplar tumour cell lines (basal and classical) is too low to demonstrate consistency in the observation. Secondly, if only PSCs respond to the conditioned medium by elevated pMAPK and not the tumour cells themselves, there must be a receptor which is specifically expressed in the PSCs and not in the tumour cells?

As suggested by this and other reviewers, we have expanded the number of tumour cell lines used for the conditioned media experiment. There was no clear trend for a differential enrichment of nuclear p-ERK when mPSCs were exposed to conditioned media from more basal-like cultures (**Supplementary Fig. 1l** of the revised version of the manuscript). We could see statistically significant differences only when mPSCs were treated with conditioned media from isogenic cell lines proficient or deficient for GATA6 expression (**Supplementary Fig. 1m** of the revised version of the manuscript). It is likely that, in our reductionistic approach, we are missing some key components of the complex and highly structured ecosystem that can be found *in vivo* and that enables the emergence of this phenotype. Therefore, we have amended the text to highlight that our observations are restricted to the *in vivo* setting. Furthermore, we could not use scRNA-seq data to infer ligand-receptor pairs, i.e. malignant cell to fibroblast communication, that might be driving this phenotype.

As we have pointed out in the result section (see page 11 of the revised version of the manuscript), similar ligand-receptor expression among CAFs subpopulations (**Supplementary Fig. 4a** of the revised version of the manuscript) and low prevalence of certain phenotypes compromises statistical power in inferring interactions that might be used as candidates for further validation. This is a known limitation of scRNA-seq data and has been evidenced by others (Shiau et al. 2023). See also response to comment#3 from reviewer# 1, comment#8 from reviewer#2.

Accordingly, we have reduced the prominence of the *in vitro* experiments and acknowledged the limitations of our study in the discussion section. To account for that, we undertook a more thorough characterization of the *in vivo* phenotype complementing scRNA-seq and multiplex IF with spatial transcriptomics.

7) In an effort to improve the understanding of MAPK activity in CAFs the authors then use scRNAseq to annotate the TME and compare control and MAPK inhibitor treated animals. The major challenge with this experiment is that tumour and microenvironment are simultaneously treated with consequential decrease in tumour cell number and CAF subtypes. Thus, delineating causal relationships becomes immensely difficult. Moreover, the distribution of cells doesn't show the variability across experimental conditions and should be followed up with flow for validation.

We agree with the reviewer about the limitation of our approach. Nevertheless, also thanks to this reviewer´s comment we now provide multiple evidence for the quantitative and qualitative changes in the epithelial and stromal compartment following MEKi. We have generated multiplex IF and FACS data on an extended set of transplantation-based models. Furthermore, data are presented at sample-level rather than in aggregate form. Overall, we show that short-term MEKi is associated with

a reduction of tumour cell number across various mouse models (**Fig. 2g,** multiplex IF data; **Supplementary Fig. 2d**, FACS). Conversely, a context- (i.e., cell line-) dependent effect for changes in stromal cell number was observed (Fig. 2h and Supplementary Fig. 2e). With the regard to CAF subtypes, we have confirmed the changes in myCAFs/iCAFs ratio using three different analyses (ISH, mIF, FACS) and additional models (**Fig 3e-g** of the revised version of the manuscript). Furthermore, we now provide evidence that changes in the fibroblast compartment are not the direct consequence of MAPK inhibition but rather a reflection of changes in the neoplastic epithelium. Indeed, direct MAPK inhibition of plastic- or TGF-β-activated mPSCs did not significantly alter expression of myCAF or iCAF markers (**Supplementary Fig. 3f** of the revised version of the manuscript). Conversely, malignant cells from MEKi treated tumours expressed lower levels of *Tgfb1* (**Supplementary Fig. 3e** of the revised version of the manuscript), and MEKi significantly reduced secretion of TGF-β1 in a panel of human cancer cell lines (**Fig. 3d** of the revised version of the manuscript).

8) I am also a little concerned with the FAP staining in Figure S2e. The stain seems a bit high and doesn't seem to align with the relative low abundance of CAFs identified (5-10% as suggested by the scRNA data)
Given the new and more quantitative data, we have removed FAP staining from our manuscript.

9) The shift observed in ration between myCAF and iCAFs (fx Fig 3b) should be followed up and validated. For example, the total number of CAFs observed is between ~5-10% as pr Fig 2e, thus a shift between myCAF and iCAF within a limited number of animals can easily be due to experimental variation and should therefore be confirmed.
We agree with the reviewer that this is a critical point of our manuscript. We now present data generated using three different methodologies, namely ISH, multiplex IF and FACS to show that short-term MEKi is associated with a decreased myCAFs/iCAFs ratio across a spectrum of transplantation-based mouse models representative of different neoplastic cell lineages (either basal-like or classical). Please, see **Fig. 3e-g** of the revised version of the manuscript. See also response to comment#7a from reviewer#1, comment#13 from reviewer#2

10) The authors propose that MAPK is essential for maintaining of myCAFs, however due to the complexity of the model system the observation could equally be caused by changes in tumour cell state, altered immune infiltration or sensitivity to cell death. Thus, the fundamental observation e.g., that MAPK activity governs myCAF identify should be tested in a simpler in vitro model system. Notably, conditioned medium from basal tumour cells increases pMAPK in PSCs to greater extend, but doesn't seem to increase myCAF signature unless TGFb is added? Doesn't this suggest that pMAPK isn't a driver (and possibly a requirement) for myCAFs unless in the context of TGFb? This should be explored in greater depth.
We agree with the reviewer that differences upon myCAFs/iCAFs ratio observed upon effective MEKi might be driven by changes into cancer-cell phenotypes. Therefore, we carefully re-analyzed single-cell RNA-seq data and investigated treatment-induced changes into expression of main drivers of CAF phenotypes, namely TGF-β and IL1a/b. We found that the treatment is specifically associated with a decrease in the expression of TGF-β in cancer cells, and an increase of Il1b expressing cells following one week of treatment. Accordingly, MEKi reduced secretion of TGF-β1 from cancer cells both at two and 7 days of treatment. Moreover, we have tested the effect of MEKi on plastic or TGF-β activated PSCs to find that effective inhibition of MAPK per se does not affect fibroblast subtype. Overall, this data suggests that MEKi induced changes into the myCAFs/iCAFs ratio are secondary to changes in the neoplastic epithelium rather than a direct effect of MAPK inhibition in CAFs. We would like to thank this reviewer as his/her comment permitted us to clarify this point.

11) Finally the authors interrogate other cohorts of scRNA seq data and confirm the MEK transcriptional signature in elevated in myCAF. They then demonstrate that the signature is also associated with outcome, but this is from bulk transcriptional data. The issue here is that the authors haven't validated the specificity of the signature in CAFs and cannot exclude other cells in the tumours e.g., tumour cells, are driving the observation.

We would like to thank the reviewer for the insightful comment. In the previous version of the manuscript, we do show that (Supplementary Fig. 4F and 5C) the signature is enriched in the stromal compartment using scRNA seq data. However, we performed a better evaluation of the stromal MEK signature (originally defined sMEK - now defined as stromal MEK inhibition signature, sMEKi) and found that, indeed, many genes were rather promiscuous as they were expressed by non CAFs cells.

Therefore, we have revised our approach. First, we used sMEKi exclusively to identify CAFs displaying elevated MAPK transcriptional activity, which was confirmed by the original PROGENy analysis. These $MAPK^{high}$ CAFs mapped almost exclusively in Clusters 3-4 of the mouse datasets, which comprise myCAF-like fibroblasts (see new **Fig. 4a** of the revised version of the manuscript). Therefore, the new mouse mapCAF signature was obtained contrasting these CAFs to the other fibroblasts. To translate our findings from mouse to human, we used a similar approach on a single-cell atlas compiled from 4 different cohorts (Chan-Seng-Yue et al. 2020; Lin et al. 2020; Peng et al. 2019; Steele et al. 2020) which we have recently used in Lupo et al. (Lupo et al. 2024). Clustering in dimensionality-reduced space revealed 9 fibroblast clusters, with the human mapCAF signature enriched in the myofibroblastic clusters 0 and 3 (**Fig. 5b**). The human mapCAF signature comprises 22 genes with no to little expression in the malignant compartment (**Supplementary Fig. 5c,d**). The specificity of this signature was further confirmed by spatial analyses where we observe that the mapCAF program (i) maps in area with prevalence of myCAFs, (ii) associates with the basal-like epithelial program; (iii) is elevated in stromal areas that stained positive for p-ERK (**Fig. 6c,d** of the revised version of the manuscript).

Thus, we hope we have sufficiently addressed the concerns about the specificity of the mapCAFs phenotype.

12) Similarly the authors observe an association with low T cell infiltration in areas with high myCAF/pMAPK CAFs. Here the number of samples analysed are low and should be increased for confidence. Moreover, the authors should be able to validate this in their scRNA data from the inhibitor treated animals where T cell infiltrate would be anticipated to increase?

We have increased the number of human tissues analyzed to correlate T cell infiltration with density of $p\text{-}ERK^{+}$ CAFs. Our new data confirm our previous observations.

Furthermore, short-term MEKi in our mouse model leads to increase $CD8^{+}$ T cells infiltration as assessed by analysing scRNA-seq data (**Supplementary Fig. 7 c**) and immunohistochemical staining of treated and untreated animals (**Fig. 7b**).

Reviewer #4 (Remarks to the Author): with expertise in PDAC

In this study, Veghini et al investigate MAPK signaling in fibroblasts in pancreatic cancer and its role in promoting the myofibroblast phenotype. They show that basal-like pancreatic tumors, known to be more aggressive than their classical counterparts, promote MAPK signaling in fibroblasts. They then proceed to test the effects of a MEK inhibitor, trametinib (MEKi), on an orthotopic KPC model that should replicate the human basal-like PDAC phenotype. MEKi treatment transiently reduced p-ERK in fibroblasts and increased the proportion of fibroblasts after 2 and 7 days of treatment. They found that MEKi treatment additionally increased the proportion of iCAFs while reducing the proportion of myCAFs. Single genes were identified that marked myCAFs and iCAFs, respectively, and were used to validate the changes in iCAFs following MEKi. A stromal MEK (sMEK) signature was then defined based on genes that were reduced in fibroblasts following MEKi. This signature was associated primarily with myCAFs and was correlated strongly with basal-like tumors. This sMEK signature predicts worse patient outcome in pancreatic cancer, bladder cancer, lung adenocarcinoma, and uveal melanoma. Additionally, p-ERK signaling in fibroblasts was associated with reduced infiltration of CD8+ T cells, suggesting that MAPK signaling in fibroblasts may be associated with an immunosuppressive microenvironment.

Overall, this paper highlights a novel role of MAPK signaling in fibroblasts that may contribute to fibroblast phenotype and drive the immunosuppressive landscape of PDAC. These results could provide new understanding of the molecular drivers of fibroblast heterogeneity within the PDAC TME. However, there are concerns related to the claims that MAPK signaling in CAFs is a driver of the myofibroblast phenotype. Additionally, it is unclear if tumor-dependent effects of MEK inhibition have been fully disentangled from the effects of MEK inhibition on the fibroblast compartment.

We would like to thank the reviewer for his/her overall positive comment. We believe that reviewers' constructive criticisms have helped us improving the overall quality of the manuscript. Moreover, we believe that we have been able to address the specific reviewer' concerns.

Comments:

1). ***The authors provide evidence to suggest that MAPK signaling in cancer-associated fibroblasts is upregulated when these fibroblasts are close in proximity to basal-like tumor cells, while there is little MAPK signaling in CAFs in classical tumors. MEK inhibition with trametinib seems to increase the proportion of iCAFs, and a stromal MAPK-high signature is associated primarily with myCAFs. The authors thus state that MAPK signaling is a key determinant of the myCAF phenotype. However, classical PDAC tumors are known to have significant myCAF populations even though the results shown in this paper would suggest that there is minimal MAPK signaling in these CAFs.***

This is also shown in Supplementary Figure 3F-G where myCAFs make up 75-95% of orthotopic tumors from classical tumor cell lines prior to MEKi treatment. It would support your hypothesis if you showed that classical PDAC tumors had a smaller population of myCAFs compared to basal-like PDAC tumors.

Otherwise, how would you explain the presence of a large myCAF population in classical PDAC tumors in the context of this study?

We agree with the reviewer that the myCAF phenotype makes the bulk of the CAFs population in PDAC, regardless of the cell state of the epithelium. However, our data suggest an increased abundance of myCAFs in proximity of basal-like tumor cells. We conducted a multiplex IF analysis of 12 PDAC cases and found that the density of $PDPN^{+}a\text{-}SMA^{+}$ cells is increased in proximity of basal-like cells (see **Fig. 1b** of the revised version of the manuscript). This data perfectly aligns with a recent

report from Moffitt and colleagues, who showed that basal-like patients had increased myCAF proportion compared to classical one (Oh et al. Nature Communications). In this work, the increased proportion of myCAFs in basal-like tumours was linked to the elevated epithelial and endothelial expression of TGFB1 in those tumours.

2. Treatment with trametinib in your model affects all the cell types found in the tumor microenvironment, including the malignant epithelial cells and the fibroblasts. How can you be sure that effects observed in the fibroblast compartment following trametinib treatment are not due to effects of MEK inhibition on the epithelial cells? This is especially important as dysregulated MAPK signaling is most associated with the malignant epithelium. I realize that the sMEK gene signature is derived from genes downregulated in the stromal compartment following MEKi treatment, but could these transcriptional changes be driven by differential tumor-CAF crosstalk after MEKi?

We thank the reviewer for this informative critique. This is a crucial point of our work and a concern raised by most reviewers. Thanks to this critique, we took several efforts and conducted a more careful analysis of the data and concluded that the changes observed in the stromal compartment are likely reflecting changes in the neoplastic epithelium.

Indeed, the short-term treatment with MEKi is associated *in vivo* with reduced expression of TGF-β in the neoplastic compartment both at two and seven days of treatment (See **Supplementary Fig. 3e** of the revised version of the manuscript). Accordingly, MEKi in a panel of human cancer cell lines (representative of the different subtypes) leads to reduced secretion of TGF-β1 both at two and 7 days of treatment (see **Fig. 3d** of the revised version of the manuscript). Conversely, direct MAPK inhibition in mouse PSCs activated either by the plastic or TGF-β treatment, does not significantly affect the expression of myCAF or iCAF markers (See **Supplementary Fig. 3f** of the revised version of the manuscript).

To address reviewer' concern about the specificity of sMEK signature, we have revised our approach to establish a new mouse signature (now called mapCAF) which is based on specific transcriptomic features of those cells displaying elevated MAPK transcriptional activity in untreated tumours (see **Fig. 4c** of the revised version of the manuscript). To translate our findings in the human setting, we used a similar approach whereby we used pathway inference to identify $MAPK^{high}$ CAFs and then contrasting those with CAFs displaying low MAPK activity to derive the human mapCAF phenotype signature. We retained genes that showed low to no expression in the malignant compartment in order to improve specificity of the signature, a prerequisite for its use in bulk RNA-seq data (**Supplementary Fig. 5c, d** of the revised version of the manuscript). The human mapCAF signature is composed by 22 genes and is enriched in basal-like tumours as shown by the analysis conducted in bulk RNA-seq, scRNA-seq analysis and spatial transcriptomics. Please, see **Fig. 5c, d; Fig. 6d** of the revised version of the manuscript)

3. In Supplementary Figure 3F-G, the authors show that orthotopic PDAC tumors derived from cell lines of classical subtype still show an increase in iCAFs following short-term MEKi treatment. They also show that p-ERK activity was limited to the epithelial cells in this model. This result suggests that the increase in iCAFs observed following MEK inhibition is driven by reduction of MAPK signaling in tumor cells as the CAFs show no appreciable MAPK signaling even prior to treatment. Is it likely that the increase in proportion of iCAFs observed in orthotopic models recapitulating the basal-like PDAC subtype also are primarily driven by MEK inhibition in tumor cells rather than the CAFs themselves?

We would like to thank the reviewer for this insightful comment and indeed, our re-analysis of the data suggests that the mapCAF phenotype as well as the change into the myCAFs/iCAFs ratio is

primarily driven by changes into the neoplastic compartment. The treatment is indeed associated with the reduced secretion of TGF-β by tumor cells, regardless of their subtype. See also response to comment#2 and **Fig. 3d, Supplementary Fig. 3e,f** of the revised version of the manuscript.

4. In Figure 4C-E and Supplementary Figure 4C, the authors show that the sMEKhigh signature is associated with both myCAFs and a hypoxia-related gene transcriptional signature. This result contrasts with recent studies which have found that hypoxia is a driver of the iCAF phenotype[1,2]. How do you reconcile your results with these other studies?

We would like to thank the reviewer for the insightful comment which gave us the opportunity to further highlight the peculiarities of our phenotype. Our data are not necessarily in contrast with previous observations. Overall, we find that hypoxia-related gene programs present high expression in both iCAFs and myCAFs. The difference lies in the different abundance of the two CAF subtypes in the PDAC TME. Indeed, myCAFs are generally more abundant and only a subset of myofibroblastic CAFs shows enrichment for hypoxic gene programs. Therefore, considering iCAFs and myCAFs as a whole, i.e. without further differentiating between myCAF subsets, hypoxia-related gene programs result particularly enriched in iCAFs vs myCAFs. We elaborate more in detail below.

The two manuscripts reporting on the association between hypoxia and the iCAFs phenotype show partially conflict results. This despite a similar experimental setup based on the injection of a hypoxia indicator few hours before harvesting of the tissue and a similar FACS panel for identification of myCAFs and iCAFs. Schwoerer and colleagues (Schworer et al. 2023) suggested that myCAFs were almost completely excluded from hypoxic tumour regions, while Mello et al. (Mello et al. 2022) reported that myCAFs are anyhow the most abundant CAFs population even in hypoxic tumour regions. Our data aligns with findings from Mello and colleagues.

When specifically looking at scRNA-seq data, Schwoerer exclusively relied on data from Elyada et al. (Elyada et al. 2019) to find enrichment of a hypoxia signature in iCAFs. Mello and colleagues (Mello et al. 2022) re-analyzed data from Steele et al (Steele et al. 2020) and identified myCAFs and iCAFs based on the expression of specific markers (APOE, DPT, C3) rather than gene signatures. We reached out to the authors who confirmed the selection of iCAFs and myCAFs based on those markers.

In this manuscript, we have analyzed scRNA-seq data from 4 different cohorts comprising 126,530 cells from 63 patients. Subclustering of the fibroblast compartment in dimensionality-reduced space identified 9 clusters which we annotated using both highly expressed markers and gene programs. One cluster could be annotated as IL1 CAF and accordingly showed enrichment of the human iCAFs signature (Please see **Fig. 5a** of the revised version of the manuscript). The markers used by Mello and colleagues identify a different subset of cells (here show the data).

We then mapped the Hallmark Hypoxia signature (the very same signature used by Mello et al.) onto the fibroblast compartment and found that it has the highest enrichment in a myCAFs clusters, C3, which interestingly shows the elevated levels of the mapCAFs signature (**Fig. 6a** and **Supplementary Fig. 6a**). In keeping with previous results, the hypoxia signature was also expressed in iCAF clusters. We would like also to point out that a certain amount of myCAFs displaying elevated level of Hallmark hypoxia signature could be seen also in the analysis from Mello and colleagues (see Fig. 1L of their manuscript). Finally, we mapped the hypoxia gene module onto spatial transcriptomics data to find that the signature is associated with the mapCAFs gene programs. Overall, we believe we have produced enough evidence to suggest that hypoxia is not an exclusive feature of iCAFs but rather identify a subset of myCAFs with elevated MAPK activity. Please, see also pages 14 and 15 (Results Section) of the revised version of the manuscript.

5. In Figure 4G it seems like TGF-beta alone strongly induces the sMEK gene transcriptional signature in mPSCs. Adding CM from classical or basal-like tumor cell lines does not seem to increase expression of this gene signature, and if anything, the CM from the classical tumor cell line seems to decrease the gene signature. Figure 4F also shows that there is a correlation between TGFB1 expression and the sMEK signature in the TCGA cohort. One way to interpret this data is that TGF-beta derived from tumor cells is a strong driver of the sMEK gene transcriptional signature and is produced more by basal-like tumors.

Treatment with MEKi would potentially reduce tumor-derived TGF-beta, reducing expression of this gene signature in the CAFs. Is there a difference in secreted TGF-beta from classical vs basal-like tumor cell lines?

Again, we would like to thank the reviewer for the insightful comment. The re-analysis of available data as well as the new experiments confirm the reviewer's original interpretation of the data. The mapCAFs phenotype is clearly a TGF-β driven phenotype. Indeed, we show that TGF-β treatment of mPSCs induces the expression of the mouse mapCAFs signature (see **Supplementary Fig. 4g** of the revised version of the manuscript) which, similarly to the original sMEK, shows positive correlation with TGF-β expression in TCGA. However, we do not think that TGF-β is the sole driver of the mapCAFs phenotype but rather than TGF-β drives increased abundance of myCAFs in basal-like tumour niches. Our spatial proteomic data clearly show an increased density of myCAFs in basal-like tumours (please see **Fig 1b, d** of the revised version of the manuscript). This data aligns with those reported recently by Moffitt and colleagues who showed that basal-like patients had increased myCAF proportion compared to classical one from scRNA-seq analysis (Oh et al. Nature Communications). In this manuscript, the authors suggest an increased proportion of myCAFs in basal-like tumours can be explained with the increased production of TGF-β by the epithelial cells with corresponding increased TGF-β response signature in the myCAFs of the classical one. However, the myCAFs signature does not distinguish basal-like from classical in bulk RNA-seq data, suggesting that the mapCAFs phenotype is not just the result of TGF-β expression by epithelial cells. To respond to the reviewer´s question about TGF-β secretion, we did measure the secretion of the cytokine in unperturbed and perturbed conditions. While we do not see differences based on cell lineage, we do find that MEKi leads to reduced TGF-β secretion which pairs with the reduced expression of epithelial TGF-β *in vivo* following MEKi (**Fig. 3d** and **Supplementary Fig. 3e** of the revised version of the manuscript). Overall, our data shows that epithelial MAPK activity sustains tumour-derived TGF-β expression and secretion as correctly suggested by the reviewer.

6. In Figure 5E-F you show that p-ERK activity in fibroblasts is inversely correlated with infiltration of CD8+ T cells. However, I think it is a stretch to say that the p-ERK+ CAFs are causing this lack of infiltrating CD8+ T cells. It could also be possible that the basal-like/squamous tumor niches where p-ERK+ CAFs are found are the primary drivers of immunosuppression and reduced CD8+ T cell accumulation. More data would be required to determine whether the MAPK-high CAFs are directly immunomodulatory.

We agree with this reviewer´s comment. We did not present mechanistic data to support a direct role of mapCAFs in excluding $CD8^+$ T cells from the tumour bed. We apologize for the inaccuracy, and we have amended the text accordingly to reduce the prominence of the conclusions that are not sufficiently supported by experimental data. See page 17, Discussion Section, of the revised version of the manuscript.

Minor Comments:

- ***Figure 1B – Are there less aSMA+ cells overall in classical PDAC tissues? Could you quantify the percent of aSMA+ cells that are also pERK+ in these tissues to show that the proportion of***

aSMA+ pERK+ cells goes up in basal-like tumors rather than just the total number of aSMA+ pERK+ cells? This would support the claim that basal-like tumors are associated with increased MAPK signaling in neighboring CAFs.

As suggested by this reviewer, we have quantified the amount of myCAFs in basal-like and classical sub-tumor microenvironment to show that, indeed, basal-like subTME present increased abundance of myCAFs. Please, see **Fig. 1b** of the revised version of the manuscript.

• ***Figure 3A-B - Even though percentage of CAFs that are myCAFs are going down, it looks like the total number of myCAFs are likely increasing. This correlates with the increase in fibroblasts seen following MEKi treatment in Figure 2E-F. This contrasts with the claim that MAPK signaling in CAFs drives the myCAF phenotype, as total number of myCAFs are increasing following MEK inhibition. The increase in the iCAF population is more than that of the myCAF population, which is why the proportion of CAFs which are iCAFs is increasing.***

We would like to thank the reviewer for the helpful comment. To specifically address this point, we have evaluated CAFs content using a more quantitative approach (i.e., flow cytometry) in an extended set of transplantation-based models, which are differently representative of the human malignant subtypes. We found that, overall, the treatment is not associated with changes in the frequency of CAFs (see **Fig. 2h** of the revised version of the manuscript). At the same time, we confirmed the treatment induced changes in myCAFs and iCAFs content using different methodologies (ISH, mIF and FACS) on the same set of models (**Fig. 3e-g** of the revised version of the manuscript).

• ***Figure 1F - Your control mPSCs show no baseline p-ERK. It is only induced with CM from classical or basal-like tumor cell lines. However, in Supplementary Figure 4E, your control mPSCs (both untreated and treated with TGF-B1) show significant p-ERK signaling, which seems to be reduced when treated with CM from either classical or basal-like tumor cell lines. Is the difference between the two due to serum starvation in the first experiment? If so, can you really say that your basal-like CM is inducing p-ERK activity in your second experiment when the levels of p-ERK are lower than your control?***

The reviewer is correct about the experimental conditions which dictate the different results observed in Fig 1f and Fig 4e of the original version of the manuscript. However, these data were removed from the revised version of the manuscript. The lack of proper modeling in our manuscript has been a concern raised by all the reviewers (see also responses to comment#3, comment#8, and comment#6 from reviewer#1, 2, and 3, respectively). As suggested by this and the other reviewers, we have clearly expanded the number of tumour cell lines used for the conditioned media experiments. There was no clear trend for a differential enrichment of nuclear p-ERK when mPSCs were exposed to conditioned media from more basal-like cultures (**Supplementary Fig. 1l** of the revised version of the manuscript), highlighting the phenotypical heterogeneity typical for PDAC and often limited value of cell-culture based experiments in small-sized sample sets. We could see statistically significant differences only when mPSCs were treated with conditioned media from isogenic cell lines proficient or deficient for GATA6 expression (**Supplementary Fig. 1m** of the revised version of the manuscript). It is likely that, in our reductionistic approach, we are missing some key components of the complex and highly structured ecosystem that can be found *in vivo* and that enables the emergence of this phenotype. Another possibility is that cultures are poorly representative of the *in vivo* cell states, which has been suggested by a multitude of studies recently. Therefore, we have amended the text to highlight that our observations are restricted to the *in vivo* setting. We have significantly reduced the prominence of the *in vitro* experiments and acknowledged

the limitations of our study in the discussion section. To account for that, we undertook a more thorough characterization of the *in vivo* phenotype complementing scRNA-seq and multiplex IF with spatial transcriptomics.

• Line 224 – Text says that the mPSCs were exposed to CM for 24 hours, while your legend for Supplementary Figure 4E says that they were treated with CM for 1 hour.
As discussed above, these data are no longer present in the manuscript.

References:
1. Schwoerer, S., Cimino, F. v., Ros, M., Tsanov, K. M., Ng, C., Lowe, S. W., Carmona-Fontaine, C., & Thompson, C. B. (2023). Hypoxia potentiates the inflammatory fibroblast phenotype promoted by pancreatic cancer cell-derived cytokines. Cancer Research. https://doi.org/10.1158/0008-5472.can-22-2316
2. Mello, A. M., Ngodup, T., Lee, Y., Donahue, K. L., Li, J., Rao, A., Carpenter, E. S., Crawford, H. C., Pasca di Magliano, M., & Lee, K. E. (2022). Hypoxia promotes an inflammatory phenotype of fibroblasts in pancreatic cancer. Oncogenesis, 11(1). https://doi.org/10.1038/s41389-022-00434-2

REFERENCES
Chan-Seng-Yue, M., J. C. Kim, G. W. Wilson, K. Ng, E. F. Figueroa, G. M. O'Kane, A. A. Connor, R. E. Denroche, R. C. Grant, J. McLeod, J. M. Wilson, G. H. Jang, A. Zhang, A. Dodd, S. B. Liang, A. Borgida, D. Chadwick, S. Kalimuthu, I. Lungu, J. M. S. Bartlett, P. M. Krzyzanowski, V. Sandhu, H. Tiriac, F. E. M. Froeling, J. M. Karasinska, J. T. Topham, D. J. Renouf, D. F. Schaeffer, S. J. M. Jones, M. A. Marra, J. Laskin, R. Chetty, L. D. Stein, G. Zogopoulos, B. Haibe-Kains, P. J. Campbell, D. A. Tuveson, J. J. Knox, S. E. Fischer, S. Gallinger, and F. Notta. 2020. 'Transcription phenotypes of pancreatic cancer are driven by genomic events during tumor evolution', *Nat Genet*, 52: 231-40.
Elyada, E., M. Bolisetty, P. Laise, W. F. Flynn, E. T. Courtois, R. A. Burkhart, J. A. Teinor, P. Belleau, G. Biffi, M. S. Lucito, S. Sivajothi, T. D. Armstrong, D. D. Engle, K. H. Yu, Y. Hao, C. L. Wolfgang, Y. Park, J. Preall, E. M. Jaffee, A. Califano, P. Robson, and D. A. Tuveson. 2019. 'Cross-Species Single-Cell Analysis of Pancreatic Ductal Adenocarcinoma Reveals Antigen-Presenting Cancer-Associated Fibroblasts', *Cancer Discov*, 9: 1102-23.
Hu, B., C. Wu, H. Mao, H. Gu, H. Dong, J. Yan, Z. Qi, L. Yuan, Q. Dong, and J. Long. 2022. 'Subpopulations of cancer-associated fibroblasts link the prognosis and metabolic features of pancreatic ductal adenocarcinoma', *Ann Transl Med*, 10: 262.
Kloesch, B., V. Ionasz, S. Paliwal, N. Hruschka, J. Martinez de Villarreal, R. Ollinger, S. Mueller, H. P. Dienes, M. Schindl, E. S. Gruber, J. Stift, D. Herndler-Brandstetter, G. A. Lomberk, B. Seidler, D. Saur, R. Rad, R. A. Urrutia, F. X. Real, and P. Martinelli. 2022. 'A GATA6-centred gene regulatory network involving HNFs and DeltaNp63 controls plasticity and immune escape in pancreatic cancer', *Gut*, 71: 766-77.
Lin, W., P. Noel, E. H. Borazanci, J. Lee, A. Amini, I. W. Han, J. S. Heo, G. S. Jameson, C. Fraser, M. Steinbach, Y. Woo, Y. Fong, D. Cridebring, D. D. Von Hoff, J. O. Park, and H. Han. 2020. 'Single-cell transcriptome analysis of tumor and stromal compartments of pancreatic ductal adenocarcinoma primary tumors and metastatic lesions', *Genome Med*, 12: 80.

Lupo, F., F. Pezzini, D. Pasini, E. Fiorini, A. Adamo, L. Veghini, M. Bevere, C. Frusteri, P. Delfino, S. D'Agosto, S. Andreani, G. Piro, A. Malinova, T. Wang, F. De Sanctis, R. T. Lawlor, C. I. Hwang, C. Carbone, I. Amelio, P. Bailey, V. Bronte, D. Tuveson, A. Scarpa, S. Ugel, and V. Corbo. 2024. 'Axon guidance cue SEMA3A promotes the aggressive phenotype of basal-like PDAC', *Gut*, 73: 1321-35.

Mello, A. M., T. Ngodup, Y. Lee, K. L. Donahue, J. Li, A. Rao, E. S. Carpenter, H. C. Crawford, M. Pasca di Magliano, and K. E. Lee. 2022. 'Hypoxia promotes an inflammatory phenotype of fibroblasts in pancreatic cancer', *Oncogenesis*, 11: 56.

Moffitt, R. A., R. Marayati, E. L. Flate, K. E. Volmar, S. G. Loeza, K. A. Hoadley, N. U. Rashid, L. A. Williams, S. C. Eaton, A. H. Chung, J. K. Smyla, J. M. Anderson, H. J. Kim, D. J. Bentrem, M. S. Talamonti, C. A. Iacobuzio-Donahue, M. A. Hollingsworth, and J. J. Yeh. 2015. 'Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma', *Nat Genet*, 47: 1168-78.

Peng, J., B. F. Sun, C. Y. Chen, J. Y. Zhou, Y. S. Chen, H. Chen, L. Liu, D. Huang, J. Jiang, G. S. Cui, Y. Yang, W. Wang, D. Guo, M. Dai, J. Guo, T. Zhang, Q. Liao, Y. Liu, Y. L. Zhao, D. L. Han, Y. Zhao, Y. G. Yang, and W. Wu. 2019. 'Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma', *Cell Res*, 29: 725-38.

Raghavan, S., P. S. Winter, A. W. Navia, H. L. Williams, A. DenAdel, K. E. Lowder, J. Galvez-Reyes, R. L. Kalekar, N. Mulugeta, K. S. Kapner, M. S. Raghavan, A. A. Borah, N. Liu, S. A. Vayrynen, A. D. Costa, R. W. S. Ng, J. Wang, E. K. Hill, D. Y. Ragon, L. K. Brais, A. M. Jaeger, L. F. Spurr, Y. Y. Li, A. D. Cherniack, M. A. Booker, E. F. Cohen, M. Y. Tolstorukov, I. Wakiro, A. Rotem, B. E. Johnson, J. M. McFarland, E. T. Sicinska, T. E. Jacks, R. J. Sullivan, G. I. Shapiro, T. E. Clancy, K. Perez, D. A. Rubinson, K. Ng, J. M. Cleary, L. Crawford, S. R. Manalis, J. A. Nowak, B. M. Wolpin, W. C. Hahn, A. J. Aguirre, and A. K. Shalek. 2021. 'Microenvironment drives cell state, plasticity, and drug response in pancreatic cancer', *Cell*, 184: 6119-37 e26.

Schubert, M., B. Klinger, M. Klunemann, A. Sieber, F. Uhlitz, S. Sauer, M. J. Garnett, N. Bluthgen, and J. Saez-Rodriguez. 2018. 'Perturbation-response genes reveal signaling footprints in cancer gene expression', *Nat Commun*, 9: 20.

Schworer, S., F. V. Cimino, M. Ros, K. M. Tsanov, C. Ng, S. W. Lowe, C. Carmona-Fontaine, and C. B. Thompson. 2023. 'Hypoxia Potentiates the Inflammatory Fibroblast Phenotype Promoted by Pancreatic Cancer Cell-Derived Cytokines', *Cancer Res*, 83: 1596-610.

Shiau, C., J. Cao, M. T. Gregory, D. Gong, X. Yin, J. W. Cho, P. L. Wang, J. Su, S. Wang, J. W. Reeves, T. K. Kim, Y. Kim, J. A. Guo, N. A. Lester, N. Schurman, J. L. Barth, R. Weissleder, T. Jacks, M. Qadan, T. S. Hong, J. Y. Wo, H. Roberts, J. M. Beechem, C. F. Castillo, M. Mino-Kenudson, D. T. Ting, M. Hemberg, and W. L. Hwang. 2023. 'Therapy-associated remodeling of pancreatic cancer revealed by single-cell spatial transcriptomics and optimal transport analysis', *bioRxiv*.

Steele, N. G., E. S. Carpenter, S. B. Kemp, V. R. Sirihorachai, S. The, L. Delrosario, J. Lazarus, E. D. Amir, V. Gunchick, C. Espinoza, S. Bell, L. Harris, F. Lima, V. Irizarry-Negron, D. Paglia, J. Macchia, A. K. Y. Chu, H. Schofield, E. J. Wamsteker, R. Kwon, A. Schulman, A. Prabhu, R. Law, A. Sondhi, J. Yu, A. Patel, K. Donahue, H. Nathan, C. Cho, M. A. Anderson, V. Sahai, C. A. Lyssiotis, W. Zou, B. L. Allen, A. Rao, H. C. Crawford, F. Bednar, T. L. Frankel, and M. Pasca di Magliano. 2020. 'Multimodal Mapping of the Tumor and Peripheral Blood Immune Landscape in Human Pancreatic Cancer', *Nat Cancer*, 1: 1097-112.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I would like to thank the authors for their clear and thorough reponse to my review. I have no further concerns to raise and support this manuscript progressing to publication.

We are very happy to hear that we have adequately addressed the reviewer's concerns. We take this opportunity to thank the reviewer, as we believe their constructive criticisms have significantly contributed to improving our work.

Reviewer #2 (Remarks to the Author):

The authors have added substantial new data that address most but not all of my comments. Given the breadth of responses to all reviewers critiques and the overall novelty of this body of work, I'm satisfied with their response to reviews. This is an interesting and important study.

We are pleased to hear that we have adequately addressed most of the reviewer's concerns and share their excitement about the potential of publishing our work.

Reviewer #3 (Remarks to the Author):

I thank the authors for addressing the queries raised. The resulting manuscript has been much improved. While I think the main question remains to elucidate the function of these mapkCAFs I also appreciate that will be beyond the scope of this manuscript. I would personally encourage follow up on this as the field is booming with new CAF phenotypes and subsets but with limited mechanistic progress.

We would like to thank the reviewer for their positive comments. We are pleased to hear that the reviewer feels the manuscript has improved. We fully agree that elucidating the function of MAPK-activated CAFs is an important step, and we are excited about the possibility of exploring this in future studies. As the reviewer noted, this area is rapidly evolving, and we are eager to contribute to the ongoing efforts to better understand CAF phenotypes and their mechanistic roles. We greatly appreciate reviewer's insights and encouragement.

I have 2 questions/analyses, which shouldn't require any additional experimentation that I would encourage the authors to address:

1. Firstly, if TGFb paracrine signalling is driving the mapCAF phenotype, and this is regulated by MEKi, it would be expected that TGFb regulated genes are also regulated in mapCAFs following MEKi. This could easily be exemplified using the data in Figure 4C

We thank the reviewer for their insightful comment. What we found and reported is that epithelial MAPK activity contributes to regulate TGFβ expression and secretion. Upon pharmacological treatment with trametinib, we observed a reduction-though not a complete ablation- of TGFβ expression (and secretion) in epithelial cells. This reduction is primarily associated with the decreased abundance of myofibroblastic CAFs (myCAFs) in MEKi treated animals. We have provided multiple lines of evidence demonstrating that mapCAFs represent a subset of myCAFs. Therefore, following reduced secretion of TGFβ, we expected a reduction of myCAFs and accordingly of mapCAFs, which is clear from our data. However, we do not necessarily expect that the remaining mapCAFs have a deregulation of TGFβ induced genes. Following reviewer's suggestion, we examined the transcriptomic profiles of the mapCAFs from MEKi- and vehicle-treated animals. As you can see in Figure R1 below (displayed here for review purposes only), there is no difference in the expression levels of both TGFβ-related genes (i.e., *Acta2, Tgfb1, Tnc, Col1a1*) or the enrichment of TGFβ-related gene sets (including one experimentally derived from TGFβ treated PSCs) between the two groups. We hope this answers reviewer's question.

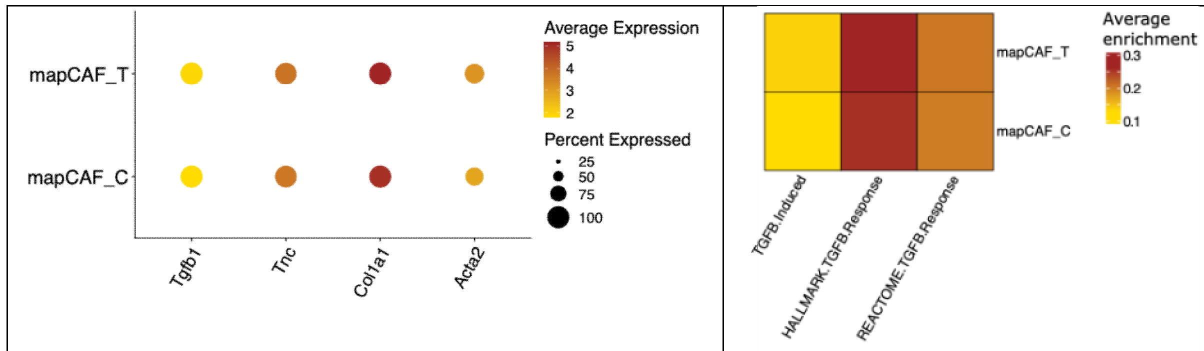

Figure R1. On the left, a bubble plot displays the average expression and fraction of cells expressing the indicated genes in mapCAFs from treated and untreated animals. On the right, a heatmap shows the average enrichment of TGFβ related gene sets in mapCAFs from vehicle- and MEKi-treated animals.

2. Secondly, in Figure 2b the number of pERK positive cells seem a bit low. To allow the reader to better interpret the data it would be beneficial to a) show individual channels and b) include additional sections/quantification.

As suggested by this reviewer, we have now provided the individual channels for the multiplex IF staining and the quantification of pERK positivity in stromal cells from several biological replicates. Please, see Figure 2b and Supplementary Figure 2b in the revised version of the manuscript.

3. Finally, just because I am puzzled:

a. In figure 2C Annotated stromal cells express RGS5, which is a pericyte marker. Why not refer to these as pericytes rather than broadly as stromal cells?

We have modified the figures as requested to annotate RGS5 expressing cells as pericytes. Please, see Figure 2c-e in the revised version of the manuscript.

b. If I understand correctly the authors include Rho Kinase inhibitors in their dissociation buffer for the single cell analysis. Would this not affect cell signalling state of cells when included for extended periods of time?

We thank the reviewer for the informative critique. For the digestion of mouse tissues, we tested several protocols before identifying the one that provided the highest cell viability, which is essential for single-cell analysis. The tissue digestions were exposed to RhoKi for 20 to 40 minutes, as we collected several fractions. It is possible that exposure to the inhibitor, as well as the digestion process itself, could affect cell signaling to some extent. However, we are confident in the robustness of our single-cell sequencing data, as the results have been validated through additional readouts, including multiplex IF and spatial transcriptomics on undigested tissues.

Reviewer #4 (Remarks to the Author):

Veghini et al have resubmitted their previous manuscript with major changes that take into account the initial reviewer comments. The core message of the paper still focuses on MAPK signaling in CAFs in the PDAC microenvironment, but the new manuscript acknowledges that MAPK signaling in malignant epithelium is a key driver of the mapCAF phenotype, predominantly through TGF-beta secretion. The following experiments and data have been added to improve the manuscript:

• Inclusion of additional cell lines and in vivo models

• Additional multiplex immunofluorescence analysis, including podoplanin as a general CAF marker

• IF and flow cytometry to verify changes in epithelial and fibroblast composition

• Measurement of TGF-B1 secretion from PDAC cell lines treated with MEKi

• Refined analysis of existing single-cell sequencing data from human PDAC patients

• Visium spatial transcriptomic analysis of 4 PDAC samples from human patients to show localization of mapCAF gene signature

• Single-cell RNA seq-based analysis of ligand-receptor interactions between cell types in the TME

The authors have responded to most of my initial concerns with a combination of new data and rewriting.

We would like to thank the reviewer for acknowledging the major changes we made in response to the initial feedback. We are glad that the reviewer feels the revised manuscript addresses most of their concerns. We truly believe that the reviewer's constructive criticisms have significantly contributed to improving the quality of our work.

I only have a few minor comments listed below:

• Can you clarify in the methods how the mapCAF gene signature, both in mouse and human, was identified? Was this based off differentially expressed genes between sMEKi-high and sMEKi-low CAFs? Could you also include a list of the mapCAF mouse genes in the Supplementary Tables? I only see the mapCAF human genes there. Also, are the mapCAF genes distinct from the sMEKi signature or are they a subset of that signature?

We thank the reviewer for the insightful comment and apologize for not including the list of genes composing the mouse mapCAFs signature in the previous version of the manuscript. We have now added this information to the Supplementary Table 5 in the revised manuscript. Following reviewer' suggestion, we have revised the method section, which now provides a more detailed description of how we identified the mapCAF signature in mice. We have also added a reference to the methods section in the results section. As noted, we initially compared the transcriptomes of mouse CAFs isolated from animals subjected to different treatments (i.e., vehicle or MEKi) for two days. We selected this time point because we observed near-complete biochemical ablation of the MAPK kinase pathway. From this, we identified a stromal MEK inhibition signature (downregulated genes only), which we considered a MAPK activity footprint. We then mapped this signature back to the single-cell transcriptomes of vehicle-treated tissues to isolate CAFs with prominent MAPK activity. To

define the mapCAF signature, we compared the transcriptomes of MAPK-high CAFs versus other CAFs. As expected, there is a substantial overlap between the sMEKi and the mapCAF signature with only 7 genes (out of 38) specific for the mapCAFs.

As described in the manuscript, we used the stromal MEKi signature to identify human CAFs with elevated MAPK activity by mapping it onto human CAF subclusters. Based on this approach, we defined the human mapCAF signature by contrasting CAFs with high versus low MAPK transcriptional activity.
We hope these clarifications address the reviewer's concerns.

- **Line 125: Should be Supplementary Fig. 1k**

We have amended the text accordingly.

- **Line 375: Should be Supplementary Fig. 7c**

We have amended the text accordingly.

- ***In Supplementary Table 6, the titles for each worksheet reference cluster 0 instead of the respective cluster being analyzed.***

We sincerely apologize to both this reviewer and the other reviewers for the oversight. During the revision process, we realized that we had mistakenly uploaded the wrong version of the table. We have since corrected this and provided the editor with the accurate version of the tables, where the issue has been addressed. Thank you for your understanding, and we appreciate your careful review of our manuscript.