

Spatial transcriptomic analysis drives PET imaging of tight junction protein expression in pancreatic cancer theranostics

Corresponding Author: Dr Katherine Ferrara

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)

Wang et al. used spatial transcriptomics (ST) and proteomics to screen for surface receptor targets in pancreatic cancer for molecular imaging and theragnostics. With the focus of a marker CLDN4, the authors developed a peptide-based molecular imaging agent. The integrative study of ST and proteomics with molecular imaging is interesting. And the data in this study were adequate and well analyzed. However, the manuscript may need some major improvement in better presenting the logic of the story. It is generally unclear of the main conclusion from each piece of the data and how it is driving the next analysis.

First of all, the authors may need to re-organize the background section to make it easier for the readers to understand the main questions/challenges to solve and how the authors advanced the field. In the current version, the authors randomly jumped back and forth among MI background, methods of the study, PDAC background, MI background, methods, etc., which made it hard to understand the logic. In addition, the paragraph of "CLDN4" seems to be standing alone without any connection to the paragraph above and below and without any explanation of why it's studied/selected and how it is important to the study. And some of the sentences may need to be re-written, as they lack inner logic to be put together. For example, it is unclear why the authors hypothesized that CLDN4 "would be exposed to a radioligand only in the presence of disease" based on the fact described earlier.

Some other comments are listed below.

On line 60, the authors may need an updated statistic for PDAC.

It is important to briefly summarize how many samples were original in this study and how many samples were acquired from the public database.

How did the authors account for batch effect when combining datasets from different centers?

Instead of using Cluster 1,2,3xxx, is there a way to annotate them as ductal/fibroblast etc. after clustering?

What does "single-annotated cells" mean here? Are they scRNAseq cohorts or annotation for the spatial data?

On line 117, a statistical test of if this % is significantly higher than in other surface markers in cluster 1, or higher than the % in other clusters may be necessary.

Is the "myCAF" one of the PDAC CAF subtypes? If so, did the authors detect iCAF, the other subtype?

Based on the data and analysis, is it possible to distinguish IPMN and PDAC? If so, what is the sensitivity and specificity?

Reviewer #2

(Remarks to the Author)

In this manuscript, the authors employed spatial transcriptomics and proteomics to identify cancer-specific surface markers. They identified CLDN4 as a potential theranostic target for pancreatic cancer and developed an Cu-64 labeled peptide to target the CLDN4-positive PDAC cells. The manuscript is generally well-written, although additional details are needed in many parts to enhance reader comprehension.

A few comments:

Line 113: It is unclear how the authors established the surfaceome score. Please provide further elaboration.

Figure 1F and Figure 4I: It is challenging to interpret the biological meaning of these figures.

Figure 2A: The images in the upper panel do not correspond with those in the lower panel, making it difficult to compare

staining results. Ideally, the authors should use a continuous section to perform staining for better comparison.

Figure 2d: Experimental data is lacking to support the proposed model.

Figure 7: The abbreviations used in the figures should be annotated in the figure legend. I assume "K" represents kidney here?

Figure 7g: It's challenging to discern any tumor in the bright field. The authors may consider including some HE staining pictures to showcase the tumor tissue.

Line 556: The sequencing data should be made publicly available.

Reviewer #3

(Remarks to the Author)

Molecular imaging enables non-invasive visualization and quantification of molecular expression levels across time in living systems. Spatial omics provide an opportunity to identify an ideal agent for MI. James Wang and colleagues applied We apply spatial transcriptomics and proteomics to select pancreatic cancer surface receptor targets for molecular imaging and theranostics. They identified CLDN4 as a theranostic target which is significantly highly expressed in cancer compared to normal pancreas. Further, they developed a peptide-based molecular imaging agent targeted to claudin-4 in a model of murine PDAC. Overall, this study makes an application of spatial omics to discover surface protein markers as agents for MI and explores their preclinical applications, which have a strong interdisciplinary investigation. The study is innovative. The reviewer has the following comments to improve the manuscript.

1. How the authors combined different ST points into UMAP, the reviewer examined the clusters projected on the HE images, cluster 1 in HT28BP1 appears to be enriched in the stromal region. If the number of samples in ST is changed, will the obtained cluster change?
2. In Fig. 1C, the Cluster identities were based on gene enrichment analysis concerning the molecular signature database. This is inappropriate, PDAC cannot recognize cell types such as hepatocytes and OPCs. They should use cell types defined by PDAC scRNA-seq.
3. The evaluation of DNA copy number variation can help the authors validate the spots with tumor cells.
4. How many cancer surfaceome genes were identified in this study? Authors should provide a supplementary table listing information on the cancer surfaceome genes they defined.
5. In Figure 3A, the HE image of a PDAC shows tumor cells dominating the entire window illustrated by the authors, such as sample STPDAC13. However, the expression of CLDN4 does not exactly match that of tumor cells. The authors quantified CLDN4 expression in tumor cell spots on each slide.
6. The clusters in Figure 4B-C show patient specificity, such as cluster 13 only in STIPMN11 and cluster 12 only in STIPMN34. Is this due to a batch effect or is it biologically significant?
7. It's not clear for the left panel and right panel of Fig 5J, the authors need to describe how to perform and interpret the results.
8. The CODEX in Figure 6 did not include the expression CLDN4. How do the results of this section relate to CLDN4?
9. Some figure numbers are written in the figures, making the label unclear, such as Fig. 2A, C, and D.

Reviewer #4

(Remarks to the Author)

Ferrara et al reported spatial transcriptomic analysis drives PET imaging of tight junction protein expression in pancreatic cancer imaging. The authors selected a tight junction protein claudin-4 as a target, and developed a peptide-based PET tracer for tumor bioimaging. However, the claudin-4 has been reported as a high expressed protein in tumor by some papers, and the peptide-based PET tracer was lacked of innovation. Hence, this work is not suitable to Nature Communications in current vision. Some comments were listed as below:

1. In the results regarding PET imaging of claudin-4 (CLDN4) in a PDAC mouse model, it seems the background uptake of ^{64}Cu -C4BP is rather high, particular in the kidneys and liver. Please provide the coronal MIP image instead of the sagittal view (otherwise I would think the PET images authors presented are rather selective), so that we can see the in vivo distribution pattern of ^{64}Cu -C4BP clearer.
2. From the ex vivo PET among different organs, I see extremely high uptake in the normal digestive system, particular for the liver, intestines, and pancreas. Since the main clinical use ^{64}Cu -C4BP would be imaging of pancreatic cancer, how can we tell the tumor lesions among such high background uptake? In Fig 7G, it's hard to say that the tumor is well visualized in the PET image (rather low tumor-to-background ratio). The translational application and resolved methods are required detailed discussion in the discussion section.
3. As a novel PET agent, I would also be curious about the in vivo distribution pattern of ^{64}Cu -C4BP in PDAC tumor bearing mice, please also provide the dynamic scan of ^{64}Cu -C4BP PET, for least 60 min p.i. In addition, the ex vivo biodistribution study is also required for evaluation of the in vivo distribution pattern of ^{64}Cu -C4BP.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have significantly improved their manuscript in this revision. Most of my previous concerns have been addressed. I just have some minor comments below.

- On Figure 3, what is the unit of gene expression? Is it normalized to 0 to 4 for each gene? If so, what is the normalization method? More generally, on all the spatial transcriptomic figures, the authors need to mention the unit of the data so that the visualization for different genes are comparable.
- And please correct a minor grammar issue between line 53-57.

Reviewer #2

(Remarks to the Author)

The rebuttal letter is well written and the authors have addressed most of my concerns, I have no more comments on this manuscript.

Reviewer #3

(Remarks to the Author)

The authors have improved the manuscript and addressed most reviewer's comments. The remained comments were as follows:

1. In the previous reviewer's comment 3, the authors performed DNA copy number variation analysis on both PDAC and IPMN samples. Do they use any spots as references?
2. Based on additional analysis of Fig. R10-11 and R14. The results showed only cluster 1 in PDAC was defined as tumor cells, while multiple clusters in IPMN were defined as tumor cells, do the different clusters of Fig. R11 come from different patients? Were the surface genes consistent in different patients or did they show different patterns?

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Spatial transcriptomic analysis drives PET imaging of tight junction protein expression in pancreatic cancer theranostics

Response to Reviewers' Comments:

We appreciate the careful and detailed comments of the reviewers and have answered each question or critique. We have performed extensive additional studies to detail the accumulation of the radiopharmaceutical over time and to compare IHC with the imaging results. Overall, the added data confirms that this radiopharmaceutical accumulates specifically in tumor tissue in proportion to Claudin-4 expression.

Reviewer #1 (Remarks to the Author): Expert in computational pancreatic cancer genomics, bioinformatics, and spatial omics

General comments:

Wang et al. used spatial transcriptomics (ST) and proteomics to screen for surface receptor targets in pancreatic cancer for molecular imaging and theragnostics. With the focus of a marker CLDN4, the authors developed a peptide-based molecular imaging agent. The integrative study of ST and proteomics with molecular imaging is interesting. And the data in this study were adequate and well analyzed. However, the manuscript may need some major improvement in better presenting the logic of the story. It is generally unclear of the main conclusion from each piece of the data and how it is driving the next analysis.

Comment 1:

First of all, the authors may need to re-organize the background section to make it easier for the readers to understand the main questions/challenges to solve and how the authors advanced the field. In the current version, the authors randomly jumped back and forth among MI background, methods of the study, PDAC background, MI background, methods, etc., which made it hard to understand the logic. In addition, the paragraph of "CLDN4" seems to be standing alone without any connection to the paragraph above and below and without any explanation of why it's studied/selected and how it is important to the study. And some of the sentences may need to be re-written, as they lack inner logic to be put together. For example, it is unclear why the authors hypothesized that CLDN4 "would be exposed to a radioligand only in the presence of disease" based on the fact described earlier.

Response: Thank you. We have revised the introduction accordingly. For the final point, CLDN4 is a tight junction protein with cell-cell junctions.

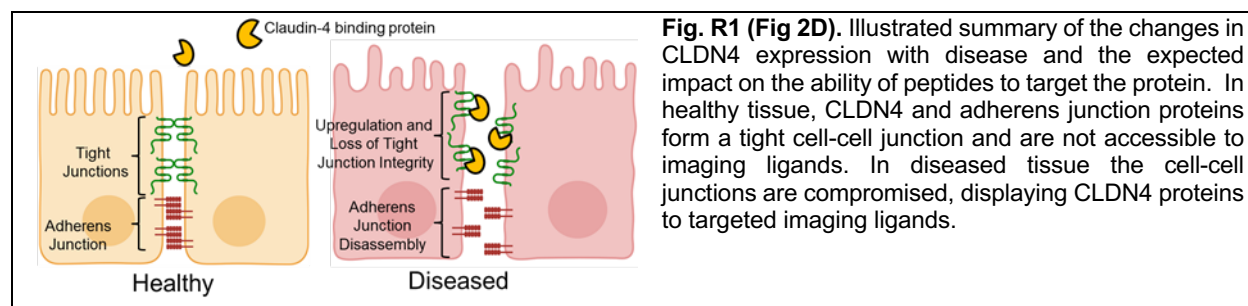


Fig. R1 (Fig 2D). Illustrated summary of the changes in CLDN4 expression with disease and the expected impact on the ability of peptides to target the protein. In healthy tissue, CLDN4 and adherens junction proteins form a tight cell-cell junction and are not accessible to imaging ligands. In diseased tissue the cell-cell junctions are compromised, displaying CLDN4 proteins to targeted imaging ligands.

Comment 2:

On line 56, the authors may need an updated statistic for PDAC.

Response:

The authors thank the reviewer for the helpful comment. Relevant text has been modified according to the latest cancer statistic for pancreatic cancer and PDAC

“Further, PDAC is the third leading cause of cancer-related deaths as of 2024¹.”

It is important to briefly summarize how many samples were original in this study and how many samples were acquired from the public database.

In line 101, the text has been modified to:

“Our discovery cohort includes 40 human surgical samples of surrounding pancreas, IPMNs² or PDAC³ acquired at Stanford (n = 12 Visium and associated CODEX and IHC data for these samples) or from publicly-available databases (n = 28 Visium)^{2,3} (**Supplementary Table 1, Fig. 1A**) and single-annotated cells from references³⁻⁵.”

Comment 3:

How did the authors account for batch effect when combining datasets from different centers?

Response:

The authors thank the reviewers for the helpful question. Batch effect was accounted for with Seurat’s integration function for different datasets, which addresses batch differences based on canonical correlation analysis and aligning datasets using mutual nearest neighbors. Relevant text has been added to line 107:

“We first integrated data from treatment-naïve PDAC samples and performed unsupervised clustering (**Supplementary Fig. 1, Fig. 1A-B**). We leveraged Seurat’s integration method, which uses canonical correlation analysis and alignment based on mutual nearest neighbors to address for batch differences⁶.”

Comment 4:

Instead of using Cluster 1,2,3xxx, is there a way to annotate them as ductal/fibroblast etc. after clustering?

Response:

The authors thank the reviewers for the question. It is important to annotate the spots after clustering. We started with distinguishing cancerous clusters based on cancer surfacesome score and enrichment in cell type molecular signatures, and then annotated the spots based on possible cell types during trajectory analysis.

Relevant text has been added at line 115:

“Given that Visium spots contain multiple cells, we then performed hypergeometric t tests (**Supplementary Fig. 1D**) to determine the significance and overlap of differentially expressed genes and single cell RNA cell signatures^{3,4}. Cluster 1 is primarily enriched in tumor cell signature genes, with secondary enrichment in fibroblasts and ductal cells, underscoring the tumor environment consisting of ductal cells and fibroblasts (**Fig. 1C**)⁷.”

And to line 195:

“Hypergeometric t tests of differentially-expressed genes with respect to single cell RNA cell type signatures^{3,4} indicated that Clusters 6, 9, 12, and 16 were enriched in tumor signature genes with p values of less than $5E^{-11}$. CNV analysis indicated that Clusters 6 and 12 had higher CNV gains (**Supplementary Fig. 3A**). Single cell RNA tumor signatures in IPMN samples confirmed that tumor signature genes clusters were upregulated in Cluster 6 and 12 (**Supplementary Fig. 3B**).”

Comment 5:

What does “single-annotated cells” mean here? Are they scRNAseq cohorts or annotation for the spatial data?

Response:

The authors thank the reviewers for the question.

The text has been changed at line 101:

“Our discovery cohort includes 40 human surgical samples of surrounding pancreas, IPMNs² or PDAC³ acquired at Stanford (n = 12 Visium and associated CODEX and IHC data for these samples) or from available databases (n = 28 Visium)^{2,3} (**Supplementary Table 1, Fig. 1A**) and annotated single cell RNA sequencing results from reference databases³⁻⁵.”

Comment 6:

On line 140, a statistical test of if this % is significantly higher than in other surface markers in cluster 1, or higher than the % in other clusters may be necessary.

Response:

The authors thank the reviewer for the important question. We have performed differential expression analysis on cluster 1 vs other clusters and filtered cancer surfacesome genes with significance. We found that CLDN4 had the highest fold change in cluster 1 compared to other clusters with an average log₂ fold change of 13.95 and an p adjusted value less than $7.6E^{-300}$. We now include a table of genes with log₂ fold change greater than 2 and low p adjusted values as a supplementary table:

Supplementary Table 3. Top fold change genes that were also cancer surfacesome genes from cluster 1 in PDAC. Differentially expressed genes were filtered to have a p adjusted value of less than 0.005, log₂ fold change of greater than 2, and only cancer surfacesome genes.

Gene	avg_log2FC	p_val_adj
CLDN4	13.9490565	<7.6E-300
MUC1	12.9688808	<7.6E-300
S100A6	12.0459834	<7.6E-300
ATP1B1	11.9892492	<7.6E-300
EPCAM	8.16324381	<7.6E-300
TSPAN3	7.09969735	<7.6E-300
TACSTD2	6.64513503	<7.6E-300
MAL2	6.62702882	<7.6E-300
SPINT2	5.69193482	<7.6E-300
TM4SF1	5.61342856	<7.6E-300
TSPAN8	5.47374558	<7.6E-300
CD151	5.10789781	<7.6E-300
CD164	3.66450756	<7.6E-300
ATP1A1	3.44909867	<7.6E-300
HSP90AA1	2.99706639	<7.6E-300

BSG	2.77598458	<7.6E-300
CLDN7	2.60022171	<7.6E-300
APLP2	2.5657688	<7.6E-300
CD9	2.41776531	<7.6E-300
VDAC1	2.16868499	<7.6E-300
TMEM123	2.08693183	<7.6E-300

We have also added the following relevant text to line 141 addressing the supplementary table:

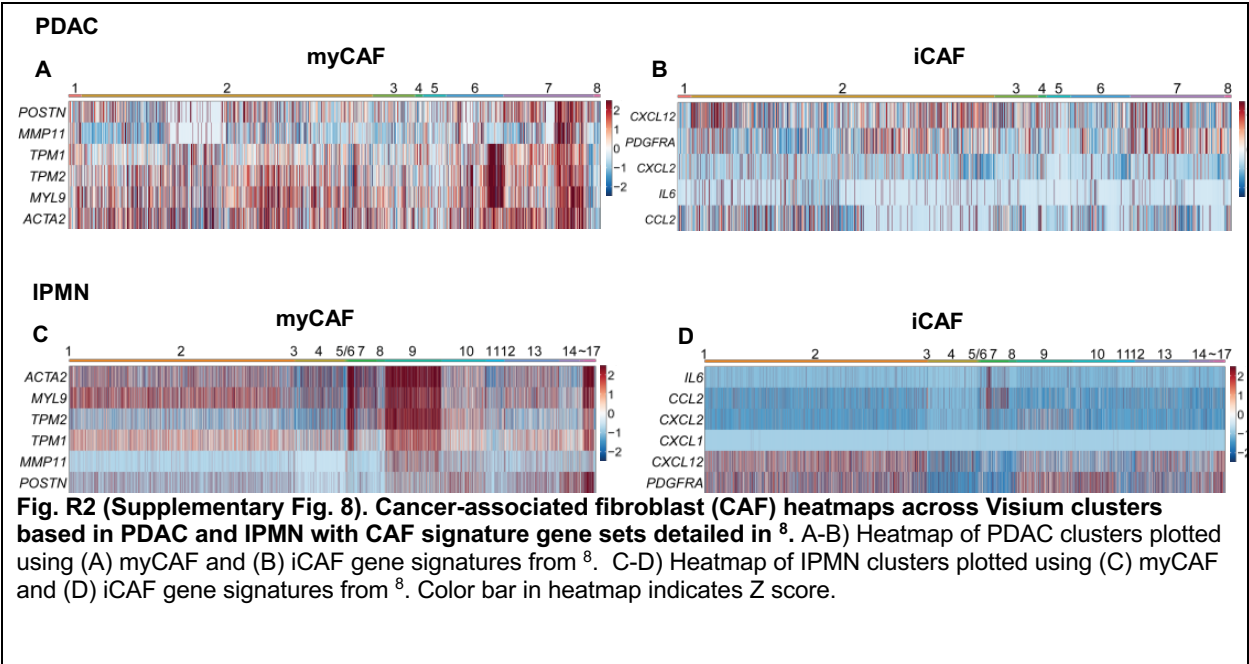
“We performed differential analysis comparing Cluster 1 with the remaining clusters and explored cancer surfacesome genes with an average log₂ fold change greater than 2 and a p value less than 0.005. *CLDN4* was upregulated to a greater extent than other genes in Cluster 1 with an average log₂ fold change of 13.9 and a p value less than 7.6E⁻³⁰⁰ (**Supplementary Table 3**).”

Comment 7:

Is the “myCAF” one of the PDAC CAF subtypes? If so, did the authors detect iCAF, the other subtype?

Response:

Thank you for this key question. We have included key gene signatures from myCAFs and iCAFs in both PDAC and IPMN to explore CAF heterogeneity in our samples. These new results are now included as a supplementary figure.



We have also added the following text at line 282:

“As a result, we further explored CAF phenotype signatures in the transcriptomic data within PDAC and IPMN samples. We removed tumor and immune cells with low *VIM* and high *EPCAM* expression or high *PTPRC* expression. Using myCAF and iCAF signatures compiled from ⁸⁻¹⁰, key myCAF genes were expressed in PDAC samples to a greater extent than iCAF genes

(Supplementary Fig. 8A-B). In the IPMN samples, myCAF genes were expressed in all clusters and to a greater extent in cluster 9 and 17 (Supplementary Fig. 8C). Expression of iCAF gene signatures was low (Supplementary Fig. 8D).”

Comment 8:

Based on the data and analysis, is it possible to distinguish IPMN and PDAC? If so, what is the sensitivity and specificity?

Response:

The authors thank the reviewer for this question. We found that there are invasive cancer phenotypes arising within IPMN locations and therefore our current database (lacking long term outcomes) is not sufficient to fully classify the lesions. This will be a goal of future work.

Reviewer #2 (Remarks to the Author): Expert in PET imaging and imaging agent synthesis

General comments:

In this manuscript, the authors employed spatial transcriptomics and proteomics to identify cancer-specific surface markers. They identified CLDN4 as a potential theranostic target for pancreatic cancer and developed an Cu-64 labeled peptide to target the CLDN4-positive PDAC cells. The manuscript is generally well-written, although additional details are needed in many parts to enhance reader comprehension.

Comment 1:

Line 126: It is unclear how the authors established the surfaceome score. Please provide further elaboration.

Response:

The authors thank the reviewer for the key question. We have strengthened the explanation for surfaceome score and modified text in line 127:

“To quantify how top cancer surfaceome atlas markers change across clusters (Supplementary Table 3), we created a cancer surfaceome score based on the total sum average of normalized and scaled expression of cancer cell surfaceome genes in each cluster.”

Comment 2:

Figure 1F and Figure 4I: It is challenging to interpret the biological meaning of these figures.

Response:

The authors thank the reviewer for the comment. Gene correlation analysis provides insight into gene expression that could infer possible biological pathways related to the disease state. We have added additional explanation in the text at line 133:

“*CLDN4* is involved in tight junction formation, which is compromised during cancer progression. Positive correlation among cancer surface protein encoding genes indicates that *CLDN4* expression changes are spatially correlated with other cancer-associated genes.”

And in line 209:

“*CLDN4*, as a tight junction protein encoding gene, correlates with cancer markers such as *S100P* and *TFF1*.”

Comment 3:

Fig. 2A: The images in the upper panel do not correspond with those in the lower panel, making it difficult to compare staining results. Ideally, the authors should use a continuous section to perform staining for better comparison.

Response:

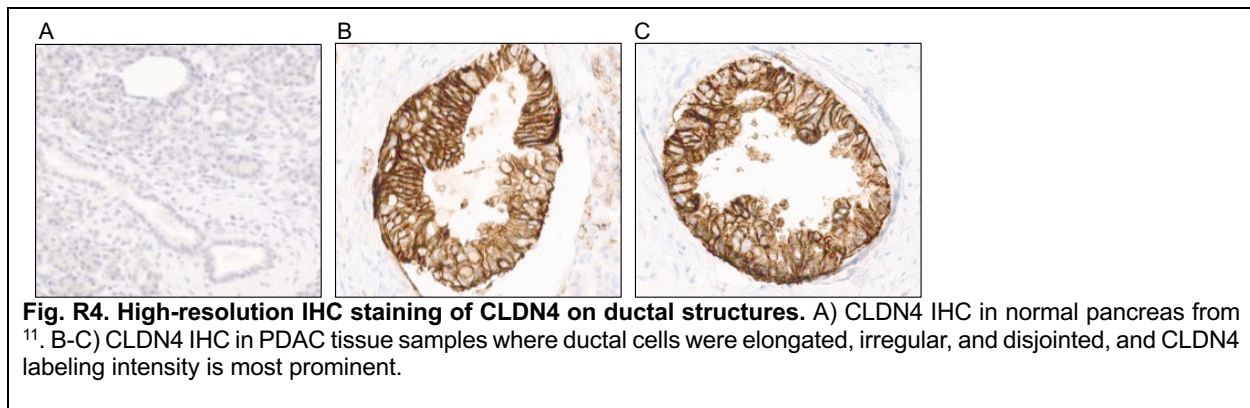
The authors thank the reviewer for the comment. Adjacent slices were selected for IHC and multiplexed IHC staining. We have removed images for which the interslice changes made the interpretation challenging.

Comment 4:

Fig. 2d: Experimental data is lacking to support the proposed model.

Response:

The authors thank the reviewer for the comment. Below, we include examples of high-resolution IHC staining (**Fig. R4**) to confirm elevated CLDN4 expression on cells lacking the normal columnar architecture and the availability of increased CLDN4 expression on the epithelial surfaces. This provides the opportunity for the peptide ligand to bind. For contrast, we have included an example from ¹¹ showing CLDN4 IHC staining in normal pancreas tissue illustrating that CLDN4 labeling was not apparent (**Fig. R4A**).



The detailed mechanisms of CLDN4-induced disruption have been documented in the literature and now included in line 81:

CLDN4 is a tight junction protein that is buried within cell-cell junctions in healthy tissue and has been suggested as a cancer biomarker¹². CLDN4 expression increases in PDAC, and enhanced CLDN4 expression disrupts other CLDN family molecules in forming polymeric strands constituting the tight junction¹³.

Comment 5:

Figure 7: The abbreviations used in the figures should be annotated in the figure legend. I assume "K" represents kidney here?

Response:

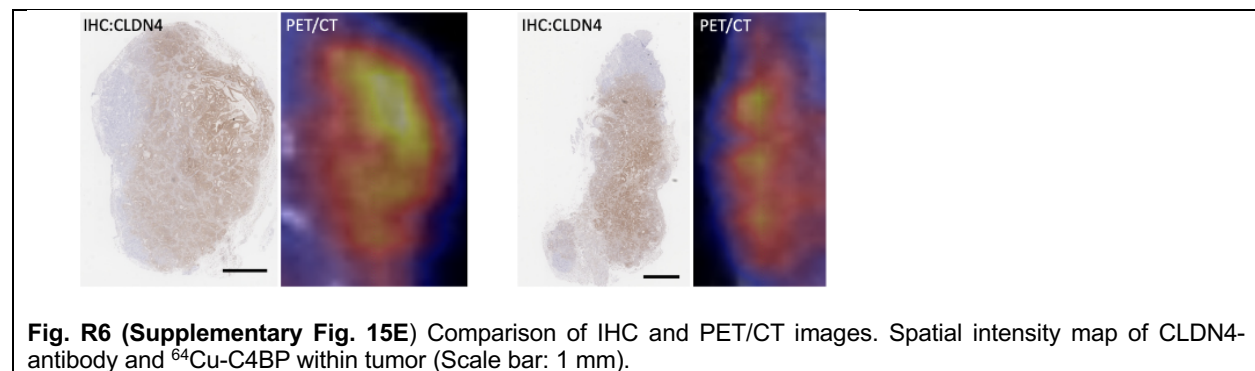
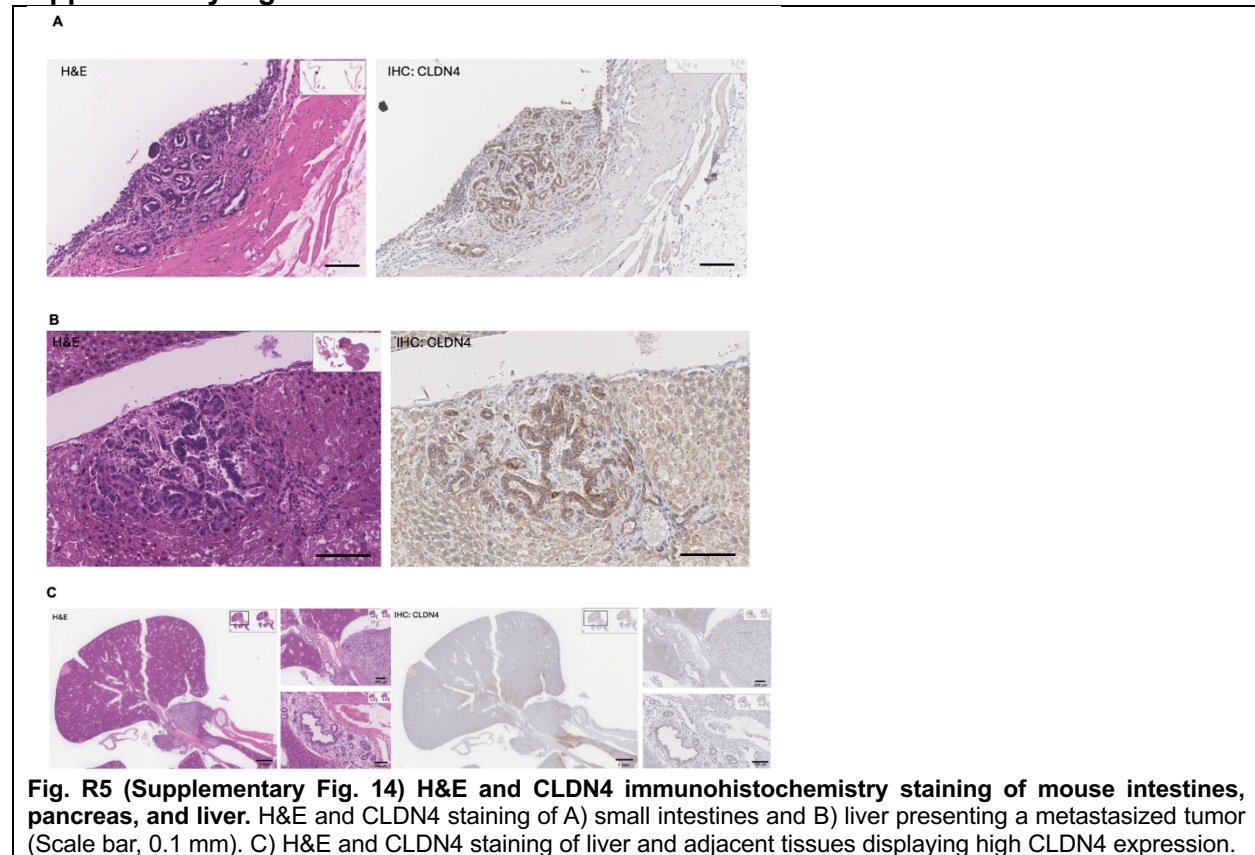
The authors appreciate this comment. The K denotes kidney, and abbreviation definitions have been updated in the caption of Fig. 7.

Comment 6:

Fig. 7g: It's challenging to discern any tumor in the bright field. The authors may consider including some HE staining pictures to showcase the tumor tissue.

Response:

The authors thank the reviewer for the comment. Additional H&E staining of tissue has been performed and is now included in the supplementary data as **Supplementary Fig. 14** and **Supplementary Fig. 15**.



Comment 7:

Line 556: The sequencing data should be made publicly available.

Response:

Thank you. We will make the data publicly available upon acceptance.

Reviewer #3 (Remarks to the Author): Expert in cancer spatial multiomics, tumour microenvironment, computational genomics, and imaging

General comments:

Molecular imaging enables non-invasive visualization and quantification of molecular expression levels across time in living systems. Spatial omics provide an opportunity to identify an ideal agent for MI. James Wang and colleagues applied We apply spatial transcriptomics and proteomics to select pancreatic cancer surface receptor targets for molecular imaging and theranostics. They identified CLDN4 as a theranostic target which is significantly highly expressed in cancer compared to normal pancreas. Further, they developed a peptide-based molecular imaging agent targeted to claudin-4 in a model of murine PDAC. Overall, this study makes an application of spatial omics to discover surface protein markers as agents for MI and explores their preclinical applications, which have a strong interdisciplinary investigation. The study is innovative. The reviewer has the following comments to improve the manuscript.

Comment 1:

How the authors combined different ST points into UMAP, the reviewer examined the clusters projected on the HE images, cluster 1 in HT28BP1 appears to be enriched in the stromal region. If the number of samples in ST is changed, will the obtained cluster change?

Response:

The authors thank the reviewer for the key question. Clustering parameters were optimized towards higher cluster homogeneity with silhouette score. The cancer associated cluster 1 pointed to stromal regions that appear to have cancer development (**Fig. R7**, H&E). Furthermore, the cancer regions were consistent with *CLDN4* and *S100P* spatial distribution (**Fig. R7**, *S100P* and *CLDN4*).

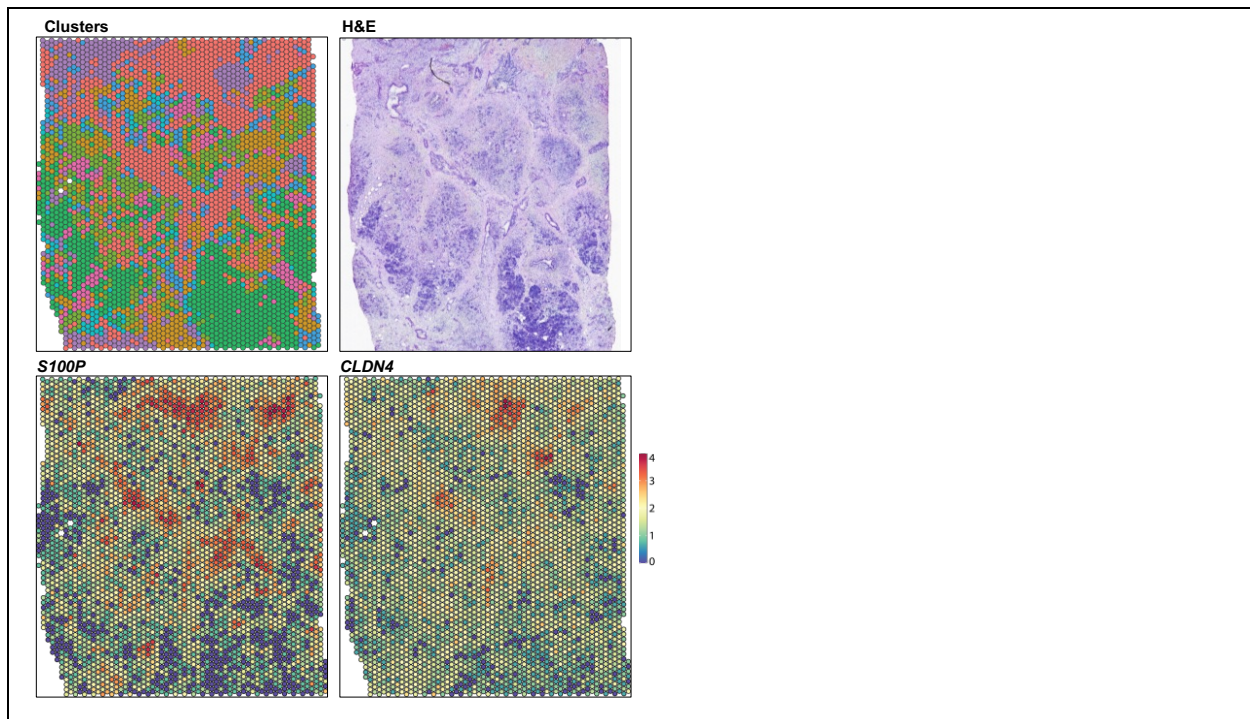


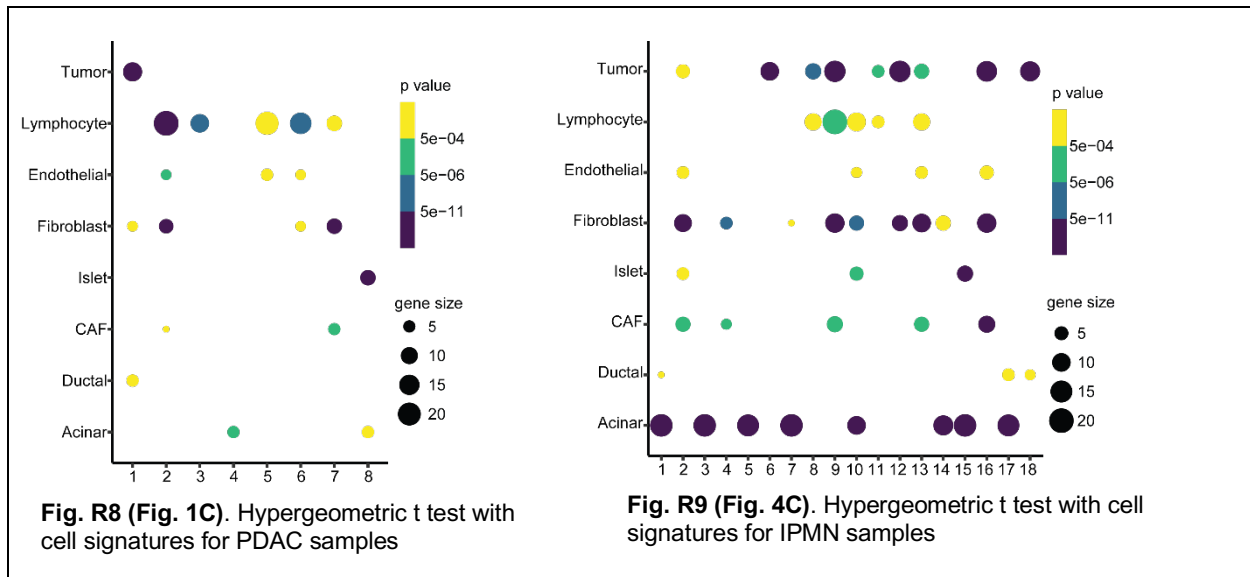
Fig. R7. Cluster regions on HT28BP1. Clusters and corresponding high-resolution H&E, *S100P* and *CLDN4* spatial expression maps.

Comment 2:

In Fig. 1C, the Cluster identities were based on gene enrichment analysis concerning the molecular signature database. This is inappropriate, PDAC cannot recognize cell types such as hepatocytes and OPCs. They should use cell types defined by PDAC scRNA-seq.

Response:

The authors appreciate this important comment. We have removed enrichment on molecular signature database from **Fig. 1C** and **Fig. 4C**. We alternatively performed hypergeometric t tests comparing cluster differentially expressed genes to cell type signatures compiled from single cell RNA sequencing in PDAC in our references. The new **Fig. 1C** and **Fig. 4C** are as follows and have been updated in the manuscript:



New relevant text to address these figures has also been added at line 115:

“Given that Visium spots contain multiple cells, we then performed hypergeometric t tests to determine the significance and overlap of differentially expressed genes and single cell RNA cell signatures^{3,4}. Cluster 1 is primarily enriched in tumor cell signature genes, with secondary enrichment in fibroblasts and ductal cells, underscoring the tumor environment consisting of ductal cells and fibroblasts (**Fig. 1C**)⁷.”

And line 195:

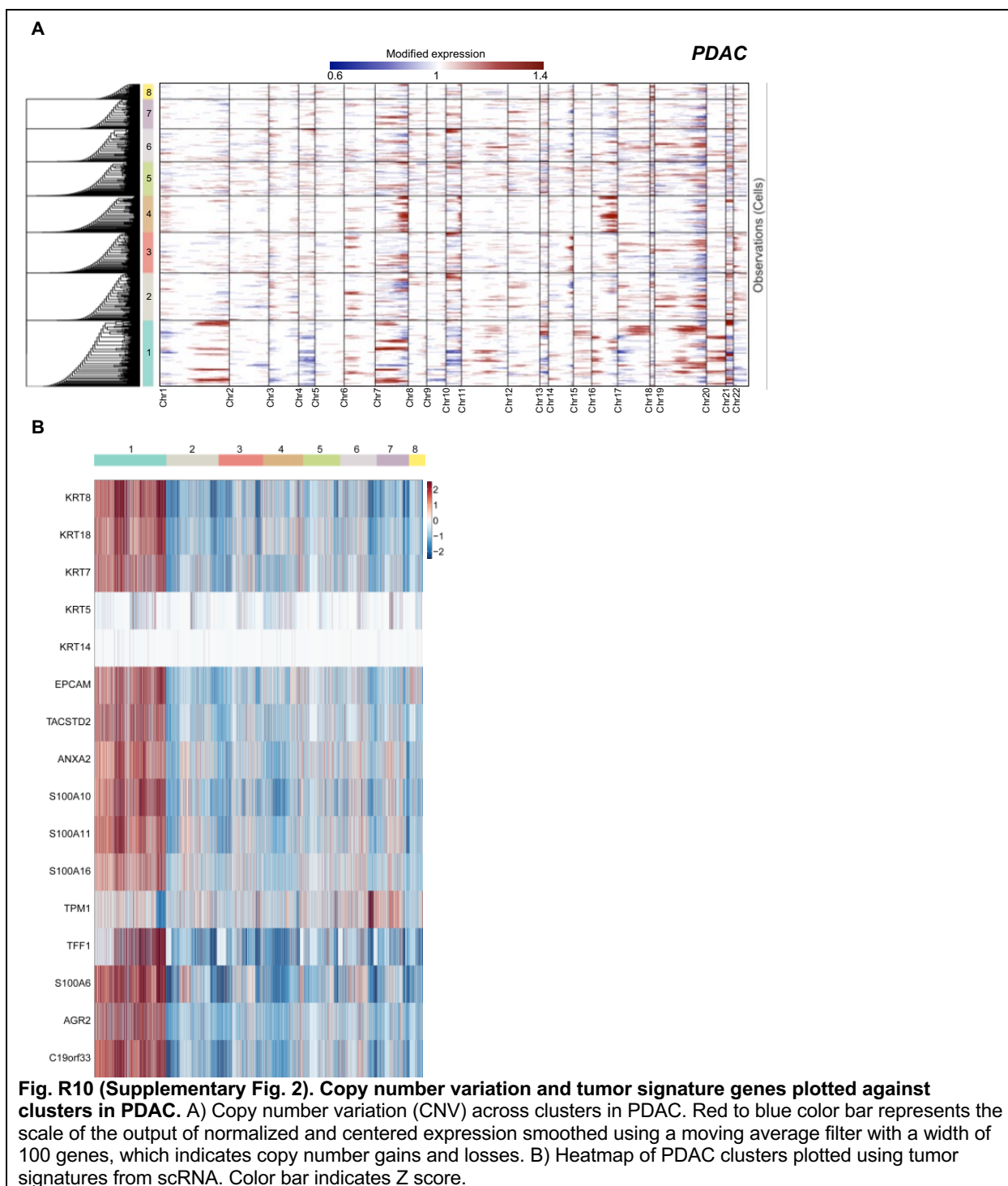
“Hypergeometric t tests of differentially-expressed genes with respect to single cell RNA cell type signatures^{3,4} indicated that Clusters 6, 9, 12, and 16 were enriched in tumor signature genes with p values of less than 5×10^{-11} .”

Comment 3:

The evaluation of DNA copy number variation can help the authors validate the spots with tumor cells.

Response:

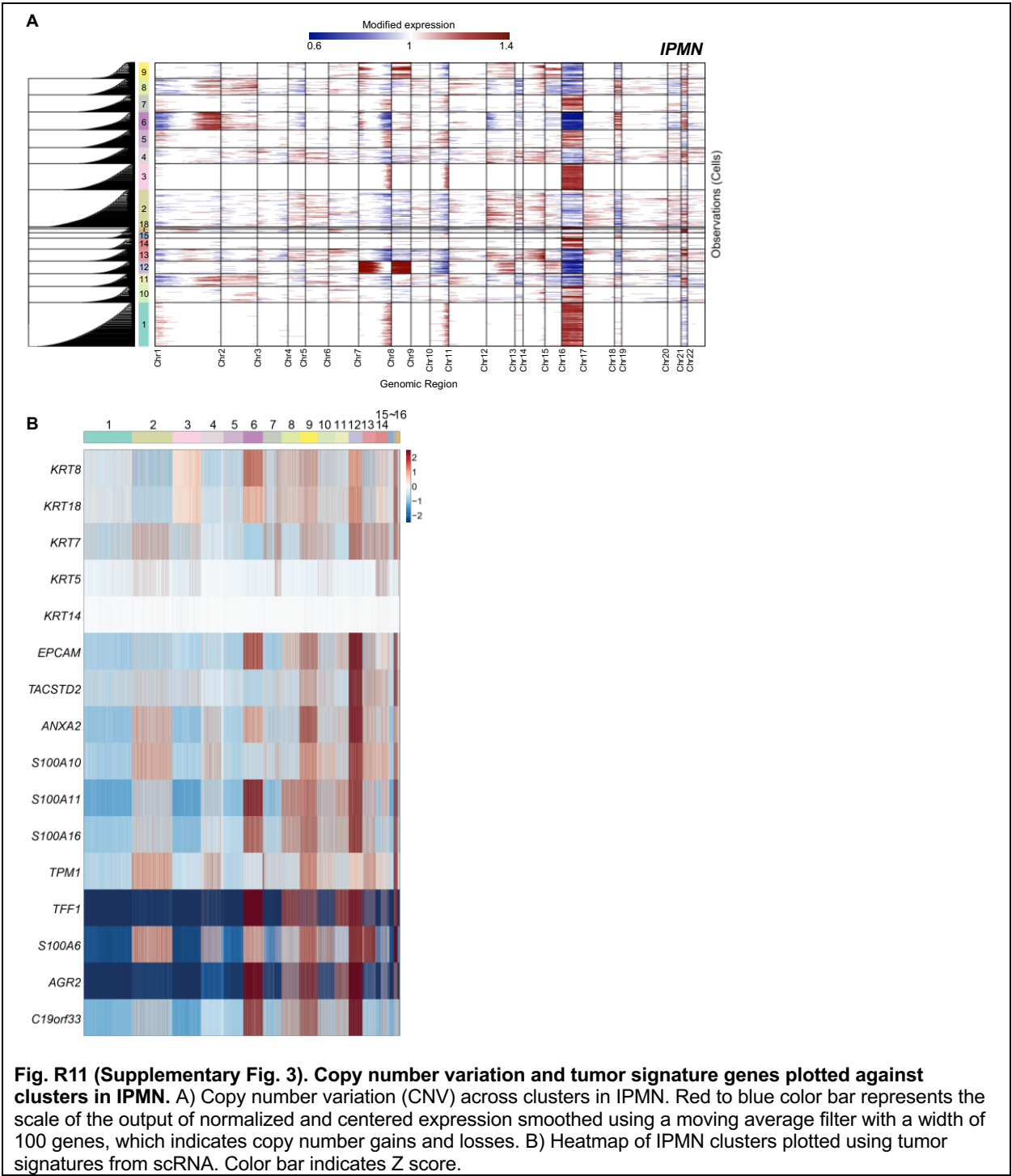
The authors thank the reviewer for the helpful comment. We performed copy number variation analysis on both PDAC and IPMN samples across clusters and have included them in the supplementary data:



We have also added relevant text at line 122:

“Since cancer development is associated with increased copy number variation (CNV), we computed CNV from our transcriptomic data across the clusters and found that Cluster 1 had increased CNV gains compared to other clusters (**Supplementary Fig. 2A**). Single cell RNA

tumor signatures^{3,4} applied across clusters also confirmed that Cluster 1 specifically upregulated tumor signature genes in PDAC samples (**Supplementary Fig. 2B**).”



We also added the following text at line 199:

“CNV analysis indicated that Clusters 6 and 12 had higher CNV gains (**Supplementary Fig. 3A**). Single cell RNA tumor signatures in IPMN samples confirmed that tumor signature genes clusters were upregulated in Cluster 6 and 12 (**Supplementary Fig. 3B**).”

Comment 4:

How many cancer surfaceome genes were identified in this study? Authors should provide a supplementary table listing information on the cancer surfaceome genes they defined.

Response:

The authors thank the reviewer for this comment. **Supplementary Table 2** listing the cancer surfaceome genes used is now included and copied below for convenience:

B2M	100.00
EEF1A1	99.99
S100A6	99.94
HLA-A	99.35
CD63	98.65
ITM2B	98.63
HLA-C	98.41
IFITM3	98.34
SPINT2	97.90
RAC1	97.73
HSP90AA1	97.55
CD74	97.29
EPCAM	96.18
TM4SF1	96.16
CYBA	96.10
CD59	95.89
RPSA	95.73
HSP90AB1	94.15
ATP1A1	93.57
ATP1B1	93.26
VAMP8	92.40
LY6E	92.10
TSPAN8	90.40
ARF1	89.77
HLA-E	89.27
RHOA	89.18
CDC42	89.04
CLDN4	88.83
CD151	88.61
TACSTD2	88.23
CIB1	87.51
IFITM2	87.32
CD164	86.53
ITGB1	86.47
APLP2	86.33
CD9	86.33
RTN4	86.27
ENO1	86.15
UBE2D3	86.12
CALR	85.88
RAB11A	85.57
APP	85.51
MUC1	85.32
TSPAN3	85.24
EZR	85.06
BSG	85.03
VDAC1	84.98
P4HB	84.87
HLA-DRA	84.85
TMEM123	84.52
TMED10	83.19
VAPA	81.93
VPS28	81.55
TMEM219	81.32
MAL2	81.02
CLDN7	80.28
HLA-B	80.05

We also modified the text to clarify this point in line 112:

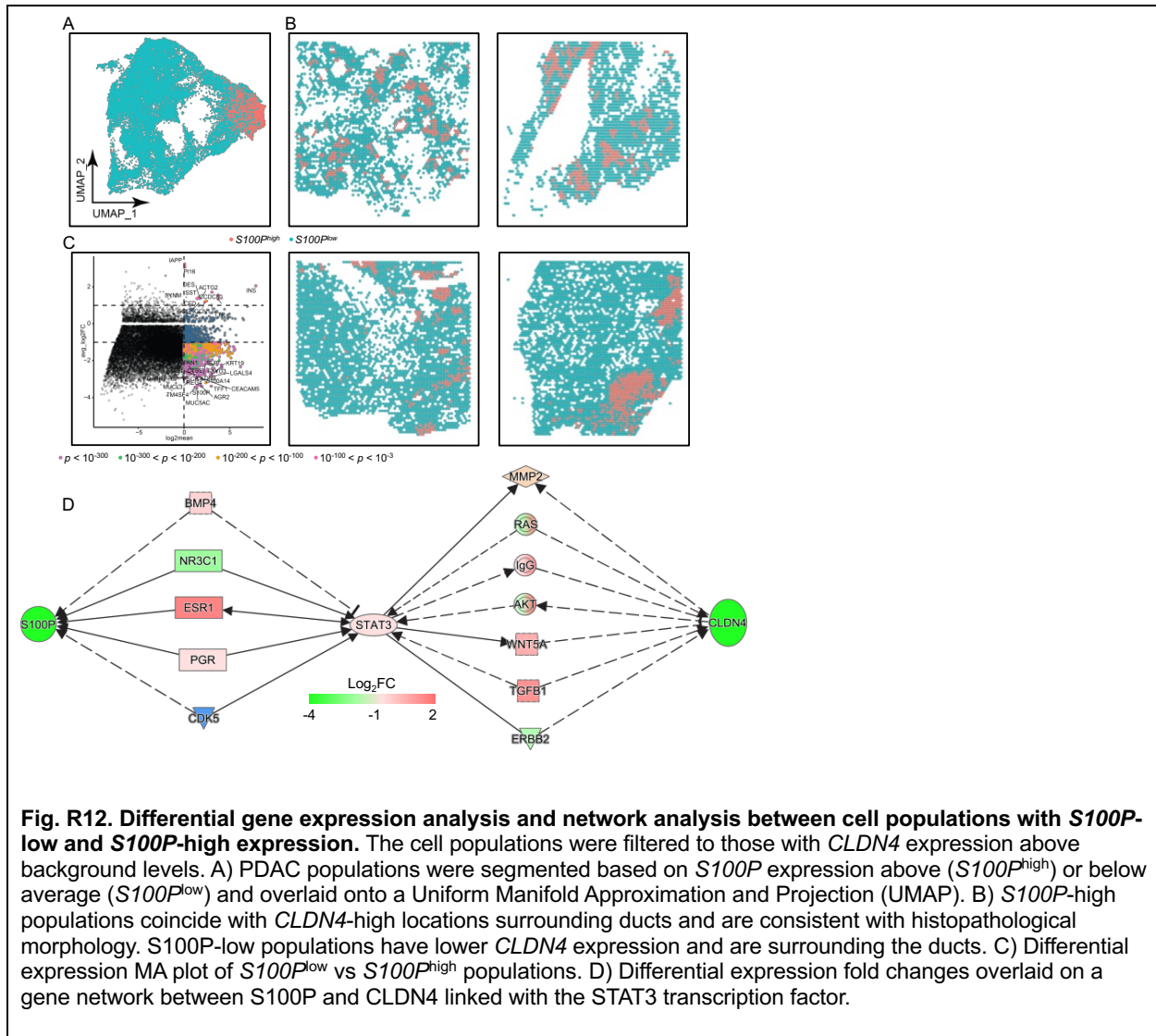
“Since we seek to develop an imaging ligand that binds to PDAC cells, we filtered the cancer surfacesome to identify genes with enhanced expression in more than 80% of PDAC cells (**Supplementary Table 2**) as our cancer surfacesome gene list.”

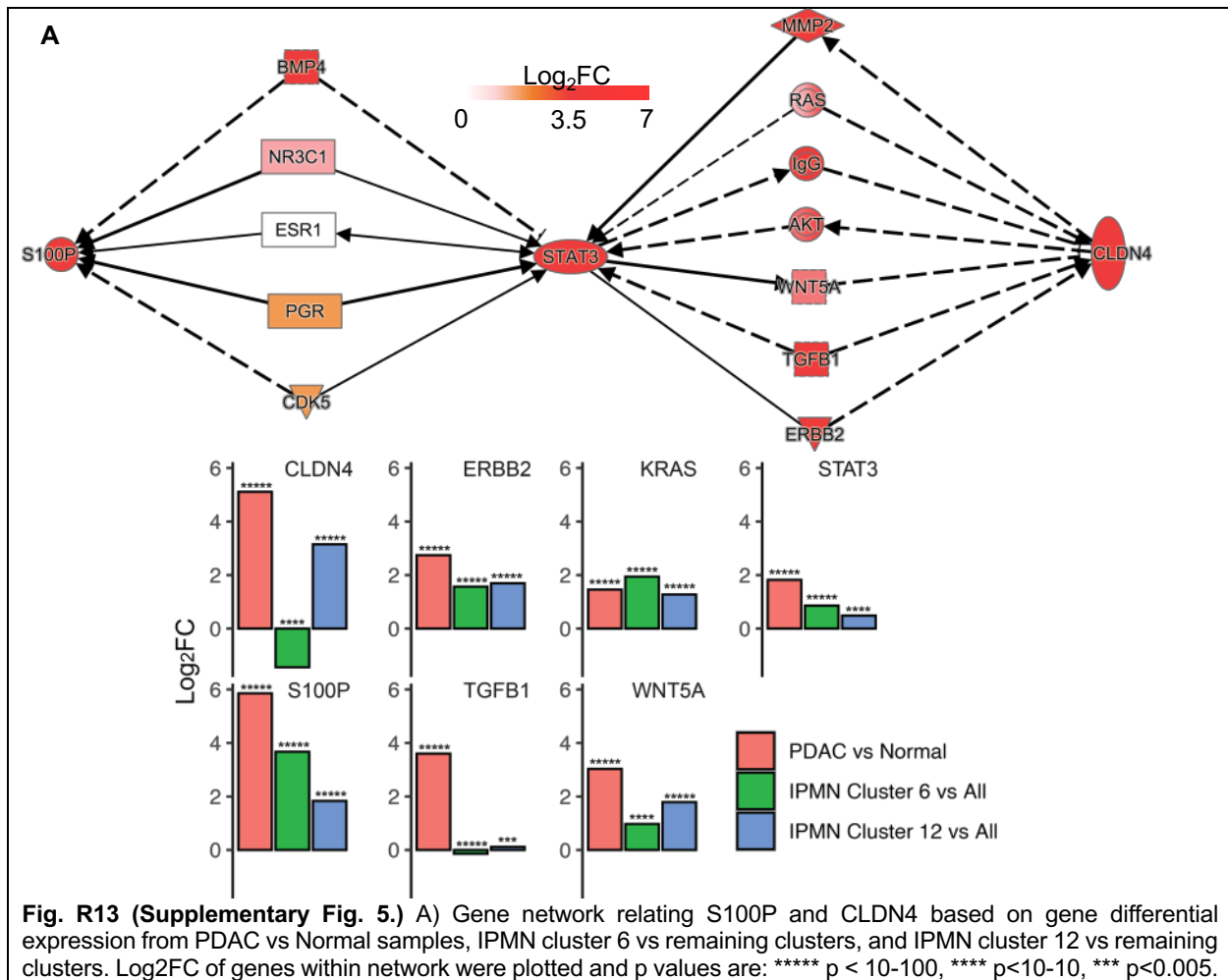
Comment 5:

In Figure 3A, the HE image of a PDAC shows tumor cells dominating the entire window illustrated by the authors, such as sample STPDAC13. However, the expression of CLDN4 does not exactly match that of tumor cells. The authors quantified CLDN4 expression in tumor cell spots on each slide.

Response:

The authors thank the reviewer for the key comment. To explore cell populations that have some *CLDN4* expression but were not necessarily tumor cells, we filtered our PDAC data based on *CLDN4* levels above background levels of expression and compared the populations with low *S100P* expression to those with high *S100P* expression. The regions with high *S100P* expression coincide with ductal and cancer regions where *CLDN4* expression is also high and aligns with pathological regions in histology. The regions with low *S100P* expression were nearby cancer regions (**Fig. R12A-B**). Differential expression between low and high *S100P* populations revealed specific genes not directly related to tumor signatures, such as *INS*, *ACTG2*, *CCN1*, and *DES* (**Fig. R12C**). To explore biological pathways in the populations with low *S100P*, we built a network based on differentially-expressed genes, *S100P*, *STAT3*, *CLDN4* (**Fig. R12D**). Our network indicated that glucocorticoid receptor genes correlated with *S100P* in downregulated expression while *ESR1* and *PGR* were upregulated in varying degrees. We hypothesize that different pathways independently regulate the *S100P* expression or *CLDN4* expression, and *CLDN4* expression is sensitive to tumor regions at higher expression levels. We did not add this figure to the paper but did add the network analysis to demonstrate the differential regulation of *S100P* and *CLDN4* in **Supplementary Fig. 5A (Fig. R13)**.



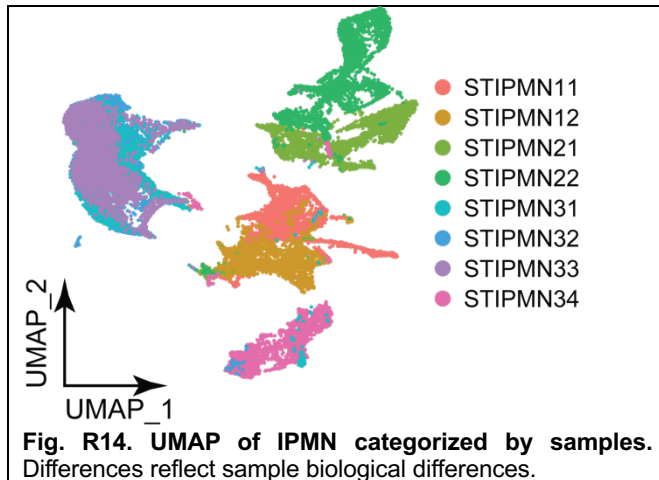


Comment 6:

The clusters in Figure 4B-C show patient specificity, such as cluster 13 only in STIPMN11 and cluster 12 only in STIPMN34. Is this due to a batch effect or is it biologically significant?

Response:

The authors thank the reviewer for the helpful question. The IPMN samples vary from each other across patients and slice location in the tumor. We plotted sample type onto our UMAP and found that, while some samples overlapped, others did not (**Fig. R14**). Specifically, normal tissue samples overlapped in clusters while samples with varying levels of pathology were clustered separately. Furthermore, in **Fig. 3**, cancer-associated genes among different samples reflect biological differences that we explored throughout the manuscript.



Comment 7:

It's not clear for the left panel and right panel of Fig 5J, the authors need to describe how to perform and interpret the results.

Response:

The authors thank the reviewer for the comment and have included a more detailed explanation to describe how the receiver operating curve (ROC) for this dataset was obtained in line 252:

“To explore whether *CLDN4* can differentiate PDAC from normal pancreas and precancer, we combined samples from our site with Ref. ³⁻⁵, which included normal pancreas, IPMN, and PDAC samples. Regions were constructed from multiple spots with similar expression levels (e.g. lower 20% or upper 20% of expression levels) and classified.”

Comment 8:

The CODEX in Figure 6 did not include the expression CLDN4. How do the results of this section relate to CLDN4?

Response:

The authors thank the reviewer for this important comment. The CODEX panels in Figure 6 were immune focused and indicated acinar cell destruction and immune cell infiltration, which together coincides with pathological ductal regions with high *CLDN4* expression. CODEX provided higher resolution to visualize CAFs and epithelial cell separation, which motivates the utility of cancer-specific imaging with *CLDN4* targeting peptides. We have now modified the text at line 306 for a better explanation:

“The spatial localization of these neighborhoods further illustrates the cellular components that promote PDAC oncogenesis and motivates the selection of *CLDN4* as an imaging and therapy target¹⁴.

Comment 9:

Some figure numbers are written in the figures, making the label unclear, such as Fig. 2A, C, and D.

Response:

The authors thank the reviewer for the comment and have fixed the figure labels.

Reviewer #4 (Remarks to the Author): Expert in pancreatic cancer imaging, in vivo imaging, and imaging agent synthesis

General comments:

Ferrara et al reported spatial transcriptomic analysis drives PET imaging of tight junction protein expression in pancreatic cancer imaging. The authors selected a tight junction protein claudin-4 as a target, and developed a peptide-based PET tracer for tumor bioimaging. However, the claudin-4 has been reported as a high expressed protein in tumor by some papers, and the peptide-based PET tracer was lacked of innovation. Hence, this work is not suitable to Nature Communications in current vision. Some comments were listed as below:

Comment 1:

In the results regarding PET imaging of claudin-4 (CLDN4) in a PDAC mouse model, it seems the background uptake of ^{64}Cu -C4BP is rather high, particular in the kidneys and liver. Please provide the coronal MIP image instead of the sagittal view (otherwise I would think the PET images authors presented are rather selective), so that we can see the in vivo distribution pattern of ^{64}Cu -C4BP clearer.

Response: Thank you for the comments and suggestions.

The kidney is the central clearance organ and as expected, there is signal during clearance. At a later time point the kidney activity is near zero (**Fig. 7E**). The pancreas is near the intestinal track and frontal kidney. Thus, in the coronal view of the maximum intensity projection, the kidney and pancreas can be difficult to separate. To clearly view the *in vivo* distribution pattern of ^{64}Cu -C4BP, we have added 3-dimensional PET/CT images as **Supplementary Video 1-4** to visualize the tumors where ^{64}Cu -C4BP accumulated in four mice.

At line 368, we have included the text:

“Three-dimensional maximum intensity projection (MIP) images (Supplementary Video 1-4, white and yellow arrows indicate tumors) and slice imaging detected the presence of tumors and metastases only in cancer-burdened mice and not in wild-type animals (Fig. 7C-D) (Supplementary Video 1-4).”

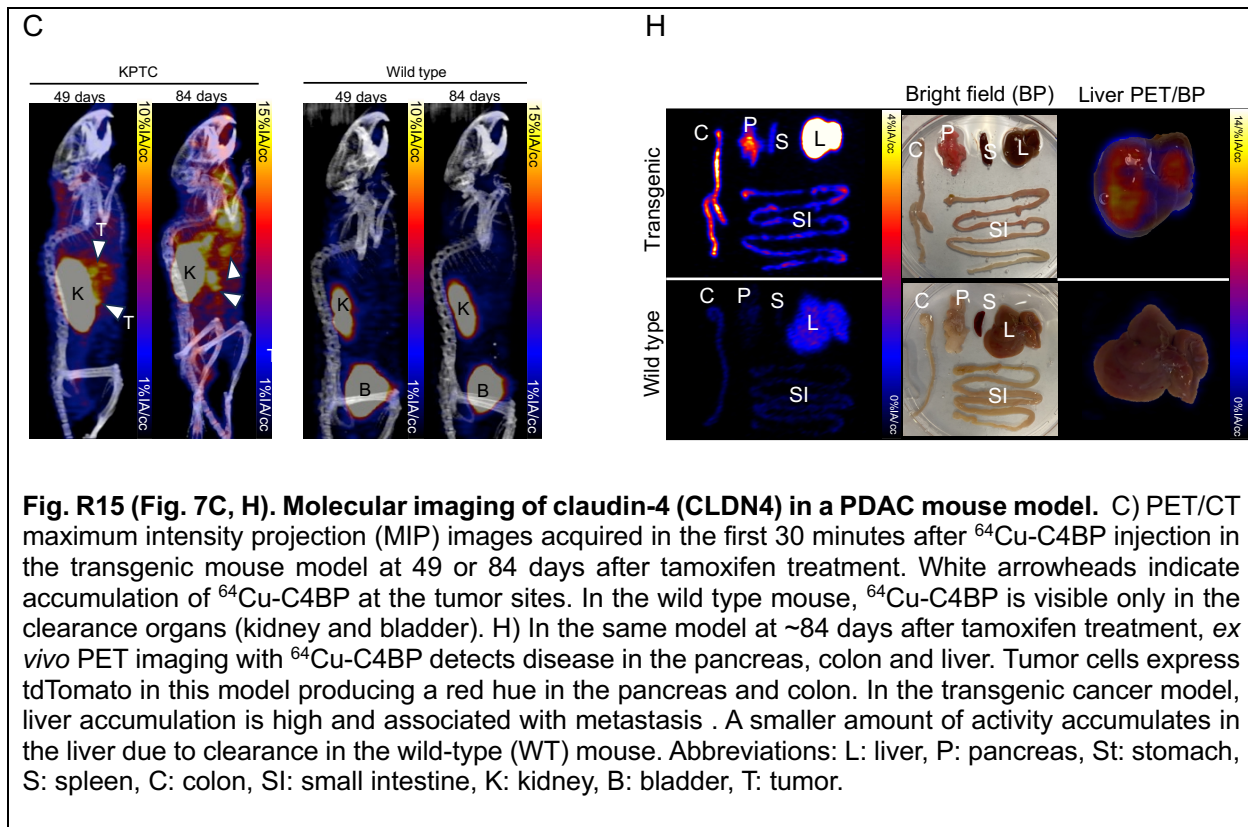
Comment 2:

From the ex vivo PET among different organs, I see extremely high uptake in the normal digestive system, particular for the liver, intestines, and pancreas. Since the main clinical use ^{64}Cu -C4BP would be imaging of pancreatic cancer, how can we tell the tumor lesions among such high background uptake? In Fig. 7G, it's hard to say that the tumor is well visualized in the PET image (rather low tumor-to-background ratio). The translational application and resolved methods are required detailed discussion in the discussion section.

Response: We thank the reviewer for asking us to clarify this question.

^{64}Cu -C4BP uptake is not increased in the wild type/normal mouse. As shown in **Fig. 7G**, the KPTC;*Ptf1a*^{CreER}; *Trp53*^{fl/fl} mouse with disseminated tumors and metastases has visible tumor lesions that are pink in color due to the tdTomato tumor signal (yellow arrowheads in the bright field image), and the radiopharmaceutical accumulates in these lesions (PET/CT middle image). These images are intended to show the *in vivo* anatomical localization of the ^{64}Cu -C4BP radiopharmaceutical within the pancreas and tumor.

Most importantly, we note that the background uptake of ^{64}Cu -C4BP in wild type mice is low as we demonstrate in **Fig. 7C** and **7H**. We are sorry for any confusion and have clarified all aspects of the paper.



Additionally, the ^{64}Cu -C4BP signal in the PET ex vivo images (**Fig. 7H**) in the digestive system of *KPTC:Kras^{LSL-G12D/+};Trp53^{fl/fl};Rosa26^{LSL-tdTomato/LSL-tdTomato};Ptf1a^{CreER}* mouse model was high in regions in which the tdTomato signal (indicating disease, bright field images) was also present. The signal is spatially correlated with the presence of disease. By comparison, in the ex vivo PET images, the ^{64}Cu -C4BP signal is not present in the wild type digestive system. The very low (background level) signal in the wild type digestive system and the relatively low signal in the wild type liver (1.7% ID/cc) are contrasted with the much higher signal in the tumor and metastases (>10% ID/cc) in this model.

In addition, transgenic mice with progressive cancer showed tumor metastases within the liver and intestine, leading to the high radioactivity in these tissues, which is visualized by ^{64}Cu -C4BP.

We have additionally added **Supplementary Fig. 14** showing the presence of metastases in the liver and intestine and we have added the following text at line 371:

“H&E histology and CLDN4 IHC stains of the intestine and liver showed metastases that aligned with the PET imaging results (**Supplementary Fig. 14A-C**).” These images are shown in this file in **Fig. R5**.

Based on the reviewer's comment, we have also added the sentence below in the discussion at line 454.

"Due to the high specificity and accumulation in tumors and metastases, we are optimistic that this CLDN4-peptide will successfully detect pancreatic cancer in human studies. While small animal imaging PET scanners have high sensitivity, with the advent of extended field-of-view scanners, the sensitivity of clinical PET imaging is increased 10 fold from earlier work and thus the feasibility of detecting small metastases will improve. Further, in human imaging, the distance between the clearance organ (kidney) and the pancreas is increased, further improving visualization."

Comment 3:

As a novel PET agent, I would also be curious about the in vivo distribution pattern of ⁶⁴Cu-C4BP in PDAC tumor bearing mice, please also provide the dynamic scan of ⁶⁴Cu-C4BP PET, for least 60 min p.i. In addition, the ex vivo biodistribution study is also required for evaluation of the in vivo distribution pattern of ⁶⁴Cu-C4BP.

Response: Thank you for this insightful comment and suggestion.

We have added longitudinal PET imaging and analysis of Capan-1 PDAC tumor bearing mice with 1-hour dynamic PET imaging of ⁶⁴Cu-C4BP. ROI analysis of the tumor from the 1-hour dynamic scan of ⁶⁴Cu-C4BP and biodistribution acquired at 2.5 h post injection are added in **Supplementary Fig. 15**. The corresponding methods and results sections are updated.

Additionally, **Supplementary Fig. 15** now includes a spatial map of the radioactivity and CLDN4 expression demonstrating the colocalization of CLDN4 and ⁶⁴Cu-C4BP. We have updated the text in the results section at line 392 to include:

"The spatial map of the radioactivity and CLDN4 expression obtained from PET and IHC tumor images confirmed colocalization of CLDN4 and ⁶⁴Cu-C4BP (**Supplementary Fig. 15E**). These images are shown in this file in **Fig. R6**.

References

- 1 Siegel, R. L., Giaquinto, A. N. & Jemal, A. Cancer statistics, 2024. *CA Cancer J Clin* **74**, 12-49 (2024). <https://doi.org/10.3322/caac.21820>
- 2 Sans, M. *et al.* Spatial Transcriptomics of Intraductal Papillary Mucinous Neoplasms of the Pancreas Identifies NKX6-2 as a Driver of Gastric Differentiation and Indolent Biological Potential. *Cancer Discovery* **13**, 1844-1861 (2023). <https://doi.org/10.1158/2159-8290.Cd-22-1200>
- 3 Cui Zhou, D. *et al.* Spatially restricted drivers and transitional cell populations cooperate with the microenvironment in untreated and chemo-resistant pancreatic cancer. *Nature Genetics* **54**, 1390-1405 (2022). <https://doi.org/10.1038/s41588-022-01157-1>
- 4 Peng, J. *et al.* Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell research* **29**, 725-738 (2019).
- 5 Consortium*, T. S. *et al.* The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. *Science* **376**, eabl4896 (2022).
- 6 Stuart, T. *et al.* Comprehensive integration of single-cell data. *Cell* **177**, 1888-1902. e1821 (2019).

- 7 Liberzon, A. *et al.* The molecular signatures database hallmark gene set collection. *Cell systems* **1**, 417-425 (2015).
- 8 Han, C., Liu, T. & Yin, R. Biomarkers for cancer-associated fibroblasts. *Biomarker Research* **8**, 64 (2020). <https://doi.org/10.1186/s40364-020-00245-w>
- 9 Elyada, E. *et al.* Cross-species single-cell analysis of pancreatic ductal adenocarcinoma reveals antigen-presenting cancer-associated fibroblasts. *Cancer discovery* **9**, 1102-1123 (2019).
- 10 Öhlund, D. *et al.* Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *Journal of Experimental Medicine* **214**, 579-596 (2017).
- 11 Nichols, L. S., Ashfaq, R. & Iacobuzio-Donahue, C. A. Claudin 4 Protein Expression in Primary and Metastatic Pancreatic Cancer: Support for Use as a Therapeutic Target. *American Journal of Clinical Pathology* **121**, 226-230 (2004).
<https://doi.org/10.1309/k144phvddupdd401>
- 12 Fujiwara-Tani, R. *et al.* Claudin-4: A New Molecular Target for Epithelial Cancer Therapy. *Int J Mol Sci* **24** (2023). <https://doi.org/10.3390/ijms24065494>
- 13 Shashikanth, N. *et al.* Tight junction channel regulation by interclaudin interference. *Nature communications* **13**, 3780 (2022).
- 14 Zhang, T., Ren, Y., Yang, P., Wang, J. & Zhou, H. Cancer-associated fibroblasts in pancreatic ductal adenocarcinoma. *Cell Death & Disease* **13**, 897 (2022).
<https://doi.org/10.1038/s41419-022-05351-1>

Spatial transcriptomic analysis drives PET imaging of tight junction protein expression in pancreatic cancer theranostics

Response to Reviewers' Comments:

We appreciate the insightful comments of the reviewers and have answered each comment. We have performed additional plots and revised the manuscript accordingly.

Reviewer #1 (Remarks to the Author): Expert in computational pancreatic cancer genomics, bioinformatics, and spatial omics

General comments:

The authors have significantly improved their manuscript in this revision. Most of my previous concerns have been addressed. I just have some minor comments below.

Comment 1:

On Figure 3, what is the unit of gene expression? Is it normalized to 0 to 4 for each gene? If so, what is the normalization method? More generally, on all the spatial transcriptomic figures, the authors need to mention the unit of the data so that the visualization for different genes are comparable.

Response:

The authors thank the reviewer for the important comment. On Figure 3, the unit for gene expression is log-normalized counts based on a negative binomial distribution calculated with Seurat package's SCTransform function and a range of 0-4 is shown. The captions for figure 3 and all other figures have been modified to include the unit for gene expression.

Comment 2:

And please correct a minor grammar issue between line 53-57.

Response:

The authors thank the reviewer for the helpful comment. We have corrected the minor grammar issue and modified lines 53-57 (now 56-59) to be as follows:

“Characterizing pancreatic nodules with imaging is significant as 30-50% of the adult population may harbor precancerous lesions ¹⁻⁴. Invasive cancer can be present within intraductal papillary mucinous neoplasms (IPMNs), and similar mutational profiles to PDAC are found in incidentally-discovered pancreatic intraepithelial neoplasms (PanINs) ².”

Reviewer #2 (Remarks to the Author): Expert in PET imaging and imaging agent synthesis

General comments:

The rebuttal letter is well written and the authors have addressed most of my concerns, I have no more comments on this manuscript.

Reviewer #3 (Remarks to the Author): Expert in cancer spatial multi-omics, tumor microenvironment, computational genomics, and imaging

General comments:

The authors have improved the manuscript and addressed most reviewer's comments. The remained comments were as follows:

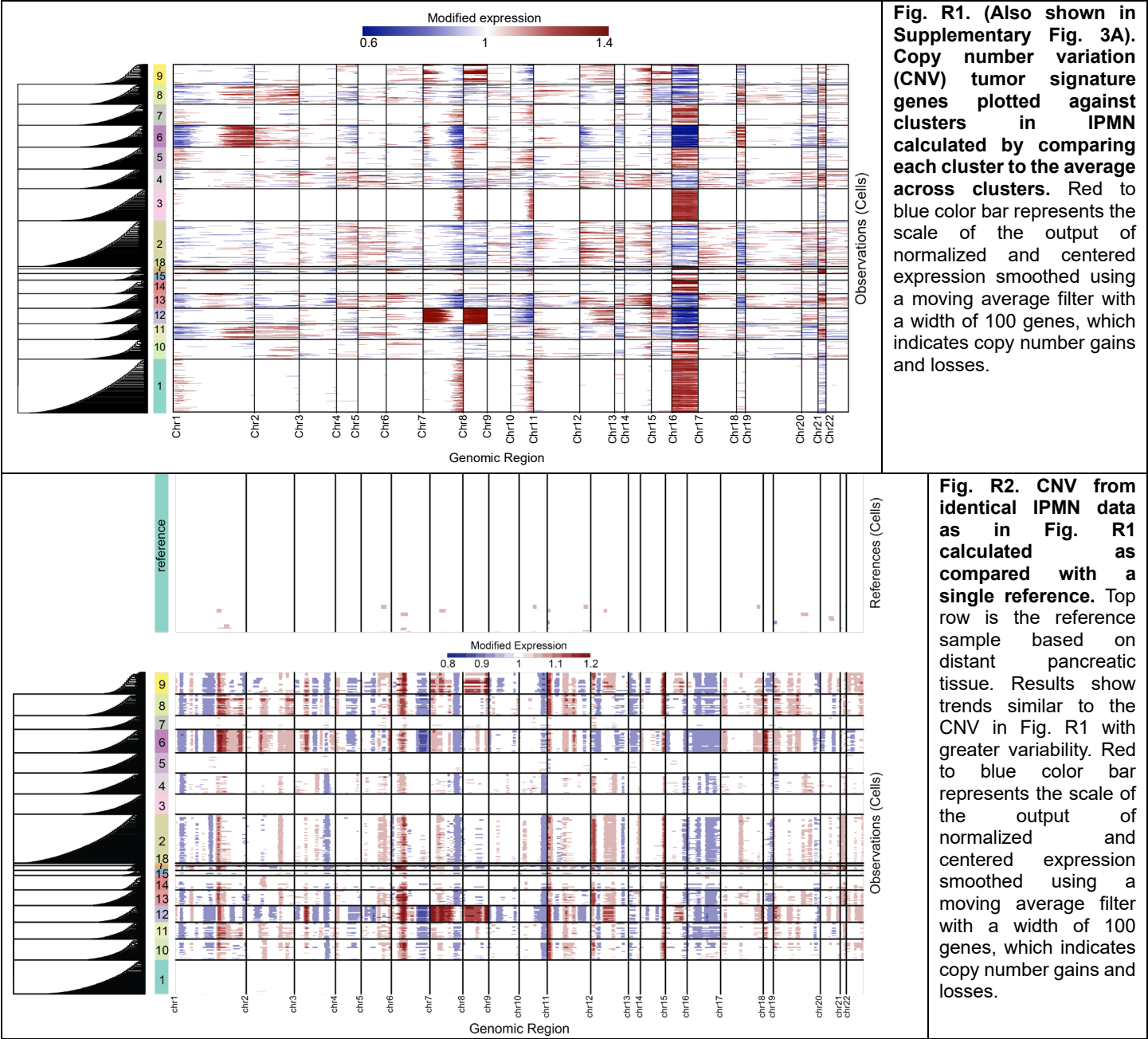
Comment 1:

In the previous reviewer's comment 3, the authors performed DNA copy number variation analysis on both PDAC and IPMN samples. Do they use any spots as references?

Response:

The authors thank the reviewer for the key question. We performed copy number variation (CNV) analysis with two methodologies and found consistent patterns across clusters. The figures in the manuscript were generated by comparing each cluster with the average of all clusters, and we have now clarified this point in the manuscript. Similarly, we attach here the results obtained by comparing regions with a reference data set of transcriptomic data from a surgical section at a distance from an IPMN lesion that was classified as histologically normal. The trends across clusters were similar although the use of the single reference from a patient with distant disease resulted in greater variability.

We show this result below for the IPMN data. Similar results were obtained for the PDAC samples.



We added additional text in the Methods section to further clarify at lines 540 – 542:

The copy number variation (CNV) calculation was performed with the infercnv package⁵⁰ with the default settings by comparing each cluster to the average of all clusters.

Comment 2:

Based on additional analysis of Fig. R10-11 and R14. The results showed only cluster 1 in PDAC was defined as tumor cells, while multiple clusters in IPMN were defined as tumor cells, do the different clusters of Fig. R11 come from different patients? Were the surface genes consistent in different patients or did they show different patterns?

Response:

The authors thank the reviewer for this question. Across the IPMN data sets, a continuum of phenotypes was detected from a pseudotime near that of histologically normal surrounding pancreas to that associated with invasive cancer. The results in the figure below illustrate variations within and between patients where the first number indicates the patient and the second indicates the sample number within that patient. Clearly there are similar patterns of gene expression across patients as well as spatial variations in expression within a patient.

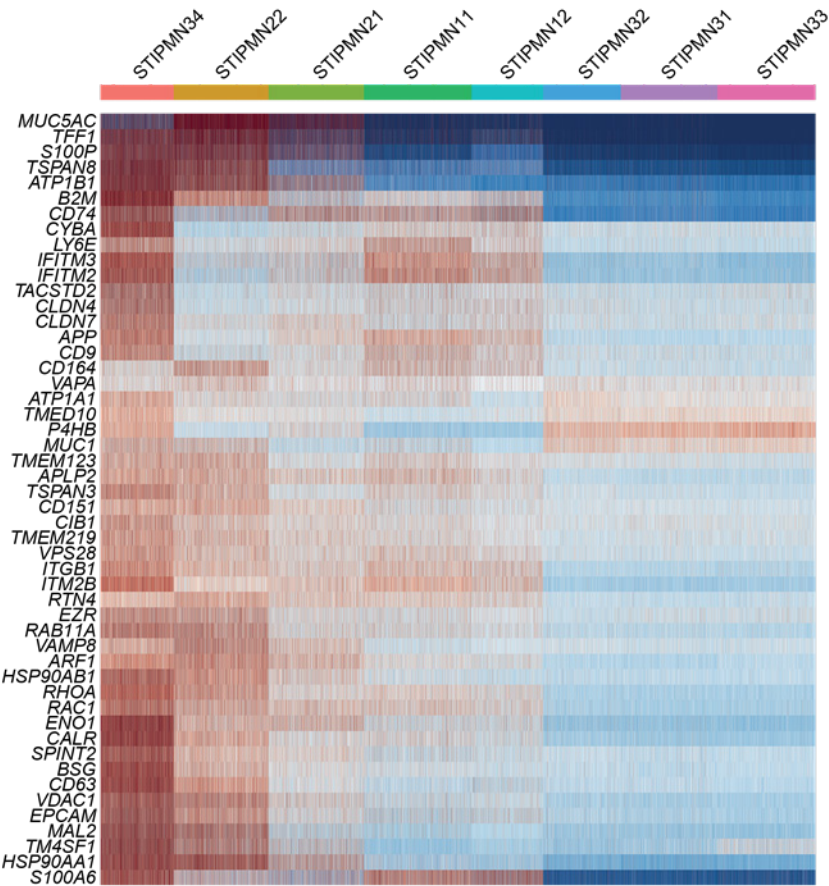


Figure R3. Cancer surfacesome with respect to samples. Cancer surfacesome gene profiles vary with patients and samples. Tumor regions exist in portions in patients 3 and 2 and to a lesser extent in patient 1. While patient 3 also contains regions with a phenotype closer to normal pancreas.

References

1 Siegel, R. L., Giaquinto, A. N. & Jemal, A. Cancer statistics, 2024. *CA Cancer J Clin* **74**, 12-49 (2024). <https://doi.org/10.3322/caac.21820>

- 2 Sans, M. *et al.* Spatial Transcriptomics of Intraductal Papillary Mucinous Neoplasms of the Pancreas Identifies NKX6-2 as a Driver of Gastric Differentiation and Indolent Biological Potential. *Cancer Discovery* **13**, 1844-1861 (2023). <https://doi.org/10.1158/2159-8290.Cd-22-1200>
- 3 Cui Zhou, D. *et al.* Spatially restricted drivers and transitional cell populations cooperate with the microenvironment in untreated and chemo-resistant pancreatic cancer. *Nature Genetics* **54**, 1390-1405 (2022). <https://doi.org/10.1038/s41588-022-01157-1>
- 4 Peng, J. *et al.* Single-cell RNA-seq highlights intra-tumoral heterogeneity and malignant progression in pancreatic ductal adenocarcinoma. *Cell research* **29**, 725-738 (2019).
- 5 Consortium*, T. S. *et al.* The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. *Science* **376**, eabl4896 (2022).
- 6 Stuart, T. *et al.* Comprehensive integration of single-cell data. *Cell* **177**, 1888-1902. e1821 (2019).
- 7 Liberzon, A. *et al.* The molecular signatures database hallmark gene set collection. *Cell systems* **1**, 417-425 (2015).
- 8 Han, C., Liu, T. & Yin, R. Biomarkers for cancer-associated fibroblasts. *Biomarker Research* **8**, 64 (2020). <https://doi.org/10.1186/s40364-020-00245-w>
- 9 Elyada, E. *et al.* Cross-species single-cell analysis of pancreatic ductal adenocarcinoma reveals antigen-presenting cancer-associated fibroblasts. *Cancer discovery* **9**, 1102-1123 (2019).
- 10 Öhlund, D. *et al.* Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *Journal of Experimental Medicine* **214**, 579-596 (2017).
- 11 Nichols, L. S., Ashfaq, R. & Iacobuzio-Donahue, C. A. Claudin 4 Protein Expression in Primary and Metastatic Pancreatic Cancer: Support for Use as a Therapeutic Target. *American Journal of Clinical Pathology* **121**, 226-230 (2004). <https://doi.org/10.1309/k144phvddupdd401>
- 12 Fujiwara-Tani, R. *et al.* Claudin-4: A New Molecular Target for Epithelial Cancer Therapy. *Int J Mol Sci* **24** (2023). <https://doi.org/10.3390/ijms24065494>
- 13 Shashikanth, N. *et al.* Tight junction channel regulation by interclaudin interference. *Nature communications* **13**, 3780 (2022).
- 14 Zhang, T., Ren, Y., Yang, P., Wang, J. & Zhou, H. Cancer-associated fibroblasts in pancreatic ductal adenocarcinoma. *Cell Death & Disease* **13**, 897 (2022). <https://doi.org/10.1038/s41419-022-05351-1>