Peer Review File

Manuscript Title: Acinar cell clonal expansion in pancreas homeostasis and carcinogenesis

Editorial Notes:

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

In this manuscript the authors studied the renewal and initiation of pancreatic ductal adenocarcinoma (PDAC) by using an elegant in vivo lineage tracing system. They identified a telomerase reverse transcriptase (TERT) 'high' subpopulation of pancreatic acinar cells in exocrine compartment, which can renew the pancreas by forming expanding clones of acinar cells. The discovery of a rare, distinct acinar cell subpopulation with elevated levels of Tert 3 mRNA distributed throughout the exocrine pancreas is a notable one. In addition, building on prior work from Jacks and others showing multiple cells of origin for PDAC, this study provides strong evidence that expression of mutant Kras in TERTHigh acinar cells increased acinar clone formation to promote PanIN and early PDAC. Furthermore, oncogenic Kras is shown to upregulate Ras/MAPK signaling and phosphorylation of ERK. This is an excellent study by an accomplished group, there are some issues relating to novelty and need for supportive data for the claims.
Major points:
Are there Tert-high cells in ductal epithelial cells that serve equivalent cell-of-origin role as Tert-high acinar cells?
It is well known that oncogenic KRAS activates ERK and thus it is expected that pERK would be present in TertCreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ cells, and that blockage of MEK by Trametinib would impair PanIN formation. In Figure 4, the data showing pERK-positive acinar cells in PDAC patients is supportive (but not direct evidence for) of acinar cell expansion in pancreatic tumorigenesis. More importantly, there is a need to more fully elucidate and explain why KRAS mutations were detected in only 5/18 pERK-positive acinar regions and, reciprocally, why mutant KRAS sequences are present in some pERK-negative samples. Moreover, given that KRAS mutation is observed in nearly all human PDAC cases, one wonders why pERK and Kras mutation are not more tightly correlated. Also, are these pERK-positive acinar cells TERT-high?
Oncogenic KRAS is known to accelerate PanIN formation. To enhance the study, it would be helpful to compare PanIN formation and acinar colony expansion between TertCreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ and Ptf1 CreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ to assess whether Tert high acinar cells expand more quickly or develop PanIN more efficiently than bulk acinar cells or other cell types in the presence of mutant Kras. Second, would it be possible to perform control experiments where Tert–low cells expressing oncogenic Kras mutation to determine the capacity of Tert-low Kras mutant acrinar cells to induce PanIN? Along these lines, is it known whether oncogenic Kras increases Tert expression in primary premalignant cells of various types in the pancreas? Third, the study would be enhanced by determination of the mechanism whereby oncogenic Kras promotes self-renewal and clonal expansion to enable induction of PanIN. Finally, can oncogenic Kras show such biological functions in Tert-low cells?
"TERT-high acinar cells may rarely sustain a Kras mutation, which in turn confers a selective growth advantage yielding expanding clones of pre-malignant cells that initially retain acinar morphology." The data are inadequate to support this point.
The title "Mutant Kras in acinar progenitor cells drives clonal expansion in early pancreas cancer" does not mention TERT which would seem central to the paper. Is oncogenic Kras alone sufficient or does oncogenic Kras* require Tert to drive clonal expansion and initiate the PanIN lesions and early PDAC? What is the functional relationship or connection between Tert and oncogenic Kras mutation in the neoplastic process? These issues need to be addressed/clarified.

As a baseline, it would be helpful to conduct injury studies in Tert wt versus Tert ko mice to see if Tert impacts clonal expansion activity.

There are several experiments that either lack alternative reinforcing methods or provide enough details

to support the claims made including
1. Beyond IF staining, additional methods are needed to determine that the RFP-labeled cell type is a subset of acinar cells, such as FACS analysis, or single cell RNA-sequencing might be useful.
2. Beyond FISH, additional methods are needed to determine TERT expression in RFP-labeled cells, such as RT-PCR, westerns, or single cell RNA-sequencing.
3. For analysis of oncogenic KRAS mutations in human PDAC patient samples, please show the PCR results and details: how many patients were examined, are the pERK negative and positive samples from the same patient or not, how many microdissections were from same patient, and what was the total list of samples used here.
4. The discovery of a small population of TERT-high acinar cells in pancreas and their clone-forming ability in vivo is significant; however, there is a lack of direct evidence for the self-renewal ability of these TERT-high cells versus TERT-low cells. Needed are in vitro assays.

Minor points:
1) The quality of extended figure 2h,i,j panels are not good.
2) No controls in extended figure 6.

Referee #3 (Remarks to the Author):

In this study, Neuhofer and colleagues provide powerful data on murine and human pancreatic tissues linking high telomerase expression in acinar cells to KRAS mutations and eventual PanIN formation. They start their work in a lineage-labeled mouse model and demonstrate that clonal expansion in acinar cells in homeostasis and repair occurs via infrequently occurring acinar cells with high TERT expression. They then show that these TERT-high cells can give rise to PanINs in a murine pancreatitis model. Finally, they translate the relevance of their studies to human samples, showing that pERK-positive acinar foci have KRAS mutations.

The experiments are well reasoned and rigorously performed, providing confidence in the resulting data. The link between TERT expression, tissue renewal, and early neoplastic transformation described in this study is quite novel and provides a new perspective on these processes. Moreover, these data provide key advances in the longstanding controversy of the "cell of origin" of pancreatic cancer, elucidating the earliest steps of pancreatic tumorigenesis, and as such will be of great interest to the field.

Still, the study could be improved with attention to the following:

1. In the first paragraph of the results, the authors state "ductal cells expressing Tomato were not detected", suggesting there are no TERT-high ductal cells. However, as the authors point out, the majority of the exocrine pancreatic cell mass consists of acinar cells, with a much smaller number of ductal cells. In order to make this assertion, the authors should provide quantitative data on the number of duct cells examined for Tomato expression. Considering the frequency of tomato-positive acinar cells in their model, did they examine enough ductal cells to be confident these cells do not occur at similar frequency? As written, it is not clear whether sampling bias (i.e. examination of a much larger number of acinar cells due to the cellular composition of the pancreas) impacts the negative result in the ductal cells.

2. In their model labeling random (rather than TERT-high) acinar cells (Ptf1a Rosa with low dose tamoxifen), the authors report a lower number of cells per clone both over time and after experimentally induced pancreatitis. However, this number is >1, suggesting that there is some division/repopulation from at least a subset of the randomly labeled acinar cells. Can the authors comment on the distribution of the cells per clone? Do most clones have 1 cell with a smaller subset with >1 in the random acinar model? This still seems consistent with their TERT model. However, if a large proportion of the clones in the random acinar model have >1 cell, it seems that TERT expression alone does label all acinar cells capable of repopulation.

3. In their mouse models, the authors show that KRAS mutation in TERT-high cells can drive PanIN formation. However, they do not show that KRAS mutations more efficiently drive PanIN formation in the TERT-high acinar cells compared to random acinar cells. It would be of interest to compare the frequency of PanIN formation in mice with KRAS limited to TERT-high acinar cells versus KRAS limited to an equivalent number of random acinar cells (i.e. their Ptfl1 Rosa low dose tamoxifen model). If such experiments are not practical, the authors should at least raise this caveat in their study.

4. In the human tissue analysis, it is intriguing that there is a higher prevalence of pERK-positive foci in the non-PDAC samples (6/9) compared to the PDAC samples (14/29). Do the authors have an explanation for this? What proportion of the non-PDAC samples came from chronic pancreatitis patients? Might repeated bouts of injury and repair to acinar cells explain this?

5. The authors state that they performed KRAS sequencing on pERK-positive PanINs in addition to pERK-positive and negative acinar cells. However, they do not present the results of the PanIN sequencing. This should be included. In addition, the authors state that pERK-positive acinar clones are frequently found near PanINs. When this happens, do they have concordant KRAS mutations in the 2 lesions? This would provide a stronger link between the observed acinar lesions and well-documented precancerous lesions.

6. The authors' data in human tissue shows KRAS mutations in acinar cells but does not link back to the original hypotheses related to TERT. Can the authors demonstrate high TERT expression in the pERK-positive acinar foci? Perhaps using the Tert RNA FISH they report in their murine model?

7. One reason for skepticism of the acinar cell of origin for human PDAC is the lack of widespread acinar-to-duct metaplasia (ADM) in human tissue samples. Can the authors comment at all about the relevance of ADM in their model and experiments? It seems that they suggest that KRAS-mutated acinar cells might transition directly to PanINs without intervening ADM, but it would be helpful to discuss this point more directly.

Author Rebuttals to Initial Comments:

In this resubmission, we have added critical new data to address important queries from the reviewers. Both reviewers appreciated the novelty in our data in having identified a subpopulation of acinar cells with unique repopulating activities in the exocrine pancreas, the finding in human pancreas tumor samples showing the existence of acinar cells harboring Kras mutations. However, both reviewers wondered whether the $TERT^{High}$ cells were more effectively transformed than randomly labeled acinar cells. We have now addressed this critical question by expressing Kras in randomly labeled acinar cells in $Ptf1^{CreER/+}$ $Rosa26^{LSLTomato/+}$ $Kras^{LSLG12D/+}$ mice. In this new cross, we used low dose tamoxifen to activate Kras in rare acinar cells, at a frequency that mimics the representation of $TERT^{High}$ cells. We find that induction of Kras in $TERT^{High}$ acinar cells results in much higher rates of metaplasia and PanIN formation, compared with induction of Kras in sparse, randomly labeled acinar cells. These data support the model that the newly identified $TERT^{High}$ acinar progenitor cells are both more regenerative than the bulk population of acinar cells and more readily transformed to PanINs when they suffer an activated mutation in Kras.

To more accurately reflect the findings in this study and to emphasize the conclusions in homeostasis and in transformation, we have changed the title to: “Acinar cell clonal expansion in homeostasis and carcinogenesis of the pancreas”. We have also addressed all of the queries from the reviewers in this Response and in the manuscript where appropriate. We have added additional new data to address the critique, including: quantification of TERT promoter activity in pancreatic duct cells; evaluation of the effects of MEK inhibition on acinar cell clonal expansion versus the effects on PanIN formation; assessment of the identify of TdTomato+ cells by qRT-PCR for acinar cell markers; and evaluation of TERT mRNA levels in TdTomato+ versus TdTomato- acinar cells. We thank the reviewers for their excellent comments which have improved our manuscript.

Referee #1 (Remarks to the Author):

1. In this manuscript the authors studied the renewal and initiation of pancreatic ductal adenocarcinoma (PDAC) by using an elegant in vivo lineage tracing system. They identified a telomerase reverse transcriptase (TERT) ‘high’ subpopulation of pancreatic acinar cells in exocrine compartment, which can renew the pancreas by forming expanding clones of acinar cells. The discovery of a rare, distinct acinar cell subpopulation with elevated levels of Tert mRNA distributed throughout the exocrine pancreas is a notable one. In addition, building on prior work from Jacks and others showing multiple cells of origin for PDAC, this study provides strong

evidence that expression of mutant Kras in TERTHigh acinar cells increased acinar clone formation to promote PanIN and early PDAC. Furthermore, oncogenic Kras is shown to upregulate Ras/MAPK signaling and phosphorylation of ERK. This is an excellent study by an accomplished group, there are some issues relating to novelty and need for supportive data for the claims.

Response: Thank you for these supportive comments.

2. Major points:
Are there Tert-high cells in ductal epithelial cells that serve equivalent cell-of-origin role as Tert-high acinar cells?

Response: We thank the reviewer for this question. To address this point directly, we quantified approximately 400 ductal cells from 5 independent *Tert*$^{CreERT2/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice each 10 days after tamoxifen injection and did not detect any Tomato+ ductal cells (Extended Data Figure 1c). We have also performed a similar analysis on pancreata from *Tert*$^{CreERT2/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$at one year. Again, we found no Tomato+ ductal cells in approximately 400 ductal cells from five independent mice at 1 year. However, we cannot rule out that TERT promoter activity in duct cells may be below the level necessary to mark cells in our TERT-CreER mice. We also cannot rule out that ductal cells replicate through another mechanism that is not based on the presence of TERT+ cells. We have now added a quantification of Tomato+ acinar cells at 10 days as well, where we find that 0.75% of acinar cells are Tomato+ by IHC.

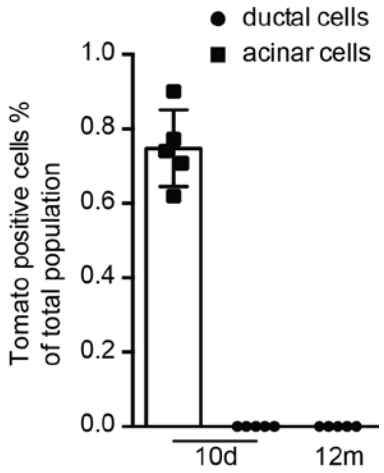

Figure 1: *Quantification of RFP+ cells in Tert$^{CreERT2/+}$; Rosa26$^{LSL\text{-}tdTomato/+}$ mice in different compartments of the pancreas at indicated time points post tamoxifen activation (n=5).*

3. It is well known that oncogenic KRAS activates ERK and thus it is expected that pERK would be present in TertCreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ cells, and that blockage of MEK by Trametinib would impair PanIN formation.

Response: The primacy of the Kras pathway in pancreas tumorigenesis is of course established. What is new here is that expression of activated Kras in TERT+ acinar progenitor cells results in pERK expression in enlarging acinar clones. We use this expression of pERK as a marker to identify regions of acinar expansion in human samples. Finally, we use trametinib to probe the requirement for Kras signaling in acinar cell expansion versus differentiation to PanIN. We find that trametinib did not impair acinar clone formation – indicating that this step in clone formation is MEK-independent. In contrast, transdifferentiation to PanIN was strongly MEK-dependent.

4. In Figure 4, the data showing pERK-positive acinar cells in PDAC patients is supportive (but not direct evidence for) of acinar cell expansion in pancreatic tumorigenesis. More importantly, there is a need to more fully elucidate and explain why KRAS mutations were detected in only 5/18 pERK-positive acinar regions and, reciprocally, why mutant KRAS sequences are present in some pERK-negative samples.

Response: We thank the reviewer for the comment. Our approach and interpretation here are shaped by the observations in the literature by others indicating that, while Kras mutations are common in human PanINs, Kras mutations have not been detected in normal appearing acinar cells or in normal appearing duct cells. Based on our findings in the mouse model showing that acinar cell clones express pERK, we screened samples from PDAC patients and found indeed that pERK-appearing regions are common in resected pancreas specimens. To my knowledge these have not been previously identified. We reasoned that there could be multiple underlying causes for the appearance of these pERK positive regions, including that: 1) they may harbor Kras mutations, 2) they may harbor mutations in other upstream genes that activate pERK or 3) they may be formed through inflammatory mechanisms involving cytokine signaling in a disease that occurs in an inflammatory milieu. For these reasons, we were not surprised that only a subset of pERK+ regions harbored Kras mutations. Furthermore, some of the pERK+ regions were relatively small and there are technical limitations that reduce the sensitivity of our sequencing. These include formalin fixation and DNA quality, low DNA yields, and mixed cell types in the lesion which could lead to false negatives.

In sequencing pERK-negative regions from a total of 34 patients, including resected specimens from PDAC patients and non-PDAC patients, we found that 89% of samples were Kras wild-type and that only

two samples showed a Kras mutation – both of these sample were from patients with a PDAC diagnosis. One was the classic G12D mutation and the other was an L6F mutation, not previously found in PDAC but occurring rarely in human colon cancer. We repeated the pERK staining on these samples and found again that they were negative. We conclude that rare Kras-mutation-harboring regions may be pERK-negative, either for biological reasons or for technical reasons related to sample processing prior to fixation and paraffin embedding. Overall, our data establish that pERK+ acinar regions in PDAC or in other pancreas diseases (chronic pancreatitis or serous cystadenoma) are enriched for the presence of activating mutations in Kras. These findings provide a central pathogenic link between normal acinar cells and PDAC supporting a model in which mutations in acinar cells in patients initiate pancreas carcinogenesis.

5. Moreover, given that KRAS mutation is observed in nearly all human PDAC cases, one wonders why pERK and Kras mutation are not more tightly correlated. Also, are these pERK-positive acinar cells TERT-high?

Response: It is important to consider that the human PDAC tumors and surrounding normal tissues are quite large and that the approaches used here, or in every previous study, analyze only a very small portion of the tumor. As such, the tumor is significantly under-sampled by analyzing only a few slides or sections. This may explain why we did not identify Kras mutations in a higher percentage of samples. This is why we conclude that the positive identification of acinar cells harboring Kras mutations is such a significant finding. Deeper analyses of tumors would very likely capture additional Kras-mutations in acinar cells. When we embarked on these experiments, we wondered whether acinar cells with Kras mutations would persist in the sample, years or decades after initiation of the tumor. It is possible that these cells could be lost or be replaced or could directly convert to PanINs and therefore be diluted or lost during pancreas tumor development. For these reasons, we conclude that the identification of Kras-mutation+ acinar cells is highly significant. These findings suggest that three different lesions can be evident in the same human tumor sample: acinar cells with Kras mutation, PanIN with Kras mutation and invasive tumor. We believe that identifying this new early lesion – acinar cells with Kras mutation – is highly informative for understanding pancreas tumorigenesis.

Regarding the link between pERK-positive acinar cells and *TERT* expression, this is very difficult to prove in human tissue as the reviewer is likely aware. TERT is very difficult to detect in both mouse and human due to low levels of mRNA and protein. This challenge in detection in part motivated our approach to develop lineage tracing for the detection and functional study of TERT+ cells. With regard to human cells or tissues specifically, human TERT is detected even at lower levels and in a more restricted pattern than is mouse TERT. The abundant RNAses in pancreas make detection of rare transcripts even more difficult. We likely have the most experience in the field in this space and we find that, for mouse, we can detect TERT mRNA in purified single cells where the entire volume of the cell is queried. However, in tissue sections these approaches have not been successful, either because signal is reduced by fixation or by analyzing only a fraction of a cell in a thin section. For these reasons, we have relied on TERT mRNA FISH in purified mouse cells, as demonstrated here (Fig.1). We have not been able to detect TERT convincingly in tissue sections in mouse, due to the underlying signal: noise problem. These issues are further exacerbated by lower levels of the TERT transcript in human, estimated at one to five mRNA molecules per cell. In mouse, we have shown that within the acinar clones TERT+ cells remain rare as the clone grows, consistent with $TERT^{High}$ cells giving rise to $TERT^{Low}$ cells. For these reasons, we are careful to avoid claims regarding the origin of the pERK+ acinar regions in human samples.

6. Oncogenic KRAS is known to accelerate PanIN formation. To enhance the study, it would be helpful to compare PanIN formation and acinar colony expansion between TertCreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ and Ptf1 CreERT2/+; KrasLSL-G12D/+; Rosa26LSL-Tomato/+ to assess whether Tert high acinar cells expand more quickly or develop PanIN more efficiently than bulk acinar cells or other cell types in the presence of mutant Kras.

Response: We thank the reviewer for this very important comment. We have now completed these genetic crosses to evaluate PanIN formation side-by-side in $Tert^{CreERT2/+}$; $Kras^{LSL\text{-}G12D/+}$; $Rosa26^{LSL\text{-}Tomato/+}$ mice and $Ptf1a^{CreERT2/+}$; $Kras^{LSL\text{-}G12D/+}$; $Rosa26^{LSL\text{-}Tomato/+}$ mice as requested (Figure 3 b, c, f and Extended

Data Fig.4). For the *Ptf1a*$^{CreERT2/+}$ cohorts, we used very low tamoxifen doses to mimic the low frequency of labeling found in *Tert*$^{CreERT2/+}$ mice. In analyzing the pancreata from these cohorts of mice, the tissues were scored in a blinded fashion by a pathologist for both the presence of mature PanINs and of metaplastic areas that represent an earlier step in the transformation of acinar cells to ductal cells. The data indicate that KRAS activation in TERT^{High} acinar cells results in more efficient formation of metaplastic areas and in a higher rate of PanIN lesion development.

First, we quantified the **number of RFP+ acinar clones** per 20x image in both genotypes at one-month post-pancreatitis. We found no difference in acinar clone number between *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice and *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice (Extended Data Fig. 4b). This result indicates that we initially label the same number of clones in both genotypes. Second, analysis of **clone size** at one-month revealed that clones are smaller in randomly labeled acinar cells in *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice compared with *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice. Third, we found a marked reduction of either **PanINs** or **metaplastic regions** in *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice compared with *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice (Fig. 3f). These data show that TERT^{High} acinar cells are more clonogenic and are more efficiently transformed to PanIN than are randomly labeled acinar cells. Importantly, we readily replicated the work of others using high tamoxifen doses in Ptf1aCreER+ mice, which leads to nearly complete transformation of the exocrine compartments (Extended Data Fig. 4 e, f). It is striking that expression of KRAS broadly in acinar cells throughout the tissue readily transforms the pancreas, whereas clonal expression of KRAS in randomly marked acinar cells fails to generate PanINs at least during the time course studied.

To address this point further, we have now aged the *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice to three months after induction of pancreatitis. We analyzed the pancreata from these mice for the presence of metaplasia and the presence of PanINs. Both metaplastic area and PanIN area for *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice at 3 months remained significantly lower than for *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice at 1 month. These findings show that clonal induction of Kras in random acinar cells results in significantly reduced transformation to PanINs.

These results reveal that outcomes in transformation are distinctly different when studying clonal initiation of tumorigenesis that more closely models the events in human cancer and suggest that widespread activation of Kras overcomes roadblocks to transformation occurring in rare acinar cells.

7. Second, would it be possible to perform control experiments where Tert–low cells expressing oncogenic Kras mutation to determine the capacity of Tert-low Kras mutant acrinar cells to induce PanIN?

Response: Our estimates of the frequency of the TERT^{High} cells is <1% of all acinar cells, based on both lineage tracing and on RNA FISH for Tert mRNA. Therefore, more than 99% of randomly labeled acinar cells are TERT^{Low}. These observations indicate that the previously described new experiment in which we activate KRAS in *Ptf1a*$^{CreERT2/+}$ acinar cells serves as an excellent response to the Reviewer's query. This experiment revealed that PanIN formation is significantly reduced in randomly marked acinar cells (TERT^{Low}) compared with TERT^{High}.

8. Along these lines, is it known whether oncogenic Kras increases Tert expression in primary premalignant cells of various types in the pancreas?

Response: This is an intriguing concept and one that we have thought about, but not investigated directly. We do note that in the literature there are a small number of papers that have suggested such a link, but the direct mechanisms underlying such a connection have not been fully revealed (Lee et al., PNAS, 2016; Liu et al., Oncotarget, 2017). We feel that this is an interesting concept but tangential to the main points raised here.

9. Third, the study would be enhanced by determination of the mechanism whereby oncogenic Kras promotes self-renewal and clonal expansion to enable induction of PanIN.

Response: Our data show that KRAS activation in $TERT^{High}$ cells enhances clonal expansion, compared with the natural rate of clonal expansion of $TERT^{High}$ cells lacking KRAS. To address the Reviewer's question, we explored whether this role of KRAS in driving acinar cell self-renewal depended on MEK. We therefore treated *Tert$^{CreERT2/+}$; Kras$^{LSL\text{-}G12D/+}$; Rosa26$^{LSL\text{-}Tomato/+}$* mice with the MEKi trametinib for 30 days. Trametinib had only a modest inhibition on KRAS-driven clonal expansion and this effect was not statistically significant (Extended Data Fig. 5). In contrast, trametinib treatment effectively blocked PanIN formation in these mice. These data reveal an interesting difference in how KRAS drives these two steps in PanIN formation. Acinar clonal expansion is largely MEK-independent, whereas transformation to PanINs is strongly MEK-dependent. These findings with MEK inhibition also correspond to our stainings with antibodies to phospho-ERK. We found that the intensity of phospho-ERK staining increased as acinar clones grew and increased further as they transformed to PanINs (Fig. 3j). Together, these data reveal important complexities in how KRAS signaling drives sequential steps in pancreas carcinogenesis and emphasize the importance of MEK signaling downstream of KRAS in the transition to PanIN.

10. "TERT-high acinar cells may rarely sustain a Kras mutation, which in turn confers a selective growth advantage yielding expanding clones of pre-malignant cells that initially retain acinar morphology." The data are inadequate to support this point.

Response: With the inclusion of new data showing that $TERT^{High}$ acinar cells are more susceptible to the transforming effects of KRAS, we feel that this statement is supported by the data. Our finding in resected human pancreas samples provides direct evidence for the 'acinar cell hypothesis' – the concept that PDAC originates in the acinar compartment and from rare progenitor cells that sustain a KRAS mutation driving acinar cell clonal expansion. This hypothesis will be further tested in the future, in particular, whether there exists a $TERT^{High}$ subpopulation in human that serves a role analogous to the cell population identified here in mouse. We cannot say whether KRAS mutations occur in a $TERT^{High}$ subpopulation in humans and we are careful to avoid this conclusion.

11. The title "Mutant Kras in acinar progenitor cells drives clonal expansion in early pancreas cancer" does not mention TERT which would seem central to the paper. Is oncogenic Kras alone sufficient or does oncogenic Kras* require Tert to drive clonal expansion and initiate the PanIN lesions and early PDAC? What is the functional relationship or connection between Tert and oncogenic Kras mutation in the neoplastic process? These issues need to be addressed/clarified.

Response: In this study, we have used TERT promoter activity to discover an important subpopulation of acinar cells that exhibits enhanced repopulating activities in homeostasis and injury. And we can now report that when these cells suffer a Kras mutation, they show enhanced conversion to PanIN lesions. Furthermore, in human pancreas cancer samples, we show evidence that acinar regions exist that harbor Kras mutations. Although leveraging the Tert promoter was critical in this process, we prefer to focus on the importance of the acinar cell subpopulation and the acinar clonal expansion step in pancreas carcinogenesis in the title. Tert is mentioned several times in the abstract and throughout the manuscript.

As a baseline, it would be helpful to conduct injury studies in Tert wt versus Tert ko mice to see if Tert impacts clonal expansion activity.

Response: We thank the reviewer for the suggestion, but we feel that interrogating the requirement for TERT in this process is beyond the scope of this paper for the reasons mentioned above. Understanding whether TERT is important in the clonal expansion process is very important for a future study.

There are several experiments that either lack alternative reinforcing methods or provide enough details to support the claims made including
1. Beyond IF staining, additional methods are needed to determine that the RFP-labeled cell type is a subset of acinar cells, such as FACS analysis, or single cell RNA-sequencing might be useful.

Response: We had previously shown by histology that together with acinar-marker co-staining supported the identify of RFP+ cells as acinar cells. As requested by the reviewer, we have now added RT-PCR analyses for acinar cell-enriched genes to show that RFP+ cells are a subset of acinar cells by comparing the expression of these acinar genes in RFP+ cells and RFP- cells from the same mice (Extended Data Fig. 1d). The technique used to purify cells from the pancreas enriches for acinar cells. Therefore RFP- are positive for acinar markers. The data show that RFP+ cells are further enriched for acinar markers by qRT-PCR, further evidence that they represent acinar cell populations.

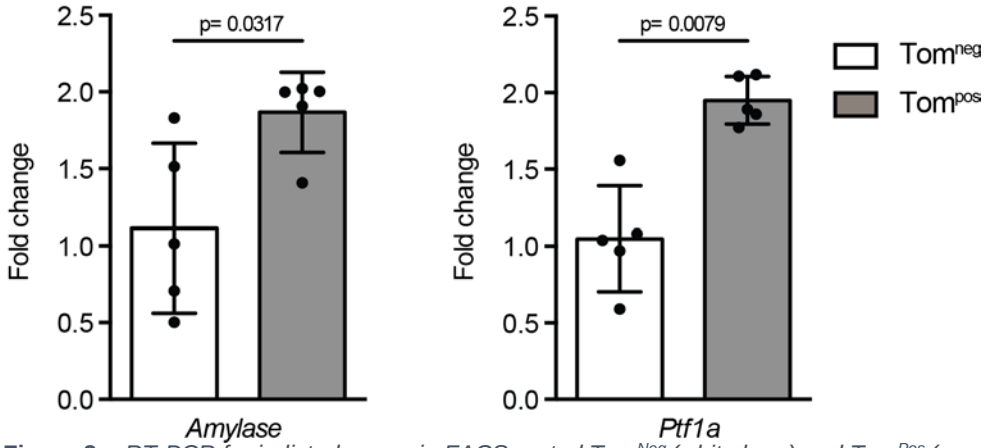

Figure 2: *qRT-PCR for indicted genes in FACS-sorted Tom^{Neg} (white bars) and Tom^{Pos} (grey bars) acinar cells of Tert$^{CreERT2/+}$; Rosa26$^{LSL\text{-}tdTomato/+}$ mice (Mann-Whitney t test).*

12. Beyond FISH, additional methods are needed to determine TERT expression in RFP-labeled cells, such as RT-PCR, westerns, or single cell RNA-sequencing.

Response: As requested by the reviewer we performed RT-PCR to determine *Tert* expression in RFP-positive cells compared to RFP-negative cells. The results show that Tert mRNA is elevated 4-fold in RFP-positive acinar cells (Extended Data Fig. 1d). These finding are consistent with our single molecular RNA FISH studies on purified RFP+ and RFP- acinar cels. (Fig. 1 h-j). Mouse TERT has not been detected by Western blot due to very low protein levels and a lack of dedicated reagents. Human TERT can only be detected by IP-Western in which TERT is purified from an amount of extract too large to run on a single SDS-PAGE lane (Venteicher et al., Science, 2009; Chen et al., Cell, 2018). Together, the RNA FISH and RT-PCR provide mutually reinforcing results indicating that TERT mRNA is elevated in the RFP+ acinar cells labeled in TERT$^{CreER/+}$ mice.

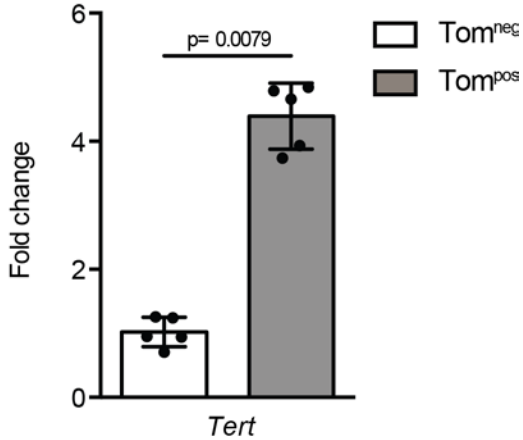

Figure 3: *qRT-PCR for indicted genes in FACS-sorted Tom^{Neg} (white bars) and Tom^{Pos} (grey bars) acinar cells of Tert$^{CreERT2/+}$; Rosa26$^{LSL\text{-}tdTomato/+}$ mice (Mann-Whitney t test).*

13. For analysis of oncogenic KRAS mutations in human PDAC patient samples, please show the PCR results and details: how many patients were examined, are the pERK negative and positive samples from the same patient or not, how many microdissections were from same patient, and what was the total list of samples used here.

Response: We examined 29 PDAC patients, 9 non-PDAC patients and 9 autopsy samples. We were not able to isolate DNA from all of the samples due to the tissue quality of the FFPE blocks and the size of some of the areas that were microdissected. The wild-type and mutation frequency, the total sequencing reads as well as the correlation to the pERK status is provided in Extended Data Table 2. We added a comment column in case there were multiple sequencing replicates for a single sample. In general, we microdissected one pERK positive area and one pERK negative area per patient but in some cases we microdissected multiple areas that are labeled as a new sample and noted in the comment column. 14/18 samples that have a pERK positive sequencing result have a corresponding pERK negative sequencing result included as a control. Additionally, we have added the diagnosis for each of the non-PDAC samples in Extended Data Table 1.

14. The discovery of a small population of TERT-high acinar cells in pancreas and their clone-forming ability in vivo is significant; however, there is a lack of direct evidence for the self-renewal ability of these TERT-high cells versus TERT-low cells. Needed are in vitro assays.

Response: We understand the reviewer's comment. However, we believe that that the clear evidence provided for clonal growth in long-term lineage tracing *in vivo* provides the most rigorous evidence for self-renewal possible. We show that these cells form clones in vivo, and that these clones continue to expand over time. While cell culture studies could in concept provide supportive evidence for these rigorous *in vivo* data, growth of acinar cells in culture has not been possible. Many published studies show that acinar cells differentiate to ductal fates in culture (in 2D, 3D and organoids; Westphalen et al. 2016, Cell Stem Cell; Wollny et al. 2016 Dev Cell; Boj et al. 2015 Cell). To address this comment further, we have now aged *Tert$^{CreERT2/+}$; Rosa26$^{LSL\text{-}Tomato/+}$* mice for two years and measured clone formation in the exocrine pancreas. We now report that Tomato+ acinar clones deriving from TERT^{High} acinar progenitors continue to expand between 12 months and 24 months and show a linear growth pattern during normal homeostasis. Together, these data provide powerful evidence for the self-renewal of TERT^{High} acinar cells during the lifetime of the animal.

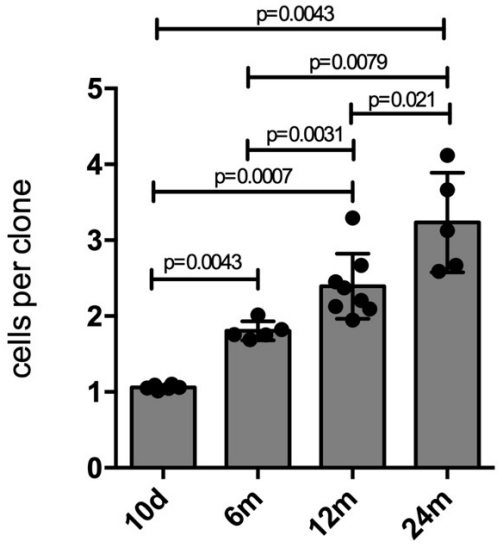

Figure 4: Quantification of RFP positive cells per clone in Tert$^{CreERT2/+}$; Rosa26$^{LSL\text{-}tdTomato/+}$ mice (gray bars)(n=6 for 10d; n=5 for 6m; n=8 for 12m and n=5 for 24m) at indicated time points (mean ± SD). Mann-Whitney t test

Minor points:
1) The quality of extended figure 2h,i,j panels are not good.

Response: We have replaced these images with higher quality images.

2) No controls in extended figure 6.

Response: Thank you. We added an IgG control of the same block (several sections later) to the figure.

Referee #3 (Remarks to the Author):

In this study, Neuhofer and colleagues provide powerful data on murine and human pancreatic tissues linking high telomerase expression in acinar cells to KRAS mutations and eventual PanIN formation. …The experiments are well reasoned and rigorously performed, providing confidence in the resulting data. The link between TERT expression, tissue renewal, and early neoplastic transformation described in this study is quite novel and provides a new perspective on these processes. Moreover, these data provide key advances in the longstanding controversy of the "cell of origin" of pancreatic cancer, elucidating the earliest steps of pancreatic tumorigenesis, and as such will be of great interest to the field.

Still, the study could be improved with attention to the following:

1. In the first paragraph of the results, the authors state "ductal cells expressing Tomato were not detected", suggesting there are no TERT-high ductal cells. However, as the authors point out, the majority of the exocrine pancreatic cell mass consists of acinar cells, with a much smaller number of ductal cells. In order to make this assertion, the authors should provide quantitative data on the number of duct cells examined for Tomato expression. Considering the frequency of tomato-positive acinar cells in their model, did they examine enough ductal cells to be confident these cells do not occur at similar frequency? As written, it is not clear whether sampling bias (i.e. examination of a much larger number of acinar cells due to the cellular composition of the pancreas) impacts the negative result in the ductal cells.

Response: Thank you for this important suggestion. We agree with the reviewer that ductal cells make up a far smaller population than the acinar cells within the exocrine pancreas. To address this point directly, we quantified approximately 400 ductal cells from 5 independent *Tert*$^{CreERT2/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice each 10 days after tamoxifen injection and did not detect any Tomato+ ductal cells (Extended Data Figure 1c). We have also performed a similar analysis on pancreata from *Tert*$^{CreERT2/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$at one year. Again, we found no Tomato+ ductal cells in approximately 400 ductal cells from five independent mice at 1 year. However, we cannot rule out that TERT promoter activity in duct cells may be below the level necessary to mark cells in our TERT-CreER mice. We also cannot rule out that ductal cells replicate through another mechanism that is not based on the presence of TERT+ cells. We have now added a quantification of Tomato+ acinar cells at 10 days as well, where we find that 0.75% of acinar cells are Tomato+ by IHC.

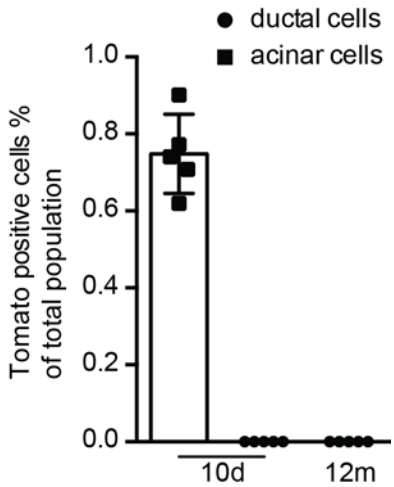

Figure 5: *Quantification of RFP+ cells in Tert*$^{CreERT2/+}$*; Rosa26*$^{LSL\text{-}tdTomato/+}$ *mice in different compartments of the pancreas at indicated time points post tamoxifen activation (n=5).*

2. In their model labeling random (rather than TERT-high) acinar cells (Ptf1a Rosa with low dose tamoxifen), the authors report a lower number of cells per clone both over time and after experimentally induced pancreatitis. However, this number is >1, suggesting that there is some division/repopulation from at least a subset of the randomly labeled acinar cells. Can the authors comment on the distribution of the cells per clone? Do most clones have 1 cell with a smaller subset with >1 in the random acinar model? This still seems consistent with their TERT model. However, if a large proportion of the clones in the random acinar model have >1 cell, it seems that TERT expression alone does label all acinar cells capable of repopulation.

Response: Thank you for raising this point. The baseline cell number per clone at 10 days for Ptf1aCreER is indeed 1.3 cells per clone. Empirically, our strategy to reduce tamoxifen levels 100-fold results in random labeling of acinar cells and 80% of 'clones' at this point are comprised of single cells. The remaining 20% are comprised mostly of 'doublets', two adjacent cells labeled together. There are rarely a small number of larger 'clones' (Ext. Data Fig. 2e). This appears to be a feature of the random labeling approach used – that there is a chance of randomly labeling cells that are immediately adjacent. Of note, this phenomenon is much less pronounced with TERT-CreER labeling, which yields ~95% single-cells at 10 days. As we aged the *Ptf1a*$^{CreER/+}$ *Rosa26*$^{LSL\text{-}Tomato/+}$ mice, we found that mean clone sizes were 1.5 and 1.4 at 6 months and 12 months, respectively. This mean clone size increases very modestly over time but the increase was not statistically significant. The modest increase in clone size with random labeling could be because: random labeling may capture rare TERT^{High} cells, but these should be very few in the random population; or there are other acinar populations capable of more limited cell division, e.g., with the ability to divide once without sufficient self-renewal capacity to form larger clones. Consistent with this latter notion, the results from the BrdU and Ki67 experiments suggest that other acinar cells can contribute to repopulating the acinar cell mass especially during regeneration. This is in line with previously published data on the Dclk1 expressing acinar cells (Westphalen et al. 2016, Cell Stem Cell) and suggests that additional acinar cell populations other than TERT^{High} acinar cells can contribute to renewal especially during regeneration.

3. In their mouse models, the authors show that KRAS mutation in TERT-high cells can drive PanIN formation. However, they do not show that KRAS mutations more efficiently drive PanIN formation in the TERT-high acinar cells compared to random acinar cells. It would be of interest to compare the frequency of PanIN formation in mice with KRAS limited to TERT-high acinar cells versus KRAS limited to an equivalent number of random acinar cells (i.e. their Ptfl1 Rosa low dose tamoxifen model). If such experiments are not practical, the authors should at least raise this caveat in their study.

Response: We thank the reviewer for this very important comment. We have now completed these genetic crosses to evaluate PanIN formation side-by-side in *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice and *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice as requested (Figure 3 b, c, f and Extended Data Fig.4). For the *Ptf1a*$^{CreERT2/+}$ cohorts, we used very low tamoxifen doses to mimic the low frequency of labeling found in *Tert*$^{CreERT2/+}$ mice. In analyzing the pancreata from these cohorts of mice, the tissues were scored in a blinded fashion by a pathologist for both the presence of mature PanINs and of metaplastic areas that represent an earlier step in the transformation of acinar cells to ductal cells. The data indicate that KRAS activation in TERT^{High} acinar cells results in more efficient formation of metaplastic areas and in a higher rate of PanIN lesion development.

First, we quantified the **number of RFP+ acinar clones** per 20x image in both genotypes at one-month post-pancreatitis. We found no difference in acinar clone number between *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice and *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice (Extended Data Fig. 4b). This result indicates that we initially label the same number of clones in both genotypes. Second, analysis of **clone size** at one-month revealed that clones are smaller in randomly labeled acinar cells in *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice compared with *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice. Third, we found a marked reduction of either **PanINs** or **metaplastic regions** in *Ptf1a*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice compared with *Tert*$^{CreERT2/+}$*; Kras*$^{LSL\text{-}G12D/+}$*; Rosa26*$^{LSL\text{-}Tomato/+}$ mice (Fig. 3f). These data show that TERT^{High} acinar cells are more clonogenic and are more efficiently transformed to PanIN than are randomly labeled acinar cells. Importantly, we readily replicated

the work of others using high tamoxifen doses in *Ptf1a$^{CreERT2/+}$; Kras$^{LSL-G12D/+}$; Rosa26$^{LSL-Tomato/+}$* mice, which when treated with cerulein leads to nearly complete transformation of the exocrine compartments (Extended Data Fig. 4 e, f). It is striking that expression of KRAS broadly in acinar cells throughout the tissue readily transforms the pancreas, whereas clonal expression of KRAS in randomly marked acinar cells fails to generate PanINs at least during the time course studied.

To address this point further, we have now aged the *Ptf1a$^{CreERT2/+}$; Kras$^{LSL-G12D/+}$; Rosa26$^{LSL-Tomato/+}$* mice to three months after induction of pancreatitis. We analyzed the pancreata from these mice for the presence of metaplasia and the presence of PanINs. Both metaplastic area and PanIN area for *Ptf1a$^{CreERT2/+}$; Kras$^{LSL-G12D/+}$; Rosa26$^{LSL-Tomato/+}$* mice at 3 months remained significantly lower than for *Tert$^{CreERT2/+}$; Kras$^{LSL-G12D/+}$; Rosa26$^{LSL-Tomato/+}$* mice at 1 month. These findings show that clonal induction of Kras in random acinar cells results in significantly reduced transformation to PanINs.

These results reveal that outcomes in transformation are distinctly different when studying clonal initiation of tumorigenesis that more closely models the events in human cancer and suggest widespread activation of Kras overcomes roadblocks to transformation occurring in rare acinar cells.

4. In the human tissue analysis, it is intriguing that there is a higher prevalence of pERK-positive foci in the non-PDAC samples (6/9) compared to the PDAC samples (14/29). Do the authors have an explanation for this? What proportion of the non-PDAC samples came from chronic pancreatitis patients? Might repeated bouts of injury and repair to acinar cells explain this?

Response: We reasoned that there could be multiple underlying causes for the appearance of these pERK positive regions, including that: 1) they may harbor Kras mutations, 2) they may harbor mutations in other upstream genes that activate pERK or 3) they may be formed through inflammatory mechanisms involving cytokine signaling in a disease that occurs in an inflammatory milieu. For these reasons, we were not surprised to find an elevated frequency of pERK-positive regions in the non-PDAC samples. Four of the six pERK-positive foci arose in non-PDAC patients that had pancreatitis. (The diagnosis is now added in Extended Data Table 1). It is well established that pancreatitis leads to activation of the MAPK pathway and this could be the reason to have a high percentage of non-PDAC samples having pERK-positive foci. The repeated rounds of damage and injury could very well contribute to these areas of pERK-positivity.

5. The authors state that they performed KRAS sequencing on pERK-positive PanINs in addition to pERK-positive and negative acinar cells. However, they do not present the results of the PanIN sequencing. This should be included. In addition, the authors state that pERK-positive acinar clones are frequently found near PanINs. When this happens, do they have concordant KRAS mutations in the 2 lesions? This would provide a stronger link between the observed acinar lesions and well-documented precancerous lesions.

Response: We did not perform sequencing on PanINs and adjacent pERK-positive acinar regions. We did sequence a small number of PanINs as controls for the presence of Kras mutations, because Kras mutations are so prevalent in these lesions. We appreciate the logic of the proposed experiment, but we do note that when others have sequenced PanINs from the same sample they have often found multiple unique Kras mutations (Kanda et al. Gastroenterology 2012). This heterogeneity may limit the utility of the experiment proposed. We do not have the data to support a genetic relationship between the acinar regions and an adjacent PanIN and for this reason, we have edited the manuscript to make sure that we do not suggest this.

6. The authors' data in human tissue shows KRAS mutations in acinar cells but does not link back to the original hypotheses related to TERT. Can the authors demonstrate high TERT expression in the pERK-positive acinar foci? Perhaps using the Tert RNA FISH they report in their murine model?

Response: Thank you for this question. Regarding the link between pERK-positive acinar cells and *TERT* expression, this is very difficult to prove in human tissue. TERT is very difficult to detect in both mouse and human due to low levels of mRNA and protein. This challenge in detection in part motivated our approach to develop lineage tracing for the detection and functional study of TERT+ cells. With regard to human cells or tissues specifically, human TERT is detected even at lower levels and in a more restricted pattern than is mouse TERT. The abundant RNAses in pancreas make detection of rare transcripts even more difficult. We likely have the most experience in the field in this space and we find that, for mouse, we can detect TERT mRNA in purified single cells where the entire volume of the cell is queried. However, in tissue sections these approaches have not been successful, either because signal is reduced by fixation or by analyzing only a fraction of a cell in a thin section. For these reasons, we have relied on TERT mRNA FISH in purified mouse cells, as demonstrated here (Fig.1). We have not been able to detect TERT convincingly in tissue sections in mouse or human, due to the underlying signal: noise problem. These issues are further exacerbated by lower levels of the TERT transcript in human, estimated at one to five mRNA molecules per cell. In mouse, we have shown that within the acinar clones TERT+ cells remain rare as the clone grows, consistent with $TERT^{High}$ cells giving rise to $TERT^{Low}$ cells. For these reasons, we are careful to avoid claims regarding the origin of the pERK+ acinar regions in human samples.

7. One reason for skepticism of the acinar cell of origin for human PDAC is the lack ofwidespread acinar-to-duct metaplasia (ADM) in human tissue samples. Can the authors comment at all about the relevance of ADM in their model and experiments? It seems that they suggest that KRAS-mutated acinar cells might transition directly to PanINs without intervening ADM, but it would be helpful to discuss this point more directly.

Response: We appreciate this point, which addresses how acinar cells transdifferentiate to ductal cells. In this resubmission, we now quantify 'Percent metaplastic area' in our Kras experiments in mice, while also reporting the 'PanIN Area'. Because the mouse models rely on cerulein-induced pancreatitis to induce PanIN, there is a very clear and synchronous induction of ADM that has been widely reported. This is particularly pronounced when Kras is expressed throughout the acinar compartment, as has been performed by many other excellent groups. In our case, clonal expression of Kras in $TERT^{High}$ acinar cells leads to a much more limited amount of ADM. We believe that this ADM step is important in the transdifferentiation from acinar to ductal to fate in mouse. There is good evidence that the ADM step is transient; Ptf1aCreER mice combined with Kras results in a wave of ADM formation after cerulein-treatment and that ADM resolves back to acinar cells, or progresses to PanIN. We speculate that the ADM step in human is transient and therefore difficult to detect. The insult that helped to induce differentiation from acinar cell to PanIN may also have occurred in the distant past, compared to the advanced time point at which we are interrogating the advanced pancreas tumor sample. Our observations showing that pERK+ acinar regions exist in human samples further suggest that additional insults or inflammation are needed to facilitate transdifferentiation to the acinar fate.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

It was good to see that the concerns of the two reviewers were similar and that the authors did a great job in addressing most of the key concerns.

The one lingering unaddressed concern relates to the connection between high TERT expression and pERK+ acinar clones in human pancreas, which is lacking. These data are critical for understanding the role of the rare TERT+ cells in human pancreatic tumorigenesis. Due to technical difficulty, they were not able to directly address the concern whether TERT expression is elevated in pERK+ human acinar cells. Previous work has established that PanIN lesions exhibit longer telomere lengths compared to normal ductal cells, which was used a surrogate for TERT reactivation during PDAC initiation. I suggest that the authors attempt telomere FISH to assess whether the rare pERK+ acinar cells possess longer telomeres relative to the normal acinar cells. This would greatly strengthen their model.

Overall, the paper is breaks new conceptual ground and is well executed with multiple levels of reinforcing data. The paper merits publication in Nature following addressing the above concern.

Referee #3 (Remarks to the Author):

The authors have thoroughly addressed my comments/concerns in the revised version.

Author Rebuttals to First Revision:

In this resubmission, we performed the telomere FISH experiment requested by reviewer #1. We did not detect any difference in telomere lengths between pERK-positive and pERK-negative acinar cells in 8 resected human PDAC samples. We believe that this result suggests that telomere shortening occurs in the PanIN phase rather than the acinar cell phase of tumor formation. Since the results are negative and since we have a large volume of data already, we prefer to not include these findings in the manuscript.

Referee #1 (Remarks to the Author):

1. It was good to see that the concerns of the two reviewers were similar and that the authors did a great job in addressing most of the key concerns.

Response: Thank you.

2. The one lingering unaddressed concern relates to the connection between high TERT expression and pERK+ acinar clones in human pancreas, which is lacking… I suggest that the authors attempt telomere FISH to assess whether the rare pERK+ acinar cells possess longer telomeres relative to the normal acinar cells. This would greatly strengthen their model.

Response: We agree with the reviewer that finding a connection between TERT and pERK+ acinar cell clones in human pancreas tissue would be important but is currently not possible due to technical limitations in detecting the very low levels of TERT transcript and protein. As suggested by the reviewer, we performed telomere FISH on multiple patient samples. While we did not detect any difference in telomere length between pERK-negative and pERK-positive acinar cells, we did replicate a significant decrease in telomere length in PanINs as previously described (Figure 1) (van Heek et al., Am. J. Pathology, 2002; Hong et al., Modern Pathology, 2011).

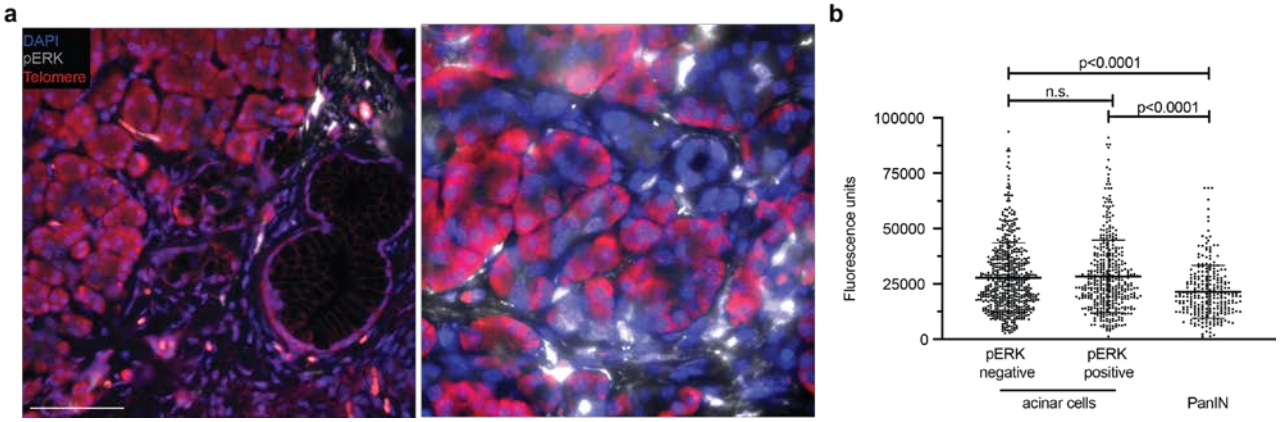

Figure 1 a, Immunofluorescence images of PDAC patient samples after staining for pERK 1/2 (gray) and telomere FISH (Red). Nuclei are counterstained with DAPI (blue). Telomere signal within the DAPI-positive nuclei represents telomere lengths. Acinar cells (dashed white line) and PanIN lesion (white arrow) are displayed. Scale bar, 50µm **b**, Quantification of telomere fluorescence signal of pERK-negative, pERK-positive acinar cells and PanIN lesions within PDAC patiens (n=8 patients, n=560 cells for pERK- negative acinar cells, n=403 cells for pERK-positive acinar cells and n=265 cells for PanINs). Mann-Whitney t test.

While it remains unclear when TERT is upregulated during pancreas carcinogenesis, the PanIN stage does appear to be the stage where significant telomere attrition occurs based on these data. This stage is consistently where investigators have consistently found short telomeres compared with adjacent stromal cells or acinar cells, and we have replicated those findings.

3. Overall, the paper is breaks new conceptual ground and is well executed with multiple levels of reinforcing data. The paper merits publication in Nature following addressing the above concern.

We appreciate the comment from the reviewer and are thankful for the comments and questions raised, improving the overall manuscript.

Referee #3 (Remarks to the Author):

1. The authors have thoroughly addressed my comments/concerns in the revised version.

Response: Thank you.