A single-cell atlas of *de novo* ß-cell regeneration reveals the contribution of hybrid ß/δ cells to diabetes recovery in zebrafish

Sumeet Pal Singh, Prateek Chawla, Alisa Hnatiuk, Margrit Kamel, Luis Delgadillo Silva, Bastiaan Spanjaard, Sema Elif Eski, Sharan Janjuha, Pedro Olivares-Chauvet, Oezge Kayisoglu, Fabian Rost, Juliane Bläesche, Annekathrin Kränkel, Andreas Petzold, Thomas Kurth, Susanne Reinhardt, Jan Philipp Junker and Nikolay Ninov
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AUTHORS: Nikolay Ninov, Sumeet Pal Singh, Prateek Chawla, Alisa Hnatiuk, Margrit Kamel, Luis Delgadillo Silva, Bastiaan Spanjaard, Sharan Janjuha, Pedro Olivares, Oezge Kayisoglu, Juliane Blaesche, Annekathrin Kraenkel, Andreas Petzold, Thomas Kurth, Susanne Reinhardt, Fabian Rost, Sema Eski, and Jan Philipp Junker

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Ninov and colleagues used sc-RNASeq approaches to characterize islet cell heterogeneity in zebrafish under homeostasis and ß -cell regeneration. They reported the presence of two distinct types of somatostatin-producing δ-cells (δ1- and δ2-cells) in the larvae and of one hybrid cell population, which shares the hormones and features of both ß- and δ1-cells. Interestingly, these ß/δ hybrid cells expand and become a prominent source of insulin-expression in well-established model of ß-cell loss and diabetes recovery in zebrafish. The authors used in vivo calcium imaging and cell tracking to study the ß/δ hybrid cells upon ß-cell loss and in the course of regeneration. Finally, they identified dkk3 for being enriched in hybrid cells and its gain-of-function increases their formation under homeostasis.

Overall, this study is interesting and well-conducted; it further expands previous knowledge of ß-cell regeneration in zebrafish and corroborates some recent observation made in the mouse.

Comments for the author

I only have some questions that should be addressed and few suggestions to improve the manuscript:
1) Zebrafish is an excellent model for studying beta-cell regeneration. Nevertheless, it would be very interesting and straightforward to address whether a similar heterogeneity (at least at the transcriptional level) in δ-cells can be found in the mouse or human islets. The authors could easily check this in available datasets.

2) The tSNE plot in Figure 1c shows that the δ1-cells are subdivided into 2 clusters, with one being much closer to the ß-cells. How similar are these two δ1 sub-clusters? does one give rise to the other? Is this visible in the pseudo-time analysis?

3) Do δ1-cells and the ß/δ hybrid cells occupy a special location in the fish islet? Can the authors visualize them in a special location or niche by IF characterization?

4) The visualization of the sc-RNAseq analysis of pancreatic cells during ß cell regeneration should be improved. In the UMAP in Fig. 5a it is difficult to appreciate the changes in clusters over time. Maybe, the clusters could be split and shown separately per day or the colours changed?
Some of the temporal changes in gene expression are actually better visible in the Supplementary Fig. S4. While the increase of δ1-cells is evident with time during regeneration, it seems that at d0 the ß-cell loss is accompanied by a loss in δ1-cells and only ß/δ hybrid cells can be detected (Fig. S4). Also, at d14 it seems that insulin expression overlaps with sst1 and there is no separate ß-cell and δ1-cell clusters, as shown instead in the control islets. How do the authors explain this? When the two separate populations reappear in the islets? Additionally, what is the ID of the cluster below the acinar and above alpha-cells (in Fig. 5a)?

5) A lineage tracing would be very helpful to track the ß/δ hybrid cells during regeneration. Could pyyb be used for lineage tracing? Is a transgenic fish line available for this?

6) Are progenitor markers reactivated during the regeneration in the ß/δ hybrid cells?

7) Minor points:
- Were the 10xGenomics and Smart-Seq2 transcriptomics done on islets of zebrafish of the same age? This should be clearly explained in the text.
- The study identifies two main somatostatin-producing δ-cells in the islet of the zebrafish. Is there a physiological reason for having two δ-cell populations? Also, looking at the sc-RNASeq dataset the overall δ-cell population seems larger than in mammalian species, where somatostatin-secreting δ-

cells comprise ~5% of the cells of the pancreatic islets (Rorsman, Husing 2018). Again, is there a reason for having such a large δ-cell representation in the fish islet? These aspects could be discussed.

Reviewer 2

Advance summary and potential significance to field

The work by Singh, Chawla and colleagues generates a single cell atlas of pancreatic cells during b-cell regeneration. Using zebrafish as a model, they identify by scRNAseq a unique population of endocrine cells exhibiting a hybrid delta-/beta-cell signature, as well as two d-cell populations (d1 and d2), present in homeostasis and increasing following b-cell ablation. Employing various expression analysis and computational approaches, as well as endocrine cell type analysis by transgenic reporters and immunohistochemistry corroborates the presence of the hybrid d1-/b-cell hybrid cells, including their responsiveness to glucose by Ca2+ imaging and measurements of fasting blood glucose levels in homeostasis and regeneration. Importantly, the study shows hybrid cells are present in the developing and adult islet, including their responsiveness to glucose. Finally, the authors test candidate gene dkk3 for its role in regulating hybrid cell identity.
The presented work is of excellent quality, high quality figures and a well written manuscript. The topic is timely and exciting, given the potential of scRNA sequencing to uncover new cell states, this study takes it a step further using elegant transgenic tools showing that the newly discovered b-/d-cell hybrid cells respond to glucose stimuli, supporting their potential for restoring glucose homeostasis after b-cell ablation. The work is suitable for publication in Development, nevertheless there are a number of points that need to be addressed to clarify and further improve the manuscript.

Comments for the author

1 - Please include information concerning the quality control parameter the single cell transcriptome data were subjected to; e.g. minimum and maximum number of genes as cut off and % content of mitochondrial gene transcripts, etc..
2 - According to the ATACseq data, the regulatory elements for some of the hybrid gene loci are open in b-cells. What does is the interpretation of this?
This is surprising, since it seems that the authors, based on the current data, conclude that the hybrid cells originate from the d1-cell population? Are comparable loci open in d1-cells?
Overall, this raises the question about the formation of hybrid cells. Do these hybrid cells represent a common progenitor? Or, do both b- and d1-cells differentiate from separate lineages and subsequently some cells adopt a bi-hormonal hybrid state? Likewise, do d1- and d2-cells arise from common progenitors or separate lineages? These points need to be addressed, for instance by detailed time-series visualizing endocrine differentiation employing lineage markers in the forming embryonic islet.
3 – Related to point 2, are hybrid cells located in specific positions or cellular niches (e.g. surrounded by d1-cells), which could influence their formation or maintenance?
4 - Is this specific endocrine cell diversity conserved in mammals? How do the two distinct delta-cell populations and in particular the hybrid cell signatures compare to those of mammalian endocrine cells?
Interrogating published single cell transcriptome data sets could be employed to address this point.
5 - Figure 4: it is suggested that hybrid cells are characterized by an enrichment of progenitor cell genes compared to differentiated b- or d1-cells. This conclusion is not easy to follow. It needs to be clarified which progenitor specific progenitor genes are enriched in hybrid cells compared to the differentiated fates.
In that case, does this suggest that there is a partial dedifferentiation during injury? Or, in fact point to a common progenitor (see above).
6 - Fig 5 D shows that not only d1- cells, but also other cluster contain subpopulations expressing insulin following b-cell ablation. For instance, a-cells (glucagon-expressing), which have previously been shown to have transdifferentiation potential for replenishing b-cells both in mammal and zebrafish (Ye, et al. Dev. 2015). Do these a-cells exhibit a comparable hybrid signature to the one described here for the d1-/b-cell hybrid cells? Do the scRNA data sets presented here inform on a potential a-cell hybrid cell population in homeostasis or regeneration? Related to this, it should be

discussed whether the mechanism of a-cell derived b-cells is thought to be distinct from the d1-hybrid cell one described in this study.
7 - The study seems to focus on the primary islet, raising the question whether secondary islets in juveniles and adults also contain hybrid cells, or is this a cell type specific to the primary islet?
8- The potential of dkk3 overexpression to increase hybrid cell numbers is interesting, however, appropriate controls are missing. Injection of for instance hsp:cherry is required. These controls may be sufficient to determine, whether an increased number of hybrid cells is next to dkk3-cherry expressing cells compared to control cells, which would allow to start elucidating paracrine signaling modalities.
9 – The result that hybrid cells increase in the adult islet until 14dpa following b-cell ablation is exciting.
Nevertheless, clarification is required as to whether bona fide beta-cells form in the regeneration process in adults like in larvae, e.g. in a subsequent step. Related to this, it should be discussed whether the process and cell-source of b-cell regeneration is the same in larvae and adults.
10 - To understand the characteristics of the hybrid cells better, please clarify the temporal order they activate the Ca2+ signaling reporter. Based on the previous work of the group, do they include the leader cell responding first, or only follower cells?

Additional points:
Why did the previous scRNAseq studies, e.g. by Lu et al J Mol Cell Biol 2019 not detect the hybrid cells described here? Discuss and cite, if appropriate.
Fig1: what was the rational for profiling pancreas from 2 months old fish, compared to older or younger?
Figure 3: panels D' and D'' seem to be mislabeled, they seem to show additional channels of C'.
Figure 5: what do the unlabeled clusters in panel B represent? Extend the labelling accordingly.
Possible mix-up: Page 8, lower paragraph: "… were enriched towards the d1-side of the pseudotime."
Shouldn't it read "b-cell side" , instead of "d1-side"?
Material and methods chapters need to be carefully checked and unrelated information concerning liver and hepatocytes removed.
Unless I missed them, legends for the movies need to be included.

Reviewer 3

Advance summary and potential significance to field

The message conveyed by this paper of Sumeet Pal Singh and co authors is quite clear and has been obtained with cutting-hedge technologies. In zebrafish, two different population of sst producing cells are present, delta1 and delta2. All along zebrafish life there is also a small beta-delta1 hybrid population of pancreatic endocrine cells; notably, this population of glucose responsive beta-delta1 hybrids cells materialise rapidly upon ablation of beta cells by de-novo formation (possibly, but not certainly) from delta1 cells. This rapid grow of glucose-responsive hybrid cells contributes to resolve hyperglycaemia in zebrafish.
The authors also show that the beta-delta1 hybrid cells expresses dkk3b (and few other fate determinants of delta and beta cells) and that over expression of dkk3b is sufficient to increase the beta-delta1 population even in the absence of beta-cell ablation.

Comments for the author

The major problem with this scientific work is that pancreatic developmental stages are used without a clear consistency. Most of the times, precise stages (adult or larval), are not clearly indicated in the main text or legends and this makes it difficult to read and understand the relevance of each finding: larval development, larval regeneration and adult regenerations can take different paths and might have different cellular sources for the formation of new beta-cells.
-Argenton et al., in 1999, using immunofluorescence, have shown that somatostatin/insulin producing cells are already present at 24 and 48 hpf: do the delta1-beta hybrids have a role also in early endocrine pancreas development? The authors claim something about this possibility in their conclusions by citing "unpublished work": to know whether embryonic pathways are recapitulated during regeneration is an issue that should be clearly stated and supported.

-Tracing delta1 to hybrids and hybrids to beta transitions in larvae (ablated or non ablated) can be performed using a sst1.1:dendra (or whatever photoconvertible FP) with transient transgenic zebrafish.
While the sense of the paper can be easily understood, there are too many insufferable typos and mistakes that need to be carefully amended. The entire list would be impossible. I 'm going to mention only some of them:
page 3: "a small proportion of glucagon expressing alpha-cells into beta-cells..."
page 5: "data was"
page 6: "interestingly, dkk3b and pyyb were also enriched in delta-cells (Fig. 2)." there is no pyyb in fig 2.
page 7: "speed and amplitude of response (n=3 islets) (Fig. 3E-F)." there is no F
....

First revision

Author response to reviewers' comments

Response to reviewers:

We would like to thank the reviewers for taking the time to provide us with insightful and constructive comments, which were invaluable in improving the manuscript. Please find below our point-by-point responses to each Reviewer. In addition, our additions to the text of the revised manuscript are shown in blue.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Ninov and colleagues used sc-RNASeq approaches to characterize islet cell heterogeneity in zebrafish under homeostasis and β-cell regeneration. They reported the presence of two distinct types of somatostatin-producing δ-cells (δ1- and δ2-cells) in the larvae and of one hybrid cell population, which shares the hormones and features of both β- and δ1-cells. Interestingly, these β/δ hybrid cells expand and become a prominent source of insulin-expression in well-established model of β-cell loss and diabetes recovery in zebrafish. The authors used in vivo calcium imaging and cell tracking to study the β/δ hybrid cells upon β-cell loss and in the course of regeneration. Finally, they identified dkk3 for being enriched in hybrid cells and its gain-of-function increases their formation under homeostasis.

Overall, this study is interesting and well-conducted; it further expands previous knowledge of β-cell regeneration in zebrafish and corroborates some recent observation made in the mouse.

Reviewer 1 Comments for the Author:
I only have some questions that should be addressed and few suggestions to improve the manuscript:

1) Zebrafish is an excellent model for studying beta-cell regeneration. Nevertheless, it would be very interesting and straightforward to address whether a similar heterogeneity (at least at the transcriptional level) in δ-cells can be found in the mouse or human islets. The authors could easily check this in available datasets.

Thanks a lot for the comment. To address the question, we compared our single-cell dataset (Salem et al., 2019) to a human pancreatic dataset (Segerstolpe, Åsa et al, 2016). The interspecies comparison revealed that the zebrafish δ2-cells clustered near to the human δ-cells, whereas the δ1-cells showed a partial overlap with the human γ-cells (shown in Figure 9). Interestingly, recent findings have highlighted the presence of cellular heterogeneity in mouse and human γ-cells with a subset of γ-cells in mice engaging in insulin production post β-cell ablation (Perez-Frances et al, 2021). In addition, another parallel study showed that *Ppy*-lineage cells could contribute to β-cells in mice during homeostasis and their number increases during diabetic conditions (Fukaishi, T. et al, 2021). These findings further corroborate our observations from the δ1-cells in zebrafish. It is possible that the δ1-cells in zebrafish

represent the ancestral precursors to the γ-cells in mammals. To highlight this idea, we have added a paragraph on the evolution of endocrine cell types across species at the end of the discussion section.

2) The tSNE plot in Figure 1c shows that the δ1-cells are subdivided into 2 clusters, with one being much closer to the ß-cells. How similar are these two δ1 sub-clusters? Does one give rise to the other? Is this visible in the pseudo- time analysis?

1. How similar are these two δ1 sub-clusters?

Thanks a lot for the comment. To address the similarity between two δ1 sub-clusters, we compared the sub- clusters and found 231 differentially expressed genes (refer to Table R1), which suggests that the clusters are trancriptomically different. Additionally, we found that the delta1_N cluster, near the ß-cells, showed expression of ß-cell related genes (subset of delta1_N cells express *ins* gene) (shown in Figure R1).

2. Does one give rise to the other? Is this visible in the pseudo-time analysis?

Using Monocle, we performed pseudo-time analysis on the ß- and two δ1 sub-clusters (delta1_N and delta1_F refer to δ1-cell clusters near and further away from ß-cell cluster, respectively). We observed that the delta1_N cells are positioned between the delta1_F and ß-cells. However, since pseudo-time analysis is a measure of the biological progression, it is difficult to elucidate the direction of the process (shown in Figure R1).

3) Do δ1-cells and the ß/δ hybrid cells occupy a special location in the fish islet? Can the authors visualize them in a special location or niche by IF characterization?

Thanks a lot for the comment. We carefully analyzed our immunofluorescence images from larval and adult zebrafish stages and observed that δ1-cells are distributed throughout the zebrafish islet and do not seem to occupy a specific location or niche. However, δ1/ß hybrid cells are majorly distributed in the islet periphery with fewer in the center (shown in supplementary figure 3).

4) The visualization of the scRNASeq analysis of pancreatic cells during ß cell regeneration should be improved. In the UMAP in Fig. 5a it is difficult to appreciate the changes in clusters over time. Maybe, the clusters could be split and shown separately per day or the colors changed? Some of the temporal changes in gene expression are actually better visible in the Supplementary Fig. S4. While the increase of δ1-cells is evident with time during regeneration, it seems that at d0 the ß-cell loss is accompanied by a loss in δ1-cells and only ß/δ hybrid cells can be detected (Fig. S4). Additionally, what is the ID of the cluster below the acinar and above alpha-cells (in Fig. 5a)? Also, at d14 it seems that insulin expression overlaps with sst1 and there is no separate ß-cell and δ1-cell clusters, as shown instead in the control islets. How do the authors explain this? When the two separate populations reappear in the islets?

1. Maybe, the clusters could be split and shown separately per day or the colors changed?

In order to improve the visualization of the UMAP plot in Figure 5a, we have provided a split-view of the different regeneration time-points used in the integrated dataset (shown in figure R2). The main figure 5 will become too crowded if we attempt to present the time course. As a result, we consider that showing the combined data is a more effective representation. Nevertheless to help readers to track the changes in gene- expression across time points, we have constructed a database in which gene-expression and cell type composition can be studied in detail for each stage of regeneration. The database is available using the link below. We believe that the changes over the course of ß-cell regeneration should be easy to follow.

URL: https://singlecell.broadinstitute.org/single_cell/reviewer_access/2e0e5103-6d56-44ba-86ee- a9650663ea63

Accession number: SCP1549

PIN:O3ZGSN7LJU

2. Additionally, what is the ID of the cluster below the acinar and above alpha-cells (in Fig. 5a)?

In the revised figure 5a, we have annotated all the clusters shown in the integrated dataset such as the ID of the cluster below the acinar and above alpha-cells is the "Smooth Muscle Cell". We have also provided a list of marker genes used for annotating different cell clusters in the form of an excel sheet (refer to Table 3).

3. Also, at d14 it seems that insulin expression overlaps with sst1 and there is no separate ß-cell and δ1-cell clusters, as shown instead in the control islets. How do the authors explain this? When the two separate populations reappear in the islets?

Only upon closer examination, we observed a tendency for separation of the *ins*/*sst1.1* bi-hormonal cells into two clusters at transcriptional level (shown in figure 6). To confirm this at the protein level, we performed IHC in the adult zebrafish pancreas at different time-points post ß-cell ablation. Surprisingly, a majority of the insulin-positive cells continue to exhibit bi-hormonal signature until 14 dpa (shown in supplementary figure 11 and 12). Therefore, while the cells attempt to resolve at the transcriptional level, this resolution remains incomplete. This may be due to an epigenetic block, which prevents the trans-differentiation to be completed. We are exploring this idea currently by chromatin accessibility analysis. The text in the manuscript has been revised to include the new information and the observed persistence of bi-hormonal cells has now been discussed.

5) A lineage tracing would be very helpful to track the ß/δ hybrid cells during regeneration. Could pyyb be used for lineage tracing? Is a transgenic fish line available for this?

Thanks a lot for the comment. Unfortunately, *pyyb* cannot be used for lineage tracing since *pyyb* is not specific only to δ1- and δ1/ß hybrid cells but is also expressed in a subset of α-cells. Therefore, *pyyb* mediated lineage tracing would not provide an unequivocal proof that δ1-cells transdifferentiate to give rise to δ1/ß hybrid cells post ß-cell loss. Also, no *pyyb* reporter line has been reported till date in the literature. Currently, we are working towards identifying an optimal short promoter for labelling the *sst1.1* expressing cells with which we would be able to address the source of the hybrid cells in the future work.

6) Are progenitor markers reactivated during the regeneration in the ß/δ hybrid cells?

This is indeed an interesting question. To address the comment we analyzed our scRNASeq datasets to assess the expression levels of progenitor markers in the δ1/ß hybrid cells over the course of ß-cell regeneration. We found that the progenitor markers such as *pdx1*, *ppdpfa*, *ppdpfb*, Wnt signaling regulators *dkk3b*, *wif1* and ERK signaling regulator *dusp2*, are expressed in the δ1/ß hybrid cells during homeostasis and over the course of regeneration (both at 2 dpa and 7 dpa). We believe that the progenitor markers are reactivated in the ß/δ1 hybrid cells post ß-cell loss (shown in figure R3).

7) Minor points:
- Were the 10xGenomics and Smart-Seq2 transcriptomics done on islets of zebrafish of the same age? This should be clearly explained in the text.

Yes, both 10X Genomics and Smart-Seq2 transcriptomic profiling of the islet were performed on 2 month post-fertilization (mpf) zebrafish. We have made the details clear in the manuscript.

- The study identifies two main somatostatin-producing δ-cells in the islet of the zebrafish. Is there a physiological reason for having two δ-cell populations? Also, looking at the sc-RNASeq dataset the overall δ-cell population seems larger than in mammalian species, where somatostatin-secreting δ-cells comprise ~5% of the cells of the pancreatic islets (Rorsman, Husing 2018). Again, is there a reason for having such a large δ-cell representation in the fish islet? These aspects could be discussed.

Thanks a lot for the comment. We were also surprised to observe such a large population of δ-cells in the zebrafish islet. As mentioned previously, we compared the transcriptome of the

zebrafish and human islet and observed that zebrafish δ2-cells cluster close to the human δ-cells and while the δ1-cells show a partial overlap with the human γ-cells. Since zebrafish islets seem to lack bonafide γ-cells, we speculate that the δ1-cells in zebrafish represent the ancestral precursors to the γ-cells in mammals, which we discussed in the revised version. We are also thinking along the same lines as the reviewer and have become interested to explore how zebrafish and other species may adapt the cellular constitution of their islets to the specific metabolic needs that the animal faces in the wild. In the future, with the development of new tools, we hope to address the role of each cell type in sugar metabolism in zebrafish.

Reviewer 2 Advance Summary and Potential Significance to Field:
The work by Singh, Chawla and colleagues generates a single cell atlas of pancreatic cells during b-cell regeneration. Using zebrafish as a model, they identify by scRNAseq a unique population of endocrine cells exhibiting a hybrid delta-/beta-cell signature, as well as two d-cell populations (d1 and d2), present in homeostasis and increasing following b-cell ablation. Employing various expression analysis and computational approaches, as well as endocrine cell type analysis by transgenic reporters and immunohistochemistry corroborates the presence of the hybrid d1-/b-cell hybrid cells, including their responsiveness to glucose by Ca2+ imaging and measurements of fasting blood glucose levels in homeostasis and regeneration. Importantly, the study shows hybrid cells are present in the developing and adult islet, including their responsiveness to glucose. Finally, the authors test candidate gene dkk3 for its role in regulating hybrid cell identity.
The presented work is of excellent quality, high quality figures and a well written manuscript. The topic is timely and exciting, given the potential of scRNA sequencing to uncover new cell states, this study takes it a step further using elegant transgenic tools showing that the newly discovered b-/d-cell hybrid cells respond to glucose stimuli, supporting their potential for restoring glucose homeostasis after b-cell ablation. The work is suitable for publication in Development, nevertheless there are a number of points that need to be addressed to clarify and further improve the manuscript.

Reviewer 2 Comments for the Author:
1- Please include information concerning the quality control parameter the single cell transcriptome data were subjected to; e.g. minimum and maximum number of genes as cut off and % content of mitochondrial gene transcripts, etc..

The cells were filtered using the following QC metrics: 3000 < nFeature_RNA > 350, 25000 < nCount_RNA > 2500 and percent.mito < 20. All the cells that passed the quality check were used for downstream analysis.

We have included the quality control parameters in the material and methods section of the manuscript.

2 - According to the ATACseq data, the regulatory elements for some of the hybrid gene loci are open in b- cells. What does is the interpretation of this?
This is surprising, since it seems that the authors, based on the current data, conclude that the hybrid cells originate from the d1-cell population? Are comparable loci open in d1-cells?
Overall, this raises the question about the formation of hybrid cells. Do these hybrid cells represent a common progenitor? Or, do both b- and d1-cells differentiate from separate lineages and subsequently some cells adopt a bi-hormonal hybrid state? Likewise, do d1- and d2-cells arise from common progenitors or separate lineages? These points need to be addressed, for instance by detailed time-series visualizing endocrine differentiation employing lineage markers in the forming embryonic islet.

The reviewer raised excellent questions that we are beginning to address. The ATAC data of ß-cells shows that ß and δ1 cells may be related at the level of chromatin accessibility. We currently do not have the ATAC data of δ1 cells in order to compare their epigenetic makeup to ß-cells, however, we do believe that the reviewer is correct in suggesting that this will turn out to be the case. We are in the process of procuring these datasets. In addition, the reviewer rightfully points out to the importance of elucidating the source of the δ1/ß hybrid cells. Presently, we are working towards developing appropriate lineage tracing tools for the sst1.1 expressing cells in the zebrafish pancreas. We believe that we would be able to address the question conclusively in the future work.

3 – Related to point 2, are hybrid cells located in specific positions or cellular niches (e.g. surrounded by d1- cells), which could influence their formation or maintenance?

We carefully analyzed our immunofluorescence images from larval and adult zebrafish stages and observed that δ1-cells are distributed throughout the zebrafish pancreatic islet and do not seem to occupy a specific location. However, ß/δ1 hybrid cells are majorly distributed in the islet periphery and fewer in the center (shown in supplementary figure 3).

4 - Is this specific endocrine cell diversity conserved in mammals? How do the two distinct delta-cell populations and in particular the hybrid cell signatures compare to those of mammalian endocrine cells? Interrogating published single cell transcriptome data sets could be employed to address this point.

Thanks a lot for the comment. To address the question, we compared our single-cell dataset (Salem et al., 2019) to a human pancreatic dataset (Segerstolpe, Åsa et al, 2016). The interspecies comparison revealed that the zebrafish δ2-cells clustered near to the human δ-cells, whereas the δ1-cells showed a partial overlap with the human γ-cells (shown in Figure 9). Interestingly, recent findings have highlighted the presence of cellular heterogeneity in the mice and human γ-cells and that a subset of γ-cells in mice engage in insulin production post conditional beta--cell ablation (Perez-Frances et al, 2021). Moreover, the γ-cells were found to often express multiple hormones, very similar to δ1-cells in fish. Also, another parallel study showed that *Ppy*- lineage cells could contribute to ß-cells in mice during homeostasis and their number increases during diabetic conditions (Fukaishi, T. et al, 2021). These findings further corroborate our observations from the δ1-cells in zebrafish. Since zebrafish islets lack bonafide γ-cells, it is possible that the δ1-cells in zebrafish represent the ancestral precursors to the γ-cells in mammals. To highlight this idea, we have added a paragraph on the evolution of endocrine cell types across species in the discussion section.

5 - Figure 4: it is suggested that hybrid cells are characterized by an enrichment of progenitor cell genes compared to differentiated b- or d1-cells. This conclusion is not easy to follow. It needs to be clarified which progenitor specific progenitor genes are enriched in hybrid cells compared to the differentiated fates. In that case, does this suggest that there is a partial dedifferentiation during injury? Or, in fact point to a common progenitor (see above).

1. It needs to be clarified which progenitor specific progenitor genes are enriched in hybrid cells compared to the differentiated fates.

As mentioned in the text referring to Figure 4, we suggested pathways associated with the endocrine progenitor maintenance such as Wnt, Fgf, Notch and Sphingolipid signaling showed a trajectory towards δ1- cells. The δ1/ß hybrid cells, compared to either ß- or δ1-cells, expressed intermediate levels of these factors and pathway component. This conclusion was drawn due to the intermediate expression level of genes such as *dusp3a*, *wif1*, *lgr4*, *s1pr1* and *her6* in the δ1/ß hybrid cells compared to either ß- or δ1-cells. We have improved the text in the manuscript to make this point clear.

2. In that case, does this suggest that there is a partial dedifferentiation during injury? Or, in fact point to a common progenitor (see above).

To address this concern from the reviewers, we performed ß-cell tracing using *Tg(ins: H2B-mEos2); Tg(ins: FLAG-NTR)* double-transgenic zebrafish larvae. These larvae express a green-to-red photoconvertible protein mEos2 (fused to histone H2B) and NTR specifically in the ß-cells. At 3 dpf, we photo labeled all pre-existing ß-cells in red, followed by Mtz treatment. At 3 dpa, the majority of the ß-cells displayed a hybrid signature, with a green nuclear label and somatostatin expression. This strongly suggests that the ins/sst bi-hormonal cells do not arise from the pre-existing ß-cells in the zebrafish larvae (shown in supplementary figure 14).

6 - Fig 5 D shows that not only d1- cells, but also other cluster contain subpopulations expressing insulin following b-cell ablation. For instance, a-cells (glucagon-expressing), which have previously been shown to have trans-differentiation potential for replenishing b-cells both in mammal and zebrafish (Ye, et al. Dev. 2015). Do these a-cells exhibit a comparable hybrid signature to the one

described here for the d1-/b-cell hybrid cells? Do the scRNASeq data sets presented here inform on a potential a-cell hybrid cell population in homeostasis or regeneration? Related to this, it should be discussed whether the mechanism of a-cell derived b-cells is thought to be distinct from the d1-hybrid cell one described in this study.

1. Do these a-cells exhibit a comparable hybrid signature to the one described here for the d1-/b-cell hybrid cells?

During homeostasis, we observe a small proportion of α-cells expressing *gcgb* and *ins* genes (α/ß hybrids) both during juvenile (~2% cells) and adult stages (~6% cells) in zebrafish (shown in figure R4). To assess the similarity between the two populations of hybrid cells, we compared the transcriptome of the δ1/ß and α/ß hybrid cells and found 928 DEGs in 2 mpf fish and 416 DEGs in the adult stages (shown in Table R2). This strongly suggests that δ1/ß and α/ß hybrid cells are trancriptomically different populations.

2. Do the scRNASeq data sets presented here inform on a potential a-cell hybrid cell population in homeostasis or regeneration?

As highlighted above, we found a small population of α/ß hybrid cells in the zebrafish pancreas under homeostasis. Additionally, some *gcg*-expressing cells displayed low-to-medium *ins* expression over the course of ß-cell regeneration. This suggests that α-cells could act as a potential source of new ß-cells post ß- cell loss in adult zebrafish. However, the δ1/ß hybrid cells are more prominent over the course of regeneration and are the major contributors of new *ins* expressing-cells. (shown in supplementary figure 8).

3. Related to this, it should be discussed whether the mechanism of a-cell derived b-cells is thought to be distinct from the d1-hybrid cell one described in this study.

To elucidate if the mechanism of α-cell derived ß-cells is different from δ1/ß hybrid cells during regeneration, we generated the transcriptome profile of the α/ß and δ1/ß hybrid cells at 7 dpa. The Gene Ontology (GO) analysis using FishEnrichr tool highlighted enrichment of genes involved in several metabolic processes, "type B pancreatic cell differentiation", "activation of MAPKKK activity" and "negative regulation of canonical Wnt signaling" in the δ1/ß hybrid cells. However, α/ß hybrid cells highlighted the enrichment of genes involved in "fatty acid elongation", "peptidyl-prolyl isomerization", "positive regulation of cytokine production and angiogenesis" and "neuropeptide signaling pathway" (shown in supplementary figure 8). This strongly suggested that the mechanisms of α-cell derived ß-cells is perhaps different from the δ1/ß hybrid cells.

7 - The study seems to focus on the primary islet, raising the question whether secondary islets in juveniles and adults also contain hybrid cells, or is this a cell type specific to the primary islet?

We performed immunofluorescence studies on the adult zebrafish pancreatic sections. Similar to the primary islet, a majority of the cells in the secondary islets showed a distinct expression of insulin and somatostatin, with a small proportion of cells expressing both insulin and somatostatin. At 7 dpa and 14 dpa, we observed a predominance of ins/sst bi-hormonal cells. This strongly suggested that the ins/sst bi-hormonal cells are not specific to the primary islet alone, but present in the whole zebrafish pancreas (shown in supplementary figures 11 and 12).

8- The potential of dkk3 overexpression to increase hybrid cell numbers is interesting, however, appropriate controls are missing. Injection of for instance hsp: cherry is required. These controls may be sufficient to determine, whether an increased number of hybrid cells is next to dkk3-cherry expressing cells compared to control cells, which would allow to start elucidating paracrine signaling modalities.

Thanks a lot for the comment. Here, we used the batch controls (hsp: *dkk3b* negative fish) which were selected post heat-shock treatment. Compared to the control, we could be certain that the increase in the hybrid cells in the zebrafish larvae was due to dkk3b overexpression.

To address the very insightful suggestion that dkk3 is implicated in paracrine regulation and that the hybrid cells are generated close to the dkk3 over-expressing cells, we will need a different set of controls and F0 DNA-injections to generate genetically mosaic islets in which the appearance of new hybrid cells can be quantified as a function of their distance front the dkk3 source. However, this will present a new line of investigation that goes beyond the current analysis.

9 – The result that hybrid cells increase in the adult islet until 14dpa following b-cell ablation is exciting. Nevertheless, clarification is required as to whether bona fide beta-cells form in the regeneration process in adults like in larvae, e.g. in a subsequent step. Related to this, it should be discussed whether the process and cell-source of b-cell regeneration is the same in larvae and adults.

1. Nevertheless, clarification is required as to whether bona fide beta-cells form in the regeneration process in adults like in larvae, e.g. in a subsequent step

Thanks a lot for the comment. We observed that in the zebrafish larvae, the newly formed insulin expressing cells which arise post ß-cell loss are either mono-hormonal or bi-hormone cells. However, during adult stages, insulin expressing cells continue to display hybrid characteristics even after glucose homeostasis is restored. The formation of mono-hormonal cells in the larvae may be due to the steady-state developmental neogenesis from ductal cells that is known to take place in larvae. In the adult regeneration, we observed that a majority of the cells are bi-hormonal, which can reflect the cessation of developmental neogenesis. This change in sources may lead to the observed prevalence of hybrid cells in adult regeneration as compared to larvae. However, it is difficult to provide evidence to support or reject these speculations currently. As we develop better lineage tracing tools, we will tackle these questions conclusively.

2. Related to this, it should be discussed whether the process and cell-source of b-cell regeneration is the same in larvae and adults.

Unraveling the cell-source and the mechanism of ß-cell regeneration is indeed an important question for the field. We are currently trying to generate new CRE reporter lines for tracing *sst1.1* expressing cells. With these tools, we would be able to provide an indisputable evidence if δ1-cells are indeed the source of new ß- cells in both larval and adult zebrafish. Additionally, we are also trying to explore the mechanism of ß-cell regeneration in larva and adult zebrafish. Since the larval regeneration may be influenced by continuous developmental neogenesis, it is difficult to ascertain at this point whether the two stages differ in the mechanisms of regeneration. We wish to address these questions in our future work.

10 - To understand the characteristics of the hybrid cells better, please clarify the temporal order they activate the Ca2+ signaling reporter. Based on the previous work of the group, do they include the leader cell, responding first, or only follower cells?

We compared the time of response of δ1/ß hybrid cells to the mono-hormonal ß-cells in the zebrafish larvae under homeostasis and observed that the δ1/ß hybrid cells were not the first-responders (or leader cells) but the follower cells (data not shown).

Additional points:
Why did the previous scRNASeq studies, e.g. by Lu et al J Mol Cell Biol 2019 not detect the hybrid cells described here? Discuss and cite, if appropriate.

Thanks a lot for referring to the study. We carefully analyzed the datasets generated by Lu et al, 2019 and found that both *sst1.1* and *dkk3b* genes were enriched in the ß-cell precursor cluster at 20 hpf, 30 hpf and 52 hpf in the zebrafish embryos. We have referred to the paper in the discussion section. This further corroborates our findings that δ1/ß hybrid cells exist at different developmental stages in the zebrafish pancreas.

Fig1: what was the rational for profiling pancreas from 2 months old fish, compared to older or younger?

We believed that the 2 month old fish with provide an adequate balance between islet plasticity and maturity as the pancreas has acquired a relatively mature state, yet proliferation is still observable. However, in our study, we have profiled islets from different stages and our findings with respect to the δ1/ß hybrid cells are quite consistent irrespective to the age of the fish i.e. a subset of cells in the zebrafish islet express both *ins* and *sst1.1* genes.

Figure 3: panels D' and D'' seem to be mislabeled, they seem to show additional channels of C'.

Thanks for pointing out the mistake. We have revised the labelling in the figure.

Figure 5: what do the unlabeled clusters in panel B represent? Extend the labelling accordingly.

We have annotated all the unlabeled clusters in the new figure 5. We have also provided a list of marker genes used for annotation in the form of an excel sheet.

Possible mix-up: Page 8, lower paragraph: "… were enriched towards the d1-side of the pseudo time." Shouldn't it read "b-cell side", instead of "d1-side"?

Thanks for pointing out the typos. We have revised the text in the manuscript.

Material and methods chapters need to be carefully checked and unrelated information concerning liver and hepatocytes removed.

We have revised the material and methods section and included the appropriate and necessary information.

Unless I missed them, legends for the movies need to be included.

We have included the legends for the movies in the manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:
The message conveyed by this paper of Sumeet Pal Singh and co authors is quite clear and has been obtained with cutting-hedge technologies. In zebrafish, two different population of sst producing cells are present, delta1 and delta2. All along zebrafish life there is also a small beta-delta1 hybrid population of pancreatic endocrine cells; notably, this population of glucose responsive beta-delta1 hybrids cells materialize rapidly upon ablation of beta cells by de-novo formation (possibly, but not certainly) from delta1 cells. This rapid grow of glucose-responsive hybrid cells contributes to resolve hyperglycemia in zebrafish.
The authors also show that the beta-delta1 hybrid cells expresses dkk3b (and few other fate determinants of delta and beta cells) and that over expression of dkk3b is sufficient to increase the beta-delta1 population, even in the absence of beta-cell ablation.

Reviewer 3 Comments for the Author:

The major problem with this scientific work is that pancreatic developmental stages are used without a clear consistency. Most of the times, precise stages (adult or larval), are not clearly indicated in the main text or legends and this makes it difficult to read and understand the relevance of each finding: larval development, larval regeneration and adult regenerations can take different paths and might have different cellular sources for
the formation of new beta-cells.

Thanks a lot for pointing this out. We have clarified the details for different developmental stages used for the experiments in the manuscript.

-Argenton et al., in 1999, using immunofluorescence, have shown that somatostatin/insulin producing cells are already present at 24 and 48 hpf: do the delta1-beta hybrids have a role also in early endocrine pancreas development? The authors claim something about this possibility in their conclusions by citing "unpublished work": to know whether embryonic pathways are recapitulated during regeneration is an issue that should be clearly stated and supported.

This is indeed an interesting question. Currently, we are trying to understand the role of δ1/ß hybrid cells, if any, in early endocrine development. If so, we would like to communicate our findings in the future work.

-Tracing delta1 to hybrids and hybrids to beta transitions in larvae (ablated or non-ablated) can be performed using a sst1.1:dendra (or whatever photo-convertible FP) with transient transgenic zebrafish.

To elucidate the source of the δ1/ß hybrid cells is indeed an important question. Presently, we are working towards identifying an optimal short promoter for labelling the sst1.1 expressing cells in the zebrafish pancreas. However, we believe we would be able to address the question conclusively in the future work.

While the sense of the paper can be easily understood, there are too many insufferable typos and mistakes that need to be carefully amended. The entire list would be impossible. I 'm going to mention only some of them:
page 3: "a small proportion of glucagon expressing alpha-cells into beta-cells..." page 5: "data was" page 6: "interestingly, dkk3b and pyyb were also enriched in delta-cells (Fig. 2)." there is no pyyb in fig 2. page 7: "speed and amplitude of response (n=3 islets) (Fig. 3E-F)." there is no F

Thanks for pointing it out the typos and the mistakes in the manuscript. We have carefully revised the text in the manuscript and corrected the typos and other mistakes.

References:

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2. Fukaishi, T., Nakagawa, Y., Fukunaka, A., Sato, T., Hara, A., Nakao, K., Saito, M., Kohno, K., Miyatsuka, T., Tamaki, M., Matsuhisa, M., Matsuoka, T., Yamada, T., Watada, H., Fujitani, Y., 2021. Characterisation of Ppy-lineage cells clarifies the functional heterogeneity of pancreatic beta cells in mice. Diabetologia 64, 2803-2816. https://doi.org/10.1007/s00125-021-05560-x
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Second decision letter

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MS TITLE: A single-cell atlas of de novo ß-cell regeneration reveals the contribution of hybrid ß/δ cells to diabetes recovery in zebrafish

AUTHORS: Nikolay Ninov, Sumeet Pal Singh, Prateek Chawla, Alisa Hnatiuk, Margrit Kamel, Luis Delgadillo Silva, Bastiaan Spanjaard, Sharan Janjuha, Pedro Olivares, Oezge Kayisoglu, Juliane Blaesche, Annekathrin Kraenkel, Andreas Petzold, Thomas Kurth, Susanne Reinhardt, Fabian Rost, Sema Eski, and Jan Philipp Junker
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.