

Heterogeneous enhancer states orchestrate β cell responses to metabolic stress

Corresponding Author: Dr Zong Wei

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the present study, Wang et al perform Paired-Tag analysis of beta cells from mice fed normal calorie (NC) and high fat diet (HFD). While some genes exhibit concordant changes in H3K4me1 and H3K27ac enhancers, others rely on either H3K4me1 or H3K27ac activity (i.e. discordant). Of note, there is loss of H3K4me1-primed enhancers in beta cells from HFD islets, with a clear trajectory toward ER stress and UPR. Finally, the authors infer ligand-receptor relationships to identify NGF as a suppressor of beta cell ER stress. Overall, the studies are interesting in that they identify discordance between H3K4me1 H3K27ac enhancer states during metabolic stress. However, the narrative of the studies is sometimes hard to follow, the relevance for human beta cell stress is unclear, and the NGF studies are early stage. Lastly, there is overreliance throughout on MIN6 cells for key validation experiments.

- Much of Figure 1 replicates previous scRNA-seq studies using mouse HFD islets, or human type 2 diabetes islets from a number of labs. This is not a bad thing per se- replication is always good- however, there needs to be some more discussion about what novelty the Paired-Tag approach confers versus previous publications.
- There are a number of issues extrapolating findings in MIN6 to primary beta cells. Firstly, heterogeneity by nature is lacking. Secondly, the cells are more vulnerable to ER stress than primary beta cells for a given insulin secretory yield. Thirdly, the cells are more plastic in response to ER stressors. Lastly, there is no transcriptomic evidence that H3K4me1 and H3K27ac contribute to discordant gene expression in MIN6 cells. Thus, interrogation of enhancer states likely needs to be performed in the same tissue type that Paired-Tag analysis was performed on.
- The link between H3K4me1 and H3K27ac activity and ligand-receptor relationships is unclear. Does NGF directly or indirectly influence H3K4me1 and H3K27ac activity? Or does NGF influence the downstream effects of H3K4me1 and H3K27ac activity i.e. increased ER stress gene expression in a beta cell subset?
- How do studies in MIN6 cells, which do not represent a HFD-induced ER-stressed beta cell subset, show any link between NGF, enhancer state and ER stress? Further studies are warranted in HFD islets, with demonstration that an ER-stressed beta cell subset/state (i.e. the major transcriptomic finding) is affected by NGF treatment.
- In general, the NGF studies felt early stage. To show a specific role of NGF, studies are needed to show presence of the receptor in the beta cell, presence of the ligand in the non-beta cell compartment, as well as antagonism (or knockdown) of the ligand/receptor etc.
- Injection of Akita mice with NGF does not demonstrate a specific ligand interaction without at least some in vitro work up showing beta cell specificity of the effect rather than via the numerous other NGF targets.
- The multi-omic studies are technically elegant, however, it is not guaranteed that the studies will pull through into human beta cell biology. Analysis of widely-available published datasets might provide evidence for some commonality in H3K4me1 and H3K27ac dis(cordance), otherwise the authors could consider using in vitro paradigms of human beta cell glucolipotoxicity.

Reviewer #2

(Remarks to the Author)

By utilizing a combinatorial index-based multi-omics platform for single-nuclei RNA and active enhancers in Langerhans

islets of lean and obese mice, Lui Wang and colleagues detected two prominent beta cell clusters and differences in the activation of enhancers and corresponding gene expression. In addition, the authors discovered a specific role of primed enhancers via FoxA2 binding for metabolism-induced stress response and identified NGF, presumably released by duct cells and fibroblasts, as novel suppressor of ER stress in beta cells.

This is a very interesting and comprehensive study providing novel insights into the molecular causes of beta cell failure in response to overnutrition-induced gluco/lipotoxicity. I have some questions and suggestions to improve the manuscript:

Major points:

The major criticism is that this is an exclusive mouse study. However, as corresponding analyses are not possible to be performed in humans, the authors should at least compare their gene lists with the results of obesity and T2D GWAS and with available scRNA data of human islets.

The authors should provide data on body weight, blood glucose and insulin concentrations of the 3 groups of mice. There is no information on the sample size; how many islet samples of the 3 groups were used for the profiling of histone modifications and gene expression on single cell basis, were samples pooled and if so from how many mice? In addition, it is necessary to refer to the mouse strain (C57BL/6J) in the results and to briefly mention that C57BL/6 mice are not prone to develop a T2D-like phenotype with beta-cell loss (PMID: 29348476).

Please explain why the two major beta-cell clusters are termed beta-hi and beta-low and what their major characteristics are. The velocity blot shown in Fig. 1D does not convince because it is not visible that beta-hi cells develop to beta-low cells.

Maybe it will be necessary to include the two time points 8 and 16 weeks of HFD feeding.

Beside velocity and pseudotime analyses the authors performed CellRank calculations. However, it is not clear how the CellRank results were incorporated in the manuscript. Did the authors integrate the results in Fig. 1F? Were any initial, intermediate or terminal states based on macrostates identified by CellRank?

Why do the authors use different latent times in Fig. 1E and Fig. 1F, shouldn't this be the same?

Concerning the beta cell clusters, I have the impression when looking at Fig. 1G that beside the proliferation cluster three clusters exist. Figure S4A gives the impression of seven beta cell clusters. The UMAP plot of Fig. 2A and 2B also indicates that rather at least two beta-hi and one beta-low low clusters exist.

According to Figure S4A the cluster pattern of males and females does not differ. However, considering the high number of differentially expressed genes (866) in beta cells in both sexes I am wondering why we do not see this in the UMAP plot. A list of genes which are repressed and induced in the three beta cell clusters (beta-hi, beta-low, Mk67-beta) in response to the HFD should be provided in the supplements.

Similarly, a list of the top 50 genes (RNAs) shown in Fig. 2E/F should be provided in the supplements.

Are the highlighted genes with a coupled RNA and H3K27ac/H3K4me1 activation that are related to ER-stress, inflammation and beta cell function (Fig. 2K/L) significantly enriched?

How many TF motifs (beside FoxA2, FOS, IRF1, NFKB2 and other listed candidates and those shown in Fig. 3 B/C) are enriched in genes which exhibit enhancer activity in beta-hi and beta-low?

I did not find NRC1 in Fig. 3F/G.

Please flip Fig. 3H and 3I or change the description in the text (lines 223-228).

Please provide a number and full list of concordant and discordant genes shown in Fig. 4A.

In the gene score map of group 3 (Fig. 4F) only Dusp10 and Kcnmb2 but not Evt1 (as described in the results line 259 and in the legend line 1171) are shown.

Is the expression of HAT, HDAC, HMT and/or KDM affected in beta cells which express discordant genes?

As H3K27ac also marks active promoters, I am wondering, if the authors took this also into consideration. Thus, isn't it in theory possible that at least some of the discordant genes can be explained by this aspect?

Are other examples of variable concordance between H3K27ac and H3K4me1 described in the literature?

For the results shown in Fig. 3 H-J and 4D it is necessary to confirm the expected effects on histone modifications.

In the UMAP plot of Fig. 5A it is very difficult to distinguish between the two time points 8 and 16 weeks. I recommend to change the color of HFD 16 weeks.

In respect to the ligands of non-beta cells (Fig. 6A) I recommend to provide information on the cells which show the differential ligand expression.

Were the results shown in Fig. 6 F reproduced or is this just one experiment? I recommend to include a quantification of at least 3 independent experiments.

The authors stated "the potential function of NGF in islets has not been characterized." This is not totally correct because in a review (PMID: 36768281) one chapter refers to "NGF in Pancreatic Beta Cells" and two other papers describe its role on insulin secretion (PMID: 27424144; PMID: 27825441). Maybe the authors highlight the new role of NGF on suppressing ER stress in islet cells.

It would be interesting to test the plasma concentration of NGF and Manf in NC and HFD fed mice as well as in Akita mice. The concentration of Manf and NGF for treatment of Min6 cells is not mentioned in the legend and for the treatment of Akita mice 1 mg/kg NGF appears quite high. I recommend to perform a concentration-response curve in Min6 cells.

In the legends of Fig. 3J, Fig. 4G and Fig. 6 E an n = 2-6 is specified; it is not appropriate to perform statistics with n = 2.

Minor points:

The authors should avoid the term "lean and obese islets" and rather use "islets of lean and obese mice".

Please provide the full name of TrkA (Tropomyosin receptor kinase A) in the abstract.

Line 127: Please correct HFD-induced

Line 328: Please correct regulates to regulate

Line 351: Please correct ameliorates

Lines 1116 and 1137: Please correct high

Line 1195, please correct FOXA2 to FoxA2.

Please adapt the font sizes in the graphs, in some cases (e.g. the legend of Fig. 1 K is much too small)

Please use a consistent spelling for beta cells, either as a Greek letter or beta, but not b cells (as in lines 1172 and 1197)

also in the figures.

Please use a consistent spelling for beta-high or beta-low (in the text it is a mixture of both versions)

Reviewer #3

(Remarks to the Author)

What are the noteworthy results?:

The authors demonstrate the relationships between enhancer states, gene expression, and over-nutrition induced metabolic dysregulation. By using a data-driven the -omics approach they identify known biological relationships and are able to identify a putative regulator of ER stress (and by extension beta-cell dysfunction) which has a significant impact on relevant traits.

Will the work be of significance to the field and related fields?:

I believe this work is certainly of significance. It is not revolutionary in that it's findings are well supported in our understanding of these phenomena. That is not to say that these results are not novel, but to say that they conform to expectations. They certainly demonstrate (and I believe well) how these tools can be leveraged to get to mechanistic candidates which can then be investigated thoroughly.

Does the work support the conclusions and claims, or is additional evidence needed?:

Their chief claims as I understood them are:

1. There are cell type specific dietary response expression profiles in pancreatic islets. Such that they observe genes differentially expressed between beta-cell states.
2. These beta-cell states are present in both chow and HFD contexts, but there is a shift in the ratio of states. Further these states are associated with expression differences.
3. Pro-transcription histone modification profiles re-capitulate the same cell clusters and show a shift in enhancer states matching the observed shift in expression between beta cell states.
4. They are able to combine histone marks and expression data to generate gene regulatory networks associated with the cell state dynamics.
5. The roles H3k27ac and H3K4me1 marks are not entirely concordant, as one would expect from a complex system.
6. Global perturbation of histone marks further supports a subset of the computationally identified GRNs.
7. Using expression profiles of all the cell types in this micro-environment, they are able to identify NGF as a potential cell-cell cross talk signal that ablates part of the effects of over-nutrition in this model.

The link between NGF and the enhancer work is not really discussed well. It feels to some extent like two projects mashed together. I feel that is a weakness in the delivery. I would like to hear more direct discussion as to whether for instance NGF is defining the enhancer landscape. Or is it an independent mechanism? I feel it should be addressed either way. In an ideal setting I would like to see matching multi-ome data generated from animals treated with NGF on the respective diets to see whether the putative GRNs respond as expected.

While I believe this would very much strengthen and unify the study, I do not believe such steps are needed to support their claims as written. They don't seem to make the claim that they are actually mechanistically linked explicitly. If they cannot be linked, then that should be explicitly stated as a limitation.

Are there any flaws in the data analysis, interpretation and conclusions?:

I find no obvious flaws in their analysis or logic. I do question some of the statistical assumptions for example correlation coefficients as pearson's correlation coefficients make assumptions about the underlying distributions of the correlated variables. That they are normally distributed and do not have substructure, I hesitate to believe that those assumptions hold here. That said, I do not believe that such potential false assumptions rise to the level that they would ultimately change the claims as the size of the effects seem substantial. I would however like a direct description of how multiple-tests corrections were performed.

Do these prohibit publication or require revision?:

No I do not believe they do, but I would want to hear more about multiple tests corrections.

Is the methodology sound? Does the work meet the expected standards in your field?

Yes and yes. I believe this study is an effective demonstration of the strengths of these methodologies.

Is there enough detail provided in the methods for the work to be reproduced?

No, please expand on the analysis methods. Was there parameter tuning? For the sake of transparency, all code should be hosted on github or something like it. Without specific parameters, re-analysis would be impossible.

Other:

Why were no repressive marks considered?

Please clarify in the text more explicitly which mouse lines were used for which experiments.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done a good job addressing this reviewer's comments. In particular, replication of key experiments in primary mouse HFD islets, use of human islet datasets, as well as better validation of NGF-TrkA, all strengthen the conclusions. One minor comment remains:

The new data on TrkA protein expression in pancreas sections is not terribly convincing, and given the challenges detecting RTKs as well as the unreliability of antibodies, this should be deleted (unless the authors can provide the necessary controls e.g. KO validation). Provision of sorted alpha cell, beta cell and delta cell transcript data is fine to support the authors' arguments.

Reviewer #2

(Remarks to the Author)

I would like to thank the authors for responding to all my questions. By this, I think the paper markedly improved.

Reviewer #3

(Remarks to the Author)

The study as written and the authors responses to my points have sufficiently addressed my concerns.

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Response to reviewers' comments:

Reviewers' comments: black and *Italic*

Authors' response: blue

Reviewer #1: *In the present study, Wang et al. perform Paired-Tag analysis of beta cells from mice fed normal calorie (NC) and high fat diet (HFD). While some genes exhibit concordant changes in H3K4me1 and H3K27ac enhancers, others rely on either H3K4me1 or H3K27ac activity (i.e. discordant). Of note, there is loss of H3K4me1-primed enhancers in beta cells from HFD islets, with a clear trajectory toward ER stress and UPR. Finally, the authors infer ligand-receptor relationships to identify NGF as a suppressor of beta cell ER stress. Overall, the studies are interesting in that they identify discordance between H3K4me1 H3K27ac enhancer states during metabolic stress. However, the narrative of the studies is sometimes hard to follow, the relevance for human beta cell stress is unclear, and the NGF studies are early stage. Lastly, there is overreliance throughout on MIN6 cells for key validation experiments.*

We thank the reviewer for summarizing the strength and novelty of our manuscript. We have included multiple new datasets to address the reviewer's concerns. Please see point-to-point responses below.

- Much of Figure 1 replicates previous scRNA-seq studies using mouse HFD islets, or human type 2 diabetes islets from a number of labs. This is not a bad thing per se- replication is always good- however, there needs to be some more discussion about what novelty the Paired-Tag approach confers versus previous publications.

We thank the reviewer for suggesting a further discussion of the technical novelty of this work. This manuscript focuses on generating the first enhancer-RNA multome in islets to interrogate the enhancer-RNA relationship in islet dysfunction. The advantage of this approach is that it establishes the link between a specific chromatin state (H3K4me1 or H3K27ac, in our case) and gene expression, revealing the heterogeneity not only at the RNA level but also at the non-coding regulatory element level. While Figure 1 focused mainly on RNA data, it defined the integrated beta cell subpopulations which is the foundation for the following integrated analysis.

In the revised manuscript, we have added the following paragraph to the discussion to emphasize the novel discovery in our study.

Several recent studies of scRNA-seq, scATAC-seq, and multiome have revealed the heterogeneity states of β cells at transcription and chromatin accessibility levels^{3,6-15}. However, the contribution of individual cis-element states, defined by combinatorial histone codes and DNA modifications, to the target gene expression has not been characterized due to technical limitations. In this study, we take advantage of Paired-Tag, which is able to quantify histone modification instead of accessibility, to interrogate the contribution of two related and distinct enhancer marks, H3K4me1 and H3K27ac, to heterogeneous gene expression in beta cell dysfunction. Though both marks are enriched at enhancers, each of them is separately regulated by a distinct set of epigenetic machinery. While our study has confirmed several principal discoveries of previous single-cell islet studies, it also reveals unexpected discrepancies of H3K27ac and H3K4me1 in regulating gene expression dynamics in beta-cell dysfunction, as further discussed below.

- There are a number of issues extrapolating findings in MIN6 to primary beta cells. Firstly, heterogeneity by nature is lacking. Secondly, the cells are more vulnerable to ER stress than primary beta cells for a given insulin secretory yield. Thirdly, the cells are more plastic in response to ER stressors. Lastly, there is no transcriptomic evidence that H3K4me1 and H3K27ac contribute to discordant gene expression in MIN6 cells. Thus, interrogation of enhancer states likely needs to be performed in the same tissue type that Paired-Tag analysis was performed on.

We thank the reviewer for pointing out the limitation of using the MIN6 cell line. We have included the following results to validate our discoveries in Paired-tag further using primary mouse islets.

1. To extend our initial validation of H3K27ac GRNs in MIN6 cells, we treated mouse islets from 16 weeks HFD mice with p300/CBP inhibitors GNE-781/C646, or the HDAC inhibitor SAHA. Among 855 genes of which RNA dynamics is coupled with H3K27ac (group 1 and group 3 in **Figure 4A**), 94 genes are upregulated upon SAHA treatment, and 320 genes are downregulated upon GNE-781 or C646 treatment (**Figure 4G**). Gene ontology of these perturbed genes (412 genes) shows the association with response to ER stress and insulin secretion (**Figure 4H**). The substantial overlap indicates that for ~50% of the RNA-H3K27ac associated genes, perturbation of H3K27ac changed gene expression.

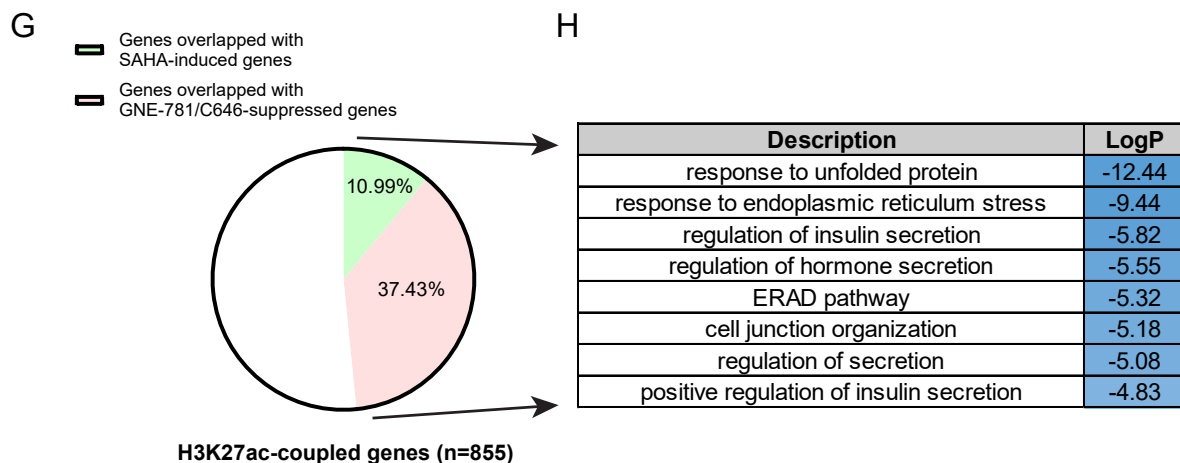


Figure 4. (G) Pie chart showing the H3K27ac-coupled genes (group 1 and 3 from (A)) that are significantly suppressed by CBP inhibitor GNE-781 or P300 inhibitor C646 (red), or induced by HDACs inhibitor SAHA (green), in primary mouse islets treated with SAHA (5 uM), GNE-781 (1 uM) or C646 (5 uM) for 24 h. (H) Gene ontology categories of 414 overlapped genes in (G).

2. To further validate our previous observation that NGF is able to reduce ER stress in MIN6 cells, we treated the islets from HFD mice with NGF and found that ER stress markers such as Ddit3 (Chop), Atf3, and Ppp1r15a (Gadd34) are significantly reduced in NGF-treated islets (**Figure 6F**).

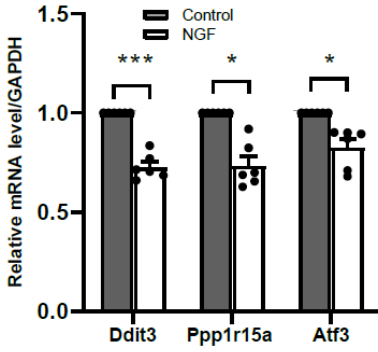


Figure 6. (F) RT-qPCR of *Ddit3*, *Ppp1r15a*, and *Atf3* expression in HFD-fed mice islets treated with or without NGF. Data shown as mean $\pm$ S.E.M. (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001.

- We further evaluated the H3K27ac perturbation on β cell heterogeneity by using a well-characterized cell surface gene, CD63, as a surrogate marker for “healthy” β cells⁸. We confirmed that CD63 is highly expressed in beta-hi cells compared to beta-low cells. (**Figure S11A**). Treatment of CBP inhibitor GNE-781, P300 inhibitor C646, or HDACs inhibitor SAHA all reduced the CD63^{hi} β cells% compared to vehicle (**Figure S11B**), suggesting that perturbation of the “optimal” H3K27ac level shifted the heterogeneity of β cells to a more dysfunctional state.

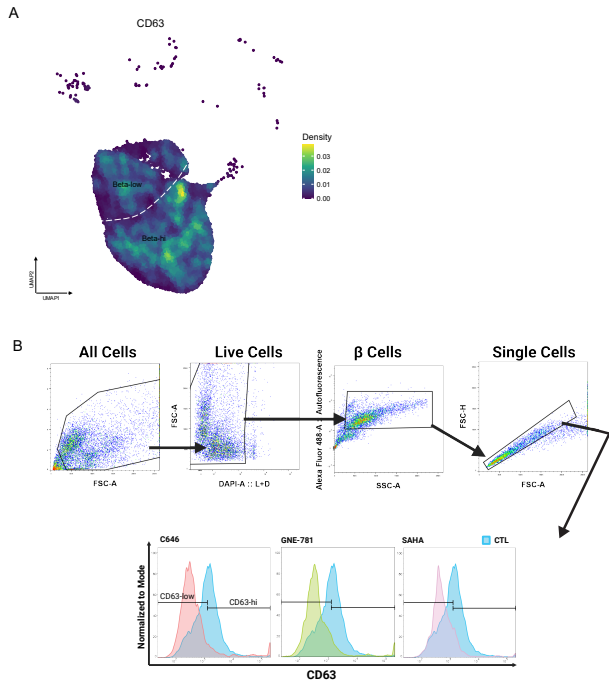


Figure S11. H3K27ac level influences the heterogeneity of β cells.

(A) UMAP showing restricted expression patterns of CD63 in β cell population. (B) Scatterplots showing the sequential gating protocol to isolate CD63-hi and CD63-low β cells from mice islets treated with SAHA, GNE-781, or C646 by FACS.

- The link between H3K4me1 and H3K27ac activity and ligand-receptor relationships is unclear. Does NGF directly or indirectly influence H3K4me1 and H3K27ac activity? Or does NGF influence the downstream effects of H3K4me1 and H3K27ac activity i.e. increased ER stress gene expression in a beta cell subset?

We thank the reviewer for pointing out this new direction of NGF signaling. NGF was identified as a potential paracrine signal using solely the snRNA data from our Paired-tag study. Therefore, there is no direct, mechanistic link between NGF and H3K27ac/H3K4me1. However, the main (novel) function of NGF here is to repress elevated ER stress genes in beta cells, which could be correlated with both changes of H3K4me1 and H3K27ac. Whether the downstream signaling of NGF directly involves H3K4me1/H3K27ac epigenetic changes may be out of the scope of this study since the primary focus of this manuscript is the enhancer heterogeneity in dysfunctional beta cells rather than the underlying mechanism of NGF signaling. We have included the potential mechanisms of NGF and perspectives for future studies in the discussion section.

- How do studies in MIN6 cells, which do not represent a HFD-induced ER-stressed beta cell subset, show any link between NGF, enhancer state and ER stress? Further studies are warranted in HFD islets, with demonstration that an ER-stressed beta cell subset/state (i.e. the major transcriptomic finding) is affected by NGF treatment.

To mimic ER stress in vitro, we treated the MIN6 cells with thapsigargin (TG), which is a widely used in vitro model for ER stress¹⁶⁻¹⁹. However, we agree with the reviewer about the limitation of TG. To further validate the function of NGF in relieving ER stress in islets, we have treated islets from HFD mice with NGF or vehicle. We found that several ER stress markers are significantly down-regulated in NGF-treated islets (**Figure 6F**, see above), suggesting that NGF is able to repress physiologically-relevant levels of ER stress.

- In general, the NGF studies felt early stage. To show a specific role of NGF, studies are needed to show presence of the receptor in the beta cell, presence of the ligand in the non-beta cell compartment, as well as antagonism (or knockdown) of the ligand/receptor etc.

To further define the NGF-TrkA signaling in islets, we have included the following results in the revised manuscript.

1. Presence of local NGF in islets: we are able to detect NGF through ELISA in pancreatic lysates from both lean and HFD mice (**Figure 6E**) and showed that similar to scRNA results in Figure 6C, NGF protein level is reduced in islets from HFD mice. Together with

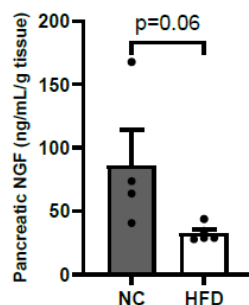


Figure 6. (E) Pancreatic NGF content in mice (B6) fed with normal chow (NC) and HFD for 16 weeks (n = 4-5).

the single cell expression data, these results support a paracrine source of NGF, confirming the results from a previous publication²⁰.

2. Presence of TrkA in beta cells: In mouse islets, Ntrk1 is highly enriched in beta cells but not alpha or delta cells (**Figure A**, data from DiGruccio MR et al. ¹), suggesting that beta cells are the main recipients of the paracrine NGF. In addition, we performed immunofluorescence staining of TrkA with pancreatic sections from lean and HFD mice (**Figure 6D**) and showed that a subset of beta cells expresses TrkA.

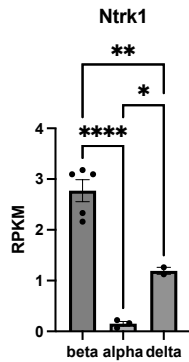


Figure A. Expression of Ntrk1(TrkA) in sorted mouse alpha, beta, and delta cells, data from DiGruccio MR et al.

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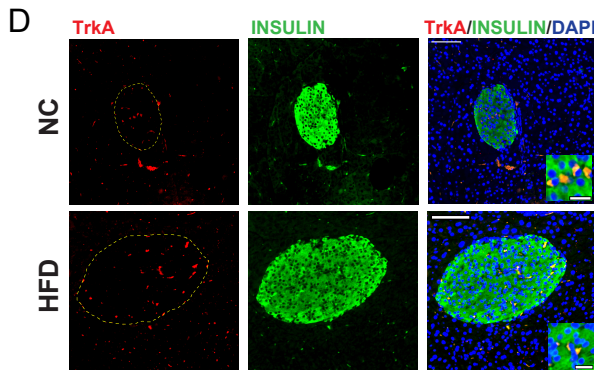


Figure 6. (D) Immunostaining of TrkA (red) and insulin (green) of pancreas section from NC and HFD mice.

3. Direct effect of NGF on primary islets: when islets from HFD mice were treated with NGF, several ER stress markers were significantly downregulated, suggesting that the anti-ER stress effects of NGF are likely through direct action on islets (**Figure 6F**, see above).

- Injection of Akita mice with NGF does not demonstrate a specific ligand interaction without at least some in vitro work up showing beta cell specificity of the effect rather than via the numerous other NGF targets.

We thank the reviewer for pointing out the confounding factors in the Akita study. We choose the Akita mice because the simplicity of the disease progression mechanism: significant elevated UPR entirely due to mutation and misfolding of insulin. Therefore, the improvement of glycemia is likely a direct consequence of enhanced beta cell health and reduced beta cell stress, similar

to the conclusion from the previous study using $\text{Ins2}^{\text{Akita}};\text{Chop}^{-/-}$ mice²¹. To further demonstrate the anti-ER stress activity of NGF, we isolated islets from HFD mice and treated the islets with NGF (see **Figure 6F** above). Although the ER stress of the HFD model is much milder than Akita mice, qPCR of ER stress markers *Ddit3*, *Ppp1r15a*, and *Atf3* showed a significant reduction, further supporting the idea that NGF is able to enhance beta cell function.

The multi-omic studies are technically elegant, however, it is not guaranteed that the studies will pull through into human beta cell biology. Analysis of widely-available published datasets might provide evidence for some commonality in H3K4me1 and H3K27ac dis(cordance), otherwise the authors could consider using in vitro paradigms of human beta cell glucolipotoxicity.

We thank the reviewer for pointing out the significance of exploring human relevance. In the revised manuscript, we used the following two approaches to determine whether our discovery in mouse islets is relevant to human beta cell development and dysfunction: 1) to cross-compare our H3K4me1-RNA association with H3K4me1 changes in human beta cell differentiation; 2) to compare our H3K27ac-RNA association to H3K27ac changes in human EndoC-betaH1 cells under inflammatory stress.

1. We determined H3K4me1-dependent gene expression in human beta-like cells by examining the published study by Vinckier et al (PMID: 32350257). In this study, they performed ChIP-seq and RNA-seq in human stem cell-derived beta cell progenitors with or without a LSD1 (H3K4me1 demethylase) inhibitor. In our reanalysis, we extracted ChIP-seq and RNA-seq data from the GEO database (accession code GSE104840) and clustered genes based on differential expression between the vehicle and LSD1-inhibited beta cell progenitors (**Figure S12**). These led to the identification of 1284 genes of which the RNA expression depends on LSD1. Notably, for 30.76% of these genes, the RNA expression also correlate with H3K4me1 changes based on our Paired-tag dataset, confirming a significant similarity between mouse and human datasets (**Figure S12B**). GO analysis of these genes confirms the biological relevance in beta cell function (**Figure S12C**).
2. We identified the list of human genes of which the expression correlates with H3K27ac using a dataset from Mireia Ramos-Rodríguez et. al. (Nat Genet 2019 Nov. PMID: 31676868, GSE133135 and GSE137136). The study performed H3K27ac ChIP-seq and RNA-seq in EndoC-bH1 treated with vehicle or cytokine mix. We identified 452 differentially expressed genes in cytokine-treated EndoC-βH1 cells and clustered them accordingly(**Figure S12D**). For 49.12% of these genes, their mouse paralogs showed significant H3K27ac-RNA association in our dataset (**Figure S12E**). GO analysis also indicates the biological significance of these overlapped genes (**Figure S12F**).

Taken together, these results (**Figure S12** below) confirmed that the association between H3K27ac/H3K4me1 and RNA is conserved between humans and mice. Our analysis confirmed that the link of gene expression to local H3K4me1/H3K27ac changes could be largely conserved in human and mouse beta cells.

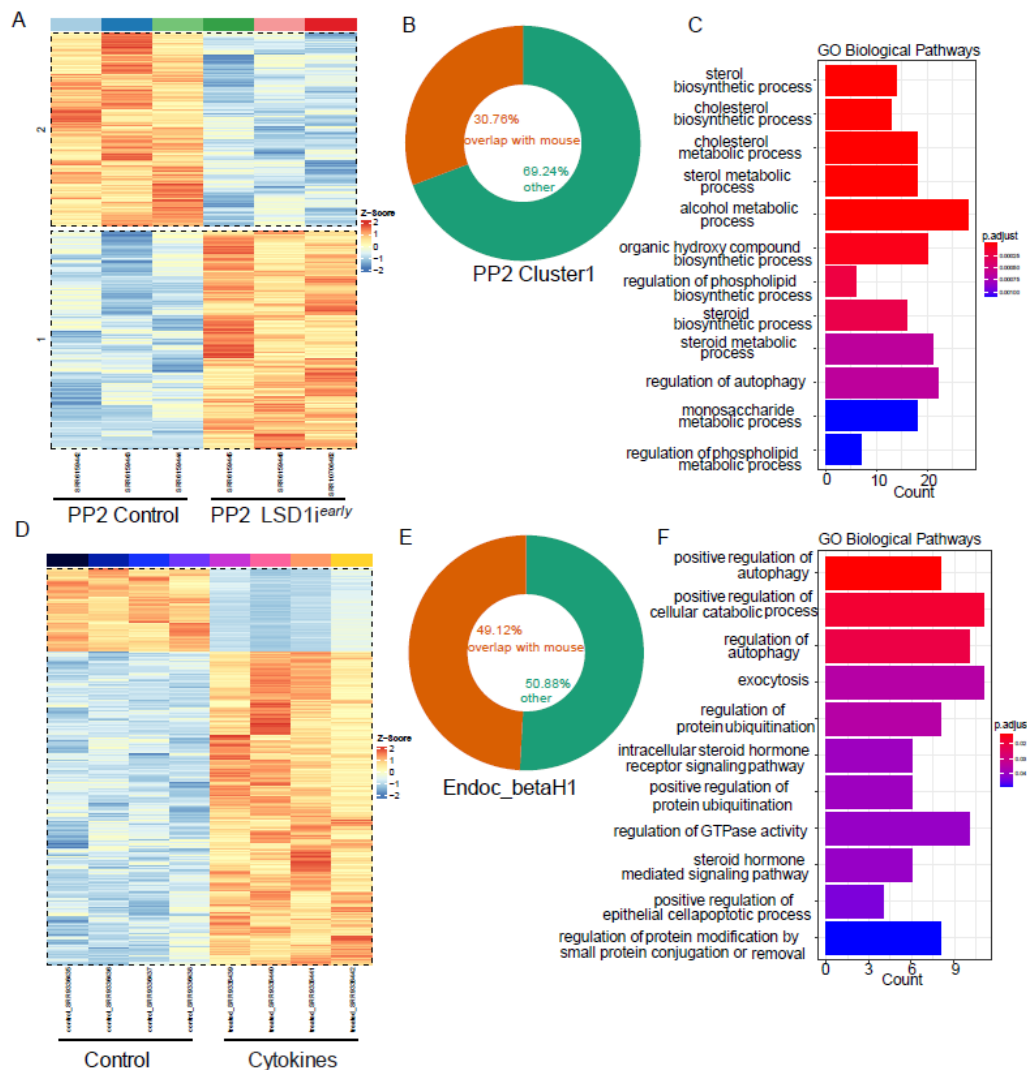


Figure S12. The H3K4me1/H3K27ac-RNA linkage is conserved in human beta cells.

(A). Heatmap showing significantly altered gene expression in PP2 cells (late pancreatic progenitor cells) after LSD1 inhibition (LSD1^{early}) in Vinckier et al.⁴. (B). Pie chart showing the percentage of LSD1^{early} induced genes (clusters1) with a mouse homolog that has significant H3K4me1-RNA association. (C). Gene ontology for genes in (B). (D). Heatmap showing differential H3K27ac peaks in EndoC-βH1 cells treated with cytokine mix (data from Ramos-Rodriguez, M et. al.⁵). (E). Pie chart showing the percentage of (D)-associated genes with a mouse homolog that has significant H3K27ac-RNA association. (F) Gene ontology for human-mouse overlapping genes in (E).

Reviewer #2: By utilizing a combinatorial index-based multi-omics platform for single-nuclei RNA and active enhancers in Langerhans islets of lean and obese mice, Lui Wang and colleagues detected two prominent beta cell clusters and differences in the activation of enhancers and corresponding gene expression. In addition, the authors discovered a specific role of primed enhancers via FoxA2 binding for metabolism-induced stress response and identified NGF, presumably released by duct cells and fibroblasts, as novel suppressor of ER stress in beta cells. This is a very interesting and comprehensive study providing novel insights into the molecular causes of beta cell failure in response to overnutrition-induced gluco/lipotoxicity. I have some questions and suggestions to improve the manuscript:

We thank the reviewer for summarizing the strength and novelty of our study. In this revision, we have included multiple new datasets based on the reviewer's advice. Please see point-to-point response below.

Major points: The major criticism is that this is an exclusive mouse study. However, as corresponding analyses are not possible to be performed in humans, the authors should at least compare their gene lists with the results of obesity and T2D GWAS and with available scRNA data of human islets.

We agree with the reviewer that connecting our discovery in mice to the existing human data is essential. To demonstrate the human relevance of genesets described in our study, we compared the overlap of our data with two different types of human studies: GWAS and human islet scRNA-seq.

We first analyzed 226 genome-wide association studies (GWAS) relevant to T2D, sourced from the GWAS Catalog (detailed description provided in revised method section), and identified 678 genes genetically linked to T2D. Next, we compiled a list of genes associated with human islet function from two recently published scRNA-seq studies (*Nature Communications* 2023, PMID: 37669939, and *Cell Reports*, PMID: 30865899).

To identify evolutionarily conserved beta cell function genes between mouse and human, we compared the pseudo time-defined dynamic mouse beta cell genes in our studies (Figure 1F) with the 4 lists above of human beta cell/T2D relevant genes and identified a total of 246 genes (Figure S4). The list includes many well-characterized T2D relevant genes, such as *Ins2*, *Tcf7l2*, *Hnf4a*, *Bach2*, *Atf3*. Thus, the significant overlap of genes between mouse and human studies confirmed the T2D relevance of our findings.

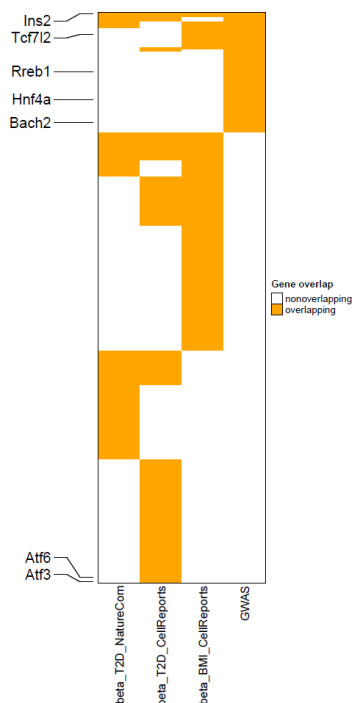


Figure S4. The overlap between top 1,000 genes in Figure 1F and T2D GWAS-associated genes and dynamic genes defined by scRNA-seq in human islets^{2,3}.

The authors should provide data on body weight, blood glucose and insulin concentrations of the 3 groups of mice. There is no information on the sample size; how many islet samples of the 3 groups were used for the profiling of histone modifications and gene expression on single cell basis, were samples pooled and if so from how many mice?

The body weight and blood glucose level are now presented as **Figure S1**. For Paired-tag experiments, we pooled islets from 8-12 mice per condition, as the assays required >3 millions nuclei. We have included this information in the methods.

In addition, it is necessary to refer to the mouse strain (C57BL/6J) in the results and to briefly mention that C57BL/6 mice are not prone to develop a T2D-like phenotype with beta-cell loss (PMID: 29348476).

We have provided the references, the phenotypic change (**Figure S1**) and limits of HFD on C57BL/6J strain in revised manuscript.

Please explain why the two major beta-cell clusters are termed beta-hi and beta-low and what their major characteristics are.

We defined the subpopulations of beta cells based on the following criteria. First, we used Seurat to unbiasedly identify clusters. This results in a total of 15 clusters (Figure S2B), 6 of which are beta cells. Several published scRNA-seq studies in islets^{2,3,7-9} chose to classify beta cells into two major populations, based on the differential expression of insulin and key beta cell marker expression. Therefore, we merged the 6 clusters into two major subpopulations and named the populations beta-hi and beta-low based on the expression level of beta cell-specific genes. The β -hi state is characterized by high expression of hormonal genes (*Ins2*, *Iapp*, *Chga*), indicative of healthier β cells. Conversely, the β -low state is marked by an increased presence of genes associated with unfolded protein response (UPR) (*Pdia3*, *Ddit3*, *Atf3*, *Herpud1*, *Hspa5*, *Eif2ak3*), ER-associated degradation (*Sel1l*, *Herpud1*) and inflammatory stress (*Nfkbiz*), suggesting these cells are under stress and potentially dysfunctional. Another population of proliferating cells expresses insulin but is distinct from the major populations (Mki67- β). Although this simplified definition may mask the minor differences within beta-hi and beta-low cells, we believe it captures the major changes in our dataset, which is a progression from functional, healthy β cells to stressed, dysfunctional β cells. To further clarify our rationale in defining cell states, we have now added the following section to the discussion.

Definition of heterogenous β cell states.

Defining the heterogenous beta cell states with clear biological features has been challenging, as previous literature suggests numerous ways of defining heterogeneity in healthy or dysfunctional beta cells. Our initial classification by Seurat reveals up to 6 potential subpopulations in the main beta cell clusters (Figure S2B). However, these statistically-defined subpopulations do not necessarily represent distinct functional states. In addition, it is challenging to define the trajectories of 6 clusters in beta cell dysfunction. Therefore, we considered the definition in multiple published single cell studies^{2,3,6-9}, and merged 6 subpopulations into two major beta cell states. It should be noted that this dichotomy is very likely an oversimplification of the complex beta cell heterogeneity. Future studies, especially with spatial-omics, single-cell electrophysiology, and lineage tracing, will be needed to further define the potential substates of beta cells.

The velocity blot shown in Fig. 1D does not convince because it is not visible that beta-hi cells develop to beta-low cells. Maybe it will be necessary to include the two time points 8 and 16 weeks of HFD feeding.

We thank the reviewer for pointing out this issue. Reanalysis of our data using the most updated version of CellRank showed a significant increase of velocity arrows direct from beta-low to beta-hi (revised Fig 1D below) to further demonstrate the transition/velocity from beta-hi to beta-low cells, as the major update in the new CellRank uses a revised method to better capture the velocity.

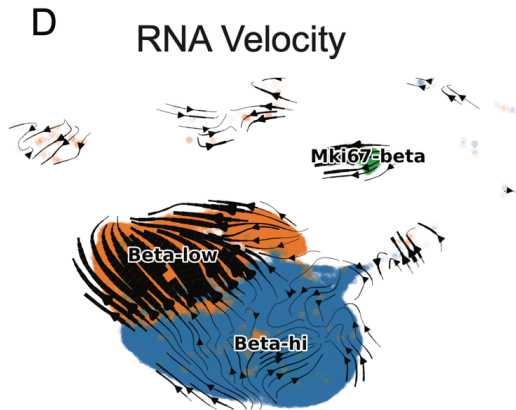


Figure 1 (D). RNA velocity flow projected in the UMAP space.

Beside velocity and pseudotime analyses the authors performed CellRank calculations. However, it is not clear how the CellRank results were incorporated in the manuscript. Did the authors integrate the results in Fig. 1F? Were any initial, intermediate or terminal states based on macrostates identified by CellRank?

Fig. 1F is generated by CellRank, which uses the snRNA-seq count matrix from all beta cells and the corresponding RNA velocity matrix as input. The macrostates are determined by CellRank, which unbiasedly infers the initial, intermediate, and terminal populations from our single nuclei RNA. Based on the inferred potentials, we used CellRank to chart gene expression dynamics as cells take on different fates and identified putative regulators. Specifically, CellRank²² computed terminal states, the number of terminal states was defined using the `cr.tl.terminal_states()` function. Then, it defined initial states with the `cr.tl.initial_states()` function. Next, fate maps were computed to determine the likelihood that an individual cell reaches a certain fate. Subsequently, the individual fate maps were aggregated to cluster-level fate maps by calculating latent time and running PAGA²³. Gene expression trends along pseudo- or latent time were calculated using a general additive model (GAM) with the `cr.pl.gene_trends()` function. Heatmaps of top 1,000 genes whose expression levels correlated best with the probability of reaching the terminal stage were plotted using the `cr.pl.heatmap()` function.

As we examined the unbiased data, we found that, as expected, the initial state featured high expression of beta cell function genes, and the terminal state featured many stress-related genes. Therefore, these unbiased states defined by CellRank are consistent with the beta-hi/beta-low states defined by velocity and pseudotime, revealing a robust trend from healthy/functional beta cells to stressed/unhealthy beta cells.

Why do the authors use different latent times in Fig. 1E and Fig. 1F, shouldn't this be the same?

We have revised Figure 1F to use the same latent times as Figure 1E for consistency.

Concerning the beta cell clusters, I have the impression when looking at Fig. 1G that beside the proliferation cluster three clusters exist. Figure S4A gives the impression of seven beta cell clusters. The UMAP plot of Fig. 2A and 2B also indicates that rather at least two beta-hi and one beta-low low clusters exist.

We thank the reviewer for pointing out this discrepancy in cluster numbers. As discussed above, we initially identified 6 clusters of non-proliferating beta cells and 1 cluster of proliferating beta cells using the FindClusters() function in Seurat. As we discussed above, although the individual clusters are defined by differential gene expression, the challenge is to 1) determine the biological features of individual clusters, and 2) reconstruct a lineage flow that reflects the transition from healthy to stressed beta cells. Therefore, we merged clusters based on the most consistent observation from a large number of published single cell and functional studies^{2,3,7-9}: that the best known biological relevant feature of beta cell heterogeneity is the differential expression of beta cell function/lineage genes and stress/inflammatory genes. We fully agree with the reviewer that it is highly likely that beta-hi and/or beta-low can be further divided into subpopulations each featuring a functional distinct group of cells. We have included an additional section in the discussion for the potential subclusters (see above, page 9).

According to Figure S4A the cluster pattern of males and females does not differ. However, considering the high number of differentially expressed genes (866) in beta cells in both sexes I am wondering why we do not see this in the UMAP plot.

The 866 genes include all statistically significant genes ($p < 0.05$) regardless of the fold change in all 3 time points. The total number is reduced to 84 once we set a threshold of 1.25 fold. The relatively smaller number of DEGs suggest a limited contribution to the reduced dimensionality in UMAP. Revised data are now presented as **Figure S6**.

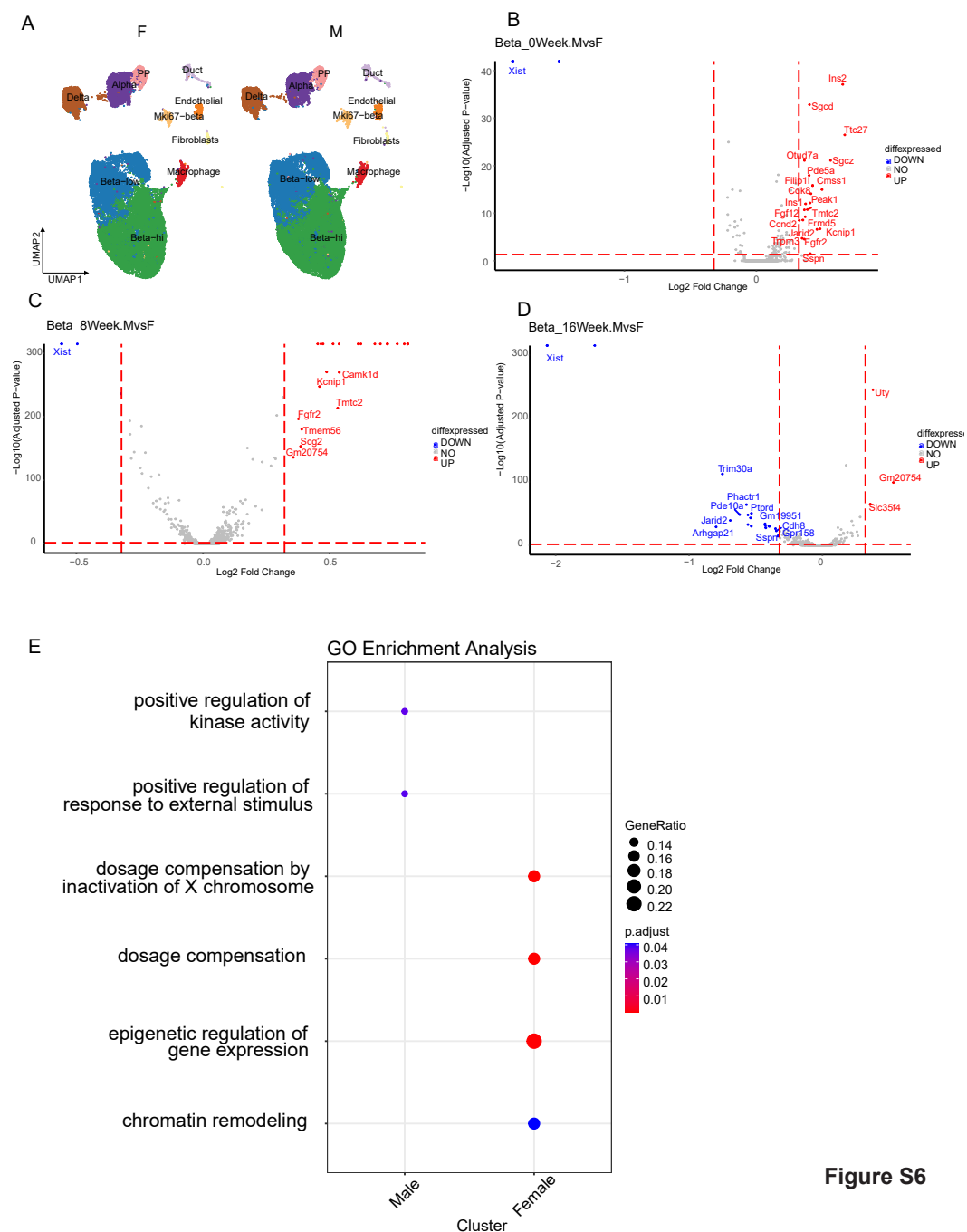


Figure S6

Figure S6. β cell transcriptome analysis reveals sexually dimorphic genes in mice.

(A). UMAP visualizations of populations defined by scRNA in islets from male (left) or female (right) mice. **(B-D)** Differential gene expression in male or female β cells in islets under 0, 8, or 16-week HFD. In the volcano plots, the male-biased and female-biased genes are labeled by blue and red dots, respectively. Differentially expressed genes were defined by adjusted p -value < 0.05 & $|\log_2(\text{fold change})| > 0.1$. **(E).** Functional enrichment analysis for male- or female-biased genes.

A list of genes which are repressed and induced in the three beta cell clusters (beta-hi, beta-low, Mki67-beta) in response to the HFD should be provided in the supplements. Similarly, a list of the top 50 genes (RNAs) shown in Fig. 2E/F should be provided in the supplements.

A list of genes that are repressed and induced in the three beta cell clusters (beta-hi, beta-low, and Mki67-beta) in response to the high-fat diet (HFD) is now included (Supplementary Tables 1). A list of the top 50 genes shown in Figure 2E/F is also included (Supplementary Tables 2 for Figure 2E & Supplementary Tables 3 for Figure 2F).

Are the highlighted genes with a coupled RNA and H3K27ac/H3K4me1 activation that are related to ER-stress, inflammation and beta cell function(Fig. 2K/L) significantly enriched?

Yes. We extracted the highlighted genes from Figure 2K/L and performed a Gene Ontology (GO) analysis. The results indicate that these genes are significantly enriched in ER-stress, inflammation, and beta cell function processes. We have now included this data as **Figure S12**.

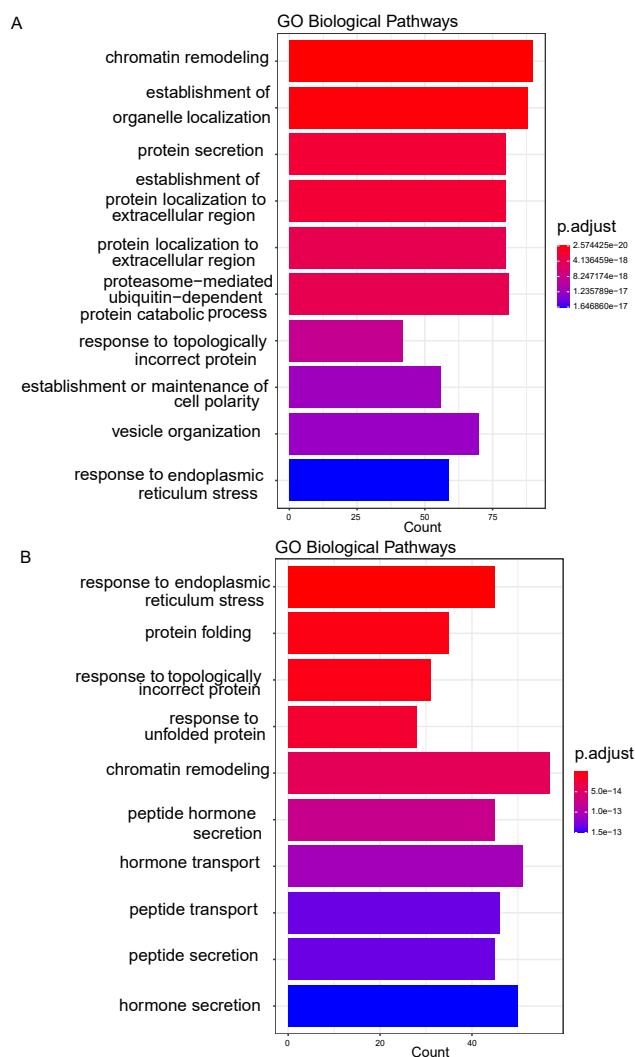


Figure S12. Gene ontology for genes in Figure 2K (A) and 2L(B) showing enrichment in distinct enriched categories related to ER-stress, inflammation, and beta cell function.

How many TF motifs (beside FoxA2, FOS, IRF1, NFKB2 and other listed candidates and those shown in Fig. 3 B/C) are enriched in genes which exhibit enhancer activity in beta-hi and beta-low?

In Figure 3B, we identified the enrichment of 76 transcription factor (TF) motifs, and in Figure 3C, 113 TF motifs are enriched. We now provide the list of TF motifs as Supplementary Tables 4.

I did not find NRC1 in Fig. 3F/G.

We apologize for this error. It should be Nr1h4 (FXR). We have revised the text accordingly.

Please flip Fig. 3H and 3I or change the description in the text (lines 223-228).

We have revised the text to reference the correct panels.

Please provide a number and full list of concordant and discordant genes shown in Fig. 4A.

In Group 1, we identified 182 concordant genes, while in Group 2 and Group 3, we observed 95 and 607 discordant genes, respectively. We now included the number in the revised manuscript and provided the full list as Supplementary Table 6.

In the gene score map of group 3 (Fig. 4F) only Dusp10 and Kcnmb2 but not Evt1 (as described in the results line 259 and in the legend line 1171) are shown.

We apologize for the error. The gene we showed in Fig 4F is Lrp11, not Evt1. The text has been corrected.

Is the expression of HAT, HDAC, HMT and/or KDM affected in beta cells which express discordant genes?

The expression of HAT/HDAC, or HMT/KDM responsible for H3K27ac and H3K4me1 remain at similar level in beta-hi and beta-low cells (Figure B).

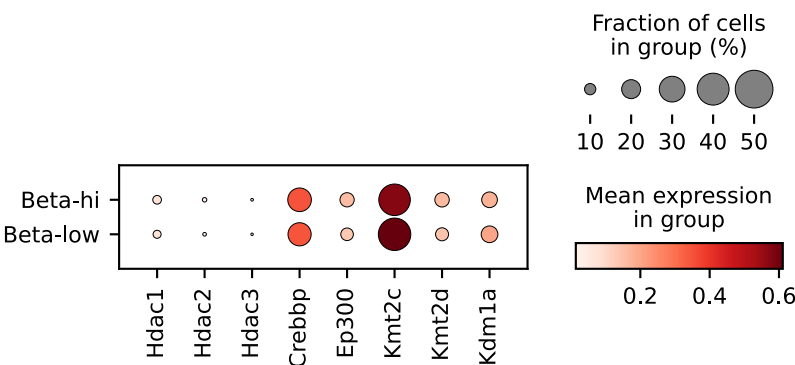


Figure B. Expression of HATs, HDACs, HMTs, and KDMs in beta-hi and beta-low cells.

As H3K27ac also marks active promoters, I am wondering, if the authors took this also into consideration. Thus, isn't it in theory possible that at least some of the discordant genes can be explained by this aspect?

We thank the reviewer for raising this significant point. We used ArchR to calculate the correlation between the gene expression and the local H3K4me1/H3K27ac level. To accommodate the nature of single-cell data, ArchR defines the “genescore” (in our case this means the H3K4me1/H3K27ac score for a certain gene) by considering H3K4me1/H3K27ac signals that fall into a default window of +/- 100kb of the gene region with a weighted normalization of distance, and then calculates the correlation between the “genescore” and RNA reads at the pseudobulk level. In other words, the H3K27ac/H3K4me1 score for any certain genes is the cumulative (and weighted) reads of multiple peaks surrounding the gene. In this case, the contribution from single active promoter reads (H3K27ac+/H3K4me1-) may be limited. To further evaluate the overall contribution of promoter to the signals, we analyzed the distribution of H3K27ac and H3K4me1 peaks in our datasets across gene regions and found that H3K27ac peaks in promoter (+/- 200bp of TSS based on the ENCODE project definition, <https://www.encodeproject.org/glossary/>) constitute only a small fraction (1.18%) of the total reads in the gene region. This suggests that the influence of H3K27ac+/H3K4me1- active promoters on the discordance is limited. We have provided the additional discussion with **Figure S10** in the revised manuscript.

A

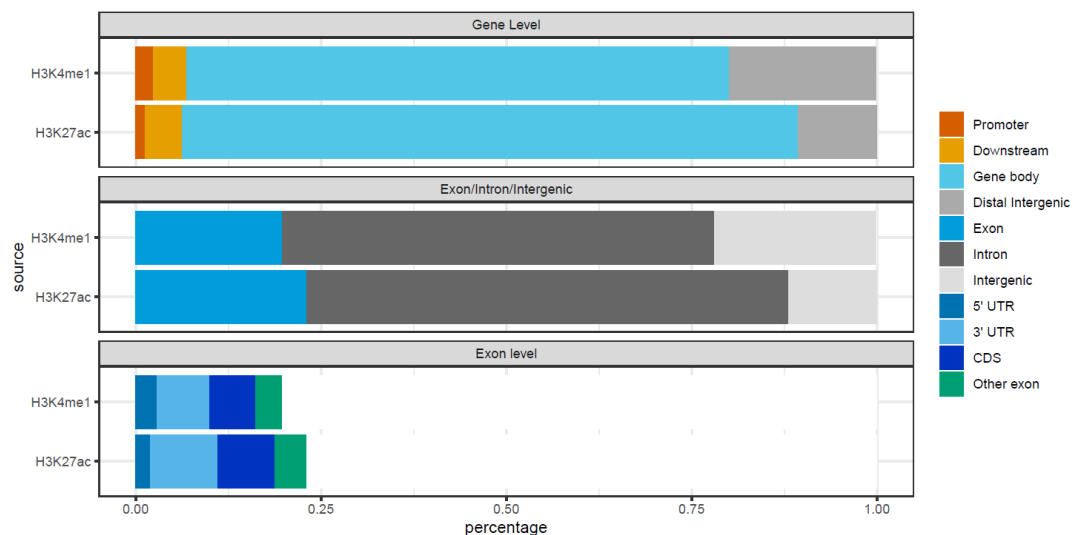


Figure S10. Stacked bar plot showing the distribution of H3K4me1 and H3K27ac peaks in different genomic regions. Promoters are defined as genomic regions encompassing 200 bp upstream and downstream of the TSS. Intragenic regions contain 5' UTR, exons, introns, and 3' UTR. Downstream means 2000 bp downstream of the transcription end site. Distal intergenic regions are genomic regions except downstream and promoter regions.

Are other examples of variable concordance between H3K27ac and H3K4me1 described in the literature?

Yes. At the bulk level, a few recent papers have documented that both H3K27ac and H3K4me1 can directly and individually contribute to gene activation. For example, a recent paper from UCSD showed that in differentiation from ESCs to neural progenitors, a subset of

developmental genes is directly controlled by H3K4me1-mediated enhancer-promoter contact (Molecular Cell 2024, PMID 38513661). On the other hand, Kang, et. al. showed that forced induction of H3K27ac enhanced eRNA transcription without altering H3K4me1 levels at the β -globin LCR HSs, highlighting the distinct roles H3K4me1 and H3K27ac played in nucleosome eviction and eRNA transcription, respectively, at enhancers (FASEB J 2021, PMID: 34309923). Therefore, while a majority of gene activation correlates with concurrent changes of H3K4me1 and H3K27ac enhancers, even at the bulk level, there are distinct requirements of H3K4me1 and H3K27ac to gene expression at specific loci. We have included these citations in the discussion of the revised manuscript.

Although the recently published bulk datasets point to a similar direction, we believe what is described in our manuscript provides a significant novel perspective. In contrast to published bulk data, we quantified the changes of association between H3K4me1/H3K27ac and RNA at the single-cell level. Importantly, we showed that the discordance may contribute to the **heterogeneous** gene expression, rather than the “all-or-none” activation in the development setting. Therefore, the discordance described here has a distinct biological implication for pathological progression.

For the results shown in Fig. 3 H-J and 4D it is necessary to confirm the expected effects on histone modifications.

Western blotting of H3K27ac in MIN6 cells treated with SAHA, GNE-781, or C646 are now provided as Figure S9E.

In the UMAP plot of Fig. 5A it is very difficult to distinguish between the two time points 8 and 16 weeks. I recommend to change the color of HFD 16 weeks.

We have revised the color pattern of **Fig. 5A** (see below) to improve the distinction between 8 and 16 weeks, in the UMAP plot.

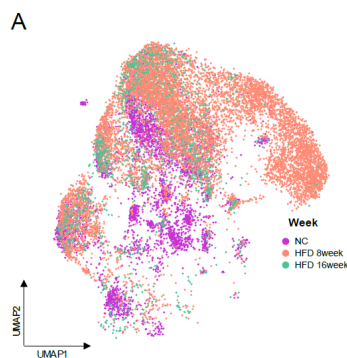


Figure 5 (A). The integrated H3K4me1-RNA UMAP of β cells at NC (0 weeks) and HFD (8 and 16 weeks) shows a distinct pattern of NC β cells.

In respect to the ligands of non-beta cells (Fig. 6A) I recommend to provide information on the cells which show the differential ligand expression.

The tissue specificity of the ligands is now provided as **Figure S14**.

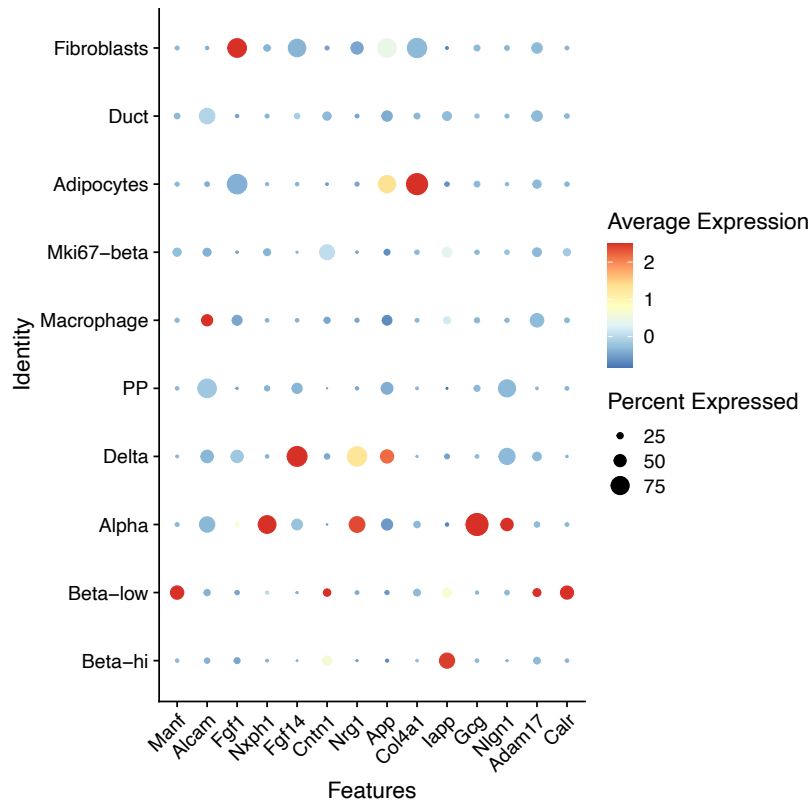


Figure S14. Expression of ligands (Figure 6A) in different cell populations.

Were the results shown in Fig. 6 F reproduced or is this just one experiment? I recommend to include a quantification of at least 3 independent experiments.

Quantification of replicates is now provided a part of **Figure 6I**.

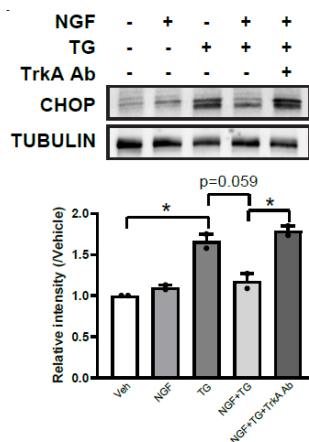


Figure 6 (I) Western blotting (top) and relative band intensity (bottom) showing CHOP (*Ddit3*) protein levels in TG-stressed MIN6 cells treated with or without NGF.

The authors stated “the potential function of NGF in islets has not been characterized.” This is not totally correct because in a review (PMID: 36768281) one chapter refers to “NGF in Pancreatic Beta Cells” and two other papers describe its role on insulin secretion (PMID: 27424144; PMID: 27825441). Maybe the authors highlight the new role of NGF on suppressing ER stress in islet cells.

We thank the reviewer for the suggestion. We have included the citations and a brief discussion of the novelty of our study in the revised manuscript.

It would be interesting to test the plasma concentration of NGF and Manf in NC and HFD fed mice as well as in Akita mice.

The NGF levels in pancreatic tissue from both lean and HFD mice are now included in the revised manuscript (**Figure 6 E**).

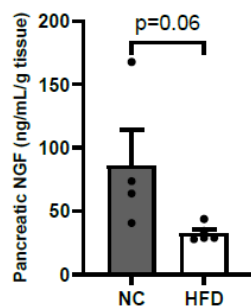


Figure 6 (E) Pancreatic NGF content in mice (B6) fed with normal chow (NC) and HFD for 16 weeks (n = 4-5).

The concentration of Manf and NGF for treatment of Min6 cells is not mentioned in the legend and for the treatment of Akita mice 1 mg/kg NGF appears quite high. I recommend to perform a concentration-response curve in Min6 cells.

We determined the concentration based on multiple previous publications that used recombinant NGF for in vitro treatment (MIN6 or INS1 (rat beta cells)), or in vivo administration^{20,24-26}. We have included the citations in the revised manuscript.

In the legends of Fig. 3J, Fig. 4G and Fig. 6 E an n = 2-6 is specified; it is not appropriate to perform statistics with n = 2.

We have repeated the experiments and increased the number of biological replicates.

Minor points:

The authors should avoid the term “lean and obese islets” and rather use “islets of lean and obese mice”.

We have changed the term to “islets from lean and obese mice” as the reviewer suggested.

Please provide the full name of TrkA (Tropomyosin receptor kinase A) in the abstract.

We have revised the abstract to include the full name of TrkA.

Line 127: Please correct HFD-induced

The text has been corrected.

Line 328: Please correct regulates to regulate

Corrected accordingly.

Line 351: Please correct ameliorates

Corrected it accordingly.

Lines 1116 and 1137: Please correct high

We have corrected it.

Line 1195, please correct FOXA2 to FoxA2.

We have revised it.

Please adapt the font sizes in the graphs, in some cases (e.g. the legend of Fig. 1 K is much too small)

We have enlarged the legends of Fig. 1G and Fig. 1K.

Please use a consistent spelling for beta cells, either as a Greek letter or beta, but not b cells (as in lines 1172 and 1197) also in the figures.

The texts have been corrected.

Please use a consistent spelling for beta-high or beta-low (in the text it is a mixture of both versions)

We have revised the manuscript accordingly.

Reviewer #3 (Remarks to the Author):

What are the noteworthy results?:

The authors demonstrate the relationships between enhancer states, gene expression, and over-nutrition induced metabolic dysregulation. By using a data-driven the -omics approach they identify known biological relationships and are able to identify a putative regulator of ER stress (and by extension beta-cell dysfunction) which has a significant impact on relevant traits.

Will the work be of significance to the field and related fields?:

I believe this work is certainly of significance. It is not revolutionary in that it's findings are well supported in our understanding of these phenomena. That is not to say that these results are

not novel, but to say that they conform to expectations. They certainly demonstrate (and I believe well) how these tools can be leveraged to get to mechanistic candidates which can then be investigated thoroughly.

Does the work support the conclusions and claims, or is additional evidence needed?:

Their chief claims as I understood them are:

- 1. There are cell type specific dietary response expression profiles in pancreatic islets. Such that they observe genes differentially expressed between beta-cell states.*
- 2. These beta-cell states are present in both chow and HFD contexts, but there is a shift in the ratio of states. Further these states are associated with expression differences.*
- 3. Pro-transcription histone modification profiles re-capitulate the same cell clusters and show a shift in enhancer states matching the observed shift in expression between beta cell states.*
- 4. They are able to combine histone marks and expression data to generate gene regulatory networks associated with the cell state dynamics.*
- 5. The roles H3k27ac and H3K4me1 marks are not entirely concordant, as one would expect from a complex system.*
- 6. Global perturbation of histone marks further supports a subset of the computationally identified GRNs.*
- 7. Using expression profiles of all the cell types in this micro-environment, they are able to identify NGF as a potential cell-cell cross talk signal that ablates part of the effects of over-nutrition in this model.*

We thank the reviewer for summarizing the strength of our manuscript. We have provided a point-to-point responses below.

The link between NGF and the enhancer work is not really discussed well. It feels to some extent like two projects mashed together. I feel that is a weakness in the delivery. I would like to hear more direct discussion as to whether for instance NGF is defining the enhancer landscape. Or is it an independent mechanism? I feel it should be addressed either way. In an ideal setting I would like to see matching multi-ome data generated from animals treated with NGF on the respective diets to see whether the putative GRNs respond as expected.

While I believe this would very much strengthen and unify the study, I do not believe such steps are needed to support their claims as written. They don't seem to make the claim that they are actually mechanistically linked explicitly. If they cannot be linked, then that should be explicitly stated as a limitation.

We thank the reviewer for raising this important point. NGF is discovered from our cell-cell communication screen using RNA data. Therefore, there is no directly connection between the NGF signaling and H3K4me1/H3K27ac changes. However, it is possible that NGF has a role in mediating the RNA and/or chromatin heterogeneity in beta cells. We have included an additional discussion section for the possible mechanisms.

Are there any flaws in the data analysis, interpretation and conclusions?:

I find no obvious flaws in their analysis or logic. I do question some of the statistical assumptions for example correlation coefficients as pearson's correlation coefficients make assumptions about the underlying distributions of the correlated variables. That they are normally distributed and do not have substructure, I hesitate to believe that those assumptions hold here. That said, I do not believe that such potential false assumptions rise to the level that

they would ultimately change the claims as the size of the effects seem substantial. I would however like a direct description of how multiple-tests corrections were performed.

For Figure S1A-B, we used deepTools' plotCorrelation function to analyze the data, which calculates the Spearman correlation coefficients of read counts. We apologize for the typo in Fig. S1 legends. This has been corrected in the revised figure legends.

For the multiple tests corrections in Figures S4 B-D, where the adjusted p-values are displayed on the y-axis, we utilized the Seurat FindMarkers function to identify differentially expressed genes between male and female mice. By default, Seurat conducts differential expression testing using the non-parametric Wilcoxon rank sum test. The adjusted p-values (p_val_adj) are calculated using the Bonferroni correction, which adjusts for all genes in the dataset.

Do these prohibit publication or require revision?

No I do not believe they do, but I would want to hear more about multiple tests corrections.

For multiple testing corrections, we employed the Bonferroni correction provided by default in Seurat.

Is the methodology sound? Does the work meet the expected standards in your field?

Yes and yes. I believe this study is an effective demonstration of the strengths of these methodologies.

We thank the reviewer for pointing out the strength of our methodology.

Is there enough detail provided in the methods for the work to be reproduced?

No, please expand on the analysis methods. Was there parameter tuning? For the sake of transparency, all code should be hosted on github or something like it. Without specific parameters, re-analysis would be impossible.

Code used for processing mouse Paired-tag data sets is now available at https://github.com/256wangliu/Multiomics_diabetes

Other:

Why were no repressive marks considered?

The major focus of this manuscript is the heterogeneity of enhancers. Therefore, we limited the scope to the two representative enhancer marks. We agree with the reviewer that the potential heterogeneity of repressive marks is a highly interesting topic for future studies.

Please clarify in the text more explicitly which mouse lines were used for which experiments.

We have revised the text to indicate specific mouse lines.

References for response letter

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7. Chiou, J., *et al.* Single-cell chromatin accessibility identifies pancreatic islet cell type- and state-specific regulatory programs of diabetes risk. *Nat Genet* **53**, 455-466 (2021).
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13. Li, J., *et al.* Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. *EMBO reports* **17**, 178-187 (2016).
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Response to reviewers

Reviewer #1 (Remarks to the Author):

The authors have done a good job addressing this reviewer's comments. In particular, replication of key experiments in primary mouse HFD islets, use of human islet datasets, as well as better validation of NGF-TrkA, all strengthen the conclusions. One minor comment remains:

The new data on TrkA protein expression in pancreas sections is not terribly convincing, and given the challenges detecting RTKs as well as the unreliability of antibodies, this should be deleted (unless the authors can provide the necessary controls e.g. KO validation). Provision of sorted alpha cell, beta cell and delta cell transcript data is fine to support the authors' arguments.

Response: We thank the reviewer for raising this important point. We have removed the immunostaining data. As suggested by the reviewer, the RNA-seq data from sorted beta cells is provided as Figure S15.