nature portfolio

Peer Review File

Pancreatic islet β-cell subtypes are derived from biochemically-distinct and nutritionally-regulated islet progenitors

Corresponding Author: Dr Guoqiang Gu

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 this manuscript, Brown et al. lineage traced the Myt1+Neurog3+ (M+N+) cells to early postnatal and later in adults. The authors found that beta cell descendants from M+N+ lineage are more proliferative, better in function, and more viable in pseudoislet culture compared with the Myt1-Neurog3+ (M-N+) population. And these differences are controlled by stable differences between these two populations genetically and epigenetically. Overall, this study represents an in-depth look on the molecular determination of beta cell subpopulation heterogeneity. However, I have some questions/concerns in data presentation and interpretation.

1. The survival of beta cells from M+N+ or M-N+ lineage was only quantified in vitro with pseudoislet culture. Does this reflect in vivo differences? Maybe the authors can label islets with cleaved caspase3 or other apoptotic markers to further substantiate the viability argument.
2. Only Figure 7 functional annotation is labelled as -log10 (adjusted p value) and all the other figures for all the functional annotations appear to plot -log10 (p value). If this is the case, these figures need to be updated to plot -log10 (adjusted p value). Furthermore, a lot of pathway annotations do not look significant. The authors need to state their significant threshold in the method.
3. It is unclear how did the authors choose the genes for functional validation. Furthermore, the methods they used for validating, including mouse model, CRISPR, and calcium imaging, are not consistent. The way the authors presented the results raise concerns on research rigor: perhaps those genes or methods not shown is because they did not work? More detailed characterizations and consistent experiments are needed. Specific questions need to be addressed include: (a) How are the candidate genes expressed in beta cells? (b) Why the authors choose to create a triple heterozygous mouse? Do Myt1, Myt1l, and St8 compensates each other so that single heterozygous does not show phenotype? If so, are they known to interact with each other? (c) Why only SyT7 was knocked down with CRISPR but not the other genes Syt genes? (d) Does the calcium differences really because of the differences in Cacna gene expression? If so, which one? Genetic modulation for individual Cacna gene combined with calcium influx is needed.
4. Figure 5E. What is the functional significance of the two DMRs? Perhaps only one of them is functional or they are bound by different factors. Overlapping with other epigenetic tracks might bring more insights.
5. Similar question as above point 4 in Figure 6J. Are the different DMRs between P2 and P60 on the same gene associated with differential motifs?
6. The argument that the epigenetic states of beta cell subtypes is stable is not supported by data shown. As the authors shown in Figure 6H, only a non-significant fraction of DMRs is stable. Also in Figure 6A, P60 and P2 do not clusters based on their DMRs. The fact that there are significant overlaps between DMR-associated genes does not provide evidence for the stability of epigenetics states because this comparison is confounded by the similarities of transcriptional states between P2 and P60.
Minor points:
1. Figure 1A. It is hard to see which cells are proliferating. Perhaps the arrows could be shown in all panels to facilitate cell registration.
2. Figures 1F-1H, only one p value is shown per figure but there are three different stimulation conditions. What is the comparison groups?
3. The authors need to clarify in the legends the images as well the FACS sorting where DAPI is used to live/dead marker

rather than nuclei markers.
4. I appreciate the authors show individual samples expression in the heatmaps of Figures 2D, 2E and 4A. However, the way the columns are organized made it hard to appreciate the differences between tdT+ and tdT- conditions. I suggest the heatmaps to be rearranged so that tdT+ (and tdT-) conditions from all four samples are placed side-by-side.
5. Line 188 and 190. Figure reference is flipped.

Reviewer #2

(Remarks to the Author)
In the present study, Brown et al use a combination of lineage-tracing, functional analysis, scRNA-seq and methylation analysis to show the existence of stable beta cell subtypes that contribute to beta cell mass and insulin release. Many studies have shown the existence of beta cell states based upon transcriptomic or functional analysis, however, the molecular underpinnings for their (in)stability has been more difficult to pin down. As such, the present studies are novel, and provide a useful resource for the field. There are however a number of issues with interpretation and/or discussion that need to be addressed.

• The rationale for the studies was not entirely clear. The authors mention that the majority of beta cell heterogeneity studies consider beta cell subtypes to be unstable. However, this depends on the exact definition of stability. For example, functional beta cell states were shown to be stable over time, ER-stressed cell states showed pseudotrajectories that imply dynamic switching, and Ins-High and Ins-Low cells undergo transitions. It would be helpful if the authors can clarify what they mean by stability and also whether or not this considers the dynamic aspect of beta cell states/function. My overall feeling is that the authors are trying to show that beta cell states are stable from birth through adulthood, which is an important topic to establish/address. However, the comparison with other studies is a bit confusing, sometimes distracting and not always accurate.
• There are a lot of different lineage-tracing models employed. While the results are clearly explained, some more hand-holding is needed for uniformed readers in terms of schematics etc.
• Islet size could relate to changes in cell size between eYFP+ versus eYFP- cells, especially since the former are loaded to the brim with GE-fluorophore.
• The mitochondrial potential and ATP/ADP assessments are crude, and don't really add to the studies, since they are not taken forward. Ideally, these assays should be done dynamically at low and high glucose, also backed up by better measures such as islet respirometry in eYFP+ cell-enriched pseudoislets.
• Along these lines, fluorophore-only controls are needed. A lot of functional analyses compare fluorophore versus fluorophore-negative populations. Since beta cell states have been defined by ER stress, and adding fluorophore to an already significant protein-load could exacerbate this, Ca2+ and insulin could all be conceivably altered in eYPF/TdT+ populations. There are dozens of published and anecdotal reports that fluorophore-positive cells do not proliferate or function normally.
• The authors conclude, based upon existence of eYFP+ cells in adults, as well as function of eYFP+ cell-enriched pseudoislets, that islet progenitors give rise to stable beta cell subtypes. Since eYFP is Cre-dependent, any progeny will be indelibly marked, but this doesn't exclude that a cell has subsequently adopted another cell state in the adult (e.g. ER stressed). Some more consideration is needed here, since the data in Fig. 1 don't entirely support the authors' claim.
• The changes in Ca2+ channel subunit expression fit with previous data on heterogeneity, but some more discussion of mouse versus human is needed given the different contributions of the various ion channels to mV and Ca2+ influx.
• Path-clamp determination of Ca2+ currents would be nice, but is not essential so long as the authors are confident that their cells cannot be distinguished by Ca2+ release from intracellular stores.
• Microscopy reporting is poor, meaning that the data will be difficult to replicate. What are the emission/excitation wavelengths? Objectives NAs etc.?
• Novel CRISPR mouse lines need full targeting and sequencing details to be provided.
• Graphs in Figure 4I are the wrong way round- control should be first?
• Quantification of glucose-stimulated Ca2+ flux is needed (AUC, amplitude).
• The manuscript title is a bit of a mouthful. Maybe consider: Pancreatic beta cell subtypes are derived from biochemically-distinct and nutritionally-regulated islet progenitors.

Reviewer #3

(Remarks to the Author)
Guqiang Gu and colleagues describe analysis of beta cell heterogeneity – the hottest topic in beta cell biology. They present provocative evidence that
1. functionally-distinct stable beta cell sub-types exist;
2. are determined early during development (derive from progenitors that are positive or negative for the transcription factor myt1);
3. have an epigenetic identity (distinct DNA methylation); and
4. that their proportions are affected by maternal nurtition in mice and modulated in human diabetes (being either cause or effet of disease).

The heart of the analysis is an elegant lineage tracing analysis using the split cre technology, whereby cells are indelibly marked based on co-expression of two markers (in this case, ngn3 and myt1). This allowed to authors to carry out a detailed comparison between labeled and unlabeled population of beta cells, including live sorting and functional analysis. They

found that the subsets of these embryonically-determined beta cells are distinct transciptionally and epigenetically, and even used CRISPR to assess the importance of few candidate genes.

Overall this is an exciting study that drives the field forward, both technologically and conceptually. Particularly notable achievements are the first-ever lineage tracing of beta cell sub-types, the demonstration of differential DNA methylation indicating stability of sub-types, and the exciting link between maternal nutrition and beta cell composition. I support publication. My comments and suggestions below are relatively minor.

1. A weak spot in the mechanistic framework proposed by the authors is the assignment of DNA methylation variation to differential expression levels of the de-novo methylase DNMT3a. This enzyme does not work in isolation – it is directed to its targets by sequence-specific factors, that are more likely to play a role in differential methylation during differentiation than DNMT3a levels. I suggest to tone down the argument that DNMT3a expression level is critical for beta cell sub-types, unless there is suportive evidence e.g. a relevant phenotype in mice heterozygous for DNMT3a or in people with a mutation in this gene (TBRS).

2. When pseudo-islets are generated from fluorescently-labeled progenitors, are the proportions of beta cells in tdT+ and tdT- islets similar? This is important to exclude functional differences resulting simply from different psecudo-islet composition. The use of ins2-apple in experiments that involved only pure beta cells is a good control, but does not exclude the need for assessment of islet cell composition.

3. I am missing images of the adult pancreas from the lineage-traced mice (stained for insulin and the tracer). Are labeled and unlabeled islets evenly distributed in the pancreas? Do they equally contribute to islets, or are there islets made of just one beta cell sub-type?

4. Sub-title in line 304 (DNA methylomes in postnatal cells are highly dynamic but…) – how do you know that methylation is dynamic, vs alterted proportion of cell types? I suggest to stick to findings.

5. The authors say in both results and end of discussion that ngn3 is re-activated in adult beta cells under metabolic stress, therefore their lineage tracing system is not workable in such conditions. The reference they cite (Talchai 2012) is known to be problematic. If you have evidence for reactivation of ngn3 in beta cells under these conditions, please show it (who are these cells?). If not, please show lineage tracing findings and functional analysis and/or discuss inconsistencies.

6. The sub-field of beta cell heterogeneity is notorious for the complete isolation of each paper from all others. I encourage the authors to search for evidence that their newly discovered sub-types are related to any previousyly reported populations, beyond the passing reference to previous papers that hit upon similar heterogeneity markers.

7. I wouldn't use terms such as "better function" for a specific sub-type of beta cells – not sure what is better. Better use objective criteria such as more glucose responsive.

8. The bottom line of the abstract is too far-fetched – in my opinion the summary of this study is a new, important insight into beta cell developmental dynamics. The potential translational implications are too speculative. Do the authors really envision global methylation changes in fetuses as a way to modify risk of diabetes?

Reviewer #4

(Remarks to the Author)
The manuscript by Brown et al., focuses on the developmental epigenetic mechanisms of postnatal beta cell heterogeneity. The authors build upon their prior work which established that the Myt1+ Neurog3+(MN+) progenitors display higher Dnmt1 expression, such that DNA methylation of cell fate-determining enhancers specifies unique endocrine-cell programs, biasing MN+ progenitors toward a beta cell fate. In the present manuscript, they utilize lineage tracing, single-cell transcriptomics, and whole genome DNA methylation profiling to show that differential DNA methylation within the embryonic endocrine progenitor pool also directs the formation of two functionally distinct beta cell subtypes. They also show that maternal overnutrition reduces the proportion of endocrine progenitors of the better functioning beta cell subtype by deregulating Dnmt3a, uncovering a potential epigenetic mechanism to explain the increased diabetes risk in the context of maternal overnutrition. The manuscript addresses a very important and timely topic, given the recent interest in the epigenetic control of beta cell heterogeneity in development and diabetes. However, the current version of this manuscript has several key issues that need to be addressed:

Major issues:

1. The authors use a split Cre model to label the M+N+ progenitors and determine the unique properties of beta cells that arise from M+N+ vs M-N+ progenitors. However, no data is presented regarding the efficiency of this Cre model.
2. In Figure 1, the authors suggest that the M-N+ have poorer survival in vitro. However, it could simply be an outcome of potentially poorer homotypic adhesion. Nonetheless, cell death needs to be quantified. It would be imperative to measure the cell-death (using TUNEL or CC3) and turnover rates of the two populations in vivo. Neonatal beta cells have high rates

of cell turnover – does one population have higher turnover (the net balance of replication and cell-death). This also begs the question: what is the relative abundance of the M+N+ and M-N+ beta cells in neonatal and adult (or immature and mature) pancreas.
3. Figure 1 M, N- data needs untreated controls.
4. The data in Figure 2 suggest that the M+N+ population has higher Dnmt3a transcript levels and the text in Figure 5 suggests this to be the case for both neonatal and adult M+N+ beta cells. It is important to determine whether these differences translate to differences in protein levels and whether they display a high-low distribution or an all or none distribution? A histological assessment is required to comment on this. Does the distribution change between pre- and post-weaning stages. Furthermore, the authors' prior work showed that the M+N+ have higher Dnmt1 levels. Given that maintenance methylation is critical in replicating cells, and that M+N+ cells are more replicative in nature, is Dnmt1 uniquely distributed in M+N+ beta cells compared to the M+N-?
5. The authors state that the adult beta cell subtypes maintain their differential gene expression. Do the M+N+ beta cells have different "maturation" trajectory compared to the M+N- cells? Out of the 919 DEGs between the two subtypes at P2, only 193 are retained in P60, and most of these are upregulated in the M+N+ subset. It may be equally important to comment on the DEG categories that are not retained between P2 vs P60 and will likely inform the differential maturation and molecular competencies of the two populations.
6. Are of any of the DEGs that appear to modulate GSIS and replication etc putative targets of Myt TFs.
7. The authors posit that various beta cell factors with motifs at the P2 DMRs are expressed at the same levels in the two subtypes but have different transcriptional activity due to differential methylation. The authors appear to conflate differences in transcript levels with protein levels and do not provide any evidence for this hypothesis. This may be a more appropriate statement for the discussion section.
8. The authors suggest that the very mild effect of maternal HFD on reducing M+N+ subtype "likely reflect the reduced beta cell subtype with better function, which is mitigated by compensatory proliferation of these cells". This is plausible given the high replicative capacity of these cells but needs to be demonstrated and quantified experimentally.
9. Do any of the M-N+ beta cell ever switch to a Myt+ fate? In other words, do any of the Tdt- (or eyfp-) cells co-stain for Myt1 (or other Myt TFs) in the juvenile or adult pancreas.
10. The section on classifying human beta cell subtypes based on murine DEGs seems to be simply tacked on. Data on human pancreatic sections is required to substantiate this. Are the authors able to identify a cell surface marker or TF that robustly distinguishes murine M+N+ beta cells from M-N+ beta cells and then use that as a benchmark for defining sub-populations.
11. Along these lines, a broader question is whether the M+N+ beta cells bear any similarity to the recently discovered H3K27me3 high CD24+ population.

Other issues:
1. Statistical measures and comparisons are not clearly noted throughout.
2. Several statements throughout the manuscript appear to overstate/over-interpret the data and need to be toned down and instead presented in the "Discussion" section.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, the authors satisfactorily addressed most of my questions. I have only one remaining issue: In Figure 4d, it is still unclear what is the functional significance of the 2 DMRs in the Arx. Furthermore, the Arx binding site is not enriched in the DMR motif analysis. Some experimental follow up should be performed.

Minor:
1. Figure 4a, the panel label should be MNYA. Furthermore, the sorted population appears to be mislabeled. Shouldn't it be eYFP-Apple+ vs eYFP+Apple+?
2. Line 303-305, "Note that mutant males displayed glucose intolerance earlier than females; results that usually occur when islet function was partially compromised". Some citations are needed here.

Reviewer #2

(Remarks to the Author)
The authors have done a good job of responding to the comments. A lot of ground is covered here, and the studies should be a valuable contribution to the field.

Reviewer #3

(Remarks to the Author)
The authors have addressed properly my concerns. I find the paper to be improved, and appropriate for publication.

Congratulations to the authors for an important study!

Please edit to fix multiple grammatical issues and typos, e.g. appearances of GISS (should be GSIS); line 45, should be “resulting in”; line 56, should be “each beta cell subset”. Line 70, should be "predisposing”; line 91, proliferation rate was (not were); title of Figure 5; title of Figure 6 (what is “index”?); and many more places.

Reviewer #4

(Remarks to the Author)
The revised manuscript by Brown and colleagues has significantly improved with the addition of new data and discussion and has addressed a large fraction of this reviewer’s concerns. Congratulations to the authors for their efforts. I especially commend them testing GSIS in both subtypes using PSIs from both MNT and MNYA (beta only PSIs) models. As all the reviews indicate, this is an excellent developmental biology study that examines in mechanisms underlying beta cell subtype allocation and maintenance.

I do have a few additional minor comments on this revision for the authors to consider:

1. The authors may consider to either delete the section on mitochondria as it does not add anything mechanistically or clearly state that mitochondrial activity does not play a role in the distinct functional behaviors of the two subtypes.
2. The data in humans still does not fully appear to confirm that these cells are the exact same subtype and ends up only detracting from the otherwise rigorous work presented in this study. The authors may consider removing this data and instead briefly discussing how to rigorously approach this question in humans.
3. Single cell data: It would be helpful to comment on whether there is underlying heterogeneity WITHIN the M+N+ and M-N+ derived beta cells, as the subtypes do not appear to segregate very clearly in the UMAP plots. It would also be helpful to integrate the scRNA-seq data in p2 and P60 and do a pseudotime and trajectory analysis to see how the trajectories of the two cell types change over the developmental timeline.
4. The data on Dnmt3a levels is not very strong. It is not clearly what the high/low levels would do. Was this done in a z-stack with MIP. As another reviewer indicated, Dnmt3a works in coordination with lineage specific TFs to guide cell fate choices.
5. The authors state “Similar to P2 gene expression, 566 of 1,867 p60 DEGs are associated with the P2 DMRs”. The following questions emerge:
• What’s the extent of conservation between these DEGs and the ones from P2 (919 DEGs of which ~300 have DMR associations). It seems there are more DEGs at P60.
• And do the associated DMRs for the conserved DEGs follow the same directionality.
6. The authors report very little overlap between DMRs at P2 and P60. “However, when we consider genes associated with P2 and P60 beta cell subtype DMRs, roughly one-third of the DMR associated genes overlaps (sic) between P2 and P60.” This suggests that there is a shift in DNA methylation dependent regulation from P2 and P60 for these genes. The directionality of “DMRs” and location of these DMRs vs gene expression directionality should be elaborated upon, as these findings will be very important in delineating how the methylation landscape of beta cell heterogeneity changes shape post-maturation.
7. The data show that both subtypes appear to undergo compensatory expansion in pups born to mothers on HFD but not highlighted in the text. This should be clearly noted in the text.
8. Minor point, multiple proof-reading errors.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed all my concerns. Congratulations!

Reviewer #4

(Remarks to the Author)
The authors have addressed all my comments in detail and the revision acknowledges all the caveats. This is overall a very insightful study that will add to the ongoing discourse on how beta cell fates and states emerge and evolve during development. This work will also provide valuable resources for the field. Many congratulations to the authors this work.

We thank all the reviewers. In this revised version, we have addressed all the critiques by adding more experimental data or by clarifying our presentation. The changes are listed below one-by-one. Note that we have extensively revised the manuscript to make space for the newly added data. We apologize for increasing the reviewing workload.

Reviewer #1 (Remarks to the Author):

Overall assessment: In this manuscript, Brown et al. lineage traced the Myt1+Neurog3+ (M+N+) cells to early postnatal and later in adults. The authors found that beta cell descendants from M+N+ lineage are more proliferative, better in function, and more viable in pseudoislet culture compared with the Myt1-Neurog3+ (M-N+) population. And these differences are controlled by stable differences between these two populations genetically and epigenetically. Overall, this study represents an in-depth look on the molecular determination of beta cell subpopulation heterogeneity. However, I have some questions/concerns in data presentation and interpretation.

Response: We thank you for the positive feedbacks.

Critique 1. The survival of beta cells from M+N+ or M-N+ lineage was only quantified in vitro with pseudoislet culture. Does this reflect in vivo differences? Maybe the authors can label islets with cleaved caspase3 or other apoptotic markers to further substantiate the viability argument.

Response: We have now replaced the in vitro data with in vivo data (revised Fig.1F-H), where we examined the apoptosis of the two beta cell subtypes from P4 – P7 mice. We did not examine apoptosis in adult mice, which has extremely rare beta-cell apoptosis, making it unfeasible for quantification. Also note that we have removed the in vitro cell death assays. As reviewer #2 (point 3) and reviewer #4 (point 2) correctly pointed out, the in vitro observations could simply reflect the different beta-cell adhesion between the beta-cell subtypes. This possibility will need new experimental designs, involving cell adhesion molecular manipulation, for clarification, beyond the scope of this current study.

Critique 2. Only Figure 7 functional annotation is labelled as -log10 (adjusted p value) and all the other figures for all the functional annotations appear to plot -log10 (p value). If this is the case, these figures need to be updated to plot -log10 (adjusted p value). Furthermore, a lot of pathway annotations do not look significant. The authors need to state their significant threshold in the method.

Response: We have now updated all the pathway analyses, presenting -log10 (adjusted p-values) in the revision. Our significance cutoff is adjusted $p<0.05$.

Critique 3. **(1)** It is unclear how did the authors choose the genes for functional validation. **(2)** Furthermore, the methods they used for validating, including mouse model, CRISPR, and calcium imaging, are not consistent. **(3)** The way the authors presented the results raise concerns on research rigor: perhaps those genes or methods not shown is because they did not work? More detailed characterizations and consistent experiments are

needed. **(4) Specific questions** need to be addressed include: **(a)** How are the candidate genes expressed in beta cells? **(b)** Why the authors choose to create a triple heterozygous mouse? Do Myt1, Myt1l, and St8 compensates each other so that single heterozygous does not show phenotype? If so, are they known to interact with each other? **(c)** Why only SyT7 was knocked down with CRISPR but not the other genes Syt genes? **(d)** Does the calcium differences really because of the differences in Cacna gene expression? If so, which one? Genetic modulation for individual Cacna gene combined with calcium influx is needed.

Response: These questions are now explained in the revised manuscript. Briefly:
Sub-point 1: We choose the Myt TFs because Myt1 is the marker used to divide the endocrine progenitors into lineage-marked sub-types. Findings could reveal whether differential Myt1 activation in embryonic Ngn3+ progenitors is linked to postnatal function.

The Ca2+ channels and Syt7 are chosen via four criteria:
1) Differentially expressed in the P2 and P60 beta-cell subtypes (193 genes identified).
2) Associated with DMRs between the beta-cell subtypes (69 genes left).
3) Implicated to regulate secretion in literature (six genes identified: Cacna1a, Gck, Gnao1, Irs2, Rab11a, and Syt7).
4) Narrowing-down: Gck is excluded, because we did not detect ATP/ADP ratio differences between our beta-cell subtypes (revised Fig. S2). Gnao1 heterozygosity has no detectable impact on insulin secretion (related to PMC3279551, Gu, Unpub. data). The haploinsufficiency of IRS2 in beta cells was reported (PMCI1716196), while Rab11a activity is not required for GSIS (10.1111/j.1365-2443.2009.01285.x.). These left us with Cacna1a and Syt7 to pursue.
Note that we did not venture into examining new players without known functions, because our goal is to test if some of the DEGs contribute to the different subtype functions, instead of new gene discovery.

Sub-point 2: We agree that it would be ideal to create heterozygous mouse models for all the genes/gene family to be manipulated. Yet this is unrealistic given all the mouse lines/strains needed for this study, especially given that we have now added manipulation of three Ca2+ channel subunits (see below).

Sub-point 3: We have not studied any other genes (except *Gnao1*, Gu, Unpub. data).

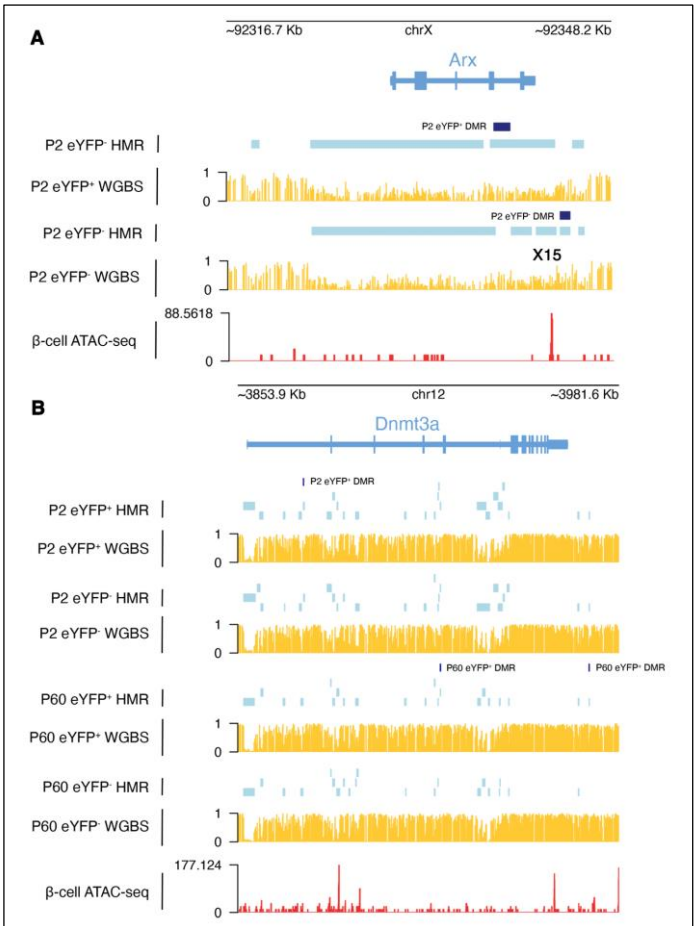
Sub-point 4, specific questions:
(a) How are the candidate genes expressed in beta cells?
Answer: If this reviewer means why these candidates are chosen, please see above. If this meant to ask the expression patterns of these genes, the data were included in now revised Tables S2, S4, revised Figs. 2E, 6A.
(b) Why the authors choose to create a triple heterozygous mouse? Do Myt1, Myt1l, and St8 compensates each other so that single heterozygous does not show phenotype? If so, are they known to interact with each other?
Answer: Pancreatic specific *Myt1*- or *St18*-single mutant islets have compromised insulin secretion (PMC2141686 and In preparation). *Myt1l*-single mutants have no detectable

beta-cell defects (not shown). There are complicated interactions amongst these paralogs: inactivating *Myt1* upregulates the transcripts of both *Myt1l* and *St18* (PMC2141686), but protein of only St18 (Unpub. data). Inactivating *Myt1l* upregulates *Myt1* transcripts and proteins (Unpub. data). Co-inactivating any two of the three will upregulate the third transcriptionally and translationally. Thus, we inactivated one allele for all three here to avoid any unforeseen complications. These complicated interactions were not included in the manuscript, which are distracting to our main message.

(c) Why only SyT7 was knocked down with CRISPR but not the other Syt genes?
Answer: Please see above for the selection of Syt7. Note that we considered other candidates as well, with Syt1, Syt5, Syt7, Syt11, and Syt13, all differentially expressed in P60 beta-cell subtypes. Syt1, Syt5, and Syt7 are Ca2+ sensors. Yet Syt7 is expressed at >5 X higher levels than Syt1 while Syt5 null mice have not detectable defects (Jax strain 008413). We did not pick Syt11 or Syt13 for the current studies because they are not Ca^{2+} sensitive. This reasoning is included in the revised manuscript.

(d) Does the calcium differences really because of the differences in Cacna gene expression? If so, which one? Genetic modulation for individual Cacna gene combined with calcium influx is needed.
Answer: We have now included gene knockdown using primary islet cells in the revised manuscript. We show that reducing the levels of *Cacna1a* and *Cacna2d1*, but not *Cana1c*, reduces glucose-induced Ca2+ influx (revised Fig. 6m-o).

Critique 4. Figure 5E. What is the functional significance of the two DMRs? Perhaps only one of them is functional or they are bound by different factors. Overlapping with other epigenetic tracks might bring more insights.



Review-Fig. 1. DMR-overlapping with chromatin-opening states using ATAC data published in PMC10108528. The DMRs were noted with black bars, followed by description.

Response: We do not know the answer. We tried to overlap the DMR patterns with published chromatin studies of beta cells by others (e.g., PMC10108528). However, read sequencing coverage at these sites is poor, so it is difficult to call accessible regions (also referred to as peaks) (see Review-Fig. 1A above). Putative TF binding sites (taken from genome-wide predicted binding

sites for TF binding profiles in the JASPAR CORE collection available through the UCSC genome browser) within these DMRs (i.e., DNA sequences covering all the related CpG dinucleotides) would suggest they have differential functions. E.g., the P2eYFP+ DMR contains TF motifs recognized by Rfx6, Hnf4a, and NeuroD1, while P2eYFP- DMR by Nkx2.2 (revised Fig. 4d). Rfx6, Hnf4a, NeuroD1, and Nkx2.2 are all required for beta-cell production and/or function. These points are included in the manuscript. Note that the DMR track overlapping with TFs can be viewed with https://genome.ucsc.edu/s/aganve/gu_betaCells.

Critique 5. Similar question as above point 4 in Figure 6J. Are the different DMRs between P2 and P60 on the same gene associated with differential motifs?

Response: Please refer to answers above - we have not found chromatin accessibility data that are dense enough to make conclusions in the DMRs (see Review-Fig. 1B). However, these DMRs have different and overlapping motifs: the DMR in P2 cells contain motifs for Insm1, Nkx2.2, and Rfx6. The P60eYFP+ DMRs contains motifs of Insm1 and Neurod1 (revised Fig. 5i) or Insm1, Neurod, and Rfx6 (revised Fig. 5i), all important regulators of beta cells. A complete list of overlapping motifs is provided in Table S8

Critique 6. The argument that the epigenetic states of beta cell subtypes is stable is not supported by data shown. As the authors shown in Figure 6H, only a non-significant fraction of DMRs is stable. Also in Figure 6A, P60 and P2 do not clusters based on their DMRs. The fact that there are significant overlaps between DMR-associated genes does not provide evidence for the stability of epigenetics states because this comparison is confounded by the similarities of transcriptional states between P2 and P60.

Response: We agree with you and our apology for not making our point clearer. What we tried to conclude is that our beta-cell subtypes remain different in the epigenetic states even as beta cells age. The implication is that these beta-cell subtypes have a mechanism to continue to build their own characteristic DNA methylation patterns even as their older DMRs are diminished and new ones built. To avoid confusion, we have removed our statement about the stability of the beta-cell DNA methylome.

Note that we retained the data about “significant overlaps between DMR-associated genes at P2 and P60” in the manuscript. These findings have implication on the mechanism(s) that prevents the DNA methylomes of the two beta-cell subtypes from converging, which is likely related to the recruitment of DNMTs and/or TET enzymes to gene regulatory elements for DNA methylation and demethylation, respectively. In other words, the features that recruit DNMTs or Tets during perinatal stages could be preserved during the maturation process, so that the P60 DMRs are preferably built in loci that were affected in P2.

Also note that we agree that the “comparison is confounded by the similarities of transcriptional states between P2 and P60”. We have done two analyses based on this concern. The first is the presented data in revised Figure 5H, where we analyzed all genome wide gene overlaps for DEGs and nearest neighbor genes to DMRs

(hypergeometric analysis) without considering gene expression. This produced a 2.2-fold enrichment at *p*=1.11E-295. The second is to only focus on the genes that are expressed in P2 and P60 beta cells, which substantially reduces the population size for hypergeometric analysis (i.e., increased stringency). The enrichment score is 2.7-fold at *p*=6.48E-287, suggesting that the detected overlap between the DMR-associated genes is not merely a result of studying similar cells, but DMR-establishment in P60 is somehow related to that in P2.

Minor points:

1. Figure 1A. It is hard to see which cells are proliferating. Perhaps the arrows could be shown in all panels to facilitate cell registration.

Response: Arrows are added and we have used enlarged panels.

2. Figures 1F-1H, only one p value is shown per figure but there are three different stimulation conditions. What is the comparison groups?

Response: We only provided p-values for those <0.05. The comparisons are now indicated by lines that cover the compared samples.

3. The authors need to clarify in the legends the images as well the FACS sorting where DAPI is used to live/dead marker rather than nuclei markers.

Response: Clarified.

4. I appreciate the authors show individual samples expression in the heatmaps of Figures 2D, 2E and 4A. However, the way the columns are organized made it hard to appreciate the differences between tdT+ and tdT- conditions. I suggest the heatmaps to be rearranged so that tdT+ (and tdT-) conditions from all four samples are placed side-by-side.

Response: Presented as suggested.

5. Line 188 and 190. Figure reference is flipped.

Response: Corrected.

Reviewer #2 (Remarks to the Author):

Overall assessment: In the present study, Brown et al use a combination of lineage-tracing, functional analysis, scRNA-seq and methylation analysis to show the existence of stable beta cell subtypes that contribute to beta cell mass and insulin release. Many studies have shown the existence of beta cell states based upon transcriptomic or functional analysis, however, the molecular underpinnings for their (in)stability has been

more difficult to pin down. As such, the present studies are novel, and provide a useful resource for the field. There are however a number of issues with interpretation and/or discussion that need to be addressed.

Response: We thank this reviewer for the positive feedback.

Critique 1• The rationale for the studies was not entirely clear. The authors mention that the majority of beta cell heterogeneity studies consider beta cell subtypes to be unstable. However, this depends on the exact definition of stability. For example, functional beta cell states were shown to be stable over time, ER-stressed cell states showed pseudo-trajectories that imply dynamic switching, and Ins-High and Ins-Low cells undergo transitions. It would be helpful if the authors can clarify what they mean by stability and also whether or not this considers the dynamic aspect of beta cell states/function. My overall feeling is that the authors are trying to show that beta cell states are stable from birth through adulthood, which is an important topic to establish/address. However, the comparison with other studies is a bit confusing, sometimes distracting and not always accurate.

Response: The reviewer's feeling is correct: we refer "stability" as "beta cell states are stable from birth through adulthood". Specifically, our conclusion is that beta cells born with different gene expression and DNA methylation patterns can have different function/proliferation/viability at perinatal stages as well as in adulthood. In this sense, these beta-cell subtypes are "stable". Note that this conclusion only applies when we grossly divide the beta cells into two subtypes. However, we cannot conclude that potential "sub-subtypes" (sub-populations within each subtype, which appear to exist – see discussion) within these subtypes can maintain their characteristic function/genetic/epigenetic levels/patterns. In other words, beta cells within our subtypes can have dynamic gene expression, epigenetic modification, and function. But some restrictions prevent them from converging for at least 8 months in mice.

Critique 2• There are a lot of different lineage-tracing models employed. While the results are clearly explained, some more hand-holding is needed for uniformed readers in terms of schematics etc.

Response: In the revised manuscript, we have used the first paragraph in the result section to describe the mouse models that are used for lineage tracing. We also included schematics in revised Fig. 1, 4, 7 to briefly explain the experimental designs.

Critique 3• Islet size could relate to changes in cell size between eYFP+ versus eYFP- cells, especially since the former are loaded to the brim with GE-fluorophore.

Response: We thank this reviewer for raising this very important issue, because it is related to whether we can use pseudo-islet size as a parameter to assay cell survival. This is also related to the critical issues raised by reviewer #1 (main point 1, suggesting the need for in vivo data) and reviewer #4 (main point 2, the possibility that pseudo-islet size and cell viability can be complicated by the poorer cell adhesion between eYFP-

cells). To address all the critiques, we have now replaced the in vitro data with in vivo cell death assays (revised Fig. 1f-j). Thus, this issue no longer applies (see response to reviewer #1 critique 1, reviewer #4 critique 2).

Critique 4• The mitochondrial potential and ATP/ADP assessments are crude, and don't really add to the studies, since they are not taken forward. Ideally, these assays should be done dynamically at low and high glucose, also backed up by better measures such as islet respirometry in eYFP+ cell-enriched pseudo-islets.

Response: We have added ADP/ATP ratios at G2.8 and G20. We also added oxygen consumption rate using newly isolated cells in seahorse assays (Fig. S2). We did not detect differences between the eYFP+ and eYFP- cells. Thus, if advised, we would be happy to remove these data from the manuscript.

Critique 5• Along these lines, fluorophore-only controls are needed. A lot of functional analyses compare fluorophore versus fluorophore-negative populations. Since beta cell states have been defined by ER stress, and adding fluorophore to an already significant protein-load could exacerbate this, Ca2+ and insulin could all be conceivably altered in eYPF/TdT+ populations. There are dozens of published and anecdotal reports that fluorophore-positive cells do not proliferate or function normally.

Response: We now add data to show that activating tdT or eYFP in adult beta cells (using Ins1Cre) did not appreciably affect insulin secretion (Fig. S1m, n).

Critique 6• The authors conclude, based upon existence of eYFP+ cells in adults, as well as function of eYFP+ cell-enriched pseudoislets, that islet progenitors give rise to stable beta cell subtypes. Since eYFP is Cre-dependent, any progeny will be indelibly marked, but this doesn't exclude that a cell has subsequently adopted another cell state in the adult (e.g. ER stressed). Some more consideration is needed here, since the data in Fig. 1 don't entirely support the authors' claim.

Response: We agree. All our conclusions are based on dividing the entire beta-cell population into two subtypes. Thus, if each subtype contains multiple sub-subtypes (which appears to be the case, see revised discussion) and these sub-subtypes drift between different states, our studies will not be able to discover them. This is made clear in the revised discussion.

Critique 7• The changes in Ca2+ channel subunit expression fit with previous data on heterogeneity, but some more discussion of mouse versus human is needed given the different contributions of the various ion channels to mV and Ca2+ influx.

Response: We have added discussions on the potential contributions of difference channels on membrane potentials and Ca2+ influx. In addition, we have performed Ca2+ channel subunit knockdown in primary mouse beta cells to show that reducing the levels of Cacna1a and Cacna2d1, but not Cacna1c, reduce the glucose-induced Ca2+ influx (revised Fig. 6m-o and Fig. S6f, g).

Critique 8• Path-clamp determination of Ca2+ currents would be nice, but is not essential so long as the authors are confident that their cells cannot be distinguished by Ca2+ release from intracellular stores.

Response: Please see response to above point 7. With knocking down of Cacna1a or Cacn2d1 showing reduced Ca2+ influx, we believe that the Ca2+ influx from extracellular source is the main cause of the tdT+ beta cells having higher Ca2+ influx.

Critique 9• Microscopy reporting is poor, meaning that the data will be difficult to replicate. What are the emission/excitation wavelengths? Objectives NAs etc.?

Response: Details added in materials and methods, for both Ca2+ recording and confocal imaging.

Critique 10• Novel CRISPR mouse lines need full targeting and sequencing details to be provided.
Response: More details are provided in M&M – note that did not used targeted mutation. Instead, it is a transgene that expresses two gRNAs for recruiting dCas9-KRAB for gene expression repression, based on transposon-aided insertion.

Critique 11• Graphs in Figure 4I are the wrong way round- control should be first?

Response: Thank you. Corrected.

Critique 12• Quantification of glucose-stimulated Ca2+ flux is needed (AUC, amplitude).

Response: Added in all relevant panels (revised Fig. 6l, n and Fig. S6g).

Critique 13• The manuscript title is a bit of a mouthful. Maybe consider: Pancreatic beta cell subtypes are derived from biochemically-distinct and nutritionally-regulated islet progenitors.

Response: The recommended title was used, with a slight modification.

Reviewer #3 (Remarks to the Author):

Overall assessment: Guqiang Gu and colleagues describe analysis of beta cell heterogeneity – the hottest topic in beta cell biology. They present provocative evidence that
1. functionally-distinct stable beta cell sub-types exist;2. are determined early during development (derive from progenitors that are positive or negative for the transcription factor myt1);
3. have an epigenetic identity (distinct DNA methylation); and
4. that their proportions are affected by maternal nurtition in mice and modulated in human diabetes (being either cause or effet of disease).

The heart of the analysis is an elegant lineage tracing analysis using the split cre technology, whereby cells are indelibly marked based on co-expression of two markers (in this case, ngn3 and myt1). This allowed to authors to carry out a detailed comparison between labeled and unlabeled population of beta cells, including live sorting and functional analysis. They found that the subsets of these embryonically-determined beta cells are distinct transciptionally and epigenetically, and even used CRISPR to assess the importance of few candidate genes.

Overall this is an exciting study that drives the field forward, both technologically and conceptually. Particularly notable achievements are the first-ever lineage tracing of beta cell sub-types, the demonstration of differential DNA methylation indicating stability of sub-types, and the exciting link between maternal nutrition and beta cell composition. I support publication. My comments and suggestions below are relatively minor.

Response: We thank you for positive comments.

Critique 1. A weak spot in the mechanistic framework proposed by the authors is the assignment of DNA methylation variation to differential expression levels of the de-novo methylase DNMT3a. This enzyme does not work in isolation – it is directed to its targets by sequence-specific factors, that are more likely to play a role in differential methylation during differentiation than DNMT3a levels. I suggest to tone down the argument that DNMT3a expression level is critical for beta cell sub-types, unless there is suportive evidence e.g. a relevant phenotype in mice heterozygous for DNMT3a or in people with a mutation in this gene (TBRS).

Response: We agree with this reviewer. It is not clear how reducing Dnmt3a dosage impacts beta cells (in both mouse models and some human diseases associated with Dnmt3a mutations). We have toned-down our interpretation. We have also added discussion on the potential pioneer factors that may help with the creation of unequal DNA methylation in early stages.

Critique 2. When pseudo-islets are generated from fluorescently-labeled progenitors, are the proportions of beta cells in tdT+ and tdT- islets similar? This is important to exclude functional differences resulting simply from different psecudo-islet composition. The use of ins2-apple in experiments that involved only pure beta cells is a good control, but does not exclude the need for assessment of islet cell composition.

Response: These data are added in revised Fig. S1d-h. Briefly, there is slight difference between the % of alpha cells in the tdT+ and tdT- pseudoislets (11% vs. 14%, respectively). There are no differences in the % of delta cells in these pseudo-islets. Note that alpha cells are proven enhancer of GSIS. Thus, these findings, together with the results from pure beta cell-derived pseudo-islets, support our conclusion that the higher GSIS in tdT+ pseudo-islets does not result from their different cellular composition.

Critique 3. I am missing images of the adult pancreas from the lineage-traced mice (stained for insulin and the tracer). Are labeled and unlabeled islets evenly distributed in

the pancreas? Do they equally contribute to islets, or are there islets made of just one beta cell sub-type?

Response: We have now added a few images showing labeled islets at different ages, with only native fluorescence (revised Fig. S1a, b) or native fluorescence and insulin IF staining (revised Fig. S1c). Briefly, when looked as pancreatic sections, we observed relatively even distribution of islet labeling (not shown because low magnification images cannot provide necessary details for this point). All recognizable islets are labeled, although the % of labeled cells varies some (ranging from ~25-80% depending on age). So far, amongst over 15,000 islets we have worked with (with more 20 cells each, for cell sorting in MNYI mice), we have not seen any with all beta cells labeled or unlabeled. There is no apparent 3-D organization patterns in the labeled or unlabeled cells.

Critique 4. Sub-title in line 304 (DNA methylomes in postnatal β cells are highly dynamic but…) – how do you know that methylation is dynamic, vs altered proportion of cell types? I suggest to stick to findings.

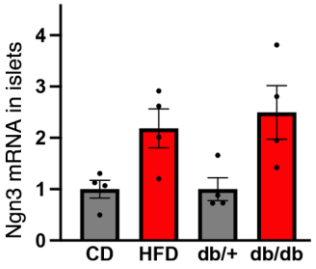
Response: We thank the reviewer for this suggestion. We cannot exclude the possibility that the DNA methylome changes between P2 and P60 arise from fast proliferation of certain beta-cell sub-populations. We have changed the title to "DNA methylomes in postnatal β cells are extensively changed from newly-born to adult stages but adult β-cell subtypes maintain DMRs that associate with their DEGs".

However, we retained the discussion on dynamic DNA methylome changes for the following reason: per Fig. 5A, unsupervised k-means clustering of total HMRs called across P2 and P60 beta-cell subpopulations (n=87792) revealed four clusters of HMRs whose methylation levels alluded to developmental stage-specific methylation changes (C1, C2, C5 and C6). Together, the total HMR count from these 4 clusters is 39278. The other two clusters, C3 and C4, do not exhibit changes in methylation fraction from P2 to P60. These results suggest P2 beta cells have altered DNA methylation patterns by P60. Given that no single beta-cell subpopulation has been found to account for most of the beta-cell expansion and beta cells on average can proliferate ~2 time from birth to adult (calculated based on clonal size, PMC2761107), it appears difficult to account for such extensive DNA methylation changes by expansion of certain beta cells.

Critique 5. The authors say in both results and end of discussion that ngn3 is re-activated in adult beta cells under metabolic stress, therefore their lineage tracing system is not workable in such conditions. The reference they cite (Talchai 2012) is known to be problematic. If you have evidence for reactivation of ngn3 in beta cells under these conditions, please show it (who are these cells?). If not, please show lineage tracing findings and functional analysis and/or discuss inconsistencies.

Response: We apologize for overly simplifying the situation. We have recently reported that certain mutations, when activating stress responses, induced Ngn3 activation in beta cells (PubMed: 39058602), like that reported by Talchai 2012. In addition, we have added a few citations to support Ngn3 reactivation in adult beta cells under metabolic stress,

including (a) (PMC4143961), (b) (PMC4067979), (c) (PMC7346095), and (d) (PMC4058003). These citations showed that hyperglycemia (a, b), immune/cellular stress (c), or inactivation of key beta-cell maintenance factors (d) reactivate Ngn3 in beta cells. Lastly, our RT-PCR analysis showed Ngn3 reactivation in islets of prediabetic DB/DB mice and HFD-fed WT mice (see Review-Fig. 2 below), consistent with a strong trend of increased Ngn3 expression in islets of diabetic Db/Db mice reported by others (p=0.096, n=3. PMID: 29542001).



Review-Fig. 2. Ngn3 induction in adult islets fed with HFD for 6 weeks (3-months of age) or in *db/db* mutants (~5-wks of age).

In the revision, we "toned down" this critique on our method, because at present, we do not know whether the induced Ngn3 levels are high enough to restore Cre activity for Cre reporter activation, which will take a substantial effort to unravel, beyond the scope of the manuscript.

Critique 6. The sub-field of beta cell heterogeneity is notorious for the complete isolation of each paper from all others. I encourage the authors to search for evidence that their newly discovered sub-types are related to any previously reported populations, beyond the passing reference to previous papers that hit upon similar heterogeneity markers.

Response: We have compared our results to two recently published heterogeneity studies (PMC10449536 and PMCID10160009). The PMC10449536 study showed that a CD63-Hi cell subtype have higher glucose response. This also contains sc-RNAseq data, allowing direct beta-cell subpopulation comparison. We found that our tdT+ subtype (with higher levels of GSIS) significantly overlaps with their CD63Hi sub-population, with our tdT+ subtype reduced in their HFD-treated samples (revised Fig. 9a-c).

The PMCID10160009 study shows that a beta-cell subtype has higher glucose response and higher H3K27me3 levels (called betaHi). We directly compared the overlaps between our and their DEGs using hypergeometric analysis (because of this Dror study did not provide sc-RNAseq). Amongst the 28 DEGs (with pAdj<0.05) from Dror, 12 overlaps with our DEGs at P60, a 4.7-fold enrichment (p=2.66E-6). In addition, we found our tdT+ cells have higher levels of K3K27me3 levels, suggesting that our tdT+ subtype significantly enriches in the reported betaHi (Fig. 9d-e).

Critique 7. I wouldn't use terms such as "better function" for a specific sub-type of beta cells – not sure what is better. Better use objective criteria such as more glucose responsive.

Response: We have avoided using "better" and instead use "more glucose responsive" or "higher-GSIS" cells.

Critique 8. The bottom line of the abstract is too far-fetched – in my opinion the summary of this study is a new, important insight into beta cell developmental dynamics. The

potential translational implications are too speculative. Do the authors really envision global methylation changes in fetuses as a way to modify risk of diabetes?

Response: Thank you. We modified the abstract and moved related topics to the discussion.

Note that we do believe that DNA methylation can be manipulated to modify the risk of diabetes. It is well documented that maternal diet can influence the risks of T2D, which is accompanied by DNA methylation changes in some cells/loci (e.g., PMC4663595, PMID:26917970, and 10.1038/ncomms4746). Thus, it is possible that manipulation of diet, such as those containing methyl-donors, can be explored for reversing the detrimental effects of diabetes-predisposing maternal diet (e.g., PMCID5297118, PMCID35629277, and PMID:37977406). Indeed, it is currently impossible for tissue/cell specific DNA methylation manipulation in human subjects to think about disease prevention/therapy. But with the advance in targeted molecule delivery, we envision this can become a reality in the future.

Reviewer #4 (Remarks to the Author):

Overall assessment: The manuscript by Brown et al., focuses on the developmental epigenetic mechanisms of postnatal beta cell heterogeneity. The authors build upon their prior work which established that the Myt1+ Neurog3+(MN+) progenitors display higher Dnmt1 expression, such that DNA methylation of cell fate-determining enhancers specifies unique endocrine-cell programs, biasing MN+ progenitors toward a beta cell fate. In the present manuscript, they utilize lineage tracing, single-cell transcriptomics, and whole genome DNA methylation profiling to show that differential DNA methylation within the embryonic endocrine progenitor pool also directs the formation of two functionally distinct beta cell subtypes. They also show that maternal overnutrition reduces the proportion of endocrine progenitors of the better functioning beta cell subtype by deregulating Dnmt3a, uncovering a potential epigenetic mechanism to explain the increased diabetes risk in the context of maternal overnutrition. The manuscript addresses a very important and timely topic, given the recent interest in the epigenetic control of beta cell heterogeneity in development and diabetes. However, the current version of this manuscript has several key issues that need to be addressed:

Response: We thank the reviewer for the overall positive comments.

Major issues:

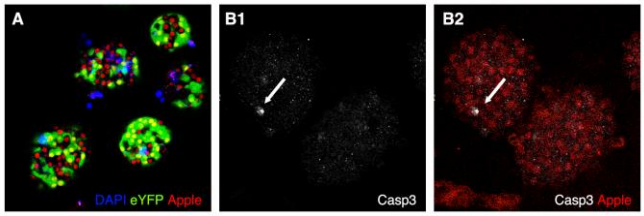
Critique 1. The authors use a split Cre model to label the M+N+ progenitors and determine the unique properties of beta cells that arise from M+N+ vs M-N+ progenitors. However, no data is presented regarding the efficiency of this Cre model.

Response: Unfortunately, we do not have Cre activity assays to directly address this. Overall, we identified ~ 25-40% Ngn3+ cells (age dependent) that are M+N+ during pancreatogenesis (PMC6327977). Corresponding to this, ~35-50% (depending on which

Cre reporter to use) of all islet cells are labeled with Cre reporters at birth (PMC6327977). In this sense, we could conclude that the split Cre-approach is quite efficient. The caveat here is that the proportion of M+N+ cells depends on the affinity of Myt1 and/or Ngn3 antibodies. In our original studies to establish this approach, we showed that when expressed at similar levels of Cre, the (nCre+cCre) can reconstitute ~1/3 of the activity of Cre (PMC2095822).

Critique 2. (1) In Figure 1, the authors suggest that the M-N+ have poorer survival in vitro. However, it could simply be an outcome of potentially poorer homotypic adhesion. Nonetheless, cell death needs to be quantified. It would be imperative to measure the cell-death (using TUNEL or CC3) and turnover rates of the two populations in vivo. Neonatal beta cells have high rates of cell turnover – does one population have higher turnover (the net balance of replication and cell-death). **(2)** This also begs the question: what is the relative abundance of the M+N+ and M-N+ beta cells in neonatal and adult (or immature and mature) pancreas.

Response: sub-point 1: We fully agree. Indeed, the cell death could result from poorer homotypic cell-cell adhesion. From our sc-RNAeq analysis, we have detected lower junctional protein expression in the M-N+ progenitor-derived beta cells (see revised Fig. 2c and 3c). It is known that cell-cell interaction is essential for cell survival. Thus, we have now replaced these in vitro analysis using in vivo data (revised Fig.1f-j. Please also see replies to reviewer #1 point 1 and reviewer #2 point 3). We did not purse whether the reduced homotypic cell-cell interaction is a reason for cell death in vitro because years of effort is needed, likely involving adhesion molecule manipulation.



Review-Fig. 3. Comparing DAPI filling-based and Cleaved Caspase 3 (Casp3)-based *in vitro* assay of beta-cell death. (A) DAPI filling in PSI. (B) Casp3-IF (average projection of Z-stacked islet images) using the same batch of PSIs as in A. Note the rareness of Casp3+ cells in B.

Note that we performed IF staining with cleaved Caspase 3 (Casp3) on the stressed pseudo-islets. The proportion of Casp3+ cells is less than ~1/10th of all the dead cells (assayed via DAPI or trypan blue uptake, see Review-Fig. 3). At presents, we do not know whether other Casp3-independent cell death processes are involved or that the dead cells in vitro quickly lose Casp3. We cannot use TUNEL staining – many dead cells were removed at the protease treatment step in the TUNEL assay process. This was the reason why we only presented DAPI-uptake for the cell death assays in the original submission.

Sub-point 2: The proportions of tdT+ beta cells at newly born and adults are presented in revised Fig. 1e.

Critique 3. Figure 1 M, N- data needs untreated controls.

Response: See above response to critique 2. These data are no longer needed because we have replaced the in vitro data with in vivo assays.

Critique 4. (1) The data in Figure 2 suggest that the M+N+ population has higher Dnmt3a transcript levels and the text in Figure 5 suggests this to be the case for both neonatal and adult M+N+ beta cells. It is important to determine whether these differences translate to differences in protein levels and whether they display a high-low distribution or an all or none distribution? A histological assessment is required to comment on this. Does the distribution change between pre- and post-weaning stages. Furthermore, the authors' prior work showed that the M+N+ have higher Dnmt1 levels. **(2)** Given that maintenance methylation is critical in replicating cells, and that M+N+ cells are more replicative in nature, is Dnmt1 uniquely distributed in M+N+ beta cells compared to the M+N-?

Response: sub-point 1: The IF staining/quantification results of Dnmt3a antibodies is included in revised Fig. 2f, g and Fig. 3i, j. In both stages, we detected higher Dnmt3a protein levels in the tdT+ beta cells.

Sub-point 2: we did not detect differential transcription of Dnmt1 between the two beta-cell subtypes, which is surprising to us as well. It is likely that the proportion of replicating beta cells is low and a small Dnmt1 level difference is diluted amongst all cells for detection. Alternatively, Dnmt1 could be expressed in a saturating level in all cells, so that its higher expression in the dividing cells is not needed.

Critique 5. The authors state that the adult beta cell subtypes maintain their differential gene expression. **(1)** Do the M+N+ beta cells have different "maturation" trajectory compared to the M+N- cells? Out of the 919 DEGs between the two subtypes at P2, only 193 are retained in P60, and most of these are upregulated in the M+N+ subset. **(2)** It may be equally important to comment on the DEG categories that are not retained between P2 vs P60 and will likely inform the differential maturation and molecular competencies of the two populations.

Response: Sub-point 1: As the original Fig. 2 showed, the few representative factors related to maturation is not differentially expressed between the beta-cell subtypes at either P2 or P60.

Sub-point 2: We have examined the P2 DEG categories that are not retained at P60. Amongst those with lower expression in P2 tdT+ cells, only two terms are found, mitochondrial inner membrane and nucleotide binding. Those with higher expression are associate with ER, Golgi, cell projection, nucleotide binding, and metal binding. Our interpretation is that the P2 tdT+ cells are poorly equipped for mitochondrial function during perinatal stages; but they gained this property faster than the P2 tdT- cells. In contrast, the P2 tdT+ cells activate some ER/Golgi related genes faster than P2 tdT- cells; yet the expression of these gene were normalized by P60, with expression of other ER/Golgi-related genes activated more in the P60 tdT+ cells (which have enriched genes for ER/Golgi as well). Thus, it appears that both subtypes are geared toward functional maturation. Because this conclusion is already supported by the findings presented in

revised Fig. 2d. We did not present these findings in the revised manuscript, which is already very bulky.

Critique 6. Are of any of the DEGs that appear to modulate GSIS and replication etc putative targets of Myt TFs.

Response: We do not have a comprehensive list of transcriptional targets of the Myt TFs due to the lack of ChIP-grade antibodies.

In our earlier studies (Huang et al., 2020), we used ChIP-PCR to identify several direct Myt TF targets involved in stress response, Atf4, Hspa1a, Hspa1b, HspH1, and Dnajb1 (Huang et al., 2020). Amongst these genes, Atf4, Hsph1, and Dnajb1 are differentially expressed in the P60 beta cell subtypes. These genes regulate beta-cell stress response.

When we expand our analyses to all Myt-dependent genes, we found significant overlaps as well. 186 of 920 DEGs between the P2 beta-cell subtypes and 408 of the 1,868 P60 DEGs overlap with the 2,864 Myt-TF-regulated genes. These represent 2.5-fold (p=2.5E-131) or 2.7-fold (p=6.9E-80) enrichment, respectively. Importantly, several Myt TF-regulated gene that are also differentially expressed between the beta-cell subtypes at both P2 and P60 are known to directly impact insulin secretion, including: Pfkm (10.1016/j.bpj.2022.01.027), Dakp3 (PMC10385282), Exoc7 (PMID: 34588186), Stxbp5l (PMID: 25002582), Hmgn3 (PMCID: PMC2747976), Cacna1a (PMID: 35918471), Dnmt3a (PMID: 36607262), Irs2 (PMID: 14617753), Ppp1r15a (PMID: 34588186), Pcsk1n (PMID: 37858332), Rbp4 (PMID: 34786060), and Pyy (PMID: 37001706).

Despite the interesting nature of these findings, we think that including them will dilute the major conclusion of the manuscript. They are therefore excluded in the revision.

Critique 7. The authors posit that various beta cell factors with motifs at the P2 DMRs are expressed at the same levels in the two subtypes but have different transcriptional activity due to differential methylation. The authors appear to conflate differences in transcript levels with protein levels and do not provide any evidence for this hypothesis. This may be a more appropriate statement for the discussion section.

Response: We apologize for the confusion. What we presented is a hypothetical condition: if a motif is differentially methylated in two cells, it may have different chromatin opening states, resulting in differential gene expression even if the TFs that bind to the motif are produced at identical levels. We have clarified this point in the revised manuscript.

Critique 8. The authors suggest that the very mild effect of maternal HFD on reducing M+N+ subtype “likely reflect the reduced beta cell subtype with better function, which is mitigated by compensatory proliferation of these cells”. This is plausible given the high replicative capacity of these cells but needs to be demonstrated and quantified experimentally.

Response: To lessen the complication caused by beta-cell proliferation, we have used new data of P1 mice. There is a significant reduction in the proportions of tdT+ beta cells in the maternal HFD-exposed pups (Revised Fig. 7c-e), accompanied by increased beta-cell proliferation of both tdT+ and tdT- beta cells (Revised Fig. 7f).

Critique 9. Do any of the M-N+ beta cell ever switch to a Myt+ fate? In other words, do any of the Tdt- (or eyfp-) cells co-stain for Myt1 (or other Myt TFs) in the juvenile or adult pancreas.

Response: Our apologies for not explaining the design clearly: Myt TF is expressed in most, if not all, islet cells from embryo to death (PMC7278035). The reason why M-N+ progenitor-derived beta cells are not labeled is that when Myt TFs are turned on in this sub-lineage, Ngn3 expression is already turned off. Thus, tDT- (or eYFP-) islet cells will express Myt TFs at all stages.

Critique 10. The section on classifying human beta cell subtypes based on murine DEGs seems to be simply tacked on. Data on human pancreatic sections is required to substantiate this. Are the authors able to identify a cell surface marker or TF that robustly distinguishes murine M+N+ beta cells from M-N+ beta cells and then use that as a benchmark for defining sub-populations.

Response: We agree that these human beta-cell related data do not add to the mechanistic insights. But we believe that they highlight a key conserved aspect of beta-cell heterogeneity between mouse and human. We therefore kept these data. However, if this reviewer/editor deems these data unnecessary, we will remove them from the manuscript.

As requested, we have added IF staining of several of our identified markers using three batches of human donor islets (with same findings): we found the differential expression of cell surface markers CD9 and CD24 (already reported by others). We also demonstrated DNMT3a, MAFB, SYT7, and TPT1 (related to cell death) as robust markers that divide human beta cells into sub-populations. These data are included in revised Fig. 8a-f. Note that we tried but could not found IF-working antibodies for RBP4, CD47, or CD79.

11. Along these lines, a broader question is whether the M+N+ beta cells bear any similarity to the recently discovered H3K27me3 high CD24+ population.

Response: See response to reviewer #3, point 6. Briefly, our gene expression overlap analyses and H3K27me3 level comparison suggest that our tdT+ subtype is enriched in the reported CD24+ beta-cellHi subtype (revised Fig. 9).

Other issues:

1. Statistical measures and comparisons are not clearly noted throughout.

Response: The details was added into each figure legend and restated in materials and methods.

2. Several statements throughout the manuscript appear to overstate/over-interpret the data and need to be toned down and instead presented in the “Discussion” section.

Response: Some over-statements are now moved to the “discussion section”.

Overall response:

We thank all the reviewers for their efforts in re-reviewing and proposing ways to improve this manuscript. We have now added more results and revisions to address all raised points.

Reviewer #1 (Remarks to the Author):

Main critique: In the revised manuscript, the authors satisfactorily addressed most of my questions. I have only one remaining issue: In Figure 4d, it is still unclear what is the functional significance of the 2 DMRs in the Arx. Furthermore, the Arx binding site is not enriched in the DMR motif analysis. Some experimental follow up should be performed.

Response: Thank you for highlighting this point, which appears to arise from our ambiguous presentation. The goal of presenting these DMRs is to show that at P2, we could detect DMRs in Arx between the descendants of Myt1+Ngn3+ (M+N+) and Myt1-Ngn3+ (M-N+) progenitors. This is consistent with our report that the DNA methylation patterns near the *Arx* locus differ between these progenitors. The implication is that the differentiated islet cells can retain at least some DNA methylation patterns from their progenitors. We are not implying that these 2 DMRs play a role in the beta cells. After differentiation, *Arx* expression in beta cells becomes nearly undetectable (10.3389/fcell.2021.648791). Thus, Arx *per se* may not be involved in setting the heterogenous function of beta-cell subtypes or in binding to the DMRs between the beta-cell subtypes. We have now clarified this point in the re-revised manuscript.

In addition, we have added experiments to test whether the hypermethylation of these DMRs can impact *Arx* transcription using an alpha cell line (AlphaTC-6). We found that hypermethylation of both DMRs significantly represses *Arx* transcription, consistent with these regions having regulatory roles in *Arx*-expressing cells. These data are now included in Fig. S4f-i.

Minor:
Critique 1. Figure 4a, the panel label should be MNYA. Furthermore, the sorted population appears to be mislabeled. Shouldn't it be eYFP-Apple+ vs eYFP+Apple+?
Response: You are correct. We have fixed the mistakes.

Critique 2. Line 303-305, "Note that mutant males displayed glucose intolerance earlier than females; results that usually occur when islet function was partially compromised". Some citations are needed here.
Response: We have a couple of relevant references (with others removed to fit the maximal number of refs we can include, hoping not to remove any citations deemed essential by this reviewer).

Reviewer #2 (Remarks to the Author):
The authors have done a good job of responding to the comments. A lot of ground is covered here, and the studies should be a valuable contribution to the field.

Response: Thank you.

Reviewer #3 (Remarks to the Author):

The authors have addressed properly my concerns. I find the paper to be improved, and appropriate for publication. Congratulations to the authors for an important study!

Please edit to fix multiple grammatical issues and typos, e.g. appearances of GISS (should be GSIS); line 45, should be “resulting in”; line 56, should be “each beta cell subset”. Line 70, should be "predisposing”; line 91, proliferation rate was (not were); title of Figure 5; title of Figure 6 (what is “index”?); and many more places.

Response: Thank you. We have used professional help to fix these problems.

Reviewer #4 (Remarks to the Author):

The revised manuscript by Brown and colleagues has significantly improved with the addition of new data and discussion and has addressed a large fraction of this reviewer’s concerns. Congratulations to the authors for their efforts. I especially commend them testing GSIS in both subtypes using PSIs from both MNT and MNYA (beta only PSIs) models. As all the reviews indicate, this is an excellent developmental biology study that examines the mechanisms underlying beta cell subtype allocation and maintenance.

Response: Thank you for your comments. We highly appreciate your insightful query of the DEG/methylation data.

I do have a few additional minor comments on this revision for the authors to consider:

Critique 1. The authors may consider to either delete the section on mitochondria as it does not add anything mechanistically or clearly state that mitochondrial activity does not play a role in the distinct functional behaviors of the two subtypes.

Response: Considering the large amount of information in this manuscript, we have deleted the mitochondria-related studies.

Critique 2. The data in humans still does not fully appear to confirm that these cells are the same subtype and only detract from the otherwise rigorous work presented in this study. The authors may consider removing this data and instead briefly discussing how to rigorously approach this question in humans.

Response: We agree we cannot conclude that our identified beta-cell subtypes in mice are the same as those in human samples (we apologize for misusing the word “equivalent” in the manuscript, if that has misled this reviewer). We tried to convey that several heterogeneity markers we found in mouse beta cells allowed us to divide normal human beta cells into subsets, whose proportions were altered in T2D. We could also detect the differential expression of several of these markers in individual human beta cells. We have made this point clear in the re-revised manuscript.

We considered removing the human beta cell-related data. However, other reviewers may disagree because this will likely lessen the significance because of the high interest in human beta-cell heterogeneity in the field. Thus, we have kept these data in the manuscript while discussing that our marker-set may not identify the same beta-cell subtypes in human samples. Yet we are open to removing these human cell-related data if all the reviewers and the editor agree.

Critique 3. Single-cell data: It would be helpful to comment on whether there is underlying heterogeneity within the M+N+ and M-N+ derived beta cells, as the subtypes do not appear to segregate very clearly in the UMAP plots. It would also be helpful to integrate the scRNA-seq data in

p2 and P60 and do a pseudo-time and trajectory analysis to see how the trajectories of the two cell types change over the developmental timeline.

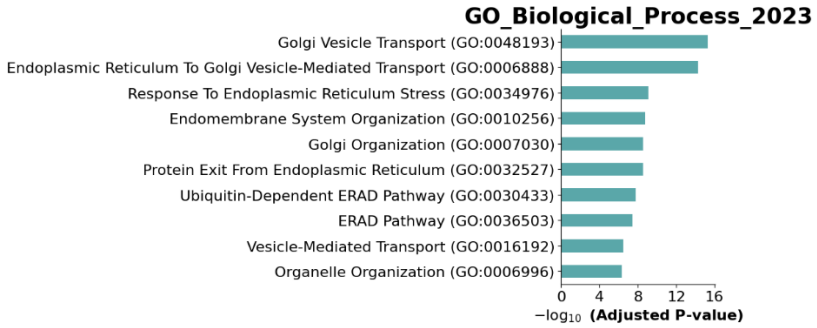
Response:

Heterogeneity

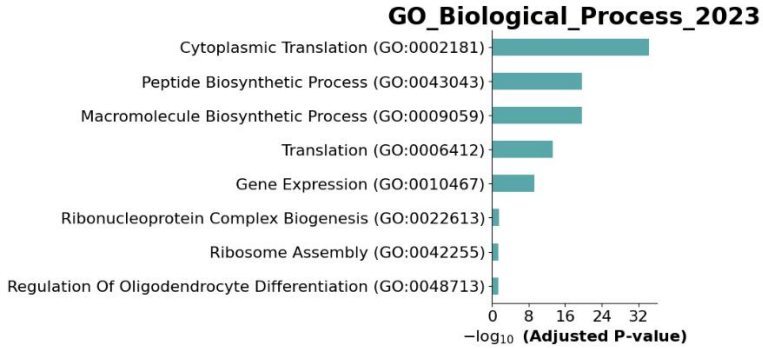
As we explained in the discussion, we believe that there is underlying heterogeneity within the M+N+ or M-N+ cell-derived beta-cell subtypes. This could explain why our cell subsets do not entirely overlap with those described by others (re-revised Fig. 9). We have made this point more explicit in the revised discussion.

Additionally, we have examined gene programs that define extreme states of M+N+ or M-N+ beta cells, as by the following gene expression enrichment analysis:

One state is enriched for transport and organization.



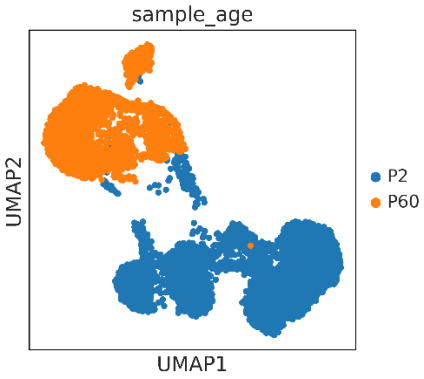
Another state is enriched for peptide and macromolecule synthesis genes.



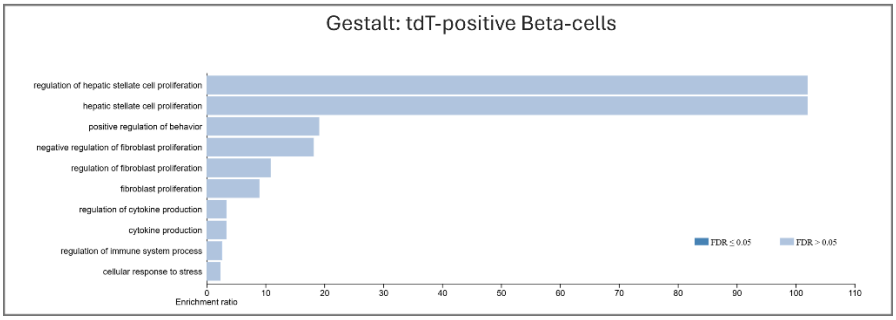
Both M+N+ or M-N+ beta cells possess the same heterogeneity in these gene programs. Thus, we conclude that beta cells are heterogeneous on different functional axes, and M+N+ or M-N+ states constitute just one of those axes.

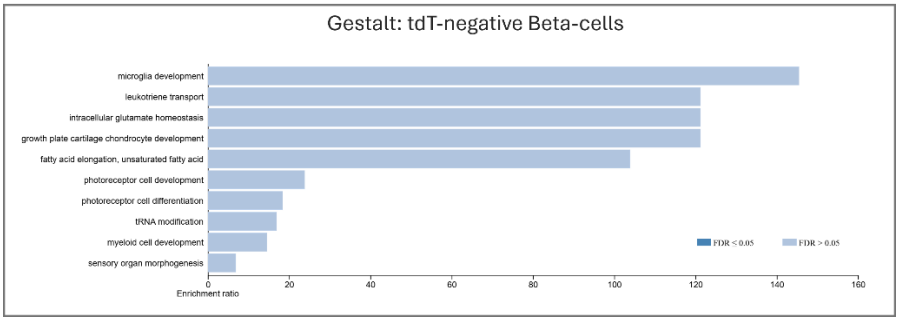
Development

As the reviewer helpfully suggested, we attempted to integrate P2 and P60 datasets to perform trajectory analysis. Unfortunately, regardless of the type of batch implementation, the data was unable to integrate (see UMAP below for an example). This indicates that the beta cells from P2 versus P60 are very different; between the two datasets, no intermediate states connect them. This situation is expected since > 50 days have passed in development between the 2 datasets.

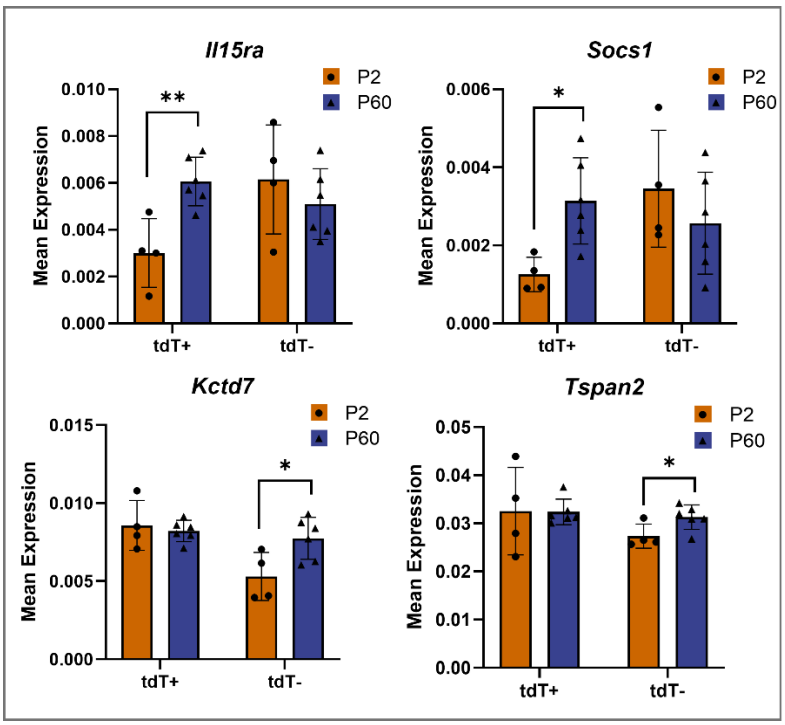


Instead, we took a different approach to examine how M+N+ and M-N+ beta-cells develop differently over P2 and P60. We first identified genes that significantly differ in expression as a function of developmental time (P2 vs P60) using pseudobulk analysis with n=4 animals. We then further categorized genes that behave differently over time between M+N+ or M-N+ beta-cells. We placed these genes through gene over-representation analysis and found two significant differences between the M+N+ or M-N+ cells: M+N+ cells are heavily associated with proliferation and immune regulation and M-N+ cells are related to neuronal differentiation.





To further examine immune and neuronal trends, we plotted a small sampling of genes found in these pathways to look at the changes between M+N+ or M-N+ beta-cells at the P2 and P60 developmental stages:



Here, *Il15ra* and *Socs1* are both associated with regulation of the immune system and *Kctd7* and *Tspan2* are both associated with neuronal differentiation and survival but are found to behave differently over the P2 and P60 time points between M+N+ or M-N+ beta-cells.

In summary, these extra analyses again highlight the difference between the two beta-cell subtypes. More intermediate stages must be sampled to fully complete trajectory analysis over the P2 and P60 developmental ages, which we will pursue in future studies. Given the vast amount of data in the manuscript, we did not include these new analyses in the revision. We hope the above findings can satisfy this reviewer critique.

Critique 4. The data on Dnmt3a levels is not very strong. It is not clearly what the high/low levels would do. Was this done in a z-stack with MIP. As another reviewer indicated, Dnmt3a works with lineage-specific TFs to guide cell fate choices.

Response: All the IF images used for quantification are average intensity projections from Z-stacked images, giving the overall level of Dnmt3a (or other factors) in the entire nucleus. Please note that heterogeneous Dnmt3a expression in adult beta cells is evident in this nice report (10.1172/JCI79956). This study showed that a few 6-week-old mouse beta cells expressed detectable Dnmt3a while others did not in the pancreatic sections. This result suggests the heterogeneity of Dnmt3a production in adult beta cells, with a caveat that the reported staining approach using sections may have a lower sensitivity for Dnmt3a detection than single cells spun onto slides (used in this new study), allowing our visualization of Dnmt3a^{Low} cells.

We agree that the pioneer factors (e.g., TFs or modified histones) that recruit Dnmt3a together with Dnmt3a guide cell fate choice and, likely, cellular heterogeneity. The question we raised here is: Is a variation in Dnmt3a level sufficient to induce (beta) cell heterogeneity by influencing the expression levels of some target gene? Future studies will address this question.

Critique 5. The authors state "Similar to P2 gene expression, 566 of 1,867 p60 DEGs are associated with the P2 DMRs". The following questions emerge:
• What's the extent of conservation between these DEGs and the ones from P2 (919 DEGs of which ~300 have DMR associations). It seems there are more DEGs at P60.
• And do the associated DMRs for the conserved DEGs follow the same directionality.

Response: For question #1, we found 59 genes common in the two DEG lists, representing ~ a 5.8-fold enrichment (p=8.17E-19, hypergeometric analysis) among beta-cell-expressed genes (to remove the influence of their being beta cells during the hypergeometric analysis). Indeed, there are more DEGs at P60 than P2, likely reflecting their functional states.

For question #2, we found that amongst the upregulated P60 DEGs (1,593), 298 or 197 are associated with lower or higher methylation in P2 beta cells derived from M+N+ or M-N+ progenitors, respectively (in addition to another 89 associated with both higher and lower methylation). This significantly (p=3.37E-6, Chi-Square test) deviates from random distribution. For the down-regulated DEGs (254), 40 or 25 are associated with lower or higher methylation (15 with both). This represents a trend of lowered methylation (p=0.055). These data are consistent with DNA methylation having repressive roles in transcription. This point was made clear in the re-revised manuscript.

Critique 6. The authors report minimal overlap between DMRs at P2 and P60. "However, when we consider genes associated with P2 and P60 beta cell subtype DMRs, roughly one-third of the DMR associated genes overlaps (sic) between P2 and P60." This suggests that there is a shift in DNA methylation dependent regulation from P2 and P60 for these genes. The directionality of "DMRs" and location of these DMRs vs gene expression directionality should be elaborated upon, as these findings will be very important in delineating how the methylation landscape of beta cell heterogeneity changes shape post-maturation.

Response: There is an overall DNA methylation level increase from P2 to P60 in the total beta-cell population, as shown in revised Fig. 5a and related text. Focusing on the 1,648 overlapped genes from P2 to P60, we found 597 or 613 that associate with DMRs with higher or lower methylation in M+N+ descendants, with another 438 having both (which is not analyzed because we do not know which is the dominant regulator). This distribution represents no significant deviation from random distribution (p=0.65). By P60, 645 or 746 genes are associated with DMRs with higher or lower methylation (278 with both). This represents a significant deviation (p=0.007) from random change of DNA methylation. These data support the reviewer's suggestion of a "shift in DNA methylation dependent regulation from P2 and P60 for these genes". This point is made clear in the re-revision.

Critique 7. The data show that both subtypes appear to undergo compensatory expansion in pups born to mothers on HFD but not highlighted in the text. This should be clearly noted in the text.

Response: This point is noted in the re-revised manuscript.

Critique 8. Minor point, multiple proof-reading errors.

Response: We have used professional help to fix these grammar issues.

Overall response:

We thank all the reviewers. We appreciate all their constructive critiques to make this manuscript the way it is now, which is much improved over the original submission.

Reviewer #1.
The authors have satisfactorily addressed all my concerns.

Response: Thank you.

Reviewer #4.
The authors have addressed all my comments in detail and the revision acknowledges all the caveats. This is overall a very insightful study that will add to the ongoing discourse on how beta cell fates and states emerge and evolve during development. This work will also provide valuable resources for the field.

Response: Thank you.