Groucho co-repressor proteins regulate β cell development and proliferation by repressing *Foxa1* in the developing mouse pancreas

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MS TITLE: Groucho co-repressor proteins regulate β cell development and proliferation by repressing Foxa1 in the developing pancreas

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

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

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Theis, et. al. entitled Â“Groucho corepressor proteins regulate beta cell development and proliferation by repressing FOXA1 in the developing pancreasÂ” represents an impressive amount of mouse genetic work and breeding with an interesting observation related to an understudied aspect of pancreatic development related to gene repressors, here the Groucho genes (GRG). Most of the studies are well done with good controls and the manuscript is well-written, with an especially well-written Discussion section. Overall this work will be interesting to readers and will be a significant contribution to the pancreatic development literature.

Comments for the author

That being said, there are some significant concerns related to the interpretation of data, as well as several minor concerns. The authors conclude that GRG4 is upregulated and compensates for the absences of GRG3. A concern here is that in general the knockdown of GRG3 as shown in supplemental Figure 1 varied from 80% to only 20%, so not great knockdown and highly variable. Secondly, the upregulation of GRG4 in Figure 2i was only about threefold. I say “only” because the authors state that at baseline GRG4 is not normally expressed at all in the developing pancreas so a threefold increase over “not at all” is still presumably extremely low and would seem unlikely to be able to compensate.

A second area of concern is the conclusion that the changes in beta cell numbers occurs late in gestation, which is the rationale for gene expression analysis at day 18.5. However, in supplemental Figure 5 the statement is made that the endocrine specification is unchanged at E16.5 in these mice. However, the percentage difference in beta cell area in supplemental Figure 5B compared with Figure 3E for post-natal day 2 would suggest that they are essentially the exact same percentage, but the standard error prevents it from being statistically significant. I think it is equally, if not more likely, that the beta cell specification is changed, and they simply have a type 2 statistical error here. This concern could greatly influence the interpretation of the data related to beta cell specification and a possible role of Groucho’s and FoxA1 and FoxA2.

There are also several minor concerns that should be easily addressed by the authors:
-Supplemental Figure 2 does not really add much to the overall glucose tolerance curve shown in the main figure.
-In Figure 1 legend there is an inadvertent insertion of a label “B” that is confusing and should be removed.
-In Figure 2 the phrasing of the description of the insulin content analysis in panel H is confusing, as they talk about insulin content as measured by GSIS? Are they referring to the insulin content of the cells or of the medium into which the cells release insulin?
-In the description of the Groucho double mutants it is expressed on line 203 that there is “early lethality”, this is a bit confusing and probably should add the word “post-natal” after early. Since later in this section the statement is made that they are born at normal Mendelian ratios.
-Supplemental Tables 1 and 2 are huge, and probably should just be referenced as a database and not shown as an actual table.
-In line 261 the authors refer to immunofluorescence in Figures 4D and E but I wonder if they meant to refer to Figure 3D and E?
-From the data in Figure 5, where we see insulin positive cells that are NeuroD1 negative, those results are convincing, but there are also NeuroD1 positive cells shown that are insulin negative. Some discussion or comment by the authors about these cells and what they may be seems indicated.

Lastly, in supplemental Figure 1A the figure legend just refers to the Groucho3 mutants, but it is not clear what the tissue is, and at what age the GRG3 mRNA analysis was done. The images shown in Supplemental Figure 1C and all other images showing double-positive hormone cells, should be documented as confocal images.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Theis et al. describe the requirement of the GRG family member to pancreatic beta-cell development and function. The authors show that pancreatic-specific deletion of Grg3, the main family member to be expressed in this organ, results in impaired beta-cell mass, insufficient insulin production and glucose intolerance of adult mice. As loss of Grg3 results in the upregulation of Grg4 in the pancreas, the authors deleted both genes in the pancreas. This exacerbates the glucose intolerance phenotype when neonatal mice display higher basal glucose levels. Double KO mice display lower beta-cell mass due to an impaired postnatal proliferation of these cells. Gene expression analysis of embryonic KO pancreata revealed ectopic expression of liver genes and Foxa1 and reduced expression of pancreatic genes, including NeuroD1. Thus the authors concluded that Grg3 acts to maintain proper beta-cell expansion and differentiation in the embryonic and neonatal pancreas. This study expands the current understanding of the complex transcriptional regulatory network of beta-cell development, and the data support its conclusions. However, the timing of Grg3 activity, as well as the relative requirement of Grg3 and 4 for beta-cell expansion and differentiation, needed to be clarified.

Comments for the author

Major concerns:

1) It is unclear whether the glucose intolerance phenotype (Fig. 2) is due to an impaired beta-cell phenotype, lower beta-cell mass, or their combination (i.e., the effect on differentiation or proliferation). What is the islet/pancreatic insulin content when normalized to beta-cell mass? What is the islets GSIS when normalized to their insulin content?

2) Related to the point above: gene expression analysis was carried on e18.5 pancreata (Fig. 5). However, as pointed by the authors, changes in expression levels could result from reduced beta-cell mass. What is the beta-cell mass at this age? Can it, by itself, account for the impaired expression of some beta-cell genes?

3) It seems beta-cell mass is unchanged in transgenic mice at e16.5 (Fig. S5). Do genes associated with beta-cell and/or hepatic differentiation already differ at this age?

4) The glucose intolerance phenotype is significantly stronger in Grg3/4 double KO mice as compared to Grg3 KO (Fig. S4). It is difficult to compare the two beta-cell mass analyses (presented in Fig. 2 and 3) as they were carried out at different ages. Still, it seems that the effect on beta-cell mass is similar when Grg3 alone or both 3 and 4 are deleted. Is this correct? When directly comparing the two KO mouse lines, are genes required for beta-cell differentiation and function differ?

Minor concerns:
1) The introduction misses references throughout (f.i., line 54, lines 68-71 and more)
2) Figures 5A and C are unclear – please replace with images with a better quality

Reviewer 3

Advance summary and potential significance to field

GRG/TLE corepressors interact with key pancreas transcription factors, but whether they have a role in pancreas development has not been well studied. In this manuscript, Theis et al. examine multiple mouse models to show that Grg3/4 are essential for β cell development. Genetic deletion of Grg3/4 with Pdx1-Cre causes a striking Foxa1 upregulation and induces expression of liver-specific genes in the mutant pancreas. The β cell transcription factor Neurod1, Ins1, Ins2 and other

islet hormones are also downregulated and the mice develop diabetes. In addition, the authors demonstrate that Grg4 compensates for loss of Grg3 in the developing pancreas.

Comments for the author

This study is very interesting with some high quality data; however, the authors do not put their findings in the context of other papers in the field. The authors should significantly update the introduction/manuscript in general, by including more relevant publications that directly relate to their study.

Major Critiques
- The introduction contains only one example of GRG/TLEs in pancreas development (Metzger, 2012). This needs to be expanded since at least a few key studies were not described (see “Grg3/TLE3 and Grg1/TLE1 Induce Monohormonal Pancreatic b-Cells While Repressing a-Cell Functions” (Metzger, 2014) and
“Expression of Groucho/TLE proteins during pancreas development” (Hoffman, 2008)). What is the expression pattern of Grg/Tle in the pancreas? Are some more highly expressed in the endocrine or exocrine pancreas? What is the phenotype of Tle-/- embryos?
- Lines 88-105: a very general/irrelevant description of transcription factor expression in the pancreas
(and detailed references), but only one example of a GRG/TLE-TF interaction at the very end (Papizan, 2011).
The study “Dynamic expression of groucho-related genes Grg1 and Grg3 in foregut endoderm and antagonism of differentiation” (Santisteban, 2010) might be relevant here. Are there more examples of GRG/TLEs interacting with key pancreas/β-cell transcription factors? In general, the introduction doesn’t build a strong rationale for further studying the role of GRG/TLEs in pancreas development.
- Throughout the study the authors use Pdx1-Cre to drive genetic deletion. This Cre deletion would occur very early in the embryo and in all pancreatic lineages, i.e. in the acinar and duct cells. What is the phenotype of these cell types? Were they examined? Are Grg2/3/4 differentially expressed in these cell types?
- “During normal development, Foxa1 expression diminishes in the pancreas and becomes restricted to the liver lineage (Li et al., 2018; Scavuzzo et al., 2018).” This seems an overly strong statement since neither of these references directly shows this or mentions it, as far as I can see. FOXA1/FOXA2 have been shown to be normally expressed in all exocrine and endocrine cells of the pancreas, as described in “Foxa1 and Foxa2 Maintain the Metabolic and Secretory Features of the Mature β-Cell” (Gao, 2010). Clarification should be made.
- The FOXA1 immunostaining in the double mutant pancreas is very striking (Fig. S7 – this should be a main figure) and shows staining in all pancreas cell types, including the acinar and duct cells. What is the phenotype in these cells?
- The authors do not discuss the interaction of GRGs with FOXA1 in the foregut endoderm. It has been previously suggested that reductions in GRG3 will promote FOXA1 expression and hepatic specification. See Santisteban, 2010 and Gordillo, 2015.
- The authors do not examine whether GRG3/4 directly regulate expression of Foxa1, Neurod1, Ins1, Ins2 etc. or whether these gene expression changes are the indirect result of altered epigenetic modifications.
Since GRG3 is known to interact with HDAC1 and DNMT3a, histone modifications and DNA methylation status should be examined/discussed.

Overall, the manuscript describes some elegant genetics, high quality data and interesting findings. The manuscript is not as well written and organized as it could be. There are lots of typos, missing information incorrect references, etc.

Minor Critiques
- Line 49: is it necessary to specify “adult” endocrine cell compartment? These cells are present in development too. What about the ghrelin-secreting β-cells?
- Line 54: a better description of diabetes is required. No mention of hyperglycemia.
- Line 59: E8.5 is quite early for MPCs. The pancreas bud is first evident at E9.5 (Pan and Wright, 2011).

See “Cellular and molecular mechanisms coordinating pancreas development” (Bastidas-Ponce, 2017) where MPCs are also described at E9.5.
- Semantics: Why is Neurogenin 3 written as NEUROGENIN3? It should be NEUROG3+ (not NGN3+) to match the gene name.
- Line 63: “endocrine cell of the islets” should be “endocrine cells”
- Line 65-66: “resulting in a fully functional adult pancreas” should be “…adult endocrine pancreas” since exocrine development was not described.
- Line 73-74: There are six mammalian Groucho homologs: Tle1-6. Why are Grg5/6 ignored? Grg is not the gene name.
- Be consistent – Grg/Tle genes. GRG/TLE proteins.
- Lines 76-79: when describing the role of GRGs and HDAC-mediated repression, the authors cite Patel 2012; however, this reference describes Polycomb-mediated repression. GRGs also function though DNMT-
mediated repression as described in Papizan, 2011.
- Lines 107-108: “…Grg2 and Grg3 were the two family members predominantly expressed during pancreas development.” Is this consistent with Hoffman, 2008?
- Line 120: listed references do not support the statement that “…downregulation of Neurod1 causes defects in β cell expansion.” Rather, deletion of Neurod1 does not alter proliferation (Gu, 2010) but causes significant apoptosis (Naya, 1997). Neither proliferation nor apoptosis is examined in Mastracci 2013a. Perhaps proliferation is only affected in the perinatal β cell expansion phase (Romer, 2019).
- Figure 1A, B: why focus on E13.5 and E15.5? What about expression before or after these stages? Why compare to Pdx1 and not Neurog3? Images in Fig. 1A are quite low resolution and lack scale bars. The Pdx1 in situ at E15.5 does not look accurate – is this an exocrine marker? Grg2/3 appear specific for the endocrine compartment but do not overlap with Pdx1. No error bars in Fig. 1B.
- Line 137: reference is missing for E15.5 RNA-seq data. Where is the adult islet data from?
- Line 148: “…a null mutation of Grg2 using a Tle2tm1a…” – mixing of Grg and Tle is confusing
- Line 148: “Tle2tm1a(EUCOMM)Hmgu” does not match the Methods section
- Line 149-150: what are the “severe” phenotypes of Grg3-/- and Grg4-/-?
- Figure 1C-F: should this be supplemental data?
- Figure 1C: all 20 exons from Tle2 were deleted?
- Figure 1D: CycB is used for reference gene expression – won’t its expression be affected by cell cycle stage? Is this a typo? Should it be Actb as described in Methods?
- Figure 1F: what stage is this? Scale bars are not legible.
- Figure 2A: scale bars?
- Figure 2B: how was pancreas area quantified? Is the pancreas the same size/mass in Pdx1-Cre; Grg3fl/fl mice?
- Figure 2C: is this E18.5 data? Whole pancreas or islets?
- Figure 2G: “significant decrease in the of number of β cells…” – not clear from the representative image and β cell number/mass was not quantified.
- Figure 2G: “no apparent effect on the other endocrine cell types.” – Is this true? GCG+ cells appear increased and infiltrated into the core of the islet.
- Figure 2G: “scale bar = 0.05&[mu]m” – is this accurate?
- Are Grg3 and Grg4 paralogs? Have others observed compensation of Grg3 by Grg4?
- Figure S4: should this be a main figure? What timepoint was this data collected? No quantification of endocrine cell numbers. How is the islet morphology/increase in GCG+ cells different than that seen in Fig.
2G with the single mutants? How many bihormonal cells were found?
- Lines 205-206: “…likely due to inefficient deletion of both Grg3 and Grg4 alleles” – was the recombination efficiency quantified?
- Lines 201-202 and lines 211-212 are contradictory. Are the mice from Fig. S4 and Fig. 3 the same genotype? Why are the Mendelian ratios different?
- Figure 3: How was pancreas area quantified? Is this accurate? What about pancreas mass – is it smaller in the double mutants?
- Line 221: Fig. S4D callout is incorrect.
- Line 238: what is Table S1? Was RNA-seq done? Which mice? How many replicates? The Table is hard to interpret as the values are not sorted by log2FC.
- Line 249: what were the control embryos?

- Some switching between Grg floxed controls and Cre-only controls
- Figure 4A: labels for significant up/downregulated genes?
- Figure 4B: genes involved in endocrine pancreas development and insulin secretion are upregulated?

This is not consistent with the rest of the data. Are the labels switched?

- Figure 4C: data is not sorted – reductions in Cpa1? Effects on exocrine pancreas?
- Table S1: Gast is highly upregulated – immature endocrine cells? Why are Tle3/4 not downregulated?
- Figure 4E: images are poor quality – is FOXA1 staining nuclear? Images in the corresponding supplemental figure are much more convincing.
- Table S4: very incomplete. Missing antibody info.
- Morphometric analysis: quantification done with 5-10 slides. How many tissue sections? Evenly spaced throughout the entire pancreas?
- RNA-seq: what kit was used for library preparation?
- Line 261: Fig. 4D, E = wrong callout
- Lines 273-274: "..Foxa1, a major regulator of hepatocyte differentiation (Lee, 2005)." – From this reference, Foxa1 and Foxa2 together promote early liver specification. FOXA1 on its own is not a regulator of hepatocyte differentiation.

First revision

Author response to reviewers' comments

Reviewer 1:

That being said, there are some significant concerns related to the interpretation of data, as well as several minor concerns. The authors conclude that GRG4 is upregulated and compensates for the absences of GRG3. A concern here is that in general the knockdown of GRG3 as shown in supplemental Figure 1 varied from 80% to only 20%, so not great knockdown and highly variable. Secondly, the upregulation of GRG4 in Figure 2i was only about threefold. I say "only" because the authors state that at baseline GRG4 is not normally expressed at all in the developing pancreas, so a threefold increase over "not at all" is still presumably extremely low, and would seem unlikely to be able to compensate.

- We agree that the GRG3 knockdown is variable, but even with this variability, these mice consistently display an overt hyperglycemic and glucose intolerant phenotype. Currently, quantifying the knockdown is only possible by Western blot since GRG3-specific antibodies do not work well for immunofluorescence in the pancreas. We were also unable to perform additional Western blots on more tissue because the specific antibody we originally used has been discontinued antibody; the currently available antibodies cross-react with the other GRG family members. Due to the variability of *Grg3* deletion, *Grg4* upregulation is also variable; however,again - it is consistently one of the most upregulated genes in all of our sequencing analyses. We attempted Western blots for GRG4 but ran into a similar issue with non-specificity of the antibody. We would also like to note that based on the increased severity of the phenotype seen in the DKO mutants, we believe this upregulation of GRG4 is sufficient to perform a compensatory role in the GRG3 single mutants.

A second area of concern is the conclusion that the changes in beta cell numbers occurs late in gestation, which is the rationale for gene expression analysis at day 18.5. However, in supplemental Figure 5 the statement is made that the endocrine specification is unchanged at E16.5 in these mice. However, the percentage difference in beta cell area in supplemental Figure 5B compared with Figure 3E for post-natal day 2 would suggest that they are essentially the exact same percentage, but the standard error prevents it from being statistically significant. I think it is equally, if not more likely, that the beta cell specification is changed, and they simply have a type 2 statistical error here. This concern could greatly influence the interpretation of the data related to beta cell specification and a possible role of Groucho's and FoxA1 and FoxA2.

- We agree that there did appear to be a decrease in beta cell area at e16.5, but we were cautious in our interpretation since the decrease did not reach significance with the previous numbers we analyzed.

However, we have now performed staining and quantification on additional samples and with these increased numbers, we also observe a significant reduction in beta cell number and proliferation at e16.5 (see updated Figures 4 and 6). However, we do believe that at least a percentage of beta cells are still being specified from the endocrine progenitor population – at least to some degree – since we still see many beta cells by immunofluorescence staining and by the presence of many critical beta cell genes at e18.5 in the RNA-seq analysis. For these reasons, we are careful in our interpretation of the possible roles of Grouchos and FoxA1 and A2 upregulation.

There are also several minor concerns that should be easily addressed by the authors:
Supplemental Figure 2 does not really add much to the overall glucose tolerance curve shown in the main figure.

- We have removed this supplemental figure.

In Figure 1 legend there is an inadvertent insertion of a label “B” that is confusing and should be removed.

- This has been corrected.

In Figure 2 the phrasing of the description of the insulin content analysis in panel H is confusing, as they talk about insulin content as measured by GSIS? Are they referring to the insulin content of the cells or of the medium into which the cells release insulin?

- We have clarified the insulin content data in the text, figure legend, and methods (Lines 201, 514-516, 647-648); we normalized insulin secreted into the media to insulin content.

In the description of the Groucho double mutants it is expressed on line 203 that there is “early lethality”, this is a bit confusing and probably should add the word “post-natal” after early. Since later in this section the statement is made that they are born at normal Mendelian ratios.

- We have changed this wording as suggested in the text (Line 226).

Supplemental Tables 1 and 2 are huge, and probably should just be referenced as a database and not shown as an actual table.

- We submitted the data tables as supplemental to provide transparency in the gene expression analysis.
  Since the raw data is available on GEO, we have removed table 1 from the supplemental information. Table 2 is not large and lists Foxa1 targets that are altered; we have chosen to leave this table in, but have streamlined the information that is provided.

In line 261 the authors refer to immunofluorescence in Figures 4D and E, but I wonder if they meant to refer to Figure 3D and E?

- We have corrected this error in call-out (Line 245).

From the data in Figure 5, where we see insulin positive cells that are NeuroD1 negative, those results are convincing, but there are also NeuroD1 positive cells shown that are insulin negative. Some discussion or comment by the authors about these cells and what they may be seems indicated.

- Neurod1 is expressed in all endocrine cell types (Chao, Loomis… Sussel, PMID17988662). We have added this information and reference to the discussion of this figure (Lines 328-329, 704-705).

Lastly, in supplemental Figure 1A the figure legend just refers to the Groucho3 mutants, but it is not clear what the tissue is, and at what age the GRG3 mRNA analysis was done. The images shown in Supplemental Figure 1C, and all other images showing double-positive hormone cells, should be documented as confocal images.

- We have added this information to the figure legend and methods (Lines 538, 721).

Reviewer #2:
It is unclear whether the glucose intolerance phenotype (Fig. 2) is due to an impaired beta-cell phenotype, lower beta-cell mass, or their combination (i.e., the effect on differentiation or proliferation). What is the islet/pancreatic insulin content when normalized to beta-cell mass?

What is the islets GSIS when normalized to their insulin content?

- We believe the glucose intolerant phenotype is due to both lower mass and an impaired beta cell phenotype; however, since mice are generally euglycemic even with a 75% reduction in mass, the impaired beta cell phenotype would be the predominant cause. GSIS data cannot be normalized to beta cell mass since GSIS was performed on isolated islets. We have clarified in the methods section that this data was cellular insulin content as measured by ELISA (Lines 201, 514-516, 647-648).

Related to the point above: gene expression analysis was carried on e18.5 pancreata (Fig. 5). However, as pointed by the authors, changes in expression levels could result from reduced beta-cell mass. What is the beta-cell mass at this age? Can it, by itself, account for the impaired expression of some beta-cell genes?

- Beta cell mass is reduced at e18.5 and could account for some of the gene expression changes. However the observed reduction in beta cell mass is not sufficient to cause a hyperglycemic phenotype, therefore there is likely a combined effect of loss of beta cells and dysfunctional beta cells, which we discuss in the text. Furthermore, we see many of the same gene changes in the single Grg3 KO – which do not have a significant decrease in beta cell mass at e18.5 (or P2). In the future, we will assess the function of Grg3/4 in beta cell function, but that is an entirely new study using beta cell specific Cre alleles.

It seems beta-cell mass is unchanged in transgenic mice at e16.5 (Fig. S5). Do genes associated with beta- cell and/or hepatic differentiation already differ at this age?

- Our previous data (n=4) showed a trending decrease without significance in insulin-positive area of mutants at e16.5 so we were conservative in our interpretation. We have now performed staining on additional samples and have updated Figure 4 to show that the decrease in insulin-positive area is significant (p=0.04). We also attempted to stain e16.5 tissue for liver proteins whose genes were upregulated in the mutant RNA-seq data and found that gene upregulation did not correspond with protein upregulation for many that we tested (*Alb, Pah, ApoB*). When staining for AAT (encoded by *Serpina1a-e* genes), we saw very few positive cells in the mutant pancreata. However, when staining for mRNA using RNA-scope technology, we were able to visualize striking upregulation of *Serpina1e* mRNA in the same pancreas samples. Therefore it appears that liver genes are upregulated, but are not translated – which is likely why we don't see a full conversion to liver lineages. We have added this data (Figure 5) and discuss it in the text (Lines 312-319, 415-426).

The glucose intolerance phenotype is significantly stronger in Grg3/4 double KO mice as compared to Grg3 KO (Fig. S4). It is difficult to compare the two beta-cell mass analyses (presented in Fig. 2 and 3) as they were carried out at different ages. Still, it seems that the effect on beta-cell mass is similar when Grg3 alone or both 3 and 4 are deleted. Is this correct? When directly comparing the two KO mouse lines, are genes required for beta-cell differentiation and function differ?

- The single Grg3 KO mice have a non-significant reduction in beta cell mass at e18.5, whereas the double KO mice have a significant reduction in beta cell mass beginning at e16.5. Although we originally did the Grg3 analysis at e18.5 to avoid any postnatal feeding variables, we chose to do the postnatal P2 timepoint for the DKO since it helped to preserve the females for multiple matings. We did perform the gene expression data at the same age (e18.5) in the single and DKO mice and show that many of the same genes are changed, although to a greater degree in the DKO mice. There does not appear to be a difference in the classes of genes that are changed. We added a table highlighting some of the most important genes and their expression changes (Supplemental Figure 4). Note that although many of the same genes have altered expression, many don't reach significance in the single KO mice.

Minor concerns:

The introduction misses references throughout (f.i., line 54, lines 68-71 and more)

- We have added in these missing references.

Figures 5A and C are unclear – please replace with images with a better quality

- We have higher-resolution images but they were affected when decreasing the PDF file size for submission - they are included in the resubmission files.

Reviewer #3:

The introduction contains only one example of GRG/TLEs in pancreas development (Metzger, 2012). This needs to be expanded since at least a few key studies were not described (see "Grg3/TLE3 and Grg1/TLE1 Induce Monohormonal Pancreatic b-Cells While Repressing a-Cell Functions" (Metzger, 2014) and "Expression of Groucho/TLE proteins during pancreas development" (Hoffman, 2008)). What is the expression pattern of Grg/Tle in the pancreas? Are some more highly expressed in the endocrine or exocrine pancreas? What is the phenotype of Tle-/- embryos?

- We added in these missing references in the introduction. In the Hoffman et al. paper, much of their analysis was based on genepaint.org data and the results are somewhat different results from our RNA *in situ* analyses - which are also supported by single cell RNA seq data. We now discuss this reference and the differences seen in our study in the text. In the second part of this comment, we assume reviewer 1 is referring to Grg1/Tle1? Simliar to our in situ analysis, single cell RNA Seq data shows that Grg1/Tle1 is primarily mesenchymal at e15.5, with lower expression in exocrine cells. To our knowledge, there is no TLE1-/- mouse KO. The Metger paper describes human TLE1-/- phenotypes.

Lines 88-105: a very general/irrelevant description of transcription factor expression in the pancreas (and detailed references), but only one example of a GRG/TLE-TF interaction at the very end (Papizan, 2011). The study "Dynamic expression of groucho-related genes Grg1 and Grg3 in foregut endoderm and antagonism of differentiation" (Santisteban, 2010) might be relevant here. Are there more examples of GRG/TLEs interacting with key pancreas/β-cell transcription factors? In general, the introduction doesn't build a strong rationale for further studying the role of GRG/TLEs in pancreas development.

- To date there are no *in vivo* GRG functional analyses done in pancreas development, which was the major rationale for our study and we have made a stronger argument for this in the introduction (Lines 89-116). The Metzger ex vivo explant studies suggested there would be a strong developmental phenotype that we wanted to pursue further, but our studies unexpected led us to the directions described in this paper and provide important new insight into the functions of Grgs/TLEs in the pancreas. Additionally, although many transcription factors have been shown to contain GRG-interaction domains and many of these interactions have been verified in other tissues, only a few have actually been tested in the pancreas. However, in addition to its interaction with NKX2.2, we have also cited papers that show the interaction of PAX family members and NKX6.1 with GRG3 (Lines 99-101).

Throughout the study the authors use Pdx1-Cre to drive genetic deletion. This Cre deletion would occur
very early in the embryo and in all pancreatic lineages, i.e. in the acinar and duct cells. What is the phenotype of these cell types? Were they examined? Are Grg2/3/4 differentially expressed in these cell types?

- Our *in situ* analysis and published single cell RNA-seq data indicates that GRG3 is expressed predominantly in beta cells, although low levels of transcripts can be found in other lineages. Morphologically, the ductal and exocrine tissue does not appear to be overtly effected and overall pancreas size was unchanged in the P2 mutant embryos, suggesting the formation exocrine tissue (which constitutes 90% of the organ) is not impaired. In adults, the overall pancreas morphology also appears normal. We performed immunofluorescent staining for acinar and duct markers to confirm this (Supplemental Figure 5). We also interrogated our RNA-seq data to see whether there are any significant molecular changes and found downregulation of some but not all acinar and ductal genes (Supplemental Figure 5). We have also added this information to the text (Lines 285-288).

"During normal development, Foxa1 expression diminishes in the pancreas and becomes restricted to the liver lineage (Li et al., 2018; Scavuzzo et al., 2018)." This seems an overly strong statement since neither of
these references directly shows this or mentions it, as far as I can see. FOXA1/FOXA2 have been shown to be normally expressed in all exocrine and endocrine cells of the pancreas, as described in "Foxa1 and Foxa2 Maintain the Metabolic and Secretory Features of the Mature ß-Cell" (Gao, 2010). Clarification should be made.

- Recently published single-cell RNA-seq (Krentz et al., 2018; Li et al., 2018) show that *Foxa1* transcript

is not detected in early pancreas stages. Prior studies were limited by the use of a FOXA1/2 antibody that did not distinguish between family members. We have changed the wording and references to better describe the expression of *Foxa1* in the pancreas and liver (Lines 104-108, 299-302, 408-412).

The FOXA1 immunostaining in the double mutant pancreas is very striking (Fig. S7 – this should be a main figure) and shows staining in all pancreas cell types, including the acinar and duct cells. What is the phenotype in these cells?

- We agree that the presence of FOXA1 in all cell lineages is striking and very interesting, however we saw no overt changes in these tissues by immunofluorescence. Our RNA-seq data did show downregulation of some (but not all) acinar and ductal genes (Supplemental Figure 5).

The authors do not discuss the interaction of GRGs with FOXA1 in the foregut endoderm. It has been previously suggested that reductions in GRG3 will promote FOXA1 expression and hepatic specification. See Santisteban, 2010 and Gordillo, 2015.

- We have added this into the discussion (Lines 415-418).

The authors do not examine whether GRG3/4 directly regulate expression of Foxa1, Neurod1, Ins1, Ins2, etc. or whether these gene expression changes are the indirect result of altered epigenetic modifications.
Since GRG3 is known to interact with HDAC1 and DNMT3a, histone modifications and DNA methylation status should be examined/discussed.

- We agree that the mechanism underlying the dysregulation of these genes remains unclear. In this study, we used the pan-pancreas *Cre* to determine overall function of GRGs in the pancreas, but this does not allow us to delve into cell-specific mechanisms of GRG-mediated gene repression (something we plan to do in the future). Since regulation could be through promoter elements or distal enhancers, it would be optimal to look for genome wide occupancy changes for HDAC1 and DNMT3a ChIP-Seq in the mutant embryonic pancreas – the caveat being that this would be on a mixed population of cells. This would be a challenging experiment in the best of times and even more so right now since we had to pare down our mouse colony due to COVID-19 and have had severe breeding depression upon reopening. While we are still very interested in the underlying mechanism of GRG3/4 function in beta cell development and function, we are unable to provide additional insights in this manuscript but plan to do so in a future publication.

Overall, the manuscript describes some elegant genetics, high quality data and interesting findings. The manuscript is not as well written and organized as it could be. There are lots of typos, missing information, incorrect references, etc.

- We thank the reviewer for his thorough reading of the manuscript. We have addressed these comments and will make the relevant changes to the text.

Minor Critiques
Line 49: is it necessary to specify “adult” endocrine cell compartment? These cells are present in development too. What about the ghrelin-secreting ε-cells?

- We specified “adult” since these are the main endocrine populations still present in the adult. Gastrin and Ghrelin cells are present during development, but largely absent from adult islets.

Line 54: a better description of diabetes is required. No mention of hyperglycemia.

- We have added this information (Lines 53-56).

Line 59: E8.5 is quite early for MPCs. The pancreas bud is first evident at E9.5 (Pan and Wright, 2011).
See “Cellular and molecular mechanisms coordinating pancreas development” (Bastidas-Ponce, 2017) where MPCs are also described at E9.5.

- Although the morphological buds are not obvious until e9.5, the pancreatic endoderm is specified around e8/8.5 (see some of the earliest Melton and Wright papers) and PDX1+ cells are first seen around e8.5/10 somite stage as reported in Ohlsson et al. 1993, Guz et al. 1995, and Ahlgren et al. 1996.

Why is Neurogenin 3 written as NEUROGENIN3? It should be NEUROG3+ (not NGN3+) to match the gene name.

- We have changed NEUROGENIN3 to “Neurogenin 3”. We have revised NGN3 to NEUROG3 throughout

the manuscript.

Line 63: “endocrine cell of the islets” should be “endocrine cells”

- We have fixed this wording (Line 64).

Line 65-66: “resulting in a fully functional adult pancreas” should be “…adult endocrine pancreas” since exocrine development was not described.

- We have amended this text (Line 65).

Line 73-74: There are six mammalian Groucho homologs: Tle1-6. Why are Grg5/6 ignored? Grg is not the gene name.

- *Grg*/GRG is a commonly used mouse nomenclature for this family of genes/proteins (Gasperowicz and Otto, 2005). Here, we use *Grg*/GRG as a way to distinguish between the mouse and human orthologues. *Grg5/6* are short forms of the gene and the protein functions are different enough that we only focus on the 4 full length forms – as we indicated in the text (Lines 76-79).

Be consistent – Grg/Tle genes. GRG/TLE proteins.

- We were trying to distinguish between the human and mouse genes, but have revised to reflect that these are all mouse genes (Lines 73-74).

Lines 76-79: when describing the role of GRGs and HDAC-mediated repression, the authors cite Patel, 2012; however, this reference describes Polycomb-mediated repression. GRGs also function though DNMT- mediated repression as described in Papizan, 2011.

- We have added these additional interacting partners and associated references (Lines 81-84). Papizan et al. implicates DNMT1 in the repression of *Arx*; however, direct interaction between GRGs and DNMTs has not been described.

Lines 107-108: “…Grg2 and Grg3 were the two family members predominantly expressed during pancreas development.” Is this consistent with Hoffman, 2008?

- Hoffman et al. described GenePaint.org data that shows *Grg2/3* expression in the e13.5 pancreatic epithelium, with *Grg1* being restricted to the surrounding mesenchyme. We have added this information (Lines 119-121, 149-150).

Line 120: listed references do not support the statement that “…downregulation of Neurod1 causes defects in β cell expansion.” Rather, deletion of Neurod1 does not alter proliferation (Gu, 2010) but causes significant apoptosis (Naya, 1997). Neither proliferation nor apoptosis is examined in Mastracci, 2013a. Perhaps proliferation is only affected in the perinatal β cell expansion phase (Romer, 2019).

- We removed the Mastracci et al. and Gu et al. references here (Line 135). Romer et al. 2019 revisits the original *Neurod1-/-* mouse phenotype from Naya et al., which originally showed a loss of beta cells and the appropriate formation of islets. Romer et al. describes a proliferation defect beginning as early as e15.5 and continuing through embryogenesis in *Neurod1-/-* mutants, which is phenocopied in the *Grg3/4* mutants we describe here.

Figure 1A, B: why focus on E13.5 and E15.5? What about expression before or after these stages? Why compare to Pdx1 and not Neurog3? Images in Fig. 1A are quite low resolution and lack scale bars. The Pdx1 in situ at E15.5 does not look accurate – is this an exocrine marker? Grg2/3 appear specific for the endocrine compartment but do not overlap with Pdx1. No error bars in Fig. 1B.

- We performed the RNA in situs at 2 stages merely to validate the RNA-Seq data and to provide spatial information. At e15.5, Pdx1 is marking the pancreatic epithelium as described by others (see Murtaugh 2007). *Grg2/3* do not overlap 100% with *Pdx1*, however, these are serial sections and they are still seen throughout the pancreatic epithelium.

Line 137: reference is missing for E15.5 RNA-seq data. Where is the adult islet data from?

- We have submitted the NGN3-EGFP data with this manuscript and the accession number can be found in the methods. The adult islet data has been published or is part of this study. The references and GEO numbers have been added to the manuscript (Line 159).

Line 148: "…a null mutation of Grg2 using a Tle2tm1a…" – mixing of Grg and Tle is confusing
- We have added *Grg2/Tle2* to the text (Line 166).

Line 148: "Tle2tm1a(EUCOMM)Hmgu" does not match the Methods section
- We have fixed this discrepancy.

Line 149-150: what are the "severe" phenotypes of Grg3-/- and Grg4-/-?
- The information could be found in the cited references, but we have also added this information to the text (lines 167-171).

Figure 1C-F: should this be supplemental data?
- This is the first reported mouse knockout of Grg2 and although there was no discernable phenotype, we believe it merits being in a figure. We are happy to move to supplemental data if necessary.

Figure 1C: all 20 exons from Tle2 were deleted?
- Yes, we have now provided a reference to the KOMP site which describes how the ES cells were generated and provide the coordinates of the deletion (Line 485).

Figure 1D: CycB is used for reference gene expression – won't its expression be affected by cell cycle stage? Is this a typo? Should it be Actb as described in Methods?
- The reference gene used in Figure 1D was *Cyclophilin B*, which is commonly used as an RNA standard.
The figure has been revised to make the distinction.

Figure 1F: what stage is this? Scale bars are not legible.
- These are 6 week islets. The figure legend has been revised to include this and the scale bars have been fixed.

Figure 2A: scale bars?
- We have added the missing scale bars to this figure.

Figure 2B: how was pancreas area quantified? Is the pancreas the same size/mass in Pdx1-Cre; Grg3fl/fl mice?
- Pancreas area was quantified by DAPI+ signal in the pancreas. There were no differences in pancreas area between mutant and controls.

Figure 2C: is this E18.5 data? Whole pancreas or islets?
- This is e18.5 whole pancreas data – we have added this information to the figure legend (Lines 641-642).

Figure 2G: "significant decrease in the of number of &[beta] cells…" – not clear from the representative image and &[beta] cell number/mass
- Beta cell mass was visually decreased in the adult single KO, but was not quantified since we focused on the DKO phenotype; we have removed the word "significant" from the text (Lines 199-200).

Figure 2G: "no apparent effect on the other endocrine cell types." – Is this true? GCG+ cells appear increased and infiltrated into the core of the islet.
- We agree that GCG+ cells appear to be increased; however mRNA and protein quantification does not support this. As in many of our other studies, we believe the infiltration of alpha cells towards the core of the islet due to a decrease in beta cell number gives the illusion that alpha cells are increased.

Figure 2G: "scale bar = 0.05&[mu]m" – is this accurate?
- We have revised this error in the figure legend.

Are Grg3 and Grg4 paralogs? Have others observed compensation of Grg3 by Grg4?
- GRG3 and GRG4 are 90% conserved in protein sequence; however, they have not been reported to act as paralogs in other systems. To our knowledge no one has reported compensation, however, it has been speculated in the literature that this is possible and makes them difficult to study in the

mammalian system.

Figure S4: should this be a main figure? What timepoint was this data collected? No quantification of endocrine cell numbers. How is the islet morphology/increase in GCG+ cells different than that seen in Fig. 2G with the single mutants? How many bihormonal cells were found?

- We have moved this figure to the main text. We did not quantify endocrine cell numbers at this stage due to difficulty collecting tissue of the extremely sick animals. We have updated the figure legend to include age of the animals.

Lines 205-206: "…likely due to inefficient deletion of both Grg3 and Grg4 alleles" – was the recombination efficiency quantified?

- The recombination efficiency was not quantified; however, the *Pdx1:Cre* allele used here is the most efficient pan-pancreas Cre available; although it has been reported to not be 100% efficient.

Lines 201-202 and lines 211-212 are contradictory. Are the mice from Fig. S4 and Fig. 3 the same genotype? Why are the Mendelian ratios different?

- We calculated Mendelian ratios perinatally (P2) and in weaned mice (3-4 weeks) and found that perinatally, double mutants had the expected Mendelian ratio but that by the time of weaning, there were significantly fewer than expected – suggesting postnatal lethality occurred after P2, but prior to weaning age.

Figure 3: How was pancreas area quantified? Is this accurate? What about pancreas mass – is it smaller in the double mutants?

- We quantified pancreas area by calculating the DAPI+ cells within the pancreas on each section; there were no significant differences between the genotypes.

Line 221: Fig. S4D callout is incorrect.

- This has been corrected.

Line 238: what is Table S1? Was RNA-seq done? Which mice? How many replicates? The Table is hard to interpret as the values are not sorted by log2FC.

- Although we uploaded the RNA-Seq data to the GEO database, we provided the analyzed data in supplemental tables for transparency purposes. We have removed the tables at the request of Reviewer

1. There were 3 replicates. All of the data is included in the methods section.

Line 249: what were the control embryos?

- We have added this information in the text.

Some switching between Grg floxed controls and Cre-only controls

- *Grg3fl/fl* controls were used during the first portion of this work as they could be easily collected as littermate controls for the *Grg3* single mutants. Due to the early lethality of *Grg3/4* mice, we could not efficiently generate double mutants with double-floxed littermate controls – therefore, we switched to using *Pdx1:Cre* as a control. No phenotypes were seen in either control used. We did use floxed littermate controls in the adult (Fig S4) data because they were readily available in those litters.

Figure 4A: labels for significant up/downregulated genes?

- The dots are color-coordinated with regard to up- or downregulation as denoted in the figure legend. We have also added a legend to the figure.

Figure 4B: genes involved in endocrine pancreas development and insulin secretion are upregulated? This is not consistent with the rest of the data. Are the labels switched?

- We have fixed the error in the labeling of this figure.

Figure 4C: data is not sorted – reductions in Cpa1? Effects on exocrine pancreas?

- The data was sorted based on gene classification; we have resorted the data based on fold change. There was no obvious phenotype in the exocrine pancreas (normal amylase staining, no changes in birth or adult weight); however, we did see changes in some, but not all, acinar and ductal genes.

We have added this data to Supplemental Figure 5.

Table S1: Gast is highly upregulated – immature endocrine cells? Why are Tle3/4 not downregulated?

- Gastrin is highly upregulated, but since we don't observe other "immaturity markers" it is difficult to speculate if this is due to immaturity. *Grg/Tle3/4* are upregulated at the RNA level; however, the floxed exons are missing in the raw reads and GRG3 protein expression is decreased.

Figure 4E: images are poor quality – is FOXA1 staining nuclear? Images in the corresponding supplemental figure are much more convincing.

- We were restricted by the size of images we were able to upload into the original document. We have provided higher quality images with resubmission.

Table S4: very incomplete. Missing antibody info.

- We have uploaded the correct version of this table with resubmission.

Morphometric analysis: quantification done with 5-10 slides. How many tissue sections? Evenly spaced throughout the entire pancreas?

- We have clarified this in the methods section (Lines 572-574).

RNA-seq: what kit was used for library preparation?

- Universal Plus mRNA-Seq with NuQuant kit was used by the Genomics Core to prepare libraries for RNA-seq. We have added this information to the Methods (Lines 589-591).

Line 261: Fig. 4D, E = wrong callout

- We have fixed this callout.

Lines 273-274: "..Foxa1, a major regulator of hepatocyte differentiation (Lee, 2005)." – From this reference, Foxa1 and Foxa2 together promote early liver specification. FOXA1 on its own is not a regulator of hepatocyte differentiation.

- We have added *Foxa2* to this sentence (lines 299-300).

Second decision letter

MS ID#: DEVELOP/2020/192401

MS TITLE: Groucho co-repressor proteins regulate &[beta] cell development and proliferation by repressing Foxa1 in the developing pancreas

AUTHORS: Alexandra Theis, Ruth A Singer, Diana Garofalo, Alexander Paul, Anila Narayana, and Lori Sussel
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

See previous review

Comments for the author

The authors have satisfactorily responded to my concerns.

Reviewer 3

Advance summary and potential significance to field

GRG/TLE corepressors interact with key pancreas transcription factors, but whether they have a role in pancreas development has not been well studied. In this manuscript, Theis et al. examine multiple mouse models to show that Grg3/4 are essential for beta cell development. Genetic deletion of Grg3/4 with Pdx1-Cre causes a striking Foxa1 upregulation and induces expression of liver-specific genes in the mutant pancreas.
The beta cell transcription factor Neurod1, Ins1, Ins2 and other islet hormones are also downregulated and the mice develop diabetes. In addition, the authors demonstrate that Grg4 compensates for loss of Grg3 in the
developing pancreas.

Comments for the author

The previous comments have been carefully addressed.