EMBO reports

Manuscript EMBOR-2013-38181

BIG3 inhibits insulin granule biogenesis and insulin secretion

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Review timeline:

Submission date:	04 November 2013
Editorial Decision:	21 November 2013
Revision received:	08 March 2014
Editorial Decision:	11 March 2014
Revision received:	14 March 2014
Accepted:	14 March 2014

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 21 November 2013

Thank you for your patience while your manuscript was peer-reviewed. We have now received the full set of referee reports that is pasted below.

As you will see, while referee 2 is more critical, both referees 1 and 3 support publication of the manuscript by EMBO reports. However, they also suggest several experiments to further strengthen the findings, which should all be performed. Upon cross-commenting on each other's reports, referees 1 and 3 agree that not all the concerns raised by referee 2 need to be addressed. Specifically, no mechanistic insight into how BIG3 inhibits insulin secretion needs to be provided, and points 1, 6, 8, 9 of referee 2 do not need to be experimentally addressed. However, both referees 2 and 3 agree that it should be investigated whether BIG3 is expressed in other islet cells, that larger EM fields should be shown and morphometric analyses of EM images performed to show BIG3 on

insulin granules and TGN, that the effect of BIG3 on insulin mRNA levels should be analyzed, that a loading control of MIN6 cell extracts should be included, that numbers of cells and mice should be added, and that all concerns should be addressed and discussed in the manuscript text, as for example the potential role of BIG3 in the brain. Referee 1 agrees with referee 2 that the antibody should be tested on BIG3 wild type and knockout islets, that insulin secretion in BKD cells should be rescued by overexpression of a BIG3 version that is insensitive to RNAi, and that better quality images should be provided for figure 7 E, F.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. The current character count largely exceeds our limits, and the manuscript text therefore needs to be shortened. Shortening may be made easier by combining the results and discussion into a single section. This may help to eliminate some redundancy that is inevitable in discussing the same experiments twice. The reference style also needs to be changed to the numbered EMBO reports style, and this will help to reduce the character count. Please note that materials and methods essential to understanding the experiments described in the main body of the manuscript must remain in the main manuscript file. Please let me know if you have questions regarding manuscript shortening.

Regarding data quantification, can you please also specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and tests used to calculate p-values in the respective figure legends? This information needs to be provided in the figure legends.

We recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate source data file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one file per figure or per figure panel.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any further questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

The study by Li et al. describes a new negative regulator of insulin secretion and provides very interesting data on its role in glucose homeostasis in mice. I am very happy to see more and more data accumulating supporting the concept in which hyperinsulinemia is causing insulin resistance rather than being a consequence of the latter, an important and very topical notion in the type 2 diabetes field.

The manuscript is well written and has a clear and logic build-up. The data are consistent and solid with very few exceptions (see below). Conclusions are raised appropriately and data are well discussed. The message of this study is clear and definitely merits publication in EMBO reports after a minor revision.

Specific comments:

The authors should discuss why enhanced insulin granule biogenesis leads to enhanced secretion/exocytosis and not only to enhanced insulin storage.

The author should also discuss the fact that they are dealing with a global BIG3 knockout despite a restricted expression pattern of BIG3 in endocrine pancreas.

In the same context, expression in skeletal muscle and adipose tissue should be added to the manuscript having particularly in mind a remarkable insulin resistance in skeletal muscle.

The authors should discuss why only stimulated insulin secretion is affected.

The authors should provide data in MIN6 cells overexpressing BIG3 to substantiate its negative regulation on stimulated insulin secretion.

The authors should cite more thoroughly work related to regulation of insulin granule biogenesis in particular in the context of ARF1 function.

Data providing evidence for enhanced granule biogenesis in Figure 7 are not very convincing; in particular in panel E and F, differences are minor.

Referee #2:

In this manuscript Li and coworkers investigated the involvement of newly identified BIG3, a member of Arf-GEF proteins in pancreatic beta cells. Based on studies in mouse insulinoma MIN6 cells depleted of BIG3 and BKO mice the authors conclude that this protein inhibits biogenesis and maturation of insulin secretory granules. The data are intriguing and potentially interesting but yet too preliminary to support these conclusions. Moreover, no mechanistic insights are provided as to how BIG3 would inhibit granule biogenesis and maturation.
Specific comments
1) The authors generated an antibody directed against BIG3 and verified its specificity by western blotting in brain lysates of wild type (Fig. S1A) and BIG3 KO mice (Fig. 3B). Oddly, no data are presented about the immunoreactivity of this antibody on pancreatic sections and islet extracts of BIG3 KO mice.
2) The immunostainings presented in Fig. 1B suggest that BIG3 expression is enriched in insulin+ cells of the pancreatic islets. What about its expression in other pancreatic endocrine cells, such as glucagon+- and somatostatin+-cells? Double labelings of BIG3 with acinar or ductal markers are also not shown.
3) The immunoelectronmicroscopy image presented in Fig. 1D is not satisfactory. To evaluate the

enrichment of BIG3 on insulin secretory granules it would be necessary to present a larger field, inclusive of other beta cell compartments and especially of the Golgi complex in view of the data presented later in Fig. 6. Moreover, these data would have to be corroborated with morphometric analysis.
4) To investigate whether BIG3 affects insulin secretion, the authors generated a stable MIN6 cell line (BKD) depleted of BIG3 (fig. 2A). The data presented in Fig. 2B suggest that upon stimulation with high glucose and high potassium insulin secretion is increased in BKD cells compared to control cells. In this case the authors should exclude an insertional effect of the construct for BIG3 knockdown by assessing insulin secretion in several independent BKD cell lines and/or in cells transiently depleted of BIG3. To exclude an off-target effect of the RNAi strategy, it should be verified whether insulin secretion in BKD cells could be restored by overexpression of a BIG3 construct insensitive to RNAi. Third, information should be provided about insulin content in BKD and control cells and thus about the insulin stimulatory index. The text or the figure legend should also specify the time interval considered to estimate total insulin secretion (AUC values; Fig. 2C). Notably, capacitance measurements suggest that membrane expansion in BKD cells is increased by 46% relative to control cells (Fig. 2E and text), while the data shown in Fig. 2B and C suggest a greater discrepancy. Reason for this apparent discrepancy should be given.
4) Fig. 4E-G. More extensive morphometric data on BIG3 and control mice should be provided, such as total number of granules/cell, total number of beta cells counted/mouse in each group, inclusive of standard error.
5) Fig. 4H-I. These data indicate that the insulin and pro-insulin content in BKO mice is increased relative to controls. For interpretation of these data it is critical to assess whether deletion of BKO alters insulin mRNA levels.
6) The data presented in Fig. 5 provide convincing evidence for increased insulin resistance in BKO mice compared to control mice. The authors suggest that this phenotype could be secondary to increase insulin release in BKO mice. Given the high expression levels of BIG3 in the brain, it cannot be excluded that some of the traits presented by BKO mice, such as increased hepatic gluconeogenesis, may not results from alterations of autonomic control of the liver by the CNS. To answer this question deletion the analysis would have to be extended to mice with beta-cell restricted deletion of BKO.
7) Fig. 6. As already indicated at point 1, enrichment of BIG3 on granules near the TGN (immature granules?) but not at the plasma would have to be corroborated by quantitative immunoelectronmicroscopy. The suggestion that BIG3 may play an inhibitory role on granule biogenesis based on the confocal data presented in Fig. 6 is entirely speculative.
8) Fig. 6B. To provide evidence for depletion of insulin granules upon stimulation with high potassium the authors would have to perform double labelings with insulin rather than with CGA. Indeed, in Fig. 6A, peripheral granules were only detected upon staining for insulin, but not for CGA. The contention that BIG3 is enriched in newly generated granules would have to be further strengthened by double labelings for furin or mannosidase receptor that are missorted into immature granules prior to their removal during granule maturation.
9) Fig. 7C does not include a control for equivalent loading of controls and BKD MIN6 cell extracts. Moreover, Fig. 7C show greater levels of β-granin in BKD cells compared to control cells, while lanes corresponding to time 0 in Fig. 7F suggest the opposite. To corroborate that BIG3 is implicated in granule biogenesis pulse-chase labeling for proinsulin-insulin conversion would have to be performed in BKO and control islets.
Minor comments:
1) The description of the methods is very generic and insufficient experimental details are provided.
2) According to EMBO policy, the "data not shown" mentioned at page 11 would have to included or not mentioned.

Referee #3:

Overall, I like this work showing the novel BIG3 to be a negative modulator of insulin granule biogeneisis and insulin secretion. There has been little progress in the area of insulin granule biogenesis, thus this is an important advance to the field. The data shown is convincing. However, I have some minor concerns, and addressing them will increase the rigour of this study and strengthen their conclusions.

1. In Fig. 1, BIG3 was convincingly shown to be in the insulin core of insulin granules in beta cells. However, they should also show if BIG3 is absent in other islet cells - alpha and delta cells. Its presence would suggest perturbation in glucagon (or somatostatin) secretion which would have effects on liver and muscle, contributing to the alteration of glucose homeostasis observed in the Big3KO mouse. If not present in non-beta cells, some explanation could be given as to why a factor important for granule biogenesis would only influence one endocrine cell type and not others within the islet.

2. Figs. 2 and 4 show BIG3 is a negative modulator of insulin secretion, whereby BIG3 KO increases insulin secretion by increased insulin granule biogenesis - granule number and insulin content. The very specific expression of BIG3 in beta cells vs the ubiquitous presence of BIG1 and BIG2, can potentially suggest that BIG3 may be competing with BIG1 or BIG2, and its absence would relieve BIG1 or 2 to manifest their actions? Levels of BIG1 and 2 should also be shown in the BIG3 KO islets or knockdwon insulinoma cell line. This may be consistent with a point they made in the Discussion that BIG3 has a non-functional catalytic motif in its Sec7 domain.

3. Fig. 5 shows that BKO mice developed insulin resistance (liver and muscle) presumably from the exposure of these tissues to the enhanced insulin release. They should also show blood insulin secretion during the IPGTT, eventhough this was shown on the islet perifusion data.

Thank you very much for editing our study by Li *et al.* for consideration of publication in the *EMBO reports*. I also would like to thank the three referees for their generous and insightful comments. We have followed their suggestions in preparing the revised manuscript. To conform to the word limit, I have made extensive changes to the text including combining the Results and Discussion sections, and moving description of certain experimental details to the Expended View section. Please see below for details:

Referee: 1
The study by Li et al. describes a new negative regulator of insulin secretion and provides very interesting data on its role in glucose homeostasis in mice. I am very happy to see more and more data accumulating supporting the concept in which hyperinsulinemia is causing insulin resistance rather than being a consequence of the latter, an important and very topical notion in the type 2 diabetes field.
The manuscript is well written and has a clear and logic build-up. The data are consistent and solid with very few exceptions (see below). Conclusions are raised appropriately and data are well discussed. The message of this study is clear and definitely merits publication in EMBO reports after a minor revision.

Specific comments:
1 The authors should discuss why enhanced insulin granule biogenesis leads to enhanced secretion/exocytosis and not only to enhanced insulin storage.

A: We thank Referee 1 for the favourable and enthusiastic comments. We also appreciate the insightful comment regarding the link between enhanced granule biogenesis and enhanced secretion. We have included the following sentence in the revised text on Page 10:

"Consistent with preferential release of newly synthesized insulin granules (Duncan et al, 2003; Gold et al, 1982; Pedersen & Sherman, 2009), increased granule biogenesis leads to larger fresh insulin granule pool, and consequently enhanced secretion upon stimulation."

2 The author should also discuss the fact that they are dealing with a global BIG3 knockout despite a restricted expression pattern of BIG3 in endocrine pancreas.

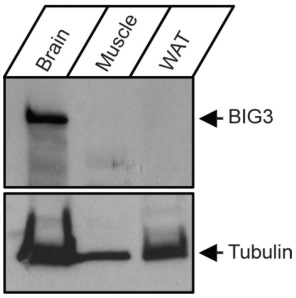
A: We appreciate this comment, and have added the following sentence in the revised version on Page 11:

"It is worth noting that BIG3 is also expressed in the brain (Figure 1A). Whether BIG3 in the CNS contributes to the observed metabolic phenotypes remains to be determined."

3 In the same context, expression in skeletal muscle and adipose tissue should be added to the manuscript having particularly in mind a remarkable insulin resistance in skeletal muscle.

A: As suggested, we have performed Western blotting experiments to examine the expression of BIG3 in skeletal muscle and adipose tissue. There is no detectable BIG3 protein in these tissues, consistent with information from relevant online resources. The lack of BIG3 in these tissues, especially skeletal muscle, further supports the notion that hyperinsulinemia may lead to insulin resistance. The new data (below) are included in the revised manuscript as Supplemental Figure S1C.

FIgure S1C



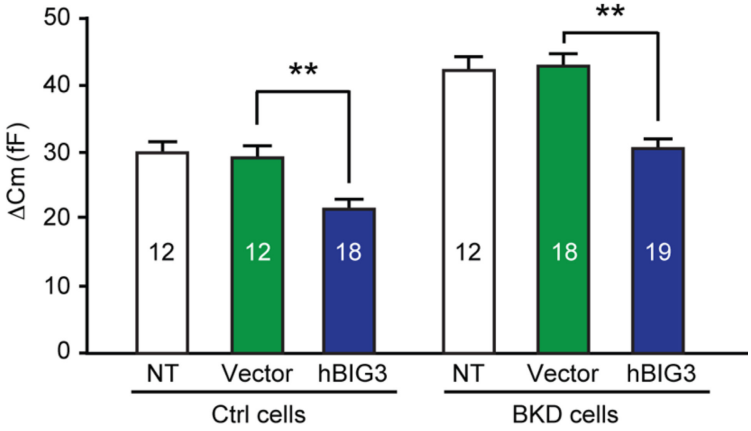
4 The authors should discuss why only stimulated insulin secretion is affected.

A: Although we observed a trend of increased basal insulin secretion from BIG3-deficient β-cells, the difference was not statistically significant. This was likely the result that basal insulin secretion in MIN6 cells and isolated islets were much smaller when compared with GSIS in our insulin secretion assays. This is consistent with the observations that the resting and fasting insulin levels were not significantly different in the BIG3 knockout and control mice. Due to the space constraint, we did not include this in the revised text. However, we would be prepared to include this in the discussion should Referee 1 feel that this is an important point.

5 The authors should provide data in MIN6 cells overexpressing BIG3 to substantiate its negative regulation on stimulated insulin secretion.

A: We thank Referee 1 for this insightful comment. We have performed overexpression experiments in control MIN6 cells, and included the results as Figure 1J (also attached below). BIG3 overexpression in control cells resulted in decreased exocytosis, consistent with the negative regulation by BIG3. We have also performed a rescue study by expressing BIG3 in BIG3 KD MIN6 cells. As shown in the figure below and Figure 1J in the revised manuscript, BIG3 expression completely rescued the KD phenotype, thus confirming the specificity of BIG3 deletion in the observed phenotypes.

Figure 1J



6 The authors should cite more thoroughly work related to regulation of insulin granule biogenesis in particular in the context of ARF1 function.

A: we have added relevant citations in the revised Introduction section on Page 3.

7 Data providing evidence for enhanced granule biogenesis in Figure 7 are not very convincing; in particular in panel E and F, differences are minor.

A: We agree with Referee 1 that more direct evidence to address granule biogenesis would be greatly helpful. This has proven to be quite technically challenging and we are still exploring the best approach to tackle the problem. The mild difference in the two panels was likely due to the short time window in the assays (30 min for Figure 7E and 15 min for Figure 7F). Understanding from the referee would be greatly appreciated.

Referee #2:
In this manuscript Li and coworkers investigated the involvement of newly identified BIG3, a member of Arf-GEF proteins in pancreatic beta cells. Based on studies in mouse insulinoma MIN6 cells depleted of BIG3 and BKO mice the authors conclude that this protein inhibits biogenesis and maturation of insulin secretory granules. The data are intriguing and potentially interesting but yet too preliminary to support these conclusions. Moreover, no mechanistic insights are provided as to how BIG3 would inhibit granule biogenesis and maturation.
Specific comments
1) The authors generated an antibody directed against BIG3 and verified its specificity by western blotting in brain lysates of wild type (Fig. S1A) and BIG3 KO mice (Fig. 3B). Oddly, no data are presented about the immunoreactivity of this antibody on pancreatic sections and islet extracts of BIG3 KO mice.

A: We thank Referee 2 for the encouraging comments. We have now included the data on pancreatic sections (supplemental Figure S3) and islet extracts (Figure 2D) of BIG3 KO mice in the revised manuscript and attached below.

Figure S3

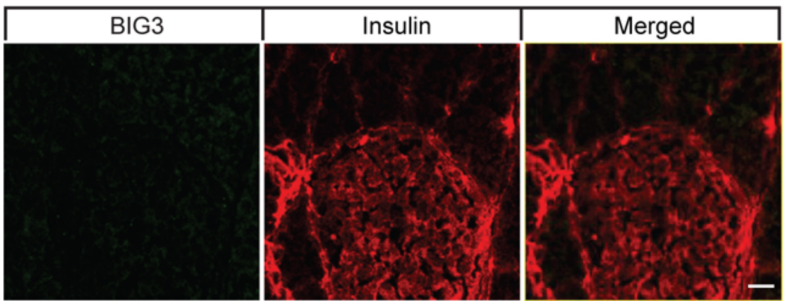
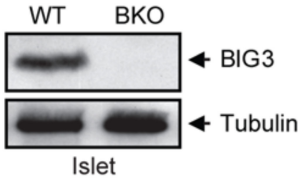


Figure 2D



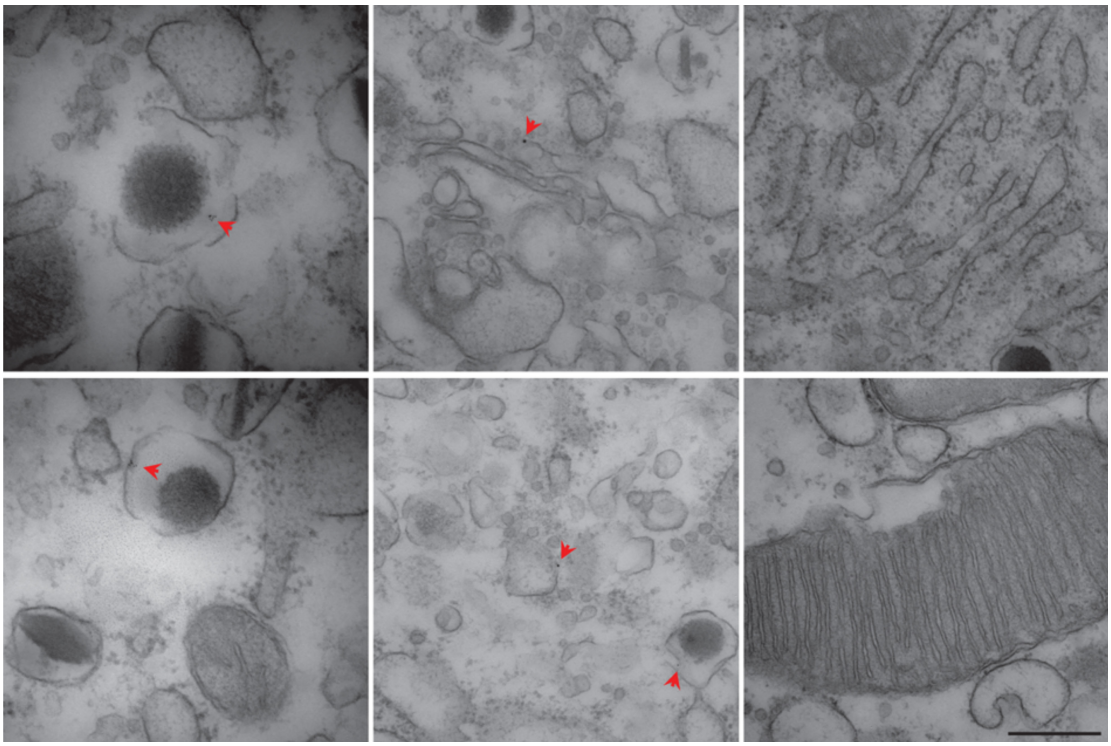
2) The immunostainings presented in Fig. 1B suggest that BIG3 expression is enriched in insulin+ cells of the pancreatic islets. What about its expression in other pancreatic endocrine cells, such as glucagon+- and somatostatin+-cells? Double labelings of BIG3 with acinar or ductal markers are also not shown.

A: We examined expression of BIG3 in α- and δ-cells, and the results are attached below. As shown below, BIG3 appeared to be expressed in glucagon-positive cells, but not in somatostatin-positive cells, or exocrine pancreatic cells. As the current study focuses on BIG3 in insulin secreting cells, and we are working on BIG3 function in glucagon secreting cells, we kindly request to keep the data below for future reports, instead of presenting them as a supplemental figure in the current study.

Data not shown

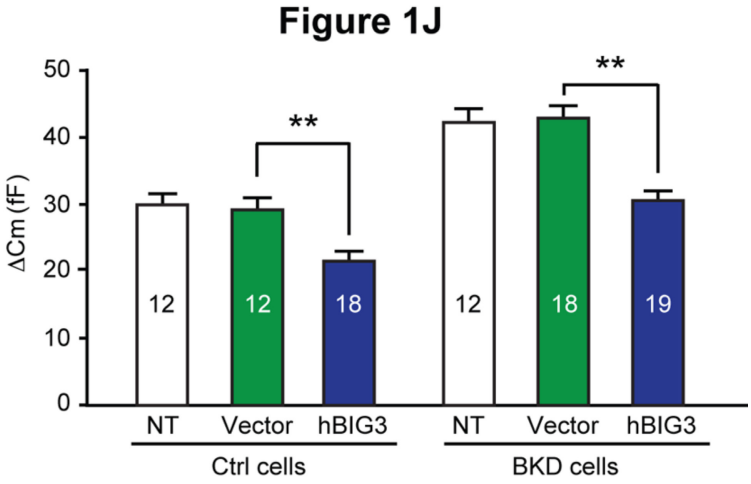
3) The immunoelectronmicroscopy image presented in Fig. 1D is not satisfactory. To evaluate the enrichment of BIG3 on insulin secretory granules it would be necessary to present a larger field, inclusive of other beta cell compartments and especially of the Golgi complex in view of the data presented later in Fig. 6. Moreover, these data would have to be corroborated with morphometric analysis.

A: We have attached below several immuno-EM images to show BIG3 localization, mostly on dense core granules, along with some signals on the Golgi. Almost no gold particles were detected in the ER, plasma membrane, mitochondria, or other organelles. The EM data qualitatively demonstrate the distribution pattern of BIG3 in β-cells.



4) To investigate whether BIG3 affects insulin secretion, the authors generated a stable MIN6 cell line (BKD) depleted of BIG3 (fig. 2A). The data presented in Fig. 2B suggest that upon stimulation with high glucose and high potassium insulin secretion is increased in BKD cells compared to control cells. In this case the authors should exclude an insertional effect of the construct for BIG3 knockdown by assessing insulin secretion in several independent BKD cell lines and/or in cells transiently depleted of BIG3. To exclude an off-target effect of the RNAi strategy, it should be verified whether insulin secretion in BKD cells could be restored by overexpression of a BIG3 construct insensitive to RNAi.

A: We thank Referee 2 for this insightful comment. We have performed the suggested experiment, and presented the results in the revised manuscript as Figure 1J and attached below. BIG3 expression completely rescued the KD phenotype, thus confirming the specificity of BIG3 deletion in the observed phenotypes.



Third, information should be provided about insulin content in BKD and control cells and thus about the insulin stimulatory index. The text or the figure legend should also specify the time interval considered to estimate total insulin secretion (AUC values; Fig. 2C). Notably,capacitance measurements suggest that membrane expansion in BKD cells is

increased by 46% relative to control cells (Fig. 2E and text), while the data shown in Fig. 2B and C suggest a greater discrepancy. Reason for this apparent discrepancy should be given. insulin stimulatory index?

A: We have now included the information on the time interval (0-60 min) in estimating total insulin secretion in the figure legend for Figure 1G (old Figure 2C) in the revised text on Page 16. The cellular content of insulin and proinsulin in islets and MIN6 cells, and insulin secretion measurements were all normalized to total protein. This is a well-accepted method and comparable to normalization to DNA content. We appreciate the comments on the discrepancy between electrophysiology and biochemical measurements of insulin granule exocytosis and insulin secretion, respectively. Capacitance measurements are limited to short time intervals (seconds) of exocytosis events, while biochemical assays cover a much longer time interval (tens of minutes). The greater difference over longer period as measured by biochemical assays may be due to the larger amount of releasable and reserve granules in BKD cells.

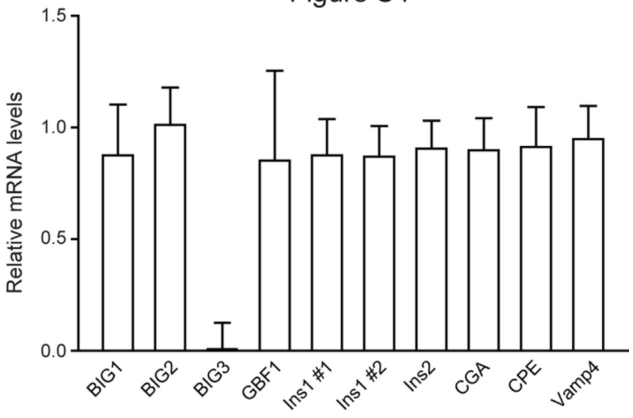
Fig. 4E-G. More extensive morphometric data on BIG3 and control mice should be provided, such as total number of granules/cell, total number of beta cells counted/mouse in each group, inclusive of standard error.

A: Morphometric data on BKO and control mice, including secretory granule density and dense core size, along with islet area, number and size, are now included in the revised manuscript as Figure 2N-2V.

5) Fig. 4H-I. These data indicate that the insulin and pro-insulin content in BKO mice is increased relative to controls. For interpretation of these data it is critical to assess whether deletion of BKO alters insulin mRNA levels.

A: We have added realtime quantitative PCR results in supplemental Figure S4 in the revised manuscript and also attached below. Insulin mRNA levels did not appear to be affected by BIG3 deletion.

Figure S4



6) The data presented in Fig. 5 provide convincing evidence for increased insulin resistance in BKO mice compared to control mice. The authors suggest that this phenotype could be secondary to increase insulin release in BKO mice. Given the high expression levels of BIG3 in the brain, it cannot be excluded that some of the traits presented by BKO mice, such as increased hepatic gluconeogenesis, may not results from alterations of autonomic control of the liver by the CNS. To answer this question deletion the analysis would have to be extended to mice with beta-cell restricted deletion of BKO.

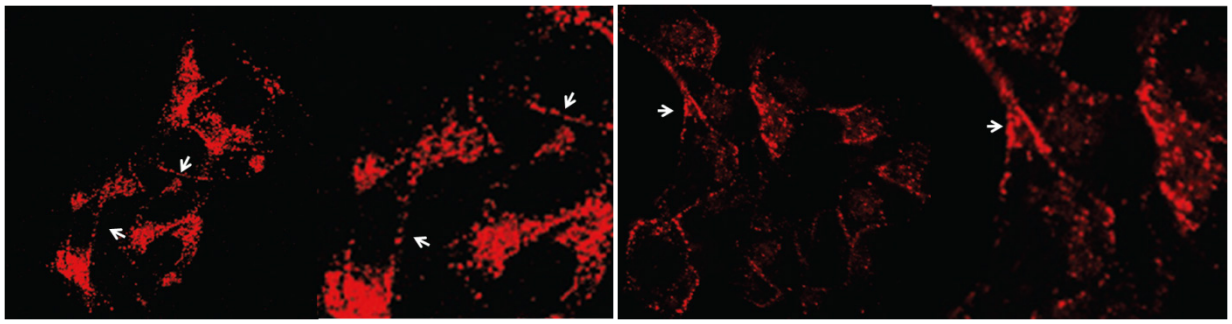
A: We appreciate the potential role of BIG3 in the brain on certain metabolic phenotypes, and have noted this in the revised text on Page 11.

7) Fig. 6. As already indicated at point 1, enrichment of BIG3 on granules near the TGN (immature granules?) but not at the plasma would have to be corroborated by quantitative immunoelectronmicroscopy. The suggestion that BIG3 may play an inhibitory role on granule biogenesis based on the confocal data presented in Fig. 6 is entirely speculative. Fig. 6 quantitative immunoelectronmicroscopy

A: We have provided additional EM images above to illustrate the BIG3 distribution pattern. In the revised manuscript, we have also performed functional measurements to show that overexpressing BIG3 in the wildtype MIN6 cells caused a reduction in insulin granule exocytosis, consistent with BIG3's inhibitory role on granule biogenesis (please see above and Figure 1J).

8) Fig. 6B. To provide evidence for depletion of insulin granules upon stimulation with high potassium the authors would have to perform double labelings with insulin rather than with CGA. Indeed, in Fig. 6A, peripheral granules were only detected upon staining for insulin, but not for CGA. The contention that BIG3 is enriched in newly generated granules would have to be further strengthened by double labelings for furin or mannosidase receptor that are missorted into immature granules prior to their removal during granule maturation.

A: As Chromogranins, such as CGA, are considered to be the aggregation factors to initial granule biogenesis, we used CGA as the marker instead of insulin. Clathrin coat, which is marked by γ-adaptin, is enriched at TGN for vesicle formation and sorted into immature secretory granules and removed during maturation. It should provide similar information as furin or mannosidase receptor to indicate the property of BIG3 positive granules. Both CGA (arrows) and insulin mark peripheral granules beneath the plasma membrane, but not BIG3 (from old Figure 6 and new Figure 4).



9) Fig. 7C does not include a control for equivalent loading of controls and BKD MIN6 cell extracts. Moreover, Fig. 7C show greater levels of β-granin in BKD cells compared to control cells, while lanes corresponding to time 0 in Fig. 7F suggest the opposite. To corroborate that BIG3 is implicated in granule biogenesis pulse-chase labeling for proinsulin-insulin conversion would have to be performed in BKO and control islets.

A: We have now included the loading control in the new Figure 5C (old Figure 7C). The relative amounts of proteins shown in Figure 5D were normalized to actin. Time 0 in Figure 7F is the time point immediately after isotope labeling, when very little, if any, mature form (β-granin, lower bands) could be converted from precursor (CGA, upper bands). As shown in subsequent time points, increased amount of β-granin was produced from CGA. At the 60 min time point, there was higher level of β-granin in BKD cells, in agreement with the observation in MIN6 cells or islets under static condition. In other words, the 60 min time point in Figure 5F would be more comparable to Figure 5C. Due to the low rate of protein synthesis and huge amount of insulin storage in isolated islets, the isotope-labeled pulse-chase and co-IP methods would be ineffective to examine the prohormone processing.

Minor comments:
1) The description of the methods is very generic and insufficient experimental details are provided.

A: Detailed methods are added in the Expended View section.

2) According to EMBO policy, the "data not shown" mentioned at page 11 would have to included or not mentioned.

A: This has been amended.

Referee #3:
Overall, I like this work showing the novel BIG3 to be a negative modulator of insulin granule biogeneisis and insulin

secretion. There has been little progress in the area of insulin granule biogenesis, thus this is an important advance to the field. The data shown is convincing. However, I have some minor concerns, and addressing them will increase the rigour of this study and strengthen their conclusions.
1. In Fig. 1, BIG3 was convincingly shown to be in the insulin core of insulin granules in beta cells. However, they should also show if BIG3 is absent in other islet cells - alpha and delta cells. Its presence would suggest perturbation in glucagon (or somatostatin) secretion which would have effects on liver and muscle, contributing to the alteration of glucose homeostasis observed in the Big3KO mouse. If not present in non-beta cells, some explanation could be given as to why a factor important for granule biogenesis would only influence one endocrine cell type and not others within the islet.

A: We thank Referee 3 for the favorable comments. As stated above, we examined expression of BIG3 in α- and δ-cells. BIG3 appeared to be expressed in glucagon-positive cells, but not in somatostatin-positive cells, or exocrine pancreatic cells. As the current study focuses on BIG3 in insulin secreting cells, and we are working on BIG3 function in glucagon secreting cells, we would like to keep the data for future reports, instead of presenting them as a supplemental figure in the current study.

2. Figs. 2 and 4 show BIG3 is a negative modulator of insulin secretion, whereby BIG3 KO increases insulin secretion by increased insulin granule biogenesis - granule number and insulin content. The very specific expression of BIG3 in beta cells vs the ubiquitous presence of BIG1 and BIG2, can potentially suggest that BIG3 may be competing with BIG1 or BIG2, and its absence would relieve BIG1 or 2 to manifest their actions? Levels of BIG1 and 2 should also be shown in the BIG3 KO islets or knockdwon insulinoma cell line. This may be consistent with a point they made in the Discussion that BIG3 has a non-functional catalytic motif in its Sec7 domain.

A: We thank Referee 3 for this insightful comment. We have added Western blots of insulinoma cells and realtime PCR analysis of islets as Figure 1E and supplemental Figure S4 in the revised manuscript. BIG1/BIG2 and GBF1 expression was not significantly changed. Measurements of BIG/GBF bioactivity in granule biogenesis in the presence or absence of BIG3 is technologically challenging. We are searching for possible assays to examine this possibility.

Figure 1E

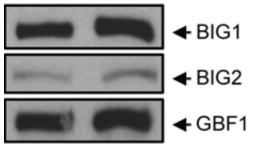
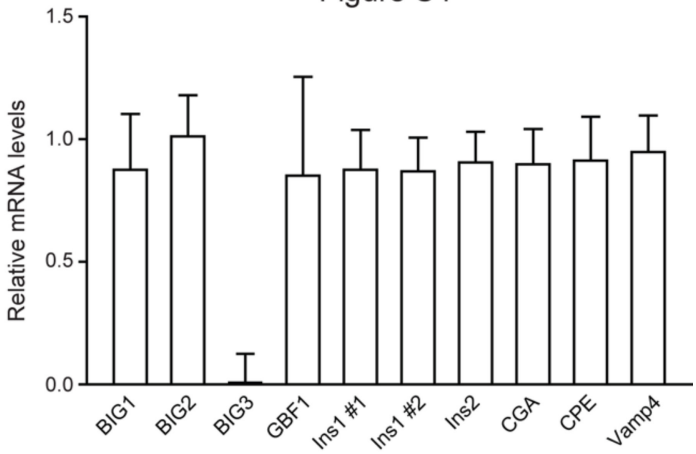


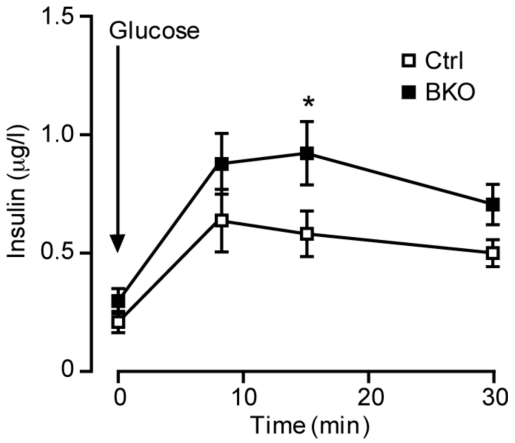
Figure S4



3. Fig. 5 shows that BKO mice developed insulin resistance (liver and muscle) presumably from the exposure of these tissues to the enhanced insulin release. They should also show blood insulin secretion during the IPGTT, eventhough this was shown on the islet perifusion data.

A: We have performed the insulin secretion measurements during the GTT and included the data as Figure 3F in the revised manuscript and attached below.

Figure 3F



Again, I would like to thank you for the support, and the referees for taking their precious time in offering the expert advice to our study. I hope that the referees find the revised version and replies to their queries satisfactory, and that our study is now ready for publication. Please feel free to contact me if I can provide any additional information.

2nd Editorial Decision 11 March 2014

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees support publication of the study in EMBO reports. Referee 3 only has a minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

I noticed that the error bars, "*" and tests to calculate p-values are not specified in the figure legends. This information must be added to the figure legends (1-3 and 5) and can be deleted from the supplementary methods. In figure S2, there is a tiny space after the first 2 columns. If the bands have been cropped from a bigger gel, or come from different gels, this needs to be clearly indicated by a white space and black line. Can you please do so? It would be best to show the entire gels and label the bands of interest.

I would like to suggest to change the title to "BIG3 inhibits insulin granule biogenesis and insulin secretion" , please let me know whether you agree.

Since January, EMBO reports papers are accompanied online by a short summary of the key findings and their significance, 2-3 bullet points highlighting key results, and a synopsis image. Can you please send us the short summary and bullet points and may be a model of your findings in the synopsis image? The image must be 211x157 pixels large, and cannot contain a lot of information due to the small size. If you prefer, the image could also show a key result instead of a model.

I look forward to receiving the newly revised manuscript and the additional information.

REFEREE REPORTS:

Referee #1:

I am satisfied with the revision and would recommend publication in EMBO reports.

Referee #3:

I am referee 3. All that I had asked for had been addressed. Importantly, the key message of this report is proving the role of BIG3 in insulin granule biogenesis, and that they have done to my satisfaction. Other aspects that need further work, such as the possible systemic effects of BIG3 i.e. brain, and precise mechanisms on insulin secretion (probably preferential release of newly synthesized insulin granules), are not within the scope of this paper.

Regarding the effects of insulin on peripheral resistance. this paper is probably the more appropriate one to cite.
Mehran AE, Templeman NM, Brigidi GS, Lim GE, Chu KY, Hu X, Botezelli JD, Asadi A, Hoffman BG, Kieffer TJ, Bamji SX, Clee SM, Johnson JD. Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metab. 2012 Dec 5;16(6):723-37.

I was asked to comment of Ref. 2 critique. These workers have addressed almost all (>90%) that this Ref. had asked. I think the evidence provided from this work is sufficiently 'mechanistic'. And overall, I would consider their responses including the new experiments done to be satisfactory. A few experiments requested I thought were a bit 'over the top' ie. off target effects of RNAi and beta-cell specific KO; these need not be done as the current evidence to prove their point on the major conclusion is sufficient.

2nd Revision - authors' response 14 March 2014

Thanks again for editing our study. I have made the requested changes and attached the files here.

1. Revised manuscript file in MS Word format, which includes updated figure legends and the suggested reference.
2. A MS word file that contains a short summary of the key findings.
3. An image file for the synopsis. The image is at a higher resolution, but with the right scale as indicated.
4. An updated "Expanded View" file in pdf format, which includes an updated Figure S2.

Please let me know if you need any additional information.

3rd Editorial Decision 14 March 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.