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Peer Review File

Spint1 disruption in mouse pancreas leads to glucose intolerance and impaired insulin production involving HEPSIN/MAFA

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Strengths:
1. The findings are original and of potential significance in the field of diabetes and islet biology.
2. Experimental design is based on novel tools including transgenic mice with Spint1-LacZ reporter and Spint1 null mice.
3. Mechanistic studies were performed to suggest that Hepsin protease and its Spint1 inhibitor play a role in regulation of GLP1 receptor and insulin secretion.
4. The manuscript is well written and data are logically presented.

Major criticisms:
1. A number of critical experiments were performed in mouse cell lines only. The studies on functional relationship between Spint1, Hepsin and MafA expression described in Figures 4 and 5 were performed using NIT-1 and Min6 cells rather than primary mouse and human beta cells. Moreover, coimmunoprecipitation study with overexpressed GLPR1, Spin1 and hepsin was performed in 293T cells At this point it is unclear whether these molecules interact with each in normal beta cells.
2. Diabetes studies are scant and performed at the time of disease onset. The authors did not measure beta cell mass in the setting of diabetes, nor developed any therapeutic approach that would suggest that Spint1 plays a role in recovery from disease. Moreover, they used 5 injections of STZ as a model, which is known to stimulate immunological response against pancreatic islets. The possibility that Spint1 might regulate this reponse has not been investigated or discussed.
3. In morphometric studies on islets the authors described use of one pancreatic section every twenty sections. Assuming that sections were 5 micrometers thick, they examined tissue levels 100 microns apart, which is less than diameter of many islets. Therefore, it is very likely that the same islets were taken for the analysis from different levels. In addition, in most experiments the groups are very small and it is unclear how many times experiments were performed. The average of independent experiments should be shown.
4. The mechanism leading to increased beta cell proliferation following inhibition of Spint1 has not been examined.
5. The manuscript lacks translational studies in human, and therefore the overall significance of described findings is limited.
6. Discussion is limited in scope. The authors did not discuss their findings in relations to what has been already published on proteases in beta cell biology and diabetes.

Additional specific comments:
1. Organization of the paper: Data from Figure 1 should go to supplement while the supplementary data on diabetes should be moved to the main body of the manuscript.
2. A finding of increased Ki67 staining confined to the large islets is interesting but should be independently confirmed by labeling cells with nucleotide analogs. Discussion should address why big rather than small and mid-size islets showed renewal activity on Spint1 null background.

3. It would be beneficial to have side by side IF, IHC or Western blot data showing expression of Spint1 and Hepsin in beta versus alpha cells in mouse and human.
4. The use of MG142 in Figure 5c should be explained.
5. The method of 293 cell transfections is not described
6. It is unclear why authors used combined IF/IHC staining to detect insulin and Ki67.
7. Aprotinin was used at 40 micrograms/mL (Figure 4e). This is very high concentration which may have caused nonspecific changes.
8. Information should be provided in figure legends as to which statistic test was used and how many independent experiments were performed for each subfigure.
9. In addition to representative images, Western blots (e.g., Figure 4g and i, and Figure 5b, c d and e) should be quantified, statistically analyzed, and their average expressed as data bars.
10. Figure 4i lacks Western blot with stained with antibody to Spint1.

Reviewer #2

(Remarks to the Author)
Subject: Disruption of Spint1 in mouse pancreatic β cells leads to glucose intolerance and 2 impaired insulin production–the involvement of HEPSIN/GLP1R/MAFA 3 signaling
Lin et al. report their study on SPINT1 glucose homeostasis and insulin production. SPINT1 is a serine protease inhibitor regulating pericellular proteolysis. The authors reported that pancreas-specific disruption of Spint1 in mice decreased MafA and insulin as well as islet size and mass, resulting in glucose intolerance. Hepsin is a Spint1 target protease, and Hepsin silencing counteracted the effect of Spint1 knockdown on MafA and Ins1. Hepsin overexpression increased GLP1R cleavage while Spint1 overexpression did the opposite. Similarly, Spint1 silencing reduced Exendin-4–induced GLP1R activation, while Hepsin knockdown restored these effects. They concluded that SPINT1 regulates glucose homeostasis and insulin production via the HEPSIN/GLP1R/MAFA signaling in β cells.
Overall, the study is interesting and well designed, and most conclusions are supported by the substantial amount data, and enough detail provided in the methods for the work to be reproduced. However, they are some concerns need to be addressed before publication.

Major concerns:
1. Is the gene expression or protein abundance of SPINT1 and HEPSIN different between Lean healthy and diabetic/insulin resistant humans? If yes, is it higher or lower in diabetic/insulin resistant humans vs Lean? Does it agree with the findings in animal or cell models in this study?
2. How many mice in each group (Spint1fl/fl and Spint1-/- islets) were used in the SILAC proteomic experiments? If only 1 mouse in each group (n=1), the quantification result will be highly unreliable, and the authors should either remove these results from the manuscript or increase the n to minimal 3. In addition, how many doubling of the NIT-1 cells before the SILAC labeling was stopped? What is the level of SILAC incorporation (e.g., >95%) observed after the doublings? Moreover, the quantification data need to be included as a supplemental table. Finally, the username and password are needed to access this dataset PXD039190.
3. Only male mice were used in the study. Please provide rationale for this and discuss potential results if female mice are used.
4. Line 499: Blood samples were kept at room temperature for 10 minutes for clotting. This may lead to degradation of insulin. It may be better to use another method to process the blood in order to measure insulin ASAP or keep the blood at 4 centigrade or lower.
5. In Figures and Supplemental Figures, some subpanels don't have sample size or P-value, and please add this information. If the sample size is n=1, the quantification result will be highly unreliable, and the authors should either remove these results from the manuscript or increase the n to minimal 3.
6. Figure 3 b, what is the P-value and # of molecules in each process? Figure 3 d, what is the # of molecules in each pathway? Figure 3 e, what is the P-value?

Minor concerns:
1. The full protein or gene name is needed when a protein or gene appears in the manuscript for the 1st time, with the gene name in a parenthesis.
2. Gene names for proteins in non-human cell lines or not in humans need to be small caps with the 1st letter capitalized, such as Mafa. The authors used this inconsistently throughout the manuscript.
3. Line 530: is it 16.5-mM?
4. In Figures and Supplemental Figures, the a, b, c, d,... need to be more obvious. Suggest changing to "a)."

Reviewer #3

(Remarks to the Author)
This manuscript looks at the role of Spint1 in pancreatic islets, proposing that hepsin is a target of Spint 1 in beta-cells, and that loss of Spint1 enhances hepsin activity to cleave GLP-1R and decrease islet function through alterations in Mafa. The rationale for these studies is questionable. Is there any evidence that Spint1 or Hepsin activity is either altered by or influences the pathogenesis of beta-cell dysfunction? Is there any evidence that GLP-1R is functionally altered through cleavage? The approach and methods have flaws, including the choice of mouse model and the use of an artificial system to interrogate the mechanism of action that is the center of this story. Overall, it is unclear what impact this work will have on

understanding the regulation of GLP-1R signaling in beta-cells or if this is a meaningful mechanism for diabetes. Some specific suggestions to help contextualize these comments are below:

Line 59 – Reference 9 does not demonstrate a downstream effect of GLP-1R is upregulation of Mafa expression. This paper looks at cAMP signaling, which is the canonical message utilized by GLP-1R, but this sentence is a stretch to say Mafa is specific for GLP-1R. A more appropriate reference is required, or a rephrasing/deletion of this sentence. References 10-12 do not directly address this either.

Line 60-63 – This concept should be considered in light of more recent data (PMIDs 28325479, 31495689), published after the 2007 reference used to support this sentence.

Line 64 – a 2004 reference to document the current landscape of GLP-1R agonists is not appropriate.

Line 65-67 – is there any rationale for assessing the proteolytic modification of GLP1R other than it hasn't been done? Is proteolytic modification of GPCRs a mechanism that regulates activity? This is alluded to with the references on line 75, but a clear focus on GPCRs is needed to provide rationale for the current investigation.

The choice of mouse model seems poor. First, Pdx1-Cre expresses strongly in the brain (PMIDs 20802254, 20824628, 23823474). Using the liver to demonstrate specificity is not appropriate. Second, with respect to the islet, Pdx1-Cre is not beta-cell specific and is expressed in other endocrine cell types, mostly notably the alpha-cell. Third, this model lacks temporal control, which seems essential given the reference literature on the developmental aspects of Spint1.

Line 524 - 36 mg/dl is not reflective of a normal blood glucose in any circumstance. 300 mg/dl is not reflective of postprandial glucose in healthy individuals.

Details on the perifusion are missing. What is the flow rate, what is the machine? The lack of GSIS in the knockouts is remarkable, but could potentially be a technical issue. It is surprising to see the samples were diluted 100-fold to get near bottom of the assay values (~0.2 ng/ml).

Details on the cDNA constructs are missing. The methods read as Glp1r cDNA was transfected, but an anti-flag antibody was used to pull down GLP-1R, making the assumption the Glp1r was flag-tagged. Is this commercially available? If constructed in house, where is the flag-tag and how does this impact GLP-1R signaling?

There is not functional assessment of the GLP-1R in the knockout models, rending the either mechanism to be supported by overexpression assays in the HEK cells. How much of this translates to primary islets.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript by Lin at al. describes a novel mechanism of regulation of GLPr1 in pancreatic beta cells. The authors propose that GLPr1 is cleaved by the serine protease, Hepsin, which in turn is regulated by the membrane-anchored inhibitor, Spint1. Spin1-deficient mice demonstrated reduced beta cell mass, impaired insulin content, lower expression of MafA transcription factor, and a somewhat worse outcome in STZ-induced diabetes. Since GLP1R agonists are or on the frontline as type 2 diabetes therapeutics, the described findings are potentially important to the broader community interested in diabetes research.

The authors made several improvements to their originally submitted work. The revised version presents a more compelling case for the interaction between GLP1R, Spint1 and Hepsin. The authors have also made the effort to perform experiments on primary mouse and human beta cells, and additional rescue experiments with siHepsin in the setting of Spint1 deficiency. Moreover, the discussion has been improved by providing an insightful explanation as to why beta cell proliferation and large, rather than small, pancreatic islets are affected by a Spint1 deficiency.

On the other hand, there are still the lingering issues of how certain experiments have been performed. First, the authors insist on examining pancreatic sections every 100 microns in their assessment of beta cell mass. While this may be fine for small islets, the larger islets, with a diameter of over 100 microns, and which account for the majority of beta cell mass, should be re-examined using sections every 300 and 600 microns to ensure that they are not counted twice. Second, the authors claim that proliferation is decreased in Spin1-deficient animals. These data are impossible for the reader to validate as the low resolution images fail to show beta cell co-staining with Ki67 or Brdu. In light of these limitations, the authors may consider flow cytometry to unequivocally demonstrate any potential differences in proliferation rates of beta and alpha cells. Third, Figure 1 convincingly shows strong positivity for Spint1 in the embryonal pancreatic ducts during. Therefore, it is possible that the primary impact of Spint1 may be on beta cell development, rather than proliferation. Finally, many of the Western blots have overexposed controls, suggesting that normalization has not been performed correctly.

Other comments:

Figure 1f describes islets with sizes of <100, 100-200 and >200 microns. Supplementary Figure 3h shows islets with sizes <50, 50-100, and >100. Is there a reason for this discrepancy?

Figure 2i. It is not mentioned how many times the experiment has been performed.

Figure 4a and b show augmented expression of Pdx1 – this finding is not mentioned in the Results or Discussion.

Figure 5 and 7: The statistical comparison between groups with siHepsin vs. siHepsin/siSpint1 is missing. It appears that the impact of siHepsin is limited when used in conjunction with siSpint1, suggesting that there are additional protease targets involved. In addition, Figure 5c does not show a group with siHepsin alone.

Supplementary Figure 6 shows peripheral distribution of Hepsin suggesting that it is primarily expressed in glucagon cells. Can the authors comment on this finding?

The paper by El Quaamarti et al is cited as both reference 34 and 45.

Reviewer #2

(Remarks to the Author)
Subject: Disruption of Spint1 in mouse pancreatic β cells leads to glucose intolerance and 2 impaired insulin production– the involvement of HEPSIN/GLP1R/MAFA 3 signaling

This is a revised manuscript and the authors have addressed most concerns adequately. However, they are some minor concerns need to be addressed before publication.

1. Figure 3b,d,e, please describe the meaning of 3/3, 6/7, 6/17, etc.
2. Figure 3b, how was the fold enrichment calculated? Why does the upper panel have x-axis with negative values?

Reviewer #3

(Remarks to the Author)
The Authors have clearly done a tremendous amount of work in revising this manuscript. However, many of the responses to the original critiques are inadequate and fail to address the underlying flaws of the overall message. For clarity, the fatal flaw in the data set is a lack of linking data from the comprehensive cell biology to a meaningful functional output. There is a knockout model in hand, and we can debate on the utility of this model, but there is no functional evidence with this reagent to demonstrate impaired GLP-1R signaling in beta-cells. Major observations from the knockout model include smaller islets that seem to be more susceptible to STZ, and an impaired glucose tolerance phenotype that is accompanied by decreases in insulin secretion. This associated with an expected phenotype of impaired GLP-1R signaling, but there is no data that directly links this. Overall, the message that can be derived from the current data set is that deletion of Spint1 at some point (perhaps during development, or perhaps in functional, mature beta-cells, or perhaps in some other cell type that is PDX1 positive) conveys a deleterious effect on beta-cell mass that confers glucose interlace. The relevance for the pathophysiology of impaired beta-cell function in diabetes is potentially there, but definitely not established. The relevance to GLP-1R activity is loosely associative at best.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is the second manuscript revision, in which authors made significant improvements in response to previous critiques. They addressed the problem of tissue sampling in morphometric analysis of pancreatic islets and convincingly discussed my suggestions regarding the possibility that Spint1 may play a role in beta cell development, and Hepsin may be expressed in glucagon-expressing cells. On the other hand, the authors have not provided an independent validation of proliferation data by flow cytometry and their Western blotting still has loading controls that appear overexposed. Setting aside these controversies, most of the data are convincing and at this time I am inclined to accept this revision as final and not requiring any additional experimental work.

Reviewer #2

(Remarks to the Author)
This is a revised manuscript and the authors have addressed almost all my concerns adequately. There is only one very minor concern need to be addressed before publication:

“Figure 3e, List of the top five diseases and disorders identified through IPA of the differentially regulated proteins in Spint1-/- islets compared to Spint1fl/fl islets. The fractions in the table indicate the proportion of genes associated with different diseases and disorders (numerator) relative to the total number of genes (denominator) in our dataset.”

It will be better to use the total number of genes (denominator) in each diseases and disorders, instead of total number of genes (denominator) in our dataset.

Reviewer #3

(Remarks to the Author)
No additional comments

Reviewer #1 (Remarks to the Author):

Major criticisms:

Comment 1. A number of critical experiments were performed in mouse cell lines only. The studies on functional relationship between Spint1, Hepsin and MafA expression described in Figures 4 and 5 were performed using NIT-1 and Min6 cells rather than primary mouse and human beta cells. Moreover, a coimmunoprecipitation study with overexpressed GLPR1, Spint1, and hepsin was performed in HEK293T cells. At this point it is unclear whether these molecules interact with each in normal beta cells.

Answer: Many thanks for your insightful comment.

1. In the revised manuscript, to address the functional relationship between *Spint1*, *Hepsin*, and *Mafa* expression in primary mouse β cells, we conducted the experiments using the primary islets of 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Q-RT-PCR analysis showed that *Spint1* depletion significantly suppressed the gene expression levels of *Ins1* and *Ins2* in the pancreatic islets of *Spint1*-knockout mice (Figure 4a in the revised manuscript, left panel). Moreover, the results from western blot analysis showed that *Spint1* deficiency reduced the protein levels of MAFA by approximately 90%, as opposed to PDX1 or NEUROD1 (Figure 4a in the revised manuscript, right panel). These data were in accordance with those obtained from *Spint1*-knockdown mouse NIT-1 and MIN6 cells (Figure 4b, 4c in the revised manuscript). Additionally, we used immunofluorescence (IF) microscopy to detect MAFA in the pancreatic islets of *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice and found that MAFA protein levels were significantly reduced in the insulin-positive regions of pancreatic islets of *Spint1*$^{-/-}$ mice (Figure 4d in the revised manuscript).
2. Moreover, in the revised manuscript, we used commercially available primary human pancreatic islets of Langerhans cells (Cat. No. ABC-TC4286, AcceGen, NJ, USA) as a primary cell model to address the role of *SPINT1* and *HEPSIN* in *MAFA* and *INS* expression through siRNA knockdown and Q-RT-PCR approaches. Similar to the results obtained from *Spint1*-knockout mice, the results also showed that *SPINT1* silencing increased *HEPSIN* expression and reduced the expression of *MAFA* and *INS* in human Langerhans islet cells (Figure 5c in the revised manuscript). Thus, data from both the primary pancreatic islets of *Spint1*-knockout mice and *SPINT1*-knockdown human Langerhans islet cells indicated that SPINT1 played a role in *Mafa*/*MAFA* and *Ins/INS* expression, at least partly *via* HEPSIN.
3. For coimmunoprecipitation study involving SPINT1, HEPSIN, and GLP1R, we have tried our best to search for proper antibodies and purchased several anti-SPINT1 (GTX114793, GTX02802, and AF1141) and anti-HEPSIN antibodies (Cayman 100022, Thermal Fisher PA5-30062, and Abcam ab189246). However, after immunoprecipitation tests, these anti-SPINT1 and anti-HEPSIN antibodies yielded limited success in pulling down endogenous SPINT1 and HEPSIN proteins from mouse primary pancreatic islets, NIT-1, and MIN6 cells. Furthermore, several lines of evidence have indicated that there is currently no useful GLP1R antibody for the detection of endogenous GLP1R in β cells through western blot analysis or immunoprecipitation[1]. Thus, it is not feasible to perform co-immunoprecipitation experiments to reveal whether these endogenous proteins interact with each other in normal β cells due to the unavailability of useful antibodies. Alternatively, since these three antibodies are suitable for immunocytochemical staining[2-4], we then performed the proximity ligation assay (PLA) using isolated normal human Langerhans

islet cells and normal human pancreas tissues to examine whether SPINT1, HEPSIN, and GLP1R directly interact each other in pancreatic β cells. The PLA results revealed that GLP1R could interact with SPINT1 and HEPSIN each other in the human Langerhans islet cells and human pancreatic slides (Figure 6a-b & Supplementary Figure 7c-d in the revised manuscript). Our findings were further corroborated by the facts that SPINT1 is widely recognized as a cognate inhibitor for HEPSIN[5], and a yeast-two hybrid assay[6] has shown the direct interaction between GLP1R and HEPSIN, while GLP1R has been found in β cells of the pancreas[7,8]. Collectively, the results of PLA from human pancreas islet cells and tissues indicated that there was close contact among GLP1R, SPINT1, and HEPSIN in pancreas β cells.

Comment 2. Diabetes studies are scant and performed at the time of disease onset. The authors did not measure beta cell mass in the setting of diabetes, nor developed any therapeutic approach that would suggest that Spint1 plays a role in recovery from disease. Moreover, they used 5 injections of STZ as a model, which is known to stimulate immunological response against pancreatic islets. The possibility that Spint1 might regulate this response has not been investigated or discussed.

Answer: Thank you for your valuable comments.

1. In response to this comment, we have performed experiments to analyze β cell mass in the context of STZ-induced diabetes. The results showed that the β cell mass of *Spint1*$^{-/-}$ mice was marginally lower than that of *Spint1*$^{fl/fl}$ mice after STZ-induced diabetes (Figure 2k in the revised manuscript). Compared to untreated mice, the β cell mass was markedly decreased in both *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice following STZ-induced diabetes (comparing Figure 2b and 2k in the revised manuscript).
2. Regarding the therapeutic approach, we have employed a serine protease inhibitor, aprotinin (Kunitz-type domain-containing protein drug used to reduce fibrinolysis and bleeding), to assess its effect in *Spint1*-silenced NIT-1 cells. Results revealed that aprotinin partially restored MAFA protein levels in *Spint1*-knockdown β cells (Figure 4f & Supplementary Figure 6c in the revised manuscript). Additionally, we also observed that incubation of SPINT1 recombinant protein, which contains two Kunitz domains similar to those in aprotinin, could partially rescue the depressed MAFA protein levels in *Spint1*-depleted β cells (Figure 4g in the revised manuscript). Please also see our answer to major Comment 5.
3. To investigate whether *Spint1* might be involved in STZ-induced immunological response against pancreatic islets, we employed immunofluorescence microscopy to examine the infiltration of immune cells in the diabetic *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mouse islets after STZ treatment. The results showed that although STZ could enhance immunological response in mouse pancreas, there was no significant difference in the amounts of infiltrating immune cells between *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice after STZ treatment (Supplementary Figure 5h in the revised manuscript). These findings suggested that SPINT1 may not play an important role in STZ-induced inflammatory response against β cells.

Comment 3. In morphometric studies on islets the authors described use of one pancreatic section every twenty sections. Assuming that sections were 5 micrometers thick, they examined tissue levels 100 microns apart, which is less than diameter of many islets. Therefore, it is very likely that the same islets were taken for

the analysis from different levels. In addition, in most experiments the groups are very small and it is unclear how many times experiments were performed. The average of independent experiments should be shown.

Answer: Thank you for your insightful comments.

1. Several lines of evidence have shown that the diameters of mouse pancreas islets range from 20 μm to 350 μm with an average diameter of 116 ± 80 μm[9-11]. Notably, one of these studies demonstrated that the average diameter of mouse islets is around 100 μm, which covers 70-80% of the islet area[9]. To accurately calculate the β cell size and mass, we measured the average diameter of mouse islets and then followed a published protocol for morphometric studies, analyzing one pancreatic section every twenty sections, as established by Marinho *et al.* [12]. While there is slight possibility that the same islets were selected for analysis from different levels, widening the gap between two sections may result in the omission of too many smaller islets. Therefore, the protocol we adopted is a balanced approach, which covers a reasonably high number of islet cells in a pancreas with less repeated islet calculation.
2. We apologize for the oversight in omitting the descriptions regarding the number of experiments conducted in the legends of the previous manuscript. In the revised manuscript, we have provided the number of independent experiment sets performed and the methodology for obtaining statistical results in the legend of each figure.

Comment 4. The mechanism leading to increased beta cell proliferation following inhibition of Spint1 has not been examined.

Answer: Thank you for the comment. In this study, we found that the knockout of *Spint1* led to a decrease in β cell proliferation rather than an increase. There were several possible mechanisms to explain this phenomenon, and two of them were elucidated in the revised manuscript.

1. In this revised manuscript, we examined whether SPINT1 affected insulin receptor signaling and its downstream effecter ERK since our *Spint1*-deficient β cells produced less insulin, and several lines of evidence have shown that insulin signaling can increase β cell growth, proliferation, and survival[13-15]. Indeed, the new results showed that *Spint1* knockout or knockdown in β cells reduced the activities of both the insulin receptor and ERK (indicated by their decreased phosphorylation levels; Supplementary Figure 8b, left panel & Supplementary Figure 8e in the revised manuscript).
2. Alternatively, it has been reported that knockout of GLP1R results in a shift in the distribution of β cells from large islets to small and medium-sized ones[16], while the treatment of GLP1R agonist Semaglutide can increase pancreatic islet size through enhancement of islet cell proliferation[17]. These results suggested that the proliferation of islet cells, including β cells, was upregulated by GLP1R signaling. Indeed, accumulative evidence has shown that GLP1/GLP1R signaling plays an important role in β cell proliferation, survival, and neogenesis, in addition to stimulating insulin expression in β cells[18-20]. Mechanistically, it has been suggested that GLP1/GLP1R may stimulate β cell proliferation *via* transactivation of the epidermal growth factor receptor (EGFR)[21]. Our results indicated that the pancreas-specific depletion of *Spint1* resulted in the upregulation of HEPSIN's enzymatic activity to inhibit GLP1R activity *via* proteolytic processing. In addition, in the revised manuscript, we also offered new results to demonstrate that the knockdown of *Spint1* in β cells caused the decreased phosphorylation of EGFR

(Supplementary Figure 8e in the revised manuscript). Thus, the reduction in β cell proliferation could possibly be attributed to the downregulation of EGFR signaling caused by diminished GLP1R activity.

Collectively, the decreased β cell proliferation following *Spint1* depletion or silencing could be attributed to both the decreased GLP1R activity caused by HEPSIN-mediated proteolytic cleavage of GLP1R and a subsequent down-regulation of insulin production and its signaling.

Comment 5. The manuscript lacks translational studies in human, and therefore the overall significance of described findings is limited.

Answer: Thank you for this critical comment. To comply with the reviewer's suggestion, we have conducted the following translational studies using human cells/tissues, aiming to strengthen the overall significance of this study further.

1. We performed PLA experiments to demonstrate the formation of hetero-complexes among GLP1R, SPINT1, and HEPSIN both in isolated normal human islet cells and in normal human pancreatic sections containing islets (Figure 6a-b & Supplementary Figure 7c-d in the revised manuscript). Moreover, our *SPINT1* knockdown experiments in human primary islet cells also supported the hypothesis that SPINT1 can modulate insulin production *via* regulating HEPSIN/GLP1R and MAFA signaling (Figure 5c in the revised manuscript).
2. More importantly, we analyzed the RNAseq data (GSE164416) from human islet donors and found that there were significant correlations of the expression between *SPINT1 vs. INS* (encoding insulin) and *SPINT1 vs. MAFA* (Supplementary Figure 6b in the revised manuscript). Additionally, we categorized the 51 patients in another human pancreatic islet-derived RNAseq data (GSE38642) into three groups based on their glycated hemoglobin (HbA1c) values [< 5.7% (non-diabetic), 5.7-6.4% (prediabetic), > 6.4% (diabetic)]. The results showed a significant increase in *SPINT1* expression levels in the prediabetic group, with a rising trend observed in the diabetic group compared to the non-diabetic group (Supplementary Figure 9a in the revised manuscript). Furthermore, we collected 66 human pancreatic tissues from patients with benign pancreatic neoplasms undergoing pancreatectomy (see the Supplementary Materials sections for details). The non-tumor parts underwent immunohistochemistry to assess the expression of SPINT1 in the pancreatic islets. We classified patients into three groups based on their HbA1c levels: non-diabetic (< 5.7%, n = 12), prediabetic (5.7-6.4%, n = 15), and diabetic (> 6.4%, n = 39). The results revealed a trend (P = 0.0591) of elevated SPINT1 expression in the islets of prediabetic patients compared to non-diabetic patients (Supplementary Figure 9b in the revised manuscript). These results suggested that β cells would increase SPINT1 to upregulate MAFA and insulin production, aiming to maintain glucose homeostasis in the presence of high blood glucose levels.
3. Regarding the therapeutic application, we have employed a serine protease inhibitor, aprotinin (a Kunitz-type protease inhibitor and also a protein-based drug to reduce fibrinolysis and bleeding), to examine whether it exhibits a potential to restore the expression of MAFA in *Spint1*-silencing NIT-1 cells. The results showed that aprotinin could partially restore the protein levels of insulin transcription factor MAFA in *Spint1*-knockdown β cells (Figure 4f & Supplementary Figure 6c in the revised manuscript). It is

noteworthy that SPINT1 has two Kunitz domains, similar to those in aprotinin. Additionally, we also observed that incubation of SPINT1 recombinant protein could partially rescue the depressed MAFA protein levels in *Spint1*-depleted NIT-1 cells (Figure 4g in the revised manuscript).

Together, these results suggested that both the Kunitz domain-containing serine protease inhibitor and recombinant SPINT1 proteins exhibited the potential to serve as an alternative agent for the treatment of diabetics and were worthy of further investigation.

Comment 6. Discussion is limited in scope. The authors did not discuss their findings in relation to what has already been published on proteases in beta cell biology and diabetes.

Answer: Thank you for the constructive suggestion. In the Discussion section of the revised manuscript (Pages 25-26, Lines 426-438), we included the following paragraph to discuss our findings in relation to what has been published on proteases in β cell biology and diabetes. "Multiple lines of evidence indicate the significance of proteases and their corresponding protease inhibitors in maintaining pancreatic β cell function. For example, trypsin facilitates pancreatic islet expansion in db/db mice by modulating components of the islet capsule matrix[22]. Similarly, α1-antitrypsin has been reported to protect β cell function by mitigating inflammatory responses[23]. Furthermore, SERPINB1 and SERPINB8 can enhance β cell proliferation and shield against β cell exhaustion in high-fat diet conditions[24-26]. Here, we include SPINT1 and HEPSIN in this list by demonstrating that SPINT1 inhibited GLP1R cleavage by suppressing HEPSIN's proteolytic function in β cells. Since GLP1R signaling is known to enhance pancreatic β cell function[27], SPINT1 reveals a new and critical role in β cells by ensuring proper GLP1R activity. This novel mechanism underscores the importance of maintaining a delicate balance between multiple serine proteases and their inhibitors to prevent β cell malfunction and hyperglycemia."

Additional specific comments:

Comment 1. Organization of the paper: Data from Figure 1 should go to supplement while the supplementary data on diabetes should be moved to the main body of the manuscript.

Answer: Thank you for the suggestion. In the revised manuscript, we have relocated most panels of the original Figure 1 to Supplementary Figure 1, except for certain images of the original Figure 1e, as these images highlight the crucial localization of *Spint1-lacZ* in the embryonic pancreatic epithelium, a key aspect that we believe is significant for this study. In addition, those Supplementary Figures on diabetes have been moved to the main body of the manuscript (Figure 2h-k in the revised manuscript).

Comment 2. A finding of increased Ki67 staining confined to the large islets is interesting but should be independently confirmed by labeling cells with nucleotide analogs. Discussion should address why big rather than small and mid-size islets showed renewal activity on the Spint1 null background.

Answer: We thank Reviewer#1 for the critical comment.

1. We did observe that *Spint1* knockout reduced the number of large islets (diameter > 200 μm) and had less $Ki67^{+}$ staining. Following the suggestion, we labeled cells in pancreas islets by treating *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice daily with BrdU (Bromodeoxyuridine, a nucleotide analog) for 14 days. After the treatment, the pancreas was subjected to immunofluorescence staining using an anti-BrdU antibody. The results showed that the BrdU incorporation in the large islet (> 200 μm) of *Spint1*$^{-/-}$ mice was decreased compared to *Spint1*$^{fl/fl}$ mice, similar to the Ki67-staining results (Supplementary Figure 6e in the revised manuscript). This indicated that SPINT1 had a greater impact on the cell proliferation of large islets compared to small and medium-sized islets.
2. To explain why the renewal activity was decreased only in larger islets of *Spint1*$^{-/-}$ mice compared to *Spint1*$^{fl/fl}$ mice, some possible explanations are provided as follows:
   a) The first possibility is based on the observation that β cells in larger islets exhibit less functional competence than those in smaller islets and result in reduced insulin production[28-30]. This disparity in local insulin levels may be exacerbated when insulin synthesis in β cells is diminished due to *Spint1* deficiency. Given that insulin can stimulate β cell proliferation[31], it is plausible that larger islets in *Spint1*$^{-/-}$ mice could manifest a noteworthy decrease in Ki67 signals when compared to *Spint1*$^{fl/fl}$ mice. This could be attributed to the diminished availability of insulin for β cells in the absence of *Spint1*.
   b) Islet endothelial cells are recognized for fostering β cell proliferation by the release of hepatocyte growth factors and other growth factors[32], influenced by the paracrine support from insulin and vascular endothelial growth factor (VEGF) secreted from β cells[32]. It has been shown that smaller islets express higher levels of VEGF-A than larger islets[33], potentially granting their endothelial cells with increased resilience against the adverse effects of reduced insulin production caused by *Spint1* deficiency. As a result, *Spint1*$^{-/-}$ β cells in larger islets may undergo a more pronounced decrease in growth factor secretion by islet endothelial cells compared to those in smaller islets, thereby contributing to reduced proliferation.
   c) GLP1R has the capacity to enhance the proliferation of β cells through its effects on other receptor tyrosine kinases, such as EGFR[34]. The decreased levels of GLP1R seem to have a more remarkable effect on large islets because *Glp1r*$^{-/-}$ mice have a lower number of large pancreatic islets than *Glp1r*$^{+/+}$ mice[16]. This is in line with our results indicating a decrease in the number of large islets in *Spint1*$^{-/-}$ mice, along with reduced GLP1R signaling in *Spint1*-deficient or -depleted β cells. We speculate that larger islets, containing β cells that are less functionally competent[28-30], might be more sensitive to alterations in GLP1R signaling and lacked efficient compensatory mechanisms. Consequently, the relatively lower GLP1R signaling in larger islets of *Spint1*$^{-/-}$ mice would likely cause a significant reduction in β cell proliferation, as indicated by Ki67 signals, compared to *Spint1*$^{fl/fl}$ mice.
   d) It is also possible that all the aforementioned factors converged and contributed together to this phenotype.

We have added the above description in the Result and Discussion section of our revised manuscript (Pages 10-11, Lines 167-171; Pages 26-27, Lines 447-473).

Comment 3. It would be beneficial to have side by side IF, IHC or Western blot data showing expression of Spint1 and Hepsin in beta versus alpha cells in mouse and human.

Answer: Thank you for the comment. In our revised manuscript, we have provided such side-by-side comparisons illustrating SPINT1 expression in mouse (Supplementary Figure 3i in the revised manuscript) and human (Supplementary Figure 9f in the revised manuscript) α and β cells, along with HEPSIN expression in mouse (Supplementary Figure 9d in the revised manuscript) and human (Supplementary Figure 9e in the revised manuscript) α and β cells. Here, we used immunohistochemistry and DAB staining for SPINT1 detection and visualization due to the absence of suitable anti-SPINT1 antibodies for immunofluorescence microscopy. The DAB staining results were pseudocolored and merged with results of immunofluorescence staining for insulin or glucagon[35].

Comment 4. The use of MG142 in Figure 5c should be explained.

Answer: We thank Reviewer#1 for the comment. The compound used in the original Figure 5c was MG132 rather than MG142. MG132 functions as a proteasome inhibitor to extend the duration of protein turnover. In our experiments, due to a high turnover rate of GLP1R, it was challenging to observe the HEPSIN-cleaved form of GLP1R without MG132. Thus, we used MG132 to slow down GLP1R turnover in *Spint1*-silencing cells, enabling the successful detection of the HEPSIN-cleaved GLP1R fragment (Figure 6c in the revised manuscript).

Comment 5. The method of 293 cell transfections is not described.

Answer: We appreciate Reviewer#1 for pointing out the negligence of including the HEK293 cell transfection protocol. In the revised manuscript, we have provided a detailed protocol of HEK293 cell transfection in the Materials and Methods section of the revised manuscript as follows: "For recombinant DNA transfection, HEK293T cells were seeded at a density of 5 x 10^5 cells per well of six-well plates in DMEM, and NIT-1 cells were seeded at a density of 1 x 10^6 cells per well of six-well plates in RPMI. The next day, the cells were transfected with control pcDNA3.1 (PLKO) or the pcDNA3.1 plasmids containing *3xFlag-Glp1r-myc*, *Spint1-his-myc*, or *Hepsin-his-myc* cDNA using Lipofectamine 3000 (STEM00015, Thermo Fisher)." (Pages 40-41, Lines 712-717).

Comment 6. It is unclear why authors used combined IF/IHC staining to detect insulin and Ki67.

Answer: Thank you for the comment. Initially, we encountered difficulties in quenching the autofluorescence present in mouse pancreas slides when we used IF to analyze the relatively weak Ki67 signals in the mouse islets. Therefore, we employed IHC with DAB staining instead to detect the Ki67 signals, as it is more sensitive than IF. By combining it with IF to detect the stronger insulin signals, we were able to locate the proliferative β cells precisely, as shown in the previous manuscript. This method, commonly referred to as dual staining, is deemed acceptable in histological analysis when two signals to be investigated are localized in distinct subcellular regions, such as Ki67 in the nucleus and insulin in the cytoplasm. In this revised manuscript, combined IF/IHC was no longer used to detect Ki67 and insulin since we have adopted more efficient

autofluorescence-quenching methods, including the use of Sudan Black B reagent, to quench the autofluorescence successfully. This enabled us to obtain clearer and more reliable double immunofluorescence signals of both Ki67 and insulin in the mouse pancreatic islets (Figure 2c in the revised manuscript).

Comment 7. Aprotinin was used at 40 micrograms/mL (Figure 4e). This is very high concentration which may have caused nonspecific changes.

Answer: We appreciate your raising this concern.

1. Aprotinin is a 58 amino acid polypeptide carrying a Kunitz domain, which functions as a serine protease inhibitor against a panel of serine proteases (*e.g.*, plasmin, kallikrein, and thrombin) with a distinct inhibitory concentration for each serine protease. For example, the Ki value of aprotinin for plasmin is 137 KIU/ml (2.88 μM, one μM = 6.5 μg/ml), and that for thrombin is ~2900 KIU/ml (61 μM or 397.1 μg/ml)[36,37]. Based on the distinct Ki value of aprotinin for each protease, we then treated si*Spint1*-treated NIT-1 cells with the following concentrations of aprotinin (0, 10, 20, 40, and 80 μg/ml). Our results showed that aprotinin exerted an induction effect on MAFA expression at 20 μg/ml, and achieved its maximal induction effect on MAFA expression in *Spint1*-knockdown cells, possibly due to the high expression of serine proteases in β cells (Supplementary Figure 6c in the revised manuscript). Based on these results, we then used 40 μg/ml aprotinin to examine its induction effect on MAFA expression in scramble siRNA-treated NIT-1 cells. The results showed that aprotinin treatment could restore the expression of MAFA protein in si*Spint1*-treated NIT-1 cells (Figure 4f in the revised manuscript). Furthermore, to address the concern of whether 40 μg/ml of aprotinin might cause some nonspecific alterations in β cells, we then analyzed the expression levels of other proteins, such as PDX1. Notably, the treatment did not cause any change in PDX1 protein (Figure 4f in the revised manuscript).
2. In addition, some studies have applied higher than 40 μg/ml of aprotinin to conduct physiological experiments. For example, Sasamoto *et al.*[38] reported the use of 40 μM (260.4 μg/ml) of aprotinin in blocking protease-induced cleavage of the extracellular loop of γENaC subunit on the rates of intracellular ENaC trafficking.

Considering the reasons mentioned above, 40 μg/ml are not a very high concentration of aprotinin for examining the potential involvement of some serine protease(s) in *Spint1* knockdown-reduced MAFA expression in β cells, and this concentration of aprotinin has no significant nonspecific change.

Comment 8. Information should be provided in figure legends as to which statistic test was used and how many independent experiments were performed for each subfigure.

Answer: Thank you for the critical comment. We apologize for omitting information regarding the number of biological replicates for certain figure panels in the previous manuscript. We have addressed this concern by providing a clear description of the statistic test, along with the number of independent experiments performed, in each figure legend of the revised manuscript.

Comment 9. In addition to representative images, Western blots (e.g., Figure 4g and i, and Figure 5b, c d and e) should be quantified, statistically analyzed, and their average expressed as data bars.

Answer: We appreciate your valuable suggestion. We have statistically quantified the representative images and western blots, presenting the results in histograms in the new Figure 4, Figure 5, and Figure 7 of the revised manuscript.

Comment 10. Figure 4i lacks Western blot with stained with antibody to Spint1.

Answer: Thank you for the comment on the western blotting results. Since the initiation of this project, we have endeavored to detect endogenous mouse SPINT1 expression in NIT-1 cells or mouse pancreatic β cells using western blotting. However, we faced formidable challenges regarding the effectiveness of anti-SPINT1 antibodies. It became evident that the antibodies available could not reliably evaluate the endogenous SPINT1 protein levels in mouse cells through western blot analysis. As a result, we opted to employ Q-RT-PCR as an alternative method to demonstrate the knockdown efficiency of *Spint1* in mouse cells (Figure 5a in the revised manuscript). This approach allowed us to accurately validate the *Spint1* knockdown effect on other proteins.

Reviewer #2 (Remarks to the Author):

We sincerely appreciate Reviewer #2 for endorsing our manuscript. His/her positive comments such as: "Overall, the study is interesting and well designed, and most conclusions are supported by the substantial amount data, and enough detail provided in the methods for the work to be reproduced" is highly appreciated. We also thank Reviewer #2 very much for his/her constructive comments. In the revised manuscript, we carefully answered the Reviewer's comments point by point as follows:

Major concerns:

Comment 1. Is the gene expression or protein abundance of SPINT1 and HEPSIN different between Lean healthy and diabetic/insulin resistant humans? If yes, is it higher or lower in diabetic/insulin resistant humans vs Lean? Does it agree with the findings in animal or cell models in this study?

Answer: Thank you for raising these important questions. Our data indicated that disruption of *Spint1* in mouse pancreatic β cells led to glucose intolerance and reduced insulin production at least partly via HEPSIN/GLP1R/MAFA signaling. The pathogenesis was closer to that of maturity-onset diabetes of the young (MODY). Nevertheless, it is an interesting question whether the gene expression or protein abundance of SPINT1 and HEPSIN differs between Lean healthy and diabetic/insulin-resistant humans, with the latter presumably representing patients with type 2 diabetes, as pancreatic islet dysfunction with dysregulation of crucial β cell genes also occurs during the development of type 2 diabetes[39]. However, studies also demonstrated that pathological changes of the islets could be different in each individual with type 2 diabetes, reflecting the diversity of pathophysiology[40]. To answer this challenging question, we performed the following two experiments:

1) we conducted a search in public gene expression datasets. We utilized GSE38642, derived from human pancreatic islets, to analyze the association between *SPINT1* and *HEPSIN* mRNA levels and human diabetes. We categorized the patients into three groups based on their clinical glycated hemoglobin (HbA1c) levels (< 5.7%, non-diabetes; 5.7-6.4%, prediabetes; > 6.4%, diabetes) and conducted a statistical analysis on the mRNA levels of *SPINT1* and *HEPSIN* in relation to these three stages of diabetes. The results showed that *SPINT1* was expressed at a significantly higher level in the islets of the prediabetic group, with an increasing trend in the diabetic group, compared to the non-diabetic subjects (Supplementary Figure 9a in the revised manuscript). However, *HEPSIN* expression had no significant association with the progression of diabetes. It suggests that elevated *SPINT1* expression in β cells may serve as a strategy to uphold glucose homeostasis, as it can boost insulin production.
2) Moreover, we collected 66 human pancreatic tissues from patients with benign pancreatic neoplasms undergoing pancreatectomy (see the Supplementary Materials sections for details). The non-tumor parts underwent immunohistochemistry to assess the expression of SPINT1 in the pancreatic islets. We classified patients into three groups based on their HbA1c levels: non-diabetic (< 5.7%, n = 12), prediabetic (5.7-6.4%, n = 15), and diabetic (> 6.4%, n = 39). The results revealed a trend (P = 0.0591) of elevated SPINT1 expression in the islets of prediabetic patients compared to non-diabetic patients (Supplementary Figure 9b in the revised manuscript).

Together, these new results aligned with our finding about the prediabetic status of the pancreas-specific *Spint1*-deficient mice in this study. They also concur with the findings in our cell models using mouse cell lines (NIT-1 and MIN6) and human primary islet cells (Figure 5c in the revised manuscript), where the presence of SPINT1 maintained the expression of insulin transcription factor MAFA to promote insulin production.

Comment 2. How many mice in each group (Spint1$^{fl/fl}$ and Spint1$^{-/-}$ islets) were used in the SILAC proteomic experiments? If only 1 mouse in each group (n=1), the quantification result will be highly unreliable, and the authors should either remove these results from the manuscript or increase the n to minimal 3. In addition, how many doubling of the NIT-1 cells before the SILAC labeling was stopped? What is the level of SILAC incorporation (e.g., >95%) observed after the doublings? Moreover, the quantification data need to be included as a supplemental table. Finally, the username and password are needed to access this dataset PXD039190.

Answer: Thank you for these important questions.

1. The number of mice that were used to collect islets for the SILAC proteomic experiments was four in each group (*Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ islets). While the quantification of SILAC incorporation after the doubling is tabularized in Excel format in Source data Figure 3c, we have also integrated some of the information (e.g., *P*-values) regarding SILAC proteomic experiments into Figure 3a of the revised manuscript.
2. To address your inquiries, we have incorporated more detailed information regarding the SILAC proteomic experiments in the Materials and Methods section of the revised manuscript. The newly added descriptions are indicated with underlining as follows: "The NIT-1 cells underwent stable isotope labelling through cultivation by seven doubling times in $^{13}C_6$-lysine/$^{13}C_6$-arginine-containing RPMI1640 media (Thermo Fisher, USA), which served as a heavy isotope-labelled group, because it is difficult to label islet cells in living organisms by isotopes[69]. To measure the level of SILAC incorporation after seven doubling times, a mass spectrometer was applied to verify the level of heavy isotope incorporation. The results showed that the level of heavy isotope incorporation in the cells was 97-98%. The *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mouse islets were classified as light isotope-labelled groups (n = 4 in each group of mice)." (Page 39, Line 686-693).
3. To access the SILAC proteomic results, please go to the database PXD039190 on the website (https://www.ebi.ac.uk/pride/) with the username: dtsh5300175@yahoo.com.tw and the password: Noodletwo2.

Comment 3. Only male mice were used in the study. Please provide a rationale for this and discuss potential results if female mice are used.

Answer: We thank the reviewer for raising this question.

1. At the beginning of our study, we did use female mice in an intraperitoneal glucose tolerance test (IGTT). Remarkably, the results obtained, as shown in Extra Supplementary Figure 1 of the revised manuscript, were parallel to those of IGTT using male mice, supporting the robustness and reproducibility of our observations. We used male mice for all the subsequent experiments due to their more stable hormonal dynamics compared to female mice, thereby minimizing unnecessary variability

related to the known effects of estrogen on blood glucose homeostasis[41]. This decision was made to ensure the consistency and reliability of our experiment results.

2. Based on our preliminary data, we infer that the results obtained with female mice would be comparable to those observed with male mice (e.g., lower serum insulin, decreased β cell mass, etc.).

Comment 4. Line 499: Blood samples were kept at room temperature for 10 minutes for clotting. This may lead to the degradation of insulin. It may be better to use another method to process the blood in order to measure insulin ASAP or keep the blood at 4 centigrade or lower.

Answer: We express our gratitude to the reviewer for providing this thoughtful reminder. The blood samples were kept at room temperature for 10 minutes to obtain the serum part. Insulin is less stable at room temperature in serum and plasma, but the degradation percentage has been found to be between 10% and 30% at 24 hours[42]. Although serum insulin is not entirely stable under these conditions, its degradation occurs gradually and at a slow pace. In a study by Nkuna *et al.*[36], serum insulin was observed to degrade by 17.68% at 4 hours under room temperature. Notably, changes exceeding the clinically significant threshold were only considered when surpassing the desirable biological variation total error of 36%. Extrapolation from their findings, we estimate the degradation of serum insulin in our cases was ~6%, which could still be in the acceptable range. In addition, in our experiments, after the serum was obtained, we did keep it immediately at 4°C or lower before subjecting them to ELISA. We will add this description in the Materials and Methods section.

Comment 5. In Figures and Supplemental Figures, some subpanels don't have sample size or P-value, and please add this information. If the sample size is n=1, the quantification result will be highly unreliable, and the authors should either remove these results from the manuscript or increase the n to minimal 3.

Answer: We sincerely appreciate the reviewer's comment and apologize for omitting information regarding the sample size and *P*-value in some subpanels. We would like to clarify that no experiment in our study was conducted with only one sample, and all the results presented in the manuscript were derived from at least three independent biological repeats. We have thoroughly reviewed all the subpanels, ensuring that the revised manuscript includes the necessary information on sample sizes and *P*-values for each.

Comment 6. Figure 3b, what is the P-value and # of molecules in each process? Figure 3d, what is the # of molecules in each pathway? Figure 3e, what is the P-value?

Answer: We sincerely appreciate the reviewer for reminding us to provide detailed statistical information on the proteomic results. The *P*-values and molecule numbers have been incorporated into the two bar charts in the revised Figures 3b and 3d, as well as the tier list in the revised Figure 3e.

Minor concerns:

Comment 1. The full protein or gene name is needed when a protein or gene appears in the manuscript for the 1st time, with the gene name in parenthesis.

Answer: We sincerely appreciate the reviewer for the thoughtful reminder. In the revised manuscript, we have ensured that the full name of each protein or gene is stated when initially mentioned in the manuscript, along with its abbreviation in a parenthesis. For subsequent references to the same protein or gene, we exclusively use the abbreviation.

Comment 2. Gene names for proteins in non-human cell lines or not in humans need to be small caps with the 1st letter capitalized, such as Mafa. The authors used this inconsistently throughout the manuscript.

Answer: We would like to express our sincere gratitude to you for the valuable reminder.

1. The gene abbreviations/symbols in this manuscript have been revised in accordance with the reviewer's suggestion and the published guidelines of the gene nomenclature of human and mouse genes, such as The HUGO Gene Nomenclature Committee Guidelines[44,45] and Mouse Genome Informatics Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat[46]. According to these guidelines, human gene symbols are presented in all capital letters and italicized, whereas mouse genes are italicized with the first letter capitalized.
2. For protein symbols, we have also adhered to the rules of the International Protein Nomenclature Guidelines[39], which describe the use of an all-uppercase gene symbol in a protein name for vertebrates. Namely, all letters for both mouse and human proteins should be in capitals without italics.

We have strived to maintain consistent usage of these symbols throughout the manuscript, with the exception of certain descriptions where it is difficult to completely avoid the popular names for some proteins (e.g., insulin and glucagon).

Comment 3. Line 530: is it 16.5-mM?

Answer: We would like to express our gratitude to the reviewer for pointing out the mistake. We have rectified the typo and changed it from 16.5-mM to the correct concentration of 16.7-mM for consistency with other descriptions.

Comment 4. In Figures and Supplemental Figures, the a, b, c, d,... need to be more obvious. Suggest changing to "a)."

Answer: We appreciate the valuable suggestion. We have followed the Reviewer's advice to make the labeling of figure panels in Figures and Supplemental Figures more obvious. To achieve this, we have enlarged the size of the characters [**a)**, **b)**, **c)**, etc.] and rendered them in bold, aligning with the stylistic convention found in articles published in Nature Communications.

Reviewer #3 (Remarks to the Author):

This manuscript looks at the role of Spint1 in pancreatic islets, proposing that hepsin is a target of Spint 1 in β-cells, and that loss of Spint1 enhances hepsin activity to cleave GLP-1R and decrease islet function through alterations in Mafa. The rationale for these studies is questionable.

Question 1. Is there any evidence that Spint1 or Hepsin activity is either altered by or influences the pathogenesis of beta-cell dysfunction?

Answer: We thank the reviewer for raising this important question.

1. *Evidence of Spint1 or Hepsin activity influencing the pathogenesis of β cell dysfunction*:
   a) Li et al. reported that *Hepsin* knockout mice had low blood glucose levels and exhibited dramatic resistance to high-fat-diet-induced obesity and hyperglycemia[47]. However, they focused on the liver pathology and did not examine the potential β cell dysfunction in their *Hepsin* knockout mice, which is highly likely and deserves further investigation.
   b) Previous studies[48,49] have shown that SPINT1 directly interacts with and inhibits HEPSIN. Furthermore, given our preliminary findings indicating abundant *Spint1* expression in the primary epithelium of the embryonic mouse pancreas (Figure 1a & Supplementary Figure 1 in the revised manuscript), we then wondered whether *Spint1* deficiency (HEPSIN upregulation) may also affect the function of the endocrine pancreas. Taking advantage of using pancreas-specific *Spint1* knockout (*Spint1*$^{-/-}$) mice, we successfully investigated this possibility. The results of the intraperitoneal glucose tolerance test (IGTT) showed that the blood glucose levels of *Spint1*$^{-/-}$ mice were significantly higher than those of *Spint1*$^{fl/fl}$ mice at 30 minutes after glucose administration and descended back to basal levels indistinguishable from those of *Spint1*$^{fl/fl}$ mice after 120-minute glucose treatment (Figure 2e in the revised manuscript). Moreover, the glucose-stimulated insulin secretion test showed that the serum insulin levels at 15 minutes after glucose stimulation were also lower in *Spint1*$^{-/-}$ mice than those in *Spint1*$^{fl/fl}$ mice (Figure 2f in the revised manuscript). The *in vitro* islet perfusion assay also showed that isolated *Spint1*$^{-/-}$ pancreatic islets had significantly flattened peaks in both insulin secretion phases that normally appeared at proper points in isolated *Spint1*$^{fl/fl}$ islets after the glucose administration (Figure 2g in the revised manuscript). Concordantly, we found that *Spint1* silencing significantly reduced insulin secretion in the mouse insulinoma cell line MIN6 (Figure 2h in the revised manuscript). We also determined whether *Spint1* deficiency increased the risk of developing diabetes. In a streptozotocin-induced diabetes test, *Spint1*$^{-/-}$ mice exhibited an earlier onset of diabetes (day 6-8, fasting glucose level > 250 mg/dL with polyuria and polydipsia) compared to *Spint1*$^{fl/fl}$ mice (Figure 2i in the revised manuscript). Together, the results indicate that *Spint1* deficiency in the mouse pancreas impairs glucose tolerance, reduces insulin secretion, and accelerates the onset of streptozotocin-induced diabetes, suggesting the significant involvement of *Spint1* in the pathogenesis of β cell dysfunction.
2. *Evidence of Spint1 or Hepsin activity being altered by the pathogenesis of β cell dysfunction*:
   To answer this question, we searched public gene expression databases to find the association of *SPINT1* and *HEPSIN* mRNA levels with human diabetes. We focused on the GSE38642 dataset[50], a gene expression profile obtained from curated DataSets in the Gene Expression Omnibus repository and

derived from the pancreatic islets of 51 patients. We categorized the patients into three groups based on their clinical glycated hemoglobin (HbA1c) levels (< 5.7%, non-diabetes; 5.7-6.4%, prediabetes; > 6.4%, diabetes) and conducted a statistical analysis on the *SPINT1* and *HEPSIN* mRNA levels in relation to different glucose status. As shown in new Supplementary Figure 9a in the revised manuscript, *SPINT1* was expressed at a significantly higher level in the islets of the prediabetic group, with an increasing trend ($P = 0.13$) in the diabetic group, compared to the non-diabetic subjects, while *HEPSIN* expression had no significant associated with the diabetic progression. It suggested that elevated *SPINT1* expression in β cells might be used by the body as a strategy to uphold glucose homeostasis by enhancing insulin synthesis. Furthermore, we collected 66 human pancreatic tissues from patients with benign pancreatic neoplasms undergoing pancreatectomy (see the Supplementary Materials sections for details). The non-tumor parts underwent immunohistochemistry to assess the expression of SPINT1 in the pancreatic islets. We classified patients into three groups based on their HbA1c levels: non-diabetic (< 5.7%, n = 12), prediabetic (5.7-6.4%, n = 15), and diabetic (> 6.4%, n = 39). The results revealed a trend ($P = 0.0591$) of elevated SPINT1 expression in the islets of prediabetic patients compared to non-diabetic patients (Supplementary Figure 9b in the revised manuscript). The results aligned with what we have found in *Spint1*$^{-/-}$ mice in our manuscript, namely that SPINT1 promoted insulin production to regulate blood glucose homeostasis.

In summary, our results indicate that pancreas-specific deficiency of *Spint1*, which likely causes aberrant elevation of HEPSIN function, may lead to hyperglycemia, reduced insulin secretion, and an earlier onset of induced diabetes in mice, which all reflect β cell dysfunction. In contrast, *Hepsin* deficiency in mice appears to lower blood glucose levels and mitigate high-fat-diet-induced hyperglycemia, manifesting almost opposite phenotypes compared to *Spint1* knockout mice. Moreover, human islet gene expression profiles also indicated that SPINT1 was upregulated in the prediabetes islets. Collectively, these data suggest that dysregulation of SPINT1 and its target serine protease HEPSIN is involved in the pathogenesis of β cell dysfunction and, to some extent, *vice versa*.

Question 2. Is there any evidence that GLP-1R is functionally altered through cleavage?

Answer: We thank the reviewer for raising this important question.

1. The answer is yes. The N-terminal signal peptide on GLP1R has been shown to be cleaved during synthesis and processing[51]. This cleavage is obligatory for its proper processing and trafficking to the cell membrane to exert its function. However, this cleavage process is different from ours.
2. Our study was not the only one to report the involvement of HEPSIN in the regulation of GLP1R activity; this was also hinted by a previous study[52]. However, we might be the first to report HEPSIN's proteolytic cleavage on GLP1R. The underlying rationale is that we identified a potential protein-protein interaction between HEPSIN and GLP1R through the analysis of the protein-protein association networks in the STRING v11 database[53]. This suggested a functional association between these two proteins. While the previous study[52] that suggested HEPSIN as an interactor of GLP1R did not delve further into the underlying functional outcome of this interaction, we sought to explore this possibility by conducting co-transfection experiments of HEPSIN and GLP1R. The result demonstrated that wild-type HEPSIN catalyzed proteolysis

on GLP1R, consequently generating two distinct fragments. In contrast, HEPSIN with a dead catalytic domain (HEPSIN S353A, HEPSIN mt.) failed to induce this cleavage (Figure 6d in the revised manuscript).

3. Functionally, we quantified the cAMP levels in GLP1R-transfected cells to assess the activity of GLP1R.
   a) *Hepsin* overexpression decreased the elevated cAMP levels caused by GLP1R (Supplementary Figure 8a in the revised manuscript).
   b) Correspondingly, *Spint1* depletion enhanced HEPSIN expression (Figure 4h-j & Figure 5 in the revised manuscript), resulting the cleavage of GLP1R (Figure 6c-d in the revised manuscript) and subsequent reduction of GLP1R activity (Figure 6f-h & Supplementary Figure 8a the in the revised manuscript). This, in turn, attenuated the phosphor-CREB-MAFA signaling pathway (Figure 7a in the revised manuscript) and decreased insulin synthesis (Figure 4a-b, Figure 5 & Figure 7b in the revised manuscript).
   c) On the contrary, *Hepsin* silencing restored the levels of CREB phosphorylation and the expression of MAFA and insulin in *Spint1*-knockdown NIT-1 cells (Figure 7a in the revised manuscript).
   d) Concordantly, *Spint1* overexpression exhibited protective effects against HEPSIN-induced GLP1R cleavage (Figure 6d-e in the revised manuscript), ultimately preserving or even improving GLP1R activity (Figure 6f-h & Supplementary Figure 8a in the revised manuscript) and increasing MAFA levels (Figure 4e in the revised manuscript).

Together, these findings further support that GLP1R is functionally deterred by HEPSIN proteolysis.

Question 3. The approach and methods have flaws, including the choice of mouse model and the use of an artificial system to interrogate the mechanism of action that is the center of this story. Overall, it is unclear what impact this work will have on understanding the regulation of GLP-1R signaling in β-cells or if this is a meaningful mechanism for diabetes. Some specific suggestions to help contextualize these comments are below:

Question 3-1. Line 59 – Reference 9 does not demonstrate a downstream effect of GLP-1R is upregulation of Mafa expression. This paper looks at cAMP signaling, which is the canonical message utilized by GLP-1R, but this sentence is a stretch to say Mafa is specific for GLP-1R. A more appropriate reference is required, or a rephrasing/deletion of this sentence. References 10-12 do not directly address this either.

Answer: Thank you for the crucial comments and suggestions.

1. We apologize for the confusion resulting from our imprecise description when citing Reference 9 in Line 59. In Reference 9, Blanchet et al.[54] stated that "CRTC2 mediated effects of incretin hormones through upregulation of *Mafa* and other CREB target genes that promote insulin gene expression" (the first sentence in their Discussion section). While incretin is recognized as a GLP1R agonist, it is accurate to note that the researchers did not establish that GLP1R is indispensably required for this signaling. They neither asserted that *Mafa* is specifically targeted by GLP1R nor did we make such a claim. We did not affirm that *Mafa* is exclusive to GLP1R's signaling; rather, we merely suggested that GLP1R activity could potentially stimulate *Mafa* expression (Supplementary Figure 8c in the revised manuscript). Indeed, *Mafa* could be regulated by various upstream signals beyond GLP1R[55,56].
2. To comply with the suggestion of the reviewer, we have rephrased this sentence as follows: "For example, GLP1 or GLP1-like drugs have been shown to stimulate the expression of *Mafa* in β cells[17]." We have also

added another reference[17] that demonstrated that Semiglutamide, a GLP1R agonist, could enhance the expression of *Mafa*. We have also deleted references 10-12 in the old manuscript and edited the sentence in the introduction of the revised manuscript. Proper references have been added to support this description. The new paragraph in the revised manuscript is as follows: "The transcription of the insulin-encoding genes is predominantly regulated by several major transcription factors[57,58], including pancreatic and duodenal homeobox 1 (PDX1)[57], neuronal differentiation 1 (NEUROD1)[58], and MAF bZIP transcription factor A (MAFA)[57], which are responsible for insulin production through the transcription of insulin-encoding genes[57]. Hierarchically, these transcription factors can respond to upstream signal pathways that mediate the environmental cues[59]. For example, GLP1 or GLP1-like drugs have been shown to stimulate the expression of *Mafa* in β cells[17,54]." (Pages 5-6, Lines 76-83)

Question 3-2. Line 60-63 – This concept should be considered in light of more recent data (PMIDs 28325479, 31495689), published after the 2007 reference used to support this sentence.

Answer: We appreciate the reviewer very much for this valuable advice. We have cited the suggested more recent papers to support the statement in our revised manuscript (Page 5, Lines 66-68) as follows: 1. The Role of Pancreatic Preproglucagon in Glucose Homeostasis in Mice [Cell Metab. 2017 Apr 4;25(4):927-934, PMID: 28325479[60]]. 2. Gut-Proglucagon-Derived Peptides Are Essential for Regulating Glucose Homeostasis in Mice [Cell Metab. 2019 Nov 5;30(5):976-986, PMID: 31495689[61]].

Question 3-3. Line 64 – a 2004 reference to document the current landscape of GLP-1R agonists is not appropriate.

Answer: We appreciate the reviewer for the comment and apologize for citing the older 2004 reference. We have substituted the 2004 reference with two recent publications to document the current landscape of GLP1R agonists in the revised manuscript (Page 5, Lines 68-69) as follows: 1. Recent Advances in Incretin-Based Pharmacotherapies for the Treatment of Obesity and Diabetes [Front Endocrinol (Lausanne). 2022 Mar 1:13:838-410, PMID: 35299971[62]]. 2. Glucagon-like Peptide-1 Receptor Agonists in the Management of Type 2 Diabetes Mellitus and Obesity: The Impact of Pharmacological Properties and Genetic Factors. [Int J Mol Sci. 2022 Mar 22;23:34-51, PMID: 35408810[63]].

Question 3-4. Line 65-67 – is there any rationale for assessing the proteolytic modification of GLP1R other than it hasn't been done? Is proteolytic modification of GPCRs a mechanism that regulates activity? This is alluded to with the references on line 75, but a clear focus on GPCRs is needed to provide rationale for the current investigation.

Answer: We express our gratitude to the reviewer for these insightful queries. Both of your questions receive affirmative responses. The rationes for evaluating the proteolytic modification of GLP1R are outlined as follows:

1. A literature study suggested that pericellular serine proteases might be involved in insulin secretion and pancreatic β cells[64], while another report identified an association between alterations in serine protease-antiprotease balance and early diabetes[65].
2. A previously published study[66] and our initial results both showed that SPINT1 was abundantly expressed in pancreatic islet cells. Moreover, our results suggested that *Spint1* deficiency caused hyperglycemia and impaired insulin synthesis in mice. HEPSIN protein, a well-known serine protease targeted by SPINT1[48], is also expressed in human islet cells at a moderate level (Human Protein Atlas, https://www.proteinatlas.org/ ENSG00000105707-HPN/ and Supplementary Figure 9e in our revised manuscript). *Hepsin* mRNA is also expressed in adult mouse pancreatic islets (Gene expression database E-MTAB-8729)[67]. GLP1R, a member of the GPCR family, has been shown to play a pivotal role in regulating the biological action of β cells by stimulating insulin production and secretion[68,69]. The favorable effects of GLP1R on β cells appear to align with those of SPINT1 in our study and may be opposite to those of HEPSIN[47]. More importantly, a protein-protein interaction between HEPSIN and GLP1R has been found and published[52]. Therefore, it is tempting to investigate whether GLP1R may undergo proteolytic modification by HEPSIN.
3. Protease-activated receptors 1-4 (PAR1-4) are well-known GPCRs activated by pericellular serine proteases[70]. For example, proteolytic activation of PAR1 by thrombin has been reported to be the most potent known trigger for platelet aggregation[71]. In addition, trypsin can cleave PAR2, creating a new N-terminus that activates PAR2, leading to the mobilization of intracellular calcium and the promotion of phosphorylation of ERK[72]. Moreover, the activation of many adhesion GPCRs[73] depends on the cleavage of their N-termini to reveal specific agonistic peptide sequences within the N-terminal stalk region, acting as tethered ligands. Finally, several GPCRs have been reported to undergo shedding after proteolytic processing by proteases. Examples include the shedding of the N-terminal functional part of GPR107 by furin[74] and the processing of GPR37 by ADAM10 and furin to shed the N-terminus[75]. Indeed, many other GPCRs, including β1-adrenergic receptor, parathyroid hormone receptor type 1, and so on, are known to undergo proteolytic processing, although the specific responsible enzymes remain unknown[67-69].
4. Even GLP1R itself is also processed by proteases before being trafficked to the cell membrane[51].

Together, these rationales prompt an intriguing question about whether pericellular proteolysis could serve as an alternative modulation on GLP1R activity, warranting further investigation. This, in turn, has motivated us to delve deeper into this inquiry.

We have added the rationale described above in the Introduction and Discussion sections of our revised manuscript (Page 5, Lines 69-75; Pages 24-25, Lines 417-425).

Question 3-5. The choice of mouse model seems poor. First, Pdx1-Cre expresses strongly in the brain (PMIDs 20802254, 20824628, 23823474). Using the liver to demonstrate specificity is not appropriate. Second, with respect to the islet, Pdx1-Cre is not β-cell specific and is expressed in other endocrine cell types, mostly notably the alpha-cell. Third, this model lacks temporal control, which seems essential given the reference literature on the developmental aspects of Spint1.

Answer: We appreciate the valuable and insightful comments provided by the reviewer.

In general, the rationale behind our choice of a pancreas-specific knockout approach over a β-cell-specific one stems from the fact that scientific studies typically commence with a broad perspective, progressively narrowing down to finer scopes through a step-by-step approach. In many studies involving gene disruption, researchers start with a whole-body analysis before narrowing their focus to specific organs or tissues and eventually to specific cell types. Considering that the whole-body knockout of *Spint1* has previously been demonstrated to be embryonically lethal and that the expression pattern of *Spint1* in the whole developing embryonic pancreatic epithelium closely aligns with that of *Pdx1*, it was logical for us then to opt for a pancreas-specific knockout approach. Furthermore, at the outset of our study, the manifestation of β cell dysfunction phenotypes (e.g., hyperglycemia) was not definitely anticipated. Consequently, targeting β cells specifically was not immediately evident at the beginning. Our decision to perform a pancreas-specific knockout was based on the available knowledge at that time to unravel the role of *Spint1* in the pancreas, not solely in β cells. However, β cell-specific knockout of *Spint1* is in our upcoming plan. We hope this helps clarify our methodology and its rationale.

The following are more specific answers to your comments:

1. First, we would like to point out that our *Pdx1-Cre* mice [*Tg(Ipf1-cre)^{1Tuv}*] contained the 4.3 kb mouse *Pdx1* promoter, which, when compared to the 5.5 kb *Pdx-1* promoter in *Tg(Pdx1-cre)$^{89.1Dam}$* mice, resulted in a significantly weaker expression of Cre in the hypothalamus, as indicated by the data in PMID 20802254[78]. To comply with the first comment, we have added a control group using the mouse hypothalamus tissue to assess the expression level of *Spint1* in this region. The results demonstrate that the endogenous *Spint1* expression in the hypothalamus did not show any significant changes between *Spint1$^{-/-}$* and *Spint1$^{fl/fl}$* mice (Figure 1b in the revised manuscript), indicating that *Pdx1-Cre* in the hypothalamus might not disrupt the *Spint1* gene efficiently. The above result is consistent with the observations that *Spint1$^{-/-}$* and *Spint1$^{fl/fl}$* mice did not differ significantly in their body weight (supplementary Figure 3d & 3f in the revised manuscript). Collectively, these findings suggest that the weak expression of *Pdx1-Cre* in the hypothalamus might not be responsible for the disruption of glucose homeostasis and defects of β cells in our *Spint1$^{-/-}$* mice.

   We have revised the corresponding paragraph in the Result section and incorporated a new sentence (Pages 8-9, Lines 128-135) to summarize the above results. "To further characterize the phenotypes of *Spint1* deficiency in the endocrine pancreas, we analyzed the morphology and function of pancreatic islets in eight-week-old adult mice. Q-RT-PCR verified that *Spint1* mRNA levels were significantly depleted in the pancreatic islets but not in the hypothalamus (HTH.) (Fig. 1b) of the *Spint1$^{-/-}$* mice compared to *Spint1$^{fl/fl}$* mice. The hypothalamus was included because it has been found to express *Pdx1* and is capable of sensing and regulating them[78]."
2. In response to the second comment, we concur with the Reviewer that *Pdx1-Cre* is not β-cell specific; therefore, we have revised the manuscript title to "Disruption of *Spint1* in mouse pancreas leads to glucose intolerance and impaired insulin production– the involvement of HEPSIN/GLP1R/MAFA signaling", removing the phrase "Disruption of Spint1 in mouse pancreatic β cells". However, we would like to refer to the review by Ebrahim et al. (PMID: 36589234)[79] for insights into *Pdx1'*s developmental

expression pattern in the pancreas. During pancreatic development, *Pdx1* is initially expressed in the foregut endoderm of the embryo. As development progresses, *Pdx1* expression becomes prominent and persists in β cells, while its expression in other exocrine and endocrine cell types gradually decreases until it disappears. This decrease in the expression of *Pdx1* is also seen in α cells. *Pdx1* expression may also be seen in γ and δ cells, but only at low levels. Given the similar expression patterns within the embryonic pancreas between *Pdx1* and *Spint1*, along with the predominant expression of *Pdx1* in the endocrine pancreas, especially in β cells during later development, the *Pdx1-Cre*-driven *Spint1*-deficient mice are suited for our original research purpose, namely the elucidation of the function of *Spint1* in the mouse endocrine pancreas.

3. In response to your third comment, we totally agree that a temporal-control gene knockout system, which theoretically allows the gene to be turned off at any desired time point, is an ideal approach. However, these inducible systems have their own limitations and disadvantages. Reported issues include inducer dose-dependent efficiency, great variations of the knockout efficiency in different organs, the toxicity of inducer agents on gestation, and spontaneous (leaky) recombinase activity[80,81].

While it is too late to initiate an entirely new *Spint1* knockout system, we have conducted additional experiments to complement our data, especially considering temporal factors. In the revised manuscript, we have included new data about the analyses of pancreatic islets of 1-week-old *Spint1*$^{-/-}$ mice. The results showed no significant change in the islet size in *Spint1*$^{-/-}$ mice compared to *Spint1*$^{fl/fl}$ mice of the same age (Supplementary Figure 3h in the revised manuscript). This suggested significant changes in islet size and function likely occurred between the second and eighth weeks. Information derived from our study in this aspect can also serve as a foundation for researchers to choose optimal time points for future inducible and β cell-specific knockout experiments of *Spint1*.

Overall, we acknowledge the advantages of cell-specific and inducible knockout models. Simultaneously, we recognize the need for caution in interpreting data from *Pdx1-Cre*-driven knockout mice, leading us to revise our title. Considering the complexity of tissues and organs comprising diverse cell types, a comprehensive understanding of gene function may necessitate comparative analyses between tissue-specific and cell-specific knockout models. Our pancreas-specific knockout model can still provide valuable insights into the overall function of the *Spint1* gene within the pancreas throughout the development, potentially complementing the findings of future β cell-specific or time-control knockout mice. Furthermore, our model may help researchers select the appropriate time point to perform inducible *Spint1* knockout. In conclusion, we acknowledge the merits of inducible and cell-specific knockout models but emphasize that our pancreas-specific knockout model is a valuable component in the broader exploration of gene function within complex tissues.

Question 3-6. Line 524 - 36 mg/dl does not reflect a normal blood glucose in any circumstance 300 mg/dl is not reflective of postprandial glucose in healthy individuals.

Answer: We appreciate the reviewer's comment and pointing out this issue. We admit that these descriptions lacked precision or appropriateness. The reason behind selecting a low glucose concentration of 2.8 mM (36 mg/dL) was that it is suitable for quantifying the insulin secreted into the supernatant under a basal or

unstimulated condition[82], not reflecting normal blood glucose. In contrast, 16.7 mM (300 mg/dL) glucose has been widely employed in many protocols to study the maximum insulin secretion capacity of the islets in response to elevated blood glucose levels[82,83], not reflecting the postprandial glucose in healthy individuals. To enhance the clarity, the abovementioned inappropriate phrases related to pancreatic islet isolation and islet perfusion have been removed in the Materials and Methods section in the revised manuscript (Pages 33-34, Lines 575-586).

Question 3-7. Details on the perfusion is missing. What is the flow rate, what is the machine? The lack of GSIS in the knockouts is remarkable, but could potentially be a technical issue. Surprisingly, the samples were diluted 100-fold to get near the bottom of the assay values (~0.2 ng/ml).

Answer: We thank the reviewer for the questions and comments. We have added the information about the machine (Pump 11 Elite / Pico Elite Plus, 70-4506 Harvard Apparatus), which was used for the measurement of glucose-stimulated insulin secretion (GSIS) in the revised manuscript. The flow rate for GSIS analysis was 150 microliters (μl) per minute[84]. In response to the second comment, we would like to clarify that GSIS was not completely absent in the knockouts, as Phase I was still faintly visible (Figure 2g in the revised manuscript). The low levels of insulin in *Spint1*$^{-/-}$ islets might be attributed to a lower synthesis of new insulin in the *Spint1*$^{-/-}$ islets rather than a technical issue of the dilution because the concentrations remained within the detection range of insulin (the lower limit of insulin detection down to 0.13 μg/L) for the ELISA (Extra Supplementary Figure 2), and both knockouts and controls were tested under the same GSIS perfusion conditions.

Question 3-8. Details on the cDNA constructs are missing. The methods read as Glp1r cDNA was transfected, but an anti-flag antibody was used to pull down GLP-1R, making the assumption that the Glp1r was flag-tagged. Is this commercially available? If constructed in house, where is the flag-tag and how does this impact GLP-1R signaling?

Answer: We thank the reviewer for these two questions.

1. The GLP1R plasmid with FLAG tag was constructed in-house by ourselves for GLP1R pulldown assays. The detailed procedure for the plasmid construction was as follows: "In constructing the FLAG-tagged GLP1R, a GLP1R cDNA (RC211333, OriGene Technologies) was inserted into a mammalian expression vector (p3xFLAG-*Myc*-CMV-24), which contained three FLAG tags at the N-terminal of GLP1R, along with a c-Myc tag at the C-terminal. DNA sequencing analysis confirmed the GLP1R sequence integrity and ensured that all nucleotides were in the correct open reading frame." (Page 41, Lines 717-722)
2. It's been reported that, although a tag conjugated on GLP1R can compromise its activity by 10-20%, tagged GLP1R still serves as a suitable experiment sample for its functional assays[85]. Our results also showed that GLP1R overexpression using this tagged GLP1R cDNA could significantly increase the levels of cAMP in HEK293 cells. This finding indicated that the exogenous GLP1R proteins with the FLAG tag retained sufficient GLP1R activity.

Question 3-9. There is not functional assessment of the GLP-1R in the knockout models, rending the either mechanism to be supported by overexpression assays in the HEK cells. How much of this translates to primary islets?

Answer: We greatly appreciate the valuable question raised by the reviewer. To address this question, we have included new data by isolating primary islets from *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice and quantifying their levels of cAMP as a functional assessment of the GLP-1R. The results demonstrated that the cAMP levels were significantly reduced in *Spint1*$^{-/-}$ islets upon Exendin-4 treatment, compared to *Spint1*$^{fl/fl}$ islets (Figure 6h in the revised manuscript). Similar results were also observed in *Spint1*-knockdown NIT-1 cells (Figure 6f in the revised manuscript), consistent with the expected opposite seen in *Spint1*-overexpressing NIT-1 cells (Figure 6g in the revised manuscript). The new results have been incorporated into the result section of the revised manuscript (Page 19, Lines 322-327).

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Answers for the Reviewers' comments

Reviewer #1 (Remarks to the Author):

Major criticisms:

The revised manuscript by Lin et al. describes a novel mechanism of regulation of GLP1R in pancreatic beta cells. The authors propose that GLP1R is cleaved by the serine protease, Hepsin, which in turn is regulated by the membrane-anchored inhibitor, Spint1. Spint1-deficient mice demonstrated reduced beta cell mass, impaired insulin content, lower expression of MafA transcription factor, and a somewhat worse outcome in STZ-induced diabetes. Since GLP1R agonists are or on the frontline as type 2 diabetes therapeutics, the described findings are potentially important to the broader community interested in diabetes research.

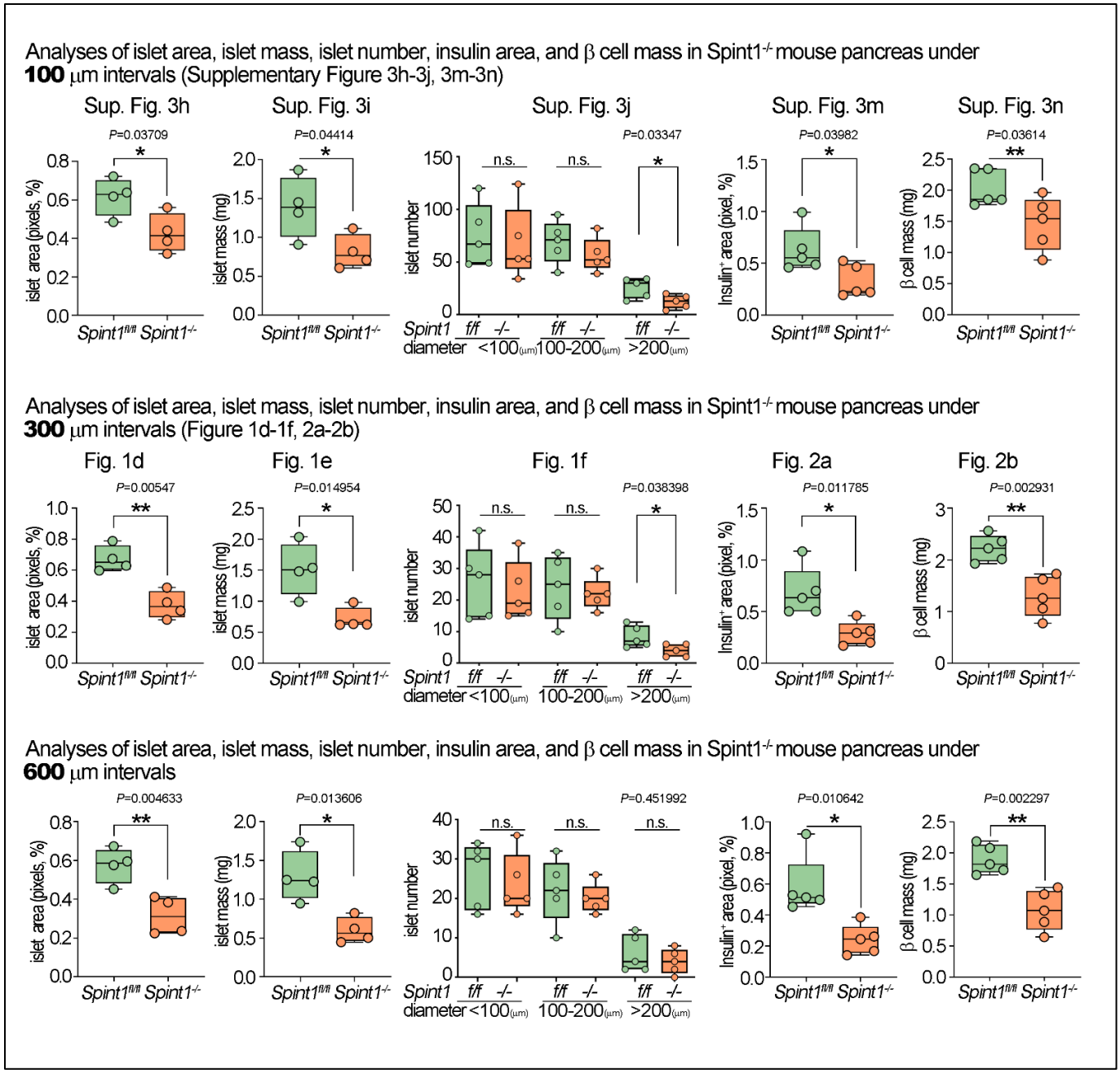
The authors made several improvements to their originally submitted work. The revised version presents a more compelling case for the interaction between GLP1R, Spint1 and Hepsin. The authors have also made the effort to perform experiments on primary mouse and human beta cells, and additional rescue experiments with siHepsin in the setting of Spint1 deficiency. Moreover, the discussion has been improved by providing an insightful explanation as to why beta cell proliferation and large, rather than small, pancreatic islets are affected by a Spint1 deficiency.

Answer: We are deeply grateful, Reviewer #1, for your positive response and encouragement! Your feedback has been instrumental in our efforts to present a more compelling case for the interaction between GLP1R, SPINT1, and HEPSIN, and to offer insightful enhancements in both results and discussion. These updated findings hold potential significance for the broader community engaged in diabetes research.

On the other hand, there are still lingering issues of how certain experiments have been performed.

Comment 1. First, the authors insist on examining pancreatic sections every 100 microns in their assessment of beta cell mass. While this may be fine for small islets, the larger islets, with a diameter of over 100 microns, and which account for the majority of beta cell mass, should be re-examined using sections every 300 and 600 microns to ensure that they are not counted twice.

Answer: We extend our sincere thanks to Reviewer #1 for their insightful comments. We re-examined the sections at intervals of every 300 and 600 μm. The findings indicated a significant reduction in islet area, mass, and β cell mass with *Spint1* depletion, consistent with previous findings at 100 μm intervals and, in some cases, even more significant. Furthermore, the data revealed a decrease in the number of islets with diameters above 200 μm at 300 μm intervals. In contrast, no significant difference in the number of large islets was observed at 600 μm intervals. This discrepancy may be due to the 600 μm intervals potentially being too broad to accurately detect the large islets, considering that the average diameter of mouse islets is reported to be 116 ± 80 μm [1, 2, 3]. Therefore, we conclude that 100 μm and 300 μm intervals are optimal for accurately capturing a representative number of islets in the mouse pancreas. In response to the reviewer's suggestion, we have included the new results in Figures 1d-f and 2a-b of the revised manuscript, based on the 300 μm intervals, and have relocated the results of 100 μm intervals to Supplementary Figure 3h-j and 3m-n of the revised manuscript for reference.



The descriptions of the revised statement in the Results and Figure Legends are as follows:

In Results:

- In Figures **1d-e,** "Our study further conducted a morphometric analysis to quantify the area, mass, size, and number of 8-week-old mouse islets. The results unveiled a significant decrease in the islet area and mass in the *Spint1*$^{-/-}$ mice (Fig. 1d-e) compared to *Spint1*$^{fl/fl}$ mice" (Pages 9, Lines 138-140)

- In Figure **1f**, "Due to variations in islet size, we quantified and categorized pancreatic islets based on their diameters (> 200 μm, 100-200 μm, and < 100 μm) within whole pancreas sections at 300 μm intervals. The results indicated that *Spint1* deficiency significantly reduced the number of islets with a diameter above 200 μm. In comparison, it had no noticeable effect on the number of islets with a diameter below 100 μm or between 100 μm and 200 μm (Fig. 1f)." (Pages 9, Lines 142-147)

- In Supplementary Fig. **3h-j,** "To avoid the omission of small islets in our calculation, we also utilized a series of whole pancreas sections at 100 μm intervals to evaluate islet size, mass, and number. Similarly, islets in *Spint1*$^{-/-}$ mice exhibited smaller area, mass, and number (in the category with diameters above 200 μm) than those in *Spint1*$^{fl/fl}$ mice (Supplementary Fig. 3h-j)." (Pages 9, Lines 147-151)

- In Figures **2a-b,** Supplementary Fig. **3m-n,** "However, the β cell area and mass of *Spint1*$^{-/-}$ mice were significantly decreased compared to that of the *Spint1*$^{fl/fl}$ mice [Fig. 2a-b (calculated by 300 μm intervals), Supplementary Fig. 3m-n (calculated by 100 μm intervals), and Supplementary Fig. 4]." (Pages 10, Lines 157-160)

In Figure Legends:

- "Figure 1**d,** Quantification of islet area percentage in 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Each pancreas was serially sectioned (300 sections per pancreas, 5 μm per section), and one out of every 60 serial sections (300 μm intervals) was taken for H&E staining to reveal islet areas. ImageJ determined the percentage of islet area in a whole pancreas area based on the merged full-view microscopic images of 6 sections per mouse (4 mice per group)." (Pages 53, Lines 1077-1082)

- "Figure 1**f,** Measurement of islet numbers in the pancreas of 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. The islets with a diameter below 100 μm were defined as small islets, those with a diameter between 100 μm and 200 μm as medium islets, and those with a diameter above 200 μm as large islets. The islet numbers with different diameters were measured using microscopic images from H&E-stained sections using ImageJ (one out of every 300 μm intervals from serial sections of each pancreas, 6 sections per mouse, 5 mice per group)." (Pages 53, Lines 1084-1091)

- "Figure 2**a-b,** Percentages of insulin-positive area in 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Mouse pancreas sections were immunohistochemically stained using an anti-insulin antibody. The merged full-view microscopic images (Supplementary Fig. 4) were analyzed to obtain the percentage of the insulin-positive area over the total pancreas area using ImageJ (one out of every 300 μm intervals from serial sections per pancreas, 6 sections per mouse, 5 mice per group). **b,** Analysis of β cell mass between 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. The β cell mass was calculated by multiplying the insulin-positive area percentage in **a** by the pancreas weight in Supplementary Fig. 3**g**." (Pages 54, Lines 1102-1110)

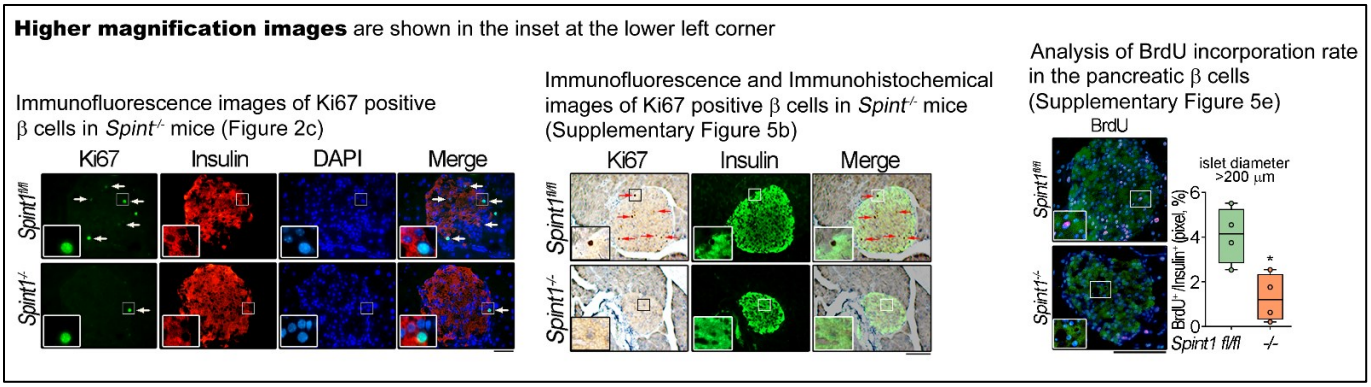
In Supplementary Figure Legends:

- "Supplementary Fig. 3**h-j,** Quantification of islet area percentage, islet mass, and islet numbers in 8-week-old *Spint1*$^{-/-}$ and *Spint1*$^{fl/fl}$ mice using sections taken at 100 μm intervals throughout the whole mouse pancreas." (Pages 8-9, Lines 94-97)

- "Supplementary Fig. 3**m-n,** Quantification of percentages of the insulin-positive area and β cell mass in 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice, using sections taken at 100 μm intervals throughout the whole mouse pancreas. **m,** Each pancreas underwent serial sections (300 sections per pancreas) in which one out of every 20 serial sections (100 μm intervals for each section) was taken for immunohistochemical stained using an anti-insulin antibody and were determined based on the merged full-view microscopic images of 15 sections per mouse (4 mice per group) using ImageJ. **n,** The β cell mass was calculated by multiplying the insulin-positive area percentage in **k** by the pancreas weight in **g**." (Pages 10, Lines 123-131)

Comment 2. Second, the authors claim that proliferation is decreased in Spint1-deficient animals. These data are impossible for the reader to validate as the low-resolution images fail to show beta cell co-staining with Ki67 or Brdu. In light of these limitations, the authors may consider flow cytometry to unequivocally demonstrate any potential differences in proliferation rates of beta and alpha cells.

Answer: Thank you for your insightful comments and suggestions. We sincerely apologize for including low-resolution images to depict the co-staining of β cells with Ki67 and BrdU in the previous manuscript. Actually, the resolution of our original images is sufficient, but it decreased greatly when we incorrectly created the figure under a compression setting. In the revised version, we have replaced them with original high-resolution images without any file compression after Ki67 and BrdU staining. Additionally, we have included high-magnification images showing Ki67-positive and BrdU-positive cells in the lower left corner of each panel (insets in Figure 2c, Supplementary Figure 5b and 5e). Detailed descriptions for each result are provided in the corresponding figure legends of the revised manuscript.



The descriptions of the revised statement in Figure and Supplementary Figure Legends are as follows:

In Figure Legends:

- "Figure **2c,** Representative immunofluorescence images of Ki67$^+$ β cells in large islets (>200 μm in diameters) of 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Pancreatic sections were subjected to immunofluorescence microscopy to detect Ki67 (green) and insulin (red, β cells). Nuclei were counterstained with DAPI (blue). Higher magnification images are shown in the inset at the lower left corner of each panel. Scale bar, 20 μm." (Pages 55, Lines 1110-1115)

In Supplementary Figure Legends:

- "Supplementary Fig. 5**b,** Detection of Ki67-positive β cells in the pancreatic islets of 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Pancreatic sections underwent IHC using an anti-Ki67 antibody (left panel) and subsequent immunofluorescence microscopy using an anti-insulin antibody (middle panel). Ki67-positive β cells are shown in the merged images (right panel). Scale bar, 20 μm. High-magnification images are shown in the insets at the lower left corner of each panel." (Pages 14, Lines 157-162)

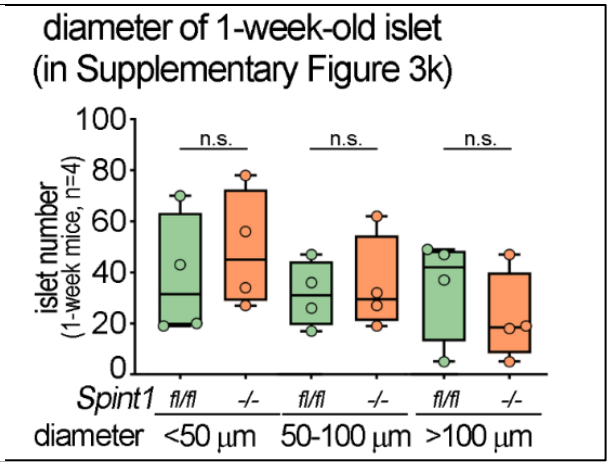
- "Supplementary Fig. 5**e,** Analysis of bromodeoxyuridine (BrdU) incorporation rate in the pancreatic β cells of 8-week-old *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice. Mice were daily treated with sterile drinking water containing BrdU (1 mg/mL) for 14 days. Representative images after immunofluorescence microscopy showed the merged signals for BrdU (pink), insulin (green), and DAPI (blue) in the left panel. Scale bar, 20 μm. The rates of BrdU incorporation in β cells were quantified in the large pancreatic islets [6 sections per pancreas

(300 μm intervals) and four mice per group], as shown in the right panel. High-magnification images are shown in the insets at the lower left corner of each panel." (Pages 15, Lines 174-182)

Comment 3. Third, Figure 1 convincingly shows strong positivity for Spint1 in the embryonal pancreatic ducts. Therefore, it is possible that the primary impact of Spint1 may be on beta cell development, rather than proliferation.

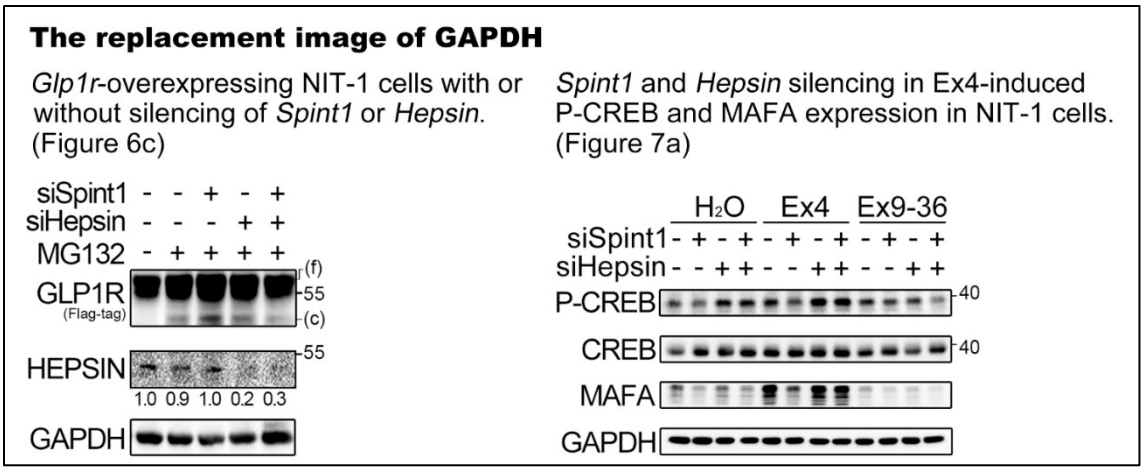
Answer: We greatly appreciate your insightful question. In our previous work, we conducted morphometric analyses on the pancreas of E17.5 mice and postnatal day 7 (P7, Supplementary Figure 3k). Our findings revealed no significant differences in pancreas morphometric parameters between *Spint1*$^{-/-}$ and *Spint1*$^{fl/fl}$ mice at E17.5 or P7. Moreover, several lines of evidence suggest that the pivotal period for increased islet mass and number occurs between week 2 and week 4 post mouse birth[4, 5, 6]. Hence, we deduce that the difference in proliferation significantly contributes to the observed islet phenotypes in 8-week-old mice. However, it is noteworthy that β cell development might also play a role, as any abnormalities in development could potentially impede the proliferation capacity of β cells during the critical period of rapid expansion in islet size and number. Your question has further highlighted the importance of our research in this area.

[figure redacted]



Comment 4. Finally, many of the Western blots have overexposed controls, suggesting that normalization has not been performed correctly.

Answer: Thank you very much for your insightful comments and suggestions. We apologize for including some overexposed loading controls in the Western blots. In the revised manuscript, we have replaced the loading control of GAPDH with images with appropriate exposure levels for normalization, as shown in Figure 6c and Figure 7a.



Reviewer #1's other comments:

Comment 1. Figure 1f describes islets with sizes of <100, 100-200 and >200 microns. Supplementary Figure 3h shows islets with sizes <50, 50-100, and >100. Is there a reason for this discrepancy?

Answer: Thank you for your insightful comments. In Figure 1f and Supplementary Figure 3j-k, we examined the islet sizes of 8-week-old and 1-week-old mice, respectively. Several lines of evidence have shown that the size of islets in 1-week-old mouse pancreas is smaller than in older mouse pancreas[4, 5, 6, 7]. Notably, one of these studies demonstrated that the average sizes of islets in 1-week-old and 8-week-old mouse pancreas are approximately 170 and 2,380 μm^3 with average diameters of 60 and 145 μm, respectively [6, 7]. According to the above findings, we then choose different criteria to categorize islet sizes at different ages: <100, 100-200, and >200 μm for the islet sizes of 8-week-old mouse pancreas (Figure 1f); Criteria 2: <50, 50-100, and >100 μm for the islet sizes of 1-week-old mouse pancreas (Supplementary Figure 3k).

Comment 2. Figure 2i. It is not mentioned how many times the experiment has been performed.

Answer: Thank you for your valuable feedback. We apologize for the oversight in failing to mention the number of times the experiment in Figure 2i. In response, we have updated the legend of Figure 2i of our revised manuscript to include the performance times of the experiments.

The descriptions of the revised statement in the legend of Figure 2i are as follows:

In Figure Legend:

- "Figure **2i,** Early onset of diabetes in streptozotocin-induced 8-week-old *Spint1*$^{-/-}$ mice. Mice were intraperitoneally injected with streptozotocin (40 mg/kg body weight) for 5 consecutive days and provided with 10% sucrose drinking water. Ante cibum blood glucose levels (AC glucose) were measured before streptozotocin treatment (day 0) and on days 6, 8, 15, and 18 after the treatment (n = 5 per group). These results were statistically calculated from three independent experiments." (Page 56, Lines 1138-1144)

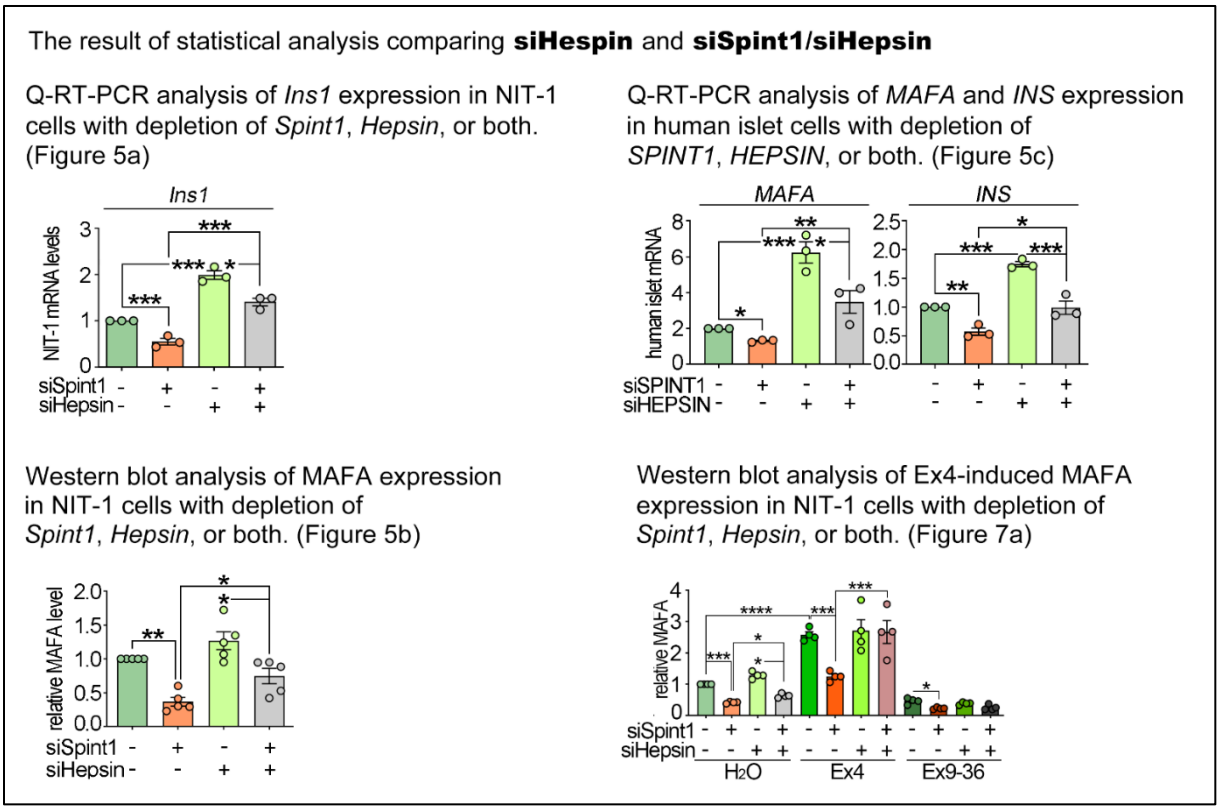
Comment 3. Figure 4a and b show augmented expression of Pdx1 - this finding is not mentioned in the Results or Discussion.

Answer: We sincerely thank Reviewer #1 for his/her insightful comments and suggestions! In our revised manuscript, we delineate the differentially augmented expression of PDX1 or NEUROD1 in *Spint1*-knockout mouse islets or *Spint1*-knockdown mouse insulinoma cells as follows: "*Spint1* deficiency led to a dramatic decrease in MAFA protein levels (Fig. 4a, right panel), accompanied by a differential augmentation of PDX1 and NEUROD1 proteins in the mouse islets, potentially acting as a compensatory mechanism[8]. Similar results were also observed in the *Spint1*-silencing NIT-1 and MIN6 cells (Fig. 4b, right panels; Fig. 4c)." (Page 15, Lines 252-256)

Comment 4. Figure 5 and 7: The statistical comparison between groups with siHepsin vs. siHepsin/siSpint1 is missing. It appears that the impact of siHepsin is limited when used in conjunction with siSpint1, suggesting that there are additional protease targets involved.

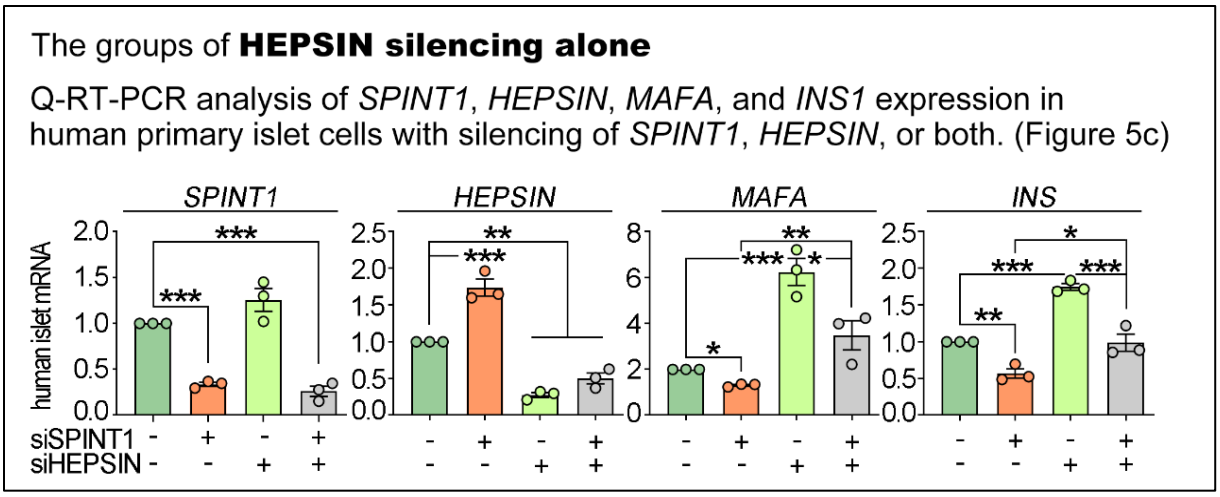
Answer: Thank you for your insightful comment. We have included the in revised Figures 5a-c, and 7a. The findings revealed that siSpint1 reduced the increased mRNA levels of siHepsin-induced *Mafa* and *Ins1* in

NIT-1 and MIN6 cells. However, it did not significantly impact the intracellular and secreted levels of insulin proteins. We fully endorse Reviewer #1's suggestion regarding potential alternative protease target(s), which influences the decreased mRNA expression of *Mafa* and *Ins1* due to *Spint1* depletion. This hypothesis is supported by SPINT1's known interactions with various serine proteases, including matriptase and prostasin[9]. The results together suggest that the possible additional proteases are involved in SPINT1/HEPSIN-mediated MAFA and insulin expression in β cells, and further investigation is necessary.



Comment 5. In addition, Figure 5c does not show a group with siHepsin alone.

Answer: We thank the reviewer for the questions. We have re-performed the experiments and showed that augmented the expression of *MAFA* and *INS* in human primary islet cells and rescued the *MAFA* and *INS* expression in *SPINT1*-knockdown human primary islet cells. The new results are shown in Figure 5c of the revised manuscript.



Comment 6. Supplementary Figure 6 shows peripheral distribution of Hepsin suggesting that it is primarily expressed in glucagon cells. Can the authors comment on this finding?

Answer: We appreciate the valuable and insightful comments provided by the reviewer. Specifically, Reviewer #1 highlighted an intriguing finding: the peripheral distribution of Hepsin is primarily observed in glucagon cells (α cells), with less expression in β cells (Supplementary Figure 9). The differential expression pattern of HEPSIN aligns precisely with our study's hypothesis: HEPSIN may inhibit GLP1R function through proteolytic modification of GLP1R, while SPINT1 enhances GLP1R function by suppressing HEPSIN. Furthermore, we observed abundant expression of GLP1R in β cells, where HEPSIN levels are comparatively lower than in α cells. Conversely, our research and previous studies [10, 11] have indicated minimal or absent GLP1R expression in mouse α cells with high HEPSIN expression. Thus, the distinct expression profiles of HEPSIN in α and β cells may serve as a natural mechanism for regulating GLP1R protein levels, with SPINT1 acting as an additional modulator by suppressing HEPSIN to enhance GLP1R levels. The primary expression of HEPSIN in α cells could potentially act as a gatekeeper to limit GLP1R expression. In contrast, the lower expression of HEPSIN in β cells may help maintain GLP1R levels to regulate insulin biosynthesis and function in response to GLP1. However, it remains plausible that HEPSIN may also play a cell-specific role, such as maintaining α cell identities, which warrants further investigations.

Comment 7. The paper by El Quaamarti et al is cited as both reference 34 and 45.

Answer: Thank you for bringing this mistake to our attention. We apologize for the repeated reference and have already rectified it in our revised manuscript.

Reviewer #2 (Remarks to the Author):

Major criticisms:

The revised Subject: Disruption of Spint1 in mouse pancreatic β cells leads to glucose intolerance and 2 impaired insulin production– the involvement of HEPSIN/GLP1R/MAFA 3 signaling

This is a revised manuscript and the authors have addressed most concerns adequately. However, they are some minor concerns need to be addressed before publication.

Comment 1. Figure 3b, d, e, please describe the meaning of 3/3, 6/7, 6/17, etc.

Answer: Thank you for your valuable comment. In response to the reviewer's suggestion, we have provided detailed explanations of the fraction's meaning in the legend of each figure in our revised manuscript.

The descriptions of the revised statement in the legends of Figure 3b-3e are as follows:

In Figure Legends:

- "Figure 3**b,** Gene Ontology (GO) analysis of the biological processes for differentially regulated proteins in the SILAC analysis of *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ islets. The biological processes significantly affected the down-regulated and up-regulated protein groups in *Spint1*$^{-/-}$ pancreatic islets compared to *Spint1*$^{fl/fl}$ islets listed in the upper and lower panels, respectively. The fractions in the histograms represent the proportion of identified genes (numerator) in our dataset that match those pathways, with the denominator indicating the total number of genes in each pathway." (Pages 57, Lines 1159-1166)

- "Figure 3**d,** Analysis of the most highly regulated signal pathways using Ingenuity Pathway Analysis (IPA) to examine the proteins differentially expressed in *Spint1*$^{-/-}$ islets relative to *Spint1*$^{fl/fl}$ islets. The top six differentially regulated signal pathways were identified using the threshold of −log (*P*-value) >3. Down-regulated pathways are represented by blue bars, while up-regulated pathways are indicated by orange bars in *Spint1*$^{-/-}$ islets compared to *Spint1*$^{fl/fl}$ islets. The fractions in the histograms show the proportion of the identified genes (numerator) in our database that match those pathways, with the denominator indicating the total number of genes in each pathway." (Pages 58, Lines 1172-1180)

- "Figure 3**e,** List of the top five diseases and disorders identified through IPA of the differentially regulated proteins in *Spint1*$^{-/-}$ islets compared to *Spint1*$^{fl/fl}$ islets. The fractions in the table indicate the proportion of genes associated with different diseases and disorders (numerator) relative to the total number of genes (denominator) in our dataset." (Pages 58, Lines 1181-1184)

Comment 2. Figure 3b, how was the fold enrichment calculated? Why does the upper panel have x-axis with negative values?

Answer: Thank you for your insightful comment. We normalized the *Spint1*$^{-/-}$ protein values relative to those of the *Spint1*$^{fl/fl}$ group. After a systematic process of filtering nonsensical and extreme values, we categorized the proteins based on one as the reference point. The proteins with ratios above one were further refined by excluding those below 1.5 before undergoing Gene Ontology (GO) analysis. Additionally, proteins with ratios below one were transformed into negative reciprocals (-1/x) and excluded if they exceeded -1.5 before GO analysis. This approach elucidates the extent of downregulation in the *Spint1*$^{-/-}$ group.

Reviewer #3 (Remarks to the Author):

Major criticisms:

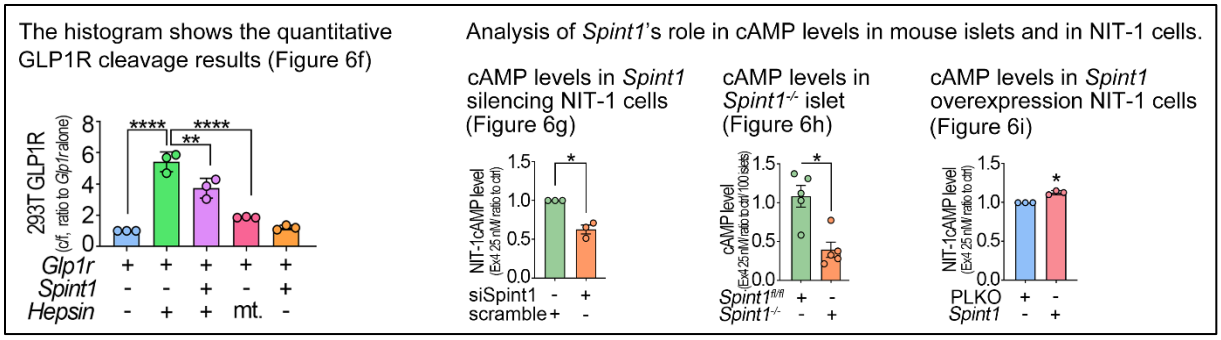
The Authors have clearly done a tremendous amount of work in revising this manuscript. However, many of the responses to the original critiques are inadequate and fail to address the underlying flaws of the overall message. For clarity, the fatal flaw in the data set is a lack of linking data from the comprehensive cell biology to a meaningful functional output. There is a knockout model in hand, and we can debate on the utility of this model, but there is no functional evidence with this reagent to demonstrate impaired GLP-1R signaling in beta-cells. Major observations from the knockout model include smaller islets that seem to be more susceptible to STZ, and an impaired glucose tolerance phenotype that is accompanied by decreases in insulin secretion. This associated with an expected phenotype of impaired GLP-1R signaling, but there is no data that directly links this. Overall, the message that can be derived from the current data set is that deletion of Spint1 at some point (perhaps during development, or perhaps in functional, mature beta-cells, or perhaps in some other cell type that is PDX1 positive) conveys a deleterious effect on beta-cell mass that confers glucose interlace. The relevance for the pathophysiology of impaired beta-cell function in diabetes is potentially there, but definitely not established. The relevance to GLP-1R activity is loosely associative at best.

Answer: We appreciate Reviewer #3 for his/her comments and acknowledgment of our substantial efforts to address his/her concerns since our initial submission. Regarding Reviewer #3's comment that "there is no functional evidence with this reagent to demonstrate impaired GLP-1R signaling in β cells.", we fully agree that establishing a connection between comprehensive cell biology and meaningful functional outcomes is crucial for scientific studies. To this end, we would like to clarify that the main purpose of this study is to elucidate SPINT1's physiological function in the pancreas, including how it regulates islet size and insulin synthesis/secretion through its downstream effector HEPSIN and the HEPSIN-mediated downregulation of MAFA-insulin. A protein interactome database unexpectedly suggested that GLP1R might be involved in SPINT1/HEPSIN action on insulin production. We proposed a hypothesis that GLP1R might act as one of the potential substrates of HEPSIN when the activity of HEPSIN is not counteracted in the absence of its inhibitor, SPINT1. This could contribute to the islet phenotypes we observed.

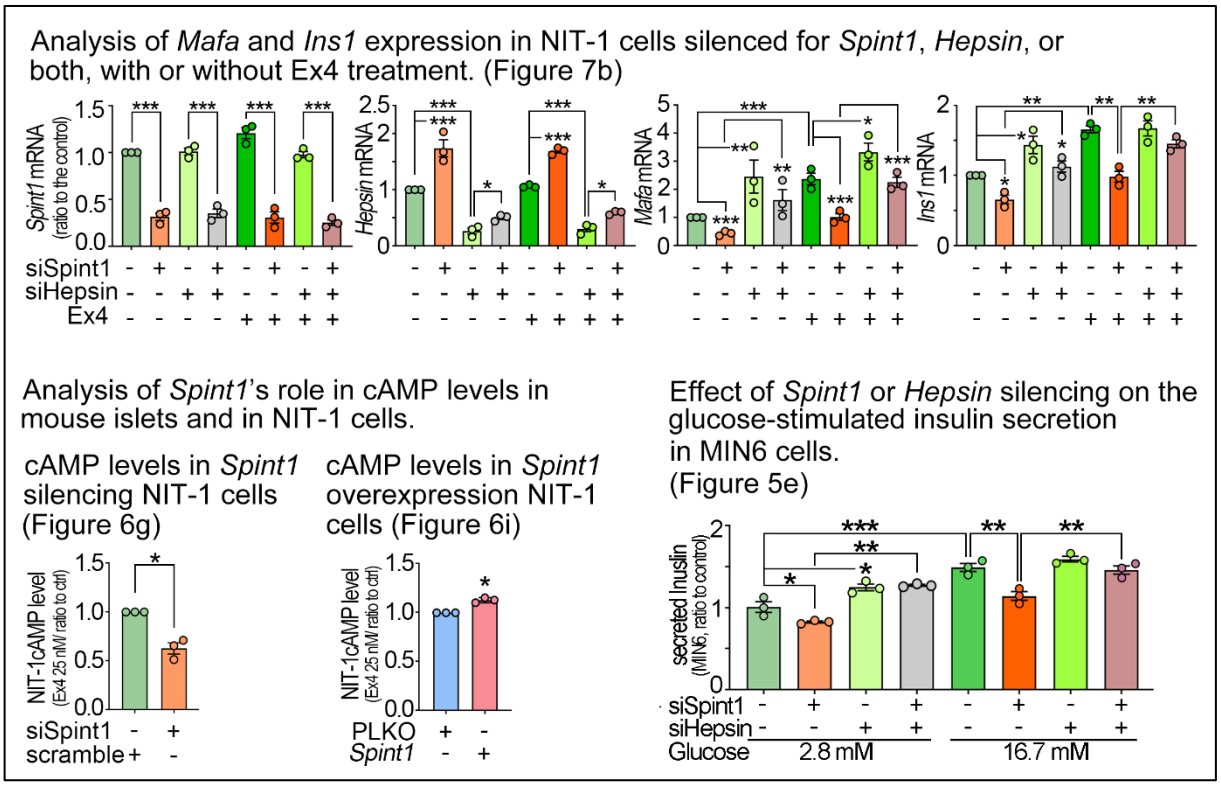
Initially, our *in vivo* proteomic approach suggested that *Spint1* deficiency might affect the function of pancreatic β cells by down-regulating G protein-coupled receptor (GPCR) pathways. Based on a published study[12] and our preliminary data showing that GLP1R is an interacting protein of HEPSIN, we sought to prove that GLP1R is a potential HEPSIN effector in SPINT1-regulated *Mafa* and *Ins1* expression. The detailed data linking cell biology to functional output regarding the roles of SPINT1 and HEPSIN in regulating GLP1R activity in islet cells are listed as follows:

1. ***Ex vivo* insulin and cAMP levels as GLP1R activity indicators**: We showed that the mRNA expression levels of *Insulin 1* and *2* were significantly reduced in isolated *Spint1*$^{-/-}$ islets compared to *Spint1*$^{fl/fl}$ islets (Figure 4A). Additionally, treatment with 25 nM GLP1R agonist exendin-4 resulted in lower cAMP production in *Spint1*$^{-/-}$ islets compared to *Spint1*$^{fl/fl}$ islets (Figure 6h). Elevated insulin expression and cAMP levels are pivotal downstream responses of GLP1R signaling in pancreatic β cells[13, 14]. Recent studies [15, 16, 17] have identified exendin-4-mediated cAMP elevation as the most common and widely used test for assessing GLP1R activity. These findings indicate a significant difference in GLP1R activity between *Spint1*$^{-/-}$ and *Spint1*$^{fl/fl}$ islets. However, in the previous manuscript, the figure legend of Figure 6

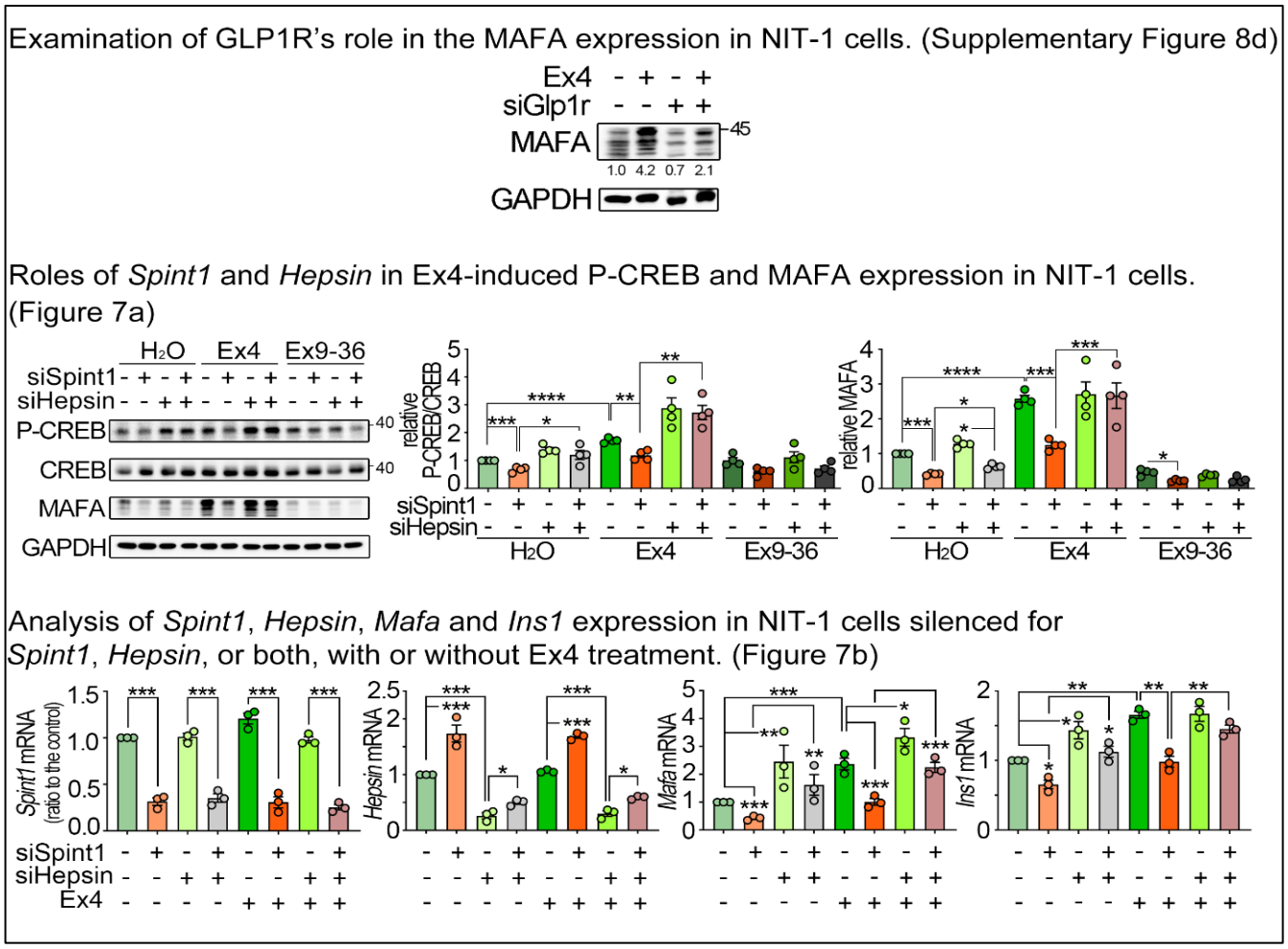
mistakenly labeled panels **f-h**) as panel **f**), which might have potentially led Reviewer #3 to overlook these important results. We sincerely apologize for any confusion this error may have caused.



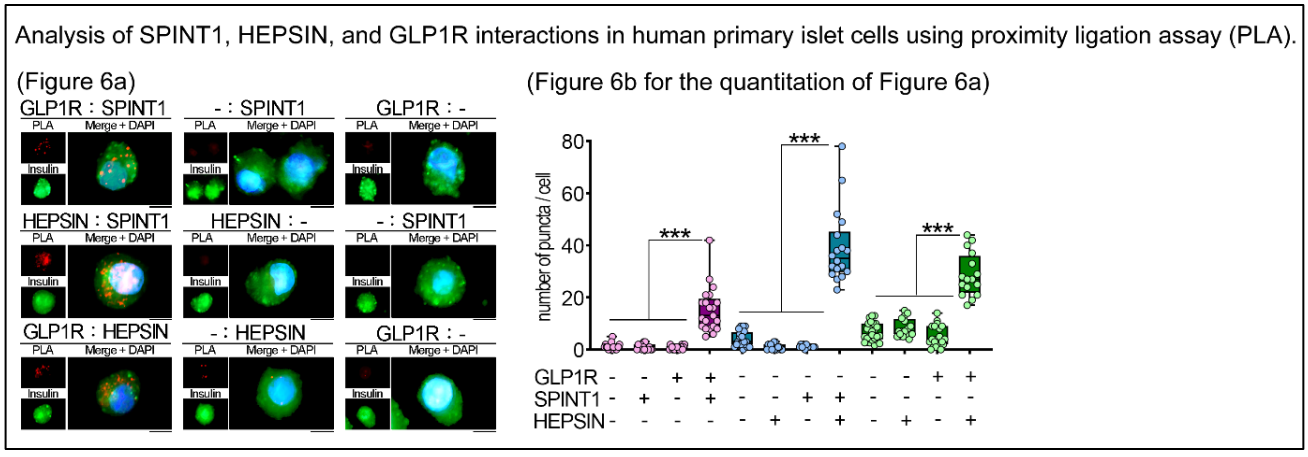
2. ***In vitro* insulin and cAMP levels as GLP1R activity indicators**: Consistent with our *ex vivo* findings, we demonstrated *in vitro* that *Spint1* depletion decreased *Insulin 1* mRNA (Figure 7b) and cAMP levels (Figure 6g) in response to exendin-4 treatment in the NIT1 mouse β cell line. Conversely, overexpression of *Spint1* in NIT-1 cells increased cAMP levels (Figure 6i). Additionally, we showed that glucose-stimulated insulin secretion in MIN6 cells was significantly reduced by *Spint1* depletion and enhanced by *Hepsin* depletion (Figure 5e).



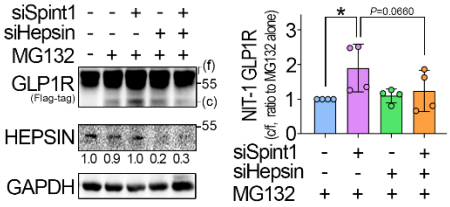
3. **MAFA and phosphorylated CREB in GLP1R signaling**: Blanchet *et al.* [18] have well demonstrated that CREB signaling mediates the effects of GLP1 agonists on β cell function by promoting MAFA expression in response to cAMP signals. Building on this, and based on our data showing that exendin-4 upregulated MAFA while *Glp1r* depletion downregulated MAFA in NIT-1 cells (Supplementary Figure 8d), we further demonstrated that *Spint1* depletion reduced the levels of phosphorylated CREB, MAFA protein (Figure 7a) and *Mafa* mRNA (Figure 7b), particularly after exendin-4 treatment. These effects were abolished by the GLP1R antagonist Ex9-36.



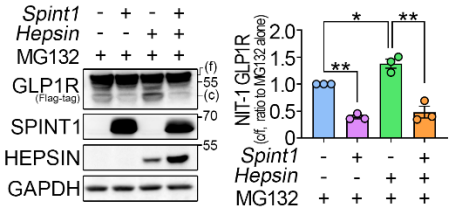
4. **SPINT1, HEPSIN, and GLP1R interaction**: To explore the mechanistic link between SPINT1 function and GLP1R activity, we screened for SPINT1 targets. Based on our results and a prior study documenting HEPSIN's ability to interact with and inhibit GLP1R[12], we hypothesized that HEPSIN, a substrate of SPINT1, mediates this regulation *via* proteolytic modification of GLP1R. Our data revealed an interaction between HEPSIN and GLP1R (Figures 6a and 6b) and demonstrated HEPSIN-mediated modification of GLP1R (Figures 6c-6f). We further found that the impaired GLP1R activity due to *Spint1* depletion, as indicated by reduced *Mafa* and *Ins1* levels, was reversed by *Hepsin* depletion (Figures 7a and 7b). Additionally, elevated cAMP levels from *Glp1r* overexpression were attenuated by *Hepsin* overexpression, an effect that was reversed by *Spint1* overexpression (Figure 6j). Moreover, our <u>new data</u> showed that *Hepsin* silencing led to increased MAFA levels, which were suppressed by Exendin 9-36 (Supplementary Figure 6f).



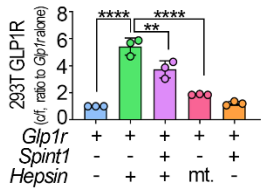
Western blot of GLP1R in *Glp1r*-overexpressing NIT-1 cells with or without **silencing** of *Spint1* or *Hepsin*.(Figure 6c)



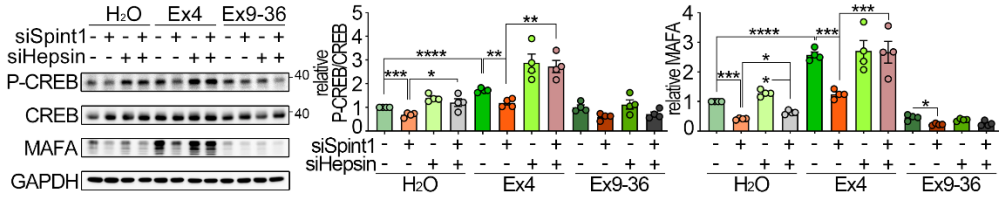
Western blot of GLP1R in *Glp1r*-overexpressing NIT-1 cells with or without **overexpression** of *Spint1* or *Hepsin*.(Figure 6d)



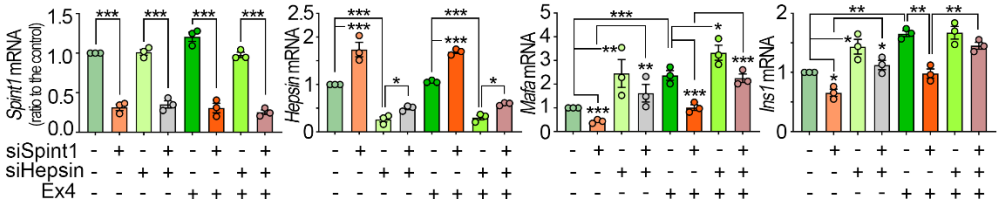
The quantitation result of immunoblot analysis of SPINT1, HEPSIN, and GLP1R in *Glp1r*-overexpressing HEK293T cells with or without the plasmids encoding cDNA for *Hepsin*, protease-null *Hepsin* mutant (mt.) or *Spint1*. (Figure 6f)



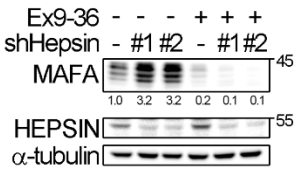
Roles of *Spint1* and *Hepsin* in Ex4-induced P-CREB and MAFA expression in NIT-1 cells. (Figure 7a)



Analysis of *Spint1*, *Hepsin*, *Mafa* and *Ins1* expression in NIT-1 cells silenced for *Spint1*, *Hepsin*, or both, with or without Ex4 treatment. (Figure 7b)



Ex9-36 treatment reversed the effect of *Hepsin* silencing on MAFA expression. (Supplementary Figure 6f)



The descriptions of the revised statement in the Results and Supplementary Figure Legends are as follows:

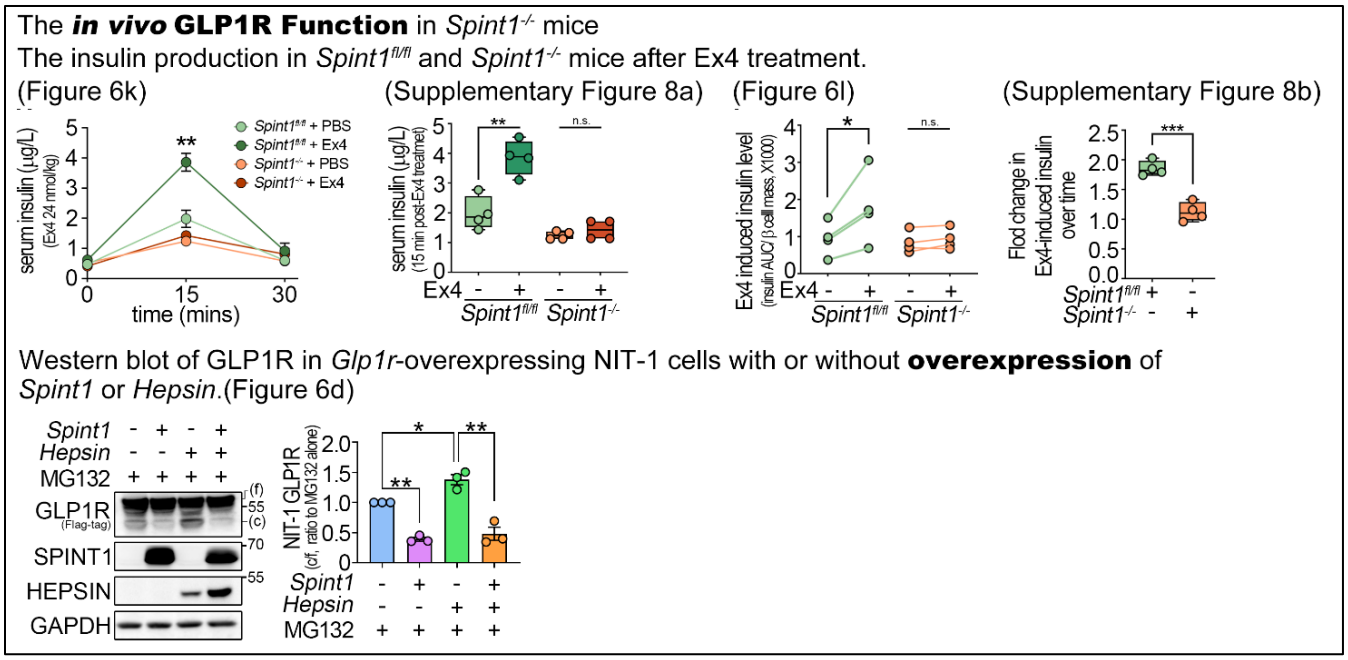
In Result:

- "Similarly, *Hepsin* silencing alone significantly increased MAFA expression in NIT-1 cells (Supplementary Fig. 6f)." (Page 17, Lines 293-294)
- "Similarly, the GLP1R antagonist (Ex9-36) suppressed the *Hepsin* silencing-induced MAFA expression in NIT-1 cells (Supplementary Fig. 6f). Thus, the results together indicate that GLP1R is involved in the action of SPINT1/HEPSIN axis on MAFA-mediated insulin production." (Page 20, Lines 350-353)

In Supplementary Figure Legends:

- "Supplementary Fig. 6**f,** Ex9-36 treatment reversed the effect of *Hepsin* silencing on MAFA expression. NIT-1 cells were infected with two different *Hepsin*-targeting shRNAs (shHepsin #1 and #2) lentivirus for 48 hours and then treated with GLP1R antagonist Ex9-36 for 24 hours. Cell lysates were collected and subjected to western blot analysis using anti-MAFA and anti-HEPSIN antibodies, with α-tubulin serving as a loading control." (Pages 18-19, Lines 234-238)

5. **New Data for *in vivo* GLP1R Function**: More importantly, in this revision, we provide new data to demonstrate further how *Spint1* affects the *in vivo* function of GLP1R on insulin production, using the approach described by Masayuki *et al.,*[19] where mice received Extendin-4 treatment to assess GLP1R's acute function on insulin secretion. The results showed that *Spint1*$^{-/-}$ mice exhibited a significant reduction in extendin-4-induced insulin secretion (new Figure 6k, Supplementary Figure 8a, Figure 6l, and Supplementary Figure 8b). Additionally, we present new data indicating that overexpression of *Hepsin* in NIT-1 cells increased the cleaved form of GLP1R, an effect abolished by *Spint1* overexpression (new Figure 6d). These new findings, together with the data from *ex vivo* (Figure 6h) and *in vitro* (Figure 6 and Figure 7, Supplementary Figure 7) analyses, strongly suggest that SPINT1 plays a critical role in modulating GLP1R-mediated insulin production in β cells through HEPSIN. Therefore, they provide a strong connection linking impaired GLP1R signaling to β cell functionals.



The descriptions of the revised statement in the result, material and method, figure and supplementary figure legends are as follows:

In Result:

- "To determine whether *Spint1* depletion affects *in vivo* GLP1R function, we analyzed serum insulin levels in *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice following treatment of exendin-4 (Ex4) and glucose oral gavage, as serum insulin levels are commonly used as an indicator for Ex4-induced GLP1R activity *in vivo*[20]. The result showed that *Spint1* deficiency suppressed the upregulation of serum insulin levels by Ex4 (Fig. 6k-l, Supplementary Fig. 8a-b), suggesting that GLP1R signaling was likely impaired in *Spint1*$^{-/-}$ mice." (Pages 19-20, Lines 338-344)

In Material and Method:

- "To assess the effects of GLP1R agonist exendin-4 (Ex4, Sigma-Aldrich) on GSIS, Ex4 was administered *via* intraperitoneally injected at a dose of 24 nmol/kg mice body weight for 30 minutes before glucose challenge, with volume-matched PBS serving as control." (Page 32, Lines 571-574)

In Figure Legends:

- "Figure 6**k,** Analysis of *in vivo* GLP1R function on stimulating insulin production in *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice after Ex4 treatment. Each mouse was intraperitoneally injected with 24 nmol/kg Ex4 for 30 minutes before an oral gavage of glucose (2 g/kg body weight). Blood samples were then collected and subjected to insulin measurement using ELISA. Control mice were injected with PBS. (n = 4 per group). **l,** Analysis of serum insulin levels normalized to β cell mass in *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice after Ex4 treatment. The Ex4-induced upregulation of insulin levels in each mouse in panel **k** were calculated as the insulin AUC (area under the curve) divided by their respective β cell mass. The connecting lines show the change in insulin levels for each mouse before and after Ex4 treatment (n = 4 per group)." (Pages 65-66, Lines 1319-1329)

In Supplementary Figure Legends:

- "Supplementary 8**a,** Effect of Ex4 on upregulating insulin level in *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice 15 minutes post-administration. We injected Ex4 into mice following oral gavage of glucose. Details of the experimental procedure are provided in Fig. 6**k**. Insulin levels were measured 15 minutes post-administration using an ELISA kit (n = 4 per group). **b,** Ex4-induced fold change in time-integrated insulin increases in *Spint1*$^{fl/fl}$ and *Spint1*$^{-/-}$ mice described in Fig. 6**l**. Ex4 upregulated the normalized insulin response over time (represented by insulin AUC/β cell mass) approximately twofold in *Spint1*$^{fl/fl}$ mice compared to that in *Spint1*$^{-/-}$ mice after Ex4 treatment." (Pages 22-23, Lines 275-282)

In summary, our data collectively provide functional evidence indicating that depletion of *Spint1* in the mouse pancreas leads to a reduction in serum insulin levels, islet mass, and the proliferation of β cells. Mechanistically, *Spint1* deficiency results in HEPSIN overactivity, which downregulates MAFA and insulin expression, at least partially through the downregulation of GLP1R activity. Therefore, we have demonstrated the link between comprehensive cell biology—specifically, GLP1R-related cAMP levels and *Mafa* expression under the conditions of exendin-4 (or exendin9-36) treatment and siRNA targeting Spint1 or Hepsin—and functional outputs, which include insulin expression levels and insulin secretion.

Due to the lack of ideal antibodies that can clearly distinguish GLP1R with or without proteolytic modifications by HEPSIN *in vivo*, for example, in immunohistochemistry, we were not able to directly demonstrate the cleavage of GLP1R protein *in vivo*. However, we did demonstrate a decrease in GLP1R-specific signaling *in vivo* in mice deficient for *Spint1*. To address your concern, we revised the abstract to highlight the definitive findings while thoroughly evaluating the potential of HEPSIN-mediated GLP1R cleavage in the Discussion, where we carefully analyze our *in vitro*, *ex vivo*, and *in vivo* data on this topic. In doing so, we aim to present this aspect of our study in a balanced manner, acknowledging its significance and current limitations. We sincerely hope that this modification will refocus the study on the SPINT1-HEPSIN-MAFA-insulin axis and adequately address the concerns of Reviewer 3. We genuinely appreciate the opportunity to address the concerns raised by Reviewer #3. Should Reviewer #3 have more specific suggestions regarding disruption of SPINT1 in mouse pancreas for glucose intolerance and impaired insulin

production, as well as the involvement of HEPSIN/GLP1R/MAFA signaling, or if there is an additional experiment Reviewer#3 deems necessary, we would wholeheartedly appreciate and commit to implementing all recommendations experimentally.

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Point-by-Point Responses to Reviewers' Questions and Comments

Reviewer #1 (Remarks to the Author):

Major criticisms:

This is the second manuscript revision, in which authors made significant improvements in response to previous critiques. They addressed the problem of tissue sampling in morphometric analysis of pancreatic islets and convincingly discussed my suggestions regarding the possibility that Spint1 may play a role in beta cell development, and Hepsin may be expressed in glucagon-expressing cells. On the other hand, the authors have not provided an independent validation of proliferation data by flow cytometry and their Western blotting still has loading controls that appear overexposed. Setting aside these controversies, most of the data are convincing and at this time I am inclined to accept this revision as final and not requiring any additional experimental work.

Answer:

We sincerely appreciate Reviewer #1 for the kind response and positive encouragement! Your feedback has been instrumental in refining our work. We are grateful that the revisions on tissue sampling and the discussions on SPINT1 and HEPSIN have addressed your suggestions effectively.

1. We fully agree with your suggestion that flow cytometry is an ideal method for analyzing cell proliferation. However, given the limited volume of mouse islets available, it would require sacrificing a substantial number of mice to obtain sufficient material for robust flow cytometry data. As an alternative, we employed high-magnification microscopy to visualize BrdU in the cell nuclei, which allows for accurate estimation of proliferation when proper random sampling and morphometric measurements are used, coupled with a sufficient number of biological replicates.

2. We appreciate your concern regarding the potential overexposure in the western blot loading controls. To address this, we have optimized the exposure times to the fullest extent possible using our current equipment, and we have provided images with the shortest achievable exposure times while maintaining clarity (e.g., Fig. 7A). Your thoughtful feedback has been invaluable in helping us improve our presentation, and we are grateful for your guidance.

Reviewer #2 (Remarks to the Author):

Major criticisms:

This is a revised manuscript and the authors have addressed almost all my concerns adequately. There is only one very minor concern need to be addressed before publication:

"Figure 3e, List of the top five diseases and disorders identified through IPA of the differentially regulated proteins in Spint1$^{-/-}$ islets compared to Spint1$^{fl/fl}$ islets. The fractions in the table indicate the proportion of genes associated with different diseases and disorders (numerator) relative to the total number of genes (denominator) in our dataset."

It will be better to use the total number of genes (denominator) in each disease and disorder, instead of total number of genes (denominator) in our dataset.

Answer:

Thank you for your valuable feedback and insightful suggestion. In response, we have replaced the original denominator (total number of genes in our dataset) with the total number of genes for each disease and disorder, as obtained from the IPA dataset. This change is reflected in our revised Figure 3e.

Top 5 of Diseases and Disordrers	
Name	(*P*-value, #Molecules)
Infectious Disease	(6.64×10^{-4}, 102/7662)
Endocrine System Disorders	(9.10×10^{-4}, 309/19654)
Tumor Morphology	(1.04×10^{-3}, 43/64)
Cancer	(1.16×10^{-3}, 371/25262)
Organismal Injury & Abnormalities	(1.18×10^{-3}, 377/33494)

The revised legend of Figure 3e is as follows:

In Figure Legend:

- "Figure **3e,** List the top five diseases and disorders identified through IPA of the differentially regulated proteins in *Spint1*$^{-/-}$ islets compared to *Spint1*$^{fl/fl}$ islets. The fractions in the table represent the number of the identified proteins associated with various diseases and disorders (numerator) relative to the total number of proteins for the corresponding disease or disorder in the IPA dataset (denominator)." (Page 60, Lines 1352-1355)

Reviewer #3 (Remarks to the Author):

No additional comments

Answer:

We are deeply grateful to Reviewer #3 for the final approval and recognition of our manuscript and response. Your insightful questions greatly contributed to improving the quality of this paper, and we sincerely thank you for your invaluable contributions.