nature portfolio

Peer Review File

Intestinal Nogo-B reduces the GLP1 levels by binding to proglucagon on the endoplasmic reticulum to inhibit PCSK1 cleavage

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Gong and colleagues report an unrecognized role for Nogo-B in the regulation of GLP-1 production and suggested that Nogo-B is a potential target for the treatment of T2DM. Given certain side-effects associated with the current GLP-1R agonists, enhancement of endogenous GLP-1 release could be an alternative therapeutic strategy. Although this work was well designed and displayed some interesting results, I have some doubts as to the interaction between Nogo-B and IP2 region of MPGF fragment. The authors hypothesized that Nogo-B might bind to the IP2 region to inhibit PCSK1-mediated cleavage of proGCG, because Nogo-B interacts with MPGF but not GLP-1 or GLP-2. Their IP-MS assay identified a peptide segment (proGCG136-156) but there is no direct evidence to indicate the binding site of Nogo-B in IP2 region. To support this hypothesis, mutations at proGCG136-146 (except for mut2 residues) should be designed to detect the interaction between the Nogo-B and the mutants in question.
In addition, the following concerns should be addressed:
1. To study the binding site of Nogo-B, Nogo-B and GFP antibodies were used to detect the interactions between Nogo-B and proGCGR fragments in Fig 3e and 3j, respectively. Why different antibodies were used for the co-IP-Western blot assay?
2. The authors compared the amino acid sequences of Nogo-B with proGCG and insulin, revealing a significant degree of sequence similarity. What was the purpose of doing this sequence alignment? What is the significance of the sequence similarities among Nogo-B and various species of proGCG and insulin?
3. Although several assays suggest that Nogo-B modulates the cleavage of proGCG by PCSK1 and ultimately reduces the production of GLP-1, does the possibility of its interaction with PCSK1 or proPCSK1 exist that may lead to functional inhibition?

Reviewer #2 (Remarks to the Author):

This is quite an interesting and timely investigation given the spotlight on GLP1 biology, especially with the current obesity and diabetes treatment paradigm shift. Common knowledge about GLP1 was focused more on clearance via DPP4, hence the novel mechanism identifying GLP1 secretion/synthesis is quite significant. Seems like the target protein Nogo-B was discovered in RNAseq screening. The authors elegantly showed the in vivo effects via multiple genetic models from siRNA, and whole-body genetic deletions to tissue-specific intestinal deletions, which are very valuable. Despite these findings would be a great addition to our understanding of GLP1 biology, this manuscript needs some revisions and addressing more questions for stronger conclusions.

First, the manuscript needs a better and more smooth flow and needs to be we-written for clear understanding. The current version, especially the conclusion is quite confusing and eclectic.

Authors truly dissociated and neglected the skeletal muscle in the phenotype and tried to

show insulin sensitivity and insulin signaling by a couple of simple qPCR or RNAseq data in the liver, which is clearly a weakness. They should show us what is canonical insulin signaling in skeletal muscle and liver by western blots, and phosphorylation assays (pIR, IR, pIRS1, pIRS2, pAKT). Additionally, they conclude insulin sensitivity via HOMA-IR in two main figures, which would be a mistake as HOMA-IR is not a validated method for mice and is already an unreliable marker of insulin sensitivity in humans. If they want to show strong effects in insulin sensitivity, they should perform a hyperinsulinemic-euglycemic clamp and show whole-body insulin sensitivity and hepatic glucose production. Because transcriptional control of glycolysis and gluconeogenesis is generally overestimated and does not represent the actual physiology. They should either measure the biochemical enzymatic activity of gluconeogenic enzymes G6p and or PCK isolated from livers.

Another point is that the authors made many graphs with a starting point that is not 0 (such as Figure 2c, 2d, etc.), which is misleading. We would suggest having levels from 0-x amount and not augmenting the difference by visualization.

In terms of insulin secretion conclusions, authors mostly used systemic insulin level measurements and concluded that Nogo-B deficiency increased insulin secretion, which is also a weak conclusion, and it is generally affected by insulin resistance levels as one can appreciate that insulin levels drop in Figure 1n with Nogo-B knockdown but elevated in Figure 2d Nogo-B KO mice. They should perform glucose-stimulated insulin secretion (GSIS) experiments to focus on insulin secretion from the pancreas.

The most novel part of the study is about the binding dynamic and cleavage of proglucagon and the synthesis of GLP-1. Their findings of binding patterns between Nogo-B, PCSK1, proGCG, and GLP1 are quite confusing. They need to use stronger and multiple binding assays such as Biacore Octet binding, radioactive binding experiments, or NMR spectroscopy for a stronger conclusion of these binding dynamics to make sure their proposed biology is accurate and not an artifact.

Another question is about the weight dependent vs independent effects in their phenotypes. They do not demonstrate the weight in Figures 1 and 2, which is problematic. We should see the weight pattern, and weekly GLP1 measurements to understand the source of the metabolic alterations whether it is fully weight dependent, GLP1-weight effect, or more acute signaling effect independent from weight phenotype. For instance, there is an interesting weight shift in Figure 1g with the Knockdown group gaining weight for 3 weeks, then losing. What is the signal for that weight shift there?

When we see lower food consumption in mice as a phenotype in metabolic cage experiments, we would prefer to see heat data and possibly make sure that mice are not simply sick and not eating, losing weight, and lowering glycemia. Systemic inflammatory markers would also be helpful to reassure the audience.

While showing the GSEA pathways for insulin signaling pathway in Figure 2 g, would be helpful to list core enriched genes and p values for 2f Enrichr data.

Again, to fully identify the systemic levels of GLP1 alterations in vivo mice models, would be

helpful to measure DPP4 activity and systemic levels to associate/establish synthesis vs clearance paradigm. Additionally, since this protein resides on ER membrane, does a deficiency of Nogo-B cause any ER dysfunction? Activation of ER stress? Authors can simply examine this with Unfolded Protein Response UPR. sXbp1, pIRE, etc.

In Figure 7, it is hard to evaluate from images. Would be helpful to analyze the image by % + staining among all slides. Using arbitrary values becomes confusing to convince the audience when image quality is poor and small images.

Possible writing suggestions.
Line 78; variety of tissues including ….. (Write the relevant organs)
Line 80; switch Nogo-B knockout to Nogo-B whole-body genetic deletion
Line 82; Nogo-B knockout mice on a normal chow diet show significant activation of the hepatic INSR-IRS-AKT pathway with no clear mechanisms.
Line 89; use full words of MPGF and proGCG as you are using these abbreviations for the first time.
Line 91; switch Knockout of intestinal Nogo-B to Tissue-specific deletion of Nogo-B in the intestine increased GLP1 and insulin levels
Line 229; Would be helpful to cite a reference paper that confirms that proGCG is going through a classical ER-golgi secretion pathway.

Reviewer #3 (Remarks to the Author):

The paper investigated the effects of Nogo-B in the context of type-2 diabetes in mice and human. The authors demonstrated with well design experiments that Nogo-B deficiency results in increased GLP-1 secretion and thereby plasma insulin levels in db/db mice and in global and intestine specific knockout mouse models. Moreover, although other groups already described the metabolic effects of Nogo-B knockout, the authors bring novelty by exploring the mechanistic effects associated to this gene in different organs. Thus, the paper brings a good contribution to the field of diabetes and to the possibility to set Nogo-B as a novel therapeutical target. However, some issues described below deserve the author's attention before the publication of the manuscript.

Major revision points:

1. The authors should explain how the hypothesis for Nogo targeting insulin and GLP-1 was generated.
1. The authors present the Knockdown of Nogo-B in db/bd mice in the mRNA level. How was the knockdown efficiency in the protein level?
2. The authors claim that the effects of Nogo-B were resulted from its action in the liver and intestine mainly. However, in the Nogo-B db/db knockdown mice the results showed a reduced food consumption. The authors should comment on the participation or not of the brain Nogos in this effect.
3. Was the knockdown in db/db mice specific for Nogo-B? How does the expression of Nogo-A and C look like?

4. Apparently, mass spectrometry was conducted at two different sites/instruments with only some generic information related to one off them (ref. 53) and no information regarding the other. Please provide detailed information regarding instrumentation, sample preparation, experimental details, instrument settings and data processing.
5. The Co-IPs data are relevant for the claims provided in the manuscript; however, the experimental conditions are not described in detail as they should allow replication. Please provide more information.
6. Overall the authors show the increased GLP-1 levels upon Nogo-B knockdown or Knockout. The effects should be resulted from reduced cleavage by PCSK1. Do the authors observed similar effects on the level of GLP-2? Were the metabolic effects associated to changes only in GLP-1? How does the GLP-2 serum levels look like in the presented animal models?
7. Line 149-150 - The authors claim that the expression of Nogo-A is lower in pancreas and liver cells. However, the western blots were done with the whole tissue. In the liver the kupfer cells are not the predominant cell type and in the pancreas the exocrine cells are the major cell type. Thus, the expression of Nogo-A could be of relevance as the kupfer cells and pancreatic islet cells compose another metabolic unit in the liver and pancreas respectively. Similarly, the expression of Nogo-A in the whole fraction of intestine could be low. However, the expression in L-cells could be totally different, what may not exclude other Nogos action in GLP-1 and 2 processing. This information should be adjusted in the paper. Moreover, the authors should provide information on the expression of Nogo-A and Nogo-B in the pancreatic islets only.
8. Line 284-285 - Sentence in the results does not correspond to what the Figure 5f shows for GLP-1 serum levels. The text describe higher levels of GLP-1 in Nogof/fVillinCre but the Figure shows lower levels compared to Nogof/f mice. What is the hypothesis for lower levels of GLP-1 in these mice?
9. Line 324 - What the authors mean by pancreatic dedifferentiation? Dedifferentiation of beta cells cannot be inferred only by stainings. The expression of beta and alpha cell markers in pancreas/islets of these mice must be provided to prove a dedifferentiation hypothesis.
10. Information on the body weight of db/db mice subjected to Nogo-B knockdown experiments and in the global knockouts should be provided as this constitute an important metabolic feature associated with the described phenotypes.
11. Regarding the overexpression of Nogo-B in HEK cells, do the authors have available cDNA/protein to verify if the retention of proglucagon in the ER activates pathways of cell recycling and markers for celular stress like activation of the UPR response?
12. Extended Data Figure 2a and b – The authors claim a significant degree of similarity of Nogo-B and proglucagon and insulin in mice and human. How was this determined?
13. Data of the blood glucose levels of intestine specific knockout mice subjected to HGD should be provided (Figure 5).
14. The determination of Nogo-B expression using stained optical density of patient intestine sections is a weak quantification method. Moreover, no detailed information is provided in the methods. Were the sections stained at the same time? How many sections were analyzed/ patient. To strengthen this finding, further information on the blood glucose, insulin, HOMA and GLP-1 levels in the selected patients should be provided.
15. Supplemental data is provided ("Liver_Pancreas_Nogo_7-10.xlsx") referring to the results of co-immunoprecipitation mass spectrometry conducted on mouse pancreas and

liver samples. The table lists iBAQ values (please provide information on how these were calculated), other parametes (Q-value of what?) and an arbitrary “score” that appears to have been used for target selection. However, among other questions related to quality control of protein identification, it remains a mystery how the authors derived the claim that Nogo-B binds insulin and GCG from the >1000 entries of the list, many of those apparently showing the same pattern in terms of row values. This is rather a substandard presentation of mass spec data and needs to be revised (and explained) substantially.
16. Data availability: It is common practice to deposit all raw data in publicly accessible databases. Unfortunately, the authors have only provided a small subset of the data (GSE236979; liver data of nogo knockouts). Please provide all data shown in the msunuscript, including the proteomics data set.

Minor revision points:

1. Figure 1e – What the Figure describes does not fit with the methods description. In the methods db/m group received scramble siRNA, while in the Figure saline is supposed to be administered to these mice only.
2. In the Figure 1h, are the comparisons of fasted blood glucose between db/db and db/db-Nogo KD non-significant or the significance p values were missed for this data point?
3. Line 109-111 - The sentence should be a rephrased removing the word “suggestion” as it seems to be associated to a degree of uncertainty.
4. Line 158-159 – Sentence repeated twice
5. Line 250 - Do the authors mean GLP-1 in this sentence?
6. Line 252 - Do the authors mean PCSK1?
7. Line 253-254 - A conclusion sentence is followed by the repetition of a result sentence
8. Line 392-393 - Where do the authors show serum GLP-1 levels in diabetic patients? It is mentioned in the text but no Figure with this data is presented.
9. Line 395 - Word homeostasis doubled
10. The authors should discuss the mechanism of body weight changes in the intestine specific Nogo-B Knockout.
11. Figure 5I – Correct a typo in the word insulin in the Figure panel
12. The term “liver knockout” (Line 406,407) may confuse the reader towards results of a liver specific knockout. The sentences should better clarify that these results are referred to effects on the liver from a global knockout of Nogo-B.
13. Extended data Figure 1 – Typo in the Y graph axis -correct to liver weight
14. In the methods the authors described that the mice were “humanely euthanized by CO_2 asphyxiation”. This is not an appropriate term - use killed by CO_2 asphyxiation [Editor note: we believe the best term to use is 'euthanized', without the "humanely" part.]

Manuscript No. NCOMMS-23-47484-T

"Intestinal Nogo-B reduces the GLP1 levels by binding to proglucagon on the endoplasmic reticulum to inhibit PCSK1 cleavage" by Dr. Ke Gong et al.

We appreciate the careful and constructive comments of the Reviewers on our manuscript. In response to each of the reviewers' comments, we have performed in vitro experiments such as point mutation and SPR assays, and repeated animal experiments for additional studies such as GSIS and hyperinsulinemic/euglycemic clamp experiments. A point-by-point response is as follows.

Reviewer #1 (Remarks to the Author):

Gong and colleagues report an unrecognized role for Nogo-B in the regulation of GLP-1 production and suggested that Nogo-B is a potential target for the treatment of T2DM. Given certain side-effects associated with the current GLP-1R agonists, enhancement of endogenous GLP-1 release could be an alternative therapeutic strategy. Although this work was well designed and displayed some interesting results, I have some doubts as to the interaction between Nogo-B and IP2 region of MPGF fragment.

The authors hypothesized that Nogo-B might bind to the IP2 region to inhibit PCSK1-mediated cleavage of proGCG, because Nogo-B interacts with MPGF but not GLP-1 or GLP-2. Their IP-MS assay identified a peptide segment (proGCG136-156) but there is no direct evidence to indicate the binding site of Nogo-B in IP2 region. To support this hypothesis, mutations at proGCG136-146 (except for mut2 residues) should be designed to detect the interaction between the Nogo-B and the mutants in question.

Response*:* Thank you for your important suggestions. We fully agree with your advice, as it helps us better explain how Nogo-B is involved in the processing and maturation of GLP1.

Based on your insightful suggestion, we have re-analyzed the co-IP-MS results to precisely identify the specific binding sites of Nogo-B with MPGF. However, we have found that the peptide sequence with the highest match to Nogo-B binding is DFPEEVAIVEELGR at positions 131-141 (**Response Figure 1a**), rather than the previously shown positions 136-156. We appreciate your suggestion, which helped us to identify the error in the analysis results.

Additionally, we consulted colleagues specializing in proteomics research to re-examine the binding sites between Nogo-B and proGCG. They confirmed that Nogo-B binds to proGCG at DFPEEVAIVEELGR (positions 131-141). We have corrected this information in **Figure 3m of the revised manuscript**.

Furthermore, subsequent co-IP experiments based on amino acid mutations also suggested that the binding between Nogo-B and the proGCG fragment existed in this region. As shown in **Response Figure 1b**, the positions 131-141 are entirely within the IP2 region.

The co-IP-MS results indicate that Nogo-B binds to the IP2 region of proGCG, which is crucial for the maturation of GLP1. Based on your valuable suggestion, this result prompts us to investigate the specific amino acid residues within the IP2 region to which Nogo-B binds. Since protein-protein interaction is typically mediated by hydrophobic amino acids, we mutated phenylalanine (F, position 132) and leucine (L, position 142) to alanine (A) within the proGCG131-144 segment. Additionally, glutamate (E) is the most frequent amino acid in this region, so we also mutated glutamate (position 134, 135, 140, and 141) to alanine (A). We then performed co-IP assay separately and assessed whether the mutated forms of MPGF bind to Nogo-B (**Response Figure c-e**). Interestingly, after mutating phenylalanine and glutamate to alanine in the region, Nogo-B still bound to MPGF. However, when leucine at position 142 was mutated to alanine, Nogo-B no longer bound to MPGF.

The above results indicate an interaction between Nogo-B and the leucine residue at position 142 of proGCG, participating in the cleavage and maturation process of GLP1. In the revised manuscript, we have added these data to the Results section **(lines 253-269 on pages 10-11)**.

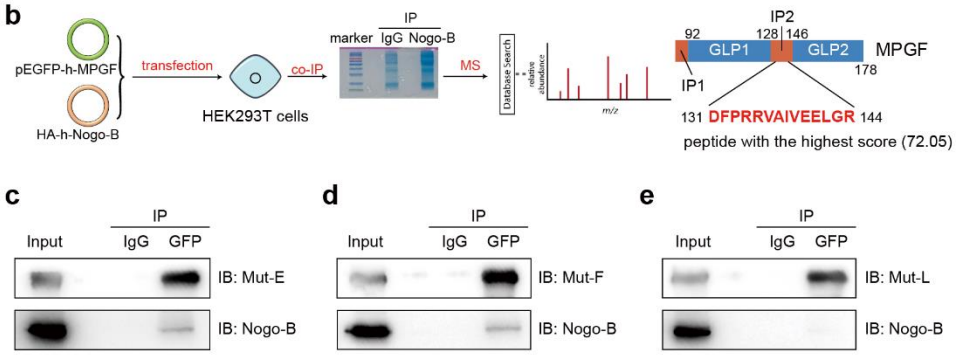
The new figures are displayed in **new Figure 3, new Extended Data Figure 7** and **Response Figure 1** as follows:

Response Figure 1

a

co-IP-MS using anti-Nogo-B antibody

the detected peptides of MPGF	position	score
K.EFIAWLVK.G	118-125	33.64
R.DFINWLIQTK.I	166-175	48.47
R.DFPEEVAIVEELGR.R	131-144	72.05
R.DFPEEVAIVEELGR.R	131-144	65.84
R.HADGSFSDEMNTILDNLAAR.D	146-165	89.34
R.HAEGTFTSDVSSYLEGQAAK.E	98-117	73.03
R.RDFPEEVAIVEELGR.R	130-144	53.07



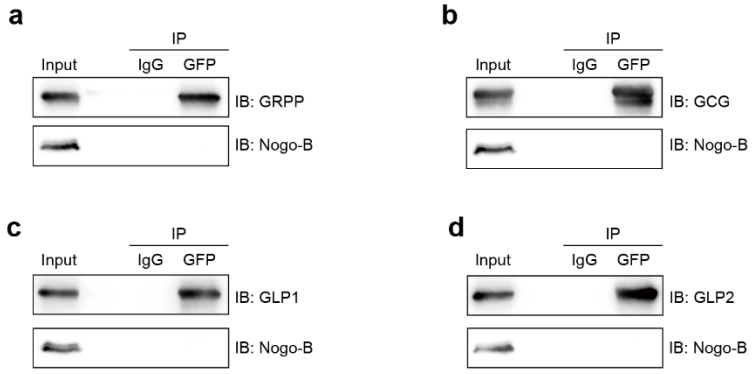
Response Figure 1. **Nogo-B binds to the IP2 region of proGCG through leucine at position 142.** Mass spectrometric detection of the peptide bound to MPGF by Nogo-B (**a**), where the peptide with the highest secondary structure similarity to the profile was in the IP2 region. The phenylalanine at position 132 was mutated to alanine (mut-F), the leucine at position 142 was mutated to alanine (mut-L), and the residues at positions 134, 135, 140, and 141 were mutated to alanine (mut-E) (**b**). Construction of EGFP-tagged expression vectors for mut-E, mut-F, and mut-L (**b**). The binding of Nogo-B to mut-E (**c**), mut-F (**d**), and mut-L (**e**) was detected using co-immunoprecipitation and Western blot.

<u>Comment 1</u>: To study the binding site of Nogo-B, Nogo-B and GFP antibodies were used to detect the interactions between Nogo-B and proGCG fragments in Fig 3e and 3j, respectively. Why different antibodies were used for the co-IP-Western blot assay?

<u>Response</u>: Thank you for pointing out this issue. We completely agree with your suggestion. In the initial experiments, we used anti-Nogo-B antibody to detect peptides that interact with Nogo-B, and then switched to anti-GFP antibody for the experiments to save costs. For the reliability and scientific validity of the experiment, it is indeed necessary to use the same IP antibody to detect the interaction between Nogo-B and different peptides of proGCG.

Based on this suggestion, we repeated the co-IP experiment and used anti-GFP antibodies to detect the interaction of different peptides of proGCG with Nogo-B (**Response Figure 2**). Consistent with previous findings, Nogo-B interacts only with the MPGF peptide, and not with GRPP, GCG, GLP1, or GLP2. The new results have been incorporated into the **Figure 3e-i of the revised manuscript**.

Response Figure 2



Response Figure 2. Nogo-B does not bind to GRPP, GCG, GLP1, and, GLP2. Immunoprecipitation assay with endogenous Nogo-B and proGCG-sheared basic short peptides (**a**: GRPP, **b**: glucagon, **c**: GLP1, and **d**: GLP2).

<u>Comment 2</u>: The authors compared the amino acid sequences of Nogo-B with proGCG and insulin, revealing a significant degree of sequence similarity. What was the purpose of doing this sequence alignment? What

is the significance of the sequence similarities among Nogo-B and various species of proGCG and insulin?

Response: Thank you for your important suggestions. The homology of protein sequences to some extent indicates their ability to bind to proteins with similar structures[1]. Since proteins with similar sequences often have similar three-dimensional structures and functions, we first assessed whether Nogo-B interacts with the receptors of these hormones by comparing sequence similarity and using co-IP-MS assays. co-IP-MS assay exhibited that GCGR, GLP1R, and INSR were not detectable, prompting us to further investigate whether Nogo-B is involved in hormone maturation processes.

Our exploration process is as follows:

1. We observed that the inhibition (in db/db and db/db-KD mice) or absence (in WT and $Nogo^{-/-}$ mice) of Nogo-B significantly affected blood glucose levels in mice which was accompanied by changes in the levels of insulin, glucagon, and GLP1 in Figure 1 and 2. This prompted us to investigate whether Nogo-B regulates blood glucose levels by modulating hormone production or function. Considering Nogo-B located in the ER and plasma membrane, and the ER is responsible for processing secreted proteins, the receptors for the hormones are located on the plasma membrane. Therefore, we hypothesize that Nogo-B may influences the production of these hormones, or competitively binds to the receptors of these hormones, thereby affecting their levels.

2. However, upon further co-IP-MS analysis, we did not detect any binding between Nogo-B and GCGR, GLP1R or INSR, which prompted us to explore another possibility. The ER is a crucial site for protein processing[2]. Additionally, in the results of co-IP-MS, we identified insulin and proGCG proteins, suggesting that the ER protein Nogo-B is likely directly involved in the processing of proGCG or insulin proteins in the ER.

3. Through co-IP experiments, we discovered that Nogo-B can bind to proGCG, rather than insulin. This result led us to investigate the binding of Nogo-B to different segments of proGCG, ultimately revealing that Nogo-B interacts with MPGF, thereby affecting the processing and maturation of GLP1.

Through the comparison of protein sequences between Nogo-B and proGCG and insulin, as well as IP-MS detection, we have ruled out the possibility of Nogo-B affecting the function of glucagon or insulin by binding to their receptor, which prompted us to focus on the direct involvement of Nogo-B in the processing and maturation of proGCG.

Based on your reasonable suggestion, we have optimized the description of the sequence alignment results in the revised manuscripts **(lines 197-202 on page 9).**

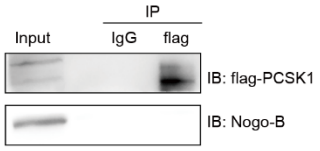
Comment 3: Although several assays suggest that Nogo-B modulates the cleavage of proGCG by PCSK1 and ultimately reduces the production of

GLP-1, does the possibility of its interaction with PCSK1 or proPCSK1 exist that may lead to functional inhibition?

***Response**:* Thank you for this comment. Based on your suggestion, we transfected human Nogo-B and PCSK1 expression vectors into HEK293t cells, and performed co-IP to investigate whether Nogo-B interacted with PCSK1 protein, which is crucial for elucidating the role of Nogo-B in regulating the cleavage of GLP1. However, we found that there was no interaction between Nogo-B and PCSK1 (**Response Figure 3**), suggesting that Nogo-B did not lead to functional inhibition by binding to the PCSK1 protein. We have added this to the Results section of the revised manuscript **(lines 283-284 on page 11)**.

The new figures are displayed in **new Extended Data Figure 7** and **Response Figure 3** as follows:

Response Figure 3



Response Figure 3. Nogo-B does not bind to PCSK1. Immunoprecipitation assay with Nogo-B and exogenous PCSK1 with a flag tag in HEK293T cells.

References:

1. Gallone G, Simpson TI, Armstrong JD, Jarman AP. Bio::Homology::InterologWalk--a Perl module to build putative protein-protein interaction networks through interolog mapping. *BMC Bioinformatics* **12**, 289 (2011).
2. McCaffrey K, Braakman I. Protein quality control at the endoplasmic reticulum. *Essays Biochem* 60, 227-235 (2016).

Reviewer #2 (Remarks to the Author):

This is quite an interesting and timely investigation given the spotlight on GLP1 biology, especially with the current obesity and diabetes treatment paradigm shift. Common knowledge about GLP1 was focused more on clearance via DPP4, hence the novel mechanism identifying GLP1 secretion/synthesis is quite significant. Seems like the target protein Nogo-B was discovered in RNAseq screening. The authors elegantly showed the *in vivo* effects via multiple genetic models from siRNA, and whole-body genetic deletions to tissue-specific intestinal deletions, which are very valuable. ***Despite these findings would be a great addition to our understanding of GLP1 biology, this manuscript needs some revisions and addressing more questions for stronger conclusions.***

Response*:* Thank you for your valuable suggestions. We have added a substantial amount of new data and have significantly reorganized and revised the manuscript. Additionally, we have optimized the Results and Discussion sections to enhance the coherence of the manuscript and to highlight the conclusions of our research.

Comment 1: First, the manuscript needs a better and more smooth flow and needs to be we-written for clear understanding. The current version, especially the conclusion is quite confusing and eclectic.

Response*:* Thank you for your important suggestions. In response to the Reviewers' suggestions, we have included new experiments and data. We have repeated animal experiments, including db/db mice injected with control siRNA and Nogo-B siRNA, $Nogo^{f/f}$ and $Nogo^{f/f}Villin^{Cre}$ mice fed a high-glucose diet, as well as STZ-treated diabetic $Nogo^{f/f}$ and $Nogo^{f/f}Villin^{Cre}$ mice. We performed experiments such as hyperinsulinemic/euglycemic clamp experiments, GSIS, measurement of serum GLP2, DPP4 and inflammatory factors, among others. In conjunction with the newly added experiments, we have reorganized the Results section and optimized the structure of each section to ensure better logical flow and coherence. We have also restructured and emphasized the conclusions to enhance the clarity and rationality of the manuscript.

Your valuable suggestions are crucial in improving the coherence and completeness of the manuscript. Based on your recommendations, we have thoroughly revised the manuscript. We believe that these revisions will make the conclusions clearer and easier for readers to understand.

Comment 2: Authors truly dissociated and neglected the skeletal muscle in the phenotype and tried to show insulin sensitivity and insulin signaling by a couple of simple qPCR or RNAseq data in the liver, which is clearly a weakness. They should show us what is canonical insulin

signaling in skeletal muscle and liver by western blots, and phosphorylation assays (pIR, IR, pIRS1, pIRS2, pAKT).

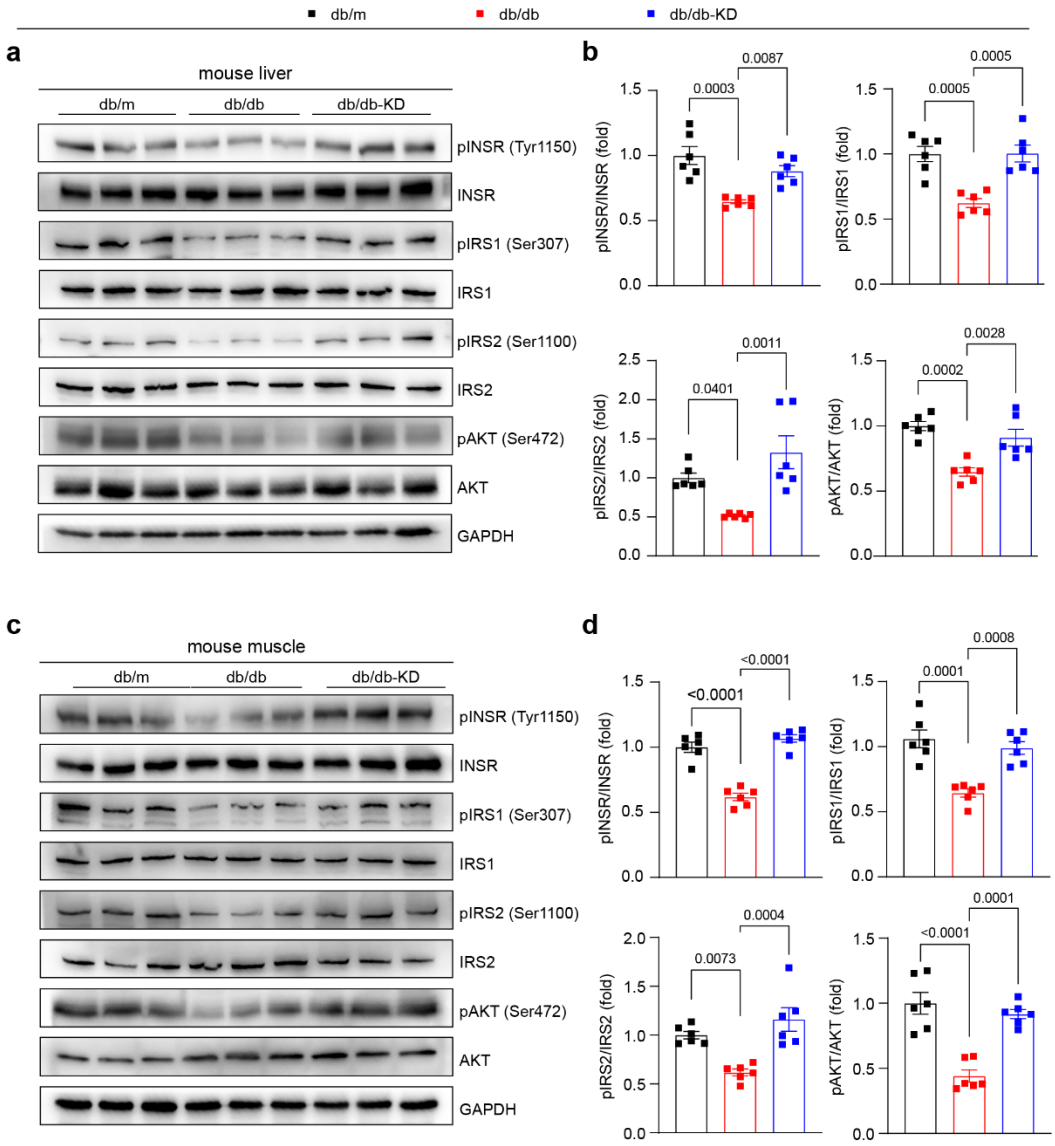
Response*:* We greatly appreciate your valuable suggestions. Skeletal muscle is the major site for the disposal of ingested glucose in individuals with normal glucose tolerance and is responsible for the majority of insulin-stimulated whole-body glucose disposal under normal conditions[1.] Following your suggestion, we detect the phosphorylation levels of IR, IRS1, IRS2, and AKT in muscle and liver by Western blot.

It is difficult to detect phosphorylated IR, IRS1, and IRS2 protein in liver and skeletal muscle by Western blot without insulin stimulation. Therefore, we needed to pre-treat the mice with insulin to assess insulin sensitivity. Since our research indicates that Nogo-B regulates insulin secretion by affecting GLP1 cleavage, injecting insulin might interfere with the results. Therefore, we repeated all animal experiments, followed by injecting each mouse with an equal volume of glucose (3 mice per group) to activate the insulin signaling pathway. After euthanizing the mice, we collected liver and skeletal muscle tissues, extracted tissue proteins, and detected molecules related to the insulin signaling pathway by Western blot.

As shown in **Response Figure 4-7**, inhibition or deficiency of Nogo-B can increase the phosphorylation levels of IR, IRS1, IRS2, and AKT in liver and skeletal muscle. This suggests that inhibition or deficiency of Nogo-B can enhance insulin sensitivity in liver and skeletal muscle by promoting intestinal GLP1 cleavage and increasing serum GLP1 levels. We have added it to the Results section of the revised manuscript **(lines139-141, 190-192, 349-350, 378-379 on page 7, 8, 14, 15).**

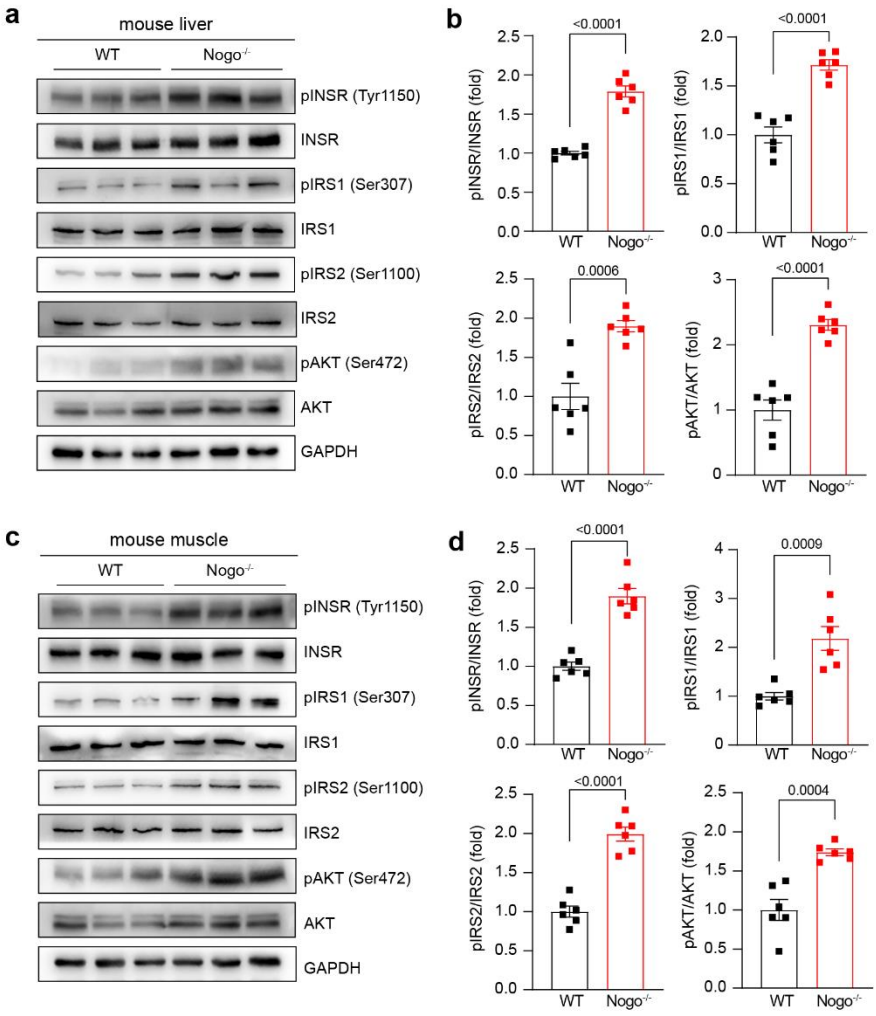
The new figures are displayed in **new Figure 1, 2, new Extended Data Figure 2, 5, 11, 13,** and **Response Figure 4-7** as follows:

Response Figure 4



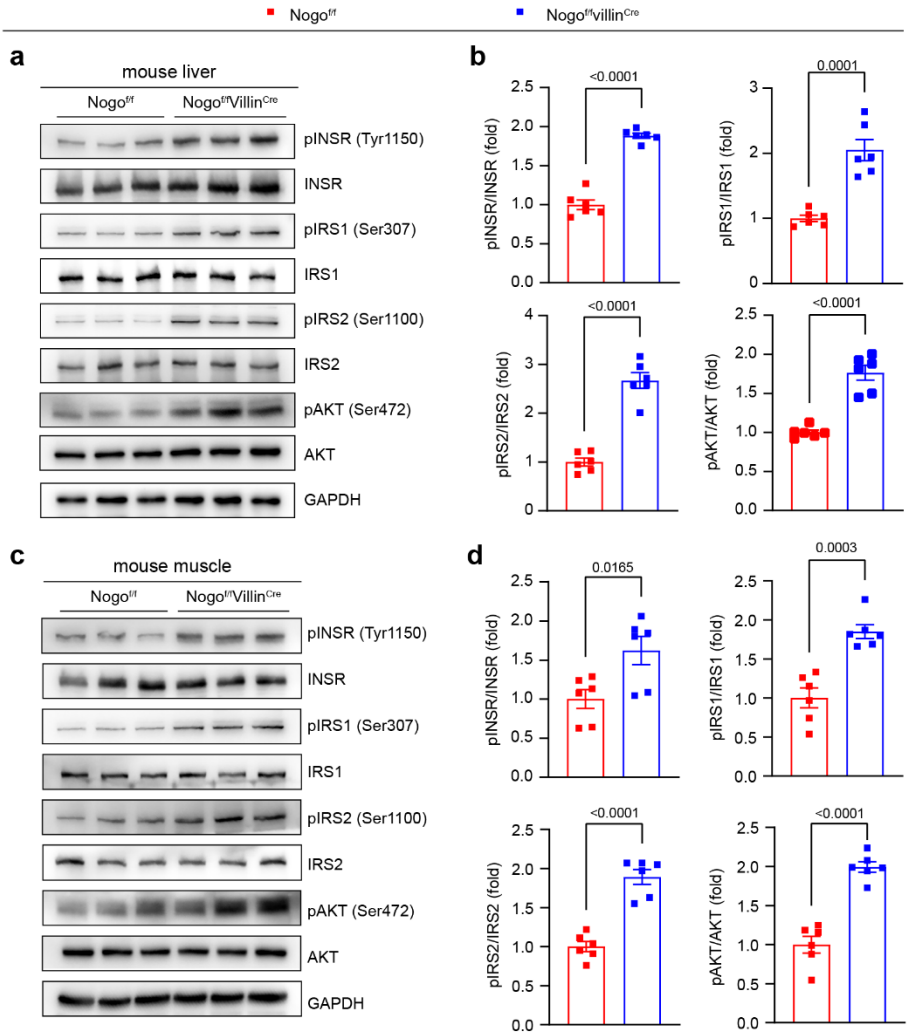
Response Figure 4. Nogo-B inhibition activates insulin signaling in the liver and muscle of mice. pINSR, INSR, pIRS1, IRS1, pIRS2, IRS2, pAKT and AKT protein levels in mouse liver (**a**，**b**) and muscle (**c, d**) of db/db mice and analysis of band density (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by one-way ANOVAs.

Response Figure 5

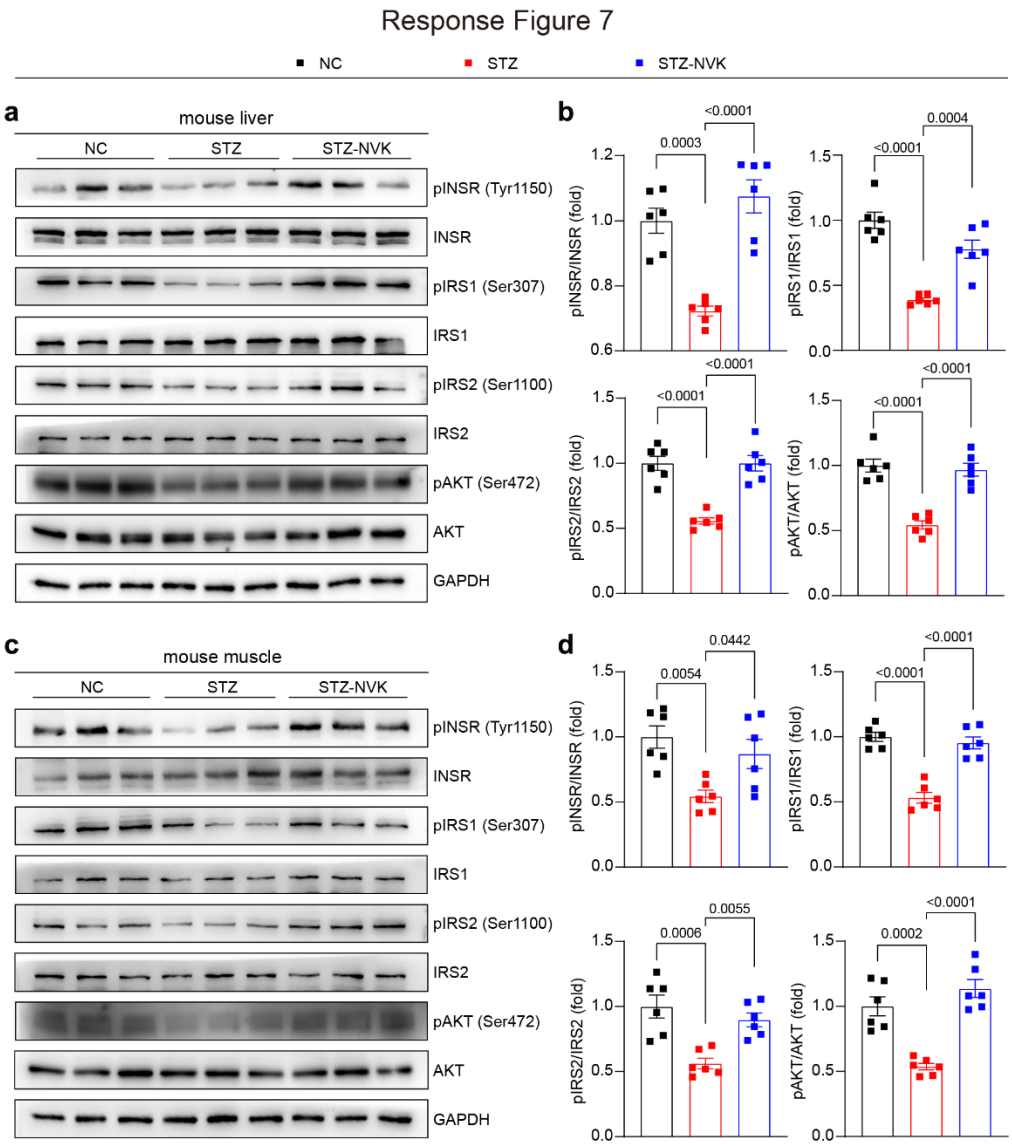


Response Figure 5. Nogo-B deficiency activates insulin signaling in the liver and muscle of mice. pINSR, INSR, pIRS1, IRS1, pIRS2, IRS2, pAKT and AKT protein levels in mouse liver (**a**，**b**) and muscle (**c, d**) of normal chow-fed WT and Nogo$^{-/-}$ mice and analysis of band density (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.

Response Figure 6



Response figure 6. Intestinal Nogo-B deficiency activates insulin signaling in the liver and muscle of mice. pINSR, INSR, pIRS1, IRS1, pIRS2, IRS2, pAKT and AKT protein levels in mouse liver (**a**，**b**) and muscle (**c, d**) of normal chow-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice and analysis of band density (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.



Response figure 7. Intestinal Nogo-B deficiency activates insulin signaling in the liver and muscle of STZ-induced diabetic mice. pINSR, INSR, pIRS1, IRS1, pIRS2, IRS2, pAKT and AKT protein levels in mouse liver (**a**，**b**) and muscle (**c, d**) of STZ-induced diabetic Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice and analysis of band density (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by one-way ANOVAs.

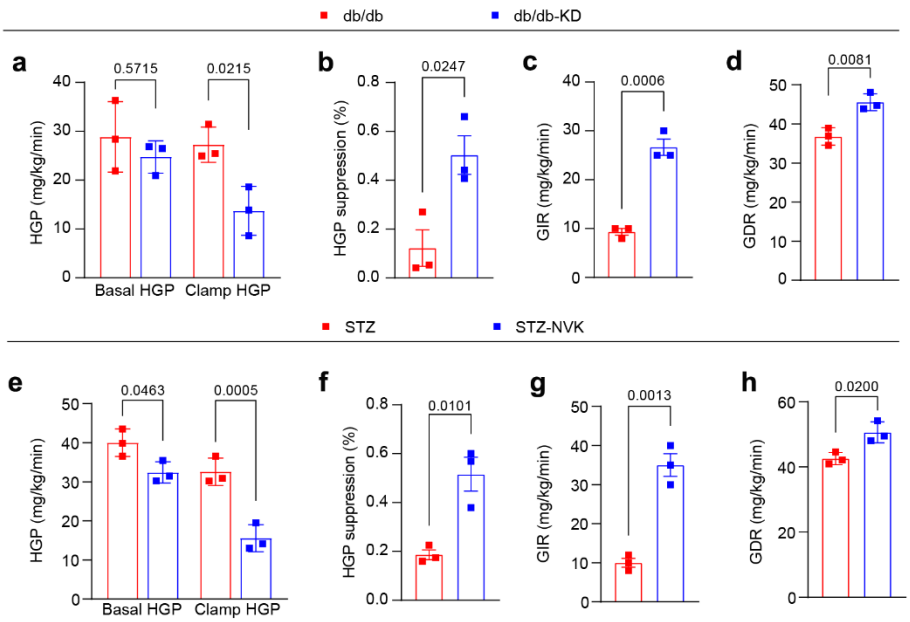
<u>Comment 3</u>: Additionally, they conclude insulin sensitivity via HOMA-IR in two main figures, which would be a mistake as HOMA-IR is not a validated method for mice and is already an unreliable marker of insulin sensitivity in humans. If they want to show strong effects in insulin sensitivity, they should perform a hyperinsulinemic-euglycemic clamp and show whole-body insulin sensitivity and hepatic glucose production.

<u>Response</u>: Thank you for your valuable suggestions. We have thoroughly investigated this issue and fully agree with your suggestion that using HOMA-

IR as a representation of insulin sensitivity is insufficient. To more accurately measure the effects of Nogo-B knockdown on insulin sensitivity, we performed hyperinsulinemic-euglycemic clamp experiments in db/db mice and STZ-treated diabetic $Nogo^{f/f}Villin^{Cre}$ mice. Inhibition or absence of Nogo-B significantly enhanced insulin-stimulated suppression of hepatic glucose production (HGP, **Response Figure 8a, b, e, f**), while increasing glucose infusion rate (GIR, **Response Figure 8c, g**) and glucose disposal rate (GDR, **Response Figure 8d, h**), reflecting whole-body insulin sensitivity. We have included these results to the Results section of the revised manuscript **(lines 141-145, 380-383 on page 7, 15).**

The new figures are displayed in **new Figure 1, new Extended Data Figure 13**, and **Response Figure 8** as follows:

Response Figure 8



Response Figure 8. **Nogo-B inhibition and intestinal Nogo-B deficiency improves mice insulin sensitivity**. **a, e**, HGP under basal and clamp conditions of mice (n = 3). **b, f**, HGP suppression of mice (n = 3). **c, g**, glucose infusion rate (GIR) of mice (n = 3). **d, h**, glucose disposal rate (GDR) of mice (n = 3). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t-test.

<u>Comment 4</u>: Because transcriptional control of glycolysis and gluconeogenesis is generally overestimated and does not represent the actual physiology. They should either measure the biochemical enzymatic activity of gluconeogenic enzymes G6p and or PCK isolated from livers.

<u>Response</u>: We greatly appreciate your valuable suggestions. We fully agree with your suggestion that assessing the enzyme activity of glucose-6-

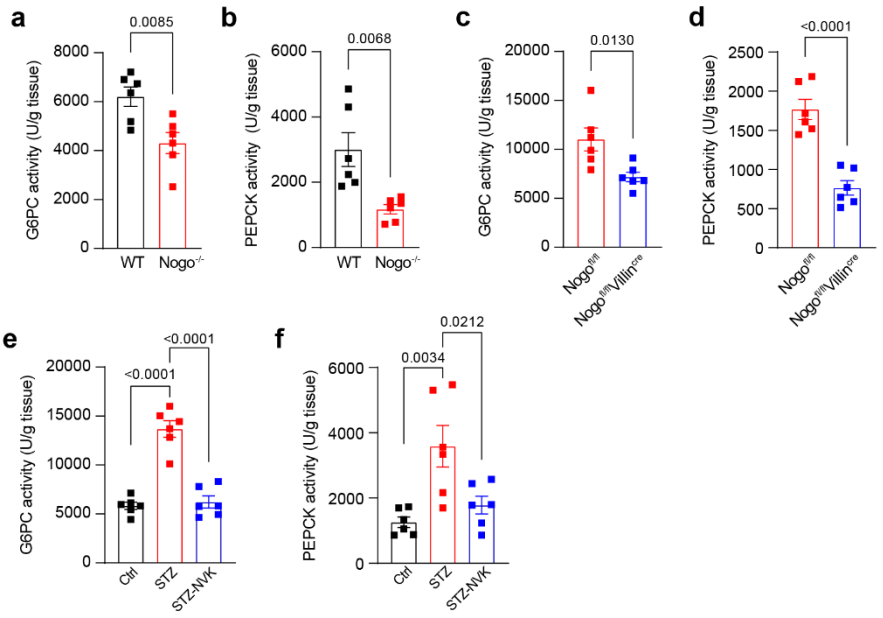
phosphatase (G6PC) and phosphoenolpyruvate carboxykinase 1 (PEPCK) would better represent the processes of gluconeogenesis.

Given the susceptibility of enzyme activity to factors such as temperature and storage time, we repeated the animal experiments. We collected fresh liver tissue immediately after euthanasia and conducted enzyme activity assays to ensure the accuracy of the results. We collected 100 mg of fresh liver tissue from mice, and conducted enzyme activity assays using G6PC and PEPCK assay kits. **As shown in the Response Figure 9**, consistent with the liver RNA-Seq enrichment results, systemic knockout of Nogo-B inhibits the enzyme activity of G6PC and PEPCK in the liver, thereby suppressing hepatic gluconeogenesis (**Response Figure 9a, b**). In addition, we observed that intestinal specific Nogo-B knockout in mice subjected to HGD (**Response Figure 9c, d**) and STZ (**Response Figure 9e, f**) treatment also inhibited the activities of G6PC and PEPCK in liver.

Research has shown that GLP1 can inhibit pancreatic α-cell secretion of glucagon, thereby suppressing hepatic gluconeogenesis[2]. Indeed, in our *in vivo* experiments, we observed that systemic or intestine-specific Nogo-B knockout elevated serum GLP1 levels and decreased serum glucagon levels, thereby inhibiting hepatic gluconeogenesis (**Figure 2b**, **Figure 5f-h**, **Figure 6d-f**). Your suggestion was very helpful for our research. We have incorporated these experimental results into the revised manuscript **(lines 187-190, 348-349, 380-381 on pages 8, 14, 15).**

The new figures are displayed in **new Figure 1, new Extended Data Figure 11, 14,** and **Response Figure 9** as follows:

Response Figure 9



Response Fig. 9. Nogo-B deficiency/inhibition inhibited hepatic gluconeogenesis. Using enzyme activity detection kits, the enzyme activities of G6PC and PEPCK in the

livers of WT and Nogo$^{-/-}$ mice fed normal chow (**a, b**, n = 6), HGD-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**c, d**, n = 6), and STZ-induced diabetic Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**e, f**, n = 6), were determined. Data are expressed as the mean ± SEM. The p values were calculated by two-tailed Student's t test.

***Comment 5*: Another point is that the authors made many graphs with a starting point that is not 0 (such as Figure 2c, 2d, etc.), which is misleading. We would suggest having levels from 0-x amount and not augmenting the difference by visualization.**

Response: Thank you for bringing this to our attention. We fully agree with the issue you raise and have subsequently readjusted and reviewed all statistical graphs to ensure that all data starts from 0 on the x-axis.

***Comment 6*: In terms of insulin secretion conclusions, authors mostly used systemic insulin level measurements and concluded that Nogo-B deficiency increased insulin secretion, which is also a weak conclusion, and it is generally affected by insulin resistance levels as one can appreciate that insulin levels drop in Figure 1n with Nogo-B knockdown but elevated in Figure 2d Nogo-B KO mice. They should perform glucose-stimulated insulin secretion (GSIS) experiments to focus on insulin secretion from the pancreas.**

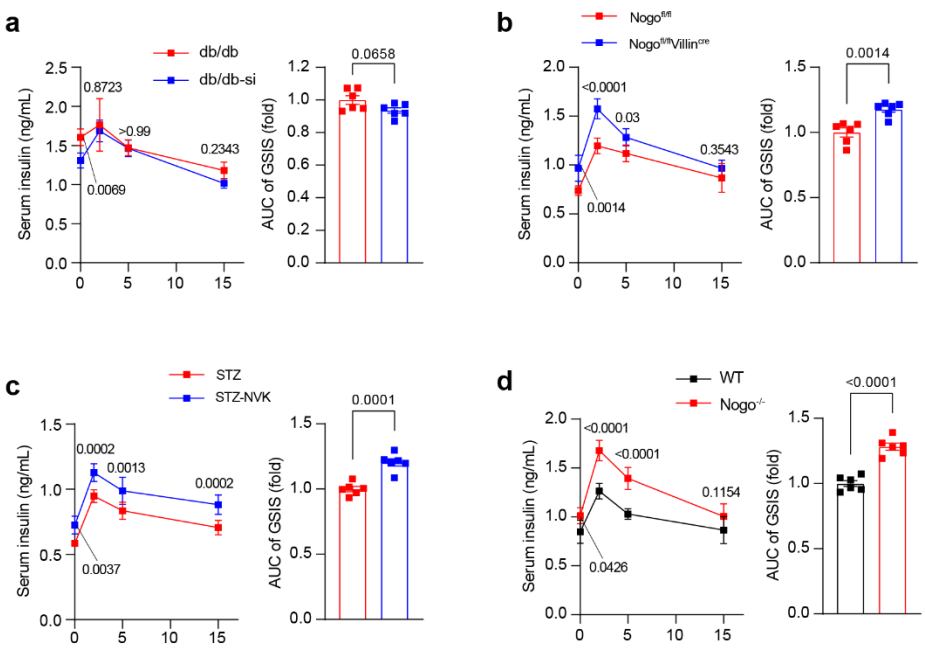
Response: Thank you for your valuable suggestions. The GSIS experiment can indeed help us to clarify the effect of Nogo-B expression on insulin secretion. Since our study focuses on the indirect regulation of insulin secretion by GLP1, we decided to perform the GSIS experiment *in vivo* rather than using isolated pancreases for in vitro experiments.

We repeated each animal experiment and performed the GSIS experiment at the end as described in the literature[3,4]. The results showed that the db/db group had higher initial insulin levels than the db/db-KD group due to insulin resistance. However, Nogo-B knockdown increased insulin secretion two minutes after glucose injection, but did not affect total secretion (**Response Figure 10a**). This shows that the mice in the db/db-KD group could secrete more insulin.

In addition, basal insulin levels were significantly higher in Nogo-B global knockout mice fed normal chow, and HGD or STZ-induced diabetic intestinal Nogo-B deficiency mice, and these mice exhibited increased GSIS (**Response Figure 10b-d**). Based on these experimental results, we can confidently conclude that global or intestinal knockout of Nogo-B stimulates insulin secretion. In the revised manuscript, we have added the GSIS results for each batch of mice in the corresponding Results section **(lines 136-138, 177-179, 347-348, 377-378 on pages 6, 8, 14, 15).**

The new figures are displayed in **new Figure 1, 2, 5, 6** and **Response Figure 10** as follows:

Response Figure 10



Response Figure 10. Nogo-B inhibition, Nogo-B global deficiency, and intestinal Nogo-B deficiency promote insulin secretion. Glucose stimulated insulin secretion (GSIS) test and AUC analysis of db/db and db/db-KD mice (**a**, n = 6), WT and Nogo$^{-/-}$ mice (**b**, n = 6), HGD-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**c**, n = 6), and STZ-induced diabetic Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**d**, n = 6). Data are expressed as the mean ± SEM. The p values were calculated by two-tailed Student's t test.

<u>Comment 7</u>: The most novel part of the study is about the binding dynamic and cleavage of proglucagon and the synthesis of GLP-1. Their findings of binding patterns between Nogo-B, PCSK1, proGCG, and GLP1 are quite confusing. They need to use stronger and multiple binding assays such as Biacore Octet binding, radioactive binding experiments, or NMR spectroscopy for a stronger conclusion of these binding dynamics to make sure their proposed biology is accurate and not an artifact.

<u>Response</u>: Thank you for recognizing the innovative nature of our research and for your valuable suggestions. Based on your suggestion, we analyzed the co-IP-MS results with the GFP antibody and identified that the Nogo-B fragment binding to proGCG may be located at positions 25-58 (**Response Figure 11a**), within the N-terminal structural domain (amino acids 1-185).

To further validate the interaction between Nogo-B and proGCG, we purified the N-terminal structural domain of human Nogo-B protein (hNogo-B-N) and proGCG protein (hproGCG), and assessed the kinetics of their binging

using enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) experiments.

In the ELISA experiments, a significant increase in absorbance was observed with increasing concentration of hNogo-B-N (**Response Figure 11b**). Non-linear regression analysis was used to characterize the affinity between hNogo-B-N and hproGCG, yielding a dissociation constant (Kd) of 21.17 μg/mL with a high goodness of fit (R^2=0.928) (**Response Figure 11c**).

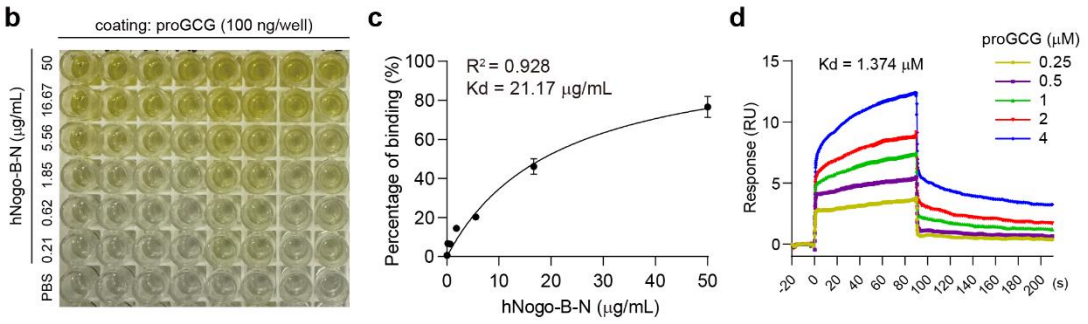
In addition, SPR analysis confirmed the specificity of the protein-protein interaction with a measured affinity constant of 1.374 μM, consistent with the expected affinity range of protein-protein interactions under physiological conditions (**Response Figure 11d**). These results further support the interaction between Nogo-B and proGCG and further strengthen this conclusion. In the revised manuscript, we have added this part of the results to the appropriate Results section **(lines 252-284 on pages 10-12).**

The new figures are displayed in **new Figure 3, new Extended Figure 7**, and **Response Figure 11** as follows:

Response Figure 11

a co-IP-MS using anti-GFP antibody

The detected peptides of Nogo-B	position	score
K.GVIQAIQK.S	234-241	39.11
K.SDEGHPFR.A	241-248	39.18
K.SDEGHPFR.A	241-248	30.12
K.YQFVR.E	22-26	23.28
K.YSNSALGHVNCTIK.E	258-272	73.84
R.AYLESEVAISEELVQK.Y	241-256	88.15
R.GPLPAAPPVAPER.Q	80-92	57.47
R.GPLPAAPPVAPER.Q	80-92	53.49
R.HQAQIDHYLGLANK.N	326-339	35.39
R.HQAQIDHYLGLANK.N	326-339	75.98
R.KPAAGLSAAPVPTAPAAGAPLM&DFGNDFVPPAPR.G	25-58	90.81
R.KPAAGLSAAPVPTAPAAGAPLM&DFGNDFVPPAPR.G	25-58	31.26
R.KPAAGLSAAPVPTAPAAGAPLMDFGNDFVPPAPR.G	25-58	100.74
R.KPAAGLSAAPVPTAPAAGAPLMDFGNDFVPPAPR.G	25-58	74.21
R.LFLVDDLVDSLK.F ! R.LFLVDDLVDSLK.A	181-206	67.44
R.QPSWDPSPVSSTVPAPSPLSAAAVSPSK.L	88-105	85.84



Response Figure 11. Nogo-B binds with proGCG. Nogo-B peptide conjugated to proGCG obtained using GFP antibody (**a**). ELISA for the binding of Nogo-B to proGCG (**b**). Non-linear fit analysis of ELISA results (**c**, n = 6). Affinity constants of Nogo-B and proGCG detected by SPR (**d**). Data are expressed as the mean ± SEM.

<u>Comment 8</u>: Another question is about the weight dependent vs independent effects in their phenotypes. They do not demonstrate the weight in Figures 1 and 2, which is problematic. We should see the weight pattern, and weekly GLP1 measurements to understand the source of the metabolic alterations whether it is fully weight dependent, GLP1-weight effect, or more acute signaling effect independent from weight phenotype. For instance, there is an interesting weight shift in Figure 1g with the Knockdown group gaining weight for 3 weeks, then losing. What is the signal for that weight shift there?

***<u>Response</u>**:* Thank you for your valuable suggestions. We fully agree with your idea. Indeed, it is crucial for us to monitor weekly body weight and serum GLP1 levels of the mice, to investigate whether the changes in body weight correspond to alterations in GLP1 levels, which is essential to determine whether Nogo-B regulation of weight changes depends on GLP1. **We kindly note that the data presented in Figure 1g pertains to blood glucose levels, not body weight.** After the third week, inhibiting Nogo-B expression leads to a decrease in blood glucose levels.

We repeated the *in vivo* experiments, recording the body weight of the mice weekly and collecting facial blood samples to measure serum GLP1 every week.

In db/db mice, **as shown in Response Figure 12a, b**, inhibition of Nogo-B expression using Nogo-B siRNA resulted in a sustained reduction in mouse body weight compared to the control group, accompanied by an increase in serum GLP1 levels.

As shown in Response Figure 12c, d, 5-week-old WT and $Nogo^{-/-}$ mice were fed normal chow for 3 weeks. The body weight of $Nogo^{-/-}$ mice remained lower than WT mice throughout the feeding period. Accordingly, the serum GLP1 levels in $Nogo^{-/-}$ mice were consistently higher than that of WT mice.

In addition, in $Nogo^{f/f}$ and $Nogo^{f/f}Villin^{Cre}$ mice fed a high-glucose diet, intestinal Nogo-B deficiency were associated with lower body weights compared to the control group, and serum GLP1 levels remained consistently higher than those in the control group **(Response Figure 12e, f)**.

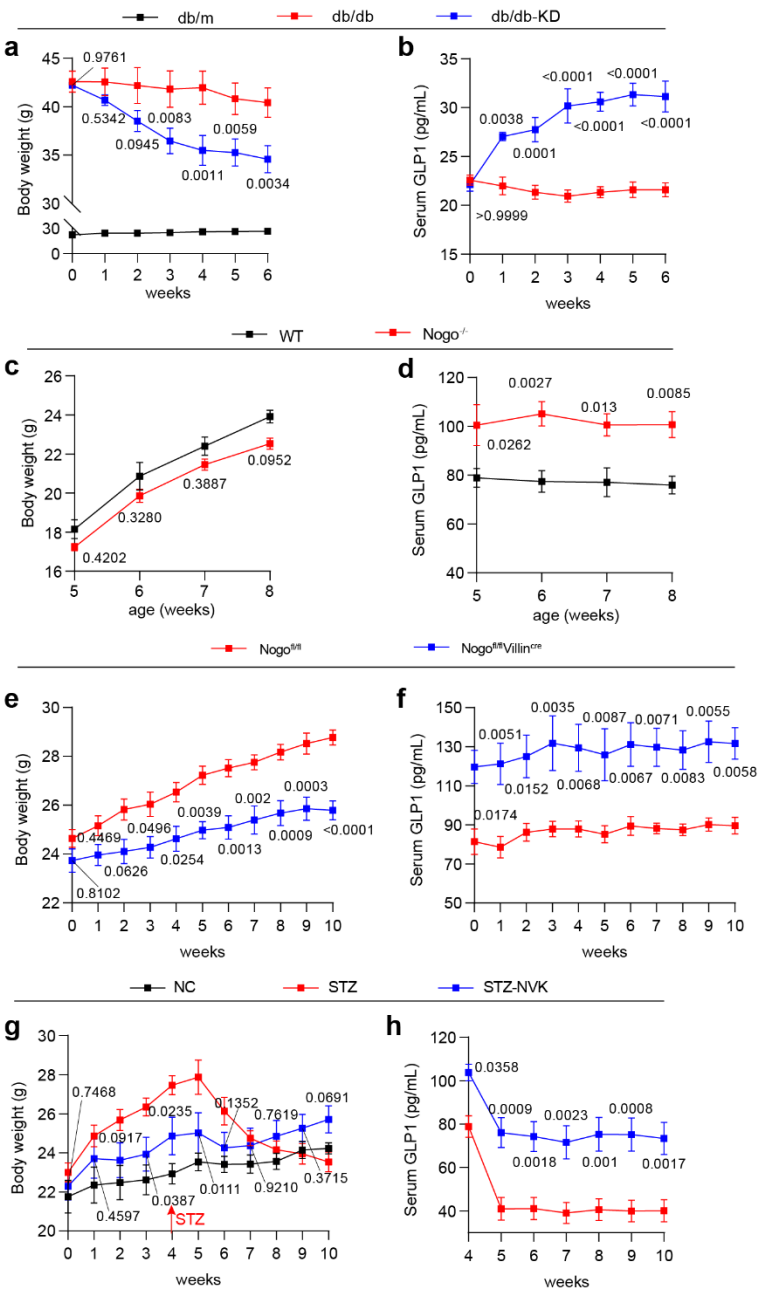
In STZ-treated $Nogo^{f/f}$ and $Nogo^{f/f}Villin^{Cre}$ mice, although serum GLP1 levels remained consistently higher in $Nogo^{f/f}Villin^{Cre}$ mice than in $Nogo^{f/f}$ mice, the body weight of $Nogo^{f/f}$ mice started to decrease from the 5th week, and the body weight of $Nogo^{f/f}$ mice was lower than that of $Nogo^{f/f}Villin^{Cre}$ mice by the 7th week (**Response Figure 12g, h)**. We speculated that this phenomenon might be due to the toxic effects of STZ. STZ can damage pancreatic beta cells and cause severe pancreatic injury. We observed symptoms of weakness and lethargy in $Nogo^{f/f}$ mice after the 5th week, but $Nogo^{f/f}Villin^{Cre}$ mice were in better condition, indicating that intestinal Nogo-B deficiency can counteract the toxic effects of STZ.

In summary, by monitoring the body weight and serum GLP1 levels in mice weekly, systemic or intestinal-specific deficiency of Nogo-B, as well as the

inhibition of Nogo-B expression, were associated with decreased body weight, corresponding to increased GLP1 levels. This suggests that the regulation of body weight by Nogo-B is dependent on GLP1. We have incorporated these findings into the revised manuscript and provided explanations and discussions on the relevant results in the Discussion section **(lines 476-482 on page 18).**

The new figures are displayed in **new Extended Data Figure 16** and **Response Figure 12** as follows:

Response Figure 12



Response Figure 12. Nogo-B deficiency/inhibition decreases body weight and GLP1 levels. Body weight of db/m, db/db and db/db-KD mice (**a**, n = 6) and measurement of serum GLP1 levels in mice weekly (**b**, n = 6). Body weight of normal chow-fed WT and

Nogo$^{-/-}$ mice (**c**, n = 6) and measurement of serum GLP1 levels in mice weekly (**d**, n = 6). Body weight of HGD-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**e**, n = 6) and measurement of serum GLP1 levels in mice weekly (**f**, n = 6). Body weight of WT, STZ-induced diabetic Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (**g**, n = 6) and measurement of STZ-treated Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice serum GLP1 levels weekly (**h**, n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test or one-way ANOVAs.

***Comment 9*: When we see lower food consumption in mice as a phenotype in metabolic cage experiments, we would prefer to see heat data and possibly make sure that mice are not simply sick and not eating, losing weight, and lowering glycemia. Systemic inflammatory markers would also be helpful to reassure the audience.**

Response: We greatly appreciate your valuable suggestion. We utilized the Brouwer equation to calculate heat production, the equation outlined is as follows[5]:

$$\text{heat production (kJ/kg}^{0.75}\text{/d)} = 16.18 \times VO_2 + 5.02 \times VCO_2$$

As shown in Response Figure 13a-c, Nogo-B knockdown had no effect on heat production in db/db mice. Serum levels of TNF-α and IL-1β were determined. Nogo-B knockdown suppressed the levels of serum inflammatory factors in db/db mice. Similarly, increased thermogenesis and reduced inflammation were observed in intestinal Nogo-B-deficient mice following STZ and high-fat diet treatment **(Response Figure 13d-f)**. Meanwhile, Nogo$^{f/f}$Villin^{Cre} mice fed a high-glucose diet showed no impact on heat production or serum inflammatory factor levels **(Response Figure 13g-i)**. We also measured serum inflammatory factor levels in WT and Nogo$^{-/-}$ mice fed a normal chow diet and found that the systemic deletion of Nogo-B does not lead to an increase in inflammatory factor levels **(Response Figure 13j, k).**

GLP1 is known to act on the nervous system to suppress appetite and consequently reduce food intake and body weight[6]. Our findings indicate that under normal physiological conditions, the weight loss observed in mice with systemic or intestine-specific knockout of Nogo-B is attributed to elevated GLP1 levels that suppress appetite and decrease food intake. The unchanged thermogenic values and inflammatory factor levels imply that the reduced food consumption is not a consequence of illness-induced meal skipping **(Response Figure 13g-k).**

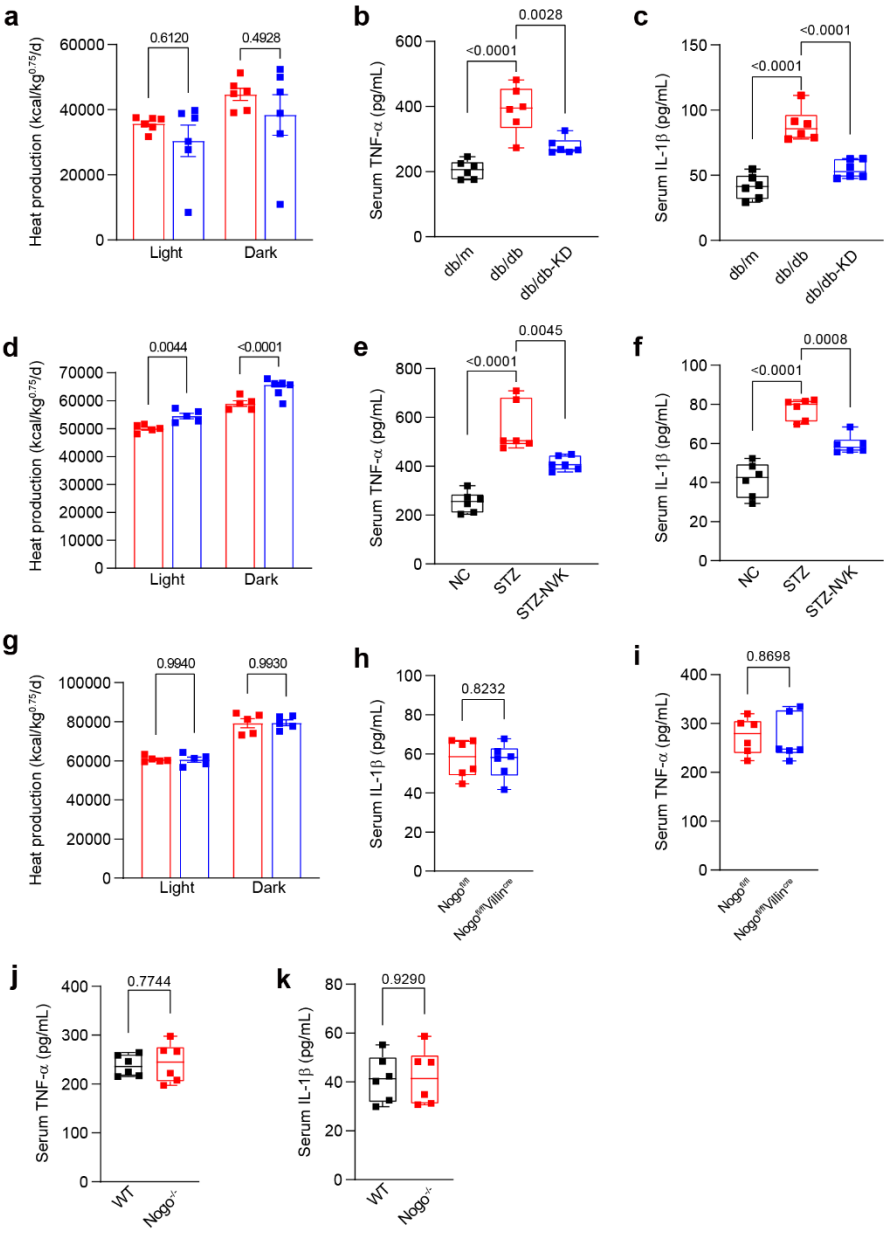
In pathological conditions, inhibition or deletion of Nogo-B ameliorates the inflammatory response in mice. However, the results regarding heat production indicate that alterations in food intake and body weight are primarily driven by changes in GLP1 levels, rather than direct effects on heat production or inflammation **(Response Figure 13a-f).**

In summary, Nogo-B inhibition/knockout does not reduce heat production or increase serum inflammatory factor levels in mice, suggesting that the reduction in food intake in Nogo-B inhibition/knockout mice is most likely related to decreased appetite, rather than inflammation, frailty, or disease. The above

data have been incorporated into the revised manuscript and discussed in the Discussion section **(lines 481-489 on pages 18-19).**

The new figures are displayed in **new Extended Data Figure 3, 4, 12, 15** and **Response Figure 13** as follows:

Response Figure 13



Response Figure 13. The effects of Nogo-B on heat production and inflammation. The heat production was calculated (**a**) and serum TNF-α (**b**) and IL-1β (**c**) levels were determined in db/db and db/db-KD mice (n = 6). The heat production was calculated (**d**) and serum TNF-α (**e**) and IL-1β (**f**) levels were determined (**d**) in HGD-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice (n = 6). The heat production was calculated (**g**) and serum TNF-α (**h**) and IL-1β (**i**) levels were determined (**f**) in STZ and STZ-NVK mice (n = 6). Serum TNF-α (**j**) and IL-1β (**k**) levels were determined in normal chow-fed WT and Nogo$^{-/-}$ mice (n = 6).

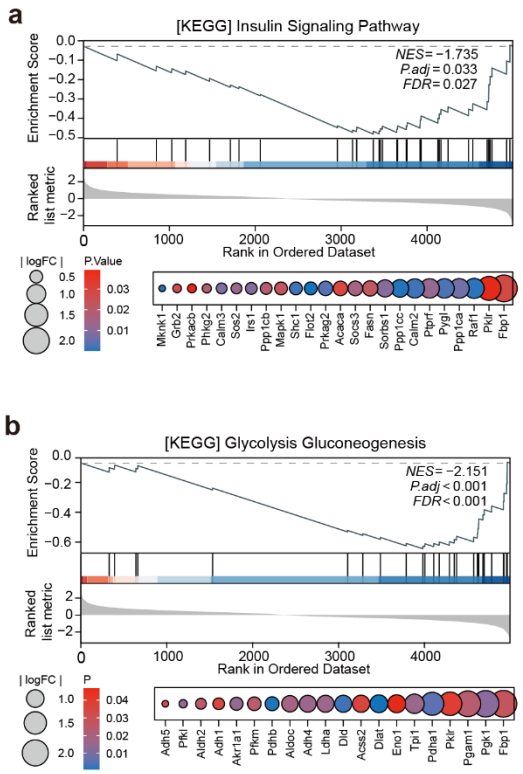
Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test or one-way ANOVAs.

<u>Comment 10</u>: While showing the GSEA pathways for insulin signaling pathway in Figure 2 g, would be helpful to list core enriched genes and p values for 2f Enricher data.

***<u>Response</u>**:* Thank you for your feedback. **As shown in Response Figure 14,** we have included the names of enriched genes and their *p* values from the GSEA pathways for insulin signaling pathway and glycolysis/gluconeogenesis **in Figure 2f, g of the revised manuscript.**

The new figures are displayed in **new Figure 2** and **Response Figure 14** as follows:

Response Figure 14



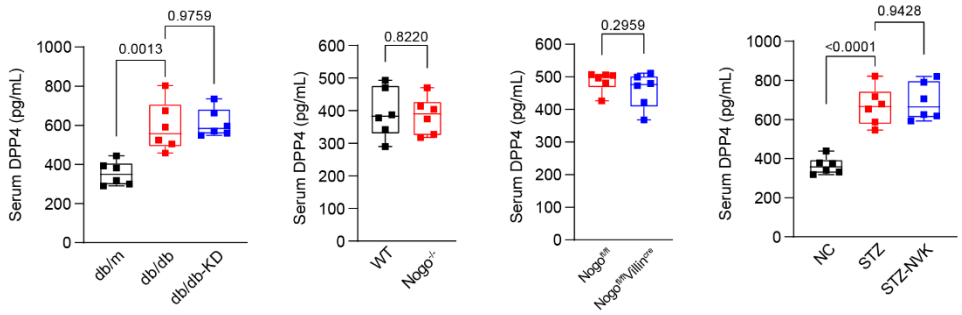
Response Figure 14. Nogo-B deficiency is involved in insulin signaling and glycolysis/gluconeogenesis. GSEA in insulin signaling pathway (**a**) and the glycolysis-gluconeogenesis pathway (**b**).

Comment 11: Again, to fully identify the systemic levels of GLP1 alterations in vivo mice models, would be helpful to measure DPP4 activity and systemic levels to associate/establish synthesis vs clearance paradigm.

Response: Thank you for your valuable suggestion. We collected serum from mice and measured serum DPP4 levels. **As shown in Response Figure 15,** serum DPP4 levels were elevated in db/db mice compared with db/m mice, which promoted GLP1 clearance and led to a decrease in GLP1 levels. Similarly, STZ induced an increase in serum DPP4 levels. However, Nogo-B global or intestine-specific knockout, as well as Nogo-B knockdown, did not affect serum DPP4 levels. This suggests that Nogo-B regulates GLP1 levels by promoting its cleavage and maturation, independent of the DPP4-mediated degradation process. We have incorporated this part in the revised manuscript **(lines 416-419 on page 16).**

The new figures are displayed in **new Extended Data Figure 16** and **Response Figure 15** as follows:

Response Figure 15



Response Figure 15. Nogo-B inhibition/deficiency has no effect on serum DPP4 levels. a-d, Effect of Nogo-B expression on DPP4 levels (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test or one-way ANOVAs.

***Comment 12*: Additionally, since this protein resides on ER membrane, does a deficiency of Nogo-B cause any ER dysfunction? Activation of ER stress? Authors can simply examine this with Unfolded Protein Response UPR. sXbp1, pIRE, etc.**

Response: We appreciate your valuable suggestions. Your suggestion inspired us to investigate whether Nogo-B affects ER stress in intestinal cells. Overexpression of Nogo-B in HEK293t cells leads to an increase in proGCG protein levels and a decrease in GLP1 protein levels, suggesting that Nogo-B promotes the accumulation of proGCG in the ER (**New Figure 4b**). The accumulation of excessive proteins in the ER can trigger the unfolded protein response (UPR) and ER stress[7]. As you suggested, it is necessary for us to detect molecules related to UPR and ER stress.

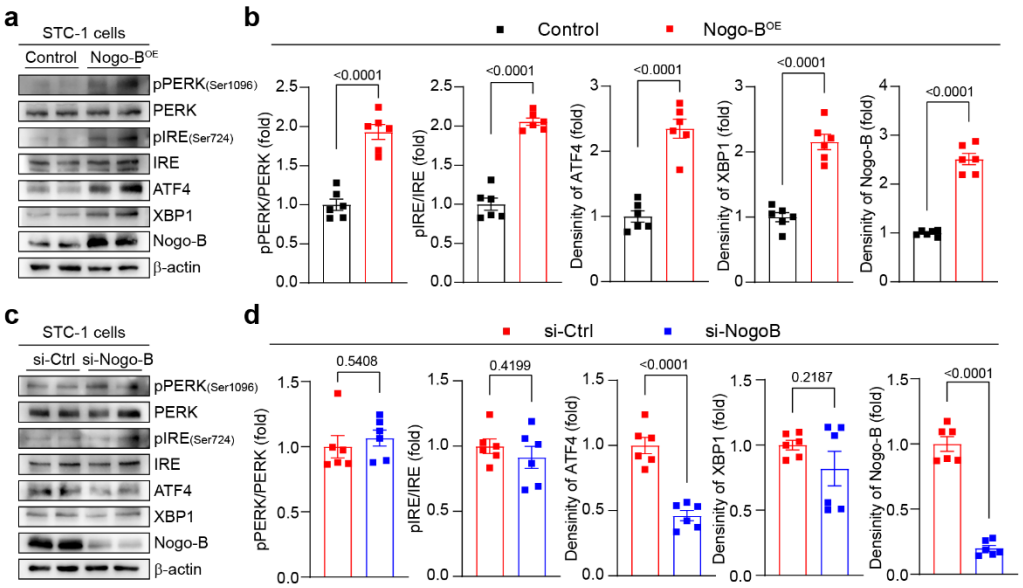
As shown in Response Figure 16, we overexpressed Nogo-B using a Nogo-B expression vector and inhibited Nogo-B expression using Nogo-B siRNA in the STC-1 enteroendocrine cell line. We then assessed ER stress and

UPR relevant molecules including protein kinase R-like endoplasmic reticulum kinase (PERK), p-PERK (Ser1096), inositol-requiring enzyme-1 (IRE), p-IRE (Ser 724), activating transcription factor 4 (ATF4), and X-box binding protein 1 (XBP1) by Western blot. We found that overexpression of Nogo-B activated the UPR and ER stress (**Response Figure 16a, b**), whereas inhibition of Nogo-B had no significant effect (**Response Figure 16c, d**).

Your valuable suggestion led us to discover that inhibiting intestinal Nogo-B expression (or Nogo-B deficiency) does not activate ER stress, which could disrupt ER function. This finding contributes to a better understanding of the role of Nogo-B in type 2 diabetes. The experimental results from the part you suggested have been incorporated into the revised manuscript as **Extended Data Figure 8 b-e (lines 308-313 on page 12).**

The new figures are displayed in **new Extended Data Figure 8** and **Response Figure 16** as follows:

Response Figure 16

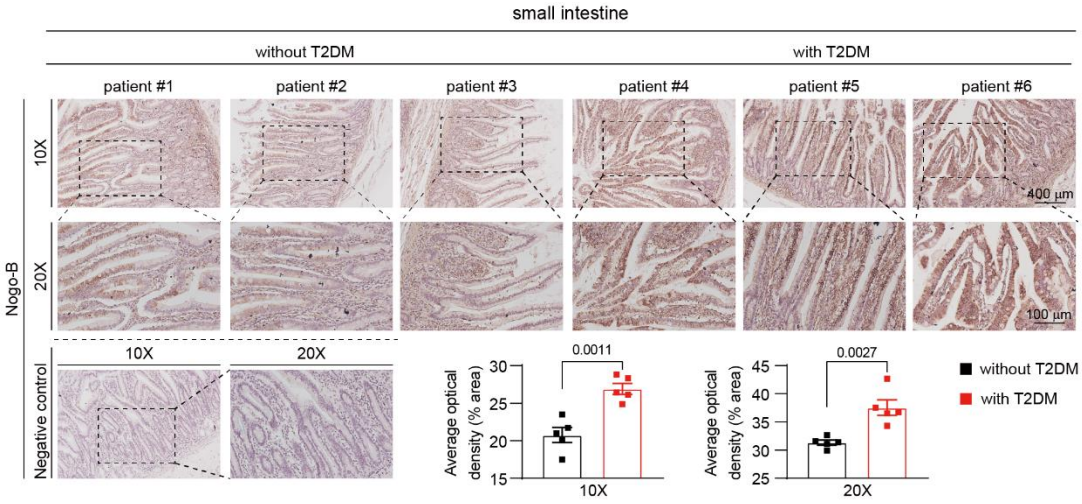


Response Figure 16. Effect of Nogo-B expression on the UPR. a-d, Effect of Nogo-B expression on protein levels of pPERK, PERK, pIRE, IRE, ATF4, and XBP1 and analysis of band density. (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.

<u>Comment 13</u>: In Figure 7, it is hard to evaluate from images. Would be helpful to analyze the image by % + staining among all slides. Using arbitrary values becomes confusing to convince the audience when image quality is poor and small images.

***Response**: Thank you for pointing this out. In our original analysis, we inadvertently omitted the percentage symbol on the vertical axis of the graph. When quantifying the immunohistochemistry results using ImageJ, we selected the positive signal areas and measured their integrated option density (IOD). We then divided the IOD by the area of the target protein distribution to calculate the average optical density (AOD, %Area). In the revised manuscript, we have corrected this oversight and added the percentage symbol to the vertical axis of **Figure 7A**.

Response Figure 17



Response Figure 17. Small intestine Nogo-B expression is increased in T2DM patients. Representative images of immunohistochemical staining of Nogo-B in small intestine from patients with or without T2DM. Quantification of Nogo-B density from 5 individual images (n = 5). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.

Possible writing suggestions.

***Comment**: Line 78; variety of tissues including ….. (Write the relevant organs)*

***Response**: Thank you for your valuable suggestions. We have supplemented the organs involved in Nogo-B expression in the revised text **(lines 85 on page 5)**.

***Comment**: Line 80; switch Nogo-B knockout to Nogo-B whole-body genetic deletion*

***Response**: Thank you for pointing out this issue. We have corrected this description in the revised text **(lines 88 on page 5)**..

***Comment*: Line 82; Nogo-B knockout mice on a normal chow diet show significant activation of the hepatic INSR-IRS-AKT pathway with no clear mechanisms.**

Response: We apologize for the inconvenience caused by our writing, and we have made changes according to your suggestions **(lines 91 on page 5)**.

***Comment*: Line 89; use full words of MPGF and proGCG as you are using these abbreviations for the first time.**

Response: Thank you for pointing out this issue. we have supplemented the full words of MPGF (major proglucagon fragment) in the revised text **(lines 99 on page 5)**.

***Comment*: Line 91; switch Knockout of intestinal Nogo-B to Tissue-specific deletion of Nogo-B in the intestine increased GLP1 and insulin levels.**

Response: Thank you for your valuable suggestions. We have made corrections in the revised text based on your suggestions **(lines 101-102 on page 5)**.

***Comment*: Line 229; Would be helpful to cite a reference paper that confirms that proGCG is going through a classical ER-golgi secretion pathway.**

Response: Thank you for your valuable suggestions. We have supplemented a reference that confirms ER-Golgi secretion pathway is necessary for the mature of proGCG in the revised text **(lines 289-290 on page 12)**.

References

1. DeFronzo RA. Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* **88**, 787-835, ix (2004).
2. Zhang Y*, et al.* GLP-1 Receptor in Pancreatic α-Cells Regulates Glucagon Secretion in a Glucose-Dependent Bidirectional Manner. *Diabetes* **68**, 34-44 (2019).
3. Zhang X*, et al.* IL18 signaling causes islet β cell development and insulin secretion via different receptors on acinar and β cells. *Dev Cell* **57**, 1496-1511.e1496 (2022).
4. Chen H, Liu J, Shi GP, Zhang X. Protocol for in vivo and ex vivo assessment of hyperglycemia and islet function in diabetic mice. *STAR Protoc* **4**, 102133 (2023).
5. Teofilo G*, et al.* Effect of feed restriction on the maintenance energy requirement of broiler breeders. *Anim Biosci* **35**, 690-697 (2022).
6. Kadouh H*, et al.* GLP-1 Analog Modulates Appetite, Taste Preference, Gut Hormones, and Regional Body Fat Stores in Adults with Obesity. *J Clin Endocrinol Metab* **105**, 1552-1563 (2020).

7. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem Sci* **40**, 141-148 (2015).

Reviewer #3 (Remarks to the Author):

The paper investigated the effects of Nogo-B in the context of type-2 diabetes in mice and human. The authors demonstrated with well design experiments that Nogo-B deficiency results in increased GLP-1 secretion and thereby plasma insulin levels in db/db mice and in global and intestine specific knockout mouse models. Moreover, although other groups already described the metabolic effects of Nogo-B knockout, the authors bring novelty by exploring the mechanistic effects associated to this gene in different organs. Thus, the paper brings a good contribution to the field of diabetes and to the possibility to set Nogo-B as a novel therapeutical target. ***However, some issues described below deserve the author's attention before the publication of the manuscript.***

Response*:* We appreciate your insightful and helpful comments. In the response, we have addressed all the concerns with a substantial amount of new data.

Major revision points:

Comment 1: The authors should explain how the hypothesis for Nogo targeting insulin and GLP-1 was generated.

Response*:* Thank you for raising this important question of logic. The following is the background of our study and our research ideas:

(1) In our previous study, we observed that systemic knockdown of Nogo-B promotes serum insulin levels while activating the hepatic insulin signaling pathway[1], which prompted us to further investigate the regulatory role of Nogo-B in relation to insulin and the insulin signaling pathway.

(2) In this study, we found that inhibition of Nogo-B in db/db mice could reduce blood glucose. At the same time, it could also affect the levels of blood glucose-regulating hormones such as insulin, glucagon, and GLP1 **(Figure 1)**. RNA-seq results from the livers of WT and Nogo$^{-/-}$ mice fed a normal diet showed enrichment in the insulin signaling pathway **(Figure 2)**.

(3) **Considering the widespread expression of Nogo-B and its localization in ER membranes and plasma membranes, we speculate whether Nogo-B directly affects the synthesis of these hormones or indirectly affects their levels by influencing the interaction with their receptors.**

(4) The similarity in amino acid sequences of proteins suggested that they may share common interacting proteins[2]. Through alignment of the amino acid sequences of Nogo-B with those of proGCG and insulin protein, we found partial sequence similarity between Nogo-B and both proGCG and insulin. Subsequently, we conducted co-IP-MS to detect proteins interacting with Nogo-B, but did not find the presence of insulin receptor (INSR), glucagon receptor

(GCGR), and GLP1 receptor (GLP1R). Interestingly, we did find proGCG and insulin proteins in the co-IP-MS results (**New Extended Data Figure 6**).

(5) We then confirmed through a series of molecular experiments that Nogo-B is able to interact with the IP2 region of proGCG, which resides proGCG in the ER thereby inhibiting the shearing of GLP1 and reducing GLP1 levels. The increase in GLP1 levels induced by Nogo-B knockdown resulted in the activation of insulin levels and the insulin signaling pathway.

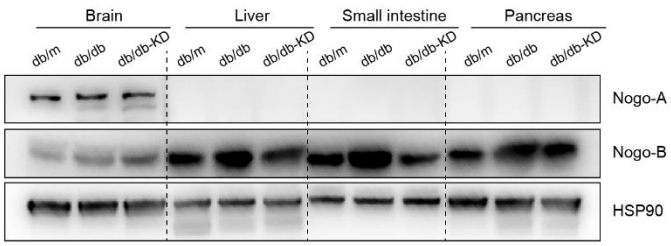
In the revised manuscript, we have reorganized the language and logic of the text to clarify the reasons why we hypothesis that Nogo-B targets these hormones.

<u>Comment 2</u>: The authors present the Knockdown of Nogo-B in db/bd mice in the mRNA level. How was the knockdown efficiency in the protein level?

***<u>Response</u>**:* We greatly appreciate your important suggestion. **As shown in Response Figure 18**, we extracted tissue proteins from db/db mice and assessed siRNA knockdown efficiency by Western blot. The results were consistent with the qPCR data, showing that Nogo-B siRNA significantly reduced Nogo-B expression in liver, small intestine, and pancreas. However, Nogo-B siRNA did not affect Nogo-B expression in the brain. Your suggestion helped us to clarify the knockdown effect of Nogo-B siRNA in db/db mice. These results have been incorporated into the revised manuscript **(lines 120-122 on page 6).**

The new figures are displayed in **new Extended Data 1** and **Response Figure 18** as follows:

Response Figure 18



Response Figure 18. The levels of Nogo-B in different tissues of db/db mice. Nogo-A and Nogo-B protein levels in different tissues of db/db mice.

<u>Comment 3</u>: The authors claim that the effects of Nogo-B were resulted from its action in the liver and intestine mainly. However, in the Nogo-B db/db knockdown mice the results showed a reduced food consumption. The authors should comment on the participation or not of the brain Nogos in this effect.

***<u>Response</u>**:* Thank you for your valuable suggestion. As described below, we discussed the relationship between Nogo-B and food consumption.

(1) As you pointed out, Nogo-B knockdown reduced food consumption in db/db mice. However, siRNA is believed to be unable to cross the blood-brain barrier[3]. **As shown in Response Figure 18**, we also found that Nogo-B siRNA did not affect the expression of Nogos in the brains of db/db mice. This suggests that Nogo-B in other tissues, rather than in the brain, indirectly regulates appetite signals in the brain, thereby affecting the food intake of the mice.

(2) In the central nervous system (CNS), GLP1 is a neurotransmitter in brain stem–hypothalamus pathways signaling satiety[4]. The appetite-suppressing effect of GLP1 relies on GLP1R, which was distributed widely in the CNS. Indeed, clinical trials have shown that intravenous administration of GLP1 peptides can suppress appetite of participants[5].

As shown in Figure 5f and Figure 6d of the revised manuscript, we found that intestinal Nogo-B deficiency can elevate serum GLP1 levels and suppress food intake in mice. This part of the results suggests that intestinal Nogo-B, rather than brain Nogos, directly regulates intestinal GLP1 production, thereby affecting appetite and food intake.

In summary, the influence of Nogo-B expression on appetite in mice is not mediated directly within the CNS. Instead, it operates through modulation of GLP1 levels. Changes in GLP1 concentration subsequently influence appetite by acting within the CNS. We have incorporated your suggestion into the Discussion section **(lines 469-476 on page 18)**.

***Comment 4*: Was the knockdown in db/db mice specific for Nogo-B? How does the expression of Nogo-A and C look like?**

Response: Thank you for your attentive review of this issue. We acknowledge that the siRNA used does not specifically knock down Nogo-B. And due to the limitations of the Nogo antibody (Novus, CAT#NB100-56681), we can only detect Nogo-A and Nogo-B.

We examined the expression of Nogos across various tissues and key cell types (intestinal L-cells, pancreatic islet cells), and found that Nogo-B was the predominantly expressed isoform, whereas Nogo-A was expressed mainly in the brain (**Response Figure 19a**), in keeping with the results of previous studies[6, 7].

Furthermore, in db/db mice, Nogo-B siRNA effectively inhibited the expression of Nogo-B in the liver, small intestine, and pancreas. However, it did not impact the expression levels of Nogo-B or Nogo-A in the brain (**Response Figure 19b**). These results underscore the knockdown efficiency of the Nogo-B siRNA and are consistent with the known expression patterns of the Nogos.

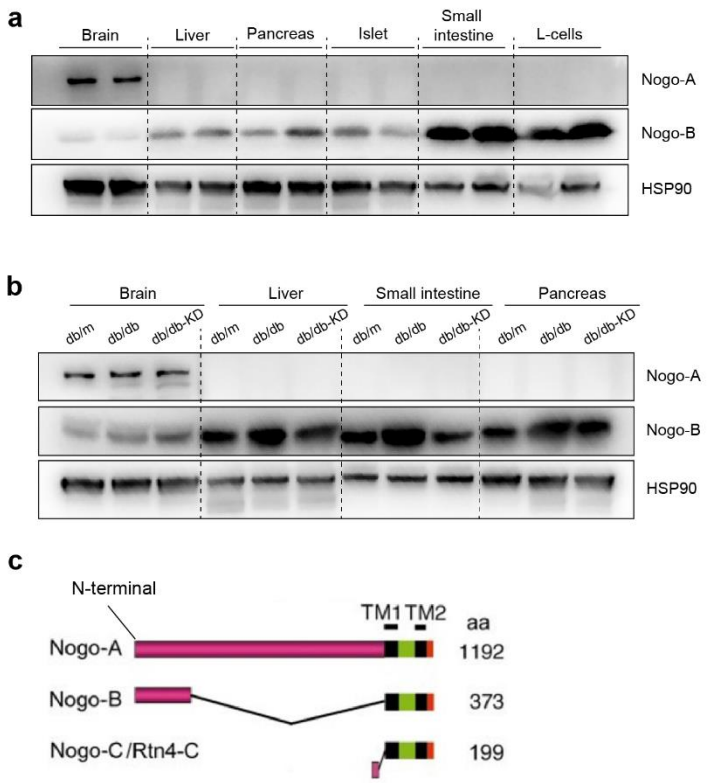
Although we did not detect levels of Nogo-C, in combination with new experimental results (**Figure 3m-s, Extended Data Figure 7g**), the Nogo-B fragment that interacts with proGCG is localized in the N-terminal structural domain (amino acids 1-185), a peptide that not present in Nogo-C (**Response Figure 19c**)[8], thus a role for Nogo-C in GLP1 maturation can be excluded. Of

course, we acknowledge that we cannot definitively rule out any effect of Nogo-C on GLP1 without further investigation, which warrants exploration in future studies.

In the revised manuscript, we have incorporated the new results and also added a discussion of the limitations of Nogo-C-related content in the Discussion section **(lines 510-514 on pages 19-20).**

The new figures are displayed in **new Extended Data 1** and **Response Figure 19** as follows:

Response Figure 19



Response Figure 19. The levels of Nogo-B in different tissues of mice. a, Nogo-A and Nogo-B protein levels in different tissues of C57BL/6J mice. **b,** Nogo-A and Nogo-B protein levels in different tissues of db/db mice. **c**, Schematic diagram of Nogo-A, Nogo-B, and Nogo-C.

<u>Comment 5</u>: Apparently, mass spectrometry was conducted at two different sites/instruments with only some generic information related to one off them (ref. 53) and no information regarding the other. Please provide detailed information regarding instrumentation, sample preparation, experimental details, instrument settings and data processing.

<u>Response</u>*:* Thanks a lot for this valuable suggestion. The detailed procedure of mass spectrometry is as follows:

Gel pieces were cut from SDS PAGE, destained with 30% ACN/100 mM NH_4HCO_3 until the gels were destained. The gels were dried in a vacuum centrifuge. The in-gel proteins were reduced with dithiothreitol (10 mM DTT/100 mM NH_4HCO_3) for 30 min at 56°C, then alkylated with iodoacetamide (200 mM IAA/100 mM NH_4HCO_3) in the dark at room temperature for 30 min. Gel pieces were briefly rinsed with 100 mM NH_4HCO_3 and ACN, respectively. Gel pieces were digested overnight in 12.5 ng/μL trypsin in 25 mM NH_4HCO_3. The peptides were extracted three times with 60% ACN/0.1% TFA. The extracts were pooled and dried completely by a vacuum centrifuge.

Each fraction was subjected to nano LC-MS/MS analysis using a Thermo Scientific Q Exactive mass spectrometer coupled to an Easy nLC system. Peptides were loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap 100, 100 μm×2 cm, nano Viper C18) and separated on a C18 analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin). The separation employed a linear gradient of buffer B (84% acetonitrile, 0.1% formic acid) in buffer A (0.1% formic acid) at a flow rate of 300 nL/min, controlled by IntelliFlow technology.

The mass spectrometer operated in positive ion mode, acquiring MS data with a data-dependent top20 method. The survey scan ranged from 300-1800 m/z, and the most abundant ions were selected for HCD fragmentation. The AGC target was set to 1×10^6, with a maximum inject time of 50 ms and one scan range. Dynamic exclusion was enabled for 30 s. For the HCD spectra, the resolution was set to 17500 at m/z 100, with an AGC target of 1×10^5. The isolation width was 1.5 m/z, micro scans set to 1, and the maximum injection time was 50 ms. The normalized collision energy was 27 eV. The underfill ratio was set to 0.1% to ensure adequate precursor ion selection at maximum fill time. Peptide recognition mode was enabled throughout the analysis.

MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) against a nonredundant International Protein Index Arabidopsis sequence database v3.85 (released at September 2011, 39679 sequences) from the European Bioinformatics Institute (http://www.ebi.ac.uk/). For protein identification, the following options were used. Peptide mass tolerance=20 ppm, MS/MS tolerance=0.1 Da, Enzyme=Trypsin, Missed cleavage=2, Fixed modification: Carbamidomethyl (C), Variable modification: Oxidation (M).

The specific information regarding mass spectrometry detection has been added to the Methods section of the revised manuscript **(lines 743-808 on pages 28-30).**

<u>Comment 6</u>: The Co-IPs data are relevant for the claims provided in the manuscript; however, the experimental conditions are not described in detail as they should allow replication. Please provide more information.

Response*:* Thank you for reviewing the text carefully and providing feedback. We performed the Co-IP experiment using rProtein G MagPoly beads and followed the protocol outlined in the product manual. We have incorporated the detailed protocol for the Co-IP experiment into the revised manuscript, as outlined below:

The rProtein G MagPoly Beads were inverted several times to ensure thorough mixing. An appropriate volume of the magnetic bead suspension was pipetted into a centrifuge tube, placed on a magnetic separator, and left to stand for about 1 min until the solution clarified, after which the supernatant was aspirated. This washing step was repeated twice. Rabbit normal IgG (3 μg) or the specific antibodies of interest (3 μg) were added to the pre-treated magnetic beads, followed by vertexing to mix and incubating on a rotator at room temperature for approximately 30 min to ensure full contact and adsorption. After incubation, the tubes were placed back on the magnetic separator until the solution clarified, and the supernatant was aspirated.

The remaining sample solution was added to the magnetic beads and gently inverted to evenly disperse the antigen and bead-antibody complexes. The tube was then placed on a rotating mixer and incubated overnight at 4°C. Once incubation was complete, the tubes were placed back on the magnetic separator and the supernatant was aspirated. Five volumes of wash buffer were added to the centrifuge tube containing the magnetic beads, vortexed to resuspend, and placed on the magnetic separator for about 1 min before the supernatant was discarded. This washing procedure was repeated five times. After washing, an equal volume of 1x SDS-PAGE Sampling Buffer was added to the magnetic beads, and the mixture was thoroughly combined. The mixture was heated at 95°C for 5 min, followed by magnetic separation to collect the supernatant for Western blot analysis.

We have also included these in the Methods section in the revised manuscript **(lines 813-833 on pages 30-31).**

Comment 7*: Overall, the authors show the increased GLP-1 levels upon Nogo-B knockdown or Knockout. The effects should be resulted from reduced cleavage by PCSK1. Do the authors observe similar effects on the level of GLP-2? Were the metabolic effects associated to changes only in GLP-1? How does the GLP-2 serum levels look like in the presented animal models?*

Response*:* Thank you for your important suggestions. **As shown in Figure 3 of revised manuscript,** Nogo-B binds to Leu142 of proGCG, which is located in the IP2 region of the MPGF fragment and also serves as a cleavage site for PCSK1. The cleavage and maturation of PCSK2 also involve the IP2 region[9]. Therefore, theoretically, Nogo-B deficiency/inhibition promotes the cleavage of PCSK1 at IP2, which would also facilitate the maturation process of GLP2.

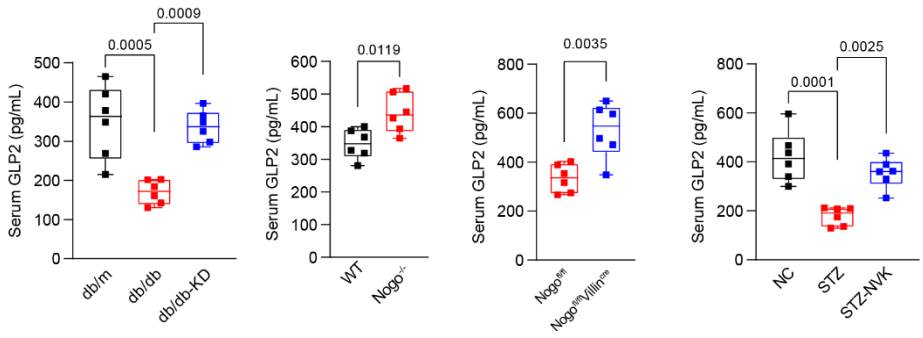
We collected serum samples from WT and Nogo$^{-/-}$ mice fed a normal chow diet, si-Ctrl and si-Nogo-B db/db mice, as well as Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice fed a high glucose diet or treated with STZ. Using a GLP2 Elisa kit, we measured serum GLP2 levels. **As shown in Response Figure 20,** systemic or intestine-specific Nogo-B deficiency, as well as Nogo-B knockdown, increased serum GLP2 levels, indicating that Nogo-B also promotes the maturation process of GLP2.

The primary function of GLP2 is to stimulate rapid growth of the small intestine and promote proliferation of crypt cells[10]. As an intestinal growth factor, GLP-2 administration was generally associated with preservation of gut mucosal structure and function in the setting of chemical, radiation, or surgically induced intestinal injury in preclinical studies[11-13]. Among GLPs, GLP1 rather than GLP-2 is primarily involved in hormone (insulin and glucagon) related metabolic processes and insulin sensitivity. Based on existing research and references, the metabolic benefits of Nogo-B deficiency or inhibition in this study are likely mediated primarily through GLP1.

However, your valuable suggestion has inspired us to shift our research focus to Nogo-B and the splicing and maturation processes GLP2, along with the corresponding intestinal growth and repair processes. We will also investigate whether Nogo-B regulates intestinal physiology through GLP2 in the future work. We have incorporated your suggestion into the Results section **(lines 415-418 on page 16).**

The new figures are displayed in **new Extended Data 16** and **Response Figure 20** as follows:

Response Figure 20



Response Figure 20. Nogo-B inhibition/deficiency decreases serum GLP2 levels. Effect of Nogo-B expression on GLP2 levels (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by one-way ANOVAs or two-tailed Student's t-test.

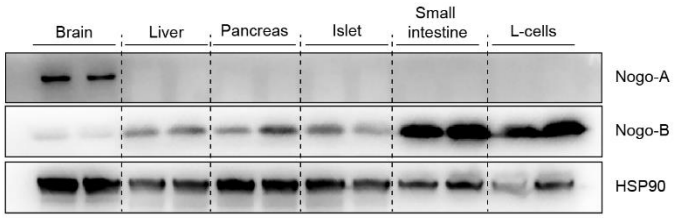
<u>Comment 8</u>: Line 149-150 - The authors claim that the expression of Nogo-A is lower in pancreas and liver cells. However, the western blots were done with the whole tissue. In the liver the Kupfer cells are not the predominant cell type, and in the pancreas the exocrine cells are the major cell type. Thus, the expression of Nogo-A could be of relevance as

the Kupfer cells and pancreatic islet cells compose another metabolic unit in the liver and pancreas respectively. Similarly, the expression of Nogo-A in the whole fraction of intestine could be low. However, the expression in L-cells could be totally different, what may not exclude other Nogos action in GLP-1 and 2 processing. This information should be adjusted in the paper. Moreover, the authors should provide information on the expression of Nogo-A and Nogo-B in the pancreatic islets only.

***Response**:* Thank you for pointing out this issue. We fully agree with your point. Our description in this section was indeed inappropriate. We have revised the manuscript accordingly to rectify this issue **(lines 120-122 on page 6)**.

As shown in **Response Figure 21**, we did not detect Nogo-A expression in mouse pancreas and islets. Nogo-B is abundantly expressed in pancreas and islets, suggesting that Nogo-B is likely to play a primary role in these tissues. Similarly, Nogo-A was undetectable in mouse intestinal tissue and intestinal L-cells, suggesting that Nogo-B is also likely to play a major role in the small intestine. In the revised manuscript, we have included the above data into **Extended Data Figure 1**. We appreciate your valuable suggestion, and will pay attention to the expression of protein isoforms in different cell types in future research, as this is crucial for the accuracy of our study.

Response Figure 21



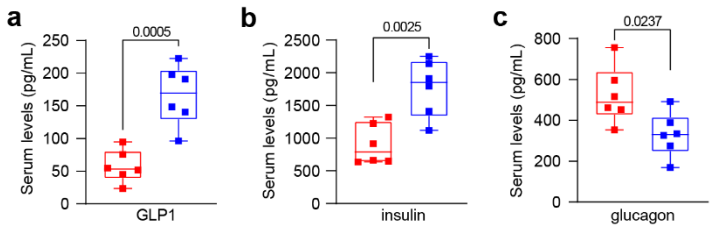
Response Figure 21. The levels of Nogo-B in different tissues of mice. Nogo-A and Nogo-B protein levels in different tissues of C57BL/6J mice.

***Comment 9**: Line 284-285 - Sentence in the results does not correspond to what the Figure 5f shows for GLP-1 serum levels. The text describes higher levels of GLP-1 in Nogo$^{f/f}$Villin^{Cre} but the Figure shows lower levels compared to Nogof/f mice. What is the hypothesis for lower levels of GLP-1 in these mice?*

***Response**:* Thank you for pointing out this issue. Due to an oversight that should have been avoided, we apologize for erroneously presenting the results of serum glucagon levels from Figure 5h in Figure 5f, which was intended to show serum GLP1 levels. In fact, **as shown in Response Figure 22**, the detection of serum GLP1 in Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice indicated that the intestinal Nogo-B absence elevated the serum GLP1 levels. In the revised

manuscript, we have corrected this section of Figure 5f and carefully reviewed all results and figures in the article to avoid such unintended problems.

Response Figure 22



Response Figure 22. Intestinal Nogo-B deficiency increases serum GLP1 levels. Levels of GLP1 (**a**), insulin (**b**) and glucagon (**c**) in $Nogo^{f/f}Villin^{Cre}$ and $Nogo^{f/f}$ mice after 10 weeks on HGD (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t-test.

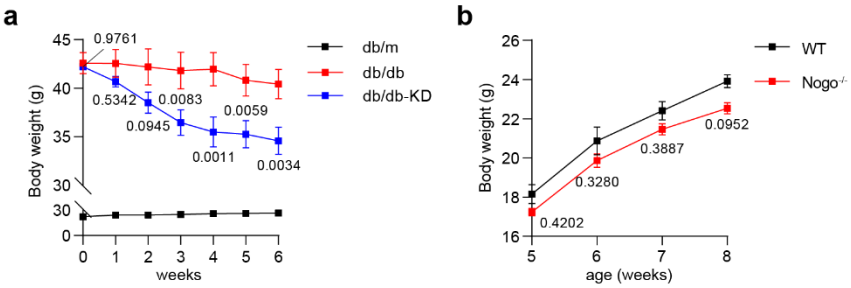
Comment 10. Line 324 - What the authors mean by pancreatic dedifferentiation? Dedifferentiation of beta cells cannot be inferred only by staining. The expression of beta and alpha cell markers in pancreas/islets of these mice must be provided to prove a dedifferentiation hypothesis.

Response**: Thank you for pointing out this issue. We fully agree with your suggestion. The use of "pancreatic dedifferentiation" here is inappropriate and lacks sufficient evidence. Therefore, we have revised this sentence to "…**increased fraction of islet β-cells.**" in the revised manuscript to avoid ambiguity in the description **(lines 385-388 on page 15).

Comment 11: Information on the body weight of db/db mice subjected to Nogo-B knockdown experiments and in the global knockouts should be provided as this constitute an important metabolic feature associated with the described phenotypes.

***Response**: Thank you for your valuable comments. We fully agree with your comments and indeed body weight is an important metabolic feature in our study. In the repeated in vivo experiments, we monitored the body weight of the mice weekly (**Response figure 23a, b**) and presented the results in **Figure 1f** and **Extended Data Figure 4b** of the revised manuscript.

Response Figure 23



Response figure 23. Nogo-B inhibition/deficiency decreases body weight. Body weight of db/m, db/db, and db/db-KD mice (**a**, n = 6), and WT and Nogo$^{-/-}$ mice (**b**, n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t-test.

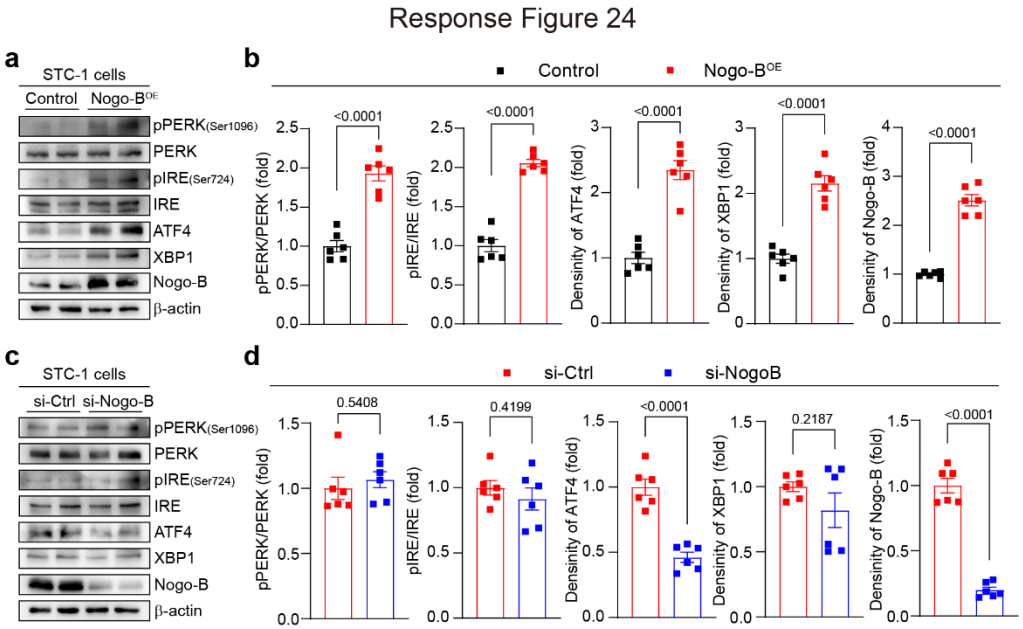
<u>Comment 12</u>: Regarding the overexpression of Nogo-B in HEK cells, do the authors have available cDNA/protein to verify if the retention of proglucagon in the ER activates pathways of cell recycling and markers for celular stress like activation of the UPR response?

<u>Response</u>: Thank you for your valuable suggestions. HEK293T cells are commonly used for transfection of eukaryotic expression vectors to express exogenous genes. Therefore, we transfected HEK293T cells with expression vectors to detect the binding of Nogo-B with proteins such as proGCG.

To simulate the effects of modulating Nogo-B expression on ER stress and UPR in intestinal cells, we treated the enteroendocrine cell line (STC-1 cells) with Nogo-B expression vectors and Nogo-B siRNA. We then detect ER stress and UPR-related molecules by Western blot, including PERK, p-PERK (Ser1096), IRE, p-IRE (Ser 724), ATF4, XBP1.

As shown in Response Figure 24, overexpression of Nogo-B in STC-1 cells increased the protein levels of p-PERK, p-IRE, ATF4, and XBP1. This increase indicates that overexpression of Nogo-B activated the UPR and ER stress (**Response Figure 24a, b**), whereas inhibition of Nogo-B had no significant effect (**Response Figure 24c, d**). The experimental results from the part you suggested have been incorporated into the revised manuscript as **Extended Data Figure 8 b-e (lines 307-311 on page 12).**

The new figures are displayed in **new Extended Data Figure 8** and **Response Figure 24** as follows:



Response Figure 24. Effect of Nogo-B expression on the UPR. a-d, Effect of Nogo-B expression on protein levels of pPERK, PERK, pIRE, IRE, ATF4, and XBP1 and analysis of band density. (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.

<u>Comment 13</u>: Extended Data Figure 2a and b – The authors claim a significant degree of similarity of Nogo-B and proglucagon and insulin in mice and human. How was this determined?

<u>Response</u>: Thank you for your question. In Extended Data Figure 2, we compared the amino acid sequences of Nogo-B with those of insulin and proGCG and found a certain degree of similarity in the amino acid sequences (identical amino acids are highlighted in red and marked with *). The amino acid sequence comparison was performed using the Multiple Sequence Comparison tool (MUltiple Sequence Comparison by Log-Expectation) to compare protein sequences (https://www.novopro.cn/tools/muscle.html)[15]. In the revised manuscript, we have added a description of the sequence comparison in the Methods section **(lines 693-696 on page 26).**

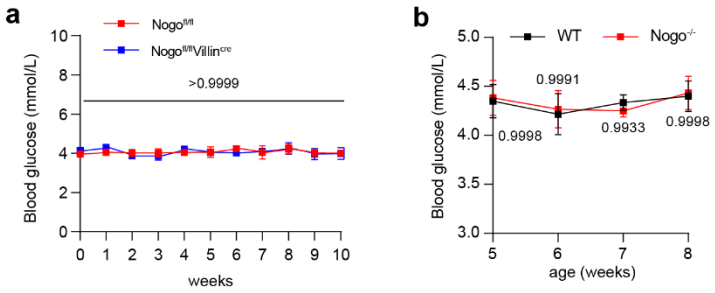
<u>Comment 14</u>: Data of the blood glucose levels of intestine specific knockout mice subjected to HGD should be provided (Figure 5).

<u>Response</u>: Thank you for your valuable suggestion. We re-fed Nogo$^{f/f}$ and Nogo$^{f/f}$Villin^{Cre} mice with HGD for 10 weeks and monitored their blood glucose levels weekly. **As shown in Response Figure 25a**, intestinal Nogo-B deficiency did not affect blood glucose levels under HGD conditions, in contrast to the effects observed in db/db mice and STZ-induced diabetic mice, but it did

result in reduced body weight. Similarly, in WT and Nogo$^{-/-}$ mice fed a normal chow diet, systemic Nogo-B deficiency resulted in decreased body weight but had no effect on blood glucose levels (**Response Figure 25b**). The experiment you suggested has been incorporated into the revised manuscript.

The new figures are displayed in **new Figure 5, new Extended Figure 4**, and **Response Figure 25** as follows:

Response Figure 25



Response figure 25. Nogo-B deficiency has no effect on blood glucose. a-b, Fasted glucose levels of mice (n = 6). Data are expressed as the mean ± SEM. The *p* values were calculated by two-tailed Student's t test.

<u>Comment 15:</u> The determination of Nogo-B expression using stained optical density of patient intestine sections is a weak quantification method. Moreover, no detailed information is provided in the methods. Were the sections stained at the same time? How many sections were analyzed/patient. To strengthen this finding, further information on the blood glucose, insulin, HOMA and GLP-1 levels in the selected patients should be provided.

***<u>Response</u>**: Thank you for your valuable suggestion. Our method of analyzing immunohistochemical staining results is a relatively common and widely used method[14]. The expression of the target protein is determined according to the coloring depth and distribution area of the target protein. Specifically, the integrated option density (IOD) value of each image is measured and divided by the area value to calculate the average optical density (AOD, %Area), which reflects the percentage of target protein per unit area.

Basic patient information is presented in **Response Table 1** below. The samples were obtained from patients with pancreatic space-occupying lesions or pancreatic tumors, and several patients unfortunately died. Therefore, the clinical data retained at the time did not include diabetes-related indicators such as HOMA, insulin, GLP1, etc., and it was also challenging for us to collect blood samples from the patients again. Fortunately, we found the blood glucose values of the patients and have displayed them in **Response Table 1**. We

apologize for not being able to provide information on patient HOMA, insulin, GLP1 levels, etc.

The samples used in this study were collected at different times during clinical procedures and then embedded for long-term storage. Paraffin sections of small bowel samples from each patient were prepared and stained simultaneously before the experiment. As there was only one small bowel sample per patient, only one section per patient was stained and analyzed. There were five patient samples in each of the control and T2DM groups. In the revised manuscript, we have added detailed information in the Methods section and a table with basic patient information **(lines 536-541 on pages 20-21).**

Response Table 1. The characteristic of patients.

		Pathology number	Gender	Age (years)	Blood glucose (mmol/L)	Height (cm)	Body weight (kg)
without T2DM	1	694826	female	68	5.64	160	63
	2	695496	female	63	5.46	160	55
	3	695761	male	48	4.8	/	/
	4	691012	male	48	4.61	/	53
	5	701180	female	44	6.12	/	58
with T2DM	6	708790	female	69	7.12	/	40
	7	718261	female	53	19.46	/	49
	8	693195	female	66	5.59	/	48.5
	9	681388	male	71	8.24	/	56
	10	685445	male	63	9.03	174	91

<u>Comment 16:</u> Supplemental data is provided ("Liver_Pancreas_Nogo_7-10.xlsx") referring to the results of co-immunoprecipitation mass spectrometry conducted on mouse pancreas and liver samples. The table lists iBAQ values (please provide information on how these were calculated), other parametes (Q-value of what?) and an arbitrary "score" that appears to have been used for target selection. However, among other questions related to quality control of protein identification, it remains a mystery how the authors derived the claim that Nogo-B binds insulin and GCG from the >1000 entries of the list, many of those apparently showing the same pattern in terms of row values. This is rather a substandard presentation of mass spec data and needs to be revised (and explained) substantially.

<u>Response</u>: Thank you for raising this question. The supplemental "Liver_Pancreas_Nogo_7-10.xlsx" is a standard protein groups output result from Maxquant software. To allow reader to better understand the results, we have added a new sheet in front of the data sheet to explain the exact meaning of each column. Specifically, iBAQ stands for intensity-based absolute

quantification of proteins, with the following formula: Σ intensity/#theoretical peptides. Q-value is the ratio of reverse to forward protein groups. Score is derived from peptide posterior error probabilities.

We greatly appreciate your inquiry regarding “how the authors concluded the interaction between Nogo-B and insulin and GCG from a list of >1000 entries, which remains a mystery”. At the outset of the experiment, hormones such as insulin, glucagon, and GLP1 had attracted our interest. However, faced with these potential clues, we utilized mass spectrometry to screen for a molecule related to Nogo-B. Insulin and GCG appeared beyond the top 1000 entries in the mass spectrometry results. Firstly, we considered that the binding between Nogo-B and Insulin or GCG might be relatively weak. Secondly, given that hormones are present *in vivo* in trace amounts and are highly efficient, the peptide/protein content of hormones within cells is very low. This possibly could explain why insulin and GCG were found among >1000 entries.

As stated in **Comment 4**, we supplemented the specific procedures and information of mass spectrometry in the revised manuscript's Methods section.

Comment 17: Data availability: It is common practice to deposit all raw data in publicly accessible databases. Unfortunately, the authors have only provided a small subset of the data (GSE236979; liver data of nogo knockouts). Please provide all data shown in the manuscript, including the proteomics data set.

***Response**:* Thank you for your valuable suggestions. We have uploaded all original datasets (including the proteomics data set) to a public database. The database link and ID have been provided in the Data Availability section.

Minor revision points:

Comment 1: Figure 1e – What the Figure describes does not fit with the methods description. In the methods db/m group received scramble siRNA, while in the Figure saline is supposed to be administered to these mice only.

***Response**:* Thank you for pointing out this issue attentively. We apologize for the inconsistency in the description in the methods section due to our oversight. We used 0.9% saline in db/m mice, and we have made the corresponding changes in the revised text **(lines 570 on page 22).**

Comment 2: In the Figure 1h, are the comparisons of fasted blood glucose between db/db and db/db-Nogo KD non-significant or the significance p values were missed for this data point?

***Response**:* Thank you for pointing out this issue. Due to our oversight, we missed the significance p values for fasted blood glucose at minute 0. It is

significant here. We added the significance p value here in the repaired manuscript **(Figure 1k)**.

***Comment 3*: Line 109-111 - The sentence should be a rephrased removing the word "suggestion" as it seems to be associated to a degree of uncertainty.**

Response: Thank you for your valuable suggestions. In the revised text, we changed the sentence to "Nogo-A and Nogo-C are predominantly expressed in the central nervous system and Nogo-B is widely expressed, which indicates that the upregulated protein in the three tissues of T2DM mice is the Nogo-B isoform" **(lines 119-122 on page 6)**.

***Comment 4*. Line 158-159 – Sentence repeated twice.**

Response: Thank you for pointing out this issue. We have removed one sentence in the revised text.

***Comment 5*. Line 250 - Do the authors mean GLP-1 in this sentence?**

Response: Thank you for pointing out this issue. We have corrected it to "GLP1" in the revised text.

***Comment 6*. Line 252 - Do the authors mean PCSK1?**

Response: Thank you for pointing out this issue. We have supplemented it with "PCSK1" in the revised text.

***Comment 7*: Line 253-254 - A conclusion sentence is followed by the repetition of a result sentence.**

Response: Thank you for this suggestion. We have made corrections in the revised text.

***Comment 8*: Line 392-393 - Where do the authors show serum GLP-1 levels in diabetic patients? It is mentioned in the text but no Figure with this data is presented.**

Response: We apologize for the inconvenience caused by our description. In the revised text, we provided separate descriptions of the results for diabetic mice and humans, which changed to "the serum GLP1 levels, intestinal proGCG and intestinal PCSK1 expression were decreased in diabetic mice (Figure 6). Similarly, intestinal proGCG and PCSK1 expression was also decreased in T2DM patients (Figure 7) **(lines 458-460 on page 18)**.

***Comment 9*: Line 395 - Word homeostasis doubled.**
Response: Thank you for pointing out this issue. We have made revisions in the revised text.

***Comment 10*: The authors should discuss the mechanism of body weight changes in the intestine specific Nogo-B Knockout.**

Response: Thank you for this suggestion. Thanks to your careful review, the logic of our text has been further improved. GLP1 can inhibit appetite and reduce food intake through its receptor GLP1R, thereby reducing weight[16]. We have integrated this part into the revised text **(lines 468-488 on pages 18-19)**.

***Comment 11*: Figure 5l – Correct a typo in the word insulin in the Figure panel.**

Response: Thank you for pointing out this issue. In the revised figure, we have made corrections to the “insulin”.

***Comment 12*: The term “liver knockout” (Line 406,407) may confuse the reader towards results of a liver specific knockout. The sentences should better clarify that these results are referred to effects on the liver from a global knockout of Nogo-B.**

Response: Thank you for pointing out this issue. Based on your suggestion, we have revised the description to " Nogo-B global knockout significantly activates hepatic INSR-INS-AKT pathway and alleviates the symptoms of NAFLD induced by high-fat or high-sugar diets " to avoid misleading the readers.

***Comment 13*: 13. Extended data Figure 1 – Typo in the Y graph axis - correct to liver weight.**

Response: Thanks for pointing out this issue. In the revised manuscript, we changed the y-axis to "liver weight".

***Comment 14*. In the methods the authors described that the mice were “humanely euthanized by CO2 asphyxiation”. This is not an appropriate term - use killed by CO2 asphyxiation [Editor note: we believe the best term to use is 'euthanized', without the "humanely" part.]**

Response: Thank you for your suggestion. We apologize for the inappropriate description. We have removed the term of “humanely” in the revised manuscript.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All my comments have been satisfactorily addressed.

Reviewer #2 (Remarks to the Author):

The authors addressed my concerns with elegant appropriate experiments and significantly increased the manuscript's rigor and findings. I am impressed by not missing a single comment and designing the necessary experimentation including glucose clamps. Hence, their conclusions and findings are stronger now, making their phenotype clearer and more comprehensive. The re-writing manuscript with additional data improves its flow and scientific merit. I am satisfied with their revision with no further reservations.

Reviewer #3 (Remarks to the Author):

The authors highlight Nogo-B as a promising new target for treating type-2 diabetes by increasing serum GLP-1 levels, thereby improving metabolic outcomes associated with T2D. The revised manuscript incorporates crucial experiments that bolster the proposed mechanism of action of Nogo-B. Specifically, western blots were included to confirm Nogo-B as a primary target. Furthermore, additional results now address its effects on food consumption and body weight reduction through GLP-1 and possibly GLP-2 actions, suggesting avenues for future research. Moreover, the manuscript includes data on blood glucose levels in global Nogo-B knockout and intestinal-specific knockout models. Interestingly, while the GLP-1 mediated by Nogo-B does not appear to affect blood glucose levels in these models, its beneficial effects are evident in diabetic mice, which warrants a comment in the discussion section. Overall, the revisions have effectively addressed all suggestions, and I recommend the manuscript for publication.