nature portfolio

Peer Review File

Defining the role of β-cell IRE1α/XBP1 pathway and its gene regulatory network components in non-obese diabetic mice

Corresponding Author: Professor Feyza Engin

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This paper aimed to uncover the mechanisms behind the protective effects of β-cell-specific deletion of XBP1 against diabetes. The study used the NOD mice as model for type 1 diabetes (T1D), and followed the contribution of XBP1 in β-cells at different stages (prediabetic and diabetic). The authors performed complex single-cell transcriptomic analysis from mice islets, coupled with histology. Moreover, as they had previously obtained single cell transcriptomics data from β-cell-specific IRE1a-deficient islets from NOD mice (Lee et al., 2020, 10.1016/j.cmet.2020.03.002), they realized comprehensive comparisons and gene regulatory network analyses to reveal the XBP1-specific regulatory networks and nodes that could be considered for therapeutic targeting.
The presented results are noteworthy, as they uncover an important transient dedifferentiation mechanism that β-cells that lack XBP1 use in order to overcome the immune attack that occurs in T1D.
The paper is well written, easy to follow, and presents results that are of great importance for the community, however, there are some aspects that need to be improved and better explained.
Major questions:
1)In Figure 1D, there are red peaks (corresponding to the Xbp1β-/- mice) that appear over the 250 mg/dl blood glucose threshold at later time points (>36 weeks), that do not show up in Figure 1F. Can the authors comment on that?
2)Previous work has shown that in β-cell-specific XBP1-deficient mice, a feedback loop occurs that hyperactivates IRE1 pathway through IRE1a phosphorylation (https://doi.org/10.1073/pnas.1105564108). This resulted in specific degradation of mRNA of different insulin-processing enzymes. It would be useful if the authors check IRE1 phosphorylation status in the Xbp1β-/- NOD mice.
3)In NOD mice (as in T1D patients), transdifferentiation was shown to occur with diabetes progression, thus it is important that the authors show how the percentages of double hormonal cells (INS&GCG and INS&SST) vary at 8w and 31w in the Xbp1fl/fl and in Xbp1β-/-, to rule out this possibility.
4)The temporary de-differentiation mechanism employed by the β-cells lacking expression of XBP1 seems to have a protective effect against the immune attack. In this study, the authors proposed it to occur via different mechanisms:
a)decreased expression of the auto-antigens
b)reduced expression of chemokines.
However, it is not very clear to the reviewer how the re-differentiated β-cells are still protected from the immune attack, at later stages. By 31w, the authors show lack of CD3 expression in the islets of Xbp1β-/- mice, which is very surprising. In the immune attack of the β-cells, macrophages and B cells also play important roles. As such, would be of interest to show immunostaining of islets with corresponding immune cells markers.
In addition, in the previous study (10.1016/j.cmet.2020.03.002), the authors showed no change in the CD4 T cells, but significantly decreased CD8 T cells in the pancreas, suggesting that this was one of the protective mechanisms in IRE1αβ-/-. Can the authors comment on the difference between the immune responses between these two models?
Minor issues:
1) In figure 2C, 2D, 2G and 2I, it is not clear how many islets/mouse were analysed in each condition. Does each dot represent the result for an islet, or it is per section (where multiple islets can occur)?
2) Figure 2E shows comparisons of H&E-stained islets sections from 8w and 31w old mice, however, in the text (row 123), it is specified “Analysis of pancreatic sections from 10 weeks of age Xbp1fl/fl and in Xbp1β-/- mice showed that knock-out mice had significantly reduced islet infiltration by immune cells.” Please revise the figure, or the text, accordingly.
3) Is it the case also for CD3-stained islets in Figure 2F? If so, please revise the text, adding the ages of mice.
4) In figures 2G and 2H are shown percentages of insulitis in 8w NOD mice, however, in the figure legend is written that the

result shown is for 5 weeks of age mice. Please revise the figure legend accordingly.
5) the y axis in Figure 2G should be put at 100% at 8w, for a better assessment of aggressiveness of insulitis progression from 8w to 31w (2G vs 2I).
6) In figure 3B, the graphs depicting expression of Dnajb9, Sec61g, Sec61a1, Fkbp11 and Dnajc3 have a different appearance than the graph that shows Xbp1 expression. It is not clear to the reviewer if there were different numbers of cells taken into the analysis, or it is just a matter of picture resolution?
7) at pg. 160, Ildr2 and H2-T23 should be in italics

Reviewer #2

(Remarks to the Author)
Review of NCOMMS-24-52041-T

In their manuscript titled “Defining the role of β-cell IRE1α/XBP1 pathway and its gene regulatory network components in NOD mice” the authors are attempting to link the dysregulation of the unfolded protein response in -cells to T1D. The same group has already published a similar study in which they showed that IRE1 deletion was protective of T1D in NOD mice (2020 Cell Metabolism paper (cit. 31). However, whether this protective effect was mediated by the loss of IRE1 ’s kinase, RIDD, or XBP1 activity was unresolved. Here using an Xbp1β- /- mice they try to answer these questions. Two observations are made 1) XBP1 deletion prior to insulitis protects NOD mice from diabetes and 2) XBP1 and IRE1 have shared and independent roles in gene regulation. Whether XBP1 is directly and solely responsible for T1D protective effects observed in IRE1 β- /- is not clearly supported.

Major comments
My major concern is that the phenotype that is presented here is very difficult to link to the autoimmune process that leads to diabetes in NOD mice. There is nearly no interrogation of the immune response in the animals. A single histology panel that examine CD3+ cells in sections is a far cry from what we would expect: inflammation, CD4, CD8, flow cytometry analysis, antigen specificity, etc..
As it stands, the work would benefit to be presented in C57Bl6 mice and focus on the physiology of insulin secretion. The early hyperglycemia peak-recovery-normoglycemia timeline is indeed puzzling and needs explanation. To throw on top of that abnormal beta cell response the immune response is difficult to justify. The phenotype that jumps to my attention is the greatly diminish insulin secretion after induced deletion. It is very likely that this diminution of insulin secretion is sufficient to blunt any autoimmunity in those mice. It is very well documented that insulin is the early antigen, and that the initiation of disease depends on it. Later events in life do not rescue the phenotype of T1D when early initiation is missed.
My second concern, even if we focus on the metabolic phenotype, is the lack of dynamic studies of the glucose/insulin response. Instead, we are taken into a lengthy analysis of a single RNAseq dataset that does not conclude very much.
It also would be necessary to evaluate the persistence of the Xbp1 knockout over time. The glycemia phenotype and recovery of beta cell mass would suggest that this phenotype is directly linked to an incomplete induction of deletion.

Reviewer #3

(Remarks to the Author)
Summary

Lee and colleagues focused on the importance of two key related UPR regulators in beta cells in the development of T1D, Ire1a (as a UPR sensor) and XBP1 (as a key UPR effector modified by Ire1a RIDD activity). Using inducible beta cell specific knockout mice on the NOD background, the authors observed that deletion of XBP1 in beta cells leads to transient hyperglycemia, beta cell dedifferentiation, reduced antigenicity/immune infiltration, and then beta cell recovery phenocopying their previously published observations in Ire1a deficient mice. They also used comparative analyses of scRNAseq from XBP1 and Ire1a knockouts, observing shared regulatory networks between these models that may underlie the protective phenotypes observed in both strains as well as some pathways that were unique between the models. The authors conclude that targeting the XBP1/RIDD axis is a key mechanism to the protection of beta cells from T1D development.

General comments

The authors present an intriguing series of physiologic, histologic, and transcriptomic studies in genetic mouse models of T1D coupled to comparative analyses of previously published results from this group. The mechanistic importance of how Ire1a deficiency or inhibition is protective to beta cells in T1D is poorly understood, hence, these studies offer unique and unappreciated insights into Ire1a activity/XBP1 function and RIDD in T1D. The work is timely, and studies were well controlled. Overall, the enthusiasm for this work is high, however, there are several necessary studies that should be included to more convincingly support the conclusions drawn by the authors.

Specific comments

1. In figures 1 and 2, the authors show that beta cell XBP1 is lost by immunostaining at 5 weeks, followed by transient hyperglycemia, and recovery later in life. What is not evident in the older XBP1 mutants (31 weeks, etc) is if the recovered beta cells still are XBP1 deficient? An alternative could be the recovery is mediated by a mosaic population of non-

recombined beta cells. Confirmation that XBP1 is still lost later in life in NOD mice would be helpful.

2. A second question is why in early life, XBP1 deficient NOD beta cells develop reduced insulin levels and hyperglycemia (similar to what is reported in B6 XBP1 beta cell knockouts), possibly due to impaired insulin processing. However, aged XBP1-deficient beta cells are later completely functional without any defects in insulin levels and glucose homeostasis. How do the authors explain that these aged beta cells have completely recovered from the XBP1 deficiency phenotype? Some assessment of proinsulin/insulin levels or processing might be helpful in young and old NOD XBP1 mutants to better reconcile these differences.

3. Single cell sequencing studies in Figure 3 provide unique insights into the pathways engaged in XBP1 mutants to explain their resistance to develop T1D. It would be useful if markers of these pathways were assessed at the protein level both in young and old XBP1 mutants to better validate these pathways.

4. Comparative studies in Figures 3 and 4 (from new work in XBP1 mutants and legacy work in the Ire1a deficient model) are used to support shared pathways from RIDD/XBP1 in beta cell protection, but these are largely correlative in nature based on phenotypes that are similar between the models. It would be helpful if a more direct study between the models could reinforce the shared pathways. For instance, is immunophenotyping in the XBP1 mutant (rather than gene expression alone) similar to that of the Ire1a mutant? Further, if adoptive transfer of T-cells from the XBP1 mutant was performed into the Ire1a mutant (and vice versa) potentially programmed from beta cell signals in these early life models, would both models still retain reduced immune activation and beta cell destruction?

5. How important is the transient hyperglycemia in XBP1 mutant NOD mice to the later protection against diabetes? If one was to reduce hyperglycemia early in life by insulin therapy or SGLT inhibition, would XBP1 deficient mice still be protected from T1D?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors made a significant effort to respond to my comments and have reasonably addressed most aspects that were raised.

Reviewer #3

(Remarks to the Author)
The authors sufficiently addressed my concerns with the manuscript.

Response to Reviewers

We would like to thank the reviewers and editor for their time and thoughtful comments. We have revised the manuscript accordingly, and specific responses to each reviewer's critiques is delineated below in BLUE text:

Reviewer #1 (Remarks to the Author):

This paper aimed to uncover the mechanisms behind the protective effects of β-cell-specific deletion of XBP1 against diabetes. The study used the NOD mice as model for type 1 diabetes (T1D), and followed the contribution of XBP1 in β-cells at different stages (prediabetic and diabetic). The authors performed complex single-cell transcriptomic analysis from mice islets, coupled with histology. Moreover, as they had previously obtained single cell transcriptomics data from β-cell-specific IRE1a-deficient islets from NOD mice (Lee et al., 2020, 10.1016/j.cmet.2020.03.002), they realized comprehensive comparisons and gene regulatory network analyses to reveal the XBP1-specific regulatory networks and nodes that could be considered for therapeutic targeting.

The presented results are noteworthy, as **they uncover an important transient dedifferentiation mechanism** that β-cells that lack XBP1 use in order to overcome the immune attack that occurs in T1D. **The paper is well written, easy to follow, and presents results that are of great importance for the community,** however, there are some aspects that need to be improved and better explained.

We thank the reviewer for their positive comments and their valuable suggestions.

Major questions:
1) In Figure 1D, there are red peaks (corresponding to the Xbp1β-/- mice) that appear over the 250 mg/dl blood glucose threshold at later time points (>36 weeks), that do not show up in Figure 1F. Can the authors comment on that?

We thank the reviewer for highlighting this. We and others define diabetes in NOD mice as "*two consecutive blood glucose measurements above 250 mg/dL*". However, the mentioned measurements showed a high glucose level only in one measurement and never showed hyperglycemia again. Therefore, we did not count that animal as diabetic.

2) Previous work has shown that in β-cell-specific XBP1-deficient mice, a feedback loop occurs that hyperactivates IRE1 pathway through IRE1a phosphorylation (https://doi.org/10.1073/pnas.1105564108). This resulted in specific degradation of mRNA of different insulin-processing enzymes. It would be useful if the authors check IRE1 phosphorylation status in the Xbp1β-/- NOD mice.

We agree with the reviewer that IRE1 hyperactivity in response to loss of XBP1 was a possibility, although various studies showed that IRE1 hyperactivation in the absence of XBP1 is context- and cell type-dependent. We also agree with the reviewer that the ideal way to test this would be to look at phospho-IRE1 levels. However, the commercially available phospho-IRE1 antibodies are notoriously problematic, and they have not worked in our hands in beta cells (I have been trying this since 2008). In addition, we tried using a Phos-tag gel approach, but this approach also failed. However, our scRNA-seq data show that almost none of the RIDD targets were upregulated in $Xbp1^{\beta-/-}$ mice, strongly suggesting that IRE1 is not hyperactivated in these mice. Also, the fact that IRE1 hyperactivation (RIDD) almost always leads to maladaptive responses and causes cell death (beta cell death in BL6 mice, PMID: 22883233, PMID: 25018104), but NOD XBP1 deletion cause beta cell survival and diabetes protection (adaptive) further support that IRE1 hyperactivation in this model is highly unlikely

(we added this to the text). Of note, although previous reports suggest IRE1α degrades insulin mRNAs, it was shown that Ire1α deletion in beta cells did not alter insulin mRNA expression either in the presence or absence of glucose stimulation (PMID: 26469762).

3) In NOD mice (as in T1D patients), transdifferentiation was shown to occur with diabetes progression, thus it is important that the authors show how the percentages of double hormonal cells (INS&GCG and INS&SST) vary at 8w and 31w in the Xbp1fl/fl and in Xbp1β-/-, to rule out this possibility.

We appreciate this thoughtful suggestion. Deletion of *Xbp1* in adult mouse beta cells led to beta cell dedifferentiation, beta-to-alpha cell transdifferentiation and increased alpha cell mass (PMID: 35316840). We acknowledge that we cannot completely rule out transdifferentiation in our models, and the best course of action to answer the reviewer's question would be to do a lineage tracing analysis, yet there are no reporter lines available on NOD background and obtaining sufficient number of animals (complex breeding scheme with multiple alleles; XBP1 flox/flox, InsCre +/, plus homozygous reporter allele) in the presence of NOD sexual dimorphism would be extremely difficult. Hence, we see this as a limitation of our study. While we appreciate the suggestion of quantification of bihormonal cells at early and late time points, detecting these cells via IF has been difficult (compared to scRNA-seq) preventing us to draw a reliable conclusion.

4) The temporary de-differentiation mechanism employed by the β-cells lacking expression of XBP1 seems to have a protective effect against the immune attack. In this study, the authors proposed it to occur via different mechanisms:
a) decreased expression of the auto-antigens
b) reduced expression of chemokines.

However, it is not very clear to the reviewer how the re-differentiated β-cells are still protected from the immune attack, at later stages. By 31w, the authors show lack of CD3 expression in the islets of Xbp1β-/- mice, which is very surprising. In the immune attack of the β-cells, macrophages and B cells also play important roles. As such, would be of interest to show immunostaining of islets with corresponding immune cells markers.
In addition, in the previous study (10.1016/j.cmet.2020.03.002), the authors showed no change in the CD4 T cells, but significantly decreased CD8 T cells in the pancreas, suggesting that this was one of the protective mechanisms in IRE1αβ-/-. Can the authors comment on the difference between the immune responses between these two models?

In response to the reviewer's request, we performed the following analyses.
a) We examined the distributions and proportions of immune cell populations involved in T1D pathogenesis within the pancreas, spleen, and pancreatic lymph nodes (PLNs) of 21-week-old control and knockout mice. Specifically, we analyzed T cells ($CD4^+$ and $CD8^+$), Tregs ($CD25^+$, FoxP3), macrophages (F4/80), B cells ($CD19^+$), and dendritic cells ($CD11c^+$). Our analysis showed significantly higher the percentage of $CD19^+$ B cells, and markedly reduced percentages of $CD4^+$ and $CD8^+$ T cells in the pancreata of $XBP1^{\beta-/-}$ mice (**Fig. 2M**). The percentage of T regulatory cells (Tregs), macrophages, or dendritic cells did not show significant alterations in the pancreas of knockout animals (**Fig. 2M**). There were no differences in any of these immune cell populations within the spleens of $Xbp1^{\beta-/-}$ mice compared to control animals (**Fig. 2N**), while a moderate reduction in $CD8^+$ T cells and a marked increase in $F4/80^+$ cells were detected in PLN of knockout animals (**Fig. 2O**). Hence, deletion of *Xbp1* in β-cells in NOD mice significantly reduced the relative representation of $CD4^+$ and $CD8^+$ T cells specifically in the pancreas. This phenotype was reminiscent of $IRE1\alpha^{\beta-/-}$ mice where the percentage of $CD4^+$ T cells showed a trend towards declining, and the percentage of $CD8^+$ T cells was significantly diminished. However, increased percentage of $CD19^+$ B cell phenotype as well as alterations in representation of $CD8^+$ T cells and $F4/80^+$ cells in PLN were unique to $XBP1^{\beta-/-}$ mice. Taken together, these data suggest

that significantly decreased islet infiltration by immune cells particularly reduced frequency of islet-reactive $CD4^+$ and $CD8^+$ T cells contributed to the β-cell survival in $Xbp1^{\beta-/-}$ mice. We added this to the text.

b) We expanded our IF from CD3 to CD4, CD8 T cells, and B cells both at 8 and 31 weeks of age (revised Figures 2H and 2L).

Minor issues:
We thank the reviewer for their careful reading of the manuscript and helping us to notice these issues.

1) In figure 2C, 2D, 2G and 2I, it is not clear how many islets/mouse were analysed in each condition. Does each dot represent the result for an islet, or it is per section (where multiple islets can occur)?

Animal numbers are indicated in the figure legends. In Fig 2C,D, each dot indicates an individual islet. We now added this information into the figure legend. In Fig 2F,G mouse numbers are indicated in the figure legends.

2) Figure 2E shows comparisons of H&E-stained islets sections from 8w and 31w old mice, however, in the text (row 123), it is specified “Analysis of pancreatic sections from 10 weeks of age Xbp1fl/fl and in Xbp1β-/- mice showed that knock-out mice had significantly reduced islet infiltration by immune cells.” Please revise the figure, or the text, accordingly.

We fixed the text accordingly.

3) Is it the case also for CD3-stained islets in Figure 2F? If so, please revise the text, adding the ages of mice.

We revised the text accordingly.

4) In figures 2G and 2H are shown percentages of insulitis in 8w NOD mice, however, in the figure legend is written that the result shown is for 5 weeks of age mice. Please revise the figure legend accordingly.

We have now revised the figure legend.

5) the y axis in Figure 2G should be put at 100% at 8w, for a better assessment of aggressiveness of insulitis progression from 8w to 31w (2G vs 2I).

We have revised the graph accordingly.

6) In figure 3B, the graphs depicting expression of Dnajb9, Sec61g, Sec61a1, Fkbp11 and Dnajc3 have a different appearance than the graph that shows Xbp1 expression. It is not clear to the reviewer if there were different numbers of cells taken into the analysis, or it is just a matter of picture resolution?

They have the same numbers, yet the quality of the images was off. We corrected the image quality.

7) at pg. 160, Ildr2 and H2-T23 should be in italics

We revised the text accordingly.

Reviewer #2 (Remarks to the Author):

Review of NCOMMS-24-52041-T

In their manuscript titled “Defining the role of β-cell IRE1α/XBP1 pathway and its gene regulatory network components in NOD mice” the authors are attempting to link the dysregulation of the unfolded protein response in β-cells to T1D. The same group has already published a similar study in which they showed that IRE1α deletion was protective of T1D in NOD mice (2020 Cell Metabolism paper (cit. 31). However, whether this protective effect was mediated by the loss of IRE1α’s kinase, RIDD, or XBP1 activity was unresolved. Here using an Xbp1β- /- mice they try to answer these questions. Two observations are made 1) XBP1 deletion prior to insulitis protects NOD mice from diabetes and 2) XBP1 and IRE1α have shared and independent roles in gene regulation. Whether XBP1 is directly and solely responsible for T1D protective effects observed in IRE1αβ- /- is not clearly supported.

Major comments
My major concern is that the phenotype that is presented here is very difficult to link to the autoimmune process that leads to diabetes in NOD mice. There is nearly no interrogation of the immune response in the animals. A single histology panel that examine CD3+ cells in sections is a far cry from what we would expect: inflammation, CD4, CD8, flow cytometry analysis, antigen specificity, etc..

We thank the reviewer for their suggestions. In the original submission, we had provided **a)** qualitative analysis of immune infiltration at 8 and 31 weeks of age by H&E staining (formerly Fig 2E), **b)** $CD3^+$ lymphocyte infiltration by immunofluorescence (IF) at 8 and 31 weeks of age (formerly Fig 2F), **c)** quantification of the extent of islet inflammation (insulitis) by performing “insulitis scoring” on H&E-stained step sections at 8 weeks of age (formerly Fig 2G, H) and 31 weeks of age (formerly Fig 2I,J), and **d)** beta cell antigen expression profile by scRNA-seq (Fig 3F). **These data suggested that reduced islet infiltration and inflammation phenotypes were shared by IRE1 and XBP1 knockout animals**.

In response to the reviewer’s request, we expanded these analyses by performing the following experiments.
a) We examined the distributions and proportions of immune cell populations involved in T1D pathogenesis within the pancreas, spleen, and pancreatic lymph nodes (PLNs) of 21-week-old control and knockout mice. Specifically, we analyzed T cells ($CD4^+$ and $CD8^+$), Tregs ($CD25^+$, FoxP3), macrophages (F4/80), B cells ($CD19^+$), and dendritic cells ($CD11c^+$). Our analysis showed significantly higher the percentage of $CD19^+$ B cells, and markedly reduced percentages of $CD4^+$ and $CD8^+$ T cells in the pancreata of $XBP1^{\beta-/-}$ mice (**Fig. 2M**). The percentage of T regulatory cells (Tregs), macrophages, or dendritic cells did not show significant alterations in the pancreas of knockout animals (**Fig. 2M**). There were no differences in any of these immune cell populations within the spleens of $Xbp1^{\beta-/-}$ mice compared to control animals (**Fig. 2N**), while a moderate reduction in $CD8^+$ T cells and a marked increase in $F4/80^+$ cells were detected in PLN of knockout animals (**Fig. 2O**). Hence, deletion of *Xbp1* in β-cells in NOD mice significantly reduced the relative representation of $CD4^+$ and $CD8^+$ T cells specifically in the pancreas. This phenotype was reminiscent of $IRE1\alpha^{\beta-/-}$ mice where the percentage of $CD4^+$ T cells showed a trend towards declining, and the percentage of $CD8^+$ T cells was significantly diminished. However, increased percentage of $CD19^+$ B cell phenotype as well as alterations in representation of $CD8^+$ T cells and $F4/80^+$ cells in PLN were unique to $XBP1^{\beta-/-}$ mice. Taken together, these data suggest that significantly decreased islet infiltration by immune cells particularly reduced frequency of islet-reactive $CD4^+$ and $CD8^+$ T cells contributed to the β-cell survival in $Xbp1^{\beta-/-}$ mice. We added this to the text.

b) We expanded our IF from CD3 to CD4, CD8 T cells, and B cells both at 8 and 31 weeks of age (revised Figures 2H and 2L).

These studies link diabetes protection phenotype in our models, at least in part, to the altered autoimmune process.

As it stands, the work would benefit to be presented in C57Bl6 mice and focus on the physiology of insulin secretion. The early hyperglycemia peak-recovery-normoglycemia timeline is indeed puzzling and needs explanation. To throw on top of that abnormal beta cell response the immune response is difficult to justify. The phenotype that jumps to my attention is the greatly diminish insulin secretion after induced deletion. It is very likely that this diminution of insulin secretion is sufficient to blunt any autoimmunity in those mice. It is very well documented that insulin is the early antigen, and that the initiation of disease depends on it. Later events in life do not rescue the phenotype of T1D when early initiation is missed.

We thank the reviewer for their suggestion. We apologize for not explaining clearly the phenotype of the beta cell-specific XBP1 and IRE1 deletion models on the C57BL6 background. Published work demonstrates that BL6 and NOD mice show completely different phenotypes in response to XBP1 and IRE1 deletion in beta cells. **Beta cell-specific XBP1 and IRE1 deletion leads to diabetes on the C57BL6 background** (PMID: 21555585, PMID: 26469762), **whereas deletion of these genes in beta cells of autoimmune T1D model NOD mice is protective against T1D.** The impact of these modifications on insulin secretion and systemic metabolism were studied in detail in the BL6 background (*see the excerpts below*).

> *Ablation of XBP1 markedly decreases the number of insulin granules in β-cells, impair proinsulin processing, increased the serum proinsulin:insulin ratio, blunt glucose-stimulated insulin secretion, and inhibited cell proliferation (PMID: 21555585).*
>
> *β cell failure upon Ire1α deletion was primarily due to reduced proinsulin mRNA translation primarily because of defective glucose-stimulated induction of a dozen genes required for the signal recognition particle (SRP), SRP receptors, the translocon, the signal peptidase complex, and over 100 other genes with many other intracellular functions (PMID: 26469762).*

We think the reason for XBP1/IRE1 deletion phenotype being different on NOD background is, at least in part, due to *Glis3* and *Xrcc4* mutations that make beta cells fragile and prone to ER stress in mice and humans (PMID: 26998692). Hence, when IRE1 or XBP1 are deleted in beta cells of NOD mice, i.e., in an already stress-sensitive background, it is highly likely that this second hit unleashes adaptive responses leading to a diabetes protective phenotype. Our previous work suggests that beta cell plasticity (loss of beta cell identity and regaining it) is an adaptive mechanism (PMID: 35941159). Hence, the lack of dedifferentiation in BL6 mice suggests impaired adaptive mechanisms in this model.

We agree with the reviewer that early reduction of insulin levels plays a critical role in T1D protection phenotype. Our mechanistic model shown in our previous publication (Lee et al Cell Metab 2020) and in this manuscript is that significantly diminished expression of insulin and other autoantigens (owing to dedifferentiation) during early stages of disease, reduced immunogenicity, and markedly reduced frequency of cytotoxic T cells in pancreas collectively contribute to diabetes protection phenotype in IRE1 and XBP1 deficient NOD mice.

My second concern, even if we focus on the metabolic phenotype, is the lack of dynamic studies of the glucose/insulin response. Instead, we are taken into a lengthy analysis of a single RNAseq dataset that does not conclude very much.

Since restoring glucose/insulin response or the metabolic phenotype would be relevant to T2D pathology but not be sufficient alone to prevent T1D in mouse models and individuals at high risk, we focused on investigating beta cell antigenicity, beta cell-driven immune regulation, and immune phenotype as possible underlying mechanisms of diabetes protection in our models. The comprehensive immunophenotyping of the revised manuscript further provides a link between diabetes protection and autoimmune process in our mice.

Our scRNA-seq data from two models were generated under the exact same experimental and environmental conditions (critically important for T1D studies), **a first for the UPR field**, to identify the beta cell-specific unique and shared molecular signatures and gene regulatory networks of these two proximal UPR genes within the same pathway. We hope that the reviewer will share our excitement and recognize the importance and the novelty of this rigorous, robust, and comprehensive data for the community.

It also would be necessary to evaluate the persistence of the Xbp1 knockout over time.

We thank the reviewer for this request. Our unpublished data show that at 17 weeks of age, long after recovery from hyperglycemia, IRE1 islets still show significantly diminished IRE1 levels (**Fig. 1**).

Figure 1. (A) Western blot image and (B) quantification of IRE1α expression level in sorted β-cell from 17-week-old euglycemic IRE1α$^{fl/fl}$ and recovered IRE1α$^{\beta-/-}$ mice (n=4/group). Data are represented as mean ± SEM. *p < 0.05

Next, we attempted to perform the same western blot analysis in beta cells of Xbp1$^{\beta-/-}$ mice. However, we noticed that islet isolation from Xbp1$^{\beta-/-}$ mice had been almost impossible starting from 7-8 weeks of age. Our efforts failed to get more than 10 islets. We found that this was due to increased collagen deposition (assessed by trichome blue staining) making our collagenase treatment inefficient in these mice (Figure 2). Indeed, this phenotype was also reported in beta cell-specific XBP1 deletion model on C57BL6 mice (PMID: 21555585). Of note, it is possible that this altered ECM contribute to diabetes protection phenotype in NOD mice by limiting islet immune cell infiltration though this is out of the scope of the current study.

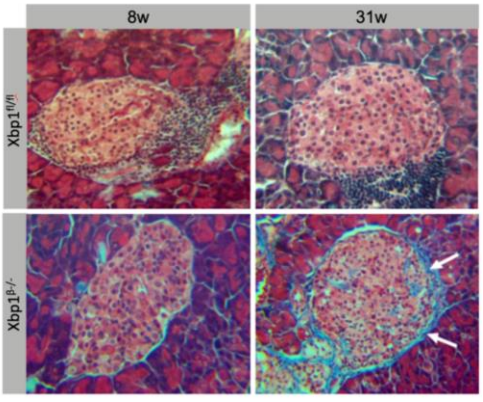

Figure 2. Representative image of Trichrome blue (light blue) staining of Xbp1$^{fl/fl}$ and Xbp1$^{\beta-/-}$ mice at indicated time points. Arrows show the unique blue staining.

Therefore, as an alternative method, we performed immunofluorescence (IF) assay on the pancreatic sections from 8- (**Fig. 3A**) and 24-week-old (**Fig. 3B**) mice using anti-XBP1 and anti-insulin antibodies. We detected significantly reduced XBP1 levels in both time points although the reduction in XBP1 levels was relatively less at the later point. However, it is important to emphasize that both in NOD mice (PMID: 24225943, PMID: 28467938) and T1D patients (PMID: 35941159), beta cells drastically lose their XBP1 expression as the disease progresses. By 10 weeks of age there is significantly reduced XBP1 levels in beta cells of NOD mice compared to 5-8 weeks of age (PMID: 24225943, PMID: 28467938). Hence, our comparison of knockout XBP1 levels to control in NOD mice at 24 weeks of age might be masking the actual deletion efficiency as control beta cells already

significantly lose their XBP1 expression by this time point. The revised figures were added as Fig.1B and 1C in the resubmission.

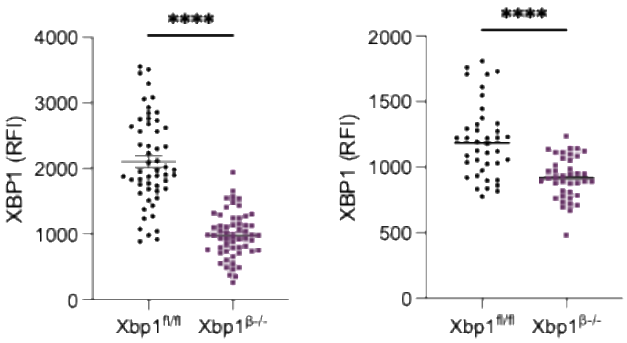

Figure 3. Quantification of pancreatic sections from (A) 8-week-old and (B) 24-week-old $Xbp1^{fl/fl}$ and $Xbp1^{\beta-/-}$ mice (n=4 per genotype at indicated time points) co-stained with anti-insulin and anti-XBP1 antibodies. Each dot represents an islet. All data are represented as mean ± SEM, with statistical analyses performed by Student's t-test, ****p<0.0001).

The glycemia phenotype and recovery of beta cell mass would suggest that this phenotype is directly linked to an incomplete induction of deletion.

Respectfully, we disagree with the reviewer that our phenotype can be explained by "the recovery of beta cell mass is directly linked to an incomplete induction of deletion". This could have been relevant to a type 2 diabetes mouse model, in which functional beta cell mass would be stable over time after the recovery of beta cell mass. However, such beta cell mass restoration prior to diabetes onset cannot explain the phenotype in NOD mice where beta cells are continuously and progressively under autoimmune attack. In other words, in such a scenario, the incomplete deletion would lead to accumulation of "XBP1-intact, "wildtype" beta cells, and due to the expression of their beta cell-specific autoantigens (insulin, proinsulin, etc.) and their intact antigen presentation machinery, these cells eventually would have been targeted and killed by cytotoxic T cells leading to diabetes phenotype (similar to what is observed in XBP1-intact NOD control mice). However, we monitored these mice up to a year and did not detect an increase in diabetes incidence. We hope that the data for quantification of beta cell IRE1 and XBP1 levels after hyperglycemia recovery (Fig.1 and 3) further alleviate the concern of the reviewer.

Reviewer #3 (Remarks to the Author):

Summary

Lee and colleagues focused on the importance of two key related UPR regulators in beta cells in the development of T1D, Ire1a (as a UPR sensor) and XBP1 (as a key UPR effector modified by Ire1a RIDD activity). Using inducible beta cell specific knockout mice on the NOD background, the authors observed that deletion of XBP1 in beta cells leads to transient hyperglycemia, beta cell dedifferentiation, reduced antigenicity/immune infiltration, and then beta cell recovery phenocopying their previously published observations in Ire1a deficient mice. They also used comparative analyses of scRNAseq from XBP1 and Ire1a knockouts, observing shared regulatory networks between these models that may underlie the protective phenotypes observed in both strains as well as some pathways that were unique between the models. The authors conclude that targeting the XBP1/RIDD axis is a key mechanism to the protection of beta cells from T1D development.

General comments

The authors present an intriguing series of physiologic, histologic, and transcriptomic studies in genetic mouse models of T1D coupled to comparative analyses of previously published results from this group. The mechanistic importance of how Ire1a deficiency or inhibition is protective to beta cells in T1D is poorly understood, hence, these studies offer unique and unappreciated insights into Ire1a

activity/XBP1 function and RIDD in T1D. **The work is timely, and studies were well controlled. Overall, the enthusiasm for this work is high,** however, there are several necessary studies that should be included to more convincingly support the conclusions drawn by the authors.

We thank the reviewer for their enthusiasm for our work and for their valuable suggestions.

Specific comments

1. In figures 1 and 2, the authors show that beta cell XBP1 is lost by immunostaining at 5 weeks, followed by transient hyperglycemia, and recovery later in life. What is not evident in the older XBP1 mutants (31 weeks, etc) is if the recovered beta cells still are XBP1 deficient? An alternative could be the recovery is mediated by a mosaic population of non-recombined beta cells. Confirmation that XBP1 is still lost later in life in NOD mice would be helpful.

We thank the reviewer for their suggestion. We think that a mosaic population of non-recombined beta cells would not explain our diabetes protection phenotype. The non-recombined beta cells with intact XBP1 upon recovery would still undergo continuous and progressive immune attack similar to control mice and develop diabetes. However, Xbp1$^{\beta-/-}$ mice are protected from T1D up to a year.

Nonetheless, in response to the reviewer's (and reviewer #2's) suggestion, we analyzed both IRE1 and XBP1 deletion at later time points after the hyperglycemia recovery. Our unpublished data show that at 17 weeks of age, long after from hyperglycemia, IRE1 islets still show significantly diminished IRE1 levels (**Fig. 1**).

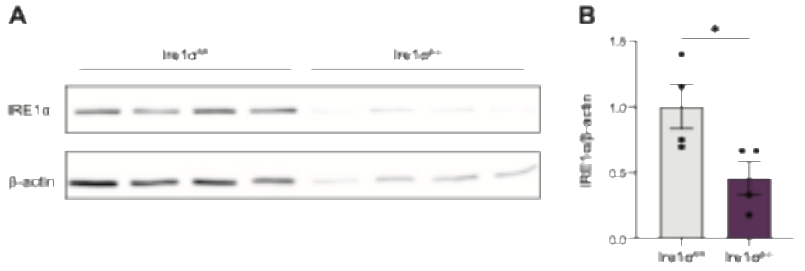

Figure 1. (A) Western blot image and (B) quantification of IRE1α expression level in sorted β-cells from 17-week-old euglycemic IRE1α$^{fl/fl}$ and IRE1α$^{\beta-/-}$ mice (n=4/group). Data are represented as mean ± SEM. *p < 0.05

Next, we attempted to perform the same western blot analysis in beta cells of Xbp1$^{\beta-/-}$ mice. However, we noticed that islet isolation from Xbp1$^{\beta-/-}$ mice had been almost impossible starting from 7-8 weeks of age. Our efforts failed to get more than 10 islets. We found that this was due to increased collagen deposition (assessed by trichome blue staining) making our collagenase treatment inefficient in these mice (Figure 2). Indeed, this phenotype was also reported in beta cell-specific XBP1 deletion model on C57BL6 mice (PMID: 21555585). Of note, it is possible that this altered ECM contribute to diabetes protection phenotype in NOD mice by limiting islet immune cell infiltration though this is out of the scope of the current study.

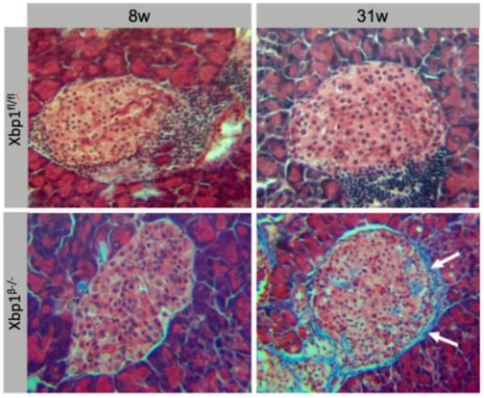

Figure 3. Representative image of Trichrome blue (light blue) staining of Xbp1$^{fl/fl}$ and Xbp1$^{\beta-/-}$ mice at indicated time points. Arrows show the unique blue staining.

Therefore, as an alternative method, we performed immunofluorescence (IF) assay on the pancreatic sections from 8- (**Fig. 3A**) and 24-week-old (**Fig. 3B**) mice using anti-XBP1 and anti-insulin antibodies. We detected significantly reduced XBP1 levels in both time points although the reduction in XBP1 levels was relatively less at the later point. However, it is important to emphasize that both in NOD mice (PMID: 24225943, PMID: 28467938) and T1D patients (PMID: 35941159), beta cells drastically lose their XBP1 expression as the disease progresses. By 10 weeks of age there

is significantly reduced XBP1 levels in beta cells of NOD mice compared to 5-8 weeks of age (PMID: 24225943, PMID: 28467938). Hence, our comparison of knockout XBP1 levels to control in NOD mice at 24 weeks of age might be masking the actual deletion efficiency as control beta cells already significantly lose their XBP1 expression by this time point. The revised figures were added as Fig.1B and 1C in the resubmission.

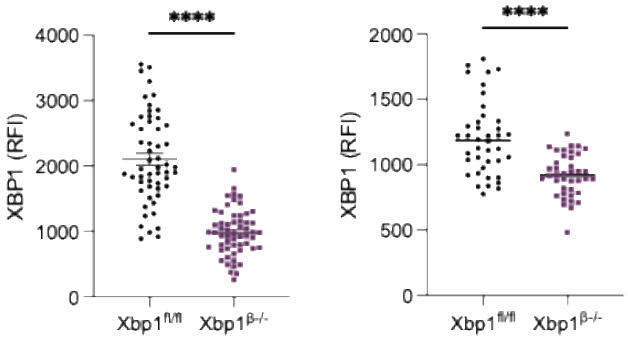

Figure 3. Quantification of pancreatic sections from (A) 8-week-old and (B) 24-week-old $Xbp1^{fl/fl}$ and $Xbp1^{\beta-/-}$ mice (n=4 per genotype at indicated time points) co-stained with anti-insulin and anti-XBP1 antibodies. Each dot represents an islet. All data are represented as mean ± SEM, with statistical analyses performed by Student's t-test, ****p<0.0001).

2. A second question is why in early life, XBP1 deficient NOD beta cells develop reduced insulin levels and hyperglycemia (similar to what is reported in B6 XBP1 beta cell knockouts), possibly due to impaired insulin processing. However, aged XBP1-deficient beta cells are later completely functional without any defects in insulin levels and glucose homeostasis. How do the authors explain that these aged beta cells have completely recovered from the XBP1 deficiency phenotype? Some assessment of proinsulin/insulin levels or processing might be helpful in young and old NOD XBP1 mutants to better reconcile these differences.

How do the authors explain that these aged beta cells have completely recovered from the XBP1 deficiency phenotype?

BL6 mice and our model show significant differences. Unlike BL6 mice, XBP1 deficient beta cells in NOD mice show dedifferentiation and the presence of bihormonal cells, suggesting beta cell plasticity. Indeed, we previously showed that stressed beta cells can undergo an immature state (lose their mature identity) as an adaptive mechanism and regain their mature identity upon resolution of stress (PMID: 35941159). Hence, it is possible that XBP1 deletion cause an adaptive loss of mature beta cell identity. However, cells can manage to recover from stress and regain their mature beta cell identity through unique mechanisms. The mechanism(s) of redifferentiation/hyperglycemia recovery phenotype is out of the scope of this brief report but will be presented as a follow up study. We are very excited about this study and wish to share our findings with the reviewer.

Some assessment of proinsulin/insulin levels or processing might be helpful in young and old NOD XBP1 mutants to better reconcile these differences.

In response to the reviewer's request, we performed ELISA (**Fig. 4**) at indicated time points. Please, see below and revised Figs. 1H-M in the resubmission.

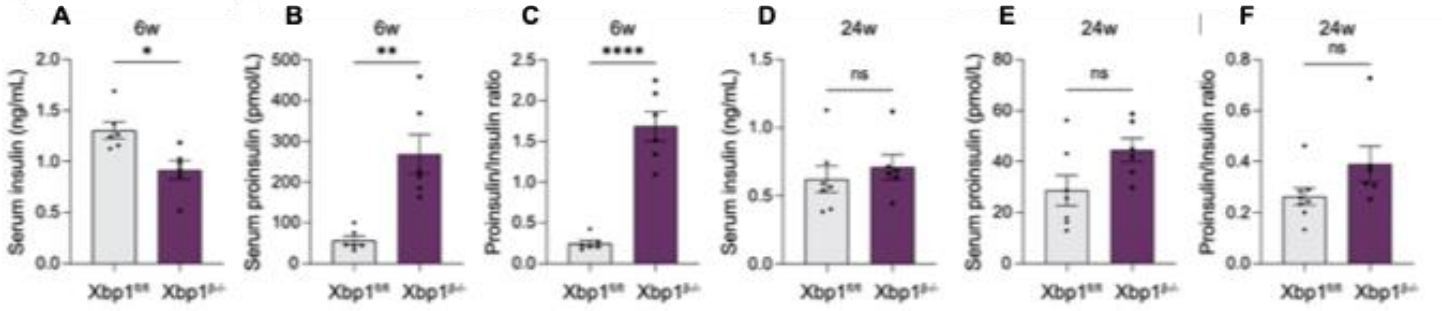

Figure 4. Serum (A) insulin, (B) proinsulin levels, and (C) proinsulin: insulin ratio of $Xbp1^{fl/fl}$ and $Xbp1^{\beta-/-}$ mice at 6 weeks of age (n=6 per genotype). Serum (D) insulin, (E) proinsulin levels, and (F) proinsulin: insulin ratio of $Xbp1^{fl/fl}$ and $Xbp1^{\beta-/-}$ mice at 24 weeks of age (n=7 for $Xbp1^{fl/fl}$ and n=6 for $Xbp1^{\beta-/-}$ mice. Each dot represents a mouse. ns, not significant. All data are represented as mean ± SEM, with statistical analyses performed by Student's t-test (*p<0.05, **p<0.01, ****p<0.0001).

3. Single cell sequencing studies in Figure 3 provide unique insights into the pathways engaged in XBP1 mutants to explain their resistance to develop T1D. It would be useful if markers of these pathways were assessed at the protein level both in young and old XBP1 mutants to better validate these pathways.

We thank the reviewer for their suggestion. Figures 3A-3F mainly focuses on the beta cell identity and disallowed genes to prove the beta cell dedifferentiation while Figure 3G focuses on immune regulation genes. We validated the dedifferentiation at the protein level by showing reduced insulin and increased glucagon levels by IF (Figures 2A-D). We also showed reduced serum insulin levels in Figure 1G. Furthermore, immunophenotyping supported the altered immune regulation in these animals. We hope the reviewer agrees that these data along with our previous publication provide compelling evidence for beta cell dedifferentiation and its role in T1D protection in IRE/XBP1 mice. However, we completely agree with the reviewer that it would be useful to complement our studies with a proteomics study ideally in time course manner to better validate select pathways in these models. We think that these studies and integration of data (requiring robust bioinformatics analysis) will enhance our understanding of the molecular signatures of XBP1deficiency in beta cells. But in this manuscript (brief report), we mainly focused on XBP1 deletion model and comprehensive gene regulatory network analysis of two different animal models.

4. Comparative studies in Figures 3 and 4 (from new work in XBP1 mutants and legacy work in the Ire1a deficient model) are used to support shared pathways from RIDD/XBP1 in beta cell protection, but these are largely correlative in nature based on phenotypes that are similar between the models. It would be helpful if a more direct study between the models could reinforce the shared pathways. For instance, is immunophenotyping in the XBP1 mutant (rather than gene expression alone) similar to that of the Ire1a mutant? Further, if adoptive transfer of T-cells from the XBP1 mutant was performed into the Ire1a mutant (and vice versa) potentially programmed from beta cell signals in these early life models, would both models still retain reduced immune activation and beta cell destruction?

is immunophenotyping in the XBP1 mutant (rather than gene expression alone) similar to that of the Ire1a mutant?

In the original submission, we had provided **a)** qualitative analysis of immune infiltration at 8 and 31 weeks of age by H&E staining (formerly Fig 2E), **b)** $CD3^+$ lymphocyte infiltration by immunofluorescence

(IF) at 8 and 31 weeks of age (formerly Fig 2F), **c)** quantification of the extent of islet inflammation (insulitis) by performing "insulitis scoring" on H&E-stained step sections at 8 weeks of age (formerly Fig 2G, H) and 31 weeks of age (formerly Fig 2I,J), and **d)** the beta cell antigen expression profile by scRNA-seq (Fig 3F**). These data suggested that reduced islet infiltration and inflammation phenotypes were shared by IRE1 and XBP1 knockout animals**.

In response to the reviewer's request, we expanded these analyses.
a) We examined the distributions and proportions of immune cell populations involved in T1D pathogenesis within the pancreas, spleen, and pancreatic lymph nodes (PLNs) of 21-week-old control and knockout mice. Specifically, we analyzed T cells ($CD4^+$ and $CD8^+$), Tregs ($CD25^+$, FoxP3), macrophages (F4/80), B cells ($CD19^+$), and dendritic cells ($CD11c^+$). Our analysis showed significantly higher the percentage of $CD19^+$ B cells, and markedly reduced percentages of $CD4^+$ and $CD8^+$ T cells in the pancreata of $XBP1^{\beta-/-}$ mice (**Fig. 2M**). The percentage of T regulatory cells (Tregs), macrophages, or dendritic cells did not show significant alterations in the pancreas of knockout animals (**Fig. 2M**). There were no differences in any of these immune cell populations within the spleens of $Xbp1^{\beta-/-}$ mice compared to control animals (**Fig. 2N**), while a moderate reduction in $CD8^+$ T cells and a marked increase in $F4/80^+$ cells were detected in PLN of knockout animals (**Fig. 2O**). Hence, deletion of *Xbp1* in β-cells in NOD mice significantly reduced the relative representation of $CD4^+$ and $CD8^+$ T cells specifically in the pancreas. This phenotype was reminiscent of $IRE1\alpha^{\beta-/-}$ mice where the percentage of $CD4^+$ T cells showed a trend towards declining, and the percentage of $CD8^+$ T cells was significantly diminished. However, increased percentage of $CD19^+$ B cell phenotype as well as alterations in representation of $CD8^+$ T cells and $F4/80^+$ cells in PLN were unique to $XBP1^{\beta-/-}$ mice. Taken together, these data suggest that significantly decreased islet infiltration by immune cells particularly reduced frequency of islet-reactive $CD4^+$ and $CD8^+$ T cells contributed to the β-cell survival in $Xbp1^{\beta-/-}$ mice. We added this to the text.

b) We expanded our IF from CD3 to CD4, CD8 T cells, and B cells both at 8 and 31 weeks of age (revised Figures 2H and 2L).

As for swapped adoptive transfer of T-cells experiments, since both IRE1 and XBP1 knockout already show diabetes protection phenotype, with their existing T cells, we think the experimental design would present an inherent limitation to see the effects of one mutant T cell's impact on the overall phenotype of the other mutant.

5. How important is the transient hyperglycemia in XBP1 mutant NOD mice to the later protection against diabetes? If one was to reduce hyperglycemia early in life by insulin therapy or SGLT inhibition, would XBP1 deficient mice still be protected from T1D?

We would like to clarify that based on our published and current work; we do not think that transient hyperglycemia alone is sufficient to cause protection against T1D. Instead, in two models we show that reduced insulin along with diminished expression of multiple autoantigens' expression (owing to beta cell dedifferentiation), altered antigen presentation machinery (scRNA-seq), cytokine/chemokine and immunoregulatory gene expression and reduced immune responses (potentially due to altered secretome or surface marker expression of key receptors/ligands in beta cells) are collectively behind the diabetes protective phenotype. Ou previous study showing dedifferentiation as an adaptive response in beta cells further supports this (PMID: 35941159). Indeed, our gene regulatory network analyses demonstrates the complexity of the phenotype in these two models. Based on this, we think the outcome of insulin therapy of XBP1 deficient mice during hyperglycemia would depend on its impact on the beta cell dedifferentiation, antigen presentation, secretome, and beta cell stress.