



Dual role of neuroplastin in pancreatic β cells: Regulating insulin secretion and promoting islet inflammation

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Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an endoplasmic reticulum (ER)-resident secretory protein that reduces inflammation and promotes proliferation in pancreatic β cells. Numerous studies have highlighted the potential of MANF as a therapeutic agent for diabetes mellitus (DM), making it essential to understand the mechanisms underlying MANF's functions. In our previous search for a molecule that mediates MANF signaling, we identified Neuroplastin (NPTN) as a binding partner of MANF that localizes on the cell surface. However, the roles of NPTN in pancreatic β cells remain unclear. In this study, we generated β cell-specific *Nptn* knockout (KO) mice and conducted metabolic characterization. NPTN deficiency improved glucose tolerance by increasing insulin secretion and β cell mass in the pancreas. Moreover, proliferation and mitochondrial numbers in β cells increased in *Nptn* KO islets. These phenotypes resulted from elevated cytosolic Ca^{2+} levels and subsequent activation of downstream molecules. Simultaneously, we demonstrated that NPTN induces the expression of proinflammatory cytokines via the TRAF6–NF- κ B axis in β cells. Additionally, NPTN deficiency conferred resistance to streptozotocin-induced diabetic phenotypes. Finally, exogenous MANF treatment in islets or β cells led to similar phenotypes as those observed in NPTN-deficient models. These results indicate that NPTN plays important roles in the regulation of insulin secretion, proliferation, and mitochondrial quantity, as well as proinflammatory responses, which are antagonized by MANF treatment. Thus, targeting the MANF–NPTN interaction may lead to a novel treatment for improving β cell functions in DM.

β cell | diabetes | endoplasmic reticulum

Pancreatic β cells are uniquely responsible for insulin production and secretion to counteract elevated blood glucose levels and are susceptible to metabolic and cytotoxic assaults. β cell dysfunction and death ultimately lead to diabetes mellitus (DM), a set of metabolic disorders characterized by dysregulated glucose homeostasis. In the etiology of type 1 diabetes mellitus (T1D), β cells are selectively targeted by autoimmunity, resulting in islet inflammation and reduced insulin production and secretion. Additionally, endoplasmic reticulum (ER) stress occurs in β cells during T1D pathogenesis, further accelerating dysfunction and apoptosis of these cells (1, 2).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an ER-resident small secretory protein that is emerging as a beneficial molecule against the pathophysiology of various diseases related to inflammation and ER stress. This includes genetic disorders such as Wolfram syndrome (3–5). Under normal conditions, MANF is retained within the ER by binding to the ER-resident molecular chaperone, BiP (6, 7). Inflammatory stimuli and ER stress cause the release of MANF from BiP, resulting in the secretion of a certain amount of MANF proteins into extracellular spaces (7–9). Simultaneously, MANF expression is also induced by unfolded protein response (UPR) signals (10–12). Accumulating evidence suggests that both ER-resident and extracellular MANF mitigate inflammation and ER stress in a variety of tissues, thereby protecting these tissues from the development and progression of disease conditions (3, 4). Notably, whole-body and pancreatic β cell-specific *Manf* knockout (KO) mice exhibited increased ER stress and β cell death, leading to the spontaneous development of diabetes (13, 14). On the other hand, local overexpression of *Manf* in pancreatic β cells inhibits or reverses diabetic phenotypes in either streptozotocin (STZ)-injected rodent models or genetic disorder models (13). In addition to its cytoprotective effects, MANF promotes the proliferation of β cells (5, 12–14). Moreover, it has been reported that the level of circulating MANF in the blood is elevated in patients with both T1D and type 2 diabetes (T2D) (15, 16). These experimental and clinical considerations suggest that MANF is a highly attractive molecule with potential for therapeutic applications.

Significance

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an endoplasmic reticulum (ER)-resident secretory protein that has the potential as a therapeutic agent for various diseases related to inflammation and ER stress, such as Type 1 diabetes mellitus. Our work shed light on the roles of a binding partner protein of MANF, Neuroplastin (NPTN), in pancreatic β cells. We demonstrated NPTN regulates Ca^{2+} dynamics and inflammation in pancreatic β cells. NPTN deficiency caused improved insulin secretion as well as the resistance to Type 1 diabetic phenotypes. We also found out that MANF treatment leads to similar phenotypes observed in NPTN deficient models through antagonizing NPTN's functions. Overall, our results provide an insight into treatment for improving β cell functions in diabetes mellitus.

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Competing interest statement: F.U. is a Founder and President of CURE4WOLFRAM, Inc., and a Chair of the Scientific Advisory Board for Emerald Biotherapeutics, Inc. F.U. is an inventor of three patents related to the treatment of Wolfram Syndrome, US 9,891,231 Soluble Manf in Pancreatic Beta Cell Disorders and US 10,441,574 and US 10,695,324 Treatment for Wolfram Syndrome and Other ER Stress Disorders.

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In the context of the molecular mechanism by which MANF exerts its cytoprotective effects, some studies have demonstrated that ER-resident MANF prevents the oligomerization of ER stress transducers, such as IRE1, which would otherwise provoke an apoptotic signal (17, 18). Another study suggested that MANF contributes to protein folding by functioning as a nucleotide exchange inhibitor for BiP (18). As for secreted MANF, a study focusing on the N-terminal saposin-like domain of MANF, which has a high affinity for lipid sulfatide present in outer-cell membrane leaflets, indicated that MANF is taken up through endocytosis and subsequently transported into the ER lumen (19). Additionally, it has been shown that treatment with recombinant MANF proteins inhibits the activation of NF- κ B, a major transcription factor of inflammatory genes (12). However, the precise mechanism, specifically how extracellular MANF modulates NF- κ B signaling within cells, remains unclear.

In pursuit of a molecule that mediates the extracellular signal of MANF into the cellular signal, we previously identified a binding partner of MANF on the cell surface, Neuroplastin (NPTN), using a proteomics strategy (20). Our previous *in vitro* studies demonstrated that NPTN induces the expression of proinflammatory cytokine genes by activating NF- κ B signaling, which is antagonized by a direct binding of MANF to NPTN (20). NPTN is an immunoglobulin superfamily glycoprotein, with two isoforms, Np65 and Np55 (21, 22). Np65 is specifically expressed in neurons of the brain, particularly the forebrain, and plays important roles in neuronal functions (23–25). On the other hand, Np55 is more ubiquitously expressed compared to Np65 (23–25). Given that MANF is indispensable for pancreatic β cell survival, NPTN is likely to be an important molecule not only in neurons but also in other types of cells in peripheral tissues, especially pancreatic β cells. However, the role of NPTN in pancreatic β cells remains unclear. Here, we generate pancreatic β cell-specific *Nptn* KO mice and *Nptn* KO Rat insulinoma cell line INS-1 832/13 to investigate the roles of NPTN in pancreatic β cells. We identify NPTN as an essential molecule for Ca^{2+} homeostasis, which leads to proper insulin secretion and proliferation of β cells. Furthermore, we demonstrate that NPTN contributes to the development of STZ-induced diabetic phenotypes, and that MANF antagonizes these NPTN roles.

Results

NPTN Deficiency Improves Glucose Tolerance by Increasing Glucose-Stimulated Insulin Secretion, Proliferation, and Mitochondrial Quantity in β Cells. To investigate the role of NPTN in pancreatic β cells, we generated pancreatic β cell-specific *Nptn* KO mice by breeding *Nptn*^{lox/lox} mice with *Ins1*^{Cre} mice (*Nptn*^{fl/fl}; *Ins1*^{Cre}) (26) (*SI Appendix, Fig. S1A*). We confirmed the robust reduction of NPTN expression in islets isolated from *Nptn*^{fl/fl}; *Ins1*^{Cre} mice (*SI Appendix, Fig. S1B*). Immunofluorescence revealed that NPTN is expressed in insulin-positive β cells as well as glucagon-positive α cells (Fig. 1A) and only the NPTN signals of β cells were depleted in *Nptn*^{fl/fl}; *Ins1*^{Cre} pancreas sections (Fig. 1A). We first identified metabolic phenotypes in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice. An intraperitoneal glucose tolerance test (IP-GTT) demonstrated that *Nptn*^{fl/fl}; *Ins1*^{Cre} mice exhibit improved glucose tolerance in both males and females at 7 wk old (Fig. 1B and *SI Appendix, Fig. S2A*). On the other hand, intraperitoneal insulin tolerance test (IP-ITT) curves were similar regardless of genotype (Fig. 1C and *SI Appendix, Fig. S2B*). IP-GTT curves of *Nptn*^{lox/lox} and *Ins1*^{Cre} mice showed no significant difference whereas *Nptn*^{fl/fl}; *Ins1*^{Cre} mice showed improved glucose tolerance as compared to the other two lines of mice, suggesting that our observation is specifically due to

Nptn KO, not *Cre* expression, in β cells (*SI Appendix, Fig. S2 C and D*). Elevation of serum insulin levels following glucose injection was higher in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice as compared to *Nptn*^{fl/fl} mice (Fig. 1D and *SI Appendix, Fig. S2E*). Basal serum insulin level was also increased in the male *Nptn*^{fl/fl}; *Ins1*^{Cre} (Fig. 1D). Static glucose-stimulated insulin secretion (GSIS) assay showed that the functional capacity of islets isolated from *Nptn*^{fl/fl}; *Ins1*^{Cre} mice was improved as compared to *Nptn*^{fl/fl} islets (Fig. 1E), whereas insulin content was similar (Fig. 1F). However, insulin content in the whole pancreas was increased in both male and female *Nptn*^{fl/fl}; *Ins1*^{Cre} mice (Fig. 1G and *SI Appendix, Fig. S2F*). Additionally, insulin-positive β cell mass was increased in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice (Fig. 1H and *SI Appendix, Fig. S2G*). These results suggested that NPTN deficiency increases GSIS as well as β cell mass of whole pancreas, which contributes to improved glucose tolerance in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice.

To better understand the impact of *Nptn* KO in β cells, we next performed bulk RNA-sequencing (RNA-seq) with islets isolated from *Nptn*^{fl/fl} and *Nptn*^{fl/fl}; *Ins1*^{Cre} mice at 7 wk old. Enrichment analyses revealed the gene sets of cell cycle were significantly up-regulated in *Nptn*^{fl/fl}; *Ins1*^{Cre} islets (Fig. 2A). Interestingly, the gene sets pertaining metabolisms in mitochondria were also up-regulated in *Nptn*^{fl/fl}; *Ins1*^{Cre} islets (Fig. 2A, *SI Appendix, Fig. S3*, and *Dataset S1 A–C*). In addition, weighted gene correlation network analysis (WGCNA) (27) showed higher expression of gene set related to citrate cycle (TCA cycle) in *Nptn*^{fl/fl}; *Ins1*^{Cre} islets (*SI Appendix, Fig. S3* and *Dataset S1D*). These findings led us to hypothesize that NPTN deficiency increases proliferation and mitochondrial function or numbers in β cells. As predicted, the percentage of the insulin-positive cells expressing a proliferation marker, Ki67, was higher in *Nptn*^{fl/fl}; *Ins1*^{Cre} pancreatic sections (Fig. 2B). It is known that the proliferation of rodent and human β cells is induced by high glucose stimulation (28–33). Consistent with the previous studies, our EdU cell proliferation assay using dispersed islet cells revealed that β cell proliferation was induced by high glucose stimulation (Fig. 2C). However, the magnitude of induction was greater in *Nptn*^{fl/fl}; *Ins1*^{Cre} β cells (Fig. 2C), indicating that NPTN deficiency leads to increased β cell proliferation. In addition, transmission electron microscopic (TEM) analyses showed that mitochondria were of greater abundance in *Nptn*^{fl/fl}; *Ins1*^{Cre} β cells as compared to *Nptn*^{fl/fl} β cells (Fig. 2D and E). Interestingly, the mitochondria in *Nptn*^{fl/fl}; *Ins1*^{Cre} β cells displayed significantly shorter and more circular morphologies, as indicated by lower aspect ratios (Fig. 2F), shorter Feret's diameters (Fig. 2G), and circularity values being closer to 1 (Fig. 2H), compared to that in *Nptn*^{fl/fl} β cells. These results imply that mitochondrial biogenesis or fission could be increased in *Nptn*^{fl/fl}; *Ins1*^{Cre} β cells. Similarly, mitochondrial DNA copy number, which reflects an abundance of mitochondria and β cell functional capacity (34–36), was increased in *Nptn*^{fl/fl}; *Ins1*^{Cre} islets (Fig. 2I). On the other hand, the expressions of β cell key genes in islets were similar regardless of genotype (*SI Appendix, Fig. S4*). In summary, NPTN deficiency in β cells causes increases of cell proliferation and mitochondria number, leading to the improved metabolic output.

NPTN Is Required for Cytosolic Ca^{2+} Homeostasis by Regulating PMCA2 Protein Levels at the Cell Surface. To investigate the factor causing the phenotypes identified in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice, we focused on cytosolic Ca^{2+} level. In pancreatic β cells, it is well known that an elevation of cytosolic Ca^{2+} level in response to glucose stimulation is crucial for insulin secretion as well as β cell proliferation (28, 31–33, 37–40). In addition, several studies demonstrated that mitochondria biogenesis and dynamics are induced by molecules functioning downstream of elevated cytosolic

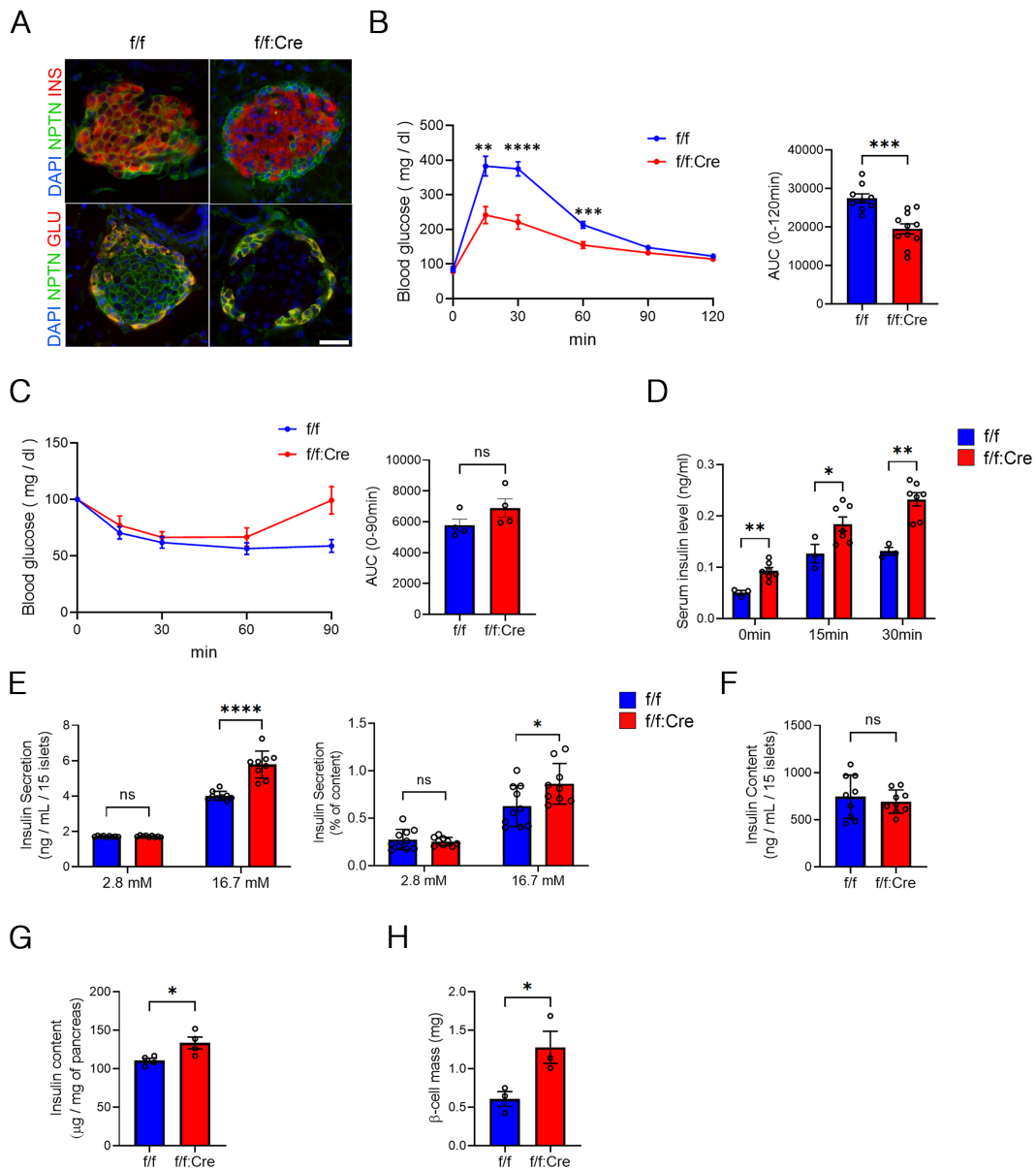


Fig. 1. NPTN deficiency increases GSIS and β cell mass. (A) Representative immunofluorescence images of NPTN, insulin, and glucagon in islets from *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* mice. (Scale bar, 25 μ m.) (B) (Left) IP-GTT with *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* male mice. (Right) AUCs of the IP-GTT (*Nptn^{fl/fl}*: n = 9, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 11, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 by the unpaired *t*-test). (C) (Left) IP-ITT with *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* male mice. (Right) AUCs of the IP-ITT (*Nptn^{fl/fl}*: n = 4, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 4). (D) Serum insulin levels following glucose injection in *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* male mice (*Nptn^{fl/fl}*: n = 3, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 7, * P < 0.05, ** P < 0.01 by the unpaired *t*-test). (E) Static GSIS per islets (Left) and normalized to insulin content (Right) in primary islets from *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* mice (*Nptn^{fl/fl}*: n = 10, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 9, * P < 0.05 and **** P < 0.0001 by the unpaired *t*-test). (F) Insulin content in the islets used in (E). (G) Insulin content of the whole pancreas from *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* male mice (*Nptn^{fl/fl}*: n = 4, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 4, * P < 0.05 by the unpaired *t*-test). (H) β cell mass of the pancreas in *Nptn^{fl/fl}* or *Nptn^{fl/fl}; Ins1^{Cre}* male mice (*Nptn^{fl/fl}*: n = 3, *Nptn^{fl/fl}; Ins1^{Cre}*: n = 3, * P < 0.05 by the unpaired *t*-test). *f/f*: *Nptn^{fl/fl}*, *f/f; Cre*: *Nptn^{fl/fl}; Ins1^{Cre}*, ns: not statistically significant.

Ca²⁺ level (41–43). We, therefore, measured cytosolic Ca²⁺ levels in *Nptn^{fl/fl}* and *Nptn^{fl/fl}; Ins1^{Cre}* islet cells. *Nptn^{fl/fl}; Ins1^{Cre}* cells showed higher basal cytosolic Ca²⁺ levels as compared to *Nptn^{fl/fl}* cells (Fig. 3A and SI Appendix, Fig. S5A). Glucose-stimulated elevation of cytosolic Ca²⁺ levels was also higher in *Nptn^{fl/fl}; Ins1^{Cre}* cells (Fig. 3B and C and SI Appendix, Fig. S5B and C). Interestingly, NPTN has been reported to stabilize plasma membrane ATPases (PMCA) through protein–protein interaction, which plays an important role for regulating cytosolic Ca²⁺ homeostasis (44–46). Moreover, it has been demonstrated that heterozygous inactivation of PMCA isoform, PMCA2, causes phenotypes that are similar to those of *Nptn^{fl/fl}; Ins1^{Cre}* mice (47). We, therefore, hypothesized that PMCA2 protein instability by loss of protein–protein interaction with NPTN would result in the metabolic phenotypes observed in *Nptn^{fl/fl}; Ins1^{Cre}* mice. As expected, PMCA2 protein level was significantly

reduced in *Nptn^{fl/fl}; Ins1^{Cre}* islets (Fig. 3D), whereas *Pmca2* mRNA level was similar regardless of genotype (SI Appendix, Fig. S5D). We also confirmed the significant reduction of PMCA2 protein levels in *Nptn* KO INS-1 832/13 cells (SI Appendix, Fig. S5E). Similar to the previous studies (44–46), the interaction between NPTN and PMCA2 was demonstrated in INS-1 832/13 cells by immunoprecipitation (Fig. 3E). Nuclear factor of activated T cells 2 (NFATc2) is one of the key transcription factors regulating β cell proliferation (48–50). The nuclear translocation of NFATc2 is regulated by calcineurin-mediated dephosphorylation, which is activated by elevated cytosolic Ca²⁺ levels (51, 52). Consistent with our EdU cell proliferation assay, the expression of NFATc2 target gene related to β cell proliferation (49), *Ccne2*, was significantly up-regulated in response to high glucose stimulation in both *Nptn^{fl/fl}* and *Nptn^{fl/fl}; Ins1^{Cre}* islets (Fig. 3F). Interestingly, the expressions of

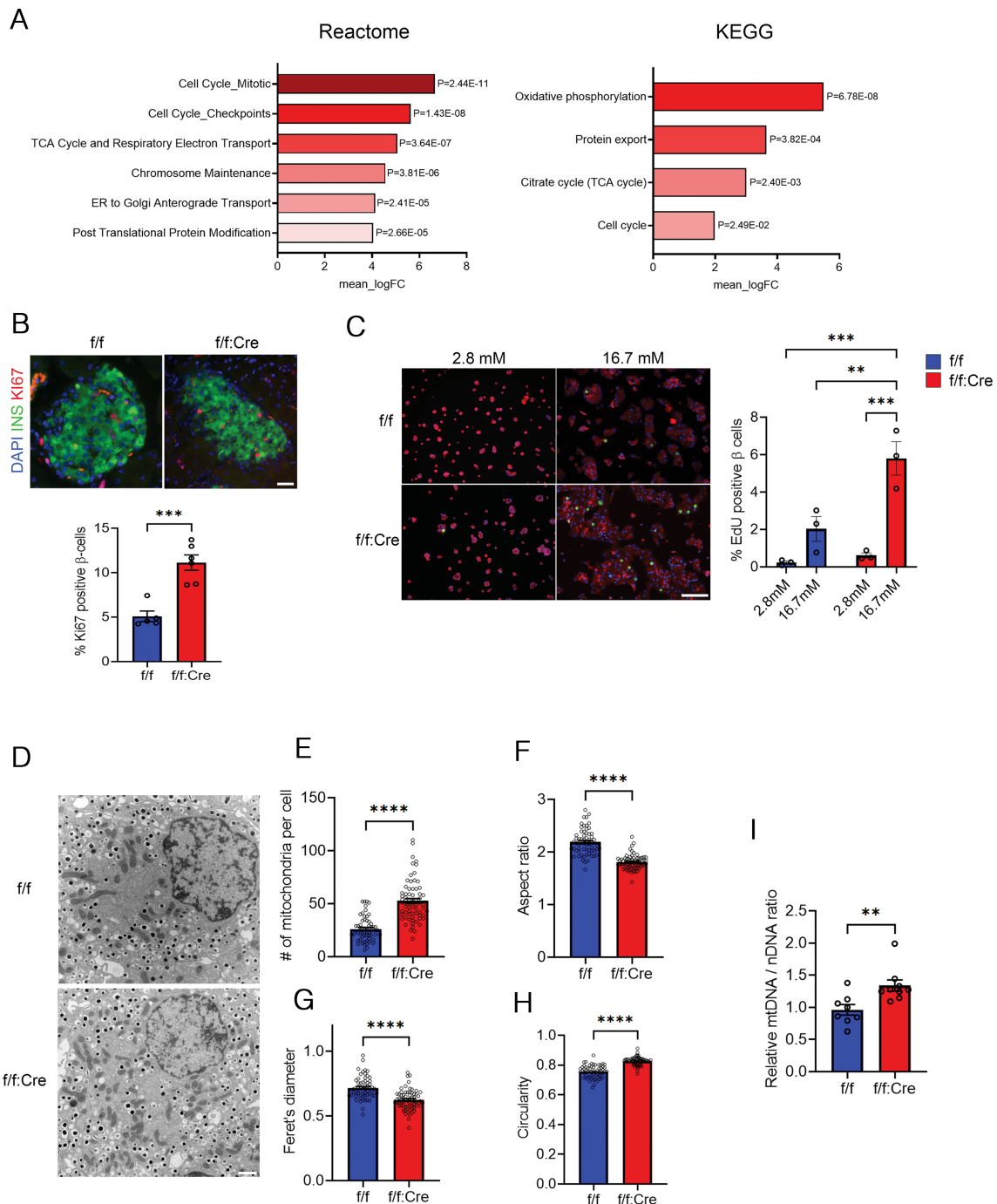


Fig. 2. NPTN deficiency increases proliferation and mitochondrial numbers in β cells. (A) Reactome and KEGG enrichment analyses for pathways up-regulated in *Nptn*^{f/f}; *Ins1*^{Cre} islets (*Nptn*^{f/f}; n = 4, *Nptn*^{f/f}; *Ins1*^{Cre}; n = 4). (B) (Upper) Representative immunofluorescence images of insulin and Ki67 in pancreas from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice. (Lower) A quantification of percentages of Ki67-positive β cells [*Nptn*^{f/f}; n = 5 (76 islets), *Nptn*^{f/f}; *Ins1*^{Cre}; n = 6 (74 islets), ****P* < 0.001 by the unpaired *t*-test]. (C) (Left) Representative immunofluorescence images of EdU and insulin in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} islet cells cultured in the medium containing 2.8 mM or 16.7 mM glucose for 3 d. (Right) A quantification of percentages of EdU-positive β cells (*Nptn*^{f/f}, 2.8 mM: n = 3, *Nptn*^{f/f}, 16.7 mM: n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, 2.8 mM: n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, 16.7 mM: n = 3, ***P* < 0.01 and ****P* < 0.001 by two-way ANOVA). (D) Representative EM images of β cells in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} islets. (E–H) A quantification of (E) mitochondrial number per cell (F) aspect ratio, (G) Feret's diameter, and (H) circularity (*Nptn*^{f/f}, n = 61 cells, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 68 cells from three mice for each genotype. *****P* < 0.0001 by the unpaired *t*-test). (I) Relative mitochondrial DNA (mtDNA) copy numbers normalized to nuclear DNA (nDNA) measured by qPCR analysis in islets from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice (*Nptn*^{f/f}, n = 8, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 9, ***P* < 0.01 by the unpaired *t*-test). f/f: *Nptn*^{f/f}, f/f:Cre: *Nptn*^{f/f}; *Ins1*^{Cre}, ns: not statistically significant.

other proliferation-related NFATc2 target genes, *Ccna2* and *Cdk2*, were also significantly increased in glucose-stimulated *Nptn*^{f/f}; *Ins1*^{Cre} islets (Fig. 3F). Moreover, the expression of *Pcna* was higher in *Nptn*^{f/f}; *Ins1*^{Cre} islets compared to *Nptn*^{f/f} islets (Fig. 3F). On the other hand, the glucose-stimulated upregulation of those genes in *Nptn*^{f/f}; *Ins1*^{Cre} islets were inhibited by treatment with a calcineurin inhibitor, FK506 (SI Appendix, Fig. S5F), suggesting that genes

involved in β cell proliferation are more transcriptionally induced by NFATc2 in *Nptn*^{f/f}; *Ins1*^{Cre} islets. Calcineurin also dephosphorylates Ser 637 of DRP1, which is an important event for an increase in the mitochondrial fission rate (42). Consistent with our TEM analysis, phosphorylation levels of DRP1 Ser 637 were reduced in *Nptn*^{f/f}; *Ins1*^{Cre} islets (Fig. 3G). Taken together, NPTN deficiency leads to higher basal and more dynamic cytosolic Ca²⁺ levels due

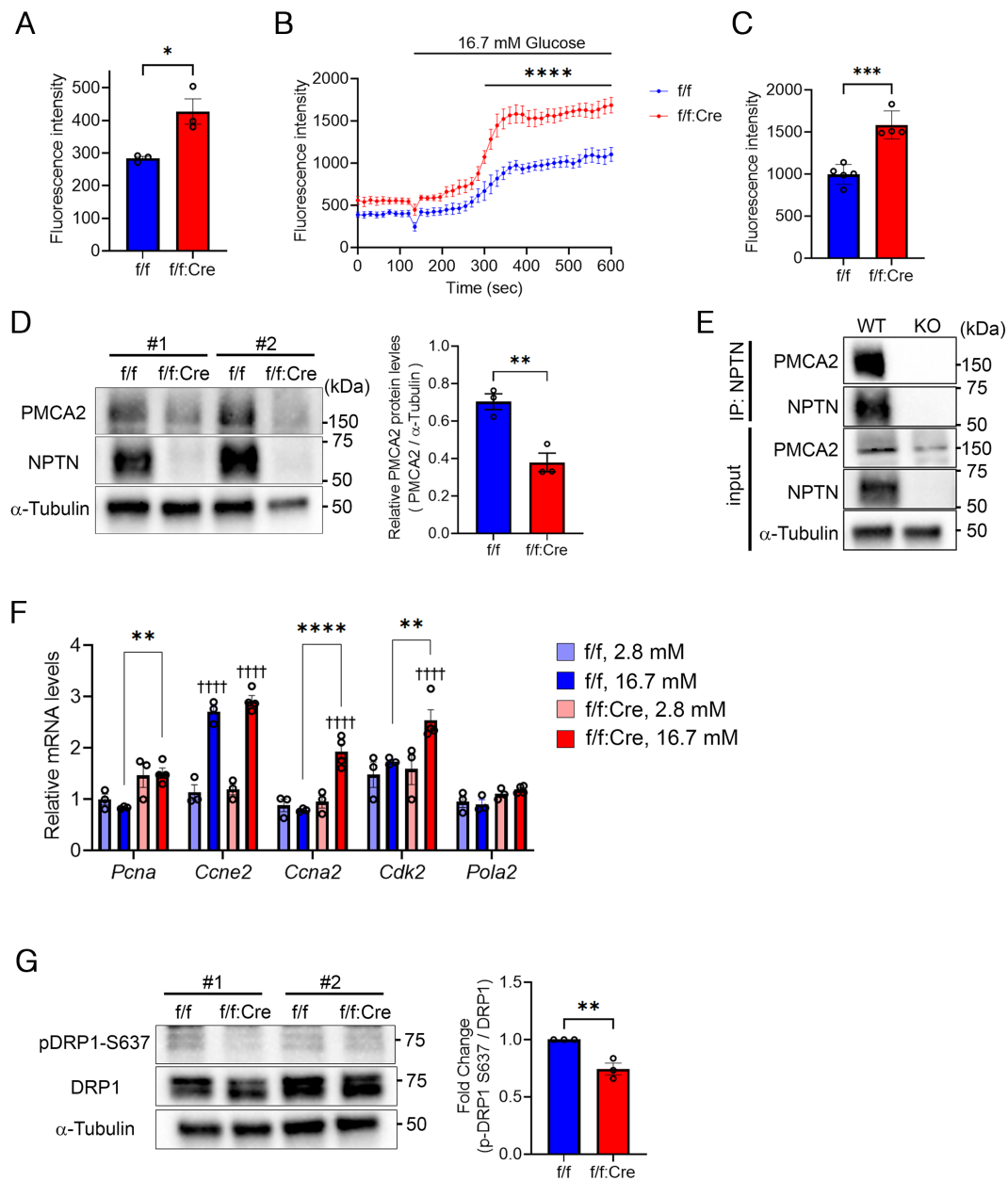


Fig. 3. NPTN stabilizes PMCA2 by the protein-protein interaction to regulate cytosolic Ca^{2+} homeostasis in β cells. (A–C) Cytosolic Ca^{2+} measurement with a calcium dye, Calbryte 520 AM. (A) Basal cytosolic Ca^{2+} levels in islet cells from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice (*Nptn*^{f/f}, n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 3. **P* < 0.05 by the unpaired *t*-test). (B) Dynamic cytosolic Ca^{2+} levels in response to glucose stimulation in islet cells from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice (*Nptn*^{f/f}, n = 5, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 4. *****P* < 0.0001 by two-way ANOVA). (C) Cytosolic Ca^{2+} levels in high glucose-treated islet cells from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice (*Nptn*^{f/f}, n = 5, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 4. ****P* < 0.001 by the unpaired *t*-test). (D) (Left) Representative blotting images of PMCA2, NPTN, and α -Tubulin in islets from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice. (Right) A quantification of PMCA2 protein levels normalized with α -Tubulin (*Nptn*^{f/f}, n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 3, ***P* < 0.01 by the unpaired *t*-test). (E) Representative blotting images of PMCA2, NPTN, and α -Tubulin in IP and input samples using *Nptn* WT or KO INS-1 832/13 cells. (F) qPCR analysis of NFATc2 target genes related to proliferation in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} islets cultured in the medium containing 2.8 mM or 16.7 mM glucose for 3 d. (*Nptn*^{f/f}, 2.8 mM: n = 3, *Nptn*^{f/f}, 16.7 mM: n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, 2.8 mM: n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, 16.7 mM: n = 4, *****P* < 0.0001 by two-way ANOVA compared to 2.8 mM for each genotype, ***P* < 0.01 and *****P* < 0.0001 by two-way ANOVA). (G) (Left) Representative blotting images of pDRP1-S637, DRP1, and α -Tubulin in islets from *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice. (Right) A quantification pDRP1-S637 protein levels normalized with DRP1 (*Nptn*^{f/f}, n = 3, *Nptn*^{f/f}; *Ins1*^{Cre}, n = 3, ***P* < 0.01 by the unpaired *t*-test). f/f: *Nptn*^{f/f}, f/f:Cre: *Nptn*^{f/f}; *Ins1*^{Cre}, ns: not statistically significant.

to reduced PMCA2 proteins at plasma membrane. Also, increased cytosolic Ca^{2+} promotes calcineurin-mediated dephosphorylation of NFATc2 and DRP1 Ser 637 contributing to increases in β cell proliferation and mitochondrial fission, respectively.

NPTN Transmits Proinflammatory Signal Via TRAF6-NF- κ B Axis. We and the other research groups have previously reported that NPTN is involved in proinflammation by activating NF- κ B signaling (20, 53). RNA-seq analysis with *Nptn*^{f/f}; *Ins1*^{Cre} islets showed lower expression of Reactome and KEGG pathways

pertaining to several inflammatory signals (Fig. 4A and Dataset S1 A and B). Overexpression of *Nptn* in INS-1 832/13 cells significantly increased mRNA levels of proinflammatory cytokine and chemokine genes, which are NF- κ B targets (54, 55) (Fig. 4B). NF- κ B signaling is activated by numerous discrete stimuli such as IL-1 β and IFN γ that contribute to β cell failure in T1D (56, 57). Appropriately, NF- κ B signaling was significantly activated after treating WT and *Nptn* KO INS-1 832/13 cells with cytokine mix (5 ng/mL IL-1 β + 100 ng/mL IFN γ), as indicated by the rapid degradation of I κ B α at 30 min after the treatment (58) (Fig. 4C).

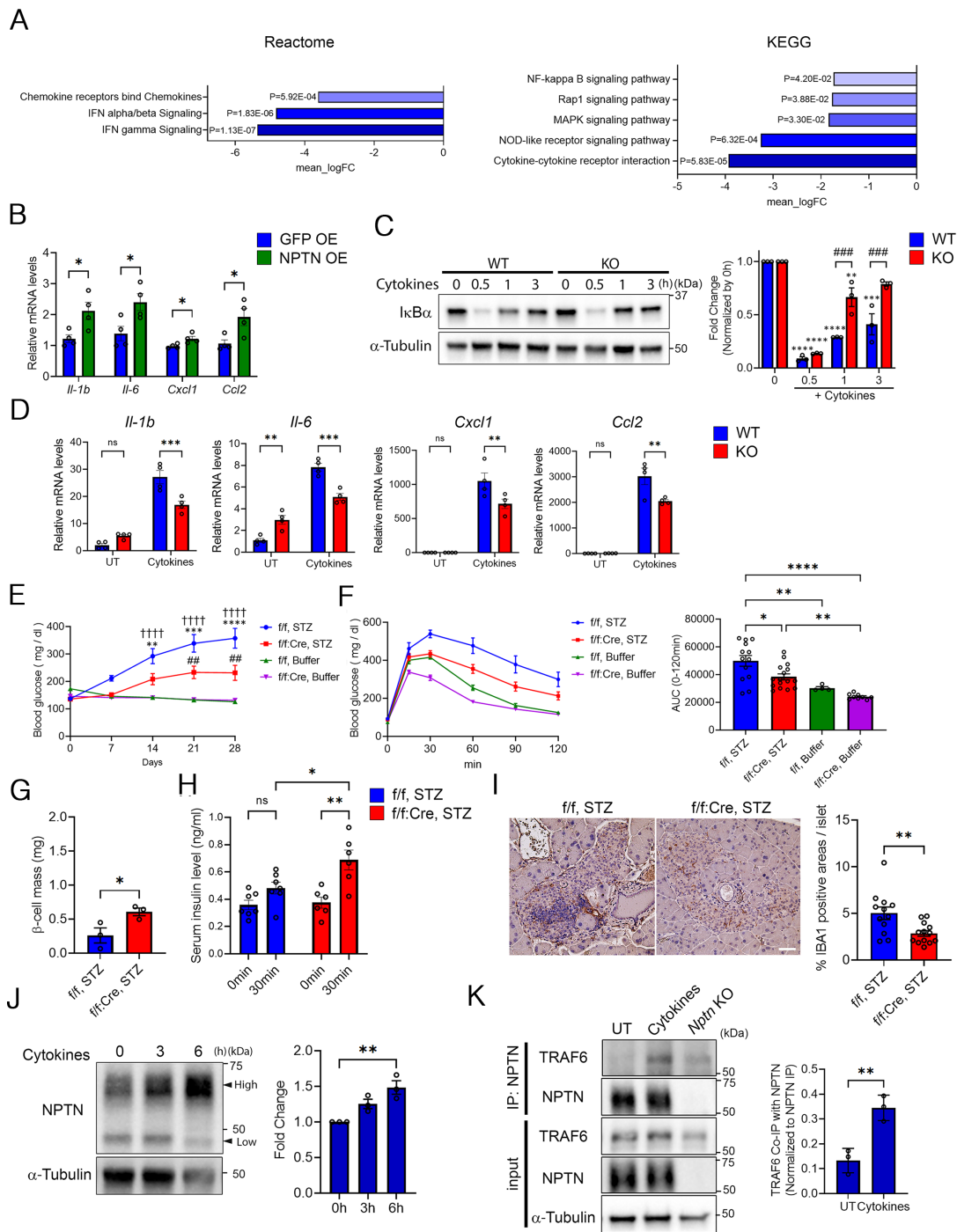


Fig. 4. NPTN deficiency inhibits islet inflammation induced by cytokines via TRAF6-NFκB axis. (A) Reactome and KEGG enrichment analyses for pathways down-regulated in *Nptn*^{f/f}; *Ins1*^{Cre} islets (*Nptn*^{f/f}; *n* = 4, *Nptn*^{f/f}; *Ins1*^{Cre}; *n* = 4). (B) qPCR analysis of NF-κB target genes related to proinflammatory cytokines in INS-1 832/13 cells with *gfp* or *Nptn* overexpression (*n* = 4, **P* < 0.05 by the unpaired *t*-test). (C) (Left) Representative blotting images of IκBα and α-Tubulin in *Nptn* WT or KO INS-1 832/13 cells treated with cytokine mix (5 ng/mL IL-1β + 100 ng/mL IFNγ) for indicated times. (Right) Fold changes of IκBα protein levels compared to 0 h. The protein levels were normalized to α-Tubulin (*n* = 3, ***P* < 0.01, and *****P* < 0.0001 by two-way ANOVA compared to 0 h for each genotype, ###*P* < 0.001 by two-way ANOVA). (D) qPCR analysis of NF-κB target genes related to proinflammatory cytokines in *Nptn* WT or KO INS-1 832/13 treated with or without cytokine mix for 6 h (*n* = 4, ***P* < 0.01 and ****P* < 0.001 by two-way ANOVA). (E) Nonfasting blood glucose levels in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice injected with STZ or buffer (*Nptn*^{f/f}; STZ, *n* = 16, *Nptn*^{f/f}; *Ins1*^{Cre}; STZ, *n* = 16, *Nptn*^{f/f}; Buffer, *n* = 7, *Nptn*^{f/f}; *Ins1*^{Cre}; Buffer, *n* = 10, ***P* < 0.01, *****P* < 0.0001, and *****P* < 0.0001 by two-way ANOVA compared to *Nptn*^{f/f}; Buffer. ###*P* < 0.01 by two-way ANOVA compared to *Nptn*^{f/f}; Buffer). (F) (Left) IP-GTT performed 25 d after the first injection with STZ or buffer. (Right) AUCs of the IP-GTT (*Nptn*^{f/f}; STZ, *n* = 13, *Nptn*^{f/f}; *Ins1*^{Cre}; STZ, *n* = 16, *Nptn*^{f/f}; Buffer, *n* = 4, *Nptn*^{f/f}; *Ins1*^{Cre}; Buffer, *n* = 9, **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001 by one-way ANOVA). (G) β cell mass of the pancreas in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice injected with STZ at 28 d after the first injection (*Nptn*^{f/f}; STZ, *n* = 3, *Nptn*^{f/f}; *Ins1*^{Cre}; STZ, *n* = 3, **P* < 0.05 by the unpaired *t*-test). (H) Serum insulin levels following glucose injection in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice injected with STZ at 28 d after the first injection (*Nptn*^{f/f}; STZ, *n* = 7, *Nptn*^{f/f}; *Ins1*^{Cre}; STZ, *n* = 6, **P* < 0.05 and ***P* < 0.01 by two-way ANOVA). (I) (Left) Representative immunohistochemistry images of the pancreas in *Nptn*^{f/f} or *Nptn*^{f/f}; *Ins1*^{Cre} mice injected with STZ at 28 d after the first injection. (Scale bar, 50 μm.) (Right) A quantification of IBA1-positive areas per islet (*Nptn*^{f/f}; *n* = 12 islets from three mice *Nptn*^{f/f}; *Ins1*^{Cre}, *n* = 13 islets from three mice, ***P* < 0.01 by the unpaired *t*-test). (J) (Left) Representative blotting images of NPTN and α-Tubulin in INS-1 832/13 cells treated with cytokine mix for indicated times. (Right) A quantification of NPTN protein levels at high MW normalized to α-Tubulin (*n* = 3, ***P* < 0.01 by one-way ANOVA). (K) Representative blotting images of TRAF6, NPTN, and α-Tubulin in IP and input samples using *Nptn* WT or KO INS-1 832/13 cells treated with or without cytokine mix for 6 h. (Right) A quantification of TRAF6 protein levels precipitated with NPTN. The protein levels were normalized to precipitated NPTN protein levels (*n* = 3, ***P* < 0.01 by the unpaired *t*-test). OE: overexpression, f/f: *Nptn*^{f/f}, f/f:Cre: *Nptn*^{f/f}; *Ins1*^{Cre}, ns: not statistically significant.

However, the recovery of I κ B α protein level was faster in *Nptn* KO INS-1 832/13 cells, suggesting that the NF- κ B signaling is attenuated more quickly in *Nptn* KO cells. Similarly, the transcriptional induction of the NF- κ B target genes by cytokine mix stimulation was inhibited in *Nptn* KO cells as compared to controls (Fig. 4D). These results suggested that NPTN contributes to proinflammation via NF- κ B signaling in pancreatic β cells. The crosstalk between β cells and immune cells in islets plays an important role in the progression of insulinitis and β cell failure (59, 60). Given that NPTN is involved in the inflammation of β cells, NPTN could be a substantial contributor of the crosstalk. To verify this idea, we investigated whether β cell-specific NPTN deficiency suppresses the development of diabetic phenotypes induced by multiple low dose-streptozotocin (MLD-STZ) injections, which is commonly used experimentally to produce a rodent model of T1D (61, 62). STZ injections increased nonfasting blood glucose levels over the weeks after the injections for the first 5 d in both *Nptn*^{fl/fl} and *Nptn*^{fl/fl}; *Ins1*^{Cre} mice (Fig. 4E). However, the rate of increase was lower in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice injected with STZ (Fig. 4E). *Nptn*^{fl/fl}; *Ins1*^{Cre} mice also showed resistance to the development of glucose intolerance (Fig. 4F). Moreover, β cell mass and serum insulin levels after glucose injection were higher in *Nptn*^{fl/fl}; *Ins1*^{Cre} mice injected with STZ as compared to controls (Fig. 4 G and H). Chemokines produced in β cells recruit circulating monocytes into islets, where they differentiate to macrophages (59, 60). Consistent with milder STZ-induced diabetic phenotypes of *Nptn*^{fl/fl}; *Ins1*^{Cre} mice, staining area of panmacrophage marker, IBA1, was reduced in pancreatic sections of *Nptn*^{fl/fl}; *Ins1*^{Cre} mice injected with STZ (Fig. 4I), indicating that NPTN contributes to islet inflammation and the development of insulinitis and diabetes.

Next, we investigated how NPTN mediates proinflammatory signaling via the NF- κ B pathway. Interestingly, western blot (WB) revealed an NPTN form of higher molecular weight (MW) which was elevated following cytokine mix stimulation, whereas the lower MW NPTN form was reduced (Fig. 4J and *SI Appendix*, Fig. S6A). NPTN protein is highly glycosylated, which occurs within ER and Golgi apparatus. WB using purified proteins existing at the plasma membrane showed only the high MW form, identifying as NPTN proteins at the plasma membrane (*SI Appendix*, Fig. S6B). On the other hand, *Nptn* mRNA levels were not altered by the cytokine mix stimulation (*SI Appendix*, Fig. S6C), which suggested that NPTN proteins are transported from ER to plasma membrane in response to cytokine stimuli. In the NF- κ B signal cascade, TNF receptor-associated factors (TRAFs), particularly TRAF2 and TRAF6, are pivotal mediators (63–65). Interestingly, a recent study has reported that the cytoplasmic region of NPTN contains TRAF6 binding motif, PxExxZ, and demonstrated an interaction upon overexpressing these proteins (66). Similarly, we also confirmed the interaction between endogenous NPTN and TRAF6 in INS-1 832/13 (Fig. 4K). This interaction was increased by cytokine mix treatment, indicating that TRAF6 transmits the proinflammatory signal from NPTN at the cell surface to the NF- κ B signaling cascade. The previous study reported S100A8 and A9 as ligands of NPTN leading to the activation of NF- κ B signaling (53). We then tested whether these ligands increase the expression of NF- κ B target genes. Transcriptional inductions of *Il-1 β* and *Il-6* were observed only in INS-1 832/13 cells treated with S100A9, but the magnitude was much smaller than that of cells treated with cytokine mix (*SI Appendix*, Fig. S6 D and E). These inductions were negated in *Nptn* KO INS-1 832/13 cells (*SI Appendix*, Fig. S6E). Taken together, NPTN is involved in proinflammation in β cells via TRAF6-mediated NF- κ B signal.

MANF Antagonizes Inflammation and Increases Insulin Secretion through NPTN. We previously demonstrated NPTN is a binding partner of MANF at the cell surface and is involved in the molecular mechanisms by which MANF inhibits inflammation (20). Therefore, we finally tested whether MANF treatment results in similar phenotypes to that observed in *Nptn* KO cells. Consistent with our previous study, cotreatment with MANF peptides inhibited the transcriptional induction of NF- κ B target genes induced by cytokine mix treatment in INS-1 832/13 cells (Fig. 5A), whereas the anti-inflammatory effect of MANF was negated in *Nptn* KO INS-1 832/13 cells (*SI Appendix*, Fig. S7A). Expectedly, the interaction between NPTN and TRAF6, which was increased by cytokine stimuli, was also reduced by MANF cotreatment (Fig. 5B). On the other hand, the NPTN protein levels in INS-1 832/13 cells treated with cytokine mix were similar regardless of MANF treatment (Fig. 5B, input), suggesting that MANF treatment does not affect the increase of NPTN proteins at the plasma membrane induced by cytokine mix stimulation. In addition to anti-inflammatory effect, we found NPTN deficiency increases GSIS in vivo and in vitro. Consecutive treatment with MANF proteins for 5 d significantly increased GSIS without affecting insulin content in the islets from *Nptn*^{fl/fl} mice (Fig. 5 C and D), whereas no increase was observed in *Nptn*^{fl/fl}; *Ins1*^{Cre} islets (*SI Appendix*, Fig. S7 B and C). In the characterization of *Nptn*^{fl/fl}; *Ins1*^{Cre} mice or islets, we concluded that improved metabolic phenotypes caused by NPTN deficiency are due to reduced PMCA2 protein levels, which alter basal and dynamic Ca²⁺ levels in β cells. Interestingly, MANF treatment for 5 d significantly reduced PMCA2 protein levels along with NPTN in the islets (Fig. 5E), suggesting that MANF treatment leads to a similar state to that of *Nptn* KO in islets. In summary, MANF reduces inflammation but also increases GSIS via NPTN in islets.

Discussion

To understand the role of NPTN in pancreatic β cells, we conducted metabolic characterization of β cell-specific *Nptn* KO mice and observed improved glucose tolerance as well as increased GSIS, proliferation, and mitochondrial number in β cells. NPTN stabilizes PMCA2 protein through protein–protein interaction, which is crucial for regulating cytosolic Ca²⁺ homeostasis. The improved metabolic phenotypes in β cell-specific *Nptn* KO mice are due to increased basal and dynamic Ca²⁺ levels caused by altered PMCA2 protein levels in β cells. In addition to β cell functions, NPTN is involved in proinflammation by activating the TRAF6–NF- κ B axis. The NPTN-mediated inflammation in β cells contributes to the development of STZ-induced diabetic phenotypes. We also demonstrated that the secretory protein MANF antagonizes these NPTN-mediated physiologies in β cells, and long-term MANF treatment affected both NPTN and PMCA2 protein levels.

NPTN is originally identified as a synaptic glycoprotein (23). Accordingly, most of earlier studies mainly focused on its physiological or disease-relevant roles in nervous systems. To date, it has been reported that NPTN has various roles in neurons such as synaptogenesis, neurite outgrowth, proper long-term synaptic plasticity, and transportation of lactate (22, 25, 45, 66–70). These roles are determined by the binding partner of NPTN and biochemical analyses suggested that NPTN proteins interact with themselves in the form of cis- or trans-homophilic binding and a variety of other proteins including PMCA2s, fibroblast growth factor receptors (FGFRs), GABA_A receptors, monocarboxylate transporter 2 (MCT2), and some cellular signal mediators such as TRAF2/6 and GRB2 (22, 25,

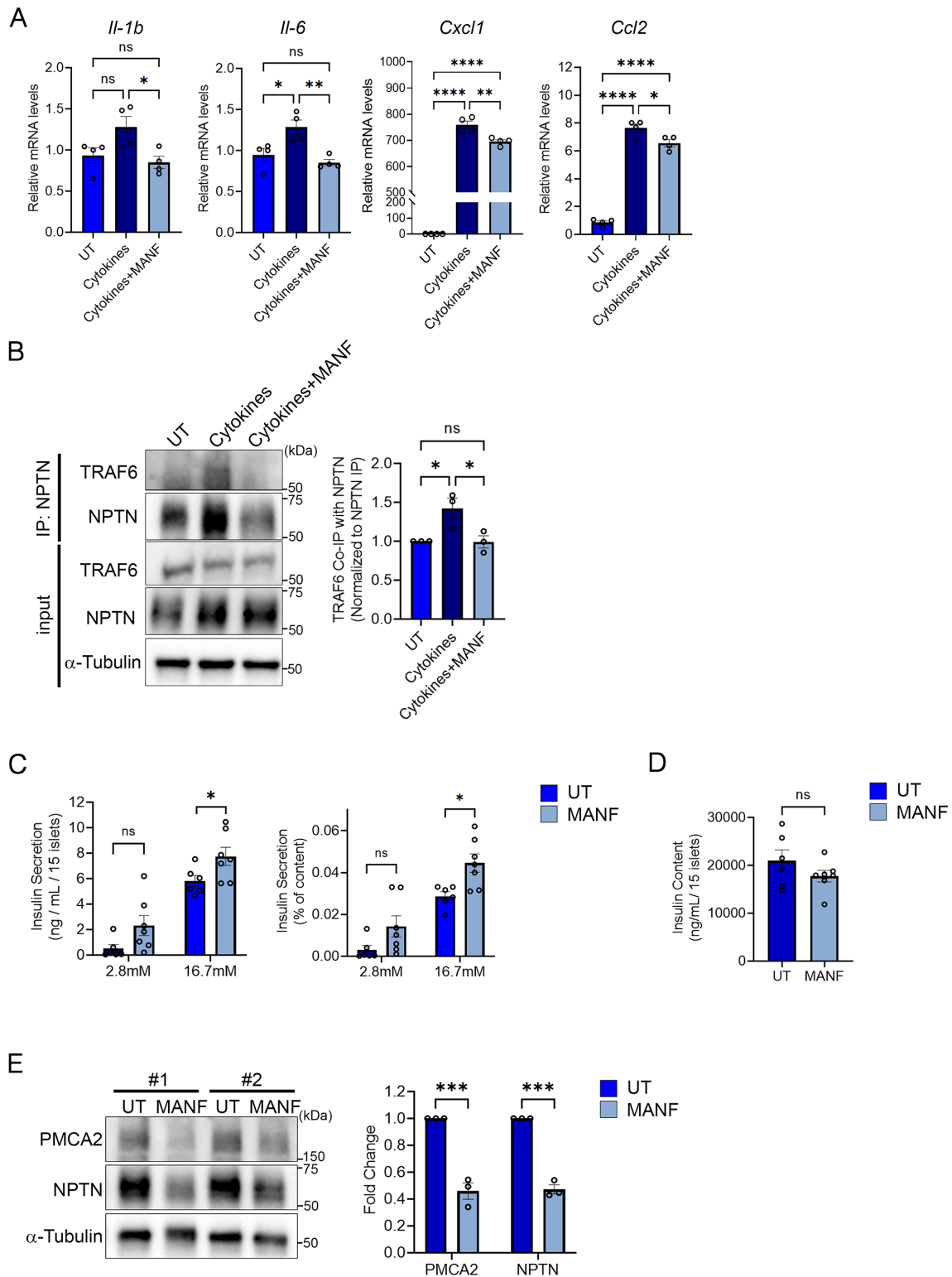


Fig. 5. MANF antagonizes NPTN roles in inflammation and insulin secretion. (A) qPCR analysis of NF- κ B target genes related to proinflammatory cytokines in *Nptn* WT INS-1 832/13 treated with or without cytokine mix (5 ng/mL IL-1 β + 100 ng/mL IFN γ) and MANF (5 μ g/mL) for 6 h (n = 4, * P < 0.05, ** P < 0.01, and **** P < 0.0001 by one-way ANOVA). (B) Representative blotting images of TRAF6, NPTN, and α -Tubulin in IP and input samples using *Nptn* WT INS-1 832/13 cells treated with or without cytokine mix and MANF proteins for 6 h. (Right) A quantification of TRAF6 protein levels precipitated with NPTN. The protein levels were normalized to precipitated NPTN protein levels (n = 3, * P < 0.05 by one-way ANOVA). (C) Static GSIS per islets (Left) and normalized to insulin content (Right) in primary islets from *Nptn*^{fl/fl} mice treated with or without MANF for 5 d (UT: n = 6, MANF: n = 7, * P < 0.05 by the unpaired *t*-test). (D) Insulin content in the islets used in (C). (E) (Left) Representative blotting images of PMCA2, NPTN, and α -Tubulin in islets from *Nptn*^{fl/fl} mice treated with or without MANF for 5 d (Right) A quantification of PMCA2 and NPTN protein levels normalized with α -Tubulin (*Nptn*^{fl/fl}, n = 3, *Nptn*^{fl/fl}; *Ins1*^{Cre}, n = 3, **** P < 0.001 by the unpaired *t*-test). UT: untreated, ns: not statistically significant.

44–46, 53, 66–70). Along with NPTN, some of these binding partners are expressed not only in nervous systems but also in peripheral tissues, implying that NPTN may have important roles

regulating physiological and pathophysiological functions in non-neuronal cells with the similar molecular mechanisms. Indeed, we demonstrated that NPTN is involved in regulating proper insulin

secretion, proliferation, and mitochondria number in β cells through the interaction with PMCA2 protein. Also, NPTN transmits inflammatory signals by the binding of TRAF6, which contributes to the development of diabetic phenotypes. Furthermore, other studies suggested that NPTN regulates T cell activation and proliferation of keratinocytes by interacting with PMCA2 and TRAF2, respectively (44, 53).

We demonstrated that NPTN interacts with PMCA2 and this interaction is important for the stabilization of PMCA2 protein and the functional capacities of β cells. Interestingly, heterozygous inactivation of PMCA2 in mice increases GSIS, proliferation, and mass in β cells (47). These phenotypes are also observed in β cell-specific *Nptn* KO mice, supporting our conclusion that the interaction between NPTN and PMCA2 proteins plays a pivotal role in regulating β cell physiology. It is well known that cytosolic Ca^{2+} is an important second messenger responsible for the transmission of physiological and pathophysiological signals modulating various cell processes. To more define NPTN roles, further investigations in the other Ca^{2+} sensitive tissues such as the muscle and kidney could be intriguing.

It was shown that NPTN recruits TRAF6 to provoke NF- κ B signaling in response to inflammatory stimuli. However, a ligand specific to NPTN has not been identified. According to genome-wide sequence searches, NPTN is most closely related to the other Ig superfamily member, CD147 (22). Interestingly, CD147 was identified as another component of a protein complex with NPTN and PMCA2, which is required for stabilization of PMCA2 protein (45). The role of CD147 in the inflammation was well studied and A100A8/9 and CyclophilinA are shown as ligands activating NF- κ B signaling via CD147 (71, 72). Sakaguchi et al. demonstrated that S100A8 and S100A9 are bound with Np65 and Np55, respectively, to induce the expression of NF- κ B target genes (53). We also confirmed an increase of cytokine gene expression in INS-1 832/13 cells treated with S100A9, whereas those expressions were similar regardless of the treatment in *Nptn* KO cells. However, the magnitude of transcriptional induction by S100A9 was much smaller than that induced by cytokine mix stimulation, indicating it is uncertain that S100A9 is a specific ligand for NPTN in β cells. As an alternative molecular mechanism by which NPTN elicits inflammatory signal, NPTN may form heterodimer/oligomeric receptor complex with cytokine receptors in a similar way to the case of PMCA2, which could modulate the inflammatory signals derived from these receptors. Meanwhile, we found an increase of NPTN proteins at the plasma membrane in response to cytokine mix stimulation. NPTN is also known as an adhesion molecule and is important for synaptogenesis (21, 22, 66). For decades, adhesion molecules have been suggested to be involved in inflammation (73, 74). Of note, recently it was reported that numbers of cell adhesion molecule 1 (CADM1)-positive islet endocrine and myeloid cells adjacent to CD8⁺ T cells, which are well known to destroy insulin-producing β cells (75), are increased in individuals with T1D (76). CADM-1 is an Ig domain-containing plasma membrane protein that mediates homotypic and heterotypic cell-to-cell contact (77, 78). Remarkably, the suppression of *Cadm1* promotes insulin secretion and β cell mass (79–81) similar to the phenotypes we observed in β cell-specific *Nptn* KO mice. β cell-specific KO mice are significantly resistant to development of STZ-induced diabetic phenotypes, therefore, NPTN could mediate cell–cell contact between islet cells and immune cells in addition to recruitment of immune cells into islets by increasing chemokine expression.

Several studies revealed effects of MANF on glucose homeostasis (4). In this study, we observed an increase of GSIS in primary islets treated with MANF proteins for 5 d, which was negated in

Nptn KO islets. However, our previous examination did not admit a significant effect of a short-term MANF treatment (24 h) on insulin secretion (5). MANF is known to promote β cell proliferation (13, 14), and it has been demonstrated that a consecutive treatment of whole islets with MANF proteins for 5 d enhances the proliferation rate of human and murine β cells (5, 14). Hence, it is suggested that our observation of increased GSIS is due to an increased β cell mass and MANF treatment for 24 h could be too short to increase enough number of β cells for improving GSIS. On the other hand, we confirmed anti-inflammatory effect in INS-1 832/13 cells treated with MANF proteins for 8 h. Given that MANF effects on GSIS and anti-inflammation were negated in *Nptn* KO islets/cells, both of the MANF effects are mediated via NPTN. However, the mechanisms how MANF suppresses NPTN roles in GSIS and inflammation could be different. Of note, islets treated with MANF proteins for 5 d exhibited decreased NPTN protein levels, whereas no change was observed in INS-1 832/13 cells treated for 8 hours. In addition to the signal transduction of MANF mediated by NPTN at plasma membrane, it is suggested that MANF is internalized through the N-terminal saposin-like domain and transferred into ER (19). Also, it is shown that MANF existing in ER stabilizes certain BiP-client complex, which may enhance the efficiency of client transfer to downstream ER quality control effectors including ER-associated degradation (ERAD) (18). Thus, as a putative mechanism, MANF might be involved in facilitating the handover of NPTN in ER to ERAD leading to the decrease in the protein levels of NPTN.

This study unveiled NPTN roles in β cells, which are important for mitigating insulin secretion and promoting islet inflammation. These roles are inhibited by MANF treatment, suggesting that MANF exerts insulin secretory and anti-inflammatory effects through binding to NPTN. Thus, inhibiting NPTN function or expression could be a good strategy to prevent or reverse β cell failure or loss in diabetes.

Methods

Animals and Cells. All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (20-0334). *Ins1^{Cre}* mice are obtained from Jackson Laboratory (RRID:IMSR_JAX:026801). *Ntpn^{fllox/fllox}* mice and *Nptn* KO INS-1 832/13 cells are generated by the Genome Engineering and iPSC Center (GEIC) at Washington University in St. Louis.

In Vivo Physiology and Pancreatic Insulin Content. IP-GTT, IP-ITT and in vivo GSIS test were performed according to standard procedures of the NIH-sponsored National Mouse Metabolic Phenotyping Centers (<http://www.mmpc.org>). To measure insulin content in whole pancreas, excised pancreata were weighed and homogenized in ice-cold acid ethanol (1.5% 12 N HCl in 70% ethanol). Homogenized pancreata were incubated at -20°C for 48 h. Pancreatic and serum insulin contents were measured by the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem; 90080). Seven-week-old mice were used in the analyses except for that with STZ-injected mice.

Islet Isolation and Dispersion. Murine islets were isolated from the mice at 7 to 12 wk old (7 wk old: RNA-seq and 8 to 12 wk old: the other analyses) as described previously (82). After isolation, islets were incubated overnight in RPMI 1640 medium (Gibco, 11875085) (11.1 mM glucose, 10% PBS, and Penicillin-Streptomycin (Gibco, 15140122) to allow them to recover from the digestion damages. For an analysis of NFATc2 target gene expression, the islets were cultured for 3 d in RPMI 1640 medium (2.8 mM or 16.7 mM glucose, 10% FBS, and Penicillin-Streptomycin) with or without 50 ng/mL FK506 (SIGMA, F4679). For MANF treatment experiments, the islets were cultured for 5 d in RPMI 1640 medium (11.1 mM glucose, 10% FBS, and Penicillin-Streptomycin) with or without 5 $\mu\text{g}/\text{mL}$ recombinant human MANF protein (R&D systems, 3748-MN). For EdU cell proliferation assay and Ca^{2+} measurement, 50 to 70 islets were collected

in a 1.5 mL tube for each well of 8-well chamber. The islets were incubated in 200 μ L Accutase solution (SIGMA, A6964) at 37 °C for 6 min after washing them with HBSS (Gibco, 14175145). The islets were dissociated by pipetting gently followed by adding 400 μ L RPMI 1640 medium. After centrifuge at 300 \times g, for 4 min, the pelleted islet cells were resuspended with RPMI 1640 medium and plated on a laminin-coated well of 8-well chamber.

Static GSIS Assay. 15 islets were equilibrated in Krebs-Ringer bicarbonate HEPES (KRBH) buffer (128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 20 mM HEPES, 5 mM NaHCO_3 , 0.1% BSA, pH7.4) at 2.8 mM glucose for 1 h at 37 °C. Then, the islets were incubated in new KRBH buffer at 2.8 mM glucose for 30 min followed by 11 mM glucose for 30 min. Supernatant was collected after the incubation in each solution. At the end of assay, the islets were lysed overnight in acid-ethanol. Secreted insulin and insulin content were measured by Ultra Sensitive Mouse Insulin ELISA Kit.

Immunofluorescence and Measurement of β Cell Mass. For β cell mass study, pancreata were weighed and fixed in 4% PFA overnight at 4 °C and paraffin-embedded for sectioning. Four groups of sections with 5 μ m thickness were made 75 μ m apart between groups. After rehydration and antigen retrieval in 10 mM sodium citrate buffer (pH 6.0), sections were permeabilized in 0.3% Triton-X prior to blocking in 2% BSA. Primary antibodies were treated overnight at 4 °C. Incubation with secondary antibodies and DAPI was for 1 h at RT. Slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen, P36970). For the other staining, pancreata were perfused with 4% PFA and fixed overnight at 4 °C. After replacement with 30% sucrose gradually, pancreata were frozen in Tissue-Tek® O.C.T. Compound (Sakura FineTek, 4583). Frozen pancreata were sectioned with 10 μ m thickness. The procedure after permeabilization was performed in the same way as insulin staining. β cell mass (mg per pancreas) was calculated by multiplying relative insulin-positive area (the percentage of insulin-positive area over total pancreas area) by pancreas weight. Seven-week-old mice were used in the analyses. All images analyses were performed with ImageJ. Antibody details can be found in [SI Appendix, Table S1](#).

EdU Cell Proliferation Assay. Dispersed islet cells were cultured in RPMI 1640 medium containing either 2.8 mM or 16.7 mM glucose supplied with 10% FBS and Penicillin-Streptomycin for 3 d. The half of medium was changed every 24 h. 10 μ M EdU was added in the culture medium for the last 24 h of stimulation. Incorporated EdU was detected by Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, C10337). To determine β cells, the cells were stained with insulin antibody and DAPI following EdU detection by a general immunofluorescence protocol. The areas for imaging were picked up randomly and the images were analyzed with ImageJ. Antibody details can be found in [SI Appendix, Table S1](#).

Ca²⁺ Measurement. Dispersed islet cells were loaded with 4 μ M Calbryte 520 AM (AAT Bioquest 20650) or 4 μ M Cal Red™ R525/650 AM (AAT Bioquest #20590) in loading solution (2 mM Glucose in KRBH buffer) at 37 °C for 1 h. After washing the cells with loading solution, 8-well chamber was inserted on the stage of the microscope with incubation system (37 °C, 5% CO₂). First, the basal cytosolic Ca²⁺ levels were measured every 15 s for 2 min. After the solution was changed to KRBH buffer containing 16.7 mM glucose, the dynamic changes of cytosolic Ca²⁺ levels were measured every 15 s up to 10 min in total. The fluorescent emission signals of Calbryte and Cal Red R525/650 were collected using the Zeiss LSM 880 Confocal Microscope equipped with a spectral detector, respectively. Images were analyzed with imageJ.

Cells and Cytokine Treatment. INS-1 832/13 cells were cultured in RPMI 1640 medium (11.1 mM glucose, 10% FBS, 1 mM sodium pyruvate (Gibco, 11360070), 2-mercaptoethanol (SIGMA, M6250), Penicillin-Streptomycin). *Nptn* WT and KO INS-1 832/13 cells were generated by GEIC at Washington University in St. Louis. For cytokine treatments, the medium was changed to serum-free RPMI 1640 medium 1 h prior to the treatment, cytokine treatment was performed with or without MANF by medium change. All recombinant proteins were obtained from R&D systems: IL-1 β (R&D systems, 501-RL), IFN γ (R&D systems, 585-IF), S100A8 (R&D systems, 9877-S8) and S100A9 (R&D systems, 2065-S9). Concentrations for each cytokine are specified in the figure legends.

Statistical Analysis. Statistical analysis was performed by unpaired and paired *t* tests and one- and two-way ANOVA with Tukey's or Dunnett's tests. Statistical tests are specified in figure legends. *P* < 0.05 was considered statistically significant. Data are shown as means \pm SEM unless otherwise noted.

Further methods details are available in [SI Appendix](#).

Data, Materials, and Software Availability. RNA-seq data has been deposited at GEO and is publicly available as of the date of publication. Accession numbers is [GSE272736](#) (83). All study data are included in the article and/or [supporting information](#).

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