Supporting Information

A distinct role of STING in regulating glucose homeostasis through insulin sensitivity and insulin secretion

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Supplemental Materials and Methods

Intraperitoneal Glucose and Insulin Tolerance Tests. The intraperitoneal glucose tolerance testing (IPGTT) was performed by intraperitoneally injection of 2 g/kg body weight of glucose after 6-hour fasting. Tail vein blood glucose was monitored at 0, 15, 30, 60, 90, and 120 min. Blood glucose levels were determined using an automatic glucometer (Roche, ACCU-CHEK, Germany) and glucose test results higher than 33.3 mmol/L were recorded as 33.3 mmol/L. The area under the curve (AUC) was calculated using the trapezoidal rule. At the same time, blood samples were collected at individual time points and serum insulin levels were determined with insulin ELISA test kits (EZassay, China) according to the manufacturer’s protocol. For intraperitoneal insulin tolerance test (IPITT), the mice were intraperitoneally injected with 0.75 U/kg body weight of insulin after a 4-hour fasting. All experiments described above were performed on age- and sex-matched cohorts.

Islet perifusion assays. For islet perifusion assays, islets were isolated and incubated overnight in RPMI medium containing 10% FBS, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37 °C in a 5% CO2 humidified environment. For each assay, 150 islet equivalents [IEQ] (approximately 100 size-matched islets) were used. Islets loaded in the perifusion chamber were first perifused with KREB’s buffer containing 2.8 mmol/L glucose for 30 min, followed by perifusion with KREB’s buffers containing 16.7 mmol/L glucose, and 2.8 mmol/L glucose. Flow rate of fluid input (2.8 mmol/L glucose and 16.8 mmol/L glucose in KREB’s buffer) was 1 ml/min and fractions were collected every minute. Insulin secretion at each time point was quantified using HTRF insulin assay kit (62INSPEC, Cisbio) according to manufacturer's instructions, and normalized to the insulin content.

Whole islet calcium imaging. After being washed twice with KRBH buffer plus 2 mmol/L glucose, islets were loaded with calcium indicator Fluo-4 AM (Thermo
Fisher Scientific, F14201) in KRBH buffer containing 5.6 µmol/L Fluo-4 AM, 0.4% Pluronic F127 (Sigma-Aldrich, P2443), and 2 mmol/L glucose for 45 min. Islets were then washed three times with KRBH buffer plus 2 mmol/L glucose, and seeded on 35 mm Glass-Bottom Dish (MatTek) for 15 min. The dish was placed into a 37 °C humidified cell culture chamber containing 5% CO₂ and mounted on a Zeiss LSM 800 inverted confocal microscope. A solid-state laser provided wavelengths of 488 nm to excite Fluo-4. Images were acquired every 10 seconds before and after adding glucose to the final concentration of 16.8 mmol/L to monitor the dynamic changes of intracellular calcium levels, and analyzed with Image J 1.52q software (NIH).

**Biochemical Assays.** Blood samples were obtained from mice, and serum was separated by centrifugation. Serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) were determined by assay kits (Nanjing Jiancheng Bioengineering Institute, China) using microplate reader (Thermo Scientific). Serum glucagon levels were determined using glucagon ELISA test kits (Ruixin Biotechnology Co., Ltd., China) according to the manufacturer’s protocol.

**Immunofluorescence and Immunohistochemical Staining.** Paraffin-embedded pancreases were cut into 5 µm sections. Immunohistochemical staining were performed using appropriate antibodies as previously described (25). For immunostaining, the sections were dewaxed and heat-based antigen retrieval in citrate buffer. After blocked with blocking solution for 1 h, the sections were incubated with anti-STING (Cat: 19851-1-AP, Proteintech, China), anti-insulin (homemade), anti-glucagon (Cat: G2654, Sigma, USA), anti-somatostatin (Cat: ab30788, abcam, USA), anti-Pax6 (Cat: 60433, Cell Signaling Technology, USA), anti-Pdx1 (Cat: 5679, Cell Signaling Technology, USA), anti-Isl1 (Cat: ab109517, Abcam, USA), and anti-Nkx6.1 (Cat: NBP1-82553, Novus, USA) as indicated overnight at 4 °C,
followed by secondary antibodies conjugated to appropriate Alexa Fluor. Fluorescent images were visualized using Axio Imager M2 (Carl Zeiss, Oberkochen, Germany).

**Transmission electron microscopy (TEM).** Fresh pancreases from 8-week-old mice were fixed with 2.5% glutaraldehyde. The pancreases were cut into pieces and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 16 h and subsequently treated with 1% osmium tetroxide at 4 °C for 2 h. The specimens were dehydrated in a graded ethanol series and then embedded in EMBed 812. Ultrathin sections were obtained using the ultra microtome (Leica EM UC7, Germany) and stained with 2% uranium acetate saturated alcohol solution and 2.6% Lead citrate and observed by TEM (HT7700, Japan).

**RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR (RT-QPCR).** Total RNA of islets was extracted using TRIzol reagent (Invitrogen, USA) and cDNA was synthesized using a PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, Japan, Cat: RR047A) following the manufacturer’s instructions. RT-QPCR was conducted with the TB Green Premix Ex TaqTM (Tli RNaseH Plus) (Takara, Japan, Cat: RR420A). GAPDH was used as standards. We normalized the data to Control = 1 for fold change which were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers were designed and chemically synthesized by TSINGKE Biological Technology (Beijing, China) Co., Ltd. The forward and reverse primer sequences used in this study were listed in supplemental Table S1.

**Protein Extraction and Western Blot Analysis.** Isolated islets were lysed in RIPA buffer. Equal amounts of protein samples were loaded on 4 - 12% NuPage gradient gels (Thermo Fisher Scientific), then proteins were transferred to nitrocellulose membrane (BioTrace NT nitrocellulose membrane, 66485, PALL, Mexico), which were blocked with 5% nonfat milk in Tris-buffered saline solution containing Tween-20 (Sigma-Aldrich) for 1 h at room temperature. After incubation with
appropriate primary antibodies and secondary antibodies, proteins were visualized using enhanced chemiluminescence (CLiNX, Shanghai, China).

**Cell culture and treatments.** The INS-1 cells (a rat insulinoma-cell line) were obtained from Zhongqiaoxinzhou Biotech Co. Ltd. (Shanghai, China), and cultured in Roswell Park Memorial Institute (RPMI, Hyclone, USA) 1640 medium containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, 100 U/mL streptomycin, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol (Gibco, USA) at 37°C in a 5% CO₂ humidified environment. The knocking down of STING expression of was achieved using specific siRNA (GenePhama, China) with Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) according to the manufacturer’s instructions. In addition, INS-1 cells were treated with Pyrrolidinedithiocarbamate ammonium (PDTC), as a NF-kB inhibitor (5μM, Cat: S3633 Selleck, USA) for 48 h. The MIN6 cells (a mouse insulinoma-cell line) were obtained from ATCC (USA), and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, USA) containing 15% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and streptomycin at 37°C in a 5% CO₂ humidified environment. MIN6 cells were treated with C-176, STING inhibitor (20μM, Cat: HY-112906, MedChemExpress, USA) for 5 h.

**siRNA transfection.** Rat STING siRNA duplexes were designed and synthesized by GenePharma (Shanghai, China). The sequences used were as follows: the sense and antisense sequences of STING siRNA were 5’-GCCUGAUCCUACUGUCAUTT-3’ and 5’-AUGACAGUAGGAUCAGGGCTT-3’. The sense and antisense sequences of negative control (NC) siRNA were 5’-UUCUCGAACGUGUCACGUTT-3’ and 5’-ACGUGACACGUUCGGAGAATT-3’. For STING silencing, the method as described earlier (25). In brief, the INS-1 cells were transfected in 12-well plates until the cells reached 70-90% confluence. Then, the medium was removed and replaced
with Opti-MEM Medium (Invitrogen, USA), in which cells were subjected to 90 pmol siRNA mixed with transfection reagent. After 6 h incubation, the supernatant was discarded and was replaced with normal medium. The efficiency of RNA knockdown was evaluated by RT-QPCR and western blotting. All RNAi experiments were repeated at least 3 times.

**Statistics.** All of the experiments above were performed independently at least three times. All data are presented as the means ± SEM. Graph-Pad Prism 8 software was used for all statistical analyses. We analyzed data using unpaired Student’s t test and used the traditional threshold $P < 0.05$ to declare statistical significance.
Supplemental Figure S1. Global deficiency of STING alleviated HFD-induced hyperlipidemia. Blood samples were used to measure the levels of lipids, including triglyceride (TG) (A), total cholesterol (TC) (B), Low-Density Lipoprotein Cholesterol (LDL-C) (C), and High-Density Lipoprotein Cholesterol (HDL-C) (D). n ≥ 5 mice/group. Values are shown as mean ± SEM. WT + ND vs WT + HFD, * P < 0.05, ** P < 0.01, WT + HFD vs STING−/− + HFD, # P < 0.05, ## P < 0.01, ns, not significant.
Supplemental Figure S2. The expression of insulin gene and amount of insulin content in islets of global STING knockout mice. (A) mRNA levels of Ins1 and Ins2 in islets from 16-week-old male mice fed with ND or HFD were measured by RT-QPCR. (B) Freshly isolated islets from 16-week-old male mice were directly lysed. Western blots were performed with anti-insulin antibody. (C) The quantification of insulin content from at least three independent experiments shown in Fig. S2B. The relative level of insulin content in islets of control mice fed with ND were set as 1. n ≥ 3 mice/group. Values were shown as the mean ± SEM. WT + ND vs WT + HFD, *** P < 0.001, WT + HFD vs STING−/− + HFD, # P < 0.05, ns, not significant.
Supplemental Figure S3. db/db mice develop diabetes and hyperlipidemia at 8 weeks of age. (A) Fast blood glucose of 8-week-old db/m and db/db diabetic mice were measured (n = 9-14 mice/group). (B) IPGTT was performed to evaluate glucose tolerance of 8-week-old db/db mice and littermate controls (n = 3-8 mice/group). (C) Serum insulin from 8-week-old db/db mice and littermate controls were measured by ELISA (n = 7-8 mice/group). (D-G) Blood samples were used to measure the levels of lipids, including triglyceride (TG) (D), total cholesterol (TC) (E), Low-Density Lipoprotein Cholesterol (LDL-C) (F), High-Density Lipoprotein Cholesterol (HDL-C) (G) (n = 10-11 mice/group). Values are shown as mean ± SEM. * P < 0.05, ** P < 0.01, **** P < 0.0001.
Supplemental Figure S4. The targeting vector was designed to remove exon 3-5 of STING by inserting a Neo cassette and two LoxP sequences flanking the region exons 3, 4 and 5.
Supplemental Figure S5. No significant differences of the STING expression in Brain and hypothalamus were observed between STING<sup>0/0</sup>; RIP-Cre and STING<sup>0/0</sup> mice. Brain and hypothalamus from 8-week-old STING<sup>0/0</sup>; RIP-Cre mice and controls were directly lysed. STING protein levels were examined by western blotting. n ≥ 5 mice/group. Values are shown as mean ± SEM, ns, not significant.
Supplemental Figure S6. STING-βKO female mice exhibited glucose intolerance and impaired insulin secretion. (A) Body weight of female STING⁺/⁻; RIP-Cre mice and controls was measured at 6 weeks of age (n = 11-13 mice/group). (B) Random blood glucose of female STING⁺/⁻; RIP-Cre mice and controls was measured at 6 weeks of age (n = 11-13 mice/group). (C) IPGTT was performed as described in Supplemental Materials and Methods, and blood glucose levels were measured to evaluate glucose tolerance of the female STING⁺/⁻; RIP-Cre mice and controls at 8 weeks of age (n = 16-21 mice/group). (D) The AUC of Fig. S6C were calculated. (E) Serum insulin in female STING⁺/⁻; RIP-Cre mice and controls during IPGTT (n = 12-13 mice/group). (F) IPITT was performed in 8-week-old STING⁺/⁻; RIP-Cre female mice and littermate controls (n = 11-15 mice/group). Values are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Supplemental Figure S7. Inhibiting STING and TBK1 impairs GSIS. (A) Freshly isolated islets from 8-week-old db/db mice and littermate controls were directly lysed. The expression of STING and TBK1, as well as phosphorylated TBK1 (p-TBK1) were determined by western blots (n = 3-5 mice/group). (B) Relative levels of STING were quantified from Fig. S7A. (C) The levels of p-TBK1 and TBK1 were quantified from Fig. S7A and the ratios of p-TBK1 and TBK1 were calculated. (D) Isolated islets from 8-12 weeks male wild-type mice were incubated overnight and treated with DMSO, TBK1 inhibitor (10μM, Cat: S8872, Selleck) and STING inhibitor (C-176, 10μM) for 24 h, respectively. Subsequently, GSIS was performed and secreted insulin was measured (n ≥ 3 mice/group). (E) INS-1 cells were treated with NF-kB inhibitor (PDTC, 5μM) for 48 h. The cells were then treated with 2.8 or 33.3 mmol/L glucose for 2 h. The secreted insulin was measured by ELISA. Values are shown as mean ± SEM from at least 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, ns, not significant.
Supplemental Figure S8. β-cell specific STING knockout does not affect the expression and nuclear localization of Pdx1, Nkx6.1, andIsl1, but inhibiting STING down-regulates the expression of Pax6. (A-C) The expression and nuclear localization of transcription factors Pdx1 (A), Isl1 (B) and Nkx6.1 (C) in 8-week-old STING^{fl/fl}; RIP-Cre mice and controls were detected by immunofluorescent staining with anti-insulin (red), anti-Pax6 (green), anti-Pdx1 (green), anti-Isl1 (green), anti-Nkx6.1 (green), and DAPI (blue). Scale bar = 50 μm (n = 3 mice/group). (D) INS-1 cells were transfected with STING siRNA (STING si) or negative control siRNA (NC si). The cells were lysed after 48 h transfection. The expression levels of Pax6 and STING were detected by western blot. (E) MIN6 cells were incubated with STING inhibitor C-176 (20 μM) for 5 h. The expression levels of STING and Pax6 were determined by western blot.
Supplemental Figure S9. The overall DNA binding activity by Pax6 is impaired in islets from the STING-βKO mice. (A) GO analysis of 2,888 genes annotated to 3,641 significantly different peaks in Fig. 8A. Most significant and nonredundant biological processes with respective gene numbers and P-values are shown. (B) Metagene heatmap of the genome-wide Pax6 CUT&Tag peaks’ occupation in islets from indicated groups. Heatmap showed signal intensity in the -5 kb flanking the Transcriptional Start Sites (TSS) to +5 kb region flanking the Transcriptional End Sites (TES). H3K4Me3 was used as the positive control for CUT&Tag assay. Scale bar indicates peak density. (C) Heatmap showed scaled, log₂ transferred normalized read-counts (as Z-Score) of 695 significantly different Pax6 bound peaks (|log₂FC| > 0.5; P < 0.05; normalized read-counts > 0) in islets between STING<sup>fl/fl</sup>, RIP-Cre and controls. (D) GO analysis of 313 genes annotated to 317 significantly down regulated peaks (log₂FC < -0.5, P < 0.05, normalized read-counts > 0) in panel C.
| Gene name       | Forward primer                  | Reverse primer                  |
|-----------------|--------------------------------|--------------------------------|
| Mouse-eGAS      | GAGGCCGCGGAAAGTCGTAAG          | TTGTCCCGTTCTTCCTCTGGA           |
| Mouse-STING     | GTCACCGCTCCAATAGTGAG           | CAGTAGTCCAAGTTCGTGCGA           |
| Mouse-TBK1      | ACTGGTGATCTCTTCTGCTGCA         | TCTTGGAAGTCCATACGCATTG          |
| Mouse-IRF3      | GAGAGCGACAGGAGGTTCAAG         | CTTCCAGTTGACAGGTCGCAA           |
| Mouse-ISG15     | ACTAACCCTAGCCGCTGCTGCA         | GTCCTCTGACAGGTCGAAGTCGAG       |
| Mouse-IFNβ      | AGCCAGCTGATGGAGAAGATCAAC       | GGAAGGCAAGGACAGTGTAAC           |
| Mouse-Ins1      | TCCCTCTCACACACCCAAAGTCC       | CAGCCTCAGTGGTTTCCACTT           |
| Mouse-Ins2      | GAAGGTGGAGGACACCAAGA          | GTCTGAAGGTGACCTGCTC             |
| Mouse-Pdx1      | GACCTTTCTCCGATGGAAACC         | GTCCTCGTGTGAAACGACC            |
| Mouse-MafA      | ATCTAATCTGCCACACCTCAC         | TGCCTTCTCCCTTGCTGAAG            |
| Mouse-NeuroD1   | CGAGGTCCGGAGGTATGAGGAG        | CATGGTTTCCCTGCAAGTCGACT        |
| Mouse-Nkx6.1    | CTGCACAGATGGCAGGAGATG         | CCGGTTATGTAAGCCCAA             |
| Mouse-Pax6      | TTCCCGAATTTCTGACGACCC         | TCTTGCTTACTCCCTCCGA             |
| Mouse-Sle2a2    | TCTTGTCTGGAGCAGAAGTATGCA      | ACATGGGAACAGTGCTGAAA            |
| Mouse-Gck       | ATGGCTGATGGATAGACTAAGGAAG     | TCCAGGCGACGCTCCATCT             |
| Mouse-Abcc8     | TGACATTGGGAAGACAGCCCCTCAT    | CACGACCGTGAAGTATGAAGTCGCA       |
| Mouse-Kcnj11    | CTCTGGAAGGCAGGAGGAGTGAAGA    | TAGAATCCCTGAGCTAGGTAGG          |
| Mouse-Kcnm2     | ATGTCTAGGCGCCATTGGGTTGGG     | CACATGTCAGGCGTGGTAGAAGAAG       |
| Mouse-Kcnm4     | GCTCAACCAAGTGCCGCTTC         | GTGATTGGAATCAGGGCAGGT           |
| Mouse-Prm4      | GAAACGCAGCTCCAGGAAAGGT       | CAGCCATCCAAGTCAAGGACT            |
| Mouse-Ptkc      | CTCTGTTTCTTCAAGCGGCCAA       | GTGAAACCACAAAAAGCTACAGACT       |
| Mouse-Atpt1a3   | TCTCAGATGTGTCGTTCTCTCT       | GGGAAAGAGAGTGAAAAGGCAAG         |
| Mouse-Adcyap1   | ACCATGTGTAAGCCGAGCAAGG       | CTGTTGCGTAAAGCTCGCTCT           |
| Mouse-Ptger3    | CGGGAGACTCTGCTGAAG            | CCCACTAAGTGCGGTGAAG             |
| Mouse-GAPDH     | ATGTTCCAGATGTGACCTCCACTCAG   | GAAGACACAGTAGCTCCACGCAGA        |
Supplemental Table S2. The information of donors.

|          | Control | T2DM     |
|----------|---------|----------|
| N        | 5       | 5        |
| Age (years) | 49.2 ± 2.22 | 51.2 ± 1.85 |
| Sex      | 1 female, 4 male | 5 male |
| BMI      | 22.87 ± 0.86 | 27.58 ± 1.83 |
| HbA1c (%) | 5.16 ± 0.16 | 7.02 ± 0.21 |