ESM Methods

Cell lines The MIN6 cell line (provided by Drs. Miyazaki and Oka , University of Tokyo, Japan) was cultured in dulbecco's modified eagle medium (DMEM) supplemented with 15% FBS, 2 mmol/l L-glutamine, 100 units /ml penicillin and 0.1mg/ml streptomycin (Life Technologies, Frederick, MD, USA). The human embryonic kidney 293 cell (293T) line (ATCC, Manassas, VA, USA) was cultured in DMEM medium supplemented with 5% FBS, 100 units /ml penicillin and 0.1mg /ml streptomycin. Pheochromocytoma-12 (PC12) cells (ATCC) were maintained in DMEM containing 5% FBS and 10% horse serum, 100 units/ml penicillin and 0.1mg /ml streptomycin. Both 293T and PC-12 cell lines were tested for authentication and mycoplasma by ATCC prior to purchasing. All cell lines were cultured at 37 °C in 5% CO2.

Mouse islets Islets from 3-4 month old sex-matched mice were isolated as previously described [1, 2] with slight modifications of the collagenase P (Roche, Indianapolis, IN, USA) manufacturer's protocol. Briefly, mice were euthanized and collagenase P solution (1 mg/ml) was injected into the common bile duct to inflate the pancreas. The inflated pancreas was then removed and incubated at 37°C for digestion until the pancreas formed a milky solution with only a few clumps. After digestion, islets were purified in density gradient Histopaque1077, 1083, and 1119 (Sigma). Finally, islets were manually selected and washed with Krebs-Ringer HEPES (KRBH) buffer, and cultured overnight in RPMI-1640 full medium (supplemented with 15% FBS, 100 U/ml penicillin, 0.1mg/ml streptomycin) containing 5mM glucose.

MicroRNA mimic and inhibitor transfection MIN6 cells, mouse islets and PC12 cells were transfected with a miR-153 mimic or inhibitor (Qiagen, Germantown, MD, USA) or scramble

control (AllStars Negative Control siRNA, Qiagen), using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. MiR-153 Mimic is chemically synthesized, double-stranded RNA which mimic the mature endogenous miRNA after transfection into cells. MiR-153 Inhibitor is chemically synthesized, single-stranded, modified RNA which specifically inhibit endogenous miRNA function after transfection into cells. Both of them target mmu-miR-153-3p (MIMAT0000163: 5'UUGCAUAGUCACAAAAGUGAUC). The procedure for transfecting islets was adapted from a previous report [3]. The transfection had been down as the following steps: 1. Shortly before transfection, seed 0.4–1.6 x 10^5 cells (or 30-40 islets) per well of a 24-well plate in 0.5 ml of an appropriate culture medium containing serum and antibiotics. 2. For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO2). 3. Dilute 1μl miRNA mimic/inhibitor in 100μl culture medium without serum (final concentration: miRNA mimic, 5nmol/l; inhibitor, 50nmol/l). Add 3μl of HiPerFect Transfection Reagent and mix by vortexing. 4. Incubate the samples for 5–10 min at room temperature (15–25°C) to allow the formation of transfection complexes. 5. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes. 6. Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time. Change the medium as required. After 72 h, the transfected cells were further processed for glucose or potassium-stimulated insulin secretion (GSIS or PSIS, respectively), gene expression analyses or western blotting.

GSIS or PSIS Insulin secretion in MIN6 cells and mouse islets was measured after the cells were transfected as described above. Cells were washed twice with basal solution (3.3 mmol/l

glucose or 5.6 mmol/l KCL in KRBH buffer), then incubated for one hour at 37°C in basal KRBH buffer to stabilize basal insulin secretion. Following incubation, MIN6 and islets were washed twice, and incubated again for one hour in basal solution and then subject to glucose or KCL stimulation for one hour in stimulating solution (25 mmol/l glucose or 50 mmol/l KCL in KRBH buffer). Supernatants were collected for insulin measurement with an ELISA kit (ALPCO Diagnostics, Salem, NH, USA) before and after stimulation. Fold change of insulin secretion was calculated by comparing insulin levels before and after stimulation.

Dopamine secretion test PC12 cells were seeded in 24-well culture plates coated with poly-l-lysine at a density of 2×10^4 cells per well and cultured for 2 days. The dopamine secretion test was conducted as previously described [4] at 72 h after transfection with either miR-153 mimic or miR-153 inhibitor. Briefly, cells were washed twice with a low K^+ buffer (4.7 mmol/l KCl) and stimulated for 3 min with low K^+ buffer, or with high K^+ buffer (25 mmol/l KCl) with or without 160 nmol/l phorbol-12-myristate-13-acetate (PMA) (Sigma-aldrich, St. Louis, MO, USA) for 6 min at 37 °C. The supernatants were collected and dopamine levels were measured with a dopamine ELISA kit (Rocky Mountain Diagnostic, Colorado Springs, CO, USA). Fold change of dopamine secretion was calculated by comparing dopamine levels before and after stimulation with high K^+ solution with or without PMA.

Generation of *Cacna1c* 3′UTR reporter construct The method for constructing the wild type (WT) and mutant luciferase reporter plasmids for miRNA target validation was adapted from a previous report [5]. Briefly, the 3′-UTR containing the WT or mutant seed sequences of *Cacna1c* were amplified and cloned into the pMIR-REPORT miRNA Expression Reporter

Vector System (Life Technologies). Total RNA was reverse transcribed into cDNA. WT *Cacna1c* 3′-UTR (860bp) was amplified by the *WT-Cacna1c* primers and mutant *Cacna1c* 3′-UTR by the four overlapping primers: *WT-Cacna1c* and *Mut-Cacna1c* (see primers sequence in ESM Table.1), and then purified using the QIAquick PCR Purification Kit (Qiagen). Purified PCR fragments were digested with *HindIII* and *SpeI* restriction endonucleases (New England Biolabs, Beverly, MA, USA) in buffer 4 overnight, gel purified, and ligated to the luciferase reporter gene in the *HindIII*- and *SpeI*-digested vector to generate the WT and mutant *Cacna1c* reporter vectors. After sequencing validation, plasmid DNA was prepared using a Plasmid Miniprep kit (Qiagen).

Target protector functional analysis Target protectors are single-stranded, modified RNA oligonucleotides complementary to specific target sites and do not bind other sequences. The protector covers the flanking region of the binding site and specifically interferes with the direct interaction between miRNA and mRNA (http://www.qiagen.com/products/miscripttargetprotectors.aspx). Target protectors enable the study of the physiological function of specific miRNA-mRNA target pairs and have no effect to other potential targets [6]. MiR-153 miScript target protector was designed to be complementary to the predicted miR-153 binding site in the *Cacna1c* 3′UTR (Qiagen). Transfection was performed according to the manufacturer's protocol. In short, MIN6 cells or islets were transfected with miR-153 mimic (final concentration of 15 nmol/l) and/ or with target protector (final concentration of 600 nmol/l). GSIS was performed 72 h later, together with quantitative real-time PCR for *Cacna1c* mRNA to assess the protective effect of the target protector.

Calcium microfluorimetry $[Ca^{2+}]_i$ was measured using the ratiometric dye fura-2/AM (a high affinity, intracellular calcium indicator). All islets (transfected with miR-153 mimic or inhibitor or scrambled control) were maintained in 5.0 mmol/l glucose to prevent transient states in their oscillatory periods due to shifts in glucose before or during the experiment. Islets were fura-loaded (30 min), washed, incubated (10–20 min), and then recorded at ~35°C in a standard external solution, containing (in mmol/l): 5.0 glucose, 135 NaCl, 2.5 $CaCl_2$, 4.8 KCl, 1.2 $MgCl_2$, and 20 HEPES, pH 7.3, followed by a solution with high KCl containing 5.0 glucose, 110 NaCl, 2.5 $CaCl_2$, 30 KCl, 1.2 $MgCl_2$, and 20 HEPES, pH 7.3. $[Ca^{2+}]_i$ measurements were made by placing mouse islets in a small volume chamber (Warner Instruments, Hamden, CT, USA). The chamber was mounted on the stage of an Olympus IX71 upright fluorescence microscope equipped with fura-2 optics (Olympus, Tokyo, Japan). Excitation light was supplied to the preparation via a light pipe using a galvanometer-driven mirror and dichroic cube. The light source was a xenon burner, which produced excitation at 340 and 380 nm (Metaflour; Molecular devices, Sunnyvale, CA, USA). Ratios were collected at 510 nm using a photomultiplier (Quantem 512SC; Photometrics, Tucson, AZ, USA) and were analyzed using IonOptix IonWizard software and standard calibration methods.

Protein extraction and western blot analysis Proteins from cells or mouse tissues were isolated with M-PER or T-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc, Rockville, MD, USA). Equivalent samples were mixed with a reducing loading buffer, heated at 70°C for 10 min, and equal amounts of protein were loaded in each lane of Novex Bis-Tris PAGE gels (Invitrogen). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and then blocked with blocking buffer (PBS/ 0.1% Tween20 (PBS-T) / 5% nonfat-

milk). After blocking for 2 h, blots were incubated over night at 4°C with rabbit anti-calcium channel, voltage-dependent, L type, α1C subunit (CACNA1C) polyclonal antibody (1:500 dilution, Santa Cruz Biotech, Dallas, TX, USA) and mouse anti-α-tubulin monoclonal antibody (1:5000 dilution, Abcam, Cambridge, MA, USA). After washing, the blots were incubated for 30 min at room temperature with HRP conjugated secondary anti-Rabbit or mouse antibody (Invitrogen). The blots then were washed and films were subsequently developed using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). Blots were quantitated using NIH Image J Software.

ESM Methods references

1. Cai T, Hirai H, Zhang G, et al. (2011) Deletion of Ia-2 and/or Ia-2beta in mice decreases insulin secretion by reducing the number of dense core vesicles. Diabetologia 54: 2347-2357
2. Ravier MA, Rutter GA (2010) Isolation and culture of mouse pancreatic islets for ex vivo imaging studies with trappable or recombinant fluorescent probes. Methods Mol Biol 633: 171-184
3. Lakey JR, Young AT, Pardue D, et al. (2001) Nonviral transfection of intact pancreatic islets. Cell Transplant 10: 697-708
4. Nishimura T, Harashima S, Yafang H, Notkins AL (2010) IA-2 modulates dopamine secretion in PC12 cells. Mol Cell Endocrinol 315: 81-86
5. Nicolas FE (2011) Experimental validation of microRNA targets using a luciferase reporter system. Methods Mol Biol 732: 139-152
6. Choi WY, Giraldez AJ, Schier AF (2007) Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science 318: 271-274