

ONLINE SUPPLEMENTAL MATERIALS

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Human islet miR-125b expression correlates with donor BMI but not with age or sex of the donors.

RT-qPCR measurements of **(A)** human islets cultured at the indicated glucose concentrations for 48h and **(B)** human islets cultured at 5.5mM glucose plotted against islet donor BMI. MiR-125b expression is normalized to that of the endogenous control miR-574-3p. **(C)** RT-qPCR measurements of miR-125b human islets cultured at 5.5mM glucose plotted against islet donor age (left hand-side panel) or sex (right hand-side panel). MiR-125b expression is normalized to that of the endogenous control miR-7d-3p. RT-qPCR measurements of **(D)** islets from β AMPKdKO (green), β LKB1KO (red) and control (C, black) male and female mice fed a chow or a ketogenic (Keto) diet for 28 days and **(E)** human islets treated with the AMPK activators C-13 (50nM) and C-991 (20nM) for 16h (left hand-side) and dissociated human islets infected with adenovirus expressing a dominant-negative AMPK protein (Ad-AMPK-DN) or empty control (Ad-GFP) at 5 MOI for 48h. MiR-125b expression is normalized to that of the endogenous control miR-574-3p. Each dot represents islets from a single mouse or human donor. Error bars represent SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way ANOVA (repeated measures) and Dunnett multiple comparisons test (A), Pearson Correlation adjusted by age and sex (B), Pearson Correlation (C), two-way ANOVA (repeated measures) and Bonferroni multiple comparisons test (D), and paired Student *t* test (E).

Supplemental Figure 2. MiR-125b regulates insulin secretion in insulinoma/ β -cell lines.

A) MiR-125b RT-qPCR in MIN6 cells transfected with 5nM miR-125b or control (C) mimics. Each dot represents an independent experiment. MiR-125b expression is normalized to that of the endogenous control miR-574-3p and presented as fold change of control-transfected cells. **B-D)** Glucose-stimulated insulin secretion presented as % of secreted insulin from total content and quantified after 30 minutes of 17 or 15 mM glucose stimulation following overnight

preincubation at 3 mM glucose of MIN6 (**B**, **C**) and EndoC β -H1 with CRISPR-mediated miR-125b knockout (**D**). MIN6 cells were transfected with 5nM miR-125b (125b) or control (C) mimics (**B**) or infected with adenovirus expressing a miR-125b inhibitor (125b-OFF) or a non-targeting control (C) at 10 MOI (**C**) 48h before the experiments. Each dot represents an independent experiment. Error bars represent SEM. ns=no significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired Student t test (A) and two-way ANOVA (repeated measures) and Sidak multiple comparisons test (B-D)

Supplemental Figure 3. Elimination of miR-125b by CRISPR-Cas9 in EndoC β -H1 cells.

A) Schematic representation of the targeting location of the gRNAs in *MIR125B-1* and *MIR125B-2* human loci. Two independent pairs of gRNAs were designed for each loci, targeting the seed of miR-125b (miR-125b-5p) or the regions essential for DROSHA and/or DICER cleavage. **B)** RT-qPCR measurements of (mature) miR-125b in Control EndoC β -H1 (containing the RIP-Cas9 vector, but no gRNAs), MIR125B1-KO1 and MIR125B1-KO2 (containing RIP-Cas9 vector with gRNAs targeting the *MIR125B1* locus, pair 1 (KO1) or pair 2(KO2)), MIR125B2-KO1 and MIR125B2-KO2 (containing RIP-Cas9 vector with gRNAs targeting the *MIR125B2* locus, pair 1 (KO1) or pair 2(KO2)) or combinations of the above gRNAs as indicated. **C)** RT-qPCR of miR-99a and miR-125a in Control (C) and MIR125B2-KO1 (KO) cells demonstrating that elimination of miR-125b using gRNAs targeting the *MIR125B2* locus does not result in reduction of the clustered miRNA miR-99a or in compensatory overexpression of the family member miR-125a. Values were normalized by the endogenous control let-7d-3p and are presented as fold change relative to control cells. Control (C) and MIR125B2-KO1 (KO) cells were **(D)** stained with Annexin V-FITC and propidium iodide (Abcam) and subjected to flow cytometry to determine the percentage of alive cells (Annexin V and PI negative cells) and **(E)** submitted to an MTT assay to determine cellular metabolic activity as absorbance values at 550-690 (Optical density). Each dot represents an independent experiment. ns=not significant, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$, paired Student t test (D) of the log[fold change] values (B, C)

Supplemental Figure 4. Experimental design and validation for the high-through identification of miR-125b target genes. **A)** Schematic overview of the steps of the experimental approach used for the identification of miR-125b targets. MIN6 cells were transfected with 5nM control of miR-125b mimics for 24h. Then, total RNA was extracted, poly(A)-enriched and prepared for sequencing (n=5, T-RNA) or RNAs present in miRISC complexes were immunoprecipitated with anti AGO2-antibodies and submitted to RNA-seq (n=5, RIP-RNA). Differential analysis of gene expression was performed for both datasets separately generating fold change gene expression (miR-125b vs control) for all genes detected. Then both lists were combined to generate a ratio of Immunoprecipitated vs Total (poly(A) RNA). **B)** Enrichment of miR-125b seed sites in the 3'UTRs (left hand-side panel) and CDSs (right hand-side panel) of the mRNAs ranked according to RIP-RNA/T-RNA. The scatter plot shows the maxima of enrichment profile. Each dot represents a 7-letter word. The Y-position shows the maximum score of an enriched word and the X-position shows the gene-index where the Z-score is maximum. Top-ranked words with a left-shift associate with gene-expression change (lower gene index represents a stronger upregulation in RIP-RNA/T-RNA upon miR-125b transfection). The sequence of mature miR-125b is shown on the right, with the seed region in red. The reverse complementary sequence (RC) is also shown in red.

Supplemental Figure 5. MiR-125b alters mitochondria morphology and genes associated with mitochondrial function. **A, B)** Quantitative analysis of mitochondria number and morphology on deconvoluted confocal images of (A) MIN6 cells transfected with miR-125b (red) or control (black) mimics or (B) EndoCβ-H1 with CRISPR/Cas9-mediated miR-125b deletion (green) or controls (black). Cells were stained with Mitotracker green. An ImageJ macro was generated and used to quantify number of mitochondria per cell, total mitochondria area and individual mitochondria perimeter. Each dot represents one acquisition (n=3 (b), n=4 (c) independent experiments). Error bars represent SEM. **p<0.01, ****p<0.0001, upaired Student t test. **C)** Gene Ontology analysis of dysregulated genes in EndoCβ-H1-MIR125B2-KO vs controls (609 and 81 genes up- and down-regulated, respectively, padj<0.1) performed

with DAVID. The graph shows enrichment scores for one representative term for each cluster grouped by semantic similarities and including terms with p_{adj} (Benjamini) < 0.05 . See Supp Table 4 for a full list of terms.

Supplemental Figure 6. β -cell-selective overexpression of miR-125b does not alter hypothalamic miR-125b expression, body weight, insulin tolerance and β -cell growth and apoptosis. **A)** RT-qPCR of miR-125b in hypothalamus from Control (RIP7-rtTA^{+/+}) and MIR125B-Tg mice (RIP7-rtTA^{+/+}, MIR125B Tg^{+/+}). **B)** Body weight of male and female MIR125B-Tg and littermate control mice monitored weekly from the age of 5 weeks. $n=5-8$ mice/genotype. **C)** Glucose tolerance test in 6 week old mice containing the transgene in the absence of rtTA (-rtTA Control, MIR125B Tg^{+/+}) and littermate controls without the transgene or rtTA, showing that the presence of the transgene doesn't affect glucose tolerance. $n=4-6$ mice/genotype. **D)** Intraperitoneal Insulin tolerance test in 7 week old MIR125B-Tg and littermate control male and female mice. $n=5-8$ mice/genotype. **E,F)** Pancreata from 11 week-old MIR125B-Tg and littermate controls were fixed and subjected to (E) immunocytochemical analysis for insulin and Ki67. The number of Ki67-positive cells is expressed as a percentage of insulin-positive cells (F) TUNEL assay for *in situ* detection of apoptotic cells. The number of TUNEL-positive cells is presented as a percentage of insulin-positive cells. Each dot represents one pancreatic section with $n=3$ mice/genotype. **G)** Insulin secretion in response to 30 minutes low (3mM, LG), high (17mM, HG) glucose or KCl (17mM KCl and 3mM Glucose) in islets from 10-11 week old Control (RIP7-rtTA^{+/+}) and MIR125B-Tg (RIP7-rtTA^{+/+}, MIR125B Tg^{+/+}) mice (left hand-side panel). Insulin and pro-insulin/insulin content in 10 week old Control (C) and MIR125B-Tg (Tg) mice quantified by HTRF (**H**) and Western blot (**I**), respectively. (**I**) shows densitometry quantification using ImageJ of the experiments presented in Figure 5, normalized by GAPDH and presented relative to the average control. **J)** Total pancreatic insulin content quantified using HTRF and presented as relative to total protein from whole pancreata of Control and MIR125B-Tg mice. **K)** Quantitative analysis of lysosomes number and total area on deconvoluted confocal images of dissociated MIR125B-Tg and Control islets

from 10-11 week old mice. Cells were stained with LysoTracker green. An ImageJ macro was generated and used to quantify number of lysosomes per cell and total lysosomal area. Each dot represents one cell (n=3 mice/genotype). ns=not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, upaired Student t test (A, E-F, I-K), paired Student t test (H) two-way ANOVA (repeated-measures), Fisher least significance different test (C-D), Bonferroni multiple comparisons test (G).

Supplemental Figure 7. Molecular and Cellular pathways altered in MIR125B-Tg mice.

Heatmaps of significantly downregulated (A) and upregulated genes (B) associated to selected cellular pathways and functions enriched in MIR125B-Tg islets following Gene Ontology analysis. Colour intensity shows magnitude of fold change. For phosphoproteins and glycoproteins, only the ~80 most up-regulated genes are displayed due to space limitations. Please see supplemental table 8 for a comprehensice list of genes.

Supplemental Figure 8. Overexpression of miR-125b in human islets. **A**, RT-qPCR of miR-125b in full human islets 48h following infection with 5 MOI of an adenovirus expressing miR-125b (pAd-MIR125B) or control (pAd-empty). Each dot represents an independent experiment with islets from different human donors **B**) Glucose-stimulated insulin secretion quantified after 30 minutes of 15 mM glucose stimulation following 1h pre-incubation at 3 mM glucose of human islets infected Ad-MIR125B or a non-targeting control (C) at 5 MOI 48h before the experiments. Each dot represents an independent experiment performed with islets from two different donors. **C**) RT-qPCR of miR-125b in dissociated human islets 24h following infection with 2 MOI of an adenovirus expressing miR-125b (pAd-MIR125B) or control (pAd-empty). Each dot represents an independent experiment with islets from different human donors **D**) Principal Component Analysis of the RNA-seq data in human dissociated islets infected with pAd-MIR125B or control (C) adenovirus. HI=Human islets. 3 independent experiments were performed with islets from three different donors (H1-3). **E**) Gene Set Enrichment Analysis (GSEA) of genes ranked by fold change in human islets infected with an adenovirus expressing miR-125 vs control during 24h. The graph shows enrichment scores

for significantly ($p < 0.05$) down- (red, top plot) and up-regulated (green, bottom plot) KEGG pathways terms. See Supp Table 8 for a full list of terms. Examples of enrichment plots (Lysosome and calcium signalling KEGG pathways) are shown in E. $**p < 0.001$, $***p < 0.0001$, $****p < 0.00001$, paired Student t test of the log[fold change] values (A, C), two-way ANOVA (repeated-measures), Bonferroni multiple comparisons test (B).

SUPPLEMENTAL RESEARCH DESIGN AND METHODS

Cells and islets culture

MIN6 cells were cultured in 4.5 g/L glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% FBS, 2mM L-glutamine, 20nM HEPES and 50 μ M β -mercaptoethanol. EndoC- β H1 cells were cultured in 1g/L glucose DMEM supplemented with 2% Bovine Serum Albumin Fraction V, Fatty Acids Free, 10mM nicotinamide, 5.5 μ g/ml transferrin 6.7 ng/ml sodium selenite and 50 μ M 2-mercaptoethanol(2). Mouse pancreatic islets were isolated with collagenase as described previously (3) and allowed for recovery from digestion overnight in culture medium (RPMI 1640, 10% FBS, L-glutamine and 11mM glucose) or directly used for experiments. Human islets were cultured in RPMI 1640, 10% FBS, L-glutamine and 5.5 mM glucose. The characteristics of the human donors are shown in Sup Table 9. Cells and islets were maintained at 37°C with 95% O₂/ 5% CO₂. AMPK activation was performed with 20 mM compound 13 (C13)(4) and 50 mM compound 991(5) as indicated in the figure legends.

Plasmids generation

To generate adenovirus expressing miR-125b, a 426-nucleotide sequence containing pre-miR-125b was amplified by PCR from C57BL6/J mice genomic DNA with the primers 5'-AGGGAGCCAGGATGTAGTCA-3' and 5'-CGGGTCACCTGATCCCATCTA-3' and subcloned into pAdTrackCMV (pAd-MIR125B). Adenovirus were then generated using the AdEasy system(6). Adenovirus containing the empty vector were used as controls.

As above, the PCR-amplified pre-miR-125b-containing region was subcloned into pBI-LTet to generate pBI-LTet-MIR125B.

For reporter assays, full length *Taz*, *M6pr*, *Tor2a1*, *Mtfp1* and *Gnpat* 3'UTRs and *Tnksbp1* and *Gnpat* CDS were amplified by PCR from MIN6 cDNA and subcloned into pmirGLO (Promega), downstream the *Firefly* ORF. MiR-125b binding sites in *M6pr* and *Mtfp1* were identified with TargetScan(7) and three and two point mutations were introduced, respectively, in the sequences complementary to the miR-125b seed using site directed mutagenesis with the primers (mutated nts in bold): *M6pr*: 5'-CTCTCCTGCTGGCT**ACT**GGAAGTGTCTGACCC-3' and 5'-GGGTCAGACACTTCC**AGT**AGCCAGCAGGAGAG-3'; *Mtfp1*: 5'-GTATTTGGGACACTCT**AGGC**AGAGTCTCTGG-3' and 5'-CCAGAGACTCTGCCT**AG**AGTGTCCCAAATAC-3'.

All the constructs were verified by sequencing.

Transfection of miRNA mimics, siRNAs and plasmidic DNA

Cells were transfected at 60-80% confluence with 5 nM/1nM (MIN6, EndoC β -H1, respectively) control or miR-125b mimics (Qiagen) or 50 nM of a mixture of four ONTARGETplus siRNAs against mouse Smad2, Smad3 or non-targeting (Horizon Discovery) as previously described(8; 9). For luciferase assays MIN6 cells were plated at ~70% confluency in 48-well plates, and, the following day, transfected with 0.4 μ l Lipofectamine 2000(ThermoFisher Scientific), 5ng plasmid and 5nM control or miR-125b mimics. After 24 h, cells were lysed and luciferase activity determined with the Dual-Glo luciferase assay system (Promega, UK), following the manufacturer's instructions.

CRISPR-Cas9-mediated deletion of miR-125b in EndoC β -H1 cells

MIR125B-1 and *MIR125B-2* sequences were downloaded from miRBase(10). Using the website tool available at <http://crispr.mit.edu/>, we designed two independent pair of gRNAs targeting the seed region of the mature miR-125b and/or the regions in the pri/pre-miR-125b essential for DROSHA and/or DICER processing (Supplemental figure 2) as in(11). Two different gRNA pairs targeting each locus were subcloned under an H1 and U6 promoter within a lentiviral vector in which hSpCas9 expression is driven by a RIP (rat *Insulin* promoter) using

the cloning strategy described in Beucher and Cebola in Nature Protocol exchange (<https://protocolexchange.researchsquare.com/article/nprot-7395>). EndoC β -H1 cells were transfected with lentivirus expressing these gRNAs and the hSpCas9 and integrating cells were selected by subculture in the presence of 4 μ g/ml puromycin, encoded by the vector. Cells infected with lentiviral vectors containing the RIP-hSpCas9 cassette but without gRNAs were used as control. RT-qPCR for mature miR-125b was performed to assess the levels of the mature miR-125b in the resultant cell populations. All the experiments were performed with controls and KO cell populations generated from at least three independent infections.

Cell viability of EndoC β -H1-MIR125B2-KO and control cells was determined by double staining with Annexin V-FITC (1:500 dilution, Abcam) and 100 μ g/ml propidium iodide (Abcam) using a Bd LSR II flow cytometer at LMS/NIHR Imperial Biomedical Research Centre Flow Cytometry Facility. Cell proliferation was assessed with a Cell Proliferation Kit I (MTT) (Roche) according to manufacturer's instructions in cells cultured in 96-well plates for 72h.

RNA extraction, reverse transcription (RT) and qPCR

Total RNA was extracted using Trizol (Thermofisher) following manufacturer's instructions. RT-qPCR was performed as previously described(8) with miRCURY LNA RT and SYBR Green PCR miRNA kits and miRCURY LNA probes for miRNAs (Qiagen) and High-capacity cDNA transcription kit and Fast SYBR Green (Qiagen) for mRNA and pri-miRNAs. For the latter, an anchored oligo dT primer was used in the RT reaction. MiR-7d-3p and miR-573-3p were used as endogenous controls due to their high stability in our system(8).

MiRISC immunoprecipitation.

MiRISC-immunoprecipitation was performed as previously described(12). Briefly, MIN6 cells were seeded in 15 cm dishes and transfected with 5 nM of control or miR-125b mimic for 24 h. Following transfection cells were washed in cold PBS, scraped in PBS and collected by centrifugation. Pellets were then resuspended in 500 μ l of lysis buffer (20mM Tris-HCl pH7.5, 150mM KCl, 0.5% NP40, 2mM EDTA, 1 mM NaF and 160 U ml⁻¹ RNAsin and protease

inhibitors) and pre-cleared with Dynabeads Protein G beads (Thermo Fisher Scientific) for 2 h at 4°C. Precleared extract was incubated with Dynabeads Protein G beads conjugated with 6µg of mouse-anti-AGO2 (clone E12-1C9, Abnova) or mouse IgG (NXA931, Merck) and 1mg/ml of heparin for at 4°C for 16 hours with end-over-end rotation. After washing, the beads-immunoprecipitate were treated with TURBO™ DNase (2 U/µL) (Thermo Fisher Scientific) and proteinase K (20mg/ml) (Thermo Fisher Scientific) for 20 min at room temperature. RNA was extracted using phenol/ chloroform and ethanol/sodium acetate precipitation.

RNA sequencing library preparation, sequencing and analysis

Quantity and integrity of total RNA from MIN6, EndoCβ-H1 and mouse islets was determined with an Agilent Bioanalyzer and mRNA enriched was performed from 500-1000ng of RNA using a NEBNext Poly(A) mRNA Magnetic Isolation Kit (NEB). Library preparation from mRNA and from miRISC-isolated RNA was performed using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) and Universal i5 and i7 primers following manufacturer's instructions. Library preparation from human islets total RNA (200ng) was performed using a NEBNext Low Input RNA Library Prep kit (NEB). Sequencing was performed on a HiSeq4000 using 75 bp paired end reads according to Illumina specifications. For total mRNA ~25-40 million reads/sample and for miRISC RNA~12 million reads/sample were obtained. FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to assess the quality of the reads obtained and reads were then mapped to the mouse or human transcriptome (GRCm38 and GRCh37, respectively) using Salmon v1.3.0(13). Differential expression analysis was performed with the R package DESeq2(14).

For miRNA target identification, Total mRNA (T-RNA) and immunoprecipitated RNA (RIP-RNA) samples were treated as separate datasets and the two resulting gene lists of differential analysis were used to calculate the ratio of immunoprecipitated RNA to total mRNA (IP-RNA/T-RNA) for each gene detected in both datasets. Genes were ranked by RIP-RNA/T-RNA ratio and subjected to motif discovery and enrichment analysis in their 3'UTRs and CDS with cWords(15). Cytoscape(16) was used to visualize genes with RIP-RNA/T-RNA >1.5

(arbitrary cut-off). Gene set enrichment analysis (GSEA) was performed for genes with a baseMean (average of the normalized count values) > 10 ranked by fold change in gene expression in human islets infected with pAd-MIR125B or pAd-empty (Control) virus against the curated KEGG gene sets collection from MSigDB (Molecular Signature Database). DAVID(17) was used for Gene Ontology enrichment analysis of genes differentially dysregulated ($p_{adj} < 0.1$) in MIR125B-Tg islets, EndoC β -H1 MIR125B2-KO and in MIN6 cells overexpressing miR-125b.

Immunoblot and Immunohistochemistry

For Western blotting, EndoC β -H1 cells, dissociated human islets and whole mouse islets lysates were prepared in RIPA buffer. 5-15 μ g of EndoC β -H1 total protein extract, ~20000 human islet cells extract or 10-20 islets extract was submitted to SDS-PAGE and transferred to 0.2 μ M PVDF membranes. Membranes were incubated overnight with anti-M6PR (sc-365196 Santa Cruz Biotechnology) or anti-Insulin (L6B10, Cell Signalling Technology).

Slides for immunohistochemistry were prepared from isolated pancreata and visualized as previously detailed(18). For β -cell mass and proliferation measurements, slides were blotted with the following primary antibodies: anti-insulin (IR00261-2, 1:4; Agilent Technologies) and anti- glucagon (g2654, 1:100; Sigma) and/or anti-Ki67 (ab15580, 1:100 Abcam) antibodies and mounted using DAPI-containing Prolong Diamond (Life Technologies). To quantify apoptosis, pancreatic sections were submitted to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using a DeadEnd Fluorometric TUNEL system kit (Promega) according to the manufacturer's instructions. Slides were visualized with an Axiovert 200M microscope (Zeiss). ImageJ software was used to quantify the area positively stained for insulin and glucagon and the number of Insulin-, Ki67-, TUNEL- and DAPI-positive cells of all visible islets. For β -cell mass we calculated the percentage of pancreatic surface that was positive, as measured in whole pancreas sections separated by at least 25 μ m in the z-axis. For β -to- α cell mass we calculated the ratio of insulin to glucagon positive area in all visible islets.

Islets for immunohistochemistry were fixed in 4% PFA and stained with anti-cathepsin (ab75852, 1:200 Abcam) and anti-LAMP1 (1D4B, 1:100 University of Iowa DSHB) antibodies. Islets were visualized with a LSM780 inverted confocal microscope (Carl Zeiss, Cambridge, UK) using a $\times 63$ 1.4 numerical aperture (NA) oil objective. Five to seven images at different z planes of the islet (top, middle, bottom) were acquired per islet. Z-stacks were prepared from this images in ImageJ and quantifications of the particles with positive staining for cathepsin D, LAMP1 or both, were performed in 3-10 individual cells per islet.

Islet fluorescence imaging

For Ca^{2+} Imaging, whole isolated islets were incubated with Cal-520 (4.5 μM ; Stratech) for 45 min at 37 °C in KRBH containing 3mM glucose. For ATP imaging, 48 hours prior to experiment, islets were infected with an adenovirus containing encoding the ATP sensor Perceval(19). Islets were incubated in KRBH buffer supplemented with 3mM glucose for 45 minutes before imaging. Excitation of the probes was achieved using 491nm and emitted light was filtered at 525nm. Islets were kept at 37°C and constantly perfused with KRBH B containing 3mM or 17mM glucose or 20mM KCl. Fluorescence live imaging was performed using a Nipkow spinning disk head and Velocity software (PerkinElmer). Data were analyzed with ImageJ software using an in-house-made macro, available upon request. For ATP/ADP ratio, the same probe and samples were used but the imaging was performed only at 3mM glucose with excitation at 491 and 405 nm to obtain ratiometric signal.

For TIRF experiments, mouse islet were infected with an adenovirus construct for NPY-venus 48 hour prior to the experiments. Before imaging, islets were dissociated using accutase, pelleted, resuspended in normal culture media and left to attach on a glass slide treated with polylysine. After incubation in KRBH buffer containing 3 mM glucose, slide was mounted in an imaging chamber. Imaging of cells expressing NPY-venus in the secreting vesicle was performed as described previously(20) using a Nikon Eclipse Ti microscope equipped with a $\times 100/1.49$ numerical aperture (NA) TIRF objective, a TIRF iLas2 module to control laser angle (Roper Scientific), and a Quad Band TIRF filter cube (TRF89902, Chroma). Acquisitions were

performed using a 488-nm laser line, and images were captured with an ORCA-Flash 4.0 camera (Hamamatsu). Metamorph software (Molecular Devices) was used for data capture, acquisition speed was set at 3Hz and the laser angle was selected for an imaged section thickness at the cell membrane of 150–180 nm. KCl at 20mM was used to stimulate insulin secretion and added to the well during acquisition after 3 minutes in KRBH 3 mM Glucose. Image analysis was performed using ImageJ to measure mean fluorescence intensity at the cell membrane over time.

For mitochondria morphology analysis MIN6 and EndoC β -H1 cells and dissociated mouse islets cultured in coverslips were pre-incubated for 45 minutes in KRBH solution supplemented with 25, 5 or 11 mM glucose, respectively, and Mitotracker GreenTM (100nM for MIN6 and islets, 70nM for EndoC β -H1; ThermoFisher) in a 37°C humidified incubator with 95% O₂/5% CO₂. Mitotracker green was washed with KRBH buffer before imaging. Cell images were acquired with a 60x oil immersion objective lens (Olympus) in a Nikon ECLIPSE Ti microscope. Dissociated islets were imaged on a LSM780 inverted confocal microscope (Carl Zeiss, Cambridge, UK) using a $\times 63$ 1.4 numerical aperture (NA) oil objective. Two to three randomly selected fields of view were taken per acquisition, containing 5-15 MIN6/EndoC β -H1 cells or a single dissociated mouse β -cell. Images were deconvoluted using Huygens software (Scientific Volume Imaging). Mitochondria morphological characteristics (i.e. number, area, elongation, circularity and perimeter) were quantified with ImageJ using an in-house macro (available upon request) as described by Wiemerslage et al (21). Quantifications were performed in z-stacks of 10 images for EndoC- β H1 and MIN6 cells and in three stacks of three images each at the top, middle and bottom of the primary β -cells.

Whole-cell voltage-clamp electrophysiology

Control and MIR125B-Tg islets were dispersed into single cells by gently titration for 30 seconds in TrypLETM Express (Thermo Fisher) and cultured overnight in RPMI-1640 medium (RPMI) with 5.6 mM glucose supplemented with 15% fetal bovine serum (FBS), 100 IU·ml⁻¹ penicillin, and 100 mg·ml⁻¹ streptomycin at 37°C, 5% CO₂. Patch electrodes (3-4 M Ω) were

backfilled with intracellular solution containing (mM) 102.0 CsCl, 10.0 TEA-Cl, 10.0 EGTA, 3.0 Na₂ATP, and 5.0 HEPES (pH 7.25 adjusted by CsOH). Voltage-clamp electrophysiology was performed on β -cells in extracellular buffer containing (mM) 119.0 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10.0 HEPES, and 17.0 glucose (pH 7.35 adjusted by NaOH). After forming a tight seal between the patch pipette and β -cell (seal resistance > 1 G Ω), whole-cell access was established and the bath solution was exchanged (3 minutes; 2 mL/minute flowrate) with extracellular buffer containing (mM) 82.0 NaCl, 5.0 CsCl, 30.0 CaCl₂, 1.0 MgCl, 0.1 EGTA, 20.0 TEA-Cl, 0.1 tolbutamide, and 17.0 glucose (pH 7.35 with NaOH). Starting from a holding potential of -80 mV, voltage-dependent Ca²⁺ (Ca_v) channel currents were generated through application of sequential 10 mV depolarizing steps ranging from -70 to +70 mV (500 ms); membrane potential was held at -80 mV for 7.5 seconds between each voltage step. Linear leak currents were subtracted using a P/4 protocol. Ca_v currents were normalized to cell capacitance and normalized peak Ca_v currents plotted as a function of applied voltage.

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