Supplementary information for
Alternative splicing encodes functional intracellular CD59 isoforms which mediate insulin secretion and are downregulated in diabetic islets.

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Extended Materials and Methods

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Anna Blom (anna.blom@med.lu.se).

Materials availability
Plasmids generated in this study, for expression of IRIS-1 and IRIS-2 are available on request, as are CRISPR/Cas9-generated CD59 knockout INS-1 and MIN6 cell clones.

Data and code availability
No coding was used, and no sequencing datasets were generated in the study.

Human Islet Donors
Human pancreatic islets were obtained from the Nordic Network for Islet Transplantation (Uppsala University, Sweden), under approval of the ethics committees at Uppsala and Lund. Informed consent was obtained from pancreatic donors or their relatives in accordance with the approval by the local ethics committee regarding organ donation for medical research. Human samples were de-identified prior to use in our study.

Human Tissue Samples
RNA samples isolated from human tissues were a kind gift from Yvonne Ceder (Lund University, Sweden). Tissue samples were either collected from patients undergoing surgical treatment, or taken at autopsy, or purchased from Clontech (USA) as described in (1). RNA samples were stored at -80 °C. cDNA was produced from 1000 ng of RNA using oligo (dT) primers, RnaseOUT and Superscript IV reverse transcriptase (Invitrogen). cDNA was further used for PCR reaction. Details about the PCR reaction and primers used can be found below (in method section about RT-PCR).

Animal Models
Rat and mice use was approved by the Malmo/Lund Animal Care and Use Committee (Permits M9-15, M87-14, 20069/2020) and abided by the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament. Apart from diabetic phenotype, animals were otherwise healthy. Animals were allocated to experimental groups by genotype/ diabetic phenotype. CD59 expression in mouse islets was assessed using RNA from isolated islets of 12-week old male db/db mice and age/ gender-matched C57BL/6J controls (Jackson lab), 3 animals in each group. For Akita mice, CD59 expression was assessed in RNA from isolated islets of 27-week old male Akita mice and non-diabetic age-matched male C57BL/6J littermate controls, with 3-5 mice in each group.

Cell lines (INS-1 832/13, MIN6, EndoC-βH1)
All cells were regularly tested for Mycoplasma, and were cultured at 37 °C in 5 % CO2.

INS-1 832/13 cells
INS-1 832/13 cells are derived from parental INS-1 cells, after stable transfection with the human proinsulin gene. INS-1 cells were derived from an x-ray induced rat insulinoma. The gender of the original animal is not reported. INS-1 832/13 cells were cultured in RPMI 1640 containing 11.1 mM D-glucose, 25 mM HEPES and L-Glutamine (HyClone) supplemented with 10 % fetal bovine serum (ATCC), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol.
**MIN6 cells**
MIN6 cells are a mouse insulinoma cell line (transfected with simian virus 40 (SV40)). These cells were grown in low glucose DMEM medium containing 25 mM HEPES and L-Glutamine (HyClone), supplemented with 20 % fetal bovine serum (ATCC), 20 mM sodium bicarbonate, 55 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin.

**EndoC-βH1 cells**
EndoC-βH1 cells are derived from human fetal pancreatic buds transduced with a lentiviral vector expressing SV40LT under insulin promoter. The transduced buds were grafted into SCID mice to develop into mature pancreatic tissue. Upon differentiation, the newly formed SV40LT-expressing β cells proliferated and formed insulinomas. The resulting β cells were transduced with human telomerase reverse transcriptase (hTERT), then grafted into other SCID mice and expanded in vitro to generate a cell line. EndoC-βH1 express many β cell–specific markers without any substantial expression of markers of other pancreatic cell types. These cells were grown in culture medium: DMEM medium (with 1 g/L of glucose) supplemented with 2 % albumin from bovine serum, 50 μM 2-mercaptoethanol, 10 mM nicotinamide, 5.5 μg/ml transferrin, 6.7 ng/ml sodium selenite, 100 U/ml penicillin and 100 μg/ml streptomycin. Neutralization medium composition: 80 % 1 x PBS, 20 % fetal bovine serum. Coating medium composition: DMEM medium (with 4.5 g/L of glucose), 2 μg/ml Fibronectin, 1 % ECM, 100 U/ml penicillin and 100 μg/ml streptomycin.

**Bacterial Strains**
DH5a E. coli used for molecular cloning were grown in LB medium at 37 °C.

**Methods details**

**Cell culture**
INS-1 832/13 cells (a gift from C. Newgard) were cultured in RPMI1640 medium (HyClone); detailed medium composition above. MIN6 cells (a gift from J. Miyazaki) were cultured in low glucose DMEM medium (HyClone); detailed medium composition above. Cells were regularly tested for *Mycoplasma* contamination.

**RT-PCR**
RNA was isolated from human and mouse islets, human and mouse liver, MIN6 cell line and mouse spleen using RNA purification kit (Qiagen). Panel of RNA from human tissues was a gift from Yvonne Ceder (Lund University, Sweden). Tissue samples were obtained either from patients undergoing surgical treatment, or taken at autopsy, or purchased from Clontech (USA) as described in (1). RNA samples were stored at -80°C. cDNA was made according to standard protocol and amplicon amplified with Red-Taq (Sigma) or DreamTaq Green PCR Master mix (Thermo Scientific) using annealing temperature 57 °C for 40 sec. GAPDH was used as a control. For detection of total human CD59 transcript primers 1 and 2 were used. To detect human IRIS-1 primers 1.3 were used and to detect human IRIS-2 primers 4 and 2 were used. Primers are listed below. For detection of mouse CD59A primers 1 and 2 were used. To detect canonical mouse CD59B primers 1 and 3 were used, and to detect mouse CD59B-IRIS-1 and IRIS-2 isoforms, primers 1 and 4 were used (products for mouse IRIS-1 and IRIS-2 can be distinguished by their differing sizes). Primers are listed below.

**Primers used for detection of human CD59 transcripts**

| Primer 1: Forward | ATCACAATGGGGAATCCAAGGAGGG |
|------------------|-----------------------------|
| Primer 2: Reverse | CTCTCCTGGTGTGACTTAGGG |
| Primer 3: IRIS-1 Reverse | CTCAGGAGAGAGGCGGAC |
| Primer 4: IRIS-2 Forward | AGTTGGGATATCATATGTTGCCC |
Primers used for detection of mouse CD59 transcripts

| Primer 1: Forward         | CAATGAGAGCTCAGAGGGGAC |
|---------------------------|-----------------------|
| Primer 2: CD59A Reverse   | AGGAGGCATCTAGGACTTAGAG|
| Primer 3: CD59B Reverse   | CTCAATGAGGAAGTTTCTGCGTTG|
| Primer 4: CD59B isoforms Reverse | CCATGAGCAAGCAGGTTTCAGG |

**RNASeq analysis**

RNAseq data from islets of over 190 human donors was assessed for presence of different splice isoforms of CD59. The dataset used is previously reported (DOI: 10.1038/s41467-020-18581-8, https://doi.org/10.1101/435743, and is deposited at the European Genome-phenome archive (EGA; https://www.ebi.ac.uk/ega/), with accession number EGAD00001005512.

**Insulin secretion INS-1 and MIN6 cells**

Before secretion, medium was changed to HBSS (Hepes-balanced salt solution; 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 25.5 mM NaHCO₃ at pH 7.2) supplemented with 2.8 mM glucose for 2 h, 37 °C. HBSS was removed and replaced with 150 µl HBSS containing 2.8/ or 16.7 mM glucose/or 2.8 mM glucose + 35 mM KCl for one hour (for INS-1 cells) and only 2.8 mM glucose + 35 mM KCl for MIN6 cells. Secreted insulin was measured by ELISA (Mercodia). Remaining cells were lysed in 100 µl of RIPA buffer, and total protein content determined by BCA protein assay kit (Thermo Scientific). Secretion is expressed as ng of insulin per mg of total protein per h.

**Insulin secretion EndoC-βH1 cells**

EndoC-βH1 cells were seeded on 48-well plate and transfected with siRNA against total human CD59 (Thermo Fisher, Silencer pre-designed siRNA against human CD59 #4392420, S269), or non-targeting negative control for 72 h (125 nM). 18 h prior to insulin secretion cells were kept in medium containing low glucose concentration (2.8 mM). Insulin secretion was done in freshly prepared 1x Secretion Assay buffer (SAB) with 0.2 % BSA, pH 7.3 (10 x SAB composition; 1.14 M NaCl, 47 mM KCl, 12 mM KH₂PO₄, 11.6 mM MgSO₄, 1M HEPES, 0.25 M CaCl₂ in H₂O). Insulin secretion was stimulated with 1 mM glucose and added to the cells for 2 h, 37 °C. SAB was removed and replaced with 250 µl SAB containing 1 mM, 20 mM glucose, or 1mM glucose + 35 mM KCl for 1 h. Secreted insulin was measured by ELISA (Mercodia). Remaining cells were lysed in 100 µl of RIPA buffer, and total protein content determined by BCA protein assay kit (Thermo Scientific). Secretion is expressed as ng of insulin per mg of total protein per h.

**Production of human anti-IRIS-1 and anti-IRIS-2 antibodies**

Antibodies against human IRIS-1 and IRIS-2 were raised by CAPRA Science Antibodies AB (Ängelholm, Sweden), by vaccinating rabbits with peptides derived from the unique C-terminals of each isoform: CQGLKTKQPGKKSAS for CD59-IRIS-1, and CELGYHYVAQAGRRQ for CD59-IRIS-2. After several rounds of vaccination, total rabbit IgG was purified from serum, and then specific antibodies positively selected by affinity chromatography using peptide-coupled columns. Antibody specificity was verified by ELISA and western blot using lysates of cells overexpressing individual isoforms or canonical CD59.

**Western blots**

Cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 % NP-40, 0.5 % deoxycholate) with addition of protease and phosphatase inhibitors (Thermo Scientific). Proteins were resolved on 15 % SDS-PAGE gel (home-made), or 4-20 % Precast Protein gels (Bio-Rad), or 15 % Tris-Tricine SDS-PAGE gel (home-made, used for detection of small proteins- mouse IRIS-2) under non-reducing (for CD59 detection) or reducing conditions, transferred onto PVDF membrane using Trans-Blot Turbo system (Bio-Rad) and blocked with Quench buffer (3 % fish gelatin in Immunowash: 50 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween 20, pH 8.0), with 5 % milk diluted in Immunowash buffer (for VAMP2 detection) or 5 % BSA diluted in Immunowash (for CD59 detection). Membranes
were probed with the following antibodies: anti-DYKDDDDK: FLAG-tag (1:1000, BioLegend, #637302), anti-β-actin (1:5000, Abcam, #Ab8226), anti-VAMP2 (1:500 Synaptic Systems, #104211), BRIC229 anti-CD59 (1:1000, IBGRL Research), anti-IRIS-1, anti-IRIS-2 (1:1000, CAPRA, home-made, #613.610, #612.609), anti-Na/K ATPase (1:100000, Abcam, #Ab76020), anti-mouse IRIS-2 (1:1000, Capra, home-made, #888.885) followed by the addition of appropriate HRP-conjugated secondary antibody (Dako) and development with ECL reagent (Santa Cruz Biotechnology).

**Cell fractionation**

INS-1 cells (6x10⁶) were harvested by scraping and separated into cytosolic and membrane and organelle fractions according to protocol from the Mem-PER Plus kit (Thermo Scientific). Proteins from each fraction were immunoprecipitated by overnight incubation (4 °C) with 10 µg of FLAG tag antibodies (BioLegend, #637302). Antibodies were immobilized on 50 µl of Sepharose G beads (GE Healthcare). Samples were run on western blot and signal detected using FLAG tag antibodies (as described above).

**Co-immunoprecipitation**

INS-1 cells (10x10⁶ cells) were incubated in Hepes-balanced salt solution with 16.7 mM glucose overnight (around 18 h). Cells were washed once with PBS and harvested by scraping from the surface. Pellets were lysed in 500 µl of lysis buffer: (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 % Triton X-100, 0.2 % NP-40, 1 mM EGTA) for 25 min on ice. Lysates were spun at 10000 x G, 4 °C for 5 min. Cleared lysates were immunoprecipitated using 10 µg of FLAG tag antibodies (BioLegend, #637302), or rat IgG2a isotype control (overnight, 4 °C with constant mixing). Sepharose protein G beads (50 µl) were then added into the lysates and incubated for 6 h, 4 °C with constant mixing. Beads were washed with lysis buffer 3 x 5 min, 4 °C. Bound proteins were eluted with 0.2 M glycine pH 3, neutralized with 1 M Tris-HCl pH 8, boiled in reducing Laemmli-sample buffer, run on 15 % SDS-PAGE gel and immunoblotted with VAMP2 antibodies as described above.

**Immunofluorescence**

Isolated human pancreatic islets were used whole/ or dispersed with Versene solution (by incubation for 10 min in 37 °C) and fixed with 4 % formaldehyde freshly diluted in filtered phosphate buffered saline pH 7.4 (PBS) for 15 min, RT. Cells were washed 3 x with 500 µl of PBS, then blocked and permeabilized with blocking buffer: 5 % normal donkey serum in 5 % Triton X-100 in PBS for 24 h, 4 °C. Cells were washed 3 x with 500 µl of PBS. Primary antibodies were diluted in blocking buffer: anti-insulin (1:200, Progen, #16049), anti-IRIS-1 (1:100, CAPRA, home-made, #613.610), anti-IRIS-2 (1:100, CAPRA, home-made, #612.609), anti-glucagon (1:200, Abcam, #Ab10988) and incubated for 2 days, 4 °C. Cells were washed with 500 µl of PBS 3 x. Secondary antibodies: anti-rabbit Alexa Fluor 546 (1:500, Molecular probes, #A11035), anti-guinea pig Cy2 conjugated (1:500, Jackson ImmunoResearch, #706-225-148), anti-mouse Alexa Fluor 647 (1:500, Thermo Fisher, #A-21235) were diluted in blocking buffer and added for 4 h, 4 °C. Cells were washed with 500 µl of PBS 3 x. PBS was removed and cells were mounted with 50 µl of mounting medium containing DAPI, single drops of cells were placed on glass slides and covered with 15 mm round cover slips. Cells were left to dry for 1-2 days. Images were taken in confocal microscope Zeiss 800.

**Immunofluorescence using peptide blocking of anti-IRIS-1 and anti-IRIS-2 antibodies to verify specificity**

Isolated human pancreatic islets were used whole/ or dispersed with Versene solution (by incubation for 10 min in 37 °C) and fixed with 4 % formaldehyde freshly diluted in filtered phosphate buffered saline pH 7.4 (PBS) for 15 min, RT. Cells were washed 3 x with 500 µl of PBS, then blocked and permeabilized with blocking buffer: 5 % normal donkey serum in 5 % Triton X-100 in PBS for 24 h, 4 °C. Cells were washed 3 x with 500 µl of PBS. Primary antibodies were diluted in blocking buffer: anti-insulin (1:200, Progen, #16049), anti-IRIS-1 (1:100, CAPRA, home-made, #613.610), anti-IRIS-2 (1:100, CAPRA, home-made, #612.609) and incubated overnight in 4 °C. Before adding to the samples
primary antibodies were pre-incubated overnight at 4 °C with constant mixing with or without the peptide-antigens used for the generation of the isof orm-specific antibodies. 1000-fold excess molar concentrations of the peptides were used for the pre-incubation and blocking of the antibodies. After incubation with primary antibodies cells were washed with 500 µl of PBS 3 x. Secondary antibodies: anti-rabbit Alexa Fluor 546 (1:500, Molecular probes, #A11035) and anti-guinea pig Cy2 conjugated (1:500, Jackson ImmunoResearch, #706-225-148) were diluted in blocking buffer and added for 1 h, RT. Cells were washed with 500 µl of PBS 3 x. PBS was removed and cells were mounted with 50 µl of mounting medium containing DAPI, single drops of cells were placed on glass slides and covered with 15 mm round cover slips. Cells were left to dry for 1-2 days. Images were taken in confocal microscope Zeiss 800.

Electron microscopy
1x10⁶ CD59 KO INS-1 cells stably overexpressing human IRIS-1, or IRIS-2, were incubated in HBSS medium containing low (2.8 mM) or high (16.7 mM) glucose for 10 minutes (IRIS-1) or overnight (IRIS-2). Cells were harvested and washed 2 x with PBS, fixed in 1 ml of 2 % paraformaldehyde diluted in 0.1 M Sorenson’s phosphate buffer for 1 h, RT. Then washed in 0.1 M Sorenson’s phosphate buffer, post-fixed in 1 % osmium tetroxide in 0.1 M Sorenson’s phosphate buffer, dehydrated and embedded on Polybed 812 Epoxyresin. Few nm sections were taken using Leica EM Uc/ ultramicrotome and mounted on a Maxaform H5 copper grid. Specimens were then blocked with PBS containing 5 % donkey serum for 30 min, RT and stained overnight in 4 °C with primary antibodies against insulin (1:200, Progen, #16049), IRIS-1 (1:100, CAPRA, home-made, #613.610), or IRIS-2 (1:100, CAPRA, home-made, #612.609). Antibodies were diluted in PBS with 5 % donkey serum. Specimens were washed 1 x with PBS for 5 min, RT and 4 x 5 min, RT with dH₂O. Secondary antibodies with 10 nm gold particles (against insulin) and 20 nm gold particles (against IRIS-1/2 isoforms) were diluted 1: 20 in PBS with 5 % donkey serum and incubated for 2 h, 4 °C. Wash steps were repeated. Grids were examined using a FEI Technai Biotwin 120 kV microscope.

Quantitative PCR
RNA was extracted using RNeasy Plus Mini kit (Qiagen) and cDNA synthesized using oligo-dT primers and SuperScript IV (Invitrogen). Quantitative PCR was performed with specific TaqMan probes (Applied Biosystem) and Viiia7 Real-Time PCR system (Thermo Fisher). Expression levels of mouse CD59A, CD59B were calculated after normalization with the geometric mean of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

VAMP2/ SNAP25- IRIS-1/ IRIS-2 ELISA detection
10x10⁶ (INS-1 CD59 KO cells stably expressing human IRIS-1 and IRIS-2) were incubated overnight in low (2.8 mM), or high (16.7 mM) glucose medium and then lysed by RIPA buffer. Lysates were cleared from insoluble materials by centrifugation at 10000 x G, 5 min, 4 °C. Protein content was determined by BCA kit (Thermo Scientific). 2, 5, 13, 25 and 50 µg of lysates were added to microtiter plates previously coated with 10 µg/ml of anti-VAMP2 (Sysy, Cat. #104211), anti-SNAP25 (Abcam, Cat. #ab5666), or isotype control antibodies (ImmunoTools) diluted in coating buffer (1.6 g Na₂CO₃, 2.9 g NaHCO₃, 500 ml H₂O, pH 9.6) and blocked with PBST (IRIS-1), or 1 % BSA in PBST (IRIS-2). After overnight incubation at 4 °C, plates were washed 4 x with PBST buffer (0.2 % Tween 20 in PBS) and incubated for 1.5 h, 37 °C with 1:500 of biotinylated anti-human IRIS-1/ IRIS-2 antibodies (CAPRA, home-made) diluted in PBST buffer. Human IRIS-1/ IRIS-2 antibodies were biotinylated using EZ-Link NHS-SS-Biotin (Thermo Scientific). After washing, plates were incubated for 1 h with 1:200 of streptavidin-horseradish peroxidase antibody (Thermo Scientific), followed by development with orthophenylenediamine (OPD; Em-Tec, Germany). Absorbance was measured at 490 nm using Varian spectrophotometer (Labx, Midland, ON, Canada).
Expression and purification of human IRIS-1 and IRIS-2 isoforms

cDNA for human IRIS-1 and IRIS-2 were cloned into the pET16b plasmid, and expressed as His-tagged proteins in BL21-RIPL E. coli cells by induction with 1mM IPTG for 3 h at 37 °C. Bacterial cell pellets were resuspended in PBS, lysed by sonication, washed 3 x in wash buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM DTT, 0.5% Triton-X 100) and remaining insoluble inclusion bodies dissolved overnight in urea buffer (6M Urea, 50 mM Tris-HCl, pH 8). IRIS-1 was purified by nickel affinity chromatography and eluted in urea buffer with 0.5 M imidazole. IRIS-1 was refolded according to a published protocol for canonical CD59 (2). Briefly, purified IRIS-1 in urea buffer was rapidly diluted by dripping into 1.5 L refolding buffer (50 mM Tris-HCl, pH 9, 0.5 M L-arginine, 1 mM EDTA, 1 mM L-cystine, 3 mM L-cysteine) and allowed to refold at 4 °C for 2 weeks. The solution was then concentrated down under compressed nitrogen in Amicon cells with 3 kDa cutoff membranes, and dialyzed into 50 mM MES, pH 6.5, before further purification on a monoS ion exchange column with elution under a NaCl gradient. The eluted peak fractions were finally dialyzed to PBS and aliquots frozen at 0.5 mg/ml. IRIS-2 could not be refolded under the same conditions, instead after IMAC purification IRIS-2 dissolved in Urea buffer was concentrated and frozen in aliquots for direct use in western blot and coating onto ELISA plates.

Silver staining of purified human IRIS-1 and IRIS-2

The purity of the purified human IRIS-1 and IRIS-2 was assessed by silver staining. The purified proteins were run under reducing conditions on a homemade 15 % Tris-Tricine SDS-PAGE gels. Gels were then used for silver staining.

Western blot for detection of purified human IRIS-1 and IRIS-2

Purified human IRIS-1 and IRIS-2 were run on a homemade 15 % Tris-Tricine SDS-PAGE gels under reducing conditions. Proteins were subsequently transferred to a PVDF membrane using Trans-Blot Turbo system (Bio-Rad) that was then cut vertically and blocked with Quench buffer. Following, the two membrane halves were incubated with primary antibodies against human IRIS-1 or IRIS-2 (1:1000, CAPRA, homemade, #613.610, #612.609) and then with HRP-conjugated goat anti-rabbit secondary antibody (Dako). The membranes were developed using Immunoblot Western Chemiluminescent HRP substrate (Millipore).

Assessing the sensitivity of the anti-IRIS-1 and anti-IRIS-2 antibodies

ELISA plates were coated overnight at 4 °C with 10 µg/ml recombinant and purified human IRIS-1 or IRIS-2 diluted in PBS. The next day, wells were washed 6 x with Immunowash and then blocked for 2 h at RT with Quench buffer. Subsequently, the wells were again washed 6 x with Immunowash before adding the anti-human IRIS-1 or IRIS-2 antibodies (CAPRA, home-made, #613.610, #612.609) diluted in Quench buffer to the wells. A seven-step two-fold dilution series with 2 µg/ml as highest concentration of the antibodies was used. Following 2 h incubation at RT with the primary antibodies, the wells were washed as previous and then incubated for 1 h at RT with an HRP-conjugated goat anti-rabbit secondary antibody (Dako). The wells were then washed as previous and developed with TMB ONE (3, 3’, 5, 5’-tetramethylbenzidine) (Kementec, Denmark). Reaction was stopped with 0.5 M H2SO4 and absorbance was measured at 450 nm using Cytation 5 imaging reader (BioTek).

Peptide-blocking of anti-IRIS-1 and anti-IRIS-2 antibodies to verify specificity

ELISA plates were coated with 10 µg/ml recombinant and purified human IRIS-1 or IRIS-2 diluted PBS overnight at 4 °C. The following day, the plates were washed 6 x with Immunowash before blocking the wells with Quench buffer for 2 h at RT. 0.5 µg/ml anti-human IRIS-1 or anti-human-IRIS-2 antibody (CAPRA, home-made, #613.610, #612.609) was then added to the wells. The antibodies had been diluted in Quench buffer and pre-incubated overnight at 4 °C with constant mixing with or without the peptide-antigens used for the generation of the isoform-specific antibodies (IRIS-1 peptide-antigen: QGLKTKQPGKKSAS, IRIS-2 peptide-antigen: ELGYHYVAQAGRRQ, GenScript Biotech). A 10-, 100-, and 1000-fold excess molar concentration of the peptides were used for the pre-incubation
and blocking of the antibodies. After a 2 h incubation at RT with the blocked or non-blocked antibodies, the wells were washed as previously and then incubated for 1 h at RT with an HRP-conjugated goat anti-rabbit secondary antibody (Dako). The wells were then washed 6 x with Immunowash and developed with TMB ONE (3, 3', 5, 5'-tetramethylbenzidine) (Kementec, Denmark). Reaction was stopped with 0.5 M H₂SO₄ and absorbance was measured at 450 nm using Cytation 5 imaging reader (BioTek).

Verifying specificity of anti-IRIS-1 and anti-IRIS-2 antibodies by Western blot and peptide-blocking
Cells were lysed with NP-40 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP-40, 5 mM EDTA), human islets were lysed with RIPA buffer (buffer composition above) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Proteins were resolved under reducing conditions in a home-made 15 % SDS-PAGE gel and transferred to a PVDF membrane using Trans-Blot Turbo system (Bio-Rad). Each sample had been loaded in two wells, one on each side of the gel. Following protein transfer, the membrane was cut vertically in the middle resulting in two halves, one for the use of non-blocked antibody against IRIS-1 or IRIS-2, and the other for the use of peptide-blocked antibody. After blocking with Quench buffer, the membrane halves were probed with a primary antibody against beta-tubulin for INS-1 cells (1:50 000, Abcam, #ab6046), against beta-actin for human pancreatic islets (1:5000, Abcam, #Ab8226) and primary antibodies against human IRIS-1 or IRIS-2 (1:1000, CAPRA, homemade, #613.610, #612.609) that had been pre-incubated overnight at 4 °C with constant mixing in the presence or absence of a 1000-fold excess molar concentration of the IRIS-1 or IRIS-2 peptide-antigens. The membranes were then incubated with HRP-conjugated goat anti-rabbit secondary antibody (Dako) and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore). Membranes incubated with non-blocked or peptide-blocked antibodies against human IRIS-1 or IRIS-2 were developed and imaged together, both for the detection of IRIS-1 or IRIS-2, and for the detection of beta-tubulin and beta-actin.

Molecular modelling
Different tools were used to develop the human and mouse protein 3D models, depending on the tasks (i.e., homology modelling, threading, simulations). In all cases, Protein Data Bank (PDB) files were manipulated with Python scripts available in PDB-Tools (3) and with the MayaChemTools Perl scripts (4). Secondary structure predictions were carried out with the PSIPRED server (5). Sequences to 3D structure alignments were computed using the PROMALS3D server (6). Simple energy minimization computations were performed for all 3D models using Chimera utilities (7) but for some models, more advanced simulation protocols were needed (see below).

Molecular modelling of human IRIS-1 and IRIS-2
Experimental structures were downloaded from the PDB (8). The structures of human CD59 PDB files 1cdr (NMR structure with the visible glycan on Asn 18) (9), structure 2, 2j8b (X-ray) (2), 2ofs (X-ray) (10) and of the related Human Secreted Ly-6/uPAR Related Protein-1 (SLURP1), PDB file 2muo (NMR) (Lyukmanova, E.N., Paramonov, A.S., Shenkarev, Z.O., Arseniev, A.S.; To be published) were analysed interactively so as to identify likely flexible loop regions. Sequence analysis was performed with the ORION web server (protein fold recognition using evolutionary hybrid profiles) (11). Visualization was carried out with the PyMOL molecular graphics system (Schrödinger, LLC). The IntFOLD suite (12) (13) was used to build a 3D model for IRIS-1. The main part of IRIS-2 3D model was directly taken from human CD59 while to build the last extra residues (~30 residues) different strategies were applied and three different models were developed. Two models were built using threading with the IntFOLD suite. In addition, a structural prediction of the C-terminal region alone was performed with PEP-FOLD3 (14) and the most likely structure grafted onto the main IRIS-2 structure. Further investigation of IRIS-2 was performed using the coarse-grained protein folding AWSEM-suite (15) with a special emphasis on the folding of the C-terminal region. Predictions of potentially locally intrinsically disordered regions as well as prediction of the possible biological
functions of these putative regions (e.g., protein-protein or protein-DNA binding) were carried out with IUPred2A (16) and the consensus approach DEPICTER (17).

Molecular modelling of mouse IRIS-1 and IRIS-2
The mouse IRIS-1 (mature CD59B_2 (Q6PBG1), 32aa) 3D homology model was built with the Swiss-Model server (18) using as template the human CD59 PDB file 2UX2 (2). This model was first energy minimized and then submitted to molecular dynamics simulations performed with the UNRES (United RESidue) simulation engine (19). The mouse IRIS-2 (mature mouse CD59B_3 (A2BI28), 65 aa) 3D model was built using a sequence to structure alignment against human CD59 carried out with PROMALS3D. Seven amino acids were missing at the C-terminal side. These residues were first built in an extended conformation with PyMol, and the resulting 3D model was then used as input for a molecular dynamics simulation performed with the UNRES tool.

Patch-clamp electrophysiology
Recordings were performed using an EPC-9 patch amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) and PatchMaster software. Patch electrodes (resistance 2-4 MΩ) were made from borosilicate glass capillaries coated with Sylgard close to the tips and fire-polished. Cells were continuously superfused with an extracellular solution containing (in mM) 138 NaCl, 5.6 KCl, 1.2 MgCl$_2$, 2.6 CaCl$_2$, 3 D-glucose, and 5 HEPES, pH 7.4 adjusted with NaOH at a rate of 0.4 ml/min, at 32°C. Voltage-dependent currents and exocytosis were measured in whole-cell voltage clamp mode with an intracellular solution containing (in mM) 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl$_2$, 0.05 EGTA, 3 Mg-ATP, 0.1 cAMP, and 5 HEPES, pH 7.2 adjusted using CsOH. For current-voltage (IV) relationships, the membrane was depolarized from −70 mV to +80 mV (10 mV steps) lasting 50 ms each. Currents were compensated for capacitive transients and linear leak using a P/4 protocol. Na$^+$ and Ca$^{2+}$ current components were separated by quantifying the initial peak current (0–5 ms) representing Na$^+$ current and the average sustained current during the latter part of the depolarization representing the Ca$^{2+}$ current. Exocytosis was detected as depolarization-dependent changes in cell capacitance using the lock-in module of Patchmaster (30 mV peak-to-peak with a frequency of 1 kHz); the protocol consisted of a train of 14 x 200 ms depolarizations to 0 mV at 1.4 Hz.

TIRF-M imaging
Cells were plated in onto 22-mm polylysine-coated coverslips and transfected using lipofectamine with the secretory granule markers NPY-EGFP or NPY-mOrange2 (20). Cells were imaged 30-36 h later using a custom-built lens-type total internal reflection microscope (TIRF-M) based on an AxioObserver Z1 with a × 100/1.45 objective (Carl Zeiss). Excitation from two DPSS lasers at 491 and 561 nm (Cobolt) was passed through a cleanup filter (zet405/488/561/640x, Chroma) and controlled with an acousto-optical tunable filter (AA-Opto). Excitation and emission light were separated using a beamsplitter (ZT405/488/561/640rpc, Chroma). The emission light was chromatically separated onto separate areas of an EMCCD camera (Roper QuantEM 512SC) using an image splitter (Optical Insights) with a cut-off at 565 nm (565 dcrx, Chroma) and emission filters (ET525/50m and 600/50m, Chroma). Scaling was 160 nm per pixel. Cells were imaged in a standard solution containing (in mM) 138 NaCl, 5.6 KCl, 1.2 MgCl$_2$, 2.6 CaCl$_2$, 10 D-glucose, 5 HEPES (pH 7.4 with NaOH). The K$_{ATP}$-channel opener diazoxide (200 μM, Sigma-Aldrich) was included to prevent spontaneous depolarizations. Exocytosis was evoked by rapidly depolarizing cells with elevated K$^+$ (75 mM KCl) equimolarly replacing NaCl in the standard solution, by computer-controlled local pressure ejection.

Quantification and statistical analysis
All experiments were performed in at least 3 independent repeats; with at least 2 duplicate samples per condition in each repeat. All western blots show a representative of at least 3 independent repeats. The mean differences between groups that have been split on two independent variables were compared with 2-way ANOVA. Student’s t-test was used in case of two independent group comparisons and 1-way ANOVA when three or more independent groups were compared. Normal distribution of data was
verified with Q-Q plot, whereas the standard deviation between the groups was visually inspected with scatter plot. Data lacking normal distribution were analysed using a non-parametric Mann-Whitney test. All statistical analyses were performed using GraphPad Prism 9. Values are expressed as a mean ± SD. Statistical tests applied in each experiment are indicated in the figure legends. The Bonferroni, Sidak, and Dunnett’s tests were used in case of multiple comparisons. In all figures, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
### Key resources table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| Guinea pig anti-insulin | EuroProxima | Cat#2263B65-1 |
| Mouse anti-beta-actin | Abcam | Cat#ab8226; RRID: AB_306371 |
| Rat anti-DYKDDDDK (FLAG-tag) | BioLegend | Cat#637302 |
| Mouse anti-VAMP2 | Synaptic Systems | Cat#104211 |
| Rabbit anti-human IRIS-1 | This paper | Cat#613.610 |
| Rabbit anti-human IRIS-2 | This paper | Cat#612.609 |
| Rabbit anti-SNAP25 | Abcam | Cat#ab5666 |
| Rabbit anti-beta tubulin | Abcam | Cat#ab6046 |
| BRIC 229 mouse anti-CD59 | IBGRL Research | Cat#9409PA |
| **Bacterial and virus strains** |        |            |
| DH5alpha | Thermo Fisher | Cat#18265017 |
| BL21-CodonPlus (DE3)-RIPL | Agilent | Part#230280 |
| **Biological samples** |        |            |
| Human pancreatic islets | Nordic Islet Network | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Diazoxide | Sigma Aldrich | Cat#D9035 |
| BAPTA-AM, cell permeant chelator | Thermo Fisher | Cat#B6769 |
| Human IRIS-1, protein | This paper | N/A |
| Human IRIS-2, protein | This paper | N/A |
| IRIS-1 peptide-antigen | GenScript Biotech | Cat#SC1848, Item number: U625QGL310_1 |
| IRIS-2 peptide-antigen | GenScript Biotech | Cat#SC1848, Item number: U625QGL310_3 |
| **Critical commercial assays** |        |            |
| Insulin secretion ELISA assay | Mercodia | Cat#10-1250-01 |
| Mem-PER Plus kit | Thermo Scientific | Cat#8942 |
| **Experimental models: Cell lines** |        |            |
| INS-1 832/13 | Lab of C Newgard | RRID: CVCL_7226 |
| MIN6 | Lab of J. Miyazaki | RRID: CVCL_0431 |
| EndoC-βH1 | Lab of R. Scharfmann | RRID: CVCL_L909 |
| **Experimental models: Organisms/strains** |        |            |
| Diabetic db/db BKS(D)-Leprdh/JOrIrJ mice | Janvier | RRID: MGI:3822315 |
| C57BL/6JRj control mice | Janvier | RRID: MGI:2670020 |
| Akita mice: C57BL/6-Ins2Akita/J | The Jackson Laboratory | JAX: 003548 |
| C57BL/6J control mice | The Jackson Laboratory | JAX: 000664 |
| **Oligonucleotides** |        |            |
| Silencer pre-designed siRNA against human CD59 | Thermo Fisher | #4392420, S269 |
| huCD59_Forward ATC ACA ATG GGA ATC CAA GGA GGG | This paper | N/A |
| huCD59_Reverse CTC TCC TGG TGT TGA CTT AGG G | This paper | N/A |
| huIRIS-1_Forward ATC ACA ATG GGA ATC CAA GGA GGG | This paper | N/A |
| Name                      | Sequence                                      | Source         | Notes   |
|---------------------------|-----------------------------------------------|----------------|---------|
| huIRIS-1_Reverse          | CTC AGG AGA GAG AGG CCG AC                   | This paper     | N/A     |
| huIRIS-2_Forward          | AGT TGG GAT ATC ACT ATG TTG CCC              | This paper     | N/A     |
| huIRIS-2_Reverse          | CTC TCC TGG TGT TGA CTT AGG G                | This paper     | N/A     |
| mouseCD59A_Forward        | CAA TGA GAG CTC AGA GGG GAC                  | This paper     | N/A     |
| mouseCD59A_Reverse        | AGG AGG CAT CTA GGA CTT AGA G               | This paper     | N/A     |
| mouseCD59B_Forward        | CAA TGA GAG CTC AGA GGG GAC                  | This paper     | N/A     |
| mouseCD59B_Reverse        | CTC AAT GAG GAA GTT TCT GCG TTG             | This paper     | N/A     |
| mouseCD59B-IRIS-1/-2_Forward | CAA TGA GAG CTC AGA GGG GAC                  | This paper     | N/A     |
| mouseCD59B-IRIS-1/-2_Reverse | CCA TGA GCA AGC AGG TTT CAG G               | This paper     | N/A     |
| Recombinant DNA           |                                               |                |         |
| pcDNA3-huCD59             |                                               | This paper     | N/A     |
| pcDNA3-huIRIS-1           |                                               | This paper     | N/A     |
| pcDNA3-huIRIS-2           |                                               | This paper     | N/A     |
| pKEVIN-huIRIS-1           |                                               | This paper     | N/A     |
| pKEVIN-huIRIS-2           |                                               | This paper     | N/A     |
| pKEVIN-mouse CD59B-IRIS-1 |                                               | This paper     | N/A     |
| pKEVIN-mouse CD59B-IRIS-2 |                                               | This paper     | N/A     |
| pET16b-human IRIS-1       |                                               | This paper     | N/A     |
| pET16b-human IRIS-2       |                                               | This paper     | N/A     |
| NPY-tdmOrange2            |                                               | Gandasi et al., 2015 |         |
| NPY EGFP                  |                                               | W Almers       | (Portland) |
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Figure S1 (relating to figures 1 and 3F): (A) Expression of human IRIS-1 and IRIS-2 was measured in various human tissues by RT-PCR (relative to GAPDH). Quantification of bands intensity is shown in Figure 1F. N=3, technical replicates. (B) Western blot verifying the specificity of novel, homemade antibodies (generated against the C-terminal domains existing in human IRIS-1 and IRIS-2, but not canonical CD59). FLAG tag immunoprecipitation was carried out on multiple clones of INS-1 WT-CD59, CD59-Knockouts, and CD59-KO cells overexpressing human IRIS-1 or IRIS-2, followed by blotting with homemade rabbit antibodies against IRIS-1 (top panel) or IRIS-2 (bottom panel). We
observe no cross-reactivity between the antibodies: antibody against human IRIS-1 does not recognize IRIS-2 and vice versa. N=4, biological replicates. (C) Western blot verifying the knockdown efficiency of total human CD59 in EndoC-βH1 cells. (D) IRIS-1 and IRIS-2 staining (orange) in intact primary human pancreatic islets. Insulin (green), glucagon (purple), nuclei stained with DAPI (blue). Islets from four different healthy human donors were used.
Figure S2 (relating to figure 5): (A) Scheme representing design of knockouts of mouse CD59B, IRIS-1 and IRIS-2 genes in MIN6 cell line. CRISPR/Cas9 system was used to produce the cells lacking either: all CD59B isoforms- marked as all CD59B KO (excising exons common to both canonical CD59B and IRIS-1, IRIS-2), or lacking canonical CD59B only (excising exon 4, present in canonical CD59B but not in mouse IRIS-1 and IRIS-2 splice isoforms). Primers used to screen potential knockouts are marked on the scheme. (B) PCR products from genomic DNA from isolated clones reveal heterozygous and homozygous knockouts of canonical mouse CD59B only, or total CD59B (including CD59B-IRIS-1 and IRIS-2). WT: wild type, heterozygous knockouts: +/-, homozygous knockouts: -/-.. Un-treated MIN6 cells were used as positive control. PCR for the Cd59a and Gapdh loci were used as positive controls for the PCR reaction. Clone A6: homozygous knockout of canonical mouse CD59B. Clone E11: homozygous knockout of total CD59B (including IRIS-1 and IRIS-2).
N=3, biological replicates. (C) Western blot (tris-tricine gel) verifying the total mouse CD59B, and canonical CD59B knockouts on the protein level. Novel, homemade antibodies generated against the C-terminal domain of mouse IRIS-2 were used. Antibodies against mouse IRIS-1 could not be generated, as non-canonical C-terminus of IRIS-1 is truncated and contains only 1aa. N=4, biological replicates.
Figure S3

(A) Silver staining of purified human IRIS-1 and IRIS-2 following Tris-Tricine SDS-PAGE. (B) Western blot of purified human IRIS-1 and IRIS-2 following Tris-Tricine SDS-PAGE. (C) Binding curves displaying the sensitivity of the anti-IRIS-1 and anti-IRIS-2 antibodies. The ELISA plates were coated with recombinant human IRIS-1 (left) or IRIS-2 (right). The graphs display the combined results of three independent experiments in which duplicates of each antibody and concentration was used. N=3. (D) The specificity of the anti-IRIS-1 (left) and anti-IRIS-2 (right) antibodies was assessed by ELISA and pre-incubation of the antibodies with the peptide-antigens used for the generation of the antibodies. Wells were coated with recombinant human IRIS-1 (left) or IRIS-2 (right). The antibodies were pre-incubated with or without a 10-, 100-, or 1000-fold excess molar concentration of either the IRIS-1 peptide antigen or the IRIS-2 peptide antigen. Both panels display the combined results of three independent experiments in which duplicate wells
were used for each antibody and peptide combination and concentration. One-way ANOVA with Dunnett’s multiple comparisons test where all groups were compared to the non-blocked anti-IRIS-1 (left) or non-blocked anti-IRIS-2 (right). N=3.
**Figure S4** (relating to figure 1, 2 and 4): (A, B) The specificity of the anti-human IRIS-1 antibody (A) and anti-human IRIS-2 antibody (B) was assessed by Western blot and antibody blocking using the IRIS-1, or IRIS-2 peptide antigen. Each cell lysate was loaded on each side of the gel (separately for IRIS-1 and IRIS-2). Following protein transfer each membrane was cut vertically into two pieces. The two halves were probed with anti-human IRIS-1, or anti-human IRIS-2 antibody pre-incubated in the presence or absence of a 1000-fold excess molar concentration of the IRIS-1, or IRIS-2 peptide antigen. The two membrane-halves were developed and imaged together for both IRIS-1 and beta-
tubulin, or IRIS-2 and beta-tubulin detection. The thin vertical line indicates where the membrane was cut prior to the addition of antibody. Representative blots (left) as well as IRIS-1, or IRIS-2 to beta-tubulin ratios quantified by densitometry from three independent experiments (right) are shown. Two-way ANOVA with Sidak’s multiple comparisons test was used for IRIS-1 (A), whereas for IRIS-2 paired t-test was used (B). N=3 for IRIS-1 (A) whereas for IRIS-2 (B) N=4, biological replicates. (C, D). The specificity of the anti-human IRIS-1 antibody (C), and anti-human IRIS-2 antibody (D) was assessed by Western blot on isolated healthy human pancreatic islets and antibody blocking using the IRIS-1, or IRIS-2 peptide antigen. Lysates from islets from three different healthy human donors (3 donors for IRIS-1, 3 donors for IRIS-2) were loaded on each side of the gel (separately for IRIS-1 and IRIS-2). Following protein transfer, the membrane was cut vertically into two pieces. The two halves were probed with anti-human IRIS-1 antibody or anti-human IRIS-2 antibody pre-incubated in the presence or absence of a 1000-fold excess molar concentration of the IRIS-1 or IRIS-2 peptide antigen. The two membrane-halves were developed and imaged together for both IRIS-1 and beta-actin, or IRIS-2 and beta-actin. Membranes for each donor for IRIS-1 (C) and IRIS-2 (D) are shown. IRIS-1 to beta-actin ratios quantified by densitometry for three different donors are shown on right on (C). IRIS-2 to beta-actin ratios quantified by densitometry for three different donors are shown on right on (D). Paired t-test was used. N=3, biological replicates.
Figure S5 (relating to figure 1, 2 and 4): (A, B) The specificity of the anti-human IRIS-1 antibody (A) and anti-human IRIS-2 antibody (B) was assessed by immunofluorescence of isolated primary human pancreatic islets and antibody blocking using the IRIS-1, or IRIS-2 peptide antigen. Primary antibodies against IRIS-1 and IRIS-2 (CAPRA, homemade, #613.610 and #612.609) were pre-incubated with or without the peptide-antigens used for the generation of the isoform-specific antibodies. 1000-fold excess molar concentrations of the peptides were used for the pre-incubation and blocking of the antibodies. Secondary antibodies anti-rabbit (IRIS-1/2) Alexa Fluor 546, and anti-guinea pig (insulin)
Cy2 conjugated were used. IRIS-1/2 showed in (white), insulin (green), nuclei stained with DAPI (blue). Images were taken in confocal microscope Zeiss 800.