SUPPLEMENTARY DATA

The endoplasmic reticulum chaperone glucose-regulated protein 94 is essential for proinsulin handling.

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SUPPLEMENTARY DATA

Supplementary materials and procedures

Small molecule inhibitors

GRP94 inhibitor (PU-WS13, GRP94i) was a gift from Dr. Gabriela Chiosis, Cornell University and The Rockefeller University, USA, and later purchased (cat. # 18409, Cayman Chemicals, Michigan, USA).

PU-WS13 prevents ATP binding and exhibits potent selectivity towards GRP94 (IC_{50} 0.22 μM) as compared to HSP90α (IC_{50} 27 μM), HSP90β (IC_{50} 42 μM) or Trap1 (IC_{50} 7.3 μM) [1]. The inhibitor was reconstituted in DMSO and used in the range of concentrations between 5-20 μM. Tunicamycin and cycloheximide were purchased as a ready to use solution (cat. # SML1287, C4859, respectively, Sigma, Søborg, Denmak) and were used at 2 µg/mL and 100 µM, respectively. Brefeldin A (cat. # B6542, Sigma) was reconstituted with DMSO and used at a concentration of 200 nM.

Cell culture

The rat insulinoma INS-1E cell line was a gift from C. Wollheim and P. Maechler, University Medical Centre, Geneva, Switzerland, and was grown in RPMI-1640 GlutaMAX medium (11 mM glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (P), 100 µg/ml streptomycin (S), 10 mM HEPES, 1 mM sodium pyruvate and 50 µmol/l β-mercaptoethanol. The GRINCH cell line (clone 8 derived from INS-1 cells, stably expressing hProCpepSfGFP) was a gift from P. Arvan, University of Michigan, Ann Arbor, USA, and maintained as in [2]. The GFP-tag is located within the C-peptide coding sequence. Following processing with PC1/3 and PC2 mature human insulin is co-stored in the secretory vesicles. Consequently, upon glucose stimulation mature insulin is co-secreted with C-peptide tagged with GFP. MIN6 β-cells were cultured in DMEM with 25 mM glucose and supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 µg/ml streptomycin, 100 units/ml penicillin and 15% FBS [3]. Cell numbers used in experiments: we have used 300.000 INS-1E cells (Fig. 3B, 6A), 200.000 INS-1E cells (Fig. 6B), 250 dispersed islets (Fig. 6C) per condition.

Generation of CRISPR/Cas9 mediated GRP94 knockout INS-1E cell lines

5×10^4 cells INS-1E cells were plated on 48-well fibronectin coated plates for 48 h (hour). To target *grp94*, Lentivirus containing the vector pLV-U6g-EPCG (coding for Clustered Regularly Interspaced Short Palindromic Repeats/Cas9) with the guide RNA (gRNA) sequence CATCCTGTTAACTTCAGCTTGG (target ID RN0000037174) targeting exon 3 of the *grp94* rat gene or non-targeting gRNA (CRISPR12V-1EA) was used (Sigma, Denmark). When cells reached ~ 60% confluence, 150 µL of Lentivirus in 350 µL of fresh medium was added. 24 h later the medium was changed, and puromycin (cat.# P9620-10ML, Sigma, Denmark) selection started after an additional 24 h. Fourteen days later cells were cloned by limiting dilution in fibronectin coated 96-well plates in 200 µL medium. GRP94 KO was confirmed in two clones by Sanger sequencing of the *grp94* gene (Genewiz Inc., Takeley, UK) and GRP94 protein expression levels evaluated by Western blotting. An additional six clones were selected as controls from non-targeting as well as *grp94* targeting cultures (*grp94* wild-type sequence and GRP94 protein level expression similar to those of parental INS-1E cells).

shRNA Lentivirus production for shRNA mediated GRP94 knock-down

Lentiviral vectors pLKO.1 shRNA (TRCN 0000071924-25-27, Mission shRNA) or empty vector (EV) (SHC001) were purchased from Sigma, Søborg, Denmark. All plasmids contained a puromycin resistance gene. HEK293FT cell-medium pre-incubated with OPTI-MEM-medium (Life Technologies, Naerum, Denmark) without antibiotics for 2 h prior to transfection and pMD2.G envelope plasmid, psPAX2 packing plasmid and the Mission shRNA were mixed in a ratio of 1:3:4, respectively, in OPTI-MEM. Lipofectamine 3000 (Life Technologies, Naerum, Denmark) was diluted with OPTI-MEM without serum. The diluted Lipofectamine 3000 and DNA were mixed gently and incubated for 20 min at room temperature. Then the plasmid/Lipofectamine 3000 mixture was added to the HEK293FT cells and incubated for 16 h. The next day transfection medium was replaced with 10 ml DMEM/glutamax medium, supplemented with 10% FBS and 50 uM β-mercaptoethanol without antibiotics, and 72 h later medium containing virus particles was collected, spun down at 1500 rpm at room temperature and filtered through a 0.45 mm filter. The filtered virus medium was concentrated for the Lentiviral particles using Lenti-X™ Concentrator (cat.# PT4421-2, Clontech, Copenhagen, Denmark) according to the manufacturer's instructions. The concentrated virus pellet was dissolved in 1 ml culture medium and stored at -80°C. Multiplicity of infection (MOI) was determined in INS-1E cells using a 10-fold serial dilution of each Lentiviral supernatant (10^2–10^6 dilutions) and puromycin-resistance selection (2 ug/ml), with untransfected cells serving as negative

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control. After 48 h, the MOI of each shRNA Lentivirus was scored by counting surviving cells under an inverted microscope.

Grp94 knockdown in INS-1E cells and dispersed human islet cells

The INS-1E cell line (passage #58; 8×10^6 cells) was seeded into 75 cm^2 culture flasks, incubated for 48 h, washed twice with HBSS buffer (with Ca^{2+} & Mg^{2+}, Life Technologies, Naerum, Denmark) and transduced with shRNA-carrying Lentivirus. After 48 h knock-down (KD) cells were selected with 2 μg/ml puromycin (Life Technologies, Naerum, Denmark), and the KD efficiency was checked by Western blotting using specific anti-GRP94 antibody. GRP94 KD cells were maintained in growth medium with addition of puromycin once weekly. Cells recovered GRP94 expression after two months in culture.

Donor human islets were received at 80% purity from the European Consortium for Islet Transplantation (ECIT). After 3 days of pre-incubation in RPMI medium supplemented with 10% FBS, 1% P/S and 5.6 mM glucose, 2,000 islets were picked, spun down at 290 rpm for 3 min, washed twice with HBSS buffer (w/o Ca^{2+} or Mg^{2+}, Life Technologies), trypsinized with 500 µl Trypsin-EDTA 0,05% (Life Technologies) and incubated at 37°C for 3 min. The digested islets were dispersed into single cell suspension by one minute of pipetting. Then, 10 ml complete medium was added to neutralize trypsin, and the dispersed cells were centrifuged at 1000 rpm for 5 min, re-suspended in complete medium, plated in ECM-coated 4-well plates (Biological Industries, EC-TC-IF-13) and incubated for 72 h. Next, islet cells were washed twice with HBSS buffer (with Ca^{2+} and Mg^{2+}) and transduced with the concentrated EV or shGRP94 Lentivirus in the presence of 8 µg/mL polybrene (cat. # TR-1003, Sigma, Denmark). After 48 h transfected cells were selected with 2 µg/ml puromycin (Life Technologies, Naerum, Denmark) and seven days later experiments performed.

***In silico* modelling of protein interactions**

The crystal structure of proinsulin (PDB ID: 2KQP), mature IGF-I (PDB ID: 1IMX) and three GRP94 crystal structures, one open (PDB ID: 5ULS) and two closed (PDB ID: 2O1U, 2O1V), were downloaded from the protein data bank [4], and the ZDOCK 3.0.2 modelling software was used to generate a prediction model of their interactions. The top-10 binding prediction complexes were visualized using the molecular visualization system, PyMOL (Schrödinger, USA).

Real-time RT-qPCR

INS-1E cells (1×10^6 per condition) were seeded in 6-cm Petri dishes 48 h prior to experiments and incubated where indicated with the IRE1a inhibitor 4q8C at 30 µM (cat. # SML0949, Sigma) for 4 h, whereafter total RNA was harvested and extracted using the Nucleo-Spin kit (Macheray-Nagel, Bethlehem, USA) according to the manufacturer's instructions. Quality and quantity of the extracted RNA was assessed using a NanoDrop 1000 (Thermo Scientific). Half-a µg total RNA was used for cDNA synthesis with the iScript™ cDNA Synthesis Kit (BioRad, Copenhagen, Denmark). Real-time qPCR was performed as follows: 15 ng cDNA with SYBR-green PCR master-mix (Life Technologies) and specific primers (supplementary figure 1) in a total volume of 10 ul were loaded in 384-well plates and run in a Real-Time PCR machine (Applied Biosystems, Naerum, Denmark) with thermal cycles as follows: hot start 95°C, 10 min; 45X amplification 95°C, 15 sec, 60°C, 1 min; dissociation curve 95°C, 15 sec, 60°C, 15 sec, 95°C, 15 sec. Gene expression levels were normalized to HPRT1.

Glucose-stimulated insulin-secretion

Three hundred-thousand INS-1E cells (control, GRP94 KD or KO) or dispersed human islet cells were seeded in 24-well plates in 2 mL complete medium. 48 h later at 80% confluence medium was removed, and cells were incubated for 2 h in Krebs-Ringer's-bicarbonate-Hepes (KRBH) buffer (149 mM NaCl, 4.4 mM KCl, 1.32 mM $NaH_2PO_4(H_2O)$, 1.32 mM $MgCl_2$, 2.75 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES [pH 7.4] and 0.1 % BSA) with 2 mM glucose to establish basal insulin secretion. Cells were subsequently incubated with KRBH buffer containing 2 mM glucose followed by 20 mM glucose for 1 h or 6 h. Supernatants were collected for measurements of proinsulin and insulin (Cat. # 10-1113-01, 10-11181-01, 10-1232-01 and 10-1250-01, Mercodia, Uppsala, Sweden) according to manufacturer's protocols. To measure insulin content, cells were washed in PBS and lysed using active lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1% NP-40, 1X Roche Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 20 mM iodoacetamide). Proinsulin and insulin contents were measured with specific ELISAs as above. 6 h collection media were concentrated using

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10.000 Da MWCO filters to remove salts and reduce volume. Proteins where then eluted from the filters with 15 uL Activated-Lysis-buffer and 5 uL NuPage LDS Loading buffer and analyzed as in Immunoblotting (see below).

Immunoblotting

Cells were washed in PBS and lysed in lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM KCl, 5 mM MgCl2, 0.5% NP-40) supplemented with protease inhibitors (Roche Indianapolis, IN, USA). After 30 min on ice, samples were spun at 13,000 x g, and the supernatant stored at -80°C. Protein concentration was determined in lysates using the Pierce BCA assay (Thermo Scientific, Copenhagen, Denmark) to adjust for protein concentration. Samples were prepared in Tricine Sample Buffer (Bio-rad) with or without β-mercaptoethanol (Sigma), and protein separated on Criterion™ Tris-Tricine gels (Bio-rad) or Nu-Page 4-12% Bis-Tris Protein Gels (for proinsulin and insulin detection), and transferred to PVDF membranes that were blocked in 5% non-fat milk in Tris-buffered saline (50 mM Tris, 150 mM NaCl; TBS). Primary antibodies were diluted in 2% BSA in TBS + 0.1% Tween (TBST) and incubated with membranes overnight at 4° C. Secondary HRP-conjugated antibodies were diluted 1:10,000 in 1% non-fat milk in TBST. Blots were developed using a chemiluminescence detection system (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific), and light emission captured using an Alphaimager system (Alpha Innotech, Broager, Denmark).
Antibodies used: anti-GRP94 rat monoclonal antibody 9G10 (cat. # ab279, Abcam, Cambridge, UK), anti-GFP (cat. # G1544, Sigma, Denmark), anti-insulin (cat. # 8138, Cell Signaling, USA) and anti-tubulin (cat. # T9026, Sigma, Denmark). For quantification of the protein bands, ImageJ software was used.

Cell viability assay

INS-1E parental cells were plated for 48 h in 48-well plates at a density of 2x10^{5} cells/well. Next, GRP94 inhibitor was added for 4 h or 24 h at a concentration of 5 or 20 μM. Finally, the staining reagent AlamarBlue (Life Technologies) was added to the cell culture for 4 h, incubated at 37°C and the resulting fluorescence was read at 570 nm and 600 nm (reference) on a plate reader.

Cell death assay

Apoptotic cell death was determined by the detection of DNA–histone complexes present in the cytoplasmic fraction of cells using the Cell Death Detection ELISAPLUS kit (Roche, Basel, Switzerland) as described by the manufacturer. Briefly, 5x10^{4} cells were plated in 48-well plates. Forty-eight h later, control (+/- 2 μg/mL of tunicamycin or 1 μM of thapsigargin present for 24 h), GRP94 KD or KO cells were washed with 1 x PBS and lysed in 400 μl lysis buffer for 30 min at room temperature. Lysates were centrifuged for 10 min at 200×g, and 20 μl supernatant and 80 μl immunoreagent (anti-DNA–POD antibody and anti-histone–biotin antibody) were added to streptavidin-coated microtiter plates and incubated for 2 h under shaking conditions (300 rev/min) at room temperature. The solution was then removed and each well washed three times with 250 μl incubation buffer, after which 100 μl ABTS solution was added. Absorbance was measured at 405 nm and 492 nm (reference).

Co-immunoprecipitation

5 x 10^{6} GRINCH or INS-1E cells (transfected 48 h earlier with 20 μg of GRP94-GFP coding plasmid [5, 6] following the Lipofectamine 3000 manufacturer's protocol; Invitrogen #L3000-008) were transferred to 20 mM KRBH for 1 h. Next, cell were lysed in 1 mL of lysis buffer: 10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% NP-40 (IGEPAL®CA-630) and protease inhibitor (Mini Protease Inhibitor Cocktail, Roche #11836153001) for 15 min and spun for 10 min at 14.000 rpm 4^{0}C. Twenty-five μL of GFP-Trap®_MA beads (Chromotek, Planegg-Martinsried, Germany) or uncoupled MA beads were blocked with 5% BSA dilution buffer (as lysis buffer but without NP-40) for 2 h. Lysates were precleared with uncoupled MA beads for 1 h in 1 mL dilution buffer. Subsequently, immunoprecipitation was performed for 1 h at 4°C. After 6x wash in dilution buffer, sample buffer was added and samples heated to 95°C for 10 min and further analyzed by SDS-PAGE and Western blotting.

Electron microscopy

Cells were grown to confluence and were kept in 2 mM glucose containing INS-1E culture media for 4 h on Thermanox coverslips and fixed with 2% v/v glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2). The samples were rinsed three times in 0.15 M phosphate buffer (pH 7.2) and subsequently post-fixed in 1% w/v OsO_4 in 0.12 M sodium phosphate buffer (pH 7.2) for 2 h. The specimens were dehydrated in graded series of ethanol,

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transferred to propylene oxide and embedded in Epon according to standard procedures. Following polymerization the Thermanox coverslips were peeled off. Sections, approximately 60 nm thick, were cut with a Leica UC7 microtome (Leica Microsystems, Vienna, Austria) and collected on copper grids with Formvar supporting membranes, stained with uranyl acetate and lead citrate, and subsequently examined with a Philips CM 100 Transmission EM (Philips, Eindhoven, The Netherlands), operated at an accelerating voltage of 80 k. Digital images were recorded with an OSIS Veleta digital slow scan 2k x 2k CCD camera and the ITEM software package, using automatic selection of the fields to eliminate bias. After manual marking of secretory granules on EM images (19 images of controls and 12 images for GRP94 KD cells, with a total of 427 and 618 granules marked, respectively). Each image was taken randomly over the prepared EM section and only images where >80% of field view was covered by a cell were further analyzed. Density quantification was performed (independently by two observers) with Zen LE software (Carl Zeiss) on 3 independent experiments.
Immunogold staining was performed with anti-insulin antibody (cat. # 8138, Cell Signaling, USA) at 1:50 dilution. Sections were incubated with primary antibody at room temperature for 4 h in 0.01 M PBS with 0.1% BSA and 0.05 M glycine, pH 7.4. Gold /protein A gold 10 nm particles were diluted in 0.05 M TBS with 0.1% BSA, 0.06 PEG, 1% fish gelatin at pH 8.2 and incubated with samples for 2 h. Finally, the samples were stained for 10 min with 0,5% uranyl acetate and the following number of cell images acquired and analyzed: 34 Ctrl, 22 GRP94i treated and 28 GRP94 KO.

Total internal reflection fluorescence (TIRF) microcopy of INS-1E cells and human islets
INS1E-cells were plated on poly-L-lysine (Sigma, Søborg, Denmark) coated coverslips and transfected with DNA plasmids using Lipofectamine 2000 (Life Technologies, Naerum, Denmark) in Opti-MEM (Life Technologies, Naerum, Denmark). Imaging was performed 24 hours after transfection. Human islets were provided by the Nordic Network for Clinical Islet Transplantation (Uppsala, Sweden) and used with ethical permission (regional ethics committee EPN, Uppsala, Sweden). Islets were cultured free-floating in sterile dishes in CMRL 1066 culture medium containing 5.5 mM glucose, 10% fetal calf serum (FCS), 2 mM L-glutamine, streptomycin (100U/ml), and penicillin (100 U/ml) at 37 °C in an atmosphere of 5% CO_2 up to two weeks. The islets were then dispersed into single cells in 2 mL cell dissociation buffer (Thermo Fisher Scientific, Denmark) supplemented with trypsin (0.005%, Life Technologies, Naerum, Denmark) by gentle pipetting for 30 seconds. The trypsin was then inhibited by adding 4 mL serum-containing medium and centrifuged for 5 minutes at 160 g. The resuspended cells were plated onto 22-mm poly-L-lysine coated coverslips and allowed to settle overnight. Cells were infected using Adenovirus adNPY-mCherry. Cells were imaged 24-30 hours post infection using custom-built lens-type total internal reflection (TIRF) microscope based on an AxioVert D1 body (Carl Zeiss) with a × 100/1.45 objective (Carl Zeiss). TIRF illumination was from two AOTF-controlled DPSS lasers at 561 nm and 491 nm (Cobolt, Stockholm, Sweden). The emission light was separated onto the two halves of a 16-bit EMCCD camera (QuantEM 512SC, Photometrics) using an image splitter (Photometrics). Filters sets were Chroma (Bellows Falls, VT, USA) parts zet405/488/561/640x, zet405/488/651/640m, ET525/50m, ET600/50m, zt405/488/561/640rpc. The decay constant of the TIRF field was 100–110 nm, calculated using exit angle of the laser from the objective (through a glass hemisphere) and assuming an index of 1.37 in the cytosol. Cells were excited simultaneously with 491 and 561 nm light and recorded in stream mode with 100 ms exposure at 10 frames s^{-1}. For imaging, cells were bathed in a 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 with NaOH). Exocytosis was not observed under these conditions. For exocytosis experiments the buffer instead contained 10 mM glucose and was supplemented with 2 µM forskolin and 200 µM diazoxide, a K-ATP channel opener that prevents glucose-dependent depolarization. Exocytosis was then evoked by computer-timed local application of elevated K^+ (75 mM KCl equimolarly replacing NaCl) through a pressurized glass electrode similar to those used for patch clamp experiments. All experiments were carried out with constant buffer perifusion at 37 °C. Granule density was calculated using a script that used the built-in ‘find maxima’ function in ImageJ (http://rsbweb.nih.gov/ij) for spot detection. Values were normalized to each cells’ contact area with the coverslip. Exocytosis events were detected manually based on sudden disappearance of the granule fluorescence.

TIRF recordings of plasma membrane PIP3 in MIN6 cells
MIN6 cells were transfected with plasmid DNA for the PIP3 translocation sensor GFP4-GRP1 [7] when seeded onto polylysine-treated 25-mm coverslips. For each coverslip, around 0.2 million cells were suspended in 100 µl Opti-MEM medium containing 0.2 µg plasmid and 0.5 µl Lipofectamine 2000. The transfection was terminated by addition of 2 ml culture medium and then the transfected cells were cultured for 24 h before imaging

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experiments. The GRP94 inhibitor PU-WS13 (20 µM) or vehicle for control was added to the culture 30 min, 4 h or 8 h before imaging.

Before imaging, the GFP4-GRP1-expressing cells were pre-incubated in experimental buffer for 30 min at 37 . The buffer contained 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 3 mM glucose, 0.1% (w/v) bovine serum albumin and 25 mM HEPES (pH adjusted to 7.40 with NaOH). Then the coverslips with attached cells were mounted in a 50-µl chamber on the stage of the microscope and superfused at 0.3 ml/min with buffer at 37 °C.Changes of the PIP3 concentration was recorded using a custom-built TIRF microscope based on an E600FN upright microscope with a 60x, NA 0.8 water immersion objective (Nikon). A diode-pumped solid-state 491 nm laser (Cobolt AB, Stockholm, Sweden) was used for excitation of GFP4-GRP1. Fluorescence was detected at 530 nm (35 nm half bandwidth interference filter, Semrock, Rochester, NY, USA) with a back-illuminated EMCCD camera (DU-897, Andor Technology, Belfast, Northern Ireland, UK) controlled by MetaFluor software (Molecular Devices, Downingtown, PA, USA). The PIP3-dependent translocation of GFP4-GRP1 to the plasma membrane was detected as increases of TIRF intensity in the cell contact area.

Confocal imaging of INS-1E and GRINCH cells

Cells were transfected with ER-mCherry (a gift from Michael Davidson, Addgene plasmid # 55041), grown to 80% confluence on coverslips coated with fibronectin and fixed in 2% formaldehyde, permeabilized with 0.1% Nonidet P-40, incubated in blocking solution, and immune-labeled with mouse monoclonal proinsulin specific antibody (GS-9A8, recognizes a junction between B and C proinsulin chains [8]) followed with fluorescein isothiocyanate-conjugated secondary antibody. GRINCH cells were not stained, but their GFP fluorescence from stably expressing hProCpepSfGFP acquired. The cover slips were mounted on Prolong Gold antifade reagent (Cat. # P36930, Invitrogen, Copenhagen, Denmark). Samples were imaged on an upright laser scanning confocal Zeiss LSM 710 microscope through a Plan-apochromat 63x/1.4 objective and analyzed using ZEN software (Carl Zeiss, Birkerød, Denmark). Three independent experiments were performed for INS-1E and GRINCH and 4-7 images with an average of 4-20 cells per image were acquired per condition.

Single-cell RNA-sequencing of pancreatic islets

The expression of the *grp94* gene (HSP90B1, Entrez ID7184) in human islet cell types was determined by reanalyzing published human islet single cell sequencing data (EBI accession number: MTAB-5061) [9]. FastQ files were downloaded from ArrayExpress (accession: E-MTAB-5061). Data was analyzed with bcbio-nextgen (https://github.com/chapmanb/bcbio-nextgen), using the hisat2 algorithm [10] to align reads to human genome version hg38 and the Salmon algorithm [11] for quantitation of gene counts. Only cells that passed the quality control in the original study [9] were maintained for further analysis. We also maintained the cell type classification from the original study.

Metabolic Labeling

INS-1E control or GRP94 KO cells were incubated in medium lacking cysteine/methionine for 30 min and then pulse labeled for 1 h with ^{35}S-Met/Cys (specific activity >1,000 Ci per mmol). At chase time 1 h, cells were washed once with PBS containing 20 mM N-ethyl maleimide (NEM) and then lysed in radioimmunoprecipitation assay buffer (25 mM Tris, pH 7.5; 1% Triton X-100; 0.2% deoxycholic acid; 0.1% SDS; 10 mM EDTA; and 100 mM NaCl) plus 2 mM NEM and a protease inhibitor cocktail. Cell lysates, normalized to trichloroacetic acid precipitable counts, were pre-cleared with zysorbin and immunoprecipitated with anti-insulin antibodies and protein-A agarose overnight at 4 °C. Immunoprecipitates were analyzed by nonreducing and reducing Tris–tricine–urea–SDS-PAGE and with phosphorimaging.

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Supplementary table 1. Rat specific primer sequences. Melting curves and cDNA template dilution-based standard curves were used for validating the specificity and efficiency (>90%) of each primer set.

primers	Forward	Reverse
Ins-1	5’ GGGGAACGTGGTTTCTTCTAC 3’	5’CCAGTTGGTAGAGGGAGCAG 3’
Ins-2	5’CAGCACCTTTGTGGTTCTCA 3’	5’CACCTCCAGTGCCAAGGT 3’
sXBP1	5’CTGAGTCCGAATCAGGTGCAG 3’	5’ATCCATGGGAAGATGTTCTGG 3’
usXBP1	5’CAGCACTCAGACTACGTGCG 3’	5’ATCCATGGGAAGATGTTCTGG 3’
ATF4	5’GTTGGTCAGTGCCTCAGACA 3’	5’CATTCGAAACAGAGCATCGA 3’
CHOP	5’CAGCGACAGAGCCAAAATAAC 3’	5’TGTGGTGGTGTATGAAGATGC 3’
BiP	5’CTGGCACTATTGCTGGACTG 3’	5’CCACCACTTCAAAGACAGCA 3’
HNF4A	5’AGATCACCTCCCCCATCTCT 3’	5’GAGCAGCACATCCTTGAACA 3’
CTCF	5’CATGCATTTCAAGCGCTATC 3’	5’CTCCTCCATTTTCCCCTTCT 3’
HSP90b1	5’GTCGGGAAGCAACAGAGAAG 3’	5’CTGGTATGCTTGTGCCTTCA 3’
HPRT1	5’GCAGACTTTGCTTTCCTT 3’	5’CCGCTGTCTTTTAGGCTT 3’

Supplementary table 2. Rat specific primer sequences for *grp94* gene, exon 3 sequencing.

Gene	**Forward**	**Reverse**
HSP90B1 (GRP94)	5’-GGAATAAGCCTCGGCATCCA-3’	5’-GGGATATCTGAAGCGGTGGG-3’

Supplementary table 3. The list of predicted GRP94 (2O1V):proinsulin interacting amino acids. Bold: GRP94 client binding site residues.

	Proinsulin aa	**GRP94 aa**
GRP94:proinsulin	**Chain B**: F1, V2, N3, H5, L17 **Chain C**: none **Chain A**: R65, Q70, T73, S74, I75, S77, L78, Y79, Q80, E82	I497, H500, R503, F540, Y575, I577, Q578, P581, R587, F588, Q651, **Y652, G653, W654, M658, M662, N676,** Y677, Y678
GRP94:proinsulin	**Chain B**: F1, V2, N3, Y16, L17, G20, E21, R22, F25 **Chain C**: R31, R32, D36, Q38, V42, A50, G51, S52, L53, Q54, P55, L56, L58, L62, R65 **Chain A**: E69, T73, S74, I75, S77, Y79, N83, N86	M85, M86, I89, E157, V160, K161, G164, T165, Y200, S225, D226, S227, N228, E229, F230 (monomer 1) H423, D424, K428, E449, T450, Q452, Q453, L494, I497, E498, H500, R503, F540, Y575, I577, Q578, A579, P581, R587, N590, K593, Q651, **Y652, W654, E659, M662, K663, Q665, A664, G670, K671, D672, I673, S674, T675, N676**, Y677, Y678, A679, S680 (monomer 2)

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Supplementary table 4. Specificity and cross-reactivity of insulin and proinsulin ELISAs provided by manufacturer Mercodia (Sweden)

	Rat Insulin ELISA 10-1250 % of reactivity	Rat/mouse Proinsulin ELISA 10-1232 % of reactivity	Human Insulin ELISA 10-1113 % of reactivity	Human Proinsulin ELISA 10-1118 % of reactivity
Rat Insulin	100	< 0.000004	0.7	nd
Human insulin	167	< 0.0000001	100	< 0.03
Rat proinsulin both genes/1/2	7/nd/nd	nd/100/145	nd	nd
Human proinsulin	75	0.5	< 0.01 Des 31-32 <0.5 Split32-33 <0.5 Des 64-65 98 Split 65-66 56	100 Des 31-32 95 Split32-33 95 Des 64-65 84 Split 65-66 90
Rat C-peptide	< 0.001	< 0.0000001 %	nd	nd
Human C-peptide	< 0.05	< 0.000001	<0.01	< 0.006

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SUPPLEMENTARY DATA

Supplementary Figure 1. GRP94:IGF-1 or :proinsulin interaction models and binding specificity in GFP-trap immunoprecipitation. **A.** GRP94-IGF-1 interaction was analyzed *in silico* by the docking computational modelling software (ZDOCK 3.0.2) with IGF-1 (1IMX, red) and open GRP94 crystal structure (5ULS). Top-scored prediction model is presented. **B.** and **C.** GRP94:proinsulin models from figure 1A with highlighted predicted interacting surfaces: proinsulin molecule surfaces marked red (B) and GRP94 surfaces marked dark blue (C). CBD of GRP94 monomers marked yellow and orange (B). **D.** GRINCH cells (INS-1E cells stably expressing hPro-CpepSfGFP) were lysed and proinsulin subjected to immunoprecipitation (via GFP-tag) and analyzed by SDS-PAGE and Western blotting for the presence of proinsulin and GRP94. Additionally, control beads without anti-GFP antibody were used to test non-specific binding to beads (representative of n=2).

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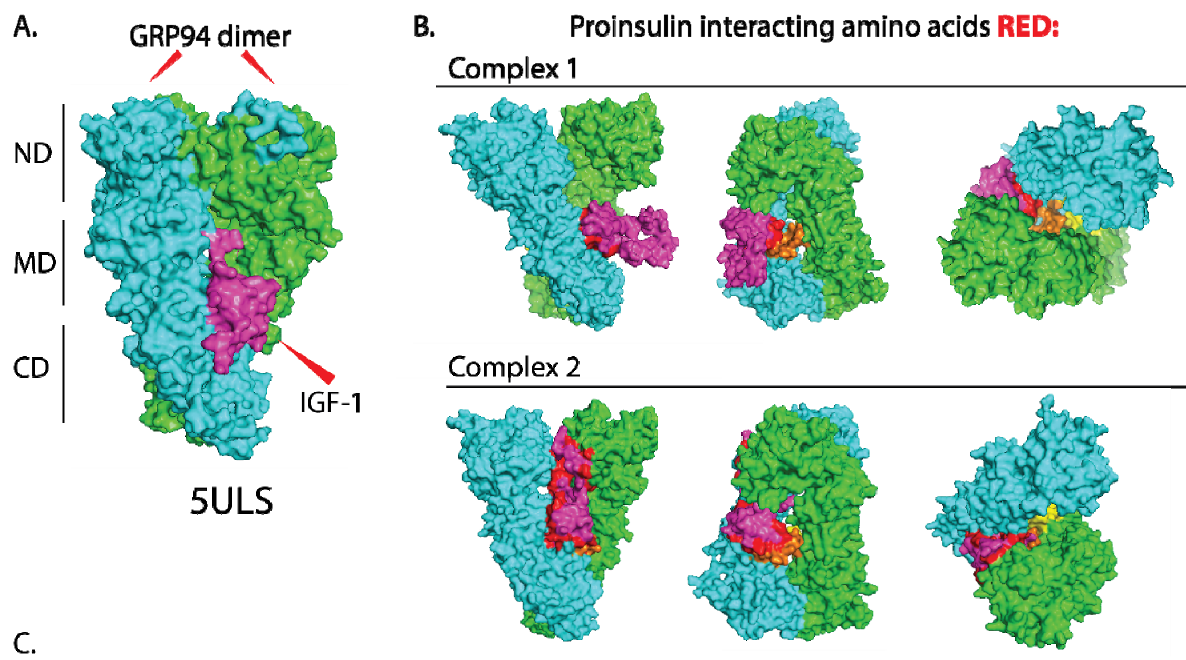

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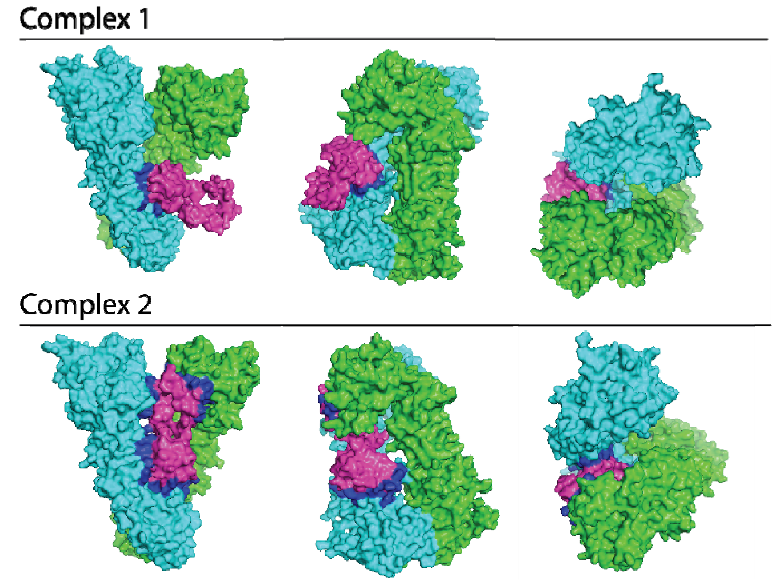

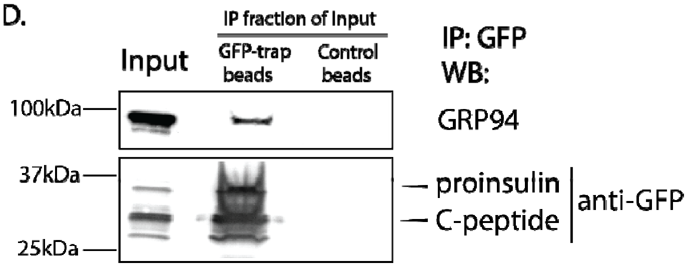

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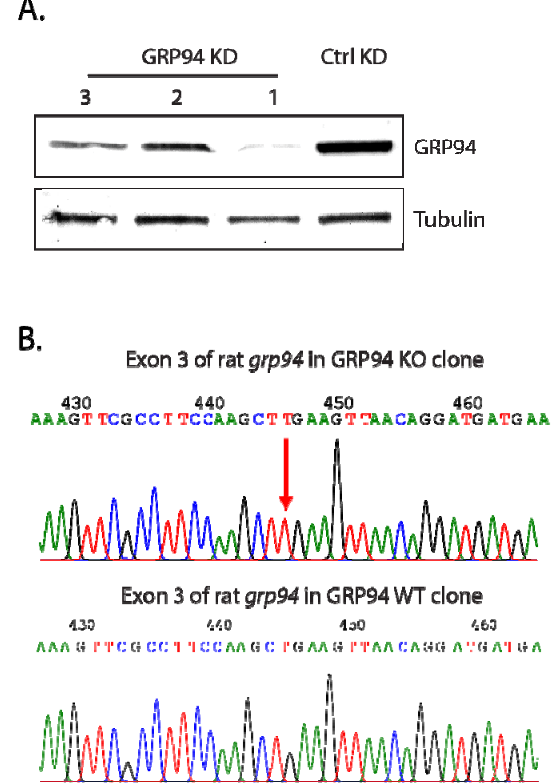
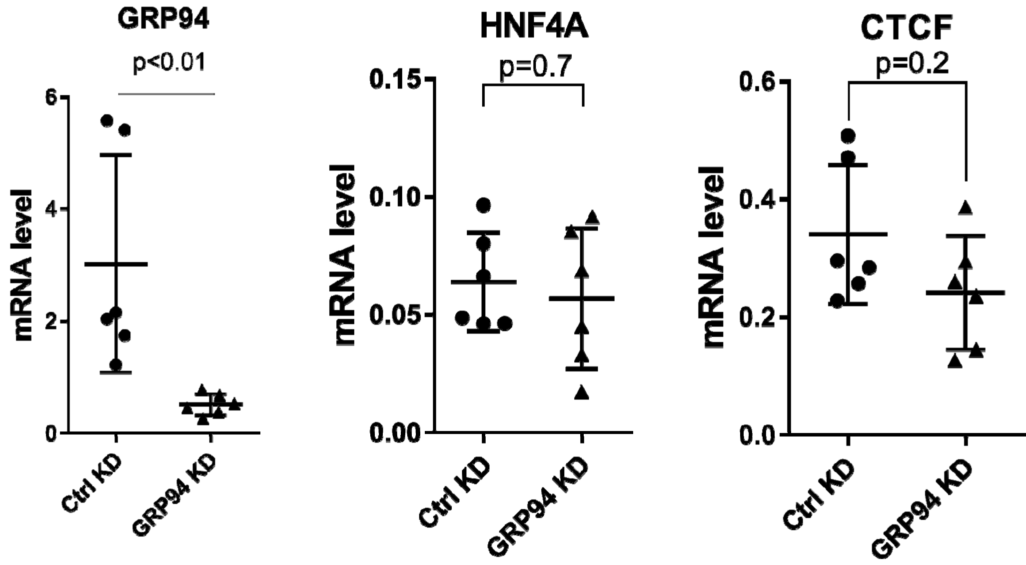
SUPPLEMENTARY DATA

Supplementary Figure 2. Efficient GRP94 KD or KO in INS-1E cells. **A.** SDS-PAGE and Western blot analysis of GRP94 expression levels in INS-1E cells two weeks after Lentiviral transduction of three different GRP94 targeting or non-targeting shRNAs, n=3. **B.** Sequencing results of exon 3 of *grp94* after CRISPR/Cas9 induced insertion of 1 bp T (indicated by red arrow) in GRP94 KO clone of INS-1E cells and its wild type counterpart in control clone.



Supplementary Figure 3. GRP94 KD does not lead to a global inhibition of transcription. mRNA levels of GRP94 and transcription factors CTCF and HNF4a were analyzed by quantitative reverse transcription-PCR (qRT-PCR) in INS-1E control and GRP94 KD cells. Data represents the means±SD analyzed by Bonferroni-corrected paired t-test of treatments versus control, n=6.



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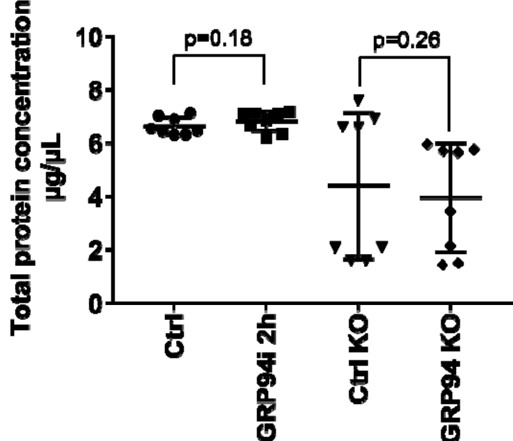
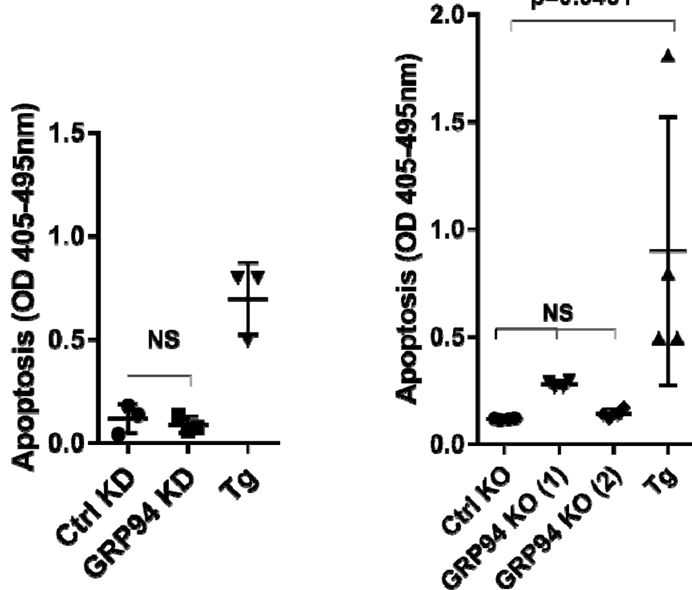
Supplementary Figure 4. Protein concentration analyzed by the bicinchoninic acid assay (BCA) of the whole cell lysates of 500.000 or 1 mln INS-1E cells lysed in 40 or 100 µL of lysis buffer. Data represents the means±SD analyzed by Bonferroni-corrected paired t-test of treatments versus control, n=8.



Supplementary Figure 5. GRP94 KD and KO do not lead to increase apoptosis in INS-1E cells. Apoptosis levels (representing internucleosomal degradation of genomic DNA) were analyzed in GRP94 KD/KO and control cells exposed for 24 h to 1 µM of thapsigargin as positive control. Raw data presenting experiments with the separate controls for KD and KO. Data represent the means±SD analyzed by Bonferroni-corrected paired t-test of treatments versus control, KD: n=4, KO n=4.



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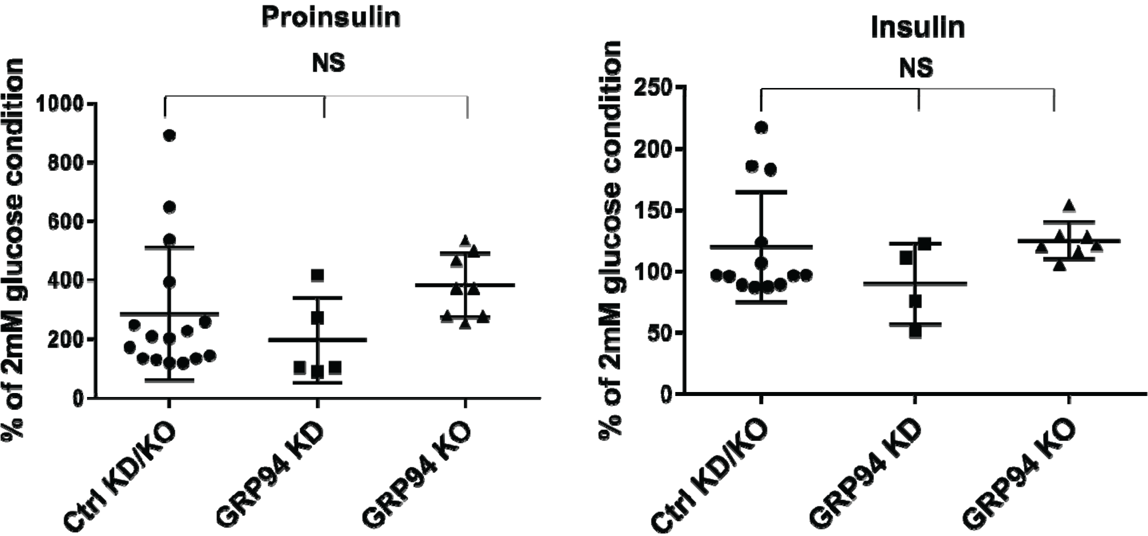
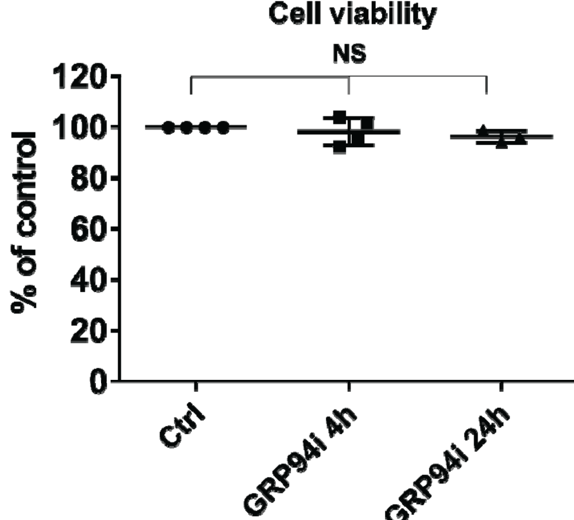
Supplementary Figure 6. Comparable glucose induced increase in intracellular proinsulin levels in control and GRP94 KD/KO cells. Intracellular proinsulin and insulin levels in control and GRP94 KD/KO INS-1E cells in response to switch in glucose concentration from 2 to 20 mM over a period of 2 h analyzed by specific ELISAs. Data analyzed by the paired t-test values of treatments versus control (Proinsulin: KD: n=5, KO: n=8, Insulin: KD: n=4, KO: n=7, graphs represent means ± SD).



Supplementary Figure 7. GRP94 inhibitor does not reduce INS-1E cells viability. The cells were treated with 20 µM of GRP94 inhibitor (PU-WS13) for 4-24 h. Staining reagent (AlamarBlue) was added to the cell culture for 4 h, incubated at 37°C, and the resulting fluorescence was read on a plate reader. The graph shows changes in fluorescence levels as % of control conditions. Data analyzed by the paired t-test values of treatments versus control (n=4 for 4h, n=3 for 24h), graphs represent means ± SD).



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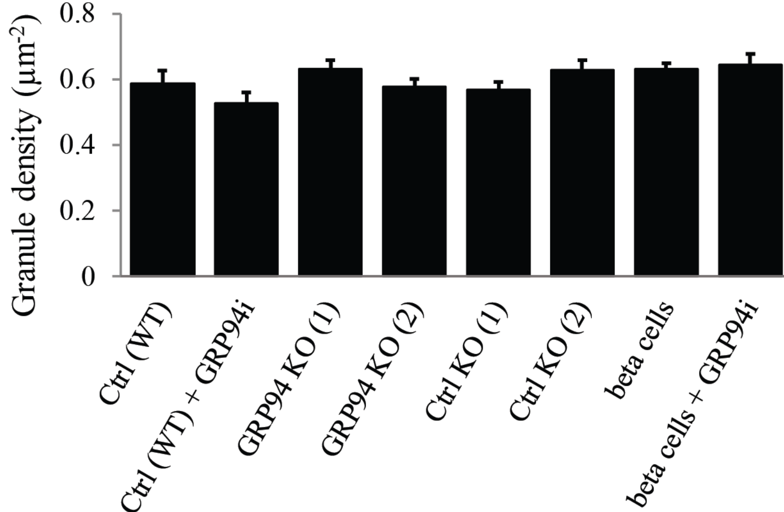
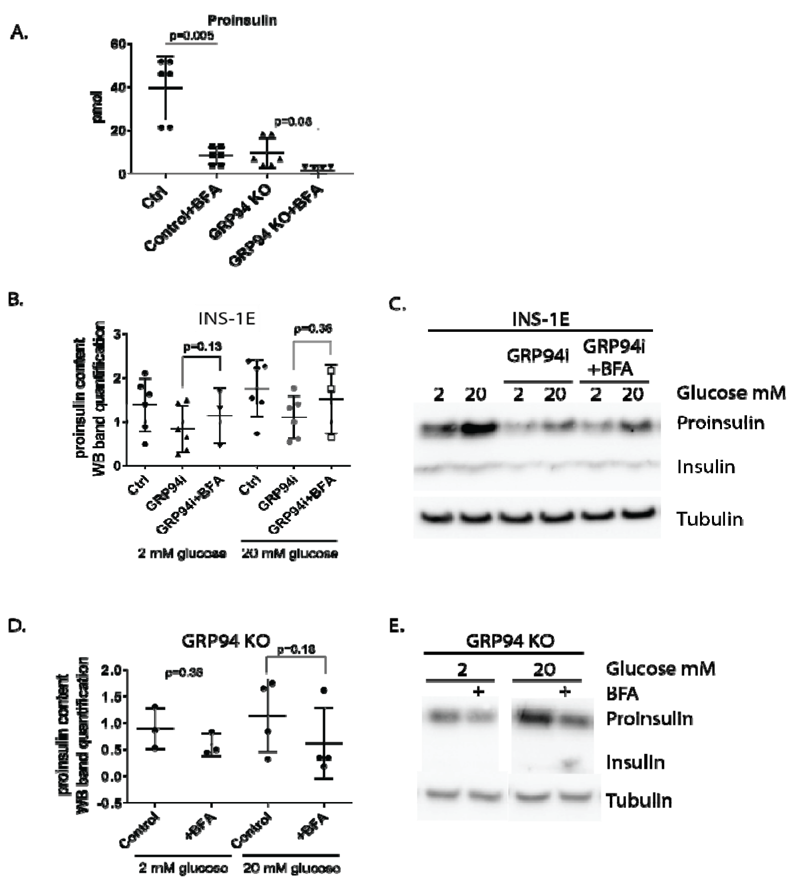
Supplementary Figure 8. Average granule density, containing NPY-GFP, normalized to footprint area of different cell types and conditions for Fig. 4 G-J.



Supplementary Figure 9. Exposure of control and GRP94 KO cells to Brefeldin A reduces proinsulin secretion but does not restore intracellular proinsulin content. **A.** Secretion of proinsulin, measured by ELISA, from KO-control and GRP94 KO cells after a 3 h exposure to 200 nM of Brefeldin A in 2 mM glucose containing KRBH, n=4. **B-E.** INS-1E cells treated with 20 µM of GRP94i or GRP94 KO cells were exposed to 200 nM of Brefeldin A for 4 h, lysed and their proinsulin content analyzed by WB (**C** and **E**). The experiments were repeated (B: GRP94i n=6 GRP94i+BFA n=3, D: 2mM glucose n=3, 20mM glucose n=4)and quantification of proinsulin bands performed (**B** and **D**). Data analyzed by the paired t-test values of treatments versus controls and graphs represent means ± SD.



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SUPPLEMENTARY DATA

Supplementary video material 1 and 2. Exocytosis events in human pancreatic β-cells transfected and expressing neuropeptide-Y (NPY)-GFP as fluorescent granule marker. Representative TIRFM videos of Ctrl (1) and GRP94i-pretreated (2) cells. The frequency of events can be observed after second two of the video, when depolarization events star.

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