SUPPLEMENTAL MATERIALS

Supplemental Methods

Genetic studies: Study protocols were approved by the Ethical Committee of the Medical School of the Jagiellonian University in Krakow, Poland, and the Joslin Diabetes Center, Boston, MA. All participants signed the informed consent forms at entry into the study. In both studies subjects were Caucasians. Type 2 diabetes was diagnosed according to World Health Organization criteria (World Health Organization, 1999), as previously described in detail (1, 2).

To be classified as type 2 diabetes, the subject's diabetes must have been managed without insulin therapy for at least two years after the diagnosis of diabetes. For both studies, we recruited only patients with type 2 diabetes diagnosed between 35 and 70 years. The control groups consisted of individuals older than 35 years with a negative history of type 2 diabetes (not taking any glucose-lowering medications) and normal fasting glucose. Subjects underwent a standardized physical examination and provided a medical history and a blood sample for biochemical measurements and DNA extraction.

Krakow case-control study: In the Polish group, study participants were residents of metropolitan Krakow, a large city in Southeastern Poland. Unrelated type 2 diabetic case subjects were recruited from among patients attending the Clinic of Metabolic Diseases at the Medical School of the Jagellonian University in Krakow, Poland. The control group consisted of non-diabetic spouses of type 2 diabetic patients and volunteers from personnel of the local University Hospital. In total, 805 unrelated individuals were enrolled into this study: 451 cases with type 2 diabetes and 354 non-diabetic controls. The study group has been

described previously (3). The clinical characteristics of the study groups used in this study are shown in the Supplemental Table 2.

Joslin case-control study: In the US group, cases with type 2 diabetes were previously used for studies on genetics of diabetic nephropathy (4). Controls consisted of non-diabetic parents of patients with type 1 diabetes who were enrolled in family studies on the genetics of diabetic nephropathy at the Joslin Clinic (5). A total of 315 unrelated cases with type 2 diabetes and 348 unrelated non-diabetic controls were included in the study. The clinical characteristics of the study groups are shown in the Supplemental Table 2. These study groups did not overlap with patients from the Joslin Diabetes Center used in the study reported by Prudente et al JCEM 2009 (6).

Sequencing: To search for polymorphisms in *TRB3*, we sequenced genomic DNA from 12 individuals (8 patients with type 2 diabetes and 4 non-diabetic controls). Reference sequence NM_021158 from GenBank was used to design primers, which covered all four exons and at least 100 bp of the exon–intron boundaries on each side of the exon as well as 400 bp of the promoter region. Bi-directional sequencing was performed using BigDye chemistry according to the manufacturer's protocol. Products of the sequencing reaction were loaded onto polyacrylamide gels, and electrophoresed on an ABI 377 sequencer (Applied Biosystems). The gel file data were analyzed using Sequencing Analysis 3.3 program. Forward and reverse sequences were compared to each other for greater accuracy using Sequencher 4 program (Gene Code Corporation) to find DNA polymorphisms.

We identified six polymorphisms (see supplemental Table 2).

Genotyping: For each of the six polymorphisms selected for this study, a separate DNA fragment was amplified using PCR protocol (Sequences of primers used in this study are available upon request). Polymorphism rs2295490/Q84R was genotyped by RFLP using Msp I enzyme. The 33 bp insertion/deletion in exon 1 was genotyped by separation of PCR products on agarose gel. Other polymorphisms were genotyped by the single base extension/fluorescence polarization method (AcycloPrime-FP SNP Detection System Wallace Victor2 Multilabel Plate Reader -Perkin Elmer) following the manufacturer's protocol. We re-genotyped 10% of the samples for quality control and there was 100% agreement between results obtained during the first and the second genotyping. To assess linkage disequilibrium (LD) between rs2295490/Q84R and other polymorphisms, we applied two methods.

First, we genotyped the initial panel of 169 cases with type 2 diabetes and 143 non-diabetic controls from Krakow for all five SNPs identified in our study. The frequencies of genotypes for the six polymorphisms in T2DM cases and controls are shown in the Supplemental Table 1.

Second, we genotyped rs2295490/Q84R in 91 CEPH (Centre d'Etude du Polymorphisme Humain) individuals that were genotyped by HapMap and analyzed the results together with all polymorphisms in the 20 kb region encompassing *TRB3* (HapMap Release#21/phaseII, Jul. 2006 http://www.hapmap.org).

Genetic data analysis: Data were analyzed with SAS software (SAS version 8.02). χ^2 and Student's t-test (2-sided) were used for comparison of categorical and continuous variables, respectively. Odds ratios and 95% confidence intervals were calculated. HaploView software (7) was used to calculate Hardy-Weinberg equilibrium, linkage disequilibrium and identify haplotype blocks and estimate the frequency of the haplotypes. Haplo-blocks were defined as described by Gabriel et al. (8)

Supplemental Results of Genetic Studies:

We sequenced 400 bp of the promoter and all 4 exons, including at least 100 bp of the flanking intronic sequences, of *TRB3* in 12 individuals (8 patients with type 2 diabetes patients and 4 controls). We found six polymorphisms with frequencies 4.2% (one in 24 chromosomes) or higher, five of which have been previously described and identified one new polymorphisms (Supplementary Table 3). In the 5'UTR of exon 1 we found a 33 nt insertion/deletion that has five alleles varying from 2 to 5 copies of the 33 nt fragment. In coding regions, we found 3 polymorphisms, all previously described. Only one in exon 2 and designated rs2295490/Q84R changed the amino acid sequence. The other two, rs6051637/Y111Y in exon 3 and rs6115830/A323A in exon 4, were synonymous. In the 3'UTR of exon 4 we found one new polymorphism, 16311CT, and one previously deposited in dbSNP, rs11537607.

Initially, all six polymorphisms were genotyped in 169 cases with type 2 diabetes and 143 non-diabetic controls from the Krakow population

(Supplementary Table 3). All of them were in Hardy-Weinberg equilibrium. The distribution of alleles or genotypes was not significantly different between cases and controls for any of the polymorphisms. However, the results for the rs2295490/Q84R polymorphism were of further interest and we genotyped a larger group of cases and controls from Krakow and Joslin. The results are shown in Supplemental Table 1. A combined analysis of the two studies using logistic regression showed the effect of R variant was significant under any of the three genetic models. However, after corrections for multiple tests the effect under the dominant genetic model was the strongest (P=0.004x3=0.012)

Linkage disequilibrium among *TRB3* polymorphisms was assessed first by analyzing genotypes in 143 non-diabetic controls from the Krakow population. rs2295490/Q84R was not a part of any haplo-block. The only polymorphism with high LD with rs2295490/Q84R was located in exon 3, rs6051637/Y111Y, D'=1 and r^2=0.1. To assess LD between rs2295490/Q84R and polymorphisms in *TRB3* locus other than those identified in our study, we used a recent HapMap database (http://www.hapmap.org). Since rs2295490/Q84R had not been genotyped in that project, we genotyped it in the same 91 CEPH (Centre d'Etude du Polymorphisme Humain) individuals that were genotyped by HapMap. We analyzed rs2295490/Q84R together with the polymorphisms in the 20 kb region encompassing *TRB3*. Again rs2295490/Q84R was not part of any haplo-block. To be certain that no other polymorphism in close proximity to rs2295490/Q84R could have been missed in the initial sequencing, we sequenced the DNA of exon 2 from 50 individuals, including 26 Q84R heterozygotes and 5 84RR

homozygotes. No new sequence differences were observed. Therefore we inferred that the increased risk of type 2 diabetes in our two Caucasian populations was due to 84R variant in *TRB3*.

Guided by the functional studies described below that suggested a diminished function in β-cells expressing the 84R variant, we analyzed clinical data for an association between 84RR genotype and β-cell function. In the Krakow group of cases with type 2 diabetes, a higher proportion of 84RR homozygotes were treated with insulin than Q84 carriers: n=10 (76.9%) vs n=205 (47.9%), p=0.04. Moreover, the level of C peptide in cases was lower in 84RR homozygotes than in Q84 carriers, mean±SD: 1.88±0.63 vs. 2.77±2.2, p=0.003.

Functional Studies:

Animal

A 420 bp fragment of the rat insulin promoter (RIP1) (9) and a 1 Kb fragment encoding mouse TRB3 cDNA were subcloned into the pWhere vector containing two H19 chromatin insulators (Invivogen). RIP-TRB3 transgenic mice were generated on the C57BL/6 backgrounds and two founders F1 and F2 were characterized. Mice were genotyped using the following primer sets: F1 RIP, GAGCTACACCTCTGGCTGCT; F WT, TCTGCCTACCTACCCCTCCT; R, GGTGTACTCTGTGCCTGTGG. Mice were housed in pathogen-free facilities and maintained on a 12 hr light/dark cycle at the Foster Biomedical Research Laboratory of Brandeis University in Waltham, MA. All protocols were approved

by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and Brandeis University and were in accordance with NIH guidelines.

MIN6 cells

MIN6 cells were used between passages 26 and 40, and grown in high-glucose DMEM as reported previously (10). For insulin (100 nM) or glucose (16.7 nM) stimulation experiments, MIN6 cells were serum starved and processed using standard protocols (10).

Physiological experiment

Blood glucose was monitored with an automated glucose monitor (Glucometer Elite, Bayer) and plasma insulin by ELISA (Crystal Chem). Glucose tolerance tests and glucose stimulated insulin secretion tests were performed 16 hr after fasting. Glucose and insulin tolerance tests were performed as described previously (11). For glucose stimulated insulin secretion tests, blood samples were collected before and after i.p. glucose injection (1.5 g/kg bw) at 2 and 5 min for insulin.

Co-immunoprecipitation

MIN6 cells expressing either Q84 TRB or 84R TRB3 were washed twice with ice-cold PBS and lysed on ice with ice-cold RIPA lysis buffer as previously described (12). Protein concentrations were determined using BCA (Pierce). Cell lysates (0.5-1mg) were pre-cleared with protein A/G agarose for 1 h rocking at 4°C. Pre-cleared cell lysates were then incubated with covalently coupled anti-Akt antibody on protein A/G agarose (13) overnight rocking at 4°C. After incubation, the immunoprecipitated proteins and the protein A/G agarose were washed 5

times with ice-cold RIPA buffer before being subjected for denaturation in samples buffer and analyzed with Western blotting.

Western blotting

MIN6 cells or dispersed human islet cells cultured in 6-well plates were washed twice with ice-cold PBS and lysed on ice with 200 μl ice-cold RIPA lysis buffer. Protein concentrations were determined using BCA (Pierce). Cell lysates (20−50 μg) were subjected to SDS−PAGE, followed by immunoblotting using specific antisera and detection with chemiluminescence (Roche). Multiple exposures were used to make sure the signal linearity. Images were quantified by ImageQuant software.

Non-radioactive pulse chase

The Click-iT™ AHA (L-azidohomoalaine) for nascent protein synthesis kit (Invitrogen) was used for these studies and experiments were performed according to manufacturer's recommendation. Briefly, MIN6 cells were seeded into 6 cm plates and allowed to grow to 80-90 % confluency. Cells were starved with methionine-free media for 1 h prior to incubation with Click-iT AHA containing methionine-free media for 2 h (pulse). Subsequently, these labeled cells were then incubated in normal serum media for the indicated periods before harvesting (chase) for analysis. Harvested cells were lysed and the cell lysates were labeled with biotin-conjugated alkyne and biotin labeled proteins were immunoprecipitated with streptavidin beads and further analyzed by Western blotting.

Insulin secretion

Human islets were dispersed as previously described (14) and infected with respective recombinant adenovirus and incubated for 24 h. For these experiments, the dispersed human islet cells were first washed with PBS and incubated in Functionality media (Cellgro) with 1 % human serum albumin and 2.8 mM glucose for 14 h, followed by incubation in Krebs-Ringer-HEPES buffer (15) (KRB, 125 mM NaCl, 4.74 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM Hepes (pH 7.4), 1% human serum albumin) with 2.8 mM glucose for 1 h. The cells were then "stimulated" with further incubated in either the same KRB buffer containing 2.8 mM glucose (basal concentration) or KRB buffer containing16.7 mM glucose (stimulatory concentration). Aliquots of the media were collected at 15, 30 or 60 min after stimulation. The insulin concentration in supernatant was measured by ELISA using rat insulin as a standard. Secretion data was normalized to total cellular protein.

MIN6 cells were seeded in 6-well plates and incubated for 24 h before infection with respective recombinant adenoviruses and incubated for an additional 24 h. Before glucose stimulated-insulin secretion (GSIS) studies, cells were incubated for 14 h with 2.8 mM glucose in DMEM with 0.1 % BSA, followed by 2.8 mM glucose in KRB buffer for 1 h. The remainder of the procedure is similar to that described for dispersed human islet cells.

For other insulin secretagogue-stimulated insulin secretion assays, the studies were performed as described above, but substituting the stimulation factor with either 10 mM of L-Arg or 50 mM of KCl instead of glucose.

Insulin content

Dispersed human islets and MIN6 cells were infected with respective recombinant adenovirus and incubated for 24 h. The medium was then removed and cells were washed twice with PBS. Cells were extracted with acid ethanol (18 % 1N HCl, 75 % Ethanol, 7 % H2O) solution for 16 h at 4°C. The insulin concentration was measured by ELISA. The insulin content was normalized to total DNA content.

Isolated islets insulin secretion experiments

Islets were obtained by collagenase digestion (11). Batches of 20 healthy size-matched islets were incubated in RPMI-1640 media containing 0.1% BSA and 2.8 mM glucose overnight. Islets were then preincubated in KRB buffer containing 0.1% BSA and 2.8 mM glucose for 1 h as basal insulin secretion. Islets were then stimulated with either 16.7 mM glucose, 10 mM L-Arg or 50 mM KCl in KRB buffer containing 0.1% BSA and 2.8 mM glucose for 1 h. Aliquots of the media were collected at 60 min after stimulation.

Immunostaining and Morphometric analyse

Mice were anesthetized, and pancreas was rapidly dissected, weighted and processed as described previously (11) and immunostained for insulin (Linco), glucagon (Sigma), somatostatin (Dako), BrdU (Dako), DAPI (Sigma) and TUNEL staining kit (Chemicon). Pancreata from WT and RIP-TRB3F1 mice were dissected, weighed, flattened by forceps, fixed in Z-fix and paraffin embedded to obtained maximal pancreatic footprint upon sectioning. Three sections (separated by 25 μM) from each pancreas were immunostained for insulin (Linco) using standard immunohistochemical method (11). The entire tissue sections

were imaged using a BX60 microscope (Olympus), from which the percentage of β-cell area relative to the total pancreatic area was measured and calculated using ImageJ software (NIH). β-cell mass was derived from the total pancreatic weight multiplied by the percentage of β-cell area.

For proliferation analysis, BrdU labeling was performed by intraperitoneal injection of BrdU (100mg/ Kg body weight) for 5 h before the pancreas was dissected, fixed in Z-fix, and co-stained with anti-BrdU (Dako) and anti-insulin antibodies. Total number of β-cells and β-cells labeled with both insulin and BrdU were evaluated by point counting morphometry. At least five thousand β-cells were counted from each of the four animals of each genotype. For apoptosis assay, staining was performed using TUNEL staining kit (Chemicon) along with anti-insulin antibody to label β-cells. Total β-cell and β-cell labeled with both insulin and TUNEL staining were evaluated by point counting morphometry. At least five thousand β-cells were counted from each of the four animals of each genotype.

Electron microscopy

Mouse pancreas was harvested from anesthetized animals and was immediately minced in a fixative solution of 2.5% gluteraldehyde in a 0.1M phosphate buffer. After several hours at room temperature the minced material was then stored at 4°C while still in the fixative solution. Islets were hand dissected from the pancreas and placed into a 0.1M phosphate buffer prior to post-fixation.

The hand dissected islets were washed several times in phosphate buffer before being post-fixed with a 2% osmium tetroxide solution in a 0.1M phosphate

buffer. The islet samples were then washed again in phosphate buffer several times before plastic embedding.

Samples were dehydrated in ascending gradations of ethanol ending with several changes of absolute ethanol. The tissues were then cleared using propylene oxide and infiltration of the tissue with Araldite epoxy resin was accomplished with a 1:1 mixture of propylene oxide and Araldite 502 epoxy resin. After two hours in sealed vials, the tissue samples were then placed into a vacuum desiccator overnight prior to embedding. Several of the hand picked islets from each sample were placed into BEEM capsules and filled with Araldite resin. The resin filled capsules were then placed into a 60*C curing oven for 48 hours.

Once cured, the resin blocks were trimmed, sectioned to produce one micron slides and stained with Toluidine Blue. The sample blocks were then re-trimmed to specific regions of interest and thin sectioned at 800 Angstroms using an LKB NOVA ultra-microtome and a Diatome Diamond Knife. The thin sections were placed on copper mesh grids, stained with uranyl acetate and lead citrate before being observed and photographed on the Philips 301 Transmission Electron Microscope. Electron micrographs of randomly selected β-cells were prepared at a magnification of ~ X17, 700. To determine the number and percentage of granules docked at the plasma membrane, all the granules in contact with the plasma membrane as well as total granules were counted by point counting morphometry as described previously (16). Subsequently, the length of the perimeter of each cell section was measured with a cartographer's

micrometer wheel (16). The docked granules were expressed as the number of granules per 10 μm of membrane. For all samples, at least 15 cells were analyzed. We took a conservative view in that granules were considered docked only if they were touching the membrane. Those that were close to the membrane and might have been in contact above or below the section under examination were not counted. While this approach could slightly under-estimate the number of docked granules it increases the consistency for each sample estimate.

Chromatin immunoprecipitation

MIN6 cells under basal condition, treated with 100 nM Thapsigargin for 6 h or 10 nM Forskolin for 1 h were fixed by addition of 37% formaldehyde to a final concentration of 1% formaldehyde and incubation at room temperature for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. Cells were then scraped and samples were prepared with SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling) according the manufacturer's protocol. The chromatin fractions were incubated in each case with 10 μg of one of the following antibodies: anti-CREB (Cell Signaling), and Normal Rabbit IgG (provided by SimpleChIP Kit) at 4 °C overnight. Input DNA and immunoprecipitated DNA were analyzed by quantitative RT-PCR with primers detecting mouse SNAP25 promoter (F 5'- gcggaatggaaacagcgtagtgaa-3', R 5'-tgttacctgccattgcttcggaga-3'). Data are normalized by input values and binding is expressed relative to the non-specific binding of IgG immunoprecipitated DNA content.

Quantitative RT-PCR analysis

Total RNA was isolated from either cultured cells or pancreatic islets using an RNeasy kit (Qiagen). cDNA was prepared from 1 μg of total RNA using the Superscript III RT-PCR kit (Invitrogen) with random hexamer primers, according to the manufacturer's instructions. The resulting cDNA was diluted 10-fold, and a 5 μl aliquot was used in a 30 μl PCR reaction (SYBR Green, PE Biosystems) containing primers at a concentration of 300 nM each. PCR reactions were run in triplicate and quantitated using the ABI Prism 7000 Sequence Detection System (ABI). Results were normalized to TATA box binding protein (TBP) expression and expressed as arbituary units. Sequences of primers used in this study are available upon request.

Synchronization and BrdU pulse study

Cells grown to 50 % confluence were incubated with 2 μM of hydroxyurea in growth medium for 22-24 h for synchronization. Cells were released from synchronization by washing twice with PBS and incubated with complex growth media for appropriate periods (17). For the BrdU pulse study, 25 μM BrdU was added to the growth media at the time of release and incubated for 2 h at 37°C.

Measurement of intracellular [Ca^{2+}]

MIN6 cells transfected with GFP-tagged TRB3 proteins were split onto 25 mm coverslips coated with Concanavalin A. After overnight recovery from trypsinization, cells were loaded with fura-2 dissolved in a standard extracellular solution (SES) containing (in mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES adjusted to pH 7.4 with NaOH and supplemented with 1 mM glucose, 20

μl ml^{-1} FBS, 1 μl ml^{-1} Pluronic F-127 and 2 μM fura-2 AM (Invitrogen). Cells were loaded in this solution for 30 min at room temperature and then washed with 1 mM glucose SES. Single-cell measurements of $[Ca^{2+}]_i$ were obtained using an inverted microscope with a dual excitation wavelength imaging system using a 40x SuperFluor lens (Nikon). The recording chamber was maintained at 35 C in a QE-1 stage platform with a TC-344B controller that also controlled an SH-27B in-line solution heater for bath perfusion (Warner Instruments) Excitation light at 340 and 380 nm (7.5 nm bandpass) was from a PTI DeltaRam monochromator and emitted light was filtered at 510 nm and detected on a Cascade 512B camera controlled with Metafluor software. The ratio of emission light intensities due to excitation at 340 and 380 nm was calculated and converted to calcium concentrations by *in vitro* calibration using pentapotassium Fura-2 and a calcium calibration buffer kit with magnesium (Invitrogen). *In Situ* calcium calibrations were also performed to check for interference between GFP and Fura-2 fluorescence. This *in situ* calibration was performed at the end of an experiment by perfusing cells with calcium-free SES containing 5 mM EGTA and 10 μM ionomycin, to obtain minimum fluorescence ratios, and then perfusing with normal SES to obtain maximum ratios. Calibration data from GFP positive cells and GFP negative cells measured from the same plates were not significantly different. Bath solutions were exchanged using a gravity feed inlet flowing at 1 – 2 ml/min with a bath volume of about 1 ml and a vacuum pump outlet.

SUPPLEMENTARY REFERENCES

1. Malecki MT, et al. The Pro12Ala polymorphism of PPARgamma2 gene and susceptibility to type 2 diabetes mellitus in a Polish population. *Diabetes Res Clin Pract*. 2003;62(2):105-111.
2. Malecki MT, et al. Homozygous combination of calpain 10 gene haplotypes is associated with type 2 diabetes mellitus in a Polish population. *Eur J Endocrinol*. 2002;146(5):695-699.
3. Wanic K, et al. Lack of association between polymorphisms in the gene encoding protein tyrosine phosphatase 1B (PTPN1) and risk of Type 2 diabetes. *Diabet Med*. 2007;24(6):650-655.
4. Ng DP, et al. Scrutiny of the glutamine-fructose-6-phosphate transaminase 1 (GFPT1) locus reveals conserved haplotype block structure not associated with diabetic nephropathy. *Diabetes*. 2004;53(3):865-869.
5. Canani LH, et al. Polymorphism in ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (ENPP1/PC-1) and early development of advanced diabetic nephropathy in type 1 diabetes. *Diabetes*. 2002;51(4):1188-1193.
6. Prudente S, et al. The TRIB3 Q84R polymorphism and risk of early-onset type 2 diabetes. *J Clin Endocrinol Metab*. 2009;94(1):190-196.
7. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005;21(2):263-265.
8. Gabriel SB, et al. The structure of haplotype blocks in the human genome. *Science*. 2002;296(5576):2225-2229.
9. Jhala US, et al. cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev*. 2003;17(13):1575-1580.
10. Borowiec M, et al. Mutations at the BLK locus linked to maturity onset diabetes of the young and {beta}-cell dysfunction. *Proc Natl Acad Sci U S A*. 2009;
11. Kulkarni RN, et al. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell*. 1999;96(3):329-339.
12. Quitsch A, et al. Postsynaptic shank antagonizes dendrite branching induced by the leucine-rich repeat protein Densin-180. *J Neurosci*. 2005;25(2):479-487.
13. Harlow E, Lane D. 1988. *Antibodies : a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
14. Josefsen K, et al. Fluorescence-activated cell sorted rat islet cells and studies of the insulin secretory process. *J Endocrinol*. 1996;149(1):145-154.
15. Tomas A, et al. Regulation of pancreatic beta-cell insulin secretion by actin cytoskeleton remodelling: role of gelsolin and cooperation with the MAPK signalling pathway. *J Cell Sci*. 2006;119(Pt 10):2156-2167.
16. Straub SG, Shanmugam G, Sharp GW. Stimulation of insulin release by glucose is associated with an increase in the number of docked granules in the beta-cells of rat pancreatic islets. *Diabetes*. 2004;53(12):3179-3183.

17. Hill BT, Whelan RD, Hemmings VJ, Franks LM. A method of synchronization of normal and malignant human cells in culture. *Cell Biol Int Rep*. 1977;1(4):379-384.

Supplemental Table 1: Risk of type 2 diabetes according to TRB3 Q84R genotypes

Genotypes	Controls N (%)	T2DM Cases N(%)	OR (95% CI)	p
Krakow study				
QQ	274(77.4)	315(69.9)	1.0 (reference)	
QR	77(21.8)	122(27.0)	1.4 (0.9-1.9)	0.06
RR	3(0.8)	14(3.1)	4.1 (1.2-14.3)	0.02
Joslin study				
QQ	265(76,1)	226(71.7)	1.0 (reference)	
QR	80(23.0)	81(25.7)	1.2 (0.8-2.0)	0.15
RR	3(0.9)	8(2.5)	3.2 (0.8-12.2)	0.07
Combined Data:				
Recessive effect*				
QQ + OR	696(99.1)	744(97.1)	1.0 (reference)	
RR	6(0.9)	22(2.9)	3.4 (1.4-8.5)	0.005 (0.015)
Dominant effect*				
QQ	539(76.7)	541(70.6)	1.0 (reference)	
QR + RR	163(23.3)	225(29.4)	1.4 (1.1-1.8)	0.004 (0.012)
Additive effect*				
Risk for 1 R allele			1.4 (1.1-1.9)	0.006 (0.018)

* Effects are estimated using logistic regression.

( ) P values corrected for number of tests

Supplemental Table 2. Clinical characteristics of the study groups.

	Krakow		Boston	
	Control subjects n=354	**Case subjects n=451**	**Control subjects n=348**	**Case subjects n=315**
men (%)	41.2	45.6	37.0	38.6
BMI (kg/m^2)	30.6 ± 7.6	31.7 ± 6.1	26.3 ± 3.8	29.3 ± 6.5
age at exam (years)	54.6 ± 11.6	59.4 ± 8.9	67.4 ± 9.2	60.3 ± 5.7
age at DM diagnosis (years)		49.9 ± 8		47.3 ± 6.6
duration of DM (years)		9.4 ± 7.6		13.6 ± 6.7
treatment with insulin (%)		48.6		74.4
HbA1c at exam (%)		7.8 ± 1.7		8.6 ± 1.5

Data are means ± SD or %.

Supplemental Table 3: Distribution of genotypes of six polymorphisms of TRB3 in initial panel of 169 T2DM cases and 143 non-diabetic controls

		T2DM cases	Controls
polymorphism	**genotype**	**n (%)**	**n (%)**
33nt I/D	AA	2 (2.3)	2 (2.5)
	AB	7 (8.2)	11 (13.6)
	AC	2 (1.2)	2 (2.47)
	AD	8 (9.4)	6 (7.4)
	BB	13 (15.3)	9 (11.1)
	BC	6 (7.1)	6 (6.2)
	BD	36 (42.3)	36 (44.4)
	CD	3 (3.5)	2 (2.5)
	DD	9 (10.6)	8 (9.9)
rs2295490(Q84R)*	**AA(QQ)***	**121 (71.6)**	**110 (76.9)**
	AG(QR)	**44 (26)**	**32 (22.4)**
	GG(RR)	**4 (2.4)**	**1 (0.7)**
rs6051637(Y111Y)*	CC	47 (30.3)	37 (26.4)
	CT	71 (45.8)	63 (45)
	TT	37 (23.9)	40 (28.6)
rs6115830(A232A)*	CC	70 (42.9)	59 (43.1)
	CT	65 (39.9)	55 (40.1)
	TT	28 (17.2)	23 (16.8)
1611 C/T	CC	135(83.3)	105(80.1)
	AC	25(15.4)	24(18.3)
	AA	2(1.2)	2(1.5)
Rs11537607	CC	88(66.1)	79(64.7)
	CT	37(27.8)	34(27.9)
	TT	8(6.0)	9(7.4)

* Indicates amino acids.

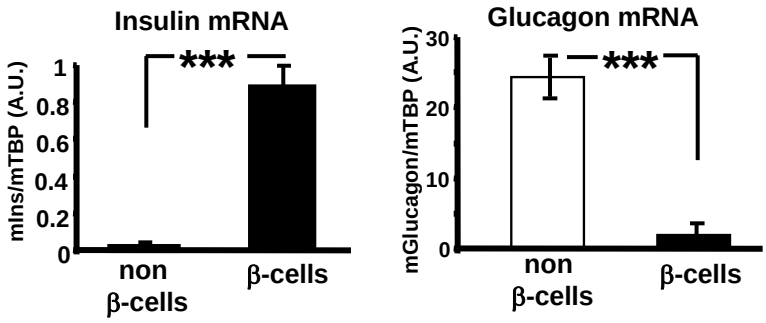


Figure S1
Purity assessment of fluorescence activated cell sorting sorted β- and non-β-cells fractions
Quantitative real time PCR of *insulin* or *glucagon* mRNA expression in FACS sorted β- and non-β-cell fractions from mouse islet cells. ***, $p<0.001$ (n=3).

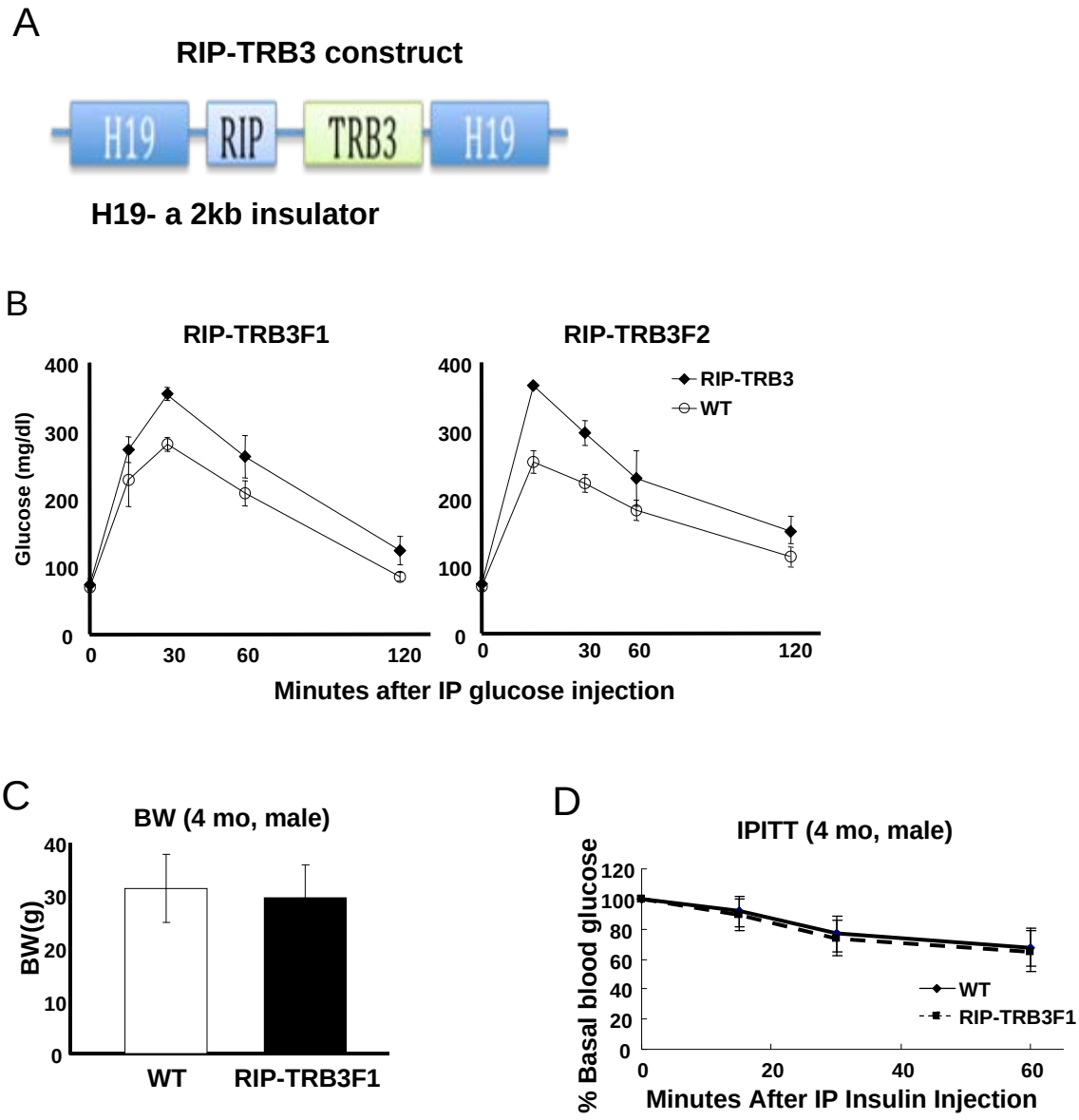


Figure S2

Characterization of RIP-TRB3 transgenic mice

(A) Schematic representation of RIP-TRB3 construct. (B) Glucose tolerance in two RIP-TRB3 founder (RIP-TRB3F1, RIP-TRB3F2) male mice (2 g/kg body weight, n=4-6). **(C)** Body weights of 4-month-old male mice. **(D)** Percent change in blood glucose after IP injection of insulin (1 U/kg body weight) in 4-month-old RIP-TRB3F1 male mice.

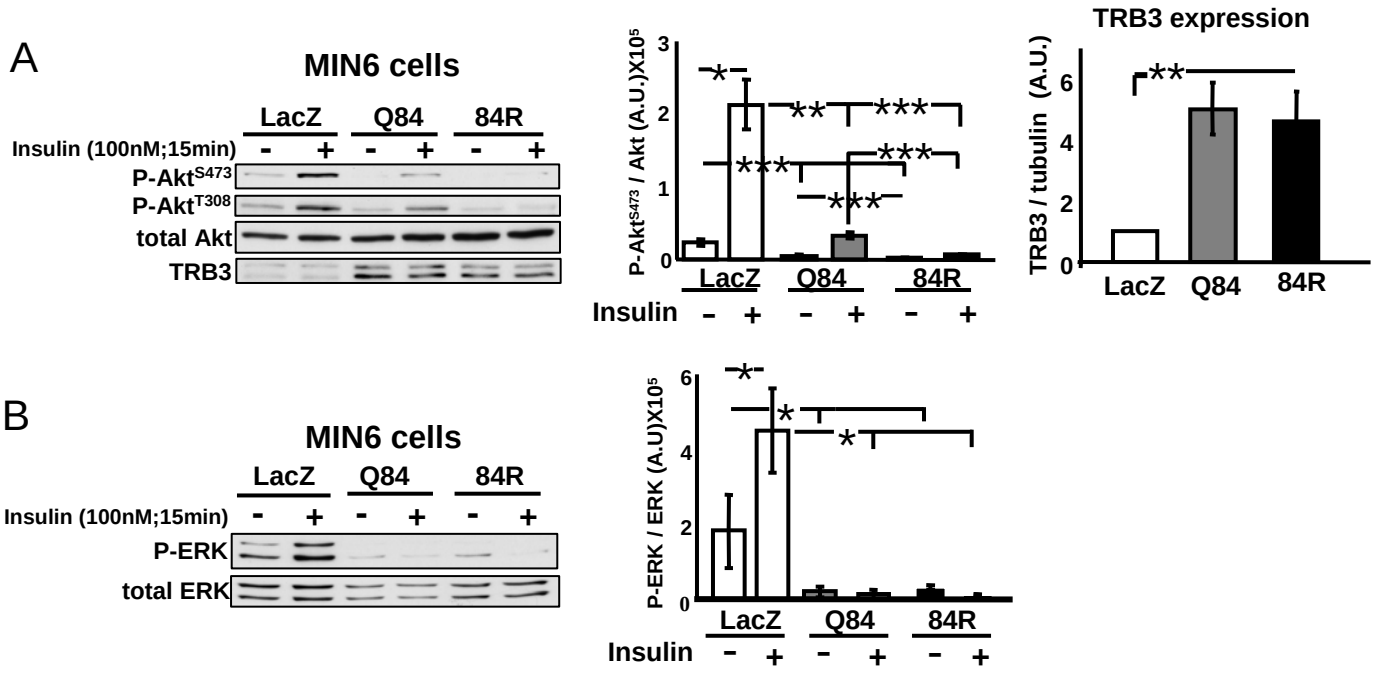


Figure S3

Effect of variant TRB3 protein expression on phosphorylation of Akt or ERK in MIN6 cells. (**A**) Western blotting of TRB3, total Akt, p-AktS473, p-AktT308 and TRB3 proteins prepared from basal or insulin-stimulated MIN6 cells expressing LacZ, Q84 or 84R TRB3. Data are depicted as a ratio of p-AktS473 to total Akt (left panel) or as a ratio of TRB3 to tubulin (right panel). *,p<0.05; **,p<0.01; ***,p<0.001 (n=4). (**B**) Western blotting of total ERK and p-ERK proteins prepared from basal or insulin-stimulated MIN6 cells expressing LacZ, Q84 or 84R TRB3. Data are depicted as a ratio of p-ERK to total ERK. *,p<0.05 (n=4). All quantitative data are presented as mean +/- SD.

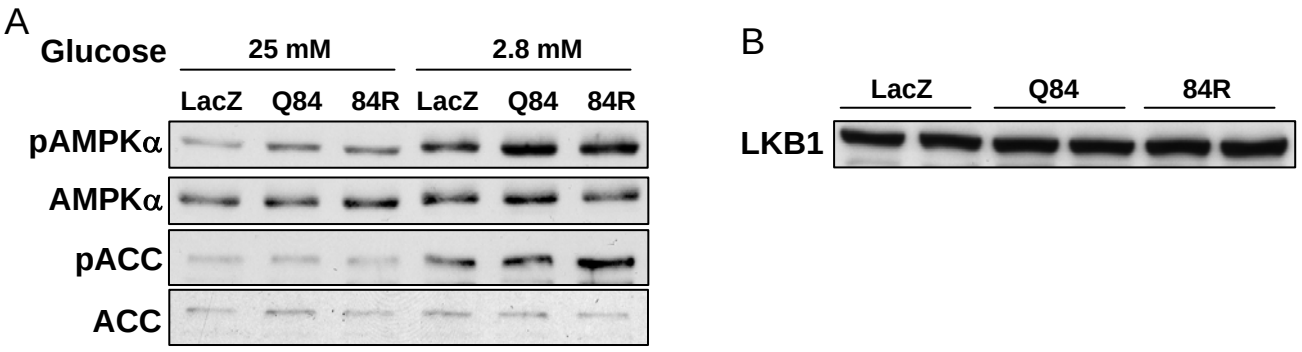


Figure S4

AMPKα and ACC phosphorylation in MIN6 cells expressing LacZ or TRB3.

(**A**) Western blotting of total AMPKα and ACC as well as p-AMPKα and p-ACC proteins from low (2.8 mM) or high (25 mM) glucose stimulated MIN6 cells expressing LacZ control, Q84 or 84R TRB3. (**B**) Western blotting of LKB1 in MIN6 cells expressing LacZ control, Q84 or 84R TRB3.

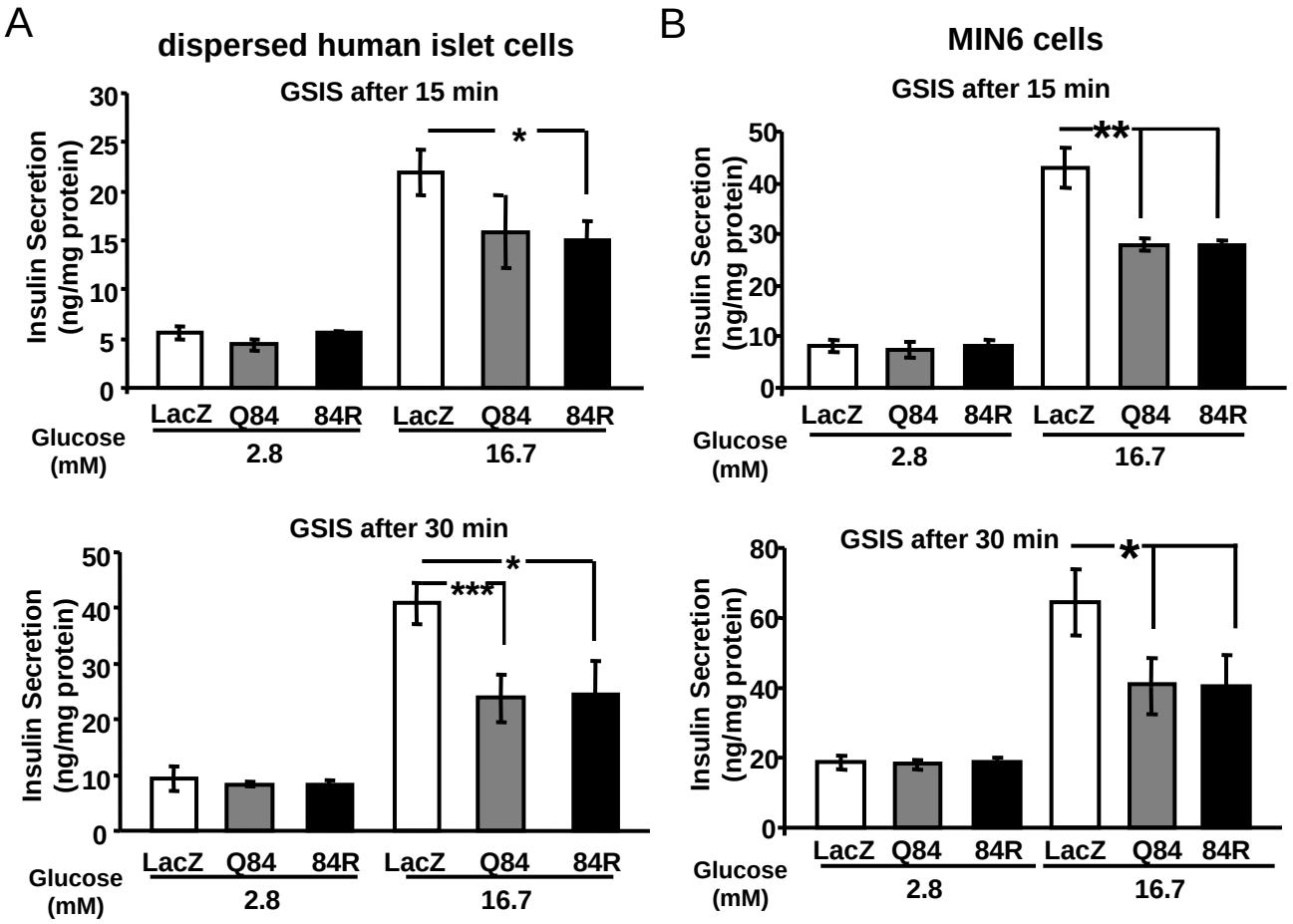


Figure S5

Reduced glucose stimulated insulin secretion by human islet cells and MIN6 cells expressing Q84 or 84R TRB3. Dispersed human islet cells (**A**) and MIN6 cells (**B**) expressing LacZ control, Q84 or 84R TRB3 were stimulated with either 2.8 mM (basal) or 16.7 mM (stimulatory) glucose and cell media was sampled at 15 (upper panel) and 30 (lower panel) for determination of insulin secretion, as described in Methods. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (n= 3).

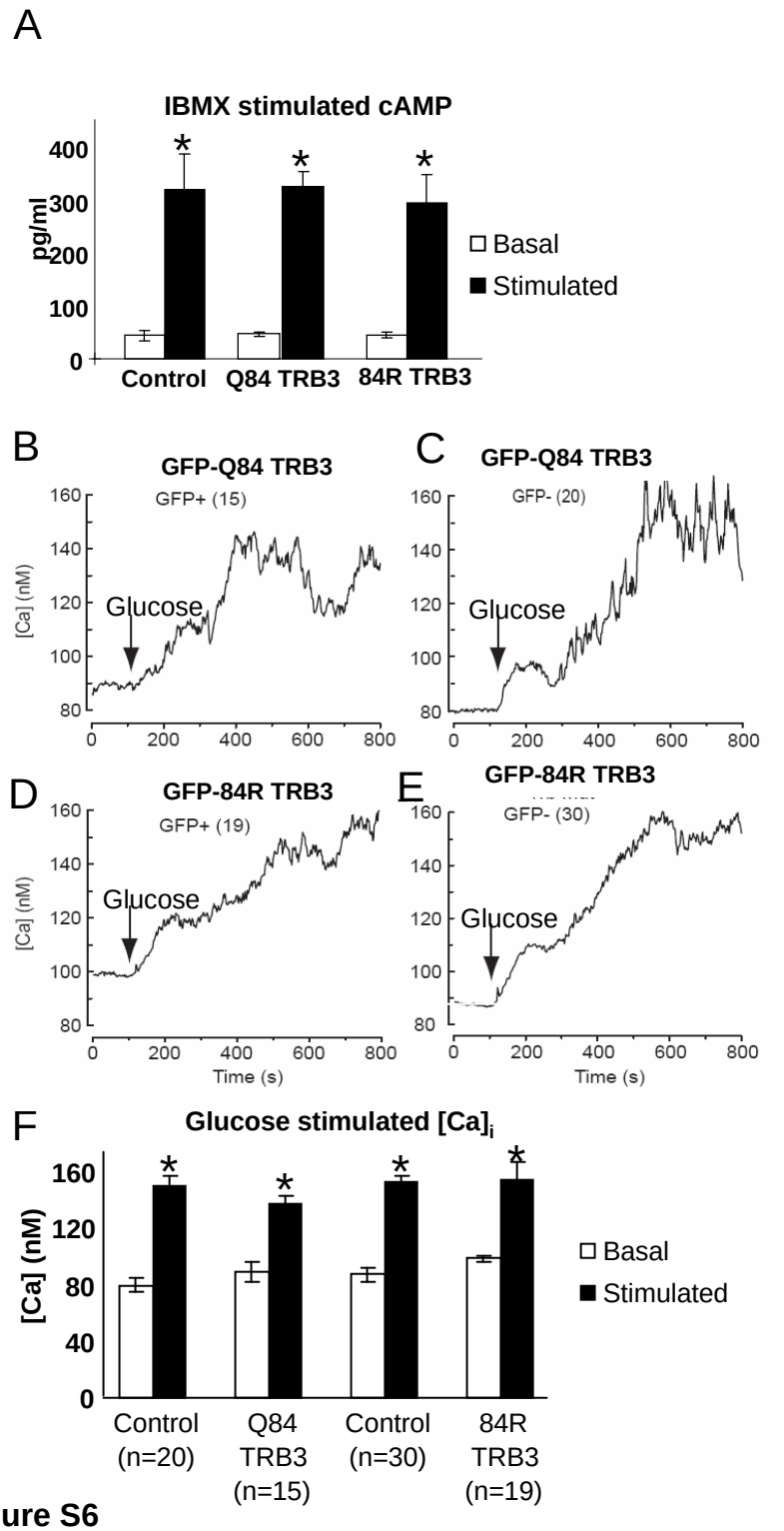


Figure S6

Intracellular $[Ca]_i$ and cAMP concentration in TRB3 expressing MIN6 cells are unaltered upon stimulation. (**A**) cAMP concentration upon stimulation with 16.7 mM glucose with 25 μM of IBMX (n=4) as described in Method. MIN6 cells transiently transfected with either GFP control, GFP-Q84 TRB3 (**B**, **C**) or GFP-84R TRB3 (**D**, **E**) were measured for $[Ca]_i$ upon stimulation with 16.7 mM of glucose (n=15-30) (**F**) Quantification of the intracellular $[Ca]_i$ recording shown in (b-e)

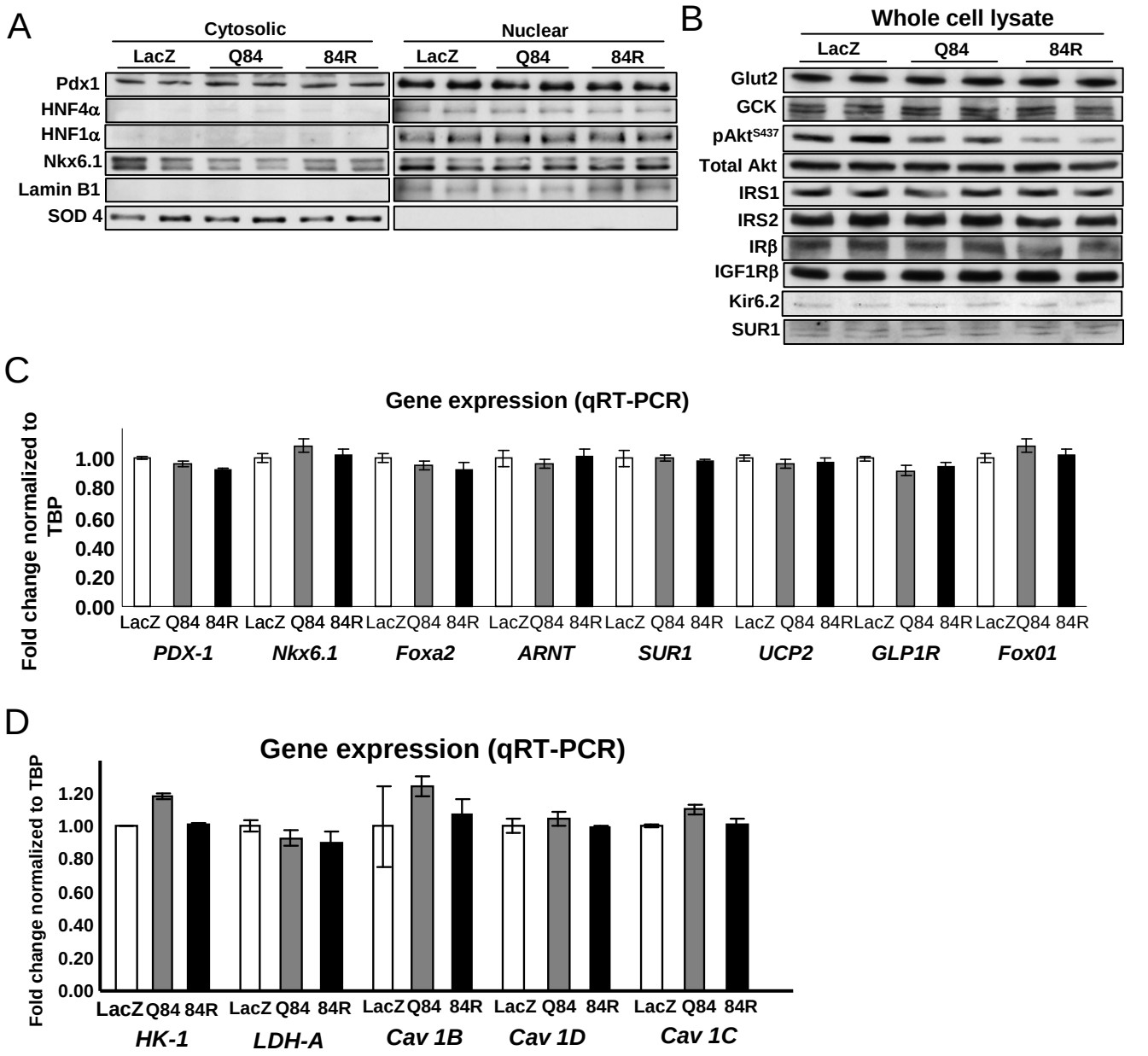


Figure S7

Protein and gene expression analyse of proteins involved in insulin biosynthesis and β-cell secretory functions. (**A**), (**B**) Western blotting of proteins involved in insulin secretion and biosynthesis pathways were assessed in cytosolic/nuclear fractions prepared from MIN6 cells expressing LacZ control, Q84 or 84R TRB3. (**C**), (**D**) Real-time quantitative RT-PCR analyse of proteins involved in insulin biosynthesis (*Pdx-1*, *Nkx6.1* and *Foxa2*), β-cell function (*ARNT* and *UCP2*), glucose metabolism (*HK-1* and *LDH-A*), stimulus-secretion coupling and calcium channels (*SUR1*, *Cav 1B*, *Cav 1D* and *Cav 1C*) in MIN6 cells expressing LacZ control, Q84 or 84R TRB3. Data are depicted as fold change normalized to TBP and expressed as arbitrary units. (n=3)

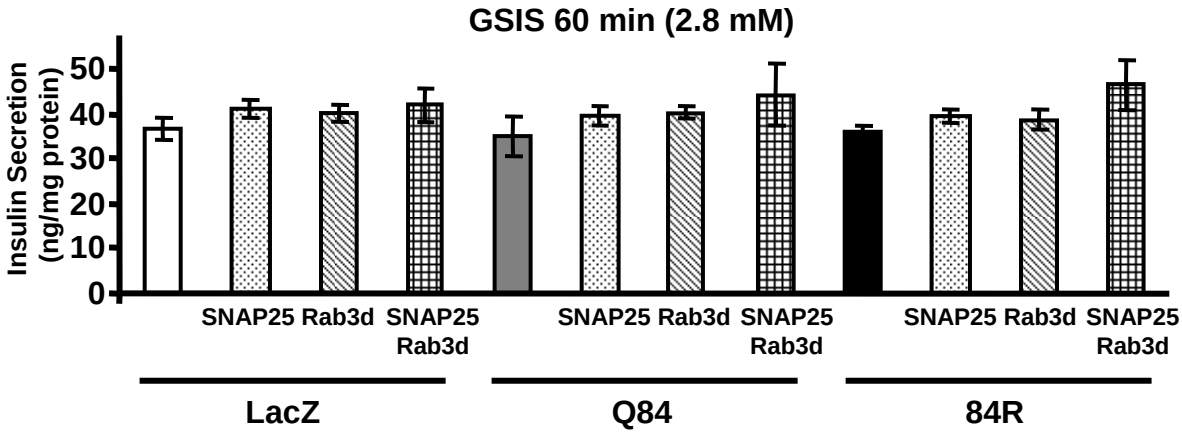


Figure S8

Baseline insulin secretion in MIN6 cells expressing Q84 or 84R TRB3 with SNAP25 and/or Rab3d. MIN6 cells expressing LacZ control alone, or with either SNAP25 or Rab3d or both; Q84 TRB3 alone or with either SNAP25 or Rab3d or both; and 84R TRB3 alone or with either SNAP25 or Rab3d or both were stimulated with 2.8 nM (basal) of glucose and media were sampled at 60 min. Data are depicted as a ratio of amount of secreted insulin (ng) to total cellular protein (mg).

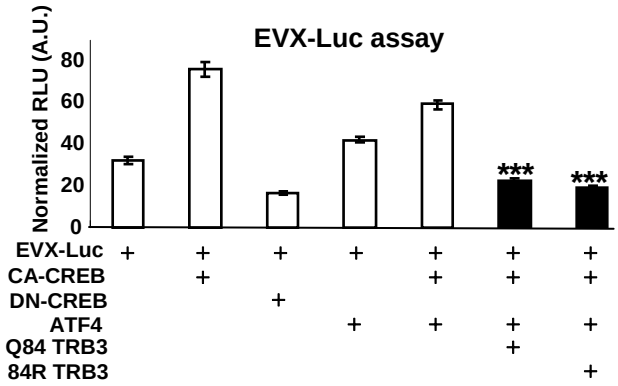
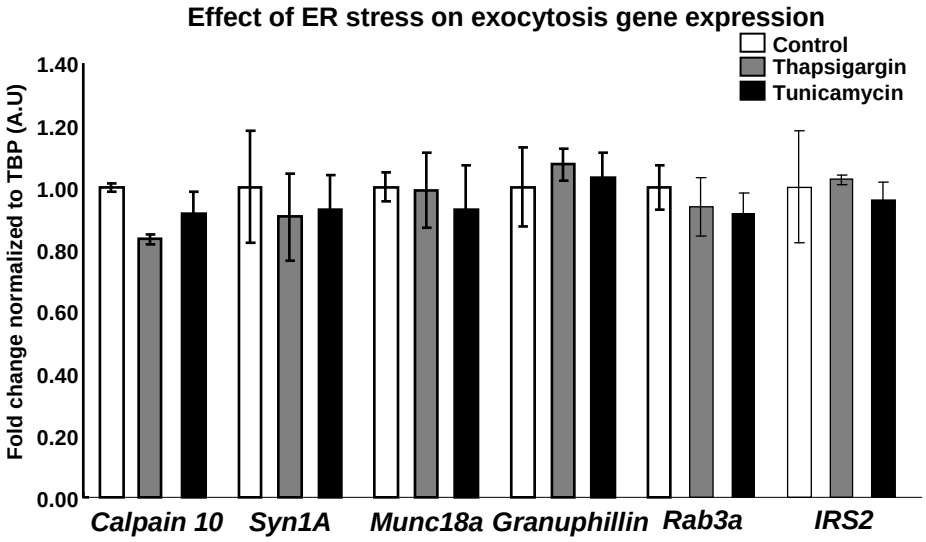


Figure S9

TRB3 inhibits CREB transcriptional activity on cAMP-response elements (CRE). HEK293 cells were transiently transfected with the indicated plasmid. EVX-Luc contains Even-skipped homeo box homolog 1 (Drosophila) promoter harboring CRE. Cells were harvested 36 h post-transfection for determination of luciferase activity. Results were normalized to *Renilla* luciferase activity and expressed as relative luciferase units (RLU). ***, $p<0.001$ vs EVX-Luc with CA-CREB. (n=4)

A



B

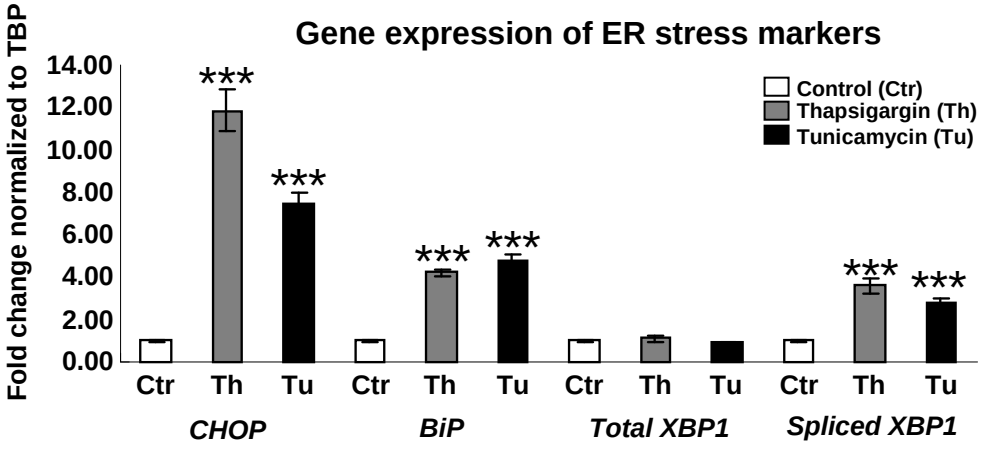


Figure S10

Gene expression analysis of proteins involved in insulin biosynthesis and β-cell secretory functions. Real-time quantitative RT-PCR analysis of proteins involved in **(A)** insulin exocytosis (*Calpain10*, *Syn1A*, *Munc18a, Granuphillin* and *Rab3a*), insulin signaling (*IRS2*) and **(B)** ER stress markers (*CHOP*, *BiP*, total *XBP1* and spliced *XBP1*) in MIN6 cells treated with either DMSO control, 100 nM of Thapsigargin or 2 μg/ml of Tunicamycin for 8 hours. Data are depicted as fold change normalized to TBP and expressed in arbitrary units. ***, $p<0.001$ (n=3)