SUPPLEMENTAL MATERIAL

Syntaxin 4 mediates NF-κB signaling and chemokine ligand expression *via* specific interaction with IκBβ

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Supplemental Table 1: Human islet donor profiles.

RRID	Sex	Age (Y)	BMI	Race	Islet purity (%)	Islet viability (%)	Expt. use	Type of islets
SAMN11483342	F	52	39.8	White	92	98	IκBα/β Degradation assay	ND
SAMN11514696	F	59	21.8	Black or African American	90	88	IκBα/β Degradation assay	ND
SAMN12500521	M	52	29.0	White	95	98	IκBα/β Degradation assay	ND
SAMN12924398	M	38	28.0	Hispanic or Latino	82	95	IκBα/β Degradation assay	ND
SAMN13254972	F	60	44.4	White	99	99	IκBα/β Degradation assay	ND
SAMN08768971	M	61	27.0	Caucasian	80	80	TMT analyses	ND

Expt. Use., experimental use; N.D., non-diabetic.

Supplemental Table 2: Primer sequences used for qRT-PCR.

Species	Gene	Primer forward (5' to 3')	Primer reverse (5' to 3')
Rat	CXCL9	CAAGGCACATTCCACTACAA	CCTTGCTGAATCTGGGTCTA
	GAPDH	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC

Supplemental Table 3: STX4 overexpression in human islets induced changes in protein levels identified by Tandem mass tag spectrometry.

Accession	Description	Coverage (%)	# of AA	M.W.	Calc. pI	Abundance ratio Sample/Control
14790124	Caspase-9 isoform alpha precursor	8	416	46.3	6.05	5.422
119372317	Xin actin-binding repeat-containing protein 2	0	3549	401.9	6.33	3.408
40255020	Chromatin modification-related protein MEAF6 isoform 1	6	201	22.7	8.28	2.79
530430991	Allergin-1 isoform X2	8	385	43.4	8.35	2.725
530422392	Coagulation factor IX isoform X2	7	418	47	5.43	2.635
16306580	Lysine-specific demethylase 2A isoform a	1	1162	132.7	7.58	2.582
578815898	Zinc finger CCCH domain-containing protein 3 isoform X2	3	1017	109.7	10.96	2.5
20149560	Syntaxin-4 isoform 3	24	297	34.2	6.28	2.498
4507285	Syntaxin-10 isoform 1	14	249	28.1	4.89	2.388
4506041	Prolargin precursor	3	382	43.8	9.38	2.367
50659084	Histone-lysine N-methyltransferase SUV420H1 isoform 2	6	393	44.6	8.7	2.265
27477113	Sterol regulatory element-binding protein 2	3	1141	123.6	8.4	2.228
66346683	Plasminogen activator inhibitor 1 RNA-binding protein isoform 3	42	393	43.1	8.44	2.136
530416982	B-cell lymphoma 3 protein isoform X1	2	650	69	10.15	2.105
256017174	Leucine-rich repeat and calponin homology domain-containing protein 1 isoform 1	2	763	84.6	5.68	2.019
103471993	Palmitoyltransferase ZDHHC17	3	632	72.6	7.5	1.983
4505777	PHD finger protein 1 isoform a	5	457	49.6	7.93	1.982
8922625	Trimethyllysine dioxygenase, mitochondrial isoform 1 precursor	4	421	49.5	7.72	1.955
38569426	Ankyrin repeat and EF-hand domain-containing protein 1	4	776	86.6	8.28	1.95
578834501	NF-kappa-B inhibitor beta isoform X1 (IκBβ)	7	416	44.9	6.09	1.942
4758040	Cytochrome c oxidase subunit 6C proprotein	8	75	8.8	10.39	1.881
578815936	Triple QxxK/R motif-containing protein isoform X2	16	86	9.7	9.36	1.878
27886568	Interferon-induced helicase C domain-containing protein 1	1	1025	116.6	5.52	1.871
530419654	GRAM domain-containing protein 4 isoform X1	5	604	69.4	9.06	1.86
23510333	Pygopus homolog 2	4	406	41.2	7.28	1.855

M.W., molecular weight; Calc.pI, calculated isoelectric point**;** Sample, Ad5-RIP-STX4; Control, Ad5-RIP-Ctrl.

Supplemental Information

Proteomic Analysis of Human Pancreatic Islets overexpressed with STX4. Human islets were immediately hand-picked for transduction using Ad5-RIP-Ctrl or Ad5-RIP-STX4. Forty-eight-hours later, islets were harvested in lysis buffer [100 mM TEAB (Tri-Triethylammonium bicarbonate), 1 mM PMSF, 0.25% (W/V) sodium deoxycholate, 1% NP40, 1X the MS-SAFE protease and phosphatase inhibitor (Cat # 78444; Thermo Fischer Scientific, Waltham, MA)]. Approximately 700 µg of each sample of lysed protein was precipitated with trichloroacetic acid. The protein pellets were washed with ice cold acetone, dried, and resuspended in 50 µl of 1:1 trifluoroethanol: 100 mM TEAB. Proteins were reduced by adding 1 µl 500 mM tris (2-carboxyethyl) phosphine and incubating at 50°C for 1 h, then alkylated by adding 3 µl of 500 mM iodoacetamide and incubating in the dark for 1 h at room temperature. Samples were then diluted with 500 µl of 50 mM TEAB. Trypsin/LysC mixture (17.5 µg) was added to each sample, and the samples were incubated overnight at 37°C with shaking. After digestion, the samples were acidified by addition of 10 µl of formic acid. Samples were centrifuged for 10 min at 14,000*g* to pellet any precipitate, and the supernatant was taken for further processing. Digested peptides were desalted using an SPE cartridge (Oasis HLB, Waters, Milford, MA) according to the manufacturer's recommendations. Purified peptides were dried in a vacuum centrifuge and resuspended in 200 µl 0.1% formic acid. Digests were quantified using a peptide quantitative colorimetric assay (Pierce, Rockford, IL). Each sample (100 µg) was taken for TMT labeling. Samples were adjusted to pH 8 with 1M TEAB, then added to 0.8 mg of TMT reagent in 41 µl anhydrous acetonitrile. After reacting for 1 h at room temperature, the reaction was quenched by adding 8 µl 5% hydroxylamine and reacting for 15 min. Samples were then combined and dried in a vacuum centrifuge. The combined samples were fractionated using a High pH Reversed-Phase Peptide Fractionation Kit (Pierce, Rockford, IL) according to the manufacturer's

instructions. Fractionated peptides were dried in a vacuum centrifuge and resuspended in 50 µl 0.1% formic acid.

Mass Spectrometry. Fractions were analyzed in triplicate using an Orbitrap Fusion Mass Spectrometer with an EasyNano 1000 nanoflow UHPLC and an EasySpray source (Thermofisher Scientific, San Jose, CA). Samples were loaded onto a 75 µm ID x 20 mm long trapping column packed with 3 µm, 100Å PepMap C18 silica at 5 µl using 100% solvent A (0.1% formic acid in water). Peptides were then eluted through a 75 µm ID x 500 mm long analytical column packed with 2 µm, 100Å PepMap C18 silica using a linear gradient from 3% to 7% solvent B (0.1% formic acid in acetonitrile) over 5 minutes, 7% to 25% solvent B over 95 min, and 25% to 95% solvent B over 5 min. Peptides were analyzed using a synchronous precursor selection MS3 method. Precursor ions were measured in the Orbitrap analyzer with a m/z range from 380 to 1500 and a resolution of 120,000. Precursor ions with charge between +2 and +6 were selected for fragmentation using the quadrupole mass filter and an isolation width of 0.7 Da, and analyzed in the ion trap using CID fragmentation with 35% relative collision energy. After being analyzed, ions were placed on an exclusion list for 70 s. Up to 10 ions from the MS/MS scan were selected for MS3 fragmentation by HCD with 65% collision energy and analyzed in the ion trap analyzer with a m/z range from 100 to 200 to measure TMT reporter ions.

Mass Spectrometry Data Analysis. Data was analyzed using Proteome Discoverer 2.1 using the Sequest HT search node (Thermofisher Scientific, San Jose, CA). Data was searched using a 5 ppm precursor ion tolerance, 0.6 Da fragment ion tolerance, and assuming full tryptic specificity, quantitative carbamidomethylation of cysteine, quantitative TMT modification of lysines and peptide amino termini, and possible oxidation of methionine. Reporter ion intensities were determined using the most confident centroid with an integration tolerance of 0.6 Da.

Protein-level relative abundances were calculated using unique and razor peptides, excluding spectra with more than 50% isolation interference, signal to noise below 10, or less than 65% of the total intensity of MS/MS ions used for the MS3 assigned to the identified sequence.

Cell culture, transient transfection and western blotting. INS-1 832/13 cells were provided by Dr. Christopher Newgard (Duke University Medical Center, Durham, NC, USA); these studies used cells within passages 52-68. INS-1 832/13 cells were transfected with pCMV-FLAG or pCMV-rat(r)STX4, or human(h)STX4 plasmid DNAs using Lipofectamine™ 2000 Transfection Reagent (Cat # 11668030, Thermo Fischer Scientific, Waltham, MA). Next, the cells were treated with cytokines (10 ng/mL TNFα, 100 ng/mL IFNγ, 5 ng/mL IL-1β), as previously described (1, 2), individually or together as a cocktail, for 1 h, 18 h, or 24 h as indicated in the text and figure legends. To evaluate IκBα, IκBβ, and STX4 levels, the cells were harvested in 1% NP-40 lysis buffer, and then cleared detergent lysates were used for immunoblotting. Tubulin was used as loading control. For pharmacological intervention studies in vitro, INS-1 832/13 cells were treated with epoxomicin (Proteasome inhibitor) (10 μM), KT5823 (3 μM; Protein kinase G inhibitor) or PDTC (NF-κB inhibitor; 250 μM) for 30 min or 1 h prior to cytokine exposure, as detailed in the figure legends.

Nitrite release assay. INS-1 832/13 cells grown in 24-well plates were transfected, then 40 h later were treated with IL-1β (5 ng/ml) and/or PDTC (250 μM). The medium was collected after 24 h and centrifuged at 100*g* for 5 min. Equal volumes of medium and Griess reagent (Sigma; St. Louis, MO) were mixed, and the absorbance was measured at 540 nm, as described previously (3).

Determination of PKG activity. Total PKG activity was determined using a non-radioactive immunoassay CycLex® Cyclic GMP dependent protein kinase (cGK) Assay Kit to measure PKG mediated phosphorylation of a synthetic substrate in INS-1 832/13 cell lysates treated with cytokine

and/or PKG*i* (KT5823 3 μM; Protein kinase G inhibitor) according to the manufacturer's directions. The results were expressed as specific activity (nmol of ADP generated/min/μg of protein).

Supplemental References

1. Ahn M, Yoder SM, Wang Z, et al. The p21- activated kinase (PAK1) is involved in diet-induced beta cell mass expansion and survival in mice and human islets. Diabetologia 2016; 59: 2145-2155.
2. Aslamy A, Oh E, Olson EM, et al. Doc2b protects β-Cells against inflammatory damage and enhances function. Diabetes 2018; 67: 1332-1344.
3. Veluthakal R, Chvyrkova I, Tannous M, et al. Essential role for membrane lipid rafts in interleukin-1beta-induced nitric oxide release from insulin-secreting cells: potential regulation by caveolin-1. Diabetes 2005; 54: 2576-2585.

Fig. S1

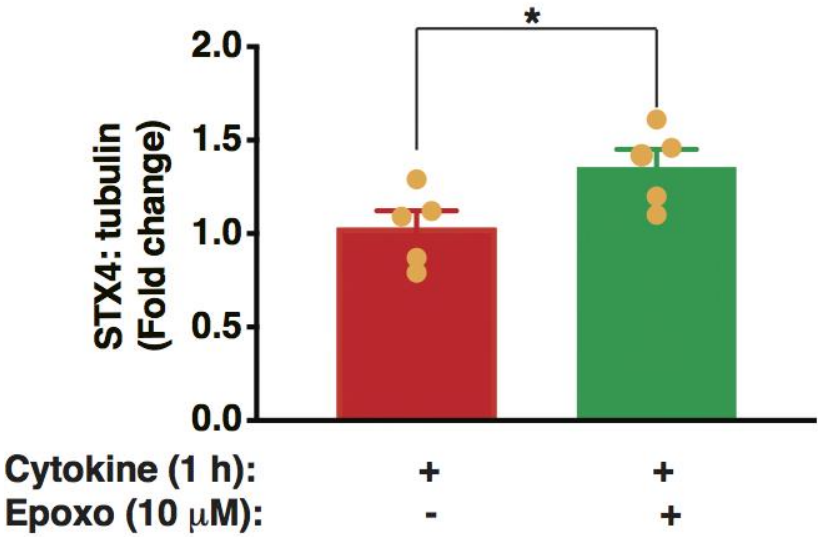

Figure S1. Epoxomicin inhibits degradation of STX4 in cytokine-exposed INS-1 832/13 cells. Proteasome inhibitor epoxomicin (Epoxo; 10 μm) was added to INS-1 832/13 cells 30 min prior to the addition of the cytokine cocktail (5 ng/mL IL-1β, 10 ng/mL TNFα, and 100 ng/mL IFNγ). Cells were harvested 1 h later for immunoblot detection of STX4 and tubulin. STX4 was normalized to tubulin in each set, where the untreated cells were set equal to 1 and all others in the set normalized thereto. Data represent the mean ± S.D. from 5 independent experiments;
*$p < 0.05$.

Fig. S2

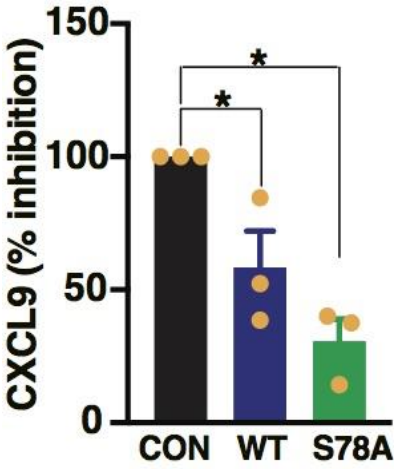

Figure S2. Enrichment of STX4 and stabilized STX4-S78A mutant decreases expression of the chemokine ligand CXCL9.

INS-1 832/13 cells were transfected with CON, hSTX4 (Wild type; WT) or hSTX4-S78A (S78A) plasmids for 48 h. Cells were left unexposed to cytokines, and harvested and RNA extracted for quantitative RT-PCR analysis for CXCL9, relative to GAPDH. In each independent experiment, CON was set equal to 100%, and other samples normalized thereto. Bars represent the mean ± S.E.M. from 3 independent experiments; *$p < 0.05$.

Fig. S3

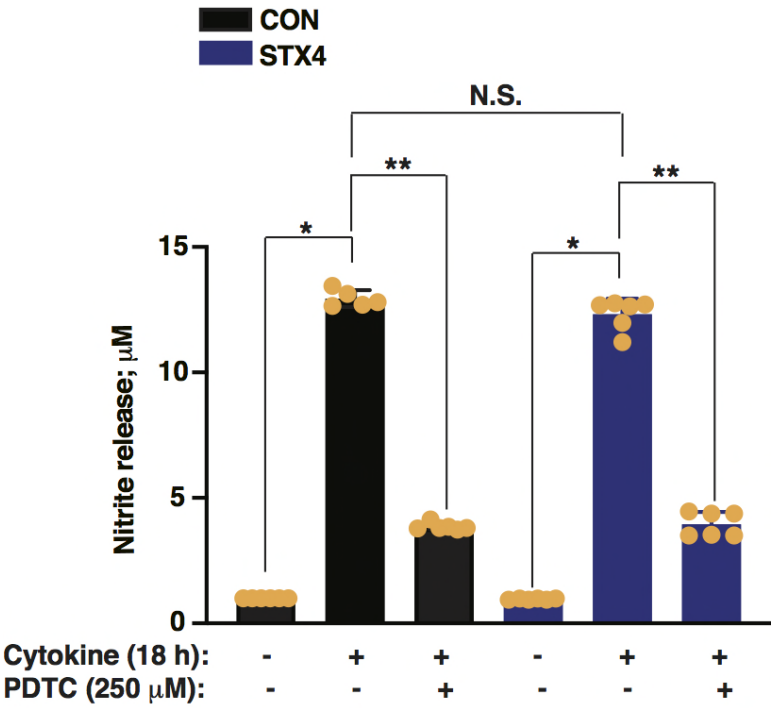

Figure S3. STX4 overexpression had no effect on NO release, whereas NF-κB inhibitor significantly attenuated NO release in INS-1 832/13 cells.

INS-1 832/13 cells were transfected with pCMV-FLAG (CON) or pCMV-rat(r)STX4 plasmid DNAs. After 48 h cells were incubated in the presence of diluent alone or cytokine cocktail (5 ng/mL IL-1β, 10 ng/mL TNFα, and 100 ng/mL IFNγ) in the presence of PDTC (added 30 min prior; 250 μM) for 18 h, as indicated. NO released into the medium was quantitated using Griess reagent. Data are means ± S.E.M. from 5-6 independent experiments *$p < 0.05$ *vs.* control. **$p < 0.05$ *vs.* cytokine-treated group.

Fig. S4

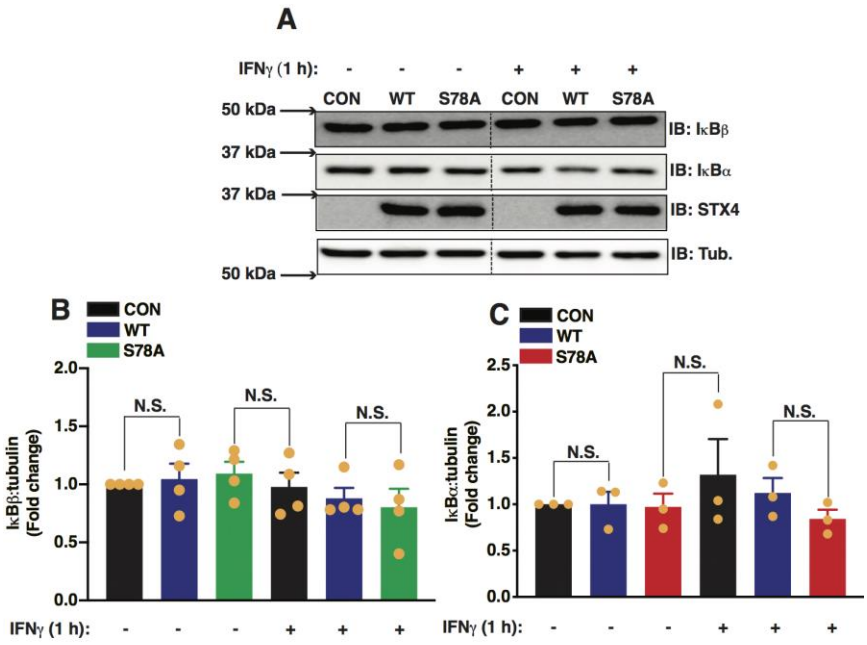

Figure S4. IFNγ did not induce significant losses of IκBβ or IκBα levels in INS-1 832/13 cells, in the presence or absence of STX4 or the S78A mutant.

INS-1 832/13 cells were transfected with empty vector (CON), hSTX4-WT (Wild type, WT) or hSTX4-S78A (S78A) plasmids followed by incubation with 100 ng/mL IFNγ for 1 h. Representative immunoblots for protein levels of IκBβ, IκBα, STX4 and tubulin, are shown. (**A)** Data represent the mean ± S.E.M. from 3-4 independent experiments with IFNγ, tabulated for levels of IκBβ, relative to tubulin (**B)**, or levels of IκBα relative to tubulin (**C**). IB, immunoblot; Tub, tubulin; N.S., non-significant. Vertical dashed lines indicate splicing of lanes from within the same gel exposure.

Fig. S5

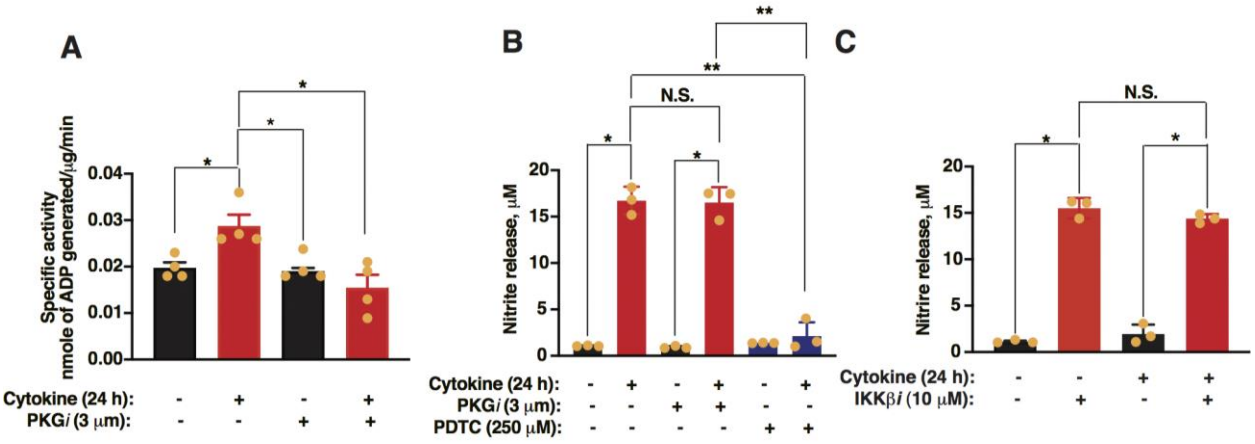

Figure S5. PKG*i* significantly attenuated PKG activity, whereas PKG*i* or IKKβ*i* had no effect on cytokine-induced NO release in INS-1 832/13 cells.

Protein kinase G inhibitor (PKG*i*; 3 μM) was added to INS-1 832/13 cells 30 min prior to the addition of the cytokine cocktail (5 ng/mL IL-1β, 10 ng/mL TNFα, and 100 ng/mL IFNγ) for 24 h. Cells were harvested 24 h later for PKG activity. **(A)** Data represent mean ± S.E.M. from 4 independent experiments and expressed as nmoles of ADP generated/min/μg of protein. (**B**) Protein kinase G inhibitor (PKG*i*; 3 μM) or IKKβ*i* (10 μM) (**C**) was added to INS-1 832/13 cells 30 min prior to the addition of the cytokine cocktail (5 ng/mL IL-1β, 10 ng/mL TNFα, and 100 ng/mL IFNγ) for 24 h. NO released into the medium was quantified using Griess reagent. Data are means ± S.E.M. from 3 independent experiments; *p < 0.05 and **p < 0.001. N.S., non-significant.

Fig. S6

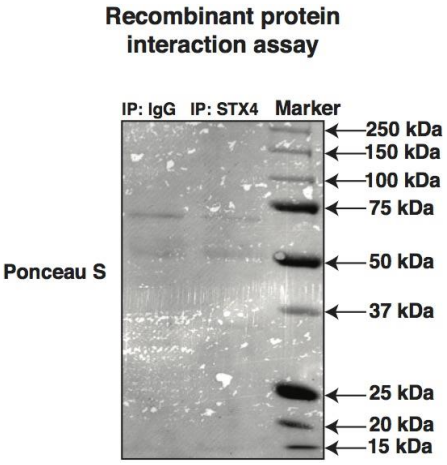

Figure S6. Ponceau S staining for protein loading in the recombinant protein interaction assay. Recombinantly expressed and purified IκBβ and STX4 proteins were combined in equimolar amounts for subsequent immunoprecipitation (IP) with rabbit anti-STX4 antibody and precipitated proteins were resolved by 10% SDS/PAGE and transferred to PVDF. The PVDF membrane was subsequently washed and total proteins stained using Ponceau S, for evaluation of protein loading and equivalent antibody heavy chain between IP reactions.