SUPPLEMENTARY DATA

SUPPLEMENTAL MATERIAL

Restoration of glucose-stimulated Cdc42-Pak1 activation **and insulin secretion by a selective Epac activator in type 2 diabetic human islets**

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Supplementary Table 1. Human islet donor profiles.
Exptl, experimental; ND, non-diabetic; GSIS, glucose stimulated insulin secretion

UNOS ID	Sex	Age (Y)	BMI	Race	Islet Purity (%)	Islet Viability (%)	Exptl use	HbA1c	Type of islets
AEDG094	M	49	25.5	White	95	95	Pak1, Cdc42	5.4	ND
AEDU239	M	53	29	Caucasian	78	94	Pak1, G-LISA, Cdc42	5.5	ND
AEDZ307	M	51	35.6	Hispanic	90	95	Pak1, G-LISA	7.10	T2D
AEEZ055	F	42	37	White	90	89	Pak1, G-LISA	6.0	T2D
AEEI285	M	16	21.8	Hispanic	80	98	Pak1, Cdc42	5.4	ND
AEEJ208	F	34	31.5	Caucasian	50	97	Pak1, Cdc42	5.7	ND
AED1377	M	43	28.2	Hispanic	75	97	Pak1, G-LISA	5.6	ND
AEGY230	F	52	42.8	Hispanic	90	95	G-LISA	6.6	T2D
AEHL151	M	59	27.7	Hispanic	85	95	Pak1	6.5	T2D
AEHG368	M	57	23.3	Hispanic	25	75	Cdc42	7.3	T2D
AELK219	f	37	38.1	Caucasian	85	94	GSIS, Cdc42	8.2	T2D
AFBE414	M	45	27.2	Caucasian	85%	95%	GSIS, Cdc42	6.5	T2D
AFBK237B	M	54	24.5	African/American	50%	90%	GSIS, Cdc42	4.8	T2D
ADHR295	M	62	35.9	Caucasian	80%	95%	Cdc42	7.4	T2D
ADHX156	M	57	34.5	Hispanic	80%	98%	Cdc42	10.4	T2D
ADDX443	M	37	30.3	Caucasian	70%	85%	Cdc42	5.3	ND

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Supplemental Information

Additional materials. Synthesis of the experimental cAMP antagonist Rp-8-Br-cAMPS-pAB was performed at the BIOLOG Life Sci. Inst. by F. Schwede (Bremen, Germany). Commercially available 8-pCPT-2'-*O*-Me-cAMP-AM (Cat. No. C 051) and PO_4-AM_3 (Cat. No. P 030) were from BIOLOG. 4-Abn-OH (Cat. No. 755339) was from Sigma-Aldrich. LRE1 was provided by Profs. Lonny Leven and Jochen Buck (Cornell University, Weill Medical College, NY).

HEK293 cell culture. HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). A HEK293 cell line stably expressing full-length sAC cloned from a rat testis cDNA library was provided by Profs. Lonny R. Levin and Jochen Buck (Cornell University, Weill Medical College, NY). Cultures were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 25 mM glucose, 10% FBS, 100 IU/ml penicillin, and 0.1 g/l streptomycin. For cells stably expressing sAC, G418 was also added to the medium.

Pull-down assay for Cdc42 activation. A protein-protein interaction pull-down technique was used to measure active Cdc42-GTP in lysates of cells treated with test solutions. Briefly, lysates (500 µg–1 mg protein/ml) were centrifuged at 4800*g* for 5 min to pellet debris. To the supernatant was added a glutathione S-transferase (GST) fusion protein of the Cdc42 (p21)-binding domain (PBD) of Pak1 that binds Cdc42-GTP. Glutathione-conjugated beads were then added to capture the Cdc42-GTP/GST-PBD complex. The beads were rotated for 1 h at 4°C, pelleted by centrifugation (4000*g* for 3 min), and washed once with lysis buffer. After centrifugation, the pellet was resuspended in buffer containing (in mM): 25 Tris (pH 7.5), 30 $MgCl_2$, 40 NaCl, and 150 EDTA. Centrifugation was performed, after which the pellet was solubilized in Laemmli sample buffer. Proteins were resolved by 12% SDS-PAGE and transferred to Immun-Blot PVDF Membrane (Cat. No. 1620177, Bio-Rad) for Western blot analysis using a 1:5,000 dilution of a primary anti-Cdc42 antibody in combination with an HRP-conjugated secondary antibody. As a loading control, GST-PBD was detected using an anti-GST antibody. Proteins were detected by enhanced chemiluminescence (ECL) using an imaging system (ChemiDoc XRS+, Bio-Rad) and an ECL/Prime Kit (Cat. No. RPN 2232, GE Healthcare Lifesciences, Pittsburgh, PA).

FRET reporter assays. AKAR3 or Epac1-camps biosensors were expressed in INS-1 832/13 cell monolayers by adenoviral transduction. Assays were performed 2 days later in a 96-well format using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). AKAR3 was provided by Prof. Jin Zhang (University of California at San Diego School of Medicine). Epac1-camps was provided by Prof. Martin Lohse (University of Wuerzburg, Germany) and Prof. Viacheslav O. Nikolaev (University of Hamburg, Germany). Reference to cite: Holz GG, Leech CA, Roe MW, *et al*. High-throughput FRET assays for fast time-dependent detection of cyclic AMP in pancreatic beta-cells. *Cyclic Nucleotide Signaling*. Xiaodong Cheng, Ed. Taylor and Francis Group (CRC Press) 2015; p35-60.

Intracellular [Ca^{2+}] measurements.

For loading of INS-1 832/13 with fura-2, cell monolayers grown on glass coverslips were equilibrated for 30 min at room temperature in a standard extracellular solution (SES) containing (in mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 mM HEPES (adjusted to pH 7.4 with NaOH) supplemented with 1 mM glucose, 1 µM fura-2-AM, and 0.01% Pluronic F-127. To allow bioactivation of intracellular fura-2-AM, the cells were then bathed for 30 min in SES to which no fura-2-AM or Pluoronic F-127 were added. Fura-2 measurements of $[Ca^{2+}]_i$ were performed at 37° C using a Nikon Ti microscope equipped with a 40X Nikon CFI Super Fluor objective, a QE-1 temperature-controlled stage (Warner Instruments, Hamden, CT) and a SH-27B in-line solution heater (Warner Instruments). The SES bath solutions were constantly perfused using a peristaltic pump at a flow rate of 2 ml/min. Excitation light at 340 nm

and 380 nm was provided by a PTI DeltaRam X monochromator (HORIBA Scientific, Edison, NJ) and the emitted light was filtered at 510 nm to be collected using a Cascade II EMCCD camera (Photometrics, Tucson, AZ) controlled by MetaFluor software (Molecular Devices, San Jose, CA). Ratio values were exported into Origin analytical software (OriginLab, Northhampton, MA) for plotting and statistical analysis.

siRNA knockdown of Cool-1/□Pix.

Silencer select pre-designed siRNA for Cool-1/□Pix (sense: 5'-gauccugaagguuauugaatt-3'; antisense: 5'-uucaauaaccuucaggauctg-3') and control siRNA (Cat. No. 4390843) were purchased from Life Technologies (Waltham, MA). INS-1 832/13 cells were cultured in RPMI-1640 containing 11.1 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 0.1 g/l streptomycin, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 10 mM HEPES (pH 7.4). At 50–60% confluence, the cells were transfected with siRNA oligonucleotides targeted against Cool-1/βPix or nontargeting siRNA using jetPrime transfection reagent (New York, NY) for 48 h incubation. After 48 h, the cells were used for GSIS or Cdc42 activation assays in the absence or presence of the Epac agonist, as described above. The rabbit polyclonal antibody #4515 (for Cool-1/βPix) was purchased from Cell Signaling Technology (Danvers, MA). The mouse monoclonal antibody AC-74 for β-actin was purchased from Sigma-Aldrich.

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Figure S1. Protein-protein interaction pull-down assay for Cdc42 activation. INS-1 832/13 cells were incubated overnight in low serum and low glucose culture media, and further incubated for 1 h with low glucose KRBH prior to each experiment. Cells were then pretreated for 20 min with low glucose solutions containing the 0.1% DMSO vehicle solution, 4-Abn-OH (10 µM), or Rp-8-Br-cAMPS-pAB (10 µM). Next, cells were exposed for 2 min to KRBH containing either low glucose (1 mM) or high glucose (20 mM) prior to their lysis. Active Cdc42-GTP in the lysates binds to GST-PBD that is affixed to glutathione-conjugated beads. Centrifugation allows recovery of Cdc42-GTP for SDS-PAGE and Western blot analysis. **(a)** Immunoblots are representative of five independent experiments **(b)** Densitometry analysis of the mean ± s.e.m. ratio of Cdc42–GTP : GST-PDB; *p < 0.05 *vs.* 1 mM glucose treated with vehicle or 4-Abn-OH; **p < 0.05 *vs.* 20 mM glucose treated with vehicle.

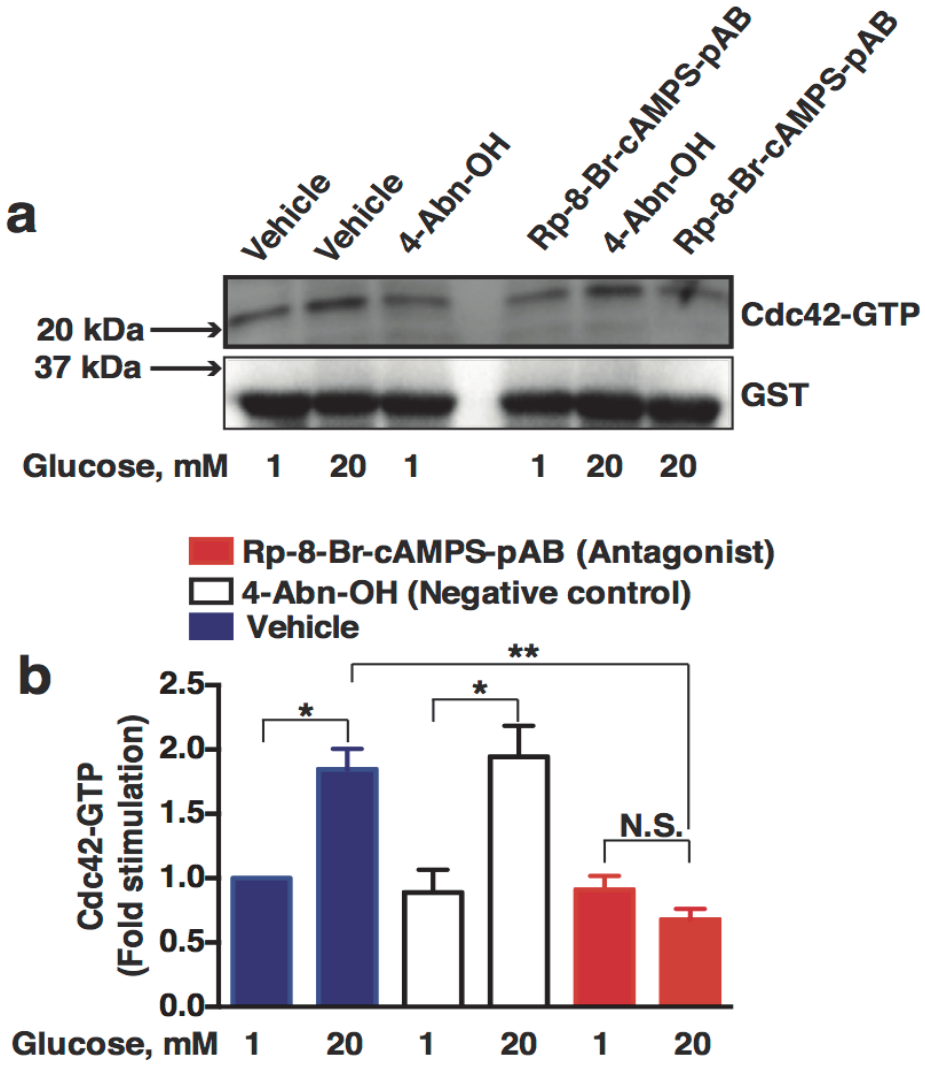

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Figure S2. cAMP analog structures. (a) 8-pCPT-2'-*O*-Me-cAMP-AM is a highly membrane permeable prodrug in which the lipophilic acetoxymethyl ester (AM-ester) undergoes hydrolytic intracellular bioactivation catalyzed by cytosolic esterases. Once the prodrug is bioactivated, free 8-pCPT-2'-*O*-Me-cAMP is liberated so that it may bind to and selectively activate Epac. High selectivity for Epac is achieved because binding of the analog to PKA is greatly reduced as a consequence of a 2'-*O*-Me substitution that is introduced on the ribose ring. **(b)** Rp-8-Br-cAMPS-pAB is a highly membrane permeable prodrug in which the lipophilic para-acetoxybenzyl ester (pAB-ester) undergoes hydrolytic intracellular bioactivation catalyzed by cytosolic esterases. Once the prodrug is bioactivated, free Rp-8-Br-cAMPS is liberated so that it may act as a cAMP antagonist by virtue of its direct binding to PKA or Epac.

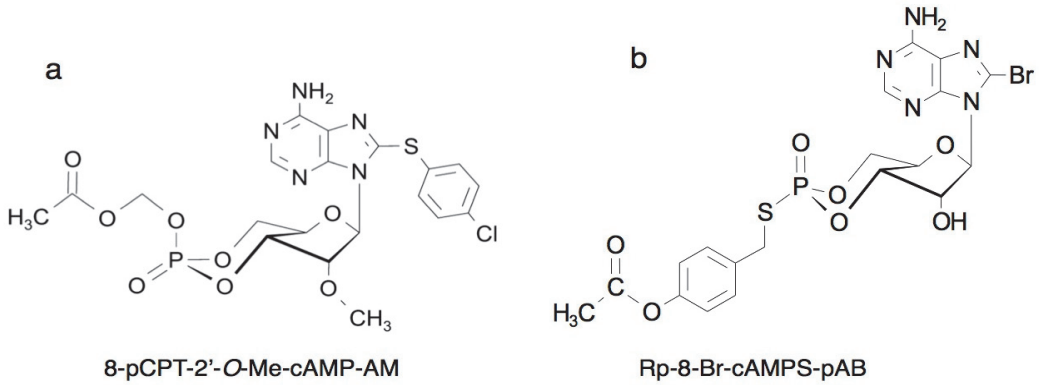

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Figure S3. Positive control for Epac1-camps.INS-1 832/13 cells **(a)** or HEK293 cells stably expressing the GLP-1 receptor (HEK-GLP-1R cells) **(b)** were virally transduced with Epac1-camps. Measurements of FRET in a 96-well format were performed using monolayers of these cells. Administration of 8-pCPT-2'-*O*-Me-cAMP-AM (10 μM) or the GLP-1R agonist Exendin-4 (a cAMP-elevating agent) produced the expected increase of the 485/535 nm emission ratio. No such ΔFRET was measured in response to the negative control PO_4-AM_3 (3.3 μM). Data for each panel indicate the mean ± s.e.m. for a single experiment that is representative of results obtained in *n = 3* independent experiments

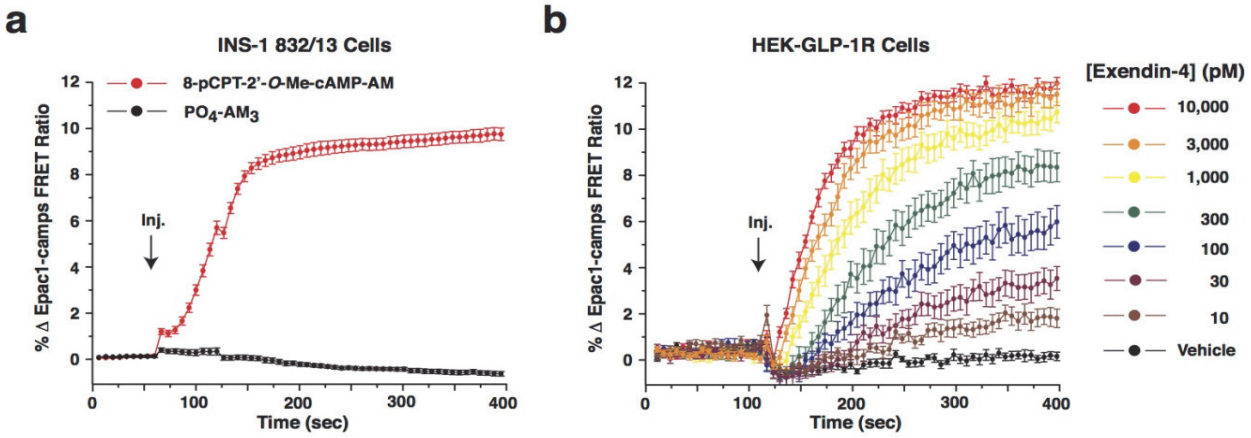

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Figure S4. Rp-8-Br-cAMPS-pAB is a potent cAMP antagonist in INS-1 832/13 cells. (a) To test the ability of Rp-8-Br-cAMPS-pAB to block cAMP action in INS-1 832/13 cells, we used adenoviral transduction to express an A-kinase activity reporter (AKAR3) that exhibits an increase of the 535/485 nm FRET ratio in response to its phosphorylation by PKA. **(b)** The cAMP-elevating agent forskolin (0.006–6 μM) dose-dependently stimulated an increase of the AKAR3 FRET ratio, whereas the negative control 0.1% DMSO vehicle solution had no effect. **(c)** Forskolin action was blocked when cells were pretreated with 0–30 μM Rp-8-Br-cAMPS-pAB. The IC_{50} for Rp-8-Br-cAMPS-pAB was approximately 0.5 μM; inhibition was evident with as little as 0.1 μM, and full block at 3 μM. **(d)** Because bioactivation of Rp-8-Br-cAMPS-pAB generates the side products acetic acid and 4-hydroxybenzyl alcohol, we took into account the possibility of a confounding side-product effect. The negative control prodrug 4-actetoxybenzyl alcohol (4-Abn-OH; 30 μM), which is hydrolyzed intracellularly to generate acetic acid and 4-hydroxybenzyl alcohol, failed to alter the action of forskolin. For each panel, these data are the mean ± s.e.m. for a single experiment, and each panel is representative of results obtained in *n = 3* independent experiments. Abbreviations: Em., emission; Ex., excitation; Fsk., forskolin; Inj., injection; Rp pAB, Rp-8-Br-cAMPS-pAB.

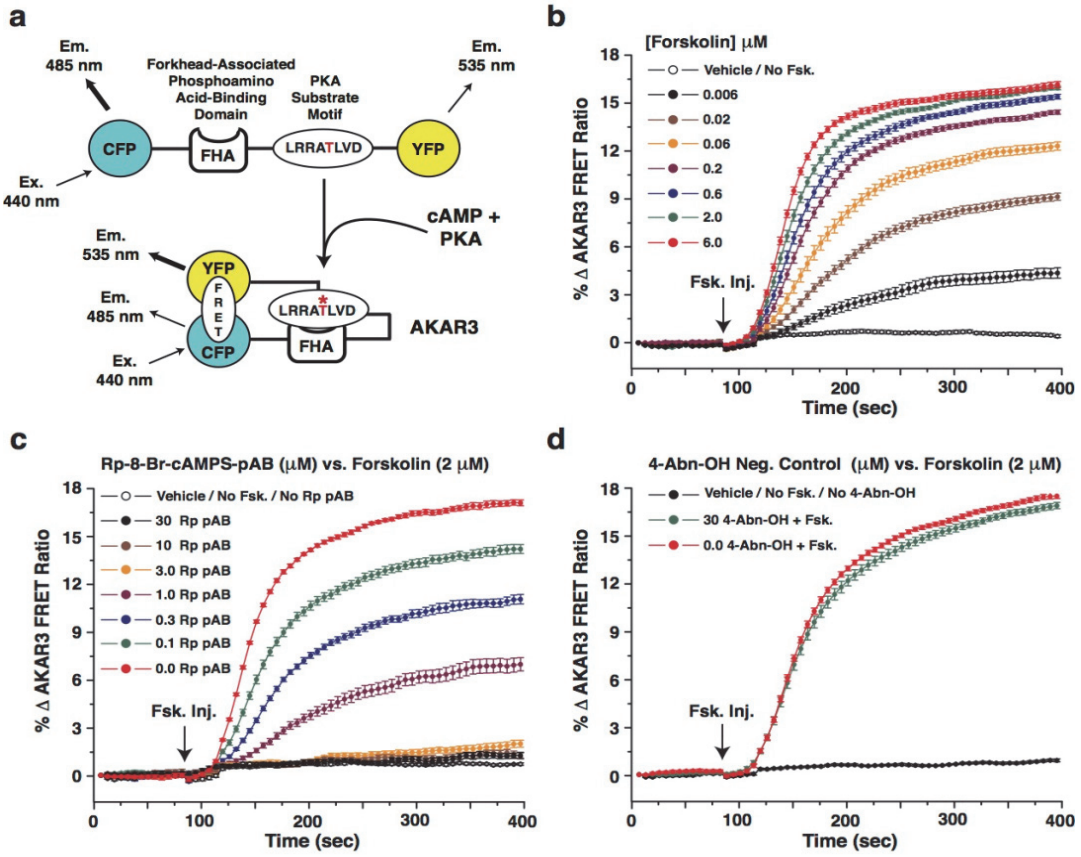

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Figure S5. Negative controls for AKAR3. The baseline AKAR3 FRET ratio in INS-1 832/13 cells was unaffected by Rp-8-Br-cAMPS-pAB **(a)** or 4-Abn-OH **(b)**, each administered alone. Data for each panel indicate the mean ± s.e.m. for a single experiment that is representative of results obtained in *n = 3* independent experiments.

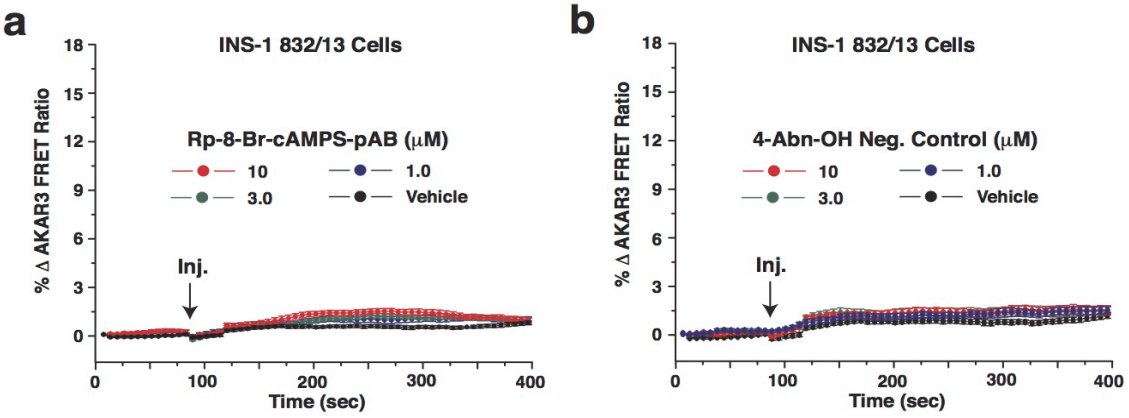

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Figure S6. Photomicrograph of pseudoislets from INS-1 832/13 cells. INS-1 832/13 cells were plated overnight at a density of approximately 80,000 cells per well using 6-well plates in which each well was coated with 1% gelatin. This procedure allowed the formation of small clumps of cells that resembled pseudoislets in their morphology, and that exhibited substantially higher rates of GSIS when compared to cells grown in monolayers.Calibration bar: 100 μm.

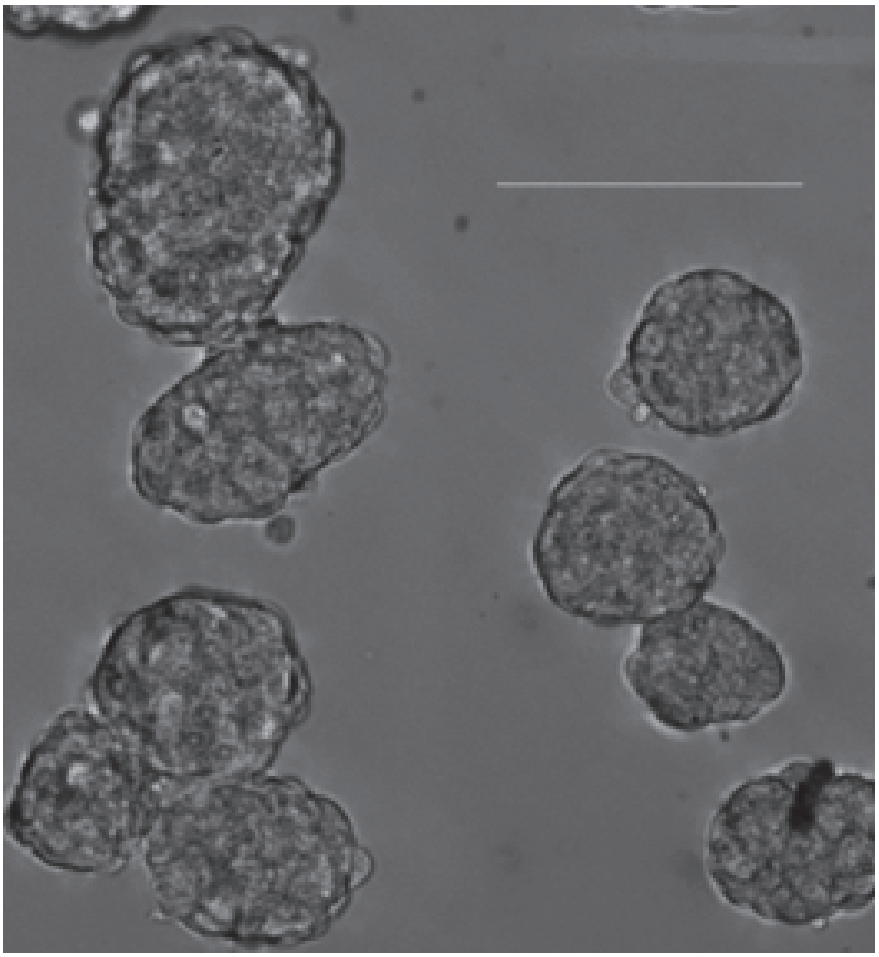

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Figure S7. AKAR3 FRET assays confirm that LRE1 inhibits sAC activity. (a) Wild-type (WT) HEK293 cells were virally transduced with AKAR3 to monitor PKA activity. No significant change in the FRET ratio was observed when these cells were treated with LRE1 (50 μM) alone. **(b)** Administration of LRE1 (50 μM) to HEK293-sAC cells led to a decrease of the FRET ratio that signifies reduced levels of cAMP owing to inhibition of sAC activity. **(c)** The cyclic nucleotide phosphodiesterase inhibitor IBMX (100 μM) failed to raise levels of cAMP in WT HEK293 cells treated or not treated with LRE1. This indicates that basal adenylyl cyclase activity is negligible in WT HEK293 cells. **(d)** IBMX (100 μM) raised levels of cAMP in HEK293-sAC cells, thereby indicating that these cells have elevated basal adenylyl cyclase activity in comparison to WT HEK293 cells. LRE1 (50 μM) counteracted this effect of IBMX, thereby demonstrating that it is an effective sAC inhibitor. Data for each panel indicate the mean ± s.e.m. for a single experiment that is representative of results obtained in $n = 3$ independent experiments

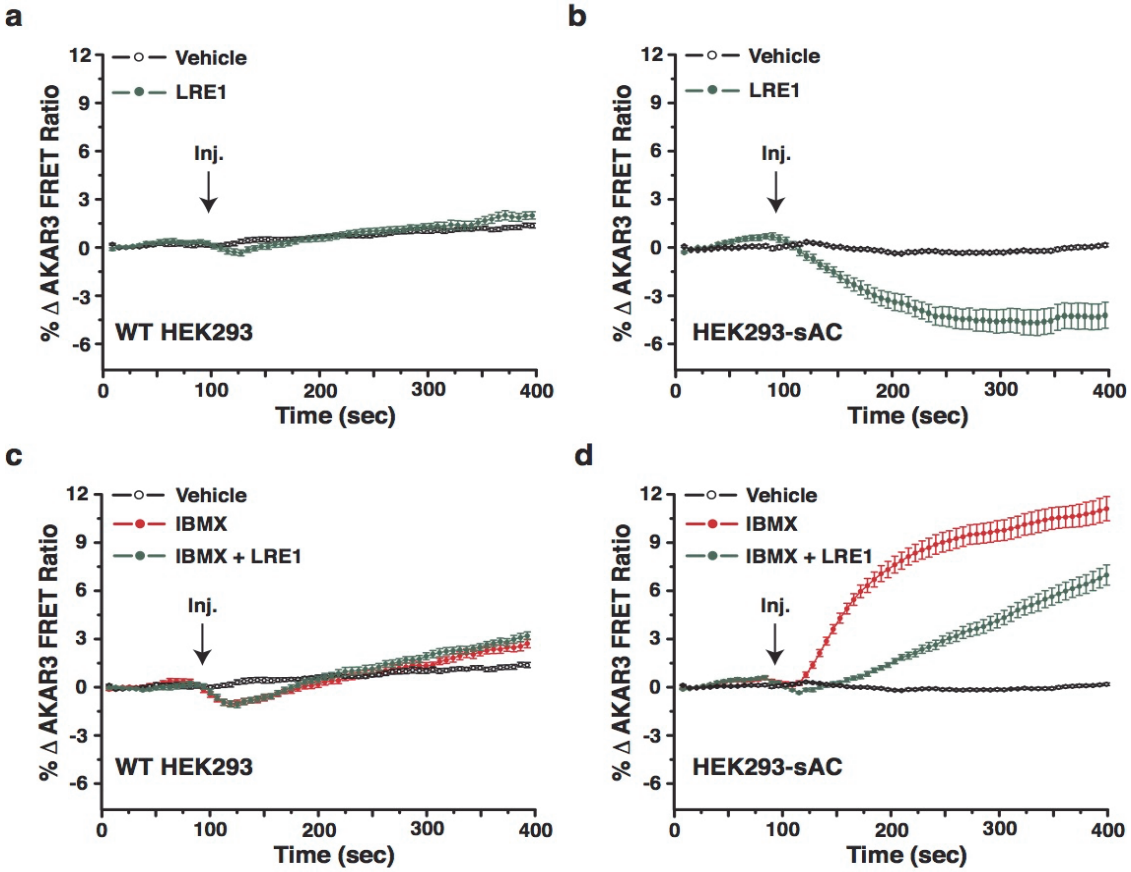

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Figure S8. siRNA knockdown studies of Cdc42 activation and GSIS. To determine if Cool-1/βPix mediates the effect of 8-pCPT-2'-*O*-Me-cAMP-AM to activate Cdc42 and to potentiate GSIS, we used siRNA to deplete endogenous Cool-1/βPix. INS-1 832/13 cells were transiently transfected with either control or Cool-1/□Pix-siRNA duplexes for 48 h and further incubated for 1 h with low glucose KRBH prior to each experiment. Cells were then pretreated for 20 min with low glucose solutions containing the vehicle, PO_4-AM_3 (3.3 μM), or 8-pCPT-2'-*O*-Me-cAMP-AM (10 μM). Cells were then exposed for an additional 2 min (for the Cdc42 activation assay) and 30 min (for GSIS) to KRBH containing either low glucose (1 mM) or high glucose (20 mM) prior to their lysis. **(a)** Representative immunoblots from one of three experiments demonstrate the knockdown of Cool-1/□Pix, when compared to control siRNA or untransfected cells. Total cellular content of actin served as a control for normalization of Cool-1/βPix levels under different experimental conditions. **(b)** Densitometry analysis of Cool-1/□Pix content relative to actin content for *n = 3* independent experiments, shown as the mean ± s.e.m.; *p < 0.05. **(c)** Data for static incubation assays of GSIS represent the mean ± s.e.m. from *n = 3* independent experiments. *p < 0.05, and **p < 0.05. (**d**) Data for Cdc42 activation assays for *n = 3* independent experiments. *p < 0.05 and **p < 0.05. Abbreviations: Con-si; Control siRNA; Cool-1/βPix-si; siRNA specific for Cool-1/βPix; N.S. not significant.

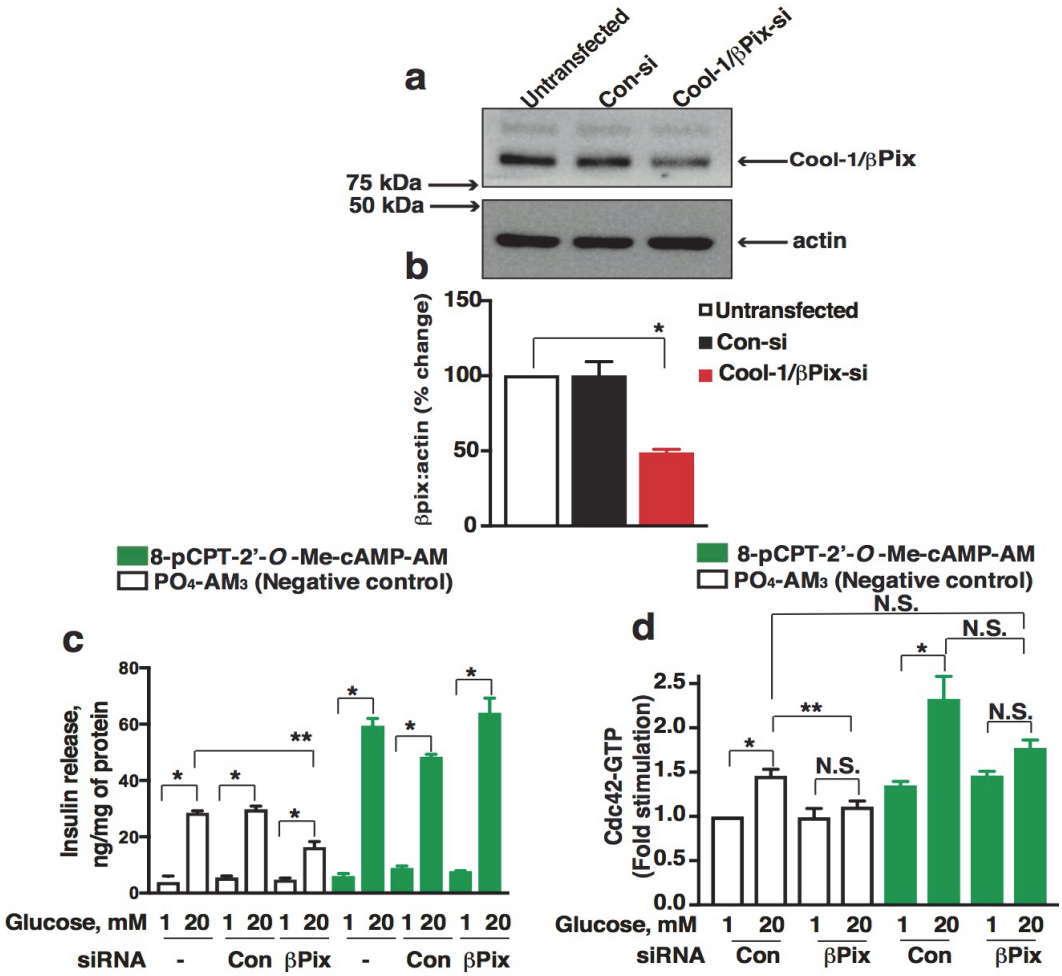

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Figure S9. Rp-8-Br-cAMPS-pAB raises levels of [Ca^{2+}] in INS-1 832/13 cells. (Top Panel) Averaged data obtained by single-cell imaging of *n* = 85 fura-2-loaded cells on a glass coverslip demonstrated that bath administration of 10 μM Rp-8-Br-cAMPS-pAB raised the $[Ca^{2+}]_i$. Although not illustrated, this action of Rp-8-Br-cAMPS-pAB was measured in single cells as an increase of $[Ca^{2+}]_i$ resulting from action potentials. **(Bottom Panel)** Averaged data obtained by single-cell imaging of *n* = 87 fura-2-loaded cells demonstrated that the negative control compound 4-Abn-OH (10 □M) failed to raise levels of $[Ca^{2+}]_i$. Ratio values are the mean + s.e.m. for both panels. The bath solution contained 16.7 mM glucose.