

## Supporting Information

### SI Methods

**Materials.** Oxotremorine-M methiodide (OXO-M), atropine sulfate, bethanechol chloride, arginine vasopressin (AVP), glibenclamide, exendin-4, sodium palmitate, and L-arginine were purchased from Sigma-Aldrich. [<sup>3</sup>H]-N-methylscopolamine ([<sup>3</sup>H]NMS; 79-83 Ci/mmol), [<sup>3</sup>H]-3-quinclidinyl benzilate ([<sup>3</sup>H]-QNB (32.9 Ci/mmol), and [ $\gamma$ -<sup>32</sup>P]ATP were from PerkinElmer Life Sciences. CX4945 was obtained from MedKoo Bioscience. Tissue culture media were from Invitrogen.

**Generation of Mutant Mice Lacking CK2 $\alpha$  in Pancreatic  $\beta$ -Cells.** The generation of homozygous floxed *CK2 $\alpha$*  mice (*fl/fl CK2 $\alpha$*  mice) has been described previously (1). These mice were obtained on a pure C57BL/6 background. We received *Pdx1-Cre-ER<sup>TM</sup>* mice with a mixed genetic background from Dr. Doug Melton (Harvard University). We backcrossed the *Pdx1-Cre-ER<sup>TM</sup>* mice for 10 generations onto a C57BL/6 background. We then crossed the backcrossed *Pdx1-Cre-ER<sup>TM</sup>* mice with the *fl/fl CK2 $\alpha$*  mice to generate *fl/fl CK2 $\alpha$ -Pdx1-Cre-ER<sup>TM</sup>* mice and *fl/fl CK2 $\alpha$*  control littermates. To induce Cre activity in  $\beta$ -cells, 8-week-old mice were injected for 6 consecutive days with tamoxifen (TMX; 1 mg i.p. per mouse per day; Sigma) suspended in corn oil (Sigma).

The following PCR primer pair was used to distinguish between the WT *CK2 $\alpha$*  allele and the floxed *CK2 $\alpha$*  allele: forward primer, 5'- GAGTCATCTGTCATCTGTAGC -3'; reverse primer, 5'- gACCACATCCTAACTATCC -3'. The use of these primers results in PCR products of the following sizes: WT allele, 500 bp; floxed allele, 600 bp. To detect the *Pdx1-Cre-ER<sup>TM</sup>* transgene, we used the following PCR primers (size of PCR product, 400 bp): forward primer, 5'-CCTGGAAAATGCTTCTGTCCG-3'; reverse primer, 5'-CAGGGTGTTATAAGCAATCCC-3'. To detect CK2 $\alpha$  protein via Western blotting, we used an anti-CK2 $\alpha$  antibody from Abcam (ab10470).

All animal experiments were conducted according to US National Institutes of Health Guidelines for Animal Research and were approved by the NIDDK Institutional Animal Care and Use Committee.

### **Generation of Transgenic Mice Overexpressing M3Rs in $\beta$ -cells ( $\beta$ -M3R Tg mice).**

We prepared a transgene construct in which the expression of the WT rat M3R was under the transcriptional control of a 650-bp fragment of the rat insulin promoter II (RIP II) (2). A 9-amino acid HA epitope tag was fused to the N-terminus of the M3R sequence. The features of the 3'-untranslated sequences of the vector have been described previously (2). The resulting 4.7-kb transgene was isolated, purified, and microinjected into the pronuclei of ova prepared from C57BL/6 mice (Taconic). Transgenic mice were identified by PCR analysis of mouse tail DNA. Specifically, mouse DNA was amplified by the following PCR primers which anneal to the 3'-untranslated region of the transgene: 5'-CTACGGGCTGCTCTACTGCTTCAGG (forward); 5'-GGCACTGGAGTGGCAACTTCCAAGG (reverse); size of PCR product: 171 bp; PCR conditions: 94 °C for 10 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s.

**RT-PCR Analysis of Transgene Expression in  $\beta$ -M3R Tg Mice.** Total RNA was prepared from several mouse tissues using the QIAzol Lysis Reagent (Qiagen), including a DNase I treatment step (RNeasy Mini Kit; Qiagen). cDNA was generated using the Superscript<sup>TM</sup> III first-strand synthesis system for RT-PCR kit (Invitrogen). In control samples, the reverse transcription step was omitted. We used the following PCR primers that are specific for the M3R transgene: forward, 5'-CCTACGACGTCCCCGACTAC (this primer anneals to the HA epitope tag); reverse, 5'-TGATGTAGGTCGTGAACAGG; size of PCR product: 405 bp. The PCR cycling conditions were as follows: 94 °C for 3 min followed by 32 cycles at 94 °C for 25 s, 54 °C for 40 s, and 72 °C for 30 s. PCRs were carried out in a final volume of 50  $\mu$ l containing 2  $\mu$ l of the RT reaction product (corresponding to  $\sim$ 0.1  $\mu$ g RNA), 5  $\mu$ l 10x *Taq* buffer containing 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Fermentas), 1.5 mM MgCl<sub>2</sub>, 1 mM of each dNTP, 200 nM of each PCR primer, and 1 unit of recombinant *Taq* DNA polymerase (Fermentas). GAPDH mRNA served as an internal control (forward primer, 5'-CGTGGAGTCTACTGGTGTCTTCACC; reverse primer, 5'-GATGGCATGGACTGTGGTCATGAGC; size of PCR product: 258 bp).

**CK2 Phosphorylation Assays With Cultured Cells.** HEK-293 cells were cultured in 175 cm flasks and transiently transfected using Lipofectamine® 2000 (Life Technologies Inc.) with pcDNA3-based expression plasmids coding for the WT mouse M3R or a phosphorylation-deficient version of this receptor (M3R-PD) (3). Both receptor constructs contained an N-terminal HA epitope tag (3). About 48 hr after transfection, cells were harvested, and membranes were prepared and re-suspended in TE buffer containing protease and phosphatase inhibitors (Complete, Roche Diagnostics).

For CK2 assays, 100  $\mu$ g membrane protein was incubated with 50  $\mu$ M ATP in CK2 assay buffer containing 5  $\mu$ ci  $\gamma$ <sup>32-P</sup>ATP with 500 units of CK2. The muscarinic agonist, OXO-M (100  $\mu$ M) and/or the CK2 inhibitor, CX4945 (10  $\mu$ M), were added as indicated. Reactions were incubated at 37°C for 30 min. Membranes were then solubilized by the addition of 750  $\mu$ l TBS buffer containing 1% NP-40, 3 mM EDTA, and protease and phosphatase inhibitors (Complete, Roche Diagnostics). Insoluble material was removed by centrifugation at 20,000 x g for 20 min. Receptors were immunoprecipitated from the resulting supernatant by addition of 25  $\mu$ l anti-HA affinity resin (Roche Diagnostics), followed by incubation at 4°C for 2 hr with mixing. Immune complexes were washed 3 times with 1 ml lysis buffer, re-suspended in 50  $\mu$ l 2 x SDS PAGE sample buffer and heated to 55°C for 5 min. Samples were separated by SDS-PAGE on 8% gels which were then dried and exposed to autoradiography film. Films were scanned and bands were quantified using AlphaImager software (Alpha Innotech). In parallel, Western blotting studies were carried out with an anti-HA antibody (Roche Diagnostics) to visualize total M3R protein (5% of the total sample was loaded).

**Use of Phos-Tag Technology to Detect CX4945-Sensitive Phosphorylation of Mouse  $\beta$ -Cell M3Rs.** We used Phos-tag methodology (4, 5) to study the effect of CX4945, a selective CK2 inhibitor, on agonist-mediated phosphorylation of HA-tagged M3Rs over-expressed by  $\beta$ -cells/islets of transgenic mice ( $\beta$ -M3R Tg mice). Islets from  $\beta$ -M3R Tg

mice were isolated by standard techniques (6) and incubated overnight at 37 °C in a 5% CO<sub>2</sub> incubator with CX4945 (10 μM) or vehicle (0.1% DMSO) in RPMI 1640 medium (Gibco) containing 5.5 mM glucose and 10% FBS. Subsequently, islets were transferred to fresh medium lacking FBS and incubated with or without CX4945 (10 μM) for 2 hr at 37 °C (5% CO<sub>2</sub>). After this incubation step, islets were stimulated with OXO-M (100 μM) for 5 min or left untreated (same buffer). Islets were then homogenized with a 150V/T Ultrasonic homogenizer. The homogenization buffer consisted of muscarinic binding buffer (11) containing 1.2% digitonin, and protease and phosphatase inhibitors (Complete, Roche Diagnostics). Islet lysates were then subjected to Phos-tag SDS-PAGE (~5 μg islet protein per lane, corresponding to ~20 islets). Gels for Phos-tag SDS-PAGE were prepared as described previously (4, 5). In brief, the resolving gel consisted of 5.5% acrylamide, apo-Phos-tag (25 μM; Wako Chemicals), Bis-Tris (357 mM), and ZnCl<sub>2</sub> (50 μM). Note that the apo-Phos-tag becomes part of the gel acrylamide matrix and interacts with Zn<sup>2+</sup> ions to generate the Zn-Phos-tag ligand that is able to interact with phosphate groups. Gels were first gently shaken for 10 min in ice-cold transfer buffer (NuPAGE, Life Technologies) containing 1 mM EDTA and then incubated at room temperature for 30 min in transfer buffer alone in order to increase transfer efficiency. Transfer was performed via trans-blot turbo technology (Bio-Rad) using 0.2 μm nitrocellulose membranes (Bio-Rad). HA-tagged M3Rs were detected with a rabbit monoclonal anti-HA antibody (Cell Signaling). This antibody detected two major M3R species. The higher molecular mass band is predicted to represent the phosphorylated form of the M3R, due to its sensitivity to CX4945 treatment. In each individual experiment, the density of the higher molecular mass band obtained in the absence of CX4945 was set equal to 100%. Immunoreactive bands were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The intensities of immunoreactive bands were quantitated using Image-J (NIH).

**MIN6 Cell Culture and siRNA-Mediated Knockdown of CK2α Expression.** MIN6 cells (a kind gift from Dr. Abner Notkins, NIH, Bethesda, MD, USA) were cultured as described previously (7). For gene silencing studies, we electroporated MIN6 cells (1.4 x 10<sup>6</sup> cells) with 150 pmol of siRNA according to the manufacturer's instructions (Amaxa Nucleofector). The following siRNA targeting the CK2α catalytic subunit of CK2 was used (8): 5'-CAUGAAUUAGAUCACGUTT. All siRNAs, including scrambled control siRNA, were obtained from Ambion. About 48 hr after electroporation, the effectiveness of siRNA-mediated knockdown of CK2α expression was confirmed via Western blotting by using a primary anti-CK2α polyclonal antibody (1:300 dilution; Santa Cruz), followed by incubation with an HRP-labeled secondary antibody (Cell Signaling Technology). Immunoreactive bands were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Transient Expression of Receptors in COS-7 Cells.** COS-7 cells were cultured and transfected with 4 μg of receptor-encoding plasmid DNA using the Lipofectamine plus kit (Invitrogen) (for details, see ref. 9).

**[<sup>3</sup>H]-NMS Radioligand Binding Assay.** [<sup>3</sup>H]-NMS saturation binding assays were performed using membranes prepared from transfected COS-7 cells as described previously (9).

**Determination of Intracellular Calcium Levels.** About 48 hr after electroporation of MIN6 cells with different siRNAs, agonist-dependent increases in intracellular calcium levels were determined by the use of FLIPR technology (Molecular Devices). All measurements were carried out in 96-well plates, as described in detail previously (10). Agonist concentration-response curves were analyzed by using Prism 4.0 software (GraphPad Software).

**Western Blotting Studies With Islet Lysates.** Western blotting studies were carried out with lysates prepared from isolated pancreatic islets by using standard techniques (6). The following antibodies were used: anti-CK2 $\alpha$  (Abcam), anti-Akt-1 (Cell Signaling), anti-Akt1-S129-P (Abcam), anti- $\alpha$ -Na/K ATPase (Abcam), anti- $\beta$ -actin (Cell Signaling), and anti-HA (rabbit mAb, Cell Signaling). Immunoreactive bands were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Protein bands were quantitated by using ImageJ software (NIH).

**Receptor Internalization and [<sup>3</sup>H]-QNB Binding Assays.** MIN6 cells were electroporated with CK2 $\alpha$  siRNA or scrambled control siRNA as described above. Cells were then transferred into 12-well plates (10<sup>6</sup> cells/well). Cells were grown for 48 hr and then incubated with OXO-M (100  $\mu$ M) for 60 min at 37 °C in FLIPR buffer (HBSS, 20 mM HEPES, pH 7.4 buffer). Following agonist treatment, cells were washed once with PBS and then scraped off the dishes with ice-cold DMEM (1 ml/well). Subsequently, cells were incubated for 2 hr at 4 °C in binding buffer (11) with a saturating concentration (2 nM) of the membrane-impermeable muscarinic radioligand [<sup>3</sup>H]-NMS (total volume: 0.5 ml). Nonspecific binding was determined in the presence of 10  $\mu$ M atropine. Protein concentrations were measured by using a bicinchoninic acid kit (Pierce), according to the manufacturer's protocol. Data were analyzed by using GraphPad Prism 4.0 software.

To determine the effect of CK2 $\alpha$  knockdown on the total number of muscarinic receptors, MIN6 cells were treated with CK2 $\alpha$  siRNA or scrambled control siRNA as outlined above. About 48 hr later, total cell lysates (~100  $\mu$ g total protein per tube) were prepared and incubated for 3 hr at room temperature in binding buffer with 0.5, 1, or 2 nM of [<sup>3</sup>H]-QNB, a membrane-permeable muscarinic radioligand (total incubation volume: 0.5 ml). [<sup>3</sup>H]-QNB binding data were obtained and analyzed as described in the previous paragraph for [<sup>3</sup>H]-NMS.

**Insulin Secretion Studies With Cultured Cells.** MIN6 cells that had been treated with CK2 $\alpha$  or control siRNA were incubated at 37 °C for 1 hr in Krebs-Ringer bicarbonate/HEPES buffer containing 3.3 mM glucose. The buffer was then removed and cells were incubated, in the same buffer, for 1 h at 37 °C with increasing concentrations of different insulin secretagogues. Insulin release was determined by measuring insulin concentrations in the incubation medium. MIN6 cells were detached from wells using

trypsin-free buffer and sonicated to liberate insulin in order to measure total insulin content. Insulin concentrations were measured via ELISA (Crystal Chem). Data were analyzed using GraphPad Prism 4.0 software.

**Insulin Secretion Studies With Mouse Isolated Pancreatic Islets.** Pancreatic islets were isolated from 14 week-old male WT mice (strain: C57BL/6NTac; Taconic Biosciences) as described (12). Batches of 12 islets were pretreated for 1 hr with CX4945 (10  $\mu$ M) or vehicle at 37 °C in Krebs-Ringer bicarbonate/HEPES buffer containing 3.3 mM glucose. After removal of the buffer solution, islets were incubated for 1 hr at 37 °C in the same buffer containing 16.7 mM instead of 3.3 mM glucose, either in the presence or absence of OXO-M (100  $\mu$ M) and/or CX4945 (10  $\mu$ M). After this incubation step, the medium was collected for insulin measurements, and the remaining islet insulin was extracted by sonication in acid/ethanol. The amount of insulin secreted during the incubation period was normalized to the total insulin content of each well (islets plus medium). Islet insulin content was measured by using an acid-ethanol method, as described in detail previously (13). Insulin concentrations were determined via ELISA (Crystal Chem).

**Human Pancreatic Islets: Source and Culture.** Human islets were received from the accredited Human Islet Resource Center at the University of Pennsylvania. The islet isolation procedure and culturing conditions have been described previously (14, 15). Isolated human islets were kept under culture conditions in medium at 5 mM glucose and 25 °C prior to transfer to the laboratory. The time that elapsed from islet isolation to transfer to the laboratory ranged from 1 to 3 days.

**Perifusion of Human Islets for Insulin Release Measurements.** Cultured human islets (180 islets) were placed on a nylon filter in a plastic perifusion chamber (Millipore) and were perfused with a flow rate of 1.2 ml per min (16). The perifusion apparatus consisted of a computer-controlled low pressure chromatography system (BIO-RAD Econo system) that allowed programmable rates of flow and glucose concentrations in the perfusate, a water bath (set at 37 °C), and a fraction collector (BIO-RAD; model 2128). The perfusate was a Krebs buffer (pH 7.4) containing (in mM): 114 NaCl, 5 KCl, 24 NaHCO<sub>3</sub>, 1 MgCl<sub>2</sub>, 6H<sub>2</sub>O, 2.2 Ca<sup>2+</sup>, 1 Pi, 10 HEPES (pH 7.4), and 1% of BSA (Sigma-Aldrich: fraction V, fatty acid-free) equilibrated with 20% O<sub>2</sub> and 5% CO<sub>2</sub> balanced with N<sub>2</sub>. In all experiments, a physiological mixture of 19 amino acids (17) was included in the perfusate. Initially, islets were perfused for 40 min with 3.3 mM glucose in the absence (control islets) or presence of CX4945 (10  $\mu$ M). The glucose concentration was then raised to 8 mM for an additional 20 min. Subsequently, an acetylcholine (ACh) ramp (0 to 1  $\mu$ M; 12.5 nM increment/min) was applied for an additional 40 min. Neostigmine (10  $\mu$ M), a cholinesterase inhibitor, was included in the perfusate to prevent ACh hydrolysis. CX4945 (10  $\mu$ M) was added to the perfusate at 3.3 mM glucose and was present during perfusion with 8 mM glucose and ACh. At the end of each experiment, KCl (30 mM) was added after a 30-min washout period. The area under curve (AUC) for ACh-stimulated insulin release was calculated using GraphPad software (Prism 6.0.) The baseline at 8 mM glucose (second phase insulin release) was used for the calculation of AUCs.

**Stimulation of Insulin Secretion by Bethanechol in Vivo.** WT mice (14 week-old males; strain: C57BL/6NTac; n=8/group) were injected with a single dose of CX4945 (25 mg/kg, i.p.) or vehicle (a mixture of DMSO/PEG400/distilled water; 0.5:4:5.5) (18), fasted for 4 hr, and then treated with the muscarinic agonist, bethanechol (2 mg/kg, s.c.). Blood was collected at different time points after bethanechol injection to measure plasma insulin levels. All experiments were carried out during the light cycle between 9 and 11 am.

**Chronic Treatment of Mice With CX4945.** WT mice or M3R-deficient mice (19) (7 week-old males and females; n=7 per group; genetic background: C57BL/6NTac) were injected twice a day for 6 days with either CX4945 (25 mg/kg, i.p.) or vehicle (5% DMSO in water). After the last CX4945 injection, mice were subjected to various metabolic tests (see the following paragraph).

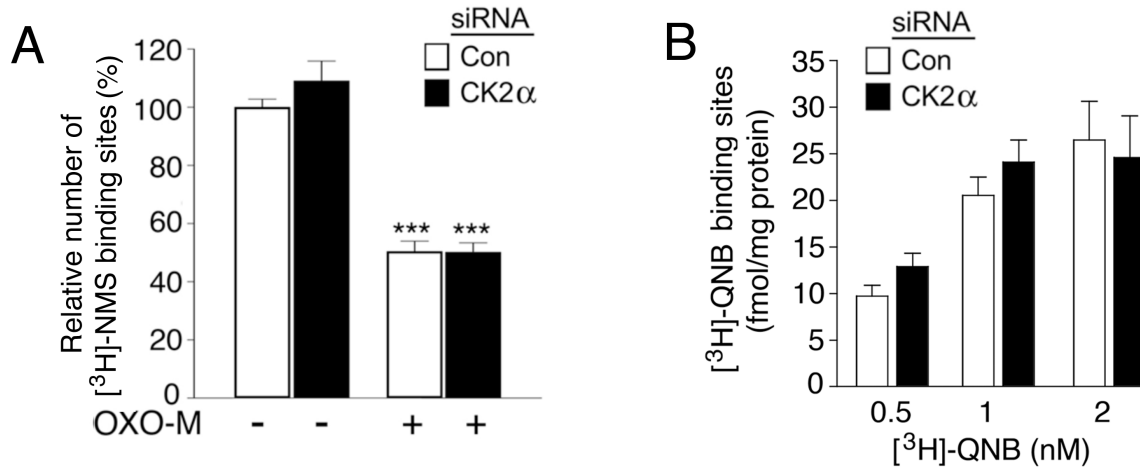
**In Vivo Metabolic Tests.** In vivo metabolic studies were performed using standard procedures. To assess i.p. glucose tolerance (IGTT), mice were fasted overnight for 12 hr. Blood samples were taken from the tail vein prior to (0 min) and 15, 30, 60, and 120 min after glucose administration (2 g i.p./kg for mice on RC; 1 g i.p./kg for mice on HFD, unless indicated otherwise). To determine peripheral insulin sensitivity (ITT), mice were fasted for 4 hr, and blood glucose concentrations were measured before and at the indicated time points after i.p. injection of human insulin (1 U/kg; Humulin, Eli Lilly). Blood glucose levels were determined using an automated blood glucose reader (Glucometer Elite Sensor; Bayer).

**Measurement of Mouse Plasma GLP-1 and Insulin Levels.** Mouse plasma GLP-1 levels were measured by using Luminex technology (Bio-Rad Bioplex; Bio-Rad), following the manufacturer's instructions. Plasma insulin concentrations were determined via ELISA (Crystal Chem).

**Statistics.** Data are expressed as means  $\pm$  SEM for the indicated number of observations. Statistical significance between groups was determined using two-tailed Student's t test or one-way analysis of variance followed by appropriate post-hoc tests.

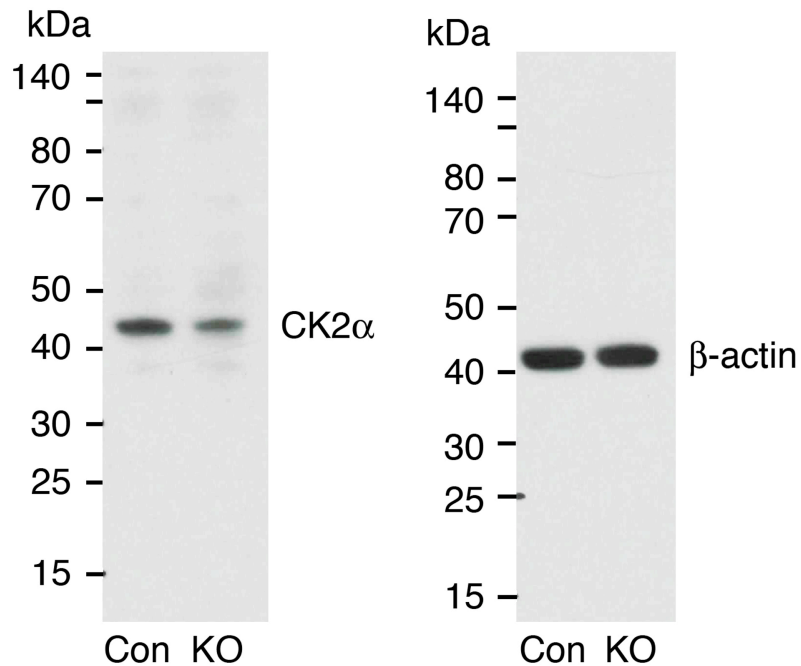
1. Rebholz H, Zhou M, Nairn AC, Greengard P, Flajolet M (2013) Selective knockout of the casein kinase 2 in D1 medium spiny neurons controls dopaminergic function. *Biol Psychiatry* 74:113-121.
2. Vasavada RC, et al. (1996) Overexpression of parathyroid hormone-related protein in the pancreatic islets of transgenic mice causes islet hyperplasia, hyperinsulinemia, and hypoglycemia. *J Biol Chem* 271:1200-1208.
3. Kong KC, et al. (2010) M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1. *Proc Natl Acad Sci USA* 107:21181-21186.
4. Kinoshita E, et al. (2009) Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat Protoc* 4, 1513-1521.

5. Kinoshita E, Kinoshita-Kikuta E (2011) Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. *Proteomics* 11:319–323.
6. Jain S, et al. (2013) Chronic activation of a designer G<sub>q</sub>-coupled receptor improves  $\beta$  cell function. *J Clin Invest* 123:1750-1762.
7. Ishihara H, et al. (1993) Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36:1139-1145.
8. Torrecilla I, et al. (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. *J Cell Biol* 177:127-137.
9. Han SJ, et al. (2005) Pronounced conformational changes following agonist activation of the M<sub>3</sub> muscarinic acetylcholine receptor. *J Biol Chem* 280:24870-24879.
10. Scarselli M, Li B, Kim SK, Wess J (2007) Multiple residues in the second extracellular loop are critical for M<sub>3</sub> muscarinic acetylcholine receptor activation. *J Biol Chem* 282:7385-7396.
11. Ruiz de Azua I, et al. (2012) Spinophilin as a novel regulator of M<sub>3</sub> muscarinic receptor-mediated insulin release in vitro and in vivo. *FASEB J* 26:4275-4286.
12. Gautam D, et al. (2006) A critical role for  $\beta$  cell M<sub>3</sub> muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. *Cell Metab* 3:449-461.
13. Duttaroy A, Zimlik C, Gautam D, Cui Y, Mears D, Wess, J. (2004). Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in M<sub>3</sub> muscarinic acetylcholine receptor-deficient mice. *Diabetes* 53, 1714-1720.
14. Deng S, et al. (2003) Insulin gene transfer enhances the function of human islet grafts. *Diabetologia* 46:386-393.
15. Deng S, et al. (2004) Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes* 53:624-632.
16. Doliba NM, et al. (2012) Glucokinase activation repairs defective bioenergetics of islets of Langerhans isolated from type 2 diabetics. *Am J Physiol Endocrinol Metab* 302:E87-E102.
17. Doliba NM, et al. (2007) Metabolic and ionic coupling factors in amino acid-stimulated insulin release in pancreatic beta-HC9 cells. *Am J Physiol Endocrinol Metab* 292:E1507-1519.
18. Son YH, Song JS, Kim SH, Kim J (2013) Pharmacokinetic characterization of CK2 inhibitor CX-4945. *Arch Pharm Res* 36:840-845.
19. Yamada M, et al. (20001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature* 410:207-212.

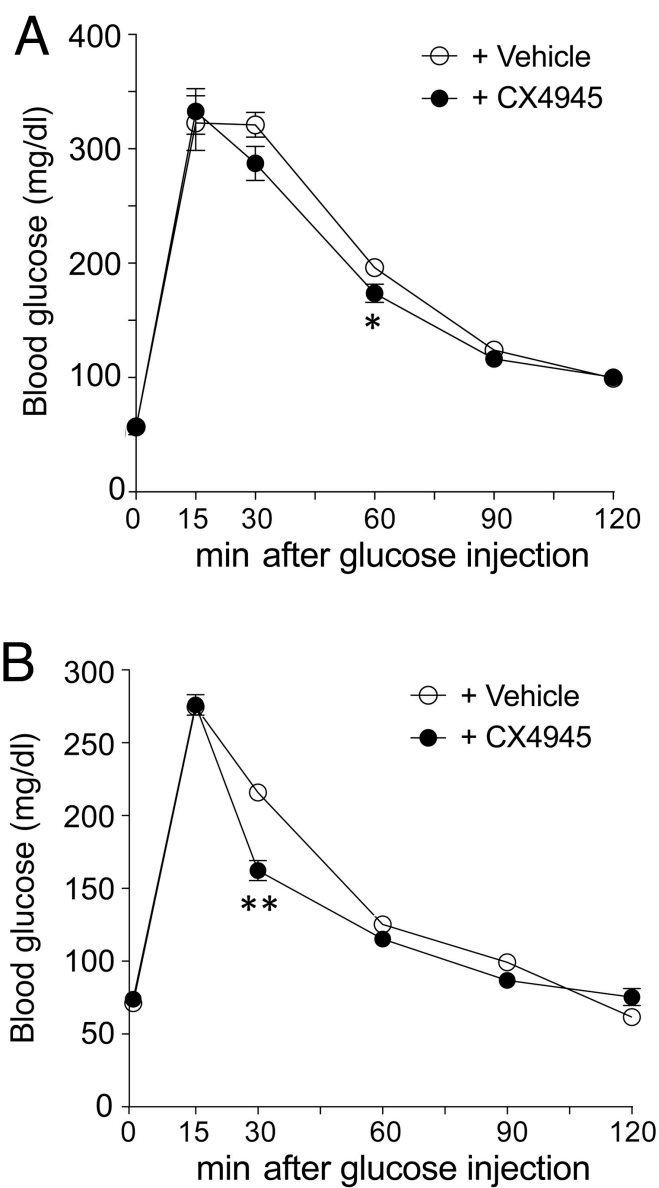


**Fig. S1.** CK2 $\alpha$  knockdown has no effect on agonist-induced M3R internalization or total M3R density. (A) Receptor internalization studies. MIN6 cells that had been electroporated with CK2 $\alpha$  siRNA or scrambled control siRNA were incubated with OXO-M (100  $\mu$ M) for 60 min at 37  $^{\circ}$ C in FLIPR buffer. Subsequently, cells were incubated for 2 hr at 4  $^{\circ}$ C in binding buffer with a saturating concentration (2 nM) of [<sup>3</sup>H]NMS, a membrane-impermeable muscarinic radioligand. In each individual experiment, M3R densities prior to OXO-M addition were set equal to 100% (mean initial receptor densities in fmol/mg: 308 $\pm$ 29). Data are given as means  $\pm$  SEM of three independent experiments. \*\*\* $p$  < 0.001, as compared to the corresponding value prior to OXO-M treatment. (B) Determination of total M3R numbers. MIN6 cells were treated with CK2 $\alpha$  siRNA or scrambled control siRNA as in (A). About 48 hr later, total cell lysates ( $\sim$ 100  $\mu$ g total protein per tube) were prepared and incubated for 3 hr at room temperature in binding buffer with the indicated concentrations of [<sup>3</sup>H]-QNB, a membrane-permeable muscarinic radioligand. Data are presented as means  $\pm$  SEM of three independent experiments.

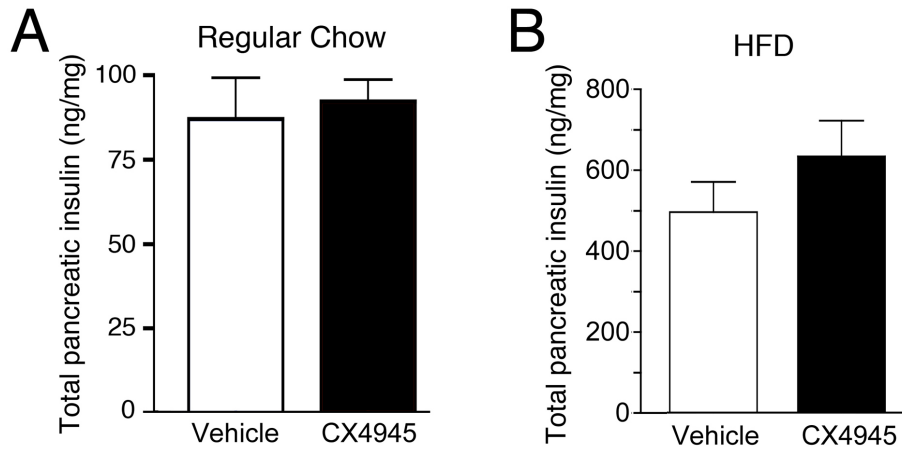




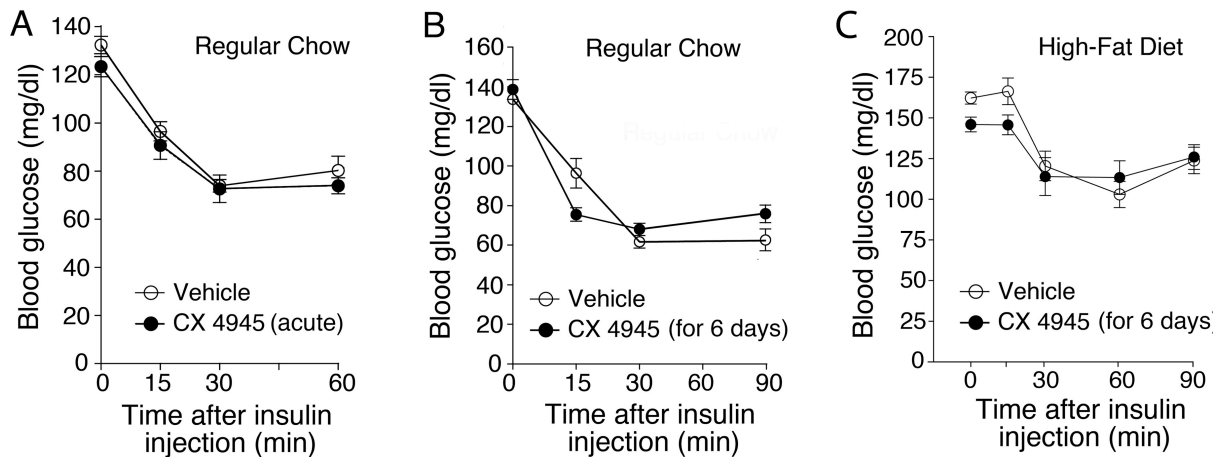
**Fig. S2.** CK2 $\alpha$  expression is strongly reduced in pancreatic islets from tamoxifen (TMX)-treated *fl/fl CK2 $\alpha$ -Pdx1-Cre-ER<sup>TM</sup>* mice. Pancreatic islets were prepared from TMX-treated *fl/fl CK2 $\alpha$ -Pdx1-Cre-ER<sup>TM</sup>* mice (' $\beta$ -CK2 $\alpha$ -KO mice'; KO) and *fl/fl CK2 $\alpha$*  control littermates (Con) (mouse age: ~11 weeks). Islet lysates were subjected to Western blotting analysis using an anti-CK2 $\alpha$  antibody (left panel). The expression of  $\beta$ -actin was monitored to ensure that equal amounts of protein were loaded (right panel). The figure shows representative immunoblots. Two additional experiments gave similar results. On average, pancreatic islets prepared from TMX-treated *fl/fl CK2 $\alpha$ -Pdx1-Cre-ER<sup>TM</sup>* mice ( $\beta$ -CK2 $\alpha$ -KO mice) showed a ~50% reduction in CK2 $\alpha$  expression, as compared to control littermates. The residual expression of CK2 $\alpha$  displayed by the KO islets is most likely due to expression of CK2 $\alpha$  in other cell types (non- $\beta$ -cells) of the endocrine pancreas ( $\alpha$ -cells,  $\delta$ -cells, etc.).



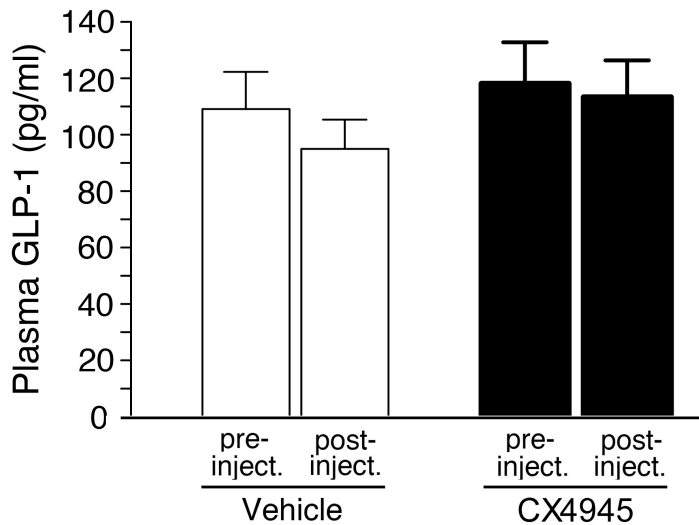
**Fig. S3.** Inhibition of CK2 slightly improves glucose tolerance in mice. (*A, B*) Intraperitoneal glucose tolerance tests (IGTT) were carried out with 7 week-old WT mice maintained on regular mouse chow (*A*, males; *B*, females). Mice were injected twice a day for 6 days with CX4945 (25 mg/kg, i.p.) or vehicle (DMSO). At the end of the 6 day injection period, IGTTs were carried out as described under SI Methods. Data are given as means  $\pm$  SEM ( $n=7$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ , as compared to the control value.



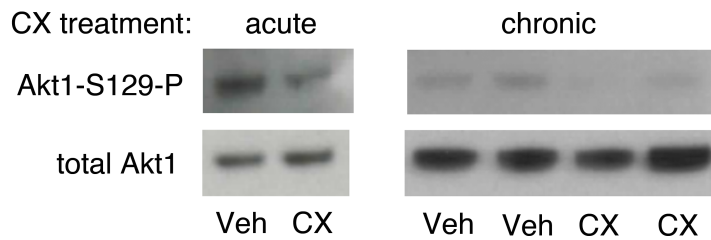
**Fig. S4.** Chronic inhibition of CK2 *in vivo* has no significant effect on total pancreatic insulin content. Adult male mice consuming regular chow (A) or a high-fat diet (HFD) (B) were injected twice a day for 6 days with CX4945 (25 mg/kg, *i.p.*) or vehicle (DMSO). At the end of the 6 day injection period, total pancreatic insulin content was determined as described under SI Methods. Data are given as means  $\pm$  SEM (n=5 per group).



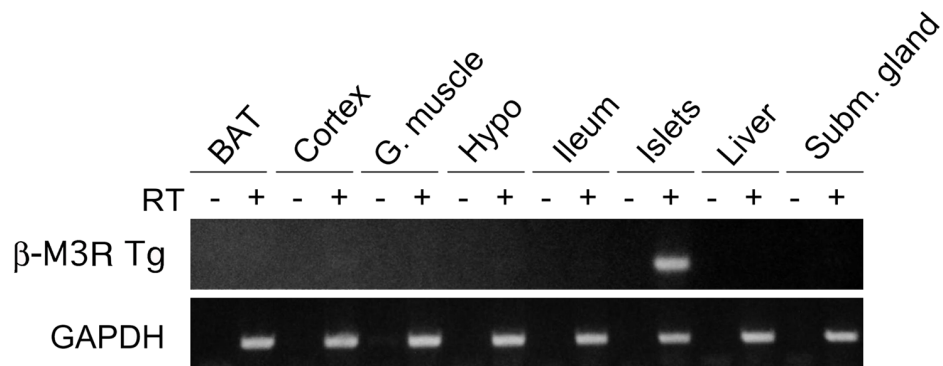
**Fig. S5.** Insulin sensitivity remains unaffected after CX4945 treatment of WT mice. Male WT mice were maintained on regular chow (A, B) or a high-fat diet (HFD) for 9 weeks (C). Insulin tolerances tests were carried out with mice that had been fasted for 4 hr (insulin dose: 1 U/kg *i.p.*). (A) Mice were co-injected with insulin and CX4945 (25 mg/kg, *i.p.*) or vehicle (DMSO) (acute CX4945 treatment). (B, C) Mice were treated twice a day for 6 days with CX4945 (25 mg/kg, *i.p.*) or vehicle (DMSO) (chronic CX4945 treatment) and then injected with a single dose of insulin (1 U/kg, *i.p.*). Blood glucose levels were determined at the indicated time points. Data are given as means  $\pm$  SEM (n=8 per group).



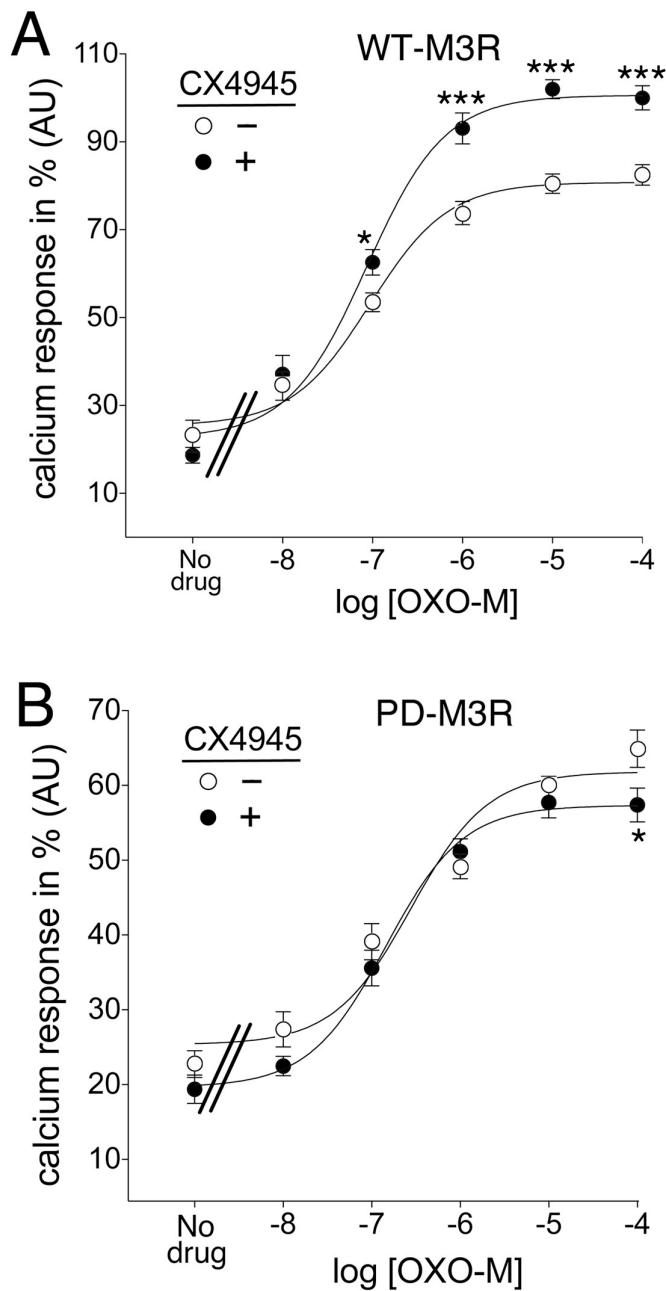
**Fig. S6.** Plasma GLP-1 levels remained unaltered after CX4945 treatment of high-fat diet (HFD) mice. Male WT mice were maintained on a HFD for 9 weeks and then injected twice a day for 6 days with CX4945 (25 mg/kg, i.p.) or vehicle (DMSO). Plasma GLP-1 levels were determined prior to and immediately after the 6-day injection period. Data are given as means  $\pm$  SEM (n=8 per group).



**Fig. S7.** CX4945 treatment greatly reduces CK2-mediated S129-phosphorylation of Akt1 in mouse pancreatic islets. Male WT mice (age: ~15 weeks) received (A) a single i.p. injection of CX4945 (25 mg/kg; CX) or vehicle (Veh; DMSO) (acute treatment) or (B) two i.p. injections per day for 6 consecutive days of CX4945 (25 mg/kg; CX) or vehicle (Veh) (chronic treatment). Pancreatic islets were isolated 4 hr after CX4945 or vehicle administration (A) or 4 hr after the last CX4945 or vehicle injection (B), respectively. Islet lysates were subjected to Western blotting analysis by using antibodies directed against total Akt1 or Akt1-S129-P (molecular mass: ~60 kDa). CX4945 treatment caused a ~40-50% reduction in Akt1-S129-P expression levels under both experimental conditions. The figure shows representative immunoblots. Two additional Western blots yielded similar results.



**Fig. S8.** Selective overexpression of the WT M3R in pancreatic islets of  $\beta$ -M3R Tg mice. A transgene-specific PCR primer pair was used to amplify cDNA prepared from the indicated tissues of adult  $\beta$ -M3R Tg mice. While transgene expression was readily detectable in islets, it was undetectable in other peripheral and central tissues. Control samples (indicated by the '-' signs above the lanes) that had not been treated with reverse transcriptase (RT) did not give any detectable RT-PCR products, confirming the absence of contaminating genomic DNA. GAPDH cDNA served as an internal control. BAT, brown adipose tissue; Cortex, cerebral cortex; G. muscle, gastrocnemius muscle (skeletal muscle); Hypo, hypothalamus; Subm. gland, submandibular gland.



**Fig. S9.** Pharmacological inhibition of CK2 does not affect calcium responses caused by stimulation of a phosphorylation-deficient (PD) mutant M3R in vitro. (A, B) COS-7 cells were transiently transfected with HA-tagged versions of the WT mouse M3R (A) or a phosphorylation-deficient mutant M3R (PD-M3R; see text for details) (B). Cells were then treated with increasing concentrations of the muscarinic agonist, OXO-M, in the presence or absence of CX4945 (10  $\mu$ M), a highly selective CK2 inhibitor. Increases in  $[Ca^{2+}]_i$  were determined via FLIPR. Data are expressed as means  $\pm$  SEM of three independent experiments, each carried out in quadruplicate. AU, arbitrary units. \* $p < 0.05$ , \*\*\* $p < 0.001$ , as compared to the corresponding control value.