Supporting Information

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SI Materials and Methods

Transfection. Cultured cells were split 24 h before transfection (30% confluence at time of transfection). Qiagen Effectene reagent and corresponding manufacturer's instructions were used to cotransfect cells with active calcium/calmodulin-dependent protein kinase II (CaMKII) (pcDNA3.1-CaMKII-T286D), pECE-SUR1 and Kir6.2-GFP, Kir6.2-T224E-GFP, Kir6.2-T224A-GFP, Kir6.2-S37E-GFP, Kir6.2-S37A-GFP, Kir6.2-S372E-GFP, Kir6.2-S372A-GFP or empty pAcGFP1N2 (Clontech) constructs. Transfection proceeded for 7 h at 37 °C and the cells were allowed to recover overnight. Transfected cells were plated on coverslips for analysis (electrophysiology).

Antibodies. The following antibodies were used for immunoblotting, immunostaining, and coimmunoprecipitation: ankyrin-G (NeuroMab), β_{IV}-spectrin, Kir6.2 (Alomone), SUR1 (Santa Cruz), CaMKIIδ (Santa Cruz), GAPDH (Fitzgerald), and ankyrin-B. Affinity-purified polyclonal antibody against phospho-Kir6.2T224 was developed (Covance) and validated.

Electrophysiology. Wild-type Kir6.2 and Kir6.2 mutants were transfected into COSm6 cells and analyzed in the absence and presence of constitutively active CaMKIIδ as described (1–3).

Immunostaining and Confocal Microscopy. Tissues were removed from mice, rinsed in 1× PBS and placed in 4% (wt/vol) paraformaldehyde (PFA) overnight. The tissues were subsequently submerged in 10%, 20%, and 30% (wt/vol) sucrose solutions before mounted in optimal cutting temperature (OCT) and sectioned to 10-μm thickness. Cryoslides were rehydrated in 1× PBS, fixed with 2% PFA/PBS, and blocked with 2 mg/mL BSA + 0.1% Triton X-100 in PBS (blocking buffer). Primary incubations were carried out in blocking buffer overnight at 4 °C and rinsed in 2 mg/mL BSA + 1% (vol/vol) Triton X-100 in PBS. Secondary incubations were carried out in blocking buffer for 2 h at 4 °C, washed, and mounted with VectaShield (no. 1 coverslips). Images were captured using a Zeiss Meta 710 confocal microscope, analyzed with accompanying Carl Zeiss software, and images were assembled using Adobe Photoshop CS5.

In Vitro Translation and Binding. CaMKII-pcDNA3.1+ was in vitro translated using the TNT Coupled Reticulocyte Lysate System (Promega) and manufacturer's protocols. In vitro translated products were incubated with immobilized GST or immobilized GST–β_{IV}-spectrin constructs overnight at 4 °C in binding buffer (PBS + 750 mM NaCl, 0.1% Triton X-100). Reactions were washed three times in binding buffer, eluted, and separated by SDS/PAGE. The gels were stained with Coomassie Blue and dried for phosphorimaging.

GST-Fusion Protein Production and Purification. cDNAs for β_{IV}-spectrin mutants: amino acids 1–699, 2015–2318, amino acids 1620–2218, and Kir6.2-CT (amino acids 160–190) were PCR-generated, cloned into pGEX6P-1 (Amersham Biosciences), and sequences were confirmed before experimentation. To facilitate cloning, all β_{IV}-spectrin constructs were engineered to contain 5′ BamHI and 3′ XhoI restriction sites and the Kir6.2-CT construct was engineered to contain 5′ BamHI and 3′ NotI restriction sites. BL21(DE3)pLysS cells (Invitrogen) were transformed with the pGEX6P-1 constructs and grown overnight at 37 °C in LB supplemented with ampicillin (50 μg/mL). The overnight cultures were subcultured for large-scale expression. Cells were grown to an optical density of 0.6–0.8 and induced with 1 mM isopropyl 1-thio-α-D-galactopyranoside (IPTG) for 2 h at 37 °C. Cells were centrifuged for 10 min at 8,000 × *g*, resuspended in PBS, and frozen at −80 °C following resuspension. Cells were lysed by thawing. The crude extract was resuspended in lysis buffer (1 mM DTT, 1 mM EDTA, 40 μg/mL 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μg/mL leupeptin, 40 μg/mL benzamidine, 10 μg/mL pepstatin) and sonicated to emulsify. Cell debris was removed by centrifugation at 11,000 × *g* for 1 h at 4 °C. Supernatants were added to 2 mL equilibrated glutathione-agarose (Amersham Biosciences) overnight at 4 °C. The glutathione-agarose solutions were washed with PBS containing 1% Triton X-100 (three times), PBS containing 500 mM NaCl (three times), and stored in PBS containing 1 mM NaN_3. Protein purification and sizes were verified by SDS/PAGE followed by Coomassie staining.

Preparation of Tissue Lysates. Tissues were harvested, flash frozen on liquid nitrogen, and proteins were solubilized as described in detail (4). Four volumes of homogenization buffer (PhosphoSafe extraction buffer; Novagen) were added to each sample and homogenized using a hand-held homogenizer. Samples were centrifuged at 100,000 × *g* for 15 min and the supernatant was stored at −80 °C. All samples were quantitated using the Pierce BCA kit before experimentation.

Islet Isolation and Dispersal. Mouse pancreatic islets were isolated and dispersed as described by our group and coworkers (5, 6). To ensure that only islets (not exocrine tissue) were used, individual islets showing smooth peripheral surface were selected from the media siliconized pipettes to avoid adherence. Viable islets were handpicked under the dissecting microscope.

Peptide Pull-Down Experiments. Biotin-conjugated β_{IV}-CTD (CTP-P) or scrambled β_{IV}-CTD (CTP-C) peptides were incubated with streptavidin beads overnight at 4 °C in PBS. The beads, along with a control of only streptavidin beads, were washed thoroughly with PBS before the addition of radiolabeled CaMKII or pancreatic lysate and protease inhibitors. The beads and proteins were incubated overnight at 4 °C in PBS. Following incubation, the beads were washed in pull-down buffer [50 mM Tris·HCl (pH 7.35), 250 mM NaCl, 0.32 M sucrose, 5 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, protease inhibitor mixture (1:100; Sigma), 1.5% (vol/vol) Triton X-100] and proteins were eluted by boiling in loading buffer plus reducing agent. The eluted proteins were separated by SDS/PAGE and either (*i*) gel dried before exposure to autoradiography film (for radiolabeled CaMKII binding) or (*ii*) transferred to nitrocellulose (for pancreatic lysate), followed by immunoblotting.

Coimmunoprecipitations. For standard coimmunoprecipitations experiments, 100 μg of tissue lysate was incubated with control Ig or experimental Ig in binding buffer [1× PBS, 0.1% Triton X-100, 250 mM NaCl, with protease inhibitor mixture (1:100; Sigma)] for 2 h at 4 °C. A total of 50 μL washed protein A sepharose beads was added and the reactions were incubated for an additional 2 h at 4 °C. Beads were washed three times in binding buffer, eluted, and the interacting proteins were separated by SDS/PAGE. Nitrocellulose blots were incubated with the appropriate antibodies and developed using standard ECL protocols. Before comparison of coimmunoprecipitation recoveries across multiple genotypes, protein levels were first quantified by standard immunoblot. Once quantified, subsequent coimmunoprecipitation experiment input loading was adjusted

to equilibrate target protein levels across genotypes to ensure that equal amounts of target protein were represented in the lysate input. For example, as Kir6.2 levels were reduced 50% in qv^{4J} islets compared with wild-type and qv^{3J} islets, the input for β_{IV}-spectrin coimmunoprecipitation experiment of Kir6.2 for the qv^{4J} input was doubled.

Pull-Downs. Immobilized GST-fusion proteins were incubated with 100 μg pancreatic lysate or radiolabeled proteins overnight in pull-down buffer at 4 °C. The samples were washed three times in pull-down buffer, eluted, and proteins were separated by SDS/PAGE. The gels were either dried and phosphorimaged (for radiolabeled binding) or transferred to nitrocellulose and immunoblotted. Nitrocellulose blots were developed using standard ECL protocols.

In Vitro Phosphorylation Assay. Active CaMKII (Millipore) was autophosphorylated by incubation in a solution of calmodulin (Millipore), ATP, $CaCl_2$, and Tris-binding buffer [20 μM Mops-NaOH (pH 7), 1 mM EDTA, 0.01% Brij-35, 5% glycerol, 0.1% 2-mercaptoethanol, 1 mg/mL BSA] on ice for 5 min. Phosphorylation reactions were performed by incubation of equal concentrations of immobilized GST-fusion protein products (including β_{2a} as a positive control and GST as a negative control) in a solution of Tris-binding buffer, calmodulin, $CaCl_2$, MgATP, ^{32}[P]γ ATP, and the autophosphorylated CaMKII on ice for 15 min. Reactions were washed five times with PBS + 5 mM EDTA, eluted, and separated by SDS/PAGE. The gel was dried and exposed to autoradiography film overnight.

Cell Culture. COSm6 cells were maintained in Dulbecco's modified eagle media, supplemented with 10% (vol/vol) FBS and 0.1% penicillin/streptomycin. Cells were cultured at 37 °C/5% CO_2. INS-1 cells were maintained in RPMI-1640 supplemented with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1 mM Na-pyruvate, 0.05 mM 2-mercaptoethanol, 0.1% penicillin/streptomycin, and 11.5 mM glucose. INS-1 cells were cultured at 37 °C/5% CO_2. For activation studies, INS-1 cells were plated to achieve 100% confluency at time of analysis. Cells were pretreated with 1 μM myristoylated autocamtide-2–related inhibitory peptide (AnaSpec) prepared in DMEM for 20 min at 37 °C. Cells were then incubated with 1 μM isoproterenol (prepared in DMEM) for 15 min at 37 °C. Media were removed and cells were immediately lysed using PhosphoSafe extraction reagent (Novagen). Control cells were incubated with DMEM alone at 37 °C.

Animals. All mice used were 8-wk-old male littermates and were housed and fed under identical conditions. C57BL/6J-$Spnb4^{qv-3J}$/J, B6ByJ;D2-$Spnb4^{qv-4J}$/J, and wild-type littermates were obtained from The Jackson Laboratory. All studies were under the auspices of the Institutional Animal Care and Use Committee (The Ohio State University Wexner Medical Center, Columbus, OH).

Statistics. Data are presented as mean ± SD. When only two groups were compared, unpaired *t* tests were used to assess significance. Differences were assumed to be significant in each case if $P < 0.05$, unless indicated; nonsignificant differences were not indicated. ANOVA and a post hoc test was used for comparison for repeated measures, such as multiple ATP concentrations of a single mutant or genotype.

1. Kline CF, et al. (2009) Dual role of K ATP channel C-terminal motif in membrane targeting and metabolic regulation. *Proc Natl Acad Sci USA* 106(39):16669–16674.
2. Koval OM, et al. (2012) CaMKII-based regulation of voltage-gated Na+ channel in cardiac disease. *Circulation* 126(17):2084–2094.
3. Li J, Kline CF, Hund TJ, Anderson ME, Mohler PJ (2010) Ankyrin-B regulates Kir6.2 membrane expression and function in heart. *J Biol Chem* 285(37): 28723–28730.
4. Mohler PJ, Davis JQ, Bennett V (2005) Ankyrin-B coordinates the Na/K ATPase, Na/Ca exchanger, and InsP3 receptor in a cardiac T-tubule/SR microdomain. *PLoS Biol* 3(12):e423.
5. Zmuda EJ, Powell CA, Hai T (2011) A method for murine islet isolation and subcapsular kidney transplantation. *J Vis Exp* (50):2096.
6. Benninger RK, et al. (2011) Defects in beta cell Ca²+ signalling, glucose metabolism and insulin secretion in a murine model of K(ATP) channel-induced neonatal diabetes mellitus. *Diabetologia* 54(5):1087–1097.

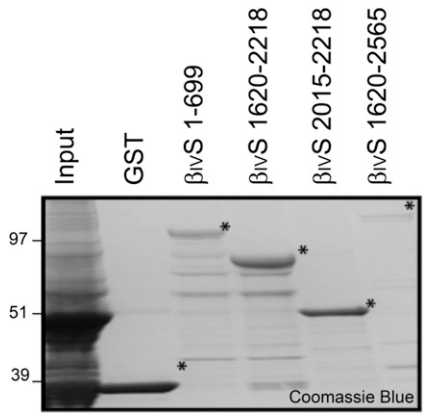

Fig. S1. Representative Coomassie Blue stained gel of β_{IV}-spectrin GST-fusion constructs used in pull-down experiments. Before binding experiments, protein concentrations were quantified by BCA assay and equal concentrations of fusion protein were used.

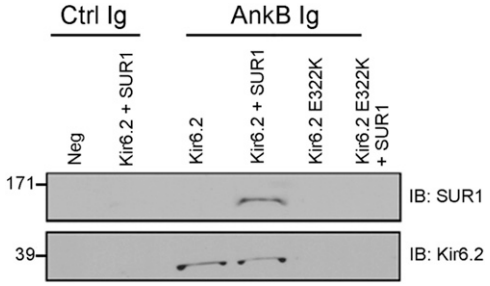

Fig. S2. Ankyrin-B immunoprecipitation of SUR1 requires Kir6.2. COSm6 cells cotransfected with Kir6.2 and SUR1 demonstrate coimmunoprecipitation with ankyrin-B Ig, but not control Ig. Conversely, disruption of the ankyrin-B/Kir6.2 association (Kir6.2 E322K) results in a loss of coimmunoprecipitation of SUR1.

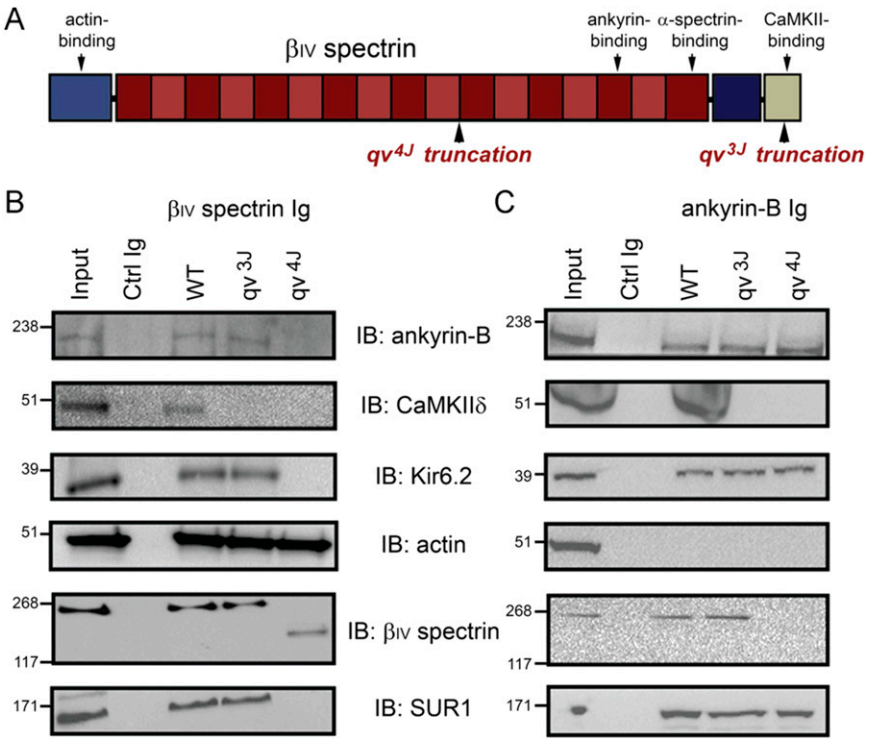

Fig. S3. β_{IV}-Spectrin mutant quivering (*qv*) mice display select loss of CaMKII and ankyrin-binding. (*A*) qv^{3J} mice harbor a premature truncation proximal to the CaMKII-binding motif in β_{IV}-spectrin, whereas qv^{4J} mice express a β_{IV}-spectrin construct truncated after spectrin repeat 10 (proximal to ankyrin-binding motif). (*B*) β_{IV}-Spectrin Ig immunoprecipitates AnkB, CaMKIIδ, Kir6.2, SUR1, and actin from WT islet lysates. In β_{IV}-spectrin qv^{3J} islets, whereas AnkB, Kir6.2, SUR1, and actin associate with β_{IV}-spectrin, the CaMKIIδ interaction is lost. In qv^{4J} islets, only the β_{IV}-spectrin/actin interaction is retained. (*C*) AnkB Ig immunoprecipitates β_{IV}-spectrin, CaMKIIδ, Kir6.2, SUR1, but not actin from islet lysates. AnkB associates with β_{IV}-spectrin Kir6.2 and SUR1, but not CaMKIIδ in qv^{3J} islets. In qv^{4J} islets, AnkB association with β_{IV}-spectrin is lost; however, Kir6.2 and SUR1 interactions are retained. Note that for data in *B* and *C*, immunoprecipitations for CaMKII used ninefold amounts of input lysate from qv^{3J} and qv^{4J} islets to compensate for ∼90% reduction in CaMKII levels in these two mouse lines (Fig. 3*F*). Immunoprecipitations for ankyrin-B, Kir6.2, and SUR1 used approximately doubled amounts of input lysate from qv^{4J} islets to compensate for ∼50% reduction in expression of the three proteins.

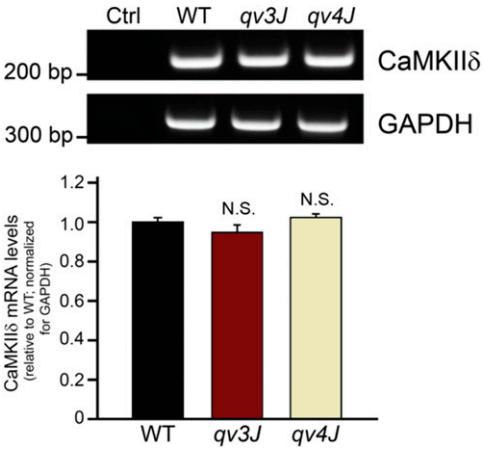

Fig. S4. β_{IV}-Spectrin qv^{3J} and qv^{4J} islets do not display altered CaMKIIδ levels. Purified pancreatic islets from age- and sex-matched WT, qv^{3J}, and qv^{4J} mice display equivalent CaMKIIδ mRNA levels (n > 150 islets per mouse, n = 3 mice per genotype). Levels are displayed as relative CaMKIIδ levels corrected for GAPDH mRNA expression.

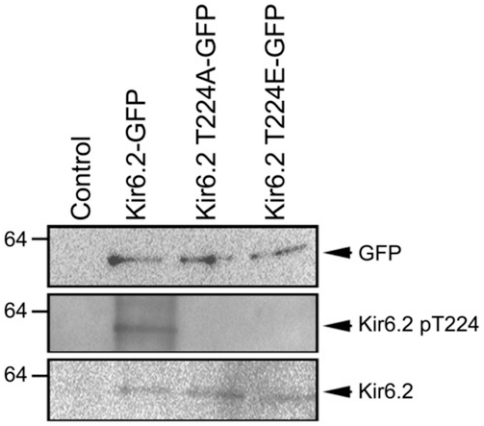

Fig. S5. Validation of affinity-purified antibody specific for phosphorylated Kir6.2 T224 (Kir6.2 pT224). Transfected COSm6 cells were stimulated with isoproterenol (1 μM), lysed, and GFP-tagged constructs were isolated by immunoprecipitation with anti-GFP Ig agarose beads. Immunoblots demonstrate that neither Kir6.2 T224A-GFP nor Kir6.2 T224E-GFP was recognized with the Kir6.2 pT224 antibody, whereas Kir6.2-GFP displayed significant immunosignal.

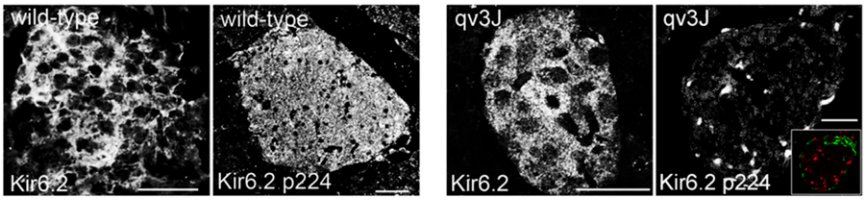

Fig. S6. CaMKII regulates Kir6.2 by direct phosphorylation of Kir6.2 T224. Immunostaining of Kir6.2 and Kir6.2 p224 in WT (*Left*) and qv^{3J} mice (*Right*). Qv^{3J} islets display normal Kir6.2 immunostaining but significant loss of Kir6.2 pT224. Note that Kir6.2 pT224 staining is sufficiently reduced that only background staining (i.e., labeled erythrocytes) is detected in these samples even at high confocal laser strength. (Scale bar, 20 μm.) *Inset* shows same sample colabeled in green for glucagon to denote boundary of islet.