Appendix VI
Methods for Chapter 7

Cell Culture. HIT-T15 cells, passage 65-79, (American Type Culture Collection) were grown in RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin, 0.1 mg/ml streptomycin (RPMI-1640 complete, Invitrogen, Carlsbad, CA) containing either 5.5 mM glucose and 19.5 mM mannitol (3.05 ml of 1 M glucose and 13.83 ml of 1M mannitol was added to 555 ml of media) or 25 mM glucose (13.88 ml of 1 M glucose was added to 555 ml of media) as indicated. Cell passages 66-79 were used for experiments. Cells were subcultured every 6-8 days. MIN6 cells, passage 25-35 (a generous gift from Dr. Marc Montminy, Salk Institute, La Hoya) were grown in DMEM + 10% FBS + 100 U/ml penicillin, 0.1 mg/ml streptomycin (DMEM complete, Invitrogen, Carlsbad, CA) containing 5.5 mM glucose for two days. After two days culture in low glucose (5.5 mM) media was changed to either low glucose (5.5 mM) DMEM complete or high glucose (25 mM) DMEM complete and cells were cultured for an additional 24 h.

Drug Treatment. Fsk treatments were performed as follows: HIT-T15 cells were used at 50-75% confluence. Cells were grown for indicated times in RPMI-1640 complete containing either 5.5 mM glucose or 25 mM glucose prepared as described above. Cells were treated with 10 μM Fsk (1 μl of 10 mM Fsk for each ml of media) or Vehicle (DMSO) for 6 h. MIN6 cells were used at 50-75% confluence. For low and high glucose treatment of MIN6 cells, cells were transferred to DMEM complete with 5.5 mM glucose for 48 h before changing the media to DMEM complete containing either 5.5 mM glucose or 25 mM glucose for the indicated times.

Human pancreatic islets were grown in RPMI-1640 complete with either 5.5 mM glucose or 25 mM glucose (10 ml/100mm dish) as indicated at 6 to 8 mg of wet weight islets per 100 mm dish at 37° C for 24 h. Islets were harvested by scraping them off the dish and 5 min centrifugation at 1000 x rpm. The cell pellet was lysed in boiling 1% SDS with protease inhibitors and phosphatase inhibitors as described in Appendix I. Protein concentration was quantified by the BCA assay (Biorad). 30-50 μg of protein per sample was resolved by SDS-PAGE and transferred to nitrocellulose. *O*-GlcNAc, IRS2 and α−tubulin were detected and quantified by immunoblotting as previously described in the Western blotting section. Relative CREB glycosylation levels were measured by ketone labeling as described in Appendix IV.

Rat pancreatic islets were isolated as described in Appendix IV. Islets were grown in RPMI-1640 complete with either 5.5 mM glucose or 25 mM glucose (10 ml/100mm dish) as indicated at 6 to 8 mg of wet weight islets per 100 mm dish at 37° C. After 20 h in culture indicated dishes were treated with either 10 mM GlcN (200 μl 0.5 M GlcN in 0.1 M HEPES pH 7.5 stock was added to 10 ml of media), 50 U/ml glucagon-like peptide-1 (10 μl 50 kU stock was added to 10 ml of media), 1 kU/ml insulin-like growth factor 1 (5 μl 2 MU stock was added to 10 ml of media) or 1 kU/ml interferon-γ (5 μl 2 MU/ml stock was added to 10 ml of media) and 100 U/ml interleukin-1β (10 μl 100kU/ml stock was added to 10 ml of media) and 1 kU/ml tumor necrosis factor-α (5 μl 2 MU/ml stock was added to 10 ml of media). Glucagon-like peptide 1, insulin-like growth factor-1, interferon-γ, interleukin-1β and tumor necrosis factor-α were purchased from PeproTech Inc. (Rocky Hill, NJ). At 29 h cells were harvested and pelleted by brief centrifugation. Cells were lysed in boiling 1% SDS with protease inhibitors and

phosphatase inhibitors as described in Appendix I. Protein concentration was quantified by the BCA assay (Biorad). 30-50 μg of protein per sample was resolved by SDS-PAGE and transferred to nitrocellulose. *O*-GlcNAc, IRS2 and α−tubulin were detected and quantified by immunoblotting as previously described in the Western blotting section.

Luciferase Assay. HIT-T15 cells were grown to ~75% confluence in 60 mm dishes with 5 ml of media for each dish. In studies of endogenous CREB activity, cells were cotransfected with 2.5 ml of 1 μg/ml pCRE-Luc (2.5 μl of a 1 mg/ml stock) (Stratagene, La Jolla CA) and 0.5 μg/ml pSV-βGal (1.25 μl of a 1 mg/ml stock) plus 3 μg/ml Targefect F2 transfection reagent (7.5 μl of a 1 mg/ml stock) in OptiMEM I. Fsk treatments were begun ~24 h post-transfection. Cells were harvested with 0.25% trypsin in Hank's balanced salt solution (0.5 ml for each 60 mm dish) (Invitrogen, Carlsbad, CA) incubated at 37° C for 2 min. Cells were were tritriated of the dish with 5 ml of media, transferred to a 15 ml conical and pelleted by centrifugation 5 min at 1000 x rpm. Harvested cells were lysed in 1xReporter Lysis Buffer (Promega, Madison, WI) with brief sonication on ice. Samples were centrifuged for 5 min at 21,000 xg. Supernatant was used for the measurement of luciferase and β-galactosidase and western blot analysis. Luciferase activity was measured using luciferase assay substrate (Promega, Madison, WI) on an Opticom 1 luminometer (MGM instruments, Inc., Hamden, CT). Luciferase activity and transfection efficiency were corrected by measurement of β-galactosidase activity.

Animal Care. 100-200 g male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA)were fed *ad libitum* and maintained by the California Institute of Technology Office of Laboratory Animal Resources. All treatments described here in were approved by the IACUC. Zucker diabetic fatty rats (ZDF) and age matched controls were purchased from Charles River Laboratories, Inc. Animals were euthanized with CO_2 according to California Institute of Technology OLAR protocol.

Streptozotocin Treatment. Animals were fasted for 12 to 16 h prior to injection of streptozotocin. Animals were anesthetized with isofluorane and administered a single intraperitoneal injection of either 16 mg/ml streptozotocin in 0.1 M sodium citrate pH 4.5 at a final concentration of 80 mg STZ/kg body weight or vehicle (0.1 M sodium citrate pH 4.5). Animals were fed *ad libitum* and given 5% glucose water (500 ml) after injection. Blood glucose levels were monitored using a Roche Diagnostics ACCU-CHEK* Glucose Monitoring System (Roche, Indianapolis, IN). Animals were euthanized as described at the indicated times.

Alloxan Treatment. Animals were anesthetized with isofluorane and administered a single intraperitoneal injection of either 28 mg/ml alloxan at a final concentration of 140 mg alloxan/kg body weight or vehicle (water). Animals were fed *ad libtum* after injection. Blood glucose levels were monitored using a Roche Diagnostics ACCU-CHEK* Glucose Monitoring System (Roche, Indianapolis, IN). Animals were euthanized as described at indicated times.

Pancreatic Islet Isolation. 150-200 g male Sprague-Dawley rats (Charles River) were euthanized with CO_2 according to California Institute of Technology OLAR protocol. Islets were isolated as described in Appendix IV.

Ketone Labeling. Ketone labeling was performed as described in Appendix IV.

Immunoprecipitation of CREB. Immunoprecipitation of CREB was performed as described in Appendix 1.

Western Blot Analysis. Total cell lysates were prepared by cell lysis in boiling 1% SDS with sonication. Samples were resolved by 10% SDS-PAGE or by 4-12% Bis-Tris PAGE and transferred to nitrocellulose. The following antibodies were used for Western blot analysis: anti-IRS2 antibody (1:1000; Upstate, Charlottesville, VA), anti-CREB antibody (1:1000; Upstate, Charlottesville, VA), anti-phospho-CREB (Ser133) antibody (1:1000; Cell Signaling, Beverly, MA) CREB, anti-IRS2 antibody (Upstate, Charlottesville, VA), anti-OGT (1:1000; Sigma-Aldrich, St. Louis, MO), anti-α−tubulin (1:5000; Sigma-Aldrich, St. Louis, MO) and anti-*O*-GlcNAc (1:5000; CTD110.6) antibody (Covance, Princeton, NJ). Blots were visualized using Supersignal West Pico chemiluminescent reagents (Pierce Biotechnology, Inc., Rockford, IL) on Hyperfilm ECL chemiluminescent film (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Specific band density was quantified by analysis of scanned images using NIH Image 1.52 software.