Appendix IV
Methods for Chapter 5

Materials. All reagents were purchased from Fisher Scientific unless otherwise specified. RPMI-1640, DMEM, Hank's Buffered Saline Solution (HBSS), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). CREB and insulin receptor substrate 2 (IRS2) antibodies were purchased from Upstate (Charlottesville VA). FLAG-M2, OGT and αTubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-*O*-GlcNAc (CTD110.6) antibody was purchased from Covance (Berkeley, CA). Anti-OGT (AL28) ascites were a generous gift of G.W. Hart (The Johns Hopkins University School of Medicine). *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) was purchased from Toronto Research Chemicals (Toronto, Canada). Forskolin (Fsk) was purchased from Axxora (San Diego, CA). Glucosamine (GlcN) was purchased from Fluka.

Cell Culture. HIT-T15 cells, passage 65-79 (American Type Culture Collection), were grown in RPMI-1640 + 10% FBS + 100 U/ml penicillin, 0.1 mg/ml streptomycin (RPMI-1640 complete). Cell passages 66-79 were used for experiments, with the best results gathered with lower passage numbers. Cells were subcultured every 6-8 days or as they reached 75-90% confluence. Cells were subcultured at a 1 to 4 ratio, dividing 1 confluent plate into 4 new plates. 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).

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 following the method reported by Konrad, Liu and Kudlow (2000) with minor modifications [1]. Briefly, the bile duct was clamped at the entrance to the duodenum and the pancreas was then perfused with 5 ml 1.5 mg/ml collagenase Type V (Sigma-Aldrich, St. Louis, MO) in ice-cold HBSS supplemented with 11 mM glucose, 1 mM L-glutamine and 0.1% BSA. All solutions were sterilized by 0.2 μm filtration. Perfused pancreata were combined and then dissociated with dissecting scissors and briefly incubated with an equal volume of HBSS containing 11 mM glucose and 1 mM L-glutamine. It should be emphasized that the supplementation of HBSS with L-glutamine is critical to maintain islet-*O*-GlcNAc glycosylation dynamics. Supernatant and fat were removed and pancreata were incubated with an equal volume of the collagenase solution for 25 min at 37° C. The incubation should be monitored, as progress of the digest beyond the break-up of the extracellular matrix will lead to islet cell death. Pancreata were dispersed by pipeting and then centrifuged 4 min 200 xg. The resulting pellet was washed twice with ice-cold modified HBSS containing 11 mM glucose and 1 mM L-glutamine and filtered through a 400 μm screen. All these steps should be performed in a sterile hood. The islets were then isolated using a simple discontinuous Histopaque-1077 (Sigma-Aldrich) gradient. Isolated islets were washed twice with ice-cold modified HBSS containing 11 mM glucose and 1 mM L-glutamine and either flash frozen for subsequent analysis or grown in low glucose DMEM (5.5 mM glucose) + 10% FBS + 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA).

Ketone Labeling. After drug treatment or isolation, cells were harvested, and the cell pellet was lysed in boiling lysis buffer (1% SDS, protease inhibitors), sonicated for 5 s, and boiled for 8 min. After centrifugation at 21,500 xg for 5 min, the supernatant was collected and protein concentration was measured using the BCA assay (Pierce, Rockford, IL). One volume of denatured cell extract was diluted into four volumes of dilution buffer (10 mM HEPES pH 7.9, 1.8% Triton X-100, 100 mM NaCl, 5-10 mM $MnCl_2$, containing protease inhibitors and 1 mM phenylmethylsulfonyl fluoride). Diluted extract was then supplemented with 1.25 mM adenosine 5'-diphosphate, 0.5 mM analogue **1** (Scheme 5) and 20 μg/mL mutant Y289L GalT. In some cases control reactions were prepared lacking enzyme or analogue **1** (Scheme 5) to control for nonspecific reactivity of streptavidin-HRP (data not shown). The reaction mixture was incubated at 4° C for 10-12 h, and dialyzed into 10 mM HEPES pH 7.9 containing 5 M urea (3 x 3 h) at 4° C. Alternatively, the reaction mixture was dialyzed via diafiltration in spin columns into the buffer indicated above. Following dialysis the sample was acidified to pH 4.8 by adding NaOAc pH 3.9 to a final concentration of 50 mM, and the amino-oxy biotin derivative was then added to a final concentration of 2.5 mM. After incubation at room temperature for 16-20 h, the sample was dialyzed 1x overnight and 2 x 2 h into CREB immunoprecipitation (IP) buffer (10 mM HEPES pH 7.9, 100 mM KCl, .2% Triton X-100 and 1mM EDTA). Alternatively, the biotinylated sample was dialyzed via spin column diafiltration into CREB IP buffer. After dialysis, the sample was centrifuged for 5 min at 21,500 xg and the protein concentration was measured. Lysate was supplemented with protease inhibitors and volumes were normalized such that equivalent amounts and concentrations of lysate were taken on for immunoprecipitation.

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

Plasmids. *E. coli* expression vector pET23b-CREB was subcloned by amplifying full-length rat $CREB_{341}$ cDNA from pRcRSV-CREB, a gift of R. Goodman (Oregon Health and Sciences University), using primers with 5' Nde1 (5'-GGAATTCCATATGACCATGGACTCTGGAGCAGACA-3') and 3' HindIII (5'-TGGTGATGCTCGAGGTGCGGCCGCAAGCTTCCCAAGCTTATCTGACTTGTGGCAGTAAAGGT-3') restriction sites and inserted into pET23b+ (Novagen) to afford pET23b-CREB. The triple alanine mutant CREB T259A/S260A/T261A construct, pET23b-CREB(AAA), was generated by standard site-directed mutagenesis using pET23b-CREB as a template using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

A mammalian Gal4 expression vector was cloned by PCR amplifying full-length Gal4, a kind gift of Dr. S. Benzer (California Institute of Technology), using 5' BamHI (5'-CGCGGATCCGGCCGATGAAGCTACTGTCTATCGAA-3') and 3' NotI (5'-CCCACCAAACCCAAAAAAGAGTAACGCGGCCGCTTTTTCCTT-3') primers and inserting Gal4 into pcDNA3.1 (Invitrogen, Carlsbad, CA).

CREB-Gal4 DNA-binding domain chimeras were generated by subcloning specified CREB domains into the BamHI and EcoRI sites of pFA-CMV (Stratagene). pFA-CMV-Q2 and pFA-CMV-Q2(AAA) were generated by PCR, amplifying the coding sequence for the Q2 transactivation domain of CREB (aa166-283) from pET23b+-CREB

and pET23b+-CREB(AAA), respectively, using 5' BamHI (5'-CGCGGATCCACCACTGTAACAGTGCCAACCC-3') and 3' EcoRI (5'-CCGGAATTCCTATGCTGCTTCTTCAGCAGGCTG-3') primers. pFA-CMV-QKQ and pFA-CMV-QKQ(AAA) plasmids were generated by PCR, amplifying the coding sequence for the full transactivation domain of CREB (aa1-283) from pET23b+-CREB and pET23b+-CREB(AAA), respectively, using 5' BamHI (5'-CGCGGATCCACCATGGACTCTGGAGCAGACA-3') and 3' EcoRI (5'-CCGGAATTCCTATGCTGCTTCTTCAGCAGGCTG-3') primers.

pFC6a-CREB and pFC6a-CREB(AAA) were generated by PCR amplifying wild-type CREB and triple alanine mutant CREB from their respective pET23b+ vectors. CREB PCR product was inserted into the pFLAG-CMV-6a *E. coli* and mammalian expression vector using EcoRI and BamHI restriction sites (Sigma-Aldrich). 5' EcoRI (5'GGCGCGAATTCCATGACCATGGA CTCTGGAGCAGACA-3') and 3' BamHI (5'- CGGGATCCTTAATCTGACTTGTGGCAGTAAAGGT-3') primers were used to amplify CREB.

***E. coli* Expression of CREB.** CREB was expressed in electrocompetent BL21(DE3) cells as described in Appendix I.

Expression and Purification of CREB and OGT from *Spodoptera frugiperda* (Sf9) and Hi-5 Insect Cells and *E. coli*. Rat CREB and human OGT were expressed and purified as described in Appendices I and II.

***In Vitro* OGT Labeling.** OGT labeling reactions were performed as described in Appendix I.

Drug Treatments. GlcN and Fsk treatments were performed as follows: HIT-T15 and MIN6 cells were used at 50-75% confluence. All HIT-T15 drug treatments were performed in RPMI-1640 complete media. All MIN6 drug treatments were performed in DMEM complete media. Cells were pretreated with 10 mM GlcN in 2 mM HEPES pH 7.5 for 3-12 h as indicated, before treatment with 10 μM Fsk or Vehicle (DMSO). GlcN was added to cells drop-wise from a freshly prepared 50x solution (0.5 M GlcN, 0.1 M HEPES pH 7.5). Media was replaced with media plus 10 μM Fsk or Vehicle (DMSO) with or without 10 mM GlcN + 2 mM HEPES pH 7.5 for indicated times. For Fsk plus GlcN media 1 μl/ml of a 1000x Fsk (10 mM in DMSO) stock and 20 μl/ml of a 50x GlcN stock solution (0.5 M GlcN, 0.1 M HEPES pH 7.5) was added to the media immediately prior to application. For Fsk media 1 μl/ml of a 1000x Fsk (10 mM in DMSO) stock was added to the media immediately prior to application. For GlcN media 1 μl/ml DMSO and 20 μl/ml of a 50x GlcN stock solution (0.5 M GlcN, 0.1 M HEPES pH 7.5) was added to the media immediately prior to application. For control media 1 μl/ml DMSO was added to the media immediately prior to application.

PUGNAC treatments were performed as follows. Cells were pretreated for 3-12 h as indicated with 100 μM PUGNAc before the addition of 10 μM Fsk for indicated times. For PUGNAc-treated cells 10 μl/ml of a 100x PUGNAc stock solution (10 mM, stored at -20° C) was added drop-wise to the cells. Media was replaced with media plus 10 μM Fsk or Vehicle (DMSO) with or without 100 μM PUGNAc for indicated times. For Fsk plus

PUGNAc media 1 μl/ml of a 1000x Fsk (10mM in DMSO) stock and 10 μl/ml of a 100x PUGNAc stock solution (10 mM, stored at -20° C) was added to the media immediately prior to application. For Fsk media 1 μl/ml of a 1000x Fsk (10 mM in DMSO) stock was added to the media immediately prior to application. For PUGNAc media 1 μl/ml DMSO and 10 μl/ml of a 100x PUGNAc stock solution (10 mM, stored at -20° C) was added to the media immediately prior to application. For control media 1 μl/ml DMSO was added to the media immediately prior to application.

For Western blot analysis cells were harvested at indicated times by aspirating off the media, scraping the cells off the dish with ~0.5 ml of media and pelleting the cells with a 1 min centrifugation at max speed on a table top centrifuge. Cell pellets were immediately lysed in boiling 1% SDS plus protease inhibitors (5 μg/ml pepstatin, 5 μg/ml chymostatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml antipain, 0.2 mM PMSF) with 3 s sonication and 5 min boiling.

For luciferase, β-galactosidase and caspase-3/7 measurements 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. Cell pellets were lysed in 1 x Reporter Lysis Buffer (Promega, Madison, WI) with brief sonication, centrifuged for 5 min at 21,000 xg at 4° C and kept on ice prior to analysis.

Luciferase Assay. HIT-T15 cells were grown to ~75% confluence in 60 mm dishes. In CREB-Gal4 chimera experiments one 60 mm dish was cotransfected with 2.5 ml of 1 μg/ml pFR-Luc (2.5 μl of a 1 mg/ml stock, Stratagene), 0.5 μg/ml pSV-βGal (1.25 μl of a

1 mg/ml stock, Promega, Madison WI) and 2 μg/ml pFA-CMV-Q2 (5 μl of a 1 mg/ml stock), 2 μg/ml pFA-CMV-Q2(AAA) (5 μl of a 1 mg/ml stock), 2 μg/ml pFA-CMV-QKQ (5 μl of a 1 mg/ml stock) or 2 μg/ml pFA-CMV-QKQ(AAA) (5 μl of a 1 mg/ml stock) plus 3 μg/ml Targefect F2 transfection reagent (7.5 μl of a 1 mg/ml stock) (Targeting Systems, Santee CA) in OptiMEM I (Invitrogen, Carlsbad, CA) following the Targefect F2 transfection reagent instructions. Cells were harvested ~28 h post-transfection.

In studies of endogenous CREB activity assays, one 60 mm dish was 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. In control Gal4 reactions, one 60 mm dish was co-transfected with 1 μg/ml pFR-Luc (2.5 μl of a 1 mg/ml stock) (Stratagene, La Jolla CA), 0.5 μg/ml pcDNA3.1-Gal4 (1.25 μl of a 1 mg/ml stock) and 0.5 μg/ml pSV-βGal (1.25 μl of a 1 mg/ml stock). Fsk/GlcN and Fsk/PUGNAc treatments were begun ~24 h post-transfection.

For studies of the effect of the triple alanine mutation (T259A/S260A/T261A) on CREB activity, HIT-T15 cells were transfected with 0.2 μg/ml pFC6a-CREB/pFC6a-CREB(AAA) (0.5 μl of a 1 mg/ml stock), 1 μg/ml pCRE-Luc (2.5 μl of a 1 mg/ml stock) 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. Drug 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.

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-CREB antibody (1:1000, Upstate, Charlottesville VA), anti-CREB(mouse) antibody (1:1000, Chemicon, Temecula, CA), anti-phospho-CREB(Ser133) antibody (1:1000, Cell Signaling, Beverly, MA), anti-α–tubulin (1:5000, Sigma-Aldrich, St. Louis, MO) and anti-O-GlcNAc (CTD110.6) antibody (1:5000, Covance, Princeton NJ). For detection of Gal4-QKQ chimera by immunoblotting, the anti-CREB(KID) antibody (1:1000, Cell Signaling, Beverly, MA) was used. 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.

Statistical Analysis. All experiments were repeated a minimum of three times. Results are presented as the mean value +/- one standard error of the mean (SEM). Statistical significance was calculated using the Student's T-test and the Tukey-Kramer HSD test where appropriate.

Bibliography

1. Konrad, R.J., K. Liu, and J. Kudlow, *A modified method of islet isolation preserves the ability of pancreatic islets to increase protein o-glycosylation in response to glucose streptozotocin*. Archives of Biochemistry and Biophysics, 2000. **381**(1): p. 92-98.