Appendix V
Methods for Chapter 6

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. 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).

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 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 and 3' HindIII 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. For primer sequence, see Appendix IV.

Drug Treatments. GlcN and Fsk treatments were performed as follows. HIT-T15 and MIN6 cells were used at 50-75% confluence. Cells were pretreated with 10 mM GlcN + 2 mM HEPES pH 7.5 for 3-12 h as indicated before treatment with 10 μM Fsk or Vehicle (DMSO) with or without 10 mM GlcN + 2 mM HEPES pH 7.5 for indicated times. 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.

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-IRS2 antibody (1:1000, Upstate, Charlottesville VA), anti-phospho-CREB (Ser133) antibody (1:1000, Cell Signaling, Beverly, MA) CREB, 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.

RNA Interference. A cocktail of three dsRNA oligonucleotides was used for OGT siRNA experiments. OGT dsRNA were based on the published sequences of Zhang et al. 2003 and Zachara et al. 2004 using mouse OGT (gi 46909606) as a template [1, 2]. OGT dsRNA sequences are as follows: OGT-H1 5'-AAGCAATCGAGCATTATCGAC-3', OGT-H2 5'-AAGTTTGAGCCCAAATCATGC-3' and OGT-K1 5'-TGGCATCGATCTCAAAGCATTTC-3'. Scrambled dsRNA 5'-ACCAGTCGCTTTGCGACTGGTT-3' was designed and purchased from Dharmacon (Lafayette, CO). OGT siRNA experiments were performed in MIN6 cells (p25-p35). MIN6 cells were grown to ~80% confluence and trypsinized. Cells were centrifuged and resuspended in Buffer T + 18% Supplement 1 (Amaxa Biosystems, Gaithersburg MD) at

a density of ~$1.5x10^7$ cells/ml. 200 μl of resuspended cells were mixed with 7 μl 60 μM dsRNA (final concentration 2 μM dsRNA). Cells were electroporated in 100 μl aliquots using program G17 on a Nucleofector II electroporator from Amaxa Biosystems. Immediately after electroporation cells were resuspended in DMEM complete and aliquoted into two 60 mm dishes. Cells were grown at 37° C for 72 h post-electroporation. Media was changed at 72 h to DMEM complete or DMEM complete supplemented with 10 mM GlcN, 2 mM HEPES pH 7.5 (20 μl of a 50x 0.5 M GlcN, 0.1 M HEPES pH 7.5 stock solution was added to 1 ml of media to afford the supplemented media) and the cells were incubated for an additional 18 h. 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 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. Cells were lysed in 1x RLB (Promega, Madison, WI) and sample protein concentration, caspase-3/7 activity and OGT expression levels were measured.

Caspase-3/7 Assay. MIN6 cells were treated with 10 mM GlcN, 2 mM HEPES pH 7.5 (20 μl of a 50x 0.5 M GlcN, 0.1 M HEPES pH 7.5 stock solution was added to 1 ml of media to afford the supplemented media) or 0.2 μg/ml camptothecin (40 μl of a 25x 5 mg/ml stock solution was added to 1 ml of media to afford the supplemented media) in DMEM complete (Sigma-Aldrich, St. Louis, MO) for 18 h. 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. Cells were lysed in 1xReporter Lysis Buffer (Promega, Madison, WI) with brief sonication on ice. Lysates were centrifuged for 3 min at 21,000 xg. Supernatant was used to measure protein concentration using the BCA assay (Pierce Biotechnology, Inc., Rockford, IL) and caspase-3/7 activity. Caspase-3/7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison WI) by following the manufacturer's protocol on a Victor3 Multilabel Plate Counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Caspase-3/7 activity measurements were normalized to the measured protein concentration for each sample.

Chromatin Immunoprecipitation (ChIP). ChIP assays were performed as described previously with the following modifications [3]. ~$7.5x10^7$ cells were fixed with media containing 1% formaldehyde for 20 min at room temperature with rocking. Formaldehyde cross-linking was arrested with the addition of 125 mM glycine (0.5 ml of 2.5 M glycine was added to 10 ml of media) and incubation at room temperature for 5 min. The cells were rinsed twice with PBS, scraped off dishes and centrifuged 5 min at ~200 xg. The cell pellet was washed once with PBS containing protease inhibitors (Roche, Indianapolis, IN). The cell pellet was resuspended in cell lysis buffer (25 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.1% NP-40) plus protease inhibitors and 0.5 mM PMSF. The cell pellet was resuspended at a concentration of ~40 mg of cell pellet wet weight in 1 ml of cell lysis buffer. The resuspended cell pellet was incubated on ice for 10 min and centrifuged for 5 min at ~21000 xg. The pellet resulting from the centrifugation is defined as the crude nuclear pellet. The crude nuclear pellet was weighed and resuspended in nuclear lysis buffer (50 mM HEPES pH 7.9, 140 mM

NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na Deoxycholate, 0.1% SDS) plus protease inhibitors and 0.5 mM PMSF at a concentration of 50 mg of crude nuclear pellet wet weight in 1 ml of nuclear lysis buffer. The resuspended crude nuclear pellet was sonicated 4 x 30 sec and 1 x 60 sec at 60 sec intervals at 55 mA and 7 W output using a Vibra cell ultrasonic processor (Sonics & Materials, Inc., Newton CT), and then centrifuged 5 min at ~21,000 xg. Supernatant was precleared twice against 5 μg/ml mouse IgG (5 μl of 1 mg/ml stock added to each ml of supernatant) (Sigma-Aldrich, St. Louis MO), 50 μg/ml sheared salmon sperm DNA (50 μl of 1 mg/ml stock added to each ml of supernatant) (Invitrogen, Carlsbad, CA) and 50 μl/ml protein A-Sepharose (Pierce Biotechnology Inc., Rockford, IL) for 2 h at 5-7^{o} C. 1 to 1.5 ml of precleared lysates were combined with 6 μg anti-CREB antibody, 6 μg AL28 anti-OGT ascites or 6 μg mouse IgG and incubated at 5-7^{o} C overnight. Samples were transferred to tubes containing 70 μl protein A-sepharose and incubated for 2 h at 5-7^{o}C. Samples were loaded onto USB reaction columns (USB Corp., Cleveland, OH) fitted with 35 μM filters. Protein A-sepharose beads were washed with gravity flow with 3 ml of nuclear lysis buffer, 2 ml of nuclear lysis buffer supplemented with 500 mM NaCl, 2 ml of Wash Buffer (20 mM Tris-Hcl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na Deoxycholate) and 2 ml of TE (20 mM Tris-Hcl pH 8.0, 1 mM EDTA). The protein A-sepharose beads were transferred to a DNase/RNase-free 1.5 ml centrifuge tube. Captured protein/DNA complexes were eluted by adding 100 μl TE + 1% SDS to the protein A-sepharose beads and incubating at 65^{o} C for 10 min. Samples were briefly centrifuged, the supernatant was collected, and an additional 150 μl of fresh TE + 1% SDS was added to the protein A-sepharose beads. Samples were incubated for 10 min at

65° C, briefly centrifuged and supernatant was combined with the initial 100 µl elution. The combined elution sample was incubated for 6 h at 65° C. Samples were diluted to 500 µl with TE and incubated with 0.29 mg/ml Proteinase K (15 µl of 10 mg/ml Proteinase K stock solution is added to the 0.5 ml sample) (Invitrogen, Carlsbad, CA) for 1 h 30 min at 37° C. 56 µl TE plus 4 M LiCl (TE supplemented with 4 M LiCl was prepared as a 1x solution and stored at room temperature) was added to each sample and DNA was extracted with Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (Invitrogen, Carlsbad, CA) followed by extraction with Chloroform. Extracted DNA samples were ethanol precipitated overnight and resuspended in RNase/DNase-free water. Primers were designed based on mouse genes using Primer3 software [4]. Mouse *IRS2* (gi 63572321) primers

5'-CCCGCCAGCACTCGCTC-3' and 5'-CGGACGTCATCAGAGCC-3' amplify a 174 bp product corresponding to bp -343 to -169.

Mouse *IRS1* (gi 94471495) primers

5'- TGCGATTGAGCTGGTATTTG-3' and 5'-CCCCTCTGGAAGTAGCGATT-3' amplify a 119 bp product corresponding to bp -587 to -465.

Mouse *Nr4A2* (gi 94471506) primers

5'-CAGCTCGAGCCACATAAACA-3' and 5'-TCCTTGTCCGCTCTCTTCAT-3' amplify a 240 bp product corresponding to bp -2515 to -2275.

Mouse *DUPS1* (gi 94471503) primers

5'- GTCTTTGCTTTTGGCTTTGG-3' and 5'- GTGCTCGCTGCTCCTAATCT-3' amplify a 242 bp product corresponding to bp -366 to -94.

Mouse 18S rRNA (gi 53990) primers

5’-CGCGGTTCTATTTTGTTGGT-3’ and 5’-AGTCGGCATCGTTATGGTC-3’ amplify a 219 bp product.

Samples were analyzed with 25-36 cycles of semi-quantitative PCR using Taq PCR_X DNA polymerase (Invitrogen, Carlsbad, CA). One 25 μl PCR reaction was composed of 3-5 μl of DNA, 1 μl 10 mM dNTP, 0.8 μl $MgCl_2$, 1 μl 100 ng/μl Coding primer, 1 μl 100ng/μl Non-coding primer, 7.5 μl PCR_x Enhancer, 2.5 μl 10x PCR_x Amplify solution, DNase/RNase-free water to bring the total volume to 24.5 μl and 0.5 μl 5 U/μl Taq PCR_x DNA polymerase. PCR reactions were run with the following cycles: 1x 95^o C 60 sec; 24-36x 95^o C 30 sec, 46^o C 60 sec, 72^o C 90 sec; 1x 72^o C 240 sec. PCR products were loaded onto 2% agarose/ethidium bromide gels and visualized on an AlphaImager 3400 (Alpha Innotech Corp., San Leandro, CA). Band density was quantified using software AlphaEaseFC software version 4.0.1 (Alpha Innotech Corp., San Leandro, CA).

RNA Isolation and RT-PCR. HIT-T15 cells were grown in 60 mm dishes to a confluence of 50-75% (~5-7x10^5 cells). Cells were pretreated with either RPMI-1640 complete supplemented with 10 mM GlcN in 2 mM HEPES pH 7.5 or RPMI-1640 complete for 3 h before treatment with RPMI-1640 complete supplemented with10 μM Fsk and 10 mM GlcN in 2 mM HEPES pH 7.5 or in RPMI-1640 complete supplemented with 10 μM Fsk for 1 h, respectively.

Cells were harvested by removing the media and scraping the cells off the dishes using a sterilized straight-edge razor. Cells were transferred to DNase/RNase-free tubes and pelleted with 30 sec centrifugation at max speed on a table-top centrifuge. RNA was

isolated using Qiagen RNeasy mini-columns following the manufacturer's procedure for the isolation of cytoplasmic RNA from animal cells (Qiagen Inc., Valencia, CA). All procedures were performed at a bench and with pipettes that had been wiped down with RNase Away solution (Fisher). Briefly, pelleted cells were resuspended in 175 µl of RLN buffer (50 mM Tris-HCl pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40) supplemented with 1 mM DTT and 0.2 U/ml Rnasin RNase inhibitor and incubated on ice for 10 min. Samples were centrifuged at 13000 x rpm for 3 min. The supernatant from the centrifugation was combined with 600 µl of RLT buffer (Qiagen) and 430 µl of ethanol. The samples were mixed with pipeting and transferred to an RNeasy spin columns (Qiagen) and centrifuged briefly. The column was washed 1x 400 µl RW1 buffer (Qiagen). 80 µl of 0.5 U/µl DNase I in 50 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 1 mM DTT was added to each column and incubated at room temperature for 1 to 2 hours. The columns were briefly centrifuged and washed 1x 500 µl of RW1 buffer and 2x 500 µl of RPE buffer (Qiagen). RNA was eluted off the columns with 80 µl of DNase/RNase-free water for each column. RNA was stored on ice and used immediately in reverse transcriptase reactions to produce cDNA. cDNA was prepared using oligo $dT_{12\text{-}18}$ primers (Invitrogen, Carlsbad, CA) and Transcriptor reverse transcriptase (Roche, Indianapolis, IN). 25 µl of RNA was combined with 3 µl of 10 mM dNTPs, 3 µl of 100 ng/µl oligo $dT_{12\text{-}18}$ primers and 8 µl 5xTranscriptor RT buffer (Roche) and incubated for 5 min at 65^{o} C. 1.5 µl of 20 U/µl Transcriptor reverse transcriptase was added to each reaction and incubated for 30 min at 42^{o} C. cDNA from the reverse transcription reactions was immediately used to set up PCR reactions. Primers for cDNA amplification were based on mouse mRNA using Primer3 software [4]. *IRS2* primers 5'-

GAGCATGGATAGACCCTGA-3' and 5'-GCAGAGGCGACCTGAACTAC-3' amplify a 211 bp product corresponding to bp +1617 to +1817. *IRS1* primers 5'-TGAGGATGTAAAACGCCACA-3' and 5'-CTTGGCCAAATCCAGGTCTA-3' amplify a 188 bp product corresponding to bp +3380 to +3567. *Nr4A2* primers 5'-AGTCTGATCAGTGCCCTCGT-3' and 5'-CTGGGTTGGACCTGTATGCT-3' amplify a 287 bp product corresponding to bp +2254 to +2540. *DUSP1* primers 5'-GAGCTGTGCAGCAAACAGTC-3' and 5'-CTTCCGAGAAGCGTGATAGG-3' amplify a 177 bp product corresponding to bp +400 to +576. Mouse *β-actin* primers 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3' amplify a 165 bp product corresponding to bp +225 to +390. Samples were analyzed with 25-38 cycles of semi-quantitative PCR using Taq PCR_X DNA polymerase (Invitrogen, Carlsbad, CA). One 25 μl PCR reaction was composed of 2.5 μl of cDNA, 1 μl 10 mM dNTP, 0.8 μl $MgCl_2$, 1 μl 100 ng/μl Coding primer, 1 μl 100 ng/μl Non-coding primer, 7.5 μl PCR_x Enhancer, 2.5 μl 10x PCR_x Amplify solution, 8.7 μl DNase/RNase-free water to bring the total volume to 24.5 μl and 0.5 μl 5 U/μl Taq PCR_x DNA polymerase. PCR reactions were run with the following cycles: 1x 95^o C 60 sec; 25-38x 95^o C 30 sec, 46^o C 60 sec, 72^o C 90 sec; 1x 72^o C 240 sec. PCR products were loaded onto 2% agarose/ethidium bromide gels and visualized on an AlphaImager 3400 (Alpha Innotech Corp., San Leandro, CA). Band density was quantified using software AlphaEaseFC software version 4.0.1 (Alpha Innotech Corp., San Leandro, CA).

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. Zhang, F., et al., *O-GlcNAc Modification is an Endogenous Inhibitor of the proteasome*. Cell, 2003. **115**: p. 715-725.

2. Zachara, N.E., et al., *Dynamic O-GlcNAc Modification of Nucleocytoplasmic Proteins in Response to Stress*. The Journal of Biological Chemistry, 2004. **279**(29): p. 30133-30142.

3. Asahara, H., et al., *Chromatin-Dependent Cooperativity between Constitutive and Inducible Activation Domains in CREB*. Molecular and Cellular Biology, 2001. **21**: p. 7892-7900.

4. Rozen, S. and H.J. Skaletsky, *Primer3 on the WWW for general users and biologist programmers*. Bioinformatics Methods and Protocols: Methods in Molecular Biology, ed. S. Krawetz and S. Misener. 2000, Totowa, NJ: Humana Press. 365-386.