**Supplemental Information for:**

# A novel chemical glycoproteomics platform reveals O-GlcNAcylation of mitochondrial voltage-dependent anion channel 2

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## Extended Experimental Procedures

***Compound synthesis and small molecule reagents***

Glyco-DIGE probes **1** and **2** were synthesized from *N*-hydroxysuccinamide (NHS) esters of Cy3 and Cy5, respectively (GE Healthcare), as follows:

Cy3-alkyne (compound **1**): Cy3 mono-NHS ester (5 mg, 6.5 μmol) was dissolved in dimethyl sulfoxide (DMSO) (80 μL). Propargyl amine (4.2 μL, 65 μmol) was added to the solution followed by addition of triethylamine (54.8 μL, 390 μmol). The reaction mixture was stirred for 21 hours in the dark. The sample was then diluted three-fold with water and purified by reversed-phase HPLC ($C_{18}$ analytical column) using a gradient of 0%-50% MeCN/0.1% TFA over 45 minutes, and the peak at retention time 30.7 minutes was collected (Figure S8A). The purified product was concentrated first *in vacuo* and then lyophilized to dryness to yield 3.96 mg of a pink solid (91% yield). HRMS (ESI): Calculated for $C_{34}H_{40}N_3O_7S_2Na_2$ $[M-H+2Na]^+$ 712.2103, found 712.2114.

Cy5-alkyne (compound **2**): Cy5 mono-NHS ester (5 mg, 6.3 μmol) was dissolved in DMSO (77 μL). Propargyl amine (4.1 uL, 63 μmol) was added to the solution followed by addition of triethylamine (53.0 μL, 378 μmol). The reaction mixture was stirred for 21 hours in the dark. The sample was then diluted three-fold with water and purified by reversed-phase HPLC ($C_{18}$ analytical column) using a gradient of 0%-50% MeCN/0.1% TFA over 60 minutes and the peak at retention time 42.3 minutes was collected (Figure S8B). The purified product was concentrated *in vacuo* and then lyophilized to dryness to yield 3.31 mg of a blue solid (72% yield). HRMS (ESI): Calculated for $C_{36}H_{42}N_3O_7S_2Na_2$ $[M-H+2Na]^+$ 738.2260, found 738.2288.

***Immunoblotting***

For 1D immunoblots, samples were separated by standard SDS-PAGE (Bio-Rad, Criterion or Mini-Protean system). For 2D immunoblots, samples were processed as in the glyco-DIGE workflow described in the Experimental Procedures to produce a 2D gel. In all cases, gels were then electroblotted onto nitrocellulose, blocked in 5% bovine serum albumin in Tris-buffered saline with Tween (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20) and analyzed by standard enhanced chemiluminescence immunoblotting methods (Thermo-Fisher). Antibodies used were: anti-O-GlcNAc (RL2, Affinity Bioreagents/Thermo-Fisher, 1:500); anti-alpha tubulin (Sigma, 1:10,000); anti-VDAC2 (Abcam, 1:500, or custom); anti-manganese superoxide dismutase (Stressgen, 1:3000); anti-caspase-3 (Cell Signaling, 1:1000); anti-HA (Sigma, 1:1000); anti-OGT (Sigma, 1:500), anti-OGA (Sigma, 1:500), anti-Ezh2 (Cell Signaling, 1:1000); anti-biotin horseradish peroxidase (HRP)-conjugate (Sigma, 1:100,000); anti-rabbit-HRP-conjugate (Southern Biotech, 1:5000 or Cell Signaling, 1:2500); anti-mouse κ light chain-HRP-conjugate (Southern Biotech, 1:5000).

***Mass spectrometric analysis***

Samples were subjected to reversed-phase chromatography with an Agilent 1200 liquid chromatography (LC) system connected in-line to either an LTQ XL mass spectrometer or an LTQ Orbitrap XL hybrid mass spectrometer. Both mass spectrometers were outfitted with a nanospray ionization source and external mass

calibration was performed prior to analysis. A binary solvent system consisting of buffer A (0.1% formic acid in water (v/v)) and buffer B (0.1% formic acid in acetonitrile (v/v)) was employed.

Proteins in glyco-DIGE gel picks were extracted and trypsin-digested (50:1 protein:enzyme, sequencing grade modified trypsin, Promega) essentially as described (Shui et al., 2008). Then, samples were resuspended in water and 30 µL aliquots were subjected to LC-MS/MS using the LTQ XL mass spectrometer. The LC was performed using a 100 µm fritted capillary pre-column (New Objective) packed in-house with 1 cm of 5 µm, 200 Å Magic C18AQ resin (Michrom Bioresources) followed by a 100 µm fused silica capillary (Polymicro Technologies) packed in-house with 10 cm of 5 µm, 100 Å Magic C18AQ resin (Michrom Bioresources). After sample injection and a 10 minute loading step in 2% buffer B, peptides were eluted using a gradient from 10% to 50% buffer B over 35 minutes, followed by a wash step in 99% buffer B for 20 minutes. A solvent split maintained a flow rate of 400 nL/minute at the column tip. Samples were subjected to a data-dependent acquisition method where each full-scan mass spectrum was recorded in positive ion mode over the *m/z* scan range of 400 to 2000. The six most intense peaks were selected for collision induced dissociation (CID) fragmentation in the linear ion trap with dynamic exclusion enabled. An isolation window of 2 Da, a minimum threshold of 500 ion counts, and normalized collision energy of 35% were used when triggering a fragmentation event.

For affinity-enriched mitochondrial proteins, samples were trypsin-digested (50:1 protein:enzyme, sequencing grade modified trypsin, Promega) at 37 °C for 16

hours and desalted using C18 Zip Tips (Millipore) or C18 spin columns (Nest Group) according to the manufacturer's instructions. Then, samples were resuspended in 50 µL water and 5 µL aliquots were subjected to LC-MS/MS using the LTQ Orbitrap XL hybrid mass spectrometer. The LC was performed using a 100 µm fritted capillary pre-column (New Objective) packed in-house with 1 cm of 5 µm, 200 Å Magic C18AQ resin (Michrom Bioresources) followed by a 100 µm fused silica capillary (Polymicro Technologies) packed in-house with 15 cm of 5 µm, 100 Å Magic C18AQ resin (Michrom Bioresources). After sample injection and a 20 minutes loading step in 2% buffer B, peptides were eluted using a gradient from 7% to 35% buffer B over 150 minutes, followed by a washing step in 99% buffer B for 20 minutes. A solvent split maintained a flow rate of 400 nL/minutes at the column tip.

Samples were subjected to one of two data-dependent acquisition methods. In the first, each full-scan mass spectrum was recorded in positive ion mode over the *m/z* scan range of 400 to 1700 using the Orbitrap mass analyzer in profile mode at a resolution of 60,000 (at 400 *m/z*). The ten most intense peaks were selected for CID fragmentation in the LTQ using a normalized collision energy of 35%. In the second, a data-dependent decision tree (Frese et al., 2011; Swaney et al., 2008) was implemented, where each full-scan mass spectrum was recorded in positive ion mode over the *m/z* scan range of 380 to 1700 using the Orbitrap mass analyzer in profile mode at a resolution of 60,000 (at 400 *m/z*). The ten most intense peaks were selected for fragmentation in the linear ion trap by either CID (set as the default fragmentation method with a normalized collision energy of 35%) or electron transfer dissociation

(ETD) (if z = +3 and $m/z \leq 650$, z = +4 and $m/z < 900$, or z = +5 and $m/z < 950$ using an activation time of 100 msec) by enabling the procedures option. For both methods, dynamic exclusion and charge state screening were enabled, rejecting ions with an unknown or +1 charge state. An isolation window of 3 Da and a threshold of 500 ion counts were used when triggering a fragmentation event.

***Database searches***

Protein identities were obtained using the SEQUEST search algorithm within Proteome Discoverer 1.3 (Thermo-Fisher). Fragmentation spectra were searched against the human database (UniProtKB/Swiss-Prot, downloaded March, 2010) appended with sequences from the common repository of adventitious proteins (Global Proteome Machine Organization, downloaded March, 2010) and the chicken avidin protein sequences (*G. gallus*, 7 isoforms, downloaded March, 2010 from the UniProtKB/Swiss-Prot database).

For the LC-MS/MS data collected for glycoDIGE gel picks, an indexed database for tryptic digests was created allowing for fully enzymatic termini, a molecular weight (MW) range of 400 to 6500 Da, two missed cleavages, one fixed modification (cysteine carboxyamidomethylation, +57.021) and up to three of the following variable modifications: methionine oxidation (+15.995), N-terminal acylation (+42.001), serine/threonine GlcNAcylation (+203.079 Da), serine/threonine GlcNAzylation (+244.081 Da), or serine/threonine GlcNAz + Cy5 dye CuAAC adduct (+937.335 Da). Precursor ion tolerance was set to 1.5 Da and fragment ion tolerances were set to 0.8

Da. The criteria used for filtering included a 1% maximum false discovery rate (FDR) obtained from decoy database searches through the percolator module (as implemented in Proteome Discoverer 1.3 with validation based on the q-value and a maximum ΔCN of 0.05), at least 2 peptides per protein, and a posterior error probability (pep) score ≤ 0.01.

For the LC-MS/MS data collected for affinity-enriched proteins from mitochondria, the non-fragment filter module (as implemented in Proteome Discoverer 1.3) was first used to simplify ETD spectra: the precursor peak was removed within a 4 Da window, charge reduced species were removed within a 2 Da window, and neutral losses from the charge reduced species were removed within a 1 Da window (maximum neutral loss was 120 Da). An indexed database for tryptic digests was created allowing for fully enzymatic termini, a MW range of 400 to 6500 Da, two missed cleavages, one fixed modification (cysteine carboxyamidomethylation, +57.021) and up to three of the following variable modifications: methionine oxidation (+15.995), N-terminal acylation (+42.001), serine/threonine GlcNAcylation (+203.079 Da), serine/ threonine GlcNAzylation (+244.081 Da), or serine/threonine GlcNAz + phosphine tag adduct (+994.391 Da). Precursor ion tolerance was set to 10 ppm and fragment ion tolerances were set to 0.8 Da. If the activation type was ETD, fragment ions were weighted (if $z \leq 2$ then the ratio of z:y:c ions was 1:0.75:0.25; if $z \geq 3$ then the ratio of z:y:c ions was 1:0.25:1). The criteria used for filtering included a 1% maximum FDR obtained from decoy database searches through the percolator module (as implemented in Proteome

Discoverer 1.3 with validation based on the q-value and a maximum ΔCN of 0.05), at least 4 peptides per protein, and a pep score ≤ 0.008.

Glycan-based modifications were not observed in any samples, likely due to fragmentation-induced destruction of the glycosidic bond during CID (Khidekel et al., 2007).

***Retroviral transduction***

HA-tagged murine VDAC2 (Cheng et al., 2003) was subcloned into the pBabe-puro retroviral backbone using standard methods (Sambrook et al., 1989). HA-VDAC2 and vector-only retroviruses were produced via the transient triple transfection method, harvested and used as described (Boyce et al., 2011) to infect VDAC2$^{-/-}$ MEFs. Stably transduced cells were selected and maintained in 3 μg/ml puromycin. Puromycin was withdrawn for phenotypic assays so as not to confound assay interpretation.

***References***

Boyce, M., Carrico, I.S., Ganguli, A.S., Yu, S.H., Hangauer, M.J., Hubbard, S.C., Kohler, J.J., and Bertozzi, C.R. (2011). Metabolic cross-talk allows labeling of O-linked beta-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. Proceedings of the National Academy of Sciences of the United States of America *108*, 3141-3146.

Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J., and Korsmeyer, S.J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science *301*, 513-517.

Frese, C.K., Altelaar, A.F., Hennrich, M.L., Nolting, D., Zeller, M., Griep-Raming, J., Heck, A.J., and Mohammed, S. (2011). Improved peptide identification by targeted fragmentation using CID, HCD and ETD on an LTQ-Orbitrap Velos. Journal of Proteome Research *10*, 2377-2388.

Frezza, C., Cipolat, S., and Scorrano, L. (2007). Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nature Protocols *2*, 287-295.

Khidekel, N., Ficarro, S.B., Clark, P.M., Bryan, M.C., Swaney, D.L., Rexach, J.E., Sun, Y.E., Coon, J.J., Peters, E.C., and Hsieh-Wilson, L.C. (2007). Probing the dynamics of O-GlcNAc glycosylation in the brain using quantitative proteomics. Nature Chemical Biology *3*, 339-348.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning, A Laboratory Manual, Second Edition edn (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Shui, W., Sheu, L., Liu, J., Smart, B., Petzold, C.J., Hsieh, T.Y., Pitcher, A., Keasling, J.D., and Bertozzi, C.R. (2008). Membrane proteomics of phagosomes suggests a connection to autophagy. Proceedings of the National Academy of Sciences of the United States of America *105*, 16952-16957.

Swaney, D.L., McAlister, G.C., and Coon, J.J. (2008). Decision tree-driven tandem mass spectrometry for shotgun proteomics. Nat Methods *5*, 959-964.

## Supplementary Figure Legends

**Figure S1. Verification of subcellular fractionation, related to Figure 2.** Wild type MEFs were fractionated as described (Frezza et al., 2007) and successful enrichment in mitochondrial and nuclear/cytoplasmic fractions was verified by immunoblot for known mitochondrial (MnSOD), nuclear (Ezh2) and cytoplasmic (tubulin) marker proteins. Similar results were obtained with Jurkat cells (not shown).

**Figure S2. VDAC2 is identified in a glyco-DIGE experiment, related to Figure 2.** Protein spots corresponding to those in Figure 2A were excised from preparative glyco-DIGE gels and analyzed by mass spectrometry. Reported are the merged tryptic peptides and their statistical indicators of quality from two proteomics experiments that identified VDAC2 as the major protein.

**Figure S3. VDAC2 immunoreactivity corresponds to VDAC2 glyco-DIGE spots, related to Figure 2.** Mitochondrial extracts were prepared from wild type MEFs and analyzed by 2D immunoblot as indicated. The anti-VDAC2 immunoreactive spots correspond to the fluorescent spots identified as VDAC2 in glyco-DIGE experiments (Figures 2A and 2B). Proteins were separated between pI 3 (left) and 11 (right) in the horizontal dimension and between MW ~115 kDa (top) and ~19 kDa (bottom) in the vertical dimension.

**Figure S4. VDAC2 is specifically enriched from mitochondrial extracts prepared from $Ac_4$GalNAz-treated cells, related to Figure 2.** Jurkat cells were treated with 100 μM $Ac_4$GalNAz or vehicle-only control for 24 hours. Mitochondrial extracts (Frezza et al., 2007) were made and reacted with phosphine-biotin, and samples were subjected to anti-biotin affinity purification. Reported are the merged tryptic peptides and their statistical indicators of quality from two $Ac_4$GalNAz-treated samples that identified VDAC2. In the vehicle-only (i.e., no $Ac_4$GalNAz) negative control sample, avidin was the only protein identified (not shown).

**Figure S5. MEFs downregulate OGT and upregulate OGA in response to Thiamet-G and glucosamine treatment, related to Figure 3.** Wild type ("WT") or VDAC2$^{-/-}$ ("**ø**") MEFs were treated with 10 μM Thiamet-G and 4 mM glucosamine or vehicle control for 26 or 50 hours and whole-cell lysates were prepared and analyzed by immunoblot as indicated. Tubulin serves as a loading control.

**Figure S6. Stable re-expression of VDAC2 in VDAC$^{-/-}$ MEFs restores sensitivity to perturbation of global O-GlcNAc, related to Figure 3.** (A) VDAC2$^{-/-}$ MEFs were stably transduced with vector only or HA-tagged VDAC2. Expression was verified by immunoblot of whole-cell lysate for HA, MnSOD (mitochondrial protein loading control) and tubulin (cytoplasmic protein loading control). (B) VDAC2$^{-/-}$ MEFs stably transduced with vector or HA-VDAC2 were treated with vehicle only or 10 μM Thiamet-G and 4 mM glucosamine for 24 hours and analyzed by caspase-3/7 activity assay to measure apoptotic protease activation. Treated samples were normalized to their corresponding vehicle-only controls. Error bars represent standard deviations.

**Figure S7. Glyco-DIGE detects DNA damage-dependent changes in O-GlcNAcylated nuclear proteins conserved across disparate human cell lines, related to Discussion.** HT1080 (left) or Jurkat (right) cells were treated with 100 μM $Ac_4$GalNAz and either 1 μg/ml doxorubicin or vehicle-only control for 24 hours. Nuclear extracts were made and reacted with **1** (vehicle-only, green) or **2** (+doxorubicin, red) and analyzed by glyco-DIGE. Arrows indicate candidate DNA damage-specific changes in O-GlcNAcylated proteins. Proteins were separated between pI 3 (left) and 11 (right) in the horizontal dimension and between MW ~190 kDa (top) and ~19 kDa (bottom) in the vertical dimension.

**Figure S8. Analytical HPLC traces for compounds 1 (A) and 2 (B), related to Extended Experimental Procedures.**