Supporting Information

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SI Text

SI Methods ***Materials for cell culture, immunoblotting, and immunoprecipitation.*** Dulbecco's Modified Eagle Medium (D-MEM) containing glucose (4.5 g/L or 1.0 g/L), L-glutamine, and 110 mg/L sodium pyruvate was purchased from Invitrogen. Fetal calf serum, penicillin/streptomycin, Dulbecco's Phosphate-Buffered Saline (DPBS), TrypLE™ Express Stable Trypsin-Like Enzyme with Phenol Red, Opti-MEM® I, and Lipofectamine™ 2000 were purchased from Invitrogen. The Invitrogen Countess Automated Cell Counter was used for cell counting. The bicinchoninic acid (BCA) Protein Assay Kit for determination of protein concentration was purchased from Pierce. For immunoblotting or immunoprecipitation, antibodies were obtained from the following sources: anti-biotin-HRP conjugate (Sigma-Aldrich), anti-O-GlcNAc (RL2, Thermo Fisher Scientific Inc., MA1-072), anti-O-GlcNAc (CTD110.6, Covance, MMS-248R), anti-SP1(PEP2) (Santa Cruz Biotechnology, SC-59), anti-TNPO1 [D45] (Abcam, ab10303), anti-NUP214 (Bethyl Lab, A300-716A), mAb414 (Covance Research Products), anti-NUP62 (BD Transduction Lab, 610497), anti-AGX1 (GeneTex, GTX103592). Anti-NUP358, a polyclonal antirabbit IgG was a generous gift from Mary Dasso (NIH) (1). Two anti-NUP153 were used. A polyclonal rabbit IgG against the zinc finger domain of human NUP153 was a generous gift from Katharine Ullman (University of Utah) (2) and was used for the experiments shown in Fig. 4, Fig. 5, and Fig. S5*D*. Anti-NUP153 (SA1) is a monoclonal mouse IgG against the C-terminal domain of NUP153. This was a generous gift from Brian Burke (Institute of Medical Biology, Singapore) and was used for the experiment shown in Fig. S4. Protein G Sepharose® (fast flow) was purchased from Sigma-Aldrich. Nitrocellulose membranes (Whatman Protran Rolls, BA79, 0.1 um) were purchased from Whatman. For chemiluminescent visualization, SuperSignal WestPico chemiluminescent substrate (ECL reagent) was purchased from Pierce. HeLa and T84 cells were obtained from the ATCC.

General materials and procedures for synthesis. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 glass plates (EM Science), staining the TLC was done by heating with ceric ammonium molybdate solution (CAM, 0.4% wt/v $Ce(SO_4)_2$, 10% wt/v ammonium molybdate tetrahydrate, and 10% sulfuric acid) or 5% phosphomolybdic acid in ethanol. Products were purified by flash column chromatography on silica gel 60 (particle size 40–63 or 60–200 μm, EM Science). ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR spectra were recorded with Varian 500 MHz spectrometers and reported in δ ppm scale. ^{1}H-NMR spectra were referenced to $CDCl_3$ (7.260 ppm). ^{13}C-NMR spectra were referenced to the center of the $CDCl_3$ triplet (77.0 ppm). Electrospray ionization mass spectrometry (ESI-MS) data were collected at the Protein Chemistry and Technology Center at UT Southwestern Medical Center. Samples were dissolved or diluted into either a solution that contained 50% acetonitrile and 5% NH_4OH or an acetonitrile:H_2O 1:1 solution that contained 0.1% formic acid. Proxeon nano-tips were used to infuse the samples into a QStar XL mass spectrometer (Applied Biosystems). Spectra were acquired with mass range m/z 50–1,000 or 300–1,000. Molecular weights were calculated using the Baysesian Protein Reconstruct tool of the Analyst QS1.1 software. High resolution ESI-TOF mass data were recorded on an Agilent ESI-TOF mass spectrometer at the Scripps Center for Mass Spectrometry, TSRI. Samples were electrosprayed into the TOF reflectron analyzer at an ESI voltage of 4000 V and a flow rate of 200 μL/min.

Synthesis of Ac_4GlcNDAz, Ac_3GlcNDAz-1-OH, Ac_3GlcNDAz-1-P, and UDP-GlcNDAz have been described elsewhere (3, 4). GlcNDAz-1-P as HPAEC standard was prepared by simple deacetylation of Ac_3GlcNDAz-1-P with MeOH:Water:TEA (5:2:1) followed by cation exchange (Dowex 50WX8-100, H^+ form) and the resulting crude aqueous elute was used in HPAEC analysis without further purification. Synthesis of standard UDP-GalNDAz was essentially the same as that described for UDP-GlcNDAz (4, 5).

Synthesis of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. To the stirred solution of Ac_3GlcNDAz-1-OH (100 mg, 0.241 mmol), and bis(S-acetyl-2-thioethyl) *N*,*N*-diisopropylphosphoramidite (0.336 mmol, 125 mg) in acetonitrile (700 μL) at 0 °C, was added ^{1}H-tetrazole (0.361 mmol, 800 μL, 0.45 M in acetonitrile) and the resulting mixture was warmed to RT. After 1 h, mCPBA (0.361 mmol, 77%, 81.0 mg) was added at 0 °C and stirred for additional 30 min at 0 °C. The resulting reaction mixture was partitioned between ethyl acetate and 10% aqueous sodium sulfite (Na_2SO_3) and the organic layer was washed with saturated aqueous sodium bicarbonate ($NaHCO_3$) and brine. After two more extractions with fresh ethyl acetate from the aqueous phases, the combined organic layer was dried (Na_2SO_4) and concentrated by vacuum evaporation. The resulting residue was purified by flash silica gel column chromatography (ethyl acetate:hexanes = 1:1 and then ethyl acetate) to provide the Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ as a colorless oil (145 mg, 86%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.00 (s, 3H, $NHCOCH_2CH_2(N=N)CH_3$), 1.68–1.79 (m, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 1.90–2.00 (m, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 2.02 (s, 6H, O-$COCH_3$), 2.16 (s, 3H, O-$COCH_3$), 2.36–2.39 (2s, 6H, S-$COCH_3$) 3.18 (m, 4H, -OCH_2CH_2SAc), 4.05–4.29 (m, 7H, -$OCH_2CH_2SAc \times 2$, H5, $H6_a$, $H6_b$) 4.47(m, 1H, H2), 5.21(m, 2H, H3 & H4), 5.56 (dd, J = 5.6, 3.3 Hz, 1H, H1), 6.31 (d, J = 9.0 Hz, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.8, 20.5, 20.6, 25.4, 29.0(m), 29.5, 30.1, 30.5 (m), 51.5(d), 61.3, 66.4(m), 67.4, 69.6, 96.3(d), 169.1, 170.5, 171.0, 171.7, 194.7, 195.1. ^{31}P NMR(200 MHz, $CDCl_3$): δ −2.28. ESI-MS: calcd. for $C_{25}H_{39}N_3O_{14}PS_2$ $[M+H]^+$ 700.16, found 700.14; calcd. for $C_{25}H_{38}N_3NaO_{14}PS_2$ $[M+Na]^+$ 722.14, found 722.13. HRMS (ESI-TOF) calcd. for $C_{25}H_{39}N_3O_{14}PS_2$ $[M+H]^+$ 700.1605, found 700.1607; calcd. for $C_{25}H_{38}N_3NaO_{14}PS_2$ $[M+Na]^+$ 722.1425, found 722.1421. NMR spectra are shown in Fig. S8 *A*–*C*.

Synthesis of p-nitrophenyl-β-D-GlcNDAz (pNP-GlcNDAz). pNP-GlcNDAz was synthesized by adapting reported protocols for related molecules (6, 7). GlcNDAz (105 mg, 0.363 mmol) was suspended in acetyl chloride (900 μL) at 0 °C and the resulting mixture was warmed to RT. After 16 h, the mixture was diluted with chloroform and poured onto ice in a separatory funnel. Saturated aqueous sodium bicarbonate ($NaHCO_3$) was added to quench the remaining acetyl chloride. After partitioning, the organic layer was washed with brine, dried (Na_2SO_4), and then concentrated by vacuum evaporation. After resuspending the dried crude mixture in dichloromethane (2 mL), tetrabutylammonium hydrogen sulfate (0.363 mmol, 123 mg), 4-nitrophenol (0.726 mmol, 101 mg), and then 1N NaOH (2 mL) were added. After vigorous stirring for 3 h, the reaction mixture was diluted with ethyl acetate (20 mL) and washed with 1 M NaOH (10 mL, five times), water (10 mL, three times), and brine. The

ethyl acetate layer was dried with Na_2SO_4 and concentrated by vacuum evaporation. The resulting concentrate was precipitated by ethyl acetate and hexane (1:2–1:3) and the precipitate was collected by filtration. The resulting precipitate was recrystallized in ethyl acetate and hexane (1:3) overnight to give 4-nitrophenyl- 3,4,6-tri-O-acetyl-β-D-GlcNDAz (60.6 mg, 0.113 mmol, 31.1% over two steps). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.96 (s, 3H, $NHCOCH_2CH_2(N=N)CH_3$), 1.69–1.79 (m, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 1.84–1.94 (m, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 2.06 (s, 3H, O-$COCH_3$), 2.08 (s, 3H, O-$COCH_3$), 3.96 (m, 1H, H5) 4.09–4.18(m, 2H, H2, $H6_a$), 4.28 (dd, J = 12.3, 5.6 Hz,1H, $H6_b$), 5.13(t, J = 9.6 Hz, 1H, H3 or H4), 5.41–5.49 (m, 2H, H1 and H4 or H3), 5.80 (d, J = 8.4 Hz, 1H, NH), 7.06 (d, J = 9.1 Hz, 2H, phenyl), 8.16 (d, J = 9.1 Hz, 2H, phenyl). ^{13}C NMR (125 MHz, $CDCl_3$): δ 19.8, 20.6, 20.7, 25.3, 29.1, 30.4, 54.7, 62.0, 68.3, 71.4, 72.2, 97.7, 116.5, 125.7, 142.9, 161.4, 169.4, 170.4, 170.8, 171.7. MALDI-TOF-MS: calcd. for $C_{23}H_{28}N_4NaO_{11}$ $[M+Na]^+$, 599.16, found 599.20; calcd. for $C_{23}H_{28}N_2NaO_{11}$ $[M+Na-N_2]^+$ 531.16, found 531.20; calcd. for $C_{23}H_{29}N_2O_{11}$ $[M+H-N_2]^+$ 509.18, found 509.22.

To the solution of 4-nitrophenyl- 3,4,6-tri-O-acetyl-GlcNDAz (46 mg, 0.086 mmol) in methanol (2.5 mL), was added NaOMe (0.1 eq., 8.6 μmol, 0.5 M in MeOH, 17 μL) and the resulting mixture was stirred for 2 h at RT. After filtration, the precipitate was washed with cold methanol to give pure pNP-GlcNDAz (12.7 mg) and the filtrate was passed through Dowex® 50WX8-100, H^+ form and then concentrated to obtain 22.8 mg of pNP-GlcNDAz (quantitative yield). ^{1}H NMR (500 MHz, CD_3OD): δ 0.97 (s, 3H, $NHCOCH_2CH_2(N=N)CH_3$), 1.65 (t, J = 7.7 Hz, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 2.06–2.18 (m, 2H, $NHCOCH_2CH_2(N=N)CH_3$), 3.43 (t, J = 9.5 Hz, 1H, H3 or H4), 3.50 (m, 1H, H5), 3.60 (dd, J = 10.2, 8.9 Hz, H4 or H3), 3.72 (dd, J = 12.15, 5.8 Hz, 1H, $H6_a$), 3.91–3.97 (m, 2H, H2 & $H6_b$), 5.21(d, J = 8.4 Hz, 1H, H1), 7.18 (d, J = 9.2 Hz, 2H, phenyl), 8.20 (d, J = 9.2 Hz, 2H, phenyl). ^{13}C NMR (125 MHz, CD_3OD): δ 19.7, 26.4, 31.4, 31.6, 57.0 62.4, 71.7, 75.6, 78.5, 99.9, 117.6, 126.6, 143.9, 163.7, 175.1. HRMS (ESI-TOF): calcd. for $C_{17}H_{23}N_4O_8$ $[M+H]^+$ 411.1516, found 411.1513; calcd. for $C_{17}H_{22}N_4NaO_8$ $[M+Na]^+$ 433.1335, found 433.1330. Stereochemistry of the anomeric position was determined based on the coupling constant of H1(d, J = 8.4 Hz). NMR spectra are shown in Fig. S8 *D* and *E*.

Synthesis of peptide substrates. Biotinylated CKII peptide [NH_2-PGGSTPVSSANMMK(biotin)-COOH] was synthesized essentially as described for other peptides (8). A modified human α-A crystallin peptide termed +P2 (Ac-AIPVSRPEK-$CONH_2$) was synthesized by standard peptide synthesis methods.

Evaluation of human OGT activity with UDP-GlcNDAz. For the lysate experiment (Fig. S1*B*), HeLa cells were transiently transfected with 24 μg of pCDNA4/TO/ncOGT-myc-His (9) using Lipofectamine® 2000. After 48 h, cells (approximately 10×10^6) were harvested and lysed in a transferase assay buffer [400 μL, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, and 1 × protease inhibitor cocktail (Complete Mini, Roche)] via sonication (five times for 5 s each, VirSonic 100, VirTis) on ice. After centrifugation (20,000 × *g*, 10 min), the supernatant was collected and mixed with 800 μL of 30% PEG8000 (in 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA) and the mixture was vortexed briefly and centrifuged (20,000 × *g*, 20 min, 4 °C). After removing the supernatant, the resulting protein pellet was resuspended in 200 μL of transferase assay buffer (25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA). After determination of the protein concentration by the bicinchoninic acid assay (BCA), the transferase assay was performed by reaction of 100 μg of the lysate, 100 μM of synthetic +P2 peptide, and UDP-sugar [0.5 mM UDP-GlcNAc, 0.5 mM UDP-GlcNDAz, or 1:1 mixture of UDP-GlcNAc and UDP-GlcNDAz (each 0.5 mM)] in 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA for 16 h at RT. After the reaction, the peptide was purified by Zip-Tip_{C18} (Millipore) and analyzed by MALDI-TOF MS using a matrix of DHB containing aniline in 50% acetonitrile.

For the competition experiment with recombinant ncOGT (Fig. S1*C*), ncOGT expression and purification were performed using a codon-optimized plasmid generously supplied by Suzanne Walker (Harvard Medical School), following the previously reported method (10). Reaction mixtures contained 2 μM recombinant ncOGT, 100 μM +P2 peptide, UDP-sugar (2 mM UDP-GlcNAc, 2 mM UDP-GlcNDAz, or 2 mM of each) in 20 mM Tris-HCl buffer pH 7.5, containing 12.5 mM $MgCl_2$ and 2 mM β-mercaptoethanol. Reaction mixtures were incubated at 37 °C for 15 h, then filtered using an Amicon® Ultra centrifugal filter unit (Millipore, 10,000 MWCO). Filtrates were analyzed by HPLC by injecting 100 μL of each onto a ZORBAX Eclipse XDB-phenyl column (4.6 × 150 mm, particle size 3.5 μm) attached to a Dynamax SD-200 HPLC system. The eluents used were 0.1% TFA in water (E1) and 0.1% TFA in acetonitrile (E2). HPLC was performed with a flow rate of 1.5 mL/ min and following gradient elution was used: T (0 min) = 5% E2, T (3 min) = 5% E2, T (5 min) = 10% E2, T (20 min) = 20% E2, T (22 min) = 100% E2, T (25 min) = 100% E2, T (28 min) = 5% E2, T (30 min) = 5% E2. Chromatograms were recorded with UV detector (Dynamax UV-C) set at 220 nm. Molar absorption coefficients (220 nm) for the O-GlcNAc-modified and O-GlcNDAz-modified peptides were estimated, based on the measured absorbance values for the unmodified peptide, GlcNAc, and GlcNDAz. Identities of the peaks corresponding to the +P2 peptide, the O-GlcNAc-modified peptide, and the O-GlcNDAz-modified peptide were confirmed by MALDI-TOF analysis of the collected fractions. The peak assigned as the O-GlcNDAz-modified peptide was also shown to be sensitive to UV irradiation.

Crosslinking O-GlcNDAz peptide with anti-O-GlcNAc antibodies. For the in vitro transfer and crosslinking experiments (Figs. S1*A* and S2), the plasmid used for recombinant ncOGT expression was a generous gift from John Hanover (NIDDK) (11). ncOGT expression and purification were performed as described previously (8, 12). In vitro OGT reactions with synthetic biotin-CK2 peptide (100 μM) were performed in 20 mM Tris-HCl pH 7.2, 12.5 μM $MgCl_2$, 715 μM β-mercaptoethanol and 100 μM of UDP-GlcNAc, UDP-GlcNDAz, or water at 37 °C for 16 h. Peptides were purified via Zip-TipC18 (Millipore) and analyzed by MALDI-TOF (Fig. S1*A*). Peptide samples were incubated overnight at 4 °C with RL2 or CTD110.6. Each sample was split in half, with one half receiving 20 min of UV irradiation (365 nm, XX-20BLB, UVP, 40 min). Samples were resolved on SDS-PAGE gels in duplicate and transferred to nitrocellulose. The blots were probed with an anti-biotin-HRP antibody (1:50,000)(Fig. S2*B*).

Mutagenesis of AGX1. Human AGX1 gene in pCMV6-XL5-UAP1 (Origene) was used as a template for constructing mutants pCMV6-XL5-AGX1(F381G) and pCMV6-XL5-AGX1(F383G) by QuikChange mutagenesis protocol (Stratagene) using the following primer sets:

AGX1(F381G) forward: CCC AAT GGA ATA AAG ATG GAA AAA GGT GTC TTT GAC ATC TTC CAG

AGX1(F381G) reverse: CTG GAA GAT GTC AAA GAC ACC TTT TTC CAT CTT TAT TCC ATT GGG

AGX1(F383G) forward: CCC ATT GGA ATA AAG ATG GAA AAA TTT GTC GGT GAC ATC TTC CAG

AGX1(F383G) reverse: CTG GAA GAT GTC ACC GAC AAA TTT TTC CAT CTT TAT TCC ATT GGG

Mutant plasmids were sequenced by the DNA sequencing core facility in McDermott Center for Human Growth & Development (University of Southwestern Medical Center).

Stable expression of AGX1(F383G) in HeLa cells. The gene encoding AGX1(F383G) was amplified from pCMV6-XL5-AGX1(F383G) by PCR using the following primers:

5′-BamHI-primer: ATAGGATCCATGAACATTAATGACCTCAAACT

3′-NotI-primer: TATGCGGCCGCTCAAATACCATTTTTCACCAGCT

The PCR product and pIRESpuro3 vector were each digested with BamH I and Not I, then ligated together. The identity of the desired plasmid (pIRESpuro3-AGX1(F383G)) was confirmed by DNA sequencing.

HeLa cells were transfected with pIRESpuro3-AGX1(F383G) using Lipofectamine-2000. After 48 h, puromycin (5 μg/mL) was added. Medium containing puromycin was replaced every two or three days. After 24 d of selection, surviving cells were maintained in DMEM containing 1 μg/ml puromycin. Those stably transfected cells were cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. HPAEC analysis of the lysates revealed UDP-GlcNDAz production, confirming stable expression of AGX1 (F383G). Overexpression of AGX1 was also determined by immunoblotting (Fig. S6*A*); assignment of bands corresponding to AGX1 and AGX2 was based on literature precedent (13).

HPAEC-PAD analysis of lysates. Lysates were prepared as described in the main text. The eluents used were 1.0 mM NaOH (E1) and 1.0 M NaOAc and 1.0 mM NaOH (E2). HPAEC was performed with a flow rate = 1 mL/ min and the following gradient elution was performed: $T_{0\ min}$ = 5% E2, T_{40} = 30% E2, T_{45} = 50% E2, T_{60} = 55% E2, T_{65} = 100% E2, T_{75} = 100% E2, T_{80} = 5% E2, T_{90} = 5% E2. GlcNDAz-1-P eluted at 20.8 min and its identity was confirmed by parallel injection of standard GlcNDAz-1-P (Fig. S3*A*). UDP-GlcNDAz eluted at 62.5 min and its identity was confirmed by parallel injection of standard UDP-GlcNDAz (Fig. S3*B*). Also, the lysate was irradiated by UV (365 nm, XX-20BLB, UVP, 45 min) and its HPAEC chromatogram was compared with that of UV-irradiated GlcNDAz-1-P standard compound or UV-irradiated UDP-GlcNDAz standard compound (Fig. S3 *A* and *B*). The peak eluting at 62.5 min from the HeLa lysate was collected, desalted by cation-exchange resin (Dowex® 50WX8-100, ammonium from) and then lyophilized for the MALDI-TOF mass analysis (Fig. S3*C*), which also confirmed the production of UDP-GlcNDAz in HeLa cells.

Crosslinking lysates with anti-O-GlcNAc antibody. HeLa cells were transiently transfected with 24 μg of pCMV6-XL5-AGX1 (F383G), 24 μg of pCDNA4/TO/ncOGT-myc-His, or 12 μg each of pCMV6-XL5-AGX1(F383G) and pCDNA4/TO/ncOGT-myc-His using Lipofectamine-2000. After 48 h, 20 μL of 50 mM Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ in DMSO or DMSO alone was added to the cells in serum-free, low-glucose (1.0 g/L) DMEM. After 19 h, cells were harvested and lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 × protease inhibitor cocktail) via vortexing (20 sec) followed by incubation in ice (30 min). Lysates were centrifuged (20,000 × *g*, 10 min). The supernatant was collected and the buffer was exchanged with fresh RIPA buffer by Zeba™ spin desalting columns (MWCO = 7,000, Pierce) following the manufacturer's protocol. After supplementing the desalted lysate with PMSF and protease inhibitor cocktail, the protein concentration was determined by the BCA method. Each 1.3 mg of lysate was rotated with RL2 (Affinity Bio Reagent, 2.6 μg) in 650 μL total volume at 4 °C. After overnight rotation, one half of each lysate was irradiated by UV (365 nm, XX-20BLB, UVP, 40 min) in 24-well dish on ice bath, while the other half was kept on ice in dark. Each sample was collected and rotated with protein G Sepharose (20 μl each, Sigma-Aldrich) at 4 °C and the beads were washed three times with the RIPA buffer. Proteins were eluted from the washed beads with 2 × loading dye by heating at 95 °C for 7 min. The eluted samples were resolved by SDS-PAGE and then transferred to nitrocellulose membrane (17 h, 30 V) at 4 °C. Immunoblotting was performed using anti-SP1 antibody (Santa Cruz) or mouse anti-NUP153 antibody (SA-1 from Brian Burke) (Fig. S4). This experiment demonstrated that cotransfection with pCDNA4/TO/ncOGT-myc-His had no effect on crosslinking efficiency, so pCDNA4/TO/ncOGT-myc-His was not included in any other experiments.

Experiments in AGX1(F383G)-expressing T84 cells. To demonstrate that crosslinking could be performed in other cell types (Fig. S5 *C* and *D*), T84 cells stably expressing AGX1(F383G) were used. T84 cells stably expressing mutant AGX1(F383G) were prepared by lentiviral transduction as follows. Site-directed mutagenesis was performed on the vector pCMV6-XL5-AGX1(F383G) to destroy an intrinsic EcoR I site located within the AGX1 gene, and the AGX1(F383G) PCR product obtained from that plasmid was then cloned into the Spe I and EcoR I restriction sites of the linearized pSIN4-EF2-IRES-Puro lentiviral vector (a kind gift from Dr. Jiang Wu at UT Southwestern) (14). The pSIN4-EF2-AGX1 (F383G)-IRES-Puro vector was utilized to assemble lentivirus with a 2nd-generation packaging system following the protocol published by the RNAi Consortium (http://www.broad.mit.edu/genome_bio/trc/rnai.html). The lentiviral packaging plasmid psPAX2 (Addgene plasmid 12260) and envelope plasmid pMD2.G (Addgene plasmid 12259) were obtained from Dr. Didier Trono. T84 cells were infected for 20 h in D-MEM/F-12 (1:1)-Hepes media (containing glucose (3.151 g/L), L-glutamine, 5% FBS, and penicillin/streptomycin) with 8 μg/mL of Polybrene (Sigma-Aldrich). Cells were infected with a targeted MOI ≪ 1 such that each cell is only likely to be infected once. Stably transduced T84 cells were subjected to selection with 7 μg/mL puromycin for 17 d before the puromycin selection was removed. For the crosslinking experiments, the T84 cells stably expressing mutant AGX1 were cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ (100 μM final concentration) or DMSO (vehicle) added at 0 h and at 24 h after the media was changed to serum-free D-MEM/F-12 (1:1)-Hepes. Cells were harvested 20 h after the second addition of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ and UV irradiated, as described in the main text. The UV-irradiated cells were lysed as described above and the lysate was resolved by SDS-PAGE and transferred to nitrocellulose membrane (17 h, 30 V) at 4 °C. Immunoblotting was performed using mAb414 or rabbit anti-NUP153 antibody (from Katharine Ullman).

In-gel digestion of silver-stained proteins. Regions of interest were excised from the silver-stained gel. The gel slices were washed three times with an acidic buffer [acetic acid:ethanol:water = 10:50:40, (v/v/v)] for 24 h. After being swollen in water twice (15 min each time), the gel bands were treated with a destaining solution containing 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. After extensive washing, the gel bands were cut into small pieces, dehydrated in acetonitrile, and then dried in a SpeedVac (Thermo Electron). A sufficient amount of trypsin (10 ng/μL in 50 mM ammonium bicarbonate) was added to cover the gel pieces and overnight digestion was performed at 37 °C. The resulting tryptic peptides were extracted sequentially with 5% TFA/50% acetonitrile/45% water (v/v/v), 0.1% TFA/75% acetonitrile/24.9% water (v/v/v), and 100% acetonitrile. The extracts were combined and dried in a SpeedVac. Desalting was achieved with C18 ZipTips (Millipore) according to the manufacturer's instructions, prior to nano-HPLC/mass spectrometric analysis.

Identification of proteins by HPLC/MS/MS analysis. Mass spectrometry was performed at the UT Southwestern Protein Chemistry Technology Center. High performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) analysis was performed on an integrated system that includes an Agilent 1100 series nanoflow LC system (Agilent) and a LTQ 2D trap mass spectrometer (Thermo Electron) equipped with a nanoelectrospray ionization (NSI) source. Tryptic peptides were reconstituted in buffer A solution (0.05% formic acid, 2% acetonitrile, 97.95% H_2O, v/v/v), and separated by a capillary HPLC column (11 cm length × 75 μm I.D.) packed in-house with Luna C18 resin (5 μm particle size, 100 Å pore diameter, Phenomenex). A 40 min gradient from 20% to 90% buffer B (0.05% formic acid, 90% acetonitrile, 9.95% H_2O, v/v/v) was used. The eluted peptides were electrosprayed directly into the LTQ ion trap mass spectrometer, which was operated in a data-dependent mode. Mascot (version 2.2, Matrix Science) was used for database search.

NCOAT specificity assay. Artificial O-GlcNAc and O-GlcNDAz substrates were used for this chromogenic assay (15, 16). pNP-GlcNAc was purchased from Sigma-Aldrich and pNP-GlcNDAz was prepared as described above. Cell lysates were prepared from nomal HeLa cells or HeLa cells that were transiently transfected with pCMV/myc-NCOAT (human NCOAT-encoding plasmid generously provided by David Vocadlo, Simon Fraser University) for 24 h with Lipofectamine-2000. Briefly, harvested HeLa cells were homogenized by 27-gauge needle (10 times) in 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 20,000 × *g* (30 min, 4 °C). The resulting supernatant was collected and concentrated using an Amicon® Ultra centrifugal filter unit (Millipore, 10,000 MWCO). 2 mM of pNP-GlcNAc or pNP-GlcNDAz was mixed with 100 μg of the HeLa lysate in 50 mM sodium cacodylate buffer (pH 6.5) containing 50 mM N-acetylgalactosamine and 150 mM NaCl. The reaction mixtures (total volume of 100 μL) were incubated at 37 °C for 30 min and then quenched with 900 μL of 0.5 M sodium carbonate. The relative amount of 4-nitrophenol released from pNP-GlcNAc or pNP-GlcNDAz was determined by measuring the UV absorbance of the solution at 400 nm (Fig. S7).

1. Joseph J, Liu S-T, Jablonski SA, Yen TJ, Dasso M (2004) The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions *in vivo*. *Curr Biol* 14:611–617.
2. Liu J, Prunuske AJ, Fager AM, Ullman KS (2003) The COPI complex functions in nuclear envelope breakdown and is recruited by the nucleoporin Nup153. *Dev Cell* 5:487–498.
3. Tanaka Y & Kohler JJ (2008) Photoactivatable crosslinking sugars for capturing glycoprotein interactions. *J Am Chem Soc* 130:3278–3279.
4. Bond MR, et al. (2011) Metabolism of diazirine-modified *N*-acetylmannosamine analogues to photo-cross-linking sialosides. *Bioconjug Chem* 22:1811–1823.
5. Bond MR (2010) Illuminating glycoconjugate interactions and glycosyltransferase substrates using photocrosslinking sugar analogs. Ph.D. (Stanford University, Stanford, CA).
6. Vocadlo DJ, Withers SG (2005) Detailed comparative analysis of the catalytic mechanisms of β-N-acetylglucosaminidases from families 3 and 20 of glycoside hydrolases. *Biochemistry* 44:12809–12818.
7. Orth R, Pitscheider M, Sieber SA (2010) Chemical probes for labeling of the bacterial glucosaminidase NagZ via the Huisgen cycloaddition. *Synthesis* 2010:2201–2206.
8. Leavy TM, Bertozzi CR (2007) A high-throughput assay for O-GlcNAc transferase detects primary sequence preferences in peptide substrates. *Bioorg Med Chem Lett* 17:3851–3854.
9. Boyce M, et al. (2011) Metabolic cross-talk allows labeling of O-linked β-N-acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. *Proc Natl Acad Sci USA* 108:3141–3146.
10. Gross BJ, Kraybill BC, Walker S (2005) Discovery of O-GlcNAc transferase inhibitors. *J Am Chem Soc* 127:14588–14589.
11. Lubas WA, Frank DW, Krause M, Hanover JA (1997) O-linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats. *J Biol Chem* 272:9316–9324.
12. Vocadlo DJ, Hang HC, Kim EJ, Hanover JA, Bertozzi CR (2003) A chemical approach for identifying O-GlcNAc-modified proteins in cells. *Proc Natl Acad Sci USA* 100:9116–9121.
13. Wang-Gillam A, Pastuszak I, Elbein AD (1998) A 17-amino acid insert changes UDP-N-acetylhexosamine pyrophosphorylase specificity from UDP-GalNAc to UDP-GlcNAc. *J Biol Chem* 273:27055–27057.
14. Zhan X, Shi X, Zhang Z, Chen Y, Wu JI (2011) Dual role of Brg chromatin remodeling factor in Sonic hedgehog signaling during neural development. *Proc Natl Acad Sci USA* 108:12758–12763.
15. Gao Y, Wells L, Comer FI, Parker GJ, Hart GW (2001) Dynamic O-glycosylation of nuclear and cytosolic proteins. *J Biol Chem* 276:9838–9845.
16. Kang HT, Ju JW, Cho JW, Hwang ES (2003) Down-regulation of Sp1 activity through modulation of O-glycosylation by treatment with a low glucose mimetic, 2-deoxyglucose. *J Biol Chem* 278:51223–51231.

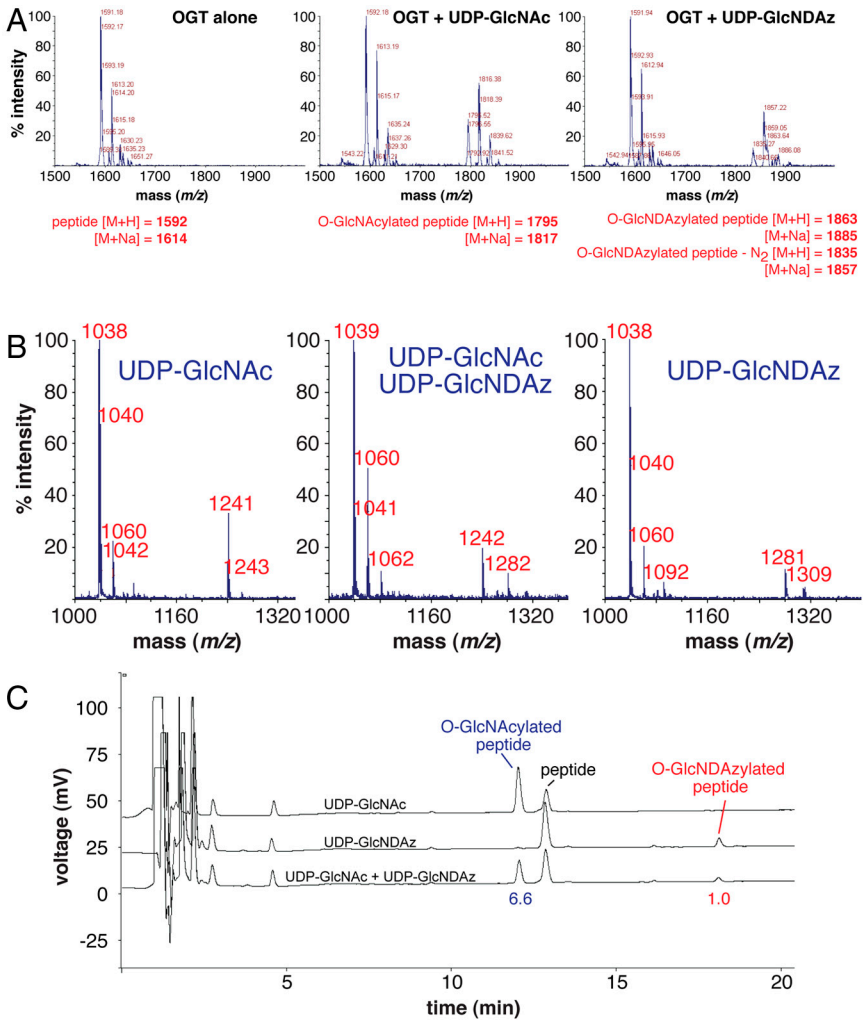

Fig. S1. Human OGT accepts UDP-GlcNDAz. (*A*) Recombinant human ncOGT was incubated with biotinylated CKII peptide alone (*Left*), with biotinylated CKII peptide and UDP-GlcNAc (*Middle*), or with biotinylated CKII peptide and UDP-GlcNDAz (*Right*). After desalting, the products of the in vitro enzymatic reaction were analyzed by MALDI-TOF analysis. Predicted masses (m/z) of the products are shown below the spectra. (*B*) HeLa cells transfected with DNA encoding human OGT were lysed and precipitated with a buffer containing 30% PEG 8000. The precipitate was resuspended in assay buffer and incubated with the +P2 substrate peptide ($m/z = 1,038$) and UDP-GlcNAc, UDP-GlcNDAz, or a 1:1 mixture of the two nucleotide sugars. Reaction products were analyzed by MALDI-TOF mass spectrometry, revealing production of the O-GlcNAc-modified peptide ($m/z = 1,241$) and the O-GlcNDAz-modified peptide (intact diazirine: $m/z = 1,309$ and photolysis product: $m/z = 1,281$). (*C*) The +P2 peptide was incubated with recombinant ncOGT and UDP-GlcNAc, UDP-GlcNDAz, or a 1:1 mixture of the two nucleotide sugars. Reaction products were analyzed by HPLC with UV detection at 220 nm. Peaks corresponding to the +P2 peptide, the O-GlcNAc-modified +P2 peptide, and the O-GlcNDAz-modified +P2 peptide were identified by collecting the peaks and analyzing them by MALDI-TOF. In the reaction containing equimolar amounts of UDP-GlcNAc and UDP-GlcNDAz, the areas of the peaks corresponding to the O-GlcNAcylated +P2 peptide and the O-GlcNDAzylated +P2 peptide were integrated and used to calculate the product ratio of O-GlcNAc-modified to O-GlcNDAz-modified peptide (relative amounts shown below trace). This analysis revealed that GlcNAc is transferred more efficiently than GlcNDAz.

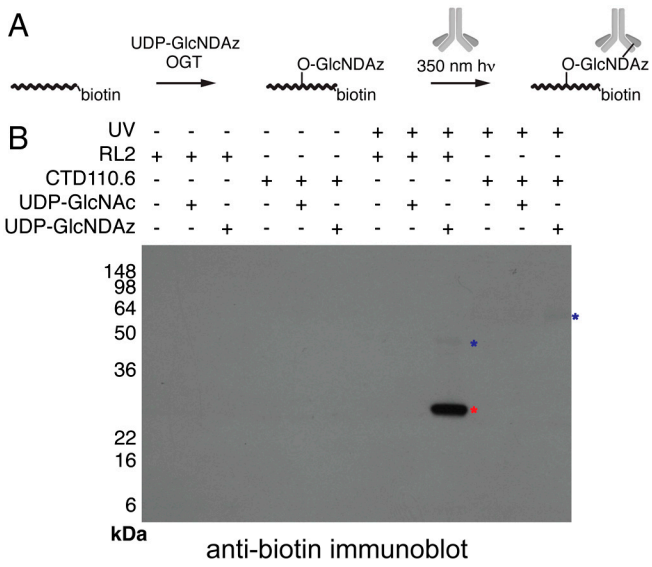

Fig. S2. Covalent crosslinking of an O-GlcNAc-mediated interaction using diazirine-modified O-GlcNAc (O-GlcNDAz). (*A*) Recombinant human ncOGT was incubated with UDP-GlcNDAz and a biotinylated CKII peptide [PGGSTPVSSANMMK(biotin)]. The partially purified peptide was incubated with an O-GlcNAc-recognizing antibody (either RL2 or CTD110.6) and irradiated with 365 nm light. (*B*) Antibiotin immunoblotting revealed a biotinylated species whose molecular weight is consistent with a covalent adduct formed from the CK2 peptide and the light chain of RL2 (red asterisk). Formation of the biotinylated product was dependent on the inclusion of UDP-GlcNDAz and administration of UV irradiation. Weaker bands corresponding to peptide crosslinking to the heavy chains of RL2 and CTD110.6 were also observed (blue asterisks). While we observe strong crosslinking of biotinylated peptide to the light chain of RL2, crosslinking to CTD110.6 was barely detectable. The reasons behind the lack of crosslinking to the CTD110.6 antibody are unclear: CTD110.6 may not recognize the CKII peptide well, the addition of crosslinker may interfere with recognition, or the orientation of the crosslinker may be poorly suited for crosslinking. We did not perform additional experiments to distinguish among these possibilities.

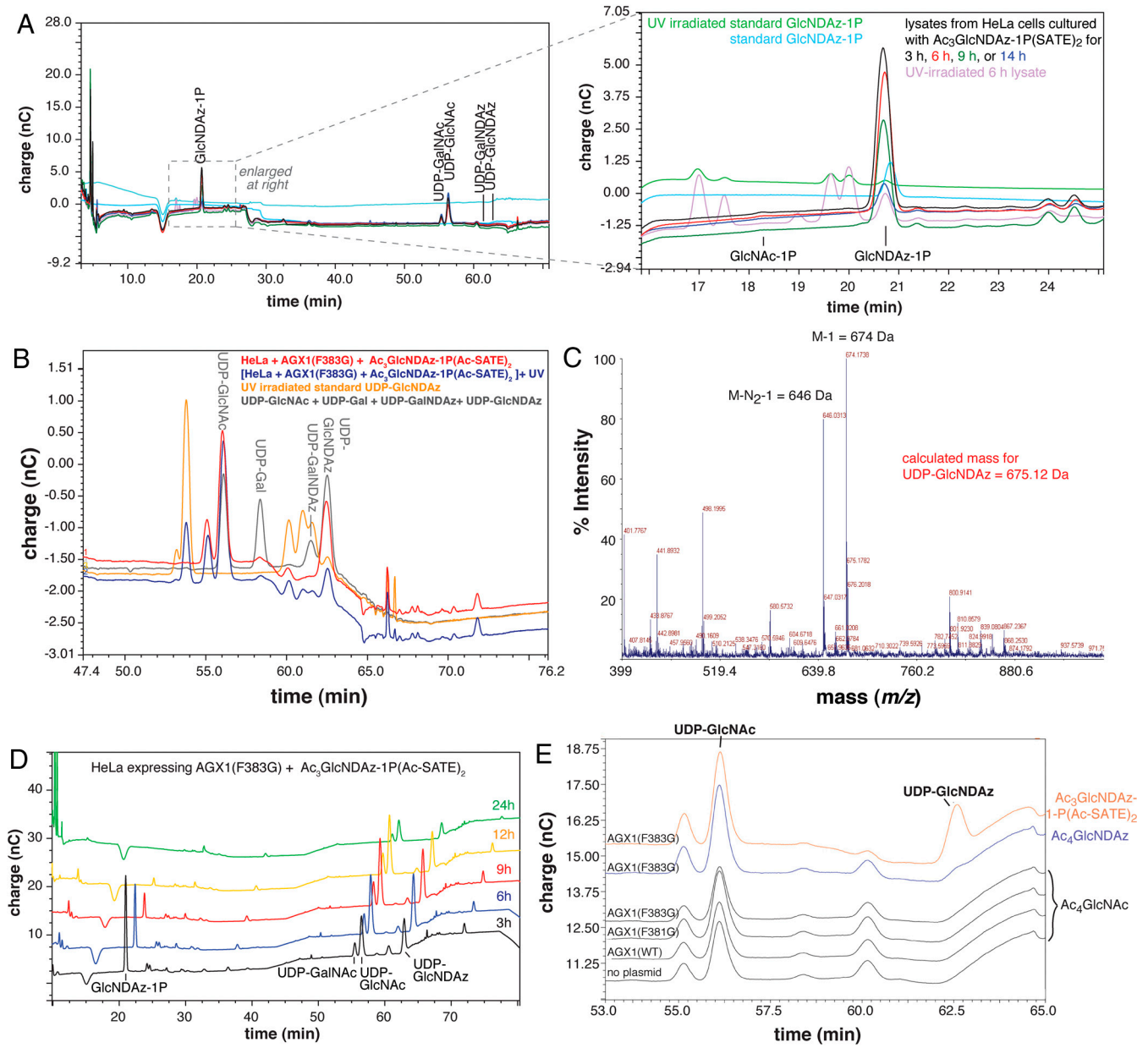

Fig. S3. Metabolism of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ to UDP-GlcNDAz. (*A*) HeLa cells were cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ for 3, 6, 9, or 14 h, then lysed. Cell lysates were analyzed by HPAEC-PAD. Both the full chromatogram and the GlcNDAz-1-P region are shown. A peak with the same retention time as standard GlcNDAz-1-P was observed rapidly (as early as 3 h) and decreased over time. UV irradiation of both the lysate and GlcNDAz-1-P standard resulted in loss of the GlcNDAz-1-P peak and production of characteristic new peaks. Production of UDP-GlcNDAz was not observed. (*B*) HeLa cells were transiently transfected with pCMV6-XL5-AGX1(F383G) and cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. HPAEC-PAD analysis of the cell lysates revealed new peak whose retention time matched that of synthetic UDP-GlcNDAz. In addition, UV irradiation of the lysate resulted in decreased intensity of the putative UDP-GlcNDAz peak, along with the production of new peaks. The new peaks were similar to those observed upon irradiation of the standard UDP-GlcNDAz molecule. (*C*) The lysate peak eluting at 62.5 min (*B*) was collected and analyzed by MALDI-TOF mass spectrometry (anionic and reflector mode), revealing a mass (*m/z*) consistent with the value predicted for UDP-GlcNDAz. (*D*) HeLa cells were transiently transfected with pCMV6-XL5-AGX1(F383G) and cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ for 3, 6, 9, 12, or 24 h. Lysates were analyzed by HPAEC-PAD. GlcNDAz-1-P levels decreased over the time frame examined, while UDP-GlcNDAz levels increased and then decreased. (*E*) HeLa cells were transiently transfected with pCMV6-XL5-AGX1(WT), pCMV6-XL5-AGX1(F381G), or pCMV6-XL5-AGX1(F383G) or no plasmid and cultured with Ac_4GlcNAc, Ac_4GlcNDAz, or Ac_3GlcNDAz-1-P(Ac-SATE)$_2$, as indicated. Only cells cultured with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ demonstrated efficient UDP-GlcNDAz production, indicated that Ac_4GlcNDAz is not efficiently metabolized to GlcNDAz-1P in these cells.

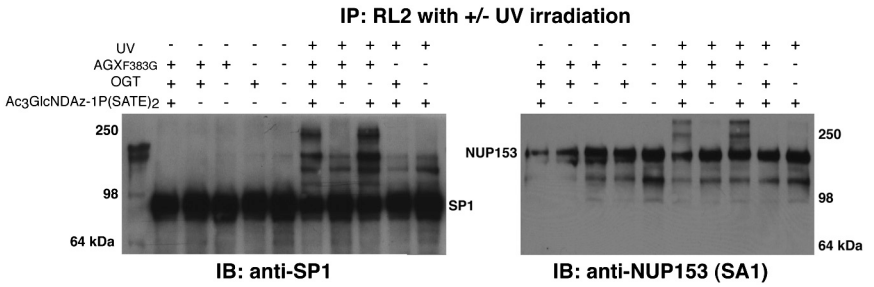

Fig. S4. Crosslinking of O-GlcNDAz-ylated proteins in lysates. HeLa cells were transiently transfected with pCMV6-XL5-AGX1(F383G) and/or pCDNA4/TO/ncOGT-myc-His. Cells were cultured with $Ac_3GlcNDAz\text{-}1\text{-}P(Ac\text{-}SATE)_2$. Cells were lysed and the lysates were incubated with RL2 (anti-O-GlcNAc antibody). The lysate-RL2 mixtures were subjected to UV irradiation (365 nm). Next, the lysates were immunoprecipitated with protein G sepharose to enrich for O-GlcNAc-modified proteins. The immunoprecipitates were resolved by SDS-PAGE and probed by immunoblotting with anti-SP1 and anti-NUP153(SA1) antibodies. Both SP1 and NUP153 are known to be O-GlcNAc-modified and both proteins were efficiently immunoprecipitated in all conditions. For both SP1 and NUP153 immunoblots, higher molecular bands were observed only in those lanes corresponding to cells that had been transfected with pCMV6-XL5-AGX1(F383G) and cultured with $Ac_3GlcNDAz\text{-}1\text{-}P(Ac\text{-}SATE)_2$. These higher molecular weight bands likely represent crosslinked complexes between RL2 and O-GlcNDAz-modified SP1 or NUP153. Alternatively, some of the bands may correspond to crosslinked complexes between endogenous proteins and O-GlcNDAz-modified SP1 or NUP153. Cotransfection with pCDNA4/TO/ncOGT-myc-His had no effect on crosslinking efficiency and was not conducted in other experiments.

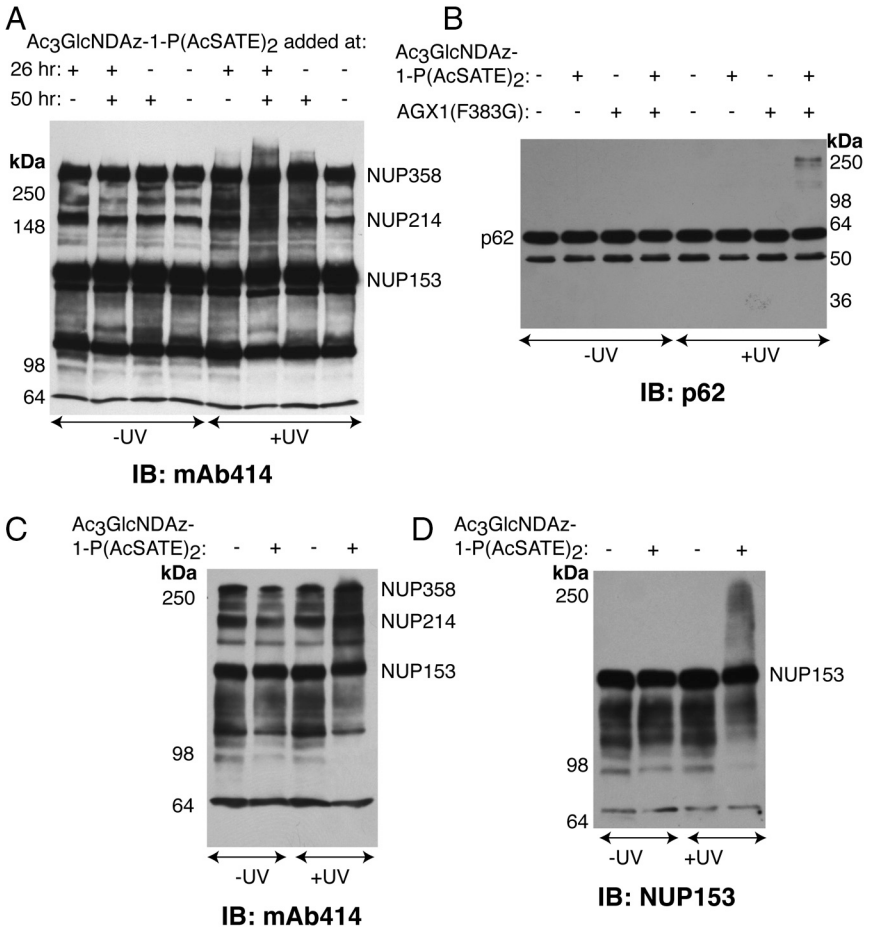

Fig. S5. Crosslinking of O-GlcNDAz-ylated proteins in intact cells. HeLa cells were transiently transfected with pCMV6-XL5-AGX1(F383G) and $Ac_3GlcNDAz\text{-}1\text{-}P(Ac\text{-}SATE)_2$ was added at the time(s) indicated (26 or 50 h after transfection). Cells were divided and half of each sample was irradiated with 365 nm light, prior to cell lysis. The lysates were analyzed by immunoblot using mAb414 (*A*). Normal HeLa cells or HeLa cells stably expressing AGX1(F383G) were treated twice with $Ac_3GlcNDAz\text{-}1\text{-}P(Ac\text{-}SATE)_2$ (at 0 and 24 h). Cells were divided and half of each sample was irradiated with 365 nm light, prior to cell lysis. The lysates were analyzed by immunoblot using an antibody recognizing NUP62 (*B*). T84 cells stably expressing AGX1(F383G) were cultured with $Ac_3GlcNDAz\text{-}1\text{-}P(Ac\text{-}SATE)_2$. The cells were divided and half of each sample was irradiated with 365 nm light, prior to cell lysis. The lysates were analyzed by immunoblot using mAb414 (*C*) or an antibody recognizing NUP153 (*D*).

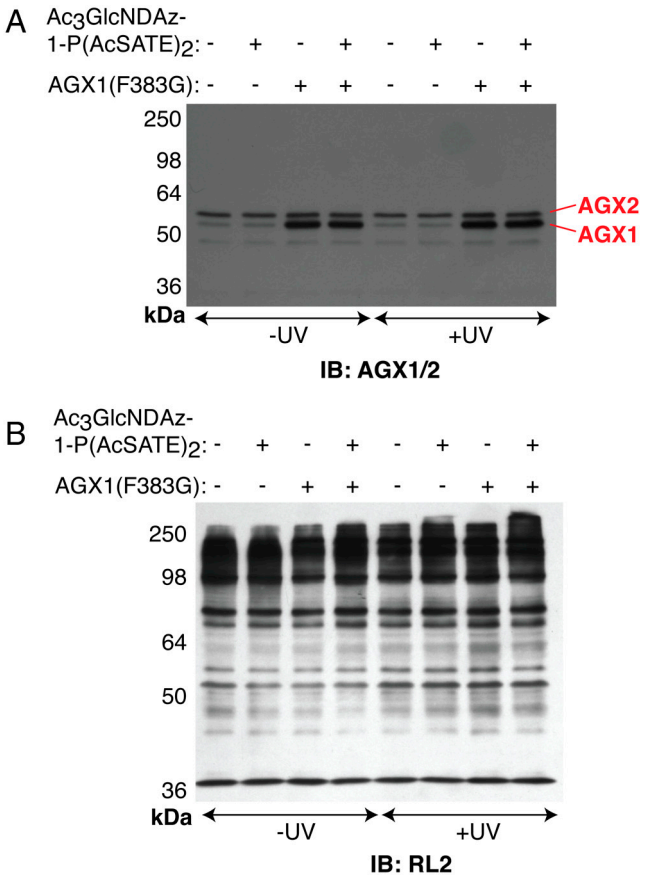

Fig. S6. Effects of AGX1(F383G) overexpression. Normal HeLa cells or HeLa cells stably expressing AGX1(F383G) were treated twice with Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ (at 0 and 24 h). Cells were divided and half of each sample was irradiated with 365 nm light, prior to cell lysis. Lysates were analyzed by immunoblot using an antibody recognizing AGX1 (*A*) or anti-O-GlcNAc antibody (RL2) (*B*). While AGX1 expression was dramatically increased (*A*), O-GlcNAcylation levels were relatively unaffected (*B*).

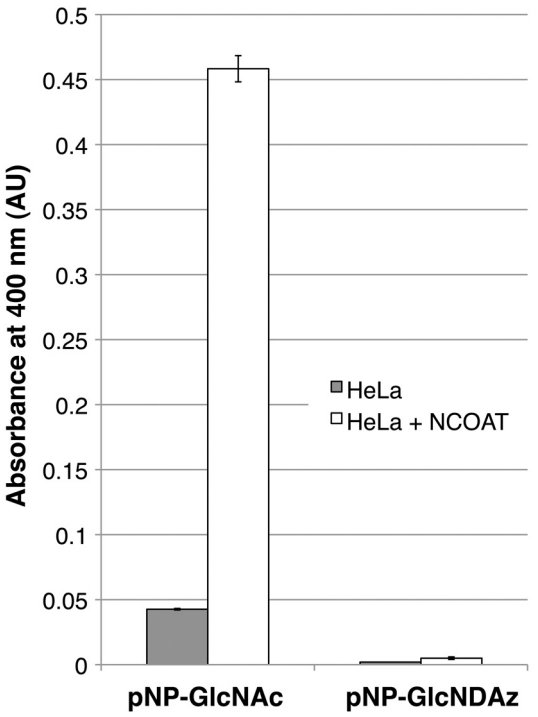

Fig. S7. Removal of O-GlcNDAz by NCOAT. Lysates from normal HeLa cells or HeLa cells overexpressing NCOAT were incubated with pNP-GlcNAc or pNP-GlcNDAz for 30 min at 37 °C. After quenching, the released amount of 4-nitrophenol was determined by UV absorption at 400 nm. Hydrolysis of pNP-GlcNAc occurred more rapidly than hydrolysis of pNP-GlcNDAz.

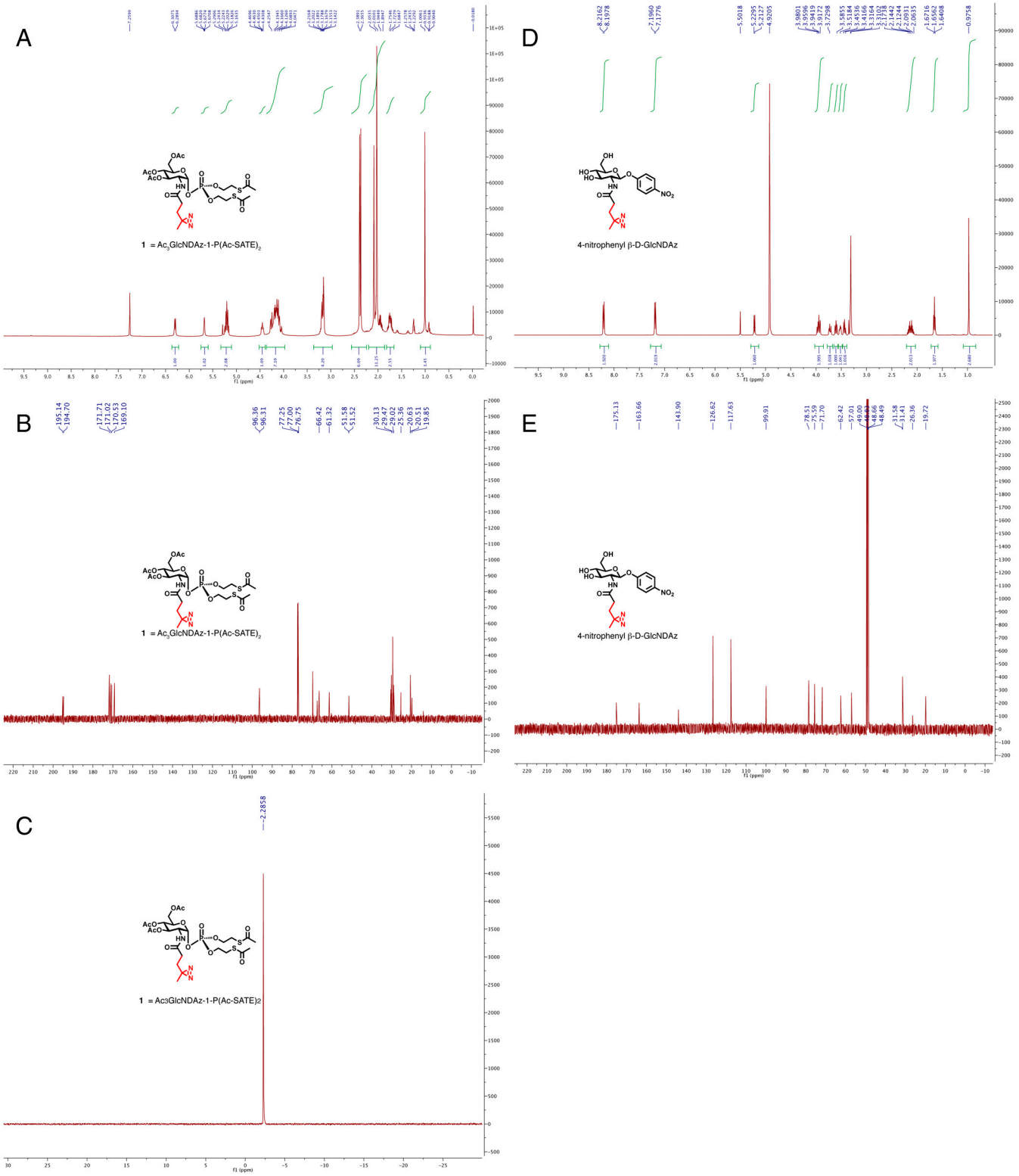

Fig. S8. NMR characterization of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ and pNP-GlcNDAz. (*A*) ^{1}H-NMR spectrum of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. (*B*) ^{13}C-NMR spectrum of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. (*C*) ^{31}P-NMR spectrum of Ac_3GlcNDAz-1-P(Ac-SATE)$_2$. (*D*) ^{1}H-NMR spectrum of pNP-GlcNDAz. (*E*) ^{13}C-NMR spectrum of pNP-GlcNDAz.

Table S1. Tryptic peptides identified by MS/MS analysis of gel slices from crosslinked mAb414 immunoprecipitate (Fig. 4*B*, lane 2)

protein name	GI#	MASCOT score	peptide sequence	ion score
Region 1 of gel				
RanBP2 (NUP358)	1009337	1108	YIASVQGSTPSPR	58
			SVELNPTQKDLVLK	30
			ELLQSFDSALQSVK	88
			SGQSALYDALFSSQSPK	55
			DTSFLGSDDIGNIDVR	52
			DTSFLGSDDIGNIDVR	45
			DTSFLGSDDIGNIDVREPELEDLTR	63
			LLVQHEINTLR	48
			IIDDSDSNLSVVK	63
			NVSGISFTENMGSSQQK	83
			APGTNVAMASNQAVR	81
			SDAGNLNFEFQVAK	60
			ELVGPPLAETVFTPK	72
			QNQTTSAVSTPASSETSK	44
			LHDSSGSQVGTGFK	59
			FGESTTGFNFSFK	57
			ERTDVIQGDDVADATSEVEVSSTSETTPK	35
			SNNSETSSVAQSGSESKVEPK	41
			SGEEDEEILFKER	80
			ITMELFSNIVPR	42
NUP214	33946327	336	SPGSTPTTPTSSQAPQK	43
			SAQGSSSPVPSMVQK	35
			TTLLEGFAGVEEAR	68
			ETLFNTLANNR	48
			LNHLVDSLQQLR	39
			QMASQAPAVNTLTESTLK	67
			NVPQVVNVQELK	40
exportin-1 (CRM1)	4507943	127	LLSEEVFDFSSGQITQVK	57
			EPEVLSTMAIIVNK	32
			EFAGEDTSDLFLEER	43
transportin-1 (TNPO1)	133925811	92	GDVEEDETIPDSEQDIRPR	37
			TLLENTAITIGR	55
Karyopherin β1 (importin subunit β1)	119615215	81	LAATNALLNSLEFTK	71
NUP153	31418202	52	IPSIVSSPLNSPLDR	54
Region 2 of gel				
RanBP2 (NUP358)	1009337	373	DVQESRELLQSFDSALQSVK	25
			ELLQSFDSALQSVK	79
			SLGGNDELSATFLEMK	22
			IIDDSDSNLSVVK	61
			ELVGPPLAETVFTPK	66
			FGESTTGFNFSFK	33
			SNNSETSSVAQSGSESKVEPK	27
			ITMELFSNIVPR	35
NUP214	119608367	192	SPGSTPTTPTSSQAPQK	25
			TTLLEGFAGVEEAR	53
			ETLFNTLANNR	55
			NVPQVVNVQELK	30
NUP153	31418202	72	IPSIVSSPLNSPLDR	67
transportin-1 (TNPO1)	133925811	70	GDVEEDETIPDSEQDIRPR	20
			TLLENTAITIGR	44
nuclear RNA export factor 1 (NXF1)	4406524	67	AQFFVEDASTASALK	62
exportin-1 (CRM1)	4507943	58	EPEVLSTMAIIVNK	20
			EFAGEDTSDLFLEER	35
gamma-catenin	29650759	57	ALMGSPQLVAAVVR	28
karyopherin β1 (importin subunit β1)	19923142	21	AAVENLPTFLVELSR	41
Region 3 of gel				
RanBP2 (NUP358)	1009337	412	ELLQSFDSALQSVK	71
			SGQSALYDALFSSQSPK	32
			DTSFLGSDDIGNIDVR	56
			ELVGPPLAETVFTPK	64
			LHDSSGSQVGTGFK	38
			FGESTTGFNFSFK	40
			ERTDVIQGDDVADATSEVEVSSTSETTPK	37
			SGEEDEEILFKER	30
NUP214	119608367	168	SAQGSSSPVPSMVQK	21
			TTLLEGFAGVEEAR	49
			LNHLVDSLQQLR	40
			QMASQAPAVNTLTESTLK	50

protein name	GI#	MASCOT score	peptide sequence	ion score
NUP153	31418202	72	IPSIVSSPLNSPLDR	56
			FVASKPLEEEEMEVPVLPK	20
			SSFNLGTIETK	37
			NVFSSSGTSFSGR	56
Region 4 of gel				
RanBP2 (NUP358)	1009337	800	ELLQSFDSALQSVK	55
			DTSFLGSDDIGNIDVR	42
			DTSFLGSDDIGNIDVREPELEDLTR	67
			LLVQHEINTLR	51
			TGSGLNSFYDQR	29
			IIDDSDSNLSVVK	74
			APGTNVAMASNQAVR	77
			SDAGNLNFEFQVAK	42
			ELVGPPLAETVFTPK	78
			GVIFGQTSSTFTFADLAK	41
			FGESTTGFNFSFK	67
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			TDVIQGDDVADATSEVEVSSTSETTPK	21
			ITMELFSNIVPR	58
			ITMELFSNIVPRTAENFR	41
NUP153	31418202	158	IPSIVSSPLNSPLDR	61
			SSFNLGTIETK	43
			NVFSSSGTSFSGR	61
NUP214	119608369	111	QMASQAPAVNTLTESTLK	22
			NVPQVVNVQELK	42
histone H2B	1568551	51	AMGIMNSFVNDIFER	54
myomesin 1	119622088	51	IIPSR	26