Supplementary Information

Versatile *O*-GlcNAc Transferase Assay for High-throughput Identification of Enzyme Variants, Substrates, and Inhibitors

Eun J. Kim[†], Lara K. Abramowitz[§], Michelle R. Bond[§], Dona C. Love[§], Dong W. Kang[□], Hans F. Leucke[¤], Dae W. Kang[‡], Jong-Seog Ahn[¶], John A. Hanover[§,*]

[†]Department of Science Education-Chemistry Major, Daegu University, Gyeongbuk 712-714, S. Korea

[§]Laboratory of Cell Biochemistry and Biology, NIDDK, National Institute of Health, Bethesda, MD 20892 U.S.A.

[□]Department of Pharmaceutical Science and Technology, Catholic University of Daegu, GyeongBuk, 712-702. S. Korea

[¤]Laboratory of Bioorganic Chemistry, NIDDK, National Institute of Health, Bethesda, MD 20892 U.S.A

[‡]Department of Biochemistry and Health Science, College of Natural Sciences, Changwon National University, Changwon 641-773, S. Korea

[¶]Chemical Biology Research Center, Bio-Therapeutics Research Institutes, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon 363-883, S. Korea

Corresponding Author

John A. Hanover

Mailing Address: Laboratory of Cell Biochemistry and Biology, NIDDK
National Institute of Health
8 Center Dr.
Building 8, Bl-27 Bethesda, MD 20892 U.S.A.
Email Address: jah@helix.nih.gov
Telephone Number: 301-496-0943 / Fax Number301-496-9431

EXPERIMENTAL PROCEDURES

Materials. All solutions were prepared using ultrapure deionized water. TAMRA-Alkyne with copper (II)-salt and reducing agent are contained in the Click-iT™ Protein Analysis Detection Kits (Invitrogen). PBS and rabbit anti-TAMRA antibody were purchased from Invitrogen (Carlsbad, CA, USA). Complete, mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN, USA). His-Trap Kit was purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). IRDye®800CW-conjugated goat anti-rabbit antibody and IRDye®800CW-Streptavidin were purchased from LI-COR Biosciences (Lincoln, NE, USA). 6×His oligopeptide, 6×His-tagged recombinant human CKII-α protein, anti *O*-linked-N-acetylglucosamine transferase (anti-OGT) antibody, and RL2 antibody were purchased from Abcam (Cambridge, MA, USA). 6×His-tagged recombinant human nuclear pore glycoprotein, p62 (Nup62) was purchased from Bioclone (San Diego, CA, USA). Rabbit reticulocyte lysates, nuclease treated were purchased from Promega (Madison, WI, USA). Ni-NTA Strips and plates with a binding capacity of 10 pmol/well for proteins were purchased from Qiagen®Sciences (Louisville, KY, USA). Plates and strips were imaged and quantified according to the manufacturer's instructions using the Odyssey Infrared Imaging System (LI-COR Biosciences). IC_{50} values were calculated using SigmaPlot 12.5 software employing "Four parameter logistic equation in the standard curve analysis."

Preparation of crude lysate containing bacterially expressed ncOGT and partially purified ncOGT. pET43.1 Ek/LIC/ncOGT was transformed into competent BL21(DE3) cells (Novagen) according to the manufacturer's instruction and cells were grown overnight on a LB Agar plate with 50 μg/mL Ampicillin (LB Agar/Amp plate) at 37°C. A single colony was inoculated in a 20 mL culture of LB broth supplemented with Ampicillin (50 μg/mL), and cultured overnight at room temperature. Cultured cells were centrifuged at 2500 × g at 4 °C for 10 min, and the supernatant was removed. The cell pellets were resuspended in 0.99 mL of OGT lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mg/mL of lysozyme, and complete mini EDTA-free protease inhibitor cocktail, and incubated at room temperature for 5 min to perform the lysozyme digestion. Then 10 μL of 10 % Triton-X100 was added to give 0.1 % final concentration of Triton-X100, and the lysate was sonicated for 15 sec on ice with 30 sec

pause in between each until DNA was completely sheared, followed by centrifugation at 14,000 × g at 4 °C for 10 min. The resulting supernatant was distributed into ~200 µL aliquots and stored at −80°C until used (40 mg/mL). Partial purification of ncOGT was performed using a His-Trap FF kit according to the manufacturer's instruction in the cold room (+4 °C). Briefly, a buffer containing 1× Phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) and 20 mM imidazole was used as binding and wash buffer. Elution was performed with 10 mL of 1× phosphate buffer containing 300 mM imidazole at a flow rate of 1 mL/min and the eluate was collected in five 1-mL fractions. The fractions were checked by measuring the absorbance of eluate at 280 nm (A_{280} nm) on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). The fractions containing protein were pooled and buffer exchanged into 100 µL of OGT assay buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 12.5 mM $MgCl_2$. Partially purified ncOGT was obtained at a concentration of 0.64 mg/mL.

Evaluation of blocking agents. 200 μL of PBS containing either 0 or 0.2 μg of 6×His-tagged Nup62 was added to the wells of a Ni-NTA strip and incubated at room temperature for 1h. Unbound Nup62 was removed by washing twice with 200 μL of PBS for 1 min. 200 μL of Odyssey blocking buffer (LI-COR Bioscience) was added to well 3, 1 μg of 6×His peptide (Abcam) in PBS to well 4, 5% BSA PBST to well 5 and 5% milk in PBST to well 6. 200 μL of PBS was added to wells 1 and 2. The strip was incubated at room temperature for 50 min. After washing the wells with 200 μL of PBST (PBS containing 0.1% Tween 20) twice, 50 μL of OGT assay buffer containing 0.64 μg of partially purified ncOGT was added to all wells and incubated at 37 °C while shaking at 100 rpm for 1h. After removing all solutions, each well was washed 4 times with 200 μL of PBST. For detection of bound ncOGT, each well was incubated with 200 μL of PBST and anti-OGT antibody (1:800 dilution) at room temperature for 1h and washed 4 times with 200 μL of PBST, followed by incubation with 200 μL of PBST and IRDye®800CW-conjugated goat anti-rabbit antibody (1:10000 dilution). Finally, each well was washed 3 times with 200 μL of PBST, filled with 200 μL of PBS and imaged on an Odyssey® Infrared Imaging System at 800 nm.

Ni-NTA Plate OGT Assay. 200 μL of PBS containing 6×His-tagged Nup62 or Casein Kinase II substrate was placed in wells as indicated in figure legends, and incubated at room temperature for 1h. After removing unbound 6×His-tagged substrate, each well was washed with

200 μL of PBS one time and blocked with 200 μL of Odyssey blocking buffer at room temperature for 1h. Subsequently, the OGT enzymatic reaction was performed using either crude lysate containing bacterially expressed human ncOGT (5 μL, 40 mg/mL), 1 μL of partially purified ncOGT (0.64 μg), or 8 μL of rabbit reticulocyte lysates, UDP-GlcNAz (40 μM) and OGT assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 12.5 mM $MgCl_2$) to a final volume of 50 μL. The plate was incubated at 37 °C and shaken at 100 rpm for 90 min. This reaction was then followed by either (1) a 'click' reaction with a TAMRA alkyne, (2) a Staudinger-ligation using Biotin-Phosphine or (3) an immunoassay probing for the incorporated GlcNAz, as indicated in figure legends.

(1) The enzymatic solutions were removed and the wells were washed 3 times with PBST. Then, the azido-labeling reaction was performed using copper-catalyzed 'click' reaction with TAMRA-Alkyne (**2**, Fig. 1). For copper catalyzed 'click' reaction with TAMRA-Alkyne (**2**, Fig. 1), all wells were sequentially treated with 31 μL of distilled water, 5 μL of 0.2% SDS, 50 mM Tris-HCl, pH 8.0, 10 μL of 2× Click-iT reaction buffer, 1 μL of $CuSO_4$ solution and 1 μL of Click-iT reaction buffer-additive 1. After 2 – 3 min incubation, 2 μL of Click-iT reaction buffer-additive 2 was added to the wells and the plate was covered with foil and incubated at room temperature for 20 min. The reactions were quenched by removing the reaction mixture and washing the wells 4 times with PBST. For detection of TAMRA-conjugated, bound Nup62, each well was incubated with 200 μL of blocking buffer containing 0.1% Tween 20 and rabbit anti-TAMRA antibody (1:1000 dilution) at room temperature for 1h, and washed 4 times with PBST, followed by incubation with 200 μL of blocking buffer containing 0.1% Tween 20 and IRDye®800CW-conjugated goat anti-rabbit antibody (1:10000 dilution) for 1h. Finally, each well was washed 4 times with PBST, filled with 200 μL of PBS, and imaged on an Odyssey® Infrared Imaging System at 800 nm.

(2) Azido-labeling reactions were performed using Staudinger ligation with Biotin-Phosphine reagent (**3**, Fig. 1) in a total volume of 100 μL. For Staudinger ligation reactions, 1 μL of 20 mM Biotin-Phosphine (**3**, Fig. 1) solution (200 μM) was added to all wells containing 99 μL PBS, and incubated at 40 °C while shaking at 100 rpm for 1h. Reactions were quenched by removing the reaction mixture and washing the wells 4 times with PBST. For detection of biotinylated, bound Nup62, each well was incubated with 200 μL of blocking buffer containing

0.1% Tween 20 and IRDye®800CW-Streptavidin (1:2000 dilution) at room temperature for 1h. Finally, each well was washed 4 times with PBST, filled with 200 μL of PBS, and imaged on an Odyssey® Infrared Imaging System at 800 nm.

(3) For detection of GlcNAz-incorporated Nup62, each well was incubated with 200 μL of blocking buffer containing 0.1% Tween 20 and RL2 antibody (1:800 dilution) at 4 °C overnight, and washed 4 times with PBST, followed by incubation with 200 μL of blocking buffer containing 0.1% Tween 20 and IRDye®800CW-conjugated goat anti-mouse antibody (1:10000 dilution) for 1h. Finally, each well was washed 4 times with PBST, filled with 200 μL of PBS, and imaged on an Odyssey® Infrared Imaging System at 800 nm.

Determination of IC_{50} values of compound 4 and 5 against human ncOGT (Fig. 5). For the inhibition assays of OGT activity, 20mM OGT inhibitors **4** and **5** were prepared in DMSO and serially diluted to give predetermined inhibitor concentrations. For compound **4**, 0.5, 1.0, 2.5, 5.0, 10, and 20 mM solutions were prepared. For compound **5**, 0.125, 0.25, 0.5, 1.0, 2.5, and 5.0 mM solutions were prepared. 200 μL of PBS containing 0.15 μg of 6×His-tagged Nup62 were placed in all wells and incubated at room temperature for 1h. After removing unbound 6×His-tagged Nup62, each well was washed with 200 μL of PBS one time and blocked with 200 μL of Odyssey blocking buffer at room temperature for 1h. Subsequently, OGT inhibition assays were performed in the absence and presence of the indicated amounts of inhibitors using 1.4 μL of crude lysate containing bacterially expressed human ncOGT, UDP-GlcNAz (40 μM) and OGT assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 12.5 mM $MgCl_2$) to a final volume of 50 μL. The plate was incubated at 37 °C and shaken at 100 rpm for 25 min. Following procedures were the same as those described above and azido-labeling reaction was performed using a Staudinger ligation using the Biotin-Phosphine reagent (**3**, Fig. 1). Inhibition assays were performed in triplicate. IC_{50} values were calculated using SigmaPlot 12.5 software employing "Four parameter logistic equation in the standard curve analysis."

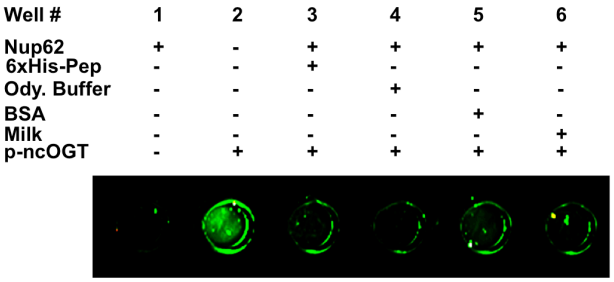

Figure S1. Evaluation of blocking agents. 6xHis-tagged Nup62 substrate was added to each well as indicated. 6xHis-peptide (6x-His-Pep), Odyssey blocking buffer (Ody. Buffer), 5% BSA (BSA), or 5% milk (milk) was used to block the Ni-NTA coated well (as indicated). Subsequently partially purified ncOGT (p-ncOGT) which is also 6XHis-tagged was incubated (as indicated). ncOGT binding to the Ni-NTA coated plate was determined using an anti-OGT antibody.

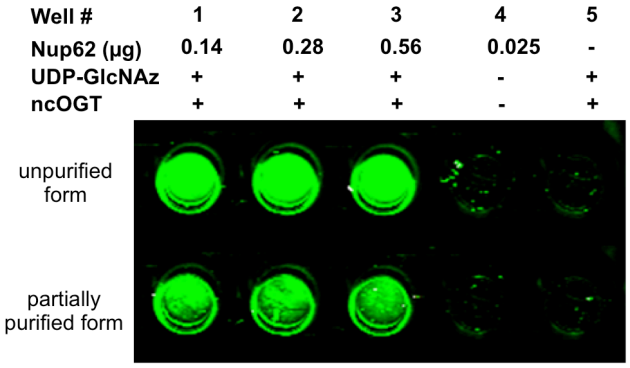

Figure S2. Determination of incorporated GlcNAz. Various amounts of the 6×His-tagged Nup62 substrate (as indicated) was used to detect activity of either unpurified or partially purified ncOGT as indicated. GlcNAz-incorporated Nup62 was detected using the RL2 antibody.

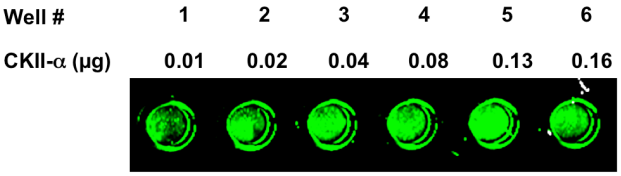

Figure S3. 6×His-tagged CKII-α as a substrate in the Ni-NTA Plate OGT Assay. Increasing amounts of the 6×His-tagged CKII-α substrate were used as indicated. Glycosyltransferase activity was detected using copper-catalyzed 'click' reaction with TAMRA-Alkyne.

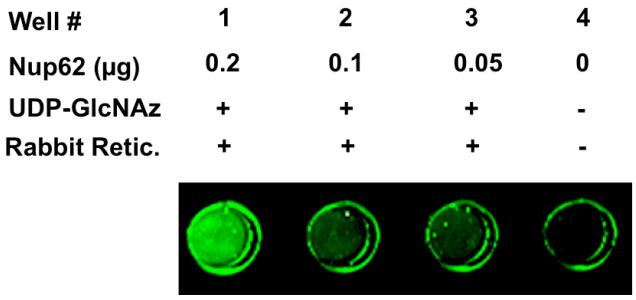

Figure S4. Assay of endogenous OGT activity in eukaryotic cell lysates. 8 μL of Rabbit reticulocyte lysates (Rab. Retic.) were used as the source of OGT enzyme with amounts of Nup62 (as indicated). Glycosyltransferase activity was detected using Staudinger ligation with Biotin-Phosphine reagent.

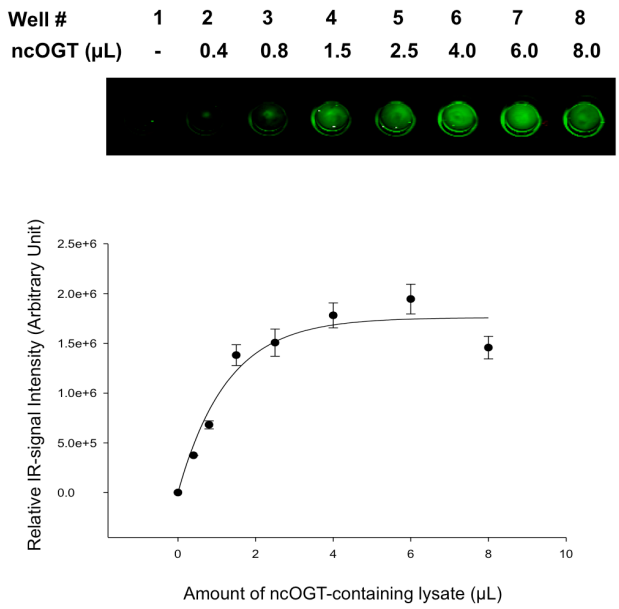

Figure S5. Amount of ncOGT-containing lysate (40 mg/mL) needed for linear enzyme activity for Nup62 substrate. Increasing amounts of ncOGT-containing lysates were used for the Ni-NTA Plate OGT Assay to measure OGT activity for the 6×Hig-tagged Nup62 substrate. Glycosyltransferase activity was detected using Staudinger-ligation with Biotin-Phosphine reagent. Experiments were performed in triplicate and quantified using the Odyssey® Infrared Imaging System at 800 nm. Error bars represent standard deviations (n=3).