Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Native detection of protein *O*-GlcNAcylation by gel electrophoresis

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Supplementary Note 1

Materials and reagents

All conventional reagents and UDP-GlcNAc were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Methanol (MeOH) and bovine serum albumin fraction V (BSA) were purchased from VWR chemicals (Hunter Boulevard, LE, UK). Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Thermo Fisher Scientific. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%) was purchased from Apollo Scientific Ltd. (Bredbury, Cheshire, UK). N,N'-Bis(acryloyl)cystamine (CAS: 60984-57-8) was purchased from Alfa Aesar (Heysham, Lancashire, UK). Amersham Protran^{TM} 0.2 µm nitrocellulose membrane was purchased from GE Healthcare. Acrylamide/Bis-acrylamide (29:1, 40% solution, used in PAGE and SOPAGE) was purchased from Geneflow Ltd. (Lichfield, Staffordshire, UK). Complete protease inhibitor cocktail tablet and phosphatase inhibitor cocktail tablet were purchased from Roche Diagnostics (Mannheim, Germany). PageRuler pre-stained protein ladder (Cat. No. 26616) was purchased from Thermo Fisher. All media used in this study for *E. coli* culture were prepared by Central Technical Services facility in the School of Life Sciences, University of Dundee.

TALON® Resin was purchased from Clontech (USA). GST resin, HiTrap Q HP 5 mL anion exchange column, HiLoad 26/600 Superdex 200 column and disposable Sephadex G-25 column (NAP-5) were purchased from GE Healthcare. Amicon ultra centrifugal filter units (0.5 mL, 4 mL or 15 mL, MWCO 3500 used in the Acryl-*Cp*OGA^{D298N} preparation and MWCO 10000 used in protein purification) were purchased from Millipore.

Plasmids encoding His-TAB1, wild-type GST-*Cp*OGA, GST-*Cp*OGA^{D298N}, hOGT (Suzanne Walker's construct), full-length ncOGT and PreScission protease were either constructed with conventional double-digestion and ligation method or maintained in our lab. All plasmids used in this study were sequenced by sequencing facility at University of Dundee to verify the correct DNA sequence. DH5α strain, BL21 strain, and BL21 (DE3) pLys strain were maintained in our lab and used to generate "in-house" high-efficiency competent cells.

The goat anti-TAB1 pAb and rabbit anti-O-GlcNAc gTAB1 (Ser^{395}) pAb were generated by Division of Signal Transduction Therapy (DSTT) at University of Dundee and used as described previously. Mouse RL2 mAb and all HRP-conjugated secondary Abs were purchased from Thermal Scientific. Rabbit anti-OGT pAb (DM-17) was purchased from Sigma-Aldrich. IRDye800 and IRDye680 labeled secondary Abs were purchased from LI-COR.

Equipment

E. coli for recombinant protein production was lysed using Sonifier (Sonics &

Materials, NewTown, CT). Anion-exchange chromatography (AIEX) and size-exclusion chromatography (SEC) were carried out on ÄKTA PURE and PRIME system (GE Healthcare). Coomassie blue-stained gels were imaged with EPSON PERFECTION V750 scanner. SDS-PAGE, SOPAGE and subsequent Western blotting were performed using Bio-Rad Mini-PROTEAN® Tetra electrophoresis system and the Mini Trans-Blot system. Blotted membranes were either documented using LI-COR Odyssey Imager or visualized using X-ray film and ECL substrate, according to the secondary Abs used.

Supplementary Note 2

All recombinant proteins used in this study were expressed and purified according to previous literatures with slight modifications.[1–5] The procedures are detailed as following.

Expression and Purification of His_6-tagged hOGT

pHEX plasmid encoding His_6-tagged hOGT (312-1032) gene (susan walker construct) was transformed into *E. coli* BL21 strain. A single clone was grown in 10 ml of LB media supplemented with Ampicillin (50 μg/mL) overnight at 37°C. 1 L of auto-induction media was inoculated with 5 ml of overnight cultures and grown at 37°C, 200 rpm until the OD_{600} reached 0.8, followed by inducing at 18°C, 200 rpm for 24 h. Cells were harvested by centrifugation (8000g, 30 min, 4°C). The cell pellet was resuspended in Lysis Buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 10% glycerol, 2 mM β-Me, 1 mM PMSF and disrupted by sonication. After clarifying by centrifugation at 4°C, 48000 g for 15 min, the supernatant was aspirated for protein purification. Protein was purified with Talon resin using standard His-tag purification protocol according to manufacturer's recommendation. All fractions containing target protein were combined and diluted ten-fold in volume with 25 mM Tris (pH 7.5) to reduce ionic strength and then loaded to HiTrap-Q column. After a 30 CV linear gradient elution from 50 mM to 500 mM NaCl in 25 mM Tris (pH 8.0) buffer, fractions containing hOGT were pooled and concentrated with Amicon concentrator (MW 10 kDa) and loaded to Superdex G-200 column equilibrated with 1×TBS (pH 7.5, with 0.5 mM TCEP). All the purified proteins in this study were supplied with 25% (v/v) glycerol (final concentration), aliquoted, analyzed by SDS-PAGE (Fig. S1) and stored at -80°C for further use. Protein concentration was determined by the Bradford method.

Expression and Purification of GST-TAB1 and tag-free TAB1

pGEX 6p-1 plasmid encoding GST-TAB1 (7-402) (referred as GST-TAB1) was transformed to *E. coli* BL21(DE3) pLys strain. The transformation, cell culture, and cell disruption were the same as that for hOGT. Lysate supernatant was then loaded onto GST-resin. The column was washed with 10 CV of Lysis Buffer, 10 CV of 1×TBS (pH 7.5), followed by elution with 10 CV of 10 mM reduced glutathione dissolved in 1×TBS (pH 7.5). Fractions were concentrated with Amicon concentrator (MW 10 kDa) and subjected to AIEX and SEC purification as described above. Tag-free TAB1 (referred as TAB1) was prepared from GST-TAB1 cell lysate through on-beads cleavage. Briefly, GST-resin was firstly loaded with GST-TAB1 lysate and thoroughly washed with 10 CV of Lysis Buffer and 5-10 CV of Cleavage Buffer containing 50 mM Tris (pH=7.0), 150 mM NaCl, 1 mM EDTA and 1 mM DTT. Each 1 ml of GST resin was re-suspended with 10 ml Cleavage Buffer, followed by adding 10 μL of home-made Prescission Protease (10 mg/ml). The column was plugged at both ends and gently rotated at 4°C. After 16 h, 10 μL each of supernatant and GST resin were analyzed by SDS-PAGE. The supernatant was subjected to next-step purification when >90% of GST-tag was cleaved. If any

uncleaved precursor was detectable, the supernatant can be subjected to another round of GST reverse pull-down with 2 ml of fresh GST resin in prior to next step. The procedures of AIEX and SEC purification were the same as above.

Expression and Purification of GST-*Cp*OGA, GST-*Cp*OGA^{D298N}, *Cp*OGA, and *Cp*OGA^{D298N}

Plasmids encoding wild-type GST-*Cp*OGA (31-618) and inactive GST-*Cp*OGA^{D298N} (31-618) was transformed to *E. coli* BL21(DE3) pLys strain. The transformation, cell culture, cell disruption and protein purification were the same as those for GST-TAB1. Tag-free *Cp*OGA and *Cp*OGA^{D298N} were prepared from GST-*Cp*OGA and GST-*Cp*OGA^{D298N}, respectively, by using the same process for tag-free TAB1.

DNA Sequence of GST-*Cp*OGA ^{D298N}:

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATC
TTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTG
AATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCC
ATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCA
ATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAA
CTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAA
AACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTAT
ACATGGACCCAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCC
ACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTT
TGGTGGTGGCGACCATCCTCCAAAATCGGATCTGGAAGTTCTGTTCCAGGGGCCCCTGGGATCCGTAG
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AGAAAATTTAAAAGAAGAGGGATATGCTTTAGTTTCAAATGATGGGAAAATAGCTATTGAGGGTAAAGAT
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TCAAGATAGATTAGATCAAATTAAATTTTATGGTGAAAATAAATTAAACACATATATTTATGCTCCTAAAGA
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TATAAATGCTTCTGCTGAAAATAAGGTTGATTTTGTTTTCGGTATTTCTCCAGGAATAGATATAAGATTTG
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AACTTGCCTTAGGACCAATGCATGGATTAGATAAAGGATTAAATCAATATGTAGATTTCTTTACTGTAAAC
CCAATGGAGCATGCTGAGCTTTCAAAAATATCAATACACACAGCTGCTGACTATAGCTGGAATATGGAT

AACTATGATTATGATAAGGCTTGGAATAGAGCAATTGATATGCTTTATGGTGATTTAGCAGAAGATATGA
AGGTATTTGCAAACCATTCAACAAGAATGGACAATAAAACTTGGGCTAAATCAGGAAGAGAAGACGCTC
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TAGATATGATTGTAGCTCAATTAAATGAAGATACAGAGGCTTATGAAAGTGCTAAAGAAATTGCACAAAA
TAAATTAAATACAGCCTTAAGTAGCTTTGCAGTTATATCTGAAAAGGTTGCTCAATCATTTATACAAGAAG
CTTTAAGTTGA

Protein sequence of GST-*Cp*OGA^{D298N} (Theoretical pI/Mw: 4.62 / 92750.40):

The Precession Protease cleavage site is underlined.

M S P I L G Y W K I K G L V Q P T R L L L E Y L E E K Y E E H L Y E R D E G D K W R N K K F E L G L E
F P N L P Y Y I D G D V K L T Q S M A I I R Y I A D K H N M L G G C P K E R A E I S M L E G A V L D I R
Y G V S R I A Y S K D F E T L K V D F L S K L P E M L K M F E D R L C H K T Y L N G D H V T H P D
F M L Y D A L D V V L Y M D P M C L D A F P K L V C F K K R I E A I P Q I D K Y L K S S K Y I A W P L Q
G W Q A T F G G G D H P P K S D L E V L F Q G P L G S V G P K T G E E N Q V L V P N L N P T P E N L
E V V G D G F K I T S S I N L V G E E E A D E N A V N A L R E F L T A N N I E I N S E N D P N S T T L I I
G E V D D D I P E L D E A L N G T T A E N L K E E G Y A L V S N D G K I A I E G K D G D G T F Y G V Q
T F K Q L V K E S N I P E V N I T D Y P T V S A R G I V E G F Y G T P W T H Q D R L D Q I K F Y G E N
K L N T Y I Y A P K D D P Y H R E K W R E P Y P E S E M Q R M Q E L I N A S A E N K V D F V F G I S P
G I D I R F D G D A G E E D F N H L I T K A E S L Y D M G V R S F A I Y W D N I Q D K S A A K H A Q V
L N R F N E E F V K A K G D V K P L I T V P T E Y D T G A M V S N G Q P R A Y T R I F A E T V D P S I
E V M W T G P G V V T N E I P L S D A Q L I S G I Y D R N M A V W W N Y P V T D Y F K G K L A L G P
M H G L D K G L N Q Y V D F F T V N P M E H A E L S K I S I H T A A D Y S W N M D N Y D Y D K A W N
R A I D M L Y G D L A E D M K V F A N H S T R M D N K T W A K S G R E D A P E L R A K M D E L W N
K L S S K E D A S A L I E E L Y G E F A R M E E A C N N L K A N L P E V A L E E C S R Q L D E L I T L A
Q G D K A S L D M I V A Q L N E D T E A Y E S A K E I A Q N K L N T A L S S F A V I S E K V A Q S F I Q
E A L S Stop

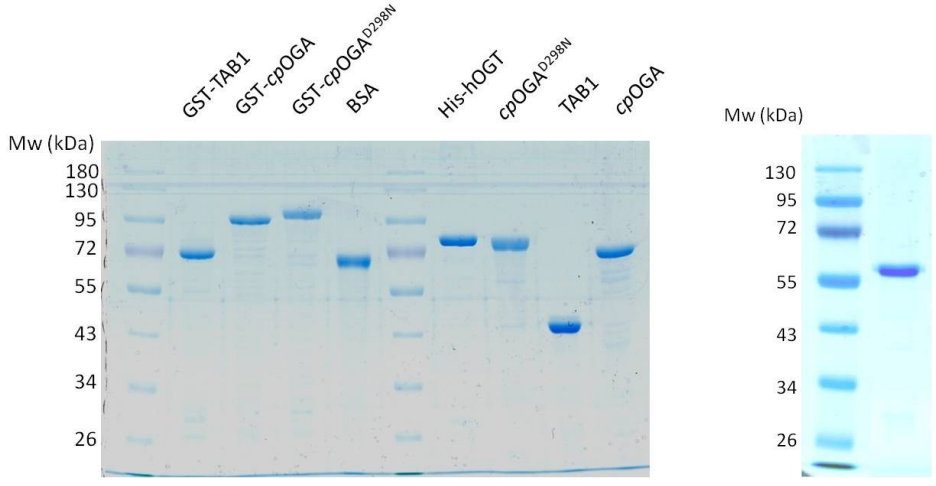

Fig. S1. SDS-PAGE analysis of purified GST-TAB1, GST-*Cp*OGA, GST-OGA^{D298N}, His-hOGT, *Cp*OGA^{D298N}, TAB1, *Cp*OGA and GST-HCF1-rep1-His.

Expression and Purification of ncOGT

Full-length human OGT (ncOGT) was expressed and purified from *E. coli* as a N-terminal His fusion protein as described previously,[6] concentrated to 10 mg/ml, mixed with equal volume of 50% glycerol prior to snap-freezing with liquid nitrogen and stored at -80 ˚C until use.

Expression and Purification of HCF-1-rep1 (867-1071)

GST-HCF1-rep1-His was encoded in pGEXmangled plasmid, and was expressed and purified as described previously.[1] Briefly, it was purified using GST-resin, Talon-resin, AIEX, and SEX procedures described above in order. Then, purified GST-HCF1-rep1-His was mixed with equal volume of 50% glycerol prior to snap-freezing with liquid nitrogen and stored at -80˚C until use.

Supplementary Note 3

Synthesis of AI-OGA

6 mg of GST-*Cp*OGA^{D298N} (20.5 mg/mL stock, in TBS buffer with 25% (v/v) glycerol) was diluted to 300 µL with 1×PBS (pH 7.4) and was passed through NAP-5 column equilibrated with 1×PBS (pH 7.4). The residual Tris was further removed by dialysis against 1×PBS (pH 7.4) at 4°C overnight, and the volume was adjusted to 3 ml with 1×PBS (pH 7.4) to bring the final protein concentration to 2 mg/ml. Subsequently, 28 µL of fresh sulfo-SMCC (10 mg/mL in water) was slowly added to GST-*Cp*OGA^{D298N} (64.7 nmol), and the mixture was incubated at r.t. for 30 min to yield maleimide-GST-*Cp*OGA^{D298N}. The volume of reaction mixture was reduced to < 0.5 ml with Amicon Concentrator (MWCO 3 kDa). Excessive and un-reacted sulfo-SMCC was further removed with NAP-5 column equilibrated with 1×MES (100 mM MES, 150 mM NaCl, pH 6.2). Meanwhile, 2.6 mg of acryloyl-cysteamine was dissolved in 1 ml of 1×MES buffer and reduced with 24 µL of 0.5 M TCEP at r.t. for 10 min. 350 µL of reduced acryloyl-cysteamine was then added to 1 ml of maleimide-GST-*Cp*OGA^{D298N}, and the reaction mixture was kept at 4°C for 2 h on a rotator. The resultant AI-OGA was dialyzed against 1×PBS, concentrated, aliquoted and stored at -20°C for future use. If undesired high molecular weight conjugates were observed by SDS-PAGE, SEC was performed to get rid of these inter-molecular conjugates. The concentration of yielded AI-OGA was 6.95 mg/mL (75 µM) measured by Bradford assay using BSA as a reference.

Function-based characterization of AI-OGA

The installation of acrylamide groups onto AI-OGA was characterized by function-based assays. As shown in Fig. S2, we cast a double-layer native PAGE gel that contains AI-OGA as an additive only in the top layer. After 2 h electrophoresis at 180 V, the gel was inspected with Coomassie Blue staining.

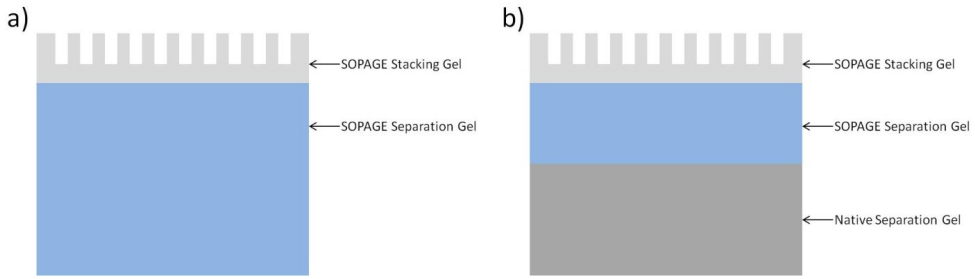

Fig. S2. Schematics of regular (a) and 2-layer SOPAGE gel (b).

Gel-shift assay to verify the affinity of AI-OGA for *O*-GlcNAc

The *O*-GlcNAc binding ability of tag-free *Cp*OGA^{D298N},GST-*Cp*OGA^{D298N} and AI-OGA under electrophoresis was evaluated by detecting the formed complex with *O*-GlcNAc TAB1. In general, 15 pmol each of *Cp*OGA^{D298N},GST-*Cp*OGA^{D298N}, and AI-OGA was mixed with 10 pmol TAB1 or gTAB1 in PBS, and the mixture was incubated at 4°C

for 1 h. Thereafter, the formed protein-protein complex was analyzed by Native-PAGE followed by Coomassie Blue staining. It is worth noting that, only GST-tagged but not the tag-free *Cp*OGA^{D298N} can form the stable complex with gTAB1. Therefore, the GST-tag was retained and GST-*Cp*OGA^{D298N} was used to prepare AI-OGA.

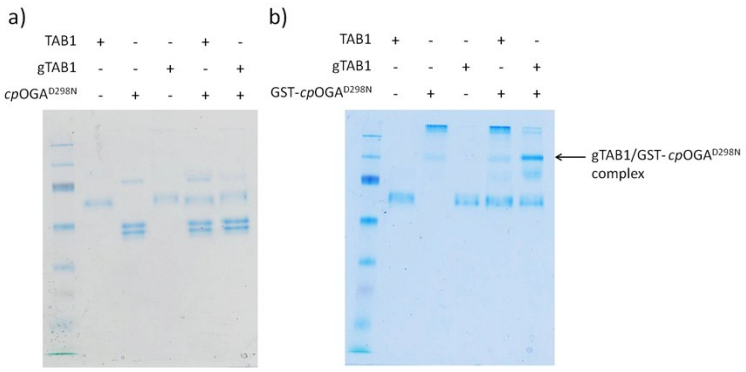

Fig. S3. Gel shift assay of a) tag-free *Cp*OGA^{D298N} and b) GST-*Cp*OGA^{D298N} in the presence of TAB1 or gTAB1. The stable complex survived under electrophoresis was visualized by Coomassie Blue staining and indicated with arrow.

Mapping acryl-modification sites of AI-OGT by MS

The original GST-*Cp*OGA^{D298N} reactant and yielded acryl-GST-*Cp*OGA^{D298N} (AI-OGA) were subjected to MS-based site-mapping. Briefly, the proteins were reduced with 17 mM DTT at 50°C for 15 min and alkylated with 35 mM IAA at r.t. for 30 min. Then, the proteins were digested with trypsin for 16 h and analyzed by nanoLC-MS. The raw data were analyzed with Maxquant software (version 1.6.1.0). Cystine carbamidomethyl was set as a fixed modification. Met Oxidation, N-term acetylation, N/Q deamidation, maleimide adduct (+219.0895 Da), and maleimide+acryl adduct (+ 350.1300 Da) were set as variable modifications. The maximum miss cleavage was set to 2. MS tolerance for MS^2 was set to 4.5 ppm.

As a result, only maleimide+Acryl adduct was identified in AI-OGA, and the maleimide adduct as a reaction intermediate was undetectable. This indicated the completion of the 2-step derivation we used. Thereafter, the structure of AI-OGA was modeled with PHYRE^2 software,[7] and all acrylamide modification sites were labelled on the model. Notably, Lys^{113} has an extremely high mod/base ratio (>40) than other sites. All details regarding MS analysis were summarized in the supplementary Excel file.

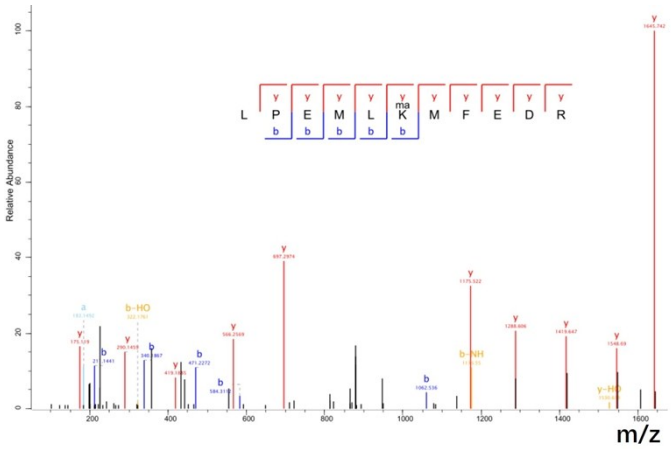

Fig. S4. Representative MS^2 spectrum of AI-OGA acrylamide site-mapping. The modification residue here is Lys^{113}.

Supplementary Note 4

Buffer recipes:

5×OGT Reaction Buffer:

125 mM Tris (pH 7.5), 62.5 mM $MgCl_2$, 0.3 mg/mL BSA and 1 mM DTT

5×OGA Reaction Buffer:

250 mM Citric Acid (pH 5.5), 625 mM Na_2HPO_4, 0.5 mg/mL BSA

Determining the dynamics and kinetics of OGT by SOPAGE

In vitro OGT reaction was carried out in 20 μL of 1×OGT Reaction Buffer containing 0.45 μM of hOGT (or ncOGT), 150 μM UDP-GlcNAc and 1.5 μM TAB1 (or GST-HCF-rep1-His), at 25°C for indicated time. For dynamics study, a 300 μL reaction was set up. Each 10 μL of the reaction mixture was stopped at the given time point by adding 30 μL of Milli-Q water and 20 uL of 3×Native Loading Buffer, followed by SOPAGE analysis, electro-transfer and blotting with appropriated antibodies. For normal SDS-PAGE and standard WB, 3×Native Loading Buffer was replaced with 5× SDS-PAGE Loading Buffer, followed by heating at 95°C for 3 min.

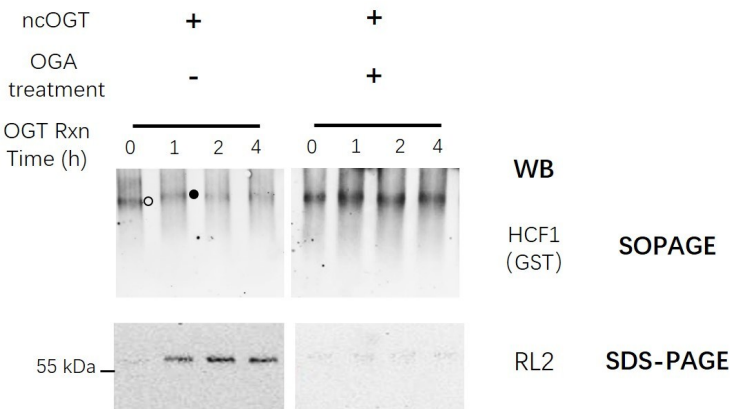

Fig. S5. Monitoring the *in vitro* glycosylation of GST-HCF1-rep1-His by ncOGT using SOPAGE. GST-HCF-rep1-His (○) and its multi- *O*-GlcNAcylated form (●) are indicated on SOPAGE picture.

Preparation of fully *O*-GlcNAcylated TAB1 (gTAB1)

gTAB1 was prepared from TAB1 using *in vitro* OGT reaction. Briefly, 300 μL of reaction containing 0.15 μM of hOGT, 150 μM UDP-GlcNAc, 1.5 μM TAB1 and 1×OGT Reaction Buffer was incubated at 25°C for 72 h. The reaction mixture was cleared by centrifugation and loaded onto Superdex G200 column equilibrated with 1×TBS (pH 7.5, with 0.5 mM TCEP) to remove hOGT. Purified gTAB1 was concentrated and stored at -80°C.

In vitro OGA treatment

If required, *O*-GlcNAc proteins were treated with 5% (w/w) of tag-free *wild-type* (active) *O-GlcNAase (Cp*OGA) in 1×OGA Reaction Buffer at 37°C for 2 h to remove *O*-GlcNAc group. Alternatively, 5% (w/w) of *Cp*OGA can be directly added to the completed *in vitro* OGT reaction and incubated for additional 2 h, since bacteria-derived *Cp*OGA has much higher activity and velocity than hOGT.

Measuring apparent K_m and V_{max} of hOGT for TAB1

Standard *in vitro* OGT assay was set up with 0.45 μM of hOGT, 150 μM UDP-GlcNAc, 1×OGT Reaction Buffer and varying concentration of TAB1 (0.167, 0.5, 1.5, 4.5, 13.5, 40.5 uM). In order to ensure no more than 10% of total TAB1 was converted to gTAB1, the reaction was carried out at 37°C for only 1 h and stopped by adding 3×Native Loading Buffer followed by snap-freezing with liquid nitrogen. To achieve a better resolution of gTAB1 from excessive TAB1, approximate 1 pmol of total TAB1 calculated from its initial concentration was loaded for SOPAGE analysis, resolved by electrophoresis, transferred, and blotted with anti-TAB1 antibody (1:3000 dilution) for 1 h. After that, the membrane was blotted with IRDye 800CW donkey anti-goat secondary Ab (1:10000 dilution) and documented with Odyssey scanner. The band intensity was quantified with Image Studio Lite software (version 5.2), and then the fraction of gTAB1 (gTAB1 %) in each sample was calculated according to the method described in the main text. Briefly, the amount of gTAB1 in each sample was calculated by multiplying initial TAB1 concentration with gTAB1%, converted to velocity V (nmol/mg/min), and plotted against TAB1 concentration. $K_{m,app}$ and V_{max} of TAB1 for hOGT were calculated by double-reciprocal plotting with GraphPad Prism 5.0 software.

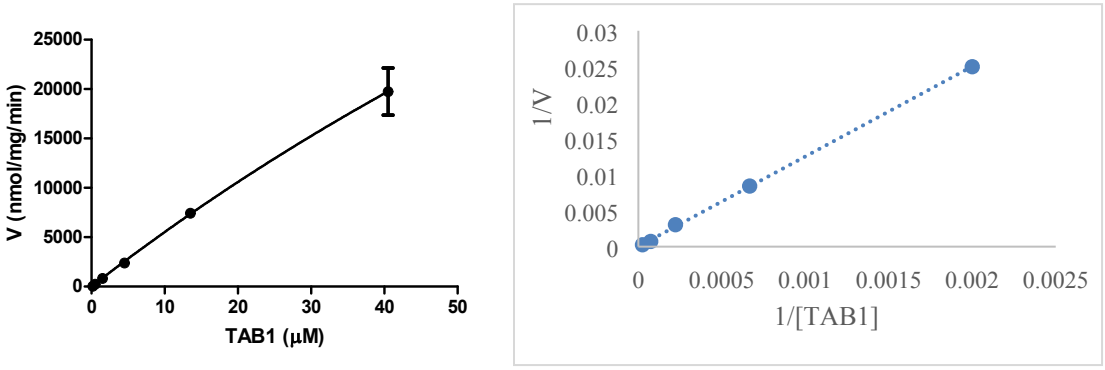

Figure S6. Steady-state kinetics of the recombinant hOGT on TAB1.

Supplementary Note 5

Buffer recipes for SOPAGE Gel

7×SOPAGE Upper Buffer (Stacking gel):

5.7 g Tris, adjust pH to 6.7 with H_3PO_4, and fill up with water to 100 mL.

4×SOPAGE Lower Buffer (Separation gel):

18.2 g Tris, adjust pH to 8.9 with HCl, and fill up with water to 100 mL.

50×SOPAGE Running Buffer:

7.5 g Tris, 36 g Glycine, and fill up with water to 250 mL.

3×SOPAGE gel Sample Buffer:

3 mL Glycerol, 0.6 mL 50× SOPAGE and Native Gel Running Buffer, 6.4 mL H_2O and 0.01% (w/v) bromophenol blue.

SOPAGE Transfer Buffer: 0.5× Towbin Buffer with 0.04% (v/v) SDS

Membrane Washing Buffer 1: 0.5× Towbin Buffer with 20% (v/v) MeOH

Membrane Washing Buffer 2: 1×TBS (pH 8.0) with 0.05% Tween-20

Membrane Blocking Buffer: 3% (w/v) BSA in 1×TBST

Casting SOPAGE Gel

The formulation of SOPAGE gel was modified from high pH Native Gel (Jonathan King Lab, MIT). To prepare 3 ml of SOPAGE Separating Gel, 1.5 ml of H_2O, 0.75 ml of 4×SOPAGE Separating Buffer, 0.75 ml of 40% Acrylamide Solution and 21 µL of 10% APS was mixed in order, and AI-OGA was added as the last step to a final concentration of 1-5 µM. The gel polymerization was initiated by adding 1.75 µL of TEMED. For 3 ml of SOPAGE Stacking Gel, 2.625 ml of H_2O, 0.5 ml of 7×SOPAGE Stacking Buffer, 0.375 mL of 40% Acrylamide Solution, and 20 µL of 10% APS were mixed in order, followed by adding 4 µL of TEMED. A two-layer gel with a bottom layer of regular native separating gel (Fig. S2b) can reduce the consumption of AI-OGA to some extent. Bio-rad mini gel glass plates with 0.75 mm or 1 mm spacer and the corresponding 10- or 15-well combs were utilized for all experiments.

General protocols for SOPAGE analysis and the follow-up WB detection

To achieve a better degree of separation, SOPAGE gel was pre-run at 180 V for 30 min in 1×SOPAGE Gel Running Buffer prior to sample loading, in order to eliminate

unpolymerized AI-OGA and other impurities. Typically, the current dropped from 12-15 mA to 8-10 mA for one piece of bio-rad MINI gel (1 mm thickness) after pre-running. The wells were then carefully flushed with 1×Running Buffer prior to sample loading. The electrophoresis condition was set to 180 V or a maximum 40 mA per gel, depending on which constraint was reached first. For electroblotting, the transfer sandwich was assembled as usual and transferred in chilled SOPAGE Transfer Buffer at 100 V for 90 min. After that, the NC membrane was first rinsed with 0.5×Towbin buffer with 20% methanol (v/v) for 1 min to remove trace SDS, followed by blocking, Ab incubation and washing procedure, which were the same as those for conventional WB.

Precautions for performing SOPAGE analysis

Notice 1:

ONLY water can be used as a cover layer during the polymerization of separation gel. Any residual organic solvents, such as ethanol, isopropanol, or butanol, can cause protein aggregation at the interface between separating and stacking gel.

Notice 2:

Before casting the stacking gel, the space between the upper edge of separating gel and the inner walls of two assembled glass plates must be THOROUGHLY rinsed with DDW and then drained. This procedure can remove any residues of unpolymerized AI-OGA that may sneak into the stacking gel, and thereby cause an uneven or curved electrophoretic band.

Notice 3:

Because the common protein ladders used for SDS-PAGE (e.g. #26616, Thermal Fisher) migrate in different modes in Native-PAGE, they can ONLY be used as a reference rather than a ruler for judging the actual protein molecular weight.

Notice 4:

It is STRONGLY recommended to first run your sample on a normal Native-PAGE to determine its migration pattern and then move forward to SOPAGE analysis.

Notice 5:

The SOPAGE transfer buffer is DIFFERENT from that used for conventional blotting, and the addition of SDS is necessary for releasing *O*-GlcNAc protein from AI-OGA by disrupting the interaction between them. SDS will also charge proteins and favor protein transfer from Gel to NC membrane. However, SDS has a negative impact on blocking and primary Ab recognition. Therefore, it is vital to perform an additional washing step with 20% MeOH prior to blocking.

Notice 6:

Keep in mind that SOPAGE is based on native PAGE, so please be aware of controlling the temperature in ALL steps, including but not limited to electrophoresis and blotting. Any overheating will denature your protein and may cause failure.

Reference:

1 F. Capotosti, S. Guernier, F. Lammers, P. Waridel, Y. Cai, J. Jin, J. W. Conaway, R. C. Conaway and W. Herr, *Cell*, 2011, **144**, 376–388.

2 S. Pathak, V. S. Borodkin, O. Albarbarawi, D. G. Campbell, A. Ibrahim and D. M. F. Van Aalten, *EMBO J.*, DOI:10.1038/emboj.2012.8.

3 K. Rafie, O. Raimi, A. T. Ferenbach, V. S. Borodkin, V. Kapuria and D. M. F. van Aalten, *Open Biol.*, DOI:10.1098/rsob.170078.

4 N. Selvan, R. Williamson, D. Mariappa, D. G. Campbell, R. Gourlay, A. T. Ferenbach, T. Aristotelous, I. Hopkins-Navratilova, M. Trost, and D. M. F. Van Aalten, *Nat. Chem. Biol.*, 2017, **13**, 882–887.

5 A. P. Willems, M. Gundogdu, M. J. E. Kempers, J. C. Giltay, R. Pfundt, M. Elferink, B. F. Loza, J. Fuijkschot, A. T. Ferenbach, K. L. I. van Gassen, D. M. F. van Aalten and D. J. Lefeber, *J. Biol. Chem.*, 2017, **292**, 12621–12631.

6 N. Selvan, S. George, F. J. Serajee, M. Shaw, L. Hobson, V. Kalscheuer, N. Prasad, S. E. Levy, J. Taylor, S. Aftimos, C. E. Schwartz, A. M. Huq, J. Gecz and L. Wells, *J. Biol. Chem.*, 2018, **293**, 10810–10824.

7 L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass and M. J. E. Sternberg, *Nat. Protoc*, 2016, **10**, 845–858.