SI Appendix

O-GlcNAcylation Regulates Epidermal Growth Factor Receptor Intracellular Trafficking and Signaling

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Experimental methods.
Figs S1-S10.
Plasmids and subcloning. To knockdown the endogenous HGS, the shRNA sequence 5’-CCGGCTGAAACGCCTCGTCTCTAACCTCGAGGTTAGAGACGAGGCGTTTCAGTTTTTG-3’ or the corresponding scramble sequence 5’-CCGGCTAAGGTTAAGTCCCTCGCTCTAGCGAGGGCGACTTAACCTTTTTTTTT-3’ was inserted the vector pLenti-FlagN-shRNA. The shRNA specifically targets 3’UTR of HGS. The vector allows for knockdown of the endogenous gene with the simultaneous expression of an exogenous gene. Flag-tagged WT HGS (cDNA clone obtained from Origene) or 3SA HGS was cloned into the same vector. Lentiviruses were produced from these constructs using a three-plasmid packing system. Cells were infected with the lentiviruses and selected for monoclonal cells with green fluorescence or with 2 μg/mL puromycin for 2 weeks.

Antibodies and reagents. All antibodies and reagents were obtained from commercial sources: anti-O-linked N-acetylglucosamine antibody (RL2, Thermo Scientific), anti-Flag (clone M2, Sigma), anti EEA1 (Sigma), anti-Myc (EarthOx), anti-HGS (Abcam), anti-EGFR (Abcam), anti-phospho-EGFR (Y1068, Cell Signaling Technology), anti-FLAG-STAT (Abcam), anti-Akt (Cell Signaling Technology), anti-phospho-Akt (Cell Signaling Technology), anti-Erk (Beyotime), anti-phospho-Erk (Cell Signaling Technology), anti-actin (Proteintech), anti-LAMP1 (Santa Cruz), anti-mouse IgG DyLight 488 (Abbkine), anti-rabbit IgG DyLight 594 (Abbkine), Thiamet G (TMG, MedChem Express), OSMI-4 (MedChem Express), cycloheximide (Sigma), hEGF (Sigma). Chymotrypsin (sequencing grade, Beijing Shengxia Proteins Scientific Ltd. China).

Immunoprecipitation and immuno-blotting analysis. For immunoprecipitation of Flag-tagged proteins, cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with complete protease inhibitors (Roche) and 10 μM TMG. Cell lysates with equal amounts of proteins were diluted with the RIPA buffer and incubated with Flag M2 magnetic beads (Sigma) with end-to-end rotation for 2 hr at 4°C. Beads were then washed three time with TBS buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl). Bound proteins were eluted with 3X Flag peptide or SDS buffer, and subjected to 8–12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE).

For immunoblotting analysis, proteins were transferred to a nitrocellulose membrane (Merck). The membrane was blocked in the buffer (5% bovine serum albumin solution and 0.1% Tween 20 in Tri-buffered saline). And then the membrane was incubated with the indicated primary and secondary antibodies. Immunoblotting signals were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).
HGS O-GlcNAcylation analysis. Analysis of HGS O-GlcNAcylation was carried out as previously described (1). Briefly, cell lysates (400 μg) were first labeled with GalNAz according to the Click-iT O-GlcNAc Enzymatic Labeling System protocol (Life Technologies) and then conjugated with an alkyn–biotin compound according to the Click-iT Protein Analysis Detection Kit protocol (Life Technologies). Control experiments in the absence of the enzyme GalT (Y289L) were carried out in parallel. Biotin labeled proteins were precipitated using methanol and chloroform, and resolubilized in 1% SDS, and neutralized with an equal volume of neutralization buffer (6% NP-40, 100 mM Na₂HPO₄, 150 mM NaCl). Lysates were further incubated with streptavidin beads (Pierce) with end-to-end rotation at 4°C overnight. Beads were then washed three times with 1 mL low-salt buffer (100 mM Na₂HPO₄, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate) and three times with 1 mL high-salt buffer (100 mM Na₂HPO₄, 500 mM NaCl, 0.2% Triton X-100). The bound proteins were eluted by boiling the beads in a buffer containing 50 mM Tris-HCl pH 6.8, 2.5% SDS, 100 mM DTT, 10% glycerol, and 20 mM biotin for 15 min. Immunoblotting was carried out with anti-HGS or anti-Flag antibodies.

HGS glycosylation site mapping. Protein samples containing Flag-tagged HGS were separated by SDS-PAGE and stained by Coomassie blue R250. Gel bands were excised and de-stained with a decolorizing buffer (50% ACN and 50% 100mM Tris-HCl, pH 7.8). After the HGS gel was completely transparent, it was chopped intensively and resuspended in 100 μL 100 mM Tris-HCl (pH 7.8) containing 10 mM CaCl₂. The HGS gel particles were digested by chymotrypsin (specifically cleaving peptide bonds at the C-termini of Tyr, Phe, Trp and Leu that are not adjacent to Pro) at a 1:50 ratio (wt/wt) and the mixture was incubated at 30 °C with shaking overnight. After digestion, the centrifuged supernatant was acidified, desalted and freeze dried for further LC-MS analysis. The peptides were trapped onto a homemade 150μm×15mm C₁₈ precolumn and washed with 15 μL buffer A (0.1% FA) automatically by a Thermo EASY nLC 1200 system (Thermo Fisher Scientific). The bound peptides were then eluted by a homemade 15cm C₁₈ column (ID 150 μm, 1.9 μm, 100 Å) over a 75 min gradient of 7-15% B in 11 min, 15-25% B in 37 min, 25-40% B in 20 min, ramping to 100% B at 69 min and held at 100% B for 7 min (buffer B was 0.1% FA in 80% ACN) at a constant flow rate of 600 nL/min. The eluted peptides were sprayed into an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The full-MS scans were taken in the Orbitrap (60000 resolution, 50ms injection time, 4e5 AGC) and the top twenty ions in each full-MS scan are then subjected to electron-transfer/higher-energy collision (EThecD) with 25% ETD collision energy and 25% HCD collision energy.
**Real-time qPCR.** Total RNA was Trizol-extracted, column-purified and reverse-transcribed using PrimeScript 1st Strand Cdna Synthesis kit (Takara). All qPCR analyses were performed using Fast SYBR Green (BioRad). The relative level of expression was calculated using β-actin as the endogenous control. Primer sequences are shown below.

| Gene   | F            | R            |
|--------|--------------|--------------|
| β-actin| CACCATTGGCAATGAGCGGTTC | AGGTCTTTGCGGATGTCCACGT |
| β-actin| AACACCCCTGGTCTGGAAGTACG | TCGTTGGACAGCCTTCAAGACC |
| EGFR   | GACAGACTCTCAGCCCATTCCT | TCGTTGGACAGCCTTCAAGACC |
| HGS    | TCATGCGGTTCCAGGAAGGTGGT | TCATGCGGTTCCAGGAAGGTGGT |

**Cell proliferation assay.** Cell proliferation was measured with the Cell Counting Kit-8 (Beyotime). The kit contains WST-8, a compound that can be reduced to orange yellow by dehydrogenases in the mitochondria in the presence of electron coupling reagents. A linear relationship can be obtained between the color intensity and the number of cells. Briefly, following the manufacturer’s instructions, 100 μL of cell suspension was added to the well in a 96-well plate, followed by the addition of 10 μL of CCK-8 solution provided, and incubated for 1 h at room temperature. Absorbance was then measured at 450 nm using a microplate reader (Biorad).

**Immunofluorescence.** Cells were seeded on 22 mm × 22 mm glass coverslips for 24 h before the experiment. Cells were stimulated with 100 ng/mL EGF for indicated time points, washed with PBS twice, and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with PBS twice, cells were permeabilized with 0.25% triton X-100, and blocked with 3% BSA for 1 h at room temperature. Afterwards, cells were incubated with primary antibodies overnight, and secondary antibody for 1 h, DAPI for 15 min, and imaged using an Olympus confocal laser-scanning microscope (FV3000) and exported to Fiji ImageJ for image processing.

**EGFR internalization and recycling assays.** For the EGFR internalization assay, the cells were serum starved for 6 h, pretreated with DMSO or OSMI-4 (20 ng/mL, 12 h), and then incubated with Alexa Fluor 488-labelled EGF (10 nM) at 4°C for 30 min. After washing, cells were incubated at 37°C to initiate internalization. After 30 min, the cells were placed on ice to stop the internalization process, washed three times with cold PBS, and then with an acid buffer (0.2 M acetic acid, 0.5 M NaCl pH 2.8). The cells were then detached from culture dishes, washed, and resuspended in the FACS buffer (2% fetal bovine serum and 0.01% sodium azide in PBS), and fixed with an equal volume of 4% formaldehyde in PBS. The fluorescence signal of internalized
EGF was detected by flow cytometry.

The EGFR recycling was measured by a FACS-based method (2). Briefly, cells were serum-starved for 4 h, and then incubated with EGF (100 ng/mL) for 1 h at 4 °C, followed by a 25-min shift at 37 °C to allow internalization (pulse). After the removal of bound EGF by mild acid/salt treatment, cells were chased at 37 °C for 40 min to allow recycling. At each time point cells were fixed in 1% formaldehyde. EGFR at the plasma membrane was labeled using an anti-EGFR antibody (Ab-1), which recognizes the extracellular portion of the receptor, and FITC-conjugated secondary antibodies, and analyzed by flow cytometry. The relative surface level (RSL) of EGFR at each time point (t) was calculated from mean fluorescence intensities (MFI) with the formula: \[ \frac{\text{MFI}(t) - \text{MFI}(\text{pulse})}{\text{MFI}(T0) - \text{MFI}(\text{pulse})} \times 100. \]

**Electron microscopy.** Stable SK cells expressing WT or 3SA HGS were pretreated with DMSO or OSMI-4 (20 ng/mL, 12 h), and then serum starved. The cells were then incubated with EGFR antibody at 4 °C for 30 min, washed three times, and incubated with 10 nm protein A-gold for 20 min. The cells were then treated with EGF (10 nM) at 37 °C for 1 h. After that, the cells were fixed in 0.1 M sodium cacodylate containing 2.0 % paraformaldehyde and 2.5 % glutaraldehyde. The MVBs were detected in Hitachi Model H-7650 TEM. Three replicates were performed for each treatment, and >2,000 μm² of cytoplasm was examined in each case. More than 50 MVBs were examined for statistical analysis for each treatment.

**Cell-free reconstitution of ILVs.** The reconstitution of ILVs and receptor sorting was performed as described previously (3,4). The cytosol was prepared from untreated SK stable cell lines expressing WT or 3SA HGS. Cells were collected and resuspended with a homogenization buffer containing a protease inhibitor mixture. Samples were subjected to sonication to disrupt membranes (5 cycles of 5 s on, 30 s off at 40W). The mixtures were centrifuged at 2000 g for 10 min at 4 °C, and the supernatants were collected. The supernatant was again centrifuged at 100,000 g for 1 h at 4°C, and the resultant supernatants were collected. 25 μg of the cytosol was used in the sorting assay.

**Protein stability assay.** Stable cells expressing WT or 3SA HGS were pretreated with 50 μM cycloheximide (CHX) to inhibit new protein synthesis, and OSMI-4 (20 ng/mL, 12 h). After treatment, cells were harvested at the indicated time points (0, 5, 10, 20 h). HGS levels were detected by immuno-blotting with anti-Flag antibody.
Mouse xenograft. The procedure of establishing xenografts in nude mice was approved by the Zhejiang University Laboratory Animal Centre. Briefly, $5 \times 10^6$ SK cells stably expressing WT or 3SA HGS were subcutaneously injected into the flanks of 6-week-old male nude BALB/c mice ($n = 5$ per group). Tumour growth was monitored every 5 days over a 6-week period. At the end of the sixth week, the tumours were harvested and weighed.

Analysis of tumor samples. Liver tumor tissues and the matched peritumoral tissues were obtained from the First Affiliated Hospital of Zhejiang University. Cell lysates were obtained from tissues and measured by the BCA method. Immunoprecipitation of HGS was performed with equal amounts of cell lysates, separated by SDS-PAGE, and immunoblotted with HGS antibody or RL-2 antibody. The ratio of the intensity of the RL-2 blot versus the intensity of the HGS blot was taken as the relative level of glycosylation.

Sorafenib treatment assay. The cell survival rate exposed to sorafenib treatment was analyzed by the MTT assay using the Cell Counting Kit-8 (Beyotime). Cells were seeded at a density of 2000 cells per well in the 96-well plate and incubated overnight. Cells were then treated with different concentrations of sorafenib and incubated for 72 h. The number of viable cells was determined. Four replications for each well were averaged. The cell survival rate was calculated by percentage of growth relative to the controls treated with DMSO.

References:
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2. Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S. & Di Fiore, P.P. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell 15, 209-219 (2008).
3. Sun, W. et al. Cell-free reconstitution of multivesicular body formation and receptor sorting. Traffic 11, 867-76 (2010).
4. Sirisaengtaksin, N. et al. UBE4B protein couples ubiquitination and sorting machineries to enable epidermal growth factor receptor (EGFR) degradation. J Biol Chem 289, 3026-39 (2014).
**Fig. S1.** Immunoblotting of HGS glycosylation levels in SK cells upon treatment with different stimuli, including glucose (A), glutamine (B), serum (C), EGF (D), and hydrogen peroxide (E). The blots are presentative of three biological replicates.
Fig. S2. Mapping glycosylation sites in HGS by tandem mass spectrometry.
Fig. S3. Analysis of cell surface expression of EGFR in stable SK cells expressing WT or 3SA HGS, as determined by flow cytometry.
Fig. S4. HGS O-GlcNAcylation does not affect its ability to target to the early endosome. a, Immunofluorescence staining of cells with Flag and EEA1 antibodies. Scale bar represents 5 μm. b, Quantification of HGS-EEA1 colocalization. Data are presented as the mean ± s.d. of n = 3 independent experiments. A two-tailed unpaired Student’s t-test was used for statistical analysis. N.S. represents not significant.
**Fig. S5.** Mapping the interacting domain of HGS with STAM. Upper panel, predicted structural domains of HGS. Lower panel, immunoblotting analysis of the interaction between various truncated HGS mutants and STAM. Immunoprecipitation was performed with the Flag antibody, and blotted with the STAM antibody. The blots are presentative of three biological replicates.
Fig. S6. Immunoblotting analysis of HGS-STAM interaction in cells expressing WT or 3SA HGS in the presence or absence of TMG treatment. Myc-tagged STAM was ectopically expressed in cells. Immunoprecipitation was performed with the Flag antibody, and blotted with the Myc antibody (A), or with the Myc antibody, and blotted with the Flag antibody (B). The blots are presentative of three biological replicates.
Fig. S7. Relative mRNA expression of HGS in stable SK cells expressing WT or 3SA HGS, in the presence of cycloheximide. The expression was normalized to the β-actin level in cells. Data are presented as the mean ± s.d. of n = 3 independent experiments. A two-tailed unpaired Student’s t-test was used for statistical analysis. **P < 0.01.
Fig. S8. HGS O-GlcNAcylation promotes HGS degradation in cells. (A) Immunoblotting of HGS expression upon transfecting different doses of OGT expression constructs in 293T cells. (B) Immunoblotting of Flag-tagged HGS in stable SK cells in the presence of cycloheximide and chloroquine. (C) Immunoblotting of Flag-tagged HGS in stable SK cells in the presence of cycloheximide and MG132. (D) Immunoblotting of the ubiquitination of Flag-tagged HGS immunoprecipitated from stable SK cells. (E) Analysis of HGS-Nedd4 interaction in cells expressing WT or 3SA HGS in the presence or absence of TMG treatment. Myc-tagged Nedd4 was ectopically expressed in cells. Immunoprecipitation was performed with the Myc antibody, and blotted with the Flag antibody. The blots are presentative of three biological replicates.
**Fig. S9.** Representative immunoblotting of EGFR expression, HGS glycosylation, and HGS expression in xenograft tumors generated from stable SK cells expressing WT or 3SA HGS. The blots are presentative of three biological replicates.
**Fig. S10.** Generation of stable cell lines using the sorafenib-resistant HepG2 cells (HepG2-R). The endogenous HGS was depleted using HGS-targeting shRNA. Then Flag-tagged WT or 3SA HGS was ectopically expressed in HGS-depleted HepG2-R cells. The blots are presentative of three biological replicates.