O-GlcNAcylation regulates epidermal growth factor receptor intracellular trafficking and signaling

Liming Wu[a,1], Yaxian Cheng[b,1], Didi Geng[b], Zhiya Fan[c] (ID), Bingyi Lin[a], Qiang Zhu[b], Jingchao Li[b], Weijie Qin[c], and Wen Yi[a,b,2] (ID)

[a]Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang Provincial Key Laboratory of Pancreatic Disease, School of Medicine, Zhejiang University, Hangzhou 310003, China; [b]Ministry of Education Key Laboratory of Biosystems Homeostasis & Protection, College of Life Sciences, Zhejiang University, Hangzhou 310058, China; and [c]National Center for Protein Sciences Beijing, State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Lifeomics, Beijing 102206, China

Ligand-stimulated epidermal growth factor receptor (EGFR) signaling plays fundamental roles in normal cell physiology, such as cell growth, cell proliferation, and cell survival. Deregulation of EGFR signaling contributes to the development and progression of diseases including cancer. Despite its essential role in biology, the mechanisms by which EGFR signaling is regulated in cells are still poorly understood. Here, we demonstrate that O-linked N-acetyl-glucosamine (O-GlcNAc) modification serves as an important regulator of EGFR intracellular trafficking and degradation. Mechanistically, O-GlcNAcylation of hepatocyte growth factor regulated tyrosine kinase substrate (HGS), a key protein in EGFR intraluminal sorting pathway, inhibits HGS interaction with signal-transducing adaptor molecule (STAM), thereby impairing the formation of endosomal sorting complex required for transport-0 (ESCRT-0). Moreover, O-GlcNAcylation increases HGS ubiquitination and decreases its protein stability in cells. Consequently, HGS O-GlcNAcylation inhibits EGFR intraluminal sorting and lysosomal degradation, leading to the accumulation of EGFR and prolonged EGFR signaling in cells. Furthermore, HGS glycosylation is demonstrated to promote tumor growth in the xenograft study and chemoresistance in liver carcinoma cells. Thus, our study reveals a role of O-GlcNAcylation in regulating receptor tyrosine kinase endocytic trafficking and signaling.

O-GlcNAcylation | EGFR | endosomal sorting | membrane receptors

As the prototypic receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR) senses extracellular growth signals and transduces the signal inside the cell to regulate various important cellular processes, including cell growth, proliferation, and survival (1). On the other hand, aberrant EGFR activity is frequently linked to diseases such as cancer (2). For example, EGFR mutations that resulted in EGFR hyper-activation have been identified clinically in several types of cancers (3, 4). Consequently, EGFR has become a therapeutic target for the development of anticancer drugs (2, 5).

EGFR expression levels and signaling strength are tightly controlled in cells. The key mechanism to terminate EGFR signaling is the intraluminal sorting and lysosomal degradation of EGFR (6, 7). Upon ligand stimulation, EGFR undergoes dimerization and autophosphorylation of the tyrosine residues, leading to the activation of EGFR. The activated EGFR is rapidly internalized through the clathrin-dependent mechanism, and targeted to early endosomes, where EGFR signaling persists. A population of EGFR molecules recycles back to the plasma membrane, while the remaining EGFR molecules are sorted into intraluminal vesicles (ILVs) of the multivesicular body (MVB). The MVB then fuses with the lysosome, which results in EGFR degradation and signal termination.

The intraluminal sorting of endosomal EGFR constitutes a key step during EGFR signal termination. This is governed by the endosomal sorting complex required for transport (ESCRT)

machineries (8, 9). ESCRT-0 consists of hepatocyte growth factor regulated tyrosine kinase substrate (HGS) and signal-transducing adaptor molecule (STAM) (10, 11). HGS is a cytosolic protein, which recognizes and recruits ubiquitinated protein cargo (including EGFR) to initiate the intraluminal sorting event. HGS is shown to interact with various proteins on the early endosomes to regulate the sorting and degradation process. Despite its critical role in EGFR endosomal trafficking and degradation, the regulation of HGS at the molecular level and how it influences EGFR signaling are poorly understood.

O-linked N-acetylglucosamine (O-GlcNAc), a monosaccharide modification of serine and/or threonine residues of cellular proteins, has recently emerged as a key regulator of various important biological processes, including gene transcription, stress response, metabolic homeostasis, and immune regulation (12, 13). In cells, a single set of enzymes—O-GlcNAc transferase (OGT) and O-GlcNAc hydrolyze (OGA)—are responsible for the addition and removal of O-GlcNAc modification, respectively. Given the critical role of O-GlcNAcylation in normal physiology, increasing evidence has now demonstrated that deregulation of O-GlcNAcylation is closely associated with the development and progression of various diseases, including

Significance

Epidermal growth factor receptor (EGFR) is one of the most important membrane receptors that transduce growth signals into cells to sustain cell growth, proliferation, and survival. EGFR signal termination is initiated by EGFR internalization, followed by trafficking through endosomes, and degradation in lysosomes. How this process is regulated is still poorly understood. Here, we show that hepatocyte growth factor regulated tyrosine kinase substrate (HGS), a key protein in the EGFR trafficking pathway, is dynamically modified by a single sugar N-acetylglucosamine. This modification inhibits EGFR trafficking from endosomes to lysosomes, leading to the accumulation of EGFR and prolonged signaling. This study provides an important insight into diseases with aberrant growth factor signaling, such as cancer, obesity, and diabetes.

neurodegeneration, cardiovascular disease, and cancer (14–16). A key question in exploring the biology of O-GlcNAcylation is to elucidate the protein-specific glycosylation function in cells, which requires detailed mechanistic studies at the molecular level.

Previously, we developed a mass spectrometry-based strategy for quantitative profiling of O-GlcNAcylated proteins using a photocleavable probe (17). HGS was reproducibly identified to possess at least twofold increase in glycosylation levels in drug-resistant liver cancer cells than drug-sensitive cells. Because the function of HGS glycosylation has not been studied before, we decided to carry out a detailed investigation. Our results demonstrate that HGS O-GlcNAcylation decreases its interaction with STAM and impairs the formation of ESCRT-0 at the early endosome. In addition, O-GlcNAcylation increases HGS ubiquitination and promotes the degradation of HGS. Consequently, HGS O-GlcNAcylation inhibits intraluminal sorting and lysosomal degradation of EGFR, leading to the accumulation of EGFR at the early endosome and prolonged EGFR signaling in cells.

Results

HGS is Dynamically Modified by O-GlcNAc. Our previous proteomic study identified HGS as an O-GlcNAcylated protein with high confidence (17). To verify the O-GlcNAcylation on HGS, we employed a widely used chemoenzymatic labeling method (18). Flag-tagged HGS was ectopically expressed in 293T cells. Proteins from cell lysates were used as substrates to perform an in vitro enzymatic reaction in which an azido-N-acetylgalactosamine (GalNAz) was transferred from the corresponding nucleotide sugar (UDP-GalNAz) onto O-GlcNAc by a galactosyltransferase. Labeled proteins were subsequently conjugated with a biotin-containing alkynl molecule via the Cu(I)-mediated [3 + 2] cycloaddition reaction. After capture and enrichment with streptavidin-coated beads, bound proteins were eluted and immunoblotted with the Flag antibody. A clear immunoblotting signal was observed in the labeled samples but was absent in the control samples in which the galactosyltransferase was omitted (Fig. 1A). Expectedly, coexpression of OGT, or inhibition of OGA with Thimet-G (TMG, a specific inhibitor of OGA), substantially increased the blotting signal (Fig. 1A). In addition, we
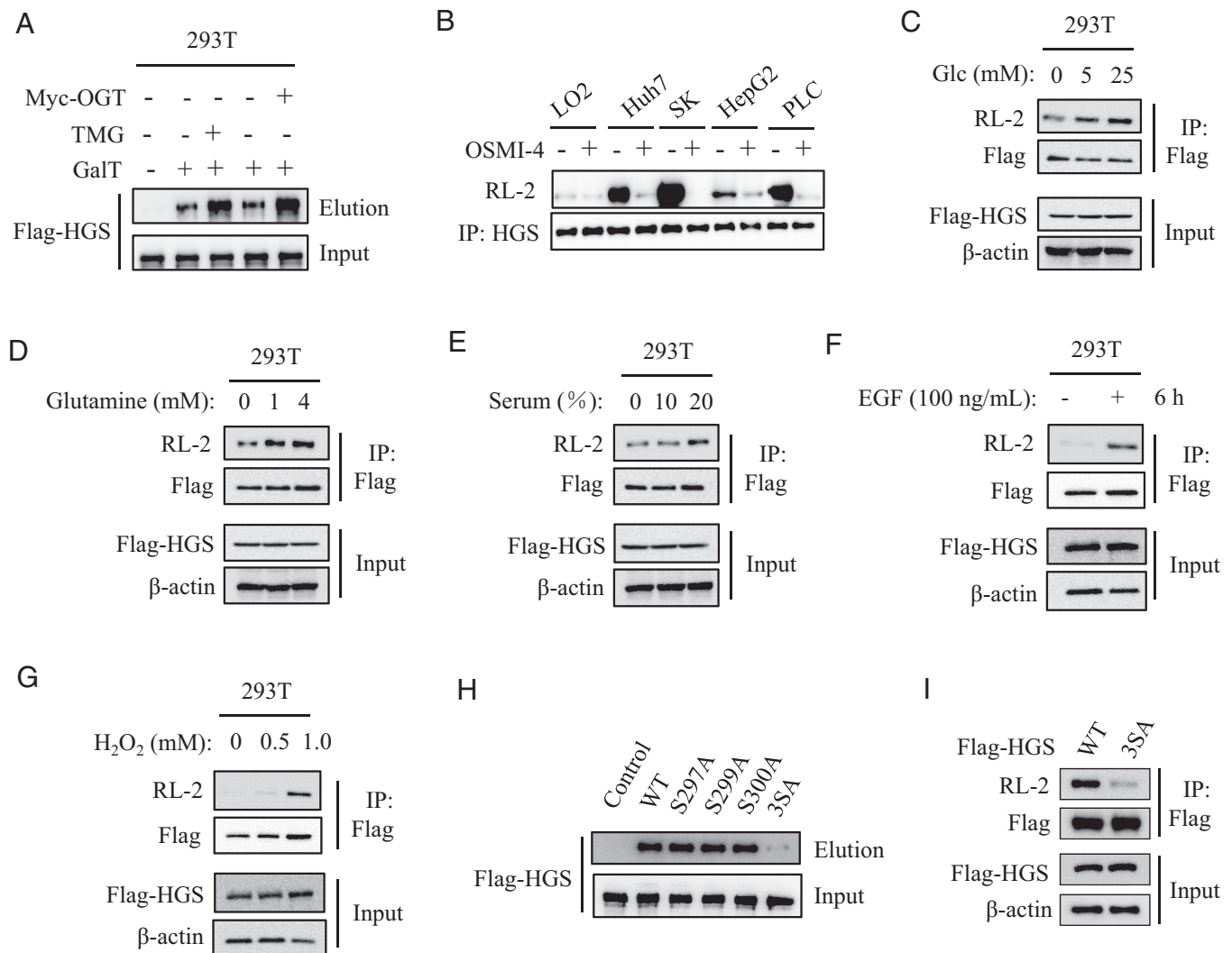
Fig. 1. HGS is dynamically modified by O-GlcNAc. (A) Validation of HGS O-GlcNAcylation using a chemoenzymatic method. Elution blots show the glycosylated form of HGS, and input blots show the total HGS. Results are representative of three biological replicates. (B) Immunoblotting of HGS glycosylation levels in various cell lines in the presence or absence of OSMI-4. HGS O-GlcNAcylation was probed by the RL-2 antibody. (C–G) HGS glycosylation levels upon treatment with different stimuli, including glucose (C), glutamine (D), serum (E), EGF (F), and hydrogen peroxide (G). The blots are presentative of three biological replicates. (H and I) Mapping glycosylation sites on HGS using the chemoenzymatic method (H) and immunoblotting with the RL-2 antibody (I). Results are representative of three biological replicates.
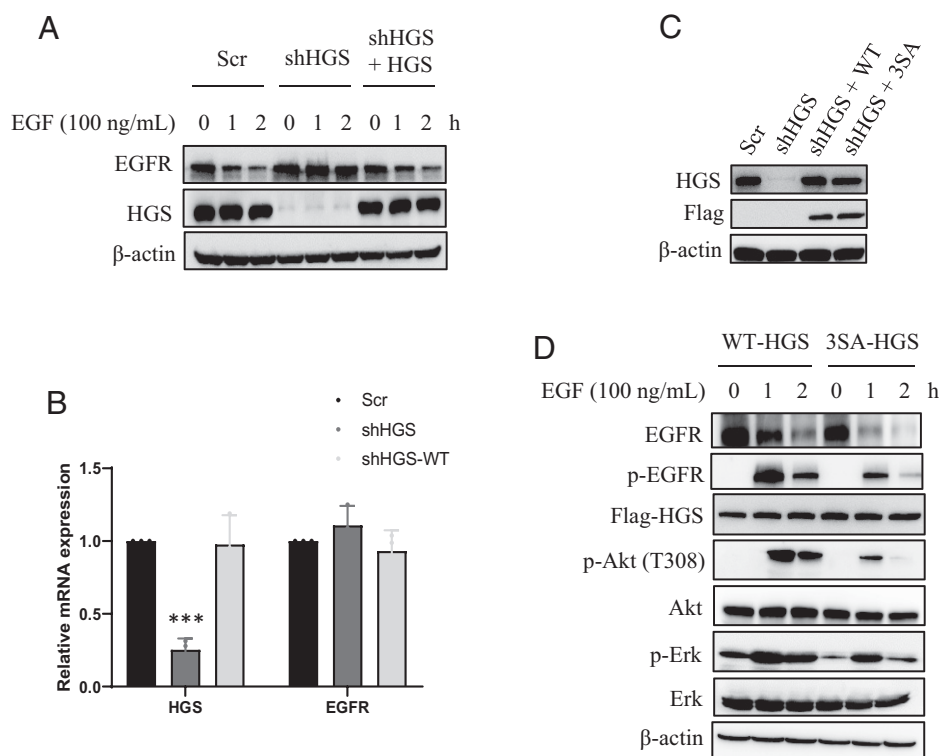
Fig. 2. HGS O-GlcNAcylation regulates EGFR degradation and signaling. (*A*) Immunoblotting of EGFR expression in the presence or absence of HGS depletion upon EGF treatment. The blots are presentative of three biological replicates. (*B*) Relative mRNA expression of EGFR upon EGF treatment. The expression was normalized to the β-actin level in cells. Data are presented as the mean ± SD of *n* = 3 independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. ***$P < 0.001$. (*C*) Immunoblotting of HGS expression in stable SK cells ectopically expressing WT or 3SA Flag-tagged HGS, with a simultaneous depletion of the endogenous HGS. (*D*) Immunoblotting of EGFR expression, EGFR activation, Akt activation, and Erk activation in engineered stable SK cells.

immunoprecipitated endogenous HGS proteins from different cell lines and readily detected immunoblotting signals with a pan-O-GlcNAc antibody (RL-2) (Fig. 1*B*). Consistently, treatment of an OGT inhibitor OSMI-4 (19) substantially reduced the blotting signals. Thus, these results confirm that HGS possesses O-GlcNAcylation in cells.

To demonstrate that HGS glycosylation is dynamically regulated, we subjected 293T cells and liver cancer cell line SK cells to different stimuli and examined HGS glycosylation levels. The results showed that HGS glycosylation levels varied when the cells were cultured under different nutrient levels or in the presence of oxidative stress (Fig. 1 *C–G* and *SI Appendix*, Fig. S1). These results were consistent with previous studies that protein O-GlcNAcylation is dynamically responsive to different environmental cues in cells (20, 21).

Using mass spectrometry, we identified three potential glycosylation sites (Ser297, Ser299, and Ser300) on HGS (*SI Appendix*, Fig. S2). To further validate glycosylation sites, we mutated each serine/threonine residue to alanine to generate singlet and triplet mutants of HGS and subsequently determined the glycosylation levels of the mutants. The results showed that while single-alanine mutants displayed comparable glycosylation levels as WT HGS, the triple-alanine mutant displayed a significant reduction of glycosylation levels (Fig. 1 *H* and *I*). The lack of appreciable change of glycosylation in the single-alanine mutants might be due to the compensatory effect by the nearby glycosylation site (22). Thus, the triple-alanine mutant (referred to as 3SA herein) will be used as the glycosylation-deficient mutant in the following study.

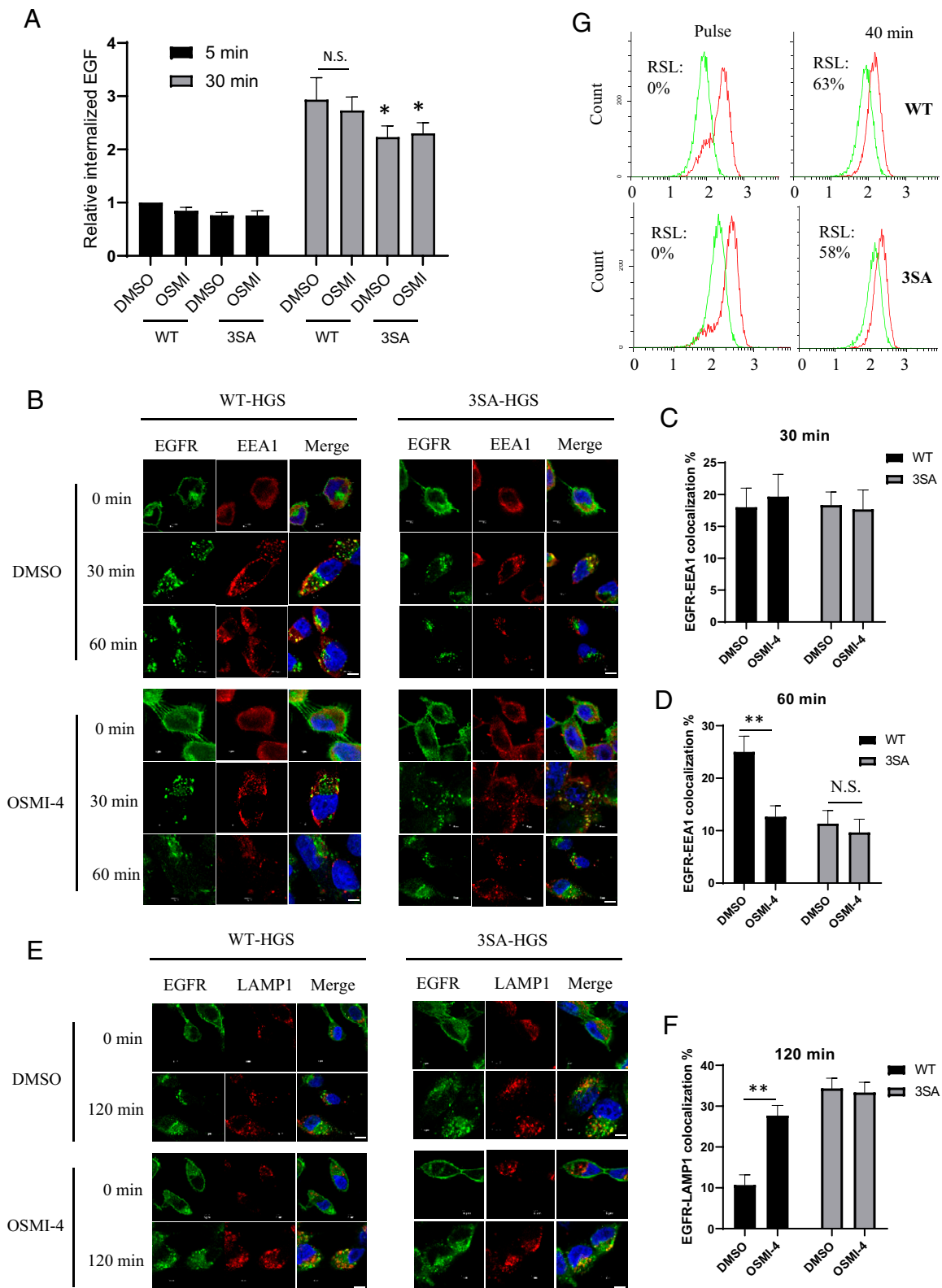**HGS O-GlcNAcylation Controls EGFR Degradation and Signaling.** HGS is a key regulator involved in RTK endosomal sorting and

lysosomal degradation (23, 24). To verify its role, we knocked down HGS expression in SK liver cancer cells with HGS-targeting small hairpin RNA (shRNA). Depletion of HGS expression blocked EGF-induced EGFR degradation (Fig. 2*A*). Re-expression of shRNA-resistant HGS in cells rescued the effect, thus verifying the specificity of the shRNA (Fig. 2*A*). EGFR mRNA levels were not altered, consistent with the notion that EGFR expression was regulated in a posttranslational manner (Fig. 2*B*).

To delineate the role of HGS O-GlcNAcylation, we generated stable rescue cell lines using SK cells, in which the WT or 3SA HGS was ectopically expressed at a comparable level with a simultaneous depletion of the endogenous HGS (Fig. 2*C*). The endogenous expression of EGFR was slightly higher in cells expressing wild-type (WT) HGS than in cells expressing 3SA HGS (*SI Appendix*, Fig. S3). In cells expressing 3SA HGS, we observed a rapid degradation of EGFR induced by EGF compared to cells expressing WT HGS (Fig. 2*D*). Consistently, EGFR autophosphorylation, ERK, and AKT activation were also reduced in cells expressing 3SA HGS (Fig. 2*D*). These results suggest that O-GlcNAcylation of HGS plays a role in EGFR degradation and signaling.

HGS O-GlcNAcylation Regulates EGFR Endosomal Trafficking and Sorting. To clarify the steps in which HGS O-GlcNAcylation regulates EGFR degradation, we first analyzed the internalization of EGFR upon EGF stimulation by quantifying the uptake of Alexa Fluor 488-labeled EGF into stable SK cells. Depletion of HGS O-GlcNAcylation whether by 3SA mutation or treatment with OSMI-4 did not block EGFR internalization (Fig. 3*A*). The amount of internalized EGF in cells expressing WT HGS was

Wu et al.
O-GlcNAcylation regulates epidermal growth factor receptor intracellular
trafficking and signaling

PNAS | 3 of 10
https://doi.org/10.1073/pnas.2107453119

Fig. 3. HGS O-GlcNAcylation regulates EGFR endosomal trafficking and sorting. (*A*) The amount of internalized EGFR quantified by the uptake of Alexa Fluor 488-labeled EGF in stable SK cells expressing WT or 3SA HGS. (*B*) Immunofluorescence staining of stable SK cells with EGFR and EEA1 antibodies at indicated time points after EGF stimulation. Scale bar, 5 μm. (*C* and *D*) Quantification of EGFR-EEA1 colocalization. Data are presented as the mean ± SD of $n = 100$ cells from three independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. **$P < 0.01$. (*E*) Immunofluorescence staining of stable SK cells with EGFR and LAMP1 antibodies 120 min after EGF stimulation. Scale bar represents 5 μm. (*F*) Quantification of EGFR-LAMP1 colocalization. Data are presented as the mean ± SD of $n = 100$ cells from three independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. **$P < 0.01$. (*G*) EGFR recycling in stable SK cells expressing WT or 3SA HGS as measured by fluorescence-activated cell sorter. Pulse, surface EGFR was measured in starved cells before the addition of EGF; 40 min, surface EGFR was measured 40 min after the pulse; RSL, relative surface level.

slightly higher than cells expressing 3SA HGS, consistent with higher EGFR levels in cells expressing WT HGS.

Next, we investigated the later endosomal trafficking steps. The internalized EGFR was targeted to the early endosome (25, 26). We analyzed the coimmunostaining of EGFR with early endosomal antigen 1 (EEA1), a cellular marker for early endosomes, after 30 min of EGF stimulation. In cells expressing WT or 3SA HGS, EGFR was colocalized with EEA1 at comparable levels, in the presence or absence of OSMI-4 (Fig. 3 *B* and *C*). This indicated that HGS O-GlcNAcylation did not affect EGFR targeting to the early endosome. However, 60 min after EGF stimulation, EGFR-EEA1 colocalization in cells expressing WT HGS was significantly greater than that in cells expressing 3SA HGS (Fig. 3 *B* and *D*). Treatment with OSMI-4 increased EGFR-EEA1 colocalization in cells expressing WT HGS, but not in cells expressing 3SA HGS (Fig. 3 *B* and *D*). These results suggest that HGS O-GlcNAcylation affects EGFR sorting from the early endosome.

From the early endosome, EGFR is further sorted to the late endosome/lysosome for degradation. Thus, we coimmunostained EGFR with lysosomal-associated membrane protein 1 (LAMP1), a cellular marker for the late endosome/lysosome (25, 26). The results showed that EGFR-LAMP1 colocalization was significantly greater in 3SA HGS-expressing cells (Fig. 3 *E* and *F*). Similar observations were shown with OSMI-4 treatment in cells expressing WT HGS, but not in cells expressing 3SA HGS. Thus, reduction of HGS glycosylation is associated with enhanced EGFR trafficking to the late endosome/lysosome, which is consistent with the increased degradation of EGFR.

Last, we quantified EGFR recycling back to the plasma membrane by flow cytometry (27). The result showed that there was no significant difference in EGFR recycling ratio between cells expressing WT or 3SA HGS, indicating that HGS O-GlcNAcylation has no effect on EGFR recycling (Fig. 3*G*).
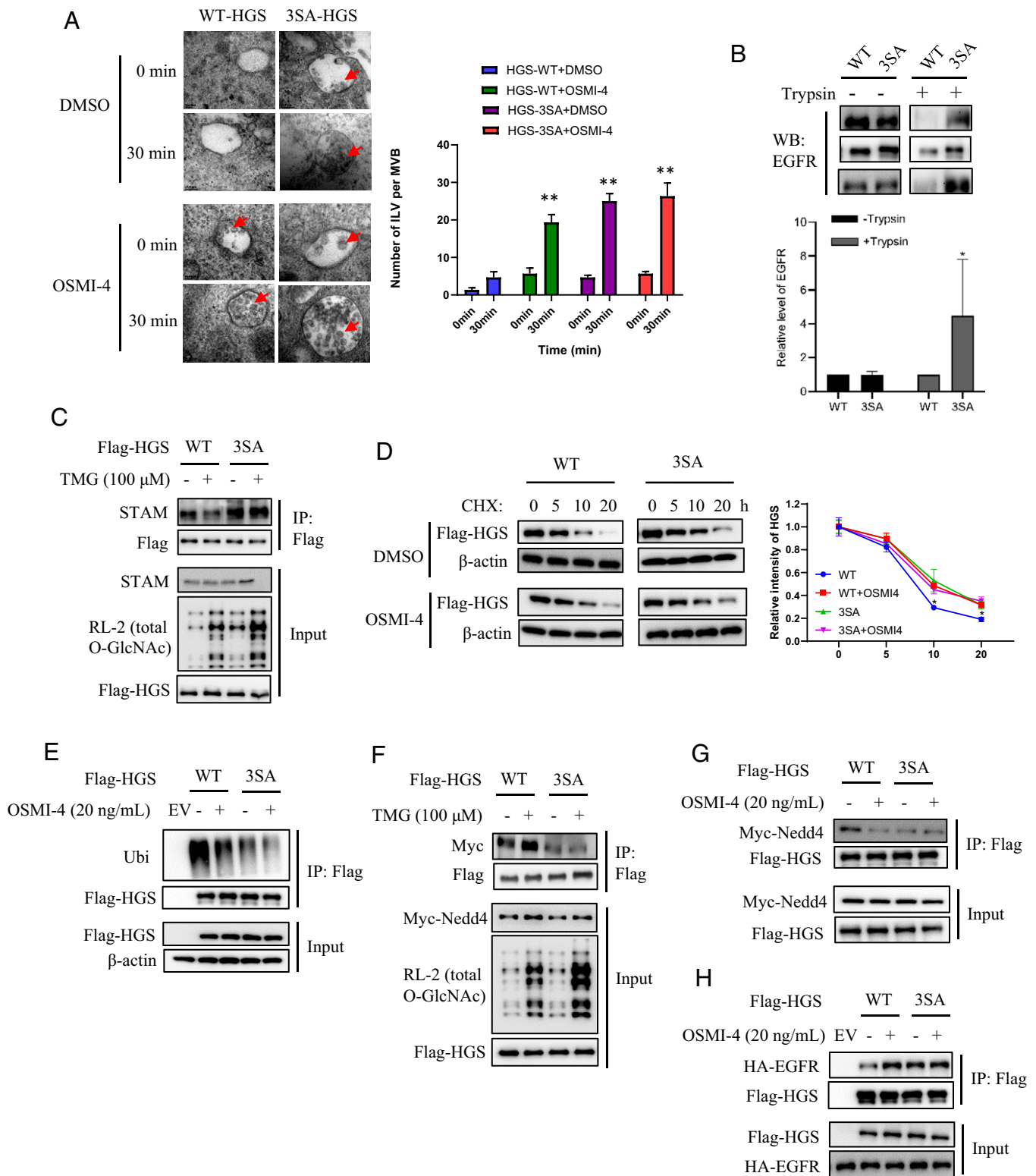
HGS O-GlcNAcylation Impairs ESCRT-0 Formation and Promotes HGS Degradation. During the trafficking from endosomes to lysosomes, EGFR is sorted into ILVs of the MVB (8). To further delineate the molecular mechanism of impeded EGFR trafficking into lysosomes, we used electron microscopy (EM) approach. Stable SK cells expressing WT or 3SA HGS were serum starved, and then treated with or without EGF for 40 min (Fig. 4*A*). EGF-induced ILV formation was significantly increased in cells expressing 3SA HGS, as compared to cells expressing WT HGS. Consistently, OSMI-4 treatment increased ILV formation in cells expressing WT HGS, but not in cells expressing 3SA HGS (Fig. 4*A*). Thus, the results indicate that sorting of EGFR into MVBs was impaired by HGS O-GlcNAcylation. To further support this notion, we performed a cell-free reconstitution assay (28, 29). In this assay, when EGFR is effectively sorted into MVB, its cytoplasmic tail domain is protected from protease digestion and can be detected using epitope-specific antibodies. In line with the EM result, the reaction system reconstituted with cytosols from cells expressing 3SA HGS showed higher EGFR immunoblotting signal in the presence of the protease (Fig. 4*B*), suggesting that more EGFR was sorted into MVB. This result further supports the notion that HGS O-GlcNAcylation impairs EGFR sorting into the MVB.

Sorting of EGFR from the early endosome into the MVB depends on the endosomal sorting complex required for transport (ESCRT) machinery, which consists of four complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III (8). HGS and STAM are two key components of ESCRT-0 that initiates the EGFR ILV sorting (10, 11). We first examined whether O-GlcNAcylation affects the targeting of HGS to the early endosome by costaining HGS with EEA1. The result showed that both WT and 3SA HGS had comparable levels of colocalization
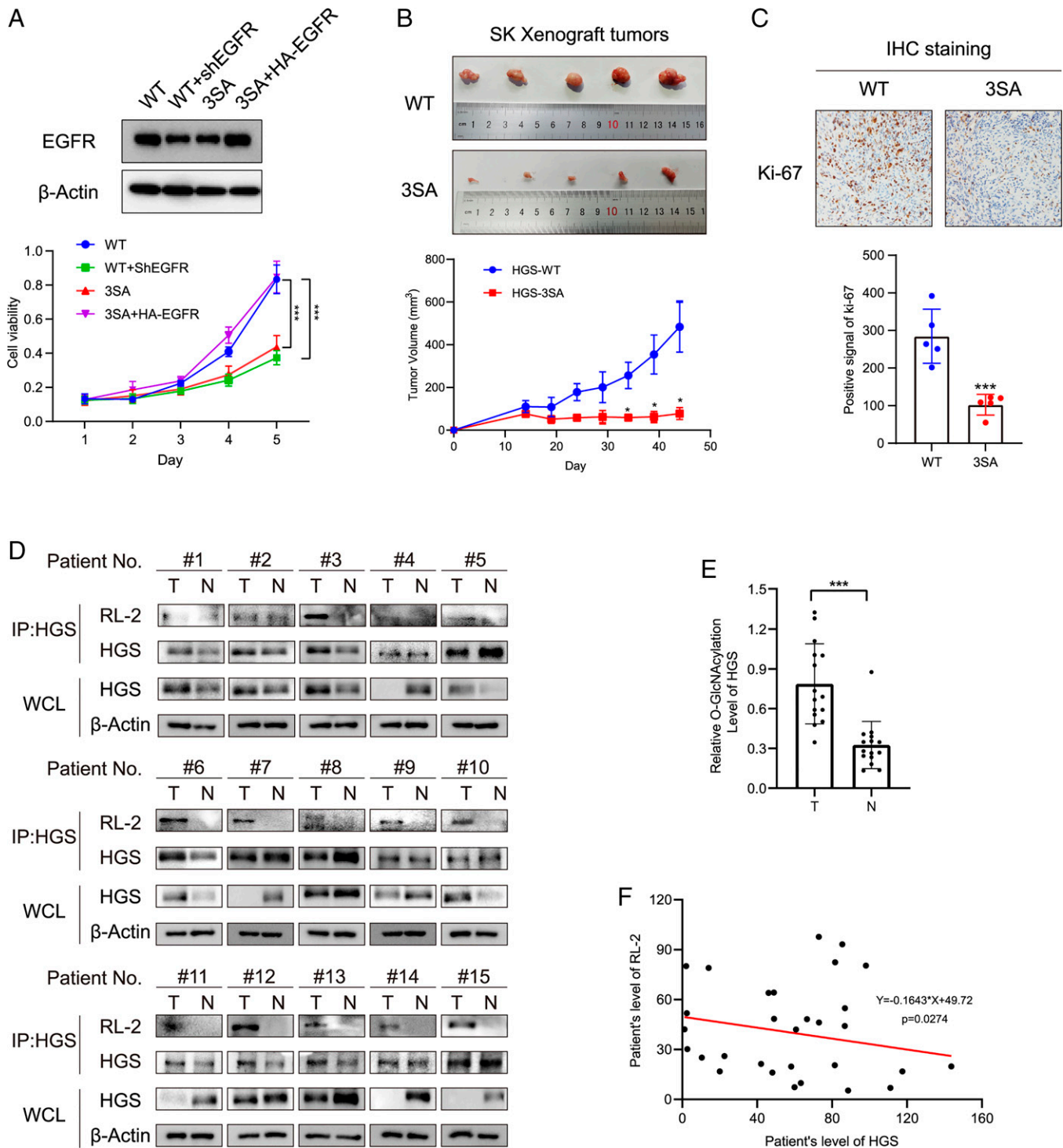
with EEA1 (*SI Appendix,* Fig. S4), indicating that O-GlcNAcylation has no effect on HGS targeting to the early endosome. Next, we examined whether HGS O-GlcNAcylation affects ESCRT-0 formation. In stable SK cell lines, the association of HGS and STAM was probed using the coimmunoprecipitation (co-IP) assay. We generated various truncated HGS proteins based on the predicted domains, and analyzed their interaction with STAM in cells (*SI Appendix,* Fig. S5). The results showed that the PSAP domain (aa 280–460) that contains the glycosylation sites (S297, S299, and S300) was indispensable for the HGS-STAM interaction. Further, 3SA HGS pulled down significantly more STAM than WT HGS did (Fig. 4*C*). Increasing O-GlcNAcylation by the treatment with TMG (30) decreased the amount of STAM being pulled down in cells expressing WT HGS, but had no effect in cells expressing 3SA HGS (Fig. 4*C*). Consistent results were obtained when the co-IP was performed in a reverse order (*SI Appendix,* Fig. S6). These results suggest that O-GlcNAcylation inhibits the HGS-STAM interaction, likely impairing ESCRT-0 formation and the subsequent ILV sorting.

We observed a higher level of mRNA expression of WT HGS, compared to 3SA HGS in stable SK rescue cell lines (*SI Appendix,* Fig. S7). Because the protein expression levels were comparable between WT and 3SA HGS (Fig. 2*C*), we speculate that O-GlcNAcylation might affect HGS protein stability. When various concentrations of OGT were transfected in cells, we observed a dose-dependent decrease in HGS protein levels with increasing OGT expression (*SI Appendix,* Fig. S8A). To further test whether O-GlcNAcylation affects HGS protein stability, we examined the degradation rate of WT and 3SA HGS in the presence or absence of OSMI-4 (Fig. 4*D*). The results showed that 3SA HGS displayed a slower degradation rate than WT HGS, and that OSMI-4 treatment slowed WT HGS degradation but had no effect on 3SA HGS, suggesting that O-GlcNAcylation promotes HGS protein degradation. Consistent with the previous report, HGS degradation was mediated through the lysosome-pathway, but not the proteosome-pathway (*SI Appendix,* Fig. S8 *B* and *C*) (31). We observed that WT HGS displayed a higher level of ubiquitination than 3SA HGS (Fig. 4*E*). Treatment with OSMI-4 significantly reduced the ubiquitination of WT HGS, but only had a small effect on 3SA HGS (Fig. 4*E*). Consistently, treatment with TMG increased the ubiquitination of WT HGS, but not on 3SA HGS (*SI Appendix,* Fig. S8D). Nedd4 was reported to be the principle E3 ubiquitin ligase for HGS in cells (25, 26). In line with the above results, we observed a stronger interaction between WT HGS and Nedd4 (Fig. 4*F* and *SI Appendix,* Fig. S8E). Treatment with TMG or OSMI-4 significantly increased or reduced WT HGS interaction, respectively, but had no effect on 3SA HGS (Fig. 4 *F* and *G*). Previous studies showed that increasing Nedd4-mediated HGS ubiquitination inhibits HGS interaction with the ubiquitinated membrane receptors, such as EGFR (32). Thus, we probed the effect of glycosylation-induced ubiquitination on HGS interaction with EGFR. Consistent with higher ubiquitination of WT HGS, WT HGS showed a weaker interaction with EGFR compared to the 3SA HGS (Fig. 4*H*). Treatment with OSMI-4 reduced WT HGS interaction, but had no effect on 3SA HGS (Fig. 4*H*). Taken together, these results suggest that O-GlcNAcylation induces HGS ubiquitination, and promotes HGS degradation in cells.

HGS O-GlcNAcylation Is Important for Cell Proliferation and Tumor Growth. To further explore the biological function of HGS O-GlcNAcylation, we performed cell proliferation assays. Stable SK rescue cells expressing WT HGS exhibited a faster cell proliferation than cells expressing 3SA HGS (Fig. 5*A*). In order to show that the difference of cell proliferation was due to the difference of EGFR expression mediated by HGS O-GlcNAcylation,

Wu et al.
O-GlcNAcylation regulates epidermal growth factor receptor intracellular trafficking and signaling

PNAS | **5 of 10**
https://doi.org/10.1073/pnas.2107453119

Fig. 4. HGS O-GlcNAcylation impairs ESCRT-0 formation and promotes HGS degradation. (*A*) ILV formation in stable SK cells expressing WT or 3SA HGS 30 min after EGF stimulation. The number of ILVs in each MVB was quantified (*n* = 50 MVBs from three biological replicates for each treatment). A two-tailed unpaired Student's *t* test was used for statistical analysis. **$P < 0.01$. (*B*) The amount of EGFR protected in a cell-free reconstitution assay with cytosols containing WT or 3SA HGS. Immunoblots of three biological replicates and the quantification were shown. (*C*) Analysis of HGS-STAM interaction in stable SK cells expressing WT or 3SA HGS in the presence or absence of TMG treatment. (*D*) Immunoblotting of HGS expression levels at different time points upon treatment with cyclohexi-mide in the presence or absence of OSMI-4 treatment. (*E*) Immunoblotting of HGS ubiquitination levels in the presence or absence of OSMI-4 treatment. EV, expression of vehicles as a negative control. (*F*) Immunoblotting of HGS-Nedd4 interaction in the presence or absence of TMG treatment. (*G*) Immunoblotting of HGS-Nedd4 interaction in the presence or absence of OSMI-4 treatment. (*H*) Immunoblotting of HGS-EGFR interaction in the presence or absence of OSMI-4 treatment. EV, expression of vehicles as a negative control. The blots are presentative of three biological replicates. Data are presented as the mean ± SD of *n* = 3 independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. *$P < 0.05$.

Wu et al.
O-GlcNAcylation regulates epidermal growth factor receptor intracellular
trafficking and signaling

Fig. 5. HGS O-GlcNAcylation promotes cell proliferation and tumor growth. (*A*) Cell proliferation analysis of stable SK cells expressing WT or 3SA HGS. (*B*) Xenograft analysis of stable SK cells expressing WT or 3SA HGS. *Top*: images of dissected tumors from mice. *Bottom*: analysis of tumor growth rate. (*C*) K_i67 staining of tumors generated from SK cells expressing WT or 3SA HGS. Quantification was shown. (*D*) Immunoblotting analysis of HGS expression and HGS O-GlcNAcylation levels from primary liver cancer tissues and the matched peritumoral tissues. (*E*) Quantification of HGS O-GlcNAcylation levels. (*F*) Analysis of the correlation of HGS O-GlcNAcylation and HGS expression levels. The blots are presentative of three biological replicates. Data are presented as the mean ± SD of $n = 3$ independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. ***$P < 0.001$.

we partially knock down EGFR in SK cells expressing WT HGS, or modestly overexpressed EGFR in SK cells expressing 3SA HGS, and further compared the cell proliferation. The results showed that EGFR knockdown reduced cell proliferation in SK cells expressing WT-HGS, while EGFR overexpression increased cell proliferation in SK cells expressing 3SA-HGS (Fig. 5*A*). Thus, this study supports the notion that the anti-proliferative effect of 3SA HGS was driven by EGFR. We next performed mouse xenograft studies to further probe the function of HGS O-GlcNAcylation on tumorigenesis in vivo. The SK rescue cell lines

Wu et al.
O-GlcNAcylation regulates epidermal growth factor receptor intracellular trafficking and signaling

PNAS | 7 of 10
https://doi.org/10.1073/pnas.2107453119

expressing WT or 3SA HGS were subcutaneously injected into immune-compromised mice, and their abilities to generate tumors were determined. Consistent with the cell proliferation results, cells expressing WT HGS formed bigger tumors and at a faster rate than cells expressing 3SA HGS (Fig. 5B). Staining with K_i-67 (a marker for cell proliferation) showed a much stronger signal in tumors generated from cells expressing WT HGS (Fig. 5C). Selected tumor tissues derived from cells expressing WT HGS showed a higher level of HGS glycosylation. The higher HGS glycosylation positively correlated with higher EGFR expression (*SI Appendix*, Fig. S9). Thus, these results demonstrate that HGS O-GlcNAcylation is important for tumor cell proliferation in vitro and in vivo.

To further investigate the clinical relevance of HGS glycosylation, we collected 15 pairs of human liver cancer tissue samples with the matched peritumoral tissue samples and compared HGS glycosylation levels. Relatively higher levels of HGS glycosylation were observed in the cancer tissues compared to the matched peritumoral tissues (Fig. 5D). Quantification confirmed the significant increase in the level of HGS glycosylation in cancer tissues (Fig. 5E). In addition, higher HGS glycosylation reversely correlated with the HGS expression levels (Fig. 5F).

HGS O-GlcNAcylation Contributes to Chemoresistance in Liver Carcinoma Cells. It was reported that aberrant activation of EGFR signaling is a crucial determinant of primary resistance to sorafenib in liver cancers (33, 34). Our previous quantitative proteomic study revealed an elevated glycosylation level on HGS in sorafenib-resistant liver cancer cell line HepG2-R, compared to the corresponding sorafenib-sensitive cell line HepG2-S (17). Considering the critical role of HGS O-GlcNAcylation in regulating EGFR degradation and signaling as revealed in this study, we postulate that HGS O-GlcNAcylation may contribute to sorafenib resistance in liver cancer cells. We first verified the upregulation of HGS glycosylation in HepG2-R cells using the chemoenzymatic labeling method. HGS O-GlcNAcylation level increased approximately threefold (with normalization of HGS expression) in the HepG2-R cells as compared to the HepG2-S cells (Fig. 6A). An estimate of 40% of HGS in the HepG2-R cells was glycosylated based on calculation of the glycosylated protein versus total HGS. Next, we investigated the effect of HGS O-GlcNAcylation on EGFR expression and downstream signaling. We used HepG2-R cells as the parental cell lines to generate stable cells expressing Flag-tagged WT or 3SA HGS with a simultaneous depletion of the endogenous HGS (*SI Appendix*, Fig. S10). Analysis of HGS glycosylation in the cell lines confirmed that 3SA HGS contained a minimal level of glycosylation (Fig. 6B). Consistent with the above results, EGFR expression and activation were significantly reduced in HepG2-R cells expressing 3SA HGS (Fig. 6C). Previous studies demonstrated that sustained activation of EGFR downstream signaling, particularly the MEK/ERK pathway, contributed to sorafenib resistance (33, 34). In line with this, the MEK/ERK pathway was found to be highly activated in HepG2-R cells, compared to HepG2-S cells (Fig. 6D). Expectedly, the MAPK activity was decreased in HepG2-R cells expressing 3SA HGS compared to cells expressing WT HGS (Fig. 6E). Finally, we investigated the effect of HGS glycosylation on sorafenib resistance. Four types of cells (HepG2-S, HepG2-R, HepG2-R/WT HGS, and HepG2-R/3SA HGS) were treated with different concentrations of sorafenib, and the cell survival was determined. The results showed that sorafenib had a comparable effect on HepG2-R cells expressing WT HGS and the parental resistant cells (Fig. 6F). HepG2-R/3SA HGS cells displayed a reduced cell viability compared to HepG2-R/WT HGS cells, indicating an enhanced drug sensitivity (Fig. 6F). Taken together, the results demonstrate that HGS

O-GlcNAcylation plays an important role in regulating sorafenib resistance of liver carcinoma cells.

Discussion

Hundreds of ubiquitinated membrane protein receptors are down-regulated through a highly conserved intracellular trafficking pathway involving endosomal targeting, MVB sorting and lysosomal degradation (35). Such mechanisms are critical for maintaining proper cell signaling and metabolism. Here, we show that O-GlcNAcylation, a prevalent form of protein glycosylation, positively regulates EGFR signaling by blocking EGF-induced EGFR intraluminal sorting and degradation (Fig. 6G). Mechanistically, O-GlcNAcylation of HGS inhibits ESCRT-0 complex formation, a key regulatory point of intraluminal sorting process, by decreasing HGS-STAM interaction and promoting lysosomal degradation of HGS. HGS has been shown to regulate the degradation of many membrane proteins, including VEGR, G protein-coupled receptors, and E-cadherin (36–38). Thus, our study suggests a general mechanism by which O-GlcNAc regulates the prosurvival signaling in cells.

The process of endosomal trafficking of activated membrane receptors is initiated by the ubiquitination of the receptors. Subsequent trafficking pathways are governed by the evolutionarily conserved ESCRT machinery. Among the various ESCRT complexes, ESCRT-0 functions to capture ubiquitinated protein cargos and target them to the early endosome. ESCRT-0 consists of two major subunits HGS and STAM. HGS is shown to recognize ubiquitinated protein cargos through its intrinsic ubiquitin-interacting motif (UIM). This interaction is critical for the initiation of the intraluminal sorting and degradation process. On the other hand, HGS itself can be ubiquitinated by the E3 ligase Nedd4. HGS ubiquitination forms an intramolecular interaction with its intrinsic UIM (35), which in turns blocks the interaction with the ubiquitinated protein cargos. Previous studies have shown that increasing HGS ubiquitination blocks the ILV sorting of EGFR by inhibiting the interaction between HGS and EGFR (25, 32). In our study, we showed that WT HGS possesses higher ubiquitination level than 3SA HGS, suggesting that O-GlcNAcylation renders HGS a better substrate for Nedd4 to promote its ubiquitination. Consistently, more Nedd4 were immunoprecipitated with WT HGS than with 3SA HGS. Consequently, the glycosylation-induced ubiquitination inhibits HGS-EGFR interaction in cells, which likely perturbs the ILV sorting of EGFR. On the other hand, higher ubiquitination renders HGS more susceptible to lysosomal degradation. Interestingly, O-GlcNAcylation has been more commonly found to stabilize protein substrates by antagonizing ubiquitination (39). Our finding that O-GlcNAcylation of HGS induces its ubiquitination and promotes HGS degradation is somewhat surprising. Notably, this destabilizing effect of O-GlcNAcylation has also been recently reported in receptor-interacting protein kinase 3 (RIPK3) (40), and forkhead box protein A2 (FoxA2) (41). Thus, our results provide an additional layer of regulation for the cell fate of membrane receptors.

O-GlcNAcylation is a dynamic and reversible chemical modification of numerous intracellular proteins. Uridine-diphospho N-acetylglucosamine (UDP-GlcNAc), the direct enzymatic substrate for O-GlcNAcylation, is biosynthesized through the metabolism of several major nutrients in cells, including glucose, glutamine, acetyl-CoA, and uridine. The cellular level of O-GlcNAcylation is highly responsive to the oscillation of nutrient concentrations, and thus, O-GlcNAcylation is considered as a nutrient-sensor in cells (12, 13). The occurrence of O-GlcNAcylation on various types of proteins such as transcription factors, metabolic enzymes, and oncogenes suggests a key mechanism to link cellular nutrient sensing to different cell functions. O-GlcNAcylation of HGS functions to inhibit
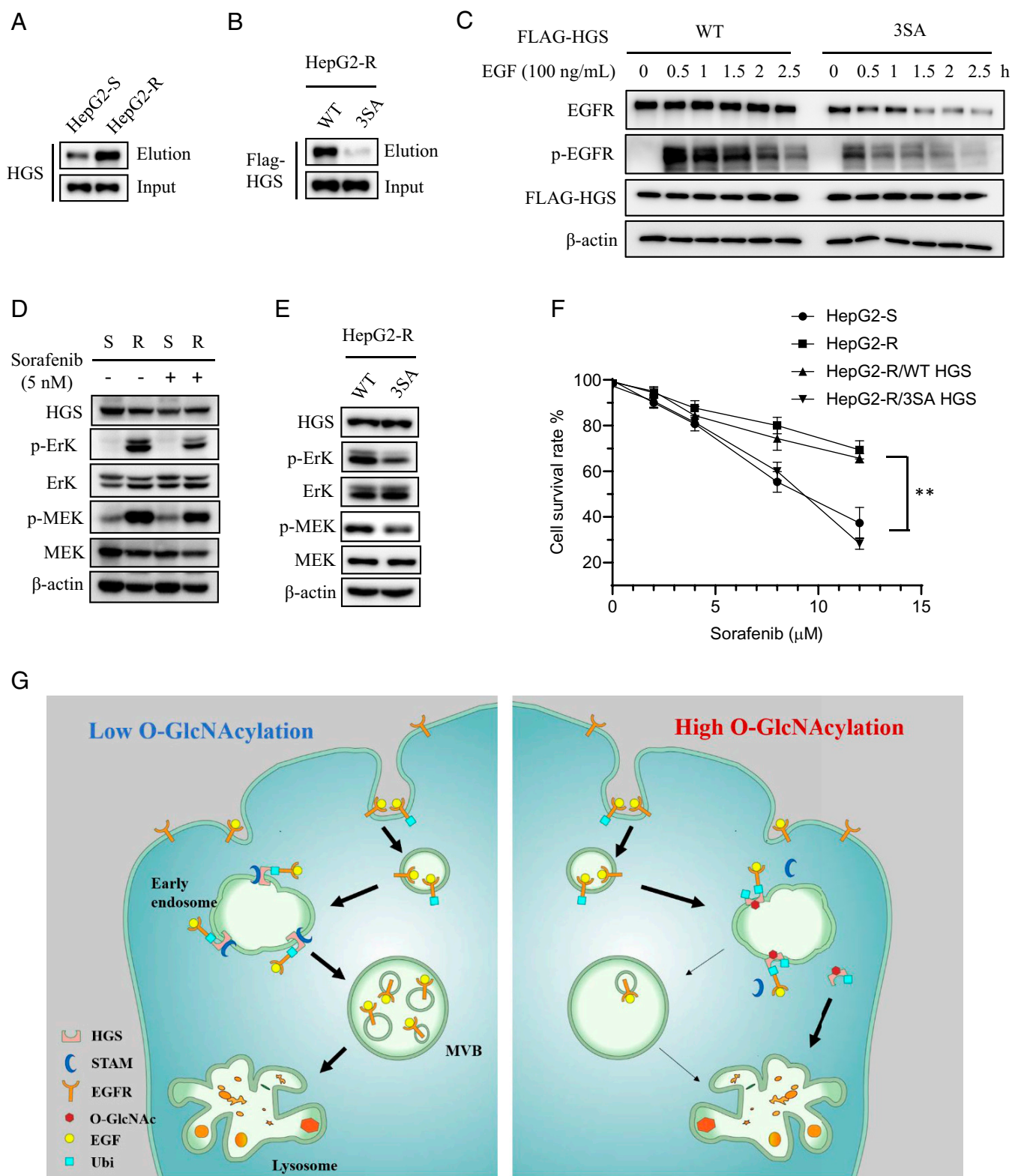
Fig. 6. HGS O-GlcNAcylation contributes to chemoresistance in liver carcinoma cells. (*A*) Analysis of HGS O-GlcNAcylation levels in sorafenib-sensitive and sorafenib-resistant HepG2 cells using the chemoenzymatic method, and blotted with an antibody against HGS. (*B*) Analysis of HGS O-GlcNAcylation levels in sorafenib-resistant HepG2 cells stably expressing Flag-tagged WT or 3SA HGS, as measured by the chemoenzymatic method and blotted with the Flag antibody. (*C*) Immunoblotting of EGFR expression and EGFR activation in HepG2-R cells stably expressing WT or 3SA HGS. (*D*) Immunoblotting of HGS expression and MAPK signaling activation in sorafenib-sensitive and sorafenib-resistant HepG2 cells. (*E*) Immunoblotting of MAPK signaling activation in HepG2-R cells stably expressing WT or 3SA HGS. (*F*) Cell survival rates of HepG2-S, HepG2-R, and HepG2-R cells stably expressing WT HGS, and HepG2-R cells stably expressing 3SA HGS, in the presence of various concentrations of sorafenib. The blots are presentative of three biological replicates. Data are presented as the mean ± SD of $n = 3$ independent experiments. A two-tailed unpaired Student's *t* test was used for statistical analysis. **$P < 0.01$. (*G*) A graphical model of the role of HGS O-GlcNAcylation on EGFR intracellular trafficking.

Wu et al.
O-GlcNAcylation regulates epidermal growth factor receptor intracellular trafficking and signaling

PNAS | 9 of 10
https://doi.org/10.1073/pnas.2107453119

downregulation of cell surface receptors to support a prolonged duration of growth factor signaling. This may represent an important mechanism linking nutrient sensing to cell growth signaling. This study also provides an important insight into the development and progression of diseases with aberrant growth factor signaling, such as cancer, obesity, and diabetes.

Methods

Cell Culture and Tumor Tissues. Cell lines 293T, SK, Hhu7, and HepG2 were all obtained from ATCC. Cell lines were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone) or high glucose Dulbecco's Modified Eagle Medium (DMEM) medium (HyClone), which contained 10% fetal calf serum (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin, in a humidified cell incubator at 37 °C with an atmosphere of 5% CO_2. Sorafenib-resistant HepG2 cells (HepG2-R) were generated by selection with sorafenib (Selleckchem) starting at 2 μM and increasing to 10 μM over the course of 3 mo. Liver tumor tissues and the matched peritumoral tissues from the same patient were obtained from the First Affiliated Hospital of Zhejiang University (Hangzhou, China). All patients were registered at the First Affiliated Hospital of Zhejiang University, and the informed consent was obtained from each patient before the study. These specimens were examined and diagnosed by pathologists. The research protocol was approved by the Ethic Committee of the School of Medicine, Zhejiang University (Hangzhou, China). The entire experimental protocol was conducted in compliance with the institutional guidelines.

Statistical Analysis. Data are presented as the means ± SDs. Statistical analyses were performed by unpaired two-tailed Student's *t* tests using GraphPad Prism 8. Data were derived from at least three independent biological replicate experiments and are presented as the mean ± SD; a value of $P < 0.05$ was considered statistically significant.

All other experimental methods and data are provided in *SI Appendix*.

1. M. A. Lemmon, J. Schlessinger, Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 (2010).
2. J. Mendelsohn, J. Baselga, Epidermal growth factor receptor targeting in cancer. *Semin. Oncol.* **33**, 369–385 (2006).
3. M. K. Nicholas, R. V. Lukas, N. F. Jafri, L. Faoro, R. Salgia, Epidermal growth factor receptor - mediated signal transduction in the development and therapy of gliomas. *Clin. Cancer Res.* **12**, 7261–7270 (2006).
4. D. S. Salomon, R. Brandt, F. Ciardiello, N. Normanno, Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.* **19**, 183–232 (1995).
5. W. Cheng, Y. Hu, R. Sheng, Development of EGFR family small molecule inhibitors for anticancer intervention: An overview of approved drugs and clinical candidates. *Curr. Med. Chem.* **21**, 4374–4404 (2014).
6. A. V. Vieira, C. Lamaze, S. L. Schmid, Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274**, 2086–2089 (1996).
7. A. Tomas, C. E. Futter, E. R. Eden, EGF receptor trafficking: Consequences for signaling and cancer. *Trends Cell Biol.* **24**, 26–34 (2014).
8. C. Raiborg, H. Stenmark, The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**, 445–452 (2009).
9. D. J. Katzmann, M. Babst, S. D. Emr, Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **106**, 145–155 (2001).
10. K. G. Bache, A. Brech, A. Mehlum, H. Stenmark, Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell Biol.* **162**, 435–442 (2003).
11. G. Prag *et al.*, The Vps27/Hse1 complex is a GAT domain-based scaffold for ubiquitin-dependent sorting. *Dev. Cell* **12**, 973–986 (2007).
12. G. W. Hart, M. P. Housley, C. Slawson, Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* **446**, 1017–1022 (2007).
13. X. Yang, K. Qian, Protein O-GlcNAcylation: Emerging mechanisms and functions. *Nat. Rev. Mol. Cell Biol.* **18**, 452–465 (2017).
14. I. O. Bolanle, K. Riches-Suman, R. Williamson, T. M. Palmer, Emerging roles of protein O-GlcNAcylation in cardiovascular diseases: Insights and novel therapeutic targets. *Pharmacol. Res.* **165**, 105467 (2021).
15. N. M. Akella, L. Ciraku, M. J. Reginato, Fueling the fire: Emerging role of the hexosamine biosynthetic pathway in cancer. *BMC Biol.* **17**, 52 (2019).
16. W. B. Dias, G. W. Hart, O-GlcNAc modification in diabetes and Alzheimer's disease. *Mol. Biosyst.* **3**, 766–772 (2007).
17. J. Li *et al.*, An isotope-coded photocleavable probe for quantitative profiling of protein O-GlcNAcylation. *ACS Chem. Biol.* **14**, 4–10 (2019).
18. J. W. Thompson, M. E. Griffin, L. C. Hsieh-Wilson, Methods for the detection, study, and dynamic profiling of O-GlcNAc glycosylation. *Methods Enzymol.* **598**, 101–135 (2018).
19. A. Estevez, D. Zhu, C. Blankenship, J. Jiang, Molecular interrogation to crack the case of O-GlcNAc. *Chemistry* **26**, 12086–12100 (2020).
20. Y. Liu *et al.*, Proteomic profiling and genome-wide mapping of O-GlcNAc chromatin-associated proteins reveal an O-GlcNAc-regulated genotoxic stress response. *Nat. Commun.* **11**, 5898 (2020).
21. M. Martinez *et al.*, Quantitative proteomics reveals that the OGT interactome is remodeled in response to oxidative stress. *Mol. Cell. Proteomics* **20**, 100069 (2021).
22. X. Li *et al.*, O-GlcNAcylation of core components of the translation initiation machinery regulates protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 7857–7866 (2019).
23. C. Raiborg, K. G. Bache, A. Mehlum, E. Stang, H. Stenmark, Hrs recruits clathrin to early endosomes. *EMBO J.* **20**, 5008–5021 (2001).
24. M. Komada, N. Kitamura, The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. *J. Biochem.* **137**, 1–8 (2005).
25. Y. Sun, A. C. Hedman, X. Tan, N. J. Schill, R. A. Anderson, Endosomal type Iγ PIP 5-kinase controls EGF receptor lysosomal sorting. *Dev. Cell* **25**, 144–155 (2013).
26. X. Tan *et al.*, LAPTM4B is a PtdIns(4,5)P2 effector that regulates EGFR signaling, lysosomal sorting, and degradation. *EMBO J.* **34**, 475–490 (2015).
27. S. Sigismund *et al.*, Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev. Cell* **15**, 209–219 (2008).
28. W. Sun *et al.*, Cell-free reconstitution of multivesicular body formation and receptor sorting. *Traffic* **11**, 867–876 (2010).
29. N. Sirisaengtaksin *et al.*, UBE4B protein couples ubiquitination and sorting machineries to enable epidermal growth factor receptor (EGFR) degradation. *J. Biol. Chem.* **289**, 3026–3039 (2014).
30. M. G. Alteen, H. Y. Tan, D. J. Vocadlo, Monitoring and modulating O-GlcNAcylation: Assays and inhibitors of O-GlcNAc processing enzymes. *Curr. Opin. Struct. Biol.* **68**, 157–165 (2021).
31. J. Zhang, J. Du, C. Lei, M. Liu, A. J. Zhu, Ubpy controls the stability of the ESCRT-0 subunit Hrs in development. *Development* **141**, 1473–1479 (2014).
32. E. R. Eden, F. Huang, A. Sorkin, C. E. Futter, The role of EGF receptor ubiquitination in regulating its intracellular traffic. *Traffic* **13**, 329–337 (2012).
33. Z. Ezzoukhry *et al.*, EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *Int. J. Cancer* **131**, 2961–2969 (2012).
34. M. J. Blivet-Van Eggelpoël *et al.*, Epidermal growth factor receptor and HER-3 restrict cell response to sorafenib in hepatocellular carcinoma cells. *J. Hepatol.* **57**, 108–115 (2012).
35. D. Hoeller *et al.*, Regulation of ubiquitin-binding proteins by monoubiquitination. *Nat. Cell Biol.* **8**, 163–169 (2006).
36. L. K. Hasseine *et al.*, Hrs is a positive regulator of VEGF and insulin signaling. *Exp. Cell Res.* **313**, 1927–1942 (2007).
37. M. Toyoshima *et al.*, Inhibition of tumor growth and metastasis by depletion of vesicular sorting protein Hrs: Its regulatory role on E-cadherin and beta-catenin. *Cancer Res.* **67**, 5162–5171 (2007).
38. A. C. Hanyaloglu, E. McCullagh, M. von Zastrow, Essential role of Hrs in a recycling mechanism mediating functional resensitization of cell signaling. *EMBO J.* **24**, 2265–2283 (2005).
39. W. Qin *et al.*, Quantitative time-resolved chemoproteomics reveals that stable *O*-GlcNAc regulates box C/D snoRNP biogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E6749–E6758 (2017).
40. B. Zhang *et al.*, O-GlcNAc transferase suppresses necroptosis and liver fibrosis. *JCI Insight* **4**, e127709 (2019).
41. H. Huang *et al.*, O-GlcNAcylation promotes the migratory ability of hepatocellular carcinoma cells via regulating FOXA2 stability and transcriptional activity. *J. Cell. Physiol.* **236**, 7491–7503 (2021).