Methods

Cell Culture and tumor tissues.

Cell lines 293T, Hela, MCF-10A, MCF-12A, Hs-578-T, MCF-7, MDA-MB-361, MDA-MB-453, MDA-MB-468 and 4T1 were all purchased from American Type Cell Culture (ATCC). MCF-10A cells were cultured in DMEM/F12 (Gibco) supplemented with 5% horse serum (Gibco), 10ug/ml insulin, 20ng/ml EGF and 0.5ug/ml hydrocortisone. MCF-12A and 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco). 293T, Hela, Hs-578-T, MCF-7, MDA-MB-361, MDA-MB-453 and MDA-MB-468 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum. All of above cells were placed in the incubator at 37°C under 5% CO_2. All cells were subjected to validation to ascertain the absence of mycoplasma contamination, and their authenticity was confirmed through short tandem repeat profiling. Breast cancer specimens and corresponding tumor-adjacent normal tissues were acquired from patients at the women's hospital, Zhejiang University School of Medicine. (Zhejiang, China). The protocols involving human participants were sanctioned by the Ethical Committee of the School of Medicine at Zhejiang University.

Immunoprecipitation and immunoblotting analysis

The cells were lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate supplemented with 20 μM Thiamet-G (MedChemExpress, HY-12588) and a protease inhibitor cocktail (Roche). Flag-tagged proteins were purified by incubation with Anti-Flag-M2 beads for 6 hours at 4°C. Then the beads were washed three times with ice-cold H150 buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, and 10% glycerol and eluted with Flag peptides obtained from GenScript (RP10586) in a PBS buffer for 6 hours at 4°C. For immunoblotting analysis, around 40 μg of protein lysate was loaded onto a 6–15% SDS-PAGE gel. The specified antibodies used in immunoblotting were listed in Supplementary Table 1. The quantification of protein band intensities was carried

out by Image J.

Immunofluorescence

Hela cells transfected with plasmids and treated with DMSO or TMG were seeded on glass coverslips or glass-bottom dishes (8001002, NEST). Cells were washed with PBS three times and fixed with 4% paraformaldehyde for 20 min at room temperature. Then cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 3% bovine serum in PBS for 1h. After incubation, cells were stained with first antibody at 4℃ overnight, followed by washed with 0.05% PBST and incubated with fluorescent dye-conjugated secondary antibodies. DAPI (Sigma-Aldrich) was used to stain the nuclei. Images were collected by Zeiss LSM980 confocal microscope system.

Recombinant protein expression and purification.

Both the wild-type BAP1 and BAP1-C91S were subcloned into the prokaryotic GST-fused expression vector pGEX-4T-1. These constructs were then transformed into E. coli strain BL21 (DE3), and recombinant protein expression was induced by adding 1 mM isopropyl-β -D-thiogalactoside for an overnight incubation at 15 °C. The cells were then collected and resuspended in a 50 ml lysis buffer (50 mM Tris-HCl at pH 8.0, 140 mM NaCl, 20 mM EDTA, 3% Triton X-100, 5 mM dithiothreitol) for 30 minutes. Followed by sonication, the lysis was incubated with GST-tag Purification Resin (Beyotime) for 6 hours at 4°C and subsequently loaded onto an affinity chromatography column. After washing three times with lysis buffer, the proteins were eluted with the elution buffer (50 mM Tris-HCl, 150 mm NaCl,10 mm GSH, pH 8.0) and quantified by coomassie staining.

Analysis of protein O-GlcNAcylation.

The analysis of BAP1 and FOXM1 O-GlcNAcylation was conducted by a chemoenzymatic labeling approach as previously described. In brief, 300 μg of cellular lysates were labeling with GalNAz using the β-1,4-galactosyltransferase GalT (Y289L). Control experiments were simultaneously executed in the absence of GalT or UDP-GalNAz. The proteins were re-suspended in 50 μL 50 mM Tris-HCl (pH 8.0) containing 1% SDS and incubated with 50 μM $CuSO_4$-BTTAA premixed complex

($CuSO_4$-BTTAA, molar ratio 1:2), 100 μM alkyne-biotin, and 2.5 mM fresh sodium ascorbate for click reaction at 37 °C for 2 h. The biotin-labeled proteins were purified using streptavidin resin (Pierce) overnight at 4 °C. The beads were washed with a low-salt buffer containing 100 mM Na2HPO4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 0.5% sodium deoxycholate, followed by an additional three washes with a high-salt buffer containing 100 mM Na2HPO4, 500 mM NaCl, and 0.2% TritonX-100. Subsequently, protein O-GlcNAcylation was detected by Western blotting.

Identification of ubiquitination sites of OGT by LC-MS/MS

Flag-tagged ΔTPR OGT was overexpressed in MDA-MB-468 cells. After cell lysis, Anti-FLAG® M2 beads (Sigma) were used to enrich OGT. Then SDS-PAGE electrophoresis was performed to prepare gel strips for MS analysis. For in-gel tryptic digestion, gel strips were destained in 25 mM NH_4HCO_3 in 50% acetonitrile (v/v) until clear. Gel strips were dehydrated with 100 μl of 100% acetonitrile for 5 min, followed by incubation with 25 mM dithiothreitol at 55 ℃ for 45 min. Gel strips were dehydrated again in 100% acetonitrile, then rehydrated with 55 mM iodoacetamide at room temperature, in the dark for 30 min. Gel strips were washed with 50 mM NH_4HCO_3 and dehydrated with 100% acetonitrile. Gel strips were resuspended with 50 mM NH_4HCO_3 containg 12.5 ng/μl trypsin and incubated on ice for 45min. Excess liquid was removed and gel strips were digested with trypsin at 37 ℃ overnight. After digestion, peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100% acetonitrile. Finally, peptides were frozen and lyophilized. Peptides were resuspended in 2% acetonitrile/0.1% formic acid for LC-MS/MS analysis.

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (30 cm, 100 μm i.d.) packed with 1.9 μm Reprosil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) . The gradient was comprised of an increase from 3% to 10% solvent B (0.1% formic acid in 98% acetonitrile) over 3 min, 10% to 24% in 37 min, 24% to 38% in 12 min and climbing to 80% in 4 min then holding at 80% for the last 4 min. All at a constant flow rate of 300 nl/min on an UltiMate 3000 nanoLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry

(MS/MS) in Orbitrap Exploris 480 (Thermo Fisher Scientific,) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 400 to 1200 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 60,000. Peptides were then selected for MS/MS using NCE setting as 27 and the fragments were detected in the Orbitrap at a resolution of 15,000. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 30s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Compensation voltage for FAIMS was set to −45V.

Analysis of metabolite by HPLC-MS/MS

A total of 2 × 10^7cells were collected and lysed in methanol through vigorous vortexing and incubated on ice for 10 minutes. The lysis was centrifuged at 18,000 g at 4 °C for 30 min and the supernatant was diluted to achieve a final composition comprising 53% LC-MS grade water in methanol. The LC-MS/MS analyses were performed using an ExionLC™ AD system (SCIEX) in conjunction with a QTRAP® 6500+ mass spectrometer (SCIEX). Samples were introduced onto a Xselect HSS T3 column (2.1×150 mm, 2.5 μm) by a 30-minute linear gradient at a flow rate of 0.4 mL/min for both positive and negative polarity modes. The eluents utilized were eluent A (0.1% Formic acid-water) and eluent B (0.1% Formic acid-acetonitrile). The QTRAP® 6500+ mass spectrometer operated in positive polarity mode with Curtain Gas maintained at 40 psi, Collision Gas set at Medium, IonSpray Voltage at 5500V, Temperature at 550 degrees Celsius, and Ion Source Gas 1 and 2 at 60 units each. In the negative polarity mode, the settings were as follows: Curtain Gas at 35 psi, Collision Gas at Medium, IonSpray Voltage at -4500V, Temperature at 550 degrees Celsius, Ion Source Gas 1 and 2 at 60 units each. The quantification of metabolite abundance was conducted by normalizing to protein levels.

Lentiviral-Mediated shRNA Interference.

Lentiviruses expressing shBAP1, shOGT, or scramble were generated by 293T cells. MDA-MB468 was chosen to establish stable BAP1 or OGT knockdown models. Infected cells were selected with 4ug/ml puromycin for 1 weeks or more. The

knockdown efficiency was measured by RT-qPCR and western blotting. All shRNA sequences were listed in Supplementary Table 2.

Generation of BAP1 reconstituted stable cell lines.

The shRNA sequence targeting the 3' untranslated region of the BAP1 gene was inserted into Plko.1 vector that affords shRNA-mediated knockdown of endogenous BAP1. To generate BAP1 reconstituted stable cell lines, the rescued BAP1 sequence (N-terminal-HA-tagged WT or 5A) was inserted into the plvx vector. The sequences were supplied in Supplementary Table 2. Lentiviruses expressing BAP1 WT or 5A were used to infect the BAP1 knockdown cells. Cells were selected with 2ug/ml puromycin for 2 weeks to obtain BAP1 reconstituted stable cell lines. Knockdown and over-expressing efficiency were detected by western blotting.

Cell proliferation assay

Cell proliferation ability was measured by Cell Counting Kit-8 (Beyotime, C0039). 2×10^3 cells were placed into a 96-well plate per well. CCK-8 solution was added followed by incubation for 2h at 37℃. Absorbance was detected at 450 nm using the Multi-Mode Plate Reader (BioTek).

Analysis of DNA synthesis

To analyze DNA synthesis, BeyoClick™ EdU-594 Cell Proliferation Assay Kit (Beyotime, C0078S) was used. A total of 5×10^5 cells were seeded in a 6-well plate per well for 24h. Then cells were incubated with 10 μM EdU buffer at 37℃ for 3h. After incubation, cells were fixed with 4% formaldehyde for 15 min and permeabilized with 0.25% Triton X-100 for 15 min at room temperature. Then the click reaction was performed followed by the staining of nuclei with Hoechst. The results were visualized by a fluorescence microscope.

Cell migration and invasion assays.

Migration and invasion assays were performed in the 24-well plates inserted with 8 μm pore size transwell filters (JET, TCS020024). For invasion assays, the filters were pre-coated with 1:10 diluted Matrigel (Corning, 356234). To eliminate the difference of proliferation rate between cells, Mitomycin C (MedChemExpress, HY-13316) was used to pretreat cells for 1 h at 37℃. 1×10^5 cells with 200 μl serum-free medium were

seeded in the upper chamber, and complete medium containing 10% FBS was added into the bottom chamber. After incubation at 37℃ for 24h (migration) or 72h (invasion), invaded cells on the underside of the membrane were fixed in 4% paraformaldehyde (Beyotime, P0099) and stained with crystal violet solution (Beyotime, C0121).

Determination of OGT half-life

To analyze the half-life of OGT, we treated PI3K WT or PI3K-H1047R cells with 50 μM cycloheximide (CHX) (MedChemExpress, 66-81-9) to suppress the synthesis of new proteins. The stated time points (0, 2, 4, 6, or 8 hours) were set to harvest the cells after treatment. Then OGT expression levels were analyzed by western blotting, and the relative half-life was calculated.

Cellular FRAP assays

FRAP experiments were conducted under a Zeiss LSM980 confocal microscope system in a live-cell incubation chamber at 37℃. Hela cells expressing FOXM1-WT-GFP or FOXM1-S394A-GFP protein were treated with or without TMG (100μM) for 24h on chambered cover glass. Then FOXM1 protein was photobleached with a 488 nm laser at 50% power for 2s. After bleaching, time-lapse images were captured for 40s in living cells.

RNA-seq analysis

The extraction of total RNA was performed using the Trizol method, followed by the evaluation of RNA sample integrity using the Qsep400 instrument. Subsequently, the construction of RNA libraries was achieved using the MGIEasy mRNA Library Prep Kit, where in 3 μg of total RNA were employed. To ensure the quality of the data, adapter sequences and low-quality reads were eliminated through the application of Cutadapt (version 1.11). The resulting clean reads were then aligned to the designated genomes using Hisat2 (version 2.1.0), permitting a maximum of two mismatches. Subsequently, these genes underwent alignment against public protein databases, specifically the NR (RefSeq non-redundant proteins) database.Estimation of transcript

abundance and normalization of expression values were performed by FeatureCount (v1.6.0), reporting values as FPKM (Fragments per kilobase of transcript per million fragments mapped). Identification of differentially expressed genes was carried out using edgeR, with stringent filtering criteria (FDR<0.05 and |log2FoldChange| > 1). The R package ClusterProfiler was harnessed for GO and KEGG enrichment analysis, leveraging the hypergeometric distribution with a Q value threshold of 0.05. The Q values derived from Fisher's exact test were subjected to multiple comparisons using FDR. The RNA sequencing data in this study have been submitted to the NCBI Gene Expression Omnibus (GEO) datasets with accession number GSE280765.

Measurement of glucose uptake

Analysis of glucose uptake in cells was performed using the Glucose Uptake Assay Kit (ab136955). In brief, cells are subjected to glucose deprivation and 2-DG is introduced to incubate for 20 minutes at 37°C to facilitate glucose uptake. Then cells are washed with PBS to eliminate extracellular 2-DG and lysed using an extraction buffer and vigorous pipetting, followed by a freeze-thaw cycle and heating at 85°C for 40 minutes to ensure complete lysis. The mixture is then cooled on ice for 5 minutes. Neutralizing buffer is added followed by centrifugation to separate the soluble fraction, which is transferred to fresh tubes. Both the supernatant samples and standard solutions are dispensed into designated microplate wells. Reaction Mix A is added and incubated at 37°C for an hour, fostering NADPH generation. An additional step involving the addition of extraction buffer and heating to 90°C for 40 minutes precedes another cooling period on ice, neutralization, and finally, the introduction of Reaction Mix B. The absorbance at 412 nm was monitored by the Multi-Mode plate reader and normalized to protein concentration.

CUT &Tag analysis

The CUT &Tag assays were conducted with NovoNGS® CUT&Tag 4.0 High-Sensitivity Kit (for Illumina®) (NovoProtein, N259-YH01). According to the manufacturer's instruction, 1×10^5 MDA-MB 468 cells (reconstituted BAP1 WT and 5A) were harvested and lysed with 100 μL ice-cold lysis buffer to collect the nuclei. Then the nuclei were washed twice and incubated with concanavalin A beads. After

incubation, the beads were incubated with primary antibody against OGT overnight, and then secondary antibody at room temperature for 1 hour. Beads were washed twice and incubated with ChiTag pAG-Tn5 for 1 h. Then tagmentation was performed at 37℃ for 1 h and stopped at 55 ℃ for 2 h with stop buffer. DNA fragments were enriched using Tagment DNA Extraction Beads and then underwent PCR amplifications. PCR products were purified using NovoNGS® DNA Clean Beads and sequencing were performed using Illumina NovaSeq 6000 platform.

CUT &Tag data processing

The raw sequencing data were cleaned by fastp (version 0.20.0) to filter low quality reads. BWA (Burrows Wheeler Aligner) and reference human genome (GRCh38.p13) were used to perform reads mapping. In addition, non-specific amplification was excluded by using *E.coli* carry-over DNA from pAG-Transposome as the spike-in. The proportion of detected ecoli data is used as a reference to normalize the data. Peaks were called using MACS2 (Yong Zhang,Tao Liu et al., 2008) and annotated using PeakAnnotator (Salmon-Divon and Dvinge et al., 2010). Heatmap showing reads around the center of OGT binding was generated using deeptools (version 3.02). ChIPseeker was used to analyze the distribution of peak regions on gene functional elements. The CUT &Tag sequencing data in this study have been submitted to the NCBI GEO datasets with accession number GSE280766.

Deubiquitination assays.

The in vitro deubiquitination assay was performed as previously described. HEK293T cells were transfected with plasmids encoding HA-tagged Ubiquitin (HA-Ub) and Flag-tagged OGT. Two days post-transfection, cells were subjected to a 6-hour incubation with 20 μM MG132 to increase production of polyubiquitinated OGT proteins. Subsequently, immunoprecipitation was carried out using Flag-M2 agarose beads to isolate Flag-tagged polyubiquitinated OGT proteins. These purified proteins were then added into a deubiquitination reaction mixture containing either GST alone, GST-BAP1, or the mutant variant GST-BAP1-C91S, all suspended in a specifically formulated deubiquitination buffer comprising 50 mM Tris-HCl (pH 8.0), 100 mM

sodium chloride, 5 mM magnesium chloride, 1 mM ATP, and 1 mM DTT. This reaction proceeded for 6 hours at 37°C, ultimately being quenched with the addition of SDS sample buffer. To ascertain the state of OGT ubiquitination post-deubiquitination reaction, western blot analysis was performed using anti-HA antibody.

Immunohistochemistry staining

Immunohistochemical (IHC) analysis was performed as previously described. Tissue specimens were first immobilized in a fixative solution composed of 4% paraformaldehyde, and subsequently, they were processed for paraffin embedding to ensure long-term preservation. Thin sections with 6 micrometers in thickness, were then carefully sliced from the embedded tissues. Sections were dewaxed and rehydrated with xylene and alcohol gradients. Antigen recovery is achieved by placing the slices in a citric acid buffer (pH = 6.0) and incubating them in a 95°C water bath for 40 minutes. Immunohistochemical staining was performed using the VECTASTAIN ABC kit (Vector Labs) according to the manufacturer's instructions. The sections were stained with primary antibodies targeting Ki67 and RL2. Quantification of the IHC results was performed by calculation of proportion of positively stained cells and the discernible staining intensity.

Quantitative Real-Time PCR Analysis.

To procure total mRNA from the cells, we employed the RNAiso Plus reagent from Takara. Briefly, 800 ng of the extracted RNA served as the template for the synthesis of complementary DNA (cDNA) using the Reverse Transcription Kit (YEASEN, 11121ES60) according to the manufacturer's recommended procedures. qRT-PCR were then carried out on the obtained cDNA templates in CFX96 Real-Time PCR Detection System (BIO-RAD). The comparative CT (ΔΔCT) method was employed to quantify the relative mRNA expression levels of the genes of interest, with the β-actin serving as an internal control for normalization purposes. The specific primers used for gene amplification were documented in Supplementary Table 2.

ChIP-qPCR assay

For the ChIP-qPCR assay, MDA-MB-468 cells were first cross-linked with 1%

formaldehyde for 10 minutes, followed by quenching with 0.125 M glycine for 5 minutes at 25°C. Cells were washed with PBS and the Nuclei were isolated by incubating the cells on ice for 10 minutes with nucleoplasm separation buffer containing 10 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$. Subsequently, the nuclei were lysed in a buffer consisting of 1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.0). DNA was fragmented via sonication to produce segments ranging from 200 to 1000 bp. The lysate was centrifuged at 13,000 rpm for 15 minutes at 4 °C to remove debris, and the supernatant was collected, with 50 μL reserved as an input sample. About 2 μg of the target-specific antibody and 20 μL of protein A/G beads were incubated with supernatant overnight at 4 °C with rotation. The beads were washed sequentially with low salt buffer (0.1% SDS, 1.0% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl), high salt buffer (0.1% SDS, 1.0% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl), LiCl wash buffer (10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1% NP-40, 1% deoxycholic acid), and TE buffer (1 mM EDTA, 10 mM Tris-HCl (pH 8.0)). The DNA was eluted with 300 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$). Then 5 M NaCl was added and incubate with the samples overnight at 65 °C to reverse cross-linking. RNaseA and Proteinase K were then used to degrade RNA and proteins, respectively. The DNA was purified using a Qiagen MinElute Reaction Cleanup Kit. Finally, qPCR was performed to quantify the enrichment of specific gene targets.

Mouse xenograft and lung metastasis model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University and conform to ethical guidelines for animal welfare. For mouse xenograft model, 1×10^6 MDA-MB-468 cells were in resuspended in Matrigel and subcutaneously injected into the flanks of 6-week-old male nude BALB/c mice (n = 5). The volume of the tumors was monitored once per five days by measuring tumor dimensions. Calculate tumor volume using the formula: V = (length × $width^2$) / 2. For combination therapy experiments, a total of 5×10^5 4T1 cells were implanted subcutaneously into the flanks of C57BL/6J mice. Upon tumors reaching

an initial volume to 400 mm^3, mice were randomized into four distinct treatment groups (n = 7 per group) to minimize bias. The mice were intraperitoneal injected with OSMI4 (100 μg/mouse) and LY294002 (200 μg/mouse), either individually or in combination. The experiment was terminated and the mice were euthanized prior to tumors reaching a volume of 2 cm^3. For mouse lung metastasis model, a total of 1×10^5 4T1 cells were into the tail vein of each mouse (n = 5). Hematoxylin and eosin (H&E) staining was performed to confirm and quantify the metastatic lesions.

Statistical analysis

The data were presented as the mean ± standard deviation (SD) derived from a minimum of three separate experiments. Statistical analyses for intergroup variations were calculated using an unpaired two-tailed t-test. $P < 0.05$ was considered to denote statistical significance.

Data availability

All data supporting the findings of this study are available from the corresponding author on reasonable request.

Supplementary Figures

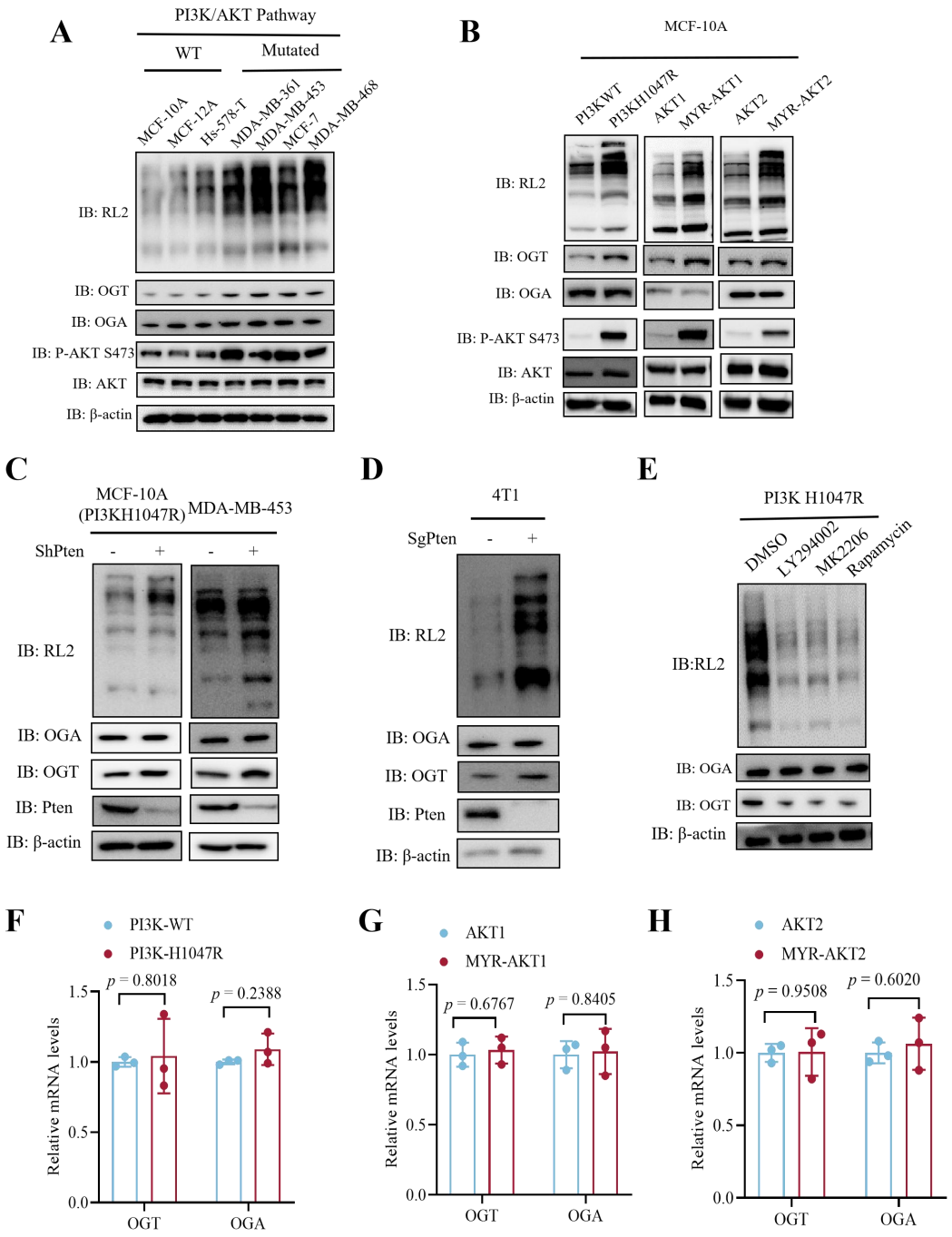


Fig. S1. A, Immunoblotting analysis of AKT phosphorylation on S437 and global O-GlcNAcylation levels in indicated cell types upon serum starvation for 24h. **B,** Immunoblotting analysis of AKT phosphorylation on S437 and global O-GlcNAcylation levels in MCF-10A cells expressing PI3K WT, PI3K H1047R, AKT1, MYR- AKT1, AKT2 or MYR- AKT2 upon serum starvation for 24h. **C,** Immunoblotting analysis of Pten expression and global O-GlcNAcylation levels in PI3K H1047R MCF-10A cells and MDA-MB-453 cells expressing Scramble or ShPten in response to serum starvation for 24h. **D,** Immunoblotting analysis of global O-GlcNAcylation

levels in WT or Pten-/- 4T1 cells in response to serum starvation for 24h. **E,** Immunoblotting analysis of OGT expression and O-GlcNAcylation levels in PI3K (H1047R) expressing MCF10A cells treated with inhibitors of PI3K (LY294002), AKT (MK2206) or mTOR (Rapamycin). **F-H,** Quantitative PCR analysis of OGT and OGA expression in MCF-10A cells expressing PI3K WT /1047R MCF-10A (**F**), AKT1/MYR-AKT1 (**G**), AKT2/MYR-AKT2 (**H**) upon serum starvation for 24h. n = 3; Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests.

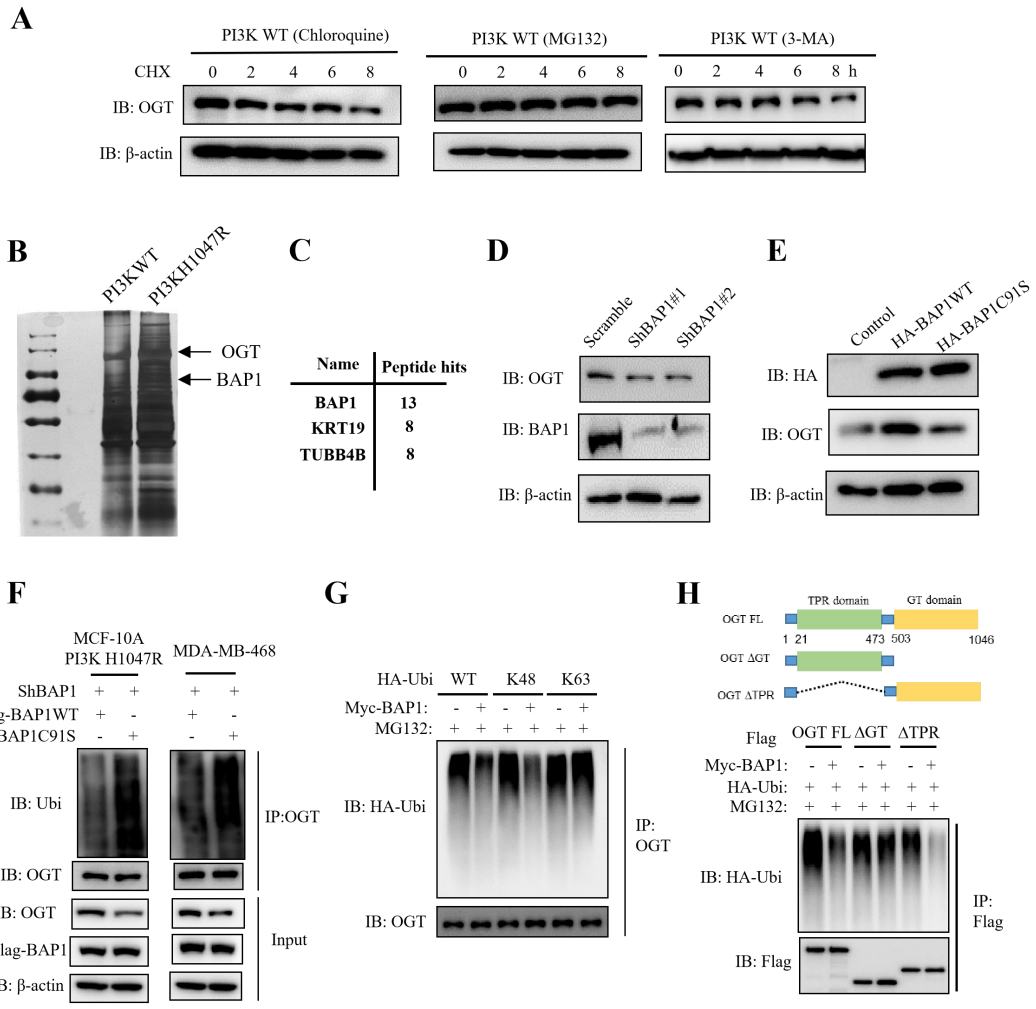
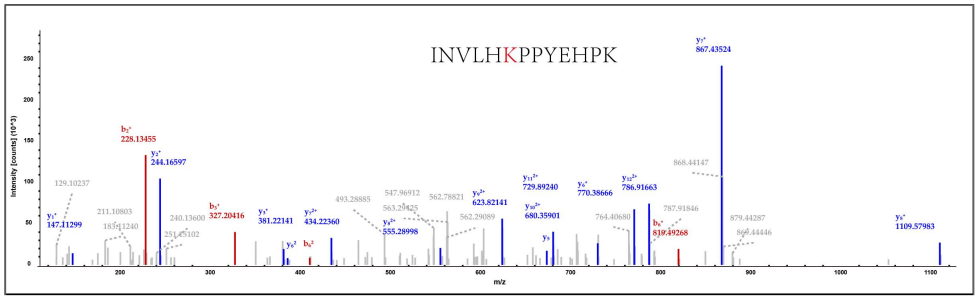


Fig. S2. A, Immunoblotting of OGT levels in PI3K WT MCF-10A cells after treatment with CHX for indicated time in the absence or presence of Chloroquine, MG132 and 3-MA. **B and C,** Coomassie Brilliant Blue staining of immunoprecipitates of OGT in PI3K WT or H1047R MCF-10A cells (**B**). Selected peptide hits of OGT immunoprecipitants only identified in PI3K H1047R cells through mass spectrometry are shown (**C**). **D**, Immunoblotting of OGT levels in

PI3K H1047R MCF-10A cells expressing Scramble, ShBAP1#1 or ShBAP1#2. **E,** Immunoblotting of OGT levels in PI3K H1047R MCF-10A cells expressing Control, HA-BAP1WT or HA-BAP1C91S. **F**, Immunoblotting of OGT ubiquitination in BAP1 depleted PI3K H1047R MCF-10A cells and MDA-MB-468 cells expressing BAP1WT or BAP1C91S. **G,** Immunoblotting of OGT ubiquitination in 293T cells transfected with ubiquitin mutants (K48 and K63). **H**, Immunoblotting of various OGT ubiquitination in 293T cells transfected with OGT constructs (ΔGT, ΔTPR, or full-length).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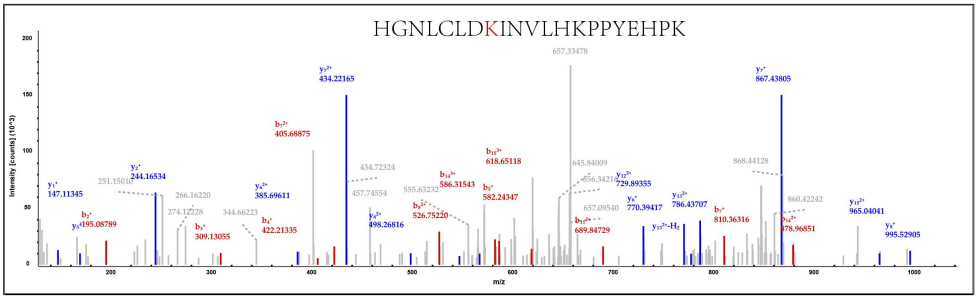
m/z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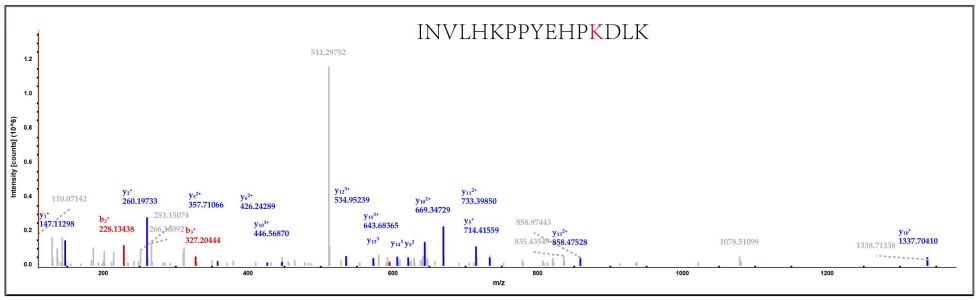
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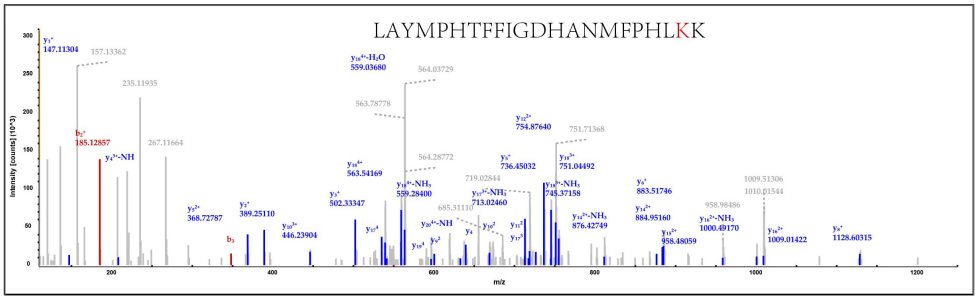
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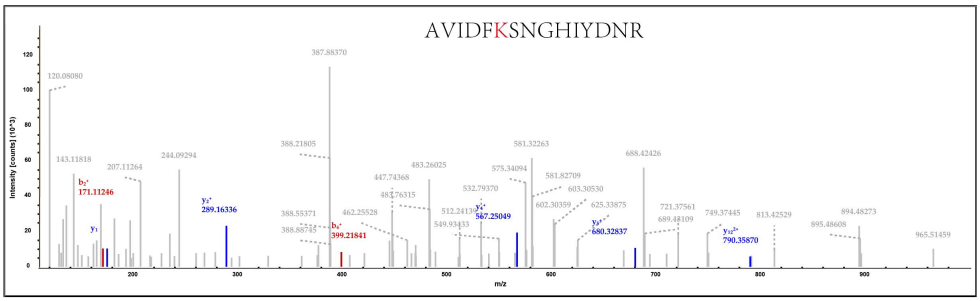
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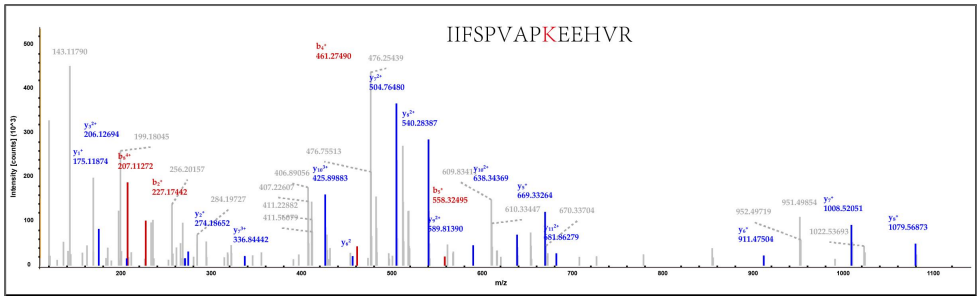
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m/z

Fig. S3. Mapping the site of ubiquitination on OGT using mass spectrometry.

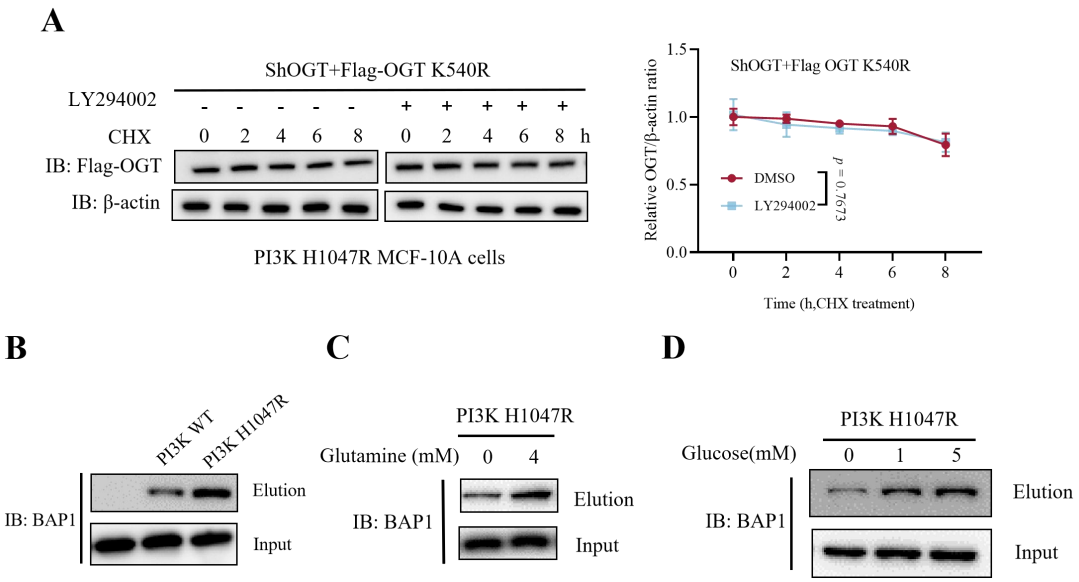

Fig. S4. A, Immunoblotting of OGT levels in PI3K H1047R MCF-10A cells after treatment with CHX for indicated time in the absence or presence of PI3K inhibitor (LY294002). n = 3; Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **B,** Immunoblotting of BAP1 O-GlcNAcylation in PI3K WT or H1047R MCF-10A cells upon serum starvation for 24h. **C and D,** Immunoblotting of BAP1 O-GlcNAcylation in H1047R MCF-10A cells upon treatment with different concentrations of glutamine (**C**) and glucose (**D**).

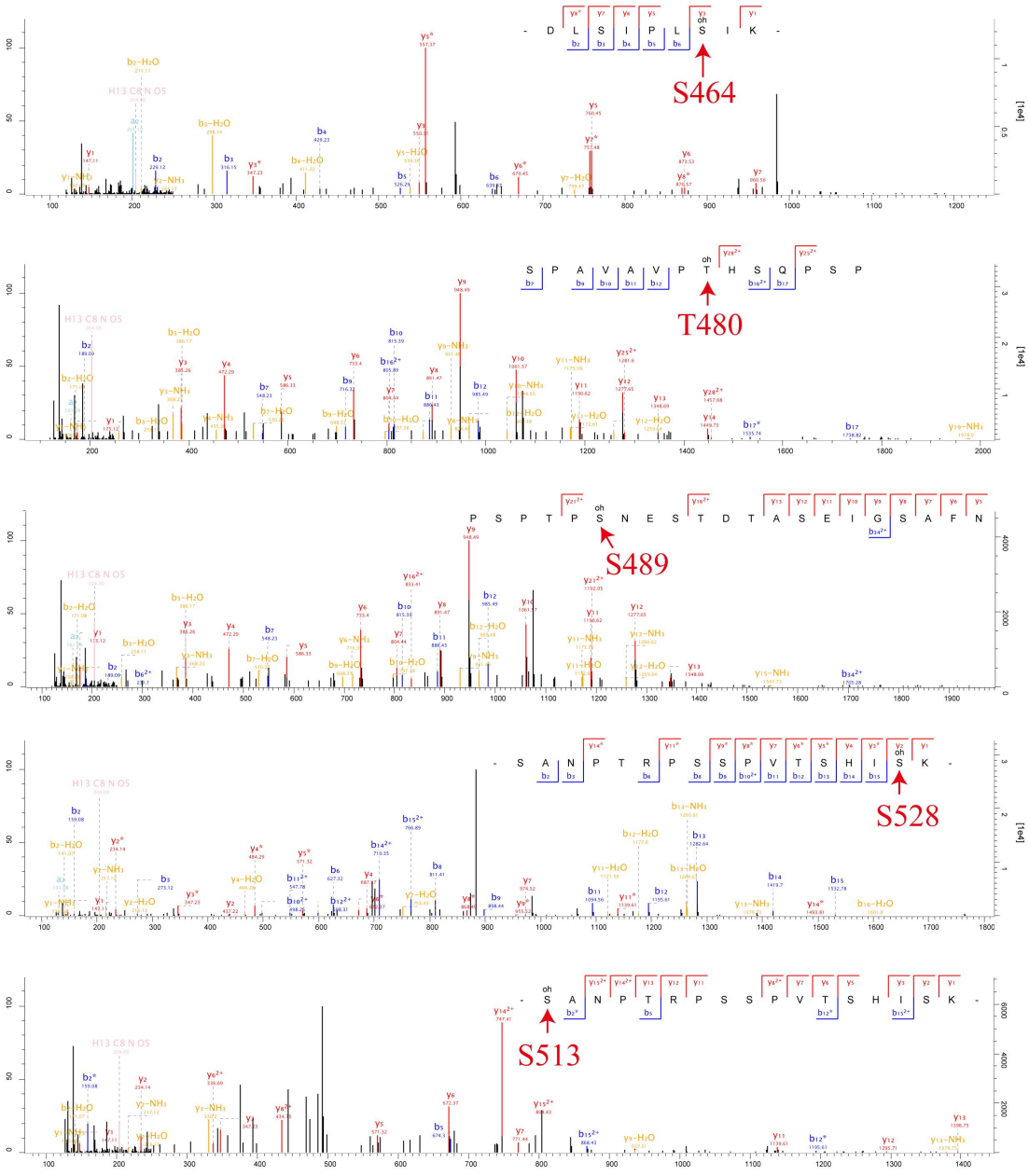

Fig. S5. Mapping the site of O-GlcNAcylation on BAP1 using mass spectrometry.

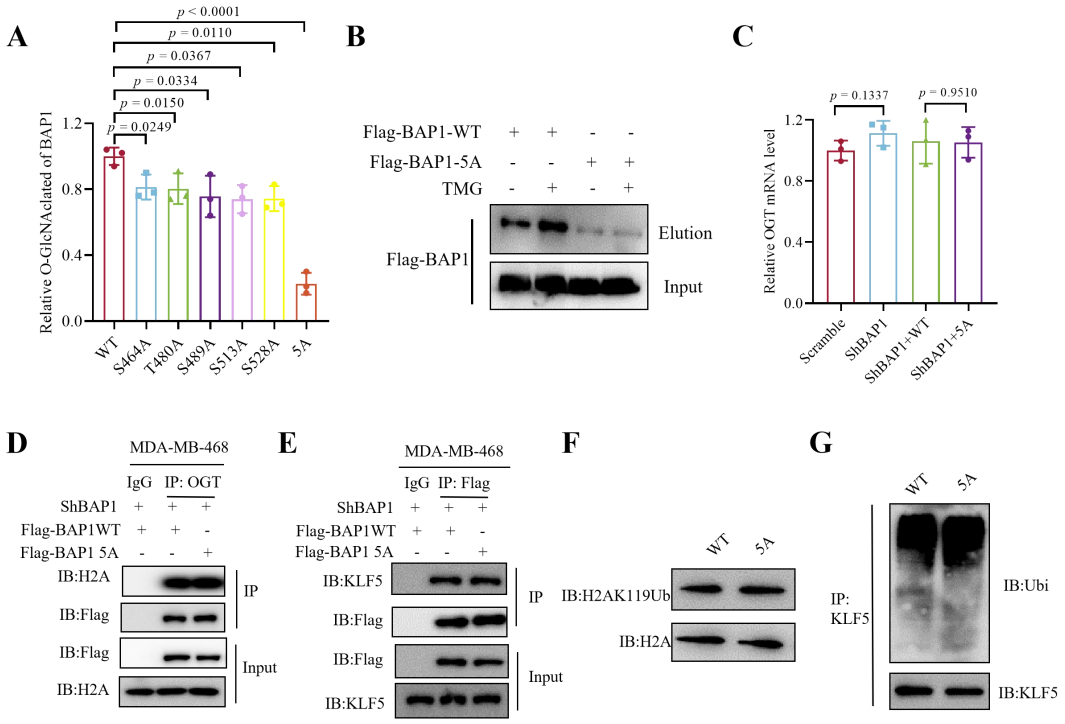

Fig. S6. A, Probing the major site of O-GlcNAcylation on BAP1 using various site-directed mutants. Quantification was shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **B,** Immunoblotting of BAP1 O-GlcNAcylation levels in the presence or absence of TMG treatment in WT or 5A BAP1-reconstituted MDA-MB-468 cells**. C,** Quantitative PCR analysis of OGT expression in MDA-MB-468 cells with BAP1 knockdown and reconstituted expression of shRNA-resistant WT and 5A BAP1**.** Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **D and E,** Analysis of H2A-BAP1(**D**) and KLF5-BAP1 (**E**) interaction in WT or 5A BAP1-reconstituted MDA-MB-468 cells. **F and G,** Analysis of H2A (**F**) and KLF5 (**G**) ubiquitination in WT or 5A BAP1-reconstituted MDA-MB-468 cells.

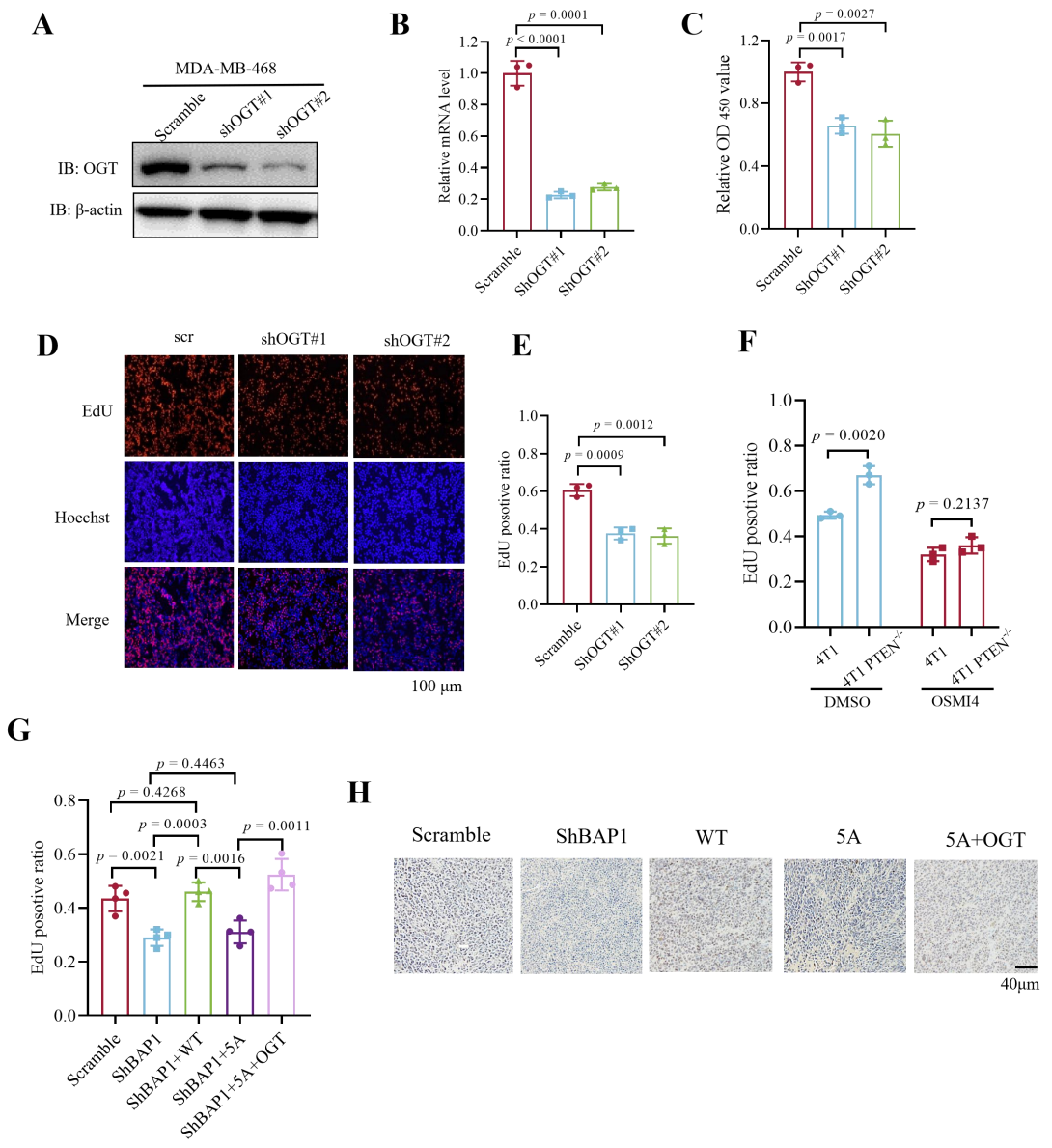

Fig. S7. A and B, Immunoblotting (**A**) and quantitative PCR (**B**) of OGT levels in MDA-MB-468 cells expressing Scramble, ShOGT#1 or ShOGT#2. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **C-E**, Analysis of cell proliferation by measuring OD450 value (**C**) and EdU absorption (**D** and **E**). Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests (Scale bar, 100 μm.). **F,** Analysis of EdU absorption in WT or Pten$^{-/-}$ 4T1 cells in the presence or absence of OSMI4 treatment. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **G,** EdU positive rate of MDA-MB-468 cells reconstituted expressing WT, 5A BAP1 or 5A BAP1 with OGT overexpression. **H**. Representative IHC images of Ki67 expression in the tumors generated from MDA-MB-468 cells stably expressing scramble,

BAP1-targeting shRNA and shRNA-resistant WT and 5A BAP1 or 5A BAP1 with OGT overexpression (Scale bar, 40 μm.).

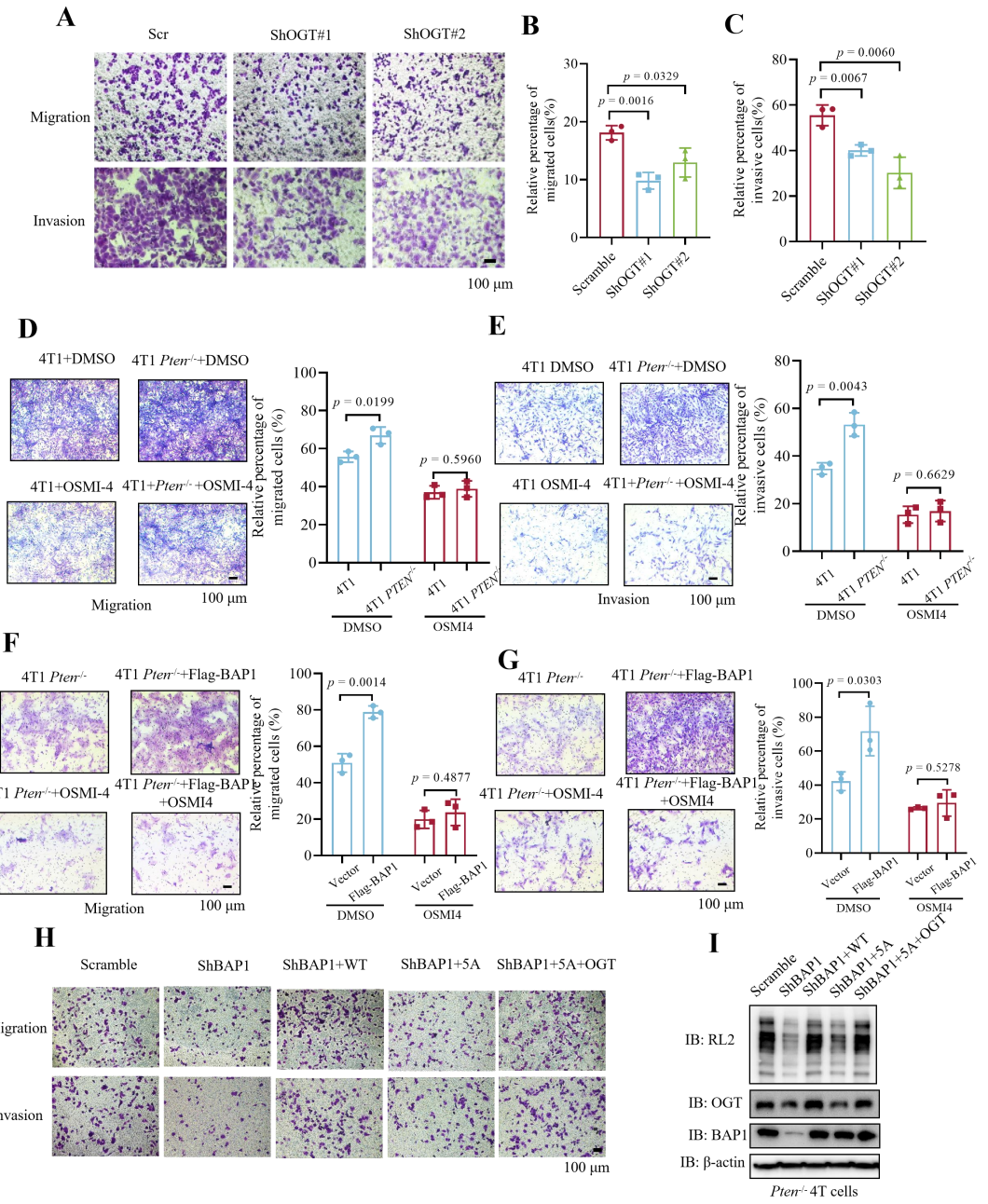

Fig. S8. A-C, Analysis of cell migration and invasion by transwell assay. Representative images (**A**) and quantification (**B and C**) were shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests (Scale bar, 100 μm.). **D and E**, Analysis of cell migration (**D**) and invasion (**E**) in WT or Pten$^{-/-}$ 4T1 cells in the presence or absence of OSMI4 treatment. Quantification was shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests (Scale bar, 100 μm.). **F and G**, Analysis of cell

migration (**F**) and invasion (**G**) in Pten-/- 4T1 cells expressing control vector or Flag-BAP1 in the presence or absence of OSMI4 treatment. Quantification was shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. (Scale bar, 100 μm.) **H,** Representative images of of cell migration and invasion by transwell assay. (Scale bar, 100 μm.) **I,** Immunoblotting of OGT expression and O-GlcNAcylation levels in Pten$^{-/-}$ 4T cells reconstituted expressing Scramble, ShBAP1, WT, 5A BAP1 or 5A BAP1 with OGT overexpression.

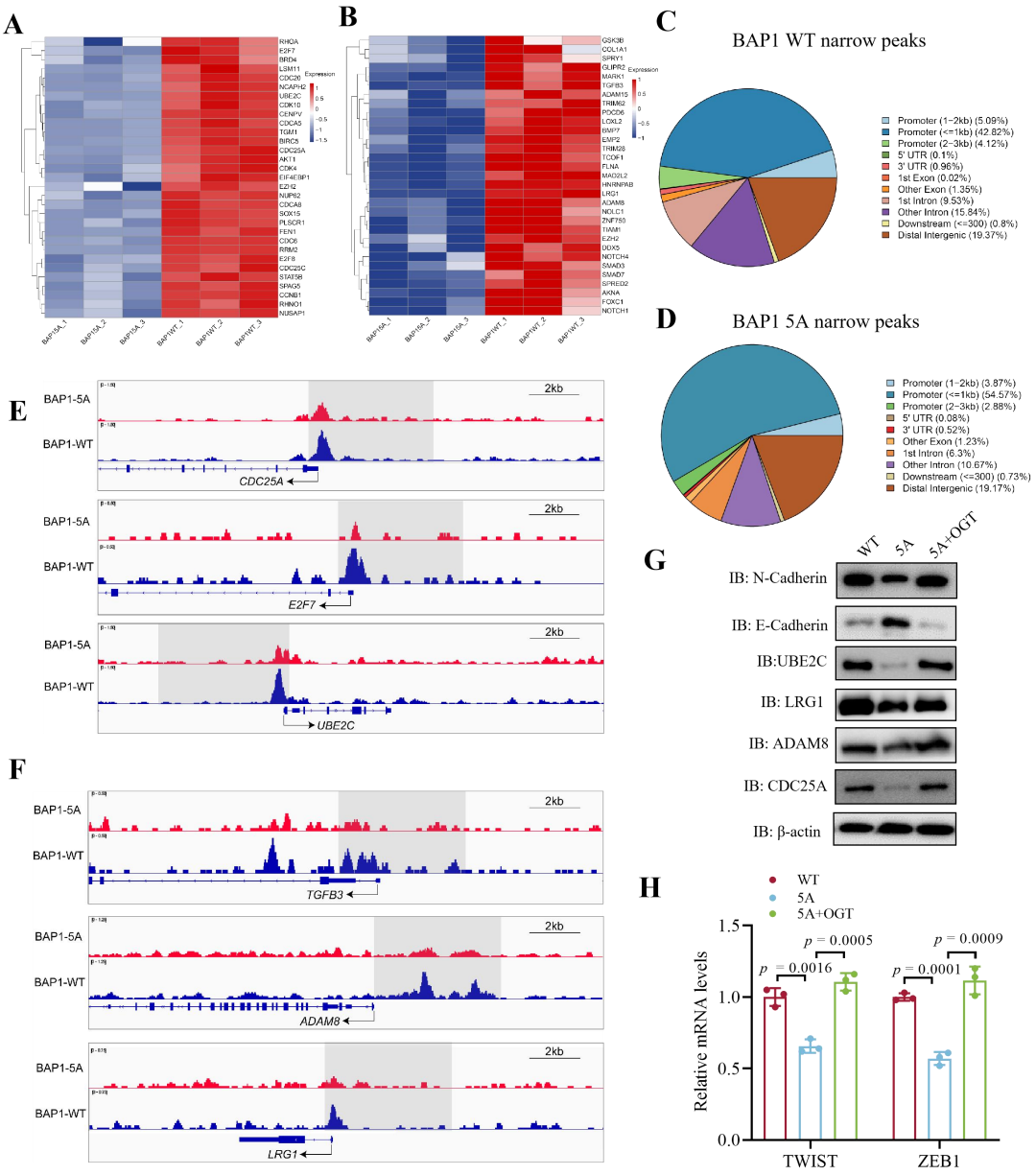

Fig. S9. A and B, Heat maps analysis of the genes differentially expressed in WT BAP1 and 5A

BAP-reconstituted MDA-MB-468 cells based on RNAseq data. **C and D**, CUT&Tag peak annotation relative to known genomic elements. **E and F**, The enrichment profiles of BAP1 WT and BAP1 5A CUT&Tag signals at gene regions. **G,** Immunoblotting of CDC25A, ADAM8, LRG1, UBE2C, E-Cadherin and N-Cadherin expression in MDA-MB-468 cells reconstituted expressing WT, 5A BAP1 or 5A BAP1 with OGT overexpression. **H,** Quantitative PCR analysis of TWIST and ZEB1 expression in MDA-MB-468 cells reconstituted expressing WT, 5A BAP1 or 5A BAP1 with OGT overexpression. n = 3; Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student’s t-tests.

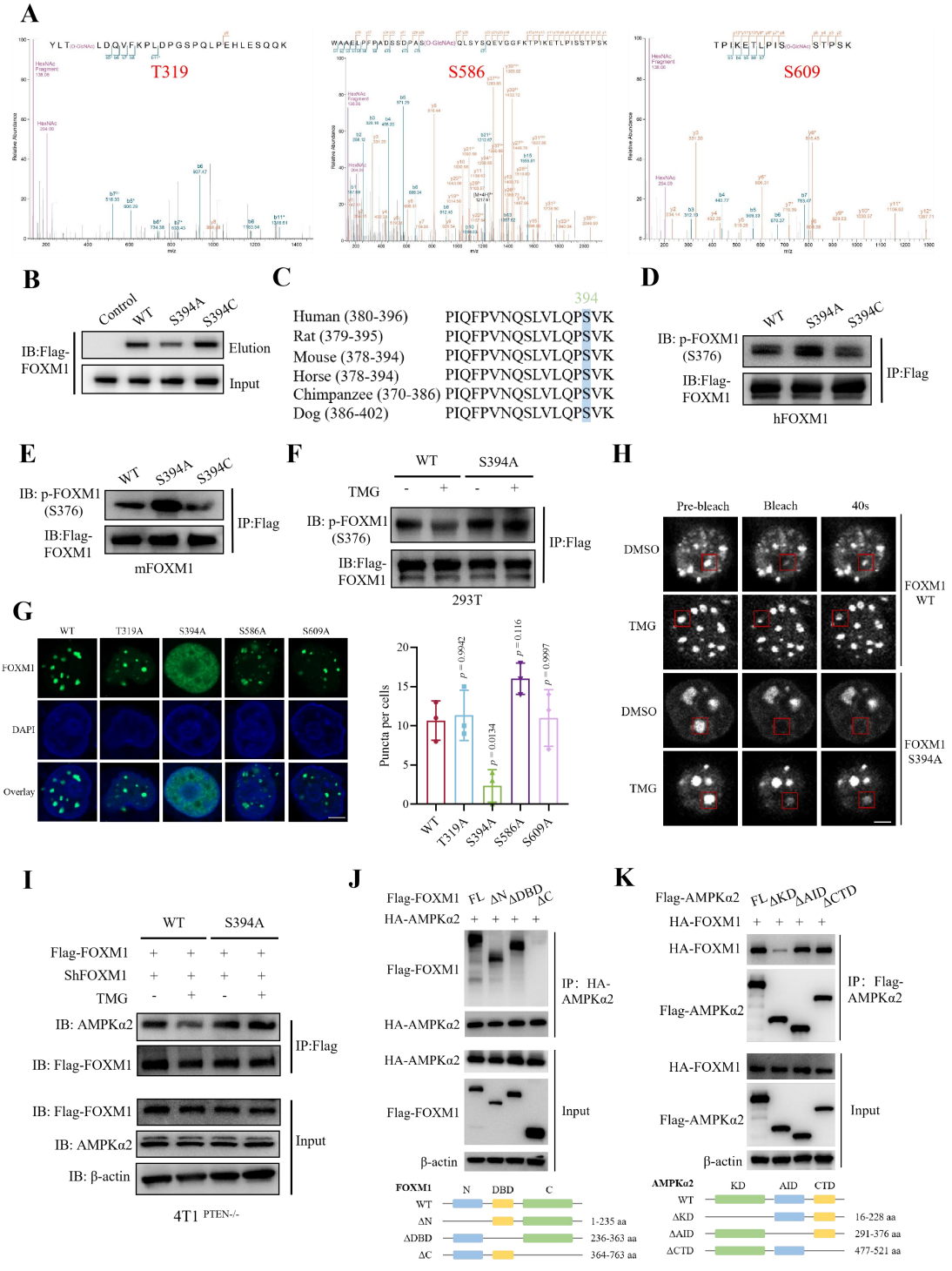

Fig. S10. A, Mapping the site of O-GlcNAcylation on FOXM1 using mass spectrometry. **B**, Immunoblotting of O-GlcNAcylation levels of FOXM1 WT, S394A, and S394C. **C**, Evolutionary conservation of the FOXM1 S394 residue across species. **D**, Immunoblotting of the phosphorylation levels (S376) of human FOXM1 WT, S394A, S394C protein. **E,** Immunoblotting of the phosphorylation levels (S376) of mouse FOXM1 WT, S394A, S394C protein. **F**, Immunoblotting of the phosphorylation levels (S376) of FOXM1 WT, S394A in 293T cells treated

with or without TMG. **G,** Immunofluorescence and DAPI staining of HeLa cells expressing GFP-tagged FOXM1 WT or corresponding mutants. Quantified average number of the nuclear FOXM1 puncta (Scale bar, 5 μm.). n = 3; Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **H,** Photo bleaching images of FOXM1 WT or S394A puncta in Hela cells treated with or without TMG (Scale bar, 5 μm.). Pictures before bleaching, under bleaching, and post bleaching (40s) were shown. **I,** Immunoblotting of the interaction between FOXM1 and AMPKα2 in Flag-FOXM1 WT, S394A-reconstituted 4T1$^{PTEN-/-}$ cells treated with or without TMG. **J,** Immunoblotting analysis of the interaction between full-length/truncated Flag-FOXM1 and HA-AMPKα2 **K,** Immunoblotting analysis of the interaction between full-length/truncated Flag-AMPKα2 and HA-FOXM1.

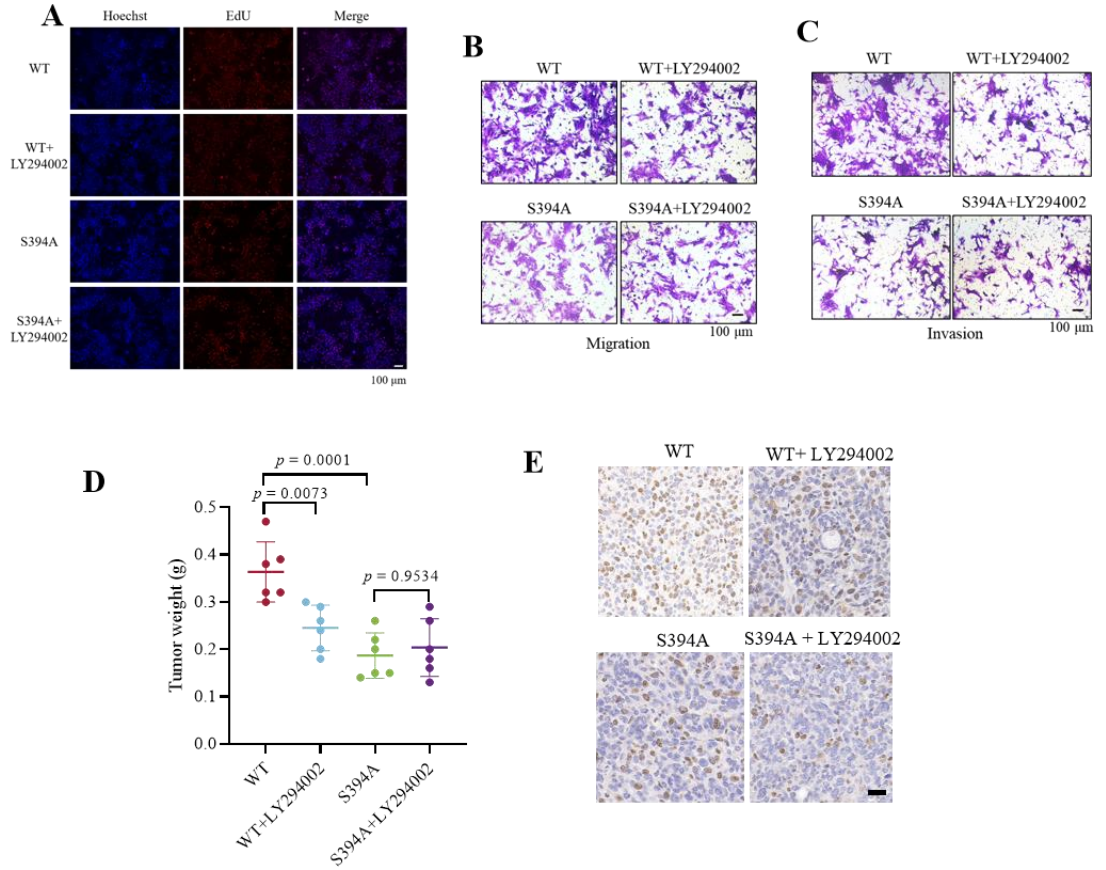

Fig. S11. A, EdU absorption of FOXM1 WT or S394A-reconstituted 4T1$^{PTEN-/-}$ cells treated with or without LY294002. **B and C,** Analysis of cell migration (**B**) and invasion (**C**) in FOXM1 WT or S394A-reconstituted 4T1$^{PTEN-/-}$ cells in the presence or absence of LY294002 treatment. **D,** Tumor weight of nude mice bearing WT or S394A-reconstituted 4T1$^{PTEN-/-}$ cells treated with

DMSO or LY294002. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. E. Representative images of KI67 staining in tumors derived from FOXM1 WT or S394A-reconstituted 4T1$^{PTEN-/-}$ cells in the presence or absence of LY294002 treatment.

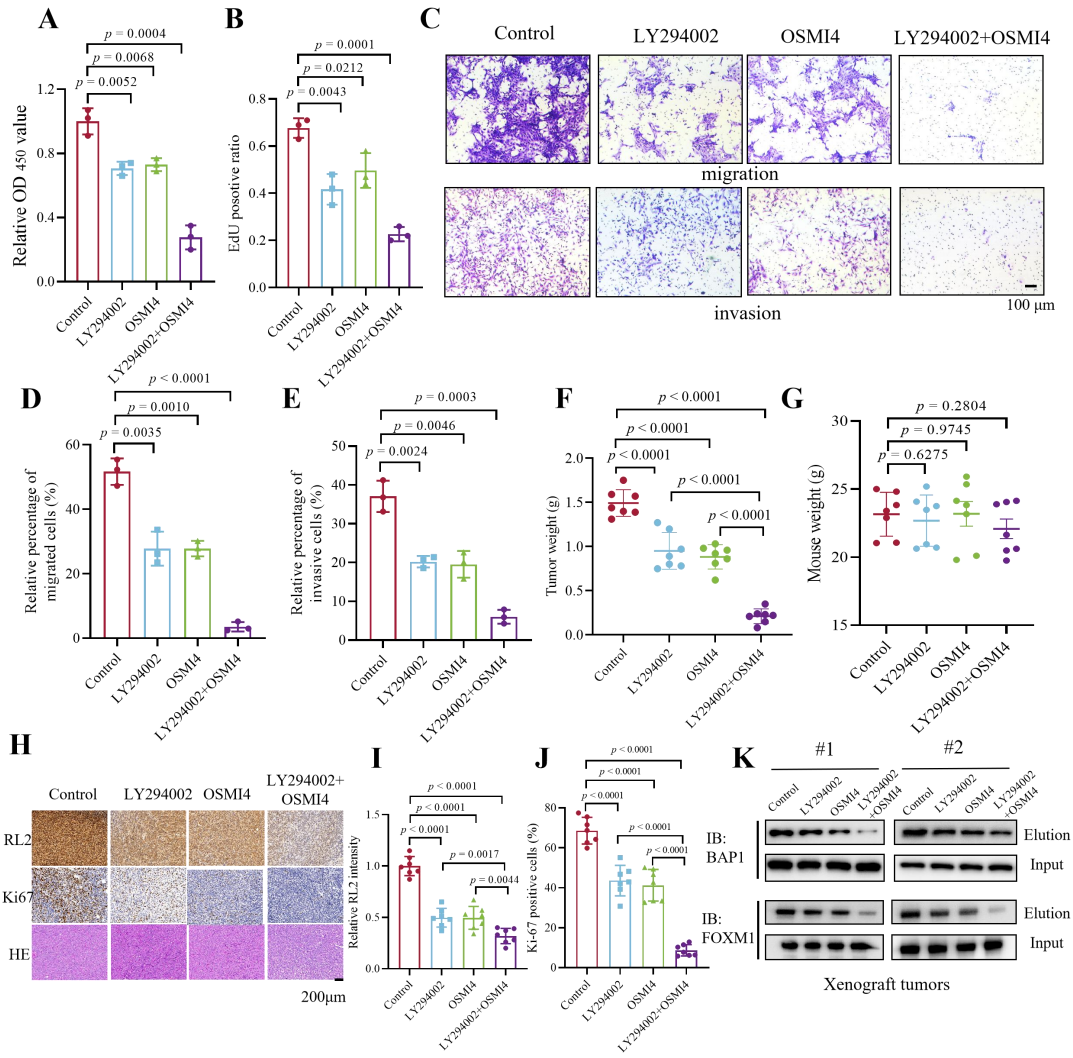

Fig. S12. A, Analysis of cell proliferation by measuring OD450 value (**A**) and EdU absorption (**B**) in *Pten*$^{-/-}$ 4T1 cells treated with LY294002, or OSMI-4, or their combination. Quantification was shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. **C-E**, Analysis of cell migration and invasion by transwell assay. Representative images (**C**) and quantification (**D and E**) were shown. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests. (Scale bar, 100 μm.). **F,** Tumors weight measured at the indicated time points. n = 7; Data are depicted as means ± SD. P-values were

calculated by unpaired two-tailed Student’ s t-tests. **G,** Analysis of mouse weight after treating with LY294002, or OSMI-4, or their combination. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student’s t-tests. **H-J,** Immunohistochemistry staining of tumor with the indicated antibodies (**H**). (Scale bar, 200 μm.) Quantification of RL2 intensity (**I**) and Ki67-positive cells (**J**) in tumor samples. n = 7; Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student’s t-tests. **K,** Immunoblotting of BAP1 and FOXM1 O-GlcNAcylation levels in tumors harvested from mice bearing *Pten*$^{-/-}$ 4T1 cells treated with LY294002, or OSMI-4, or their combination.

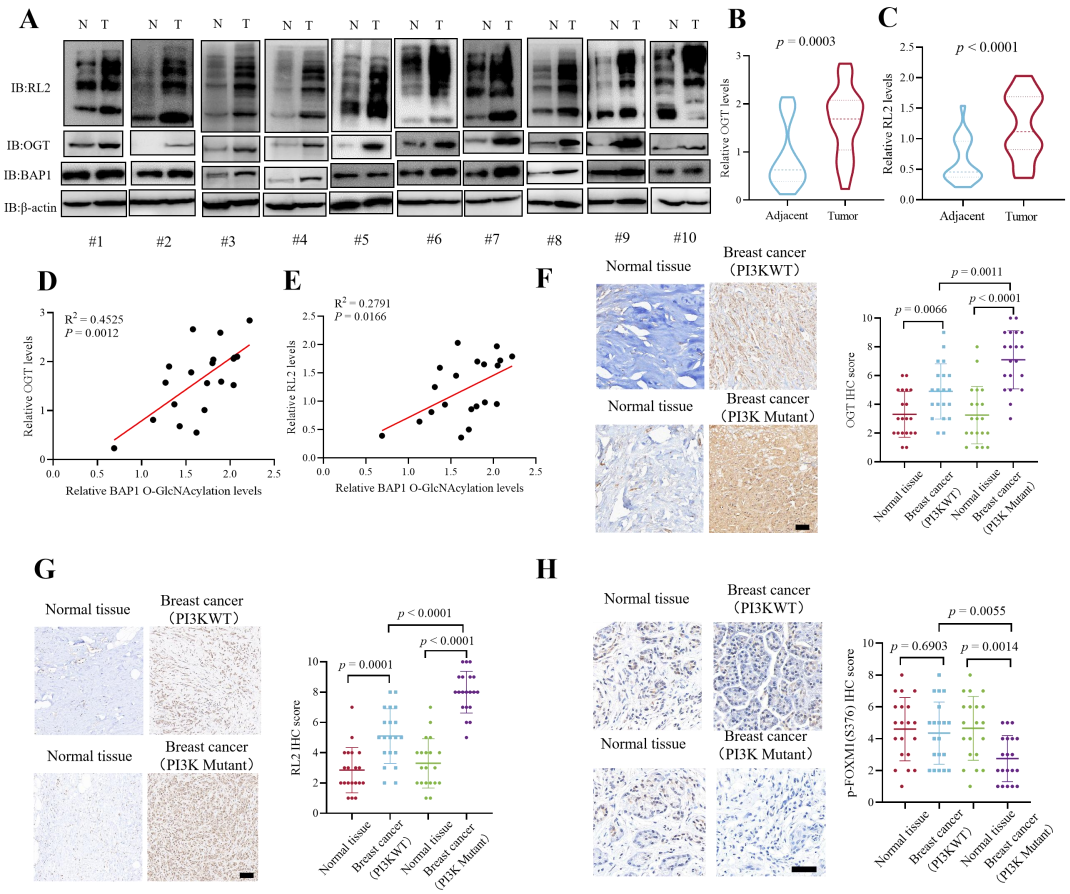

Fig. S13. A-C, Immunoblotting of OGT, RL2 and BAP1 expression in paired breast malignant tumor tissues and their corresponding adjacent normal tissues (n=10) (**A**). Quantification of OGT (**B**) and RL2 (**C**) were shown. Data are depicted as means ± SD. P-values were calculated by paired two-tailed Student’s t-tests. **D and E,** Analysis of the correlation of BAP1 glycosylation with OGT (**D**) and RL2 (**E**) levels in clinical breast cancer tissues. Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student’s t-tests. **F-H,**

Immunohistochemistry staining of OGT (**F**), RL2 (**G**), and p-FOXM1(S376) (**H**) in breast malignant tumor tissues with PI3K WT and mutant and their corresponding adjacent normal tissues (n=20) (Scale bar, 50 μm). Data are depicted as means ± SD. P-values were calculated by unpaired two-tailed Student's t-tests.

Supplementary Table 1: Information about antibodies used in this study.

Antibody	Brand	Cat#	Dilution
anti-UAP1	Abcam	ab95949	1:1000
anti-GFPT2	Abcam	ab190966	1:1000
anti-GFPT1	Abcam	ab125069	1:1000
anti-PTEN	Abcam	ab32199	1:1000
anti-BAP1	Abcam	ab199396	1:1000
anti-P-S473-AKT	Cell Signaling Technology	#4060	1:1000
anti-AKT	Cell Signaling Technology	#9272	1:1000
anti-Ubi	Abcam	ab137031	1:1000
anti-Ki67	Abcam	ab15580	1:1000
anti-RL2	Abcam	ab2739	1:1000
anti-OGT	Abcam	ab177941	1:1000
anti-OGA	Abcam	ab124807	1:1000
anti-KLF5	Abcam	ab137676	1:1000
anti-H2A	Abcam	ab177308	1:1000
anti-H2AK119Ub	Abcam	ab193203	1:1000
anti-HA tag	Cell Signaling Technology	#54062S	1:1000
anti-β-actin	Cell Signaling Technology	#4970	1:1000
anti-Flag	Abcam	ab125243	1:1000
anti-c-Myc tag	Abcam	ab9132	1:1000
anti-CDC25A	Proteintech	55031-1-AP	1:1000
anti-E-cadherin	Proteintech	20847-1-AP	1:1000
anti-ADAM8	Invitrogen	PA5-27082	1:1000
anti-E2F7	Abcam	ab245655	1:1000
anti-LRG1	Abcam	ab178698	1:1000
anti-FOXM1	Cell Signaling Technology	#20459	1:1000
anti-FOXM1 pS376	Acquired from Zhang's Lab		1:1000
anti-AMPKα2	HUABIO	A6A10	1:500

Supplementary Table 2: Sequences of primes used in this study.

Prime	Sequence
β-actin/qPCR/F	CATGTACGTTGCTATCCAGGC
β-actin/qPCR/R	CTCCTTAATGTCACGCACGAT
GFPT1/qPCR/F	AACTACCATGTTCCTCGAACGA
GFPT1/qPCR/R	CTCCATCAAATCCCACACCAG
GFPT2/qPCR/F	ATGTGCGGAATCTTTGCCTAC
GFPT2/qPCR/R	ATCGAGAGCCTTGACTTTCCC
GNPNAT/qPCR/F	ACTCCTATGTTTGACCCAAGTCT
GNPNAT/qPCR/R	TCTGTTAGCTGACCCAATACCT
PGM1/qPCR/F	CCAAACCGACTGAAGATCCGT
PGM1/qPCR/R	CATGTTTCGATCCCCATCTCC
UAP1/qPCR/F	AATGACCTCAAACTCACGTTGT
UAP1/qPCR/R	GCTCTGCATAAAGTTCTACCTGT
OGT/qPCR/F	TCCTGATTTGTACTGTGTTCGC
OGT/qPCR/R	AAGCTACTGCAAAGTTCGGTT
OGA/qPCR/F	GAAGGAGAGTCAAGCGACGTT
OGA/qPCR/R	TCCATAACCCAAGGTCTTCCAT
GLUT1/qPCR/F	ATTGGCTCCGGTATCGTCAAC
GLUT1/qPCR/R	GCTCAGATAGGACATCCAGGGTA
GLUT4/qPCR/F	GCCATGAGCTACGTCTCCATT
GLUT4/qPCR/R	GGCCACGATGAACCAAGGAA
E2F/qPCR/F	ACGCTATGAGACCTCACTGAA
E2F/qPCR/R	TCCTGGGTCAACCCCTCAAG
LRG1/qPCR/F	AGAACCTGAGCGACCTCTATC
LRG1/qPCR/R	CACAGCGCGTGTCATTCTG
CDC25A/qPCR/F	GTGAAGGCGCTATTTGGCG
CDC25A/qPCR/R	TGGTTGCTCATAATCACTGCC
ADAM8/qPCR/F	GAGGGTGAGCTACGTCCTTG
ADAM8/qPCR/R	CAGCCGTATAGGTCTCTGTGT
TGFB3/qPCR/F	ACTTGCACCACCTTGGACTTC
TGFB3/qPCR/R	GGTCATCACCGTTGGCTCA
CENPV/qPCR/F	AGAGATGTGGCGTTCAGAGC
CENPV/qPCR/R	CAAAGTTGCTGGCCCCAATC
CCNB1/qPCR/F	GCACTTCCTTCGGAGAGCAT
CCNB1/qPCR/R	TGTTCTTGACAGTCCATTCACCA
MMP2/qPCR/F	AGTCTGTGTTGTCCAGAGGC
MMP2/qPCR/R	CCTTGGGGCAGCCATAGAAG
ShOGT-#1F:	CCGGGCCCTAAGTTTGAGTCCAAATCTCGAGATTTGGACTC AAACTTAGGGCTTTTTG
ShOGT-#1R:	AATTCAAAAAGCCCTAAGTTTGAGTCCAAATCTCGAGATTT GGACTCAAACTTAGGGC
ShOGT-#2F:	CCGGTGTTGCAGATGGGTGATATATCTCGAGATATATCACCC ATCTGCAACATTTTTG

ShOGT-#2R:	AATTCAAAAATGTTGCAGATGGGTGATATATCTCGAGATATA TCACCCATCTGCAACA
ShBAP1-#1F:	CCGGCGTCCGTGATTGATGATGATACTCGAGTATCATCATCA ATCACGGACGTTTTTG
ShBAP1-#1R:	AATTCAAAAACGTCCGTGATTGATGATGATACTCGAGTATCA TCATCAATCACGGACG
ShBAP1-#2F:	CCGGGCCACCATGTTGACATAAGTTCTCGAGAACTTATGTCA ACATGGTGGCTTTTT
ShBAP1-#2R:	AATTCAAAAAGCCACCATGTTGACATAAGTTCTCGAGAACTT ATGTCAACATGGTGGC
ShFOXM1-#1F:	CCGGAGGACCACTTTCCCTACTTTACTCGAGTAAAGTAGGGAAA GTGGTCCTTTTTTG
ShFOXM1-#1R:	AATTCAAAAAAGGACCACTTTCCCTACTTTACTCGAGTAAAGTA GGGAAAGTGGTCCT