Supplementary

Materials and Methods

Cell culture and transfection

Human HCC cell lines (HCCLM3, MHCC-97H, Huh7 and Hep3B) and HepaRG cell lines were purchased from the cell bank of the Chinese Science Academy and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA). 293T cells obtained from ATCC were cultured in DMEM supplemented with 10% FBS. The mycoplasma-free status was confirmed by STR profiling. To induce endoplasmic reticulum stress, tunicamycin (4 μg/ml) was administered in DMEM. To adjust the UPR pathway, Sal003 (20 μM) and ISRIB (0.2 μM) were administered into DMEM. To examine drug sensitivity, bortezomib was administered into DMEM at the indicated concentration. To inhibit protein degradation, MG-132 (100 nM) and bafilomycin A1 (100 nM) were administered into DMEM. To inhibit protein synthesis, cycloheximide (0.6 μM) was administered into DMEM.

To knock down SEC24C expression, lentiviral plasmid vectors encoding short hairpin RNAs (shRNAs) targeting SEC24C or scramble shRNA were generated and designated sh-SEC24C and sh-NC, respectively. To knock down NPM1 expression, small interfering RNAs (siRNAs) targeting NPM1, SEC24A, SEC24B, SEC24D or scramble siRNA were generated and designated siNPM1 and siNC, respectively. To knock out SEC24C expression, small guide RNAs (sgRNAs) targeting SEC24C or PERK were generated and designated sgSEC24C, sgPERK.

Human SEC24C shRNA KD1: 5′- ACTTATGTTATCGAGTCAATG-3′
Human SEC24C shRNA KD2: 5′- TTGATGTAAAGCGACTAATAT-3′
Human SEC24C shRNA NC: 5′- TTCTCCGAACGTGTCACGT -3′
Human SEC24C sgRNA KO: 5′- GTGTCACGAACAGCCTTCAC-3′
Human PERK sgRNA KO: 5′- CGCAACTCTGTCTCATCGTC-3′
Mouse SEC24C sgRNA KO: 5′- CATGACATTCATCG-3′
Human NPM1 siRNA KD1: 5′- AACAAUGUGCAACUCAUCCTT-3′
Human NPM1 siRNA KD2: 5′- AAUGUCUUCUACAGAACUAGG-3′
Human SEC24A siRNA KD: 5′-CCAAGAAGGUAUUACAUCA-3′
Human SEC24B siRNA KD: 5′-GGGAAAGGCUGUGACAAUA -3′
Human SEC24D siRNA KD: 5′-GAGGAACCCUUUACAAAUA-3′
Human siRNA NC: 5′- UUCUCCGAACGUGUCACGU-3′

Transfection of cells was conducted according to the manufacturer's instructions, and the efficiency of transfection was validated by qRT–PCR. Then, the cells were subjected to RNA extraction or functional assays.

RNA Extraction and Quantitative real-time PCR

Total mRNA was extracted from cells using TRIzol reagent (Invitrogen), and equal amounts of RNA were reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Transcript levels were measured in duplicate by quantitative reverse transcription PCR (ABI 7900; Life Technologies). Expression levels were calculated relative to GAPDH. The primer pairs used in SYBR Green reactions are listed below.

SEC24C Forward 5′- TGATGGTTGTGTCTGATGTGG-3′
Reverse 5′- TGTCTCTGTTTCCCTTGTGTC-3′
GAPDH Forward 5′- ACAGTCAGCCGCATCTTCTT-3′
Reverse 5′- ACGACCAAATCCGTTGACTC-3′
CHOP Forward 5′- GGAAACAGAGTGGTCATTCCC-3′
Reverse 5′- CTGCTTGAGCCGTTCATTCTC-3′
GADD34 Forward 5′- ATGATGGCATGTATGGTGAGC -3′
Reverse 5′- AACCTTGCAGTGTCCTTATCAG-3′
NPM1 Forward 5′- GGAGGTGGTAGCAAGGTTCC-3′
Reverse 5′- TTCACTGGCGCTTTTTCTTCA -3′

Cell proliferation, migration, invasion and apoptosis assay

The proliferation ability of HCC cells was tested by clone formation assay and Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Sal003, ISRIB and bortezomib were purchased from Selleck. Cell migration and invasion assays were conducted using a Transwell chamber (8 μm pore size; Millipore). After starvation for 12 hours, cells suspended in 200 μl serum-free DMEM were seeded in the upper chambers, and the bottom chambers were filled with 500 μl DMEM containing 10% FBS. For the migration assay, the upper chambers were 24-well hanging cell culture inserts. For the invasion assay, the Transwell chambers were coated with 50 μl of Matrigel. After 24 h of incubation, migrating cells (through the membrane or Matrigel) were stained with 0.1% crystal violet (Beyotime, Nantong, China) for 5 min. The invaded cells were photographed in five random fields with a microscope at 200× magnification. For apoptosis analysis, cells were stained with Annexin V-FITC/PI and then detected by flow cytometry according to the manufacturer's instructions (Vazyme, Nanjing, China).

Immunoblotting and Antibodies

Cells and liver samples were homogenized and ruptured using ice-cold RIPA buffer containing fresh protease and phosphatase inhibitors (Beyotime), and the protein concentration was measured using the BCA assay (Beyotime). Subcellular fractionations are acquired with the instruction of NE-PER™ kit (ThermoFisher, #

78835). After denaturation, protein samples were subjected to SDS–PAGE, followed by immunoblotting with antibodies. Primary antibodies against SEC24C (1:1000; NBP1-81550, Novus), Bip (1:1000; 3183s, Cell Signaling Technology), LC3A/B (1:1000; 4108s, Cell Signaling Technology), REEP5 (1:500, ab167405, Abcam), NPM1 (1:1000; 3542s, Cell Signaling Technology), RTN3 (1:1000, 12055-2-AP, Proteintech), PERK (1:1000; 3192s, Cell Signaling Technology), Phospho-PERK (1:1000; AP0886, ABclonal), eIF2α (1:1000, 5324s, Cell Signaling Technology), Phospho- eIF2α (1:1000, ab32157, Abcam), ATF4 (1:1000, 11815s, Cell Signaling Technology), CHOP (1:1000; 2895s, Cell Signaling Technology), Flag (1:2000; 14793s, Cell Signaling Technology), SEC24A (1:1000, 15958-1-AP, Proteintech), SEC24B (1:1000, A304-876A-T, Thermo) SEC24D (1:1000, 13673-1-AP, Proteintech), GAPDH (1:2000, 60004-1-Ig, Proteintech), β-actin (1:2000, Beyotime, AA128) were applied. Subsequently, the blots were incubated with proper secondary antibodies conjugated to horseradish peroxidase. When multiple target proteins were analyzed in the same experiment, in which not all the target proteins could be analyzed by a single immunoblot, aliquots of the same sample were analyzed by separate SDS-gel electrophoreses with their own loading controls, followed by separate immunoblotting analyses. The data of multiple immunoblots along with one representative loading control were placed in a signal figure panel. Blots were visualized with an enhanced chemiluminescence detection kit (Pierce, Thermo Scientific, USA) and the ChemiDoc MP Imaging System (Bio-Rad).

Immunoprecipitation and mass spectrometric analysis

For immunoprecipitation analysis, antibodies were mixed and incubated on a rotating platform for an hour, and then elution buffer and quenching buffer were used to wash the magnetic beads and antibodies. The diluted lysate solution was added to the tube containing the antibody-coupled magnetic beads and incubated according to the manufacturer's recommendations by using a Direct Magnetic IP kit (Thermo). The SEC24C protein complexes were immunoprecipitated with anti-SEC24C antibody-conjugated beads by SDS–PAGE and then stained with Coomassie brilliant blue. The Coomassie brilliant blue-stained bands were determined by LC–MS/MS with desalting using a famos autosampler. The sample spectra were evaluated with the National Center for Biotechnology Information protein database with Mascot and Sequest.

Plasmid constructs and lentiviral packaging

The human SEC24C gene was cloned into pLVX-CMV-IRE-Puro to generate pLVX-SEC24C-Flag. The SEC24C fragments comprised residues 1 to 421, 422 to 498, 499 to 747, 748 to 843, 844 to 961, and 962 to 1094. SEC24C fragments were synthesized by Anhui General Biological Co. Ltd. based on the sequence. Then, the sequences were subcloned into the pCDH-CMV-MCS-EF1-copGFP-T4A-Puro vector plasmid by NheI/BamHI and further packaged for lentiviral particles according to a previously described method. In brief, the candidate plasmid was cotransfected with psPAX2 and

pMD2.G in the 293 T-cell line by using Lipofectamine® 3000 transfection reagent (Invitrogen Life Technologies, CA, USA) per the manufacturer's recommendations. The supernatant was collected after culturing for 48 h. The virus supernatant was concentrated through ultracentrifugation. The human SEC24A, SEC24B and SEC24D gene was cloned into pLVX-CMV-IRE-Puro to generate pLVX-SEC24A, pLVX-SEC24B, pLVX-SEC24D.

The Cas9 expression construct pLV1-CMV-Cas9-Puro-U6-sgRNA was used for the expression of sgRNAs in Huh7 cells. Empty pLV1-CMV-Cas9-Puro-U6-sgRNA plasmid was used as a control (sgNC). Puromycin (1 μg/ml, Merck, 540411) was added 24 h after transfection. Cells were collected 72 h after transfection.

pCW57-CMV-ssRFP-GFP-KDEL was a gift from Noboru Mizushima (Addgene plasmid # 128257; http://n2t.net/addgene:128257; RRID: Addgene_128257)

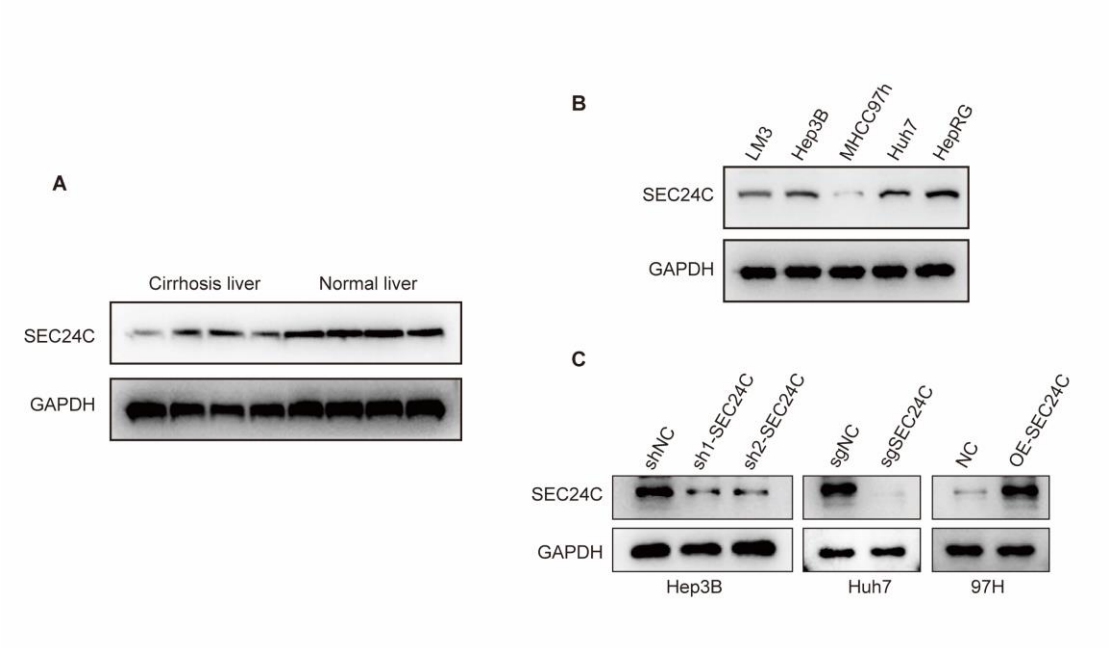
Immunohistochemistry and immunofluorescence

Paraffin-embedded and formalin-fixed HCC samples were used for immunohistochemistry detection as previously described (Jiang, R. et al. Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. Hepatology 54, 900–909 (2011). Slides were incubated with 3% hydrogen peroxide and blocking buffer for 5 min and 30 min, respectively, followed by primary antibody overnight at 4°C. Primary antibodies against SEC24C (1:300; NBP1-81550, Novus), PERK (1:300; ab217322, Abcam), phospho-PERK-T982 (1:200; AP0886, ABclonal), CHOP (1:1000; A20987, Abclonal), and Ki67 (1:800; 9129s, Cell Signaling Technology) were used according to the manufacturer's instructions. The SEC24C expression level was determined by integrating the percentage of positive tumor cells and the intensity of positive staining. The intensity of staining was scored as follows: negative (score 0), bordering (score 1), weak (score 2), moderate (score 3), and strong (score 4). We scored the staining extent according to the percentage of positively stained tumor cells in the field: negative (score 0), 0–25% (score 1), 26–50% (score 2), 51–75% (score 3), and 76–100% (score 4). The staining was observed and assessed by two independent pathologists without knowing the identity of the samples.

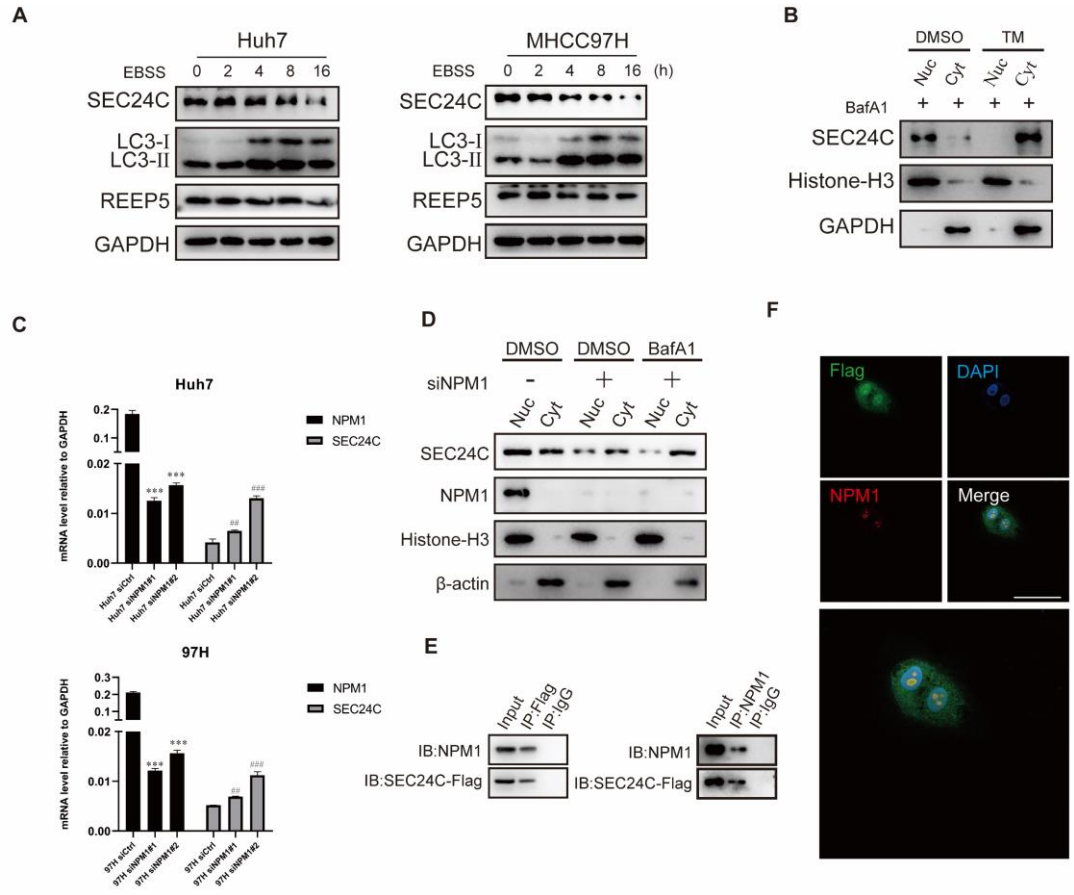
For immunofluorescence analysis, cells were washed twice with PBS, fixed with paraformaldehyde, and then permeabilized in 0.1% Triton X-100 at room temperature for 10 minutes. After blocking, the cells were incubated with the respective primary antibodies overnight at 4°C. The primary antibodies used in the present study were as follows: NPM1 (1:300, A17983, Abcloanl), SEC24C (1:300; NBP1-81550, Novus), PERK (1:300, A18196, ABclonal), Calreticulin (1:300, #20181, Promab) and LAMP1 (1:300, sc-20011, Santa Cruz). Subsequently, the cells were incubated with proper secondary antibodies conjugated with the indicated fluorescent dye for 1 h at room temperature. DAPI was used to label the nuclei, and ER-Tracker was used to label the endoplasmic reticulum. Positive cells were quantified using Image-Pro Plus software (Media Cybernetics, MD, USA) and detected by confocal microscopy (Zeiss,

Oberkochen, Germany).

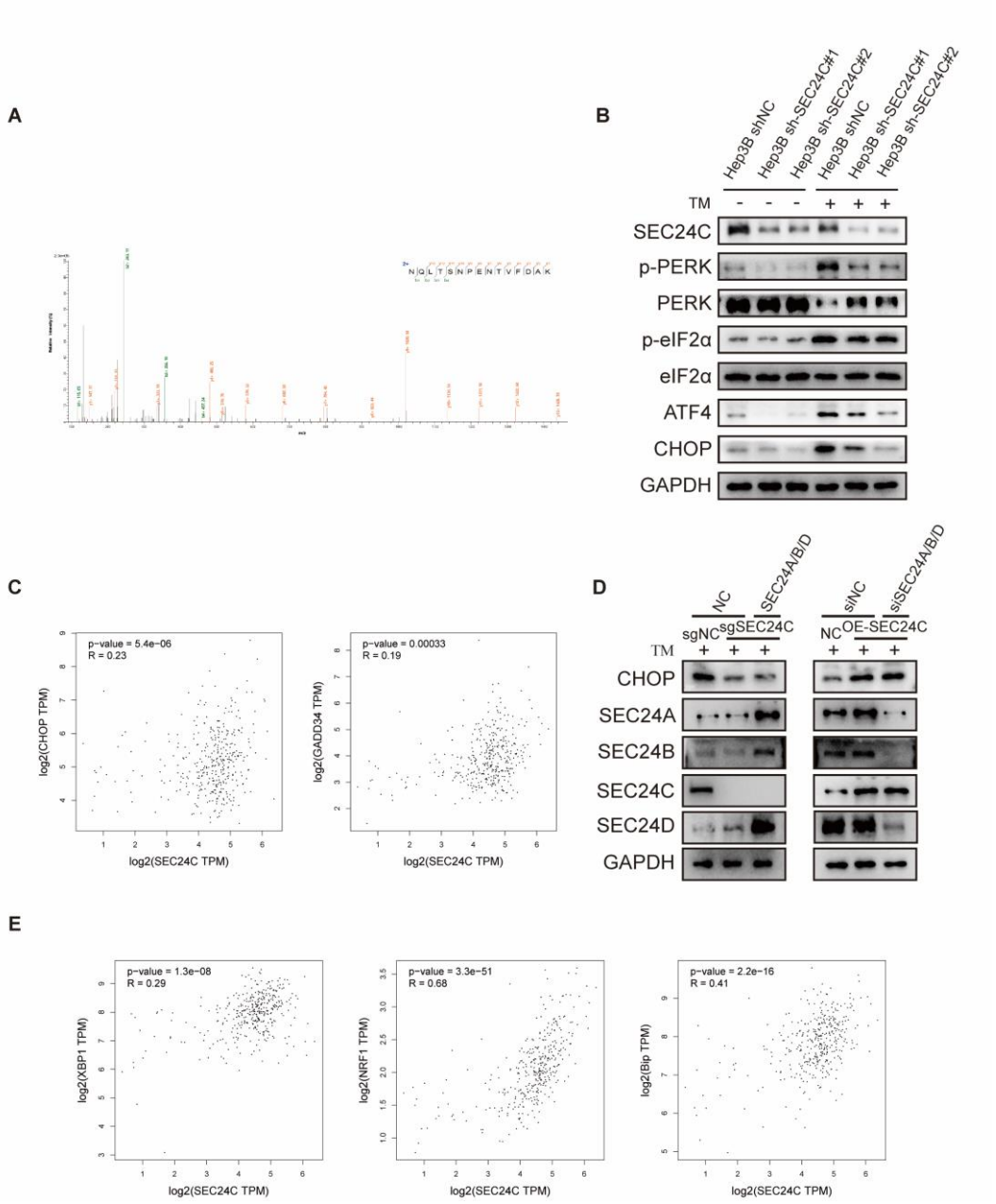
Supporting Figures:



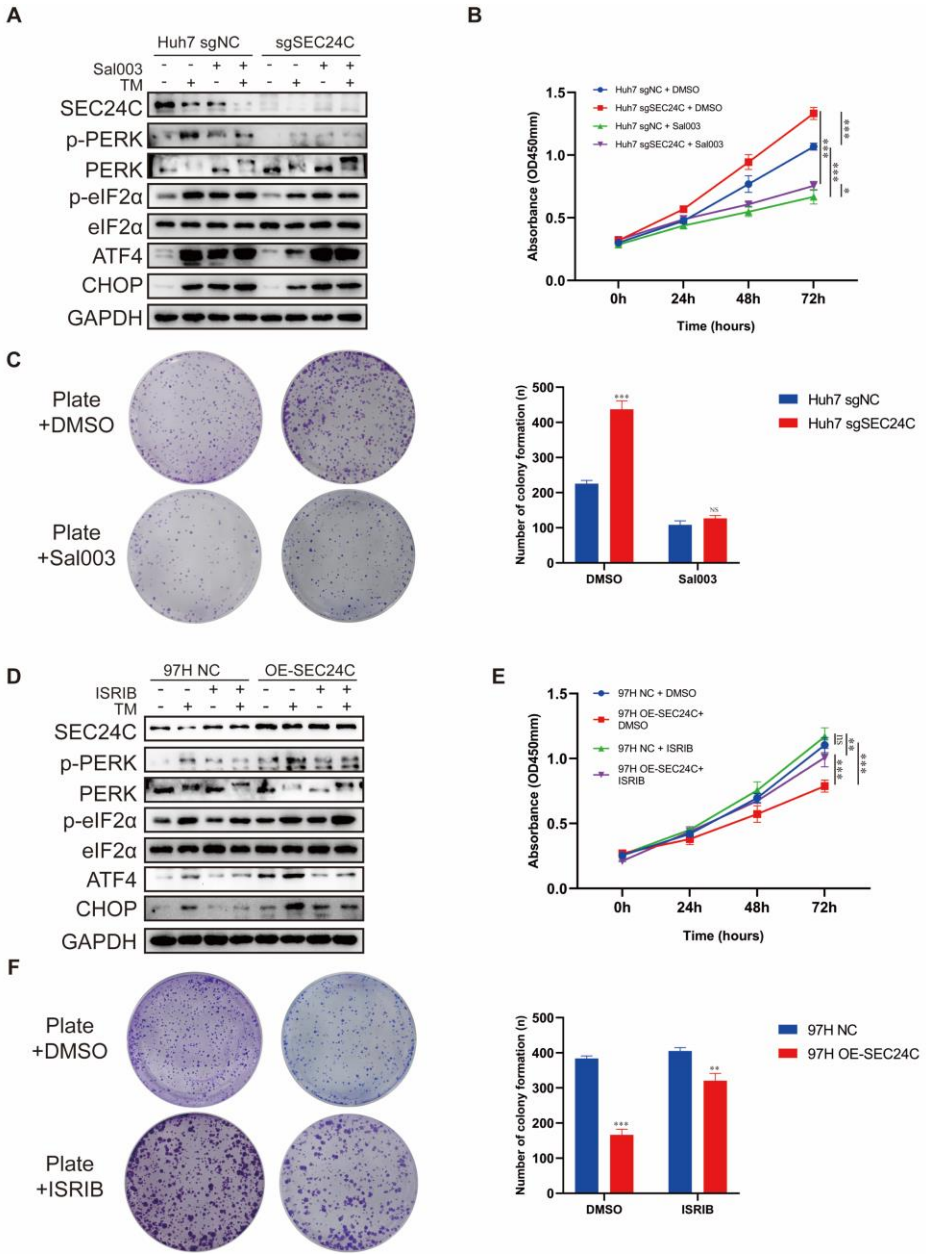
Supporting Figure S1 SEC24C expression in the different HCC cell lines. (A) Representative expression levels of SEC24C protein in cirrhosis liver and normal liver tissues analyzed by WB. (B) SEC24C protein levels in HepaRG, Huh7, MHCC-97H, HCCLM3, and Hep3B cell lines respectively. (C) Western blot analysis of the efficiency of SEC24C knockdown in Hep3B cells, SEC24C knockout in Huh7 cells and SEC24C overexpression in MHCC97H cells.



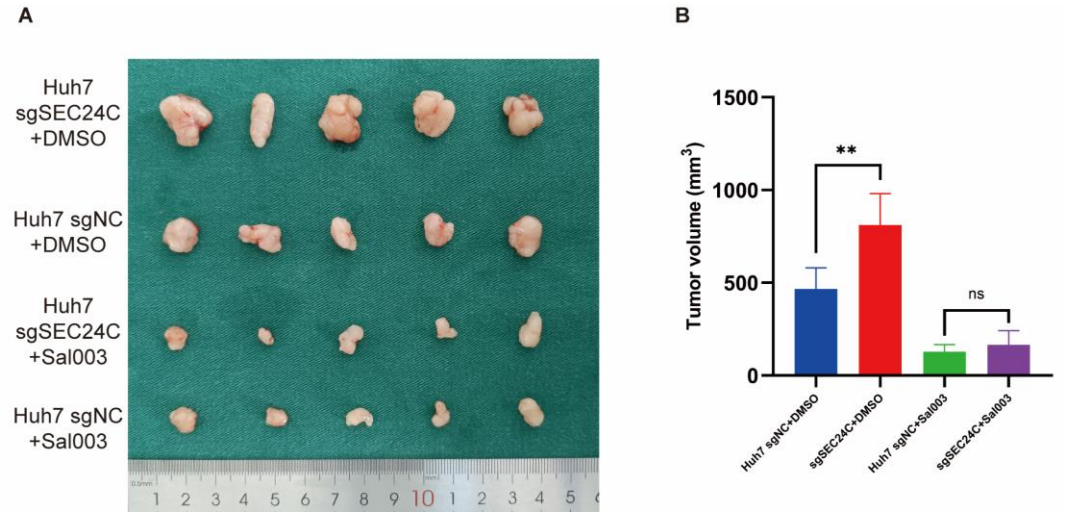
Supporting Figure S2 Shuttling and degradation of SEC24C under the starvation conditions in HCC cells. (A) Western blot analysis of the protein levels of LC3-I/II and REEP5 after SEC24C knockout or overexpression under the starvation. (B) Western blot analysis of the indicated proteins obtained from the subcellular fractionations of Hep3B cells treated with DMSO or TM. (C) qRT–PCR was used to examine the mRNA levels of SEC24C and NPM1 after NPM1 knockdown in Huh7 and MHCC97H cells. All the data has been expressed as the mean ± SD. (***P < 0.001; $^{\#\#}$P < 0.01; $^{\#\#\#}$P < 0.001) (D) Western blot analysis of the indicated proteins obtained from the subcellular fractionations of Hep3B cells transfected with siNC or siNPM1 upon treatment with DMSO or BafA1. (E) SEC24C-Flag interacts with NPM1, as evidenced by a Co-IP assay with Flag antibody. (F) Representative images depicting the co-localization of SEC24C-Flag and NPM1 with Flag antibody. Abbreviations: Cyt, cytosolic fraction; Nuc, nuclear fraction.



Supporting Figure S3 SEC24C modulated the activation of the UPR independent of its secretory function in HCC cells. (A) A representative Bip tryptic peptide identified by mass spectrometry (MS) analysis of SEC24C-interacting proteins. (B) Western blot analysis of the protein levels of phosphorylated PERK, PERK, phosphorylated eIF2α, eIF2α, ATF4 and CHOP after SEC24C knockdown following tunicamycin treatment. (C) TCGA database showed the significant positive correlation between the mRNA levels of SEC24C and CHOP or GADD34. (D) Western blot analysis of the protein levels of CHOP upon SEC24C, SEC24A, SEC24B or SEC24D change after the treatment with tunicamycin. (E) TCGA database showed the significant positive correlation between the mRNA levels of SEC24C and X-box binding protein 1 (XBP1), Nuclear Respiratory Factor 1 (NRF1) or Bip.



Supporting Figure S4 Restraining eIF2α activation can reverse the suppressive role of SEC24C in HCC progression. (A) The protein levels of the PERK-eIF2α-ATF4 axis and CHOP after the treatment with tunicamycin, Sal003 or both in Huh7 sgNC and Huh7 sgSEC24C cells. CCK8 assays (B) and colony formation assays (C) of Huh7 sgNC and Huh7 sgSEC24C cells treated with DMSO or Sal003. The numbers of the colonies were calculated and have been shown in the right bar graph. (D) The protein levels of the PERK-eIF2α-ATF4 axis and CHOP with tunicamycin, ISRIB or both in MHCC97H NC and MHCC97H OE-SEC24C cells. CCK8 assays (E) and colony formation assays (F) of MHCC97H NC and MHCC97H OE-SEC24C cells treated with DMSO or Sal003. The numbers of colonies were calculated and have been shown in the right bar graph. All experiments were performed in triplicate, and data has been expressed as the mean ± SD. (*P < 0.05; **P < 0.01; ***P < 0.001; ns as no significance).



Supporting Figure S5 Sal003 inhibited HCC progression in vivo independent of SEC24C expression. (A) Representative images have been shown for the tumors derived from the nude mice induced by Huh7 sgSEC24C and Huh7 sgNC cells after the tail vein injection of vehicle or Sal003. The tumor weight and volume of each group of mice have been summarized in the bar graph (B). All the data has been expressed as the mean ± SD. (**P < 0.01; ns as no significance).