Materials and methods

Cell culture

The Huh7 human HCC cell line (Shanghai Institutes for Biological Sciences, CAS, China) was maintained in minimum essential media (MEM, Gibco) containing 10% (volume fraction) fetal bovine serum (FBS, Gibco) and 1% (volume fraction) penicillin-streptomycin, and incubated at 37 °C with 5% CO_2 (Thermo Scientific, USA).

Animal experiments

All animal experiments received ethical approval from the Institutional Animal Care and Use Committee (IACUC) at The First Affiliated Hospital of Zhejiang University School of Medicine. C57BL/6J mice (GemPharmatech Co., Ltd., Nanjing, China) were housed in a specific pathogen-free (SPF) facility with regulated humidity, temperature, and 12-h light/dark cycles, with free access to food and water. All procedures complied with the institutional ethical guidelines.

In order to evaluate the role of siWTAP in protein kinase B (AKT)/Ras-driven HCC and its therapeutic potential, 8-week-old C57BL/6J mice (n=8) were randomized into three groups. Hydrodynamic tail vein injections were administered using a plasmid cocktail containing 5 µg pT3-elongation factor-1α (EF1α)-myristoylated AKT (myr-AKT), 5 µg NRasV12/pT2-CAGGS, and 0.8 µg pCytomegalovirus-sleep beauty (pCMV/SB) dissolved in 2 mL saline. Four weeks post-AKT/Ras induction, the mice received daily oral sorafenib (12.5 mg/kg, dissolved in corn oil) for 10 d, followed by intravenous siWTAP or scramble small interfering RNA (siRNA) (1.5 mg/kg) every 3–4 d for two weeks.

RNA interference

WTAP-targeting siRNAs and negative control (siControl) were synthesized commercially (GenePharma, Shanghai, China). Transient transfections were carried out using the jetPRIME Polyplus Kit (France) following standard protocols. The cells were collected for immunoblot analysis 48 h post-transfection. All the siRNA sequences are listed in Table S1.

Table S1　Target sequences of siRNAs

Gene	Sequences (5'→3')
SiWTAP-1	GCUUUGGAGGGCAAGUACATT
SiWTAP-2	GGUUCGAUUGAGUGAAACATT

Cell counting kit-8 (CCK-8) assay

Cell proliferation was quantified via CCK-8 assay (Dojindo Laboratories), wherein Huh7 cells

were plated at 2×10^3 cells/well in 96-well plates and incubated for 24 h under standard conditions. The cells were then exposed to sorafenib at gradient concentrations ranging from 1.25 to 20 μmol/L for 96 h. A volume of 10 μL CCK-8 reagent was added to each well, followed by 1 h incubation. The absorbance at 450 nm was measured, and the 50% inhibitory concentration (IC_{50}) was calculated.

Western blot analysis

Protein lysates were extracted using ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Biyuntian) containing protease/phosphatase inhibitors (Thermo Scientific), quantified via detergent-compatible (DC) protein assay (Thermo Scientific), and resolved electrophoretically on 4%–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (GenScript). After being transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), the membranes were incubated overnight at 4 °C with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibody detection and chemiluminescent visualization (Bio-Rad). All the antibodies used are listed in Table S2.

Table S2   Antibodies

Antigens	Host	Source	Identifier
GAPDH	Mouse	Proteintech	CAT#60004-1-Ig
WTAP (WB)	Mouse	Proteintech	CAT#60188-1-Ig
MAPK	Rabbit	Cell Signaling Technology	CAT#4695
P-MAPK	Rabbit	Cell Signaling Technology	CAT#4370

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; WTAP: Wilms' tumor 1-associated protein; WB: western blotting;

MAPK: mitogen-activated protein kinase; P-MAPK: phosphorylated MAPK.

Immunohistochemistry (IHC) and TUNEL assay

Formalin-fixed, paraffin-embedded tissue blocks were sectioned into 5 μm-thick slices for histological analysis. After deparaffinization and antigen retrieval, the slides were incubated with primary antibodies and visualized using the Real Envision Detection System (DAKO). Apoptosis was assessed via the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Kit (Abcam). A quantitative assessment of positively stained regions or cell counts was conducted through systematic sampling of six randomized fields per histological section. The subsequent statistical evaluation was performed using ImageJ software equipped with the IHC analyzer module.

Gene set enrichment analysis (GSEA)

WTAP-associated pathways were analyzed using The Cancer Genome Atlas-liver hepatocellular carcinoma (TCGA-LIHC) data via GSEA software (https://www.gsea-msigdb.org). Hallmark and KEGG_C2 gene sets were evaluated to compute enrichment score (ES), normalized ES (NES), and significance (*P*-value).

Molecular docking

The three-dimensional (3D) structural coordinates of WTAP (UniProt ID: Q15007) and mitogen-activated protein kinase (MAPK) (UniProt ID: P28482) were obtained from the AlphaFold Database (https://alphafold.ebi.ac.uk). The docking results of the proteins with their targets were visualized using PyMOL software (https://pymol.org/2). In the visualization, WTAP is represented in red, MAPK in blue, and the docking interface in yellow.

Exploration of the model in chemotherapy response

Sorafenib IC_{50} values were calculated using the R package "oncoPredict." The IC_{50} value signifies the concentration of a substance required to inhibit a specific biological or metabolic process by 50%. To ascertain differences between groups, the Wilcoxon signed-rank test was utilized.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9.5.1 and R programming language (version 4.4.1). All experimental data were presented as mean values with standard error of the mean (SEM). For comparisons between two experimental groups, unpaired Student's *t*-test was applied, while one-way analysis of variance (ANOVA) was utilized for multi-group analyses. The statistical significance thresholds were defined as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.