SUPPLEMENTARY MATERIALS AND METHODS

Cell lines and clinical samples

The Huh-7 cell line was obtained from the Japanese Collection of Research Bioresource (JCRB, Osaka, Japan). The HCCLM3 cell line was purchased from the BeNa Culture Collection (Suzhou, China). The HEK-293T cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Huh-7 cells with UBE2S knockout (Huh-7$^{KO(UBE2S)}$) were constructed by Cyagen Biosciences Inc. using the CRISPR/Cas9 technique. The gRNA sequences were as follows: gRNA-A1-GCTGATGGCCTGAAGCATCC-TGG and gRNA-A2-GGTACTCTAGATTTGAAGCA-AGG. All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO_2 incubator. A tissue microarray containing 90 HCC samples for immunohistochemistry was purchased from Shanghai Outdo Biotech Co. Ltd. (HLivH180Su16), and the study was approved by the Ethical Committee of Shanghai Outdo Biotech Company (YB M-05-02).

Quantitative PCR analysis

Total RNA was extracted using the Total RNA kit II (Omega, USA) and reverse-transcribed into complementary DNA by PrimeScript™ RT reagent kit (TaKaRa, Japan). Quantitative PCR assays were performed using SYBR Premix Ex-Taq™ reagent (TaKaRa, Japan) in a Mx3005P Real-Time PCR system (Agilent, Santa Clara, CA, USA), according to the manufacturer's instructions. The primer sequences are listed in Supplementary Table 2.

Western blot analysis

Cells or tissues used for Western blot analysis were lysed with RIPA lysis buffer. Proteins were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The membranes with proteins were blocked with 5% skimmed milk for 1 h and incubated with primary antibodies overnight at 4°C and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the proteins were visualized using the ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). The antibodies used in this study are listed in Supplementary Table 3.

Chromatin immunoprecipitation (ChIP)

Huh-7 or HCCLM3 cells transfected with E2F2 overexpressing plasmids were crosslinked with 1% formaldehyde for 10 min and then incubated with glycine for 5 min at room temperature. The cells were washed twice with cold PBS and harvested in lysis buffer using a ChIP Assay Kit (9005; Cell Signaling Technology, Danvers, MA, USA). Micrococcal nucleases and ultrasound were used to break up the nucleus and splice chromatin. Chromatin samples were divided equally and incubated with anti-FLAG, negative control IgG, or positive control Histone H3 antibodies overnight. Then, protein G magnetic beads were added and incubated for 2 h at 4°C. Thereafter, the beads were washed thrice using the low-salt buffer and once using the high-salt buffer at 4°C rotation. The eluted chromatin was then incubated with ChIP elution buffer containing NaCl and proteinase K for 2 h at 65°C. Finally, DNA samples were purified for qPCR analysis. The primers used for ChIP-qPCR are listed in Supplementary Table 2. The antibodies used are listed in Supplementary Table 3.

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Animal studies

Animal studies were approved by the Animal Ethics Committee of National Translational Science Center for Molecular Medicine, Fourth Military Medical University (2021-NTSCMM-ID013). All mice were housed under pathogen-free conditions in a controlled temperature (22–24°C) and light (12 hours light/dark cycle) environment.

To detect the effect of UBE2S on the tumorigenicity of HCC *in vivo*, *UBE2S*$^{loxP/loxP}$ mice with a C57BL/6N background were generated using CRISPR/Cas9, and hepatocyte-specific UBE2S knockout mice (*UBE2S*$^{-/-}$) and control mice (*UBE2S*$^{+/+}$) were produced by crossing *UBE2S*$^{loxP/loxP}$ mice with albumin-Cre (Alb-Cre) mice (Biocytogen, Beijing, China). The genotypes of the mice were identified by PCR using the genomic DNA of the mouse tail, and the primer sequences of the genotypes are listed in Supplementary Table 4. To induce HCC, male Alb-Cre^{+}/*UBE2S*$^{loxP/loxP}$ mice (*UBE2S*$^{-/-}$) and Alb-Cre^{-}/*UBE2S*$^{loxP/loxP}$ mice (*UBE2S*$^{+/+}$) were injected intraperitoneally with 25 mg/kg diethylnitrosamine (DEN; Sigma-Aldrich, St Louis, MI, USA) on postnatal day 14. Thereafter, both groups of mice were intraperitoneally injected with 0.5 μl/g CCl_4 once a week for a total of 14 times starting at postnatal week 5. The mice were sacrificed and liver tissues were collected on week 32 after birth.

To evaluate the therapeutic effects of cephalomannine and PX-478 *in vivo*, Huh-7 cells stably expressing firefly luciferase were subcutaneously injected into the flanks of male nude mice aged 6–8 weeks. Two weeks after inoculation, the nude mice were divided into four groups based on the tumor volume. The mice were then intraperitoneally injected with cephalomannine (10 mg/kg) and PX-478 (50 mg/kg), either alone or in combination, once every three days for a total of five times. In addition, the mice were intraperitoneally injected with D-luciferin potassium before and after treatment for *in vivo* imaging using the Carestream Molecular Imaging System according to the manufacturer's instructions.

Seahorse XF glycolysis stress test

The extracellular acidification rate (ECAR) of different HCC cell lines was monitored using a Seahorse Analyzer XF96 (Agilent) according to the manufacturer's instructions. Briefly, HCC cells (80 μL, 1×10^4 cells/well) were cultured in an XF96 cell culture plate containing medium. The XF calibrator was added to each well of the XF probe plate pretreated with sterile water overnight, and incubated in a CO_2-free incubator at 37°C for 45–60 min. The cells were washed with XF assay medium thrice and were cultured in a CO_2-free incubator at 37°C for 60 min. Glucose, oligomycin, and 2-deoxy-D-glucose (2-DG) were successively added to each well of the XF probe plate, and the ECAR of the wells in the XF96 cell culture plate was assessed.

Dual-luciferase reporter assay

Different HCC cells or HEK-293T cells were seeded in 24-well plates and transfected with a luciferase reporter plasmid containing the UBE2S promoter together with the E2F2 overexpression plasmid or control plasmid in the presence of pRL-TK for 24 h. After the cells were lysed with PLB buffer for 15 min, LAR II and Stop & Glo® reagents were added successively to measure the luciferase activity using the Dual-Glo Luciferase Assay System (Glomax 20/20 Luminometer; Promega, Madison, WI, USA).

Cytotoxicity assay

Huh-7 cells (100 μL, 5×10^3 cells/well) or HCCLM3 cells (100 μL, 3×10^3 cells/well) were seeded into 96-well plates and cultured overnight at 37°C in a 5% CO_2 incubator. The cells were then treated with different concentrations of cephalomannine or PX-478 for 72 h, and the absorbance at 450 nm was measured using the CCK-8 reagent (Engreen Biosystem, Beijing, China) according to the manufacturer's instructions.

Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation assays were performed to detect the interaction between UBE2S and VHL in different HCC cell lines according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the target protein antibody and control IgG antibody was immobilized on an amino link-coupled resin. HCC cells were then lysed with IP lysis buffer and incubated overnight at 4°C with the resin. The protein complex was further eluted with elution buffer and boiled with loading buffer for subsequent Western blot analysis.

Surface plasmon resonance (SPR) assay

SPR analysis of the interaction between VHL and UBE2S (1–156 aa) as well as the binding kinetics were performed using a Biacore T200 instrument. The recombinant VHL protein was immobilized on a CM5 chip (Biacore; GE Healthcare). Purified UBE2S (1–156 aa) protein was injected at indicated concentrations at a flow rate of 30 μL/min. The resulting data were fit using a mathematical model of a simple 1:1 interaction in the Biacore T200 evaluation software.

Immunoprecipitation (IP) assay

Immunoprecipitation assay was performed according to the manufacturer's instructions (Thermo Fisher Scientific). Briefly, Huh-7 or HCCLM3 cells transfected with indicated plasmids for 48 h were lysed with IP lysis buffer and then incubated overnight at 4°C with the primary antibody attached to protein A/G agarose. The complex was then washed with IP lysis buffer and conditioning buffer. Finally, the sample was eluted with elution buffer and boiled with loading buffer for subsequent Western blot analysis.

In vitro ubiquitination assay

For *in vitro* ubiquitination assay, reaction mixtures (50 μL) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, 5 mM ATP, 10 μg of ubiquitin, 0.2 μg of UBE1, 0.2 μg of UBE2S, and 0.5 μg of VHL were incubated at 37°C for 2 h. The reactions were stopped by boiling in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting using anti-Ub antibody or anti-VHL antibody.

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