Supplementary Information

Supplementary Materials

Immunoblotting (IB) assays.

The immunoblotting experiments were performed in accordance with established procedures. In summary, the cell lysates underwent separation using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique and were subsequently transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were probed using antibodies specific to RBMS3 (1:500, Abcam, ab272612), ANGPT2 (1:1000, Proteintech, 24613-1-AP), TRIM21 (1:1000, Proteintech, 12108-1-AP) and Flag (1:2000, Sigma,F1804), HA (1:2000, Proteintech, 66006-2-Ig) overnight incubation at a temperature of 4°C, followed by a subsequent incubation for one hour at room temperature using the specified secondary antibodies (PIERCE) conjugated with horseradish peroxidase. The blotting membranes were subjected to stripping and then probed using an antibody against α-Tubulin (1:1000 Abcam, ab7291) or an antibody targeting GAPDH (1:1000, Proteintech, 60004-1-lg).

Vectors, retroviral infection and transfection.

The pCDH-EF1a-Puro retroviral vector was employed to create the pCDH-EF1a-Puro/RBMS3 recombinant plasmid by subcloning the human RBMS3 coding sequence, which had been amplified via PCR, into the pCDH-EF1a-Puro vector. The Plko.1-EGFP-puro vector was employed to clone two shRNA oligonucleotides, leading to the production of RBMS3-sh#1 and RBMS3-sh#2, respectively, with the aim of efficiently suppressing the expression of endogenous RBMS3. The Supplementary Table S3 contains the comprehensive sequences of siRNA oligonucleotides. The stable cell lines underwent a 10-day selection process using puromycin (0.5μg/ml) starting 48 hours after infection. The protein expression

of RBMS3 in the specified stable cell lines was confirmed through SDS-PAGE gel analysis following a ten-day selection period.The plasmids HA-K63, HA-K48, HA-K48R, HA-K63R were subcloned into the pCDH-EF1avector, respectively. In this study, Lipofectamine 3000 (Life Technologies) or RNAimax (Life Technologies) was employed for conducting recombinant plasmid or siRNA transfections as per the guidelines provided by the manufacturer.

Reverse transcription (RT) and real-time PCR.

In this investigation, TRIzol (Life Technologies) was employed to extract total RNA from cultured cells or tissue samples according to the guidelines provided by the manufacturer. To perform reverse transcription (RT) of total mRNA, a PrimeScript RT Reagent kit (TaKaRa) was utilized. The cDNAs were amplified and quantified using the Bio-Rad CFX qRT-PCR detection system from Applied Biosystems. The FastStart Universal SYBR Green Master kit (ROX) provided by Roche (Toronto, ON, Canada)was utilized for amplification and quantification. Measurement was carried out utilizing the ABI Prism 7500 Sequence Detection System also from Applied Biosystems. The expression levels of the data were standardized by referencing the housekeeping gene GAPDH to account for any variations. The standardization was determined using the formula $2^{-[(C_t \text{of gene}) - (C_t \text{of } GAPDH)]}$, where Ct represents the threshold cycle for each transcript. The primer sequences, derived from the Genome database, can be found in Supplementary Table S3 under the Primers and Oligonucleotides table.

CCK8 assay

Cell survival was assessed by a Cell Counting Kit-8 (KGA317, KeyGen Biotech, China) assay. Briefly, HCC cells were seeded into a 96-well plate at a density of 3000 cells per well. After a 48-hour incubation, 10 μL of CCK-8 reagent was added

to each well. The cells were then returned to the incubator at 37°C for an additional 2 hours. The absorbance at 450 nm was measured using a microplate reader.

ELISA.

The concentration of ANGPT2 protein in the supernatant of the cell culture was quantified using ELISA kits that were specifically developed for detecting human ANGPT2 (Boster Biological Tecnhology Co., LTD, Pleasanton, CA, USA, EK0657) , according to the manufacturer's protocol.

Immunoprecipitation-Mass Spectrometry (IP-MS)

he immunoprecipitation and mass spectrometry analyses were conducted in accordance with our previous investigations[1, 2]. Lysates from the designated cells were incubated with agarose beads that were conjugated with Flag (Sigma-Aldrich). In order to perform the mass spectrometry (MS) analysis, specific bands were excised and subsequently subjected to LC-MS/MS analysis.

Co-immunoprecipitation (Co-IP) assay

Co-immunoprecipitation and western blot analysis were performed using standard protocols. The following antibodies were used: anti-Flag or anti-HA. IB assays were performed with the objective of elucidating the interactions between proteins involving the target protein..

Chemical reagents

Puromycin (S7417), Sorafenib (S7397), Anti-ANGPT2antibody (MEDI3617) (A2770) and MG132(S2619)were purchased from selleck (Houston, US). Cycloheximide (C7698) was purchased from Sigma-Aldrich (Darmstadt, Germany).

CRISPR/Cas9 system.

The Huh7/R3 knockout cells were created using the CRISPR/Cas9 system,

following established protocols[2, 3]. The corresponding single guide RNAs were designed and inserted into the GV392 plasmid (RBMS3 sgRNA: TTGCTGTGCAAATTCGCTGA). In brief, $3*10^5$ Huh7 cells were infected with lenti-CRISPR virus at a multiplicity of infection (MOI) of 2. After 24 hours, puromycin selection was applied to the infected cells at a concentration of 0.5ug/ml for duration of 7 days. Subsequently, Huh7/Cas9 cells were re-infected with GV392-GFP-RBMS3 gRNA lentivirus at an MOI of 4 to ensure that over 95% of the cells expressed the target gene. Genomic DNA was isolated from Huh7/RBMS3 KO#1 cells using a QIAGEN genomic DNA Kit and amplified to obtain the target sequence. Knockout and Mutation Detection Kit (GeneChem) was utilized for heterodimerization and digestion as per manufacturer's instructions. Additionally, genomic DNA underwent amplification and sequencing on HiSeq 2500 (Illumina, San Diego, CA, USA) with a single-end run consisting of reads measuring100-bp in length. The sequencing data obtained from HiSeq 2500 underwent analysis and normalization following previously described methods

Determination of protein degradation.

The designated time points were chosen to administer CHX or MG132 treatment to the cells, after which total protein was extracted. The quantification of IB assays was then utilized to calculate the protein half-lives.

References

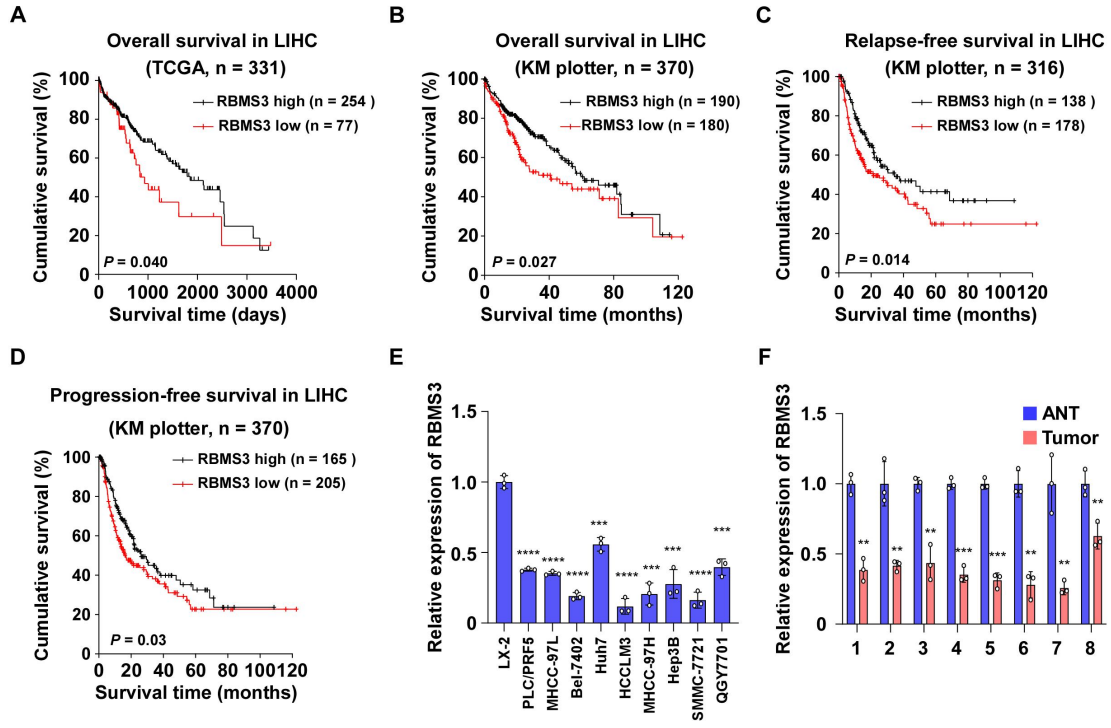
1 Cao L, Wu G, Zhu J, Tan Z, Shi D, Wu X *et al*. Genotoxic stress-triggered beta-catenin/JDP2/PRMT5 complex facilitates reestablishing glutathione homeostasis. Nature communications 2019; 10: 3761.

2 Wu G, Song L, Zhu J, Hu Y, Cao L, Tan Z *et al*. An ATM/TRIM37/NEMO Axis Counteracts Genotoxicity by Activating Nuclear-to-Cytoplasmic NF-kappaB Signaling. Cancer research 2018; 78: 6399-6412.

3 Wu G, Cao L, Zhu J, Tan Z, Tang M, Li Z *et al*. Loss of RBMS3 Confers Platinum Resistance in Epithelial Ovarian Cancer via Activation of miR-126-5p/beta-catenin/CBP signaling. Clinical cancer research : an official journal of the American Association for Cancer Research 2019; 25: 1022-1035.

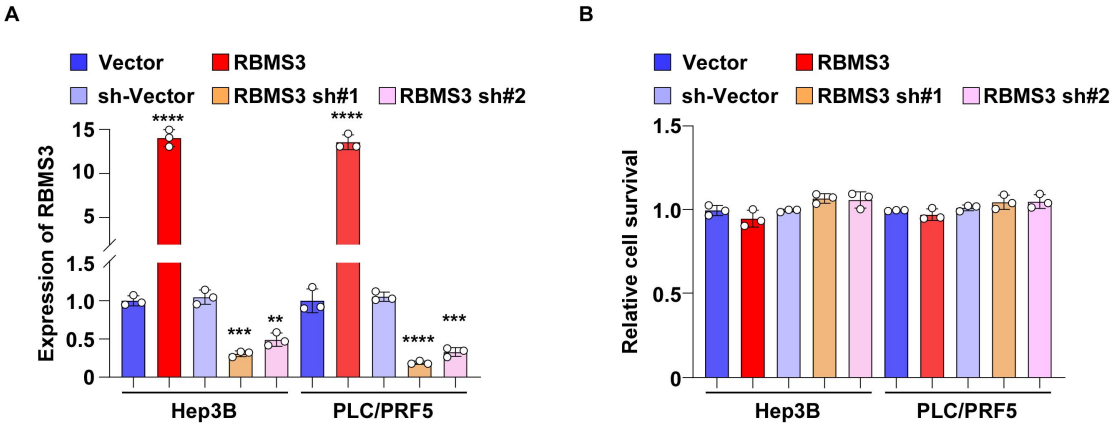
Supplemental Figure legends

Supplemental Figure 1



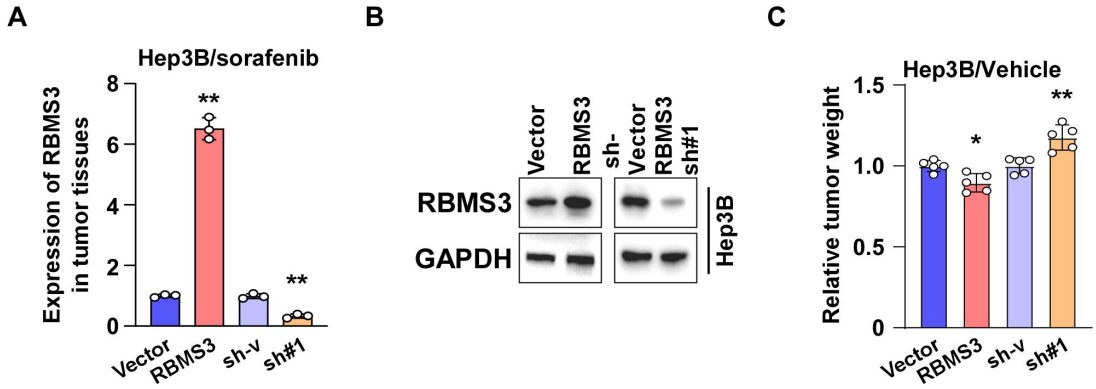
Supplemental Figure 1. RBMS3 expression is decreased in HCC and is associated with poor prognosis. A. Overall survival analysis in TCGA-LIHC patients with low and high expression of RBMS3. B-D. Overall survival (B), relapse-free survival (C) and progression-free survival (D) analysis in KM-plotter-LIHC patients with low and high expression of RBMS3. E. The mRNA expression of RBMS3 in HCC cell lines compared with hepatic stellate cells. F. The mRNA expression of RBMS3 in HCC tissues compared with ANT tissues.

Supplemental Figure 2



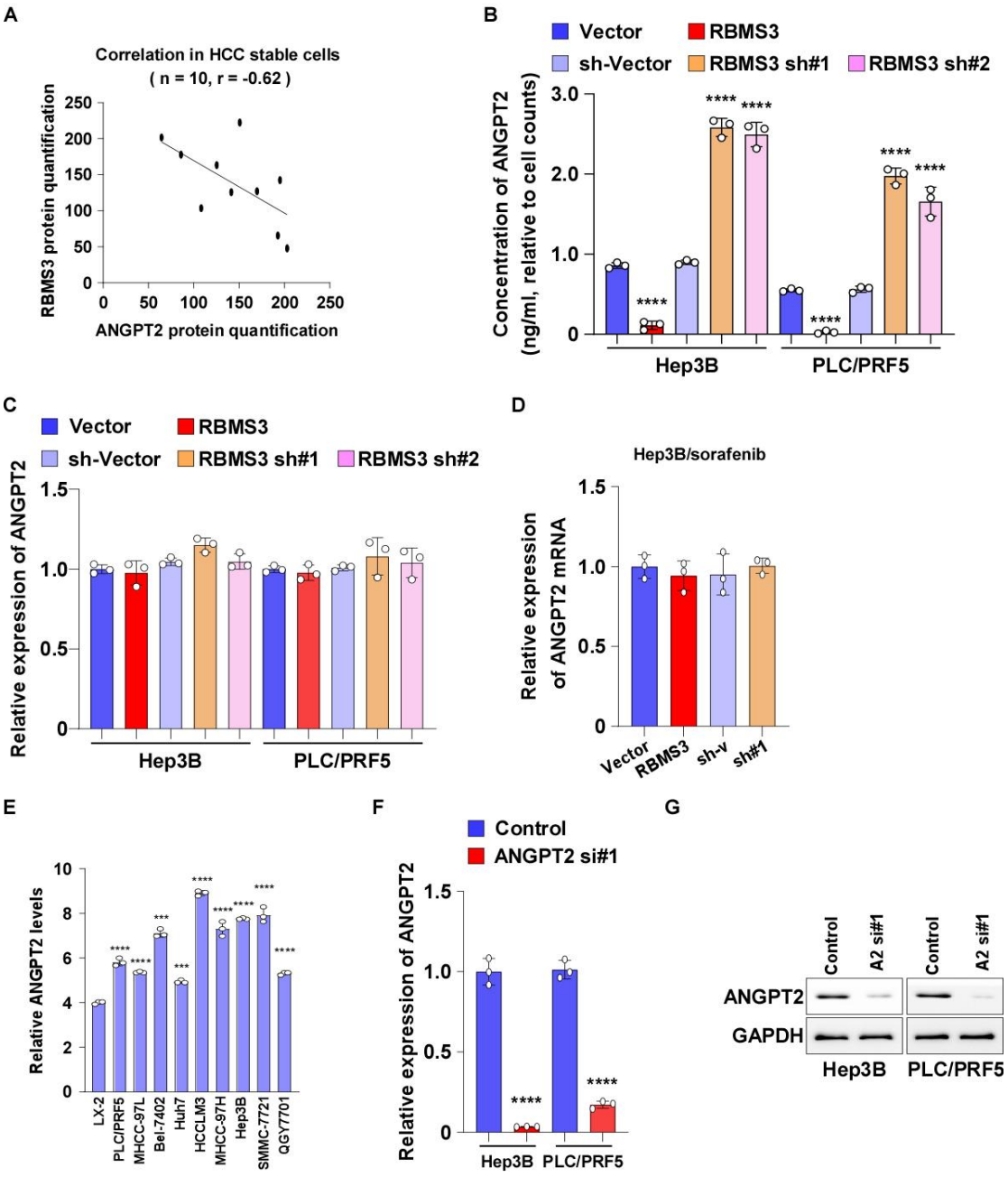
Supplemental Figure 2. Verification of RBMS3 expression and cell survival in indicated cells. A. The RNA expression of RBMS3 in indicated HCC stable cell lines. B. The cell survival of indicated HCC stable cell lines was conducted by CCK8 assays following sorafenib treatment.

Supplemental Figure 3



Supplemental Figure 3. The expression of RBMS3 in subcutaneous tumor from mice. **A.** The RNA expression of RBMS3 in xenograft tumors form by indicated HCC stable cell lines. B. The protein expression of RBMS3 in xenograft tumors form by indicated cells. C. The tumors weight of mice in indicated group.

Supplemental Figure 4

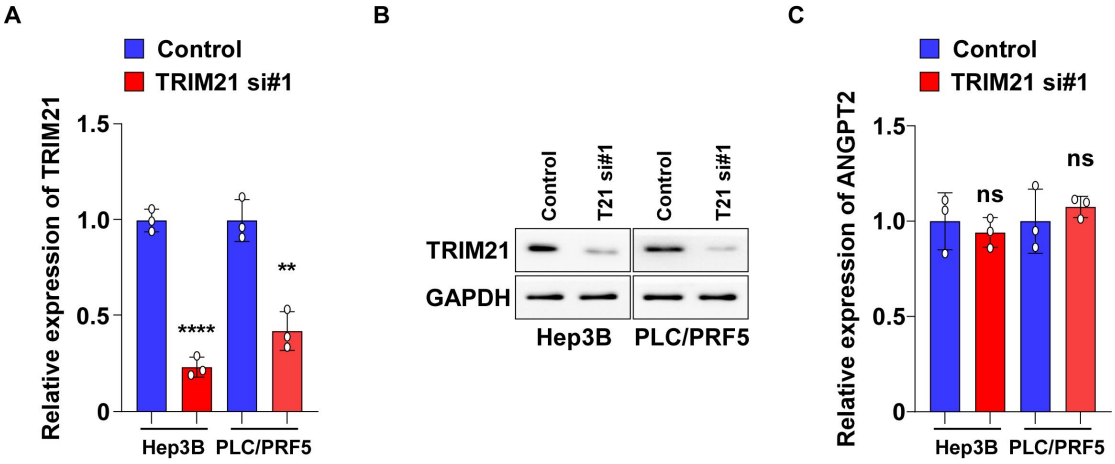


Supplemental Figure 4. The expression of ANGPT2 in indicated cells and tissues.

A. Correlation analysis of protein expression in indicated HCC stable cell lines. B. The concentration of ANGPT2 secreted by the specified HCC cell lines, with equal cell counts, was quantified using ELISA. C. The mRNA expression of ANGPT2 in indicated HCC stable cell lines following sorafenib treatment. D. The mRNA expression of ANGPT2 in xenograft tumor tissues form by indicated HCC stable cell

lines. E. ANGPT2 ELISA was performed to quantify ANGPT2 protein levels in supernatants of indicated HCC cell lines. F-G. The mRNA (D) and protein (E) expression of ANGPT2 in HCC cells transfected with ANGPT2 siRNA or control.

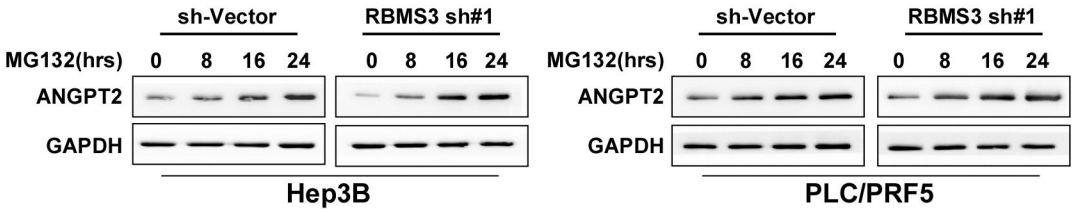
Supplemental Figure 5



Supplemental Figure 5. Verification of TRIM21 and ANGPT2 expression in TRIM21 knockdown cells.A-B. The mRNA (A) and protein (B) expression of TRIM21 in HCC cells transfected with TRIM21 siRNA or control. C. The mRNA expression of ANGPT2 in HCC cells transfected with TRIM21 siRNA or control. HCC cells in this figure were treated with sorafenib before harvested.

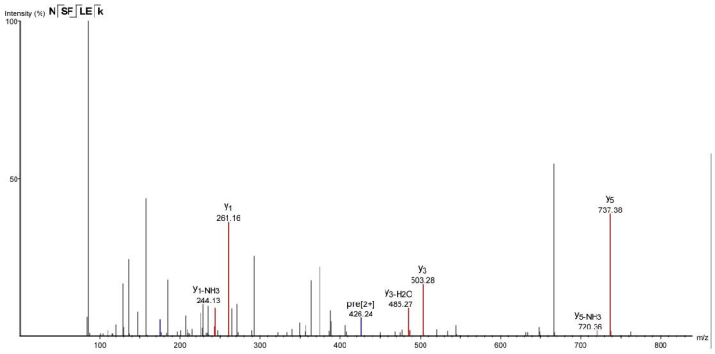
Supplemental Figure 6

A



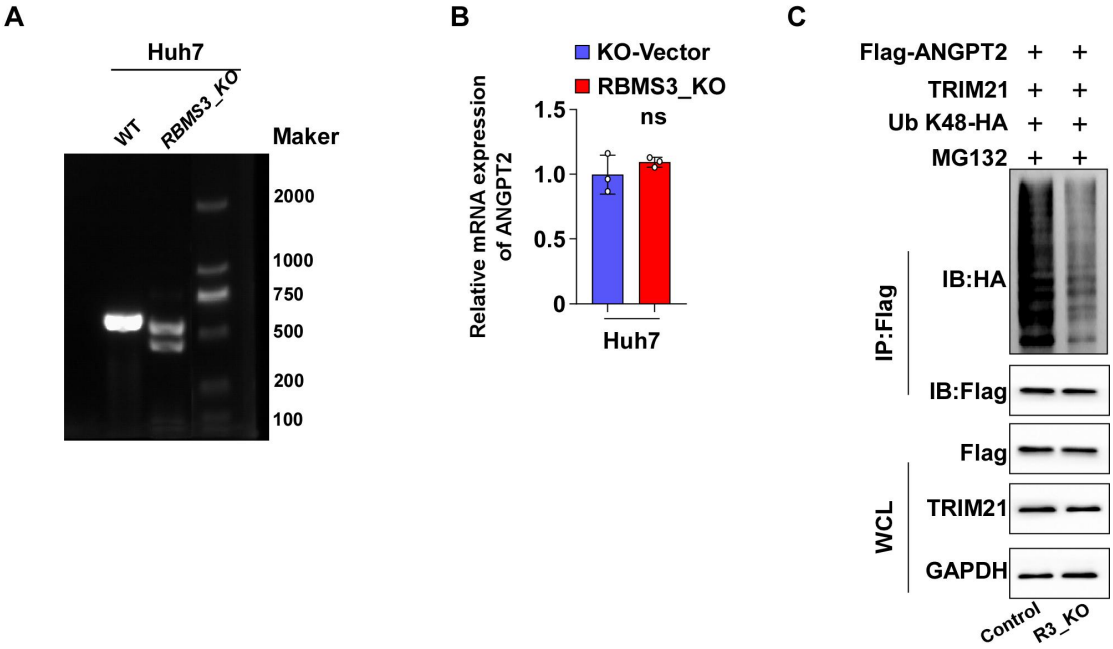
B

Protein	Protein Position	Modified Peptide Sequence Window	Modifications
ANGPT2	K193	KLQDKNSFLEkKVLAMEDKHI	Ub



Supplemental Figure 6. The protein degradation of ANGPT2 with CHX treatment. A. Protein expression (left) of degrading curve (right) of ANGPT2 in indicated HCC cells at various time points after treated with MG132 and sorafenib. The expression levels were quantified and normalized against loading control GAPDH. B. An ubiquitination site of ANGPT2 on the K193 lysine residue has been predicted by analyzing the protein mass spectrometry data.

Supplemental Figure 7



Supplemental Figure 7. Verification of RBMS3 knockout and ANGPT2 expression in RBMS3_KO cells. A. Verification of RBMS3 knonkout by utilizing Knockout and Mutation Detection Kit. B. The mRNA expression of ANGPT2 in RBMS3_KO cells compared with control cells. C. Cells transfected with indicated plasmids were treated with MG132 for 4 h before harvested. Flag-ANGPT2 were pull-downed and subjected to IB analysis with indicated antibodies.

Supplementary Table S1. The relationship of RBMS3 expression in 87 liver cancer patients with clinical pathological characteristics.

Characteristics	Subgroup	Patients, Number(%)	RBMS3 expression		*P* value
			Low(53)	High(34)	
Gender					
	Male	81 (93.1)	49	32	0.564
	Female	6 (6.90)	4	2	
Age (years)					
	⩽50	54 (62.1)	33	21	0.570
	>50	33 (37.9)	20	13	
AFP, μg/L					
	< 300	35 (40.2)	24	11	0.165
	≥ 300	52 (59.8)	29	23	
Relapse					
	Yes	59 (67.8)	42	17	0.005
	No	28 (32.2)	11	17	
ANGPT2 expression					
	High	47 (54.0)	33	14	0.044
	Low	40 (46.0)	20	20	

Supplementary Table S2. Univariate and multivariate analysis of different prognostic parameters in patients with HCC by Cox-regression analysis

	Univariate analysis		Multivariate analysis	
	P	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**
Age (years) ⩽50 >50	0.493	1.172 (0.745-1.842)	0.110	1.457 (0.918-2.313)
AFP, μg/L < 300 ≥ 300	0.008	1.876 (1.178-2.9897)	0.089	1.621 (0.716-3.668)
Relapse Yes No	< 0.001	4.220 (2.377-7.493)	< 0.001	3.458 (1.846-6.477)
RBMS3 level Low expression High expression	0.002	0.463 (0.287-0.747)	0.036	0.577 (0.345-0.964)

Supplementary Tables S3 Primers and Oligonucleotides used in this study.

Primer used for subcloning and plasmid construction:	
RBMS3-forwards	GAGGAATCCTCTAGGTCGACCTGGATCCGCCACCA TGGGCAAACGCCTGGATCAG
RBMS3-downwards	CGCAGAGATGCGGCCGCGTCGACGAATTCTCACTT GTCATCGTCATCCTTGTAG
ANGPT2-forwards	CTTAAGCTTGGTACCGAGCTCGGATCCGCCACCAT GTGGCAGATTGTTTCTTTAC
ANGPT2-downwards	ATCACCGTCATGGTCTTTGTAGTCCTCGAGGAAAT CTGCTGGTCGGATCAT
TRIM21-forwards	ACTTAAGCTTGGTACCGAGCTCGGATCCGCCACCA TGGCTTCAGCAGCACGCTTGAC
TRIM21-downwards	ATCAGGTACGTCGTATGGGTACTCGAGATAGTCAG TGGATCCTTGTGATCCAATATTC
Primer used for real-time RT-PCR	
RBMS3-up	GGGGAACAGTTGAGTAAAACCA
RBMS3-dn	ACAATTTTTCCATACGGTTGGCA
GAPDH-up	TGTGGGCATCAATGGATTTGG
GAPDH-dn	ACACCATGTATTCCGGGTCAAT
ANGPT2-up	AACTTTCGGAAGAGCATGGAC
ANGPT2-dn	CGAGTCATCGTATTCGAGCGG
TRIM21-up	TCAGCAGCACGCTTGACAAT
TRIM21-dn	GGCCACACTCGATGCTCAC
Oligonucleotides for siRNAs	
ANGPT2 siRNA	UGAUAGAAAUAGGGACAAATT
TRIM21 siRNA	GUUGGCUGAGAAGUUGGAATT
RBMS3 KO #1	
RBMS3 sgRNA	TTGCTGTGCAAATTCGCTGA