Supporting Information

Supplementary Materials and Methods

RNA sequencing

Prior to RNA-seq library construction, rRNA was depleted from total RNAs as previously described[1, 2]. Then, ribo– RNA-seq libraries were prepared by using Illumina TruSeq RNA Sample Prep Kit V2 and subjected to deep sequencing with Illumina HiSeq 2000 at CAS-MPG Partner Institute for Computational Biology Omics Core, Shanghai, China.

Cell lines and Lentivirus-mediated stable cell line construction

The normal-type hepatocyte QSG7701 cell as well as the HCC cell lines Huh-7, HCC-LM3 (LM3) and SMMC-7721 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were maintained at 37℃ in an atmosphere containing 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SMMC-7721 and HCC-LM3 shcircZKSCAN1 stable transfectants and circZKSCAN1 overexpression transfectants were established using the lentivirus expressing shcircZKSCAN1 and circZKSCAN1 expression vector as described[3]. To generate circZKSCAN1 overexpression system, relative plasmids were purchased from Addgene Company, including pcDNA3.1(+) ZKSCAN1 nt 400-1782 delta440-500 delta1449-1735 and pcDNA3.1(+) ZKSCAN1 Sense. Then they were used to generate circZKSCAN1 overexpression lentivirus. by GenePharma Company (Shanghai, China). Lentiviral infection was carried out according to the manufacturers, instructions. Cells were infected with lentivirus at a multiplicity of infection (MOI) of 20 in the presence of 8 ug/mL polybrene (Sigma) for 6 hours. After 24 hours, the original medium was replaced with fresh medium.

Small interfering RNA (siRNA) transfection

The siRNAs (sense and antisense strands) were synthesized by Biotend (Shanghai, China). siRNA target sequences are listed in Supplementary Table 1. In vitro transfection in HCC cells was carried out using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's protocol. The final siRNA concentration used was 100 nM.

Animal experiments The Nude mice used in our experiment was purchased from Model Animal Resource Information Platform (Nanjing, China). It is BALB/c-Foxn1em12Cd178/Nju, which strain background is BALB/c-nu/Nju [N000020]. Adeno-associated virus expressing LV-circZKSCAN1 and green fluorescent protein were purchased from Genechem (Shanghai, China). The intratumoral injection was applied in multiple sites within one xenografted tumor with a total amount of 10^{11} titer of adenovirus.

RT-qPCR and Western Blot

Total RNA was isolated from *cells and liver tissues* using the Trizol method according to the

manufacturer's protocols. Real-time PCR analyses were performed using an ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green PCR kit (Applied TaKaRa, Otsu, Shiga, Japan). The ΔCt method was used with 18s and β-actin mRNA as an endogenous control for normalization of the results. Primers used in this study are shown in the Supplementary table. 6. Cells and Liver tissues were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM sodium fluoride, 5 mg/ml of aprotinin, 20 mM leupeptin and 1 mM sodium orthovanadate) and centrifuged at 12 000g for 15 min. Protein concentrations were determined via the BCA assay kit according to manufacturer's protocols. Specific primary antibodies used were as follow: antibody against Histone H3, FMRP and QKI were purchased from ABCAM. Antibodies against beta-catenin were obtained from Santa Cruz Biotechnology (CA). Antibodies against CCAR1 was obtained from Novusbio. The dilutions were 1:1000 in 5% BSA. After incubating with the fluorescein-conjugated secondary antibody, the immunocomplexes were detected using an Odyssey fluorescence scanner (Li-Cor, Lincoln, NE).

In vivo/in vitro limiting dilution assay

For the in vivo limiting dilution assay (LDA), constructed cell lines were dissociated into single cells, diluted serially to the desired doses (10^4, 10^5 and 10^6/ml), and then mixed with Matrigel at a ratio of 1:1 and injected subcutaneously into NOD-SCID mice. After two months, the number of tumors was counted. As for in vitro, constructed cell lines and control cells were seeded into 96-well ultra-low attachment culture plates at various cell numbers and incubated for 7 days. CSC frequencies were analyzed using Extreme Limiting Dilution Analysis (ELDA) software as described[4].

Immunochemistry

Paraffin-embedded liver sections (5 μm thick) were used for immunohistochemical detection of EpCAM, Ki67, and CCAR1. Tissue microarray slides were deparaffinized and rehydrated in ethanol and then treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. To detect EpCAM, the antigen retrieval method was performed using EDTA and EGTA (pH 8.0). To detect Ki67 and CCAR1, the antigen retrieval method was performed using 10 mM sodium citrate (pH 6.0). After blocking nonspecific antigens with 2% BSA in PBS for 30 min, the sections were incubated with 1:100 diluted mouse anti-EpCAM(Cell signaling), rabbit anti-Ki67 (ABCAM) or anti- CCAR1 polyclonal antibody (Novusbio) at 4°C overnight. Corresponding secondary antibodies were used at 4°C for one hour, followed by diaminobenzidine (DAB) staining (Dako, Carpinteria, CA). Sections were counterstained with hematoxylin for staining of nuclei. The assessment of immunostaining was performed using the ImageScope software (Media Cybernetics, Inc., Bethesda, MD) according to the staining intensities and the percentage of positively-stained cells.

Spheroid assay

HCC cells were plated in 96-well ultra-low attachment culture dishes (Corning Incorporated Life Sciences) at 500 cells per well and cultured in DMEM/F12 (Gibco) supplemented with 1% FBS, 20 ng/mL bFGF and 20 ng/mL EGF for seven days. The number of spheroids was counted and representative views were pictured.

Cell proliferation analysis

In total, 5×10^3 cells were cultured in each well of 96-well plates in 10% FBS/DMEM. ATP activity was measured using a Cell Counting Kit-8 and a Synergy 2 micro-plate5 reader at indicated time points. The results are presented as proliferation index relative to control cells.

Patient-derived xenograft(PDX) models

Fresh tumor tissue was sterilely obtained from primary hepatocellular carcinoma tissue that was undergoing surgical excision. Portions were frozen or placed in formalin and embedded in paraffin for later analyses. Fresh tumor tissue was kept on ice in DMEM medium for transport and minced into 1 to 2 millimeters fragments and then injected subcutaneously on nude mice(BALB/CJNju [N000020]).

Flow cytometry analysis

HCC cells were incubated with APC-conjugated anti-EpCAM (Biolegend), with APC conjugated anti-mouse IgG (Miltenyi Biotec) as the secondary antibody followed by flow cytometry analysis using a Moflo XDP flow cytometer from Beckman Coulter.

Luciferase reporter assay

Approximately 24 h after transfection of the indicated reporter plasmids, the cells were harvested and washed with PBS once; then 50 μl of passive lysis buffer was added, and the cells were incubated on ice for 30 min. The cells were then spun at 5,000 rpm for 4 min to remove debris, and 10 to 20 μl was used to assay for luciferase activity using a dual luciferase reporter assay (Promega) in a single-injector luminometer. The luciferase reporter constructs containing multiple TCF/LEF1 binding sites (pGL3-OT) and a negative control construct (pGL3-OF) were used. A Dual-Luciferase reporter assay as carried out according to the manufacturer's suggestions (Promega, USA). RL-TK (Promega, USA) was co-transfected with each reporter construct to normalize
for transfection. Relative b-catenin activation was determined by the OT/TK ratio.

Data analysis

All data in this study was represented at least three experiments and they are expressed as the mean±SEM. Differences between groups were compared using Student's t-test or Two Way ANOVA (as indicated in each Figure/Table). Statistical significance was determined as $P<0.05$. The analysis was performed using GraphPad Prism software (San Diego, CA, USA).

RNase R treatment

RNase R digestion reaction as performed as previously reported[3]. Total RNA (5 μg) was incubated for 15 min at 37 °C with or without 3 $U\bullet\mu g^{-1}$ RNase R (Epicentre Biotechnologies, Madison, WI, USA). The RNA was subsequently purified by phenol-chloroform extraction and then subjected to RT-qPCR.

RNA isolation

Total RNAs from cell lysates were isolated using TRIzol reagent (Life Technologies, Carlsbad, CA). The nuclear and cytoplasmic fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The RNA then subjected to RT-qPCR To quantify the

amount of mRNA and circRNA.

References:

1. Yang L, Duff MO, Graveley BR, Carmichael GG, Chen LL. Genomewide characterization of non-polyadenylated RNAs. Genome Biol. 2011; 12: R16.
2. Yin QF, Chen LL, Yang L. Fractionation of non-polyadenylated and ribosomal-free RNAs from mammalian cells. Methods Mol Biol. 2015; 1206: 69-80.
3. Yao Z, Luo J, Hu K, Lin J, Huang H, Wang Q, et al. ZKSCAN1 gene and its related circular RNA (circZKSCAN1) both inhibit hepatocellular carcinoma cell growth, migration, and invasion but through different signaling pathways. Mol Oncol. 2017; 11: 422-37.
4. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009; 347: 70-8.

Supplementary Table 4: Baseline characteristics of the patients with HCC (n = 112)

Variables	Patients (%)
Age*	50.88 ± 11.682
Gender (Female)	9 (8.03)
Preoperative AFP*	
> 500 ng/ml	45 (40.18)
≤ 500 ng/ml	58 (51.79)
HBV infection	104 (92.86)
HBV DNA	72 (64.29)
Total bilirubin*	17.88 ± 8.83
Albumin*	41.19 ± 5.20
ALT*	77.02 ± 151.19
AST*	81.72 ± 138.01
Portal hypertension	28 (25.00)
Hepatic metastases	102(91.07)
Cirrhosis	20 (17.86)
Tumor size (cm)*	6.90 ± 4.57
Vascular invasion	24 (21.43)
Tumor capsule	44 (39.29)
Tumor differentiation*	
Well or moderate	87 (77.68)
Poor	16 (14.29)
Ascites	15 (13.39)
circ*ZKSCAN1* expression	58 (51.79)
CCAR1 expression	46 (41.07)

AFP, alpha fetoprotein; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase

*Data were not available for all patients.

Supplementary Table 5: Clinicopathologic factors and circ*ZKSCAN1* expression level in HCC tissue-microarrays

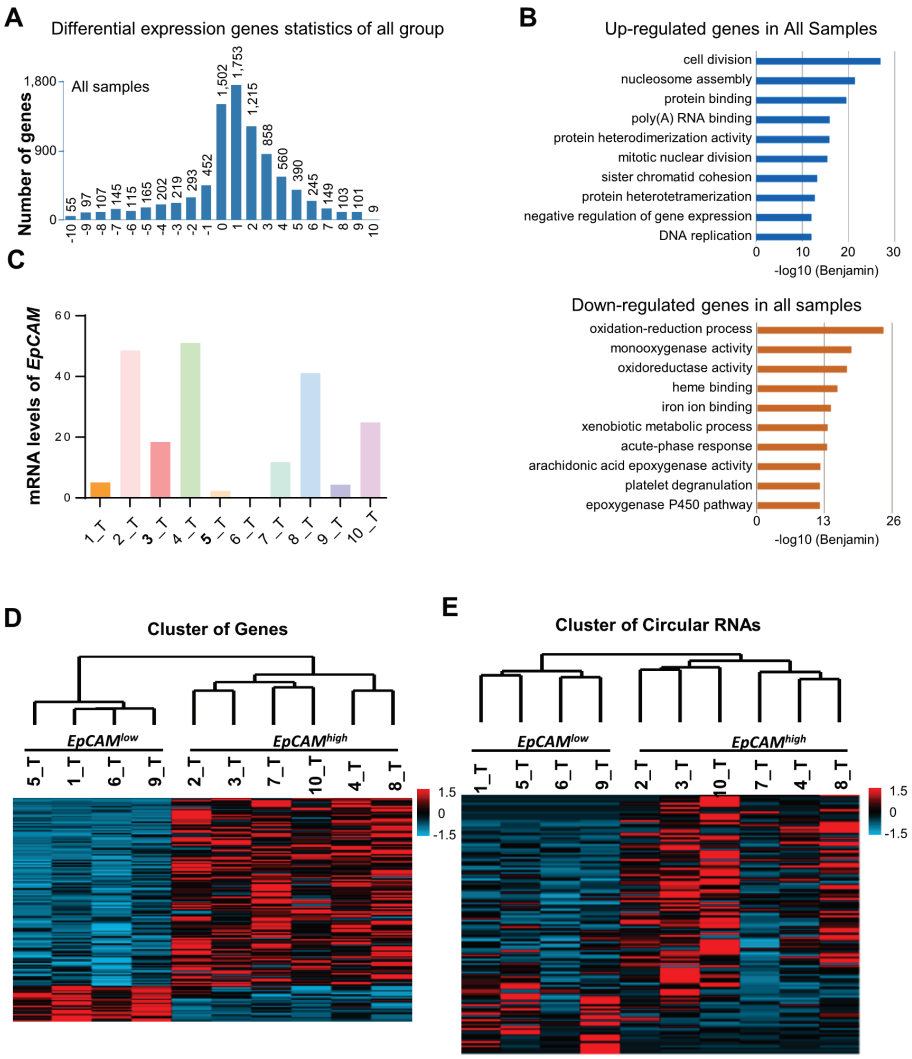
samples (n=112)

Variables		circZKSCAN1		P value
		low expression	high expression	
Age	<30	1	1	0.425518
	30-44	12	21	
	45-59	23	19	
	60-74	11	15	
	>75	1	0	
gender	Male	50	53	0.81342
	female	4	5	
HBV DNA	negative	13	6	0.049065
	positive	31	41	
AFP	< 500 ng/ml	27	31	0.990746
	>= 500 ng /ml	21	24	
Total bilirubin	< 20 μmol/L	32	40	0.59991
	>= 20 μmol/L	16	16	
Albumin	< 35g/L	3	1	0.237923
	>= 35g/L	45	55	
ALT	< 50U/L	24	36	0.141549
	>= 50U/L	24	20	
AST	< 40U/L	17	32	0.026914
	>= 40U/L	31	24	
Vascular invasion	negative	33	47	0.067023
	positive	15	9	
Portal hypertension	negative	34	43	0.595005
	positive	14	14	
Ascites	negative	40	48	0.571795
	positive	8	7	
Extrahepatic spread	negative	0	1	0.357257
	positive	47	55	
Diameter	< 5cm	19	30	0.214577
	>= 5cm	28	27	
Tumor capsule	negative	19	32	0.211071
	positive	22	22	
Differentiation	Poor	9	7	0.440675
	moderate	38	48	
	Well	0	1	
Cirrhosis	negative	42	42	0.043493
	positive	5	15	
CCAR1	low expression	25	41	0.00874
	high expression	29	17	

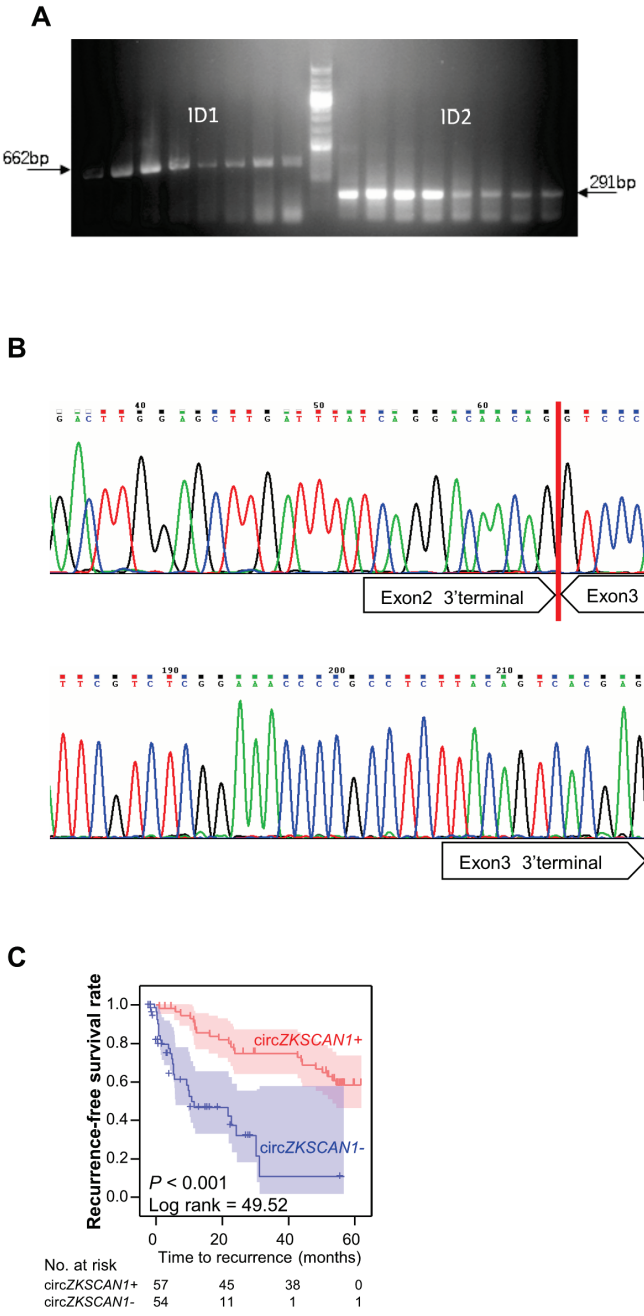
Note: P<0.05 by Chi-square test or Student t test

Supplementary table 6: Primers used for real-time PCR

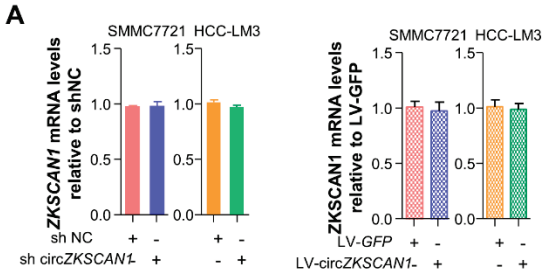
Primer names （human）	Sequences	
	Forward (5'~3')	Reverse (5'~3')
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
circRNA-ZKSCAN1	CCTCGAGCTTTGACCTTCATCACG	CTCACCTTTATGTCCTGGGAGGT
ZKSCAN1	CCTCGAGCTTTGACCTTCATCACG	CTACCCTCATGAGGGGGTGC
ID1	CCTCGAGCTTTGACCTTCATCACG	GAACTGGATCCAGAGGCACCAT
ID2	AGGTCTGGCTGCAGGAATACCG	CTCACCTTTATGTCCTGGGAGGT
FUS	ATGGCCTCAAACGATTATACCCA	GTAACTCTGCTGTCCGTAGGG
DGCR8	GCAGAGGTAATGGACGTTGG	AGAGAAGCTCCGTAGAAGTTGAA
ELAVL	GGGTGACATCGGGAGAACG	CTGAACAGGCTTCGTAACTCAT
EIF4A3	GGGGCATCTACGCTTACGG	GCGATGACATCTCTCCCTTTGA
FMRP	TATGCAGCATGTGATGCAACT	TTGTGGCAGGTTTGTTGGGAT
PTB	AGCGCGTGAAGATCCTGTTC	CAGGGGTGAGTTGCCGTAG
IGF2BP1	GCGGCCAGTTCTTGGTCAA	TTGGGCACCGAATGTTCAATC
IGF2BP2	AGTGGAATTGCATGGGAAAATCA	CAACGGCGGTTTCTGTGTC
LIN28A	TGCGGGCATCTGTAAGTGG	GGAACCCTTCCATGTGCAG
LIN28B	CATCTCCATGATAAACCGAGAGG	GTTACCCGTATTGACTCAAGGC
ABCG2	CAGGTGGAGGCAAATCTTCGT	ACCCTGTTAATCCGTTCGTTTT
BMI-1	CCACCTGATGTGTGTGCTTTG	TTCAGTAGTGGTCTGGTCTTGT
CD133	AGTCGGAAACTGGCAGATAGC	GGTAGTGTTGTACTGGGCCAAT
CD90	ATCGCTCTCCTGCTAACAGTC	CTCGTACTGGATGGGTGAACT
C-MET	AGCAATGGGGAGTGTAAAGAGG	CCCAGTCTTGTACTCAGCAAC
C-MYC	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
EpCAM	AATCGTCAATGCCAGTGTACTT	TCTCATCGCAGTCAGGATCATAA
KLF4	CCCACATGAAGCGACTTCCC	CAGGTCCAGGAGATCGTTGAA
NANOG	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
NOTCH1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
OCT4	CTGGGTTGATCCTCGGACCT	CCATCGGAGTTGCTCTCCA
SOX2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT
CCAR1	CAACAACAGTTACAGCAACCCC	GCTGAGGAGTAGACAGGCTAAG
ARHGAP21	CATGGCACAGCCAGTTGAAAT	CCCTACCAGGTTGTGTCAGTA
ZHX3	TACTGCGATTTCAGATCCCATGA	GTTCCACACAAAGCTGGCTTC
PPP4R1	CACAAGATGAAATGTTGACGCC	CAAGGTATCGAGCAAACTCCG
ANKRD18B	AAAGGGCAATGCAGGCAATAG	TCCATCCGCTCTTTCTCCATA
QKI	AAGCCCACCCCAGATTACCT	ACTCTGCTAATTTCTTCGTCCAG

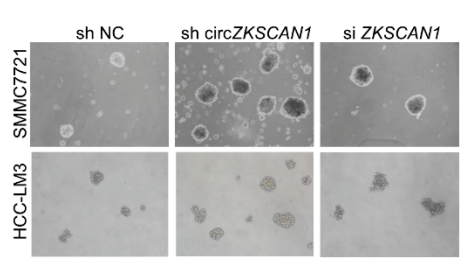


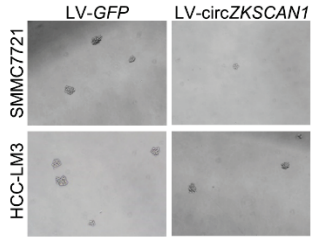
Supplementary Figure1. (a) Differential expression genes statistics of all group from an RNA-seq of 10 HCC samples. (b) Go enrichment analysis of differential expressed genes participated in a wide range of crucial cellular activities. (c) The relative EpCAM mRNA expression level of 10 sequenced samples based on the data of RNA-sequencing. (d) Differentially expressed genes of $EpCAM^{low}$ or $EpCAM^{high}$ subgroup. n = 10, red is higher and blue is lower expression. (e) Circular RNAs cluster analysis of $EpCAM^{low}$ or $EpCAM^{high}$ subgroup. n = 10, red is higher and blue is lower expression.

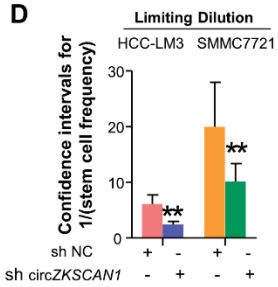


Supplementary Figure 2. (a) Verification of ZKSCAN1 circularization. Two primers were designed respectively to verify whether the circulant sequence of circZKSCAN1 has an intron insertion between exon 2 and 3. (b) Sequencing result of ID2 primer amplification products. (c) Kaplan-Meier analysis of the correlation between circZKSCAN1 expression levels and recurrence-free survival (RFS).



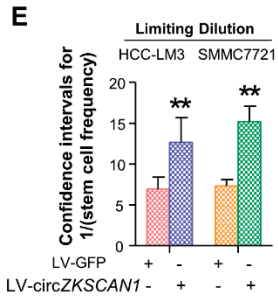






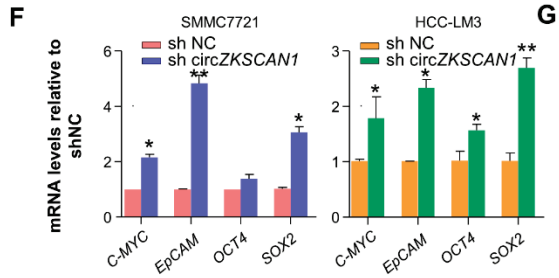
In vitro limiting dilution

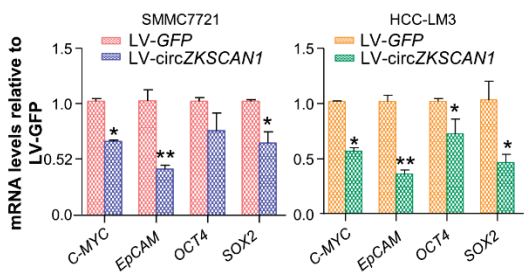
Frequency of colony formation		Number of cells seeded each well			Confidence intervals for 1/(stem cell frequency)	
		5	3	1	Estimate	Upper and lower Limits
SMMC7721	sh NC	31/48	18/48	4/48	1:5.89	1:7.73-1:4.49
	sh circZKSCAN1	45/48	40/48	3/48	1:2.37	1:2.97-1:1.89
HCC-LM3	sh NC	16/48	4/48	1/48	1：18.26	1:27.94-1:11.94
	sh circZKSCAN1	26/48	8/48	2/48	1:9.66	1:13.373-1:6.98

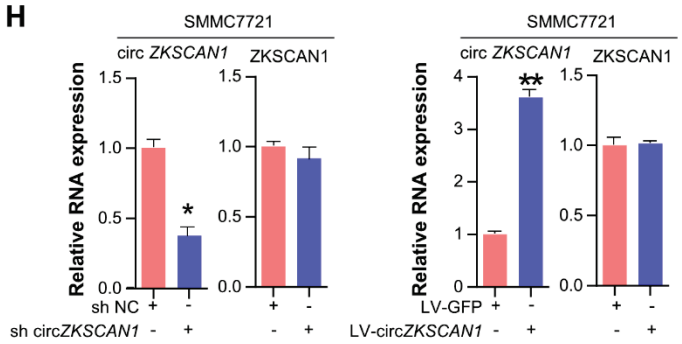


In vitro limiting dilution

Frequency of colony formation		Number of cells seeded each well			Confidence intervals for 1/(stem cell frequency)	
		10	8	5	Estimate	Upper and lower Limits
SMMC7721	LV-GFP	45/48	30/48	18/48	1:6.8	1:8.41-1:5.49
	LV-circZKSCAN1	38/48	19/48	7/48	1:12.3	1:15.7-1:9.6
HCC-LM3	LV-GFP	36/48	32/48	17/48	1：6.54	1:8.15-1:6.54
	LV-circZKSCAN1	31/48	22/48	8/48	1:10.33	1:17.10-1:13.31



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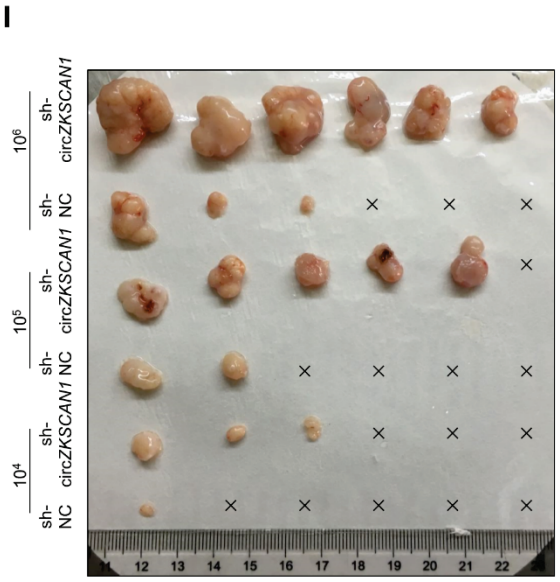
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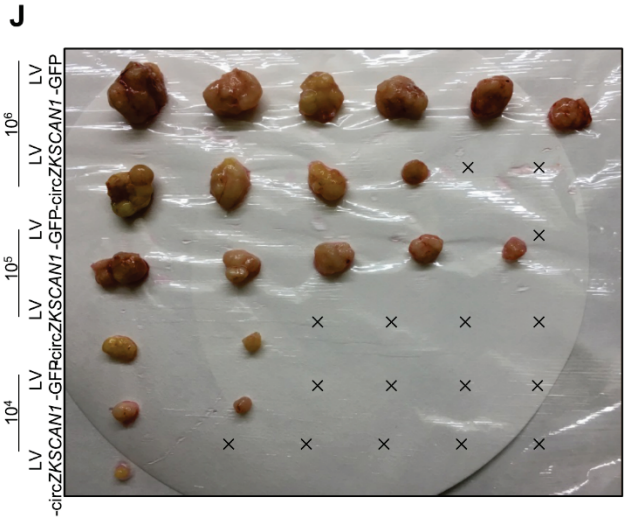
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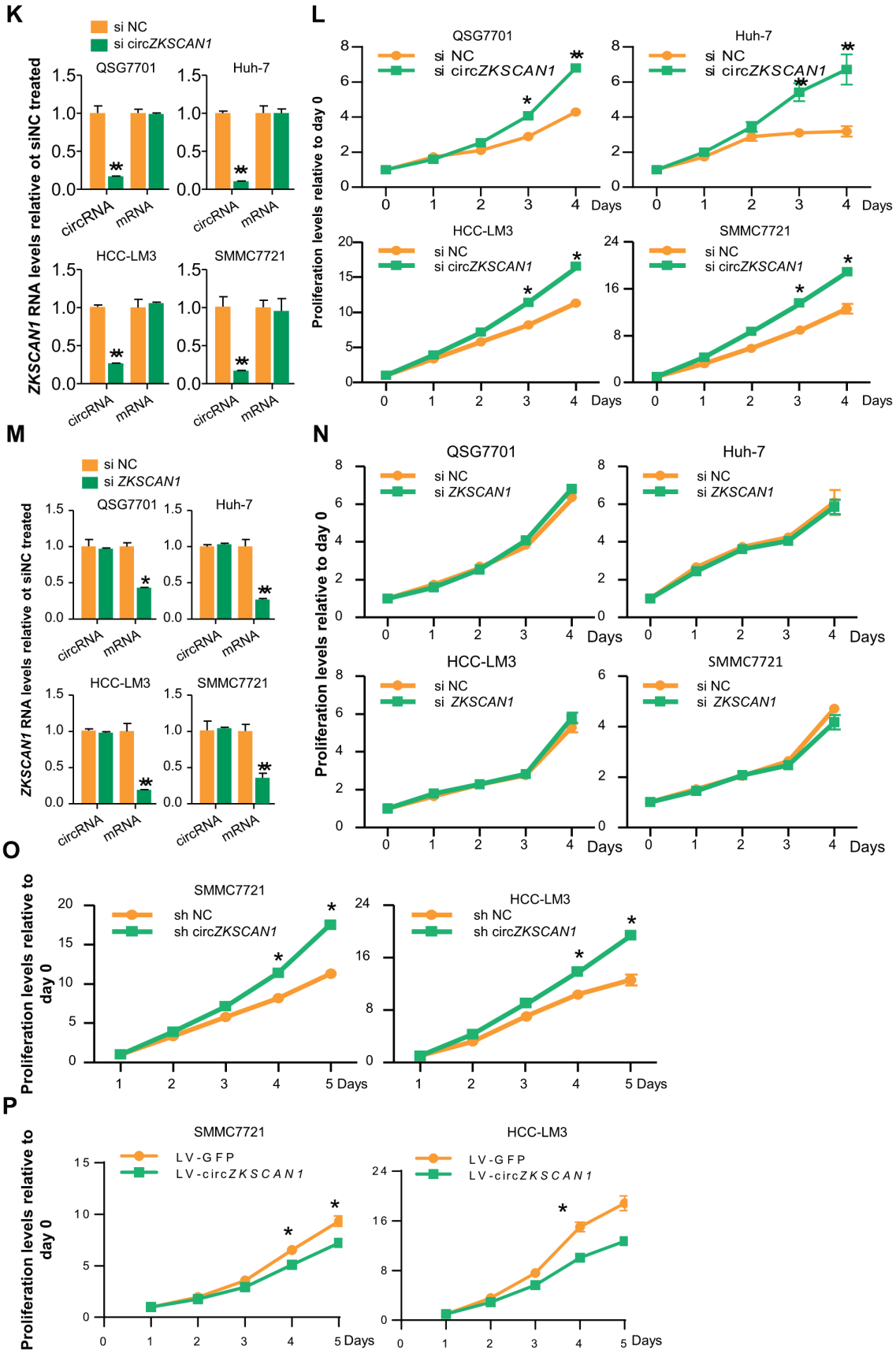
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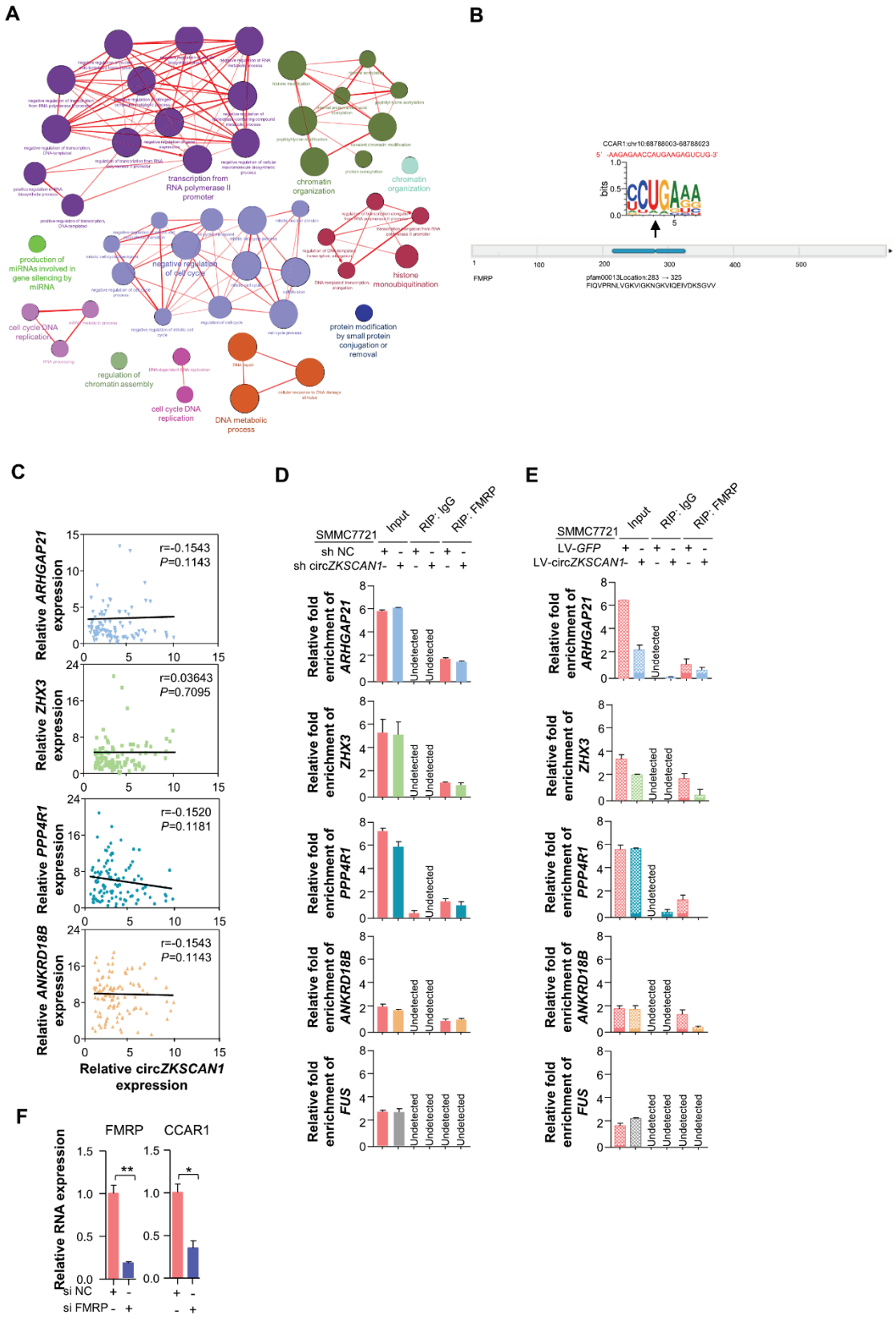
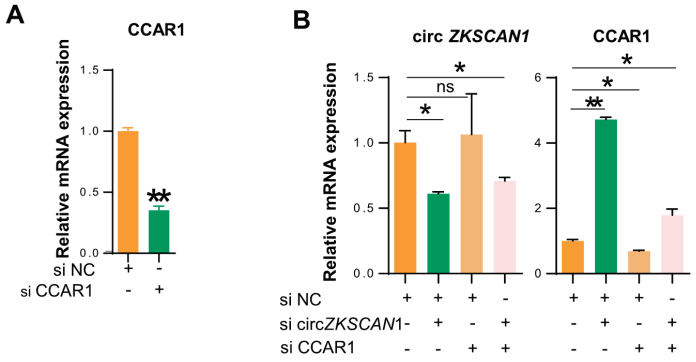
Supplementary Figure 3. (a) Expression of *ZKSCAN1* mRNA was quantified by real-time PCR in SMMC7721/HCC-LM3 after circZKSCAN1 knocked-down or overexpressed using lentivirus, respectively. Data are presented as mean ± SEM (n=4). NS, not significant. (b)&(c) Exemplary microscopic images of the sphere formation assay. (d)&(e) Cells were seeded into 96-well ultra-low attachment culture dishes at the cell doses of 5, 3,1 or 10, 8, 5. Cells were incubated under the spheroid condition for 14 days. Colony formation was assessed by visual inspection and the frequency of cancer stem cell was evaluated by a limiting dilution assay. **p<0.01. (f)&(g) CSCs markers expression levels assessed by real-time RT-PCR analysis in circZKSCAN1 knocked-down or overexpressed cell lines . Data are presented as mean ± SEM (n=4). *P<0.05, **p<0.01. (h) *ZKSCAN1* circRNA and mRNA expression levels assessed by real-time RT-PCR analysis in SMMC7721 xenograft tumor. Data are presented as mean ± SEM (n=4). *P<0.05, **p<0.01. (i)&(j) Representative pictures of xenograft tumor from circZKSCAN1 knocked-down or overexpressing cell lines. The engraftment rates of tumor across mice were counted during the 2 or 4 months observation. (k)&(m) Expression of *ZKSCAN1* circRNA and mRNA was quantified by real-time PCR in QSG7701/Huh-7/HCC-LM3/SMMC-7721 after transfected with two siRNAs against circZKSCAN1(ZKSCAN1) or control siRNA (siNC), respectively. (l)&(n) HCC cells were transfected with two siRNAs against circZKSCAN1(ZKSCAN1) or control siRNA (siNC) and the cell viability was detected by CCK-8 assay at indicated times. Data are presented as mean ± SEM (n=4). The different degrees of significance was indicated as follows in the graphs: NS, not significant; *P<0.05; **P<0.01. (o)&(p) The cell viability of HCC cell lines were detected by CCK-8 assay at indicated times. Data are presented as mean ± SEM (n=4). The different degrees of significance was indicated as follows in the graphs: NS, not significant; *P<0.05.

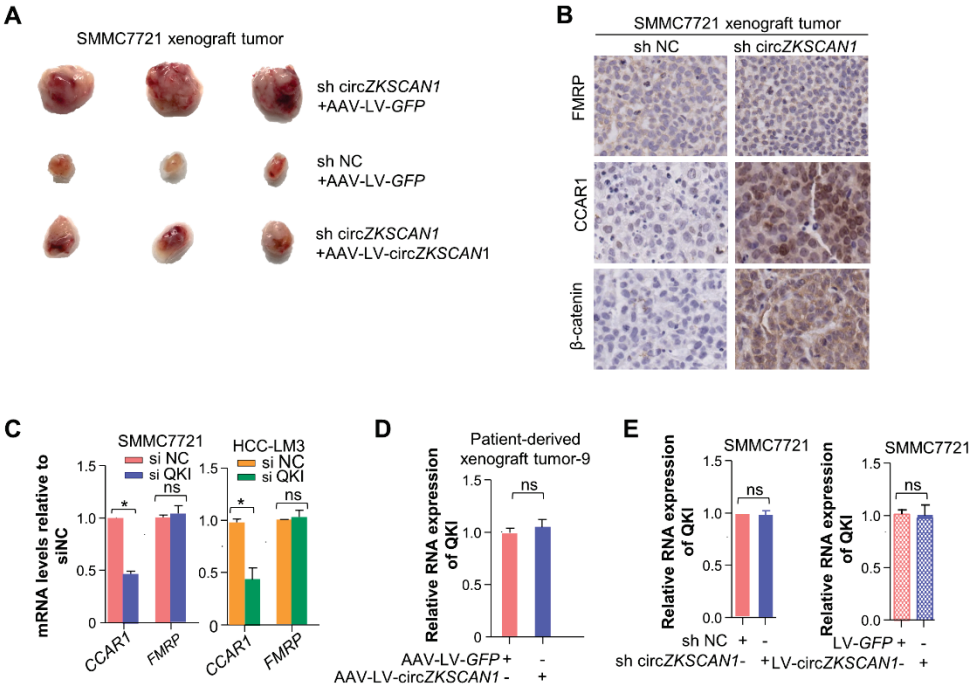


Supplementary Figure 4. (a) GO analysis of the RIP-target genes of FMRP. (b) Graphic

abstract of the physical binding between FMRP protein and CCAR1 mRNA (c) The correlation of the mRNA level of 4 FMRP target genes and circZKSCAN1 expression level using 112 HCC frozen tissues was determined by qPCR. (d)&(e) Whole-cell RNA extract was precipitated with input (positive control), IgG (negative control) and FMRP antibody and the precipitation were then verified by Western Blot. Four genes that might be combined with FMRP level of the precipitations was confirmed by qPCR. FUS, a negative control. (f) The expression change of CCAR1 after FMRP knockdown was determined by RT-PCR.



Supplementary Figure 5. (a) The knockdown efficiency of small interfering RNA targeting CCAR1 was determined by qPCR. Data are presented as mean ± SEM (n=4). The different degrees of significance was indicated as follows in the graphs: ***P*<0.01. (b) The expression level of circZKSCAN1 and CCAR1 in the context of single circZKSCAN1, CCAR1 knockdown or double knockdown was determined by qPCR. Data are presented as mean ± SEM (n=4). The different degrees of significance was indicated as follows in the graphs: **P*<0.05; ***P*<0.01.



Supplementary Figure 6. (a) Images of xenograft tumor with or without circZKSCAN1

overexpression were presented. (b) IHC detection of FMRP, CCAR1 and β-catenin in xenograft tumors. (c) The expression changes in CCAR1 and FMRP after QKI knockdown was detected via RT-PCR. (d) The expression change of QKI in PDX-9 with or without circZKSCAN1 overexpression was detected via RT-PCR. (e) The expression change of QKI in xenograft tumors with or without circZKSCAN1 overexpression was detected via RT-PCR.