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°C 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 polybrevne (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].

**Immunohistochemistry**

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, $5 \times 10^3$ 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-plate 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 steriley 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/C Jnu [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 β-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·μg⁻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.
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)   |
| circZKSCAN1 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 circZKSCAN1 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      |         |
### Supplementary table 6: Primers used for real-time PCR

| Primer names | Sequences |
|--------------|-----------|
| **(human)**  | Forward (5’~3’) | Reverse (5’~3’) |
| **18S**      | CGGCTACCACATCCAAGGAA | GCTGGAATTACCACGGCCCT |
| **circRNA-ZKSCAN1** | CCTCGAGCTTTGACCTTCATCAG | CTCACCTTTATGTCTGAGGCT |
| **ZKSCAN1**  | CCTCGAGCTTTGACCTTCATCAG | CTACCCTCAATGAGGGGTGC |
| **ID1**      | CCTCGAGCTTTGACCTTCATCAG | GAACTGGATCCAGAGGCACTC |
| **ID2**      | AGGTCCTGGCTCAGGAGAATACC | CTCACCTTTATGTCTGAGGCT |
| **FUS**      | ATGGCCTCAGGGAACCACATCCA | GTAACCTCTGTCTCAGGAGG |
| **DGCR8**    | GCAGAGGTAATGGACGTTGG | AGAGAAGCTCCGTAAGGCT |
| **ELAVL**    | GGGTGCACAGGGAGGAGAGG | CTGAACAGGCTCAGGAGG |
| **EIF4A3**   | GGGGTTCCACATGCTGACGTTGG | CCGAGATCCATCTCAGGAGG |
| **FMHRP**    | TATGCTGAGCTTTGAGTTTCGT | TTGGCAAGCTCAGGAGG |
| **PTB**      | AGCCGCAGTGAAGATGGAATAG | CAGGGTCTCGAGGAGG |
| **IGF2BP1**  | GCCGCGGATCTTTGCTTGG | TTGGGACAGGATGAGG |
| **IGF2BP2**  | AGTGGAGTGCTGAGGAGGAAATCA | CAACGCCGGTTCTGTAG |
| **LIN28A**   | TTGGGACAGGATGAGG | CACGAGGAGGTCGGAGG |
| **LIN28B**   | CATCTCCATGATAAAGGGGAGG | GTTACCCGTTAAGCTCAGG |
| **ABCG2**    | CAGGGTGGAGGCAATCTTGG | ACCCTGTAACCTCAGGAGG |
| **BMI-1**    | CCACCTGTAATGCTGTTTGG | TCCAGTGTGCTGTCAGGAGG |
| **CD133**    | AGCTGGAAGCTCGAGTAGTTGG | GGAACCTCAGGAGGAGG |
| **CD90**     | ATCCGCTCTCTGCTAAGAGCTC | TCCCTGACGAGGAGGAGG |
| **C-MET**    | AGCTGTGCTGACTTTGAAGGAGG | CCAGAGGGATGGTAGAACAG |
| **C-MYC**    | GCCGCTCTGGAACAGGGAAGTCA | AGGTGAGTGCTGAGG |
| **EpCAM**    | AATGTGCAAGGCTTACTCATT | TCTCATCGAGTACAGATCATA |
| **KL4**      | CACCATGGACATGGCTTCCC | CAGGTGAGGAGGATGGAGG |
| **NANOG**    | TTTGTGGGCTCTGAAAGAAC | AGGGTGGTCTCAGGAAGAAG |
| **NOTCH1**   | GAGGGTTGGAGGCAATGATGC | TCTGTGACGAGGAGGAGG |
| **OCT4**     | CTGGTGATCTGGAGGACCTT | CCATGGAGTTGCTCAGGAGG |
| **SOX2**     | GCCGAGGTGGACACCTTGGCG | GGCAGGCTGTACTTTCCTTT |
| **CCAR1**    | CAACACAGGATACGACACACC | GCTGAGGAGGTAGACGAGG |
| **ARHGAP21** | CATGGGCAGCAGGCTGGAATT | CCGTACCAGGTGTGTGAGG |
| **ZH3X**     | TACTGCGATTCTCACTCATTGA | GTTCCACAAAGGCTGCTC |
| **PPP4R1**   | CAAGAGATGAAATGTTTGGC | CAAAGGATCCAGGAGGAG |
| **ANKRD18B** | AAAGGCGCAATGGCGGAAATAG | TCCATCGGTCTTTCCTGAG |
| **QKI**      | AAGGCCCACCCAGATACCT | ACTGCTGTTATCTTGGCAG |
**Supplementary Figure 1.**

(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\textsuperscript{low} or EpCAM\textsuperscript{high} subgroup. \(n = 10\), red is higher and blue is lower expression. (e) Circular RNAs cluster analysis of EpCAM\textsuperscript{low} or EpCAM\textsuperscript{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).
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.