Supplementary file

Materials and Methods

Patient samples

A total of 95 patients with primary HCC from The University of Hong Kong-Queen Mary Hospital (HKU-QMH) were included in this study. The samples of these 95 patients were used to detect the FTO mRNA levels with qPCR. The overall and disease-free survival data were available in 88 and 69 of these patients, respectively. RNA-seq data of 41 patients was from our other independent HKU-QMH patient sample cohort and used to show the FTO mRNA levels. All patients are ethnic Chinese and had surgical resection at Queen Mary Hospital, Hong Kong. Primary HCC tumor samples and their corresponding non-tumorous (NT) liver samples were collected immediately after surgery and stored at -80°C. The use of clinical samples in this study was approved by the institutional review board of The University of Hong Kong and Hong Kong Hospital Authority.

Chemicals and antibodies

CS2 were obtained from Sigma-Aldrich (St. Louis, USA). Mouse anti-PD-1 antibody (clone: LTF-2) and IgG (clone: RMP1-14) control were purchased from BioXcell (Lebanon, NH, USA). Anti-YTHDF2, anti-beta Catenin, and anti-syndecan-4 antibodies were purchased from Abcam (Cambridge, UK). Syndecan-4 (5G9) blocking antibody was purchased from Santa Cruz (California, USA). Anti-m6A antibody was purchased from Synaptic Systems (Göttingen, Germany). Anti-human ERK1/2, Anti-human Phospho-ERK1/2, Anti-human AKT, Anti-human Phospho-AKT (Ser473) were purchased from Cell Signaling Technology (Danvers, USA). PMA (phorbol 12-myristate 13-acetate), IFNγ, Il-4 and IL-13 were obtained from Sigma-Aldrich.

Cell lines

Human HCC cell lines (HepG2, Hep3B, HuH7, PLC), normal liver cell line MIHA, mouse hepatoma cell line Hepa1-6 and human monocyte cell line THP-1 were obtained from American Type Culture Collection. Human HCC cell line 97L was a gift from Dr ZY Tang (Fudan University, Shanghai, China) and the STR authentication confirmed no contamination. HepG2, Hep3B and PLC were cultured in MEM containing 10% fetal bovine serum (FBS), while the others were cultured in DMEM supplemented with 10% FBS. Human bile duct carcinoma cell line HuCC-T1 and human colorectal adenocarcinoma cell line HCT-15 were also obtained from American Type Culture Collection. These cell lines were cultured in RPMI1640 containing 10% FBS.

Stable KD of HCC cells

A lentiviral-mediated approach was used to construct stable FTO or GPNMB KD HCC cell lines. Human ON-TARGETplus SMARTpool siRNA duplexes which target FTO or GPNMB and non-target control were purchased from Sigma-Aldrich. pLKO.1-puro vectors containing shRNAs targeting FTO (shFTO) or GPNMB (shGPNMB) and shNTC were stably transduced into HCC cell lines. Puromycin selection was performed to obtain stable expression of shRNAs and shNTCs. Sequences of all shRNAs are listed (Supplementary Table 3).

Animal models.

Limiting dilution assay was done by subcutaneously injecting 2×10^3, 2×10^4 and 2×10^5 HCC cells into the flanks of male BALB/cAnN-nu nude mice for 25-30 days. The tumor initiating capacity was analyzed by the confidence intervals (CIs) for 1/(stem cell frequency) using extreme limiting dilution analysis. Tumor incidence and tumor mass were recorded.

Orthotopic liver injection model was employed to investigate the tumor growth and progression. Briefly, 1×10^6 luciferase-labelled MHCC-97L cells were injected into the left lobes of livers of nude mice, whereas 3×10^6 luciferase-labelled mouse hepatoma Hepa1-6 cells were injected into left lobes of livers of C57BL/6 mice. Each experimental group had at least 6 mice. After 6 weeks for nude mice and 2 weeks for C57BL/6 mice, the mice were sacrificed and Xenogen IVIS 100 Imaging System (IVIS Spectrum In Vivo Imaging System, PerkinElmer, Waltham, MA) was utilized to visualize the liver tumor size and lung metastasis. Tumor mass and lung metastasis rate were recorded. Tumors from C57BL/6 mice were dissociated for detecting tumor infiltrating immune cells by flow cytometry.

For immune check point blockade (ICB) therapy, 3×10^6 Hepa1-6 cells were orthotopically injected into liver lobe of C57BL/6 mice. Five days later, all the mice were administrated with 250μg IgG control (LTF-2; BioXCell) or anti-PD-1 inhibitor (RMP1-14; BioXCell) intraperitoneally every 3 days for 8 days. Each experimental group had at least 6 mice. Xenogen IVIS 100 Imaging System was utilized to visualize the liver tumor size and lung metastasis. Tumor mass and lung metastasis rate were recorded.

For hydrodynamic tail-vein injection (HDTVi)-induced HCC model, sterile plasmid mix with a total volume corresponding to 10% of body weight was injected into lateral tail vein of 8-week old male C57BL/6 mice in 6-8 s. A total of 30 μg of CRISPR-Cas9 vector system carrying sgRNA targeting Trp53 ($Trp53^{KO}$) and EF1α overexpression vector carrying c-Myc ($C\text{-}Myc^{OE}$) and sleeping beauty (SB) transposon system was injected into lateral tail vein of 8-10-week old male C57BL/6 mice, as previous described [1]. Two weeks after delivery of plasmids, mice were treated intraperitoneally with vehicle control, anti-PD1 inhibitor (250μg per mouse), CS2 (5mg/kg) and combination of anti-PD1 and CS2 every 3 days for another 2 weeks. Each experimental group had at least 6 mice. Liver

weight was utilized to represent tumor mass here due to the multi-foci tumor formation of $Trp53^{KO}$ C-Myc^{OE} HDTVi model.

To deplete mouse CD8 T cells in vivo, $Trp53^{KO}$ C-$Myc^{OE\text{-}luc}$ HDTVi model was used. Two weeks after delivery of plasmids, C57BL/6 mice were administered with 2 doses of anti-CD8 antibody (BE0004-1, BioXcell) (250μg/mouse; i.p. injection; every 3 days) 5 days prior to CS2 (5mg/kg; i.p. injection; every 3 days) and/or anti-PD1 administration (250μg/mouse, i.p. injection; every 3 days). Mice were sacrificed at week 5 and Xenogen IVIS 100 Imaging System (IVIS Spectrum In Vivo Imaging System, PerkinElmer, Waltham, MA) was utilized to visualize the liver tumor size.

Flow cytometry for tumor infiltrating immune cell detection

Briefly, 2×10^5 dissociated tumors cells were stained by corresponding antibody for 1 hour in dark at room temperature according to manufacturer's instruction. The stained cells were washed with PBS buffer (2%FBS in PBS) and suspended in 200μl PBS buffer for detection by BioRad ZE5™ Cell Analyzer (Bio-Rad Laboratories, California, USA). Sources of the antibodies are provided in Supplementary Table 4.

Human T cell isolation and expansion

$CD8^+$ T cells were isolated from human PBMCs of healthy donors using commercial kit (Cat. No. 130-096-495; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction and expanded in vitro by adding recombinant IL2 (Cat.no.CTP0021; Thermofisher, Massachusetts, USA) and CD3/CD28 Dynabeads (Cat.no.11161D; Thermofisher).

T cell activation assay

1-2×10^5 isolated T cells were co-cultured with NTC or FTO-KD HCC cells for 5 days. T cells were harvested and the proportion of effector memory T cells ($CD44^+CD62L^-$) was detected by BioRad ZE5™ Cell Analyzer (Bio-Rad Laboratories, California, USA). Sources of the antibodies are provided in Supplementary Table 5.

T cell proliferation assay

1-2×10^5 isolated T cells were stained with 2μM of carboxyfluorescein succinimidyl ester (CSFE; Cat.no. C34554, Thermofisher) for 10 min and subsequently co-cultured with human HCC cells at a 1:1 ratio for 3 days. The counts of divided T cells were analyzed by flow cytometry with 488nm excitation, followed by gating the numbers of proliferated cells using FlowJo software and normalized to NTC group

Macrophage polarization assay

1×10^6 human monocyte THP-1 cells were induced to M0 differentiation with 200ng/ml PMA (phorbol 12-myristate 13-acetate) for 24 hours. Differentiated M0 macrophages were co-cultured with human HCC cells with or without FTO KD for 72 hours. mRNA expression of M1 and M2 markers was detected by qPCR. The primers are listed in Supplementary Table 2.

Macrophage recruitment assay

1×10^6 human monocyte THP-1 cells were induced to M0 differentiation with 200ng/ml PMA (phorbol 12-myristate 13-acetate) for 24 hours. Differentiated M0 macrophages were induced to M1 or M2 polarization by adding 20ng/mL IFNγ/LPS (Sigma-Aldrich) or 20ng/mL IL-4/IL-13 (Sigma-Aldrich), respectively, for another 48 hours. 2×10^5 M1 or M2 macrophages were seeded in the

upper chamber of transwell and co-cultured with human HCC cells in the lower chamber at a ratio of 1:1 for 72 hours. The macrophages in the upper chamber were fixed in methanol and stained with crystal violet. The cells having migrated through the upper chamber were counted by software Image J (National Institutes of Health, Bethesda, USA).

Histology

Mouse liver and lung tissues were dissected and sectioned for formalin fixation and paraffin embedding. Slides were stained with haematoxylin and eosin for histological analysis.

RNA extraction, reverse transcription PCR, and quantitative real-time PCR

Total RNA was extracted from clinical specimens or HCC cell lines using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The RNA was then reverse-transcribed by the GeneAmp PCR Reagent Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was performed with the use of Power SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primer sets and normalized to the internal control HPRT. The qPCR primer sequences are provided in Supplementary Table 2. All qRT-PCR reactions were done in triplicates.

Colony formation assay

For colony formation assay, 1000 cells were seeded into 6-well plates. After two weeks, formed HCC colonies were counted. Anchorage-independent growth was detected similarly as colony formation with plates coated with soft agar.

Sphere formation assay

1,000 cells were cultured in 0.25% methyl cellulose (Sigma-Aldrich) supplemented DMEM/F12 medium with 20ng/mL EGF (Life Technologies), 10ng/mL basic FGF, B27 (1:50, GIBCO), and 4μg/mL insulin in 24-well plates, which were coated with poly HEMA (Sigma-Aldrich). The cells were replenished with 30 μL supplementary medium every other day.

Cell proliferation assay

The proliferation rates of HCC cells were measured by cell counting. 1×10^3 cells in 100ul full medium were seeded in triplicate in 96-well culture plates. On the day of cell counting, cells were fixed with 50ul methanol for 5 minutes followed by staining with 50ul DAPI solution for 5 minutes. The cell counting was performed on ImageXpress pico system.

Transwell migration and invasion assays

For migration assay, 500ul conditioned medium containing 10% FBS from vector or shNTC control cells were collected and used as the chemoattractant in the bottom chamber in the 24-well plate. 1×10^5 cells were re-suspended in 100μl serum-free medium and seeded into the upper transwell with an 8um-pore size membrane (Millipore). After 18 hours of incubation, the migrated cells were fixed with methanol for 15 minutes and stained with crystal violet for 20 minutes. For cell invasion assay, the transwells were coated with Matrigel (BD Biosciences, San Jose, CA) on the upper surface of transwell chamber and kept in the incubator for 1 hour before cell seeding. Photographs of three randomly selected fields of the stained migrated or invaded cells were captured and the total numbers of migrated and invaded cells were counted. The experiments were repeated independently three times.

Isolation of sEVs from cell culture medium

For isolation of sEVs from cell culture supernatants, HCC cells were cultured in medium with 10% sEV-depleted FBS, which was prepared by 100,000×g centrifugation overnight (⩾12h) at 4 °C (Himac, CP100NX Ultracentrifuges). sEVs were purified by differential centrifugation after the cell culture supernatant were collected. Briefly, cell culture supernatants were centrifuged at 2000×g for 15 min to remove cell debris and dead cells. The supernatant was centrifugated at 20,000×g for 30 min at 4 °C to remove microvesicles and passed through 0.22 μm filter followed by ultracentrifugation at 100,000×g for 2 h at 4 °C to collect sEVs. The sEVs were washed with PBS and collected by ultracentrifugation at 100,000×g for another 2 h at 4 °C.

sEV characterization

The morphology and integrity of sEVs were observed by electron microscopy (Philips CM100 transmission electron microscope; FEI Company). Target proteins present on EVs were determined by immunogold staining followed by visualizing by transmission electronic microscopy. Protein of isolated sEVs was examined by western blotting with sEV specific markers CD63 (Sigma-Aldrich), CD81 (Abcam), CD9 (Abcam), HSP70 (Abcam), Alix (Santa Cruz), and sEVs negative markers GM130 (Abcam). The size distribution of sEVs and particle concentration was measured by ZetaView BASIC NTA PMX-120 (Particles Metrix GmbH).

Proteinase K treatment

A total of 25 μg of sEVs were subjected to Proteinase K treatment, which effectively stripped surface proteins without compromising the integrity of the sEVs. Proteinase K was stocked at a

concentration of 20 mg/mL and was diluted 1000-fold to a working concentration of 20 μg/mL prior to use. Each sample was brought up to a total volume of 100 μL and incubated at 37℃ for exactly 5 minutes. The reaction was stopped by adding 1 mL of sEV-free full medium to each sample. The sample was then purified by ultracentrifugation at 100,000×g for 1.5 hours, after which the supernatant was completely removed, and the pellet was dissolved in 50 μL of PBS. To prepare for Western blotting, 10 μL of 6×loading buffer was mixed with each sample and boiled at 95℃ for 10 minutes. An equal volume of samples was loaded for Western blotting. Tetraspanin protein CD63 and heat shock protein HSP70 were used as representative membrane and inner proteins, respectively. The pattern of the target protein was compared to the representative proteins to determine the location of the target protein on the sEVs.

Western blotting analysis

Quantified protein lysates were resolved on SDS-PAGE, transferred onto a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad), and blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBST) for 1 h at room temperature. The blocked membrane was then incubated with primary antibody diluted in 5% bovine serum albumin in TBST at 4 °C overnight. Band intensities of western blot were analyzed using ImageJ. Antibodies used in this study are listed in Supplementary Table 4.

m6A Dot Blot assay

To determine the global m6A abundance, m6A dot blot assays were employed with total RNA as described previously[2]. In brief, 50 ul RNA samples was mixed with 150 ul RNA incubation buffer, followed by denatured at 65 °C for 5 minutes. 200ml of chilled 20×SSC buffer was added and

mixed well before samples were loaded onto the Amersham Hybond-N+ membrane (RPN303B, GE Healthcare) with a Bio-Dot Apparatus (Bio-Rad). After crosslinking under 254 nm UV for 1 h, the membrane was stained with methyl blue and the image was captured. The membrane was then washed with 1×PBST buffer (PBST01-02, Bioland Scientific LLC), blocked with 5% non-fat milk and incubated with rabbit anti-m6Aantibody (1:2000, 202003, Synaptic Systems) overnight at 4°C. After washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (ab6721, Abcam) for 1 hour at room temperature and was developed with Amersham ECL Prime Western Blotting Detection Reagent (45-010-090, Fisher Scientific).

Transcriptome sequencing

Transcriptome sequencing of stable FTO KD HepG2 and 97L cells and their non-target controls (NTC) were performed with HiSeq-1500. PolyA$^+$ messenger RNA (mRNA) library was constructed with KAPA Stranded mRNA-Seq Kit. RNA-data were analyzed as previously described[3-5]. In brief, sequencing reads were aligned to reference human genome (hg38) by HISAT2. Transcripts were assembled by StringTie and the expression level of individual genes was identified as fragments per kilobase per million (FPKM). Gene ontology and pathway analyses were carried out.

m6A-methylated RNA immunoprecipitation (MeRIP) assay and m6A sequencing

Total RNAs were first extracted from stable FTO KD HepG2 and 97L cells and their corresponding NTCs. RNAs were treated with DNase according to TURBO DNA-freeTM Kit (ThermoFisher) protocol to avoid DNA contaminations. RNA concentration was adjusted to 1μg/μl with nuclease-free water. RNA was chemically fragmented into ~100nt size and fragmented RNA was incubated with m6A antibody for immunoprecipitation according to the standard protocol of Magna MeRIP™

m6A Kit (Merck Millipore). For high-throughput sequencing, purified RNA fragments from m6A-MeRIP were used for library construction with NEBNext Ultra RNA library Prep kit from Illumina and sequenced with Illumina HiSeq 2000. Library preparation and high-throughput sequencing were done by Novogene, Beijing, China.

All the IP and Input samples were sequenced by Illumina Novaseq platform with paired end 150-bp read length. Quality control of raw data was performed with FastQC (v0.11.8) according to Q30 standards[6]. Clean fastq reads were aligned to the human reference genome (GRCh38/hg38; Ensemble version 103) via HISAT2 (v2.2.1)[7] aligner with default settings after quality control by Cutadapt (v2.10)[8] and Trimmomatic (v0.38)[9]. Only the reads with mapping quality score (MAPQ) ≥20 were kept for the downstream analysis. For MeRIP-seq, the MACS3 (v 3.0.0a7; p-value < 0.05, call-summit, no model, extsize 50)[10] software was used to identity m6A methylation sites (peaks) based on its paired m6A-RIP/input data from the aligned reads with fold changes cut-off > 1.5. All m6A peaks were intersected in a pairwise fashion among two or three replicates or between two conditions using the BedTools package[11]. The m6A-enriched motifs were identified by using HOMER (v4.7) findMotifsGenome.pl with the parameter '-rna -len 5,6'. All the peaks annotated in mRNAs were used as target sequences, and exon sequences except for the peak-containing sequences were used as the background sequences. The p-values for all motifs were calculated and reported by Homer under the assumptions described at the Homer website (http://homer.ucsd.edu/homer/motif/). Visualization of m6A peaks were performed by IGV (Integrative Genomics Viewer) software (v2.14.0)[12].

Statistics

Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc.). One-way ANOVA with Dunnett comparison test for more than two groups or Student's t tests were used to compare the mean values of two groups. For in vitro functional assays, data were expressed as mean ± SD. For in vivo experiments, data were expressed as mean ± SD followed by either Unpaired t-test or Mann-Whitney test. Statistical significance was defined as *P<0.05, ** P<0.01, and ***P<0.001.

Supplementary Figure legend

Supplementary Figure S1. **Expression FTO in different HCC cell lines and expression of cancer stemness markers upon FTO KD.** (A) TCGA database shows FTO expression in different types of cancer. (B) The etiology of HCC patients. (C) STR authentication of 97L cells. (D) FTO mRNA and protein level in five HCC cell lines and an immortalized normal liver cell line (MIHA) by qPCR and western blotting. (E) The FTO KD efficiency at mRNA level in 97L and HepG2 cells by qRT-PCR. (F) The mRNA levels of cancer stemness genes upon FTO KD. (t-test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001)

Supplementary Figure S2. Enhanced immune response in tumor microenvironment upon FTO KD in vivo. (A)-(C) Representative flow cytometry histogram and quantification of the proportion of tumor infiltrating macrophages ($CD45^+/F4/80^+$), M1 ($CD45^+F4/80^+CD86^+$), and M2 macrophages ($CD45^+F4/80^+CD163^+$), respectively. (D) CSFE staining showing FTO enhanced human $CD8^+$ T cell proliferation upon co-culturing with FTO KD HCC cells. (E) mRNA expression of M1 and M2 macrophage-related genes upon co-culturing with HCC cells (NTC, shFTO#1 and shFTO#2). (t-test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001)

Supplementary Figure S3. RNA-seq and MeRIP assays with FTO KD in 97L and HepG2 cells. (A) Venn diagram and (B) GO Biological Processes Annotation Analysis showing upregulated genes by >1.5 folds upon FTO KD in HepG2 and 97L cells. (C) Successful overexpression FTO-WT and FTO-MUT at protein level in PLC cells by western blotting. (D) m6A motif detected by the HOMER motif analysis with m6A-seq data in 97L cells with or without FTO KD. (t-test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001)

Supplementary Figure S4. Promotion of HCC tumor growth by FTO was dependent on its demethylase activity. (A) The TCGA database shows significant upregulation of GPNMB expression in multiple cancer types. (B) GPNMB expression was positively correlated with FTO expression in TCGA HCC cohort. (C) Limiting dilution assay showing tumors derived from subcutaneous xenografts by injection of 2×10^3, 2×10^4 and 2×10^5 PLC cells. (D) The tumor incidence rate for each group was recorded at the end of the experiments after 4 weeks. The tumor initiating capacity was analyzed by the confidence intervals (CIs) with the formula of CI = 1/ (stem cell frequency). (E) KD efficiency of YTHDF2 siRNA in 97L cells was measured by qRT-PCR. (t-test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001)

Supplementary Figure S5. Successful KD of GPNMB in HepG2 and 97L cells. (A), (B) Stable GPNMB KD models established with two independent shRNA sequences in 97L and HepG2 cells. The protein level of GPNMB was detected by western blotting. (C) Liver with tumors derived from orthotopic injection of 97L cells (NTC and shGPNMB) and the tumor mass. n=6 in each group. (D) Bioluminescence images of mice with quantification of their bioluminescent intensities. (t-test, mean ± SD, *P < 0.05, **P < 0.01)

Supplementary Figure S6. FTO promoted cell proliferation and sphere formation through upregulating GPNMB in HCC. (A) Successful overexpression of GPNMB in FTO-KD HCC cells, detected by western blotting. (B) Migration and (C) sphere formation assays showing abrogation of the suppression by FTO KD with overexpression of GPNMB. (D), (E) Quantification for bands in the Western blots. (F) GPNMB expression on sEVs derived from HCC patients' and healthy donors' plasma. (G) Quantification for bands in the Western blots. (H) Size distribution of sEVs derived from 97L and PLCPRF/5 cells, as measured by ZetaView Particle Tracking Analyzer. (I), (J) Degree of activation of effector memory T cells ($CD44^{+}CD62L^{-}$) by flow cytometry.

Supplementary Figure S7. CS2 sensitized HCC cells to Sorafenib treatment in vivo. (A) A schematic summary of Sorafenib treatment in vivo. Orthotopic 97L cell-derived xenograft tumors were treated with vehicle control, sorafenib, CS2, and combined sorafenib and CS2. (B) Tumors in the livers and the tumor masses. (C) Protein expression of GPNMB, AKT/p-AKT and ERK/p-ERK in mouse tumor tissues by western blotting.) (D) Proportions of macrophages in tumor tissues by flow cytometry. (t-test, mean ± SD, *P < 0.05, **P < 0.01).

Supplementary Figure S8. Depletion of CD8 T cells in mice abrogated the anti-tumor effect of CS2/anti-PD1. (A) A schematic summary of anti-CD8, FTO inhibitor CS2 and anti-PD-1 treatment in hydrodynamic tail vein injection (HDTVi) (p53 KO/c-Myc-luc) model. (B), (C) Bioluminescent images and quantification of their bioluminescent intensities. (one-way ANOVA followed by Dunnett comparison test, mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001).

Supplementary References:

1 Ho, D. W. H. *et al.* Single-cell RNA sequencing shows the immunosuppressive landscape and tumor heterogeneity of HBV-associated hepatocellular carcinoma. *Nature Communications* **12**, doi:ARTN 368410.1038/s41467-021-24010-1 (2021).

2 Su, R. *et al.* R-2HG Exhibits Anti-tumor Activity by Targeting FTO/m6A/MYC/CEBPA Signaling - ScienceDirect. (2018).

3 Ho, D. W.-H. *et al.* Single-cell transcriptomics reveals the landscape of intra-tumoral heterogeneity and stemness-related subpopulations in liver cancer. *Cancer letters* **459**, 176-185 (2019).

4 Ho, D. W.-H. *et al.* Single-cell RNA sequencing shows the immunosuppressive landscape and tumor heterogeneity of HBV-associated hepatocellular carcinoma. *Nature Communications* **12**, 3684 (2021).

5 Ho, D. W.-H., Lam, W.-L. M., Chan, L.-K. & Ng, I. O.-L. Investigation of Functional Synergism of CENPF and FOXM1 Identifies POLD1 as Downstream Target in Hepatocellular Carcinoma. *Frontiers in Medicine* **9**, 860395 (2022).

6 Andrews, S. (Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom, 2010).

7 Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. *Nature methods* **12**, 357-360 (2015).

8 Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* **17**, 10-12 (2011).

9 Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120 (2014).

10 Zhang, Y. *et al.* Model-based analysis of ChIP-Seq (MACS). *Genome biology* **9**, 1-9 (2008).

11 Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841-842 (2010).

12 Thorvaldsdóttir, H., Robinson, J. T. & Mesirov, J. P. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in bioinformatics* **14**, 178-192 (2013).

Supplementary Table 1.

Clinicopathological correlation of FTO overexpression in HCC patient samples.

Parameters	FTO overexpression (n=37)	FTO normal and underexpression (n=56)	*P* *
Gender			0.238
Male	30 (32.3%)	39 (41.9%)	
Female	7 (7.5%)	17 (18.30%)	
Mean age (range) ^	51. 6(16-74)	56.3 (24-74)	0.081
Tumor size			0.347
>5cm	18 (22.8%)	33 (41.8%)	
≤5cm	13 (16.5%)	15 (19.0%)	
Background liver disease			0.115
Normal and Chronic hepatitis	12 (15.0%)	27 (33.8%)	
Cirrhosis	20 (25.0%)	21 (26.3%)	
Liver invasion			0.639
Presence	14 (17.7%)	17 (21.5%)	
Absence	18 (22.8%)	30 (38.0%)	
Tumor microsatellite formation			0.385
Presence	22 (25.6%)	27 (31.4%)	
Absence	13 (15.1%)	24 (27.9%)	
Tumor encapsulation			0.644
Presence	12 (14.3%)	16 (19.0%)	
Absence	21 (25.0%)	35 (41.7%)	
Venous invasion			0.529
Presence	23 (24.7%)	31 (33.3%)	
Absence	14 (15.1%)	25 (26.9%)	
Cellular differentiation			0.358
Edmondson grade I-II	16 (20.2%)	18 (22.8%)	
Edmondson grade III-IV	16 (20.2%)	29 (36.7%)	
TNM staging			**0.000029***
I-II	2 (2.4%)	25 (30.1%)	
III-IV	31 (37.3%)	25 (30.1%)	

* Fisher exact test

^ t-test

Supplementary Table 2

Primer sequences used for qRT-PCR analysis.

Target gene		Sequence (5'-3')
Human FTO	Forward	ACAACGGACAAGATGAAGTGGA
	Reverse	ATCCCTGCCTTCGAGATGAG
Human AKT3	Forward	TGGATGCCTCTACAACCCATCA
	Reverse	TGTGTGCCACTTCATCCTTTGC
Human PDGFB	Forward	CTCGATCCGCTCCTTTGATGA
	Reverse	CGTTGGTGCGGTCTATGAG
Human RORO4	Forward	CCCTGTGCTTGGAACTCAGTG
	Reverse	CGCTGATGTACCCATAGGTGG
Human GPNMB	Forward	AAGTGAAAGATGTGTACGTGGTAACAG
	Reverse	TCGGATGAATTTCGATCGTTCT
Human FZD8	Forward	TGGAGTGGGGTTACCTGTTG
	Reverse	AGCGGCTTCTTGTAGTCCTC
Human SEMA3G	Forward	GGGTCTGTGCTCAAAGTCATCG
	Reverse	AAGTCCCACTGCCTCTTCTTCC
Human YTHDF2	Forward	TAGCCAACTGCGACACATTC
	Reverse	CACGACCTTGACGTTCCTTT
Human CD68	Forward	ATTCACCAGTTCTGCCCACC
	Reverse	GCTTCCCTGGACCTTGGTTT
Human CD80	Forward	TGCTGGCTGGTCTTTCTCAC
	Reverse	GTCCGGTTCTTGTACTCGGG
Human CD86	Forward	CCCCAGTGCACTATGGGAC
	Reverse	CAGGGTCCAACTGTCCGAAT
Human CD204	Forward	CGAAAGTTCGACTGGTCGGT
	Reverse	TGTCCCCCATTGCCGAATTT
Human CD206	Forward	CATCAGGGTGCAAGGAAGGT
	Reverse	TCCATCCGTCCAAAGGAACG
Human CD163	Forward	TCCTTGTGGGATTGTCCTGC
	Reverse	ATGGGAATTTTCTGCAAGCCG
Human SMO	Forward	TGGTCACTCCCCTTTGTCCTCAC
	Reverse	GCACGGTATCGGTAGTTCTTGTAGC
Human NANOG	Forward	CCTGTGATTTGTGGGCCTG
	Reverse	GACAGTCTCCGTGTGAGGCAT
Human OCT4	Forward	CTTGCTGCAGAAGTGGGTGGAGGAA
	Reverse	CTGCAGTGTGGGTTTCGGGCA
Human GAPDH	Forward	ACAACGGACAAGATGAAGTGGA
	Reverse	ATCCCTGCCTTCGAGATGAG
Human HPRT	Forward	CTTTGCTGACCTGCTGGATT
	Reverse	CTGCATTGTTTTGCCAGTGT

Supplementary Table 3.
List of shRNA sequences used in this study.

Target gene	Species	Sequence (5'-3')
shFTO#1	Human	CCCATTAGGTGCCCATATTTA
shFTO#2	Human	TCACCAAGGAGACTGCTATTT
shGPNMB#1	Human	CACAAGGAATACAACCCAATA
shGPNMB#2	Human	CGCACAAGTGAAAGATGTGTA
shFTO#1	Mouse	TTGAAAGAGGAGCCCTATTTC
shFTO#2	Mouse	GTCTCGTTGAAATCCTTTGAT
shNTC	H & M	CAACAAGATGAAGAGCACCAA

Supplementary Table 4.

List of antibodies used in this study.

Antibody	Application	Source	Cat No.
m6A (N6-methyladenosine) antibody	Dot bolt, WB	Synaptic Systems	202003
Anti-FTO antibody	WB	Abcam	ab126605
Recombinant Anti-GPNMB antibody	WB, Co-IP, Immunogold	Abcam	ab235873
Recombinant Anti-beta Catenin antibody	WB	Abcam	ab32572
Anti-Syndecan 4 antibody	WB	Abcam	ab74139
Syndecan-4 Antibody (5G9)	Neutralization	Santa Cruz	sc-12766
Anti-Akt	WB	Cell signaling	9272
Anti-phospho-Akt (Ser473)	WB	Cell signaling	9271
Anti-p44/42 MAPK (ERK1/2)	WB	Cell signaling	4695
Anti-Phospho-p44/42 MAPK (ERK1/2)	WB	Cell signaling	4370
Anti-CD63	WB	Sigma-Alrich	SAB2109138
Anti-CD63	Immunogold	Abcam	ab271286
Anti-CD9	WB	Abcam	ab92726
Anti-CD81	WB	Abcam	ab79559
Anti-ALIX	WB	Santa Cruz	53540
Anti-HSP70	WB	Abcam	ab181606
Anti-GM130	WB	Abcam	ab52649
Anti-GFP (B-2)	WB, Co-IP	Santa Cruz	sc-9996
Donkey Anti-Rabbit IgG H&L	Immunogold	Abcam	ab105294
Goat Anti-Mouse IgG H&L	Immunogold	Abcam	ab27241
Normal rabbit IgG	Co-IP	Santa Cruz	sc-2027
Normal mouse IgG	WB	Santa Cruz	sc-3877

Human CD44	Flow cytometry	Miltenyibiotec	130-113-337
Human CD62L	Flow cytometry	Biolegend	304840
Mouse CD45	Flow cytometry	Biolegend	103128
Mouse CD8	Flow cytometry	Biolegend	126606
Mouse CD4	Flow cytometry	Biolegend	100451
Mouse CD279 (PD-1)	Flow cytometry	Biolegend	135216
Mouse F4/80	Flow cytometry	Biolegend	157304
Mouse CD86	Flow cytometry	Biolegend	105110
Mouse CD163	Flow cytometry	Biolegend	155320
Isotype Control In vivo blockade (IgG)	In vivo treatment	BioXcell	BP0090
InVivoMAb anti-mouse PD-1 (CD279)	In vivo treatment	BioXcell	BE0146
InVivoMAb anti-mouse CD8α	In vivo treatment	BioXcell	BE0004-1

Supplementary Table 5.

List of reagents used in this study.

Antibody	Source	Cat No.
RNA Fragmentation Reagents	Thermo Fisher	AM8740
Methyl Blue	Millipore Sigma	M6900
Amersham Hybond-N+ membrane	GE Healthcare	RPN119B
Brequinar (CS2)	Sigma-Alrich	508321
Magna MeRIP m6A Kit	Millipore	17-10499
CD8+ T Cell Isolation Kit, human	Miltenyi biotec	130-096-495
Dynabeads™ Human T-Activator	Thermofisher	11131D
CD3/CD28	Thermofisher	PHC0021
Recombinant Human IL-2 Protein	Goldbio	Luck-1G
D-Luciferin Firefly, potassium salt		