Materials and methods

Cell culture, transfection, lentivirus infection and reagents

Human HCC cell lines (SK-HEP-1, HepG2, Hep3B, Li-7) and Murine HCC cell line (H22) were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The authenticity of all cells was verified by short-tandem repeat (STR) profiling. SK-HEP-1, HepG2 and Hep3B were maintained in MEM medium (Biological Industries), and Li-7 cells and H22 cells were cultured in RPMI 1640 medium (Biological Industries). All the above media were supplemented with 10% fetal bovine serum (Wisent), 60 μG /mL penicillin G, 100μg /mL streptomycin, and 50μg/mL gentamicin (Solarbio). The culture conditions were 37℃ in a humidified atmosphere containing 5% CO_2. Polyplus jetPRIME transfection reagent was used for transient siRNA and DNA transfections following manufacturer's protocol (Invitrogen). Lentivirus infection was performed as the procedures previously.[1] Plasmids and lentivirus were purchased from Shandong Vigene and Zhejiang REPOBIO. Lentivirus siRNAs against TP53 and overexpression of TP53 and E2F1 are packaged by lentiviral plasmids. In order to avoid off-target effect, we used 2 single siRNAs targeting E2F1, TSC1 and Beclin1. All siRNA and plasmid sequences were provided in Table 1. All reagents were provided in Table 2.

Patients and samples

This study was approved by the Institutional Review Board of The First Affiliated Hospital, Zhejiang University School of Medicine strictly under the guidelines of the Ethics Committee of the hospital and the 2013 Declaration of Helsinki. All the patients HCC tissues were obtained from The First Affiliated Hospital, Zhejiang University School of Medicine between January 2015 and December 2016. After screening, a total of 97 patients who underwent surgery were finally included in the analysis. Clinical data were obtained (Supplement Table). Patient eligibility included histologically or cytologically confirmed primary HCC. The exclusion criteria included extrahepatic metastasis or large blood vessels invasion, survival time after surgery less than 90 days, tumor cellrecurrence time after surgery less than 60 days.

Whole-exome sequencing

Murine HCC cell line H22 underwent whole-exome sequencing. Poor quality reads were filtered out and the remaining high-quality filtered reads were aligned to the human reference genome (hg19) using the BurrowsWheeler Alignment tool (BWA, version 0.7.5a).[2] SAMtools were used to produce the Sequence Alignment and Mapping (SAM) files into Binary Alignment and Mapping (BAM) files.[3] Polymerase chain reaction (PCR) duplicates were removed from the BAM files by Picard and SAMtools before variant calling. The Genome Analysis Toolkit (GA TK, version 2.4.7) was used to recalibrate base quality and optimize local realignment. Single nucleotide variants (SNVs) and indels were invoked using MuTect (version 1.1.4) and V arscan2 (version 2.3.5) with default parameter settings.[4-6] CONTRA(version 2.0.4) was used to detect copy-number variations.[7]

The filtered variants were annotated using ANNOV AR, including gene, chromosomal

information, exonic function (synonymous, nonsynonymous, stop gain, nonframeshift, or frameshift indel), amino acid changes, and allele frequencies extracted from public databases such as the 1000 Genomes Project (2012 February version) and dbSNP (version 132).

Variants located in exonic regions with sufficient coverage (minimum depth of coverage ≥ 8) and variant allele frequency (V AF ≥ 0.1) were selected for further statistical analyses. Synonymous variants were filtered out. Read alignments were manually investigated using the Integrative Genomic Viewer (http://www.broadinstitute.org/igv/).

We used Fisher's exact test to analyze mutations and polymorphic variants separately to identify variants enriched in patients with a good outcome. P-values < 0.05 were considered significantly different. R version 3.0.2 (http://www.R-project.org/) and R package (ggplot2) were used for all statistical analyses and for generating heat maps and plots.

RNA-seq and gene set analysis

Total RNA was isolated from human HCC cell lines, then mRNA was selected using poly(A) selection protocol. The RNA-seq library preparation and sequencing were performed by RiboBio.[8] Intratumor adipogenesis pathway scores were measured by the Gene set Variation Analysis (GSVA)[9] Bioconductor software package using the "Hallmark_adipogenesis" gene set from the Molecular Signature database (MSigDB)[10], similar to our method of measurement of scores for several other signaling pathways.[11] In the gene set enrichment analysis (GSEA)[12], a false discovery rate (FDR) less than 25% recommended by the GSEA software was defined as statistically significant.

Transposase-accessible chromatin by sequencing (ATAC-seq)

Freshly sorted cells were extracted and library preparation was performed according to the instructions provided in the ATAC-Seq Kit (Active Motif 53150). Sequencing data were analyzed by Galaxy software (https://usegalaxy.org/) or basepair software (https://www.basepairtech.com/) and were mapped to the human genome (hg38), using bowtie.

Sanger sequencing

TP53 mutations in HCC tissues from patients were detected by Sanger sequencing. It was performed on exons 2 - 11 of TP53 (primer sequences listed in Table 3).

Co-immunoprecipitation assay and western blot analysis

2 x 10^6 SK-HEP-1 and HepG2 cells were planted in a 10 cm plate and cultured for 24h. Above cells were transfected with lentivirus E2F1, after 48h, and treated with puromycin for anther 48h. Tissue protein extraction, cell lysis, immunoprecipitation, western blotting, and blocking were performed as described previously.[1] The primary antibodies included rabbit anti-human E2F1(CST, 37425; Proteintech, 66515-1-1g), rabbit anti-human Phospho-p70 S6 Kinase (Thr389) (CST,9234), anti-PD-L1 (CST,

60475; CST,13684T; Abcam,ab205921) and mouse anti-human β-actin (Proteintech, 60008-1-Ig). The bands were incubated with diluted primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies for 1h at room temperature. The bands were visualized by chemiluminescence. All antibodies were diluted appropriately according to manufacture' protocols in TBST buffer containing 5% bovine serum albumin for western blotting. The related information for all antibodies were displayed in Table 4.

Chromatin Immunoprecipitation

Cells were treated with 1% methanol for 10 min at 37 ℃, and cross-linking was terminated with glycine (125 mM) at room temperature. The cross-linked cells were lysed and sonicated to obtain sheared DNA.[13] Lysates were incubated with appropriate antibody with agarose beads at 4℃ overnight. DNA was eluted from the pellets and cross-linked with 0.2 M NaCl solution at 65℃. The Quantitative PCR was used to quantify the related gene expression levels.

RT-qPCR analysis

RNeasy Mini Kit was used to collect and isolate RNA from HCC cells and human HCC tissues according to the manufacturer's procedures (QIAGEN). RNA purity was assessed using the ND-1000 Nanodrop. The cDNA was synthesized using the Hiscript ® II RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme# R223-01). Quantitative Real-time PCR assays were performed in a 7500 real-time PCR system (Applied Biosystems) using ChamQ SYBR Color qPCR Master Mix (Vazyme) and appropriate primers (sequences are shown in Table 5). The amount of target cDNA was analyzed through the conversion of the threshold cycle (CT) and was calculated normalized to the amount of β-actin.

Immunofluorescence

1.5 x 10^5 cells were cultured on glass coverslips attached to 6-well plates. Cell slides were immersed in 4% paraformaldehyde for 10 min and incubated with PBS containing 0.5% Triton X-100 for 10 min. After blocking with goat serum, diluted primary antibodies were dropped on the coverslips and incubated overnight at 4°C. Target proteins were visualized with appropriate fluorescently labeled secondary antibodies. DAPI (300nM) staining was applied for nuclear localization. The fluorescence was monitored by Laser-scanning confocal microscope (Olympus).

Luciferase reporter assay

3 x 10^3 – 5 x 10^3 HCC cells were planted in 96-well plates. After 24h incubation, cells were transfected with pGL3-HRE-luciferase (Luc) (150 ng) or pGL3-E2F1-promoter-luciferase with pSV40-renilla (100 ng). After cultivated in 50nM Everolimus 48h. luciferase reporter assay was conducted according to the manufacture's protocol (Promega). The relative Luc activity was normalized by renilla activity.

Flow cytometry staining and analysis

All antibodies used for flow cytometry are listed in Table 6. Single-cell suspensions were prepared. For surface marker staining, cells were stained with the Zonbie NIR Dye (BioLegend) and a cocktail of antibodies specific for cell-surface markers for 30 min at 4 °C in the dark. All samples were sorted and analyzed by BD FACSCanto™ II flow cytometry. We obtained fluorescence data from at least 50,000 cells. the FlowJo software (FlowJo, Ashland, Oregon, USA) was used for data analysis.

Immunohistochemistry

Tumors were obtained from C57BL/6J mice, BALB/c mice and the patients, and processed formalin fixation and paraffin embedment. Microarrays were established from 97 HCC samples. Immunohistochemical staining was performed according to the protocol of the manufacturer of the immunohistochemical assay kit (Proteintech). The immunohistochemical staining were scanned by Laser-scanning confocal microscope (Olympus). The immunostaining levels were evaluated by immunoreactive score (IRS). The IRS was determined by multiplying the percentage of positive cells and staining intensity. The gradations were performed as described previously.[1] IRS (≤7) was defined as low and IRS (≥8) was defined as high.

Multicolor fluorescence staining.

Liver tissue sections of patients were deparaffinized using xylene, rehydrated in gradient ethanol (100%, 95%, 90%, 80%, 70%) and distilled water. The sections were antigenic repair was performed using EDTA antigen repair buffer (PH 9.0), incubated in 3% H_2O_2 to eliminate endogenous peroxidase activity, and then blocked with 3% BSA working solution at room temperature. Primary antibody was then added drop-wise to the sections. Sections were placed in a wet box and incubated at 4°C overnight. After primary antibody incubation, sections were washed with PBS, and then incubated with the secondary antibody at room temperature in dark condition. Visualization reagent was utilized for signal amplification. Then, the procedure of blocking and incubation with primary and secondary antibody was repeated as described. These primary antibodies included antibodies to CD8, PD-L1, E2F1. Cell nuclei were counterstained with DAPI and the sections were treated with antifluorescence quenching and sealing tablets. Finally, Image acquisition was performed with a Nikon inverted fluorescence microscope.

Transmission electron microscope

Each experimental group of HCC cells was examined under a transmission electron microscope for autophagy. To obtain the cell samples, the HCC cells were digested with 2.5g/L trypsin, centrifuged at 3000 r/min, washed with PBS three times and collected in 1.5ml EP tubes. Then the cells were fixed with 25g/L glutaraldehyde for 24h, with 10g/L citric acid, and dehydrated by graded ethanol, infiltrated and embedded in epoxy resin. Ultramicrotome slices were stained with uranyl acetate and lead citrate, then observed under a transmission electron microscope.

Animal experiment

C57BL/6J mice and BALB/c mice were obtained from the Animal Facility of Zhejiang University. Animal care and experiments were performed in strict accordance with the "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals".

Three HCC mice models were used in this study. H22 cells ($1x10^6$) were resuspended in 100μl sterile PBS and basement membrane matrix (1:1), and subcutaneously injected into right flank of eight weeks old male BALB/c mice to establish subcutaneous tumor xenograft model. A total of 20μL of PBS containing H22 cells ($1x10^6$) and basement membrane matrix (1:1) was injected into the right liver lobe of eight weeks male BALB/c mice to establish an orthotopic tumor model. 2mL mixture of 5μg NRASG12V, 5μg c-MYC, and 0.5μg SB100 in PBS was injected via tail vein to establish the plasmid-induced mouse HCC model. In addition, Trp53 knockout mice were constructed through injecting with AAV vector pAV-U6-shRNA-CMV-GFP via tail vein.

After 1 week of tumor growth in xenograft model and orthotopic model, in order to confirm the antitumor effectiveness of Everolimus (Selleck, S1120) and Anti-PD-L1 antibody (Selleck, A2115), mice were randomly assigned to each group. The Everolimus was dissolved in DMSO, and then administered into mice at a dose of 0.5 mg/kg for six consecutive days with a day off on the seventh day for 2 weeks. Anti-PD-L1 or IgG isotype (Selleck, A2116) was administrated by intraperitoneal injection at dose of 10 mg/kg for every other day for 2 weeks. Each animal was earmarked and followed individually throughout the experiment. The width and length of the tumors and the body weight of mice were measured at the time of each injection until the end of the experiments. Tumor volume was calculated with formula: length x $width^2$ x 0.5 (mm^3).

CyTOF staining and data acquisition

Cells were washed once with 1xPBS and then stained with 100μL of 250nM cisplatin (Fluidigm) for 5min on ice to exclude dead cells, and then incubated in Fc receptor blocking solution before stained with surface antibodies cocktail for 30 min on ice. Cells were washed twice with FACS buffer (1xPBS+0.5%BSA) and fixed in 200μL of intercalation solution (Maxpar Fix and Perm Buffer containing 250nM 191/193Ir, Fluidigm) overnight. After fixation, cells were washed once with FACS buffer and then perm buffer (eBioscience), stained with intracellular antibodies cocktail for 30 min on ice. Cells were washed and resuspend with deionized water, adding into 20% EQ beads (Fluidigm), acquired on a mass cytometer (Helios, Fluidigm).

CyTOF data analysis

Data of each sample were debarcoded from raw data using a doublet-filtering scheme[14] with unique mass-tagged barcodes. Each .fcs file generated from different batches were normalized through bead normalization method.[15] Manually gate data using a FlowJo software to exclude to debris, dead cells and doublets, leaving live,

single immune cells. Apply the Phenograph clustering algorithm[16] to all cells to partition the cells into distinct phenotypes based on marker expression levels. Annotate cell type of each cluster according to its marker expression pattern on a heatmap of cluster vs marker. Use the dimensionality reduction algorithm t-SNE to visualize the high-dimensional data in two dimensions and show distribution of each cluster and marker expression and difference among each group or different sample type. Perform T-test statistical analysis on the frequency of annotated cell population.

Statistical analysis

SPSS V.21.0 statistical software was used for statistical analysis. The significance of the differences between groups was determined using the Student's t-test. Values of $p < 0.05$ were considered to be significantly different. $\chi 2$ test was used to analyze the correlation between quantitative data. For survival analysis, overall survival was estimated using the Kaplan–Meier method.

Table 1

Oligonucleotides	Company
siRNA targeting sequence TP53 1# 1# CACTACAACTACATGTGTA	Vigene
siRNA targeting sequence E2F1 1# GCAUCUAUGACAUCACCAA(dT)(dT) E2F1 2# GCUGGACCACCUGAUGAAU(dT)(dT) E2F1 3# CCUCUUCGACUGUGACUUU(dT)(dT)	Vigene
siRNA targeting sequence TSC1 1# CGGCTGATGTTGTTAAATA ; TSC1 2# GTGGCCCTATGCTTGTAAA	Tsingke Biotechnology
siRNA targeting sequence BECN1 1# GCTTGGGIGTCCTCACAATTT; BECN1 2# CCCGTGGAATGGAATGAGATT	Tsingke Biotechnology
mus-Trp53-si-2: GUAAACGCUUCGAGAUGUU	Vigene
pLent-EF1a-FH-CMV-Blasticidin-Trp53	Vigene
pGL3-HRE-luciferase	This study
pGL3-E2F1-promoter-luciferase	This study
pSV40-renilla	This study
pLVX-flag-Puro-E2F1	This study
pT3-EF1α-NRASG12V	Vigene
pT3-EF1α-c-MYC	Vigene
pCMV(CAT)T7-SB100	Vigene

Table 2

Reagents	Company	Product code
Puromycin	Solarbio	Cat# P8230
Rapamycin	Selleck	Cat# S1039
Everolimus	Selleck	Cat# S1120
MG132	Selleck	Cat# S2619
HiScript II Q RT SuperMix for qPCR (+gDNA wiper)	Vazyme	Cat# R223-01
2 × ChamQ Universal SYBR qPCR Master Mix	Vazyme	Cat# Q711-02
RNeasy Mini Kit (50)	Qiagen	Cat# 74104
Nuclear and Cytoplasmic Protein Extraction Kit	Proteintech	Cat# PK10014
Chloroquine diphosphate salt	sigma-aldrich	Cat# C6628
jetPRIME	Polyplus	Cat# 101000046
Anti-fluorescence quench sealing tablets	HaoKe	Cat# HK1421
EDTA antigen recovery solution (9.0)	HaoKe	Cat# HKI0004
DAPI	HaoKe	Cat# HK1032

Table 3

Exon	Forward primer (5'-3')	Reverse primer (5'-3')
TP53 Exon 2-3	TCTCATGCTGGATCCCCACT	AGTCAGAGGACCAGGTCCTC
TP53 Exon 4	CGTTCTGGTAAGGACAAGGG	AAGGGTGAAGAGGAATCCCA
TP53 Exon 5	GTTTGTTTCTTTGCTGCCGT	AGAGGCCTGGGGACCCT
TP53 Exon 6	GACAGGGCTGGTTGCCC	TCATGGGGTTATAGGGAGGTC
TP53 Exon 7	CCTGCTTGCCACAGGTCT	GTGATGAGAGGTGGATGGGT
TP53 Exon 8-9	CAAGGGTGGTTGGGAGTAGA	CCCCAATTGCAGGTAAAACA
TP53 Exon 10	GCAACAGAGTGAGACCCCAT	TGAAGGCAGGATGAGAATGGA
TP53 Exon 11	AGACCCTCTCACTCATGTG	TGACGCACACCTATTGCAAG

Table 4

Antibodies	Company	Product code
E2F1 Monoclonal antibody	Proteintech	Cat# 66515-1-1g
E2F1 antibody	Cell Signaling Technology	Cat# 3742S
p53 antibody	Abcam	Cat# ab32389
p53 antibody	Abcam	Cat# ab26
p53 antibody	Cell Signaling Technology	Cat# 2524T
PD-L1 antibody	Cell Signaling Technology	Cat# 13684T
PD-L1 antibody	Cell Signaling Technology	Cat# 60475S
PD-L1 antibody	Abcam	Cat# ab205921
Rabbit Anti-p70 S6 Kinase Monoclonal Antibody	Cell Signaling Technology	Cat# 2708
Phospho-p70 S6 Kinase (Thr389)	Cell Signaling Technology	Cat# 9205
4E-BP1 Rabbit mAb	Cell Signaling Technology	Cat# 9644S
P-4E-BP1 (Thr37/46) Rabbit mAb	Cell Signaling Technology	Cat# 2855T
P62 Polyclonal antibody	Proteintech	Cat# 18420-1-AP
LC3 Polyclonal antibody	Proteintech	Cat# 14600-1-AP
Beclin1 Polyclonal antibody	Proteintech	Cat# 11306-1-AP
Rabbit IgG	Abcam	Cat# ab172730
Beta Actin Monoclonal antibody	Proteintech	Cat# 60008-1-Ig
Goat anti-mouse IgG (H+L)	Proteintech	Cat# SA00001-1
Goat anti-rabbit IgG (H+L)	Proteintech	Cat# SA00001-2
Lamin B1 Monoclonal antibody	Proteintech	Cat# 66095-1-Ig
Mouse IgG2b	Cell Signaling Technology	Cat# 53484S
Mouse IgG1	Cell Signaling Technology	Cat# 5415S
CD8a	Cell Signaling Technology	Cat# 98941S

Table 5

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	CATCCACGAAACTACCTTCAACTCC	GAGCCGCCGATCCACACG
TP53 Human	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
TP53 Mice	CCCCTGTCATCTTTTGTCCCT	AGCTGGCAGAATAGCTTATTGAG
PD-L1 Human	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
PD-L1 Human	GGAAAGGCAAACAACGAAGA	GTTAGTGAATGGGCCCAAGA

Table 6

Antibodies	Company	Product code
Anti-human PD-L1	eBioscience	Cat# 12-5983-42
Anti-mouse PD-L1	eBioscience	Cat# 12-5982-82
Mouse IgG1 kappa isotype	eBioscience	Cat# 12-4784-82
Zonbie NIR Dye	Biolegend	Cat# 77184
Anti-mouse CD45	TONBO	Cat# 35-0451-U100
Anti-mouse CD3e	TONBO	Cat# 50-0031-U100
Anti-mouse CD4	TONBO	Cat# 75-0042-U100
Anti-mouse CD8a	TONBO	Cat# 65-0081-U100

References

1. Ling S, Li J, Shan Q*, et al.* USP22 mediates the multidrug resistance of hepatocellular carcinoma via the SIRT1/AKT/MRP1 signaling pathway. *Mol Oncol* 2017; **11**:682-95.
2. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010; **26**:589-95.
3. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* 2011; **27**:2987-93.
4. DePristo MA, Banks E, Poplin R*, et al.* A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* 2011; **43**:491-8.
5. Cibulskis K, Lawrence MS, Carter SL*, et al.* Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol* 2013; **31**:213-9.
6. Koboldt DC, Zhang Q, Larson DE*, et al.* VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 2012; **22**:568-76.
7. Li J, Lupat R, Amarasinghe KC*, et al.* CONTRA: copy number analysis for targeted resequencing. *Bioinformatics* 2012; **28**:1307-13.
8. Zhuang Q, Li W, Benda C*, et al.* NCoR/SMRT co-repressors cooperate with c-MYC to create an epigenetic barrier to somatic cell reprogramming. *Nat Cell Biol* 2018; **20**:400-12.
9. Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013; **14**:7.
10. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* 2015; **1**:417-25.

11. Oshi M, Newman S, Tokumaru Y*, et al.* Inflammation Is Associated with Worse Outcome in the Whole Cohort but with Better Outcome in Triple-Negative Subtype of Breast Cancer Patients. *J Immunol Res* 2020; **2020**:5618786.
12. Subramanian A, Tamayo P, Mootha VK*, et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005; **102**:15545-50.
13. Xing B, Ma J, Jiang Z*, et al.* GLP-1 signaling suppresses menin's transcriptional block by phosphorylation in β cells. *J Cell Biol* 2019; **218**:855-70.
14. Zunder ER, Finck R, Behbehani GK*, et al.* Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. *Nat Protoc* 2015; **10**:316-33.
15. Finck R, Simonds EF, Jager A*, et al.* Normalization of mass cytometry data with bead standards. *Cytometry A* 2013; **83**:483-94.
16. Levine JH, Simonds EF, Bendall SC*, et al.* Data-Driven Phenotypic Dissection of AML Reveals Progenitor-like Cells that Correlate with Prognosis. *Cell* 2015; **162**:184-97.