**β-catenin activation promotes immune escape and resistance to anti-PD-1 therapy in hepatocellular carcinoma**

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**SUPPLEMENTARY METHODS**

***Western Blot Analysis***

Cell or tissue samples were lysed and Western blot analysis was done as described previously (1). Cells were washed with PBS and lysed at 4°C in 200 μl lysis buffer. Phospho-lysis buffer (50 mM Tris pH 7.5, 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% Glycerol) supplemented with phosphatase and protease inhibitors (cOmplete and PhosSTOP tablets, Roche) was used for cell lysis and protein concentration was determined using the BCA Protein Assay Kit (Pierce Biochemicals). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were immunoblotted with antibodies against MYC (Abcam Ab32072), β-catenin (BD Biosciences 610153), Axin2 (Abcam ab-32197), β-actin (Santa Cruz Biotechnology sc-47778), and vinculin (Sigma-Aldrich V9131). Blots were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit (SIGMA-ALDRICH A4914) or goat anti-mouse IgG (Santa Cruz Biotechnology sc-516102), developed using ECL Plus chemifluorescent reagent (Amersham Biosciences) and imaged using the ChemiDoc MP system (Biorad). All immunoblots were performed independently at least twice.

### *RNA extraction and qRT-PCR*

Total RNA from mouse liver tissue and cultured tumor cells was isolated using Trizol® (Invitrogen) followed by digestion with DNase I (Roche) and purification with the RNeasy Kit (Qiagen). RNA was reverse-transcribed using the SuperScriptTM IV First-Strand Synthesis System (Invitrogen), and qPCR was performed with a CFX384 Touch™ Real-Time PCR Detection System (Bio Rad) with TaqMan Universal PCR Master Mix (Applied Biosystems) and specific TaqMan probes for *Axin2* (Mm00443610\_m1), *Ccl5* (Mm01302427\_m1), *Pd-l1* (Mm03048248), and *Gapdh* (Mm99999915\_g1) (TaqMan Gene expression Assays, Applied Biosystems). For detecting *luciferase* (luc), *OS*, and *Gapdh* transcripts, we used the SYBR green method and the following primers: luc-F (ATCCATCTTGCTCCAACACC), luc-R (TTTTCCGTCATCGTCTTTCC), OS-F (AAGCAGGCAGAGAGGTGGTA), OS-R (TCCAACACATCTGCCAAAGA), Gapdh-F (AAGCTCATTTCCTGGTATGACA), Gapdh-R (GGAGATGCTCAGTGTTGGGG). The expression of the target gene was calculated by the cycle threshold method and results were expressed as relative fold change. Values were normalized to *Gapdh* mRNA levels.

***Immunofluorescence***

Tissues were frozen in O.C.T. compound and stored at -80°C. 10μm-tissue sections were fixed and blocked for 1h in PBS solution supplemented with 2% fetal calf serum and 0.5% bovine serum albumin. Sections were then stained with a conjugated primary antibody for CD3 (purchased from Biolegend, anti-CD3-AlexaFluor(AF)488). Antibody incubation step was conducted for a minimum of 2h in a dark, humidifier chamber at 4°C. Tissue sections were visualized under a Carl Zeiss Axioimager Z1 microscope. The analysis of immunofluorescence staining and CD3+ cell numbers was performed with ImageJ.

***Immunohistochemistry***

Immunohistochemical labeling of HCC patient samples was performed using 4 μm-thick sections from formalin-fixed and paraffin-embedded tissues. Slides were deparaffinized with serial xylene treatments and subjected to antigen retrieval by heated treatment in citrate solution (pH 6.0). All immunohistochemical labeling was performed on the automated Ventana Benchmark XT system using the biotin-free Ventana OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). The following antibodies were applied at indicated dilutions: β-catenin (monoclonal mouse M3539, dilution 1:250, Dako, Carpinteria, CA, USA) and CD8 (mouse monoclonal C8/144B, dilution 1:40, Dako, Carpinteria, CA, USA). Appropriate positive and negative controls were included with each immunolabeling procedure. Interpretation of immunohistochemical stains was performed as follows. With β-catenin, positive expression was defined as abnormal immunolabeling in at least 10% of neoplastic tumors cells while negative expression was defined as an absence of nuclear staining and preserved membranous expression. The number of CD8+ T cells was counted using a 40X objective and 10X ocular (400X). Both peritumor and intratumor areas were counted separately and within three representative foci. Final scores for CD8+ T cells represent an average of three foci per high power field.

### *Isolation of immune cells and flow analysis*

The liver was perfused with 2 mM PBS-EDTA, chopped, and enzymatically digested using a buffer composed of HBSS (Corning) supplemented with 1 mg/ml collagenase IV (SIGMA) and DNase I 0.02 mg/ml (SIGMA) for 30 minutes at 37 °C. The digestion was neutralized with HBSS and the liver suspension was strained through a 70 μm nylon mesh (Corning) and centrifuged at 30 g at room temperature for 3 minutes. The supernatant was transferred to a new tube and centrifuged at 600 g at 4 °C for 5 minutes and the pellet, containing hepatocytes, was stored at -80 °C for downstream experiments. After centrifugation, the pellet was re-suspended in 36% HBSS-percoll and centrifuged at 800 g at 4°C for 20 minutes. The resultant pellet was incubated with ACK lysing buffer (Gibco) to lyse erythrocytes. For testing successful T cell depletion, blood was collected and incubated with ACK lysing buffer (Gibco) to lyse erythrocytes. One million of cells were resuspended in blocking solution (2% BSA in PBS), Fc receptors were blocked with Biolegend TruStain FcX PLUS anti-mouse CD16/32 antibody per manufacturer instructions. Pentamer staining (SIINFEKL) was carried out at room temperature protected from light for 15min. Cells were then stained with remaining antibodies on ice protected from light for 30 min. Fixation was done with the eBioscience Fixation/Permeabilization kit (Thermofisher) according to manufacturer´s instructions. Samples were acquired using an LSRFortessa flow cytometer (BD Biosciences) from the Tisch Cancer Institute Flow Cytometry Core. Doublets were excluded using height versus area dot plots and samples were additionally gated on viable leukocytes by DAPI exclusion. Data analysis was performed using FlowJo software (Tree Star). We detected the following antigens: MHCII (APC/Cy7, M5/114.15.2), CD169 (FITC, 3D6.112), F4/80 (APC, BM8), CD11c (PE-Cy7, N418), Ly6G (PB, 1A8), CD45 (BV510, 30-F11), CD11b (AF700, M1/70), Ly6C (PerCP-Cy5.5, HK1.4), NK1.1 (APC/Cy7, PK136), CD3 (FITC, eBio500A2), B220 (FITC, RA3-6B2), Gr-1 (FTIC, RB6-8C5), CD24 (PE-Cy7, M1/69), CD103 (PerCP-Cy5.5, 2E7), FoxP3 (PE-Cy7, FJK-16s), CD8 (PB, 53-6.7), Tcrb (PerCP-Cy5.5, H57-597), CD4 (biotin, GK1.5), streptavidin (BV650, nn), SIINFEKL (PE), and CD45 (AF-700, I3/2.3). All antibodies were purchased from BioLegend or eBioscience. To study splenocytes, we mechanically disaggregated spleens using two slides.

***Patient cohort and evaluation of treatment response***

Patients receiving nivolumab at ISMMS were eligible to be enrolled in the study if they had a confirmed histologic diagnosis of HCC and viable tumor tissue (either biopsy or archival sample) prior to the start of immunotherapy. Once local Institutional Review Board (IRB) approval was granted, written informed consent for tumor profiling was obtained from each patient on a retrospective protocol (IRB number 17-01728). Initial diagnosis of HCC was made following the clinical practice guidelines from the European Association for the Study of the Liver (8). All included patients presented an advanced (BCLC-C) or intermediate (BCLC-B) stage with prior progression to surgery and/or loco-regional therapies at the moment of immunotherapy initiation. Nivolumab was administered at a dose of 240 mg every 2 weeks and was continued until toxicity, progression, or death, according to the treating physician. Assessment of response was conducted at least 3 months after treatment initiation and performed by mRECIST criteria (9). Treatment response was defined as follows: CR (complete response: disappearance of any intratumor arterial enhancement in all target lesions); PR (partial response: at least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions); PD (progressive disease: an increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started); SD (stable disease: any cases not qualifying for either partial response or progressive disease). The electronic medical records were reviewed to extract information on patient’s gender, age, race, etiology, date of diagnosis, specimen location (liver, local recurrence, or extrahepatic metastasis), extent of disease, treatment history, type, number and dates of systemic therapy with radiographic response, date of progression, and the last date of follow-up or date of death.

***DNA extraction and detection of CTNNB1 mutations***

Prior to DNA extraction from FFPE (formalin-fixed paraffin-embedded) samples, all tissue sections were macrodissected to avoid contamination from non-cancerous tissue. Genomic DNA was isolated from 9 macrodissected 5 μm-thick FFPE sections using QIAamp DNA FFPE Tissue Kit (Qiagen). DNA quantity was assessed using nanodrop. Presence of hot-spot mutations in *CTNNB1* gene was assessed by polymerase chain reaction (PCR) and subsequent Sanger sequencing. PCR was performed in a volume of 25 µL reaction mixture containing 1.5 mmol/L MgCl2, 0.2 mmol/L of each deoxynucleoside triphosphate, 0.125 mmol/L of each primer, and 1 U of Platinum Taq DNA Polymerase (Invitrogen), and using the following protocol: 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 30 seconds, 60°C annealing for 30 seconds, and 72°C extension for 2 minutes, followed by a 10-minute final extension at 72°C. The primers used were 5’-GATTTGATGGAGTTGGACATGG-3’ (forward) and 5’-TGTTCTTGAGTGAAGGACTGAG-3’ (reverse). PCR products were then run on a 2% agarose gel and the band of interest was extracted using PureLink Quick Gel Extraction kit (Invitrogen). The DNA was submitted to Macrogen USA for standard Sanger sequencing.

**TABLES**

**Supplementary Table S1. Oncogenic signatures analyzed by GSEA in *MYC-luc;sg-p53* (n = 3) and *MYC-lucOS;sg-p53* (n = 3) murine HCCs.** NES, normalized enrichment score.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **NAME** | **SIZE** | **NES** | **NOM p-val** | **FDR q-val** |
| **Enriched in *MYC-lucOS;sg-p53*** |  |  |  |  |
| BCAT\_BILD\_ET\_AL\_DN | 42 | 1.4017792 | 0.10763209 | 1 |
| EIF4E\_DN | 93 | 1.392698 | 0 | 1 |
| PKCA\_DN.V1\_UP | 146 | 1.3392494 | 0 | 1 |
| PRC2\_SUZ12\_UP.V1\_DN | 159 | 1.3373437 | 0 | 1 |
| SRC\_UP.V1\_DN | 135 | 1.314339 | 0 | 1 |
| PRC2\_EZH2\_UP.V1\_DN | 171 | 1.3004566 | 0 | 1 |
| CYCLIN\_D1\_UP.V1\_DN | 168 | 1.2758791 | 0.108606555 | 1 |
| ESC\_V6.5\_UP\_EARLY.V1\_UP | 156 | 1.2605222 | 0 | 1 |
| PRC1\_BMI\_UP.V1\_DN | 159 | 1.2478946 | 0 | 1 |
| YAP1\_UP | 39 | 1.2333175 | 0.10966543 | 1 |
| BCAT\_GDS748\_DN | 36 | 1.2273605 | 0.10309278 | 1 |
| ATF2\_UP.V1\_DN | 167 | 1.2068361 | 0.106589146 | 1 |
| ATF2\_S\_UP.V1\_UP | 163 | 1.1988376 | 0 | 1 |
| SNF5\_DN.V1\_UP | 159 | 1.1929619 | 0.29411766 | 1 |
| IL2\_UP.V1\_DN | 157 | 1.181506 | 0.10557769 | 1 |
| JAK2\_DN.V1\_UP | 150 | 1.1734569 | 0.100603625 | 1 |
| RAF\_UP.V1\_DN | 172 | 1.1659428 | 0.10456274 | 1 |
| CSR\_LATE\_UP.V1\_DN | 141 | 1.1586678 | 0.2004008 | 1 |
| E2F3\_UP.V1\_UP | 161 | 1.1514105 | 0 | 1 |
| RB\_DN.V1\_UP | 127 | 1.1475664 | 0.31610337 | 1 |
| CTIP\_DN.V1\_DN | 113 | 1.1460432 | 0.1037182 | 1 |
| VEGF\_A\_UP.V1\_UP | 170 | 1.1454589 | 0.19573644 | 1 |
| RB\_P130\_DN.V1\_UP | 124 | 1.1440784 | 0.10076046 | 1 |
| DCA\_UP.V1\_DN | 151 | 1.1434256 | 0.20892495 | 1 |
| NOTCH\_DN.V1\_UP | 159 | 1.1419442 | 0.093023255 | 1 |
| SIRNA\_EIF4GI\_DN | 80 | 1.1409672 | 0.27592954 | 1 |
| TBK1.DF\_DN | 268 | 1.1384729 | 0 | 1 |
| EGFR\_UP.V1\_DN | 166 | 1.1378766 | 0.09631147 | 1 |
| PRC1\_BMI\_UP.V1\_UP | 149 | 1.135967 | 0.11111111 | 1 |
| NFE2L2.V2 | 357 | 1.1327188 | 0 | 0.98569196 |
| HOXA9\_DN.V1\_DN | 168 | 1.1245518 | 0 | 1 |
| ATM\_DN.V1\_DN | 126 | 1.1187239 | 0.21705426 | 1 |
| BRCA1\_DN.V1\_DN | 114 | 1.1179175 | 0.21325052 | 1 |
| RAPA\_EARLY\_UP.V1\_DN | 167 | 1.117071 | 0.09221312 | 0.98034066 |
| ATF2\_UP.V1\_UP | 171 | 1.1141201 | 0.31073445 | 0.9624811 |
| MEK\_UP.V1\_DN | 166 | 1.1134163 | 0 | 0.9424216 |
| P53\_DN.V2\_DN | 134 | 1.1122364 | 0.22007722 | 0.92009795 |
| JAK2\_DN.V1\_DN | 102 | 1.1079501 | 0.19959678 | 0.92482454 |
| LTE2\_UP.V1\_DN | 169 | 1.1070931 | 0.21714285 | 0.9055889 |
| GLI1\_UP.V1\_UP | 24 | 1.1065828 | 0.19547325 | 0.88422346 |
| BCAT.100\_UP.V1\_DN | 30 | 1.1011986 | 0.18832392 | 0.88810575 |
| RPS14\_DN.V1\_UP | 165 | 1.1011381 | 0.1891892 | 0.8681739 |
| KRAS.KIDNEY\_UP.V1\_DN | 115 | 1.1006219 | 0.1988417 | 0.8526014 |
| BMI1\_DN.V1\_DN | 125 | 1.0992688 | 0.10020877 | 0.84051496 |
| IL15\_UP.V1\_DN | 150 | 1.0971199 | 0.30350193 | 0.8311692 |
| MTOR\_UP.V1\_UP | 155 | 1.087465 | 0.30753565 | 0.8620225 |
| P53\_DN.V1\_DN | 174 | 1.0863683 | 0.2857143 | 0.84970194 |
| GCNP\_SHH\_UP\_LATE.V1\_UP | 168 | 1.070296 | 0.09343936 | 0.9172781 |
| PRC2\_EED\_UP.V1\_DN | 175 | 1.058468 | 0.33204633 | 0.9546363 |
| WNT\_UP.V1\_UP | 165 | 1.0490048 | 0.21115538 | 0.97910887 |
| BCAT.100\_UP.V1\_UP | 44 | 1.0344719 | 0.530888 | 1 |
| LTE2\_UP.V1\_UP | 170 | 1.031249 | 0.31320754 | 1 |
| AKT\_UP.V1\_UP | 159 | 1.0312377 | 0.42857143 | 1 |
| KRAS.AMP.LUNG\_UP.V1\_UP | 109 | 1.0287954 | 0.41117764 | 1 |
| KRAS.PROSTATE\_UP.V1\_DN | 117 | 1.0287348 | 0.41431263 | 1 |
| RELA\_DN.V1\_DN | 121 | 1.0249478 | 0.3806706 | 1 |
| STK33\_SKM\_UP | 231 | 1.0247042 | 0.30857143 | 1 |
| PDGF\_ERK\_DN.V1\_UP | 129 | 1.0244198 | 0.2804642 | 0.9887706 |
| BCAT\_GDS748\_UP | 43 | 1.0215358 | 0.3976834 | 0.9907023 |
| MYC\_UP.V1\_DN | 126 | 1.0210608 | 0.4165103 | 0.9779101 |
| TBK1.DN.48HRS\_UP | 48 | 1.0205667 | 0.50884086 | 0.9627148 |
| GCNP\_SHH\_UP\_EARLY.V1\_UP | 158 | 1.0149257 | 0.48393574 | 0.9706773 |
| MEL18\_DN.V1\_DN | 133 | 1.0125246 | 0.41984734 | 0.95792234 |
| JNK\_DN.V1\_UP | 162 | 1.0108569 | 0.4950884 | 0.9634183 |
| PRC2\_EED\_UP.V1\_UP | 171 | 1.0001575 | 0.39688715 | 0.99614275 |
| ESC\_J1\_UP\_EARLY.V1\_UP | 160 | 0.99411565 | 0.5283401 | 1 |
| NRL\_DN.V1\_DN | 124 | 0.99041003 | 0.51132077 | 0.9995861 |
| KRAS.PROSTATE\_UP.V1\_UP | 117 | 0.9871208 | 0.5096525 | 1 |
| VEGF\_A\_UP.V1\_DN | 179 | 0.9847527 | 0.51132077 | 1 |
| CSR\_EARLY\_UP.V1\_DN | 121 | 0.98436934 | 0.5259117 | 0.98721075 |
| KRAS.AMP.LUNG\_UP.V1\_DN | 118 | 0.98219126 | 0.51039696 | 0.9801646 |
| KRAS.DF.V1\_UP | 175 | 0.98135054 | 0.42857143 | 0.96989334 |
| KRAS.DF.V1\_DN | 165 | 0.9811951 | 0.6040892 | 0.95730627 |
| IL21\_UP.V1\_DN | 149 | 0.9795952 | 0.49325627 | 0.95468265 |
| STK33\_NOMO\_DN | 230 | 0.9769808 | 0.4730539 | 0.94997543 |
| RPS14\_DN.V1\_DN | 169 | 0.9753664 | 0.38381743 | 0.9421662 |
| AKT\_UP.V1\_DN | 174 | 0.97350436 | 0.50760454 | 0.9385424 |
| JNK\_DN.V1\_DN | 163 | 0.9714143 | 0.5239923 | 0.9356709 |
| ALK\_DN.V1\_DN | 119 | 0.9694547 | 0.620229 | 0.92877084 |
| STK33\_NOMO\_UP | 259 | 0.96674323 | 0.39688715 | 0.9234195 |
| CTIP\_DN.V1\_UP | 109 | 0.9641593 | 0.38899803 | 0.92058593 |
| KRAS.LUNG.BREAST\_UP.V1\_DN | 122 | 0.96399724 | 0.32239383 | 0.9099816 |
| STK33\_UP | 249 | 0.95148385 | 0.39688715 | 0.9552862 |
| BRCA1\_DN.V1\_UP | 112 | 0.94709015 | 0.4990138 | 0.96054935 |
| BMI1\_DN\_MEL18\_DN.V1\_DN | 127 | 0.94233626 | 0.6223507 | 0.96787256 |
| PTEN\_DN.V1\_DN | 141 | 0.94142264 | 0.5931864 | 0.9598608 |
| AKT\_UP\_MTOR\_DN.V1\_UP | 170 | 0.9307445 | 0.41984734 | 0.9953192 |
| PDGF\_UP.V1\_UP | 132 | 0.92620015 | 0.41965973 | 1 |
| ERB2\_UP.V1\_DN | 167 | 0.9226707 | 0.6052104 | 1 |
| CYCLIN\_D1\_KE\_.V1\_UP | 179 | 0.92098385 | 0.68761903 | 1 |
| ESC\_J1\_UP\_LATE.V1\_UP | 179 | 0.92096597 | 0.51224107 | 0.99635804 |
| MTOR\_UP.V1\_DN | 167 | 0.91216433 | 0.6164122 | 1 |
| PKCA\_DN.V1\_DN | 152 | 0.91129607 | 0.6949807 | 1 |
| LEF1\_UP.V1\_DN | 166 | 0.9078109 | 0.51039696 | 1 |
| NOTCH\_DN.V1\_DN | 145 | 0.90701383 | 0.60076046 | 0.99238926 |
| RB\_P107\_DN.V1\_UP | 131 | 0.90561086 | 0.624498 | 0.98446447 |
| ATF2\_S\_UP.V1\_DN | 165 | 0.90371376 | 0.7992278 | 0.98133415 |
| CAHOY\_ASTROCYTIC | 96 | 0.9035401 | 0.710728 | 0.97183996 |
| PDGF\_UP.V1\_DN | 103 | 0.89992595 | 0.5929368 | 0.97926885 |
| RAPA\_EARLY\_UP.V1\_UP | 160 | 0.8990163 | 0.81474483 | 0.9729798 |
| TGFB\_UP.V1\_DN | 156 | 0.8953074 | 0.6969112 | 0.98398226 |
| MTOR\_UP.N4.V1\_DN | 139 | 0.8866626 | 0.8125 | 0.9946288 |
| PIGF\_UP.V1\_DN | 165 | 0.8859802 | 0.71705425 | 0.98546726 |
| CRX\_DN.V1\_UP | 130 | 0.88224345 | 0.9028571 | 0.9840761 |
| CAHOY\_NEURONAL | 92 | 0.87803376 | 0.5192308 | 0.9897636 |
| CRX\_NRL\_DN.V1\_UP | 134 | 0.8763141 | 0.6177024 | 0.9855067 |
| PTEN\_DN.V2\_UP | 111 | 0.8734614 | 0.5096525 | 0.9912974 |
| DCA\_UP.V1\_UP | 141 | 0.87205136 | 0.90267175 | 0.98816633 |
| KRAS.LUNG\_UP.V1\_UP | 109 | 0.8691194 | 0.6191406 | 0.98817736 |
| GCNP\_SHH\_UP\_EARLY.V1\_DN | 155 | 0.86826855 | 0.80080485 | 0.98230976 |
| CAHOY\_OLIGODENDROCUTIC | 88 | 0.86803854 | 0.79844964 | 0.9743929 |
| PTEN\_DN.V1\_UP | 158 | 0.8656979 | 0.6223507 | 0.97012717 |
| ATM\_DN.V1\_UP | 126 | 0.8610628 | 0.6970874 | 0.9742069 |
| KRAS.600.LUNG.BREAST\_UP.V1\_DN | 233 | 0.8429205 | 0.9015444 | 1 |
| P53\_DN.V1\_UP | 173 | 0.84079987 | 0.7936803 | 1 |
| ESC\_V6.5\_UP\_LATE.V1\_UP | 173 | 0.8264602 | 0.6967985 | 1 |
| E2F1\_UP.V1\_DN | 175 | 0.8210408 | 0.7992278 | 1 |
| KRAS.BREAST\_UP.V1\_UP | 122 | 0.81700385 | 0.8146718 | 1 |
| KRAS.600\_UP.V1\_DN | 241 | 0.81512326 | 0.7953668 | 1 |
| CYCLIN\_D1\_KE\_.V1\_DN | 172 | 0.813803 | 0.8986083 | 1 |
| RAF\_UP.V1\_UP | 177 | 0.81208795 | 0.80190474 | 1 |
| KRAS.LUNG\_UP.V1\_DN | 115 | 0.80594957 | 0.90304184 | 1 |
| RELA\_DN.V1\_UP | 135 | 0.8027304 | 0.90192306 | 1 |
| RB\_DN.V1\_DN | 117 | 0.7977357 | 0.6065259 | 1 |
| KRAS.BREAST\_UP.V1\_DN | 118 | 0.7954092 | 0.7953668 | 1 |
| WNT\_UP.V1\_DN | 159 | 0.7828065 | 0.9015444 | 1 |
| E2F1\_UP.V1\_UP | 184 | 0.77684474 | 0.67196816 | 1 |
| PRC2\_EZH2\_UP.V1\_UP | 168 | 0.76569974 | 0.70689654 | 1 |
| PRC2\_SUZ12\_UP.V1\_UP | 151 | 0.7644362 | 0.8084291 | 1 |
| IL21\_UP.V1\_UP | 163 | 0.76069796 | 0.9017341 | 1 |
| RB\_P130\_DN.V1\_DN | 124 | 0.7536666 | 0.7808765 | 1 |
| IL2\_UP.V1\_UP | 166 | 0.7495987 | 0.8084291 | 1 |
| SNF5\_DN.V1\_DN | 156 | 0.7461306 | 0.90304184 | 1 |
| CAMP\_UP.V1\_DN | 183 | 0.74465007 | 0.9043152 | 1 |
| SRC\_UP.V1\_UP | 141 | 0.7425978 | 0.90267175 | 1 |
| E2F3\_UP.V1\_DN | 115 | 0.7415406 | 0.90192306 | 1 |
| KRAS.50\_UP.V1\_UP | 45 | 0.74136823 | 0.90304184 | 1 |
| NRL\_DN.V1\_UP | 129 | 0.7380713 | 0.90077823 | 1 |
| TBK1.DF\_UP | 263 | 0.7378989 | 0.9035917 | 1 |
| CSR\_LATE\_UP.V1\_UP | 152 | 0.7298146 | 0.7248996 | 1 |
| ERB2\_UP.V1\_UP | 171 | 0.7248244 | 0.9043152 | 1 |
| CRX\_DN.V1\_DN | 130 | 0.72226053 | 0.90304184 | 1 |
| KRAS.LUNG.BREAST\_UP.V1\_UP | 119 | 0.7166102 | 0.90304184 | 1 |
| IL15\_UP.V1\_UP | 164 | 0.6999477 | 0.90304184 | 1 |
| KRAS.600.LUNG.BREAST\_UP.V1\_UP | 232 | 0.6921234 | 0.90267175 | 1 |
| P53\_DN.V2\_UP | 125 | 0.6916389 | 0.90077823 | 1 |
| PTEN\_DN.V2\_DN | 122 | 0.68751204 | 0.9052045 | 1 |
| CAHOY\_ASTROGLIAL | 95 | 0.6793365 | 0.9015444 | 1 |
| TGFB\_UP.V1\_UP | 173 | 0.6574584 | 0.9035917 | 1 |
| BMI1\_DN.V1\_UP | 138 | 0.6522988 | 0.8022814 | 1 |
| EGFR\_UP.V1\_UP | 173 | 0.6438653 | 0.9035917 | 0.9983661 |
| MEK\_UP.V1\_UP | 178 | 0.58459365 | 0.7917448 | 1 |
| CORDENONSI\_YAP\_CONSERVED\_SIGNATURE | 57 | 0.5716204 | 0.90229887 | 0.9993452 |
| KRAS.300\_UP.V1\_UP | 123 | 0.569192 | 0.90229887 | 0.99318665 |
| **Enriched in *MYC-luc;sg-p53*** |  |  |  |  |
| EIF4E\_UP | 83 | -1.4387141 | 0 | 0.35398757 |
| MYC\_UP.V1\_UP | 151 | -1.3524488 | 0 | 0.6023484 |
| CAMP\_UP.V1\_UP | 178 | -1.2764359 | 0.10114504 | 0.7650757 |
| AKT\_UP\_MTOR\_DN.V1\_DN | 175 | -1.0420941 | 0.3863179 | 1 |
| SINGH\_KRAS\_DEPENDENCY\_SIGNATURE\_ | 17 | -1.0410426 | 0.57873213 | 1 |
| KRAS.50\_UP.V1\_DN | 37 | -0.9987784 | 0.4990099 | 1 |
| TBK1.DN.48HRS\_DN | 49 | -0.9802907 | 0.6046065 | 1 |
| ESC\_J1\_UP\_LATE.V1\_DN | 174 | -0.9654259 | 0.48856547 | 1 |
| STK33\_DN | 237 | -0.96170026 | 0.3960396 | 1 |
| CRX\_NRL\_DN.V1\_DN | 118 | -0.9090561 | 0.6701245 | 1 |
| GCNP\_SHH\_UP\_LATE.V1\_DN | 167 | -0.9050568 | 0.7886179 | 1 |
| RB\_P107\_DN.V1\_DN | 118 | -0.90208894 | 0.8110687 | 1 |
| YAP1\_DN | 36 | -0.8940934 | 0.78904665 | 1 |
| SIRNA\_EIF4GI\_UP | 84 | -0.89096653 | 0.6069364 | 1 |
| CYCLIN\_D1\_UP.V1\_UP | 171 | -0.8771659 | 0.796334 | 1 |
| CSR\_EARLY\_UP.V1\_UP | 150 | -0.85643077 | 0.68541664 | 1 |
| MTOR\_UP.N4.V1\_UP | 183 | -0.8251165 | 0.90267175 | 1 |
| STK33\_SKM\_DN | 228 | -0.81998175 | 0.89375 | 1 |
| ESC\_V6.5\_UP\_LATE.V1\_DN | 168 | -0.8199236 | 0.7815126 | 1 |
| HINATA\_NFKB\_IMMU\_INF | 15 | -0.8046907 | 0.68958336 | 1 |
| KRAS.300\_UP.V1\_DN | 120 | -0.80333334 | 0.7908903 | 1 |
| PDGF\_ERK\_DN.V1\_DN | 137 | -0.8009526 | 0.78333336 | 1 |
| PIGF\_UP.V1\_UP | 174 | -0.7875572 | 0.79880476 | 1 |
| MEL18\_DN.V1\_UP | 133 | -0.75563574 | 0.6785714 | 1 |
| ALK\_DN.V1\_UP | 117 | -0.75266373 | 0.7881874 | 1 |
| ESC\_J1\_UP\_EARLY.V1\_DN | 162 | -0.7504191 | 0.89440995 | 1 |
| LEF1\_UP.V1\_UP | 176 | -0.7470636 | 0.6785714 | 1 |
| KRAS.KIDNEY\_UP.V1\_UP | 131 | -0.73605025 | 0.89484537 | 1 |
| HOXA9\_DN.V1\_UP | 164 | -0.7205349 | 0.8954918 | 1 |
| BMI1\_DN\_MEL18\_DN.V1\_UP | 135 | -0.69712275 | 0.78333336 | 1 |
| GLI1\_UP.V1\_DN | 24 | -0.6916324 | 0.89759034 | 1 |
| KRAS.600\_UP.V1\_UP | 240 | -0.66240746 | 0.8952772 | 1 |
| ESC\_V6.5\_UP\_EARLY.V1\_DN | 163 | -0.63960505 | 0.7815126 | 1 |
| BCAT\_BILD\_ET\_AL\_UP | 40 | -0.6081982 | 0.89285713 | 0.98957974 |

**Supplementary Table S2. Gene sets analyzed by GSEA in murine and human tumors.** NES, normalized enrichment score.

**NAME NES NOM p-val Group1 Group2 Species**

*CTNNB1-*mutant human HCCs 1.61 0.03 *MYC-luc;sg-p53* *MYC;CTNNB1* Mouse

n = 3 n = 12

*CTNNB1-*mutant human HCCs 2.27 < 0.001 *CTNNB1-WT CTNNB1-mut* Human

n = 263 n = 97

**Supplementary Table S3. Immune cell-related genes in murine HCCs.** A, *MYC-luc;sg-p53* (n = 3); *B,* *MYC-lucOS;sg-p53* (n = 3); C, *low-Axin2 MYC-lucOS;sg-p53* (n = 4); D, *MYC-luc;CTNNB1* (n = 7); E, *MYC-lucOS;CTNNB1* (n = 5). The p values indicate Mann-Whitney test.

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**Supplementary Table S4. Immune cell-related genes in human HCCs, TCGA**. A, *CTNNB1* wild-type (WT; n = 263); B, *CTNNB1*-mutant (mut; n = 97). C, low *CTNNB1*-mutant HCC signature (CTNNB1 low, n = 120, first tertile); D, intermediate (CTNNB1 inter, n = 120, second tertile); E, high (CTNNB1 high, n = 120, third tertile). The p values indicate Mann-Whitney test.

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**Supplementary Table S5. List of murine chemokines differentially expressed in *MYC-luc;sg-p53* tumors (n = 3) and β-catenin-driven tumors (*MYC-lucOS;CTNNB1* and *MYC-luc;CTNNB1,* n = 12*).***

|  |  |
| --- | --- |
| **Gene symbol** | **p value** |
| Ccl1 | 0.6374 |
| Ccl11 | 0.3648 |
| Ccl12 | 0.3912 |
| Ccl17 | 0.044 |
| Ccl19 | > 0.9999 |
| Ccl2 | 0.4484 |
| Ccl20 | 0.0242 |
| Ccl21b | > 0.9999 |
| Ccl22 | 0.233 |
| Ccl24 | 0.0879 |
| Ccl25 | 0.5363 |
| Ccl26 | > 0.9999 |
| Ccl27a | 0.6505 |
| Ccl28 | 0.0484 |
| Ccl3 | 0.8396 |
| Ccl4 | 0.2945 |
| Ccl5 | 0.0308 |
| Ccl6 | 0.3165 |
| Ccl7 | 0.5363 |
| Ccl8 | 0.3165 |
| Ccl9 | 0.2945 |
| Cxcl1 | 0.0044 |
| Cxcl10 | 0.0088 |
| Cxcl11 | 0.7516 |
| Cxcl12 | 0.1802 |
| Cxcl13 | 0.4484 |
| Cxcl14 | 0.4703 |
| Cxcl15 | 0.1758 |
| Cxcl16 | 0.9451 |
| Cxcl17 | > 0.9999 |
| Cxcl2 | 0.1363 |
| Cxcl3 | 0.5077 |
| Cxcl5 | 0.9473 |
| Cxcl9 | 0.2022 |

**Supplementary Table S6. List of human chemokines differentially expressed in *CTNNB1-*wild-type (n = 263) or mutant tumors (n = 97).**

|  |  |
| --- | --- |
| **Gene symbol** | **p value** |
| CCL14 | 0.333 |
| CCL14-CCL15 | 0.5216 |
| CCL15 | 0.0602 |
| CCL16 | < 0.0001 |
| CCL18 | 0.7953 |
| CCL19 | < 0.0001 |
| CCL2 | < 0.0001 |
| CCL20 | < 0.0001 |
| CCL21 | < 0.0001 |
| CCL22 | < 0.0001 |
| CCL28 | 0.0003 |
| CCL3 | 0.8103 |
| CCL3L1 | 0.1293 |
| CCL4 | 0.4075 |
| CCL4L2 | 0.2389 |
| CCL5 | 0.0035 |
| CXCL1 | < 0.0001 |
| CXCL10 | 0.7102 |
| CXCL11 | 0.1966 |
| CXCL12 | 0.0016 |
| CXCL14 | 0.0012 |
| CXCL16 | < 0.0001 |
| CXCL2 | 0.05 |
| CXCL3 | < 0.0001 |
| CXCL9 | 0.3877 |

**REFERENCES**

1. Huang CH, Lujambio A, Zuber J, Tschaharganeh DF, Doran MG, Evans MJ*, et al.* CDK9-mediated transcription elongation is required for MYC addiction in hepatocellular carcinoma. Genes Dev **2014**;28(16):1800-14.