Supplementary Materials

Dyrk2 gene transfer suppresses hepatocarcinogenesis by promoting the degradation of Myc and Hras

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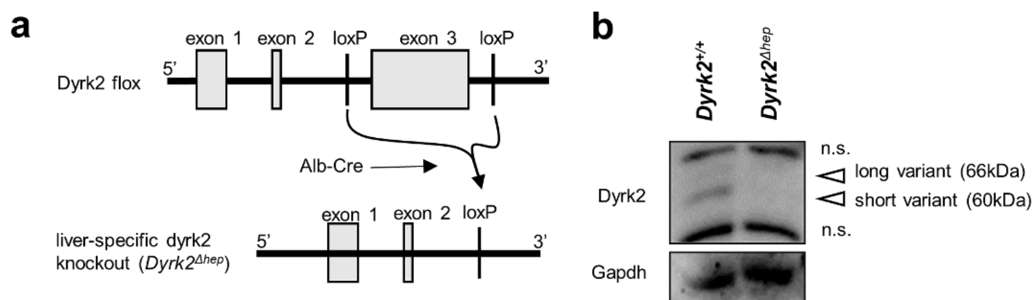

Fig. S1. Liver-specific Dyrk2-knockout mice (*Dyrk2$^{\Delta hep}$*) were generated. (a) Schematic representation to generate *Dyrk2$^{\Delta hep}$* mating Dyrk2 flox mice with Alb-cre mice (*Dyrk2$^{+/+}$*). (b) Western immunoblotting of Dyrk2 (arrowhead) in *Dyrk$^{+/+}$* and *Dyrk2$^{\Delta hep}$* livers. Non-specific bands were represented as “n.s.”. Gapdh was used as an internal control.

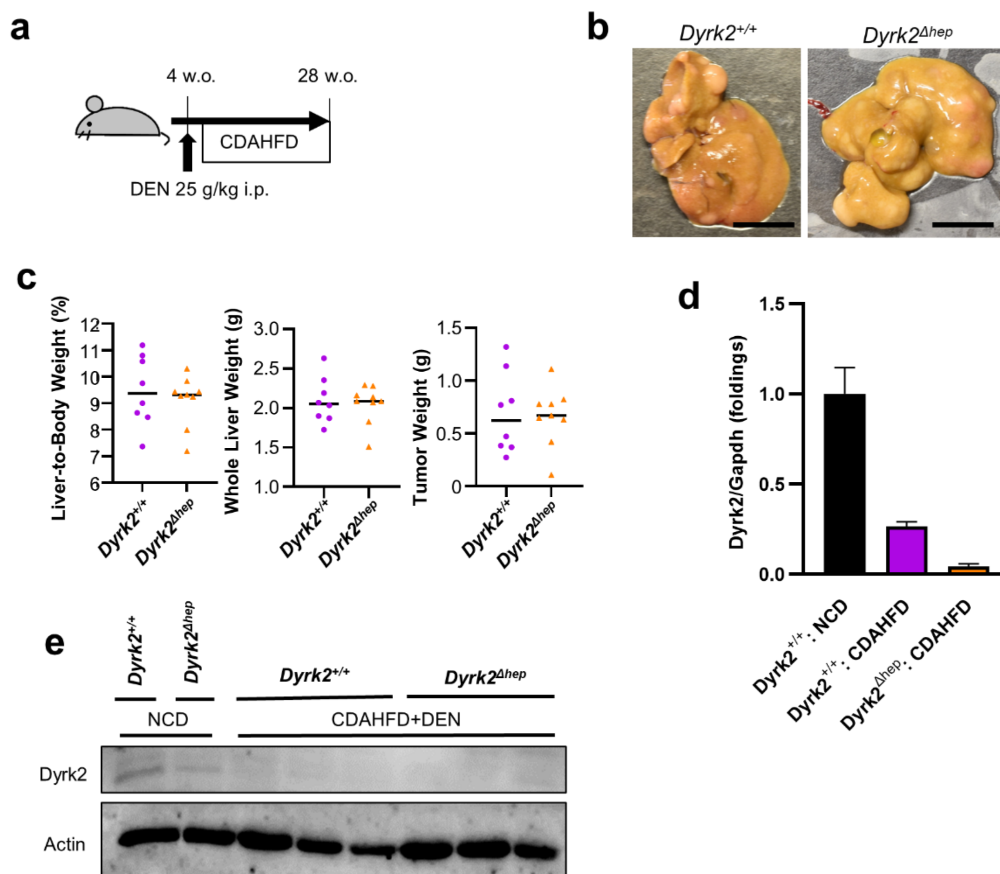

Fig. S2. Feeding CDAHFD inhibited the expression of Dyrk2 in the liver. (a-d) NASH-like hepato-carcinogenetic model for *Dyrk2*$^{+/+}$ (n = 8) and *Dyrk2*$^{\Delta hep}$ (n = 9), injecting with DEN intraperitoneally at 4 weeks old and feeding CDAHFD until 28 weeks old. (a) Schematic representation of the protocol. (b) Gross appearances of livers (scalebar, 1 cm). (c) The graphs of liver-to-body weight percentages, whole liver weights, and tumor weights (There was no significant difference). (d) qRT-PCR analysis of relative *Dyrk2* mRNA expression in *Dyrk2*$^{+/+}$ and *Dyrk2*$^{\Delta hep}$ using CDAHFD and DEN at 28 weeks old (n = 4-5 in each group). (e) Western immunoblotting of Dyrk2 in livers of *Dyrk2*$^{+/+}$ or *Dyrk2*$^{\Delta hep}$ using NCD, or CDAHFD and DEN. Actin (pan) was used as an internal control.

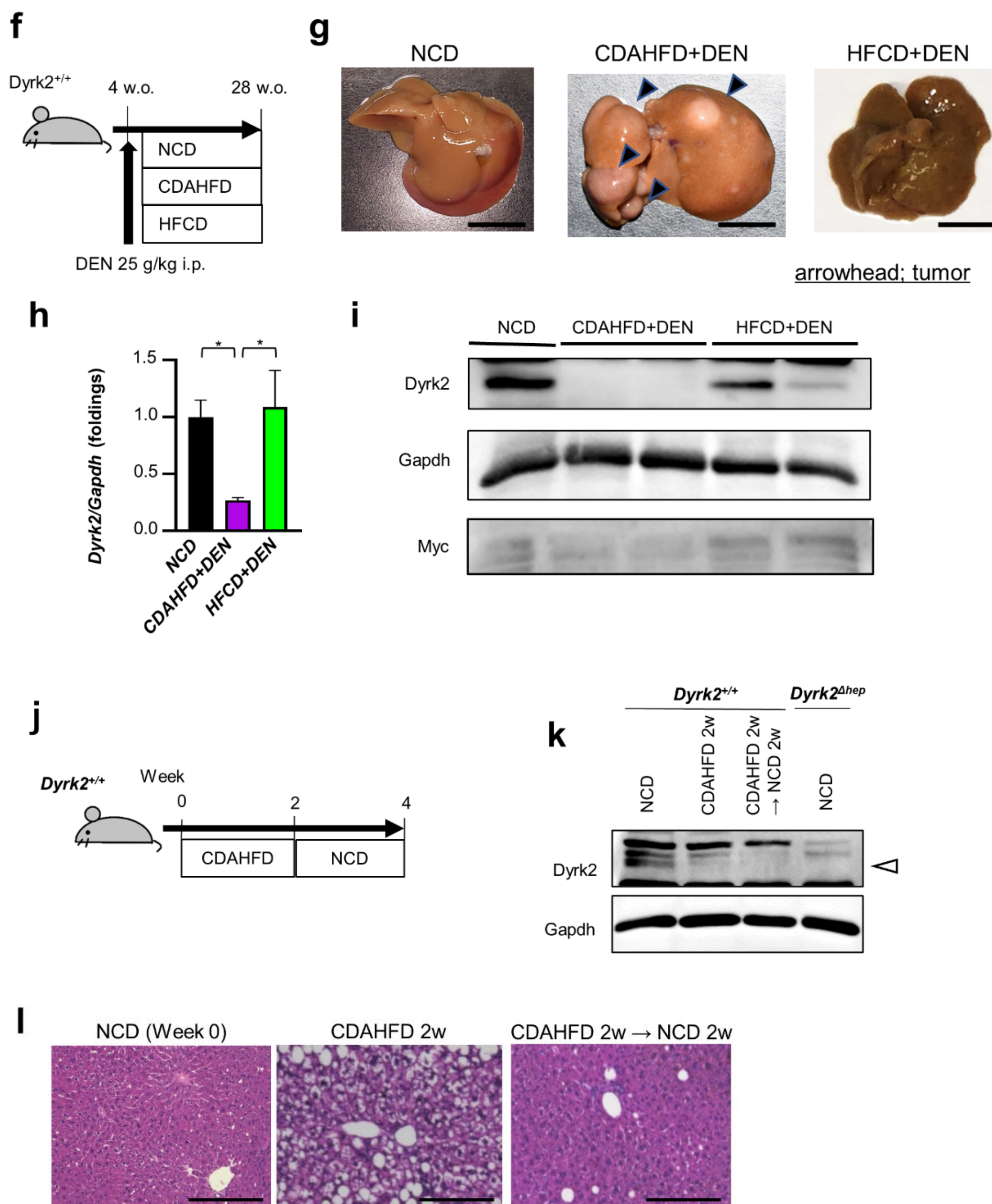

Fig. S2 continued

A comparison of Dyrk2 expression in *Dyrk2*$^{+/+}$ mice treated with DEN and fed either normal diet (NCD), CDAHFD or HFCD for 24 weeks. (f) Schematic representation of protocol. (g) Gross appearances of livers (arrowhead; tumors). Development of liver cancer was only observed in the DEN+CDAHFD group and not in the HFCD or NCD groups (h) qRT-PCR analysis of relative *Dyrk2* mRNA expression. (i) Western immunoblotting of Dyrk2 in livers. (j-k) *Dyrk2*$^{+/+}$ was fed CDAHFD for 2 weeks without DEN and then fed NCD. (j) Schematic representation of protocol. (k) H&E staining of livers of mice fed NCD, CDAHFD for 2 weeks, and mice fed CDAHFD for 2 weeks, and then fed NCD for 2 weeks. (scale bar; 100 μm). Livers of mice fed CDAHFD for 2 weeks appeared inflammatory cell infiltration and fat droplet

formation while those of mice fed NCD at 2 weeks after CDAHFD almost improved. (I) Western immunoblotting of Dyrk2 (arrowhead). Gapdh was used as an internal control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

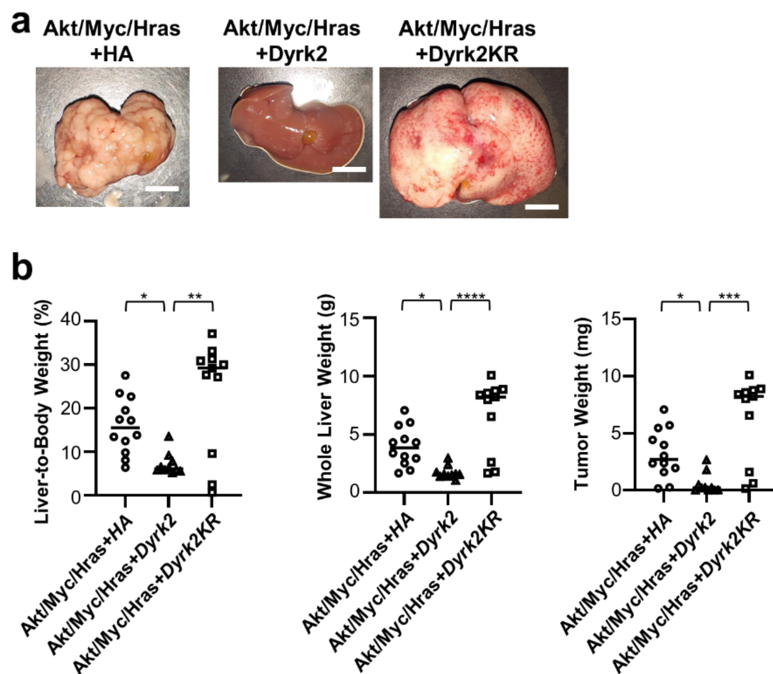

Fig. S3. Forced expression of Dyrk2 suppressed carcinogenesis in *Dyrk2*$^{+/+}$. Liver auto-carcinogenic model for *Dyrk2*$^{+/+}$, injected with the HA- (n = 12), Dyrk2- (n = 10), or Dyrk2KR-expressing plasmid (n = 11) in addition to Sleeping Beauty transposase- and 3 oncogenes-expressing plasmids by HTVi. (a) Gross appearances of livers (scale bar, 1 cm). (b) Liver-to-body weight percentages, whole liver weights, and tumor weights about each group 2 weeks after HTVi. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

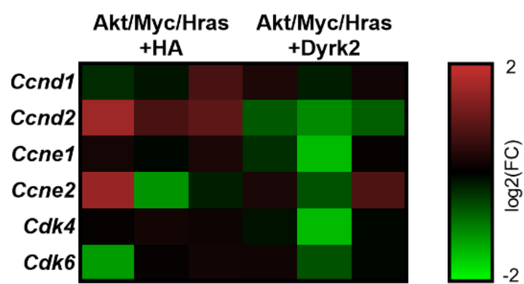

Fig. S4. The heatmap of genes related to cell cycle. Microarray analysis of HA- and Dyrk2-expressing tumors in Dyrk2$^{\Delta hep}$ (n=3 in each group).

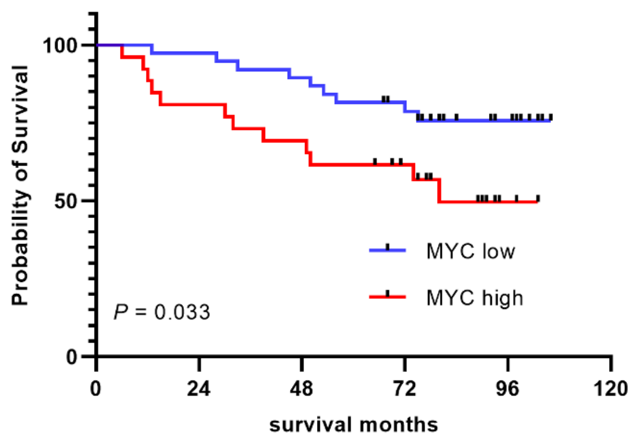

Fig. S5. Overall survival of HCC patients categorized with MYC expression.

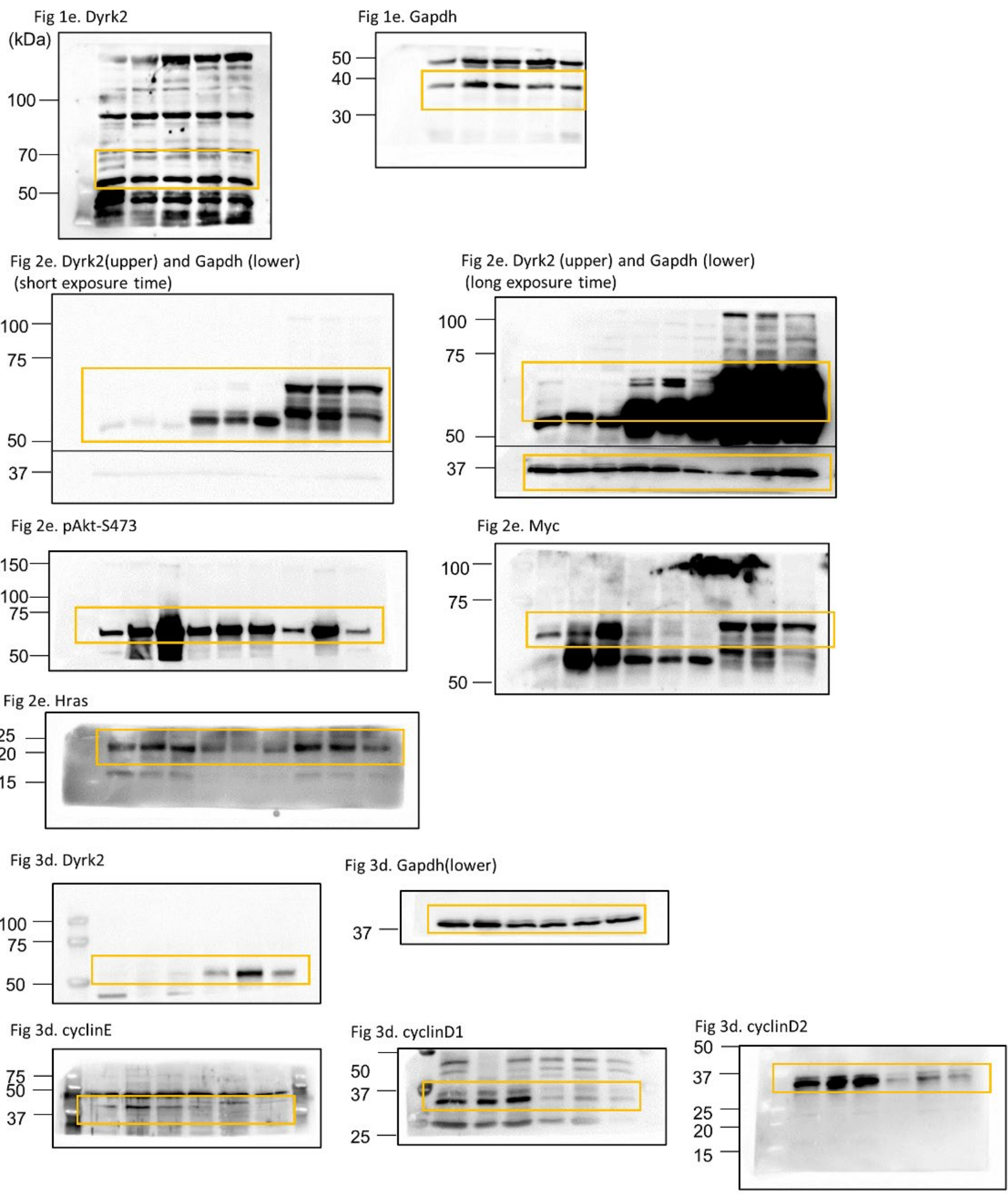

Fig. S6. Images of membranes for Western immunoblotting. The rectangles mean used by other Figures in this report, and the line in a membrane indicates that the membrane has been sliced.

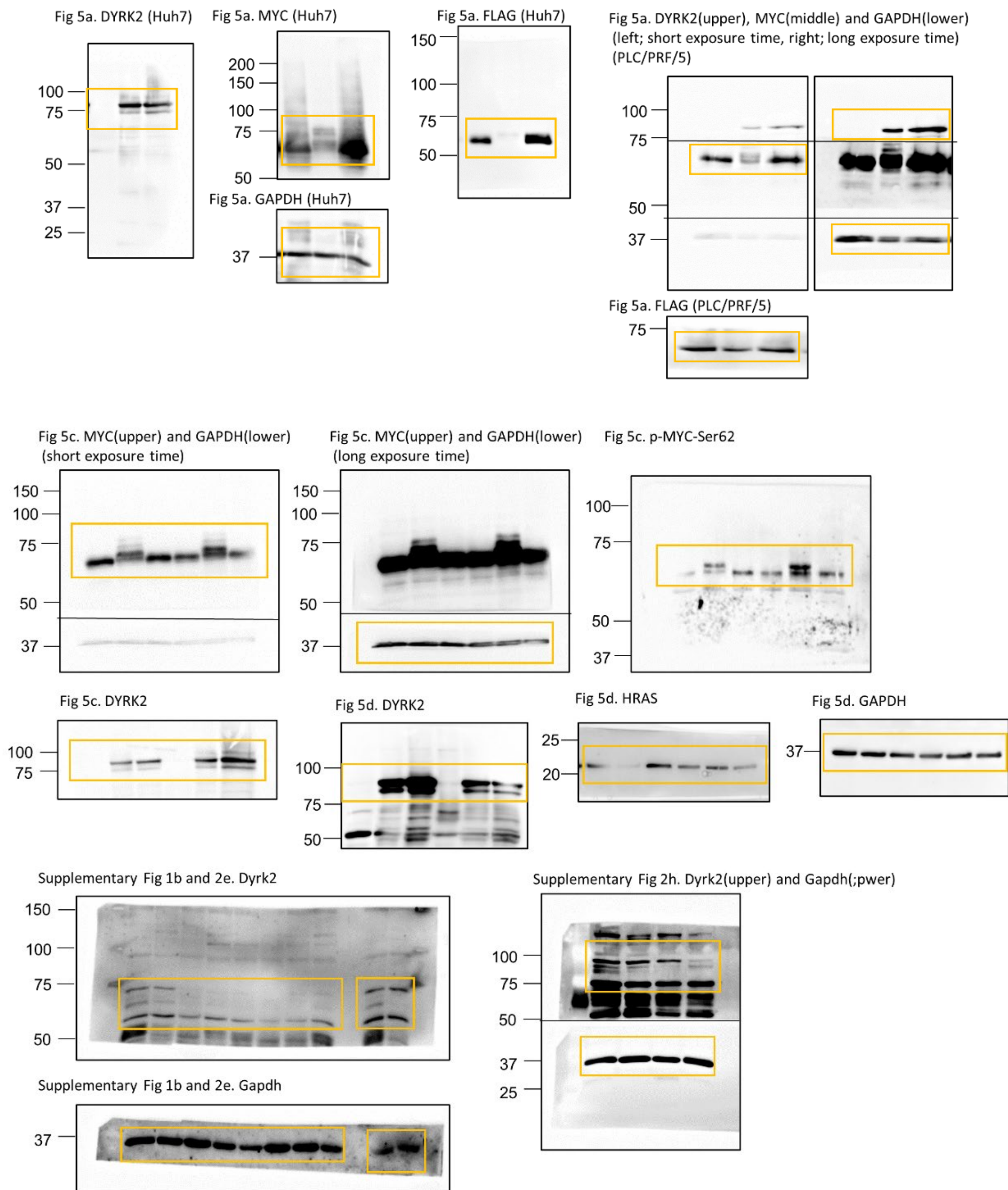

Fig. S6 continued.

Table S1. Clinicopathological Characteristics of DYRK2^{high}+MYC^{low} HCC and DYRK2^{low}+MYC^{high} HCC Patients

	DYRK2^{high}+MYC^{low}	**DYRK2^{low}+MYC^{high}**	*P* value
	n = 20	n = 19	
Age (years)	63 (56 – 68)	66 (64 –75)	0.115
Gender			
male/female	17/3	16/3	1.000
Etiology			
virus/NBNC	13/7	13/6	1.000
Liver cirrhosis			
yes/no	6/14	12/7	0.056
Histological grade			
well or moderately/poorly	20/0	16/3	0.106
Tumor size			
<5cm/≥5cm	15/5	16/3	0.695
Multinodular			
yes/no	15/5	14/5	1.000
Vascular invasion			
yes/no	1/19	2/17	0.605
Extrahepatic metastasis			
yes/no	0/20	1/18	0.487
Tumor marker			
AFP			
<20ng/mL/≥20 ng/mL	14/6	13/6	1.000
DCP			
<40 mAU/mL/≥40mAU/mL	8/12	9/10	0.751
HCC staging			
UICC stage			
I or II/III or IV	19/1	16/3	0.342

Data are shown as median (interquartile range) or number.

AFP, α-fetoprotein; DCP, Des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; NBNC, non-B non-C; UICC, Union for International Cancer Control.

Table S2. List of qRT-PCR primer sequences

gene	species	primer sequence
GAPDH (*Gapdh*)	human/mouse	F: 5'-TCAAGGCTGAGAACGGGAAG-3' R: 5'-ATGGTGGTGAAGACGCCAGT-3'
DYRK2	human	F: 5'-GGGGAGAAAACGTCAGTGAA-3' R: 5'-TCTGCGCCAAATTAGTCCTC-3'
Dyrk2	Mouse	F: 5'-CTACCACTACAGCCCACACG-3' R: 5'-TCTGTCCGTGGCTGTTGA-3'
AKT1 (*Akt1*)	human/mouse	F: 5'-TGGACTACCTGCACTCGGAGAA-3' R: 5'-GTGCCGCAAAAGGTCTTCATAG-3'
MYC (*Myc*)	human/mouse	F: 5'-TCAAGAGGCGAACACACAAC-3' R: 5'-GGCCTTTTCATTGTTTTCCA-3'
HRAS (*Hras*)	human/mouse	F: 5'-GACAGAATACAAGCTGGTGGT-3' R: 5'-GGCACGTCTCCCCATCAATG-3'
Slc2a1	Mouse	F: 5'-ATGGATCCCAGCAGCAAG-3' R: 5'-CCAGTGTTATAGCCGAACTGC-3'
Hk2	Mouse	F: 5'-TGATCGCCTGCTTATTCACGG-3' R: 5'-AACCGCCTAGAAATCTCCAGA-3'
Srebf1	Mouse	F: 5'-CAGGAGAACCTGACCCTACG-3' R: 5'-TCATGCCCTCCATAGACACA-3'
Scd1	Mouse	F: 5'-CATTCAATCCCGGGAGAATA-3' R: 5'-TAGTCGAAGGGGAAGGTGTG-3'

Supplementary material and methods

Immunohistochemistry

Mice tissues were fixed with phosphate-buffered 4% paraformaldehyde (Nacalai Tesque Inc., Kyoto, Japan) overnight, dehydrated by VIP 5 Jr (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and embedded in paraffin. The embedded sample was sliced into 4 μm thick and dried up on an extender. After deparaffinized and hydrated, sections were stained with hematoxylin and eosin (H&E staining). Or the hydrated sections were performed antigen retrieval with HistoOne-VT (Nacalai Tesque Inc., Kyoto, Japan) or citrate buffer pH 6 at 105°C for 15 min in an autoclave for immunohistochemistry. The Sections were blocked in 0.5% bovine serum albumin at room temperature for 60 min. The sections were incubated with the following primary antibodies overnight at 4°C; anti-ki-67 (Nichirei Co., Tokyo, Japan) and above antibodies used in immunoblotting. After blocking internal HRP with 3% H_2O_2 and washed with PBS three times, the sections were incubated with ImmPRESS Detection Kit (Vector Laboratories, Newark, USA) at room temperature for 1 hour and detected with ImmPACT DAB EqV Peroxidase Substrate (Vector Laboratories, Newark, USA) for 1-3 min. For immunofluorescence staining, the sections after blocking internal HRP and washing were incubated with Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology) at room temperature for 1 hour. Images were collected with a microscope BZ-X800 (Keyence Co., Tokyo, Japan). ImageJ Fiji (version 1.53) was used to count total and stained-positive cells.

Sphere formation assays

For sphere formation assays, 1×10^3 cells were seeded into each well of 24-well coated with ultra-low attachment surfaces (Corning, Lowell, MA, USA) and cultured with serum-free DMEM. After 1 week, the number of spheres (>200 μm for Huh7 / >100μm for PLC/PRF/5)

was counted.

Migration assays

For migration assays, 2 x 10^5 cells were seeded into each well of a 24-well plate. Cells were transfected with pFLAG-MYC and either pEGFP, pEGFP-DYRK2 or pEGFP-DYRK2^{K239R} overnight, and culture media was replaced. The epithelial monolayer sheets of more than 90% confluent were formed after transfections, then, wounds were generated by scraping the cell monolayers with 200 µm pipet tips. Wounded cells were cultured for 2 days. After 0 and 48 hours, images were collected with a microscope BZ-X800, and wound areas were measured with ImageJ Fiji.

Seahorse XF real-time ATP rate and glycolytic rate assays

Huh7 cells were transfected with pcDNA3-DYRK2-HAC (DYRK2) or pcDNA3-HAC (Empty) overnight. Then, cells were trypsinized and inoculated onto XF24 Cell Culture Microplates (4 x 10^4 cells/well). 24 hours later, the culture medium was changed to XF DMEM medium with 7.4 pH containing 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. The glycolytic activity was assayed by measuring extracellular acidification rate (ECAR), quantification of basal glycolysis and compensatory glycolysis using the XFe24 Extracellular Flux Analyzer according to the manufacturer instructions (Agilent, Santa Clara, USA). Briefly, the plate was incubated at 37 °C in a non-CO2 incubator for 1 hour before analysis. After baseline measurements, the parameters of real-time glycolytic ATP production were calculated using 1.5 µM of Oligomycin, an inhibitor of oxidative phosphorylation and 0.5 µM of Rotenone/Antimycin A (Rot/AA), a mix of Complex-I, III-dependent respiration inhibitors, while glycolytic parameters were calculated using 0.5 µM of Rot/AA and 5 µM of 2-DG (2-deoxy-D-glucose) as an inhibitor of glycolysis (hexokinase inhibitor) to confirm pathway specificity.