Supplementary figure and legends

Figure S1:

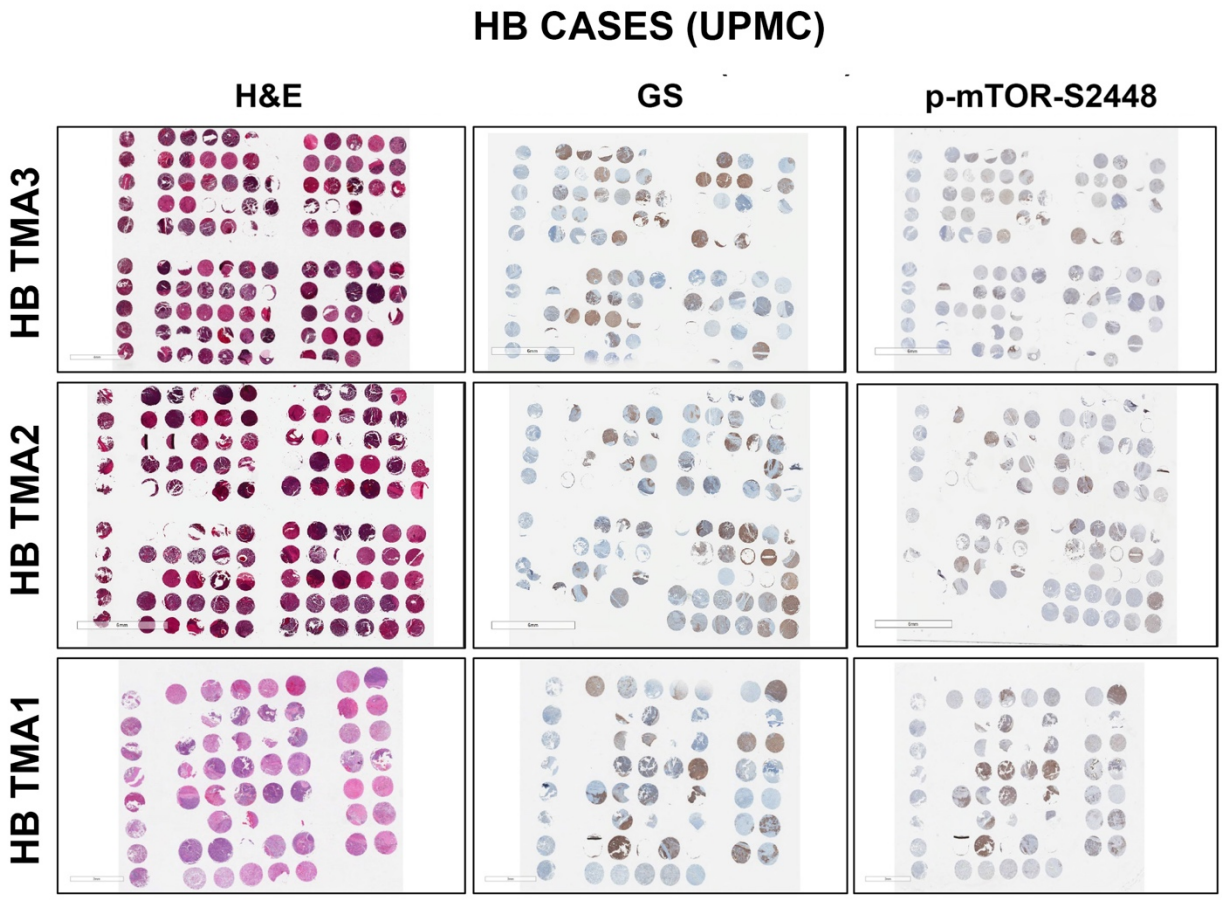

Figure S1: Related to Figure 2. Hepatoblastoma (HB) tissue microarrays (TMAs) stained for GS and p-mTOR-S2448. Three HB TMAs with 55 usable samples were stained for GS and p-mTOR-S2448 to address any correlation between the two proteins. Each HB had multiple histological components including fetal, crowded fetal, embryonal and others. A total of 113 components were identified in the 55 cases. H&E was done to address histology and is presented in the left column for the corresponding TMAs.

Figure S2:

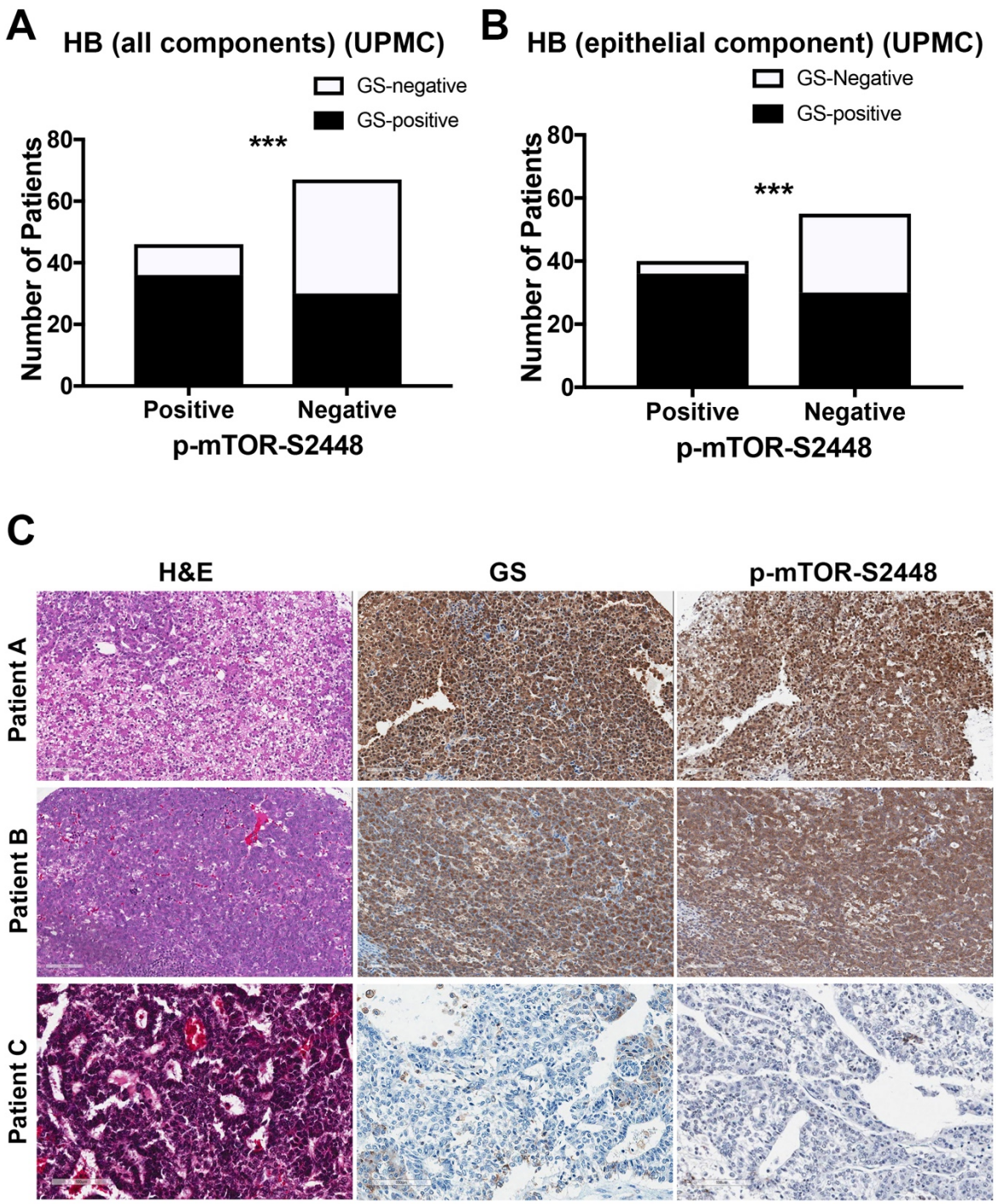
A
HB (all components) (UPMC)
GS-negative
GS-positive
***
Number of Patients
80
60
40
20
0
Positive
Negative
p-mTOR-S2448
B
HB (epithelial component) (UPMC)
GS-Negative
GS-positive
***
Number of Patients
80
60
40
20
0
Positive
Negative
p-mTOR-S2448
C
H&E
GS
p-mTOR-S2448
Patient A
Patient B
Patient C

Figure S2: Related to Figure 2. Significant correlation between IHC for GS and p-mTOR-S2448 in HB patients.

A. 32% of all HB components (n=113) within the 55 HB cases at Children's Hospital Pittsburgh, were simultaneously positive for GS and p-mTOR-S2448. Bar graph representing Fisher's Exact test showed a significant correlation between GS and p-mTOR-S2448 staining in these samples (***p=4.52E-4; 2-sided test).

B. 38% of only epithelial components of the HB (n=95) in the 55 cases showed simultaneously positivity for GS and p-mTOR-S2448 while 25 cases were negative for both these markers. Fisher's Exact test showed a significant correlation between GS and p-mTOR-S2448 (***p=2.36E-4, 2-sided test).

C. Representative IHC of HB samples from the Chidren's Hospital Pittsburgh showing simultaneous positivity (Patient A and B) or negativity (Patient C) for GS and p-mTOR-S2448 (50x).

Figure S3:

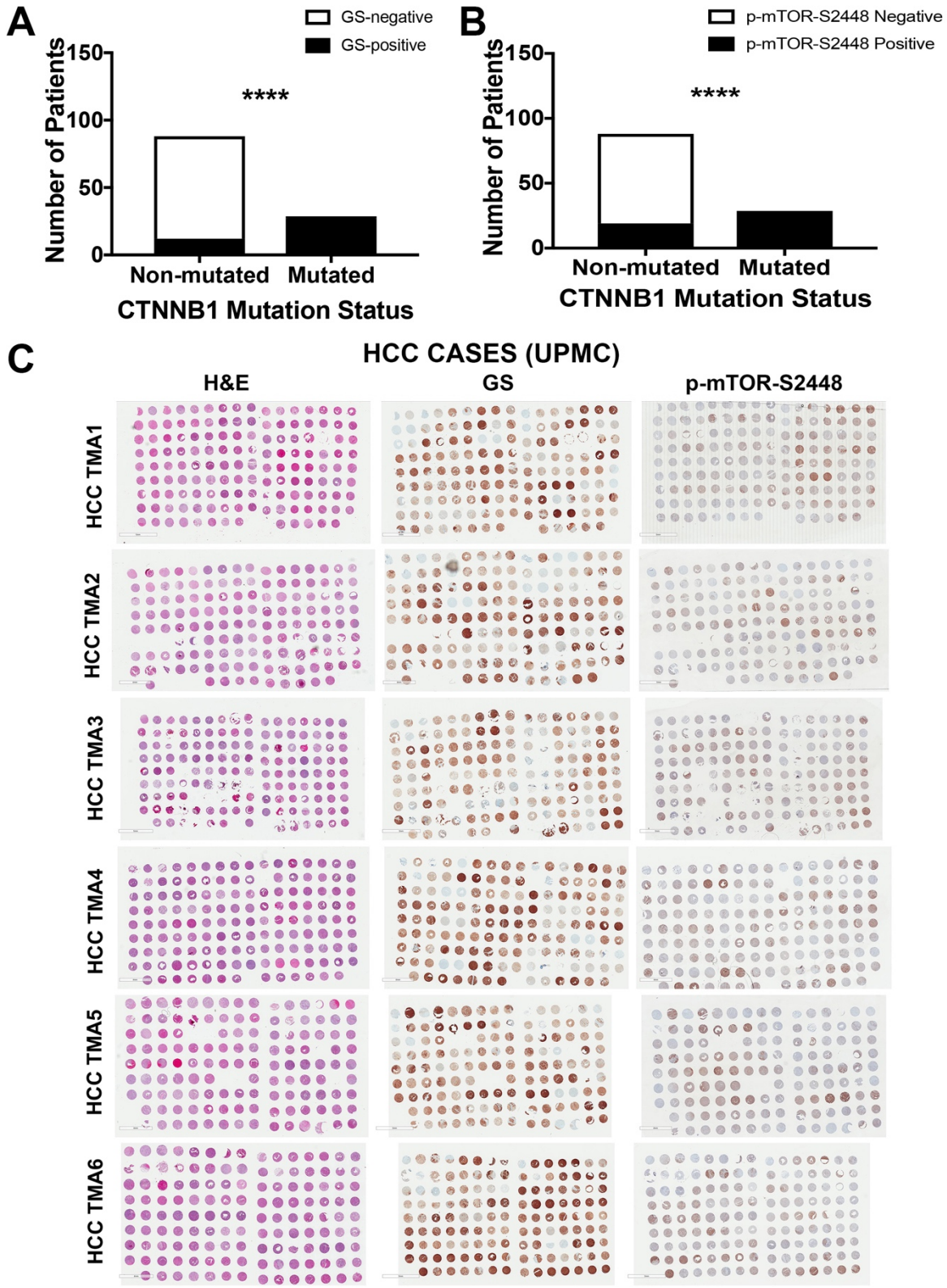
A
GS-negative
GS-positive
Number of Patients
150
100
50
0
****
Non-mutated
Mutated
CTNNB1 Mutation Status
B
p-mTOR-S2448 Negative
p-mTOR-S2448 Positive
Number of Patients
150
100
50
0
****
Non-mutated
Mutated
CTNNB1 Mutation Status
C
HCC CASES (UPMC)
H&E
GS
p-mTOR-S2448
HCC TMA1
HCC TMA2
HCC TMA3
HCC TMA4
HCC TMA5
HCC TMA6

Figure S3: Related to Figure 2. HCC TMAs from University of Pittsburgh Medical Center stained for GS and p-mTOR-S2448. Six HCC TMAs with 252 usable samples were stained for GS and p-mTOR-S2448 to address any correlation between the two proteins. H&E was done to address histology and is presented in the left column for the corresponding TMAs.

Figure S4:

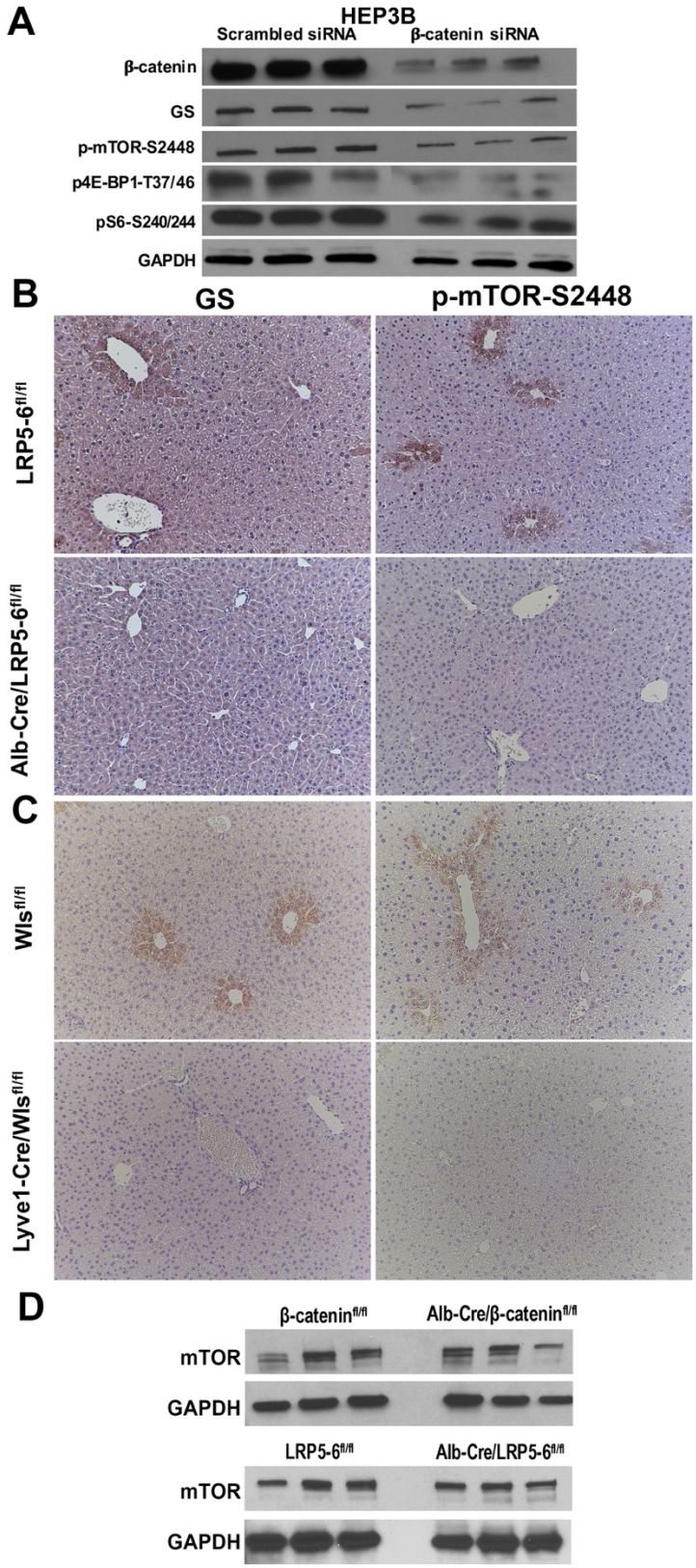
A
HEP3B
Scrambled siRNA
β-catenin siRNA
β-catenin
GS
p-mTOR-S2448
p4E-BP1-T37/46
pS6-S240/244
GAPDH
B
GS
p-mTOR-S2448
LRP5-6fl/fl
Alb-Cre/LRP5-6fl/fl
C
Wlsfl/fl
Lyve1-Cre/Wlsfl/fl
D
β-cateninfl/fl
Alb-Cre/β-cateninfl/fl
mTOR
GAPDH
LRP5-6fl/fl
Alb-Cre/LRP5-6fl/fl
mTOR
GAPDH

Figure S4: Related to Figure 3. Loss of Wnt/β-catenin signaling affected GS and p-mTOR-S2448 protein while no change in total mTOR levels were evident.

A. SiRNA-mediated knockdown of β-catenin gene for 48 hours in the Hep3B cells resulted in notable loss of β-catenin, GS, p-mTOR-S2448, p-S4E-BP1-T37/46 and pS6-S240/244 while GAPDH verified comparable protein loading in the WB.

B. Conditional deletion of Wnt co-receptors LRP5-6 from hepatocytes in the Alb-Cre/LRP5-6$^{fl/fl}$ mice leads to absent staining of the pericentral hepatocytes for not only GS but also for p-mTOR-S2448 (bottom), which are both evident in the livers from littermate LRP5-6$^{fl/fl}$ mice (top) (100x).

C. Conditional deletion of Wntless from hepatic endothelial cells in the Lyve1-Cre/Wls$^{fl/fl}$ leads to absent staining of the pericentral hepatocytes for not only GS but also for p-mTOR-S2448 (bottom), which are both evident in the livers from littermate Wls$^{fl/fl}$ mice (top) (100x).

D. WB using whole cell lysates from the livers of Alb-Cre/β-catenin$^{fl/fl}$ and β-catenin$^{fl/fl}$ mice or Alb-Cre/LRP5-6$^{fl/fl}$ and LRP5-6$^{fl/fl}$ mice shows no change in total mTOR protein levels by WB. Comparable loading was verified by GAPDH.

Figure S5:

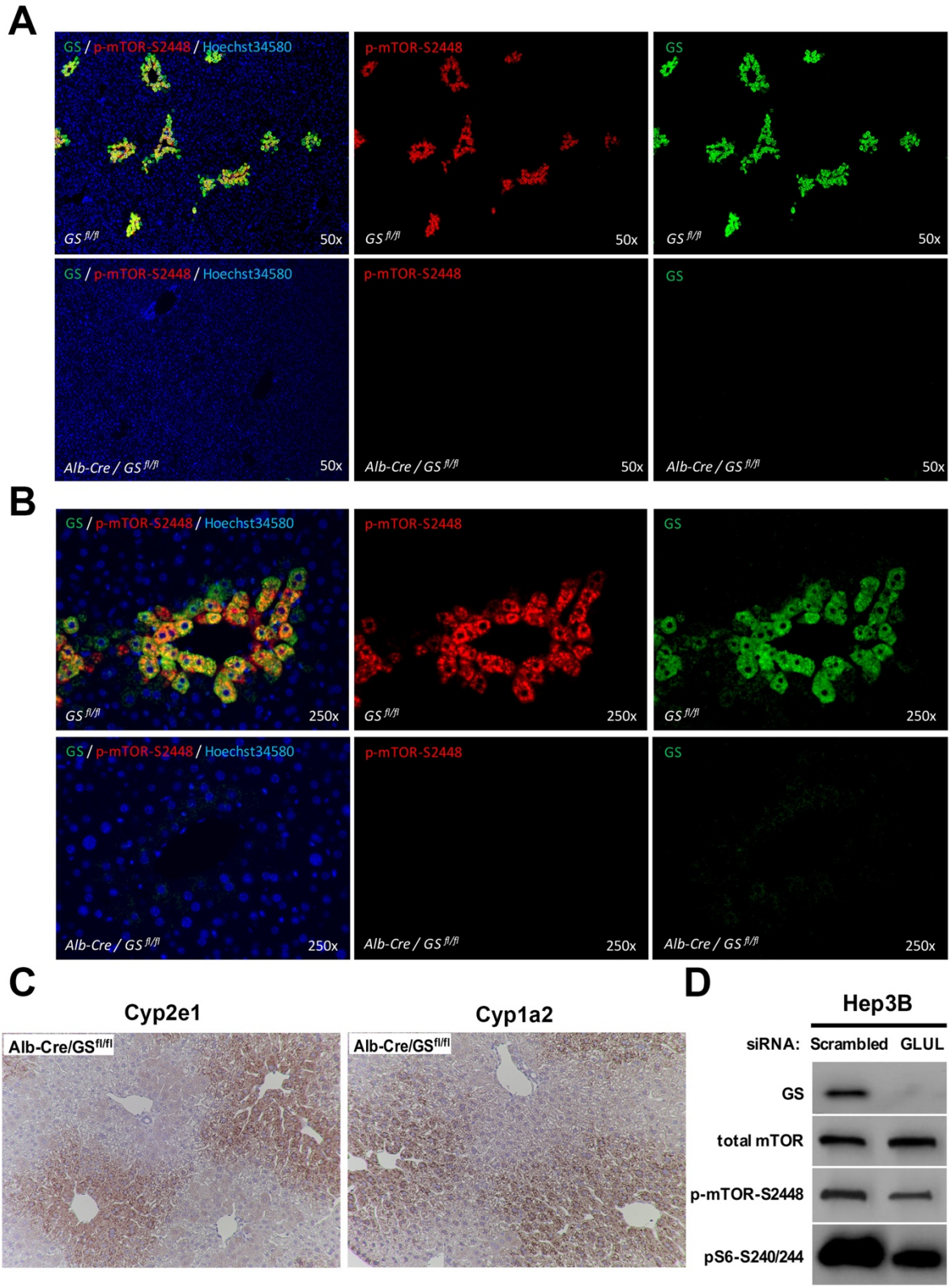
A
GS / p-mTOR-S2448 / Hoechst34580
p-mTOR-S2448
GS
GS fl/fl
50x
Alb-Cre / GS fl/fl
B
250x
C
Cyp2e1
Cyp1a2
Alb-Cre/GSfl/fl
D
Hep3B
siRNA: Scrambled GLUL
GS
total mTOR
p-mTOR-S2448
pS6-S240/244

Figure S5: Related to Figure 4. Loss or knockdown of GS from liver cells affects p-mTOR-S2448 levels and signaling without affecting other Wnt/β-catenin signaling targets, both *in vitro* and *in vivo.*

A. Double immunofluorescence shows colocalization of GS and p-mTOR-S2448 in the $GS^{fl/fl}$ mice (top), while no staining is seen for either in the Alb-Cre/$GS^{fl/fl}$ livers (bottom) (50x).

B. Double immunofluorescence shows colocalization of GS and p-mTOR-S2448 in the $GS^{fl/fl}$ mice (top), while no staining is seen for either in the Alb-Cre/$GS^{fl/fl}$ livers (bottom) (200x).

C. IHC on the Alb-Cre/$GS^{fl/fl}$ livers for the Wnt/β-catenin targets cyp2e1 and cyp1a2 shows intact staining in several layers of hepatocytes around central vein showing normal Wnt/β-catenin catenin signaling in the absence of GS in hepatocytes.

D. SiRNA-mediated knockdown of GS gene (Glul) for 48 hours in the Hep3B cells resulted in notable losses of GS, p-mTOR-S2448 and pS6-S240/244 without affecting total mTOR levels as shown in a representative WB. GAPDH verified comparable protein loading.

Figure S6:

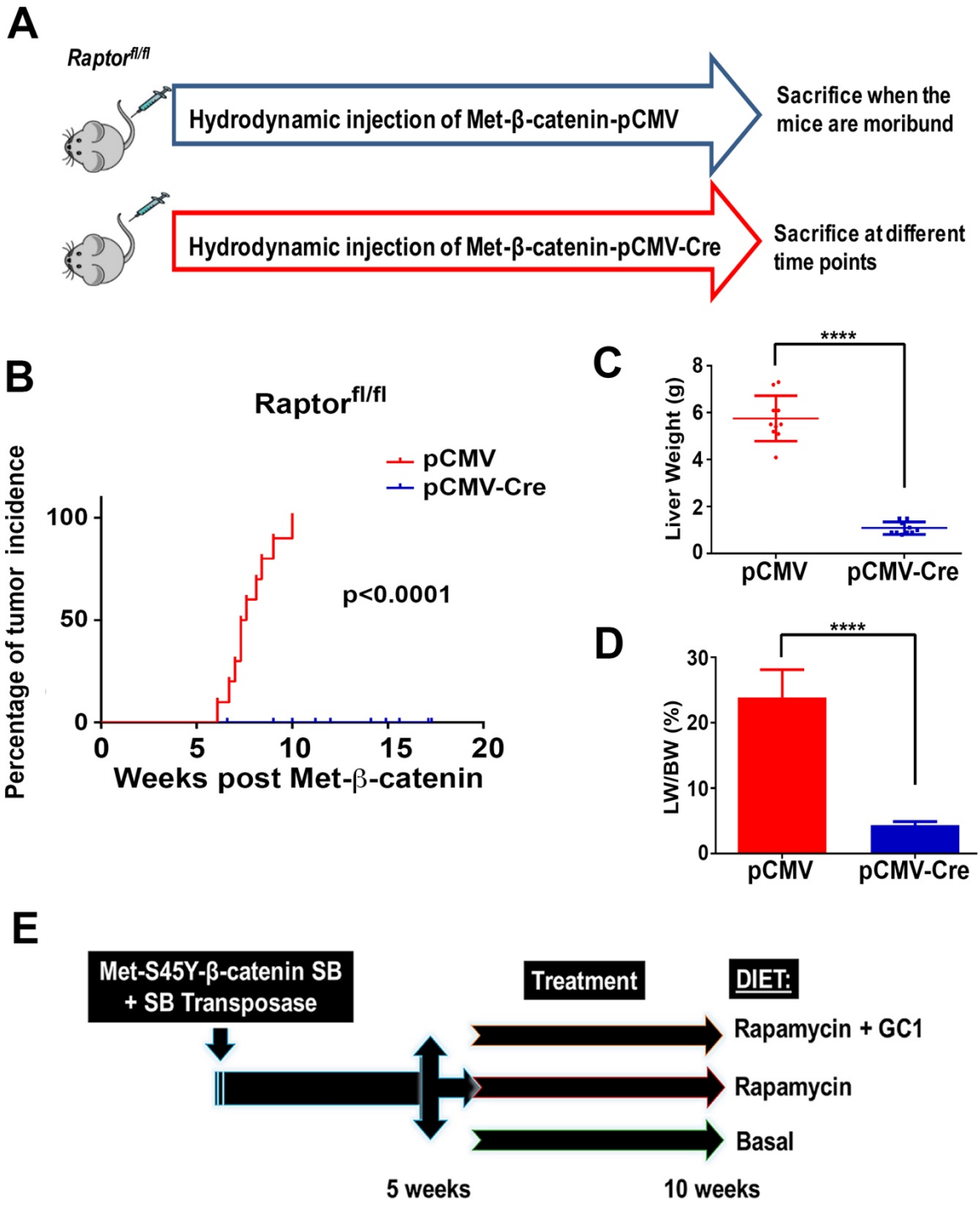
A
Raptor fl/fl
Hydrodynamic injection of Met-β-catenin-pCMV
Sacrifice when the mice are moribund
Hydrodynamic injection of Met-β-catenin-pCMV-Cre
Sacrifice at different time points
B
Raptor fl/fl
pCMV
pCMV-Cre
p<0.0001
Percentage of tumor incidence
Weeks post Met-β-catenin
C
Liver Weight (g)
pCMV
pCMV-Cre
****
D
LW/BW (%)
pCMV
pCMV-Cre
****
E
Met-S45Y-β-catenin SB + SB Transposase
Treatment
DIET:
Rapamycin + GC1
Rapamycin
Basal
5 weeks
10 weeks

Figure S6: Related to Figures 5, 6 and 7. Schematic showing establishment of Met-β-catenin models for mTORC1 inhibition using genetic or drug treatment approaches.

A. Study design showing use of Raptor$^{fl/fl}$ (Exon-6) mice for delivering Met-β-catenin via SB-HTVI along with pCMV or pCMV-cre in 10 mice for each group for assessing effect of mTORC1 inactivation on HCC development.

B. A significant decrease in tumor incidence in Met-β-catenin-pCMV-Cre injected Raptor$^{fl/fl}$ mice as compared to Met-β-catenin-pCMV injected Raptor$^{fl/fl}$ mice (n=10/group) ($p<0.0001$).

C. A significant decrease in the liver weight of the Raptor$^{fl/fl}$ mice injected with Met-β-catenin-pCMV-Cre as compared to the Met-β-catenin-pCMV-injected group. Data are presented as mean ± SD. (****$p<0.0001$).

D. A significant decrease in the liver weight/body weight ratio (LW/BW %) of the Raptor$^{fl/fl}$ mice injected with Met-β-catenin-pCMV-Cre as compared to the Met-β-catenin-pCMV-injected group. Data are presented as mean ± SD. (****$p<0.0001$).

E. Schema depicting establishment of the Met-β-catenin HCC model and randomization at 5 weeks after injection of the plasmids into three groups. The control group had access to basal diet (n=5), and experimental groups had access to either Rapamycin containing diet (18mg/kg) only (n=8) or access to a combination diet containing Rapamycin (18mg/kg) and Sobiterome or GC1 (5mg/kg) (n=8). All mice were euthanized at 5 weeks after treatment for analysis.

Figure S7:

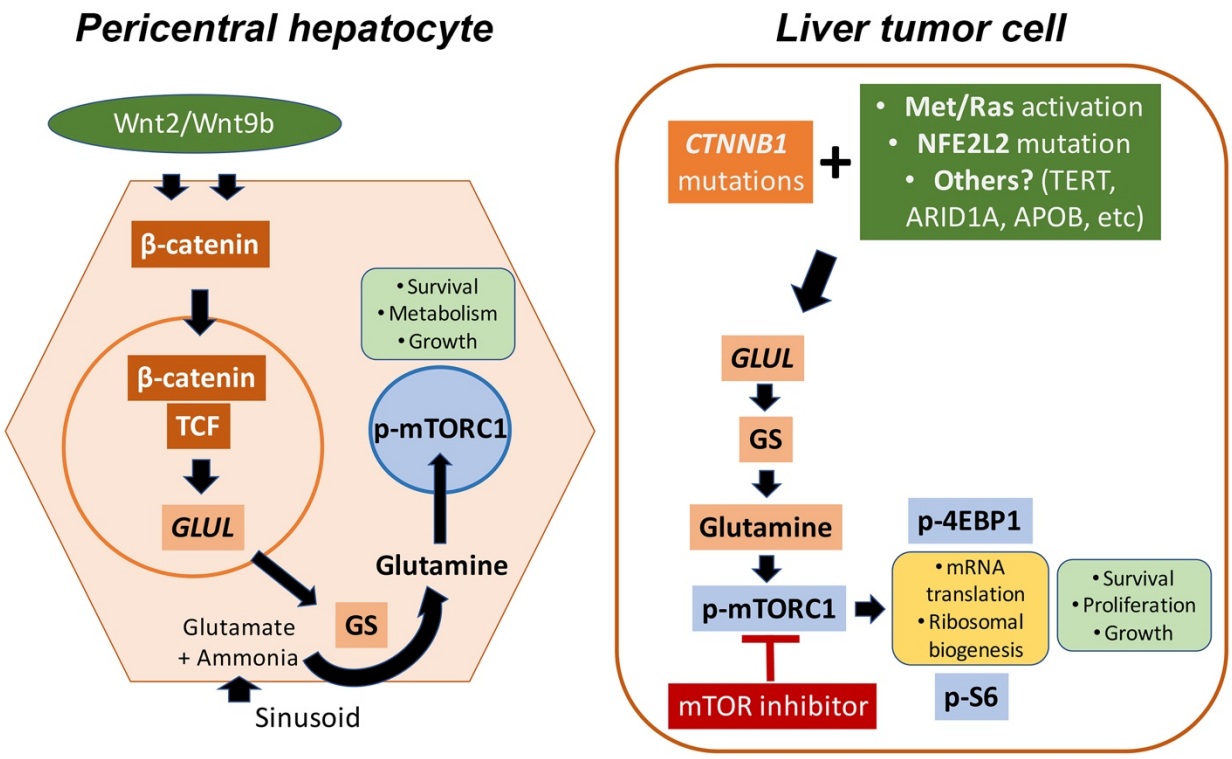

Figure S7: Related to Figures 2, 3 and 5. Schematic showing summary of Wnt-β-catenin-GS-Glutamine-mTORC1 axis in hepatic pathophysiology. Left panel shows the Wnt-mTORC1 axis in pericentral hepatocyte where the most upstream Wnts (2 and 9b) are released constitutively from central vein endothelial cells to persistently stabilize and activate β-catenin-TCF-dependent expression of *GLUL* and in turn increased GS which condenses ammonia taken up from sinusoidal blood with Glutamate yielding Glutamine. Glutamine can translocate into lysosome to directly induce phosphorylation of p-mTOR at S2448 and cause mTORC1 activation and may contribute to several biologic processes inherent to zone 3 of liver lobule. The right panel represents liver tumor cell in which mutations in β-catenin gene cooperate with other aberrations to also lead to GS upregulation which eventually leads to increased Glutamine that in turn

constitutively activated mTORC1. Such metabolic addiction of *CTNNB1*-mutated HCC to mTORC1 can be therapeutically exploited by use of mTOR inhibitors.

Supplementary tables

Table S1: Related to Figure 2. Demographics and additional information on hepatoblastoma patients represented on the 3 tissue microarrays from Children's Hospital, Pittsburgh.

S. No.	HB. No.	Age	Gender	Biopsy/ Resection	Histology	GS	p-mTOR -S2448	Miscellaneous
1	HB2B	9m	F	R	CF	3+	2+	AWOD
					E	2+	Neg	
2	HB3B	51M	F	R/M	CF	2+	3+	AWOD
	HB3C				CF	3+	3+	
3	HB6A	6M	F	B	CF	3+	2+	DOD
					E	Neg	Neg	
	HB6B				WDF	3+	1+	
	HB6C				CF	3+	2+	
					E	Neg	Neg	
4	HB9A	48M	M	R	CF-MT	3+	2+	AWOD
					PF	Neg	Neg	
5	HB10	15M	M	R	CF	3+	2+	AWOD
					E	Neg	Neg	
6	HB14	108M	F	R	CF	3+	3+	DOD
7	HB16	12M	F	R	CF	2+	2+	AWOD
					E	Neg	Neg	
8	HB17A	8M	F	R	WDF	3+	1+	AWOD
	HB17B				CF	3+	1+	
9	HB21	66M	F	R	CF	3+	1+	AWOD
					E	Neg	Neg	
10	HB22				CF	3+	3+	
					E	2+	1+	
					SCU	Neg	Neg	
11	HB24	108M	M	R	E	Neg	Neg	AWOD
12	HB26B	6M	M	R	F	3+	1+	AWOD
13	HB29	24M	M	R	CF	2+	Neg	AWOD
					E	Neg	Neg	
					Blastema	Neg	Neg	
14	HB30	12M	F	R	Tera-gl	Neg	3+	AWOD
15	HB31	24M	M	R	E	Neg	Neg	DOD
					CF (2%)	3+	Neg	
					SCU/BI	Neg	Neg	
16	HB32B	12M	F	R	CF	3+	1+	AWOD
17	HB34	36M	M	R	Tx F	3+	3+	AWOD
					CF	2+	1+	

18	HB36	18M	M	R	WDF	3+	2+	AWOD
19	HB35	24M	F	R	WDF	3+	2+	No FU
					CF	2+	Neg	
20	HB39	36M	M	R	CF	1+	1+	AWOD
					E	Neg	Neg	
21	HB41B	72M	M	R	CF	3+	3+	AWOD
22	HB48	36M	M	R	CF	3+	Neg	AWOD
					Tera NE	Neg	Neg	
23	HB49	24M	F	R	CF	2+	1+	AWOD
					Mesen	Neg	Neg	
					Cholagio	Neg	3+	
24	HB52	12M	M	R	PF	3+	3+	AWOD
25	HB53A	6M	F	R	Cf	3+	3+	AWOD
					E	Neg	3+	
26	HB53B				WDF	3+	1+	
					Mesen.	Neg	Neg	
27	HB54	24M	M	R	Cf	2+	1+	AWOD
					E	Neg	Neg	
28	HB55	11M	F	R	wdf	1+	Neg	AWOD
					CF	2+	Neg	
					E	Neg	Neg	
29	HB56	84M	M	R	CF	3+	3+	AWOD
30	HB57A	12M	M	R	WDF	3+	3+	AWOD
	HB57B				WDF	3+	3+	
31	HB60A	12M	F	R	CF	3+	3+	AWOD
					E	Neg	2+	
	HB60B				CF	3+	3+	
32	HB61	72M	M	R	WDF	3+	3+	AWOD
33	HB63	60M	F	R	CF	2+	2+	AWOD
34	HB65	43M	F	R	CF	3+	1+	AWOD
					Blast	Neg	Neg	
					Cholangio	Neg	3+	
					E	N1+	1+	
35	HB66B	24M	M	R	Cf	3+	3+	AWOD
36	HB67B	11M	F	R	CF	3+	3+	AWOD
					E	Neg	2+	
					Blastema	Neg	Neg	
37	HB68B	36M	M	R	CF	3+	2+	AWOD
					Tera-Gl	Neg	2+	
38	HB70A	24M	F	R	CF	3+	3+	AWOD
					E	Neg	2+	

	HB70B				Cf	2+	2+	
39	HB72	36M	M	R	CF	1+	Neg	AWOD
					E	Neg	Neg	
40	HB74	12M	M	R	CF	2+	1+	AWOD
41	HB75	24M	M	R	E	Neg	Neg	AWOD
					CF	2+	Neg	
42	HB76	12M	M	R	CF	3+	3+	AWOD
					E	1+	Neg	
43	HB79	12M	M	R	CF	2+	1+	AWOD
44	HB81A	12M	M	B	CF	3+	3+	AWOD
	HB81B			R	F	3+	1+	
45	HB82B	24M	M	R	E	1+	Neg	AWOD
					CF	3+	1+	
					Cholangio		3+	
					Blastema	Neg	Neg	
	HB82D				CF	2+	2+	
					E	Neg	Neg	
					Cholangio	Neg	3+	
46	HB83A	8M	M	R	CF	3+	3+	AWOD
	HB83B				CF	3+	1+	
47	HB84B	12M	M	R	CF	3+	1+	AWOD
48	HB85	12M	F	R	WDF	3+	3+	AWOD
					CF	2+	1+	
					E	1+	Neg	
					Blastema	neg	neg	
49	HB87B	24M	F	R	CF	3+	1+	AWOD
					E	Neg	Neg	
50	HB88B	60m	F	R	CF	3+	3+	AWOD
51	HB91B	12M	F	R	CF	2+	w	AWOD
					E	Neg	w	
					Ter-gl	Neg	neg	
					Blastema	Neg	neg	
52	HB95	48M	M	R	WDF	3+	3+	AWOD
53	HB96	36M	F	R	F	3+	1+	No FU
54	HB102	10M	M	R	CF	3+	3+	AWOD
55	HB104B	12M	F	R	CF	2+	1+	AWOD

Abbreviations: HB-hepatoblastoma; F-fetal; E-embryonal; Nal; CF-crowded fetal; WDF-well differentiated fetal; SCU-small cell undifferentiated; PF-pure fetal; Mesen-Mesenchymal; Cholangio-cholangiocarcinoma; Tera-Gl- Teratoid; AWOD-alive without disease; DOD-death of disease; No FU- no follow-up; B- Biopsy; R- Resection; Neg- Negative.
Scoring of staining: 1+ is up to 10%; 2+ is 10-50%; 3+ = >50%.