ONLINE SUPPLEMENT.

A. METHODS:

Mice and hydrodynamic tail vein injections. A list of all mice used in the study is provided in Supplementary table 1. Wild-type FVB/N mice were obtained from the Jackson Laboratory (Harbor, ME). Hydrodynamic injection was performed as described previously (1, 2). Briefly, 20 μg of pT3-EF5α-G12D-mutant-K-Ras and pT3-EF5α-S33Y-mutant-β-catenin-Myc-Tag or pT3-EF5α-G12D-mutant-K-Ras and pT3-EF5α-S45Y-mutant-β-catenin-Myc-Tag along with SB transposase in a ratio of 25:1 were diluted in 2 ml of 0.9% NaCl, filtered through 0.22 μm filter (Millipore), and injected into the lateral tail vein of 6 to 8 weeks old FVB/N mice in 5-7 seconds. The injections were repeated for each group in combination with pT3-EF5α-dnTCF4 or pT3 plasmid only. Mice were housed, fed, and monitored in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. All animals were carefully monitored for signs of morbidity or discomfort. Close attention was paid to the abdominal girth. Animals were euthanized at specific time points after injection or at the earliest signs of morbidity.

Immunohistochemistry. Liver specimens were fixed in 10% buffered and embedded in paraffin. Hematoxylin & Eosin (H&E) staining on 4μm liver sections was performed to note the appearance and characteristics of preneoplastic and neoplastic foci at different times after tail vein injection. Immunohistochemistry was performed on these sections as well as described previously (1, 2). Briefly, deparaffinized sections were incubated in 3% H_2O_2 dissolved in 1X phosphate-buffered saline (PBS) for 30 minutes to quench the

endogenous peroxidase. For antigen retrieval, slides were microwaved in 10 mM citrate buffer (pH 6.0) for 12 minutes. Subsequently, slides were incubated with primary antibodies overnight at 4°C. Primary antibodies used in the study include Ki-67 (NM-sP6; Thermo Fisher Scientific), TUNEL (Kit; S7100; EMD Millipore), Myc-Tag (SC-788; Santa Cruz), Cyclin-D1 (Rb-9041-P; Thermo Fisher Scientific) and GS (SC-74430; Santa Cruz). After washes, the sections were incubated in the appropriate biotin-conjugated secondary antibody (Chemicon, Temecula, CA), for 30 minutes at room temperature. Signal was detected using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and developed using DAB (Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with Shandon Hematoxylin solution (Thermo Fisher Scientific, Pittsburgh, PA), subjected to dehydration process and covered-slipped. For negative control, the sections were incubated with secondary antibodies only or with control IgG.

For quantification of TUNEL staining, 5 random fields at 50x magnification were counted from each section after 3rd cycle of Scr. LNP and CTNNB1-LNP treatment for TUNEL-positive cells. Statistical comparison was performed by unpaired T test using Prism 6 for Mac OS X (GraphPad Software Inc., La Jolla, CA).

Protein isolation and western blots. Liver tissues from age matched control FVB/N mice or various treatment groups were homogenized in lysis buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS] containing the Complete Protease and Phosphatase Inhibitor Cocktail (Roche Molecular Biochemicals). Protein concentrations were determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Aliquots of 50 μg lysates were

denatured by boiling in Tris-Glycine SDS Sample Buffer (Life Technologies, Carlsbad, CA), resolved by SDS PAGE, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) using the Biorad transfer apparatus. Membranes were blocked in 5% BSA in Tris-buffered saline containing 0.1% Tween 20 for 1 hour and probed with various primary antibodies in Supplementary Table 2. Each primary antibody was followed by incubation with horseradish peroxidase-secondary antibody diluted 1:10,000 for 1 hour. After appropriate washing, the signal was developed with the Super Signal West Pico or Super Signal West Femto (Thermo Fisher Scientific, Waltham, MA).

Microarray analysis. Liver tissues from control male FVB mice (n=4) and tumor bearing G12D-K-Ras-S45Y-β-catenin mice (n=4; Range-6-7 weeks post injection) and G12D-K-Ras-S33Y-β-catenin mice (n=4; Range-7-8 weeks post injection) were used to isolate mRNA and preparation of probes for hybridization on the Affymetrix R430 2.0 mouse array as described previously (2). Analysis of array data was performed in R statistical package. Microarray data was normalized using the expresso function from the "affy" Bioconductor package (3) with parameters as follows: normalize.method="quantiles", bgcorrect.method="rma", pmcorrect.method="pmonly", summary.method="medianpolish". The probes were mapped to genes using the custom brainarray CDF (4). Differential expression between normal and tumor samples was determined using the "limma" Bioconductor package (5), using the age of the mouse as a covariate. We compared both the G12D-K-Ras-S45Y-β-catenin and G12D-K-Ras-S33Y-β-catenin mutants against normal livers and found the two mutations to induce highly similar transcriptional changes and samples bearing the two mutations were pooled for subsequent analysis. We also compared the pooled data from G12D-K-Ras-

S45Y-β-catenin and G12D-K-Ras-S33Y-β-catenin mutants against the pooled data from previously published hMet-S45Y-β-catenin and hMet-S33Y-β-catenin mutants (2).

Animal treatment with lipid nanoparticle (LNP) formulation containing Dicer substrate small interfering RNA (DsiRNA) against β-catenin gene. The generation of lipid nanoparticle (LNP) carrying DsiRNA against CTNNB1 was described in a previous publication (6). To test efficacy of this agent in suppressing β-catenin and its affect on HCC, mice were injected with pT3-EF5α-G12D-mutant-K-Ras and pT3-EF5α-S45Y-mutant-β-catenin-Myc-Tag along with SB transposase as described above. Mice were randomized into three groups at 5 weeks based on treatment, group 1 received PBS (n=7), group 2 received LNP with DsiRNA against a scrambled sequence (Scr. LNP; 3mg/kg/dose) (n=11), and group 3 received LNP with DsiRNA against CTNNB1 (3mg/kg/dose) (n=12). Treatments were performed every 24 hours intravenously via lateral tail vein injection for 3 days followed by no injection for 4 days. Similar cycles were repeated 2 or 3 times. Mice were harvested after 1, 2 and 3 cycles (n≥3/group) and compared for LW/BW ratio, β-catenin signaling and Ras signaling by WB analysis and immunohistochemistry.

Statistical analysis. Where applicable all data is presented as mean ± standard error (SE). Statistical differences among the various groups were assessed with Tukey-Kramer's test and p value < 0.05 was considered statistically significant. All statistics were performed with Prism 6, version 6.0 (GraphPad Software Inc., La Jolla, CA).

B. REFERENCES:

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C. ONLINE SUPPLEMENTARY TABLES

Supplementary Table 1: Mice used in the study.

No.	Mouse	Plasmid	Sex	Strain	Harvest (Weeks post-inj.)	Liver phenotype	Body weight (gm)	Liver weight (gm)	LW/BW (%)
101	Kras- beta-catenin s33y M1w	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	1	Normal	22.905	1.519	6.631739795
102	Kras- beta-catenin s33y M2w	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	2	Normal	24.985	1.509	6.039623774
103	Kras- beta-catenin s33y M3w	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	3	Normal	25.002	1.636	6.543476522
104	Kras- beta-catenin s33y M4w	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	4	Normal	24.709	1.712	6.92864948
105	Kras- beta-catenin s33y M7W	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	7	Tumor	30.203	5.549	18.37234712
106	Kras- beta-catenin s33y M8W-1	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	8	Tumor	28.938	5.897	20.37804962
107	Kras- beta-catenin s33y M8W-2	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	8	Tumor	30.867	5.781	18.72873943
108	Kras- beta-catenin s33y M8W-3	Kras(20ug)-beta-catenin s33y(20ug)-sb(1.6ug)	Male	FVB	8	Tumor	28.025	6.043	21.56289028
109	kras beta-catenin s33y PT3 M7W-1	Kras(20ug)-beta-catenin s33y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	7	Tumor	30.749	5.558	18.07538457
110	kras beta-catenin s33y PT3 M7W-2	Kras(20ug)-beta-catenin s33y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	7	Tumor	28.28	5.598	19.79490806
111	kras beta-catenin s33y PT3 M7W-3	Kras(20ug)-beta-catenin s33y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	7	Tumor	29.943	7.142	23.85198544
112	kras beta-catenin s33y PT3 M7W-4	Kras(20ug)-beta-catenin s33y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	7	Tumor	25.834	5.786	22.39684137
113	kras beta-catenin S33Y dnTCF4 M9W	Kras(20ug)-beta-catenin s33y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	9	Normal	28.73	1.453	5.057431257
114	kras beta-catenin S33Y dnTCF4 M10W	Kras(20ug)-beta-catenin s33y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	10	Normal	30.823	1.809	5.868993933

115	kras beta-catenin S33Y dnTCF4 M12W-1	Kras(20ug)-beta-catenin s33y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	12	Normal	29.923	1.482	4.952711961
116	kras beta-catenin S33Y dnTCF4 M12W-2	Kras(20ug)-beta-catenin s33y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	12	Normal	26.984	1.401	5.191965609
117	Kras- beta-catenin s45y M1w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	1	Normal	27.995	1.845	6.590462583
118	Kras- beta-catenin s45y M2w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	2	Normal	25.44	2.091	8.219339623
119	Kras- beta-catenin s45y M3w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	3	Small tumors	23.343	2.709	11.60519213
120	Kras- beta-catenin s45y M4w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	4	Small tumors	25.283	3.202	12.66463632
121	Kras- beta-catenin s45y M6W-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	30.118	6.476	21.50209177
122	Kras- beta-catenin s45y M6W-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	29.951	7.691	25.67860839
123	Kras- beta-catenin s45y M6W-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	31.183	4.672	14.98252253
124	Kras- beta-catenin s45y M6W-4	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	27.584	6.15	22.29553364
125	kras beta-catenin s45y PT3 M6.4W-1	Kras(20ug)-beta-catenin s45y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	6.4	Tumor	29.402	6.673	22.69573498
126	kras beta-catenin s45y PT3 M6.4W-2	Kras(20ug)-beta-catenin s45y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	6.4	Tumor	30.113	6.772	22.48862617
127	kras beta-catenin s45y PT3 M6.4W-3	Kras(20ug)-beta-catenin s45y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	6.4	Tumor	30.467	6.592	21.63652476
128	kras beta-catenin s45y PT3 M6.4W-4	Kras(20ug)-beta-catenin s45y(20ug)-PT3MCS(60ug) - sb(4.0ug)	Male	FVB	6.4	Tumor	28.612	7.176	25.08038585
129	kras beta-catenin s45y dnTCF4 M9W	Kras(20ug)-beta-catenin s45y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	9	Normal	30.442	1.581	5.193482688
130	kras beta-catenin s45y dnTCF4 M10W	Kras(20ug)-beta-catenin s45y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	10	Normal	26.54	1.389	5.233609646
131	kras beta-catenin s45y dnTCF4 M12W-1	Kras(20ug)-beta-catenin s45y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	12	Normal	33.196	1.386	4.175201832

132	kras beta-catenin s45y dnTCF4 M12W-2	Kras(20ug)-beta-catenin s45y(20ug)-dnTCF4(60ug) - sb(4.0ug)	Male	FVB	12	Normal	32.075	1.402	4.371005456
137	kras control1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7.8	Tumor	30.206	7.918	26.2133351
138	kras control2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7.8	Tumor	28.307	5.827	20.58501431
139	kras control3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	8.6	Tumor	22.931	3.856	16.81566438
140	kras control4	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	8.6	Tumor	29.586	5.857	19.79652538
141	kras drug 1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7.8	Small tumors	28.807	1.908	6.623390148
142	kras drug 2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	8.6	Tumor	29.627	3.587	12.10719951
143	kras drug 3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	9.6	Small tumors	30.501	1.923	6.304711321
144	kras pbs 5w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Small tumors	28.253	3.805	13.46759636
145	kras pbs 5w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Small tumors	27.268	3.745	13.73404723
146	kras pbs 5.8w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	27.369	5.329	19.47093427
147	kras pbs 5.8w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	26.125	3.529	13.50813397
148	kras pbs 5.8w-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	27.319	4.491	16.43910831
149	kras pbs 6w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	22.952	4.508	19.64098989
150	kras pbs 7w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7	Tumor	29.403	4.572	15.54943373
151	kras control 5w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Small tumors	27.859	3.595	12.90426792
152	kras control 5w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Small tumors	27.896	2.496	8.947519358
153	kras control 5.8w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	27.791	6.029	21.69407362
154	kras control 5.8w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	27.201	4.296	15.793537
155	kras control 5.8w-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	29.962	2.387	7.966757893

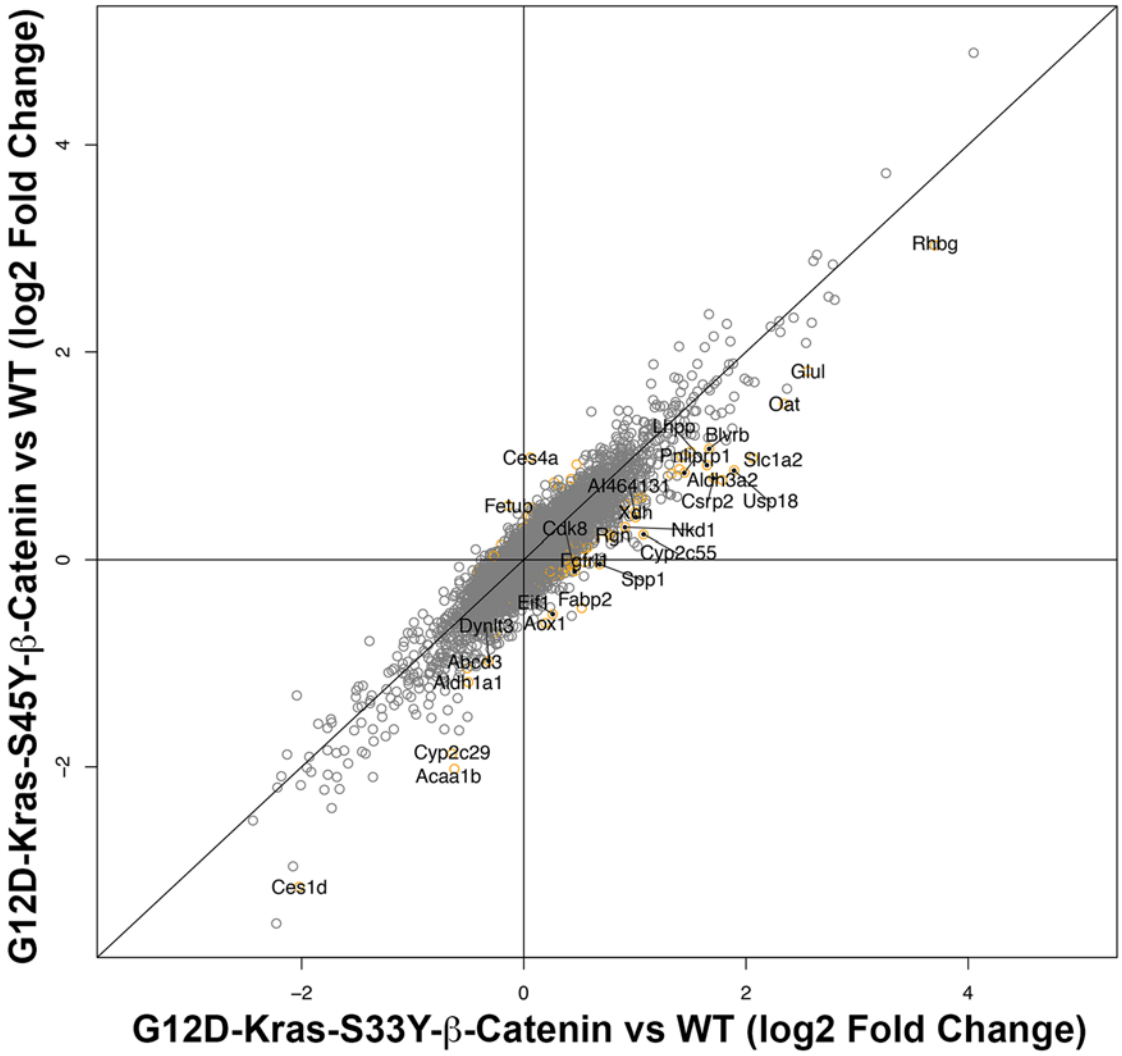
156	kras control 6w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	6	Tumor	28.204	6.17	21.8763296
157	kras control 7w	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7	Tumor	29.115	6.892	23.67164692
158	kras drug 5w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Normal	25.893	1.803	6.963271927
159	kras drug 5w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Normal	24.495	1.795	7.328026128
160	kras drug 5w-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5	Normal	27.119	1.952	7.197905527
161	kras dug 5.8w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Tumor	27.953	4.279	15.30783816
162	kras dug 5.8w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Small tumors	29.192	2.12	7.262263634
163	kras dug 5.8w-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	5.8	Small tumors	26.922	1.901	7.061139588
164	kras drug m7w-1	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7	Small tumors	32.374	3.363	10.38796565
165	kras drug m7w-2	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7	Small tumors	33.194	2.016	6.073386757
166	kras drug m7w-3	Kras(20ug)-beta-catenin s45y(20ug)-sb(1.6ug)	Male	FVB	7	Small tumors	25.92	1.856	7.160493827

Supplementary Table 2: Antibodies used for Western blots.

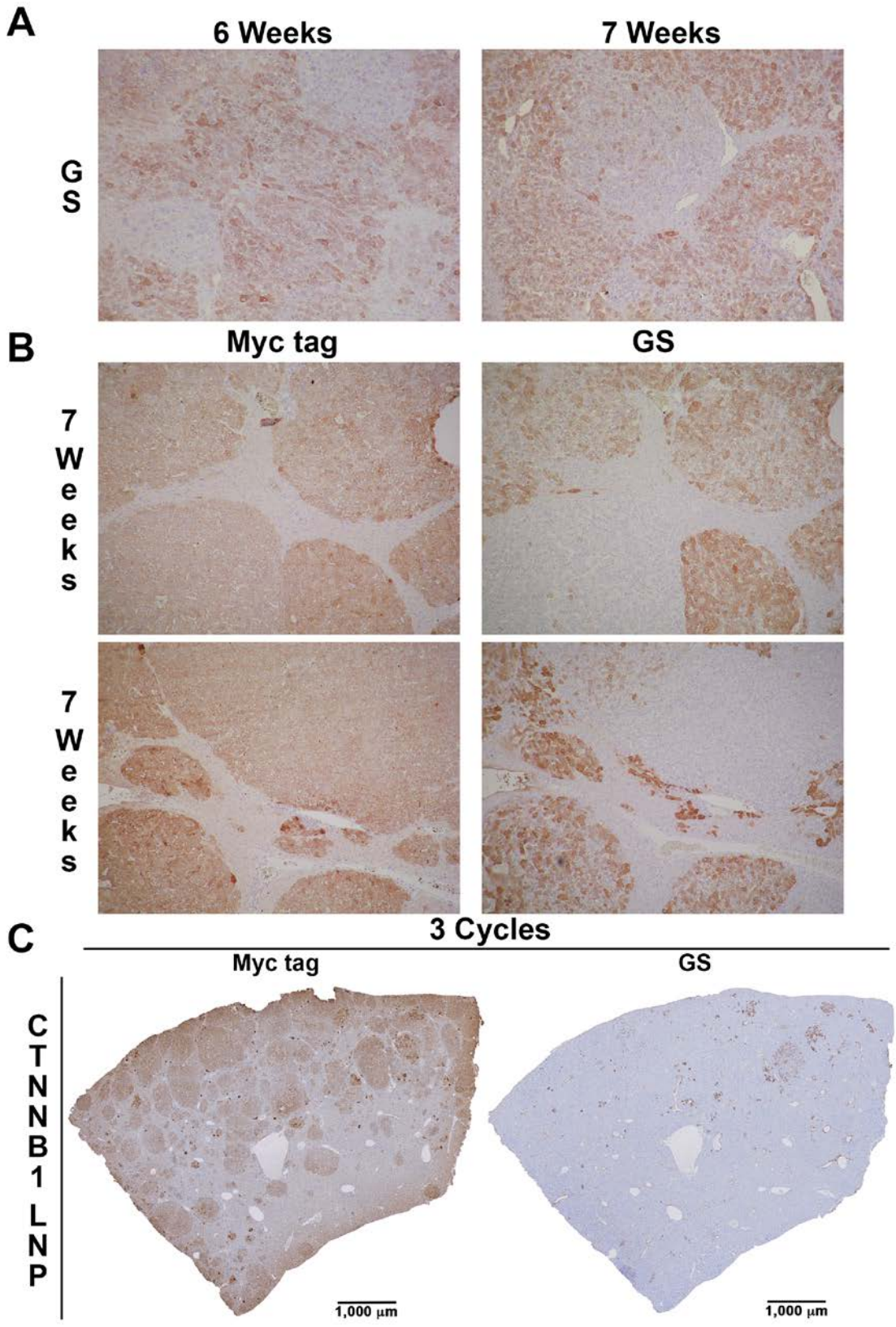
Antibody	Catalogue Number	Company	Species	Dilution
GAPDH	sc-25778	Santa Cruz	Rabbit	1:1000
Glutamine synthetase	sc-74430	Santa Cruz	Mouse	1:1000
Regucalcin	sc-130344	Santa Cruz	Mouse	1:1000
Cyclin-D1	sc-753	Santa Cruz	Rabbit	1:1000
β-Catenin	sc-7199	Santa Cruz	Rabbit	1:1000
LECT2	sc-99036	Santa Cruz	Rabbit	1:500
Myc-tag	2276	Cell Signaling	Mouse	1:1000
ERK	4695	Cell Signaling	Rabbit	1:1000
P-ERK	4370	Cell Signaling	Rabbit	1:1000
AKT	4685	Cell Signaling	Rabbit	1:1000
P-AKT Ser473	4060	Cell Signaling	Rabbit	1:1000
mTOR	2983	Cell Signaling	Rabbit	1:1000
P-Ser2448 mTOR	2971	Cell Signaling	Rabbit	1:1000
P-Ser2481 mTOR	2974	Cell Signaling	Rabbit	1:1000
P-EIF4E	9741	Cell Signaling	Rabbit	1:1000
EIF4E	9742	Cell Signaling	Rabbit	1:1000
P-4E-BP1	9644	Cell Signaling	Rabbit	1:1000
P-235/236 S6 Ribosomal Protein	4585	Cell Signaling	Rabbit	1:1000
P-240/244 S6 Ribosomal Protein	5364	Cell Signaling	Rabbit	1:1000

D. ONLINE FIGURES

Online Figure 1.



Online Figure 2.



E. ONLINE SUPPLEMENTARY FIGURE LEGENDS:

ONLINE FIGURE 1: High molecular similarity between G12D-Kras-S33Y-β-catenin and G12D-Kras-S45Y-β-catenin models. The correspondence between fold change in the G12D-Kras-S33Y-β-catenin and G12D-Kras-S45Y-β-catenin models is depicted as a scatter plot. Changes in both models were qualitatively similar but there were 82 genes that were significantly different (Q-value FDR <0.2) between the two models. There is a general trend towards the Kras+S45Y model producing changes of smaller magnitude. All significantly different genes are marked orange and the top 30 are labeled. The Pearson correlation coefficient is 0.91 (p-value <1e-16).

ONLINE FIGURE 2: Rare mouse shows incomplete response to CTNNB1-LNP due to tumor heterogeneity and expansion of GS-negative tumors in K-Ras-β-catenin.

(A) Immunohistochemical staining of liver sections with GS from G12D-K-Ras-S45Y-β-catenin mice at 6 and 7 weeks after injection showed occasional tumor nodules lacking GS staining.

(B) Immunohistochemical staining of serial liver sections with GS or Myc-tag from two G12D-K-Ras-S45Y-β-catenin mice at 7 weeks after injection showed that all tumor nodules were Myc-tag positive, but a small subset of these mutant β-catenin containing tumor nodules were GS negative.

(C) Immunohistochemical staining of representative serial liver sections with GS or Myc-tag from G12D-K-Ras-S45Y-β-catenin mice after 3 cycles of CTNNB1-LNP treatment

showed that the remained tumor nodules in one liver lobe were Myc-positive but almost exclusively GS-negative.