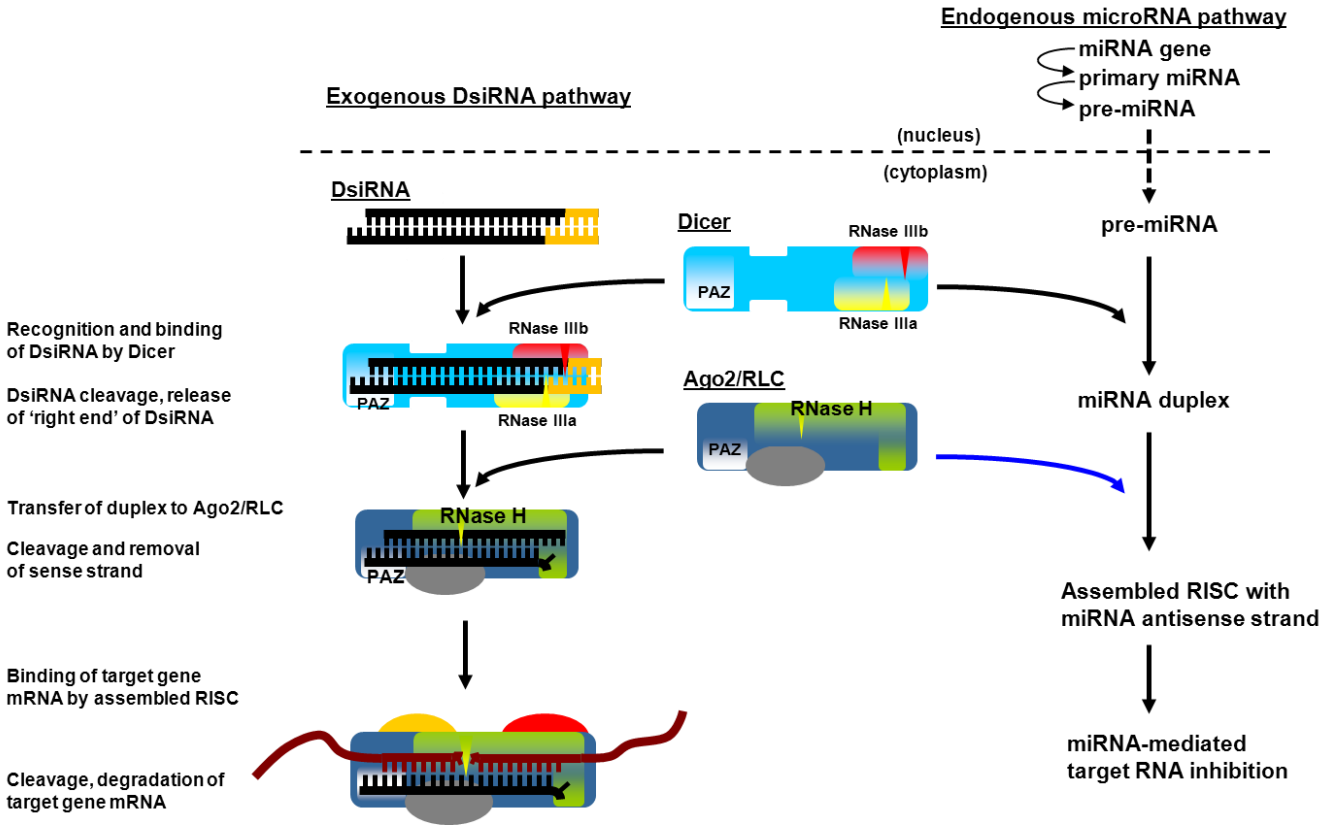
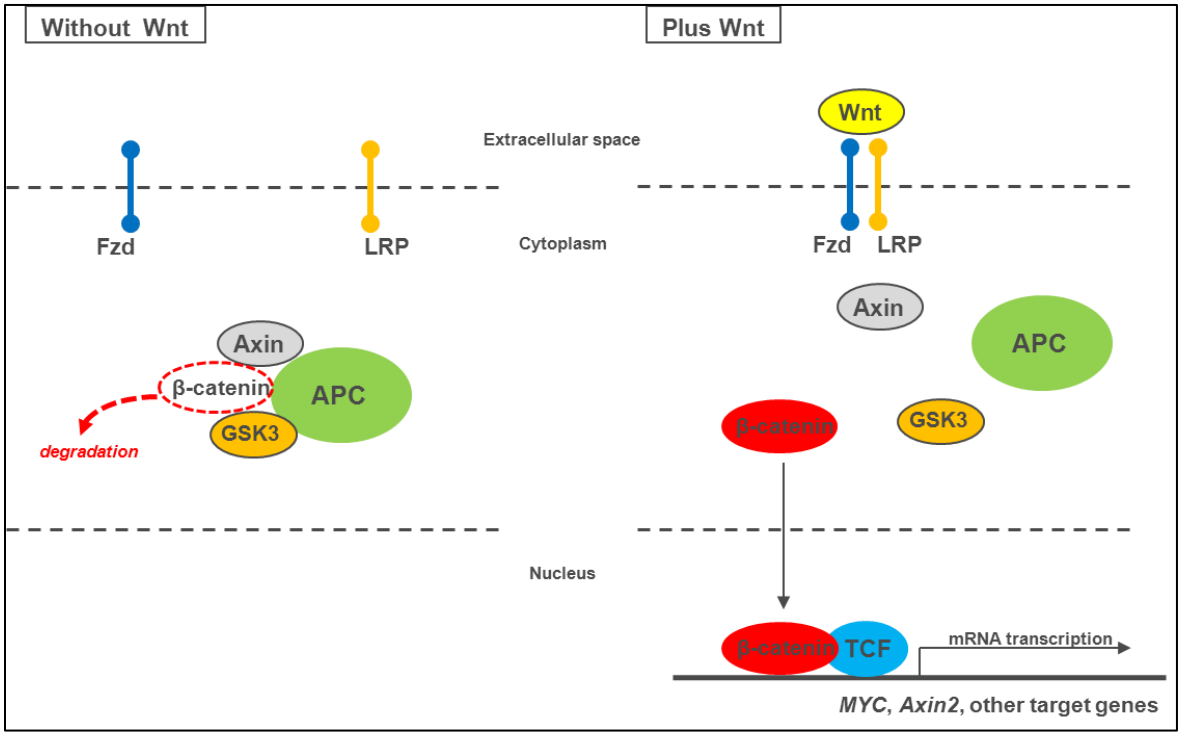
Supplementary Material: Dudek et al.
Knockdown of β-catenin with Dicer-Substrate siRNAs Reduces Liver Tumor Burden In Vivo

Fig. S1 DsiRNAs and the endogenous RNAi pathway



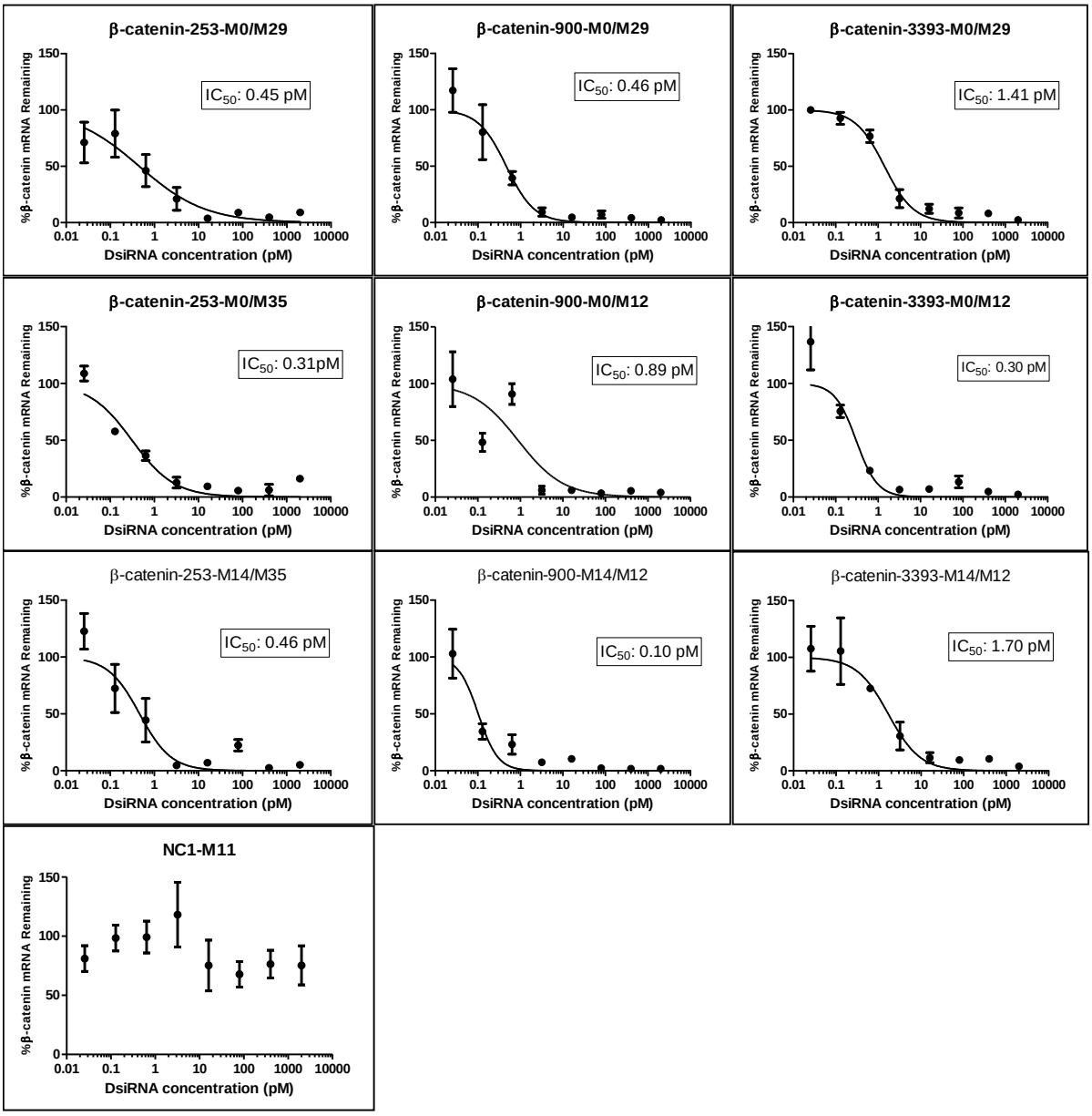
DsiRNAs are processed in a manner similar to that which normally occurs during the maturation of endogenous cellular microRNAs. DsiRNAs, like pre-miRNAs, are recognized and cleaved by Dicer to yield RNA duplexes that are incorporated into the RNA-induced silencing complex (RISC). DsiRNA incorporation into RISC leads to cleavage and degradation of the target cellular mRNA, whose sequence anneals to the DsiRNA antisense strand in RISC. The asymmetric structure of DsiRNAs (two base single-stranded overhang at one end, blunt at the other end) promotes an oriented loading of DsiRNAs into RISC, such that the correct antisense strand of the DsiRNA is used as the antisense strand in RISC, leading to efficient recognition and cleavage of the target mRNA [Rose, SD, et al. (2005). *Nucleic acids research* 33: 4140-4156; Amarzguioui, M, et al. (2006). *Nature protocols* 1: 508-517; Zhou, J, et al. (2012). *Molecular therapy Nucleic acids* 1: e17].

Fig. S2 The canonical Wnt signaling pathway



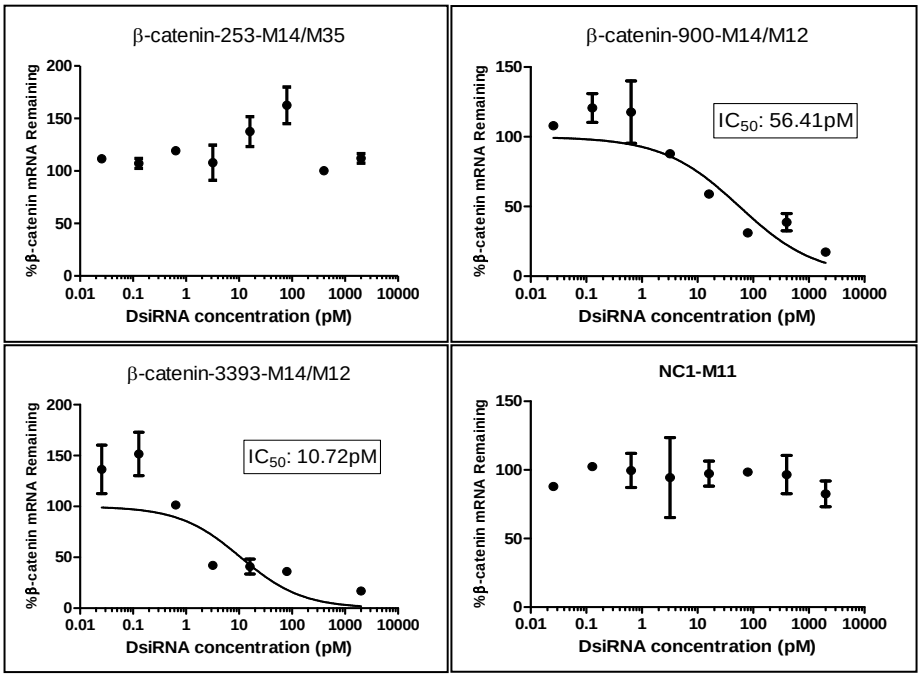
A simplified summary of the canonical Wnt-β-catenin signaling pathway. In the absence of Wnt ligand, a cytoplasmic complex including APC (Adenomatous polyposis coli), Axin, and GSK3 (Glycogen synthase kinase 3) promotes constitutive degradation of β-catenin. Upon binding of Wnt ligands to Fzd (Frizzled) and LRP family members, the APC complex no longer causes β-catenin degradation. Accumulated β-catenin translocates into the nucleus and binds with TCF. β-catenin and TCF together activate transcription of target genes including *MYC* and *Axin2*. In addition to mutation of β-catenin itself, several components of the Wnt-β-catenin pathway are mutated or mis-expressed in cancer. For example, *APC* and *Axin* are mutated in various cancers, leading to overexpression of β-catenin and activation of β-catenin target genes. DsiRNA-induced knockdown of β-catenin would block pathway activation due to mutation of β-catenin itself, or due to mutations in Axin, APC, or other components upstream of β-catenin.

Fig. S3 β-catenin DsiRNA RNA knockdown IC_{50} curves in Hep 3B human cells in vitro



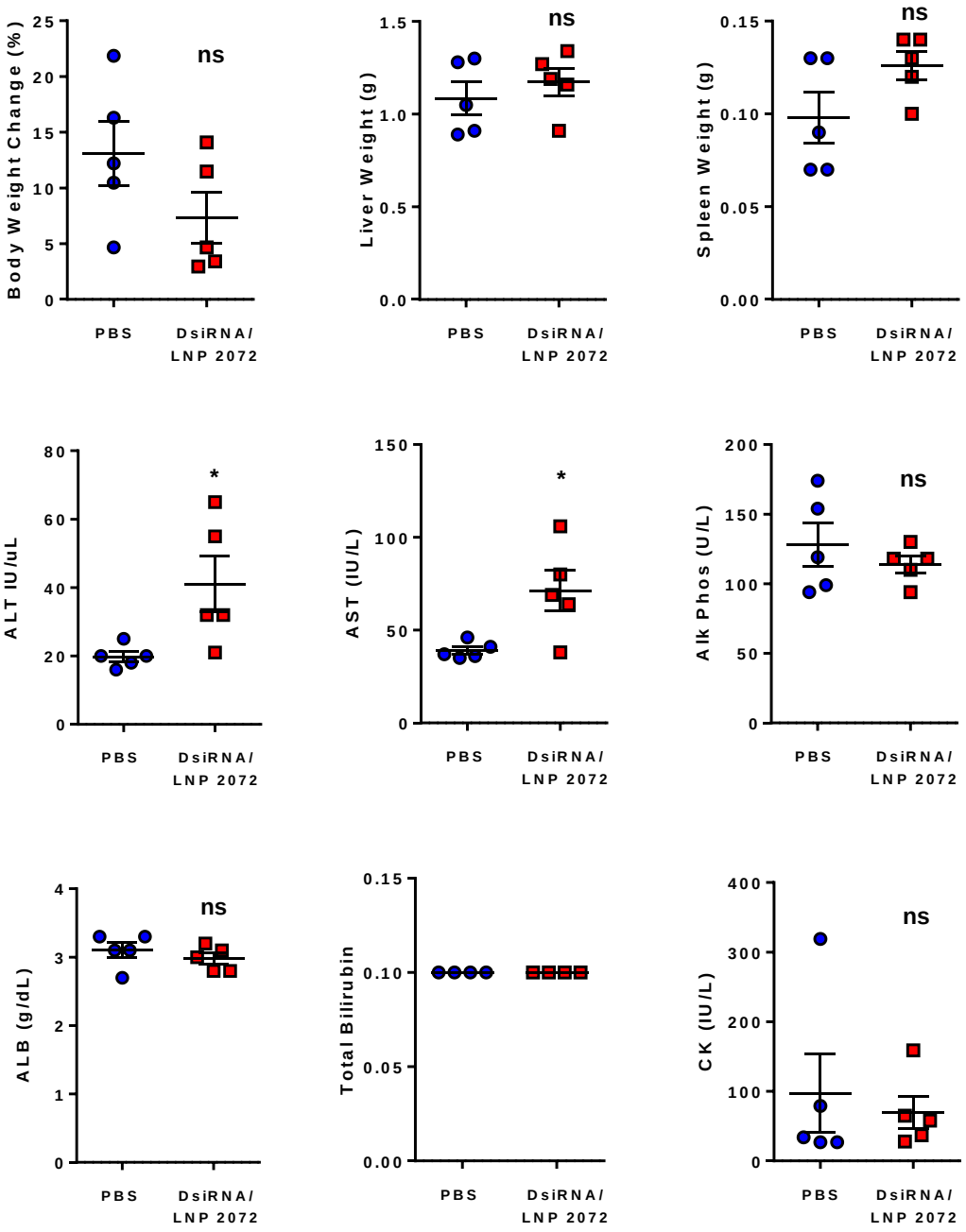
β-catenin mRNA knockdown activity of three DsiRNA lead sequences, β-cat-253, β-cat-900, and β-cat-3393 is shown. The DsiRNA suffix, such as M0/M29, indicates the sense/antisense strand 2'-OMe pattern combination, such as M0 sense strand pattern combined with M29 antisense strand. The activity of three 2'-OMe modified versions is shown: the duplex tested in the 1° screen, in the top row (S-M0/AS-M29, for all); a version with a 3° screen 2'-OMe pattern, in the second row (with only antisense strand 2'-OMe modification), and the lead DsiRNA identified from the 4° screen, in the third row (with both sense and antisense strands modified). The percent mRNA remaining for β-catenin (relative to Mock transfection with RNAiMAX transfection reagent without DsiRNA), is shown. All DsiRNAs retained high activity through 2'-OMe modification, yielding potent leads, some with femtomolar IC_{50} values. NC1-M11 is a nonspecific control sequence DsiRNA, and therefore did not knock down β-catenin mRNA. Means ± standard deviation are shown.

Fig. S4 β-catenin DsiRNA RNA knockdown IC_{50} curves in Hepa1-6 mouse cells in vitro



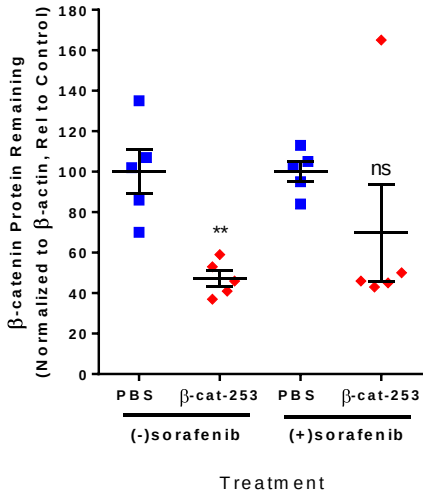
β-catenin mRNA knockdown activity of three DsiRNA lead sequences, β-cat-253, β-cat-900, and β-cat-3393 in Hepa1-6 mouse cells is shown. The percent mRNA remaining for β-catenin, after transfection with β-catenin DsiRNAs and relative to Mock transfection (RNAiMAX lipid transfection reagent without DsiRNA), is shown. β-cat-900-M14/M12 and β-cat-3393-M14/M12 retained high activity with 2'-OMe modification (with higher IC_{50} values than in Hep 3B cells, due to the lower transfection efficiency in Hepa1-6 cells). β-cat-253 is a human sequence-specific DsiRNA (not matching mouse β-catenin sequence), and therefore did not show high activity in Hepa1-6 mouse cells. NC1-M11 is a nonspecific control sequence DsiRNA, and therefore did not knock down β-catenin mRNA. Means ± standard deviation are shown.

Fig. S5: Lack of in vivo toxicity of LNP2072



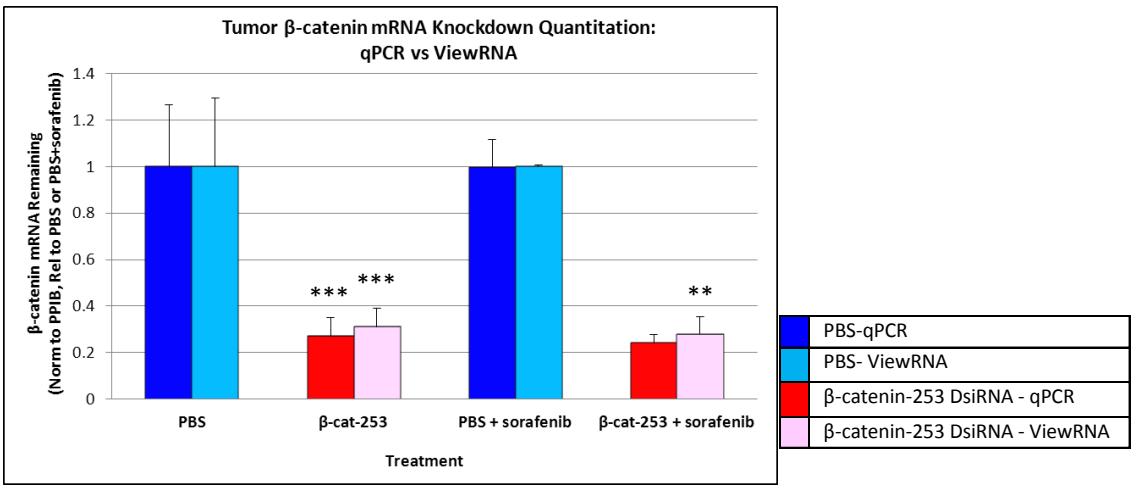
CD-1 mice were dosed iv with PBS or the DsiRNA HPRT1-716-M21/M36 in LNP2072 (5 mg/kg, biw x2 = four doses over two weeks). One day after the fourth and final dose, mice were sacrificed and body weight, liver and spleen weights, and the indicated serum markers were measured (ALT, alanine aminotransferase; AST, aspartate aminotransferase; Alk Phos, alkaline phosphatase; ALB, albumin; CK, creatine kinase). There were no significant changes for most parameters, and very minor increases in ALT and AST (≤ 2-fold; Means ±SEM; *, P<0.05).

Fig. S6: Quantitation of knockdown of β-catenin protein in Hep 3B tumors



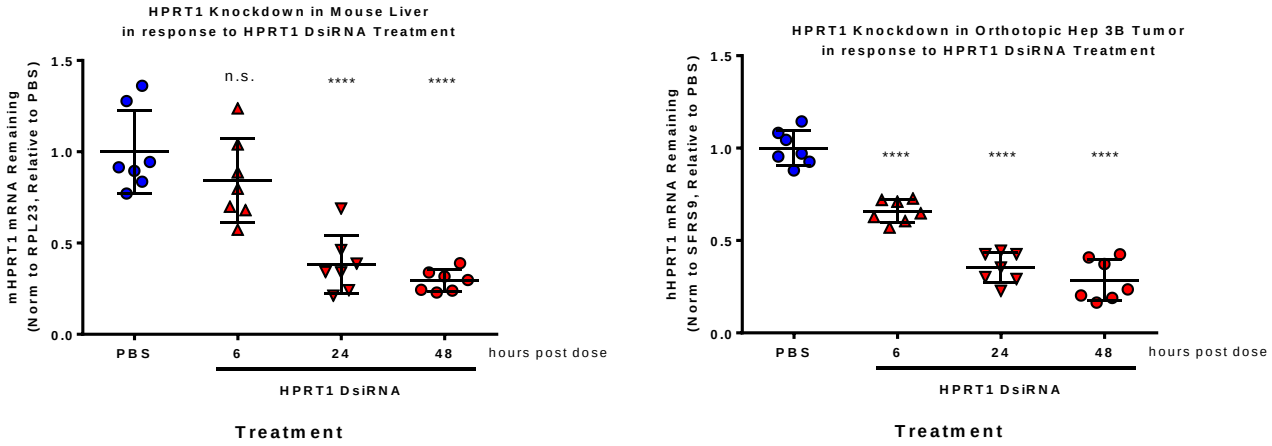
As shown in Fig 4c, tumor tissue samples were analyzed by immunblotting for β-catenin and β-actin. A VersaDoc Imager (BioRad, Hercules, CA) was then used to quantify the intensity of the β-catenin band versus β-actin band (for normalization) in each sample; Means ±SEM are shown (**, P<0.01 versus PBS by t-test; note, β-cat-253 (+)sorafenib is significant versus PBS (+)sorafenib by t-test, P<0.0001, if single outlier is excluded).

Fig. S7: Knockdown of β-catenin mRNA in Hep 3B tumors quantitated by ViewRNA vs. qPCR



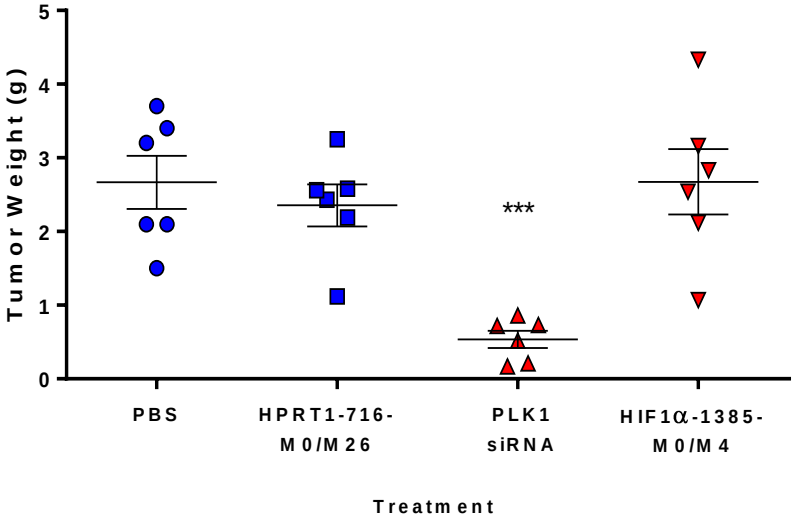
For quantitation of β-catenin mRNA expression using ViewRNA, three regions from four tumor sections were counted for each mouse, normalized to *PPIB* gene expression, and fold change was calculated relative to the average PBS value for the group. Values for tumors were averaged for each treatment group and Means + standard deviation are shown. Data from ViewRNA quantitation was graphed alongside β-catenin mRNA knockdown determined by qPCR (same data as in Fig 4b). ViewRNA and qPCR showed similar levels of β-catenin mRNA Knockdown. **P<0.01; ***P<0.001

Fig. S8: Time course of in vivo target gene knockdown in response to DsiRNA-LNP treatment



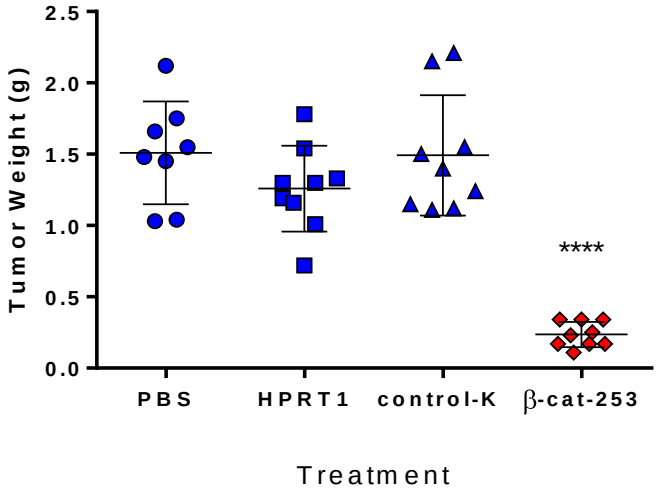
Mice bearing Hep 3B tumors orthotopically in the liver were dosed iv with HPRT1-716-M21/M36 DsiRNA in LNP2072 (10 mg/kg, single dosing). At the indicate times, mice were sacrificed and liver and tumor tissues were isolated and analyzed for gene expression by qPCR. *HPRT1* expression was reduced within hours, for both liver and Hep 3B tumor, after treatment with this human-mouse common sequence *HPRT1* DsiRNA (****, P<0.0001).

Fig. S9: Lack of Hep 3B tumor inhibition by DsiRNAs to *HPRT1* and *HIF1α*



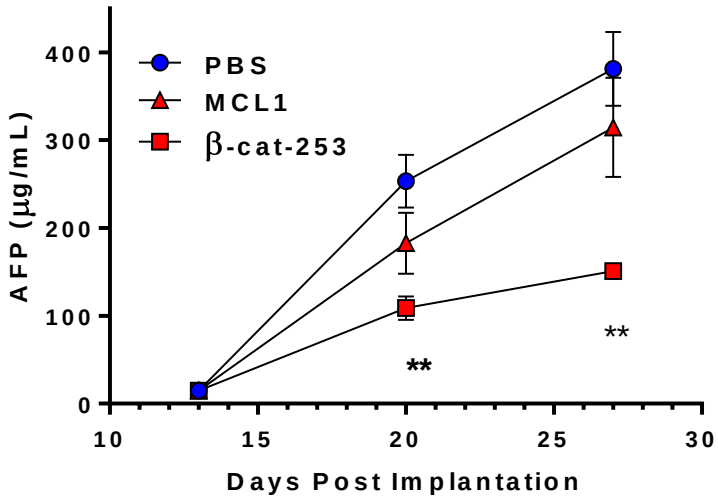
Mice bearing Hep 3B orthotopic liver tumors were dosed iv as indicated using LNP2072 (5 mg/kg, six doses over two weeks). Two days after the final dose, mice were sacrificed and tumors were weighed. Hep 3B tumor growth was inhibited by an siRNA to *PLK1* previously shown to be efficacious against Hep 3B [PLK1424-2/A in Judge, AD, et al. (2009). *The Journal of clinical investigation* **119**: 661-673; ***, P<0.0001], but was not inhibited by DsiRNAs to *HPRT1* or *HIF1α*.

Fig. S10: Lack of Hep 3B tumor inhibition by a DsiRNA to *HPRT1* and control DsiRNA control-K



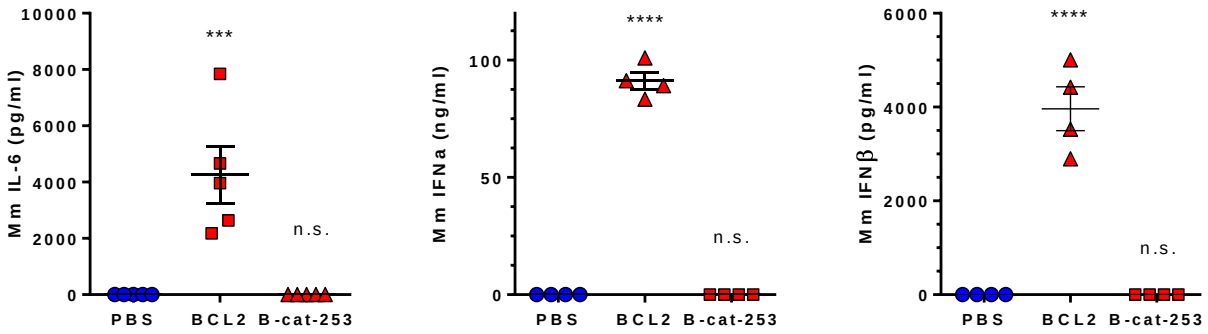
Mice bearing Hep 3B orthotopic liver tumors were dosed iv as indicated with PBS or HPRT1-716-M21/M36, control-K-M16/M11, or β-cat-253-M14/M35 using LNP2072 (5 mg/kg, five doses over two weeks). Two days after the final dose, mice were sacrificed and tumors were weighed. Hep 3B tumor growth was inhibited by β-cat-253 (****, P<0.0001), but not by the control *HPRT1* DsiRNA or the nonspecific control DsiRNA control-K.

Fig. S11: Reduced tumor-derived AFP levels in mice treated with β-cat-253 DsiRNA



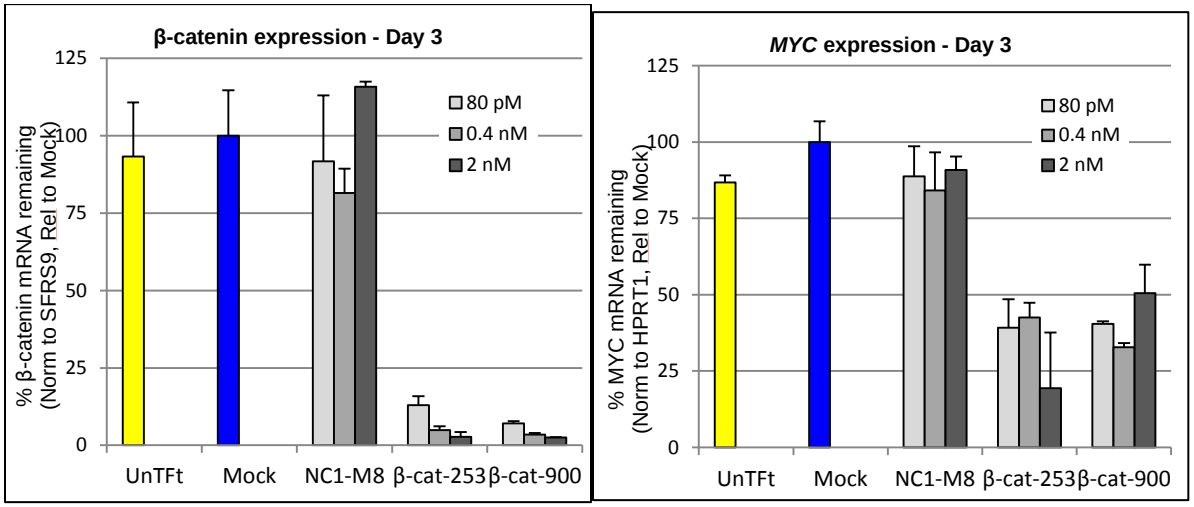
DsiRNAs in LNP2072 were dosed at 5 mg/kg, in 5 doses over 15 days, starting approximately two weeks after implant [same study as in Figure 5b; (-)sorafenib groups]. On the indicated days, serum was isolated and assayed for AFP levels. AFP levels were significantly reduced by β-cat-253-M14/M35 treatment, (**, P<0.01).

Fig. S12: Lack of cytokine induction in mice treated with β-cat-253 DsiRNA



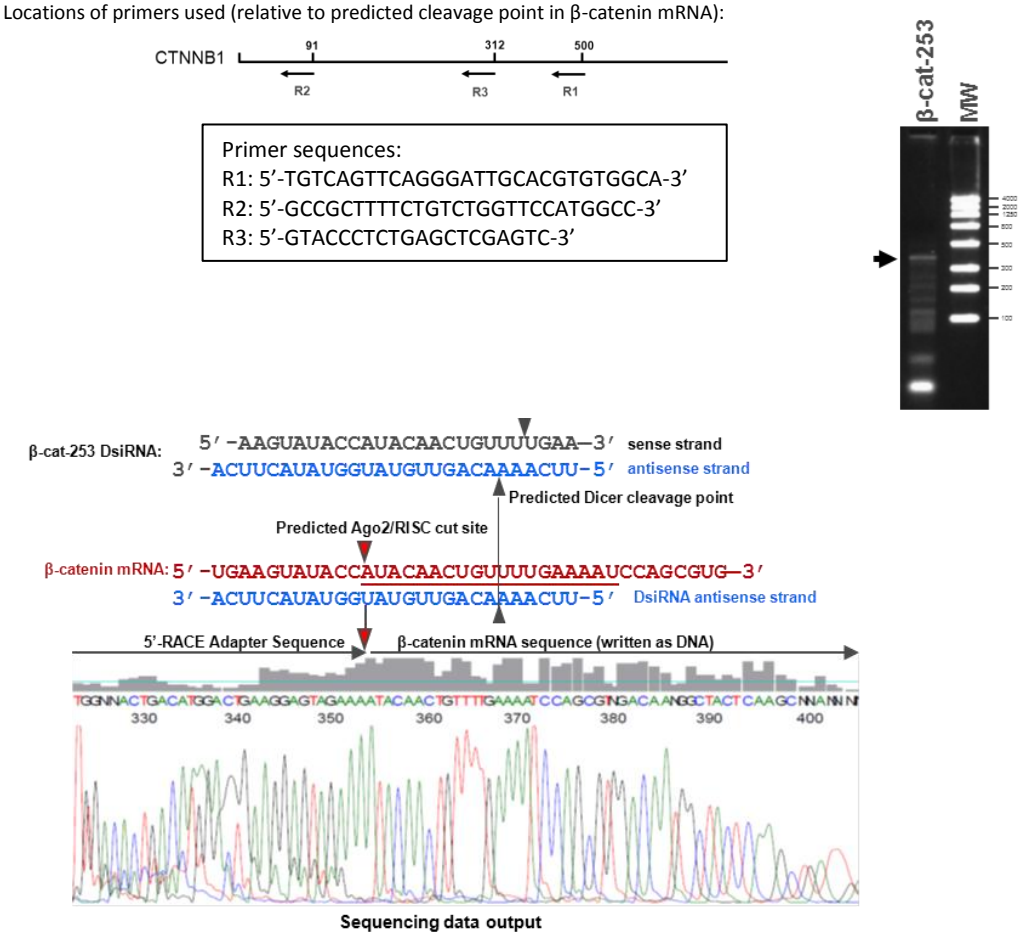
BALB/c mice received a single iv dose of PBS or 10mg/kg β-cat-253 or an immunostimulatory siRNA based on a published siRNA to *BCL2* (Yano et al. Clin Cancer Res, 10, 7721–7726, 2004; sense, 5'-GUGAAGUCAACAUGCCUGU-dTdT-3'; antisense, 5'-GCAGGCAUGUUGACUUCAC-dTdT-3'), in LNP2072. Five hours post-dose, serum was isolated and assayed for cytokine expression by ELISA (BD Biosciences kit for IL-6, VeriKine kit from PBL Interferon Source for IFN). Cytokines were significantly induced by the *BCL2* siRNA, but not by the β-cat-253 DsiRNA (***, P<0.001; ****, P<0.0001).

Fig. S13: Inhibition of expression of *MYC* by β-catenin DsiRNAs in Hep 3B cells



Hep 3B cells were transfected with the DsiRNAs β-cat-253-M14/M35, β-cat-900-M14/M12, or NC1-M8 (a nonspecific control sequence DsiRNA), Mock transfected, or left untransfected (UnTft). After three days, RNA was isolated for qPCR. Both β-catenin DsiRNAs potently knocked down β-catenin mRNA, and caused reduced expression of the downstream target gene *MYC* (Means + standard deviation are shown).

Fig. S14: Detection of β-catenin mRNA cleavage product resulting from in vivo Dicer processing of β-cat-253-14/35 and RISC-mediated mRNA cleavage



Tumor RNA was isolated after treatment with the β-cat-253-14/35 DsiRNA, from the experiment shown in Figure 6. An oligonucleotide was ligated to RNA 5' ends using RNA ligase, for identification of the 5' end of the cleaved β-catenin mRNA by 5' RACE (Rapid Amplification of cDNA End; Invitrogen GeneRacer kit with SuperScript III RT). RNA was reverse-transcribed into cDNA using primer R1. qPCR was performed using a primer specific for the 5' oligonucleotide in combination with R3, yielding the expected 356 (312 + 44) bp fragment indicated by the arrow after agarose gel electrophoresis. The fragment was purified and nested PCR was performed using the RACE 5' nested primer with R2. The gel-purified PCR product was directly Sanger sequenced using the R2 primer, and analysis was done using FinchTV (Geospiza, PerkinElmer, Seattle, WA), yielding the sequence shown at the bottom. This sequence included both the 5'oligo sequence and the underlined β-catenin sequence, indicating the 5' terminus (cleavage point) of the mRNA. The predicted cleavage of the 25/27mer DsiRNA by Dicer into a 21 nucleotide duplex removes six nucleotides from the 5' end of the antisense DsiRNA strand. The resultant 21 nucleotide antisense strand, after incorporation into RISC, is predicted to direct cleavage of the β-catenin mRNA at a site located between nucleotides 10 and 11 from the 5' end of the β-cat-253 21 nucleotide antisense strand. The detected β-catenin mRNA terminus was at this point, confirming both Dicer processing of the β-cat-253 25/27mer, and RISC-mediated cleavage of the β-catenin mRNA in vivo. An identical cleavage point was detected for all four analyzed tumor-bearing mice.

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Table S1. Primers and Probes used in Real-Time qPCR

FAM is 6-carboxyfluorescein, IBFQ is Iowa Black dark quencher and ZEN is an internal quencher.

Gene, oligo name	DNA sequence (5'-3')
qPCR assays used in screening	
Human β-catenin	
Assay 1 Forward primer	GCTGAAGGTGCTATCTGTCT
Assay 1 Reverse primer	GACGTTGACTTGGATCTGTC
Assay 1 probe	HEX-CCGGCTATT-ZEN-GTAGAAGCTGGTGGAAT-IBFQ
Assay 2 forward primer	GCAGGTGGATCTATTTCATGTT
Assay 2 reverse primer	GCATCGTATCACAGCAGGTT
Assay 2 probe	FAM-TGGGTAGGG-ZEN-TAAATCAGTAAGAGGTGT-IBFQ
Human HPRT1	
F517 Forward primer	GACTTTGCTTTCCTTGGTCAG
R591 Reverse primer	GGCTTATATCCAACACTTCGTGGG
P554 Probe	FAM-ATGGTCAAG-ZEN-GTCGCAAGCTTGCTGGT-IBFQ
Human SFRS9	
F569 Forward primer	TGTGCAGAAGGATGGAGT
R712 Reverse primer	CTGGTGCTTCTCTCAGGATA
P644 Probe	HEX-TGGAATATG-ZEN-CCCTGCGTAAACTGGA-IBFQ
Additional qPCR assays used for in vitro assays	
Human β-catenin	
F3453 Forward primer	TAGACAAATAGAAAATGGTCC
Reverse primer	TCTTGAAGCATCGTATCACAG
probe	FAM-ATCAGTAAG-ZEN-AGGTGTTATTTGGAACC-IBFQ
Human SFRS9	
F569 Forward primer	TGTGCAGAAGGATGGAGT
R712 Reverse primer	CTGGTGCTTCTCTCAGGAT
P644 Probe	MAXN-TGGAATATGCCCTGCGTAAACTGGA-IBFQ
Mouse β-catenin	
F3339 Forward primer	TAAGCAGGTGGATCTATTTC
R3521 Reverse primer	CCATTTCTATAACCGCATCTG
P3690 probe	FAM-CGTGCGGTA-ZEN-GGGTAAATCAGTAAGAG-IBFQ
Mouse HPRT1	
F576 Forward primer	CAAACTTTGCTTTCCCTGGT
R664 Reverse primer	CAACAAAGTCTGGCCTGTATC
P616 probe	HEX-TGGTTAAGGTTGCAAGCTTGCTGGTG-IBFQ
Human MYC	
F514 Forward primer	CTGCTTAGACGCTGGATTT
R620 Reverse primer	GTCGTAGTCGAGGTCATAGTTC
P567 probe	FAM-ACGATGCCC-ZEN-CTCAACGTTAGCTTC-IBFQ
Human HPRT1: *same as above, except probe:*	
P554 Probe	Cy5-ATGGTCAAGGTCGCAAGCTTGCTGGT-IBRQ
Additional qPCR assays used for in vivo analysis	
Mouse RPL23	
F139 Forward primer	CTGTGAAGGGAATCAAGGGA
R249 Reverse primer	TGTCGAATTACCACTGCTGG
P162 Probe	Cy5-CTGAACAGACTTCCTGCTGCTGGTG-IBRQ

Supplemental Experimental Procedures

Testing for in vivo immunostimulation activity

DsiRNAs were formulated in LNP F30.1 as follows. Lipids containing CHOL/DOTAP/DODMA/DSPE-PEG2000 (28/30/17/25, weight ratio) were mixed in the presence of 8mg Tween-80 and then dissolved in ethanol at 160mg/mL. DsiRNAs in water were added to lipids at a weight ratio of 1:12 and then diluted with PBS under vortex condition to yield a milky suspension. After centrifugation at 1,000g for 10 minutes, the supernatant was collected and 0.20μm filtered. The final formulation typically contains DsiRNA at a concentration of 1mg/mL with encapsulation above 80%, determined using UPLC and Picogreen assay (Life Technologies, Grand Island, NY), respectively. Male BALB/C mice were dosed with DsiRNA at 10 mg/kg iv in LNP F30.1. At 7 days post-dose, serum was collected and analyzed for immune response, as determined through appearance of IgM antibodies to PEG. PEG IgM titer was measured using streptavidin-coated assay plates with immobilized biotinylated PEG. A standard curve was generated using a commercially available IgM antibody against PEG (ANP Technologies, Newark, DE).

Lipid Nanoparticles

In brief, LNP2072 is composed of 14 mol% DSPC, 3 mol% DSPE-PEG(2000), 3 mol% DMPE-PEG(2000), 33 mol% cholesterol, and the ionizable lipids DODMA (26 mol%) and DL-036 (21 mol%). DL-036 is a novel lipid developed by Dicerna Pharmaceuticals containing dilinoleoyl tail groups linked by an amide directly to a dimethyl amine head group. The final DsiRNA-loaded particles that comprised LNP2072 were formed in three steps: 1) DODMA and DMPE-PEG(2000) were mixed in water and extruded through polycarbonate membranes to form particles approximately 70 nm in diameter; 2) DsiRNA in water was then added with constant stirring; and then 3) cholesterol, DSPC, DSPE-PEG(2000) and DL-036 in a 90% ethanol solution was added, again with constant stirring, to form the final surface composition of the particles. The particles were then exchanged into PBS by diafiltration. The final LNP2072 particles had an average diameter of 105 nm with a polydispersity index (PDI) of < 0.1. A complete manufacturing process description and physical characterization of LNP2072 will be presented in a separate publication.

DsiRNA hybridization assay for in vivo distribution studies

The hybridization assay uses a capture probe linked to the assay plate to capture the antisense strand of β-cat-253-M14/M35, and utilizes a biotinylated hybridization probe for detection. Capture probe (5'-mCmAmAmCmUmGmUmUmUmUmGmAmAmUmU-NH2-3', where 'm' indicates 2'-OMe-modified RNA) is first conjugated onto an NHS activated assay plate (via the NH_2- group). Tissue is lysed in 4M guanidine thiocyanate, 0.5% sodium *N*-lauroyl sarcosine, 1 mM DTT, and 25mM sodium citrate. Detection probe with a biotin label (5'-Biotin mUmUmUmGmAmAmGmUmAmUmAmCmCmAmU-3') is mixed with tissue lysates and heat denatured. This mixture is then added to the assay plate. Upon incubation, the β-cat-253-M14/M35 antisense strand-detection probe hybrid binds to the plate through hybridization with the capture probe. After washes with PBS, the bound hybrid is detected by streptavidin-conjugated horseradish peroxidase. Quantitation is done by comparison to a standard curve of β-cat-253-M14/M35 prepared in corresponding blank tissue lysate.

ViewRNA

Manufacturer's protocol (Affymetrix, Santa Clara, CA) was followed with the exceptions noted below. Formalin-fixed/paraffin embedded tissue sections were cut to 5 microns and stored in

dessicated container until use. A humidified oven and dry oven were used for the incubations. Slides were washed using the Little Dipper (SciGene, Sunnyvale, CA, 1080-65-1). After melting the paraffin, Histo-Clear (Electron Microscopy Sciences, Hatfield, PA, 64110-04) was used to wash it away. Pretreatment of tumor tissue was for 15 minutes at 95°C. Protease digestion of tumor tissue was 15-20 minutes at 40°C. Slides were mounted in Vectashield Mounting Media with DAPI (Vector Labs, Burlingame, CA, H-1200). Analysis of slides was performed using a Nikon Microscope with a 20x objective with an NA of 0.75. Signal intensity and area of signal were measured using Nikon Elements software.

Western analysis of tissue samples

Frozen tissue was diced into small pieces using a razor blade. 500 ul of PBS plus Protease Inhibitors Cocktail (Roche, Indianapolis, IN, # 11836170001) was added to approximately 50 mg of frozen tissue and tissue was crushed using a bead homogenizer (Bullet Blender 24, Next Advance Inc, Averill Park, NY). Samples were centrifuged at 1000 x g at 4˚C for 30 seconds and the supernatant and loose tissue pellet were transferred, leaving the beads behind. The sample was then again centrifuged at 1000 x g, at 4˚C for 30 seconds, after which the clear supernatant was removed and discarded, leaving the loose pellet. An equal volume of 2X RIPA Buffer (Boston Bioproducts, Ashland, MA, #BP-115-5X) plus Protease Inhibitors Cocktail was added to the pellet. Samples were gently mixed at 4˚C for 30 minutes, then centrifuged at 10000 xg, and 4˚C for 30 minutes. The supernatant was recovered, and BCA protein assay (Pierce, Rockford, IL, #23227) was performed for protein measurement. For western analysis, tissue lysates containing 50 ug of total protein were loaded per well of 4-20% Tris-Glycine gels. Primary antibodies for β-catenin (NB110-56924) and β-actin (NB600-501) were from Novus Biologicals (Littleton, Colorado) and fluorescent labeled anti-IgG secondary antibodies (Life Technologies, Grand Island, NY) were used for detection. A VersaDoc Imager (BioRad, Hercules, CA) was used to quantify the intensity of the β-catenin band versus β-actin band (for normalization) in each sample.

Tissue RNA isolation and qPCR analysis

Approximately 50 mg of sample was homogenized in 0.5 ml guadinium thiocyanate-phenol using a Tissuelyser II (Qiagen , Valencia, CA). The homogenate was chloroform extracted and 0.1 ml of the aqueous phase was loaded onto an SV96 Total RNA Isolation binding plate (Promega , Madison, WI). RNA was then purified according to manufacturer instructions. RNA was quantitated using spectrometry at 260 and 280 nm. For human *Axin2* qPCR, a TaqMan assay (Hs01063168_m1) from Applied Biosystems (Carlsbad, CA) was used. For mouse *Axin2*, a TaqMan assay (Mm01265779_m1) from Applied Biosystems was used; for both mouse β-catenin and *Axin2*, multiplex qPCR of tissue mRNA was done with a qPCR assay to *RPL23* for normalization. All analyses of target gene expression included standard normalization versus an appropriate housekeeping gene (*RPL23* or *HPRT1* for mouse, *SFRS9* or *HPRT1* for human) to control for sample processing and measure specific effects on expression of the target gene of interest.