Supporting Information for

Targeting cancer gene dependencies with anthrax-mediated delivery of peptide nucleic acids

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I. SUPPLEMENTAL FIGURES

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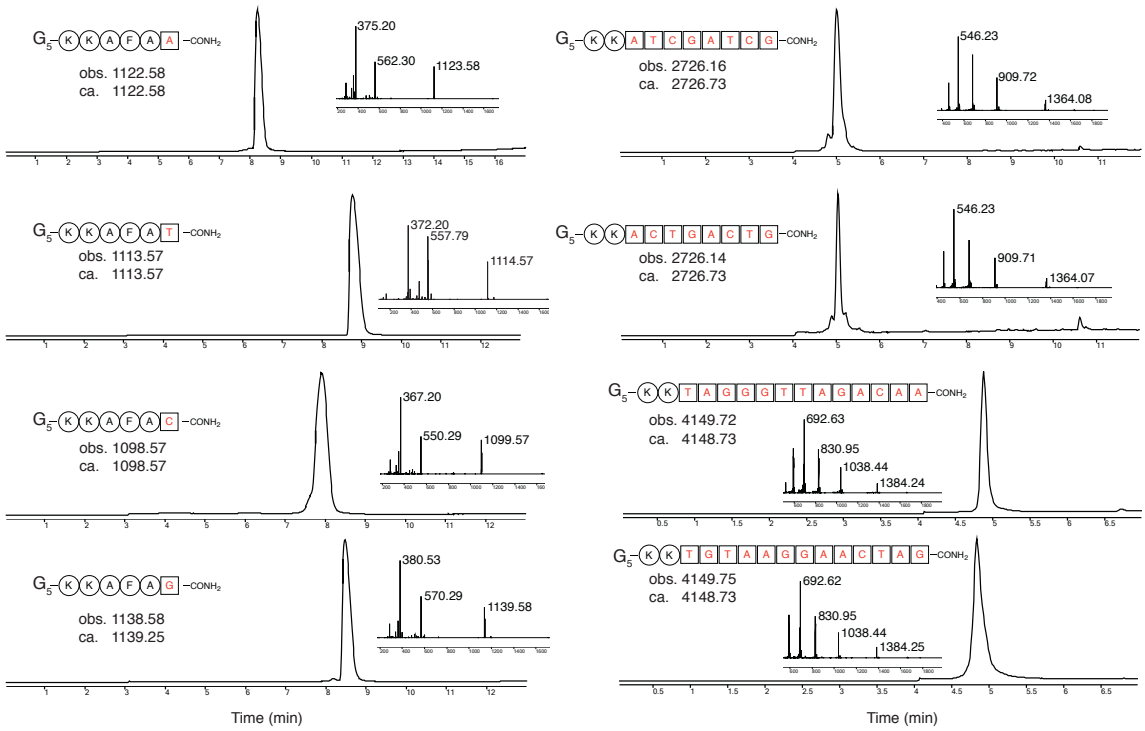

Figure S1. LC/MS analysis of G_5-PNAs **2**–**5** (left column) and **7**–**10** (right column), which comprise five Gly and two Lys residues on the N-terminus that are followed by the corresponding PNA building blocks. RP-HPLC analysis was performed on an Agilent 1260 system using an Agilent Zorbax 5 µm 300SB-C3 column (2.1 x 150 mm) with a gradient of 1–91% or 1–61% CH_3CN in H_2O with 0.1% formaic acid over 12 min, depending on the G_5-PNA solubility. MS (ESI) analysis was performed with an Agilent 6520 Accurate-Mass quadrupole time-of-flight (Q-TOF) liquid chromatography-mass spectrometry system.

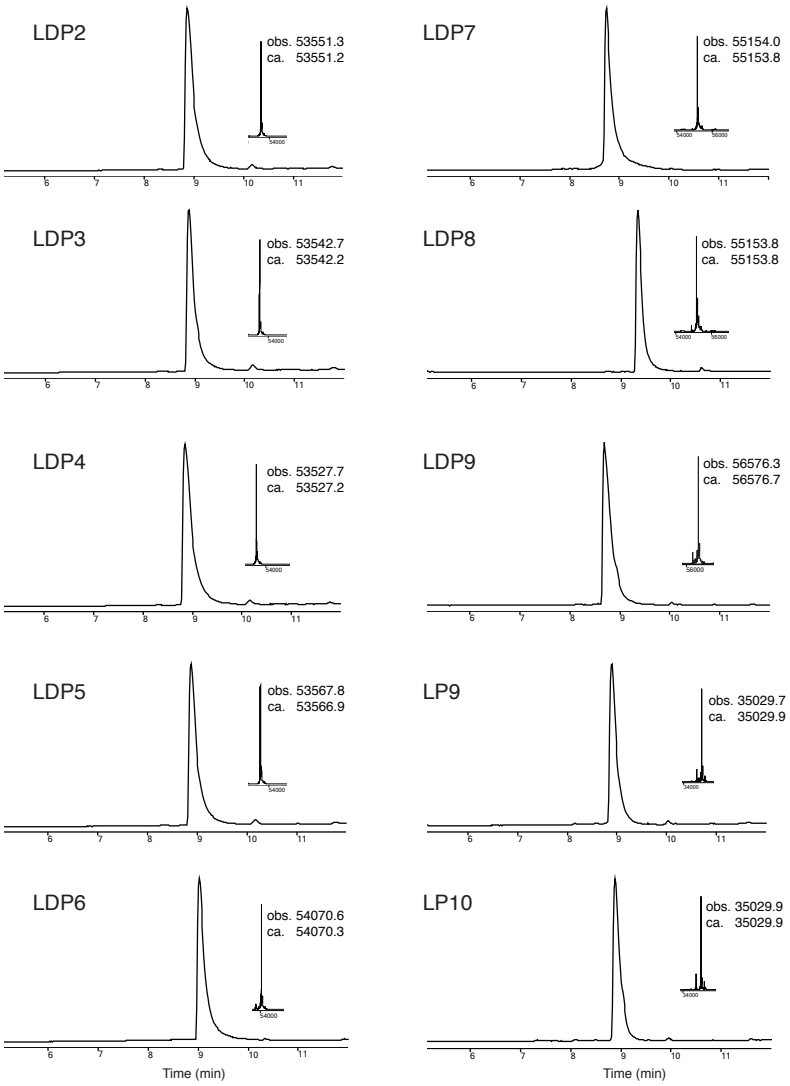

Figure S2. LC/MS analysis of LDPs **2**–**9** and LPs **9** and **10**. RP-HPLC analysis was performed on an Agilent 1260 system using an Agilent Zorbax 5 μm 300SB-C3 column (2.1 x 150 mm) with a gradient of 1–91% or 1–61% CH_3CN in H_2O with 0.1% formaic acid over 12 min, depending on the G_5-PNA solubility. MS (ESI) analysis was performed with an Agilent 6520 Accurate-Mass quadrupole time-of-flight (Q-TOF) liquid chromatography-mass spectrometry system. The deconvoluted mass spectra are shown.

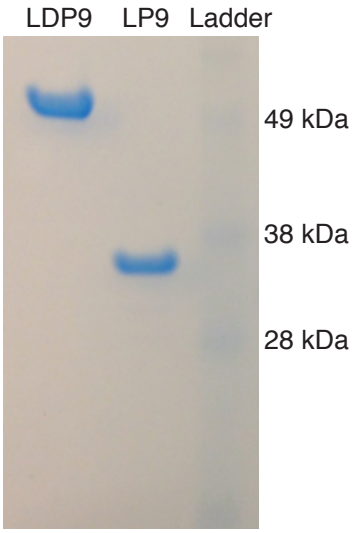

Figure S3. Coomassie-stained SDS-PAGE gel of LDP **9** and LP **9**.

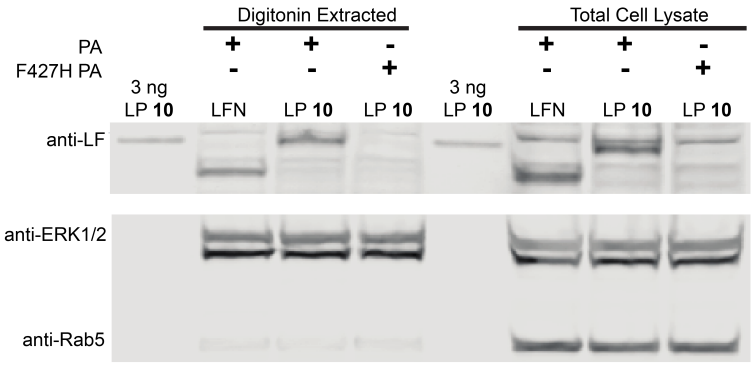

Figure S4. Western blot with HEK-293T cell lysates that shows translocation of LP **10**. Cells were incubated with 250 nM LP **10** in the presence of 40 nM (wild-type) WT or F427H PA for 12 hours, followed by the treatment with a digitonin extraction or total lysis buffer (~1 million cells per lane). Purified LP **9** (3 ng) was also run on the gel for the purpose of quantification.

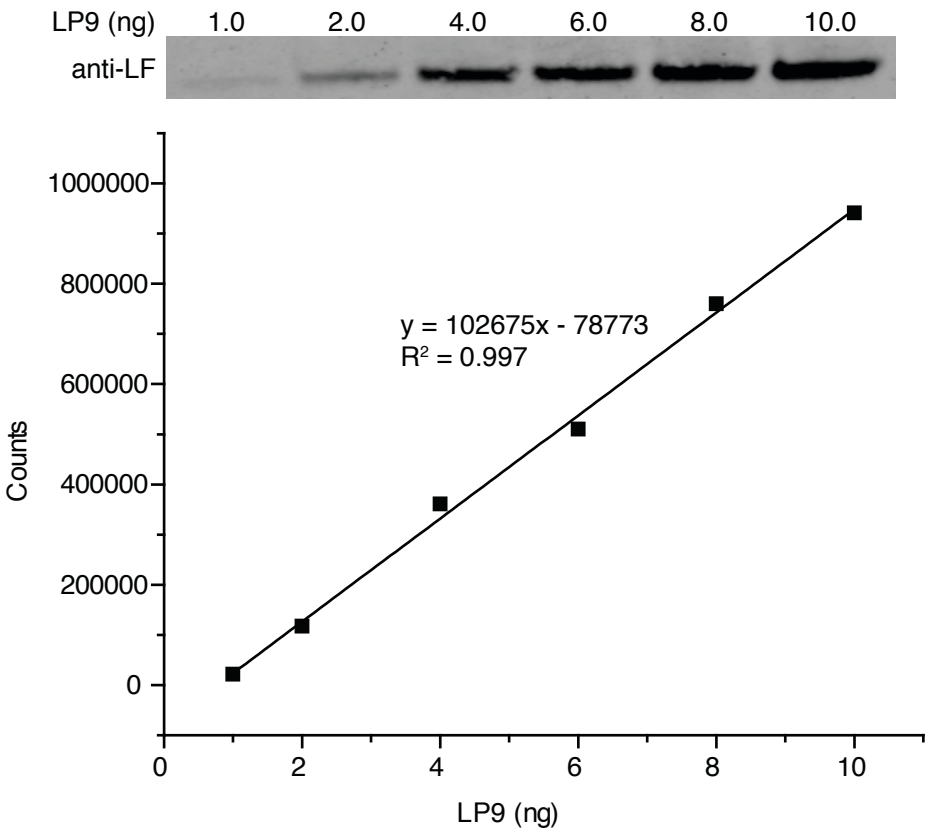

Figure S5. Linear relationship between western blot band intensity (counts) and protein amount loaded (ng). Amounts of 1.0 to 10.0 ng of pure LP **9** were loaded on the gel and the signal intensity of each band was analyzed by Image Studio (LI-COR Biosciences).

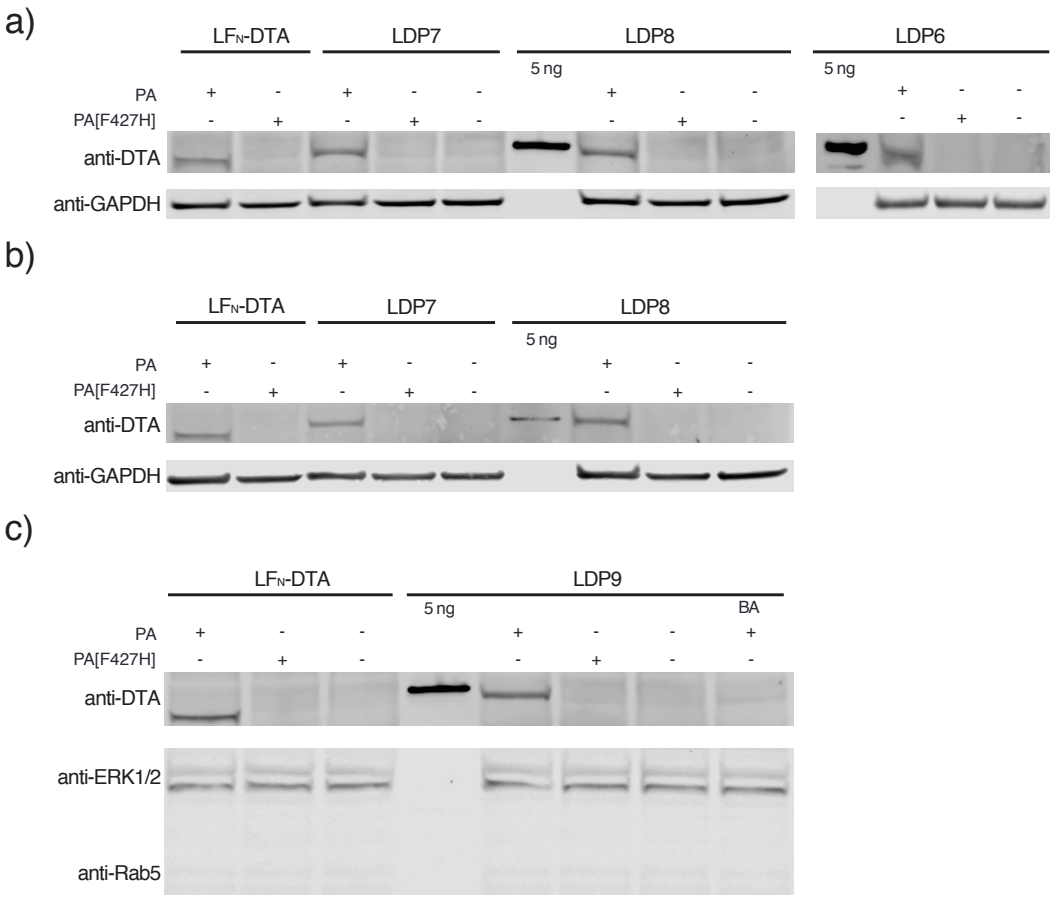

Figure S6. Western blots with HEK-293T and CHO-K1 cell lysates that show translocation of LDPs **6**–**9**. Cells were treated with 100 nM of LDP and 20 nM of PA or PA[F427H] for 12 hours. The cells were subsequently subjected to digitonin extraction. Purified LDP was also run on the gel as a loading control. a) Translocation of LDP **6**–**8** in HEK-293T cells. b) Translocation of LDP **7** and LDP **8** in CHO-K1 cells. c) Translocation of LDP **9** in HEK-293T cells. BA stands for Bafilomycin A1, which inhibits the acidification of the endosome. It was used as a negative control to validate the translocation pathway.

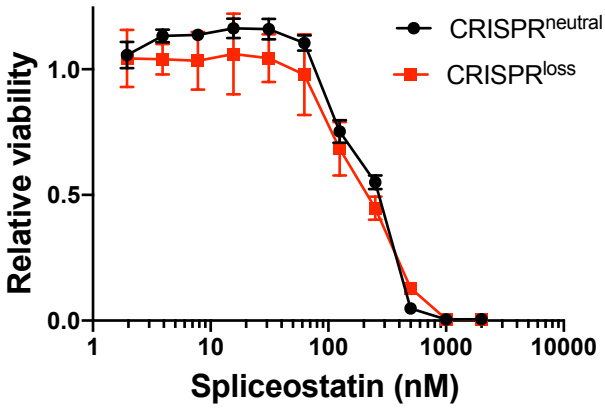

Figure S7. Dose response curve for CRISPR^{neutral} (black) and CRISPR$^{copy\text{-}loss}$ cells (red) upon treatment with the splicing modulator, Spliceostatin A. Relative viability was quantified by Cell titer glo and normalized to DMSO vehicle control. Data represent the mean of three replicate wells ± the standard deviation (± SD).

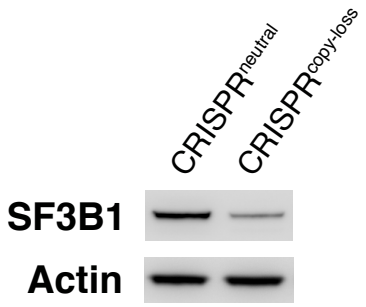

Figure S8. SF3B1 immunoblot in CRISPR^{neutral} and CRISPR$^{copy\text{-}loss}$ cells. Isogenic CAL-51 cells treated with transient transfection of Cas9 sgRNAs upstream and downstream of the *SF3B1* locus and screened for single copy *SF3B1* deletion. Cells were then lysed and evaluated for the effects of partial *SF3B1* copy-number loss on protein levels.

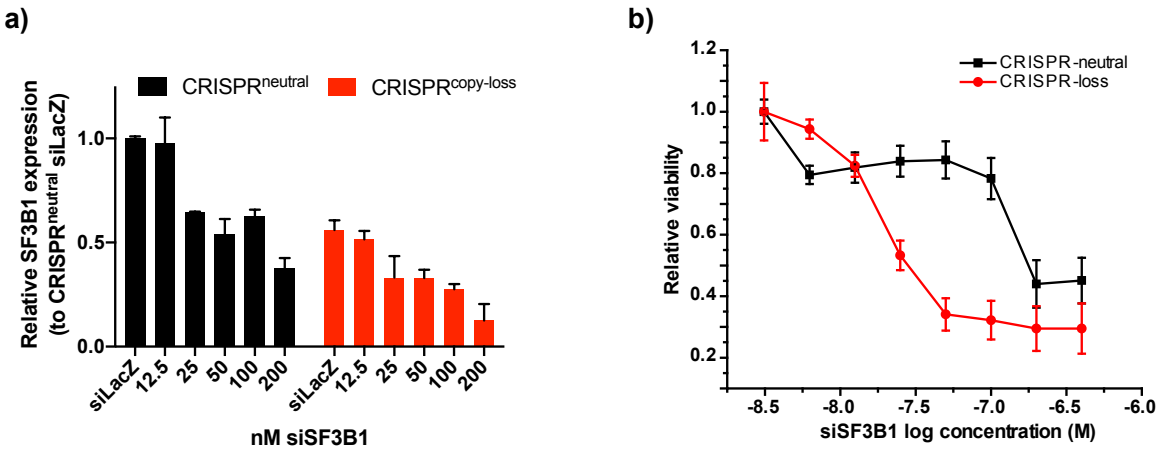

Figure S9. siRNA suppression of SF3B1 in CRISPR^{neutral} and CRISPR$^{copy\text{-}loss}$ CAL-51 cells. (a) SF3B1 mRNA expression level upon siRNA transfection by quantitative PCR with the indicated concentrations of siRNA. Relative SF3B1 expression was normalized to siLacZ transfected cells in CRISPR^{neutral} cells (black) and CRISPR^{loss} cells (red). (b) Relative cell viability of CRISPR^{neutral} (black) and CRISPR$^{copy\text{-}loss}$ (red) cells upon siRNA-mediated suppression of SF3B1. Cell viability calculated as the fold change in Cell Titer-Glo luminescence relative to a siLacZ control. Data represent the mean of three replicate wells ± the standard deviation (± SD).

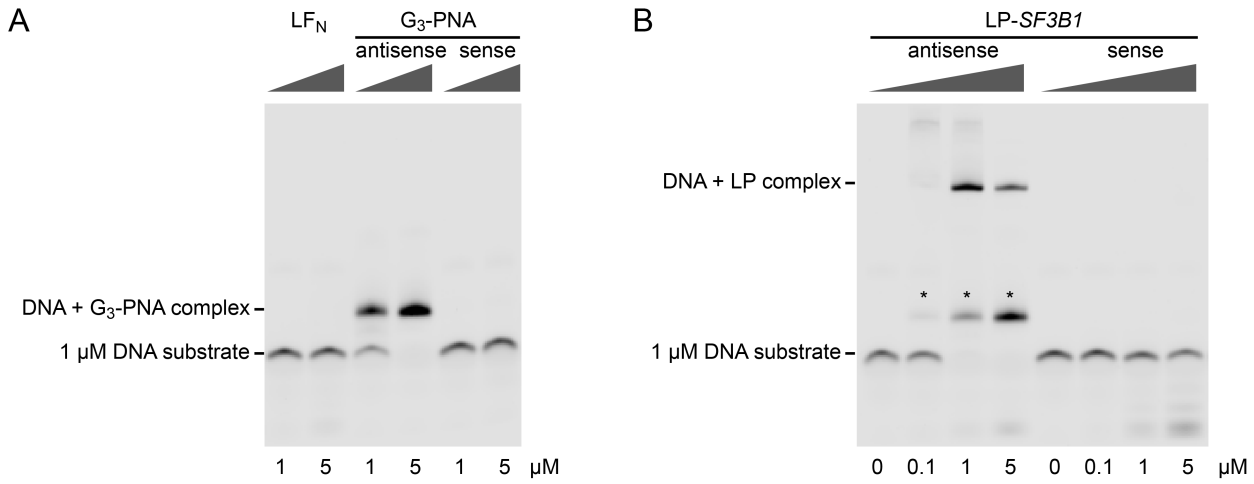

Figure S10. Binding analysis of sense and antisense *SF3B1* PNAs to a DNA substrate by an electrophoretic mobility shift assay. 1 μM of the DNA-Cy3 probe was co-incubated with the indicated sense or antisense *SF3B1* PNA construct for 2 hours at room temperature. The mixture was then subjected to agarose gel electrophoresis, imaged with a Typhoon FLA 7000 scanner (GE), and analyzed based on the shift in the band for the DNA-Cy3 substrate. (A) Analysis of DNA-Cy3 binding to: (A) LF_N only, antisense G_3-PNA-*SF3B1*, and sense G_3-PNA-*SF3B1*; (B) antisense and sense LP-*SF3B1*. The asterisks (*) indicate bands from DNA-Cy3 that form a complex with residual G_3-PNA from the sortase-mediated ligation reaction.

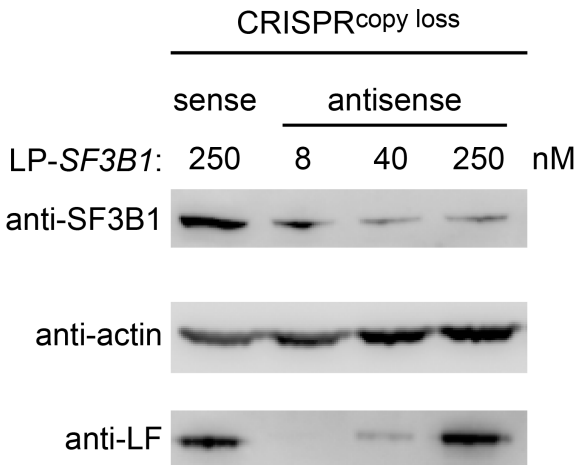

Figure S11. Immunoblot showing dose-dependent suppression of the SF3B1 protein in CRISPR$^{copy\text{-}loss}$ cell lysate after incubation with 8, 40, or 250 nM sense or antisense LP-*SF3B1* and 50 nM PA. Cell lysates were collected after a four-day incubation time. The western blot was visualized with anti-SF3B1, anti-LF, and anti-actin (as a loading control).

II. METHODS

Identification of cancer gene dependencies

Cancer gene dependencies were identified from analyses using a genome-scale shRNA viability screen from project Achilles (version 2.4.3, https://portals.broadinstitute.org/achilles/datasets/5/download). 216 cell lines were profiled for their sensitivity to suppression of 5711 genes and had mutation calls from hybrid capture sequencing of commonly mutated cancer driver genes (*KRAS*, *PIK3CA*, *BRAF*, *NRAS*, *CTNNB1* and *EGFR)*. Each cell line was classified as sensitive to suppression of each gene using an ATARiS[50] gene dependency threshold of >-0.75. For each gene, we performed a two-class comparison between cell lines, with and without mutation of each of the driver genes above. Statistical significance was calculated using a Fisher's exact test and the false discovery rate (FDR)-adjusted p values were calculated using the Benjamini and Hochberg method.[51] Gene dependencies identified from the mutation of those driver genes were then combined with a list of copy-number associated gene dependencies, which were previously identified by our group.[2]

Estimation of the druggability of cancer gene dependencies

Classification of candidate cancer gene dependencies in traditionally druggable protein target families was facilitated by integrating information from The British Pharmacological Society (BPS) and the International Union of Basic and Clinical Pharmacology (IUPHAR) database on targets and families accessed on March 23rd 2017 (http://www.guidetopharmacology.org/DATA/targets_and_families.csv). Cancer gene dependencies that were found in a traditionally druggable protein family were designated a "potentially druggable target", but were otherwise designated "challenging to drug".

Protein expression and purification

His_6-SUMO-LF_N-DTA(C186S)-LPSTGG-His_6, His_6-SUMO-LF_N-LPSTGG-His_6, SrtA*-His_6, wild-type (WT) protective antigen (PA), and PA[F427H] were expressed in *E. coli* BL21 (DE3) cells at New England Regional Center of Excellence/Biodefense and Emerging Infectious Diseases (NERCE). The cells were resuspended in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer containing Roche protease inhibitor cocktail and lysed by sonication. The supernatant was subsequently loaded on the HisTrap FF crude Ni-NTA column (GE Healthcare, UK) and eluted with 500 mM imidazole. The protein buffer was then changed into 20 mM Tris-HCl, 150 nM NaCl, pH 8.5 buffer using a HiPrep 26/10 Desalting column (GE Healthcare). WT PA and PA[F427H] were purified from the periplasm of *E. coli* BL21 (DE3) cells.

Synthesis of peptide nucleic acids (PNAs)

Peptide nucleic acids (PNAs) were synthesized with Fmoc-based PNA synthesis on a 0.05 mmol scale using aminomethyl resin with a Rink-amide linker. After the synthesis, the PNAs were cleaved from resin with a mixture of TFA/m-cresol (4:1) for 2 h, then dried under a stream of N_2 (g). After the PNAs were dry, they were triturated with cold ether (3x), suspended in a solution of 50:50 H_2O/CH_3CN with 0.1 % TFA, and lyophilized. All PNAs contained two Lys residues to increase solubility and contained three or five Gly residues at their N-terminus to perform a subsequent sortase-mediated ligation.

Peptide and PNA purification

The crude peptides and PNAs were dissolved in 99:1 H_2O/CH_3CN that contained 0.1% TFA, then were purified by semi-preparative RP-HPLC. The compounds were loaded on an Agilent

Zorbax 300SB-C18 column (9.4 x 250 mm, 5 μm) at a flow rate of 4 mL/min, then eluted with a gradient of 1-31% CH_3CN over 100 min. The clean fractions were pooled, lyophilized, and analyzed by either MALDI or LC/MS. The final isolated yields ranged from 5% to 10%.

Sortase-mediated ligation

The sortase-mediated ligation reactions were performed using SrtA* according to the previously reported conditions.[23] LF_N-DTA-LPSTGG-H_6 or LF_N-LPSTGG-H_6 (100 μM) was combined with a G_5-PNA (500 μM) and SrtA* (10 μM) in the sortase reaction buffer (10 mM $CaCl_2$, 50 mM Tris, 150 mM NaCl, pH 7.5). After gently rotating the reaction mixture for 30 min., Ni-NTA beads were added to the reaction mixture (for removal of GG-H_6, H6-SrtA*, and any remaining starting material). The supernatant was then subjected to 4 rounds of buffer exchange (20 mM Tris, 150 mM NaCl, pH 7.5) to remove the excess G_3-PNA or G_5-PNA.

Analytical liquid chromatography-mass spectrometry (LC-MS)

LC/MS analysis was performed on an Agilent 6520 Accurate-Mass quadrupole time-of-flight (Q-TOF) liquid chromatography-mass spectrometry system. Analytical RP-HPLC was performed on an Agilent Zorbax 300SB-C3 column (2.1 x 150 mm, 5 μm) with H_2O and CH_3CN that contained 0.1% formic acid, and an elution gradient of either 5 to 65% CH_3CN over 15 min or 1 to 61% CH_3CN over 12 min, depending on the solubility of the construct.

Protein synthesis inhibition assay

The CHO-K1 cells were maintained in F-12K media supplemented with 10% (v/v) fetal bovine serum at 37 °C and 5% CO_2. The cells were plated at 30K cells per well in a 96-well plate one day

before the assay. On the day of experiment, the cells were treated with the LDPs in the presence of 20 nM PA for 30 min at 37 °C and 5% CO_2. The cells were then washed with PBS and incubated with medium supplemented with 1 μCi/mL ^{3}H-leucine (Perkin Elmer, MA) for 1 h at 37 °C. The radiation from incorporation of ^{3}H-Leu into cellular proteins was measured to determine the inhibition of protein synthesis with a scintillation counter. All counts were normalized to cells treated with only PA and all experiments were done in triplicate.

Cytosolic protein extraction and western blot

CHO-K1 or HEK-293T cells were incubated with 250 nM of LDPs or LPs and 40 nM of PA or PA[F427H] for 12 h at 37 °C and 5% CO_2. The cells were then trypsinized and washed with PBS. For cytosolic extraction, ~1 million cells were resuspended in 50 - 100 μg/mL digitonin in 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose supplemented with Roche protease inhibitor cocktail on ice for 10 min and spun down at 13,000 rpm for 5 min at 4 °C. For total cell lysis, lysis buffer (pH 7.5) with 25 mM Tris, 150 mM NaCl, 1% v/v NP-40 supplemented with Roche protease inhibitor cocktail was used. Cells were lysed on ice for 30 min and spun down for 10 min at 4 °C. Subsequently, the supernatants from both methods were run on an SDS-PAGE gel and then transferred onto a nitrocellulose membrane soaked in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol) using a TE 70 Semi-Dry Transfer Unit (GE). After the transfer was finished, the membrane was blocked with LI-COR blocking buffer at room temperature for 2 h before it was blotted against anti-LF or anti-DTA or anti-ERK1/2 and anti-Rab5 in TBST overnight at 4 °C. The membrane was then washed and incubated with appropriate secondary antibodies for 1 h at room temperature and imaged with the LI-COR Odyssey infrared imaging system. The signal intensity of each band was quantified by drawing a rectangle around

it and measuring the average signal within the rectangle using the analysis tool in Image Studio (LI-COR Biosciences).

mRNA gene transcript for *SF3B1* (NCBI Reference Sequence: NM_012433.2)

The **bolded** region indicates the target for the sense and antisense LP-*SF3B1* constructs. The underlined region indicates the translated region.

GGAAGTTCTTGGGAGCGCCAGTTCCGTCTGTGTGTTCGAGTGGACAAAATGGCGAAGATCGCCAAGACTCACGAA
GATATTGAAGCACAGATTCGAGAAATTCAAGGCAAGAAGGCAGCTCTTGATGAAGCTCAAGGAGTGGGCCTCGA**T**
TCTACAGGTTATTATGACCAGGAAATTTATGGTGGAAGTGACAGCAGATTTGCTGGATACGTGACATCAATTGCT
GCAACTGAACTTGAAGATGATGACGATGACTATTCATCATCTACGAGTTTGCTTGGTCAGAAGAAGCCAGGATATC
ATGCCCCTGTGGCATTGCTTAATGATATACCACAGTCAACAGAACAGTATGATCCATTTGCTGAGCACAGACCTCC
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Generation of isogenic SF3B1^{loss} cells by CRISPR Cas9

The diploid breast cancer cell line, CAL-51, was used to create daughter cell lines that differed only in *SF3B1* copy number. We designed gRNAs that target intergenic loci upstream and downstream of the *SF3B1* locus to delete the entire gene. The 5' gRNA targeted one allele of a heterozygous SNP (rs3849362) to bias towards heterozygous *SF3B1* deletion because homozygous SF3B1 deletion is lethal. Oligonucleotides with BbsI overhangs were annealed and cloned into pX458 that also encodes Cas9 and GFP (Addgene plasmid #48138). For the 5' guide targeting rs3849362, 5' CACCGCGCATTATAGATTATGGCCC (forward) and 5' AAACGGGCCATAATCTATAATGCGC (reverse). For the 3' targeting guide: 5'CACCGCGGAGTTTCATCCGTTACAC (forward), AAACGTGTAACGGATGAAACTCCGC (reverse). pX458 plasmids with either the 5' or 3' targeting guides were co-transfected into cells and GFP+ single cells were plated at 1 cell per well by FACS.

PNA and siRNA delivery for cell viability assays using CellTiter-Glo

Estimation of cell viability was performed by measuring ATP-promoted luminescence. Prior to the experiment, the cell suspensions were seeded (100 μL) in 96-well plates at a density of 1.0 × 10^4 cells/mL. After 4 to 16 h, the LF_N and PA components were added and the cells were incubated

at 37°C and 5% CO_2. After seven days, the cell viability was measured with the CellTiter-Glo luminescence assay. For siRNA transfection, the same plating conditions were used, except a pool of four SF3B1 siRNAs were transfected in a 6 well plate for 24 h prior to plating using Oligofectamine. The sequences for the siRNAs were first reported in (Massiello et al., 2006)[52] and are as follows:

siSF3B1 #1 (sense) 5'-GGA AUU AGA UGC UAU GUU CUU, (antisense) 5'-GAA CAU AGC AUC UAA UUC CUU.

siSF3B1 #2 (sense) 5'-GCA AAC GAG UCA AAC CAU AUU, (antisense) 5'-UAU GGU UUG ACU CGU UUG CUU

siSF3B1 #3 (sense) 5'-GAA CCG CUA UUG AUU GAU GUU, (antisense) 5'-CAU CAA UCA AUA GCG GUU CUU

siSF3B1 #4 (sense) 5'-GUA GAA UGU UGC AAU AUU GUU, (antisense) 5'-CAA UAU UGC AAC AUU CUA CUU

siLacZ control (sense) 5'-UGU UCG CAU UAU CCG AAC CUU, (antisense) 5'-GGU UCG GAU AAU GCG AAC AUU

Cellular sensitivity to chemical modulation of the spliceosome

Cells were plated at 10,000 cells per well in 96-well plates. 24 hours after seeding, cells were treated with the indicated concentrations of splicing modulator. Relative cell viability was expressed as the percent change in CellTiter-Glo luminescence normalized to vehicle (DMSO) treated cells. Spliceostatin A was a gift from Dr. Minoru Yoshida.[30]

Splicing luciferase reporter assays

Polyclonal, stable CRISPR^{neutral} and CRISPR$^{copy\text{-}loss}$ cells were generated by transfection and hygromycin selection. Cells expressed either CMV-LUC2CP/intron/ARE (Luc-I in this manuscript, Addgene plasmid # 62858) or CMV-LUC2CP/ARE (Luc-ORF in this manuscript, Addgene plasmid # 62857) which were gifts from Gideon Dreyfuss.[53] To calculate the fold change in splicing activity, the ratio of Luc-I:Luc-ORF was calculated after normalizing to sense SF3B1 PNA treated cells.

III. SUPPLEMENTAL NOTES

Definitions of terms:

Gene dependency – A gene that, when suppressed by RNAi, results in decreased cellular fitness.

Somatic genetic alteration – A genetic change that deviates from the normal germline DNA sequence, including mutations or copy number changes. These alterations accumulate in cells and contribute to cellular transformation.

Cancer gene dependency – A gene dependency that is associated with a cancer specific somatic genetic alteration. The association with a genetic alteration suggests the gene is only required for cancer, but not normal, cell survivial because the normal cells lack the associated genetic event

Driver gene – A gene, that when altered by a somatic genetic alteration (i.e. mutation or copy number change), contributes to cellular transformation and cancer.