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Supplemental Information

Centyrin ligands for extrahepatic delivery of siRNA

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Supplemental data

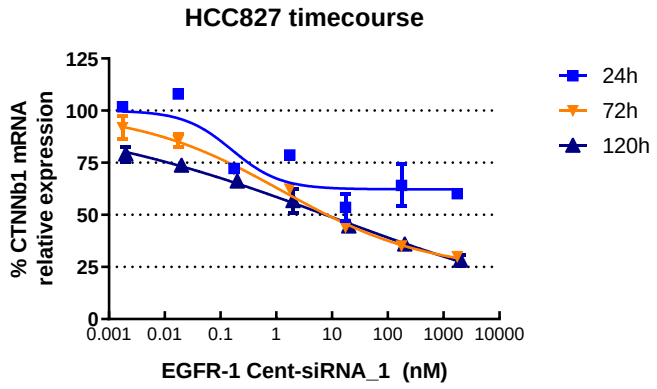
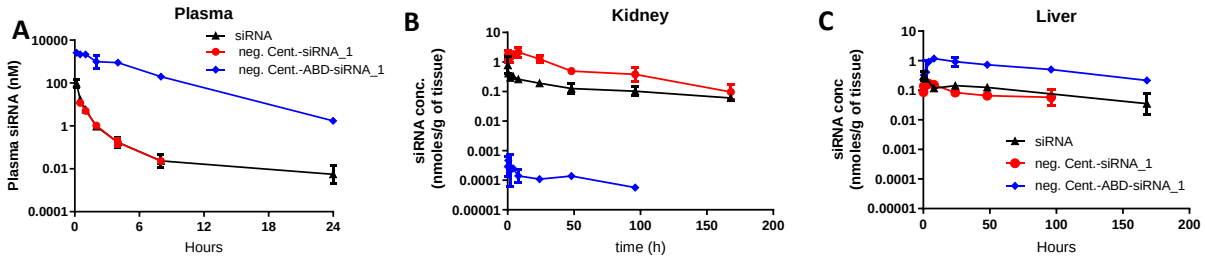


Figure S1: HCC827 cells were treated with a dose range of and EGFR-CTNNb1 siRNA conjugate (EGFR-1 Cent-siRNA_1) for 24h, 72h, or 120 and CTNNb1 mRNA expression was measured by RT/qPCR. Knockdown was detected as early as 24h after treatment and maximal at 72h. mRNA knockdown persisted at least through 120h.



D

Area under the curve (nM*h)	Plasma	Kidney	Liver
siRNA	30.4	22.0	17.2
neg. Cent.-siRNA_1	16.3	106.0	8.1
neg. Cent.-ABD-siRNA_1	9339.0	0.01	100.2

Figure S2: BALB/c mice were dosed intravenously with a single dose of free siRNA or non-binding Centyrins-siRNA conjugates with (neg. Cent-ABD-siRNA_1) or without an ABD (neg-Cent-siRNA_1), where all compounds contained 3mpk siRNA. siRNA levels in A) plasma, B) kidney and C) liver were detected at various timepoints after dosing by stem-loop PCR. D) Area under curve for each tissue are shown. N=3 per group. Data represent average +/-SEM.

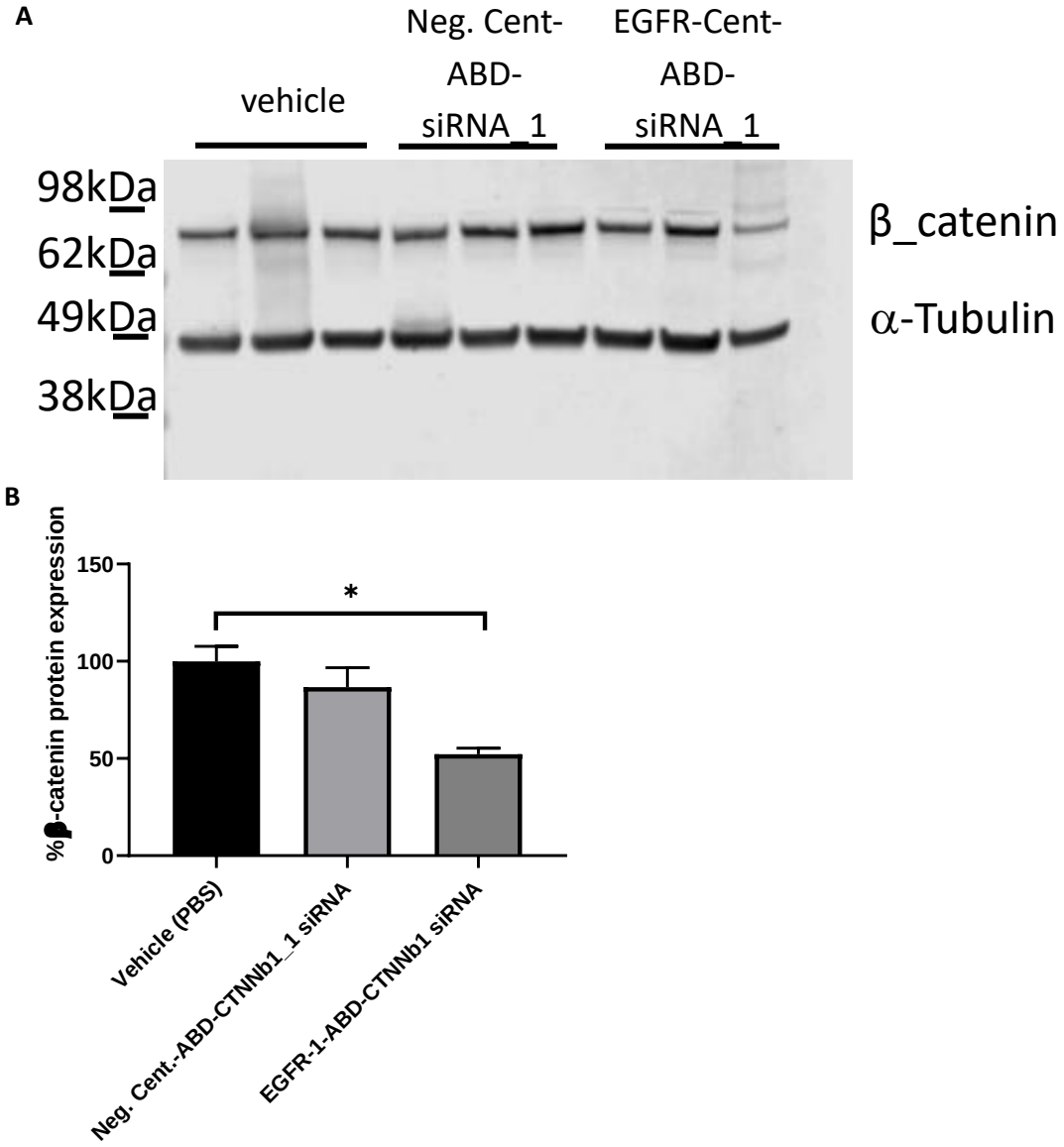


Figure S3: Mice bearing A431 tumor xenografts were dosed three times every other day with targeted or non-targeted siRNA conjugates containing 10mpk siRNA. Tumors were excised 72h following the final dose and b-catenin was assessed by western blot following tissue lysis (N=3 mice per group). A) Protein from tumor lysates were stained for b-catenin (top band) or alpha-tubulin (bottom band). B) Relative b-catenin protein levels were normalized to alpha-tubulin by band intensity analysis. Nearly 50% loss of b-catenin protein was detected in tumors isolated from mice dosed with EGFR-1-ABD-CTNNb1 siRNA. Data are plotted as average +/- SEM. *indicates $p<0.05$ vs vehicle.

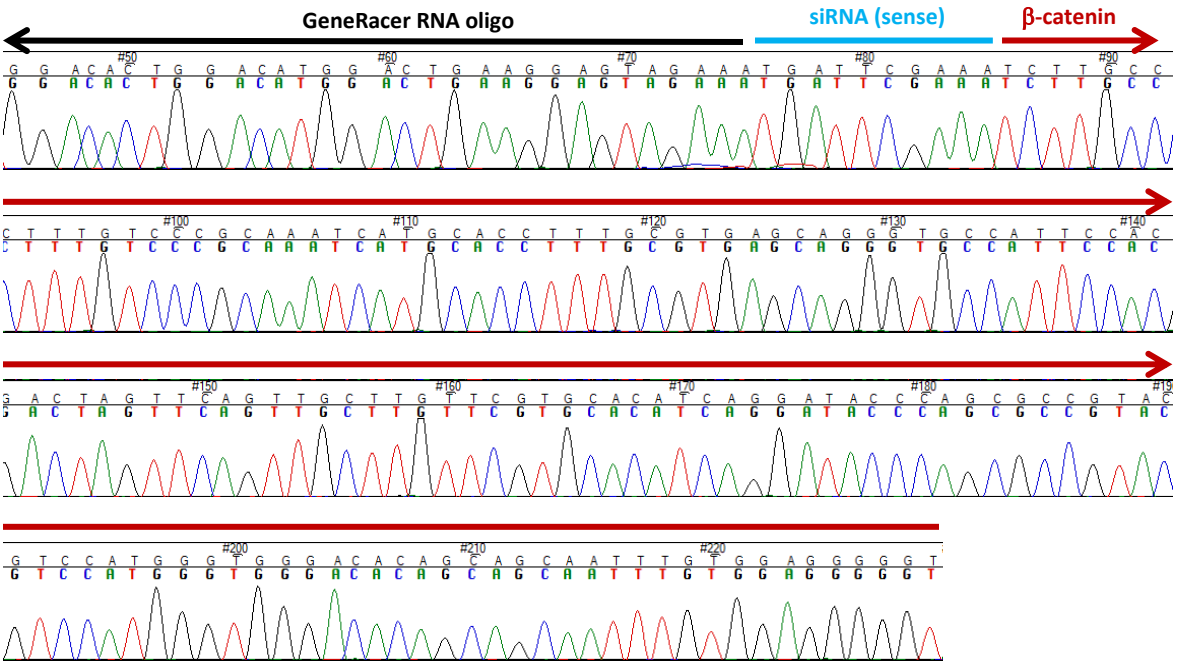


Figure S4: Mice bearing A431 tumor xenografts were treated with three doses (every other day) of Centyrin-siRNA conjugates containing 10mpk siRNA. mRNA cleavage fragments were evaluated from RNA isolated from EGFR-ABD-siRNA-1 treated tumor tissue with a 5' RACE assay. The PCR products were run on an agarose gel and mRNA was extracted from the 200bp band. RNA sequencing revealed a nearly perfect match with the target cleavage site, as indicated. The CTNNb1 gene sequence is indicated by the red lines, siRNA binding sequence is indicated by the blue line and a portion of GeneRacer RNA adapter amplified by inner primer and the inner gene specific primers is shown with black arrow. Remaining sequence was from the cloning vector but is not shown.

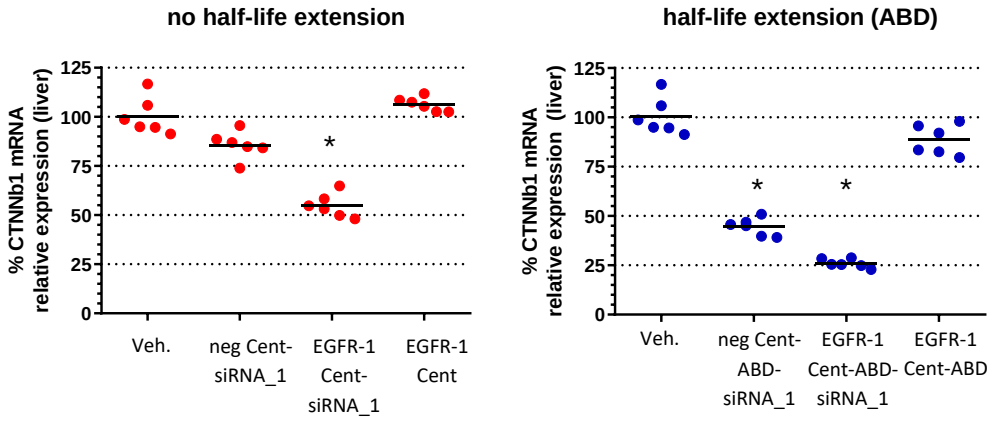
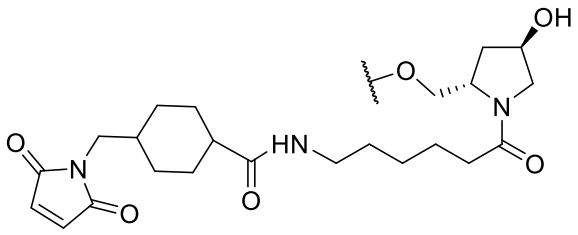
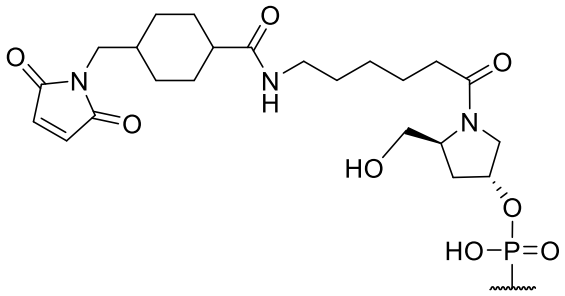


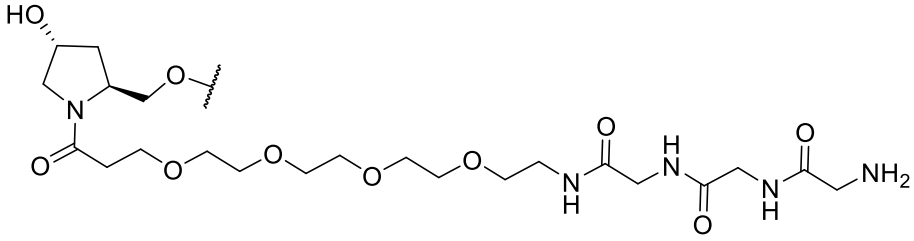
Figure S5: Mice bearing A431 tumor xenografts were treated with three doses (every other day) of Centyrin-siRNA conjugates containing 10mpk siRNA or equimolar levels of Centyrin only. Tissue was extracted 72h after final dose and RNA was isolated for assessment of mRNA knockdown by RT/qPCR. Knockdown is shown in liver from mice dosed with conjugates A) without half-life extension and B) containing an ABD for half-life extension. N=6 per group. Data represents average +/- SEM. * indicates $p<0.001$ vs. vehicle controls.



Structure of L1: 3’ maleimide linker



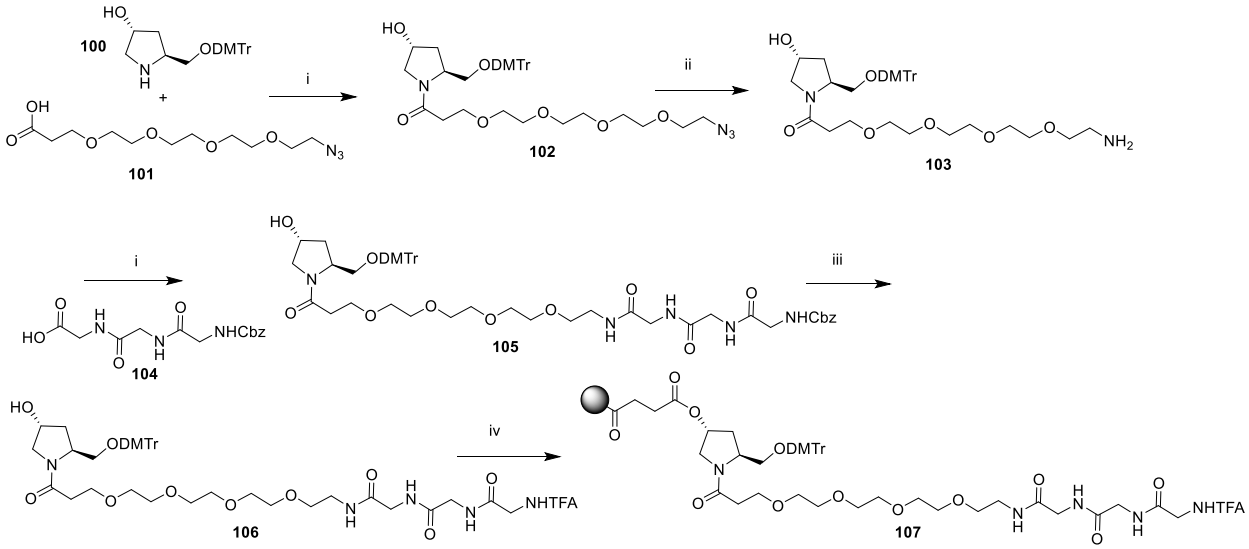
Structure of Q: 5’ maleimide linker



Structure of L2: 3’ Gly_3 linker

Synthesis of L2 CPG Loaded support:

Scheme 1



i) HBTU, DIEA, DMF; ii) H2, Pd/C (5% wet, Degussa type), EtOAc/MeOH iii) a. H2, Pd/C (5% wet, Degussa type), EtOAc/MeOH b. Ethyl trifluoroacetate, Triethylamine, EtOAc/MeOH; iv) a. Succinic anhydride, DMAP, Triethylamine, DCM; b. HBTU, DIEA, LCAA CPG, Acetonitrile; c. Acetic anhydride, Triethylamine, Pyridine

Compound **102**: Azido carboxylic acid (7.00g, 24.04 mmol) was dissolved in 90 mL of DMF in a round bottom flask under argon and cooled the solution in an ice bath. HBTU (9.12g, 24.04 mmol) and DIEA (8.36 ml, 48.05 mmol) were added and stirred mixture for 5 minutes. A solution of amine **100** (10.07g, 24.04 mmol) in 15 mL of DMF was added and mixture stirred overnight. TLC checked and the reaction mixture was poured into an ice-water, extracted with ethyl acetate, washed the organic layer with brine, dried over anhydrous sodium sulfate and removed the solvents. Residue was purified by silica gel chromatography using mixture of dichloromethane/methanol (gradient elution). Fractions were collected and evaporated under reduced pressure to give **102** (16.00g, 96%) as yellow viscous liquid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.40 – 7.13 (m, 8H), 6.90-6.80 (m, 5H), 5.05 – 4.84 (m, 1H), 4.37 (q, *J* = 4.7 Hz, 1H), 4.22 – 4.06 (m, 1H), 3.72 (s, 6H), 3.66 – 3.19 (m, 22H), 3.12 (dd, *J* = 8.8, 5.4 Hz, 1H), 3.06 – 2.96 (m, 1H), 2.10 – 1.75 (m, 2H). Mass calc. for $C_{37}H_{48}N_4O_9$ 692.34; found 693.345 (M+H).

Compound **103**: Azido derivate **102** (11.00g, 15.88 mmol) was dissolved in a mixture of EtOAc/MeOH (4:1, 220 mL), degassed the mixture with argon and Pd/C (1.00g, wet Degussa type 5 wt%). The mixture was hydrogenated under balloon pressure of hydrogen overnight. Reaction was monitored by TLC, once the reaction is over, filtered the solution over celite, washed the cake with EtOAc and MeOH. Solvents were removed and the residue dried under high vacuum overnight to get **103** as pale-yellow viscous liquid. This was used for next reaction without any further purification. Mass calc. for $C_{37}H_{50}N_2O_9$ 666.35; found 667.352 (M+H).

Compound **105**: Carboxylic acid **104** (4.52g, 14.00 mmol) was dissolved in 80 mL of DMF in a round bottom flask under argon and cooled the solution in an ice bath. HBTU (5.57g, 15.30 mmol) and DIEA (7.67 ml, 44.10 mmol) were added and stirred mixture for 5 minutes. A solution of amine **103** (10.20g, 15.30 mmol) in 20 mL of DMF was added and mixture stirred overnight. TLC checked and the reaction mixture was poured into an ice-water bath, extracted with ethyl acetate, washed the organic layer with brine, dried over anhydrous sodium sulfate and removed the solvents. Residue was purified by silica gel chromatography using mixture of dichloromethane/methanol (gradient elution). Fractions were collected and evaporated under reduced pressure to give **105** (10.10g, 70%) as a pale-yellow viscous liquid. ^{1}H NMR (400 MHz, DMSO) δ 8.22 – 8.06 (m, 2H), 7.85 (t, *J* = 5.6 Hz, 1H), 7.49 (t, *J* = 6.0 Hz, 1H), 7.41 – 7.13 (m, 13H), 6.90-6.75 (m, 5H), 5.11 – 4.87 (m, 3H), 4.38 (q, *J* = 4.7 Hz, 1H), 4.14 (td, *J* = 5.3, 2.6 Hz, 1H), 3.80 – 3.54 (m, 14H), 3.54 – 3.27 (m, 16H), 3.27 – 2.90 (m, 5H), 2.07 – 1.78 (m, 3H). Mass calc. for $C_{51}H_{65}N_5O_{14}$ 971.45; found 972.448 (M+H).

Compound **106**: Cbz derivative **105**(6.40g, 6.58 mmol) was dissolved in a mixture of EtOAc/MeOH (1:1, 200 mL), degassed the mixture with argon and Pd/C (0.700g, wet Degussa type 5 wt%). The mixture was hydrogenated under balloon pressure of hydrogen overnight. Reaction was monitored by TLC, once the reaction is over, filtered the solution over celite, washed the cake with EtOAc and MeOH. Solvents were removed and the residue dried under high vacuum overnight to get amine as pale-yellow viscous liquid. Crude amine (5.80g) was dissolved in a mixture of DCM/Methanol (1:1, 100 mL) under argon. Ethyl trifluoroacetate (3g, 21 mmol) and triethylamine (3 mL, 22 mmol) were added and stirred the mixture overnight. TLC checked and solvents were removed under reduced pressure. Crude residue was dissolved in dichloromethane, transferred to a separatory funnel and washed successively with water, aqueous bicarbonate solution and brine. Organic layer was dried over anhydrous sodium sulfate and solvents were removed. Residue was purified by silica gel chromatography using mixture of dichloromethane/methanol (gradient elution). Fractions were collected and evaporated under reduced pressure to give **106** (5.5g, 90%, two steps) as a pale-yellow viscous liquid. ^{1}H NMR (400 MHz, DMSO) δ 9.63 (s, 1H), 8.37 (t, *J* = 5.7 Hz, 1H), 8.15 (t, *J* = 5.9 Hz, 1H), 7.86 (t, *J* = 5.7 Hz, 1H), 7.38 – 7.10 (m, 8H), 6.77-6.90 (m, 5H), 5.05 – 4.85 (m, 1H), 4.47 – 4.07 (m, 2H), 3.87 (s, 3H), 3.82 – 3.53 (m, 11H), 3.53 – 3.27 (m, 18H), 3.25 – 2.94 (m, 4H), 2.29 – 1.76 (m, 2H). Mass calc. for $C_{45}H_{58}F_3N_5O_{13}$ 933.40; found 933.411 (M+H).

Compound **107**: Compound **106** (5.70g, 6.10 mmol), succinic anhydride (1.23g, 12.20 mmol), DMAP (0.786 g, 12.20 mmol), and Et_3N (1.64 mL, 12.20 mmol) were dissolved in DCM (100 mL) and stirred for 24 h. The reaction mixture was transferred to separatory funnel, diluted with DCM, and washed with saturated brine. The organic layer was dried over anhydrous Na_2SO_4. Solvents and volatiles were removed under reduced pressure. The residue was dried under high vacuum overnight to obtain the Et_3N salt of the hemi-succinate as an off-white solid (6.20g, crude). The hemi-succinate (6.00g, 5.80 mmol) and HBTU (2.30g, 5.85 mmol) were dissolved in acetonitrile (250 mL). Diisopropylethylamine (DIEA, 3.02 mL, 17.4 mmol) was added to the solution, and the mixture was swirled for 3-4 min followed by addition of LCAA-CPG support (50 g, amine content: 152 μmol/g). The suspension was gently shaken at room temperature on a wrist-action shaker for 24 h then filtered, and washed with DCM, 10% MeOH in DCM, DCM and ether. The solid support was dried under vacuum for 24 h. The unreacted amines on

the support were capped by stirring with 30% acetic anhydride/pyridine containing 1% Et_3N at room temperature for 3 h. The washing of the support was repeated as above. The solid was dried under vacuum for 24 h to yield solid support **107** (53.00 g, 71.00 μmol/g loading).