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Supplemental Information

Engineering highly efficient backsplicing and translation of synthetic circRNAs

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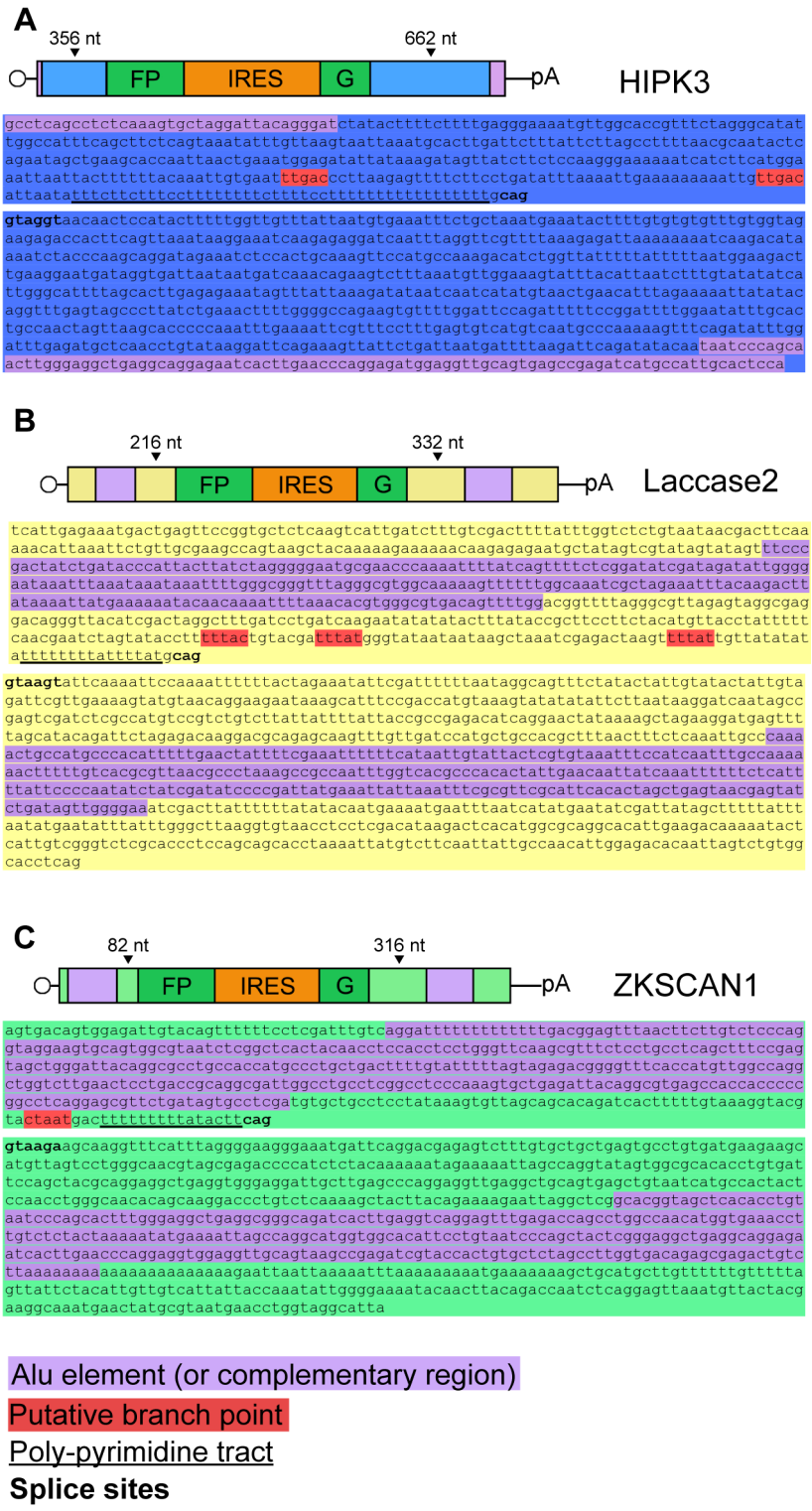
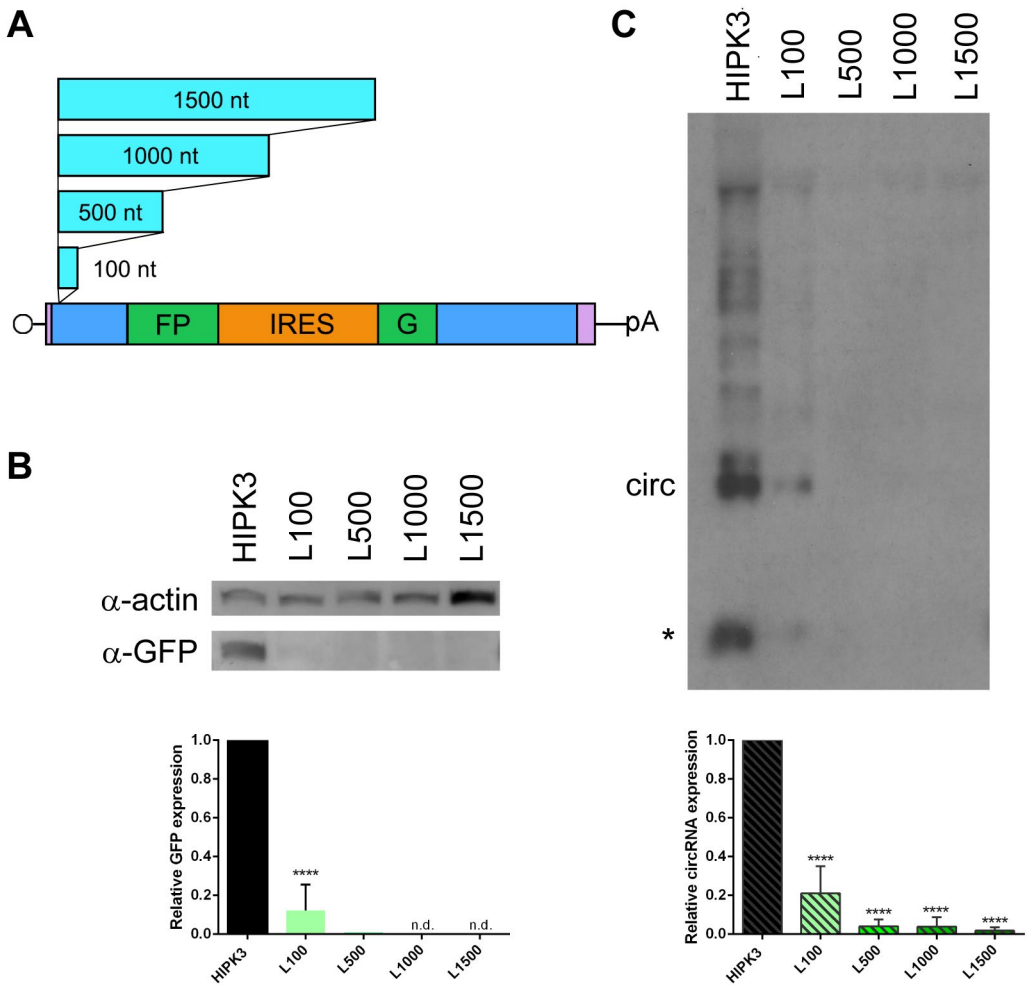
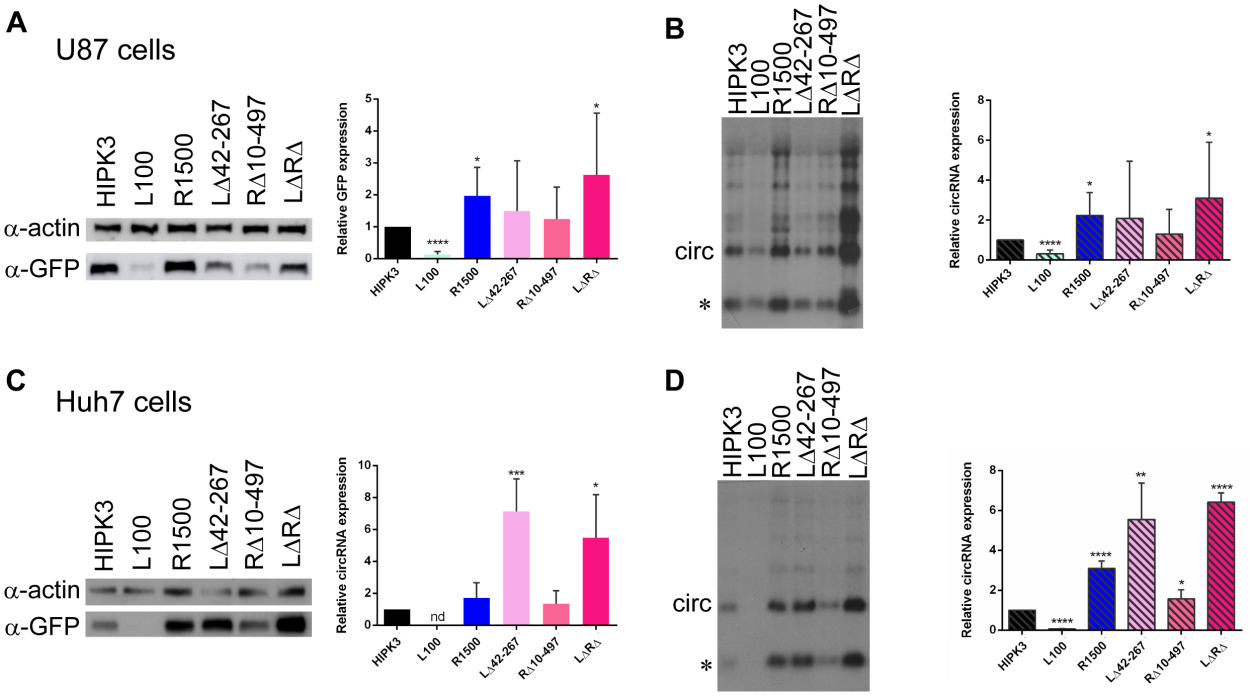


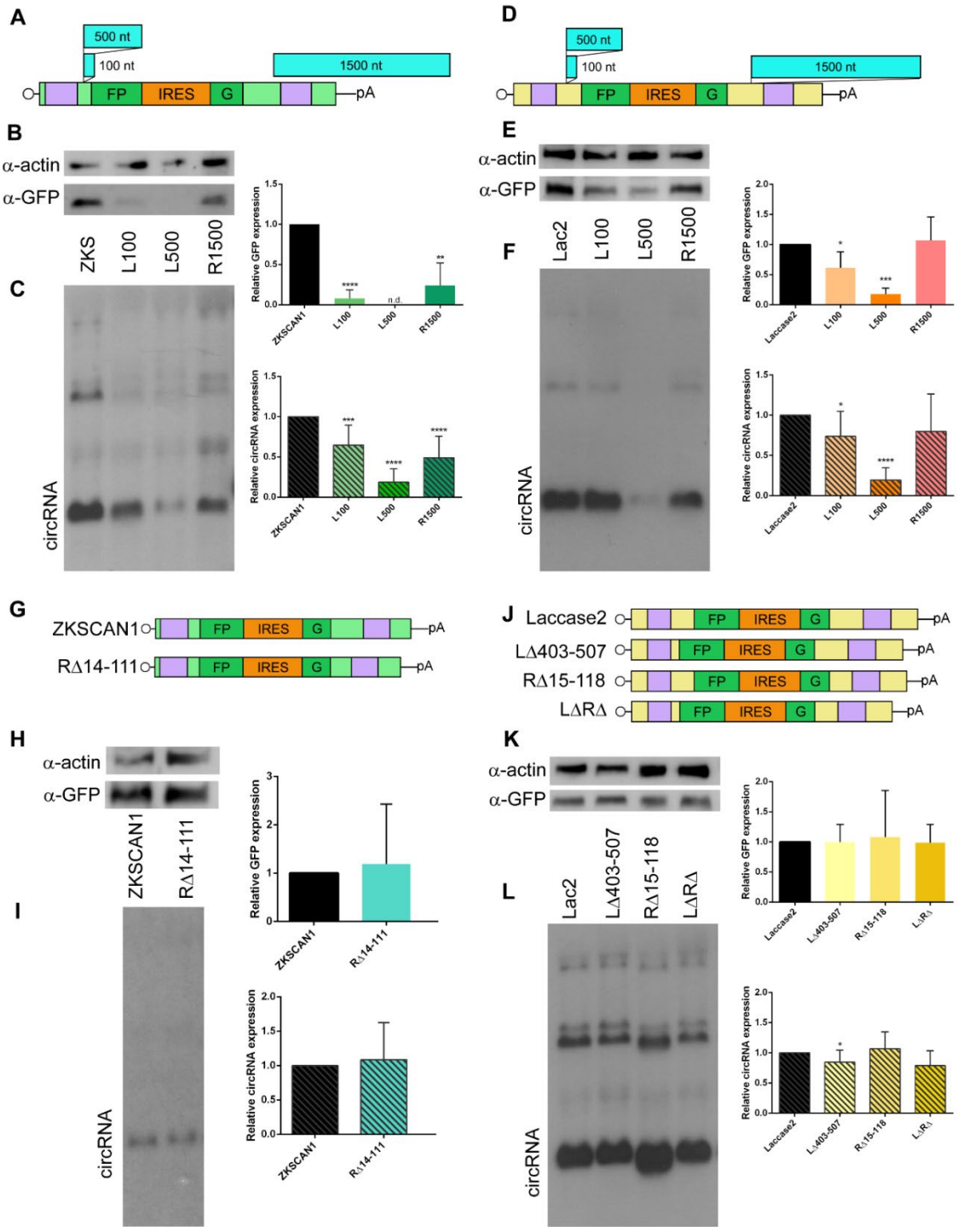
Supplemental Figure 1: Sequence information for HIPK3, Laccase2, and ZKSCAN1 intron pairs. Sequence information for the (A) HIPK3, (B) Laccase2, and (C) ZKSCAN1 intron pairs. Distances between the complementary region and splice sites are noted on top of the schematic. Complementary regions and splice site information are overlaid on the sequences.



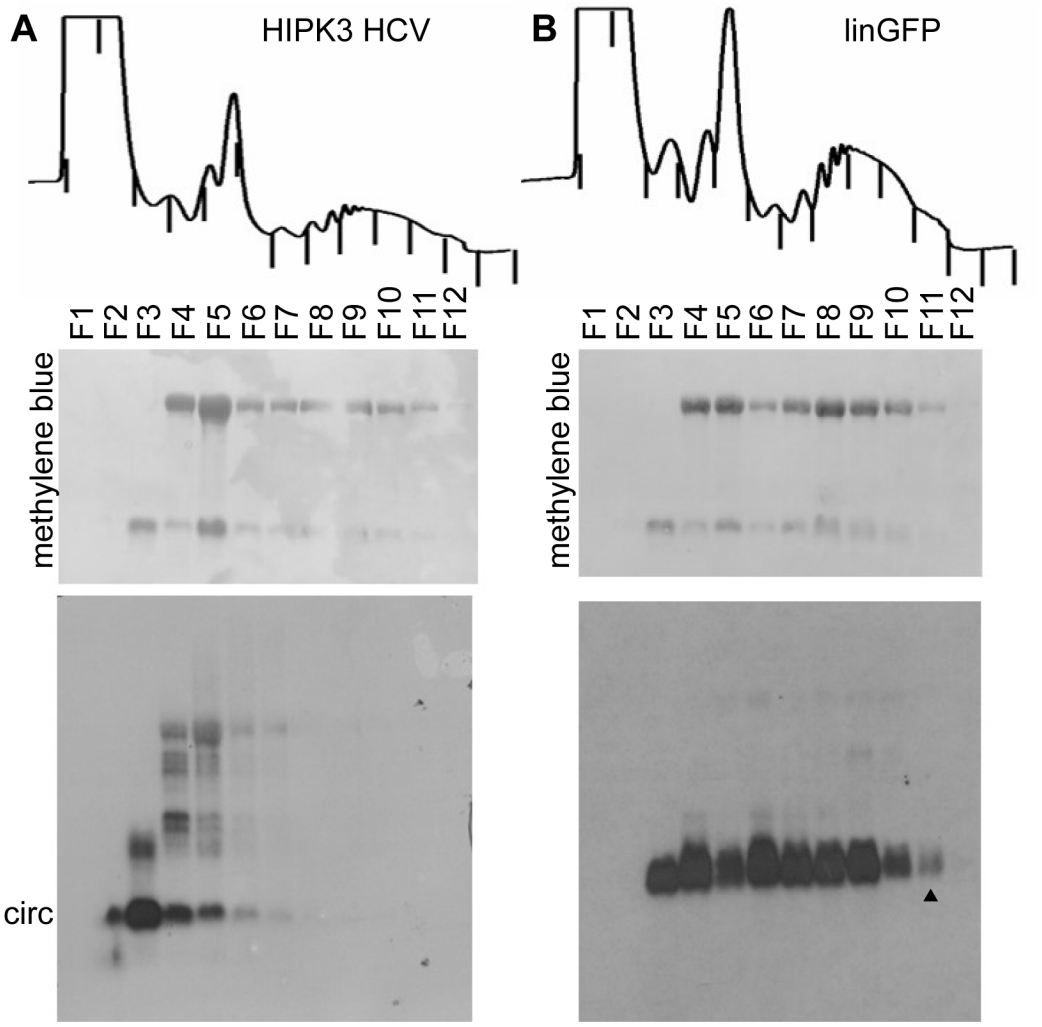
Supplemental Figure 2: Increased distance between the left *Alu* and splice acceptor is harmful even when inserted distal to the splice site. (A) Sequence ranging from 100nt to 1500nt was inserted into the left HIPK3 intron at a site distal to the splice site. Constructs were transfected into HEK293 cells and expression assayed at 4 days post-transfection by (B) Western blot analysis, with actin as a loading control (quantification below), and (C) Northern blot analysis, probing for GFP sequences (quantification below). On Northern blots, the * refers to an additional circular band. Western and Northern blots were quantified as detailed in the methods and graphed relative to the unchanged HIPK3 intron construct. Student's t-test was performed to test for statistical significance. Where indicated, * = $p<0.05$; ** = $p<0.005$; *** = $p<0.0005$; **** = $p<0.00005$.



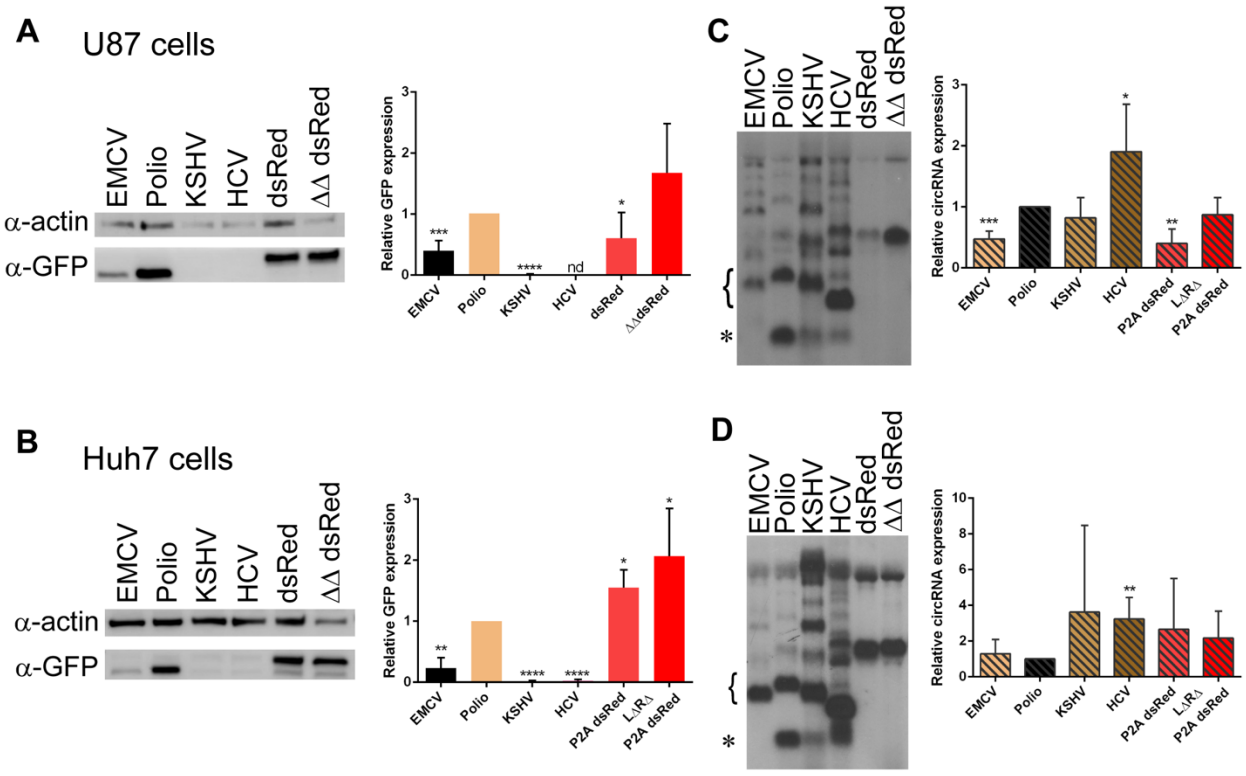
Supplemental Figure 3: The effect of insertions and deletions is conserved in glioblastoma and hepatocarcinoma cell lines. The indicated insertion/deletion constructs were transfected into either (A,B) U87 or (C,D) Huh7 cells and assayed at 4 days post-transfection by (left) Western blot analysis, with actin as a loading control and (right) Northern blot analysis, probing for GFP sequences. The * refers to an additional circular band. Western and Northern blots were quantified as detailed in the methods and graphed relative to the unchanged HIPK3 intron construct. Student's t-test was performed to test for statistical significance. Where indicated, * = p<0.05; ** = p<0.005; *** = p<0.0005; **** = p<0.00005.



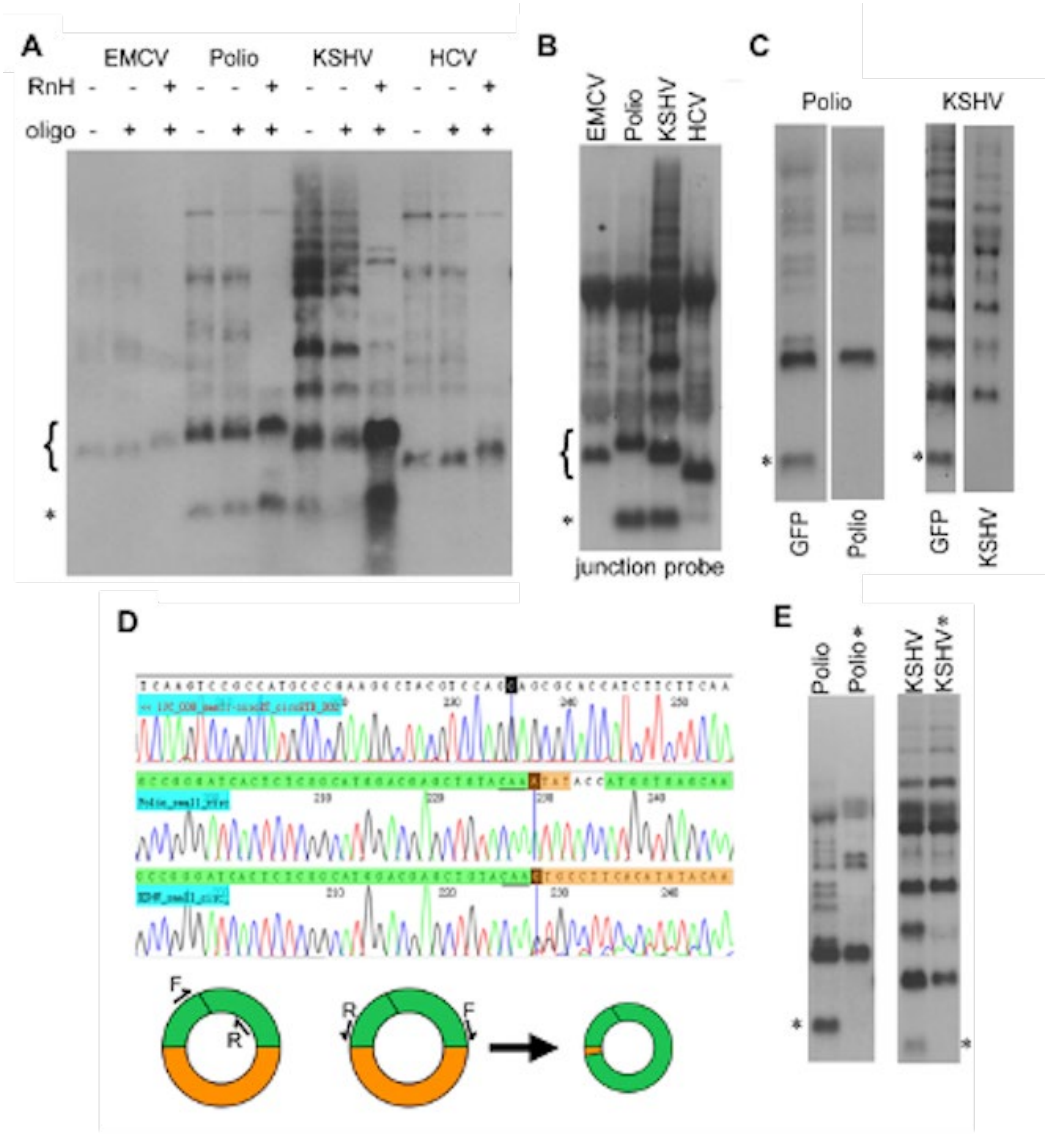
Supplementary Figure 4: Intronic spacing effects on circRNA formation are conserved in Laccase2 and ZKSCAN1 intron pairs. Randomized sequences of the indicated sizes were inserted into (A) the ZKSCAN1 backbone or (D) the Laccase2 backbone. Constructs were transfected into HEK293 cells and expression assayed at 4 days post-transfection by (B,E) Western blot analysis, with actin as a loading control, and (C,F) Northern blot analysis, probing for GFP sequences. The indicated sequences were deleted in (G) the ZKSCAN1 backbone or (J) the Laccase2 backbone. Constructs were transfected into HEK293 cells and expression assayed at 4 days post-transfection by (H,K) Western blot analysis, with actin as a loading control, and (I,L) Northern blot analysis, probing for GFP sequences. Western and Northern blots were quantified as detailed in the methods and graphed relative to the unchanged HIPK3 intron construct. Student's t-test was performed to test for statistical significance. Where indicated, * = $p<0.05$; ** = $p<0.005$; *** = $p<0.0005$; **** = $p<0.00005$.



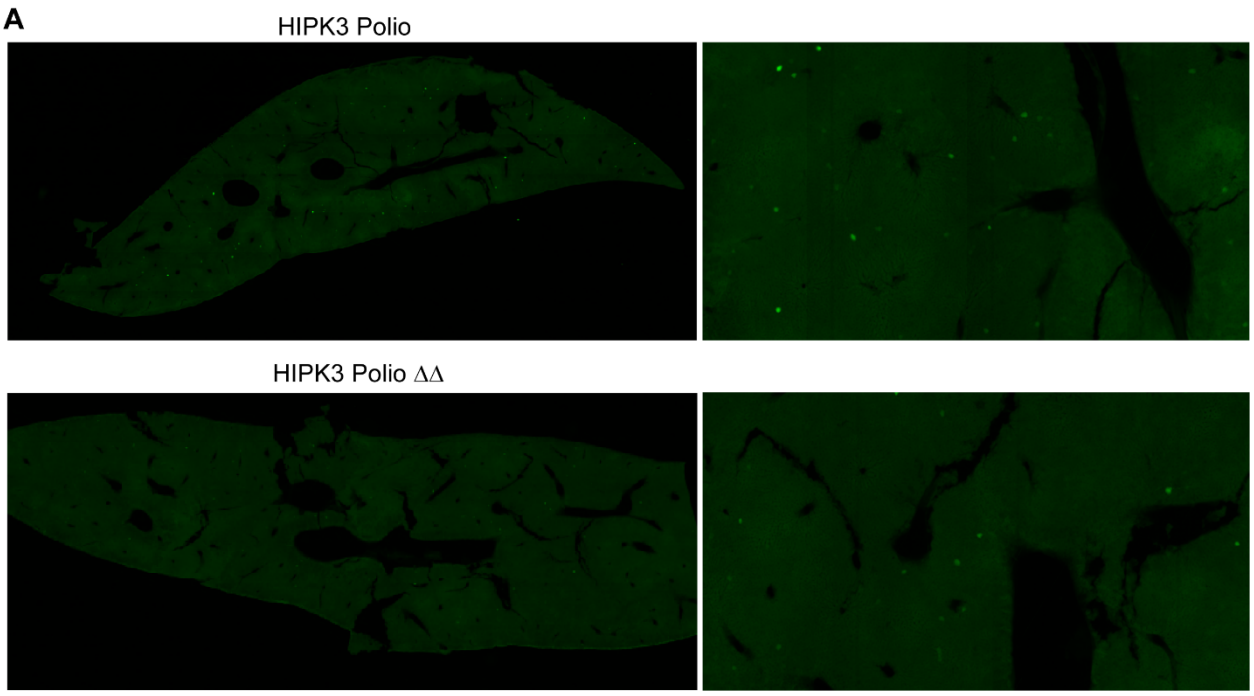
Supplemental Figure 5: Translation efficiency with the HCV IRES and a control linear RNA. To assay translation efficiency, the indicated constructs were transfected into HEK293 cells and harvested in cycloheximide followed by a sucrose gradient and fractionation. Top: OD trace of the gradient, with fractions marked by lines. Middle: RNA was extracted from gradients, separated by gel electrophoresis, transferred to a membrane, and stained with methylene blue to visualize the ribosomal RNA. Bottom: The same membrane was probed for GFP sequences. The arrowhead marks the last fraction in which the circRNA is detected. The above was carried out for (A) the HIPK3 HCV construct or (B) a cap-driven linear GFP mRNA.



Supplemental Figure 6: IRES-mediated translation efficiency and circRNA expression varies in glioblastoma and hepatocarcinoma cell lines. The indicted IRES/exon constructs were transfected into either (A,C) U87 or (B,D) Huh7 cells and assayed at 4 days post-transfection by (left) Western blot analysis, with actin as a loading control and (right) Northern blot analysis, probing for GFP sequences. The * refers to an additional circular species. Western and Northern blots were quantified as detailed in the methods and graphed relative to the unchanged HIPK3 intron construct. Student's t-test was performed to test for statistical significance. Where indicated, * = p<0.05; ** = p<0.005; *** = p<0.0005; **** = p<0.00005.



Supplemental Figure 7: IRES elements affect splicing and impact formation of additional RNA species. (A) RNase H digestion was performed with an oligonucleotide targeting the back-splice junction. Samples were analyzed by Northern blot and probed against GFP sequences. (B) RNA from the indicated constructs was analyzed by Northern blot and probed with an oligonucleotide spanning the back-splice junction. (C) RNA from the indicated constructs was analyzed by Northern blot and probed for GFP and the specific IRES sequences. (D) A Virtual Northern blot was performed and RT-PCR with primers spanning either the back-splice junction (top) or the IRES sequence (middle, Poliovirus IRES; bottom, KSHV vFLIP IRES) confirm the presence of both the back-splice and a linear splice in the small circRNA band. (E) Mutations to the splice donor site identified in (D) modify splicing to remove the small circRNA band, as analyzed by Northern blotting, probing for GFP. On all Northern blots, the * refers to the small circular species.



Supplemental Figure 8: CircRNA reporters express low GFP protein levels in the liver. (A) Immunofluorescent staining was performed on sectioned liver tissue to visualize GFP expression.