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Supplemental information

Endogenous spacing enables co-processing of microRNAs and efficient combinatorial RNAi

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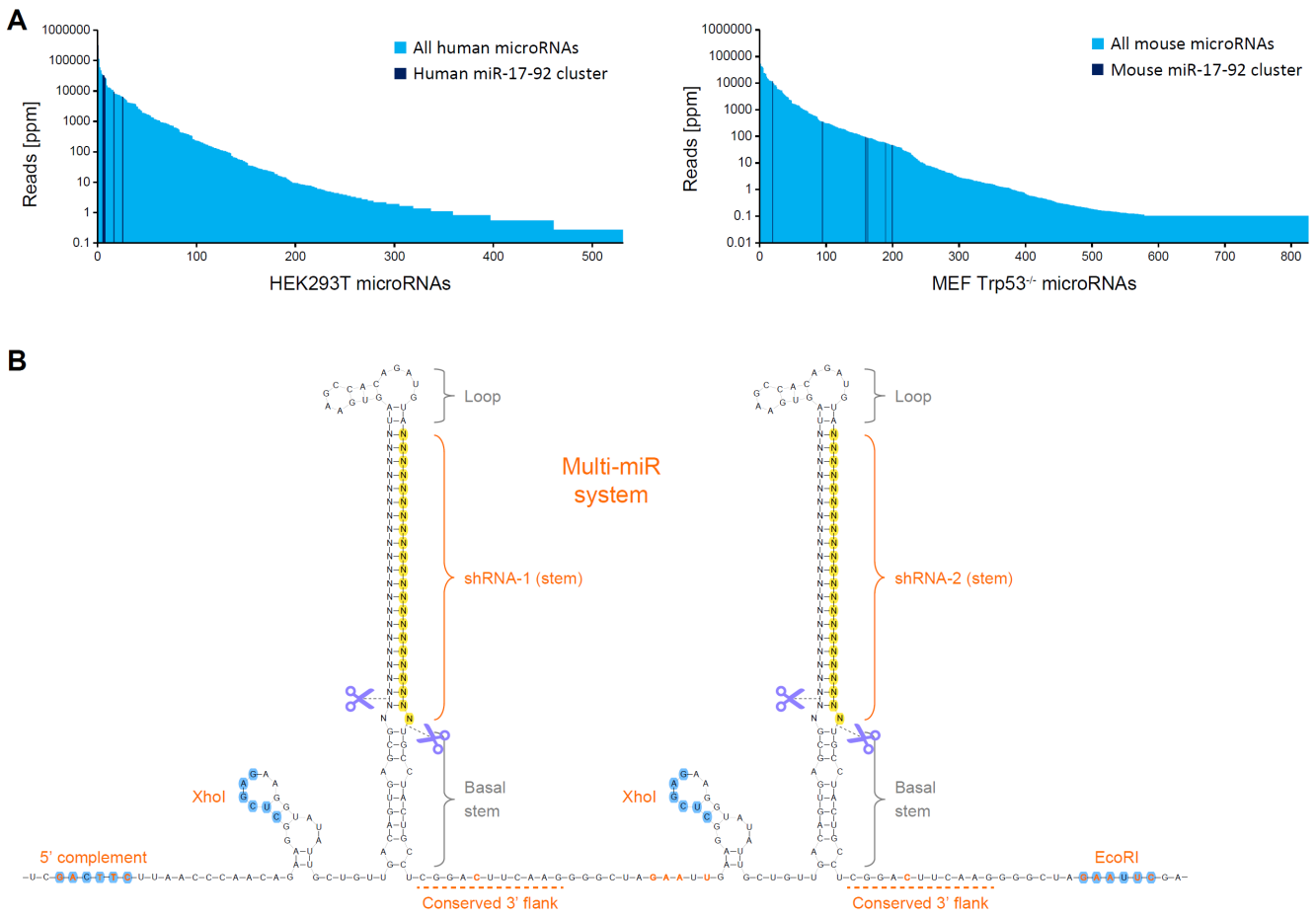
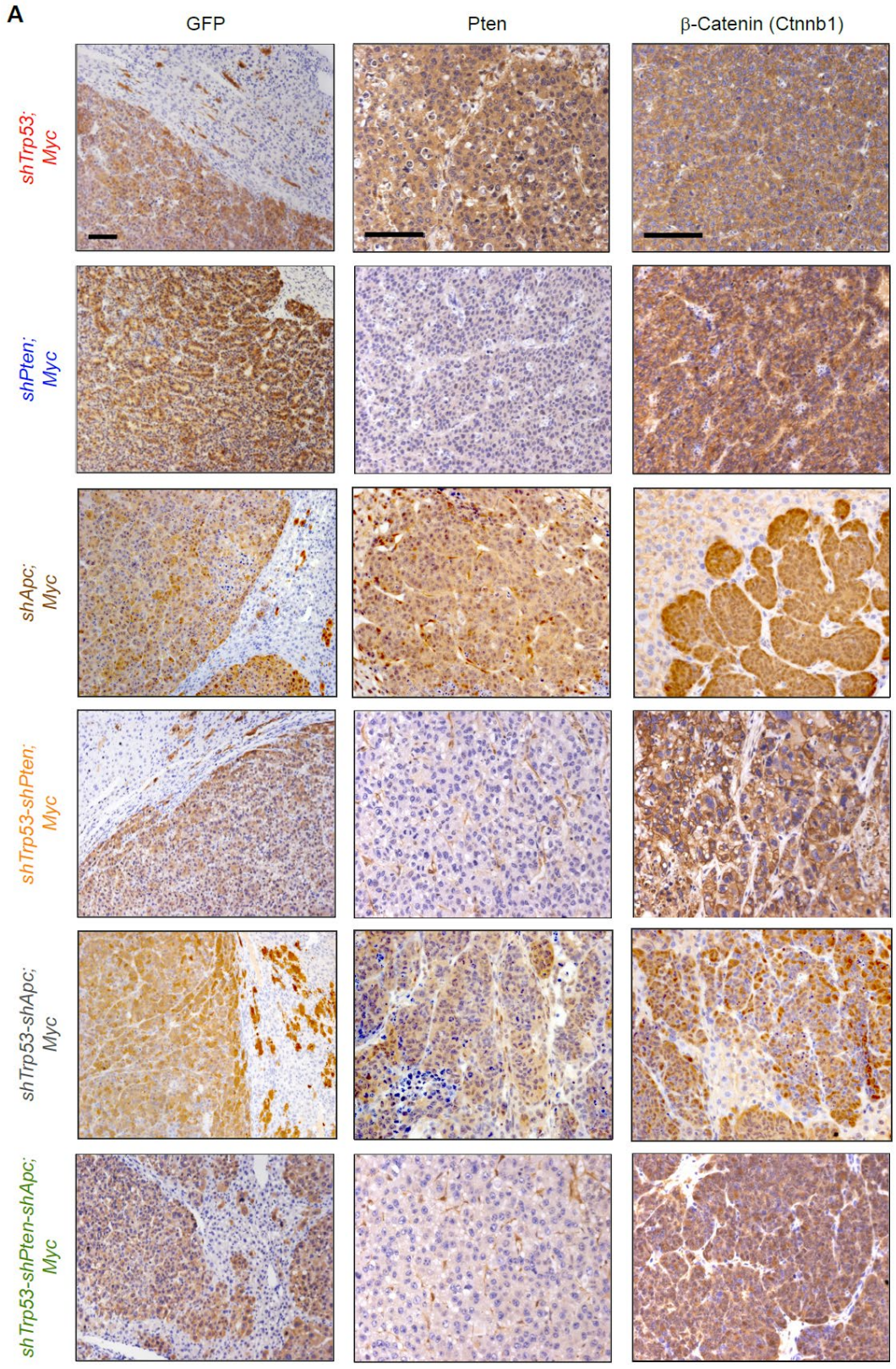


Supplementary Figure S1 Architecture of the Multi-miR system. Related to Figure 1.

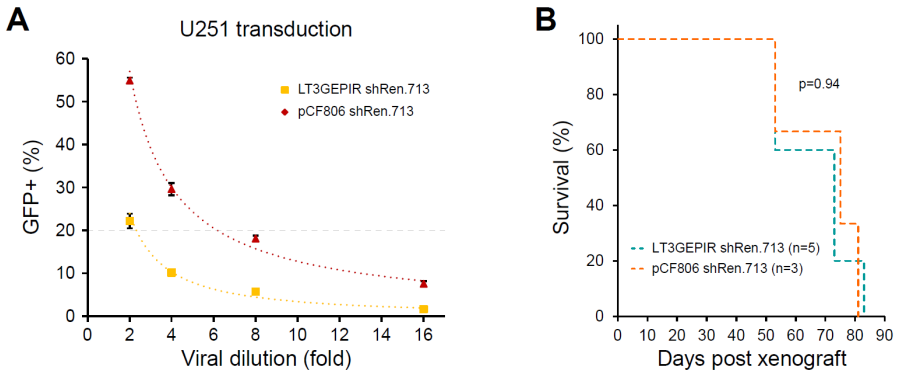
(A) MicroRNA sequencing reads in human (HEK293T) and mouse (MEF) cells, highlighting the representation of microRNAs from the respective miR-17-92 cluster.

(B) Schematic of the Multi-miR architecture, showing the concatenation of two miR-E scaffolds. Conserved 3' flank sequences, including the 5'-DCNNC-3' (D = A, G, U) motif required for efficient pri-miRNA processing, are highlighted. XhoI and EcoRI restriction sites used for cloning are indicated. Violet scissors mark canonical Drosha cleavage sites. The sequence (spacing) between the 3' Drosha cleavage site of shRNA-1 and the 5' Drosha cleavage site of shRNA-2 is 74 nucleotides long.



Supplementary Figure S2 Multi-miR mediated target suppression *in vivo*. Related to Figure 3.

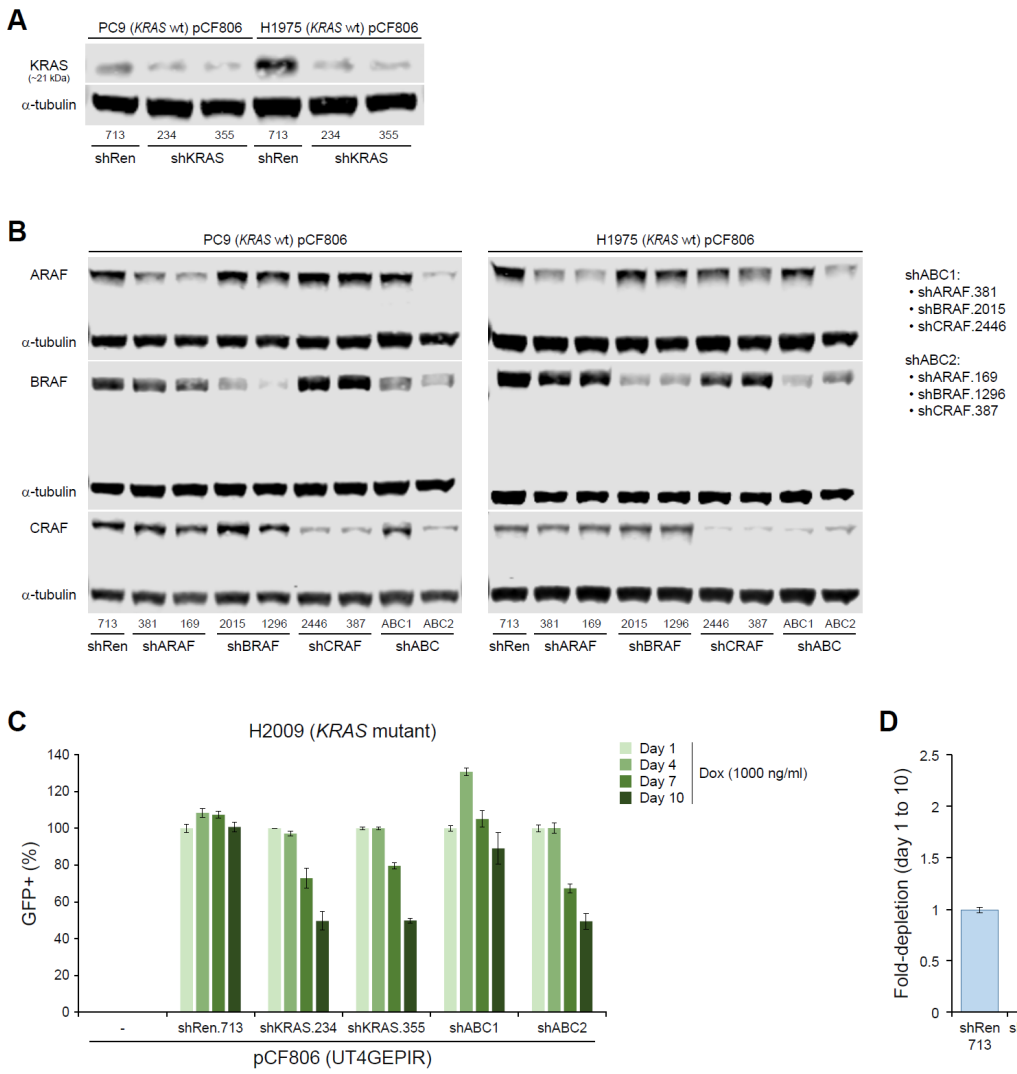
(A) Representative immunohistochemical images of liver and tumor tissues in C57BL/6 mice co-injected with the *Myc* transposon and the indicated shRNAs. Sections were stained for the indicated markers (GFP, Pten, Ctnnb1 – a.k.a. β-Catenin). Scale bars, 25 μm (left column) or 100 μm (middle and right columns).



Supplementary Figure S3 *In-vivo* assessment of Multi-miR vectors. Related to Figure 4.

(A) Comparison of viral titers. U251 cells were transduced at the indicated dilutions with supernatant from HEK293T packaging cells transfected with LT3GEPIR and pCF806 (UT4GEPIR) vectors. Target U251 cells were treated with doxycycline (1000 ng/ml) at day two post-transduction, and the percentage of GFP+ cells quantified after 48 hours of treatment. Note, pCF806 yielded approximately 4-fold higher viral titers than LT3GEPIR did. Error bars show the standard deviation (n=3).

(B) Kaplan-Meier survival curve of mice expressing a negative control shRNA (shRen.713) from LT3GEPIR or pCF806 (UT4GEPIR) vectors. Significance was calculated using the log-rank test.



Supplementary Figure S4 Suppressing synthetic-lethal gene nodes. Related to Figure 5.

(A) Validation of KRAS knockdown by immunoblotting in *KRAS* wild-type (wt) non-small cell lung cancer (NSCLC) PC9 and H1975 cells expressing shRNAs targeting *KRAS* (shKAS.234, shKRAS.355) or a negative control (shRen.713). All shRNAs were expressed from pCF806 vectors transduced at single copy genomic integration. Cells were treated with doxycycline (1000 ng/ml) for 72 hours to induce shRNA expression prior to harvest.

(B) Validation of ARAF, BRAF and CRAF (a.k.a. RAF1) knockdown by immunoblotting in *KRAS* wild-type (wt) PC9 and H1975 cells expressing shRNAs targeting *ARAF* (shARAF.381, shARAF.169), *BRAF* (shBRAF.2015, shBRAF.1296), *CRAF* (shCRAF.2446, shCRAF.387), all three RAFs (shABC1 = shARAF.381-shBRAF.2015-shCRAF.2446, shABC2 = shARAF.169-shBRAF.1296-shCRAF.387) or a negative control (shRen.713). Cells were transduced and treated as described above.

(C) Competitive proliferation assay assessing the growth effects of KRAS and triple A/B/C-RAF knockdown in *KRAS* mutant NSCLC H2009 cells. Cells were transduced at single copy with pCF806 vectors expressing shRNAs targeting *KRAS* (shKRAS.234, shKRAS.355), all three RAFs (shABC1, shABC2) or a negative control (shRen.713). The percentage of transduced (GFP+) cells was monitored by flow cytometry at day 1, 4, 7 and 10 of doxycycline (1000 ng/ml) treatment and normalized to day 1.

(D) Quantification of data shown in (C). Fold-depletion of cells expressing the indicated single- or multi-shRNA constructs. Significance was calculated using the two-tailed Student's t-test with alpha = 0.05. ns, not significant.