Supplemental File 2 Legend.

Multiple cloning regions for vectors described in this manuscript. (A) restriction map of pHB vector (in pBluescript sk(+) backbone). **(B)** restriction map of pNE vector (in pUAST backbone). **(C)** restriction map of pNE3 or p*attB*-NE3 vectors. *SpeI** indicates that this site is only unique in pNE3, not p*attB*-NE3. All multiple cloning regions are ~175 base-pairs in length, including the shmiR.

Supplemental File 3 Legend.

Recommended shmiR-chaining procedure. **(A)** shmiR-chaining method for pHB-based vectors is shown. Here, separate siRNA sequences are cloned into at least two *HindIII-BamHI*-linearized pHB scaffolds. The resultant constructs are then digested as follows; construct #1 is excised from pHB following a *XbaI-SpeI* double-digest, generating an ~150-bp fragment. This fragment is ligated into construct #2, linearized by *XbaI* digest. This results in a construct bearing tandem hairpins in the pHB vector. We remove these hairpins as an *EcoRI-XhoI* fragment (~350 bps). **(B)** shmiR-chaining method for pNE2-based vectors is shown. Here, siRNA sequence #1 is cloned into the pHB-cloning vector, while shmiR sequence #2 is cloned into pNE (in pUAST backbone). Similar to above, an ~150-bp fragment containing shmiR-sequence #1 is excised by a *XbaI-SpeI* double digest from pHB. pNE2 containing shmiR sequence #2 is concurrently linearized with *XbaI*, allowing efficient ligation to the previously generated *XbaI-SpeI* shmiR sequence #1 fragment. Proper insertion is performed by screening for ~350-bp fragments generated upon subsequent *XbaI/SpeI* double digest. **(C)** Directional shmiR-

chaining method for pNE3-based vectors (in pUAST or p*attB*-UAST backbone) is shown. Here, sequence #1 is cloned into pHB, as above, and sequence #2 is cloned into pNE3, as above. A ~160-bp pHB shmiR element is subsequently removed via *KpnI/SpeI* double digestion. Concurrently, pNE3 is linearized by *KpnI/SpeI* double digestion. Sequence #1 from pHB is then ligated directionally into the transgenic-ready vectors, and if needed, directionality can be screened by *XbaI/SpeI* double digestion, and appearance of a ~350-bp product. Please note that the *SpeI* cloning site in p*attB*-NE3 is not unique to the p*attB*-UAST vector.

Supplementary File 4 Legend

Similar knockdown efficiency between shmiRs and long dsRNA *in vitro*. ~450 ng of GFP, GFP-shmiR-Luc1+2, or a dsRNA encompassing ~500-bps of the firefly Luciferase coding region (Luc dsRNA) were transfected into S2 cells, along with 25 ng/each firefly or *Renilla* Luciferase in 24-well plates. Luciferase activity was measured 48 hrs after transfection.

Supplemental File 5 Legend

Confirmed expression of miR* and dpp2mutant siRNA sequences from Drosophila larvae. Sequentially probed Northern blot (DNA probes) showing expression of mature *dpp*2, predicted *dpp*2*, and *dpp*2HBmutant siRNAs. ~10 ug of total larval RNA from described crosses was analyzed, per lane, by 20% denaturing PAGE. Note dramatic

difference in detectability between the *dpp*2 and *dpp*2* (black arrow) siRNAs. 25 and 17-nt RNAs were used as size markers.

Supplemental File 6 Legend

***dpp* phenotypic series in the fly wing generated by a single siRNA using multiple transgenic lines**. All wings shown were dissected from transgenic flies crossed to the A9-GAL4 driver at 25ºC. **(A-B)** UAS-shmiR-*dpp*2 expression from transgenic line #5 (distinct from line #2 shown in Figure 4) displays a moderate dpp^{shv} phenotype in females (A) that becomes more pronounced in males (B). **(C-D)** UAS-shmiR-*dpp*2HB expression from transgenic line #1 (distinct from line #6 shown in Figure 4) displays a more severe, dpp^{d5} mutant phenocopy. Resultant wings from females (C) of this cross are reduced in size compared to wild type and are devoid of all vein tissue, while males (D) have nearly complete loss of wing and vein tissue altogether. **(E)** Frequency of *dpp* mutant phenocopy is presented for transgenic lines created with a particular shmiR construct. Number of total lines established per construct is noted in parentheses.

Supplemental File 7 Legend

Quantitative Real-Time PCR measurement of dpp mRNA in shmiR-*dpp*2HB expressing strains. Relative expression of *dpp* mRNA to the endogenous control *RP49* mRNA is displayed. UAS-NLS-GFP, as well as two unique UAS-shmiR-*dpp*2HB strains (noted as lines #3 and #6) were crossed to da-GAL4. qPCR was performed with RNA extracted from 2nd and 3rd instar larvae. Displayed is an average of duplicate

measurements from a single RNA collection for UAS-NLS-GFP and triplicate measurements for the UAS-shmiR-*dpp*2HB transgenics.

Supplemental File 8 Legend

RNAi directed against *dpp* using the Valium-based dsRNA expression system. **(A-B)** Examples of *dpp* knockdown phenotype resulting from UAS-*dpp*-dsRNA X A9-GAL4 in females. **(C-D)** Examples of dpp knockdown phenotype resulting from UAS-*dpp*-dsRNA X A9-GAL4 in males. A thin or shortened wing vein is marked by the black arrowhead. Similar results were obtained using UAS-*dpp*-dsRNA line 48A.1 (data not shown).