Supporting Information

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SI Text

PCR Cloning Primers. ***ftz intron.*** Cloned as separate parts into pCR2.1-TOPO and then transferred to pAc5.1_B.

Part I. F: CCA CTA GTG ACA GCC CCG AAC GGA GCC
R: CCT CTA GAG GTA CCC ATA TTT TCA ATA TTT TCA AAG TGT ATT TTT AGG GG

Part II. F: CCA CTA GTC TCG AGT TTG TAT ACA TTT TTG ATA TTT TCA AAC AAT ACG CA
R: CCT CTA GAC TCC AGG GTC TGG TAG CGG G

ftz intron in p*attB*-UAST (PCR amplified from *ftz* intron in pAc5.1)
F: CCG AAT TCG ACA GCC CCG AAC GGA GCC
R: CCT CTA GAC TCC AGG GTC TGG TAG CGG G

tomosyn intron. Cloned as separate parts into pCR2.1-TOPO and then transferred to pAc5.1_B.

Part I. F: CCA CTA GTC AAA GCG TTC AGC CCC AAG CCA CAC
R: CCT CTA GAG GTA CCA CAG ATG CCT TTC GAT TAT GGG CAC

Part II. F: CCA CTA GTC TCG AGC GTT GTC TAA GAG CTT CAA AAT CGA TAA TAA C
R: CCT CTA GAG GAT GTG GGC GAG TAT GGA AAG CC

Reporter Genes. ***eGFP/mCherry.*** F: CCT CTA GAA TGG TGA GCA AGG GCG AGG
R: CCA CTA GTG CTA GCT TAC TTG TAC AGC TCG TCC ATG CC

eGFP +1 (will introduce frameshift if *ftz* intron is spliced into shmiR hairpin) (Fig. S6)
F: CCT CTA GAC ATG GTG AGC AAG GGC GAG G
R: CCA CTA GTG CTA GCT TAC TTG TAC AGC TCG TCC ATG CC

Renilla luciferase. F: CCT CTA GAA TGA CTT CGA AAG TTT ATG ATC CAG AAC AAA GGA AAC
R: CCG CTA GCA ATT ATT GTT CAT TTT TGA GAA CTC GCT C

miR-1 cloning primers. F: CCA AGT GAG TAG TGC CAC
R: CGG TTC TAC TTC TGT TTC AAT C

RT-PCR Primers (Performed in Fig. S6). ***ftz intron.*** F: CCT CTA GAA TGG TGA GCA AGG GCG AGG
R: AGC TCC TCG CCC TTG CTC AC

Northern blot probes. *dpp* shmiR: CCA CTC TAG TCG AGA TCG AGA

dpp-5 shmiR: GGT GAC TAG TCG AGA TCG AGA

2S RNA: TAC AAC CCT CAA CCA TAT GTA GTC CAA GCA

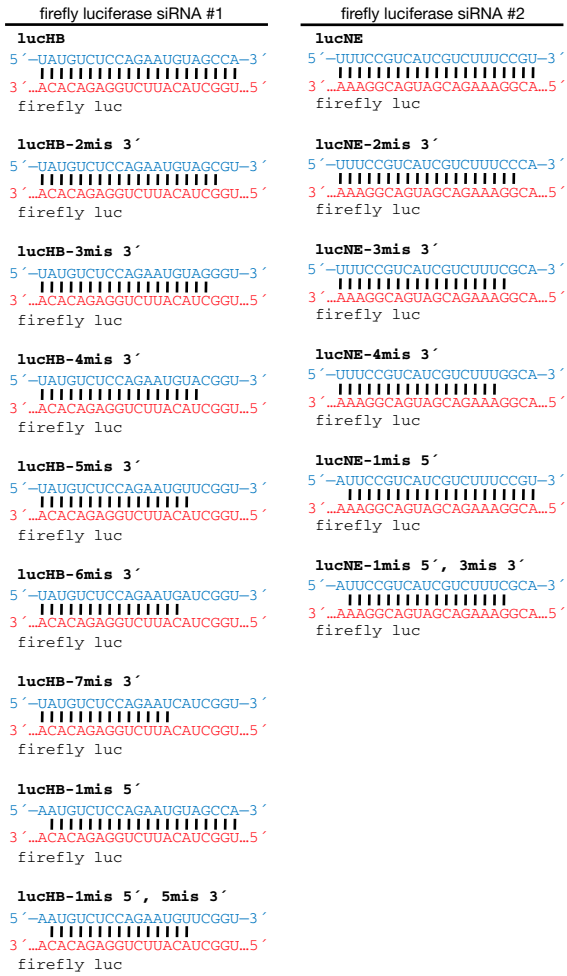

Fig. S1. Alignments of firefly luciferase siRNAs #1 and #2 with their respective target sites on the GL3 (Promega) coding region. For each alignment, the siRNA sequence is shown in blue, and the target site is shown in red.

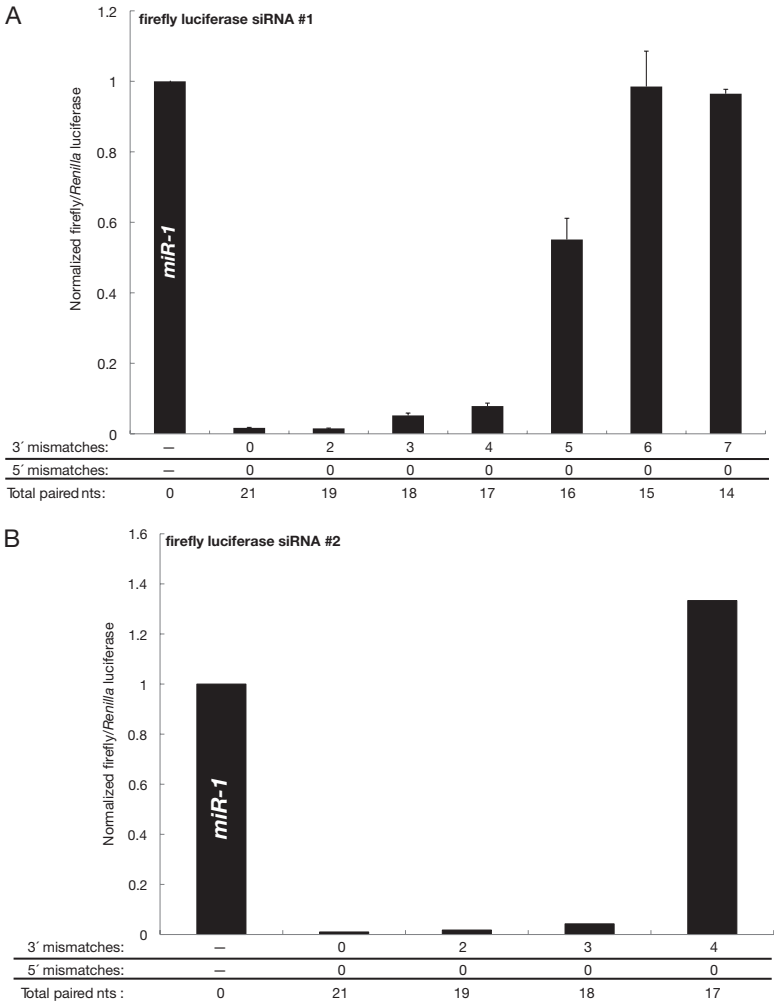

Fig. S2. Bulk 3′ mismatching between a siRNA and target mRNA does not severely affect gene silencing in target excess conditions. The previously described series of firefly luciferase siRNAs #1 (*A*) and #2 (*B*) were expressed in a 1:8 ratio of shmiR:firefly luciferase in *Drosophila* S2 cells. siRNAs with highest efficiency in the shmiR excess conditions remained highly efficient.

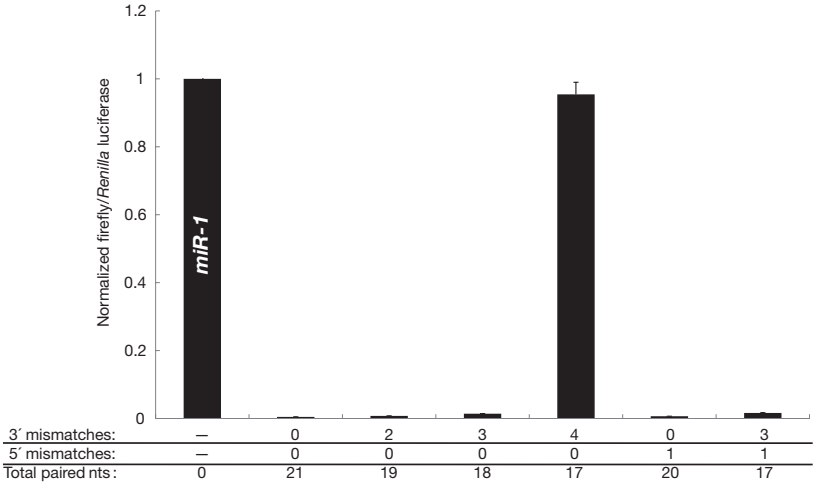

Fig. S3. Base pairing of nucleotides 2–18 is sufficient to induce complete gene silencing of a reporter gene in a *Drosophila* S2 cell-culture assay. As in Fig. 1, a single firefly luciferase-targeting shmiR (firefly luciferase siRNA #2) was mutated to express siRNAs harboring combinations of 3′ and 5′ mismatches from the perspective of the siRNA. shmiRs were coexpressed along with firefly and Renilla luciferase plasmids in an 8:1 ratio of shmiR:luciferase. All siRNA sequences in relation to the target mRNA are shown in Fig. S1.

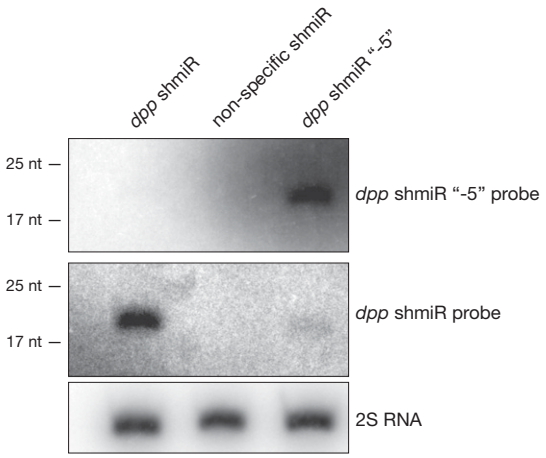

Fig. S4. Specific and efficient expression of *dpp*-targeted siRNAs in transgenic flies. A sequentially probed Northern blot assay was used to evaluate expression of a fully complementary *dpp*-targeted siRNA (*dpp* shmiR) or expression of a *dpp*-targeted siRNA with five 3′ mismatches (*dpp* shmiR-5). Equal loading of total larval RNA for each lane (~15 μg RNA/lane) was assessed by 2S ribosomal RNA expression.

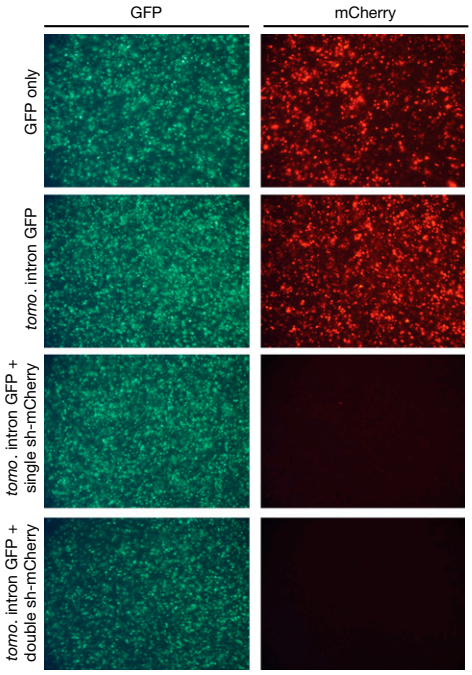

Fig. S5. Efficient reporter expression and knockdown from a 5′ *tomosyn* intron-eGFP shmiR expression construct. The *Drosophila tomosyn* (tomo.) intron was chosen as a potential intronic shmiR expression system, because it natively houses *miR-970*. *miR-970* was removed from the *tomo*. intron region by sequential PCR cloning steps and was modified to include a *KpnI*/*Xho*I cloning site as described for the *ftz* intron (Fig. 3). Expression of a 5′ modified *tomo*. intron-eGFP construct was compared with eGFP alone by visualization of green fluorescence in an S2 cell-transient transfection assay, as shown in Fig. 3. shmiR expression and function were assayed by placing a single or tandem mCherry-targeting shmiR construct in the 5′ modified *tomo*. intron. Expression of this construct was confirmed by the presence of eGFP. shmiR function was confirmed by loss of mCherry expression after coexpression of either 5′ modified *tomo*. intronic shmiR-eGFP constructs or mCherry.

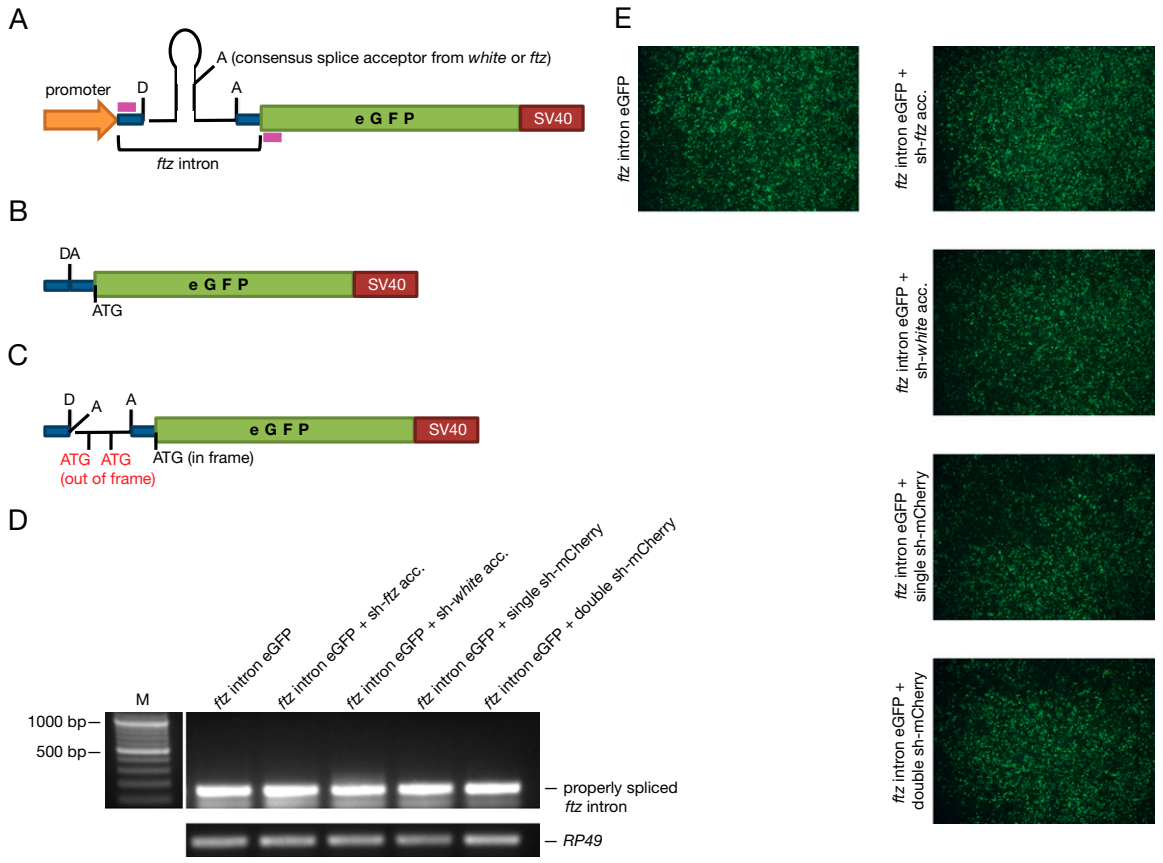

Fig. S6. Strong splice acceptors in the intronic shmiR stem loop do not affect splicing of the host intron. (*A*) A cartoon representation of the 5′ *ftz* intronic shmiR-eGFP construct. Here, the stem loop segment that will be excised and processed into an siRNA contains either a *ftz* or *white* splice acceptor sequence. Appropriate splicing is monitored by RT-PCR of total RNA, with primers marked by purple rectangles. (*B*) An appropriately processed and spliced intronic shmiR transcript is shown and will generate an ∼150-bp product by RT-PCR. (*C*) Inappropriate splicing into the shmiR-localized splice acceptor will generate a longer transcript than *B*, resulting in an ∼210-bp product by RT-PCR. In addition, this construct contains two potential start codons (red ATG) downstream of the shmiR stem loop. If the shmiR-localized acceptor site is used, along with either alternate start codon, the eGFP ORF will be out of frame, and eGFP will not express. (*D*) RT-PCR was performed on randomly primed total RNA with the described *ftz*-intron configurations 72 h after transfection into S2 cells. Intronic shmiRs containing the *ftz* or *white* splice acceptor sites were compared with an empty *ftz* intron or a single or tandem mCherry-targeting, intronic shmiR as a control. RT-PCR of *RP49* mRNA was used as a loading control. (*E*) eGFP expression from various 5′ intronic shmiR configurations was assayed using the same cells (transfection conditions and constructs) used for RNA collections described in *D*. Presence of eGFP confirms that all 5′ ftz-intronic shmiR constructs are appropriately translated.

Table S1. shLuciferase (GL3) 1

Oligonucleotide sequences used to clone Luciferase-targeting shmiRs

lucHB siRNA		
TATGTCTCCAGAATGTAGCCA		
lucHB-2	pHB top	agcttagtACGCTACATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATGTAGCGTgcg
TATGTCTCCAGAATGTAGCGT	pHB bot	gatccgcACGCTACATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATGTAGCGTacta
lucHB-3	pHB top	agcttagtACCCTACATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATGTAGGGTgcg
TATGTCTCCAGAATGTAGGGT	pHB bot	gatccgcACCCTACATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATGTAGGGTacta
lucHB-4	pHB top	agcttagtACCGTACATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATGTACGGTgcg
TATGTCTCCAGAATGTACGGT	pHB bot	gatccgcACCGTACATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATGTACGGTacta
lucHB-5	pHB top	agcttagtACCGAACATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATGTTCGGTgcg
TATGTCTCCAGAATGTTCGGT	pHB bot	gatccgcACCGAACATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATGTTCGGTacta
lucHB-1(5′)	pHB top	agcttagtTGGCTACATTGTGGAGACAATtagttatattcaagcataAATGTCTCCAGAATGTAGCCAgcg
AATGTCTCCAGAATGTAGCCA	pHB bot	gatccgcTGGCTACATTCTGGAGACATTtatgcttgaatataactaATTGTCTCCACAATGTAGCCAacta
lucHB-1(5′), -5	pHB top	agcttagtACCGAACATTGTGGAGACAATtagttatattcaagcataAATGTCTCCAGAATGTTCGGTgcg
AATGTCTCCAGAATGTTCGGT	pHB bot	gatccgcACCGAACATTCTGGAGACATTtatgcttgaatataactaATTGTCTCCACAATGTTCGGTacta
lucHB-6	pHB top	agcttagtACCGATCATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATGATCGGTgcg
TATGTCTCCAGAATGATCGGT	pHB bot	gatccgcACCGATCATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATGATCGGTacta
lucHB-7	pHB top	agcttagtACCGATGATTGTGGAGACAAAtagttatattcaagcataTATGTCTCCAGAATCATCGGTgcg
TATGTCTCCAGAATCATCGGT	pHB bot	gatccgcACCGATGATTCTGGAGACATAtatgcttgaatataactaTTTGTCTCCACAATCATCGGTacta

Table S2. shLuciferase (GL3) 2

Oligonucleotide sequences used to clone Luciferase-targeting shmiRs

lucNE siRNA		
TTTCCGTCATCGTCTTTCCGT		
lucNE-2	pNE top	ctagcagtTGGGAAAGACCATGACGGATAtagttatattcaagcataTTTCCGTCATCGTCTTTCCCAgcg
TTTCCGTCATCGTCTTTCCCA	pNE bot	aattcgcTGGGAAAGACGATGACGGAAAtatgcttgaatataactaTATCCGTCATGGTCTTTCCCAactg
lucNE-3	pNE top	ctagcagtTGCGAAAGACCATGACGGATAtagttatattcaagcataTTTCCGTCATCGTCTTTCGCAgcg
TTTCCGTCATCGTCTTTCGCA	pNE bot	aattcgcTGCGAAAGACGATGACGGAAAtatgcttgaatataactaTATCCGTCATGGTCTTTCGCAactg
lucNE-4	pNE top	ctagcagtTGCCAAAGACCATGACGGATAtagttatattcaagcataTTTCCGTCATCGTCTTTGGCAgcg
TTTCCGTCATCGTCTTTGGCA	pNE bot	aattcgcTGCCAAAGACGATGACGGAAAtatgcttgaatataactaTATCCGTCATGGTCTTTGGCAactg
lucNE-5	pNE top	ctagcagtTGCCTAAGACCATGACGGATAtagttatattcaagcataTTTCCGTCATCGTCTTAGGCAgcg
TTTCCGTCATCGTCTTAGGCA	pNE bot	aattcgcTGCCTAAGACGATGACGGAAAtatgcttgaatataactaTATCCGTCATGGTCTTAGGCAactg
lucNE-1(5′)	pNE top	ctagcagtACGGAAAGACCATGACGGATTtagttatattcaagcataATTCCGTCATCGTCTTTCCGTgcg
ATTCCGTCATCGTCTTTCCGT	pNE bot	aattcgcACGGAAAGACGATGACGGAATtatgcttgaatataactaAATCCGTCATGGTCTTTCCGTactg
lucNE-1(5′),-3	pNE top	ctagcagtTGCGAAAGACCATGACGGATTtagttatattcaagcataATTCCGTCATCGTCTTTCGCAgcg
ATTCCGTCATCGTCTTTCGCA	pNE bot	aattcgcTGCGAAAGACGATGACGGAATtatgcttgaatataactaAATCCGTCATGGTCTTTCGCAactg

Table S3. shDpp2 (*dpp*^{shmiR}) all placed in p*attb*-NE3 backbone

Oligonucleotide sequences used to clone *dpp*-targeting shmiRs

dpp2 siRNA	pNE top	ctagcagtCCACTCTAGTGGAGATCGACAtagttatattcaagcataTCTCGATCTCGACTAGAGTGGgcg
TCTCGATCTCGACTAGAGTGG	pNE bot	aattcgcCCACTCTAGTCGAGATCGAGAtatgcttgaatataactaTGTCGATCTCCACTAGAGTGGactg
dpp2-2 siRNA	pNE top	ctagcagtGGACTCTAGTGGAGATCGACAtagttatattcaagcataTCTCGATCTCGACTAGAGTCCgcg
TCTCGATCTCGACTAGAGTCC	pNE bot	aattcgcGGACTCTAGTCGAGATCGAGAtatgcttgaatataactaTGTCGATCTCCACTAGAGTCCactg
dpp2-3 siRNA	pNE top	ctagcagtGGTCTCTAGTGGAGATCGACAtagttatattcaagcataTCTCGATCTCGACTAGAGACCgcg
TCTCGATCTCGACTAGAGACC	pNE bot	aattcgcGGTCTCTAGTCGAGATCGAGAtatgcttgaatataactaTGTCGATCTCCACTAGAGACCactg
dpp2-4 siRNA	pNE top	ctagcagtGGTGTCTAGTGGAGATCGACAtagttatattcaagcataTCTCGATCTCGACTAGACACCgcg
TCTCGATCTCGACTAGACACC	pNE bot	aattcgcGGTGTCTAGTCGAGATCGAGAtatgcttgaatataactaTGTCGATCTCCACTAGACACCactg
dpp2-5 siRNA	pNE top	ctagcagtGGTGACTAGTGGAGATCGACAtagttatattcaagcataTCTCGATCTCGACTAGTCACCgcg
TCTCGATCTCGACTAGTCACC	pNE bot	aattcgcGGTGACTAGTCGAGATCGAGAtatgcttgaatataactaTGTCGATCTCCACTAGTCACCactg
dpp2MUT siRNA	pNE top	ctagcagtCCACTCTAGACCAGAAGCACAtagttatattcaagcataTCTGCTTCTGCTCTAGAGTGGgcg
TCTGCTTCTGCTCTAGAGTGG	pNE bot	aattcgcCCACTCTAGAGCAGAAGCAGAtatgcttgaatataactaTGTGCTTCTGGTCTAGAGTGGactg
dpp2-1(5′),-4 siRNA	pNE top	ctagcagtGGTGTCTAGTGGAGATCGACTtagttatattcaagcataACTCGATCTCGACTAGACACCgcg
ACTCGATCTCGACTAGACACC	pNE bot	aattcgcGGTGTCTAGTCGAGATCGAGTtatgcttgaatataactaAGTCGATCTCCACTAGACACCactg

Table S4. sh*ftz* splice acceptor and sh*white* splice acceptors originally cloned into p*attB*-NE3 vector

Oligonucleotide sequences used to clone splice acceptor-containing shmiRs*

ftz acceptor shmiR	pNE top	CTAGCAGTTGCAATCTGTTAGCATAAGGATAGTTATATTCAAGCATATGCTTATGCTTACAGATTGCAGCG
TGCTTATGCTTACAGATTGCA	pNE bot	AATTCGCTGCAATCTGTAAGCATAAGCATATGCTTGAATATAACTATCCTTATGCTAACAGATTGCAACTG
white acceptor shmiR	pNE top	CTAGCAGTGCAACCATCTCCAAATTAATATAGTTATATTCAAGCATATTTTAATTTGCAGATGGTTGCGCG
TTTTAATTTGCAGATGGTTGC	pNE bot	AATTCGCGCAACCATCTGCAAATTAAAATATGCTTGAATATAACTATATTAATTTGGAGATGGTTGCACTG

*From Fig. S6.