Cell Reports, Volume *7*

Supplemental Information

A Rapid Genome-wide MicroRNA Screen Identifies *miR-14* as a Modulator of Hedgehog Signaling

Kevin Kim, Arunachalam Vinayagam, and Norbert Perrimon

SUPPLEMENTAL METHODS

***Drosophila* strains and genetics.** All stocks were maintained and crossed at 25°C with the exception of RNAi crosses and *miR-14* overexpression crosses, which were performed at 29°C. The *miR-14* homozygous deletion mutant used is *miR-14*$^{\Delta 1}$ (Xu et al., 2003). *UAS-miR-14* is described in (Bejarano et al., 2012) and *hh-Gal4* in (Tanimoto et al., 2000). *SRF-Gal4*, *UAS-EGFP* was kindly provided by Dr. Mark Krasnow. *UAS-hh*, *UAS-ptc*, *and btl-Gal4* were kindly provided by Dr. Chrysoula Pitsouli. *ci-Gal4*, *ap-Gal4*, and *ptc-Gal4* were kindly provided by Dr. Dong Yan. *w*^{1118}, *sd-Gal4*, and *hh*^{AC} were obtained from the Bloomington Stock Center (http://flybase.bio.indiana.edu). RNAi lines from the TRiP facility at Harvard Medical School (http://www.flyrnai.org/TRiP-HOME.html) are: *hh-RNAi* (HMS00492), *ptc-RNAi* (JF032230), and *smo-RNAi* (JF02363).

Quantification of adult wing size and *hh*^{Mrt} phenotype analysis. Wings clipped from adult male flies, were mounted in 50% Xylene and 50% Permount (Fisher) solution and imaged using Zeiss Axioskop 2. For quantification of wing size, wing area was measured using ImageJ (NIH). We used Microsoft Excel to calculate the average values and the corresponding standard deviation, and to perform the *t*-test analysis.

Western blots. Lysates prepared from whole pupae were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and subjected to Western analysis using antibodies against Hh (1:1000; kindly provided by Dr. Xinhua Lin), Ptc (1:250; kindly provided by Dr. Matt Scott), Smo (1:500; (Denef et al., 2000); kindly provided by Dr.

Dong Yan) and α-Tubulin (1:1000; Sigma). Blots were subsequently incubated with HRP-conjugated goat secondary antibody (Amersham), and processed for chemiluminescence (Pierce). For quantification of band intensity, the raw images were analyzed using ImageJ (NIH).

qPCR. Total RNA was prepared from whole pupae. RNA was extracted using TRIzol Reagent (Invitrogen) and treated with DNAse I (Promega). cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad) and qPCR was performed using iQ SYBR Green Supermix (Bio-Rad). *gapdh* and *rp49* were used to normalize the RNA levels. Relative quantification of mRNA levels was calculated using the comparative CT method. qPCR primer sequences are available upon request.

SUPPLEMENTAL REFERENCES

Bejarano, F., Bortolamiol-Becet, D., Dai, Q., Sun, K., Saj, A., Chou, Y.T., Raleigh, D.R., Kim, K., Ni, J.Q., Duan, H.*, et al.* (2012). A genome-wide transgenic resource for conditional expression of Drosophila microRNAs. Development *139*, 2821-2831.

Denef, N., Neubuser, D., Perez, L., and Cohen, S.M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell *102*, 521-531.

Rehmsmeier, M., Steffen, P., Hochsmann, M., and Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. RNA *10*, 1507-1517.

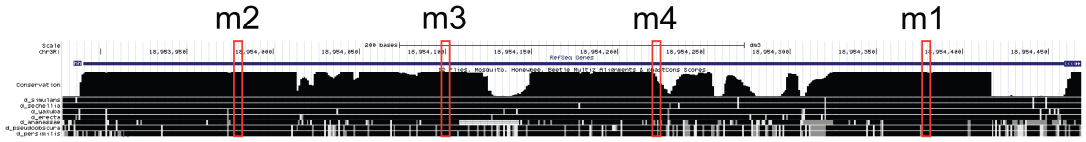
Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. Mol Cell *5*, 59-71.

Xu, P., Vernooy, S.Y., Guo, M., and Hay, B.A. (2003). The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol *13*, 790-795.

SUPPLEMENTAL FIGURES

A

hh 3' UTR



m1 mfe: -10.7 kcal/mol position 78

```
target 5'                U      U 3'
                          GAUUGA
                          CUGACU
miRNA  3' UAUCCUCUCUCUUUUU        5'
```

m2 mfe: -16.0 kcal/mol position 455

```
target 5' U    A   ACCAUUAUACU      A 3'
            AUAG AGA           GACUGA
            UAUC UCU           CUGACU
miRNA  3'      C   CUCUUUUU           5'
```

m3 mfe: -18.3 kcal/mol position 343

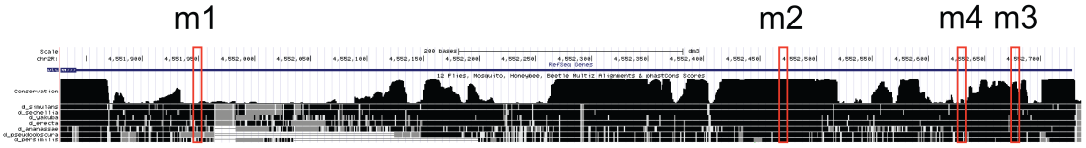
```
target 5'   C     ACAC   C     U 3'
              GGAGA    AGA GACUG
              CCUCU    UUU CUGAC
miRNA  3' UAU     CUCU   U     U 5'
```

m4 mfe: -10.6 kcal/mol position 224

```
target 5'      C  AAU     C     U 3'
                GA   GAAAA  ACUG
                CU   CUUUU  UGAC
miRNA  3' UAUCCU  CU      UC    U 5'
```

B

ptc 3' UTR



m1 mfe: -14.4 kcal/mol position 91

```
target 5'  C  C   UCU UU       U  3'
             AG GGG   G  GAGGAUU
             UC CUC   C  UUUCUGA
miRNA  3' UA      UCU UU       CU 5'
```

m2 mfe: -12.2 kcal/mol position 611

```
target 5'             C      U   A 3'
                       AAGGAC UGA
                       UUUCUG ACU
miRNA  3' UAUCCUCUCUCUU            5'
```

m3 mfe: -16.4 kcal/mol position 817

```
target 5'             C         C 3'
                       GAAGACUGA
                       UUUCUGACU
miRNA  3' UAUCCUCUCUCUU           5'
```

m4 mfe: -14.9 kcal/mol position 758

```
target 5'   U   AAAC  AGC      C      U 3'
              GGA    GA   GAGGAA  GAUUG
              CCU    CU   CUCUUU  CUGAC
miRNA  3' UAU                  UU     U 5'
```

C

Figure S1. Detailed locations of predicted *miR-14* MREs within the 3' UTRs of 3 target genes. (A-C) Alignments and conservation data were produced by the UCSC Genome Center. The *miR-14* and 3' UTR alignments were predicted by RNAHybrid (Rehmsmeier et al., 2004). Boxed sequences show the seed sequence alignments. **(C)** Note that a single nucleotide polymorphism (SNP) is present in the predicted MRE (m6) of the cloned *smo* 3' UTR. Genomic sequences were verified by sequencing and confirmed that the SNP is real and not the result of a PCR error. The SNP is labeled with red colored font.

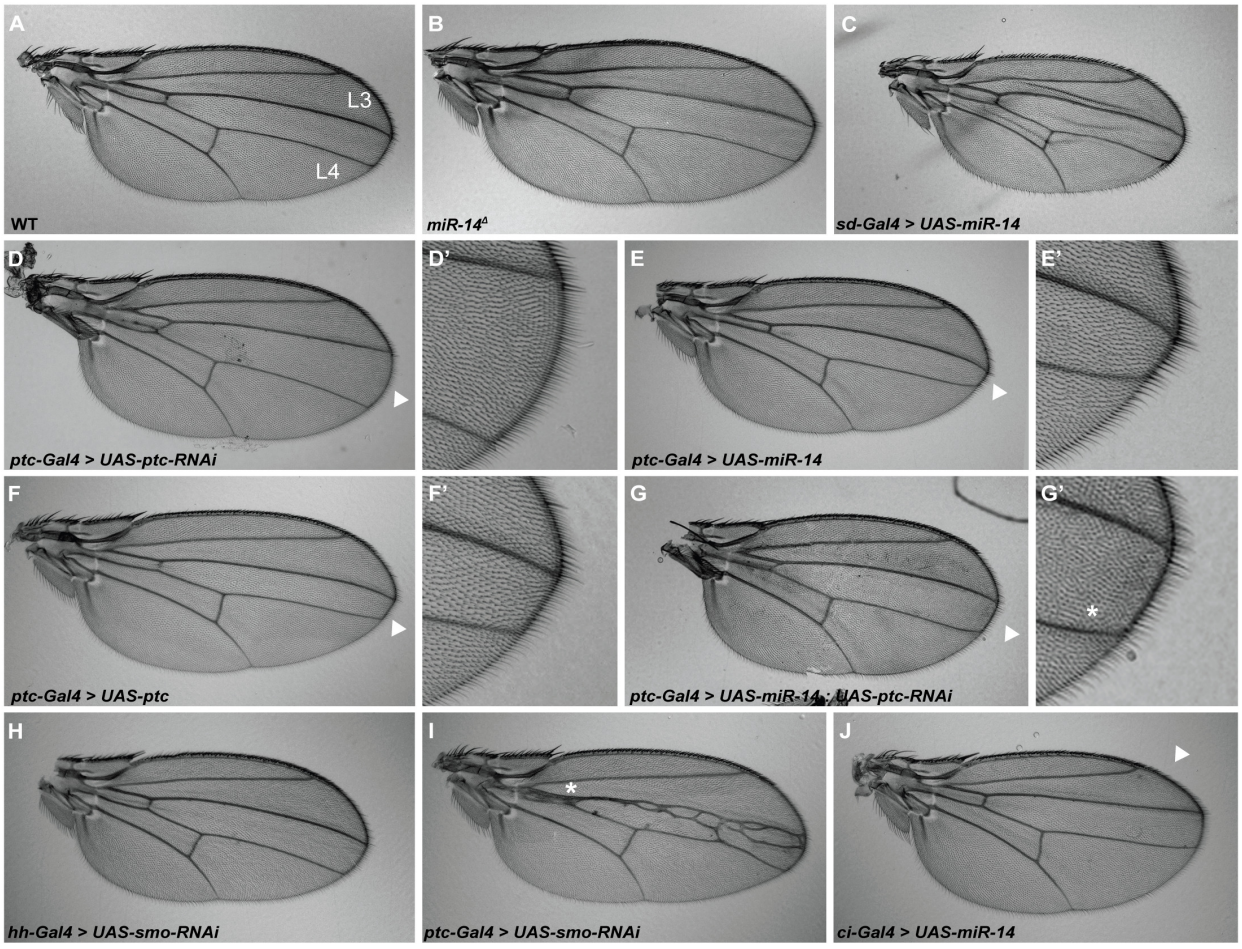


Figure S2. Adult wing phenotypes. (A) Wild-type wing. L3 and L4 are two major veins that mark the A-P boundary of the wing. **(B)** *miR-14* mutant wing. **(C)** Overexpression of *miR-14* using *sd-Gal4*, which is uniformly expressed throughout the wing pouch, results in small wing. **(D-D')** RNAi against *ptc* at the A-P boundary causes overgrowth of the region, resembling hyperactivation of Hh signaling. **(E-E')** Overexpression of *miR-14* reduces the distance between veins L3 and L4 (marked by arrowhead). **(F-F')** Overexpression of *ptc* at the A-P boundary phenocopies overexpression of *miR-14*. **(G-G')** Overexpression of *miR-14* along with *UAS-ptc-RNAi* at the A-P boundary partially phenocopies overexpression of *miR-14* alone. Note the slight curving of L4 vein towards

the anterior direction of the wing (marked by asterisk) **(G')**. **(H)** RNAi against *smo* in the posterior compartment by *hh-Gal4* does not disrupt the development of the wing. **(I)** Reducing the level of *smo* in the A-P region results in a clear decrease in the L3 and L4 intervein region (marked by asterisk), consistent with reduced Hh signaling. **(J)** Overexpression of *miR-14* in the A compartment reduces the overall size of the A compartment causing the wing to curve in the anterior direction (marked by arrowhead).

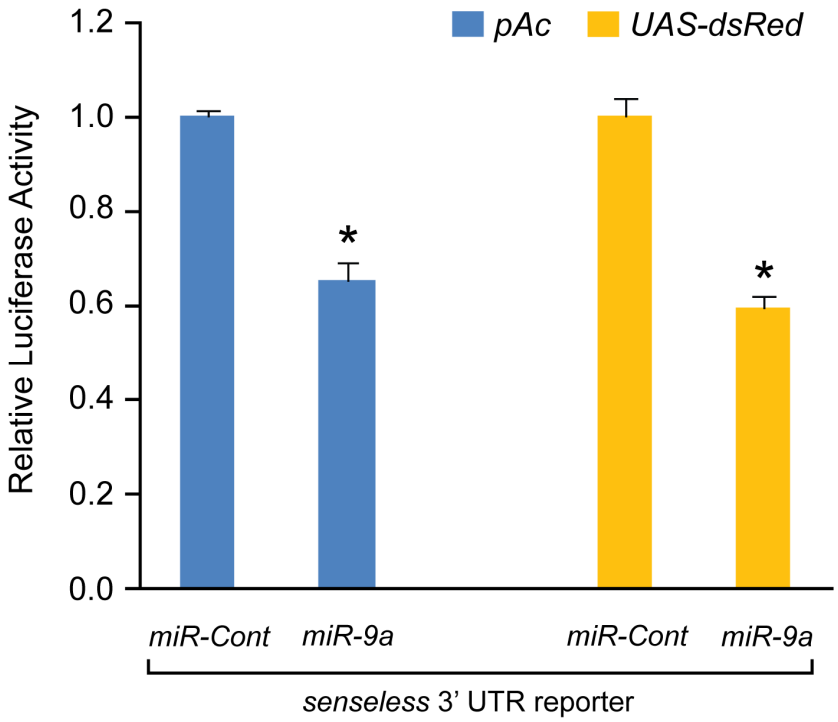


Figure S3. Comparing the miRNA activities of *pAc* and *UAS-dsRed* vectors. Both *pAc* and *UAS-dsRed-miR-9a* are equally effective at repressing the *senseless* 3' UTR luciferase reporter activity.

SUPPLEMENTAL TABLES

Genes	3' UTR (bp)	Forward Primer	Reverse Primer
cubitus interruptus (*ci*) (FBgn0004859)	279	CGGAATTCAAAATGTTATCTAGCTAACAC	CCGAGCTCGCCGGTATTAAGGGGAAAAT
casein kinase Iα (*CKIα*) (FBgn0015024)	1,341	CGGAATTCGAGCTGCAGCGCATTCAGACG	CCGAGCTCAATTAAATGTTGGGGCTCAAAT
costa (*Cos2*) (FBgn0000352)	1,033	CGGAATTCATACGAATTTAACCATTTCCA	CCGAGCTCTGATTAAACACTGATTGATTAGT
Fused (*Fu*) (FBgn0001079)	774	CTAGCTAGCCCGGCACTTTCTTTTATTGG	CCGAGCTCCACCATAGAGCCATTGGTGA
shaggy (*GSK3*) (FBgn0003371)	1,016	CGGAATTCGGGAAATAGTAACATACATAC	CCGAGCTCAAACAGTTTCGCTTTCGCTTTTGCT
hedgehog (*hh*) (FBgn0004644)	569	CGGAATTCGATGGAATCCTGGAAGAGCGA	CCGAGCTCGCGCTCATTTGAATAACCTGA
patched (*ptc*) (FBgn0003892)	874	CGGAATTCCACTAGCACTAGTTCCTGTAG	CCGAGCTCGATACAGCGAAAACTTCCGTTTC
smoothened (*smo*) (FBgn0003444)	654	CGGAATTCCAAGACTAAATAAGCAATTGATGC	CCGAGCTCTTAAAGCAAGGCTAGGACTCG
Suppressor of fused (*Su(fu)*) (FBgn0005355)	159	CGGAATTCTGGTGTCCATTGGTTAGCTAGT	CCGAGCTCATCAAAGGCCCGTCGAGT

Table S1. List of Hh pathway genes, the size and the primer sequences used to amplify the 3' UTRs used in the screen. Red colored sequence represents restriction sites and green colored sequence represent flanking sequences added for efficient restriction enzyme digestion.

Supplemental Tables S2-S4 are provided as Microsoft Excel Files

Table S2. List of miRNA overexpression plasmids used for the screen and normalized luciferase levels from three separate experiments for 9 Hh pathway genes. For each miRNA-target interaction pair, a negative log2 median fold-change (LMF) score was calculated, where the higher LMF score corresponds to stronger repression of the target gene by the miRNA.

Table S3. Compiled list of miRNAs predicted to regulate 3' UTRs of Hh pathway components from the screen. For each predicted miRNA-target pair, confidence score assigned by individual tools were extracted. We used the least stringent cutoff values for each tool to compile all possible miRNA-target predictions. High confidence miRNA-target interaction scores for each tool are: TargetScan, Branch-Length score ≥ 0.8; miRanda, mirSVR score ≤ -0.5; and DIANA, miTG score ≥ 0.5.

Table S4. Analysis of the true positive rate (TRP) and false positive rate (FPR) values for various LMF score cutoff values using positive reference set (PRS) and random reference set (RRS).