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Supplemental Data

The Mirtron Pathway Generates MicroRNA-Class Regulatory RNAs in *Drosophila*

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Supplemental Experimental Procedures

1. Genomic pri-mirtron expression constructs.

We used the following primer pairs to amplify ~400 nt pri-miRtron fragments from Canton S genomic DNA. These were cloned into the NotI/XbaI sites in the 3' UTR of UAS-DsRed (Stark et al., 2003) and sequence verified.

mir-1003 for: GGGgcggccgcGTGAACGACTGCAACAGCA
rev: GggtctagaCTTGTGCGTCTCCTCCTTTC

mir-1010 for: GGGgcggccgcAAGGGCGACCTTATCGATGT
rev: GggtctagaGGTTGAGAATGCCCAGGTAA

mir-1004 for: GGGgcggccgccgagggtattcccatgtacg
rev: Gggtctagagaggcaggcatcctttagaa

2. “Intron” mirtron expression constructs.

We first generated a UAS-DsRed-myc vector with in-frame cloning sites between the DsRed and 2xmyc coding sequences. The top strand is shown below.

```
         DsRed2                Asc                      Not I            stop XhoI
5'CGC CAC CAC CTG TTC CTG GCG CGC Cag  insert  atG CGG CCG Cag 2xmyc TAA CTCGAG GGG 3'
   R   H   H   L   F   L   A   R   Q           M   R   P   Q
```

The top strand sequence of 2xmyc is:

ATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAA

In the “empty” vector, the insert position is occupied by the small *white* intron:

GTGAGTTTCTATTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAG

We then cloned the following oligo pairs into AscI/NotI-digested UAS-DsRed[intron]myc vector. Note that the AscI and NotI sites are destroyed in the process; SpeI was introduced as a marker site.

```
miR-1003
CGCGaactagtacacagGTGGGTATCTGGATGTGGTTGGCTCTGGCGGTCCTCTCACATTTACATATTCACAGatcaaac
    ttgatcatgtgtcCACCCATAGACCTACACCAACCGAGACCGCCAGGAGAGTGTAAATGTATAAGTGTCtagtttgCCGG

miR-1003Gmut
CGCGaactagtacacagcTGGGTATCTGGATGTGGTTGGctctggcggtccTCTCACATTTACATATTCACAGatcaaac
    ttgatcatgtgtcgACCCATAGACCTACACCAACCgagaccgccaggAGAGTGTAAATGTATAAGTGTCtagtttgCCGG

miR-1008
CGCGaactagtacacagGTAAATATCTAAAGTTGAACTtggccaatggcaagTCACAGCTTTTTGTGTTTACAGatcaaac
    ttgatcatgtgtcCATTTATAGATTTCAACTTGAaccggttaccgttcAGTGTCGAAAAACACAAATGTCtagtttgCCGG

miR-1008Gmut
CGCGaactagtacacagcTAAATATCTAAAGTTGAACTtggccaatggcaagTCACAGCTTTTTGTGTTTACAGatcaaac
    ttgatcatgtgtcgATTTATAGATTTCAACTTGAaccggttaccgttcAGTGTCGAAAAACACAAATGTCtagtttgCCGG
```

3. Hybrid pri-miRNA/mirtron construct.

The miR-1003 sequence was substituted into a pri-miR-6-1 fragment, with appropriate changes on the miRNA* sequence to maintain pairing. The following sequence (top strand only shown) was digested and cloned into the NotI and Xba sites of UAS-DsRed.

5' GGG GTCGAC GCGGCCGC
ttacttaaacttaatcacagcctttaatgtctGTGGATATGTgCATGTGCGGTAagttaatataccatatcta**TC**
TCACATTTACATATTCACAG gtacctaaagtgcctaacatcattatttaattt
CTCGAG TCTAGA GGG 3'

4. Luciferase sensor constructs.

We modified psiCheck2 (Promega) by inserting the following oligo pair into SalI/NotI-digested vector.

```
    SalI SacI  NotI   XbaI   SalI EcoRI EcoRV XhoI   SpeI
5' TCGACGAGCTCGCGGCCGCTCTAGAGTCGACGAATTCGATATCCTCGAGACTAGTC 3'
    3' GCTCGAGCGCCGGCGAGATCTCAGCTGCTTAAGCTATAGGAGCTCTGATCAGCCGG 5'
```

Sensors were then cloned by inserting the following oligo pairs into the NotI/XhoI sites of modified psiCheck.

```
miR-1003 sensor
```

```
GGCCGCctgtgaatatgtaaatgtgagaaatcacctgtgaatatgtaaatgtgagac
    CGgacacttatacatttacactctttagtggacacttatacatttacactctgagct

miR-1003 seed mut sensor
GGCCGCctgtgaatatgtaaatCtCaCaaatcacctgtgaatatgtaaatCtCaCac
    CGgacacttatacatttaGaGtGtttagtggacacttatacatttaGaGtGtgagct

miR-1004 sensor
GGCCGCctgtgagggaagtgatgtgagaaatcacctgtgagggaagtgatgtgagac
    CGgacactcccttcactacactctttagtggacactcccttcactacactctgagct

miR-1004 seed mut sensor
GGCCGCctgtgagggaagtgatCtCaCaaatcacctgtgagggaagtgatCtCaCac
    CGgacactcccttcactaGaGtGtttagtggacactcccttcactaGaGtGtgagct
```

For the miR-1010 miRNA-type sensor (in which the three central bases are unpaired), we cloned a 4-copy multimer of the following oligos into the Sal/XhoI sites of modified psiCheck.

```
miRtron-2 mi-sensor
tcgacaaa CTGCAAATGGAAatcTAGGTGAAA aaac
    gttt GACGTTTACCTTtagATCCACTTT tttgagct
```

5. GFP sensor transgenes.

The oligo pairs for miR-1003 and miR-1004 sensors were cloned into the NotI/XhoI sites in the 3' UTR of tub-GFP-SV40 3'UTR (Brennecke et al., 2005).

6. pLitmus templates for synthesis of dsRNA.

The following PCR primers were used to clone fragments of *Ldbr*, *Drosophila Exp5*, *Ago1* and *Ago2* into the XhoI-XbaI sites of pLitmus (NEB). This vector contains opposing T7 promoters flanking the cloning site and were used to synthesize dsRNA using the Megascript kit (Ambion).

dDBR:
XhoI-dDBR-1125+ AGAGCTCGAGTGAAGACGAGGAAAGGGAGA
XbaI-dDBR-1549- AGAGGTCTAGACGTATTGCTCTGGGGGTTTA

Exp5N:
XhoI-Exp5-110+ AGAGCTCGAGCCTGTGAGCGGTTTAAGGAG
XbaI-Exp5-487- AGAGGTCTAGAGTCGCAAGAAGACCAGAAGC

Exp5C:
XhoI-Exp5-2917+ AGAGCTCGAGCTGGAGGATCAGCTCAATCG
XbaI-Exp5-3406- AGAGGTCTAGAGACGGAGCAGCTCGTAGAAC

Ago1:
XhoI-Ago1-2497+ AGAGCTCGAGTCTCCGAGGGACAATTCC
XbaI-Ago1-2896- AGAGGTCTAGAGCCACTAAATGGGCGTAGT

Ago2:
XhoI-Ago2-2475+ AGAGCTCGAGTATGGTGAAGAACGGGTCGT
XbaI-Ago2-2928- AGAGGTCTAGACTTGTGGTTGATGCCGTTC

Other templates in pLitmus were obtained from Phil Zamore (Forstemann, 2005).

7. Quantitative reverse transcription-PCR analysis (Q-PCR).

Q-PCR was performed with Thermo-X One Step RT-PCR Platinum Taq HiFi (Invitrogen) and SYBR Green (ABI) using a MyiQ Real-Time PCR Detection System (BioRad) and the included software package.

Primers for Q-PCR analysis of the *actin* intron.

Act5Cint-103+ CGGGTTCCAATTCGAGTTTT
Act5Cint-226- GAAAAGGGAGGGGAGAAGC

Primers for control amplification of the *rp49* gene.

rp49 A2 ATCGGTTACGGATCGAACA
rp49 B2 ACAATCTCCTTGCGCTTCTT

8. Ago-1 co-immunoprecipitation assay.

Assay was modified from a published protocol (Miyoshi et al., 2005). 0-10 h embryos (~400ul) were collected and dechorionated in bleach. Embryos were then homogenized with 5-6 strokes of a chilled dounce homogenizer (Kontes) in 2ml of Hepes-NP40 buffer (30 mM HEPES (pH 7.4), 100 mM sodium chloride, 2 mM magnesium acetate, 5mM DTT and 0.1% NP40). Ago1 complex was purified from embryo lysate using anti-Ago1 antibody immobilized on Gamma-Bind beads (Amersham Bioscience). Sodium chloride was added to the lysates to 800 mM just before immunoprecipitation was started. The reaction mixtures were rocked at 4°C for 1.5 h,

and the beads washed extensively with a buffer containing 30 mM HEPES (pH 7.4), 800 mM sodium chloride, 2 mM magnesium acetate, 5 mM DTT and 0.1% NP40. The final wash was carried out with Hepes-NP40 buffer containing 100mM NaCl. Proteins were eluted with SDS sample buffer without DTT. DTT was added to the eluate up to 100mM before boiling. RNAs were isolated from immunoprecipitate with Phenol/Chroloform/Isoamylalcohol (Sigma). Anti-T7 tag mouse monoclonal antibody (Novagen) was used as a control antibody in parallel immunoprecipitations. 20% (Protein) or 15% (RNA) of the input and supernatants and 100% of the immunoprecipitates were analyzed by Western or Northern blot.

Figure S1. Additional examples of well-conserved and rapidly-evolving mirtrons

Alignments and conservation data were produced by the UCSC Genome Center (http://genome.ucsc.edu/). Gold tracks depict the positions of cloned small RNAs, the blue track depicts the exon/intron structure of host genes, and the black tracks at bottom depict nucleotide conservation of the region across 12 Drosophilid species. Greater height of the "conservation" track reflects deeper sequence conservation. Note that the terminal loop region (red arrows) exhibits accelerated divergence relative to the mirtron hairpin arms in both well-conserved (A) and poorly-conserved (B) mirtrons.

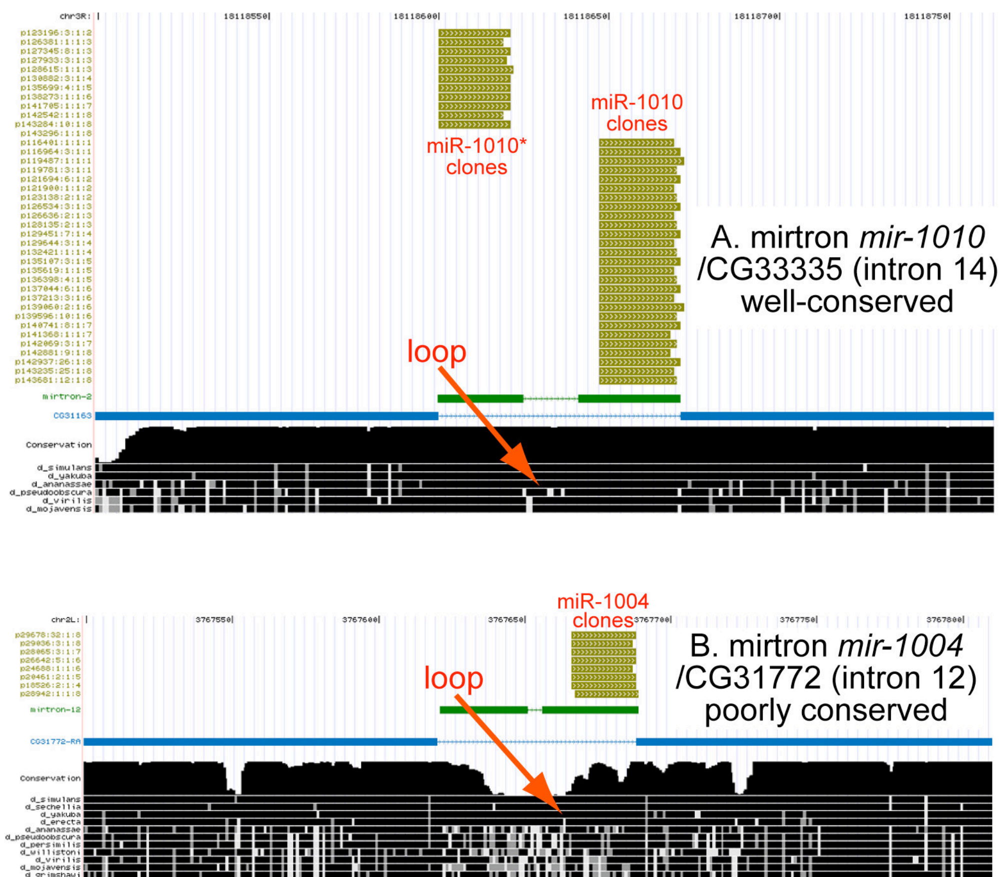


Figure S2. Confirmation of dsRNA knockdowns

A. Effective knockdown of Dcr-1 and Drosha in S2 cells. Western blot for Dcr-1 and Drosha accumulation showed little effect with non-cognate dsRNAs, but >90% knockdown with cognate dsRNAs. α-tubulin was used as a loading control.

B. Ldbr knockdown. (Left) S2 knockdown. Cells were treated with dsRNAs against *GFP*, *Drosha* or *Ldbr*. Quantitative rt-PCR demonstrated accumulation of the *actin* intron relative to an *rp49* control in *Ldbr* knockdown cells. Threshold cycle and baseline curves were determined using the software included with the MyiQ real-time PCR instrument (BioRad) and used to calculate mean fold changes from 4 independent samples of dsRNA-treated cells. Error bars represent standard deviations. (Right) Transgenic knockdown. RNA was extracted from three independent samples each of Canton S (control) or *da-Gal4, UAS-Ldbr*^{RNAi} late third instar larvae and analyzed in the same way.

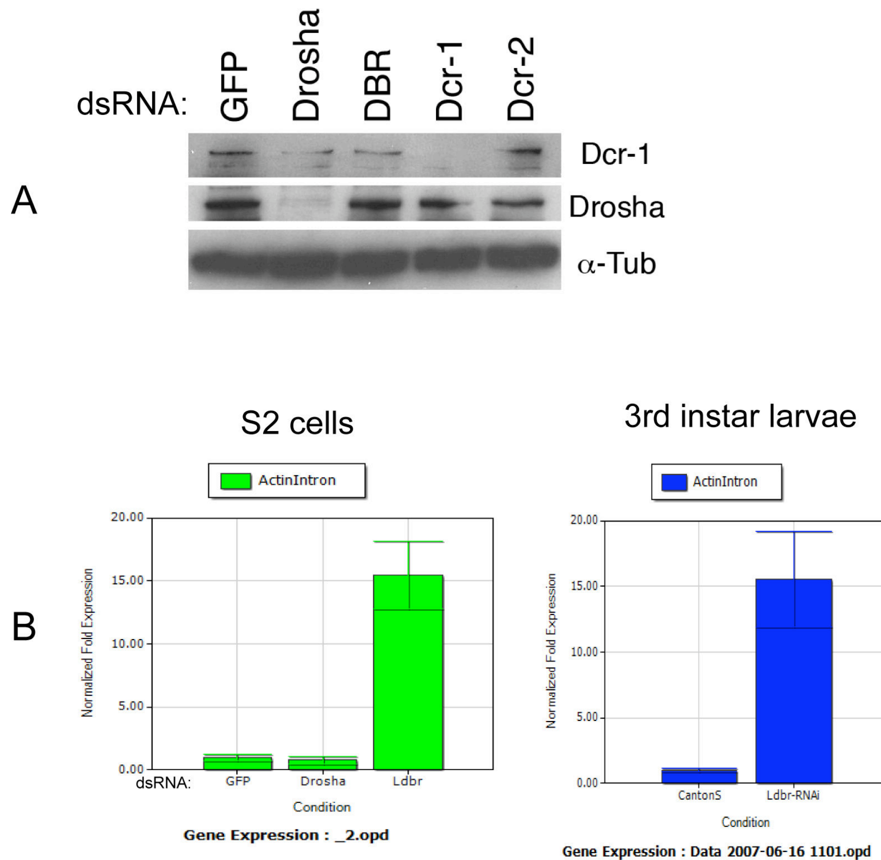


Figure S3. Quantification of the effect of *Ldbr* knockdown on mirtron maturation in transgenic animals

Three separate isolates of RNA from Canton S (control) and *da-Gal4>UAS-Ldbr*^{RNAi} larvae were blotted and probed sequentially for miR-1010 and miR-1. Phosphorimager analysis was performed, and the mean ratio of mature miR-1010 to miR-1 was calculated; this ratio was set to unity in control samples. Error bar represents standard deviation.

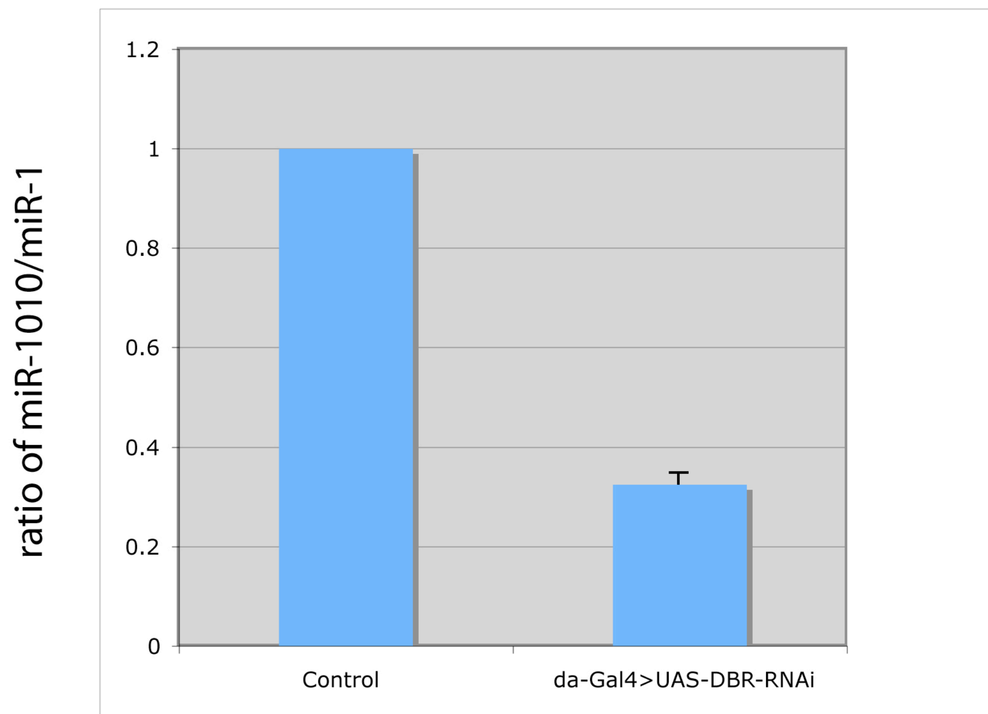


Figure S4. Effect of *Exportin-5* dsRNA on the maturation of endogenous miRNAs and exogenous mirtrons

S2 cells were treated with dsRNAs against *GFP* or two different regions of *Exportin-5* (*Exp5-N* and *Exp5-C*), then transfected with *ub-Gal4* and *UAS-DsRed-small RNA* expression constructs. Treatment with either *Exp5* dsRNA decreased the level of mature miR-2b (lanes 1-3), as reported previously (Shibata et al., 2006). *Exp5* dsRNAs also reduced the steady state levels of hairpin and mature small RNAs derived from *mir-1003* (lanes 4-6) and *mir-1010* (lanes 7-9) mirtron expression constructs.

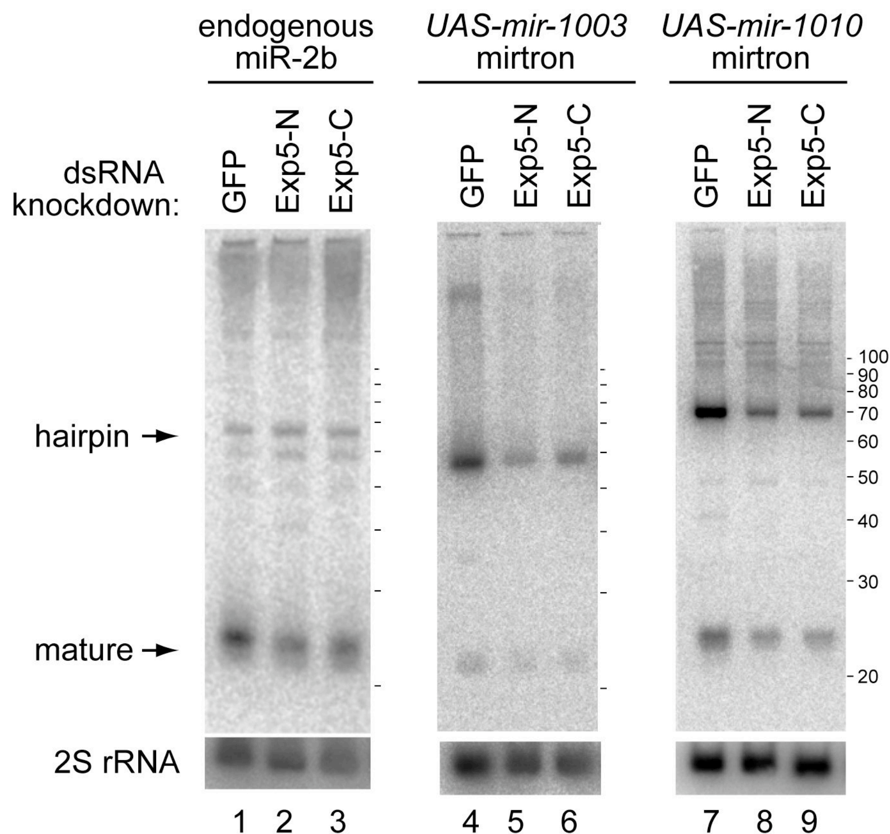
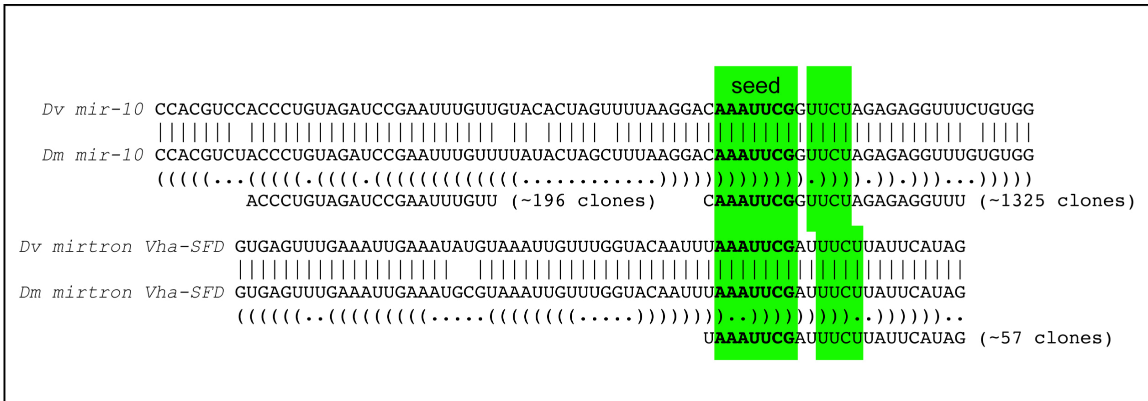


Figure S5. Similarities between *mir-10* and a mirtron located in *VhaSFD*

Shown are alignments of the precursors of the *mir-10* and *Vha-SFD* mirtron genes between the highly diverged species *D. virilis* (*Dv*) and *D. melanogaster* (*Dm*). The clone numbers are given as approximate since ~10% of clones have different termini (likely due to end degradation). The major, mature small RNAs produced from these loci are highly related across their 5' halves (green shade), and share an identical seed (AAAUUCG). Note that the predominant small RNA produced from *Drosophila mir-10* is not the left arm, “5p” miRNA (Aravin et al., 2003) but rather the right arm, “3p” miRNA (Schwarz et al., 2003).



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