Identification of Drosophila MicroRNA Targets

Alexander Stark*, Julius Brennecke*, Robert B. Russell, Stephen M. Cohen*

European Molecular Biology Laboratory, Heidelberg, Germany

MicroRNAs (miRNAs) are short RNA molecules that regulate gene expression by binding to target messenger RNAs and by controlling protein production or causing RNA cleavage. To date, functions have been assigned to only a few of the hundreds of identified miRNAs, in part because of the difficulty in identifying their targets. The short length of miRNAs and the fact that their complementarity to target sequences is imperfect mean that target identification in animal genomes is not possible by standard sequence comparison methods. Here we screen conserved 3' UTR sequences from the Drosophila melanogaster genome for potential miRNA targets. The screening procedure combines a sequence search with an evaluation of the predicted miRNA–target heteroduplex structures and energies. We show that this approach successfully identifies the five previously validated let-7, lin-4, and bantam targets from a large database and predict new targets for Drosophila miRNAs. Our target predictions reveal striking clusters of functionally related targets among the top predictions for specific miRNAs. These include Notch target genes for miR-7, proapoptotic genes for the mir-2 family, and enzymes from a metabolic pathway for mir-277. We experimentally verified three predicted targets each for mir-7 and the mir-2 family, doubling the number of validated targets for animal miRNAs. Statistical analysis indicates that the best single predicted target sites are at the border of significance; thus, target predictions should be considered as tentative until experimentally validated. We identify features shared by all validated targets that can be used to evaluate target predictions for animal miRNAs. Our initial evaluation and experimental validation of target predictions suggest functions for two miRNAs. For others, the screen suggests plausible functions, such as a role for mir-277 as a metabolic switch controlling amino acid catabolism. Cross-genome comparison proved essential, as it allows reduction of the sequence search space. Improvements in genome annotation and increased availability of cDNA sequences from other genomes will allow more sensitive screens. An increase in the number of confirmed targets is expected to reveal general structural features that can be used to improve their detection. While the screen is likely to miss some targets, our study shows that valid targets can be identified from sequence alone.

Introduction

MicroRNAs (miRNAs) are small RNAs, typically of approximately 21–23 nt, that direct posttranscriptional regulation of gene expression by binding to messenger RNAs (mRNAs). Many endogenously encoded miRNAs have been cloned from plants and animals (Lagos-Quintana et al. 2001, 2002; Lau et al. 2001; Lee and Ambros 2001; Mourrelatos et al. 2002; Reinhart et al. 2002; Ambros et al. 2003; Aravin et al. 2003; Lim et al. 2003). Combining these data with computational cross-genome comparison predicts 100–120 miRNA-encoding genes in Caenorhabditis and Drosophila and approximately 250 in mouse and human (Ambros et al. 2003; Grad et al. 2003; Lai et al. 2003; Lim et al. 2003a, 2003b). However, functions have been assigned to only four animal miRNAs (Reinhart et al. 2000; Brennecke et al. 2003; Lee et al. 1993; Wightman et al. 1993; Xu et al. 2003), in part owing to the difficulty in identifying mutations in such small genes. A method to identify the target genes that are regulated by miRNAs would greatly help the study of miRNA function in animals (Ambros 2001).

Two modes of miRNA-directed target inhibition have been demonstrated. The same small RNA can cause degradation of its target mRNA or block its translation depending on the degree of miRNA–target sequence complementarity (Hutvagner and Zamore 2002; Doench et al. 2003). Target RNAs containing sequences with perfect complements of the miRNA (or exogenously supplied short interfering RNA [siRNA]) are cleaved by the RNA-induced silencing complex (RISC) ribonuclease (Hutvagner and Zamore 2002; Martinez et al. 2002; Zeng et al. 2002). Endogenous plant miRNAs have been shown to regulate target RNAs by RNA interference (RNAi) involving perfect or near-perfect target site complementarity (Llave et al. 2002b; Kasschau et al. 2003; Palatnik et al. 2003; Tang et al. 2003; Xie et al. 2003). Targets for plant miRNAs have been identified on a genome-wide scale by searches that require a high degree of sequence complementarity (Rhoades et al. 2002). However, this approach did not find targets for known animal miRNAs. The animal miRNAs tested until now pair imperfectly with their targets and act to control translation. Indeed, systematic analysis of the complete C. elegans miRNA complement has confirmed the absence of targets with perfect or near-perfect sequence complementarity (Ambros et al. 2003).

To date targets have been experimentally validated for just three animal miRNAs: the lin-4 targets lin-14 and lin-28 (Wightman et al. 1993; Ha et al. 1996; Moss et al. 1997; Olsen...
and Ambros 1999; Seggerson et al. 2002), the let-7 targets lin-41 and lin-57/hbl-1 (Reinhart et al. 2000; Slack et al. 2000; Abrahante et al. 2003; Lin et al. 2003), and the bantam target hid (Brennecke et al. 2003). These miRNA–target duplexes contain mismatches, gaps, and G:U basepairs at different positions. Even allowing for G:U basepairs, the longest contiguous alignments in these examples range from 8 to 10 nt. Such limited information content makes it difficult to identify targets within whole-genome or transcriptome databases, since standard alignment methods produce many false positives with such short variable sequences. Furthermore, the small number of validated examples makes the development and benchmarking of a generally applicable computational method problematic at present. Here we present a screen for miRNA targets in Drosophila that identifies all of the previously known miRNA targets and we demonstrate that it successfully predicts new targets that we validate experimentally.

Results

Database Design

For each of the validated miRNA–target pairs, functional target sites are located in the 3′ untranslated region (UTR) of the miRNA and are conserved in the 3′ UTRs of the homologous genes from related species (Wightman et al. 1993; Moss et al. 1997; Pasquinelli et al. 2000; Brennecke et al. 2003). We used pairwise comparison of the 3′ UTRs of orthologous genes in related genomes to identify conserved 3′ UTR sequences. Figure 1A shows the resulting pattern of 3′ UTR conservation for the known targets in worms and flies. The vast majority of miRNA target sites (red bars in Figure 1A) are located in blocks of conserved sequence (white blocks in Figure 1A). Figure 1B shows cross-genome conservation of these miRNA target sites. A striking pattern of uninterrupted conservation emerges at the end of the target sequences that pair with the 5′ end of the miRNAs.

To permit genome-wide searches for targets of Drosophila miRNAs, a conserved 3′ UTR database was prepared by comparison of Drosophila melanogaster and Drosophila pseudoobscura 3′ UTRs. As very few 3′ UTRs are defined by cDNA sequence data in D. pseudoobscura, we used genomic sequence following the last exon of the D. pseudoobscura gene as the orthologous UTR (see Materials and Methods). Last exons were reliably detected in D. pseudoobscura for approximately two-thirds of D. melanogaster genes. On average, 22% of the D. melanogaster 3′ UTR sequence is conserved in the predicted D. pseudoobscura 3′ UTR. Much of this reflects isolated blocks of very high conservation interspersed among less-conserved sequence. Use of conserved 3′ UTRs reduces the expected number of sequence matches that would occur at random by 4- to 5-fold in relation to full-length 3′ UTRs, and severalfold further compared to the full transcriptome. We considered using the Anopheles gambiae genome to extend the cross-species comparison. Although genome annotation identifies orthologs for two-thirds of D. melanogaster genes (Zdobnov et al. 2002), we were unable to identify the last D. melanogaster exon for approximately half of these. We therefore chose not to require conservation in Anopheles, but use it as an additional level of validation for predicted Drosophila targets, where possible.

Screening Strategy

We have adopted a two-step approach to target identification that combines a sensitive sequence database search with an RNA folding algorithm to evaluate the quality of the RNA duplex formed between the miRNA and its predicted targets. We examined the known target sites for lin-4, let-7, and bantam for common features. All of these sites showed better complementarity to the 5′ end of the miRNA, with no obvious common features elsewhere (Figure 2A and 2B). There were few sequence mismatches or G:U basepairs in the alignment of the first eight residues at the 5′ end of the miRNA. We used the alignment tool HMMer (Eddy 1996) to search for sequences complementary to the first eight residues of the miRNA, allowing for G:U mismatches. Where possible, the corresponding sites were also identified in the D. pseudoobscura 3′ UTR, and the sites from both genomes were considered, since the regions outside of the sequence match can vary between the two organisms, leading to differences in subsequent steps (see below).

The identified sequences were extended to the length of the miRNA plus five residues to allow for bulges and were evaluated for their ability to form energetically favorable RNA–RNA duplexes with the miRNA using Mfold, which combines knowledge of known RNA structures with thermodynamic parameters, such as those involved in basepairing to evaluate the free energy of folding (ΔG) (Mathews et al. 1999; Zuker et al. 1999). Mfold requires a single linear sequence as input, so each predicted target was linked to the miRNA using a standard hairpin-forming linker sequence (GCGGAGAC-GC). An example of the Mfold output is shown in Figure 2C for the top-scoring bantam miRNA target site that we had previously identified in the 3′ UTR of hid (Brennecke et al. 2003).

The Mfold free energy of folding (ΔG) was determined for each predicted target, which allows predicted sites to be ranked according to ΔG. However, ΔG depends on miRNA length and GC content, so it is not possible to distinguish systematically real targets from random matches using the raw ΔG score or to compare different miRNAs. Instead, we calculated Z-values as a measure of nonrandomness, with an average random site scoring $Z = 0$ ($Z = \text{standard deviations} / \text{SD}$ above the mean of background matches). Figure 2D shows the distribution of folding energies for predicted targets of the bantam miRNA compared to 10,000 randomly selected target sequences.

Most of the previously validated targets have more than one predicted miRNA-binding site in their 3′ UTRs. Use of the Z-value allows us to add the scores of several sites within one UTR by selecting only those scores that are different from background matches. This is not possible with ΔG alone because even average random matches have favorable energy values (Figure 2D) and the sum of several average random matches in a UTR could score better than a single true site. We have selected $Z \geq 3$ as a cutoff value, as folding energies of more than 3 SD above the mean ($Z \geq 3$) are expected to occur for only 0.3% of random matches. Use of a higher Z-value increases the likelihood that predictions are correct, but also increases the risk of missing out contributions from real sites of lower folding energy. For example, only three of the five conserved bantam sites previously identified in the hid 3′ UTR score $Z \geq 3$ (with the best site at $Z = 7.4$). We evaluated our
Figure 1. Features of Known miRNA Target Sequences
(A) Comparison of sequence conservation in the 3’ UTRs of miRNA target genes. For lin-14, lin-28, lin-41, and lin-57, comparison was between C. elegans and C. briggsae. For hid, comparison was between D. melanogaster and D. pseudoobscura. White regions indicate conservation, and black regions are not conserved under the conditions used for producing the 3’ UTR database (see Materials and Methods for details). The positions of predicted miRNA target sites from the literature are shown in red. Most of these UTRs contain multiple predicted target sequences, and while regulation of the UTR has been experimentally validated in each case, most individual sites have not been tested for function.

(B) Detailed comparison of the pattern of sequence conservation in the conserved sites. Target site length is miRNA length plus 5 nt. For lin-57 we excluded three of the eight previously predicted sites that were not located in conserved sequence blocks and included a newly identified ninth site that is conserved. White type on black indicates residues that are not identical in the target sites in the two genomes. Black type on white indicates identity. All residues basepairing with positions 2–8 of the miRNA are identical in the conserved sites in both genomes.

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predictions by the best single site in the 3' UTR ($Z_{\text{max}}$) and by the sum of sites with $Z \geq 3$ ($Z_{\text{UTR}}$).

Tests with Previously Validated Targets

Table 1 summarizes the performance of the method in predicting the known targets of the *C. elegans* miRNAs *lin-4* and *let-7* and the *Drosophila* miRNA *bantam*. The *Drosophila hid* gene ranked first of all predicted *bantam* targets sorted by the single best site ($Z_{\text{max}}$) or by the sum of sites ($Z_{\text{UTR}}$). All of the known targets of *lin-4* and *let-7* were found when their 3' UTRs were added to the *Drosophila* 3' UTR database. Like *hid*, the *let-7* target *lin-57* ranked near the top of the list by both measures. With several sites predicted of $Z \geq 3$, *lin-57* ranked first by $Z_{\text{UTR}}$. Its best single site ranked in position 2 ($Z = 6.8$). *C. elegans lin-14* was predicted to contain multiple *lin-4* sites (Wightman et al. 1993). Three of these scored $Z \geq 3$, *lin-14* ranked first when the list of predicted *lin-4* targets was sorted for $Z_{\text{UTR}}$—although the best single site in *lin-14* placed it in position 20 ($Z = 4.3$). The *lin-4* target *lin-28* and the *let-7* target *lin-41* ranked highly when the lists were sorted for the best single site, but ranked lower when multiple sites were summed because they had few high-scoring sites. The *Drosophila* homolog of *lin-41*, *dpld*, also ranked high among *let-7* targets ($Z = 5.6$; see below). We compared our results with previous target predictions from the literature that have not been experimentally validated (Table 1). Our screen supports some of them (e.g., *let-7* regulating *lin-14*), but we consider others unlikely because they rank very low on their lists or have no sites of $Z \geq 3$ (e.g., *let-7* and *lin-28* or *miR-4* and *m4*).

The predicted *miR-14* target *Drice* (Xu et al. 2003) is unlikely to be valid because the site is not conserved in the predicted *Drice* 3' UTR from *D. pseudoobscura*.

This analysis shows that all known targets were detected and ranked among the top-scoring predictions in genome-wide searches. This suggests that other valid targets should rank among the small number of best predictions that can be tested experimentally. Of particular interest were three miRNAs for which we predicted clusters of functionally related targets: *miR-7*, the *miR-2* family, and *miR-277* (Table 2; Table 3). Clustering of top-scoring sites in a group of related genes is likely to be significant when it arises from an unbiased genome-wide analysis. *miR-7* and *miR-2* were selected for target validation.

**miR-7 Regulates Notch Targets**

Among the top 10 predictions for *miR-7*, we found *Enhancer of split* (*E(spl)*) and *Bearded* (*Brd*) complex genes (Figure 3A). 

**Figure 2. miRNA Target Prediction Strategy**

(A) *let-7*, *lin-4*, and *bantam* miRNA sequences showing the pattern of basepairing to their known targets. Yellow indicates a conventional basepair. Orange indicates a G:U basepair. Blue indicates a mismatch. The black bars indicate the positions of loops in the target sequence. Note that the extra bases that form the loops in the target sequences are not shown. Sequences are shown at the length of the miRNA.

(B) Quantitation of the data from (A). This comparison shows that the 5' ends of the miRNA are always involved in good pairing with target sequences and suggests that searches for complementarity to the first eight residues of the miRNA would select all known targets.

(C) Graphic representation of the Mfold output for the sequences and suggests that searches for complementarity to the first eight residues of the miRNA would select all known targets.

(D) Plot of the Mfold free energy distribution for 10,000 random sequences (green) and for predicted targets of the *bantam* miRNA (red). X-axis: ΔG calculated for each site by Mfold.

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tional repressor; Tom and m4 encode Brd family proteins. The 
small repressor hairy was also among the top 10. These sites 
were conserved in the orthologous genes from *Anopheles*
when those could be identified. This prompted us to examine 
all the genes in *E(spl)* and *Brd* complexes for *miR-7* sites. We 
found possible target sites in many of them. Alignment of 
these sites showed a pattern of 5' end conservation quite 
similar to that for validated targets, with no mismatches 
and few G:U basepairs for about half of these genes (Figure 3B).

To assess the validity of some of the predicted targets, 
transgenic flies expressing the *miR-7* miRNA and several 
sensor transgenes were prepared. A genomic fragment 
containing the *miR-7* hairpin was cloned into the 3' UTR of 
a UAS–DSRed2 plasmid to allow GAL4-dependent expression 
of *miR-7*. The 3' UTRs of *HLHm3*, m4, and hairy were cloned 
to a tubulin promoter–EGFP (enhanced green fluorescent protein) 
reporter plasmid. As a control, a specific *miR-7* sensor 
transgene was produced by cloning two copies of a 
perfect complement of the *miR-7* miRNA sequence into the 3' 
UTR of the tubulin promoter–EGFP reporter. The *miR-7* 
sensor was expressed uniformly in the wing imaginal disc. 
GAL4-dependent expression of *miR-7* miRNA reduced expression of *miR-7* green fluorescent protein (GFP) sensor 
transgene (Figure 3C). As the target sites in the sensor 
construct are perfect complements of the *miR-7* miRNA, this 
is expected to be due to RNAi. GAL4-dependent expression of 
*miR-7* also reduced expression of the m4 3' UTR sensor 
transgene (Figure 3D). The *miR-7* site in m4 is identical in *D.
* pseudoobscura and conserved in *Anopheles*.

Expression of *miR-7* also caused a clear downregulation of the 
hairy 3' UTR sensor transgene, although its overall level of 
expression was lower in the wing disc (Figure 3E). The hairy 
gene has been cloned and cDNAs sequenced from two 
additional insect genomes: the flour beetle *Tribolium casta-
naeum* and *Drosophila simulans*. The predicted *miR-7*–binding 
site is conserved in these genomes, as well as in *Anopheles*, 
and shows striking conservation of alignment at the 5' and 3' ends 
of the predicted miRNA-binding site (Figure 3F). The level of 
expression of the *HLHm3* 3' UTR sensor was too low to be 
reliably studied, but also showed regulation by *miR-7*. Again, 
the *miR-7* site in *HLHm3* is identical in *D. pseudoobscura*. 
These observations validate the utility of the screen in predicting 
new miRNA targets.

To assess *miR-7* function in vivo, we examined wings in 
which *miR-7* was overexpressed under ptc–Gal4 control. 
Nothing of the wing margin was observed (Figure 3G), 
which is characteristic of reduced *Notch* signaling (de Celis 
and Garcia Bellido 1994; Diaz-Benjumea and Cohen 1995; 
Rulifson and Blair 1995; Micchelli et al. 1997). The *Notch* 
target *cut* was expressed at reduced levels in the *miR-7-*

| miRNA–Target Pair | ΔG  | Z_{Max} | Rank Z_{Max} | # Z ≥ 3 | Rank Z_{UTR} | References |
|-------------------|-----|---------|--------------|---------|--------------|-----------|
| **Confirmed Pairs** |     |         |              |         |              |           |
| *lin-4/lin-14*     | −29.9 | 4.3     | 20           | 3       | 1            | Wightman et al. (1993); Ha et al. (1996) |
| *lin-4/lin-28*     | −30.9 | 4.6     | 8            | 1       | 15           | Moss et al. (1997) |
| *let-7/lin-41*     | −32.3 | 6.4     | 3            | 2       | 20           | Reinhart et al. (2000); Slack et al. (2000) |
| *let-7/lin-57* (hbl-1) | −33.4 | 6.8     | 2            | 14      | 1            | Abrahante et al. (2003); Lin et al. (2003) |
| *bantam/hid*       | −37.4 | 7.4     | 1            | 3       | 1            | Brennecke et al. (2003) |
| **Predicted Pairs** |     |         |              |         |              |           |
| *lin-4/lin-41*     | −28.9 | 4.0     | 32           | 1       | 36           | Slack et al. (2000) |
| *lin-4/lin-57*     | −21.6 | 1.7     | 361          | 0       | —            | Abrahante et al. (2003); Lin et al. (2003) |
| *let-7/lin-14*     | −35.1 | 7.2     | 1            | 13      | 2            | Reinhart et al. (2000) |
| *let-7/lin-28*     | −20.6 | 2.8     | 861          | 0       | —            | Moss and Tang (2003) |
| *miR-13a/hb*       | —     | —       | —            | 0       | —            | Abrahante et al. (2003) |
| *miR-4/hb*         | —     | —       | —            | 0       | —            | Abrahante et al. (2003) |
| *miR-3/hb*         | —     | —       | —            | 0       | —            | Abrahante et al. (2003) |
| *miR-11/HLHm8*     | −29.4 | 4.7     | 27           | 1       | 46 (predicted UTR) | Lai (2002) |
| *miR-4/m4*         | −21.5 | 2.1     | 272          | 0       | —            | Lai (2002) |
| *miR-7/HLHm3*      | −37.3 | 7.0     | 2            | 1       | 16           | Lai (2002) |
| *miR-7/Tom*        | −34.5 | 6.1     | 5            | 2       | 1            | Lai (2002) |
| *miR-14/Dric*      | —     | —       | —            | 0       | (Site not conserved) | Xu et al. (2003) |

Table 1. Assessment of Predictions for Known and Predicted miRNA Targets

Confirmed pairs indicates experimentally validated target 3' UTRs. ΔG, Z_{Max}, and Z_{UTR} are defined in the text. Predicted pairs indicates examples predicted in the literature for which there was no experimental validation. The *let-7/lin-14* pair ranks very high on the list of *let-7* predictions and is likely to be a functional target. The *lin-4/lin-41* pair requires experimental validation. The other *C. elegans* predictions cannot be distinguished from random matches. The 5' end of the K box shows sequence complementarity to the miR-2/miR-13 family and to miR-6 and miR-11 (Lai 2003). The prediction of *HLHm8* as a target for *miR-11* seems plausible (using predicted UTR), as do the two *miR-7*–GY box-based predictions. None of the conserved sites predicted for *Drosophila* hunchback (Abrahante et al. 2003) were on our lists because of interrupted 5' alignments. DOI: 10.1371/journal.pbio.00000600001
Table 2. Top Ten Predictions for miR-7 and miR-2a

| miR-7   | ΔG  | Zmax  | # Z ≥ 3 | ZcTR  | Gene          | Alignment                      | Flags | Ag Z ≥ 3 |
|---------|-----|-------|---------|-------|--------------|--------------------------------|-------|----------|
| 1       | −38.7  | 7.47   | 1       | 7.47  | CG14989-RB   | AGACGCAAGAACCCG-GGCG          | −     | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 2       | −37.3  | 7.03   | 1       | 7.03  | HLHm3        | GCAAGAAGUCCUGCUU-GGUCUCCA     | −     | NF       |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 3       | −35.3  | 6.39   | 1       | 6.39  | CG17657-RA   | ACAGCGUUGUAGGGCUCUCUCA        | −     | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 4       | −35.0  | 6.29   | 1       | 6.29  | hairy        | AGACGAAAACUGU-GAAGCAGGGCUGU   | −     | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 5       | −34.5  | 6.13   | 2       | 11.78 | Tom          | UAGCCGAUAGUCUAGUCUCCA         | −     | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 6       | −33.9  | 5.94   | 1       | 5.94  | hep          | GCAAGAAGAAGUCG-GGUCUCCA       | 5’ helix NF | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 7       | −33.8  | 5.91   | 1       | 5.91  | CG8944-RA    | AGCGAAGAAGUCCUGCUU-GGUCUCCA   | 5’ helix NF | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 8       | −33.1  | 5.68   | 1       | 5.68  | CG10540-RA   | CGACAAAGCGCCUUAAGUCUCCA       | −     | −        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 9       | −31.8  | 5.27   | 1       | 5.27  | CG10444-RA   | GGCACC-AAAA-CAG-AUGCGUCCA     | −     | NF       |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |
| 10      | −31.5  | 5.17   | 1       | 5.17  | m4           | -CAUCAUCU-GAUGCUAGGG-AGUCCA   | −     | +        |
|         |      |        |         |       |              | UUGUGUUGUGUGUGAGGG-AGGAGGAGG   |       |          |

| miR-2a  | ΔG  | Zmax  | # Z ≥ 3 | ZcTR  | Gene          | Alignment                      | Flags | Ag Z ≥ 3 |
|---------|-----|-------|---------|-------|--------------|--------------------------------|-------|----------|
| 1       | −39.0  | 6.78   | 2       | 11.85 | CG1969-RB    | -GCU-GCC-GGCGCGG-GUGG          | 5’ helix mispairing | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 2       | −38.6  | 6.66   | 1       | 6.66  | CG4269-RA    | GCUCCUGUG--CAU-GGAUUGGUGUGUUA | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 3       | −38.0  | 6.49   | 1       | 6.49  | reaper       | -CAGCAAGAACACG-GUUGUGUAGGA     | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 4       | −34.3  | 5.42   | 1       | 5.42  | Glaz         | GCA-GUCUGUAGUA GUUGUGUAGUA     | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 5       | −33.5  | 5.19   | 1       | 5.19  | BGS058999.3 | GCGAGAGACCGUUGUGCGGCG           | 5’ helix NF | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 6       | −33.2  | 5.1    | 1       | 5.1   | Sce          | GCGUCGUCG-GAGGUG-GUGGGGCGGUGG   | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 7       | −33.0  | 5.04   | 1       | 5.04  | hbs          | -CAACAGCGCC-GCUUGAGGCGUAGUAGU   | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 8       | −32.8  | 4.99   | 1       | 4.99  | amon         | GCGAGAGGUGAGGCGUUGUGUAGUAGU   | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 9       | −32.5  | 4.9    | 1       | 4.9   | gnn          | GCGAGAGAAGUGGCGUUGUGUAGUAGU   | −     | −        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |
| 10      | −32.1  | 4.78   | 1       | 4.78  | CG7187-RA    | GCUUAGUAGUA-GAUGCUAGUGUAGUAGU   | 5’ helix | +        |
|         |      |        |         |       |              | CGAGUAGUUGUUGUUGUAGCGGAGCGAGCGAGG   |       |          |

ΔG, Zmax, and ZcTR are explained in the text. Alignment: The target site is shown on top. Conventional basepairs are indicated by asterisks and GU basepairs by plus signs; mismatches and gaps are indicated by dashes. Flags: The “5’ conservation” (5’ cons) flag identifies sites that differ in the two genomes at any residue complementary to positions 2–7 of the miRNA. The “S helix” (helix) flag identifies sites that do not have at least six contiguous basepairs in positions 1–8. The “CDS+” (CDS+) flag indicates that the predicted site overlaps coding sequence on the same strand; “CDS−” (CDS−) indicates that the overlap is on the opposite strand. In some cases, Mfold structures include basepairs that are not between the miRNA and its target. “Mispairing” (mispairing) flags sites with artificially high folding energies. Ag (Z ≥ 3): Anopheles genes that cannot be reliably identified by our criteria are indicated as “NF.” For the cases in which the orthologous Anopheles gene was found, the presence of a target site with a Z score ≥ 3 is indicated by a plus sign. Absence of a site or a Z score < 3 is indicated by a minus sign. Heavy outlining indicates those loci that would pass stringent filtering of the lists using the flags and requiring lack of a conserved target in an Anopheles ortholog.

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expressing cells at the wing margin (Figure 3E); wingless expression was also reduced (data not shown). Although bHLH transcription factors and Brd-like genes of the E(spl) complex are not strictly required for all aspects of Notch activity at the wing margin, they are required for cut expression (Ligoxygakis et al. 1999). miR-7 expression might provide a means to simultaneously downregulate these and other proteins, which might otherwise function redundantly to mediate Notch activity in the wing margin. We also noted reduced spacing of veins 3 and 4 in these wings, which may also reflect reduced Notch activity in controlling tissue growth (Baonza and Garcia-Bellido 1999). E(spl) genes are also expressed in proneural clusters, where they are required for sense organ determination and bristle patterning. Clones also expressed in proneural clusters, where they are required for growth (Baonza and Garcia-Bellido 1999).

We also noted reduced Notch activity in controlling tissue growth (Baonza and Garcia-Bellido 1999). E(spl) genes are also expressed in proneural clusters, where they are required for growth (Baonza and Garcia-Bellido 1999).

miR-2 Regulates Proapoptotic Genes

The proapoptotic genes reaper and grim were among the top predictions for miR-2a and miR-2b (see Table 2). reaper, grim, and the third proapoptotic gene sickle are clustered in the genome and show blocks of high conservation in their 3’ UTRs, which include the miR-2 family target sites (underlined in Figure 4A). Alignment of these sites shows a very similar pattern of predicted miRNA binding (Figure 4B). The corresponding sites are highly similar in D. pseudoobscura, but the orthologous genes cannot be identified in Anopheles. To evaluate these predictions, we made 3’ UTR sensor transgenes for reaper, grim, and sickle. The expression level of the reaper 3’ UTR sensor transgene was too low to be reliably studied in transgenic flies, so we used an in vitro assay to assess its function. Drosophila Schneider S2 cells express the miR-2 family of miRNAs (Lagos-Quintana et al. 2001). S2 cells were transfected with the reaper 3’ UTR construct or with a version of the construct in which the miR-2-binding site was mutated (the residues shown in Figure 4B were replaced by a NoI site). A low level of GFP expression was detected in immunoblots of cells transfected with the reaper 3’ UTR construct (Figure 4C, lane 2). The level of GFP expression was much higher in cells transfected with the mutated UTR construct, suggesting that the endogenous miR-2 family miRNAs in S2 cells can repress expression of a reporter construct via the reaper 3’ UTR. The grim and sickle 3’ UTR sensor transgenes were expressed at detectable levels in transgenic flies and were both downregulated by expression of miR-2b in the wing disc (Figure 4D and 4E). The miR-13 family is similar in sequence to the miR-2 family. Experimental validation will be needed to determine whether reaper, grim, and sickle are also regulated by miR-13. Identification of reaper, grim, and sickle as targets suggests that miR-2 family miRNAs may be involved in control of apoptosis.

Statistical Evaluation of Target Predictions

Although a number of the top-ranking sites identified in our screen have been experimentally validated, we wanted to assess the likelihood that sites with equivalent scores can be found by chance. To do so, we calculated E-values for the

| Rank | Gene     | Function                      | Enzyme        | # Z > 3 | Z_{UTR} |
|------|----------|-------------------------------|---------------|---------|---------|
| 1    | CG31651  | Protein GlcNAc transferase     | EC2.4.1.41    | 2       | 7.31    |
| 2    | CG5599   | Val Leu Ile degradation       | EC2.3.1-      | 2       | 6.53    |
| 3    | CG1673   | Val Leu Ile degradation       | EC2.6.1.42    | 1       | 5.75    |
| 4    | fz       | Cell polarity                 |               | 1       | 4.89    |
| 5    | CG8199   | Val Leu Ile degradation       | EC1.2.4.4     | 1       | 4.53    |
| 6    | CG18549  | —                             |               | 1       | 4.23    |
| 7    | CG1140   | Val Leu Ile degradation       | EC2.8.3.5     | 1       | 3.9     |
| 8    | scu      | Val Leu Ile degradation       | EC1.1.1.35    | 1       | 3.81    |
| 9    | CG15093  | Val Leu Ile degradation       | EC1.1.1.31    | 1       | 3.79    |
| 10   | CG7740   | Membrane protein              |               | 1       | 3.64    |
| 11   | CG17896  | Val Leu Ile degradation       | EC1.2.1.27    | 1       | 3.61    |

Required: Z > 3 for D. melanogaster, D. pseudoobscura, Anopheles. DOI: 10.1371/journal.pbio.0000060.t003
bantam miRNA based on the tail of the cumulative distribution of $\Delta G$ values for 10,000 random matches. An $E$-value predicts the number of background matches with a similar or better score ($E$-values scale with database size and are applicable to any distribution profile). Values greater than 1 are not significant, while those close to 0 are very significant. The results are presented on a logarithmic scale for UTRs containing one, two, or four sites of a given $\Delta G$ value (Figure 5). The best single bantam site in the hid UTR had an $E$-value of 1.3. This means that background matches reach RNA–duplex energies similar to the best sites, even in the smaller conserved 3′ UTR database. Indeed, target sites predicted using shuffled bantam miRNA sequences give folding energy distributions very similar to the native sequence (data not shown).

The lack of statistical significance for even the best single site means that they require experimental validation.

Additional Validation by Cross-Genome Comparison

One means to improve the significance of the predictions would be to require conservation in a third genome. The two Drosophila species are separated by an estimated 30 million years. The mosquito A. gambiae is separated from Drosophila by 250 million years. Orthologous mosquito genes have been defined for approximately two-thirds of Drosophila genes; however, systematic comparison showed great differences in length between orthologous gene pairs (Zdobnov et al. 2002). Indeed, we were able to identify orthologous last exons with confidence for only half of these pairs, or one-third of D. melanogaster genes. We have therefore chosen to use conservation in Anopheles to provide more stringent evaluation of target site conservation, instead of requiring it generally. The presence of a conserved site with a high $Z$ score across all three genomes increases the confidence that the site is functional. To illustrate the utility and limitations of this, we examined the top 100 predictions for miR-7 and miR-2. The
Anopheles orthologs were identified for 52 of the top 100 predicted miR-7 targets. Of these, 11 had conserved target sites (Z ≥ 3), including four of the top ten predictions: hairy, Tom, m4, and CG14989 (see Table 2). For miR-2a, forty of the top hundred predictions had a detectable ortholog in Anopheles. Of these sites, five were conserved in Anopheles (Z ≥ 3), and none of these were among the top ten predictions. Conservation in Anopheles can be used to enrich for sites with a higher probability of being valid, but increases the risk of missing real targets. It is only useful in cases where the orthologous UTR region can be identified, which, for example, is not the case for the validated miR-2a targets grim, reaper, and sickle.

miR-277: A Metabolic Switch?

Table 3 shows predicted miR-277 targets that are conserved (Z ≥ 3) in Anopheles. Of the top 11, seven are enzymes involved in branched chain amino acid degradation (Figure 6). At more relaxed stringency (Z ≥ 2), two additional enzymes were identified (Figure 6) along with a number of unrelated loci. This striking clustering of functionally related enzymes suggests that miR-277 regulates the pathway for valine, leucine, and isoleucine degradation by downregulating many of its enzymes and thus acts as a metabolic switch. The degradation of these essential amino acids is presumably regulated under conditions of starvation or excess dietary intake. miR-277 expression has so far only been detected in adult flies (Aravin et al. 2003; Lai et al. 2003), suggesting a role in regulating metabolic responses to environmental conditions. Interestingly, the human homolog of CG8199 is mutated in maple syrup urine disease. It remains to be determined whether these enzymes are regulated by miRNAs in vertebrates.

Features Shared by Validated Targets

Comparison of the five previously validated targets and the six new targets validated here revealed three features shared by all sites. First, cross-genome comparison showed perfect sequence identity in the target site residues that basepair with residues 2–8 of the miRNA (see Figure 1B). This was also true for the newly validated target sites (data not shown). Second, the pattern of basepairing between the miRNAs and their targets, shown in Figure 2A, suggested that a continuous helix of at least six of the first eight basepairs might be required (allowing G:U basepairs). This was also true for the newly validated target sites (see Figures 3B and 4B). Third, many transcripts in the D. melanogaster genome overlap other transcripts on the same strand or on the opposite strand of the DNA. There are many examples of alternate splicing that produce alternate 3' UTRs so that one UTR variant may include coding exons from another variant. In such cases, the basis for the sequence conservation between genomes is unclear. None of the validated sites from Drosophila overlaps coding sequence (CDS) on either strand (this feature was not examined for the C. elegans sites).

Target sites that do not share these features are indicated in Table 2. These features can be used to increase the stringency of the screen, by discounting sites that differ from validated targets. For miR-7 this would eliminate two of the top ten predictions so that the validated targets would constitute three of the remaining top eight predictions. For miR-2a this would eliminate four of the top ten predictions, so that the validated targets reaper and grim would rank in positions 2 and 6. We have chosen not to implement the flags as filters to exclude predictions because they are based on a relatively small set of 11 validated targets, although we note...
Figure 6. Valine, Leucine, and Isoleucine Catabolic Pathway
Enzymes identified as miR-277 targets are boxed and identified by CG number. Red boxes required $Z > 3$ in Anopheles. Blue boxes required $Z > 2$ in Anopheles. In addition to the predicted targets, the other enzymes for which the gene has been identified in Drosophila are shaded in green. The metabolic pathway chart is from www.genome.ad.jp/kegg/pathway/map/map00280.html.

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that all nine predicted miR-277 targets would pass such a filter. When more targets are validated, we will learn whether these features have a general predictive value.

**Discussion**

One of the major limitations in studying animal miRNA function is the difficulty in identifying miRNA targets. Our screening strategy has proven to be useful for predicting new miRNA targets. Three new targets have been experimentally validated for miR-7 and for miR-2, bringing the total number of validated targets of animal miRNAs to 11. In addition, we predict a number of miRNA–target pairs or target families that seem likely to be valid, but require experimental validation. Our study depended on the high-quality annotation of the *D. melanogaster* genome and the availability of the *D. pseudoobscura* genome sequence. Where possible, we have extended the analysis to include evaluation of predicted sites in the *A. gambiae* genome. More complete annotations of the fly and mosquito genomes, aided by cDNA sequencing projects, will increase the number of genes for which orthologous UTR sequences can be defined. This will permit more sensitive and more extensive cross-genome comparison. We also expect improvements to come from further knowledge of the structural requirements of miRNA-target pairing.

**Evaluation of Target Predictions**

In designing the screening strategy, we considered the balance between sensitivity and specificity. We chose a search strategy that was based on the known examples, but generalized to allow detection of similar targets. By doing so, we risk missing fewer valid targets at the expense of including more false positives, as indicated by our statistical analysis (see Figure 5). To help distinguish false positives from potentially valid targets, we identify features shared by valid targets and, where possible, test predictions for conservation in a third, more distantly related, genome. Both positive and negative results in tests of new predictions will provide a better understanding of how miRNAs bind their targets, perhaps highlighting positions that are particularly critical. This may permit us to achieve both high sensitivity and high specificity in target prediction.

Complete tables of target site predictions are available as Dataset S1 and at www.mirna.embl.de. These tables report Z scores and sequences for the *D. melanogaster* and *D. pseudoobscura* target sites and, where possible, for the *Anopheles* target site. The tables contain flags to identify sites that share the features described above. We recommend using these flags to filter the predictions, but note that this may exclude valid targets. (Filtered tables containing the top 100 predictions are available as Dataset S2 and at www.mirna.embl.de) We recommend making use of the *Anopheles* data to discount predictions where the orthologous gene is identified and the site is absent or has a low Z score (Z < 2). We emphasize that the absence of an identified orthologous 3′ UTR in the mosquito should not be taken as evidence that a target prediction is not valid.

The presence of a conserved site in all three genomes increases the confidence that a predicted site is valid, as in the case of the miR-7 sites in *hairy* and *Tom*. Also, *dpld*, the *Drosophila* homolog of the *let-7* target *lin-41*, ranks second among *Drosophila let-7* targets when conservation in *Anopheles* was required. A number of other target predictions that meet these requirements look quite promising. We have high confidence that the cluster of enzymes in the branched chain amino-acid degradation pathway will prove to be valid miR-277 targets. Another promising candidate is the predicted miR-9a target *Lyra*. *Lyra* contains two predicted miR-9a sites. The best *Lyra* site ranks first among all predicted miR-9a targets that are conserved in *Anopheles*. Intriguingly, mutations affecting the *Lyra* 3′ UTR lead to a dominant phenotype and to increased *Lyra* protein levels, an observation that strongly suggests that *Lyra* is subject to translational regulation. miR-9 is an excellent candidate to mediate this regulation. Many other miRNA–target pairs are identified with sites of a similar quality to those mentioned here (examples include four conserved sites for miR-309 in *Ets65a* at Z ≥ 2). As a cautionary note, we wish to emphasize that conservation of target sites in *Anopheles*, while compelling, should not be taken as sufficient evidence of function without experimental validation.

Although it is more difficult to distinguish functional sites from false positives in the cases where only two genomes are compared, we have made use of the clustering of related genes to identify real targets. *reaper*, *grim*, and *sickle* have been validated as miR-2 targets. We note that the Netrin receptor *unc-5* and *Netrin-A* rank second and fourth among predicted miR-288 targets. We also noted an abundance of transcription factors among the predicted targets of miR-9, miR-279, and miR-286 for which orthologous UTRs were not identified in *Anopheles*. These predictions merit further study.

**Single versus Multiple Sites**

Our statistical analysis shows that the very best single predicted target sites are not statistically significant, even though we have used a reduced database consisting of conserved 3′ UTR sequences. This means that prediction of any single target site cannot be taken as evidence for regulation of a transcript by a miRNA without experimental validation. Sites that are not statistically significant alone can be significant when combined. For example, although none of the *bantam* sites are significant individually, their combined scores are highly significant and supported by experimental validation. 3′ UTRs with multiple predicted target sites are likely to be valid targets for regulation by the miRNA, particularly if their best single sites also rank high in the lists of predicted targets.

Despite the advantages conferred by multiple sites, single miRNA target sites can mediate regulation in vivo. The *C. elegans lin-4* miRNA appears to regulate its target *lin-28* through a single site (Moss et al. 1997). We have presented evidence that miR-2 family miRNAs can regulate expression of transgenes containing the 3′ UTRs of *reaper* and *grim*, which have one predicted target site, as well as the *sickle* 3′ UTR, which has two predicted sites. Similarly, miR-7 can regulate expression of transgenes containing the *HLHm3*, m4, and *hairy* 3′ UTRs, which have one predicted target site. Further work will be needed to gain insight into what makes some single sites functional and others not. One possibility is that a single site for one miRNA might function in conjunction with independent target sites for other miRNAs in the same UTR. Indeed, a survey of our lists of target predictions indicates that many 3′ UTRs are predicted to contain binding sites for more than one miRNA.
Materials and Methods

Conserved 3' UTR database. D. melanogaster 3' UTRs were obtained from the Berkeley Drosophila Genome Project (www.fruitfly.org/annot/release3.html) and those of >50 nt were selected. Duplicate UTRs, those with adjacent copies of the same transcript were removed. For each of the resulting 10,196 nonredundant 3' UTRs, we mapped the last 50 amino acids of the corresponding open reading frame to the D. pseudoobscura genome sequence with TBLASTN (E ≤ 10⁻⁵; hgsc.bcm.tmc.edu/projects/Drosophila). We selected UTR matches that included the last 10 residues and had a sequence identity >80% or E ≤ 10⁻¹⁰ and compared these UTRs to the 3,000 nt downstream of the putative D. pseudoobscura ortholog with BLASTN (word size = 7; E ≤ 10,000, assuming a database the size of the whole D. pseudoobscura genome). Nonconserved nucleotides or those outside the matched regions were replaced by Ns in the D. melanogaster 3' UTR database to produce the conserved 3' UTR database. The D. pseudoobscura genome has not been fully assembled. This means that some D. pseudoobscura genes are located close enough to the end of a contig that the UTR sequences may be missed. 389 D. melanogaster genes mapped to the D. pseudoobscura genome less than 1 kb from a contig end; 189 mapped less than 500 nt from a contig end. UTR conservation may be underestimated for these genes. For 3,564 genes, we did not detect a suitable ortholog using this protocol. Of these, 571 are known genes; the others are predicted genes about which little is known. For the 4,662 D. melanogaster genes lacking annotated UTRs, we assumed 3' UTRs of 2 kb after of the stop codon and built a separate database of predicted UTRs. The search for Anopheles orthologs was done using TBLASTN for the last 50 amino acids of each D. melanogaster ORF. Owing to the more extensive sequence divergence, a lower cutoff threshold was allowed (E < 0.05) if the last exon of the predicted ORF mapped to the same location (±1 kb) in the annotated genome as the orthologous gene (Zdobnov et al. 2002). If not, the cutoff was E ≤ 10⁻⁶, as for D. pseudoobscura. The second, more stringent step of comparing the last 10 amino acids was omitted. composer search, HMmer (Eddy 1996) profiles were constructed for each of two alignments per miRNA containing copies of the reverse complement of the first (5') 8 nt of the miRNA. The first alignment contained five copies of the exact complement; the second had an additional five copies with C replaced by T and A replaced by G to allow for GU mismatches. We searched the conserved 3' UTR database with both profiles and lenient domain bit score threshold (domT ≥ 3) and combined the results. Sequence matches were extended to miRNA length plus 5 nt, the hairpin loop and miRNA sequence were added, and the sequence was evaluated using Mfold. For Anopheles, predicted UTRs were searched for the presence of residues 2–7 of the predicted target site. The target sequences were extended and evaluated using Mfold. Only the best site in the Anopheles UTR was reported.

Statistics. For each miRNA, we calculated the mean and SD of a background distribution, i.e., the Mfold free energy of 10,000 randomly selected sequences from the conserved UTR database with lengths of miRNA plus 5 nt. For each prediction, we calculated the Z score as the number of SDs above the mean. To compute E-values, we fit an exponential function to the cumulative background distributions and used this to give a value for any observed energy and database size. E-values are not restricted to normal distributions and scale with database size, so different searches can be compared.

Supporting Information

Dataset S1. Complete Lists

These contain all predicted targets for each miRNA. There is one file per miRNA. No filtering has been done. From left, the columns are as follows:

gene: FlyBase identifier.
same: if there is one, in addition to the identifier.
GO term: gene ontology information about the gene product.
Z (me): score for the D. melanogaster site.
d(Gme): folding energy for the D. melanogaster site.
Alignment (me): of the miRNA to the target site; asterisk, conventional basepair; plus sign, G-U basepair; minus sign, mismatch.
start and stop: position of the site in the 3' UTR.
miRNA (me): the target site, linker, and miRNA sequence as submitted to Mfold.
#sites: total number of sites of Z ≥ 3 in that UTR.
Z(UTR): sum of Z for all sites ≥ 3 in that UTR.
Z(max): the best Z score for the UTR.
#Z ≥ 3: total number of sites of Z ≥ 3 found in that UTR.

UTR: whether the site is an experimentally defined or predicted UTR.
5' conservation, 5' helix, CDS+, CDS-, and mismatching flags are described in text and in Table 2.
Z(UTR): Z score for the corresponding site from D. pseudoobscura.

Anopheles: D. melanogaster ortholog (explained in the text).
(); as above, for the D. pseudoobscura site.
Mfold (ps): as above, for the D. pseudoobscura site.
Ano-blastn: TBLASTN score for the Anopheles ortholog (explained in the text).
(); as above, for Anopheles.
ALN (an): as above, for Anopheles.
(); as above, for Anopheles.
(); as above, for Anopheles.

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Dataset S2. Top 100 List

This dataset presents our recommended filtering of the lists. Only sites in experimentally validated UTRs were included. The 5' conservation, 5' helix, CDS+, and CDS- flags were used to discard sites that we consider less likely to be valid. This allows the use of a lower Z score cutoff (Z ≥ 2). Sites of Z < 2 were removed and Z(UTR) includes all sites Z ≥ 2. Columns are as above, except the flags have been removed.

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Accession Numbers

The accession numbers for the miRNAs discussed in this paper are listed in Table 2. The accession numbers for the target genes discussed in this paper are listed in Table 3. The accession numbers for the miRNAs discussed in this paper are listed in Table 2. The accession numbers for the target genes discussed in this paper are listed in Table 3. The accession numbers for the miRNAs discussed in this paper are listed in Table 2. The accession numbers for the target genes discussed in this paper are listed in Table 3. The accession numbers for the miRNAs discussed in this paper are listed in Table 2. The accession numbers for the target genes discussed in this paper are listed in Table 3.
References

Abrahante JE, Daud AL, Li M, Volk ML, Tennessen JM, et al. (2005) The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev Cell 4: 625–637.

Ambros V (2001) MicroRNAs: Tiny regulators with great potential. Cell 107: 823–826.

Ambros V, Lee RC, Lavanway A, Williams PT, Jewell D (2003) MicroRNAs and other tiny endogenous RNAs in C. elegans. Curr Biol 13: 807–818.

Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, et al. (2003) The small RNA profile during Drosophila melanogaster development. Dev Cell 5: 357–360.

Baona A, Garcia-Bellido A (1999) Notch signaling directly controls cell proliferation in the Drosophila wing disc. Proc Natl Acad Sci U S A 97: 2669–2674.

Blochinger K, Jan LY, Jan YN (1993) Post-embryonic patterns of expression of Notch, a locus regulating sensory organ identity in Drosophila. Development 117: 441–450.

Brennecke J, Hamer J, Hiepnier DR, Stark A, Belo C, et al. (2003) The identification of microRNAs and miRNA function. Dev Cell 4: 205–217.

Chen C, He C, Hannon GJ (2003) Linking microRNAs and the Dicer complex to P-element-induced wavy/let-7 processing in Caenorhabditis elegans. Cell 110: 513–520.

Chen X, He C, Hannon GJ (2003) A microRNA regulatory circuit mediates negative posttranscriptional regulation of lin-29. Genes Dev 17: 438–442.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.

Chen X, He C, Hannon GJ (2003) Positive regulation of let-7 by lin-14. Nat Genet 34: 377–380.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.

Chen X, He C, Hannon GJ (2003) Negative regulation of let-7 by lin-14. Nat Genet 35: 535–539.