

RESEARCH COMMUNICATION

A single Hox locus in *Drosophila* produces functional microRNAs from opposite DNA strands

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MicroRNAs (miRNAs) are ~22-nucleotide RNAs that are processed from characteristic precursor hairpins and pair to sites in messages of protein-coding genes to direct post-transcriptional repression. Here, we report that the miRNA *iab-4* locus in the *Drosophila* Hox cluster is transcribed convergently from both DNA strands, giving rise to two distinct functional miRNAs. Both sense and antisense miRNA products target neighboring Hox genes via highly conserved sites, leading to homeotic transformations when ectopically expressed. We also report sense/antisense miRNAs in mouse and find antisense transcripts close to many miRNAs in both flies and mammals, suggesting that additional sense/antisense pairs exist.

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Hox genes are highly conserved homeobox-containing transcription factors crucial for development in animals (Lewis 1978; for reviews, see McGinnis and Krumlauf 1992; Pearson et al. 2005). Genetic analyses have identified them as determinants of segmental identity that specify morphological diversity along the anteroposterior body axis. A striking conserved feature of Hox complexes is the spatial colinearity between Hox gene tran-

scription in the embryo and the order of the genes along the chromosome (Duboule 1998). Hox clusters also give rise to a variety of noncoding transcripts, including microRNAs (miRNAs) *mir-10* and *mir-iab-4/mir-196*, which derive from analogous positions in Hox clusters in flies and vertebrates (Yekta et al. 2004). miRNAs are ~22-nucleotide (nt) RNAs that regulate gene expression post-transcriptionally (Bartel 2004). They are transcribed as longer precursors and processed from characteristic pre-miRNA hairpins. In particular, Hox miRNAs have been shown to regulate Hox protein-coding genes by mRNA cleavage and inhibition of translation, thereby contributing to the extensive regulatory connections within Hox clusters (Mansfield et al. 2004; Yekta et al. 2004; Hornstein et al. 2005; Ronshaugen et al. 2005). Several Hox transcripts overlap on opposite strands, providing evidence of extensive antisense transcription, including antisense transcripts for *mir-iab-4* in flies (Bae et al. 2002) and its mammalian equivalent *mir-196* (Mainguy et al. 2007). However, the function of these transcripts has been elusive. Here we show that the *iab4* locus in *Drosophila* produces miRNAs from opposite DNA strands that can regulate neighboring Hox genes via highly conserved sites. We provide evidence that such sense/antisense miRNA pairs are likely employed in other contexts and a wide range of species.

Results and Discussion

Our examination of the antisense transcript that overlaps *Drosophila mir-iab-4* revealed that the reverse complement of the *mir-iab-4* hairpin folds into a hairpin reminiscent of miRNA precursors (Fig. 1A). Moreover, 17 sequencing reads from small RNA libraries of *Drosophila* testes and ovaries mapped uniquely to one arm of the *iab-4* antisense hairpin (Fig. 1B). All reads were aligned at their 5' end, suggesting that the *mir-iab-4* antisense hairpin is processed into a single mature miRNA in vivo, which we refer to as miR-*iab-4*AS. For comparison, we found six reads consistent with the known miR-*iab-4*-5p (or miR-*iab-4* for short) and one read for its star sequence (miR-*iab-4*-3p). Interestingly, the relative abundance of mature miRNAs and star sequences for *mir-iab-4*AS (17:0) and *mir-iab-4* (6:1) reflects the thermodynamic asymmetry of the predicted miRNA/miRNA* duplexes (Khvorova et al. 2003; Schwarz et al. 2003). Because they derived from complementary near palindromes, miR-*iab-4* and miR-*iab-4*AS had high sequence similarity, only differing in four positions at the 3' region (Fig. 1B). However, they differed in their 5' ends, which largely determine miRNA target spectra (Brennecke et al. 2005; Lewis et al. 2005): miR-*iab-4*AS was shifted by 2 nt, suggesting targeting properties distinct from those of miR-*iab-4* and other known *Drosophila* miRNAs.

We confirmed robust transcription of *mir-iab-4* sense and antisense precursors by *in situ* hybridization to *Drosophila* embryos (Fig. 1C). Both transcripts were detected in abdominal segments in the posterior part of the embryo, but intriguingly in nonoverlapping domains. As described previously (Bae et al. 2002; Ronshaugen et al. 2005), *mir-iab-4* sense was expressed highly in abdominal segments A5–A7, showing modulation in levels within the segments: *abdominal-A* (*abd-A*)-expressing cells (Fig. 1D; Karch et al. 1990; Macias et al. 1990) ap-

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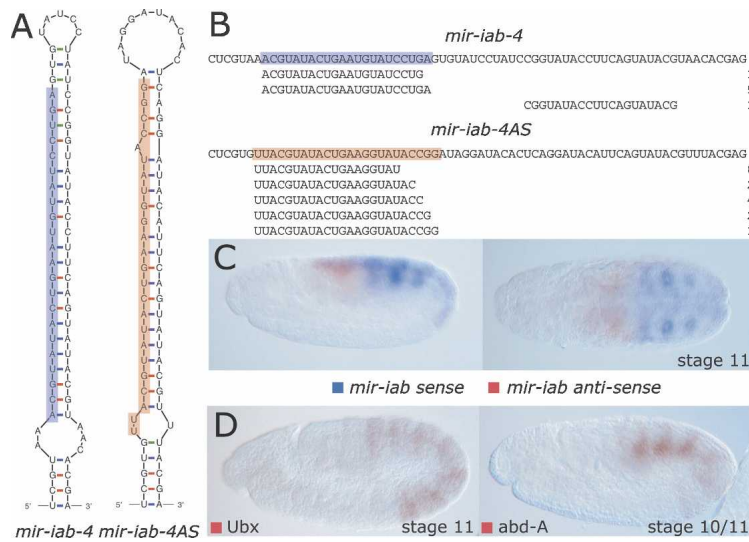


Figure 1. *Drosophila iab-4* contains sense and antisense miRNAs. (A) *mir-iab-4* sense and antisense sequences can adopt fold-back stem-loop structures characteristic for miRNA precursors (structure predictions by Mfold [Zuker 2003]; mature miRNAs shaded in blue [miR-*iab-4*] and red [miR-*iab-4AS*]). (B) Solexa sequencing reads that uniquely align to the *mir-iab-4* hairpin sequence (top) or its reverse complement (bottom; numbers on the right indicate the cloning frequency for each sequence). The mature miRNAs have very similar sequences that are shifted by 2 nt and are different in only four additional positions. (C) Expression of primary transcripts for *mir-iab-4* (blue) and *mir-iab-4AS* (red) in nonoverlapping abdominal segments determined by in situ hybridization (lateral [left panel] and dorsal [right panel] view of embryonic stage 11, anterior is to the left). (D) Lateral views of stage 10/11 embryos in which *Ubx* and *abd-A* proteins are visualized (anterior is to the left, and dorsal is upwards).

peared to have more *mir-iab-4*, whereas *Ultrabithorax* (*Ubx*)-positive cells appeared to have little or none (Fig. 1D; Ronshaugen et al. 2005). In contrast, *mir-iab-4AS* transcription was detected in the segments A8 and A9, where *Abdominal-B* (*Abd-B*) is known to be expressed (Fig. 1C; Yoder and Carroll 2006). Primary transcripts for *mir-iab-4* and *mir-iab-4AS* were also detected by strand-specific RT-PCR in larvae, pupae, and male and female adult flies (Supplemental Fig. S1), suggesting that both miRNAs are expressed throughout fly development.

To assess the possible biological roles of the two *iab-4* miRNAs, we examined fly genes for potential target sites by searching for conserved matches to the seed region of the miRNAs (Lewis et al. 2005). We found highly conserved target sites for miR-*iab-4AS* in the 3' untranslated regions (UTRs) of several Hox genes that are proximal to the *iab-4* locus and are expressed in the neighboring more anterior embryonic segments: *abd-A*, *Ubx*, and *Antennapedia* (*Antp*) have four, five, and two seed sites, respectively, most of which are conserved across 12 *Drosophila* species that diverged 40 million years ago (Fig. 2A; Supplemental Fig. S2; *Drosophila* 12 Genomes Consortium 2007; Stark et al. 2007a). More than two highly conserved sites for one miRNA is exceptional for fly 3' UTRs, placing these messages among the most confidently predicted miRNA targets and suggesting that they might be particularly responsive to the presence of the miRNA. The strong predicted targeting of proximal Hox genes was reminiscent of previously characterized miR-*iab-4* targeting of *Ubx* in flies and miR-196 targeting of *HoxB8* in vertebrates (Mansfield et al. 2004; Yekta et al. 2004; Hornstein et al. 2005; Ronshaugen et al. 2005).

To test whether miR-*iab-4AS* is functional and can directly target *abd-A* and *Ubx*, we constructed Luciferase reporters carrying the corresponding wild-type 3' UTRs and control 3' UTRs in which each seed site was disrupted by point substitutions. *mir-iab-4AS* potently repressed reporter activity for *abd-A* and *Ubx* (Fig. 2B). This repression was specific to the miR-*iab-4AS* seed sites, as expression of the control reporters with mutated sites was not affected. We also tested whether *mir-iab-4AS* reduced expression of a Luciferase reporter with the *Abd-B* 3' UTR, which has no seed sites. As expected, *mir-iab-4AS* expression did not affect reporter activity,

consistent with a model where miRNAs do not target genes that are coexpressed at high levels (Farh et al. 2005; Stark et al. 2005). In addition to demonstrating specific repression dependent on the predicted target sites, these assays confirmed the processing of the *mir-iab-4AS* hairpin into a functional mature miRNA.

If miR-*iab-4AS* were able to potently down-regulate

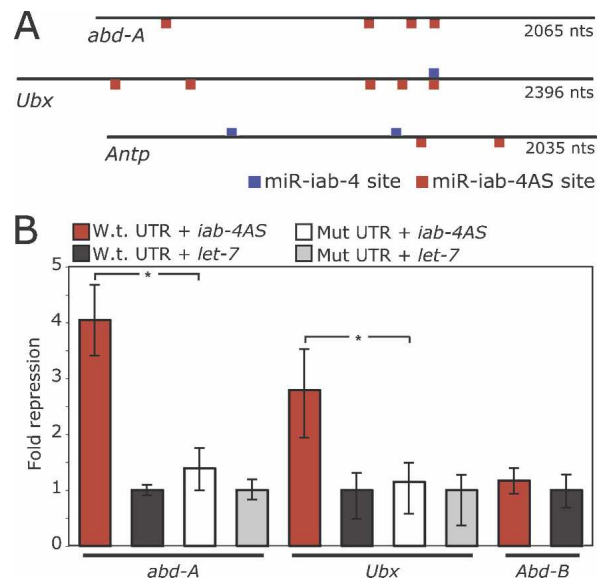


Figure 2. miR-*iab-4AS* targets neighboring Hox genes. (A) miR-*iab-4AS* has five 3' UTR seed sites (red) in *Ubx*, four in *abd-A*, and two in *Antp* of which three, four, and one are conserved across 12 *Drosophila* species, respectively (Supplemental Fig. S2). miR-*iab-4* has one 3' UTR seed site (blue) in *Ubx* and two in *Antp*, while *abd-A* has no such sites. (B) miR-*iab-4AS* mediates repression of luciferase reporters through complementary seed sites in 3' UTRs from *abd-A* and *Ubx*, but not *Abd-B* (*Antp* was not tested). Luciferase activity in S2 cells cotransfected with plasmid expressing the indicated miRNA with either wild-type luciferase reporters or mutant reporters bearing a single point mutation in the seed. Bars represent geometric means from 16 replicates, normalized to the transfection control and noncognate miRNA control [*let-7*; see Materials and Methods]. Error bars represent the fourth largest and smallest values from 16 replicates [*] $P < 0.0001$, Wilcoxon rank-sum test).

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Ubx in the fly, its misexpression should result in a *Ubx* loss-of-function phenotype, a line of reasoning that has often been used to study the functions and regulatory relationships of Hox genes. *Ubx* is expressed throughout the haltere imaginal disc, where it represses wing-specific genes and specifies haltere identity (Weatherbee et al. 1998). When we expressed *mir-iab-4AS* in the haltere imaginal disc under *bx-Gal4* control, a clear homeotic transformation of halteres to wings was observed (Fig. 3). The halteres developed sense organs characteristic of the wing margin and their size increased severalfold, features typical of transformation to wing (Weatherbee et al. 1998). Consistent with the increased number of miR-iab4AS target sites, the transformation was stronger than that reported for expression of *iab-4* (Ronshaugen et al. 2005), for which we confirmed changes in morphology but did not find wing-like growth (Fig. 3D).

We conclude that both strands of the *iab-4* locus are expressed in nonoverlapping embryonic domains and that each transcript produces a functional miRNA *in vivo*. In particular, the novel *mir-iab-4AS* is able to strongly down-regulate neighboring Hox genes. Interestingly, vertebrate *mir-196*, which lies at an analogous position in the vertebrate Hox clusters, is transcribed in the same direction as *mir-iab-4AS* and most other Hox genes, and targets homologs of both *abd-A* and *Ubx* (Mansfield et al. 2004; Yekta et al. 2004; Hornstein et al. 2005). With its shared transcriptional orientation and homologous targets, *mir-iab-4AS* appears to be the functional equivalent of *mir-196*.

The expression patterns and regulatory connections between Hox genes and the two *iab-4* miRNAs show an intriguing pattern in which the miRNAs appear to reinforce Hox gene-mediated transcriptional regulation (Fig. 4A). In particular, miR-iab-4AS would reinforce the posterior expression boundary of *abd-A*, *Ubx*, and *Antp*,

supporting their transcriptional repression by *Abd-B*. *mir-iab-4* appears to support *abd-A*- and *Abd-B*-mediated repression of *Ubx*, reinforcing the *abd-A/Ubx* expression domains and the posterior boundary of *Ubx* expression. Furthermore, both *iab-4* miRNAs have conserved target sites in *Antp*, which is also repressed by *Abd-B*, *abd-A*, and *Ubx*. The *iab-4* miRNAs thus appear to support the established regulatory hierarchy among Hox transcription factors, which exhibits "posterior prevalence," in that more posterior Hox genes repress more anterior ones and are dominant in specifying segment identity (for reviews, see McGinnis and Krumlauf 1992; Pearson et al. 2005). Interestingly, *Abd-B* and *mir-iab-4AS* are expressed in the same segments, and the majority of *cis*-regulatory elements controlling *Abd-B* expression are located 3' of *Abd-B* (Boulet et al. 1991). This places them near the inferred transcription start of *mir-iab-4AS*, where they potentially direct the coexpression of these genes. Similarly, *abd-A* and *mir-iab-4* may be coregulated as both are transcribed divergently, potentially under the control of shared upstream elements.

Our data demonstrate the transcription and processing of sense and antisense *mir-iab-4* into functional miRNAs with highly conserved functional target sites in neighboring Hox genes. In an accompanying study (Bender 2008), genetic and molecular analyses in *mir-iab-4* mutant *Drosophila* revealed that the proposed regulation of *Ubx* by both sense and antisense miRNAs occurs under physiological conditions and, in particular, the regulation by miR-iab-4AS is required for normal development. These lines of evidence establish miR-iab-4AS as a novel Hox gene, being expressed from within the Hox cluster and regulating Hox genes during development.

The genomic arrangement of two miRNAs that are expressed from the same locus but on different strands

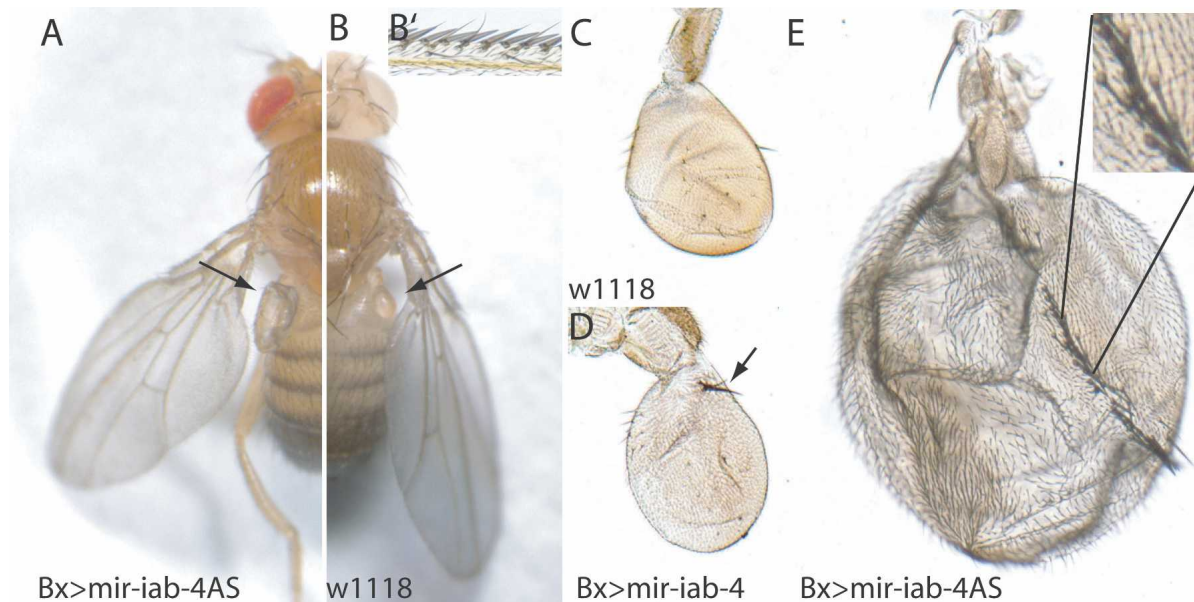


Figure 3. Misexpression of miR-iab-4AS transforms halteres to wings. (A,B) Overview of an adult wild-type *Drosophila* (B) and an adult expressing *mir-iab-4AS* using *bx-Gal4* (A). The halteres, balancing organs of the third thoracic segment, are indicated by arrows. (C) Wild-type haltere. (D) Expression of *mir-iab-4* using *bx-Gal4* induces a mild haltere-to-wing transformation. Sensory bristles characteristic of wild-type wing margins (shown in B') are indicated by an arrow. (E) Expression of *mir-iab-4AS* using *bx-Gal4* induces a strong haltere-to-wing transformation, displaying the triple row of sensory bristles (inset) normally seen in wild-type wings (shown in B'). Note that C–E are at the same magnification.

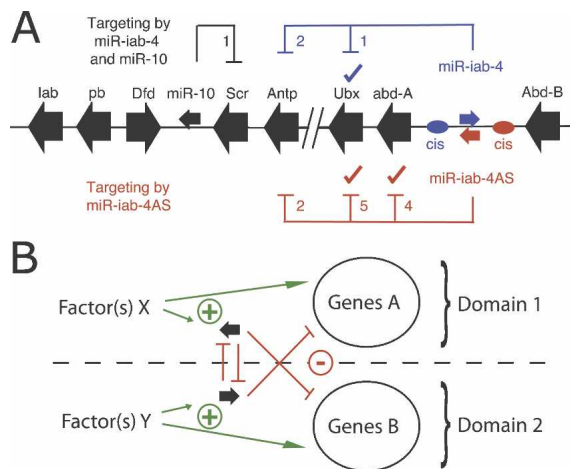


Figure 4. Regulation of gene expression by antisense miRNAs. (A) miRNA-mediated control in the *Drosophila* Hox cluster. Schematic representation of the *Drosophila* Hox cluster (Antennapedia and Bithorax complex) with miRNA target interactions (check marks represent experimentally validated targets). miR-iab-4 (blue) and miR-iab-4AS (red) target anterior neighboring Hox genes and miR-10 (black) targets posterior *Sex-combs-reduced* (*Scr*) (Brennecke et al. 2005). *abd-A* and *mir-iab-4* and *Abd-B* and *mir-iab-4AS* might be coregulated from shared control elements (*cis*). Note that *mir-iab-4AS* is expressed in the same direction as most other Hox genes and its mammalian equivalent, *mir-196*. (B) General model for defining different expression domains with pairs of antisense miRNAs (black). Different transcription factor(s) activate the transcription of miRNAs and genes in each of the two domains separately (green lines). Both miRNAs might inhibit each other by transcriptional interference or post-transcriptionally (vertical red lines), leading to essentially nonoverlapping expression and activity of both miRNAs. Further, both miRNAs likely target distinct sets of genes (diagonal red lines), potentially re-enforcing the difference between the two expression domains.

might provide a simple and efficient means to create nonoverlapping miRNA expression domains (Fig. 4B). Such sense/antisense miRNAs could restrict each other's transcription, either by direct transcriptional interference, as shown for overlapping convergently transcribed genes (Shearwin et al. 2005; Hongay et al. 2006), or post-transcriptionally, possibly via RNA-RNA duplexes formed by the complementary transcripts. Sense/antisense miRNAs would usually differ at their 5' ends and thereby target distinct sets of genes, which might help define and establish sharp boundaries between expression domains. Coupled with feedback loops or coregulation of miRNAs and genes in *cis* or *trans*, this arrangement could provide a powerful regulatory switch. The *iab-4* miRNAs might be a special case of tight regulatory integration in which miRNAs and proximal genes appear coregulated transcriptionally in *cis* and repress each other both transcriptionally and post-transcriptionally.

It is perhaps surprising that no antisense miRNA had been found previously, even though, for example, the intriguing expression pattern of the *iab-4* transcripts had been reported nearly two decades ago (Cumberledge et al. 1990; Bae et al. 2002), and *iab-4* lies in one of the most extensively studied regions of the *Drosophila* genome. The frequent occurrence of antisense transcripts (Yelin et al. 2003; Katayama et al. 2005) suggests that more antisense miRNAs might exist. Indeed, up to 13% of known *Drosophila*, 20% of mouse, and 31% of human

miRNAs are located in introns of host genes transcribed on the opposite strand or are within 50 nt of antisense ESTs or cDNAs (Supplemental Table S1). These include an antisense transcript overlapping human *mir-196* (see also Mainguy et al. 2007). However, because of the contribution of noncanonical base pairs, particularly G:U pairs that become less favorable A:C in the antisense strand, many miRNA antisense transcripts will not fold into hairpin structures suitable for miRNA biogenesis, which explains the propensity of miRNA gene predictions to identify the correct strand (Lim et al. 2003). Nonetheless, in a recent prediction effort, 22 sequences reverse-complementary to known *Drosophila* miRNAs showed scores seemingly compatible with miRNA processing (Stark et al. 2007b). Deep sequencing of small RNA libraries from *Drosophila* confirmed the processing of small RNAs from four of these high-scoring antisense candidates (Ruby et al. 2007), and the ovary/testes libraries used here showed antisense reads for an additional *Drosophila* miRNA (*mir-312*) (see Supplemental Tables S2, S3). In addition, using high-throughput sequencing of small RNA libraries from mice, we found sequencing reads that uniquely matched the mouse genome in loci antisense to 10 annotated mouse miRNAs. Eight of the inferred antisense miRNAs were supported by multiple independent reads, and two of them had reads from both the mature miRNA and the star sequence (Supplemental Table S2). These results suggest that sense/antisense miRNAs could be more generally employed in diverse contexts and in species as divergent as flies and mammals.

Materials and methods

Plasmids

3' UTRs were amplified from *Drosophila melanogaster* genomic DNA and cloned in pCR2.1 for site-directed mutagenesis. The following primer pairs were used to amplify the indicated 3' UTR: *abd-A* (tc tagaCGCGTCAGCAAAGTCAACTC; gtcgacATGGATGGGTCTCGT TGCAG), *Ubx* (tctagaATCCTTAGATCCTTAGATCCTTAG; ctcgag ATGGTTTGAATTTCCACTGA), and *Abd-B* (tctagaGCCACCACCT GAACCTTAG; aactcgagCGGAGTAATGCGAAGTAATTG). Quick-Change multisite-directed mutagenesis was used to mutate all miR-iab-4AS seed sites from ATACGT to ATAGGT, per the manufacturer's directions (Stratagene). Wild-type and mutated 3' UTRs were subcloned into pCJ40 between *SacI* and *NotI* sites to make *Renilla* luciferase reporters. Plasmid pCJ71 contains the *abd-A* wild-type 3' UTR, pCJ72 contains the *Ubx* wild-type 3' UTR, pCJ74 contains the *Abd-B* wild-type 3' UTR, pCJ75 contains the *abd-A* mutated 3' UTR, and pCJ76 contains the *Ubx* mutated 3' UTR fused to *Renilla* luciferase. The control *let-7* expression vector was obtained by amplifying *let-7* from genomic DNA with primers 474 base pairs (bp) upstream of and 310 bp downstream from the *let-7* hairpin and cloning it into pMT-puro. To express miR-iab-4 and miR-iab-4AS, a 430-bp genomic fragment containing the miR-iab-4 hairpin was cloned, in either direction, downstream from the tubulin promoter as described in Stark et al. (2005). For the UAS-miR-iab-4 and UAS-miR-iab-4AS constructs, the same 430-bp genomic fragment containing the miR-iab-4 hairpin was cloned downstream from pUAST-DSred2 (Stark et al. 2003) in either direction.

Reporter assays

For the luciferase assays, 2 ng of p2129 (firefly luciferase), 4 ng of *Renilla* reporter, 48 ng of miRNA expression plasmid, and 48 ng of p2032 (GFP) were cotransfected with 0.3 μ L Fugene HD per well of a 96-well plate. Twenty-four hours after transfection, expression of *Renilla* luciferase was induced by addition of 500 μ M CuSO_4 to the culture media. Twenty-four hours after induction, reporter activity was measured with the Dual-Glo luciferase kit (Promega), per the manufacturer's instructions on a Tecan Safire II plate reader.

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The ratio of *Renilla*:firefly luciferase activity was measured for each well. To calculate fold repression, the ratio of *Renilla*:firefly for reporters cotransfected with *let-7* was set to 1. The Wilcoxon rank-sum test was used to assess the significance of changes in fold repression of wild-type reporters compared with mutant reporters. Geometric means from 16 transfections representing four replicates of four independent transfections are shown. Error bars represent the fourth highest and lowest values of each set.

Drosophila strains

UAS-miR-iab-4 and *UAS-miR-iab-4AS* flies were generated by injection of the corresponding plasmids into *w¹¹¹⁸* embryos. *bx^{MS1096}-GAL4* flies were obtained from the Bloomington Stock Center.

In situ hybridization and protein stainings

Double in situ hybridization for the miRNA primary transcripts was performed as described in Stark et al. (2005). Probes were generated using PCR on genomic DNA with primers TCAGAGCATGCAGAGACAT AAAG, TTGTAGATTGAAATCGGACACG for *iab-4* sense and ATTT TACTGGGTGTCTGGGAAAAG, TAGAACTGAGACGGAGAAGCAG for *iab-4* antisense. Protein stainings were performed as described in Patel (1994). Antibodies used were mouse anti-Ubx (1:30), mouse anti-abd-A (1:5), and HRP-conjugated goat anti-mouse (Dianova, 1:3000).

RT-PCRs

Total RNA was isolated using Trizol (Invitrogen), treated with RQ1 DNase (Promega), and used for strand-specific cDNA synthesis with SuperScript III (Invitrogen). Primers for cDNA synthesis were CATATAA CAAAGTGCTACGTG (*iab-4* sense) and CTTTATCTGCATTTG GATCCG (*iab-4* antisense). Both primers were used for subsequent amplification.

Small library sequencing

Drosophila small RNAs were cloned from adult ovaries and testes as described previously (Brennecke et al. 2007) and sequenced using Solexa sequencing. A total of 657,251 sequencing reads uniquely matched known *Drosophila* miRNAs (Rfam release 9.2), and the 69 miRNAs with unique matches had 1011 matches on average (Stark et al. 2007b). Two miRNAs had unique matches to the antisense hairpin (Supplemental Tables S2, S3). Mouse small RNAs were cloned from wild-type and c-kit mutant ovaries (Supplemental Table S4; G. Hannon, pers. comm.) and from Comma-Dbgeo cells, a murine mammary epithelial cell line (Ibarra et al. 2007), and were sequenced using Solexa sequencing. A total of 4,217,883 reads uniquely matched known mouse miRNAs (Rfam release 9.2), and the 286 miRNAs with unique reads showed 256 reads on average. Sequencing reads matching to the plus and minus strand of known mouse miRNAs with antisense reads are listed in Supplemental Table S3.

Multiple sequence alignments and target site prediction

The multiple sequence alignments for the indicated Hox 3' UTRs were obtained from the University of California at Santa Cruz (UCSC) genome browser (Kent et al. 2002) and were slightly manually adjusted. We predicted target sites according to Lewis et al. (2005) by searching for 3' UTR seed sites (reverse-complementary to miRNA positions 2–8 or matching to "A" + reverse complement of miRNA positions 2–7).

Antisense transcripts near known miRNAs

To assess the fraction of *Drosophila*, human, and mouse miRNAs that are also putatively transcribed on both strands and might give rise to antisense miRNAs, we determined the number of miRNAs that are near known transcripts on the opposite strand. We obtained the coordinates of all introns of protein-coding genes and all mapped ESTs or cDNAs for the three species from the UCSC genome browser (Kent et al. 2002). We intersected them with the miRNA coordinates from Rfam (release 9.2; Griffiths-Jones et al. 2006), requiring miRNAs and transcripts to be on opposite strands and at a distance of at most 50 nt. For each miRNA, we recorded the number of antisense transcripts and their identifiers. Note that some of the transcripts might have been mapped to more than one place in the genome, such that the intersection represents an upper estimate based on the currently known transcripts.

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