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

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Abstract

MicroRNAs (miRNAs) are ~22-nucleotide RNAs that are transcribed as longer precursors and processed from characteristic pre-miRNA hairpins. They pair to sites in protein-coding messages to direct post-transcriptional repression of these mRNAs. Here, we describe a miRNA locus, iab-4, that is transcribed convergently from both DNA strands, giving rise to two distinct functional miRNAs. Iab-4 is located in the *Drosophila* Hox cluster and both miRNAs target neighboring Hox genes, leading to homeotic transformations when ectopically expressed. Sense and anti-sense miRNA pairs constitute an elegant way to establish non-overlapping expression domains. We found 9 sense/anti-sense miRNAs in mouse and anti-sense transcripts in the proximity of other miRNAs, suggesting that additional sense/anti-sense pairs are likely employed in other contexts.

Introduction

Hox genes are highly conserved homeobox-containing transcription factors crucial for development in animals ((Lewis 1978), reviewed in (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 co-linearity between Hox gene transcription in the embryo and the order of the genes along the chromosome (Duboule 1998). Hox clusters also give rise to a variety of non-coding 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 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 anti-sense transcription, including anti-sense 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.

Results and Discussion

Our examination of the anti-sense 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 anti-sense hairpin (Fig. 1B). All reads were aligned at their 5'end, suggesting that the *mir-iab-4* anti-sense hairpin is processed into a single mature miRNA *in vivo*, which we refer to as miR-iab-4AS. 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). Because they derived from complementary near palindromes, miR-iab-4 and miR-iab-4AS had high sequence similarity, only differing in 4 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-4AS was shifted by 2 nucleotides, suggesting targeting properties distinct from those of miR-iab-4 and other known *Drosophila* miRNAs.

We confirmed robust transcription of iab-4 sense and anti-sense 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 non-overlapping domains. As described previously (Bae et al. 2002; Ronshaugen et al. 2005), iab-4 sense was expressed highly in abdominal segments A5-A7, showing modulation in levels within the segments. The *abdominal-A* (*abd-A*)-expressing cells (Karch et al. 1990; Macias et al. 1990) appeared 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. 1D, (Yoder and Carroll 2006)).

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'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 4, 5, and 2 seed sites respectively, most of which are conserved across 12 *Drosophila* species that diverged 40 Million years ago (Fig. 2A; *Antp* not shown). 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 strikingly 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-iab4AS is indeed 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 all seed sites were 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 co-expressed genes (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 *Ubx* in the fly, its mis-expression 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 several fold, 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 non-overlapping 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* appeared 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 (reviewed in (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 co-expression of these genes. Similarly, *abd-A* and *mir-iab-4* appear co-expressed may be co-regulated as both are transcribed divergently, potentially under the control of shared upstream elements.

Our data clearly demonstrate the transcription and processing of sense and anti-sense miR-iab-4 into functional miRNAs with highly conserved functional target sites in neighboring Hox genes. In an accompanying manuscript (Bender in preparation), genetic and molecular analyses in miR-iab-4 mutant *Drosophila* revealed that the proposed regulation of *Ubx* by both sense and anti-sense miRNAs occurs under physiological conditions and in particular the regulation by miR-iab-4AS is required for normal development.

The intriguing genomic arrangement of two miRNAs that are expressed from the same locus but on different strands might provide a simple and efficient means to create non-overlapping miRNA expression domains (Fig. 4B). Such sense/anti-sense 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/anti-sense 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 co-regulation 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, where miRNAs and proximal genes appear co-regulated transcriptionally in *cis*, and repress each other both transcriptionally and post-transcriptionally.

It is perhaps surprising that no anti-sense 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 anti-sense transcripts (Yelin et al. 2003; Katayama et al. 2005) suggests that more anti-sense 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 nucleotides of anti-sense ESTs or cDNAs (Table S1). These include an anti-sense transcript overlapping human *mir-196* (see also (Mainguy et al. 2007)). However, because of the contribution of non-canonical basepairs, particularly G:U pairs which become non-compatible A/C in the anti-sense strand, many miRNA anti-sense 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 gene-prediction effort, 22 sequences with reverse-complementary to known *Drosophila* miRNAs had scores seemingly compatible with miRNA processing (Stark et al. 2007), and deep sequencing of small RNA libraries from *Drosophila* confirmed the processing of small RNAs from four of these high-scoring anti-sense candidates (Ruby et al. 2007). In addition, using high-throughput sequencing of small RNA libraries from mice, we found sequencing reads that uniquely matched the mouse genome anti-sense to 9 known mouse

miRNAs. 6 of the inferred anti-sense miRNAs were supported by multiple independent reads, and 2 of them had reads from the mature miRNA and the star sequence (Table S2). These results suggest that sense/anti-sense miRNAs are more generally employed in diverse contexts and in species as divergent as flies and mammals.

Materials and Methods

Plasmids

3´ UTRs were amplified from *D. 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 (tctagaGCGGTCAGCAAAGTCAACTC; gtcgacATGGATGGGTTCTCGTTGCAG), Ubx (tctagaATCCTTAGATCCTTAGATCCTTAG; ctcgagATGGTTTGAATTTCCACTGA), and Abd-B (tctagaGCCACCACCTGAACCTTAG; aactcgagCGGAGTAATGCGAAGTAATTG). Quickchange multi-site directed mutagenesis was used to mutate all miR-iab-4AS seed sites from ATACGT to ATAGGT, per manufacturers directions (Stratagene, CA). Wild-type (w.t.) and mutated (mut) 3´ UTRs were subcloned into pCJ40 between SacI and NotI sites to make *Renilla* luciferase reporters. Plasmid pCJ71 contains the abd-A w.t. 3´ UTR, pCJ72 contains the Ubx w.t. 3´ UTR, pCJ74 contains the Abd-B w.t. 3´ UTR, pCJ75 contains the abd-A mut 3´ UTR, and pCJ76 contains the Ubx mut 3´ UTR fused to *Renilla* luciferase. The control *let-7* expression vector was obtained by amplifying *let-7* from genomic DNA with primers 474 bp upstream and 310 bp downstream of the let-7 hairpin and cloning it into pMT-puro. To express miR-iab-4 and miR-iab-4AS, a 430bp genomic fragment containing the miR-iab-4 hairpin was cloned, in either direction, downstream of the tubulin promoter as described in (Stark et al. 2005). For the UAS-miR-iab-4 and UAS-miR-iab-4AS constructs, the same 430bp genomic fragment containing the miR-iab-4 hairpin was cloned downstream of pUAST-DSred2 (Stark et al. 2003) in either direction.

Reporter assays

For the Luciferase assays, 2 ng p2129 (firefly luciferase), 4 ng *Renilla* reporter, 48 ng miRNA expression plasmid, and 48 ng p2032 (GFP) were cotransfected with .3 µl FuGENE HD per well of a 96 well plate. Twenty-four hours after transfection, expression of *Renilla* luciferase was induced by addition of 500 µM $CuSO_4$ to the culture media. Twenty-four hours induction, reporter activity was measured with the Dual-Glo luciferase kit (Promega, WI), per manufacturers instructions on a Tecan Safire II plate reader.

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 w.t. reporters compared to 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 w^{1118} embryos. bx^{MS1096}*-GAL4* flies were obtained from the Bloomington Stock Center.

In situ hybridization and protein stainings

Double in situ hybridization was performed as described in (Stark et al. 2005). 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).

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.

Mouse small RNAs were cloned from wild-type and c-kit mutant ovaries, and from mouse mammary epithelia (CommaD cells) and sequenced using Solexa sequencing.

Multiple sequence alignments

The multiple sequence alignments for the indicated Hox 3'UTRs were obtained from the UCSC genome browser (Kent et al. 2002) and partly manually adjusted.

Anti-sense 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 anti-sense 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 ((Griffiths-Jones et al. 2006) Release 9.2), requiring miRNAs and transcripts to be on opposite strands and at a distance of at most 50 nts. For each miRNA, we recorded the number of anti-sense 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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Figure Legends

Fig. 1: *Drosophila* iab-4 contains sense and anti-sense miRNAs.
(A) *mir-iab-4* sense and anti-sense sequences can adopt fold-back stem loop structures characteristic for miRNA precursors (structure predictions by Mfold, (Zuker 2003); mature miRNAs shaded in blue and red, respectively). (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 nucleotides and different in only 4 additional positions. (C) Expression of pre-cursor transcripts for *mir-iab-4* (blue) and *mir-iab-4AS* (red) in non-overlapping abdominal segments determined by *in situ* hybridization (lateral (left) and dorsal (right) view of embryonic stage 11, anterior is to the left). (D) Lateral views of stage 10/11 embryos in which *Ubx* protein (B) and *abd-A* protein are visualized (C; anterior is to the left, dorsal upwards).

Fig. 2. miR-iab-4AS targets the Hox genes *abd-A* and *Ubx*.
(A) miR-iab-4AS has 5 seed sites in *Ubx* and 4 in *abd-A* (red) of which 3 and 4 are conserved across 12 *Drosophila* species (*Drosophila melanogaster*, *simulans*, *sechellia*, *yakuba*, *erecta*, *ananassae*, *pseudoobscura*, *persimilis*, *willistoni*, *mojavensis*, *virilis*, *grimshawi*), respectively. 2 sites in the 3'UTR of *Ubx* show extended complementarity such that they are likely also functional for miR-iab-4 (blue), while *abd-A* contains 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*. 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. Error bars represent the fourth largest and smallest values from 16 replicates (asterisk, $P < 0.0001$, Wilcoxon rank-sum test).

Fig. 3. Mis-expression of miR-iab-4AS transforms halteres to wings.
(A, B) Overview of an adult wildtype *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) Wildtype 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 (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 (inlet) normally seen in wildtype wings (B'). Note that C-E are at the same magnification.

Fig. 4 Regulation of gene expression by anti-sense 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 (checkmarks represent experimentally validated targets). miR-iab (blue) and miR-iabAS (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 co-regulated 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 anti-sense 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

non-overlapping 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.

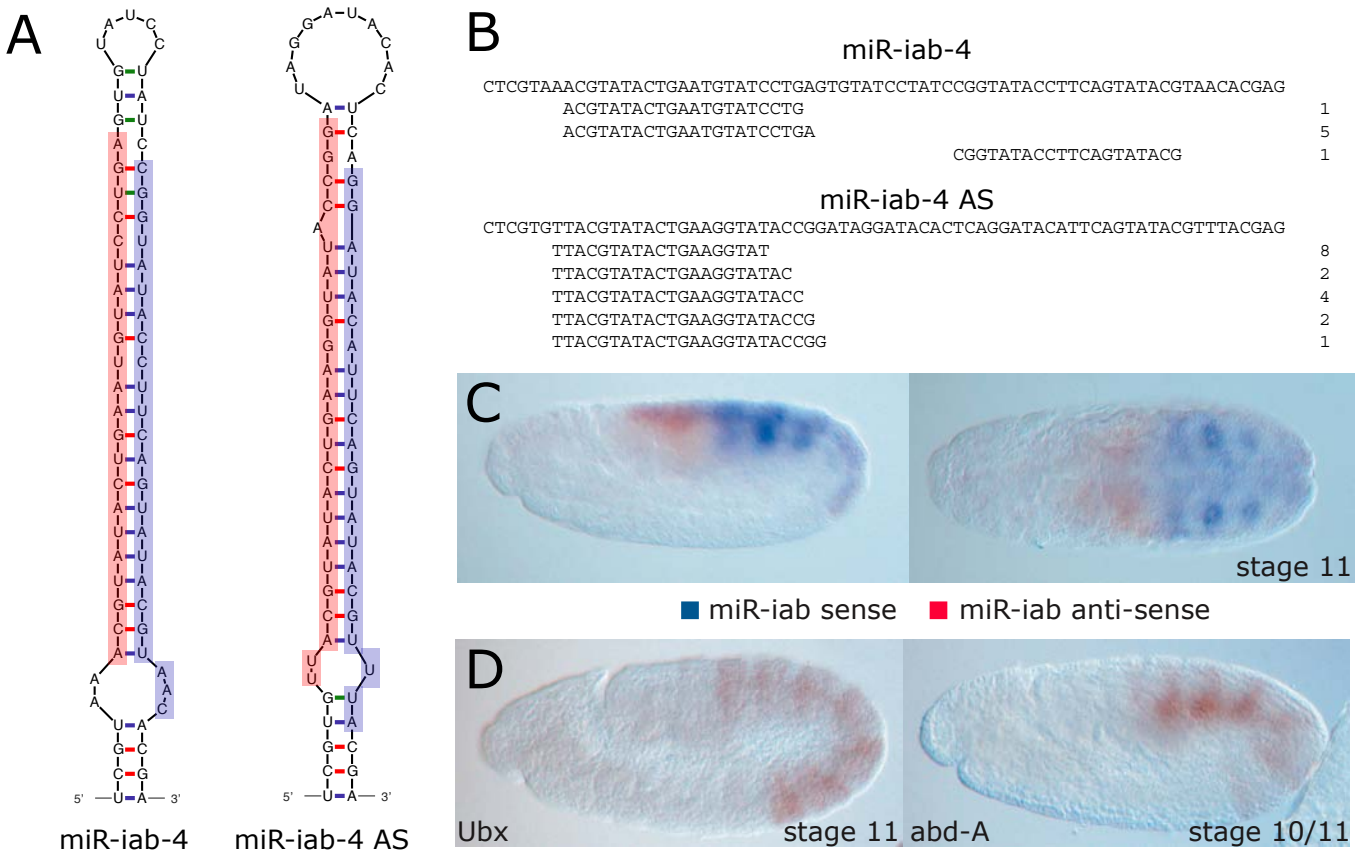

Figure 1

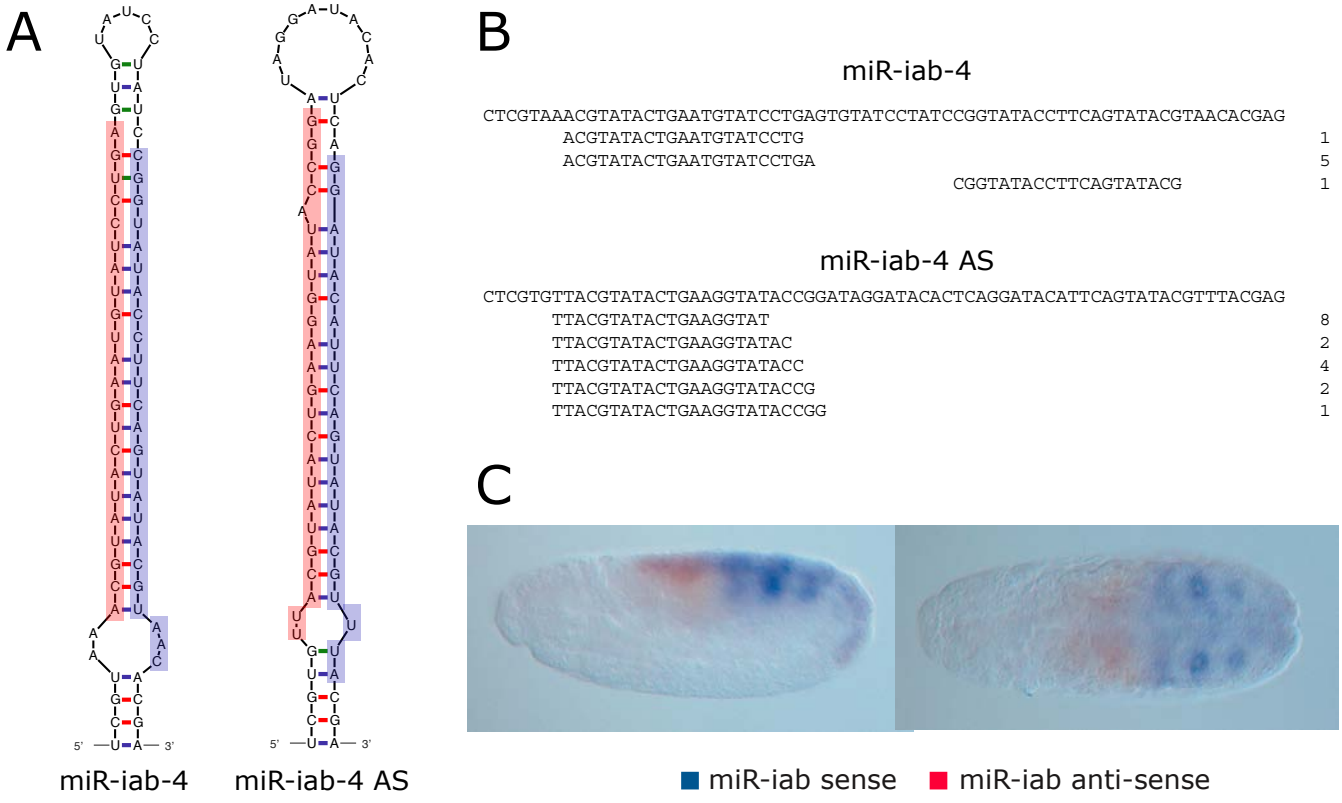

Figure 1 (alternative)

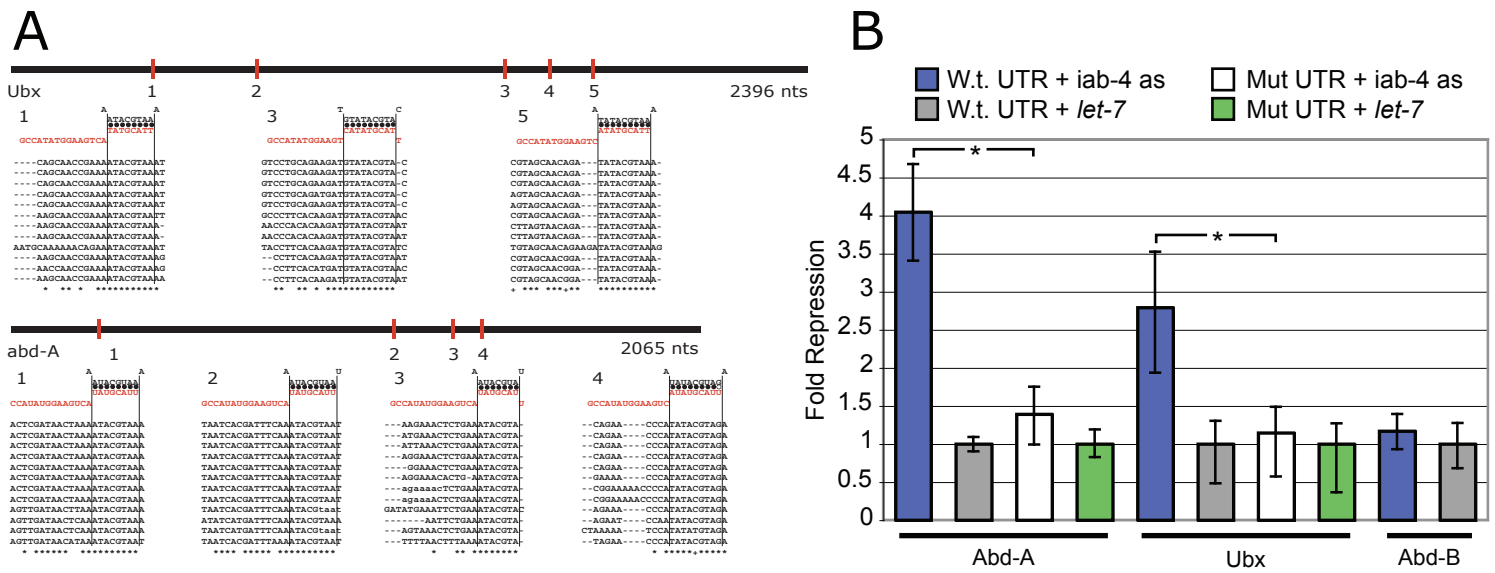
A
Ubx
1
2
3
4
5
2396 nts
abd-A
1
2
3
4
2065 nts
B
W.t. UTR + iab-4 as
W.t. UTR + let-7
Mut UTR + iab-4 as
Mut UTR + let-7
Fold Repression
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
*
*
Abd-A
Ubx
Abd-B

Figure 2

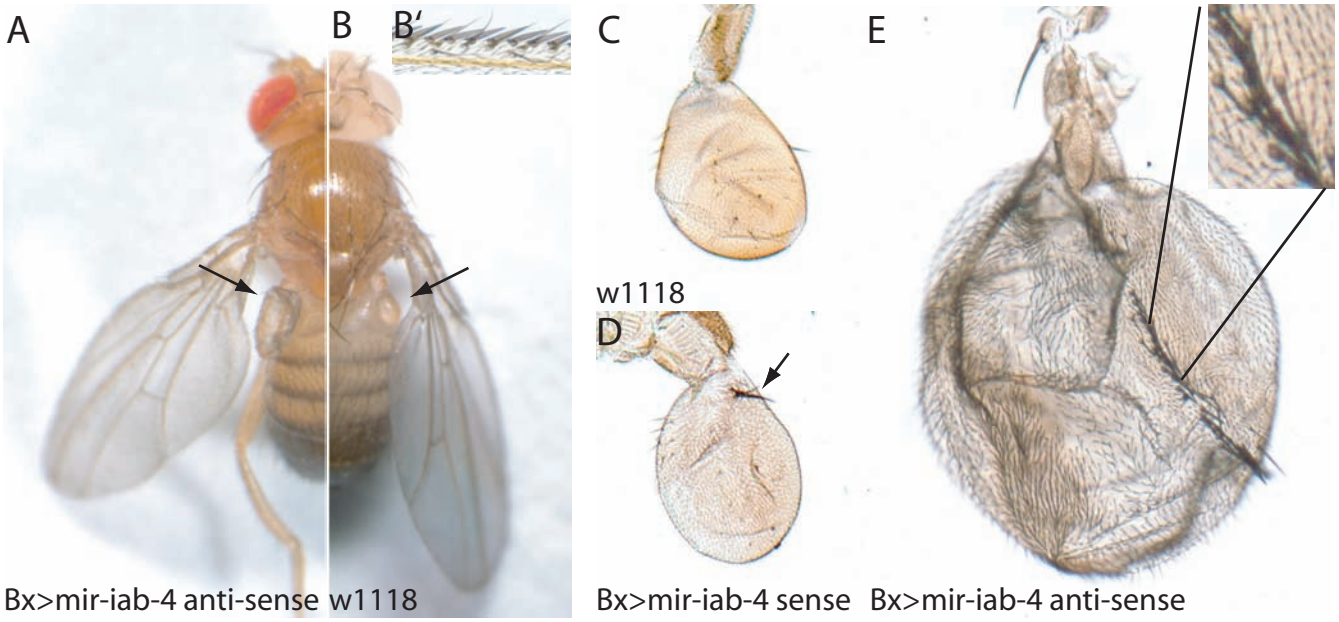
A
B
B'
C
E
w1118
D
Bx>mir-iab-4 anti-sense
w1118
Bx>mir-iab-4 sense
Bx>mir-iab-4 anti-sense

Figure 2

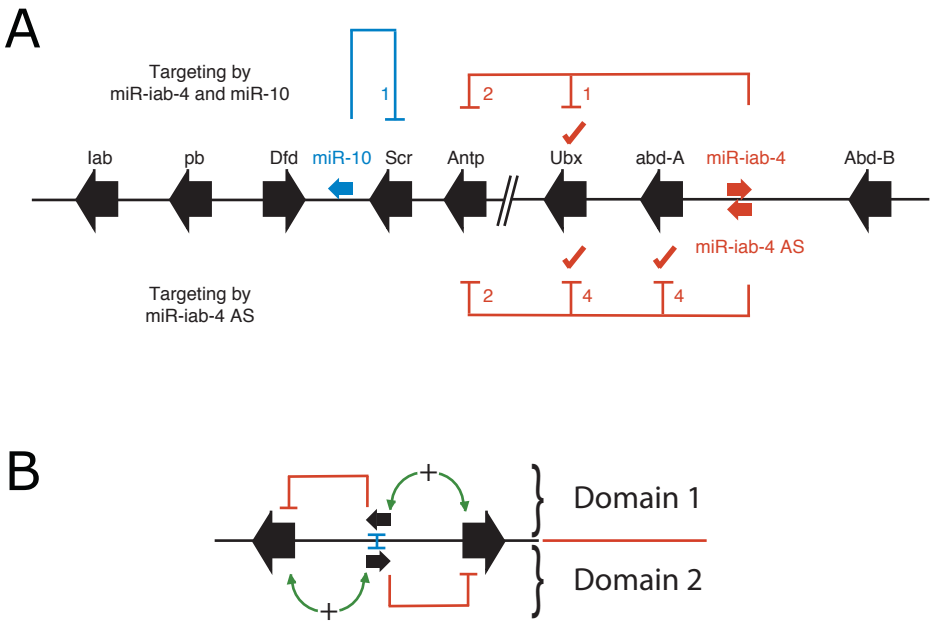
A
Targeting by
miR-iab-4 and miR-10
1
2
1
Iab
pb
Dfd
miR-10
Scr
Antp
Ubx
abd-A
miR-iab-4
Abd-B
miR-iab-4 AS
Targeting by
miR-iab-4 AS
2
4
4
B
Domain 1
Domain 2

Figure 4