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**abd-A Regulation by the iab-8 Noncoding RNA**

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**Abstract**

The homeotic genes in *Drosophila melanogaster* are aligned on the chromosome in the order of the body segments that they affect. The genes affecting the more anterior segments repress the more posterior genes. This posterior dominance rule must be qualified in the case of abdominal-A (abd-A) repression by Abdominal-B (Abd-B). Animals lacking Abd-B show ectopic expression of abd-A in the epidermis of the eighth abdominal segment, but not in the central nervous system. Repression in these neuronal cells is accomplished by a 92 kb noncoding RNA. This “iab-8 RNA” produces a micro RNA to repress abd-A, but also has a second, redundant repression mechanism that acts only “in cis.” Transcriptional interference with the abd-A promoter is the most likely mechanism.

**Introduction**

Genome wide surveys for RNA transcription units in a variety of eukaryotes have revealed a surprising number of transcripts that are not traditional messenger RNAs. A variety of functions have been suggested for these “noncoding” RNAs (ncRNAs), although the large majority have no known purpose (reviewed in ref [1]). In *Drosophila melanogaster*, primary transcripts cover at least 60% of the genome [2]. Many of these transcripts do not correspond to defined genes, but they are evolutionarily conserved. Particular attention has been given to ncRNAs in the bithorax complex (BX-C), a cluster of three homeobox-containing transcription factors required for segment identity (reviewed in ref. [3]). Although much of the ∼300 kb of BX-C DNA is transcribed, the BX-C contains only one other protein coding sequence [4]. Lipshitz et al. [5] first described apparent ncRNAs from the BX-C in the bithorax regulatory region. They suggested that such transcripts could reflect nonspecific initiation by RNA polymerase near a strong enhancer, a possibility that still remains attractive. Several other ncRNAs in the BX-C have been identified by Northern blots or RNA in-situ hybridizations [6-8]. It has been suggested that such transcripts might block silencing by the Polycomb Group proteins [9], but this idea is not yet supported by the analysis of existing mutations. A readthrough product of the *bithoraxoid* ncRNA transcription unit may repress features of early transcription from the Ultrabithorax (Ubx) promoter [10], and the iab-4 and iab-8 ncRNAs are the likely precursors for micro RNAs (miRNAs) [11–13]. Otherwise, these ncRNAs still lack functions.

Prior indications of the iab-8 noncoding RNA

Here, we focus on the iab-8 ncRNA. Several lines of evidence have suggested the existence of a 90 kb-long transcription unit, extending between Abd-B and abd-A, with a likely start site within the iab-8 regulatory region. RNA in-situ hybridizations to embryos, using genomic DNA fragment from the iab-2 through the iab-8 regulatory regions as probes, detect an RNA in the 8th and 9th abdominal segments (parasegments 15 & 14). Strand-specific probes revealed that it is transcribed in a distal-to-proximal direction (from Abd-B towards abd-A) [7,8,14,15]. This transcript is first seen at about stage 6 [11] in the epidermis, but from stage 14 onward (germband shortening), the RNA is detected only in the developing central nervous system (CNS). A promoter for an uncharacterized RNA was independently mapped to the iab-8 region, just downstream of the Abd-B transcription unit [16].

Additionally, a transcript starting in the iab-8 region has been suggested as the precursor for a micro RNA, called miR-iab-8 or miR-iab-4AS [11–13]. This miRNA is transcribed from the iab-3 regulatory region in the distal-to-proximal direction, and strand specific genomic probes from this region indicate that the precursor is made in the 8th and 9th abdominal segments, as described above. This miRNA is required for male and female fertility, and complementation tests with a series of rearrangement breakpoints suggest that the start site of this RNA is in the iab-8 regulatory region, downstream of Abd-B [11].

Here, we characterize the structure and function of the 92 kb long “iab-8 ncRNA”. This ncRNA represses the expression of the homeotic gene abd-A in the posterior CNS. This repression...
Author Summary

Although long, noncoding RNAs have been found in many organisms, it has been difficult to assign to them any molecular function. The homeotic gene clusters in the fruit fly, *Drosophila melanogaster*, contain many such noncoding RNAs. We have characterized one such noncoding RNA, a 92 kb transcription unit from within the bithorax complex. This transcript, called the iab-8 ncRNA, is made in the cells of the central nervous system in the eighth abdominal segment, along with the homeotic transcription factor Abdominal-B. Another homeotic transcription factor, abdominal-A, is repressed in these cells. It has generally been assumed that abdominal-A repression in these cells is mediated by the Abdominal-B protein. However, here we show that it is not Abdominal-B that represses abdominal-A, but the iab-8 ncRNA. This repression is accomplished by two redundant mechanisms; the iab-8 precursor produces a micro RNA, which targets the abdominal-A mRNA, and iab-8 transcription interferes with the abdominal-A promoter, which lies just downstream of the iab-8 ncRNA poly(A) site.

Results/Discussion

Repression of *abd-A* in the 8th abdominal segment

In wild type embryos, *abd-A* expression is detected in the epidermis and CNS of PS7 to PS12 but not in PS13 (Figure 1A). *Abd-B* is strongly expressed in PS13, and it was initially claimed that *Abd-B* represses *abd-A* in PS13 [17], just as *abd-A* represses *Ubx* and *Ubx* represses *Antp* [18]. This repression hierarchy can account for the dominance of posterior homeotic genes over anterior ones, often called “posterior prevalence” [19]. Indeed, embryos homozygous for *Df(3R)C4*, which removes *Abd-B*, show *abd-A* expression throughout PS13 (Figure 1B). However, the *Df(3R)C4* deficiency extends downstream of the *abd-A* transcription unit, removing all of the *iab-7* regulatory region and part of *iab-8* (Figure 2). Surprisingly, embryos homozygous for an *Abd-B* null point mutation, *Abd-B*\exp14, show *Abd-A* derepression in PS13 of the epidermis, but not in the CNS (Figure 1C). Homozygotes for *Abd-B*\exp14, a deletion removing all of the *Abd-B* coding sequences (Figure 2), show the same *Abd-A* expression pattern (not shown). This unexpected repression of *Abd-A* in the CNS can be seen most dramatically in the *Abd-B*\exp14 mutation. *Abd-B*\exp14 deletes the promoter for the *Abd-B* “m” transcript [20], expressed from PS10 through PS13, but leaves the promoters for the “v” transcripts expressed in PS14. In the CNS of *Abd-B*\exp14 homozygotes, *abd-A* does not fill in the gap left by the absence of *Abd-B* in PS13 (Figure 1D). Clearly then, there must be some function deleted by *Df(3R)C4* that is not affected by *Abd-B*\exp14 or more subtle *Abd-B* mutations. Our attention turned to the iab-8 region deleted in *Df(3R)C4*.

Mapping the iab-8 ncRNA exons

The spliced product of the iab-8 ncRNA was initially uncovered by a fortuitous insertion of an exon-trap mobile element. This element, a derivative of the *Minos* mobile element, is called Hostile takeover (*Mh*/*Ho-WP*); Genbank #JN049612. An insertion was recovered in the *iab-6* domain of the BX-C (“TA” target site bases 83,277 & 85,278), named *Mh*/*Ho-WP*\_LNP or simply *LNP*, for short (Figure 2). 3’ RACE products were amplified with primers within *LNP* and within the 3’ exon of *abd-A*. The sequence of the product revealed the exon structure diagramed in Figure 2. The sequence included 5 novel exons before it spliced into *abd-A*, at the 5th exon of the predominant splice form of the *abd-A* mRNA [4,17]. Many of these exons match those of a cDNA designated MIP06894 [Genbank BT099824.1] (Figure 2), identified by the Berkeley *Drosophila* Genome Project.

The exons of the *LNP* cDNA were used to generate primers for 5’ and 3’ RACE, using total RNA from Oregon R embryos. Figure 2 diagrams the predominant splicing product, which spans ~92 kb. An RT/PCR product was recovered and sequenced that extended from exon 1 through exon 8, as well as one that extended from exon 1 through exon 7, and then included exons 5, 6, and 7 of the *abd-A* transcription unit. Figure 2 also shows three alternate 5’ exons and five alternate 3’ splicing patterns. RT/PCR products included extensions of exons 2 or 4, ending at sites of genomic poly(dA) stretches; these were likely derived from splicing intermediates. Rare clones were also recovered that skipped exons, splicing from exons 1, 2, or 6 into *abd-A* exons 5 or 6. Exon 1 had two start sites separated by 135 bases; the upstream start was ~3 fold more abundant. Exon 4 included only 6 bases, although rare products included an alternate 3’ extension of 92 bases. Quantitative PCR was also used to show that termination at exon 8 was ~500-fold more common than splicing into *abd-A*. The sequences of the predominant and alternate exons are given in Figure S1. Two recent genome-wide searches for novel non-coding transcripts in embryos have uncovered some of these same
transcripts [2,21]. Graveley et al. [2] also reported transcripts from adult males with most of the same exons, but an alternate start site, in the iab-6 region.

The promoter for the iab-8 RNA maps distal to the Fab-8 boundary, in the iab-8 regulatory region [22]. The iab-8 region should be under Polycomb Group repression in parasegments 1–12, which explains why the transcript is only expressed in PS13 and 14 [11]. Exons 1–7 appear to be evenly spaced across the abdominal region of the bithorax complex, with one in each of the iab cis-regulatory domains. A comparison with the genomic sequences of various Drosophila species suggests that the sequences of the exons are not more conserved than those of the introns. However, the existence of the exons does appear to be conserved, in that the splice junctions are among the most conserved features of the exons. This is illustrated in Figure 3A for exon 3, in the iab-6 region. The embryonic expression pattern is also conserved; expression is restricted to PS13 and 14 in D. pseudoobscura and D. virilis, as it is in D. melanogaster (see Figure S2).

The spliced product of the iab-8 RNA is non-coding by traditional criteria, but the possibility of small peptides [23,24] cannot be ruled out. In particular, exon 8 includes a potential 9 amino acid peptide, with appropriate translation initiation and termination signals, and the coding potential for this peptide is well conserved in D. ananassae, D. pseudoobscura and D. willistoni (Figure 3B), although it is not found in D. virilis and more distantly related species.

**abd-A repression by the iab-8 RNA**

There are many chromosome rearrangements, mostly from the collection of E. B. Lewis, which interrupt the iab-8 ncRNA transcription unit. These can be used to test whether truncated versions of the iab-8 RNA can repress abd-A. Rearrangement

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**Figure 2. Map of the abdominal half of the bithorax complex.** The horizontal bar indicates the DNA sequence map, numbered in kb according to Martin et al. [4] (Genbank U31961). Base #1 corresponds to base 12,809,162 on chromosome 3R in release 5.37 of the Drosophila genome. The coordinates proceed distal to proximal on chromosome 3R, which is opposite in orientation to the whole genome numbering. The regulatory domains iab-2 through iab-8 are color coded; the domain borders are defined by deletion mutations (Fab8 [22]; Fab7, [41]; Mcp, [44]; iab-3/iab-4, L. Sipos personal communication), or inferred from the binding sites of the CTCF factor [45]. Below the DNA bar are shown the splicing patterns of abd-A and Abd-B (in black), a cDNA derived from the Mi(Hto-WP)LNP insertion (red), and the MIP06894 cDNA (green). At the bottom, the splicing pattern for the iab-8 ncRNA is shown in dark blue, with numbered exons, and alternate 5’ or 3’ extensions indicated with light blue lines. Mutant lesions are indicated above the DNA bar. The rearrangement breakpoints are color coded according to their phenotypes when heterozygous with the mfs5649 insertion.

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**Figure 3. Evolutionary conservation.** A. A comparison of exon 3 and neighboring bases with the homologous regions from the genomes of three Drosophila species. B. Potential nine amino acid peptide within exon 8 of the iab-8 ncRNA. The D. melanogaster sequence is compared to that of D. ananassae. The initial methionine codon is preceded by a perfect translation start consensus sequence [46], and there are two stop codons after the 9th amino acid. The three bases altered in D. ananassae are highlighted in red; only one changes the predicted amino acid.

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breaks that truncate the iab-8 RNA near its start site cause a dramatic derepression of abd-A in the CNS of the 8th abdominal segment, indistinguishable from that seen in Df(3R)C4 homozygotes (Figure 1). Rearrangements with this effect include iab-7 (PS256), iab-6 (11), iab-2,3, iab-4 (25), and iab-5 (25). The same spread of ABD-A into the CNS of PS13 is seen with the FabD deletion, which removes the iab-8 ncRNA promoter (Figure 2). Interestingly, embryos homozygous for chromosome breaks mapping closer to abd-A show a much more subtle derepression of abd-A. In iab-3(MO22) homozygotes, for example, weak misexpression is limited to a few cells (Figure 4). Similar weak misexpression is seen in homozygotes of iab-4,5,13 and iab-4 (25) (Figure 2). Finally, embryos homozygous for the iab-3(MO22) rearrangement show abd-A misexpression in only a very few CNS cells in the most anterior part of the 8th abdominal segment (Figure 4). This break lies downstream of the poly(A) addition site of the major iab-8 transcript, but upstream of the abd-A transcription start site.

The difference between the two classes of breakpoints seems to be the expression of miR-iab-8. The iab-4 (35) break, maps just upstream (within 3 kb) of the miR-iab-8 coding region and shows complete loss of abd-A repression in PS13. In contrast, the iab-3(MO22) break maps ~5 kb downstream of miR-iab-8 and shows only slight misexpression. Thus, one might guess that miR-iab-8 is responsible for most of the repression of abd-A, especially since the 3’ UTR of abd-A includes sequences homologous to the “seed” region of miR-iab-8 [12,13]. However, embryos homozygous for a deletion of miR-iab-8 (ΔmiR-iab-8) do not show a dramatic misexpression of ABD-A in the PS13 CNS [11]. A closer examination of these homozygous embryos does reveal a weak misexpression of abd-A in a small number of nuclei in anterior PS13 (Figure 4), but clearly not the strong and widespread misexpression of iab-4 (35). Thus, it appears that miR-iab-8 does repress abd-A in the PS13 CNS, but there must be a second, redundant function of the iab-8 RNA to completely repress abd-A. UBX expression in embryos is apparently not affected by this deletion, which removes the iab-8 ncRNA promoter (Figure 2).

Fertility function of the iab-8 ncRNA

A deletion of the miR-iab-8 causes sterility in both sexes [11]. Thus, we expected that any combination of alleles that failed to make the miR-iab-8 micro RNA would be sterile, including, for example, an iab-7 break (iab-7(PS256) or iab-7(PS26)) homozygous with ΔmiR-iab-8 [11]. There is an insertion of the “PZ” P element ~4.2 kb downstream of the iab-8 RNA start site, designated mfs5649 (here called mfs5649; Figure 2). Homozygotes are sterile in both sexes, and the females show the same phenotype (blockage of the oviduct) as is seen in ΔmiR-iab-8 homozygotes [25]. We assume the mfs5649 insertion truncates the iab-8 RNA, since it fails to complement with ΔmiR-iab-8 for the sterility phenotype. The FabD deletion (derived from the mfs5649 P element; Figure 2; [22]) is also sterile as a homozygote or as a heterozygote with ΔmiR-iab-8.

We tested rearrangement breakpoints in the iab-2,3, and 4 regions, downstream of the miR-iab-8 template, for fertility when heterozygous with the mfs5649 P element. Surprisingly, many rearrangement breakpoints 3’ to the miR-iab-8 template have a sterile sterility phenotype when heterozygous with mfs5649 (Figure 2); males of these genotypes are fertile. These sterile females show a failure of mature oocytes to move through the oviduct, much like mfs5649 homozygotes or the ΔmiR-iab-8 homozygotes. It does not seem likely that breakpoints downstream of the miR-iab-8 template interfere with the proper processing of the micro RNA, because these same breakpoints are fertile when heterozygous to ΔmiR-iab-8. It is possible that the subtle misexpression of ABD-A in PS13 seen in iab-3 breaks is responsible for the female sterility, especially if the misexpression it is more dramatic at later times in development. Not all breakpoints give this female sterility phenotype, and there is no apparent order to

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**Figure 4. ABD-A expression in rearrangements truncating the iab-8 ncRNA.** Embryos homozygous for the indicated mutations were doubly stained for ENGRAILED (green) and ABD-A (red), and the CNS’s were dissected and photographed. The posterior end of each CNS is shown; the ENGRAILED stripes mark the parasegmental boundaries. The iab-4 and iab-7 breaks cause widespread misexpression of ABD-A in PS13, but iab-3 breaks show only subtle misexpression in a few nuclei. Embryos homozygous for a deletion of the iab-8 miRNA also show misexpression in only a few nuclei.

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the fertile and sterile breakpoint alleles (Figure 2). Some of the rearrangements may fuse the iab-3 region with novel transcription units, restoring the repression of abd-A in the critical cells.

**Mechanism of repression**

The iab-8 ncRNA could make a product, such as another miRNA, that represses abd-A. Indeed, there is a secondary structure hairpin in exon 6 of the spliced transcript that could serve as a miRNA precursor. The iab-8 ncRNA could also code for tiny peptides, as noted above (Figure 3B). These possibilities prompted us to misexpress the iab-8 ncRNA spliced product. A cDNA cassette, representing the major splicing product (Figure 2) plus 236 bp of genomic DNA downstream of the poly(A) addition site, was cloned into the pUAST vector [26]. P element transgenes were recovered and crossed to flies expressing the yeast GAL4 activator in abdominal segments 3–8 (parasegments 8–13). However, embryos containing both the GAL4 activator and the UAS/iab-8 cDNA target showed no apparent reduction in the ABD-A levels in the segments expressing GAL4 (not shown).

The cDNA misexpression experiment does not rule out a product made from an intron, such as an RNA component of a diffusible repressive complex, as alleged for non-coding RNAs in mammalian HOX complexes [27]. If the putative second repressor involves a diffusible molecule, it should be able to act on both chromosomes, even if it is only produced by one. The miR-iab-8 micro RNA should be diffusible in this way, and so, to examine the second repression function, we needed to test genotypes lacking miR-iab-8. Specifically, heterozygotes were made with the ΔmiR-iab-8 deletion on one chromosome, and with a mutation truncating the iab-8 RNA upstream of the miRNA template (mfs5649, iab-7[510], or iab-5[145]) on the other chromosome. Such embryos make the iab-8 RNA from only one chromosome, and cannot make the micro RNA from either. As shown in Figure 5, these embryos showed strong ABD-A misexpression in the CNS of PS13 (the 8th abdominal segment), suggesting that the iab-8 RNA can only repress the copy of abd-A on the chromosome from which it is transcribed. To control for a potential effect of haploinsufficiency of the iab-8 RNA, the ΔmiR-iab-8 deletion was also tested over DfP9, a deletion that removes the entire bithorax complex. These ΔmiR-iab-8/DfP9 embryos show no apparent misexpression of ABD-A in PS13. Thus, the second iab-8 RNApressive function must act only in cis.

In a similar test, we employed a duplication for the proximal two thirds of the complex, Dp(3;2)D109, which extends into the iab-5 region (at ~110 kb) [28]. This duplication includes abd-A, but lacks the iab-8 RNA promoter. Embryos homozygous for the ΔmiR-iab-8 deletion but containing this duplication also show ABD-A misexpression in the PS13 CNS (Figure S3). Thus, there are two mechanisms by which the iab-8 RNA represses abd-A, first, through production of the iab-8 miRNA (acting in trans), and second, a repressive function acting only in cis. The Supplementary Table S1 summarizes which genotypes supply which repressive functions.

The cis-repression of one transcription unit by another is often termed transcriptional interference. This term, however, encompasses several possible molecular mechanisms [29]. An example of a long, ncRNA involved in transcriptional cis-repression is the XIST RNA, involved in mammalian X chromosome inactivation [30] (A recent report suggests that the XIST RNA can also work in trans [31]). Nascent transcripts are involved in repression in RNAi silencing of heterochromatin in fission yeast [32] and in RNA-directed DNA methylation in Arabidopsis [33]. By analogy to these systems, the iab-8 RNA could recruit gene silencing machinery to the site of its transcription. The RNA sequences required for such recruitment might be mapped by examination of deletions in the BX-C. Ideally, the iab-8 miRNA should be removed to have a clear assay for the cis repressor. We have checked embryos homozygous for the Fab3.5[50] deletion (Figure 2), which covers the site of the iab-8 miRNA precursor; they still show abd-A repression in the posterior CNS. Likewise, a double deletion chromosome, with ΔmiR-iab-8 and Fab7[7], also retains the cis repression. The Fab7[7] deletion (Figure 2) was tested because it removes a Polycomb Response Element [34,35] which is coincident with exon 2 of the cDNA. Two other deletions have been examined which span the iab-4 through iab-7 regions, although both retain the iab-8 miRNA (iab-4,5,6[10] and iab-6,7[10]; Figure 2). In these, we looked for more subtle misexpression, such as that seen in iab-3 breaks (Figure 4), but no such misexpression was seen. This analysis does not yet cover the iab-2 and iab-3 regions, nor does it exclude the possibility of multiple redundant sequences throughout the transcription unit that could recruit repressive factors.

A more likely repression mechanism, perhaps, is that the RNA polymerase transcribing the iab-8 RNA somehow interferes with the abd-A promoter. Examples of this type of transcriptional interference come from budding yeast, where the GAL7 gene is repressed by the upstream GAL10 transcript [36], and the SER3 gene is repressed by the upstream, noncoding SRG1 transcript [37]. In these cases, the 3’ ends of the upstream transcripts are close to the downstream promoters, suggesting repression by occlusion of the downstream promoters or their proximal enhancers. If the iab-8 RNA interferes with an abd-A enhancer, that enhancer must lie downstream of the iab-4[10] breakpoint, since abd-A is totally derepressed in the PS13 CNS in embryos homozygous for this break (Figure 4). The abd-A promoter seems like the most likely target of interference, since the major poly(A) site of the iab-8 RNA lies only 1.1 kb upstream of the initiation site of abd-A, and the iab-8 RNA primary transcript likely continues past its poly(A) addition site [38]. In any case, minor splice variants

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**Figure 5. Test for trans repression by the iab-8 ncRNA.** CNS’s, stained for ABDA, were dissected from embryos of the indicated genotypes. In ΔmiR/mfs5649 embryos, only one chromosome makes a full length iab-8 ncRNA, and neither chromosome produces the iab-8 miRNA, as diagrammed. The strong expression of ABDA in PS13 in this genotype shows that the abd-A gene on the mfs5649 chromosome is not repressed, i.e. the iab-8 ncRNA acts only in cis. doi:10.1371/journal.pgen.1002720.g005
clearly do continue past the poly(A) site and into the \textit{abd-A} transcription unit (Figure 2).

The function of the iab-8 ncRNA fits with the rule of posterior dominance - it blocks expression of a more anterior homeotic gene in more posterior segments. The repression of \textit{Ubx} by the \textit{bcd} ncRNA [10], although subtle, fits the same pattern. The novel aspect, here, is that this posterior repression can be accomplished by noncoding transcription units, in addition to DNA binding proteins. The mechanism of transcriptional interference would fix the arrangement of these ncRNAs in the bithorax complex. It seems possible that the ancestral HOX complex turned off proteins. The mechanism of transcriptional interference would fix by noncoding transcription units, in addition to DNA binding aspect, here, is that this posterior repression can be accomplished ncRNA [10], although subtle, fits the same pattern. The novel Fab864

\textbf{Materials and Methods}

\textbf{Drosophila strains}

Wild type stocks were Canton S or Oregon R. Mutations included \textit{abd-A}dxx1, \textit{igl-577}, \textit{igl-582}, \textit{igl-585}, \textit{igl-72662}, \textit{igl-740}, \textit{Abd-B}116, \textit{Abd-B}214, \textit{Df(3R)C4, Df(3)P9} (ref [39]); \textit{igl-3}, \textit{igl-34i} (ref [17]) \textit{igl-64i} ref [40]; \textit{Fab7} ref [41]; \textit{msf}(3)55694 [ref [25], Fab8074 ref [22]; \textit{Fab8075} ref [42]; \textit{igl-57054}, \textit{igl-57568}, \textit{igl-446}, \textit{igl-345}, \textit{iab-9} (ref [11]; \textit{T(3;2)Dp1109} [28] and \textit{M{\textit{i}/Hto-WP}} (described here).

\textbf{Antibody staining}

Embryos were fixed, stained, and mounted as described by [17]. Primary antibodies used were mouse anti-\textit{ABD-B} (1:2 dilution, developed by S. Celniker, Developmental Studies Hybridoma Bank), mouse anti-\textit{UBX} (1:10, developed by R. White, Developmental studies Hybridoma Bank), rabbit anti-\textit{B-galactosidase} (1:1500, Cappel/MP Biomedicals), mouse anti-\textit{B-galactosidase} (1:1000, Promega), rabbit anti-\textit{En} (1:500, Santa Cruz Biotechnology, rabbit anti-\textit{En} (1:500, Santa Cruz Biotechnology), mouse anti-\textit{ABD-A} (1:500, 6A18.12, gift of I. Duncan), and goat anti-\textit{ABD-A} (1:100, Santa Cruz Biotechnology). Secondary antibodies were donkey anti-mouse, donkey anti-goat, and donkey anti-rabbit, coupled to either Alexa 488 or Alexa 555 (1:500, Invitrogen), and HRP coupled goat anti-mouse (1:1000, Bio-Rad).

The CNS’s were hand dissected with tungsten needles and placed on a glass slide in a drop of Immu-Mount (for HRP staining, Shandon) or Vectashield with DAPI (for fluorescence, Vector Laboratories), and then gently flattened under a coverslip. Fluorescence images were taken with a Leica SP2 AOBS confocal microscope; the fluorescence pictures show free projection averages of stacks of images, after scanning through the depth of the tissue. Homozygous embryos were identified by the absence of lacZ staining from the \textit{T3M3 flz-LacZ} balancer.

\textbf{Fertility tests}

Each of ten mutant virgin females was placed in a vial with three wild type males. Likewise ten mutant males were mated, each with three wild type virgin females. Vials were maintained at 25° for five days, and then examined for the presence of larvae.

cDNA analysis

Adults heterozygous for \textit{M{\textit{i}/Hto-WP}} and \textit{Hsp70-Gal4} (Bloomington stock #1799) were heat shocked for 45 min. at 37° to induce \textit{GAL4} expression, and then left to express the \textit{LNP} transcript at room temperature for 4 h. RNA was then isolated using TRI reagent (Sigma) and reverse transcribed with MMLV reverse transcriptase (Promega) using an adaptor primer (GACAAGACACCCGACT18V). PCR was then performed using a forward primer in \textit{Hto} and a reverse primer in the 6th exon of \textit{abd-A}. The resulting ampliplex was sequenced to identify the splicing pattern.

Total RNA from Oregon R embryos was prepared using the RNeasy-PCR kit (Ambion), and 3’ RACE and RNA ligase-mediated 5’ RACE reactions were performed using the First-Choice RLM-RACE kit (Ambion). The 5’ RACE procedure was designed to recover only capped 5’ ends. Gel-isolated products were sequenced directly, or cloned first into the PCR-Blunt vector (Invitrogen). Quantitative PCR reactions used cDNA prepared from 6–12 h old embryos. The initial cDNA products were compared to measured dilutions of amplified cDNA products covering the relevant exons.

\textbf{DNA in situ hybridization and embryo staining}

The production of digoxigenin-labeled probes and the hybridization of embryos was as described by Fitzgerald and Bender [11], except that acetone treatment [43] was used instead of proteinase K for permeabilization of the embryos. Clones spanning exon 8 from \textit{D. melanogaster}, \textit{D. pseudobscura} and \textit{D. virilis} were recovered after PCR reaction on genomic DNAs with the following pairs of oligonucleotides: \textit{D. melanogaster} 5’GGTTGTTAAGGGTTACAGGCGG3’ and 5’GGATGTGGTGGGGTGGG’ generating a fragment of 1013 bp; \textit{D. pseudobscura} 5’CAGGACATTACGTAACACGGCG3’ and 5’GGATGTGGTGGGGTGGG’ generating a fragment of 1477 bp, \textit{D. virilis} 5’CGTTCTCGGTCTAATTCAGGCGG3’ and 5’CCGATCTCTGGTGTC3’ generating a fragment of 1364 bp.

\textbf{Supporting Information}

\textbf{Figure S1} Sequences of iab-8 ncRNA exons. The first and last bases of each exon are numbered according to the SEQ89E coordinates of Martin et al. [4] (Genbank U31961). (PDF)

\textbf{Figure S2} Conserved iab-8 noncoding RNA expression patterns in \textit{D. melanogaster}, \textit{D. pseudobscura} and \textit{D. virilis} embryos. The top 3 panels show embryos at stage 8, while the bottom panels show embryos at stages 14–17. (TIF)

\textbf{Figure S3} Additional test for repression by the iab-8 ncRNA. Males of the genotype \textit{T(2;3) Dp1109, AmiR, Fab/TM2} were crossed to \textit{AmiR/LacZ} females. The \textit{T3M3}-containing embryos were recognized by their \textit{LacZ} expression. Among the remaining embryos, half showed no apparent ABD-A expression in the CNS of \textit{PS13} (presumed to be \textit{T2/AmiR}), and half gave clear \textit{PS13} misexpression (presumed \textit{Dp1109, AmiR/AmiR}). Thus, the repression fails to act in \textit{trans} on the duplication. The \textit{PS13} misexpression is weaker than the PS7-12 level, because the former derives from only the one \textit{abd-A} copy on the duplication, but the latter represents three doses of the \textit{abd-A} gene. \textit{Dp1109, +/AmiR} embryos produced in a control cross displayed little, if any, ABD-A misexpression in \textit{PS13}. (TIF)

\textbf{Table S1} Two mechanisms, iab-8 miRNA \textit{trans-repression} and \textit{cis-repression} mediate \textit{abd-A} repression in \textit{PS13} of the CNS. The table summarizes which of these 2 mechanisms is/are affected in the various mutant alleles. Note that complete ectopic expression in \textit{PS13} is only observed when both mechanisms are affected. (TIF)
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