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miR-9a prevents apoptosis during wing development by repressing *Drosophila LIM-only*

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Abstract

Loss of *Drosophila mir-9a* induces a subtle increase in sensory bristles, but a substantial loss of wing tissue. Here, we establish that the latter phenotype is largely due to ectopic apoptosis in the dorsal wing primordium, and we could rescue wing development in the absence of this microRNA by dorsal-specific inhibition of apoptosis. Such apoptosis was a consequence of de-repressing *Drosophila LIM-only* (*dLMO*), which encodes a transcriptional regulator of wing and neural development. We observed cell-autonomous elevation of endogenous dLMO and a *GFP-dLMO* 3'UTR sensor in *mir-9a* mutant wing clones, and heterozygosity for *dLMO* rescued the apoptosis and wing defects of *mir-9a* mutants. We also provide evidence that *dLMO*, in addition to *senseless*, contributes to the bristle defects of the *mir-9a* mutant. Unexpectedly, the upregulation of dLMO, loss of Cut, and adult wing margin defects seen with *mir-9a* mutant clones were not recapitulated by clonal loss of the miRNA biogenesis factors Dicer-1 or Pasha, even though these mutant conditions similarly de-repressed miR-9a and dLMO sensor transgenes. Therefore, the failure to observe a phenotype upon conditional knockout of a miRNA processing factor does not reliably indicate the lack of critical roles of miRNAs in a given setting.

Introduction

Dominant alleles of invertebrate genes associated with loss of 3' untranslated regions (3' UTRs) were harbingers of the existence of a regulatory universe mediated by ~22 RNAs known as microRNAs (miRNAs). For example, 3' UTR mutants of *C. elegans lin-14* that induced defects in developmental timing were critical in illuminating its repression by the founding miRNA lin-4 (Lee et al., 1993; Wightman et al., 1991; Wightman et al., 1993). In addition, 3' UTR mutants of the *Drosophila* Notch pathway genes *E(spl)m8* and *Bearded*, which affect eye and bristle specification (Klämbt et al., 1989; Leviten et al., 1997; Leviten and Posakony, 1996), permitted the 7-mer regulatory logic of miRNA binding sites to be elucidated (Lai, 2002; Lai et al., 1998; Lai and Posakony, 1997; Lai et al., 2005). These genes, along with a handful of targets analyzed more recently, demonstrate that the miRNA-mediated repression of certain genes can be critical to organismal phenotype (Flynt and Lai, 2008).

On the other hand, computational and quantitative profiling methods indicate that a majority of animal transcripts are directly targeted by one or more miRNAs, with individual miRNAs

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often targeting hundreds of transcripts via highly conserved binding sites (Bartel, 2009). Since the phenotypes of many miRNA loss-of-function mutants are relatively subtle (Smibert and Lai, 2008), presumably very few individual targets are regulated by miRNAs in a manner that is critically required for gross aspects of development or physiology (Flynt and Lai, 2008). Knowledge of such critical miRNA targets, whose slight overactivity is not tolerated, is especially relevant to understanding how miRNA dysfunction contributes to disease.

The development of *Drosophila* wings requires the coordinated action of several signaling pathways and positional information systems, which yield precise control over cell survival, proliferation, and specification (Cadigan, 2002; Milan and Cohen, 2000). Genetic analysis of mutants that perturb wing development revealed diverse insights into mechanisms of tissue patterning and growth, including many concepts that embody fundamental principles of gene regulation and animal development. Amongst *Drosophila* wing mutants, dominant *Beadex* (*Bx*) alleles causing loss of adult wing tissue were identified over 80 years ago (Mohr, 1927; Morgan, 1925). In the past decade, *Bx* mutants were recognized to result from gain-of-function of *Drosophila LIM-only* (*dLMO*) (Milán et al., 1998; Shoresh et al., 1998; Zeng et al., 1998). Curiously, most *Bx* alleles are caused by transposon insertions that disrupt its 3' UTR, which hinted at critical post-transcriptional repression of *dLMO*. Another gene that affects wing development is *mir-9a*. Deletion of this highly conserved miRNA results in fully penetrant loss of posterior wing margin, along with a small number of ectopic sensory organs (Li et al., 2006).

In this report, we demonstrate a critical role for miR-9a in suppressing apoptosis in the developing wing, and show that the wing morphology defect of animals lacking this miRNA can be fully rescued by inhibiting apoptosis during wing development. While miR-9a has ~200 target genes that are deeply conserved across Drosophilid radiation (http://www.targetscan.org/), we find that its major functional requirement is to suppress *dLMO* in the developing wing pouch. We observed that dLMO is ectopically expressed in *mir-9a* mutant wing primordia, is directly repressed via its 3' UTR by endogenous miR-9a in the developing wing, and that heterozygosity for *dLMO* fully rescues the *mir-9a* wing defect. Our findings confirm and extend the recent report of *dLMO* as an important target of miR-9a in the wing (Biryukova et al., 2009), and collectively highlight the disproportionate functional impact of de-repressing certain transcripts within the collective pool of thousands of miRNA targets. Unexpectedly, the phenotype of miR-9a wing pouch clones is demonstrably stronger in certain respects than is clonal loss of the miRNA biogenesis factors Pasha and Dcr-1. This has consequences for interpreting the lack of certain phenotypes upon removing "all" miRNAs in certain settings.

Materials and methods

Drosophila strains

We used the following previously described strains: *pasha[KO]* (Martin et al., 2009); *mir-9a* stocks (*mir-9a[J22]*, *mir-9a[E39]*, and *UAS-mir-9a*) provided by Fen-Biao Gao (Li et al., 2006); *senseless* stocks (*sens[E2]*, *Lyra*, and *UAS-sens*) obtained from Hugo Bellen (Nolo et al., 2000); *dLMO* stocks (*Bx[1]* and *hdpR26*, and *UAS-dLMO*) from Marco Milan (Milán et al., 1998); *dcr-1[Q1147x]* from Richard Carthew (Lee et al., 2004); and *UAS-Diap1*, *UAS-p35*, *ptc-Gal4* and *ap-Gal4* obtained from the Bloomington Stock Center. For clonal analysis, we recombined *mir-9a* alleles onto FRT80B and generated clones using *hs-FLP* (Bloomington Stock Center), *vg-FLP* (gift of Konrad Basler) or *ubx-FLP* (obtained from David Bilder). Clones were marked by absence of *arm-lacZ* (from Stephen Cohen) or *ubi-GFP* (Bloomington Stock Center).

UAS-DsRed-mir-9a was generated by amplifying the *mir-9a* locus with the following primers and cloning into pENTR (Invitrogen), and then transferring the insert into *UAS-DsRed* (Stark et al., 2003). miR-9A_F: CACCTAACTTAACATAAATAATAGAC; miR-9A_R: TCTAGATTGCCAAAGCAGTTGGCCG. The miR-9a sensor contained two antisense target sites, and was generated by annealing the oligos below and cloning into the NotI and XhoI restriction sites of *tub-GFP-SV40* (gift of Julius Brennecke and Stephen Cohen). miR-9a sensor F Not: GGCCTCATACAGCTAGATAACCAAAGAAATCACACTCATACAGCTAGATAACCAAAG A; miR-9a sensor R Xho: TCGATCTTTGGTTATCTAGCTGTATGAGTGTGATTTCTTTGGTTATCTAGCTGTATGA. The *dLMO* sensor was made by amplifying the *dLMO* 3' UTR and ~200 bp downstream of the poly-adenylation signal from *w[1118]* genomic DNA using the oligos below, followed by cloning into the NotI and XhoI restriction sites of *tub-GFP-SV40*. dLMO UTR F Not: GATCgcggccgcAATAAAGCCCTGGGCATGGG; dLMO UTR R Xho: GATCctcgagTGCCCTCTAGCTCCTCTAGCTCC. Transgenic *Drosophila* were made using standard injection with delta2–3 helper transposase (BestGene Inc.) and multiple lines were analyzed for each construct.

Indirect immunofluorescence

To analyze imaginal discs, we used standard fixation in 4% paraformaldehyde, as described previously (Lai and Rubin, 2001). We used the following primary antibodies: mouse anti-Cut (1:10, Developmental Studies Hybridoma Bank-DSHB), guinea pig anti-Sens (1:2000, gift of Hugo Bellen), rat anti-dLMO (1:100, gift of Stephen Cohen), mouse anti-Wg (1:10, DSHB), rabbit anti-cleaved caspase-3 (1:50, Cell Signaling Technology), rabbit anti-GFP (1:600, Molecular Probes), mouse anti-βgalactosidase (1:10, DSHB). We used Alexa-488, -568 and -647-conjugated secondary antibodies (1:600, Molecular Probes).

Results

The major role of miR-9a during wing development is to suppress apoptosis

In contrast to their mild PNS defects, flies lacking *mir-9a* exhibit substantial and completely penetrant wing notching (Li et al., 2006). We examined the null alleles *mir-9a[J22]* and *mir-9a [E39]* in more detail, both as homozygotes and as trans-heterozygotes. All three genotypes lack ~50% of the posterior wing margin in all individuals (Fig. 1A, B), and a small fraction of animals (~10%) further exhibit mild loss of anterior wing margin (Fig. 1C). Thus, the posterior margin is more sensitive to miR-9a activity.

Notch pathway activity at the dorsoventral boundary of the wing pouch is necessary to activate Wingless signaling to specify wing margin cells. To assay whether this accounted for the *mir-9a* mutant phenotype, we examined the expression of Wingless (Wg), Cut, and Senseless (Sens) proteins. Wg serves as an early marker of specified wing margin cells, and its expression was normal in *mir-9a* mutant wing discs (Fig. 2A, F). On the other hand, we observed highly penetrant breaks in wing margin-associated Cut in the posterior compartment, and occasional gaps in anterior compartment (Fig. 2B, G). These patterns were consistent with the observed penetrance of adult wing notching (Fig. 1B, C). Cut-expressing cells induce the expression of Sens in two flanking rows of cells, and we observed corresponding breaks in Sens expression in *mir-9a* mutants (Fig. 2C, H).

Since expression of Wg at the wing margin was uninterrupted in the absence of miR-9a, we inferred that initially deficient margin specification was not the major cause of *mir-9a* wing loss. Another mechanism by which wing notching might arise is through excess cell death. We tested this by staining for apoptotic cells using antibodies that recognize cleaved (activated)

caspase-3. In wildtype, only a small number of dying cells are seen in third instar wing imaginal discs (Fig. 2D). In contrast, we observed abundant cell death specifically in the wing pouch in all three *mir-9a* mutant genotypes, but not in the pro-notum region of the disc (Fig. 2I and data not shown). In summary, wing notching is the predominant morphological defect caused by lack of *mir-9a*, and this is associated with mildly defective margin specification and a high degree of ectopic cell death in the wing primordium.

Previously, the *mir-9a* wing defect was rescued by expressing a *UAS-mir-9a* transgene throughout the wing pouch using *vg-Gal4* (Li et al., 2006). Curiously, we observed substantially more cell death in the dorsal compartment of the wing pouch, compared to the ventral compartment (Fig. 2I and Supplementary Fig. 1). We therefore asked whether the dorsal-specific expression of miR-9a was sufficient to rescue the loss of adult wing tissue. Indeed, activation of *UAS-mir-9a* using *ap-Gal4*, which is exclusively active in the dorsal compartment, completely restored the continuity of the adult wing margin in the *mir-9a* mutant (Fig. 1D). We used this regimen to check for the consequence of blocking cell death in *mir-9a* mutants. In fact, misexpression of either *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) (Fig. 1E), or the baculovirus inhibitor of apoptosis P35 (data not shown) could fully rescue *mir-9a* wing notching. We clearly observed cell-autonomous rescue of apoptosis in these backgrounds, since the dorsal-specific activity of *ap-Gal4* did not rescue ventral apoptosis in *mir-9a* mutants (Fig. 2K–M). Nevertheless, the small amount of remaining ectopic cell death was tolerated to permit the emergence of normal adult wings (Fig. 1E). We conclude that the main phenotypic requirement for *Drosophila* miR-9a is to suppress apoptosis in the wing primordium, and that dorsal wing development is especially sensitive to *mir-9a* dosage and apoptosis.

miR-9a represses dLMO to prevent apoptosis in the wing primordium

It was previously reported that miR-9a targets the zinc finger transcription factor encoded by *senseless* (*sens*) to control bristle and wing development (Li et al., 2006). The rationale was that ectopic Sens induces extra sensory organs and loss of wing margin (Fig. 1F) (Nolo et al., 2000; Nolo et al., 2001), similar to phenotypes seen in *mir-9a* loss-of-function animals. Notably, heterozygosity for *sens[E58]* could partially rescue *mir-9a* wing phenotypes, consistent with the inference of de-repressed Sens in this mutant (Li et al., 2006).

We tested this further by recombining the *sens[E2]* null allele with *mir-9a* alleles and evaluating genetic interactions. We observed a mild dominant suppression of wing notching (Fig. 1I), which we quantified in cohorts of 50 wings as a ~35% reduction of *mir-9a*-associated wing margin loss in the *sens* heterozygote (Fig. 1K). However, *mir-9a* mutant wing discs did not accumulate ectopic Sens in the third instar wing disc (Fig. 2H), during which time widespread apoptosis is manifest (Fig. 2I); Instead, we observed loss of wing margin-associated Sens in the posterior compartment. This contrasts with abundant ectopic Sens observed in the wing pouch in dominant alleles of *sens* that exhibit wing notching, referred to as *Lyra* mutants (Nolo et al., 2001), and with the observation that *Lyra* wing discs do not exhibit ectopic apoptosis (Fig. 3A–C).

Invertebrate and vertebrate Senseless proteins exhibit anti-apoptotic activity (Jafar-Nejad and Bellen, 2004), and *Drosophila* Sens specifically inhibits apoptosis by repressing pro-apoptotic genes such as *reaper* (Chandrasekaran and Beckendorf, 2003). We verified this by misexpressing Sens in the wing pouch, which yielded hundreds of ectopic sensory organ precursors, but not aberrant apoptosis (data not shown). Finally, *mir-9a* mutant wing discs that were heterozygous for *sens* still exhibited abundant apoptosis (Fig. 3D–F). Together, these observations suggested that the derepression of other targets might mediate the apoptotic phenotype of *mir-9a* mutants.

Amongst the many other predicted conserved targets of miR-9a is the transcriptional regulator encoded by *Drosophila LIM-only* (*dLMO*) (e.g http://www.targetscan.org/fly_12/). dLMO can sequester Chip, a LIM-domain cofactor of the LIM-homeodomain factor Apterous, which together specify the dorsal-ventral axis of the developing wing (Milan and Cohen, 2000). Excess dLMO activity, as seen in *Beadex* (*Bx*) alleles, titrates Chip protein and consequently interferes with wing margin development (Milán et al., 1998; Shoresh et al., 1998; Zeng et al., 1998). As is the case with *mir-9a* loss-of-function mutants, the development of the posterior wing margin is more sensitive than the anterior wing margin to increased dLMO (Fig. 1G, H), and *Bx* gain-of-function mutants of dLMO exhibit apoptosis in the developing wing pouch (Fristrom, 1969). We confirmed that *Bx[1]* heterozygous discs (i.e. female larvae, selected on the basis of gondal morphology to distinguish them from *Bx[1]/Y* hemizygous male larvae) exhibit excess apoptosis and breaks in wing margin Cut expression (Fig. 3G–I), similar to *mir-9a* mutants (Fig. 2G, I). These genetic observations raised dLMO as an attractive candidate target of miR-9a.

In wildtype, dLMO is expressed throughout the wing pouch but is elevated in the dorsal compartment (Fig. 2E). In contrast to Sens, dLMO protein was upregulated in *mir-9a* mutant wing pouches (Fig. 2J). The mutant tissue exhibited uniform dLMO across the dorsal and ventral compartment, indicating greater elevation of dLMO in the ventral compartment *of mir-9a* mutant discs. However, analysis of *mir-9a* mutant clones, which provide an internal control for accumulation of dLMO in neighboring non-clonal tissue, demonstrated a cell-autonomous increase in dLMO in both compartments (Fig. 2N–P).

We next tested whether *dLMO* exhibited genetic interactions with *mir-9a*. Impressively, loss of a single allele of *dLMO* (*hdp[R26]*) in females fully rescued the adult wing margin defect of both *mir-9a[J22]* homozygotes as well as *mir-9a[J22]/[E39]* transheterozygotes (Fig. 1J and data not shown). A similar genetic interaction was recently reported by Heitzler and colleagues (Biryukova et al., 2009). Heterozygosity of dLMO also strongly suppressed the apoptotic defect in the third instar wing pouch, and restored nearly normal expression of Wg, Cut (compare Fig. 2G, I to Fig. 3J–L) and Sens (data not shown) along the wing margin. Altogether, these observations indicate that repression of *dLMO* by miR-9a is critical to prevent apoptosis in the developing wing primordium.

dLMO is directly targeted by miR-9a

In principle, the effects of *mir-9a* loss-of-function on dLMO could be indirect. However, the *dLMO* 3' UTR contains an 8mer site for miR-9a (ACCAAAGA) in its 3’ UTR that is deeply conserved across the sequenced Drosophilid species (Biryukova et al., 2009), along with a nearby poorly-conserved G:U seed site (Supplementary Fig. 2). As well, loss of the *dLMO* 3' UTR is highly correlated with dLMO gain-of-function phenotypes (Biryukova et al., 2009; Shoresh et al., 1998). To assess the direct response of dLMO to miR-9a in vivo, we generated transgenic miR-9a and *dLMO* 3' UTR sensors fused to ubiquitously expressed *GFP*. We analyzed the behavior of these sensors in wing disc clones lacking the miRNA biogenesis enzyme Dicer-1 (Dcr-1), as well as clones specifically lacking miR-9a.

We first observed that the miR-9a sensor was upregulated in *dcr-1* clones (Fig. 4A–A"), indicating that it is repressed by miRNAs in the wing disc. This sensor was specifically repressed by miR-9a, since *mir-9a[E39]* clones also upregulated the sensor (Fig. 4B–B"). Detailed examination revealed the exquisite sensitivity of the sensor to the endogenous level of the miRNA. We observed a perfectly inverse relationship of endogenous miR-9a dosage and miR-9a sensor activity: homozygous *mir-9a* mutant cells expressed the highest levels of the sensor, their twinspot wildtype clones displayed the lowest levels of the sensor, and *mir-9a* heterozygous cells exhibited an intermediate sensor level (Fig. 4C–C").

We next tested whether the *dLMO* sensor behaved similarly. Indeed, it was de-repressed in both *dcr-1* clones (Fig. 4D–D") and *mir-9a* clones (Fig. 4E–E"), demonstrating that miR-9a directly represses *dLMO* via its 3' UTR in vivo. Finally, we performed reciprocal gain-of-function assays to examine the expression of the *dLMO* sensor in wing discs ectopically expressing *UAS-DsRed-miR-9a* under control of *ptc-Gal4*. We observed mild suppression of *GFP-dLMO* in the *ptc-Gal4* domain (Fig. 4F–F"). Together with the phenotypic data, these in vivo sensor assays demonstrate that *dLMO* is the key direct target of endogenous miR-9a whose repression prevents apoptosis during *Drosophila* wing development.

Both *sens* and *dLMO* contribute to sensory organ defects in *mir-9a* mutants

Recently, dLMO was shown to act as a neural pre-patterning factor, working in concert with Pannier and Chip to promote the expression of proneural bHLH genes in dorsocentral (DC) proneural clusters (Asmar et al., 2008). In this capacity, it functions well upstream of *sens*, which is expressed in sensory organ precursor cells only following their Notch-mediated selection from proneural clusters. We therefore wondered whether deregulation of dLMO contributed to the development of ectopic sensory organs in *mir-9a* mutants.

We first re-evaluated adult sensory organ phenotypes of *mir-9a* mutants in detail. As reported earlier, *mir-9a* mutants displayed increased density of stout mechanosensory bristles along the anterior wing margin and a mild increase in anterior scutellar (aSC) bristle organs (Li et al., 2006). However in our cultures, the aSC phenotype was not consistent across the three *mir-9a* null genotypes (n=50 females for each test, data not shown). Since laboratory stocks such as *w[1118]* are prone to exhibiting ectopic aSC bristles, miR-9a may not be the sole determinant for the aSC phenotype. We also observed a mild increase in notum microchaete density in *mir-9a[J22]* homozygotes and *mir-9a[J22]/[E39]* trans-heterozygotes, but not *mir-9a[E39]* homozygotes. Such variability of phenotypes in the various *mir-9a* genotypes is consistent with the notion that miR-9a functions as a neural "robustness" factor (Li et al., 2006), and that variation in genetic background may influence the penetrance of mild miR-9a-dependent phenotypes. We did, however, reproducibly observe some ectopic dorsocentral DC and anterior postalar (aPA) macrochaete bristles in all three genotypes, which we interpreted to be directly caused by miR-9a loss (Fig. 5).

To minimize confounding background effects, we focused subsequent genetic modifier tests on the DC and aPA positions in *mir-9a* deletion trans-heterozygotes. We observed that *sens* heterozygosity could partially suppress ectopic neurogenesis at both DC and aPA positions (Fig. 5C, D). *dLMO* heterozygosity could partially suppress ectopic aPA macrochaetes, but had only mild effects at the DC position (Fig. 5C, D). Therefore, de-repression of dLMO and Sens are both relevant to the wing margin and the neurogenesis defects of *mir-9a* mutants, placing them amongst the most vital targets of miR-9a. At the same time, the phenotypic importance of these miR-9a:target interactions in different settings is distinct: repression of dLMO is more critical for wing development (since *dLMO* heterozygosity provided much better rescue of wing apoptosis and margin development), while restriction of Sens is more critical for bristle patterning (since *sens* heterozygosity provided much better rescue at the DC position).

dcr-1 and *pasha* clones do not phenocopy *mir-9a* mutant clones

Since the detailed biology of few miRNA genes is presently known, many researchers examine conditional knockouts of miRNA biogenesis factors to assess the plausibility of miRNA-mediated control in a given setting. We were curious to see if the defects of *mir-9a* mutants were indeed recapitulated by clonal loss of *dcr-1* (Lee et al., 2004), which is required for the biogenesis of all *Drosophila* miRNAs. Adult wings carrying *mir-9a[E39]* clones exhibited notching along the posterior wing margin (Fig. 6A, D), similar to homozygous mutant wings

(Fig. 1B). On the other hand, wings carrying *dcr-1* clones were not flat and exhibited blistering, likely due in part to uneven growth of the wing surfaces caused by the lack of growth-promoting miRNAs such as bantam in *dcr-1* mutant cells (Fig. 6B) (Brennecke et al., 2003; Friggi-Grelin et al., 2008; Martin et al., 2009). Surprisingly, we did not observe the tissue loss typical of wings bearing *mir-9a* clones amongst >100 wings carrying high frequency *dcr-1* clones generated with either *vg-FLP*, *ubx-FLP*, or *hs-FLP*, even though large sectors of mutant wing margin tissue exhibiting defective non-sensory bristle differentiation were commonly evident (Fig. 6B, E and data not shown). To test this further, we analyzed clones of *pasha* (Martin et al., 2009), which encodes an obligate nuclear cofactor for the primary miRNA processing factor Drosha. Again, the presence of these mutant clones caused abnormal wing growth, but they always exhibited continuous wing margin (data not shown).

We next asked if phenotypic differences between the different mutant clones were apparent at the third instar. We observed that small *mir-9a[E39]* clones contained apoptotic cells (Fig. 6F–F''), consistent with the whole disc *mir-9a* mutants. We observed that similarly-sized *dcr-1* clones also preferentially contained apoptotic cells (Fig. 6G–G''). Because of the growth defect of *Dcr-1* clones, we sensitized this assay by generating large clones using the *Minute* technique. Mutant clones of both *mir-9a* and *Dcr-1* in this background exhibited high incidence of reactivity with cleaved Caspase-3, but there was also substantial non-autonomous apoptosis (data not shown), likely due to cell competition induced in the *Minute* background (Morata and Ripoll, 1975; Tyler et al., 2007). In any case, the excess apoptosis in *dcr-1* mutant disc clones was consistent with the apoptotic phenotype of conditional knockout of mammalian *Dicer* in various organs (Damiani et al., 2008; De Pietri Tonelli et al., 2008; Harfe et al., 2005; Schaefer et al., 2007). In addition to loss of miR-9a, it is certainly conceivable that loss of other miRNAs contributes to the apoptotic defect of *dcr-1* clones.

However, when we examined the expression of Cut at the wing margin, we observed a clear difference between *mir-9a* and *dcr-1* clones. *mir-9a[E39]* clones that overlapped the central region of the posterior wing margin reliably exhibited loss of Cut (Fig. 6H–H'' and Supplemental Figure 3A), consistent with the results from *mir-9a* mutant discs (Fig. 2G). Note that Cut was not necessarily lost throughout the clone, as expected from the fact that whole mir-9a mutant discs typically lost only a portion of Cut at the posterior wing margin (Fig. 2G). In contrast, similarly-positioned *dcr-1* clones frequently maintained Cut (Fig. 6I–I''). We noted examples of wing pouches bearing posterior *dcr-1* clones that exhibited a decrease or loss of Cut (Supplemental Figure 3B, C). However, such loss was not particularly associated with the clone boundary, and often extended into the wild-type tissue. The basis of this variable phenotype is not clear, but conceivably may be a consequence of the altered growth properties of *dcr-1* mutant cells, leading to uncoordinated wing margin development. In any case, we could clearly distinguish that *mir-9a* mutant clones that overlapped the central region of the posterior wing margin consistently lacked Cut, whereas *dcr-1* mutant clones did not.

We next studied the expression of dLMO, which we showed to be a key direct target that is de-repressed in *mir-9a* mutant wings. We focused here on dLMO in the ventral compartment of the wing pouch, since its elevation in *mir-9a* mutant cells was especially evident in ventral clones (Fig. 2N–P and Fig. 6J–J''). Surprisingly, we did not observe comparable increases in dLMO staining in *dcr-1* mutant clones (Fig. 6K–K''). We sensitized this further by generating *dcr-1*, *pasha* double mutant clones, but these still did not exhibit the defect in dLMO accumulation (Fig. 6L–L'') seen in wing disc cells lacking only miR-9a.

These results indicated that loss of miR-9a was more dentrimental to normal expression of Cut and dLMO in the wing pouch than was loss of Dcr-1 and/or Pasha, consistent with the observation that *mir-9a* but not *dcr-1* or *pasha* mutant cells reliably exhibit loss of adult wing tissue. These data are perhaps especially unexpected given that cells that are mutant for miRNA

biogenesis factors clearly de-repress both miR-9a and dLMO sensors (Fig. 4), and are thus are demonstrably deficient in miR-9a activity (along with most if not all other miRNAs). We conclude that the failure to observe certain mutant phenotypes upon conditional knockout of core miRNA biogenesis factors does not rule out critical roles for miRNAs in a given setting.

Conclusions

dLMO is an essential genetic switch target of *Drosophila* miR-9a

Because of their relative ease of detection, dominant alleles and X-linked mutants constituted a high proportion of the classical spontaneous mutants isolated by Morgan and colleagues. Bridges isolated *Bx[1]* in 1923 (Morgan, 1925), and genetic tests by Green in the early 1950s established that *Bx* was due to overactivity of the locus (Green, 1952). In fact, the recessive allele *Bx[r]* was associated with a duplication of the region, indicating that as little as a two-fold increase in *Bx* activity could interfere with wing development. In 1979, Lifschytz and Green further proposed that *Bx* might be due to a mutation in a cis-acting repressor site in the *heldup* locus (Lifschytz and Green, 1979). Indeed, the cloning of *Bx* by the Cohen, Jan, and Segal labs in 1998 finally revealed that *Bx* and *heldup* were gain- and loss-of-function alleles of the *dLMO* gene, respectively (Milán et al., 1998; Shoresh et al., 1998; Zeng et al., 1998). Moreover, most spontaneous *Bx* alleles proved to be transposable element insertions in the *dLMO* 3’ UTR, and new *Bx* mutants were easily obtained by imprecise excisions of a downstream transposable element, so as to delete *dLMO* 3’ UTR sequence (Shoresh et al., 1998). Collectively, these 85 years of research indicated that 3'UTR-mediated post-transcriptional repression of *dLMO* is critical for normal development.

A small number of other gain-of-function mutants in *Drosophila* and *C. elegans* result from the loss of 3’ UTR regulatory elements, and many of these are now appreciated to be key genetic switch targets of miRNAs (Flynt and Lai, 2008). Our studies, together with concurrent work from Heitzler and colleagues (Biryukova et al., 2009), establish dLMO as one of a handful of genes whose loss of miRNA-mediated repression leads to a severe morphological defect. We found that the lack of *mir-9a* results in upregulation of dLMO, aberrant apoptosis in the wing pouch, and failure to completely specify and develop the wing margin. Importantly, dorsal-specific expression of miR-9a, dorsal-specific inhibition of cell death (using p35 or Diap1), or heterozygosity for *dLMO*, all strongly reduced ectopic apoptosis and restored adult wing development in *mir-9a* null animals.

Ectopic apoptosis in the wing pouch has previously been reported to result in loss of wing margin (Delanoue et al., 2004; Smith-Bolton et al., 2009; Sotillos and Campuzano, 2000; Yoshida et al., 2001). However in other cases, the disc is able to compensate for cell loss in the face of ectopic apoptosis, so that no loss of margin is observed in the adult wing (Cifuentes and Garcia-Bellido, 1997; Ng et al., 1995; Perez-Garijo et al., 2004). In the *mir-9a* mutant, we show that excess apoptosis is coupled with a margin specification defect. Despite our ability to rescue the mutant by inhibiting apoptosis, we do not rule out that ectopic apoptosis by itself might be insufficient to induce adult margin loss; perhaps it requires the sensitized background evidenced by the demonstrable failure to fully activate Cut in the third instar. We also note that *Bx* was recently reported not to be suppressed by inhibiting apoptosis (Bejarano et al., 2008), which might be at odds with our conclusions. However, that study examined *Bx/Y* hemizygotes, which are substantially stronger in phenotype than *Bx/X* heterozygotes. It is clear that elevation of dLMO yields a variety of patterning defects that are not seen in *mir-9a* mutants (Milán et al., 1998; Zeng et al., 1998). We infer that loss of miR-9a results in apoptosis and wing margin defects that are attributable to de-repression of dLMO, but that elevation of dLMO can clearly generate developmental phenotypes that are not simply due to excess apoptosis.

A minor, but quantifiable, consequence of lacking *mir-9a* is the development of a small number of ectopic sensory organs. This is demonstrably due to the de-repression of the proneural factors Sens and dLMO. Therefore, even though computational approaches provide evidence for hundreds of conserved miR-9a targets, including compelling "anti-target" relationships with a large number of neural genes (Stark et al., 2005), the bulk of its morphologically evident phenotypes can be accounted for by the failure to repress only two target genes, *sens* and *dLMO* (Biryukova et al., 2009; Li et al., 2006). In addition to miR-9a, Drosophilid species encode miR-9b and miR-9c, as well as the ancestrally related miR-79 (Aravin et al., 2003; Lai et al., 2003; Lai et al., 2004). The function of these miRNAs remains to be studied, but conventional knowledge of miRNA targeting suggests that they may have overlapping target capacity since they have similar seeds. It is conceivable that the analysis of double or triple *mir-9* mutants may reveal additional targets that mediate compelling phenotypes. Nevertheless, it is clear that miR-9a serves a function to repress *dLMO* and *sens* that cannot be substantially compensated by the remaining miR-9-related genes.

Implications for the regulation of human dLMO genes

dLMO and miR-9 are both highly conserved between invertebrates and vertebrates. However, vanishingly few miRNA:target interactions have been preserved over this evolutionary distance, indicating that these post-transcriptional target networks are much more plastic than the genes themselves (Chen and Rajewsky, 2006). Therefore, the existence of a key miR-9a:*dLMO* regulatory connection in flies does not necessary imply that human miR-9 regulates LMO genes, and human LMO genes lack conserved canonical miR-9 seed sites (http://www.targetscan.org/). Heitzler and colleagues proposed that mammalian *LMO2* is a conserved target of miR-9 (Biryukova et al., 2009). However, the candidate site contains a G:U seedpair, a feature that is detrimental to, although not necessarily incompatible with miRNA targeting (Brennecke et al., 2005; Doench and Sharp, 2004; Lai et al., 2005). Directed studies are needed to assess whether this site alone confers repression by miR-9.

On the other hand, the necessity of restricting LMO activity might well prove to be a conserved feature of invertebrate and vertebrate biology. As in *Drosophila*, vertebrate LMO proteins can dominantly interfere with LDB:Islet complexes, indicating that its overactivity is especially "dangerous". Indeed, elevation of LMO proteins has myriad consequences for downstream transcriptional networks, and *LMO2* is in fact a T-cell oncogene (Rabbitts, 1998). Intriguingly, *LMO2*, which normally regulates hematopoetic development (Warren et al., 1994), has a highly conserved 8mer seed for miR-223 (http://www.targetscan.org/). Recent studies demonstrated that *mir-223* mutant mice exhibit hematopoetic defects, and that *mir-223* deletion has consequences for the neutrophil transcriptome and proteome (Baek et al., 2008; Johnnidis et al., 2008). While the depth of peptide sampling was insufficient to report on LMO2 status, the microarray data demonstrated *LMO2* to be the 60th most-upregulated mRNA across the transcriptome of *mir-223* knockout cells. Indeed, Marziali and colleagues recently reported that suppression of *LMO2* by miR-223 regulates erythropoiesis (Felli et al., 2009). We note that its paralog *LMO1* contains a highly conserved canonical site for miR-181, another miRNA with a demonstrated function in the hematopoietic system (Chen et al., 2004). These observations suggest that the regulation of vertebrate LMO genes by hematopoietic miRNAs, and its potential relevance to cancer, deserves further study.

Implications for studying conditional loss of miRNA biogenesis factors

To date, relatively few *Drosophila* or vertebrate miRNA genes have been analyzed using bona fide mutant alleles (Smibert and Lai, 2008). As an approximation, many researchers have taken to analyzing the effects of conditional knockout of miRNA biogenesis factors, such as Dicer. This manipulation is presumed to break all miRNA regulatory links, thereby serving as a plausibility test of whether miRNAs might be required in a given setting. Acknowledged

drawbacks of this approach include uncertainty as to whether one or many miRNAs might contribute to a given phenotype, and whether phenotypes are a direct or indirect cause of miRNA loss. However, a caveat that is little considered is the potentially canceling effects of removing "all" miRNAs, so that loss of one miRNA might be compensated for by the concomitant loss of another miRNA(s). While such an outcome might seem to require highly unlikely coincidences, it may be plausible if we consider that most biological processes are under both positive and negative control, and that most genes are themselves miRNA targets.

During development of the *Drosophila* wing primordium, we showed that clones lacking *mir-9a* upregulate dLMO and induce wing notching, whereas *dcr-1* and *pasha*-mutant clones do not (Fig. 6). Additionally, *mir-9a* mutant clones exhibit a more severe phenotype than *dcr-1* mutant clones with respect to loss of wing margin, both in the third instar wing pouch and in the adult wing. Although the cells analyzed were homozygous mutant for substantial periods of time (72–96 hours), perdurance of miRNAs on account of Dcr-1 or Pasha proteins inherited by mutant cells conceivably contributes to the phenotypic disparity. For example, perdurance may explain the incomplete phenocopy of *bantam* mutant discs by *dcr-1* or *pasha* "whole disc" mutants (Brennecke et al., 2003;Martin et al., 2009). However, potential perdurance is not reconciled with the comparable upregulation of miR-9a and dLMO sensor activity in *dcr-1* and *mir-9a* homozygous mutant cells (Fig. 4), which report on similar loss of miR-9a activity in these clones. Together, these data suggest that *mir-9a* mutant cells exhibit phenotypes that are intrinsically different from those of *dcr-1* or *pasha* mutant cells.

In summary, the failure to observe a phenotype in cells or tissues that are mutant for a general miRNA biogenesis factor cannot reliably be taken as evidence that miRNAs lack substantial roles in the setting of interest. Reciprocally, our observation that loss of miRNA-mediated regulation from a single target gene (e.g. failure to repress *dLMO* in *mir-9a* mutant wings) can be of greater phenotypic impact than loss of "all" miRNA-mediated regulation (e.g, in *dcr-1, pasha* double mutant wing clones) highlights the disproportionate consequence of releasing particular miRNA targets from amidst a regulatory web that is inferred to encompass most animal transcripts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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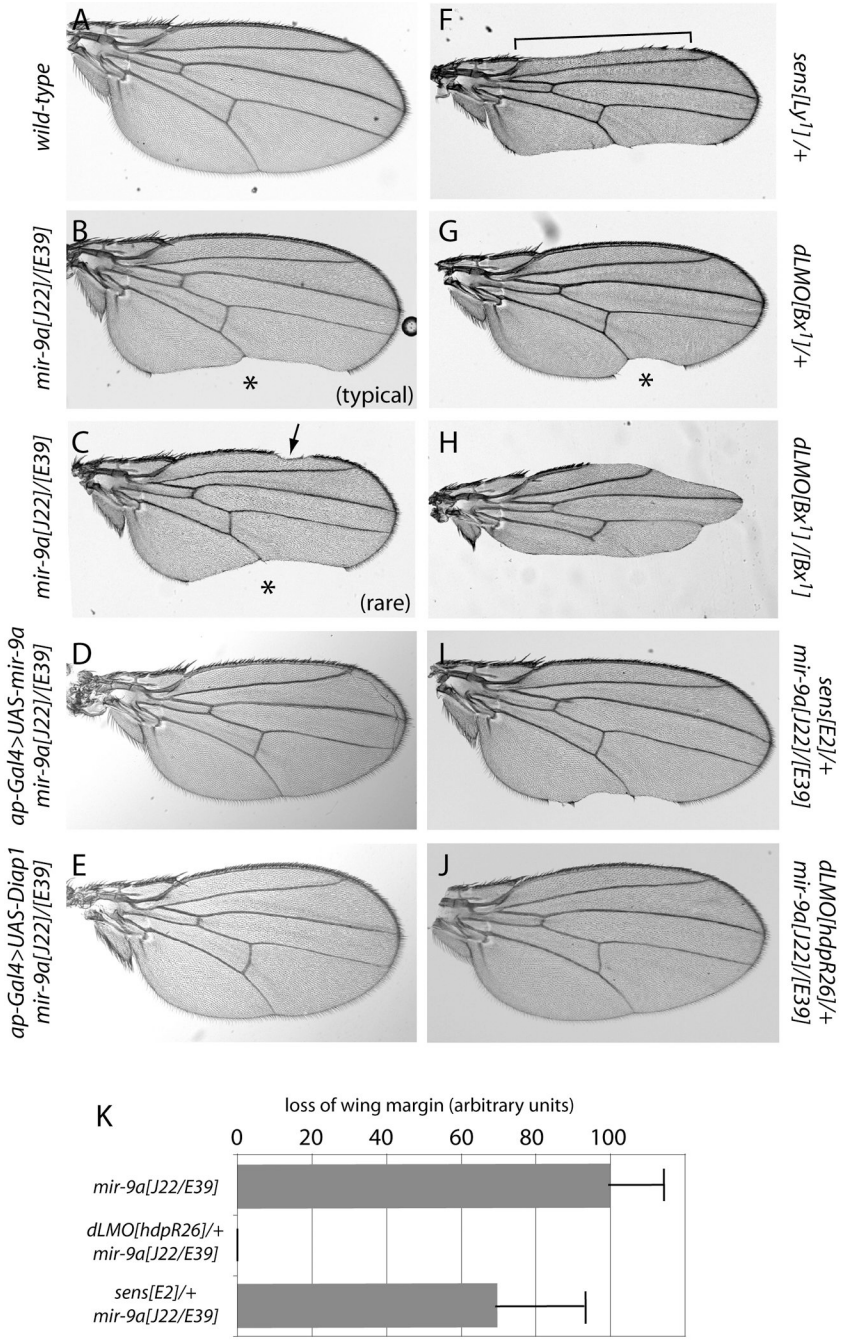

Figure 1.

Genetic interactions amongst *mir-9a, senseless*, and *dLMO* in wing morphology. Shown are wings of adult females, oriented with anterior to the top and posterior to the bottom. (A) Wild-type. (B) Transheterozygotes of *mir-9a[J22]/[E39]* null alleles exhibit completely penetrant notching along the posterior margin (asterisk). (C) About 10% of *mir-9a[J22]/[E39]* flies also show notching along the anterior wing margin (arrow). (D) Wing notching in *mir-9a* mutants can be rescued by reintroduction of miR-9a in the dorsal compartment using *ap-Gal4*. (E) Wing notching in *mir-9a* mutants can also be rescued by ectopic expression of *Drosophila inhibitor of apoptosis 1* (*Diap1*). (F) Heterozygotes of the gain-of-function allele of senseless, *Lyra [1]*, exhibit loss of both anterior (bracket) and posterior wing margin. (G) Heterozygotes of

the gain-of-function allele of *dLMO*, *Bx[1]*, show loss of only posterior margin (asterisk). (H) *Bx[1]* homozygotes exhibit loss of both anterior and posterior margin. (I) Heterozygosity for the null allele *sens[E2]* partially suppressed *mir-9a* wing notching. (J) Heterozygosity for the null allele *dLMO[hdpR26]* completely rescued *mir-9a* wing defects. (K) Quantification of wing margin loss; each bar depicts the mean and standard deviation across 50 female wings.

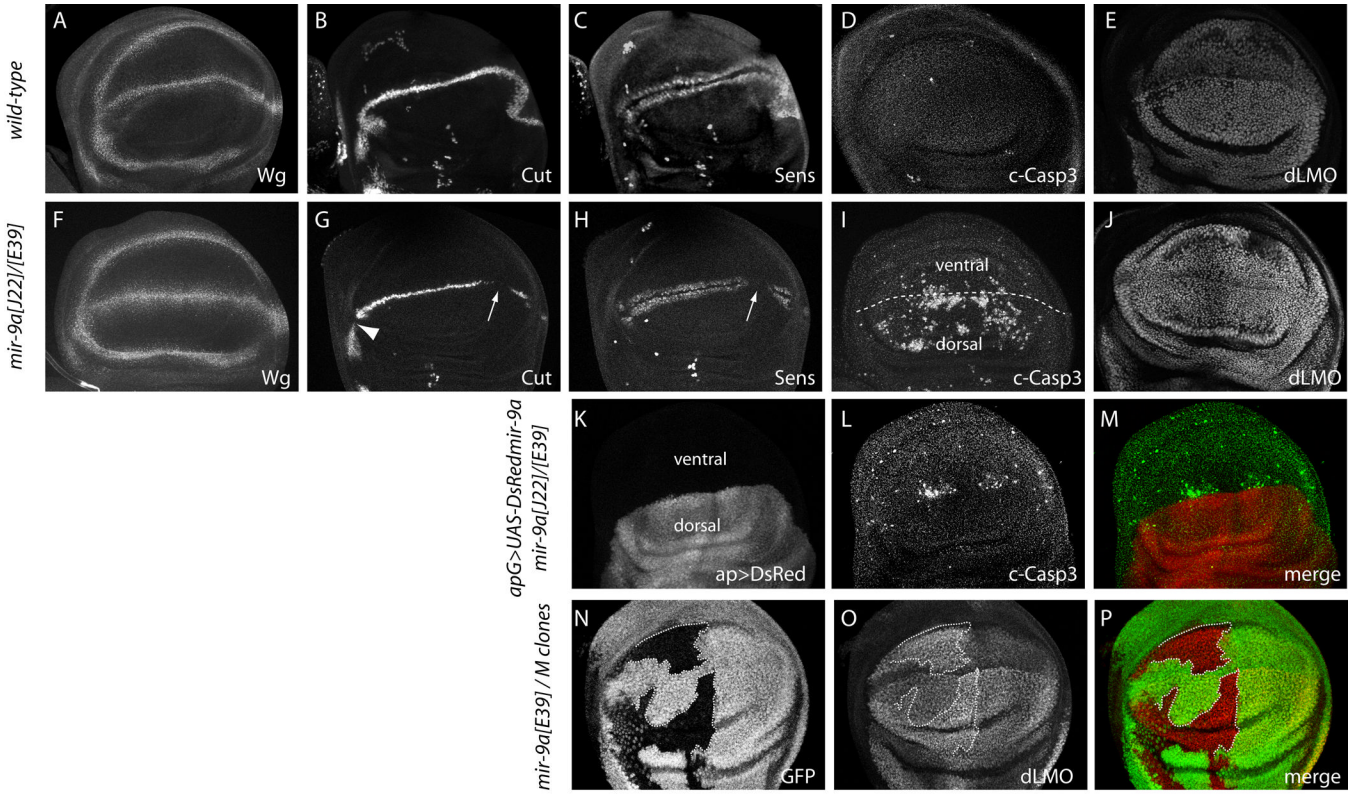

Figure 2.

Cellular basis of *mir-9a* mutant phenotypes. All panels depict the wing pouch region of third instar wing imaginal discs stained for the indicated markers, oriented with anterior to the left, posterior to the right, ventral to the top and dorsal to the bottom. (A–E) Wildtype, (F–J) *mir-9a [J22]/[E39]*, (K–M) *ap-Gal4>UAS-DsRed-mir-9a* in *mir-9a[J22]/[E39]*, (N–P) *mir-9a* clones generated in *hs-FLP; FRT80B M ubi-GFP/FRT80B mir-9a[J22]*. The following panels are double stainings of the same tissue: B and C, G and H, F and I, K–M and N–P. (A, F) The expression of Wingless (Wg) is fairly normal in *mir-9a* mutants. (B, G) *mir-9a* mutants exhibit posterior breaks in Cut at the wing margin (arrow), and occasional anterior breaks (arrowhead). (C, H) *mir-9a* mutants exhibit breaks in wing margin-associated expression of Sens, particularly in the posterior compartment (arrow); the isolated stained cells are sensory organ precursors. (D, I) *mir-9a* mutants exhibit a high degree of apoptosis in the wing pouch as marked by cleaved Caspase-3 (c-Casp3), mostly in the dorsal compartment. (E, J) *mir-9a* mutants accumulate higher levels of dLMO. (K–M) Dorsal-specific expression of miR-9a suppresses the majority of the apoptotic defect of *mir-9a* mutants; a small amount of ectopic ventral apoptosis remains. (N–P) Clonal analysis using *Minute* technique demonstrates cell-autonomous elevation of dLMO in wing pouch cells lacking miR-9a.

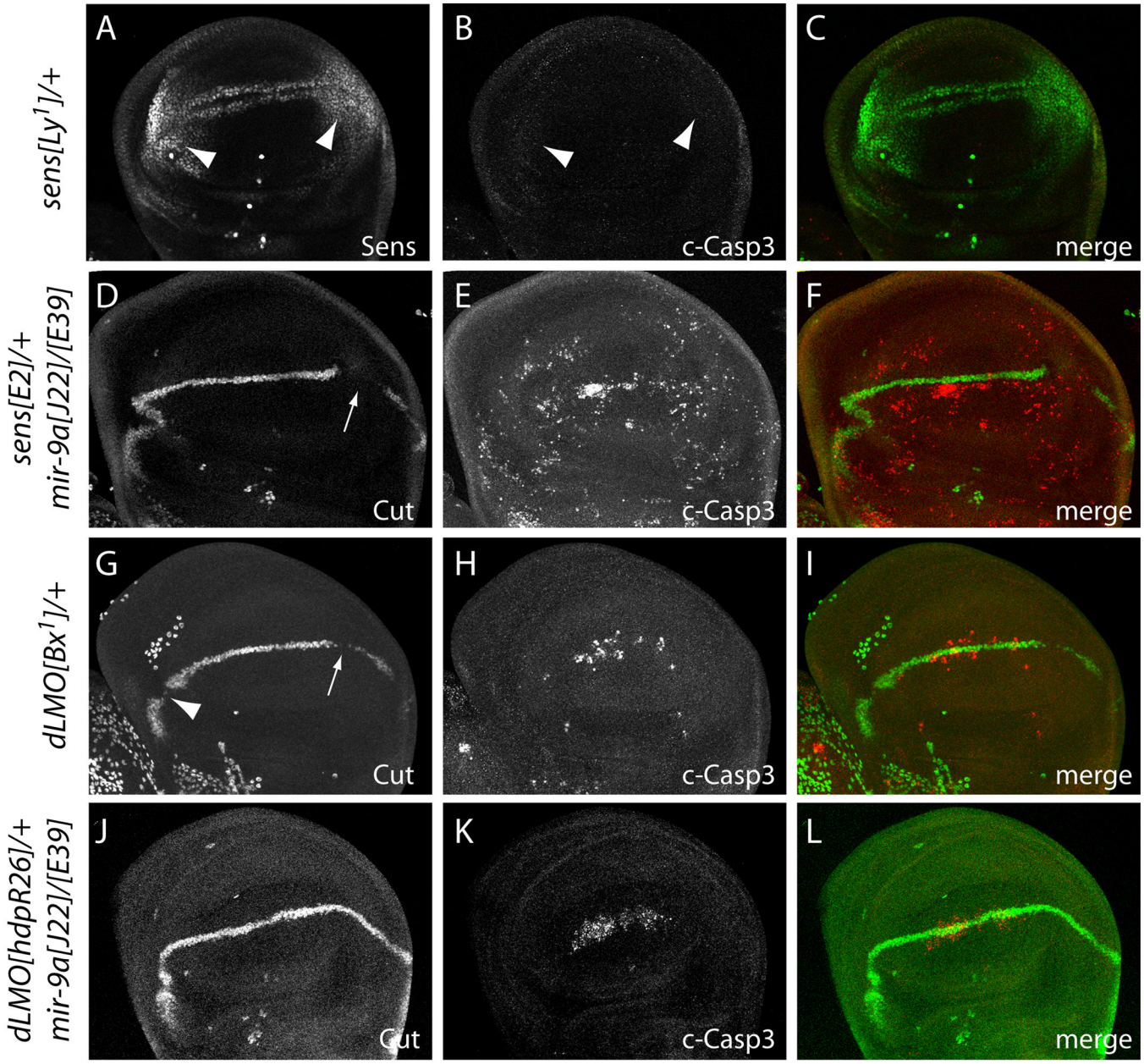

Figure 3.

Rescue of *mir-9a* phenotypes by heterozygosity for *dLMO*. (A–C) Strong ectopic accumulation of Sens in a heterozygote of the gain-of-function allele *Ly[1]* (A, arrowheads) is not associated with ectopic cell death (B, arrowheads). (D–F) Heterozygosity for the null allele *sens[E2]* did not rescue the Cut or apoptosis defects of the *mir-9a* mutant. (G–I) Female heterozygote of the dLMO gain-of-function allele *Bx[1]* exhibits breaks in wing margin expression of Cut and ectopic apoptosis, similar to *mir-9a* mutant discs (compare with Fig. 2G, I). (J–L) Female heterozygosity for the null allele *dLMO[hdpR26]* rescued continuity of Cut expression at the wing margin and strongly suppressed ectopic apoptosis caused by loss of *mir-9a*.

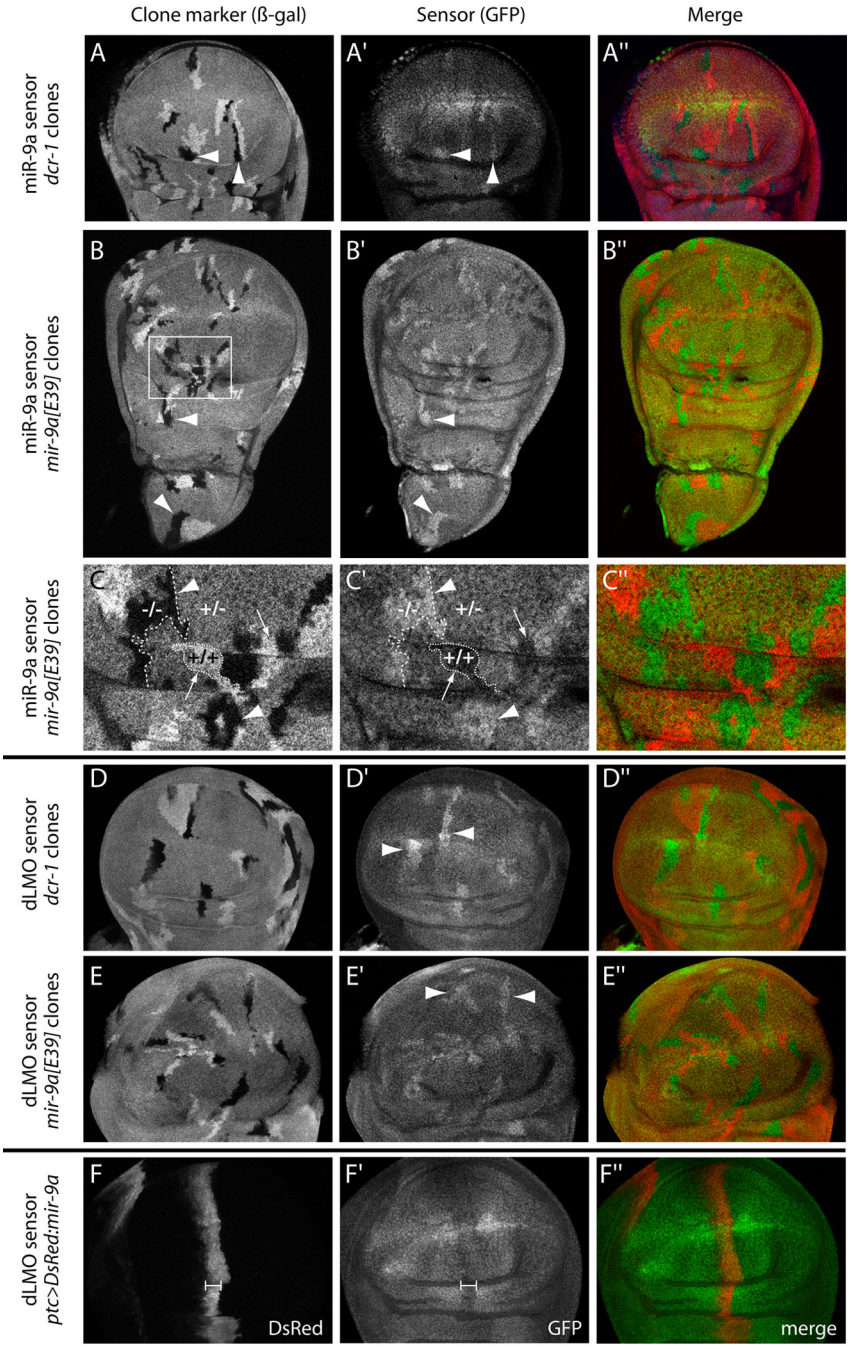

Figure 4.

dLMO is a direct in vivo target of miR-9a in the *Drosophila* wing disc. (A–A") *hs-FLP; tub-GFP-mir-9a/+; FRT82B, dcr-1[Q1147x]/FRT82B, arm-lacZ* disc carrying somatic clones marked by the absence of β-galactosidase (arrowheads). *dcr-1* homozygous mutant cells exhibit elevated levels of the miR-9a GFP sensor. (B–B") *hs-FLP; tub-GFP-mir-9a/+; mir-9a [E39], FRT80B/arm-lacZ, FRT80B* disc bearing β-gal-negative clones (arrowheads). *mir-9a* homozygous mutant cells exhibit increased levels of the miR-9a GFP sensor throughout the wing disc, in both the wing pouch and the presumptive notum. (C–C") Magnification of the region boxed in (B) highlights the sensitivity of the miR-9a sensor to *mir-9a* dosage. Homozygous mutant cells (−/−) exhibit highest sensor activity while homozygous wildtype

twinspots (+/+) exhibit lowest sensor activity; the remaining heterozygous (+/−) cells express an intermediate level of miR-9a sensor. (D–D") *hs-FLP; tub-GFP-dLMO 3' UTR/+, FRT82B, dcr-1[Q1147x]/FRT82B, arm-lacZ* disc; dLMO sensor is upregulated in *dcr-1* mutant cells. (E–E") *hs-FLP; tub-GFP-dLMO 3'UTR/+; mir-9a[E39], FRT80B/arm-lacZ, FRT80B* disc; dLMO sensor is upregulated in *mir-9a* mutant cells. (F–F") *ptc-Gal4, tub-GFP-dLMO 3' UTR; UAS-DsRed-mir-9a* disc; the dLMO sensor is suppressed in the domain of ectopic miR-9a (bracket).

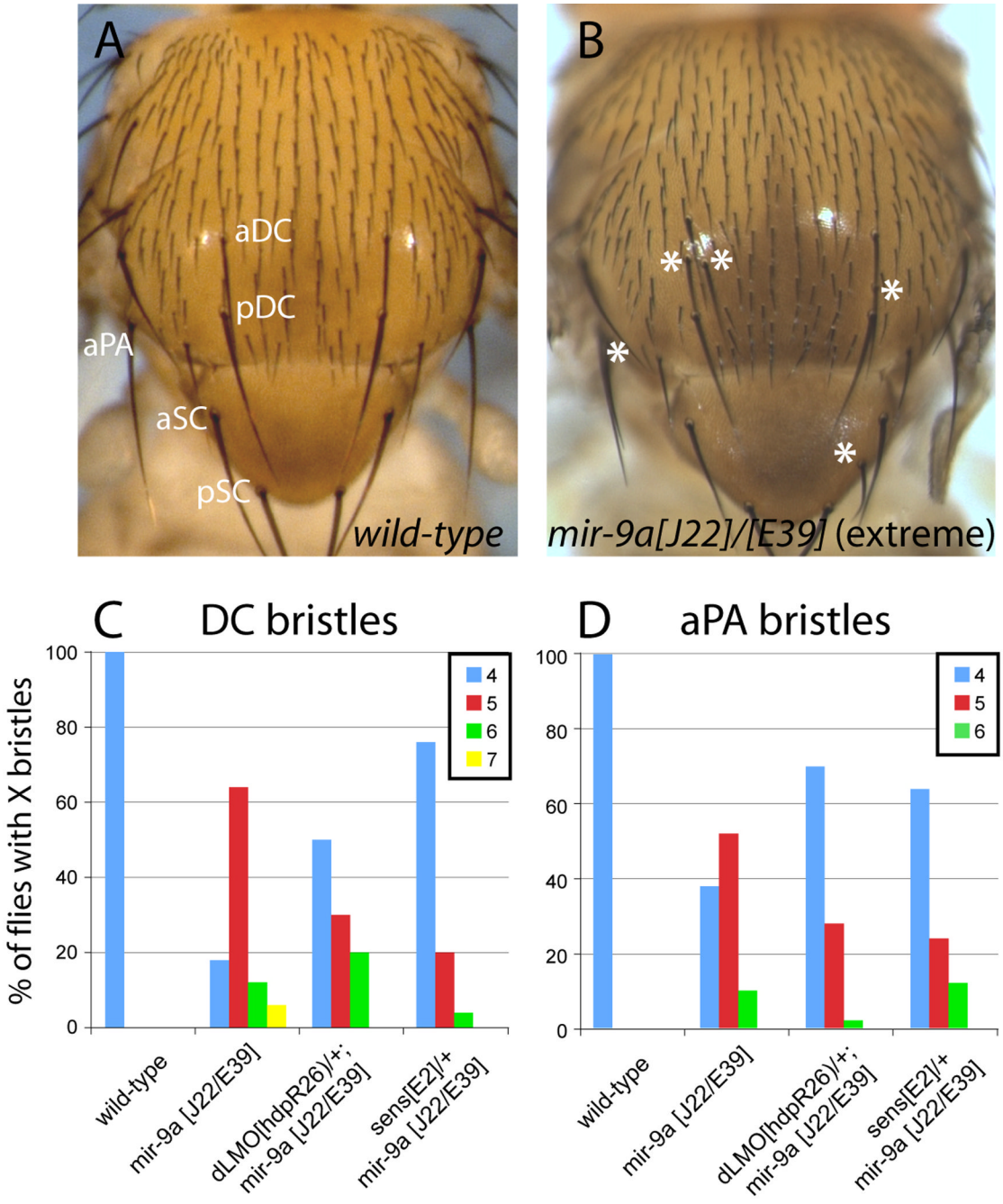

Figure 5.

Contribution of dLMO and Sens to ectopic neurogenesis in *mir-9a* mutants. (A) Notum of a wild-type animal shows an orderly array of microchaete sensory organ bristles and precise positioning of the larger macrochaete bristles; aDC and pDC: anterior and posterior dorsocentrals; aSC and pSC: anterior and posterior scutellars; aPA: anterior postalar bristle. (B) Notum of *mir-9a[J22]/[E39]* mutant exhibits slightly increased density of microchaete bristles and ectopic DC, aSC and aPA bristles. Note that an extreme phenotype is shown for illustration purposes; many *mir-9a* mutant animals exhibit no ectopic macrochaetes or only a single extra bristle across all positions. (C) Heterozygosity for *sens* substantially suppresses the ectopic DC phenotype of *mir-9a* mutants. (D) Heterozygosity for either *dLMO* or *sens*

partially suppressed the ectopic aPA phenotype of *mir-9a* mutants. Bristle counts were performed for 50 females in each genotype.

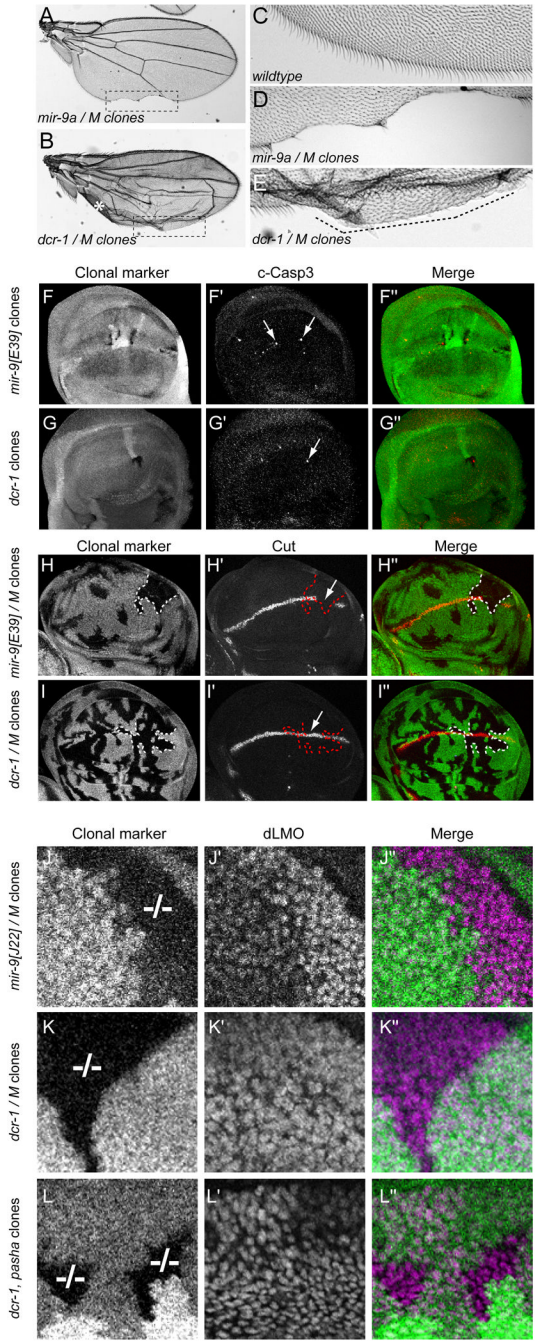

Figure 6.

dcr-1 and *pasha* clones do not phenocopy *mir-9a* clones. (A) Wing carrying large *mir-9a [E39]* clones induced with *hs-FLP* and the *Minute* technique exhibit notching along the posterior wing margin. (B) Wing carrying large *dcr-1[Q1147x]* mutant clones induced with *hs-FLP* and the *Minute* technique. The uneven surface of the wing prevented a flat mounting, and the asterisk indicates a section of the wing that folded over on itself. Nevertheless, no loss of margin tissue was observed. (C) Closeup of the wildtype posterior wing margin highlights a regular array of non-sensory bristles. (D) Magnification of the posterior wing margin in panel A (boxed region) illustrates substantial loss of tissue in wing bearing *mir-9a* clones. (E) Magnification of the posterior margin of the wing bearing *dcr-1* clones in panel B (boxed

region); the differentiation of posterior wing margin bristles is highly disturbed (dotted line), but the margin remained continuous. (F–L) Clonal analysis of wing pouch development. Mutant clones are marked by their absence of β-gal or GFP in the "clonal marker" channel; antigens of interest are shown in F'–L', and merged images are shown in F"–L". Panels F, G, I, K and L depict *lacZ* clonal markers while panels H and J used GFP markers; the choice of marker did not affect the results. The *Minute* technique ("*M*" genotype) was used in panels H, I, J and K. (F–G) Staining for cleaved caspase-3 in *mir-9a* and *dcr-1* clones showed that both exhibit apoptotic cells. (H–I) *mir-9a/M* clones that overlap the middle region of the posterior wing margin reliably showed a break in Cut expression (H', arrow), whereas similarly positioned *dcr-1/M* clones frequently maintained Cut expression (I', arrow). (J–L) dLMO staining of ventral wing pouch clones of *mir-9*, *dcr-1* and *pasha* mutant alleles; these are shown at higher magnification than panels F–I. Homozygous mutant clones are indicated by "−/− ". (J) *mir-9a[E39]/M* clones reliably exhibit strong upregulation of dLMO in the ventral wing pouch. (K) *dcr-1[Q1147x]/M* ventral wing clones do not upregulate dLMO. (L) *dcr-1 [Q1147x]*, *pasha[KO]* double mutant ventral clones express normal levels of dLMO.