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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 *Caenorhabditis 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, 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 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

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~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 *Not*I and *Xho*I restriction sites of *tub-GFP-SV40* (gift of Julius Brennecke and Stephen Cohen). miR-9a sensor F Not: GGCCTCATACAGCTAGATAACCAAAGAAATCACACTCATACAGCTAGATAACCAAAGA; 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-β-galactosidase (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 (Figs. 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 (Figs. 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 (Figs. 2B, G). These patterns were consistent with the observed penetrance of adult wing notching (Figs. 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 (Figs. 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 pronotum 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 (Figs. 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