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**Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis**

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The *Drosophila* let-7-Complex (let-7-C) is a polycistronic locus encoding three ancient microRNAs: let-7, miR-100, and fly lin-4 (miR-125). We find that the let-7-C locus is principally expressed in the pupal and adult neuromusculature. let-7-C knockout flies appear normal externally but display defects in adult behaviors (e.g., flight, motility, and fertility) as well as clear juvenile features in their neuromusculature. We find that the function of let-7-C to ensure the appropriate remodeling of the abdominal neuromusculature during the larval-to-adult transition is carried out predominantly by let-7 alone. This heterochronic role of let-7 is likely just one of the ways in which let-7-C promotes adult behavior.

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Mutations in heterochronic genes in *Caenorhabditis elegans* cause cells in particular lineages to express their stage-specific fates earlier or later than normal [Ambros and Horvitz 1984]. Detailed analysis of these genes has revealed a regulatory pathway of heterochronic genes that specifies the timing of cellular development in diverse cell types and thereby ensures a coordinated schedule of developmental events throughout the worm [for review, see Rougvie 2005; Moss 2007]. The existence of the heterochronic gene pathway in worms and the conservation of some of its components through animal evolution suggest that functionally analogous pathways could also coordinate developmental timing in higher organisms (Pasquinelli et al. 2000). Two of these highly conserved components of the heterochronic pathway, let-7 and lin-4, are microRNAs [miRNAs], a class of small RNAs that post-transcriptionally modulate the expression of target transcripts [for review, see Jackson and Standart 2007]. The sequences and developmentally regulated expression profiles of let-7 and lin-4 are conserved among diverse bilaterians [Pasquinelli et al. 2000; Sempere et al. 2003]. For example, *Drosophila* let-7 and miR-125 (fly lin-4) are robustly up-regulated during metamorphosis, as is another highly conserved miRNA, miR-100 [Pasquinelli et al. 2000; Sempere et al. 2002, 2003; Bashirullah et al. 2003]. All three of these ancient miRNAs are encoded in a 1-kb region of the *Drosophila* genome [Fig. 1; Sempere et al. 2003], and their clustered organization has been conserved and duplicated in vertebrates [Supplemental Fig. S1; Sempere et al. 2003; Prochnik et al. 2007]. These findings suggest that miR-100, let-7, and miR-125 coordinately control gene expression to regulate developmental timing in animals. To test this hypothesis, we analyzed the roles of miR-100, let-7, and miR-125 in *Drosophila* and find that these miRNAs are required for normal adult behavior, suggesting roles in neural development and/or function. let-7 in particular is required for remodeling of the fly neuromusculature during the larval-to-adult transition, confirming that a general developmental timing function of let-7 has been evolutionarily conserved from worms to flies.

**Results and Discussion**

The clustered organization of *Drosophila* miR-100, let-7, and miR-125 suggests that these miRNAs are co-transcribed as a single polycistronic transcript. To test this hypothesis, we isolated cDNAs generated from genomic regions between miR-100 and let-7 and between let-7 and miR-125 using 5' and 3' rapid amplification of cDNA ends (RACE). This analysis identified two overlapping cDNA fragments that corresponded to a 2435-nucleotide [nt] primary transcript that encoded the ~70-nt hairpin sequences of miR-100, let-7, and miR-125, and was comprised of three exons that spanned 17,400 kb of genomic DNA [Fig. 1A]. We conclude that miR-100, let-7, and miR-125 are cotranscribed from a single locus, which we refer to as the let-7-Complex (let-7-C) since let-7 was the first of these miRNAs identified in *Drosophila* [Pasquinelli et al. 2000]. We infer that the miR-100, let-7, and miR-125 clusters in the genomes of other animals [Supplemental Fig. S1] also represent single polycistronic loci. It should be noted that cotranscribed let-7-C miRNAs may not always be coexpressed, given that post-transcriptional processing of mature miRNAs from primary transcripts can be subject to developmental regulation [Thomson et al. 2006; Wulczyn et al. 2007; Viswanathan et al. 2008].

To investigate whether let-7-C miRNAs collectively regulate developmental timing in *Drosophila*, we generated two independent let-7-C knockout strains, let-7-C\(^{C\text{KO}}\) and let-7-C\(^{C\text{GKI}}\) [Fig. 1B,C; Supplemental Fig. S2]. Both strains lack expression of the mature processed forms of miR-100, let-7, and miR-125 [Fig. 1B,C]. To reduce the potentially complicating effects of genetic background on our study, we analyzed the let-7-C\(^{C\text{KO}}\) and let-7-C\(^{C\text{GKI}}\) knockout alleles in *trans* and refer to this trans-heterozygous let-7-C\(^{C\text{KO/GKI}}\) null strain as let-7-C\(^{C\text{KO/GKI}}\). We found that ~43% of let-7-C\(^{C\text{KO/GKI}}\) animals died prematurely during the course of development, with the majority [74%] of these arresting at the very end of meta-
Figure 1. *Drosophila* *let*-7-*C* locus, knockouts, and rescuing transgenes. (A) The *Drosophila* *let*-7-*C* locus, located at cytological location 36E on chromosome 2, encodes a 2435-nt primary transcript containing three evolutionarily conserved miRNAs: miR-100, *let*-7, and miR-125. (B) The *let*-7-*CGI* and *let*-7-*CGK* mutations contain 1071- and 991-base-pair (bp) deletions respectively, removing miR-100, *let*-7, and miR-125. The CG10283 gene, located proximally to *let*-7-*C*, is also disrupted by the *let*-7-*CGK* mutation. The *let*-7-*CGK* mutation contains Gal4 coding sequences driven by the *let*-7-*C* locus. Derivative rescue constructs rescue *let*-7-*C* null function. (C) Expression of *let*-7-CKO1, *let*-7-CGKI, and derivative rescuing transgenes. (D) Expression of miR-100, *let*-7, and/or miR-125 RNA is eliminated in the *let*-7-*CGI*, *let*-7-*CGK*, and *let*-7-*CGKI/GKI* strains and is restored by P*W8*, *let*-7-*C* and derivative rescuing transgenes. Samples of total RNA from 1-d-old male flies of the following genotypes were analyzed by Northern blot: wild type in lane 1, *let*-7-*CGK* in lane 2, *let*-7-*CGKI* in lane 3, *let*-7-*CGKI/GKI* in lane 4, *let*-7-*CGI/GKI* in lane 5, *let*-7-*CGKI* in lane 6, *let*-7-*CGIK* in lane 7, *let*-7-*CGIK/GKI* in lane 8, and *p*UAS-*let*-7-*C*; *let*-7-*CGIK/GKI* in lane 9. Northern blots were probed for miR-100, *let*-7, and miR-1*F* RNAs, and miR-1 expression was used as a loading control.

morphosis. The remaining 57% of *let*-7-*CGI/GKI* mutants eclosed as adults, but displayed chronic defects in adult function, including severely reduced motility, flight, and fertility (Fig. 2). *let*-7-*CGI/GKI* mutants that carried a transgene that restored *let*-7-*C* miRNA expression were fully rescued for developmental viability and adult functions (Fig. 2; data not shown). Despite their developmental and behavioral defects, *let*-7-*CGI/GKI* mutant pupae and adults appeared morphologically normal (Supplemental Fig. S3), indicating that *let*-7-*C* miRNAs are not required for the morphogenesis of the adult exterior. These data indicated that *let*-7-*C* expression is predominantly required for adult behavior and are consistent with the hypothesis that *let*-7-*C* miRNAs play an essential role in regulating the developmental remodeling of internal tissues during metamorphosis.

To test whether the activity of each of the *let*-7-*C* miRNAs is required for *let*-7-*C* function, we analyzed the phenotypes of three different *let*-7-*C* derivative strains in which the expression of miR-100, *let*-7, or miR-125 had been eliminated individually (Fig. 1C; Supplemental Material). We refer to these singly mutant strains as miR-100, *let*-7, and miR-125, respectively. miR-100 mutants functioned normally in all behavioral assays (Fig. 2), indicating that miR-100 was not solely responsible for any of the identified *let*-7-*C* functions. None of the single mutant strains displayed strong male fertility or climbing defects [Fig. 2D], suggesting that for normal male fertility and climbing behavior, the combinatorial action of any two *let*-7-*C* microRNAs could suffice. In contrast, *let*-7* and miR-125 mutants displayed severely reduced spontaneous locomotion as well as partial defects in flight (Fig. 2A–C). The normal climbing and nearly normal flight of *let*-7* and miR-125* mutants suggested that their severely impaired spontaneous locomotory activity was not simply the consequence of physically or metabolically impaired mobility, but rather likely reflected a behavioral deficit of neurological origin. Finally, *let*-7* mutants alone displayed moderately severe defects in female fertility and oviposition [Fig. 2E,F], indicating that *let*-7* was required for an essential function to promote female reproductive behavior.

To identify the specific place where *let*-7-*C* miRNAs may function to promote adult behavior, we examined the spatiotemporal expression pattern of the *let*-7-*C* locus. We used the *let*-7-*C* strain, in which the yeast
transcriptional activator Gal4 had been inserted into the let-7-C locus [Fig. 1B], to drive expression of Gal4-dependent transgenes encoding membrane-bound or nuclear forms of GFP. A UAS-let-7-C transgene placed under the control of the let-7-C::Gal4 insertion restored miR-100, let-7, and miR-125 expression [Fig. 1D, lane 9] as well as climbing activity [Fig. 3A] to let-7-CCKO1/GKI mutants. Three characteristics of the let-7-C::Gal4 expression pattern are outlined below. First, let-7-C::Gal4 was expressed in neurons that innervated structures throughout the adult (Fig. 3C), including sensory organs in the head, flight muscles in the thorax, and the alimentary tract, the male and female reproductive tracts, and the male and female genitalia in the abdomen (data not shown). We noted that let-7-C::Gal4 was very densely expressed in the posterior tip of the adult abdominal ganglion (Fig. 3B), as well as in motoneurons that projected posteriorly and innervated two distinct sets of abdominal muscles, the dorsal internal oblique muscles (DIOM) and the dorsal muscles (DM) [Fig. 3C]. The DIOMs are remnants of the larval body wall that persist through metamorphosis (presumably to function in the process of eclosion) and in the wild type are fated to die within 12 h of eclosion (Crossley 1978; Kimura and Truman 1990). In contrast, the DMs are the adult body-wall muscles and are derived from larval myoblasts that undergo myogenesis during metamorphosis [Miller 1950; Currie and Bate 1991, 1995]. Third, let-7-C::Gal4 was not only expressed in motoneurons but in muscle cells as well, including the DIOMs and DMs [Fig. 3E, data not shown]. Taken together, the expression of let-7-C::Gal4 in pupal and adult neurons and muscles is consistent with the hypothesis that the behavioral phenotypes of let-7-C mutant adults are the consequence of defects in the metamorphosis of the neuromusculature.

To test whether let-7-C miRNAs play a role in specifying the configuration of the adult neuromusculature, we examined the abdominal muscle system of let-7-CCKO1/GKI mutants since, as shown above, let-7-C is expressed in abdominal motoneurons and muscles. We found two very clear and highly penetrant defects [Fig. 4A,B]. First, the DIOMs that ordinarily decay during post-eclosion maturation of wild-type flies failed to disappear in older let-7-CCKO1/GKI mutants [Fig. 4A,B]. We quantified this phenotype by scoring the presence of six DIOMs in aged wild-type and let-7-CCKO1/GKI mutant flies. Two-day-old wild-type males (n = 10) retained none of these DIOMs, whereas 2-d-old let-7-CCKO1/GKI males (n = 10) retained 89.9% ± 11.7% of these DIOMs. Nineteen-day-old male let-7-CCKO1/GKI mutants (n = 6), the oldest cohort of let-7-CCKO1/GKI mutants examined, retained 91.5% ± 9.3% of these DIOMs. Second, the DMs of let-7-CCKO1/GKI mutant adults were clearly smaller than those in age-matched wild-type controls [Fig. 4A–D]. We quantified this phenotype by measuring the width and number of nuclei present in a set of approximately six to
Figure 4. The adult neuromusculature of let-7-C\textsuperscript{CKO1/GKI} mutants display persistent pupal as well as immature adult characteristics. [A,B] Dorsal sections of abdominal segments 3–5 (A3-A5) from 2-d-old wild-type [A] and let-7-C\textsuperscript{CKO1/GKI} mutant [B] males stained for rhodamine phalloidin. The dorsal vessel (dv), which runs in an anterior–posterior orientation along the dorsal midline of the abdominal cavity, bisects the segments. [B] In the let-7-C\textsuperscript{CKO1/GKI} mutant, a persistent DIOM is seen in each hemisegment [white asterisks]. [A,B] In addition, let-7-C\textsuperscript{CKO1/GKI} mutant DMs are smaller than wild type [arrowheads]. Male-specific muscles are apparent in A5 hemisegments in both wild type and mutant [black asterisks]. [C,D] Adult let-7-C\textsuperscript{CKO1/GKI} mutant DMs and DM neuromuscular junctions (NMJs) do not grow to wild-type size. DMs from 2-d-old let-7-C\textsuperscript{CKO1/GKI}, P[UAS-nls-GFP]/+ [A and let-7-C\textsuperscript{CKO1/GKI}, P[UAS-nls-GFP]/+ [B] and let-7-C\textsuperscript{CKO1/GKI}, P[UAS-mCD8-GFP]/+ [C] and let-7-C\textsuperscript{CKO1/GKI}, P[UAS-mCD8-GFP] [D] males triple-labeled for rhodamine phalloidin [red], GFP [green], and HRP [blue]. Rhodamine phalloidin-only, GFP-only, and HRP-only channels are also shown. let-7-C\textsuperscript{CKO1/GKI} mutant DMs are narrower [brackets], contain fewer nuclei, and are contacted by shorter NMJs [arrowheads]. [E,F] let-7-C mutant DMs and innervating neurons persist into adulthood. DIOMs from 0-h-old let-7-C\textsuperscript{CKO1/GKI}, P[UAS-mCD8-GFP]/+ [E] and 12-h-old let-7-C\textsuperscript{CKO1/GKI}, P[UAS-mCD8-GFP]+ [F] males double-labeled for rhodamine phalloidin [purple] and GFP [green]. GFP-only channels are also shown. Bars: A,B, 100 µm; C,D, 10 µm; E,F, 50 µm.

eight distinct DMs close to the dorsal vessel per A4 hemisegment per 2-d-old wild-type (n = 7) or let-7-C\textsuperscript{CKO1/GKI} (n = 7) male. Wild-type DMs were 20.2 ± 2.7 µm in width and contained 13.9 ± 1.2 nuclei, whereas let-7-C\textsuperscript{CKO1/GKI} DMs were 12 ± 1.6 µm in width and contained 10.6 ± 0.8 nuclei [Fig. 4C,D]. Restoration of let-7-C expression rescued both the DIOM and DM phenotype; let-7-C\textsuperscript{CKO1/GKI} mutants that carried the let-7-C transgene (n = 6) retained none of the six DIOMs scored above, and their DMs were 16.5 ± 1.4 µm in width and contained 12.8 ± 0.6 nuclei. To test whether let-7-C\textsuperscript{CKO1/GKI} mutant muscle phenotypes were the consequence of defects apparent prior to the onset of metamorphosis, we examined the musculature and myoblasts of let-7-C\textsuperscript{CKO1/GKI} larvae and found that both appeared normal [Supplemental Fig. S4]. We therefore concluded that the abdominal muscle system of let-7-C\textsuperscript{CKO1/GKI} mutant adults failed to complete its larval-to-adult remodeling, displaying both persistent pupal as well as immature adult characteristics. We interpreted this as a heterochronic phenotype, since let-7-C\textsuperscript{CKO1/GKI} mutant adults exhibited both juvenile features [e.g., muscle system morphology] as well as mature adult traits [e.g., external appearance] at the same time.

To test whether let-7-C affects the remodeling of other internal tissues, we examined the morphogenesis of the CNS during metamorphosis in let-7-C\textsuperscript{CKO1/GKI} mutants and found that at a gross level, CNS development appeared to have proceeded normally [data not shown]. To examine the results of nervous system remodeling in finer detail, we focused on the morphology of motoneurons that innervate the DIOMs or the DMs. DIOMs are innervated by DIOM motoneurons, which also degenerate after eclosion. The DIOMs and their DIOM motoneurons, however, are triggered to die at different times and therefore may be controlled by independent signals [Kimura and Truman 1990]. Interestingly, we found that the neuromuscular junctions [NMJs] connecting DIOMs and their innervating motoneurons failed to decay in let-7-C\textsuperscript{CKO1/GKI} mutant adults [Fig. 4 E,F], indicating that the DIOMs and DIOM motoneurons persisted together. These data suggested that let-7-C functioned to coordinate the fates of DIOMs and DIOM motoneurons. Similarly, the reduced size of let-7-C\textsuperscript{CKO1/GKI} mutant DMs was reflected in clear defects in let-7-C\textsuperscript{CKO1/GKI} mutant DM NMJs, which were either completely absent, shorter in length than wild-type NMJs, or devoid of boutons, appearing as long, thin processes along the length of the DM [Fig. 4C,D]. To quantify this phenotype, we measured the length of DM NMJs containing boutons in the same set of approximately six to eight DM muscles of wild-type (n = 7), let-7-C\textsuperscript{CKO1/GKI} (n = 7), and rescued let-7-C\textsuperscript{CKO1/GKI} (n = 6) flies; wild-type NMJs were 55 ± 7.8 µm in length, let-7-C\textsuperscript{CKO1/GKI} mutant NMJs were 11.2 ± 1.6 µm in length, and rescued let-7-C\textsuperscript{CKO1/GKI} mutant NMJs were 60.5 ± 6.4 µm in length. We concluded that the heterochronous abdominal muscle defect was reflected in a corollary nervous system defect, supporting the hypothesis that disruption of neuromusculature remodeling could underlie at least some of the let-7-C\textsuperscript{CKO1/GKI} mutant behavioral phenotypes.

We note the striking similarity between let-7-C\textsuperscript{CKO1/GKI} mutant phenotypes and the phenotypes associated with manual denervation of abdominal muscles prior to metamorphosis, reported by Currie and Bate in 1995. In both cases, adult DM muscles fail to grow to wild-type width, contain fewer nuclei, and display aberrant NMJs. However, the let-7-C mutation and denervation differ in at least one respect: their effect on the male-specific muscle of Lawrence [MOL] [Lawrence and Johnston 1986]. MOLs are present in let-7-C\textsuperscript{CKO1/GKI} adult males but absent in manually denervated adult males [Supplemental Fig. S5; Currie and Bate 1995]. Interestingly, Currie and Bate did not report the persistence of DIOMs in denervated adults, which could mean either that DIOM degeneration is unaffected by denervation or that DIOMs degenerate precociously when denervated and were therefore not observed. In either case, the overall simi-
The functional dissection of *Drosophila* let-7-C presented here indicates that let-7-C is required for adult behavior and that defects in neuromusculature remodeling correlate with some aspects of this requirement. We note that the perdurance of juvenile features in adult *Drosophila* let-7 mutants is analogous to the reiteration of larval cell fates in adult *Caenorhabditis elegans* let-7 mutants [Reinhart et al. 2000], confirming the suggestion by Pasquinelli et al. in 2000 that let-7 might control developmental transitions in diverse bilateria (Pasquinelli et al. 2000). Future work in flies should extend this analysis to identify the relevant mRNA targets that *Drosophila* let-7 regulates in its heterochronic role and to examine how this heterochronic function is integrated into the more general requirements of the let-7-C locus in promoting adult behavior. For the most part, the set of targets predicted for *Drosophila* let-7 are distinct from those predicted for *C. elegans* let-7 [Grun et al. 2005; Lall et al. 2006]. Our unpublished observations indicate that one of *Drosophila* let-7’s targets is the transcription factor *abrupt* [Hu et al. 1995], although we also find that ectopic expression of *abrupt* in a let-7-CGal4-driven pattern is not sufficient to recapitulate the let-7-C phenotype. The conservation of the genomic clustering as well as neuronal expression of let-7, mir-125, and mir-100 from flies to vertebrates [Supplemental Fig. S1; Wienholds et al. 2005; Ason et al. 2006; Wulczyn et al. 2007] suggests that let-7-C loci could function in neuromuscular and/or neuronal remodeling in mammals. Future work on let-7-C should reveal how its diverse effects on temporal cell fates, developmental timing, and neuronal remodeling are related.

**Materials and methods**

*Drosophila* strains and genetics

Fly stocks were maintained at 25°C on standard media on a 12-h light, 12-h dark cycle. *Canton S* and/or *w^1118* stocks were used as wild-type controls. Transgenic animals were generated using standard methods. Detailed descriptions of methods used to generate let-7-C mutant flies can be found in the Supplemental Material.

**Histology**

Adult brains or abdomens were fixed in 4% paraformaldehyde for 1 h or 15 min, respectively. Samples were washed in PBT, blocked for 1 h with 5% goat serum in PBT, and incubated overnight with primary antibodies, including rabbit anti-GFP [1:1000; Molecular Probes], mouse anti-ncl2 [1:10; Developmental Studies Hybridoma Bank], rabbit anti-twist [1:4000; Siegfried Roth], and rabbit anti-HRP [1:500; Jackson Laboratories]. Samples were washed in PBT and incubated with AlexaFluor 488 or 568 conjugated anti-mouse or anti-rabbit secondary antibodies [Invitrogen] and/or additional stains, including rhodamine phallolidin [1:1000; Sigma], Cy5 conjugated anti-HRP antibodies [1:200; Jackson Labs], and DAPI [1:10,000; Molecular Probes]. Samples were washed and mounted in Vectashield (Vector Laboratories). Images were collected on a Leica confocal microscope.

The cDNA sequence of *pri-let-7-C* has been deposited in GenBank under accession number EU624487. Complete methods can be found in the Supplemental Material.

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