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

Nicholas S. Sokol  
*Dartmouth College*

Peizhang Xu  
*Howard Hughes Medical Institute*

Yuh-Nung Jan  
*Howard Hughes Medical Institute*

*See next page for additional authors*

Follow this and additional works at: [https://escholarship.umassmed.edu/pmm_pp](https://escholarship.umassmed.edu/pmm_pp)

Part of the Biochemistry Commons, Developmental Biology Commons, Molecular Biology Commons, and the Molecular Genetics Commons

Repository Citation
Sokol, Nicholas S.; Xu, Peizhang; Jan, Yuh-Nung; and Ambros, Victor R., "Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis" (2008). *Program in Molecular Medicine Publications and Presentations*. 37.
[https://escholarship.umassmed.edu/pmm_pp/37](https://escholarship.umassmed.edu/pmm_pp/37)

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Program in Molecular Medicine Publications and Presentations by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis

Authors
Nicholas S. Sokol, Peizhang Xu, Yuh-Nung Jan, and Victor R. Ambros

Keywords
let-7, let-7-Complex, microRNA, heterochronic developmental timing, neuromuscular junction

Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial 4.0 License

Rights and Permissions
Publisher PDF posted as allowed by the publisher’s author rights policy at http://genesdev.cshlp.org/site/misc/terms.xhtml.

This article is available at eScholarship@UMMS: https://escholarship.umassmed.edu/pmm_pp/37
**Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis**

Nicholas S. Sokol,1,3,6 Peizhang Xu,2 Yuh-Nung Jan,2 and Victor Ambros1,4,5

1Department of Genetics, Dartmouth Medical School, Hanover, New Hampshire 03755, USA; 2Howard Hughes Medical Institute, Department of Physiology, and Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143, USA

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.

Supplemental material is available at http://www.genesdev.org.

Received March 10, 2008, revised version accepted April 11, 2008.

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<sup>C<sub>KO1</sub></sup>* and *let-7-C<sup>C<sub>KO2/GKI</sub></sup>* [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<sup>C<sub>KO1</sub></sup>* and *let-7-C<sup>C<sub>KO2/GKI</sub></sup>* knockout alleles in *trans* and refer to this trans-heterozygous *let-7-C*-null strain as *let-7-C<sup>C<sub>KO1/GKI</sub></sup>*. We found that ~43% of *let-7-C<sup>C<sub>KO1/GKI</sub></sup>* animals died prematurely during the course of development, with the majority (74%) of these arresting at the very end of meta-

---

**Keywords:** *let-7*, *let-7-Complex*, microRNA, heterochronic, developmental timing, neuromuscular junction

Present addresses: 1Indiana University, 1001 E. 3rd St., Jordan Hall A502, Bloomington, IN 47405, USA; 2University of Massachusetts Medical School, Program in Molecular Medicine, 373 Plantation St., Two Biotech, Suite 306, Worcester, MA 01605, USA. Corresponding authors.

<sup>3</sup>E-MAIL rambros@gmail.com; FAX (508) 856-5657.

<sup>6</sup>E-MAIL nsokol@indiana.edu; FAX (812) 855-6082.

Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1671708.
The remaining 57% of let-7-CKO1/GKI mutants eclosed as adults, but displayed chronic defects in adult function, including severely reduced motility, flight, and fertility (Fig. 2). let-7-CKO1/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-CKO1/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-100Δ, let-7Δ, and miR-125Δ, respectively. miR-100Δ 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-7Δ and miR-125Δ 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-7Δ and miR-125Δ 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-7Δ mutants alone displayed moderately severe defects in female fertility and oviposition [Fig. 2E,F], indicating that let-7Δ was required for an essential function to promote female reproduction.

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-CGR1 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-C/let-7-C 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-C KO/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-C KO/GKI mutants (Fig. 4A,B). We quantified this phenotype by scoring the presence of six DIOMs in aged wild-type and let-7-C KO/GKI mutant flies. Two-day-old wild-type males (n = 10) retained none of these DIOMs, whereas 2-d-old let-7-C KO/GKI males (n = 10) retained 89.9% ± 11.7% of these DIOMs. Nine-day-old male let-7-C KO/GKI mutants (n = 6), the oldest cohort of let-7-C KO/GKI mutants examined, retained 91.5% ± 9.3% of these DIOMs. Second, the DMs of let-7-C KO/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
eight distinct DMs close to the dorsal vessel per A4 hemi-segment per 2-d-old wild-type (n = 7) or let-7-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-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-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-CKO1/GKI mutant muscle phenotypes were the consequence of defects apparent prior to the onset of metamorphosis, we examined the muscleature and myoblasts of let-7-CKO1/GKI larvae and found that both appeared normal [Supplemental Fig. S4]. We therefore concluded that the abdominal muscle system of let-7-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 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-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-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. Similarily, the reduced size of let-7-CKO1/GKI mutant DMs was reflected in clear defects in let-7-C 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-CKO1/GKI (n = 7), and rescued let-7-CKO1/GKI (n = 6) flies; wild-type NMJs were 55 ± 7.8 µm in length, let-7-CKO1/GKI mutant NMJs were 11.2 ± 1.6 µm in length, and rescued let-7-CKO1/GKI mutant NMJs were 60.5 ± 6.4 µm in length. We concluded that the heterochronic 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 mutant behavioral phenotypes.

We note the striking similarity between let-7-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 mutant adults 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-
larity between the effects of genetic depletion of let-7-C and muscle denervation during metamorphosis supports the hypothesis that let-7-C is required to regulate an interaction between muscles and motorneurons during neuromusculature remodeling. To test whether the activities of miR-100, let-7, or miR-125 are required individually for neuromusculature remodeling, we examined the abdominal muscle pattern as well as DM NMJs in miR-100\textsuperscript{+}, let-7\textsuperscript{+}, and miR-125\textsuperscript{+} single mutants (Fig. 5). We found that 2-d-old miR-100\textsuperscript{+} (n = 6) and miR-125\textsuperscript{+} (n = 7) males retained none of the six DIOMs, while let-7\textsuperscript{+} males (n = 7) retained 61% ± 28.5% of DIOMs. Although the frequency of complete DIOM retention is lower in let-7\textsuperscript{+} mutants compared to let-7-C\textsuperscript{KO/KR} mutants, we noted that 83% ± 25% of let-7\textsuperscript{+} mutant DIOMs had arrested at some stage in the process of degeneration. With respect to both the DM and DM NMJ phenotype, we similarly found that miR-100\textsuperscript{+} (n = 5) and miR-125\textsuperscript{+} (n = 6) mutants appeared normal, whereas let-7\textsuperscript{+} mutants (n = 6) phenocopied let-7-C\textsuperscript{KO/KR} mutants. miR-100\textsuperscript{+} and miR-125\textsuperscript{+} DMs were 18.3 ± 0.8 µm and 16.8 ± 0.8 µm in width, respectively, while let-7\textsuperscript{+} DMs were 12 ± 1.8 µm in length (Fig. 5A–C). Similarly, miR-100\textsuperscript{+} and miR-125\textsuperscript{+} NMJs were 45.9 ± 10.2 µm and 55.1 ± 13.3 µm in length, respectively, while let-7\textsuperscript{+} NMJs were 12.5 ± 5 µm in length (Fig. 5D,E). For the sake of consistency, all the morphological data quantified in this study were collected from adult males. However, let-7-C\textsuperscript{KO/KR} and let-7\textsuperscript{+} mutant females exhibited DM and DIOM phenotypes identical to their male siblings (data not shown), suggesting that the reduced egg-laying displayed by let-7\textsuperscript{+} mutant females (Fig. 2F) might be a consequence of defects in their abdominal neuromusculature. From these data, we concluded that the activity of let-7 alone was predominantly responsible for let-7-C-dependent remodeling of the abdominal neuromusculature, and therefore that a heterochronic let-7 role in regulating developmental transitions had been evolutionarily conserved from worms to flies.

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 retention 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 bilaterians ([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; Laß 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-C::Gal4-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 *w1118* 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.

**Histochemistry**

Adult brains or abdomens were fixed in 4% paraformaldehyde for 1 h or 12-h dark cycle. * Canton S* and/or *w1118* 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.

**Acknowledgments**

We thank the Developmental Studies Hybdridoma Bank, Yang Hong, and Jeff Sekelsky for reagents; members of the Ambros lab for illuminating discussions; and Yashi Ahmed and Claudio Pikielny for use of their fly room and injection facility. N.S.S. was supported by a postdoctoral fellowship from the Damon Runyon Cancer Research Fund (1729-02) and an equipment grant from the Hitchcock Foundation. P.X. was supported.
by a grant from the National Institute of Health (ROI NS40929) to Y.N.J., who is an HHMI investigator. The Drosophila project in V.A.’s lab was supported by a National Institute of Health grant (GM066826).

References


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

Nicholas S. Sokol, Peizhang Xu, Yuh-Nung Jan, et al.

Genes Dev. 2008 22: 1591-1596
Access the most recent version at doi:10.1101/gad.1671708

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2008/06/11/22.12.1591.DC1.html

References
This article cites 22 articles, 10 of which can be accessed free at:
http://genesdev.cshlp.org/content/22/12/1591.full.html#ref-list-1

Articles cited in:
http://genesdev.cshlp.org/content/22/12/1591.full.html#related-urls

Related Content
A matter of timing: microRNA-controlled temporal identities in worms and flies
Manfred Frasch

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.