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Temporal Regulation of Metamorphic Processes in *Drosophila* by the *let-7* and *miR-125* Heterochronic MicroRNAs

Elizabeth E. Caygill[1] and **Laura A. Johnston**[1,*]

[1]Department of Genetics and Development College of Physicians and Surgeons, Columbia University, New York, New York 10032

Summary

Background—The *let-7* and *lin-4* microRNAs belong to a class of temporally expressed, noncoding regulatory RNAs that function as heterochronic switch genes in the nematode *C. elegans*. Heterochronic genes control the relative timing of events during development and are considered a major force in the rapid evolution of new morphologies. *let-7* is highly conserved and in *Drosophila* is temporally coregulated with the *lin-4* homolog, *miR-125*. Little is known, however, about their requirement outside the nematode or whether they universally control the timing of developmental processes.

Results—We report the generation of a *Drosophila* mutant that lacks *let-7* and *miR-125* activities and that leads to a pleiotropic phenotype arising during metamorphosis. We focus on two defects and demonstrate that loss of *let-7* and *miR-125* results in temporal delays in two distinct metamorphic processes: the terminal cell-cycle exit in the wing and maturation of neuromuscular junctions (NMJs) at adult abdominal muscles. We identify the *abrupt* (*ab*) gene, encoding a nuclear protein, as a bona fide *let-7* target and provide evidence that *let-7* governs the maturation rate of abdominal NMJs during metamorphosis by regulating *ab* expression.

Conclusions—*Drosophila let-7* and *miR-125* mutants exhibit temporal misregulation of specific metamorphic processes. As in *C. elegans*, *Drosophila let-7* is both necessary and sufficient for the appropriate timing of a specific cell-cycle exit, indicating that its function as a heterochronic microRNA is conserved. The *ab* gene is a target of *let-7*, and its repression in muscle is essential for the timing of NMJ maturation during metamorphosis. Our results suggest that *let-7* and *miR-125* serve as conserved regulators of events necessary for the transition from juvenile to adult life stages.

Introduction

The heterochronic genes of *C. elegans* form a regulatory network that temporally regulates specific cell fates [1]. Mutations in heterochronic genes alter the timing of stage-specific processes, resulting in reiteration of larval patterns or execution of precocious adult fates. Two of the best-studied heterochronic genes in *C. elegans* are *lin-4* and *let-7*, which serve as early and late developmental timers, respectively [2,3]. Both *let-7* and *lin-4* are microRNAs, small noncoding RNAs that posttranscriptionally regulate gene expression by binding to semicomplementary sequences on mRNA targets and thus cause their destruction or prevent their translation into protein [4,5]. In the worm, only a few targets of these microRNAs have been identified, and each target participates in the heterochronic gene network [1,6].

*Correspondence: lj180@columbia.edu.

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In *C. elegans*, *lin-4* is required to downregulate target genes in early larval stages, whereas *let-7* acts in the last larval stage, just prior to the transition to adulthood, and loss of either microRNA alters the timing of decisions affecting cell fates [1]. Notably, the defects of heterochronic mutants are limited to specific tissues and specific developmental stages. Loss of *let-7*, for example, prevents the programmed cell-cycle exit of hypodermal blast cells at the L4-adult transition, causing them to reiterate an earlier program of cell division and preventing their differentiation at the appropriate time; however, cell division is unaffected in other tissues or at earlier stages of development [1,3].

The sequence of the mature 21–22 nucleotide (nt) *let-7* microRNA is 100% conserved among bilaterians [7]. Temporal regulation of *let-7* is also conserved, and its expression is largely limited to developmental stages corresponding to the transitional period between the juvenile and adult in the nematode, fly, zebrafish, and mouse [7,8]. *Drosophila let-7* is coexpressed with the *lin-4* homolog, *miR-125*, in numerous tissues beginning late in the third larval instar (L3) and peaking in pupae during metamorphosis [7,9,10]. Their coregulation arises from a common genomic locus on the left arm of the second chromosome that also includes another microRNA, *miR-100* [11,12]. All three microRNAs are expressed together in one primary transcript. Interestingly, paralogs of *let-7* and *miR-125* are also clustered in the mouse genome and are expressed with temporal overlap [8,13], and in *C. elegans*, certain *let-7* and *lin-4* family members are expressed at the same time [14]. The overlapping expression of *let-7* and *miR-125* and their genomic clustering in many species suggests that these microRNAs share a subset of functions. Computational analyses of microRNAs have predicted hundreds of targets for *let-7* and for *miR-125*, although none have been functionally validated within any organism other than *C. elegans*.

We sought to determine whether the heterochronic functions of *let-7* and *miR-125* are conserved in *Drosophila* and here analyze a mutation that eliminates both microRNAs. Loss of *let-7* and *miR-125* leads to widespread defects during metamorphosis. The most severe of these, impaired eclosion of newly formed adult flies, results in 70% lethality. We map this defect to a delay in maturation of abdominal neuromuscular junctions (NMJs) and establish as its cause the persistence of a *let-7* target, Abrupt, in mutant muscle cells. In addition, we demonstrate that *let-7* is critical for the appropriate timing of cell-cycle exit of developing wing cells; this role is remarkably similar to a *let-7* function in *C. elegans*.

Results

Loss of *let-7* and *miR-125* Leads to Defects Specifically during Metamorphosis

In *Drosophila*, *let-7* and the *lin-4* homolog, *miR-125*, are coregulated along with *miR-100* (Figure 1A). All three miRNAs are expressed as one primary, poly-adenylated RNA (Figure S1 available online) that is processed by the enzymes Dicer and Drosha to yield the functional miRNAs [9, 15]. Expression of the three miRNAs is temporally regulated, and the 21–22 nt RNAs are observed in larvae beginning at the L3-pupal transition and peaking in pupae during metamorphosis [7, 9].

We initiated studies of *Drosophila let-7* and *miR-125* to determine whether their role in timing developmental processes was conserved. Using the Gal 4-UAS expression system [16], we found that extensive misexpression of *let-7* or *miR-125* at early stages arrested development at the L1 or L2 stage and led to lethality (Table S1), confirming the importance of temporal restriction of their expression to late in development. To examine the physiological role of these microRNAs, we generated a mutant by using homologous recombination [17]. We replaced a 36 bp region of the *let-7* precursor hairpin with the *white* eye-color gene and verified the deletion by PCR (Figures 1A–1C). Northern blot analysis indicated that expression of both *let-7* and *miR-125* was lost as a result of the *white* insertion (Figure 1D).

Consistent with the timing of their expression, loss of *let-7* and *miR-125* did not alter embryonic or larval development. All mutant animals entered the pupal stage with normal timing and viability and developed as superficially normal pharate adults (Figures 1E–1G). Strikingly, however, the majority of mutant pharates were unable to eclose, suggesting underlying metamorphic defects (Tables 1 and 2; see below). Moreover, animals successful at eclosion had severely shortened life-spans; in longevity assays, 50% of homozygous mutant females died just 8 days after eclosion (Figure 1H). In addition, we observed many locomoter and behavioral defects in the mutants prior to their death (Table 1 and Figure S4). Close examination of eclosed mutant animals also revealed that their wings were significantly smaller than normal (Figure 2D).

let-7 Is Required for Terminal Exit from the Cell Cycle in Wing Imaginal Discs

In *C. elegans*, loss of *let-7* prevents the programmed cell-cycle exit of the hypodermal blast cells at the L4-adult transition, causing them to reiterate an earlier program of cell division [3]. Expression of *let-7* at earlier stages is sufficient to force precocious exit from the cell cycle and differentiation of these cells [3,18]. The wing phenotype in our *Drosophila* mutant suggested a functional parallel with *C. elegans let-7* in cell-division control, and we therefore examined wings from *let-7*, *miR-125* mutants for proliferation defects. Prior to wing differentiation, cell division in the primordial wing imaginal disc ceases approximately 24 hr after puparium formation (APF), concomitant with the expression of the miRNAs (Figure 2A) [19,20]. Although control wing discs had few mitotic cells at 24 hr APF, age-matched wing discs from *let-7*, *miR-125* mutant pupae contained numerous dividing cells (Figures 2A–2C). This led to significantly more cells in adult mutant wings (Figure 2D). In mosaic wings, clones of *let-7*, *miR-125* mutant cells contained 25% more cells than wild-type clones in control wings (Figure 2E). These data indicate that most *let-7*, *miR-125* mutant cells continued to divide when wild-type cells had exited the cell cycle in preparation for differentiation. Strikingly, the mutant cells were significantly smaller than wild-type controls (Figures 2D and 2E), suggesting that the extra divisions were uncoupled from cellular growth. We also found that cell death was increased in pupal wings of mutant animals compared to controls (Figure 2C), and early events of wing metamorphosis such as the proximal-distal extension that separates the anterior and posterior crossveins also appeared to be delayed Figure S2A). These data suggest that the small wings of mutant animals resulted from a combination of small cell size and increased cell death during pupal development.

Given the precedent from *C. elegans*, the failure of the double-mutant cells to exit the cell cycle at the appropriate time suggested that loss of *let-7* was responsible for this defect. We tested this idea by examining whether misexpression of *let-7* was sufficient to induce cell-cycle withdrawal in larval-stage wing discs. Indeed, clonal expression of UAS-*let-7* via a “flp-out” Gal 4 cassette forced cells in the wing disc to arrest after only 30 hr of growth (Figure 3A). Moreover, broad misexpression of *let-7* in larval wing discs significantly reduced their size relative to controls (Figure 3B) and led to markedly undersized adult wings (Figure 3C). The effect of *let-7* was specific; expression of UAS-*miR-125* did not alter wing disc or adult wing size (Figures 3B and 3C). The correspondence between the misexpression and loss-of-function experiments suggests that loss of *let-7* is responsible for the cell division defect in *let-7*, *miR-125* mutant wings. Taken together, these data suggest that *let-7* is required for enforcing the terminal cell-cycle arrest in pupal-stage wing cells and that *Drosophila let-7* functions in a conserved heterochronic role as a stage-specific timer of cell-cycle exit.

Neuromuscular Junctions Mature Slowly in *let-7*, *miR-125* Mutants

To determine whether other abnormalities in the *let-7*, *miR-125* mutants were temporal in nature, we focused on the eclosion defect. We found that 100% of *let-7*, *miR-125* mutant animals initiated eclosion behavior with the same timing as age-matched controls (wild-type,

n = 16; mutant, n = 15), indicating that the circadian and endocrine triggers for eclosion were intact [21,22]. However, although *let-7*, *miR-125* mutants carried out the typical pattern of abdominal and thoracic contractions [22,23], the contractions were largely unproductive. Up to 70% of *let-7*, *miR-125* mutants were unable to generate the force required to emerge from the pupal case (Table 2), suggesting the flies suffered from severe muscle weakness.

Muscle weakness can result from reduced synaptic strength, and we therefore examined the NMJs of wild-type and mutant abdominal muscles at different times during pupal development. Midway through the pupal stage, at 54 hr APF, the extent of nascent NMJs on dorsal abdominal muscles of mutant and wild-type animals was similar (Figures 4A and 4B). However, although small boutons formed over the next 42 hr, mutant NMJs grew at a significantly slower rate than controls and were 50% smaller than NMJs from aged-matched controls at each time point (Figure 4C). The immaturity of mutant dorsal abdominal NMJs was such that at 96 hr APF their size approximated that of NMJs in wild-type animals that were 18 hr younger (Figure 4C). Staining with antibodies against the synaptic bouton marker, cysteine string protein (CSP) [24, 25], in animals just prior to eclosion revealed that the NMJs in abdominal muscles of mutant flies contained significantly fewer and visibly smaller boutons (Figures 5F–5H).

The *abrupt* Gene Is an In Vivo *let-7* Target that Persists Aberrantly in *let-7*, *miR-125* Mutants

miRNAs function by binding to and targeting specific mRNAs for translational repression or destruction [26], and both miRNAs are predicted by several databases to individually target hundreds of mRNAs [27,28], although none of these have been verified in vivo. To get at the mechanism by which loss of *let-7* and *miR-125* could reduce the size and elaboration of abdominal NMJs, we searched for candidate targets of *let-7* and *miR-125* activity that might bring about the NMJ defect. The *abrupt* (*ab*) gene, which encodes a nuclear, BTB-POZ, Zn-finger containing protein, is a highly ranked candidate whose 3′ untranslated region (UTR) contains several predicted *let-7* binding sites (Figure 6A) and is regulated by *let-7* expression in cell-culture assays [29]. *ab* (also called *clueless* (*clu* [30]) is widely expressed during fly development and is required for diverse functions, including neuromuscular targeting and dendrite arborization in embryos [31–33], and the patterning of the fifth longitudinal vein (LV5) of the wing [34].

Ab protein is expressed throughout the wing disc in wild-type late L3 animals (the highest levels are in cells that will form LV5) (Figure 6B) [34], but its expression is reduced to barely detectable levels early in the pupal stage (Figure 6C). Strikingly, however, Ab protein continued to be expressed specifically in *let-7*, *miR-125* mutant cells, particularly in the LV5 region, in mosaic pupal-stage wing discs (Figures 6D–6E). We took advantage of the wing expression of Ab to test for microRNA specificity by using Gal 4-mediated misexpression. Expression of UAS-*let-7* in L3 wing disc cells was sufficient to repress Ab expression prematurely (Figures 6F–6G). In contrast, Ab expression was not altered by expression of UAS-mut-*let-7*, a mutated form of *let-7* (Figure 6H), or by expression of UAS-*miR-125* (Figure 6I). Thus, the persistent expression of Ab in mutant pupal wing-disc cells was likely due to loss of *let-7*. The data indicate that *let-7* is both necessary and sufficient for the appropriate temporal repression of Ab in wing discs and establishes *ab* as a bona fide, in vivo target of *Drosophila let-7*.

We then examined whether Ab was involved in the abdominal NMJ defects in *let-7*, *miR-125* mutants. Ab is expressed in the nuclei of both wild-type and mutant adult myoblasts within the first 26 hr APF (Figures 5A–5C). However, Ab was rarely observed in freshly eclosed wild-type adults, indicating that its expression is lost from abdominal muscles as pupae mature (Figure 5D). In contrast, many nuclei from abdominal muscles of freshly eclosed *let-7*, *miR-125* mutants contained high levels of Ab protein (Figure 5E). Taken with our observation that expression of UAS-*let-7*, but not UAS-*miR-125*, is sufficient to repress Ab

expression (Figures 6G and 6I), these data suggest that *let-7* functions during pupal development to repress Ab expression in muscle nuclei.

let-7-Mediated Repression of Abrupt Expression in Muscle Nuclei Is Required for NMJ Growth and for Eclosion

Retrograde signaling from muscle to motoneuron controls NMJ development in the *Drosophila* adult [35]. We reasoned that the persistence of Ab expression in mutant muscles could interfere with this signaling, cause the delay in NMJ maturation, and possibly, form the basis of the eclosion defect. Although Ab was present in the pupal brain of wild-type and *let-7*, *miR-125* mutants (Figures S3C and S3D), we did not detect Ab in abdominal ganglia or motoneurons from either genotype (Figures S3A and S3B), supporting this idea. We attempted to phenocopy the eclosion defect by expressing a UAS-*ab* transgene in muscle or neuronal lineages with Gal 4 drivers specific to these cell types. Expression of *ab* in muscle cells was lethal at the L1 or L2 stage, as was expression in cholinergic neurons. In contrast, a pan-neuronal or a motoneuron driver allowed animals to eclose (Table S2).

As a more definitive test of whether persistent Ab expression in *let-7*, *miR-125* mutant muscles reduced their ability to form mature NMJs, we carried out genetic suppression experiments by using mutant alleles of *ab*. Bouton numbers were significantly increased in abdominal NMJs of *let-7*, *miR-125*, ab^{1D}/+ adults (Figures 5F–5I). Moreover, reduction of *ab* gene dosage significantly suppressed the eclosion defect. *let-7*, *miR-125* mutants carrying one copy of the strong ab^{1D} allele eclosed at a frequency nearly double that of *let-7*, *miR-125* alone (Table 2). These data provide compelling evidence that the persistence of Ab in the abdominal muscles of *let-7*, *miR-125* mutants underlies the NMJ growth defect and leads to muscle weakness that impairs adult eclosion.

We also tested for a causal role for Ab in other defects in the *let-7*, *miR-125* mutants. Ab was expressed in cells of the larval wing disc and was downregulated early in the pupal stage (Figures 6B and 6C). Interestingly, in a wild-type backgound, one copy of the strong ab^{1D} or ab^{clu1} allele produced a dominant increase in wild-type wing size (Figure S4A and data not shown). Thus, although reducing the genetic dose of *ab* led to an increase in *let-7*, *miR-125* mutant wing size, this probably does not reflect an epistatic genetic relationship. In support of this interpretation, cell size was not altered in *let-7*, *miR-125*, ab^{1D}/+ wings (Figure S4A; or ab^{clu1}, data not shown). Reducing the dose of *ab* also did not suppress the defects in locomotion (Figure S4B), the sterility of either female or males (Figure S4C), or the reduced lifespan (Figure S4D) of *let-7*, *miR-125* mutants. Therefore, these defects must be due to other *let-7* targets, to loss of *miR-125*, or to contributions from a combination of the microRNAs.

Discussion

Our results indicate that in *Drosophila*, loss of the *let-7* and *miR-125* microRNAs leads to numerous defects that alter the morphology and behavior of adult flies. The expression of these microRNAs is restricted to the metamorphic stage of development, and we show that both loss and premature expression of *let-7* and *miR-125* are detrimental to animal survival, indicating that temporal restriction of their expression is essential. Many defects in the mutants affect sensory and motor behaviors, and our results indicate that neuromuscular connectivity in abdominal muscles is severely hampered and that there are direct consequences on the rate of adult eclosion. Both the NMJ and eclosion defects result from deregulated expression of a single *let-7* target in abdominal muscles. Control of NMJ growth represents a new regulatory role for microRNAs in general, and given the extensive neurological problems of *let-7*, *miR-125* mutants, it is possible that synapse development is broadly regulated by these particular micoRNAs.

let-7-Regulated Repression of *abrupt* Is Required for Timing NMJ Maturation During Metamorphosis

Our data clearly implicate regulation of *ab* by *let-7* as a critical factor in the rate of NMJ development during *Drosophila* metamorphosis. The aberrant persistence of Ab in muscle nuclei in the mutant significantly slows NMJ development, perhaps by interfering with synapse-strengthening signals exchanged between muscle and motoneurons [35]. Because Ab expression is widespread during *Drosophila* development, the need for its downregulation late in development is in striking contrast to the requirement for Ab at earlier stages: in muscle for neuromuscular targeting and for epidermal attachment of specific muscle groups [31] and in a class of embryonic and larval sensory neurons to limit dendritic branching [32,33]. Based on its dose sensitivity, Ab has been proposed to function in a concentration-dependent manner [31]. By examining the expression of Ab protein directly, we find that it is downregulated to nearly undetectable levels in most abdominal-muscle nuclei by the pharate adult stage (Figure 5D and data not shown). Its decline in the myoblasts may begin as early as 26 hr APF (Figure 5B), a time when *let-7* expression is rising [10,11]. However, our experiments indicate that deregulation of Ab cannot account for all of the mutant defects, indicating that other targets of *let-7* and/or *miR-125* must function as effectors for these processes (Figure S4). To date, Ab is the only target of *Drosophila let-7* to be verified in vivo, and no *miR-125* targets have been authenticated.

Temporal Control of Cell Division by *Drosophila let-7*

In addition to its role in NMJ maturation, our results implicate *let-7* in a wing defect that causes cells to continue to divide after wild-type wing cells have exited the cell cycle. This phenotype is strikingly similar to the reiterated divisions of hypodermal blast cells in *C. elegans let-7* mutants [3]. In both cases, the extra divisions do not continue indefinitely, nor do they occur in all tissues (data not shown) [1]. Little is known about the mechanism of cell-cycle regulation by *let-7* in either organism. In *C. elegans*, persistent expression of *lin-41*, encoding a RBCC (ring B-box coiled-coil) protein, in *let-7* mutants accounts for much of the mutant phenotype [36]. *Drosophila* homologs of *lin-41* include *dappled* (shown to be a *let-7* target in cell-culture assays [37]) and *brat* (*brain tumor*), a translational inhibitor [38]. *brat* encodes a potent tumor suppressor whose absence causes metastatic brain tumors [39] and that is predicted by one computational program to contain *let-7* binding sites [13]. Interestingly, overexpression of *brat* suppresses wing growth [38]. Thus, a plausible hypothesis for the wing defect in *let-7*, *miR-125* mutants is that loss of *let-7* simultaneously deregulates a cell-cycle regulator(s) and brat and that the former drives additional cell divisions in the wing while the latter suppresses growth.

Practical and Evolutionary Implications of *let-7 and miR-125* Function during Metamorphosis

The expression of many genes required during larval stages is downregulated during metamorphosis, in part because their presence during pupal development hinders the ordered progression of events such as the primary and secondary responses to ecdysone, the hormone that controls insect metamorphosis [40,41]. Thus, a plausible role for *let-7* and *miR-125* is to rid cells of unnecessary larval mRNAs quickly at the transition to pupal development; such a role might be similar to the clearing of maternal mRNAs at the maternal–zygotic transition as recently demonstrated in zebrafish and in *Drosophila* [42,43]. Heterochrony facilitates rapid evolution of new morphologies through changes in the timing of developmental processes [44,45]. The pleiotropic phenotype associated with *let-7, mir-125* mutants suggests that each microRNA contributes to multiple processes during metamorphosis, and the identification of additional targets of both is therefore an important future quest that should clarify their contribution to developmental timing and morphological evolution.

Conclusions

We have generated a mutant of *Drosophila let-7* and *miR-125* that exhibits temporal misregulation of specific metamorphic processes. *Drosophila let-7* is both necessary and sufficient for the appropriate timing of a specific cell-cycle exit and thus functions as a conserved heterochronic microRNA. The *abrupt* gene is a target of *let-7*, and its repression in muscle is essential for the timing of NMJ maturation during metamorphosis. *let-7* and *miR-125* could serve as conserved regulators of processes necessary for the transition from juvenile to adult life stages.

Experimental Procedures

Fly Strains: *yw*;+;*hsflp hsp70*-I-SceI/TM6B, *yw ey*flp, *w; En*Gal 4, *w; Dpp*Gal 4, *w; Tub*Gal 4, *w; Vg*Gal 4, *yw; Act>y+>*Gal 4, *w; MEF2*Gal 4, *w; 24B*Gal 4, *w;CHA*Gal 4, *w*; UAS-GFP, and *w*; UAS-P35 [46] were obtained from the Bloomington Stock Center. $w;ab^{1}$, $w;ab^{1D}$, and $w;clu^{1}$ were gifts of S. Crews. C155Gal 4 and OK6Gal 4 were gifts of B. McCabe. *yw hsflp*; FRT40A, Ubi-GFP, *yw hsflp*; FRT40A *let-7*, and *yw hsflp*; FRT40A *let-7 ck* were generated in this work. Our control strain (designated +/+) was *yw*; +; +, which was used to isogenize all other strains.

Fly Husbandry: Eggs from appropriate crosses were collected on yeasted grape plates for 2 hr periods. Larvae were staged from egg deposition and after hatching were raised in uncrowded vials containing yeasted molasses and cornmeal food at 25°C for defined periods of time. Pupae were staged from white pre-pupa formation as in [47] and were raised on glass slides at 25°C for defined periods of time. Adult viability was measured daily from vials of ten flies each. Fertility was assessed by observation of progeny production from single-pair crosses with wild-type partners.

Homologous Recombination: Genomic fragments (5.1 kb) flanking *let-7* were PCR amplified and cloned into an ends-out homologous-recombination vector kindly provided by S. Chen and G. Struhl. Standard procedures were used for injecting the plasmid into w^{1118} embryos and isolating transformants. Virgin female flies carrying the targeting construct on the X chromosome were crossed to *yw hsflp hsp70*-I-SceI-expressing males. Progeny were collected for 24 hr periods and heat shocked for 1 hr at 37°C each day for three consecutive days. Eclosing progeny were crossed to *yw eyflp*-expressing males, and female progeny that retained eye color were PCR screened for the insertion of the mini-white gene into the *let-7* locus. Primer set 1: forward, CAAACCACCT AGCAAAAAGGA; reverse, TAGGGTCTCAGGGAATCAGC. Primer set 2: forward, TTCCGCAAAAATGGGTTTTA; reverse, AGGCTTCCCCTGCTATCATT.

Molecular Biology: The UAS-*let-7* and UAS-*miR-125* lines were generated by PCR amplification of 366 bp and 188 bp fragments, respectively, and cloning into pUAST [16]. The UAS-mut-*let-7* line was generated by site-directed mutagenesis of the UAS-*let-7* construct, with G-C transitions at nucleotides 4 and 5 of the mature *let-7* miRNA and complementary mutations on the opposing side of the hairpin. Standard procedures were used for injecting the plasmids into w^{-} embryos and isolating transformants. Northern analysis confirmed the production of processed *let-7* and *miR-125* RNAs from Gal 4 activation (data not shown).

Reverse-Transcription PCR: Poly (A) + RNA was generated from total RNA isolated from whole 42 hr APF pupae. Primers flanking all three miRNAs were then used for amplification of a 1400 bp product. Primer set 3: forward, AAAAGGGGTTGCTTTTCGAT; reverse, CCTTCCTTTGCAGTCAAAATG.

Northern Blotting: Total RNA was isolated from 42 hr APF pupae, separated on a 15% denaturing polyacrylamide gel, and transferred to Genescreen Plus membrane (Perkin Elmer). Membranes were probed with either Starfire (Integrated DNA technologies) or LNA (Exiqon) probes, radioactively labeled according to the manufacturers' instructions.

Immunocytochemistry: Fixation and immunocytochemistry of imaginal discs and epidermal preparations was carried out as described [48]. The following primary antibodies and dilutions were used: rabbit anti-Abrupt, 1:500 (gift of E. Bier) [34], mouse anti-abrupt 1:100 (DSHB), rabbit anti-H3P, 1:1000 (Upstate Biotechnology), mouse anti-CSP, 1:200 (DSHB); rabbit anti-Twist, 1:5000 (gift of M. Baylies). Secondary antibodies were as follows: goat anti-rabbit Alexa 488 (1:2000), donkey anti-mouse Cy-5 (1:600), and donkey anti-rabbit Cy3 (1:600) (Jackson ImmunoResearch). Alexa 647-Phalloidin (Molecular Probes) was used at 1:100 for labeling F-actin, and Hoechst 33258 (Sigma) was used for marking DNA. Cy3-HRP (1:200, Molecular Probes) was used for marking neurons. TUNEL assays (a specific protocol is available upon request) were carried out with Apoptag Red (Chemicon International). Images were captured on a Zeiss Axiophot equipped with Apotome or Bio-Rad 1000 or Leica confocal microscopes and processed in Adobe Photoshop or Image J.

Measurements of Wing and Cell Size: The Gal 4-UAS system [16] was used for misexpression of *let-7* and *miR-125*. For measurement of wing and disc size, images were captured on a Zeiss Axiophot; size was measured in microns with Axiovision software (Zeiss). Cell size was calculated from a count of trichome number in two defined areas per wing and subsequent calculation of an average cell size (each trichome corresponds to one cell [49]). Cell number per wing was extrapolated from these measurements. For mitotic clones in adult wings, trichomes (marked with *crinkled (ck)* in both control and mutant wings) were counted as above. Clone size was determined from the inclusive area of *ck* mutant cells; conditions were optimized so that between 2 and 6 clones per wing were obtained. Cell size in clones was calculated from the number of cells per clone/clone size. All measurements were done with female wings.

Generation of Clones and Cell-Proliferation Measurements: For the cell-number assay, Gal 4 “flp-out” clones expressing UAS-GFP ± UAS-*let-7* were generated by heat-shock-induced Flp recombinase at 60 hr AEL and dissected 20, 30, or 40 hr later [50]. Clone cell number was determined from a count of GFP-positive nuclei. For analysis of Ab expression, clones expressing UAS-GFP ± UAS-*let-7*, UAS-mut-*let-7*, or UAS-*miR-125* were generated at 70 hr AEL and dissected at 100 hr AEL. For mutant clones, mitotic recombination was induced at 72 hr AEL; the *ck* mutation was used for marking control and *let-7*, *miR-125* mutant clones in the adult wing.

Bouton Measurements: Abdomens of female flies were dissected down the ventral midline, pinned, fixed with 4% paraformaldehyde, and stained with anti-CSP, Cy3-HRP, and Alexa-647-conjugated Phalloidin. CSP-positive boutons were examined for size and counted in two medial muscle groups of one hemitergite of abdominal segment IV for each fly.

Locomotor Assay: Groups of ten 2-day-old adult flies were tapped to the bottom of an empty vial. The percentage of flies that successfully climbed back to the top of the vial in 15 s was recorded and averaged from ten consecutive trials.

Statistical Analysis: Two-tailed Student's t tests with unequal variance were used for significance assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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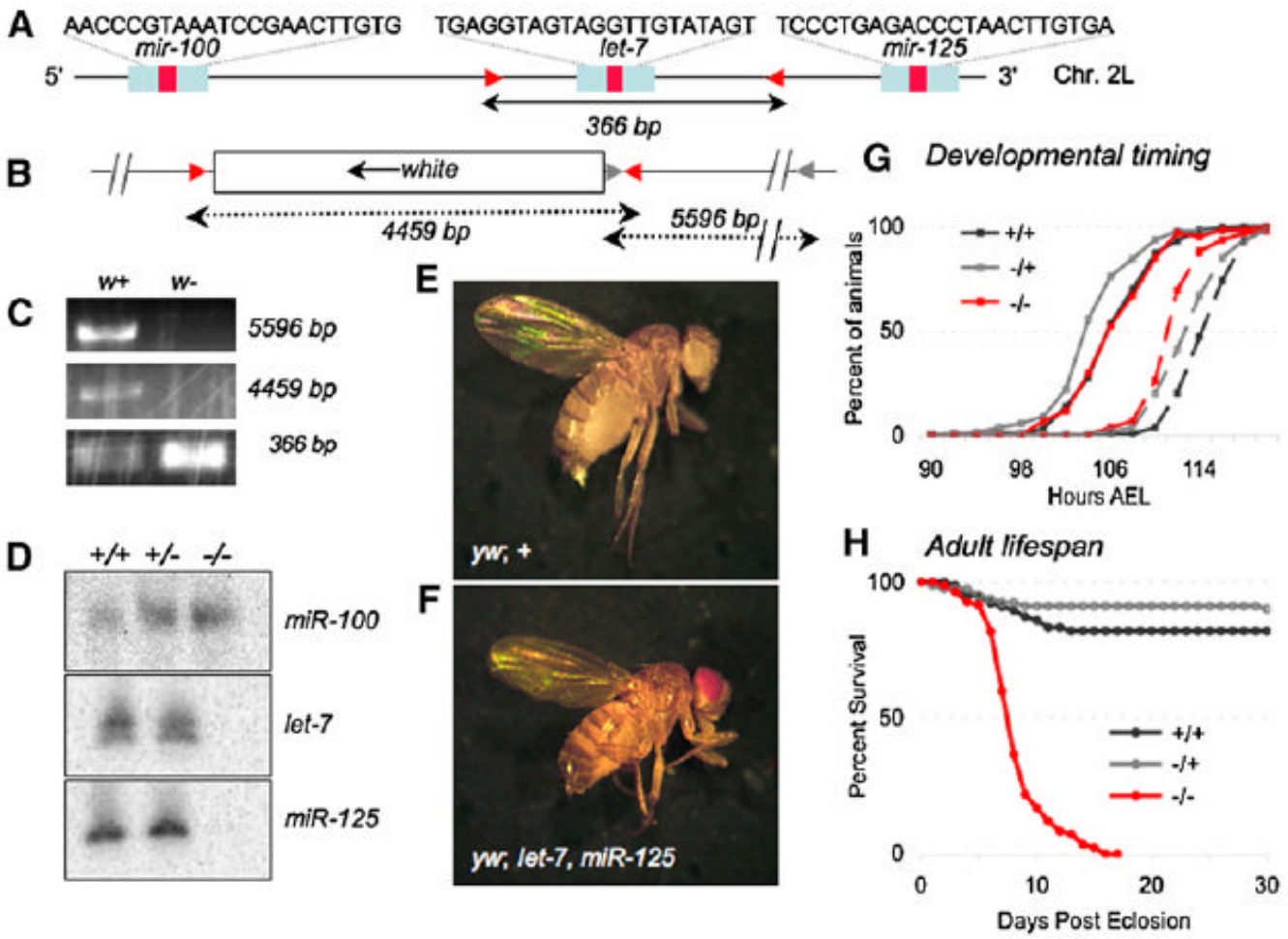

Figure 1. Generation of a *Drosophila let-7, miR-125* Mutant

(A) The wild-type *let-7, miR-125* locus. Red arrowheads indicate positions of primers (set 1, see Experimental Procedures) that amplify a 366 bp product in both wild-type and heterozygous mutants (C).

(B) The mutated *let-7, miR-125* locus, primer set 1 (red arrow heads), now amplifies an additional 4459 bp product in heterozygous mutants; gray arrowheads indicate the positions of primer set 2, which amplifies a 5596 bp product in heterozygous mutants (C).

(C) PCR analysis (using the primer sets described in [A] and [B]) of sibling *w*+ (heterozygous mutant) and *w*− (non mutant) flies confirms the deletion of *let-7* (loss of the 366 bp band; one copy is left in the heterozygous mutant) and insertion of the mini-*w* gene (gain of the 4459 bp and 5596 bp bands).

(D) Northern analysis of total pupal RNA from animals 42 hr APF. Probes for *miR-100, let-7*, and *miR-125* confirm the absence of *let-7* and *miR-125*.

(E and F) Wild-type female fly (E) and female *let-7, miR-125* homozygous mutant fly (F).

(G) Developmental timing of larval wandering (solid lines) and pupariation (dashed lines) of *let-7, miR-125* (−/−, n = 61) is unchanged relative to wild-type (+/+, n = 244) and heterozygous mutant (−/+, n = 92) larvae.

(H) Lifespan of *let-7, miR-125* (−/−, n = 82) adult female flies is severely shortened relative to the wild-type (+/+, n = 77) and the heterozygous mutants (−/+, n = 79).

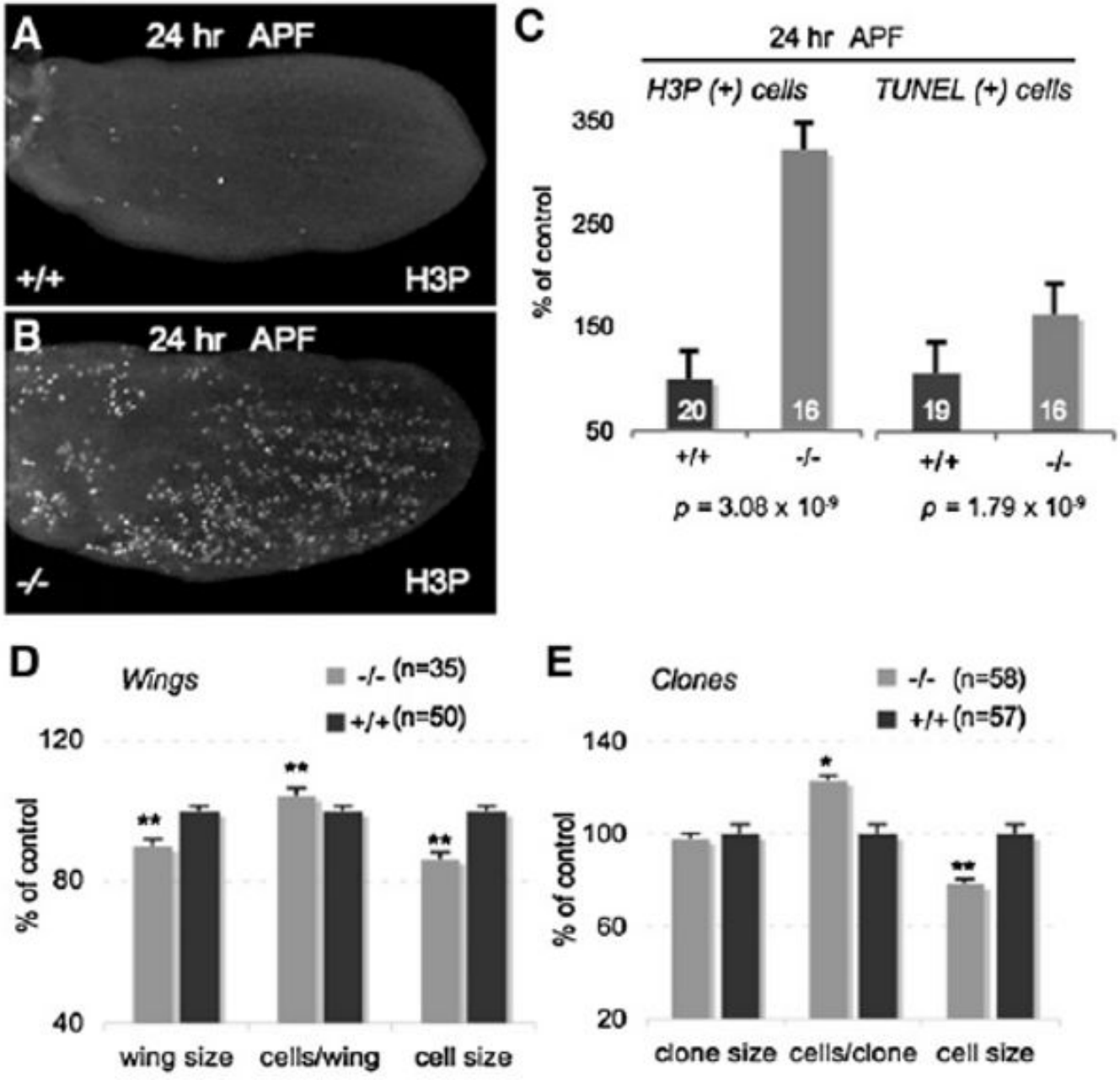

Figure 2. *let-7* Is Required to Time the Terminal Cell-Cycle Exit in the Wing

(A and B) Most cell division has ceased by 24 hr APF in wild-type wing discs (A), whereas many cells continue to divide in *let-7*, *miR-125* mutant wing discs at 24 hr APF (B). Discs in (A) and (B) are oriented with anterior up and are stained for phospho-Histone 3 (H3P), a marker of mitosis. Wing discs are magnified 1000×.

(C) Mutant wing discs at 24 hr APF contain more mitotic or dying cells than wild-type controls. Mitotic cells, marked by H3P (left), and dying cells, marked by TUNEL assay (right), in mutant wings at 24 hr APF, expressed as a percentage of wild-type controls. Error bars represent standard error.

(D and E) Wings from eclosed *let-7*, *miR-125* mutants (−/−) are significantly smaller and contain more, smaller cells than wild-type (+/+) wings (D). Clones mutant for *let-7*, *miR-125*

contain 25% more and smaller cells than wild-type clones (E). Number of wings or clones examined is indicated next to the graph legend. * $p < 0.05$; ** $p < 0.001$, compared to wild-type; error bars represent standard error.

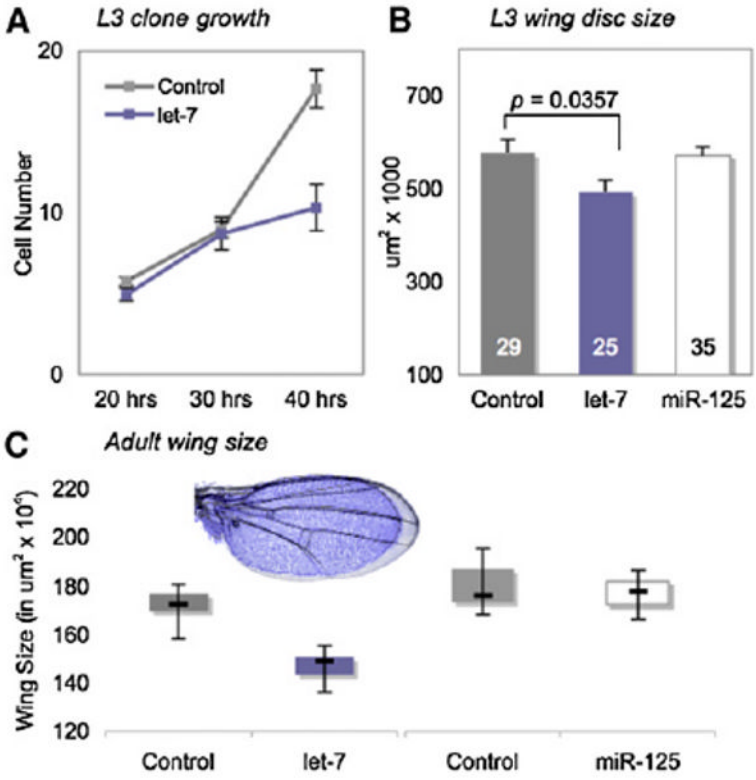

Figure 3. *let-7* Misexpression Causes Premature Cell-Cycle Exit in the Wing

(A) Cell number in "flp-out" clones of cells misexpressing UAS-*let-7* and UAS-GFP; control clones express UAS-GFP alone (see Experimental Procedures). Most *let-7*-expressing cells stop dividing after 30 hr of clonal growth. Controls: n = 52 at 20 hr, n = 41 at 30 hr, and n = 34 at 40 hr; UAS-*let-7*: n = 34 at 20 hr, n = 31 at 30 hr, and n = 31 at 40 hr. Error bars represent standard error.

(B and C) The wing-specific driver, Vestigial (Vg) Gal4, was used to misexpress UAS-GFP alone (control), or in combination with UAS-*let-7* or UAS-*miR-125* in larval wing discs. UAS-*let-7* expression significantly reduced disc size, whereas UAS-*miR-125* had no effect. Numbers in each bar represent discs measured. (C) Misexpression of UAS-*let-7* under Vg Gal4 control significantly reduces adult wing size compared to controls that express UAS-GFP alone ($p < 0.001$; control: n = 50; UAS-*let-7*: n = 50). In contrast, misexpression of UAS-*miR-125* does not alter wing size ($p > 0.05$; control: n = 20; UAS-*miR-125*: n = 20). Boxes represent second- and third-quartile values; whiskers extend to the maximum and minimum values. Inset shows the size difference of overlaid UAS-*let-7*-misexpressing (blue) and control (gray) wings.

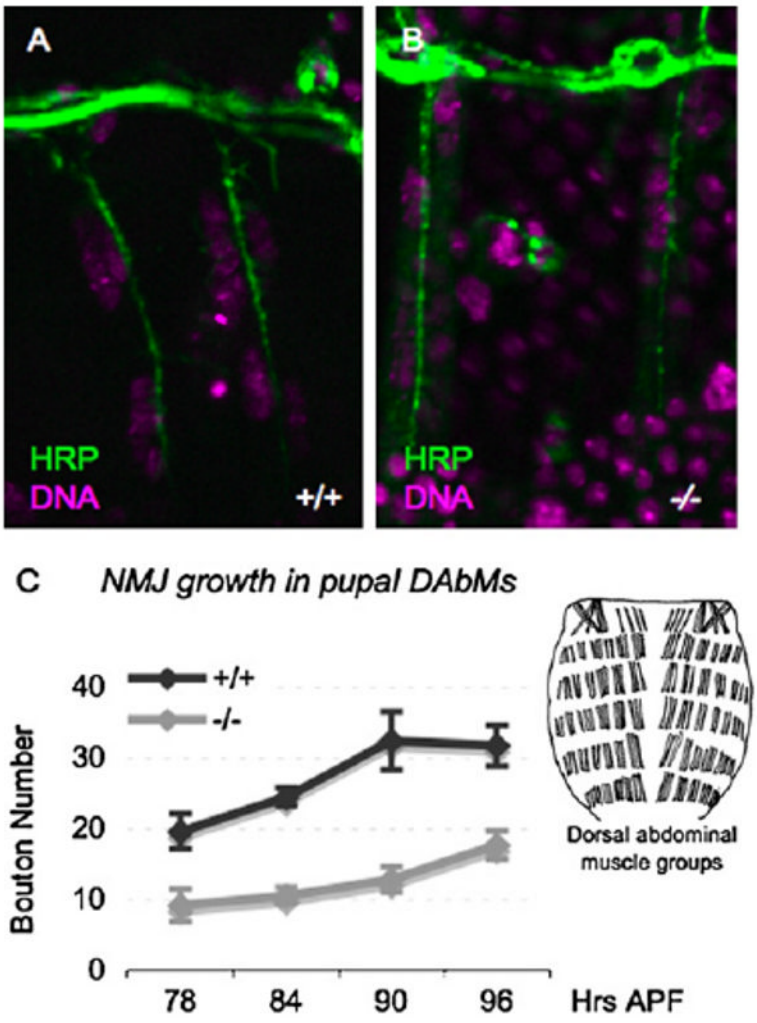

Figure 4. NMJ Maturation Is Delayed in *let-7, miR-125* Mutants

(A and B) NMJ growth begins with normal kinetics in *let-7, miR-125* mutants. Nascent NMJs containing a similar extent of boutons are present in both wild-type (A) and *let-7, miR-125* (B) dorsal abdominal muscles at 54 hr APF. Images are magnified 4000×.

(C) NMJ growth (measured by increase in bouton number during pupal development) is significantly delayed in *let-7, miR-125* mutants (−/−) relative to wild-type (+/+). Ninety-six hour APF corresponds to the onset of eclosion. Error bars represent standard error. Inset: representation of dorsal abdominal muscles of the adult fly (modified with permission from [23]).

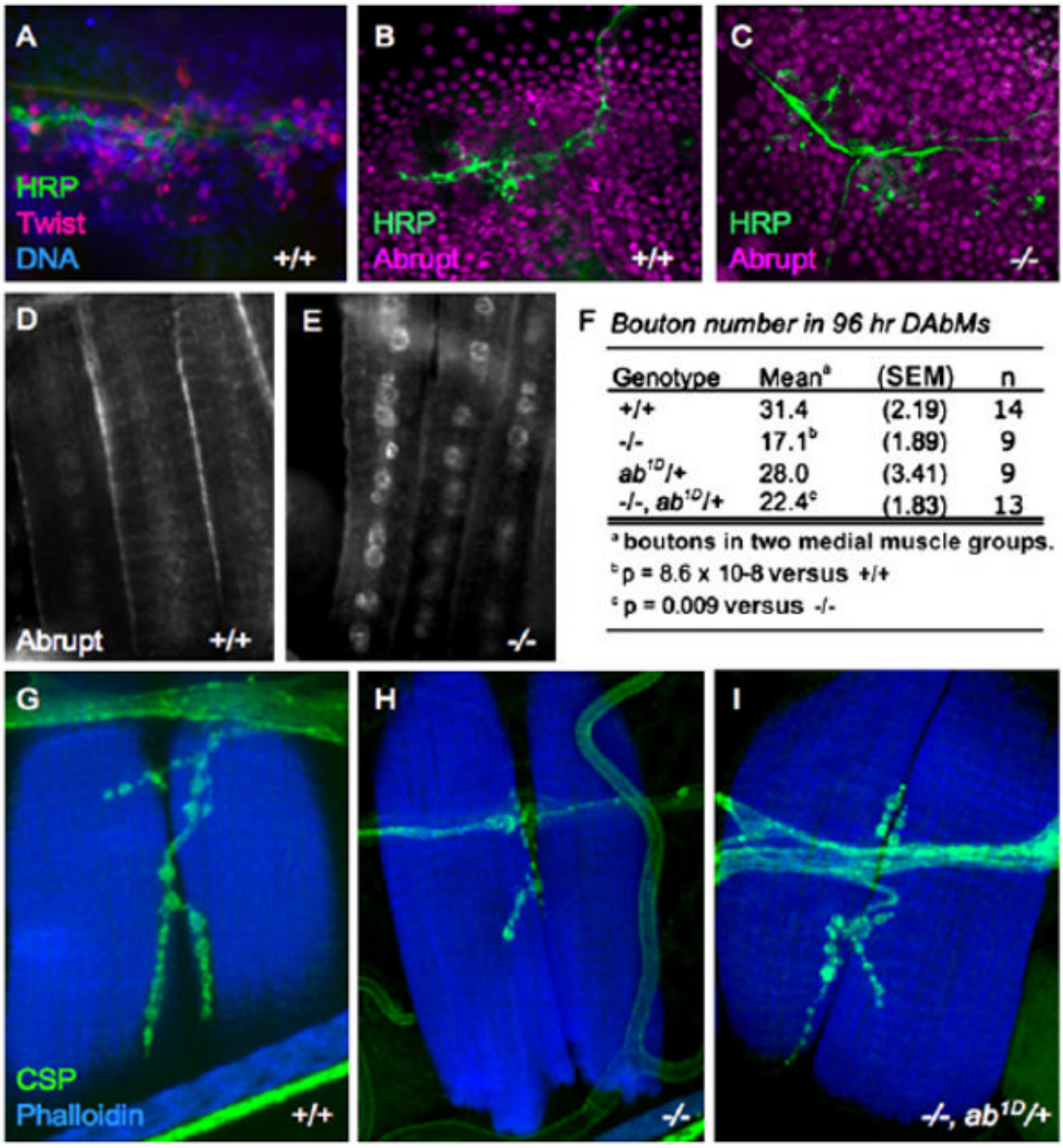

Figure 5. The Eclosion Defect and Delayed NMJ Maturation in *let-7, miR-125* Mutants Are Due to Persistent Expression of Abrupt

(A) Wild-type pupal epidermis at 24 hr APF, stained with Twist antibodies to show the myoblasts, which reside within the histoblast nests (marked in blue with Hoechst stain of DNA) and migrate along Horseradish peroxidase (HRP)-positive neurons (green). Images are magnified 4000×.

(B and C) Ab is expressed in myoblasts and histoblasts at 26 hr APF in both wild-type (B) and *let-7, miR-125* mutants (C). HRP marks the growing neurons (green). Images are magnified 4000×.

(D and E) Ab expression is downregulated in the nuclei of freshly eclosed wild-type adult dorsal abdominal muscles (D), but nuclear Ab expression persists in dorsal muscles from *let-7*, *miR-125* mutant adults (E). Images are magnified 2000×.

(F–I) NMJs of *let-7*, *miR-125* mutants at 96 hr APF have significantly fewer boutons than do controls (F–H). Reducing the dose of *ab* with the strong ab^{1D} allele (which alone has no effect on NMJs) significantly rescues bouton number in the mutants; SEM denotes standard error of the mean.

(G–I) Dorsal abdominal muscles (blue, Phalloidin) and synaptic boutons (green, CSP) from wild-type (G), *let-7*, *miR-125* mutant (H), and *let-7*, *miR-125*, ab^{1D}/+ double mutant (I) adult females. Images are magnified 4000×.

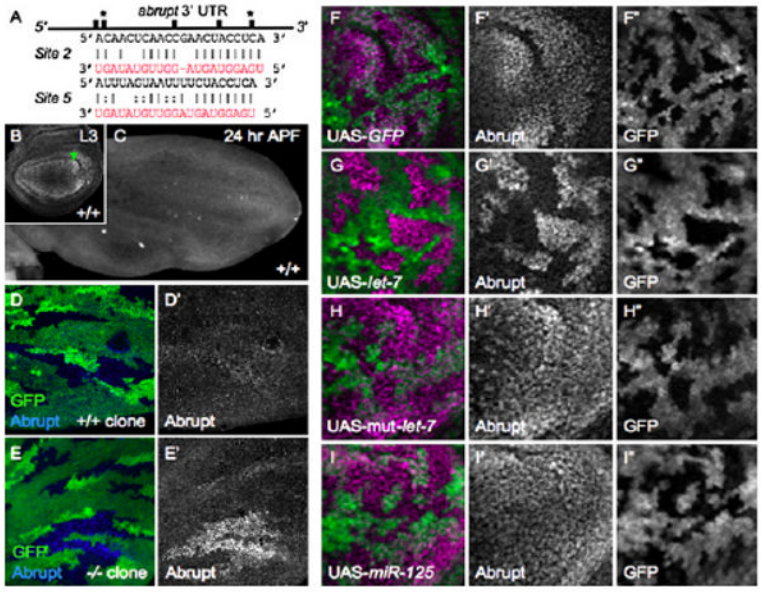

Figure 6. Abrupt Is a Bona Fide *let-7* Target

(A) Representation of the 3′ UTR of the *abrupt* mRNA, depicting the positions of five predicted *let-7* binding sites as black bars. The sequences of two sites (*) are shown. The *let-7* sequence is shown in red; lines indicate complementarity, and dots indicate G:U wobble basepairs.

(B and C) Ab is expressed broadly in mature wild-type (+/+) L3 wing discs, with highest expression in the region of LV5 ([B], arrowhead; n = 25); its expression is lost from the discs by 24 hr APF ([C], n = 10).

(D and E) Loss of *let-7*, *miR-125* prevents downregulation of Ab in 24 hr APF wing discs. (D and D′) Control wing-disc clones generated by mitotic recombination, showing merged (D) and single-channel (D′) images. Clones are marked by the absence of GFP (lack of green). One hundred percent of control clones show downregulation of Ab (n = 10 discs), whereas in *let-7*, *miR-125* mutant clones (E and E′), Ab expression (blue) persists, most strongly in the LV5 region (92%, n = 13 discs).

(F–I) Misexpression of *let-7*, but not *miR-125*, is sufficient to repress Ab expression. L3 wing discs with "flp-out" clones expressing UAS-GFP (green) alone (F–F″ n = 13), or in combination with UAS-*let-7* (G–G″, n = 17), a mutated form of UAS-*let-7* (H–H″, n = 9), or UAS-*miR-125* (I–I″; n = 14), are stained for Ab (purple). Images are magnified 2000× in (B), (D), and (E) and 4000× in (F)–(I). Image in (C) is magnified 1000×.

Table 1

Defects in *let-7*, *miR-125* Mutants

Defect	Description
Eclosion	Low eclosion frequency (Table 2)
NMJ size	Fewer, smaller boutons in dorsal abdominal (DAb) muscles (Figure 5)
Lifespan[a]	Reduced longevity (Figure 1H)
Fertility[a]	Sterile, morphologically normal gonads, no oviposition
Locomotion	No flight, uncoordinated movements, trembling[b]
Behavior	Aberrant grooming, aborted courtship, male-male courtship
Wing and cell size	Small wings, small cells (Figure 2)

[a] Both males and females.

[b] Some defects increase in severity with age.

Table 2

Genetic Suppression of Eclosion Defect

Genotype[a]	% eclosed[b]	n[c]
yw; +	97	259
yw; let-7 miR-125	31	185
w; ab^{1}/ab^{1D}	87	124
w; ab^{1}/ab^{clu1}	100	126
w; let-7 miR-125 ab^{1D}/+	60	133
w; let-7 miR-125 ab^{1}/ab^{1D}	53	227
w; let-7 miR-125 ab^{1}/ab^{clu1}	59	227

[a] Includes males and females for each genotype.

[b] Percentages are of expected genotype from each cross.

[c] n = number of flies.