

## Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and Broad-Complex gene activity

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### Abstract

*lin-4* and *let-7* are founding members of an extensive family of genes that produce small transcripts, termed microRNAs (miRNAs). In *Caenorhabditis elegans*, *lin-4* and *let-7* control the timing of postembryonic events by translational repression of target genes, permitting progression from early to late developmental programs. To identify *Drosophila melanogaster* miRNAs that could play similar roles in the control of developmental timing, we characterized the developmental expression profile of 24 miRNAs in *Drosophila*, and found 7 miRNAs that are either upregulated or downregulated in conjunction with metamorphosis. The upregulation of three of these miRNAs (*mir-100*, *mir-125*, and *let-7*), and the downregulation of a fourth (*mir-34*) requires the hormone ecdysone (Ecd) and the activity of the Ecd-inducible gene *Broad-Complex*. Interestingly, *mir-125* is a putative homologue of *lin-4*. *mir-100*, *-125*, and *let-7* are clustered within an 800-bp region on chromosome 2L, suggesting that these three miRNAs may be coordinately regulated via common *cis*-acting elements during metamorphosis. In S2 cells, Ecd and the juvenile hormone analog methoprene exert opposite effects on the expression of these four miRNAs, indicating the participation of both these hormones in the temporal regulation of *mir-34*, *-100*, *-125*, and *let-7* expression in vivo.

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### Introduction

Complex temporal cues are integrated within developing animals to generate appropriate body structures on schedule. Genetic analysis has revealed molecular pathways controlling the temporal regulation of postembryonic development in *Caenorhabditis elegans* and metamorphosis in *Drosophila melanogaster* (Rougvie, 2001; Thummel, 2001). In *C. elegans*, *lin-4* and *let-7* are essential components of the heterochronic gene pathway (Ambros, 2000). This pathway dictates temporal decisions of cell fate from one larval stage to the next. Upregulation of *lin-4* during the

first larval stage (L1) triggers progression to later developmental programs by translationally repressing its target genes *lin-14* and *lin-28* (Ruvkun and Giusto, 1989; Moss et al., 1997; Feinbaum and Ambros, 1999; Olsen and Ambros, 1999; Seggerson et al., 2002). Similarly, upregulation of *let-7* at the fourth larval stage (L4) promotes the transition from larval to adult programs by repressing its target gene *lin-41* (Slack et al., 2000). *lin-4* and *let-7* are two small noncoding RNAs of 22 and 21 nucleotides, respectively (Lee et al., 1993; Reinhart et al., 2000). *lin-4* inhibits the translation of *lin-14* and *lin-28* by base-pairing to partially complementary sites in the 3'-UTRs of their mRNAs (Olsen and Ambros, 1999; Seggerson et al., 2002). *let-7* is thought to inhibit *lin-41* expression in a similar fashion through binding to complementary sites in its 3'UTR (Slack et al., 2000). *let-7* RNA has been widely conserved throughout the

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Table 1  
*Drosophila* miRNAs

Gene	Probe sequence <sup>A</sup>	Location	Notes
<i>mir-1</i>	CATACTTCTTTACATTCCA	38D1	B
<i>mir-2a</i>	GCTCATCAAAGCTGGCTGTGATA	37E3-38A2	B, C, D
<i>mir-2b</i>	GCTCCTCAAAGCTGGCTGTGATA	28B-29X, 37E3-38A2	B, C, D
<i>mir-2c</i>	GCCCATCAAAGCTGGCTGTGATA	88E7-88F4	C, E, F
<i>mir-3</i>	TGAGACACACTTTGCCAGTGA	56D7-56E3	C, D
<i>mir-4</i>	TCAATGGTTGTCTAGCTTTAT	56D7-56E3	C, D
<i>mir-5</i>	CATATCACAAACGATCGTTCCCTTT	56D7-56E3	C, D
<i>mir-6</i>	AAAAAGAACAGCCACTGTGATA	56D7-56E3	C, D
<i>mir-7</i>	ACAACAAAATCACTAGTCTTCCA	57A3-57B3	D
<i>mir-8</i>	GTCATCTTTACCTGAGTATTA	53D2-53E10	D
<i>mir-9</i>	TCATACAGCTAGATAACCAAAGA	76B3-76D1	D
<i>mir-10</i>	ACAAATTCGGATCTACAGGGT	84A5-84C1	D
<i>mir-11</i>	GCAAGAACTCAGACTGTGATG	93E4-93F3	D
<i>mir-12</i>	ACCAGTACCTGATGTAATACTCA	13C5-13E14	D
<i>mir-13a</i>	ACTCATCAAAATGGCTGTGATA	88E7-88F4	D
<i>mir-13b</i>	ACTCGTCAAAATGGCTGTGATA	8C7-8D10, 88E7-88F4	C, D
<i>mir-14</i>	TAGGAGAGAGAAAAAGACTGA	45C5-45F4	C, D
<i>mir-34</i>	CAACCAGCTAACACACTGCC	85F10-85F12	E, G
<i>mir-79</i>	GCTTTGGTAATCTAGCTTTAT	36A6-36A7	E, G
<i>mir-84a</i>	ACAATATTACATACTA <b>ATGAT</b>	U	G
<i>mir-84b</i>	ACAATATTACATACTA <b>CATTC</b>	39E1-40A2	G
<i>mir-87</i>	CACACCTGAAATTTTGTCTCA	30C7-30F4	E, H
<i>mir-92</i>	CAGGCCGGGACAAGTGCAATG	96F-96F	E, I
<i>mir-100</i>	CACAAGTTCCGATTTACGGGTT	36E5-36F2	C, E, I
<i>mir-124b</i>	ATAAGGCACGGGTGAATGCCAA	36D-36D	E, J
<i>mir-125b</i>	TCACAAGTTAGGGTCTCAGGGA	36E5-36F2	C, J
<i>mir-133</i>	ACAGCTGGTTGAAGGGGACCAA	38C-38D	E, J
<i>let-7</i>	ACTATACAACCTACTACCTCA	36E5-36F2	C, K, L

<sup>A</sup>StarFire (IDT) DNA oligonucleotides probe sequences were used for Northern detection of miRNAs. Bold bases indicate mismatches between the original and *Drosophila* miRNA sequences.

<sup>B</sup>Cloned from *C. elegans* (Lau et al., 2001; Lee and Ambros, 2001).

<sup>C</sup>*mir-2a-1*, *-2a-2*, and *-2b-2* sequences are clustered within 400 bp in 37E3-38A2; *mir-3*, *-4*, *-5*, *-6-1*, *-6-2*, and *-6-3* within 900 bp in 56D7-56E3; *mir-2c*, *-13a*, and *-13b-1* within 400 bp in 88E7-88F4; *mir-100*, *-125*, and *let-7* within 800 bp in 36E5-36F2.

<sup>D</sup>Cloned from *Drosophila* and expression confirmed by Northern blot analysis (Lagos-Quintana et al., 2001).

<sup>E</sup>Expression of predicted conserved miRNAs in *Drosophila* confirmed by Northern blot analysis in this work.

<sup>F</sup>*mir-2c* sequence was predicted by BLAST homology to *mir-2a*.

<sup>G</sup>Cloned from *C. elegans* and predicted to be conserved in *Drosophila* (Lau et al., 2001).

<sup>H</sup>Cloned from *C. elegans* and predicted to be conserved in *Drosophila* (Lee and Ambros, 2001).

<sup>I</sup>Cloned from *Homo sapiens* and predicted to be conserved in *Drosophila* (Mourelatos et al., 2002).

<sup>J</sup>Cloned from *Mus musculus* and predicted to be conserved in *Drosophila* (Lagos-Quintana et al., 2002).

<sup>K</sup>*let-7* was initially cloned from *C. elegans* by classical genetics methods using transformation rescue of *let-7* mutant phenotype (Reinhart et al., 2000).

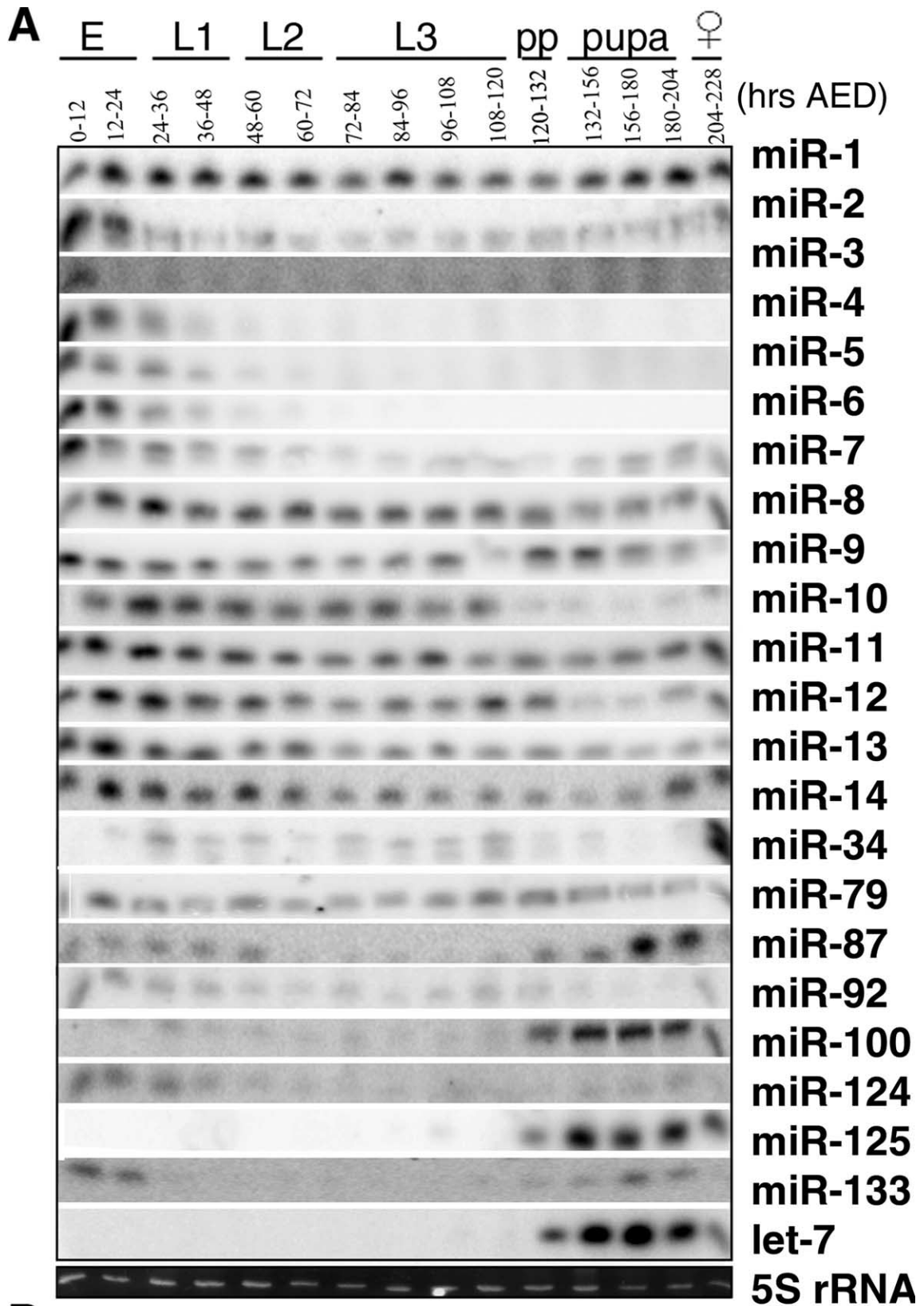
<sup>L</sup>Expression of predicted conserved *let-7* in *Drosophila* confirmed by Northern blot analysis (Pasquinelli et al., 2000).

bilaterian clade from mollusks to flies to humans (Pasquinelli et al., 2000). As in worms, *let-7* RNA is developmentally upregulated in *Drosophila* and other invertebrates at the larval to adult transition. In vertebrates, *let-7* is also developmentally upregulated, but without a common temporal pattern among species (Pasquinelli et al., 2000; L.F.S. and V.A., unpublished observations). Although the *lin-4* RNA sequence is unique to nematodes, a putative *lin-4* homologue, *miR-125*, has recently been identified in flies

and mammals that is only slightly divergent from the *lin-4* sequence (Lagos-Quintana et al., 2002).

*lin-4* and *let-7* belong to an extensive class of genes with small noncoding RNA transcripts (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001, 2002; Mourelatos et al., 2002), termed microRNAs (miRNAs). miRNAs are 18–25 nucleotides in length and result from the processing of a  $\approx 70$ -nucleotide hairpin by Dicer-dependent cleavage (Grishok et al., 2001; Hutvagner et al., 2001; Lee

Fig. 1. Developmental profile of miRNAs in the wild type. (A) Synchronously developing Canton-S flies were harvested from embryos (E), larvae (L1–L3), prepupae (pp), pupae (pupa), and adult females (♀) at hours after egg deposition (AED) indicated at top. Our hybridization conditions could not distinguish among very closely related miRNAs (see Table 1), so miR-2 and miR-13 signals represent a composite of miR-2a,b,c, and miR-13a,b, respectively. 5S rRNA was used as loading control. (B) Schematic representation of 20-hydroxyecdysone (Ecd) and juvenile hormone (JH) titer fluctuations during development (adapted from Riddiford, 1993).



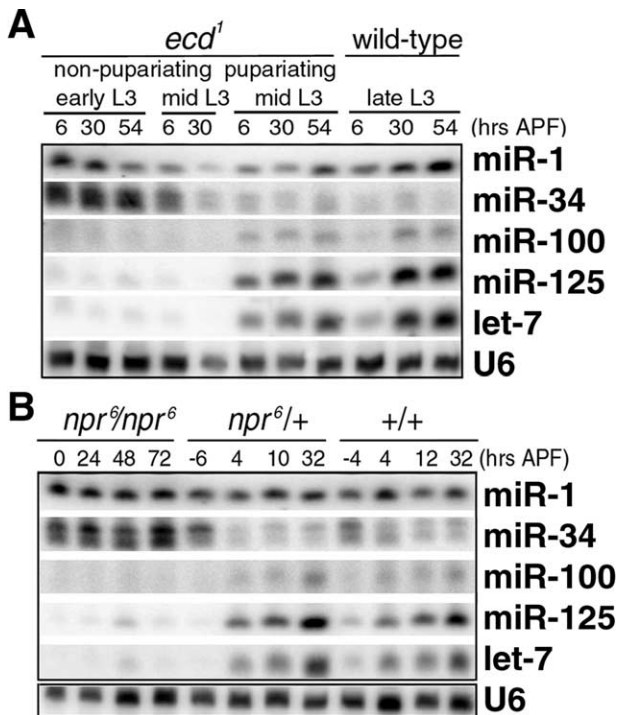


Fig. 2. miR-34 levels increase, and miR-100 and miR-125 levels decrease in *ecd*<sup>1</sup> and *npr*<sup>6</sup> mutants. (A) *ecd*<sup>1</sup> animals reared at 20°C were transferred to 29°C during early and mid-L3 stage and harvested at time intervals after puparium formation (APF). The time of PF for *ecd*<sup>1</sup> animals upshifted in the early L3 was defined by the time of PF of *ecd*<sup>1</sup> animals grown in parallel at 20°C. *ecd*<sup>1</sup> animals upshifted in mid L3 were harvested in two separate populations (nonpupariating and pupariating). The profile of miRNA RNAs accumulation in wild-type animals upshifted during the late L3 is shown for comparison. (B) Synchronously developing *npr*<sup>6</sup>/*npr*<sup>6</sup> and *npr*<sup>6</sup>/+ siblings were harvested at the late L3 and at time intervals during prepupal and pupal stages, relative to the time of PF in *npr*<sup>6</sup>/+ animals. The profile of miRNA RNAs in the wild type is shown for comparison. *mir-1*, which is constitutively expressed during development, was unaffected by *ecd*<sup>1</sup> and *npr*<sup>6</sup> mutations. U6 was used as loading control.

and Ambros, 2001). A number of the known miRNAs are evolutionary conserved from nematodes and insects to mammals, suggesting that they carry out important and perhaps conserved functions (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001, 2002; Mourelatos et al., 2002).

In *Drosophila*, the sesquiterpenoid juvenile hormone (JH) and steroid 20-hydroxyecdysone (Ecd) control temporal aspects of development from embryogenesis to adulthood (Riddiford, 1993; Thummel, 1995, 1996; Richards, 1997). Cellular and molecular responses to Ecd have been studied extensively in *Drosophila* through the genetic analysis of Ecd-induced metamorphosis (Thummel, 1996; Richards, 1997). The rise of Ecd titer at the end of L3 is transduced via an Ecdysone Receptor (*EcR*)/Ultraspiracle (*USP*) heterodimeric complex (Thomas et al., 1993; Yao et al., 1993) which, in turn, activates a primary cascade of transcriptional factors or early-inducible genes: *E74*, *E75*, *Broad-Complex* (*BR-C*) (Burtis et al., 1990; Segaves et al., 1990; Thummel

et al., 1990; DiBello et al., 1991). These “early-genes” coordinate the temporal and spatial activation of late genes, which either directly or indirectly, carry out distinct metamorphic processes such as puparium formation, programmed cell death of larval tissues, remodeling of the central nervous system, and proliferation and morphogenesis of imaginal discs (Ashburner et al., 1974; Richards, 1981; Thummel, 1996). The *BR-C* locus encodes a family of zinc-finger transcriptional factors (Bayer et al., 1997) and plays a pivotal role in the initiation and progression through metamorphosis (Kiss et al., 1988; DiBello et al., 1991; Karim et al., 1993). The misexpression of *BR-C* during L2 precociously activates pupal-cuticle genes, suggesting that *BR-C* is sufficient to specify some of the processes during pupal development (Zhou and Riddiford, 2002). In addition, the exogenous application of a JH analog at the onset of metamorphosis phenocopies loss-of-function *BR-C* mutations (Restifo and Wilson, 1998). Thus, at the end of L3, *BR-C* activity integrates signals resulting from a pulse of Ecd and a decrease of JH titer, to effect a switch from larval to pupal temporal programs.

We have previously reported that the regulation of *let-7* RNA expression during metamorphosis in *Drosophila* requires both the Ecd pulse at the end of the L3 as well as *BR-C* activity (Sempere et al., 2002). Thus, we were interested in investigating whether other miRNAs were temporally regulated and, furthermore, whether this regulation was also mediated by hormones. In this report, we surveyed the developmental expression profile of 24 miRNAs cloned from or predicted to be conserved in *Drosophila*. *Drosophila* miRNAs exhibit a variety of in vivo developmental profiles and most appear unresponsive to Ecd or JH in S2 cells. However, the expression of four miRNAs, *mir-34*, *mir-100*, *mir-125*, and *let-7*, undergo temporal changes at larval-to-pupal or pupal-to-adult stages, and respond to Ecd and/or JH in S2 cells. Ecd and JH have opposite effects on expression of these miRNAs, consistent with their opposing roles in vivo. Furthermore, from studies of mutants defective for either Ecd biosynthesis (*ecd*<sup>1</sup>) or *BR-C* activity (*npr*<sup>6</sup>), we show that Ecd and *BR-C* are required for the temporal upregulation of *mir-100*, *mir-125*, and *let-7*, and the temporal downregulation of *mir-34* expression during metamorphosis. This coregulation of *mir-100*, *mir-125*, and *let-7* expression may result in part from their tight chromosomal linkage in *Drosophila*.

## Materials and methods

### *Drosophila*-stocks, S2 cells, and hormonal treatments

*Drosophila* stocks were maintained on a standard cornmeal-yeast-agar medium at 25°C. *npr*<sup>6</sup> and *ecd*<sup>1</sup> animals were handled as described in Sempere et al. (2002). For developmental expression profiles, synchronous developing embryos and larvae or pupae were harvested every 12 or

24 h, respectively. Animals were synchronized from egg laying; after hatching, first-instar larvae were hand-collected in groups of 50–70 animals and placed on a standard agar media supplied with a yeast paste. Animals were synchronized again at puparium formation; pupariating animals in a 2-h interval were collected in groups of 50 and placed on petri dishes with a moist paper towel to maintain a humid environment.

*Drosophila* S2 cells were cultured in Schneider's medium (Gibco) supplemented with 10% fetal bovine serum at 23°C. Hormonal treatments were as follows: cells were plated in 25-cm<sup>2</sup> flasks containing 4 ml of medium and allowed to grow for 48 h, when they reach 80% of confluence. Then, 20-hydroxyecdysone (Sigma) was added to a final concentration of  $5 \times 10^{-6}$  M, and/or methoprene (ZR515; gift from Dr. Cerf at Sandoz) was added to a final concentration of  $1 \times 10^{-4}$  M. Control cells were treated with an equal volume of solvent.

#### Northern blot analysis

Total RNA was extracted from animals or S2 cells by using Trizol (Gibco). Animals were homogenized in 2 mL of Trizol with a dounce glass homogenizer prior to extraction. Northern blots were conducted as described in Sempere et al. (2002), with the difference that GeneScreen Plus (New England Nuclear) membrane was used. Radioactive-labeled StarFire (Integrated DNA Technologies) oligonucleotide probes used for miRNA detection are listed in Table 1. Membranes were stripped in 0.1% SDS boiling solution for 30 min, and hybridized with different probes. Membranes were stripped up to five times without a noticeable decrease in intensity or quality of signal detection. Radioactive signals were quantified with ImageQuant software package (Molecular Dynamics). The relative levels of miRNA transcripts were represented as the ratio of the miRNA and U6 signals and were normalized to a 0–1 scale.

## Results and discussion

### miRNAs display diverse developmental expression profiles in *D. melanogaster*

To test whether other miRNAs are temporally regulated in *Drosophila*, we examined the developmental expression profile of 24 miRNAs. Fourteen of these miRNAs (*mir-1* to *mir-14*) had been identified by cDNA cloning from *Drosophila* RNA (Lagos-Quintana et al., 2001), while the other ten (*mir-34*, -79, -84, -87, -92, -100, -124, -125, -133, and *let-7*) had been cloned from other species (Reinhart et al., 2000; Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2002; Mourelatos et al., 2002) (see Table 1). Of these latter miRNAs, *mir-34*, -79, -84, -87, -92, -124

(2001; Lagos-Quintana et al., 2002; Mourelatos et al., 2002) and *mir-100* and *mir-133* (this work) were predicted to be conserved based on homology of miRNA sequence and hairpin fold of their precursors, while *mir-125* and *let-7* had been reported to be expressed in *Drosophila* (Pasquinelli et al., 2000; Lagos-Quintana et al., 2002). By Northern blot analysis, we confirmed the expression of *mir-1* to *mir-14* and validated the conservation of 7 out of these 8 predicted miRNAs: *mir-34*, -79, -87, -92, -100, -124, and -133 (Fig. 1). We could not detect any signal with probes for *mir-84* (data not shown). Most of the miRNAs that were originally cloned from 0- to 6-h *Drosophila* embryos (*mir-1* to *mir-14*) are either embryonically enriched or constitutively expressed throughout development, as shown previously (Lagos-Quintana et al., 2001). The miRNAs that were originally identified in other species exhibit more diverse expression patterns. Twelve of these 23 expressed miRNAs are developmentally upregulated or downregulated in association with embryonic to larval (*mir-3*, -4, -5, and -6), larval to pupal (*mir-10*, -34, -87, -92, -100, -125, -133, and *let-7*), or pupal to adult transitions (*mir-34*). Some miRNAs are arranged in clusters on the genome; for example, *mir-2b2/2a-1/2a-2* are located in 37E3-38A2 and *mir-3/4/5/6-1/6-2/6-3* are located in 56D7-56E3 (Lagos-Quintana et al., 2001), *mir-100/let-7/mir-125* are located in 36E5-36F2 and *mir-2c/13a/13b* are located in 88E7-88F4 (see Table 1). In general, the members of a miRNA cluster seem to have very similar expression patterns. In particular, *mir-100*, *mir-125*, and *let-7* transcripts are clustered in an 800-bp region and are oriented in the same direction, suggesting that the transcription of *mir-100* and *mir-125* is likely coregulated with that of *let-7* by common *cis*-acting elements.

### Ecdysone and Broad Complex are required for regulation of *mir-34*, *mir-100*, and *mir-125* expression during metamorphosis

A rise in Ecd titers at the end of L3 triggers the initiation of metamorphosis, marked by puparium formation (Thumel, 1996; Richards, 1997). During this same developmental stage, *mir-100* and *mir-125* expression increases, while *mir-34* expression decreases (Fig. 1). To test whether Ecd was required for *mir-34*, -100, and -125 regulation, we used the temperature-sensitive *ecd<sup>l</sup>* strain that is impaired in the biosynthesis of Ecd at the restrictive temperature (29°C) (Garen et al., 1977). In *ecd<sup>l</sup>* animals blocked from pupariation by a transfer at 29°C during early or mid L3, miR-34 was detected at notably higher levels, whereas miR-100 and miR-125 were detected at dramatically lower levels compared with wild type (Fig. 2A). In *ecd<sup>l</sup>* animals that escaped the pupariation block during mid L3, miR-34, -100, and -125 were detected at levels similar to wild type animals. *Broad-Complex (BR-C)* is an early Ecd-inducible gene that plays a pivotal role in relaying the Ecd signal at the end of L3 to downstream genes, initiating a genetic cascade that leads to the execution of metamorphic programs (Kiss et al.,

1988; DiBello et al., 1991; Karim et al., 1993; Zhou and Riddiford, 2002). *npr<sup>6</sup>* animals, which lack all *BR-C* transcriptional factor isoforms, never pupariate, and remain as larvae for 5 to 10 days past the normal time of pupariation. To test whether *BR-C* played a role in the regulation of *mir-34*, *-100*, and *-125*, we examined the expression profile of these miRNAs in *npr<sup>6</sup>* animals by Northern blot analysis. miR-100 and miR-125 were detected at much lower levels in nonpupariating homozygous *npr<sup>6</sup>* animals than in *npr<sup>6</sup>/+* or wild-type animals. By contrast, miR-34 was detected at higher levels in *npr<sup>6</sup>* compared with *npr<sup>6</sup>/+* or wild-type animals (Fig. 2B). These results suggest that Ecd and *BR-C* activity are required for the temporal upregulation of *mir-100* and *mir-125* expression and the downregulation of *mir-34* during pupal development. The expression pattern of *mir-1*, which is constitutively expressed during development, was not affected by *ecd<sup>1</sup>* or *npr<sup>6</sup>* mutations, indicating that disruption of Ecd gene pathway has not a general effect on miRNA biogenesis.

*Ecdysone and Juvenile hormone exert opposite effects on the expression of mir-34, mir-100, mir-125, and let-7 in S2 cells*

Although Ecd is the major ecdysteroid produced at the end of L3, other ecdysteroids are also present in the hemolymph and may elicit some developmental signals at the time of pupariation (Fisk and Thummel, 1998). Moreover, since biosynthesis of all ecdysteroids is blocked in *ecd<sup>1</sup>* mutants (Warren et al., 1996), we could not definitively attribute the effect of *ecd<sup>1</sup>* on miRNA expression to decreased levels of Ecd alone. To test whether Ecd was sufficient to regulate *mir-34*, *-100*, and *-125* expression, S2 cells (Schneider and Blumenthal, 1978; Vitek and Berger, 1984; Cherbas and Cherbas, 1981, 1998) were incubated with Ecd for 24–42 h. Total RNA was extracted from samples harvested at 6-h intervals and analyzed by Northern blots. Longer incubation times of S2 cells with Ecd correlated with increased levels of miR-100 and miR-125 (Fig. 3A). In contrast, miR-34 was detected at very low levels at all times examined compared to untreated cells. Thus, the addition of Ecd results in induction of *mir-100* and *mir-125* and repression of *mir-34* expression. These observed effects of Ecd in S2 cells are consistent with repression of *mir-34* and activation of *mir-100*, *mir-125*, and *let-7* expression by an Ecd signal during *Drosophila* development.

Juvenile hormone (JH) can induce the expression of some genes as well as repress expression of other genes activated by Ecd (Berger et al., 1992; Restifo and Wilson, 1998; Feng et al., 1999; Dubrovsky et al., 2000, 2002; Zhou and Riddiford, 2002). Conditional mutants affecting JH biosynthesis are currently unavailable, so we tested whether application of JH to cultured cells could affect expression of these developmentally regulated miRNAs. S2 cells were treated with the JH analog, methoprene, or a combination of methoprene and Ecd and incubated for 24–42 h (Fig. 3A).

We found that *mir-34*, *mir-100*, *mir-125*, and *let-7* expression responded to JH treatment. miR-34 accumulated in cells treated with methoprene to levels higher than in untreated cells. Significant induction of *mir-34* expression by methoprene could be observed after 8 h of incubation (data not shown). This indicates that JH acts positively on *mir-34* expression, possibly by stimulating *mir-34* transcription. The enhanced expression of *mir-34* induced by methoprene was suppressed when S2 cells were incubated with both methoprene and Ecd, indicating that JH and Ecd have opposite effects on *mir-34* expression. *mir-100*, *-125*, and *let-7* RNA levels were significantly reduced in cells treated with both methoprene and Ecd compared with cells treated with Ecd alone, indicating that the JH analog inhibits the Ecd-induction of *mir-100*, *-125*, and *let-7*. Since the temporal expression profiles of *mir-34*, *-100*, *-125*, and *let-7* overlap major changes in Ecd and JH titers during development, the results reported here suggest that their developmental regulation could be modulated by opposing JH/Ecd input signals in vivo (Fig. 4B). Previous studies have shown that exogenous application of JH can antagonize some functions of *BR-C* (Restifo and Wilson, 1998; Zhou and Riddiford, 2002). Thus, these opposite effects of JH and Ecd signals on *mir-34*, *-100*, *-125*, and *let-7* expression could be mediated by *BR-C* (Fig. 4B).

The expression of *mir-87* and *mir-133*, like *mir-100*, *-125*, and *let-7*, is clearly upregulated during metamorphosis in wild type animals (Fig. 1). Similarly, the expression of *mir-10* and *mir-92*, like *mir-34*, decreases during pupal development. However, unlike *mir-34*, *-100*, *-125*, and *let-7*, our experimental manipulation of Ecd or JH signaling in S2 cells did not affect the expression of *mir-10*, *-87*, *-92*, or *-133* (data not shown), suggesting that temporal signals, other than Ecd and JH, regulate the developmental expression of these miRNAs.

*Broad-Complex mediates the ecdysone-dependent regulation of mir-100 and mir-125 expression in S2 cells*

The 24-h lag between the addition of Ecd to cultured S2 cells and the expression of *mir-100* and *mir-125* suggest that the initial Ecd signal activates *mir-100* and *mir-125* expression indirectly via intermediate regulators. One such intermediate could be *BR-C*, which we have shown is required for *mir-100* and *mir-125* expression in animals. To test whether *BR-C* activity was required for the Ecd-induced expression of *mir-100* and *mir-125* in S2 cells, *BR-C* activity was inhibited by RNAi using a 700-nucleotide dsRNA corresponding to a common region of all *BR-C* isoforms (Sempere et al., 2002). S2 cells were incubated for 30 min with *BR-C* dsRNA or mock dsRNA corresponding to unrelated *C. elegans* sequence. Then, the transfected and non-transfected cultures were treated with Ecd and harvested 32, 40, and 48 h later. The levels of *mir-100* and *mir-125* RNAs were detected at considerably lower in *BR-C* RNAi cells compared with nontransfected or mock RNAi cells (Fig.

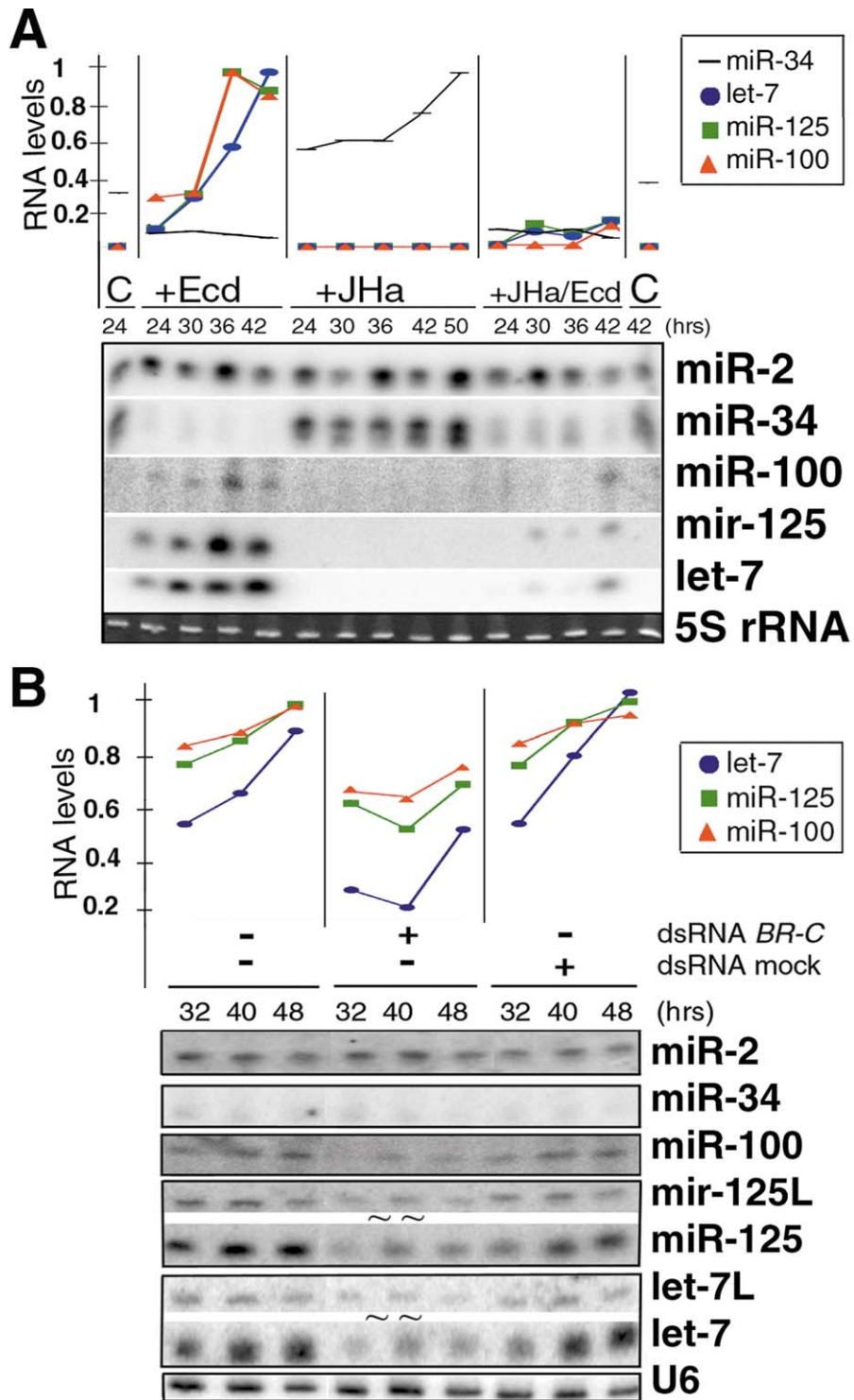


Fig. 3. Ecd and JH signaling pathways exert opposite effects on *mir-34*, *-100*, *-125*, and *let-7* expression in S2 cells. (A) S2 cells were harvested after 24–42 h of incubation with 20-hydroxyecdysone (+Ecd), the JH analog, methoprene (+JHa), or Ecd and methoprene (+JHa/Ecd), or just solvent (C). 5S rRNA was used as loading control. (B) S2 cells were transfected with 40  $\mu$ g of dsRNA corresponding to either a conserved region of all *BR-C* mRNA isoforms or a nonspecific dsRNA (mock) from *C. elegans*. Then, transfected and control cells were treated with Ecd, harvested 32, 40, and 48 h later, and probed for *miR-34*, *-100*, *-125*, and *let-7* RNA. U6 was used as loading control. *mir-2*, which is constitutively expressed in S2 cells, was used as a control gene for the specificity of a hormonal and RNAi effect. (A, B) Above blot panels, quantification of miRNA levels is expressed in relative signal units.



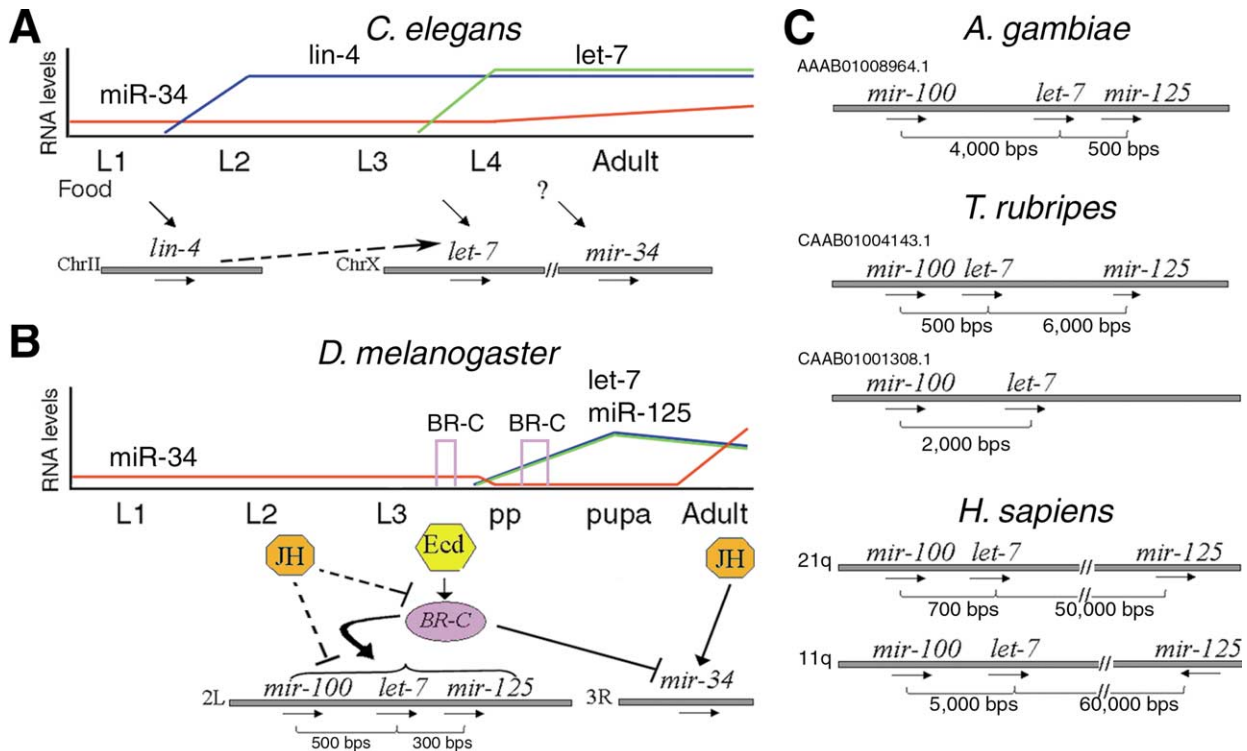


Fig. 4. Model for temporal and hormonal regulation of miRNAs. (A) In *C. elegans*, the *lin-4* and *let-7* genes are unlinked and exhibit distinct temporal expression profiles (Lee et al., 1993; Reinhart et al., 2000). The upregulation of *lin-4* near the end of L1 sets an early clock that dictates the execution of L2 and L3 developmental programs. This early clock controls the upregulation of *let-7* at the late L3, which sets a late clock that dictates the transition from L4 to adult programs. (B) In *Drosophila*, intermediate levels of JH and Ecd correlate with moderate induction of *mir-34* expression during larval stages. A pulse of Ecd at the end of L3 acts via *BR-C* to trigger the coordinated upregulation of the *mir-100*, *-125*, and *let-7* gene cluster and downregulation of *mir-34* expression. Our results could not distinguish whether JH opposes the Ecd-induced expression of *mir-100*, *mir-125*, and *let-7* via *BR-C* or acts independently of *BR-C*. At adult eclosion, rising levels of JH correlate with full induction of *mir-34* expression. (C) *mir-100*, *-125*, and *let-7* are clustered in the genomes of the mosquito *Anopheles gambiae*, the Puffer fish *Takifugu rubripes* and humans. Clusters are shown on BAC sequences for the unassembled genomes of *A. gambiae* and *T. rubripes*, and on chromosomes for humans. In the CAAB01001308.1 cluster, either *mir-125* is not present or it is beyond the end of clone sequence, which is about 60,000 bp downstream of *let-7*.

3B). This result further supports the conclusion that *BR-C* is required to mediate the activation of *mir-100* and *mir-125* by an Ecd signal in vivo (Fig. 4B). This result also argues against the possibility that *mir-100*, *mir-125*, and *let-7* RNAs were detected at very low levels in nonpupariating *ecd<sup>1</sup>* and *npr<sup>6</sup>* mutants (Fig. 2) simply because these mutant animals were arrested in a stage before *mir-100*, *mir-125*, and *let-7* are normally upregulated.

It should be noted that *BR-C* RNAi did not result in complete loss of *mir-100* and *mir-125* expression, suggesting that RNAi treatment was not fully effective. Consistent with an incomplete knockdown of *BR-C* by RNAi, *mir-34* levels were unaffected by *BR-C* RNAi in Ecd-treated cells (Fig. 3B). Based on our results with *npr<sup>6</sup>* mutant animals, one would have expected that *mir-34* expression should be derepressed by *BR-C* RNAi in Ecd-treated cells. Since *BR-C* activity may not have been completely eliminated by RNAi, we could not assess the requirement for *BR-C* activity in the repression of *mir-34* in S2 cells.

#### *The clustered arrangement of mir-100, let-7, mir-125 genes is evolutionary conserved in bilaterians*

Interestingly, the sequence of *mir-125* is quite similar to the sequence of *lin-4* (Lagos-Quintana et al., 2002), suggesting that they may be homologues. The evolutionary conservation of microRNAs such as *mir-125/lin-4*, *mir-100*, and *let-7* implies the conservation of multiple complementary target sequences for each of the miRNAs. In *C. elegans*, *lin-4* translationally downregulates LIN-14 and LIN-28 protein expression by base-pairing to partially complementary sites in the 3'-UTR of their mRNAs (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Feinbaum and Ambros, 1999; Olsen and Ambros, 1999; Seggerson et al., 2002). Translational repression of *lin-14* and *lin-28* activities by *lin-4* is required for the temporal transition from early to late developmental programs, eventually leading to the adult differentiation of hypodermis and vulva. Although *lin-28* is a member of an evolutionary conserved gene fam-



ily (Moss et al., 1997), no obvious *Drosophila* or human homologues of *lin-14* have been found and the targets of *mir-125* in flies and vertebrates are yet to be identified.

The finding that the *mir-100*, *-125*, and *let-7* gene cluster is coregulated in *Drosophila* suggests that these three genes may act in concert to control the translation of target genes involved in adult morphogenesis and differentiation. In a recent study, Bashirullah et al. (2003) similarly found that *mir-125* and *let-7* are coordinately upregulated at the onset of metamorphosis (Bashirullah et al., 2003; in press). *mir-100*, *-125*, and *let-7* are quite distinct in sequence, and so they probably base-pair to distinct cognate binding sites in their target mRNAs. These three miRNAs could have unique target mRNAs that they repress in parallel, and/or they could act together to repress particular targets that contain the appropriate combination of cognate sites.

With the exception of *C. elegans*, the chromosomal clustering of *mir-100*, *mir-125/lin-4*, and *let-7* appears to be widely conserved in animal phylogeny (Fig. 4). In the mosquito *Anopheles gambiae*, *mir-100*, *-125*, and *let-7* are clustered within 5000 bp and oriented in the same direction (Fig. 4C). In vertebrates, *mir-100*, *-125*, and *let-7* are similarly clustered, although with somewhat greater spacing (Fig. 4C). Remarkably, *mir-100* and *let-7* are located together in two distinct chromosomal locations within 500 and 2000 bp in the Puffer fish *Takifugu rubripes*, and within 700 bp and 5000 bp in humans. The conservation of the clustered arrangement of *mir-100*, *mir-125*, and *let-7* suggests that important aspects of their regulation may also be evolutionarily conserved.

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