MicroRNAs and the Evolution of Insect Metamorphosis

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Abstract

MicroRNAs (miRNAs) are involved in the regulation of a number of processes associated with metamorphosis, either in the less modified hemimetabolan mode or in the more modified holometabolan mode. The miR-100/let-7/miR-125 cluster has been studied extensively, especially in relation to wing morphogenesis in both hemimetabolan and holometabolan species. Other miRNAs also participate in wing morphogenesis, as well as in programmed cell and tissue death, neuromaturation, neuromuscular junction formation, and neuron cell fate determination, typically during the pupal stage of holometabolan species. A special case is the control of miR-2 over Kr-h1 transcripts, which determines adult morphogenesis in the hemimetabolan metamorphosis. This is an elegant example of how a single miRNA can control an entire process by acting on a crucial mediator; however, this is a quite exceptional mechanism that was apparently lost during the transition from hemimetaboly to holometaboly.

miRNA: microRNA

20E: 20-hydroxyecdysone

JH: juvenile hormone

EcR: ecdysone receptor

bHLH-PAS: basic helix–loop–helix–PER–ARNT–SIM

INTRODUCTION

Insect metamorphosis originated in the early Devonian, approximately 400 million years ago, with the innovation of wings and emergence of the Pterygota (33). Hemimetaboly, arguably the most primitive mode of metamorphosis, establishes the essential adult body plan during embryogenesis; changes occurring during postembryonic development are limited to general growth during nymphal instars and complete morphogenesis of wings and genitalia during the adult molt (6). The phylogenetically more basal pterygote insects, from Palaeoptera to Condylognatha (33), present hemimetabolan metamorphosis. Holometabolan metamorphosis originated in the Carboniferous, approximately 350 million years ago. In this mode, the adult body plan is formed in the pupal stage rather than throughout embryo development. Thus, juveniles can develop a body plan capable of exploiting resources that differ dramatically from those exploited by adults (6). This accounts for the spectacular evolutionary success of holometabolan insects, which represent more than half of the world's eukaryotic biodiversity (18).

Despite the important role of metamorphosis in the evolutionary success of insects, the mechanisms that they use to regulate the process are not completely understood. The best-known layer of regulation is the endocrine, which is based on the effects of juvenile hormones and ecdysteroids, for which our knowledge is not limited to just chemical and physiological aspects but extends to molecular mechanisms of action (see below). Another layer of regulation recently discovered is based on microRNAs (miRNAs), which are short (ca. 22 nucleotides), single-stranded, noncoding RNAs that operate by blocking target mRNA through base pairing between the miRNA and its complementary match sequence in the mRNA (3). Data available indicate that miRNAs must be taken into account when trying to understand the finely tuned regulation of any biological process (4, 20). Evidence that miRNAs influence the regulation of insect metamorphosis has been accumulating over the last 10 years and recent reviews on insect miRNAs (2, 7, 30) have evaluated information available in a general context. The aim of the present review is to integrate the specific information regarding miRNAs and metamorphosis into a coherent scheme, comparing hemimetabolan and holometabolan species and inferring general trends in an evolutionary frame.

ENDOCRINE-BASED MECHANISMS REGULATING INSECT METAMORPHOSIS

Endocrine regulation of metamorphosis is based on two hormones: (*a*) 20-hydroxyecdysone (20E), which is the most common biologically active form of ecdysone, and (*b*) juvenile hormone (JH). Mostly, 20E promotes molting, whereas JH prevents metamorphosis; thus, when 20E acts in concert with JH, molting leads to a juvenile stage, and when it acts in the virtual absence of JH it triggers metamorphosis (52). At the molecular scale, both hormones act through a cascade of transcription factors that transduce the hormonal signal. In the case of 20E, its heterodimeric receptor is composed of two nuclear receptors: the ecdysone receptor (EcR), to which 20E actually binds, and the ultraspiracle (USP). Upon binding, the 20E-receptor complex activates the expression of a hierarchy of genes that code for transcription factors (e.g., *HR3*, *HR4*, *HR39*, *Broad complex*, *E75*, and *FTZ-F1*). These transcription factors in turn lead to the regulation of genes that determine the cellular changes associated with molting and metamorphosis (26, 35).

The MEKRE93 Pathway

The molecular action of JH is mediated by its receptor, a heterodimer composed of two bHLH-PAS proteins: Methoprene-tolerant (Met), to which JH actually binds, and the coreceptor Taiman.

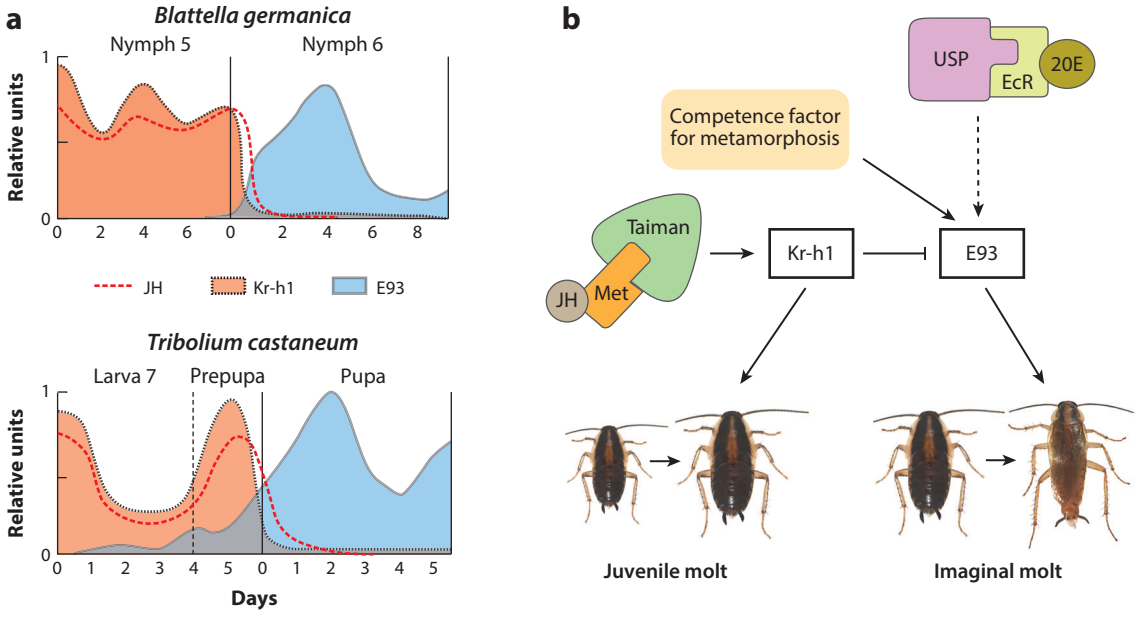

Figure 1

Hormones and metamorphosis. (*a*) mRNA expression pattern of *Krüppel homolog 1* (*Kr-h1*) and *E93* in the last juvenile instars of the hemimetabolan *Blattella germanica* and the holometabolan *Tribolium castaneum*; data are from Belles & Santos (8) and Ureña et al. (54). Profile of circulating juvenile hormone (JH) is also indicated; data for *B. germanica* are from Treiblmayr et al. (51). As there are no data available on JH in *T. castaneum*, the profile shown corresponds to the lepidopteran *Manduca sexta* measured in the indicated stages; data from Riddiford et al. (40). (*b*) The MEKRE93 pathway. The JH-receptor complex, composed of Methoprene-tolerant (Met) and Taiman, induces the expression of the transcription factor *Kr-h1* that subsequently represses the expression of *E93*, which is the trigger of metamorphosis (8, 24). *E93* expression would be promoted by ecdysone signaling, initiated by the binding of 20-hydroxyecdysone (20E) to the ecdysone receptor (EcR) and the formation of the heterodimeric receptor with ultraspiracle (USP). A competence factor for metamorphosis of unknown nature should also contribute to promote the expression of *E93*.

The JH-receptor complex induces the expression of the transcription factor Krüppel homolog 1 (Kr-h1). This subsequently represses the expression of gene *E93*, which is the trigger of metamorphosis (8, 54). The essential axis Met → Kr-h1 ⊣ E93, or MEKRE93 pathway (8, 24), is conserved in both hemimetabolan and holometabolan species and switches metamorphosis on and off in late juvenile instars when the insect is competent to undergo metamorphosis (46). Briefly, at the beginning of the last juvenile instar, JH production ceases, *Kr-h1* transcription declines, and *E93* expression increases (possibly under the action of 20E signaling along with the action of a competence factor related to critical size); collectively, these changes promote metamorphosis (**Figure 1**).

miRNAs INVOLVED IN METAMORPHOSIS: THE miR-100/let-7/miR-125 CLUSTER

The first miRNAs to be described, lin-4 and let-7, were discovered in the nematode *Caenorhabditis elegans*. Further studies showed that 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 miRNA alters the timing of cell fate determining events (34). In the fruit fly *Drosophila melanogaster*, let-7 is coexpressed with the lin-4 homolog, miR-125, in numerous tissues beginning late in the third larval instar and peaking in pupae during metamorphosis (38, 45). Notably, a

Kr-h1: Krüppel homolog 1

mutation that eliminates let-7 and miR-125 leads to widespread defects during metamorphosis (11). In most insects investigated to date, both let-7 and miR-125 are encoded by the same primary transcript along with a third miRNA, miR-100 (42). Thus, although a number of different miRNAs have been studied in relation to insect metamorphosis, the miR-100/let-7/miR-125 cluster is repeatedly associated with the regulation of this process, as reported in a number of model species from cockroaches to flies. Interestingly, the members of this cluster are among the evolutionarily oldest miRNAs, as miR-100 and miR-125 were acquired in Eumetazoa and let-7 in Bilateria (12, 49), and they are conserved in mammals, including humans (19).

miRNAs AND METAMORPHIC HORMONES

Effects of Hormones on miRNAs

In the hemimetabolan species *Blattella germanica*, high-throughput sequencing of miRNAs identified miR-100, let-7, and miR-125 as the most differentially expressed during the last (metamorphic) nymphal instar with respect to the previous (nonmetamorphic) nymphal instar. Moreover, treatment with 20E tended to increase their expression, whereas simultaneous treatment with 20E and JH had an inhibitory effect (44).

Among holometabolan species, a study using the temperature-sensitive *ecd*^{1} strain of *D. melanogaster* showed that miR-100, let-7, and miR-125 levels were much lower when ecdysteroid synthesis was impaired (45). The role of the *Broad complex*, an early 20E response gene in the ecdysteroid cascade, was also studied using *npr*^{6} specimens of *D. melanogaster*, which lack all Broad complex factors. Results showed that miR-100, let-7, and miR-125 had much lower levels in homozygous *npr*^{6} flies than in *npr*^{6}/+ or wild-type flies, which indicates that ecdysteroid signaling acts through the Broad complex for temporal upregulation of this miRNA cluster (45). Additional experiments with *Drosophila* S2 cells showed that incubation with 20E for periods that extended beyond 30 h correlated with increased levels of miR-100, let-7, and miR-125, whereas the addition of Methoprene, a JH analog, reduced the stimulatory effect of 20E on miR-100, let-7, and miR-125 expression (45). These results bear a close similarity to those obtained by Rubio et al. (44) in the hemimetabolan species *B. germanica*.

Effects of miRNAs on Hormones

In *D. melanogaster*, ecdysteroid signaling through EcR involves a positive autoregulatory loop that increases EcR levels (25). miR-14 modulates this loop by limiting the expression of EcR, whereas ecdysteroid signaling through EcR downregulates miR-14 (55). This modulatory action of miR-14 may be crucial due to the intrinsic lability of the positive autoregulatory loop that controls ecdysteroid signaling. There are no data available on the possible action of miRNAs on JH synthesis or reception, but a study (29) reports on the action of miR-2 on Kr-h1, which is crucial for hemimetabolan metamorphosis, as described in detail below.

miRNAs AND HEMIMETABOLAN METAMORPHOSIS

In insects, the endonuclease dicer-1 processes the miRNA precursor for the formation of the mature miRNA (27). Thus, a straightforward method for exploring whether or not miRNAs influence a biological process is to deplete *dicer-1* expression and examine the effects on the process. This approach was followed in the cockroach *B. germanica* using RNAi experiments, which showed that dicer-1 depletion prevented metamorphosis and thus the last instar nymphs

molted to supernumerary giant nymphs instead of adults (21). This suggests that miRNAs are involved in the metamorphosis of hemimetabolan insects.

Roles of the miR-100/let-7/miR-125 Cluster in Wing Morphogenesis

Comparison of miRNA libraries in the penultimate and last nymphal instars of *B. germanica* revealed that miR-100, let-7, and miR-125 were differentially expressed in the latter stage (44). Thus, the first functional studies focused on these three miRNAs. When miR-100 was depleted with a specific anti–miR-100 in this last instar, the wings of the resulting adults were slightly reduced in size and presented vein pattern malformations, like partial fusion of cross-veins in the anterior area of the remigium, and aberrant bifurcations of those in the posterior area (**Figure 2**). Specific depletion of let-7 elicited the same vein pattern malformations in the adult wings (**Figure 2**), whereas equivalent depletion of miR-125 had no apparent effect (43). Interestingly, the wing phenotype obtained after depleting let-7 and miR-100 was similar to that caused by depleting *Broad complex* expression (23). This suggests that Broad complex directly or indirectly induces or coactivates the expression of the miR-100/let-7/miR-125 cluster. In any case, these results also showed that this miRNA cluster was not responsible for the total suppression of metamorphosis triggered by dicer-1 depletion (21).

Global Control of Metamorphosis Through Kr-h1 and JH Signaling

The supernumerary nymph obtained after dicer-1 depletion in *B. germanica* was identical to that obtained after ectopically administering JH in last-instar nymphs, which triggers a dramatic upregulation of *Kr-h1* expression (28). This suggests that Kr-h1 might be a key target of miRNAs during metamorphosis. Notably, the decrease of Kr-h1 transcript levels in freshly emerged last-instar nymphs of *B. germanica* is very abrupt (28) and does not follow the slower decline in JH production that occurs during the same period (51) (**Figure 1**).

Further work indicated that dicer-1 depletion results in an increase in Kr-h1 mRNA levels; depletion of *Kr-h1* expression in dicer-1 knockdown animals rescues metamorphosis; and the 3′ untranslated region (UTR) of Kr-h1 mRNA contains a functional binding site for miR-2 (29). These data suggest that the inhibition of metamorphosis caused by dicer-1 and miRNA depletion was due to the deregulation of a factor promoting the decrease of Kr-h1 expression and that this factor might be miR-2. This hypothesis was corroborated by treating the last nymphal instar with a miR-2 inhibitor (which impaired metamorphosis) and by treating dicer-1–depleted animals with a miR-2 mimic (which allowed nymph-to-adult metamorphosis to proceed) (**Figure 3**). Taken together, the observations indicate that miR-2 miRNAs scavenge *Kr-h1* transcripts when the transition from nymph to adult stage should proceed, thus crucially contributing to the correct culmination of metamorphosis.

miRNAs AND HOLOMETABOLAN METAMORPHOSIS

Depletion of dicer-1 in the red flour beetle *Tribolium castaneum* did not completely inhibit metamorphosis; instead, it only resulted in wing extension defects (50). In the same beetle, argonaute-1 (ago-1) interference in larvae produced developmental defects and impaired pupation (50). Argonaute proteins are the direct binding partners of miRNAs and coordinate downstream gene silencing events by interacting with other protein factors (31). In *D. melanogaster*, and probably in other insects, miRNA precursors are predominantly processed by dicer-1 (27) and loaded into ago-1 (13, 37).

ago-1: argonaute-1

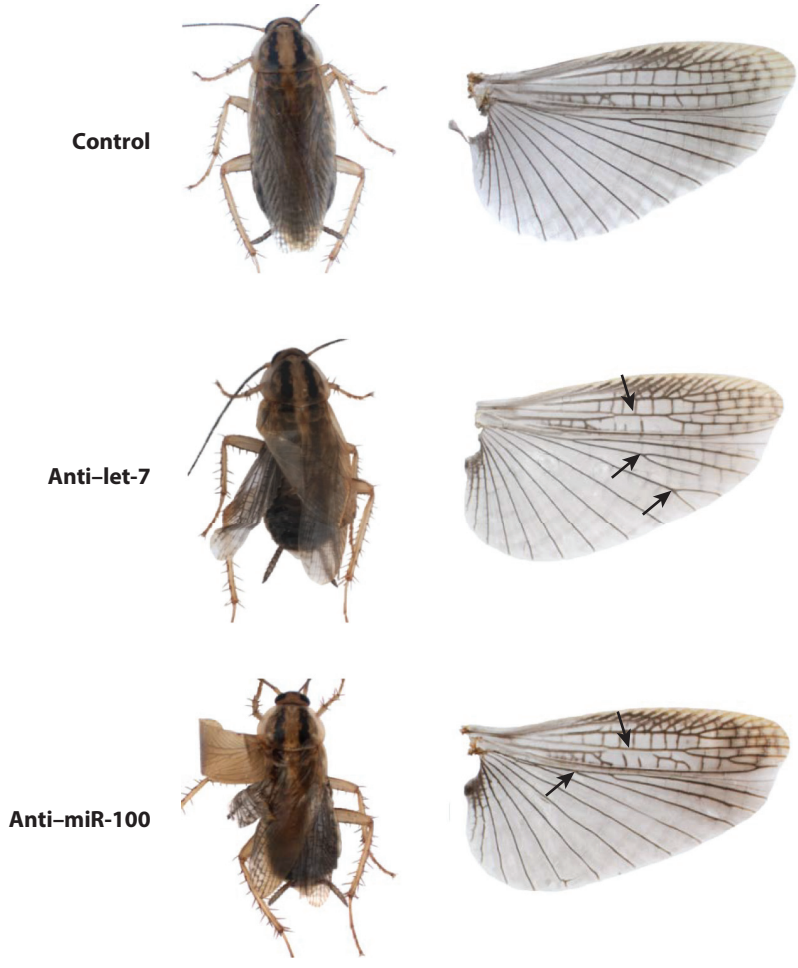

Figure 2

The miR-100/let-7/miR-125 cluster and wing formation in hemimetabolan species. Effects of let-7 and miR-100 depletion on hindwing venation in *Blattella germanica*. Images show partial fusion of cross-veins in the anterior area of the remigium, and aberrant bifurcations of those in the posterior area. Original data and experiments are described in Rubio & Belles (43); the figure is adapted, with permission, from Reference 43.

However, ago-1 functions extend beyond small RNA–guided posttranscriptional gene regulation. Indeed, ago-1 has been implicated in mRNA translation and decay, as well as in other cellular processes, such as transcriptional regulation and splicing (31). And so it comes as no surprise that in *T. castaneum*, dicer-1 depletion produces a weaker phenotype than ago-1 depletion (50). Therefore, the phenotypes observed after dicer-1 depletion suggest that the mechanism by which miR-2 scavenges *Kr-h1* transcripts and controls metamorphosis does not operate in this holometabolan species. Indeed, the 3′ UTR of Kr-h1 mRNA in *T. castaneum* does not contain any binding sites for miR-2, and this is true of other holometabolan species examined, including *D. melanogaster* and the silkworm *Bombyx mori* (29).

Conversely, the wing extension defects observed after dicer-1 depletion in *T. castaneum* (50) could derive not only from intrinsic mechanisms of wing formation modulated by miRNAs but

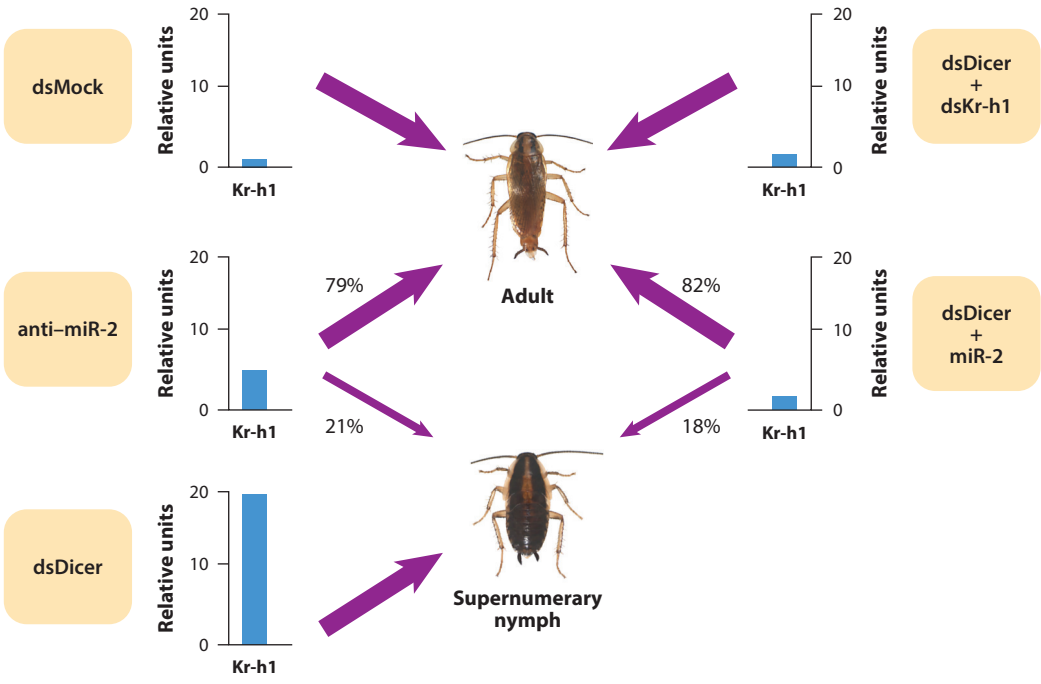

Figure 3

miR-2 scavenges Kr-h1 during the last nymphal instar and controls metamorphosis in *Blattella germanica*. RNAi depletion of dicer-1 (dsDicer-1 treatment) in the sixth (last) nymphal instar inhibits metamorphosis, triggering the formation of a supernumerary seventh nymphal instar (N7); the treatment impairs the decrease in Kr-h1 transcripts observed in controls (dsMock treatment). Depletion of Kr-h1 (dsKr-h1 treatment) expression in dsDicer-1–treated animals rescues metamorphosis. Administration of a miR-2 inhibitor (anti–miR-2 treatment) impairs metamorphosis. Administration of a miR-2 mimic (miR-2 treatment) in dsDicer-1–treated animals rescues metamorphosis. Original data and experiments are described in Lozano et al. (29); the figure is adapted, with permission, from Reference 29.

also from problems associated with deficiencies in ecdysis (see, e.g., 16). This implies that miRNAs may be involved in neuromuscular coordination, and the data available for *D. melanogaster* support this conjecture, as described below.

Diverse miRNAs Acting on Wing Formation

The pioneering paper by Caygill & Johnston (11) showed that loss of let-7 and miR-125 results in delays in cell cycle exit in maturating wing cells of *D. melanogaster*. This led to the conclusion that let-7 is crucial to the timing for the exit of wing imaginal disc cells from the cell cycle approximately 24 h after pupariation. The *abrupt* gene was shown to be a target in vivo of let-7 in pupal wing discs, where the appropriate temporal repression of *abrupt* expression by let-7 seems to be a requirement for correct wing development (11). More recent work indicates that another miRNA, miR-7, modulates cell growth and cell cycle progression during wing development by targeting the cell cycle regulator Dacapo and the Notch signaling pathway (1).

With regard to wing formation, the *Drosophila LIM-only* gene (*dLMO*) encodes a transcription cofactor that represses the expression of *apterous*, a gene required to determine wing dorsal identity (14). During wing development, miR-9 regulates *dLMO* mRNA through a specific target site; thus,

BIDIRECTIONAL TRANSCRIPTION OF MicroRNA LOCI PRODUCES DISTINCT FUNCTIONAL MicroRNAs

miRNA precursors adopt a hairpin structure from which mature microRNAs are processed. The opposite DNA strands thus have the complementary sequence capable of forming the equivalent hairpin structure. In the fruit fly *Drosophila melanogaster*, the miRNA iab-4 locus is part of the Bithorax complex that additionally contains three Hox genes, including *Ultrabithorax* (*Ubx*). Ectopic expression of iab-4 results in a haltere-to-wing homeotic transformation; iab-4 is responsible for this transformation by directly binding to the 3′ UTR of Ubx, thus blocking Ubx activity in vivo. Notably, transcription of the opposite DNA strand produces another mature mRNA (termed miR-iab-8, but very similar to iab-4) that strongly represses Ubx. This notable example shows that antisense transcription and processing of a miRNA locus can add a fascinating new level of functional diversity to miRNA genes.

mutants without the miR-9 site express high levels of *dLMO* mRNA and protein and exhibit a wing phenotype characterized by a lack of margins (9). Data suggest that miR-9 ensures a precise dosage of dLMO for wing development during *D. melanogaster* metamorphosis.

Still in the context of *D. melanogaster* and wing development, the Hox gene *Ultrabithorax* (*Ubx*) determines the formation of halteres in the metathorax instead of flying wings, and ectopic expression of the miRNA iab-4 triggers a haltere-to-wing homeotic transformation (41) (**Figure 4**). This effect is mediated by iab-4 binding to the *Ubx* mRNA, thus blocking its translation. Interestingly, the iab-4 locus is part of the Bithorax complex that contains up to nine homeotic genes, including *Ubx* (41). Notably, antisense transcription of this iab-4 locus generates a symmetric hairpin containing a miRNA that has been named iab-8 (53), or iab-4AS (48), which is almost identical to iab-4 and thus also targets *Ubx* mRNA (see the sidebar titled Bidirectional Transcription of MicroRNA Loci Produces Distinct Functional MicroRNAs). Unsurprisingly, ectopic expression of iab-8 triggers haltere-to-wing transformation, which is even more efficient than the transformation induced by iab-4 because iab-8 is a stronger repressor of *Ubx* (48, 53). Although the above data suggest that two largely redundant miRNAs, namely, iab-4 and iab-8 (iab-4AS), finely modulate the expression of *Ubx* to correctly produce the metathoracic halteres during metamorphosis, they also show that the mechanism generating the miRNAs can be so sophisticated as to involve sense and antisense transcription and processing of the same locus.

As in other processes in animal development, an important aspect of wing morphogenesis is a subdivision of proliferating tissues into adjacent compartments. In this context, cell interactions mediated by the Notch receptor have been implicated in the specification of compartment boundaries in *D. melanogaster* (32, 39). Notch mediates boundary formation in the fly wing partly through repression of bantam miRNA. Cell proliferation is triggered by bantam, and the actin regulator enabled has been identified as its target. Increased levels of enabled and reduced proliferation rates contribute to maintenance of the dorsal-ventral affinity boundary (5).

The Metamorphosis of the Abdomen in *Drosophila*

In *D. melanogaster*, the adult abdomen is formed through a process by which larval epidermal cells are replaced by adult cells. The cells that will form the epidermis of the adult abdomen, the abdominal histoblast cells, are specified during embryogenesis and remain quiescent during larval development, without being encapsulated in imaginal discs. During pupal development, the abdominal histoblast cells proliferate and migrate to replace the larval abdominal cells. A recent work by Verma & Cohen (56) shows that miR-965 controls histoblast proliferation and migration

Ubx: Ultrabithorax

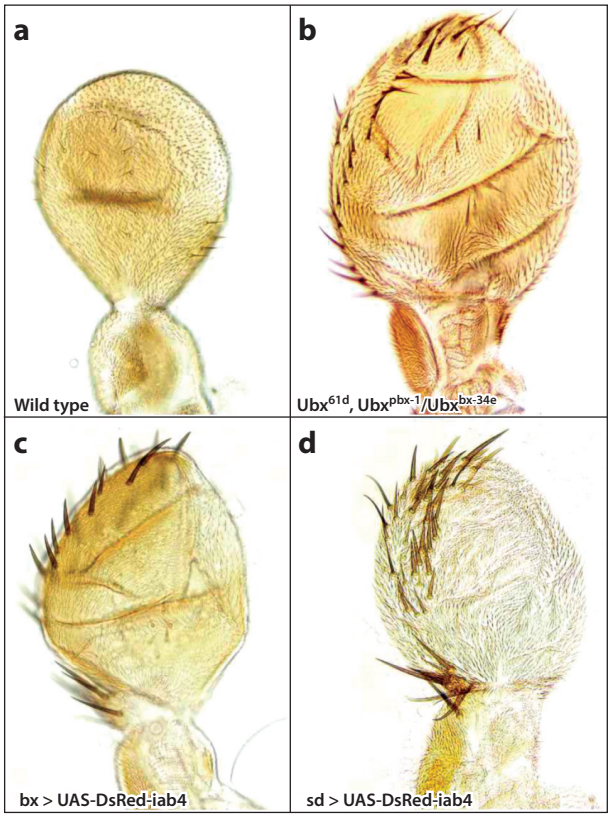

Figure 4

Haltere-to-wing homeotic transformation triggered by directed expression of iab-4 in *Drosophila melanogaster*. (*a*) Wild-type haltere, which contains small lightly pigmented sensilla but lacks the triple row of sensory bristles seen in wings. (*b*) Haltere-to-wing transformation in a mild *Ubx* loss-of-function mutant background. Misexpression of *iab-4* miRNA hairpin in (*c*) *bx-Gal4/Y*, *UAS-DsRed-iab-4* and (*d*) *sd-Gal4*, *UAS-DsRed-iab-4* animals induces a similar haltere-to-wing transformation. Adapted from Ronshaugen et al. (41) with permission from Cold Spring Harb. Lab. Press and Eric C. Lai.

via string and wingless factors. Interestingly, ecdysone signaling downregulates miR-965 at the beginning of pupariation, linking activation of the histoblast cells to the endocrine control of metamorphosis.

Cell and Tissue Death

A process associated with holometabolan metamorphosis is the dramatic destruction of practically all tissues and organs and the formation of new ones that take place in the pupal stage. The first miRNA studied in this context was bantam, when it was identified as a key modulator of apoptosis through the control of the proapoptotic gene *hid* in *D. melanogaster* (10). It was subsequently shown that bantam mediates the interaction between the epidermal growth factor receptor (EGFR) and Hippo growth control pathways in different tissues of the fly, notably in wing and eye development (15, 22). The Hippo signaling pathway acts via the transcriptional coactivator Yorkie and p53 to control the expression of the proapoptotic gene reaper; Yorkie further modulates reaper levels

EGFR: epidermal growth factor receptor

posttranscriptionally through regulation of miR-2 to prevent apoptosis during tissue growth and metamorphosis (59).

An example of cell and tissue death during metamorphosis is the complete disintegration of *D. melanogaster* larval salivary glands during pupal formation. In this process, loss of miR-14 specifically prevents the induction of autophagy during salivary gland cell death, whereas misexpression of miR-14 prematurely induces autophagy specifically in salivary glands (36). It is worth noting that miR-14 regulates this context-specific autophagy through its target, inositol 1,4,5-trisphosphate kinase 2, thereby affecting inositol 1,4,5-trisphosphate signaling and calcium levels during salivary gland cell death.

Neuromaturation, Neuromuscular Junctions, and Neuron Cell Fate

D. melanogaster mutants devoid of let-7 and miR-125 expression display defects in the maturation of neuromuscular junctions of abdominal muscles as adults (11). The gene *abrupt*, which encodes a nuclear protein expressed in muscle cells, was identified as a let-7 target, and evidence that let-7 regulates the maturation of abdominal neuromuscular junctions during metamorphosis by controlling *abrupt* mRNA was provided (11). A parallel study reported that let-7 knockout flies look morphologically normal but have juvenile neuromuscular features and display defects in adult abilities, such as flight and motility (47). This suggests that let-7 contributes to regulation of the appropriate remodeling of abdominal neuromusculature during metamorphosis. Also with respect to neurogenesis and neuromaturation, a more recent study reported that let-7 and miR-125 act on the transcription factor Chinmo, regulating temporal cell fate in the mushroom body lineage (58). Significantly, let-7 is activated in postmitotic neurons formed during pupal morphogenesis, when transitions among three mushroom body subtypes take place.

miRNAs AND THE EVOLUTION OF INSECT METAMORPHOSIS

Influence on the Endocrine Context

The positive effect of ecdysteroids on miRNA expression and the negative effect of JH, especially on the preadult instar, are common to both hemimetabolan and holometabolan species, suggesting that these hormonal properties were selected with the emergence of metamorphosis. At least in the case of the miR-100/let-7/miR-125 cluster, the stimulatory action of ecdysteroids appears to be mediated by Broad complex, in both *D. melanogaster* (45) and *B. germanica* (43), thus suggesting that the transducing action of Broad complex also appeared with the emergence of metamorphosis (**Figure 5**).

The action of miR-14 on the autoregulatory loop that increases EcR levels has been reported in *D. melanogaster* (55), but it is not known whether miR-14 has this effect in hemimetabolan species. A notable example is the control of miR-2 over the JH signaling pathway through Kr-h1 in *B. germanica*, which ensures that metamorphosis proceeds correctly (29). The *Kr-h1* mRNA of hemimetabolan species that have been studied in this context possesses a conserved miR-2 site in the 3′ UTR, suggesting that the mechanism of miR-2 control over Kr-h1 during metamorphosis is common to all hemimetabolans. Conversely, the holometabolan species studied do not present a miR-2 target site in their *Kr-h1* mRNA (29), which implies that the above mechanism was lost in holometabolans (**Figure 5**).

In hemimetabolan species, transition to the adult stage directly succeeds the last nymphal instar, requiring the virtual disappearance of Kr-h1 and the onset of *E93* expression. In holometabolans, the formation of the adult succeeds the last larval instar and pupal stages, and these transitions

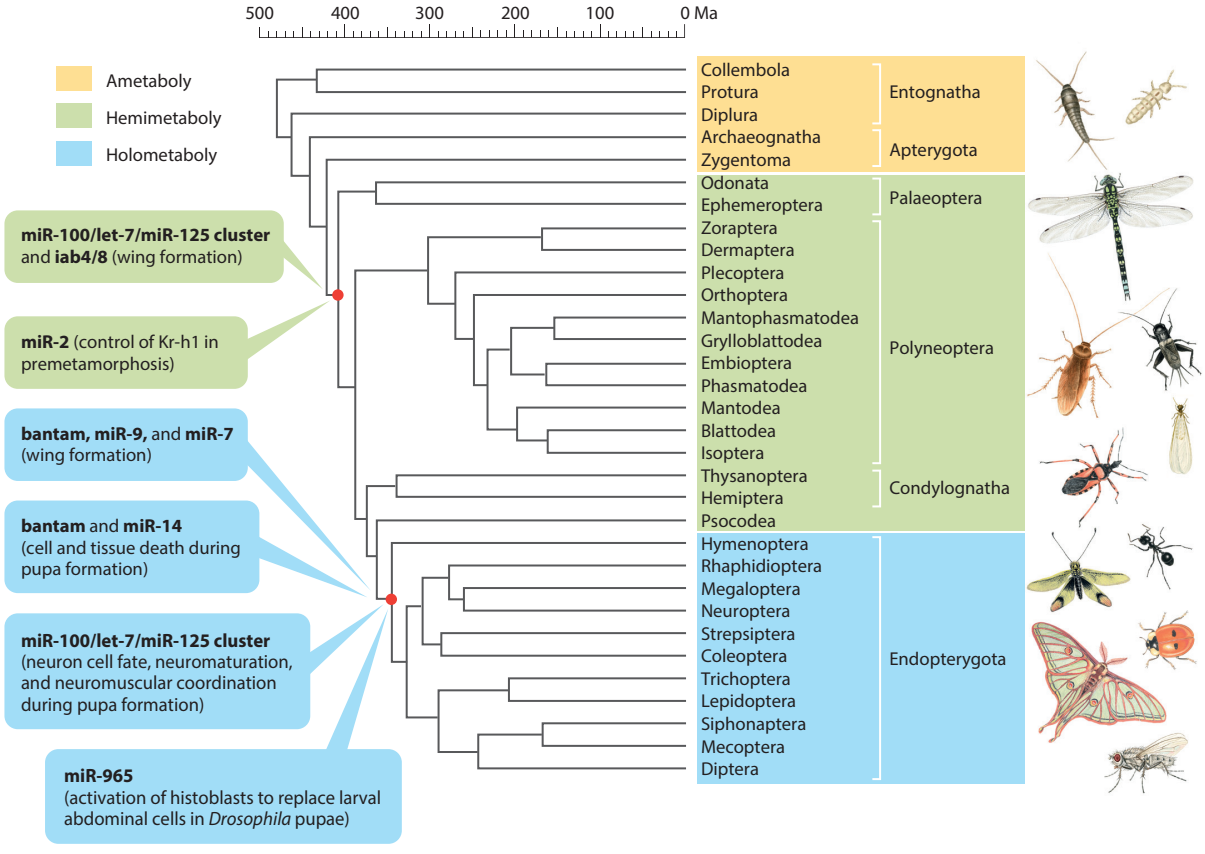

Figure 5

MiRNAs and metamorphosis during insect evolution. MiRNAs and functions associated with the origin of pterygotes (and the emergence of hemimetaboly) and of endopterygotes (and the emergence of holometaboly) are indicated. The function of miR-2 as a *Kr-h1* mRNA scavenger appears to have disappeared in endopterygotes. Phylogenetic reconstruction according to Misof et al. (33).

require a transient decrease of Kr-h1 toward the end of the last instar larvae, which triggers the pupal stage, and a subsequent reexpression of *Kr-h1*, which prevents the formation of adult features in the pupae (8) (**Figure 1**). Thus, a miRNA scavenging *Kr-h1* transcripts could be unsuitable for modulating the sinuous pattern of *Kr-h1* expression of holometabolan metamorphosis. As E93 represses *Kr-h1* expression in hemimetabolan and holometabolan species (8, 54), it is possible that modulation of *Kr-h1* expression during holometabolan metamorphosis relies on the reciprocally repressive Kr-h1/E93 loop (8). Moreover, other more specific regulatory mechanisms of Kr-h1 expression cannot be ruled out, for example, the one mediated by the Orthodenticle homeobox protein acting in conjunction with EcR to directly repress *Kr-h1* expression (17), a crucial pathway for photoreceptor maturation regulation during *D. melanogaster* pupariation.

Wing Development

The functions of the miR-100/let-7/miR-125 cluster related to wing formation are common to hemimetabolan and holometabolan insects (11, 43); thus, they probably originated with the emergence of wings and metamorphosis (**Figure 5**).

The hypothesized regulation of Ubx dosage by iab-4/8 has only been studied in *D. melanogaster*. Interestingly, iab-4/8 is arthropod-specific (49), and Ronshaugen et al. (41) noticed that the *iab-4/8* locus is conserved in both sequence and genomic location not only in *D. melanogaster* but also in the mosquitoes *Anopheles gambiae* and *Aedes aegypti*, the honey bee *Apis mellifera*, and the red flour beetle *T. castaneum*, suggesting a conserved evolutionary mechanism of *Hox* gene regulation by iab4/8 in holometabolan species.

We examined the genomic location of *Ubx* and *iab-4/8* in available genomes of hemimetabolan species (**https://i5k.nal.usda.gov**), and results revealed that both loci are in the same scaffold in the dragonfly *Ladona fulva* (Odonata), the mayfly *Ephemera danica* (Ephemeroptera), the German cockroach *B. germanica* (Blattodea), the western flower thrips *Frankliniella occidentalis* (Thysanoptera), the bed bug *Cimex lectularius* (Hemiptera), and the glassy-winged sharpshooter *Homalodisca vitripennis* (Homoptera). Conversely, in the apterygote *Catajapyx aquilonaris* (Diplura), *iab-4/8* and *Ubx* are in different scaffolds. Taken together, the data suggest that the mechanism by which iab-4/8 controls the dosage of Ubx and the morphogenesis of the metathoracic wings is also present in hemimetabolan insects and could have emerged with the origin of wings and metamorphosis (**Figure 5**).

Other miRNAs involved in wing development, such as miR-9, which affects wing margin formation (9), and bantam, which contributes to wing compartment boundary formation (5), have only been studied in *D. melanogaster* and it is not known whether they have these functions in hemimetabolan species.

Tissue Degradation and Reconstruction in the Pupae

Dramatic cell and tissue deconstruction and reconstruction are characteristic of the pupal stage. Thus, miRNA functions related to cell death studied in the pupae of *D. melanogaster*, such as the role of bantam in controlling the proapoptotic *hid* gene (10) in the context of the EGFR and Hippo pathways (15, 22, 59) and that of miR-14 in the degradation of the larval salivary gland (36), might have emerged with the origin of Holometabola. Following the same line of reasoning, as significant nervous and muscular remodeling is characteristic of the pupal stage, we can hypothesize that the functions of the miR-100/let-7/miR-125 cluster in neuron cell fate, neuromaturation, and neuromuscular coordination described in *D. melanogaster* (11, 58) also originated with holometaboly (**Figure 5**).

CONCLUSIONS AND PROSPECTS

The available data have provided us with a general understanding of the role of miRNAs in insect metamorphosis regulation and evolution. There are ancient miRNAs, such as the miR-100/let-7/miR-125 cluster, that have conserved functions in all insects studied to date and other miRNAs with specific roles related to particular processes in holometabolan species that were coopted to play these roles in the transition from hemimetaboly to holometaboly. A special case is the control over *Kr-h1* transcripts exerted by miR-2, which is crucial in hemimetabolan metamorphosis and represents a singular example of a single miRNA controlling a process by acting on its main mediator. However, this is quite an exceptional mechanism that was apparently lost in holometabolans, as the *Kr-h1* mRNA of the species studied from this group does not contain miR-2 binding sites. Usually, miRNAs play subtle fine-tuning roles that ensure the correct amount of the relevant mRNAs in processes associated with metamorphosis. Further studies will likely reveal other miRNAs associated with additional functions, and we can predict that miRNAs are behind every detail of the complex regulatory network that leads to metamorphosis, in either the hemimetabolan or holometabolan modes.

From a more general perspective, it is surprising that there are no miRNAs recorded as appearing with the origin of endopterygotes. Tarver et al. (49) mention two miRNAs, miR-11 and miR-932, but the first belongs to the miR-2 family (19) and the second has recently been reported in hemimetabolan species (57). One can conjecture that the innovation of the pupal stage and the dramatically derived morphology exhibited by holometabolan larvae would require the concourse of new miRNA families, as new miRNA families are required for body plan evolution (12). Establishing the miRNA catalog of selected hemimetabolan and holometabolan species on the basis of rigorous methods (like those described in Reference 19) will likely unveil new miRNA families that emerged with the Holometabola. The study of these new miRNAs will surely reveal new and specific functions in holometabolan metamorphosis. In turn, this would complete the mechanistic description of metamorphosis regulation and reconstruction of the evolutionary transition from hemimetaboly to holometaboly.

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