microRNA *miR-275* is indispensable for blood digestion and egg development in the mosquito *Aedes aegypti*

Bart Bryant, Warren Macdonald, and Alexander S. Raikhel[1]

Department of Entomology, Institute for Integrative Genome Biology, University of California, Riverside, CA 92521

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2009.

Contributed by Alexander S. Raikhel, November 4, 2010 (sent for review October 1, 2010)

The mosquito *Aedes aegypti* is the major vector of arboviral diseases, particularly of Dengue fever, of which there are more than 100 million cases annually. Mosquitoes, such as *A. aegypti*, serve as vectors for disease pathogens because they require vertebrate blood for their egg production. Pathogen transmission is tightly linked to repeated cycles of obligatory blood feeding and egg maturation. Thus, the understanding of mechanisms governing egg production is necessary to develop approaches that limit the spread of mosquito-borne diseases. Previous studies have identified critical roles of hormonal- and nutrition-based target of rapamycin (TOR) pathways in controlling blood-meal–mediated egg maturation in mosquitoes. In this work, we uncovered another essential regulator of blood-meal–activated processes, the microRNA *miR-275*. The depletion of this microRNA in *A. aegypti* females after injection of its specific antagomir resulted in severe defects in blood digestion, fluid excretion, and egg development, clearly demonstrating that *miR-275* is indispensable for these physiological processes. *miR-275* exhibits an expression profile that suggests its regulation by a steroid hormone, 20-hydroxyecdysone (20E). In vitro organ culture experiments demonstrated that *miR-275* is induced by this hormone in the presence of amino acids, indicative of a dual regulation by 20E and TOR. This report has uncovered the critical importance of microRNAs in controlling blood-meal–activated physiological events required for completion of egg development in mosquito disease vectors.

dicer | ecdysone | small RNA | argonaute

The mosquito *Aedes aegypti* is the major vector for dengue, yellow fever and, recently, chikungunya virus (1). It is an anautogenous mosquito, and the female needs to obtain a blood meal to produce eggs, hence the underlying mechanism for spread of devastating diseases to humans. Therefore, understanding the biology of how this mosquito vector reproduces is vital to devising new approaches to fight devastating diseases brought on by arboviruses. Because of the extensive body of knowledge amassed regarding the reproductive biology of *A. aegypti*, this mosquito also serves as a model system for investigating blood-meal–initiated events in other arthropod vectors that transmit disease pathogens (2, 3). Central to egg maturation is vitellogenesis, a process of production of massive amounts of yolk protein precursors (YPPs) by the fat body, a tissue analogous to the mammalian liver and adipose tissue. YPPs are secreted into the hemolymph and subsequently accumulated by developing oocytes via receptor-mediated endocytosis (4). After blood feeding, mosquito vitellogenesis is activated by a cooperative action of nutritional amino acid/target of rapamycin (AA/TOR) and steroid hormone (20-hydroxyecdysone, 20E) pathways (5, 6).

MicroRNAs (miRNAs) are small noncoding RNAs of approximately 21–24 nt in length that have been shown to be responsible for posttranscriptional regulation of mRNAs in both plants and animals (7, 8). miRNAs act mostly on the 3′ UTR of target mRNAs and either degrade or inhibit translation of multiple transcripts (7–10). miRNAs are transcribed by RNA pol II to form a pri-miRNA molecule. pri-miRNAs are cleaved by Drosha to form the premiRNAs, which are then exported into the cytoplasm by Exportin-5. premiRNAs are cleaved by Dicer1 (Dcr1), resulting in a miRNA/miRNA duplex. The mature miRNA molecule is then loaded into an Argonaute (Ago) complex, which targets multiple mRNAs for either destruction or inhibition of translation (9). In addition, some miRNAs are found in introns of genes and bypass Drosha processing (9). Since the discovery of miRNAs in *Caenorhabditis elegans* (10, 11), numerous studies have demonstrated their essential role in regulating development, cell differentiation, apoptosis, and other critical biological events in both animals and plants (12, 13). It is estimated that nearly 30% of human mRNAs are targeted by miRNAs (13).

Although multiple biological functions have been identified as targets of miRNAs in model organisms, including the fruit fly *Drosophila melanogaster* (14–21), information concerning possible roles of miRNAs in mosquito biology is limited. Both conserved and mosquito-specific miRNAs have been found in *Anopheles stephensi* and *A. aegypti* (22, 23). RNAi depletion of either *Dcr1* or *Ago1* was shown to result in a higher infection level by *Plasmodium berghei* parasites in the midgut of *Anopheles gambiae* mosquitoes (24). In *Aedes albopictus* and *Culex quinquefasciatus*, miRNAs were cloned and sequenced, and two were found to be up-regulated owing to West Nile virus infection (25). It is of particular importance to decipher what roles miRNAs might play in regulating blood feeding, female reproduction, and antipathogen immunity, because these functions constitute an interconnected triangle defining the ability of a female mosquito to transmit disease pathogens in consecutive reproductive cycles. In *A. aegypti*, several miRNAs have shown stage-specific expression, and their level was enhanced in the midgut of blood-fed females (23), suggesting miRNA involvement in blood-meal–associated events. However, the precise roles of miRNAs in mosquitoes remain unresolved.

The significance of our present study is the identification of a particular miRNA required for both proper blood digestion and egg development in the anautogenous mosquito *A. aegypti*. Specific antagomir depletion of *miR-275* resulted in a dramatic defect in intake and digestion of blood: in mosquitoes examined 24 h post-blood meal (PBM), blood was not digested in the posterior midgut, and it filled a specialized anterior portion of the digestive system called the crop, which is normally used for storing nectar. The overall volume of engorged blood remained very large, suggesting a defect in fluid excretory function. In these mosquitoes, egg development was profoundly inhibited; primary follicles representing future eggs were small and heterogeneous in size; in contrast, their nurse cells remained intact, which is characteristic of early stages of egg development.

Author contributions: B.B. and A.S.R. designed research; B.B. and W.M. performed research; B.B. and A.S.R. analyzed data; and B.B. and A.S.R. wrote the paper.

The authors declare no conflict of interest.

See Profile on page 22381.

[1]To whom correspondence should be addressed. E-mail: alexander.raikhel@ucr.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016230107/-/DCSupplemental.

Moreover, expression of *miR-275* was elevated by AAs and 20E, indicating that it is under the control of AA/TOR and 20E regulatory cascades initiated by blood feeding. This report clearly illustrates the critical importance of miRNAs in controlling blood-meal–activated physiological events required for completion of egg development in mosquito disease vectors.

Results

Temporal Expression Profiles of miRNAs in the Female Mosquito Fat Body During Vitellogenesis. We analyzed the expression of 27 conserved miRNAs that have been implicated in various developmental events in *Drosophila* and *C. elegans* in the mosquito female fat body during vitellogenesis. The analysis was performed by means of the Qiagen miScript PCR System, as described in *Methods* (Fig. S1). The expression level of vitellogenin (*Vg*), the gene encoding the major YPP, was used to monitor the physiological stage of vitellogenic female mosquitoes (Fig. 1 *A* and *B* and Fig. S1). Expression of the *Dcr1* gene was also assayed during vitellogenesis (Fig. 1*AII* and Fig. S1). This analysis has allowed allocation of the examined miRNAs into four groups according to their expression patterns in the vitellogenic fat body: *miR-9a* represents group one (Fig. 1*AIII*), which was down-regulated during vitellogenesis; *miR-275*, group two (Fig. 1*AIV*), up-regulated at 12 h PBM; *miR-1*, group three (Fig. 1*AV*), up-regulated at 24 h PBM; and *miR-7*, group four (Fig. 1*AVI*), which did not change expression until 36 h PBM. All 27 miRNAs peaked at 36 h PBM, correlating with *Dcr1* expression, which was low during the vitellogenic period but peaked at the time of termination of *Vg* gene expression (Fig. 1A and Fig. S1). Significantly, the relative amounts of three miRNAs—*miR-8*, *miR-14*, and *miR-275*—were considerably higher than any other miRNA in the vitellogenic fat bodies (Fig. S1).

We further investigated expression patterns of three miRNAs during pupal and adult stages of female mosquitoes. Three particular miRNAs were chosen for the following reasons: *bantam* is a well-characterized miRNA known to be involved in cell-death developmental processes in *Drosophila* (14, 16); *miR-8* has been shown to be involved in activation of PI3K in *Drosophila* (21) and was highly expressed in the mosquito fat body; a highly expressed *miR-275* was selected because of its pattern of expression of being elevated 7.2-fold from 0 to 12 h PBM, compared with other miRNAs that were only up-regulated in the range of 1- to 2.5-fold. Of note, *miR-305* exhibited a 28-fold up-regulation during the same time; however, its overall expression level was low. We obtained RNA from female pupae days 1–3 (representing the pupal developmental period), female fat bodies after eclosion, days 1–3 (representing the previtellogenic period), and female fat bodies 24, 36, and 48 h PBM (representing the vitellogenic period). This range of samples was subjected to expression analysis for *Vg*, *bantam*, *miR-275*, and *miR-8*. All three miRNAs analyzed were highly expressed during the pupal developmental period (Fig. 1*BII–IV*). However, their expression profiles were different: *miR-275* was high at the beginning of pupal development and declined precipitously thereafter; *bantam* and *miR-8* exhibited a peak at mid-pupal stage. Expression of *bantam* was low during pre- and vitellogenic periods; *miR-8* expression, however, was still high during previtellogenesis (1–3 d), declined by 24 h PBM, and increased again by 48 h PBM, which corresponds to the termination of vitellogenesis. *miR-275* was severely down-regulated during the previtellogenic period and

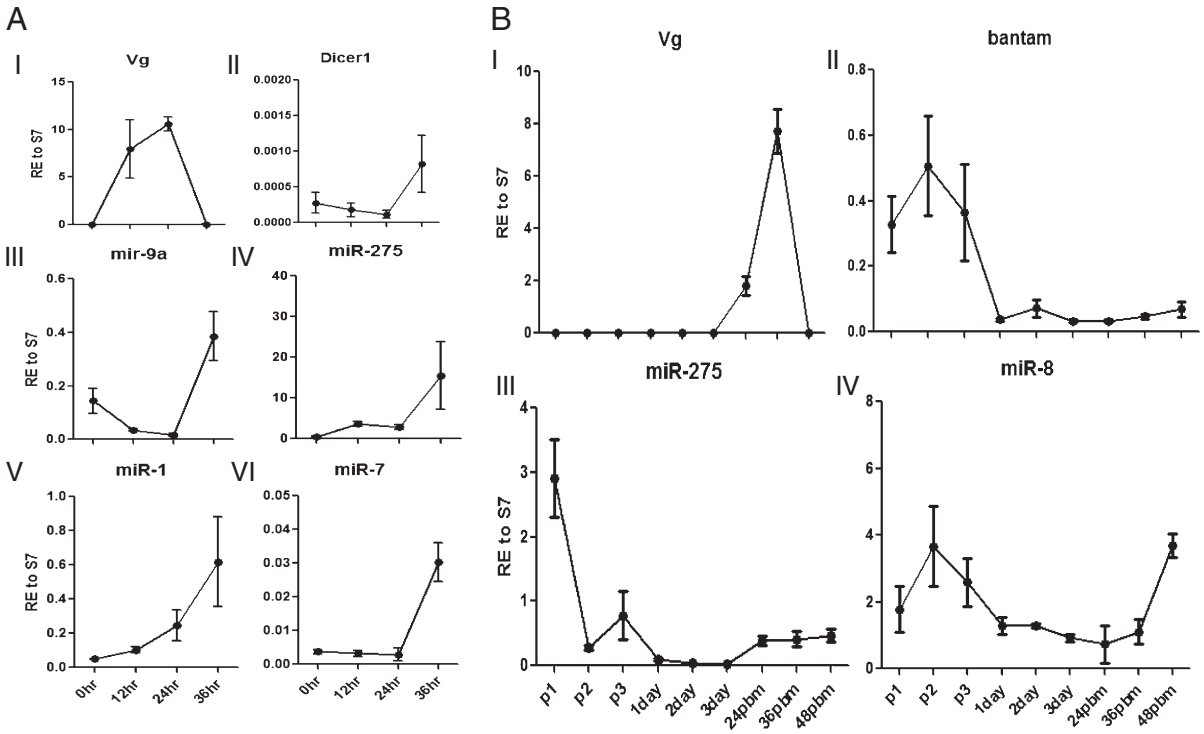

Fig. 1. Temporal expression profiles of miRNAs in the female mosquito fat body during vitellogenesis. (*A*) Fat bodies were analyzed for expression of miRNAs at time points 0, 12, 24, and 36 h PBM. Four patterns of expression were identified for the miRNAs analyzed in relation to expression of *Vg* (*I*) and *Dcr1* (*II*). (*III*) *miR-9a* represents the group of miRNAs that is down-regulated during vitellogenesis; (*IV*) *miR-275* represents the group of miRNAs that is up-regulated at 12 h PBM; (*V*) *miR-1* represents the group of miRNAs that is up-regulated at 24 h PBM; and (*VI*) *miR-7* represents the group of miRNAs that did not change until 36 h PBM. Expression of miRNAs analyzed peaked at 36 h PBM. (*B*) A broader expression profile for miRNA expression was analyzed at the following time points: pupae day 1, 2, 3; fat bodies from female mosquitoes posteclosion days 1, 2, 3; and fat bodies from female mosquitoes 24, 36 and 48 h PBM. (*I*) *Vg*, (*II*) *bantam*, (*III*) *miR-275*, and (*IV*) *miR-8*. Data shown are three biological replicates and are illustrated as ±SEM.

up-regulated during the vitellogenic period (Fig. 1*BII–IV*). Although in this set of samples *Vg* expression peaked at 36 h PBM (Fig. 1*BI*) compared with 24 h PBM in the previous experiment (Fig. 1*AI*), expression patterns of miRNAs had the same trend with respect to *Vg* expression and peaked at the time of *Vg* expression termination (Fig. 1 *A* and *B*). Taken together, these data suggest that the expression of *miR-275* and several other miRNAs are regulated during the vitellogenic cycle of the female mosquito and may play a role in regulating these vitellogenic events.

20E and AAs Activate Gene Expression of *miR-275* in the Female Mosquito Fat Body. We decided to focus on characterization of *miR-275* because of its high level of expression, shown to be elevated after the initiation of vitellogenesis by a blood meal (Fig. 1 *AIV* and *BIII*). This expression pattern suggests that *miR-275* may be regulated by signals elicited by blood feeding, including 20E and AA/TOR (3, 5). To test this hypothesis, we used in vitro fat body culture (5, 26). We dissected fat bodies from female mosquitoes 3–5 d after eclosion and incubated them in culture media containing different combinations of AAs and/or 20E for 6 h. Fat bodies were then harvested for RNA, which was converted to cDNA and used to monitor expression of selected miRNAs and mRNAs. We analyzed *Vg*, *miR-275*, and *bantam*. The *Vg* gene is activated by a blood meal via cooperative action of the nutritional AA/TOR and 20E regulatory pathways (3, 5, 6). It has been shown that for activation of its expression in vitro, both 20E and AAs are required (5). As expected, we found that *Vg* was highly induced in the culture medium containing 20E and AAs, whereas *bantam* did not significantly change its expression level in any of the culture media conditions (Fig. 2). Significantly, *miR-275* reacted similarly to *Vg* in that it was induced in the medium containing both 20E and AAs (Fig. 2). Thus, this in vitro analysis, in addition to the in vivo expression profiling (Fig. 1), indicates that mosquito *miR-275* is a 20E-regulated miRNA that is also under the control of the nutritional AA/TOR pathway activated by blood feeding.

miRNA Expression in Dcr1:RNAi, Ago1:RNAi, and miR-275-Antagomir Backgrounds. To decipher a possible role for miRNAs in blood-meal–activated events leading to egg production, we first examined the effect of RNAi depletion of genes encoding major components of the miRNA pathway *Dcr1* and *Ago1*. *A. aegypti* genes encoding *Dcr1* and *Ago1* have been reported in a comparative analysis of genes involved in small RNA pathways in mosquitoes (27). This previous study identified two gene copies for *Aedes Ago1*. In our analysis, aligning the proposed *Ago1* isoforms revealed an extra sequence only at the N terminus in the putative B isoform and not in the A isoform, with the rest of the Ago1 AA sequence sharing 100% similarity. Aligning of their nucleotide sequences showed 99% identity when the 5′ end of the putative B isoform was removed. These results are highly suggestive of haplotypes instead of two individual genes for *Ago1*. We updated the gene model for *Aedes Ago1*, which shares 87% identity with *Drosophila Ago1* (Fig. S2).

The updated *Ago1* gene model along with *Dcr1* was used to design dsRNA for RNAi depletion of these genes. The appropriate dsRNA molecules were injected into the thorax of female mosquitoes 1 to 2 d after eclosion from the pupal stage. RNAi depletion of a bacterial *Mal* gene was used as a negative control, as described previously (5). Mosquitoes were allowed to recover for 4 to 5 d before blood feeding. miRNA expression levels were measured in fat bodies of female mosquitoes 24 h PBM. The level of *bantam* was reduced 6.8-fold and 4.5-fold in the Dcr1:RNAi background and the Ago1:RNAi background, respectively, compared with the *Mal* control (Fig. 3). The level of *miR-275* decreased 6.7-fold in the Dcr1:RNAi background and 9.5-fold in the Ago1:RNAi background. Despite the obvious effect of RNAi depletion of either *Dcr1* or *Ago1* on the levels of

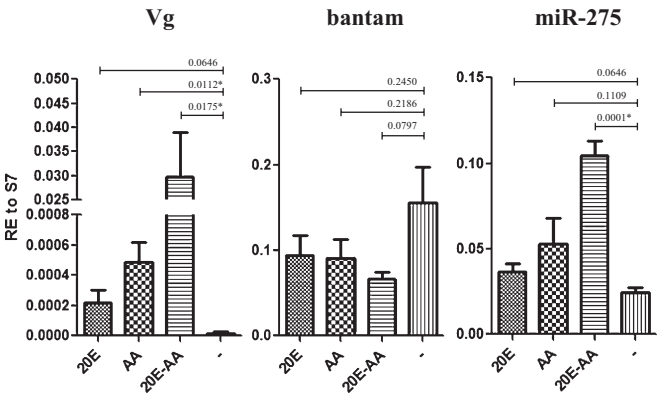

Fig. 2. In vitro fat body analysis of expression of *Vg*, *bantam*, and *miR-275*. Fat bodies from 3- to 5-d-old previtellogenic female mosquitoes were dissected and incubated in culture media with indicated conditions: 20E_culture medium supplemented with 20E only; AA_culture medium supplemented with AA only; 20E-AA_culture medium supplemented with both 20E and AAs; and -_culture medium containing neither 20E nor AAs. The data shown are two biological replicates with two technical replicates and are illustrated as ±SEM. An unpaired Student's *t* test was used for comparison, and *P* values are shown. *Significant difference.

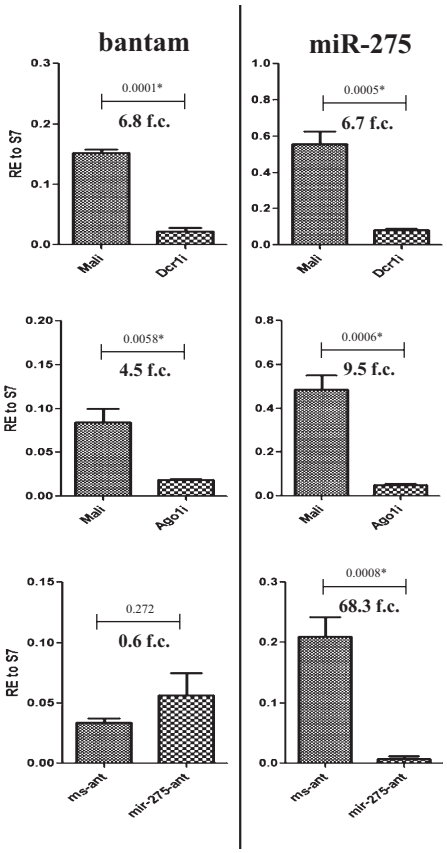

Fig. 3. *bantam* and *miR-275* expression in Dcr1:RNAi (Drc1i), Ago1:RNAi (Ago1i), and miR-275-antagomir (mir-275-ant) backgrounds in mosquito fat bodies 24 h PBM. Mali, (-) control for RNAi; ms-ant, missense antagomir (-) control. Statistical results are illustrated above the bracket, and fold changes are below the bracket. Data shown are two biological replicates with two technical replicates and are illustrated as ±SEM. An unpaired Student's *t* test was used for comparison, and *P* values are shown. *Significant difference.

miRNAs, no visible defects in egg development or blood digestion were observed under these conditions. A possible explanation for the lack of any physiological phenotype is that although Dcr1:RNAi and Ago1:RNAi both broadly affected miRNA expression, they resulted in a relatively low level of down-regulation of miRNA expression.

Next, we used an approach based on an antisense-mediated depletion of a particular miRNA by a sequence-specific antisense oligonucleotide (ASO). Specific depletion of individual miRNAs by ASOs in HeLa cells (28), *C. elegans* (28), mice (29), and *Drosophila* (30) has been documented. We have selected for our experiments the antagomir version of the ASOs (31, 32). Unlike other ASOs, the antagomir has a 3′ conjugation of cholesterol, which has been shown to be approximately eightfold more potent in *Drosophila* S2 cells (31). We designed an antagomir consisting of the reverse complement of *miR-275*, which we termed "miR-275-ant," and an antagomir directed against a scrambled *miR-275* sequence, which we termed "ms-ant" (see *Methods* for details). To determine an appropriate concentration we did a titration of the miR-275 antagomir and found 100 μM to be optimal (Fig. S3). Both antagomirs were injected into 2- to 3-d-old mosquito females, and the levels of *bantam* and *miR-275* were evaluated in the fat body from mosquitoes 24 h PBM. miR-275-ant had no significant effect on *bantam* expression (0.6-fold change), but *miR-275* was dramatically decreased by 68.3-fold compared with the ms-ant control 24 h PBM (Fig. 3). These results demonstrate that we have developed an efficient approach based on an antisense-mediated depletion of a particular miRNA by means of the antagomir technology for mosquitoes.

Specific Antagomir Depletion of miR-275 Drastically Affects both Blood Digestion and Egg Development. Following the same procedure outlined above, we specifically depleted mosquito females of *miR-275* by means of the antagomir approach to study its role in egg development. Such depletion resulted in dramatic defects in intake and digestion of blood in mosquito females. The digestive system of the adult mosquito female contains a large extension of the esophagus, called the crop, which is used to store nectar; a narrow tubular anterior portion of the midgut; and a large flask-like posterior region or stomach (Fig. 4 *A*, *C*, and *E*). During blood feeding, ingested blood passes through the esophagus and the midgut anterior portion into the stomach, bypassing the crop (2). In the stomach, the blood forms a bolus surrounded by a chitin–protein envelope called the peritrophic matrix, within which the ingested blood undergoes digestion (33–35). Assayed 24 h PBM, the stomach of the wild-type mosquito female contained a compact dark brown bolus of digested blood, whereas its crop and the anterior midgut were void of any blood (Fig. 4*E*). The midgut of the ms-ant mosquito had a similar morphology (Fig. 4*C*). Females with the miR-275-ant background demonstrated no obvious defects in host-seeking behavior and blood feeding, fully filling their stomachs, as did wild-type controls. The examination of these females 3 h PBM revealed no clear defects in the digestive system—the crop remained clear, and the blood concentrated in the posterior midgut (not shown). In contrast, at 24 h PBM, the midgut of a female with the miR-275-ant background was filled with bright red blood that did not form a bolus mass, suggesting that the blood was not digested and the peritrophic matrix had not formed normally. Strikingly, the undigested blood was present in the anterior midgut and filled up the crop, dramatically extending its volume (Fig. 4*A* and Fig. S4). In addition, as shown on images with actin staining, the junction between the anterior midgut and the stomach was not constricted in a female with the miR-275-ant background compared with those in the wild-type and the ms-ant mosquitoes (Fig. 4 *B*, *D*, and *F*). The overall volume of blood in the digestive system of these mosquitoes was very large, similar to that of newly fed mosquitoes, indicating a failure of the excretory machinery to effectively remove fluids from ingested blood.

Another developmental defect in miR-275-ant mosquitoes was inhibition of egg development. Paired mosquito ovary consists of approximately 150 individual ovarioles, each of which in turn contains a primary follicle (egg chamber) and a germarium with germ and somatic stem cells and an undifferentiated secondary follicle. During the first gonadotrophic cycle, only primary follicles develop into mature eggs, permitting secondary follicles to grow in the second cycle (2). Within a mosquito meroistic follicle, a cluster of seven nurse cells, which synthesize proteins and RNAs for a developing oocyte, and a single oocyte are surrounded by a layer of cells called the follicular epithelium. During the previtellogenic stage, primary follicles grow from 40 to 110 μm in length; at this stage, the nurse cells occupy approximately three quarters of the follicle volume. After bloodmeal–activated vitellogenesis, the oocyte grows exponentially, accumulating large amounts of YPPs and progressively occupy-

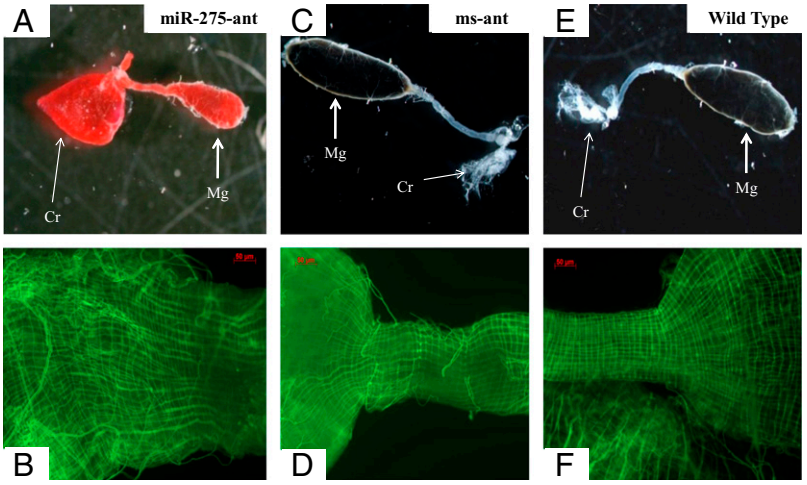

Fig. 4. Depletion of *miR-275* drastically affects blood digestion. (*A* and *B*) miR-275-ant background; (*C* and *D*) ms-ant background; (*E* and *F*) wild-type background. Midguts were obtained 24 h PBM from different backgrounds and analyzed for blood digestion defects. *A*, *C*, and *E* are bright-field observations of the backgrounds, and *B*, *D*, and *F* are actin staining of the backgrounds. Images for actin staining have a 50-μm scale.

ing larger portions of the primary follicle; in contrast, the nurse cells shrink in their volume and, by 24 h PBM, undergo cell death and cytoplasmic dumping into the oocyte (2). In wild-type mosquitoes examined 24 h PBM, primary follicles were 222 μm in length on average and were mostly occupied by yolk-filled oocytes and remnants of apoptotic nurse cells visible at the apex of each follicle (Fig. 5 *E* and *F*). The ms-ant mosquitoes had a similar ovarian phenotype (Fig. 5 *C* and *D* and Fig. S5). In contrast, ovarian development was severely compromised in mosquitoes with the miR-275-ant background. In these mosquitoes examined 24 h PBM, primary follicles were small (135 μm on average) and heterogeneous in size (Fig. 5*A* and Fig. S5). Their nurse cells were large relative to oocytes; nuclear staining clearly revealed that round nuclei of these nurse cells showed no signs of degeneration, which is characteristic of early stages of egg development (Fig. 5 *A* and *B*). Another defect in the miR-275-ant background ovaries was premature differentiation of secondary follicles; each of these follicles, 40–50 μm in size, was clearly separated from the germarium, contained large, presumably polyploid, nuclei of differentiating nurse cells, and was surrounded by a well-defined follicular epithelium (Fig. 5 *A* and *B*). In contrast, ovarioles of the wild-type or ms-ant mosquitoes at 24 h PBM had a small secondary follicle that still remained undifferentiated and located within the germarium (Fig. 5 *C*–*F*).

Although the size of oocytes in primary follicles in the miR-275-ant background ovaries varied considerably, they contained some yolk, suggesting that YPP production by the fat body was not completely compromised in miR-275-ant mosquitoes (Fig. 5*A*). Unlike *Drosophila* ovaries, those in mosquitoes do not produce YPPs. This function solely relies on the fat body (6). To determine whether *miR-275* affected *Vg* production, we used both in vitro fat body culture and in vivo analysis of fat bodies from blood-fed females 24 h PBM. Under both conditions, we found that *miR-275* was not needed for successful production of *Vg* (Fig. S6). *Vg* production was induced by 20E and AAs in all of the backgrounds analyzed (*miR-275* depleted, ms-ant control, and wild type) in in vitro fat body culture analysis (Fig. S6*A*). As controls, *miR-275* was successfully knocked down (Fig. S6*B*), whereas expression of *bantam* was not affected in any of the backgrounds (Fig. S6*C*). Of note, depletion of *miR-275* resulted in two phenotypes: a positive phenotype, as described above, and a negative phenotype in which both blood digestion and egg development were normal, as further described below. For in vivo analysis we analyzed both positive and negative phenotypes, and in all backgrounds tested this analysis showed similar results in that Vg production was not affected in miR-275–depleted mosquitoes. We also analyzed what effect lack of *miR-275* might have on TOR activation, because the AA signaling, which is required for Vg production, is mediated by TOR (5). We found no difference in TOR activation by analysis of S6K phosphorylation, a TOR downstream effecter (Fig. S6*D*).

Although the *miR-275* depletion phenotype was very drastic, its penetrance was relatively low (Table S1). We repeated the experiment of depleting *miR-275* in female mosquitoes eight times and observed a percentage range of this phenotype (Table S1). This experiment has been done by two independent researchers using multiple independent batches of antagomirs obtained from Dharmacon. Regardless of the hands and/or the batch of antagomirs, the phenotype held. To determine whether variability of the penetrance of the phenotype was due to variations in levels of *miR-275* inhibition after *miR-275* antagomir injections, we compared *miR-275* expression levels in mosquitoes exhibiting the phenotype with the negative phenotypes and found no statistical difference between the two ($P = 0.1412$) (Fig. S7). In addition, there was no statistical difference between wild-type and ms-ant ($P = 0.1797$) for *miR-275* expression and, as expected, there was a significant difference between wild-type and miR-275–depleted mosquitoes—$P = 0.0002$ for wild-type vs. positive phenotype and $P = 0.0003$ for wild-type vs. negative phenotype (Fig. S7).

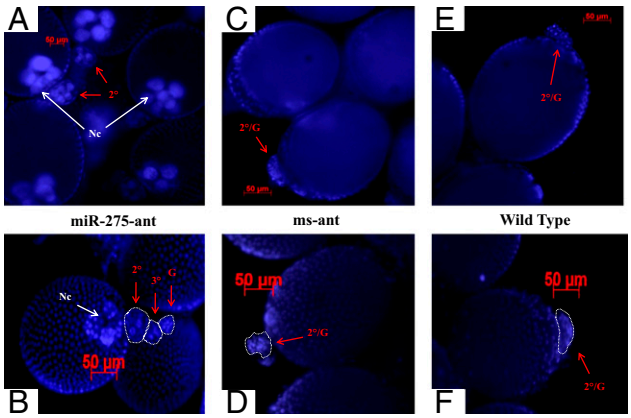

Fig. 5. Depletion of *miR-275* drastically affects egg development. (*A* and *B*) miR-275-ant background; (*C* and *D*) ms-ant background; (*E* and *F*) wild-type background. Ovaries were obtained 24 h PBM from different backgrounds and stained with Hoescht nuclear stain. White arrows point to nurse cells, and red arrows point to follicles. Nc, nurse cells; 2°, secondary follicle; 3°, tertiary follicle; 2°/G, secondary follicle within germarium. *B*, *D*, and *F* illustrate the secondary follicle in the different backgrounds, and a white dashed line encircles the follicles. All images have a 50-μm scale.

Discussion

Our results show that, in the mosquito *A. aegypti*, loss of *miR-275* results in severe defects linked to inability to digest blood, excrete excessive fluids, and properly develop eggs. miRNA repertoires in *A. aegypti* and other mosquitoes have been elucidated (22, 23, 25), but their functional roles in events critical for hematophagy and subsequent egg development have not been deciphered. The present work, therefore, represents a significant step toward defining regulatory roles of miRNAs in hematophagous disease vector arthropods. Although involvement of other miRNAs cannot be ruled out, our present study implicates a single miRNA, *miR-275*, in the regulation of major events of the gonadotrophic cycle. *miR-275* is found in multiple arthropods, including hematophagous species, and future research should establish how universal *miR-275* is in regulating events of blood digestion and egg maturation in disease vector arthropods.

Specific antagomir depletion of *miR-275* resulted in dramatic impairment of digestion of blood in mosquito females: the midgut of a female with miR-275-ant background was filled with bright red blood and did not form a bolus mass, suggesting that the blood was not digested and the peritrophic matrix not formed normally. In addition, undigested blood was present in the anterior midgut and filled up the crop, which is normally used only for nectar/sugar storage. The latter phenotype suggests a possible defect in muscle function in the alimentary canal of a female with the miR-275-ant background that results in regurgitating undigested blood into the anterior midgut and the crop. Indeed, the anterior midgut was considerably wider in these females, suggesting muscle weakening. In addition, the overall volume of the digestive system of these mosquitoes, dramatically extended by undigested blood, is suggestive of a disruption of the excretory function, which effectively removes fluids under normal conditions after blood feeding. However, the feeding behavior of females with miR-275-ant background was not affected, indicating the specificity of *miR-275* action. Egg maturation was severely compromised in mosquitoes with the miR-275-ant background, depletion of which disrupted normal progression of ovarian development. The most notable feature of primary follicles from these ovaries was the lack of apoptosis of nurse cells, which normally occurs by 24 h PBM. This

process is regulated by developmental as well as stress and nutritional signals (36). Nutrient deprivation in *Drosophila* has been shown to initiate premature follicle apoptosis in midoogenesis (37). In contrast, nurse cells in mosquitoes with the miR-275-ant background are well developed, indicating that despite defects in blood digestion in these mosquitoes, there is no premature nutrient-deprived activation of apoptosis. Whether *miR-275* has any effect on genes preventing apoptosis remains to be established.

Premature differentiation of secondary follicles in the ovaries of miR-275-ant background mosquitoes is another hallmark of disruption of normal communication within the ovariole. Induction of follicle formation and their early separation from the germarium were observed after 20E application to mosquito females before blood feeding, when the level of endogenous ecdysteroids is normally low (38). This effect of follicle development was independent of juvenile hormone and presumably from peptide hormones. In *Drosophila*, production of follicles depends on Notch signals received from the germline; JAK-STAT and Notch signal formation of stalk cells separating one follicle from another (39, 40). TSC1/2 tumor suppressor complex, which is an upstream inhibitor of TOR, has been implicated in maintaining *Drosophila* germline stem cells by preventing their differentiation (41). Moreover, overexpression of *Ago1* in the germaria lead to overproliferation of germline stem cells in the *Drosophila* ovary, suggesting that an Ago1–miRNA pathway plays an instructive role in differentiation of egg chambers (42).

It is not clear whether observed defects in ovarian development are due to the requirement of this miRNA to its targets in the ovary or as a consequence of the disruption of the digestive system functions linked to the failure of blood digestion. Interestingly, in vivo and in vitro monitoring of *Vg* showed that YPP production by the fat body was not compromised in miR-275-ant mosquitoes; and 20E and AA could elicit their respective signals via hormonal and nutritional regulatory pathways, respectively. This suggests that, although the digestive function was severely disrupted in these mosquitoes, some elements of the regulatory circuitry, both nutritional and hormonal, remained intact after *miR-275* depletion, permitting YPP production to proceed. Thus, genes encoding 20E regulatory hierarchy as well as those encoding the AA/TOR pathway are unlikely targets of *miR-275*. In *Drosophila*, *miR-14* is involved in a regulatory feedback controlling the level of the ecdysone receptor, which is a 20E binding partner of a functional 20E receptor (17). Although, *miR-8* has been implicated in regulation of the insulin branch of the TOR pathway (21), no information is available on the role of miRNAs in control of the nutritional AA branch of this pathway. Whether *miR-8* and *miR-14* play similar roles during blood-meal–activated egg development of mosquitoes remains to be determined.

Further studies are required to establish *miR-275* targets in the mosquito gonadotrophic cycle at the molecular level. Deciphering miRNA targets in a nonmodel organism, such as the mosquito, is a challenging task because of the complexity underlying regulatory roles of these molecules. Genetic analyses in animal model organisms have revealed that certain miRNAs possess defined gene-, cell-, and organ-specific roles (10, 18, 43). In addition, other miRNAs have been predicted to have multiple gene targets, signifying importance of these molecules in regulatory networks controlling principal biological functions (43, 44).

We found that *miR-275* itself is a target of 20E and AA regulation. *miR-275* was up-regulated after blood feeding, correlating with the elevating ecdysteroid titer in female mosquitoes (45). Our in vitro fat body culture assay has provided definitive proof that both 20E and AAs are required for activation of expression of *miR-275*. In addition, several other miRNAs were also up-regulated by 12 h PBM—in particular *let-7*, *miR-100*, *miR-125*, and *miR-305* (Fig. S1*III*). Whether these mosquito miRNAs are also regulated in a manner similar to *miR-275* needs to be established. In *Drosophila* they are strongly activated during pupal development, the time when 20E levels are high (46, 47). *Let-7* and *miR-125* are required for metamorphic processes in *Drosophila* (47). 20E and juvenile hormone have opposite effects on *let-7*, *miR-100*, and *miR-125*, which is in agreement with opposite roles of these two hormones during insect development and metamorphosis (46). Analyses of RNAi depletion of the 20E early gene *broad* in *Drosophila* S2 cells and *Drosophila broad* mutant resulted in inhibition of induction of *let-7*, *miR-100*, and *miR-125*, implicating the 20E gene regulatory hierarchy in hormonal control of miRNA expression (46). Experiments showing that RNAi depletion of the ecdysone receptor inhibits expression of *let-7* and *miR-125* by 20E in *Drosophila* S2 cells further substantiate this conclusion (48). Further studies are warranted to investigate the molecular basis of *miR-275* expression during mosquito egg development.

In this work, we applied an antagomir approach to deplete *miR-275* in the mosquito *A. aegypti*. This resulted in a 10-fold more efficient decrease of the *miR-275* level than observed after either *Dcr1* or *Ago1* depletion. Moreover, the antagomir depletion was highly specific for *miR-275*. Several studies in *Drosophila* and other insects have reported significant depression of miRNA levels and clear phenotypes after depletion of major miRNA biogenesis factors (31, 49–51). However, despite appreciable decrease of *miR-275* in *Dcr1*- and *Ago1*-depleted backgrounds, no phenotypes attributed to this miRNA in the mosquito were observed. The fact that antagomirs result in higher specific knockdown efficiency for a given miRNA possibly explains why we observed no defects in blood digestion and egg development in either the Dcr1:RNAi or the Ago1:RNAi backgrounds. Bejarana et al. (18) have reported that, in *Drosophila*, specific depletion of *mir-9a* results in a phenotype different from that of *Dcr-1* or *Pasha*, miRNA biogenesis factors. Taken together, these data suggest that the failure to observe a phenotype in cells or tissues that are depleted of a general miRNA biogenesis factor cannot reliably be taken as an indicator of the lack of miRNA involvement in a process under-investigation.

In conclusion, this study has established a fundamental role of miRNAs in regulation of gonadotrophic events in mosquitoes. Future studies are warranted to find targets of *miR-275* and determine what roles they normally play in blood digestion and egg development. This report has opened the door for exploration of miRNA in regulation of critical physiological functions specific to vector arthropods, such as blood-meal–activated gonadotrophic cycles, which are ultimately linked to the transmission of disease pathogens.

Methods

Animals. The mosquito *A. aegypti* UGAL/Rockefeller strain was raised as described previously (26). Female mosquitoes 3–5 d after eclosion were fed on the blood of anesthetized white rats to initiate egg development.

mRNA and miRNA Expression Analysis. *A. aegypti* miRNA sequences available at miRBase (http://www.mirbase.org/index.shtml) were examined for their location in the genome using BLAST searches at the Broad Institute site (http://www.broadinstitute.org/annotation/genome/aedes_aegypti/). miRNA expression was analyzed quantitatively by means of the miScript PCR System from Qiagen that includes the miScript reverse transcription kit (catalog. no. 218061; Qiagen), miScript SYBR Green PCR kit (catalog no. 218073, Qiagen), and QuantiTect Primer Assays. The miScript allows expression analysis of miRNAs by real-time PCR. This kit contains a blend of enzymes containing a poly(A) polymerase and a reverse transcriptase. A poly(A) polymerase adds a poly(A) tail to the 3′ end of miRNAs. Reverse transcriptase then converts the RNA to cDNA using oligo-dT and random primers. The oligo-dT primers have a universal tag sequence on the 5′end. This tag allows amplification in the real-time PCR step of analysis. Initially, we used specific primers provided by QuantiTect Primer Assays for *let-7*, *miR-125*, and *miR-100* quantification. Once we obtained the amplicons for all three miRNAs from real-time PCR analysis, we cloned and sequenced all of the amplicons using TOPO-TA

(Invitrogen) and discovered the Qiagen signature sequence that was added to the 3′ end of three miRNAs. On the basis of this signature sequence, we designed our own common-region reverse primer, termed Universal primer 5′ GAATCGAGCACCAGTTACGC 3′. Forward primers for expression analysis were the sequence of the mature miRNA, as long as the Tm was ≈58 °C. If Tm for the mature miRNA sequence was <58 °C, extra sequences were added to the 5′ end of the forward primers. Once this expression analysis was optimized, we used our own primers instead of the primers provided by QuantiTect Primer Assays. First, RNA was isolated by TRIzol (Invitrogen) extractions from fat bodies of blood-fed female mosquitoes at various time points. RNA was then digested with DNase I (catalog no. 18068015; Invitrogen) and subjected to cDNA production with the miScript reverse transcription kit from Qiagen. cDNA obtained from this was then subjected to expression analysis with the miScript SYBR Green PCR kit from Qiagen. The PCR condition was as follows: Step 1, 95 °C for 15 min; Step 2, 94 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s for 50 cycles; Step 3, 95 °C for 1 min. This was then followed by melt curve analysis. The miScript PCR System was also used to analyze *Vg* and *Dicer1* transcripts using *S7* as an internal control. Expression data were plotted using $2^{-\Delta Ct}$ whereby the cycle threshold (Ct) for the gene of interest is compared with the Ct of the internal control gene, in this case *S7* (52). All expression analysis is plotted as relative expression to S7 (52). *Vg* primers are Vg-Forward primer 5′ CCAGAAGACGTCAGCATTCA 3′ and Vg-Reverse primer 5′ TGGCGCAGATGATAGAACAG 3′. *S7* primers are S7-Forward primer 5′ TCAGTGTACAAGAAGCTGACCGGA 3′ and S7-Reverse primer 5′ TTCCGCGCGCGCTCACTTATTAGATT 3′. *Dicer 1* primers are Dcr1-Forward primer 5′ TGCAATCTGCTGATAGGCAC 3′ and Dcr1-Reverse primer 5′ ATGATATGCGGTGGAAGCTC 3′.

In Vitro Fat Body Culture. In vitro fat body culture was performed as previously described (5, 26). Reagents used include 20E, from Sigma (catalog no. H5142), dissolved in ethanol. In brief, fat bodies from 3- to 5-d-posteclosion female mosquitoes were dissected and incubated in one of four conditions: (*i*) no 20E and no AAs, (*ii*) 20E and no AAs, (*iii*) no 20E and AAs, and (*iv*) 20E and AAs. Fat bodies were kept under these conditions for 6 h at 27 °C. After these incubations, fat bodies were harvested, RNA was isolated by TRIzol, and miRNA/mRNA expression was determined as explained above.

RNA Interference. Gene models for *Dicer1* (*Dcr1*) and *Argonaute 1* (*Ago1*) in *A. aegypti* have been reported (27); however, when we compared the two proposed *Ago1* genes, we considered these to be haplotypes rather than individual genes. A new *Ago1*gene model is shown in Fig. S2 and was sent to ImmunoDB (http://cegg.unige.ch/Insecta/immunodb). For RNAi experiments, PCR products were cloned into the TOPO-TA vector from Invitrogen (catalog no. 45-0640) according to the manufacturer's specific instructions. Once the plasmids were verified by sequencing, they were used to make dsRNA. For *Dcr1* and *Ago1*, 449-bp and 546-bp regions were obtained, respectively, using RT-PCR. Primers for *Dcr1* are Dcr1-Forward primer 5′ TCCGAAAAGTTCATTCCCTG 3′ and Dcr1-Reverse primer 5′ ATACATCGGCCAGACTCCAC 3′. Primers for *Ago1* are Ago1-Forward primer 5′ CCCGGAAAGACACCTGTTTA 3′ and Ago1-Reverse primer 5′ CGTGATACCCGGCTTGTAGT 3′. To obtain an amplicon for use as a substrate for a dsRNA reaction, the following primers were used:

Universal TOPO Forward primer 5′ taatacgactcactatagggGATCCACTAGTAACGGCCG 3′ and Universal TOPO Reverse primer 5′ taatacgactcactatagggGTGTGATGGATATCTGCAGAATTCG 3′. Uppercase correlates to a common region outside of the PCR product insertion site of the pCR II vector. Lowercase correlates to the T7 primer sequence. PCR products using the above-mentioned plasmids as templates and the universal primers result in both 5′ and 3′ addition of T7 primer sequence. This PCR product was then used to make dsRNA with the MEGAscript kit from Ambion (catalog no. AM1334) according to the manufacturer's specific instructions.

Mosquitoes were CO_2 anesthetized 1 to 2 d after eclosion and injected into the thorax at a volume of 0.5 μL with appropriate dsRNA molecules at ≈1.5–2 μg/μL. *Mal* was used as a negative control, as described previously (5). Mosquitoes were allowed to recover for 4 to 5 d before blood feeding.

Synthesis and Application of Antagomirs. Antagomirs were obtained from Dharmacon using the custom RNA module at http://www.dharmacon.com/rna/rna.aspx. Antagomir to *miR-275* was 5′ mC* mG* mC mG mC mG mC mU mA mC mU mU mC mA mG mG mU mA mC mC* mU* mG* mA* mA 3′. The control antagomir termed missense was 5′ mC* mG* mC mU mU mU mC mG mU mG mG mU mU mC mU mG mG mU mA mC* mC* mU* mU* mA 3′. "*" is a PS backbone instead of the usual PO backbone. "m" is an OCH_3 group on the 2′ end of the base instead of the usual OH group. Finally, there is a 3′ cholesterol group added to each RNA oligo for potency reasons (31). Antagomirs were constructed as outlined in ref. 32. They were injected into female mosquitoes at a dose of 100 μM in a volume of 0.5 μL—using the same method as mentioned above for dsRNA molecules—and delivered ≈50 pg of antagomir per mosquito. Unlike with dsRNA, we found a relatively high lethality (approximately 25%) in mosquitoes injected 1 to 2 d after emergence with any of the antagomirs. However, utilization of slightly older mosquitoes, 2 to 3 d after emergence, for antagomir depletion experiments brought mortality rate to the same level as in dsRNA experiments, which have been routinely used in mosquito biology (5, 26).

Actin Staining of Midguts and DNA Staining of Oocytes. For fluorescence staining of midguts, mosquitoes were intrathoracically injected with and allowed to incubate for 30 min in a solution containing 0.165 μM phalloidin Alexa Fluor 488 (A12379; Invitrogen), 1% Triton-X 100, and 8% formaldehyde, whereby APS (*Aedes* physiological saline) was the diluent according to conditions detailed by Glenn et al. (53). Midguts were then dissected and washed in APS-T (*Aedes* physiological saline with 0.3% Triton-X 100) and mounted using mounting media from VectaShield (H1000).

For fluorescence staining of oocytes, mosquitoes were dissected and ovaries incubated in APS-T for 10 min. The ovaries were then put into a DNA staining solution containing 5 μM Hoescht 33342 (H1399; Invitrogen) in APS for 10 min. All imaging was performed using a Zeiss microscope, AxioObserver A1, and images were obtained using AxioVision software.

Western Blot Analysis. Protein analysis of Vg and TOR signaling were done according to ref. 54. Briefly, fat bodies from blood-fed female mosquitoes were obtained 24 h PBM. These fat bodies were homogenized and lysed and run on Tris-Glycine gels (Invitrogen) and transferred to PVDF membranes. These membranes were probed with Vg, S6K-P, and native S6K antibodies according to ref. 54.

ACKNOWLEDGMENTS. This study was supported by National Institutes of Health Award R37 AI244716.

1. Weaver SC, Reisen WK (2010) Present and future arboviral threats. *Antiviral Res* 85:328–345.
2. Clements AN (2000) *The Biology of Mosquitoes Volume 1: Development, Nutrition and Reproduction* (CABI, Wallingford, Oxfordshire, United Kingdom), pp 304–408.
3. Attardo GM, Hansen IA, Raikhel AS (2005) Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. *Insect Biochem Mol Biol* 35:661–675.
4. Sappington TW, Raikhel AS (1998) Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem Mol Biol* 28:277–300.
5. Hansen IA, Attardo GM, Park JH, Peng Q, Raikhel AS (2004) Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. *Proc Natl Acad Sci USA* 101:10626–10631.
6. Raikhel AS, Brown M, Belles X (2005) *Endocrine Control of Reproductive Processes: Comprehensive Molecular Insect Science*, eds Gilbert L, Gill S, IatrouK (Elsevier, Amsterdam), Vol 3, pp 433–491.
7. Smibert P, Lai EC (2008) Lessons from microRNA mutants in worms, flies and mice. *Cell Cycle* 7:2500–2508.
8. Carrington JC, Ambros V (2003) Role of microRNAs in plant and animal development. *Science* 301:336–338.
9. Liu N, et al. (2008) The evolution and functional diversification of animal microRNA genes. *Cell Res* 18:985–996.
10. Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75:843–854.
11. Reinhart BJ, et al. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403:901–906.
12. Chen X (2009) Small RNAs and their roles in plant development. *Annu Rev Cell Dev Biol* 25:21–44.
13. Bushati N, Cohen SM (2007) microRNA functions. *Annu Rev Cell Dev Biol* 23:175–205.
14. Hipfner DR, Weigmann K, Cohen SM (2002) The bantam gene regulates *Drosophila* growth. *Genetics* 161:1527–1537.
15. Xu P, Vernooy SY, Guo M, Hay BA (2003) The *Drosophila* microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* 13:790–795.
16. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell* 113:25–36.
17. Varghese J, Cohen SM (2007) microRNA miR-14 acts to modulate a positive autoregulatory loop controlling steroid hormone signaling in *Drosophila*. *Genes Dev* 21:2277–2282.

18. Bejarano F, Smibert P, Lai EC (2010) miR-9a prevents apoptosis during wing development by repressing *Drosophila* LIM-only. *Dev Biol* 338:63–73.
19. Iovino N, Pane A, Gaul U (2009) miR-184 has multiple roles in *Drosophila* female germline development. *Dev Cell* 17:123–133.
20. Sokol NS, Ambros V (2005) Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev* 19: 2343–2354.
21. Hyun S, et al. (2009) Conserved MicroRNA miR-8/miR-200 and its target USH/FOG2 control growth by regulating PI3K. *Cell* 139:1096–1108.
22. Mead EA, Tu Z (2008) Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, *Anopheles stephensi*. *BMC Genomics* 9:244.
23. Li S, Mead EA, Liang S, Tu Z (2009) Direct sequencing and expression analysis of a large number of miRNAs in *Aedes aegypti* and a multi-species survey of novel mosquito miRNAs. *BMC Genomics* 10:581.
24. Winter F, Edaye S, Hüttenhofer A, Brunel C (2007) Anopheles gambiae miRNAs as actors of defence reaction against *Plasmodium* invasion. *Nucleic Acids Res* 35: 6953–6962.
25. Skalsky RL, Vanlandingham DL, Scholle F, Higgs S, Cullen BR (2010) Identification of microRNAs expressed in two mosquito vectors, *Aedes albopictus* and *Culex quinquefasciatus*. *BMC Genomics* 11:119.
26. Roy SG, Hansen IA, Raikhel AS (2007) Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* 37: 1317–1326.
27. Campbell CL, Black WC, 4th, Hess AM, Foy BD (2008) Comparative genomics of small RNA regulatory pathway components in vector mosquitoes. *BMC Genomics* 9:425.
28. Hutvágner G, Simard MJ, Mello CC, Zamore PD (2004) Sequence-specific inhibition of small RNA function. *PLoS Biol* 2:E98.
29. Krützfeldt J, et al. (2005) Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 438:685–689.
30. Leaman D, et al. (2005) Antisense-mediated depletion reveals essential and specific functions of microRNAs in *Drosophila* development. *Cell* 121:1097–1108.
31. Förstemann K, Horwich MD, Wee L, Tomari Y, Zamore PD (2007) *Drosophila* microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell* 130:287–297.
32. Horwich MD, Zamore PD (2008) Design and delivery of antisense oligonucleotides to block microRNA function in cultured *Drosophila* and human cells. *Nat Protoc* 3: 1537–1549.
33. Shao L, Devenport M, Jacobs-Lorena M (2001) The peritrophic matrix of hematophagous insects. *Arch Insect Biochem Physiol* 47:119–125.
34. Lu SJ, Pennington JE, Stonehouse AR, Mobula MM, Wells MA (2006) Reevaluation of the role of early trypsin activity in the transcriptional activation of the late trypsin gene in the mosquito *Aedes aegypti*. *Insect Biochem Mol Biol* 36:336–343.
35. Isoe J, Rascón AA, Jr., Kunz S, Miesfeld RL (2009) Molecular genetic analysis of midgut serine proteases in *Aedes aegypti* mosquitoes. *Insect Biochem Mol Biol* 39:903–912.
36. Pritchett TL, Tanner EA, McCall K (2009) Cracking open cell death in the *Drosophila* ovary. *Apoptosis* 14:969–979.
37. Hou YC, Chittaranjan S, Barbosa SG, McCall K, Gorski SM (2008) Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during *Drosophila melanogaster* oogenesis. *J Cell Biol* 182:1127–1139.
38. Beckemeyer EF, Lea AO (1980) Induction of follicle separation in the mosquito by physiological amounts of ecdysterone. *Science* 209:819–821.
39. López-Schier H, St Johnston D (2001) Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during *Drosophila* oogenesis. *Genes Dev* 15:1393–1405.
40. Assa-Kunik E, Torres IL, Schejter ED, Johnston DS, Shilo BZ (2007) *Drosophila* follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. *Development* 134:1161–1169.
41. Sun P, Quan Z, Zhang B, Wu T, Xi R (2010) TSC1/2 tumour suppressor complex maintains *Drosophila* germline stem cells by preventing differentiation. *Development* 137:2461–2469.
42. Yang L, et al. (2007) Argonaute 1 regulates the fate of germline stem cells in *Drosophila*. *Development* 134:4265–4272.
43. Smibert P, Lai EC (2010) A view from *Drosophila*: Multiple biological functions for individual microRNAs. *Semin Cell Dev Biol* 21:745–753.
44. Herranz H, Cohen SM (2010) MicroRNAs and gene regulatory networks: Managing the impact of noise in biological systems. *Genes Dev* 24:1339–1344.
45. Hagedorn HH, et al. (1975) The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc Natl Acad Sci USA* 72:3255–3259.
46. Sempere LF, Sokol NS, Dubrovsky EB, Berger EM, Ambros V (2003) Temporal regulation of microRNA expression in *Drosophila* melanogaster mediated by hormonal signals and broad-Complex gene activity. *Dev Biol* 259:9–18.
47. Caygill EE, Johnston LA (2008) Temporal regulation of metamorphic processes in *Drosophila* by the let-7 and miR-125 heterochronic microRNAs. *Curr Biol* 18:943–950.
48. Garbuzov A, Tatar M (2010) Hormonal regulation of *Drosophila* microRNA let-7 and miR-125 that target innate immunity. *Fly (Austin)* 4:306–311.
49. Miyoshi K, Okada TN, Siomi H, Siomi MC (2009) Characterization of the miRNA-RISC loading complex and miRNA-RISC formed in the *Drosophila* miRNA pathway. *RNA* 15: 1282–1291.
50. Liu X, et al. (2007) Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. *RNA* 13:2324–2329.
51. Gomez-Orte E, Belles X (2009) MicroRNA-dependent metamorphosis in hemimetabolan insects. *Proc Natl Acad Sci USA* 106:21678–21682.
52. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.
53. Glenn JD, King JG, Hillyer JF (2010) Structural mechanics of the mosquito heart and its function in bidirectional hemolymph transport. *J Exp Biol* 213:541–550.
54. Shiao SH, Hansen IA, Zhu J, Sieglaff DH, Raikhel AS (2008) Juvenile hormone connects larval nutrition with target of rapamycin signaling in the mosquito *Aedes aegypti*. *J Insect Physiol* 54:231–239.