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Juvenile hormone regulation of microRNAs is mediated by E75 in the Dengue vector mosquito *Aedes aegypti*

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MicroRNAs (miRNAs) are small noncoding RNAs that play critical roles in controlling posttranscriptional gene regulation and have a profound effect on mosquito reproduction and metabolism. Juvenile hormone (JH) is critical for achieving reproductive competence in the main vector of human arboviral diseases, *Aedes aegypti*. We report a JH-mediated mechanism governing miRNA expression. Using a transcription factor screen with multiple primary miRNA (pri-miRNA) promoters, we identified that the Ecdysone-induced protein E75 (E75) isoform (E75-RD) induced miRNA gene promoter activity. E75 binding sites were determined in miRNA promoters by means of cell transfection assay. E75-RD was found to be up-regulated by JH, as shown by the JH application and RNA interference (RNAi) of the JH receptor Methoprene-tolerant (Met). Small RNA sequencing from RNAi of Met and E75 displayed an overlapping miRNA cohort, suggesting E75 to be an intermediate component within the JH hierarchical network controlling miRNAs. Further experiments confirmed that E75-RD positively regulates several miRNAs including miR-2940. Reducing *miR-2940* resulted in the arrest of follicle development and number of eggs laid. Performing miRNA target predictions and RT-qPCR from antagomir Ant-2940-3p–treated fat body tissues identified the mRNA target Clumsy (*AAEL002518*). The molecular interaction between this gene target and miR-2940 was confirmed using an in vitro dual luciferase assay in *Drosophila* S2 cells and in *Ae. aegypti* Aag2 cell lines. Finally, we performed a phenotypic rescue experiment to demonstrate that miR-2940/Clumsy is responsible for the disruption in egg development. Collectively, these results established the role of JH-mediated E75-RD in regulation of miRNA gene expression during the mosquito reproductive cycle.

small noncoding RNA | hormone | isoform

Mosquitoes are responsible for transmitting numerous disease pathogens to humans due to their obligatory blood feeding for reproduction. The *Aedes aegypti* mosquito is a carrier of multiple arboviral diseases, such as dengue fever, yellow fever, chikungunya, and Zika virus. Limited treatment is available for these diseases and thus vector population control can be an effective method to curb disease transmission. Since *Ae. aegypti* is also capable of transmitting multiple viruses that circulate in the same geographic region they inhabit, removing or reducing the vector population effectively could inhibit the spread of all viral diseases (1). During a single gonotrophic cycle, each mosquito lays ∼100 to 150 eggs and can undergo this reproductive cycle up to approximately three times during her lifespan. Thus, inhibiting the reproductive cyclicity could have a significant impact on the immediate and future population densities and provides an effective target for biological control efforts. A greater understanding of the underlying molecular mechanisms that regulate mosquito reproduction is needed in order to develop such vector control methods.

In insects, hormones play critical roles in development, metamorphosis, reproduction, longevity, differentiation, and migratory behavior (2, 3). In adult female mosquitoes, juvenile hormone III (JH) mediates the posteclosion (PE) developmental phase and is involved in tissue maturation required for subsequent reproductive events, such as production of yolk protein precursors and egg development, following acquisition of host blood. The JH hemolymph titer increases over time, reaching peak levels at ∼48 to 50 h PE and declining thereafter (4). JH binds its receptor, Methoprene-tolerant (Met), a member of the basic helix–loop–helix-Per-Arnt-Sim (BHLH-PAS) transcription factors, leading to a cascade of events that results in transcriptional activation and repression of JH target genes. Previous research has centered on the impact of JH on transcriptional regulation of messenger RNA genes (4, 5). This had resulted in the identification of two Met-regulated transcription factors, Krüppel homolog 1 (Kr-h1) and Hairy, and their role in repression of JH-regulated mRNA transcripts (6–8).

One of the critical organs that undergoes JH-activated maturation is the fat body, an analog to the vertebrate liver and adipose tissue. The fat body is the insect center for metabolism and immunity. Interference with the JH-dependent fat body maturation process inhibits the ribosomal biogenesis pathway and results in insufficient production of lipids and yolk, leading to an inhibition of egg development (9).

MicroRNAs (miRNAs) belong to a class of small noncoding RNAs that are posttranscriptional regulators of mRNA expression and have been linked to different mosquito biological processes. *miR-275* was shown to target *sarco/endoplasmic reticulum* Ca^{2+} *adenosine triphosphates* (*SERCA*) to control proper blood digestion in the midgut, and *miR-8* regulates the Wingless signaling pathway in the fat body for secretion of lipids into the hemolymph during vitellogenesis (10–12). Inhibition of both miRNAs results in biological

Significance

***Aedes aegypti* mosquitoes transmit numerous viral pathogens to humans. Targeting mosquito reproduction could be critical in controlling the spread of these pathogens. MicroRNAs (miRNAs) control key physiological processes during mosquito reproduction. The insect hormone juvenile hormone (JH) is essential for a mosquito to achieve reproductive competence. JH governs expression of several miRNAs through the transcription factor Ecdysone-induced protein 75 (E75-RD). Blocking E75 by RNA interference impairs miR-2940, which in turn affects ovarian follicle formation and egg maturation. We identified the messenger RNA of the glutamate receptor Clumsy as a miR-2940 target and showed its involvement in reproduction. This study establishes E75-RD to be an intermediate factor within the JH hierarchical network that is involved in regulation of miRNAs in mosquitoes.**

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The authors declare no competing interest.

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defects that lead to egg inhibition and prevention of mosquito reproduction. Newer advancements in genome-editing tools using CRISPR/Cas9 have allowed for the generation of miRNA-specific mutations in mosquitoes that have had a more-profound impact on reproductive physiology than antagomir-based treatments. This technique has been applied to generate miRNA-specific mutations for *miR-309*, responsible for targeting the Homeobox gene *SIX4*, and for *miR-277*, responsible for targeting both the *insulin-like peptide-7* and *insulin-like peptide-8* (13, 14). The phenotypic outcome from these mutagenesis experiments completely inhibits egg development and thus is a promising technique for a population control tool.

Regulation of miRNA gene expression in mosquitoes has received limited attention. Several studies in *Drosophila melanogaster* have highlighted the potential for the insect hormone 20-hydroxyecdysone (20E) to regulate miRNA expression of the primary miRNA (pri-miRNA) containing miR-100, miR-125, and Let-7 through the ecdysone receptor (15, 16). This miRNA locus is similarly regulated by the 20E-inducible transcription factor Broad in the cockroach *Blattella germanica* during metamorphosis (17). Interestingly, the combined treatment of JH and 20E will repress activity of this loci, suggesting that both insect hormones have a functional role in miRNA regulation (18). Recently, a study on the hormonal control of miRNAs in the mosquito gut implicated both JH and 20E in this process (19). However, the molecular mechanism governing JH control of miRNA gene expression has not been elucidated. Thus, we explored the role of the JH-mediated transcription factors and their potential to regulate miRNA expression in the mosquito fat body during the PE developmental phase. We provide evidence that JH mediates the expression of the transcription factor Ecdysone-Induced Protein 75 (E75). We demonstrate that a specific E75 isoform (E75-RD) is an intermediate component of the JH hierarchical transcriptional network and acts as a direct activator of miRNA expression. We further show the functional role of one E75-regulated miRNA, *miR-2940-3p*, as a direct regulator of the glutamate receptor Clumsy. Blockage of *miR-2940-3p* results in an overexpression of *clumsy* mRNA transcripts, leading to a reproductive defect in egg development. The presented work demonstrates a miRNA aspect of gene expression mediated by JH regulation through E75-RD. This research expands our knowledge of insect development and highlights a potential for the expansion of an alternative mosquito control strategy.

Results

RNAi Treatment of Drosha Leads to pri-miRNA Enrichment. Identifying the candidate transcription factors for miRNA expression regulation can be challenging due to the scarcity of information on promoter elements located upstream of miRNA start sites to which the transcription factors would typically bind. This issue arises in large part due to the nature of the miRNA biogenesis. Mature miRNAs originate from a pri-RNA strand that is quickly degraded after cleavage by Drosha in the miRNA biogenesis pathway (20–22). As a result, pri-miRNAs are seldom detected in a standard small RNA sequencing (RNA-seq) application and, consequently, are missing from the genome annotation data. A methodology to enrich for pri-miRNAs addressing this specific issue has been described using a Drosha-knockout human cell line (23). We utilized this methodology using RNA interference (RNAi) targeted against the *Ae. aegypti drosha* transcript, followed by RNA-seq analysis for the identification of novel pri-miRNAs and their potential start sites. We performed RNAi for targeting Drosha (*AAEL008592*) expression from adult female mosquitoes and collected fat body tissue at 72 h PE. We evaluated the knockdown efficiency of our treatment using RT-qPCR and found *drosha* levels to be ∼80% lower in treatment group than in Luciferase RNAi control group (iLuc) (Fig. 1*A*). To confirm an enrichment of potential pri-miRNA transcripts within our *Drosha* knockdown group, we subsequently measured gene expression levels of the long noncoding transcript *AAEL027655*. *AAEL027655* is currently the only transcript within *Ae. aegypti* genome adjacent to a premiRNA sequence, in this case premiRNA-276, and we hypothesize this is to be a representative of an authentic pri-miRNA transcript. When we measured the transcript level of *AAEL027655* within our iDrosha group, we found a higher level of the transcript than in the iLuc control ($P < 0.05$; *SI Appendix*, Fig. S1*A*), confirming that the RNAi treatment was both sufficient in reducing *drosha* mRNA levels and led to an enrichment of the pri-miRNA transcripts.

Identification of pri-miRNA Transcripts in the *Ae. aegypti* Genome. We next performed RNA-seq analysis on the iDrosha and iLuc samples in an attempt to discover *Ae. aegypti* pri-*miRNAs*. Three biological replicates were included in treatment and control groups, and each biological sample was sequenced to the depth of ∼20 million reads (2 × 100) (*SI Appendix*, Table S1). Reads were mapped to the *Ae. aegypti* reference genome (L5.1) and expression levels were compared between the iLuc and iDrosha groups. *AAEL027655* (fold change, +3.8 at 72 h PE) displayed an upregulation within the iDrosha dataset, consistent with the RT-qPCR findings (*SI Appendix*, Dataset S1). Using the Integrative Genomics Viewer (24), we manually scanned for reads mapping upstream of *premiRNA* genomic locations to identify potential pri-miRNAs. Using this approach, we discovered an enrichment of reads located upstream to the precursor miRNA (pre-miRNA) of *miR-2940* and *miR-252* in the iDrosha samples that was absent in the iLuc samples. (Fig. 2 and *SI Appendix*, Fig. S2). Based off this enrichment, using RT-qPCR, we confirmed the enrichment of both pri-miRNAs and selected three pri-miRNAs (*miR-276*, *miR-2940*, and *miR-252*) for further functional promoter analysis (Fig. 1*B* and *SI Appendix*, Fig. S1 *A* and *B*).

Analysis of pri-miRNA Promoters with JH-induced Transcription Factors in Cell Transfection Assay. To determine potential regulators of pri-miRNAs, we utilized a cell-based luciferase assay in *Drosophila* S2 cells. Promoter regions of *miR-276*, *miR-2940*, and *miR-252* were subcloned using PCR into the promoterless firefly luciferase vector (pGL4.17) and transfected with or without protein expression vectors (pAc5.1). Initially, we measured luciferase levels from different *Ae. aegypti* transcription factors that have previously been characterized during the PE developmental phase, which include Met and

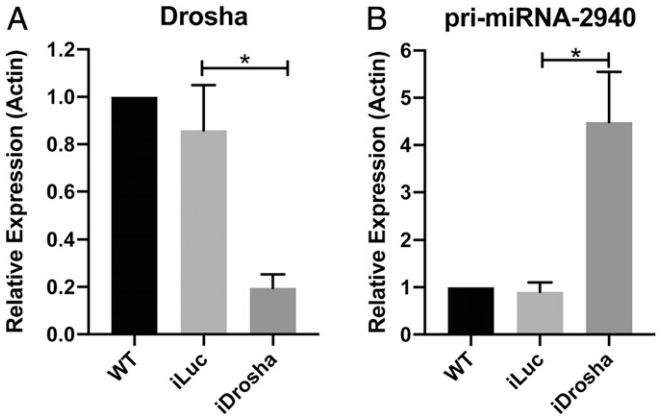

Fig. 1. RNAi treatment of Drosha leads to enrichment of pri-miRNA transcripts. (*A* and *B*) Relative gene expression levels of drosha and pri-miRNA-2940 from fat body tissues at 72 h PE after treatment with dsRNA targeting Drosha (iDrosha). WT and dsLuciferase (iLuc) are controls. For gene expression analysis, each group represents an average of three independent experiments that included three to four biological samples per experiment. Statistical significance was calculated using a one-way ANOVA. *$P < 0.05$. The data are shown as average ± SEM.

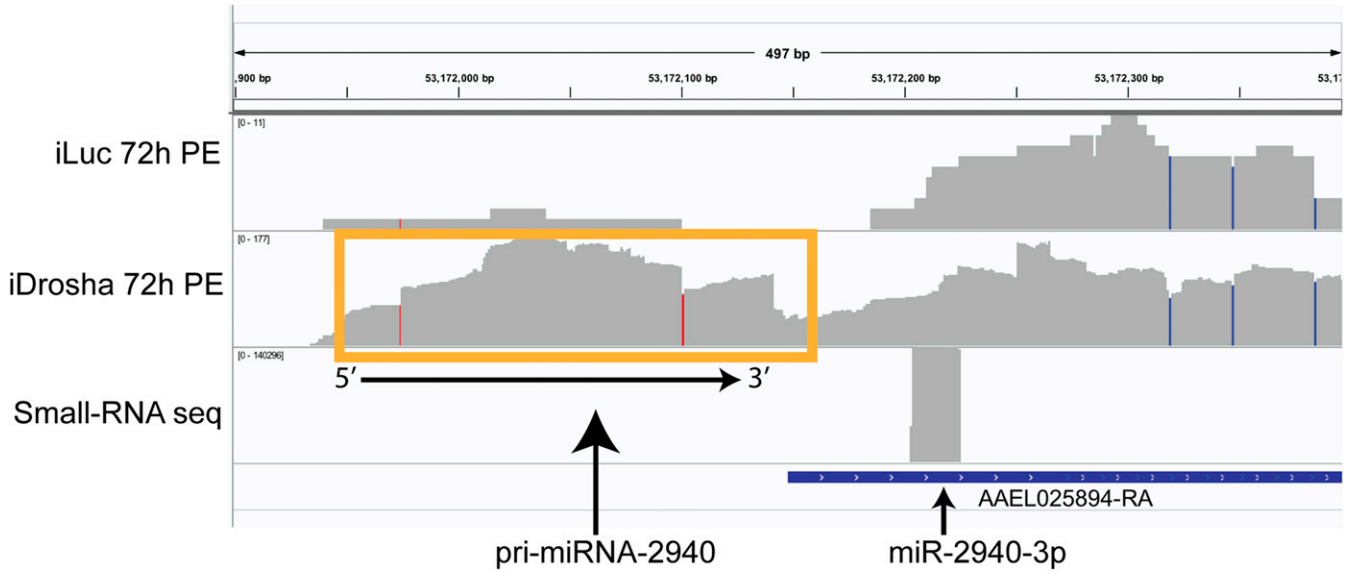

Fig. 2. IGV illustrations for pri-miRNA enrichment for *mir-2940* in iDrosha RNA-Seq datasets.

Taiman, Hairy and Groucho, or Krüppel-homolog 1 (Kr-h1), on each of the individual pri-miRNA promoters. The overexpression of each transcription factor had no effect on the amount of luciferase produced by any of the three candidate promoters tested (Fig. 3*A* and *SI Appendix*, Fig. S4 *A* and *B*). Thus, we reasoned that an additional factor was likely responsible for pri-miRNA regulation. We searched previous fat body–specific RNA-seq data during the PE phase to identify additional transcription factors expressed during this phase. E75 (*AAEL007397*) was found to have a relative expression level value equal to that of Kr-h1; thus, we speculated it could have a regulatory function during this phase (6). E75 is a nuclear receptor comprised of three major isoforms—E75-RA, E75-RC, and E75-RD (previously referred to as E75A, E75B, and E75C, respectively) (25). We examined the expression profile of each individual isoform during the PE and post-blood meal (PBM) phases and reasoned that since E75-RD was the most abundant isoform during the PE phase (26) and displayed a temporal profile peaking at 60 h PE, it is likely associated with JH regulation during the PE phase (*SI Appendix*, Fig. S3*A*). We cloned the E75-RD isoform into the pAc5.1 protein expression vector and repeated the luciferase assay. Interestingly, the expression of E75 increased the amount of luciferase produced by all three candidate promoters of *miR-2940*, *miR-252*, and *miR-276* (Fig. 3*A* and *SI Appendix*, Fig. S4 *A* and *B*), suggesting that this transcription factor is a key activator of miRNA regulation in the JH pathway.

Identification of E75 Interaction with pri-miRNA Promoters. We next utilized a progressive deletion strategy in order to identify the specific DNA motif bound by E75 on the promoter region for all three of these candidate promoters. The 5′ region of each promoter plasmid was truncated until the luciferase activity was equivalent to the promoterless pGL4.17 plasmid. We found the respective upstream regions for *miR-2940* (165 to 115), *miR-276* (570 to 560), and *miR-252* (325 to 280) to contain the DNA region responsible for E75 binding (Fig. 3*B* and *SI Appendix*, Fig. S5 *A* and *B*). To confirm this result, we performed a site-directed mutagenesis assay on the specific DNA region from the wild-type (WT) plasmid and found activity levels equivalent to the empty control vector. Mutating a 6–base pair (bp) region on the *miR-2940* promoter (Fig. 3 *C* and *D*) and 13-bp region on the *miR-252* promoter alleviated E75 activity (*SI Appendix*, Fig. S5*C*). Thus, both these in vitro experiments suggest that E75 is an activator of miRNAs and confirm the specific DNA binding site on the promoter for E75 regulation of *miR-2940 and miR-252* expression. We next set to perform a series of in vivo experiments to determine whether E75 regulation of miRNA expression was occurring within the fat body tissue during the PE developmental phase.

Evaluating the Impact of JH on E75 Transcription Levels. As E75 is largely thought to be an ecdysone-responsive gene (27, 28), we set out to establish its role within the JH hierarchical network. Two

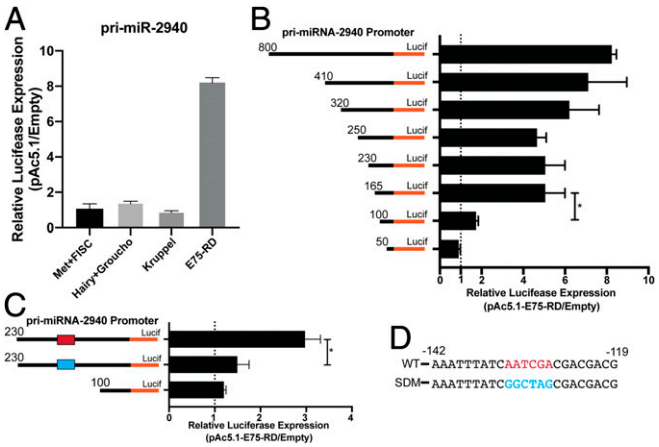

Fig. 3. E75-RD is an activator of the pri-miRNA-2940 promoter. (*A*) Luciferase reporter assay after cotransfection with either Met and FISC (Taiman), Hairy and Groucho, Kruppel, or E75-RD protein expression vectors and the pri-miR-2940 promoter firefly reporter construct. Pri-miRNA promoter firefly luciferase measurements were divided by measurements from a promoterless firefly vector and the respective candidate protein expression. (*B*) Luciferase measurements from a serial deletion series of the pri-miRNA-2940 promoter with E75-RD protein expression. (*C*) Luciferase measurements of the pri-miRNA-2940 promoter with a mutated DNA sequence between the 135 and 130 DNA region. (*D*) Putative E75-RD binding site with flanking regions within the pri-miRNA-2940 promoter. The red DNA sequence corresponds to the WT promoter, and the blue sequence corresponds to the mutation. Firefly luciferase measurements are normalized to an internal Renilla luciferase control. Transfection experiments were completed independently three times with each experimental group having three technical replicates. Statistical significance was calculated using a Student's *t* test. $*P < 0.05$. The data are shown as average ± SEM.

experiments were completed to support the hypothesis that E75-RD is regulated by JH. Topically applying JH to the abdomen of newly emerged adult mosquitoes can result in an artificial activation of JH pathway in the fat body tissue (29). Accordingly, we applied JH III topically to the abdomen of female mosquitoes at 0 to 3 h PE and dissected fat body tissues at 6 h posttreatment. We analyzed *E75-RD* expression and noted that fat body samples treated with JH III had higher levels than the acetone treatment alone and WT controls (Fig. 4 *A* and *B*). Next, we performed RNAi for the JH receptor Met. Removal of Met through double-stranded RNA (dsRNA) knockdown has been shown to be an effective method to prevent JH activation, blocking the induction of both Hairy and Kr-h1 (6, 30). We reasoned that *E75-RD* would also display a lower expression level in the absence of Met. We injected adult female mosquitoes at 0 to 24 h PE with dsRNA targeting either *met* (iMet) or *luciferase* (iLuc) and subsequently measured *E75-RD* expression levels in the fat body tissue 4 d later. On treatment with iMet, we found that *E75-RD* was lower than in the iLuc control group (Fig. 4 *C* and *D*). Taken together these experiments indicate that the E75-RD isoform is regulated by JH through Met.

E75 Acts as an Intermediate Component within the JH/Met Hierarchical Network in Regulating miRNAs. To investigate the role of E75 and as an intermediate component of the JH hierarchical network, we performed small RNA-seq from iMet and iE75 fat body samples. Identification of transcriptions shared between both knockdown groups would provide insight into the E75 regulatory role during this phase. We purified total RNA from iLuc, iMet, and iE75 fat body samples and prepared small RNA libraries for next-generation sequencing (*SI Appendix*, Fig. S6 and Table S2). Three independent biological samples from each treatment group were used for the differential expression analysis. We examined the global small RNA profile (transfer RNA, ribosomal RNA, pre-miRNA, long noncoding RNAs, and small nuclear RNAs) and found large expression changes in the iMet and iE75 groups but not in the iLuc control. The proportion of reads mapping to rRNAs, lncRNAs, snRNAs, and tRNAs was higher in the iMet and iE75 groups, while that related to premiRNAs was lower in both treatment groups, suggesting a global shift for the regulation of RNA transcripts (*SI Appendix*, Fig. S7). In terms of the percentage of genes shared between the two datasets, we selected genes (fold change < 0.8 for down-regulation and >1.2 for up-regulation) between iMet and iE75 for comparison. We found that ~55% of the genes that were up-regulated in the iE75 dataset were also up-regulated in the iMet dataset, while about 73% of the genes that were down-regulated in the iE75 dataset also appeared in the iMet dataset (Fig. 4 *E* and *F* and *SI Appendix*, Fig. S8*A*). The down-regulated set of genes contained 40 of the 52 premiRNA transcripts, with the remaining 12 not being differentially expressed and on the low end of detection.

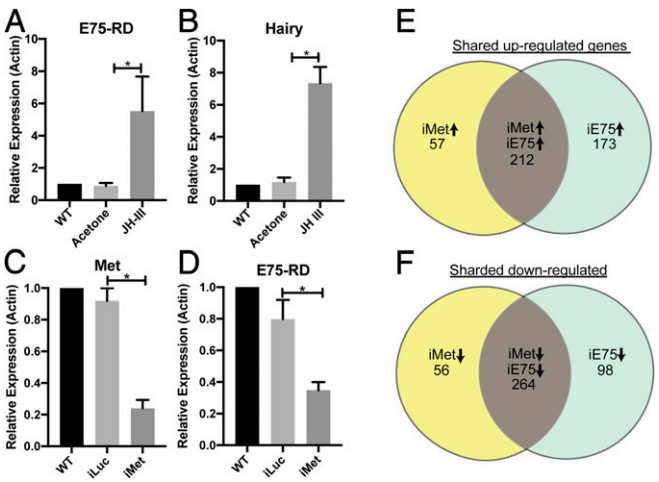

Fig. 4. E75 acts an intermediate component within the JH/Met hierarchical network. (*A* and *B*) In vitro application of JH III impacts E75 and Hairy. Relative gene expression levels of E75-RD and Hairy from WT-, acetone-, and JH III–treated fat body tissues dissected at 6 h post treatment, respectively. (*C* and *D*) RNAi treatment of Met reduces expression of isoform E75-RD. Relative gene expression levels of Met and E75-RD from WT, iLuc, and iMet from fat body tissues dissected at 72 h PE. (*E* and *F*) Illumina small RNA-seq analysis of Met and E75 RNAi-depleted *Ae. aegypti* mosquito fat body at 72 h PE. Venn diagram analysis of Met and E75 RNAi small RNA-seq analysis showing 55% of genes up-regulated in iE75 are also up-regulated in iMet, and 77% of genes down-regulated in iE75 are also down-regulated iMet, respectively. Circles containing only iMet↑ or iE75↑ indicate genes specifically up-regulated (fold change > 1.2) within that dataset. Circles containing only iMet↓ or iE75↓ indicate genes specifically down-regulated (fold change < 0.8) within that respective dataset. The intersection containing either iMET↑/iE75↑ or iMet↓/iE75↓ indicates genes shared within the respective datasets. For gene expression analysis, each group is the average from three independent experiments that included three to four biological samples per experiment. Statistical significance was calculated using a one-way ANOVA. **P* < 0.05. The data are shown as average ± SEM.

As the majority of the shared premiRNA transcripts between iMet and iE75 were down-regulated, we next focused our analysis on the individual expression of mature miRNAs in the iMet and iE75 datasets (*SI Appendix*, Dataset S3). As our luciferase-based, pri-miRNA promoter analysis experiments demonstrated only E75-RD to be capable of directly activating pri-miRNA expression, we speculated that iE75 treatment would have a larger impact on miRNA expression than iMet. We indeed found that 60% (60/102) of mature miRNAs identified had a greater reduction (fold change < 0.8) in the iE75 group than in iMet, suggesting that iE75 knockdown had a more-impactful change on miRNA expression than iMet knockdown (Fig. 5 and *SI Appendix*, Table S3).

When we examined the specific expression profiles of *miR-276-3p*, *miR-252-5p*, and *miR-2940-3p* in the iMet dataset, their expression was 25, 23, and 25% lower than in the iLuc control, respectively (*SI Appendix*, Dataset S3). This expression was then even further reduced in the iE75 dataset by 52, 48, and 39% when compared with the iLuc control, respectively. The expression levels for miRNAs were validated using RT-qPCR from independent E75 RNAi treatment groups ($P < 0.05$; *SI Appendix*, Figs. S9 *A–C*). These results establish E75 to be an intermediate component within the JH/Met hierarchical network.

miR-2940-3p Is Critical for Primary Ovarian Follicle and Egg Development. We examined the functional roles of *miR-2940-3p*, *miR-276-3p*, and *miR-252-5p* during the gonotrophic cycle. Synthetic RNA probes complementary to the mature miRNA strand, termed antagomirs (Ant), were designed and injected into adult female mosquitoes 6 to 24 h PE and measured for efficiency 4 d postinjection ($P < 0.05$; *SI Appendix*, Figs. S10 *A–C*) using RT-qPCR from fat body tissue. After confirmation of miRNA depletion, female mosquitoes were provided a blood meal and subsequently examined for primary follicle development 24 h postfeeding. We found that treatment with Ant-2940-3p impaired primary follicle development growth (average length: 182 μM) relative to that in WT and antagomir control groups (average lengths: 220 and 221 μM, respectively, $P < 0.05$; *SI Appendix*, Fig. S11*A*). Concordantly, we measured egg-laying numbers and found that Ant-2940-3p females deposited fewer eggs (average number: 57 eggs per female) than the WT and antagomir control groups, both of which laid an average of 90 eggs per female ($P < 0.05$; *SI Appendix*, Fig. S11*B*). Treatment with either Ant-276-3p or Ant-252-5p had no major impact on the reproductive cycle when similarly analyzed. These experiments highlight the importance of *miR-2940-3p* in egg production during the gonotrophic cycle.

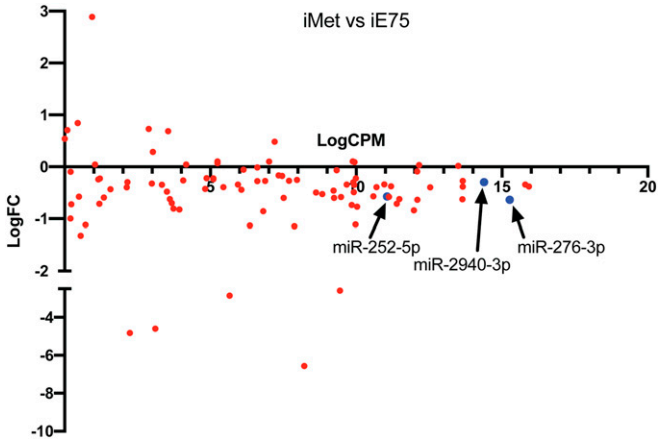

Fig. 5. MA plot depicting small RNA-seq results from iE75 analysis. The *x*-axis represents the log mean average value for each miRNA expression (counts-per-million), and the *y*-axis represents the log ratio between the iMet and iE75 samples. Each point is representative of an individual gene, and the different colors represent different classes of noncoding RNAs.

Computation and In Vivo Analyses Indicate miR-2940-3p Targets Clumsy. To identify the molecular target of *miR-2940-3p*, we coupled RNA-seq analysis from iE75 knockdown fat body samples with an in silico approach using different bioinformatics miRNA target prediction programs to produce a highly selective list of potential mRNA targets. As our previous results indicated that *miR-2940-3p* is regulated through the transcription factor E75, RNAi treatment of E75 should reduce miRNA-2940-3p levels and accordingly alleviate its repressive effect on potential mRNA targets. As a result, we performed RNA-seq analysis from iE75 fat body samples and specifically selected the 3′untranslated regions (UTR) from those genes with up-regulated expressions (fold change >1.3) to be used as the reference dataset for miRNA target prediction (*SI Appendix*, Table S4 and Dataset S4). Using this approach greatly restricted the starting list of the potential 14,718 transcripts within the *Ae. aegypti* transcriptome to only 342 transcripts found to be up-regulated within the E75 knockdown fat body samples. The predicted mRNA–miRNA interactions from these 342 transcripts were evaluated using the two bioinformatics programs, miRanda (31) and RNA hybrid (32), producing a total of nine genes predicted by both programs with a *miR-2940-3p* binding site (*SI Appendix*, Table S5). To confirm an authentic mRNA target, we screened for elevated gene expression levels in vivo using RT-qPCR from fat body tissue treated with Ant-2940-3p. Of the nine predicted mRNAs on the target list, only *AAEL002518* was demonstrated to have a higher gene expression level than in the WT and Ant-Scr groups (scrambled) (Fig. 6*A*). The UniProt database defines *AAEL002518* as a glutamate ion channel, containing both a transmembrane domain and ligand-binding site for glutamate or glycine. Using a protein Basic Local Alignment Search Tool suggested that *AAEL002518* is homologous to the *D. melanogaster* gene *clumsy*. These data suggest that mosquito *clumsy AAEL002518* is either directly or indirectly regulated through the actions of *miRNA-2940-3p.*

In Vitro Analysis Indicates miR-2940-3p Directly Targets Clumsy. To assess whether *clumsy* is the authentic target of *miR-2940-3p,* we utilized a cell culture–based luciferase assay. The 3′UTR of *AAEL002518* was cloned downstream of the *Renilla* translational stop codon within the psiCheck-2 vector to generate a 3′UTR-fused luciferase reporter. The reporter vector was then transfected into *Drosophila* S2 cells along with a *miR-2940-3p* mimic, and luciferase activity was measured 24 h posttransfection. We found that the addition of the *miR-2940-3p* mimic resulted in a 20% lower luciferase activity than in the treatment group containing the Allstar mimic (negative siRNA control) (Fig. 6*B*). Our bioinformatics predictions identified two potential binding sites for *miR-2940-3p* within the 3′ UTR of *clumsy,* referred to as Site-1 and Site-2 (*SI Appendix*, Fig. S12). To identify the specific site to which *miR-2940-3p* binds to on the UTR transcript, we performed a site-directed mutagenesis assay to scramble the seed sequence to abolish the miRNA–mRNA interaction and alleviate luciferase repression. The mutation in Site-1 alleviated the repressive effect by the *miR-2940-3p* mimic, while that in Site-2 still resulted in a ∼20% lower luciferase activity than the Allstar control group (Fig. 6 *C* and *D*). This result confirms that *miR-2940-3p*–mediated repression of *AAEL002518* is directed through Site-1 on the 3′UTR.

As *miR-2940-3p* is a Culicinae subfamily–specific miRNA, we additionally performed the transfection experiments in the *Ae. aegypti* Aag2 cell line using endogenous miRNA as a substitute for the miRNA mimic. Transfection of Aag2 cells with psiCheck-2 vector containing the 3′UTR of *clumsy* resulted in a ∼15% decrease of luciferase activity in comparison with the empty psiCheck-2 vector. Mutating Site-1 restored luciferase levels to the empty psiCheck-2 vector, while mutating Site-2 still resulted in luciferase reduction

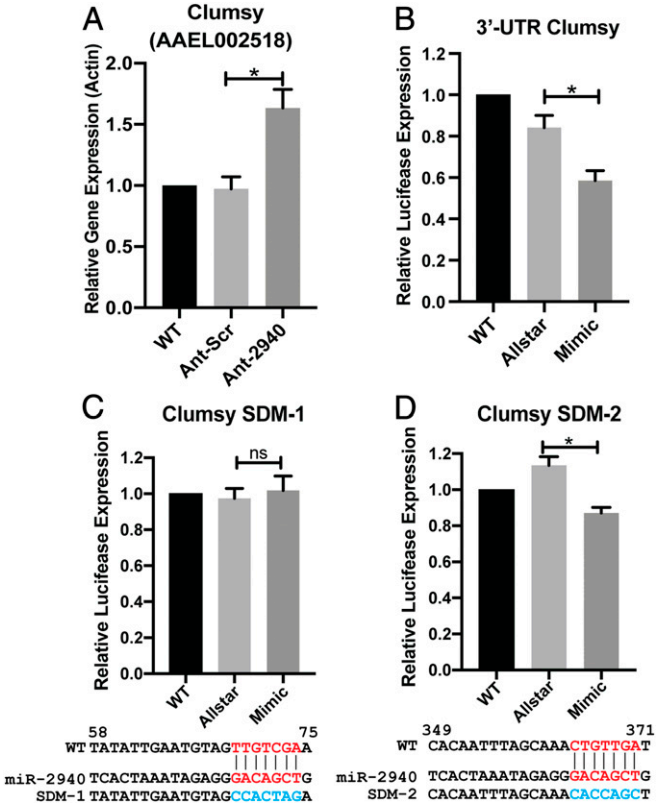

Fig. 6. miR-2940-3p directly targets Clumsy (AAEL002518). (*A*) Relative gene expression level for clumsy measured by RT-qPCR from fat body tissue in WT-, Ant-Scr–, and Ant-2940–treated individuals. (*B*) Dual luciferase reporter assay for clumsy in *Drosophila* S2 cells. (*C* and *D*) Dual luciferase reporter assay for clumsy using mutated binding sites in *Drosophila* S2 cells. Independent plasmids were generated with mutations on the 3′UTR in the potential "seed" sequence for the predicted miR-2940-3p binding site. The seed sequences are highlighted in blue, and the corresponding substitutions on the mutated plasmid are highlighted in red. For gene expression results, each experiment consisted of three to four biological samples, and each experiment was repeated a total of three independent times. Statistical significance was calculated using a one-way ANOVA. $*P < 0.05$. For luciferase transfection experiments, results are expressed as the relative ratio of Renilla luciferase activity to Firefly luciferase activity. Statistical significance was calculated using an unpaired Student's *t* test. The data represent three biological replicates with three technical replicates and are shown as average ± SEM.

($P < 0.05$; *SI Appendix*, Fig. S13). This finding further supports our results obtained using the S2 cell line, where Site-1 was found to be responsible for mediating transcript repression. The combination of both the in vitro S2 and Aag2 cell-based results further strengthens our findings that *AAEL002518* is an authentic target of *miR-2940-3p*.

Clumsy RNAi Rescues Ant-2940-3p Treatment. We next confirmed that up-regulation of *AAEL002518* is responsible for the reproductive defects we noted in the *Ant-2940-3p* treatment experiments. We performed a phenotypic rescue experiment by simultaneous knockdown of *miR-2940-3p* and *AAEL002518* in the same individuals. We synthesized dsRNA targeting *AAEL002518* ($P < 0.05$; *SI Appendix*, Fig. S14) and mixed it with synthetic *Ant-2940-3p*, using final concentrations of 2 μg/μL and 200 μmol/μL, respectively. Depleting *AAEL002518* along with *Ant-2940-3p* treatment partially restored primarily follicle development (average length: 193 μM) in mosquitoes to the level noted in the Ant-Scr/iLuc and WT control groups (average length: 205 and 213 μM, respectively), confirming that up-regulation of *AAEL002518* is responsible for the reproductive defects caused by *Ant-2940-3p* treatment (*SI Appendix*, Fig. S15*A*). We additionally measured the number of eggs laid and found *clumsy* RNAi (69 eggs per female) to restore egg deposition more than Ant-2940-3p treatment (59 eggs per female, $P < 0.05$; *SI Appendix*, Fig. S15*B*).

AAEL002518 is an *Ae. aegypti* homolog of *clumsy*, originally discovered in *D. melanogaster*, as an ionotropic glutamate receptor belonging to the subtype kainate receptors. Glutamate receptors contain a ligand-binding domain, responsible for binding the amino acid glutamate and a transmembrane channel for trafficking different ions across the cell membrane (33). We attempted to mimic our phenotypic rescue experiments using AP-5, a synthetic antagonist of kainate receptors (34), as a substitute for dsRNA targeting *AAEL002518*. The mixture of Ant-2940-3p and AP-5 was able to rescue both primary follicle growth (average length: 209 μM) and egg development, suggesting that Clumsy was effectively inhibited by the chemical treatment. These experiments demonstrate that the up-regulation of Clumsy is responsible for the reproductive defect noted with Ant-2940-3p treatment.

Discussion

miRNAs play a critical role in multiple physiological processes across different insect systems. Our study focused on the control of miRNAs due to their importance as regulators of gene expression in reproduction in the disease vector *Ae. aegypti*. We investigated the effect of the JH pathway on miRNAs during the PE phase of the mosquito reproductive cycle. This reproductive phase is critical for nutrient mobilization, energy homeostasis, as well as for acquisition of reproductive competence required for egg production during the vitellogenic phase.

Most of the research focusing on miRNA regulation in insects has centered on the pri-miRNA transcript responsible for encoding the miRNA cluster of Let-7, miR-125, and miR-100. This locus was originally identified in *D. melanogaster* to be inducible by 20E treatment and later demonstrated to be specifically regulated through the ecdysone-specific Broad complex (15, 16). These findings were shown to extend to a less-derived insect system, the cockroach *B. germanica*, suggesting that the mechanism of 20E regulation on miRNA expression is highly conserved in insect species (17). While these studies have centered on role of 20E and Broad in larva-to-adult transitionary phase, the JH pathway transcription factors mediating miRNA expression are largely unexplored. Both Let-7 and miR-278 have been demonstrated to be authentic targets of the JH transcription factor Kr-h1 in *Anopheles gambiae* and *Locusta migratoria* (35, 36). Inhibition of Let-7 leads to an increase of Kr-h1, resulting in reduced vitellogenin transcripts and an abolishment of ovarian development.

Our study identified E75 (specifically the isoform E75-RD) as a factor in the juvenile hormone hierarchical network that regulates miRNAs. E75 has been classified as an "early" responsive gene under the ecdysone signaling pathway (27); however, there are in vitro studies utilizing S2 cells that do suggest the possibility of JH regulating specific E75 isoforms. Applying JH III to culture medium induced both the expression of E75A and an additional isoform currently unidentified in *Ae. aegypti* (37, 38). An additional follow-up study identified the transcription factor fushi tarazu Transcription 1 (β-FTZ-F1) as a potential regulator of JH III–regulated E75A expression (39). JH III has also been implicated in β-FTZ-F1 regulation in *Ae. aegypti* fat body tissue (40); and thus, our future studies will examine the potential role of β-FTZ-F1 on E75-RD regulation during the PE developmental phase.

Two methodologies used to identify miRNA–mRNA interactions are in silico bioinformatic predictions and the in vivo Cross-Linking Immunoprecipitation-Seq approach targeting Ago1. While both techniques are effective at identifying authentic miRNA–mRNA interactions, both have significant drawbacks. In silico predictions generally have an extremely high false-positive rate and require the use of multiple of bioinformatic programs, and CLIP-Seq techniques can be still technically challenging despite improvements in RNA ligation and library preparation. Here, we utilized the information that E75 is a regulator of miR-2940 to generate a highly curated list of potential mRNA targets for miR-2940-3p from E75 knockdown RNA-seq analysis. This approach removed ~95% of potential mRNA transcripts that may have appeared as false-positive result following our in silico bioinformatic analysis. This allowed for selecting a very limited list of potential mRNA genes that aided the identification of Clumsy as an authentic target of miR-2940-3p.

While our antagomir-based treatments of miRNAs revealed that miR-2940-3p has a functional role in female reproduction, miR-276-3p and miR-252-5p likely play in other aspects of mosquito physiology. The depletion of miR-252-3p impacted nymphal development in *B. germanica* (18), and the depletion of miR-276-5p resulted in enhanced female fertility in *Anopheles gambiae* (41). Since E75-RD has been identified as the regulator of the pri-miR-276 and pri-miR-252 transcripts, it is likely having a functional role in regulating those miRNA-specific processes.

The underlying molecular mechanism of how the overactivation of Clumsy during the PE developmental phase leads to an inhibition of primary ovarian and egg development still needs to be examined. The downstream signaling response activated by Clumsy and the impact on fat body development can be critical to designing a novel reproductive control strategy. Although glutamate receptors are highly conserved in eukaryotic systems, the potential ligands have evolutionarily differed between vertebrate and insect systems (34). This underlines a prospective control strategy where a chemical agonist could be used to specifically target *Ae. aegypti* Clumsy and would be ineffective in activating the vertebrate homolog.

Overall, the present work not only highlights a previously unidentified role for E75-RD as a factor within the JH hierarchical network but also demonstrates E75-RD as a critical regulator of miRNA expression in the mosquito.

Materials and Methods

Mosquito Rearing. The *Ae. aegypti* was reared at 27 °C and 80% humidity, as described previously (42). Blood feeding of adult mosquitoes was performed using White Leghorn chickens.

Double-Stranded RNAi and Antagomir Injections. DsRNAs were synthesized using the MEGAscript Kit (Ambion), and antagomirs were obtained from Dharmacon, as previously described (11). Further details are described in *SI Appendix, Supplemental Materials and Methods*.

RNA Extraction and qPCR Analysis. RNA Extraction, complementary DNA synthesis, and qPCR analysis were performed as previously described (4).

Oligos are listed in Dataset S5. Further details are described in *SI Appendix, Supplemental Materials and Methods*.

Juvenile Hormone In Vivo Application. Newly emerged female mosquitoes (3 h PE) were collected and chilled on ice, and a 0.3-μL aliquot of 1 μg/mL JH III (Sigma) or solvent (acetone) was topically applied to the abdomen. Fat body tissue (~5 to 6 per biological replicate) was collected 6 h posttreatment and subjected to RNA extraction and RT-qPCR.

miRNA Computational Target Predictions. The 3′UTRs were extracted from the AaegL5.1 genome database. UTRs were specifically selected from genes that displayed a >1.3 Log2 fold change from the iE75 dataset. The miRNA–mRNA target prediction programs miRanda and RNAhybrid were then used to determine best match with the mature aae-miR-2940-3p sequence.

Cell Culture Conditions. *Drosophila* S2 (Invitrogen) and mosquito Aag2 cells were maintained at 26 °C in Schneider's *Drosophila* medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies) and 1× Penicillin–Streptomycin (ThermoFisher Scientific).

Promoter Luciferase Assay. The promoter luciferase assay was performed as previously described (6). DNA fragments were cloned into the pGL 4.17 vector (Promega), and coding domain sequence regions for protein expression were cloned into pAc5.1-V5 vector (Invitrogen). Full details on experimental protocol are contained in *SI Appendix, Supplemental Materials and Methods*.

In Vitro Validation of miR-2940-3p Interaction with Clumsy 3′UTR. In vitro target validation was performed as previously described (43). Full details on experimental protocol are contained in *SI Appendix, Supplemental Materials and Methods*.

Chemical Injections. For the AP-5 rescue experiment, mosquitoes were coinjected with a mixture of AP-5 and antagomir at a final concentration of 50 nM and 200 μM, respectively. Mosquitoes were allowed to recover for 3 to 4 d on both H_20 and sugar water before blood feeding.

RNA-seq Library Preparation and Analysis. Total RNA samples were prepared as described above and sent to University of California Riverside Gencore for Illumina next-generation sequencing on the NextSeq 550 platform. Full details on library construction and bioinformatics analysis are contained in *SI Appendix, Supplemental Materials and Methods*.

Small RNA Library Preparation and Analysis. A total of 3 μg total RNA was run on a Novex TBE-Urea 15% (Invitrogen) at 100 V for 1 h. The gel was stained with SYBR Gold for 10 min and visualized under ultraviolet light. The portion containing small RNAs was excised with a razor and purified using a ZR small-RNA Polyacrylamide Gel Electrophoresis recovery kit (Zymo). Confirmation of small RNAs was completed using a small RNA bioanalyzer chip (Agilent) and used for the small RNA library prep kit (NEB). Library preparation followed the manufacturer's protocol, with no changes. Small RNA reads were trimmed to 18 bp (fastx-tool kit) and mapped to the *Ae. aegypti* genome, allowing one mismatch using Bowtie (44). Mapped reads were counted using HT-Seq (45), and differential expression analysis was completed using EdgeR (46). Mature miRNA read counts were generated using miRDeep2 software (47).

Data Availability. All study data are included in the article and/or *SI Appendix*.

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1. A. Baly *et al*., Cost effectiveness of *Aedes aegypti* control programmes: participatory versus vertical. *Trans. R. Soc. Trop. Med. Hyg.* **101**, 578–586 (2007).
2. M. Jindra, S. R. Palli, L. M. Riddiford, The juvenile hormone signaling pathway in insect development. *Annu. Rev. Entomol.* **58**, 181–204 (2013).
3. H. F. Nijhout, *Insect Hormones* (Princeton University Press, 1994).
4. Z. Zou *et al*., Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E2173–E2181 (2013).
5. S. W. Shin, Z. Zou, T. T. Saha, A. S. Raikhel, bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 16576–16581 (2012).
6. T. T. Saha *et al*., Hairy and Groucho mediate the action of juvenile hormone receptor Methoprene-tolerant in gene repression. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E735–E743 (2016).
7. T. T. Saha *et al*., Synergistic action of the transcription factors Krüppel homolog 1 and Hairy in juvenile hormone/Methoprene-tolerant-mediated gene-repression in the mosquito *Aedes aegypti*. *PLoS Genet.* **15**, e1008443 (2019).
8. R. Ojani, X. Fu, T. Ahmed, P. Liu, J. Zhu, Krüppel homologue 1 acts as a repressor and an activator in the transcriptional response to juvenile hormone in adult mosquitoes. *Insect Mol. Biol.* **27**, 268–278 (2018).
9. J. L. Wang, T. T. Saha, Y. Zhang, C. Zhang, A. S. Raikhel, Juvenile hormone and its receptor methoprene-tolerant promote ribosomal biogenesis and vitellogenesis in the *Aedes aegypti* mosquito. *J. Biol. Chem.* **292**, 10306–10315 (2017).
10. B. Bryant, W. Macdonald, A. S. Raikhel, microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 22391–22398 (2010).
11. K. J. Lucas *et al*., MicroRNA-8 targets the Wingless signaling pathway in the female mosquito fat body to regulate reproductive processes. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1440–1445 (2015).
12. B. Zhao *et al*., MicroRNA-275 targets sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) to control key functions in the mosquito gut. *PLoS Genet.* **13**, e1006943 (2017).
13. L. Ling, V. A. Kokoza, C. Zhang, E. Aksoy, A. S. Raikhel, MicroRNA-277 targets *insulin-like peptides 7* and *8* to control lipid metabolism and reproduction in *Aedes aegypti* mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E8017–E8024 (2017).
14. Y. Zhang *et al*., microRNA-309 targets the Homeobox gene SIX4 and controls ovarian development in the mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E4828–E4836 (2016).
15. L. F. Sempere, N. S. Sokol, E. B. Dubrovsky, E. M. Berger, V. Ambros, Temporal regulation of microRNA expression in *Drosophila melanogaster* mediated by hormonal signals and broad-complex gene activity. *Dev. Biol.* **259**, 9–18 (2003).
16. G. Chawla, N. S. Sokol, Hormonal activation of let-7-C microRNAs via EcR is required for adult *Drosophila melanogaster* morphology and function. *Development* **139**, 1788–1797 (2012).
17. M. Rubio, X. Belles, Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolan metamorphosis. *J. Insect Physiol.* **59**, 1089–1094 (2013).
18. M. Rubio, A. de Horna, X. Belles, *MicroRNAs in Metamorphic and Non-Metamorphic Transitions in Hemimetabolan Insect Metamorphosis* (Elsevier-Academic Press, Amsterdam, 2012).
19. X. Zhang, A. S. Raikhel, Hormonal regulation of microRNA expression dynamics in the gut of the yellow fever mosquito *Aedes aegypti*. *RNA Biol.* (2020).
20. S. Asgari, MicroRNA functions in insects. *Insect Biochem. Mol. Biol.* **43**, 388–397 (2013).
21. K. Lucas, A. S. Raikhel, Insect microRNAs: Biogenesis, expression profiling and biological functions. *Insect Biochem. Mol. Biol.* **43**, 24–38 (2013).
22. X. Belles, A. S. Cristino, E. D. Tanaka, M. Rubio, M.-D. Piulachs, "Insect MicroRNAs: From molecular mechanisms to biological roles" in *Insect Molecular Biology and Biochemistry*, L. I. Gilbert, Ed. (Elsevier-Academic Press, Amsterdam, 2012), pp. 30–56.
23. G. Jeong, Y. H. Lim, Y. K. Kim, Precise mapping of the transcription start sites of human microRNAs using DROSHA knockout cells. *BMC Genomics* **17**, 908 (2016).
24. H. Thorvaldsdóttir, J. T. Robinson, J. P. Mesirov, Integrative genomics viewer (IGV): High-performance genomics data visualization and exploration. *Brief. Bioinform.* **14**, 178–192 (2013).
25. B. J. Matthews *et al*., Improved reference genome of *Aedes aegypti* informs arbovirus vector control. *Nature* **563**, 501–507 (2018).
26. J. Cruz, D. H. Sieglaff, P. Arensburger, P. W. Atkinson, A. S. Raikhel, Nuclear receptors in the mosquito *Aedes aegypti*: Annotation, hormonal regulation and expression profiling. *FEBS J.* **276**, 1233–1254 (2009).
27. W. A. Segraves, D. S. Hogness, The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4**, 204–219 (1990).
28. M. Bialecki, A. Shilton, C. Fichtenberg, W. A. Segraves, C. S. Thummel, Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Dev. Cell* **3**, 209–220 (2002).
29. J. Zhu, J. M. Busche, X. Zhang, Identification of juvenile hormone target genes in the adult female mosquitoes. *Insect Biochem. Mol. Biol.* **40**, 23–29 (2010).
30. M. Li, E. A. Mead, J. Zhu, Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 638–643 (2011).
31. D. Betel, A. Koppal, P. Agius, C. Sander, C. Leslie, Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90 (2010).
32. J. Kruger, M. Rehmsmeier, RNAhybrid: MicroRNA target prediction easy, fast and flexible. *Nucleic Acids Res.* **34**, W451–W454 (2006).
33. S. F. Traynelis *et al*., Glutamate receptor ion channels: Structure, regulation, and function. *Pharmacol. Rev.* **62**, 405–496 (2010).
34. Y. Li *et al*., Novel functional properties of *Drosophila* CNS glutamate receptors. *Neuron* **92**, 1036–1048 (2016).
35. J. Song *et al*., The microRNAs let-7 and miR-278 regulate insect metamorphosis and oogenesis by targeting the juvenile hormone early-response gene *Krüppel-homolog 1*. *Development* **145**, dev170670 (2018).
36. X. Fu, P. Liu, G. Dimopoulos, J. Zhu, Dynamic miRNA-mRNA interactions coordinate gene expression in adult *Anopheles gambiae*. *PLoS Genet.* **16**, e1008765 (2020).

37. E. B. Dubrovsky, V. A. Dubrovskaya, E. M. Berger, Hormonal regulation and functional role of Drosophila E75A orphan nuclear receptor in the juvenile hormone signaling pathway. *Dev. Biol.* **268**, 258–270 (2004).
38. V. A. Dubrovskaya, E. M. Berger, E. B. Dubrovsky, Juvenile hormone regulation of the E75 nuclear receptor is conserved in Diptera and Lepidoptera. *Gene* **340**, 171–177 (2004).
39. E. B. Dubrovsky *et al*., The Drosophila FTZ-F1 nuclear receptor mediates juvenile hormone activation of E75A gene expression through an intracellular pathway. *J. Biol. Chem.* **286**, 33689–33700 (2011).
40. J. Zhu, L. Chen, A. S. Raikhel, Posttranscriptional control of the competence factor betaFTZ-F1 by juvenile hormone in the mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13338–13343 (2003).
41. L. Lampe, M. Jentzsch, S. Kierszniowska, E. A. Levashina, Metabolic balancing by miR-276 shapes the mosquito reproductive cycle and *Plasmodium falciparum* development. *Nat. Commun.* **10**, 5634 (2019).
42. S. G. Roy, I. A. Hansen, A. S. Raikhel, Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **37**, 1317–1326 (2007).
43. S. Liu, K. J. Lucas, S. Roy, J. Ha, A. S. Raikhel, Mosquito-specific microRNA-1174 targets serine hydroxymethyltransferase to control key functions in the gut. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 14460–14465 (2014).
44. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, R25 (2009).
45. S. Anders, P. T. Pyl, W. Huber, HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
46. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).
47. J. An, J. Lai, M. L. Lehman, C. C. Nelson, miRDeep*: An integrated application tool for miRNA identification from RNA sequencing data. *Nucleic Acids Res.* **41**, 727–737 (2013).