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Authors

He, Ya-Zhou
Aksoy, Emre
Ding, Yike
et al.

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Hormone-dependent activation and repression of microRNAs by the ecdysone receptor in the dengue vector mosquito *Aedes aegypti*

Ya-Zhou He[a,b], Emre Aksoy[a,c], Yike Ding[a,b], and Alexander S. Raikhel[a,b,1]

[a]Department of Entomology, University of California, Riverside, CA 92521; [b]Institute of Integrative Genome Biology, University of California, Riverside, CA 92521; and [c]Graduate Program in Genetics, Genomics, and Bioinformatics, University of California, Riverside, CA 92521

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Female mosquitoes transmit numerous devastating human diseases because they require vertebrate blood meal for egg development. MicroRNAs (miRNAs) play critical roles across multiple reproductive processes in female *Aedes aegypti* mosquitoes. However, how miRNAs are controlled to coordinate their activity with the demands of mosquito reproduction remains largely unknown. We report that the ecdysone receptor (EcR)–mediated 20-hydroxyecdysone (20E) signaling regulates miRNA expression in female mosquitoes. EcR RNA-interference silencing linked to small RNA-sequencing analysis reveals that EcR not only activates but also represses miRNA expression in the female mosquito fat body, a functional analog of the vertebrate liver. EcR directly represses the expression of clustered *miR-275* and *miR-305* before blood feeding when the 20E titer is low, whereas it activates their expression in response to the increased 20E titer after a blood meal. Furthermore, we find that SMRTER, an insect analog of the vertebrate nuclear receptor corepressors SMRT and N-CoR, interacts with EcR in a 20E-sensitive manner and is required for EcR-mediated repression of miRNA expression in *Ae. aegypti* mosquitoes. In addition, we demonstrate that miR-275 and miR-305 directly target *glutamate semialdehyde dehydrogenase* and *AAEL009899*, respectively, to facilitate egg development. This study reveals a mechanism for how miRNAs are controlled by the 20E signaling pathway to coordinate their activity with the demands of mosquito reproduction.

small RNA | steroid hormone | vector-borne disease | corepressor | coactivator

Female hematophagous mosquitoes serve as vectors for numerous pathogens of harmful human diseases, causing more than half a billion illnesses and close to a million deaths annually, worldwide. The *Aedes aegypti* mosquito is one of the most dangerous vectors, transmitting dengue fever, yellow fever, chikungunya, Zika, and other arboviral diseases (1–3). Due to the lack of effective vaccines, growing drug resistance in pathogens and insecticide resistance in vectors, reduction of the vector population through targeting mosquito reproduction is a promising strategy for preventing disease transmission. Reproduction in most female mosquitoes is intrinsically linked to their ability to transmit disease pathogens due to the requirement of blood feeding for initiating egg development. Acquisition of blood activates numerous processes and genes essential for digestion of blood in the midgut, massive synthesis and secretion of yolk protein precursors in the fat body (FB), a functional analog of the vertebrate liver, accumulation of yolk proteins in developing oocytes, and eventual egg production (4). Therefore, deciphering the molecular mechanisms underlying blood-feeding–triggered egg development is of great significance in the development of novel approaches to control mosquito populations and, thus, mosquito-borne diseases.

MicroRNAs (miRNAs) are small noncoding RNAs, 21–24 nt in length, that have emerged as key components of posttranscriptional gene expression regulation. Most animal miRNAs imperfectly base pair with sequences in the 3′-untranslated region (UTR) of target mRNAs and inhibit protein synthesis by either repressing translation or promoting mRNA decay (5, 6). In mammals, miRNAs participate in the regulation of almost every cellular process investigated so far, and their dysregulation is often associated with human diseases, such as cancer and neurodevelopmental disorders (7). Functional studies have revealed the critical roles of miRNAs across multiple reproductive processes in female *Ae. aegypti* mosquitoes (8–11). However, how miRNAs are controlled to coordinate their activity with the demands of mosquito reproduction remains largely unknown. This is crucial for developing a full understanding of the regulatory mechanisms of mosquito reproduction and may pave the way toward the utilization of these small molecules for control of mosquito-borne disease.

Canonical animal miRNAs are encoded in the genome as individual genes or as clusters containing several different miRNAs. Clustered miRNAs are generally transcribed together as polycistronic transcripts, which are processed to the individual mature miRNAs. Some miRNAs reside in the introns of protein-coding genes and usually share the promoter of the host gene (12). The biogenesis of miRNAs is a multistep process. miRNA genes are transcribed mainly by RNA polymerase II as structured primary miRNAs (pri-miRNAs), which are processed into precursor miRNAs (pre-miRNAs) and finally into mature miRNA duplexes by two members of the RNase III family of enzymes, Drosha and Dicer. The duplex is loaded onto the Argonaute (AGO) proteins,

Significance

Mosquitoes transmit numerous devastating human diseases. MicroRNAs play important roles in mosquito reproduction, but how their expression is controlled remains poorly understood. We demonstrate that ecdysone receptor (EcR) dynamically regulates the expression of clustered *miR-275* and *miR-305* to coordinate their activity with the demands of the female *Aedes aegypti* reproduction. EcR represses their expression at the low 20-hydroxyecdysone (20E) level before blood feeding by interacting with the corepressor SMRTER, the insect analog of the vertebrate nuclear receptor corepressors SMRT and N-CoR. In contrast, it activates their expression at the high 20E level after a blood meal via replacement of SMRTER with the coactivator Taiman. Our study gives valuable clues for miRNA utilization to control mosquito populations and, thus, mosquito-borne diseases.

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The authors declare no competing interest.

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[1]To whom correspondence may be addressed. Email: alexander.raikhel@ucr.edu.

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and one strand of the duplex remains as mature miRNA, whereas the other is discarded from AGO (12–14). Transcription of miRNA genes is regulated in a similar manner to that of protein-coding genes and is a major level of control responsible for tissue- or development-specific expression of miRNAs (6, 15).

The steroid hormone 20-hydroxyecdysone (20E) is a key regulator in the coordination of multiple developmental processes, including embryogenesis, metamorphosis, and reproduction in insects (16–18). The action of 20E is mediated by the ecdysone receptor (EcR) complex, a heterodimer consisting of two nuclear receptors, EcR and the retinoid X receptor homolog Ultraspiracle (USP) (16). The EcR/USP complex recognizes sequence-specific DNA motifs located in the regulatory regions of target genes, known as ecdysone response elements (EcREs) (19). In the absence of 20E, EcR/USP binds DNA and represses transcription via interaction with transcriptional corepressors (20). After binding to 20E, the complex recruits transcriptional coactivators, displaces the corepressors, and, thereby, activates transcription (16, 21).

20E, which is synthesized in the ovary of blood-fed females and secreted into the hemolymph, is the major regulator of blood-feeding–activated reproductive processes in female mosquitoes. Its titer is low before blood feeding, increases after a blood meal, peaking at 18–24 h post blood meal (PBM), and decreases rapidly thereafter (22). Our previous small RNA sequencing (sRNA-seq) analyses have shown that many miRNAs increase or decrease in expression in the female *Ae. aegypti* FB after a blood meal (23), suggesting that the 20E pathway may be involved in governing miRNA gene expression in mosquitoes. Here, we examined the role of the EcR-mediated 20E signaling in miRNA expression by means of EcR RNA interference (RNAi) silencing linked to sRNA-seq analysis and found that EcR not only activates but also represses miRNA expression in the female mosquito FB. Importantly, we show that EcR directly represses the expression of clustered *miR-275* and *miR-305* by interacting with the corepressor SMRTER (SMRT-related ecdysone receptor-interacting factor) before blood feeding, whereas it activates their expression with the aid of the steroid receptor coactivator Taiman after a blood meal. miR-275 and miR-305 contribute to blood-feeding–triggered egg development through targeting *glutamate semialdehyde dehydrogenase* (*GSD*) and *AAEL009899*, respectively. Thus, our results reveal a molecular mechanism of how miRNAs are controlled by the 20E signaling pathway to coordinate their activity with the demands of mosquito reproduction.

Results

Transcriptome-Wide Screening of EcR-Regulated miRNAs in the Female Mosquito FB. To examine whether the 20E pathway is involved in governing miRNA gene expression, we conducted transcriptome-wide screening of differentially expressed miRNAs after EcR RNAi. Female mosquitoes were injected with either ds*EcR* (iEcR) or ds*Luciferase* (iLuc) as a control at 24 h posteclosion (PE), and were fed blood 3 d later. FBs were dissected at 24 h PBM, when the titer of 20E is high, and used for sRNA-seq (Fig. 1*A*). Compared with the control, *EcR* mRNA levels were 63% lower in the FBs of iEcR mosquitoes (*SI Appendix*, Fig. S1*A*). Small RNA libraries were constructed with three biological replicates for each treatment and sequenced to the total depth of 163 million tags, 91.42% of which were mapped to the *Ae. aegypti* genome (*SI Appendix*, Fig. S1*B*). Size distribution analysis showed that most of the small RNA reads exhibited lengths of 21–23 nt (*SI Appendix*, Fig. S1*C*), the expected size for insect miRNAs. Tag counts for 146 *Ae. aegypti* mature miRNAs in miRBase v22 were normalized to numbers of genome-mapped tags in each library, expressed as tags per million (TPM) (Dataset S1). Correlation analysis showed that all three biological replicates correlated well in the iEcR treatment, whereas one replicate was separated from the others in the iLuc group (*SI Appendix*, Fig. S1*D*). Averages of three replicates were used to represent expression levels under each treatment. To identify differentially expressed miRNAs, we analyzed mature miRNAs with a total normalized value greater than 10 TPM. We found 9 down-regulated [$\log_2$ (iEcR/iLuc) less than −0.5] and 21 up-regulated miRNAs [$\log_2$ (iEcR/iLuc) > 0.5] in the FBs of iEcR mosquitoes relative to iLuc control (Fig. 1*B* and Dataset S2). Quantitative reverse-transcription PCR (qRT-PCR) analysis of four down-regulated (miR-275-3p, miR-305-5p, miR-263a-5p, and miR-279) and four up-regulated (miR-988-3p, miR-2a-3p, miR-278-3p, and miR-2b) miRNAs confirmed the sRNA-seq data (*SI Appendix*, Fig. S1*E*). These results indicate that EcR plays a critical role in controlling miRNA gene expression in the female mosquito FB.

To better understand the role of EcR-mediated 20E signaling in coordinating miRNA expression, we analyzed the expression profiles of these iEcR-responsive miRNAs in the FB after a blood meal using our previous sRNA-seq data (23). We found that all the iEcR–down-regulated miRNAs normally increase in expression after a blood meal, peaking at 24 h (miR-275-3p, miR-275-5p, miR-305-5p, miR-305-3p, miR-263a-5p, miR-263a-3p, miR-279, and miR-989) or 36 h PBM (miR-277-3p) and decreasing thereafter (Fig. 1*C*). Thus, EcR is required to activate the expression of these miRNAs after a blood meal. Of the 21 iEcR–up-regulated miRNAs, 4 (miR-988-3p, miR-2765, miR-981, and miR-34-3p) normally decrease in expression during the first 24 h PBM (Fig. 1*D*), indicating that EcR is required to shut down the PE expression of these miRNAs. Intriguingly, three (miR-278-5p, miR-1174, and miR-252-3p) of the iEcR–up-regulated miRNAs increase rapidly in expression after a blood meal, peaking at 6 h PBM and dropping dramatically at 24 h PBM (Fig. 1*D*), suggesting that EcR is also required to shut down miRNA expression at the early stage of the PBM phase. In addition, for these iEcR–up-regulated miRNAs, nine normally increase in expression after a blood meal and peak at either 36 h (miR-282-3p, miR-375, and miR-278-3p) or 48 h PBM (miR-281-3p, miR-2a-3p, miR-2b, miR-2c, miR-2940-5p, and bantam-5p) (Fig. 1*D*), indicative of a role of EcR in keeping these miRNAs from being precociously activated during the PBM phase. Taken together, we conclude that EcR-mediated 20E signaling not only activates but also represses miRNA expression in the female mosquito FB after a blood meal.

20E Activates *miR-275* and *miR-305* Transcription in the FB. To elucidate the mechanisms of EcR regulation of miRNA gene expression and to explore the physiological relevance of the regulation in mosquitoes, two of the iEcR-responsive miRNAs, miR-275 and miR-305, were selected for further investigation. They were chosen for the following reasons: 1) Both miRNAs are highly expressed in the FB and dramatically down-regulated in response to EcR RNAi (*SI Appendix*, Fig. S1*E*); 2) precursors of *miR-275* and *miR-305* (pre–miR-275 and pre–miR-305) are closely clustered in the genome (Fig. 2*A*), suggesting their cotransactivation by the 20E pathway; 3) previous studies have shown that miR-275 is essential for blood digestion and egg development in *Ae. aegypti* (8) and that miR-305 is involved in susceptibility of *Anopheles gambiae* to the *Plasmodium* parasite (24).

A time course analysis of pre–miR-275 and pre–miR-305 levels in the female mosquito FB showed that they were positively correlated with the titers of 20E (Fig. 2*B*), suggesting that these two miRNAs may be activated by 20E. To test this hypothesis, we first performed an in vivo 20E injection assay. Because the target of rapamycin-mediated amino acid (AA) signaling also plays an important role in activating PBM events in female mosquitos (17, 25), we injected 20E alone or with addition of a mixture of AAs. The levels of pre–miR-275 and pre–miR-305 in the FBs of female mosquitoes at 96 h PE (when endogenous 20E level is low) were significantly higher than the control (solvent) at 6 h after 20E injection, but not changed by AA injection (Fig. 2*C*). We then conducted an in vitro FB culture assay. After culture of 96 h PE

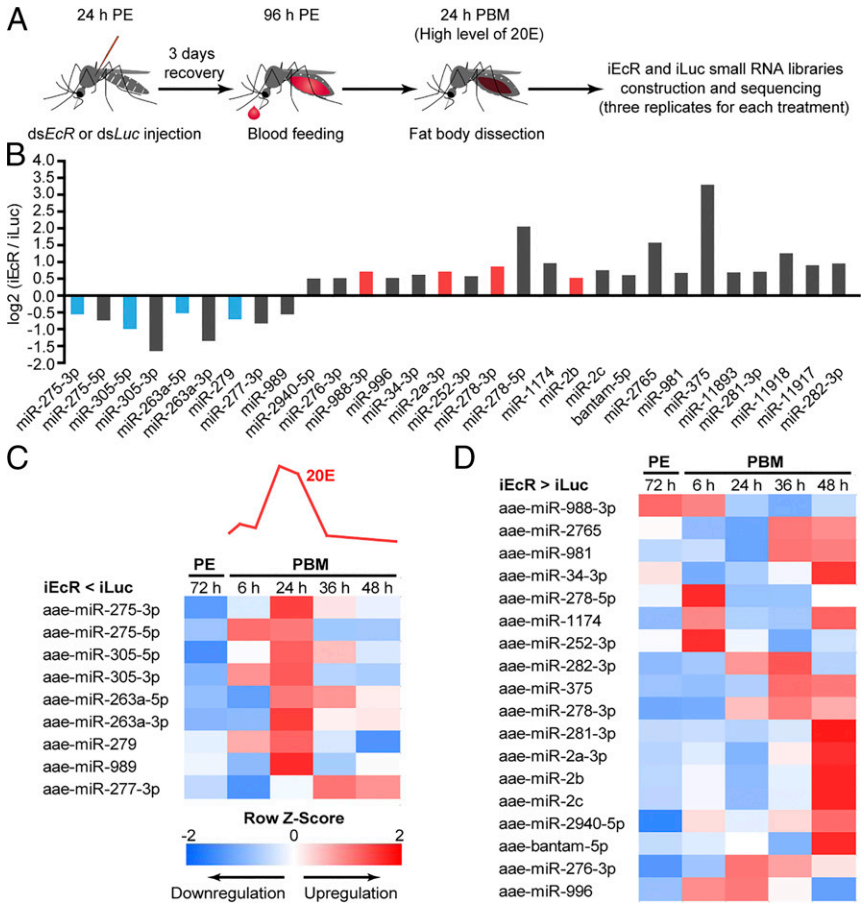

Fig. 1. Transcriptome-wide screening of ecdysone receptor (EcR)-regulated miRNAs in the female mosquito fat body (FB). (*A*) Schematic representation of the study design. (*B*) Differentially expressed miRNAs in the FBs of iEcR females at 24 h post blood meal (PBM) compared with iLuc control. Filter condition, $\log_2$ (iEcR/iLuc) greater than 0.5 or less than −0.5. qRT-PCR validation is highlighted by blue for down-regulation and red for up-regulation. (*C* and *D*) Time course expression profiles of iEcR–down-regulated (*C*) and –up-regulated (*D*) miRNAs in the female mosquito FB after a blood meal. Data are derived from Zhang et al. (23). Expression levels are represented by averages of normalized expression from three biological replicates.

FBs in medium containing 20E for 6 h, the pre–miR-275 and pre–miR-305 levels were greater than in the solvent control (Fig. 2*D*). Similar results were obtained with the mature miRNAs (*SI Appendix*, Fig. S2 *A* and *B*). Therefore, 20E activates *miR-275* and *miR-305* expression in the FB.

To investigate whether an intermediate factor(s) is required for the 20E-dependent activation of *miR-275* and *miR-305*, we utilized a classic approach using the protein synthesis inhibitor cycloheximide (Chx). As expected, Chx inhibited the activation of two late-response genes, *vitellogenin* (*Vg*) and *vitellogenic carboxypeptidase* (*VCP*), but not of two early-response genes, *E74* and *E75* (17), by 20E in FB culture assays (*SI Appendix*, Fig. S2*C*). Chx was ineffective in preventing the 20E activation of *miR-275* and *miR-305* (Fig. 2*E*), suggesting direct control of *miR-275* and *miR-305* by the 20E pathway. We further silenced the expression of the two early transcription factors E74 (iE74) and E75 (iE75) using RNAi (*SI Appendix*, Fig. S3*A*), and examined the effect on *miR-275* and *miR-305* expression. The levels of pre-miRNAs and mature miRNAs of *miR-275* and *miR-305* in the FBs of iE74 or iE75 mosquitoes were comparable to that in the iLuc control. In contrast, they were significantly lower in the FBs of iEcR mosquitoes than the control (*SI Appendix*, Fig. S3*B*). Together, these results indicate that 20E directly activates *miR-275* and *miR-305* transcription through the ecdysone receptor complex (EcR/USP).

Precise Mapping of the Transcription Start Sites of *miR-275* and *miR-305*. Unlike traditional mRNA genes, the promoter regions of miRNA genes are widely unknown in *Ae. aegypti*. To gain insight into the genomic interaction of EcR/USP with *miR-275* and *miR-305*, we mapped their transcription start sites (TSSs) using the rapid amplification of cDNA ends (RACE) technique. Previous studies have shown that silencing of Drosha in human cells prevents cleavage and enriches for pri-miRNAs, which enables easier identification of the gene structure of intact pri-miRNAs (26). Therefore, we first silenced *Ae. aegypti* Drosha (AAEL008592) using RNAi (*SI Appendix*, Fig. S4*A*). Compared with the iLuc control, *Drosha* mRNA level was 50% lower in the FBs of ds*Drosha*-injected (iDrosha) females at 24 h PBM (*SI Appendix*, Fig. S4*B*). The pre-miRNA levels of *miR-275* and *miR-305* in the FBs of iDrosha mosquitoes were higher, but the mature miRNA levels were lower than in the control (*SI Appendix*, Fig. S4 *C* and *D*). Moreover, the level of primary transcript of *miR-276* (pri–miR-276, AAEL027655), the only miRNA gene with complete genome annotation in *Ae. aegypti*, was significantly higher in iDrosha females than in the control (*SI Appendix*, Fig. S4*E*), confirming enrichment of pri-miRNAs in the FBs of iDrosha mosquitoes.

Next, the total RNA from FBs of iDrosha mosquitoes at 24 h PBM was used for 5′-RACE. To pinpoint the TSS of *miR-275*, a cDNA library was prepared using a pre–miR-275–specific reverse-transcription primer and used for PCR amplification with a GeneRacer 5′ primer (GR5P) and a pre–miR-275–specific primer

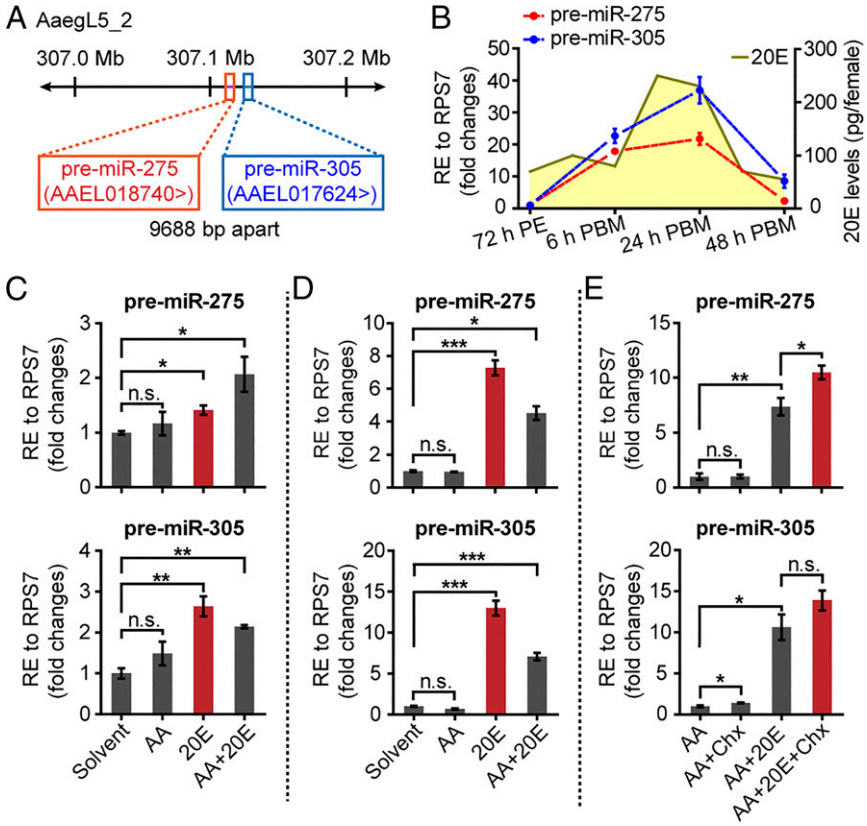

Fig. 2. 20E activates *miR-275* and *miR-305* transcription in the FB. (*A*) Schematic diagram showing the genomic location of pre–miR-275 and pre–miR-305. (*B*) Time course expression profiles of pre–miR-275 and pre–miR-305 in the female mosquito FB posteclosion (PE) and PBM. The 20E titers in *Ae. aegypti* females after a blood meal are derived from Hagedorn et al. (22) and are shown in yellow. (*C*) Relative levels of pre–miR-275 and pre–miR-305 in the FBs at 6 h after injection of the indicated reagents. A 0.5-μL aliquot of injection solution containing either ethanol (solvent), ethanol and amino acids (AA), 20E (10^{-4} M) dissolved in ethanol, or amino acids and 10^{-4} M 20E (AA + 20E) was injected into female mosquitoes at 96 h PE. (*D* and *E*) Relative levels of pre–miR-275 and pre–miR-305 in the FBs. (*D*) FBs from 96-h PE female mosquitoes were incubated in culture solution containing either ethanol (solvent), ethanol and amino acids (AA), 20E (10^{-6} M) dissolved in ethanol, or amino acids and 10^{-6} M 20E (AA + 20E) for 6 h. (*E*) FBs from 96 h PE female mosquitoes were incubated in amino acids plus culture solution containing either ethanol (AA), ethanol and 10^{-3} M Chx (AA + Chx), 10^{-6} M 20E (AA + 20E), or 10^{-6} M 20E and 10^{-3} M Chx (AA + 20E + Chx) for 6 h. (*B–E*) Mean ± SEM from three independent experiments; n.s., not significant; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (independent-samples *t* test).

(275SP). A single band between 1 and 1.5 kb was obtained and subcloned. A total of 12 clones were sequenced and two TSSs with a single-nucleotide spacer were identified. Sixty-seven percent of the clones started from TSS1, which was 992 bp upstream of pre–miR-275 (*SI Appendix*, Fig. S4*F*). To determine whether *miR-275* and *miR-305* were cotranscribed as a single transcript, another cDNA library was prepared using a pre–miR-305–specific reverse-transcription primer and used for PCR amplification with the same primer pair, GR5P and 275SP. A single band of the same size as mentioned above was amplified, subcloned, and sequenced. The same two TSSs were identified, and 58% of the 12 sequenced clones started from TSS1. Therefore, the two clustered miRNAs were indeed transcribed together as a single transcript. Since more clones started from TSS1, it was regarded as the TSS of *miR-275* and *miR-305* in the following experiments.

EcR Represses Expression of the *miR-275/miR-305* Cluster at the Low 20E Level before Blood Feeding, whereas It Activates Its Expression at the High 20E Level after a Blood Meal. We cloned a 5′ upstream regulatory region (−2002 to +98) of the *miR-275/miR-305* cluster into the pGL4.17 luciferase reporter vector (miR-275-Luc) and performed luciferase assays. *Drosophila* Schneider 2 (S2) cells were transfected with the reporter construct and the expression vectors for EcR and USP. Compared with the ethanol control, cells transfected with the reporter construct alone responded to 20E, with a 31-fold luciferase activity induction, which was likely mediated by the endogenous *Drosophila* EcR/USP complex. When EcR and USP expression vectors were transfected into cells, the activation of the reporter gene was sevenfold stronger in response to 20E than to the ethanol control (Fig. 3*A*), indicating that EcR/USP transactivates the *miR-275/miR-305* promoter in the presence of 20E. Interestingly, the basal expression of the reporter gene was repressed by the overexpression of EcR but not by USP when 20E was absent (Fig. 3*A*), suggesting that EcR represses the *miR-275/miR-305* promoter in the absence of 20E.

To test whether the roles of EcR and USP observed in S2 cells also exist in mosquitoes, we silenced EcR and USP in mosquitoes using RNAi (*SI Appendix*, Fig. S5*A*), dissected the FBs at 96 h PE, and cultured them for 6 h in medium containing either 20E or ethanol. In the absence of 20E, the expression levels of *miR-275* and *miR-305* were increased by EcR knockdown, but not markedly affected by USP knockdown compared with the control. In the presence of 20E, the expression levels of miRNAs were reduced by either EcR or USP knockdown (Fig. 3*B*). In *Ae. aegypti*, the 20E titer is low before blood feeding, but increases after a blood meal and peaks at 18–24 h PBM (22). We then analyzed the effect of EcR and USP knockdown on *miR-275* and *miR-305* expression before and after a blood meal. At 96 h PE,

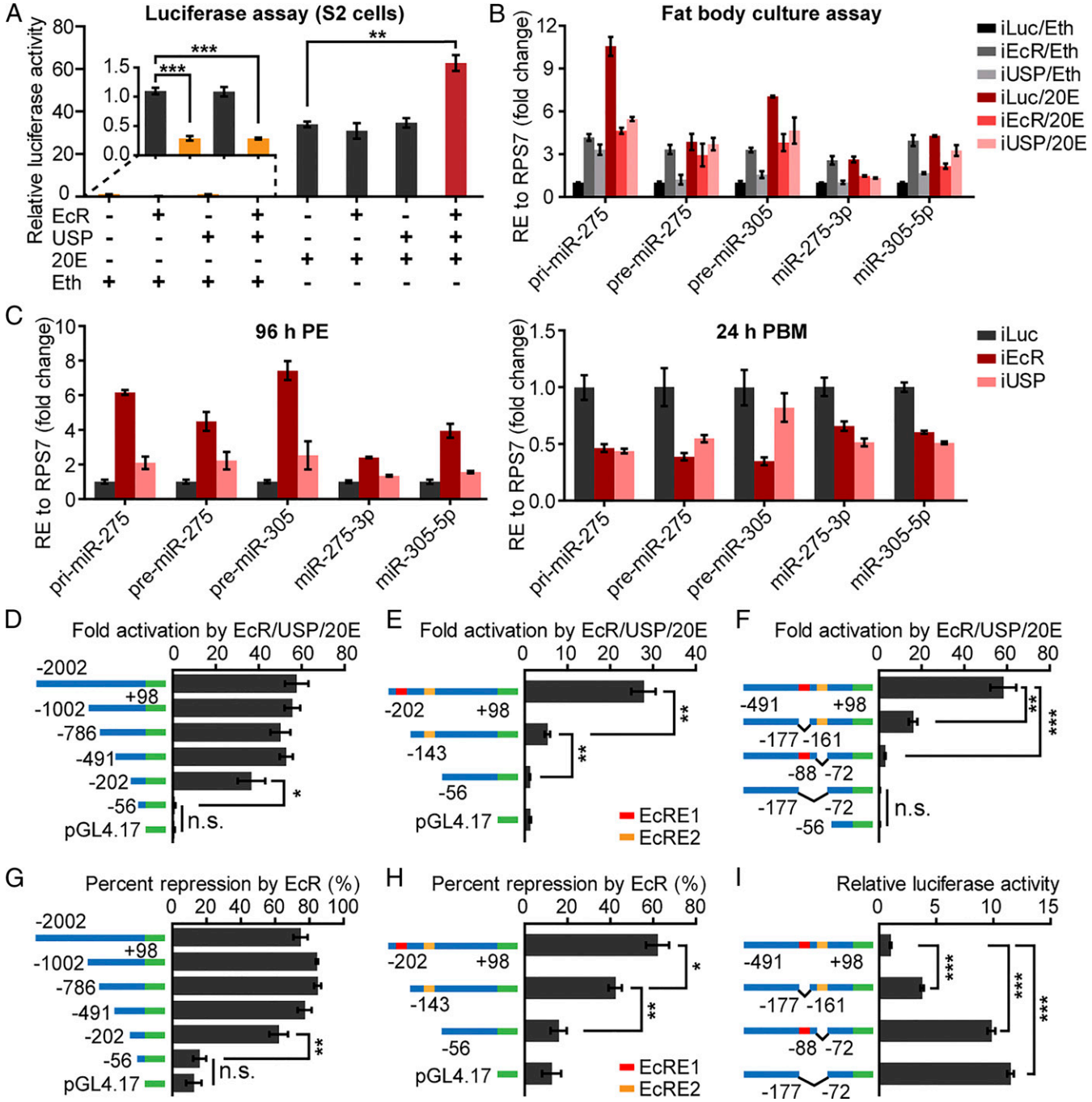

Fig. 3. EcR represses expression of the *miR-275* and *miR-305* cluster at the low 20E level before blood feeding, whereas it activates its expression at the high 20E level after a blood meal. (*A*) Luciferase assays in S2 cells transfected with the pGL4.17 reporter plasmid carrying the *miR-275/miR-305* promoter (−2002 to +98) conjugated to firefly luciferase (miR-275-Luc) and the expression vectors for EcR and/or Ultraspiracle (USP). Treatments with empty pAc5.1 expression vector served as controls. A *Renilla* luciferase reporter construct was cotransfected in each well as a reference. Transfected cells were exposed to 20E (10^{-6} M) dissolved in ethanol (Eth) or Eth alone. Data represent normalized luciferase activity (firefly/*Renilla*) and are shown as mean ± SEM from three independent experiments. (*B* and *C*) Relative levels of primary transcript (pri–miR-275), pre-miRNAs and mature miRNAs of *miR-275* and *miR-305* in the FBs. (*B*) FBs were dissected from iLuc, iEcR, or iUSP female mosquitoes at 96 h PE and incubated in culture solution containing 20E (10^{-6} M) dissolved in Eth or Eth alone for 6 h. (*C*) FBs were dissected from iLuc, iEcR, and iUSP female mosquitoes at 96 h PE and 24 h PBM. (*D–F*) Luciferase assays in S2 cells transfected with the expression vectors for EcR and USP and the desired reporter constructs. Transfected cells were exposed to 20E (10^{-6} M) dissolved in Eth or Eth alone. Data represent fold induction of normalized luciferase activity from EcR/USP/20E-treated cells over that of pAc5.1/Eth-treated cells. (*G–I*) Luciferase assays in S2 cells transfected with the expression vector for EcR and the desired reporter constructs. (*G* and *H*) Data represent percent reduction of normalized luciferase activity from EcR-treated cells compared with pAc5.1-treated cells. (*I*) Data represent normalized luciferase activity. (*A–I*) Mean ± SEM from three independent experiments; n.s., not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (independent-samples *t* test).

the expression levels of both miRNAs increased in the FBs of iEcR mosquitoes but were not markedly changed in those of iUSP mosquitoes compared with the iLuc control. At 24 h PBM, the expression levels of miRNAs decreased with both iEcR and iUSP treatments compared with the control (Fig. 3*C*). We conclude that EcR represses *miR-275* and *miR-305* expression before blood feeding, when 20E level is low, whereas it activates their expression after a blood meal, when 20E level is high. USP appears to be essential for 20E-induced activation, but dispensable for EcR-mediated repression of miRNAs.

The *Ae. aegypti* Taiman, also known as FISC (βFTZ-F1 interacting steroid receptor coactivator), is a key factor required for both juvenile hormone- and 20E-regulated gene expression (27, 28). It interacts with EcR/USP in a 20E-dependent manner and is indispensable for the activation of 20E-inducible protein-coding genes (27). We thus examined whether Taiman is involved in the activation of the *miR-275/miR-305* cluster by EcR/USP in response to 20E. We first performed a luciferase assay in S2 cells with the miR-275-Luc reporter. The results showed that Taiman potentiates transcriptional activation of the *miR-275/miR-305* promoter by EcR/USP in response to 20E (*SI Appendix*, Fig. S5*B*). We then silenced Taiman in mosquitoes using RNAi (*SI Appendix*, Fig. S5*C*), dissected the FBs at 96 h PE, and cultured them in medium containing either 20E or ethanol for 6 h. Compared with the control, Taiman knockdown significantly reduced the extent of induction of *miR-275* and *miR-305* expression by 20E (*SI Appendix*, Fig. S5*D*). These data indicate that the EcR/USP/Taiman complex is also involved in 20E-induced activation of miRNA expression.

EcR Binds to EcREs of the *miR-275/miR-305* Promoter Both before and after a Blood Meal. The EcR/USP complex usually binds to sequence-specific motifs, known as EcREs, in the promoter region

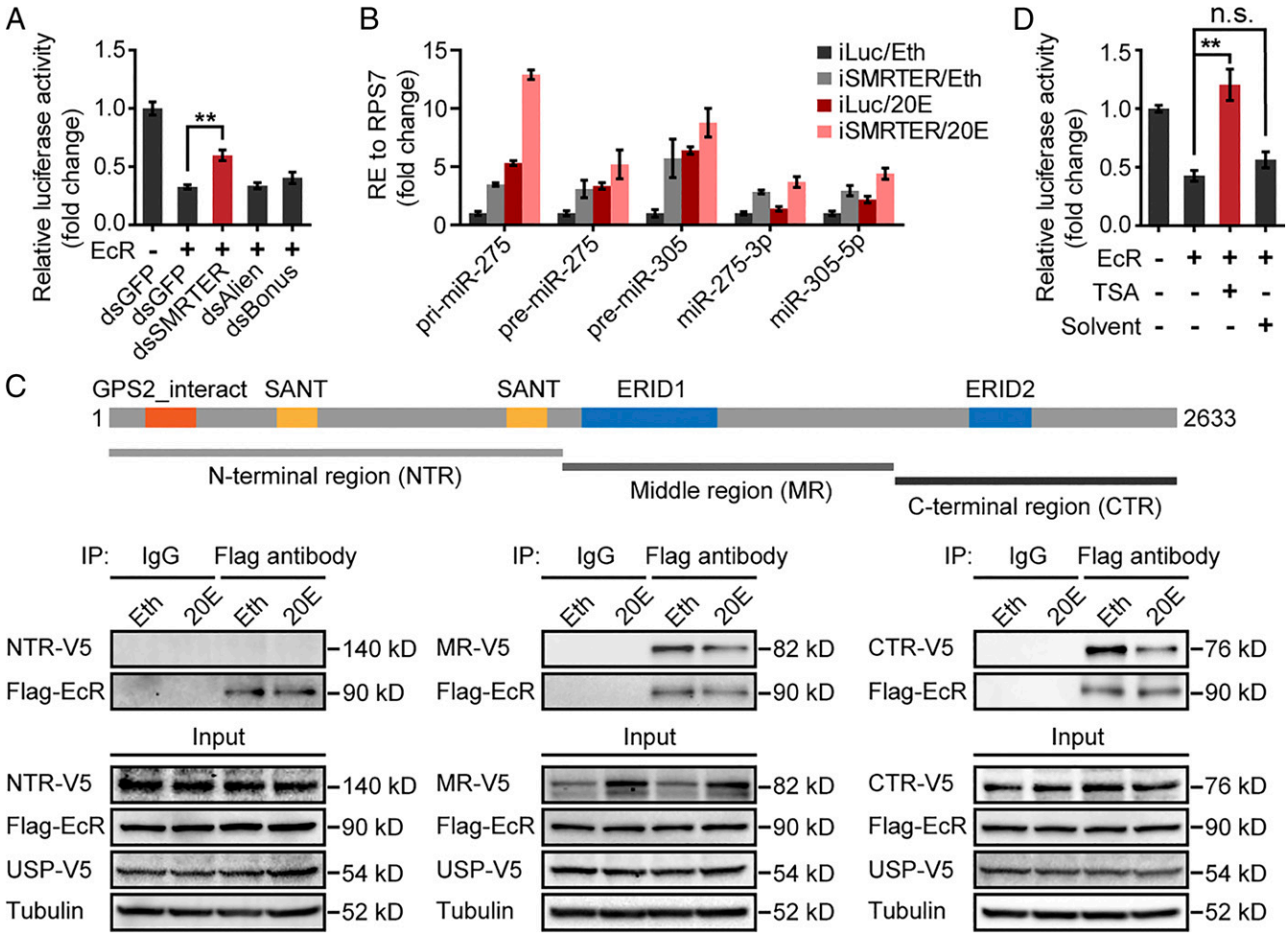

Fig. 4. The corepressor SMRTER is involved in repression of miRNA expression by EcR. (*A*) SMRTER is required for EcR-mediated repression. dsRNA-treated Aag2 cells were transfected with the miR-275-Luc reporter construct and the expression vector for EcR. Treatment with empty expression vector served as a control. A *Renilla* luciferase reporter construct was cotransfected in each well as a reference. Data represent normalized luciferase activity (firefly/*Renilla*). (*B*) Relative levels of primary transcript, pre-miRNAs, and mature miRNAs of *miR-275* and *miR-305* in the FBs dissected from iLuc or iSMRTER female mosquitoes at 96 h PE and incubated for 6 h in culture solution containing 20E (10^{-6} M) dissolved in Eth or Eth alone. (*C*) SMRTER interacts with EcR in a hormone-sensitive manner. (*Top*) Schematic representation of SMRTER domain structure and depletion constructs used for interaction assay. The two corresponding regions of the *Drosophila* SMRTER EcR-interacting domains (ERID1 and ERID2) are indicated by blue. (*Bottom*) S2 cells were transfected with the expression vectors for V5-tagged SMRTER fragments, Flag-EcR and V5-USP. Transfected cells were exposed to 20E (10^{-6} M) dissolved in Eth or Eth alone. Extracts were immunoprecipitated (IP) with Flag antibody or IgG, and pellets were analyzed by immunoblotting using Flag and V5 antibodies. Input (10%) was analyzed using Flag, V5, and Tubulin antibodies. Molecular masses are indicated on the *Right*. (*D*) Transcriptional repression by EcR requires histone deacetylase activity. Aag2 cells were transfected with the miR-275-Luc reporter construct and the expression vector for EcR. Transfected cells were exposed to trichostatin A (TSA) (10^{-5} M) dissolved in DMSO or DMSO alone (solvent). Data represent normalized luciferase activity. (*A*, *B*, and *D*) Mean ± SEM from three independent experiments; n.s., not significant; $^{**}P < 0.01$ (independent-samples *t* test).

of target genes (19). Serial deletion analysis of the *miR-275/miR-305* promoter revealed that the sequence between −202 and −57 is crucial for the EcR/USP/20E-induced activation of the reporter gene (Fig. 3*D*). This sequence was then searched against the *Drosophila* EcR/USP binding matrix using the JASPAR online server (jaspar.genereg.net/), and two putative EcREs were predicted: EcRE1, −176 to −162, and EcRE2, −87 to −73 (*SI Appendix*, Fig. S6*A*). To test the functionality of these potential EcREs, we deleted EcRE1 from the reporter plasmid and evaluated the effect on 20E responsiveness. Deletion of EcRE1 reduced the extent of 20E induction. However, a significant level of 20E responsiveness was retained by this reporter construct (Fig. 3*E*), indicating that these two EcREs may work in concert to endow full 20E responsiveness of the *miR-275/miR-305* promoter. To verify this hypothesis, we further constructed reporter plasmids with one or both of the EcREs deleted. Deletion of either EcRE1 or EcRE2 significantly but not completely reduced the extent of induction by 20E. However, double deletion of EcRE1 and EcRE2 resulted in a complete loss of 20E inducibility (Fig. 3*F*), clearly demonstrating that responsiveness to 20E was attributed to these two sites. Surprisingly, transfection assays with these deletion constructs showed that the sequence between −202 and −57 is also critical for EcR-mediated repression of the reporter gene in the absence of 20E (Fig. 3*G*). Moreover, both EcRE1 and EcRE2 were required for the EcR-mediated repression, as deletion of either EcRE1 or EcRE2 reversed repression of the reporter gene (Fig. 3 *H* and *I*). To determine whether EcR binds to these two EcREs in mosquitoes, we performed chromatin immunoprecipitation (ChIP) assays. The results demonstrate that EcR is associated with the two EcREs (amplimer 3) of the *miR-275/miR-305* promoter both before (96 h PE) and after (18 h PBM) a blood meal (*SI Appendix*, Fig. S6*B*).

The Corepressor SMRTER Is Involved in Repression of miRNA Expression by EcR. Transcriptional repression by nuclear receptors usually occurs via association with corepressors (29). So far, no corepressor of EcR has been reported in *Ae. aegypti*. However, several EcR corepressors have been identified in *Drosophila*, including SMRTER (20), Alien (30), and Bonus (31). To determine whether such corepressors are involved in the repression of miRNA expression by EcR, we identified the *Ae. aegypti* homologs of these *Drosophila* corepressors (*SI Appendix*, Fig. S7*A*) and analyzed their roles in EcR-mediated repression. First, we examined whether EcR could also repress the *miR-275/miR-306* promoter in *Ae. aegypti* Aag2 cells using luciferase assays. Similar to the results in S2 cells, EcR repressed the basal activity of the miR-275-Luc reporter construct in the absence of 20E, whereas it activated the reporter gene in the presence of USP and 20E (*SI Appendix*, Fig. S7*B*). Next, we silenced these potential corepressors in Aag2 cells by soaking cells for 72 h in medium containing dsRNA corresponding to *SMRTER*, *Alien*, *Bonus*, or *GFP* (*SI Appendix*, Fig. S7*C*). When compared with the ds*GFP* control, a significantly higher luciferase activity was detected only in ds*SMRTER*-treated cells (Fig. 4*A*), indicating that SMRTER is required for EcR-mediated repression in Aag2 cells.

Time-course expression analyses showed that SMRTER and Bonus were stably expressed in the female mosquito FB before and after a blood meal, while *Alien* transcript increased at 6 h PBM but decreased to the level before blood feeding at 24 h PBM (*SI Appendix*, Fig. S7*D*). We then silenced their expression in mosquitoes using RNAi (*SI Appendix*, Fig. S7*E*) and analyzed the expression levels of *miR-275* and *miR-305* in the FBs at 96 h PE, when the two miRNAs were repressed by EcR (Fig. 3*C*). Although the expression levels of these two miRNAs in iAlien and iBonus mosquitoes were not changed, they were significantly higher in iSMRTER mosquitoes than in iLuc control (*SI Appendix*, Fig. S7*F*). Further culture of the 96 h PE FBs from iSMRTER and iLuc mosquitoes in medium containing either 20E or ethanol for 6 h showed that the expression levels of *miR-275* and *miR-305* in iSMRTER treatment were higher than the iLuc control, both in the absence and presence of 20E (Fig. 4*B*). Together, these results strongly suggest the involvement of SMRTER in miRNA repression by EcR.

SMRTER Interacts with EcR in a Hormone-Sensitive Manner. In *Drosophila*, SMRTER interacts with EcR via two independent EcR-interacting domains, ERID1 and ERID2 (20). Alignment of ERID1 and ERID2 with *Ae. aegypti* SMRTER identified two corresponding domains in the middle and C-terminal regions, respectively (*SI Appendix*, Fig. S8). We then performed coimmunoprecipitation assays to examine whether *Ae. aegypti* SMRTER interacts with EcR and which parts mediate the interaction. The V5-tagged N-terminal, middle, or C-terminal region of SMRTER (Fig. 4*C*) was expressed together with Flag-tagged EcR and V5-tagged USP in S2 cells. Flag antibody but not the control IgG precipitated Flag-EcR from protein extracts of both ethanol- and 20E-treated cells. The middle and C-terminal regions of SMRTER were precipitated with Flag-EcR, whereas the N-terminal region was not. Moreover, the interactions of EcR with the middle and C-terminal regions of SMRTER were weakened by 20E (Fig. 4*C*), suggesting that SMRTER interacts with EcR in a hormone-sensitive manner.

Transcriptional Repression by EcR Requires HDAC Activity. SMRTER induces gene silencing by interacting with the repressor dSin3A (20), which forms a complex with histone deacetylase (HDAC) that can modify histones and remodel chromatin (32). We then tested whether HDAC activity is required for EcR-mediated repression in mosquitoes. Aag2 cells were transfected with the miR-275-Luc reporter construct and the expression vector for EcR. After transfection, the cells were incubated in medium containing the HDAC inhibitor trichostatin A (TSA) or DMSO (solvent) as a control. Whereas the repression of reporter activity by EcR was not affected by the solvent treatment, it was markedly abrogated by the TSA treatment (Fig. 4*D*), indicating that transcriptional repression by EcR requires HDAC activity.

The EcR/SMRTER Complex Is Also Involved in Repression of EcR-Regulated Protein-Coding Genes. The above results indicate that the EcR/SMRTER complex represses miRNA gene expression in mosquitoes. We next asked whether the EcR/SMRTER complex could also repress the expression of protein-coding genes. We analyzed the transcript levels of two EcR-regulated protein-coding genes, *E74* and *E75*, in the FBs of dsRNA-injected female mosquitoes at 96 h PE after being cultured in medium containing either 20E or ethanol for 6 h. As expected, knockdown of either EcR or USP resulted in lower transcript levels of *E74* and *E75* in the presence of 20E than in iLuc control. In the absence of 20E, higher transcript levels of these two genes were seen with EcR knockdown but not USP knockdown (*SI Appendix*, Fig. S9*A*). Importantly, SMRTER knockdown resulted in higher transcript levels of both genes, regardless of the presence of 20E (*SI Appendix*, Fig. S9*B*). These data indicate that the EcR/SMRTER complex represses not only miRNA genes but also protein-coding genes in mosquitoes.

miR-275 and miR-305 Promote Egg Development in Female Mosquitoes by Regulating Target Genes Expression. A spatiotemporal expression analysis of miR-275 and miR-305 revealed that both miRNAs were highly expressed in the midgut, FB, and ovary of female mosquitoes after a blood meal (*SI Appendix*, Fig. S10*A*). To explore the physiological relevance of these two miRNAs, female mosquitoes were injected with antagomirs for miR-275-3p (Ant-275) or miR-305-5p (Ant-305) at 24 h PE, and were fed blood 3 d later. Mosquitoes injected with a randomly scrambled "missense" antagomir (Ant-Con) were used as controls. miR-275-3p and miR-305-5p were depleted by 99.1% and 75.9%, respectively, in the

FBs of Ant-275– and Ant-305–treated mosquitoes at 24 h PBM compared with the control (*SI Appendix*, Fig. S10*B*). Ant-275 treatment resulted in a dramatic defect in ovary development, which is consistent with our previous study (8). A severe growth arrest of the ovary was also observed in Ant-305–treated mosquitoes, as evidenced by the much smaller primary follicles (157 μm on average) than in the Ant-Con–treated and wild-type (WT) mosquitoes (217–224 μm on average) at 24 h PBM (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S10*C*). Furthermore, both Ant-275– and Ant-305–treated females displayed reduced fecundity, laying significantly fewer eggs (84 and 81 eggs per female on average for Ant-275 and Ant-305 treatment, respectively) than the Ant-Con–treated and WT controls (124–129 eggs per female on average, Fig. 5*C*). Collectively, both miR-275 and miR-305 promote egg development in female mosquitoes.

Several potential target genes of miR-275-3p and miR-305-5p in the female mosquito FB have been isolated through AGO 1 cross-linking and immunoprecipitation followed by high-throughput sequencing in our previous study (*SI Appendix*, Table S1) (23). Moreover, the targeting of two genes, *GSD* and *grainyhead* (*GRH*), by miR-275-3p was validated by luciferase assays in human 293TRex cells (23). However, whether they are authentic targets of these two miRNAs in mosquitoes is unknown. We first assessed the regulating ability of miR-275-3p and miR-305-5p on the expression of these candidate targets in vivo. Of the four potential targets of miR-275-3p, the *GSD* mRNA level was significantly higher, whereas the *Ribose-phosphate pyrophosphokinase 1* (*PRPS1*) mRNA level was significantly lower in the FBs of Ant-275–treated mosquitoes than in control (Fig. 6*A*). Of the three candidate targets of miR-305-5p, only *AAEL009899* mRNA level was markedly higher with Ant-305 treatment than the control (Fig. 6*B*). Next, using luciferase assays, we tested whether the WT 3′-UTRs of these genes could respond to miR-275-3p or miR-305-5p. Compared with S2 cells cotransfected with the control mimic (Con-mimic), the luciferase activity of *GSD* 3′-UTR containing reporter was 30.3% lower in cells cotransfected with miR-275-3p mimic. However, the activities of *PRPS1* 3′-UTR containing reporter were comparable between the miR-275-3p mimic and con-mimic treatments. The activity of the reporter containing the *AAEL009899* 3′-UTR was 33.5% lower when cotransfected with miR-305-5p mimic than with the con-mimic (Fig. 6*C*). Several in silico tools (RNAhybrid, PITA, and miRanda) were used to predict miRNA binding sites in the 3′-UTRs of *GSD* and *AAEL009899* mRNAs, and 7-mer and 8-mer binding sites for miR-275-3p and miR-305-5p, respectively, were identified (*SI Appendix*, Fig. S11 *A* and *B*). To functionally validate the predicted binding sites, luciferase reporters with mutated (Mut) binding sites were constructed. As expected, the luciferase activities were recovered when S2 cells were cotransfected with the Mut constructs and miRNA mimics (Fig. 6*D*). Since miR-275 and miR-305 are conserved miRNAs, the levels of endogenous miR-275-3p and miR-305-5p in S2 cells could be lowered by Ant-275 and Ant-305, respectively (*SI Appendix*, Fig. S11 *C* and *D*). When cells were transfected with the WT constructs, the repression of expression of the reporter gene was reversed by Ant-275 or Ant-305 treatment compared with the Ant-con treatment. In contrast, the same treatment with Ant-275 or Ant-305 did not modify the activities of Mut constructs (*SI Appendix*, Fig. S11 *E* and *F*). These data clearly demonstrate that *GSD* and *AAEL009899* are authentic targets of miR-275-3p and miR-305-5p, respectively.

Finally, we conducted phenotypic rescue experiments through RNAi silencing of GSD (iGSD) and AAEL009899 (iAAEL009899) in miR-275– and miR-305–depleted female mosquitoes (*SI Appendix*, Fig. S12*A*), respectively. The defects in ovary development and egg deposition caused by miR-275-3p or miR-305-5p depletion significantly recovered after the knockdown of their respective targets. The average follicle size in Ant-275/iGSD–treated mosquitoes was significantly larger than that in the Ant-275– or Ant-275/iLuc–treated females at 24 h PBM. Moreover, Ant-275/iGSD–treated insects laid a dramatically higher number of eggs than the Ant-275– or Ant-275/iLuc–treated groups (Fig. 6 *E*–*G* and *SI Appendix*, Fig. S12*B*). Similarly, both the follicle size and egg deposition number greatly increased in Ant-305/iAAEL009899–treated mosquitoes compared with the Ant-305 and Ant-305/iLuc treatments (Fig. 6 *H*–*J* and *SI Appendix*, Fig. S12*C*). Therefore, the dysregulation of *GSD* and *AAEL009899* could explain, at least in part, the adverse phenotypes caused by miR-275 and miR-305 depletion, respectively.

Discussion

miRNAs have been shown to regulate a variety of physiological processes such as blood digestion, egg development, metamorphosis, and defense against pathogens in mosquitoes (33). However, detailed information concerning regulation of miRNA expression in mosquitoes is limited. Our results showed that nine miRNAs were down-regulated in the female mosquito FB at 24 h PBM after EcR RNAi. Furthermore, EcR directly activated the transcription of clustered *miR-275* and *miR-305* in response to 20E. Therefore, EcR-mediated 20E signaling plays an important role in the activation of miRNA expression in mosquitoes. In *Drosophila*, 20E and EcR directly activate transcription of the *let-7-complex* locus, which encodes the cotranscribed miR-100, let-7, and miR-125 (34). Moreover, direct activation of the *miR-275* and *miR-305* cluster by EcR and 20E has also been found in the embryogenic cell line of *Bombyx mori* (35). Thus, direct activation of miRNA expression by EcR and 20E is quite conserved among insects. Notably, 21 miRNAs were up-regulated in the FB at 24 h BPM after EcR RNAi, suggesting a significant role of the 20E pathway in the repression of miRNA expression in

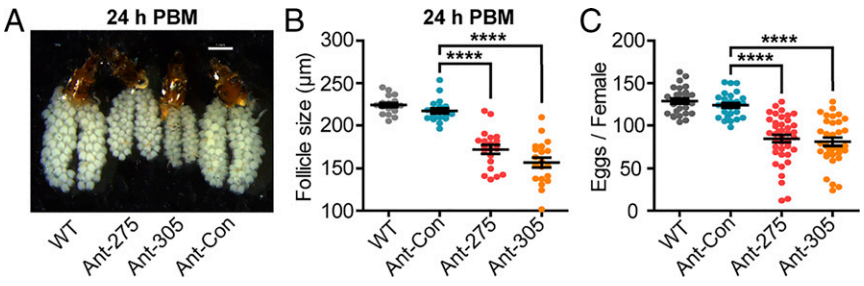

Fig. 5. miR-275 and miR-305 promote egg development in female mosquitoes. (*A*) Effect of miR-275-3p and miR-305-5p depletion on ovary development observed at 24 h PBM. Ovaries were visualized using the Leica M165FC stereo microscope. (Scale bars, 1 mm.) (*B*) Average follicle size of ovaries isolated from wild-type (WT), Ant-Con–, Ant-275–, and Ant-305–injected mosquitoes at 24 h PBM. (*C*) Egg numbers deposited per female by WT, Ant-Con–, Ant-275–, and Ant-305–injected mosquitoes. (*B* and *C*) One dot represents one female mosquito. Data represent three biological replicates with 6–14 individuals in each replication. Mean ± SEM is shown. ****$P < 0.0001$ (nonparametric Mann–Whitney *U* test).

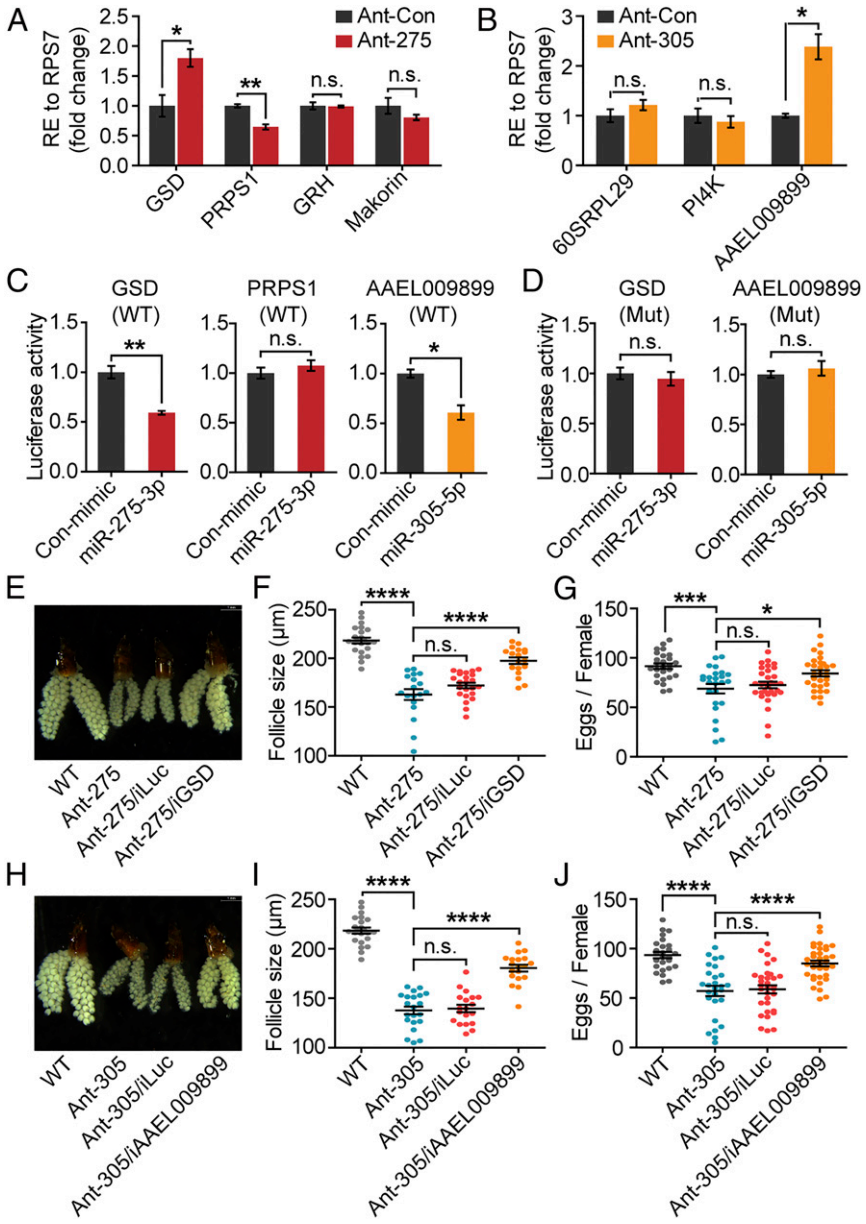

Fig. 6. Identification of miR-275 and miR-305 target genes. (*A* and *B*) Relative mRNA levels of potential miR-275-3p (*A*) and miR-305-5p (*B*) target genes in the FBs of Ant-Con– and Ant-275– or Ant-305–treated female mosquitoes at 24 h PBM. (*C*) Luciferase activities in S2 cells transfected with the reporter construct containing the WT 3′-UTR of *glutamate semialdehyde dehydrogenase* (*GSD*), *Ribose-phosphate pyrophosphokinase 1* (*PRPS1*), or *AAEL009899* and the desired miRNA mimics. (*D*) Luciferase activities in S2 cells transfected with the mutant (Mut) reporter construct of *GSD* or *AAEL009899* and the desired miRNA mimics. (*C* and *D*) Data represent normalized luciferase activity (*Renilla*/firefly). (*A*–*D*) Mean ± SEM from three independent experiments; n.s., not significant; **P* < 0.05, ***P* < 0.01 (independent-sample *t* test). (*E*–*G*) GSD RNAi rescues miR-275 depletion phenotypes. Ovaries (*E*), average follicle size (*F*), and egg numbers deposited per female (*G*) were analyzed from WT, Ant-275–, Ant-275/iLuc–, and Ant-275/iGSD–treated mosquitos. (*H*–*J*) AAEL009899 RNAi rescues miR-305 depletion phenotypes. Ovaries (*H*), average follicle size (*I*), and egg numbers deposited per female (*J*) were analyzed from WT, Ant-305–, Ant-305/iLuc–, and Ant-305/iAAEL009899–treated mosquitos. (*E* and *H*) Ovaries were visualized using the Leica M165FC stereo microscope. (Scale bars, 1 mm.) (*F*, *G*, *I*, and *J*) One dot represents one female mosquito. Data represent three biological replicates with 6–14 individuals in each replication. Mean ± SEM is shown. n.s., not significant; **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 (nonparametric Mann–Whitney *U* test).

mosquitoes. The mechanism of miRNA repression by EcR in the presence of 20E in mosquitoes is still unknown. In *Drosophila*, it has been shown that 20E indirectly represses *miR-8* transcription through two early-response genes, *E74* and *Broad-complex* (36). Future investigations of the molecular basis of miRNA repression by EcR and 20E in mosquitoes would improve our understanding of hormonal regulation of miRNA expression in insects.

The mammalian type II nuclear receptors retinoic acid receptor (RAR) and thyroid hormone receptor (TR) bind DNA constitutively and repress target gene transcription in the absence of ligand via association with the corepressors SMRT and N-CoR, each of which forms large complexes that include Sin3 and HDAC (37, 38). On binding to ligands, these nuclear receptors recruit coactivators, displace the corepressors, and thereby induce transcription of the target gene (39). In *Drosophila*, the EcR/USP complex binds DNA and represses transcription in the absence of 20E by interacting with corepressors, including SMRTER (20), Alien (30), and Bonus (31). 20E exposure increases transport of

EcR and USP into the nucleus and conformational changes lead to the replacement of corepressors with coactivators and gene activation (16). A similar dual role of the mosquito EcR was observed in our study. Moreover, we showed that the *Ae. aegypti* SMRTER interacts with EcR in a hormone-sensitive manner and is required for EcR-mediated repression in the absence of hormone. SMRTER is the insect analog of the vertebrate nuclear receptor corepressors SMRT and N-CoR (20). The *Drosophila* SMRTER has been shown to interact with the repressor dSin3A (20), which forms a complex with HDAC that can modify histones and remodel chromatin (32). Notably, the implication of HDAC in EcR-mediated repression in mosquito cells was confirmed by treatment with the HDAC inhibitor TSA. These results strongly indicate that the molecular basis of gene repression by nuclear receptors is quite conserved between vertebrates and insects.

While SMRTER is functionally similar to the vertebrate SMRT and N-CoR, its primary structure is quite divergent from its vertebrate counterparts. Overall, the *Drosophila* SMRTER is ∼13.5% and the *Ae. aegypti* SMRTER 16.8% identical to SMRT and N-CoR. Moreover, the defined receptor interaction domains and repression domains in SMRT and N-CoR are not conserved in SMRTER (20, 38, 40). Two independent EcR-interacting domains, ERID1 and ERID2, have been identified in *Drosophila* SMRTER (20). Our results showed that the middle and C-terminal regions of *Ae. aegypti* SMRTER interact with EcR. Comparative analysis revealed that the middle and C-terminal regions contain a sequence corresponding to ERID1 and ERID2, respectively. Therefore, the EcR-interaction domains of SMRTER may be conserved in insects. Interestingly, although the nuclear receptor interaction domains of SMRTER are divergent from those of SMRT and N-CoR, SMRTER interacts with vertebrate nuclear receptors RAR and TR, and, reciprocally, vertebrate SMRT also interacts with EcR (20). Future study of critical amino acids in the nuclear receptors and corepressors that are responsible for their interactions would shed light on how this family of nuclear receptor corepressors have evolved.

The reproductive cycle of adult female mosquitoes is divided into two phases by the acquisition of a blood meal. The expression of 20E-dependent genes is silenced prior to blood feeding, but activated in response to increased 20E titer after a blood meal. Previous studies have shown that EcR and USP proteins are abundant in the nuclei of the *Ae. aegypti* FB both before and after a blood meal (41). However, HR38, the *Ae. aegypti* homolog of vertebrate nerve growth factor-induced protein B orphan nuclear receptor, blocks the dimerization of EcR and USP via directly binding to USP before blood feeding (42). Here, we found that both EcR and SMRTER are essential for repressing the transcription of 20E-dependent miRNA and protein-coding genes in the absence of hormone, whereas USP is largely dispensable. After a blood meal, USP binds to EcR (42), and the complex recruits the steroid receptor coactivator Taiman to activate the expression of target genes. The competence factor βFTZ-F1 plays an important role in facilitating the recruitment of Taiman to the EcR/USP complex in a 20E-dependent manner (28). Consistently, our results showed that Taiman is required for 20E-dependent activation of miRNA by EcR/USP. Based on the results from previous works and our study, we propose a model of blood-feeding–triggered switch of EcR binding partners for the control of target gene expression in the female mosquito FB. Before blood feeding, USP dimerizes with HR38, whereas EcR recruits the corepressor SMRTER to repress the transcription of target genes. After a blood meal, the increased 20E titer leads to the dissociation of USP/HR38 complex and the formation of EcR/USP heterodimer, which recruits βFTZ-F1 and Taiman, displaces SMRTER, and activates the transcription of target genes (*SI Appendix*, Fig. S13).

Here, we demonstrated that miR-275 directly targets *GSD* in the FB. GSD, also known as pyrroline-5-carboxylate synthase, is the key enzyme in the synthesis of proline in higher eukaryotes (43). Female mosquitoes must use amino acids derived from blood meal protein to produce lipids and carbohydrates, as well as to provide energy through the oxidation of amino acid carbon skeletons (44, 45). These processes lead to the release of ammonia, which is highly toxic to mosquito tissues. An important mechanism to detoxify ammonia in mosquitoes is to utilize proline as a temporary nitrogen sink to store ammonia (46). The FB is implicated as the main tissue involved in ammonia detoxification in *Ae. aegypti* females (45). Our results thus indicate that miR-275 plays an important role in ammonia detoxification in female mosquitoes through targeting *GSD* in the FB. miR-305 promotes egg development through targeting *AAEL009899* in the FB. Unfortunately, AAEL009899 is an uncharacterized protein. Structure analysis revealed the presence of two transmembrane domains in this protein (*SI Appendix*, Fig. S12*D*), suggesting that AAEL009899 is a membrane protein. Future study of the function of AAEL009899 in mosquitoes would improve our understanding of the important role of the miR-305–AAEL009899 interaction in mosquito reproduction.

Materials and Methods

A detailed description of the materials and methods is given in *SI Appendix, Materials and Methods*. In brief, dsRNA-mediated gene silencing, qRT-PCR, FB culture, luciferase reporter assay, and miRNA antagomir treatment were performed according to the protocols described previously (8, 11, 28). Small RNA extracted from FBs was sequenced on a BGISEQ-500 platform. Total RNA from FBs was used to generate cDNA for 5′-RACE using the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. ChIP assays were performed using the Magna ChIP G Tissue Kit (Sigma-Aldrich) following the manufacturer's instructions. Coimmunoprecipitation assays were performed using the anti-Flag M2 affinity agarose (Sigma-Aldrich) according to the manufacturer's instructions. Primers used in this study are shown in Dataset S3.

Data Availability. Short-read sequence data of sRNA-seq have been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA694226). All other relevant data are included in the main text and supporting information.

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1. A. D. Barrett, S. Higgs, Yellow fever: A disease that has yet to be conquered. *Annu. Rev. Entomol.* **52**, 209–229 (2007).
2. K. A. Tsetsarkin, R. Chen, S. C. Weaver, Interspecies transmission and chikungunya virus emergence. *Curr. Opin. Virol.* **16**, 143–150 (2016).
3. S. C. Weaver *et al*., Zika virus: History, emergence, biology, and prospects for control. *Antiviral Res.* **130**, 69–80 (2016).
4. G. M. Attardo, I. A. Hansen, A. S. Raikhel, Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. *Insect Biochem. Mol. Biol.* **35**, 661–675 (2005).
5. J. C. Carrington, V. Ambros, Role of microRNAs in plant and animal development. *Science* **301**, 336–338 (2003).
6. J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet.* **11**, 597–610 (2010).
7. L. F. R. Gebert, I. J. MacRae, Regulation of microRNA function in animals. *Nat. Rev. Mol. Cell Biol.* **20**, 21–37 (2019).
8. 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).
9. 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).
10. 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).
11. 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).
12. T. Treiber, N. Treiber, G. Meister, Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. *Nat. Rev. Mol. Cell Biol.* **20**, 5–20 (2019).

13. Y. Lee *et al*., MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).
14. M. Ha, V. N. Kim, Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **15**, 509–524 (2014).
15. M. J. Turner, F. J. Slack, Transcriptional control of microRNA expression in *C. elegans*: Promoting better understanding. *RNA Biol.* **6**, 49–53 (2009).
16. N. Yamanaka, K. F. Rewitz, M. B. O'Connor, Ecdysone control of developmental transitions: Lessons from *Drosophila* research. *Annu. Rev. Entomol.* **58**, 497–516 (2013).
17. S. Roy, T. T. Saha, Z. Zou, A. S. Raikhel, Regulatory pathways controlling female insect reproduction. *Annu. Rev. Entomol.* **63**, 489–511 (2018).
18. L. Swevers, An update on ecdysone signaling during insect oogenesis. *Curr. Opin. Insect Sci.* **31**, 8–13 (2019).
19. L. Cherbas, K. Lee, P. Cherbas, Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev.* **5**, 120–131 (1991).
20. C. C. Tsai, H. Y. Kao, T. P. Yao, M. McKeown, R. M. Evans, SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Mol. Cell* **4**, 175–186 (1999).
21. V. A. Francis, A. Zorzano, A. A. Teleman, dDOR is an EcR coactivator that forms a feed-forward loop connecting insulin and ecdysone signaling. *Curr. Biol.* **20**, 1799–1808 (2010).
22. H. H. Hagedorn *et al*., The ovary as a source of alpha-ecdysone in an adult mosquito. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3255–3259 (1975).
23. X. Zhang, E. Aksoy, T. Girke, A. S. Raikhel, F. V. Karginov, Transcriptome-wide microRNA and target dynamics in the fat body during the gonadotrophic cycle of *Aedes aegypti*. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E1895–E1903 (2017).
24. N. J. Dennison, O. J. BenMarzouk-Hidalgo, G. Dimopoulos, MicroRNA-regulation of *Anopheles gambiae* immunity to *Plasmodium falciparum* infection and midgut microbiota. *Dev. Comp. Immunol.* **49**, 170–178 (2015).
25. I. A. Hansen, G. M. Attardo, J.-H. Park, Q. Peng, A. S. Raikhel, Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10626–10631 (2004).
26. 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).
27. P. Liu, X. Fu, J. Zhu, Juvenile hormone-regulated alternative splicing of the *taiman* gene primes the ecdysteroid response in adult mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7738–E7747 (2018).
28. J. Zhu, L. Chen, G. Sun, A. S. Raikhel, The competence factor β Ftz-F1 potentiates ecdysone receptor activity via recruiting a p160/SRC coactivator. *Mol. Cell. Biol.* **26**, 9402–9412 (2006).
29. X. Hu, M. A. Lazar, Transcriptional repression by nuclear hormone receptors. *Trends Endocrinol. Metab.* **11**, 6–10 (2000).
30. U. Dressel *et al*., Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* **19**, 3383–3394 (1999).
31. R. Beckstead *et al*., Bonus, a *Drosophila* homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit betaFTZ-F1-dependent transcription. *Mol. Cell* **7**, 753–765 (2001).
32. L. A. Pile, D. A. Wassarman, Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. *EMBO J.* **19**, 6131–6140 (2000).
33. M. Hussain, K. Etebari, S. Asgari, Functions of small RNAs in mosquitoes. *Adv. Insect Physiol.* **51**, 189–222 (2016).
34. 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).
35. X. Jin *et al*., 20-Hydroxyecdysone-responsive microRNAs of insects. *RNA Biol.* **17**, 1454–1471 (2020).
36. H. Jin, V. N. Kim, S. Hyun, Conserved microRNA miR-8 controls body size in response to steroid signaling in *Drosophila*. *Genes Dev.* **26**, 1427–1432 (2012).
37. T. Heinzel *et al*., A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43–48 (1997).
38. L. Nagy *et al*., Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373–380 (1997).
39. D. P. Edwards, The role of coactivators and corepressors in the biology and mechanism of action of steroid hormone receptors. *J. Mammary Gland Biol. Neoplasia* **5**, 307–324 (2000).
40. H. Li, C. Leo, D. J. Schroen, J. D. Chen, Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. *Mol. Endocrinol.* **11**, 2025–2037 (1997).
41. S. F. Wang *et al*., Differential expression and regulation by 20-hydroxyecdysone of mosquito ultraspiracle isoforms. *Dev. Biol.* **218**, 99–113 (2000).
42. J. Zhu, K. Miura, L. Chen, A. S. Raikhel, AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO J.* **19**, 253–262 (2000).
43. I. Pérez-Arellano, F. Carmona-Álvarez, A. I. Martínez, J. Rodríguez-Díaz, J. Cervera, Pyrroline-5-carboxylate synthase and proline biosynthesis: From osmotolerance to rare metabolic disease. *Protein Sci.* **19**, 372–382 (2010).
44. G. Zhou *et al*., Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. *J. Insect Physiol.* **50**, 337–349 (2004).
45. P. Y. Scaraffia, J. Isoe, A. Murillo, M. A. Wells, Ammonia metabolism in *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **35**, 491–503 (2005).
46. D. A. Goldstrohm, J. E. Pennington, M. A. Wells, The role of hemolymph proline as a nitrogen sink during blood meal digestion by the mosquito *Aedes aegypti*. *J. Insect Physiol.* **49**, 115–121 (2003).