Supplemental Results

The molecular mechanism regulating E93 gene expression by the JH pathway in adult female mosquitoes. To elucidate the role of Hairy in the control of *E93* gene expression, the 1.9-kb promoter of *E93* gene was PCR-amplified and subcloned into the pGL4.17 reporter vector. 300 ng of desired reporter plasmids and 20 ng of the *Renilla* control luciferase reporter in the pCopia vector were co-transfected into the S2 cells along with 200 ng each of the expression plasmids Hairy-Flag and Groucho1-V5 (Gro1-V5), which were previously cloned (1). The empty vector pAc5.1 was added to normalize the concentration of transfected plasmids in each well. Transfection of *Drosophila* S2 cells was performed using FuGENE® HD kit (Promega) following the manufacturer's instructions. This analysis has confirmed that Hairy is involved in the *E93* gene repression.

To elucidate Kr-h1 interaction with the *E93* gene, we analyzed the *E93* upstream regulatory region (nt -1900 to -1). A putative Kr-h1 binding element (KBS: GAGGTCA) is present in this region. Subsequently, we performed the ChIP-qPCR analysis (Fig. 2*E* and 2*F*). For each replicate, FBs were collected from 50 adult female mosquitoes at 72 h PE. The reaction was performed in triplicate. Immunoprecipitation was carried out using the Kr-h1 antibody. Nonspecific IgG was used as a negative control.

Molecular mechanism of E93 gene regulation by the 20E pathway in adult female mosquitoes. In the CHX applied groups, FBs collected from female mosquitoes at 96 h PE were pretreated with complete medium containing 10 μM CHX for 30 min and then incubated either with or without 10 μM 20E for additional 10 h. FBs in the medium containing only 20E or control (ethanol) were incubated for 10 h directly. Transcript levels of *E93* were highly induced after incubation with 20E, suggesting direct control of the *E93* gene by 20E.

To investigate the interaction of EcR and E93, we first analyzed the *E93* upstream regulatory region (nt -1900 to -1). A putative EcR binding element (EcRE: ATAGTTGTTGAACTT) is present in this region. Thus, we subcloned the cDNA sequences of *EcR* (nt 1-2022) and its partner *USP* (nt 1-1377) to pAc5.1b/V5 vector to express EcR-V5 and USP-V5 fusion proteins in *Drosophila* S2 cells. The promoter sequence of *E93* in the recombinant luciferase vector ($E93_{1.9kb}$-Luc) in this experiment was the same as used in Fig. 2*B*. Subsequently, we co-transfected pAc5.1b/EcR-V5 and pAc5.1b/USP-V5 along with $E93_{1.9kb}$-Luc into S2 cells with or without 2 μM 20E to determine the activation of *E93* by the 20E/EcR signaling. Cells transfected with empty vector pAc5.1b and $E93_{1.9kb}$-Luc plasmid served as the controls. Notably,

the luciferase activity significantly increased in the presence of 20E (Fig. S4*D*), confirming direct activation of the *E93* gene by the EcR/USP complex.

E93 regulates autophagy in female Aedes mosquitoes. Given the indicative function of ATG8 during autophagy, the regulatory mechanism of E93 on the *ATG8* expression was explored. The *ATG8* promoter region (nt -1861- -56) was subcloned into the luciferase vector pGL4.10 and the *E93* cDNA sequence (nt 1477 - 4608) was subcloned into the pAc5.1b/V5 vector to produce a fusion protein E93-V5. pGL4.73 vector carrying *Renilla* luciferase served as a control. A dual luciferase reporter assay was then carried out after co-transfection with pAc5.1b or pAc5.1b/E93 along with the pGL4.10-*ATG8* (ATG8-Luc) reporter plasmid into *Drosophila* S2 cells for 48 h. Cells without transfection or transfected with an empty vector pAc5.1b and ATG8-Luc plasmid served as the controls. After overexpressing E93-V5 fusion protein in S2 cells, the luciferase activity was significantly higher than that in control groups (Fig. 7*D*). This assay demonstrates the activation of *ATG8* gene by E93.

Materials and Methods

Mosquito Rearing. The *Aedes aegypti* mosquitoes were cultured at 27 ℃ with 80% humidity, as described previously (2-4). The use of all the vertebrate animals was approved by the Animal Care and Use Committees of either the Chinese Academy of Sciences, Institute of Zoology (IOZ20190062) or the University of California, Riverside.

***In Vitro* FB Culture.** *In vitro* FB culture assays were conducted as previously described (1, 5). Briefly, the FBs with adhered abdominal walls were dissected from female mosquitoes within 1 h PE (first cycle) or 3 h post FPBM (second cycle). These were cultured in insect complete medium with Methoprene (20 nM) or with ethanol (as control). For CHX treatments, 20 μM CHX was added to the culture medium with or without Methoprene. All incubations lasted for 8 h, after which, samples were collected for qPCR analysis. In the experiments of 20E addition, FB tissues collected at 96 h PE (first cycle) or 7 d post FPBM (second cycle) were incubated in the complete culture medium supplemented with 20E (10 μM) or with ethanol (as control) for 10 h. For CHX treatments, FBs were pretreated with culture medium containing 10 μM CHX for 30 min, then incubated either with or without 20E for an additional 10 h. After incubation, samples were collected for further qPCR analysis. The experiments described above were performed in triplicate under the same conditions.

RNAi Assays. dsRNAs prepared according to the instructions of the T7 RiboMAX Express RNAi system (Promega) were injected into ice-anesthetized female mosquitoes within 24 h PE using a Nanoliter 2000 injector (World Precision Instruments). RNAi FB samples of *Met, Kr-h1, Hairy and (Kr-h1+Hairy)* were collected 3-4 d after injection, while samples of *EcR, E93* RNAi were fed with blood 3 d post injection. Then FBs of EcR were collected at 24 h FPBM, and FBs of E93 were collected at 18 h, 24 h, 36 h or 44 h FPBM. For the study of second gonadotrophic cycle, the remaining iE93 mosquitoes from the same batches of injection were given a second blood meal after laying their eggs (mosquitoes received only one dsRNA injection before the first blood meal). FBs used for the second gonadotrophic cycle experiments were obtained at 18 h, 24 h, 36 h or 44 h SPBM. Bacteria *luciferase* gene RNAi (iLuc) was served as the control in *Kr-h1* and *Hairy* microinjection assays. And the coding region of the *green fluorescent protein* (*GFP*) gene was used to generate the control dsRNA (dsGFP) in the remaining RNAi assays. Primers used in dsRNA synthesis and RNAi efficiency tests are shown in Table S2.

RNA Extraction and qPCR. FB tissues derived from 10 – 15 female mosquitoes were homogenized using Tissuelyser-24 machine and then fully lysed using TRIzol reagent (Invitrogen). Total RNA was extracted following the method presented previously (2). cDNA was prepared using the FastQuant RT Kit (Tiangen). qPCR experiments were performed using SYBR Green SuperReal PreMix (Tiangen) in quadruplicate on the applied biosystems qPCR machine (Thermo Fisher Scientific). Endogenous reference ribosomal protein S7 (*rps7*) was used to normalize different templates. Primers for qPCR are shown in Table S2.

Cell Culture and Transfection. *Drosophila* S2 cells were cultured in Schneider's *Drosophila* Medium (Gibco) with 10% fetal calf serum (Gibco) and kept at 27 ℃. FuGENE® HD kit (Promega) and Lipofectamine™ 3000 (Invitrogen) were used in the cell transfection assay.

Luciferase Reporter Assay. Luciferase reporter assays were performed according to our previous paper (2). Briefly, promoters of *E93* and *ATG8* were amplified from *A. aegypti* genome and subcloned into the pGL4.10 reporter vector. The pGL4.73 vector carried *Renilla* luciferase acted as a control. Coding regions of *E93*, *EcR* and *USP* were subcloned to the pAc5.1b expression vector and overexpressed as fusion proteins in S2 cells. Dual-luciferase reporter assay system (Promega) and a GloMax 96 Microplate Luminometer (Promega) were used to determine the luciferase activity after each assay. In the luciferase assay for the repression of *E93* by Hairy, the *E93* promoter was subcloned into the pGL4.17 reporter vector. The *Renilla* luciferase cloned into the *pCopia* reporter vector served as control. Hairy-Flag and Groucho1-V5 plasmids have been cloned previously (1).

Western Blotting. 3 d post-injection, *E93* RNAi-depleted female mosquitoes were given a blood meal and then dissected to collect FBs at 24 h FPBM or 36 h FPBM for the western blot analysis. *GFP* knock down mosquitoes acted as a control. For the cells overexpression detection, corresponding plasmids were transfected into S2 cells and harvested after transfection for 48 h. Proteins were extracted using RIPA lysis buffer with 1 × protease inhibitors.

ChIP Quantitative PCR (ChIP-qPCR). ChIP assays were performed using Magna ChIP™ G Tissue Kit (Sigma-Aldrich) following the manufacturer's instructions. In brief, FBs with adhered abdominal walls were ground in liquid nitrogen and homogenized in ice-cold stabilizing buffer. Formaldehyde at 1% final concentration was added to the buffer and incubated for 10 min at room temperature. Crosslinking was stopped by adding glycine, and chromatin was sheared by sonication with Covaris Ultrasonicator. Immunoprecipitation was conducted using either Kr-h1 or EcR antibody. Nonspecific IgG was used as the negative control. DNA was purified using a QIAquick Spin PCR purification Kit (Qiagen, CA). At last, DNA precipitated from the above protocol was used to analyze by qPCR, using the primers listed in Table S2.

EMSA analysis. FBs with adhered abdominal walls were dissected from female mosquitoes at 72 h PE (for Kr-h1 analysis) and 36 h PBM (for EcR analysis). Nuclear protein extracts from FBs were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific). The biotin labelled, unlabeled and mutant *E93* probes were synthesized and purified by BGI. EMSA was carried out according to the instructions of the Pierce LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

Transcriptomic analysis. FB tissues dissected at 24 h or 36 h FPBM were used to isolate the total RNA. After mRNA enriching and inverse transcription, pair-ended cDNA libraries were sequenced using Illumina platform. Raw data obtained from the sequencing were preprocessed to generate clean data through removing adaptor sequences and reads of low quality. Subsequently, the clean data were used to perform bioinformatic analysis. HISAT2 (6) was employed to align reads to the corresponding transcripts, and StringTie (7) was used to assemble and quantify the mapped reads. Transcript levels were estimated by Fragments Per Kilobase of transcript per Million fragments mapped (FPKM). DESeq2 (8) was used for differential expression analysis between groups, and genes were considered as statistically significant with FC > 2 and FDR < 0.05. The hierarchical clustering analysis was plotted using pheatmap packages in R environment. Venn diagram was calculated at website http://bioinformatics.psb.ugent.be/webtools/Venn/. KOBAS software (9) was used to test the statistical enrichment of differential expression genes in KEGG pathways.

Immunofluorescence Assay and Imaging. 3 d after the *E93* RNAi knockdown, mosquitoes were given a blood meal. FBs dissected from 36 h FPBM were fixed in 4% paraformaldehyde for 30 min. Subsequently, they were immersed in 0.5% Triton X-100 to penetrate the cell membrane. After blocking in 3% BSA solution for 1 h at room temperature, rabbit anti-ATG8 antibody was used to stain FBs overnight at 4 ℃. Goat anti-rabbit secondary antibody Alexa Fluor® 594 (Invitrogen) was then applied to detect the ATG8 protein distribution in FBs. Nuclei were stained with Hoechst 33342 (Invitrogen). Results were visualized under a Zeiss LSM 710 confocal microscope.

Statistical Analysis. Except for transcriptome data, statistical significance analysis of the remaining data presented in this paper were calculated in GraphPad Prism 6.0 software. Student's t test was used to evaluate the significance between different samples and expressed as a *p* value less than 0.01 or 0.05. Data are shown as mean ± SEM.

Sources of antibodies. IgG used in the ChIP-qPCR assay was purchased from Merck Millipore; antibodies against V5 and GAPDH were purchased from Invitrogen and CoWin Biosciences, respectively; ATG8 and Vg antibodies used in the western blotting were the same as previously reported (10); and for the EMSA experiments, the antibody against Histone H3 was purchased from Easybio. The Kr-h1 antibody was purified at the Animal Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, and the EcR antibody was purified by GL Biochem.

References:

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7. M. Pertea *et al.*, StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol.* 33, 290-295 (2015).
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9. X. Mao, T. Cai, J. G. Olyarchuk, L. Wei, Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics.* 21, 3787-3793 (2005).
10. B. Bryant, A. S. Raikhel, Programmed autophagy in the fat body of *Aedes aegypti* is required to maintain egg maturation cycles. *PLoS. One.* 6, e25502 (2011).

Supplemental Figures

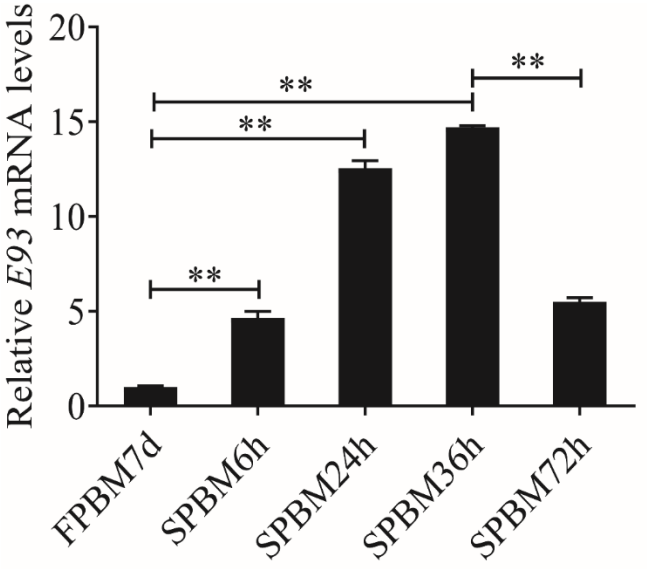

Fig. S1. The cyclic pattern of *E93* expression across the second gonadotrophic cycles. Time course of *E93* gene expression during the second cycle: including second post-blood meal (SPBM) period (6 h, 24 h, 36 h and 72 h). The expression of *E93* at FPBM 7 d was used as controls during the second cycle. Data are shown as Mean ± SEM. ** $p < 0.01$.

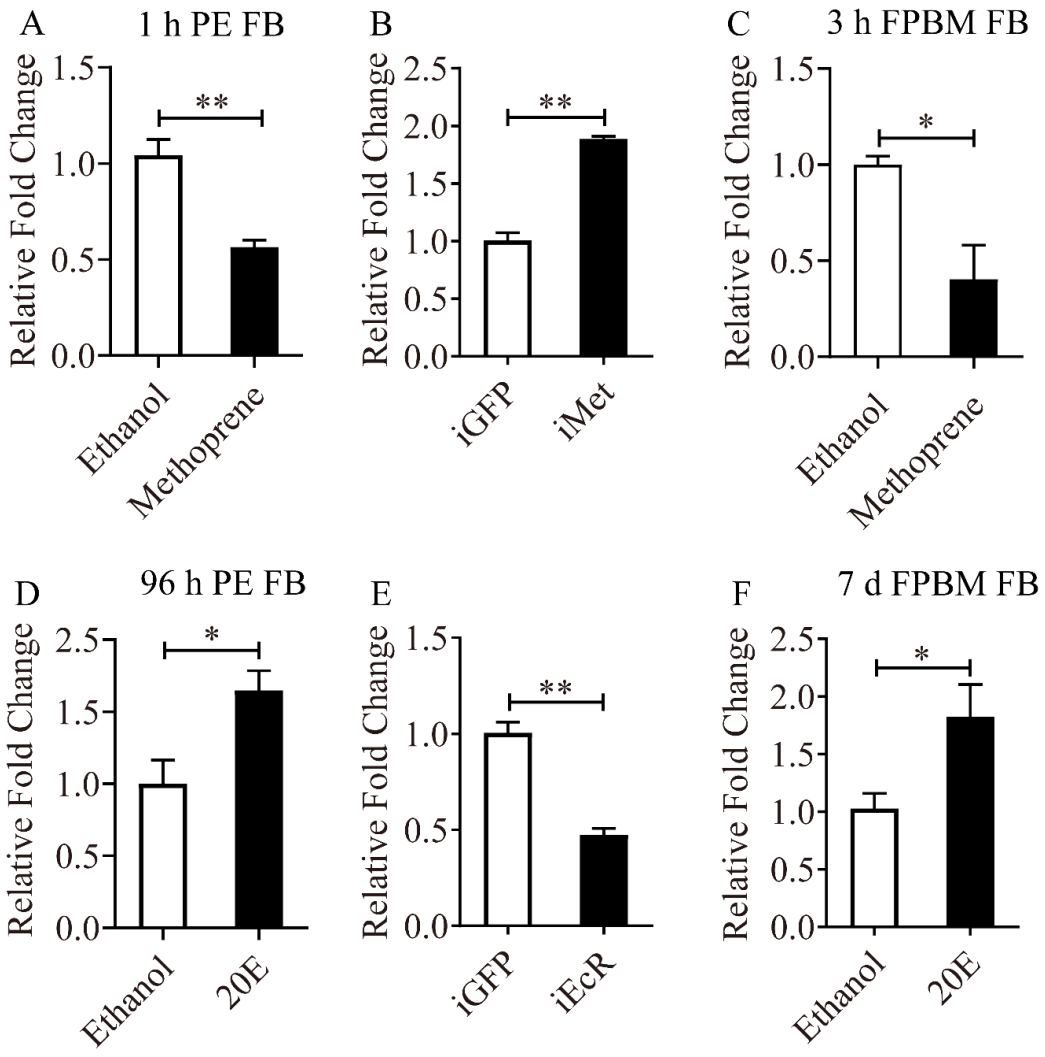

Fig. S2. Effects of JH, 20E and their receptors, Met and EcR, on *E93* gene expression. (A) An *in vitro* FB culture assay showing Methoprene repressive effect on *E93* gene expression during the first gonadotrophic cycle. (B) RNAi showing the action of JH receptor Met on *E93* gene expression. (C) *In vitro* FB culture assay showing Methoprene's effect on *E93* gene expression during the second gonadotrophic cycle. (D) *In vitro* FB culture assay showing the effect of 20E on *E93* gene expression during the first gonadotrophic cycle. (E) RNAi showing the action of EcR on *E93* gene expression. (F) *In vitro* FB culture assay showing the effect of 20E on *E93* gene expression during the second gonadotrophic cycle. The collecting time of FBs for culture assay are shown at the top of the corresponding graphs. Data are shown as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

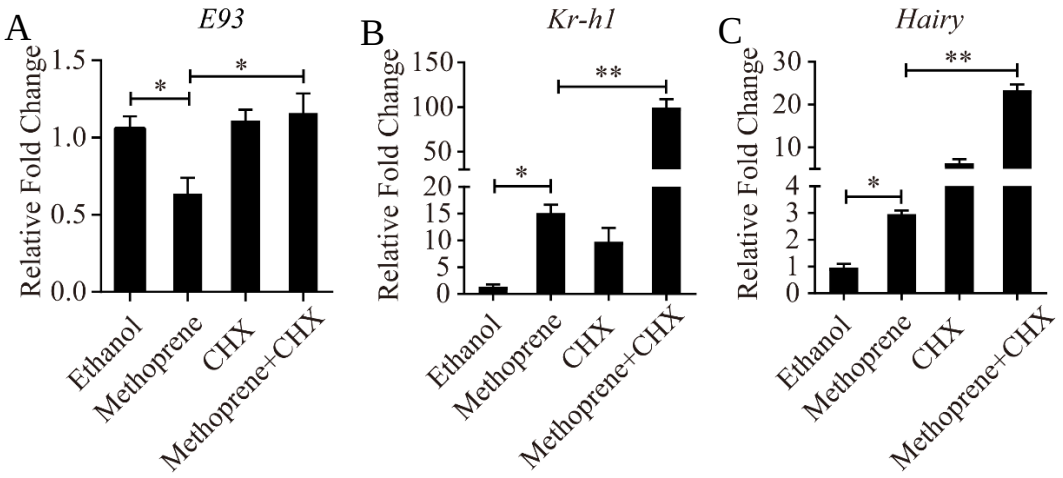

Fig. S3. The CHX effect on JH-mediated gene expression. (A) *In vitro* FB culture assays showing the effect of CHX on the Methoprene-mediated expression of the *E93* gene. The repressive effect of Methoprene on *E93* gene expression was compromised by the addition of CHX to the Methoprene-containing medium, as detected using qPCR. FB samples incubated with either ethanol (solvent) or CHX alone were used as controls. CHX was ineffective in preventing the activating effect of Methoprene on (B) *Kr-h1* and (C) *Hairy*. Medium containing ethanol and CHX only (CHX) served as controls. Data are shown as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

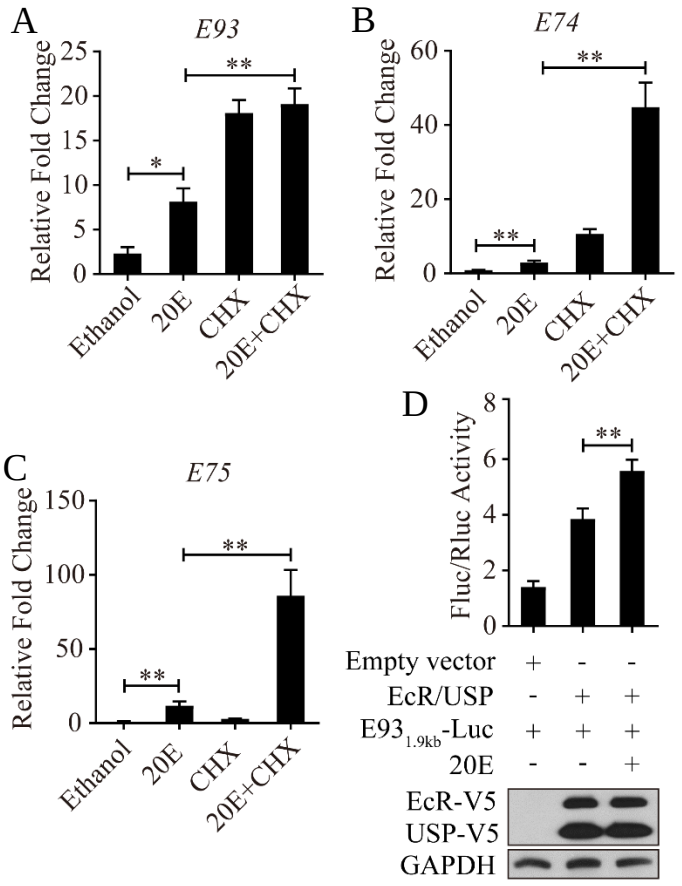

Fig. S4 *E93* is the 20E-regulated primary response gene. (A) *In vitro* FB culture assays showing the effect of CHX on the 20E-mediated expression of the *E93* gene. CHX was ineffective in preventing the activating effect of 20E on the *E93* gene. Medium containing ethanol or CHX only (CHX) were used as controls. CHX effect on the 20E-mediated gene expression of (B) *E74* and (C) *E75*. CHX was ineffective in preventing the 20E activating effect on *E74* and *E75*. Medium containing ethanol (solvent) or CHX only (CHX) served as controls. (C) Dual luciferase reporter assay performed after co-transfection of pAc5.1b/EcR-V5 plus pAc5.1b/USP-V5 with the reporter vector containing the *E93* promoter sequence (nt -1900 to -1) ($E93_{1.9kb}$-Luc). Cells transfected with the pAc5.1b empty vector were used as controls. The *Renilla* luciferase vector pGL4.73 served as an expression control. The western blot showed the protein levels of EcR-V5 and USP-V5 fusion proteins after different transfection in S2 cells for 48 h using anti-V5 monoclonal antibody. GAPDH antibody was used as a loading control. Data are shown as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

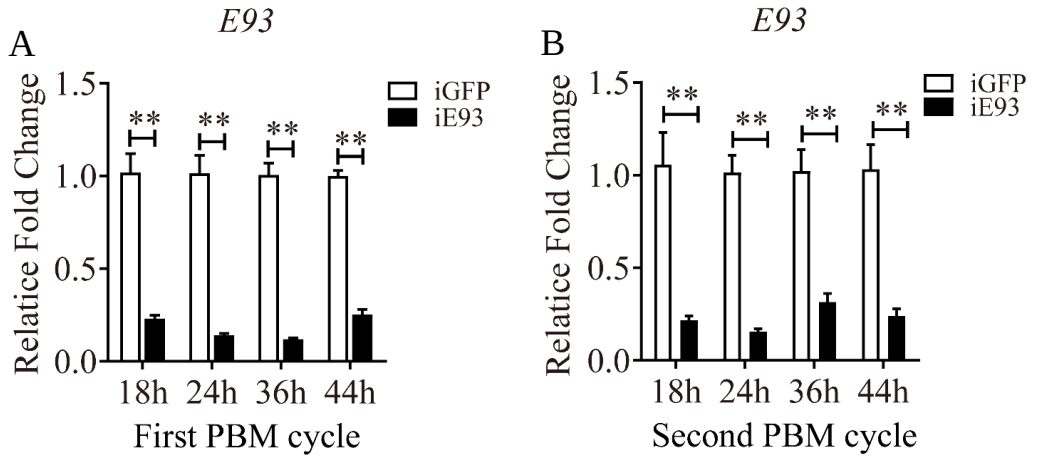

Fig. S5. *E93* transcript levels after *E93* RNAi during the first and second reproductive cycles. (A) Female mosquitoes injected with dsE93 or dsGFP within 24 h PE were given the first blood meal 3 d post injection, and the *E93* transcript levels were examined at 18 h, 24 h, 36 h and 44 h FPBM. (B) Another mosquito batch also injected at 24 h PE was given the second blood meal after laying eggs, and the *E93* transcript levels were examined at 18 h, 24 h, 36 h and 44 h SPBM. *E93* transcript levels were quantified using qPCR. Each sample was normalized to its internal control *rps7* mRNA. All the data in iE93 mosquitoes were normalized to that in iGFP mosquitoes, which were represented as 1. Data are shown as Mean ± SEM. ** $p < 0.01$.

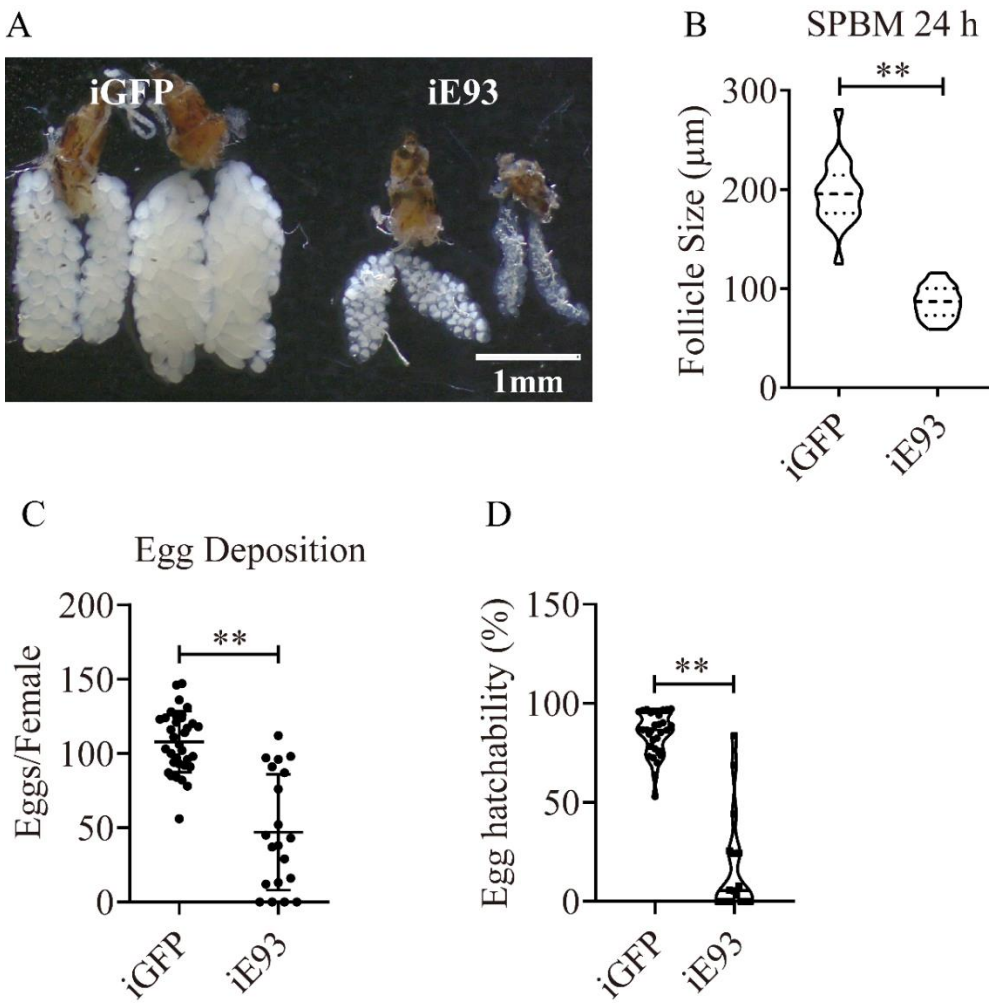

Fig. S6. *E93* RNAi negatively affects the development and maturation of mosquito ovaries during the second gonadotrophic cycle. Female mosquitoes were injected with dsE93 or dsGFP within 24 h PE and ovarian development was examined 24 h SPBM. (A) The ovary phenotype of iGFP and iE93 mosquitoes were shown. Images were captured under a 5-megapixel, high-definition CMOS camera built in to a Leica EZ4W stereoscopic microscope. (B) The follicle length (24 h SPBM) of iGFP and iE93 mosquitoes was measured in ImageJ software. Egg deposition (C) and Egg hatchability (D) in iE93 were compared with that in iGFP mosquitoes. Data shown in B, C and D were analyzed by the Graphpad Prism 6 and shown as mean ± SEM. ** $p < 0.01$.

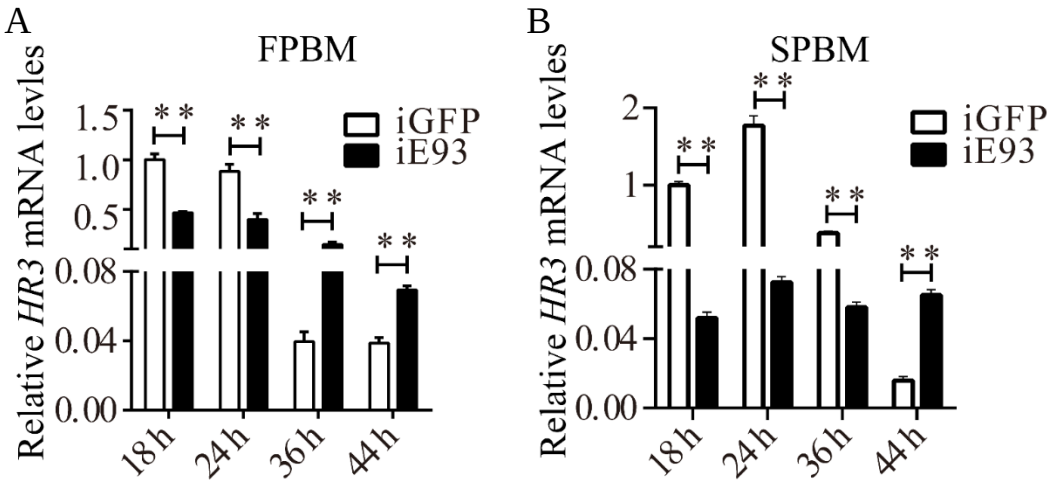

Fig. S7. Effect of *E93* depletion on the *HR3* mRNA abundance during the first and second gonadotrophic cycles of female *A. aegypti* mosquitoes. (A) Female mosquitoes, injected with dsRNA of *E93* or *GFP* within 24 h PE, were given the first blood meal 3 d post-injection. The *HR3* transcript levels were examined at 18 h, 24 h, 36 h, and 44 h FPBM. (B) Another mosquito batches were given a second blood meal after laying eggs, and the *HR3* transcript levels were examined at 18 h, 24 h, 36 h, and 44 h SPBM. Transcript levels of *HR3* were quantified using qPCR. Each sample was normalized to its internal control *rps7*. All the data in iE93 mosquitoes were normalized to that in iGFP mosquitoes, which were represented as 1. Data are shown as Mean ± SEM. ** $p < 0.01$.

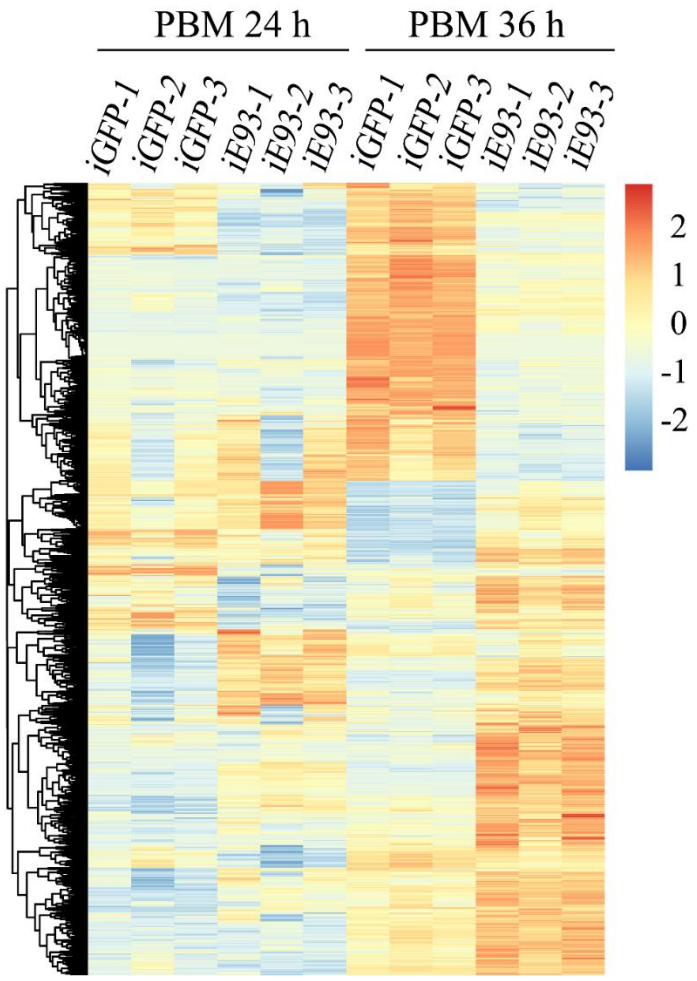

Fig. S8. Hierarchical clustering analysis of the DEGs after *E93* RNAi.

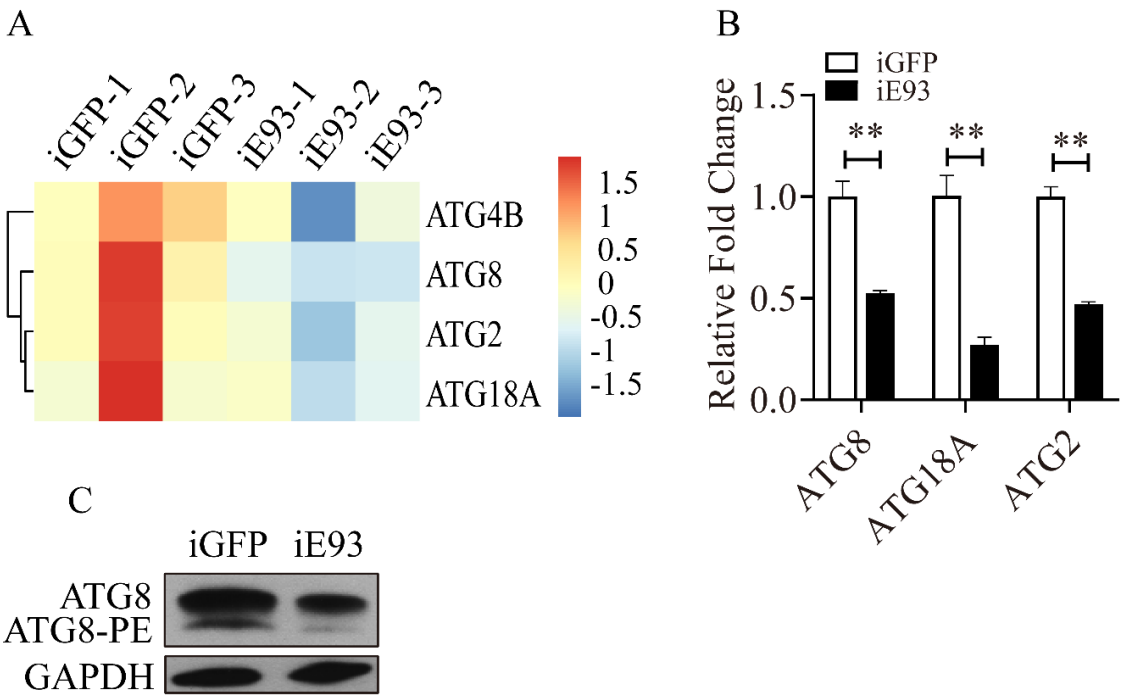

Fig. S9. Autophagy of mosquitoes is affected by E93 at 24 h FPBM. (A) Hierarchical clustering analysis of autophagy-related genes. (B) The qPCR analysis of the mRNA levels of autophagy-related genes in *E93* RNAi mosquitoes. (C) The protein levels of the autophagy protein 8 (ATG8) in iE93 and control mosquitoes. ATG8-PE represents the lipidated ATG8. PE is phosphatidylethanolamine. GAPDH was used as the loading control. Data are shown as Mean ± SEM. ** $p < 0.01$.

Table. S1. Differentially expressed genes after *E93* RNAi.

Table. S2. Primers used in this study.