Juvenile hormone (JH) controls many biological activities in insects, including development, metamorphosis, and reproduction. In the *Aedes aegypti* mosquito, a vector of dengue, yellow fever, chikungunya, and zika viruses, the metabolic tissue (the fat body, which is an analogue of the vertebrate liver) produces yolk proteins for developing oocytes. JH is important for the fat body to acquire competence for yolk protein production. However, the molecular mechanisms of how JH promotes mosquito reproduction are not completely understood. In this study we show that stimulation of the JH receptor methoprene-tolerant (Met) activates expression of genes encoding the regulator of ribosome synthesis 1 (RRS1) and six ribosomal proteins (two ribosomal large subunit proteins, two ribosomal small subunit proteins, and two mitochondrial ribosomal proteins). Moreover, RNAi-mediated depletion of *RRS1* decreased biosynthesis of the ribosomal protein L32 (Rpl32). Depletion of *Met, RRS1*, or *Rpl32* led to retardation of ovarian growth and reduced mosquito fecundity, which may at least in part have resulted from decreased vitellogenin protein production in the fat body. In summary, our results indicate that JH is critical for inducing the expression of ribosomal protein genes and demonstrate that *RRS1* mediates the JH signal to enhance both ribosomal biogenesis and vitellogenesis.

As a vector of dengue, yellow fever, chikungunya, and zika viruses, the *Aedes aegypti* mosquito causes severe morbidity and increased economic costs (1–4). Due to the lack of effective vaccines, controlling these dangerous vector-borne diseases remains a big challenge. Female hematophagous mosquitoes rely on blood intake to activate vitellogenesis, which involves the production of vitellogenin (Vg) the major yolk protein precursor (YPP) in the fat body, and is controlled by ecdysteroid 20-hydroxyecdysone (20E) (5, 6). A sesquiterpenoid juvenile hormone (JH) plays an essential role in stimulating mosquito reproduction during the previtellogenic preparatory period (7). Unraveling the molecular mechanisms regarding hormone control of reproduction will aid in development of novel strategies for prevention of vector-borne diseases.

JH, as an endocrine regulator, plays crucial roles in controlling insect development, metamorphosis, and reproduction. JH hemolymph level increases over the first 2 days posteclosion (PE), reaches its peak at 48–54 h PE, and maintains a high level in female *A. aegypti* before a blood meal (8). JH-dependent PE development is vital for a female fat body to become competent for the production of massive YPPs. The process is always associated with dramatic enlargement of nucleoli, development of Golgi complexes, and ribosome proliferation (9, 10). Removal of the corpora allata (CA; the source of JH) in newly emerged female mosquitoes blocks activation of fat body nucleoli for ribosomal and ribosomal RNA production, whereas re-implantation of CA or topical application of JH rescues the phenotypes (10, 11). This indicates that JH controls ribosome proliferation during PE development. However, the molecular mechanisms underlying JH regulation of ribosome proliferation are not well understood.

JH exerts its genomic function through the receptor methoprene-tolerant (Met), a member of the family of the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors (12, 13). A transcriptional steroid receptor coactivator (FISC/SRC/Taiman (Tai)) has been identified in the silkworm as a functional partner of Met (14–16). JH induces
the heterodimerization of Met with its partner FISC/SRC/Tai to bind E-box-like motifs in the regulatory regions of JH-target genes and activate the transcriptions (13–17).

The microarray screen combined with Met RNA interference (RNAi) has shown Met repression of early PE (at 6 h PE) and mid PE (at 24 h PE) genes but activation of late PE (at 66 h PE) genes (18). Met involvement in gene repression mediated by Hairy and a corepressor Groucho has been recently clarified (19). In the study, Saha et al. (19) performed a RNAi-based transcriptomic screen to identify genes co-regulated by Met and Hairy in the fat body. As a result, 1613 transcripts were shown to be activated, whereas 538 were suppressed after RNAi depletion of Met (iMet). In the current study we have shown that among 538 iMet-suppressed transcripts, many are those involved in ribosome biogenesis. One of them is a gene termed RRS1 (regulator of ribosome synthesis 1, AAEL012185) that is the homolog protein of RRS1p identified in Saccharomyces cerevisiae that regulates ribosome synthesis. RRS1p, as an essential nuclear protein, is required for maturation of 25S rRNA and functions in assembly of the 60S ribosomal subunits (20, 21). RRS1p, along with Rpf2p, recruits ribosomal proteins RpL5 and RpL11 as well as 5S rRNA into preribosomes (22). However, studies of RRS1 in insects are limited. Here, we have demonstrated that A. aegypti RRS1 and six ribosomal protein genes are activated by Met, and Met directly regulates the transcription of RRS1. RNAi-mediated depletion of RRS1 resulted in a decreased level of ribosomal protein large subunit 32 (RpL32) protein. Met, RRS1, or RpL32 depletion caused retardation of ovarian growth, which may at least partly result from a decreased Vg expression in the fat body. Our study provides new insight into JH-dependent ribosome proliferation and vitellogenesis.

**Results**

**Met RNAi depletion suppressed the expression of genes involved in ribosome biogenesis**

We previously identified 2151 differentially expressed transcripts controlled by Met in the fat body of female A. aegypti mosquitoes at the end of the PE phase, with 1613 transcripts down-regulated and 538 up-regulated (19). Among the latter, a total of 91 transcripts (17%) are involved in ribosome biogenesis; of these, 47 encode ribosomal protein large subunits, 30 encode ribosomal protein small subunits, and 14 encode ribosome biogenesis-related factors (Fig. 1A and supplemental Table S1). From these 14 genes, we selected the gene AAEL012185 encoding regulator of ribosome synthesis 1 (RRS1), which has been shown to regulate ribosome biosynthesis in S. cerevisiae (20).

To validate the RNA-seq results, RRS1 along with two genes encoding ribosomal protein large subunits (AAEL003396, AAEL003324) and two genes encoding ribosomal protein small subunits (AAEL010168, AAEL014903) were selected for quantitative real-time PCR (qRT-PCR) analysis. As shown in Fig. 1, B–E and H, the qRT-PCR results confirm the RNA-seq experiments, with all tested genes down-regulated after Met RNAi depletion (iMet). We also measured the expression of two mitochondrial ribosomal protein genes (AET-4427, AET-7465), and the results indicate that they are suppressed in iMet (Fig. 1, F and G). Efficiency of Met RNAi depletion was also confirmed (Fig. 1I).
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Figure 2. Developmental profiles of six ribosomal protein genes and RRS1 were analyzed in the fat bodies of adult female A. aegypti using qRT-PCR. The color indicates the -fold change of the gene abundance in the form of a logarithm. The dendrograms are constructed based on an algorithm of hierarchical clustering.

Expression profiles of genes involved in ribosome biogenesis in the fat body correlated with the endogenous JH titers of adult female mosquitoes during the PE phase

JH secretion occurs soon after adult emergence. JH titers increase to their peak at 48–54 h PE and remain high until blood feeding (8). To clarify the relationship between the expression of genes involved in ribosome biogenesis and endogenous JH concentration, we measured the expression profiles of these genes at different developmental stages. Transcripts of all genes involved in ribosome biogenesis are closely correlated with the changes of endogenous JH titers. As shown in Fig. 2, expression levels of all ribosomal protein genes increased along with PE development, reached their highest level at 72 h PE or 12 h post blood meal (PBM), and declined to a low level, then began to increase again. RRS1 transcript exhibited its highest level at 48 h PE and remained at the same high level at 60 h PE, decreasing by 72 h PE. The active transcription of RRS1 was concomitant with the peak of endogenous JH, suggesting that RRS1 responds to JH immediately.

RRS1 is transcriptionally regulated by the JH-receptor complex

To understand whether the action of Met on genes involved in ribosome biogenesis is direct or mediated by an intermediate factor, we employed in vitro fat body culture analyses and used the protein biosynthesis inhibitor cycloheximide (CHX). The expression levels of six ribosomal protein genes were considerably elevated by JH, and CHX inhibited JH-mediated induction of ribosomal protein genes (Fig. 3, A–F), suggesting that JH induced the expression of these ribosomal protein genes indirectly. However, CHX could not inhibit JH-mediated induction of RRS1 (Fig. 3G), suggesting that JH induced the expression of RRS1 directly. Krüppel homolog 1 (Kr-h1, AAE002390), which has previously been identified as a direct target of Met (23), served as a control; its expression, which was activated by JH, was not inhibited by CHX (Fig. 3H).

To further determine whether Met directly regulates RRS1 transcription, we conducted luciferase reporter assays. Mosquito bHLH-PAS transcription factors Met and FISC form a heterodimer in the presence of JH or JH analogs to act on JH response element (JHRE) and directly activate transcription of JH target genes (14). Here, the full lengths of Met (amino acid 1–977) and FISC (amino acid 1–1488) were expressed in S2 cells, with a C-terminal Myc tag or FLAG tag, respectively (Fig. 4A). Because E-box or E-box-like are characteristic motifs recognized by bHLH-PAS transcription factors (24), we cloned the upstream regulatory sequences of RRS1, which included an E-box-like motif (CACCGG) into the pGL4.17 vector. To diminish the possible influence of endogenous Met, Gce (germ cell-expressed), and Tai in S2 cells, Drosophila Met (DmMet) and Tai (DmTai) double-stranded RNAs (dsRNAs) were added to the transfection mixture. After transfection, the expression of Met or FISC or Met plus FISC was confirmed by Western blot using anti-Myc or anti-FLAG antibodies (Fig. 4B). Treatment with DmMet and DmTai dsRNAs led to a significant reduction of endogenous Met, Gce (the paralog of Met, the dsRNA sequences targeting DmMet sharing 44% identity with that of DmGce), and Tai in S2 cells (Fig. 4C). However, the transcripts of transfected Met-Myc or FISC-FLAG were not influenced (Fig. 4D). In endogenous Met-, Gce-, and Tai-suppressed S2 cells, JH did not induce RRS1 reporter activity in S2 cells. No protein expression or expression of either Met or FISC alone in S2 cells could not induce RRS1 reporter activity. Co-expression of Met and FISC could not induce reporter activity either. However, after the addition of JH III or JH analog methoprene, co-expression of Met and FISC significantly induced reporter activity (Fig. 4B), which indicates that the JH-Met-FISC complex is required for RRS1 transcription.

To determine whether the JH-receptor complex physically interacts with the RRS1 promoter, we performed electrophoretic mobility shift assay (EMSA) using nuclear extracts from S2 cells overexpressing both Met-Myc and FISC-FLAG fusion proteins followed by JH III treatment. As shown in Fig. 4E, a band shift was detected in samples incubated with the labeled RRS1 probe; this was abolished after preincubation with unlabeled RRS1 probe, suggesting that the protein-DNA interaction is specific. The band was diminished when nuclear extracts were preincubated with anti-Myc or anti-FLAG antibody, suggesting that Met-Myc and FISC-FLAG constitute the binding complex. The band shift disappeared after mutation of E-box-like motif (Fig. 4F), suggesting this motif is crucial for the interaction.

RRS1 depletion reduced Rpl32 protein but not transcript levels in the fat body of adult female mosquitoes

To elucidate the relationship between RRS1 and ribosomal proteins, we performed RNAi-mediated depletion analyses. When compared with the control groups, newly emerged female mosquitoes (0–6 h PE) treated with RRS1 dsRNA (iRRS1) displayed an efficient depletion of the RRS1 transcript in the fat body at 72 h PE (Fig. 5B). We selected Rpl32 to test. The abundance of the Rpl32 transcript showed no significant difference between iRRS1 and the control groups (Fig. 5A). However, depletion of RRS1 resulted in decreased Rpl32 protein in fat bodies at both 72-h PE and 24-h PBM (Fig. 5C), which suggests that Rpl32 is regulated by RRS1.

RRS1 or Rpl32 depletion resulted in abnormal ovarian development that mimics the phenotype of ovaries caused by depletion of Met

To assess the function of RRS1 and Rpl32 in the adult female mosquito, we conducted RNAi-mediated depletion of RRS1 (iRRS1) or Rpl32 (iRpl32) in newly emerged female mosqui-
toes and checked the ovaries dissected at both 72 h PE and 24 h PBM. We also performed RNAi depletion of Met (iMet) as a control, as it has been reported that iMet results in retardation of ovarian growth at 72 h PE (18, 19). Analysis of ovaries from non-injected (NI) female mosquitoes at 72 h PE and those injected with luciferase dsRNA (iLuc) demonstrated average primary follicle lengths of 105 μm and 106 μm, respectively. However, similar to previously shown iMet retardation of ovarian follicle growth (with an average primary follicle length of 67 μm), iRRS1 and iRpL32 were also shown to stall the growth, with average primary follicle lengths of 76 μm and 70 μm, respectively, which is much smaller than that of the controls (Fig. 6A).

In addition to 72 h PE, we evaluated the ovarian development at 24 h PBM. As shown in Fig. 6, B–D, ovaries from NI or iLuc at 24 h PBM developed normally, with follicle lengths reaching an
average of 214 μm and 211 μm, respectively. However, iMet, iRRS1, and iRpL32 significantly inhibited the ovarian development, with average primary follicle lengths of 103 μm, 132 μm, and 84 μm, respectively. In addition, mosquitoes of NI and iLuc laid on average 121 and 113 eggs per female, respectively. However, iMet, iRRS1 and iRpL32 dramatically reduced fecundity, resulting in significantly fewer eggs laid: an average of 14 eggs per female with iMet, 26 with iRRS1, and 4 with iRpL32 (Fig. 6E).

Met, RRS1, or RpL32 RNAi depletions resulted in the decreased level of Vg protein in the fat body of adult female mosquitoes

During mosquito vitellogenesis, Vg (the major YPP) is synthesized in and secreted from the fat body and is subsequently accumulated in developing oocytes (5). To check whether Met, RRS1, or RpL32 is involved in Vg synthesis and rule out the possible involvement of other tissues after blood feeding, we performed in vitro fat body culture analyses. Newly emerged female mosquitoes were injected with dsRNA, and fat bodies isolated at 72 h post injection (during this stage, the fat body is reproductively competent) were subjected to in vitro fat body culture. Depletion of Met or RpL32 was confirmed by means of Western blots (Fig. 7, E and F), whereas depletion of RRS1 was confirmed using qRT-PCR (Fig. 7G). Fat bodies from mosquitoes of iMet, iRRS1, or iRpL32 exhibited a much lower Vg protein level than those from mosquitoes of NI or iLuc (Fig. 7D).

Depletion of Met significantly suppressed the abundance of Vg transcripts (Fig. 7A). However, iRRS1 and iRpL32 did not result in significant differences of the Vg transcript level when compared with the controls (Fig. 7, B and C).

Met, RRS1, or RpL32 RNAi depletions resulted in a decreased level of polysomes in the fat body of adult female mosquitoes

To check whether the decreased level of Vg protein in the fat body of iMet, iRRS1, or iRpL32 is due to the decreased ribosomal biogenesis, we isolated polysomes using sucrose gradient sedimentation and determined the amount as the material absorbing at 254 nm. As shown in Fig. 8A, the amount of polysomes from fat bodies of iMet, iRRS1, or iRpL32 is much lower than that of the control group, suggesting the involvement of Met, RRS1, or RpL32 in ribosomal biogenesis.

Discussion

Vg synthesis can be induced by JH in many insects (25–27). In mosquitoes, the massive production of Vg only occurs after blood feeding and is primarily governed by 20E (28). However,
Depletion of Met or RRS1 results in decreased expression of RpL32 proteins in the female fat body. A, the transcripts of RpL32 exhibited no significant differences in RRS1-depleted (iRRS1) fat bodies of female mosquitoes at 72 h PE. B, qRT-PCR confirming the depletion of RRS1. NI, mosquitoes without injection; iLuc, dsRNA for the luciferase gene. All expression values are calculated against the housekeeping gene β-actin. Data are the mean ± S.E. from three biological replicates. **, p < 0.01 (t test). C, RNAi-mediated depletion of Met or RRS1 resulted in decreased expression of RpL32 in the female fat bodies at both 72 h PE and 24 h PBM. Shown are Western blot analyses using antibodies against RpL32 and β-actin (as control) in fat bodies from non-injected (NI), luciferase dsRNA-treated (iLuc), Met dsRNA-treated (iMet), or RRS1 dsRNA-treated (iRRS1) mosquitoes. Both NI and iLuc served as the controls.

Figure 6. Depletion of Met, RRS1, or RpL32 resulted in decreased follicle length and dramatically reduced egg number. A, primary ovarian follicle length (in μm) measured in iMet, iRRS1, iRpL32, iLuc, and non-injected (NI) mosquitoes at 72 h PE. B and C, female mosquito ovaries at 24 h PBM. D, average follicle length at 24 h PBM. E, egg numbers deposited by each female mosquito. NI, non-injected; iLuc, luciferase dsRNA-treated; iMet, Met dsRNA-treated; iRRS1, RRS1 dsRNA-treated; iRpL32, RpL32 dsRNA-treated mosquitoes. Error bars represent ± S.D. ***, p < 0.0001 (t test).

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tor) led to decreased ribosomal protein L34 (29, 30). Mutation of the RRS1 gene in yeast leads to a compromised 60S subunit production by affecting ribosome assembly and thus decreased polysomal content (20). A similar decrease in the level of polysomes was observed in RRS1-depleted A. aegypti in our study. We speculate that the absence of RRS1 leads to impaired ribosomal assembly in mosquitoes as well. The newly synthesized unassembled RpL32 protein is unstable and is rapidly degraded by the system (31), resulting in the observed decreased protein levels. However, this hypothesis needs to be tested in the future.

We would like to stress that we presume this to be the general mechanism by which RRS1 affects the level of the ribosomal proteins. Although providing additional immunoblots for other RpLs is beyond the scope of this manuscript, we furnish the results for RpL32 as a representative.

Drastically smaller primary ovarian follicles usually represent that YPP synthesis, secretion, and/or uptake are compromised. For example, smaller ovarian follicles have resulted from depletion of miR-8, which is responsible for the proper secretion of lipophorin and Vg by the fat body (35). Considering that RpL32 is a component of the translational apparatus, we speculate that the smaller primary ovarian follicles caused by depletion of Met, RRS1, or RpL32 may have resulted from the decreased YPP synthesis in fat bodies. Because RNAi depletion has a systemic effect on the target gene, we performed RNAi combined with in vitro fat body culture experiments to rule out the possible involvement of other tissues. The results demonstrated that depletion of Met, RRS1, or RpL32 dramatically reduced the expression of Vg proteins, which may at least partly account for the decreased primary follicles and reduced fecundity. Furthermore, depletion of Met significantly reduced the abundance of Vg transcripts, similar to the observation in the linden bug, where Met RNAi suppressed Vg gene expression in the fat body and blocked ovarian development (36). This result is reasonable as some factors identified downstream of JH-Met signaling pathway control Vg mRNA expression. For example, βFTZ-F1, which could be stimulated by JH in the fat body, is involved in regulating the expression of Vg transcripts as a competence factor (7). However, depletion of RRS1 or RpL32 led to insignificant differences in Vg transcripts, suggesting that RRS1 or RpL32 may only contribute to the translational apparatus and be responsible for Vg translation. The protein translation efficiency is associated with the ribosomes bound to form polysomes. Depletion of Met, RRS1, or RpL32 decreased the level of polysomes, indicating that these genes are involved in ribosomal biogenesis and further explaining why a decreased
expression of Vg protein was observed when depleting these genes.

In conclusion, we demonstrated that JH could regulate many ribosomal protein genes, and JH-receptor complex acts on the promoter of RRS1 to regulate its transcription. RRS1 transduces JH signal in mediating ribosomal biogenesis and vitellogenesis through regulation of the expression of RpL32 proteins.

**Experimental procedures**

**Mosquito rearing**

*A. aegypti* mosquitoes were maintained at 27 °C and 86% relative humidity, as described previously (19). Adult mosquitoes were fed water and a 10% sucrose solution continuously via wicks. Blood feeding was conducted using white Leghorn chickens following instructions approved by the Animal Care and Use Committee, University of California, Riverside, CA. All dissections were carried out in *Aedes* physiological solution (38).

**RNAi**

The dsRNAs of Met, RRS1, and RpL32 were synthesized using the MEGAscript kit (Ambion, Austin, TX) as described previously (38). The bacterial luciferase gene was used as a template to produce control iLuc dsRNA, and synthesized dsRNAs were purified using MEGAClear kit (Ambion). Subsequently, the corresponding dsRNA was quantified to 4 μg/μl, and 300-nl samples of dsRNA were microinjected into the thoraces of CO2-anesthetized newly emerged female mosquitoes (0–6 h PE) using Picospritzer II (General Valve Corp., Fairfield, NJ). Primers used for dsRNA synthesis are listed in supplemental Table S2.

**In vitro fat body culture**

Fat body culture analyses were performed according to a method described previously (38). To test the effects of JH (Sigma) or CHX (Sigma) on gene expression, fat bodies dissected from newly emerged female mosquitoes (0–6 h PE) were incubated in culture medium with 1 × 10⁻⁶ M JH III or solvent (acetone) or incubated in the medium with 1 × 10⁻⁷ M CHX combined with or without JH. After incubation for 12 h, samples were harvested for qRT-PCR analysis.

To analyze the expression of Vg resulting from RNAi depletion of Met, RRS1, or RpL32, fat bodies were dissected from female mosquitoes 72 h after dsRNA injection. Subsequently, fat bodies were incubated in a complete culture medium supplemented with 1 × 10⁻⁶ M 20E (Sigma) and amino acids. After incubation for 6 h, fat bodies were collected for qRT-PCR or Western blot analyses.

**RNAi in S2 cells and luciferase reporter assay**

The dsRNAs of *DmMet* and *DmTai* were synthesized using the MEGAscript kit (Ambion). The promoter region of *RRS1* (nucleotides −3389 to −2597) was synthesized (GenScript, Piscataway, NJ) and subcloned into reporter vector pGL4.17 (Promega, Madison, WI). *A. aegypti* Met (nucleotides 1–2931) and FISC (nucleotides 1–4464) DNA fragments were amplified and subcloned into a pAc5.1 vector (Invitrogen) fused with Myc and FLAG tag, respectively. The primers used for dsRNA synthesis and PCR amplification are listed in supplemental Table S2. All recombinant plasmids were confirmed by sequencing. Transient transfections were performed in *Drosophila* S2 cells using FuGENE HD transfection reagent (Promega) following the protocol. *DmMet* and *DmTai* dsRNAs were added together with plasmids in the transfection mixture according to a method described previously (39). Each well of S2 cells was co-transfected with 100 ng of desired reporter plasmid, 10 ng of the control *Renilla* luciferase reporter plasmid *pCopia*, and 1.5 μg of each dsRNA. In addition, some wells were also co-transfected with *pAc5.1/Met-Myc* or *pAc5.1/FISC-FLAG* or both. *DmTai* dsRNA was incubated in culture medium with 10 ng of the control *Renilla* luciferase reporter plasmid *pCopia*, and 1.5 μg of each dsRNA. In addition, some wells were also co-transfected with *pAc5.1/FISC-FLAG* or both.

**qRT-PCR**

Total RNAs of fat bodies from different developmental stages (PE: 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h; PBM: 12 h, 24 h,
36 h, 48 h, 60 h, and 72 h), RNAi, or in vitro culture treatments were extracted using TRizol (Invitrogen) following the manufacturer’s instructions. Total RNAs from S2 cells were also extracted. Approximately 2 μg of total RNA was first treated with DNase I and then applied to synthesize cDNAs using SuperScript II reverse transcriptase (Invitrogen). qRT-PCR reactions were processed in 96-well plates with iQ SYBR Green Supermix (Bio-Rad) using the iCycler iQ system (Bio-Rad). Quantitative measurements for each gene were performed in triplicate and normalized against the reference gene β-actin using the 2-ΔΔCT calculation method. The expression profiles of genes at different developmental stages are shown in the heatmap generated by R3.2.0 software based on the mean value of 2-ΔΔCT of each time point. Primers designed for qRT-PCR are listed in supplemental Table S2.

Western blot

Total proteins from fat bodies after RNAi treatment, in vitro culture treatments, or S2 cells were extracted, quantified, and applied to Western blot analyses as described previously (38). Aliquots of protein samples were resolved on 4–20% mini-protein TGX pre-cast gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. Rabbit polyclonal antibodies against RpL32 (Abcam, Eugene, OR) were used at a 1:5000 dilution to detect RpL32 proteins followed by the secondary anti-rabbit HRP (Abcam). Mouse monoclonal antibodies against Vg (40) were used at a 1:5000 dilution to detect Vg followed by the secondary anti-mouse HRP (Abcam). Rabbit anti-Myc antibodies (Sigma) were used to detect Met-Myc fusion proteins, whereas rabbit anti-FLAG antibodies (Sigma) were used to recognize FISC-FLAG. Monoclonal antibodies against β-actin (Sigma) were used as a loading control.

EMSA

RRS1 probe (5’-CACTTTTTGTGTCTCAATAGGCGAAGGCAAGA-3’) and RRS1 mutant probe (5’-CACTTTTTGTGTCTCAATAGGCGAAGGCAAGA-3’) were designed for EMSA assays. We performed EMSA according to a method described previously (18). Briefly, the annealed DNA oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega). Nuclear protein extracts from S2 cells were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Chino, CA). The DNA-protein complex was resolved on 5% TBE Criterion Precast Gel (Bio-Rad). Subsequently, the gel was dried, incubated with phosphor imaging screen, and visualized using autoradiography. A 50-fold amount of unlabeled RRS1 probe was preincubated with the nuclear extracts before the addition of labeled probe in the competition assays. The anti-Myc antibody (Sigma), anti-FLAG antibody (Sigma), or the rabbit IgG (Sigma) was preincubated with nuclear extracts before the addition of labeled probe in the supershift assays.

Determination of polysomes

We isolated polysomes according to a method described previously (37). Fifty fat bodies dissected from female mosquitoes of each dsRNA treatment were immediately frozen in liquid nitrogen and homogenized in 1 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 140 mM KCl, 1.5 mM MgCl2, 1% Triton X-100, 1% sodium deoxycholate, 0.2 units/ml RNase inhibitor, 1 mg/ml heparin, and 1 mM dithiothreitol). The homogenate was then centrifuged for 10 min at 12,000 × g. The supernatant was layered on a 20–60% linear sucrose gradient prepared in 20 mM Tris-HCl (pH 8.0), 140 mM KCl, 1.5 mM MgCl2, and 0.1 mg/ml CHX. The gradients were then centrifuged at 120,000 × g for 3 h at 4 °C. The fractions containing polysomes were pooled, and the amount of polysomes was determined as the material absorbing at 254 nm.

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