Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression

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Juvenile hormone (JH) plays crucial roles in many aspects of insect life. The *Methoprene-tolerant* (*Met*) gene product, a member of the bHLH-PAS family of transcriptional regulators, has been demonstrated to be a key component of the JH signaling pathway. However, the molecular function of Met in JH-induced signal transduction and gene regulation remains to be fully elucidated. Here we show that a transcriptional coactivator of the ecdysteroid receptor complex, FISC, acts as a functional partner of Met in mediating JH-induced gene expression. Met and FISC appear to use their PAS domains to form a dimer only in the presence of JH or JH analogs. In newly emerged adult female mosquitoes, expression of some JH responsive genes is considerably dampened when Met or FISC is depleted by RNAi. Met and FISC are found to be associated with the promoter of the early trypsin gene (*AaET*) when transcription of this gene is activated by JH. A juvenile hormone response element (JHRE) has been identified in the *AaET* upstream regulatory region and is bound in vitro by the Met-FISC complex present in the nuclear protein extracts of previtellogenic adult female mosquitoes. In addition, the *Drosophila* homologs of Met and FISC can also use this mosquito JHRE to activate gene transcription in response to JH in a cell transfection assay. Together, the evidence indicates that Met and FISC form a functional complex on the JHRE in the presence of JH and directly activate transcription of JH target genes.

development | endocrinology | chromatin immunoprecipitation

Juvenile hormones (JHs) are sesquiterpenoid molecules synthesized and secreted by the corpora allata in insects. JHs are essential for development, reproduction, diapause, caste differentiation, migratory behavior, and longevity in many insect species (1–4). The prominent role of JH is maintaining the status quo in juvenile insects and preventing an insect from precociously turning into an adult. During larval development, ecdysone (the molting hormone) causes larval–larval molts in the presence of JH in the hemolymph. After the corpora allata stop secreting JH in the final larval instar, insect tissues change their commitment, and ecdysone triggers the larval–pupal and pupal–adult molts (5).

JH appears to harness a variety of signal transduction pathways to exert its function. Some effects of JH are mediated via membrane receptors and the protein kinase C signaling pathway (4, 6), whereas more evidence suggests that JH acts through intracellular receptors to modulate gene expression (7–10). In some cases, JH seems to exert its functions by modulating the ecdysteroid signaling pathway (11–17).

A leading candidate for the JH receptor (or a component of the receptor) is the product of the *Methoprene-tolerant* (Met) gene, which was originally isolated in *Drosophila melanogaster* (18). Met belongs to the basic helix–loop–helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors that also includes the hypoxia inducible factor 1α (HIF-1α), aryl hydrocarbon receptor (AhR), aryl hydrocarbon nuclear translocator (Arnt), and CLOCK proteins. In vitro-synthesized *Drosophila* Met protein binds to JH-III with high affinity (19). Flies carrying the *Met* mutations show resistance to both the toxic and morphogenetic effects of JH and several JH analogs, including methoprene (18).

Recent studies suggest that *Met* and its paralogous gene in *Drosophila*, *germ cell expressed* (*gce*), have overlapping but not identical functions in JH signaling (20, 21). In *Tribolium castaneum*, it has been clearly demonstrated that the Met ortholog of this beetle (TcMet) plays an essential role in mediating the classical antimetamorphic effect of JH during molting. RNAi suppression of TcMet expression causes larvae to pupate prematurely, before reaching their final instar (22). It remains unclear how Met protein mediates JH signaling at the molecular level.

JH plays important roles in the control of various aspects of adult reproduction in mosquitoes (23). Secretion of JH-III begins soon after emergence of the adult. JH-III levels increase during the first 2 d and remain high until a blood meal is taken. Upon blood feeding, the hemolymph JH-III titers drop precipitously, whereas 20E titers begin to rise and reach their maximum level at 18–24 h after a blood meal (23). Our previous study has shown that the mosquito ortholog of Met is required for the JH-induced expression of the *Krüppel homolog 1* (*AaKr-h1*) gene and the *early trypsin* (*AaET*) gene in newly emerged adult female mosquitoes (24). Although the function of AaKr-h1 is unknown in mosquitoes, AaET is a female-specific protease involved in initial digestion of blood proteins in the midgut (25). Transcription of *AaET* is activated by JH after adult eclosion, but the AaET protein is produced only after blood ingestion (26). Here we report that Met binds to another bHLH-PAS domain protein only in the presence of JH. Both proteins are required for the proper expression of JH target genes after adult emergence. We also provide evidence indicating that the heterodimer directly binds to the regulatory regions of the target genes and activates their transcription in response to JH.

Results

Identification of a Met-Interacting Protein. bHLH-PAS proteins tend to use the bHLH-PAS domains to form homodimers or heterodimers with other bHLH-PAS proteins (27). DmMet has been shown to form Met–Met and Met–GCE dimers in vitro, although formation of the two protein complexes are greatly reduced in the presence of JH or JH analogs (28). We performed a yeast two-hybrid screening to isolate mosquito proteins that are specifically associated with Met only in the presence of JH. A chimeric protein consisting of the bHLH-PAS domain of the *Aedes aegypti* Met (AaMet[1-505]) and the Gal4 DNA-binding domain was used as bait. The yeast transformants were selected on solid medium contained 10^{-6} M methoprene. The screening yielded a single clone encoding the bHLH-PAS domain (amino acid residues 1–539) of FISC, a mosquito protein which has been previously characterized as a coactivator of the ecdysteroid receptor (29).

The yeast two-hybrid assay indicated that the interaction between the bHLH-PAS domains of AaMet and AaFISC was methoprene-dependent as the cotransformants were unable to grow on the selection medium without the presence of methoprene (Fig. S1). To examine the potential AaMet–AaMet interaction, we cloned AaMet[1-505] into the pGAD10 Gal4 activation domain fusion vector and used it in the two-hybrid assays for comparison. It appeared that AaMet formed a homodimer only in the absence of methoprene, consistent with a previous report by Godlewski et al. (28).

JH-Dependent Protein Interaction. To validate the Met-FISC interaction in insect cells and to characterize its dependence on juvenile hormone, we used a modified two-hybrid system developed by Peter and Lucy Cherbas' laboratory (30). pCMA-GAD and pCMA-GBD are expression vectors for recombinant proteins fused to the GAL4 activation domain and binding domain, respectively. A cDNA fragment encoding the amino terminal bHLH-PAS domain of AaMet was cloned into pCMA-GBD, whereas the bHLH-PAS domain of AaFISC was cloned into pCMA-GAD.

The GAD-AaFISC fusion was not able to activate the UAS×4–188-cc-Luc reporter gene, regardless of the presence of JH-III (Fig. 1A). GBD-AaMet was activated by JH-III and stimulated expression of the luciferase reporter gene, reminiscent of the transcriptional activity of DmMet in a similar experiment (19). The JH-dependent activation of the reporter gene by GBD-AaMet was further boosted when the GBD-AaMet and GAD-AaFISC fusion proteins were expressed together, suggesting a physical interaction between the bHLH-PAS regions of AaMet and AaFISC. In contrast, similar experiments implied that AaMet did not form either a homodimer or a heterodimer with other bHLH-PAS proteins, such as AaTgo (the mosquito ortholog of *Drosophila* Tango) in the L57 cells (Fig. 1A).

Next, we used the same cell transfection system to study the hormone-specificity and JH dose–response of the protein interaction between AaMet and AaFISC. Formation of the AaMet-AaFISC dimer was induced by JH-III and two JH agonists (methoprene and pyriproxyfen), but not by farnesol (a biosynthetic intermediate for JH-III) (Fig. 1B). In subsequent hormonal treatment experiments, farnesol was used as negative control. Marked activation of the reporter gene by the AaMet-AaFISC interaction was observed when the transfected cells were exposed to JH-III at a concentration of 10^{-7} M, and the reporter activity continued to increase in a JH-dose dependent manner (Fig. 1C). Together, these results demonstrated that the AaMet-AaFISC interaction is a JH-specific response.

PAS Domains Essential for the Met-FISC Interaction. Having demonstrated that the bHLH-PAS regions of AaMet and AaFISC were sufficient for their JH-dependent dimerization, we started to delineate the functional domains in the bHLH-PAS regions. Derivatives of the pCMA-GBD-AaMet[1-596] vectors were generated to produce GBD-Met fusion proteins with truncations of bHLH, PAS-A or PAS-B domains (Fig. 2A). Similar deletion mutations in GAD-AaFISC[1-609] fusion protein were created. Two-hybrid assays were performed in the L57 cells as described above with these unique expression vectors. In the absence of bHLH domain, GBD-MetΔbHLH showed even stronger binding to GAD-AaFISC[1-609] (Fig. 2B), indicating this domain in AaMet is not required for the JH-dependent Met-FISC interaction. Truncations of the two PAS domains in AaMet all significantly diminished formation of the Met-FISC complex, implicating the PAS domains in binding of JH and/or in protein–protein interaction. On the other hand, the bHLH, PAS-A and PAS-B domains of AaFISC all seemed to contribute to the Met-FISC interaction in response to JH, although the PAS-A and PAS-B
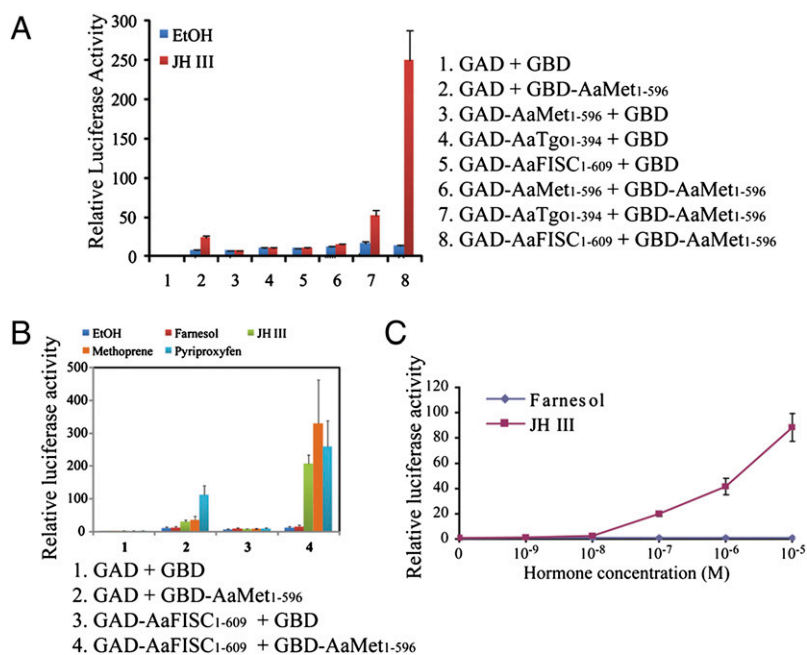

Fig. 1. Interaction between AaMet and AaFISC in *Drosophila* L57 cells. (A) Modified two-hybrid assays. cDNA fragments encoding the bHLH-PAS domains of AaMet, AaFISC, and AaTgo were cloned into the pCMA-GBD and pCMA-GAD vectors. L57 cells were transfected by the reporter construct UAS×4–188-cc-Luc together with the indicated GAD and GBD fusion constructs. Transfected cells were cultured in the presence of 5×10^{-6} M JH-III or ethanol (solvent; EtOH) for 16 h. Activity of the reporter gene was measured by dual luciferase reporter assay. The two-hybrid assays were also used to examine the hormone specificity (B) and JH dose-dependence (C) of the AaMet-AaFISC interaction. L57 cells were cotransfected by pCMA-GBD-AaMet, pCMA-GAD-AaFISC and UAS×4–188-cc-Luc. Transfected cells were cultured in the presence of 5×10^{-6} M JH-III, methoprene, pyriproxyfen, farnesol, or ethanol (B) or in medium with indicated concentrations of JH-III or farnesol (C). The mean average of three independent experiments is shown, with error bars representing SD.
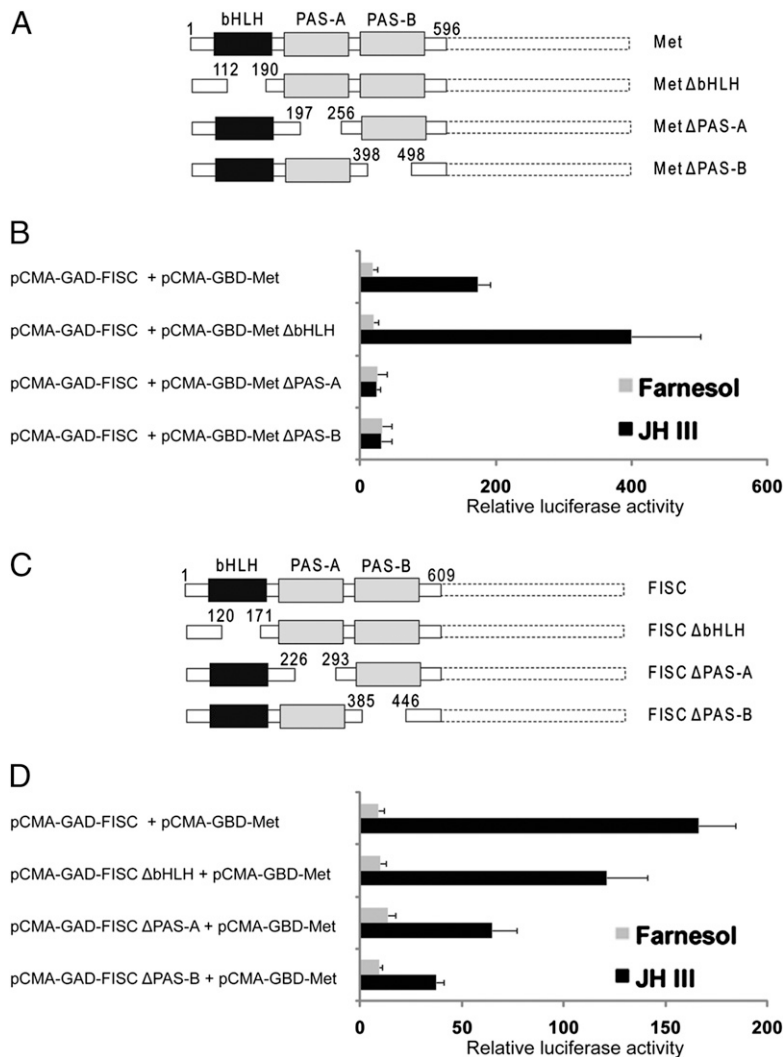
Fig. 2. Roles of bHLH and PAS domains of AaMet and AaFISC in their JH-dependent dimerization. (*A*) Schematic diagram of truncations introduced into the bHLH-PAS region of AaMet. Similar truncations were introduced into the corresponding regions of AaFISC (*C*). L57 cells were transfected by the reporter construct UAS×4–188-cc-Luc, together with the indicated expression vectors that produced the truncated GBD-Met (*B*) or GAD-FISC (*D*). Transfected cells were cultured in medium with 5×10^{-6} M of JH-III or farnesol.

domains appeared to play a bigger role in the binding of AaFISC to AaMet (Fig. 2*D*).

Roles of AaMet and AaFISC in Expression of the JH Target Genes. Our previous studies have detected expression of AaMet and AaFISC genes in the fat body, midgut, and ovaries of adult female mosquitoes during posteclosion development (24, 29). AaMet and AaFISC, two bHLH-PAS family transcription factors, form a heterodimer in response to JH, suggesting that the AaMet-AaFISC complex may function in modulating transcriptional response to JH. After injecting double-stranded RNA corresponding to AaMet or AaFISC into adult female mosquitoes within 30 min after eclosion, we examined expression of four JH target genes that are normally up-regulated in the midgut after eclosion (24). Knockdown of either AaMet or AaFISC caused a considerable decrease in mRNA transcripts of *AaET* and *AaKr-h1* in the midgut (Fig. 3*A* and Fig. S2). Expression of *AAEL002576* and *AAEL002619* were not markedly reduced in the *AaMet RNAi* mosquitoes, whereas impaired function of AaFISC affected the mRNA levels of *AAEL002619*, but not *AAEL002576*. Consistent with a diminished JH response, RNA interference of AaMet and AaFISC also significantly reduced the number of eggs oviposited

by each female mosquito after a blood feeding (Fig. S3). These results indicated that both AaMet and AaFISC play important roles in modulating JH-regulated gene expression in adult female mosquitoes.

Detection of AaMet and AaFISC on a JH-Activated Promoter. To examine whether AaMet and AaFISC directly regulate the promoter of *AaET*, we performed ChIP assays. The presence of AaMet and AaFISC in the proximal regulatory regions of *AaET* was at a background level at 2 h posteclosion (Fig. 3 *C* and *D*), when endogenous JH concentration had not yet increased in the newly emerged mosquitoes. At 30 h posteclosion, when the JH titers were near their peak, occupancy of the *AaET* promoter by either AaMet or AaFISC increased significantly. The association of AaMet and AaFISC with the *AaET* proximal promoter was concomitant with the active transcription of *AaET* at this stage (26). Binding of either AaMet or AaFISC to the *AaET* promoter went down to the background level again at 4 h after a blood meal (Fig. 3 *C* and *D*), when the JH concentrations declined precipitously and transcription of *AaET* was shut down. These results showed that AaMet and AaFISC act directly on the *AaET* promoter to activate its transcription.
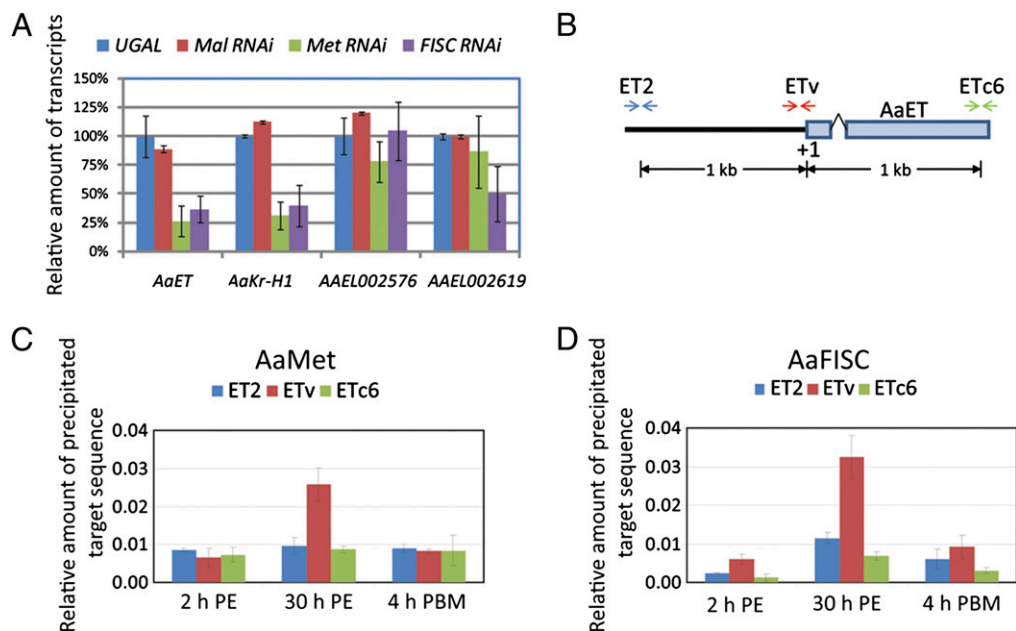
Fig. 3. AaMet and AaFISC are required for expression of JH target genes in the midgut of adult female mosquitoes. (*A*) Double-stranded RNA (dsRNA) induced gene knockdown. A 0.5-μg quantity of dsRNA for either AaMet or AaFISC was injected into newly emerged female mosquitoes within 30 min after eclosion. DsRNA for bacterial *malE* gene was used as control. Then, 4 d after injection, midguts were collected from the mosquitoes. Total RNA was extracted and subjected to quantitative RT-PCR analysis. Results are expressed as percentage of mRNA levels in the uninjected (*UGAL*) mosquitoes. (*B*) Schematic structure of the AaET gene. Three pairs of primers were designed to amplify the distal upstream region (ET2), the proximal promoter region (ETv), and the coding region of *AaET* (ETc6). Association of AaMet (*C*) and AaFISC (*D*) with the *AaET* promoter was measured by chromatin immunoprecipitation assays. Amount of immunoprecipitated DNA in each sample was represented as signal relative to the total amount of input chromatin. PBM, post blood meal; PE, posteclosion.

Identification of a JH Response Element. We cloned a 2.0-kb promoter region of *AaET* into the pGL3 basic luciferase reporter vector, and used transient transfection assays to test whether AaMet and AaFISC activated the *AaET* promoter in response to JH-III. Expression of either AaMet or AaFISC alone in L57 cells had no substantial effect on the activity of the *pAaET-Luc* reporter gene (Fig. S4*A*). When the two proteins were expressed together, the reporter gene was activated significantly if JH-III was present in the cultural medium. Serial deletion analysis of the promoter region revealed that the proximal region (nt −540 to −165) was crucial for the JH-induced activation of the reporter gene (Fig. S4*B*). Bioinformatic analysis of this region revealed a sequence (CCACACGCGAAG) similar to the binding site of the mammal AhR/Arnt bHLH-PAS heterodimer (Fig. S5). To test the function of this DNA element, we inserted four copies of this sequence and the minimal core promoter of *AaET* into the pGL3 basic luciferase reporter vector. Although the minimal core promoter alone was not responsive to JH treatment (Fig. S6), expression of the unique reporter gene (4×JHRE-luc) was considerably activated in L57 cells by the AaMet-AaFISC complex in the presence of JH-III (Fig. 4*A*), suggesting that this 12-nucleotide sequence acted as a juvenile hormone response element (JHRE). Furthermore, gel shift assays suggested the existence of a protein complex containing both AaMet and AaFISC in the nuclear extracts of adult female mosquitoes (Fig. 4*B*). Binding of the protein complex to JHRE was abolished by antibodies against either AaMet or AaFISC, presumably by blocking dimerization or DNA binding of these two proteins. The protein complex was detected in mosquitoes at 30 h after eclosion, but not in the newly emerged mosquitoes or the blood-fed mosquitoes. The appearance of the AaMet-AaFISC complex seems to correlate well with endogenous JH concentrations and the expression profile of *AaET* in the adult female mosquitoes.

Conserved Mechanism for JH Signaling and Transcriptional Regulation. The JHRE shares a high degree of sequence similarity with a common motif that has been previously identified in a group of *Drosophila* JH-responsive promoters (9). Using transient transfection assays, we tested the functions of *Drosophila* Met, GCE, and Taiman (TAI; the *Drosophila* ortholog of AaFISC) in mediating JH signaling in the L57 cells. None of the three bHLH-PAS proteins alone had any significant effect on the expression of the 4×JHRE-luc reporter gene (Fig. 5). Coexpression of DmTAI with either DmMet or DmGCE led to significant induction of the reporter gene by JH-III. In contrast, the combination of DmMet and DmGCE was not able to activate the reporter gene in response to JH-III. This evidence suggests that binding of the Met-FISC complex to the JHRE is a conserved mechanism in activating expression of JH target genes.

Discussion

Genetic studies have shown that Met is required for proper expression of JH target genes in fruit flies, red flour beetles, and mosquitoes (10, 24, 31). Although the protein structure of Met suggests that it may act as a JH-activated transcriptional regulator, the binding of Met to JH-responsive promoters has not been definitively demonstrated so far. In this study, a chromatin immunoprecipitation experiment indicated that Met was indeed associated with the early trypsin promoter when this gene was activated by endogenous juvenile hormone in the newly emerged adult female mosquitoes. This is a unique demonstration of Met directly regulating a JH target gene.

To elucidate the molecular roles of Met in JH signaling, a number of proteins have been tested in vitro or in the cultured insect cells for their abilities to bind Met (9, 28, 32). The protein interactions with Met were largely independent of the presence of JH, or even repressed by JH. Using a library screening approach, we have identified a mosquito bHLH-PAS protein (FISC) that
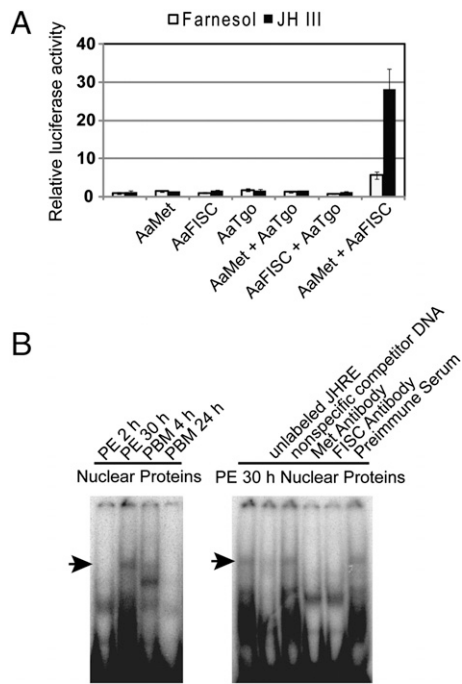
Fig. 4. AaMet and AaFISC bind to JHRE identified in *AaET* upstream regulatory region. (*A*) L57 cells were transfected with the 4×JHRE-luc reporter plasmid, together with the indicated expression vectors. After transfection, cells were cultured in medium with 5×10^{-6} M JH-III or farnesol. (*B*) EMSA experiments. Nuclear proteins were extracted from abdomens of adult female mosquitoes at the indicated time points and incubated in vitro with JHRE labeled by [γ-^{32}P] ATP. For competition reactions, nuclear proteins were incubated with an approximate 100× molar excess of unlabeled probe or a nonspecific double-stranded oligonucleotide for 20 min before incubation with labeled probe. Identity of complex was verified by directly adding polyclonal antibodies against AaMet and AaFISC to the binding reactions.

binds to Met in a JH-dependent manner. EMSA and ChIP experiments have demonstrated that the Met-FISC complex forms in vivo and binds to a JH-regulated promoter in previtellogenic mosquitoes only in the presence of high titers of juvenile hormone. This observation is consistent with the RNAi results showing that both Met and FISC are required in adult mosquitoes for activation of JH target genes, such as *AaET* and *AaKr-h1*. In Fig. 1, the GBD-Met fusion (without the GAD-FISC fusion) activated the UAS×4–188-cc-Luc reporter gene after the JH treatment. This activation also relied on the endogenous
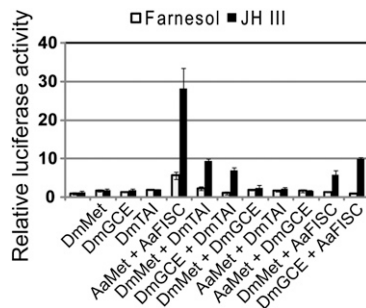

Fig. 5. JH-induced transcriptional activation by the *Drosophila* homologs of Met and FISC. L57 cells were transfected by 4×JHRE-luc and expression vectors for the indicated proteins. Transfected cells were cultured in medium with 5×10^{-6} M JH-III or farnesol. Average results of three independent experiments are shown, with error bars representing SD.

Taiman protein in the L57 cells as the JH induction was severely dampened when Taiman was depleted by RNAi (Fig. S7). Formation of the Met-FISC complex thus constitutes a key step in signal transduction of juvenile hormone. It is also worth noting that not all of the JH target genes are affected by RNAi knockdown of Met or FISC (Fig. 3*A*), implying that JH might act through several distinct pathways even in a single tissue at a particular developmental stage.

Transient transfection and gel shift assays indicated that Met-FISC activated the *AaET* promoter by binding to the JHRE. It is currently under investigation whether the two proteins are directly binding to the JHRE or are recruited to the JHRE via protein interaction with other transcription factors. Because of the relative large sizes of the two proteins, it is difficult to obtain full-length and functional recombinant Met and FISC proteins. EMSA experiments using in vitro-synthesized proteins turned out to be problematic, because both rabbit reticulocyte lysate and wheat germ extract displayed high background binding to the labeled JHRE. In a separate experiment, our preliminary study showed that the JH-induced transcriptional activation by Met-FISC was completely abolished in cell transfection assays if the DNA binding domain (bHLH region) of either Met or FISC was truncated. However, we cannot rule out the possibility that the bHLH regions are also required for interactions with other proteins.

A distal regulatory region of *AaET* was also shown to be indispensable for JH-dependent activation of the *AaET* promoter (Fig. S4). Intriguingly, when four copies of JHRE were placed upstream of the minimal promoter (TATA box) of *AaET*, the JHRE seemed to be sufficient for the Met-FISC mediated JH activation (Fig. S6). This discrepancy implies that regulation of JH target genes is more sophisticated than the binding of Met-FISC to JHRE. More studies are needed to elucidate the underlying molecular mechanisms.

In vitro experiments have shown that Met can bind to both EcR and USP, two components of the ecdysteroid receptor (32). Here we find that FISC, a coactivator of the EcR/USP, also binds to Met and plays an important role in juvenile hormone signaling. Whether these protein interactions are involved in the crosstalk of ecdysone and JH signaling is awaiting further experimental evidence. Because the binding of FISC to EcR/USP and Met relies on the presence of 20-hydroxyecdysone and juvenile hormone respectively, the shuffling of FISC between the two signaling pathways may account for the antagonistic actions of these two hormones.

A sequence similar to the *AaET* JHRE is also found in the promoter region of *AaJHA15*, another JH-regulated gene in adult female mosquitoes (33). The common motif 2 discovered in a group of JH-activated *Drosophila* promoters also shares high sequence similarity with the *AaET* JHRE, suggesting an evolutionarily conserved mechanism underneath the JH-induced transcriptional activation. Indeed, the *Drosophila* Met and Taiman activated the 4×JHRE-luc reporter gene in a JH-dependent manner. Although DmMet-AaFISC appeared comparable to DmMet-DmTAI in mediating JH-induced gene expression, AaMet-DmTAI was completely unable to activate expression of the reporter gene after JH treatment. This observation suggests that the intricate protein interactions between Met and FISC/TAI determine the affinity of the dimers to the JHRE and/or their ability to activate transcription of the JH target genes.

Unlike mosquitoes, two Met-like genes (Met and gce) exist in fruit flies. Combination of gce and Taiman also led to considerable activation of the reporter gene in response to JH. This observation is in line with a recent report showing that gce can partially substitute for Met in vivo (20). It would be interesting to test next whether Met-TAI and gce-TAI preferentially bind to distinct JH responsive promoters in vivo.

Materials and Methods

Yeast Two-Hybrid Screen. A yeast two-hybrid cDNA library was constructed in the pGAD10 Gal4 activation domain vector, according to the manufacturer's instructions (Clontech), using a total of 10 mg poly(A)+ RNA from abdomens of adult female mosquitoes. AaMet[1-505] (bHLH-PAS domain) was cloned into the Gal4 DNA-binding domain vector pGBKT7 (Clontech). Yeast strain AH109 was sequentially transformed with pGBKT7-AaMet[1-505] and with the mosquito cDNA library. All of the selection medium contained 10^{-6} M methoprene dissolved in DMSO. Colonies that appeared on the SD/-Trp/-Leu/-His plates (medium stringency) were transferred to the SD/-Trp/-Leu/-His/-Ade/Xα-Gal plates (high stringency). The library plasmids from positive clones that expressed HIS3, Ade and LacZ reporters were recovered and retransformed into yeast cells, together with the original bait, for testing the specificity of protein–protein interactions.

Transient Transfection Assay. *Drosophila* L57-3–11 cells were transfected according to the instructions of Hu et al. (30). pCMA was used as the expression vector for all of the proteins described in the transfection assays. Truncated proteins were expressed by deletional mutagenesis using a method described by Li et al. (34). The ORFs for AaTgo, DmMet, and Dmgce were cloned by RT-PCR based on the cDNA sequences in the GenBank. Juvenile hormone III, methoprene, pyriproxyfen and farnesol (Sigma Aldrich) were dissolved in ethanol.

Double-Stranded RNA-Induced Gene Silencing. RNAi knockdown of AaMet and AaFISC was performed as described previously (24, 29). Briefly, 0.5 μg dsRNA was injected into the newly emerged female *Ae. aegypti* mosquitoes within 30 min after eclosion. The mosquitoes were then maintained in the insectary under normal conditions. Then, 3–4 d after injection, the mosquitoes were dissected, and the mRNA extracted from the midgut was examined by quantitative RT-PCR (24).

Chromatin Immunoprecipitation Assay. Polyclonal antibodies for AaMet and AaFISC have been reported previously (24, 29). *Ae. aegypti* mosquito abdomens were homogenized in PBS on ice, followed by the addition of formaldehyde to a final concentration of 1% and incubation at 37 °C for 10 min. Chromatin immunoprecipitation assays were performed using a QuikChIP kit (IMGENEX) according to the instruction manual. Mock immunoprecipitations using preimmune sera for each antibody were included as negative controls to determine the baseline of the nonspecific background. The precipitated DNA and DNA input were analyzed by using quantitative RT-PCR. PCR primers were as follows: ET2-F, 5′-GCTTGGTAGAACAGTCAA-TGGGTCAG-3′; ET2-R, 5′-AGAGTCCATCGGATAGGCATCACG-3′; ETv-F, 5′-GT-TTTGAAATTACCCATCCCACACG-3′; ETv-R, 5′-GTCCATTCCTATGATGCGGAT-TCTT-3′; ETc6-F, 5′-GTAAGGATTCTTGCCAGGGAGACTC-3′; and ETc6-R, 5′-AT-CCATTGGCGAACAGTGGACAC-3′.

Electrophoretic Mobility Shift Assay. Abdomens were collected from 200 adult female *Ae. aegypti* mosquitoes for each time point. Nuclear protein extraction was carried out as described by Miura et al. (35). Detailed method for EMSA experiments was described in *SI Text*.

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