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The Role of Juvenile Hormone Receptor Methoprene Tolerant in Reproductive Development of Adult Female Mosquito, *Aedes aegypti*

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by

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ABSTRACT OF THE DISSERTATION

The Role of Juvenile Hormone Receptor Methoprene Tolerant in Reproductive Development of Adult Female Mosquito, *Aedes aegypti*

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Dr. Alexander S. Raikhel, Chairperson

Vector mosquitoes require vertebrate blood for their egg maturation, and repeated blood feeding cycles result in acquisition and transmission of pathogens which course numerous devastating human diseases. Elucidation of molecular basis of blood meal-activated reproductive cycles is essential for developing future mosquito control strategies. The juvenile hormone (JH) controlled post-eclosion (PE) developmental phase is required for female mosquitoes to attain competence for egg development. Time course microarray analysis of *Aedes* fat body revealed a high transcriptional activity in this tissue during the PE development. The hierarchical clustering identified two major gene clusters: 1843 early (EPE) genes maximally expressed at 6 h PE and 1815 late (LPE) genes at 66 h PE. Functionally, the EPE and LPE clusters are markedly different, with the former genes involved in metabolic pathways and the latter those regulating transcription and translation processes. RNA interference screen of the JH receptor Methoprene-tolerant (MET) resulted in up-regulation of EPE gene expression and down-regulation of LPE genes. Overrepresentation of putative e-box-like MET binding sites was observed in

upstream regions of MET-activated (LPE), but not MET-repressed genes (EPE). Electrophoretic mobility shift assays (EMSA), utilizing a combination of mutational and anti-Met antibody super-shift analyses, confirmed binding properties of the MET consensus motif variants in a subset of MET activated genes. We tested an indirect model of gene repression by JH/MET hierarchy. The JH/MET-activated bHLH protein Hairy has been established as an intermediate factor mediating the JH/MET gene repression by means of RNAi/RNA-sequencing. 79% of Hairy-repressed genes showed an overlap with MET-repressed transcriptome. Upstream regions of 20% of these MET/Hairy repressed genes harbored a Hairy interacting putative *cis*-regulatory module (hCRM) containing Hairy-binding sites. Lastly, Cycle (CYC) was identified as a bHLH-PAS binding partner of MET. Here, we characterize the MET/CYC heterodimer mediated JH-dependant circadian expression of *Krüppel homolog-1* and *Hairy*. The activation of these genes not only depends on JH but on the ratio of light:dark periods as well. These studies have elucidated the molecular mechanism of gene regulation by JH/MET signaling pathway.

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INTRODUCTION

Juvenile hormone (JH) was first discovered by Wigglesworth in 1934 (1) in the blood-sucking hemimetabolous insect, *Rhodnius prolixus*, as a factor preventing metamorphosis of nymphs to adults. Subsequent studies have revealed that JH controls the onset of metamorphosis in holometabolous insects. In most species, exogenous application of JH results in reiteration of its juvenile stage, whereas experimental removal of JH causes an insect to metamorphose prematurely (2). Thus, JH has aptly being named the “status quo” hormone.

Wigglesworth later showed that JH is necessary for egg maturation in many adultinsects(3). Subsequent work by various researchers has demonstrated multiple important functions of JH in the insect life cycle. JH plays a role in egg development, reproductive maturation of male insects, morphostasis (cell division without differentiation), seasonal polyphenism and behavioral responses (2). It is an insect-specific hormone whose function has been diversified in the process of evolution to play a critical role in regulation of many key biological processes.

JH is synthesized by a small pair of glands called Corpora allata (CA) situated just below the insect brain. First isolated in 1956 from the moth *Hyalophora cecropia* (4), its structure was elucidated by Roller et al. in 1967(5). JH is a sesquiterpenoid - a structure new to natural product chemistry at the time of its discovery and is unique amongst hormones of either invertebrates or vertebrates. JH shows a variety of forms in insects.

JH0, JHI, JHII are found only in Lepidopterans while JHIII is by far the most widespread amongst all in insects (6) (Fig. 1).

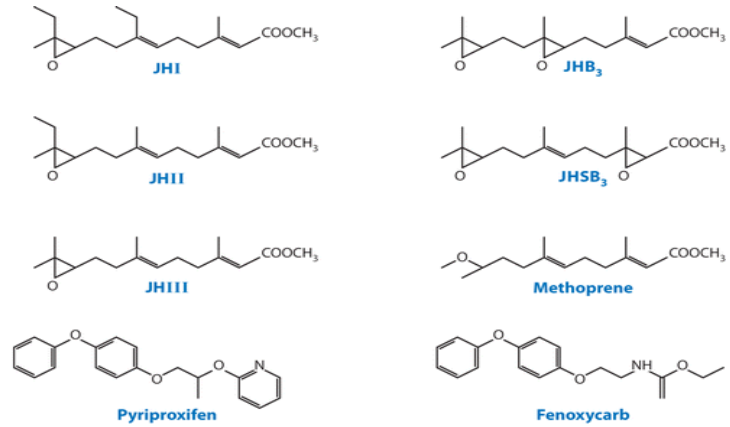
Despite its importance, the molecular basis of JH action is only partially understood. Working to the disadvantage of JH researchers was the fact that the model insect *Drosophila* does not show classical response to JH (7). In this insect, ectopic JH application cannot induce extra larval stages and it blocks only adult differentiation of the abdominal histoblast, with rest of the adult body being insensitive to JH.

Fig. 1 legend:

Structure of various forms of JH and its analogs:

Structures of the major naturally occurring JHs and three of the many JH analogs, methoprene, pyriproxifen and fenoxycarb. Abbreviations: JH, juvenile hormone; JHB_3, JH bisepoxide; $JHSB_3$, JH skipped bisepoxide. JHIII is the most widespread form of JH and the only form found in mosquito *Aedes aegypti*. From Jindra et al. (2013) (20).

Fig. 1.



Search for JH receptor - role of Methoprene tolerant (MET) in JH action:

How JH achieves its function has remained unclear, mainly because a JH receptor has long eluded identification. The lipophilic nature of the sesquiterpenoid JH suggests involvement of an intracellular receptor, yet none of the known insect nuclear hormone receptors have been linked with the biological function of JH.

Ultraspiracle (USP) is a member of nuclear receptor family that is orthologous to the vertebrate retinoid X receptor (RXR). In insects USP is best known as the heterodimeric partner of the ecdysone receptor (EcR). EcR binds the ligand 20E with USP being an orphan nuclear receptor without any ligand. USP was shown to bind JH apparently as a homodimer (8) and was proposed to be the JH receptor important for preventing metamorphosis. But the same group later demonstrated that USP binds JH with at least 100 times lower affinity than what is expected for a receptor (9). Thus, binding of JH to USP is likely an artifact rather than an interaction with a bona-fide receptor.

A leading candidate for the JH receptor is the product of the *Methoprene-tolerant* (*MET*) gene, which was identified by Wilson and Fabian in 1986 in chemical mutagen screens of *Drosophila*, as mutants that were able to survive toxic doses of JH or JH analog (JHA) methoprene (10). The same group later showed that MET encodes a transcription factor of the basic helix loop helix (bHLH)-Per –Arnt-Sim (PAS) domain family (11) that has about 40% homology to the dioxin receptor translocator (ARNT) that is necessary to transport the dioxin receptor (AhR) to the nucleus. bHLH-PAS domain proteins are particularly interesting signaling molecules, because many of them act as

sensors of external stimuli such as hypoxia, xenobiotics or hormones. The *MET* null mutants in *Drosophila* show a greater than 10-fold resistance to morphogenetic effects of JH at the time of pupariation and delayed egg production (12). By means of immunocytochemistry, MET was found to reside in the nucleus in most insect tissues (13). Recombinant *Drosophila* MET binds JH at physiological (nanomolar) concentrations and to mediate a weak JH- and methoprene-dependent transcriptional activation *in vitro* (14). These data suggested a key role that MET it may be the elusive JH receptor. However, *MET*-null mutants in *Drosophila melanogaster* were viable and fertile leaving the notion that MET is a putative JH receptor unsupported with an anticipated developmental phenotype. Thus, although Drosophila is an excellent genetic tool which revealed the identity of such important genes as *MET*, it failed to reveal its role in the fundamental function of JH: maintaining "status quo" in juvenile insects.

The evidence for MET's "status quo" action came nearly 20 years after the discovery of the gene itself, from Konopova and Jindra who worked with red flower beetle *Tribolium*. It was shown that deficiency of MET in young larvae leads to premature pupariation and precocious metamorphosis. Suppression of *MET* expression by the injection of double-stranded (ds) *MET* RNA in the third or fourth larval instars causes precocious metamorphosis two instars later (*i.e.* after 5^{th} or 6^{th} instars, respectively) rather than after seventh or eighth instar (15). Moreover, beetle pupae that had *MET* dsRNA were unresponsive to methoprene and JH and most formed normal adults rather than second pupae as the control JH-treated pupae. Their study not only established MET's function and supported its putative receptor role but also established *Tribolium* as a

model organism to address question regarding JH action. Unlike *Drosophila* exogenous application of JH to *Tribolium* leads to reiteration of juvenile stages, *i.e.* it shows a classical JH response. *Tribolium castaneum* has a sequenced genome and reverse genetics is easy to perform and most importantly, a single ortholog of *Drosophila MET* and *germ cell expressed* is present in the genome. Indeed, binding of JH to MET and JH dependent MET mediated transactivation of target genes were characterized in *Tribolium*, establishing MET as a JH receptor (16).

Germ cell expressed (GCE), a second bHLH-PAS domain protein that has 70-86% identity to MET in its conserved region, was identified from *Drosophila* (17). The presence of GCE might give the possible explanation to the paradox that MET null mutants are fully viable in *Drosophila*. It was shown that MET forms homodimer and MET and GCE form heterodimer in the absence of JH leading to some speculation that MET may function differently during larval life and metamorphosis (17). Latest reports show that, in *Drosophila*, Met might functionally overlap with its paralog, encoded by the *GCE* gene. *GCE* can increase sensitivity of *MET*-null mutants to methoprene (18). Recently, premature degeneration of the fat body was observed in *Drosophila* larvae that either were deprived of JH or lacked both MET and GCE; and addition of a JH mimic (pyriproxyfen) could remedy only deficiency of JH but not the loss of MET and GCE (19).

Identification of bHLH-PAS binding partners of MET:

MET is a member of basic helix loop helix (bHLH) –Per-Arnt-Sim (PAS) domain transcription factor family. Members of this family play role in varied biological functions including cell proliferation, apoptosis and tissue differentiation specifically during development of animals and plants. Structurally, all members of this superfamily contain bHLH domain of 60 amino acids consisting of two regions: a DNA binding basic region of 15 amino acids in length, and a helix-loop-helix region. These proteins tend to use the bHLH-PAS domains to form homo- or hetero-dimers with other bHLH-PAS proteins to attain biological functionality and triggering the expression of target genes. *Drosophila* MET has been shown to form MET-MET and MET-GCE dimers *in vitro*, but the formations of the two protein complexes are greatly reduced in the presence of JH or its analogs (17). Considering other evidences, it is highly unlikely that MET-MET/ MET-GCE dimers will be involved in mediating downstream JH action (20)

Scientists kept searching for JH dependent protein-protein interaction of MET. A mosquito bHLH-PAS protein- βFtz-F1 interacting steroid receptor co-activator (FISC) has been identified as a binding partner of MET in yeast two hybrid screening approach. The binding has also been shown to be dependent on the presence of JH and in a dose dependant manner (21). This observation is backed by RNAi results showing that both MET and FISC are required in adult mosquitoes for activation of JH responsive genes such as *Aedes Early Trypsin* (*AaET*) and *Krüppel homolog-1*(*Kr-h1*). Luciferase reporter assays further provided evidence for the binding of MET and FISC in *Aedes* mosquito (21). It was also shown that the PAS domain of MET is necessary for binding. This study

not only established FISC as the binding partner of MET but also established the Dengue vector mosquito, *Aedes aegypti* as a potent model to address complicated questions regarding JH action. In *Aedes*, however, most of the questions concerning biological function of JH are directed towards adult development and reproductive maturation instead of metamorphosis.

FISC cDNA was screened out in yeast two hybrid assays aiming to identify the binding partner of orphan nuclear receptor Ftz-F1 in mosquito *Aedes* (22). FISC closely resembles *Drosophila* Taiman and belongs to the p160 steroid receptor co-activator (SRC) family. SRC functions as a potent co-activator of ecdysone receptor-dependent transcription (23). Interestingly, in *Tribolium* SRC has been recently identified as the binding partner of MET (24, 16) thus establishing the involvement of *Drosophila* Taiman homologs as a MET binder across various insect taxa. However, from the existing body of evidence, it would be inappropriate to conclude that the entire MET downstream effects are brought about as a result of its interaction with Taiman, or in other words that Taiman is the only bHLH-PAS partner of MET.

Interaction of MET with other nuclear proteins:

Recent study conducted by Dubrovsky et al (25) in *Drosophila* S2 cells, has shown that MET and GCE both interact with orphan nuclear receptor Ftz-F1. This finding was further confirmed by insect two hybrid assay (26). Ftz-F1 is required for induction of JH responsive genes E75A in S2 cell lines. These interactions are severely reduced when helix 12 of the FTZ-F1 activation function 2 (AF2) is removed, implicating AF2 as an interacting site. In this detailed structural study docking simulations revealed

hetero-dimer formation by a typical NR box-AF2 interaction and this relies primarily on hydrophobic contacts, including a unique interaction with helix 4 of Ftz-F1. These findings suggest that a novel NR box enables MET and GCE to interact JH-dependently with the AF2 of FTZ-F1 (26). The intracellular nature of JH signaling was also proved in this study by expressing an intracellular form of JH esterase, an enzyme known to degrade JH, leading to the shutting down of JH target gene E75A (25).

Drosophila MET has been shown to bind with nuclear proteins FKBP39 and Chd64 (27). The complex binds to DNA element in the promoter of certain JH responsive genes in Drosophila and honey bee, *Apis mellifera*. But, these findings need further confirmations and functional tests before its significance could be ascertained. There are some reports that Drosophila Met binds EcR and USP, but this interactions does not depend on JH (27, 28)

Binding of JH to the receptor MET:

One of the most critical evidence for a receptor candidate is its binding of the respective ligand molecule. Until recently this evidence was largely missing for JH receptor candidate MET. bHLH-PAS proteins typically form heterodimeric transcription factors. The vertebrate aryl hydrocarbon receptor (AhR) requires activation by a ligand bound to its C-terminal PAS domain (PAS-B) to combine with the AhR nuclear translocator (Arnt) and to activate transcription (29). In *Drosophila* there is some evidence of high affinity binding of JH to MET (14). But this remained unverified in *Drosophila* and across various species. Consequently, it could not be ascertained whether the JH-dependent interaction between MET and Taiman requires the hormone to be

actually bound to a specific ligand-binding site. Recently, a low but reproducible binding of JH to *Drosophila* MET protein has been reported (16). Unexpectedly, the activity of MET appears much weaker compared with its paralog GCE, which was not previously tested. The capacity of both *Drosophila* MET and GCE to bind JH agrees with the ability of GCE to restore methoprene sensitivity in *MET*-null flies (18) and supports the view that MET and GCE functionally overlap (19). MET binding to its ligand was tested and proved in other insect species such as the beetle, *Tribolium* and firebrat, *Thermobia domestica* (16).

Assaying for JH binding with truncated *Tribolium* MET protein, it is shown that PAS-B domain of MET is necessary and sufficient for specific, high-affinity ligand binding and discriminates between biologically active and inert compounds (16). When PAS- B domain was structurally modeled using crystal structure of hypoxia-inducible factor 2 α- PAS-B domain as a template, a distinct hydrophobic ligand binding pocket was predicted to be present. Computational docking studies of JH with this hydrophobic pocket led to several solutions with best docking results corresponding to a theoretical affinity of -7.4 Kcal/mol. JH binding to Met was abolished by mutations in key amino acids in the ligand binding pocket of PAS-B domain thus proving the ligand receptor interaction (16). This group went a step further and probed the ligand dependency of the protein-protein interaction of MET. MET-MET homo-dimeric complex was previously shown to dissociate in the presence of methoprene (17). A single point mutation in the ligand binding pocket of MET renders this MET-MET complex resistant to the effects of methoprene. MET mutants incapable of binding JH were also found to be severely

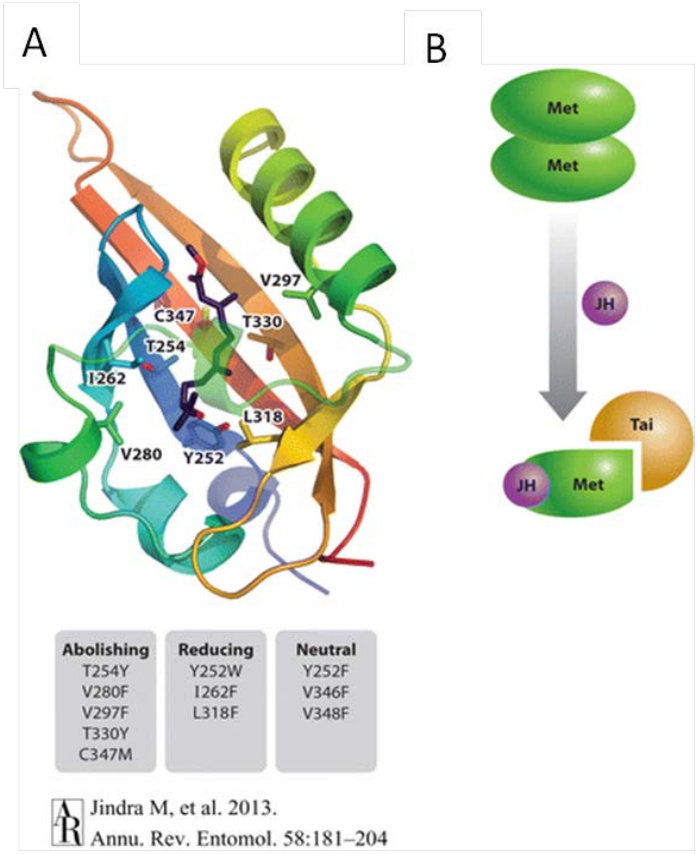
compromised in its ability to respond to Methoprene and bind its heterodimeric partner Taiman. Thus, the effect the ligand plays in either MET-MET or MET-Taiman complex formation strictly requires its binding to PAS-B domain of MET (Fig. 2).

Fig. 2 legend:

A model of the interaction of Met with JH and Taiman:

(*A)* Based on the crystal structure of HIF2α, the PAS-B fold of *Tribolium* MET was modeled with computationally docked JH III. Mutations of residues pointing toward the hormone ligand abolished or reduced JH III binding *in vitro*. Y252F and mutations of two valine side chains pointing away from the ligand had no effect (16). Image by Thomas Iwema. (*B*) Upon JH binding, Met undergoes a conformational change that liberates it from a Met-Met complex and allows its interaction with another bHLH-PAS protein, Taiman. From Jindra et al. (2013) (20).

Fig. 2.



Identification of juvenile hormone target genes:

In the past few decades there has been .several studies characterizing molecular targets of JH at the individual gene level. Several candidates from various life stages of different insect has been identified including the established marker genes *Kr-h1*, *Broad*, *Early Trypsin*, *E75*, *JHA15*, *Juvenile hormone inducible protein*, *Juvenile hormone easterase* (20, 25, 30-32).With advent of modern genomic tools and the availability of sequenced genomes of various insects recent efforts are focused on identifying JH targets at genome wide level.

Targets genes of JH during mosquito adult development were characterized by Zhu et al. in 2010 (33). This was also the first attempt to address this issue at a genomic scale. Using microarray specially designed for *Aedes* this group did a comparison between mRNA collected from mosquito abdomens treated with or without JH (33). By applying strict filtering conditions and confirmations by real time PCR six JH target genes were identified. The six genes thus identified are mosquito homologs of *Early Trypsin*, *Trypsin*, *Sodium/solute symporter*, *Peritrophic membrane chitin binding protein* and transcription factors *Hairy* and *Krh-1*(33). Their temporal expression and tissue and JH specificity were characterized. The temporal pattern of the six genes varied indicating involvement of various JH control methods for the induction of these genes. All the members showed induction by JH or its analogs but not fernesol (control). The involvement of MET in expression of 4 of the 5 genes tested was confirmed (33). *Peritrophic membrane chitin binding protein* was found to be a JH regulated gene not under MET control thus indicating alternative JH signaling pathway.

Another attempt towards genome wide expression analysis specifically focused on dual regulation by JH and Met in beetle, *Tribolium castaneum* (24). Beetles were injected with control dsRNA or *MET* dsRNA and then were treated with either JH analog Methoprene or solvent alone. RNA was collected from these four samples and subjected to Microarray transcriptome analysis. Comparisons between JH treated control and *MET* injections revealed the desired gene sets. In 37 genes a 50 % reduction in Methoprene mediated induction was reported in MET depleted insects while 32 genes showed a similar induction in Methoprene mediated reduction in *MET* RNAi insects (24). With the ontology of these genes worked out, this study gives a firsthand overview of MET mediated transcriptome in *Tribolium*.

Identification of juvenile hormone response element (JHRE):

High titer of JH alters the expression level of significant number of genes. It is assumed that JH would bind a protein or more probably a protein complex, which directly interacts with a DNA response element to bring about alteration in gene expression of a good percentage of JH response genes. This DNA sequences termed juvenile hormone response element (JHRE) has been the focus of research for several insect physiologists. The first such attempt was with *Locusta migratoria* gene *jhp21*, which is co-expressed with *Vitellogenin* during JH induced ovarian maturation. The sequence identified is a partial palindrome that binds JH induced fat body nuclear protein (34). Yeast one hybrid assays identified a putative lipid-binding protein, tfp1 to bind to this JHRE in presence of JH (35). In another attempt a DR4 sequence was identified as JHRE on spruce budworm (*Choristoneura*) *JH esterase* (*jhe*) gene that is induced by JH

in final larval instar (36). In a more thorough study by Li et al. a 29 nucleotide (nt) JHRE was identified in the promoter region of 13 of 16 genes regulated by JH in both *Drosophila* L57 cells and honey bee brain. This sequence, which is unique from previously identified JHREs, was shown to bind nuclear extracts from JH treated L57 cells (27). Two nuclear proteins – FK509-binding protein (FKBP39) and a calponin-like protein Chd64 demonstrated binding with this JHRE (27). Interestingly yeast two hybrid assays showed that these two proteins also interact with MET, EcR and USP to varying degree prompting the group to propose a model on the switch between JH and 20E action during metamorphosis. The initial efforts to identify JHRE are mostly fragmented and largely remain unverified by further research.

As previously mentioned Li et al has shown that mosquito MET and FISC form heterodimer in a JH dependant manner. RNAi knockdown of either MET or FISC results in considerable decrease in mRNA transcripts of JH inducible genes *Early Trypsin* (*ET*) and *Krh-1* in adult *Aedes* mosquito (21). Chromatin Immunoprecipitation studies have shown that MET and FISC binds to the proximal regulatory region of *ET* gene in mosquito. These DNA-protein interactions were found to be significantly enhanced at a developmental stage with high JH titer. With *ET* expression level corresponding well with JH titer during mosquito adult development, it can be concluded that MET and FISC act directly on the *ET* promoter to activate its transcription. Luciferase assay proved that indeed 2 kb upstream promoter of ET could result in activation of the reporter gene only when both the proteins MET and FISC are expressed in L57 cells in the presence of JH (21). A serial deletion of the promoter region was done to identify the JHRE and nt -540

to -165 was found to be crucial. In this region a sequence (CCACACGCGAAG) similar to the binding site of the mammalian AhR/Arnt bHLH-PAS heterodimer was identified and subjected to further analysis. Through reporter assays and electrophoretic mobility shift assays, the binding of this 12 nt JHRE with MET and FISC was confirmed (21).

Recently, a MET binding motif has been characterized from the promoter region of *Bombyx Kr-h1* gene (termed *k*JHRE) as 'GGCTCCACGTG'(37). Such motifs occur in upstream of *Kr-h1* genes *Tribolium*, *Drosophila*, and *Apis*. Interestingly, there are two MET proteins in *Bombyx*, of which only one activated transcription from the *Kr-h1* JHRE in response to JH when transfected to a human cell line. However, this activation also required addition of a *Bombyx* ortholog of Taiman that, like in *Aedes* and *Tribolium*, interacted with MET in a JH-dependent manner (37). Interestingly, the identified *k*JHRE harbored a E-box motif 'CACGTG', already known to be a binding sequence for bHLH-PAS factors. Looking back at the identified JHRE from *Aedes ET* gene promoter, it can be said that this JHRE includes an E-box-like motif ('CACGCG') with a C in position 5 instead of a T.

Dubrovsky et al, on the other hand, identified Ftz-F1 as the binding partner of MET in *Drosophila* S2 cells (25). The two dimmers activate transcription of target gene through binding with upstream Ftz-F1 response element FIRE (25).

Juvenile hormone signaling pathway:

The role of MET in the JH signaling pathway has already been established. JH binding of MET is also confirmed and it is now considered a receptor of JH in the signaling pathway. Protein binding partner MET- Taiman and Ftz-f1 has been identified.

All these factors have been found to play a significant role in regulating JH mediated gene expression. All this have been discussed in detail above. Apart from this, two other transcription factors have been identified to act downstream JH signal- Kr-h1 and Broad.

Krh-1 is a JH responsive gene. Transcription factor Krh-1 is induced by exogenous application of JH during pupal-adult transition in *Drosophila* (30). Krh-1 shares homology in zinc-finger motifs and amino acid connecting zinc-finger motifs with the segmentation gene *Krüppel*. Expression of *Krh-1* was induced during embryonic and larval stages and disappeared during pupal and adult stages in *Tribolium*. Suppression of JH biosynthesis by dsRNA mediated knock-down of JH biosynthetic pathway enzymes lead to a reduction in the transcript level of *Kr-h1* (38). This phenotype can be rescued by exogenous application of JH or its analog. However, RNAi mediated knockdown of Krh-1 causes precocious larval-pupal transition which could not be prevented by JH treatment (39), indicating that Krh-1 is involved in anti-metamorphic functions of JH and that it is required to maintain a larval state. Further, Krh-1 has also been shown to be involved in pupal JH response. RNAi mediated knockdown of Krh-1 could counteract the formation of an extra pupal stage as a result of exogenous JH treatment. The transcript level of Krh-1 was high in this extra pupal stage upon JH treatment (39). Again these results provide evidence of Kr-h1's involvement in anti-metamorphic action of JH, this time during pupal development. Krh-1 transcripts were significantly down-regulated in *MET* RNAi background in both larval and pupal stages showing that Krh-1 works downstream of MET (39, 24). Involvement of Krh-1 in metamorphosis of has been shown in some other insect holometabolous insects taxa (37) including few hemimetabolous insects like

Pyrrhocoris apterus, Rhodnius prolixus (40) and *Blattella germanica* (41). Krh-1 has been found to play a role in JH signaling pathway downstream of MET during adult development in mosquito (33), thus indicating that this mode of JH signaling is conserved during metamorphosis and reproductive development in insects.

Broad-Complex (BR-C) gene encodes a Broad-Complex-Tramtrack-Bric-a-brac (BTB) domain with one of four alternatively spliced C2H2 zinc-finger motifs Z1-Z4. In *Manduca sexta,* BR-C is involved in JH signaling pathway during pupal metamorphosis in insects (32, 42). Exogenous application of JH or its analogs leads to induction of BR-C gene in *Tribolium* (43). Knockdown of MET results in reduction in BR-C transcript level leading to the conclusion that MET activated BR-C gene (43). BR-C also functions downstream of MET and Krh-1 in JH signaling pathway during *Tribolium* pupal development (39). The role of Broad has been probed in some other insects (42, 44, 45), but the results have failed to establish a unified mechanism of BR-C action in JH signaling. Consequently, BR-C's involvement in JH function needs further verification.

Rational for Current Research

JH's role in adult reproductive development in mosquito *Aedes aegypti* females:

*Aedes aegypti*is the vector mosquito for several devastating human diseases, primarily the Dengue and Yellow fevers. It is an excellent model organism to address questions regarding JH's role in reproductive maturation and adult development of insects. Being a floodwater mosquito *Aedes* eggs could survive periodic stretches of desiccation and thus are easy to rear in the laboratory (47). Its genome has been

sequenced and annotated (48). Moreover, there is only a single copy of the gene encoding the JH receptor MET in *A. aegypti*. JH plays a pivotal role in adult reproductive development in female *A. aegypti* mosquitoes (49). The first phase of the reproductive cycle, also referred to as post eclosion (PE) period, starts just after the eclosion of the female mosquito from its pupal stage. Synthesized by the CA, JH hemolymph titer peaks two days after eclosion (Fig. 3.). JH prepares the mosquito for blood feeding and makes is responsive to signals associated with 20-hydroxyecdysone (20E), the principal hormone governing the vitellogenesis. As a result of the JH action in the fat body it is remodeled into an efficient protein factory. The ovary in response to JH action becomes equipped with the endocytic machinery for massive and specific protein uptake and becomes responsive to ovarian ecdysteriogenic hormone (OEH) secreted from the brain. The female mosquito becomes receptive to mating and is responsive to host stimuli. The other molecular event associated with this PE developmental phase is increase in the ploidy level in the fat body trophocytes. Trophocytes becomes 4n from 2n, while another 20% becomes octaploid by 3rd day post-eclosion (47). In addition ribosomes begin to accumulate. Total RNA increases by about 50% over the first 3 days after eclosion. Also there is development of endoplasmic reticulum and Golgi complexes (47). Female mosquito can take several blood meals and develop corresponding batch of eggs. Before every subsequent blood meal there is a JH governed developmental period absolutely necessary for the proper maturation of that batch of eggs (49). Similar physiological processes mentioned above in this paragraph occur during subsequent JH governed

periods. *In vitro* fat body culture experiments have reinforced the necessity of JH exposure of fat bodies for 20E response and expression of target gene *Vitellogenin* (50).

JH has been shown to have a direct effect on PE follicular maturation in *Aedes*. Shortly after eclosion, a batch of synchronously developing follicles separate from the germarium (51). The follicles, called primary follicles, grow to approximately 100µm in length and by 72 hr enter a morphologically quiescent "resting stage", in which they remain until the female feeds on blood. In some key experiments it has been shown that when the CA was surgically removed (allatectomy) from newly emerged mosquitoes thus removing the source of JH, the primary follicles were underdeveloped. Activation of fat body nucleoli for ribosomal RNA production and ribosomal production itself is blocked by allatectomy of newly eclosed females. But it can be restored by either implantation of CA or topical application of JH to allatectomized females (49, 51).

Though there has been some research on the physiology of JH dependant PE period in *Aedes,* this developmental period largely remains unexplored. With the recent burst of scholarly activity in JH and several key discoveries regarding molecular mode of JH action, this is an exciting time to address questions regarding JH regulation of adult development in mosquitoes.

Using molecular biology, high through put screening techniques, functional genomics and bioinformatics we are trying to address several specific questions concerning the molecular basis of JH action in the *A. aegypti* female mosquitoes. What role does JH play in transcriptional regulation genes during JH dependant PE period in *Aedes* females? What is the mechanism of MET action? Are there any novel downstream

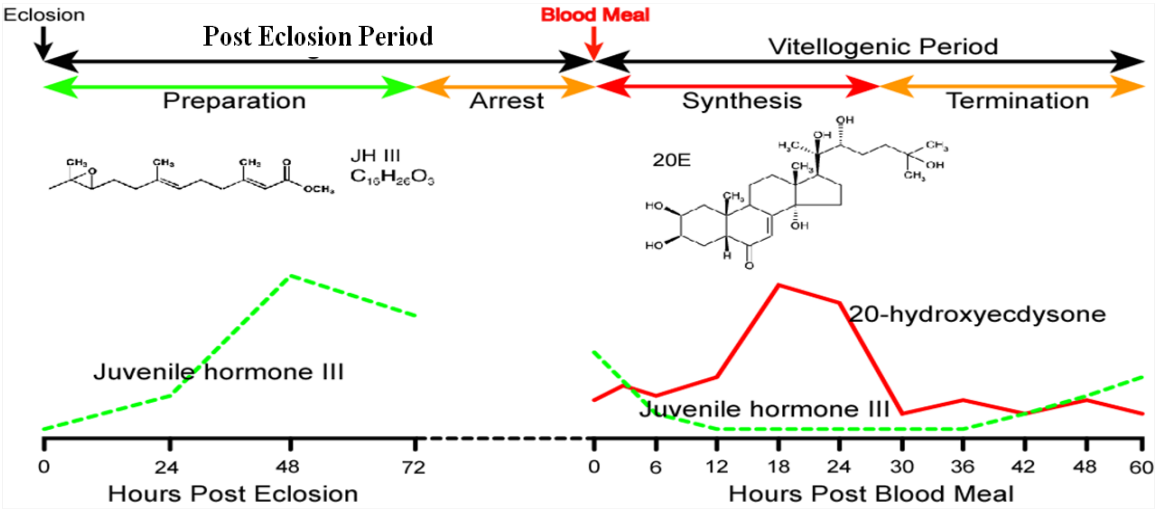
transcription factors effecting JH signaling? How do they transmit the JH/MET signal? Does MET have any additional bHLH factor(s), other than that of Taiman, as its partner(s)?

Fig. 3 legend:

Hormonal titers during the first vitellogenic cycle of the anautogenous mosquito, *A. aegypti.*

Adult reproductive development in mosquito *Aedes aegypti* is controlled by alternating titers of two hormones Juvenile hormone (JH) and 20-hydroxyecdusone (20E). JH controls mosquito development in newly eclosed adult *Aedes* females preparing the mosquito for blood meal regulated events including responsiveness to 20E. 20E is the principal hormone governing the vitellogenesis in mosquitoes. As soon as the mosquito takes blood the JH titer in the haemolymph falls to a basal level. Even small amounts of JH at the vitellogenic synthesis phase have been found to be detrimental for the insect (47). Modified from Zhu et al. (2003) (49).

Fig.3.



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Chapter I

Regulation of gene expression dynamics by juvenile hormone receptor Methoprene tolerant: How JH controls reproductive development in *Aedes aegypti* female mosquitoes

ABSTRACT:

Juvenile hormone (JH) plays a key role in regulating the reproduction of female mosquitoes. Microarray time-course analysis revealed dynamic changes in gene expression during posteclosion (PE) development in the fat body of female *Aedes aegypti*. Hierarchical clustering identified three major gene clusters: 1,843 early-PE (EPE) genes maximally expressed at 6 h PE, 457 mid-PE (MPE) genes at 24 h PE, and 1,815 late-PE (LPE) genes at 66 h PE. The RNAi mediated depletion of the JH receptor Methoprene-tolerant (MET) showed that 27% of EPE and 40% of MPE genes were up-regulated whereas 36% of LPE genes were down-regulated in the absence of this receptor. MET repression of EPE and MPE and activation of LPE genes were validated by an in vitro fat body culture experiment using MET RNAi. Sequence motif analysis revealed the consensus for a 9-mer MET-binding motif, $CACG^{C}/_{T}G^{A}/_{G}{}^{T}/_{A}G$. MET-binding motif variants were overrepresented within the first 300 bases of the promoters of MET activated (LPE) genes but not in MET repressed (EPE) genes. EMSAs using a combination of mutational and anti-MET antibody supershift analyses confirmed the binding properties of the MET consensus motif variants. The major gene functions regulated by EPE/MPE and LPE cluster are strikingly different. While, carbohydrate,

lipid, and xenobiotics metabolism were found to be enriched in EPE/MPE clusters, transcription and translation gene functions dominated the LPE cluster. This study represents a significant advancement in the understanding of the regulation of gene expression by JH and its receptor MET during female mosquito reproduction.

INTRODUCTION:

A unique aspect of female mosquito reproduction is its JH-dependent posteclosion (PE) development, a period critical for a female to become responsive to 20E and digest blood, subsequently resulting in egg maturation. The JH hemolymph titer increases after adult eclosion, reaching a peak at 48–50 h PE and declining slowly over the next 5 d in females not given a blood meal (1). Several aspects of the JH-dependent PE development have been characterized: Ovarian primary follicles grow double in length, their nurse cells increase in ploidy, ovaries develop an endocytic complex of coated vesicles and receptors, and the follicular epithelium undergoes differentiation (2, 3, 4). Likewise, extensive JH-dependent cellular remodeling occurs in the fat body, an insect analog of the vertebrate liver and adipose tissue. This remodeling involves dramatic enlargement of nucleoli, accumulation of ribosomes, and the development of both Golgi complexes and extensive invaginations of the plasma membrane (5, 6). JH also controls translation of the nuclear receptor betaFTZ-F1 (*fushi tarazu* binding factor 1), known as the competence factor (7). However, our understanding of molecular mechanisms underlying JH regulation of female mosquito PE development has been limited.

Recent studies have established that Methoprene-tolerant (MET), a member of the family of basic helix–loop–helix (bHLH)-Per-Arnt-Sim (PAS) transcription factors, is the

JH receptor. In *Drosophila melanogaster*, null mutants of the *MET* gene convey potent resistance to JH and the insecticide methoprene, one of the JH analogs (8, 9). MET binds to JH with a high-affinity–inducing reporter gene transcription (10, 11, 12). RNAi-mediated depletion of MET in the beetle *Tribolium castaneum* leads to precocious pupation, suggesting an anti-metamorphic action of MET (13). In addition, Parthasarathy et al. (14) showed that MET depletion also results in precocious formation of adult features in pupae. Members of bHLH-PAS family transcription factors require the formation of homo- or heterodimers for DNA binding and transcriptional regulation (15). Studies in *Aedes aegypti*, *Tribolium castaneum*, and the silkworm *Bombyx mori* have shown that MET interacts with a bHLH-PAS domain-containing steroid receptor coactivator, SRC [also known as betaFTZ-F1 interacting steroid receptor coactivator ("FISC") and "Taiman"] (12, 16, 17). Several studies have reported identification of the MET-binding response motifs; however, no unified consensus has been reached (12, 16, 18).

We believe that JH receptor MET plays an importnat role during adult PE development in *Aedes aegypti*. To investigate this hypothesis, we first characterized the developmental dynamics of genes expressed during JH-regulated PE development of the female *A. aegypti* fat body, a tissue central to female reproduction. The microarray analysis revealed an unexpectedly high level of gene activity, with 6,146 genes expressed in functionally distinct temporally regulated gene clusters. MET RNAi microarray screens showed a significant disruption of the normal PE gene expression patterns with depletion of MET resulting in repression of genes expressed early during PE and

activation of those that showed maximal expression during late PE stage. In vitro, fat-body culture experiment using MET RNAi validated the direct role of MET in mediating JH action in altering the expression of selected genes. Bioinformatics analysis and EMSA delineated a consensus for a 9-mer MET-binding motif, CACG$^{C}/_{T}$G$^{A}/_{G}$$^{T}/_{A}$G, which was present in the promoters of a number of MET–activated genes. We thus have provided substantial evidence for the central role of JH and its receptor MET in the regulation of female mosquito reproductive biology.

MATERIALS AND METHODS:

Experimental animals:

The mosquito *A. aegypti* UGAL/Rockefeller strain was raised as described previously (19). Adult mosquitoes were fed continuously on water and 10% (wt/vol) sucrose solution. All dissections were performed in Aedes physiological solution at room temperature (19).

Microarray assays:

Single-color hybridizations of custom-made Agilent microarrays with 15,321 *A. aegypti* genes were conducted at the University of Chicago Core Instrument Facility following standard protocols (20). Three independent biological replicates were performed for each treatment. The raw expression data were processed using Agilent Feature Extraction Software (21). Subsequent analysis steps were performed in the statistical programming environment R using Bioconductor packages (22). The expression data were background corrected and quantile normalized with functions from the Agi4 × 44 PreProcess and Limma libraries (23). Statistical analysis of DEGs was

performed with the Limma library. When filtering for DEGs, we used a fold change of ≥1.75 (0.8 in log2 scale) and an adjusted *P* value of ≤0.01, in accordance with our previously used filtering criterion (20). The latter was adjusted for multiple testing with the Benjamin–Hochberg method to determine false-discovery rates (24). Hierarchical clustering with complete linkage was performed with the hclust function in R. The distance matrix required for this method was obtained by computing distance-transformed Spearman correlation coefficients among the expression profiles of all DEGs. Discrete clusters were obtained by cutting the resulting cluster dendrogram with the cutree function using a visually determined height value.

Preparation of JH-deficient mosquitoes:

We used a previously described approach, in which the head and the JH-producing endocrine gland, the corpora allata, were isolated from the rest of a female mosquito body by severing the metathorax and abdomen (3). The wound was sealed with paraffin, and the abdomens then were attached with paraffin to a microscopic slide. Slides with adhered abdomens were placed in Petri dishes with a wet filter paper and kept at 27 °C. These JH-deprived mosquito abdomens were prepared at 6 h PE, a time of very low JH titer (1). The abdomens were treated topically with three applications of 500 pg JH III (Sigma-Aldrich) in acetone (solvent) or with acetone alone (3). JH response was characterized 24 h after hormonal treatment. The effectiveness of eliminating JH was evaluated by measuring the length of ovarian follicles. In JH-deprived mosquitoes treated with acetone (control solvent) only, follicles remained small (50–60 μm), similar to those early in PE, indicating a low level of JH in these preparations. Application of JH III

resulted in growth of ovarian follicles to about 80–90 μm, clearly showing that this treatment was effective in rescuing the arrested follicular growth phenotype. Total RNA was extracted from fat bodies 24 h after hormonal treatment. The experiment was repeated three times under the same conditions.

In vitro fat-body culture:

Female mosquitoes were injected with MET dsRNA or Luc dsRNA within 0–6 h PE. Fat bodies were dissected from mosquitoes 72 h after injection and were incubated in a complete culture medium supplemented with amino acids for 8 h, as described previously (19). JH (10 μg/mL JH III) or solvent (acetone) was added to the culture medium. Total RNA was isolated and analyzed using qPCR. The experiment was repeated three times under same conditions.

RNA extraction:

For the microarray transcriptome analysis, RNA samples collected at nine time points at 6-h intervals after eclosion of female mosquitoes were selected. For MET RNAi analysis, fat body samples were collected 72 h after injection. RNA was extracted from fat bodies of 10 female mosquitoes using the TRIzol method (Invitrogen) according to the manufacturer's protocol. It was concentrated using the RNeasy MiniElute cleanup kit (Qiagen) for further processing.

RNA interference mediated knockdown of MET:

To synthesize MET dsRNA, we followed a method described previously (19). In brief, dsRNA of a specific gene template was synthesized using the MEGAscript kit (Ambion). The luciferase gene was used to generate control iLuc dsRNA. After dsRNA

synthesis, samples were subjected to phenol/chloroform extraction and ethanol precipitation. dsRNA then was suspended in RNase-free water to a final concentration of 5 µg/µL. At 24 h PE, female mosquitoes were injected with 300 nL dsRNA. The Picospritzer II (General Valve) was used to introduce corresponding dsRNAs into the thorax of CO_2-anesthetized female mosquitoes.

Quantitative RT-PCR analysis:

cDNAs were synthesized from 2 µg total RNA using the Omniscript Reverse Transcriptase kit (Qiagen). RNA was treated with DNase I (Invitrogen) before cDNA synthesis. PCR was performed using the Platinum High Fidelity Supermix (Invitrogen). qRT-PCR was performed using the iCycler iQ system (Bio-Rad) and an IQ SYBR Green Supermix (Bio-Rad). Quantitative measurements were performed in triplicate and normalized to the internal control of S7 ribosomal protein mRNA for each sample. Real-time data were collected from the software iCycler v3.0. Raw data were exported to Excel (Microsoft) for analysis. Primers used for qRT-PCR are provided in Table 1.

Table 1 legend:

Primers for qRT-PCR and RNA interference mediated knockdown of gene:

Shown below is a list of primers used for Real time quantitative PCR and RNAi knockdown. All of primers are in 5'-3' direction. The Real time quantitative PCR primer leads to amplification of product 80-140bp in length. The RNAi primers have primers have a T7 RNA polymerase binding sequence at the 5' end.

Table 1.

Primers used for Quantitative RT-PCR and RNAi	
Primers for Q-PCR	
AAEL002704 Forward	AATGGAACGTCCAAGCTGTC
AAEL002704 Reverse	GGGACTGGTTCATGGAGTTG
AAEL002720 Forward	CGATGTACGCGTGGATAATG
AAEL002720 Reverse	GGAAGGTCATCGATTCGGTA
AAEL012131 Forward	ATGGCGTTAAAGAATCCACG
AAEL012131 Reverse	AGCCGGATCACAGTTCATTC
AAEL000693 Forward	AATGCGTTCCGTACTTGTCC
AAEL000693 Reverse	GCTGCTGGAACAAGAACTCC
AAEL010222 Forward	CCCTCAATCTCGGATGAAAA
AAEL010222 Reverse	CGACATGGAAACCACAGTTG
AAEL002576 Forward	GGTGTACTTCGACCTGGGAA
AAEL002576 Reverse	GAGCGTTCCAACGAAGAGTC
AAEL000765 Forward	CAAGCAGCTGCAATACCTGA
AAEL000765 Reverse	GTTACGGACGACGGTGTTCT
AAEL002390 Forward	CAGGTCGTCAATCTGAGCAA
AAEL002390 Reverse	GGTCTGGTGGAAGTTCGTGT
AAEL005480 Forward	CAAGTGCACATTTTGCCATC
AAEL005480 Reverse	GGATCAACTGAACGCTGGAT
AAEL011145 Forward	CGATGGTTTGTTCTCCACCT
AAEL011145 Reverse	GACACTCTTTCAGGGCTTCG
AAEL002080 Forward	ACGGAGGGAAAATGGATACC
AAEL002080 Reverse	TCTTCCCCAATGCTATCGTC
AAEL008621 Forward	GAGAAGGGTTCTGCATCGAG
AAEL008621 Reverse	CCAATCCAAAATCAGCCACT
AAEL002343 Forward	GGAAAAGGGATGCAGCAATA
AAEL002343 Reverse	TATCGATTCCCTGGTTCTCG
AAEL008789 Forward	ACGACGAAGTTGTGGAAACC
AAEL008789 Reverse	GTCCAAAATGCCTGAGTGCT
AAEL013990 Forward	ATATGCGGATGACCAGTTCC
AAEL013990 Reverse	TTTGGAGGCATCTTCAATCC
AAEL001746 Forward	GGACGACAGCTCAAAGAAGG
AAEL001746 Reverse	TCACTTTCATCGGGGAGTTC
AAEL001917 Forward	GAACAGCACAAGCGAATGAA
AAEL001917 Reverse	TTGGCCTTGAGAGTCGTCTT
AAEL000300 Forward	GAAAGACGAACGAAAGCGAC
AAEL000300 Reverse	TCACGATCTCCATCCATCAA
AAEL009642 Forward	GAGGGAAAGTTCGATGTGGA
AAEL009642 Reverse	AATCCCACATCCACCCAGTA
AAEL008596 Forward	TGCCACTCAACAGCAGAATC

AAEL008596 Reverse	CACTTGTGGAGATCCACCCT
AAEL004284 Forward	CAACTGCACAAGCTGAAGGA
AAEL004284 Reverse	ACGTGCGATAGCTTCTTCGT
AAEL002334 Forward	ACGCAACCTCTCTCTCCGTA
AAEL002334 Reverse	CCTTCTGCTTACGCTCAACC
AAEL006586 Forward	ATCTTCCCGGTGTTGACTTG
AAEL006586 Reverse	CTTTCCCTTCTTCACCACCA
AAEL000703 Forward	GCCTGGATATGGCAACAACT
AAEL000703 Reverse	GTTACGATCCAACACGGCTT
AAEL011114 Forward	GAGGAGAAAGCAGCACAACC
AAEL011114 Reverse	TCGAATGGCTTTTCCATTTC
AAEL005093 Forward	GAAGTTATGGTTGCGGAGGA
AAEL005093 Reverse	TTCTAACACGCAAAGCATCG
AAEL008364 Forward	CGTGGCAGACTGAGTTCAAA
AAEL008364 Reverse	GGACGTGACATCCGAGTTTT
AAEL010481 Forward	AAGTGTCGGACCTGTTCACC
AAEL010481 Reverse	GGTGTAGAAAATGACCGCGT
AAEL003619 Forward	CGTTATCCCGTATTTGGTGG
AAEL003619 Reverse	CCTCTGCTCGAAAACTGACC
AAEL002655 Forward	AACAATCGCTACACGGAACC
AAEL002655 Reverse	AACAGTGCAGAGTCGTGGTG
AAEL007568 Forward	AGATTGCAGATGCAGTCACG
AAEL007568 Reverse	ACTTTAGCTGCCTCCACGAA
AAEL015432 Forward	ATCTTCCCGGTGTTGACTTG
AAEL015432 Reverse	CTTTCCCTTCTTCACCACCA
AAEL000001 Forward	AGGCCGACATGGAATACAAG
AAEL000001 Reverse	CCACCTCTACTGCTGCTTCC
AAEL012876 Forward	GATTTCGTTGGGAAAAGCAA
AAEL012876 Reverse	CTCCTTGAGCGATTTCTTCG
AAEL001249 Forward	CCACCGGAGAGTTGAACATT
AAEL001249 Reverse	GGTCGCGTTTTATCGGAGTA
AAEL006860 Forward	ATTCATCAGCGAGCAGAACC
AAEL006860 Reverse	TATCGCAATCTCCTGGCTTC
AAEL010970 Forward	AAAGCTCCGAAAGACAACCA
AAEL010970 Reverse	CCCGCTTCTTCTTCAGTTTG
AAEL000674 Forward	GAGAATTTGGTCGCGATTGT
AAEL000674 Reverse	GGCGAACAGGACACGTAAAT
Primers for RNAi	
MET RNAi Forward	TAATACGACTCACTATAGGGTCAATTTGTTCGACTCTGCG
MET RNAi Reverse	TAATACGACTCACTATAGGGATACACAAAGTCGCCCGTTC

Computational analyses of regulatory gene regions of MET-depletion transcriptome:

The upstream regions of the MET-depleted genes were extracted from the database for the *A. aegypti* genome (http://aaegypti.vectorbase.org/), and pattern searches were performed with an in-house PerlScript for the different 9-mers (25). The 2-kb regions were divided into twenty 100-base windows to check if the patterns show a bias for a certain region within the entire search space. The actual GC content of the MET-depleted up- and down-regulated genes were calculated with the help of another in-house PerlScript. These values were used to calculate the probability of finding the 9-mers by random chance. A pattern search allowing one base mismatch, N (A/T/C/G), for each of the 9-mers was used to check for the degeneracy. The reverse complements for the 9-mers were used to search the "+" strand to account for the "–" strand, and the frequencies of hits from both strands were added. The frequencies of observation by random chance within both stands were calculated when results were plotted.

Electrophoretic mobility shift assays (EMSA):

The annealed deoxyoligonucleotide of each motif was purified from 15% TBE Criterion Precast Gel (Bio-Rad) and labeled with γ-^{32}P ATP. EMSA was performed using a gel-shift assay system (Promega) with the fat-body nuclear extract of female mosquitoes 48 h PE. Nuclear extracts were prepared as described previously (26). The DNA–protein complex was separated on 5% TBE Criterion Precast Gel (Bio-Rad). After electrophoresis, the gel was dried and incubated with phosphor imaging screen in the cassette overnight to reach highest exposure and was visualized in the Personal Molecular Imager (Bio-Rad) by auto-radiography. For the competition assay, 50-fold unlabeled E-

box–like motif or unlabeled AP-2 motif (nonspecific competitor oligonucleotides; Promega) was incubated with nuclear extract for 10 min and then was incubated further with the labeled probe for 20 min. Identity of a complex was verified by directly adding polyclonal antibodies against *A. aegypti* MET.

Table 2 legend:

Oligonucleotides used for EMSA assay:

E-box and E-box like motifs and their flanking sequences from the promoter regions of indicated genes used for gel mobility shift assays. The oligonucleotides used as mutated versions of original promoters for this study are also provided.

Table 2.

Probes for EMSA		
Probe Name	***Probe Sequence***	***GeneID***
T1	TGGCTGCGATGGCGCACGCGGAGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCTCCGCGTGCGCCATCGCAGCCA	
T2	TAAATTGGATTATTCACGTGTCATTACAGTAAATCGTC	AAEL000674
	GACGATTTACTGTAATGACACGTGAATAATCCAATTTA	
T3	AATATTAGTCCCAACACTTGGAGATTGAGGAAAAATA	AAEL007568
	TATTTTTCCTCAATCTCCAAGTGTTGGGACTAATATT	
T4	TTGACTGACCCACCACTCGGTGCATGACTGATCGG	AAEL007568
	CCGATCAGTCATGCACCGAGTGGTGGGTCAGTCAA	
T1-mut(mutated)	TGGCTGCGATGGCGTCAATAGAGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCTCTATTGACGCCATCGCAGCCA	
B1	TGGCTGCGATGGCGCACGCGAAGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCTTCGCGTGCGCCATCGCAGCCA	
B2	TGGCTGCGATGGCGCACGCGGTGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCACCGCGTGCGCCATCGCAGCCA	
B3	TGGCTGCGATGGCGCACGCGATGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCATCGCGTGCGCCATCGCAGCCA	
S21	TGGCTGCGATGGCGCACGTGATGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCATCACGTGCGCCATCGCAGCCA	
S22	TGGCTGCGATGGCGCACGTGGTGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCACCACGTGCGCCATCGCAGCCA	
S23	TGGCTGCGATGGCGCACGTGAAGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCTTCACGTGCGCCATCGCAGCCA	
S24	TGGCTGCGATGGCGCACGTGGAGGATTTCCGGTTGTGG	AAEL006860
	CCACAACCGGAAATCCTCCACGTGCGCCATCGCAGCCA	

RESULTS:

Gene expression analysis of mosquito *A. aegypti* female fat body during JH dependant PE development:

We used custom-made Agilent microarray chips that contained probe sets corresponding to 15,321 *A. aegypti* genes (20) and examined fat-body samples collected at nine time points spanning the entire PE developmental period of female mosquitoes, from 0–6 h until 72–78 h (Fig. 1*A*). Differentially expressed gene (DEG) sets were established by comparing transcripts from each of the eight PE time points with that from 0–6 h PE using a minimum fold change of ≥1.75 (0.8 in a log2 scale) as the confidence threshold and a false-discovery rate (*P* value) of ≤0.01, as has been used previously (20). There was an unexpected increase of gene expression activity in the PE fat body of female *Aedes* mosquitoes (Fig. 1*A*). The number of DEGs increased dramatically over the course of PE development, reaching a maximum at 60–66 h PE, with 6,146 genes showing differential expression (Fig. 1*A*).

To analyze the gene-expression dynamics during PE development, we performed hierarchical clustering of the DEGs identified in the previous step (Fig. 1*B*). Partitioning of the resulting gene dendrogram identified 11 clusters. From these 11 clusters, three major clusters with expression trends relevant for fat-body PE development were identified: early posteclosion (EPE), mid posteclosion (MPE), and late posteclosion (LPE) (Fig. 1*B* and *C*). The 1,843 genes of the EPE (clusters 1 and 4) have the highest expression around 0–6 h PE, followed by a gradual decline throughout PE development (Fig. 1*C*). The MPE (cluster 10) contained 457 genes. Their expression levels peaked at

18–24 h PE and declined thereafter. For LPE (cluster 2) with 1,815 genes; transcript levels were low at 0–6 h PE, rose throughout PE to reach peak expression at 60–66 h, and declined moderately at 72–78 h PE (Fig 1*C*). Quantitative RT-PCR (qPCR) analysis of selected EPE, MPE, and LPE genes showed correlation with microarray data (Fig 2*A*-*L*).

Therefore, our data suggests that LPE genes are showing an expression peak at a developmental time period when hemolymph titer of mosquito is high. On the other hand, EPE gene transcripts are abundant when there is little JH in the hemolymph and decreases with a gradual increase in the level of JH. MPE genes were maximally expressed at intermediate JH level. Because JH is the principal hormone governing the development of PE mosquitoes, we hypothesize that its titers are responsible for differential gene expression. Moreover, we suggest that the JH receptor MET mediates these effects of JH.

Fig. 1 legend:

The microarray analysis of the fat-body transcriptome during PE development of *A. aegypti* female mosquitoes:

(*A*) Correlation between the JH titer and number of differentially regulated genes throughout the PE developmental period. Number of significantly up-regulated and down-regulated genes in the microarray experiment [fold change ≥1.75 (0.8 in log2 scale) and a false-discovery rate (*P* value) of ≤0.01] referred in a chronological time order (h PE). JH titers (dashed line), labeled on the right *y*-axis, are from Shapiro et al. (8). (*B*) Hierarchical clustering result of DEGs. The heatmap shows the expression values (mean among three replicates) for the nine different time points. Each row represents data for one probe, and each column corresponds to a time point between 0–6 h and 72–78 h PE, with 6-h intervals. Cutting the gene dendrogram identified 11 discrete clusters, which are color coded on the left. Clusters 1 and 4 were designated as EPE, cluster 10 as the MPE, and cluster 2 as the LPE gene cohorts. (*C*) Schematic expression profiles of EPE, MPE, and LPE gene sets during the 78-h PE period.

Fig. 1.

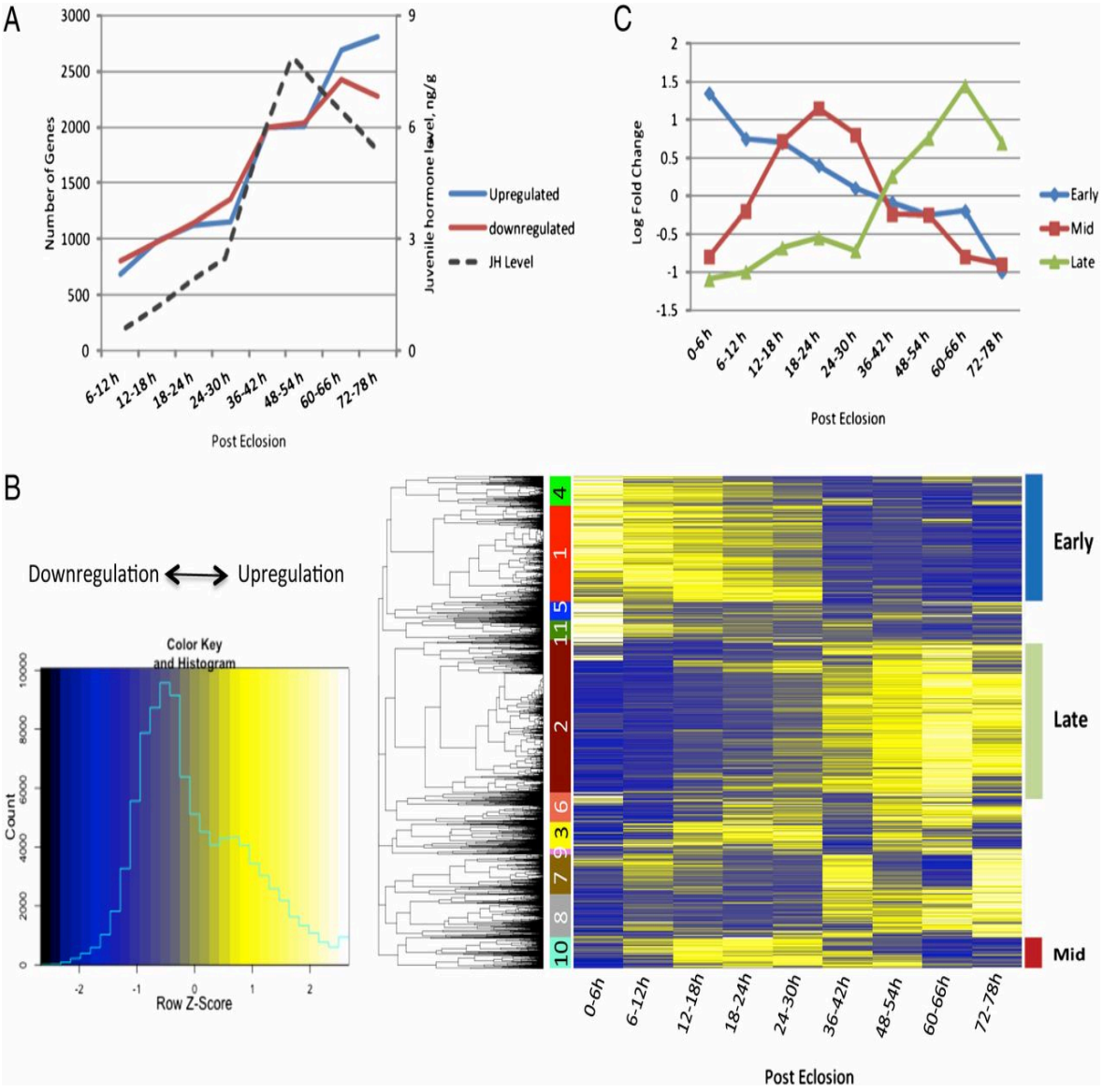
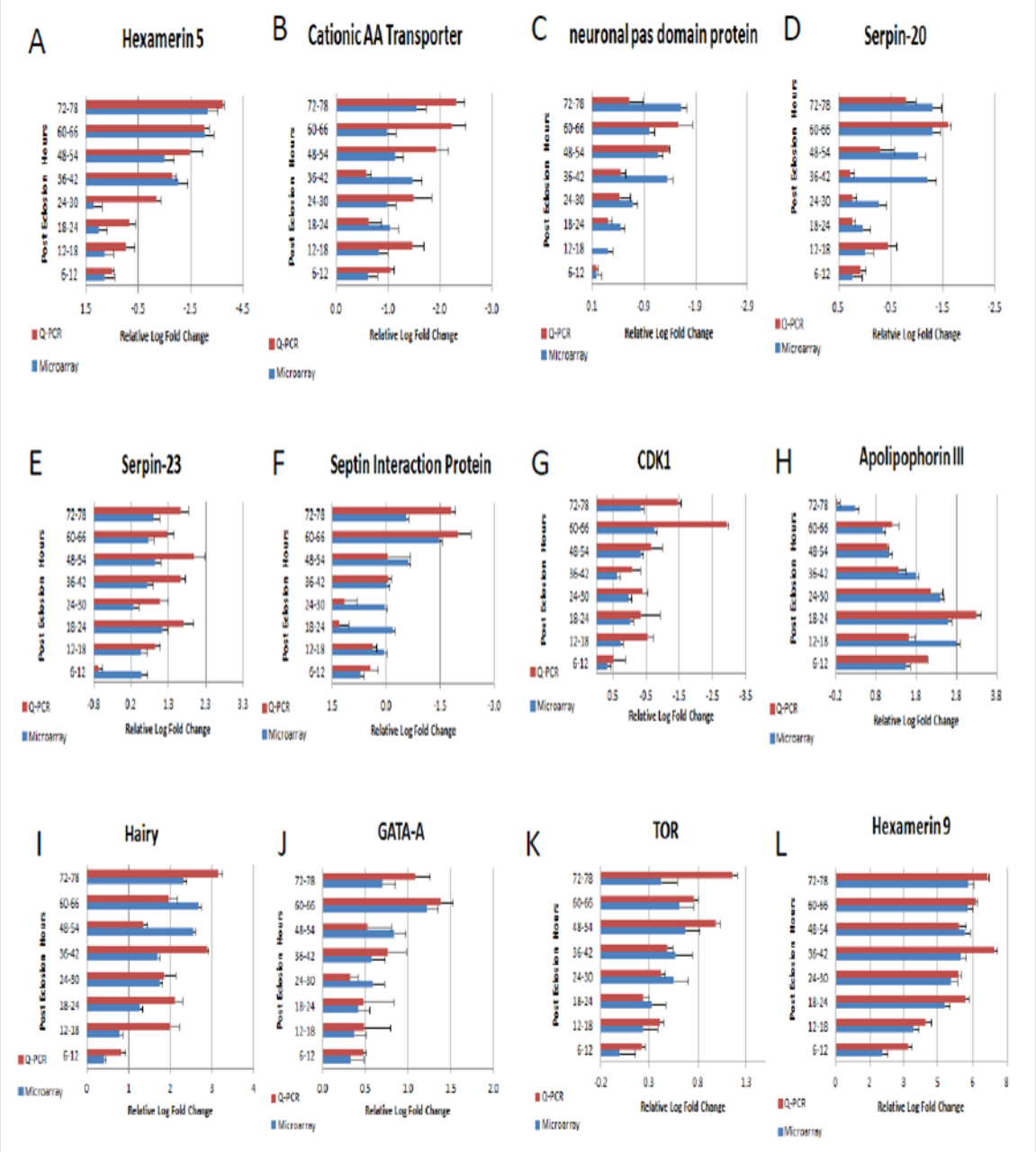

A
Number of Genes
Juvenile hormone level, ng/g
Upregulated
downregulated
JH Level
6-12 h
12-18 h
18-24 h
24-30 h
36-42 h
48-54 h
60-66 h
72-78 h
Post Eclosion
C
Log Fold Change
Early
Mid
Late
Post Eclosion
B
Downregulation
Upregulation
Color Key and Histogram
Count
Row Z-Score
Early
Late
Mid
0-6h
6-12h
12-18h
18-24h
24-30h
36-42h
48-54h
60-66h
72-78h
Post Eclosion

Fig. 2 legend:

Quantitative real time-PCR (qRT-PCR) validation of expression profiles of genes selected from the microarray data analysis:

(*A–D*) Comparison using four genes from the early-posteclosion (EPE) cluster: *hexamerin 5* (*A*), *cationic AA transporter* (*B*), *neuronal Per-Arnt-Sim (PAS) domain protein* (*C*), and *serpin-20* (D). (*E–H*) Comparison using four genes from the mid-posteclosion (MPE) cluster: *serpin-23* (*E*), *septin interaction protein* (*F*), *cyclin-dependent kinase 1* (CDK1) (*G*), and *apolipophorin III* (H). (*I–L*) Comparison using four genes from the late-posteclosion (LPE) cluster: *Hairy* (*I*), *GATA-binding transcription factor activator (GATA-A)* (*J*), *target of rapamycin* (*K*), and *hexamerin 9* (*L*). Red bars show qPCR data, and blue bars show microarray data. Hours posteclosion and relative log2 fold change are shown in the y- and x-axes, respectively.

Fig. 2.


A
Hexamerin 5
B
Cationic AA Transporter
C
neuronal pas domain protein
D
Serpin-20
E
Serpin-23
F
Septin Interaction Protein
G
CDK1
H
Apolipophorin III
I
Hairy
J
GATA-A
K
TOR
L
Hexamerin 9
Post Eclosion Hours
72-78
60-66
48-54
36-42
24-30
18-24
12-18
6-12
Q-PCR
Microarray
Relative Log Fold Change

Testing JH sensitivity of EPE and LPE genes:

Selected genes from EPE and LPE cluster were tested for JH sensitivity. For this purpose, we used preparations of isolated mosquito abdomens deprived of JH. The specimens were prepared as described in *Materials and Methods*. Total RNA was isolated 24 h after preparing JH-deprived mosquitoes, and transcript levels were measured by qRT-PCR for seven EPE and nine LPE genes (Table 3). The expressed EPE gene transcripts in the JH-deprived abdominal isolations could be inhibited by exogenous JH application for selected candidates (Fig 3*A-D* and Table 3). In contrast, LPE genes transcripts could be induced by JH application (Fig 3*E-H* and Table 3). The transcript levels of testes LPE genes were found to be at basal level in the absence of JH. Thus, for subset of EPE and LPE genes the respective inhibitory and activating effect of JH was confirmed lending further support to our hypothesis that identified PE cluster profile are, at least in part, JH linked.

Fig. 3 legend:

JH sensitivity of EPE and LPE genes:

JH deficient isolated abdomens were prepared by severing the body between meso- and meta-thorax at 6 h PE. Then, meta-thorax/abdomen-containing bodies were sealed by paraffin and topically treated with JH III or solvent. Total RNA was extracted 24 h post-treatment and analyzed by means of qRT-PCR. Target genes were selected from the time-series PE transcriptome gene clusters. The EPE gene encoding *Hexamerin-5*, *Glycogen phosphorylase*, *Metalloproteinase* and *Sodium channel* were repressed (*A, B, C, D*), while the LPE gene encoding for *Hairy*, *Cathepsin B, JH-inducible protein*, and *Serine/threonine protein kinase* was induced by JH (*E. F G, H*). Data were normalized using S7 as an internal control. Relative log2-fold changes were established by comparing transcript levels of each sample to 0–6 h PE mosquito sample. Asterisk (*) represents $P < 0.01$ (*t* test).

Fig. 3.

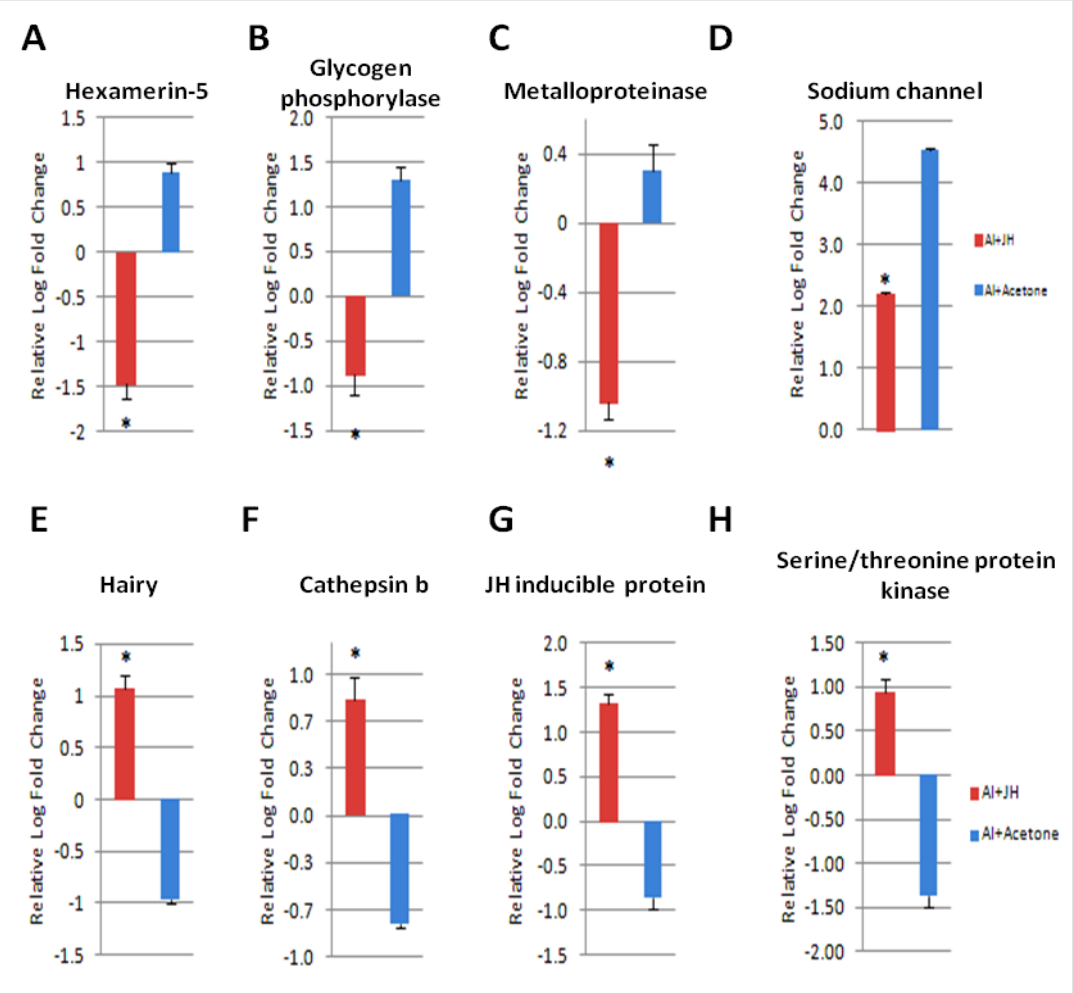


Table 3 legend:

Complete list of EPE and LPE genes showing JH response:

JH deficient 'isolated abdomens' were prepared as mentioned in *Material and Methods*. 'Isolated abdomens were treated with either JH or solvent alone. Experimental details can be found in Fig. 3 legend. Genes in bold are depicted in Fig. 3(*A-H*).

Table 3.

VectorbaseID	Gene Name	Cluster of PE development	JH responsiveness
AAEL000765	***Hexamerin 5***	**EPE**	**Repressed**
AAEL000703	***Glycogen phosphorylase***	**EPE**	**Repressed**
AAEL002655	***Matrix metalloproteinase***	**EPE**	**Repressed**
AAEL003619	***Sodium channel***	**EPE**	**Repressed**
AAEL006586	*Serine protease*	EPE	Repressed
AAEL010481	*Sugar transporter*	EPE	Repressed
AAEL015432	*Trypsin, putative*	EPE	Repressed
AAEL005480	***Hairy protein***	**LPE**	**Induced**
AAEL009642	***Cathepsin b***	**LPE**	**Induced**
AAEL001833	***Juvenile hormone-inducible protein, putative***	**LPE**	**Induced**
AAEL011114	***Serine/threonine-protein kinase rio2***	**LPE**	**Induced**
AAEL001917	*Ribosome biogenesis protein brix*	LPE	Induced
AAEL000300	*Wd-repeat protein*	LPE	Induced
AAEL004284	*Mitochondrial ATPase inhibitor, putative*	LPE	Induced
AAEL002334	*Eukaryotic translation initiation factor 3 subunit*	LPE	Induced
AAEL010434	*Vitellogenin A1*	LPE	Induced

The effect of MET depletion on the fat body transcriptome in *Aedes* female mosquitoes PE:

MET, being the receptor of JH, is expected to play a central role in mosquito PE development. In preparation for the planned downstream experiments we generated MET depleted mosquito by injecting *MET* dsRNA to 6-12 h PE female mosquitoes. Luciferase (Luc) dsRNA was injected as control as mosquitoes lacked this gene in its genome. *MET* transcript level was measured in this knockdown mosquitoes 72 h post injection as a quality control. Compared with the *iLuc* control, the *MET* transcript levels, visualized using reverse transcriptase-PCR, was very low in the female mosquito fat body after the injection of *MET* dsRNA (Fig. 4*D*), giving confidence in the knockdowns. As previously reported, deprivation of JH results in disruption of PE development in mosquitoes which is manifested by retardation of normal ovarian follicular growth (2, 3). Strikingly, depletion of MET resulted similar follicular phenotype indicating PE developmental anomalies and lending strong support to the hypothesis that MET is key player in JH signaling cascade. (Fig. 4 *A* and *B*). Further, the transcript abundance of MET marker genes- *Sodium solute symporter, Krüppel homolog -1, Hairy* were also tested with qRT-PCR (Fig. 4*C*).

The MET depleted mosquitoes were profiled for its transcriptome. Fat body RNA was isolated from both MET dsRNA-injected and *Luc* dsRNA-injected (control) mosquitoes at 72 h post injection and were subjected to microarray analysis Downstream processing was performed as mentioned before. Transcripts with differential expression of ≥1.75 folds (0.8 in a log 2 scale) and a false-discovery rate (*P* value) of ≤0.01 were

selected for further examination. With this filtering criterion, 1,385 and 1,169 genes were found to be repressed and activated by *MET* respectively. The microarray could be validated with qRT-PCR for 11 selected genes (seven down-regulated and four up-regulated) from MET-depleted fat bodies (Fig. 5*A* and *B*).

Comparison of the fat-body transcriptomes of MET-depleted mosquitoes with that of the PE developmental time course revealed that iMet results in up-regulation of 491 (27%) and 181 (40%) of EPE and MPE genes, respectively (Table 4; Fig. 6*A* and *B*). On the other hand, 645 (36%) of LPE genes were down-regulated by iMet (Table 4; Fig. 6*C*).Statistically, the two-tailed *P*-values of < 0.001 (Fisher's exact test) suggests that the overlaps (associations) in all the three cases are highly significant. If MET is involved in regulation of PE gene expression profiles, MET depletion would result in repression of LPE and activation of EPE and MPE genes. For significant portion of LPE (36%), EPE (40%) and EPE (27%) genes this holds true. In contrast, only 4% of LPE genes were up-regulated*; and* a mere 1% of EPE and 3% of MPE genes were down-regulated by MET depletion (Table 4). Taken together, these experiments clearly establish JH receptor MET as a significant mediator of normal PE gene expression in the female fat body of *A. aegypti* mosquito.

Fig. 4 legend:

Validation of Methoprene-tolerant (MET) RNAi depletion (iMet):

(*A*) iMet caused retardation of (post eclosion) PE ovarian growth. Follicle length (in micrometers) was measured from ovaries for UGAL-strain (wild type) mosquitoes 6 h PE and from control luciferase-depleted (iLuc) mosquitoes and MET-depleted (iMet) mosquitoes (both 72 h PE). Data are shown for individual follicle sizes (n = 24), P values (t test) are shown. (*B*) Microscopic images of ovarian follicles of iLuc and iMet mosquitoes 72 h PE. (Magnification: 10×). (*C*) Quantitative RT-PCR analysis of MET marker genes in MET depleted mosquito fat body. *Sodium solute symporter 1*, *Kr-h1* and *Hairy* were used. Statistically significant differences (two sample t-test; P value ≤ 0.01) between samples are labeled. (*D*) RT-PCR analysis of RNA levels in fat bodies of UGAL, iLuc, and iMet female mosquitoes at 72 h PE.

Fig. 4.

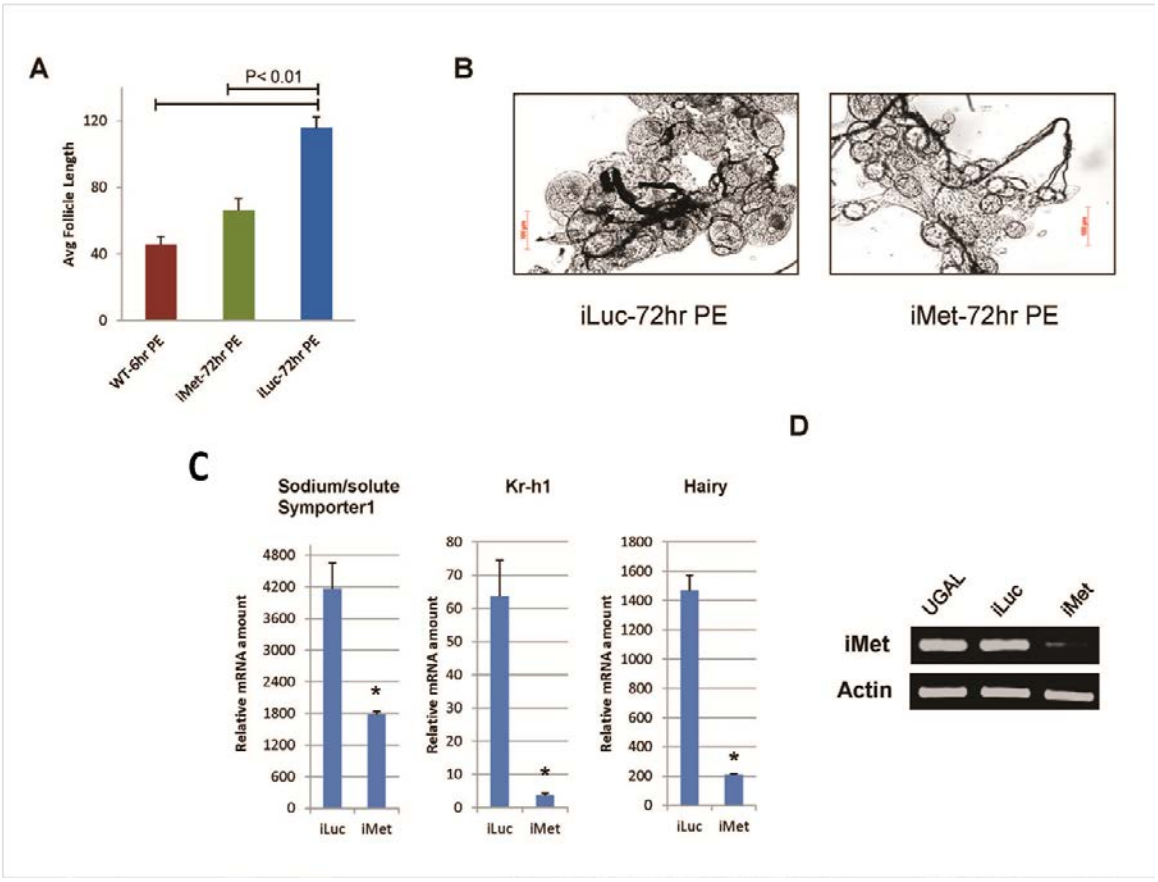

A
P< 0.01
Avg Follicle Length
120
80
40
0
WT-6hr PE
iMet-72hr PE
iLuc-72hr PE
B
iLuc-72hr PE
iMet-72hr PE
C
Sodium/solute Symporter1
Kr-h1
Hairy
Relative mRNA amount
iLuc
iMet
D
UGAL
iLuc
iMet
iMet
Actin

Fig. 5 legend:

Validation of MET depleted mosquito transcriptome:

Quantitative RT-PCR validation of microarray analysis data of gene-expression profiles from the fat body of MET RNAi-depleted mosquitoes. Genes for the qRT-PCR analysis were chosen from the iMet down-regulated (*D*) and the iMet up-regulated (*E*) gene repertoires. The y-axis shows the AAEL numbers for the genes, and the x-axis shows the relative log fold change. Red bars show qRT-PCR data, and blue bars show microarray data.

Fig. 5.

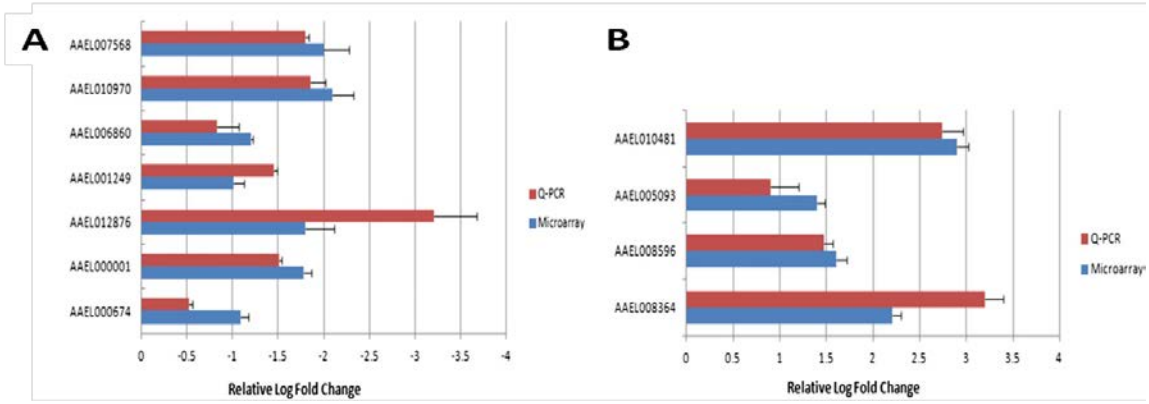


Table 4 legend:

Comparison between MET regulated and PE transcriptome:

All the eleven clusters generated by the hierarchical clustering of differentially expressed genes from the fat bodies of mosquitoes spanning the PE developmental period were compared with MET activated and repressed gene sets Cluster 1 and 4 were combined together as Early Post Eclosion (EPE) and therefore are represented together. The total number of genes belonging to each cluster and the corresponding number of genes in cluster affected as result of MET depletion are mentioned. The percentage of total number of genes in each cluster altered by MET depletion has also been indicated.

Table 4.

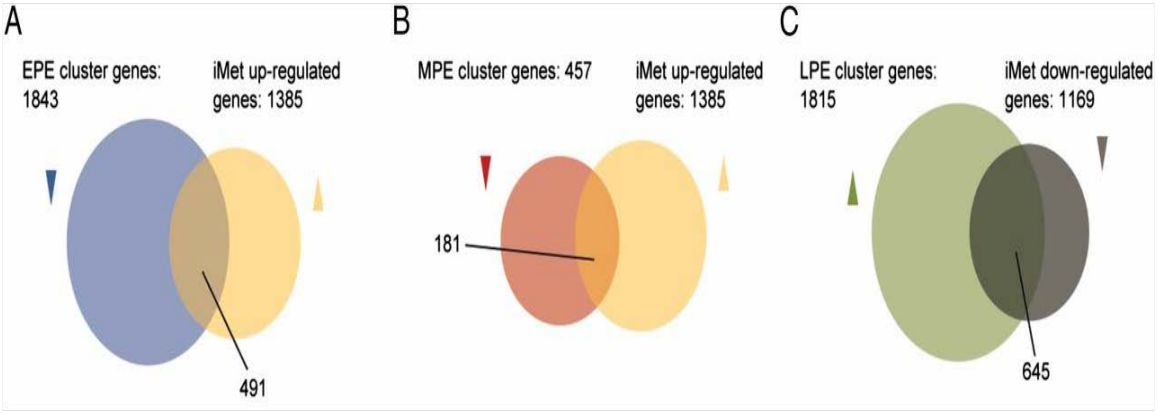
	EPE	MPE	LPE	Cluster 8	Cluster 3	Cluster 6	Cluster 7	Cluster 9	Cluster 5	Cluster 11
Total no. of genes in the cluster	1843	457	1815	480	227	238	603	23	178	293
MET **Repressed**	**491**	**181**	**76**	21	92	34	31	4	16	27
% of total cluster genes **Repressed** by MET	**27%**	**41%**	**4%**	4%	41%	14%	5%	17%	9%	9%
MET **Activates**	**20**	**12**	**645**	66	4	15	11	0	6	22
% of total cluster genes **Activated** by MET	**1%**	**3%**	**36%**	14%	2%	6%	2%	0%	3%	7%

Fig. 6 legend:

Venn diagrams showing the overlap between MET depleted transcriptome and EPE, MPE and LPE clusters:

(*A* and *B*) Venn diagrams comparing the up-regulated genes from the MET RNAi-depleted transcriptome with (*A*) the EPE cluster and (*B*) the MPE cluster. (*C*) Venn diagram comparing the down-regulated genes from the MET RNAi-depleted transcriptome with the LPE cluster.

Fig. 6.



Determining the role of MET in JH mediated gene regulation:

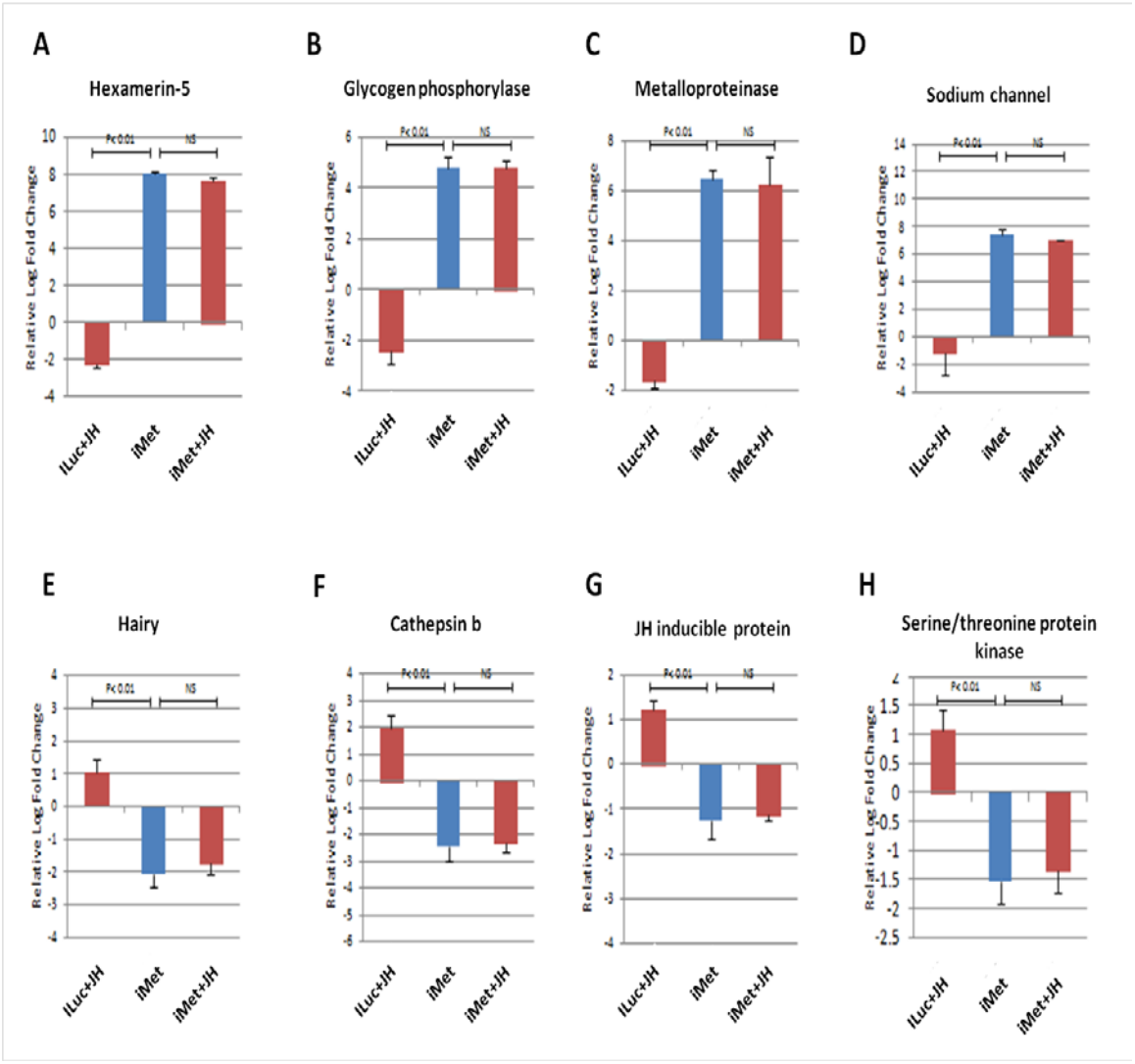
To verify MET's involvement in this JH regulation *in vitro* fat body culture experiments were performed. Fat bodies from MET dsRNA- and Luc dsRNA-injected mosquitoes were incubated in a complete culture medium supplemented with either JH or solvent (acetone) for 3h. Already identified JH regulated genes belonging to EPE and LPE cluster were used for this analysis. RNA was extracted from the incubated fat bodies and tested using qRT-PCR for transcript abundance. In fat bodies from *iLuc* control mosquitoes, these genes showed a significant response to the presence of JH in the culture medium correlating with the effect of JH application on these genes in JH-deprived 'isolated abdomens'. As expected, JH had an inhibitory and activatory effect on the expression of the selected sets of EPE and LPE genes respectively (Fig 7*A*-*H*). However, the JH sensitivity of these genes was severely compromised in fat body cultures of mosquitoes lacking MET, clearly establishing the MET's role in JH dependant regulation of these genes (Fig 7*A*-*H*).

Fig. 7 legend:

MET mediates the JH regulation of EPE and LPE genes:

(*A-H*) MET RNAi renders JH target genes insensitive to JH. Fat bodies 72 h after injection of MET dsRNA were dissected and incubated with or without JH (10 μg/ml JH III) for 3 h at 27°C. Identified JH target genes (Fig. 7) were characterized by means of quantitative qRT-PCR for gene expression level. Data were normalized using S7 as an internal control. Relative log2-fold changes were established by comparing transcript levels of dsMET (iMet) sample to dsLuc (iLuc) control mosquito sample. Statistically significant differences (two sample *t*-test; *P* value ≤0.01) between samples are labelled.

Fig. 7.


A
Hexamerin-5
B
Glycogen phosphorylase
C
Metalloproteinase
D
Sodium channel
E
Hairy
F
Cathepsin b
G
JH inducible protein
H
Serine/threonine protein kinase
Relative Log Fold Change
P< 0.01
NS
lLuc+JH
iMet
iMet+JH

Kyoto Encyclopedia of Genes and Genomes (KEGG) based functional genomic analysis of PE and MET-regulated fat body transcriptomes:

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we examined the ontology of the genes belonging to the EPE, MPE, and LPE clusters (20). We found that the genes could be categorized into 13 functional groups based on the biological processes and molecular functions (Fig. 6*A*). Our results clearly indicated that functionally, the genes belonging to EPE/MPE are distinctly different from the ones grouped under LPE. The major function categories from the EPE/MPE clusters encoded enzymes involved in carbohydrate and lipid metabolism. Other notable functional gene groups were xenobiotics metabolism, organismal systems, cellular processes, and folding, sorting and degradation (Fig. 8*A*). LPE, on the other hand, comprised of genes involved in translation machinery, amino acid metabolism, transcription machinery and replication Translation happens to be largest LPE functional group which has minimal representation in EPE/MPE clusters. In addition to above mentioned functional categories, each of these clusters contained a large proportion of genes belonged to diverse or unknown functional classes, defined as having either insufficient information or no significant matches to other organisms.

Functional categories for *iMet*–up-regulated and –down-regulated genes were found to be significantly different as well. The functional groups that showed significant overrepresentation ($P < 0.01$ in a hypergeometric distribution) among the *iMet*–up-regulated genes but not among the *iMet*–down-regulated genes were carbohydrate and lipid metabolism, cellular processes, xenobiotics biodegradation and metabolism,

organismal systems, biosynthesis of other secondary metabolites and signal transduction (Fig. 8*B*). On the other hand, functional groups of replication and repair, translation, transcription, nucleotide metabolism, and folding sorting and degradation were enriched ($P < 0.01$ in a hypergeometric distribution) among the *iMet*–down-regulated genes but not among the *iMet*–up-regulated genes (Fig. 8*B*). Amino acid metabolism was the only functional group that was overrepresented within both gene sets.

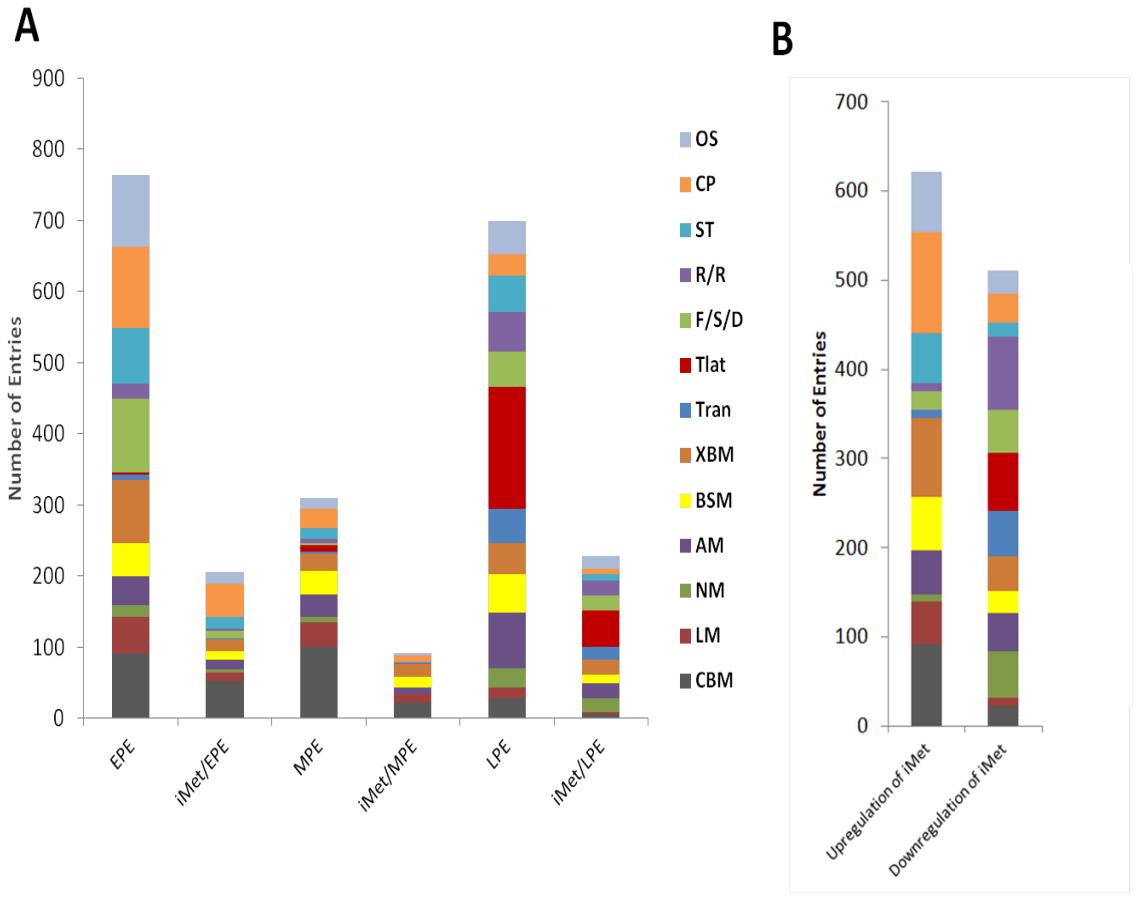
Large overlaps between iMet–up-regulated and EPE genes were observed in the functional categories cellular processes and carbohydrate metabolism; the two-tailed $P < 0.001$ (Fisher's exact test) confirms the very high statistical significance of the overlaps. Significant overlaps ($P < 0.01$) were observed in case of carbohydrate metabolism, xenobiotics, biodegradation and metabolism, and biosynthesis of other secondary metabolites when the *iMet*–up-regulated and the MPE gene sets were considered (Fig. 8*A*). Genes down-regulated in the MET-depleted transcriptome largely overlapped with those of the LPE cluster, especially those encoding for the transcription and translational machinery ($P < 0.001$) (Fig. 8*A*). Additionally, the genes encoding for the DNA replication system were found to be down-regulated by *MET* knockdown (Fig.8*A*).

Fig. 8 legend:

Gene ontology analysis of mosquito PE and MET depleted transcriptomes:

(*A*) Gene ontology analysis of gene clusters expressed in the female fat body PE. EPE, early posteclosion gene cohort; MPE, mid-posteclosion gene cohort; LPE, late posteclosion gene cohort. iMet/EPE, iMet/MPE, and iMet/LPE, comparison of the Met RNAi-depleted transcriptome with respective clusters. (*B*) Distribution of gene functional groups within up- and down-regulated MET RNAi-depleted transcriptome. Functional group abbreviations are OS, organismal systems; CP, cellular process; ST, signal transduction; R/R, replication and repair; F/S/D, folding sorting and degradation; Tlat, translation; Tran, transcription; XBM, xenobiotics biodegradation and metabolism; BSM, biosynthesis of other secondary metabolites; AM, amino acid metabolism; NM, nucleotide metabolism; LM, lipid metabolism; CBM, carbohydrate metabolism.

Fig. 8.


A
B
Number of Entries
900
800
700
600
500
400
300
200
100
0
EPE
iMet/EPE
MPE
iMet/MPE
LPE
iMet/LPE
OS
CP
ST
R/R
F/S/D
Tlat
Tran
XBM
BSM
AM
NM
LM
CBM
700
600
500
400
300
200
100
0
Number of Entries
Upregulation of iMet
Downregulation of iMet

Identification of consensus MET-binding motif in MET-regulated genes from *Aedes* mosquitoes:

Utilizing the expertise brought into our Laboratory by the Bioinformatics specialist, Dr Sourav Roy, we searched for putative MET-binding motifs in upstream regulatory regions of MET-dependent genes identified in this study. Previously published data provided experimental evidence that two sequences—CCACACGCGAAG and CACGCGGTG—in the promoters of *A. aegypti* midgut-specific *early trypsin* (*ET*) and *kruppel homolog 1* (*Kr-h1*) genes, respectively, bind MET (16, Chapter III). Both of these MET-binding sequences shared the sub-pattern CACGCG, which is similar to but not identical with the palindromic canonical E-box motif, CACGTG, with thymine replaced by cytosine at the fifth position. E-box is a characteristic signature of recognition for the family of transcription factors containing the bHLH-PAS protein structural motif (17). The JH response element from the *B. mori Kr-h1* gene (*k*JHRE)—GGCTCCACGTG—contains the canonical E-box sequence (12). The responsiveness of the *k*JHRE to MET has been confirmed experimentally in a cell-culture transfection assay (12). However, a putative JH binding motif sequence reported for *Drosophila melanogaster* and the honey bee *Apis mellifera* was suggested to be significantly different and contained neither the E-box nor any E-box–like motifs. Moreover, direct interaction of this motif with MET has not been confirmed (18).

We used a pattern-search approach to identify the presence of putative MET-binding sites in MET-coregulated gene cohorts. We extracted 2-kbp regions upstream of the translation start sites (TSSs) for 1,154 of the 1,169 MET–activated genes from the

Vectorbase database. These regions were divided into twenty 100-base windows and were searched for the presence of the known mosquito MET-binding motifs CACGCGGTG and CACGCGAAG. There were only 8 and 16 exact matches for these two MET-binding motifs, respectively, within the search space of 1,154 genes. This result suggested that the consensus for the MET-binding site might be more degenerate.

Based on the bioinformatics analysis and prior experimental evidence, we propose, that the consensus for the MET-binding motif in *A. aegypti* is CACG$^{C}/_{T}$G$^{A}/_{G}{}^{T}/_{A}$G (CACGYGRWG) (Fig. 9*A* and *B*). This definition is inclusive of the experimentally proven MET-binding motifs in *A. aegypti*, the JH response element in *B. mori*, and the palindromic canonical E-box motif. Of the 1,154 MET-depleted genes that were down-regulated by a fold change of ≥1.75 (0.8 in log2 scale), 68 were found to carry the putative MET-binding motif CACGYGRWG, in either the "+" or "–" strand. The motif appeared twice within the 2-kb upstream regions of five of these sequences. The GC content for the 1,154 sequences searched was ~40%. Therefore, the probability of finding the consensus within each of the 100-base windows for all 1,154 sequences by random chance is approximately 3. It was observed that the occurrence of the putative MET-binding motif CACGYGRWG within the first 300 bp from the TSSs was three (1–100 bases; 201–300 bases) to four (101–200 bases) times more, which is significantly higher (P = < 0.1 and < 0.05 respectively; chi square test) than what one would expect by random chance (Fig. 9*C*) and suggests a positional bias for this region. For the 1,334 of 1,385 MET genes that were repressed by a fold change of ≥1.75 (0.8 in log2 scale), no such pronounced peaks were observed when 2 kbp upstream of the TSS of these genes

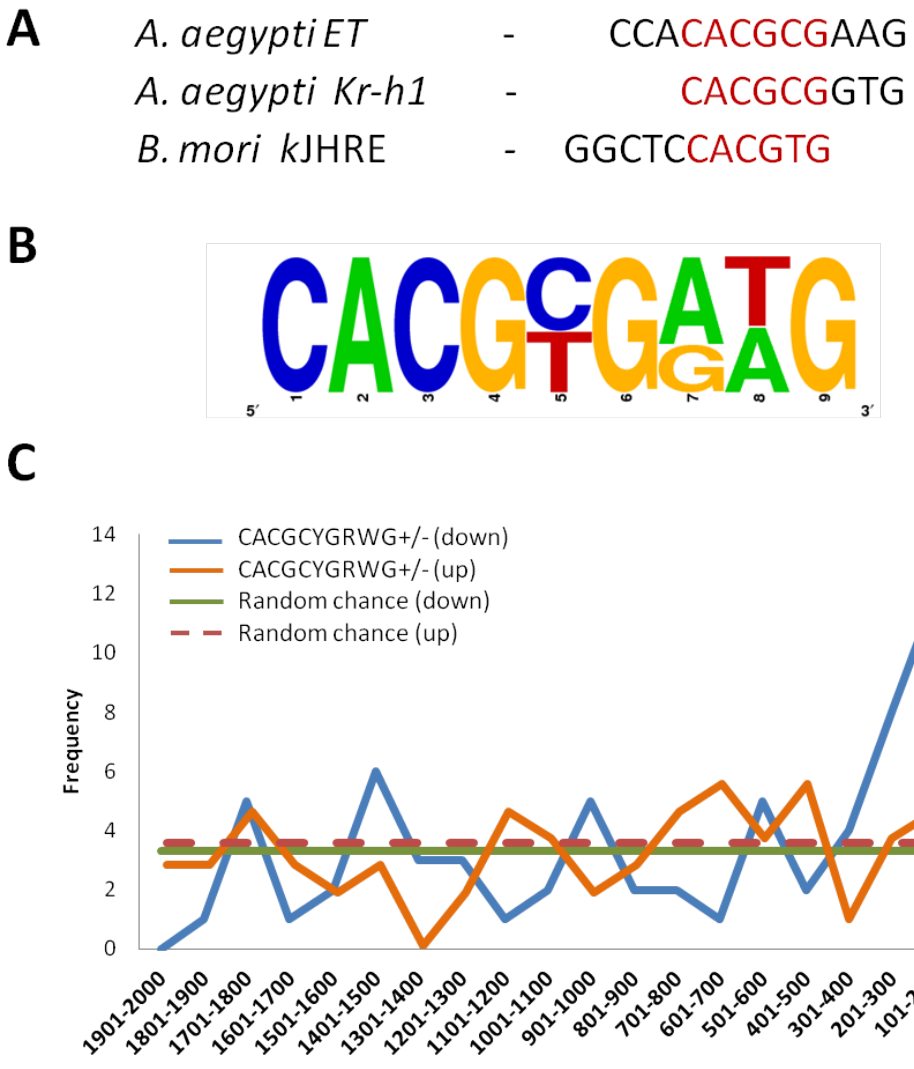
were searched (Fig. 9*C*). Two of the experimentally proven MET-binding sites have five and three bases, respectively, upstream of the E-box–like 6-mer (12, 16), we therefore aligned each of the 73 CACGYGRWG motifs detected, along with 10 bases upstream of their start sites, but this alignment did not yield any meaningful consensus.

Fig. 9 legend:

Computational analysis of gene promoters from the MET-depleted transcriptome:

(*A*) Previously identified MET binding motifs from *A. aegypti* and *B. mori*. The E-box or E-box-like 6-mer has been indicated in Red. Both the *A. aegypti* genes harboured a E-box-like motif 'CACGCG', while the *B.mori* had a canonical E-box 'CACGTG' *(B)* Characterization of a MET-binding motif. Based on prior characterizations the putative MET-binding motif was defined as CACGYGRWG. The nine-base consensus sequence has three degenerate positions, with cytosine/thymine at position five, adenine/guanine at position seven, and thymine/adenine at position eight. (*C*) Distribution of putative MET-binding sites within the upstream regions of up- and down-regulated genes from the MET RNAi-depleted transcriptome. The occurrence of the putative MET-binding motifs was enriched within the first 300 bp from the TSSs of iMet–down-regulated genes.

Fig. 9.


A
A. aegypti ET - CCACACGCGAAG
A. aegypti Kr-h1 - CACGCGGTG
B. mori kJHRE - GGCTCCACGTG
B
5′
3′
C
CACGCYGRWG+/- (down)
CACGCYGRWG+/- (up)
Random chance (down)
Random chance (up)
Frequency
0
2
4
6
8
10
12
14
1901-2000
1801-1900
1701-1800
1601-1700
1501-1600
1401-1500
1301-1400
1201-1300
1101-1200
1001-1100
901-1000
801-900
701-800
601-700
501-600
401-500
301-400
201-300
101-200
1-100

Validation of MET-Binding Consensus Using EMSA:

EMSA was used to evaluate the consensus characterized by the computational analysis. We used 35–38 bases from three *Aedes* gene promoters within the MET–activated gene repertoire, which carried nine base motifs that either matched or were very close to the consensus defined above. The promoter of the gene encoding *ribosomal protein S28* (RPS28, AAEL006860) carried the motif CACGCGGAG (T1) 158 bases upstream of the TSS that matched the consensus. That of *RNA m5u methyltransferase* (AAEL000674) had CACGTGTCA (T2) 1,477 bases upstream, and the *DNA-directed RNA polymerase E1* (AAEL007568) promoter carried CACTCGGTG (T3) and CACTTGGAG (T4) 401 and 1,501 bases, respectively, upstream of the TSS (Fig. 10*A* and Table 2). EMSA showed that T1 formed a dense band for the DNA–protein complex with nuclear extracts isolated from the fat body of mosquitoes 48 h PE (Fig. 10*B*). Binding was greatly reduced for T3 and T4 (Fig. 10*B*), each of which had one base mismatch to the consensus in the fourth position of the six-base in E-box-like motif. In the case of T2, in which there were three mismatches at the 3′ end of the six-base in E-box-like motif, binding was almost nonexistent (Fig. 10*B*). We then mutated the entire six-base in E-box-like motif of T1, leaving the three 3′-end bases intact, and observed a prominent reduction in binding (Fig. 10*C*). These results showed that all nine bases are important for proper binding. The presence of MET in the DNA-binding complex for T1 was tested using polyclonal antibodies against *A. aegypti* MET (AaMET AB) in EMSA with nuclear extracts from fat bodies of female mosquitoes 48 h PE. There was interference in the formation of the DNA–protein complex when the AaMET AB was

added to the EMSA reaction mixture (Fig. 10*D*). The formation of the complex was not disrupted by nonspecific (NS) antibody. This result proved the presence of MET within the complex formed by the T1 sequence.

The putative MET-binding consensus has three degenerate bases at positions 5, 7, and 8 (Fig 9*B*). Therefore, EMSA was used further to study the binding properties of all other possible variations of the MET-binding consensus, three with cytosine in the fifth position and four with thymine in the same position. To examine the formation of DNA-binding complexes to possible sequences with cytosine in the fifth position, the T1 sequence was mutated in the seventh and eighth positions, resulting in three sequences designated B1, B2, and B3 (Fig. 11*A* and Table 2). In all three cases, dense bands were observed in EMSA using fat-body nuclear extracts (Fig. 11*B* and *C*). The binding specificity was confirmed by competition with a 50× concentration of their respective unlabeled specific probes (Fig. 11*B*). The presence of MET in DNA–protein complexes was proven by interference of the DNA–protein complex formation when the reactions were performed with the addition of AaMET AB (Fig. 11*C*). Next, we checked binding properties of the four possible combinations with thymine in the fifth position. T1 was mutated accordingly resulting in 37 base probes with varying bases in position 7, 8 and every combination of them. This resulted in four sequences designated as S21, S22, S23 and S24 (Fig. 11*A* and Table 2). We found that the bands formed by these sequences differed from each other in EMSA with the fat-body nuclear extract (Fig. 11*D*). The S22 and S23 sequences formed dense bands similar to that of the T1 motif. Addition of AaMET AB but not NS serum to the EMSA reaction mixture interfered with the binding

of both the S22 and S23 motifs (Fig. 11*E*). Formation of the DNA–protein complex was reduced substantially in EMSA with the S21 sequence, in which adenine and thymine were present in the seventh and eighth positions, whereas guanine and adenine in these same positions in the S24 sequence diminished binding to an undetectable level. Taken together, our results suggest that the first six bases of the nine-base motif to which MET can bind are similar to the E-box or E-box–like sequences with a degenerate fifth position (either cytosine or thymine). The three bases downstream of the E-box–like motif are important, and two of these bases can be degenerate with invariable guanine in the ninth position. The binding properties are similar for all tested sequence combinations with cytosine in the fifth position but not for those with thymine in that position.

Fig. 10 legend:

Validation of the MET-binding consensus:

Four motifs from three iMet–down-regulated genes designated as T1 (*Ribosomal protein S28*), T2 (*RNA m5u methyltransferase*), T3, and T4 (*DNA-directed RNA polymerase E1*) were tested using EMSA with fat-body nuclear extract from female mosquitoes 48 h PE. (*A*) The motif sequences used for EMSA analysis. (T1-T4) with the 9mer MET binding consensus highlighted in red. Any deviation from the identified consensus has been marked in blue. The mutated T1 sequence (T1-mut) has also been provided with the mutated E-box-like motif highlighted in bold. (B) Binding between the motifs and fat body nuclear extracts from female 48 h PE mosquitoes. Only T1 formed a dense band indicating DNA-protein interaction.(*C*) The six-base, E-box–like motif CACGCGGAG of T1 was mutated to the T1 mutant, TCAATAGAG, and was tested using EMSA with the fat-body nuclear extract. (*D*) The presence of MET in the DNA–protein complex formed by T1 in EMSA with the fat-body nuclear extract was confirmed by adding polyclonal antibody against MET. NS, nonspecific antibody against *Actin*. Lower panels in *B–D* represent loading controls of unbound probes.

Fig. 10.

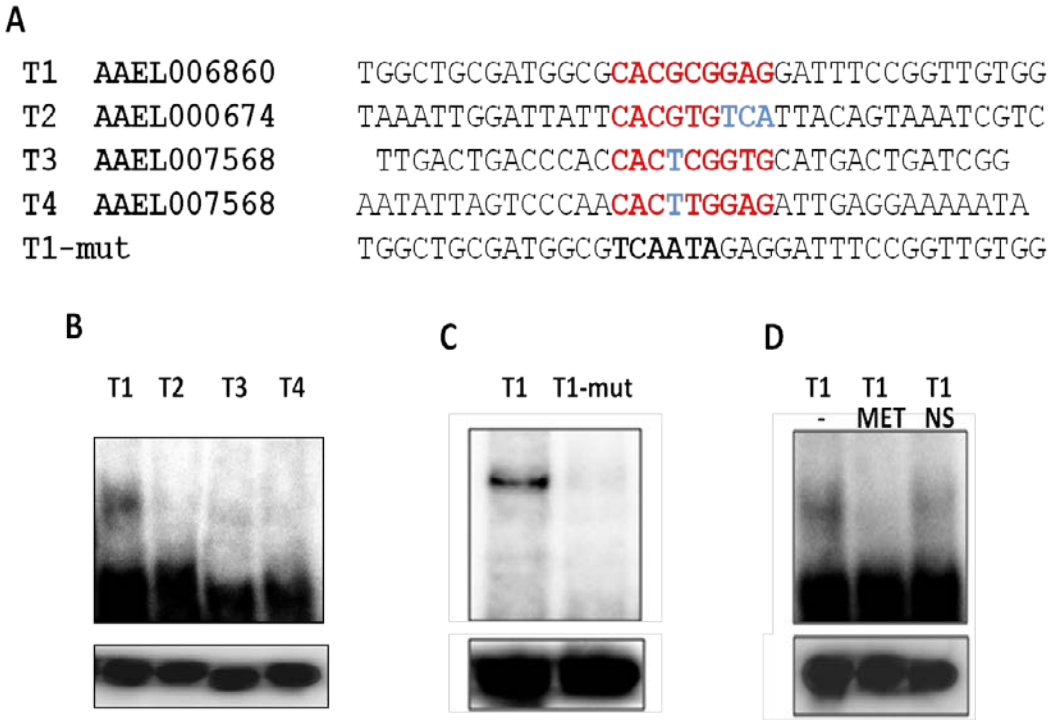

A
T1 AAEL006860 TGGCTGCGATGGCGCACGCGGAGGATTTCCGGTTGTGG
T2 AAEL000674 TAAATTGGATTATTCACGTGTCATTACAGTAAATCGTC
T3 AAEL007568 TTGACTGACCCACCACTCGGTGCATGACTGATCGG
T4 AAEL007568 AATATTAGTCCCAACACTTGGAGATTGAGGAAAAATA
T1-mut TGGCTGCGATGGCGTCAATAGAGGATTTCCGGTTGTGG
B
T1 T2 T3 T4
C
T1 T1-mut
D
T1 T1 T1
- MET NS

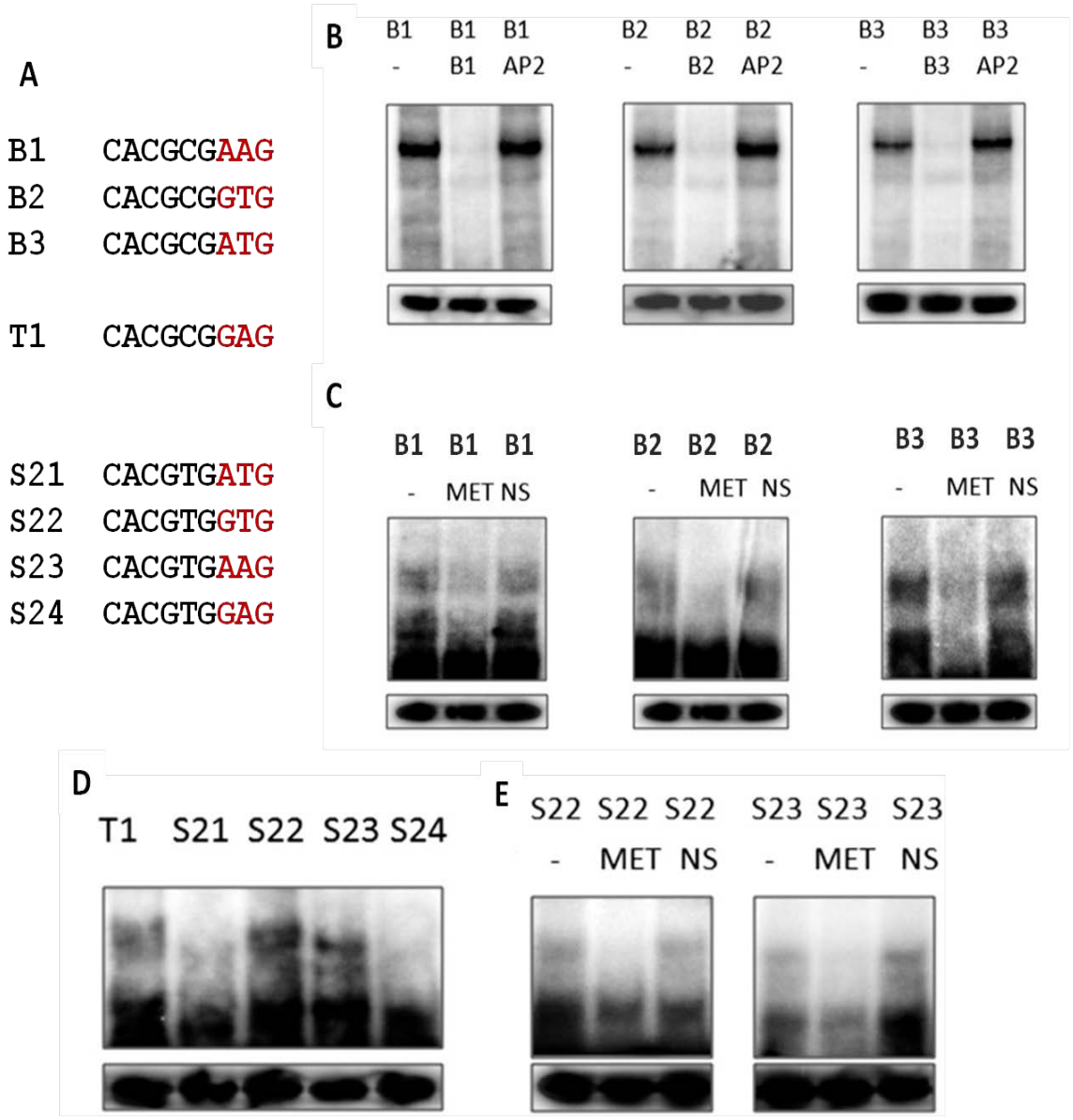
Fig. 11 legend:

Binding properties of motifs with degenerate bases in 7th and 8th position in 9mer MET binding consensus:

(*A*) Sequences representing different combinations of the consensus for the putative MET-binding sites with cytosine at position five (B1-B3, T1) and thymine at the fifth position (S21-S24) in the probes used for the EMSA analysis. (*B* and *C*) Analysis of the binding affinity for the different combinations of the consensus MET-binding motif with cytosine at the fifth position. The consensus 9mer MET binding site in T1:CACGCGGAG was mutated to CACGCGAAG (B1), CACGCGGTG (B2), and CACGCGATG (B3), keeping the flanking sequence intact. (*B*) For competition analysis, a 50× molar excess of unlabeled probe or a nonspecific double-stranded oligonucleotide (AP2) was added to the binding mixtures. This experiment proved that the bands were specific. (*C*) The presence of MET in the DNA–protein complex was confirmed by adding polyclonal antibody to the binding reaction (nuclear extract from 48-h PE mosquito fat body). Nonspecific antibody (NS, Actin) does not affect binding in any of the reactions. (*D* and *E*) Analysis of the binding affinity for the different combinations of the consensus MET-binding motif with thymine at the fifth position. The consensus MET binding motif of T1 was mutated to CACGTGATG (S21), CACGTGGTG (S22), CACGTGAAG (S23), CACGTGGAG (S24), keeping the flanking sequence intact. (*D*) EMSA demonstrated that bands for variations of the motif S22 and S23 were the same size as the T1 motif. Binding capacity was highly reduced for S21 and S24. (*E*) The presence of MET in the DNA–protein complex formed by S22 and by S23 was confirmed

by adding polyclonal antibody to the binding reaction (nuclear extract from 48-h PE mosquito fat body). Nonspecific antibody (NS, Actin) does not affect binding in either of the two reactions. Lower panels in *B*–*E* represent loading controls of unbound probes.

Fig. 11.



DISCUSSION:

This transcriptome study combined with the MET RNAi has revealed important biological features related to the fat body of female *A. aegypti* mosquitoes. Very high transcriptional activity was observed in this tissue during the JH-dependent PE development. Although we anticipated finding putative JH gene targets using microarray analysis during PE development, such a spectacular rise in transcription was unexpected and reflects the great importance of JH-regulated periods in governing gonadotrophic cycles in mosquitoes. Gene sets with distinct gene expression profiles having clearly defined peaks could be identified for this developmental period. Three significantly large gene sets were named Early, Mid and Late PE (EPE, MPE, LPE) depending on temporal peaks of the expression profiles. Further characterization was focused on these three sets (EPE, MPE and LPE) leaving aside other clusters for future investigation. JH being the principal hormone governing this developmental stage is expected to play a key role in mediating the expression of genes in EPE, MPE and LPE.

Perhaps the best way to cross check this hypothesis would be to have transcriptome of JH deficient mosquitoes. Unfortunately, our efforts to engineer JH-less mosquitoes by knockdown of the pathway enzymes for JH biosynthesis failed. We anticipate that there might be some penetrance issues with the dsRNA to the tiny JH producing gland corpora allata. Following this we focused our research on the newly characterized JH receptor MET. Ovarian follicular phenotyopes of MET depleted mosquitoes matched that of previously characterized JH-deficient mosquitoes, giving us further support to pursue this direction of research. MET depletion resulted in alteration

of 27%, 40% and 36% of EPE, MPE and LPE clusters respectively. Patricularly, EPE and MPE genes were found to be induced in iMet mosquitoes, while LPE genes were repressed. Thus, MET depletion resulted in significant disruption of normal PE gene expression. This results strongly suggest that PE gene expression (EPE, MPE and LPE) is regulated by JH and mediated by its receptor MET. However, a large percentage of EPE, MPE, and LPE genes appear not to be controlled by MET. Future research should investigate the regulation of expression of these genes.

Differential JH sensitivity was proved for selected genes from EPE and LPE cluster. Tested EPE were inhibited by ectopic JH application, reflecting physiological conditions where a high JH titer would result in shutting off the expression of EPE genes. The effect of JH on LPE genes were quite the opposite, activating the genes tested. Considering the titer of the hormone during PE developmental period, it can be concluded that the LPE genes would require the involvement of JH for their induction. *In vitro* fat body culture experiments coupled with MET RNAi knockdowns have demonstrated the absolute necessity of MET in mediating the JH regulation of the genes. In these experiments, MET RNAi knockdowns rendered the repressive and activating actions of JH on EPE and LPE genes ineffective.

A 9-mer consensus for the MET-binding motif CACG$^{C}/_{T}$G$^{A}/_{G}$$^{T}/_{A}$G was identified using pattern-search and EMSA analyses. The core of this motif is conserved between the mosquito *A. aegypti* and the moth *B. mori*. However, further analysis is needed to establish conservation of this MET consensus sequence among other insects. Along with conservation, the binding sites of many transcription factors show bias for a certain

position within the upstream regions, because these transcription factors can act most effectively when at a certain distance from the TSS (27, 28). The effect of positional bias for transcription factor binding sites has been observed in insects (29), vertebrates (30, 31), and plants (25). In our case, the *in silico* analysis determined that MET-binding motif variants showed a strong positional bias for the first 300 bases of the promoters of iMet–down-regulated genes. This bias suggests that MET might be directly involved in the up-regulation of some LPE genes. No such bias was detected in the iMet–up-regulated genes indicates that the EPE and MPE genes might entirely be controlled indirectly by MET. Several transcription factors have been shown to be activated by MET including *Hairy* and *Kr-h1*. *These factors might be involved in indirect gene regulation by MET.* But the targets of these factors have not been characterized. Overall, the complexity of JH/MET transcriptional regulation requires additional investigation.

The insect fat body is a storage depot of energy reserves and is a center of metabolism, immunity, detoxification, and production of yolk protein precursors during reproduction. It responds to changing the physiological needs of an insect organism by altering its functions. Gene ontology analysis has provided insight into the biological significance of the fat body in female mosquitoes and identified large and diverse sets of differentially expressed genes during PE development. The overrepresentation of metabolic genes (carbohydrate, lipid, and xenobiotics metabolism) in EPE and MPE clusters and their distinct functional difference from LPE cluster, which is primarily dominated by translation, transcription and amino acid metabolism, reflects the complexity of fat-body activities that accommodate the requirements of reproducing

female mosquitoes. A newly eclosed female mosquito needs a rapid increase in carbohydrate and lipid metabolism to support nectar feeding and energy-consuming host seeking. By the end of PE development, a female mosquito prepares for the intense demands of post–blood-feeding reproductive events. The high expression level of the genes involved in translation, transcription, and amino acid metabolism in the fat body at the end of PE development reflects a distinctive adaptation of mosquitoes as hematophagous insects. MET down- and up-regulated genes that show significant overlaps with EPE and LPE, respectively, also share the major functional ontologies with these clusters. This study advances our understanding of JH regulation of mosquito reproduction and provides important insight into the control of gene expression by JH and its receptor MET.

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Chapter II

Characterizing the role of Hairy in gene repression by juvenile hormone/Methoprene tolerant signaling pathway

ABSTARCT:

We have shown that Hairy is an integral part of the juvenile hormone/Methoprene tolerant (JH/MET) hierarchy down-regulating gene expression. We conducted RNAi mediated depletions for MET and Hairy combined with RNA sequencing analysis (RNA-seq) of the *A. aegypti* female fat body at the JH-regulated PE phase. This analysis has revealed that 79 percent of 311 Hairy-repressed genes were also found in the Met-repressed transcriptome. Analysis of selected genes from this cohort has demonstrated that they are indeed repressed by JH. Moreover, the JH repressive action on these genes was prevented by the addition of a protein synthesis inhibitor cycloheximide (CHX) to the fat body culture, strongly suggesting the existence of an indirect regulatory hierarchy. RNAi depletions of either MET or Hairy renders JH ineffective in repressing these genes in the fat body tissue culture assay. The MEME analysis of the upstream sequences of genes repressed by both MET and Hairy showed the presence of two non-coding DNA elements (CNEs), one of which contained a putative Hairy-binding motif that interacted with Hairy in the electrophoretic gel mobility assay (EMSA). Authenticity of Hairy-binding motif was confirmed by its mutagenesis and super-shifts with antibodies against *Aedes* Hairy. RNAi knockdown of a co-repressor Groucho phenocopied the effect of Hairy RNAi knockdown indicating that it is also involved in the JH/Met/Hairy hierarchy.

Taking all these evidences together from our study, along with the fact that Hairy is MET induced transcription factor, it can be said that Hairy is a component of JH/MET hierarchy in gene repression.

INTRODUCTION:

Recently, significant progress has been achieved in understanding JH regulation of gene expression in *A. aegypti* female mosquito during PE development (1, 2, 3). Microarray analysis has shown that gene expression activity during the PE phase in the *A. aegypti* female fat body is very high (2). Using hierarchical clustering method three major gene expression profiles were identified and named Early PE (EPE) Mid PE (MPE) and Late PE (LPE) depending on the highest expression point of clustered gene sets. Depletion of JH receptor MET results in significant disruption of EPE, MPE and LPE gene expression. Selected sets of EPE and LPE genes were found to be regulated by JH, and this JH sensitivity was dependant on the presence of MET, supporting the hypothesis of JH/MET signaling pathway in controlling EPE and LPE gene expression (2). Through bioinformatic analysis, an overrepresentation of the consensus 9-mer MET-binding motif CACG$^{C}/_{T}$G$^{A}/_{G}$$^{T}/_{A}$G was observed in promoters of 68 MET activated (LPE) genes, indicating a direct gene regulation model. Electrophoretic mobility shift assays (EMSA), utilizing a combination of mutational and anti-Met antibody super-shift analyses, confirmed binding properties of the Met consensus motif variants (2). No statistical overrepresentation was observed for MET repressed (EPE) gene sets suggesting an indirect mode of gene repression involving intermediate transcription factors. However, the identity of such an additional factor in

JH/MET mediated gene repression remains elusive. Considering that this question is of general importance for understanding of JH action in insects at the molecular level, we used *A. aegypti* mosquitoes as a system to investigate this question.

In *A. aegypti*, JH and MET highly activate several transcriptional repressors, including *Krüppel homolog-1* (*Kr-h1*, and AAEL002390) and *Hairy* (*AAEL005480*). Kr-h1 has been implicated in mediating the JH and Met signaling during development and metamorphosis (4), while the role of Hairy has remained unclear.

Drosophila Hairy is a pair-rule gene that involved in early patterning of the embryo (5,6) and plays a key role in the segmentation gene hierarchy (7). During embryo segmentation, Hairy represses a downstream pair-rule gene, *fushi tarazu* (8, 9). Hairy also regulates other developmental processes: sex determination (10), peripheral nervous system development during larval development (11, 12), morphogenetic furrow progression in the developing eye (13) and additionally has a role in metabolic suppression in hypoxia-tolerance (14).

Hairy belongs to the evolutionary conserved Hairy/Enhancer of split/Deadpan (HES) subclass of repressor bHLH proteins (15). As a long-range repressor, *Drosophila* Hairy has the ability to inhibit activities of activators located over 1 kb away from the gene transcription start site (16). It can induce a widespread histone deacetylation and inhibits the recruitment of basal transcription machinery without inducing chromatin compaction in the repression of *fushi tarazu* gene expression (17). Most bHLH proteins bind to a consensus DNA sequence of CANNTG, known as an

E-box. Additional binding specificity is determined by the two middle bases as well as by bases flanking the E-box (18). *Drosophila* Hairy has been shown to bind preferentially to a sequence of CGCGTG (11, 12).

Here, we show that Hairy plays an essential role in the JH/Met gene repressive hierarchy in an adult insect. RNAi mediated depletion of MET and Hairy combined with high throughput RNA sequencing (RNA-seq) analysis of the *A. aegypti* female fat body has revealed that 79 percent of 311 Hairy-repressed genes were also repressed by MET. Markers from MET/Hairy repressed gene cohorts are responsive to JH. However their JH mediated repression requires translation of intermediate factors as evidenced from cycloheximide (protein synthesis blocker) assays. JH repression of the marker genes are also compromised by RNAi of either MET or Hairy in fat body tissue culture experiments *in vitro*. There data strongly support the hypothesis for the existence of JH/MET/Hairy hierarchy in gene repression. MEME analysis of the upstream sequences of genes repressed by both MET and Hairy showed the presence of two non-coding DNA elements (CNEs), one of which contained a putative Hairy-binding motif that interacted with TNT expressed *A. aegypti* Hairy as tested by EMSA. Authenticity of Hairy-binding motif was confirmed by its mutagenesis and super-shifts with antibodies against *Aedes* Hairy. RNAi knockdown of a co-repressor Groucho phenocopied the effect of Hairy depletion indicating that it is also involved in the JH/Met/Hairy hierarchy. Thus, our study has established that Hairy mediates the repressive action of JH and MET possibly working in coordination with co-repressor Groucho.

MATERIALS AND METHODS:

Experimental animals:

The mosquito *A. aegypti* UGAL/Rockefeller strain was raised as described previously (19). Adult mosquitoes were fed continuously on water and 10% (wt/vol) sucrose solution. All dissections were performed in *Aedes* physiological solution at room temperature (19).

RNA interference mediated gene knockdown:

To synthesize dsRNA for desired gene, we followed a method described previously (19). In brief, dsRNA of a specific gene template was synthesized using the MEGAscript kit (Ambion). The luciferase gene was used to generate control iLuc dsRNA. After dsRNA synthesis, samples were subjected to phenol/chloroform extraction and ethanol precipitation. dsRNA then was suspended in RNase-free water to a final concentration of 5 μg/μL. At 24 h PE, female mosquitoes were injected with 300 nL dsRNA. The Picospritzer II (General Valve) was used to introduce corresponding dsRNAs into the thorax of CO_2-anesthetized female mosquitoes.

RNA extraction:

For the high throughput transcriptome sequencing, RNA from fat body was collected from RNAi knockdown mosquitoes 72hr post injection. RNA was extracted from fat bodies of 10 female mosquitoes using the TRIzol method (Invitrogen) according to the manufacturer's protocol. It was concentrated using the RNeasy MiniElute cleanup kit (Qiagen) for further processing.

Library preparation for sequencing:

For purpose of library preparation for high throughput sequencing, TruSeq RNA sample prep V2 kit (Illumina) was used. Library preparation was done following the protocol provided along with the Kit. Tagging of the cDNA samples were performed as described in (20). Appropriate library specific adapters were added as tags for multiplexing the sequencing samples. The quality of the library was checked using the Agilent 2100 Bioanalyzer.

RNA-seq analysis:

Data analysis and processing of reads were performed using the statistical programming language R. The RNA-seq data processing packages Cufflinks and CummeRebund were used to assemble the reads into meaningful contigs, which were then aligned to the *A. aegypti* transcript sequence database (AaegL1.3 geneset, Vectorbase) using the R package Bowtie2. The resulting count table was transformed into FPKM (fragments per Kbp of transcript per million fragments mapped) values.

***In vitro* fat-body culture:**

Female mosquitoes were injected with MET dsRNA or Luc dsRNA within 0–6 h PE. Fat bodies were dissected from mosquitoes 72 h after injection and were incubated in a complete culture medium supplemented with amino acids for 8 h, as described previously (19). JH (10 μg/mL JH III) or solvent (acetone) was added to the culture medium. Total RNA was isolated and analyzed using qPCR. The experiment was repeated three times under same conditions.

Quantitative RT-PCR analysis:

cDNAs were synthesized from 2 µg total RNA using the Omniscript Reverse Transcriptase kit (Qiagen). RNA was treated with DNase I (Invitrogen) before cDNA synthesis. PCR was performed using the Platinum High Fidelity Supermix (Invitrogen). qRT-PCR was performed using the iCycler iQ system (Bio-Rad) and an IQ SYBR Green Supermix (Bio-Rad). Quantitative measurements were performed in triplicate and normalized to the internal control of S7 ribosomal protein mRNA for each sample. Real-time data were collected from the software iCycler v3.0. Raw data were exported to Excel (Microsoft) for analysis. Primers used for qRT-PCR are provided in Table 1.

Table 1 legend:

Primers for qRT-PCR and RNA interference mediated knockdown of gene:

Shown below is a list of primers used for Real time quantitative PCR and RNAi knockdown. All of primers are in 5'-3' direction. The Real time quantitative PCR primer leads to amplification of product 80-140bp in length. The RNAi primers have primers have a T7 RNA polymerase binding sequence at the 5' end.

Table 1.

Primers used for Quantitative RT-PCR and RNAi	
Primers for Q-PCR	
AAEL002655 Forward	AACAATCGCTACACGGAACC
AAEL002655 Reverse	AACAGTGCAGAGTCGTGGTG
AAEL005004 Forward	GGTCGCTACACCGTCAATTT
AAEL005004 Reverse	CGTGGAGCGATGGTAAACTT
AAEL000703 Forward	GCCTGGATATGGCAACAACT
AAEL000703 Reverse	GTTACGATCCAACACGGCTT
AAEL003619 Forward	CGTTATCCCGTATTTGGTGG
AAEL003619 Reverse	CCTCTGCTCGAAAACTGACC
AAEL003060 Forward	GGTAGCTTTCCTGCTCGTTG
AAEL003060 Reverse	CACTGCAGCGAGATTTGGTA
AAEL011542 Forward	GGGAACGATAATGCACGAGT
AAEL011542 Reverse	AAATTGTGCTGATGCTGCTG
AAEL002495 Forward	ATCGATCCCACCTAAGGACC
AAEL002495 Reverse	ATATTTGGAGCAGTCCGACG
AAEL013775 Forward	CTGTCGTAGCAGATGCCAAA
AAEL013775 Reverse	GAAAAGTGTCTTTCGCGAGG
Primers for RNAi	
MET RNAi Forward	TAATACGACTCACTATAGGGTCAATTTGTTCGACTCTGCG
MET RNAi Reverse	TAATACGACTCACTATAGGGATACACAAAGTCGCCCGTTC
Hairy RNAi Forward	TAATACGACTCACTATAGGGTGACCGTGAAACATTTGGAA
Hairy RNAi Reverse	TAATACGACTCACTATAGGGCGGTCTCCAAGGTTTGTCAT
Groucho RNAi Forward	TAATACGACTCACTATAGGGGACCGCGATAGTGTTGGTTT
Groucho RNAi Reverse	TAATACGACTCACTATAGGGGACGTATTTGGTTGGGTTGG

Electrophoretic mobility shift assays (EMSA):

The annealed deoxyoligonucleotide of each motif was purified from 15% TBE Criterion Precast Gel (Bio-Rad) and labeled with γ-^{32}P ATP. EMSA was performed using a gel-shift assay system (Promega) with *in vitro* TNT Hairy. The DNA–protein complex was separated on 5% TBE Criterion Precast Gel (Bio-Rad). After electrophoresis, the gel was dried and incubated with phosphor imaging screen in the cassette overnight to reach highest exposure and was visualized in the Personal Molecular Imager (Bio-Rad) by auto-radiography. For the competition assay, 50-fold unlabeled E-box–like motif or unlabeled AP-2 motif (nonspecific competitor oligonucleotides; Promega) was incubated with nuclear extract for 10 min and then was incubated further with the labeled probe for 20 min. Identity of a complex was verified by directly adding polyclonal antibodies against *A. aegypti* Hairy.

RESULTS:

Identification of MET and Hairy regulated genes by RNA-seq:

To understand whether Hairy is involved in the JH gene regulatory hierarchy, we conducted an transcriptomic analysis of MET and Hairy depleted mosquito. Illumina RNA-seq technology was used to generate the transcriptional profiles of genes affected by RNAi-mediated depletion of MET (iMet) and Hairy (iHairy) in the fat body of *A. aegypti* female mosquitoes. RNAi for Luciferase (iLuc) served as a control. Female mosquitoes 12 h after eclosion were injected separately with each corresponding dsRNA. Three days later, RNA-seq libraries were made from poly(A)-RNA extracted from 10

dsRNA-treated mosquito fat bodies for each RNAi treatment - iMet, iHairy and iLuc. Three biological replicates of RNA-seq libraries were constructed for each RNAi treatment. Each RNA-seq library generated between 62 and 241 million reads (101 nucleotide sequences for 1st and 2nd replicates; 51 for 3rd) (Fig. 1*A*). The reads were aligned with Bowtie2 against *A. aegypti* transcript sequence database (AaegL1.3 geneset, Vectorbase). The resulting count table was transformed into FPKM (fragments per kbp of transcript per million fragments mapped) values. The relative abundance of transcripts was defined by the sum of FPKM values of the three samples (iLuc, iMet, and iHairy), and transcripts for each RNAi treatment were sorted from highest to lowest level of abundance. 10,000 transcripts with highest abundance from each RNAi treatment were then selected for further analysis.

The differentially expressed transcripts were defined by a two-fold increase or decrease of FPKM values. Among the 10,000 abundantly present transcripts, a total of 2,151 transcripts were identified as having different expressions between the MET-depleted and control mosquitoes, with 538 transcripts decreasing more than two-fold following RNAi depletion of MET (MET-activated transcripts) and 1,613 transcripts showing a more than two fold increase (MET-repressed transcripts) (Figure 2*A*). A total of 455 transcripts showed a more than two-fold differential expression between Hairy-depleted and control mosquitoes, with 311 iHairy-upregulated (Hairy-repressed) transcripts and 138 iHairy-downregulated (Hairy-activated) transcripts (Figure 2*A*).

Significantly, there was a 79% (of all iHairy up-regulated genes) overlap between iMet and iHairy-activated transcripts. 247 genes were up-regulated by either iMet or

iHairy, suggesting that these two factors work in a hierarchical fashion in mediating JH repressive action (Fig. 2*A*). On the other hand, the overlap between iMet and iHairy repressed genes were much lower, with only 47 genes showing down-regulation in both iMET and iHairy transcriptomes, pointing to the absence of systemic MET-Hairy hierarchy for JH-dependent gene activation (Fig. 2*A*). The reproducibility of results in three biological replicates was verified by comparison of genes co-activated in both iMet and iHairy (Fig. 1*B*). Consequently, the data from the first experiment was used for all subsequent analyses.

qRT-PCR was employed to validate the RNA-seq analysis and simultaneously establish marker genes for MET/Hairy co-regulation. Candidates were selected on the basis of high differential expression in both iMet and iHairy samples. Four genes- *Matrix metalloproteinase* (AAEL002655), *Conserved hypothetical protein* (function unknown; AAEL005004), *Phosphorylase* (AAEL000703), *Amino acid transporter protein* (AAEL003619) for MET/Hairy repression and four more- *Serine protease* (AAEL003060), *Metalloproteinase, putative* (AAEL011542), *Chitin binding protein* (AAEL002495), *Hypothetical protein* (function unknown; AAEL013775) for MET/Hairy activation were confirmed (Fig. 2*B* and *C*). These marker genes have subsequently been used in our research for various molecular analyses.

Fig. 1 legend:

RNA-seq analysis of MET and Hairy depleted adult *A. aegypti* fat body:

(*A)* Table showing a summary of RNA-seq experiments. Three biological replicates of RNA-seq libraries were constructed from poly(A)-RNA extracted from 10 mosquito fat bodies, which were respectively treated with dsRNA of luciferase control (iLuc), Met (iMet), and Hairy (iHairy). The number and quality of the reads originating from each libraries are indicated. (*B*) Reproducibility of the parallel three experiments. Venn diagrams showing the up-regulated transcripts by the dsRNA-mediated depletion of both MET and Hairy. The RNA-seq experiments from three biological replicates produced the similar results for the selection of transcripts with differential expression, which was defined as more than two fold difference in 10,000 abundantly expressed transcripts. More than 74% of transcripts were simultaneously characterized in three replicates as the transcripts whose expression were differentially up-regulated by both dsRNA-mediated Met and Hairy, clearly indicating the reproducibility of the parallel three experiments.

Fig. 1.

A

Replicates	Sample ID	length	# Reads	% of >= Q30 Bases (PF)	Mean Quality Score (PF)
1st	iLuc 1	101	241,496,528	89.76	34.97
	iMet 1	101	125,120,346	89.85	35.04
	iHairy 1	101	214,169,272	88.58	34.66
2nd	iLuc 2	101	71,899,072	95.9	37.18
	iMet 2	101	72,167,840	95.7	37.1
	iHairy 2	101	66,299,014	95.82	37.16
3rd	iLuc 3	51	120,748,264	Not tested	Not tested
	iMet 3	51	62,560,173	Not tested	Not tested
	iHairy 3	51	107,084,636	Not tested	Not tested

B

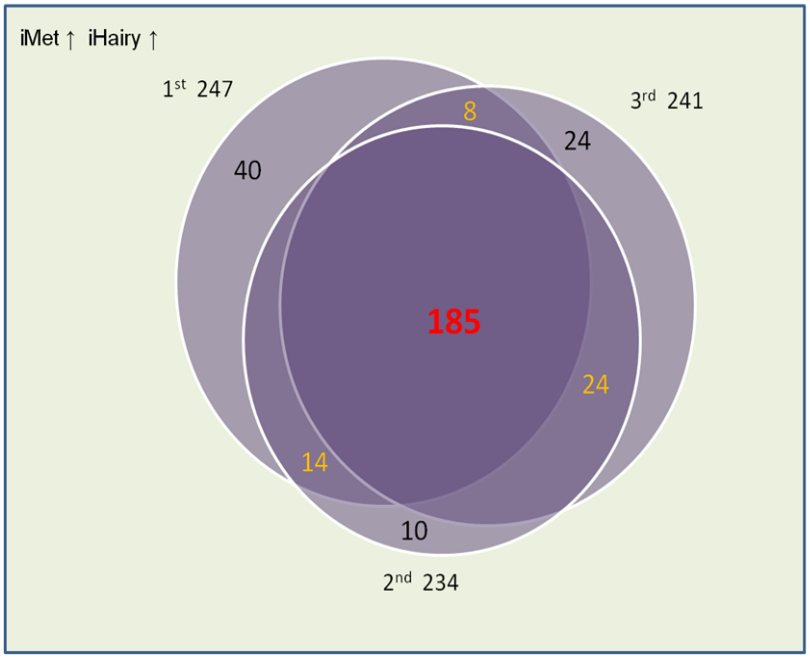


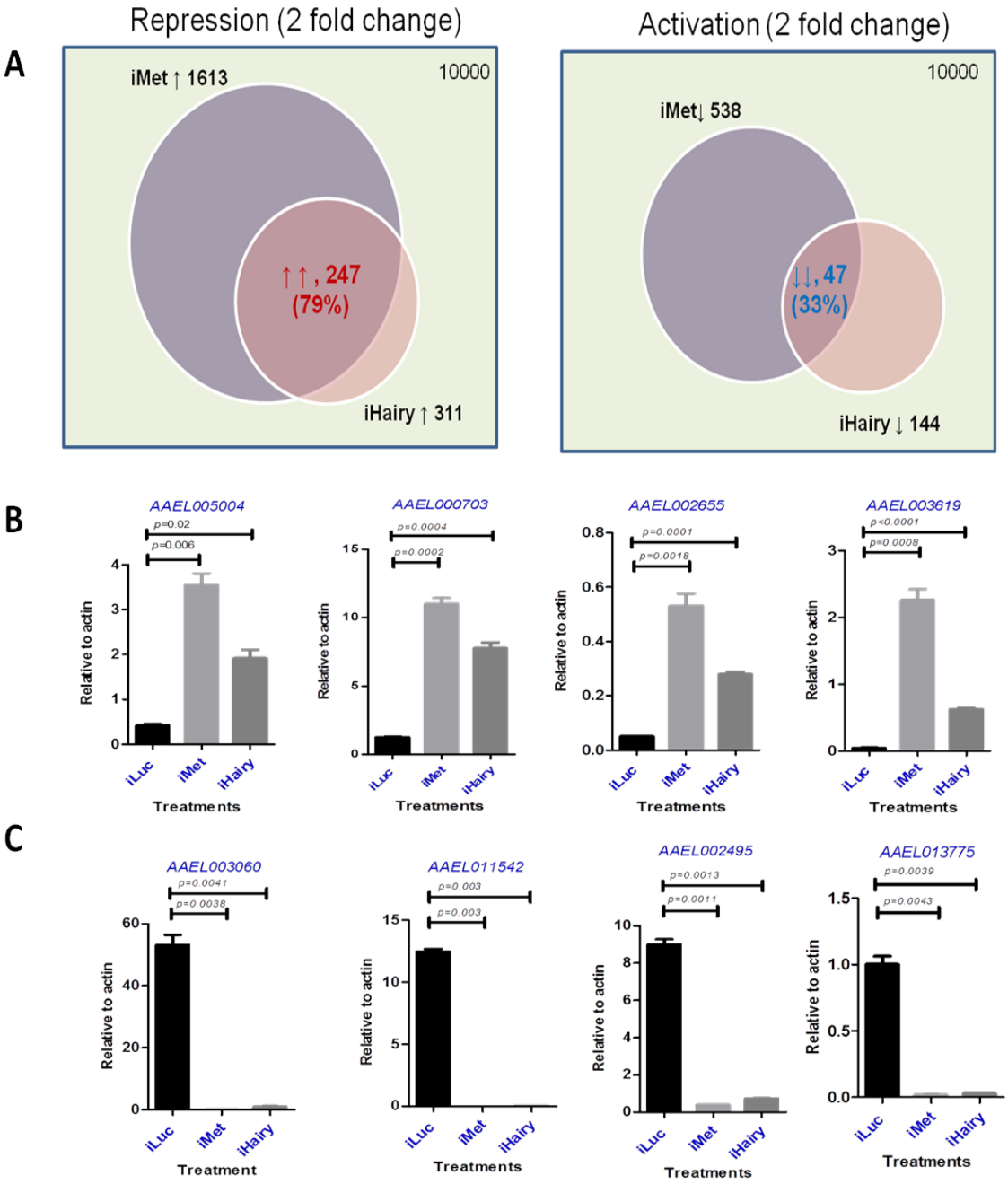
Fig. 2 legend:

Comparison of MET and Hairy transcriptomes:

(*A*)Venn diagrams comparing the differentially expressed transcripts from the MET RNAi-depleted transcriptome with the Hairy-depleted one. iMET↑, more than 2 fold up-regulation by RNAi depletion of Met (Met-repressed); iHairy↑, more than 2 fold up-regulation by RNAi depletion of Hairy (Hairy-repressed); ↑↑, more than 2 fold up-regulation by both RNAi depletion of MET and Hairy (Met/Hairy-repressed); iMet↓, more than 2 fold down-regulation by RNAi depletion of MET (Met-activated); iHairy↓, more than 2 fold down-regulation by RNAi depletion of Hairy (Hairy-activated); ↓↓, more than 2 fold down-regulation by both RNAi depletion of MET and Hairy (Met/Hairy-activated). (*B*-*C*) Identification of MET and Hairy co-regulated marker genes. RNAi mediated knockdowns were performed as previously described, for Luc, MET and Hairy. Tissues were collected 3d post injection and subjected to qRT-PCR based gene expression analysis. Candidates were selected on the basis of high differential expression in both iMet and iHairy mosquito fat body (*B*) Four genes- *Matrix metalloproteinase* (AAEL002655), *Conserved hypothetical protein* (function unknown; AAEL005004), *Phosphorylase* (AAEL000703), *Amino acid transporter protein* (AAEL003619) with a high fold change for MET and Hairy repression were confirmed. (*C*) Four more- *Serine protease* (AAEL003060), *Metalloproteinase, putative* (AAEL011542), *Chitin binding protein* (AAEL002495), *Hypothetical protein* (function unknown; AAEL013775) for MET and Hairy activation were confirmed. All experiments were done in triplicates. *P*-

value was calculated on the basis of two-tailed pair end *t*-test using software package GraphPad Prism 5.0.

Fig. 2.



Functional genomics of MET and Hairy regulated transcriptome:

For the examination of the functional ontology of the genes, we utilized the eggNOG v3.0 database (21). Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG) is a database of orthologous groups of genes, which was constructed through identification of reciprocal best BLAST matches and triangular linkage clustering. The orthologous groups (OGs) are annotated with functional descriptions with functional categories which were derived from the original Clusters of Orthologous groups (COG)/EuKaruotic Orthologous Groups (KOG) categories. We made two changes in assignments of orthologous groups (OGs) for *A. aegypti* genes. Among 5,944 OG assigned genes (out of a total of 10K abundantly expressed genes), 4.3% (256) genes were assigned to various OG's under a parental OG group. To simplify the ontology analysis, all the genes belonging to the sub-groups under an OG were reassigned to the parental OG group (Table 2*A*), based on the specific gene information. A second change was made to the OG of [V] Defense mechanisms. We manually annotated 47 genes encoding antimicrobial peptides (AMPs), Gram-negative binding proteins (GNBPs), Fibrinogen and fibronectin proteins, Galactoside-binding lectins, Peptidoglycan recognition proteins (PGRPs), Toll and IMD pathway components, lysozymes and prophenoloxidases (PPOs) into the simplified [V] OG, which included the original OG assignment of [V], [TV] and [VW] (Table 2*B*).

The OG analysis based on adjusted NOG revealed that the functional distribution of MET-activated and MET-repressed genes were very different (Fig. 3*A*) confirming our

previous results from microarray data. The OGs that showed significant overrepresentation (P<0.01 in a hypergeometric distribution) in MET-activated transcriptome are only two in number belonging to the category of Information Storage and Process – [J] Translation, ribosomal structure and [A] RNA processing and modification (Fig. 3*C* and *B*). Contrastingly, MET-repressed transcripts were mostly represented in the other two categories of Cellular Process and Signaling and of Metabolism (Fig. 3*A*). The significantly overrepresented OGs in MET-repressed genes were four in Metabolism category – [G] Carbohydrate transport and metabolism, [I] Lipid transport and metabolism, [P] Inorganic ion transport and metabolism, and [Q] Secondary metabolites biosynthesis, transport and metabolism – and four in Cellular Process and Signaling category – [V] Defense mechanisms, [T] Signal transduction mechanisms, [Z] Cytoskeleton, and [W] Extracellular structure (Fig. 3*B*).

Functionally, Hairy-repressed and MET-repressed transcriptomes are similar., with most transcripts mapping to OG's in the categories of Cellular Process and Signaling and of Metabolism (Fig. 3*A*). The OGs that were significantly overrepresented (P<0.01 in a hypergeometric distribution) in Hairy-repressed transcripts were three in Metabolism category – [G] Carbohydrate transport and metabolism, [E] Amino acid transport and metabolism, and [Q] Secondary metabolites biosynthesis, transport and metabolism – and three in Cellular Process and Signaling category – [V] Defense mechanisms, [W] Extracellular structure, and [O] Posttranslational modification, protein turnover, chaperones (Fig. 3*C*). On the other hand, the overrepresented groups in Hairy-activated transcripts were not only significantly different from those in Hairy-repressed transcripts,

but also from those in MET-activated transcripts. [E] Amino acid transport and metabolism and [F] Nucleotide transport and metabolism are the only two OG's showing statistical relevance for Hairy activated transcriptome.(Figure 4). Indeed, none of Hairy-activated transcript belonged to the OGs of [J] and [A], statistically associated with MET-activated transcriptome. There were no functional group significantly overrepresented in both MET and Hairy activated genes (Figure 4), thus leaving the idea of MET/ Hairy hierarchy for gene activation unsupported by functional analysis.

To enhance the annotation and gene mapping, we built contigs of *A. aegypti* transcripts by using the Trinity *de novo* assembly method and utilized the sequence information of these contigs to supplement gene sequences available in Vectorbase. We then annotated MET/Hairy co-repressed genes. During annotation, the isoform-specific and gene duplicated transcripts were removed, resulting in 193 MET/Hairy-repressed genes. The composition of functional groups amongst these 193 MET/Hairy co-repressed genes was similar to those from MET and Hairy repressed transcriptomes, mostly represented by the categories of Cellular Process and Signaling and of Metabolism (Fig. 2). About half of MET/Hairy-repressed genes belonged to the Metabolism category. Two OGs in the category – [G] Carbohydrate transport and metabolism and [Q] Secondary metabolites biosynthesis, transport and metabolism – were not only the groups with most number of MET- and Hairy-repressed genes, but were also the overrepresented OGs in MET/Hairy co-repressed genes (Fig. 2). Another large number of genes belonged to a group of protease/peptidase; 38 genes encoding serine proteases (SPs) and 7 genes encoding other types of protease/peptidase. Protease/peptidase belongs to the OG of [O]

category - posttranslational modification, protein turnover, chaperones in NOG functional grouping, but, some SPs also have a role in proteolytic signaling of immune response-related serine protease cascade [V]. Thus, this OG analysis has reinforced the hypothesis of Met and Hairy working in the same hierarchy mediating JH repressive action

Table 2 legend:

Changes in assignments of orthologous groups (OGs) for *A. aegypti* genes:

(*A*) The various sub-categories under the parental OG group. genes belonging to the original functional groups by eggNOG were clustered together in simplified parental OG categories. Three major categories Metabolism, Cellular process and signaling, and Information storage and processing are also mentioned. (*B*) Defining the gene group [V] Defense mechanism by manual annotation. 47 genes encoding antimicrobial peptides (AMPs), Gram-negative binding proteins (GNBPs), Fibrinogen and fibronectin proteins, Galactoside-binding lectins, Peptidoglycan recognition proteins (PGRPs), Toll and IMD pathway components, lysozymes and prophenoloxidases (PPOs) were annotated to the simplified [V] OG, which included the original OG assignment of [V], [TV] and [VW].

Table 2.

A

	The simplified functional groups	The original functional groups by eggNOG
METABOLISM	[Q] Secondary metabolites biosynthesis, transport and catabolism	Q
	[P] Inorganic ion transport and metabolism	P, PT
	[I] Lipid transport and metabolism	I, IJT , IKT , IM , IN, IO, IOTUV , IU
	[H] Coenzyme transport and metabolism	H
	[F] Nucleotide transport and metabolism	F
	[E] Amino acid transport and metabolism	E, EG , EH , EI , EJ , ET , EU
	[G] Carbohydrate transport and metabolism	G, CG
	[C] Energy production and conversion	C, CD , CH , CO
CELLULAR PROCESSES AND SIGNALING	[O] Posttranslational modification, protein turnover, chaperones	O, KOT , OU
	[U] Intracellular trafficking, secretion, and vesicular transport	U, CU , KU , UZ
	[W] Extracellular structures	W
	[Z] Cytoskeleton	Z
	[N] Cell motility	N, DN , NT , NTZ
	[M] Cell wall/membrane/envelope biogenesis	M, MO , MOT , MU , MW
	[T] Signal transduction mechanisms	T, IT , KT , TU , TUZ , TW , TZ
	[V] Defense mechanisms	V, TV , VW
	[Y] Nuclear structure	Y, UY
	[D] Cell cycle control, cell division, chromosome partitioning	D, BD , BDL , BDLT , BDT , DO , DP , DT , DTUZ , DTZ , DU , DUZ , DZ
INFORMATION STORAGE AND PROCESSING	[B] Chromatin structure and dynamics	B, BL , BT
	[L] Replication, recombination and repair	L
	[K] Transcription	K, AK , BK , DK , DKL , DKLT , JK , KL , KLO , KO
	[A] RNA processing and modification	A, ABO , AT
	[J] Translation, ribosomal structure and biogenesis	J, JO , JT , JU

B

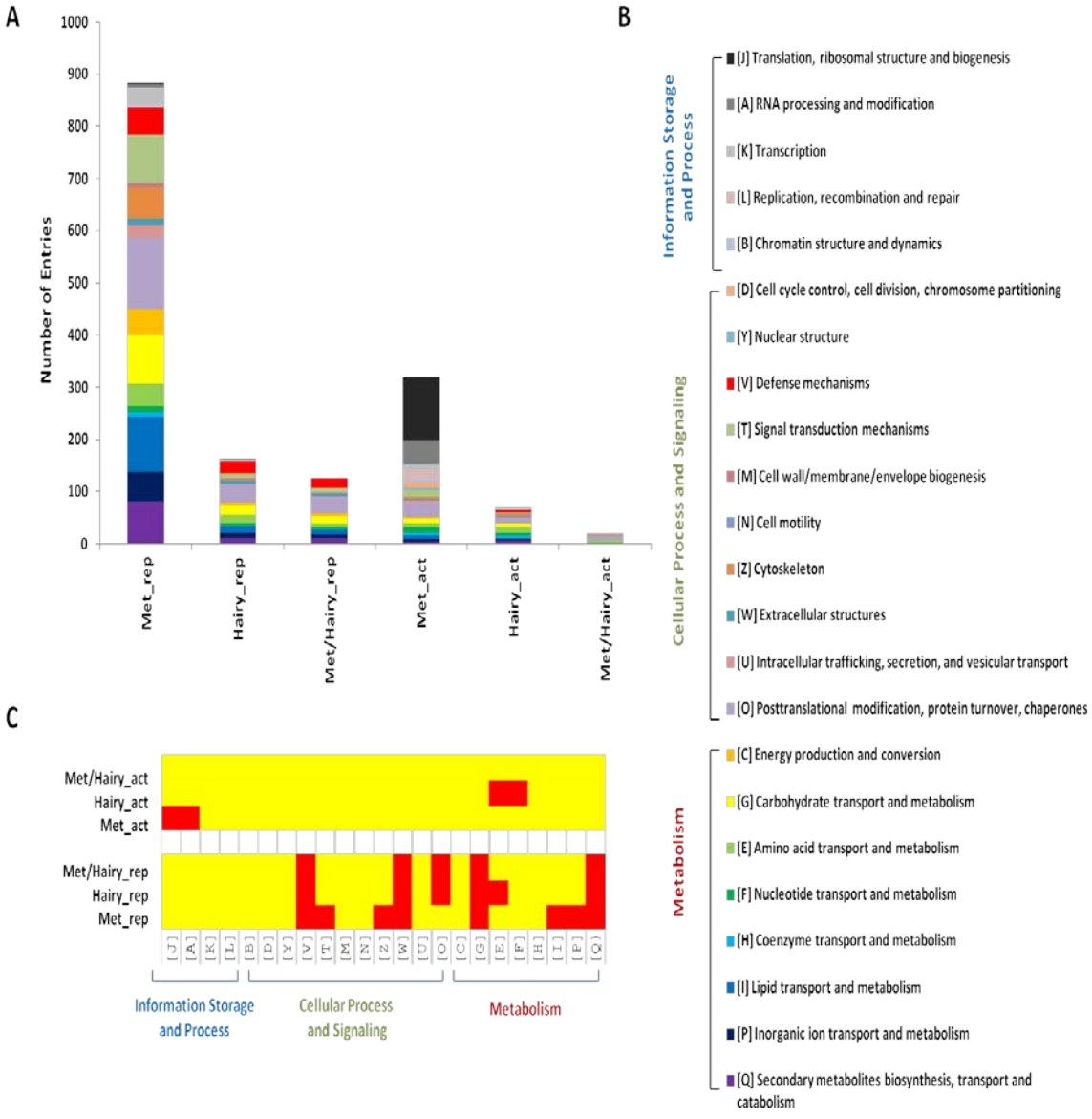
Putative immune genes	original NOG assignment	Changed to
AMPs	S	V
GNBPs	G	V
Fibrinogen and fibronectin proteins	R	V
Galactoside-Binding Lectins	W	V
IMD pathway components	T	V
Toll pathway components	NA	V
Rel transcription factors	K	V
Lysozyme proteins	S	V
Peptidoglycan Recognition Proteins	NA	V
Prophenoloxidases	NA	V

Fig. 3 legend:

Gene ontology analysis of MET and Hairy depleted mosquito transcriptomes:

(*A*) Gene ontology analysis of gene sets differentially expressed in the female fat body after RNAi depletion of MET and Hairy. Six different gene cohorts- MET repressed, Hairy repressed, MET/Hairy co-repressed, MET activated, Hairy activated and MET/Hairy co-activated are analyzed. The OGs are color coded full nomenclature of the OGs could be found in Fig. X*B*. (*B*)Three major categories and the functional groups with corresponding abbreviations were indicated. (*C*) OG's significantly overrepresented ($P<0.01$ in a hypergeometric distribution) in the above mentioned six gene cohorts are marked in red. The detailed nomenclature for OG abbreviations can be found in Fig. 3*B.*

Fig. 3.


A
B
C
Number of Entries
1000
900
800
700
600
500
400
300
200
100
0
Met_rep
Hairy_rep
Met/Hairy_rep
Met_act
Hairy_act
Met/Hairy_act
Information Storage and Process
[J] Translation, ribosomal structure and biogenesis
[A] RNA processing and modification
[K] Transcription
[L] Replication, recombination and repair
[B] Chromatin structure and dynamics
Cellular Process and Signaling
[D] Cell cycle control, cell division, chromosome partitioning
[Y] Nuclear structure
[V] Defense mechanisms
[T] Signal transduction mechanisms
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[Z] Cytoskeleton
[W] Extracellular structures
[U] Intracellular trafficking, secretion, and vesicular transport
[O] Posttranslational modification, protein turnover, chaperones
Metabolism
[C] Energy production and conversion
[G] Carbohydrate transport and metabolism
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport and catabolism
Met/Hairy_act
Hairy_act
Met_act
Met/Hairy_rep
Hairy_rep
Met_rep
[J] [A] [K] [L] [B] [D] [Y] [V] [T] [M] [N] [Z] [W] [U] [O] [C] [G] [E] [F] [H] [I] [P] [Q]
Information Storage and Process
Cellular Process and Signaling
Metabolism

Establishing MET/ Hairy hierarchy in JH mediated gene repression:

We utilized *in vitro* fat body culture system to test for JH sensitivity of verified MET/Hairy regulated genes. *In vitro* fat body tissue culture experiments were performed as mentioned previously. The minimal culture medium was supplemented with JH (treatment) or solvent alone (control), incubated for 3 h followed by tissue collection, RNA extraction, cDNA synthesis and qRT-PCR analysis. All the genes tested showed strong response to JH (Fig. 4*B*). MET/Hairy repressed marker genes identified from RNA-seq study were suppressed (Fig. 4*B*) by JH action.

In classic experiments, Ashburner utilized a protein synthesis inhibitor cycloheximide (CHX) to show the existence of an indirect ecdysone (20E) gene hierarchy. CHX prevents translation of intermediate factors in signaling pathway thereby preventing the hormone from triggering its usual response (22). We utilized this elegant model to probe the hypothesis about involvement of intermediate factors in JH/MET mediated gene repression. We conducted *in vitro* fat body experiments using fat bodies isolated from 3-6 h PE female mosquitoes and incubated them with different combinations of CHX and JH (Fig. 4). Transcript levels of four JH inhibited marker genes (MET/Hairy repressed in RNA-seq study) were tested. The sensitivity of this established JH response genes were completely compromised by the addition of CHX in the culture medium (Fig. 4*B*). *Krüppel homolog-1* (*Kr-h1*, and AAEL002390) and *Hairy* (*AAEL005480*), two genes directly regulated by MET, has been used as controls for this experiment (Fig. 4*A*). This experiment clearly demonstrated that there is a factor downstream of JH/MET, which

needs to be translated, for the repression of the tested marker genes by JH/MET signaling pathway.

We then employed tandem experiments of dsRNA mediated knockdown and *in vitro* fat body cell culture to track down the downstream factor in JH/MET signaling pathway regulating the marker gene expression. RNAi depletions of MET and Hairy was performed as described above. Fat bodies from mosquitoes with depleted Luc, MET and Hairy were incubated in the presence or absence of JH. After a 3 hr incubation period the tissues were collected and subjected to gene expression analysis by qRT-PCR. JH treatment resulted in characteristic repression of four marker genes (MET/Hairy repressed from RNA-seq study) (Fig. 5 *A*-*D*). MET is an integral part of JH signaling, and expectedly depletion of MET renders these genes insensitive to JH (Fig. 5 *A*-*D*). Depletion of Hairy phenocopied this effect of MET in marker gene expression, establishing Hairy as a mediator of JH/MET signaling pathway (Fig. 5 *A*-*D*).

Taken together, the results from RNA-seq data, the ontology study and *in vitro* fat body tissue culture experiments strongly support the hypothesis that Hairy is an intermediate component in the JH/MET repressive hierarchy.

Fig. 4 legend:

Novel protein synthesis is an absolute requirement for the regulation of gene expression by JH:

(*A*) Protein synthesis inhibitor cycloheximide (CHX) cannot block the JH mediated induction of *Krüppel homolog-1* (*Kr-h1*, and AAEL002390) and *Hairy* (*AAEL005480*). *Kr-h1* and Hairy are direct targets of MET and therefore does not require translation of additional factors for their induction (*B*) iMet/iHairy activated genes *Matrix metalloproteinase* (AAEL002655), *Conserved hypothetical protein* (function unknown; AAEL005004), *Phosphorylase* (AAEL000703) and *Amino acid transporter protein* (AAEL003619) are repressed by JH (10 μg/mL of JHIII). Addition of CHX to the minimal culture medium for *in vitro* fat body culture results in loss of JH sensitivity indicating the requirement of an intermediate component. All experiments were done in triplicates. *P*-value was calculated on the basis of two-tailed pair end *t*-test using software package GraphPad Prism 5.0.

Fig. 4.

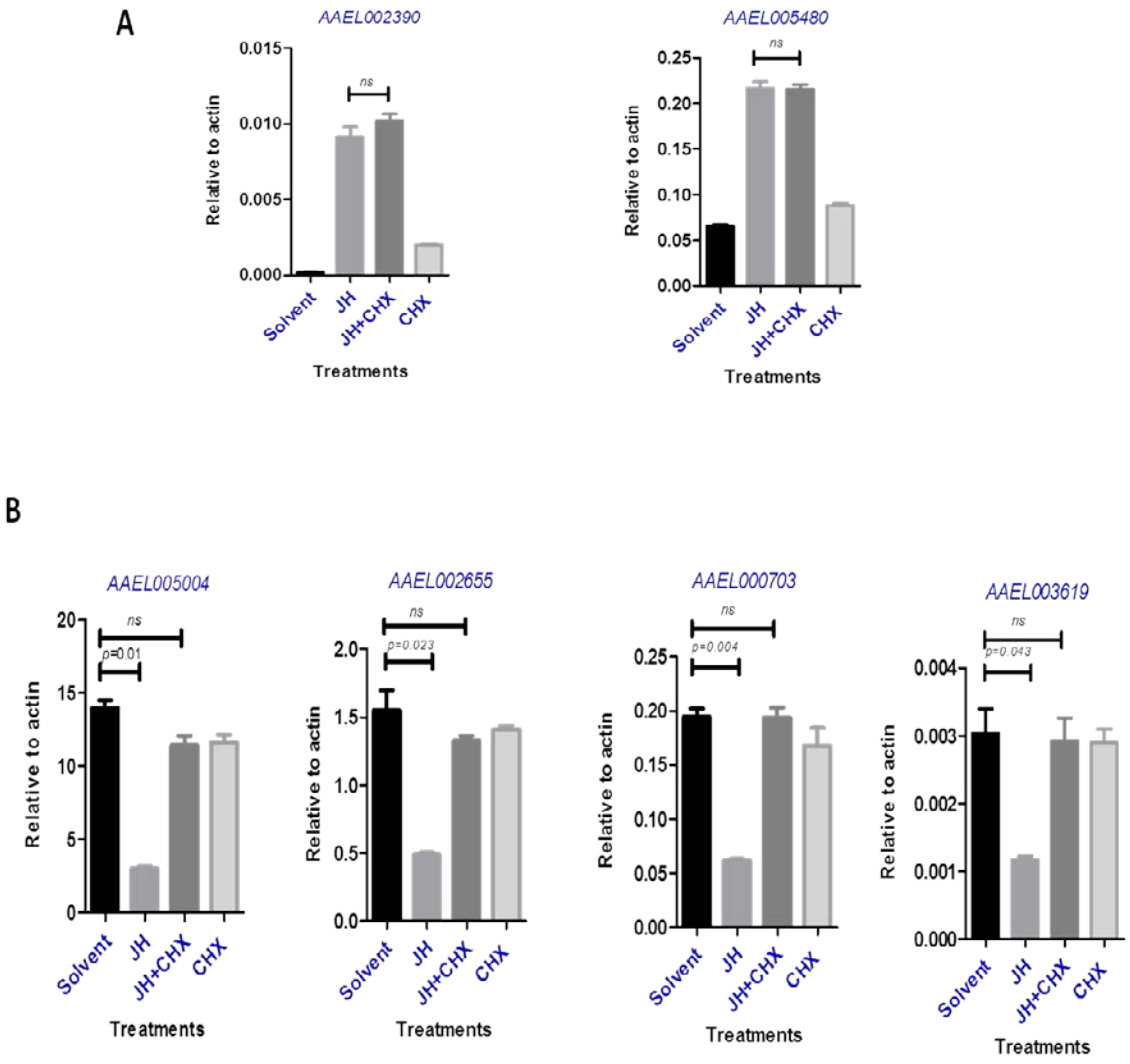
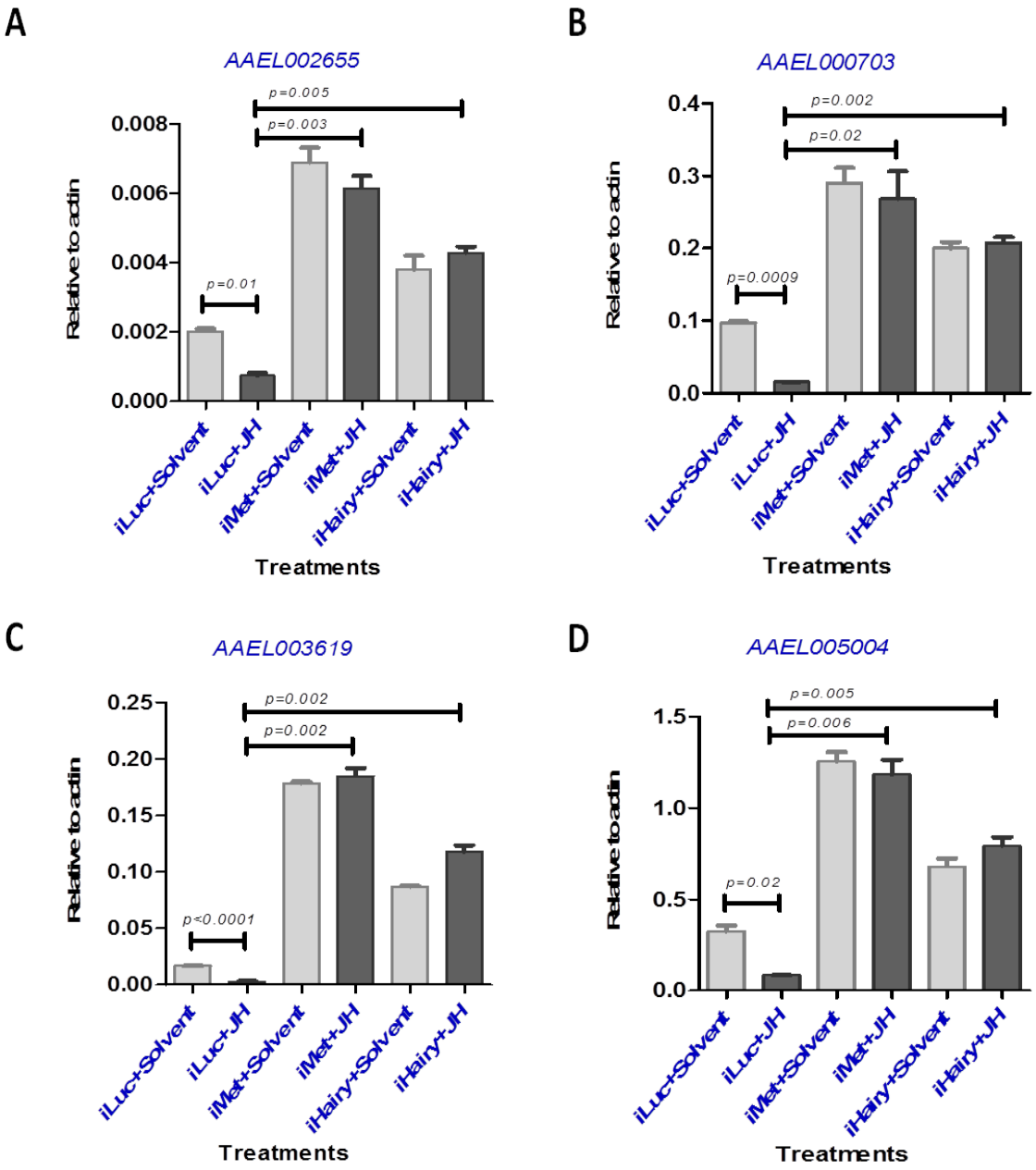

A
AAEL002390
Relative to actin
0.015
0.010
0.005
0.000
ns
Solvent
JH
JH+CHX
CHX
Treatments
AAEL005480
Relative to actin
0.25
0.20
0.15
0.10
0.05
0.00
ns
Solvent
JH
JH+CHX
CHX
Treatments
B
AAEL005004
Relative to actin
20
15
10
5
0
ns
p=0.01
Solvent
JH
JH+CHX
CHX
Treatments
AAEL002655
Relative to actin
2.0
1.5
1.0
0.5
0.0
ns
p=0.023
Solvent
JH
JH+CHX
CHX
Treatments
AAEL000703
Relative to actin
0.25
0.20
0.15
0.10
0.05
0.00
ns
p=0.004
Solvent
JH
JH+CHX
CHX
Treatments
AAEL003619
Relative to actin
0.004
0.003
0.002
0.001
0.000
ns
p=0.043
Solvent
JH
JH+CHX
CHX
Treatments

Fig. 5 legend:

Hairy is the downstream factor regulating gene repression in JH/MET signaling pathway:

(*A* and *B*) Tandem experiments with RNAi knockdown of MET followed by *in vitro* fat body culture with or without JH treatment. Fat bodies collected from 3 d post injection iLuc, iMet and iHairy mosquitoes were cultured in minimal medium with or without JH (10 μg/mL of JHIII) for 3h. (*A*-*D*) qRT-PCR based trascriptional profiling of JH/MET/Hairy repressed genes (*A*)*Matrix metalloproteinase* (AAEL002655), (*B*) *Phosphorylase* (AAEL000703), (*C*) *Amino acid transporter protein* (AAEL003619), and (*D*) *Conserved hypothetical protein* (function unknown; AAEL005004). Hairy knockdowns phenocopies the effect of MET knockdowns in rendering the candidates insensitive to repressive action of JH. All experiments were done in triplicates. *P*-value was calculated on the basis of two-tailed pair end *t*-test using software package GraphPad Prism 5.0.

Fig. 5.


A
AAEL002655
p=0.005
p=0.003
p=0.01
0.008
0.006
0.004
0.002
0.000
Relative to actin
iLuc+Solvent
iLuc+JH
iMet+Solvent
iMet+JH
iHairy+Solvent
iHairy+JH
Treatments
B
AAEL000703
p=0.002
p=0.02
p=0.0009
0.4
0.3
0.2
0.1
0.0
Relative to actin
Treatments
C
AAEL003619
p=0.002
p=0.002
p<0.0001
0.25
0.20
0.15
0.10
0.05
0.00
Relative to actin
Treatments
D
AAEL005004
p=0.005
p=0.006
p=0.02
1.5
1.0
0.5
0.0
Relative to actin
Treatments

Groucho is a possible co-repressor of Hairy in JH/Met signaling pathway:

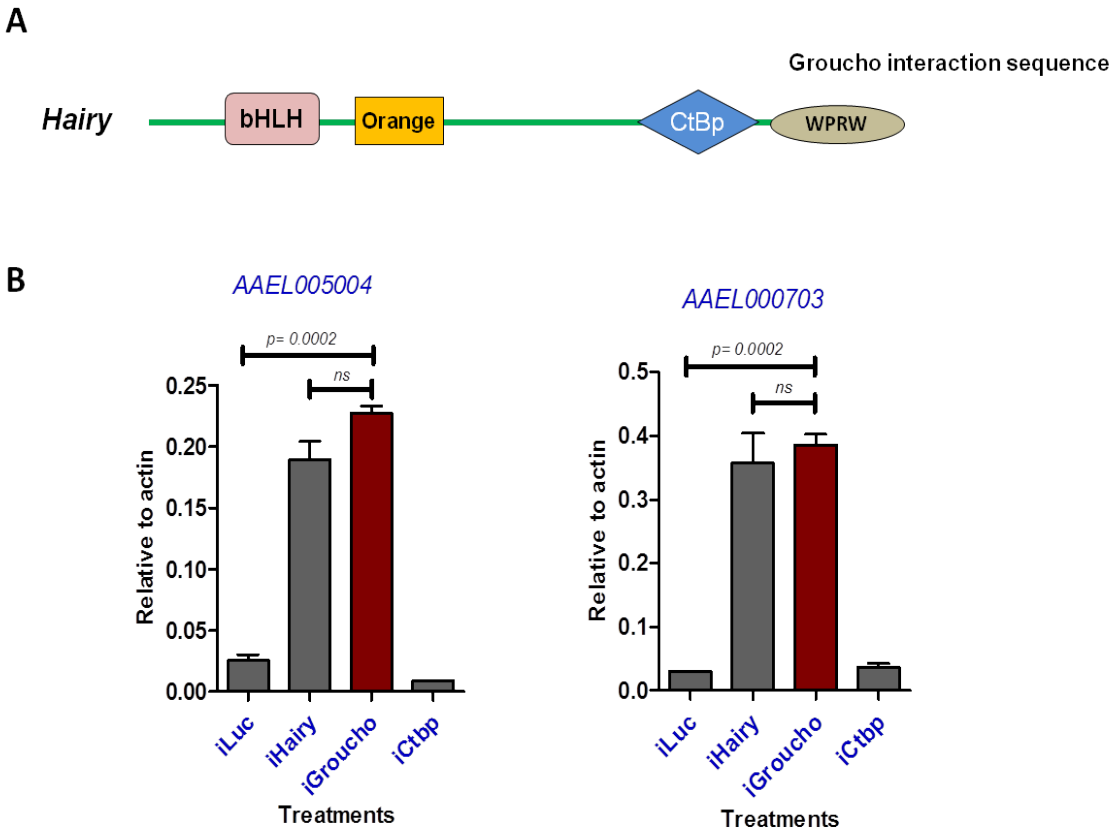
We then focused our attention to JH/MET/Hairy hierarchy in gene repression and analyzed the Hairy protein for recognition sequences of addition co-repressors. Hairy protein carried a binding domain for Groucho (binds to WRPW tetrapeptide domain) and C-terminal-binding protein (CtBP) (Fig. 6*A*). We tested potential involvement of these co-repressors in the JH/Met/Hairy mediated repression of particular genes. RNAi depletion of Groucho, but not CtBP, resulted in induction of *Conserved hypothetical protein* (function unknown; AAEL005004) and *Phosphorylase* (AAEL000703) genes; a phenotype exactly similar to the Hairy knockdown (Fig. 6*B*). This results indicate that Groucho might serve as a co-repressor of Hairy in regulating the JH/MET signaling pathway targets like *Conserved hypothetical protein* (function unknown; AAEL005004) and *Phosphorylase* (AAEL000703).

Fig. 6 legend:

Depletion of Groucho results in similar molecular phenotypes as that of Hairy knockdowns:

(*A*) Structure of the Hairy protein with various functional protein domains. Hairy is bHLH, Orange domain containing protein which harbors binding sites for two additional co-repressors Groucho (binds to WPRP domain) and C-terminal-binding protein (CtBp). (B) RNAi mediated knockdown of Luc, Hairy, Groucho and CtBp was performed as mentioned before. Tissues were collected 3d post injection and subjected to qRT-PCR expression analysis for profiling *Conserved hypothetical protein* (function unknown; AAEL005004) and *Phosphorylase* (AAEL000703). Depletion of Groucho and not CtBp, results in similar molecular phenotypes as the Hairy knockdowns. All experiments were done in triplicates. *P*-value was calculated on the basis of two-tailed pair end *t*-test using software package GraphPad Prism 5.0.

Fig. 6.


A
Groucho interaction sequence
Hairy
bHLH
Orange
CtBp
WPRW
B
AAEL005004
p= 0.0002
ns
Relative to actin
0.25
0.20
0.15
0.10
0.05
0.00
iLuc
iHairy
iGroucho
iCtbp
Treatments
AAEL000703
p= 0.0002
ns
Relative to actin
0.5
0.4
0.3
0.2
0.1
0.0
iLuc
iHairy
iGroucho
iCtbp
Treatments

Analysis of 5' upstream regions of MET/Hairy co-repressed genes

To find binding sequences responsible for Hairy-mediated repression, we analyzed 5-kb long 5' upstream UTRs of MET/Hairy co-repressed genes. Promoter sequences of 193 candidate genes, extracted using Biomart program from the *A. aedes* genome, were analyzed by the Multiple Em for Motif Elicitation (MEME) motif sequence analysis tool. We identified two putative non-coding DNA elements (CNEs) in the promoter region of these genes. The MET/Hairy-A (MH-A) modules, composed of 11 motifs with a total length of 260-290 bp, were represented in 57 of 193 genes (Figs. 7 and 8). The MET/Hairy-B (MH-B) modules were 550–650 bp in length and consisted of 19 motifs. 20 of 193 genes contained MH-B modules in their 5' upstream regions (Figs. 7 and 8). We have also chosen 143 ribosomal protein (RP) genes as a control for the MEME promoter analysis, because the expression of RP genes was activated by MET but were unaffected by Hairy depletion. MEME analysis of 5-kb upstream sequences of 143 RP genes showed the presence of three modules Ribosomal Protein A-C (RP-A, RP-B, and RP-C), which had completely different nucleotide sequences from those of MH-A and MH-B modules of 193 Met-Hairy repressed genes (Fig. 8)

When we tested the occurrence of the modules in upstream sequences of both gene groups by using blastn search with the nucleotide sequences of those modules against upstream sequences of MET/Hairy-repressed genes and those of RP genes, the results clearly showed that the modules were preferentially present in the upstream of MET/Hairy repressed or ribosomal protein genes, respectively (Table 3). The MH-A sequence of AAEL000025 aligned with 36 upstream sequence sites of MET/Hairy

repressed genes and 9 sites of RP genes, respectively (Table 3). Whereas, the RP-A sequence of AAEL013116 aligned with 9 sites of MET-Hairy repressed genes and 29 sites of RP genes (Table 3). Other domains of MH-B, RP-B, and RP-C also showed preferential presence between MET/hairy-repressed and RP genes. These control experiments gives confidence in identified modules.

Fig. 7 legend:

The MEME analysis of 5-kb upstream sequences of 193 MET/Hairy-repressed genes: This figure represents a part of the result covering upstream region of 43 genes. The two identified conserved modules, MH-A in 14 gene and MH-B in 4 gene promoters respectively amongst these 43 genes, are highlighted here. Each MH-A or MH-B module are indicated as red-lined or green-lined rectangular box. The gene ID from VectorBase annotations are mentioned to the left.

Fig. 7.

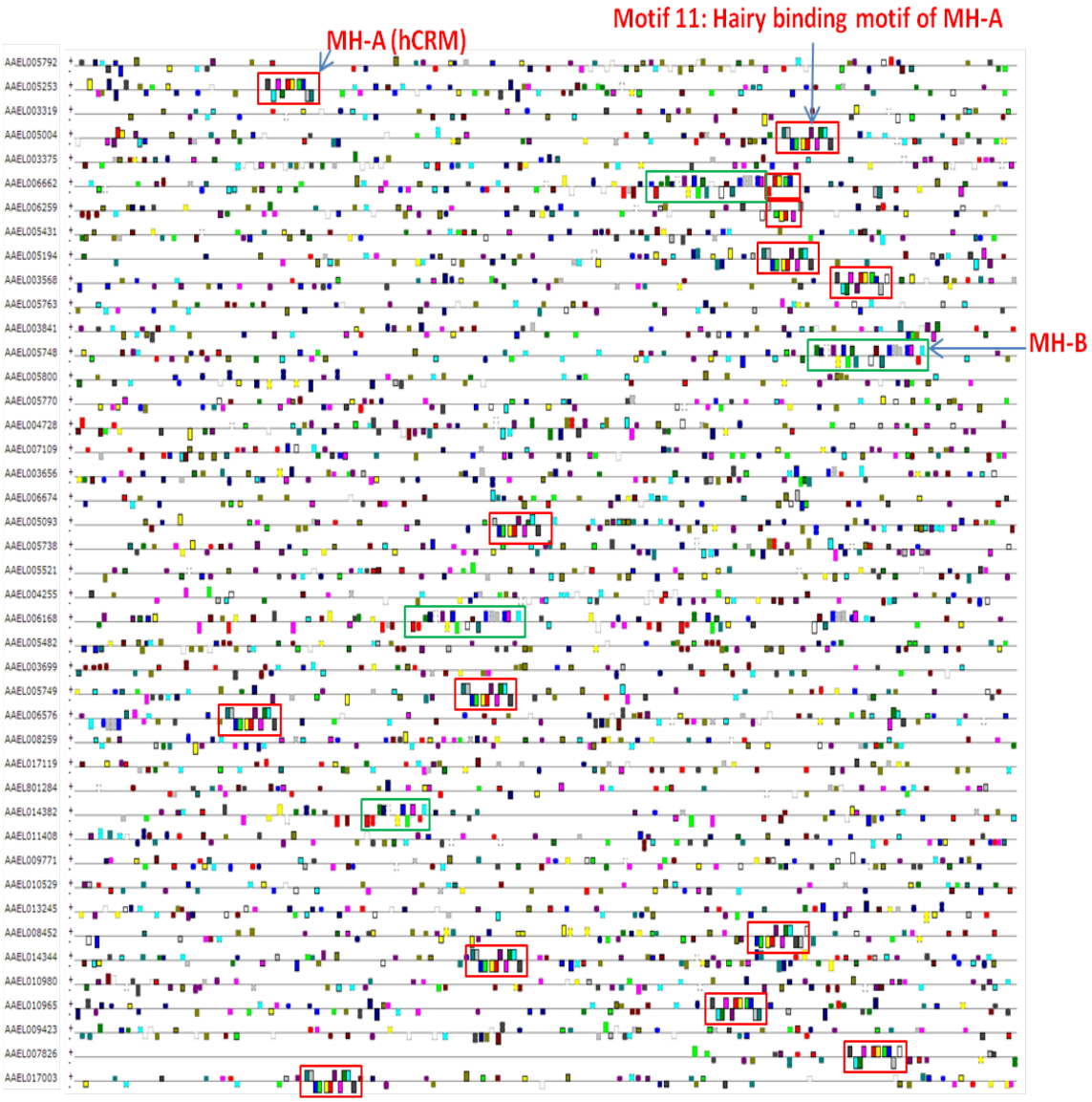


Fig. 8 legend:

Representative sequences of the identified modules from MET/Hairy repressed and control Ribosomal protein gene groups:

Sequence for MET/Hairy module A and B, and Ribosomal protein module A,B and C from selected candidate genes are provided. Specific gene ID for the gene whose sequence are used and the length of the sequence are also indicated. In the MH-A module from AAEL005004 5'-upstream region, the putative Hairy binding motif corresponding to a E-box-like motif has been highlighted. No E-box-like motif could be detected within the MH-B, RP-A, RP-B and RP-C module.

Fig. 8.

>MH-A AAEL005004 (278 nt long)
GGGCCCACATAGCCGTAGCGGTAAACGCACAGCTATTCAGCAAGACCAAGCTGAGGGTCATGGGTTCGAATTCCACCGGTCGAGGATCTTTTCGGGTTGGAAAT
TTTCTCGACTTCCCAGAACATAGAGTATCTTCGTACCTGCCACACGATAT**ACACATGC**AAAAATGGTCATTGGCAAAGTAAGCTCTCAGTTAATAACTGTA
GAAGTGCTCATAAGAACACTTAGCTGAGAAGCAGGCTCTGTCCCAGTGGGGACGTAACGCCAGAAAGAAGAAG
>MH-B AAEL801283 (639 nt long)
GCGGCCAAGACAAAGTACATGCTAGCTGGTGGGGCCGAGCGCGACAGGGCTCGCCTAGGTTGCAGTGTTACGATAGACGGGGATACGTTCGAGGTGGTCGACGA
GTTCGTCTACCTTGGATCCTTGCTGACGGCTGACAATAACGTTAGCCGTGAAATACGGAGGCGCATCATCAGTGGAAGTCGGGCCTACTATGGCCTCCAGAAGA
AGCTGCGGTCAAAAAAGATTCACGCCCGCACCAAATGTACCATGTACAAAACGCTCATAAGGCCGGTAGTCCTCTACGGGCATGAAACGTGGACGATGCTCGAG
GAGGACTTGCAAGCACTTGGAGTCTTCGAACGTCGGGTGCTTAGGACGATTTTCGGCGGTGTGCAGGAGAACGGTGTGTGGCGGCGAAGGATGAACCACGAGCT
CGCCCAACTCTACGGCGAACCCAGTATCCAGAAGGTGGCCAAAGCTGGAAGGATACGATGGGCAGGGCATGTTGCAAGAATGCCGGACAGCAACCCTGCAAAGA
TGGTATTCGCTTCGGATCCGGTTGGTACAAGAAGGCGTGGAGCGCAGCGAGCTAGATGGGCGGATCAAGTGCGTATCGATTTGGCGAGCGTGGGATGGAGAGAT
GCGGCCACGAACCGA
>RP-A AAEL013116 (426 nt long)
AATTGGGTCCTAAAATTTTTAATGAAATCTTGTTTTTAATTAATAACACGAAAAAGCATGTTACTGTAACTACTTTAGCAATTTTTCCCGCTCAAATAATGGCT
ATATCATGTTTAAACTTTAATCTAAAAATTTAGTTCATAAATGAACCTTGACACTTTTGATCATGTTTGACGTTCGCTTAGTCGACAAACACACCACAGGGGTT
TTAGTTTGACCACTGGGGTTGTTCCTATCTGACATTTCGTGAGGGACACGGAAAACAAAATACACCCAAAATTTGAGTTTGAGCCAAAGGACGTGACAAAATCT
AAAAAAAAAAATTGGGCTTAAACCGAAGGAAAACATTAGAAAATTGAGTAAATATGTGTTTTTGGCCTAAACTTAAGTGTTTGGCACTAAAATTGGGACAGGGC
TTTAGGACCC
>RP-B AAEL009151 (76 nt long)
ATTCATACAAAAAGCGTTACAAGGGGGTGGGTGGGTGTCAAAAATAGCCATTTTTAGCGTTATGAAATTTGTGAAT
>RP-C AAEL005817 (254 nt long)
CACAGACAAACAGACGTAACACTTAGAACAAATCTCGATCAAAATCATAGTCACGAGGACATGTACGCCCAATGCTAAAATCCGCGTGTTTGGCCGACGGGCCA
ACAGATGGCGGTAGTGTGTAAACGTCAAACACGAACAAAAACGAAGCAAGCGCTGTGGGTGGCGGATTGGCCACCTAACATATTTTTGAATCGACCGTTAAAAA
GGTGGTCGATGGACAATGATGAGAGTGTGACGTCTGTTTGTCTGTG

Table 3 legend:

The preferential presence of MH-A and MH-B module in the upstream region of MET/Hairy repressed genes:

Frequency of MET/Hairy modules A and B in the upstream region of 143 ribosomal genes were tested by blastn. Similarly, the occurrences of Ribosomal protein nodules A, B and C in the upstream region of 193 MET/Hairy repressed genes were also tested as a control. The gene ID for the candidates from which module sequences were used for the blastn search are indicated. The cutoff (E-value) used to select hits for the blastn search are also mentioned.

Table 3.

Domain_type	gene_ID	base pairs	cutoff (E-value)	193 MET/Hairy-repressed genes	143 Ribosomal protein genes
MH-A	AAEL000025	284	1.00E-50	36	9
MH-A	AAEL005004	278	1.00E-50	23	8
MH-B	AAEL801283	639	1.00E-50	13	3
RP-A	AAEL013116	426	1.00E-50	9	29
RP-B	AAEL009151	76	1.00E-10	2	22
RP-C	AAEL005817	254	1.00E-50	0	7

Analysis of Hairy binding to E-box-like motif in MH-A modules:

The canonical E-box motif (CANNTG) is represented by two sequence variants: type A motifs with the CA$^{G}/_{C}$CTG and type B motifs with the CAC$^{G}/_{A}$TG consensus. Each motif type binds to a different subclass of bHLH proteins (23). *Drosophila* Hairy specifically recognizes the canonical type B motif with a sequence CACGTG, but also a non-canonical site CGCGTG (11, 12).

Motif 11 identified in the MH-A module harbored an E-box-like motif. Among 57 upstream sequences of the conserved module MH-A, 42 contained motif 11. Majority of E box-like motifs in the MH-A module consisted of the sequence with a consensus C$^{A}/_{G}$CATG (11 sequences for CACATG, 12 for CGCATG totaling to 23).This consensus C$^{A}/_{G}$CATG has only one substitution of G to A in 4th nucleotide of the C$^{A}/_{G}$CGTG sequence of *Drosophila* Hairy binding site (11, 12). This kind of variation has been reported to be present in the type B motif of bHLH protein-binding E-box, CAC$^{A}/_{G}$TG (23) suggesting that C$^{A}/_{G}$CATG might be the Hairy binding motif in *A. aegypti*. All of the C$^{A}/_{G}$CATG sequences except one (the sequence from AAEL010965 MH-A) were flanked by A and C at the 5' and 3' ends respectively. Thus, the putative mosquito hairy binding site was refined to consensus sequence AC$^{A}/_{G}$CATGC

Of the remaining 19 motif 11-containing MH-A (total no. of MH-A with motif 11 is 42) modules, 14 harbored the sequence CGAATG. This sequence is flanked by an A in the 5'end for all 14 CGAATG, while 10 of them had a C in 3' end. Consequently, the consensus motif was modified to ACGAATGN. Whether this sequence motif along with

its flanking sequence is capable of binding Hairy, remains to be tested. Thus 88% (37 out of 42) motif 11 containing MH-A modules harbors an E-box-like motif, which are present in three variant forms.

Next, the binding between TNT translated *A. aegypti* Hairy and its putative binding sequence ACACATGC was investigated. ACACATGC motif and its flanking sequences obtained from the *Conserved hypothetical protein* (function unknown, AAEL005004) gene, which is co-repressed by MET and Hairy, were tested by means of EMSA (Fig. 10). The result clearly showed the specific binding between Hairy and this motif. Addition of the cold motif itself blocked the binding, but not by the non-specific competitor SP1. Addition of polyclonal antibodies against *Aedes* Hairy resulted in the super-shift of the binding complex, clearly indicating the presence of Hairy in it. The mutation of E-box-like sequence (AAEL005004m1) from class B-like sites to class A-like sites almost completely abolished the binding (Fig. 10), showing that class A E-box are incapable of binding to mosquito Hairy. However, when we mutated two base pair of ACACATGCAAA to ACACATGCAGG (AAEL005004m2), the binding was still intact, showing the conserved sequence of right flanking sequence was not important for Hairy binding (Fig. 10).

We already showed that there were three major variation of E-box-like sequences in motif 11 of MH-A (Fig. 9 *A*-*C*). We tested the binding of the variants to Hairy by mutating the AAEL005004 (CACATG) to AAEL005004m3 (CGCATG) or AAEL005004m4 (CGAATG). A clear binding between all of three variant motifs and

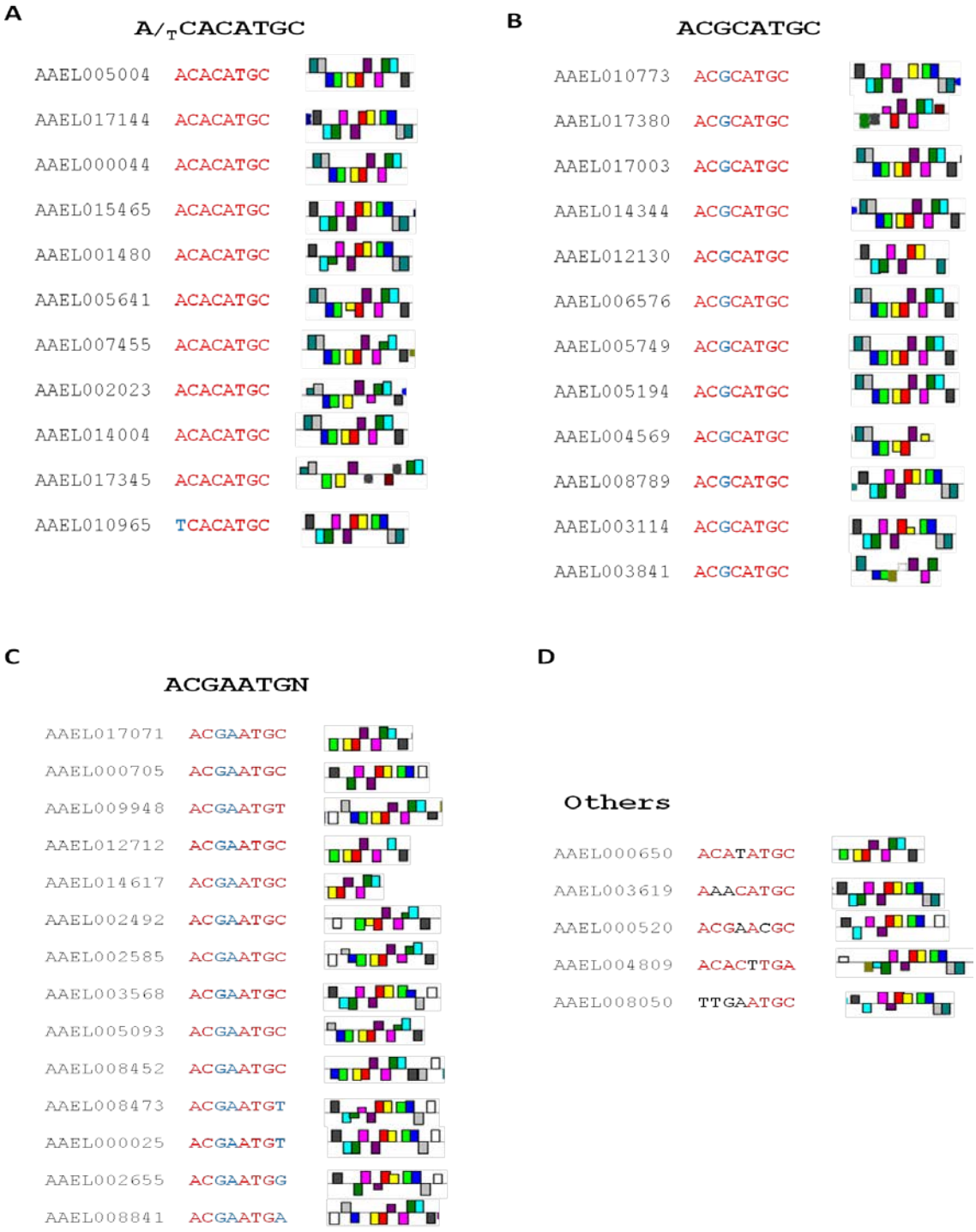
Hairy could be detected in EMSA. These results showed that a majority of MH-A motifs 11 binds to Hairy, suggesting MH-A is a Hairy-interacting putative *cis*-regulatory module (hCRM). Promoters of 37 (11 sequences for CACATG, 12 for CGCATG 14 for CGAATG) out of the 193 (about 20%) MET/Hairy repressed genes harbored the hCRM. The presence of modular structure flanking the identified Hairy binding motif in these genes suggests a complex mechanism of gene regulation.

Fig. 9. legend:

Variation in the E-box-like binding motif harbored in the MH-A module:

Of the 57 gene promoters containing MH-A module 37 harbored an E-box-like motif. There are three major variants of E-box-like motif found in MH-A: (*A*) CACATG (11 sequences), (*B*) CGCATG (12 sequences) and (*C*) CGAATG. The putative Hairy binding motif for all the 37 E-box-like containing MH-A modules along with the MEME predicted motif structures (right) are provided. The E-box-like sequence is harbored within the MEME predicted motif 11 in MH-A module. 42 amongst the 57 MH-A module has a motif 11. Of this 42, 37 (88%) harbors E-box-like motif. (*D*) The 5 gene which do not have a characteristic E-box-like motif along with MEME predicted modular structures are provided.

Fig. 9.


A
A/TCACATGC
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AAEL000044 ACACATGC
AAEL015465 ACACATGC
AAEL001480 ACACATGC
AAEL005641 ACACATGC
AAEL007455 ACACATGC
AAEL002023 ACACATGC
AAEL014004 ACACATGC
AAEL017345 ACACATGC
AAEL010965 TCACATGC
B
ACGCATGC
AAEL010773 ACGCATGC
AAEL017380 ACGCATGC
AAEL017003 ACGCATGC
AAEL014344 ACGCATGC
AAEL012130 ACGCATGC
AAEL006576 ACGCATGC
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AAEL008789 ACGCATGC
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AAEL003841 ACGCATGC
C
ACGAATGN
AAEL017071 ACGAATGC
AAEL000705 ACGAATGC
AAEL009948 ACGAATGT
AAEL012712 ACGAATGC
AAEL014617 ACGAATGC
AAEL002492 ACGAATGC
AAEL002585 ACGAATGC
AAEL003568 ACGAATGC
AAEL005093 ACGAATGC
AAEL008452 ACGAATGC
AAEL008473 ACGAATGT
AAEL000025 ACGAATGT
AAEL002655 ACGAATGG
AAEL008841 ACGAATGA
D
Others
AAEL000650 ACATATGC
AAEL003619 AAACATGC
AAEL000520 ACGAACGC
AAEL004809 ACACTTGA
AAEL008050 TTGAATGC

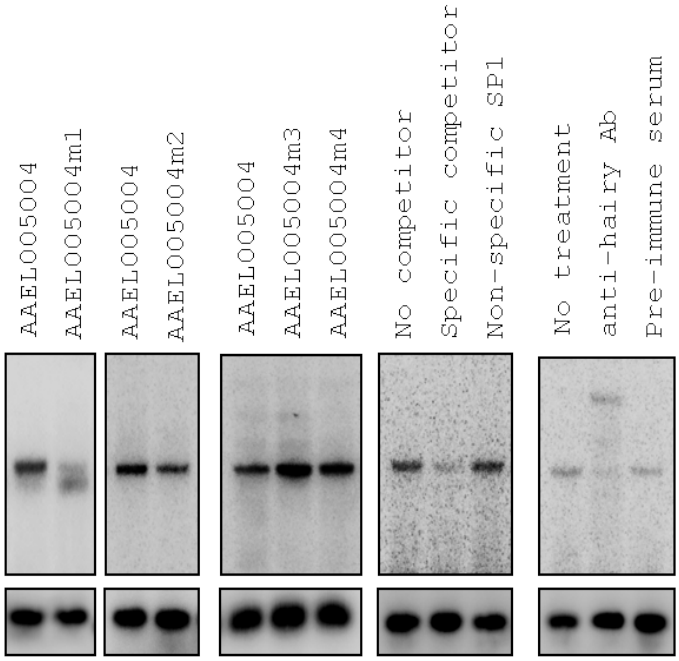
Fig. 10 legend:

EMSA analysis of putative Hairy binding motif within MH-A module:

The binding of *in vitro* translated Hairy to the E-box-like motif and its flanking sequence from the promoter region of AAEL005004, harbored within the motif 11 of MH-A module. Each probe with corresponding flanking sequences has been indicated in the lower panel. The presence of Hairy in the DNA-protein complex formed by the motif in EMSA with *in vitro* translated Hairy was confirmed by adding polyclonal antibody against *A. aegypti* Hairy, resulting in super-shift of the DNA-protein complex. The binding properties of mutated AAEL005004 - AAEL005004m1 (motif mutated to class A E-box motif), AAEL005004m2 (mutation in the 3' flank), AAEL005004m3 and AAEL005004m4 (motif mutated to variants of the E-box-like motif observed within MH-A module) were also tested.

Fig. 10.

AAEL005004 (putative Hairy binding motif, TNT-hairy)



AAEL005004	CAGCATATACACATGCAAAAATGG
AAEL005004m1	CAGCATATACAGCTGCAAAAATGG
AAEL005004m2	CAGCATATACACATGCAGGAATGG
AAEL005004m3	CAGCATATACGCATGCAAAAATGG
AAEL005004m4	CAGCATATACGAATGCAAAAATGG

DISCUSSION:

Methoprene tolerant (MET), the receptor of JH (24, 25), is found to play a critical role during post eclosion (PE) developmental phase in adult mosquitoes (1, 2). Transcriptionally, PE is hyper active phase and MET significantly contributes in controlling the gene expression of PE female mosquito. Our previous studies have demonstrated that MET-repressed genes do not harbor any MET-binding putative motifs in the promoter region (2) suggesting the absence of a direct action of MET in gene repression. Thus, an important question has remained unanswered concerning the modulation mechanism JH/MET repressive action on gene expression. Here, we have identified an intermediate factor of JH/MET signaling pathway Hairy, which functions in repression of target genes. Hairy is a bHLH transcription factor with an Orange domain and C-terminal Groucho and CtBP binding domains (15). Hairy is highly induced as result of MET depletion in adult mosquito (1, 2). The MET/Hairy hierarchy in gene repression is very apparent with 79% of Hairy repressed genes overlapping with the ones suppressed by MET. Functional genomics provides support to this hypothesis. Using the classic protein synthesis inhibitor cycloheximide (CHX) the necessity of intermediate factors for JH/MET mediated gene repression was characterized for a set of marker genes. Combining RNAi and *in vitro* fat body tissue culture the existence of JH/MET/Hairy hierarchy for gene repression could be validated for these marker genes. This study is the first report of JH/MET/Hairy heirarchy JH signaling pathway regulating gene repression. However, the 247 MET/Hairy co-repressed genes identified from RNA-seq study only accounts for 15% of the total number of MET repressed genes indicating involvement of

other mechanisms and transcription factors. *Krüppel homolog-1* (*Kr-h1*, and AAEL002390), a zinc finger domain containing protein, which is activated by MET (1, 2) can be a good target to probe in the future. Also, it should be pointed out that here that knockdown of MET results in much greater induction in all the tested genes in comparison to iHairy. Similar difference in expression level exists between iMet and iHairy in RNA-seq data. With our current understanding, we cannot explain this observation.

Clustering behavior of transcription factor binding sites has been studied for more than a decade, following the availability of genome sequences of higher animals (26). This cluster of *cis*-regulatory information found in many key developmental genes was organized into modular units of a few hundred base pairs. These *cis*-regulatory modules (CRMs) were observed in many developmental genes in *Drosophila* and in other organisms. Most CRM studies have focused on the conserved non-coding DNA elements (CNEs) identified by multi-species comparative genomic studies (27). These CNEs are abundantly found in regions around genes that encode transcriptional regulators involved in embryonic development (28) and can act as CRMs and drive gene expression in embryos (29). In this study we have identified a putative hairy interacting CNE (hCNE) in promoter regions of 20% of the MET/Hairy co-repressed genes. The hairy interaction of E-box-like motifs in this putative CNE has been established through EMSA analysis. The biological role of such complex modular structure is perplexing. All this indicates a complex mode of gene regulation which needs further investigation

Hairy is an established gene repressor in *Drosophila*, which functions by recruiting several other co-repressors. Our results suggests the role of Groucho as a co-repressor for Hairy in mediating JH/MET dependant gene repression. Hairy protein has a C-terminal conserved WRPW tetrapeptide that is necessary and sufficient for the recruitment of Groucho. Groucho is a WD-repeat containing protein that is not able to bind DNA on its own. But when brought to an endogenous or heterologous promoter it serves as a strong repressor of transcription (30). Groucho, in turn, has been proposed to recruit Rpd3, a class I histone deacetylase, suggesting a mechanism involving chromatin remodeling (17). Recruitment of Groucho, however, does not account for all of Hairy's repressor properties. Hairy has been shown to repress transcription in the absence of Groucho, presumably through a number of chromatin independent mechanisms (30).

Activation by JH/MET signaling pathway in general remains a mystery. Apart from a proposed direct model for small set of MET activated genes the entire mechanism is a black box. Our results indicate that there is no systemic hierarchy between MET and Hairy in gene activation. Although, a small number of genes are co-activated by both MET and Hairy and this was confirmed by qRT-PCR for selected candidates. It would be interesting to probe the mechanism of regulation of this small set of genes. Further research is needed before we can assemble a picture for JH/MET dependant activation of genes.

There is a cohort of gene, which are activated by Hairy and not by MET. The mechanism of regulation of this set of genes can also take us to novel directions. Of particular curiosity is the question how Hairy, an established repressor also acts as an

gene activator under specific conditions? Different set of co-regulators might play a role in switching Hairy from a repressor to an activator, a hypothesis which remains unexplored.

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Chapter III

Circadian expression of juvenile hormone-induced genes in mosquito *Aedes aegypti*: the role of bHLH-PAS heterodimer of methoprene-tolerant and cycle

ABSTRACT:

The juvenile hormone (JH) receptor methoprene tolerant (MET), being a bHLH-PAS factor, requires formation of homo- of heterodimer with another bHLH-PAS factor to render its biological effects. The mosquito the steroid receptor coactivator SRC/FISC (*Drosophila* TAI homolog) hasbeen previously reported to interact with MET regulating JH-mediated gene expression. Recent study in our laboratory has identified *Aedes* Cycle (CYC, AAEL002049), another bHLH-PAS factor, as a binding partner of MET. Here, we show that in adult female *A. aegypti* mosquitoes JH III control of gene expression is mediated by a heterodimer of two bHLH-PAS proteins-MET and CYC. In newly eclosed female mosquitoes, the expression of two JH-responsive genes, *Kr-h1* and *Hairy*, was dependent on both the ratio of light to dark periods and JH III. Their expression was compromised by *in vivo* RNA interference (RNAi) depletions of CYC, MET, and FISC. Moreover, an *in vitro* fat body culture assay has shown that JH III was not effective in induction of *Krh1* and *Hairy* gene expression in in female mosquitoes with RNAi-depleted CYC, MET or FISC . A sequence containing an E-box–like motif from the *Aedes Kr-h1* gene promoter specifically interacted with a protein complex, which included MET and CYC from the female mosquito fat body nuclear extract. These results

indicate that a MET/CYC heterodimer mediates JH III activation of *Kr-h1* and *Hairy* genes in the context of light-dependent circadian regulation in female mosquitoes during posteclosion development. This study provides an important insight into the understanding of the molecular basis of JH action.

INTRODUCTION:

The role of basic helix–loop–helix (bHLH)-Per-Arnt-Sim (PAS) protein Methoprene-tolerant (MET) in mediating juvenile hormone (JH) response has been established (1). Binding of MET to its ligand JH III has also been probed at great detail (2, 3) leading scientists to accept MET as the JH receptor. As a bHLH-PAS protein, MET requires either a homo- or heterodimeric partner for its activity (4). The first identified bHLH-PAS binding partner of MET was MET itself and its paralog germ cell expressed (GCE) from *Drosophila* (5). But, the MET/MET homodimer formation was reported to be disrupted in the presence of JHIII (5) and it is unlikely that MET/MET or MET/GCE complexes are involved in transduction of JH signal. As previously mentioned, yeast two hybrid screens in mosquito *Aedes aegypti* identified bHLH-PAS factor Ftz-F1-interacting steroid receptor coactivator (FISC) (6) as a MET partner. FISC was confirmed to be a binding partner of MET in beetle *Tribolium*, co-regulating several JH responsive genes and was named SRC because of its homology to the vertebrate steroid receptor coactivator-1 (SRC-1, synonymous to p160 or NCoA-1) (7). The closest *Drosophila* relative of FISC/SRC is Taiman (TAI); a known EcR coactivator (8). Recently, MET-TAI interaction has also been reported in *Bombyx* mori (9). Thus, the question was asked whether an additional bHLH transcription factor with DNA-binding properties serves as

MET partner. In our Laboratory, research led by Dr. Shin focused on finding novel bHLH-PAS binding partners of MET using yeast two-hybrid (Y2H) library screening system. The bait plasmid contained *Aedes* $MET^{122–977}$ that included the bHLH, PAS-A, and PAS-B domains and the 477-long C-terminal region (Fig. 1*A*). A prey library was prepared from mRNA isolated from *A. aegypti* female mosquitoes, 1–2 d PE. The screening led to the identification of *A. aegypti* bHLH-PAS protein CYC (AAEL002049) and the protein-protein interaction was found to be JHIII dependant (Fig. 1*B*). The VectorBase annotation of *Aedes* CYC was truncated; therefore, full length cDNA was cloned by rapid amplification of both cDNA ends, followed by DNA sequencing. The full-length cDNA of 3,122 nucleotides encoded a 744 amino acid-containing protein, adding another 90 amino acids to the N-terminal end to the sequence found in VectorBase. The particular clone (Y24) identified in the Y2H screen was found to include a mosquito cDNA sequence that encoded a partial CYC^{17-678} protein.

Following the lead, the binding between *Aedes* $MET^{122–977}$ and the full-length *Aedes* $CYC^{1–744}$ was tested. As controls, the *Aedes* ortholog of *Drosophila* Tango (TGO) was used. $MET^{122–977}$, $CYC^{1–744}$, and $TGO^{1–570}$ exhibited only a background binding to $MET^{ET22–977}$ in the absence of JHIII. In the JH III-containing selective growth media, $MET^{122–977}$ and $CYC^{1–744}$ were found to show a strong binding resulting in complementation of yeast cell growth (Fig. 1*C*). However, $TGO^{1–570}$ showed only a background binding level to $MET^{122–977}$ even in the presence of JH III. Likewise, a MET/MET homodimer formation was not observed, irrespective of the presence or absence of JH III (Fig 1*C*).

Furthermore, the effect of the ligand concentration on MET/CYC binding was established using the Y2H β-galactosidase assay in yeast cells. The level of MET/CYC and MET/MET binding was evaluated using β-galactosidase reporter assays, following the addition of either JH III or JH analog methoprene to the yeast cells (Fig. 1*D*). While JHIII strongly induced binding between $MET^{122–977}$ and $CYC^{1–744}$ in a dose-dependent manner, methoprene did not enhance protein-protein interaction even at high ligand concentrations. (Fig. 1*D*). Although these experiments confirmed the involvement of JHIII in mediating the heterodimer formation, it raised some questions regarding methoprene action. It was expected that the JH analog methoprene would behave in similar fashion as JHIII, a contradiction which cannot be explained with our current understanding. There was no MET/MET interaction in the presence of either JH III or methoprene, validating our previous results (Fig. 1*D*).

MET/CYC interaction could be confirmed by coimmunoprecipitation (co-IP) experiments using tagged c-myc-$MET^{122–977}$ and HA-$CYC^{1–744}$ fusion proteins, coexpressed in *Drosophila* S2 cells. After immunoprecipitation with the anti-c-myc antibody, HA-$CYC^{1–744}$ was detected with anti-HA antibody as a co-IP product, demonstrating interaction between MET and CYC (Fig. 1*E*).The protein–protein interaction between MET and CYC in the Co-IP reaction did not require the presence of JH III. This difference with JH III-dependent MET and CYC binding in the Y2H system is not clear. Nevertheless, the co-IP experiment serves as a confirmation of MET/CYC interaction.

In *Drosophila*, CYC is a bHLH-PAS protein that plays a central role in regulating circadian rhythms via heterodimerization with another bHLH-PAS protein Clock (CLK) (10). In this chapter, we investigate the role of CYC and MET in circadian gene expression of adult *Aedes* female mosquitoes. Depletion of either *CYC* or *MET b*y means of RNA interference (RNAi) impaired the circadian activation of *Kr-h1* and *Hairy* genes. Moreover, JH III was not effective in induction of *Kr-h1* and *Hairy* gene expression *in vitro* in the fat body of female mosquitoes with RNAi-depleted *CYC*, *MET*, or *SRC/FISC* in contrast to wild-type and control RNAi mosquitoes. We provide evidence that the MET/CYC heterodimer specifically binds to a sequence containing the E-box–like motif in the regulatory region of the *Kr-h1* gene. These results indicate that the MET/CYC heterodimer mediates JH III regulation of circadian gene expression in the mosquito *A. aegypti* and provide an important insight into the mode of action of this key insect hormone.

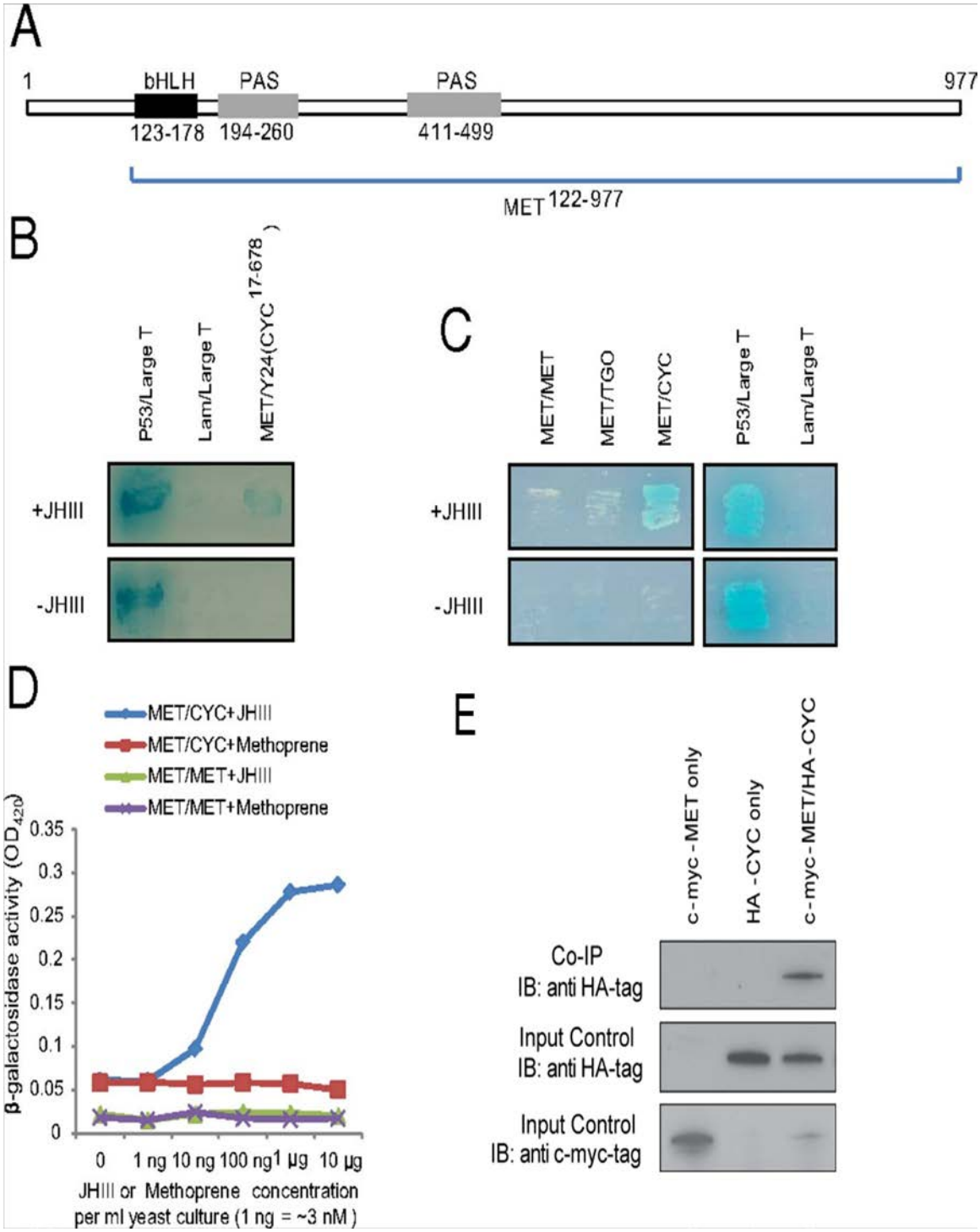
Fig. 1. Legend:

CYC binds to MET in a JH III-dependent manner:

(*A*) Schematic diagram of MET used to construct yeast bait. $MET^{122–977}$ contained bHLH, PAS-A and PAS-B domains, and the full C-terminal region. (*B*) A MET-binding Y2H clone (Y24) encodes *A. aegypti* CYC. JH III (10 μg/mL) was necessary for the growth of the yeast clone. (*C*) Full-length CYC specifically bound to MET in the presence of 10 μg/mL JH III. No significant binding was observed between MET and MET in the presence of JH III (MET/MET). *Aedes* TGO bound to MET at the background level in the presence or absence of JH III (MET/TGO). In both *B* and *C*, the yeast colony transformed with pGBKT7-53 and pGADT7-T plasmids was used as a positive control. The two positive control plasmids were a fusion protein of GAL4 DNA-BD with murine p53 and a fusion of GAL4-AD with large T-antigen, respectively. Murine p53 and large T-antigen specifically bound to each other, irrespective of the presence of JH III (P53/Large T). For the negative control, pGBKT7-Lam expressing human lamin C was used in place of the pGBKT7-53 plasmid and to test its interaction with murine p53 as a control for fortuitous interactions. This control was negative under all conditions (Lam/Large T). (*D*) JH III, but not methoprene, mediated binding between MET and CYC in a dose-responsive manner. Measurements were performed by means of the quantitative yeast β-galactosidase assay. (*E*) Coimmunoprecipitation of MET and CYC. Complementary DNAs encoding A. aegypti MET122–977 or CYC1–744 were amplified by means of PCR, inserted into pAC5.1/V5/HisA vector (Invitrogen) with a specific 5′-terminal tag (c-myc for MET and HA for CYC). These two plasmids were transfected

separately or together in Drosophila S2 cells. Cell lysates from these three samples were collected by centrifugation at 15,000 × g for 10 min at 4 °C and treated with anti-c-myc mouse monoclonal antibody, which was followed by precipitation with protein-G-agarose. The precipitated samples were subjected to Western blotting with anti-HA rabbit polyclonal antibody. For input controls, c-myc-MET and HA-CYC are transfected separately or together in Drosophila S2 cells and detected by Western blotting with either anti-HA rabbit polyclonal or anti-c-myc mouse monoclonal antibodies.

Fig. 1.


A
1
bHLH
PAS
PAS
977
123-178
194-260
411-499
MET 122-977
B
P53/Large T
Lam/Large T
MET/Y24(CYC 17-678)
+JHIII
-JHIII
C
MET/MET
MET/TGO
MET/CYC
P53/Large T
Lam/Large T
+JHIII
-JHIII
D
MET/CYC+JHIII
MET/CYC+Methoprene
MET/MET+JHIII
MET/MET+Methoprene
β-galactosidase activity (OD420)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1 ng
10 ng
100 ng
1 µg
10 µg
JHIII or Methoprene concentration
per ml yeast culture (1 ng = ~3 nM)
E
c-myc-MET only
HA-CYC only
c-myc-MET/HA-CYC
Co-IP
IB: anti HA-tag
Input Control
IB: anti HA-tag
Input Control
IB: anti c-myc-tag

MATERIALS AND METHODS:

Experimental Animals:

The UGAL/Rockefeller strain of *A. aegypti* mosquitoes was maintained in the laboratory as described previously (*Material and methods*, Chapter I). Adult mosquitoes were provided with water and a 10% sucrose solution. To initiate egg development, mosquitoes were blood fed on white rats. The University of California at Riverside Institutional Animal Care and Use Committee approved all procedures for use of vertebrate animals.

RNAi Approach:

For RNAi-mediated depletion, dsRNAs were synthesized using T7 RNA polymerase. Synthesis of dsRNAs was accomplished by simultaneous transcription of both strands of template DNA using the MEGAscript kit (Ambion). A Picospritzer II (General Valve) was used to introduce dsRNA into the thorax of CO_2-anesthetized mosquito females at 1–2 D PE. Mosquitoes were assayed 4D after dsRNA injections. Mosquitoes injected with luciferase dsRNA (iLuc) served as controls. The primers for RNAi are provided here (Table 1).

Table 1 legend:

Primers used for RNA interference mediated knockdown of genes:

Shown below is a list of primers used for RNAi knockdown of specific genes. All of primers are in 5'-3' direction. All the primers have a T7 RNA polymerase binding sequence at the 5' end.

Table 1.

PRIMER NAME	SEQUENCE
MET forward	TAATACGACTCACTATAGGGTCAATTTGTTCGACTCTGCG
MET reverse	TAATACGACTCACTATAGGGATACACAAAGTCGCCCGTTC
CYC forward	TAATACGACTCACTATAGGGCCAGAGTGCAAGGAATGTCA
CYC reverse	TAATACGACTCACTATAGGGAGTCGCACAAACCCATTTTC
FISC forward	TAATACGACTCACTATAGGGCGGCCGAGTGAACTTCTTAG
FISC reverse	TAATACGACTCACTATAGGGTGGATGTCTTGCTGACTTGC
Spineless forward	TAATACGACTCACTATAGGGACACATTCCTACCGGCTGAC
Spineless reverse	TAATACGACTCACTATAGGGTTGGATCGGCATGTAGACAA
Similar forward	TAATACGACTCACTATAGGGCCACCAGTGTGACCATGAAG
Similar reverse	TAATACGACTCACTATAGGGAACGATGCTGTGAGGCTTCT

RNA Preparation, Northern Analyses, and Real-Time RT-PCR:

Total RNA was prepared using TRIzol (GIBCO/BRL). For Northern blotting, 5 μg of total RNA from each sample was separated on a formaldehyde gel, blotted, and hybridized with the corresponding ^{32}P-labeled DNA probe. Real-time RT-PCR experiments were performed, as described previously (*Material and methods*, Chapter I). Primers used for the gene expression analysis are provided here (Table 2).

Table 2 legend:

Primers used for Real time quantitative PCR and semi-quantitative RT-PCR:

Shown below is a list of primers used for Real time quantitative PCR and semi-quantitative RT-PCR. All of primers are in 5'-3' direction. The Real time quantitative PCR primers leads to amplification of product 80-140bp in length. Semi-quantitative RT-PCR products are 500-1000bp in length.

Table 2.

PRIMER NAME	SEQUENCE
Real Time Quantitative PCR	
MET forward	GGACGACAGCTCAAAGAAGG
MET reverse	TCACTTTCATCGGGGAGTTC
CYC forward	TGGGCACCAGTATGTACGAA
CYC reverse	GTCGCACAAACCCATTTTCT
Kr-h1 forward	CAGGTCGTCAATCTGAGCAA
Kr-h1 reverse	GGTCTGGTGGAAGTTCGTGT
Hairy forward	CAAGTGCACATTTTGCCATC
Hairy reverse	GGATCAACTGAACGCTGGAT
Br-Z4 forward	TTAAACCACTCCAGCGGCGG
Br-Z4 reverse	GGTTGCAGGTGCTGTTGCTC
AAEL002619 forward	GGTGCCTTCAATCGAATGTT
AAEL002619 reverse	GGCGTTCTCAGGAAAGTCAC
FISC forward	CGGCCGAGTGAACTTCTTAG
FISC reverse	TGGATGTCTTGCTGACTTGC
Kr-h1 forward	CGCTTACCCAGCTATGCTTC
Kr-h1 reverse	CTCTTGAACGTTTCATCGCA
Hairy forward	TGACCGTGAAACATTTGGAA
Hairy reverse	CGGTCTCCAAGGTTTGTCAT

***In Vitro* Fat Body Culture:**

The *in vitro* fat body culture experiments were performed as described previously (*Material and methods*, Chapter I). Fat bodies were dissected 4D after dsRNA injections of *Aedes* female mosquitoes. These fat bodies were incubated for 6 h in a complete culture medium supplemented with either 1 µg/mL JH III or a solvent (acetone). Incubation plate wells were siliconized before incubation with JH to prevent its absorption by plastic.

Rapid Amplification of c-DNA ends:

Rapid Amplification of cDNA Ends (RACE) was performed using the SMART RACE cDNA Amplification Kit (Clontech) to obtain the 5′ and 3′ ends of the cDNA sequences of *A. aegypti Kr-h1*. Reverse transcription was carried out using an Omniscript reverse-transcriptase kit (Qiagen) with oligo (dT) primers. PCR was performed using Platinum High Fidelity Supermix (Invitrogen). Primers used are provided (Table 3)

Table 3 legend:

Oligonucleotides used for RACE:

Shown below is a list oligonucleotides used for RACE analysis: All of the primers/oligonucleotides are in 5'-3' direction.

Table 3.

PRIMER NAME	SEQUENCE
Kr-h1 first	GCGATTGACGCCGCTGCCAAC
Kr-h1 nest	CGTTGGCATGGCCCTTGATGTGG

Electrophoretic Mobility Shift Assays:

The annealed deoxyoligonucleotide of each E-box–like motif was purified from 15% TBE Criterion Precast Gel (Bio-Rad). Double-stranded oligonucleotides were labeled by γ-^{32}P ATP. EMSA was performed using a gel-shift assay system (Promega) with the nuclear extract of the fat body of 1- to 2-d-old mosquitoes or one of *Drosophila* Schneider S2 cells (Active motif). The DNA–protein complex was separated on 5% TBE Criterion Precast Gel (Bio-Rad) and visualized by means of autoradiography. For competition assay, 50-fold unlabeled E-box–like motif or unlabeled AP-2 motif was incubated with the nuclear extract for 10 min and then further incubated with labeled motif for 20 min. Identity of the complex was verified by directly adding polyclonal antibodies against *A. aegypti* MET (a gift from Jinsong Zhu, Virginia Tech, Blacksburg, VA), *Drosophila* CYC (Abcam), or *A. aegypti* FISC to the binding reactions.

RESULTS:

Expression of *Kr-h1 and Hairy* genes under light–dark cycles in newly enclosed female mosquitoes:

The *Drosophila Krüppel homolog 1 (Kr-h1)* gene encodes a zinc-finger motif transcription factor, which has been implicated in larval–pupal metamorphosis (11). It is now used as a representative marker gene under the regulation of JH and MET (9, 12-14). The *Hairy* gene encodes a bHLH protein with an orange domain and C-terminal Groucho interacting motifs (15). Expression of *Kr-h1* (AAEL002390) and *Hairy* (AAEL005480) genes is JH III-dependent during the PE development of *A. aegypti* female mosquitoes (12). When female mosquitoes were maintained under a 12-h dark/12-h light (12D:12L)

cycles until 4 d (4D) PE, *Kr-h1* and *Hairy* gene transcript abundance exhibited periodic fluctuations, increasing from circadian time 0 (CT0) to CT8 in each light–dark cycle (Fig. 2 *A* and *B*). The overall transcript levels of both genes reached a maximum at 4D PE, which is in an accord with our previous results. However, when PE female mosquitoes were maintained under constant dark (24D:0L) conditions, there were no significant differences in either *Kr-h1* or *Hairy* gene expression at CT0 and CT8, and unlike those under the 12D:12L cycle (Fig. 2 *A* and *B*) the overall transcript levels of both *Kr-h1* and *Hairy* genes did not increase by 4D PE.

We investigated whether the light/dark-dependent expression of *Kr-h1* and *Hairy* genes was JH III-dependent. We analyzed the expression of these JH-dependent genes in newly emerged female mosquitoes (1-d PE) because their endogenous JH levels were reportedly low during this period (16). Female mosquitoes 1-d PE were treated with a 0.2-μL topical application of either 1 μg/mL JH III in acetone solution or a solvent (acetone) in the early morning (CT0), the time of the transition from dark to light, and then maintained on sugar solution until next morning (CT24). Total RNA was collected at 4-h intervals, and transcript abundance of *Kr-h1* and *Hairy* was determined using quantitative real-time PCR (qPCR). Expression profiles of these genes in solvent-treated mosquitoes were similar, peaking at CT12, declining to the basal level by CT16, and rising again by CT24 (Fig. 2 *C* and *D*). Topical treatment with exogenous JH III resulted in an enhanced abundance of *Kr-h1* and *Hairy* transcripts within several hours; the levels of both transcripts were considerably higher in JH III-treated female mosquitoes than in those treated with a solvent (Fig. 2 *C* and *D*). The *Kr-h1* transcript level exhibited a bell-

shaped curve, peaking at CT12 and returning to a background level by CT24 (Fig. 2*C*). In the same JH III-treated mosquitoes, *Hairy* transcript level peaked at CT8, maintaining high level of expression until CT20 (Fig. 2*D*).

Northern blot analysis confirmed our qPCR data that Kr-h1 expression depends on the light/dark cycle and is strongly enhanced by the application of exogenous JH III (Fig. 3). At the same time, CYC and MET transcripts exhibited no enhancement from application of exogenous JH III, but their transcript abundance gradually increased by CT24 (Fig. 3).

Fig. 2. legend:

Circadian- and JH-dependent expression of *Kr-h1* and *Hairy* genes:

Kr-h1 (*A*) and *Hairy* (*B*) genes showed the circadian rhythmic pattern of gene expression when mosquitoes were kept under a 12 h dark–12 h light (12D:12L) cycle, with a higher expression level at circadian time 8 (CT8) than CT0 on each day. Overall, expression levels of both genes reached maximum at 4D PE. This circadian expression was not observed in the mosquitoes kept in constant darkness (24D:0L) and transcripts remains low at 4D PE. (*C* and *D*) Female mosquitoes 1-d PE were collected at CT0, the transition time from dark to light in a 12D:12L cycle, and each mosquito was treated with 0.2 μL of either 1 μg/mL JH III in acetone solution or acetone only. RNA samples were isolated at 4-h intervals until CT24 (same time point as CT0 2D PE) and applied to qPCR for quantifying the gene expression of *Kr-h1* (*C*) and *Hairy* (*D*). Statistical significance between samples was evaluated using the Student *t* test (GraphPad 5.0).

Fig. 2.

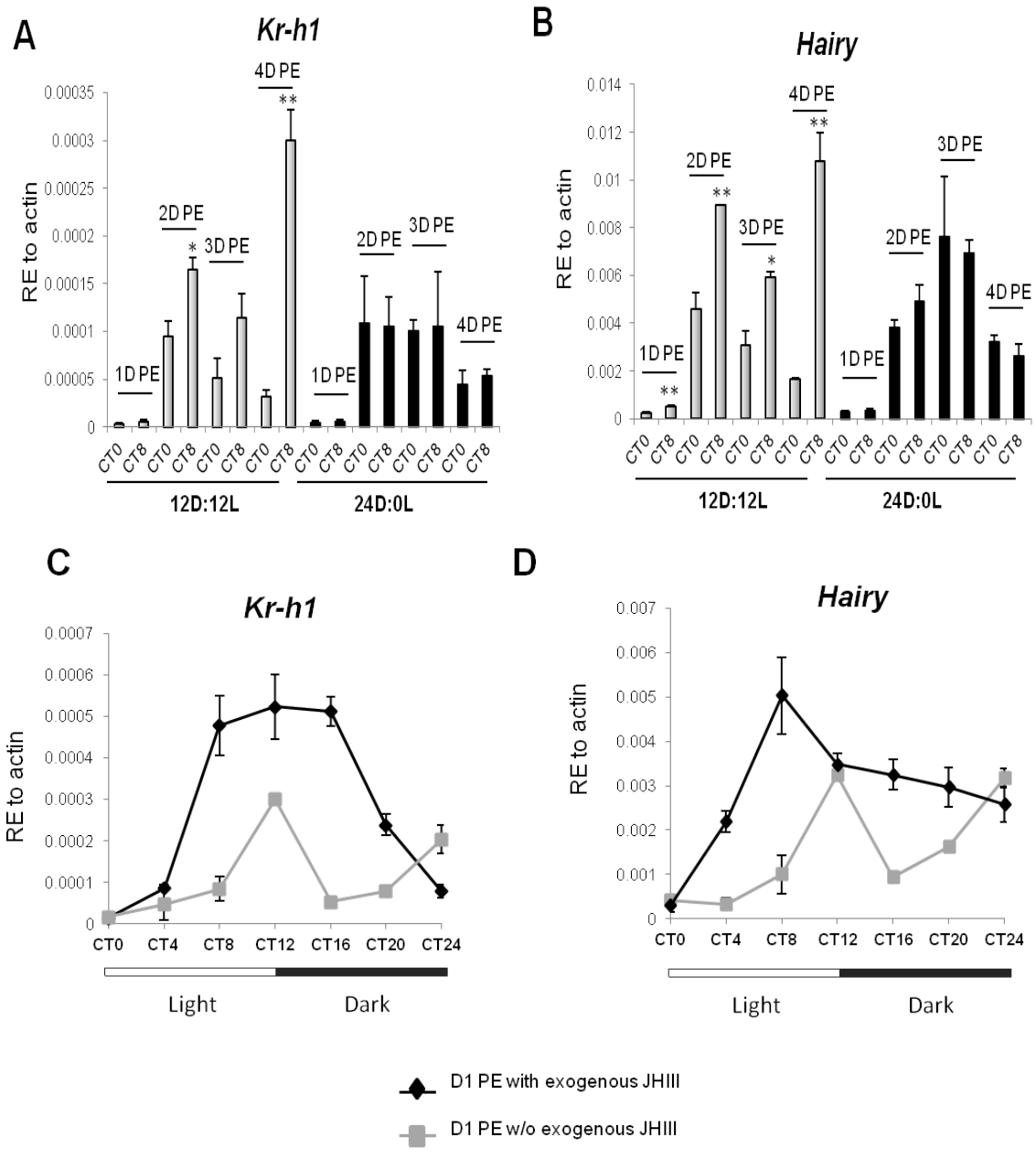
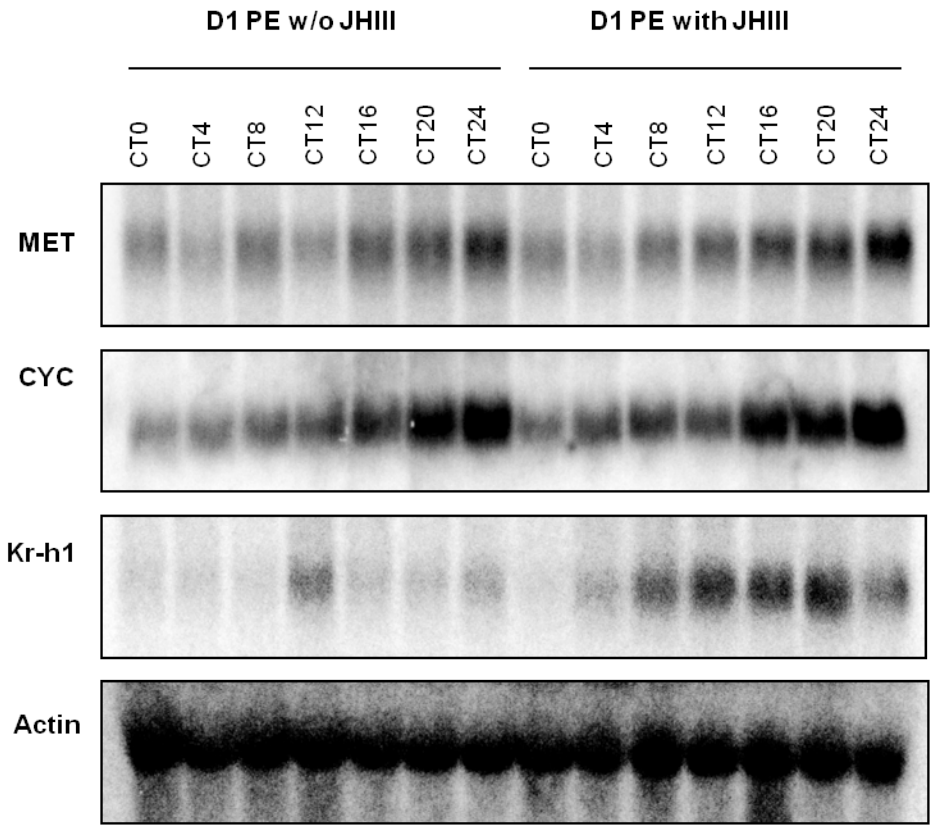

A
Kr-h1
RE to actin
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
1D PE
2D PE
3D PE
4D PE
**
*
CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8
12D:12L
24D:0L
B
Hairy
RE to actin
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
1D PE
2D PE
3D PE
4D PE
**
*
CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8 CT0 CT8
12D:12L
24D:0L
C
Kr-h1
RE to actin
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
CT0 CT4 CT8 CT12 CT16 CT20 CT24
Light
Dark
D
Hairy
RE to actin
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
CT0 CT4 CT8 CT12 CT16 CT20 CT24
Light
Dark
D1 PE with exogenous JHIII
D1 PE w/o exogenous JHIII

Fig. 3. legend:

Transcript profiling of *MET, CYC, Kr-h1* in mosquitoes 1-d PE by means of Northern Blotting:

Mosquitoes 1-d PE were collected at CT0 and each mosquito was treated with 0.2 μL of either 1 μg/mL JHIII in acetone solution or acetone only. RNA was isolated in 4-h intervals until CT24 and used in Northern analysis with respective probes. Actin transcript was used as a loading control.

Fig. 3.



Characterizing the role of MET, CYC and FISC in JH mediated circadian expression of *Kr-h1* and *Hairy* genes:

We then tested effects of RNAi depletions of CYC, MET, and FISC on the abundance of *Kr-h1* and *Hairy* transcripts. Efficiency of RNAi for each factor is presented in Fig. 4*A*. In the control iLuc mosquitoes, the level of *Kr-h1* and *Hairy* transcripts at CT12 increased compared with CT0, which is in an agreement with the circadian PE expression of these genes. In contrast, depletion of CYC resulted in a significant decrease of the abundance of *Kr-h1* and *Hairy* transcripts at CT12, although it did not markedly reduce the expression of these genes at CT0 (Fig. 5 *A* and *B*). Depletions of MET and FISC lowered transcript levels of both genes at CT0 and CT12. These results indicate that CYC, MET, and FISC are required for circadian regulation of JH-dependent *Kr-h1* and *Hairy* genes. However, MET and FISC are also needed for expression of these genes irrespective of light/dark cycles (Fig. 5 *A* and *B*). RNAi depletion of CLK did affect expression of either *Kr-h1* or *Hairy*, suggesting that the CLK/CYC/PER circuit was not directly involved in the JH III-dependent regulation of *Kr-h1* and *Hairy* (Fig. 4*B*).

An *in vivo* RNAi depletion is systemic and cannot completely rule out a possibility of an indirect effect on circadian regulation of JH-dependent *Kr-h1* and *Hairy* genes. Therefore, we conducted *in vitro* experiments to determine the effect of RNAi depletions of MET, CYC, and FISC on JH III-dependent level of *Kr-h1* and *Hairy* transcripts. Fat bodies from female mosquitoes 4D after double-stranded RNA (dsRNA) treatments were dissected and incubated in the complete culture medium supplemented

with either JH III or solvent (acetone). The level of *Kr-h1* and *Hairy* transcripts was equally low in fat bodies from all dsRNA treatments after incubation in the culture medium with acetone. In fat bodies from iLuc control mosquitoes, the levels of *Kr-h1* and *Hairy* transcripts highly increased in the presence of JH III (Fig. 5 *C* and *D*). In contrast, there was no such JH III mediated elevation of the level of *Kr-h1* and *Hairy* transcripts in fat bodies from females mosquitoes with RNAi depletions of MET, CYC, and FISC (Fig. 5 *C* and *D*), clearly showing that these factors are directly involved in JH-dependent regulation of circadian expression of *Kr-h1* and *Hairy* genes

Fig. 4. legend:

Efficiency of RNAi for MET, CYC and FISC:

(*A*) RNAi-mediated depletions of MET, CYC, or FISC. Transcript abundance of MET, CYC, or FISC in female mosquitoes after treatments with their respective dsRNAs (iMET, iCYC, and iFISC) was measured by quantitative RT-PCR. iLuc was used as a control. (*B*) RNAi-mediated depletions of CLK. Transcript abundance of *Kr-h1*, *Hairy*, and *Actin* in female mosquitoes after treatments with CLK dsRNAs was measured by semi quantitative RT-PCR. dsRNA injection of Luc (iLuc) was used as a control.

Fig. 4.

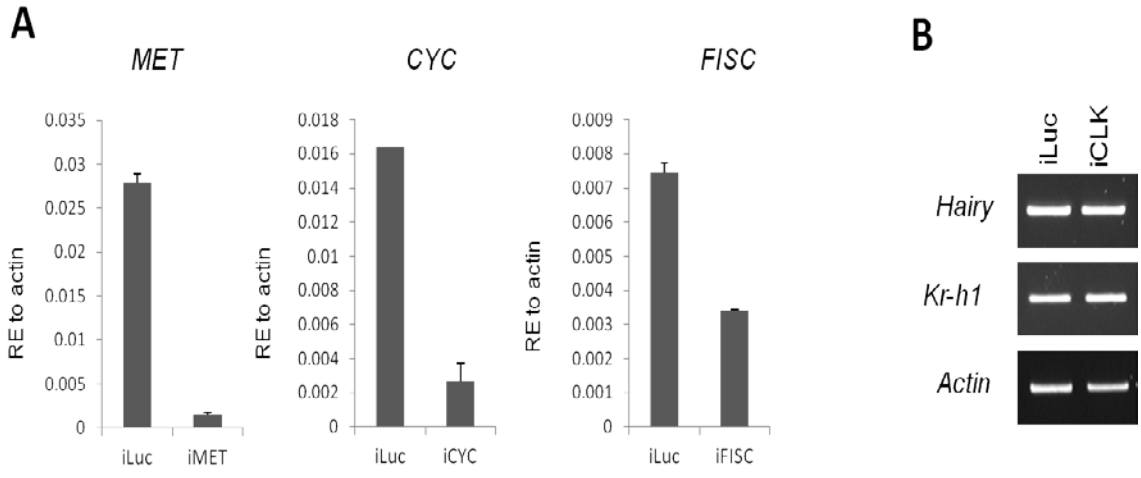
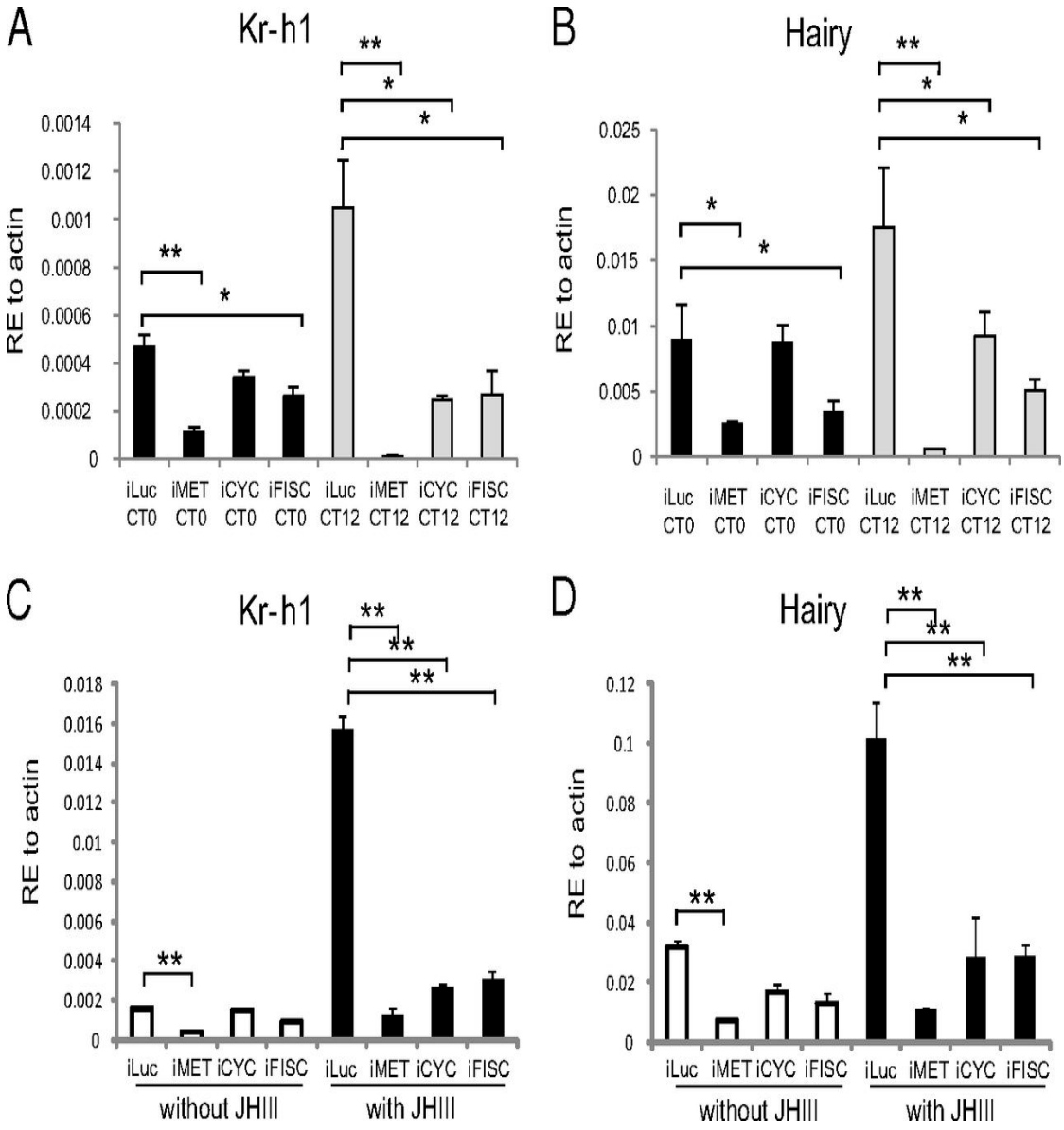


Fig. 5 legend:

MET, CYC, and FISC mediates the circadian activation of *Kr-h1* and *Hairy* genes: RNAi depletion of CYC (iCYC), MET (iMET), or FISC (iFISC) compromised the circadian activation of *Kr-h1* and *Hairy* genes in fat bodies of *Aedes* female mosquitoes. After injection of iMET, iCYC, iFISC, and control dsRNA (iLuc) into the newly emerged female mosquitoes (1-d PE), they were subjected to a 12D:12L cycle for 4 d. Total RNA from these 5D PE mosquitoes was extracted at CT0 and CT12 and subjected to qPCR analysis using either *Kr-h1* or *Hairy* gene-specific primers. (*C* and *D*) Four days after dsRNA injection of iMET, iCYC, iFISC, or control iLuc into 1 PE-old *Aedes* female mosquitoes, fat bodies were dissected and incubated in culture medium either in the presence of 1 μg/mL JH III or solvent (acetone). RNA samples were isolated 6 h after incubation and the transcript levels were quantified by means of qPCR for *Kr-h1* (*C*) and *Hairy* (*D*). Results were normalized against the β-*actin* transcript. All experiments were performed in triplicates. Statistical significance between samples was evaluated using the Student *t* test (GraphPad 5.0).

Fig. 5.


A
Kr-h1
RE to actin
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
iLuc CT0
iMET CT0
iCYC CT0
iFISC CT0
iLuc CT12
iMET CT12
iCYC CT12
iFISC CT12
B
Hairy
RE to actin
0.025
0.02
0.015
0.01
0.005
0
C
Kr-h1
RE to actin
0.018
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0.004
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iLuc iMET iCYC iFISC iLuc iMET iCYC iFISC
without JHIII
with JHIII
D
Hairy
RE to actin
0.12
0.1
0.08
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0.04
0.02
0
iLuc iMET iCYC iFISC iLuc iMET iCYC iFISC
without JHIII
with JHIII

Investigating the binding properties of MET and CYC to a sequence containing an E-box–like motif from the *Kr-h1* gene promoter:

The gene encoding *Kr-h1* factor (AAEL002390) has been annotated in the *A. aegypti* genome. In this study, we identified two additional exons encoding 5′-UTR of *Kr-h1* by means of 5′-RACE PCR (Fig. 7*A* and Fig. 6). As a result, we predicted a putative promoter located about 85-kb upstream from the annotated *Kr-h1* gene. It harbored four tandem motifs, designated as K1, K2, K3, and K4, with core sequences resembling E-box sites (Fig. 7*A* and Fig. 6). The motif K3 had "T" in a position 5, whereas other sequences had a "C." This motif K3 was similar to a recently reported MET-interacting sequence from *B. mori* (9), whereas K1, K2, and K4 "CACGCG" motifs were similar to a MET-binding sequence (ETv) from the JH III-regulated *Aedes* gut-specific *early trypsin (ET)* gene (Fig. 7*A*) (6).

We used the electrophoretic mobility shift assay (EMSA) to evaluate binding properties of these four *Kr-h1* promoter sequences. When we tested binding between each of these sequences and the nuclear extract collected from fat bodies of female mosquitoes 2D PE, only the K1 sequence formed a dense band (Fig. 7*B, left*). There was interference in formation of this complex when we added the antibodies against either *Aedes* MET or *Drosophila* CYC, indicating that both MET and CYC were components of this DNA–protein complex (Fig. 7*B, Center*). However, the addition of *Aedes* FISC antibody did not affect the formation of the complex, suggesting that FISC was not in the complex under these conditions (Fig. 7*B, Center*). Addition of a nonspecific serum had a weak effect on the formation of the complex. Binding specificity was confirmed by

competition with the 50-fold excess of unlabeled K1 probe, which eliminated the K1 complex. In contrast, addition of the 50-fold excess of unlabeled nonspecific probe (AP2) had little effect (Fig. 7*B*, *Right*).

The EMSA between the nuclear extract from *Drosophila* Schneider L2 cells and the *Kr-h1* promoter sequences K1–K4 confirmed the results. Only the K1 sequence formed a specific binding complex (Fig. 7*C*, *Left*). Formation of the complex was affected by addition of *Drosophila* anti-CYC antibodies, suggesting that CYC is one of the components of the binding complex with the *Aedes Kr-h1* K1 sequence (Fig. 7*D*, *Left Upper*). The specificity of the K1 complex was confirmed by competition with the 50-fold excess of unlabeled K1 probe, which eliminated the K1 complex. In contrast, addition of 50-fold excess of unlabeled AP2 nonspecific probe had no effect (Fig. 7*D*, *Right Upper*).

The mobility of the ETv complex in the EMSA assay with the nuclear extract from *Drosophila* Schneider L2 cells was dissimilar from that of the K1 complex, suggesting that the compositions of these complexes are different (Fig. 7*C*, *Right*). The mobility of the ETv complex was not affected by the addition of the anti- CYC antibodies, indicating that CYC was not a part of the ETv complex (Fig. 7*D*, *Left Lower*). Moreover, addition of the 50-fold excess of either unlabeled K1 or ETv probes displaced only their respective complexes (Fig. 7*D*, *Left*).

Fig. 6 legend:

Molecular cloning of full length *Kr-h1* cDNA:

Nucleotide sequences of 5′-end of *Aedes Kr-h1* cDNA and predicted 2-kb promoter region. RACE primers were designed based on the VectorBase genome annotation (AAEL002390). Our RACE analysis supplemented the annotated sequence of AAEL002390 with two more exons, indicated as blue-colored (exon 1) and green-colored (exon 2) highlights. A 502 nucleotide sequence in 5′ end encoding additional 67 N-terminal amino acids with 5′-UTR and a 78 3′-UTR in 3′ end. Four highlighted and underlined sequences indicate three sequences (K1, K2, K3, and K4) containing imperfect Ebox– like motifs. The predicted translation start site (ATG) is indicated as an underlined bold.

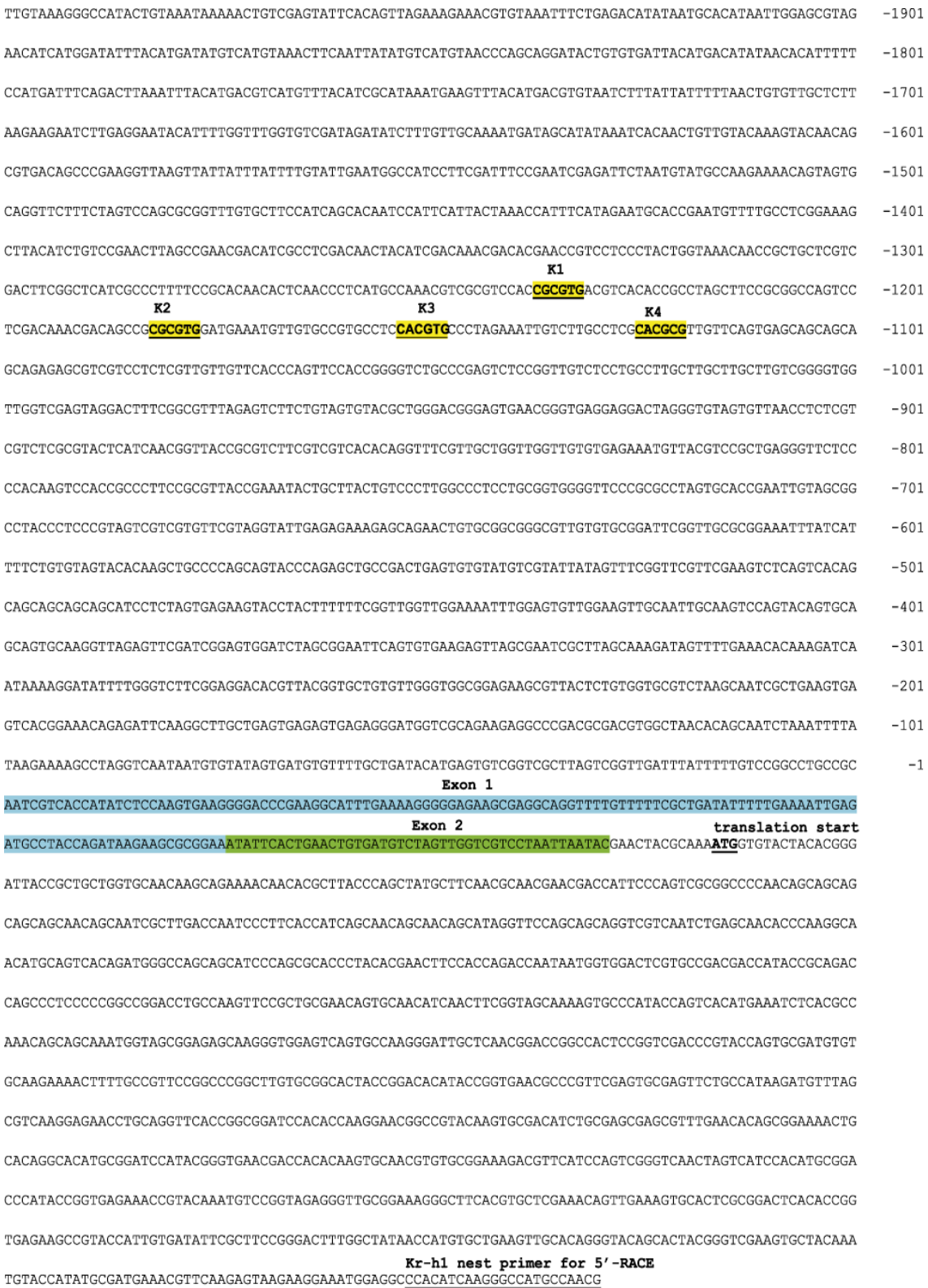

-1901
-1801
-1701
-1601
-1501
-1401
-1301
K1
-1201
K2
K3
K4
-1101
-1001
-901
-801
-701
-601
-501
-401
-301
-201
-101
-1
Exon 1
Exon 2
translation start
Kr-h1 nest primer for 5'-RACE

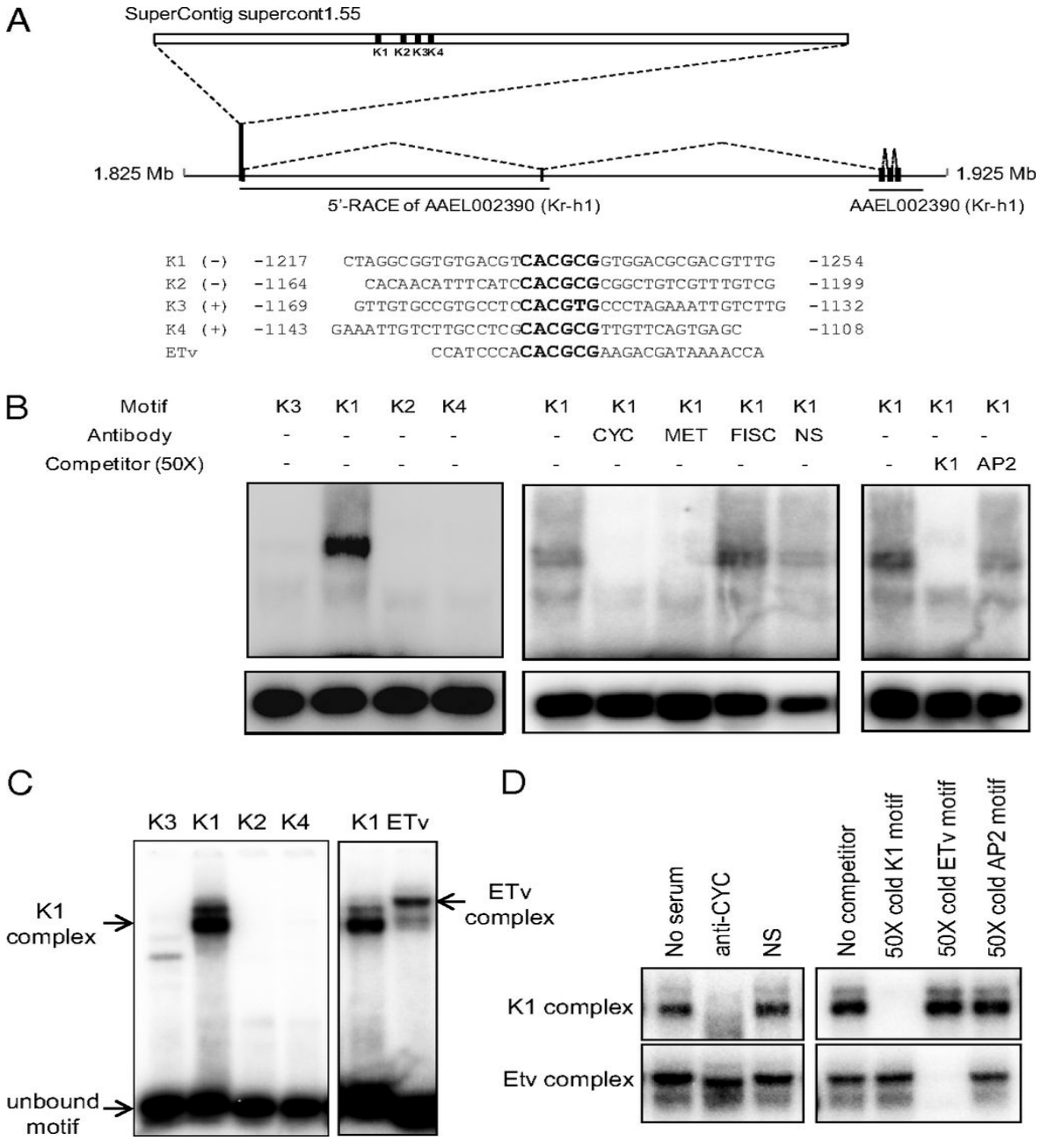
Fig. 7 legend:

Binding of CYC and MET to the E-box-like motif from the *Kr-h1* gene promoter:

(*A*) A map of the 2-kb of 5′ regulatory region of the *Kr-h1* gene. It was cloned by means of 5′-RACE PCR based on the nucleotide sequence of AAEL002390 (*Kr-h1*). It harbored three imperfect E-box-like (CACGCG) motifs—K1, K2, and K4—and a single K3 CACGTG motif. E-box–like motifs (indicated in bold) and their flanking sequences, used for gel mobility shift assay (EMSA) experiments (*B*), are indicated. (*B*) The EMSA revealed the presence of a DNA–protein complex between the *Kr-h1* K1 sequence and the nuclear extract from fat bodies of female mosquitoes (*left panel*). The presence of MET and CYC, but not FISC, in the DNA–protein complex was confirmed by adding polyclonal antibodies against each of these proteins to the binding reactions (*central panel*). NS – nonspecific serum control. For competition tests (*Right*), a 50-fold molar excess of unlabeled probe or a nonspecific double-stranded oligonucleotide (AP2) was added to the binding mixtures. (*C* and *D*) Gel mobility shift assay using nuclear extracts from Schneider *Drosophila* S2 cells. (*C*) The assay revealed the presence of a DNA–protein complex between the *Kr-h1* K1 sequence and the nuclear extract from Schneider *Drosophila* S2 cells (*Left*). This K1 complex shows a different mobility from that of the complex, formed by binding between the ETv motif and the nuclear extract (*Right*). (*D*) The presence of CYC in the *Kr-h1* K1 complex, but not in the ETv complex, was confirmed by adding polyclonal antibody against *Drosophila* CYC to the binding reactions (*Left*). For competition tests (*Right*), an ∼50-fold molar excess of unlabeled

probe or a nonspecific double-stranded oligonucleotide (AP2) was added to the binding mixtures.

Fig. 7.



DISCUSSION:

The bHLH-PAS transcription factors function as obligatory dimers, mainly as heterodimers, binding DNA at specific promoter elements and recruiting cofactors (4). DNA-binding properties of bHLH-PAS transcription factors are determined by the bHLH domains located in their N-termini, whereas their PAS domains are essential for dimerization (4, 17). The PAS domains of bHLH-PAS transcription factors are also essential for recruitment of cofactors, including bHLH-PAS NCoA/SRC coactivators (17). The JH receptor MET recruits a bHLH-PAS nuclear transcriptional coactivator SRC/FISC for JH-activated gene expression (3, 6-9). Similar to other bHLH-PAS proteins, MET contains two PAS domains, PAS-A and PAS-B (3, 6). The PAS-B domain of MET is necessary and sufficient for JH III binding (3). It is also adequate for dimerization of *Tribolium* MET with SRC (3). However, both PAS-A and PAS-B domains are needed for MET interaction with SRC/FISC in *A. aegypti* (6). Thus, these studies have demonstrated that MET possesses functions characteristic of members of the bHLH-PAS transcription factor family by being capable of recruiting coactivators and additional transcription factors to initiate a ligand mediated gene expression. However, whether MET has additional bHLH-PAS binding partner has not been investigated previously.

Dr. Shin's research led to the identification of *Aedes* CYC as a JH III-dependent MET-interacting protein based on Y2H screening of the library prepared from female mosquitoes during JH-mediated PE. In direct Y2H tests, MET bound CYC only in the presence of JH III. This protein–protein interaction was additionally confirmed by means

of Co-IP of these proteins expressed with specific tags. No such interaction was detected for MET/MET in Y2H tests, irrespective of the presence or absence of JH III. Similar to the vertebrate bHLH-PAS transcription factor ARNT (HIF-beta), its *Drosophila* ortholog, TGO, serves as a partner for several bHLH-PAS transcription factors (4, 17, 18). No interaction between *Aedes* TGO and MET was found under the screening and assaying conditions used in our lab.

Using Y2H β-galactosidase reporter experiments, the dose dependent JH III–induced binding between MET and CYC was shown. Interestingly, methoprene did not enhance MET/CYC interaction, even at the high concentration. Based on our current understanding, it is hard to explain this contradictory result. One possible reason for this contradictory results may be the use of $MET^{122–977}$ rather than previously used $MET^{1–596}$ (6). The long C-terminal of MET used in our construct might contribute to the specificity of JH III binding to the heterodimers MET/CYC. Despite extensive studies of bHLH-PAS transcription factors, there is still much to learn about the functional significance of their long C-termini (17). The glutamine-rich C-terminal domain, C-TAD, of the aryl hydrocarbon (dioxin) recruits several coactivators, including CBP/p300 (17). Further studies are required to understand the functional role of the MET C-terminal region.

We have shown here that, in *A. aegypti* female mosquitoes, expression of JH-dependent *Kr-h1* and *Hairy* genes requires light/dark daily rhythms and oscillated, gradually rising to the maximal level by the fourth day of PE development. Genome-wide profiling of PE females of the mosquito *Anopheles gambiae* has demonstrated that at least 16% of genes with various biological functions are under light/dark rhythm control

(19). However, whether JH III is involved in mosquito circadian gene regulation has not been characterized. In this work, we showed that CYC, MET, and the steroid receptor coactivator FISC are required for the JH-mediated regulation of circadian expression in the female mosquito PE. Deciphering the precise cross talk between the JH-mediated regulation of circadian gene expression and the circadian clock molecular machinery represents an exciting goal for the future research.

The composition of the MET DNA-binding complex was further investigated using EMSA to analyze the nuclear extract collected from fat bodies of female mosquitoes PE. In this EMSA screening, a specific band was formed with the K1 sequence containing an E-box–like motif from the JH-regulated *Kr-h1* gene. Addition of antibodies against either *Aedes* MET or *Drosophila* CYC resulted in displacement of the band, indicating that both MET and CYC are components of this MET DNA-binding protein complex. When using EMSA to analyze *Drosophila* cell line nuclear extracts, both the K1 sequence from the JH-regulated *Kr-h1* gene and the Etv sequence from *Aedes ET* gene formed specific bands, although of different mobility. Significantly, *Drosophila* CYC antibody effectively displaced the K1 sequence-protein complex but had no effect on mobility of the Etv sequence-protein band. The latter experiments suggest that CYC is not a component of a DNA-binding complex forming with the Etv sequence from the *Aedes* gut-specific ET gene. The K1 sequence from *Aedes Kr-h1* gene that bound MET/CYC contained a core sequence resembling imperfect E-box site. The E-box motif with a consensus CANNTG is a characteristic signature for recognition binding sites in genes regulated by bHLH-PAS transcription factors (17, 18). However,

this K1 motif “CACGCG” had C in a position 5 that was similar to a MET-binding sequence ETv from the JH III-regulated *Aedes ET* gene (6). A recently reported MET-interacting site from *B. mori* had T in a position 5 (9). Further large-scale bioinformatics analyses are required to develop a unified consensus for MET binding sites in insect genes.

JH is an insect-specific hormone responsible for a plethora of functions and processes (20). JH receptor is a heterodimer of two DNA-binding bHLH-PAS transcription factors, with MET as its obligatory component. This suggests that MET is capable of recruiting different DNA-binding bHLH-PAS partners in a context of various sex-, stage-, tissue-, cell-, and gene-specific conditions. Our study provides an important insight into one specific JH function - circadian control of gene expression mediated by the formation of MET-CYC complex. The ability of the JH receptor MET to heterodimerize with different partners is likely a key to the pleiotropic action of its ligand. Future research should explore this important hypothesis.

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CONCLUSIONS

Vertebrate blood acts as the primary source of amino acids for the process of Vitellogenesis (synthesis of yolk proteins for egg maturation) in mosquitoes like *Aedes aegypti*. For each batch of eggs an adult female produces, she requires a prior blood meal (1). This repeated blood feeding makes her an exceptional disease vector transmitting pathogens for devastating human diseases like Dengue and Yellow fever. A newly enclosed adult mosquito goes through a developmental process which prepares it for egg production in response to blood feeding. This period of mosquito reproductive cycle is called post eclosion (PE) development and is controlled by an insect specific juvenile hormone (JH) (1). JH, a sesquiterpenoid by structure, acts on the mosquito fat body (the tissue analogous to vertebrate lever) enhancing its metabolic machinery and protein synthesizing capacity thus priming the fat body for vitellogenesis. JH governed PE development is an absolute necessity for reproductive cycles of mosquito and consequently the spread of vector population.

Though there are some studies focusing on individual gene expressions at PE (2), global transcriptomic analysis on a genome wide scale were completely missing for this vitally important developmental phase in adult female *A. aegypti*. Using Microarray technique, we have demonstrated, for the first time that PE is hyperactive transcriptionally resulting in altered expression of nearly 40% (6146 genes of total 15321 tested) of all mosquito genes currently annotated in *A. aegypti* genome. This high transcriptional activity is a testimony to the dramatic physiological changes a newly

eclosed mosquito goes through in preparation for the intense process of blood feeding and vitellogenesis. Through hierarchical clustering, these differentially expressed genes (DEGs) identified from time course microarray, were grouped together based on their expression profiles. Three major gene clusters with distinct expression peaks at early (6h PE), mid (24h PE) and late (66h PE) PE (named EPE, MPE and LPE respectively) were identified. The EPE and MPE genes are primarily involved in carbohydrate and lipid metabolism whereas the LPE are primarily protein synthesis machinery genes. The JH haemolymph titer increases after adult eclosion reaching a peak at 48hr PE and then declining slowly over the next 5 days in females not fed a blood meal (3). Thus, the gene expression peaks EPE, MPE and LPE cluster correspond to low, intermediate and high JH titers.

To determine how JH might be involved in regulating these gene groups, we took advantage of newly identified JH receptor Methoprene tolerant (MET) (4, 5). MET is a member of basic helix–loop–helix (bHLH) - Period (Per)-Aryl hydrocarbon nuclear translocator (Arnt)-Singleminded (Sim) (PAS) domain family of transcription factors (6). Supression of MET expression via injection of double stranded RNA (dsRNA) against MET transcripts resulted in ovarian developmental arrest. This lack of development of primary follicles is a characteristic phenotype of JH deprivation (7, 8). When the transcriptome of the MET RNAi-treated fat bodies was analyzed, 1,385 genes were up-regulated and 1,169 were down-regulated suggesting involvement of MET in PE gene regulation. Interestingly, iMET up-regulated genes had a 27% and 40% overlap with EPE and MPE genes respectively while 36% of LPE genes were included in iMET down-

regulated gene group. This results showed that a significant portion of the EPE, MPE and LPE genes are regulated by MET and possibly, by JH.

Insects have an open circulatory system and breathe through a series of tracheal tubes at the side of their thorax and thus can survive quite well in a headless condition for at least 24hr. This works to the benefit of JH researchers who can surgically remove the head along with the associated JH producing gland Corpora allata (CA), to produce JH-less 'Isolated abdomens'. This system could be utilized to probe for JH responsive molecular targets *in vivo*. JH acted as inhibitors of tested EPE genes, and as an activator for LPE genes thus supporting our hypothesis. Using tandem experiments of RNAi knockdowns followed by in vitro tissue culture of knockdown fat bodies, MET's role in mediating expression of these JH responsive genes were accessed. The characteristic JH response (inhibitory for EPE and activatory for LPE) was severely compromised with MET depletion, thus establishing the JH/MET signaling pathway in regulation of EPE and LPE genes. MET suppressed and activated transcriptomes had a similar composition of functional groups with that of EPE and LPE gene sets, as revealed by gene ontology analysis. These studies established the central role of JH/MET signaling pathway in controlling the PE gene expression of adult female mosquito.

Previous studies in *Aedes* and *Bombyx* had identified similar MET binding motifs from the promoter regions of *Early Trypsin* and *Krüppel homolog-1* genes respectively. We were able to develop a consensus for MET binding motif as a 9-mer ‘CACG$^{C}/_{T}$G$^{A}/_{G}$$^{T}/_{A}$G’ and verify that with elctrophoretic mobility shift assays. This 9-mer

consensus showed an overrepresentation in the first 300 bases upstream region of 68 MET activated genes indicating a direct gene regulation model. However, no statistically significant overrepresentation was observed for MET repressed gene sets suggesting an indirect mode of gene repression involving intermediate transcription factors.

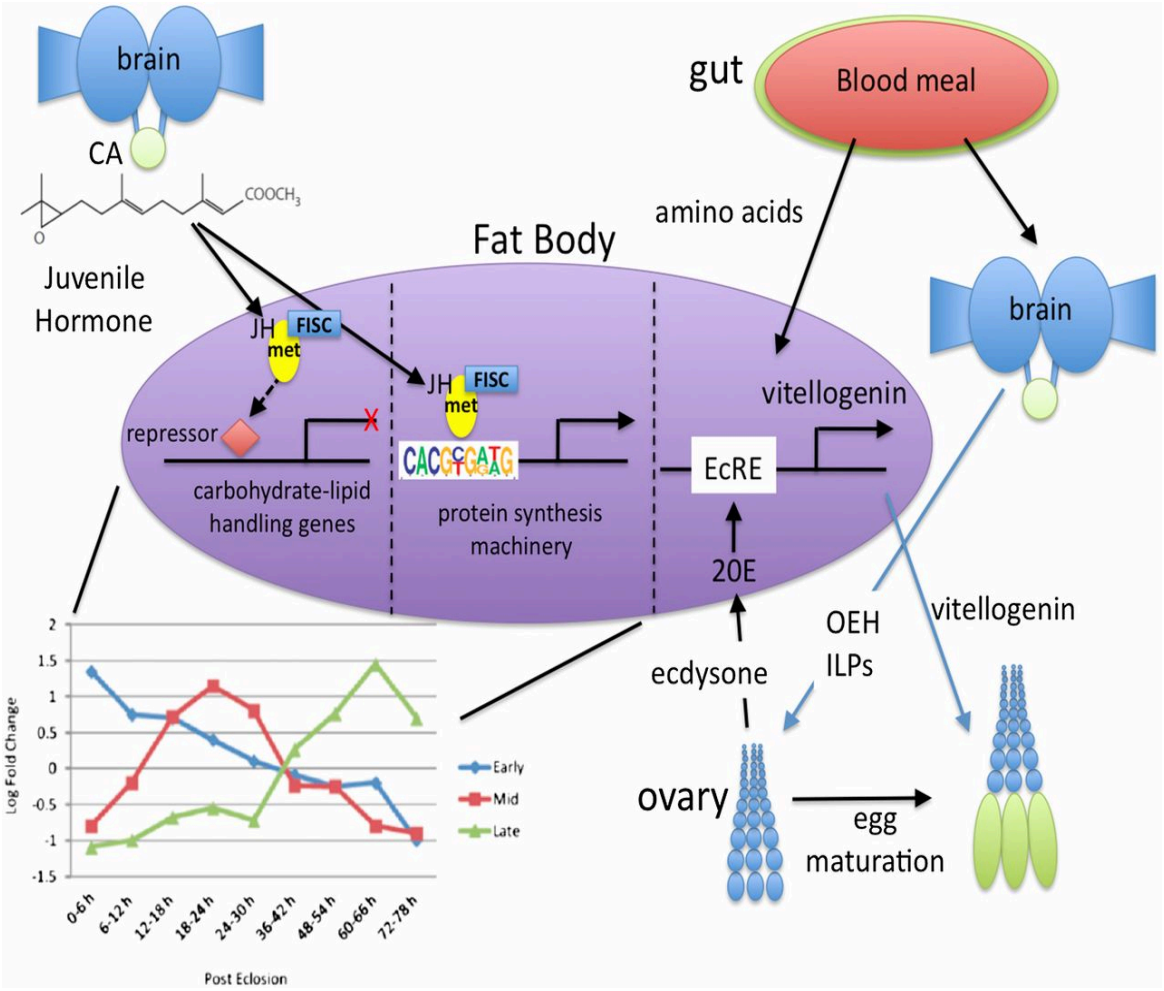
The findings of this study have recently been published. The paper can be accessed here- http://www.ncbi.nlm.nih.gov/pubmed/23633570. Following the publication an excellent commentary on this paper was published, summarizing the results and its implication in this field of research. This commentary can be accessed here- http://www.ncbi.nlm.nih.gov/pubmed/23720307. The following figure and figure legend summarizing chapter I of my thesis are directly from this commentary.

Fig. 1. legend:

Diagram of the hormonal control of reproductive maturation in the female mosquito:

The corpora allata (CA) secrete JH III that causes posteclosion development of the fat body by regulating the expression of three gene clusters, as portrayed in figure 1*C* from Zou et al. (2). The early and middle gene clusters regulate carbohydrate and lipid metabolism and are suppressed by JH, whereas the late genes are activated by JH and the JH receptor Met, binding directly to the genes via the consensus sequence shown (from figure 4*A* of ref. 2). These genes make the protein synthesizing machinery so that after the blood meal the fat body responds to the ecdysteroid 20-hydroxyecdysone (20E) binding with Ultraspiracle (USP) to the ecdysone response element (EcRE) and activating vitellogenin production for yolk in the eggs. The blood meal provides amino acids for this production and signals the brains to secrete the neuropeptides ovarian ecdysteroidogenic hormone (OEH) and several insulin-like peptides (ILPs), which cause the ovarian follicle cells to secrete ecdysone that is converted to the active 20E in the fat body and other peripheral tissues. [Figure prepared by James W. Truman (Howard Hughes Medical Institute, Ashburn, VA).]

Fig. 1.



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We found that about 15% of the genes repressed by JH/MET signaling pathway are controlled by an intermediate transcription factor, Hairy. Hairy is a transcription factor of bHLH Orange domain family which is strongly induced by JH and MET. Our RNA-seq analysis of MET and Hairy depleted fat body transcriptomes revealed that a remarkable 79% (247) of all Hairy repressed genes (311 total) overlap with that of MET repressed transcriptome. Using *in vitro* fat body tissue culture technique with addition of protein synthesis blocker cycloheximide (CHX) in the culture medium, it was shown that novel protein translation is an absolute requirement for JH/MET mediated gene repression. RNAi Hairy knockdowns rendered the JH ineffective in the suppression of marker genes, a phenotype similar to MET knockdowns, thus establishing the existence of JH/MET/Hairy signaling pathway. No systemic hierarchy for activation of genes was observed between MET and Hairy.

Functional genomic analysis of genes regulated by MET and Hairy provided further support to our hypothesis. MET up and down-regulated genes are functionally different, primarily controlling metabolism and protein synthesis machinery respectively. This observation is in accord with our previous results from microarray study. Hairy repressed genes, though much smaller in number than MET, has very similar composition of functional groups with MET repressed transcriptome. Amongst the 193 curated MET/Hairy co-repressed genes, statistically significant overlaps were observed in carbohydrate and secondary metabolite metabolism and in defense mechanism and extracellular structure orthologous groups (OGs). In contrast, none of the OG's showed an overlap between MET and Hairy activated gene sets with statistical significance.

Hairy is an established repressor, which functions by recruiting additional co-repressors. Hairy protein has a binding motif for co-repressor Groucho (WPRP tetrapeptide) and CtBp (9). Groucho depletion, but not CtBp, results in a molecular phenotype similar to Hairy knockdowns pointing to Groucho as a co-repressor of Hairy in JH/MET gene repression. However this hypothesis requires further confirmation.

Based on previously identified Hairy binding motif in *Drosophila*, we looked into the 5' upstream regions of 193 MET/Hairy repressed genes to identify possible hairy binding sites., We found that mosquito Hairy binds to E-box-like motifs- '$C^{A}/_{G}CATG$' and 'CGAATG'. Interestingly, in nearly 20% of MET/Hairy co-repressed genes, these Hairy binding motifs were embedded inside a 260-290bp long *cis*-regulatory module termed MET/Hairy-A (MH-A). Transcription factor binding sites have been known to clusters together (10) in the promoter regions of many developmental genes in *Drosophila* (11) and in other organisms. MEME analysis showed that this Hairy interacting MH-A module is composed of eleven motifs. Amongst these, motif 11 deserves a special mention because the Hairy binding E-box-like motif was always found to be a part of it. We believe that MH-A (present in 57of 193 MET/Hairy repressed genes) is a hairy interacting *cis*-regulatory module (hCRM) in mosquito *A aegypti*. The presence of such clustered transcription factor binding sites also suggests complexity in the mechanism of gene repression by Hairy, which needs to be explored.

Taken together, our studies in chapter I and II clearly establishes that JH/MET plays a dual role during PE adult development in mosquito *A. aegypti*. a) JH/MET has an

inhibitory effect on the EPE and MPE cluster genes. The mechanism of gene repression is indirect acting through intermediate transcription factors. One such factor contributing to JH/MET gene repression is Hairy, accounting for 15% of MET repressed genes. The mechanism of gene repression through Hairy appears to be complex, with the involvement of co-repressors, and additional transcription factors which function by binding to the identified *cis*-regulatory module from upstream region of a subset of MET/Hairy repressed genes. How MET represses the rest of the 85% of genes remains unclear. We believe that other transcription factors, like Hairy, might play a role. One good candidate to focus our studies in the future can be the zinc finger transcription factor *Krüppel homolog-1*(*Kr-h1*). b) JH/MET activates LPE genes, and for a subset of MET activated genes, the effect seems to be direct. Based on previous studies and our investigation a MET binding consensus motif could be defined and validated. 68 MET activated genes harbored this binding consensus with appositional bias for the proximal promoter region of the transcription start site. However, the mechanism of MET mediated activation for the rest of the genes remains a black box. There is some (47 genes) overlap between MET and Hairy activated gene, no hierarchy between MET and Hairy for gene activation could be established. Overall, MET mediated activation requires extensive research before any conclusion can be drawn.

In the 3rd chapter of my thesis, I probed an entirely different aspect of MET regulation in adult insects. MET, being a bHLH-PAS domain protein, functions by forming heterodimers with other bHLH-PAS factors. Studies in *Aedes* (12) and *Tribolium* (13) have identified Ftz-F1-interacting steroid receptor co-activator (FISC), a *Drosophila*

homolog of co-activator Taiman, as the bHLH-PAS partner of MET. In our lab, research led by Dr. Sang Woon Shin has identified another bHLH-PAS factor Cycle (CYC) an MET binder in addition to FISC. In *Drsosphila,* CYC plays a central role in regulating circadian rhythms genes via heterodimerization with other bHLH-PAS proteins (10).

In Chapter III, we investigated the role of JH in circadian gene expression of adult *Aedes* female mosquitoes. We found that known JH target *Krüppel homolog-1*(*Kr-h1*) and *Hairy* shows circadian rhythmic expression pattern when kept under 12h dark: 12h light (12D:12L) cycles. Overall transcript levels for these two targets reached a maximum at 4d PE, which is in accord with our previous results. Mosquito maintained under constant darkness (24D:0L) not only lost the rhythmic gene expression pattern but also failed to induce the transcripts at 4d PE establishing the role of dark:light cycles in the induction of these genes. JH was found to greatly enhance the expression of these genes without altering the diurnal periodic fluctuations in expression. Thus we found that both JH and adequate light exposure are necessary factors for expression *Kr-h1* and *Hairy in vivo.* MET, CYC and FISC directly mediates this JH dependant circadian expression of *Kr-h1* and *Hairy.* MET and CYC were shown to be a part of the DNA-protein complex that binds to E-box-like motifs identified at the promoter region of *Kr-h1* gene indicating how MET and CYC might act in regulating this gene. However, FISC which was found to be a mediator of JH dependant circadian expression of *Kr-h1,* was not detected in the protein complex that binds the *Kr-h1* promoter motif. With our current understanding we were unable to explain this contradiction.

This study provides an important insight into one specific JH function - circadian control of gene expression mediated by the formation of MET-CYC complex. Deciphering the precise cross talk between the JH-mediated regulation of circadian gene expression and the circadian clock molecular machinery represents an exciting goal for the future research.

Chapter III is part of the paper published in 2012. the link can be found here.
http://www.ncbi.nlm.nih.gov/pubmed/23012454

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