Molecular Mechanisms Underlying Functions of Juvenile Hormone Receptor

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biochemistry

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November 21, 2013
Blacksburg, Virginia

Keywords: juvenile hormone, receptor, gene regulation, DNA-binding, methoprene-tolerant, FISC

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Abstract

Juvenile hormone (JH) is one of the principal hormones that regulate insect development and reproduction. Accumulating evidence suggests that Methoprene-tolerant (Met) protein is a nuclear receptor of JH. Work by others has shown that Met is capable of binding JH at physiological concentration. An RNAi knockdown of Met causes down-regulated expression of JH-responsive genes and a phenotype similar to that observed in JH-deficient insects, suggesting that Met is required for mediating physiological and molecular responses to JH.

The work in this report aims to understand the mechanisms underlying gene regulation by JH via Met. Met is a bHLH-PAS (basic-helix-loop-helix Per-ARNT-Sim) family protein. Many proteins in this family function as heterodimers formed with other proteins of this family. In a yeast two-hybrid screening, we discovered that another bHLH-PAS family protein, FISC, interacts with Met in the presence of JH. FISC is also required for JH functions as an RNAi knockdown of FISC down-regulated JH-responsive genes. To elucidate how Met and FISC mediate JH functions in gene regulation, we employed molecular biology techniques and characterized the formation of a JH-Met-FISC complex and its actions in activating gene expression using mosquito *Aedes aegypti* as a model. My results demonstrated that Met and FISC forms a complex when JH is present via their conserved N-terminal domains. The complex then binds to E box-like sequences presented in the promoter of JH-responsive genes to activate gene expression. This mechanism also applies to the fruit fly *Drosophila melanogaster*, suggesting that it is a conserved action of JH in insects. Further studies showed that DNA-

binding by Met and FISC requires the basic regions of the bHLH domains of both proteins. Lastly we identified a consensus binding-site of Met and FISC.

Acknowledgement

I would like to give special thanks to my advisor, Dr. Jinsong Zhu, for giving me the opportunity to work in the laboratory. His kind support, patient guidance, and friendly assistance have contributed much to the success of this project. Besides research, I appreciate his advice on career development as well as his help with my adapting to the life here. I also would like to thank my committee members, Dr. Glenda Gillaspy, Dr. Tim Larson, and Dr. Jake Tu for their discussion, ideas, and support, which not only helped a lot with the project but also broadened my view in the biology field. I also thank people in the laboratory, Xing Zhang, Andy Mead, Pengcheng Liu, Reyhaneh Ojani, and Xiaonan Fu, for their help, interesting discussions, and endless humor.

Attribution

Chapter 2: Jinsong Zhu, Ph.D, principal investigator. Dr. Zhu performed the yeast two-hybrid screening, RNAi experiments, and gel-shift assays in the paper.

Chapter 2: Edward A. Mead, Ph.D, Dr. Jinsong Zhu laboratory, biochemistry department at Virginia Tech, is currently a postdoc at Rutgers University. Dr. Mead was a co-author on this paper and performed the JH-binding assays.

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Chapter 1

Introduction

1.1 Mosquito life cycle and mosquito-transmitted diseases

Mosquitoes are probably the best-known group of insects because they are vectors of some of the most devastating human diseases. Over 3500 species and subspecies of mosquitoes are classified in the family Culicidae in the order Diptera. They are found everywhere with standing water and on every continent except Antarctica. Mosquitoes are holometabolous insects undergoing complete metamorphosis. There are four stages in mosquito life cycle, namely egg, larva, pupa, and adult (Fig. 1.1). Mosquitoes are aquatic until adult eclosion. In some species, eggs are laid on water surface for quick hatch; in other species, eggs are laid on moist soil and will hatch when flooded. Mosquito larvae feed on small plant and animal particles by filtering water through their brush-like mouth. When a larva grows to a point that it is too large for the hard exoskeleton cuticle, it sheds the old exoskeleton and produces a new larger one. This process is called molting. The stage between two molting events is called instar. A mosquito larva goes through four instars before it becomes a pupa and then emerges as an adult at the surface of the water. Molting from last instar larva to pupa to adult is called metamorphosis.

Female adults of most mosquito species take blood from vertebrate animals including human beings, to obtain amino acids and other nutrition for egg production. Eggs are laid a few days later and the female mosquitoes are ready for taking a second blood meal to produce another batch of eggs. Female mosquitoes typically take multiple blood meals in the adult stage and produce several batches of eggs. During repeated biting, parasites can be taken up from one person and transmitted to the next person (Carpenter *et al.*, 1965; Eldridge, 2005).

Mosquito-borne diseases are among the most widely spread diseases in the world. It is estimated that approximately three billion people are at risk of malaria, which is caused by the parasite *Plasmodium* transmitted by *Anopheles* mosquitoes. Every year there are more than 300 million clinical cases and about one million deaths, most of whom are children under five years old in Africa (Enayati *et al.*, 2010; Jelinek, 2010). The vector-borne diseases, yellow fever and dengue fever, are transmitted primarily by mosquitoes of genus *Aedes*. Estimation by the World Health Organization shows that there are approximately 200 million clinical reports and 30,000 deaths worldwide caused by yellow fever every year (Reiter, 2010). More than 50 million dengue fever cases are reported every year in over 100 countries with 3.5 billion people at risk (Jelinek, 2010). Because of the essential role of mosquito vectors in disease transmission, vector control has drawn much attention as a strategy to prevent mosquito-borne diseases. With the critical roles in development and reproduction, juvenile hormone, a natural growth regulator found only in insects, and has been proposed as a target for vector control.

1.2 Juvenile hormone in insect development and reproduction

Development

Insect development and reproduction are orchestrated primarily by two hormones, juvenile hormone (JH) and ecdysone (Fig. 1.1). During development, molting is triggered by critical titers of ecdysone. Ecdysone is a steroid hormone released primarily by the prothoracic gland and is then converted to 20E, the principal molting hormone, in periphery tissues (Gilbert *et al.*, 2002; Litwack, 2005). While ecdysone is responsible for initiating molting, the nature of the molting is governed by JH. JH is secreted from corpora allata (CA), a pair of endocrine glands posterior to the brain and directly innervated by the brain (Tobe *et al.*, 1985). JH is

produced throughout the late embryo stage and most of the larval stages, with its titer fluctuates at different developmental stages (Bownes *et al.*, 1987; Sliter *et al.*, 1987).

When JH is present, it prevents the development of mature traits and limits ecdysteroid-induced molting to another larval stage. When the final instar nymphs (larvae of insects that do not have a pupa stage, i.e., hemimetabolous insects) of the kissing bug *Rhodnius prolixus* are implanted with active CA, JH released by the CA prevents metamorphosis and the nymphs produce another set of larval instead of adult traits (Wigglesworth, 1934). A similar result has been observed in the silkworm *Bombyx mori* (Bounhiol, 1938). On the other hand, when JH is removed from larvae, insects undergo precocious metamorphosis and usually die before adult eclosion. Removal of CA (allatectomy) from larvae of the cotton leaf-worm *Spodoptera littoralis*, leading to JH-deficiency, results in death of the insects as midget pupate (El-Ibrashy, 1971). Genetic ablation of CA cells in *Drosophila* also causes pupal lethality (Liu *et al.*, 2009). The evidence demonstrates that JH has a "status quo" effect in insect development.

JH titers drop to an undetectable level early in the last larval stage in holometabolous insects (Baker *et al.*, 1987; Grossniklaus-Bürgin *et al.*, 1990). A small peak of ecdysteroid in the absence of JH induces onset of metamorphosis and cell commitment to producing pupal tissues (Riddiford, 1976; Weeks *et al.*, 1990). An ensuing large peak of ecdysteroid elicits metamorphosis. JH is also released for a short period at the rise of the large peak of ecdysteroid to prevent development of adult characteristics. JH is then depleted prior to formation of a pupa. In the absence of JH, ecdysone triggers eclosion of an adult from a pupa (Williams, 1961).

Reproduction

Insects adopt various reproduction strategies and functions of JH in insect reproduction vary considerably across species. However, many essential reproduction events are common in insects and JH plays an important role in governing these events. In the anautogenous mosquito *Ae. aegypti*, the first gonotrophic cycle is divided into two phases separated by ingestion of blood meal, the previtellogenic phase of the first few days after adult emergence and the following vitellogenic phase. A cornerstone in insect reproduction is vitellogenesis, involving synthesis of large amounts of yolk precursor proteins. Vitellogenins (Vg) are a large group of high molecular weight glycolipoproteins. They are primarily synthesized and processed in insect fat body. Other important reproduction events include follicle development and egg maturation in the ovary (Raikhel, 1992).

During the previtellogenic phase, fat body trophocytes become competent for synthesis of Vg, which is initiated by blood ingestion. Primary follicles double their size in the first 2-3 days and reach the resting stage where a blood meal is required for further development of the follicles. In the vitellogenic phase, initiated by ingestion of blood, digestive enzymes are released into midgut for breaking down proteins in the blood meal. The amino acid pool generated is used to produce Vg, which is released into hemolymph and taken up by maturing follicles and oocytes (Klowden, 1997; Wyatt *et al.*, 1996).

Many aspects of the post-eclosion development in *Ae. aegypti* are regulated by JH. JH titer starts to rise after adult eclosion and remains high during the previtellogenic phase. JH titer quickly decreases after blood meal and stays at a low level until the end of the vitellogenic phase (Shapiro *et al.*, 1986). Therefore, the previtellogenic phase is the primary period in which JH exerts its functions in regulating mosquito reproduction. JH in the previtellogenic phase is

required for the fat body to become competent for Vg synthesis in response to blood meal. Competence is achieved after JH-stimulated fat body differentiation, which includes proliferation of ribosomes and increase in ploidy (Dittmann *et al.*, 1989; Raikhel *et al.*, 1990). A high concentration of JH is also essential for the differentiation and growth of follicles during the previtellogenic phase. The follicles in mosquitoes allatectomized at emergence could not grow to the resting stage and the arrested growth can be rescued by application of JH (Gwadz *et al.*, 1973). Although uptake of yolk proteins into oocytes occurs during the vitellogenic phase, the process depends on the endocytotic complex which appears at the oocyte membrane during the previtellogenic phase. Allatactomy at eclosion of female adults prevents production of the endocytotic complex and development of the complex is restored by JH application (Raikhel *et al.*, 1985). These results demonstrate that JH is required for development of follicles during the previtellogenic phase and for priming mosquito fat body to become competent for Vg synthesis, as well as for oogenesis in the vitellogenic phase.

1.3 Chemistry of juvenile hormones

Naturally occurring JHs

JHs are lipophilic sesquiterpenoids (Fig. 1.2) (Röller *et al.*, 1967). Several different forms of JHs are secreted by insects. Each of them bears a terminal epoxide group and methyl ester group. JH III has the simplest structure with three branches at C3, C7, and C11 of the carbon skeleton are methyl groups. Replacement of one, two, or three of the methyl groups with ethyl groups results in JH II, JH I, and JH 0 (Riddiford, 1994). JH III is the only JH form identified in the majority of insects, including mosquitoes (Cusson *et al.*, 2013). JH 0, JH I, JH II, as well as 4-methyl JH I are also found in Lepidoptera, although functions of JH 0 and 4-methyl JH I are

not known (Bergot *et al.*, 1980; Judy *et al.*, 1973; Meyer *et al.*, 1968; Schooley *et al.*, 1984). In addition to JH III, the *in vitro* cultured corpora allata cells of some species of flies, such as fruit fly and blowfly, also release a bisepoxide form of JH III, JHB_3, which is active as shown in *Drosophila* S2 cells (Richard *et al.*, 1989; Wang *et al.*, 2009). A JH precursor that lacks expoxide groups, methyl farnesoate (MF), has been isolated from cockroach embryos and larvae (Brüning *et al.*, 1985; Cusson *et al.*, 1991). Subsequent studies show that MF is also released by CA cells of some other insects, such as *D. melanogaster* and *Ae. aegypti* (Jones *et al.*, 2007; Jones *et al.*, 2010). Although lacking the characteristic expoxide groups of JH, bioassays in *Drosophila* indicate that MF has JH activity, as the application of MF affects metamorphosis. (Harshman *et al.*, 2010; Jones *et al.*, 2010).

JH agonists

As an important insect growth regulator, JH and its analogues are proposed as "third-generation pesticides" that would provide more specific alternatives to the previous generations of insecticides exemplified by arsenate of lead and DDT. It is predicted that resistance to these insecticides would not evolve as insects cannot become resistant to their own hormone (Williams, 1967). More than 4000 JH analogs have been synthesized and bioassayed. Active JH analogues encompass compounds of great structural diversity. Modeling of a set of active JH agonists shows that an electronegative atom, oxygen or nitrogen, at both ends of the molecule is critical and the distance between the electronegative atoms is important for their biological activity (Liszekova *et al.*, 2009). One of the most active JH analogs is methoprene (Fig. 1.2B), which is structurally similar to the natural JHs but lacks the unstable epoxide and methyl ester. High biological activity of methoprene is due to its binding to JH receptors, thus functioning as a JH

agonist (Henrick, 2007). Methoprene has been registered for commercial use because of its low mammalian toxicity, wide range of insect pest targets, and short environmental half-life (Cusson *et al.*, 2000; Henrick, 2007; Minakuchi *et al.*, 2006). However, the prediction that insecticides mimicking JH functions are proof against resistance by insects has proven wrong as resistance to methoprene has been observed both in laboratory and under field conditions (Crowder *et al.*, 2007; Wilson *et al.*, 2006).

1.4 Methoprene-tolerant is a JH receptor

Methoprene-tolerant

In an effort to search for genes important for JH functions, Wilson *et al.* performed an ethyl methane sulfonate mutagenesis screening in *Drosophila* and selected a mutant strain, which is resistant to the toxic and morphogenetic effects of JH and methoprene. The gene responsible for the resistance was mapped to the X chromosome and named *methoprene-tolerant* (*Met*) (Wilson *et al.*, 1986). Cloning of *Met* shows that it codes for a protein of the basic-helix-loop-helix Per-Arnt-Sim (bHLH-PAS) family (Fig. 1.3). With its homology to the dioxin receptor, Met is implied to be a JH receptor and a transcription factor (Ashok *et al.*, 1998). Immunostaining in fruit flies shows that Met is located in cell nuclei of known JH target tissues (Pursley *et al.*, 2000). Nuclear localization of Met is confirmed by fluorescence microscopy of cell lines expressing Met protein fused with GFP or YFP (Greb-Markiewicz *et al.*, 2011; Miura *et al.*, 2005). *In vitro* synthesized *Drosophila* Met binds JH at physiological concentration (Miura *et al.*, 2005). Binding assays and mutagenesis with *Tribolium* Met demonstrated that the JH binding pocket is located in PAS B domain (Charles *et al.*, 2011). The observations support the idea that Met is a nuclear receptor of JH. Moreover, RNAi knockdown of Met in *Tribolium*

larvae leads to precocious development of mature trait, which resembles the phenotype seen in JH-deficient insects, suggesting that Met plays a critical role in JH functions in regulating metamorphosis (Konopova *et al.*, 2007). Furthermore, Met is required for expression of JH-inducible genes, such as *kr-h1* and *AaET*. RNAi experiments in *Tribolium* show that knockdown of Met leads to down-regulation of *kr-h1* (Parthasarathy *et al.*, 2008). The result is confirmed in an independent study in *Tribolium* that induction of *kr-h1* by methoprene treatment in animals injected with *Met* dsRNA is weaker than that in control animals (Minakuchi *et al.*, 2009). Expression of a JH-inducible gene, *AaET*, is down-regulated in mosquitoes injected with *Met* dsRNA (Zhu *et al.*, 2010). Although mounting evidence demonstrates that Met actions are in line with JH functions, it has been documented that Met could counteract JH functions. JH inhibits programmed cell death (PCD) in the fat body during larval organ remodeling. However, Met is shown to induce PCD. Overexpression of Met leads to precocious and enhanced PCD, and the effect could be suppressed by topical application of methoprene (Liu *et al.*, 2009).

GCE

Since JH prevents metamorphosis and ablation of CA leads to precocious puparation and death before adult eclosion, it is expected that *Met* null mutation would be lethal. It was a surprise that fruit flies with *Met* null mutation show full viability and apparent normal development (Wilson *et al.*, 1986). The paradox was solved by the identification of a paralog gene of *Met* in *Drosophila*, *germ cell-expressed* (*GCE*) (Moore *et al.*, 2000). Only one *Met*-like gene presents in other insects of the Diptera order such as mosquitoes, suggesting that the paralog gene stems from gene duplication after divergence of *Drosophila* from other insects (Wang *et al.*, 2007). GCE is also a bHLH-PAS protein, sharing 68% to 86% identity with Met in

the conserved domains (Moore *et al.*, 2000). Like Met, GCE is capable of binding JH *in vitro*, supporting that these proteins function redundantly to mediate JH actions (Charles *et al.*, 2011). Fly mutants with *gce* null mutation are also insensitive to ectopic application of JH, just like *Met* null mutants. Although neither mutation is lethal, flies with *Met* and *gce* double mutation die as prepupae (Abdou *et al.*, 2011).

Conserved domains of Met and GCE

Met, as well as GCE, consists of three conserved domains, a bHLH domain and two PAS domains, PAS A and PAS B. The bHLH proteins are a large group of transcription regulators found in almost all eukaryotic organisms. They are involved in a diverse array of critical developmental processes, including sex determination, neurogenesis, and cellular differentiation (Massari *et al.*, 2000). The bHLH domain is comprised of ~60 amino acids and can be divided into two highly conserved and functionally distinct regions, the basic region and HLH region (Fig. 1.4B&C). The basic region at the N-terminal end is comprised of 13 amino acids, rich in arginine and lysine residues. The basic region binds to DNA with a hexanucleotide core called E-box (CANNTG) (Fig. 1.4D). The C-terminus HLH region contains two alpha-helices separated by a loop of variable length. The helices promote formation of homo- or heterodimers, which bring basic regions of two proteins together to bind DNA (Sailsbery *et al.*, 2012). The bHLH proteins are classified into six groups (A-F) according to their sequence conservation, DNA binding, and the presence of additional domains. Proteins in group C contain additional C-terminal domains such as PAS domain, represented by Clock, hypoxia-inducible factor (HIF), and aryl hydrocarbon receptor (AhR) (Jones, 2004).

The PAS domain is named by the first letter of the first three proteins identified in the family: Period, aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded (Sim). Hundreds of PAS domain-containing proteins have been identified in all kingdoms of life, with roles in various aspects of life such as development, environmental adaptation, and circadian rhythm (McIntosh *et al.*, 2010). A PAS domain usually encompasses approximately 100-120 amino acids. The average sequence identity is below 20%, however resolved structures of PAS domains from bacteria to vertebrates show that 3D conformation of these proteins is very similar (Hefti *et al.*, 2004).

A canonical PAS domain contains five beta strands and a few alpha helices. The beta strands form a central antiparallel beta sheet, which is highly conserved in 3D structure, while the alpha helices vary considerably in numbers of helices and their 3D structures. The beta sheet together with alpha helices and several loops form a globular PAS domain. Some PAS domains are capable of binding ligands, typically through a binding pocket formed between the beta sheet and a couple of helices. Structures of the helices involved in ligand binding vary considerably, allowing PAS domains to perceive various types of signals such as oxygen, chemicals, and light (Moglich *et al.*, 2009). Like bHLH domains, PAS domains also promote protein-protein interaction to form either homodimer or heterodimer with another PAS domain protein. The outer surface of the beta sheet usually forms hydrophobic interface for dimerization while sequences flanking the PAS core also contribute to stabilizing the quaternary structure (Moglich *et al.*, 2009; Taylor *et al.*, 1999).

Protein partner of Met

Since bHLH-PAS family proteins usually function as homodimer or heterodimer formed with another bHLH-PAS protein, it is of particular interest to test whether Met acts in a similar way. Pull-down assays show that Met is capable of forming homodimer as well as forming heterodimer with GCE. However, dimerization is dramatically reduced when JH or JH analogs are present, suggesting that Met-Met and Met-GCE dimers are not the active Met-containing complex mediating JH functions (Godlewski *et al.,* 2006). A survey of *Tribolium* genome identified 11 bHLH-PAS proteins. When knocked-down by RNAi in larvae, reduced expression in 6 of 11 proteins affects growth and development of the beetles, suggesting their roles in development and as potential partners of Met (Bitra *et al.*, 2009b).

1.5 JH-responsive genes

To identify genes regulated by JH, microarray assays have been carried out in cell lines and tissues of many insects, including fly, mosquito, and beetle (Li *et al.*, 2007; Minakuchi *et al.*, 2008; Zhu *et al.*, 2010; Zou *et al.*, 2013). Many genes are found to be regulated by JH in these studies; however, only a few genes are independently identified as JH-responsive genes. Two of the most studied JH-inducible genes are *Krüppel homolog 1* (*kr-h1*) in *Bombyx mori*, *Drosophila melanogaster* and *Tribolium castaneum*, and the *early trypsin* (*AaET*) gene in *Ae. aegypti*.

kr-h1

kr-h1 was originally identified as a homologous gene to *Krüppel (Kr),* a segmentation gene of *Drosophila* (Schuh *et al.*, 1986). *Drosophila kr-h1* produces three putative transcripts with distinct promoters and first exons. There are two main isoforms with the beta isoform being

expressed as the predominant isoform during embryonic development and the alpha isoform is the major isoform during post-embryo development. Both alpha and beta isoforms of Kr-h1 proteins contain eight C2H2-type zinc fingers as well as an N-terminal polyQ domain and a C-terminal Serine/Threonine-rich-domain, implying their functions as transcription factors (Pecasse *et al.*, 2000). Kr-h1 beta is specifically expressed in neurons. Misexpression caused problems in neuron pathfinding and altered expression profiles of ecdysone-responsive genes (Beck *et al.*, 2004; McGovern *et al.*, 2003). The alpha isoform is expressed during larval stages until the early pupal stage (Pecasse *et al.*, 2000). The major function of Kr-h1 in post-embryo development is to mediate metamorphosis, while other functions such as regulating neuron morphogenesis in fly larvae are also documented (Riddiford, 2008; Shi *et al.*, 2007).

Although *kr-h1* has been implicated in mediating insect metamorphosis for many years, recent work brought *kr-h1* into the realm of JH study. In 2008, Minakuchi *et al.* found in a microarray study that expression of *kr-h1* was induced by topical JH treatment (Minakuchi *et al.*, 2008). Mounting evidence demonstrates that Kr-h1 plays an important role in JH signaling. When ectopically expressed in abdominal epidermis of *Drosophila* pupae, Kr-h1 prolonged the expression of BR protein, a critical metamorphosis suppressor, in the pleura and the dorsal tergite. Misexpression of *kr-h1* also led to missing or truncated bristles in the dorsal midline. Both effects induced by Kr-h1, prolonged expression of *br* and missing bristle, resemble the effects observed after JH treatment (Minakuchi *et al.*, 2008; Zhou *et al.*, 2002b). Regulation of *kr-h1* expression by JH and function of Kr-h1 in mediating JH actions in preventing metamorphosis has been confirmed in early larval stages of fruit fly and in the red flour beetle (Huang *et al.*, 2011; Minakuchi *et al.*, 2009). Subsequent studies show that Kr-h1 functions downstream of Met. Met RNAi in the beetle at both larval and pupal stages led to decrease in

expression of *kr-h1* (Minakuchi *et al.*, 2009; Parthasarathy *et al.*, 2008). Reduced expression of *kr-h1* was also observed in the fat body of fly larvae with *Met* and *gce* double null mutation (Abdou *et al.*, 2011). These results show that *kr-h1* works downstream of *Met* and upstream of *br* in mediating JH functions in preventing metamorphosis. Studies in hemimetabolous insects, such as the true bug *Pyrrhocoris apterus* and the western flower thrips *Frankliniella occidentalis*, suggest that the mechanism of Kr-h1 function is conserved in both holometabolous and hemimetabolous insects (Konopova *et al.*, 2011; Minakuchi *et al.*, 2011).

AaET

In most mosquito species, adult females take blood from vertebrate hosts in order to acquire necessary amino acid nutrients for producing egg proteins. Mosquitoes can take blood meals of more than their body weight, which need to be digested quickly. Digestion of blood occurs in the midgut by exo- and endoproteolytic enzymes, of which serine proteases are the principle enzymes for blood digestion.

AaET is a female specific serine protease expressed in the midgut. *AaET* mRNA is undetectable in mosquito larvae, pupae, and newly-emerged adults. Transcription of *AaET* occurs in the midgut of female mosquitoes shortly after emergence of adults and *AaET* mRNA reaches its maximal level in 3-7 days (Noriega *et al.*, 1996a; Noriega *et al.*, 1997). The expression profile of *AaET* correlates well with rising titer of JH in adult mosquitoes. Noriega *et al.* shows that expression of *AaET* is induced by topically applied JH in the newly emerged mosquitoes when the endogenous JH is still at background level (Noriega *et al.*, 1997). On the other hand, when JH titer is reduced *in vivo* by overexpression of JH esterase, the major enzyme responsible for JH degradation *in vivo*, expression of *AaET* is repressed. The effect could be reversed by application of a JH analog, methoprene (Edgar *et al.*, 2000). The results indicate that

expression of *AaET* in the midgut is regulated by JH. Furthermore, recently it has been shown that Met is required for expression of *AaET*. *Met* RNAi by dsRNA injection in female mosquitoes significantly reduced *AaET* expression in the midgut (Zhu *et al.*, 2010).

1.6 Research aims

It has been more than 75 years since the first evidence suggested that JH is an insect growth regulator. A great deal of research has been dedicated to understanding the physiological roles of JH and the underlying mechanisms of JH actions. Many aspects of physiological function of JH have been outlined, yet understanding of the molecular mechanisms just began emerging in recent years. Met has been shown to be a nuclear receptor of JH. It mediates many physiological functions of JH as well as induction of JH-responsive genes. However, how Met does so is only speculative.

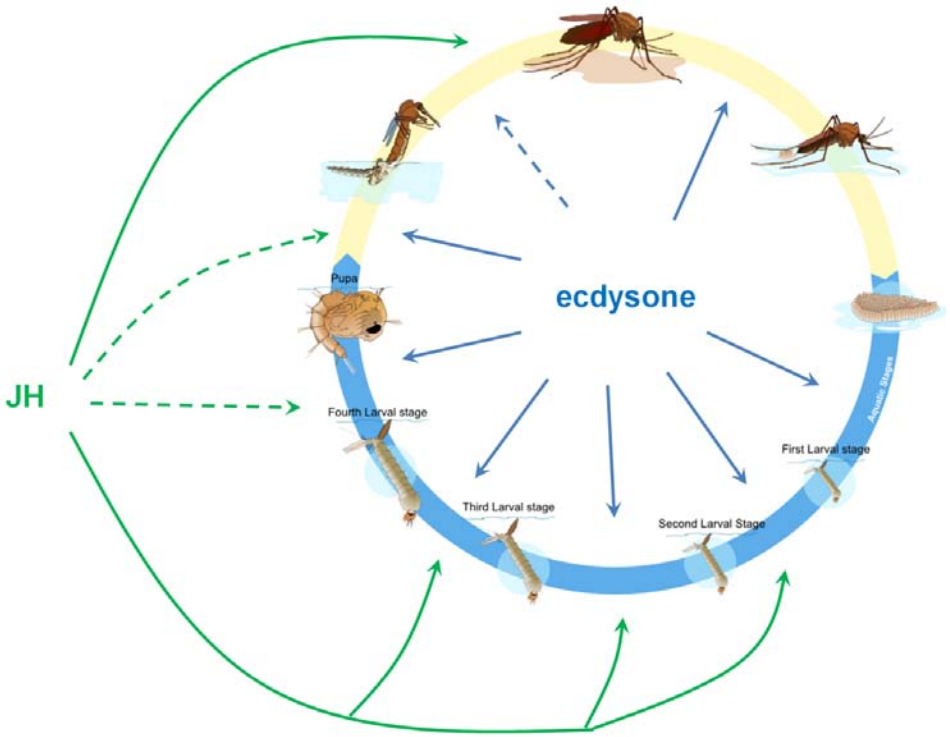
JH analogs, the so-called third-generation pesticides, are thought to be proof against evolution of resistance in insects because of the essential role of JH in insect development and reproduction. However, mutant strains resistant to a commercial insecticide, the JH agonist methoprene, have emerged in several species of insects. JH agonists target insects only during a very short period during metamorphosis, after the crop-eating pests have already done their damage. Alternatively, JH antagonists would target the much longer and earlier larval stages. Therefore, JH antagonists would be more effective than JH agonists as insecticides. However, no JH antagonist has been found so far. Antagonists for PAS domain proteins are possible, as exemplified by the identification of antagonists for this family of proteins AhR and HIF-2 (Bisson *et al.*, 2009; Rogers *et al.*, 2013).

Studying the molecular mechanisms of Met actions would shed light into JH functions on the molecular level. It would also provide insight into designing new insecticides mimicking or blocking JH functions. The objective of the present body of work is to understand how Met mediate JH functions in gene regulation. The two specific aims are:

Aim 1. Investigate whether Met functions as a transcription activator and that directly regulates expression of JH-inducible genes.

Aim 2. Elucidate how Met is recruited to the JH-responsive promoters.

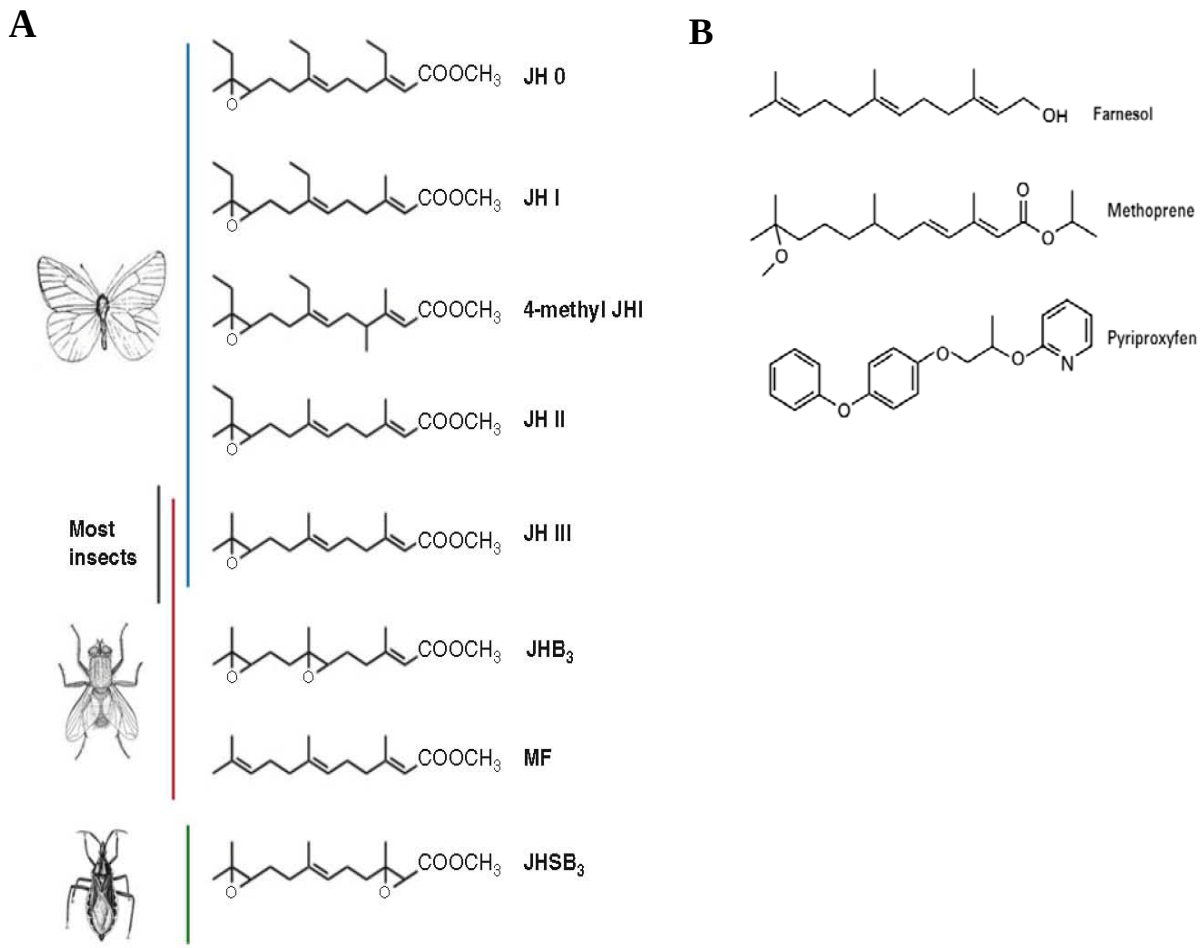
Figure 1.1



Mosquito life cycle and regulation of development and reproduction by JH and ecdysone. Stages of mosquito shown in the figure are (clockwise): eggs, four larval stages, pupa, emergence of adult, female adult taking blood, female mosquito laying eggs. Regulation by hormones is shown by arrows. Dash arrows means the hormone concentration is low.

The life cycle of mosquito is adapted from (Villarreal, 2010) and the regulation by JH and ecdysone is added by the author according to several publications (Riddiford, 1994; Wyatt *et al.*, 1996). Used under fair use, 2013. Permission statement from author: "This work has been released into the public domain by its author, LadyofHats. This applies worldwide. In some countries this may not be legally possible; if so: LadyofHats grants anyone the right to use this work for any purpose, without any conditions, unless such conditions are required by law."

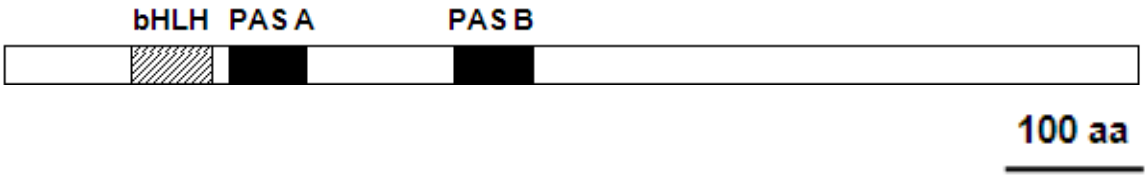
Figure 1.2



Structures of natural JHs and related chemicals. A) Chemical structures of natural JHs and their distribution in insects. The insects shown from top to bottom represent classes Lepidoptera, Diptera, and Hemiptera. JHB_3, JH III bisepoxide. MF, methyl farnesoate. $JHSB_3$, JH III skipped bisepoxide. B) Structures of JH-related compounds used in this work. Farnesol is an inactive JH precursor. It is used as a negative control of JH. Methoprene and pyriproxyfen are two active JH analogs.

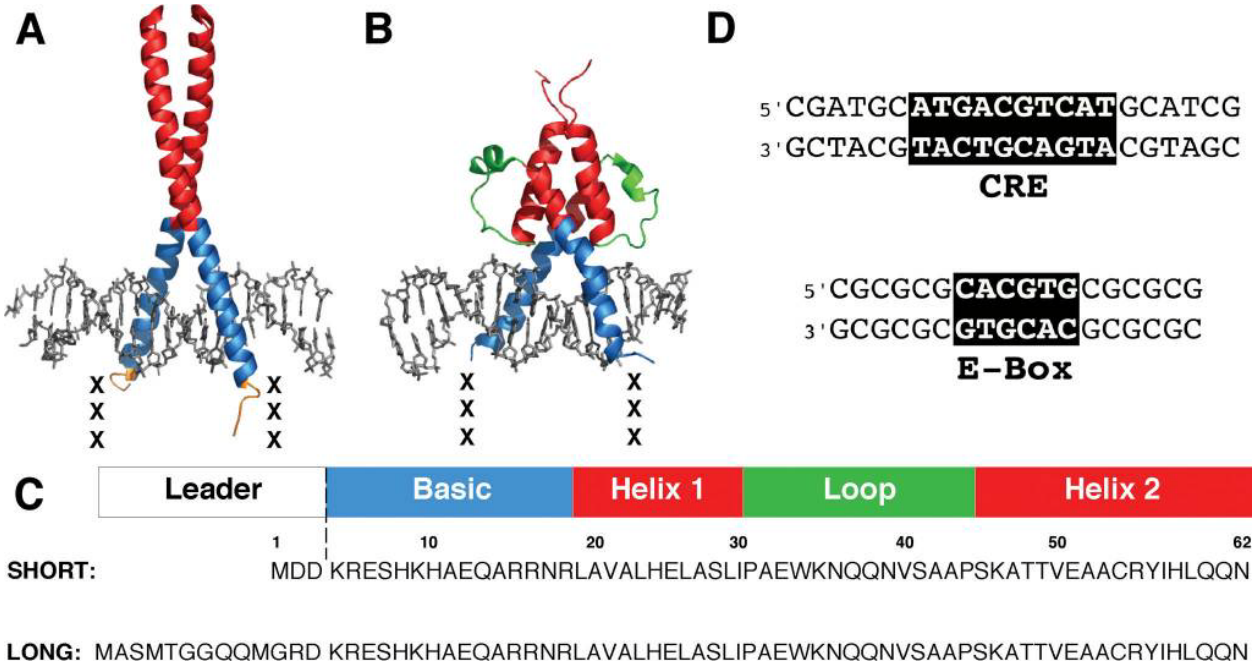
The figure 1.2A is from published material (Cusson *et al.*, 2013). Used with permission of publisher.

Figure 1.3



Schematic diagram of the structure of Met protein. *Ae. aegypti* Met has 977 amino acids. Boxes represent the three N-terminal conserved domains, bHLH and two PAS domains.

Figure 1.4



Canonical structure of bHLH dimer binding to E box. B) A structural model of a dimer of bHLH domains binding to DNA. Sub-domains are colored as in C), basic region in blue, helix in red, and loop in green. D) A perfect palindrome E box sequence.

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Chapter 2

A heterodimer of two bHLH-PAS proteins mediates the juvenile hormone-induced gene expression

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Proc Natl Acad Sci U S A 108(2): 638-643. 2011

2.1 Abstract

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

response to JH in a cell transfection assay. Together, the evidence indicates that Met and FISC form a functional complex on the JHRE in the presence of JH and directly activate transcription of JH target genes.

2.2 Introduction

Juvenile hormones (JHs) are sesquiterpenoid molecules synthesized and secreted by the corpora allata in insects. JHs are essential for development, reproduction, diapause, caste differentiation, migratory behavior and longevity in many insect species (Flatt *et al.*, 2005; Nijhout, 1994; Riddiford, 2008; Wyatt *et al.*, 1996). The prominent role of JH is maintaining the status quo in juvenile insects and preventing an insect from precociously turning into an adult. During larval development, ecdysone (the molting hormone) causes larval-larval molts in the presence of JH in the hemolymph. After the corpora allata stop secreting JH in the final larval instar, insect tissues change their commitment and ecdysone triggers the larval-pupal and pupal-adult molts (Riddiford *et al.*, 2003).

JH appears to harness a variety of signal transduction pathways to exert its function. Some effects of JH are mediated via membrane receptors and the protein kinase C signaling pathway (Wyatt *et al.*, 1996; Yamamoto *et al.*, 1988), while more evidence suggests that JH acts through intracellular receptors to modulate gene expression (Comas *et al.*, 1999; Dubrovsky *et al.*, 2000; Li *et al.*, 2007; Minakuchi *et al.*, 2008). In some cases, JH seems to exert its functions by modulating the ecdysteroid signaling pathway (Dubrovskaya *et al.*, 2004; Henrich *et al.*, 2003; Parthasarathy *et al.*, 2007; Richards, 1978; Zhou *et al.*, 1998a; Zhou *et al.*, 1998b; Zhou *et al.*, 2002b).

A leading candidate for the JH receptor (or a component of the receptor) is the product of the *Methoprene-tolerant* (Met) gene, which was originally isolated in *Drosophila melanogaster* (Ashok *et al.,* 1998). Met belongs to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors that also includes the hypoxia inducible factor 1α (HIF-1α), aryl hydrocarbon receptor (AhR), aryl hydrocarbon nuclear translocator (Arnt), and CLOCK proteins. *In vitro*-synthesized *Drosophila* Met protein binds to JH-III with high affinity (Miura *et al.,* 2005). Flies carrying the *Met* mutations show resistance to both the toxic and morphogenetic effects of JH and several JH analogs, including methoprene (Ashok *et al.*, 1998). Recent studies suggest that *Met* and its paralogous gene in *Drosophila*, *germ cell expressed* (*gce*), have overlapping but not identical functions in JH signaling (Baumann *et al.*, 2010; Liu *et al.*, 2009). In *Tribolium castaneum,* it has been clearly demonstrated that the Met ortholog of this beetle (TcMet) plays an essential role in mediating the classical anti-metamorphic effect of JH during molting. RNAi suppression of TcMet expression causes larvae to pupate prematurely, before reaching their final instar (Konopova *et al.,* 2007). It remains unclear how Met protein mediates JH signaling at the molecular level.

JH plays important roles in the control of various aspects of adult reproduction in mosquitoes (Hagedorn, 1994). Secretion of JH-III begins soon after emergence of the adult. JH-III levels increase during the first two days and remain high until a blood meal is taken. Upon blood feeding, the hemolymph JH-III titers drop precipitously, while 20E titers begin to rise and reach their maximum level at 18-24 h post blood meal (Hagedorn, 1994). Our previous study has shown that the mosquito ortholog of Met is required for the JH-induced expression of the *Krüppel homolog 1* (*AaKr-h1*) gene and the *early trypsin* (*AaET*) gene in newly emerged adult female mosquitoes (Zhu *et al.*, 2010). While the function of AaKr-h1 is unknown in mosquitoes,

AaET is a female-specific protease involved in initial digestion of blood proteins in the midgut (Noriega *et al.*, 1996b). Transcription of *AaET* is activated by JH after adult eclosion, but the AaET protein is produced only after blood ingestion (Noriega *et al.*, 1996a). Here we report that Met binds to another bHLH-PAS domain protein only in the presence of JH. Both proteins are required for the proper expression of JH target genes after adult emergence. We also provide evidence indicating that the heterodimer directly binds to the regulatory regions of the target genes and activates their transcription in response to JH.

2.3 Materials and methods

Mosquitoes

The *Aedes aegypti* mosquito Liverpool strain was reared at 27 ^{o}C and 80% relative humidity with a photoperiod cycle of 16 h light/8 h dark. Larvae were fed Tetramin tropical flakes (Bian *et al.*, 2008), and adults were maintained on a 10% sucrose solution by wick.

Plasmids

pCMA-GAD, pCMA-GBD, and UAS×4-188-cc-Luc are from Hu (Hu *et al.*, 2003). pRL-CMV is from Promega.

Plasmids carrying inserts with any mutation or deletion were generated by site-directed mutagenesis as described in details in the "Site-directed mutagenesis" section below.

Plasmid	Description of construction
pGAD10-AaMet$^{1-505}$	Vector: pGAD10. cDNA fragment coding for AaMet amino acid 1-505 was inserted at *Bam*H I restriction site.
pGBKT-AaMet$^{1-505}$	Vector: pGBKT. cDNA fragment coding for AaMet amino acid 1-

	505 was inserted at *Bam*H I site.
pCMA-GAD-AaTgo$^{1-394}$	Vector: pCMA-GAD. cDNA fragment coding for AaTgo amino acid 1-394 was inserted at *Not* I site.
pCMA-GBD-AaMet$^{1-596}$	Vector: pCMA-GBD. cDNA fragment coding for AaMet amino acid 1-596 was inserted at *Not* I site.
pCMA-GAD-AaFISC$^{1-609}$	Vector: pCMA-GAD. cDNA fragment coding for AaFISC amino acid 1-609 was inserted at *Not* I site.
pCMA-AaMet	Vector: pCMA. Full-length cDNA of AaMet was inserted between restriction sites *Hin*d III and *Xho* I.
pAc5.1-AaFISC	Vector: pAc5.1 V5-His A. Full-length cDNA of AaFISC was inserted between restriction sites between restriction sites *Bam*H I and *Xba* I.
pCMA-AaTgo	Vector: pCMA. Full-length cDNA of AaTgo was inserted between restriction sites between restriction sites *Xba* I and *Xho* I.
pAaET-luc	Vector: pGL3 basic. -2075 to +61 section of *AaET* was inserted between restriction sites *Xho* I and *Nco* I.
4×JHRE-luc	Vector: pGL3 basic. Four tandem repeats of JHRE (CCACACGCGAAG) was ligated to -77 to +61 section of *AaET* by *Hin*d III. The chimerical fragment was inserted between restriction sites *Kpn* I and *Nco* I of the vector.

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described (Carrigan *et al.*, 2011). Primers containing point mutation were used in PCR to amplify full length of template plasmids. PCR product was cleaned up with PCR purification kit (Qiagen) followed by digested with *Dpn* I at 37^{o}C for 1 hour to remove template plasmid. The DNA was purified again and about 200 ng of the DNA was used to transform *E. coli* NEB 10-beta competent cells (New England Biolabs) following manufacturer's instructions. Primers for mutagenesis are shown below.

Plasmid	Primer	Sequence (5'- 3')
pCMA-GBD-AaMet$^{1-596}$ΔbHLH	Fwd	TTCCTCGAGGACAGAAGACCGAAGTGC
	Rev	TTCCTCGAGAAGCAGCTTTGGAGTCTTC
pCMA-GBD-AaMet$^{1-596}$ΔPAS A	Fwd	CGACGTTCCTCGAGGAATGCTGGTGCCCAACAACTTAGTCAA
	Rev	CAGCATTCCTCGAGGAACGTCGTGCACTTCGGTCTTCTGTC
pCMA-GBD-AaMet$^{1-596}$ΔPAS B	Fwd	TTCCTCGAGGAAACGATCAACACCCTAGTCTCGGACGAAGAA
	Rev	GTGTTGATCGTTTCCTCGAGGAATCTGCAAGCCTCAATCAAT
pCMA-GAD-AaFISC$^{1-609}$ΔbHLH	Fwd	AAATCAACAAGTGCAACAACGGAAACCGAGATTTGACC
	Rev	GTTGTTGCACTTGTTGATTTGCGACTGTGGCTTAGTATCT
pCMA-GAD-AaFISC$^{1-609}$ΔPAS A	Fwd	GAGCTAAACTGGGATCAAAATGAGTTTCTACAACCACCA
	Rev	TTTTGATCCCAGTTTAGCTCCTCGAAATAGGCCGACTTT
pCMA-GAD-AaFISC$^{1-609}$ΔPAS B	Fwd	GGACAACGGTGCGGCCCAGGTGTCGTCGTATCGGTTGC
	Rev	CCTGGGCCGCACCGTTGTCCAGCGTCTGTGGCATAGTG
pAaET-Luc Δ-2075- -1530	Fwd	TTATGTTCCGGATTAGGTGGATAACCCAAATTTGATGTCT
	Rev	CTAATCCGGAACATAAACGCGTAAGAGCTCGGTACCTAT
pAaET-Luc Δ-1530- -969	Fwd	TGAACATTATGTTCCGATGGACTCTGCATCTGTCTTCAGA
	Rev	CCATCGGAACATAATGTTCACAAATTCTAGGGCAGATTTACC
pAaET-Luc Δ-969- -540	Fwd	GTCTTCAGATCCGGAATAACTACGAAACCACCTAGACC
	Rev	TATTCCGGATCTGAAGACAGATGCAGAGTCCATCGGAT
pAaET-Luc Δ-540- -165	Fwd	ATATCTCCGAAAATCCATCTGCACGTGTGTACCGTAATC
	Rev	ATGGATTTTCGGAGATATGGTCTAGGTGGTTTCGTAGTT

Yeast two-hybrid screen

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

Transient transfection assay

Drosophila L57-3-11 cells were transfected according to the instructions of Hu et al. (Hu *et al.*, 2003). pCMA was used as the expression vector for all the proteins described in the transfection assays. Truncated proteins were expressed by deletional mutagenesis using a method described by Li et al. (Li *et al.*, 2008). The ORFs for AaTgo, DmMet and Dmgce were cloned by RT-PCR based on the cDNA sequences in the GenBank. Juvenile hormone III, methoprene, pyriproxyfen and farnesol (Sigma Aldrich) were dissolved in ethanol.

Double-stranded RNA-induced gene silencing

RNAi knockdown of AaMet and AaFISC was performed as described previously (Zhu *et al.*, 2010; Zhu *et al.*, 2006). Briefly, 0.5 μg of dsRNA was injected into the newly emerged female *Ae. aegypti* mosquitoes within 30 minutes after eclosion. The mosquitoes were then maintained in the insectary under normal conditions. 3–4 days after injection, the mosquitoes were dissected and the mRNA extracted from the midgut was examined by quantitative RT-PCR (Zhu *et al.*, 2010). PCR primers for generating DNA template for synthesizing dsRNA are listed below.

Gene	Primer	Sequence (5'- 3')
malE		The DNA template was obtained by PCR amplification of plasmid LITMUS28i-Mal with T7 promoter primer: TAATACGACTCACTATAG
AaMet	Fwd	TAATACGACTCACTATAGGGGCTGTTAAAAGTGGATGATGATAC
	Rev	TAATACGACTCACTATAGGGAATCGGCACCTTGGTAGAACGATC
AaFISC	Fwd	TAATACGACTCACTATAGGGAGCCAATTGATGCAACAACAGCTG
	Rev	TAATACGACTCACTATAGGGGTTGGGATGGACTCGGAGGTCCTG
GFP	Fwd	TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC
	Rev	TAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTCG
DmTai	Fwd	TAATACGACTCACTATAGGGTTCTATGAACAACTCCCGGC
	Rev	TAATACGACTCACTATAGGGGCCAGCGCTAATGAGGTTAC

Quantitative RT-PCR

RNA extraction, reverse transcription and quantitative real-time PCR were carried out as previously described (Chen *et al.*, 2004). Dissected tissues were homogenized with a motor-driven pellet pestle mixer (Kontes, Vineland, New Jersey, USA) and lysed by Trizol reagent (Invitrogen). RNA was isolated following the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). RT was carried out using an Omniscript reverse transcriptase kit (Qiagen) in a 20 μl reaction mixture, containing random primers and 1 μg total RNA at 37 ^{o}C for 1 h. qPCR reactions were run using the SYBR GreenER qPCR Supermix (Invitrogen) on the ABI prism 7300 sequence detection system (Applied Biosystems). Real-time PCR was performed in triplicate and normalized to rpS7 mRNA expression for each sample. All experiments were performed at least three times independently and representative data are shown. Real-time PCR primers are listed in table below.

Gene	Primer	Sequence (5'- 3')
rpS7	Fwd	CCCGGAGCCCTACCTATAAACTAT
	Rev	GCAGCACAAAGATGATTTATGCAC
AaET	Fwd	AATACAGATCCTGCGGCCTA
	Rev	CCATTATACTGCGGGTGAGG
AaKr-h1	Fwd	TGCGGAGAGCTTGGCAATA
	Rev	AAACACCCTTTGCTTTCGTTCA
AAEL002576	Fwd	TGCGCTCGGAGGTATGAAG
	Rev	GCCGTCATGCTAAGACTTTGG

AAEL002619	Fwd	CACCACAGTGGCACCTACGA
	Rev	GCAACACCACGGCAAAAAT

Chromatin immunoprecipitation assay

Polyclonal antibodies for AaMet and AaFISC have been reported previously (Zhu *et al.*, 2010; Zhu *et al.*, 2006). *Ae. aegypti* mosquito abdomens were homogenized in PBS on ice, followed by addition of formaldehyde to a final concentration of 1% and incubation at 37°C for 10 minutes. Chromatin immunoprecipitation assays were performed using a QuikChIP kit (IMGENEX) according to the instruction manual. Mock immunoprecipitations using preimmune sera for each antibody were included as negative controls to determine the baseline of the nonspecific background. The precipitated DNA and DNA input were analyzed by using quantitative RT-PCR. PCR primers are shown below.

Position	Primer	Sequence (5'- 3')
ET2	Fwd	GCTTGGTAGAACAGTCAATGGGTCAG
	Rev	AGAGTCCATCGGATAGGCATCACG
ETv	Fwd	GTTTTGAAATTACCCATCCCACACG
	Rev	GTCCATTCCTATGATGCGGATTCTT
ETc6	Fwd	GTAAGGATTCTTGCCAGGGAGACTC
	Rev	ATCCATTGGCGAACAGTGGACAC

Preparation of nuclear protein extracts (NE)

Abdomens were collected from 200 adult female *Ae. aegypti* mosquitoes for each time point. Nuclear protein extraction was carried out as described by Miura et al. (Miura *et al.,* 1999). Dissected mosquito abdomens were frozen in liquid nitrogen. The 200 abdomens were grounded using a mortar and a pestle to fine powder in liquid nitrogen. The powder was homogenized in 2 ml of homogenization buffer (10 mM Hepes, pH 7.9 containing 1 mM EDTA, 150 mM NaCl, 0.6% (v/v) Triton X-100, 1% (v/v) ethanol, 4 mM DTT, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), 5 μg/ml each of leupeptin, pepstatin and antipain, 2 mM benzamidine, and 9 μg/ml of aprotinin) with a Dounce tissue homogenizer and a type B pestle, loose fit. The sample was centrifuged at 500×g for 30 s and the supernatant was transferred to a fresh ice-cold 15 ml falcon tube. After 5 min incubation, the sample was centrifuged at 1,500×g for 10 min at 4°C. Supernatant was removed and the pellet was resuspended in 100 μl of the lysis buffer (20 mM Hepes, pH 7.9 containing 0.2 mM EDTA, 420 mM NaCl, 1.2 mM MgCl2, 25% (v/v) glycerol, 1% (v/v) ethanol, 1 mM DTT, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), 5 μg/ml each of leupeptin, pepstatin and antipain, 2 mM benzamidine, and 9 μg/ml of aprotinin). After incubation on ice for 20 min, the sample was centrifuged at 16,000×g for 5 min in a microcentrifuge at 4°C. The supernatant was transferred into fresh ice-cold 1.5 ml tubes and frozen in liquid nitrogen.

Electrophoretic mobility shift assay

A 10-pM quantity of doublestranded oligonucleotide was end labeled with T4 DNA kinaseand 50 μCi [γ-^{32}P] ATP (PerkinElmer). The unincorporated dradioactivity was removed through a Sephadex G-25 (Amersham Pharmacia Biotech) spin column. Reactions were carried

out in a 20-μL volume containing 4 μg nuclear extracts, 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 4% (vol/vol) glycerol, and 1 μg poly(dI-dC)·poly(dI-dC). Nuclear proteins were preincubated with 100-fold excess of unlabeled competitor DNA or 3 μg antibodies. After 20-min incubation at 4 °C, 0.05 pmol of [^{32}P] labeled DNA probe (~10,000 cpm) was added, and the incubation continued for another 30 min at room temperature. The reaction mixture was resolved using a 6% nondenaturing polyacrylamide gel at a constant voltage of 100 V for 90 min at 4 °C. The gel was dried, and the protein–DNA complexes were visualized by PhosphorImager analysis. Oligonucleotides (only sense strands are shown) used to generate the probe and competitor DNA were as follows:

AaET JHRE: 5′-CCATCCCACACGCGAAGACGATAAAACCA-3′

Nonspecific competitor: 5′-GATCCAGATTAGGATAGCATATGCTACCCAGATATA

2.4 Results

Identification of a Met-interacting protein

bHLH-PAS proteins tend to use the bHLH-PAS domains to form homodimers or heterodimers with other bHLH-PAS proteins (Partch *et al.*, 2010). DmMet has been shown to form Met-Met and Met-GCE dimers *in vitro*, although formation of the two protein complexes are greatly reduced in the presence of JH or JH analogues (Godlewski *et al.*, 2006). We performed a yeast two-hybrid screening to isolate mosquito proteins that are specifically associated with Met only in the presence of JH. A chimeric protein consisting of the bHLH-PAS domain of the *Aedes aegypti* Met (AaMet$^{1\text{-}505}$) and the Gal4 DNA-binding domain was used as bait. The yeast transformants were selected on solid medium contained 10^{-6} M methoprene. The screening yielded a single clone encoding the bHLH-PAS domain (amino acid residues 1-539) of

FISC, a mosquito protein which has been previously characterized as a coactivator of the ecdysteroid receptor (Zhu *et al.*, 2006).

The yeast two-hybrid assay indicated that the interaction between the bHLH-PAS domains of AaMet and AaFISC was methoprene-dependent as the co-transformants were unable to grow on the selection medium without the presence of methoprene (Figure 2.S1). To examine the potential AaMet-AaMet interaction, we cloned AaMet$^{1\text{-}505}$ into the pGAD10 Gal4 activation domain fusion vector and used it in the two-hybrid assays for comparison. It appeared that AaMet formed a homodimer only in the absence of methoprene, consistent with a previous report by Godlewski et al. (Godlewski *et al.*, 2006).

JH-dependent protein interaction

To validate the Met-FISC interaction in insect cells and to characterize its dependence on juvenile hormone, we used a modified two-hybrid system developed by Drs. Peter and Lucy Cherbas (Hu *et al.*, 2003). pCMA-GAD and pCMA-GBD are expression vectors for recombinant proteins fused to the GAL4 activation domain and binding domain, respectively. A cDNA fragment encoding the amino terminal bHLH-PAS domain of AaMet was cloned into pCMA-GBD, while the bHLH-PAS domain of AaFISC was cloned into pCMA-GAD.

The GAD-AaFISC fusion was not able to activate the UAS×4-188-cc-Luc reporter gene, regardless of the presence of JH-III (Fig. 2.1A). GBD-AaMet was activated by JH-III and stimulated expression of the luciferase reporter gene, reminiscent of the transcriptional activity of DmMet in a similar experiment (Miura *et al.*, 2005). The JH-dependent activation of the reporter gene by GBD-AaMet was further boosted when the GBD-AaMet and GAD-AaFISC fusion proteins were expressed together, suggesting a physical interaction between the bHLH-PAS

regions of AaMet and AaFISC. In contrast, similar experiments implied that AaMet did not form either a homodimer or a heterodimer with other bHLH-PAS proteins, such as AaTgo (the mosquito ortholog of *Drosophila* Tango) in the L57 cells (Fig. 2.1A).

Next, we used the same cell transfection system to study the hormone-specificity and JH dose-response of the protein interaction between AaMet and AaFISC. Formation of the AaMet-AaFISC dimer was induced by JH-III and two JH agonists (methoprene and pyriproxyfen), but not by farnesol (a biosynthetic intermediate for JH-III) (Fig. 2.1B). In subsequent hormonal treatment experiments, farnesol was used as negative control. Marked activation of the reporter gene by the AaMet-AaFISC interaction was observed when the transfected cells were exposed to JH-III at a concentration of 10^{-7} M, and the reporter activity continued to increase in a JH-dose dependent manner (Fig. 2.1C). Together, these results demonstrated that the AaMet-AaFISC interaction is a JH-specific response.

PAS domains essential for the Met-FISC interaction

Having demonstrated that the bHLH-PAS regions of AaMet and AaFISC were sufficient for their JH-dependent dimerization, we started to delineate the functional domains in the bHLH-PAS regions. Derivatives of the pCMA-GBD-AaMet$^{1-596}$ vectors were generated to produce GBD-Met fusion proteins with truncations of bHLH, PAS-A or PAS-B domains (Fig. 2.2A). Similar deletion mutations in GAD-AaFISC$^{1-609}$ fusion protein were created. Two-hybrid assays were performed in the L57 cells as described above with these new expression vectors. In the absence of bHLH domain, GBD-MetΔbHLH showed even stronger binding to GAD-AaFISC$^{1-609}$ (Fig. 2.2B), indicating this domain in AaMet is not required for the JH-dependent Met-FISC interaction. Truncations of the two PAS domains in AaMet all significantly diminished

formation of the Met-FISC complex, implicating the PAS domains in binding of JH and/or in protein-protein interaction. On the other hand, the bHLH, PAS-A and PAS-B domains of AaFISC all seemed to contribute to the Met-FISC interaction in response to JH, although the PAS-A and PAS-B domains appeared to play a bigger role in the binding of AaFISC to AaMet (Fig. 2.2D).

Roles of AaMet and AaFISC in expression of the JH target genes

Our previous studies have detected expression of AaMet and AaFISC genes in the fat body, midgut and ovaries of adult female mosquitoes during post-eclosion development (Zhu *et al.*, 2010; Zhu *et al.*, 2006). AaMet and AaFISC, two bHLH-PAS family transcription factors, form a heterodimer in response to JH, suggesting that the AaMet-AaFISC complex may function in modulating transcriptional response to JH. After injecting double-stranded RNA corresponding to AaMet or AaFISC into adult female mosquitoes within 30 minutes after eclosion, we examined expression of four JH target genes that are normally upregulated in the midgut after eclosion (Zhu *et al.*, 2010). Knockdown of either AaMet or AaFISC caused a considerable decrease in mRNA transcripts of *AaET* and *AaKr-h1* in the midgut (Fig. 2.3A and Fig. 2.S2). Expression of *AAEL002576* and *AAEL002619* were not markedly reduced in the *AaMet RNAi* mosquitoes, while impaired function of AaFISC affected the mRNA levels of *AAEL002619*, but not *AAEL002576*. Consistent with a diminished JH response, RNA interference of AaMet and AaFISC also significantly reduced the number of eggs oviposited by each female mosquito after blood feeding (Fig. 2.S3). These results indicated that both AaMet and AaFISC play important roles in modulating JH-regulated gene expression in adult female mosquitoes.

Detection of AaMet and AaFISC on a JH-activated promoter

To examine whether AaMet and AaFISC directly regulate the promoter of *AaET*, we performed chromatin immunoprecipitation (ChIP) assays. The presence of AaMet and AaFISC in the proximal regulatory regions of *AaET* was at a background level at 2 h post-eclosion (Fig. 2.3C and 2.3D), when endogenous JH concentration had not yet increased in the newly emerged mosquitoes. At 30 h post eclosion when the JH titers were near their peak, occupancy of the *AaET* promoter by either AaMet or AaFISC increased significantly. The association of AaMet and AaFISC with the *AaET* proximal promoter was concomitant with the active transcription of *AaET* at this stage (Noriega *et al.*, 1996a). Binding of either AaMet or AaFISC to the *AaET* promoter went down to the background level again at 4 h post blood meal (Fig. 2.3C and 2.3D), when the JH concentrations declined precipitously and transcription of *AaET* was shut down. These results showed that AaMet and AaFISC act directly on the *AaET* promoter to activate its transcription.

Identification of a JH response element

We cloned a 2.0-kb promoter region of *AaET* into the pGL3 basic luciferase reporter vector, and used transient transfection assays to test whether AaMet and AaFISC activated the *AaET* promoter in response to JH-III. Expression of either AaMet or AaFISC alone in L57 cells had no substantial effect on the activity of the *pAaET-Luc* reporter gene (Fig. 2.S4A). When the two proteins were expressed together, the reporter gene was activated significantly if JH-III was present in the cultural medium. Serial deletion analysis of the promoter region revealed that the proximal region (nt -540 to -165) was crucial for the JH-induced activation of the reporter gene (Fig. 2.S4B). Bioinformatic analysis of this region revealed a sequence (CCACACGCGAAG)

similar to the binding site of the mammal AhR/Arnt bHLH-PAS heterodimer (Fig. 2.S5). To test the function of this DNA element, we inserted 4 copies of this sequence and the minimal core promoter of *AaET* into the pGL3 basic luciferase reporter vector. While the minimal core promoter alone was not responsive to JH treatment (Fig. 2.S6), expression of the new reporter gene (4×JHRE-luc) was considerably activated in L57 cells by the AaMet-AaFISC complex in the presence of JH-III (Fig. 2.4A), suggesting that this 12-nucleotide sequence acted as a juvenile hormone response element (JHRE). Furthermore, gel shift assays suggested the existence of a protein complex containing both AaMet and AaFISC in the nuclear extracts of adult female mosquitoes (Fig. 2.4B). Binding of the protein complex to JHRE was abolished by antibodies against either AaMet or AaFISC, presumably by blocking dimerization or DNA binding of these two proteins. The protein complex was detected in mosquitoes at 30 h after eclosion, but not in the newly emerged mosquitoes or the blood-fed mosquitoes. The appearance of the AaMet-AaFISC complex seems to correlate well with endogenous JH concentrations and the expression profile of *AaET* in the adult female mosquitoes.

Conserved mechanism for JH signaling and transcriptional regulation

The JHRE shares a high degree of sequence similarity with a common motif that has been previously identified in a group of *Drosophila* JH responsive promoters (Li *et al.*, 2007). Using transient transfection assays, we tested the functions of *Drosophila* Met, GCE and Taiman (TAI; the *Drosophila* ortholog of AaFISC) in mediating JH signaling in the L57 cells. None of the three bHLH-PAS proteins alone had any significant effect on the expression of the 4×JHRE-luc reporter gene (Fig. 2.5). Co-expression of DmTAI with either DmMet or DmGCE led to significant induction of the reporter gene by JH-III. In contrast, the combination of DmMet and

DmGCE was not able to activate the reporter gene in response to JH-III. This evidence suggests that binding of the Met-FISC complex to the JHRE is a conserved mechanism in activating expression of JH target genes.

2.5 Discussion

Genetic studies have shown that Met is required for proper expression of JH target genes in fruit flies, red flour beetles and mosquitoes (Minakuchi *et al.*, 2008; Parthasarathy *et al.*, 2008; Zhu *et al.*, 2010). Although the protein structure of Met suggests that it may act as a JH-activated transcriptional regulator, the binding of Met to JH-responsive promoters has not been definitively demonstrated so far. In this study, a chromatin immunoprecipitation experiment indicated that Met was indeed associated with the early trypsin promoter when this gene was activated by endogenous juvenile hormone in the newly emerged adult female mosquitoes. This is the first demonstration of Met directly regulating a JH target gene.

To elucidate the molecular roles of Met in JH signaling, a number of proteins have been tested *in vitro* or in the cultured insect cells for their abilities to bind Met (Bitra *et al.*, 2009a; Godlewski *et al.*, 2006; Li *et al.*, 2007). The protein interactions with Met were largely independent of the presence of JH, or even repressed by JH. Using a library screening approach, we have identified a mosquito bHLH-PAS protein (FISC) that binds to Met in a JH-dependent manner. EMSA and ChIP experiments have demonstrated that the Met-FISC complex forms *in vivo* and binds to a JH-regulated promoter in previtellogenic mosquitoes only in the presence of high titers of juvenile hormone. This observation is consistent with the RNAi results showing that both Met and FISC are required in adult mosquitoes for activation of JH target genes, such as *AaET* and *AaKr-h1*. In Figure 2.1, the GBD-Met fusion (without the GAD-FISC fusion)

activated the UAS×4-188-cc-Luc reporter gene after the JH treatment. This activation also relied on the endogenous Taiman protein in the L57 cells as the JH induction was severely dampened when Taiman was depleted by RNAi (Fig. 2.S7). Formation of the Met-FISC complex thus constitutes a key step in signal transduction of juvenile hormone. It is also worth noting that not all the JH target genes are affected by RNAi knockdown of Met or FISC (Fig. 2.3A), implying that JH might act through several distinct pathways even in a single tissue at a particular developmental stage.

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

A distal regulatory region of *AaET* was also shown to be indispensable for JH-dependent activation of the *AaET* promoter (Fig. 2.S4). Intriguingly, when four copies of JHRE were placed upstream of the minimal promoter (TATA box) of *AaET*, the JHRE seemed to be sufficient for the Met-FISC mediated JH activation (Fig. 2.S6). This discrepancy implies that regulation of JH

target genes is more sophisticated than the binding of Met-FISC to JHRE. More studies are needed to elucidate the underlying molecular mechanisms.

In vitro experiments have shown that Met can bind to both EcR and USP, two components of the ecdysteroid receptor (Bitra *et al.*, 2009a). Here we find that FISC, a coactivator of the EcR/USP, also binds to Met and plays an important role in juvenile hormone signaling. Whether these protein interactions are involved in the crosstalk of ecdysone and JH signaling is waiting for further experimental evidence. Since the binding of FISC to EcR/USP and Met relies on the presence of 20-hydroxyecdysone and juvenile hormone respectively, the shuffling of FISC between the two signaling pathways may account for the antagonistic actions of these two hormones.

A sequence similar to the *AaET* JHRE is also found in the promoter region of *AaJHA15*, another JH-regulated gene in adult female mosquitoes (Bian *et al.*, 2008). The common motif 2 discovered in a group of JH-activated *Drosophila* promoters also shares high sequence similarity with the *AaET* JHRE, suggesting an evolutionarily conserved mechanism underneath the JH-induced transcriptional activation. Indeed, the *Drosophila* Met and Taiman activated the 4×JHRE-luc reporter gene in a JH-dependent manner. While DmMet-AaFISC appeared comparable to DmMet-DmTAI in mediating JH induced gene expression, AaMet-DmTAI was completely unable to activate expression of the reporter gene after JH treatment. This observation suggests that the intricate protein interactions between Met and FISC/TAI determine the affinity of the dimers to the JHRE and/or their ability to activate transcription of the JH target genes.

Unlike mosquitoes, two Met-like genes (Met and gce) exist in fruit flies. Combination of gce and Taiman also led to considerable activation of the reporter gene in response to JH. This

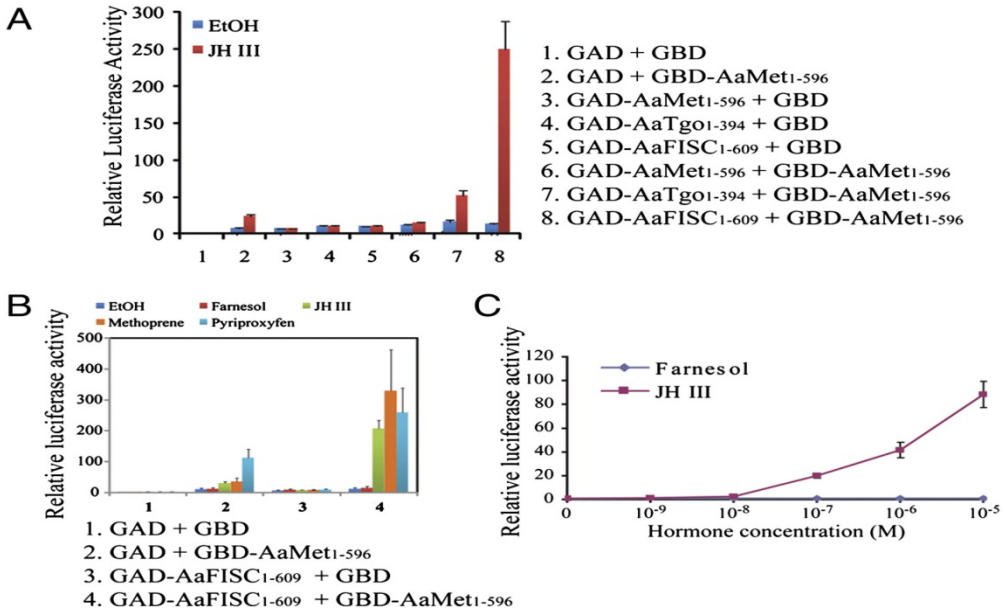
observation is in line with a recent report showing that gce can partially substitute for Met *in vivo* (Baumann *et al.,* 2010). It would be interesting to test next whether Met-TAI and gce-TAI preferentially bind to distinct JH responsive promoters *in vivo*.

2.6 Acknowledgement

We thank Dr. Lucy Cherbas for providing the pCMA, pCMA-GAD, pCMA-GBD and UAS×4-188-cc-Luc plasmids, and Dr. Denise J. Montell for supplying the cDNA clone and antibodies for Taiman. We also thank Dr. Honglin Jiang for helping with the EMSA experiments. This work was partly supported by the startup fund for J.Z. from Virginia Tech and a grant (J-929) from the Thomas F. and Kate Miller Jeffress Memorial Trust (to J.Z.).

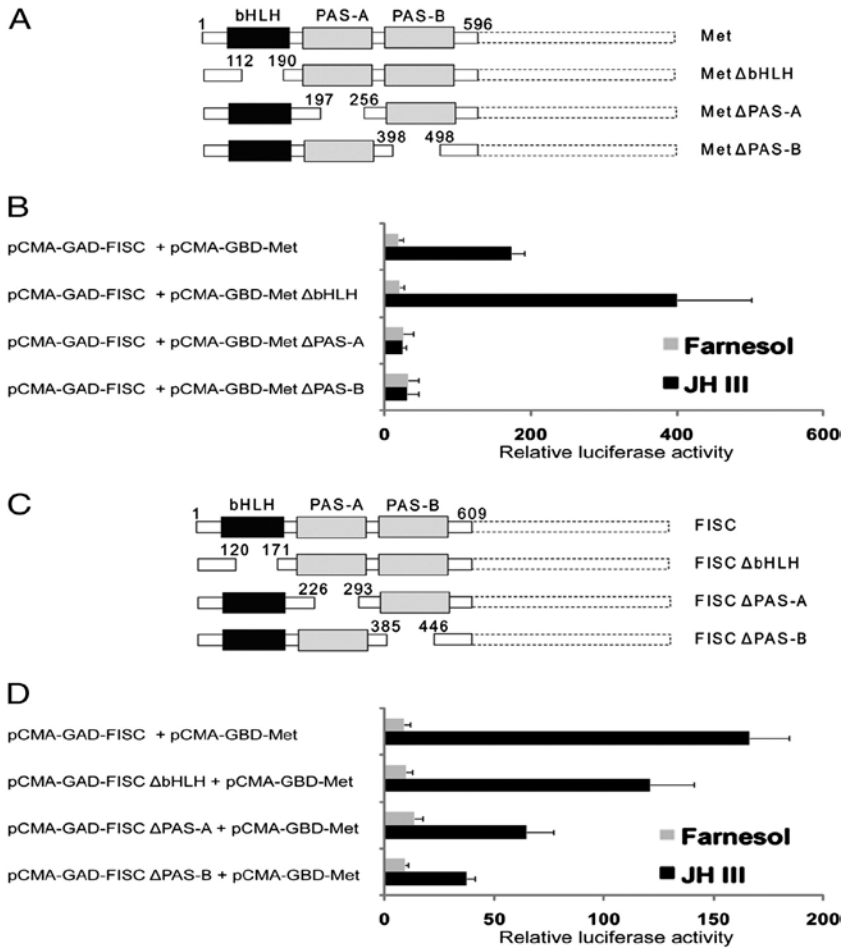
2.7 Figure legends and supplemental information

Figure 2.1



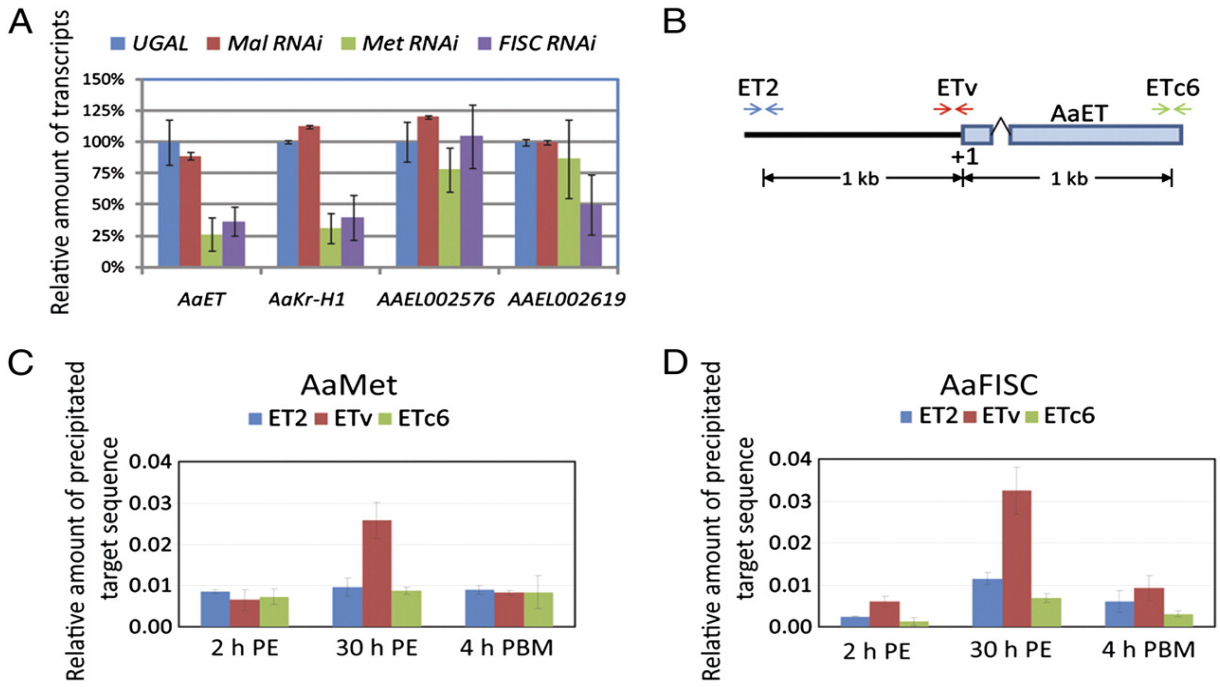
Interaction between AaMet and AaFISC in *Drosophila* L57 cells. (A) Modified two-hybrid assays. cDNA fragments encoding the bHLH-PAS domains of AaMet, AaFISC, and AaTgo were cloned into the pCMA-GBD and pCMA-GAD vectors. L57 cells were transfected by the reporter construct UAS×4–188-cc-Luc together with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

Figure 2.2



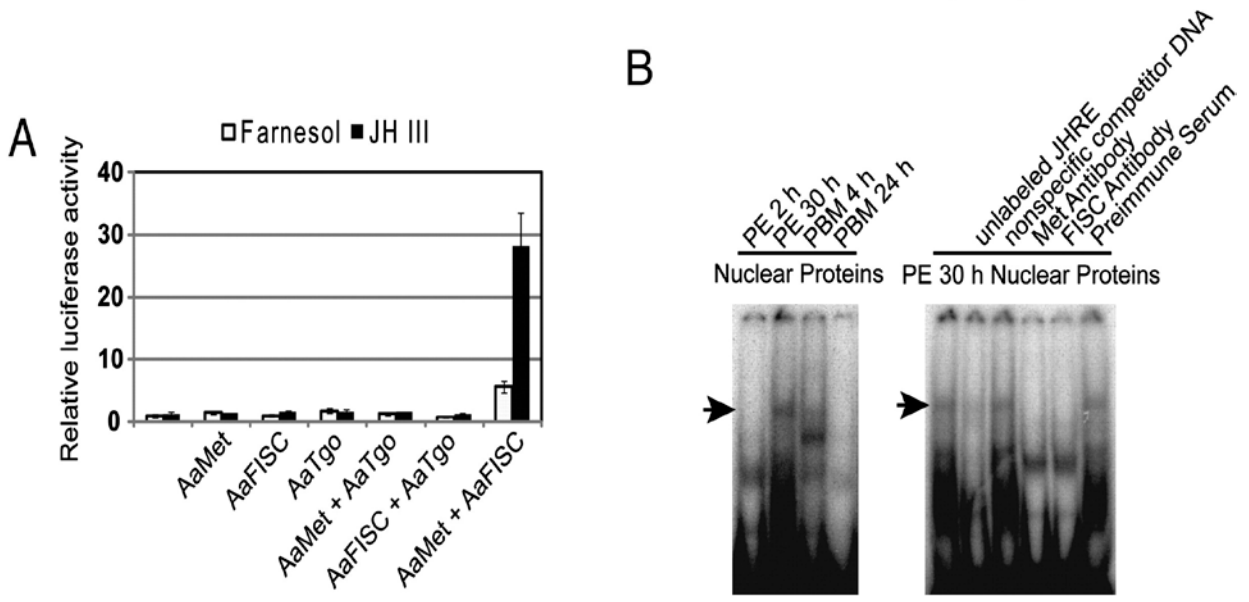
Roles of bHLH and PAS domains of AaMet and AaFISC in their JH-dependent dimerization. (A) Schematic diagram of truncations introduced into the bHLH-PAS region of AaMet. Similar truncations were introduced into the corresponding regions of AaFISC (C). L57 cells were transfected by the reporter construct UAS×4–188-cc-Luc, together with the indicated expression vectors that produced the truncated GBD-Met (B) or GAD-FISC (D). Transfected cells were cultured in medium with 5×10^{-6} M of JH-III or farnesol.

Figure 2.3



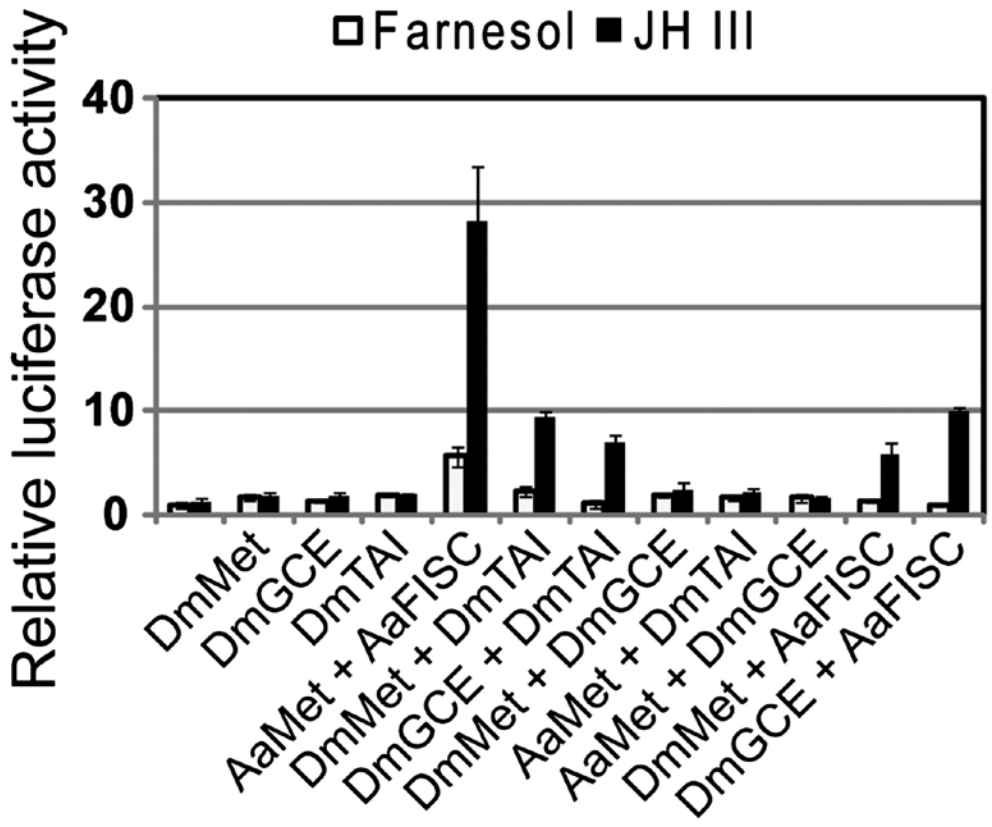
AaMet and AaFISC are required for expression of JH target genes in the midgut of adult female mosquitoes. (A) Double-stranded RNA (dsRNA) induced gene knockdown. A 0.5-µg quantity of dsRNA for either AaMet or AaFISC was injected into newly emerged female mosquitoes within 30 min after eclosion. DsRNA for bacterial *malE* gene was used as control. Then, 4 d after injection, midguts were collected from the mosquitoes. Total RNA was extracted and subjected to quantitative RT-PCR analysis. Results are expressed as percentage of mRNA levels in the uninjected (UGAL) mosquitoes. (B) Schematic structure of the *AaET* gene. Three pairs of primers were designed to amplify the distal upstream region (ET2), the proximal promoter region (ETv), and the coding region of *AaET* (ETc6). Association of AaMet (C) and AaFISC (D) with the *AaET* promoter was measured by chromatin immunoprecipitation assays. Amount of immunoprecipitated DNA in each sample was represented as signal relative to the total amount of input chromatin. PBM, post blood meal; PE, posteclosion.

Figure 2.4



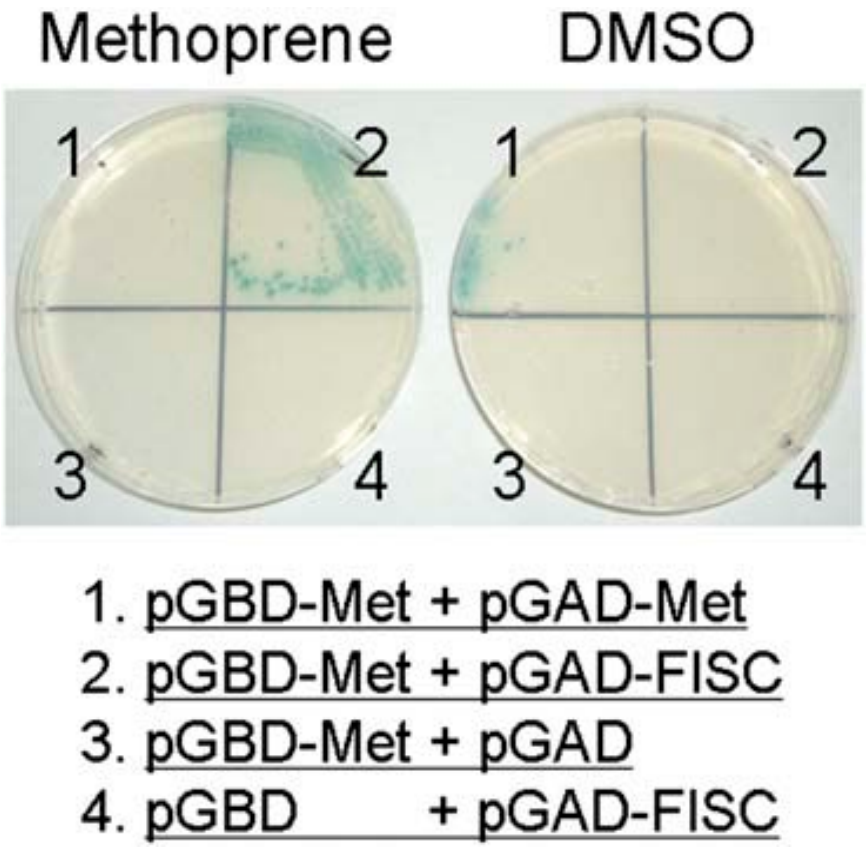
AaMet and AaFISC bind to JHRE identified in *AaET* upstream regulatory region. (A) L57 cells were transfected with the 4×JHRE-luc reporter plasmid, together with the indicated expression vectors. After transfection, cells were cultured in medium with 5 × 10−6 M JH-III or farnesol. (B) EMSA experiments. Nuclear proteins were extracted from abdomens of adult female mosquitoes at the indicated time points and incubated in vitro with JHRE labeled by [γ-32P] ATP. For competition reactions, nuclear proteins were incubated with an approximate 100× molar excess of unlabeled probe or a nonspecific double-stranded oligonucleotide for 20 min before incubation with labeled probe. Identity of complex was verified by directly adding polyclonal antibodies against AaMet and AaFISC to the binding reactions.

Figure 2.5



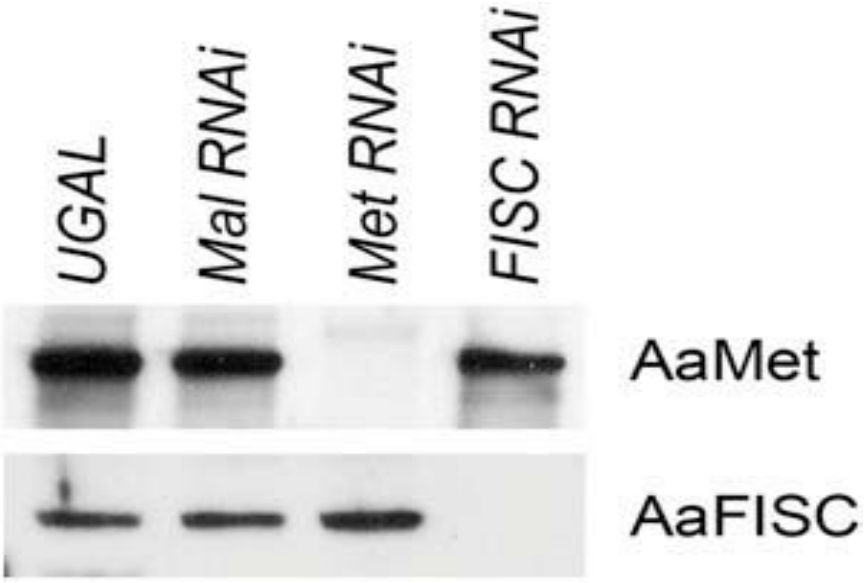
JH-induced transcriptional activation by the *Drosophila* homologs of Met and FISC. L57 cells were transfected by 4×JHRE-luc and expression vectors for the indicated proteins. Transfected cells were cultured in medium with 5×10^{-6} M JH-III or farnesol. Average results of three independent experiments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

Figure 2.S1



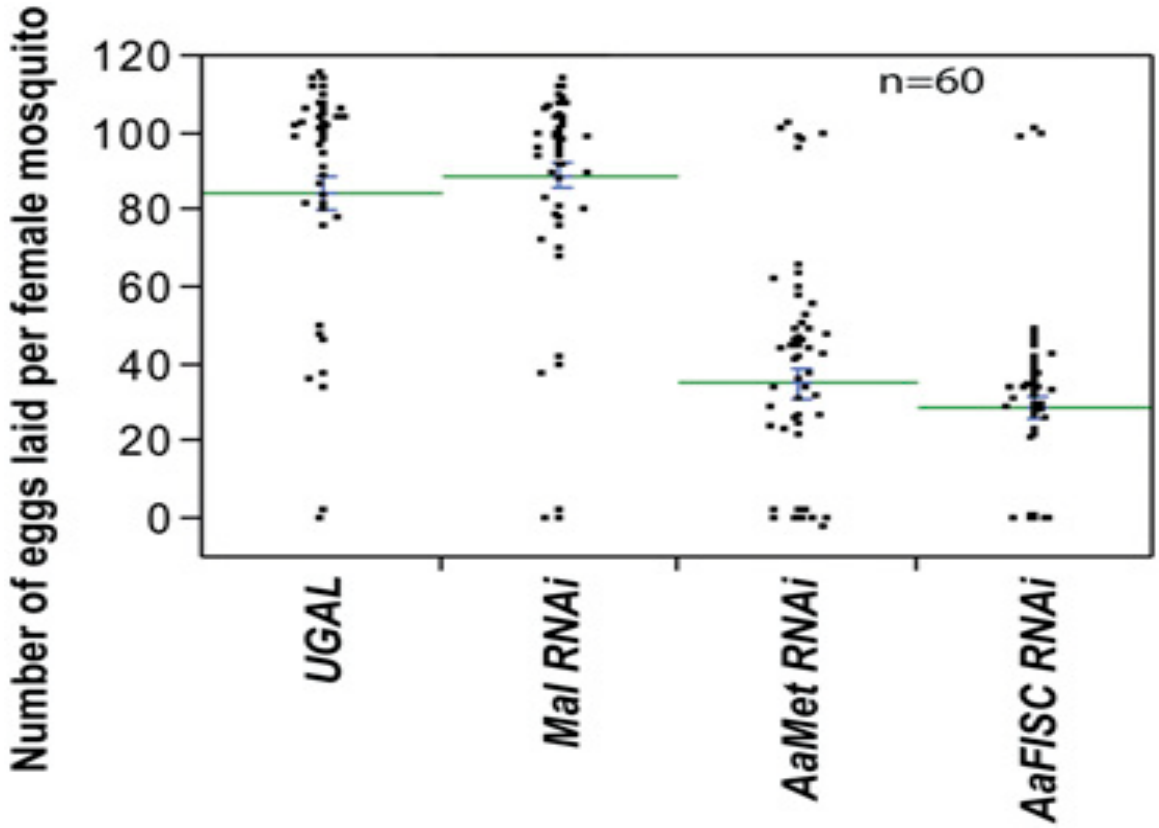
AaMet-AaFISC and AaMet-AaMet interactions detected in yeast two-hybrid assays. The indicated plasmids were cotransformed into AH109 and plated on SD/-Trp/-Leu/-His/-Ade/Xα-Gal plates containing either 10^{-6} M methoprene or DMSO (solvent) only. After ~5d, blue colonies appeared on the plate.

Figure 2.S2



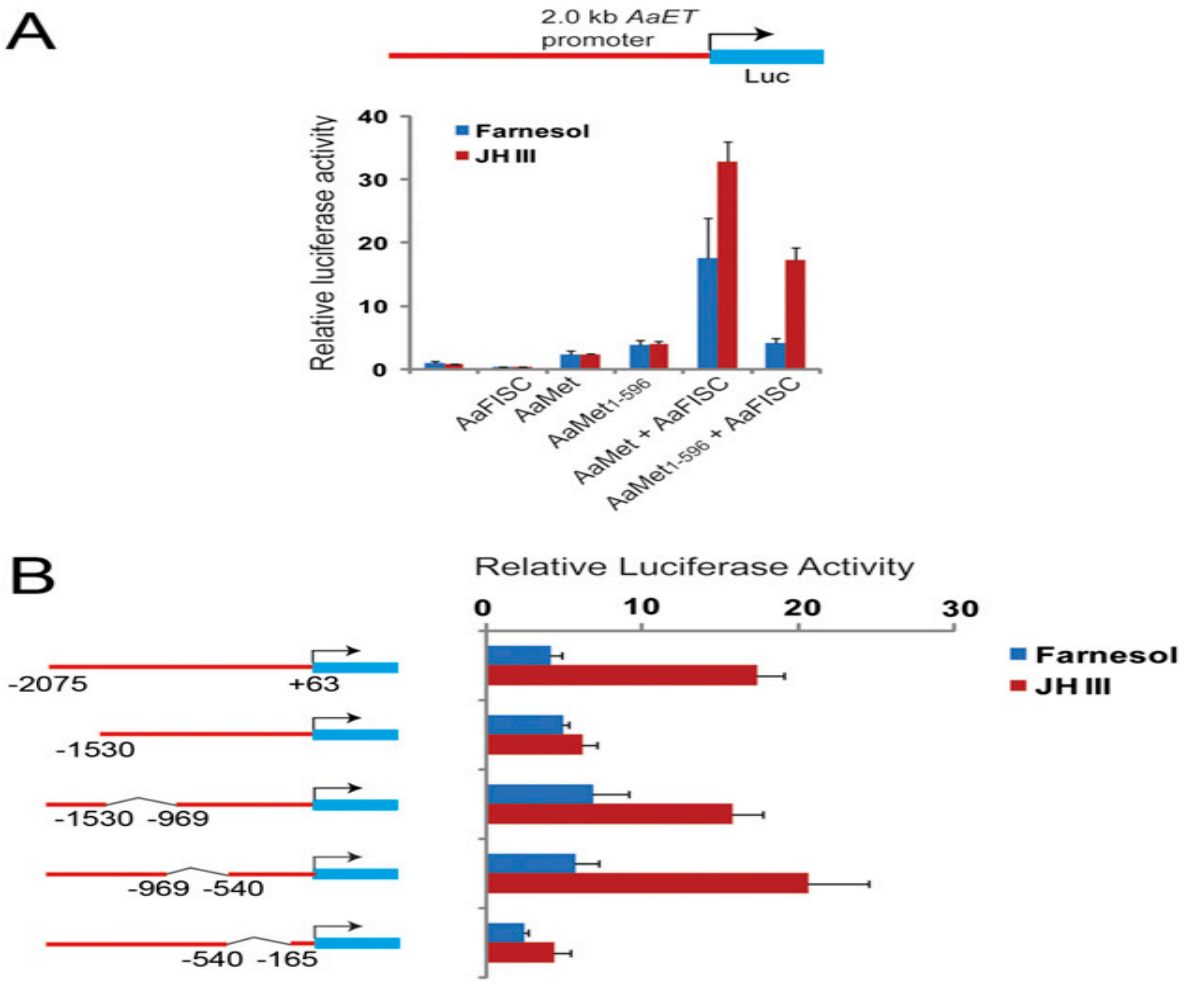
Depletion of AaMet and AaFISC in adult mosquitoes by RNAi. Newly emerged adult female mosquitoes were injected with dsRNAs corresponding to *AaMet*, *AaFISC*, or bacterial *MalE* gene. Uninjected (*UGAL*) mosquitoes were also used as control. Then, 4 d after injection, midguts were collected from the mosquitoes. Protein extracts were analyzed by immunoblotting.

Figure 2.S3



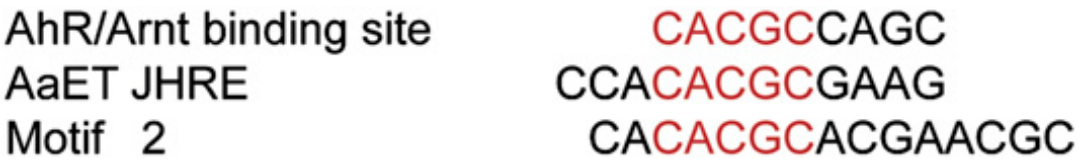
RNAi-mediated knockdown of AaMet and AaFISC decreases egg deposition. Newly emerged adult female mosquitoes were injected with dsRNAs corresponding to *AaMet*, *AaFISC* or bacterial *MalE* gene. Dots represent egg counts for individual mosquitoes within 10 d after the first blood meal. Green bars represent median number of eggs oviposited from three replicates; short blue bars indicate SEs. AaMet-and AaFISC-depleted mosquitoes lay significantly fewer eggs ($P < 0.001$) than Mal RNAi mosquitoes and untreated control mosquitoes (*UGAL*). Data were analyzed using JMP8 software.

Figure 2.S4



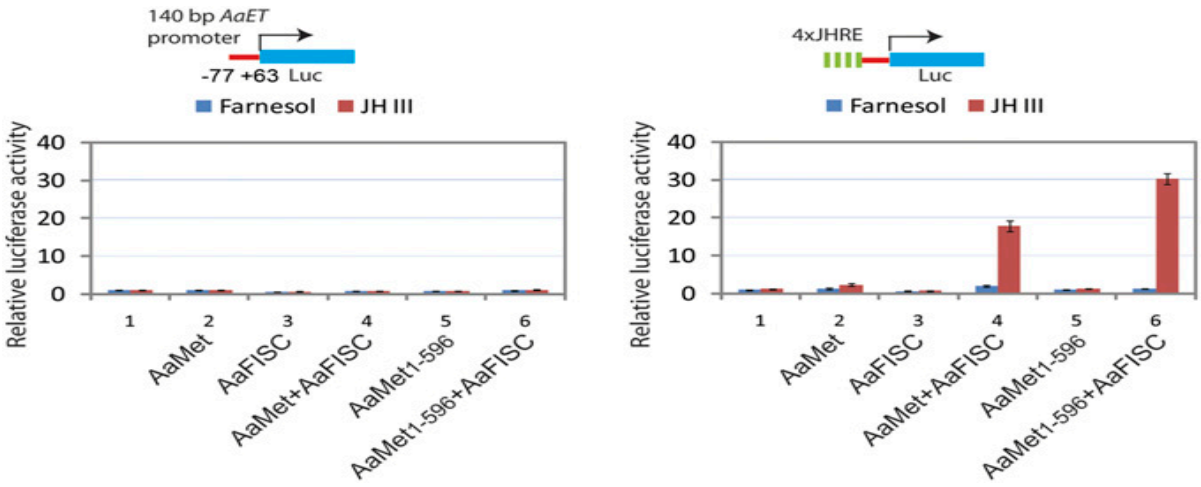
Functional analysis of *AaET* promoter. (A) The 2.0-kb upstream regulatory region of *AaET* was cloned into the pGL3 basic vectors. L57 cells were transfected by the reporter plasmid and expression vectors for the indicated proteins. After transfection, cells were cultured in medium with 5×10^{-6} M JH-III or farnesol. (B) L57 cells were transfected by the expression vectors for AaMet and AaFISC, together with the indicated derivative reporter constructs. Activity of reporter gene was measured by dual luciferase reporter assay.

Figure 2.S5



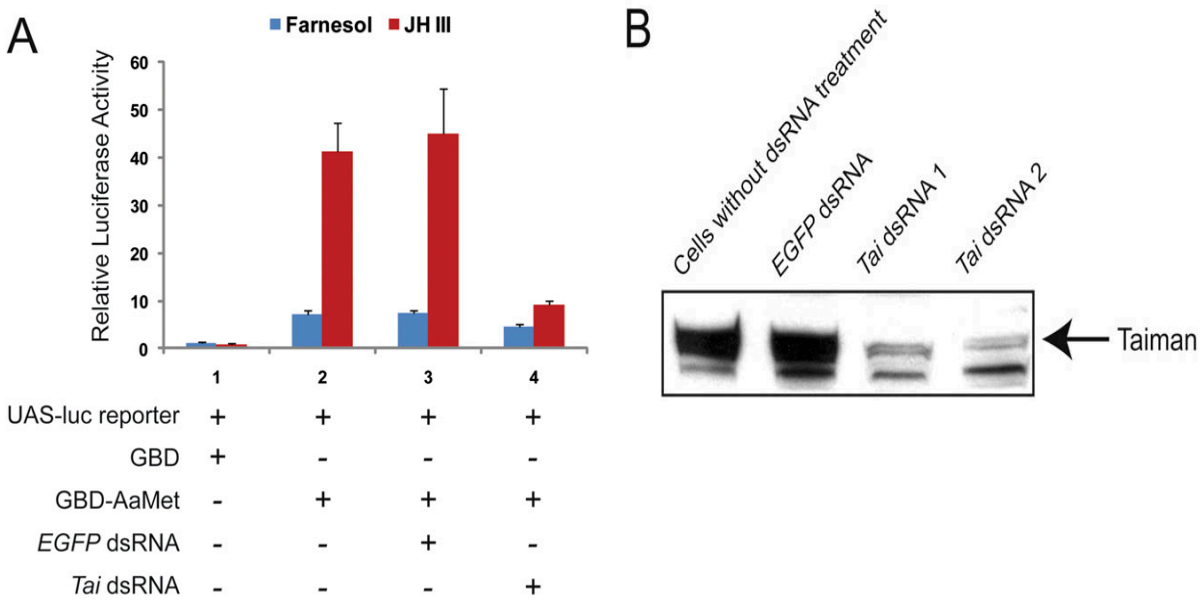
Sequence alignment of JHRE and an AhR/Arnt binding site. AhR/Arnt is a mammalian bHLH-PAS dimer that activates the transcription of a battery of genes encoding proteins involved in xenobiotic metabolism. AhR/Arnt binding site (5′ GCGTG 3′) is from version 8.3 of TRANSFAC. JHRE sequence is also similar to motif 2 identified in JH -inducible promoters in the *Drosophila* L57 cells and in the honey bee, *Apis mellifera* (Li *et al.*, 2007).

Figure 2.S6



JH response of *AaET* core promoter. Two reporter genes were constructed using the pGL3 basic reporter vector. Thefirst gene contains a 140 -bp (nt −77 to +63) core promoter of *AaET*; the second gene carries four copies of JHRE in addition to the core promoter sequence. L57 cells were transfected by the expression vectors for AaMet and AaFISC, together with one of the reporter constructs. After transfection, cells were cultured in medium with 5×10^{-6} M JH-III or farnesol.

Figure 2.S7



Taiman in L57 cells affects the transactivation function of AaMet. L57 cells were diluted to 2 × 10^6 cells/mL in serum-free medium. A 100μL quantity of suspension was mixed with 38 nM dsRNA corresponding to *EGFP* or *DmTaiman* and transferred to a single well of a 48-well cell culture plate. After 1 h incubation at room temperature, 200 μL medium containing 7.5% FBS was added to the cells. Three days later, the cells were transfected by the UAS×4–188-cc-Luc plasmid together with the indicated expression vectors (A). After transfection, cells were cultured in medium with 5 × 10^{-6} M of JH-III or farnesol. Dual luciferase assays were performed to measure the reporter activity. Depletion of Taiman was confirmed by Western blot analysis (B). Polyclonal antibodies against Taiman were a kind gift from Denise J. Montell. Tai dsRNA 1 and Tai dsRNA 2 represent samples from two independent biological replicates.

Chapter 3

FISC acts as the DNA-binding partner of methoprene-tolerant in juvenile hormone signaling

3.1 Abstract

JH governs diverse events in insect development and reproduction. Methoprene-tolerant (Met) protein is a nuclear receptor of JH. We have previously reported that JH-bound Met recruits another protein, FISC, to form an active JH receptor complex to regulate JH-responsive genes. However, the molecular mechanism of the binding of the Met-FISC complex to the target promoters remains elusive. Here we show that FISC is the obligatory partner of Met in binding to JH-response elements (JHREs). Purified recombinant Met and FISC proteins are used in gel-shift assays. Met and FISC together bind a previously identified JHRE in a JH-dependent manner, while Met or FISC cannot do so alone. Mutagenesis analysis of the two basic helix-loop-helix (bHLH)-PAS proteins suggests that both Met and FISC directly bind DNA and their basic regions in the bHLH domain are in direct contact with DNA. Furthermore, employing an in vitro selection and amplification method, we identified a consensus sequence recognized by Met and FISC. The core sequence CACGTGC, is similar to the JHREs that have been previously discovered in several JH target genes.

3.2 Introduction

JH plays critical roles in regulating a great diversity of events in insect development and reproduction (Riddiford, 1994). During development, JH has a “status quo” effect that maintains larva status of an insect. It disappears from insect hemolymph at the end of the larval stage, allowing metamorphosis to occur. Reappearance of JH in adult insects is required for reproduction in most insects. In some insects, JH also regulates various other physiological

processes, such as diapauses. It is usually required for maintaining larval diapauses while rising titers of JH terminates adult diapauses (Danks, 1987).

Met protein is a nuclear receptor of JH. Met has been shown to mediate many JH functions. Null-mutation of the *Met* gene in *Drosophila melanogaster* renders flies resistant to toxic effects of topically applied JH III or a chemical analog, methoprene (Wilson *et al.*, 1986). RNAi knockdown of *Met* in the red flour beetle, *Tribolium castaneum* causes precocious development of mature traits, which is phenotypically similar to premature development in JH-deficient insects (Konopova *et al.*, 2007; Tan *et al.*, 2005). Met is located in the nuclei of tissues regulated by JH (Pursley *et al.*, 2000). Sequence analysis shows that Met is a bHLH-PAS family protein that consists of three conserved domains, an N-terminal bHLH domain followed by two PAS domains, PAS A and PAS B (Ashok *et al.*, 1998). *In vitro* synthesized Met binds JH with relatively high affinity through a binding pocket formed by the PAS B domain (Charles *et al.*, 2011; Miura *et al.*, 2005). Mechanism studies show that Met is essential for the induced expression of JH-responsive genes, such as *kr-h1* in several insect species and *AaET* in the yellow fever mosquito *Aedes aegypti* (Li *et al.*, 2011; Minakuchi *et al.*, 2009; Zhang *et al.*, 2011; Zhu *et al.*, 2010).

Met is a bHLH-PAS domain protein. Proteins in this family typically function as either homodimer or heterodimer formed with another bHLH-PAS protein (Gu *et al.*, 2000). Our previous work finds that another bHLH-PAS family protein, FISC, forms a functional complex with JH-bound Met to regulate expression of *AaET*. Injection of FISC dsRNA down-regulates expression of *AaET*. When JH titer is high to induce expression of *AaET in vivo*, both Met and

FISC associate with the proximal promoter region of *AaET*, as shown in ChIP-qPCR result. Furthermore, Met and FISC can activate gene expression via a JHRE identified from promoter of *AaET* (Li *et al.*, 2011). The requirement of FISC in JH signaling has been confirmed by subsequent studies. RNAi knockdown of FISC in mosquito *Aedes aegypti* Aag2 cell line reduces JH-induced expression of *kr-h1* (Zhang *et al.*, 2011). FISC and its *Drosophila* ortholog, TAI, have been previously characterized as steroid receptor coactivators (SRCs). They function as coactivators of the ecdysone receptor (EcR) and they are required for gene expression induced by ecdysone (Bai *et al.*, 2000; Zhu *et al.*, 2006). Although possessing a putative DNA-binding domain, SRCs are characterized as transcription coactivators, interacting with transcription factors and recruiting downstream effectors including histone acetyltransferases and protein methyltransferases (Xu *et al.*, 2009). Although Met/FISC complex is shown to be essential for expression of JH-responsive genes, it is not clear how the complex is recruited to the target promoters and whether Met and FISC directly binds DNA. Several models have been proposed that either FISC does not directly bind DNA but functions as a coactivator of Met or another unidentified protein is actually required for the DNA binding of Met (Kayukawa *et al.*, 2013; Riddiford, 2013). The molecular role of FISC in JH action is not clearly defined.

JH response elements (JHRE) are also not well-characterized. A few JHREs have been identified in the past few years (Kayukawa *et al.*, 2012; Kayukawa *et al.*, 2013; Kethidi *et al.*, 2004; Li *et al.*, 2011; Li *et al.*, 2007; Shin *et al.*, 2012; Zhou *et al.*, 2002a; Zou *et al.*, 2013). A subset of these JHREs contains E box-like sequences, which are typically bound by dimer of bHLH proteins. Our previous study of the *AaET* promoter in *Aedes aegypti* revealed a JHRE, which contains an imperfect palindrome E-box, CACGCG. Kayukawa *et al.* identified a perfect

palindrome E-box, CACGTG, from the promoter and first intron of the *Bombyx mori kr-h1* gene (Kayukawa *et al.*, 2012; Kayukawa *et al.*, 2013). A recent bioinformatic study of the upstream sequences of JH-regulated genes in *Aedes aegypti* found similar E box-like sequences in a small subset of genes (Zou *et al.*, 2013). However, binding of Met and FISC (or their orthologs) to these E box-like sequences is not confirmed. A comprehensive screening of sequences preferably bound by Met and FISC is lacking.

To investigate the role of FISC in DNA-binding by JH-Met-FISC complex, we carried out gel-shift assays with *E. coli*-expressed Met and FISC proteins, and with the JHRE from *AaET*. Results indicate that Met and FISC are required and sufficient for binding to JHRE, and both Met and FISC directly bind JHRE through their basic regions located in the bHLH domain. Furthermore, utilizing a selection and amplification protocol, we performed a comprehensive screening of sequences preferably bound by Met and FISC. A consensus sequence, GCACGTG, was found to bind Met and FISC with high affinity. Luciferase reporter assay in insect cells demonstrated that the consensus sequence was capable of conveying induction of gene expression by JH-Met-FISC, indicating that it is a functional JHRE. This study significantly advances our understanding of the JH-activated gene expression by Met and FISC in molecular details.

3.3 Materials and methods

Plasmids

pCMA-AaMet, pCMA-GBD-AaMet$^{1\text{-}596}$, pAc5.1-FISC, UAS×4-188-cc-Luc, pRL-CMV, and 4xJHRE-luc are described in Chapter 2.

4xMFBS-luc: similar to 4xJHRE-luc. Four tandem repeats of a MFBS-containing DNA fragment (5'-GCCGCACGTGTC-3') was ligated to an AaET fragment spanning -77 to +61 section by *Hin*d III. The chimerical fragment was inserted between restriction sites *Kpn* I and *Nco* I of the vector.

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described (Carrigan *et al.*, 2011). Primers containing point mutation were used in PCR to amplify full length of template plasmids. PCR product was cleaned up with PCR purification kit (Qiagen) followed by digested with *Dpn* I at 37^{o}C for 1 hour to remove template plasmid. The DNA was purified again and about 200 ng of the DNA was used to transform *E. coli* NEB 10-beta competent cells (New England Biolabs) following manufacturer's instructions. Primers for mutagenesis are shown below.

1) primers for mutagenesis of pCMA-Met and pCMA-GBD-AaMet$^{1-596}$

Mutation	Primer	Sequence (5'- 3')
Met-R119Q	Fwd	ATCCTAAATGGCCAGGAAGCGCGGAACCGAGCGGAGAAGAA
	Rev	GCTTCCTGGCCATTTAGGATTTTACAAGCAGC
Met-R122Q	Fwd	CAGAACCGAGCGGAGAAGAACCGCCGCGACAAACTTA
	Rev	CTTCTCCGCTCGGTTCTGCGCTTCCCGGCCATTTAGGA
Met-R124Q	Fwd	CGGAACCAAGCGGAGAAGAACCGCCGCGACAAACTTA
	Rev	CTTCTCCGCTTGGTTCCGCGCTTCCCGGCCATTTAGGA
Met-K127Q	Fwd	CGGAACCGAGCGGAGCAGAACCGCCGCGACAAACTTA
	Rev	CTGCTCCGCTCGGTTCCGCGCTTCCCGGCCATTTAGGA

Met-R129Q	Fwd	CAACGCGACAAACTTAACGGCTCCATACAGGAACT
	Rev	CCGTTAAGTTTGTCGCGTTGGTTCTTCTCCGCTCGGTT
Met-R130Q	Fwd	CGCCAAGACAAACTTAACGGCTCCATACAGGAACT
	Rev	CCGTTAAGTTTGTCTTGGCGGTTCTTCTCCGCTCGGTT
Met-K132Q	Fwd	CGCCGCGACCAACTTAACGGCTCCATACAGGAACT
	Rev	CCGTTAAGTTGGTCGCGGCGGTTCTTCTCCGCTCGGTT

2) primers for mutagenesis of pGEX-6P-1-Met$^{1-597}$:

The difference between this series of primers and the primers listed above is that the sequences of the Metr series of primers below correspond to the cDNA of Met with codon optimization for expression in *E. coli*, while the primers above correspond to wild-type Met sequence.

Mutation	Primer	Sequence (5'- 3')
Met-K132Q	Fwd	CGTCGCGATCAACTGAATGGCAGCATTCAGGAACT
	Rev	CCATTCAGTTGATCGCGACGGTTTTTCTCCGCGCGGTT
Met-R130Q	Fwd	CGTCAAGATAAACTGAATGGCAGCATTCAGGAACT
	Rev	CCATTCAGTTTATCTTGACGGTTTTTCTCCGCGCGGTT
Met-R129Q	Fwd	CAACGCGATAAACTGAATGGCAGCATTCAGGAACT
	Rev	CCATTCAGTTTATCGCGTTGGTTTTTCTCCGCGCGGTT
Met-K127Q	Fwd	CGTAACCGCGCGGAGCAGAACCGTCGCGATAAACTGA
	Rev	CTGCTCCGCGCGGTTACGAGCTTCGCGACCGTTCAGGA
Met-R124Q	Fwd	CGTAACCAAGCGGAGAAAAACCGTCGCGATAAACTGA

	Rev	TTTCTCCGCTTGGTTACGAGCTTCGCGACCGTTCAGGA
Met-R122Q	Fwd	CAGAACCGCGCGGAGAAAAACCGTCGCGATAAACTGA
	Rev	TTTCTCCGCGCGGTTCTGAGCTTCGCGACCGTTCAGGA
Met-R119Q	Fwd	ATCCTGAACGGTCAGGAAGCTCGTAACCGCGCGGAGAAAAA
	Rev	GCTTCCTGACCGTTCAGGATTTTACAGGCCGC

3) for mutagenesis of pAc5.1-FISC and pRSET A-FISC$^{1-609}$:

Mutation	Primer	Sequence (5'- 3')
FISC-K117Q	Fwd	AAATCAACCAGTGCAACAACGAGAAACGTCG
	Rev	GTTGTTGCACTGGTTGATTTGCGACTGTGGCTTA
FISC-K122Q	Fwd	AACGAGCAACGTCGCCGGGAGCTGGAGAATGAGTACATCG
	Rev	TCCCGGCGACGTTGCTCGTTGTTGCACTTGTTGATTT
FISC-R123Q	Fwd	AACGAGAAACAGCGCCGGGAGCTGGAGAATGAGTACATCG
	Rev	TCCCGGCGCTGTTTCTCGTTGTTGCACTTGTTGATTT
FISC-R124Q	Fwd	AACGAGAAACGTCAGCGGGAGCTGGAGAATGAGTACATCG
	Rev	TCCCGCTGACGTTTCTCGTTGTTGCACTTGTTGATTT
FISC-R125Q	Fwd	AACGAGAAACGTCGCCAGGAGCTGGAGAATGAGTACATCG
	Rev	TCCTGGCGACGTTTCTCGTTGTTGCACTTGTTGATTT

Protein purification

Met:

The codon usage of Met cDNA was optimized for bacterial expression (see supplemental information 1 for the optimized sequence). A cDNA fragment coding for amino acid sequence 1-

597 was cloned into expression vector pGEX-6P-1 (GE Healthcare) between restriction sites *Bam*H I and *Not* I, resulting in a expression plasmid for Met, pGEX-6P-1-Met$^{1-597}$. BL21(DE3) strain transformed with the plasmid was cultured in LB medium at 37^{o}C to approach OD_{600} of 0.6. The temperature was then changed to 25^{o}C. When OD_{600} reached 0.8, IPTG was added to a final concentration of 0.5 mM. Culture was harvested 3 hours after IPTG induction. Cell pellets were resuspended in lysis buffer (20 mM sodium phosphate, pH 7.3, 150 mM NaCl, 2 mM DTT, 1 mM PMSF, and 1×Halt protease inhibitor cocktail (Thermo Scientific)). Cells were lysed using DeBEE high pressure homogenizer (BEE international) and cleared by centrifugation at 30,000 × g for 30 min. Proteins in the supernatant were affinity-purified using AKTA prime and GSTrap FF column (GE Healthcare) at 4^{o}C with binding buffer (20 mM sodium phosphate, pH 7.3, 150 mM NaCl, 2 mM DTT) and elution buffer (50 mM Trhis-HCl, pH8.0, 10 mM reduced glutathione, 2 mM DTT). Purified protein was dialyzed in PBS buffer containing 2 mM DTT and 10% glycerol, and was stored at -80^{o}C.

FISC:

A cDNA region coding for amino acid sequence 1-609 of FISC was cloned into expression vector pRSET A (Invitrogen) between restriction sites *Bam*H I and *Kpn* I, resulting in plasmid pRSET A-FISC$^{1-609}$. Recombinant FISC was expressed as a His-tag fusion protein under the control of T7 promoter in *E. coli* BL21(DE3) pLysS strain. Bacterial cells were grown in LB medium at 37^{o}C. Temperature was lowered to 20^{o}C when OD_{600} reached 0.4. IPTG was added to a final concentration of 0.2 mM when OD_{600} reached 0.6. Cells were cultured for two more hours and were then pelleted by centrifugation. FISC was purified using AKTA prime and HisTrap FF column according to the standard protocol provided by GE Healthcare. Buffers used for FISC

protein purification are: lysis buffer (20 mM sodium phosphate, pH 7.4, 0.5 mM NaCl, 20 mM imidazole, 2 mM DTT, 1 mM PMSF, and 1×Halt protease inhibitor cocktail); binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 mM NaCl, 20 mM imidazole, 2 mM DTT); elution buffer (20 mM sodium phosphate, pH 7.4, 0.5 mM NaCl, 0.5 M imidazole, 2 mM DTT). Dialysis in PBS and protein storage was conducted as described above for Met.

Gel-shift assay

Double-stranded DNA oligonucleotides were end-labeled by T4 Polynucleotide Kinase (New England Biolabs) and [γ-^{32}P] ATP (PerkinElmer), followed by purification with Bio-Spin 6 column (Bio-Rad). For binding, 0.5 µg of purified Met, FISC, or both proteins was added to buffer solution (20 mM sodium phosphate (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, 100ng/µl BSA, 50 ng/µl poly(dA-dT), and 10 µM JH III or DMSO carrier). After 10 min incubation at room temperature, 20 fmol labeled probe (~20,000 cpm) was added to make a total volume of 20 µl. The solutions were incubated for 20 more minutes followed by electrophoresis at 120V for 50 min with a 6% polyacrylamide DNA retardation gel (Invitrogen) in 0.5x TBE buffer. The gel was dried and the ^{32}P-labeled DNA was visualized by autoradiography. For competition experiments, 50-fold molar excess of unlabeled specific or nonspecific competitor was added to buffer solution 10 minutes before addition of probe. In super-shift experiments, 3 µg of GST antibody, His tag antibody or mock IgG was added 20 minutes after addition of probe, and the solution was incubated for an additional 20 minutes before electrophoresis.

In vitro selection and amplification of DNA binding site

Screening for DNA binding site was modified from a method described previously by Swanson *et al.* (Swanson *et al.*, 1995). A single-stranded DNA library, 5'-CCACCAACAACAACATCAGC-$(N)_{17}$-CTTCCGATGGATACTGGAGG-3', was synthesized. It contained all possible 17-bp DNA sequences ($4^{17} \approx 1.7 \times 10^{10}$ different sequences) flanked by adaptor sequences. To generate double-stranded DNA, the single-stranded DNA library was annealed to a primer complementary to the 3' adaptor sequence, followed by DNA extension with Taq polymerase at 72 ^{o}C for 30min. The reaction products were resolved in 2.5% agarose gel and the double-stranded DNA was recovered. Purified DNA was end-labeled with [γ-^{32}P] ATP and T4 Polynucleotide Kinase, followed by purification as described above.

Gel-shift assays were conducted by incubating 0.5 μg each of purified Met and FISC, 1 ng of labeled DNA in 20 μl binding buffer (20 mM sodium phosphate, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 5 mM DTT, 100 ng/μl BSA, 100 ng of sonicated salmon sperm DNA (GE healthcare), and 10 μM JH III). After electrophoresis and autoradiography, shifted band was cut from the gel. The gel slice was placed in 200 μl water and kept in a shaker at 700 rpm at 4^{o}C overnight. Forty microliters of the eluent were used as DNA template for PCR amplification to generate an enriched pool of selected oligonucleotides for the next round of selection. A total of ten rounds of selection were conducted. After the last selection, DNA was cloned into pCR2.1 TOPO TA cloning vectors (Invitrogen) and subjected to sequencing analysis. Consensus sequence was identified by MEME algorithm (Bailey *et al.*, 2009).

Luciferase reporter assay

Reporter assay was performed as previously described (Li *et al.*, 2011). Briefly, about 0.5 million L57 cells were plated in each well of a 48-well plate. Transfection was carried out

according to the manufacturer's instructions with 2 μl cellfectin (Invitrogen) and 320 ng of DNA (100 ng of firefly luciferase reporter plasmid, 100 ng of each plasmid for expressing Met and FISC or empty control vector, and 20 ng of internal control plasmid pRL-CMV (Promega)). Hormones were added to the culture medium at 24 hours after transfection. Cells were harvested at 48 hours after transfection and reporter activity was measured using Dual Luciferase Assay kit (Promega).

Measuring the dissociation constant of binding of Met and FISC to DNA

The apparent equilibrium dissociation constants (K_d) for the binding of Met and FISC to JHREs were measured as described (Riechmann *et al.*, 1996). Gel-shift assays were carried out with a fixed amount of purified Met and FISC proteins (0.5 μg each) and increasing amounts of probes. Probes were used at six concentrations, 1 nM, 2.5 nM, 5 nM, 10 nM, 25 nM, and 50 nM. After gel electrophoresis, the bound and free probe was quantitated with a phosphorimager (Molecular Dynamics). The data were used to calculate the apparent K_d by the method of Scatchard (Scatchard, 1949).

3.4 Results

Met and FISC bind to JHRE as a complex

To investigate whether Met and FISC proteins are sufficient for binding to JHRE in the presence of JH III, we carried out gel-shift experiments with purified recombinant Met and FISC proteins. The bHLH-PAS domain of Met (amino acid 1-597) was expressed in *E. coli* as a fusion protein with a N-terminal GST tag. The bHLH-PAS domain of FISC (amino acid 1-609) was expressed with a N-terminal 6×His tag (Fig. 3.1A). After affinity purification, there was only one

major protein band corresponding to the 6×His-FISC fusion (Fig. 3.1C). For GST-Met fusion, we have tried many different purification procedures and conditions, and still could not completely separate two major proteins with size of approximately 93 kD and 60 kD (Fig. 3.1B). Mass spectrometry analysis showed that the 93 kD polypeptide was the expected GST-Met fusion, while the 60 kD protein was a derivative of GST-Met that lacked the C-terminal portion of the Met PAS domain. The mixture of the 93 kD and 60 kD proteins was used as GST-Met in subsequent DNA binding assays.

A 29-bp DNA fragment from the *AaET* promoter, containing a JHRE (CACGCG), was used as probe in the gel-shift assay (Fig. 3.2A). As shown in Fig. 3.2B, neither Met nor FISC alone was able to bind the *AaET* JHRE. The purified Met and FISC proteins together showed weak binding to JHRE. The binding was enhanced considerably when JH was added to the incubation mixture. DNA binding specificity was demonstrated by competition experiments. The binding was abrogated by addition of unlabeled *AaET* JHRE at 50-fold molar excess, but not by 50-fold excess of unlabeled nonspecific competitor. To verify that both Met and FISC were present in the observed DNA-protein complex, we performed a super-shift experiment. Antibodies against the GST or His tags were added to the DNA binding reactions. Addition of either GST antibody or His antibody resulted in formation of a larger DNA-protein complex while addition of non-specific IgG did not have similar effect (Fig. 3.2C), indicating that the purified Met and FISC proteins bind JHRE together.

The basic regions of both Met and FISC proteins are involved in DNA binding

The basic regions of bHLH proteins are usually involved in DNA binding, with the basic residues (i.e., Arginine and Lysine) often forming direct contact with DNA bases (Jones, 2004).

Seven and five basic residues exist in the basic regions of AaMet and FISC, respectively (Fig. 3). These residues are highly conserved among *Aedes aegypti*, *Drosophila melanogaster*, *Tribolium castaneum* and *Bombyx mori*. To test whether the putative DNA binding domains of both Met and FISC were required for their binding to JHRE, Met and FISC mutants were created by introducing point mutations in the bHLH domains to replace the basic animo acids individually with Glutamine, which is structurally similar to Arginine and Lysine but has no charge. The mutants were then tested for their abilities to activate JH-inducible promoters in transient transfection assays and their abilities to bind JHRE *in vitro*.

For reporter assay, plasmids expressing wild-type or mutant Met and FISC were transfected into L57 cells together with a firefly luciferase reporter driven by four copies of *AaET* JHRE. While some mutations had no or little effect, R122Q, R129Q, and R130R mutations in AaMet all led to a dramatic decrease in the JH-induced expression of the reporter gene (Fig. 4A). Likewise, K117Q, R124Q, and R125Q mutations in FISC also displayed a similar effect (Fig. 4B). The result implied that these basic residues played an important role in the binding of Met and FISC to JHRE. It is also possible that these mutations affect dimerization or transactivation activity of Met and FISC. To test the later two possibilities, we carried out another reporter assay, in which Met was expressed as a fusion to the Gal4 DNA binding domain (GBD) and the firefly luciferase reporter gene was under the control of the upstream binding sites for Gal4 (UAS). In this system, we expect Met and FISC to form a heterodimer in the presence of JH and use the GBD domain to bind UAS of the reporter gene. GBD-Met and FISC indeed activated expression of the UAS-driven reporter gene when JH was added to the culture medium after transfection. Mutations of the basic residues in Met and FISC showed no marked negative effect on the JH-induced reporter expression (Fig. 5), indicating that these basic

residues are not essential for dimerization and transactivation of Met and FISC. Therefore, these two reporter assays together suggest that the basic regions of both Met and FISC are required for binding of the Met-FISC complex to JHRE.

To test this hypothesis, we performed gel-shift assays with purified wild-type and mutant proteins of AaMet and FISC that were expressed in *E. coli*. There was a very good correlation between the reporter assay and the *in vitro* DNA-binding assay. Mutations in Met and FISC that reduced the JH-induced expression of 4×JHRE-Luc in the reporter assay, such as Met^{R122Q}, Met^{R129Q}, Met^{R130R}, $FISC^{K117Q}$, $FISC^{R124Q}$, and $FISC^{R125Q}$, also significantly diminished the binding of Met and FISC to *AaET* JHRE (Fig. 6). In conclusion, the results demonstrated that both Met and FISC directly bind JHRE through the basic regions of their bHLH domains.

Identification of consensus DNA sequence bound by the JH receptor complex

To identify consensus sequence bound by Met and FISC, we screened a random DNA library using a selection and amplification method (Swanson *et al.*, 1995). We synthesized a 57 nt DNA library containing a random set of 17 nucleotides flanked by PCR priming sequences. The double-stranded DNA was end-labeled with ^{32}P and incubated with JH III and purified Met and FISC in a gel-shift experiment. After electrophoresis and autoradiography, DNA was retrieved from the shifted band and amplified by PCR to obtain an enriched DNA pool for a second round of selection. After 10 rounds of selection, the enriched DNA was analyzed by DNA sequencing. Among the 70 sequences that we obtained, 34 were unique sequences. The consensus motif was identified by the MEME motif discovery algorithm as an E box-like sequence, GCACGTG (Fig. 7). This motif exists in 67 of the 70 sequences. For future reference, the consensus sequence was numbered from -4 to +3 as shown in Fig. 7B. The most abundant

sequence, GGCCGCACGTGTCGTTG, was chosen for further study and it was named MFBS1 (Met-FISC binding site 1).

To validate the binding selectivity, gel-shift assay was conducted using the purified Met and FISC recombinant proteins. As shown in Fig. 8A, Met and FISC were able to bind MFBS1 when JH was present. We went on introducing point mutations into the consensus motif and using the MFBS1 derivatives as probes in gel-shift assay. Individual point mutation at any position within the sequence CACGTG considerably decreased or abolished the binding of Met-FISC (Fig. 8A). Competition experiments with 5-, 10-, and 20-fold molar excess of unlabeled competitors with a different nucleotide base at the -4 position showed that DNA with a base G at the -4 position bound Met-FISC with the highest affinity (Fig. 8B). The results indicate that the consensus sequence is a true binding site of Met and FISC.

Met and FISC bind consensus sequence with high affinity

To compare the binding affinity of Met-FISC to MFBS1 and several naturally occurring E box-like JHREs, we measured the apparent equilibrium dissociation constants (K_d) of the binding of Met and FISC to specific DNA fragments. The K_d of specific protein-DNA binding was determined by a series of gel-shift assays with a fixed amount of Met and FISC proteins and increasing amounts of DNA probe. Free and bound DNA probes were quantitated and the data were used to calculate the apparent K_d. The gel-shift assays and calculation of K_d of Met and FISC binding to MFBS1 are shown in Fig. 9 as an example.

A second E box-like sequence was discovered in the promoter of *AaET*. It is named *AaET*-JHRE2 and the JHRE we previously found is named *AaET*-JHRE1. The K_d of binding of Met-FISC to these two JHREs and MFBS1 were measured. The Met-FISC complex showed the

strongest binding to MFBS1, with K_d at 5.8 nM. The K_d for AaJHRE2 is 13.4 nM, which is very close to MFBS1. The binding of Met-FISC to the JHRE of *AaET* was much weaker, with a K_d of 103 nM (Table 3.1). A difference within the E box region among the three DNA probes is at the +2 position, with a T in MFBS1 and AaJHRE2 and a C in AaJHRE1. To test whether the difference in affinity of the three DNAs to Met-FISC is due to the difference at this position, the K_d of mutant DNAs of MFBS1 and AaJHRE2 carrying T to C mutation and AaJHRE1 carrying C to T mutation was measured. Mutation of T to C in the MFBS1 and AaJHRE2 abolished binding to Met-FISC, indicating that T at this position is critical for binding to Met-FISC. However, the AaJHRE1 and its mutant carrying a T at the +2 position both bound Met-FISC, with similar low affinity. This suggests that the sequences flanking the E box also contribute to association with Met-FISC.

The consensus sequence is a functional JHRE

To test whether the consensus sequence isolated by *in vitro* screening actually functions as JHRE, we performed reporter assays in *Drosophila* L57 cells similar to those in Figure 2.4. A reporter was constructed with a luciferase reporter driven by four copies of a shorter version of MFBS1, GCCGCACGTGTC. The reporter was readily induced by JH III when the *Aedes aegypti* Met and FISC were over-expressed in L57 cells, indicating that the consensus sequence is recognized by Met and FISC and that it functions as a JHRE in cells (Fig. 10).

3.5 Discussion

Met and FISC are sufficient to bind JHRE

Our previous work has demonstrated Met and FISC play critical role in mediating JH-regulated expression of the *AaET* gene in the mosquito *Ae. aegypti*. The conclusion is supported by subsequent studies that both Met and FISC (or its ortholog) are required for expression of another JH-responsive gene, *kr-h1*, in *Ae. aegypti*, *T. castaneum*, and *B. mori* (Kayukawa *et al.*, 2012; Shin *et al.*, 2012; Zhang *et al.*, 2011). It has been shown that Met and FISC form a complex through their N-terminal bHLH-PAS domains in a JH-dependent manner and that the hormone-bound protein complex occupies the promoter of *AaET* when the gene is transcriptionally up-regulated by JH. Moreover, the JH-Met-FISC complex could activate gene expression through short DNA sequences, JHREs, in insect cell lines.

Although mounting evidence supports that Met and FISC are required for expression of JH-inducible genes, a recent study suggests that they are not sufficient to bind JHRE. In *Drosophila* S2 cells, full-length TcMet and TcSRC (*T. castaneum* ortholog of Met and FISC) activated a luciferase reporter gene that was under the control of JHRE from *B. mori kr-h1* (*k*JHREc) in a JH-dependent manner. However, in a similar experiment conducted in mammalian HEK293 cells only marginal activation of the reporter was observed. Since JH-dependent dimerization of the two proteins was observed in both cell lines, the functional difference of TcMet and TcSRC between insect cells and mammalian cells prompts the authors to postulate that additional factors intrinsic to insect cells are required for DNA binding.

To test whether Met and FISC are sufficient for DNA binding, we performed gel-shift assays with *E. coli*-expressed recombinant Met and FISC proteins. We demonstrate that *in vitro* binding of Met to JHRE requires the presence of FISC and JH, and Met and FISC are sufficient

for their binding to JHRE. The weak JH response in the aforementioned experiment in mammalian cells does not necessarily mean additional factors are required for DNA-binding of Met-FISC. Low transcription efficiency of the insect-derived promoter in mammalian cells could be the real reason.

Both Met and FISC directly bind DNA

FISC, as well as its *Drosophila* ortholog, TAI, has been previously characterized as a steroid receptor coactivator (SRC) of the p160 family. They function as a coactivator of ecdysone receptor (EcR), enhancing gene expression induced by ecdysteroids (Bai *et al.*, 2000; Zhu *et al.*, 2006).. Although possessing a putative DNA-binding domain, SRCs function as coactivators, interacting with transcription factors and recruiting downstream effectors including histone acetyltransferases and protein methyltransferases (Xu *et al.*, 2009). While a great deal of evidence has demonstrated that FISC forms a complex with Met and that both proteins are recruited to JHRE, there are several different views concerning whether FISC functions as a coactivator of Met or it directly binds JHRE together with Met (Kayukawa *et al.*, 2012; Riddiford, 2013).

To elucidate the molecular function of FISC in JH signaling, we explored whether the conserved basic helix-loop-helix region in FISC is a functional DNA binding domain. Mutation in the basic region of FISC led to a dramatic decrease in the binding of Met/FISC to JHRE and the JH-induced transactivation activity of Met/FISC, suggesting that the basic region in FISC forms direct interaction with JHRE and that FISC functions as an obligatory DNA-binding partner of Met in mediating gene regulation by JH.

Some bHLH-PAS family proteins can function as both transcription factors and transcription cofactors in distinct pathways. For example, the aryl hydrocarbon receptor nuclear translocator protein (ARNT) binds DNA together with aryl hydrocarbon receptor (AhR) to regulate gene expression in response to xenobiotics and acts as a coactivator of estrogen receptor in the absence of AhR (Lindebro *et al.*, 1995; Rüegg *et al.*, 2008). Here we show that aside from a transcription coactivator of EcR in ecdysone signaling, FISC functions as a transcription factor in the JH pathway. As FISC plays an important role in both ecdysone and JH pathways, it is intriguing to study whether FISC is involved in the crosstalk between the two pathways.

Other protein partners of Met

Met was previously reported to form homodimer and heterodimers with several proteins, such as GCE, EcR, and Ultraspiracle (USP) (Bitra *et al.*, 2009a). It is not clear whether these protein complexes occur *in vivo* and if so what their functions are. FISC is the first JH-dependent Met-interacting protein ever discovered and the functional importance of the dimer has been confirmed by several laboratories. A recent yeast two-hybrid screening identified another bHLH-PAS protein, Cycle (CYC), which interacts with Met when JH is present. The Met-CYC complex binds a JHRE found in *Aakr-h1* and the binding does not require FISC (Shin *et al.*, 2012). While the Met-Cycle complex regulates the expression of *kr-h1* in adult mosquitoes, knockdown of Cycle in *T. castaneum* does not affect *kr-h1* expression during development of beetles (Bitra *et al.*, 2009b). The evidence suggests that Met might form complexes with different protein partners at various stages in different insect species to mediate diverse JH functions.

JHREs

We have previously reported an E box-like JHRE discovered from the promoter of *AaET*. JH can induce gene expression via the JHRE and both Met and FISC are required. Similar JHREs have been identified from the promoters of *kr-h1* in several insect species and studies of these JHREs confirmed our results. Since only *AaET* and *Kr-h1* have been shown to be under the direct control of Met and FISC, it is not clear whether the E box-like sequence we have identified represents a typical binding site of Met/FISC. *In vitro* unbiased selection from random DNA library indicates that E-box-like sequences are indeed the DNA binding sites of Met and FISC. Several copies of the E-box-like sequences have been identified in the upstream regulatory regions of *AaET* and *Kr-h1*. Our study indicates that each individual JHRE in *AaET* has a distinct binding affinity to Met/FISC. The copy numbers and the spacing of the multiple JHREs may define the expression pattern of the target genes, activating the regulated genes precisely at the proper concentrations of JH.

Besides E box-like sequences, earlier studies discovered several very different JHREs. Two JHREs were discovered by mutagenesis analysis of the promoters of JH-responsive genes. Both of them contain sequences similar to steroid hormone response elements (Kethidi *et al.*, 2004; Zhou *et al.*, 2002a). Although JH can strongly activate expression of reporter genes that are driven by the two JHREs, it is unknown how JH functions via these JHREs and whether Met and FISC are involved. In an analysis of the promoters of JH-regulated genes common in fruit fly and honeybee, Li *et al*. found a 29-bp consensus motif that can be activated by JH III in *Drosophila* L57 cells. This JHRE is also different from E-box. Two proteins, FKBP39 and Chd64, were found to interact with each other and bind this JHRE. The two proteins also interact with EcR, USP, and Met (Li *et al.*, 2007). Although my current research focuses on Met, FISC,

and E box-like JHRE, these different groups of JHREs suggest that different mechanisms might be employed by JH to regulate gene expression.

Measuring the apparent K_d

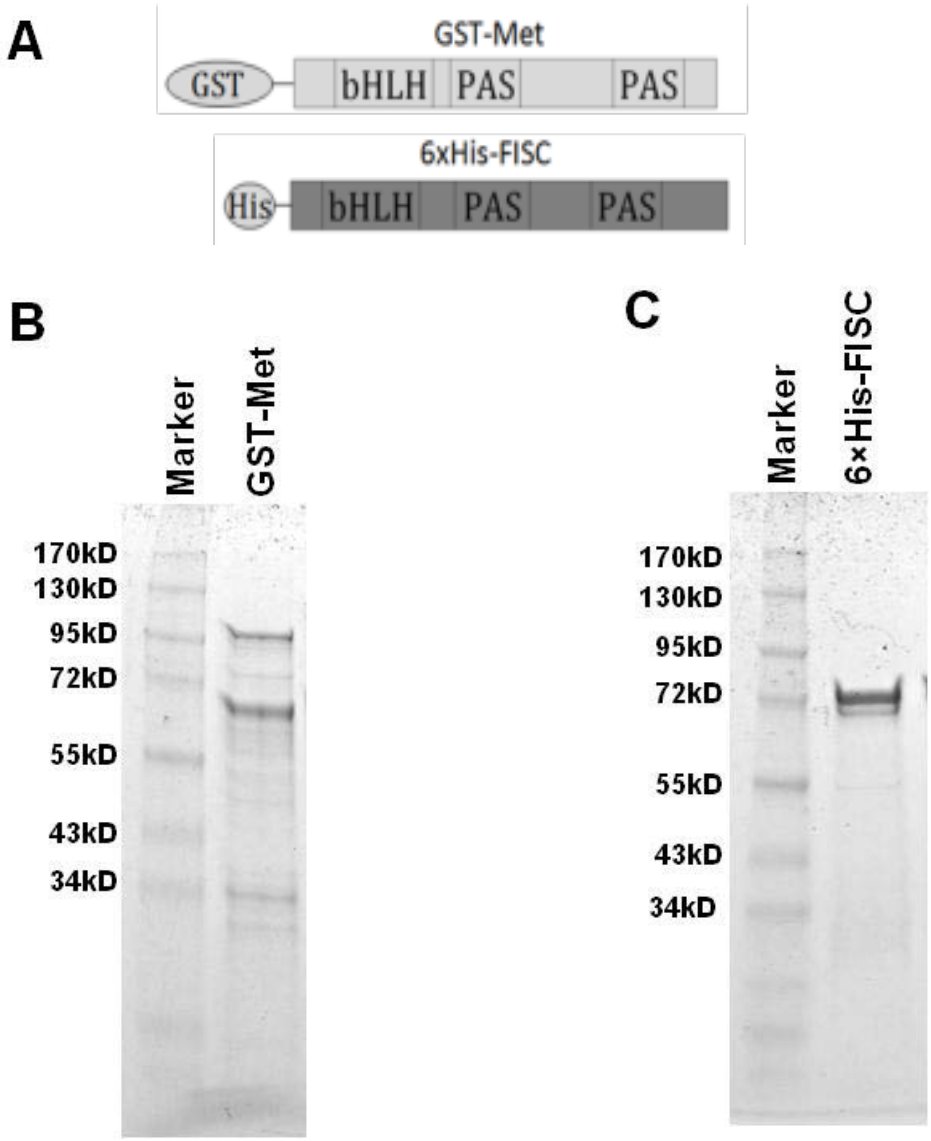
Several methods have been used to measure K_d of protein-DNA binding *in vitro*, such as gel-shift assay and fluorescence polarization. These experiments are usually conducted using a fixed amount of DNA probe and increasing amounts of protein. As the concentrations of protein increase, more DNA is bound by the protein, resulting in either more shifted band in gel-shift assays or higher fluorescence anisotropy in fluorescence polarization. K_d is determined as the concentration of the protein needed for half of the DNA probe bound by protein. These methods usually work well in situations where DNA is bound by a single unliganded protein. However, in the scenarios where DNA is bound by a protein complex or ligand-protein complex, the efficiency of forming active DNA-binding complex needs to be taken into account and these methods could overestimate the K_d. The method used in this body of work to estimate K_d of protein-DNA binding is to use a fixed amount of proteins and increasing concentrations of DNA probe. Although it is unknown how much active JH-Met-FISC complex is formed in the solution, the amount is expected to be constant in all solutions. In addition the method used to calculate K_d does not require knowing the exact amount of active DNA-binding complex. Therefore, this method should give a more accurate estimate of protein-DNA binding affinity.

3.6 Acknowledgment

We thank Dr. Richard Helm for his help with mass spectrometry.

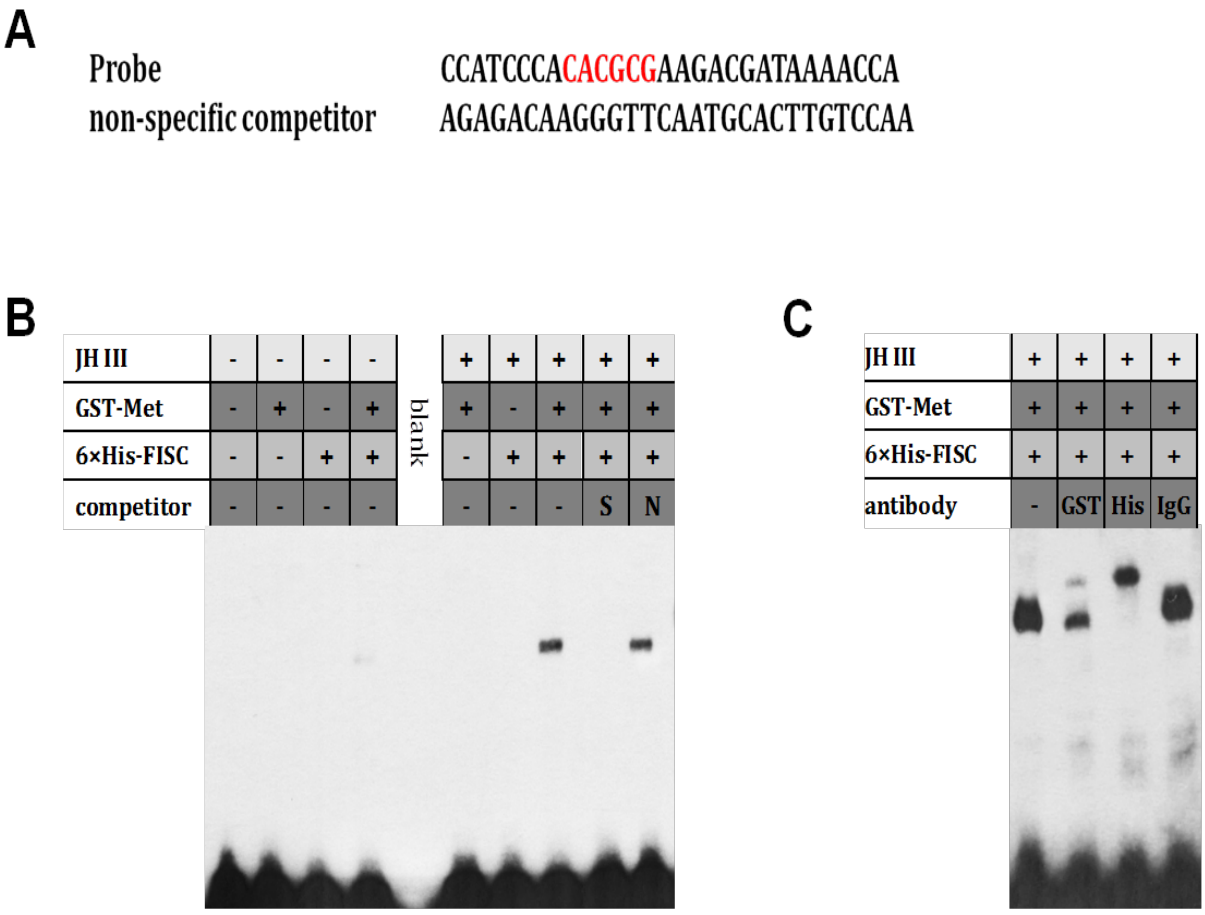
3.7 Figures and tables

Figure 3.1



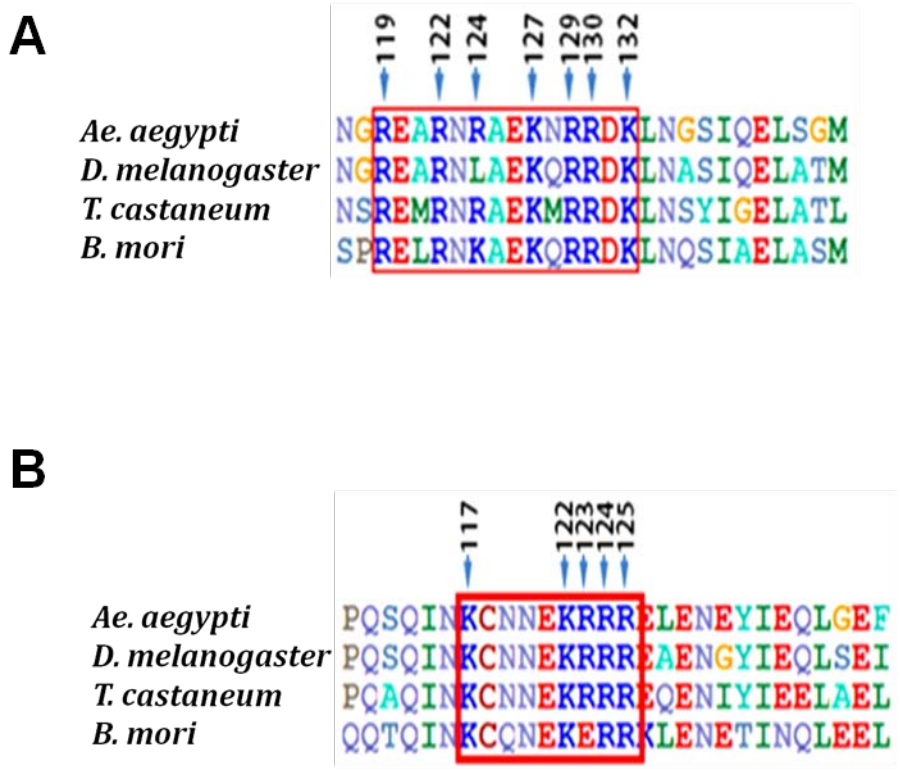
Purified recombinant Met and FISC proteins. A) Schematic representation of the GST-Met and 6×His-FISC fusion proteins. Purified Met B) and FISC C) were electrophoresized in 4-20% SDS-PAGE gels followed by Coomassie blue staining.

Figure 3.2



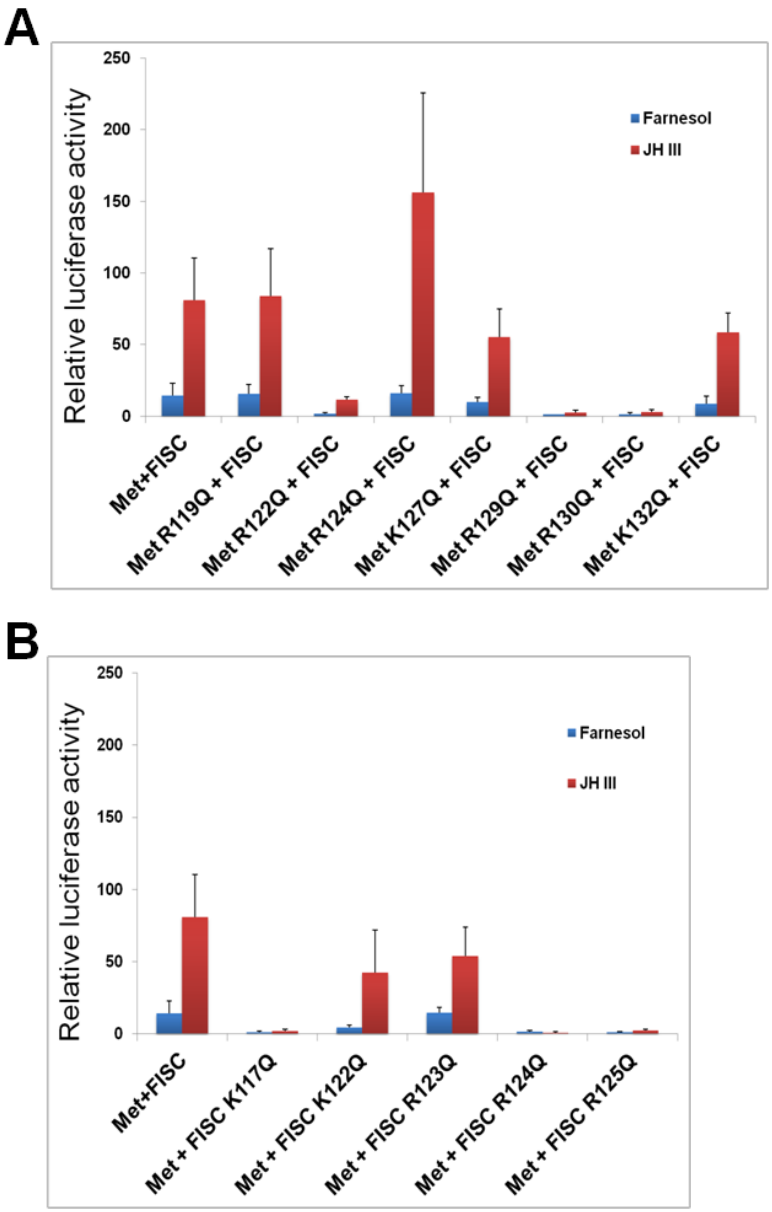
Purified Met and FISC proteins are sufficient for *in vitro* binding of JHRE. A) DNA probe and non-specific competitor used for gel-shift assay. The E box-like sequence in the probe is show in red. B) Gel-shift assay with the recombinant proteins. Met or FISC, or either protein alone, were incubated with the DNA probe in the presence or absence of 10^{-6} M JH III for 20 min followed by electrophoresis. For competition, 50-fold molar excess of unlabeled specific or non-specific competitor DNA, was mixed with proteins 20 min before addition of the probe. C) Super-shift assay. After incubating Met and FISC proteins with the DNA probe for 20 min, antibodies for GST tag or His tag were added to the reaction and the incubation was extended for 20 more min. Non-specific IgG was used in parallel as a control.

Figure 3.3



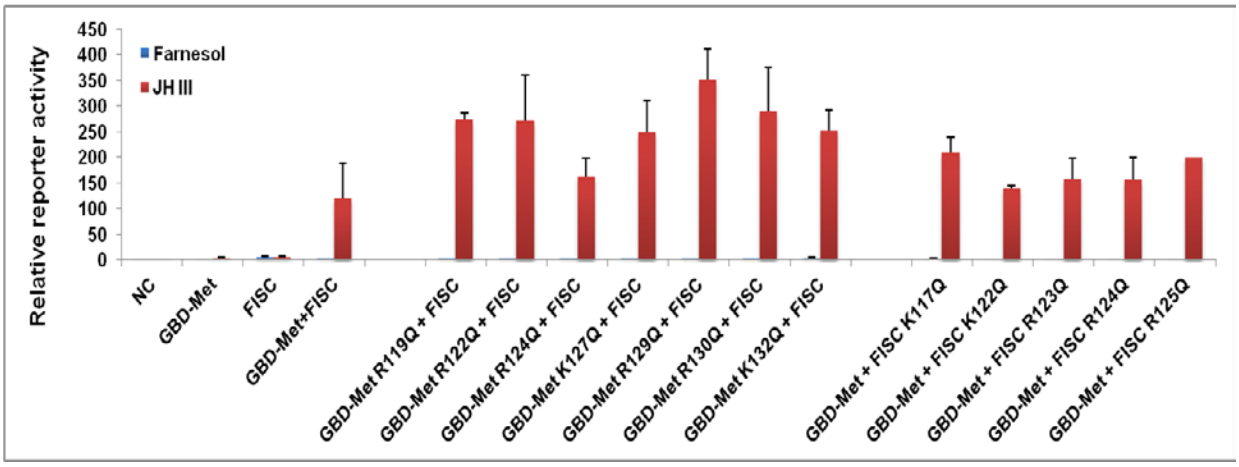
Sequence alignment of the first helix of the bHLH domains of Met and FISC. Sequences from four insect species, mosquito *Ae. aegypti*, fruit fly *D. melanogaster*, red flour beetle *T. castaneum*, and silkworm *B. mori*, were aligned using clusterW. Basic regions are shown in rectangles. Numbers indicate positions of the basic residues in the *Ae. aegypti* A) Met and B) FISC.

Figure 3.4



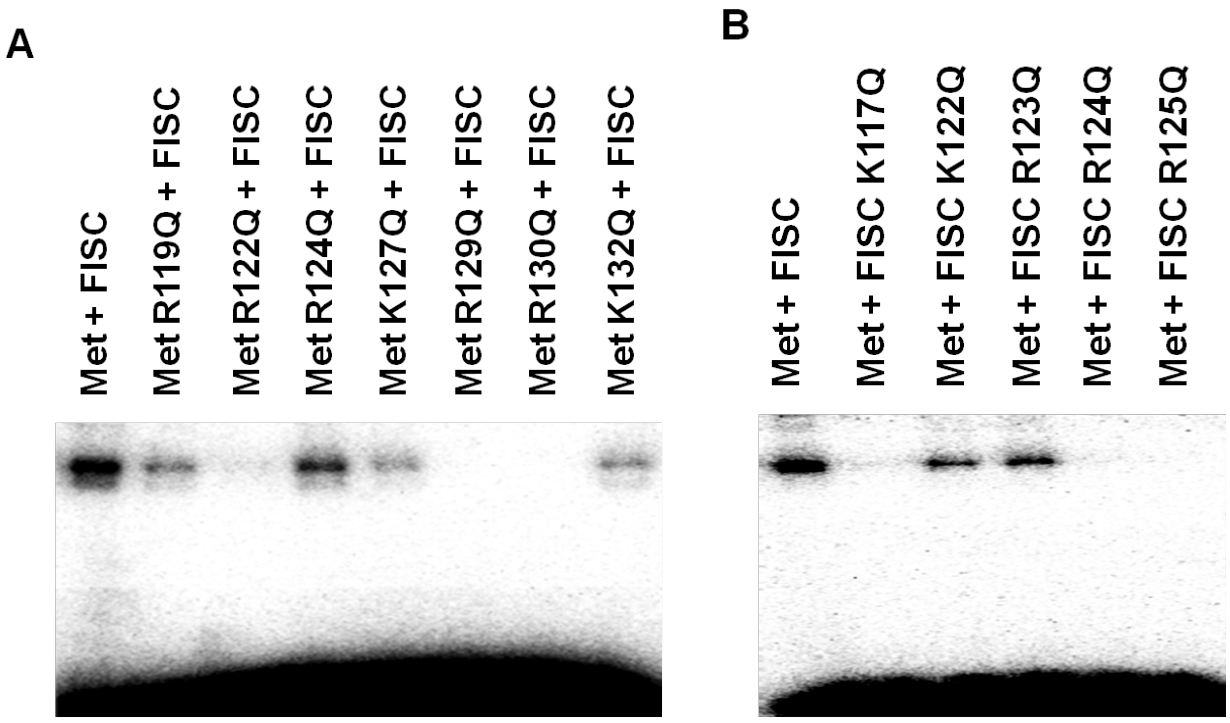
The basic regions of Met and FISC are involved in DNA binding. Luciferase reporter assays were conducted in L57 cells. Cells were treated with 5 µM JH III or farnesol control 24 hours after transfection. A) The 4×JHRE-luc reporter gene was activated by intact FISC and various derivatives of Met. B) The 4×JHRE-luc reporter gene was activated by intact Met and various derivatives of FISC. The error bars represent standard deviation, n=3.

Figure 3.5



Mutation of basic residues in the basic regions of AaMet and FISC has no negative effect on dimerization of the two proteins or on their JH-dependent transactivation activity. Plasmids expressing GBD-Met and FISC and the 5×UAS-luc reporter construct were introduced into L57 cells by transient transfection. Controls and mutants are as labeled. Cells were treated with 5 µM JH III or farnesol control 24 hours after transfection. The error bars represent standard deviation, n=3.

Figure 3.6



The basic regions of Met and FISC directly bind JHRE. A) Gel-shift assay with intact FISC and various derivatives of Met. B) Gel-shift assay with purified Met protein and various derivatives of FISC. Wild-type and mutants of Met and FISC are as labeled. In each experiment, the same amount of Met and FISC proteins were incubated with DNA probe in the presence of 10^{-6} M JH III for 20 min followed by electrophoresis.

Figure 3.7

A

ATCGGA AGGCACGTGCCA CCCCTCTGCT
TCAG CGGCACGTGTCA GCTTCTCTTC
TCAG CGGCACGTGCCA AACCTGCTTC
GGAAGGT CGGCACGTGCCA TGCGCTGA
GGA AGGCACGTGTCG ACATACGGCT
CGGA AGGCACGTGTCG CACTGGCTGA
GGA AGGCACGTGCCG AGTTGCCGCT
GAAGTGGTCC AGGCACGTGCCG CTGA
ACAT CAGCACGTGTCG CCCCCATCCC
ACAT CAGCACGTGCCG GGGCGGGGGC
GGAAG CCGCACGTGCCA ACTTCGCT
CGGAAGG GGGCACGTGTCA TACAGCTGAT
TCAGCGAAC ACGCACGTGCCG CCTTCC
GGA AGGCACGTGCAG CACAAGGGCT
GGAAG GGGCACGTGCCG GTGTGGCTGA
TCAG CGGCACGTACCA AACCTGCTTC
TCAGCTGGA CAGCACGTGTAG CCTTAC
ATCAGC ACGCACGTGTAA CTGACCTTCC
CCATCGG AAGCACGTACCA CGTAACCGCT
ACATCAG CAGCACGTATCA AACCTGCTTA
CTTCGT AAGCACGTGTCC TGGGGACGGC
ACATCAG CGGCACGTGACA CATGGGCTTC
TCAGCCGAAG CAGCACGTGCCC CTTCC
GGAAGTACCA CGGCACGTGTCT GCTGA
TCAGCGC GCGCACGTGCCG TCCCTTCC
CAGCTGTAGA AGGCACGTGCAC TTCCGAT
TCAGCGCGGC CGGCACGTGTGG CTTCC
ATCAGCCGGT GCGCACGTGTAG CCTTCC
GTAAGG CCGCACGTGGCG TTGGGCTGA
TCAGCCGAA GCGCACGTGCGA CCTTCC
GGAAGAT CGTCACATGCCA TGCGCTGA

In vitro selection of DNA sequences bound by Met and FISC. A) 31 unique sequences after iterative cycles of enrichment and amplification were analyzed by the MEME algorithm.

B) The top-scoring motif generated by MEME motif discovery algorithm (Bailey *et al.*, 2009). The common motif, GCACGTG, was numbered from -4 to +3.

B

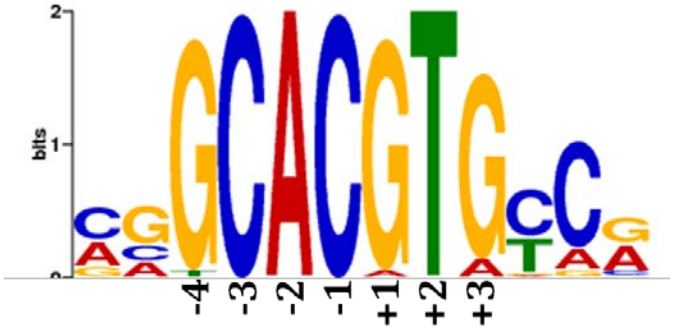
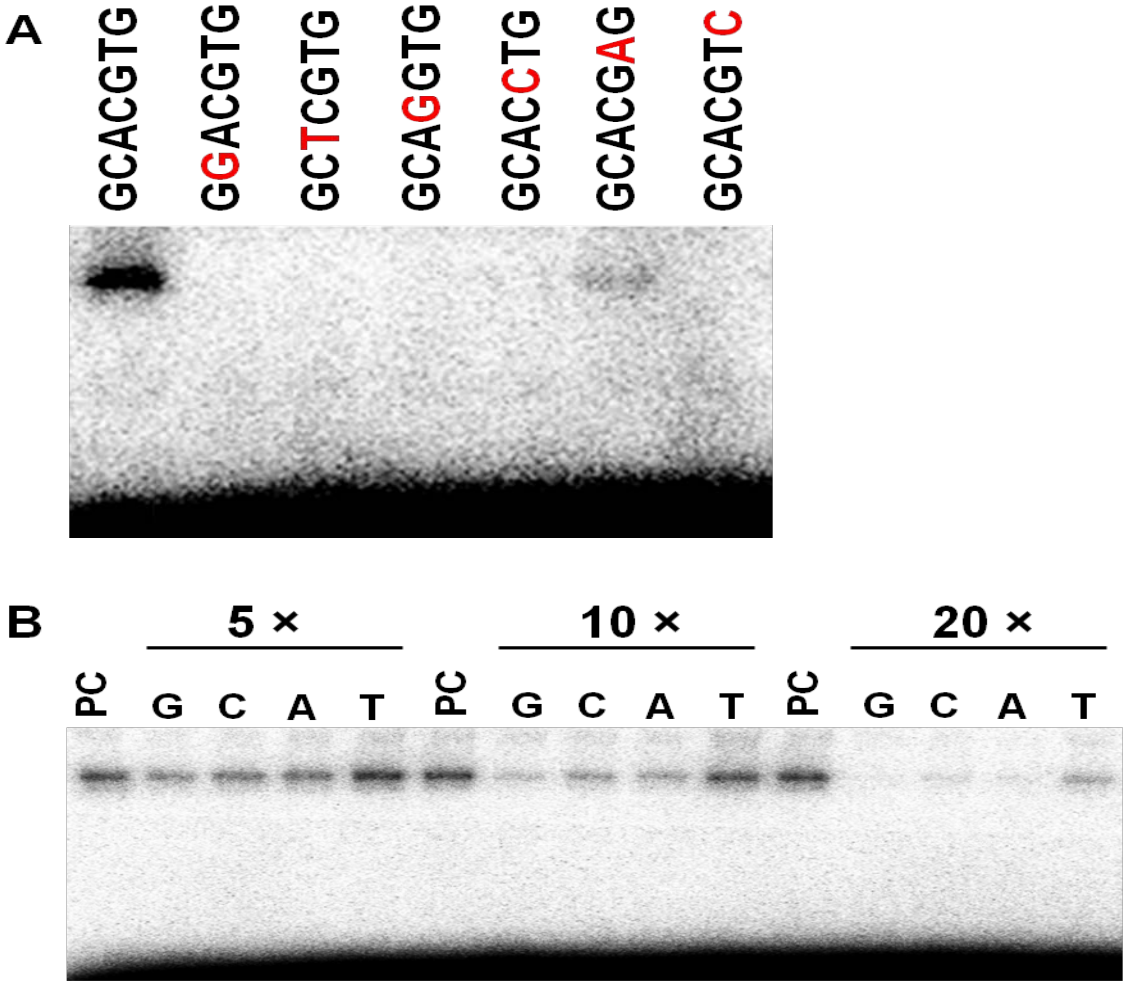
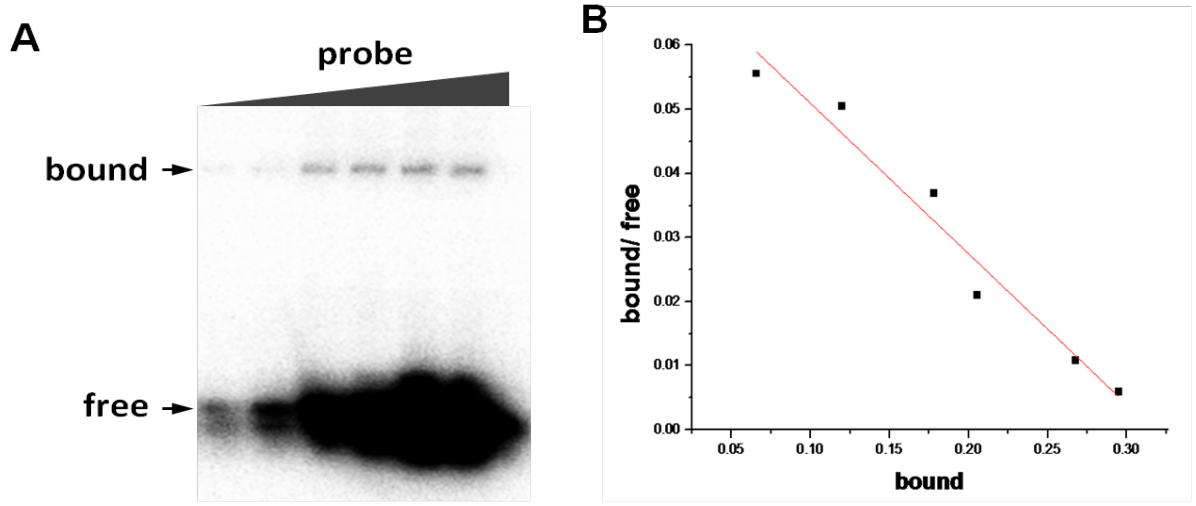


Figure 3.8



Validation of the consensus sequence. Gel-shift assays were conducted with purified Met and FISC proteins in the presence of 10^{-6} M JH III. A) MFBS1 and its mutants were labeled individually and used as probes in the experiment. Only the consensus sequence region is shown here. Point mutation in each mutant is shown in red. 2) Competition experiments were conducted with MFBS1 as probe. Competitors with G, C, A, or T at the -4 position in 5-, 10-, or 20-fold molar excess were added in solution 20 minutes before addition of probe. PC represents control without competitor.

Figure 3.9



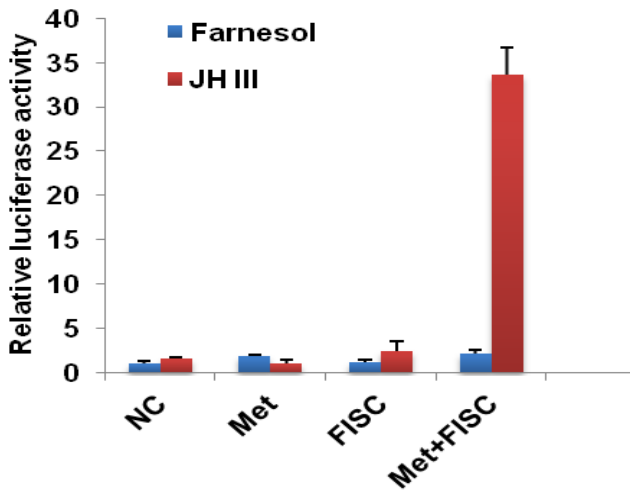
An example of determining apparent K_d of protein-DNA binding by gel-shift assays. A) Gel-shift assay was conducted with a fixed amount of Met and FISC proteins and with six increasing amount of DNA probe. Bound and free probe in each sample was quantitated. B) Data were plotted as bound probe/ free probe against bound probe. Linear regression was created with Origin software. K_d = -1/ slope. Three replicates were conducted for each probe.

Table 3.1

DNA probe	Sequence	Kd/ nM
MFBS1	GCCGCACGTGTCGTTGG	5.8±2.0
MFBS1 T+2C	GCCGCACGCGTCGTTGG	ND
AaJHRE2	ATCTGCACGTGTGTACC	13.4±6.3
AaJHRE2 T+2C	ATCTGCACGCGTGTACC	ND
AaJHRE1	GTCTTCACGCGTGGGAT	103±28
AaJHRE1 C+2T	GTCTTCACGTGTGGGAT	162±24

The consensus sequence is a preferred binding site of Met and FISC. K_d of the binding of Met/FISC to several DNA sequences were measured as shown in Fig. 9. The E box region is shown in red with the +2 position shown in blue. K_d was shown as mean±SD, n=3. ND represents not detectable.

Figure 3.10



The consensus sequence is a functional JHRE. A luciferase reporter driven by four copies of MFBS was transfected into L57 cells together with plasmids expressing Met and FISC or empty vector control. Transfected cells were either treated with 10^{-6} M JH III or Farnesol. Error bar represents standard deviation, n=3.

Chapter 4

Summary and Future Perspectives

4.1 Summary

The work described in this thesis is an effort to gain insight into the molecular mechanisms underlying gene regulation by JH. For the first time gene regulation by JH was shown to be mediated by a dimer of two bHLH-PAS proteins, Met and FISC, formed in the presence of JH. The work was conducted in mosquito *Ae. aegypti*, the yellow fever mosquito. Understanding the mechanisms of how JH regulates mosquito development and reproduction could help with future design of new insecticides for better vector control. Since JH signaling is highly conserved in insects, the knowledge gained from mosquito could also be applied to other insects.

Yeast two-hybrid screening using Met as bait revealed a JH-dependent dimerization partner of Met. The protein, FISC, was previously characterized as a steroid hormone receptor coactivator, which typically are recruited by transcription factors to enhance gene expression. To test whether FISC is involved in gene regulation by JH, RNAi experiments were conducted in mosquitoes. The JH-inducible gene, *AaET*, is activated by the rising titer of JH in the midgut of adult female mosquitoes. Depletion of FISC by injecting dsRNA reduced the mRNA level of *AaET* significantly, indicating FISC plays an important role in JH signaling. Luciferase assays in L57 cells showed that FISC can act together with Met to activate gene expression in a JH-dependent manner.

To examine how FISC is recruited by Met, two-hybrid assays were performed in L57 cells. The bHLH-PAS domains of Met and FISC showed protein-protein interaction in a JH-dependent manner. The interaction is specific as no interaction was observed between Met and

another bHLH-PAS protein, Tgo. Deletion mutations of Met and FISC provided some insight into the interaction between the two proteins. Deletion of either PAS A or PAS B domains of Met abolished the interaction, suggesting that these are the dimerization domain of Met. However, since the dimerization requires JH and the PAS B domain is the JH-binding domain, we could not rule out the possibility that the effect of deletion of PAS B domain is due to affecting JH-binding of Met. Deletion of the bHLH domain of Met led to a stronger signal in the assay than that of the wild-type Met, suggesting it is not essential for dimerization. This is surprising since in the canonical model of bHLH-PAS family proteins the bHLH domain is required for stabilizing protein dimmers. As for FISC, truncation of any of the three conserved domains affected its interaction with Met. Deletion of the bHLH domain had less effect than the PAS domains, suggesting that each domain contributes differently to the dimerization.

To investigate the roles of Met and FISC in expression of JH-inducible genes *in vivo*, RNAi experiments were performed in mosquitoes. In adult female mosquitoes injected with FISC dsRNA, expression of JH-inducible genes, *AaET* and *Aakr-h1*, in the midgut is dramatically reduced compared to that in control mosquitoes. The effect is similar to Met knockdown, indicating both Met and FISC are involved in gene regulation by JH.

To determine whether Met and FISC directly regulate expression of JH-inducible genes, ChIP experiments followed by qPCR were carried out to examine whether Met and FISC occupy promoter of *AaET* when the gene is transcribed. Analysis of the genomic DNA fragments co-immunoprecipitated with Met and FISC shows that the proximal promoter region of *AaET* is associated with Met and FISC, while neither the further upstream sequence nor the downstream sequence of *AaET* is occupied by the two proteins. In addition, the association was only observed at a time point when *AaET* was transcribed, but not at other time points when the mRNA level of

AaET stays low or is decreasing. The result suggests that *AaET* is directly regulated by Met and FISC by recruiting the proteins to its promoter.

A reporter assay in L57 cells was conducted to test whether Met and FISC could activate gene expression when recruited to the promoter. In the assay, Met and FISC could induce expression of the reporter gene driven by *AaET* promoter with JH present. Both proteins were required for the function as no reporter expression was observed when only one protein was present in the cells. The result is consistent with the RNAi experiments. Truncation of the *AaET* promoter in the reporter shows that deletion of the proximal promoter region diminished induction of the reporter by Met and FISC. This indicates that the two proteins are associated with this promoter region to activate gene expression, confirming our ChIP results.

An E box-like sequence, CACGCG, was found in the proximal promoter region of *AaET*. The sequence is similar to a previously identified DNA motif from upstream sequences of JH-inducible genes in *Drosophila*. To test whether the sequence functions as a JHRE, another luciferase reporter driven by four copies of short DNA fragment containing the E box-like sequence was constructed and tested in cell lines. The reporter was induced by JH when both Met and FISC were present, indicating that the sequence is a JHRE. The result also suggests that Met and FISC together bind to the JHRE to activate gene expression. When *Aedes* Met and FISC were replaced by *Drosophila* homologs, the reporter was also induced by JH, suggesting a conserved mechanism between mosquitoes and fruit flies. Association of Met and FISC with the JHRE was confirmed by gel-shift assays using a JHRE-containing DNA fragment as probe. A shifted band was observed when the probe was incubated with nuclear protein extract from adult female mosquitoes at the time point when *AaET* was transcribed and the band was abolished when either Met antibody or FISC antibody was added in the binding solution.

It has been suggested that other proteins besides Met and FISC are also required for gene regulation by JH. To determine whether Met and FISC are sufficient for binding DNA, gel-shifted assays were conducted using purified recombinant bHLH-PAS domains of Met and FISC. While neither Met nor FISC could bind the DNA probe containing the JHRE, the two proteins together are sufficient to bind the DNA probe when JH is present. Competition experiments demonstrate that the binding is specific as the binding was diminished by addition of excess unlabeled specific competitor DNA but not by non-specific competitor DNA. The super-shift experiment confirms that both proteins are required for DNA binding, as addition of antibody of either Met or FISC protein resulted in a super-shift band.

FISC was previously reported to function as a coactivator of a steroid hormone receptor. It is not clear whether FISC functions as a coactivator of Met or it directly binds DNA together with Met. To address the question, effects of mutation in the putative DNA-binding domains of Met and FISC were tested by reporter assays and gel-shift assays. The basic regions in the bHLH domains are typically responsible for DNA binding and the basic residues in this region could form direct interaction with DNA. Reporter assay with wild-type and mutant Met and FISC shows that mutation of some basic residues in the basic region of both Met and FSIC resulted in no induction of reporter. A modified reporter assay demonstrated that the mutation did not affect dimerization of Met and FISC or their ability to activate gene transcription. Therefore, the results suggest that mutation of these basic residues disrupted protein-DNA interaction. To confirm this, Met and FISC proteins carrying mutation of the basic residues were purified and tested in gel-shift assays. Consistent with the reporter assays, gel-shift assays show that mutation of these basic residues affected DNA-binding by Met and FISC. These results strongly support the

mechanism that both Met and FISC directly bind DNA and that DNA binding is through the basic regions of their bHLH domains.

A few JHREs have been reported so far. However, they are identified either from a single JH-inducible gene or from promoters, which are not experimentally confirmed, of a group of JH-inducible genes, many of which could be indirectly regulated by JH. A comprehensive screening would provide better insight into the consensus sequence bound by Met and FISC. *In vitro* selection of DNA sequences bound by Met and FISC resulted in a 7-bp consensus sequence containing a perfect palindrome E box, G<u>CACGTG</u>. The consensus sequence was validated by mutagenesis followed by gel-shift assay. Mutation of any position within the E box led to considerably weaker binding by Met and FISC. Competition experiments show that at the position outside the E box the G is preferred to other bases. Affinity of two naturally occurring JHRE and the consensus sequence to Met and FISC was compared by measuring their apparent dissociation constants. The result also confirms that the consensus sequence is the preferable binding-site of Met and FISC. In addition, it suggests that sequence outside the E box region also contribute to binding to the two proteins.

To examine whether the consensus sequence is a functional JHRE, a reporter driven by four copies of a short DNA fragment containing the consensus sequence was constructed and tested in L57 cells. In the experiment, the luciferase reporter is strongly induced by JH when both Met and FISC are present, indicating that the consensus sequence is capable of conveying response to JH. Searching in the promoters of *AaET* and *kr-h1* discovered the consensus sequence from both genes, suggesting that the consensus sequence is the true JHRE *in vivo* that recruits Met and FISC to activate gene expression when JH is present.

This body of work advanced our understanding in the molecular mechanisms of gene regulation by JH. Met had been shown to be a nuclear receptor of JH and it was implicated in mediating JH functions in regulating gene expression, yet the mechanism was poorly understood. We show here for the first time that this process requires additional proteins to form active hormone-protein complex. As the first protein partner of Met discovered, FISC has been demonstrated to be essential in regulating several JH-responsive genes. The function depends on JH-dependent dimerization of Met and FISC via their N-terminal bHLH-PAS domains. Dimerization brings the DNA-binding domains of the two proteins into close proximity. Together they bind to DNA, which contains an E box-like sequence, to activate gene expression.

4.2 Future perspectives

Protein partners of Met

FISC has been demonstrated to be a dimerization partner of Met in mediating gene regulation by JH during both insect metamorphosis and reproduction. RNAi experiments in adult female mosquitoes show that Cycle and Met also form protein complex when JH is present to regulate the expression of the JH-inducible gene *kr-h1* in a light-dependent fashion in the fat body (Shin *et al.*, 2012). Met, FISC, and Cycle are all bHLH-PAS family proteins, which usually function as dimers to regulate gene expression. Therefore it is intriguing to test whether other bHLH-PAS proteins could form complexes with Met in a JH-dependent manner. A genome-wide survey in *T. castaneum* identified eight additional bHLH-PAS proteins, including Tgo and Clock (Bitra *et al.*, 2009b). Just like Met and FISC, these bHLH-PAS family proteins probably are all conserved in insects. Protein-protein interaction with Met could be examined by two-hybrid assay in either yeast or insect cell lines. FISC and Cycle can be used as positive controls in the

experiments. Tgo is unable to interact with Met, therefore it can be used as a negative control. It is also interesting to further explore functions of the complexes formed by Met and different protein partners. Does Met recruit different partner to mediate specific JH functions? Is formation of the complexes stage- and tissue-specific?

Recent studies indicate that the mechanisms of JH signaling via Met and other bHLH-PAS proteins are more complicated. Expression of *kr-h1* in the fat body of adult female mosquitoes requires Met, Cycle, and FISC (Shin *et al.*, 2012). Similarly, in the linden bug *Pyrrhocoris apterus*, Met, Cycle, and Clock are all required for expression of genes that are involved in JH-induced termination of adult diapause, while the bug ortholog of FISC does not play a role in this JH function as shown in RNAi experiment (Bajgar *et al.*, 2013). It is unknown in these scenarios how three bHLH-PAS family proteins mediate JH signaling. Do they form large protein complexes, maybe together with even more proteins, or do they bind as dimers to different sites on a promoter to regulate gene expression synergistically?

In addition to bHLH-PAS family proteins, several proteins in the ecdysone cascade, such as EcR, USP, and FTZ-F1, have also been shown to interact with Met (Bernardo *et al.*, 2012; Bitra *et al.*, 2009a). Interaction of Met with EcR and USP is through the interface formed by the bHLH-PAS domains of Met, which is also involved in its interaction with FISC. However, interaction of Met with EcR and USP is JH-independent and it is unknown whether the complexes are functional in regulating gene expression (Bitra *et al.*, 2009a). Interaction of Met with FTZ-F1 does not involve the N-terminal bHLH-PAS domains. Instead, the interaction is through the C-terminal leucine-rich motifs of Met. Dimerization of Met and FTZ-F1 is JH-dependent and JH functions through the dimer to induce expression of *E75A* in *Drosophila* S2 cells (Bernardo *et al.*, 2012; Dubrovsky *et al.*, 2011). Since JH modulates ecdysone signaling

during insect development, the importance of the interactions between Met and the players in the ecdysone pathway needs to be examined *in vivo* to understand the crosstalk between JH and ecdysone signals.

Non-genomic effects of JH

Besides direct regulation of gene expression through nuclear receptors, many steroid hormones also have non-genomic effects on cells, which are characterized by rapid onset (within seconds to minutes), short duration (minutes to a few hours), and insensitivity to inhibitors of transcription and protein synthesis (Falkenstein *et al.*, 2000). Non-genomic effects of JH also have been documented. Pratt *et al.* observed more than 40 years ago that when JH was applied to dissected vitellogenic follicles of *Rhodnius prolixus*, large spaces appeared between the follicular epithelial cells (Pratt *et al.*, 1972). The shrinkage of the follicular epithelial cells and appearance of spaces between cells, called "patency", have also been observed in other insects, such as locust *Locusta migratoria* (Davey *et al.*, 1993; Pszczolkowski *et al.*, 2005a; Webb *et al.*, 1997). Patency can be induced by physiological concentration of JH in minutes and it does not require *de novo* macromolecular synthesis as inhibitors of transcription and translation do not affect the response (Abu-Hakima *et al.*, 1977a; Abu-Hakima *et al.*, 1977b), indicating that it is a non-genomic response to JH. Subsequent studies show that patency involves cytoskeleton reorganization, Ca^{2+} release, and protein kinase C (PKC)-mediated activation of JH-sensitive Na^{+}/K^{+}-ATPase (Abu-Hakima *et al.*, 1977a; Abu-Hakima *et al.*, 1979; Ilenchuk *et al.*, 1987; Pszczolkowski *et al.*, 2008; Sevala *et al.*, 1989). Although called non-genomic effects, in a broad sense, these effects also include regulated transcription and translation in response to hormones, which are, distinct from the traditional model of direct regulation via nuclear receptors, indirectly

regulated through intracellular signal transduction pathways (Meyer *et al.*, 2009). In this sense, JH-regulation of protein synthesis in the male accessory glands (MAGs) of *Drosophila melanogaster* has been proposed to be a non-genomic effect. The MAGs produce proteins that are components of seminal fluid and that are important for female reproductive behavior. Protein synthesis in the MAGs can be induced rapidly by JH *in vitro* and *in vivo*. The response requires calcium and PKC and it could be mimicked by activators of PKC, suggesting a membrane-mediated effect of JH (Yamamoto *et al.*, 1988).

Steroid hormones can elicit their non-genomic effects via membrane receptors. For example, a membrane G-protein-coupled estrogen receptor, GPER, mediates non-genomic effects of estrogen (Han *et al.*, 2013). It is unclear whether JH has membrane receptors. An experiment using pharmacological inhibitors shows that JH-induced patency functions via the inositol triphosphate/diacylglycerol signaling pathways, which are downstream of G protein-coupled receptors (GPCRs) (Pszczolkowski *et al.*, 2005b). This suggests that JH might bind GPCRs on the cell membrane. Moreover, a protein in the membrane preparations from vitellogenic follicle cells of locusts specifically binds JH III at high affinity. The size of the protein is about 35kD as estimated by SDS-PAGE electrophoresis, similar to the size of GPER. Membrane preparation from the MAGs of *R. prolixus* also specifically binds JH (Sevala *et al.*, 1995). However, there is currently no evidence linking the membrane proteins to any cellular responses. There are approximately 200 GPCRs in fly and mosquito genomes (Suwa *et al.*, 2011). A large scale RNAi screening might be useful to identify the JH-binding GPCRs. Large scale RNAi screening in insects might turn out to be impractical. However, if certain insect cell lines retain the non-genomic effects of JH, such as the JH-induced rapid calcium release or PKC

activation, cell lines could be a simpler system than animals for studies of the underlying mechanisms.

Estrogen also induces non-genomic effects via a membrane-associated estrogen receptor (mER), although the mechanisms remain elusive. It has been shown that the mER and the conventional nuclear receptor ER are encoded by the same transcript (Meyer *et al.*, 2009). Therefore it will be interesting to investigate whether Met has dual functions like ERs. Intracellular localization experiments show that Met is primarily a nuclear protein (Greb-Markiewicz *et al.*, 2011). However, current evidence from immunostaining and fluorescent microscopy do not rule out that a subpopulation of Met could be associated with cell membrane. In the case of mER, the number of mER is only 2%-3% of that of the nuclear receptor ER expressed in the same cells (Razandi *et al.*, 1999), which might not be readily visible by immunostaining and fluorescent microscopy. To test whether Met is a receptor of JH in mediating the non-genomic effects, RNAi could be conducted in cell lines or in insect animals to examine whether the JH effects could be diminished by the depletion of Met.

Function of Met in the absence of JH

In almost all JH functions examined, the functions of Met are in line with those of JH. An exception is the effects of JH and Met on programmed cell death (PCD) of *Drosophila* larval fat body. During metamorphosis, ecdysteroids induce expression of caspase genes, *Dronc* and *Drice*, which are involved in PCD in larval organs. As an anti-metamorphosis agent, JH inhibits this induced expression so that the caspase-dependent PCD only occurs during larval-pupal transition when JH concentration is low. Flies topically treated with JH show down-regulated expression of *Dronc* and *Drice* and a decrease in PCD. *Met*-overexpressing animals exhibit upregulation of the

caspases as well as enhanced PCD, which are suppressed by topical application of JH analogue (Liu *et al.*, 2009). As a unique observation in JH signaling, the underlying mechanism of JH counteracting Met in preventing PCD of larval fat body is unknown. The results suggest that Met has JH-independent functions. In fact, opposite functions of unliganded and lignaded nuclear receptors have been demonstrated in many endocrine pathways (Santos *et al.*, 2011). For example, thyroid receptor (TR) exhibits dual functions in frog development (Shi, 2013). When thyroid hormone is absent, TR recruits corepressor complex to repress gene expression. Upon binding thyroid hormone, TR releases the corepressor complex and recruits coactivator complex to activate gene expression. Similarly, while unliganded glucocorticoid receptor (GR) binds the *BRCA1* promoter to activate its transcription, the activation is abolished when glucocorticoid is present (Ritter *et al.*, 2012).

There are stages during insect life cycles when Met is expressed and JH is absent. It is possible that the unliganded Met plays a role in certain physiological events. Investigation of what proteins the unliganded Met interacts with and which genes are regulated by unliganded Met in those events will greatly advance our understanding of the functions of bHLH-PAS proteins in insects.

Appendix A

cDNA sequence of the *AaMet* bHLH-PAS domain with codon optimization for expression in *E. coli*

ATGAAAGAAGAATCAAATCCGAAACCGTCTCCGCCGACCTCTGGCTCTGGTAGTGGT
AACTCCTCGCTGCTGGAACGTGGTGCTGCGGTCGCGATCGTGGGCGGTAGTCAGATT
GCCGTTTTTCCGGGCGCATCCGGTCTGACCGCTCTGCAACGTCCGCCGCGCACGATT
AGTTGCACCTCCTATGATTCAGAATCGGATGACTGTATGCAGCGTAGCGGCCCGAGC
AGCGTGGCAACCACCACCGCAACCGAACCGGGTGAAAAACTGGATGCAAGCGCTTC
TGCGGGTAACAATAGTGCCTCCAAATACGATTGCGAAGACTCCAAAGCGGCCTGTA
AAATCCTGAACGGTCGCGAAGCTCGTAACCGCGCGGAGAAAAACCGTCGCGATAAA
CTGAATGGCAGCATTCAGGAACTGTCTGGTATGGTTCCGCATGTCGCGGAAAGCCCG
CGTCGCGTTGACAAAACCGCCGTCCTGCGTTTTTCCGCCCACGCACTGCGCCTGAAA
TATGTTTTCGATACGGAACAGGAACAAACCAAACAGGAACCGTCTGAAAACGCAGC
TGGCCAAAAAACCGAAGTGCATGATGCACTGTTTCGTATGCTGAACGGCTTTCTGCT
GACGGTCACCTGCCGCGGTCAGATCGTCCTGGTGTCAGCTTCGGTGGAACATTTCCT
GGGCCACTGTCAGACCGATCTGTACGGTCAAAACCTGTTCAATCTGATTCATCCGGA
TGACCACAACCTGCTGAAACAGCAACTGGTGCCGAACAATCTGGTGAACCTGTTTGA
TTCAGCTGTTAGCGCCCCGAGCACGTCTCGTACCCCGAGTGGCACGGAAACCTCCGC
GGAAGAACAGCAACGCAAATCACAGGATGAAGAAGACGAAATCGATCGTAAACTG
CGCCAAGATCGTCGCAAATTTACCCTGCGTATTGCTCGCGCGGGTCCGCGTTCTGAA
CCGACCGCGTATGAACTGGTGACCATCGATGGCTTTTTCCGTCGCGCGGATGCCGCA
CCGCGTGGTGAACGTCCGAGCGGCCCGAGCGGCCTGCAACTGCTGCGTCGCGCCCG
TGGCCGCGATGACGGTATTACCCTGCAAAGCATCAACGGCAATGACATTGTTCTGGT
CGCCGTGGCACGTGTGCAGAAAGTTCCGACGATCTGCGATCGTCTGATTGAAGCATG
TCGCTATGAATACAAAACCCGTCATCTGATTGACGGTCGCATCGTGCAGTGCGATCA
CCGCATCAGCGTGGTTGCGGGCTATCTGACGACCGAAGTTAGTGGTCTGTCCCCGTT
TACCTTCATGCACAAAGATGACGTGCGTTGGGTTATTGTCGCGCTGCGCCAGATGTA
TGACTACTCACAAAACTATGGCGAATCGTGCTACCGTCTGATGACGCGCACCGGCG
ATTTTGTTTATCTGAAAACCCGTGGCTATCTGGAAGTCGATGACAGTTCCAAAAAAG
TCCAGTCATTCGTGTGTATTAATACCCTGGTCTCGGACGAAGAAGGTCGTCGCCTGG
TGCGCGAAATGAAACATAAATTTAGCGTGATCGTTGAAGCCGATGAACTGCCGGAC
GAATCTGATGAACCGGCAGTGGAAAACCCGACGCAGATTGAAAAAGCTGTTCTGAA
CCTGCTGACCAATCTGCACAGCGAAGATGACGAACCGTCTGAACGCGCACTGCCGT
CAAATACGTCGCAGGAAACCGATGGTAGCGAAGGCTCTCAACTGGCAATCATCGCC
CCGTCCTCAAAAGCGGTGAAATCGGCAATCGTGAAA

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