Molecular Mechanisms Underlying Juvenile Hormone (JH) Signaling Pathway

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ABSTRACT

Juvenile hormone (JH) is an important insect hormone that controls diverse biological processes in postembryonic development and adult reproduction. JH exerts its effects through the nuclear receptor Methoprene-tolerant (MET). MET is a transcription factor of the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family. In the presence of JH, MET forms a heterodimer with its DNA-binding partner Taiman (TAI). The MET-TAI complex directly binds to the regulatory regions of some JH target genes and regulates their transcription. However many questions remain unanswered regarding the JH-regulated gene expression. The work in this report aims to determine the role of protein kinase C (PKC) in JH signaling in adult mosquitoes and to find the direct target genes of Krüppel homolog 1 (Kr-h1), a zinc finger transcription factor encoded by a JH early response gene.

We discovered that PKC is an essential component of a membrane-initiated JH signaling pathway. PKC was activated by JH in a phospholipase C (PLC)-dependent manner. Inhibition of PKC activity dramatically decreased the JH-induced gene expression. RNAi experiment indicated that several PKC isoforms

were involved in the JH action in adult female mosquitoes. We showed that PKC modulated the transactivation activity of MET by enhancing the binding of MET and TAI to the promoters of JH target genes.

Kr-h1 is rapidly upregulated by JH in newly emerged mosquitoes. RNAi-mediated depletion of AaKr-h1 caused a substantial decrease in oviposited eggs, indicating that this protein plays an essential role in mosquito reproduction. We combined chromatin immunoprecipitation (ChIP) with cloning of the generated DNA and have identified chromatin binding sites of AaKr-h1 in *Aedes aegypti*. After adult emergence, binding of AaKr-h1 to its *in vivo* targets increased with the JH-induced increase in AaKr-h1. Interestingly, depletion of AaKr-h1 in newly emerged mosquitoes led to considerable upregulation of some AaKr-h1 target genes but downregulation of other target genes. The results suggest that AaKr-h1 acts downstream of AaMET to regulate gene expression in response to JH and that AaKr-h1 can activate or repress the expression of individual target gene.

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GENERAL AUDIENCE ABSTRACT

Vector-borne diseases such as malaria, dengue fever, yellow fever and Zika virus infection have considerable economic, ecological and public health impacts. These diseases are transmitted by the bites of infected mosquitoes. For example, dengue is the most rapidly spreading mosquito-borne disease in the world. Dengue incidence has increased 30-fold within the past 50 years. World Health Organization estimates that more than 2.5 billion people (about 40% of the world's population) are at the risk of dengue. An estimated 500,000 people with severe dengue need hospitalization each year and about 5% of those affected die.

Currently, there are no effective vaccines or drugs for some vector-borne diseases such as dengue, dengue haemorrhagic fever and Zika virus infection. The most effective method to control these diseases is to limit transmission by using vector control tools such as chemical insecticides. However, many vector species have developed resistance to insecticides. Thus there is an urgent need to expand our knowledge of insect biology with an aim to design new insecticides. The juvenile hormone (JH) of insects plays a vital role in insect life and controls insect development and reproduction. Since JH only exist in arthropods, it provides a

good target for the design of environmentally safe insecticides. Although significant progress has been made in recent years, the molecular mechanism of JH signaling remains to be elucidated. Here we studied the JH signaling pathway in *Ae. aegypti* mosquitoes, the major vector for dengue virus and Zika virus. We identified two key components in mosquito cells that are required for initiation of egg development in response to the JH signal. The results also revealed new layers of complexity in the JH action in mosquitoes. This information would provide the basis for the development of new insecticides for vector control.

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Attribution

Chapter 2: Protein kinase C modulates transcriptional activation by the juvenile hormone receptor Methoprene-tolerant

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For chapter 2:

Pengcheng Liu, Ph.D, Dr. Jinsong Zhu laboratory, biochemistry department at Virginia Polytechnic Institute and State University, is currently a postdoc in Dr. Jinsong Zhu laboratory was a co-author on this paper and helped with PKC inhibition assay and kinase activity assay in mosquito fat bodies and also ChIP assay in mosquito cultured fat bodies.

Xiaonan Fu, Dr. Jinsong Zhu laboratory, biochemistry department at Virginia Polytechnic Institute and State University, is currently a graduate student in Dr. Jinsong Zhu laboratory was a co-author on this paper and helped with RNA sequencing.

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List of Abbreviations

Lists of abbreviations used in this dissertation

20E	20-hydroxyecdysone
BR-C	Broad-complex
CaMKII	Calcium/calmodulin-dependent protein kinase II
ChIP	Chromatin immunoprecipitation
DAG	Diacylglycerol
EcR	Ecdysone receptor
ET	Early trypsin
Gce	Germ cell expressed
IP3	Inositol 1,4,5-triphosphate
JH	Juvenile hormone
JHA	Juvenile hormone analogues
JHRE	Juvenile hormone response element
KBS	Kr-h1 binding site
Kr-h1	Krüppel homolog 1
MET	Methoprene-tolerant
PBM	Post blood meal
PE	Post eclosion
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

TAI	Taiman
USP	Ultraspiracle protein

Lists of abbreviations of insect species names used in this dissertation

Aa	*Aedes aegypti*
Ae. aegypti	*Aedes aegypti*
B. germanic	*Blattella germanic*
B. mori	*Bombyx mori*
Bm	*Bombyx mori*
D. melanogaster	*Drosophila melanogaster*
Dm	*Drosophila melanogaster*
L. migratoria	*Locusta migratoria*
P. apterus	*Pyrrhocoris apterus*
R. prolixus	*Rhodnius prolixus*
T. castaneum	*Tribolium castaneum*
Tc	*Tribolium castaneum*

Chapter 1: Introduction

1.1. The urgent need for new insecticides to control mosquito-transmitted diseases

Mosquito-borne infections are diseases that are transmitted to humans through the bites of infected mosquitoes. Blood feeding is necessary for mosquito vectors to obtain nutrients needed for energy and reproduction, and different viruses (arboviruses) and parasites have evolved to exploit this requirement as a way to move between hosts. Mosquito-borne diseases have significant public health impact, mostly in tropical and sub-tropical regions. Dengue is the most rapidly spreading mosquito-borne disease and the *Ae. aegypti* mosquito is the world's most important vector for the dengue viruses (Severson et al., 2004). Over 40% of the world's population are now at risk and the World Health Organization (WHO) reports 50-100 million new dengue infection occur annually worldwide (WHO, 2014). An estimated 500,000 people with severe dengue need hospitalization each year and about 5% of those affected die (Guzman et al., 2010).

Vector control interventions aim to reduce the number of infectious vectors in order to prevent or reduce disease transmission. These strategies include: biological control, using chemical insecticides and environmental management (Raghavendra et al., 2011). Vector control is currently the main strategy against some diseases like dengue and chagas (Townson et al., 2005). Insecticides are widely used to control *Aedes* population. However, resistance to carbamates, organochlorines, organophosphates and pyrethroids has been developed in *Ae. aegypti* and this resistance becomes an important problem for vector control (Ranson et al., 2010). Thus there is an urgent need to expand our knowledge of insect biology with an aim to design new insecticides.

1.2. Juvenile hormone (JH) signaling pathway as a target for vector control

1.2.1. Juvenile hormone (JH)

Juvenile hormone is an insect sesquiterpenoid hormone that controls many physiological aspects of insect life, including growth, development, reproduction and diapause (Jindra et al., 2013a). In some species, JH also regulates different polyphenisms and caste determination behavior (Gilbert, 2011; Hartfelder and Emlen, 2012; Nijhout, 1998). Since JH only exists in arthropods, it provides a good target for the design of environmentally safe insecticides (Ramaseshadri et al., 2012).

JH is secreted by a pair of endocrine glands called corpora allata, which have neural connections with the insect's brain (Tobe and Clarke, 1985). Eight forms of JH have been identified. They are the products of mevalonate pathway and are structurally related with a methyl-esterified farnesol backbone and a C10 epoxide group; the difference is in the branches of the carbon skeleton at C3, C7 and C11. JH III is the most common and is present in most insects, whereas JH I and II are the major forms of JH in Lepidoptera (Bergot et al., 1981; Goodman and Granger, 2009; Röller et al., 1967; Schooley and Baker, 1985).

Drosophila melanogaster produces a bis-epoxide JH III (JHB III) (Richard et al., 1989). JH skipped bisepoxide (JHSB III) is another bis-epoxide form of JH III that is isolated from heteropteran insects (Kotaki et al., 2009, 2011) (Figure 1.1.A). With an estimated more than 2.5 million insect species on earth (Mora et al., 2011), it will not be surprising if new forms of JH are discovered in future.

1.2.2. JH agonists

In holometabolous insects, the commitment for a larval-pupal molt is induced by 20E in the absence of JH (Riddiford, 1996). Analogues of the JH are used as insecticides, preventing the larvae from developing into adult insects.

Since the early 1970s, numerous analogs of JH have been tested for insecticidal activity (Retnakaran et al., 1985). These analogues are categorized as the "third generation pesticides" and it was proposed that insects are not able to develop resistance to molecules that mimic their own hormones. However, this presumption turns out to be not true. Many instances of JHA resistance have been documented. For example, resistance to pyriproxyfen has been observed in the sweetpotato whitefly, *Bamesia tabaci* (Horowitz and Ishaaya, 1994). Using an ethyl methane sulfonate mutagenesis screen, resistance to methoprene was found in a *D. melanogaster* mutant (*methoprene-resistant*) (Wilson and Fabian, 1986).

Most of the early analogs, such as methoprene [isopropyl(*E,E*)-(*R,S*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate], resemble JH in their basic terpenoid structure. Methoprene has the same structure as JH except it lacks the unstable epoxide and methyl ester (Figure 1.1.B). This compound has low mammalian toxicity, wide range of insect pest targets and short environmental half-life (Cusson and Palli, 2000; Henrick, 2007). Several highly active compounds including pyriproxyfen [phenoxyphenoxy(*R,S*)-2-(2-pyridyloxy)propyl ether] were synthesized later. These compounds are structurally distinct from natural juvenile hormones (Figure 1.1.B). These non-terpenoidal JH mimics usually have greater stability in the environment and are more active *in vivo* compared to the terpenoid ones (Beckage et al., 2000).

1.2.3. JH in metamorphosis

The most well known role of JH is in the regulation of insect metamorphosis. Metamorphosis is a crucial event in insect life, which involves several developmental changes such as cell proliferation, differentiation and programmed cell death. Insect metamorphosis can be classified into three types: ametaboly, hemimetaboly and holometaboly. In the most primitive wingless insects (apterygotes) like the silverfish *Lepisma*, there is almost no change in form during growth from juvenile to adult stages. These are known as ametabolous insects. Hemimetabolous insects develop from the nymph into the adult, while holometabolous insects develop from larva to pupa and then to adult.

Post-embryonic development of insects involves several molting, in which an insect sheds its old exoskeleton and grows a new and bigger one to replace. Molting process is regulated by two hormones: JH and ecdysone (Riddiford, 1994). Ecdysone is a steroid hormone, which is secreted by prothoracic gland and is then converted peripherally to the active form 20-hydroxyecdysone (20E).

It was in 1934 that Wigglesworth *et al* first found the anti-metamorphic action of JH. They reported that decapitation of *Rhodnius prolixus* (a hemimetabolous insect) larvae caused the formation of premature adult. So they concluded that JH was necessary to maintain the larval phase (Wigglesworth, 1934). Similar experiment was performed on *Bombyx mori* and the similar results confirmed the anti-metamorphic action of JH (Fukuda, 1944). Genetic ablation of corpora allata in *D. melanogaster* led to precocious metamorphosis and pupal lethality due to JH deficiency (Liu et al., 2009). When the final instar nymphs of *R. prolixus* were implanted with active corpora allata (that was dissected from third or fourth stage nymphs and implanted in the

abdomen of fifth stage nymphs), the JH produced by the glands prevented metamorphosis (Wigglesworth, 1934).

In holometabolous insects corpora allata secret JH in early larval instars. In the presence of JH, ecdysone induces a larva-to-larva molt. In the last larval instar, when corpora allata stop release of JH, ecdysone induces larval-pupal transition (Nijhout, 1998; Riddiford, 1994) (Figure 1.2).

In pre-pupal stage, when ecdysone secretion initiates metamorphosis, JH level goes up for a short period of time and this prevents immature insects from undertaking a precocious adult differentiation (Champlin and Truman, 1998; Kiguchi and Riddiford, 1978).During the pupal stage, JH level drops again to allow ecdysone to trigger adult commitment (Riddiford and Ajami, 1973; Williams, 1961).

1.2.4. JH in reproduction

JH controls many aspects of reproduction in insects. In female mosquitoes that require a blood meal to produce eggs, the first gonotrophic cycle is composed of two phases: previtellogenic phase (before blood feeding) and vitellogenic phase (after blood feeding). A key step in insect reproduction is vitellogenesis in which large amount of vitellogenin (Vg) (the yolk protein) is produced in insect fat body. In *Ae. aegypti* mosquito, JH titer goes up in the newly emerged adult and signals the beginning of reproductive maturation (Hagedorn, 1994; Klowden, 1997). Previtellogenic stage is the main period in which JH controls mosquito reproduction. In this stage JH affects many different tissues such as the fat body, midgut, and ovaries (Klowden, 1997). JH elicits oocyte growth (Feinsod and Spielman, 1980; Gwadz and Spielman, 1973), proliferation of ribosomes in the fat body (Raikhel and Lea, 1990), and expression of the *early trypsin (ET)* gene in the midgut (Edgar et al., 2000). When corpora allata were surgically removed in newly emerged mosquitoes, the follicles stopped growing, but the arrested growth

could be rescued through JH application (Gwadz and Spielman, 1973). The uptake of vitellogenin into oocytes requires endocytotic complexes that emerge at the oocyte membrane during previtellogenic phase. *In vivo* experiments have demonstrated that formation of the endocytic complexes is controlled by JH (Raikhel and Lea, 1985). Removal of corpora allata from newly emerged female mosquitoes disrupts formation of the endocytotic complexes, but that phenotype could be rescued by JH application (Raikhel and Lea, 1985). JH titer remains high during the previtellogenic phase and drops quickly after a blood meal, reaching the lowest level at 24h after blood feeding (Shapiro et al., 1986). In contrast, 20E level increases after blood ingestion and reaches its peak at 18-24 h after blood meal (Hagedorn, 1994). In simple word, the high JH level during adult stage prepares female mosquitoes for blood meal digestion and oogenesis (Noriega, 2004).

1.3. Molecular mechanism of JH action

1.3.1. Methoprene-tolerant (MET) is a JH nuclear receptor

A major challenge in studying molecular mechanism of JH action was identifying the JH receptor. Finding the JH receptor was not easy as this hormone can bind to various protein carriers and catabolic enzymes. JH receptor was first discovered in 1986 by Wilson *et al* in a mutagenesis study in *D. melanogaster* (Wilson and Fabian, 1986). When the JH mimic methoprene was applied to fruit flies at the onset of metamorphosis, it showed a lethal effect. This study led to selection of a mutant, which showed resistance to the lethal effect of methoprene. The genetic locus was named *Methoprene-tolerant (Met)* (Wilson and Fabian, 1986).

If the MET protein acts as the JH receptor, the *Met* null mutation is expected to be lethal. However, the *Met* mutation displayed no obvious effect on the development of *D. melanogaster,*

hindering the acceptance of MET as the JH receptor. The paradox was resolved when a paralog gene for *Met*, *germ cell expressed (Gce)*, was found later in *D. melanogaster* (Moore et al., 2000). The DmGCE protein shares 68%-86% identity with DmMET. Both GCE and MET are able to bind to JH *in vitro* (Charles et al., 2011; Moore et al., 2000). Only one *Met*-like gene is present in other insects of the Diptera order, suggesting that the *Met* and its paralog *Gce* came from gene duplication during higher fly evolution (Baumann et al., 2010; Wang et al., 2007). *Tribolium castaneum* has a single *Met/Gce*-like gene and exhibits robust systemic RNA interference (RNAi), making this insect a good model to examine the function of MET (Tomoyasu and Denell, 2004). MET knockdown in *T. castaneum* undergoes precocious metamorphosis, which phenocopies the allatectomy in larvae. RNAi knockdown of TcMET also resultd in the down-regulation of JH target genes such as *Kr-h1* (Konopova and Jindra, 2007). Later it was shown that in *D. melanogaster,* simultaneous mutation of *Met* and *Gce* caused lethal effect during larval-pupal transition (Abdou et al., 2011).

MET belongs to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors. The bHLH domain is approximately 60 amino acids long and contains a DNA-binding basic region and a helix-loop-helix region. The HLH region is needed for formation of homodimers and heterodimers (Gu et al., 2000).The bHLH transcription factors bind to hexameric consensus sequence called E-box (CANNTG) located in the promoter of target genes (Sailsbery et al., 2011). Immunostaining in *D. melanogaster* showed that MET is in the nuclei of JH target tissues (Pursley et al., 2000). Nuclear localization of MET was further confirmed in cell lines in which MET was expressed as fusions with green or yellow fluorescent protein (Greb-Markiewicz et al., 2011; Miura et al., 2005).

Several studies in different insect species, including the *Ae. aegypti* mosquito, demonstrated that MET binds to JH at physiological concentration (nanomolar) (Charles et al., 2011; Jindra et al., 2015; Li et al., 2014a; Miura et al., 2005). High affinity binding of JH, expression in JH target tissues and JH-dependent transcriptional activity all support the conclusion that MET mediates the effect of JH and acts as the hormone receptor (Liu et al., 2009; Miura et al., 2005; Pursley et al., 2000).

1.3.2. Protein partners of MET

Proteins of bHLH-PAS family usually function as homo- and heterodimers (Furness et al., 2007; Partch and Gardner, 2010). Till now, two bHLH-PAS proteins have been identified as the protein partners of MET: the steroid receptor coactivator Taiman (TAI) and circadian clock protein Cycle (CYC) (Shin et al., 2012). One is the homolog of the vertebrate steroid receptor coactivator (SRC-1/NCoA-1/p160), which is called Taiman in *D. melanogaster* (Bai et al., 2000), FISC in *Ae. aegypti* (Zhu et al., 2006b) and SRC in other insects (Guo et al., 2014; Kayukawa et al., 2012a; Zhang et al., 2011a).

In a yeast two-hybrid (Y2H) screening using *Ae. aegypti,* TAI was found to be associated with AaMET in the presence of JH (Li et al., 2011b). RNAi knockdown of either AaMET or AaTAI in adult female mosquitoes disrupted the expression of some JH target genes including the *AaET* and the *AaKr-h1*. A juvenile hormone response element (JHRE) was found in the upstream regulatory region of *AaET* and *in vitro* study showed that the AaMET-AaTAI complex bound specifically to this JHRE (Li et al., 2011b). The MET-TAI interaction was also verified in *T. castaneum* (Zhang et al., 2011a). RNAi-mediated knockdown of all 11 bHLH-PAS proteins in *T. castaneum* showed TcMET and TcSRC (the *T. castaneum* ortholog of AaTAI) were required for

the expression of JH target genes. The physical interaction between TcMet and TcSRC was further confirmed using a yeast two-hybrid assay (Zhang et al., 2011a).

The second is circadian clock protein Cycle (CYC), which dimerizes with MET and induces circadian-rhythmic expression of JH target genes in *Ae. aegypti* mosquitoes (Shin et al., 2012). CYC was identified in a separate yeast two-hybrid screening as another JH-dependent partner of AaMET. RNAi-mediated knockdown of either AaMET or AaCYC disrupted the circadian activation of JH target genes in adult *Ae. aegypti* mosquitoes. It was also shown that AaMET and AaCYC bound to a DNA sequence with an E-box like motif in the regulatory region of *AaKr-h1* gene (Shin et al., 2012).

1.3.3. Non-genomic action of JH

It has been suspected for a long time that besides a nuclear receptor, JH may also function through a cell-surface initiated pathway (Davey, 2000). A recent study in our lab confirmed the existence of a JH signaling cascade that is triggered through a yet unknown JH membrane receptor. In this pathway, phospholipase C (PLC) is activated by JH and results in an increase in the intracellular levels of diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP_3) and calcium (Ca^{2+}), leading to the activation of calcium/calmodulin-dependent protein kinase II (CaMKII) (Liu et al., 2015a). The non-genomic branch of JH signaling leads to enhanced MET and TAI phosphorylation, which is required for their DNA binding to JHRE. The cell surface-initiated JH pathway may be a mechanism to regulate the function of the intracellular JH receptor in a tissue/stage specific manner. As the activation of PLC increases the concentration of Ca^{2+} and DAG and these two second messengers have been known to activate PKC, PKC is expected to play a role in membrane initiated JH signaling (Figure 1.3).

1.3.4. JH target genes

One of the most important steps towards acquiring more information about JH signaling pathway was finding JH target genes. Newly emerged adult female *Ae. aegypti* mosquitoes were topically treated with exogenous JH; their transcriptome was examined before and after treatment (Zhu et al., 2010a). At three hours after the JH treatment, 16 genes were up-regulated and 33 genes were down-regulated. At 12 hours, 72 genes were up-regulated and 76 genes were down-regulated. One of the genes induced by JH was *AaKr-h1* (Zhu et al., 2010a). Further RNAi studies of Aag-2 cells (an *Ae. aegypti* cell lineage of embryonic origin) (Lan and Fallon, 1990; Peleg, 1968) showed that the JH-induced up-regulation of AaKr-h1 relied on AaMET and AaTAI; the expression of AaKr-h1 dropped by 80% when either AaMET or AaTAI was depleted by RNAi (Zhu et al., 2010a). In a subsequent microarray analysis, 6146 JH response genes were identified in female *Ae. aegypti* mosquitoes (Zou et al., 2013). Those genes were categorized into three groups: early post eclosion (EPE) genes with maximum expression at 6 h post eclosion (PE), mid post eclosion (MPE) genes that reach their expression peaks at 24 h PE, and late post eclosion (LPE) genes that climax at 66 h PE. MET mediates some of the JH regulation of gene expression. MET depletion caused up-regulation of some EPE and MPE genes, and also caused down-regulation of some LPE genes (Zou et al., 2013).

1.4. The zinc finger transcription factor Krüppel homolog 1 (Kr-h1)

One of the most important JH-response genes is *Kr-h1*. This gene was first identified as a homolog of a segmentation gene called *Krüppel* in *D. melanogaster* (Schuh et al., 1986). These two proteins are homologous in the zinc fingers and the spacers between the zinc finger motifs. *Kr-h1* was classified as a JH-regulated gene in a microarray analysis using *D. melanogaster* (Minakuchi et al., 2008a). In that genome-wide study of JH-target genes, *D. melanogaster*

abdominal integument was treated with pyriproxyfen (a JH mimics) at pupariation and it was found that 217 genes were up-regulated and 114 genes were down-regulated by that treatment. One of the JH-induced genes was *Kr-h1* (Minakuchi et al., 2008a).

1.4.1. Structure of Kr-h1

Kr-h1 is a zinc finger protein with eight C_2H_2 type zinc fingers. The zinc finger region is conserved in Kr-h1 homologs of both holo- and hemi-metabolous insects, however, the N-and C-terminal portions are less conserved (Konopova et al., 2011; Pecasse et al., 2000).In *D. melanogaster*, there are three Kr-h1 isoforms that differ in their N-terminal regions (Pecasse et al., 2000). Two important isoforms are named as α and β. DmKr-h1β is mainly expressed in neuronal cells during the embryonic stage (Beck et al., 2004). DmKr-h1α is highly expressed during larval stage and declines sharply after pupariation. The α isoform is functioning in metamorphosis and mutants lacking DmKr-h1α die at the pupariation time (Pecasse et al., 2000).Two Kr-h1 isoforms (α and β) were detected in *B. mori*; the transcription start site of BmKr-h1β was in the first intron of isoform α (Kayukawa et al., 2012a).

In *Ae. aegypti* mosquitoes, two Kr-h1 isoforms have been identified (Cui et al., 2014). They were named as AaKr-h1α and AaKr-h1β, which include two alternative exons of 35 and 126 nucleotides respectively. AaKr-h1α is the predominant isoform in Aag-2 cells and studies of *Ae. aegypti* mosquitoes showed that AaKr-h1α mRNA levels are always higher than the β isoform during all developmental stages (Cui et al., 2014).

1.4.2. JH induces Kr-h1 expression

The induction of Kr-h1 expression by JH has been confirmed in several studies using different insect species (Kayukawa et al., 2012a; Konopova et al., 2011; Lozano and Belles, 2011b;

Minakuchi et al., 2009; Minakuchi et al., 2011; Minakuchi et al., 2008b; Shpigler et al., 2010; Zhu et al., 2010a) and insect cell lines (Kayukawa et al., 2012a; Zhang et al., 2011a).

Expression of Kr-h1 in *B. mori* larvae is closely correlated with the change of endogenous JH concentrations (Kayukawa et al., 2012a). Removing corpora allata from *B. mori* larvae dramatically decreased Kr-h1 expression, however the botched expression of Kr-h1 was rescued by topical application of the JH analog methoprene (Kayukawa et al., 2012a). A study of NIAS-Bm-aff3 cells (originated from *B. mori* fat body) showed that JH treatment rapidly induced Kr-h1 expression within 30 min (Kayukawa et al., 2012a). The JH induction did not need *de novo* protein synthesis as cycloheximide (CHX) treatment (an inhibitor of protein synthesis) had no adverse impact on the expression of Kr-h1 (Kayukawa et al., 2012a).

In *T. castaneum*, the expression profile of Kr-h1 matches with the expression profile of juvenile hormone acid methyltransferase (JHAMT) (a rate limiting enzyme in JH biosynthesis) (Galkina et al., 2010). Moreover, when JH biosynthesis was suppressed in *T. castaneum* larvae by injection of TcJHAMT dsRNA, Kr-h1 expression decreased considerably but was restored by topical application of methoprene to the larvae (Galkina et al., 2010). When methoprene was applied to the newly emerged *T. castaneum* pupae, it caused a quick increase in the Kr-h1 transcripts (Galkina et al., 2010).

1.4.3. JH induces *Kr-h1* expression through the MET-TAI complex

The JH-MET-Kr-h1 cascade is well conserved in holometabolous and hemimetabolus insects (Kayukawa et al., 2013).In a cell transfection experiment, ectopical expression of *B. mori* Met2 and SRC was sufficient to activate the *BmK-h1* promoter in mammalian HEK293 cells which are believed to have no receptor for JH (Kayukawa et al., 2012a)

Two JHREs has been identified in the *T. castaneum* Kr-h1 gene; one is up-stream of the transcription start site (TSS) and the other is located in the first intron of *Kr-h1* gene (Kayukawa et al., 2013). In Tc81 cells (a cell line generated from *T. castaneum* embryos), *Kr-h1* is transcriptionally activated by JH (Kayukawa et al., 2013). Knockdown of TcMET or TcSRC in Tc81 cells significantly reduced the JH-dependent expression of Kr-h1, suggesting that the hormone induces Kr-h1 expression through the MET-SRC complex.

In a study using female *Ae. aegypti* mosquitoes, RNAi experiment showed knockdown of MET or TAI decreases the expression of *Kr-h1* in the midgut (Li et al., 2011b). In another study using Aag-2 cells, it was shown that JH rapidly (within 1.5 h) induces *AaKr-h1* expression and RNAi experiment showed that MET is required for the induction of *AaKr-h1* (Cui et al., 2014).

Studies in *Pyrrhocoris apterus* showed in MET knocked down larvae, Kr-h1 transcript level drops by 90% (Konopova et al., 2011).

1.4.4. Kr-h1 mediates the anti-metamorphic action of JH

Several studies confirmed that JH prevents precocious metamorphosis by inducing *Kr-h1* expression (Kayukawa et al., 2014; Konopova et al., 2011; Lozano and Belles, 2011b; Minakuchi et al., 2009; Minakuchi et al., 2008a). Depletion of MET or Kr-h1 by RNAi causes precocious metamorphosis both in holometabolous *T. castaneum* (Konopova and Jindra, 2007; Minakuchi et al., 2009) and hemimetabolous *P. apterus* (Konopova et al., 2011) and *Blattella germanica* (Lozano and Belles, 2011b) insects. Minakuchi *et al* showed that in *D. melanogaster* Kr-h1 plays an important role to mediate JH action (Minakuchi et al., 2009). During adult development, ectopic expression of *Kr-h1* in the abdominal epidermis of *D. melanogaster* caused loss of abdominal bristles and prolonged expression of *broad (br)* gene, a phenotype similar to that caused by ectopic application of JH (Minakuchi et al., 2008b).

In *T. castaneum,* expression of *Kr-h1* gene is continuous in the larval stage but is shut down during pupal stage (Minakuchi et al., 2009).

Disrupting JH biosynthesis pathway in *T. castaneum* larvae prevented *Kr-h1* expression and caused precocious metamorphosis (Minakuchi et al., 2009). Down regulation of *TcKr-h1* by RNAi in the larvae similarly caused precocious larval-pupal transition and this effect could not be rescued by application of methoprene. RNAi depletion of *Kr-h1* in pupae prevented the formation of second pupal cuticle in response to the exogenous application of JH (Minakuchi et al., 2009). These findings suggest that JH in *T. castaneum* larvae prevents precocious metamorphosis through expression of *Kr-h1* and that in pupae exogenous application of JH re-activates the expression of *Kr-h1*, which in turn triggers the formation of abnormal second pupae (Galkina et al., 2010).

1.4.5. Kr-h1 acts upstream of broad (br)

Broad complex consists of different isoforms of C_2H_2 zinc finger transcription factors that belong to the Broad- Tramtrack-Bric-a-brac (BTB) protein family (Bayer et al., 1996; DiBello et al., 1991). This ecdysone-induced transcription factor is required in metamorphosis to activate pupae-specific genes (Zhou and Riddiford, 2002). The amorphic *br* mutant in *D. melanogaster* cannot undergo metamorphosis (Kiss et al., 1976). The importance of br for pupal development has also been studied in *T. castaneum* (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008) and *B. mori* (Uhlirova et al., 2003). RNAi knockdown of *br* in the imaginal discs and primordial of *B. mori* prevents normal metamorphosis (Uhlirova et al., 2003).

In *D. melanogaster*, misexpression of Kr-h1α results in re-expression of *br* in the imaginal epidermis (Minakuchi et al., 2008b). However, misexpression of *br* did not affect Kr-h1 expression, suggesting that Kr-h1 acts upstream of br (Minakuchi et al., 2008b).

Recently Kayukawa *et al* found Kr-h1 binding site (KBS) in the promoter of *br* gene using a *B. mori* cell line and they confirmed the *in vitro* binding of Kr-h1 to that KBS through electrophoresis mobility shift assay (Kayukawa et al., 2016).

1.5. Research aims

JH regulates a wide variety of processes in insects during post-embryonic development and adult reproduction. During past decades, studies in a variety of insects have expanded our knowledge of the physiological functions of JH, but how this hormone works at the molecular level is not well understood. We know that the bHLH-PAS protein MET is the JH nuclear receptor. Upon binding of JH, MET dimerizes with TAI and induces transcription of target genes such as *Kr-h1*. Several questions still remain regarding the JH-regulated gene expression: Is the transactivation activity of MET regulated by mechanisms other than direct ligand binding? Does the JH signaling employ a regulatory hierarchy in which the JH primary-response genes control the expression of secondary-response genes?

Several lines of evidence have suggested that JH action involves some form of transmembrane signaling (Davey, 2000). Protein kinase C (PKC) has been implicated in those JH-initiated transmembrane signaling in a number of studies (Ilenchuk and Davey, 1987; Sevala and Davey, 1989; Sevala et al., 1995). In this study my first objective is to determine the role of PKC in the JH-regulated post-emergence development in adult mosquitoes, and to test the hypothesis that PKC connects the membrane-initiated JH signaling pathway with the nuclear pathway through modulating transactivation activity of the MET-TAI complex.

There is a big gap in our knowledge about the signaling events downstream of MET, especially about the function of Kr-h1. Kr-h1 has been shown to mediate the anti-metamorphic action of JH during post-embryonic development. This gene is also expressed in the adult stage, but so far

there is no study on its role in the JH regulation of insect reproduction. My second objective is to test whether Kr-h1 functions as a transcription factor and modulates gene expression in response to JH in adult mosquitoes. For this purpose, I isolated the *in vivo* chromatin binding sites of Kr-h1 and subsequently identified a few Kr-h1 target genes in adult mosquitoes. Finding Kr-h1 target genes will provide more information about various gene regulation events that are controlled by JH and shed light on the "cross-talk" between the JH pathway and the 20E or insulin signaling pathways. Overall it will advance our understanding of the molecular events downstream of MET in the JH pathway.

My objectives:

1. Determine the role of protein kinase C (PKC) in the JH signaling pathway in adult mosquitoes.
2. Identify the direct target genes of Kr-h1 in adult mosquitoes.

1.6. Figures

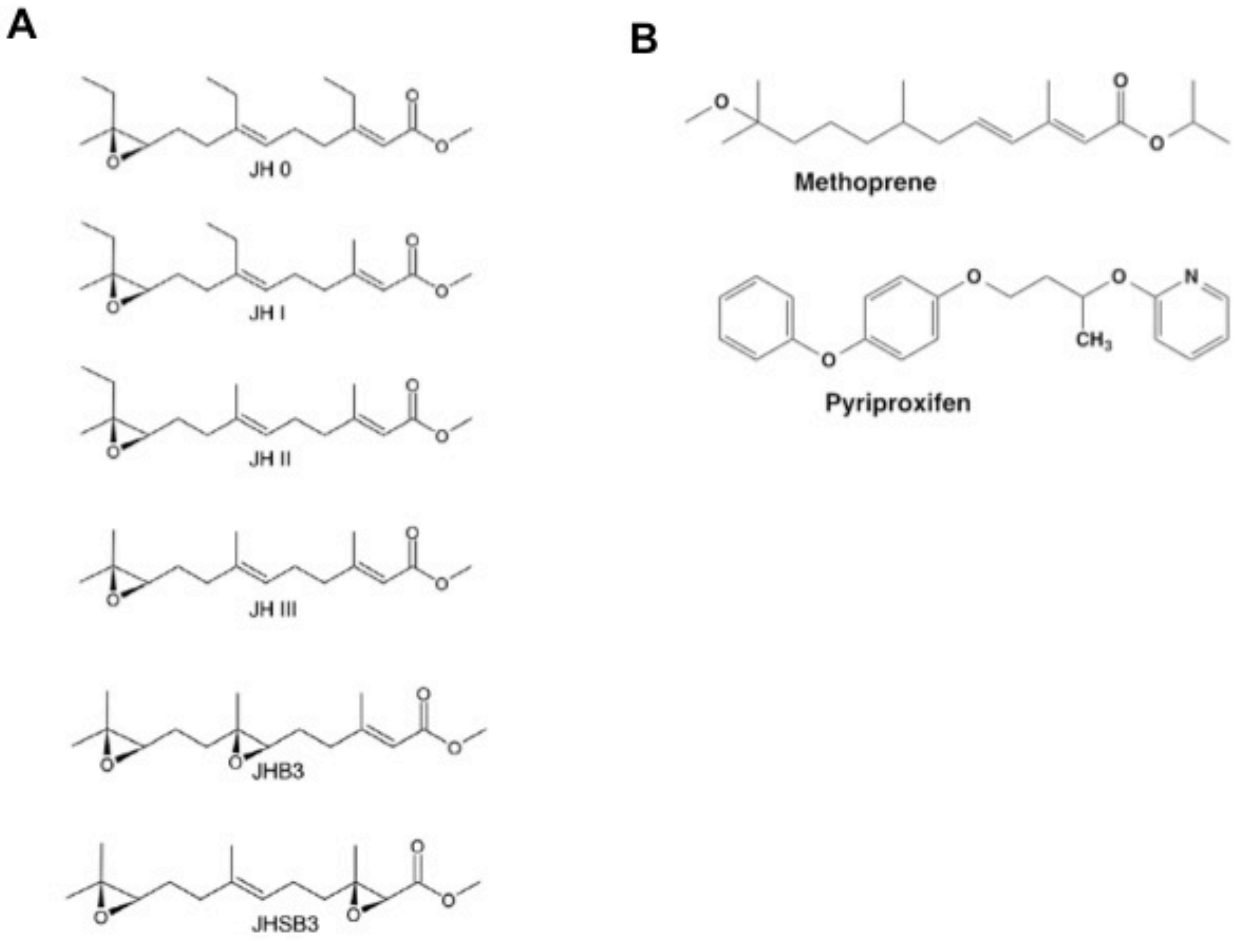

Figure 1.1. (*A*) Chemical structure of natural Juvenile hormones and (*B*). Juvenile hormone analogs.

* Used with permission of Elsevier publisher. Figure 1.1. (*A*) is adapted from (Dubrovsky and Bernardo, 2014) and Figure 1.1. (*B*) is adapted from (El-Sheikh et al., 2015) *

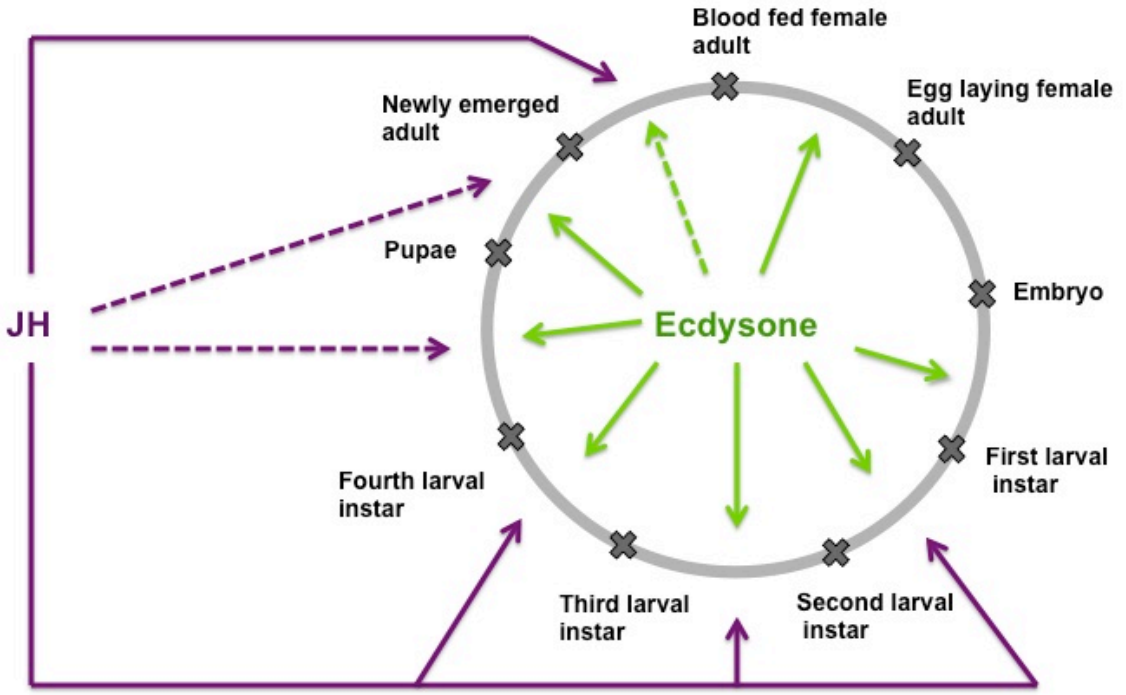

Figure 1.2. Hormonal control of metamorphosis. To control metamorphosis, JH works in cooperation with another hormone called ecdysone (molting hormone). In the presence of JH, ecdysone triggers larval to larval molt but in the last larval instar, in the absence of JH, ecdysone induces larval to pupal molt.

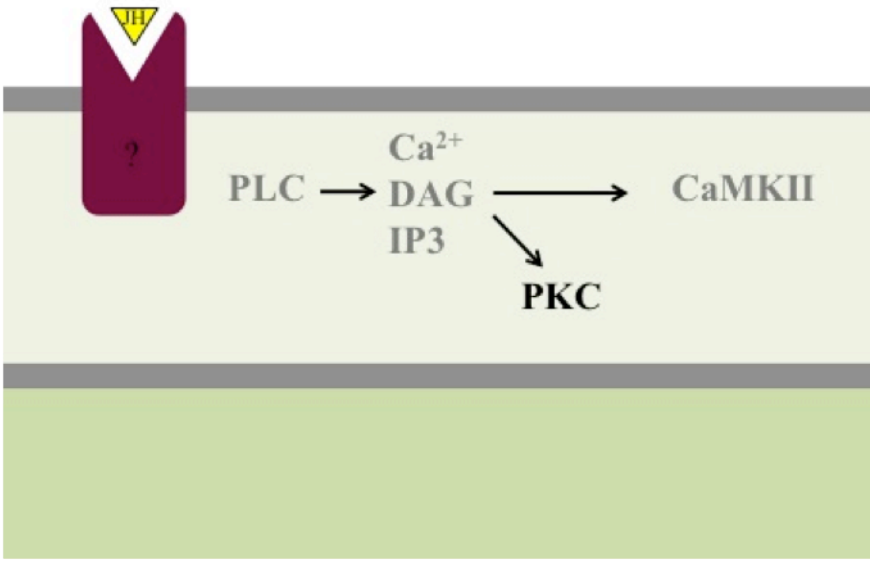

Figure 1.3. Non-genomic action of JH. Besides a nuclear receptor, JH also functions through a yet unknown cell-surface receptor. In this pathway, PLC is activated by JH and results in an increase in the intracellular levels of DAG, IP3 and Ca2+, leading to the activation of CaMKII. Activation of PKC as another important mediator of this cell membrane initiated pathway is expected.

Chapter 2: Protein kinase C modulates transcriptional activation by the juvenile hormone receptor Methoprene-tolerant

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2.1. Abstract

Juvenile hormone (JH) controls many biological events in insects by triggering dramatic changes in gene expression in target cells. The Methoprene-tolerant (MET) protein, an intracellular JH receptor, acts as a transcriptional regulator and binds to the promoters of tissue- and stage-specific JH target genes when JH is present. Our recent study has demonstrated that the transcriptional activation by MET is modulated by a membrane-initiated JH signaling pathway, involving phospholipase C (PLC) and calcium/calmodulin-dependent protein kinase II (CaMKII). Here we report that protein kinase C (PKC) is another essential intermediate of this pathway. PKC was activated by JH and this action was PLC-dependent. Inhibition of the PKC activity substantially weakened the JH-induced gene expression in mosquito cells. RNAi experiments indicated that several PKC isoforms were involved in the JH action during the post-emergence development of adult female mosquitoes. JH treatment considerably increased the binding of MET to the promoters of JH response genes in cultured mosquito abdomens that were collected from newly emerged female adults. The JH-induced DNA binding of MET was hindered when the abdomens were treated with a PKC inhibitor and JH. Therefore, the results

suggest that PKC modulates the transactivation activity of MET by enhancing the binding of MET to JHRE in the JH target genes. This mechanism may allow for variable and stage- and tissue-specific genomic responses to JH.

2.2. Introduction

Juvenile hormones (JH) are a group of acyclic sesquiterpenoids secreted in insects by the corpora allata, a pair of endocrine glands located on the posterior side of the brain (Tobe and Stay, 1985). JH is responsible for the regulation of insect metamorphosis and reproduction (Goodman and Cusson, 2012; Nijhout, 1994; Riddiford, 1994). In addition, this pleiotropic master hormone also governs many other aspects of insect life such as caste differentiation, migratory behavior, diapause and longevity (Flatt et al., 2005; Nijhout, 1994; Wyatt and Davey, 1996). Several signaling pathways have been postulated so far to be involved in the action of JH, but the mechanistic details largely remain to be elucidated (Jindra et al., 2013b; Wheeler and Nijhout, 2003).

Many effects of JH rely on its intracellular receptor MET, the product of the *Methoprene-tolerant (Met)* gene (Ashok et al., 1998b; Jindra et al., 2013b). MET contains an N-terminal basic helix-loop-helix (bHLH) DNA recognition motif, followed by two spaced Per- ARNT-Sim (PAS) domains (PAS-A and PAS-B) (Ashok et al., 1998b). In the presence of JH, MET binds to another bHLH-PAS domain protein called Taiman (TAI) (also known previously as FISC in *Ae. aegypti* and SRC *in T. castaneum*) (Charles et al., 2011; Li et al., 2011a; Zhang et al., 2011b). TAI acts as the obligatory DNA binding partner of MET; the MET-TAI complex recognizes an E-box like sequence (5'-GCACGTG-3') in the regulatory regions of JH target genes, leading to their transcriptional activation (Kayukawa et al., 2012b; Li et al., 2014b). In newly emerged adult *Ae. aegypti* mosquitoes, expression of the *AaET* gene and the *AaKr-h1* gene is induced by

JH via MET-TAI complex (Li et al., 2014b; Li et al., 2011a; Zhu et al., 2010b). After adult emergence, JH induces the transcription of *AaET* in the midgut, but the transcript will not be translated until the mosquito takes the blood meal (Noriega et al., 1996; Noriega et al., 1997). Kr-h1 is a zinc finger transcription factor that mediates the anti-metamorphic action of JH in phylogenetically distant insect orders (Konopova et al., 2011; Lozano and Belles, 2011a; Minakuchi et al., 2009; Minakuchi et al., 2008a).

In addition to binding to an intracellular JH receptor, several lines of evidence have suggested that JH action involves some form of transmembrane signaling (Davey, 2000). In the ovary of *R. prolixus* and *Locusta migratoria*, JH causes the follicle cells to shrink and create large intercellular spaces, thereby facilitating the transport of yolk proteins into the developing oocytes (Davey and Huebner, 1974; Davey et al., 1993). This rapid hormonal response is initiated by the binding of JH to a plasma membrane protein and involves activation of a Na^+/K^+ ATPase via a Protein kinase C (PKC)-dependent pathway (Ilenchuk and Davey, 1987; Sevala and Davey, 1989; Sevala et al., 1995). PKC has also been implicated in the JH-stimulated protein synthesis in male accessory glands of *D. melanogaster*. Yamamoto *et al.* showed that addition of JH caused an increase in protein synthesis in cultured accessory glands from the wild-type flies, but not from flies with mutation in PKC (Yamamoto et al., 1988).

We have recently reported that JH activates the phospholipase C (PLC) pathway in *Ae. aegypti* mosquitoes and triggers an increase in the concentration of diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP_3) and intracellular calcium (Ca^{2+}), which in turn activates calcium/calmodulin-dependent protein kinase II (CaMKII) (Liu et al., 2015b). This membrane-initiated JH signaling modulates phosphorylation of MET and TAI, and substantially enhances DNA binding of the MET-TAI complex to JHRE. Besides CaMKII, PKC is another intracellular mediator normally

associated with the PLC pathway. Here we provide the first evidence that PKC is also activated by JH in a PLC-dependent manner in *Ae. aegypti*. PKC plays a critical role in transcriptional activation of JH target genes. Activation of PKC by JH is essential for effective binding of MET-TAI to JHRE. This study significantly advances our understanding of the function of PKC in the action of JH.

2.3. Materials and Methods

2.3.1. Chemicals

JH-III was purchased from Sigma Aldrich and was dissolved in ethanol. In all inhibition experiments, cells were pre-incubated with inhibitors for 1 h before the addition of JH-III. Final concentrations of inhibitors in all cell-culture studies were as follows: calphostin C (Santa Cruz Biotechnology), 5 μM; RO 31-8220 (Santa Cruz Biotechnology), 10 μM; Gö 6983 (Santa Cruz Biotechnology), 10 μM; KT 5720 (Santa Cruz Biotechnology), 10 μM; U73122 (EMD Millipore), 1 μM. Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Aldrich and was used at a final concentration of 10 μM.

2.3.2. Cell culture

Ae. aegypti Aag-2 cells (Lan and Fallon, 1990; Peleg, 1968) were maintained at 28°C in Schneider's *Drosophila* media (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). Passages of cells were conducted every 4 days with a 1:5 dilution of cells.

2.3.3. Mosquito rearing and tissue culture

The *Ae*. *aegypti* Liverpool strain was maintained in an insectary at 28°C and 60–70% humidity, with a 14/10 h day/night light cycle. Larvae were fed with pulverized fish food (TetraMin Tropical Flakes) and adults were maintained on a 10% sucrose solution. Female mosquitoes (7 days post eclosion) were fed on defibrinated sheep blood using an artificial membrane feeder to produce eggs. All tissue dissections were performed in *Aedes* physiological saline (APS) (Hagedorn et al., 1977). Tissues were collected from female mosquitoes within 30 min after eclosion. *In vitro* tissue culture was carried out as previously described (Deitsch et al., 1995; Raikhel, 1997). The mosquito abdomens were cut open and placed on top of the fat body culture medium. Fat bodies attached to the inner wall of cuticles were in the medium and the outside surface of cuticle was exposed to air. Three groups of five mosquitoes were used for each treatment. When inhibitors were used, dissected tissues were pre-incubated with the inhibitors for 1 h before the addition of JH-III.

2.3.4. Real-time PCR

Total RNA was extracted from Aag-2 cells or mosquitoes using TRIzol reagent (Life technology), according to the manufacturer's instruction. RNA was primed with oligo (dT) primer and reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed in triplicate on an ABI 7300 system (Applied Biosystem) using the GoTaq qPCR Master Mix (Promega) and gene-specific primers (Table 2.1). Transcript abundance was normalized to that of *RpS7*.

2.3.5. RNA sequencing (RNA-seq) and data analysis

mRNAs were purified using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). RNA integrity of all samples was assessed by Agilent 2100 Bioanalyzer. RNA-seq libraries were prepared using the NEBNext mRNA Library Prep Reagent Set (New England Biolabs), and were loaded onto flow cell channels of the Illumina MiSeq platform. Paired-end reads (100 nt × 2) were aligned to the *Ae. aegypti* reference genome (AaegL3, Vectorbase) using TopHat (Trapnell et al., 2009).The normalized mRNA abundance was calculated as FPKM values (Fragments Per Kilobase of transcript per Million mapped reads) using Cufflinks (Trapnell et al., 2012).We used a FPKM cutoff of 1 for transcripts to be considered reliable for further analysis. All the data have been submitted to NCBI SRA database (accession number: SRS1128317).

2.3.6. Double-stranded RNA (dsRNA)-induced gene silencing (RNAi)

DsRNAs were synthesized by *in vitro* transcription of PCR-generated DNA templates (Table 2.2) and injected into *Ae. aegypti* mosquitoes as described previously (Zhu et al., 2003). Briefly, female mosquitoes were injected with 0.5 μg of dsRNA within 1 h post-eclosion. DsRNA for green fluorescent protein (GFP) was used as a negative control. Three days after dsRNA injection, RNA was extracted for real-time PCR analysis.

For RNAi in the cell line, Aag-2 cells were cultured in a 6-well plate and incubated overnight to allow cells to adhere to the plate. Complete media was replaced with FBS- and antibiotic-free media containing 5 μg dsRNA per well. Cells were then incubated for 3 days before adding JH-III (5 μM) and incubating for an additional hour, followed by RNA extraction and real-time PCR analysis.

2.3.7. Luciferase reporter assay

pCMA-GAL4 and UAS×4-188-cc-Luc were from Dr. Lucy Cherbas (Hu et al., 2003). Construction of pCMA-AaMET, pCMA-AaTAI and 4×JHRE1-luc has been explained previously (Li et al., 2014b; Li et al., 2011a). Aag-2 cells were cultured in 48-well plates and were transfected with pCMA-AaMET (0.2 μg per well), pCMA-AaTAI (0.2 μg per well), 4×JHRE1-luc reporter plasmid (0.2 μg per well). Cellfectin II reagent (Life technology) was used for transfection according to the manufacturer's instruction. The Renilla luciferase construct pRL-CMV (0.1 μg per well) was used as an internal control of transfection efficiency. At 24 h after transfection, cells were treated with JH-III and various inhibitors for 4 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined as the ratio of firefly luciferase activity to Renilla luciferase activity. Data are presented as the mean ± S.D. of three independent experiments.

2.3.8. Western blot analysis

Whole cell lysates were extracted from Aag-2 cells or cultured mosquito tissues using a modified RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Protein concentrations were measured using the bicinchoninic acid (BCA) assay. An equal amount of protein was loaded in each lane and separated on a SDS-PAGE gel in all experiments. Production of polyclonal antibodies against AaMET and AaTAI has been reported previously (Zhu et al., 2010b; Zhu et al., 2006a). Anti-Phospho-(ser) PKC substrate antibody was purchased from Cell Signaling Technology.

2.3.9. Chromatin immunoprecipitation assay (ChIP)

The ChIP assay was performed using SimpleChIP Plus Enzymatic Chromatin Immunoprecipitation Kit (Cell signaling Technology). After Aag-2 cells were treated with ethanol, JH-III, or JH-III together with a PKC inhibitor, cells were washed and resuspended in PBS. Formaldehyde was added to a final concentration of 1%, and crosslinking was performed for 10 minutes at 37°C. Crosslinking was stopped by adding 1.25 M glycine to reach a final concentration of 125 mM. After nuclei preparation, chromatin digestion was performed using 0.25 µl of Micrococcal nuclease for 5 min at 37°C. Immunoprecipitation was carried out using the anti-AaMET antibody or anti-AaTAI antibody. Non-specific goat IgG (ImmunoReagents Inc) was used for mock immunoprecipitation. DNA was purified from the pellets according to the manufacturer's protocol and analyzed by real-time PCR. For cultured mosquito abdomens, the tissues were homogenized in PBS on ice and then incubated at 37°C with 1% formaldehyde for 10 min. The ChIP assay was then performed as described above for the Aag-2 cells. Primers used in the ChIP assays are listed in Table 2.3.

2.4. Results

2.4.1. PKC is required for the JH-induced expression of AaKr-h1

A previous study has shown that JH rapidly induces *AaKr-h1* expression in mosquito Aag-2 cells (Cui et al., 2014). The effect of JH and its analogs (Methoprene and Pyriproxifen) on *AaKr-h1* was tested using Aag-2 cells and a similar result was observed (Figure 2.1). JH, Methoprene and Pyriproxifen rapidly induce *AaKr-h1* expression within 1h, while ethanol (control solvent) had no effect on gene expression (Figure 2.1). To examine the involvement of PKC in the JH signaling pathway, we inactivated PKC in Aag-2 cells using specific inhibitors and then measured the expression level of *AaKr-h1* after JH treatment. RO31-8220, Gö6983 and

calphostin C are non-isoform-selective PKC inhibitors (Beltman et al., 1996; Gschwendt et al., 1996; Kobayashi et al., 1989). Incubation of Aag-2 cells with 5 μM JH-III for 1 h resulted in a 70-fold increase in the *AaKr-h1* expression (Figure 2.2.A). Pre-incubation of Aag-2 cells with RO31-8220, Gö6983 or calphostin C all substantially abolished the stimulatory effect of JH on *AaKr-h1* expression, indicating an involvement of PKC in this JH response. In contrast, the protein kinase A (PKA) inhibitor KT5720 did not markedly affect the JH-induced expression of *AaKr-h1* (Figure 2.2.A). The role of PKC in JH signaling was also investigated using *in vitro*-maintained fat bodies of newly emerged adult female mosquitoes, in which JH has been shown to considerably upregulate the expression of *AaKr-h1* and *AAEL002576* (another JH target gene) via the PLC pathway (Liu et al., 2015b). The JH-induced expression of both genes was decreased by more than 70% when the fat bodies were pre-incubated with RO31-8220 or U73122, a PLC inhibitor. Conversely, inactivation of PKA by KT5720 did not show a marked inhibitory effect in this experiment (Figure 2.2.B). Therefore, the results suggest that functional PKC is required in the JH signaling pathway.

To test whether the PKC inhibitors used in this study adversely affect global gene expression in mosquito cells, we performed transcriptome analyses in Aag-2 cells treated with JH or with JH and RO31-8220. mRNA levels of the majority of genes were not altered by the addition of RO31-8220 to the medium (Figure 2.3), suggesting that the inhibitors did not adversely affect the global gene expression under our experimental conditions and that PKC plays a specific role in the JH-regulated gene expression. To assess the role of PKC in the JH signaling pathway, we treated Aag-2 cells with the PKC activator PMA (Phorbol-12-Myristate-13-Acetate). In the absence of JH-III, PMA was not able to cause any increase in the amount of *AaKr-h1* mRNA (Figure 2.4), suggesting that the activation of PKC alone was not sufficient to induce the

expression of JH target genes. When the cells were treated simultaneously with PMA and JH-III, PMA did not further enhance the JH-induced expression of *AaKr-h1* (Figure 2.4). This observation implied that PKC was almost fully activated after the cells were exposed to JH.

2.4.2. JH induces PKC activity in Aag-2 cells and in mosquito fat bodies

After demonstrating that PKC plays an essential role in the JH-regulated gene expression, we examined whether the kinase activity of PKC was increased by JH in mosquito cells. Phosphorylation of mosquito proteins by PKC was detected by Western blot analysis using a specific antibody that recognizes the phosphorylated serine residues in conserved PKC phosphorylation sites. Although identities of the detected protein bands were not known, the overall intensity of signal on the blot was proportional to the activity of PKC. Compared with cells treated with ethanol, JH-III substantially increased the phosphorylation of a group of proteins in Aag-2 cells within 30 minutes after the JH treatment (Figure 2.5.A). The kinase activity were stronger at 2 h after the hormone treatment. Interestingly, when PLC was inactivated by its inhibitor (U73122), phosphorylation of those proteins after the JH treatment was not detected in the Western blot. When the fat bodies of newly emerged female mosquitoes were exposed to JH-III, the PKC activity was also considerably enhanced in a PLC-dependent fashion (Figure 2.5.B). Based on these results, we conclude that PKC is activated in the presence of JH and that PKC acts downstream of PLC in the JH signaling pathway.

2.4.3. Roles of individual PKC isoforms in the regulation of JH-inducible genes

PKC is a family of serine/threonine protein kinases. Based on homology with the PKC isoforms in *D. melanogaster* (Shieh et al., 2002), we identified five putative PKC isoforms in *Ae. aegypti*: a conventional PKC (AaPKC1), two novel PKCs (AaPKC2 and AaPKC3), an atypical PKC (AaPKC4) and a PKC-related kinase (AaPKC5) (Table 2.1). Five isoform-specific dsRNAs were

synthesized and dsRNA against GFP was used as control. Knockdown of each PKC isoform in Aag-2 cells displayed minor effect on the JH-regulated expression of *AaKr-h1* (Figure 2.6.A and Figure 2.9). However, when a combination of dsRNAs for all five PKC isoforms was applied to the cells at the same time, the JH induced expression of *AaKr-h1* decreased by 59% compared with the dsGFP-treated cells, implying potential functional redundancy of the PKC isoforms in the JH signaling.

The role of PKC was also investigated in newly emerged adult female mosquitoes. The depletion of AaPKC3, AaPKC4 and AaPKC5 showed the most dramatic effect and reduced the expression of *AaKr-h1* by 74%, 62% and 73%, respectively (Figure 2.6.B and Figure 2.9). Depletion of AaPKC1 and AaPKC2 caused a mild decrease (by 38% and 23%, respectively) in *AaKr-h1* expression. In contrast, the mosquitoes injected with dsRNA for GFP exhibited similar levels of *AaKr-h1* mRNA transcripts to the uninjected controls. The influence of PKC was not exclusive to *AaKr-h1* as the RNAi knockdown of PKCs had similar impact on the expression of *AaET*, another well-characterized JH target gene (Figure 2.6.B). The involvement of PKC isoforms from different subfamilies implies synergistic action of PKC isoforms in cellular response to JH.

2.4.4. Inactivation of PKC decreases the transcriptional activation by MET in response to JH

The JH-induced expression of *AaET* and *AaKr-h1* has been shown to be mediated by the AaMET-AaTAI complex (Li et al., 2011a). To investigate the connection between PKC and the intracellular JH receptor AaMET, we performed a reporter assay using a luciferase gene that was placed under the control of a minimal promoter and the JHRE identified in *AaET*. When AaMET and AaTAI were over-expressed in Aag-2 cells, JH-III increased the reporter activity by 92-fold over treatment with ethanol (Figure 2.7.A). Pre-incubation with PKC inhibitors (RO 31-8220, Gö

6983 and calphostin C) either completely abolished or substantially weakened the JH-induced expression of the luciferase reporter, indicating that the PKC activity is required for proper function of the AaMET-AaTAI complex on JHRE. In the control experiment, we explored the role of PKC in activation of the UAS-luc reporter gene by GAL4. Inactivation of PKC by its inhibitors under similar experimental conditions showed no remarkable effect on the GAL4-activated luciferase expression, suggesting that the JH-induced PKC activity specifically modulates function of the AaMET-AaTAI complex on JHRE (Figure 2.7.B).

2.4.5. PKC activity is essential for binding of the MET-TAI complex to JHRE

To test the possibility that inactivation of PKC causes a decrease in binding of MET to JHRE, ChIP assay were performed. We have recently shown that AaTAI is the obligatory DNA-binding partner of AaMET in regulating JH response genes (Li et al., 2014b). Therefore, binding of AaMET to JHRE *in vivo* is expected to be accompanied with the DNA binding of AaTAI. In Aag-2 cells at 30 minutes after addition of JH-III to the culture medium, the binding of AaMET and AaTAI to the proximal promoter of *AaKr-h1* increased by 20.4- and 26.7-fold, respectively, compared with the cells just exposed to ethanol (Figure 2.8.A and B). Binding of AaMET and AaTAI to the 3' UTR of *AaKr-h1* remained at the background level regardless of the presence of JH. Interestingly, inhibition of PKC by RO 31-8220 decreased the JH-induced DNA binding of AaMET and AaTAI by 69% an 73%, respectively (Figure 2.8.A and B). Adding RO 31-8220 to the cell culture did not markedly change the nuclear protein levels of AaMET and AaTAI in Aag-2 cells (Figure 2.10), suggesting that PKC regulates the transactivation activity of AaMET and AaTAI by modulating DNA binding property of the AaMET-AaTAI heterodimer.

The role of PKC in the DNA binding of AaMET and AaTAI was also assessed in the mosquito abdomens that were dissected from newly emerged adult female animals and cultured *in vitro*.

The ChIP results indicated that binding of AaMET and AaTAI to the *AaKr-h1* promoter in the JH-treated abdomens was 8.2- and 6.1-fold stronger, respectively, than the ethanol-treated abdomens (Figure 2.8.C and D). However, this JH-induced specific DNA binding was dramatically repressed when PKC was inhibited by RO 31-8220 in the cultured abdomens. Likewise, inhibition of the PKC activity adversely affected the recruitment of AaMET-AaTAI to the JHRE of *AaET* (Figure 2.8.E and F), suggesting that the activation of PKC by JH is a general mechanism to modulate the DNA binding activity of AaMET and AaTAI.

2.5. Discussion

Accumulating evidence has indicated that JH regulates insect metamorphosis, reproduction and other biological processes via a MET-dependent pathway (Jindra et al., 2013b). Our recent study has demonstrated that DNA binding of the MET-TAI complex is governed by a membrane-initiated PLC pathway. Activation of this PLC pathway considerably increases the intracellular levels of IP_3, DAG and Ca^{2+}, leading to phosphorylation of MET and TAI which is presumably a requisite for the binding of MET and TAI to JHRE (Liu et al., 2015b). CaMKII is a component of the PLC pathway. It is activated by the JH-triggered elevation of intracellular Ca^{2+}; its activity is essential for the proper binding of MET and TAI to JHRE (Liu et al., 2015b). In current study, we demonstrate that PKC is another component of the JH-activated PLC pathway. The PKC activity is also critically important for the DNA binding of MET and TAI.

The membrane-initiated JH signaling was discovered in the study of the development of ovarial patency in *R. prolixus*. JH causes the ovarian follicle cells to shrink within minutes after application of JH (Davey and Huebner, 1974). This process is not affected by inhibitors of transcription, indicating that it is a non-genomic hormone response (Abu-Hakima and Davey, 1977). *In vitro* studies have suggested that JH binds to an unknown membrane protein on *R.*

prolixus follicle cells (Ilenchuk and Davey, 1985) and activates PKC, which then phosphorylates the alpha subunit of Na^+/K^+ ATPase, leading to the decrease in cell volume (Davey, 1996). In *Heliothis virescens,* JH-I regulates the patency development primarily via a G-protein coupled receptor (GPCR) and a cyclic adenosine monophosphate (cAMP)-dependent pathway (Pszczolkowski et al., 2005), while JH-II and JH-III presumably evoke patency by stimulation of the PLC-PKC pathway (Pszczolkowski et al., 2008). In *Ae. aegypti*, current evidence indicates that JH activates the PLC-PKC pathway via a receptor tyrosine kinase on the cell membrane to modulate gene expression during post-emergence development in adult mosquitoes (Liu et al., 2015b). These membrane-initiated signal transduction events bear some resemblance.

PKC has been previously reported to curb DNA binding of several transcription factors involved in the JH-controlled gene expression (Kethidi et al., 2006; Zhou et al., 2002). A DNA-binding protein in *L. migratoria* was implicated in the JH-activated synthesis of yolk proteins in the fat body (Zhou et al., 2002). *In vitro* assays indicated that PKC activity present in the fat body nuclear extract inhibited binding of this locust protein to a JHRE. Similarly, the PKC activity in *D. melanogaster* L57 cells was shown to repress binding of a *Drosophila* nuclear protein (or a protein complex) to a JHRE identified from the JH esterase gene of *Choristoneura fumiferana* (Kethidi et al., 2006). In both cases, the JHREs do not resemble the E-box sequence that is recognized by MET-TAI, suggesting that the nuclear proteins bound to those JHREs are transcription factors other than MET. Although the regulation of PKC activity by JH has not been fully elucidated in *L. migratoria* and *D. melanogaster,* these examples imply that PKC may either increase or decrease the DNA binding of different transcription factors involved in the JH signaling. The JH-activated PLC-PKC pathway thus may alter the amplitude and dynamics of gene expression to facilitate stage- and tissue-specific biological responses to JH.

Integration of extranuclear and intranuclear signaling is common in steroid responses in mammals (Hammes and Davis, 2015). The biological effects of steroids are primarily mediated by binding to their classical intranuclear receptors (Evans and Mangelsdorf, 2014). The liganded receptors act as transcription factors and together with coregulators bind to specific DNA response elements located in the promoters of target genes, causing activation or repression of transcription (Tsai and O'Malley, 1994). However, a vast body of work indicates that cellular responses to steroids may be mediated by a small population of classical steroid receptors located at the plasma membrane, which interact with G proteins, signaling kinases and other G protein-coupled receptors, to mediate rapid effects of the hormones (androgen, estrogen, progesterone, etc.) (Mani et al., 2012). The nongenomic steroid activity typically involves the rapid activation of second messenger systems and activation of PKC, PI3K and mitogen-activated protein kinase (Hammes and Levin, 2007; Pietras and Szego, 1975; Szego and Davis, 1967). This rapid action initiates at the cell surface and can be readily induced by cell-impermeable hormone conjugates (Zhao et al., 2005; Zheng et al., 1996).

Recent reports have demonstrated in mammals that normal function of both extranuclear and nuclear steroid signaling is required for many hormonal responses in important biological processes (Pedram et al., 2009; Pedram et al., 2014; Roforth et al., 2014). The membrane-initiated signaling promotes phosphorylation of the nuclear steroid receptors and their co-regulators, modulates recruitment of these transcription factors to target promoters, and imposes an additional control in the regulation of transcription by the nuclear receptors (Marino et al., 2006). Therefore, the genomic and non-genomic pathways are inextricably linked. The rapid, non-classical hormonal responses are often associated with and likely required for more slow-acting (but long-lasting) genomic hormonal effects. The integration is believed to provide

plasticity for cell response to steroids (Marino et al., 2006). Steroids could activate discrete signaling pathways via their membrane receptors and associated proteins, depending on the cellular type and on the physiological status of the cell. This should lead to different gene expression in various types of target cells and different biological outcomes in response to the same hormone (Hammes and Davis, 2015). Similar mechanism may be used by insects in response to JH, although this hypothesis needs to be tested in additional tissue- and stage-specific JH actions.

In our study, the activation of PKC by JH led to enhanced binding of AaMET and AaTAI as a complex to JHRE. The phosphorylation targets for PKC in the JH signaling pathway are yet unknown. We have shown that JH induces phosphorylation of both AaMET and AaTAI, and that this JH action is mediated by the PLC pathway (Liu et al., 2015b). Whether the phosphorylation of both proteins relies on the function of PKC awaits further investigation. A bioinformatic analysis with the GPS 2.1 software (Xue et al., 2011) revealed dozens of putative PKC phosphorylation sites in AaMET and AaTAI. While it is tempting to speculate that AaMET and AaTAI are direct phosphorylation targets of PKC, there is no evidence so far to substantiate this hypothesis. AaMET purified by immunoprecipitation from the JH-treated Aag-2 cells was not detectable by a phospho-(Ser) PKC substrate antibody (Figure 2.11). Although we could not rule out the possibility that AaMET is phosphorylated by PKCs on threonine or serine residues that could not be recognized by this antibody, it is conceivable that PKC uses another kinase as an intermediate to regulate the phosphorylation of AaMET. Mass spectrometry analysis of AaMET and AaTAI from Aag-2 cells that are treated with ethanol, JH alone, or JH together with a PKC inhibitor is expected to reveal the PKC-dependent phosphorylation sites. This information will enable us to examine whether the JH-induced phosphorylation of AaMET affects its binding to

JH, and determine whether phosphorylation of both proteins affects their dimerization and their binding to JHRE. It is important to note that phosphorylation of AaMET and AaTAI may require the synergistic action of PKC, CaMKII and other unidentified kinases. This may explain why the PKC activator was not able to enhance the expression of AaKr-h1 in Aag2 cells (Figure 2.4).

PKC sits at the crossroads of divergent intracellular signaling pathways. Several studies have indicated that PKC plays a critical role in response to the steroid hormone 20-hydroxyecdysone (20E) in insects. The nuclear receptor complex for 20E is composed of the ecdysone receptor (EcR) and ultraspiracle protein (USP). In *D. melanogaster,* phosphorylation of USP is regulated by 20E in the salivary glands during early third instar (Song et al., 2003). *In vitro* studies have indicated that the *Drosophila* USP is phosphorylated by PKC on Ser35 (Wang et al., 2012b). Inactivation of PKC in the salivary glands *in vivo* leads to a decrease in USP phosphorylation and attenuates 20E signaling in *D. melanogaster* (Wang et al., 2012b). In the lepidopteran insect *Helicoverpa armigera*, 20E activates the PLC-PKC pathway via a G protein-coupled 20E receptor (Wang et al., 2015). The 20E-induced phosphorylation of USP promotes binding of the EcR-USP complex to ecdysone-response elements and is essential for the 20E-induced gene expression (Liu et al., 2014). The fact that PKC is capable of modifying DNA binding of the ecdysteroid receptor complex and the JH nuclear receptor complex obviously raises several questions: (1) Are the putative membrane receptors for JH and 20E present simultaneously in the same cell or tissue? (2) Is phosphorylation of USP and MET mediated by the same PKC isoforms? (3) Is USP phosphorylated by PKC when cells are exposed to JH but not to 20E? (4) Does the phosphorylation of MET and USP affect formation of the MET-TAI and EcR-USP complexes, respectively? Addressing these questions will significantly advance our

understanding of the crosstalk between 20E and JH signaling pathways, and the anti-metamorphic function of JH.

2.6. Figures, tables and supplemental information

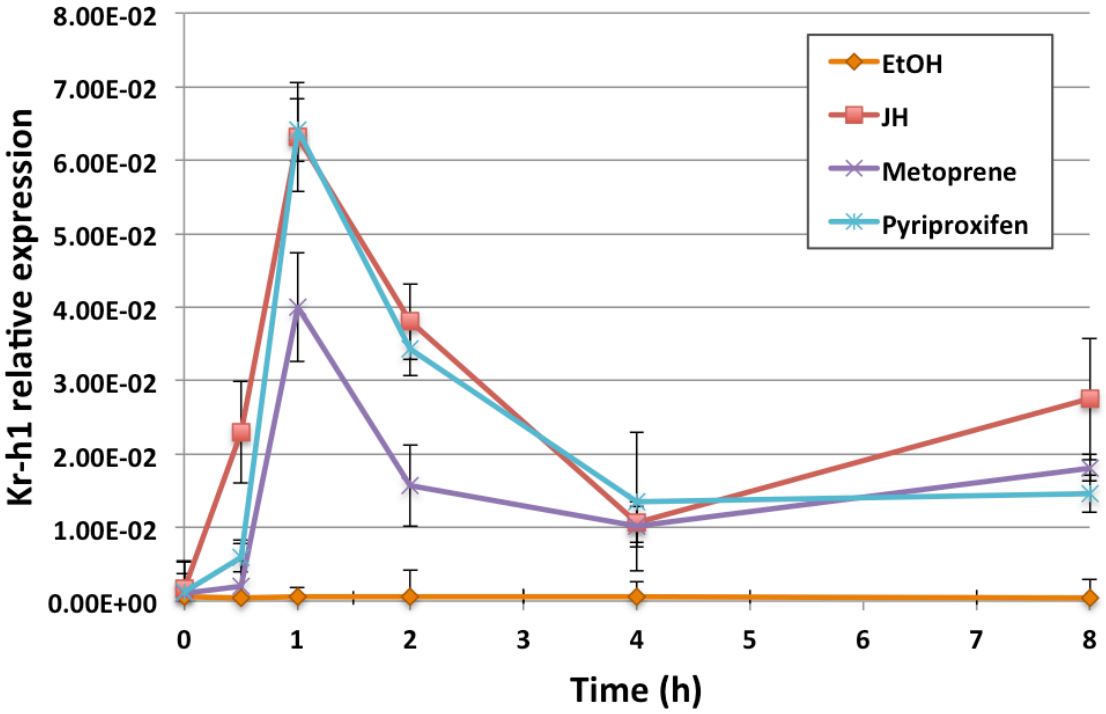

Figure 2.1. JH induces *AaKr-h1* expression in Aag2 cells. Aag-2 cells were treated with JH III, Methoprene or Pyriproxifen for 1 h. Ethanol was used as a negative control. The expression level of *AaKr-h1* was measured using real-time PCR. Results are the mean ± S.D. of three independent experiments.

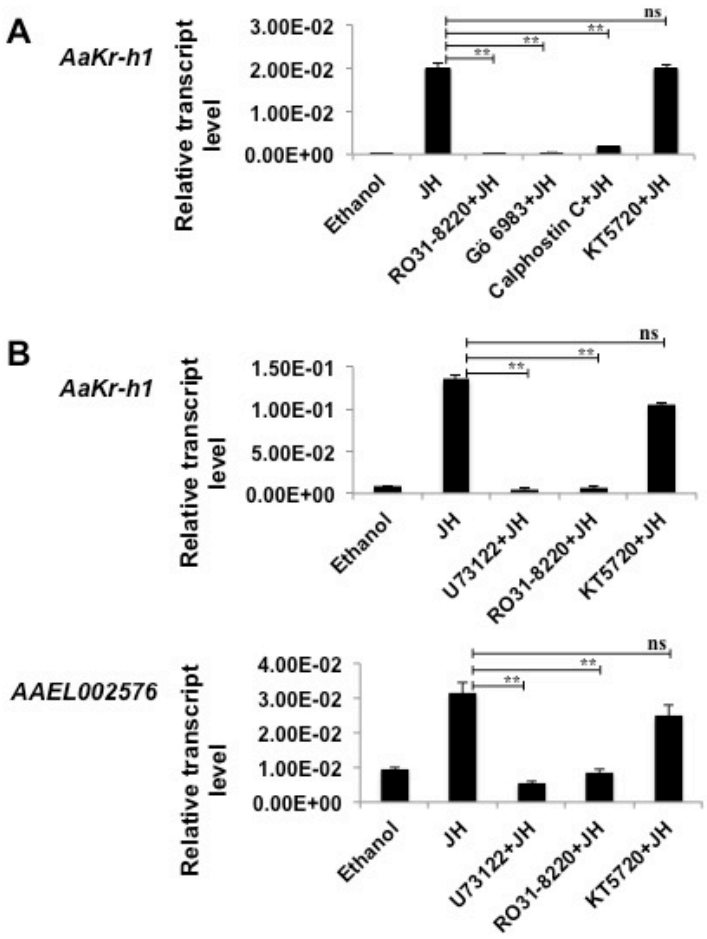

Figure 2.2. PKC activity is required for the JH-induced expression of *AaKr-h1* in mosquito cells. (*A*) Aag-2 cells were pretreated with the three PKC inhibitors (RO31-8220, Gö6983 and calphostin C) or the PKA inhibitor KT5720 for 1 h. Ethanol was used as a negative control. JH-III was added to the culture medium at a final concentration of 5 μM and cells were incubated for an additional hour. The expression level of *AaKr-h1* was measured using real-time PCR. Results are the mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p<0.05$; **, $p<0.01$; ns, not significant, if $p>0.05$). (*B*) Fat bodies from newly emerged *Ae. aegypti* mosquitoes were cultured *in vitro* and incubated with the indicated inhibitors for 1 h. After JH-III (5 μM) was added to the culture medium, the fat bodies were cultured for another hour and collected for RNA extraction. The expression levels of *AaKr-h1* and *AAEL002576* were measured by real-time PCR. Results are the mean ± S.D. of three independent experiments.

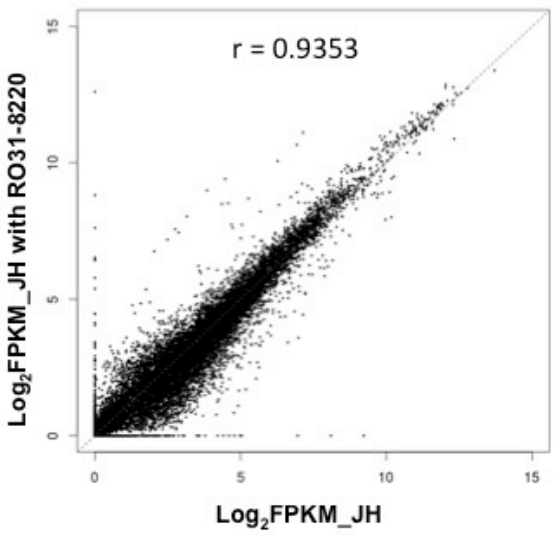

Figure 2.3. Inhibition of PKC does not alter global gene expression in mosquito cells. Aag-2 cells were cultured in the presence of ethanol (as a negative control), JH III (5 μM), or JH III together with the PKC inhibitor (RO31-8220 (10 μM) for 1h.RNA sequencing was performed to compare transcriptomes of the cells after different treatments. The scatter plot shows normalized read counts for each gene between the treatment of JH and the treatment of JH with RO31-8220. FPKM - fragments per kilobase of exon per million fragments mapped.

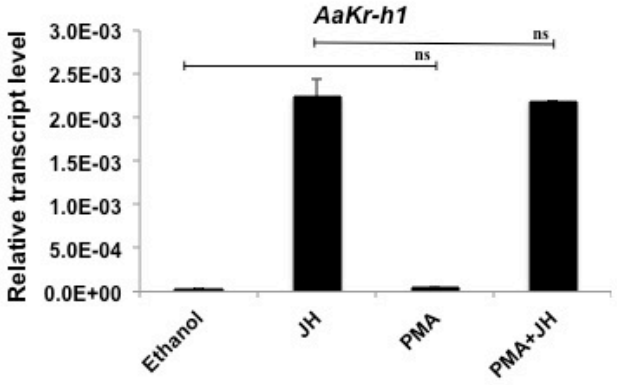

Figure 2.4. Activation of PKC alone is not sufficient to induce *AaKr-h1* expression. Aag-2 cells were treated with ethanol, JH-III (5 μM), PKC activator PMA (10 μM), or a combination of PMA and JH-III. Total RNA was extracted from the cells one hour after the treatment to measure abundance of the *AaKr-h1* mRNA using real-time PCR. Data represent mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test. ns, not significant, $p>0.05$.

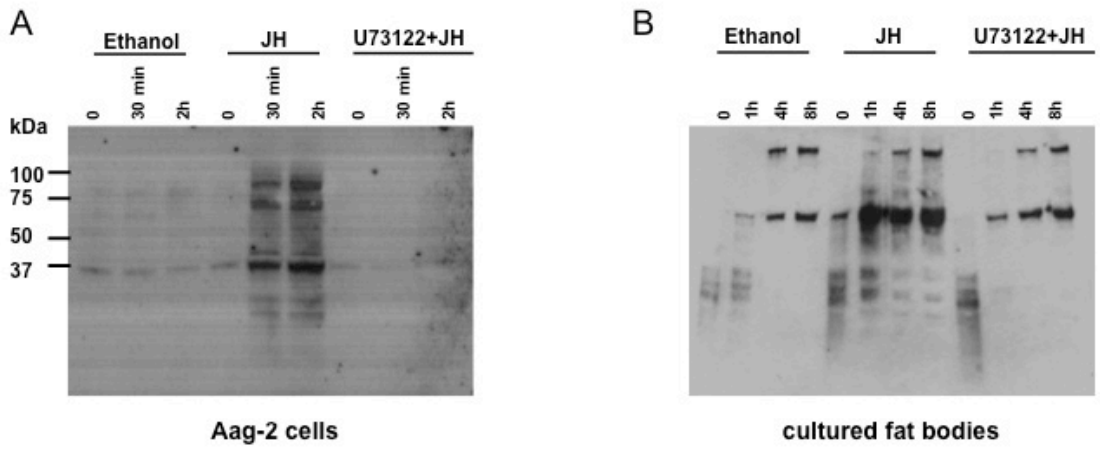

Figure 2.5. JH treatment activates the kinase activity of PKC. (*A*) Whole cell lysate was extracted from Aag-2 cells treated with ethanol, JH-III (5 μM), and the PLC inhibitor U73122 (1 μM) together with JH-III. Equal amount of proteins (20 μg/lane) was loaded into the wells of the SDS-PAGE gel. Western blot analysis was conducted using a phospho-(Ser) PKC substrate antibody. (*B*) Fat bodies from newly emerged mosquitoes were cultured *in vitro* with ethanol, JH-III (5 μM) and U73122 (1 μM) for the indicated time periods. To inactivate PLC, fat bodies were pre-incubated with U73122 for 1 h before JH-III was added to the culture. Protein extracts were subjected to Western blot analysis using the phospho-(Ser) PKC substrate antibody.

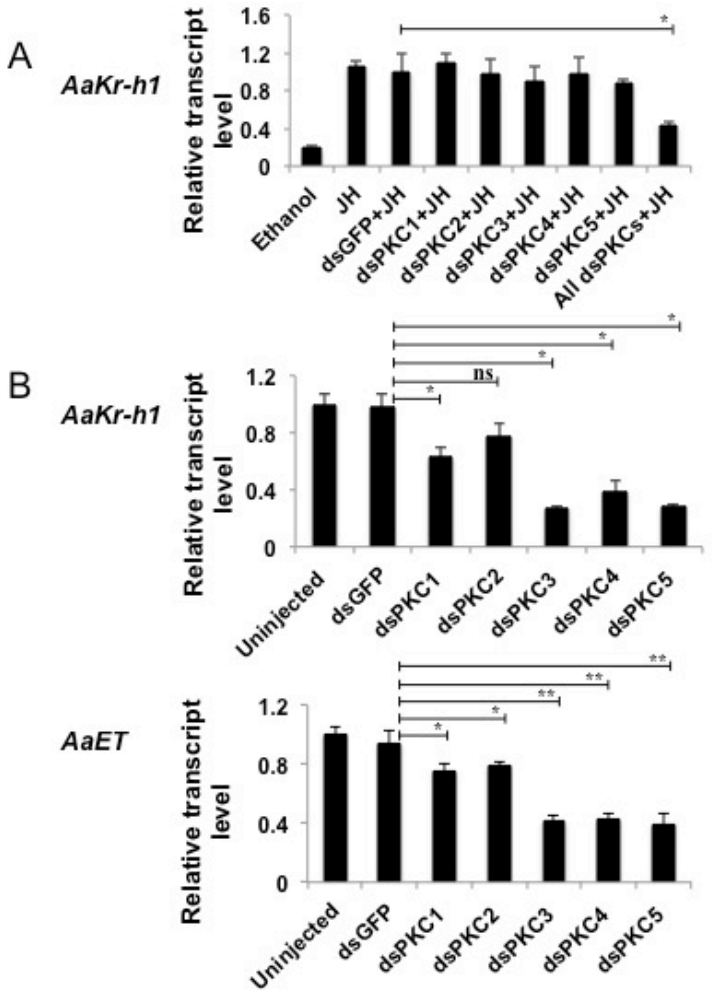

Figure 2.6. RNAi analysis of the *Ae. aegypti* PKC genes in the genomic response to JH. (*A*) Aag-2 cells in a 6-well plate were treated with dsRNAs (5 μg per well) for individual PKC isoforms (AaPKC 1 to 5) or a mix of dsRNAs (a total of 5 μg per well) for all five PKC isoforms. DsRNA for GFP was used as negative control. After incubating with the dsRNAs for 72 h, cells were treated with JH-III (5 μM) for 1 h. Total RNA was then extracted from the cells and the expression of *AaKr-h1* was measured using real-time PCR. For comparison, expression in the cells treated with the dsRNA for GFP was set as 1. Data represent mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p<0.05$; **, $p<0.01$; ns, not significant, $p>0.05$). (*B*) Newly emerged female mosquitoes were injected with dsRNAs for individual PKC isoforms within 1 h after eclosion. At 72 h after injection, total RNA was extracted from the mosquitoes. The amount of *AaKr-h1* or *AaET* transcripts in the uninjected control mosquitoes was set as 1.

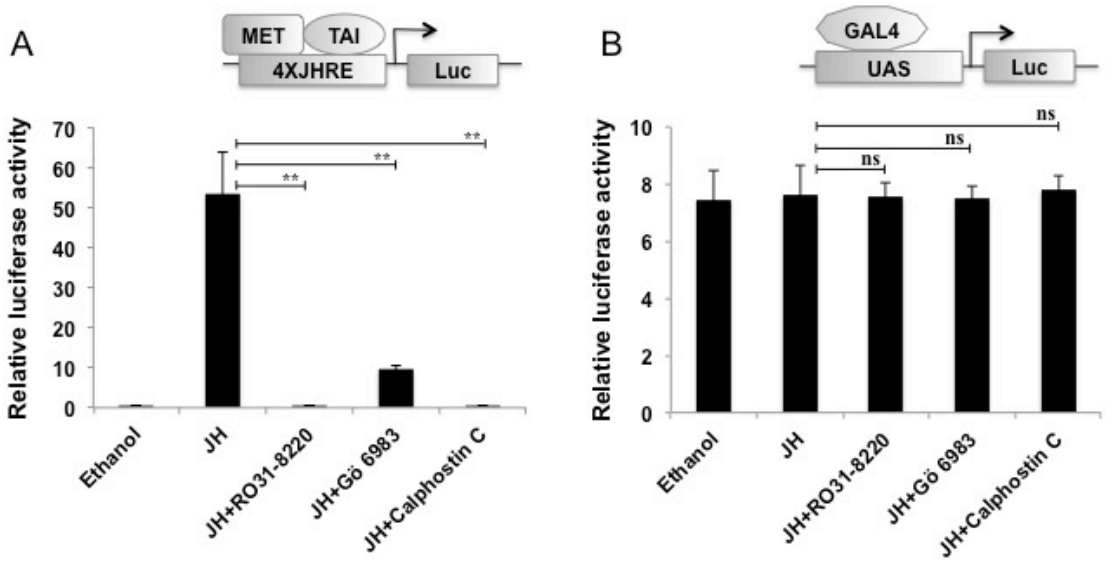

Figure 2.7. Inhibition of PKC lessens JH-induced gene activation by the MET-TAI complex. (*A*) Aag-2 cells were transfected with the following plasmids: pCMA-AaMET, pCMA-AaTAI, 4×JHRE-luc firefly luciferase reporter construct, and a constitutively expressed Renilla luciferase plasmid (pRL-CMV, Promega). At 24 h after transfection, cells were treated with the inhibitors for 1 h before JH-III (5 μM) was added to the medium; the culture was extended for additional 4 h. Firefly luciferase activity was normalized to the Renilla luciferase activity of each sample. Results represent mean ± S.D. for three independent experiments. Statistical analysis was conducted by paired t-test (**, $p<0.01$; ns, not significant, $p>0.05$). (*B*) Aag-2 cells were transfected with pCMA-GAL4, UAS×4-188-cc-Luc and pRL-CMV. Transfected cells were treated with JH-III and the PKC inhibitors under the same conditions as described in (*A*).

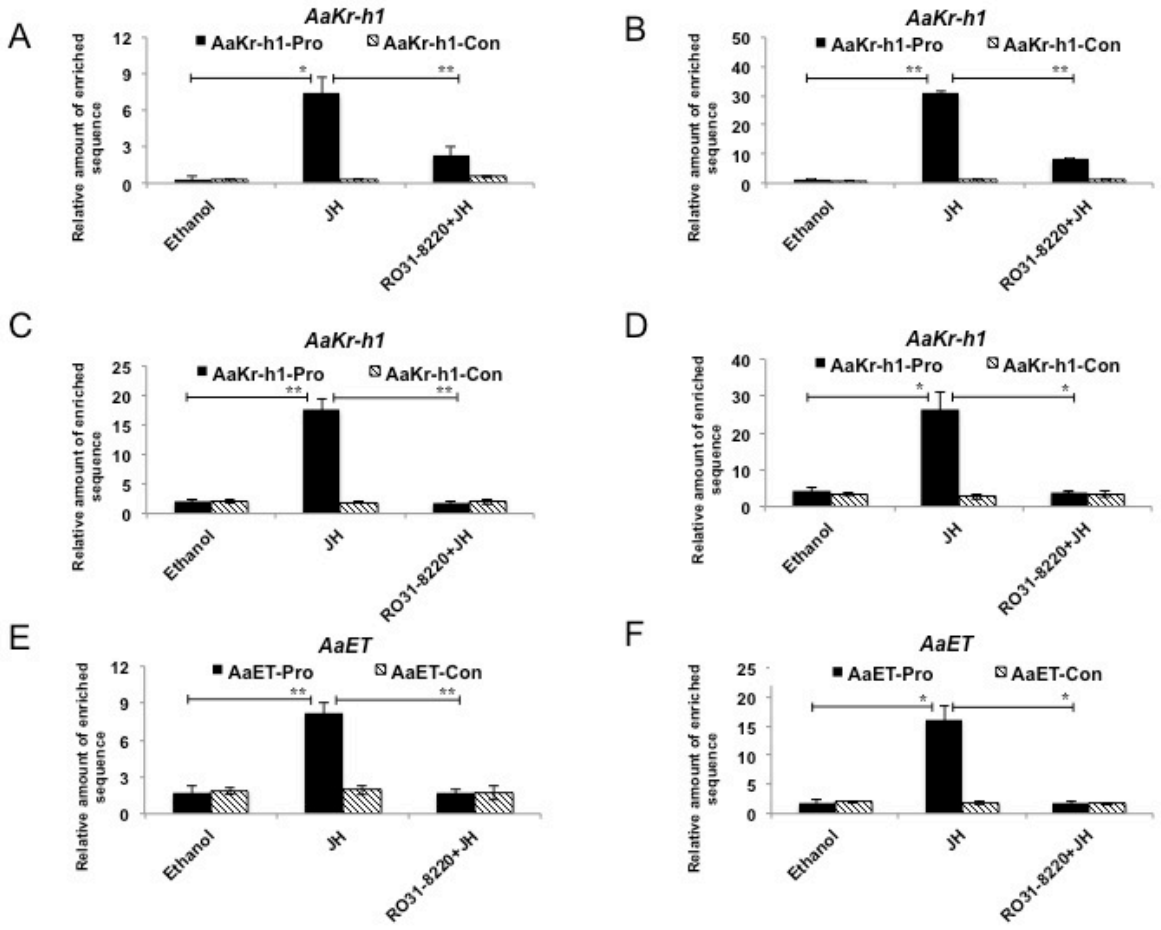

Figure 2.8. The JH-induced PKC activity is required for MET to bind to JHRE. ChIP assays were performed using antibodies against AaMET (*A, C* and *E*) and AaTAI (*B, D* and *F*). (*A* and *B*) Aag-2 cells were treated with ethanol, JH-III and the PKC inhibitor RO31-8220 as indicated. (C-F) Abdomens of newly emerged mosquitoes were cultured *in vitro* and treated with ethanol, JH III and RO31-8220 as indicated. After chromatin immunoprecipitation, the precipitated DNA was analyzed using real-time PCR. For each JH target gene, two pairs of primers were designed to amplify the proximal promoter (Pro) region and a control (Con) region. Results are shown as a percentage of input chromatin and represent mean value ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (*, $p<0.05$; **, $p<0.01$).

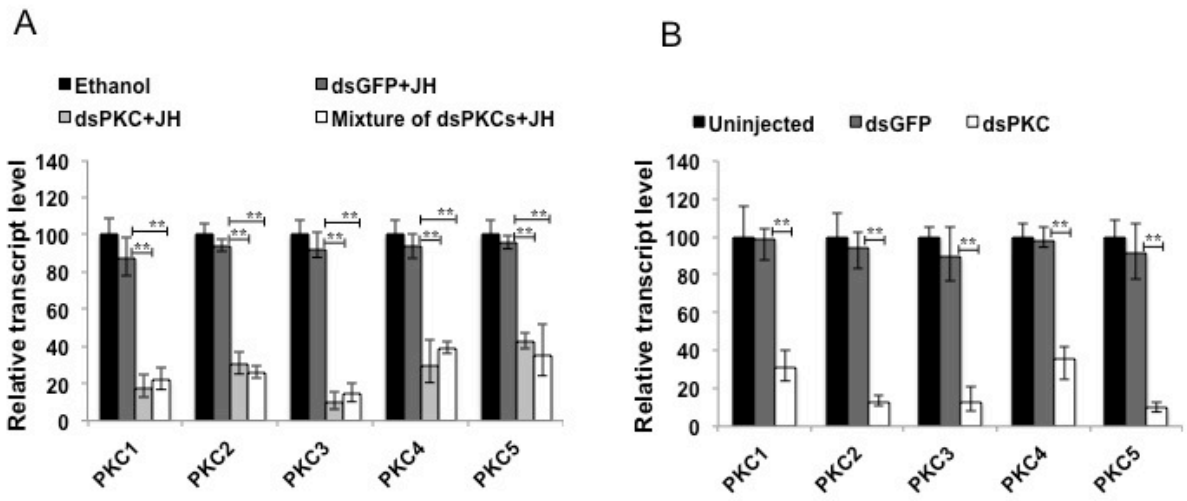

Figure 2.9. The efficiency of gene knockdown by RNAi. Effective knockdown of the PKC isoforms in Aag-2 cells (A) and in the injected mosquitoes (B) was verified by real-time PCR analysis. The amount of PKC transcripts in the untreated control group was set as 100%. Results are the mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test. **, $p<0.01$.

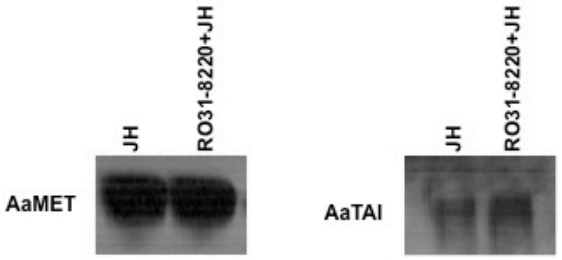

Figure 2.10. The protein levels of AaMET and AaTAI in Aag-2 cells were not affected by inhibition of PKC. Aag-2 cells were treatedwith JH alone or JH together with the PKC inhibitor RO31-8220. The nuclear proteins were extracted and analyzed by Western blot using antibodies for AaMET and AaTAI. An equal amount of protein (30 μg/lane) was loaded in each lane.

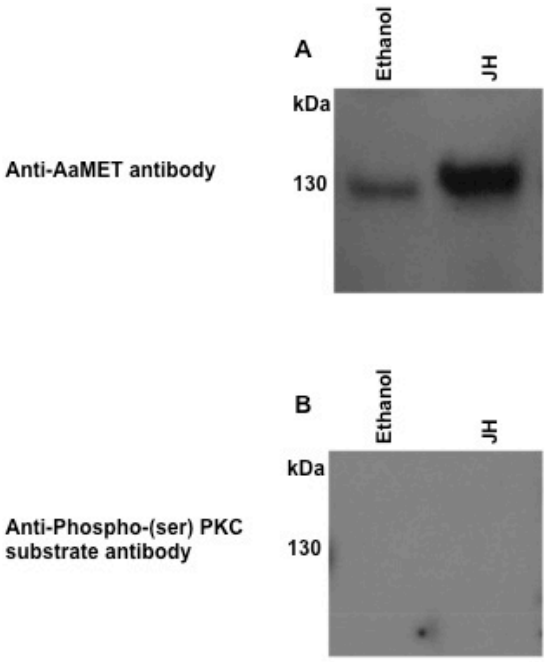

Figure 2.11. Examination of the phosphorylation of MET by PKC. Nuclear proteins were extracted from Aag-2 cells 1h after adding JH-III (5 μM) to the cultural medium, AaMET was purified by immunoprecipitation with AaMET antibody. The pellets were analyzed by Western blot using AaMET antibody (A) and the phosphor-(Ser) PKC substrate antibody (B).

Table 2.1. Primers used in real-time PCR.

Gene	**Primer**	**Sequence (5'-3')**
AarpS7	Fwd	TCAGTGTACAAGAAGCTGACCGGA
	Rev	TTCCGCGCGCGCTCACTTATTAGATT
AaKr-h1	Fwd	TTCTCGCAACAACAGCAACATCCG
	Rev	TCATCAGATCCATTGACGCTGGGT
AaET	Fwd	AATACAGATCCTGCGGCCTA
	Rev	CCTCACCCGCAGTATAATGG
AaPKC1	Fwd	CGTCAGTTCACTTCGGAGAA
	Rev	GATGTGTTGCACAAATTCCG
AaPKC2	Fwd	GGGTGATTTGATGTTCCACA
	Rev	TCAGTCCGGACACGATTTC
AaPKC3	Fwd	ACGCGACATACGATGACATT
	Rev	TTGTGGTGGATCGTTCTATGA
AaPKC4	Fwd	GAAGCATCTACCGAAGAGGTG
	Rev	GTGGCAGAACGCACAGAA
AaPKC5	Fwd	ATCAAATTCCTCAACCCGAT
	Rev	CGTTGATGTTCATCTGTTTCG

Table 2.2. Primers for making DNA templates to synthesize dsRNAs.

Gene	**Primer**	**Sequence (5'-3')**
AaPKC1	Fwd	TAATACGACTCACTATAGGGAGCAGCAGTCCGTTTAGCATAGATT
	Rev	TAATACGACTCACTATAGGGAGACAAGGTGCCACATTAAATAGTAGA
AaPKC2	Fwd	TAATACGACTCACTATAGGGAGACAGGAAGGGTATCGTTTATCGG
	Rev	TAATACGACTCACTATAGGGAGGTTGTTCGGGTTGGTGTAGGTG
AaPKC3	Fwd	TAATACGACTCACTATAGGGAGAAAGAAAGATGAGACCAGACCCAC
	Rev	TAATACGACTCACTATAGGGAGATCACCTTACCGAACGAACCCT
AaPKC4	Fwd	TAATACGACTCACTATAGGGAGACAAACCGTGCAGCAACGAACA
	Rev	TAATACGACTCACTATAGGGAGGACTCCCAGAGCCCACCAATCC
AaPKC5	Fwd	TAATACGACTCACTATAGGGAGTGAAGCGAAACACCAATACACCG
	Rev	TAATACGACTCACTATAGGGAGACCGCCTCCCATAAGCACCACT
GFP	Fwd	TAATACGACTCACTATAGGGAGACGTAAACGGCCACAAGTTC
	Rev	TAATACGACTCACTATAGGGAGTGCTCAGGTAGTGGTTGTCG

Table 2.3. Primers used in real-time PCR for ChIP assay.

Gene	**Primer**	**Sequence (5'-3')**
AaKr-h1-ChIP	Fwd	TTCCGCGGCCAGTCCTCGACAAA
	Rev	TCTCTGCTGCTGCTGCTCACTGA
AaKr-h1-Con	Fwd	TTCTGGAATGTGGATTGTTGA
	Rev	CCTTTGCTTTCGTTCACTCA
AaET-ChIP	Fwd	GTTTTGAAATTACCCATCCCACACG
	Rev	GTCCATTCCTATGATGCGGATTCTT
AaET-Con	Fwd	GTAAGGATTCTTGCCAGGGAGACTC
	Rev	ATCCATTGGCGAACAGTGGACAC

Chapter 3: Krüppel homolog 1 can activate and repress the expression of juvenile hormone response genes in mosquitoes

3.1. Abstract

Juvenile hormone (JH) regulates insect metamorphosis and reproduction through the intracellular receptor Methoprene-tolerant (MET). In the presence of JH, MET forms a heterodimer with its DNA-binding partner Taiman (TAI). This complex binds to JHREs in JH target genes and activates their transcription. *Kr-h1* is a JH early response gene that is under the direct control of MET and TAI. Kr-h1 encodes a zinc finger protein that represses insect metamorphosis. The molecular function of Kr-h1 during metamorphosis remains elusive. The role of Kr-h1 in JH-regulated reproduction is largely unknown. Here we report that AaKr-h1 functions as an important transcription regulator in adult *Ae. aegypti* mosquitoes. The amount of Kr-h1 proteins increases with rising JH levels in newly emerged adult mosquitoes, reaches its peak at 48 h after eclosion, then decreases gradually and disappears after blood feeding. RNAi-mediated depletion of AaKr-h1 substantially reduced egg production after blood feeding. Using a ChIP-cloning approach, we identified *in vivo* AaKr-h1 chromatin binding sites in adult female *Ae. aegypti*. RNAi mediated knockdown of AaKr-h1 leads to up-regulation or down-regulation of individual AaKr-h1 target genes, suggesting that AaKr-h1 functions as both an activator and a repressor in mediating the JH response in mosquitoes.

3.2. Introduction

The sesquiterpenoid juvenile hormone (JH) is known for its anti-metamorphic action. JH delays metamorphosis of immature larvae until they have reached a proper size and stage. In the last

larval instar, JH titer goes down and in the absence of JH, the molting hormone (20-hydroxyecdysone (20E)) induces metamorphosis (Nijhout, 1998)

JH also plays important roles in the adult stage of insect life. It is involved in many aspects of reproduction, including the previtellogenic development, vitellogenesis and oogenesis (Hartfelder, 2000). A critical step in egg production is vitellogenesis, in which the yolk protein precursor vitellogenin (Vg) is synthesized in the fat body and is later taken up by the developing oocyte. In *Ae. aegypti,* JH is required during previtellogenic phase to make the fat body to become competent for Vg synthesis (Dittmann et al., 1989; Raikhel and Lea, 1990). In *T. castaneum*, JH regulates Vg synthesis in the fat body; knockdown of the JH receptor MET causes a dramatic decrease in Vg expression and blocks oocyte maturation (Parthasarathy et al., 2010).

The molecular mechanism underlying JH action has been partially elucidated only in recent years. An important transducer in the JH signaling pathway is its nuclear receptor Methoprene-tolerant (MET). MET was first identified in a *D. melanogaster* mutant that was resistant to the toxic dose of JH (Ashok et al., 1998a; Wilson and Fabian, 1986). It belongs to the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors (Ashok et al., 1998a; Charles et al., 2011; Jindra et al., 2015). Studies in *T. castaneum* showed that depletion of MET induces larvae to undergo precocious metamorphosis, indicating that JH exerts its anti-metamorphic action through MET (Konopova and Jindra, 2007). In response to JH, MET forms a heterodimer with another bHLH-PAS protein, Taiman (TAI) (Li et al., 2011b). TAI acts as the DNA-binding partner of MET and the MET-TAI complex recognizes an E-box like sequence (5'-GCACGTG-3') in the regulatory regions of JH-responsive genes, leading to the transcriptional activation of these genes (Li et al., 2014a). MET-regulated genes in *Ae. aegypti* have been

identified by microarray analysis of adult mosquitoes after knockdown of MET (Zou et al., 2013).

Another key component in JH signal transduction is the Kr-h1 protein. *Kr-h1* was identified in 2008 as a JH target gene in the JH-treated abdomen of *D. melanogaster* (Minakuchi et al., 2008b). The JH-induced expression of *Kr-h1* has since been reported in other insects including *T. castaneum* (Kayukawa et al., 2013; Minakuchi et al., 2009), *B. mori* (Kayukawa et al., 2012a; Wang et al., 2012a), *Ae. aegypti* (Li et al., 2011b; Shin et al., 2012; Zhang et al., 2011a), *P. apterus* (Konopova et al., 2011) and *B. germanica* (Lozano and Belles, 2011b). JH activates *Kr-h1* expression via MET as knockdown of MET in *Tribolium* disrupted the JH-induced *Kr-h1* expression (Minakuchi et al., 2009; Parthasarathy et al., 2008). The JHREs that are recognized by the MET-TAI complex has been found in the regulatory regions of *Kr-h1* gene in *T. castaneum* (Kayukawa et al., 2013), *B. mori* (Kayukawa et al., 2012a) and *Ae. aegypti* (Shin et al., 2012; Zou et al., 2013). Knockdown of Kr-h1 in young *Tribolium* larvae caused formation of premature pupa and the exogenous JH application couldn't rescue that phenotype (Minakuchi et al., 2009). The role of Kr-h1 in mediating the anti-metamorphic action of JH is conserved in holometabolous insects such as *D. melanogaster* (Minakuchi et al., 2008a) and *T. castaneum* (Minakuchi et al., 2009) and in hemimetabolous insects such as *B. germanica* (Lozano and Belles, 2011b) and *P. apterus* (Konopova et al., 2011).

Kr-h1 contains eight C_2H_2 type zinc fingers that are highly conserved among holo-, hemi- and ametabolous insects. This protein also contains less conserved Glutamine-rich and Proline/Serine/threonine rich regions at N- and C- termini, respectively (Konopova et al., 2011; Pecasse et al., 2000). Proteins that contain C_2H_2 type zinc fingers have quite diverse functions,

which range from interactions with DNA/RNA to protein-protein interactions to membrane association (Laity et al., 2001).

Because of its eight zinc fingers, it has been suggested that Kr-h1 functions as a transcription factor and mediates the effect of JH on downstream gene expression (Kayukawa et al., 2016). Recently, a Kr-h1 binding site (KBS) was identified in the promoter of the *broad-complex* gene (*BR-C*) and the direct binding of Kr-h1 to that KBS was shown in an electrophoresis mobility shift assay (Kayukawa et al., 2016). Although that information is valuable, it is not supported by *in vivo* binding or interaction of Kr-h1with other JH regulated genes.

Here we aim to find chromatin binding sites and target genes of Kr-h1 in adult *Ae. aegypti* mosquitoes. Here using ChIP-cloning assay, we reported the *in vivo* binding of AaKr-h1 to several locations in *Ae. aegypti* genome. Maximal binding of Kr-h1 to those locations takes place at 48 h post eclosion, roughly coincident with the peak of the Kr-h1 protein. Using RNAi experiments we showed that AaKr-h1 functions as a transcription factor, regulates the expression of its direct target genes and plays an essential role in egg production. Interestingly, we showed that depletion of Kr-h1 caused upregulation of some of its target genes but led to downregulation of some other target genes, suggesting that Kr-h1 can both activate and repress gene expression in response to JH.

3.3. Materials and Methods

3.3.1. Mosquito rearing and Cell culture

Ae. aegypti mosquitoes of the Liverpool strain were maintained at 28°C and 60–70% humidity, with a 14/10 h day/night light cycle. Mosquito larvae were fed on pulverized fish food (TetraMin Tropical Flakes) and adults were provided with a 10% sucrose solution. Female mosquitoes (7 days post eclosion) were fed on anesthetized mice to stimulate egg production.

Drosophila S2 cells (Schneider, 1972) and *Ae. aegypti* Aag-2 cells (Lan and Fallon, 1990) were cultured at 28°C in Schneider's *Drosophila* media (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). Cells were passaged every two to three days to maintain the exponential growth.

3.3.2. Plasmids and Cloning

The expression vector pGEX-6p-1 was purchased from GE healthcare. The codon usage of *AaKr-h1* cDNA was optimized for bacterial expression. The optimized cDNA for the N-terminal (amino acid residues 1-461) and C-terminal (amino acid residues 363-702) Kr-h1 was cloned separately into pGEX-6p-1 between BamHI and XhoI restriction sites (Table 3.2), resulting the following expression plasmids: NKrh-pGEX-6p-1 and CKrh-pGEX-6p-1.

pAc5.1/V5-His A was purchased from Invitrogen. cDNA of full-length *AaKr-h1* was cloned between Kpn I and Xho I restriction sites of pAc5.1/V5-His A vector (Table 3.3).

3.3.3. Expression and purification of recombinant AaKr-h1

NKrh-pGEX-6p-1 and CKrh-pGEX-6p-1 plasmids were transformed into *Escherichia coli* BL21 (DE3). The cells were cultured in Luria-Bertani (LB) medium at 37°C to reach an OD_{600} of 0.8. After that isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the media to a final concentration of 0.1 mM and the cultures were grown at 28°C for six more hours. The cell pellet was re-suspended in lysis buffer (150 mM NaCl, 20 mM sodium phosphate, pH 7.3, 2 mM DTT, 1 mM PMSF, 1× Halt protease inhibitor (Thermo Scientific)). DeBEE high pressure homogenizer (BEE international) was used to lyse the cells. Affinity purification was carried out using ÄKTA prime and GSTrap FF columns (GE Healthcare). The buffers used for this protein purification were: binding buffer (20 mM sodium phosphate, pH 7.3, 150 mM NaCl, 2mM DTT) and elution buffer (50 mM Tris-HCl, pH 8, 10 mM reduced glutathione, 2 mM DTT). Purified

proteins were dialyzed in PBS buffer and then concentrated using Pierce concentrators. One milligram each of NKr-h1 and CKr-h1 were combined and sent out to Thermo scientific for antibody production using rabbit.

3.3.4. Affinity purification of Kr-h1-specific antibody

The polyclonal antibody was antigen affinity purified from the rabbit antisera using Amino link plus coupling resin column (Thermo scientific) according to the manufacturer's instruction. The specificity of the AaKr-h1 antibody was tested in western blot using S2 cells that were transfected with the pAc5.1/V5-His A-Full AaKr-h1 plasmid to express V5-tagged full-length AaKr-h1. Transfected S2 cells were lysed using RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Rabbit IgG (as control) and AaKr-h1 antibody were used to precipitate AaKr-h1-V5 from lysates of the transfected cells. The pellet was analyzed by western blot using V5 antibody.

3.3.5. S2 cells transfection

Drosophila S2 cells were cultured in 6-well plates and allowed to settle overnight. For each well, in 100 µl of medium without supplementation, 20 ng/µl final concentration of plasmid (pAc5.1/V5-His A-Full AaKr-h1) were mixed with FuGENE HD Transfection Reagent (Promega) at a ratio of 3:2 (DNA:FuGENE). The mixture was incubated at room temperature for 15 min and was then added into each well. After two days' incubation, the cells were collected and lysed using RIPA buffer.

3.3.6. Western blot

Whole cell lysates were extracted from S2 cells or adult female *Ae. aegypti* mosquitoes, using RIPA buffer. Protein concentrations were measured using the bicinchoninic acid (BCA) assay. An equal amount of protein was loaded in each lane and separated on a SDS-PAGE gel in all experiments. Anti V5 antibody (used at 1:5000 dilution) was purchased from Thermo Scientific. Anti-GAPDH antibody (Thermo Scientific) was used at 1:5000 dilution as a loading control for immunoblotting.

3.3.7. ChIP-cloning

3.3.7.1. Chromatin immunoprecipitation (ChIP)

Abdomens were collected from 100 adult female mosquitoes for each ChIP-cloning experiment. The tissue was grounded into a fine powder in liquid nitrogen. The ChIP assay was performed using SimpleChIP Plus Enzymatic Chromatin Immunoprecipitation Kit (Cell signaling Technology) according to the manufacturer's instruction. Briefly, the tissue was homogenized in PBS on ice. Formaldehyde was added to a final concentration of 1%, and crosslinking was performed for 10 minutes at 37°C. Crosslinking was stopped by adding 1.25 M glycine to reach a final concentration of 125 mM. After nuclei preparation, chromatin digestion was performed using 0.25 µl of Micrococcal nuclease for 5 min at 37°C. Immunoprecipitation was carried out using the purified AaKr-h1 antibody.

3.3.7.2. Cloning, sequencing, and analysis of AaKr-h1-binding fragments

After eluting the DNA, T4 DNA ligase (New England Biolabs) was used to set up the blunting reaction according to the manufacturer's instruction. Briefly, 2.5 µg of DNA and 0.75 µl of T4 DNA ligase enzyme was used to set up the blunting reaction and incubated for 15 minutes at

12°C. To stop the reaction 10mM EDTA was added to the tube and incubated for 20 minutes at 75°C. QIGEN mini-elute column was used to clean the DNA. TOPO cloning reaction was set up as indicated in the manufacturer's instruction (TOPO TA cloning kit for sequencing (Thermo Scientific)). The produced plasmid library was then transformed into 10-beta competent *E. coli* cells and cultured in LB-kanamycin agar plates. Plasmid DNA was isolated from sixty randomly picked clones and was sent for DNA sequencing. The results were analyzed using *Ae. aegypti* genome database.

3.3.8. Quantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Life Technologies) from *Ae. aegypti* mosquitoes or Aag-2 cells. The first strand cDNA was synthesized with oligo (dT) primer and reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed in triplicate on an ABI 7300 system (Applied Biosystem) using the GoTaq qPCR Master Mix (Promega). Transcript abundance was normalized to that of RpS7. The primers used in qRT-PCR are listed in (Table 3.4).

3.3.9. Double-stranded RNA (dsRNA)-induced gene silencing (RNAi)

DsRNAs were synthesized by *in vitro* transcription of PCR-generated DNA templates (Table 3.5) and were injected into *Ae. aegypti* mosquitoes as described previously (Zhu et al., 2003). Briefly, female mosquitoes were injected with 0.5 μg of dsRNA within 30 min post-eclosion. DsRNA for green fluorescent protein (GFP) was used as a negative control. Four days after dsRNA injection, RNA was extracted and the effect of AaKr-h1 knockdown on the expression of candidate genes was analyzed by real-time PCR.

3.4. Results

3.4.1. *AaKr-h1* expression profile in adult *Ae. aegypti* mosquitoes

In order to obtain the expression profile, qRT-PCR was used to measure the mRNA levels of *AaKr-h1* in adult female mosquitoes collected at 0 h, 12 h, 24 h, 36 h, 48 h, 96 h post eclosion (PE) and also at 12 h post blood meal (PBM). As expected, the mRNA profile of *AaKr-h1* correlated well with the JH titer in mosquito (Figure 3.1.A). During post-emergence development, JH titer increases 10-fold within 48 h and after that it gradually declines (Shapiro et al., 1986). The mRNA level of *AaKr-h1* raised gradually after eclosion and increased 2.8 fold ($p<0.01$) to the highest level at 48 h after eclosion. After blood ingestion, the expression of *AaKr-h1* decreased dramatically; the amount of AaKr-h1 mRNA at 12 h PBM was lower than that at eclosion (Figure 3.1.A).

The AaKr-h1 protein was also examined in adult female mosquitoes collected from the abovementioned time points. For this purpose an antibody was raised against AaKr-h1. As the full-length AaKr-h1 was not expressed in soluble form, the N-terminal (amino acid residues 1-461) and the C-terminal (amino acid residues 363-702) fragments of AaKr-h1 were expressed in *E.coli* as fusion proteins with a GST tag (Figure 3.4). After affinity purification, these two protein fragments were combined and sent to Thermo scientific for custom-antibody production using rabbits. Polyclonal AaKr-h1 antibodies were affinity purified from the rabbit antisera, using immobilized antigens.

Western blot was performed using anti-AaKr-h1 antibody. The AaKr-h1 protein, undetectable in mosquitoes at 0 h and 12 h post eclosion, became evident at 24 h (Figure 3.1.B). The protein level continued to increase, peaked at 48 h and decreased considerably at 96 h post eclosion. At 12 h after a blood meal, AaKr-h1 was undetectable again (Figure 3.1.B). The *AaKr-h1* mRNA

profile correlated well with its protein profile and was consistent with the change of JH titers in adult mosquitoes (Shapiro et al., 1986).

3.4.2. JH induces *AaKr-h1* expression via AaMET

JH activates *AaKr-h1* expression in Aag-2 cell line. Aag-2 cells treated with 5 μM JH-III for 1 h resulted in a 11-fold increase in the *AaKr-h1* mRNA, compared with the cells treated with ethanol ($p<0.01$) (Figure 3.2.A). To examine the involvement of AaMET in the JH-induced expression of *AaKr-h1*, newly emerged adult female mosquitoes were injected with dsRNA against AaMET or GFP (as control). Three days later, the mRNA level of *AaKr-h1* was measured by qRT-PCR in the un-injected, dsGFP- and dsMet-injected mosquitoes. The result showed that depletion of AaMET reduced the expression of *AaKr-h1* by 88% ($p<0.01$) (Figure 3.2.B), confirming that JH regulates the expression of *AaKr-h1* via AaMET.

3.4.3. AaKr-h1 is required for egg production in *Ae. aegypti* mosquitoes

JH plays an important role in controlling insect reproduction (Riddiford, 2012). To examine the role of AaKr-h1 in egg production in *Ae. aegypti*, the expression of *AaKr-h1* was knocked down using RNAi. Newly emerged adult female mosquitoes were injected with dsRNA for *AaKr-h1* or *GFP*. Five days after injection, un-injected, dsGFP-injected and dsKr-h1-injected mosquitoes were blood-fed on anesthetized mice. The number of eggs laid by each female mosquito was counted and analyzed using GraphPad software (Figure 3.3.A). The results indicated that dsKr-h1-injected mosquitoes produced 46% fewer eggs compared with the un-injected ones ($p<0.001$). However there was no significant difference in the number of eggs between the un-injected and dsGFP-injected controls ($p>0.1$). These results showed that AaKr-h1 plays an important role in JH-regulated mosquito reproduction. The successful knockdown of AaKr-h1 was confirmed by

qRT-PCR using mosquitoes randomly picked from the un-injected, dsGFP-injected and dsKr-h1-injected groups (three mosquitoes from each group) (Figure 3.3.B).

3.4.4. Identification of AaKr-h1 chromatin binding sites and target genes in adult mosquitoes

To find genes that are directly regulated by AaKr-h1, a ChIP experiment was performed to clone the DNA binding sites of AaKr-h1 in mosquitoes. To test whether the purified AaKr-h1 antibody can bind specifically to native AaKr-h1, S2 cells were transfected with pAc5.1/V5-His A-Full AaKr-h1 vector to express full length V5-tagged AaKr-h1. The cell lysates were incubated with the AaKr-h1 antibody or nonspecific rabbit IgG (as control). The precipitated pellets were analyzed by western blot using the V5 antibody. When the immunoprecipitation (IP) experiment was performed with the control IgG, no band was detected in the western blot (Figure 3.5). However, when the IP experiment was performed with the AaKr-h1 antibody, the AaKe-h1-V5 fusion was detected at the expected size (Figure 3.5). This data confirmed that the antibody specifically recognize AaKr-h1 and can be used in ChIP-cloning experiment.

Abdomens were collected from one hundred female *Ae. aegypti* mosquitoes at 48 h PE. Regular ChIP procedure was performed in which formaldehyde was used to covalently stabilize protein-DNA complexes. After chromatin was fragmented through nuclease digestion, the specific antibody was used to capture AaKr-h1 with its associated genomic DNA. Cross-linking reversal was then performed and the DNA was purified. The DNA fragments were cloned into TOPO cloning vector. Several hundred colonies were obtained and plasmid DNA was purified from 60 randomly picked clones for DNA sequencing. Figure 3.6 summarizes ChIP cloning procedure.

Bioinformatics analysis was performed using the *Ae. aegypti* genome database to localize the DNA fragments associated with AaKr-h1 and subsequently identify the AaKr-h1 target genes.

After positioning an AaKr-h1-binding sequence in the *Ae. aegypti* genome, the nearest gene to that sequence was considered as the AaKr-h1 target gene. The distances between the AaKr-h1-binding regions and their closest annotated genes are listed in the Table 3.1.

To verify the DNA binding of AaKr-h1 that was identified by ChIP-cloning, two ChIP experiments were performed. In the first assay, the abdomens of female *Ae. aegypti* mosquitoes were collected at 48 h PE. The ChIP assay was conducted with the AaKr-h1 antibody and Rabbit IgG (as control). Enrichment of four identified AaKr-h1 DNA-binding sites (regulatory regions of *AaEL000741*, *AaEL003068*, *AaEL005648* and *AaEL003080*) was compared between the DNA precipitated by the AaKr-h1 antibody and the nonspecific rabbit IgG. Significant higher enrichment was observed for all four binding sites when ChIP was performed with the AaKr-h1 antibody (Figure 3.7.A). In the second assay, ChIP was performed using the AaKr-h1 antibody to analyze the binding of AaKr-h1 to different regions of the mosquito gene *AaEL005957*, in which a sequence in the 5' upstream regions was identified in the ChIP-cloning experiment. After DNA purification, qPCR was carried out to amplify the 5' regulatory region and the coding sequence of *AaEL005957*. The result showed that binding of AaKr-h1 to the identified regulatory region was 8.1 fold stronger than the control region ($p<0.05$) (Figure 3.7.B). Theses two experiments verify the DNA binding of AaKr-h1 to the sequences that were identified in ChIP-cloning.

3.4.5. The DNA binding patterns of AaKr-h1 on individual target genes

The dynamics of AaKr-h1 binding to the candidate genes was investigated in female mosquitoes collected at 0 h, 12 h, 24 h, 36 h, 48 h, 96 h post eclosion (PE) and at 12 h post blood meal (PBM). ChIP experiment was performed using the AaKr-h1 antibody, and rabbit IgG was used as control. qPCR analysis of the eluted DNA showed that for all six sequences examined (regulatory regions of *AaEL005810*, *AaEL013177, AaEL005957, AaEL014226*, *AaEL004444*

and *AaEL005545*), the *in vivo* binding of AaKr-h1 increased after eclosion and reached to the highest level within 48 h (Figure 3.8). Also, for all examined sequences, the binding of AaKr-h1 decreased considerably at 96 h post eclosion and dropped further at 12 h post blood meal to the lowest level after eclosion (Figure 3.8). These results are consistent with the protein levels of AaKr-h1 that was previously described in section 3.4.1. In the control experiment with rabbit IgG, background binding remained at relatively low level at different time points (Figure 3.8).

3.4.6. Expression of the AaKr-h1 target genes in previtellogenic adult mosquitoes

If expression of the putative target genes is indeed regulated by AaKr-h1, then there should be a correlation between the mRNA profiles of the target genes and *AaKr-h1*. To test this hypothesis, the mRNAs of six candidate genes were measured by qRT-PCR at 0 h, 12 h, 24 h, 36 h, 48 h, 96 h post eclosion (PE) and 12 h post blood meal (PBM). These target genes, which include *AaEL005810*, *AaEL013177, AaEL005957, AaEL014226*, *AaEL004444* and *AaEL005545,* have been previously reported to be under the control of AaMET (Zou et al., 2013). The expression profiles of *AaEL005810*, *AaEL013177* and *AaEL005957* correlate well with the mRNA profile of *AaKr-h1*. The mRNA levels of these genes peaked at 48 h PE and increased by 1.8, 1.8 and 2.5 fold respectively after eclosion. The mRNAs then decreased to the lowest levels at 12 h PBM (Figure 3.9.A).

Interestingly, the expression of three other genes (*AaEL014226*, *AaEL004444* and *AaEL005545*) showed a different pattern when they were compared with the mRNA profile of *AaKr-h1*. Their mRNA levels generally decreased after adult emergence, dropped to the lowest amounts at 48 h PE, and gradually went up after that (Figure 3.9.B). These data suggested that AaKr-h1 may positively regulate some target genes and negatively regulate other target genes.

3.4.7. Knockdown of AaKr-h1 has opposite effects on individual AaKr-h1 target genes

To assess the effect of AaKr-h1 knockdown on the expression of its target genes, newly emerged female *Ae. aegypti mosquitoes* were injected with dsRNA for *AaKr-h1* or *GFP* (as control). Four days later expression of the six AaKr-h1 target genes (*AaEL005810*, *AaEL013177, AaEL005957, AaEL014226*, *AaEL004444* and *AaEL005545*) were measured in the un-injected, dsGFP-injected and dsKr-h1-injected mosquitoes. The results showed that the RNAi-mediated depletion of AaKr-h1 decreased the expression of *AaEL005810*, *AaEL013177* and *AaEL005957* by 2.9, 1.8 and 1.9 fold, respectively, compared to the un-injected mosquitoes (Figure 3.11.A). However, dsGFP injection did not show any significant effect on the expression of AaKr-h1 target genes (p>0.1). This data indicated that AaKr-h1 functions as an activator for *AaEL005810*, *AaEL013177* and *AaEL005957*, the mRNA profiles of which were all similar to that of *AaKr-h1*. Interestingly, knockdown of AaKr-h1 increased the expression of *AaEL014226*, *AaEL004444* and *AaEL005545* by 2, 2.6 and 1.6 fold, respectively, compared with the un-injected control (Figure 3.11.B). DsGFP injection did not show any significant effect on the expression of these genes (p>0.1). This data showed that AaKr-h1 functions as a repressor on *AaEL014226*, *AaEL004444* and *AaEL005545*. Figure 3.10 conforms the successful knock down of AaKr-h1 in the injected mosquitoes.

3.5. Discussion

Our understanding of the molecular action of JH has increased substantially in recent years: JH is perceived by its nuclear receptor MET; JH-bound MET interacts with its DNA-binding partner TAI to form the JH receptor complex, which regulates the expression of JH response genes such as *Kr-h1*. Regulation of *Kr-h1* expression by JH and MET has been reported in several insects.

The properties of Kr-h1 as a mediator of the anti-metamorphic action of JH has been shown in both hemimetabolous insects and holometabolous ones.

Kr-h1 contains eight highly conserved C_2H_2 type zinc fingers and it has been suggested that Kr-h1 functions as a transcription factor. But yet the properties of Kr-h1 as a transcriptional regulator have not been demonstrated. There is a big gap in our knowledge regarding to the molecular function of this protein. For example, the target genes of Kr-h1, its binding motif and its possible protein partner(s) are still unknown.

The function of Kr-h1 as a repressor has been recently reported in two studies. A study of a *B. mori* cell line has shown that Kr-h1 directly binds to an upstream sequence of *Broad-complex* gene (*BR-C*) and represses its expression (Kayukawa et al., 2016). BR-C is required for larval to pupal transition and its expression is induced by 20E. During larval stage, JH prevents 20E-induced expression of *BR-C*. RNAi-mediated knockdown of *Kr-h1* prevented the JH-mediated repression of *BR-C*, indicating that Kr-h1 exerts its anti-metamorphic action through repressing *BR-C* expression (Kayukawa et al., 2016). A Kr-h1 binding site has been identified in *BR-C* promoter through a reporter assay and the binding of Kr-h1 to that sequence has been confirmed in gel shift assay.

In another study it was found that Kr-h1 represses the expression of an ecdysone-dependent transcription factor called E93 in *B. germanica* (Belles and Santos, 2014). E93 has been shown to trigger adult morphogenesis in several insects including *B. germanica*, *T. castaneum* and *D. melanogaster* (Ureña et al., 2014).

The evidence presented in our current study clearly indicates that Kr-h1 binds to DNA and functions as a bifunctional transcription factor, which is able to activate and repress gene expression. Although most of transcription factors act either as activators or repressors, several

different krüppel-like factors that have been reported to exert both activation and repression functions. For example human EZF (Krüppel-like zinc finger protein that is expressed in vascular endothelial cells and contains three C-terminal zinc fingers) (Yet et al., 1998), GLIS3 (a member of the GLIS subfamily of kruppel like proteins that has five C_2H_2 type zinc fingers) (Kim et al., 2003) and EKLF (Erythroid kruppel like factor in macrophage) (Luo et al., 2004).

In a reporter assay using a series of plasmids containing various fragments of hEZF it was found that there is an activation domain between amino acids 91 and 117 of hEZF and the repression domain exists within the zinc finger domain (amino acid 181-388) (Yet et al., 1998). It was shown that EKLF enhanced the expression of interleukin (IL)-12 p40 in unstimulated macrophage cells while repressed the expression of IL-12 p40 in cells stimulated by IFN-γ/LPS. That dual function of EKLF has been proposed to be through the recruitment of either co-activators or co-repressor (Luo et al., 2004).

The transition from the activator of transcription to repressor is an interesting subject to study. This transactivation activity of Kr-h1 may be converted by its conformational change after binding to different sequence, the combinatory effect of other DNA binding proteins, posttranslational modification of Kr-h1, and interaction with other proteins. We have conducted yeast two hybrid screening, however no meaningful protein interaction involving Kr-h1 was detected under our experimental condition.

We have recently reported that JH activates the phospholipase C (PLC) pathway in *Ae. aegypti* mosquitoes and causes an increase in the concentration of diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP3) and intracellular calcium (Ca^{2+}), which subsequently activates calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Liu et al., 2015a; Ojani et al., 2016). It is possible that Kr-h1 serves as the direct or indirect target of

CaMKII or PKC and that phosphorylation modification switches its function between an activator and a repressor.

MET has been shown to regulate a large number of genes in adult mosquitoes in response to JH (Zou et al., 2013). The early response genes, which include genes encoding transcription factors such as Kr-h1 and Hairy, are likely controlled directly by MET. MET may regulate many other genes indirectly via the action of the early gene products. Indeed, Hairy has been reported to mediate the action of MET in gene repression (Saha et al., 2016). The Hairy target genes identified by RNAi in the fat body of female *Ae. aegypti* mosquitoes overlapped substantially with the JH-repressed genes identified by depletion of MET (Saha et al., 2016).

This is the first report showing that Kr-h1 can function as both an activator and a repressor. To elucidate how Kr-h1 exerts opposite functions, we need to identify more Kr-h1 target genes using ChIP-seq. For that purpose, RNA-seq analysis of AaKr-h1 RNAi mosquitoes is also desired. Having more Kr-h1 target sequences will help to identify Kr-h1 binding motifs and to determine whether that motifs are different between the genes that are activated by Kr-h1 and the ones that are repressed by Kr-h1. Finding the binding motif of Kr-h1 will also open the door to investigate the mechanism of its recruitment on chromatin as it is still unknown whether Kr-h1 directly binds to DNA or it is recruited to DNA via interaction with other DNA-binding proteins.

3.6. Figures, tables and supplemental information

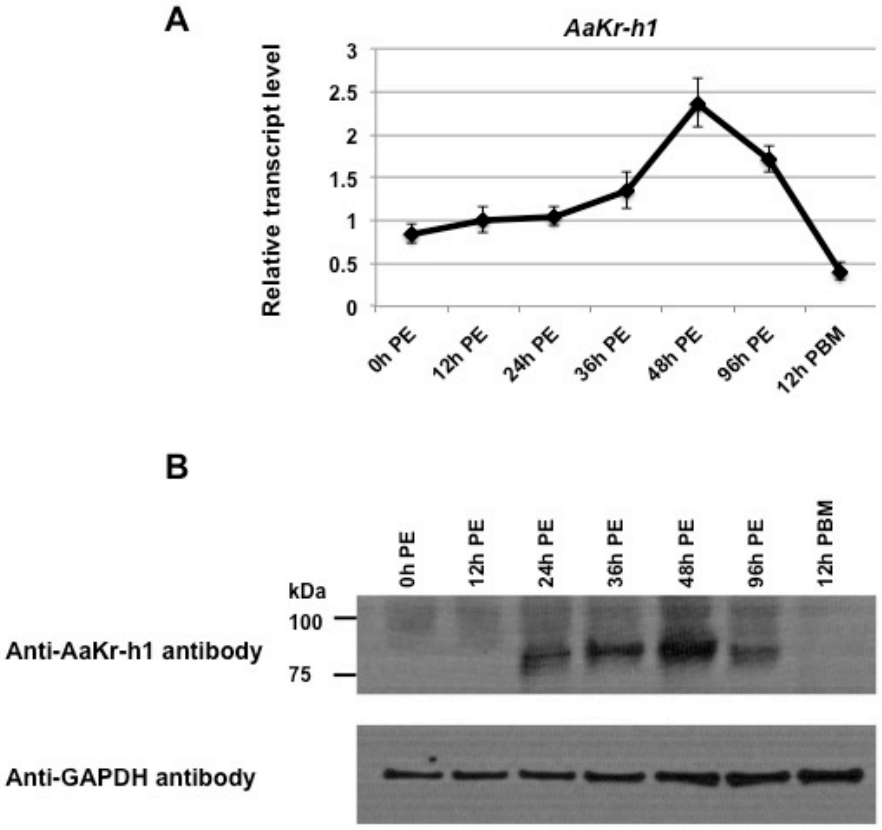

Figure 3.1. *AaKr-h1* mRNA profile in *Ae. aegypti* mosquitoes. (*A*) Adult female mosquitoes were collected at the indicated time-points. The expression level of *AaKr-h1* was measured using real-time PCR. Results are the mean ± S.D. of three replicates. PE, post eclosion; PBM, post blood meal. (*B*) Protein profile of AaKr-h1 in *Ae. aegypti* mosquitoes Adult female mosquitoes were collected at the indicated time points. RIPA buffer was used to extract proteins. Equal amount of proteins were loaded into the wells of the SDS-PAGE gel. Western blot analysis was conducted using the AaKr-h1 antibody. The GAPDH antibody was used for the loading control.

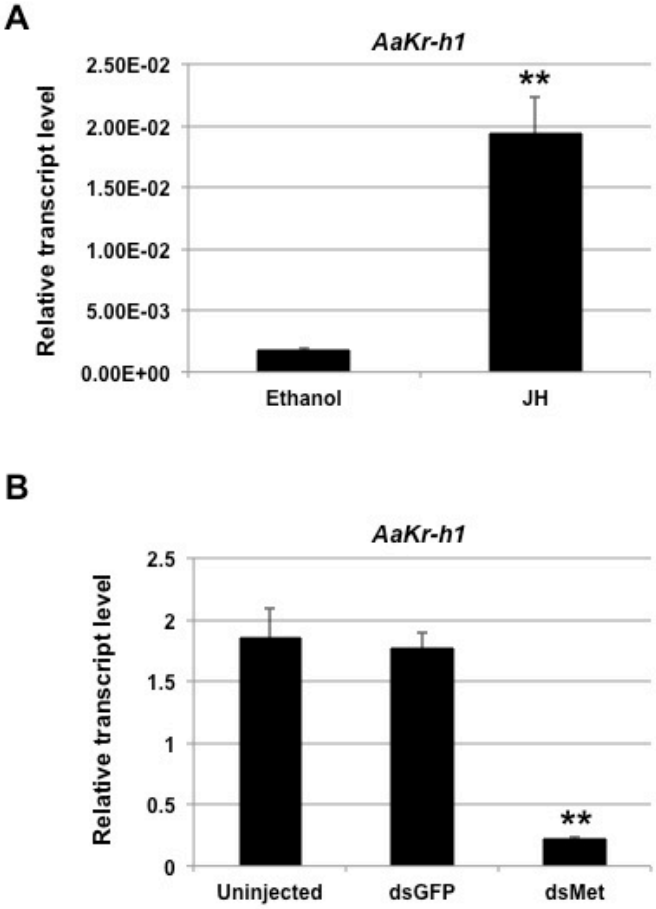

Figure 3.2. JH induces *AaKr-h1* expression. (*A*) Aag-2 cells were treated with ethanol (solvent control) and JH-III (5 μM) for one hour. Total RNA was extracted and the mRNA level of *AaKr-h1* was measured by real-time PCR. Results are the mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (**, $p<0.01$). (*B*) JH induces *AaKr-h1* expression via AaMET. Newly emerged female *Ae. aegypti* mosquitoes were injected with dsRNAs for *AaMET* or *GFP* (as control) within 1 h after eclosion. At 72 h after injection, total RNA was extracted from the mosquitoes and the expression of *AaKr-h1* was measured using real-time PCR. Data represent mean ± S.D. of three independent experiments. Statistical analysis was conducted by paired t-test (**, $p<0.01$).

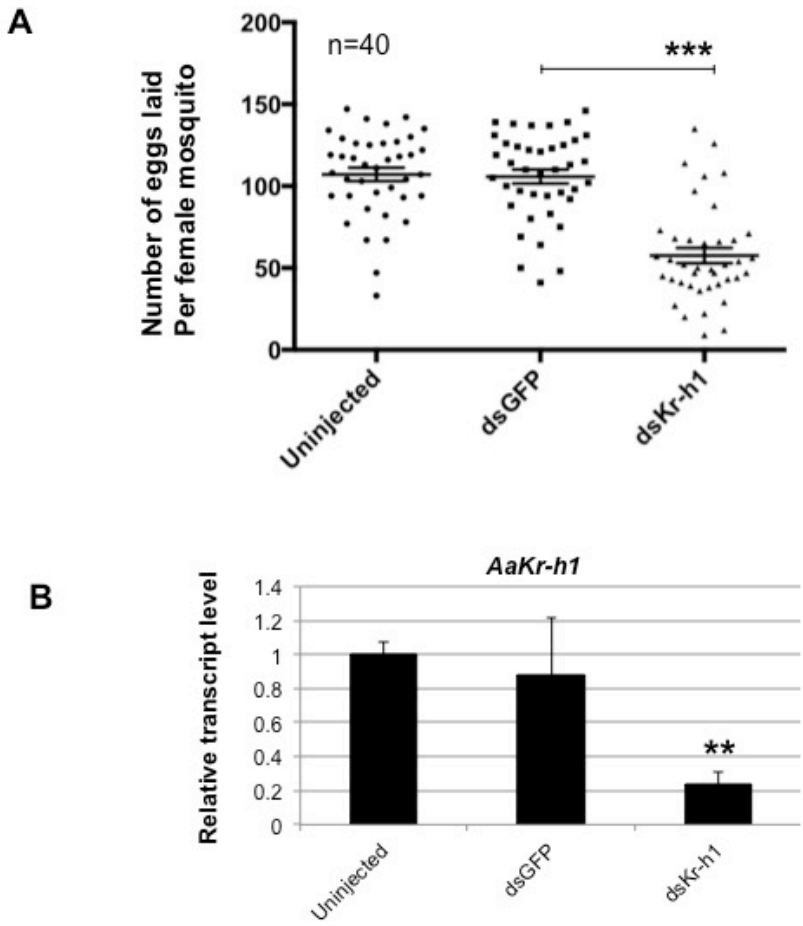

Figure 3.3. RNAi-mediated knockdown of AaKr-h1 decreases egg deposition in *Ae. aegypti* mosquitoes. (*A*) Newly emerged adult female mosquitoes were injected with dsRNAs for *AaKr-h1* or *GFP* (as control). Dots represent egg counts for individual mosquitoes within 5 days after the blood meal. Lines represent mean number of eggs oviposited from three replicates; bars indicate SEMs. Data was analyzed using GraphPad software. Statistical analysis was conducted by paired t-test (***, $p<0.001$). (*B*) AaKr-h1 was successfully knocked down in *dsKr-h1* injected mosquitoes. A group of three mosquitoes were randomly picked from un-injected, *dsGFP*- and *dsKr-h1*-injected mosquitoes. Total RNA was extracted and the expression level of *AaKr-h1* was measured by real-time PCR. Results are the mean ± S.D. of three replicates. Statistical analysis was conducted by paired t-test (**, $p<0.01$).

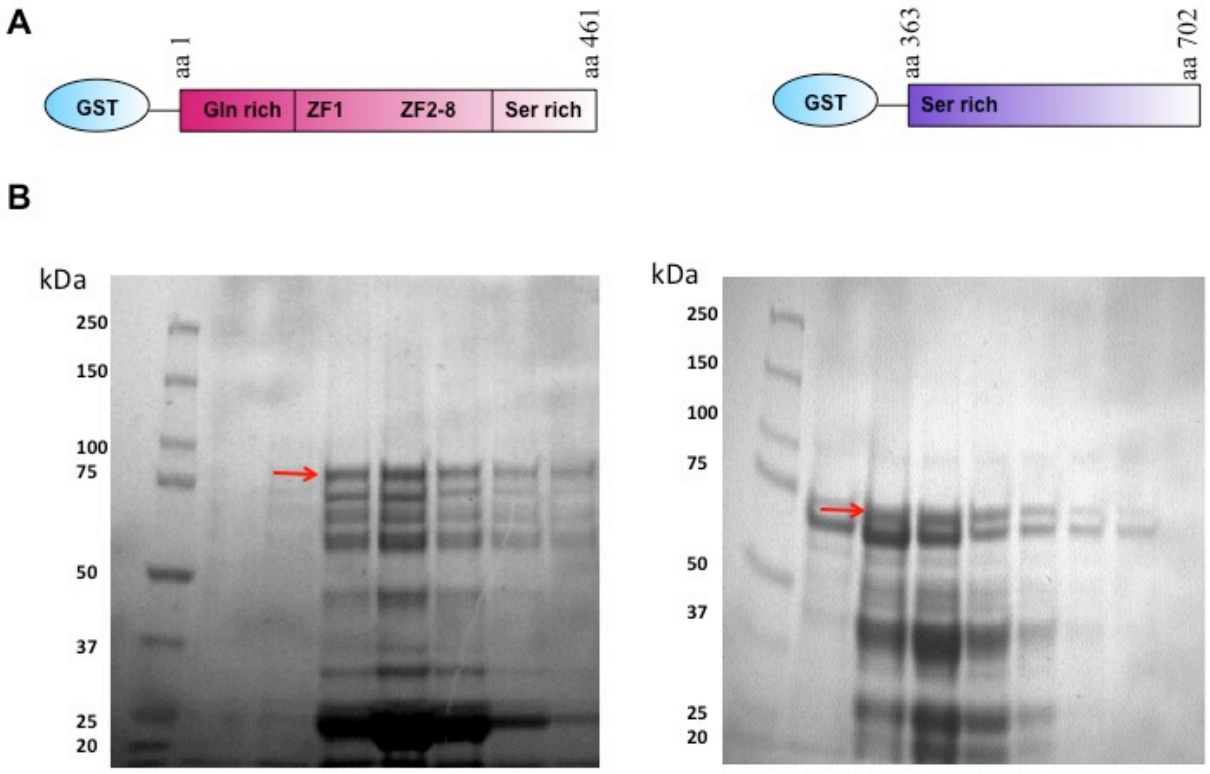

Figure 3.4. Purified recombinant AaKr-h1 proteins. (*A*) Schematic representation of the GST-tagged N-terminal (amino acid residues 1-461) (left) and C-terminal (amino acid residues 363-702) (right) fragments of AaKr-h1. (*B*) Partially purified GST-N-AaKr-h1 (left) and GST-C-AaKr-h1 (right) were separated on SDS-PAGE gels, followed by Coomassie blue staining.

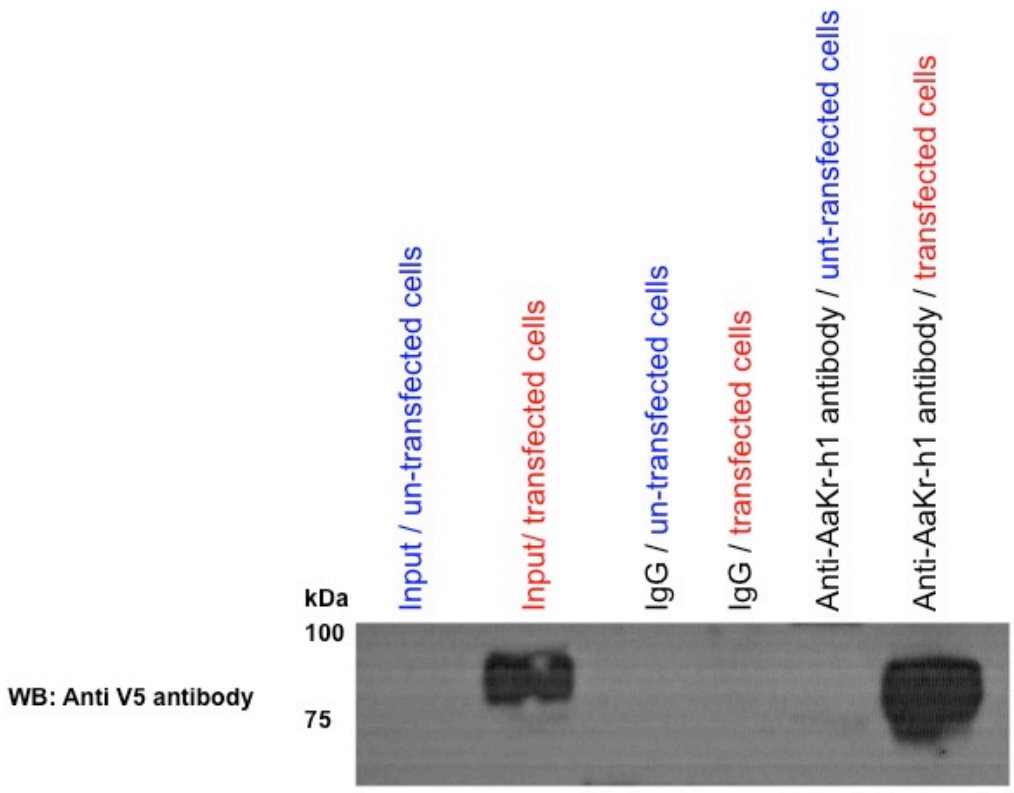

Figure 3.5. Purified AaKr-h1 antibody is able to specifically immunoprecipitate the AaKr-h1 protein. *Drosophila* S2 cells were transfected with pAc5.1/V5-His A-Full AaKr-h1 vector. RIPA buffer was used to extract proteins from both transfected and un-transfected cells. The purified AaKr-h1 antibody and rabbit IgG (as control) were used for immunoprecipitation. Anti-V5 antibody was used for western blot.

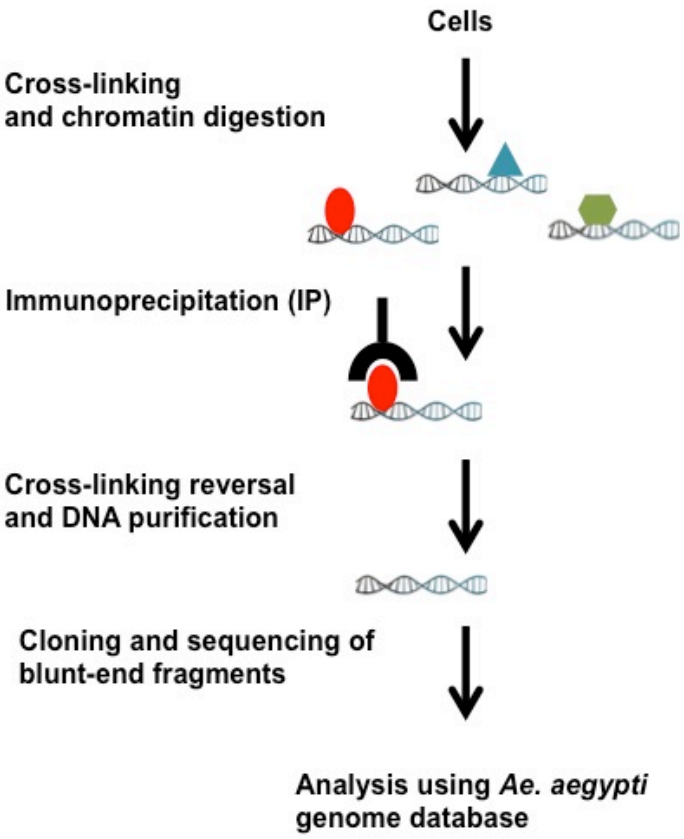

Figure 3.6. Schematic representation of ChIP-cloning process. ChIP cloning is a method to clone the individual DNA fragments bound by the protein of interest. Sequencing of the cloned DNA fragments will lead to identification of novel binding sites.

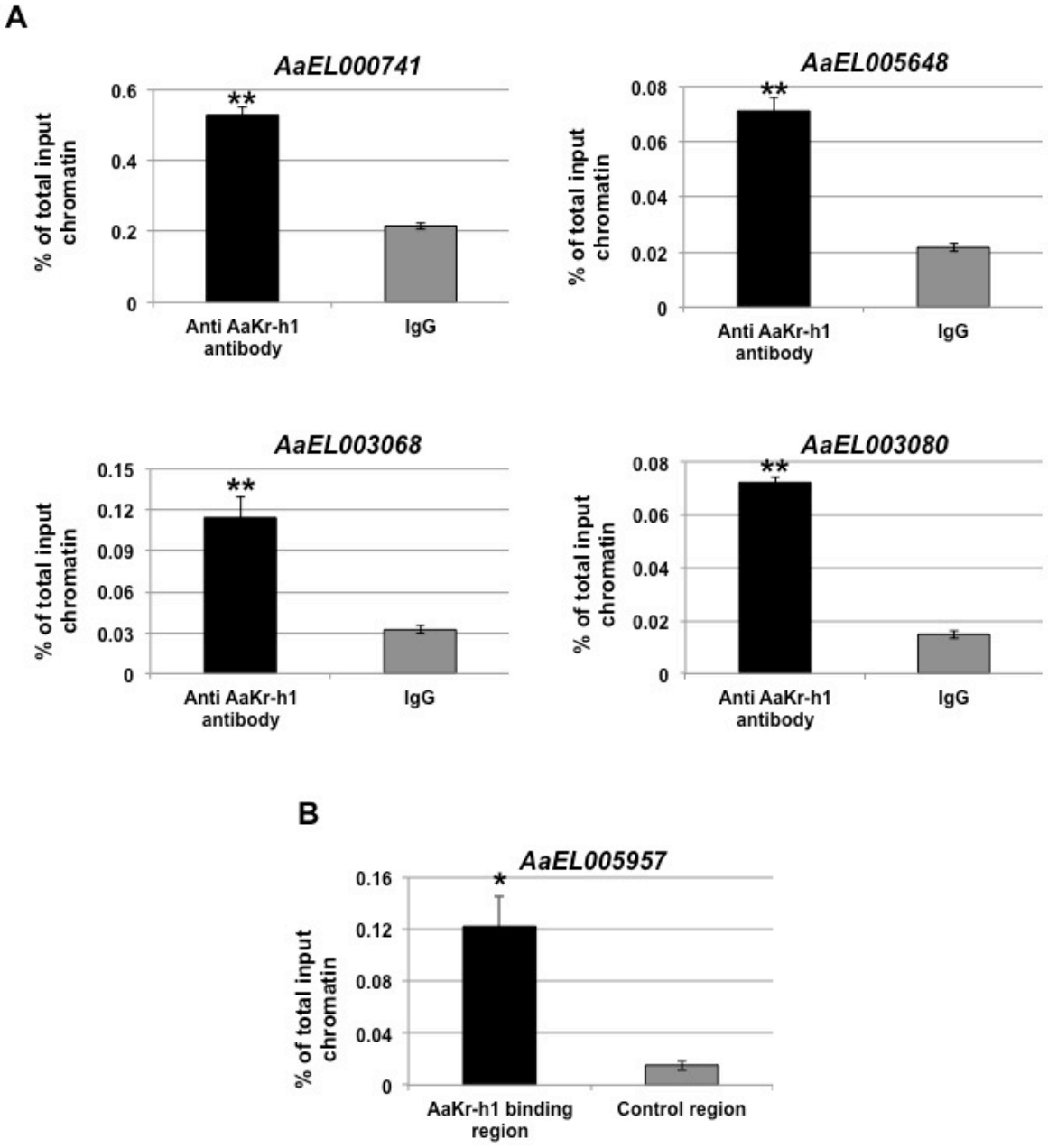

Figure 3.7. Verification of the *in vivo* binding of AaKr-h1 to the DNA sequences that were identified by ChIP-cloning. (*A*) Enrichment of four sequences were confirmed in an independent ChIP assay using mosquito abdomens collected at 48 h PE. The AaKr-h1 antibody and Rabbit IgG (as control) were used this ChIP experiment. Real time PCR was performed to compare the enrichment of the AaKr-h1 binding sequences (regulatory regions of *AaEL000741*, *AaEL003068*, *AaEL005648* and *AaEL003080*) between the immunoprecipitations with the AaKr-h1 antibody and with rabbit IgG. Results are shown as a percentage of input chromatin and represent mean value ± S.D. of three replicates. Statistical analysis was conducted by paired t-test (**, $p<0.01$).

(*B*) Selective binding of AaKr-h1 to the regulatory region of *AaEL005957*. After chromatin immunoprecipitation with the AaKr-h1 antibody, the precipitated DNA was analyzed using real-time PCR to compare the enrichment of the regulatory region identified by ChIP-cloning and a control region in the coding sequence of *AaEL005957*. Results are shown as a percentage of input chromatin and represent mean value ± S.D. of three replicates. Statistical analysis was conducted by paired t-test (*, $p<0.05$).

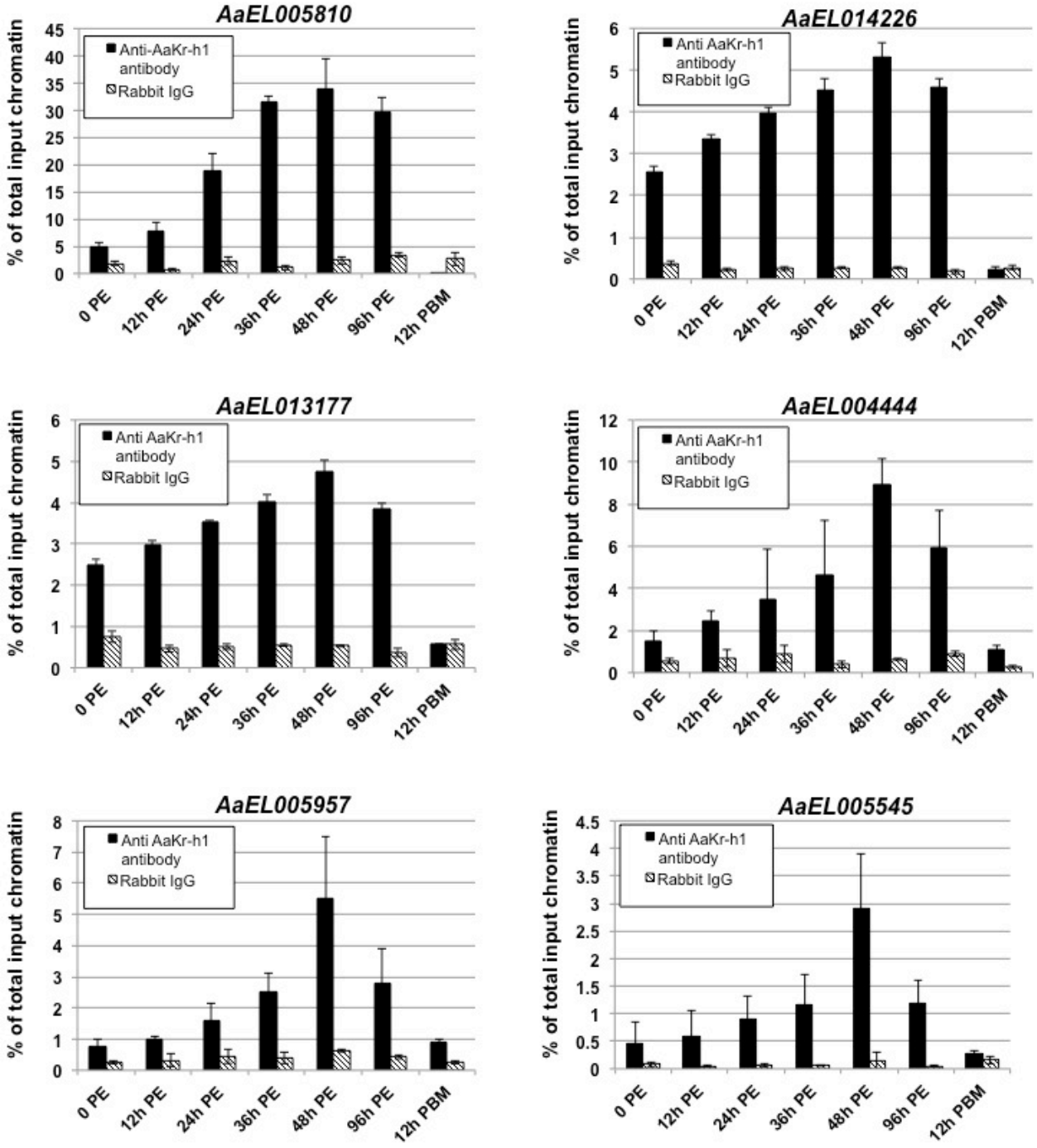

Figure 3.8. Binding of AaKr-h1 on the regulatory region of the individual AaKr-h1 target gene. One hundred female mosquito abdomens were collected from the indicated time points. The ChIP experiment was performed using ant-AaKr-h1 antibody or normal rabbit IgG (as control). After chromatin immunoprecipitation, the enrichment of the relevant AaKr-h1 binding sequence was determined using real-time PCR. Results are shown as a percentage of input chromatin and represent mean value ± S.D. of three replicates. PE, post eclosion; PBM, post blood meal.

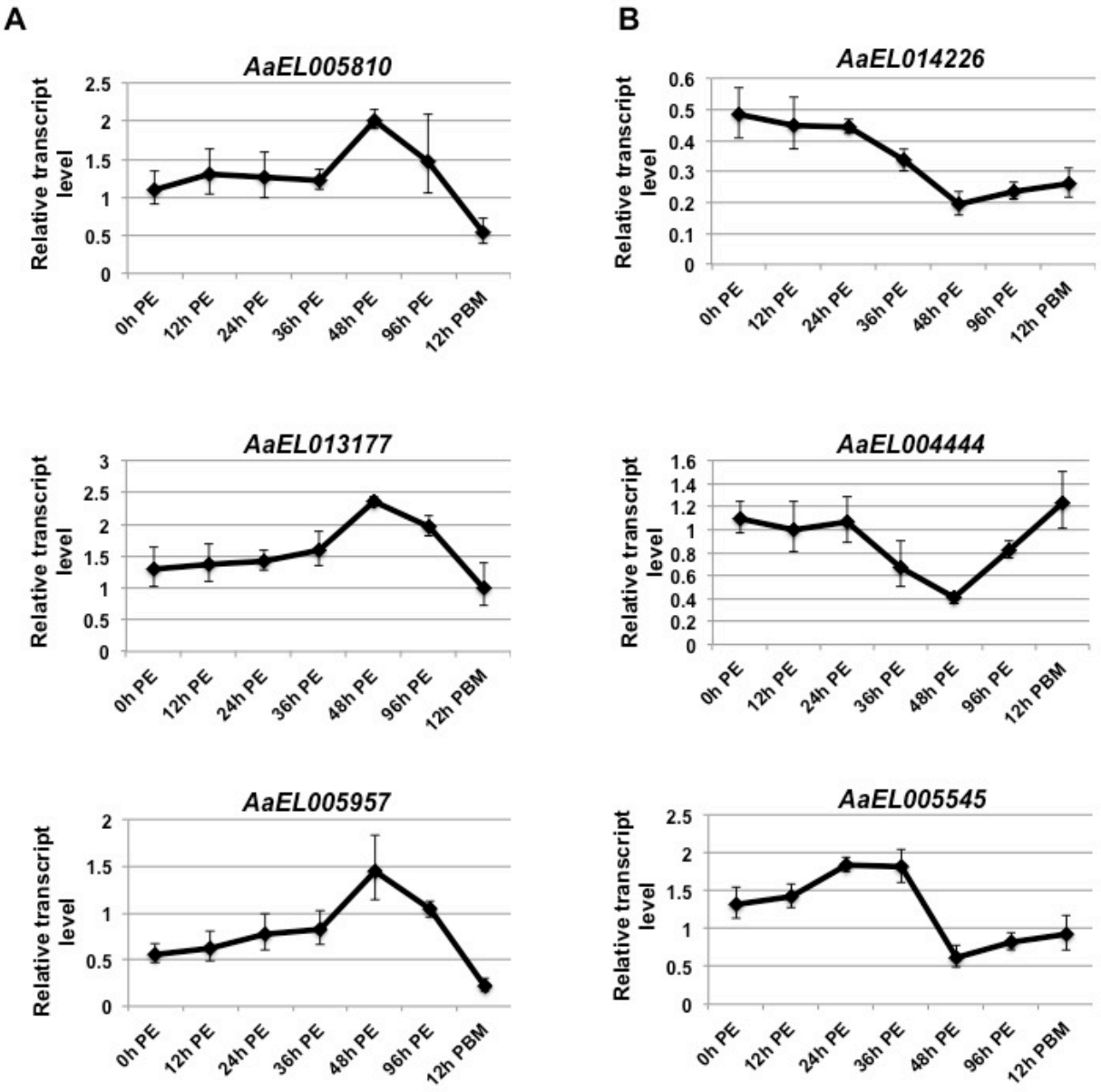

Figure 3.9. The expression profiles of AaKr-h1 target genes in previtellogenic adult mosquitoes. (*A* and *B*) Adult female *Ae. aegypti* mosquitoes were collected at the indicated time-points. Total RNA was extracted from the mosquitoes and the expression of AaKr-h1 target genes were measured using real-time PCR. Data represent mean ± S.D. of three replicates. PE, post eclosion; PBM, post blood meal.

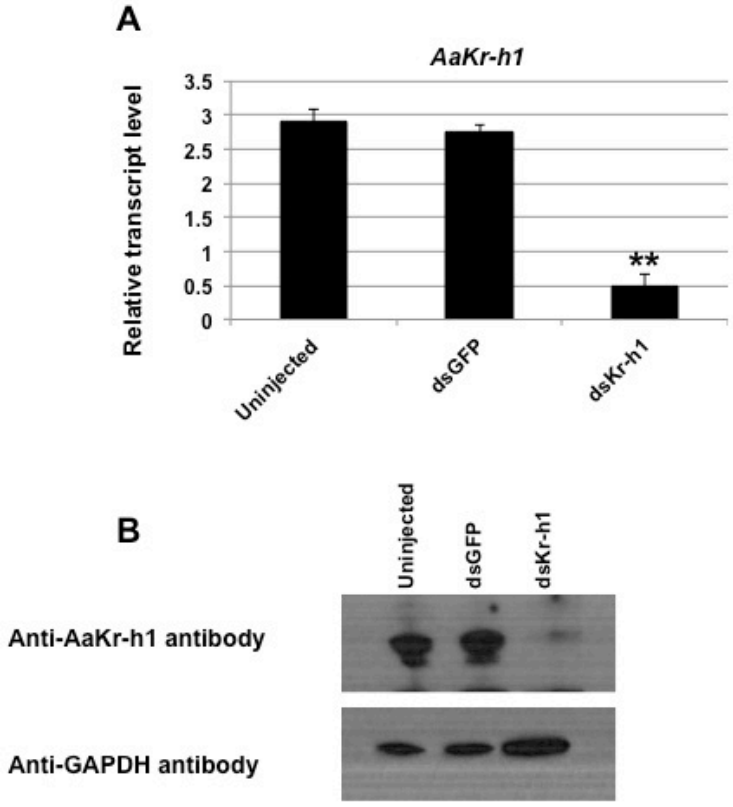

Figure 3.10. *AaKr-h1* was successfully knocked down in *dsKr-h1* injected mosquitoes. (*A*) Total RNA was extracted from un-injected, *dsGFP*- and *dsKr-h1*-injected mosquitoes at 4 days after dsRNA injection. The mRNA levels of *AaKr-h1* were measured by real-time PCR. Results are the mean ± S.D. of three replicates. Statistical analysis was conducted by paired t-test (**, $p<0.01$). (*B*) Knockdown of AaKr-h1 was confirmed by western blot. RIPA buffer was used to extract proteins from un-injected, *dsGFP*- and *dsKr-h1*-injected mosquitoes. Equal amount of proteins were loaded on a SDS-PAGE gel. Western blot analysis was conducted using anti-AaKr-h1 antibody. Anti-GAPDH antibody was used for the loading control.

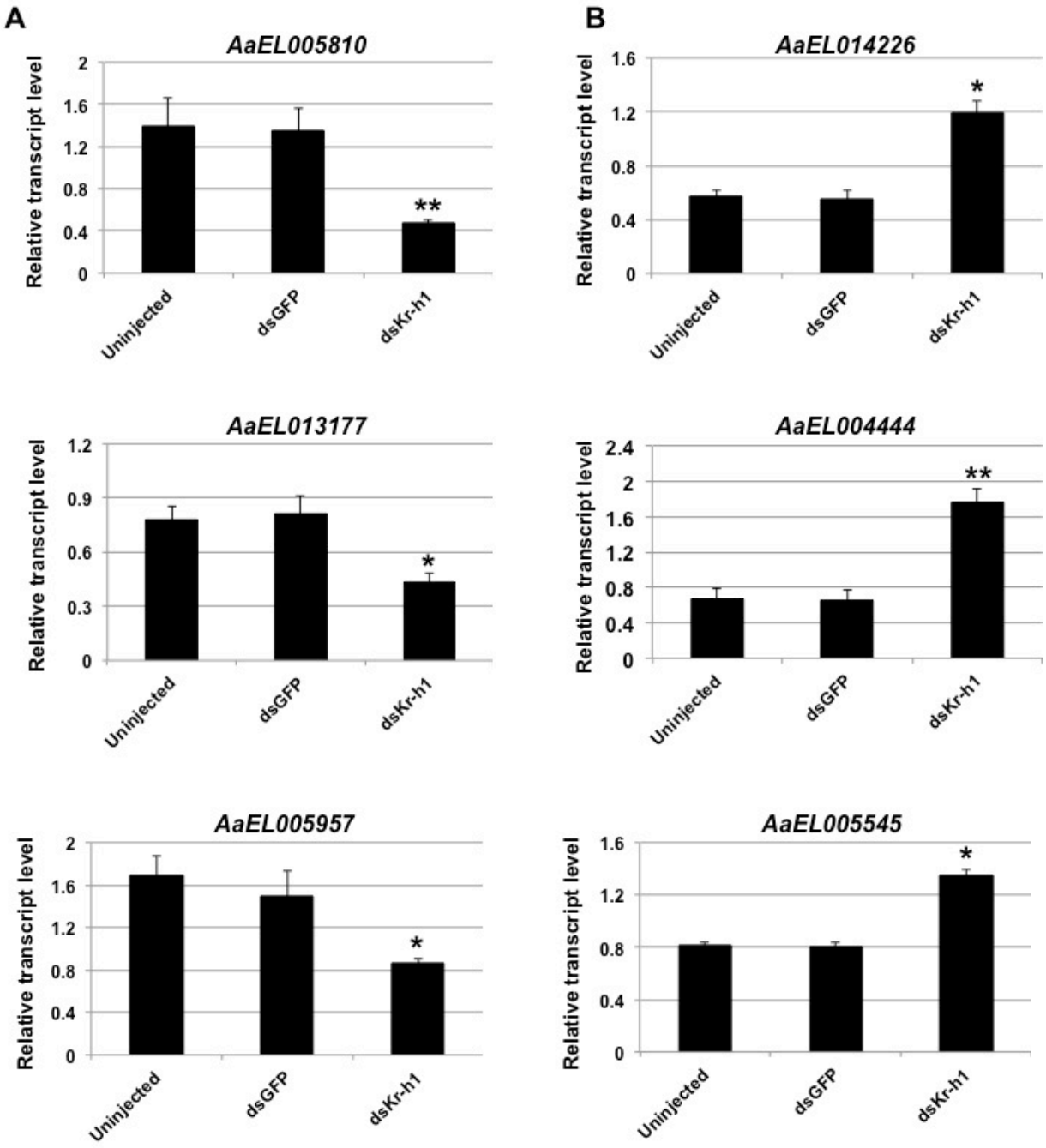

Figure 3.11. RNAi-mediated knockdown of *AaKr-h1* leads to downregulation (*A*) or upregulation (*B*) of individual AaKr-h1 target genes. Newly emerged female mosquitoes were injected with dsRNAs for *AaKr-h1* or *GFP* within 30 min after eclosion. At 4 days after injection, total RNA was extracted from the mosquitoes. The amount of transcript in the uninjected, dsGFP-injected and dsKr-h1-injected mosquitoes was measured by quantitative RT-PCR. Data represent mean ± S.D. of three replicates. Statistical analysis was conducted by paired t-test (*, $p<0.05$; **, $p<0.01$).

Table 3.1. ChIP-cloning results.

Clone	Blast hit	Distance	Accession number	Gene function
1	supercont1.678 83033- 83183	51 kb	*AaEL012328*	Hypothetical protein
2	supercont1.309 1123192-1122398		No gene	No gene
3	supercont1.107 238744- 239418	56 Kb	*AaEL004093*	Hypothetical protein
4	supercont1.65 2536115- 2535172	1193 Kb	*AaEL002727*	Diacylglycerol kinase
5	supercont1.201 1048528- 1049084	134 Kb	*AaEL006341*	Hypothetical protein
6	supercont1.445 320989- 321529	161 Kb	*AaEL009992*	Hypothetical protein
7	supercont1.1081 65616- 65375	176 Kb	*AaEL014338*	Hypothetical protein
8	supercont1.15 19197-19725	36 Kb	*AaEL000741*	Ecdysone-induced protein 74EF
9	supercont1.840 186485- 185133	60 Kb	*AaEL013414*	Hypothetical protein
10	supercont1.76 2041931- 2042480	38 Kb	*AaEL003068*	Hypothetical protein
11	supercont1.536 204079- 203408	35 Kb	*AaEL018210*	Hypothetical protein
12	supercont1.168 920752-920920	3.9 Kb	*AaEL005648*	Clip-Domain Serine Protease family B. Protease homologue
13	supercont1.284 490399- 490057	69 Kb	*AaEL007821*	Signalosome, subunit 2, CSN8, putative
14	supercont1.248 1494624 - 1495531	78 Kb	*AaEL007316*	Hypothetical protein
15	supercont1.4 3367362-3366347	222 Kb	*AaEL000242*	Voltage-gated potassium channel
16	supercont1.119 81050-81549	17 Kb	*AaEL004444*	Zinc finger protein
17	supercont1.76 62379- 63323	13 Kb	*AaEL003080*	Hypothetical protein
18	supercont1.271 314220- 313573	900 bp	*AaEL007652*	Hypothetical protein
19	supercont1.175 567643- 568161	14 Kb	*AaEL005822*	Hypothetical protein

20	supercont1.15 4021282- 4020480	56 Kb	*AaEL018121*	Hypothetical protein
21	supercont1.500 58530- 57637	12 Kb	*AaEL010717*	Ecdysone receptor isoform-B (EcRB)
22	supercont1.182 1220096- 1219257	10 Kb	*AaEL005957*	Phospholipase b, plb1
23	supercont1.454 394164- 393982	59 Kb	*AaEL010117*	Fibrinogen
24	supercont1.434 925469- 925747		No gene	No gene
25	supercont1.1131 133448- 133780	2.5 Kb	*AaEL014487*	Hypothetical protein
26	supercont1.175 275807-276101	1.2 Kb	*AaEL005810*	Hypothetical protein
27	supercont1.383 1005986- 1006368	4.2 Kb	*AaEL009241*	Translation initiation factor if-2
28	supercont1.14 2376768- 2377520		No gene	No gene
29	supercont1.162 628545- 629795	900 bp	*AaEL005545*	Tetraspanin, putative
30	supercont1.123 427346- 427574	18 Kb	*AaEL004556*	Hypothetical protein
31	supercont1.122 2163511- 2162654	24 kb	*AaEL004522*	Gambicin anti-microbial peptide
32	supercont1.204 795129- 795831	51 Kb	*AaEL006411*	Timeless circadian protein
33	supercont1.55 1040168- 1040026	612 bp	*AaEL002403*	Hypothetical protein
34	supercont1.73 993813- 993954	18 kb	*AaEL002980*	U1 small nuclear ribonucleoprotein A
35	supercont1.801 206028- 206193	9 kb	*AaEL013177*	Nucleotide-binding protein, putative
36	supercont1.21 601222- 600701	27 kb	*AaEL001018*	Hypothetical protein
37	supercont1.283 193494- 193644	23 kb	*AaEL007797*	Hypothetical protein
38	supercont1.86 68722- 70069	111 kb	*AaEL003432*	Hypothetical protein
39	supercont1.638 463996- 463831	55 kb	*AaEL011969*	Protein-s isoprenylcysteine o-methyltransferase
40	supercont1.801 206028- 206193	9 kb	*AaEL013177*	Nucleotide-binding protein, putative

41	supercont1.545 535344- 536084	173 kb	*AaEL011164*	Cadherin
42	supercont1.1044 164199- 164352	4 kb	*AaEL014226*	Hypothetical protein
43	supercont1.21 601222- 600701	27 kb	*AaEL001018*	Hypothetical protein
44	supercont1.411 865481- 864750	163 kb	*AaEL009595*	Hypothetical protein
45	supercont1.123 650714- 651119	138 kb	*AaEL004578*	Hypothetical protein
46	supercont1.348 194475- 194302	130 kb	*AaEL008784*	Serine-type enodpeptidase
47	supercont1.221 351203- 352060	34 Kb	*AaEL018044*	Hypothetical protein
48	supercont1.526 746852- 747579		No gene	No gene
49	supercont1.297 121372- 121175	27 kb	*AaEL017171*	Hypothetical protein
50	supercont1.179 1278338- 1278612	54 kb	*AaEL005913*	Hypothetical protein
51	supercont1.265 324813- 324982	144 kb	*AaEL007584*	Hypothetical protein
52	supercont1.11 2073246- 2072323	51 kb	*AaEL000580*	Hypothetical protein
53	supercont1.2 1888060- 1888388		No gene	No gene
54	supercont1.50 2047109- 2047800	107 kb	*AaEL002172*	Tyrosine-protein kinase

Table 3.2. Primers used to clone N-*AaKr-h1* and C-*AaKr-h1* in pGEX-6p-1.

Plasmid	**Primer**	**Sequence (5'-3')**
NKrh-pGEX-6p-1	Fwd	CGCGGATCCATGGTCTACTACACG
	Rev	CCGCTCGAGTCAAACAGACTGTTC
CKrh-pGEX-6p-1	Fwd	CGCGGATCCGAAATCCCGGATGAA
	Rev	CCGCTCGAGTCACGACGCCTTGG

Table 3.3. Primers used to clone full length *AaKr-h1* in pAC5.1/V5-His A.

Plasmid	**Primer**	**Sequence (5'-3')**
pAC5.1/V5-His A-Full AaKr-h1	Fwd	CGGGGTACCACCATGGTGCATCATCACCATCACC ATGTGTACTATACGGGATTACCGCTGCTGATGCA ACAAGCAGAG
	Rev	CCGCTCGAGGGATGCCTTGGCGTACTGAATGAC

Table 3.4. Primers used in qRT-qPCR.

AaKr-h1 binding site	**Primer**	**Sequence (5'-3')**
supercont1.119	Fwd	CGCATTACCTGATGTTCTGG
81050-81549	Rev	TCAGAAAGCCCAGTAGCTGA
supercont1.182	Fwd	TTTCGACTCGCTCTCTAGCA
1220096- 1219257	Rev	AACGCACCAACAGACAGGTA
supercont1.175	Fwd	GAAGTGCTCATTAAACACTAAGCTG
275807-276101	Rev	TTCTTCTTCTTGGCATTACGTC
supercont1.162	Fwd	CCCTAAGTTCACCGAACCAT
628545- 629795	Rev	GAACAAACTCACCGGATTGA
supercont1.801	Fwd	TGCACGTCTGCTGTCAGATA
206028- 206193	Rev	GAGATCAGCCACTTCTTCCC
supercont1.1044	Fwd	GACAGGATGGAGGATCGTTT
164199- 164352	Rev	ATAGCCGAATAGCCTCTCCA
supercont1.15	Fwd	TTTCACTGATCCGGCTGTTA
19197-19725	Rev	GGGAAGCAAAGTGACGAAGT
supercont1.76	Fwd	ATGTCAGCTACTGGGCCTTT
2041931- 2042480	Rev	CAGTAGGCCATTGCAAGCTA
supercont1.168	Fwd	AATGACAGCCTCGTGTATGC
920752-920920	Rev	TTCATGTGACAGGTCCGTCT
supercont1.76	Fwd	TGAGCCATAATTTCATCCCA
62379- 63323	Rev	TCGTTCATGGCTCGTTATGT
Gene	**Primer**	**Sequence (5'-3')**

AaEL004444	Fwd	AGCAGATCACACCGTAGTGC
	Rev	TGAAATTGCAGGGTTGTTGT
AaEL005957	Fwd	CATACCATTCATGGCACGTT
	Rev	GGACGTTCCATAGCACACAC
AaEL005810	Fwd	AAAGATCCGATCGGTCAAAC
	Rev	AGATGAGCGTCTTGTTGACG
AaEL005545	Fwd	GCGGCGTCAATAACTATCAA
	Re	GTGTTGAATGGAACGCACTC
AaEL013177	Fwd	GACGAGCACAACCTGATGAT
	Rev	GTTTCCACCAGAAGACCCAT
AaEL014226	Fwd	GTGCCTTTGTTCAGGACTCA
	Rev	GACGGTAATGGAGGTGGAGT
AarpS7	Fwd	TCAGTGTACAAGAAGCTGACCGGA
	Rev	TTCCGCGCGCGCTCACTTATTAGATT
AaKr-h1	Fwd	TTCTCGCAACAACAGCAACATCCG
	Rev	TCATCAGATCCATTGACGCTGGGT

Table 3.5. Primers for making DNA templates to synthesize dsRNAs.

Gene	**Primer**	**Sequence (5'-3')**
AaKr-h1	Fwd	TAATACGACTCACTATAGGGAGTAATGACCTCAAATGGTGCAGATCG
	Rev	TAATACGACTCACTATAGGGAGGCCTTGGCGTACTGAATGACGGAG
GFP	Fwd	TAATACGACTCACTATAGGGAGACGTAAACGGCCACAAGTTC
	Rev	TAATACGACTCACTATAGGGAGTGCTCAGGTAGTGGTTGTCG
AaMet	Fwd	TAATACGACTCACTATAGGGGCTGTTAAAAGTGGATGATGATAC
	Rev	TAATACGACTCACTATAGGGAATCGGCACCTTGGTAGAACGATC

Chapter 4: Summary

4.1. General review

The goal of this study was to understand the molecular mechanism of JH action. This hormone regulates many aspects of insect life, including metamorphosis and reproduction. Since it is unique to insects, its signaling pathway has been studied as a target for new insecticide development. In this study, our model organism was *Ae. aegypti*, the vector for transmitting several diseases including dengue, chikungunya and yellow fever.

We have demonstrated that protein kinase C (PKC) is a component of a cell membrane-initiated JH signaling cascade, and that PKC connects to the previously known JH nuclear signaling pathway by modulating the ability of the JH nuclear receptor MET in regulating gene expression. We have also elucidated for the first time the role of AaKr-h1 as a bifunctional transcription regulator that acts downstream of MET in the JH signaling pathway.

4.2. Review of chapter 2

Several studies have demonstrated that JH, a lipophilic signal molecule, regulates gene expression via its nuclear receptor MET. Our study showed a cell surface-initiated PLC pathway activates PKC. Activation of PKC plays a crucial role in JH-regulated gene expression, as it is required for proper binding of the JH nuclear complex (MET-TAI) to DNA. The involvement of PKC in the cellular response to JH was shown in both Aag-2 cells and in *in vitro* cultured fat bodies from newly emerged *Ae. aegypti.* Pre-treatment of the cultured fat bodies with different PKC inhibitors abolished the JH-induced expression of *AaKr-h1* and *AaEL002576*. The kinase activity of PKC after the JH treatment was confirmed in western blot using a specific antibody that recognizes the phosphorylated serine residues in PKC target proteins. In that experiment it

was also depicted that PKC acts downstream of PLC in the JH signaling pathway. RNAi-mediated knockdown of five different PKC isoforms, in the Aag-2 cells and in the adult mosquitoes, indicated that multiple PKC isoforms are involved in cellular response to JH. In Aag2 cells, a reporter assay showed that inactivation of PKC considerably reduced the JH-induced transcription activation by the MET-TAI complex, suggesting that PKC modulates the function of the MET-TAI complex on JHRE. Subsequent ChIP experiment demonstrated that activation of PKC by JH is required for effective DNA binding of AaMET and AaTAI in Aag2 cells and the cultured mosquito tissues.

4.3. Future perspectives of chapter 2

Our data suggest the existence of a membrane-initiated JH signaling pathway, but yet no protein has been identified as the JH membrane receptor. Preliminary studies in our lab suggest that JH activates the PLC-PKC pathway through receptor tyrosine kinases. There are 30 genes coding for receptor tyrosine kinases in the *Ae. aegypti* genome and RNAi-mediated individual knockdown of those genes will help to find the JH membrane receptor.

A G-protein coupled receptor has been identified in *Helicoverpa armigera* as the cell surface receptor for 20E, which is also a lipophilic steroid (Cai et al., 2014). Finding the JH membrane receptor will provide more information regarding the crosstalk between the JH and 20E signaling pathways.

The nuclear receptor for 20E is composed of the ecdysone receptor (EcR) and ultraspiracle protein (USP). In *Drosophila*, USP is phosphorylated by PKC on Ser35 (Wang et al., 2012b). The 20E-induced phosphorylation of USP promotes binding of the EcR-USP complex to ecdysone-response elements and is essential for the 20E-induced gene expression (Liu et al., 2014). It will be interesting to test whether the same PKC isoforms are involved in the

phosphorylation of MET and USP, and whether MET and USP are both phosphorylated when insect cells are exposed simultaneously to 20E and JH.

It is important to determine whether PKC directly phosphorylates MET. Finding the exact amino acid residue(s) that are phosphorylated after JH treatment will provide more clues. MET and TAI will be purified from Aag-2 cells that receive different treatments: ethanol, JH and JH plus a PKC inhibitor. Mass-spectrometry analysis of the MET and TAI proteins will lead to identification of the PKC-dependent phosphorylation sites. A detailed mutagenesis study of the identified residues will reveal the effect of phosphorylation on formation of the MET-TAI complex and on its DNA binding.

As the preliminary studies in our lab suggest that the JH cell surface receptor is a receptor tyrosine kinase, it is attracting to find the involvement of other components of the receptor tyrosine kinase activated signaling pathway, such as MAP kinase in JH pathway and test whether they can modulate the transactivation activity of MET-TAI complex.

4.4. Review of chapter 3

Expression of Kr-h1 is activated rapidly in response to JH and Kr-h1 mediates the anti-metamorphic action of JH. Little is known about the function of Kr-h1 in the JH-regulated reproduction in insects. Our study showed that in adult mosquitoes JH induces the expression of AaKr-h1 via MET. Kr-h1 plays an essential role in mosquito reproduction; depletion of AaKr-h1 in female mosquitoes leads to a dramatic decrease in egg production.

4.5. Future perspectives of chapter 3

Kr-h1 gene is upregulated by the JH-activated MET-TAI complex. It encodes a well-conserved zinc finger protein that represses the larval-pupal metamorphosis. Although Kr-h1 plays a key role in JH signaling pathway, the molecular function of Kr-h1 is poorly understood. While our

ChIP-cloning approach has yielded some AaKr-h1 target genes, the genome-wide DNA-binding sites for Kr-h1 will be identified next by combining ChIP with high-throughput DNA sequencing (ChIP-Seq). This study will provide a better understanding of the cellular response to JH and will help us identify the consensus DNA sequence for Kr-h1 binding. Our data showed that AaKr-h1 up-regulates the expression of some target genes and down-regulates the expression of others. ChIP-Seq data will reveal whether these two groups of target genes carry different Kr-h1 binding sequences. Hairy also functions downstream of MET and has been reported to mediate the action of MET in gene repression. Using ChIP-Seq data we can also investigate whether Kr-h1, MET and Hariy work together on the same JH target genes.

The cross-talk of JH and 20E signaling pathways has been an interesting subject to study for a long time. Kr-h1 is a key component that connects these two signaling pathways together, at least partially through regulating the expression of broad. We have shown that Kr-h1 is required for mosquito egg production. It will be interesting to investigate the effect of Kr-h1 knockdown on the expression of 20E target genes.

In our current study, AaKr-h1 exerts different function on individual target genes, suggesting that it may recognize different DNA sequences with various DNA-binding partners or recruit other proteins such as coactivators and corepressors. Identification of Kr-h1-interacting proteins will undoubtedly advance our understanding of the molecular mechanism underlying JH action.

4.6. Summary

In conclusion, we found PKC is activated by JH and functions downstream of PLC as another component of the cell surface-initiated JH signaling pathway. A recent study from our lab suggested that in addition to passing through the plasma membrane as a lipophilic signal molecule, JH acts on cell surface via a yet unknown receptor (Liu et al, 2015). Liu *et al*

suggested that this membrane receptor is a receptor tyrosine kinase and they showed that binding of JH to the membrane receptor induces PLC activation. PLC hydrolyses the membrane phospholipid PIP_2 (phosphotidylinositol-4,5-bisphosphate) to IP_3 and DAG. DAG remains in the membrane but IP_3 diffuses to the endoplasmic reticulum (ER), binds to its receptor (a calcium ion channel) and releases Ca^{2+} from internal stores in the ER to the cytoplasm. In this report, we showed PKC is functioning downstream of PLC and its function is required for DNA binding of the JH nuclear receptor complex (MET-TAI) to JHRE. MET purified by immunoprecipitation from the JH-treated Aag-2 cells was not detectable by a phospho-(Ser) PKC substrate antibody. Although we could not rule out the possibility that AaMET is phosphorylated by PKCs on threonine or serine residues that could not be recognized by this antibody, it is possible that PKC indirectly regulates the phosphorylation of MET through an intermediate kinase (Ojani et al, 2016). Binding of the MET-TAI complex to JHRE activates the expression of JH target genes such as *Kr-h1*. Kr-h1 itself is a transcription factor that can activate or repress the expression of downstream JH-regulated genes (Figure 4.1). The mechanism by which Kr-h1 switches its function is not clear yet. Conformational changes after binding to different DNA sequences, combinatory effects of other DNA binding proteins and post-translational modifications of Kr-h1 all could contribute to this functional transition.

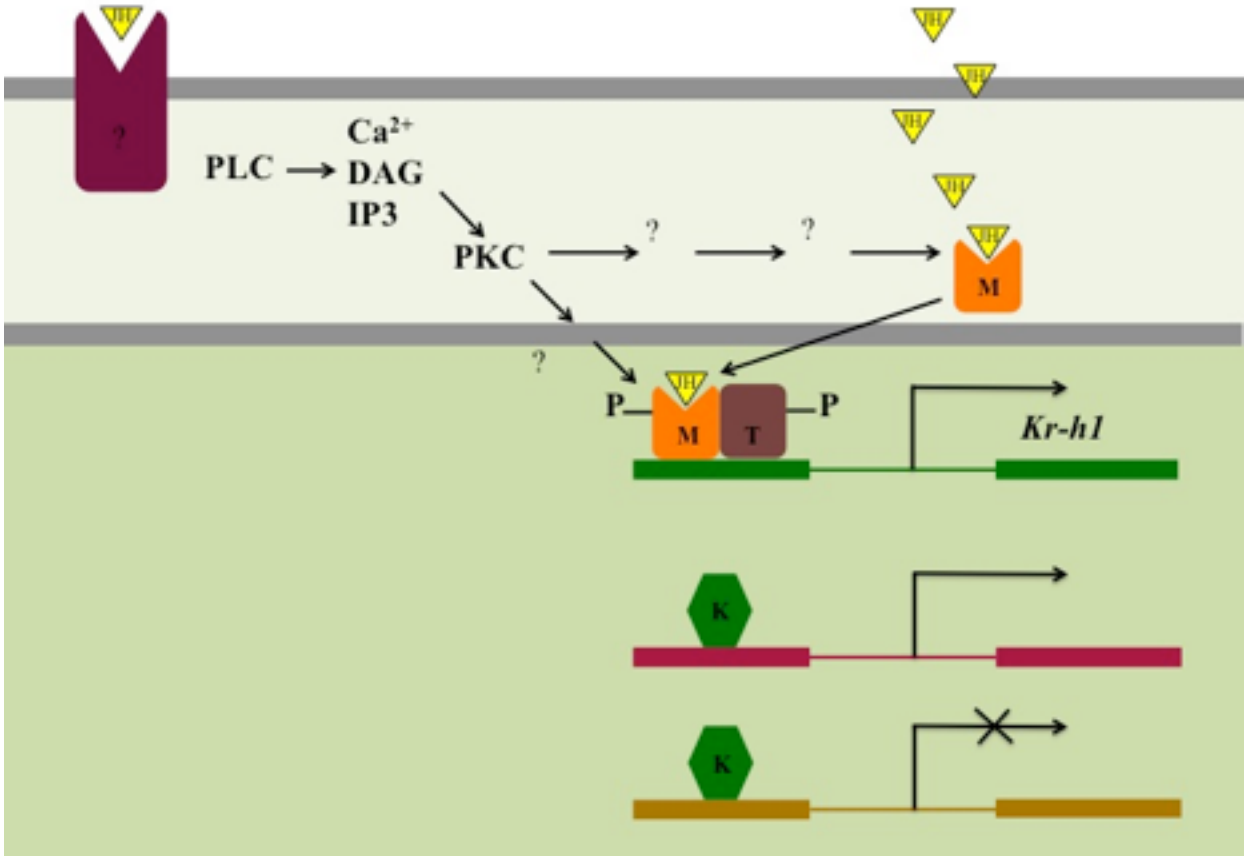

Figure 4.1. Graphical abstract represents the model proposed in this study for JH signaling pathway.

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