Methoprene-tolerant 1 regulates gene transcription to maintain insect larval status

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Abstract

Insect molting and metamorphosis are regulated by two hormones: 20-hydroxyecdysone (20E) and juvenile hormone (JH). The hormone 20E regulates gene transcription via the nuclear receptor EcR to promote metamorphosis, whereas JH regulates gene transcription via its intracellular receptor methoprene-tolerant (Met) to prevent larval–pupal transition. However, the function and mechanism of Met in various insect developments are not well understood. We propose that Met1 plays a key role in maintaining larval status not only by promoting JH-responsive gene transcription but also by repressing 20E-responsive gene transcription in the Lepidopteran insect Helicoverpa armigera. Met1 protein is increased during feeding stage and decreased during molting and metamorphic stages. Met1 is upregulated by JH III and a low concentration of 20E independently, but is downregulated by a high concentration of 20E. Knockdown of Met1 in larvae causes precocious pupation, decrease in JH pathway gene expression, and increase in 20E pathway gene expression. Met1 interacts with heat shock protein 90 and binds to JH response element to regulate Krüppel homolog 1 transcription in JH III induction. Met1 interacts with ultraspiracle protein 1 (USP1) to repress 20E transcription complex EcRB1/USP1 formation and binding to ecdysone response element. These data indicate that JH via Met1 regulates JH pathway gene expression and represses 20E pathway gene expression to maintain the larval status.

Key Words

- methoprene-tolerant 1
- juvenile hormone
- gene transcription
- 20-hydroxyecdysone

Introduction

Insect growth, molting, and metamorphosis are coordinately regulated by two hormones, namely juvenile hormone (JH) and 20-hydroxyecdysone (20E): 20E promotes molting and metamorphosis and JH prevents larval–adult transition until insects have attained an appropriate stage (Riddiford et al. 2003). The hormone 20E binds to its nuclear receptor EcR and forms a transcription complex with the heterodimeric partner ultraspiracle protein (USP) in Drosophila (Yao et al. 1992). The EcR/USP transcription complex binds to ecdysone response element (EcRE) to promote gene transcription in the 20E pathway in Drosophila (Riddihough & Pelham 1987), such as transcription factor Broad (Br) that initiates metamorphosis in Manduca sexta (Zhou & Riddiford 2001) and transcription factor hormone receptor 3 (HR3) to promote molting and metamorphosis in M. sexta (Lan et al. 1999).

Methoprene-tolerant (Met) gene is cloned in a Drosophila melanogaster mutant that showed resistance to toxic doses of JH or JH analog (Ashok et al. 1998). Met protein...
has a typical basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) domain. As Met binds to JH III with a high affinity through its C-terminal PAS domain (Charles et al. 2011) and regulates gene transcription in the JH pathway in *Drosophila*, Met is considered as the JH intranuclear receptor (Jindra et al. 2013). In a recent paper, De Loof et al. have advanced arguments in favor of the view that the still missing membrane receptor of JH is probably nothing else than the Ca^{2+}-homeostasis system of cells. Indeed, at least some isoforms of Ca^{2+}-ATPase have a binding site for farnesol-like sesquiterpenoids, the family of compounds to which JH belongs. These authors think that a low cytoplasmic Ca^{2+} concentration is causal to the status quo effect of JH (De Loof et al. 2014).

Met forms homodimers with Met or germ cell expressed (Gce) without hormone regulation in *Drosophila* (Godlewski et al. 2006). JH regulates the formation of the complex of Met with the steroid co-activator p160/SRC in *Tribolium castaneum* (Zhang et al. 2011, Jindra et al. 2013), which is known as FISC in *Aedes aegypti* (Li et al. 2011), Taiman in *D. melanogaster* (Charles et al. 2011), and Ncoa/Src1/p160 in mammals (Beischlag et al. 2002). In *A. aegypti*, Met interacts with Cycle (CYC) (Shin et al. 2012) and βFtz-F1 (Zhu et al. 2003) by the induction of JH. Met2 interacts with SRC and binds to JH response element (kJHRE) in the Krüppel homolog 1 gene (*Krh1*) to promote *Krh1* transcription to repress metamorphosis in *Bombyx mori* (Kayukawa et al. 2012). However, Met is also involved in the 20E pathway. Met RNAi prevents tissue remodeling, which results in lethality and disruption of the 20E-triggered transcriptional cascade reaction in *B. mori* (Guo et al. 2012). In *Drosophila*, the overexpression of Met in fat body results in precocious and enhanced programmed cell death and cell dissociation (Liu et al. 2009). Met can also interact with EcR and USP in insect cells in an ecodysteorid or in a JH-regulation-independent manner, suggesting that the interaction among Met, EcR, and USP mediates the crosstalk between these two important hormones (Bitra & Palli 2009). Therefore, the basic function of Met in various insects is not really clear.

*Helicoverpa armigera* (Noctuidae, Lepidoptera) is a severe agricultural pest throughout the world. We reveal that Met1 has an important function in maintaining the larval status in this insect. Knockdown of *Met1* causes precocious pupation by repressing the JH pathway gene expression and promoting 20E pathway gene expression. The mechanism used by Met1 in controlling 20E and JH pathways is studied in this work.

## Materials and methods

### Experimental animal

*H. armigera* larvae were raised on the artificial diet, which was made from powder of wheat germs and soybeans at 27 °C with 60–70% relative humidity. The illumination condition consisted of 14 h light:10 h darkness cycles in an insectarium (Zhao et al. 1998).

### *H. armigera* epidermal cell line culture

The *H. armigera* epidermal cell line (HaEpi) was established in our laboratory (Shao et al. 2008). Cells were cultured as a loosely attached monolayer and maintained at 27 °C in tissue culture flasks. The tissue culture flasks had an area of 25 cm² with 4 ml of antibiotic-free Grace’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were subcultured weekly to a near confluent monolayer.

### Bioinformatic analyses of Met1

The translation and prediction of Met1 were analyzed by Expasy Proteomics Server (http://www.expasy.ch/tools/). The nucleotide sequence of Met1 has been submitted to GenBank with GenBank Number: KJ184572. Protein domain prediction was performed using SMART (http://smart.embl-heidelberg.de/). The phylogenetic tree was obtained using the neighbor-joining method in MEGA 3.1 (http://www.megasoftware.net/).

### Recombinant expression and polyclonal antibody preparation

The fragment of Met1 81 aa–277 aa was expressed in *Escherichia coli* Rosetta host cells by the vector pGEX-4T-1, as inclusion body with the primers (sequence-Met1–F1–BamHI: TACTCAGGATCCCGGAGCCAATACAACTCT; Met1–R1–XhoI: TACTCAGTGGATCCAATCCACCTG-GATGA). Protein was purified by SDS–PAGE. The antisera was prepared by the method described previously (Dong et al. 2013). The specificity of the antisera was determined by western blot analysis.

### Quantitative real-time RT-PCR

Total RNA of larvae was extracted using Unizol reagent (Biostar, Shanghai, China). The RNA was reverse transcribed into the first-strand cDNA as the template for quantitative real-time RT-PCR (qRT-PCR) with the primers.
listed in Supplementary Table S1, see section on supplementary data given at the end of this article. The 10 μl mixture, which was used to perform qRT-PCR, comprised 5 μl SsoFast EvaGreen Supermix (Bio-Rad), 1 μl cDNA (1:50 diluted), 2 μl of 1 μmol/l forward primer, and 2 μl of 1 μmol/l reverse primer in 1 ml FBS-free Grace’s medium for 12 h. β-actin was used for quality control. Data were analyzed using the following formula: 

\[ R = 2^{-\left(\Delta C_{\text{sample}} - \Delta C_{\text{control}}\right)} \]

where \( R \) is the relative expression level, \( \Delta C \) sample is the difference between the Ct of the gene and the average β-actin in the experimental sample, and \( \Delta C \) control is the difference between the Ct of the gene and the average of β-actin in the control sample.

**Hormone treatment on larvae**

Stock solutions (20 mM) of 20E and JH III (Sigma) were prepared in dimethyl sulfoxide (DMSO). The 20E and JH III stock solute were diluted to 0.1 mg/ml with PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, and 2.7 mM KCl, pH 7.4). The hormone 20E or JH III (0.1 mg/ml) was injected in the 6th instar 6 h larvae (500 ng/larva). The untreated larvae were injected with an equivalent amount of DMSO.

**Western blot analysis**

Tissues were homogenized in Tris-buffered saline (TBS, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl), and protein quantification was performed using a β-actin antibody. The proteins were separated in SDS–PAGE and then transferred onto a nitrocellulose membrane. After being blocked with 2% non-fat milk in TBS for 1 h at room temperature, the membrane was incubated with the antibody for 12 h at 4 °C. The membrane was washed with TBST (0.1% Tween 20 in TBS) for 15 min thrice. Subsequently, the blot was probed with the HRP-conjugated goat anti-rabbit IgG (1:10 000 in the blocking solution). To visualize the peroxidase activity, 4-chloro-1-naphthol was used as a HRP substrate.

**RNAi in the HaEpi cell line and larvae**

MEGA-script RNAi Kit (Ambion, Inc., Austin, TX, USA) was used to synthesize dsRNA with the T7 promoter-containing PCR primers (Met1-RNAi-F and Met1-RNAi-R sequence in Supplementary Table S1). HaEpi was cultured to 80% confluence at 27 °C in Grace’s medium supplemented with 10% FBS. Approximately 1 μg dsRNA and 125 μl FBS-free Grace’s medium with 8 μl lipofectamine were mixed to incubate cells in 1 ml FBS-free Grace’s medium for 12 h. Subsequently, cells were cultured in Grace’s medium supplemented with FBS for 12 h and incubated with 20E or JH III for 12 h. For the RNAi experiment in larvae, dsRNA was diluted in nuclease-free water to 100 ng/μl. Approximately 5 μl of dsRNA were injected in a sixth instar 6 h larva. The control larvae were injected with 5 μl dsGFP individually. Experiments were carried out in 30 larvae and repeated thrice for statistical analysis.

**Hematoxylin–eosin staining**

The dewaxed histological sections were stained with hematoxylin for 10 min at room temperature. The sections were washed consecutively with running water and Scott liquid for 1 min. The sections were then differentiated with 1% hydrochloric acid in alcohol for 20 s, washed with Scott liquid for 1 min, stained with 0.5% water-soluble eosin dye solution for 30 s, and washed with running water. After sealing, the sections were observed under an Olympus BX51 fluorescence microscope.

**Co-immunoprecipitation**

The open reading frame (ORF) of the genes was cloned into the pLEX-4-vector fused with red fluorescent protein (RFP) or green fluorescent protein (GFP) using the primers (Supplementary Table S1) respectively. USP1–RFP–His, EcRB1–His, Met1–GFP–His, and Met1–RFP were over-expressed in HaEpi cells via transfection with Cellfectin II Reagent (Invitrogen) according to the method described previously (Hou et al. 2012). After 48 h of culture, cells were incubated with 1 μM 20E or JH III for 2 h. DMSO treatment was used as the control, and RFP–His was expressed as the control. The proteins were extracted from cells using the RIPA buffer, three wells of six well plate/800 μl (0.1 M Tris–HCl buffer, pH 8.0 containing 0.15 M NaCl, and 1% NP-40) and harvested by centrifugation at 12 000 g for 20 min at 4 °C. Approximately 30 μl to 40 μl of supernatant were used as input proteins. The rest of the supernatant was added the antiserum against Met1 (1:100), incubated for 3 h with gentle shaking at 4 °C, and then incubated with 40 μl Protein A resin for 3 h with gentle shaking at 4 °C. After centrifugation at 12 000 g for 10 min at 4 °C, the supernatant was discarded. After being washed with PBS, the resin was treated with the SDS–PAGE loading buffer and boiled for 10 min for western blot analysis with various antibodies. The molecular masses of the proteins are shown in Supplementary Figure S3 (see section on supplementary data given at the end of this article).
Electrophoretic mobility shift assay

Various proteins were overexpressed in HaEpi cells. The nuclear proteins were extracted from HaEpi cells and diluted to 1 μg/μl. *T. castaneum Khr1–JHRE–digoxin-labeled double-stranded probe contains E-box (sequences: 5’-GGCCCTCCAGTGTGCAGGAGG-3’ and 3’-CGTTCGA-CACGTGGAGGCCACCGC-5’). The E-box ‘CACGTG’ is conserved in *Apis mellifera*, *Acrystosiphon pisum*, *B. mori*, *D. melanogaster*, *N. vitripennis*, and *T. castaneum* (Kayukawa et al. 2012). *H. armigera* HR3-EcRE (Liu et al. 2014) digoxin-labeled double-stranded probe (sense 5’-gctctgggatagtgaag-3’ and antisense 3’-tcagacttcgaaccctggg-5’) was synthesized by Shengong (Shanghai, China). The probes were incubated with nuclear proteins for 20 min at room temperature in a binding buffer (Beyotime, Shanghai, China). The detailed method was described in our previous paper (Liu et al. 2014). The 100 fmol probes were incubated with 5 μg nuclear proteins for 20 min at room temperature in 15 μl binding buffer (Beyotime, Shanghai, China). A fifty-fold amount of non-labeled-probe was used as specific competitor. For antibody mediated super shift assay, antiserum (1:2000) was incubated with the nuclear proteins in reaction mixture for 20 min, followed by incubation with a digoxin-labeled probe for another 20 min. The protein-DNA complex was separated from the free probe in 5.75% non-denaturing polyacrylamide gel.

Results

The full cDNA sequence of Met1 is 2410 bp, with an ORF of 1581 bp, and encodes 526 amino acid proteins with a molecular mass of 59 kDa. Met1 has the typical characteristics of b-HLH-PAS family proteins with four structural domains of b-HLH, two PAS, and one PAC. Met1 of *H. armigera* is similar to Met1 of *B. mori* (Supplementary Figure S1, see section on supplementary data given at the end of this article). HLH is a DNA-binding domain (Murre et al. 1989). PAS is used as a signal sensor domain (Taylor & Zhulin 1999). PAC is a motif C-terminal to PAS, which is proposed to contribute to the PAS domain fold by SMART analysis (http://smart.embl-heidelberg.de/).

Met1 expression level increased during feeding but decreased during metamorphosis

To study the function of Met1, we examined the expression profile during development from fifth instar to prepupae. Met1 showed a higher expression level at the fifth instar feeding stage (F) and the sixth instar feeding stage (F) compared with the fifth instar molting stage (M) and the metamorphic molting stage (MM) in the integument, midgut, and fat body (Fig. 1A and a). This expression pattern suggests that Met1 expression level is regulated during larval development.

Considering that JH titer is higher during feeding and 20E is higher during molting and metamorphosis in *M. sexta* (Riddiford et al. 2003), we injected sixth instar 6 h larvae with JH III and 20E to analyze the hormone regulation in Met1 expression. Results showed that 20E and JH III upregulated the expression of Met1 in the midgut independently. However, the higher concentration of 20E repressed Met1 expression (Fig. 1B and b). These results suggest that Met1 expression is coordinately regulated by JH and 20E.

Knockdown of Met1 causes precocious pupation by changing the gene expression

Met1 was knocked down by injecting dsRNA of Met1 into 6th instar 6 h larvae to examine the function of Met1 in

**Figure 1**

Western blot analysis showing Met1 expression profiles during larval development. The specificity of the antibody against *H. armigera* Met1 is shown in Supplementary Figure S2, see section on supplementary data given at the end of this article. (A) Profile of Met1 expression in the integument, midgut, and fat body. F, feeding; M, molting; MM, metamorphic molting. β-actin was used as the loading control. (a) Quantification of Met1 expression in (A) using the ImageJ software.
the larval development. The knockdown of Met1 caused precocious pupation (Fig. 2A and B). The pupation time from 6th instar 0 h to pupa in dsMet1-injected larvae was 92 h, which was 28 h more precocious than the 120 h in dsGFP-injected control larvae (Fig. 2C). These results suggest that Met1 is involved in maintaining the larval status.

JH III was injected in the 6th instar 6 h larval hemocoel to examine the involvement of Met1 in JH III function of preventing metamorphosis. In the DMSO-injected control, the midgut performed normal remodeling by separating from the imaginal midgut 70 h after the first injection. By contrast, injection of JH III prevented midgut remodeling. dsGFP injection did not repress the JH III-prevented midgut remodeling. However, dsMet1 injection repressed the JH III-prevented midgut remodeling (Fig. 2D). These results suggest that Met1 is involved in the JH III function to prevent metamorphosis.

To address the mechanism of Met1 knockdown causing precocious metamorphosis, we examined a set of gene expression after Met1 knockdown in larvae, including the 20E pathway genes EcRB1, USP1, HR3, and Br, and JH pathway gene Krh1 using qRT-PCR. EcRB1, USP1, HR3, and Br significantly increased in the midgut after Met1 knockdown compared with the dsGFP-injected control. However, Krh1 was downregulated after Met1 knockdown (Fig. 2E). These results suggest that Met1 knockdown caused precocious metamorphosis because of the change in gene expression, and Met1 is a promoter of JH pathway gene expression and a repressor of 20E pathway gene expression.

**Knockdown of Met1 in HaEpi cells represses gene expression in JH pathway and promotes gene expression in 20E pathway**

To confirm the conclusion regarding the Met1 knockdown in larvae, the function of Met1 in JH and 20E pathways was examined in HaEpi cells by RNAi. JH III increased expression of Met1, USP1, Br, and Krh1 transcripts. However, after knockdown of Met1, JH III could not increase expression of these gene transcripts (Fig. 3A). By contrast, 20E increased expression of Met1, EcRB1, USP1, HR3, and Br to a greater extent (Fig. 3B). These results confirm that JH via Met1 promotes USP1, Br, and Krh1 expression, and Met1 inhibits 20E-induced EcRB1, USP1, HR3, and Br expression.

**Overexpression of Met1 in HaEpi cells promotes JH III-induced gene expression and represses 20E-induced gene expression**

To corroborate the data in Met1 knockdown in HaEpi cells, we induced overexpression of Met1 in HaEpi cells by fusing
Met1 interacts with Hsp90 and binds to Krh1-JHRE

Met binds to the E-box ‘CACGTG’ in the core region of kJHRE (GGCCTCCACGTG) and the E-box is conserved in six species (A. mellifera, A. pisum, B. mori, D. melanogaster, N. vitripennis, and T. castaneum) (Kayukawa et al. 2012). To confirm that JH III regulates Krh1 transcription via Met, we overexpressed Met1–RFP in HaEpi cells and performed an electrophoretic mobility shift assay (EMSA) experiment using kJHRE (GGCCTCCACGTG) in the Krh1 promoter of Tribolium as a probe. In the RFP-expressing cells, the nuclear extracts from normal cells, or DMSO- or 20E-treated cells could not change the mobility of kJHRE (Fig. 5A and lane 1, 2, and 3). However, the nuclear extracts from JH III-treated RFP-expressing cells changed the mobility of kJHRE (Fig. 5A and lane 4), suggesting that JH III-induced nuclear proteins can bind to kJHRE. In the Met1–RFP-expressing cells, the nuclear extracts from DMSO- or 20E-treated cells could not change the mobility of kJHRE (Fig. 5A and lanes 5 and 6). However, the nuclear extracts from JH III-treated cells changed the mobility of kJHRE and increased the amount of mobilized kJHRE compared with RFP-expressing control cells (Fig. 5A and lane 7 compared with lane 4). When the 50-fold excess of unlabeled kJHRE was added, the amount of the mobilized kJHRE decreased (Fig. 5A and lane 8 compared with lane 7), suggesting that Met1–RFP binds to kJHRE. After the addition of the antibodies against either Hsp90 or Met1, the mobilized kJHRE band disappeared (Fig. 5A and lane 9 and 10). The data were statistically analyzed (Fig. 5B). These results confirm that Met1–RFP binds to kJHRE, and Hsp90 is necessary for this binding.

Given that Met1 can interact with Hsp90 to regulate the gene expression in the JH pathway in H. armigera (Liu et al. 2013), Co-IP was conducted to confirm the interaction between Met1 and Hsp90 in JH induction. Hsp90 was co-precipitated by an anti-Met1 antibody from the JH III-treated cells, but not from the 20E-induced cells (Fig. 5C and D). These findings suggest that Met1 interacts with Hsp90 and binds to kJHRE to promote Krh1 transcription in the JH pathway.

Met1 represses EcRB1–USP1 interaction

Considering that the overexpression of Met1 repressed 20E-induced gene transcription, we performed an EMSA experiment by examining the binding of EcRB1 to EcRE in H. armigera HR3 (5′-GGGGTCAATGAACTG-3′ and 3′-CAGTTCATTGACCCC-5′) (Liu et al. 2014) to investigate the mechanism. In the RFP-expressing control cells, when the nuclear extracts from DMSO- or JH III-induced RFP-expressing cells were incubated with Dig-EcRE, no mobilized Dig-EcRE was detected (Fig. 6A and lanes 2 and 4). However, Dig-labeled EcRE was mobilized by the nuclear extracts from 20E-treated cells (Fig. 6A and lane 3
compared with lane 2), and the non-Dig-labeled EcRE competitor could compete to decrease the amount of mobilized Dig-EcRE (Fig. 6A and lane 5 compared with lane 3), suggesting 20E-induced nuclear extracts binding to the Dig-EcRE probe. The overexpressed Met1–RFP decreased the 20E-induced amount of mobilized Dig-EcRE (Fig. 6A and lane 7 compared with lane 3). The non-Dig-labeled EcRE competitor decreased the amount of mobilized Dig-EcRE (Fig. 6A and lane 8 compared with lane 7). The antibody against Met1 increased the amount of mobilized Dig-EcRE (Fig. 6A and lane 9 compared with lane 7). JH III repressed the binding of nuclear extracts to the Dig-EcRE probe (Fig. 6A and lane 10). The EcRB1–His was overexpressed in cells to confirm 20E-induced binding of EcRB1 to EcRE. The amount of mobilized Dig-EcRE by 20E-induced nuclear extracts (Fig. 6C and lane 3) was decreased using an anti-His antibody that recognizes EcRB1–His (Fig. 6C and lane 4), indicating that the nuclear protein in the nuclear extracts binding to EcRE was EcRB1. Statistical analysis showed the significant difference in the amount of the mobilized Dig-EcRE in various treatments (Fig. 6B and D). These results suggest that Met1 represses the EcRB1 binding to EcRE.

To address the mechanism of overexpressed Met repressing the EcRB1 binding to EcRE, we induced overexpression of EcRB1–His, USP1–RFP, and Met1–GFP in HaEpi cells simultaneously. Three proteins were equally expressed in cells, as shown in the input proteins (Fig. 6E, Input). From the co-immunoprecipitates (Co-IP) by anti-RFP that precipitated USP1–RFP as well as the interacting proteins, Met1–GFP and USP1–RFP, were detected either in the DMSO- or JH III-treated cells, whereas, EcRB1–His was detected together with Met1–GFP and USP1–RFP in 20E-treated cells. After addition of JH III to cells, the amount of 20E-induced EcRB1–His was reduced and that of Met1–GFP was increased (Fig. 6E, Co-IP). Statistical analysis revealed that the differences in the protein amounts in various treatments were significant (Fig. 6E and F). These results suggest that Met1 interacts with USP1-independent hormone regulation; however, 20E promotes the interaction between EcRB1 and USP1, and JH increases the interaction between USP1 and Met1.

Given that USP is phosphorylated by 20E induction and the phosphorylation of USP is essential for its function in gene transcription (Sun & Song 2006, Wang et al. 2012), the phosphorylation status of USP1 was examined by a 7.5% low-concentration SD–PAGE gel to demonstrate the mechanism of EcRB1 and USP1 interaction in 20E induction. The overexpressed USP1–His in HaEpi cells partially increased the molecular mass 6 h after 20E induction.
treatment, compared with DMSO and JH III treatments. The increased molecular mass of USP1–His was decreased upon lambda protein phosphatase treatment, indicating that it was the phosphorylated form of USP1 (Fig. 6G). These results suggest that 20E regulates USP1 phosphorylation to interact with EcRB1, and JH III maintains USP1 nonphosphorylation to interact with Met1.

Discussion

Met1 is undoubtedly an important transcription factor with a crucial function in insect development. However, the function and mechanism of Met in regulating insect development are not well understood because of its complicated roles in either the JH pathway or the 20E pathway. In this research, we demonstrated that Met1 promotes JH-regulated Krh1 transcription and represses 20E-induced EcRB1, USP1, Br, and HR3 transcription to maintain the larval status. The expression level of Met1 is upregulated in larval life when JH is present, i.e. during the feeding stages. It is downregulated during molting and metamorphosis, thus in stages when the JH titer is lowered or even zero. In these stages, the ecdysteroid titer is increased. JH mediates Met1 binding to kJHRE via interaction with Hsp90 to promote Krh1 transcription. Met1 represses EcRB1/USP1 binding to EcRE for EcRB1, USP1, Br, and HR3 transcription by interacting with USP1.

Met1 promotes Krh1 transcription to maintain the larval status

_Tribolium_ Met functions in JH response to mediate the antimetamorphic effect of JH (Konopova & Jindra 2007). Double mutation of Met and gce in _Drosophila_ larvae totally blocks the Krh1 expression (Abdou et al. 2011). Krh1 is a JH early-inducible gene identified in _D. melanogaster_ (Minakuchi et al. 2008). Knockdown of _Tribolium_ Krh1 causes precocious metamorphosis (Minakuchi et al. 2009). In our study on _H. armigera_, Met1 knockdown causes precocious pupation and midgut remodeling. Correlated with the precocious pupation, Met1 knockdown repressed the Krh1 expression. These results are similar to the observations in _Drosophila_ and _Tribolium_. Therefore, Met1 promotes the JH pathway in _H. armigera_. The function of Met1 at the level of the nucleus is the maintenance of the larval status.
JH regulates PKC-phosphorylated Hsp90 and interaction with Met1 to participate in Krh1 expression (Liu et al. 2013). In this study, we further revealed that the complex of Hsp90 and Met1 binds to kJHRE to promote Krh1 transcription. Considering the critical function of Krh1 in the suppression of insect metamorphosis (Kayukawa et al. 2012), we proposed that JH maintains the larval status by upregulating Met1 expression and then directing the Krh1 expression.

The fact that JH acts not only through a nuclear receptor but through a membrane receptor(s) as well should not be overlooked. According to De Loof et al. (2014), a low cytoplasmic Ca$^{2+}$ concentration is causal to maintaining the larval status of holometabolous insects. At least some of the proteins that constitute the Ca$^{2+}$ homeostasis system have a binding site for sesquiterpenoids, JH being one of them. At a higher Ca$^{2+}$ concentration, Ca$^{2+}$-induced programed cell death, a typical aspect of metamorphosis, is activated. JH may play a role through the membrane receptor and protein kinase C in the accessory gland of male fruit flies and the follicle of vampire bugs (Sevala & Davey 1993). However, the specific membrane receptor of JH needs to be elucidated.

**Met1 represses the transcription of a set of 20E-responsive genes to prevent metamorphosis**

Met1 represses a set of 20E-responsive gene transcription, including EcRB1, USP1, HR3, and Br, EcRB1 and USP1 form a transcription complex and bind to EcRE to initiate gene transcription (Jindra et al. 1996). HR3 is a reporter gene in the 20E pathway that is involved in molting and metamorphosis (Lam et al. 1999). Br is a metamorphosis initiator that promotes programed cell death gene expression (Cakouros et al. 2002). Knockdown of Met1 helps 20E to induce EcRB1, USP1, HR3, and Br

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**Figure 6**

Overexpressed Met1 represses EcRB1 binding to EcRE. (A) EMSA analysis of nuclear protein binding to EcRE. Lane 1, the nuclear extracts from normal cells; lanes 2, 3, 4, and 5, the nuclear extracts from the RFP-overexpressing control cells treated with DMSO, 20E, JH III (1 μM for 6 h), and a competitor (non-labeled probe EcRE, 50-fold molar excess of Dig-labeled probe EcRE); and lanes 6, 7, 8, 9, and 10, the nuclear extracts from Met1–RFP-overexpressing cells treated with DMSO, 20E, competitor, anti-Met1, and JH III. (B) Statistical analysis of data in (A) using Student’s t-test (asterisks indicate significant differences (P < 0.05) between the groups based on three independent experiments for all the following statistical analyses). (C) EMSA analysis of EcR binding to EcRE in the induction of 20E. (D) Statistical analysis of data in B by Student’s t-test. (E) Co-IP showing the protein interaction among Met1, EcRB1, and USP1 (12.5% SDS-PAGE gel). pIEX-4T1–EcRB1–His, pIEX-4T1–USP1–RFP, and pIEX-4T1–Met1–GFP plasmids were co-transfected in cells. Input: anti-His detected EcRB1–His; anti-GFP detected Met1–GFP; anti-RFP detected USP1–RFP; and anti-β-actin detected β-actin in various treatments. Co-IP: USP1–RFP was immunoprecipitated with an anti-RFP monoclonal antibody. Co-precipitated EcRB1–His and Met1–GFP were detected by anti-His or anti-GFP antibodies, respectively, in cells treated with DMSO, 20E, JH III, and 20E plus JH III (1 μM for 6 h). JH III was used as the loading control in the experiments. (F) Statistical analysis of data in (E) by Student’s t-test. (G) Western blot analysis showed the phosphorylation of USP1 in the induction of 20E (7.5% SDS-PAGE gel). USP1–His-overexpressing cells were treated with DMSO, 20E, or JH III (1 μM for 6 h). The 20E-treated protein was incubated with Lambda protein phosphatase (λ-phosphatase) with β-actin as the loading control. (H) Statistical analysis of data in (G) by Student’s t-test.
transcription, thus promoting precocious metamorphosis, suggesting that Met1 is a repressor of the 20E pathway.

By EMSA experimental analysis, the overexpressed Met1 can repress EcRB1 binding to EcRE. This might be due to the fact that Met1 interacts with USP1 that disturbs the formation of EcRB1/USP1 complex and its binding to EcRE. USP1 is the heterodimeric partner of EcRB1 in the formation of a transcription complex for gene transcription in the 20E pathway. JH also binds to USP1 and participates in JH-regulated gene transcription, suggesting its role in JH pathway (Jones & Sharp 1997). We found that 20E regulates USP1 partially phosphorylated to interact with EcRB1. JH maintains USP1 nonphosphorylation to repress its interaction with EcRB1 and increases its interaction with Met1. However, the interaction between Met1 and USP1 may not rely on JH regulation because the overexpressed Met1 is co-immunoprecipitated with nonphosphorylated USP1 in DMSO-treated cells also. The 20E- or JH hormone-independent interactions between Met and USP and between Met and EcRB1 has been reported in D. melanogaster (Bitra & Palli 2009) and B. mori (Guo et al. 2012) respectively. Therefore, Met1 might repress 20E pathway gene transcription by repressing the formation of EcRB1/USP1 transcription complex and its binding to EcRE.

**Expression of Met1 is coordinately regulated by JH and 20E**

In *Tribolium*, Met is continuously expressed throughout embryonic development (Konopova & Jindra 2007). In *Bombyx*, the developmental profiles of Met1 and Met2 from day 2 of the 4th instar to day 2 of the prepupal stage, when the 20E titer is high, have been demonstrated by Guo et al. (2012). In *H. armigera*, Met1 is expressed during the entire larval stage from the 5th instar stage up to the prepupal stage. However, the expression levels increased during the feeding stage and decreased at molting and later metamorphic stages. The different expression profiles in various insects may be attributed to species specificity. JH III and 20E may independently upregulate Met1 expression. However, a higher concentration of 20E represses Met1 expression. Thus, Met1 is coordinately regulated by the two hormones.

**Conclusion**

This study deepens our understanding of how interactions among a number of key transcription factors contribute, at the level of the nucleus, to the maintenance of the larval status by JH. JH upregulates the expression of Met1 and interaction with phosphorylated Hsp90 and nonphosphorylated USP1, which binds to kJHRE to promote *Krh1* transcription for larval growth. The hormone 20E mediates the interaction between EcRB1 and phosphorylated USP1. Met1 interacts with USP1 to repress the formation of EcRB1/USP1 complex and its binding to EcRE and, therefore, to repress 20E-induced gene transcription and metamorphosis (Fig. 7).

The challenge for future experiments is to uncover which genes are activated by the JH- and 20E-dependent transcription factors. Perhaps, the transcription of genes coding for some of the proteins that make up the Ca^{2+}-homeostasis system, which seems to play a crucial role in realizing some JH-dependent effects, is controlled this way.

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**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0019.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
W-L Z performed the major experiments, C-Y L cloned the gene, W L helped on EMSA, D W performed overexpression experiments, J-X W directed the study, and X-F Z designed the study and wrote the final version of the manuscript.

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