MET Is Required for the Maximal Action of 20-Hydroxyecdysone during Bombyx Metamorphosis

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Abstract

Little is known about how the putative juvenile hormone (JH) receptor, the bHLH-PAS transcription factor MET, is involved in 20-hydroxyecdysone (20E; the molting hormone) action. Here we report that two MET proteins found in the silkworm, Bombyx mori, participate in 20E signal transduction. Met is 20E responsive and its expression peaks during molting and pupation, when the 20E titer is high. As found with results from RNAi knockdown of Ecr-USP (the ecdysone receptor genes), RNAi knockdown of Met at the early wandering stage disrupts the 20E-triggered transcriptional cascade, preventing tissue remodeling (including autophagy, apoptosis and destruction of larval tissues and generation of adult structures) and causing lethality during the larval-pupal transition. MET physically interacts with EcR-USP. Moreover, MET, Ecr-USP and the 20E-response element (EcRE) form a protein-DNA complex, implying that MET might modulate 20E-induced gene transcription by interacting with EcR-USP. In conclusion, the 20E induction of MET is required for the maximal action of 20E during Bombyx metamorphosis.

Introduction

The molting hormone, 20-hydroxyecdysone (20E), and juvenile hormone (JH) coordinate control insect molting and metamorphosis. Overall, 20E orchestrates the molting process, whereas JH determines the nature of the molt. In the fruitfly, Drosophila melanogaster, Methoprene-tolerant (MET), a bHLH-PAS transcription factor [1], binds JH at physiological concentrations in vitro [2] and is postulated to be the JH receptor [3]. MET forms homodimers or heterodimers with its paralog, germ-cell expressed (GCE), and JH reduces this dimerization [4]. Although Met and gce null single mutants are fully viable, Met gce double mutants die during the larval-pupal transition [3,5], resembling what is seen in JH-deficient animals [7]. Functionally, MET/GCE mediates JH action to prevent 20E-triggered apoptosis of larval fat body [6,7] and differentiation of the optic lobe of the adult brain [8]. In the beetle Tribolium castaneum, MET plays a similar key role in JH action during the larval-pupal metamorphosis [9,10]. Recently, the ligand binding properties of MET were confirmed in Tribolium, suggesting strongly that MET is the actual JH receptor [11].

A great deal more is known about the 20E signal transduction pathway. The 20E nuclear receptor complex is a heterodimer composed of ecdysone receptor (EcR) and ultraspiracle (USP) [12,13]. The heterodimeric EcR-USP is known as the ecdysone receptor and binds the 20E-response element (EcRE) with the assistance of a molecular chaperone complex [14]. In the absence of 20E, the ecdysone receptor associates with transcriptional co-repressors. When 20E binds to the ecdysone receptor, the co-repressors dissociate [15,16]. The ligand-receptor complex (20E-ecdysone receptor complex) then recruits transcriptional co-activators to induce gene expression through the EcRE [17]. 20E triggers a transcriptional cascade, including transcription of the 20E-primary-response genes (i.e. transcription factor genes Br-C, E74, E75, and E93) and, subsequently, the 20E-secondary-response genes [18]. Moreover, Br-C, E74, E75, E93 and other 20E response genes positively impact 20E signaling. For example, E93 binds to many 20E response genes and cell death genes on polytene chromosomes. The expression of these genes is defective in E93 mutants, while E93 overexpression results in the upregulation of these genes [19].

One major function of JH is to inhibit some of the actions of 20E [3]. The p160/SRC/NCoA-like molecule, TAIMAN in Drosophila [20] and FISC in the mosquito, Aedes aegypti [21], which also belongs to the bHLH-PAS family of transcriptional regulators, is a transcriptional co-activator of the 20E-ecdysone receptor complex through physical interaction with EcR. Moreover, the p160/SRC/NCoA-like molecule directly associates with MET and is involved in JH action in Aedes, Drosophila, and Tribolium, suggesting a role in enhancing JH-20E crosstalk [22,23]. It has been shown that the orphan nuclear receptor BFTZ-F1, which is a
Results

The two Met genes are 20E responsive

There are two Met genes, Met1 and Met2, in the Bombyx genome (GenBank accession numbers: Met1, EU249371; Met2, EU249372) (Figure S1A) [30]. Met1 and Met2 mRNA expression in the fat body was measured from day 2 of the 4th instar to day 2 of the prepupal stage by quantitative real-time PCR (qPCR). The developmental profiles show that Met mRNA levels reach a small peak during the 4th larval molt and are very high during the larval-pupal transition (Figure 1A), suggesting that they are upregulated at stages when the 20E titers are high [31]. Met1 and Met2 mRNA levels as well as the MET1 protein level were increased in the fat body 6 hr after 20E injection into day 2 of the 5th instar larvae (Figure 1B and S1B). They were also decreased 24 hr after RNAi at the initiation of the early wandering stage (Figure S1C). The fat body and generating adult structures during larval-pupal metamorphosis. To further substantiate this, premise RNAi studies were conducted.

Met RNAi results in lethality

Met RNAi (10 μg dsRNA per larva) was performed at the initiation of the early wandering stage. Met RNAi resulted in lethality during the larval-pupal-adult metamorphosis, with a higher percentage of lethality occurring from Met2 RNAi (∼80%) compared to Met1 RNAi (∼50%) (Table 1). Although most of the Met RNAi treated silkworms were able to spin, their cocoons were much thinner (Figure S2A), and the larval-pupal transition was delayed significantly (∼24 hr) (Figure 2A). Some Met RNAi treated silkworms died during the wandering stage (Figure 2A) or during pupation (Figure 2B), while some arrested during the mid-pupal stage lacked adult structures (Figure 2C). Overall, Met RNAi results in lethal phenotypes similar to Ecr-USP RNAi treated animals [27], demonstrating that MET is functionally important during Bombyx metamorphosis.

Met RNAi prevents tissue remodeling

Through the ecdysone receptor, the 20E-triggered transcriptional cascade is important in removing obsolete larval tissues via programmed cell death (PCD, mainly apoptosis and autophagy) and generating adult structures from progenitor cells during metamorphosis [18,32]. Since Met RNAi treated animals result in lethal phenotypes similar to those observed in Ecr-USP RNAi treated animals, we investigated the effects of Met RNAi on larval tissue remodeling during Bombyx metamorphosis to determine the possible role of Met in PCD. Eighteen hr after treatment, Met RNAi significantly prevented apoptotic events in the fat body as estimated by TUNEL labeling and quantification of caspase 3 activity [29] (Figure 3A). By 24 hr, Met RNAi nearly abolished autophagy, as estimated by Lysotracker staining [33,34] (Figure 3B). Twenty-four hr after pupation, Met RNAi dramatically inhibited fat body cell dissociation (Figure 3C). The inhibitory effects on fat body tissue remodeling by Met RNAi were stronger than for Met1 RNAi (Figure 3A–C). Similar to the fat body, silk gland lysis was also prevented by Met RNAi 24 hr after pupation. In this tissue, the inhibitory effects of Met2 RNAi were also stronger than for Met1 RNAi (Figure S2B).

Met RNAi also affected adult structure formation. Most of the surviving Met RNAi pupae did not fully develop legs or wings during the late pupal stage (Figure 2D). Since half of the Met1 RNAi treated larvae survived to the adult stage, we closely examined developmental defects of their adult structures. Many of the surviving Met1 RNAi adults failed to emerge normally (pupal cuticle remained attached to the head or abdomen) and they had shortened and distorted legs or unexpanded wings (Figure 2E and Figure S2C). These results demonstrate that Met is required for proper tissue remodeling during Bombyx metamorphosis, including PCD of obsolete larval tissues and generation of adult structures. We next examined the question of the mechanism of Met action.

Met RNAi disrupts the 20E-triggered transcriptional cascade

Since the Met RNAi effects resemble those of Ecr-USP RNAi at the phenotypic level, we investigated whether Met RNAi also disrupts the 20E-triggered transcriptional cascade in the fat body. As determined by qPCR, the 20E-response genes EcR, USP, Br-C, and E74A were significantly downregulated 24 hr after Met RNAi. Similar to the phenotypic effects of Met RNAi, the inhibitory effects on gene expression by Met2 RNAi (70–95%) were stronger than for Met1 RNAi (50–90%) (Figure 4A). Moreover, Western blots for MET1, USP, and Br-C (Figure 4B) as well as immunohistochemistry for USP and Br-C (Figure 4C) revealed that protein levels were decreased by Met RNAi. To avoid the possibility of off-targeting, we generated two other sets of Met dsRNAs (Figure S1A) which exhibited similar but stronger inhibiting effects on gene expression when higher concentrations (30 μg of dsRNA per larva) were used (Figure S3A). Since three different sets of Met dsRNAs were used, the off-targeting problem should be largely minimized. To be consistent with the above experimental data (Figures 2, 3, 4, and S2), we used the first set of Met dsRNAs in the following experiments.

To verify the above results, samples of fat body were explanted 24 hr after RNAi treatment and cultured for an additional 6 hr in the presence of 20E. 20E treatment caused significant upregulation of all 6 genes in the egfp RNAi treated fat body, while this upregulation was dramatically decreased when Met2 RNAi was performed (Figure S3B). We therefore conclude that Met RNAi disrupts the 20E-triggered transcriptional cascade in the fat body during larval-pupal metamorphosis.

To prevent effects of JH, Met RNAi experiments were performed ∼6 hr after pupation, a stage where JH is absent and 20E is present [30]. Surprisingly, both Ecr-USP RNAi and Met RNAi at this stage did not cause lethality. However, the 20E-response genes EcR, USP, Br-C, and E74A were significantly downregulated 24 hr after Met RNAi treatment, and the inhibitory effects of Met2 RNAi were stronger than for Met1 RNAi (Figure 4D).
To further insure that the interference was not due to JH, we performed Met RNAi followed by the addition of 20E to Bombyx DZNU-Bm-12 cells [35] which should lack JH. Met2 mRNA levels were very low in these cells and the efficiency of Met2 RNAi was poor, but Met1 RNAi decreased Met1 mRNA levels by about 90%. Six hr after 20E treatment, all the 20E-response genes were significantly upregulated in egfp RNAi treated cells, but this upregulation was significantly decreased in Met1 RNAi treated cells (Figure S3C). These data show conclusively that MET is required for the maximal ability of 20E to induce gene expression, and thus tissue remodeling, in Bombyx.

In addition, RNAi knockdown of either EcR or Met in Tribolium during the early quiescent stage resulted in lethality, delayed larval-pupal transition, and disrupted the 20E-triggered transcriptional cascade (Figure S4), demonstrating that MET is also required for the maximal action of 20E during metamorphosis in Tribolium. We then turned to the question of transactivation of the 20E-ecdysone receptor complex by MET in Bombyx.

MET, EcR-USP and EcRE are components of a protein-DNA complex

It has been reported that Drosophila MET physically interacts with EcR-USP [36,37]. A CytoTrap yeast two-hybrid experiment was carried out to investigate whether such direct associations among MET1, Met2, EcR and USP occur in Bombyx. As expected, EcR (the EcR-B1 isoform was used throughout the paper) and USP strongly associate with one another. Weak associations were observed between MET1 and Met2, MET1 and MET1, and Met2 and Met2, while intermediate associations were formed between the two MET proteins and the ecdysone receptor (Figure 5A). To confirm the yeast two-hybrid
results that MET associates with EcR-USP, we performed immunoprecipitation experiments. When the HA-EcR, FLAG-USP, and V5-Met1 constructs were co-transfected into HEK 293 cells, MET physically interacts with EcR-USP, while 20E treatment had little or no stimulating effects on the physical interactions between MET1 and EcR-USP (Figure 5B) confirming the results reported in [36,37]. As negative controls, IgG was not able to pull down endogenous HA-EcR, FLAG-USP, or V5-MET1 (Figure S5A).

Since MET physically interacts with the ecdysone receptor, we investigated whether MET, ecdysone receptor and EcRE form a protein-DNA complex when the HA-EcR, FLAG-USP, V5-Met1, and cMyc-Met2 constructs were co-transfected into HEK 293 cells. As expected, the overexpressed MET was not able to bind EcRE in the electrophoretic mobility shift assay (EMSA) but EcR-USP did (Figure S5B). Addition of the HA or FLAG antibody resulted in a shift of EcRE by HA-EcR or FLAG-USP (Figure 5C). When the V5 or cMyc antibody was added, binding of the ecdysone receptor-EcRE complex was also shifted by V5-MET1 or cMyc-MET2 (Figure 5D), demonstrating that MET, ecdysone receptor, and EcRE form a protein-DNA complex.

Subsequently, Met1 RNAi and EcRE-Luc transfections were simultaneously conducted in DZNU-Bm-12 cells, followed by 20E treatment and measurements of EcRE-driven luciferase activity. Met1 RNAi had no apparent effects on basal luciferase activity, but significantly decreased 20E-induced EcRE-driven luciferase activity. Moreover, co-transfection with EcR and USP increased 20E-induced EcRE-driven luciferase activity, and this induction was again significantly decreased by Met1 RNAi (Figure 5F), suggesting that MET is required for the maximal action of 20E in inducing gene expression via physical interaction with the ecdysone receptor and EcRE.

**Discussion**

In this study, we demonstrate that MET is required for the maximal action of 20E in *Bombyx*. Although the Met1 mRNA level in the fat body is much higher than the Met2 mRNA level, with the first set of dsRNAs, the inhibiting effects of Met2 RNAi on 20E-triggered gene expression and tissue remodeling were stronger than for Met1 RNAi. However, results from the other two sets of dsRNAs showed that Met1 RNAi had higher inhibiting effects than Met2 RNAi. Met RNAi performed at different developmental stages and in cultured cells confirmed the major conclusion that MET is required for the maximal action of 20E in *Bombyx*. Unfortunately, it is difficult to determine with certainty which MET is more important and whether the two Met genes are functionally redundant in the 20E signal transduction pathway when only using RNAi methodology. One reason is that RNAi knockdown of one Met gene downregulates the other Met gene, which is 20E responsive. Our preliminary data suggest that the Met1 mRNA level in the midgut was more abundant than the Met2 mRNA level and that using the first set of dsRNAs, Met1 RNAi resulted in a more severe inhibitory effects on midgut remodeling than Met2 RNAi. Since the systemic RNAi approach might result in non-tissue-autonomous effects, we suppose that performing RNAi with the binary GAL4/UAS system in *Bombyx* might be more useful for understanding which MET is more important in terms of different tissues or developmental stages. Mutation of the two Met genes, both separately and together, using...
MET Is Involved in 20E Action

A gene-targeting method [40] should be able to eventually resolve the problem in the future.

Both Bombyx Met genes are 20E responsive, exhibiting similar expression patterns to other 20E-response genes, including Br-C, E74, E75 and E93. RNAi knockdown of each of these genes also interrupts the 20E-triggered transcriptional cascade to different levels (unpublished data). Thus, it appears that the 20E induction of those 20E-response genes (including the Met genes) is required for the maximal action of 20E to induce gene expression in Bombyx. In Drosophila, mutation of Br-C, E74, E75, or E93 interrupts the 20E signaling, with more pronounced effects in E93 mutants. It has been well documented that the 20E induction of E93 determines a PCD response by positively impacting the 20E signaling [19]. The feedback regulation of 20E signaling should be common in insects.

Previously, we demonstrated that MET and GCE are functionally redundant in transducing JH signal to induce Kr-h1 expression and to antagonize 20E-induced Br-C expression [6,7]. However, our preliminary experiments suggest that MET and GCE might not be the same in modulating 20E signaling using the Met and gce mutants. It might be incomparable between the function of Met and gce in Drosophila and that of Met1 and Met2 in Bombyx, since the duplication events in these two insect species are evolutionary independent [6,41]. Nevertheless, in Tribolium, Met RNAi during the early quiescent stage also disrupts the 20E-triggered transcriptional cascade, showing that the single MET protein in Tribolium and the two MET proteins in Bombyx have similar functions in modulating 20E signaling during metamorphosis.

We have also tried to dissect out the molecular mechanism how MET is required for the maximal action of 20E in Bombyx. Our preliminary results suggest that MET might bind transcriptional co-activators, including CBP/p300 [42], which is important for transactivation of the 20E-ecdysone receptor complex via EcRE. The family of bHLH-PAS transcriptional regulators, consisting of transcription factors and co-activators, are critical components of gene expression networks that underlie essential developmental and environmental processes [43,44]. The mammalian bHLH-PAS transcription factors, such as the dioxin receptor (DR), recruit many transcriptional co-activators, including CBP/p300, p160/SRC/NCoA, p140, and CARM1/PRMT1 for transactivation [45]. Ligand-activated nuclear receptors (i.e., estrogen receptor; ER) also recruit these transcriptional co-activators for transactivation [45]. Thus, it might be a common mechanism that a bHLH-PAS transcription factor recruits histone-modifying transcriptional co-activators to liganded nuclear receptors for transactivation [43,45]. CBP/p300 RNAi attenuated, but did not abolish, 20E-induced luciferase activity driven by EcRE, suggesting that the receptor complex may consist of other histone-modifying transcriptional co-activators. A good candidate is p160/SRC/NCoA, which has been demonstrated in Aedes, Drosophila, and Tribolium [20,21,22,23].

Very recently, it has been documented that the Bombyx MET2 might act as a JH receptor. In the presence of JH, MET2 associates with SRC, the p160/SRC/NCoA-like molecule in Bombyx, to interact the JHRE in inducing Kr-h1 expression [31]. Considering the MET function in both JH and 20E actions, we propose that MET plays a role mediating JH-20E crosstalk, and that the detailed molecular mechanism is surely worthy of further investigation.

Figure 3. Met RNAi prevents fat body remodeling. The inhibitory effects on fat body remodeling by Met2 RNAi during the initiation of the early wandering stage were stronger than for Met1 RNAi (A–C). egfp dsRNA was used as a control. (A) Met RNAi prevented the apoptotic events, estimated by TUNEL (left panel, green) and measured by caspase 3 activity (right panel) 18 hr after RNAi treatment. The inset shows that TUNEL (green) and DAPI (blue) co-localize in nuclei (Bar: 50 μm). (B) Met RNAi prevented autophagy, estimated by LysoTracker (red) 24 hr after RNAi treatment. The inset shows that the LysoTracker (red) and DAPI (blue) stain the cytoplasm and the nuclei, respectively (Bar: 50 μm). (C) Met RNAi dramatically prevented cell dissociation of the fat body 24 hr after pupation (Bar: 50 μm).

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Insects and cell lines

*Bombyx* (P50) [28,29], *Tribolium* [9] were reared as previously described. *Bombyx* DZNU-Bm-12 cells [35] were maintained in TNM-FH (Sigma) medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) at 27°C. And human HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone).

Conventional molecular, biochemical, and cellular approaches

The full-length *Met1* and *Met2* cDNA sequences (GenBank accession numbers: EU249371 and EU249372) were cloned using RACE. Details of qPCR and Western blotting were previously described [7,27,28,46]. Caspase 3 activity was determined according to the manufacturer's instructions (Beyotime, Shanghai, China). TUNEL labeling (Beyotime) and LysoTracker staining (Invitrogen) were used to estimate apoptosis and autophagy, respectively, and monitored with an Olympus Fluoview FV1000 confocal microscope. Primers used here and elsewhere are listed in Table S1.

Figure 4. *Met* RNAi disrupts the 20E-triggered transcriptional cascade. RNAi was performed during initiation of the early wandering stage (A–C) and ~6 hr after pupation (D). The RNAi knockdown efficiency by *Met2* RNAi is higher than for *Met1* RNAi, and the downregulation rate of *Met1* by *Met2* RNAi is higher than for *Met2* by *Met1* RNAi. The #1 set of *Met* dsRNA was used. *egfp* dsRNA was used as a control. (A) *Met1* and *Met2*, and the 20E-response genes *EcR*, *USP*, *E74A* and *Br-C*, as determined by qPCR, were significantly downregulated 24 hr after *Met* RNAi. *RpL23* is used as a negative control of 20E-response gene. (B) *MET1*, *USP* and *Br-C* protein levels, as determined by Western blots, significantly decreased 24 hr after *Met* RNAi. *Tubulin* was used as a loading control. (C) *USP* and *Br-C* protein levels, as estimated by immunohistochemistry, significantly decreased 24 hr after *Met* RNAi. Localization of *USP* (green) and *Br-C* (red) were restricted to nuclei (Bar: 50 μm). (D) RNAi was performed ~6 hr after pupation. The rest is as in (A).

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dsRNAs were generated using the T7 RiboMAX™ Express RNAi System (Promega). Preliminary data showed that the P50 strain of Bombyx (the Chinese strain variation, Dazao) was more sensitive to RNAi treatments than the other tested strains [47], and the P50 strain of Bombyx was used throughout this study. RNAi

RNAi and hormone treatment

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Figure 5. Physical interaction between MET and EcR-USP. (A) The CytoTrap yeast two-hybrid analyses revealed direct associations among MET1, MET2, EcR and USP. Strong associations between bait and prey proteins led to more yeast colonies. (B) When the HA-EcR, FLAG-USP, and V5-Met1 constructs were co-transfected into human HEK 293 cells, 20E treatment for 6 hr at a final concentration of 1 μM had little or no stimulating effects on the physical interactions between MET and EcR-USP. In the immunoprecipitation experiments, the bottom Western blot is input, IP, immunoprecipitate; Blot, Western blot. (C) The HA-EcR, FLAG-USP, V5-Met1, and cMyc-Met2 constructs were co-transfected into the human HEK 293 cells. After nuclear extracts were bound with biotin-labeled EcRE, the protein-DNA complexes were separated on a 5% native PAGE gel followed by EMSA. Addition of the HA or FLAG antibody resulted in a shift of EcRE. In (C) and (D), the shift was indicated by a black arrow in comparison with a gray arrow. (D) The HA-EcR, FLAG-USP, V5-Met1, and cMyc-Met2 constructs were co-transfected into human HEK 293 cells. After nuclear extracts were bound with biotin-labeled EcRE, the protein-DNA complexes were separated 5% native PAGE followed by EMSA. When the V5 or cMyc antibody was added, binding of EcR-USP-EcRE was shifted by MET1 and MET2 in EMSA showing that MET, EcR-USP and EcRE form a protein-DNA complex. (E) Met1 RNAi and transfection were simultaneously conducted in Bombyx DZNUI-Bm-12 cells for 48 hr, followed by 20E treatment for 6 hr at a final concentration of 1 μM, and measurements of EcRE-driven luciferase activity were done. MET is required for 20E function to induce gene expression via the ecdysone receptor and EcRE. The bars labeled with different lowercase letters are significantly different (P<0.05, ANOVA).
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knockdown was performed at two developmental stages, including initiation of the early wandering stage and 6 hr after pupation. After the RNAi treatment (10 or 30 μg of dsRNA per animal), fat body from the abdominal segments was collected for bioassays. Three biological replicates were used, each of which consisted of 10 silkworms. The details of hormone treatment in vivo (1 μg 20E per animal; Sigma Aldrich) were previously described [28,29,48].

RNAi knockdown in DZNU-Bm-12 cells was performed using the Effectene transfection reagent (Qiagen) for 48 hr at a final concentration of 2 μg/ml dsRNA. To determine the 20E primary-response genes, 1 μM 20E and 10 μg/ml cycloheximide (Sigma Aldrich) were used [48].

Antibodies and immunohistochemistry

The Bombyx MET1 and Br-C antibodies were produced by the Abnart Company (Shanghai). A cDNA fragment encoding amino acids 151M to 350Q of MET1 and the full-length Abmart Company (Shanghai). A cDNA fragment encoding amino acid sequence was provided by Dr. K.F. Kafatos (Harvard University). Br-C were generated. The AB11 USP-specific monoclonal antibody was provided by Dr. K.F. Kafatos (Harvard University). Antibodies and immunohistochemistry

Antigen-purified rabbit polyclonal antibodies against MET1 and Br-C were generated. The AB11 USP-specific monoclonal antibody was provided by Dr. K.F. Kafatos (Harvard University). The monoclonal antibodies against the V5 tag (Sigma Aldrich), cMyc tag (Santa Cruz), and Tubulin (Invitrogen) were also used. USP and Br-C were detected in explanted fat body from the 5th abdominal segment by immunohistochemistry with the above primary antibodies. The fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch) were FITC-conjugated Affini-pure Goat Anti-Mouse IgG for USP and Cy3-conjugated Affini-pure Goat Anti-Rabbit IgG for Br-C. Fluorescence signals were detected using the Olympus Fluoview FV1000 confocal microscope.

Yeast two-hybrid assay

Yeast two-hybrid assays were carried out using the CytoTrap system (Stratagene), which is based on the ability of human Sos to complement a temperature-sensitive cdc25 allele (cdc25H) in yeast when Sos is targeted to the plasma membrane through bait-prey interactions. This system has been well characterized for protein-protein interaction studies between transcription factors and their associated proteins [SR6]. First, the full length MET1, MET2, Ecr-B1 (Ecr) and USP1 (USP) were amplified from silkworm genome, then these genes were cloned into bait or prey vector. MET1, MET2, Ecr or USP were expressed as a fusion protein with human Sos as the bait protein. On the other hand, MET1, MET2, Ecr and USP were expressed as prey antibodies fused with a myristoylation (Myr) signal, targeting the proteins to the cell membrane. Expression of the prey is controlled by the GAL1 promoter, which is induced on galactose, and repressed on glucose medium. When bait and prey are co-transformed into the cdc25H strain, the only cells capable of growing at restrictive temperatures on galactose medium are those that have been rescued by the bait-prey interactions that recruit Sos to the cell membrane.

Transient transfection assay

Transient transfection assay in DZNU-Bm-12 cells was carried out for 48 hr using Effectene according to the manufacturer’s instructions. The final DNA concentration was 2 μg/ml, and the DNA:Effecene ratio was 1:25. The vector used to overexpress V5-Met1 and cMyc-Met2 was pEGFP-N1 (Clontech) under the control of the sel promoter. After transfection, cells were treated with 1 μM 20E, followed by immunoprecipitation, qPCR, and luciferase assay.

Transient transfection assays in HEK 293 cells were performed using Lipofectamine 2000 (Invitrogen). The full length MET1, MET2, Ecr-B1 (Ecr) and USP were amplified from the silkworm genome, and the HA, FLAG, V5, cMyc tags were fused in their 3-ends, respectively and cloned into the pcDNA 3.1(+) vector (Invitrogen). After transfection, the cells were harvested for immunoprecipitation, luciferase assay and EMSA.

Immunoprecipitation

After treatment, DZNU-Bm-12 cells and HEK 293 cells were harvested and lysed in ice-cold NP-40 lysis buffer (Beyotime). Lysates were incubated with FLAG, V5, or cMyc antibody or IgG for 4 hr, followed by incubation with protein G (GE Healthcare) overnight at 4°C. After extensive washing with cold NP-40 buffer, the samples were treated with RIPA lysis buffer (Beyotime) about 15min on the ice. Then immunoprecipitates were separated by SDS-PAGE and analyzed by Western blots after measured the protein concentration by the enhanced BCA protein assay kit (Beyotime).

Luciferase assay

Luciferase assays were carried out using the Dual Luciferase Assay System (Promega) and a Modulus Luminometer (Turner BioSystems). The reporter pGL3 vector (Promega) containing four repeated EcRE sequences (GACAAGGGTTCAATG-CACCTTGTC) and a hsp70 mini promoter was used for the luciferase reporter. And the reference pRL vector (Promega) carrying Renilla-luciferase driven by actin3 promoter was co-transfected into the cell with the reporter vector. The dual luciferase double reporter assay system and substrates were purchased from Promega.

EMSA

The HA-Ecr, FLAG-USP, V5-Met1, and cMyc-Met2 constructs were co-transfected into HEK 293 cells and nuclear extracts were prepared by the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). The minimal EcRE (sense: AGTT-CAATGGGCCT; anti-sense: AGGCCATTGAACCT) was biotin-labeled as a probe using the Biotin 3’ End DNA Labeling Kit (Pierce). After binding, the nuclear extract (15 μg) containing the biotin-labeled EcRE and the protein-DNA complexes were separated on 5% nondenaturing PAGE gel. HA, FLAG, V5, and cMyc antibodies were added to the nuclear extract to detect the shift of EcRE. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce).

Supporting Information

Figure S1 The diagram of the three sets of Met dsRNA and confirmation of the MET and Br-C antibodies. (A) The diagram illustrates the three sets of Met dsRNA. Red bar: #1 set of Met1 (491–916) and Met2 (491–916) dsRNA; green bar: #2 set of Met1 (141–586) and Met2 (1925–2336) dsRNA; yellow bar: #3 set of Met1 (948–1348) and Met2 (245–669) dsRNA. (B and C) Western blotting confirmation of the MET1 and Br-C antibody after Met1 and Br-C RNAi. The arrow points to the MET1 protein and the Br-C protein isoforms with ideal molecular weights. egfp RNAi was used as a control. Tubulin was used as a loading control. (PDF)

Figure S2 Met RNAi prevents removal of obsolete larval tissues and generation of adult structures. dsRNA (10 μg per larva) was injected into larvae during initiation of the early wandering stage. More than 30 silkworms were included in each group. egfp dsRNA was used as a control. (A) Met RNAi larvae
form thinner cocoons. Cocoon images were collected after the silkworms stopped spinning. (B) Met RNAi prevented silk gland lysis 24 hr after pupation. The inhibiting effects, particularly on the middle silk gland, by Met2 RNAi were stronger than Met1 RNAi. (C) Met RNAi affected adult structure formation. Many of the surviving Met1 RNAi treated adults exhibited shortened and distorted legs (left panel) or unexpanded wings (right panel). (PDF)

**Figure S3** Met RNAi disrupts the 20E-triggered transcriptional cascade during the early wandering stage and in DZNu-Bm-12 Cells. Three biological replicates were used and one was represented (A–C). In each biological replicate, more than 10 larvae were used (A and B), egfp dsRNA was used as a control. (A) The other two sets (#2 and #3) of Met dsRNA (30 μg per larva) also disrupted the 20E-triggered transcriptional cascade during initiation of the early wandering stage. See Figure S1A for the locations of the three sets of Met dsRNA. (B) 20E treatment fails to induce expression of 20E-response genes in fat body explanted from the Met2 RNAi silkworms during the early wandering stage. (C) Met1 RNAi disrupts the 20E-triggered transcriptional cascade, except Met2 whose expression level is extremely low, in Bombyx DZNu-Bm-12 cells. RNAi knockdown was performed using the Effective transfection reagent (Qagen) for 48 hr at a final concentration of 2 μg/ml dsRNA. The cells were treated with 20E for 6 hr at a final concentration of 1 μM. (PDF)

**Figure S4** Met is required for 20E action in Tribolium. RNAi knockdown of either Er or Met (~4 ng per larva) in Tribolium during the early quiescent stage resulted in lethality (A), significantly delayed the larval-pupal transition (A and B), and disrupted the 20E-triggered transcriptional cascade (C). egfp dsRNA was used as a control. (A) Larval, prepupal, and pupal numbers were counted 24 and 48 hr after RNAi treatment. Total lethality caused by egfp, Met and Er dsRNAs was compared. (B) Phenotypic images were collected from the above experimental animals 24 (left) and 48 hr (right) after RNAi treatment. (C) Met, Er, USP, E74, and Br-C mRNA levels, as determined by qPCR, were significantly down-regulated 24 hr after Met RNAi. (PDF)

**Figure S5** The negative controls for the IP and EMSA experiments. (A) The HA-Er, FLAG-USP, and V5-Met1 constructs were co-transfected into human HEK 293 cells, the cells were treated by 20E for 6 hr at a final concentration of 1 μM. The negative control IgG was not able to pull down HA-Er, FLAG-USP, and V5-Met1. IP, immunoprecipitate; Blot, Western blot. (B) The HA-Er and FLAG-USP or V5-Met1 and cMy-Met2 constructs were co-transfected into the human HEK 293 cells. After nuclear extracts were bound with biotin-labeled EcR, the protein-DNA complexes were separated on a 5% native PAGE gel followed by EMSA. The shift was indicated by a black arrow in comparison with a gray arrow. (PDF)

**Table S1** A list of all primers used in this paper. (DOC)

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**Author Contributions**

Conceived and designed the experiments: S. Li YC JW. Performed the experiments: EG QH S. Liu LT ZS QP JG MS KL. Analyzed the data: EG S. Li. Wrote the paper: S. Li EG YC JW LG.

**References**


