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ORIGINAL ARTICLE

The knockdown of *Ha-GRIM-19* by RNA interference induced programmed cell death

Du-Juan Dong · Peng-Cheng Liu · Jin-Xing Wang · Xiao-Fan Zhao

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Abstract *GRIM-19* (genes associated with retinoid-IFN-induced mortality-19) is a subunit of mitochondrial respiratory complex I in mammalian systems. However, its function in vivo is not really understood. We cloned *GRIM-19* and explored its function and hormonal regulation in insect, the cotton bollworm, *Helicoverpa armigera*. The results showed that *Ha-GRIM-19* was highly expressed during the larval stage. Its transcript levels could be upregulated by juvenile hormone (JH) analog methoprene or by methoprene plus 20E. The methoprene-upregulated transcription enhancement of *Ha-GRIM-19* was mediated by the transcription factor *Ha-Met1*, the putative receptor of JH. Other transcription factors *Ha-USP1* and *Ha-Br-Z2* suppressed the action of methoprene in inducing *Ha-GRIM-19* expression, but *Ha-Br-Z2* introduced interaction between 20E and methoprene in upregulation of *Ha-GRIM-19*. The knockdown of *Ha-GRIM-19* by RNA interference in larvae and in insect cell line induced programmed cell death. These data imply that *Ha-GRIM-19* plays role in keeping the normal cellular growth and it is able to be upregulated by methoprene through putative JH receptor *Met*.

Keywords GRIM-19 · JH · Mitochondria · Cell death · Metabolism

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number: HM369463.

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D.-J. Dong · P.-C. Liu · J.-X. Wang · X.-F. Zhao (✉)
The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Science, Shandong University, Jinan 250100, China
e-mail: xfzhao@sdu.edu.cn

Introduction

Mitochondria are subcellular organelles that play pleiotropic roles in cells. They are the major source of energy which generates ATP through oxidative phosphorylation via the mitochondrial respiratory chain consisting of four complexes and two electron carriers, coenzyme Q10 and cytochrome *c* (Newmeyer and Ferguson-Miller 2003; Hatefi 1985). Mitochondria also regulate apoptosis through the release of cytochrome *c* and subsequent caspase activation (Lawen 2003). These two outwardly disparate functions of mitochondria required the involvement of GRIM-19 (genes associated with Retinoid-IFN-induced mortality-19) (Huang et al. 2007).

GRIM-19 was found to be co-purified with mitochondrial NADH: ubiquinone oxidoreductase (complex I) in the bovine heart (Fearnley et al. 2001). Through gene targeting in mice, the GRIM-19 was further demonstrated that it was critical for assembly and enzymatic activity of complex I (Huang et al. 2004). *GRIM-19* was initially identified as interferons (IFNs)-β and all-*trans*-retinoic acid (ATRA)-inducible gene with pro-apoptotic nature in human cancer cell lines (Angell et al. 2000). ATRA, a metabolite of retinol (vitamin A), is a kind of terpenoid. It is a potent suppressor of human tumor cell growth when combined with interferons (IFN) (Lindner et al. 1997). As a pro-apoptotic factor GRIM-19 regulates IFN/RA-induced cell death by producing reactive oxygen species (ROS) (Huang et al. 2007). However, GRIM-19 was also found protecting cells from apoptosis triggered by the classical death reagents such as UV and staurosporine through maintaining the mitochondrial membrane potential ($\Delta\Psi_m$) (Lu and Cao 2008). GRIM-19 appears, therefore, as a dual function protein involved in cell death and normal mitochondrial metabolism (Maximo et al. 2008). However, till recently,

studies on the roles of GRIM-19 were mainly carried on in vitro. The roles in the growth and development in vivo were not deeply investigated yet. Apart from vertebrates, GenBank documented GRIM-19 homologues in *Drosophila melanogaster*, *Bombyx mori*, *Caenorhabditis elegans* and so on (Angell et al. 2000; Fearnley et al. 2001), but the function and hormonal regulation of GRIM-19 are not known.

JH is a sesquiterpenoid with a molecular structure similar to the terpenoid ATRA (Nakamura et al. 2007). JH coordinately orchestrates insect growth and development with the molting hormone 20-hydroxyecdysone (20E) during insect life cycles. Methoprene is a synthetic analogue of JH that is used as a research tool (Nishiura et al. 2005; Parthasarathy and Palli 2007) and an insecticide in the field (Staal 1975). Years of research have provided us an understanding of the 20E signaling pathway, the molecular basis of JH action and the process of how JH mediates the function of 20E, however, remain unclear (Wheeler and Nijhout 2003), especially with uncertain receptors of JH and JH-regulated target genes. Currently, there are two candidate genes of the JH receptor: *ultraspiracle* (*USP*) (Jones et al. 2006; Jones and Sharp 1997) and *methoprene-tolerant* (*Met*) gene (Miura et al. 2005). USP is the insect ortholog of the vertebrate retinoid-X receptor (Thummel and Chory 2002), which could bind to JH and JH-like ligands with low affinity (Jones et al. 2006). *Met* as a JH receptor is based on the genetic evidence that *Met* null mutants are more resistant to the effects of JH (Wilson 1996). However, because of the considerable low capacity of JH binding, USP is disputed as a JH receptor, and the apparent normal development of *Met* null mutants challenged the classification of *Met* as a JH receptor as well (Riddiford 2008).

Konopova and Jindra (2008) demonstrated that the ecdysteroid-responsive gene *Broad-Complex* (*BR-C*) acts downstream of *Met* in JH signaling to coordinate primitive holometabolous metamorphosis in *Tribolium castaneum*. *BR-C* is a key factor in the crosstalk between 20E and JH in *D. melanogaster* (Dubrovsky 2005). However, most of the studies on JH receptors, and the crosstalking regulator between JH and 20E focus on *D. melanogaster*. The function of these genes and the mechanism of JH regulating insect development in the Lepidoptera are less known.

To investigate the function of *GRIM-19* and its hormonal regulation in the insect development, we cloned and characterized *GRIM-19* from the cDNA library of *Helicoverpa armigera* and designated it as *Ha-GRIM-19*. Northern blot analysis shows that the transcript levels of the gene at the larval stage were higher compared with those at the pupal stage. *Ha-GRIM-19* was upregulated by JH analog methoprene or by methoprene and 20E combination. The knockdown of *Ha-GRIM-19* by RNA interference (RNAi) induced programmed cell death (PCD) in larvae and HaEpi cells established in our laboratory (Shao et al. 2008). These data imply that Ha-GRIM-19 plays role in keeping the normal cellular growth and is upregulated by methoprene through Ha-Met1. In addition, we reveal that *USP1* was a suppressor of methoprene regulated *Ha-GRIM-19* expression. *Ha-Br-Z2* also suppressed the function of methoprene in inducing *Ha-GRIM-19* expression, but it introduced interaction between 20E and JH.

Materials and methods

Insects

Cotton bollworms were cultured in the laboratory with an artificial diet composed mainly of wheat and soybean; the insects were reared under light:dark conditions at 14:10 h (Zhao et al. 2005).

Sequence analysis

Gene translation and prediction of deduced protein were performed using the ExPASy Proteomics Server (http://www.expasy.ch/tools/) to include the computation of p*I*/Mw, NetPhos, NetNGlyc, and NetOGlyc. Signal sequence and motif prediction was conducted using SMART (http://smart.embl-heidelberg.de/). Alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw/index.html) and GENDOC computer programs (http://www.psc.edu/biomed/genedoc/).

Northern blot analysis

The *Ha-GRIM-19* cDNA was cloned in the pGEM-T Easy Vector (Promega Corp., Madison, WI, USA). The DIG RNA labeling kit (Roche, Mannheim, Germany) was used to transcribe the digoxigenin-tagged antisense probe in vitro. Unizol reagent was used to isolate total RNA from various tissues, including those from the integument, midgut, fat body, and hemocytes, and from worms at different development stages.

Total RNA (10 μg) was denatured and electrophoresed on a formaldehyde-containing agarose gel, and then transferred into a nylon membrane (IMMOBILON-NY+, Millipore, Milford, MA, USA). The target mRNA was hybridized with a digoxigenin-labeled antisense RNA probe (100 ng/mL) in 50% formamide (68°C) and washed stringently (68°C). Anti-DIG-phosphatase antibody was used to detect the probe, which was then visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (Zhao et al. 2002). Prehybridization,

hybridization, washing, and color development were performed in accordance with the manufacturer's protocols.

Hormonal regulation of *Ha-GRIM-19* gene

20E (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at a storage concentration of 20 mM (9.6 mg/mL). Final concentration was 1 μM in the cell culture in Grace's medium. Stock solution of methoprene (synthetic JH mimic; Dr. Ehrenstorfer GmbH, Augsburg, Germany) was prepared as a 100 mM (31 mg/mL) solution. The final methoprene concentration was 5 μM in Grace's medium.

The HaEpi cells were seeded in 6-well cell culture plates for 48 h with Grace's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Medgenics, St. Louis, MO, USA). 20E and methoprene were then directly added to the medium, separately or in combination. Control cells were treated with equal volumes of DMSO. Total RNA was extracted from cells at time intervals of 0.5–12 h using Unizol reagent according to the manufacturer's protocols for the synthesis of cDNA. The differences between the control and challenged samples were compared by RT-PCR with gene specific primers GRIMF (5′-atggaagcggctgtagcc-3′) and GRIMR (5′-ttaagagcgcagcttgag-3′). The following procedure was employed: one cycle (94°C, 2 min), then 26 cycles (94°C, 30 s; 53°C, 45 s; 72°C, 45 s), followed by a final cycle (72°C, 10 min). The *β*-actin gene was used for normalization. Ratios of *Ha-GRIM-19* to *β*-actin were calculated with Quantity One (Bio-Rad, Hercules, CA, USA). Each experiment was repeated three times independent of each other, and the data from three repeats were statistically analyzed.

The stock solutions of 20E and methoprene were diluted separately in 0.1 mg/mL phosphate buffered saline (10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; 140 mM NaCl; and 2.7 mM KCl, pH 7.4), then injected into the larvae. The sixth instar 6 h larvae were injected with 20E (500 ng/larva) or methoprene (500 ng/larva), separately or in combination. Untreated controls were injected with equivalent amounts of DMSO. Total RNA of the midgut was extracted from the injected worms at different developmental periods. A comparison of the differences between the control and challenged samples was performed by RT-PCR, as cited earlier.

RNAi by feeding bacterially expressed dsRNA on larvae

We fed bacterially expressed *Ha-GRIM-19* dsRNA to the *H. armigera* larvae to knock down *Ha-GRIM-19* in vivo. The primers of GRIM-19ERNAiF (5′-tactca**gcgg ccgc**atggaagcggctgtagcc-3′), GRIM-19ERNAiR (5′-tactc a**ctcgag**ttaagagcgcagcttgag-3′), GFPERNAiF (5′-tactca **gcggccgc**tggtcccaattctcgtggaac-3′), and GFPERNAiR (5′-t actca**ctcgag**cttgaagttgaccttgatgcc-3′) were used for PCR to amplify the gene fragments (bold letters indicate *Not*I and *Xho*I sites). The dsRNA expression and feeding procedure for the larvae were performed according to the previous studies (Tian et al. 2009; Timmons et al. 2001). Both plasmid pPD129.36 (L4440) and strain HT115 were presented by Dr. Marek Jindra (Biology Center ASCR, Czech Republic). Bacterial cells that expressed dsRNA were collected from 100 mL IPTG (Isopropyl *β*-D-1-Thiogalactoside)-induced culture, resuspended in 0.4 mL sterile water. The artificial diets were cut into 1 cm × 1 cm × 0.2 cm and 0.5 g in weight. Each piece of diet was overlaid with a 50 μL suspension of bacterial culture containing bacteria expressing dsRNA of Ha-GRIM-19. The diets of control group were covered with a 50 μL suspension of bacterial culture containing bacteria expressing dsRNA of GFP (green fluorescence protein), or 50 μL ddH_2O. All diets were replaced daily. The differences between the control and treated samples were compared by RT-PCR. The primers for RT-PCR are listed in Sup. 4.

Paraffin section and terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The midguts and fat bodies were dissected in PBS and fixed for 10 h in 4% paraformaldehyde at 4°C, then embedded in wax after dehydration. 5 μm cryosections were cut and placed on glass slides. The sections were then dried overnight. The TUNEL assay was carried out using In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany) as per the instruction manual. The nuclei were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/mL in water, San Jose, USA) for 10 min. Fluorescence was detected with an Olympus BX51 fluorescence microscope (Japan).

RNAi in the HaEpi cell line

All primers of dsRNA for RNAi are listed in Sup. 3. The primers for RT-PCR are listed in Sup. 4. The dsRNA was synthesized using the MEGAscript RNAi kit (Ambion Inc., Austin, TX, USA). For dsRNA transfection, a lipophilic transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was employed according to the manufacturer's instructions. The final concentration of dsRNA was 3 μg/ml in the medium. After incubation at 27°C for 10 h post-dsRNA transfection, the cells were rinsed and then replenished with a medium containing hormone for an another 12 h incubation. The final hormone concentration was as cited earlier. The RNA was extracted

from the cells for RT-PCR analysis. Detailed methods were described in the earlier work of our laboratory (Shao et al. 2008; Zheng et al. 2009). GFP was used as control.

Results

Sequence analysis of *Ha-GRIM-19*

A 645 bp full-length cDNA was obtained from the cDNA library of *H. armigera*, which includes a 51 bp 5′ untranslated region (UTR), a 456 bp open reading frame (ORF), and a 138 bp 3′ UTR. The 3′ UTR contained a 19 bp polyadenylation $(A)^+$ tail. The ORF encoded a 151 amino acid protein. The calculated molecular mass of the protein was 17.8 kDa with a predicted isoelectric point of 9.4. The protein includes one putative protein kinase C phosphorylation site and one presumed casein kinase II phosphorylation site (123–126) (Sup. 1).

Ha-GRIM-19 is a highly conserved gene in insects

The result of BLASTX analysis suggests that *Ha-GRIM-19* has certain similarities to various genes, including GRIM-19 from *B. mori* (79%), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 13 from *T. castaneum* (64%), CG3446 from *D. melanogaster* (62%), AGAP009652-PA from *Anopheles gambiae* (62%), and mitochondrial NADH: ubiquinone oxidoreductase B16.6 subunit from *Aedes aegypti* (55%) (Sup. 2). SMART predicted that Ha-GRIM-19 protein contains a GRIM-19 domain (3–139 aa).

Ha-GRIM-19 was expressed during the larval stage in various tissues

To study the tissue distribution of *Ha-GRIM-19*, the transcripts of *Ha-GRIM-19* in the integument, midgut, fat body, and hemocytes were examined by Northern blot. The results show that *Ha-GRIM-19* was expressed in the four tissues. However, the expression levels of the gene at the feeding stage (F) were higher than those at the molting (M) and metamorphic stages (W) (Fig. 1a).

To examine the developmental expression patterns of *Ha-GRIM-19*, the transcripts of *Ha-GRIM-19* from the fifth instar 12 h larvae to 8 days pupae were analyzed by Northern blot. *Ha-GRIM-19* expression were not observed during the pupal stage in all the detected tissues (Fig. 1b). In the integument and hemocytes, *Ha-GRIM-19* was expressed during the larval stage with higher expression at the fifth instar 12 h and sixth instar 48 h feeding larvae and metamorphically committed larvae (Fig. 1b). However, in the midgut and fat body, the transcripts of *Ha-GRIM-19* decreased when the larvae were metamorphically committed.

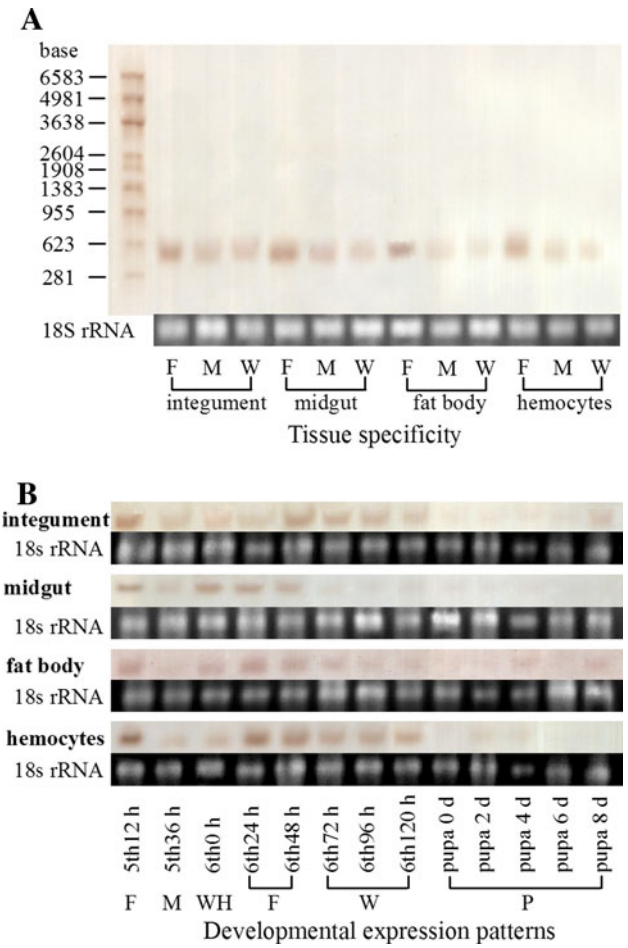

Fig. 1 Expression profiles of *Ha-GRIM-19* detected by northern blot analysis. **a** Expression of *Ha-GRIM-19* in various tissues of the integument, midgut, fat body, and haemocytes. *F* 5th instar feeding stage; *M* molting stage (HCS); *W* wandering stage. **b** Expression patterns of *Ha-GRIM-19* in the integument, midgut, fat body, and haemocytes during development. *WH* white head (0 h after ecdysis), sixth instar at 0 h larvae; *P* pupae. Total RNA (10 μg) was used in each lane, and 18S ribosome-RNA was used as loading control

These data indicate that *Ha-GRIM-19* is a larval growth-related gene.

Ha-GRIM-19 was upregulated by JH analog methoprene and enhanced by mixing with 20E

To examine the effect of ecdysone and JH on *Ha-GRIM-19* expression, the HaEpi cells were treated with 20E and JH-mimicking methoprene, separately or in combination. Compared with the DMSO-treated cells, 20E alone had no significant effect on *Ha-GRIM-19* expression. However, the methoprene enhanced the expression of *Ha-GRIM-19* by 2.5-folds at 1 h. In addition, the methoprene and 20E combination increased the expression of *Ha-GRIM-19* by four-folds at 1 h after the challenge (Fig. 2a). These results indicate that *Ha-GRIM-19* was induced by JH-mimicking methoprene with the induction enhanced by 20E.

The results of hormonal regulation of *Ha-GRIM-19* in vivo confirmed the conclusion. The 20E injection alone did not affect the expression of *Ha-GRIM-19* in the midgut. However, compared with the control, methoprene injection increased the expression of *Ha-GRIM-19* by fivefolds at 6 h, and the combined injection of methoprene and 20E enhanced the expression of *Ha-GRIM-19* by ninefolds at 6 h after the challenge (Fig. 2b).

Met1, *USP1*, and *Br-Z2* participated in the hormonal regulation of *Ha-GRIM-19*

To investigate the roles of *Met1*, *USP1,* and *Br-Z2* in the hormonal regulation of *Ha-GRIM-19*, each gene was knocked down separately in the epidermal cell line of HaEpi. Then, their effects on *Ha-GRIM-19* expression were checked. Semi-quantitative RT-PCR results revealed that the expression of *Ha-GRIM-19,* compared with that in the dsGFP control, could not be induced after the knockdown of *Ha-Met1,* whether the cells were treated with methoprene or with a combination of methoprene and 20E (Fig. 3a). After the knockdown of *Ha-USP1*, the expression of *Ha-GRIM-19* was increased by methoprene or by the methoprene and 20E combination compared with that in the dsGFP control (Fig. 3b). When the *Ha-Br-Z2* was knocked down, the expression of *Ha-GRIM-19* increased unlike that in the dsGFP control after induction with methoprene. However, *Ha-GRIM-19* decreased compared

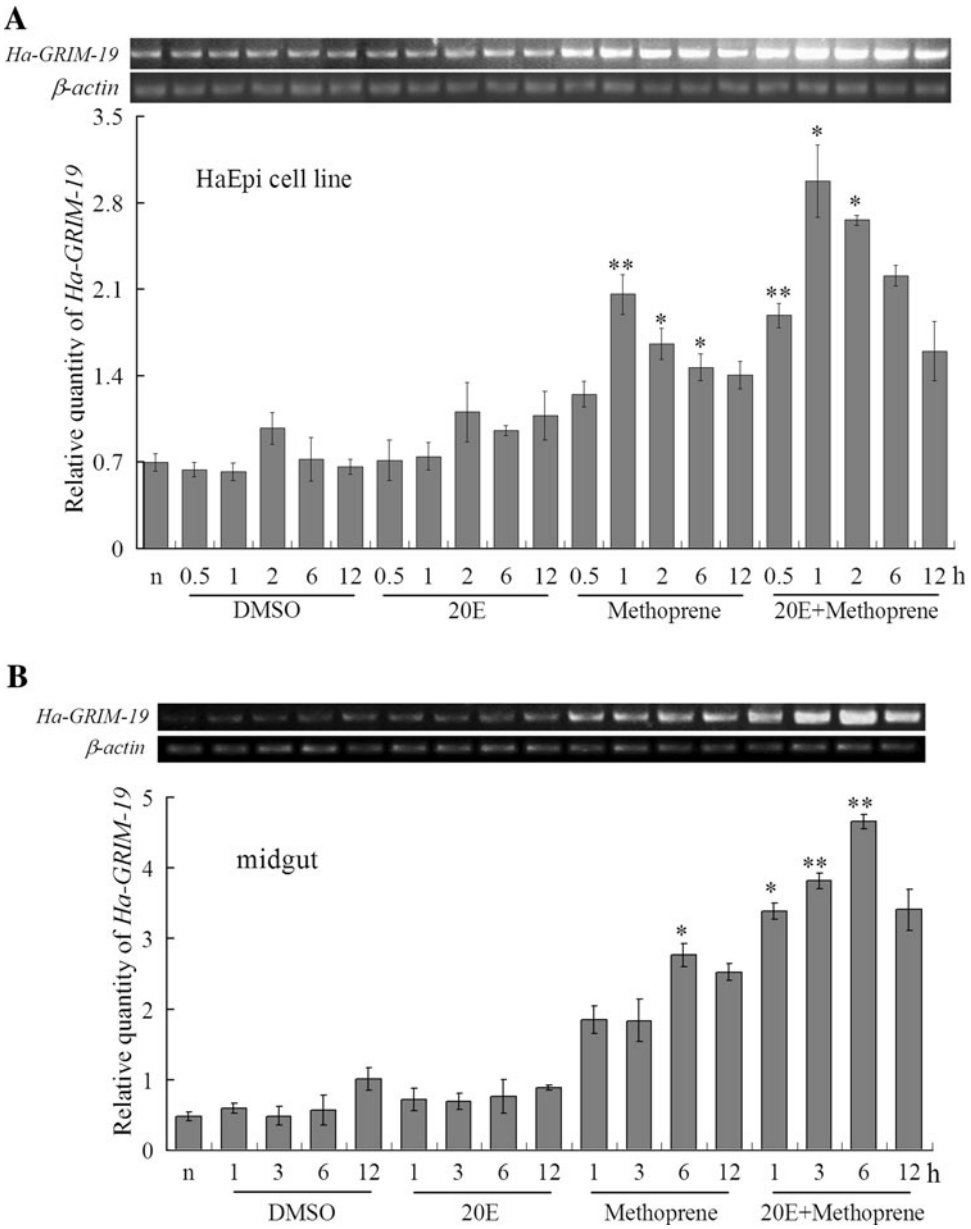

Fig. 2 Hormonal regulation of *Ha-GRIM-19* expression. **a** Hormonal regulation of *Ha-GRIM-19* in the HaEpi cell line at 0.5, 1, 2, 6, and 12 h after treatment with hormone. *n* normal cells without treatment. **b** Hormonal regulation of *Ha-GRIM-19* in the midgut at 1, 3, 6, and 12 h after treatment with hormone. *n* sixth instar at 6 h larvae. Equal amounts of diluted DMSO were used as solvent control for the hormones. *β*-actin was used as quantitative control. *Error bars* represent the standard deviation in three replicates. *Asterisks* indicate significant differences (Student's *t* test, $*P < 0.05$, $**P < 0.005$)

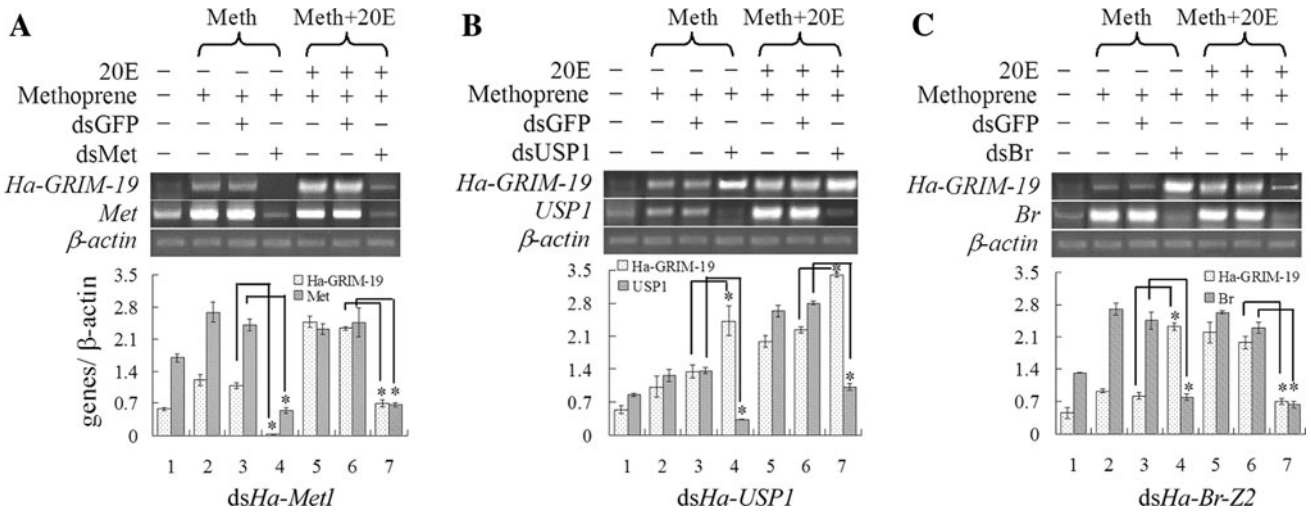

Fig. 3 Semi-quantitative RT-PCR to show the effects of knockdown *Ha-Met1* (**a**), *Ha-USP1* (**b**), and *Ha-Br-Z2* (**c**) on the expression of *Ha-GRIM-19*. The numbers *1–7* are: normal cells; cells incubated with methoprene; cells treated with dsRNA of GFP and incubated with methoprene; cells treated with dsRNA of *Met1* (**a**)/*USP1* (**b**)/*Br-Z2* (**c**) and incubated with methoprene; cells incubated with combined methoprene and 20E; cells treated with dsRNA of GFP and incubated with combined methoprene and 20E; cells treated with dsRNA of *Met1* (**a**)/*USP1* (**b**)/*Br-Z2* (**c**) and incubated with combined methoprene and 20E. *Error bars* represent the standard deviation in three replicates. *Asterisks* indicate significant differences (Student's *t* test, $*P < 0.05$)

with that in the dsGFP control after the cells were treated with combined methoprene and 20E (Fig. 3c). These results indicate that the JH analog methoprene upregulated *Ha-GRIM-19* expression through *Ha-Met1*. *Ha-USP1* suppressed the induction of methoprene to *Ha-GRIM-19* expression. Although *Ha-Br-Z2* suppressed the induction of methoprene to *Ha-GRIM-19* expression, it also induced the interaction between 20E and JH in upregulation of *Ha-GRIM-19*.

Knockdown of *Ha-GRIM-19* induced cell death

RNAi was performed to further understand the function of *Ha-GRIM-19* in vivo. Semi-quantitative RT-PCR shows that *Ha-GRIM-19* was significantly knocked down via feeding of the third instar larvae with bacteria that expressed dsRNA of *Ha-GRIM-19*, as opposed to the feeding of the diet-containing bacteria expressing dsRNA of *GFP* (Fig. 4c). The results show that after continuous feeding for 9 days, the midgut of control larvae fed with diet-containing bacteria expressing dsRNA of *GFP* was still full of food and the fat body showed white strips (Fig. 4a, c, e, g) at the sixth instar at 52 h. However, the larvae feeding on diet-containing bacteria expressing dsRNA of *Ha-GRIM-19* exhibited an obvious cell death phenotype at the sixth instar at 52 h. The entire body shrank, the midgut became red, and the fat body began to undergo histolysis (Fig. 4a, b, d, f, h). Inside the midgut and fat body, TUNEL staining suggests that PCD occurred in the dsGRIM-19 treated larvae (Fig. 4a, n, p). Statistical analysis showed that 86.1% of the larvae exhibited cell death phenotype after knockdown of *Ha-GRIM-19*. In contrast, the control group did not appear as abnormal exhibition (Fig. 4b). These results reveal that *Ha-GRIM-19* was required for the midgut and fat body cell survival in the final larval instar.

Further results of knockdown of *Ha-GRIM-19* in the epidermal cell line confirmed the conclusion. Compared with the control, 48 h after knockdown of *Ha-GRIM-19*, apoptotic body appearance was seen under inverted microscope (Fig. 5b). TUNEL assay also showed positive staining (Fig. 5d).

To demonstrate the mechanism of the phenomenon caused by RNAi, we detected the expressions of several related genes after the knock down of *Ha-GRIM-19*. Semi-quantitative RT-PCR shows that the expression of apoptosis inhibitor gene *survivin* decreased in the midgut or fat body, whereas the expression of *caspase-1*, the effector genes of PCD and *matrix metalloproteinase* (*MMP*), increased (Fig. 6). These results indicate that *Ha-GRIM-19* knockdown led to PCD.

Discussion

From *H. armigera,* we identified *Ha-GRIM-19,* a 645 bp full-length *GRIM-19* gene that encodes 151 amino acids. BLAST results show that *Ha-GRIM-19* had 79% homology with the *GRIM-19* from *B. mori*. *Ha-GRIM-19* expression was upregulated by JH and enhanced by mixing with 20E.

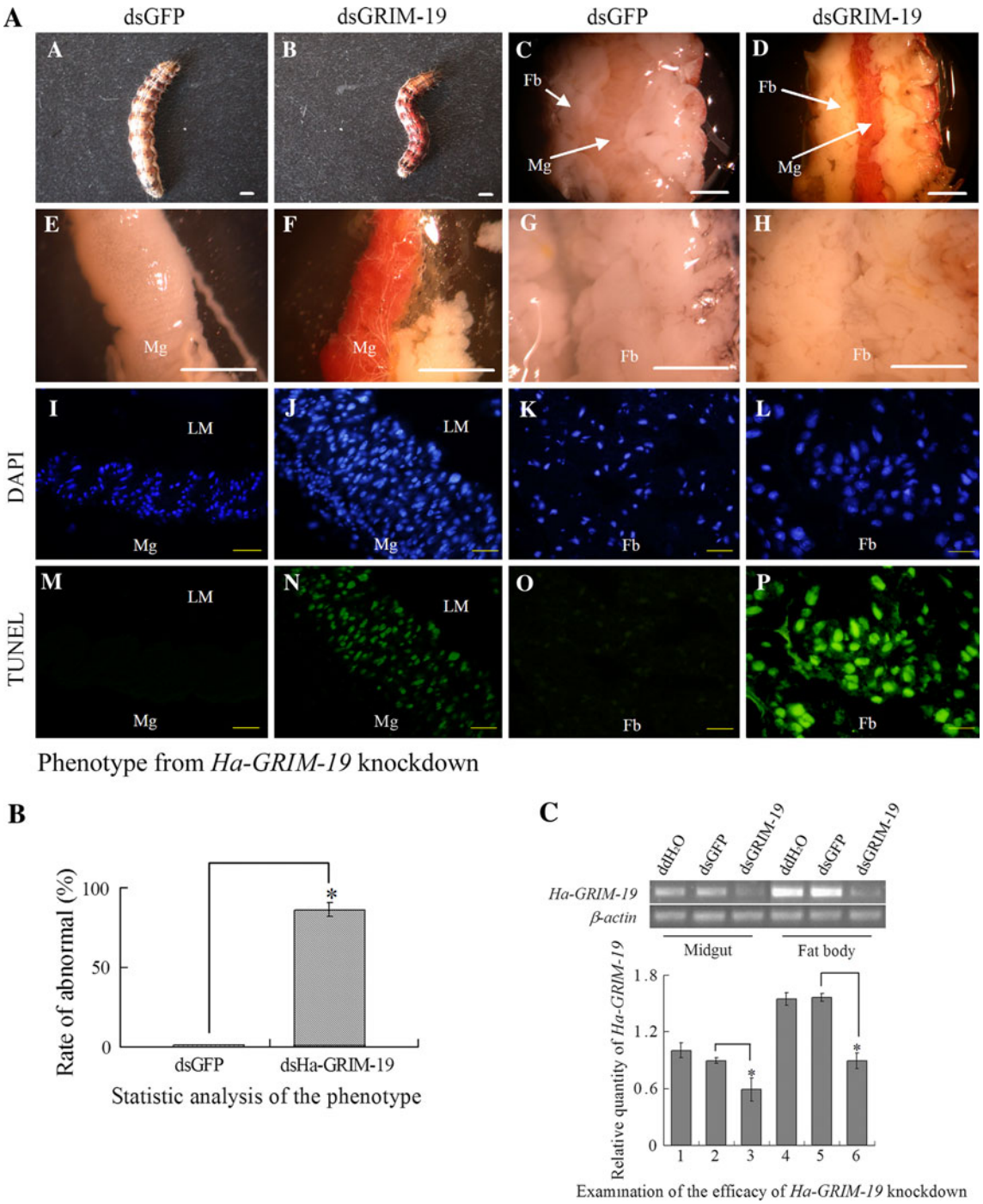

Fig. 4 RNA interference of *Ha-GRIM-19* in vivo. **a** Phenotype from *Ha-GRIM-19* knockdown. *A*, *C*, *E*, *G*, *I*, *K*, *M* and *O* are from larvae fed on dsGFP; *B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P* are from larvae fed on dsHa-GRIM-19. Nuclear staining was performed by DAPI (*I–L*). Cells undergoing PCD were detected by tunel assay (*M–P*). *Mg* midgut; *Fb* fat body; *LM* lumen of midgut. Scale bar (*white*) = 0.25 cm, scale bar (*yellow*) = 50 μm. **b** Statistical analysis of the phenotype. **c** Examination of the efficacy of *Ha-GRIM-19* knockdown by semi-quantitative RT-PCR. *Error bars* represent the standard deviation in three replicates. *Asterisks* indicate significant differences (Student's *t* test, $*P < 0.05$) based on 30 larva samples and three replicates

The knockdown of *Ha-GRIM-19* by RNAi-induced cell death, which implies that *Ha-GRIM-19* plays role in keeping the normal cellular growth and it is upregulated by methoprene through putative JH receptor *Met*.

Ha-GRIM-19 is necessary in maintaining cell growth

The developmental expression patterns suggest that *Ha-GRIM-19* is a larval growth-related gene. It was expressed

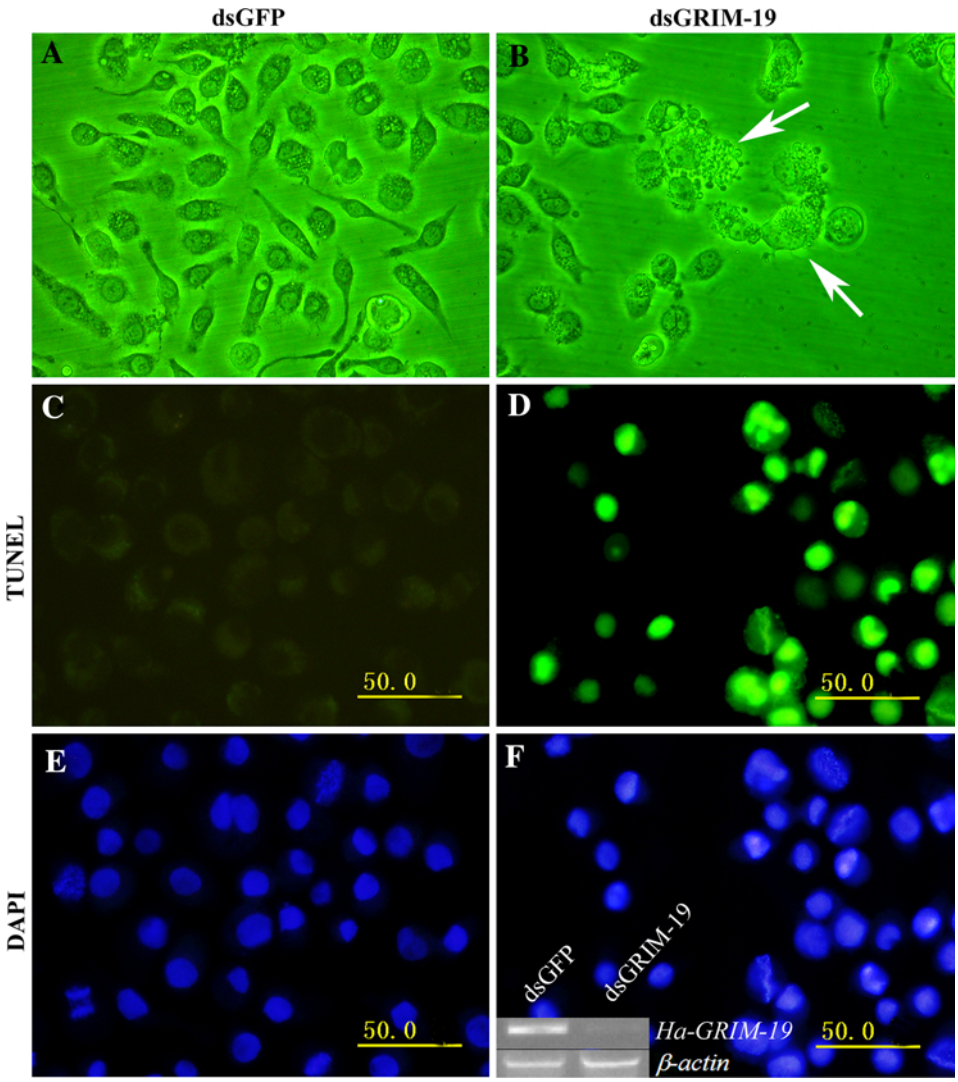

Fig. 5 RNA interference of *Ha-GRIM-19* on the HaEpi cell line. **a** Control, morphology of the HaEpi cells treated with dsGFP; **b** morphology of the HaEpi cells treated with dsHa-GRIM-19; **c** control, TUNEL assay analyzes the HaEpi cells treated with dsGFP; **d** TUNEL assay analyze the HaEpi cells treated with dsHa-GRIM-19. The nuclei were stained by DAPI (**e**, **f**). *White arrow* in (**b**) indicated the apoptotic bodies formed after cell undergoing the apoptosis. Scale bar (*yellow*) = 50 μm. The HaEpi cells were incubated with dsRNA (3 μg/mL) and lipofectamine 2000 (500 ng/mL) at 27°C for 48 h

in the larval stage in various tissues. *Ha-GRIM-19* expression stopped during the pupal stage, a period of the larval tissues undergo programmed cell death and the adult tissues remodel in insects (Wigglesworth 1972), in most detected tissues. *Ha-GRIM-19* also highly expressed at metamorphically committed stage in integument and hemocytes, which might be related to the overlapped JH titer and 20E titer. *H. armigera* belongs to Lepidoptera: Noctuidae and should have similar 20E and JH titer to *Manduca sexta*. Riddiford et al. (2003) showed that JH still existed and overlapped with 20E during wandering stage in *M. sexta*. The decreased expression of *Ha-GRIM-19* in the midgut and fat body during metamorphosis might be because of tissue specificity. These data suggest that as a functional subunit of mitochondrial respiratory complex I, GRIM-19 plays important roles in maintaining cell growth at larval feeding stages.

Knockdown of *Ha-GRIM-19* lead to PCD

The knockdown of *Ha-GRIM-19* resulted in the suppression of *survivin* and rises of the PCD effector genes *Ha-Caspase-1* and *MMP*. *Survivin* is a member of the inhibitor of apoptosis protein family, a major class of apoptosis regulators, which inhibit apoptosis by binding to caspases (Deveraux and Reed 1999). Members of the caspase family play a central and evolutionary role in PCD, removing unwanted, damaged, and dangerous cells during development to maintain homeostasis (Lockshin and Zakeri 2001). *Ha-Caspase-1*, a member of the caspase family, was upregulated by an ecdysone agonist RH-2485, implying that *Ha-Caspase-1* possibly participates in ecdysone-regulated PCD during larval molting and metamorphosis (Yang et al. 2008). The *MMP* is a family of extracellular proteases, and has been reported as a requirement in the tissue remodeling

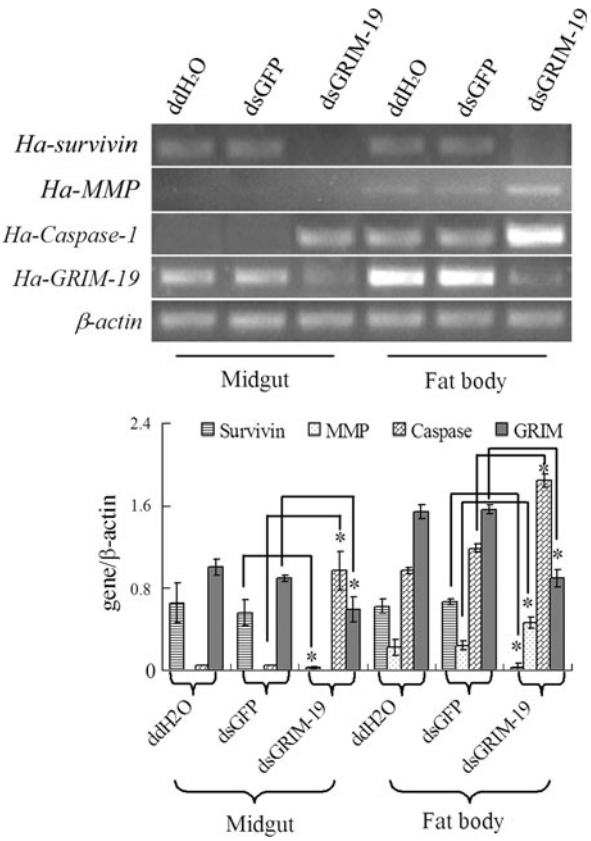

Fig. 6 Semi-quantitative RT-PCR analysis the effect of *Ha-GRIM-19* knockdown on *Ha-survivin, Ha-MMP,* and *Ha-Caspase-1*. Sample sequence from left to right is as follows: midgut from larvae fed on diet containing ddH_2O, dsGFP, and dsHa-GRIM-19; fat body from larvae fed on diet containing ddH_2O, dsGFP, and dsHa-GRIM-19. *Error bars* represent the standard deviation in three replicates. *Asterisks* indicate significant differences (Student's *t* test, $*P < 0.05$)

during metamorphosis in *Drosophila* (Page-McCaw et al. 2003), *Galleria mellonella* (Altincicek and Vilcinskas 2008), and *Tribolium* (Knorr et al. 2009). These indicate that the inactivity of *Ha-GRIM-19* triggered the cell death. This is reasonable because GRIM-19 is a functional subunit of complex I, which is not only important for complex I assembly (Fearnley et al. 2001) but also essential for its electron transfer activity (Lu and Cao 2008). In addition, GRIM-19 has a particular role in the maintenance of $\Delta\Psi_m$ (Lu and Cao 2008). Low $\Delta\Psi_m$ was reported in early apoptotic process in many systems, and cells with a decrease of $\Delta\Psi_m$ underwent spontaneous apoptosis (Zamzami et al. 1995; Cohen 1997). Knockdown of GRIM-19 during feeding larval stage probably causes a metabolic stress and a loss of $\Delta\Psi_m$ that can eventually lead to cell death. This phenomenon is consistent to the report that GRIM-19 was noticed protecting cells from apoptosis triggered by the classical death reagents such as UV and staurosporine through maintaining the mitochondrial membrane potential (Lu and Cao 2008).

Ha-GRIM-19 expression was upregulated by JH and enhanced by mixing with 20E

GRIM-19 is a terpinoid combined with IFN-inducible gene with pro-apoptotic nature in human cancer cell lines (Angell et al. 2000). Similarly, our results show that the JH analog methoprene could also upregulate the expression levels of *Ha-GRIM-19*, but its function is to maintain cell growth. Therefore, larval tissues and cells undergo programmed cell death when *Ha-GRIM-19* expression stopped at the metamorphic stage or pupal stage when JH decreased. The fact that *Ha-GRIM-19* is not inducible by 20E suggests that 20E is not a direct regulator of *Ha-GRIM-19*. However, 20E could enhance the expression of Ha-GRIM-19 induced by JH, which might explain the interaction of these two hormones in the larval developmental stage, in this stage, JH and 20E co-exist (Riddiford et al. 2003).

Methoprene through Met upregulated *Ha-GRIM-19* expression

The expression of *Ha-GRIM-19* decreased after the knockdown of *Ha-Met1,* whether the cells were treated with methoprene or with combined methoprene and 20E. This result supports that methoprene performed its effect on *Ha-GRIM-19* through Met, the candidate receptor of JH (Miura et al. 2005).

JH analog methoprene did not upregulate the expression of *Ha-GRIM-19* through USP. USP was considered as a possible JH nuclear receptor candidate (Jones et al. 2006; Jones and Sharp 1997). However, our data suggest that *Ha-USP1* was a suppressor in the JH pathway in terms of regulating *Ha-GRIM-19* expression. This was confirmed by the fact that the mRNA levels of *Ha-GRIM-19* increased after the knockdown of *Ha-USP1*, which indicates that *USP1* had a negative impact on the induction of *Ha-GRIM-19* by methoprene or by methopren plus 20E.

Our results further proved that *Br* suppressed the methoprene pathway and played roles in regulating the interaction between JH and 20E. The *BR-C* gene encodes a conserved N-terminal Broad-Complex-tramtrack-Bric-a-brac domain with one of several alternative C-terminal zinc-finger motifs in *D. melanogaster* and *T. castaneum* (Bayer et al. 1996; DiBello et al. 1991; Konopova and Jindra 2008). BLASTX analysis indicated that we obtained one gene which has certain similarities to *BR-C* isoform Z2 in *H. armigera* (data not yet published). We prepared the dsRNA according to the sequence of the common region of *Ha-Br-Z2* to knock down *Ha-Br*. The fact that knockdown of *Ha-Br-Z2*, *Ha-GRIM-19* was increased by methoprene but decreased by combined methoprene and 20E suggests that *Ha-Br-Z2* not only acted as a suppressor in the JH

pathway, but also served as a mediator in the JH and 20E crosstalk. This conclusion is consistent with *Br* acting as a jointer in JH and 20E crosstalks (Dubrovsky 2005; Riddiford et al. 2003; Zhou and Riddiford 2002).

In summary, *Ha-GRIM-19* was identified as a larval growth-related gene. It served as a suppressor of PCD to maintain cell growth regulated by JH in insect. It was upregulated by JH analog methoprene and enhanced by mixing with 20E. *Met* was an inductor for methoprene by upregulating *Ha-GRIM-19* expression. USP served as a suppressor for *Ha-GRIM-19* expression in the JH pathway. *Br* appeared to participate in the 20E enhancement on the expression of *Ha-GRIM-19* induced by methoprene, which proposed a model to study the hormonal interactions in the lepidoteran insect.

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