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Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*

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Juvenile hormone (JH) regulates many developmental and physiological events in insects, but its molecular mechanism remains conjectural. Here we report that genetic ablation of the corpus allatum cells of the *Drosophila* ring gland (the JH source) resulted in JH deficiency, pupal lethality and precocious and enhanced programmed cell death (PCD) of the larval fat body. In the fat body of the JH-deficient animals, *Dronc* and *Drice*, two caspase genes that are crucial for PCD induced by the molting hormone 20-hydroxyecdysone (20E), were significantly upregulated. These results demonstrated that JH antagonizes 20E-induced PCD by restricting the mRNA levels of *Dronc* and *Drice*. The antagonizing effect of JH on 20E-induced PCD in the fat body was further confirmed in the JH-deficient animals by 20E treatment and RNA interference of the 20E receptor *EcR*. Moreover, MET and GCE, the bHLH-PAS transcription factors involved in JH action, were shown to induce PCD by upregulating *Dronc* and *Drice*. In the *Met*- and *gce*-deficient animals, *Dronc* and *Drice* were downregulated, whereas in the *Met*-overexpression fat body, *Dronc* and *Drice* were significantly upregulated leading to precocious and enhanced PCD, and this upregulation could be suppressed by application of the JH agonist methoprene. For the first time, we demonstrate that JH counteracts MET and GCE to prevent caspase-dependent PCD in controlling fat body remodeling and larval-pupal metamorphosis in *Drosophila*.

KEY WORDS: Juvenile hormone, 20-hydroxyecdysone, *Dronc* (*Nc*), *Drice* (*Ice*), *Met*, *gce*, Fat body, Metamorphosis, Programmed cell death, *Drosophila melanogaster*

INTRODUCTION

The molting hormone 20-hydroxyecdysone (20E) and juvenile hormone (JH) coordinately control insect development and metamorphosis. Although the molecular mechanism of JH action remains elusive (Riddiford; 2008), a great deal is known about 20E action (Riddiford et al., 2000; Yin and Thummel, 2005; Zitnan et al., 2007). The 20E receptor complex is a heterodimer composed of two nuclear proteins, Ecdysone receptor (EcR) and Ultraspiracle (USP). In the absence of 20E, EcR-USP associates with co-repressors, binds to the 20E-response elements, and represses transcription of the 20E primary response genes. After binding 20E to form the 20E-EcR-USP complex, this ligand-receptor complex recruits co-activators and then induces the 20E-triggered transcriptional cascade, which includes the 20E primary and secondary response genes (Gilbert et al., 2000; Riddiford et al., 2003; Palli et al., 2005).

During the larval-pupal metamorphosis of holometabolous insects, larval organs undergo programmed cell death (PCD), including type I PCD apoptosis, type II PCD autophagy and eventually histolysis, whereas the adult progenitor cells undergo cell proliferation, differentiation and organogenesis to give rise to the adult organs (Edgar and Orr-Weaver, 2001; Ward et al., 2003). This process is largely controlled by 20E. The molecular mechanism of how 20E controls larval organ remodeling is relatively well understood in *Drosophila* (Yin and Thummel, 2005; Neufeld and Baehrecke, 2008). First, the 20E-EcR-USP complex and the 20E primary response genes [including *Br-C* (*broad* – FlyBase) *E74* (*Eip74EF*), *E75* (*Eip75B*) and *E93* (*Eip93F*)] induce expression of several 20E secondary response genes that account for PCD, including the caspases *Dronc* (*Nc*) and *Drice* (*Ice*) (Cakouros et al., 2004; Kilpatrick et al., 2005) and the death activators *reaper* and *Hid* (*Wrinkled*) (Yin and Thummel, 2005). Second, Reaper and HID prevent Dronc and Drice from ubiquitin-regulated protein degradation, and Dronc and Drice activate each other by protein cleavage (Hay and Guo, 2006; Dorstyn and Kumar, 2008). Third, Reaper, HID, Dronc and Drice promote IAP1 (Thread) to undergo ubiquitin-regulated protein degradation, and vice versa (Hay and Guo, 2006). Fourth, *E93* is a key determinant of autophagy, partially acting through *Dronc* (Lee et al., 2000). Last, the 20E signal blocks Phosphotidylinositol 3 kinase (PI3K) and Target of rapamycin (TOR) activity, which in turn inhibits autophagy (Rusten et al., 2004; Columbani et al., 2005). Overall, the initiator caspase Dronc and the effector caspase Drice play important roles in regulating the 20E-induced caspase-dependent PCD in *Drosophila*.

JH regulates many physiological and developmental events in insects (Riddiford, 1994; Wyatt and Davey, 1996). In the larvae of many insect orders, particularly in Coleoptera, Orthoptera and Lepidoptera, the larval-pupal metamorphosis results from a low titer of JH and a high titer of 20E. In these insects, application of JH, or JH agonists, can prevent normal metamorphic events, resulting in a supernumerary larval molt. For this reason, JH is referred to as the ‘status quo’ hormone (Riddiford, 1994; Riddiford et al., 2003). It has

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been shown that JH plays an important role by preventing 20E-induced PCD during midgut remodeling of the mosquito *Aedes aegypti* (Wu et al., 2006; Parthasarathy and Palli, 2007b) and the moth *Heliothis virescens* (Parthasarathy and Palli, 2007a). At the molecular level, JH modifies or suppresses the 20E-triggered transcriptional cascade and downregulates caspase genes (Wu et al., 2006; Parthasarathy and Palli, 2007a; Parthasarathy and Palli, 2008). In addition, JH can directly affect gene expression independent of 20E (Kethidi et al., 2004; Li et al., 2007). Unfortunately, the molecular mechanism by which JH regulates gene expression remains unknown (Gilbert et al., 2000; Riddiford, 2008).

Methoprene-tolerant (MET) was thought to be a possible JH receptor (JHR). *Met* encodes a transcription factor of the bHLH-PAS family (Ashok et al., 1988) and *Drosophila Met* mutants are resistant to the JH agonist methoprene (Wilson and Fabian, 1986). MET forms homodimers or heterodimerizes with its paralog Germ-cell expressed bHLH-PAS (GCE) in a JH-sensitive manner (Godlewski et al., 2006). Global overexpression of *Met* causes high mortality during larval life (Barry et al., 2008). Although the *Met*-null mutant is fully viable (Wilson and Ashok, 1998), animals receiving *gce* RNAi in a *Met*-null background die during the pupal-adult transition, and overexpressed *gce* can substitute for *Met* function (T.G.W., unpublished). It was also reported that *Drosophila* MET binds JH at physiological concentrations in vitro and that the transcriptional activity of MET is dependent on JH concentration (Miura et al., 2005). In terms of JH signal transduction, *Met* interacts with *Br-C* to regulate *Drosophila* development (Zhou and Riddiford, 2002; Wilson et al., 2006). However, it is not known how *Met* regulates JH-responsive genes in the control of physiological and developmental events. In the beetle *Tribolium castaneum*, *Met* plays a key role in JH action by preventing the premature development of adult structures during larval-pupal metamorphosis (Konopova and Jindra, 2007; Parthasarathy et al., 2008b), and *Met* acts upstream of *Br-C* (Konopova and Jindra, 2008; Suzuki et al., 2008; Parthasarathy et al., 2008a). There is no doubt that *Met* plays a crucial role in JH action and lies upstream in the JH signal transduction pathway (Riddiford, 2008), but whether MET is the bona fide JHR remains inconclusive.

In *Drosophila*, a high titer of JH at the wandering stage and a high titer of 20E during pupariation both cause and mediate the larval-pupal metamorphosis (Dubrovsky, 2005). Application of JH or methoprene does not cause supernumerary larval molts, even when fed continuously throughout larval life (Wilson and Fabian, 1986; Riddiford and Ashburner, 1991). However, JH is required for reproduction, including protein synthesis in the male accessory gland (Yamamoto et al., 1988) and endocytotic uptake of vitellogenin by oocytes (Postlethwait and Weiser, 1973). In this paper, we show that the larval fat body of JH-deficient animals undergoes precocious and enhanced caspase-dependent PCD. Strikingly, JH prevents 20E-induced caspase-dependent PCD by counteracting MET and GCE and not via the suppression of the 20E-triggered transcriptional cascade. For the first time, we demonstrate that JH counteracts MET and GCE to prevent caspase-dependent PCD in *Drosophila*.

MATERIALS AND METHODS

Fly strains and genetic experiments

Met^{w3}::*UAS-Met* (Barry et al., 2008), *Met^{w3}*; *UAS-gceRNAi* (T.G.W., unpublished) and *UAS-jhamt* were generated in our laboratories. Four GAL4 lines were used: *Aug21-GAL4* [*Aug21*> (Mirth et al., 2005)], *Adh-GAL4* [*Adh*> (Grönke et al., 2003)], *FB-GAL4* [*FB*> (Grönke et al., 2003)] and *Act-GAL4* (*Act*>). The UAS-death activator line used was *UAS-grim* (Wing et al., 1998). *UAS-Dronc* was obtained from S. Kumar (Quinn et al., 2000). Flies from the Bloomington *Drosophila* Stock Center included: (1) *w^{1118}*, (2) *Act*>, (3) *hs-EcR-RNAi*, (4) *UAS-mcd8GFP* (*UAS-GFP*), (5) *Adv/Cyo*::*arm-GFP*, (6) *TM6B/TM3*:: *arm-GFP* and (7) *SP/Cyo*; *TM3/TM6B*.

Aug21>::*UAS-GFP* and *Aug21*>::*hs-EcR-RNAi* animals were produced by recombination of *Aug21*> with *UAS-GFP* and *hs-EcR-RNAi*, respectively. Homozygous *Met^{w3}*; *UAS-gceRNAi* females were crossed with *Act*>/*Cyo, arm-GFP* males to produce *Met^{w3}*/*Y*; *Act*>/*UAS-gceRNAi* males. Homozygous *Met^{w3}*::*UAS-Met* females were crossed with *FB*> males to produce *Met^{w3}*::*UAS-Met/Y*; *FB*> males.

Hormones

Juvenile hormone acid methyl transferase (JHAMT) activity in the brain-RG complex was measured as previously described (Li et al., 2003b; Sheng et al., 2008). JH synthesis by the brain-RG complex was monitored using a modification of the radiochemical assay (Richard et al., 1989) and reversed-phase HPLC separation (Li et al., 2003a). Third instar larvae were topically treated with 0.5 μl of a variety of concentrations (0-3 μg/μl) of methoprene dissolved in acetone (Wilson and Fabian, 1986). Treatment with 20E was performed on second or third instar larvae, which were fed on yeast mixture containing different concentrations (0-3 μg/μl) of 20E (McBrayer et al., 2007).

Fluorescence microscopy

GFP- and non-GFP-containing embryos were separated under an Olympus SZX16 fluorescence stereomicroscope. Apoptosis was measured using the Caspase 3&7 Apoptosis Detection Kit (green nuclei) according to the manufacturer's instructions (Invitrogen). For determining whether the cell membrane was disrupted, apoptosis was also detected by propidium iodide staining (red nuclei) and nuclei with Hoechst 33342 (blue) (Beyotime). The staining was monitored under an Olympus Fluoview FV1000 confocal microscope or an Olympus IX71 inverted fluorescence microscope using the same conditions for the control and experimental samples.

2D-DIGE/MS analysis

The two-dimensional fluorescence difference gel electrophoresis/mass spectrum analysis (2D-DIGE/MS) was performed by Shanghai Applied Protein Technology (Jia et al., 2007). Using 2D-DIGE, fat body protein profiles were compared between *Aug21*>; *UAS-grim* and *Aug21*> at three developmental stages: early wandering (EW), white prepupa (WPP) and 6 hours after pupariation (6AP). MALDI-TOF (Applied Biosystems) and LTQ (Thermo Finnigan) MS analyses were used to identify the proteins differentially expressed between the two lines (Alban et al., 2003; Sun et al., 2007).

Biochemical and molecular methods

SDS-PAGE electrophoresis and western blot analysis for FBP1 were as previously described (Sun et al., 2007). Quantitative real-time PCR (qPCR) was performed in a Rotor-Gene 2000 thermocycler (Corbett Research) using *rp49* (*RpL32*) for normalization (Sheng et al., 2008). Details of the qPCR primers are available upon request.

Statistics

Experimental data were analyzed by ANOVA and Student's *t*-test using an SAS program.

RESULTS

Ablation of the corpus allatum results in JH deficiency leading to pupal lethality

The cells comprising the corpus allatum (CA) are located within the ring gland (RG) and are responsible for JH biosynthesis in *Drosophila* (Richard et al., 1989; Dai and Gilbert, 1991). To assess the physiological roles of JH in *Drosophila*, the CA was genetically ablated using the UAS-GAL4 system (Brand and Perrimon, 1993). *Aug21*> is a GAL4 driver that specifically targets gene expression to the CA (Colombani et al., 2005; Mirth et al., 2005). Driven by *Aug21*>, *UAS-grim* (Wing et al., 1998) was expressed in the CA resulting in cell ablation. All of the *Aug21*>; *UAS-grim* animals died during early pupal life after normal pupariation. In addition, larval

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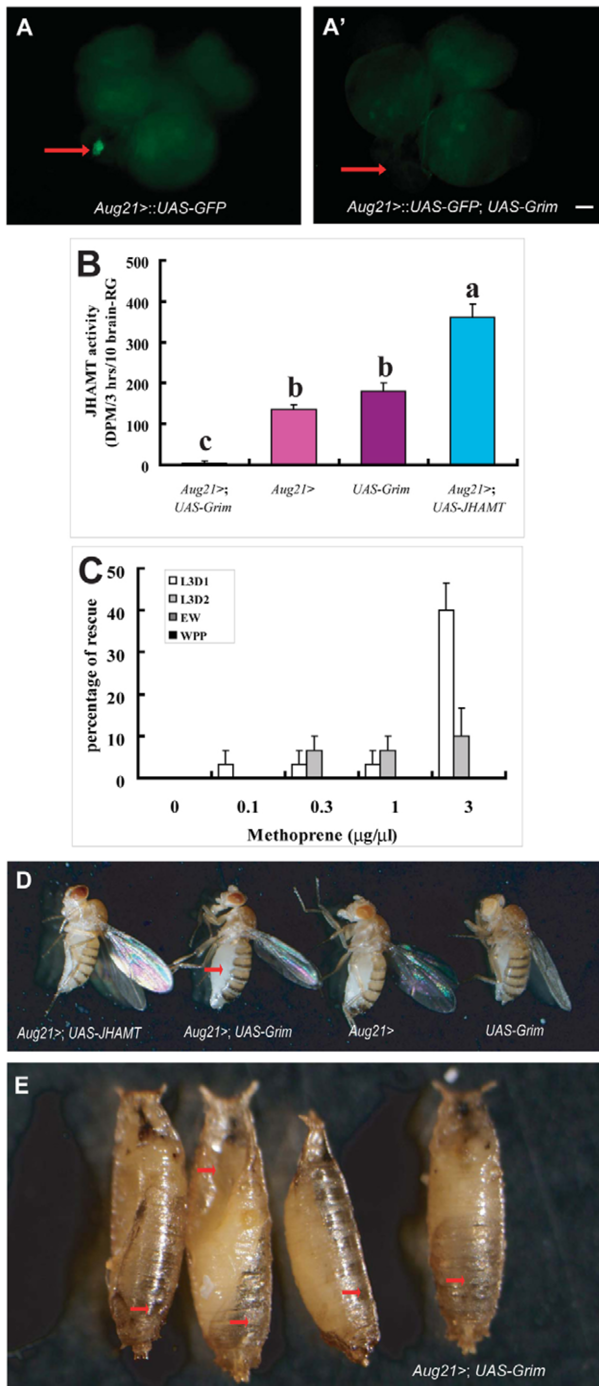

Fig. 1. Ablation of the corpus allatum results in juvenile hormone (JH) deficiency leading to pupal lethality. (**A**,**A′**) In comparison to *Aug21*>::*UAS-GFP* (A), all GFP-labeled corpus allatum (CA) cells were ablated in *Aug21*>::*UAS-GFP*; *UAS-grim* (A′) *Drosophila* larvae at the early wandering (EW) stage. Arrows point to the CA, or former position of the CA, in the brain-ring gland (RG) complex. Scale bar: 100 μm. (**B**) JHAMT activity in the brain-RG complex at the EW stage. The bars labeled with different lowercase letters are significantly different ($P<0.05$, ANOVA). (**C**) Methoprene application to *Aug21*>; *UAS-grim* larvae rescued pupal lethality. L3D1, day 1 of the third instar; L3D2, day 2 of the third instar; WPP, white prepupa. (**D**) Methoprene-rescued *Aug21*>; *UAS-grim* adults were reproductively competent. Arrow denotes the developing eggs in the abdomen of a methoprene-rescued *Aug21*>; *UAS-grim* female. (**E**) *Aug21*>; *UAS-grim* died during early pupal life. Arrows point to empty portions of the pupae.

development of *Aug21*>; *UAS-grim* animals was delayed and the body weight reduced (see Fig. S1 in the supplementary material). *UAS-GFP* was then included in the background of the *Aug21*> flies to create *Aug21*>::*UAS-GFP* for monitoring the timing and extent of CA ablation. In comparison to *Aug21*>::*UAS-GFP* (Fig. 1A), all GFP-labeled CA cells were ablated in *Aug21*>::*UAS-GFP*; *UAS-grim* by early wandering (EW) (Fig. 1A′), although some cells were still present at earlier larval stages (data not shown). To determine whether JH titers were affected by CA ablation, three indirect assays were conducted. First, JHAMT activity (Shinoda and Itoyama, 2003) in the brain-RG complex was measured, as JHAMT overexpression in *Drosophila* results in elevated JH levels (Niwa et al., 2008) (W.G.B. and S. S. Tobe, unpublished) and JHAMT is a key regulatory enzyme for JH biosynthesis (Sheng et al., 2008). JHAMT activity measured at EW was undetectable in *Aug21*>; *UAS-grim*, whereas it was 2- to 2.5-fold higher in *Aug21*>; *UAS-jhamt* than in the two control lines *Aug21*> and *UAS-grim* (Fig. 1B). Second, the rate of in vitro JH biosynthesis (Yagi and Tobe, 2001) by the brain-RG complex, a determining regulator of JH titer, was measured at EW. No in vitro JH biosynthesis was detected in *Aug21*>; *UAS-grim*, and the rate of in vitro JH biosynthesis in *Aug21*>; *UAS-jhamt* [~900 disintegrations per minute (DPM) in 3 hours from five brain-RG] was ~2.5-fold higher than in the two control lines. Third, the JH agonist methoprene was tested for its ability to rescue developmental lethality. Methoprene treatment was able to rescue *Aug21*>; *UAS-*

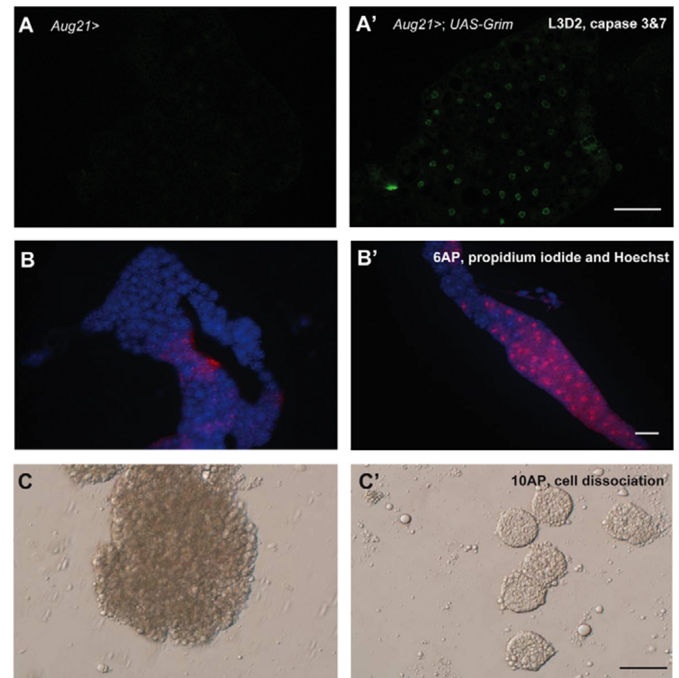

Fig. 2. The fat body in JH-deficient animals undergoes precocious and enhanced programmed cell death (PCD) and cell dissociation. Apoptosis and cell dissociation in the fat body were compared between the control line *Aug21*> (**A-C**) and the JH-deficient line *Aug21*>; *UAS-grim* (**A′-C′**) at several developmental stages: L3D1, L3D2, EW, WPP, 6 hours after pupariation (6AP) and 10AP. (A,A′) Caspase 3&7 apoptosis detection (green nuclei) at L3D2. (B,B′) Propidium iodide staining for cell membrane disruption (red nuclei) and staining of nuclei with Hoechst 33342 (blue) at 6AP. (C,C′) Cell dissociation at 10AP. The staining was monitored by confocal (A,A′) or inverted fluorescence (B-C′) microscopy with the same conditions for control (A-C) and experimental (A′-C′) samples. Scale bars: 100 μm.

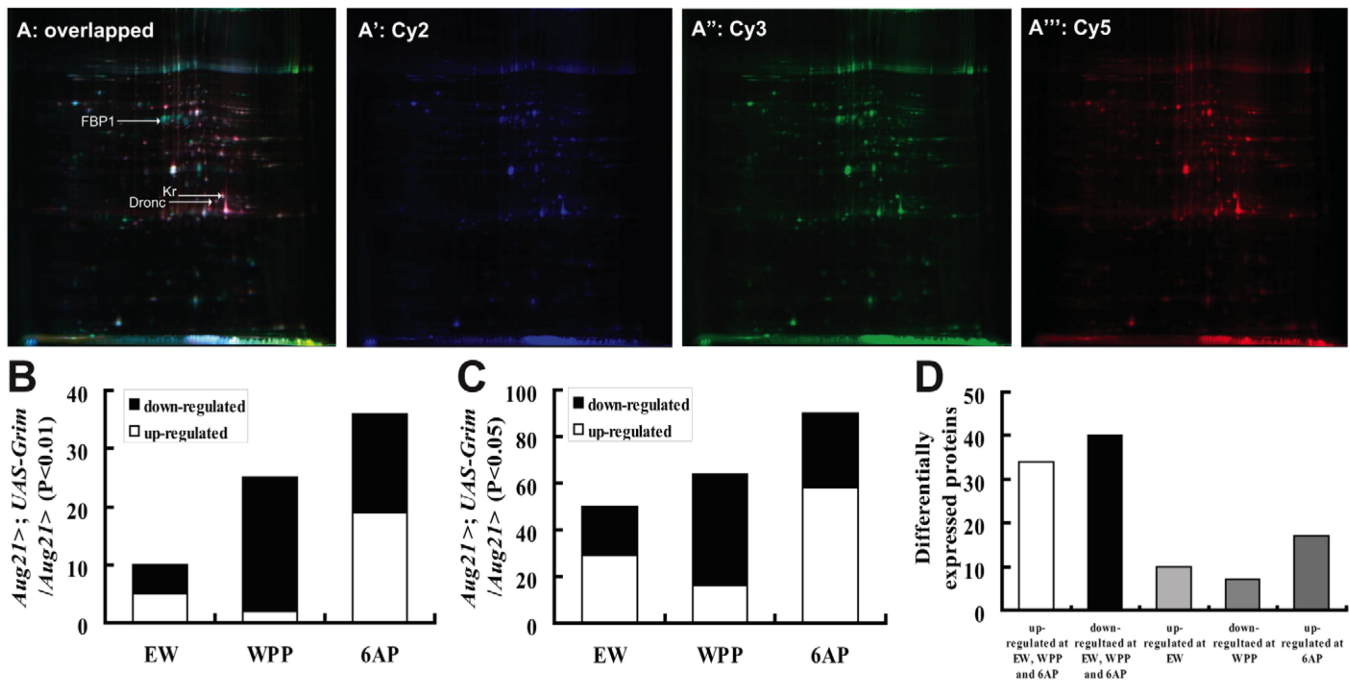

Fig. 3. 2D-DIGE analysis of differentially expressed proteins in the fat body of the JH-deficient line *Aug21>; UAS-grim* and the control line *Aug21>*. (**A-A‴**) A representative 2D-DIGE image with merged Cy2, Cy3 and Cy5 (A, arrows point to the differentially expressed proteins Dronc, FBP1 and KR) and the individual images of Cy2 (A′, blue), Cy3 (A″, green) and Cy5 (A‴, red). (**B**,**C**) Ratio of differentially expressed protein spots (B, *P*<0.01; C, *P*<0.05; ANOVA) in the fat body of the JH-deficient line *Aug21>*; *UAS-grim* and the control line *Aug21>* at EW, WPP and 6AP. (**D**) The five groups of differentially expressed protein spots.

grim development to the adult stage, depending on the dose of methoprene used and the stage of the larvae treated (Fig. 1C). The application of low doses of methoprene (0.1, 0.3 or 1 μg/μl) on day 1 or 2 of the third instar (L3D1 or L3D2) rescued 0-7% of the pupae to adults, whereas treatment with 3 μg/μl of methoprene on L3D1 was able to rescue ~40% of the pupae to adults that were reproductively competent (Fig. 1D). However, once *Aug21>*; *UAS-grim* larvae reached the EW stage, methoprene failed to rescue the JH-deficient pupae to the adult stage, even at higher concentrations (>3 μg/μl). These results demonstrated that CA ablation results in JH deficiency leading to pupal lethality.

We then carefully observed the JH-deficient *Aug21>*; *UAS-grim* animals for developmental defects during the larval-pupal transition. Although a small proportion (~10%) of the JH-deficient pupae underwent head eversion successfully, the adult organs of these animals initiated development but never completed it. As visualized beneath the cuticle by microscopy, internal portions of the pupae were seen to progressively retract from the cuticle (apolysis), creating an apparently empty space beginning 6 hours after pupariation (6AP) (Fig. 1E). During the larval-pupal metamorphosis of *Drosophila*, the fat body undergoes a remodeling process but remains in the posterior part of the pupa (Nelliot et al., 2006; Liu et al., 2009). The posterior portion of the JH-deficient pupae often appeared to be empty, suggesting that JH has an important role in the control of fat body development during the larval-pupal transition.

JH prevents PCD during fat body remodeling

Similar to other larval organs, the *Drosophila* larval fat body undergoes massive destruction by PCD and necrosis (Hoshizaki, 2005; Liu et al., 2009). As predicted, fat body remodeling in the JH-deficient line was altered dramatically and differed significantly from w^{1118}, *UAS-grim*, *Aug21>* and the JH-overexpressing line *Aug21>*; *UAS-jhamt*. Since no significant differences in fat body remodeling were observed in the latter four lines, in the following studies only experimental data for one control line, *Aug21>*, are presented. Apoptosis and cell dissociation in the fat body of the JH-deficient line *Aug21>*; *UAS-grim* and the control line *Aug21>* were compared at several developmental stages: L3D1, L3D2, EW, white prepupa (WPP), 6AP and 10AP. At L3D1, L3D2 and EW, apoptosis of fat body cells was almost undetectable in the control (L3D2; Fig. 2A) but was pronounced in the JH-deficient animals (L3D2; Fig. 2A′) when stained using the Caspase 3&7 Apoptosis Detection Kit. From EW to WPP, apoptosis became stronger in the control but weaker in the JH-deficient animals (data not shown). At WPP and 6AP, the majority of the fat body cells in the JH-deficient animals died as a result of apoptosis, showing a disrupted cell membrane when stained with propidium iodide (6AP; Fig. 2B′), but only a small portion of cells of the control were stained by propidium iodide (6AP; Fig. 2B). At 10AP, fat body cells in the control appeared to round up and began to lose their tight associations with one another (Fig. 2C), but nearly all fat body cells in the JH-deficient animals were completely dissociated into individual cell masses (Fig. 2C′). In conclusion, the fat body in the JH-deficient pupae underwent precocious and enhanced PCD and eventually failed to complete the remodeling process, demonstrating that JH plays a crucial role in the control of fat body remodeling in *Drosophila* by preventing PCD.

Caspase genes are upregulated in the fat body of JH-deficient animals

The important role of JH in preventing PCD in the *Drosophila* fat body prompted us to compare the protein profiles in the fat body of the JH-deficient line *Aug21>*; *UAS-grim* and the control line

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Table 1. Differentially expressed proteins identified by 2D-DIGE/MS

Group	CG number	Protein description	MW (Da)	pI	MASCOT score
Group 1: upregulated at EW, WPP and 6AP	8091	Dronc, initiator caspase (Nedd2-like caspase)	51141	6.6	108
	1803	Regucalcin*	33680	6.0	105
	5261	Dihydrolipoyllysine-residue acetyltransferase	44118	8.9	149
	9780	ATPase, Neprilysin	67766	9.1	72
Group 2: downregulated at EW, WPP and 6AP	17285	Fat body protein 1 (FBP1)	119350	5.8	109
	33102	Hexokinase-t1 (HEX-t1)	52918	6.1	67
	3481	Alcohol dehydrogenase (ADH)*	27727	7.7	121
	7176	Isocitrate dehydrolase (IDH)*	52955	7.2	205
	16936	Glutathione transferase (GST)	25446	5.9	70
	3752	Aldehyde dehydrogenase (ALDH)*	57325	6.4	122
	6084	Aldehyde reductase	–	–	–
	6180	Phosphatidylethanolamine binding	28706	9.0	420
	17237	ATPase, Ca^{2+} binding	21813	9.5	68
	3092	Unknown	44565	6.3	99
Group 3: upregulated at EW	12051	Actin 42A	41824	5.3	250
	3922	Ribosomal protein S17	15332	10	77
	1065	Succinyl coenzyme A synthatase, alpha subunit	34766	9.1	106
Group 4: downregulated at WPP	3340	Krüppel (KR)	54715	7.1	120
Group 5: upregulated at 6AP	4027	Actin 5C*	42194	5.3	96
	14792	Stubarista (STA)	30266	4.8	78
	7592	Odordant-binding protein 99b	17505	6.1	123

Those proteins marked with an asterisk were detected twice, in two different protein spots. MASCOT scores >60 are significant (P<0.05).

Aug21> at three developmental stages (EW, WPP and 6AP) using 2D-DIGE/MS analysis. A representative 2D-DIGE image, with merged Cy2 (blue), Cy3 (green) and Cy5 (red) fluorescence is shown in Fig. 3A-A‴. Across all the loading samples, ~1500 protein spots were reproducibly detected in each gel. The number of differentially expressed protein spots between the JH-deficient and the control animals gradually increased (>1.5-fold) from EW to WPP to 6AP. Most of the differentially expressed protein spots in the fat body of the JH-deficient animals at WPP (90%, P<0.01; 70%, P<0.05%) were downregulated, whereas more than half of them were upregulated at EW or 6AP (Fig. 3B,C). The 111 differentially expressed protein spots (P<0.05) between the two lines can be divided into five groups (Fig. 3D). Approximately 70% of the protein spots were either upregulated (34, group 1) or downregulated (40, group 2) at the three developmental stages in the JH-deficient animals (Fig. 3D).

Twenty-six of the most differentially expressed protein spots were subjected to MALDI-TOF and/or LTQ MS analysis. Twenty-one proteins were identified and five of them were detected twice, in different spots (Table 1). The mRNA levels of ten of the proteins were then analyzed by qPCR. The most significant protein in group 1 is Dronc (Fig. 4A,A′), an initiator caspase that can be upregulated by the 20E-EcR-USP complex, Br-C and E75 (Cakouros et al., 2004), which was increased at all three stages in the JH-deficient animals. The mRNA level of *Drice* (Fig. 4B), an effector caspase that can be upregulated by Br-C and activated by Dronc (Kilpatrick et al., 2005), was also increased at all three stages in the JH-deficient animals. Regucalcin, a molecular marker of senescence (Fujita, 1999), was also identified in group 1, suggesting senescence of fat body cells (see Fig. S2A,A′ in the supplementary material). The protein FBP1 in group 2 (Fig. 4C,C′) was decreased at the three stages studied in the JH-deficient animals. Western blot analysis showed that in the JH-overexpressing line *Aug21*>; *UAS-jhamt*, the estimated, elevated levels of JH were able to convert FBP1 from an inactive form (P69) to an active form (P50) (Fig. 4C″). Changes in the levels of *Lsp2* and *Fbp1* mRNAs followed a similar pattern (see Fig. S2B′ in the supplementary material). Most proteins in group 2 (Table 1) are involved in energy metabolism and detoxification. The mRNA levels of all the tested genes were downregulated in the JH-deficient animals at WPP and upregulated in the JH-overexpressing animals at EW (see Fig. S2C-M in the supplementary material). The one protein identified in group 4 was Krüppel (KR), which was downregulated in the JH-deficient animals at WPP (Fig. 4D,D′). The mRNA levels of two *Kr* paralogous genes, *Kr-H1* and *Kr-H2*, shared a similar pattern to that of *Kr* (see Fig. S2N′,O′ in the supplementary material). The 2D-DIGE/MS and qPCR analyses indicate that the failure of fat body remodeling in the JH-deficient animals is a result of multiple developmental defects, including precocious and enhanced caspase-dependent PCD. Importantly, the two caspase genes *Dronc* and *Drice*, which can be upregulated and activated by 20E action (Cakouros et al., 2004; Kilpatrick et al., 2005), were significantly upregulated in the fat body of the JH-deficient animals, suggesting that JH antagonizes 20E-induced caspase-dependent PCD.

JH does not suppress the 20E-triggered transcriptional cascade in preventing caspase-dependent PCD of the fat body

Previous reports have shown that JH also elicits the expression of several 20E response genes, including *E75* in *Drosophila* S2 cells (Dubrovsky et al., 2004), *E74B* in late third instar larval organs (Beckstead et al., 2007) and *Kr-H1* in the abdominal integuments of prepupae or pupae (Minakuchi et al., 2008). The above 2D-DIGE/MS and qPCR analyses revealed that JH elicited the expression of several 20E response genes, including *Fbp1*, *Lsp2* and *Kr-H1* (Fig. 4; see Fig. S2 in the supplementary material). Furthermore, the qPCR analysis demonstrated that the 20E-triggered transcriptional cascade was reduced in the fat body of the JH-deficient animals at WPP and was enhanced in the fat body of the JH-overexpressing animals at EW (data not shown). In contrast to

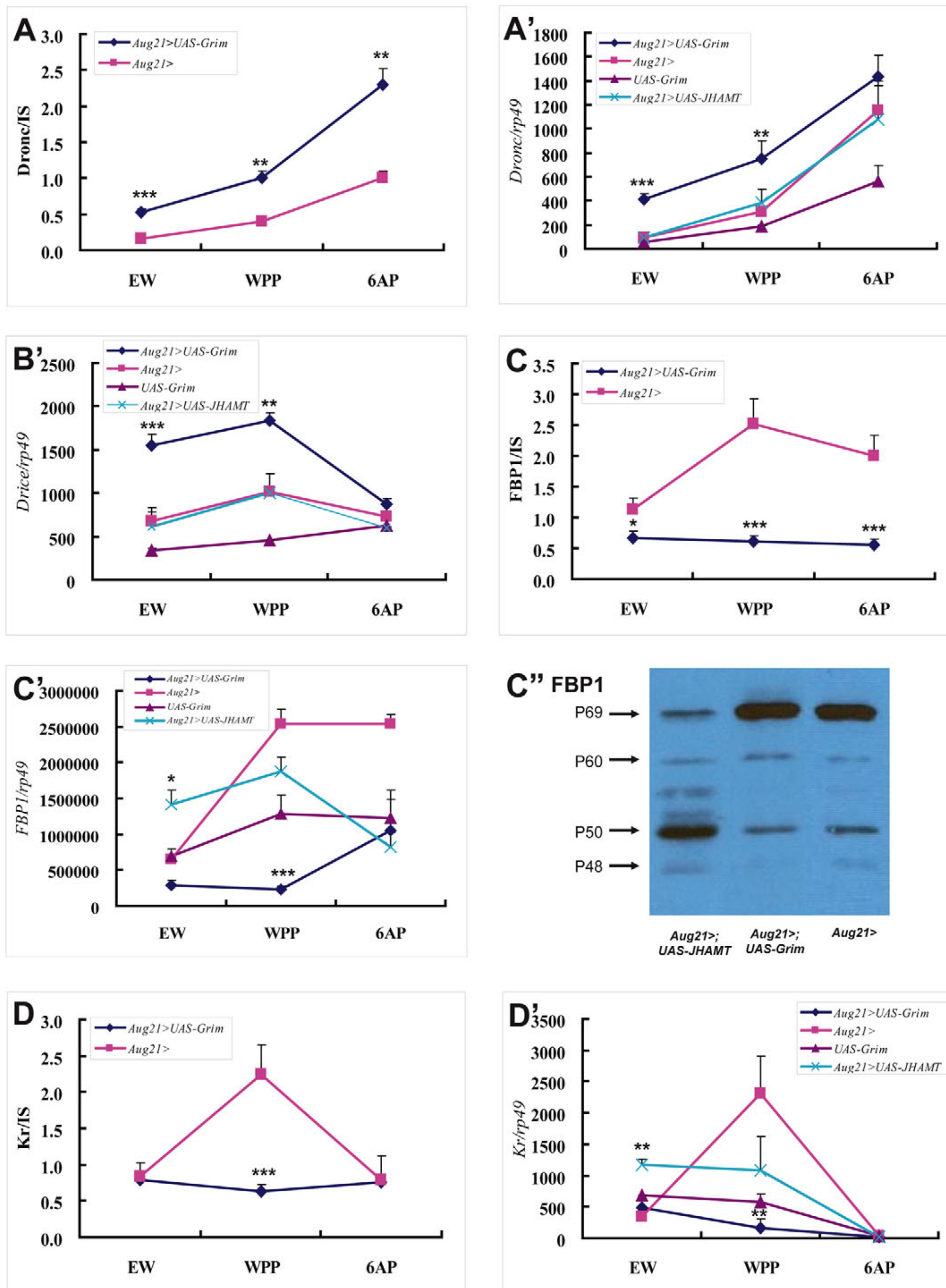

Fig. 4. Differentially expressed protein and mRNA profiles in the fat body. The fat body protein profiles of the JH-deficient line *Aug21>*; *UAS-grim* and the control line *Aug21>* were compared at three developmental stages (EW, WPP and 6AP) by 2D-DIGE/MS analysis. The internal standard (IS) is the mean value of the protein in all of the fat body samples and is used for normalization. qPCR was used to assess the fat body mRNA profiles of (1) the JH-deficient line *Aug21>*; *UAS-grim*, (2) the control line *Aug21>*, (3) the control line *UAS-grim*, and (4) the JH-overexpressing line *Aug21>*; *UAS-jhamt* at EW, WPP and 6AP. *rp49* was used for normalization. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ANOVA. (**A**,**A'**) The protein (A) and mRNA (A') profiles of Dronc. (**B**) The mRNA profile of *Drice*. (**C-C''**) The protein (C) and mRNA (C') profiles and western blotting (C'') of FBP1. Note the inactive (P69) and active (P50) forms of FBP1. (**D**,**D'**) The protein (D) and mRNA (D') profiles of Krüpple (KR).

the caspase genes *Dronc* and *Drice* (Fig. 4A-B), but similar to other genes in the 20E-triggered transcriptional cascade, the death activator genes *reaper* (Fig. 5A) and *Hid* (Fig. 5B), as well as the autophagy genes *Atg8A* (Fig. 5C) and *Atg8B* (Fig. 5D), were downregulated in the fat body of JH-deficient animals at WPP, and *Hid* and *Atg8A* were upregulated in the JH-overexpressing animals at EW. The mRNA level of *Iap1*, another important gene involved in preventing PCD, was not altered in the fat body of the JH-deficient and JH-overexpressing animals (data not shown). Based on the above data, we conclude that JH does not suppress the 20E-triggered transcriptional cascade in preventing caspase-dependent PCD in the fat body of JH-deficient animals.

JH antagonizes 20E-induced caspase-dependent PCD to regulate larval-pupal metamorphosis

To further support the hypothesis that JH antagonizes 20E-induced caspase-dependent PCD in the fat body, we genetically manipulated the 20E signal in the JH-deficient line *Aug21>*; *UAS-grim*. The JH-deficient larvae were highly sensitive to 20E treatment. When second instar larvae were fed on the yeast mixture containing 1 μg/μl 20E, only ~10% of the JH-deficient animals pupariated and the rest died. However, under these conditions, ~60% of the three control lines pupariated precociously and the pupae were smaller (Fig. 6A,B). Decreasing the 20E signal partially rescued the JH-deficient pupae. Using

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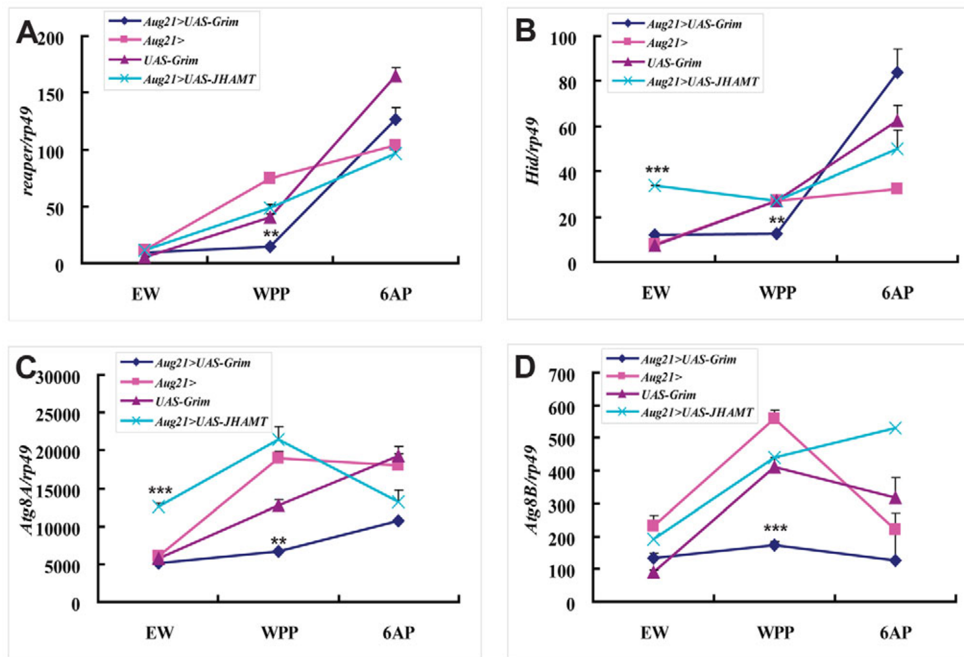

Fig. 5. The mRNA profiles of several 20E-induced PCD-related genes in the fat body. The gene expression profiles of the death activator genes *reaper* (**A**) and *Hid* (**B**) and of the autophagy genes *Atg8A* (**C**) and *Atg8B* (**D**) were compared in the fat body of (1) the JH-deficient line *Aug21>*; *UAS-grim*, (2) the control line *Aug21>*, (3) the control line *UAS-grim*, and (4) the JH-overexpressing line *Aug21>*; *UAS-jhamt* at EW, WPP and 6AP. *rp49* was used for normalization. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ANOVA.

EcR RNAi to disrupt 20E signaling, heat shock-induced expression of *EcR* dsRNA causes a high degree of lethality in *hs-EcR-RNAi* animals (Lam and Thummel, 2000). When reared without heat-shock induction, ~10% of *Aug21>::hs-EcR-RNAi*; *UAS-grim* (*hs-EcR-RNAi* on the JH-deficient background) animals survived to the pre-adult stage (Fig. 6C) and less than half of those emerged as adults (Fig. 6C′). These genetic experiments confirmed the hypothesis that JH antagonizes 20E-induced PCD to regulate larval-pupal metamorphosis in *Drosophila* (Fig. 6D).

Overexpression of *Met* specifically in the fat body results in precocious and enhanced PCD

As concluded above, JH does not suppress the 20E-triggered transcriptional cascade to prevent caspase-dependent PCD in the fat body. Since MET and GCE play a crucial role in JH action and lie upstream in the JH signal transduction pathway in *Drosophila* (Riddiford, 2008), we studied whether *Met* and *gce* mediate the prevention of PCD by JH. Most of the *Met*/*gce*-deficient Met^{w3}/Y; *Act>/UAS-gceRNAi* animals die during the pupal-adult transition (T.G.W., unpublished). Surprisingly, precocious and enhanced PCD was never observed in the larval fat body of the *Met*/*gce*-deficient animals (data not shown). Moreover, the *Met*/*gce*-deficient animals showed a similar lethality phenotype to that of the global JH-overexpressing *Act>*; *UAS-jhamt* animals, which also die during the pupal-adult transition (Niwa et al., 2008).

Previous studies have shown that global overexpression of *Met* in Met^{w3}::*UAS-Met/Y*; *Act>* causes high mortality during larval life (Barry et al., 2008). Similarly, nearly all Met^{w3}::*UAS-Met/Y*; *Adh>* animals, in which *Met* is overexpressed specifically in the fat body, died during larval life. Less than 10% of the *Met*-overexpressing animals were able to survive to the EW stage and ~1% pupariated but never emerged as adults. Moreover, larval development of the *Met*-overexpressing animals was greatly delayed and their body weight dramatically reduced (Fig. 7A). Once the *Met*-overexpressing larvae reached the EW stage, larval fat body cells began to dissociate (Fig. 7B) from each other and underwent dramatic apoptosis (Fig. 7C). The developmental defects of the *Met*-overexpressing animals are similar to, but much stronger than, those of the JH-deficient animals (Figs 1 and 2). The phenotypic and genetic data for these animals strongly suggest that JH counteracts MET and GCE.

Overexpression of *Met* upregulates *Dronc* and *Drice* leading to PCD and this upregulation can be suppressed by methoprene application

We then studied whether *Met* induces PCD via increasing the mRNA levels of the caspases *Dronc* and *Drice*. In comparison to male w^{1118} animals at WPP, the mRNA levels of *Dronc* (Fig. 8A) and *Drice* (Fig. 8B) were downregulated in the fat body of the *Met*/*gce*-deficient animals and the downregulation was less significant in the *Met* mutant Met^{w3}/Y.

Because it is difficult to collect sufficient larval fat body from Met^{w3}::*UAS-Met/Y*; *Act>* or Met^{w3}::*UAS-Met/Y*; *Adh>*, we used Met^{w3}::*UAS-Met/Y*; *FB>*, in which *Met* is also specifically overexpressed in the fat body. Met^{w3}::*UAS-Met/Y*; *FB>* exhibited better survival and less significant developmental defects than Met^{w3}::*UAS-Met/Y*; *Adh>*. In comparison to male w^{1118} animals at WPP, *Dronc* (Fig. 8C) and *Drice* (Fig. 8D) were dramatically upregulated in the fat body of the *Met*-overexpressing animals. Importantly, application of methoprene (1 μg/μl) at the EW stage significantly downregulated *Dronc* (Fig. 8C) and *Drice* (Fig. 8D) at WPP. However, application of methoprene to Met^{w3}/Y; *Act>/UAS-gceRNAi* had no significant effects on *Dronc* and *Drice* at WPP (data not shown). Together, these experiments demonstrated that JH is epistatic to MET and GCE.

We then investigated whether overexpression of *Dronc* in the fat body causes lethality and PCD. Approximately 95% of *Adh>*; *UAS-Dronc* animals, in which *Dronc* is overexpressed in the fat body, died during different larval stages (Fig. 8E), with significant apoptosis at EW (Fig. 8F). The remaining animals died at the pupal stage.

Altogether, the data in this paper demonstrate that JH counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent PCD in *Drosophila* (Fig. 8G).

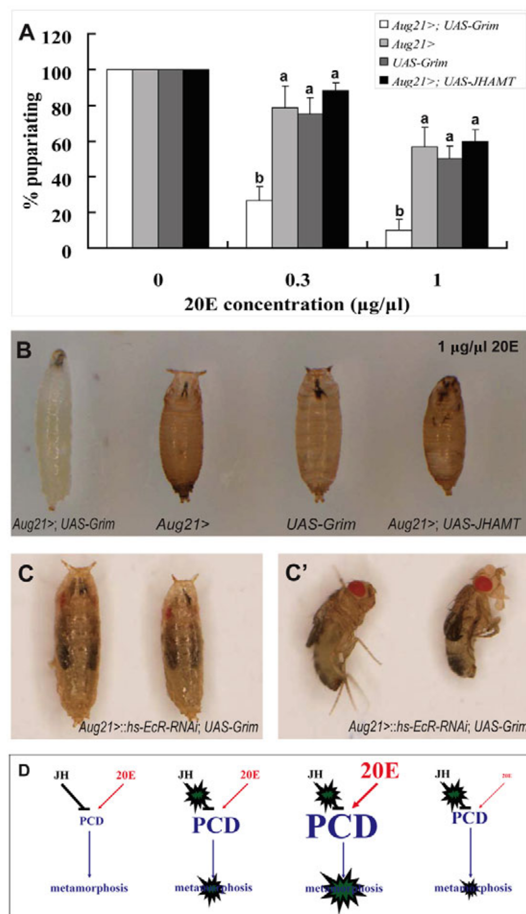

Fig. 6. JH antagonizes 20E-induced PCD to regulate larval-pupal metamorphosis. (**A**) JH-deficient *Aug21>; UAS-grim Drosophila* larvae were highly sensitive to 20E treatment. Three concentrations (0, 0.3 and 1 μg/μl) of 20E were applied to the four fly lines at the second instar stage and the percentage pupariating measured. Bars labeled with different lowercase letters are significantly different ($P<0.05$, ANOVA). (**B**) Typical lethal stages of the four fly lines after feeding 1 μg/μl 20E at second instar. (**C**,**C′**) Without heat-shock induction, ~10% of *Aug21>::hs-EcR-RNAi*; *UAS-grim* larvae survived to the pre-adult stage (C) and about half of that 10% emerged as adults (C′). (**D**) Model showing how JH antagonizes 20E-induced PCD and thus regulates *Drosophila* metamorphosis. Text size conveys magnitude of treatment and response.

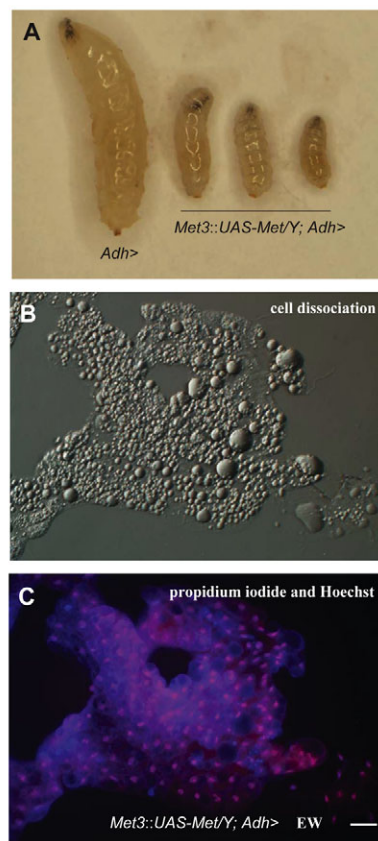

Fig. 7. Overexpression of *Met* specifically in the fat body results in precocious and enhanced PCD and cell dissociation. (**A**) The body weight of the *Met*-overexpressing Met^{w3}::*UAS-Met/Y*; *Adh>* animals was dramatically reduced, in comparison to *Adh>*. (**B**,**C**) Fat body cells of the *Met*-overexpressing animals at WPP began to dissociate from each other (B) and underwent significant apoptosis (C). Apoptosis was detected by propidium iodide (red nuclei) and nuclei were stained with Hoechst 33342 (blue). Scale bar: 100 μm.

DISCUSSION

JH has 'status quo' actions in *Drosophila*

The status quo action of JH has been well documented in several insect orders, particularly in Coleoptera, Orthoptera and Lepidoptera, in which JH treatment causes supernumerary larval molting and JH deficiency triggers precocious metamorphosis (Riddiford et al., 2003). However, as JH does not cause supernumerary larval molting in flies (Srivastava and Gilbert, 1968; Wilson and Fabian, 1986; Riddiford and Ashburner, 1991), evidence for the status quo action of JH in *Drosophila* has remained elusive. From past studies and from the experimental data presented here, we conclude that the status quo hypothesis does indeed apply to JH action in *Drosophila*. First, although JH application during the final larval instar or during the prepupal stage has little effect on the differentiation of adult head and thoracic epidermis in *Drosophila*, it does prevent normal adult differentiation of the abdominal epidermis. After JH treatment, a second pupal, rather than an adult, abdominal cuticle is formed in Diptera (Srivastava and Gilbert, 1968; Zhou and Riddiford, 2002). Second, JH or a JH agonist applied to *Drosophila* at the onset of metamorphosis results in lethality during pupal-adult metamorphosis (Madhaven, 1973). Similarly, global overexpression of *jhamt* results in severe defects during the pupal-adult transition and eventually death (Niwa et al., 2008). Third, CA ablation leading to JH deficiency caused precocious and enhanced fat body PCD (Fig. 2). Fourth, JH deficiency resulted in pupal lethality (Fig. 1A) and delayed larval development (see Fig. S1 in the supplementary material), although JH deficiency was not sufficient to cause precocious metamorphosis. The composite data demonstrate that JH in *Drosophila* does have status quo actions on the abdominal epidermis during pupal-adult metamorphosis and on the fat body during larval-pupal metamorphosis. We conclude that the status quo action of JH in *Drosophila* is functionally important, but more subtle than that in Coleoptera, Orthoptera and Lepidoptera. However, it is not clear whether JH is essential for embryonic and earlier larval development because the CA cells are not completely ablated in the JH-deficient animals until the EW stage. To address this question, it would be necessary to generate a mutant (i.e. of *jhamt*) that interrupts JH but not the farnesyl pyrophosphate biosynthesis pathway.

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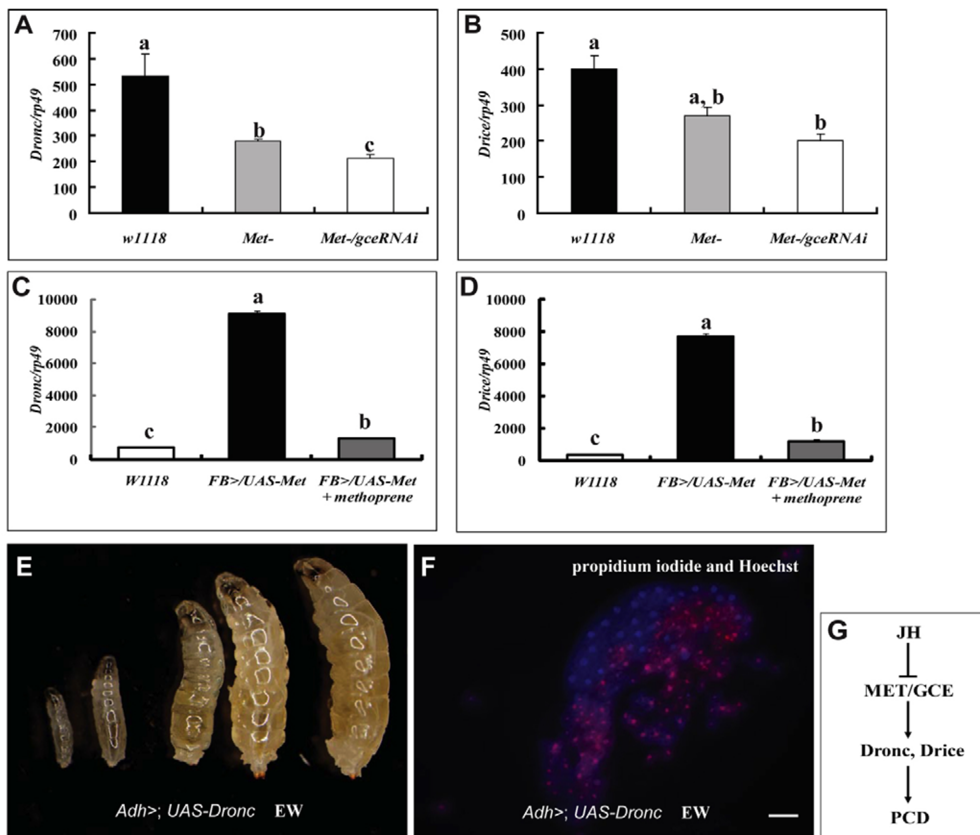

Fig. 8. JH counteracts MET/GCE to prevent caspase-dependent PCD. (**A**,**B**) The expression levels of *Dronc* (A) and *Drice* (B) in the fat body at WPP were compared in the male fat body of the control line w^{1118}, in the *Met* mutant Met^{w3}/*Y* (*Met*–), and in the *Met*/*gce*-deficient line Met^{w3}/*Y*; *Act*>/*UAS-gceRNAi* (*Met/gceRNAi*). Bars labeled with different lowercase letters are significantly different (P<0.05, ANOVA). (**C**,**D**) The expression levels of *Dronc* (C) and *Drice* (D) in the fat body at WPP were compared in the male fat body of the control line w^{1118}, in Met^{w3}::*UAS-Met/Y*; *FB*> (*FB*>; *UAS-Met*) in which *Met* is specifically overexpressed in the fat body, and in Met^{w3}::*UAS-Met/Y*; *FB*> treated with methoprene (1 μg/μl) at EW (*FB*>; *UAS-Met*+methoprene). (**E**) Approximately 95% of *Adh*>*UAS-Dronc* larvae, in which *Dronc* is overexpressed in the fat body, died during different larval stages. (**F**) Significant apoptosis was detected in the fat body of *Adh*>*UAS-Dronc* at EW. Scale bar: 100 μm. (**G**) Model showing how JH counteracts MET/GCE to prevent caspase-dependent PCD.

JH prevents caspase-dependent PCD in controlling fat body remodeling and larval-pupal metamorphosis in *Drosophila*

The insect fat body is analogous to vertebrate adipose tissue and liver and functions as a major organ for nutrient storage and energy metabolism (Hoshizaki, 2005; Liu et al., 2009). In response to 20E pulses, *Drosophila* larval organs undergo a developmental remodeling process during metamorphosis (Ward et al., 2003). Blocking the 20E signal specifically in the fat body during the larval-pupal transition (*Lsp2*>; *UAS-EcR*^{DN}) prevented the fat body from undergoing PCD (our unpublished data) and cell dissociation (Cherbas et al., 2003).

The experimental data in this paper demonstrated that JH prevents caspase-dependent PCD in the fat body during the larval-pupal transition in *Drosophila*. First, JH deficiency in *Aug21*>, *UAS-grim* resulted in the fat body undergoing precocious and enhanced PCD and cell dissociation (Fig. 2). Precocious and enhanced apoptosis appeared as early as L3D1 in the JH-deficient animals (Fig. 2A,B). Methoprene application on L3D1 was able to rescue ~40% of the pupae to adults, but it failed to rescue post-EW (Fig. 1D). Second, 2D-DIGE/MS and qPCR analyses indicated that the fat body in the JH-deficient animals has multiple developmental defects. The upregulation of the caspase genes *Dronc* and *Drice* (Fig. 4A-B) should account for the PCD in the fat body, as overexpression of *Dronc* in the fat body causes PCD, cell dissociation, and thus lethality (Fig. 8E,F; data not shown). As demonstrated previously, overexpression of *Dronc* (Dorstyn et al., 1999; Lee et al., 2000) or *Drice* (Kilpatrick et al., 2005) in cells and tissues is sufficient to cause caspase-dependent PCD. Third, the 20E-triggered transcriptional cascade in the fat body was downregulated in the JH-deficient animals (Fig. 5), indicating that JH does not suppress the 20E-triggered transcriptional cascade in preventing caspase-dependent PCD in the fat body.

The antagonizing effect of JH on 20E-induced PCD in the fat body was further confirmed in the JH-deficient animals by 20E treatment and RNA interference of *EcR* (Fig. 6). One might expect that perfect timing, titer and receptor response of JH and 20E are required to ensure accurate PCD in a tissue- and stage-specific manner during *Drosophila* metamorphosis (Ward et al., 2003). In the JH-deficient animals, the upregulation of *Dronc* and *Drice* resulted in precocious and enhanced PCD, such that the JH-deficient animals are committed to die during the larval-pupal transition (Fig. 1A). This hypothesis was strengthened by overexpression of *Dronc* specifically in the fat body, which caused larval lethality (Fig. 8E). Taken together, we conclude that JH antagonizes 20E-induced caspase-dependent PCD in controlling fat body remodeling and larval-pupal metamorphosis in *Drosophila* (Fig. 6D).

JH counteracts MET and GCE to prevent caspase-dependent PCD in *Drosophila*

Based on the phenotypes and gene expression profiles in the four fly lines used, we conclude that JH counteracts MET and GCE to prevent caspase-dependent PCD (Fig. 8G). First, the *Met*-overexpressing animals died during larval life (Barry et al., 2008) (Fig. 7A), with precocious and enhanced PCD and cell dissociation in the fat body (Fig. 7B,C; data not shown). Dramatic upregulation of *Dronc* and *Drice* was observed when *Met* was specifically overexpressed in the fat body and this upregulation was significantly decreased by methoprene application (Fig. 8C,D) demonstrating that JH is epistatic to MET and GCE. Moreover, the

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Dronc-overexpressing animals (Fig. 8E,F) exhibited similar phenotypes to the *Met*-overexpressing animals. Second, in the fat body of the JH-deficient animals, PCD (Fig. 2) and the expression of *Dronc* and *Drice* (Fig. 4A-B) were upregulated but not as significantly as in the *Met*-overexpressing animals. This might explain why the JH-deficient animals did not die until early pupal life (Fig. 1E). Third, both the global JH-overexpressing animals (Niwa et al., 2008) and the *Met*/*gce*-deficient animals (T.G.W., unpublished) died during the pupal-adult transition. In these animals, *Dronc* and *Drice* were downregulated and caspase-dependent PCD was decreased in the fat body (Fig. 8A,B; our unpublished data), implying that these animals died from a lack of caspase-dependent PCD. Weak mutants of *Dronc* (Xu et al., 2005) and *Drice* mutants (Muro et al., 2006) die during pupal life, showing that caspase-dependent PCD is essential for *Drosophila* metamorphosis. In addition, we also observed that methoprene application at the onset of metamorphosis results in delayed fat body remodeling (our unpublished data).

In the future, it will be crucial to elucidate the detailed molecular mechanism of how JH counteracts MET and GCE to prevent caspase-dependent PCD. In *Drosophila* S2 cells, the transcriptional activity of MET is dependent on the JH concentration (Miura et al., 2005) and both MET-MET and MET-GCE interactions can be greatly diminished by JH (Godlewski et al., 2006). The bHLH-PAS transcription factors typically function as hetero- or homodimers (Gu et al., 2000). If MET/GCE is the JHR, the transcriptional activities of the dimerized MET/GCE and the JH-MET/GCE complex should differ. In other words, the dimerized MET/GCE should induce transcription of *Dronc* and *Drice* and, in turn, JH binding to form the JH-MET/GCE complex should reduce this induction. Although, to our knowledge, there are no examples in the literature in which a receptor, without ligand, acts as a transcriptional activator and the transcriptional activity of the receptor is diminished when the ligand is bound, we could speculate that the JHR is a unique hormone receptor and perhaps that is the reason why it has yet to be isolated and characterized. Unfortunately, the two experiments described above (Miura et al., 2005; Godlewski et al., 2006) were conducted in *Drosophila* S2 cells, where the possibility of an endogenous JHR could not be eliminated. Although MET/GCE is definitely a key component in the JH signal transduction pathway, whether MET/GCE is the bona fide JHR remains conjecture.

It is very likely that MET cross-talks with EcR-USP via a large molecular complex (Li et al., 2007). One can hypothesize that MET promotes 20E action in the absence of JH and suppresses 20E action in the presence of JH, a model which we favor. *Drosophila* FKBP39 (FK506-BP1) could be a key component in this complex because it physically interacts with MET, EcR and USP, and binds the *D. melanogaster* JH response element 1 (Li et al., 2007). Moreover, *Drosophila* FKBP39 inhibits 20E-induced autophagy (Juhász et al., 2007). Further analysis of the complex will be crucial to precisely define the molecular mechanism of cross-talk between the action of JH and 20E.

In summary, we conclude that JH counteracts MET and GCE to prevent caspase-dependent PCD in controlling fat body remodeling and larval-pupal metamorphosis in *Drosophila*. The *Drosophila* fat body has provided an excellent model for studying the long-standing question of JH signal transduction. To finally settle the question of the bona fide JHR and to understand the precisely defined molecular mechanism of JH action requires further research at a variety of levels in several species of insects that can be genetically manipulated, such as *Drosophila*, *Bombyx* and *Tribolium*.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/12/2015/DC1

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