ABSTRACT

Title of Document: **JUVENILE HORMONE BIOSYNTHESIS AND SIGNALING PATHWAY IN DROSOPHILA MELANOGASTER**

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Insect development and metamorphosis are controlled by two major hormones; 20-hydroxyecdysone (20E) and juvenile hormone (JH). 20E signaling pathway is well recognized while JH signaling is still ambiguous. For a better understanding of JH biosynthesis and signaling we worked on two parallel projects; reverse genetic and forward genetic studies.

In the reverse genetic study, we have tested the potential functional redundancy between *Methoprene-tolerant* (*Met*) and *germ cell-expressed* (*gce*), two paralog bHLH-PAS transcription factors in *Drosophila* that were suggested to be JH receptors. *Met* null mutants are viable, resistant to JH and low fecundity. No *gce* mutant was available at the begening of this project. We generated a *gce* null allele and found that it phenocopies *Met* mutants. *Met-gce* double mutants are lethal at prepupal stage, which is similar to the JH-deficient flies. *Krüppel homolog1* (*Kr-h1*)

and *broad* (*br*) are two known JH signaling componets. Further investigations revealed that *Met-gce* double mutant diminishes *Kr-h1* expression, induces precocious *br* expression, and causes premature and enhanced caspase-dependent programmed cell death. Therefore, we conclude that Met and Gce are functionally redundant in transducing JH signals.

Expression of *br* is induced by 20E, but its induction can be suppressed by JH. In the forward genetic study, we designed and conducted a novel genetic screen to isolate mutations that can de-repress *br* expression at early larval stages. From 4,400 lethal lines, 55 mutations were isolated based on the precocious *br* expression in 2nd instar larvae. Genes associated with these 55 mutations include *apterous*, *InR*, *NMAR1*, *Fpps* and *Kr-h1*, which are known to be involved in JH biosynthesis or signaling. Other genes encode proteins with various molecular functions, including enzymes, signal transduction molecules, and transcriptional factors. Among them, there are three Wnt signaling components, *Axin* (*Axn*), *supernumerary limbs* (*slmb*), and *naked cuticle* (*nkd*) and two TGF-β signaling components, *thick vein* (*tkv*) and *mothers against Dpp* (*mad*). We further demonstrated that Wnt signaling mediates JH signaling by regulating *Met* and *gce* expression, and that TGF-β signaling controls JH biosynthesis by upregulating transcription of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH biosynthesis.

JUVENILE HORMONE BIOSYNTHESIS AND SIGNALING PATHWAY IN DROSOPHILA MELANOGASTER

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

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Dedication

To my wife, Ola, my children Hossam, Sara, and the coming baby for their support and their patience during my pursuit of this endeavor.
To my parents and my sisters for their love and encouragement.

Acknowledgements

I would like to thank many persons who have helped me to improve myself as a scientist and a person for last five years at University of Maryland. It has been very exciting, adventurous, and productive times in my life and will last in my mind forever.
It has been a long road getting here, and to everyone who has helped me get through this. I will always be indebted to you all. There are no words to describe how thankful I am for you all to be by my side, through the good and the bad.

I am indebted to my supervisor, Dr. Jian Wang, for his guidance and support throughout my PhD studies. When my confidence was shaken during bouts of frustrating results, a brief consultation with him allowed me to regain my carriage, to try again and ultimately to succeed. I am especially appreciative of the opportunity to attend scientific meetings and speak with key scholars in our field. His mentorship afforded me valuable opportunities to explore and grow within the laboratory, and encouraged development of a self-motivated attitude that will no doubt serve me in the future.

I express sincere gratitude and thanks to the members of my dissertation committee, Drs. Raymond John St. Leger, Jonathan D. Dinman, David J. Hawthorne, Judd O. Nelson, for their comments and suggestions during committee meetings and for their generous help in editing my dissertation.

I would like to thank all past and present members of the Wang lab for their help and input. I am extremely grateful for Dr. Jianhua Huang's instruction in various molecular techniques that were instrumental to my ability to design and perform much of the work presented herein. Thanks to Dr. Jianhua Huang, Dr. Cheng Peng, Ola Zyaan, and Ying Wang who were involved in the JH biosynthesis and signaling projects.

I would like to thank Dr. Sheng Li and his lab members in the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China, for their collaboration.

I would also like to thank the entire Department of Entomology at the University of Maryland, professors, staff and students. This department is truly an exceptional place and I am fortunate to have been a part of it.

Finally and lovely, I would also like to thank my wife, Ola, for her support hand in hand in the lab and endless words of encouragement and motivation towards the goal. My parents and sisters for encouraging me on and letting me know that this higher level of learning was within my reach.

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Prepupal stage: from pupariation to head eversion
Early pupal stage: from head eversion to yellow eyes
Late pupal stage: from yellow eyes to eclosion
Adult stage: after eclosion.

Fig 4.8. CA-specific knockdown of *dpp*, *tkv*, *mad*, *Nmdar1*, or *jhamt* induces precocious *br* expression. *GAL4-Aug21* flies were crossed with *UAS-dpp RNAi*, *UAS-tkv RNAi*, *UAS-mad RNAi*, *UAS-Nmdar1 RNAi*, and *UAS-jhamt RNAi*. The FBs of their progeny were dissected at the 2nd instar larval stage and stained with Br-core antibody (red) and DAPI (blue).

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Fig 4.11. A model for the function of TGF-β signaling in controlling JH biosynthesis and insect metamorphosis. Proposed model as described in the text illustrating the function of TGF-β signaling in controlling JH biosynthesis and insect metamorphosis. The genes and proteins involved in this study are highlighted in red.

Chapter 5:

Fig 5.1. A genetic screen identifies that Axn, Slmb and Nkd regulate *br* expression. GFP images show the expression of *GAL4-PG12>UASmCD8GFP* in 2nd instar larvae. GFP was only expressed in the salivary gland of the wild type [A], but widely expressed in all tissues of *Axn* [B], *slmb* [C] and *nkd* [D] mutant larvae. (A'-D') White light images of the same organisms are shown in [A-D].

Fig 5.2. Precocious *br* expression in *Axn*, *slmb* and *nkd* mutants is not prevented by JHA. Wild type and the *Axn*, *slmb*, and *nkd* mutants were reared on normal (-JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Fat bodies of the 2nd instar larvae were stained with a Br-core antibody (red), and nuclei were labeled with DAPI (blue).

Fig 5.3. Expression of *Met*, *gce* and *Kr-h1* is reduced in the *Axn*, *slmb* and *nkd* mutants.

(A) Total RNAs were extracted from wild type, *Axn*, *slmb* and *nkd* 2nd instar larvae. The mRNA levels of *Met*, *gce* and *Kr-h1* were assessed by quantitative real-time PCR and normalized to *rp49* mRNA. Values shown are the means of 4 independent experiments ± standard deviations.

(B) The same total RNAs described in [A] were used as the templates for a 30-cycle reverse transcriptional PCR. The RT-PCR products were analyzed by DNA agarose gel electrophoresis.

Fig. 5.4. Gain-of-function *arm* mutation induces precocious *br* expression. (A-C) Fat bodies of 2nd instar larvae were stained with a Br-core antibody (red) and DAPI (blue).

Fig. 5.5. Gain-of-function *arm* mutation suppresses *Met*, *gce* and *Kr-h1*expression Total RNA was extracted from the 2nd instar larvae. The mRNA levels of *Met*, *gce* and *Kr-h1* were assessed by qRT-PCR and normalized to *rp49* mRNA. Values shown are the means of 4 independent experiments ± standard deviations. Genotypes include: wild type; *arm-GAL4/UAS-arm*^{S10} and *arm-GAL4/UAS-arm*^{S10}*,UAS-gce/+*.

Fig. 5.6. As described in the text, the proposed model illustrates the cross-talk between the Wnt and JH signaling pathways.

List of Abreviation

Abbreviation	Full form
20E	20-hydroxyecdyson
Ap	*Apterous*
AST	Allatostatin
AT	Allatotropin
Axn	*Axin*
bp	base pairs of DNA
br	*broad*
BR-C	Broad complex
BTB	Broad-tramtrack-Bric-a- Brac
CA	Corpora allata
cDNA	complementary DNA
CyO	Curly of Oster
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dscam	*Drosophila down syndrom cell adhesion molecule* gene
dsRNA	Double-stranded RNA
dsRNA	Double strand RNA
E75A	nuclear receptor encoded by the *E75* early ecdysone-inducible gene
FB	Fat body
FISC	Ftz-F1 interacting steroid-receptor coactivator
FLP	Is a gene under the control of hsp70 produces Flpase which is recombinase catalyzes recombination between FRT sites
Fpps	Farnesyl diphosphate synthase
FRT	Flippase Recognition Target
Gce	*Germ cell-expressed*
GFP	Green fluorescent protein
GOF	Gain of function
hs	Heatshock promoter
Hsp70	Heat shock promoter no. 70
InR	*Insulin receptor*
JH	Juvenile hormone
JHA	Juvenile hormone analogue
JHAD	Juvenile hormone acid diol
Jhamt	*Jh acid methyltransferase*
JHBP	Juvenile hormone binding protein
JHE	Juvenile hormone esterase

JHEH	Juvenile hormone epoxide hydrolase
JHM	Juvenile hormone mimics.
Kr-h1	*Kruppel homolog1*
Mad	*Mothers against Dpp*
MARCM	Mosaic analysis with a repressible cell marker
mCD8	Mouse cluster of differentiation 8
Met	*Methoprene-tolerant*
NGS	Normal goat serum
Nkd	*Naked cuticle*
NMDAR	N-methyl-d-aspartate subtype of glutamate receptors
OR	Oregon R
PBS	Phosphate buffered saline
PBT	PBS + 1% Triton X-100
PCD	Programmed cell death
PCR	polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
RNAi	RNA interference
SAM	S-adenosyle-l-methionine
Slmb	*Supernumerary limbs*
Tai	Taiman
TGFβ	Transforming growth factor ***beta***
Tkv	*Thick vein*
UAS	Upstream activating sequence
Vcp	Vitellogenic carboxypeptidase
Vg	Vitellogenins
w	*white*

Chapter 1

Introduction

Juvenile Hormone Biosyntheses and Signaling Pathways

Insect development is controlled by two major hormones; the steroid 20-hydroxyecdysone (20E or Ec) and the sesquiterpenoid juvenile hormone (JH). The later was discovered by Wigglesworth (1936) as an anti-metamorphic humoral factor in the blood sucking bug, *Rhodnius prolixus*. Further studies reveal that JH has many vital functions in controlling insect development, reproduction and behavior. For example, JH is required for the maintenance of cuticular identity during larval molts, polyphenisms of aphids and locusts, caste determination in social insects, control of behavior in honeybee colonies, larval and adult diapause regulation, ovarian development, vitellogenesis, and maintenance of morphostasis (cell division without differentiation) in imaginal discs of holometabolous insects during the larval intermolt growth period (Nijhout, 1994; Truman et al., 2006). Here, we summarize the current understanding of chemical structures, physiological functions, biosynthesis, and signaling pathways of JH.

1.1. Chemical Structures of JH

The chemical structure of JH was first identified by Röller *et al.* (1967) showing that it is formed of noncyclic sesquiterpenoids structure carrying an epoxide group near one end and a methyl ester on the other. Over the years JH was found to have

different forms such as JH0, JH I, JH II, JH III, and JH III bisepoxide (JHB_3) (Fig. 1.1) (reviewed in Nijhout, 1994). So far, JH has been identified in about 100 different insect species representing ten insect orders with JH III being the predominant form (Baker, 1990). JH 0, I, II, and III have been isolated from different members of order Lepidoptera while JH 0 was isolated only from lepidopteran embryos (Bergot et al., 1980; Bergot et al., 1981; Schooley et al., 1984). JH bisepoxide (JHB3) was first reported as an unknown juvenoid biosynthesized *in vitro* by the adult corpora allata (CA) of the female black blow fly, *Phormia regina* (Liu et al., 1988), and its structure is similar to JH III except for the 6, 7-epoxy group with the probability that it could be an oxidation product of JH III.

An *in vitro* study revealed that 95% of juvenoids produced by the isolated ring glands from third instar of *Drosophila melanogaster* are JHB_3, while JH III is only a minor product (Richards *et al.*, 1989). In addition, the isolated ring glands from other cyclorrhaphous dipteran larvae also produce JHB_3 almost exclusively. However, corpora allata from mosquito larvae produce only JH III, signifying that JHB_3 production may be restricted to the higher Diptera. The suggestion that JHB_3 is a fly juvenile hormone was supported when the synthetic JHB_3 was topically applied to the newly formed *D. melanogaster* white puparia and caused developmental responses similar to those obtained with JH Ill (Richards *et al.*, 1989).

1.2. Physiological functions of JH

1.2.1. Function of JH in Metamorphosis

JH has conserved functions in hemimetabolous and holometabolous insects where it keeps the ‘status quo’ moults in these two groups of insects and maintains the former

morphology in the new resulted instars in addition to its ability to prevent differentiation without interfering with growth (Riddiford, 1994). By applying JH analogs to the final nymphal instar of hemimetabolous insects, both supernumerary nymphal molts and nonviable "monstrous" larval/adult mosaics may result (Nijhout, 1994). As first shown by Wigglesworth (1934), when the last stage *Rhodnius* nymphs is exposed to active corpora allata, a supernumerary molting occurs which ended by 6^{th} nymph instar instead of adult.

The phenomenon also occurs in the holometabolous insects. For example, when methoprene is topically applied at the beginning of the eighth (final) *Tribolium* larval instar, it results in supernumerary larvae. After repeated JH treatment, the supernumerary larvae can reach their eleventh instar (i.e., they completed 10 postembryonic ecdyses, rather than 7 ecdyses in wild type) (Konopova and Jindra 2007). Moreover, when pupae of *Tenebrio molitor* are exposed to JH analogs, these pupae produce supernumerary (second) pupae instead of adults (Socha and Sehnal 1972). Application of high doses of juvenile hormone (JH) mimics to mature lepidopteran larvae also induces prolongation of last instar larval duration of *Bombyx mori* (Akai and Kobayashi 1971) or the production of super larvae of *Ephestia kühniella* (Hong 1975). However, in higher dipterans such as *Drosophila*, exogenous JH does not cause supernumerary larval molting, even when they are fed with JH continuously throughout larval life, but causes the formation of a pharate adult with a pupa-like abdomen (Riddiford and Ashburner, 1991). In contrast, reduction of JH in the early immature stages of insects usually causes precocious metamorphosis. If the corpora allata are removed (allatectomy) from a third-instar *Rhodnius* nymph, the

next molt successively turns the nymph into a precocious adult (smaller than normal adult) (Wigglesworth, 1936). In the tobacco hornworm, *Manduca sexta*, as in other Lepidoptera, allatectomy of the penultimate instar larva causes precocious pupation (Kiguchi and Riddiford 1978).

1.2.2. Functions of JH in Adults

In addition to the roles of JH in the immature stages, JH plays essential roles in adult reproductive biology. In the females of different insect species, JH plays distinct roles in controlling oocyte development. Studies in lower Diptera such as *Aedes aegypti* revealed that JH triggers the vitillogenesis. Synthesis of vitellogenins (Vg), the yolk protein precursors, in the fat body is induced by 20E, while JH III prepares newly emerged mosquitoes to become competent to respond to the 20E induction (Clements, 1992). On the contrary, when the gypsy moth, *Lymantria dyspar*, are treated with JH analogue (JHA), the females show retardation in vitellogenesis process (Davis *et al.,* 1990). In *Drosophila melanogaster* females, loss of JH does not affect Vg production, but the deposition of Vg into the oocytes is blocked (Bownes, 1989; Gavin and Williamson, 1976).

In males, JH controls male accessory gland functions and courtship. JH exists in the *Hyalophora cecropia* accessory gland in high amount, suggesting a role in male reproductive biology (Williams, 1956). It was reported that JH can induce protein synthesis in *Drosophila melanogaster* male accessory gland (Yamamoto et al., 1988) and the *ap* mutant males, which have JH biosynthesis deficiency, showed less courtship activity with the females than the wild type males (Tompkins, 1990).

Moreover, *Drosophila* males mutant for *Met* (the potential JH receptor) exhibited low protein accumulation in male accessory glands and low mating behavior (Wilson, et al., 2003).

1.2.3. Other Functions of JH

In the tobacco hornworm, *Manduca sexta*, corpora allata secrete the inactive metabolite of JH (JH acid) early in metamorphosis and the imaginal discs then locally convert the JH acid to JH (active form) using methyl transferase to prevent them from undergoing precocious adult differentiation during the larval-pupal transition (Sparagana et al., 1985). The local production of JH-catabolic enzyme (JH esterase) was activated in the wing discs of the lepidopteran *Galleria mellonella* to reduce the JH levels in this particular tissue early in the final larval instar, prior to the enzyme appearing in the hemolymph which allowed the imaginal tissues to escape from the JH suppressive action (Reddy et al., 1980).

JH was found to have an important role in insect polymorphisms. JH has a role in morph determination of *Aphis fabae* for photoperiod-mediated wing polymorphism, where the application of JH at the critical phase sensitive to photoperiod resulted in change in the development from winged to the flightless form (Hardie and Lees 1985).

JH has many roles in cast determination in social insects. In honey bees, queen larvae which were feed on high amount of royal jelly have high rates of JH synthesis by the CA, while the worker larvae which were feed on mixed diet of royal jelly, pollen and nectar have low rates of JH synthesis and the food quality and quantity signals are believed to be transmitted via the stomatogastric nervous system of honey bee larvae

(Boleli et al., 1998).The mode of action of JH in honey bee caste differentiation is highly pleiotropic where JH affects cell proliferation in the developing ovaries during a critical phase in the last larval instar, and reduces induction of somatic cells in the developing female gonad (Schmidt and Hartfelder 1998).

Insect diapause, which is a physiological rather than a behavioral response to unfavorable environmental conditions, is controlled by JH in both larval and adult stages. In some cases the pupal molt was prevented by high titers of JH accompanied by low ecdysteroid titers. However, low titers of JH in adults prevent reproduction and may induce searching behavior for suitable overwintering sites. Low JH titers during diapause are maintained either by repression of CA activity or by elevated levels of JH esterase (Denlinger 1985).

1.3. JH Biosynthesis and Degradation

The titer of JH is controlled by the relative rates of JH biosynthesis and degradation. JH is synthesized in a special pair of glands, the corpora allata (CA) (Tobe and Stay, 1985; Gilbert et al., 2000).

1.3.1. Biochemical Pathways of JH Biosynthesis

Biochemical process of JH biosynthesis consists of two pathways, mevalonate pathway and JH biosynthesis pathway (Fig. 1.2). The mevalonate pathway is a conserved metabolic route based on reductive polymerization of acetyl-CoA and results in isoprenoid compounds (Belles, et al., 2005). The latter are used as precursors by different organisms to produce distinct final products, such as

cytokinins and phytoalexins in plants, steroid hormones in mammals (Cane, 1999), and defensive secretions, pheromones, and JH in insects (Seybold and Tittiger, 2003). The insect mevalonate pathway has two important peculiarities: the absence of the sterol branch and the synthesis of juvenile hormone (JH) (Clark and Bloch 1959; Schooley and Baker, 1985). Isopentenyl diphosphate produced by the mevalonate pathway is further modified to JH by JH biosynthesis pathway (Fig. 1.2). Many enzymes involved in JH biosynthesis pathway, such as Farnesyl diphosphate synthase (FPPS) and JH acid methyltransferase (JHAMT), are well characterized in *Drosophila* and other insects.

In insects, farnesyl pyrophosphate is converted to juvenile hormone (JH) via a conserved pathway consisting of isoprenoid derived metabolites (Manuela 1999). FPPS catalyzes the first step of this pathway by the condensation of dimethylallyl diphosphate with two molecules of isopentenyl diphosphate, producing farnesyl diphosphate (FPP) (Yong lei Zhang 2008). To date, FPPS has been characterized in many insect species, including *Agrotis ipsilon* (Castillo-Gracia and Couillaud, 1999); *Choristoneura fumiferana* and *Pseudaletia unipuncta* (Cusson et al., 2006); *Bombyx mori* (Kinjoh et al., 2007); *Drosophila melanogaster* (Sen et al., 2008); and *Anthonomus grandis* (Taban et al., 2009).

JHAMT is an enzyme that converts JH acids or inactive precursors of JHs to active JHs at the final step of JH biosynthesis pathway in insects. It transfers a methyl group from S-adenosyl-l-methionine (SAM) to the carboxyl group of JH acids to produce

JHs in the corpora allata (Shinoda and Itoyama, 2003). Studies reveal that JHAMT is predominantly expressed in corpora allata and its developmental expression profile correlates with changes in the JH titer, indicating that it is a key regulatory enzyme for JH biosynthesis (Niwa et al., 2008; Sheng et al., 2008).

1.3.2. Regulation of JH Biosynthesis

JH biosynthesis is regulated at three closely linked steps. In the first step, developmental, environmental and physiological cues are received by the central nervous system, which somehow determines the appropriate rate of JH synthesis (Riddiford et al., 1993). In the second step, the brain transfers these signals into the JH biosynthesis in an endocrine gland, corpus allatum (CA). It has long been thought that JH biosynthesis is regulated primarily by two neuropeptides secreted by brain neurosecretory cells: allatotropin (AT) and allatostatin (AST) that stimulates or inhibits JH synthesis, respectively (Stay, 2000; Weaver and Audsley, 2009). In the final step of JH biosynthesis regulation, the brain signals received in the CA should be translated into changes of the expression and/or activity of key regulatory JH biosynthetic enzymes, which directly determine the rate of JH biosynthesis.

In different insect species and at different stages of development, three allatostatin families (A-, B-, and C-type allatostatins), and two structurally unrelated allatotropins have been characterized (Weaver and Audsley, 2009). A-type ASTs were first identified from the viviparous cockroach species *Diploptera punctata* (Pratt et al., 1989; Woodhead et al., 1989), and then discovered in widely across other insect

orders, including Diptera, Lepidoptera and Orthoptera. All A-type allatostatins share the same C-terminal motif (Y/F)XFG(L/I)-NH2, which also forms the core active region of the peptide. Usually, one insect species has 4-14 A-type allatostatins which are encoded in a single neuropeptide precursor. B-type ASTs, also termed the W(X)6Wamide ASTs, were first isolated in crickets. They are C-terminally amidated peptides with tryptophan in the second and ninth positions. The *Manduca sexta* allatostatin (Manse-AS) is a representative of C-type allatostatins. It is a single 15-amino-acid peptide with the nonamidated C-terminal pentapeptide P-I-S-C-F. Orthologues of this peptide have also been deduced from genomic sequence data of the fruit fly, mosquito, and several lepidopteran species (Stay and Tobe, 2007).

The first AT to be structurally characterized is a 13-amino acid amidated peptide, which stimulates JH synthesis in isolated CC-CA of Lepidoptera. Identical or similar peptides have since been identified in other orders, including Diptera, Orthoptera, and Coleoptera. However, thus far, no AT-like neuropeptides and AT receptor genes have been found in the *Drosophila* genome (Nassel 2002; Hauser et al., 2006; Liu et al., 2006; Yamanaka et al., 2008).

Several other regulation mechanisms independent to AT and AST have been found to control JH biosynthesis. First, it is reported that brain may directly control JH biosynthesis through neurotransmitters. For example, studies in cockroach and *Drosophila* revealed that glutamatergic nerves innervate the CA cells and *N*-methyl-d-aspartate subtype of glutamate receptors (NMDAR) are expressed in both brain and CA. Additionally, glutamate and NMDA were demonstrated to stimulate JH synthesis *in vitro* (Chiang et al., 2002). Second, genetic analysis observed that *Drosophila*

insulin signaling genes, such as *Insulin receptor* (*InR*) and *chico*, are required for normal JH biosynthesis.

InR mutants are slow to develop, small, infertile, and long-lived. Additionally, they have reduced JH synthesis in the young adults, and that normal longevity and vitellogenesis are restored by topical application of a JH analog (Tatar et al., 2001). Another studies in *D. melanogaster* revealed that *chico* homozygous mutant genotypes reduced JH synthesis by 67% of the wild type and without influencing the ratio of JH subtypes. (Tu et al., 2005).

1.3.3. JH binding proteins

After the active form is released into the hemolymph, a specific protein, Juvenile hormone binding protein (JHBP), binds to JH. JHBP functions to keep JH in solution in the hemolymph, prevents non-tissue-specific uptake and degradation of JH, and assists in the interaction between JH and JH specific degradation enzymes (Goodman et al., 1990; Trowell, 1992). JHBPs are different among insect orders. In Lepidoptera this protein has low molecular weight and binds JH I and II with high affinity (Whitmore and Gilbert, 1972; Dillwith et al., 1985; Lerro and Prestwich, 1990). In Blattodea, Isoptera, Hymenoptera, Diptera and Coleoptera, a high molecular weight protein functions as JHBP with high binding activities for JH III (de Bruijn *et al.*, 1986; de Kort *et al.*, 1987, de Kort and Koopmanschap, 1987; King and Tobe, 1992; Sevala *et al.*, 1997). A very large hexameric protein with 6 binding sites with high affinity for JH III serves as the JHBP in Orthoptera (Koopmanschap and de Kort, 1988; Braun and Wyatt, 1996).

1.3.4. JH Degradation

JH degradation is attributed to juvenile hormone esterase (JHE) and to juvenile hormone epoxide hydrolase (JHEH) in the hemolymph and tissues (DeKort and Granger, 1996). JHE proteins have been isolated and purified from *D. melanogaster* (Campbell et al., 1992). JHE hydrolyzes the ester of JH to produce JH acid. JHEH hydrolyzes the epoxide of JH to produce JH diol (JHD), but JHEH only functions in cells (Halarnkar et al., 1993). The cumulative activities of the two enzymes convert JH to the juvenile hormone acid diol (JHAD) for which no function has been discovered. Most JH is bound to JHBP and hence JH is protected from degradation by non-specific esterases with low binding affinities (Touhara et al., 1993; Touhara and Prestwich, 1993; Touhara et al., 1995). JHE is the only enzyme in the hemolymph that has a high affinity for JH, and hence is the only hemolymph esterase considered important in JH degradation (Gilbert et al., 2000).

1.4. JH Signaling Pathways

At the physiological level, Ec induces molting, whereas the "status quo" hormone JH titer determines the nature of the molt. At a high JH titer, JH antagonizes the 20E-induced physiological and developmental events to assure larval molting and to prevent larval-pupal-adult metamorphosis (Riddiford, 1994; Buszczak and Segraves, 2000; Gilbert et al., 2000; Thummel, 2001; Dubrovsky, 2005; Truman and Riddiford, 2007; Riddiford, 2008). At a low JH titer, 20E promotes larva-pupa or pupa-adult metamorphosis (Gilbert et al., 2000; Riddiford et al., 2003). During the last two decades, dramatic progress has been made towards understanding the molecular

mechanisms underlying 20E signaling (Thummel, 2002). In contrast, relatively little is known about the molecular action of JH.

1.4.1 Met and Gce as the potential JH receptors

Methoprene-tolerant (*Met*) gene, which is also known as *Resistance to Juvenile Hormone* (*Rst(1)JH*), was discovered by Wilson and Fabian (1986) while screening mutagenized *Drosophila* for resistance to methoprene, a JH analogue used as an insecticide. The *Met* encodes a bHLH-PAS protein family member (Wilson and Ashok, 1998). Several different studies propose MET as a component of the elusive JH receptor. For example, *Met* null mutants are greater than 10-fold more resistant to methoprene (Wilson and Fabian, 1986). Recombinant Met prepared in an *in vitro* transcription-translation system binds JH III with high affinity (Miura et al., 2005). In *Tribolium*, suppression of *Met* expression by injection of double-stranded (ds) *Met* RNA causes precocious metamorphosis (Konopova and Jindra, 2007), further suggesting its involvement in JH signaling.

However, null *Met* mutants of *Drosophila* are completely viable, which is not what one would expect if Met is a JH receptor. One reasonable explanation is that redundancy may exist between *Met* and another *Drosphila* bHLH-PAS gene, *germ cell-expressed* (*gce*), which has more than 50% homology to *Met* (Moore et al. 2000; Godlewski et al., 2006). Met can either form homodimers or can heterodimerize with Gce *in vitro* and this dimerization can be reduced by JH (Fig. 1.3) (Godlewski et al., 2006). Interestingly, there is only one *Met* homologous gene in mosquitoes and beetles, which is more similar to the fruit fly *gce* than to *Met* (Wang et al., 2007; Konopova and Jindra, 2007).

Flies receiving *gce* RNAi in a *Met* null background die during the pupal-adult transition (Liu et al., 2009; Baumann et al., 2010). Overexpression of *Met* causes precocious and enhanced PCD in larval tissues, resulting in high mortality during larval life (Barry et al., 2008; Liu et al., 2009). Meanwhile, overexpressed *gce* can substitute for *Met* function (Baumann et al., 2010). During the larval-pupal transition, Met/Gce mediates JH action to prevent 20E-triggered programmed cell death (PCD) of larval tissues (Liu et al., 2009) and cell differentiation of adult tissues (Riddiford et al., 2010). Many groups have suggested that when a *gce* mutant becomes available, its phenotype could help evaluate the functions of *Met* and *gce* in insects.

1.4.2. Broad and JH-20E cross-talk

There is a cross-talk between JH and Ec signaling pathways through the insect life cycle. During larva-to-larva molts, the simultaneous presence of 20E and JH leads to rapid accumulation of some early 20E-inducible proteins, such as E74 and E75. These proteins perform two functions. They facilitate activation of secondary 20E-inducible genes which induce larva molting process and repress their own expression. However, at the end of larval development, 20E in the absence of JH activates a large group of early response genes (BR-C, E74 and E75), thus initiating the onset of metamorphosis.

After 20E activates the expression of BR-C, JH is no longer able to prevent pupation. Finally the differentiation of an adult occurs in the presence of high 20E titer, in the absence of JH and of BR-C activity, suggesting that two transcription factors, BR-C and E75A, contribute to the cross-talk between the two hormones. It appears that BR-C is a key target of JH status quo action, and E75A is a part of the mechanism

whereby JH prevents BR-C activation (Dubrovsky, 2005). On the other hand, although some progress regarding JH signal transduction has been made recently, the detailed molecular mechanism of JH action remains speculative because of the JH receptor (JHR) has not been identified yet in *Drosophila* or any other insect species.

The recent progress in our understanding of JH molecular action identified only three well investigated JH signaling genes, *broad* (*br*), *Methoprene-tolerant* (*Met*), and *Krüppel-homolog 1* (*Kr-h1*). Briefly, Br is a critical molecule at the cross talk between Ec and JH signaling (Fig. 1.4) (Zhou and Riddiford, 2002), *Met* gene encodes a bHLH-PAS protein family member, which is suggested to be a component of the elusive JH receptor (Ashok et al., 1998), while *Kr-h1* is a primary JH response gene that acts downstream of *Met* in repressing *br* expression (Minakuchi et al., 2008, 2009).

The *Broad* gene and its responses to JH actions:

Br is an early Ec-inducible gene, previously known as *Broad-Complex* (*Br-C*), and it is necessary for the onset of insect metamorphosis and specification of pupal development (Karim et al., 1993; Bayer et al., 1996; Zhou and Riddiford, 2002). *Drosophila br* encodes four transcriptional factors that contain a common N-terminal domain and four pairs of different DNA-binding zinc finger domains that belong to the Broad-Tramtrack-Bric-a-Brac (BTB) family (DiBello et al., 1991; Bayer et al., 1996). *Broad* null mutants can develop into 3rd instar and die before pupal formation (Kiss et al., 1976, 1988). Ectopic expression of *br* in the early second instar larvae of *Drosophila* induces premature pupal formation (Zhou et al., 2004). Therefore, *br* is necessary and sufficient for the initiation of insect metamorphosis. For this reason, *br*

is thought of as the JH-dependent "pupa identifier" to specify pupal development and to mediate the 'status quo' action of JH (Zhou and Riddiford, 2002; Dubrovsky, 2005).

Consistent with its function, Br is predominantly expressed during the larval-pupal transition in all holometabolous insects examined (Dubrovsky, 2005). Previous studies in *Manduca*, *Bombyx*, and *Tribolium* suggested that the temporal pattern of *br* expression is the result of 20E and JH interaction. 20E directly induces *br* expression, but this induction can be prevented by JH in young larvae. Therefore, expression of *br* is specifically restricted to the larval-pupal transition when 20E is high but JH is low or absent (Fig. 1.5) (Zhou et al., 1998; Reza et al., 2004; Konopova and Jindra, 2008). However, unlike in moths and beetles, JH cannot prevent the larval-pupal transition or induce extra larval instars in the fly (Riddiford and Ashburner, 1991; Restifo and Wilson, 1998).

1.4.3.- *Kruppel homolog1* and its functions in mediating JH signaling:

The *Drosophila Kruppel homolog1* (Kr-h1), a C_2H_2-type zinc finger transcription factor, is a 20E-response gene (Pecasse et al., 2000; Beck et al., 2004). *Kr-h1* mutants die at the prepupal-pupal transition (Beck et al., 2004). In both *Drosophila* and *Tribolium*, *Kruppel-homolog1* (*Kr-h1*) mRNA levels are high during embryonic stage and continuously expressed in the larvae, and then disappears during pupal and adult development (Pecasse et al., 2000; Minakuchi et al., 2009). In a microarray analysis when JH was topically applied to newly emerged mosquitoes, *Aedes aegypti*, (before the normal endogenous increase in JH after eclosion), transcriptional changes were detected in a group of JH target genes including *Kr-h1* (Zhu, et al., 2010).

Kr-h1 expression can be induced in the abdominal integument by JH application at pupariation (Minakuchi et al., 2008). Suppression of *Kr-h1* by dsRNA in the early larval instars of *Tribolium* causes precocious *br* expression and premature metamorphosis after one succeeding instar. Thus, KR-H1 is necessary for JH to maintain the larval state during a molt through suppressing *br* expression (Fig. 1.6) (Minakuchi et al., 2009).

Importantly, JH can induce *Kr-h1* expression as well and Kr-h1 lies upstream of Br-C (Minakuchi et al., 2008). Although sufficient evidence is lacking in *Drosophila*, in both *Tribolium* (Minakuchi et al., 2009) and the mosquito, *Aedes aegypti* (Zhu et al., 2010), *Met* is required for JH action to induce *Kr-h1* expression. It is likely that *Kr-h1* is an early JH-response gene that mediates JH action by linking *Met*/*gce* and *Br-C* (Minakuchi et al., 2009). Another study in hemimetabolous insects was conducted to test the expression profiles of *Kr-h1* in two thrips species, the western flower thrips and a predatory thrips belonging to order Thysanoptera. The data showed that *Kr-h1* expression profiles in post-embryonic development were comparable to those in holometabolous insects. In addition, when they consistently applied exogenous JHM in distinct developmental stages, a lethality in the propupal stages (non-feeding stages called propupa and pupa between the larval and adult stages), prolonged expression of *Kr-h1* was quantified when the newly molted propupae treated with exogenous JHM suggesting that that *Kr-h1* could be involved in JH actions in thrips' metamorphosis (Minakuchi et al., 2011).

1.4.4.- Taiman is required for both JH and 20E signaling pathways:

Taiman (*tai*) is a ligand-dependent nuclear receptor (HLH-PAS family) that encodes a steroid hormone receptor coactivator which activates transcription in conjunction with a ligand-dependent nuclear receptor from a RNA polymerase II promoter rather than binding DNA directly. Ecdysone signaling depends on the tissue type, the developmental stage, and the EcR/USP complexes with Taiman co-activator. In the *Drosophila* ovary, mutations in *tai* caused defects in the migration of specific follicle cells, the border cells, and the mutant cells exhibited abnormal accumulation of E-cadherin, beta-catenin, and focal adhesion kinase. TAI protein colocalized with the ecdysone receptor in vivo and augmented transcriptional activation by the ecdysone receptor in cultured cells, introducing a new function for steroid hormones and the requirement of this type of coactivator (TAI) for cell motility and in stimulating invasive cell behavior, independent of effects on proliferation (Bai et al., 2000)

Zhu and his colleagues (2006) identified mosquito protein FISC (β **F**tz-F1 **i**nteracting **s**teroid-receptor **c**oactivator). FISC, the mosquitoes ortholog of *DmTaiman*, was found to functionally link to β*Ftz-F1* during transcriptional activation of *E74B*, *E75A*, *Vg* (*vitellogenin*), and *VCP* (*vitellogenic carboxypeptidase* in the stage-specific 20E response. They concluded that, in the mosquito fat body, βFtz-F1 defined the 20E response after a blood meal by enhancing the recruitment of FISC to the EcR/USP complex at the regulatory sites of their target genes and this is achieved through protein-protein interaction with FISC.

FISC was found to bind to Met *in vivo* in a JH-dependent manner and to act as a functional partner of Met in mediating JH-induced gene expression, such as *AaET*

and *AaKr-h1*, indicating that, Met-FISC complex constitutes a key step in signal transduction of juvenile hormone (Li et al., 2011)

1.5. JH Analogs as Insecticides:

Williams (1967) first suggested that JH analogs (JHAs) might be used as insecticides to disturb insect growth and development. Since then, many of JH analogs and JH agonists have been commercialized as insecticides. For example, methoprene is a JH analog which has been used for decades as an insecticide (Staal 1975, Retnakaran 1985). It is essentially nontoxic to humans and vertebrates when ingested or inhaled. Therefore, it is approved by the WHO for use in drinking water cisterns to control mosquito larvae and is used in the production of a number of foods including meat, milk, mushrooms, peanuts, rice and cereals. It also has several uses on domestic animals (pets) for controlling fleas.

Juvenile hormone mimics are the main components of the third generation of insecticides which are called Insect Growth Regulators (IGRs). JH mimics can be classified into two classes of hormone-based IGRs include: juvenoids (JH mimics, JH agonists and antagonists and JH analogs) and anti-JH agents. Larvae are not able to complete the final molt into adult resulting in death when JH synthesis is completely inhibited by using anti-JHs such as precocenes which inhibit JH biosynthesis by destroying cells of the CA (Bowers et al., 1976). In contrast, continuous application of JH analogs cause the larvae to molt into abnormal and reproductively-incompetent adults (Bowers, et al., 1980). Several JH analogs have been discovered and synthesized and are commercially available as insecticides against household pests and agricultural pests. These include methoprene (Henrick et al., 1973), fenoxycarb

(Dorn et al., 1981, Masner et al., 1981), diofenolan (Sechser et al., 1994), and pyriproxyfen (Hatakoshi et al., 1986, Kawada et al., 1989)

Although JH mimics are specific and effective in disrupting both insect embryonic development and metamorphosis (Riddiford and Williams 1967, Staal 1975, Dhadialla et al., 2005), unfortunately they also negatively affect caste differentiation in social insects and some are toxic to beneficial aquatic predator insects such as the dragonfly and the back swimmer (Dhadialla 1998), and toxic to crustaceans such as shrimp, crabs and lobsters (Tuberty 2005, Walker 2005). In general, the JHAs have low acute toxicity to fish, birds, mammals, and human. For example, the acute 50% lethal dose (LD_{50}) for fenoxycarb in rat is >10 g/kg (oral), >2 g/kg (dermal), and >480 mg/m^3 for 4-h exposure (inhalation) (Grenier and Grenier 1993).

More recently, persistent efforts by the agrochemical industry have led to the discovery of several new and much more chemically diverse insecticidal agents with JH or ecdysteroid modes of action. Such compounds have much greater metabolic and environmental stability than earlier analogs and are better suited to agriculture. In addition, some display remarkable target pest selectivity and some of them are among the most environmentally-friendly types of insecticides (Retnakaran et al., 1985)

1.6. Directions and research goals

Since the discovery of the *Met* gene by Wilson and Fabian (1986), it has been confirmed that *Met* mutant flies are resistant to JHA, and have reduced fecundity, e.g. delayed egg laying by females and other phenotypes. However, the important point here is that homozygous mutant flies for *Met* are viable. This contradicts the expectation that a JH receptor such as Met should be lethal due to the importance of JH signaling in immature stages as well as in the adult stages. To explain this apparent contradiction, it was hypothesized that another functionally redundant gene exists, which may function simultaneously with *Met* downstream of JH actions. The discovery by Moore and colleagues (2000) identified the *Dm-gce* gene which encodes bHLH PAS proteins and named it according to its expression in a subset of embryonic germ cells. In this study we performed a bioinformatics analyses to align *Met* and *gce Drosophila* amino acids, and found an overall identity and similarity 50% and 61% respectively, and 68 -86% identities and 86-88% similarities in the conserved domains bHLH, PAS-A, and PAS-B. A *gce* null allele was thus required to test the redundancy hypothesis between *Met* and *gce* in order to further ourunderstanding of JH signaling. In parallel, there are few known genes which are recognized to be involved in either the cross-talk between Ec and JH signalings or in JH signaling alone. Thus, in order to identify more genes, a genetic screening method had to be undertaken to achieve this goal. The present body of work aims to answer questions regarding the JH receptor candidates and identify new genes participating in the JH synthesis or signaling pathway.

The two primary approches of this dissertation research are:

1- Reverse genetics approaches applied to candidate genes such as *br*, *Kr-h1*, and *Met*, and generation of a *gce* null allele.

2- Forward genetics approaches effected by conducting a saturation genetic screen in order to identify genes involved in JH synthesis or signaling based on our previous knowledge of the key genes such as *br*, which plays important roles in the cross-talk between Ec and JH signaling pathways.

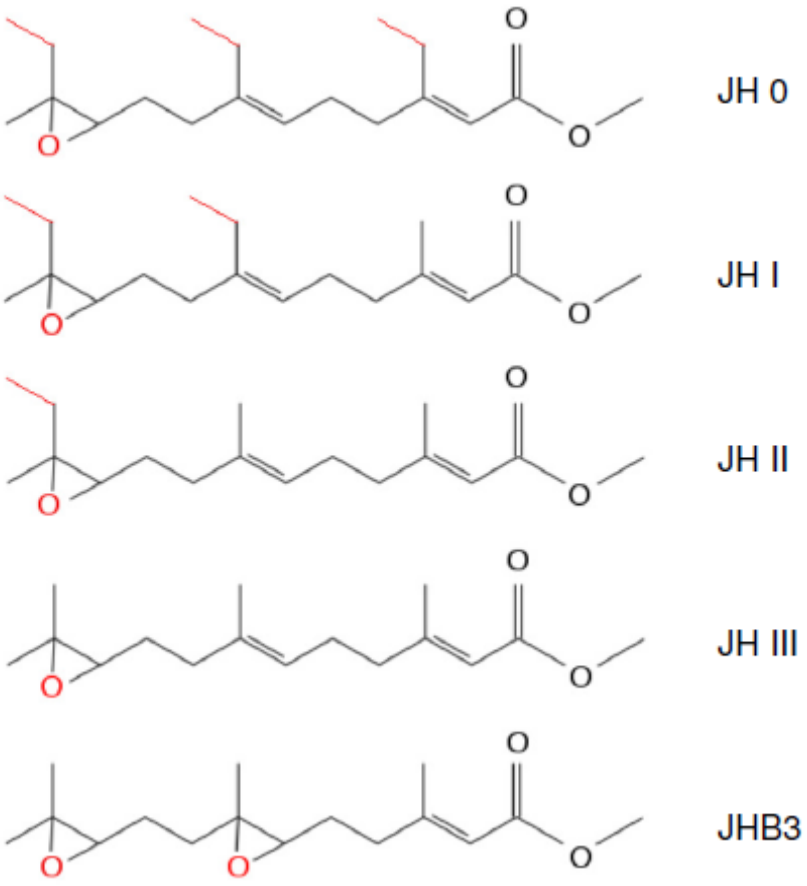

Figure 1.1: Chemical structure of Juvenile Hormone homologues; JH 0, JH I, JH II, JH III, JHB3 (JH III bisepoxide). Figure reproduced from Nijhout (1994)

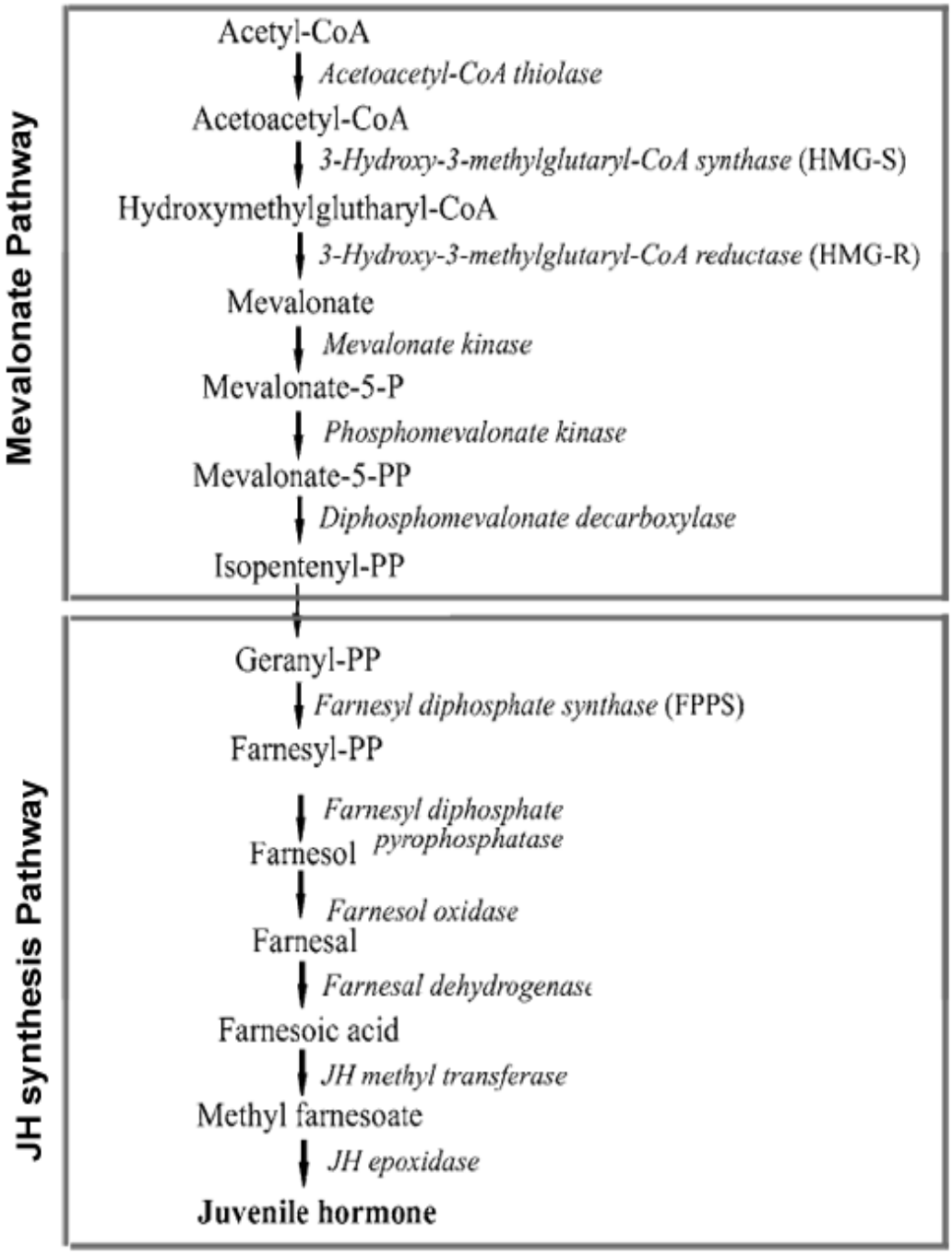

Figure 1.2: Model of JH Biosynthesis pathways through the mevalonate pathway starting from acetyle-CoA in insects (Modified from Bellés et al, 2005)

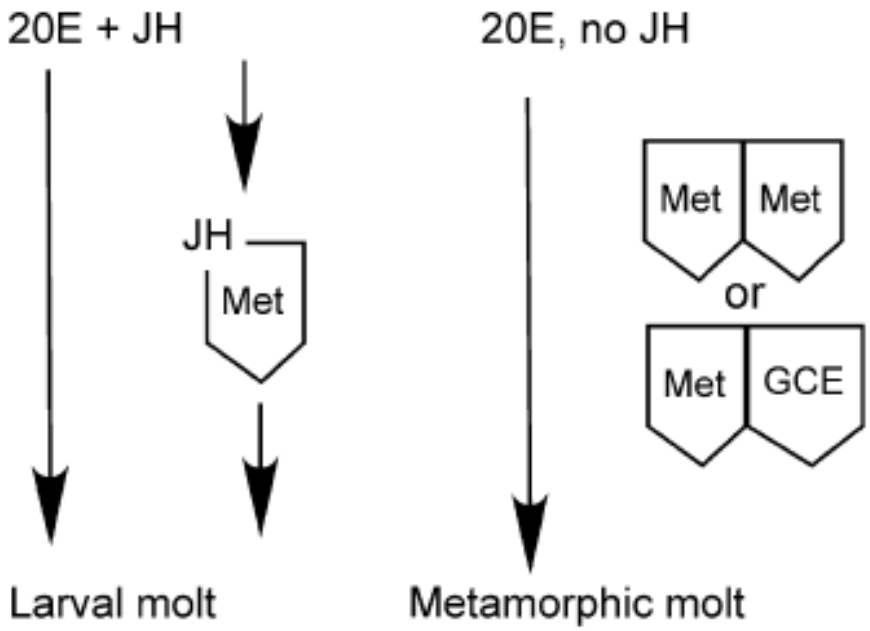

Figure 1.3: Model for Methoprene-tolerant (Met) as the JH receptor based primarily on Godlewski et al. (2006) and Miura et al. (2005). When JH is present, Met binds JH and remains as a monomer and regulates larval genes. When JH is absent, Met either homodimerizes or heterodimerizes with germ cells-expressed (gce) and metamorphosis proceeds in response to 20E. (Reviewed in Riddiford 2008)

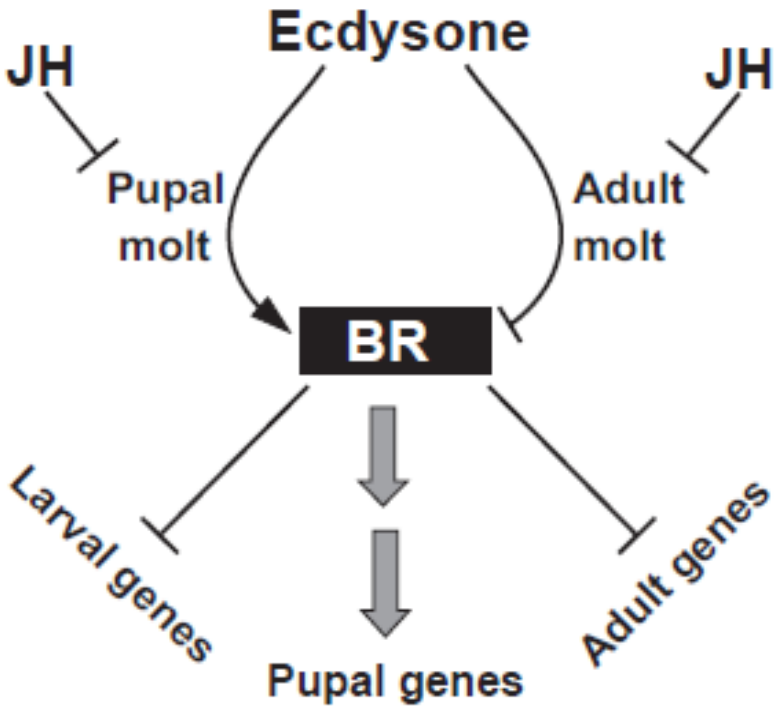

Figure 1.4: Summary diagram of the hormonal regulation of Broad (BR) protein expression and its role in the specification of pupal development based on studies of its action on cuticle genes. Juvenile hormone (JH) prevents the pupal molt, by preventing the activation of the br gene by ecdysone and prevents the adult molt by preventing the suppression of br by ecdysone in JH-sensitive tissues. BR is sufficient to activate the pupal program and to suppress both the larval and the adult programs (Zhou and Riddiford 2002)

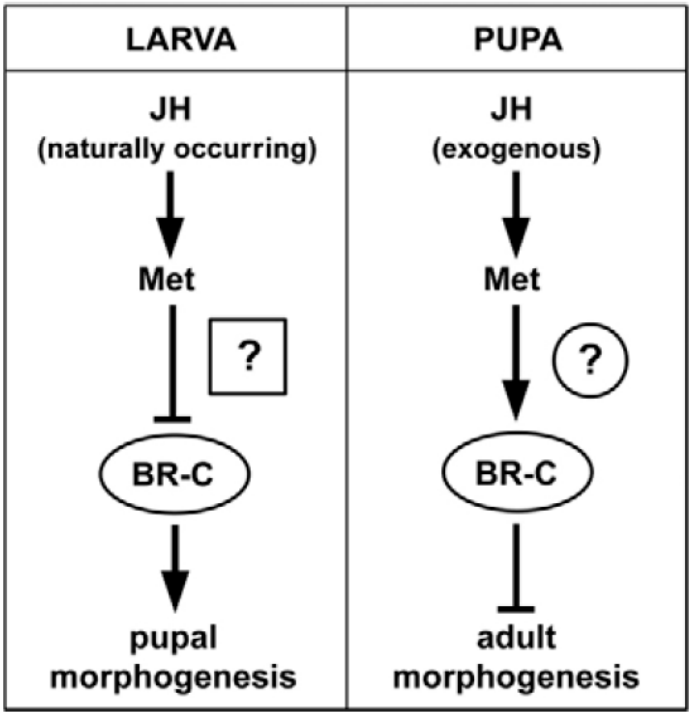

Figure 1.5: Model for functioning of BR-C in Tribolium metamorphosis. In young larvae, naturally occurring JH blocks pupal differentiation by repressing BR-C. JH is absent in early pupae, and its addition blocks adult morphogenesis by causing ectopic BR-C activation and death after a supernumerary pupal cuticle deposition. As both effects of JH on BR-C expression require Met, unknown stage-specific factors must modulate Met function (Konopova and Jindra 2008)

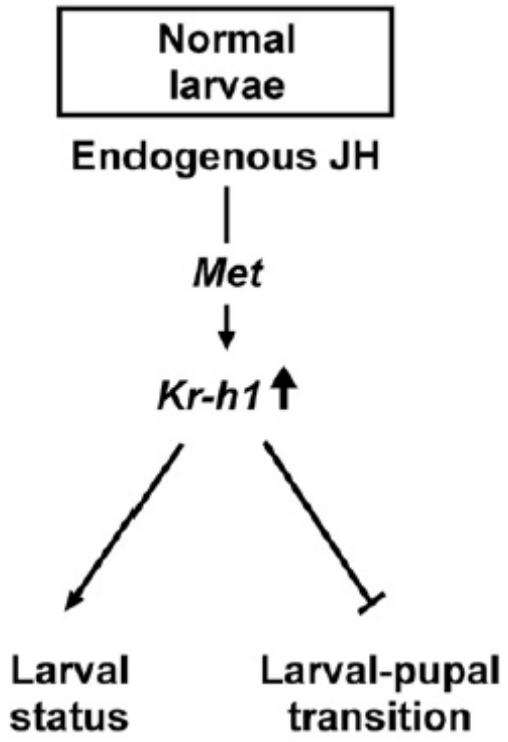

Figure 1.6: Models of JH signaling pathway in insect molting and metamorphosis in normal larvae (Minakuchi et al., 2009)

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Chapter 2

MET and GCE are functionally redundant in transducing the "status quo" action of juvenile hormone

ABSTRACT

Methoprene-tolerant (MET) and Germ cell expressed (GCE), two bHLH-PAS transcription factors in *Drosophila*, lie upstream of the juvenile hormone (JH) signal transduction pathway. Here we report that MET and GCE are functionally redundant in transducing the "*status quo*" action of JH. Both *Met* and g*ce* null single mutants are fully viable, but the *Met-gce* double mutant, Met^{27}-$gce^{2.5k}$, dies during the larval-pupal transition. Precocious and enhanced caspase-dependent programmed cell death (PCD) appears in fat body cells of Met^{27}-$gce^{2.5k}$ during the early larval stages. Expression of *Kr-h1*, a JH response gene that inhibits 20-hydroxyecdysone (20E)-induced *broad* (*br*) expression, is abolished in Met^{27}-$gce^{2.5k}$ during larval molts. Consequently, expression of *br*, which induces caspase-dependent PCD predominantly during the larval-pupal transition and is prevented by JH during larval molts, occurs precociously in Met^{27}-$gce^{2.5k}$. Defective phenotypes and gene expression changes in Met^{27}-$gce^{2.5k}$ are similar to the JH-deficient animal, *Aug21-GAL4>UAS-grim*. Importantly, exogenous application of JH agonists restored the JH signal in *Aug21-GAL4>UAS-grim,* but not in Met^{27}-$gce^{2.5k}$. Our results demonstrate that *Drosophila* MET and GCE redundantly transduce JH action to prevent 20E-induced caspase-dependent PCD during larval molts by inducing *Kr-h1* to inhibit *br* expression.

INTRODUCTION

It has been nearly 80 years since the discovery of juvenile hormone (JH) by V. B. Wigglesworth. Despite its important developmental and physiological roles, how JH functions at the molecular level is still not well understood, largely because the JH receptor has not been identified. The most likely candidate is Methoprene-tolerant (Met), which was first identified from the fruit fly, *Drosophila melanogaster*, in a genetic screen for mutants resistant to methoprene, a JH agonist (JHA) (Wilson and Fabian, 1986). Met is a typical bHLH-PAS transcription factor (Ashok et al., 1988), which binds JH (Shemshedini and Wilson, 1990; Miura et al., 2005). Met forms homodimers or heterodimers with its paralog, Germ-cell expressed (Gce), while JH reduces their dimerization (Godlewski et al., 2006). Although the *Met* null allele is fully viable (Wilson and Ashok, 1998), animals subjected to RNAi knockdown of *gce* in a *Met* null background die during the pupal-adult transition (Liu et al., 2009; Baumann et al., 2010). Overexpression of *Met* causes precocious and enhanced programmed cell death (PCD) in larval tissues (Liu et al., 2009), resulting in high mortality during larval development (Barry et al., 2008). Unlike *Met*, *gce* overexpression does not cause lethality; globally overexpressed *gce* can partially substitute for *Met* function (Baumann et al., 2010). During the larval-pupal transition, Met and Gce mediate JH action to prevent 20E-triggered caspase-dependent PCD of larval tissues (Liu et al., 2009) and differentiation of adult imaginal discs (Riddiford et al., 2010). In the beetle, *Tribolium castaneum*, which has only a single *Met*-like ortholog, Met plays a key role in JH action by maintaining proper larval molting and

preventing the premature development of adult structures during the larval-pupal metamorphosis (Konopova and Jindra, 2007; Parthasarathy et al., 2008a).

JH has many vital functions in insect development and reproduction, one of which is to modulate the action of the molting hormone, 20-hydroxyecdysone (20E), to coordinate insect molting and metamorphosis. Overall, 20E orchestrates the molting process, whereas JH determines the nature of the molt (Riddiford et al., 2003; Riddiford, 2008). Broad (Br), previously known as Broad-complex, is a zinc finger transcription factor involved in JH-20E crosstalk (Zhou et al., 2004). In both *Drosophila* and the moth, *Manduca sexta*, *br* expression is directly induced by 20E via its nuclear receptor complex, EcR-USP, but this induction can be prevented by JH during larval molts (Zhou et al., 1998; Zhou and Riddiford, 2002), resulting in *br* expression predominantly during the larval-pupal transition (Emery et al., 1994; Huang et al., 2011). Ectopic expression of *br* in the *Drosophila* 2nd instar larvae is sufficient to induce pupal formation (Zhou et al., 2004), while the *br* null alleles develop normally to the final larval instar but fail to undergo pupal formation (Kiss et al., 1976, 1988). Therefore, Br is called a "pupa specifier" in the initiation of metamorphosis (Zhou et al., 2002; 2004). During metamorphosis, Br induces apoptosis by up-regulating *Dronc* and *Drice*, two caspase genes responsible for apoptotic programmed cell death (PCD) (Cakouros et al., 2002; Kilpatrick et al., 2005). In *Tribolium*, Met mediates JH action to prevent 20E-induced *br* expression during larval molts, but is also required for exogenous JH to induce *br* expression during the pupal stage (Konopova and Jindra, 2008; Suzuki et al., 2008; Parthasarathy et al., 2008b).

Krüppel homolog1 (Kr-h1), another zinc finger transcription factor, is a key component in the JH signal transduction pathway. It has been documented that JH induces *Kr-h1* expression in *Drosophila* (Minakuchi et al., 2008). In *Tribolium* (Minakuchi et al., 2009) and the mosquito *Aedes aegypti* (Zhu et al., 2010), Met is required for JH action to induce *Kr-h1* expression. Moreover, the *Tribolium Kr-h1* is a JH early-response gene that mediates JH action by linking *Met* and *br* (Minakuchi et al., 2009).

In a previous study, it was demonstrated that a "*status quo*" action of JH is to prevent 20E-induced PCD in *Drosophila*, which is subtle, but functionally important (Liu et al., 2009). To understand how Met and Gce mediate the "*status quo*" action of JH, we generated a *Met-gce* double mutant, Met^{27}-$gce^{2.5k}$, which dies during the larval-pupal transition, exhibiting lethal and defective phenotypes similar to the JH-deficient animal, *Aug21-GAL4>UAS-grim*. Furthermore, we show that Met and Gce are functionally redundant in transducing the "*status quo*" action of JH through *Kr-h1* induction, which inhibits *br* expression and precludes 20E-induced caspase-dependent PCD during larval molts. This study lays a foundation for further elucidation of the molecular mechanism of JH action in *Drosophila*.

MATERIALS AND METHODS

Fly strains and genetics

All *Drosophila* strains were grown on standard cornmeal/molasses/yeast food at 25°C. *Aug21-GAL4*, *UAS-grim*, *UAS-gce*, and Met^{27} flies are as described previously (Wilson and Ashok, 1998; Liu et al., 2009; Baumann et al., 2010). Three lines of *UAS-Met-dsRNA, tubulin-GAL4*, and *arm-GAL4* flies were obtained from the VDRC Stock Center and Bloomington *Drosophila* Stock Center, respectively. *Oregon*-R and/or w^{1118} were used as wild type controls.

Preparation of fly food: The fly food was prepared in a Groen® steam kettle and mixed by a LEESON motor.

For the preparation of 10 – 12 trays of fly food; 750 ml Molasses (Food Service Direct) is added to 14 Liters hot water and mixed well. Corn meal (157g) (Food Service Direct) is added slowly to avoid lumps, followed by 80g of Agar (Moorehead & Co.) and 900g yeast (Food Service Direct). The food is boiled for 30 min, and after cooling for 45 min, the preservatives and anti-fungal agents 188 g Tagosept and 68 ml Propionic acid (Fisher Scientific) are added and mixed well. About 15-20 ml is added to each vial.

Generation of gce deletion lines by imprecise excision: To generate the *gce* deletion line, a *Minos*-insertion line *Mi{ET1}MB07696* inserted in the 4^{th} intron of *gce* (stock # 25565) was obtained from the Bloomington *Drosophila* Stock Center. This transposable element has $GFP^{E.3xP3}$ eye marker. To transpose the *Minos*-insertion

element, another fly line (stock # 24613) was obtained from Bloomington *Drosophila* Stock Center. This fly has a *P*-element insertion (*P{hsILMiT}*), a Hsp70 promoter driving expression of an intronless *Dhyd\Minos\Ths.PF* gene. *Minos* transposase-induced imprecise excision was carried out according to the reported procedure (Metaxakis et al., 2005) with modifications as follow:

About 5 – 7 virgin females carrying *Minos*-insertion transposable element (Stock # 25565) were crossed with males carrying the *P{hsILMiT}* insertion (stock # 24613) in 100 independent vials. The progenies of those crosses were heat shocked for 30 min. at 38^{o}C two times a day for three days starting from 2nd instar larvae. To screen for successful excision, 1,000 F1 males were crossed with 8,000 virgin females that carried the *Fm7c* balancer chromosome with one male and eight females in each vial. One F2 female that carried the *Fm7c* balancer chromosome and probably has successful excision marked by the white and GFP negative eye was picked from each F1 cross and mated with *Fm7c/Y* males in 1,000 independent vials. The progenies of the last cross were used to isolate the genomic deletion lines by PCR.

Genomic DNA was extracted from individual fly lines carrying the *Minos*-excised chromosome over an *FM7c* balancer according to previous described protocol (Bender et al., 1983) with some modifications as follow:

About 20 flies in a 1.5 ml Eppendorf tube are homogenized in 600 µl buffer A (0.1 M Tris HCI (pH 7.5) - 0.1 M EDTA - 0.1 M NACl - 1% SDS). Add 1 µl 100 ug/ml RNase A and incubate at 65^{o}C for 30 min. Add 90 µl buffer B (1:2.5 5 M KAc – 6 M LiCl), shake, and incubate on ice for 30 min. Centrifuge at 14,000 rpm for 15 min.

Transfer 550 supernatant into a new 1.5 ml Eppendorf tube, add 550 µl Chloroform centrifuge for 10 min after mixing. Transfer 500 supernatant to a new Eppendorf tube again and add 500 µl Isopropanol and centrifuge for 15 min, decant supernatant and wash the pellet with 800 µl 70% ethanol. Centrifuge for 5 min at 14,000 rpm and decant supernatant, air dry for 5 min and resuspend the pellet in 100 µl TE buffer (10 mM Tris-HCl (pH 7.5) – 1 mM EDTA pH 8). Store at -20^{o}C.

Genomic DNA amplifications for deletion screening were carried out using primers designed from the DNA sequence of *gce* obtained from Flybase.com to amplify a 4-kb fragment flanking the *Mi{ET1}MB07696* insertion site. The primers used for the analytical PCR reactions were 5'-CAGAACGTGATCATTGCACTCGAATC-3' and 5'-GACCGAACGAGAAGTAACCCTGA-3'.

PCR amplification was performed with 1 µl genomic DNA obtained from each excision line as a template, 2 µl of 10X PCR reaction buffer, 1µl of 25mM $MgCl_2$, 1 µl (10 µM) of each primer, 0.1 µM of each dNTP, and 0.25 U *Taq* DNA polymerase (Invitrogen) in a final volume of 20 µl.

PCR amplification was carried out in a 200 µl Eppendorf cycler with an initial denaturation step at 94 °C for 2 minutes. Amplifications were achieved through 33 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 4 min. A final extension step was carried out for 10 min at 72 °C. PCR products were loaded in 1% Agarose gel for electrophoresis analysis and stained with Ethidium Bromide.

About 400 F2 lines were screened for deletions and 4 lines were selected based on the reduced length of PCR products. One line, $gce^{2.5k}$, gave the shortest, 1.5-kb, PCR product. DNA sequencing demonstrated that $gce^{2.5k}$ carries a 2.5-kb deletion that removes exons 5–8 and part of exon 9 of *gce*.

Generation of Met^{27}- $gce^{2.5k}$ *double mutant:* The Met^{27}- $gce^{2.5k}$ double mutant was generated by genetic recombination and verified by PCR and DNA sequencing.

Since *Met* and *gce* genes are located in positions 10D and 13B on the first chromosome, respectively, the recombination technique was applied to combine $Met^{27}$and $gce^{2.5k}$ mutations in one chromosome. Virgin Met^{27} females were crossed with $gce^{2.5K}$ males. Met^{27} / $gce^{2.5K}$ transheterozygous virgin females were collected from the F1 progeny and crossed with $Fm7G/Y$ male. Because no obvious phenotype is associated with either Met^{27}or $gce^{2.5k}$ mutations, 200 $Fm7G$-balanced virgin females were randomly collected from F2 progeny and each of them was crossed with $Fm7G/Y$ male separately to set up 200 independent lines. Genomic PCR and DNA sequencing were used to detect Met^{27} and $gce^{2.5K}$ mutations for all 200 lines. The presence of $gce^{2.5K}$ was assessed by genomic DNA PCR with the same pair of primers used in the *gce* deletion line screening. The presence of Met^{27} was confirmed by genomic PCR followed by DNA sequencing. Met^{27} carries three point mutations and/or DNA single-nucleotide polymorphisms within its intron (G-A-T in Met^{27} and A-G-C in the wild type of the genome position 11,512,216 - 11,512,229 - 11,512,230, respectively).

Lethal phase determination for Met^{27}- $gce^{2.5k}$ double mutants: Because Met^{27}-$gce^{2.5K}$ double mutants are lethal, we determined their lethal phase. Met^{27}- $gce^{2.5k}$ /*FM7c,act-GFP* females were crossed with *FM7c,act-GFP/Y* males. 100 newly hatched larvae of Met^{27}- $gce^{2.5k}$ / Y male progeny were picked up based on the lack of GFP and were reared in one vial of fly food. Lethal phase of these larvae was closely monitored.

Generation of p{Met} transgenic flies: To generate *p{Met}* transgenic flies, BACR29A04 was obtained from BACPAC Resources, Children's Hospital Oakland, CA. This BAC clone carries 160 kb genomic DNA of *D. melanogaster* in the BAC vector and is transformed into DH10B *E.coli* host. To extract the DNA from the *E.coli* host, one single colony was inoculated into 100 ml LB media (10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl dissolved in 1 liter H_2O; pH 7.0) supplemented with 25µg/ml kanamycin antibiotics and cultured at 37^oC in shacking incubator at 250 rpm over night (16-18 hrs). The culture was centrifuged for 10 min. at 3,000 rpm and the supernatant was discarded. The pellet was resuspended (vortex) in 15 ml P1 solution buffer (50mM Tris, pH 8 - 10 mM EDTA - 100 ug/ml RNase A; filter sterilized, 4^oC). 15 ml P2 solution buffer (0.2N NaOH - 1% SDS; filter sterilized, room temp) was added and mixed gently by converting the tube about 6 times and left at room temperature for 5 min. Slowly, 15 ml P3 solution buffer (3M KOAc, pH 5.5; autoclaved, 4^oC) was added and gently shaked to mix the contents and left on ice for 10 min, and then the sample was spun at 10,000 rpm for 10 min. at 4^oC. Ice-cold isopropanol was added to the supernatant in the ratio of 1:1 and mixed and kept on ice for 20 min. or -20^oC overnight, and then centrifuged at 10,000 rpm

for 15 min at 4^{o}C. The pellet was washed using 5 ml 70% ethanol, centrifuged, air dried at room temperature, and resuspended in 2 ml TE buffer.

Because Met is flanked by SspI restriction cites, about 20 µl BAC DNA was digested with SspI restriction enzyme (Invitrogen), and the products were separated by 1% agarose gel electrophoresis and stained by ethidium bromide. An approximately 5.7-kb DNA fragment containing the *Met*-encoding region and its putative promoter was cut from the gel using DNA ladder Plus marker (Invitrogen), and purified by gel purification kit (QIAGEN). The purified fragment was sub-cloned into the *pBluescript KS* plus cloning vector (Invitrogen), digested by SmaI (blunt end); 3 µl plasmid victor, 12 µl insert, 4 µl 5X ligation buffer, and 1 µl TD4 DNA Ligase enzyme (Invitrogen), and transformed into DH5α *E. coli* competent cells as follow: 5 µl overnight ligation mix was mixed gently with 100 µl competent cells and incubated in ice for 45 min., the mix was heat shocked for 90 s. and then returned to ice for 3 min. 700µl LB was added and the suspension incubated at 37°C for 1 hour. The suspension was streaked out on LB agar plates with 1 µg/µl Ampicillin, 40 µl X-Gal, 4 µl IPTG, and incubated overnight at 37°C. The positive colonies (white) were selected and each colony was cultured in 5 ml LB with 1 µg/µl Ampicillin and incubated overnight at 37°C. Different independent plasmids were isolated by Plasmid Mini Kit (QIAGEN) according to the manufacture protocol.

The correct sub-cloned plasmid containing the 5.7 kb DNA fragment was detected by digestion screening of about 50 isolated plasmids and confirmed by sequencing analysis using M13 F & M13 R primers.

The 5.7 kb *Met* fragment was excited from the *pBluescript-Met* plasmid by EcoRI/NotI and following gel purification was subcloned into *pCaSpeR-4* using the same enzymes. A positive clone *pCaSpeR-4–Met* was selected by restriction enzyme digestion as well as DNA sequencing analysis.

To isolate plasmid DNA for Drosophila transformation, the correct *pCaSpeR-4–Met* construct was re-transformed into DH5α E-coli competent cells and cultured on LB agar plates for overnight. A single colony was isolated and cultured in LB for large scale plasmid extraction using HiSpeed® Plasmid Midi Kit (QIAGEN) following the manufacture protocol.

Transgenic fly lines were generated by *P*-element-mediated germline transformation. *pCaSpeR-4–Met* DNA prepared using HiSpeed® Plasmid Midi Kit was sent to Rainbow Transgenic Flies, Inc (Camarillo, CA) for embryo injection. *pCaSpeR-4–Met* together with a helper plasmid that carries *P*-transposase gene was injected into ~250 0-1 hrs w^{1118} embryos. The surviving injected eggs (embryos) were reared to adulthood at 25°C. Single white-eyed (w^-) adult males and 3 females were crossed with *w^-; Pin/CyoY* virgin females or males, respectively. F1 progenies were screened for the red eye flies (w^+) that carry the integrated *P-element* insertion. A single red eye male with *Pin* or *CyoY* marker was re-crossed with 5-7 virgin *Pin/CyoY* females. In order to know which chromosome received the *P-element* insertion, we observed the F2 progenies as follow:

- If all males are white eye and all females are red eye, the *P-element* insertion is on the first chromosome (X).

- If all red eye flies are associated with either *Pin* or *CyoY*, but all *Pin/CyoY* flies are white eye, the *P-element* insertion is on the second chromosome (2^{nd}).
- If some *Pin/CyoY* males are red eye flies, the *P-element* insertion is on the third chromosome (3^{rd}).

JHA treatment

The JHA pyriproxyfen (Sigma-Aldrich) was dissolved in 95% ethanol to give a 300 ppm stock solution. JHA-containing fly food was prepared by adding JHA stock solution to the standard food at 50–55°C to a final concentration of 0.03–3 ppm. Parental flies were transferred on the JHA food. Their progeny were reared at 25 ^{o}C.

JHA resistance assay: About 100 wild type, Met^{27}, or $gce^{2.5K}$ parental flies were placed in a large fly food bottle with a small side opening plugged with a piece of cotton for aeration. Each bottle was supplied with a grape juice media plate carrying a small piece of yeast past in the middle to enhance the egg laying production. The grape juice medium was prepared as follow: To prepare 1 Liter grape juice medium, 473 ml Welch's 100% Grape Juice, 455 ml ddH2O, and 29.3 g granulated Agar are mixed together and boiled for 8 min. or until the agar has completely dissolved. When the mixture has cooled to 65^{o}C, 9.9 ml 95% ethanol and 9.5 ml Glacial Acetic Acid are added and mixed well. The medium is poured in the small petri dish plates, kept in wet chamber (sealed plastic boxes or large petri-dishes with wet Kimwipe), and stored in the refrigerator at 4^{o}C. The yeast past was freshly made by mixing about 1 g dry yeast with 1 ml 0.5% proponic acid. The parental flies from each genotype were

left in the large bottle (with grape juice plate and yeast past) for 3 – 7 hrs to collect enough eggs and then the plates were replaced by new ones. The plates with eggs were kept in wet champers at 25^{o}C overnight. 100 newly hatched wild type, *Met*^{27}, or *gce*$^{2.5k}$ larvae were collected from the grape juice plates and transferred into vials of food containing 0.03, 0.1, 0.3, 1 or 3 ppm pyriproxyfen. The same amount of the solvent (ethanol) was mixed into the food for the control (0 ppm) vials. Seven repeats of each type of flies were tested on the 6 treatments. Survival rates were calculated based on the numbers of flies developing to adulthood.

JHA treatment for Br-antibody staining: To create larvae lacking *Met* and *gce* expression, *Met*^{27}- *gce*$^{2.5k}$ /*FM7c,act-GFP* females were crossed with *FM7c,act-GFP/Y* males on food containing 0.1 ppm pyriproxyfen. GFP-negative larvae were selected at the 2nd instar for FB dissection and Br-antibody staining.

Immunohistochemistry and microscopy

In order to observe the expression of *br*, fat bodies were dissected from flies of different genotypes and treatments at indicated developmental stages. Immunohistochemistry was performed as described previously (Patel, 1994) with the following modifications. The larvae were dissected in glass dissecting plates contains phosphate buffered saline (PBS; 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 to 1L distilled H_2O - pH 7.4) and fixed in 4% Para-formaldehyde in PBS on shaker for 30 min at room temperature. The samples were washed 3 times with PBT buffer (PBS + 1% Triton X 100) each for 30 min. The tissues were blocked by

5% Normal Goat Serum (NGS) in PBT (1:20 dilution) for 30 min. The Br- core antibody (25E9.D7, from the DSHB at the University of Iowa) against endogenous Br proteins was added to the samples (1:100 in PBT + NGS) and kept overnight at 4^{o}C. The samples were then washed 3 times with PBT each for 20 min. Tissues were then stained with the second antibody goat-anti-mouse-Cy3 (Jackson ImmunoResearch, PA) using a 1:300 dilution for 2 hrs. The samples were rinsed with PBT 3 times for 30 min. Then, the tissues were mounted in VECTASHIELD Mounting Medium with DAPI that specifically labels DNA (Vector Laboratories, INC., CA). Apoptosis was measured using the Caspase 3 & 7 Apoptosis Detection Kit (Invitrogen, CA). Cell membrane disruption was detected using the Propidium Iodide Staining Kit (Beyotime, Shanghai, China). Fluorescence signals were captured with Leica SP5 X laser scanning Confocal Microscope under the same conditions (40X oil immersion lens).

Female fecundity assay

One newly eclosed virgin female of *Oregon-R*, Met^{27}, or $gce^{2.5K}$ was crossed with one *Oregon-R* male in each vial with ten replicates. The flies were transferred into new vials and the number of eggs was counted for each cross every three days in the same time for 30 days. The cumulative numbers of eggs laid by a single female were calculated as the mean of the ten independent experiments for each type of flies. The average age when females started to lay eggs were also monitored and recorded.

Biochemical and molecular methods

Caspase 3 activity assay: Caspase 3 activity was determined using the Beyotime Caspase 3 Activity Kit, following the manufacturer's instructions (Beyotime, Shanghai, China).

Total RNA isolation: Total RNAs were isolated from 2nd instar larvae using the RNeasy Mini Kit (Qiagen) at room temperature as follow:

Homogenize ten 2nd instar larvae using RNase free pellet pestle (Kimble Chase) in 350 ul lysis buffer (RLT) and 3.5 ul β-Mercaptoethanol using motor mixer. Centrifuge lysate for 3 min at maximum speed in a microcentrifuge (≥8000 x g), and transfer the supernatant into a new RNase free Eppendorf tube. Add 1 volume (350 µL) of 70% ethanol to the cleared lysate, and mix well. Apply 700 µL of the sample to an RNeasy mini spin column sitting in a 2-mL collection tube. Centrifuge for 15 sec at maximum speed. Discard flow- through, and reuse the collection tube. Add 350 µL Buffer RW1 (washing buffer 1) onto the RNeasy column, and centrifuge for 15 sec at maximum speed. Discard flow-through and reuse the collection tube.

In a new RNase free Eppendorf tube mix gently 10 µl DNase I stock solution (RNase-Free DNase, Qiagen) with 70 µl Buffer RDD (reaction buffer) by inverting the tube. Centrifuge briefly. Add DNase I incubation mix (80 µl) directly to RNeasy column membrane, and incubate at room temp. for 15 min. Add 350 µl Buffer RW1 to RNeasy column, centrifuge for 15 s at ≥8000 x g, and discard flow-through. Add 500 µl Buffer RPE (washing buffer 2) to the RNeasy spin column, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through. Add another 500 µl Buffer RPE to the

RNeasy spin column, and centrifuge for 1 min at ≥8000 x g. Place the RNeasy spin column in a new 2-ml collection tube, and centrifuge at full speed for 1 min to dry the membrane. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane, and centrifuge for 1 min at ≥8000 x g to elute the RNA. The RNA sample can be stored at -80^{o}C for up to one year.

Synthesis of the first strand cDNA: In order to produce templates for qualitative RT-PCR (visualized by agarose gel electrophoresis) or quantitative RT-PCR on the LightCycler® 480 System, the first strand of cDNAs were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as follow:

Thaw all frozen reagents before use, briefly centrifuge them before starting the procedure, and keep all reagents on ice while setting up the reactions. Mix fresh 3 µg total RNA with 1 µl anchored-oligo$(dT)_{18}$ primer (50 pmol/µl) and add PCR-grade water to 13 µl final volume.

To the tube containing the template-primer mix, add 4 µl 5× conc. Transcriptor Reverse Transcriptase Reaction Buffer (8 mM $MgCl_2$), 0.5 µl Protector RNase Inhibitor (40 U), 2 µl Deoxynucleotide Mix (10 mM each), and 0.5 µl Transcriptor Reverse Transcriptase (20 U). Mix the reagents in the tube carefully and do not vortex.

Incubate the RT reaction for 30 min at 55°C, inactivate Transcriptor Reverse Transcriptase by heating to 85° for 5 min., and stop the reaction by placing the tube

on ice. At this point the reaction tube may be stored at +2 to +8°C for 1-2 h or at -15 to -25°C for longer periods.

Qualitative reverse transcriptional PCR (RT-PCR): In order to compare qualitatively different genes transcription levels (mRNA level) in mutant flies with the wild type flies, the qualitative RT-PCR analyses were performed as follow: Three micro-litter cDNA of mutant or wild type animals as template was mixed with 2 µl of 10X PCR reaction buffer, 1µl of 25mM $MgCl_2$, 1 µl (10 µM) of each primer, 0.1 µM of each dNTP, and 0.25 U *Taq* DNA polymerase (Invitrogen) in a final volume of 20 µl.

PCR amplification was carried out in a 200 µl Eppendorf cycler with an initial denaturation step at 94°C for 2 minutes. Amplifications were achieved through 23 cycles to avoid the saturation point at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. A final extension step was carried out for 5 min at 72°C. PCR products were loaded in 1% Agarose gel electrophoresis stained by Ethidium Bromide.

The photos were taken from the gel by Alpha Innotech Imaging Station and processed by Photoshop.

Quantitative real-timePCR (qRT-PCR): Q-RT-PCR was performed in a LightCycler® 480 Instrument (Roche) using *rp49* for normalization (Sheng et al., 2008).

The reaction mixture was performed in 6 replicates for each gene as 20 µl total volume in 96-Multiwell-Plate using LightCycler® 480 SYBR Green I Master Kit (Roche) as follow:

Thaw one vial of "LightCycler® 480 SYBR Green I Master" and Water, PCR-grade, and Keep the Master mix away from light. In each well of the 96-Multiwell-Plate add 3 µl PCR-grade water, 2 µl PCR primer mix (10 µM), and 10 µl 2X master mix. Add 5 µl of the corresponding cDNA template and mix by pipetting up and down. Seal the Multiwell Plate with LightCycler® 480 Multiwell Sealing Foil.

Load the Multiwell Plate into the the LightCycler® 480 Instrument and start the PCR program as follow:

One cycle of Pre-Incubation at 95^{o}C for 5 min., 45 cycles of Amplification (95^{o}C for 10 s., 60^{o}C for 10 s, 72^{o}C for 10 s.), 1 cycle for Melting Curve (95^{o}C for 5 s., 65^{o}C for 1 min., 97^{o}C for the Continuous Acquisition Mode), and 1 Cooling cycle at 40^{o}C for 10 s.

The data were analyzed by using LightCycler® 480 Software, Version 1.5 (Roche) and the fit points were calculated as CP values. The fold increase or decrease was calculated according to the following equation:

$$A/B = 2^{\Delta (CP_A - CP_B)}$$

Where:

A is the percentage increase or decrease in the tested sample

B is the control sample (wild type) which counted as 100%

CP_A is the CP value of the tested sample

CP_B is the CP value of the control sample (wild type)

The primers used were synthesized in *Integrated DNA Technologies*, Inc. included:

Kr-h1: 5'-GAATACGACATAACAGCC-3' and

5'-CGATTTCCGTGAATATGTTCT-3'

Met: 5'-GCCAGAACCCTATCAGTTGG-3' and

5'-AGCAGACGGTAGCAGCTCTC-3'

gce: 5'-ACGGATCCATCCAGGAACTA-3' and

5'-CATGGCAGGTGAGTGTGAGA-3'

Rp49: 5'-GACAGTATCTGATGCCCAACA-3' and

5'-CTTCTTGGAGGAGACGCCGT-3'

RESULTS

The gce null mutant phenocopies the Met-null mutant

In a previous study (Baumann et al., 2010), it was shown that global expression of *gce* dsRNA in *tubulin-GAL4>UAS-gce-dsRNA* results in severe lethality during the pupal stage. Similarly, we found that global expression of three independent *UAS-Met-dsRNA* lines in the *tubulin-GAL4>UAS-Met-dsRNA* also resulted in 95–100% pupal lethality. This result is in conflict with the fact that the *Met* null allele, Met^{27}, is fully viable (Wilson and Ashok, 1998). A possible explanation for the lethal phenotypes of the *gce*- and *Met*-RNAi animals is dsRNA off-targeting, which means RNA interference can silence not only the target gene, but also other genes with a similar sequence (Kulkarni et al., 2006).

Met and gce were found to have highly similar amino acids sequences especially in the three conserved domains; bHLH, PAS-A, and PAS-B (Fig. 2.1)

In order to clarify the function of Met and Gce in JH signaling, we created a *gce* deletion line, $gce^{2.5k}$, using the *Minos* element-induced imprecise excision technique (Metaxakis et al., 2005). The original *Minos* element transgenic line, *Mi{ET1}MB07696*, which carries an insertion on the X chromosome within the fourth intron of *gce,* is viable and fertile and shows no phenotypic differences from wildtype animals. After imprecise excision, a 2.5-kb DNA fragment encoding the bHLH and both

PAS domains was completely deleted in $gce^{2.5k}$ flies (Fig. 2.2). No *gce* transcript was detected in these flies, demonstrating that $gce^{2.5k}$ is a *gce* null allele (Fig. 2.5).

Like the Met^{27} mutant, the $gce^{2.5k}$ mutant is fully viable and fertile, which further indicates that the pupal lethality of *Met*- and *gce*-RNAi flies results from dsRNA off-targeting. As *Met* mutants are resistant to JHA (Wilson and Fabian, 1986), we first tested whether $gce^{2.5k}$ also confers JHA resistance. One hundred wildtype, Met^{27} and $gce^{2.5k}$ newly hatched larvae were reared on the standard diet supplemented with different concentrations of a potent JHA, pyriproxyfen, over multiple trials. When flies were reared in the food containing 0.3 ppm pyriproxyfen, more than 90% of the wildtype flies died just prior to eclosion (as expected); by contrast, about 95% of $gce^{2.5k}$ flies developed to adulthood, implying that the $gce^{2.5k}$ allele conferred ~3–5-fold resistance to pyriproxyfen *versus* wildtype. The JHA resistance of $gce^{2.5k}$ flies was significant, but much weaker than that seen in Met^{27}, which was ~30 times more resistant to pyriproxyfen than wildtype (Fig. 2.3). Similar results were obtained with methoprene, another common JHA.

In *Drosophila*, JH is the major gonadotropic hormone regulating the uptake of yolk proteins and the maturation of sexual behavior (Kelly et al., 1987; Gruntenko et al., 2010). *Met* mutant females show a delayed onset of vitellogenic oocyte development and oviposition (Wilson and Fabian, 1986). In order to evaluate the effects of a *gce* null allele on vitellogenic oocyte development and reproduction, we examined the fecundity of wildtype, Met^{27}, and $gce^{2.5k}$ females. Wildtype females started to lay eggs at about 3 ± 0.4 days after eclosion. In contrast, Met^{27} and $gce^{2.5k}$ females began ovipositing to lay eggs at 6 ± 0.5 and 4.6 ± 0.7 days after eclosion, respectively. After 24 days, the number of eggs laid by a single Met^{27} and $gce^{2.5k}$ female reduced to 27% and 65% of wildtype (Fig. 2.4). All of these results suggest

that Met and Gce have similar functions, but that the physiological role of Met in JH action is predominant for these two bHLH-PAS transcription factors.

Met and gce double mutations cause prepupal lethality

Since both *Met* and *gce* genes are on the X chromosome, we generated a *Met-gce* double mutant, Met^{27}-$gce^{2.5k}$, by genetic recombination to further characterize the potential functional redundancy of these genes. Met^{27} and $gce^{2.5k}$ mutations in the double mutant were verified by PCR and DNA sequencing. Moreover, as confirmed by reverse transcription PCR, neither the *Met* nor *gce* transcript was detectable in Met^{27}-$gce^{2.5k}$ (Fig. 2.5). Compared to wildtype, onset of metamorphosis (larval wandering) of Met^{27}-$gce^{2.5k}$ was delayed ~12 hours (Fig. 2.6), and its body weight reduced (Fig. 2.7). Importantly, Met^{27}-$gce^{2.5k}$ died ~24 hours after pupariation and failed to undergo head eversion (Fig. 2.8A). When reared on food containing a high JHA concentration (i.e., 1 ppm pyriproxifen), all wildtype animals died at the pharate adult stage. However, when Met^{27}-$gce^{2.5k}$ mutants were reared on food containing the same high dosage of JHA, their development was not affected. They still died ~24 hours after pupariation (Fig. 2.8A).

Of note, pupal lethality seen in Met^{27}-$gce^{2.5k}$ flies can be fully rescued by either a single copy of the transgene that carries a 5.7-kb *Met* genomic fragment or by global *gce* overexpression (Fig. 2.8B), indicating that loss of both *Met* and *gce* is required to cause lethality. This finding confirms the hypothesis that Met and Gce are functionally redundant in *Drosophila*.

Precocious and enhanced caspase-dependent PCD in *Met^{27}-gce$^{2.5k}$*

The lethal phase and defective phenotypes of *Met^{27}-gce$^{2.5k}$* were similar to, but slightly stronger than, those of the JH-deficient *Aug21-GAL4>UAS-grim* flies. For example, the posterior portion of *Met^{27}-gce$^{2.5k}$* prepupae becomes progressively empty during the larval-pupal transition (Fig. 2.8A). This phenotype is identical to that observed in the JH-deficient animal, *Aug21-GAL4>UAS-grim*, in which it was shown to be caused by precocious and enhanced caspase-dependent PCD (Liu et al., 2009). Therefore, we further tested whether the same developmental defects of fat body remodeling observed in the JH-deficient animals also occur in *Met^{27}-gce$^{2.5k}$* flies.

Four different assays were employed to detect the apoptotic PCD and/or cell dissociation of fat body cells. First, when the Caspase 3 & 7 Apoptosis Detection Kit was used to label apoptotic cells in the fat body of 2nd instar larvae, PCD was undetectable in wildtype flies, but apparent in *Aug21-GAL4>UAS-grim* and *Met^{27}-gce$^{2.5k}$* (Fig. 2.9). Second, when Caspase 3 activity in the fat body of 2nd instar larvae was tested, it increased by ~60% in *Aug21-GAL4>UAS-grim* and ~100% in *Met^{27}-gce$^{2.5k}$* (Fig. 2.10). Third, when propidium iodide was used to mark the disrupted cell membrane (a characteristic feature of the final stage of cell death) in the pupal fat body at 4 hours after pupariation, the majority of fat body cells in *Aug21-GAL4>UAS-grim* and *Met^{27}-gce$^{2.5k}$*, but few in wildtype, were labeled (Fig. 2.11). Finally, we assessed fat body cell dissociation 8 hours after pupariation. Fat body cells in the wildtype larvae still associated with one another, but those in *Aug21-GAL4>UAS-grim* and *Met^{27}-gce$^{2.5k}$* were mostly dissociated into individual cells (Fig.

2.12). Therefore, all results indicate that precocious and enhanced caspase-dependent PCD occurs in both *Aug21-GAL4>UAS-grim* and Met^{27}-$gce^{2.5k}$ animals. Consistent with the lethal phenotypes, the developmental defects in fat body remodeling in Met^{27}-$gce^{2.5k}$ double mutants were slightly stronger than that in *Aug21-GAL4>UAS-grim* (Fig. 2.9, 2.10, 2.11, 2.12).

Importantly, when reared on food containing an intermediate concentration of JHA (0.1 ppm pyriproxyfen), precocious and enhanced caspase-dependent PCD and cell dissociation phenotypes were prevented in *Aug21-GAL4>UAS-grim,* but not in Met^{27}-$gce^{2.5k}$ (Figs. 2.9 – 2.12), demonstrating that Met and Gce redundantly mediate the "*status quo*" action of JH to prevent caspase-dependent PCD during larval molts.

Diminished *Kr-h1* expression in Met^{27}-$gce^{2.5k}$

In *Drosophila*, it has been documented that exogenous JHA induces *Kr-h1* expression during the larval-pupal transition, and *Kr-h1* lies upstream of *br* in the JH signaling pathway (Minakuchi et al., 2008). Recently, we observed precocious *br* expression in early larval stages of *Kr-h1* mutants (Huang et al., 2011). Next, we chose to examine roles for Met and Gce in the JH-induced *Kr-h1* expression. Quantitative real-time PCR (qRT-PCR) revealed that *Kr-h1* was highly expressed during the 2nd larval molt of wildtype animals, and that this expression level was not enhanced by exogenous JHA (Fig. 2.13). However, the level of *Kr-h1* mRNA in JH-deficient *Aug21-GAL4>UAS-grim* larvae was decreased to ~40% of the wildtype level, which could be restored to wildtype levels by exogenous JHA (Fig. 2.13). These results suggest that *Kr-h1* expression in early larvae is up-regulated by

endogenous JH to a saturated level; excess JH does not further stimulate *Kr-h1* expression, but loss of JH reduces *Kr-h1* expression.

Importantly, there are two differences regarding *Kr-h1* expression in Met^{27}-$gce^{2.5k}$ and *Aug21-GAL4>UAS-grim*. First, revealed by qRT-PCR, *Kr-h1* mRNA was nearly abolished in Met^{27}-$gce^{2.5k}$ larvae (Fig. 2.13). When RT-PCR was carried out through 30 cycles, *Kr-h1* mRNA was obviously reduced in both $Met^{27}$and $gce^{2.5k}$ larvae and was totally undetectable in Met^{27}-$gce^{2.5k}$ larvae (Fig. 2.14). Second, JHA treatment restored *Kr-h1* expression in *Aug21-GAL4>UAS-grim* but not in Met^{27}-$gce^{2.5k}$ (Fig. 2.13).

These results demonstrate that Met and Gce are functionally redundant in transducing JH action to induce *Kr-h1* expression in *Drosophila*.

Precocious *br* expression in Met^{27}-$gce^{2.5k}$ animals

It has been reported that 20E-induced expression of the caspase *Dronc* during hormone-dependent PCD in *Drosophila* is regulated by the "pupa specifier" Broad (Cakouros et al., 2002). Recently, we demonstrated that *br* is precociously expressed during early larval stages in *thick veins* (*tkv*), *Mother-against dpp* (*Mad*) and *Kr-h1* mutant backgrounds, which disrupt JH biosynthesis or JH signaling (Huang et al., 2011). We inferred that the precocious and enhanced caspase-dependent PCD in *Aug21-GAL4>UAS-grim* and Met^{27}-$gce^{2.5k}$ could, at least partially result from precocious *br* expression caused by the decrease in *Kr-h1* expression during larval molts. To test this hypothesis, we assessed the presence of endogenous Br proteins in the fat body cells of 2nd instar larvae by immunohistochemistry with a Br-core

antibody (Emery et al., 1994). As expected, Br proteins were not detected in wildtype larvae. However, precocious *br* expression was observed in *Aug21-GAL4>UAS-grim* and Met^{27}-$gce^{2.5k}$, which was indicated by accumulation of Br proteins in the nuclei. When the larvae were reared on food containing JHA, precocious *br* expression was prevented in *Aug21-GAL4>UAS-grim* but not in Met^{27}-$gce^{2.5k}$ (Fig. 2.15).

Taken together, our data show that (1) *Drosophila* Gce has similar functions as its paralog, Met; (2) the *Met-gce* double mutant dies during the larval-pupal transition and causes phenotypes similar to the JH-deficient animals; and (3) *Kr-h1* expression is eliminated and expression of *br* is precociously triggered during the larval molts, which induces precocious PCD. In conclusion, *Drosophila* Met and Gce, the redundant paralogs, transduce the "*status quo*" action of JH to prevent 20E-induced caspase-dependent PCD during larval molts by inducing *Kr-h1* expression, which in turn inhibits *br* expression (Fig. 2.16).

DISCUSSION

Based on the similar functions of Met and Gce, the lethality of Met^{27}-$gce^{2.5k}$, together with the previous findings cited in the introduction (Godlewski et al., 2006; Barry et al., 2008; Liu et al., 2009; Baumann et al., 2010), we conclude that Met and Gce are functionally redundant in *Drosophila*.

Kr-h1 was first identified as a JH-response gene in *Drosophila* (Minakuchi et al., 2008). Given the previous reports that *Kr-h1* mediates JH action by linking *Met* and *br* in *Tribolium* (Minakuchi et al., 2009) and *Aedes* (Zhu et al., 2010), it is not surprising that Met and Gce transduce JH signal to induce *Kr-h1* expression in

Drosophila. Down-regulation of *Kr-h1* in the JH-deficient animals and in the *Met-gce* double mutant during larval molts could at least partially account for precocious *br* expression and the resulting caspase-dependent PCD.

It has been shown that JH induces *Kr-h1* expression in a rapid and reversible manner in *Tribolium* (Minakuchi et al., 2009). A similar phenomenon has been observed in *Drosophila* Kc cells (data not shown). This observation is consistent with the previous finding that JH rapidly and reversibly reduces Met-Gce dimerization (Godlewski et al., 2006). Recent studies in *Aedes*, *Drosophila* and *Tribolium* have demonstrated that the p160/SRC/NCoA-like molecule is also required for JH action to induce expression of *Kr-h1* and other JH response genes (Li et al., 2011; Zhang et al., 2011). Importantly, Met and the p160/SRC/NCoA-like molecule, Taiman in *Drosophila* and FISC in *Ades*, form a JH-dependent functional complex with the JH response element to directly activate transcription of JH target genes (Li et al., 2011). Taken together, one might assume that JH reduces Met-Gce dimerization, after which the Met or Gce monomer forms a heterodimer with Taiman to induce *Kr-h1* expression in *Drosophila*. However, to elucidate the detailed molecular mechanism of how Met, Gce, Taiman, and other possible or unknown transcriptional regulators transduce the JH signal to induce *Kr-h1* expression in a rapid and reversible manner will require substantial studies at the biochemical and genetic levels.

The ligand-receptor complex, 20E-EcR-USP and the 20E primary response gene *br* induce expression of *Dronc* and *Drice* to stimulate apoptotic PCD (Cakouros et al., 2004; Kilpatrick et al., 2005). It is generally accepted that the major role of JH is to antagonize 20E action during larval molts (Riddiford, 2008), so that the 20E-induced

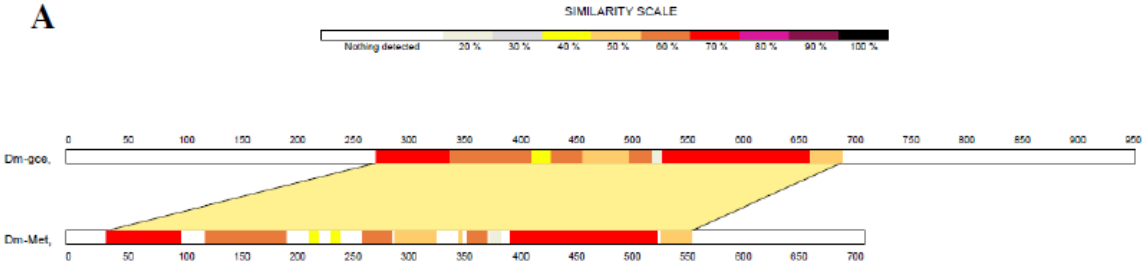
caspase-dependent PCD takes place predominantly during larval-pupal metamorphosis (Yin and Thummel, 2005). This has been shown in JH-deficient animal, *Aug21-GAL4>UAS-grim* larvae, in which the 20E-induced expression of *Dronc* and *Drice* and subsequent apoptosis occurs precociously. Moreover, it has been verified genetically that JH antagonizes 20E action in the JH-deficient animal by 20E application and *hs-EcR-RNAi* rescuing experiments (Liu et al., 2009). In this study, we have further demonstrated precocious caspase-dependent PCD as early as the 2nd larval molt in the JH-deficient animal. Similarly, double mutations of *Met* and *gce* result in precocious and enhanced caspase-dependent PCD during larval molts, which results from the loss of JH signal opposing the 20E action.

We have previously reported that Met overexpression also accelerates 20E-triggered PCD and that JH counteracts Met and Gce to prevent 20E-triggered PCD during the larval-pupal transition in *Drosophila* (Liu et al., 2009). Accordingly, we observed the same phenotypes in both gain-of-function and loss-of-function of *Met* and *gce*. One reasonable explanation is that Met and Gce involve both 20E and JH signaling pathways. They are more critical for JH action during larval molts, while they are more important for 20E action during the larval-pupal transition. Although further investigations are required to understand the detailed molecular mechanisms of how Met and Gce mediate the JH-20E crosstalk, there are currently at least two lines of supporting evidence. First, Met physically binds EcR and USP (Li et al., 2007); second, Met binds the p160/SRC/NCoA-like molecule, a critical transcriptional co-activator for both JH and 20E to induce gene expression (Bai et al., 2000; Zhu et al., 2006; Li et al., 2011; Zhang et al., 2011). We assume that Met, Gce,

Taiman, and other unknown transcriptional regulators not only transduce JH signal to induce *Kr-h1* expression but also mediate JH-20E crosstalk in *Drosophila*.

Taken together, we conclude that Met and Gce are functionally redundant in transducing the *status quo* action of JH in *Drosophila*. This study lays a foundation to finally elucidate the JH signal transduction pathway and to understand the intricate JH-20E crosstalk. At present, probably the most urgent and critical issue is to identify whether Met and Gce are the actual JH receptors. Since Met binds JH at physiological concentrations *in vitro* (Miura et al., 2005), JH has no "*status quo*" action in the *Met* and *gce* double mutant, which dies during the larval-pupal transition, the most persuasive evidence to demonstrate that Met and Gce are the JH receptors could be a co-crystallization of JH and Met (and/or Gce) showing a hydrophobic JH binding pocket within the JH receptor.

A



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Dm-Met,       36 NGREARNLAEKQRRDKLNASIQELATMVPHAAESSRRLDKTAVLRFATHGLRLQYVFGKS
                 ****** *** ****** ***** ***** *** ** ********* * ***   ** *

Dm-gce,      337 LMQQRPQ---------------------ITDTLMDMLDSFFLTLTCHGHILLISASIEQ
Dm-Met,       96 ASRRRKKTGLKGTGMSASPVGDLPNPSLHLTDTLMQLLDCCFLTLTCSGQIVLVSTSVEQ
                    *                          *****  **  ****** * * * * * **

Dm-gce,      375 HLGHCQSDLYGQSIMQITHPEDQNMLKQQLIPTELENLFDAH------------------
Dm-Met,      156 LLGHCQSDLYGQNLLQITHPDDQDLLRQQLIPRDIETLFYQHQHHQQQGHNPQQHSTSTS
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Dm-gce,      417 ---GDSDAEGEP---------RQRSKAEED-------------------AIDRKLREDRR
Dm-Met,      216 ASTSGSDLEEEEMETEEHRLGRQQGEADDDEDHPYNRRTPSPRRMAHLATIDDRLRMDRR
                      ** * *          **   *  *                     **  ** ***

Dm-gce,      446 SFRVRLARAGPRSEPTA-YEVVKIDGCFRRSDEAPRGVRSNHFSSNLQLIRRTRGRD---
Dm-Met,      276 CFTVRLARASTRAEATRHYERVKIDGCFRRSDSSLTGGAAANYPIVSQLIRRSRNNNMLA
                 * ******  * * *  ** **********    *          ***** *

Dm-gce,      502 ----------------DVIP---LHTISGNDIILTGCARIIR---PPKIASRLI------
Dm-Met,      336 AAAAVAAEAATVPPQHDAIAQAALHGISGNDIVLVAMARVLREERPPEETEGTVGLTIYR
                           * *    ** ****** *   **  *   **

Dm-gce,      534 --DANTLEYKTRHLIDGRIIDCDQRIGIVAGYMTDEVRNLSPFTFMHNDDVRWVIVALRQ
Dm-Met,      396 QPEPYQLEYHTRHLIDGSIIDCDQRIGLVAGYMKDEVRNLSPFCFMHLDDVRWVIVALRQ
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Dm-gce,      592 MYDCNSSYGESTYRLFTRNGNIIYLQSKGYLEIDKETNKVHSFVCVNTLLGEEEGKRRVQ
Dm-Met,      456 MYDCNSDYGESCYRLLSRNGRFIYLHTKGFLEVDRGSNKVHSFLCVNTLLDEEAGRQKVQ
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Dm-gce,      652 EMKKKFSVIINTQIP--QSTIDVPASEHPALLEKAVLRLIQNLQKS
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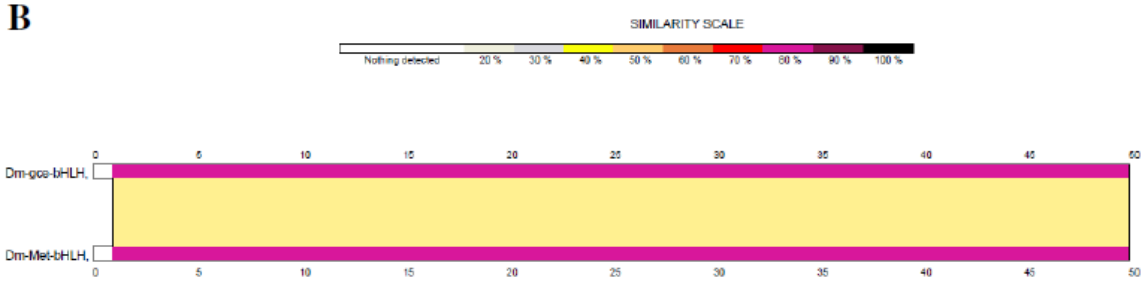
B

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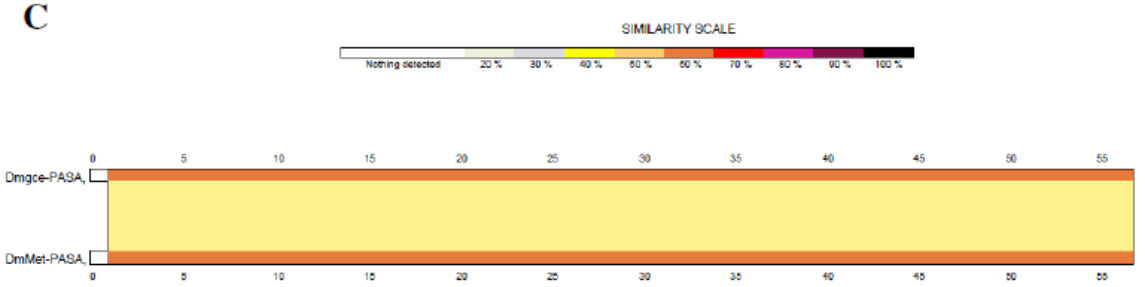
C

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DmMet-PASA      1 LMQLLDCCFLTLTCSGQIVLVSTSVEQLLGHCQSDLYGQNLLQITHPDDQDLLRQQL
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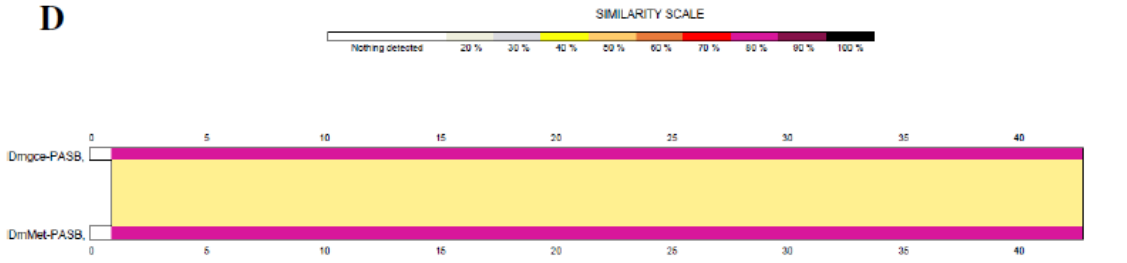


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86.0% identity in 43 residues overlap; Score: 198.0; Gap frequency: 0.0%

Dmgce-PASB      1 EYKTRHLIDGRIIDCDQRIGIVAGYMTDEVRNLSPFTFMHNDD
DmMet-PASB      1 EYHTRHLIDGSIIDCDQRIGLVAGYMKDEVRNLSPFCFMHLDD
                  ** ******* ********* ***** ********* *** **
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Fig. 2.1. Amino acid sequences comparison between *Dm-gce* and *Dm-Met* using SIM - Alignment Tool for protein sequences and the graphic view was made by LALNVIEW program. (A) the whole amino acids alignment, (B) bHLH domain alignment, (C) PAS-A domain alignment, (D) PAS-B domain alignment. (http://www.expasy.ch/tools/sim-prot.html).

Asterisks denote perfect identities in the alignment positions.

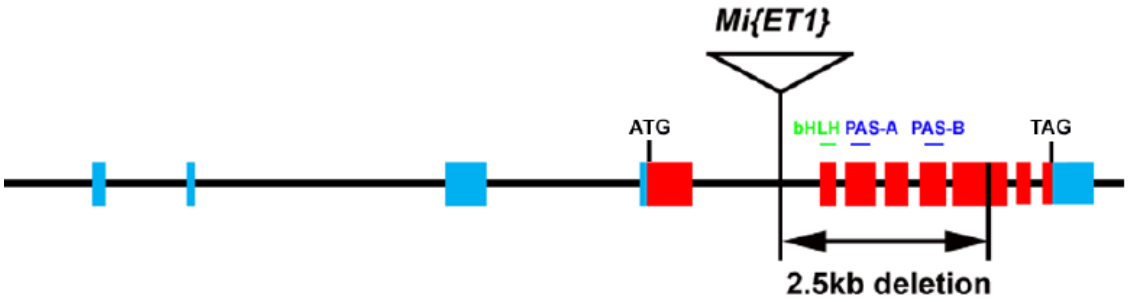

Fig. 2.2. The diagram of *gce* gene structure shows the insertion of *Mi{ET1}MB07696* and position of the 2.5-kb deletion in the $gce^{2.5k}$ allele. The *gce* coding sequence is highlighted in red. The *gce* exons that encode the functional motifs of bHLH, PAS-A, and PAS-B are marked.

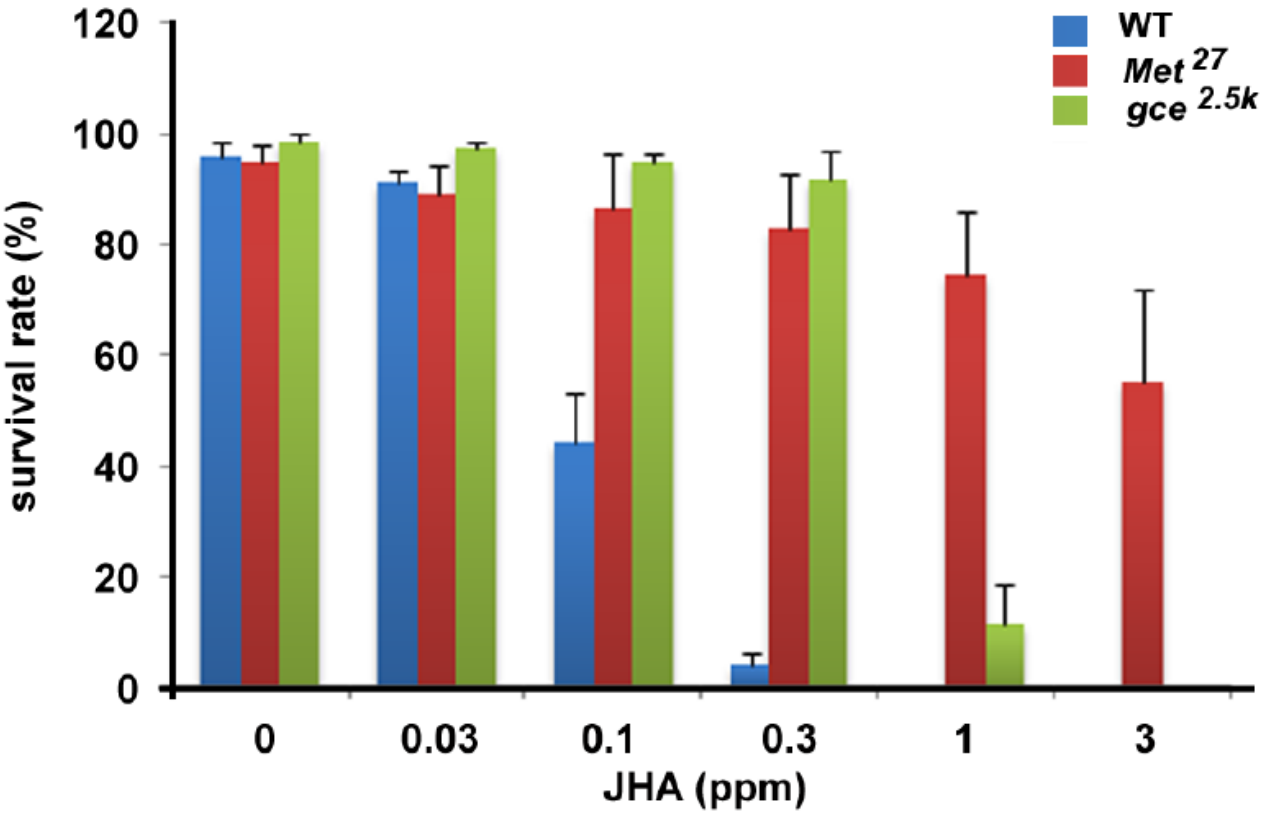

Fig. 2.3. A null allele of *gce* is resistant to JHA. One hundred newly hatched larvae of wildtype, Met^{27}, and $gce^{2.5k}$ were reared on normal food or food containing different concentrations of JHA, pyriproxifen. The percentages of individuals that develop into adults are shown as the mean of 10 replicates ± standard deviation.

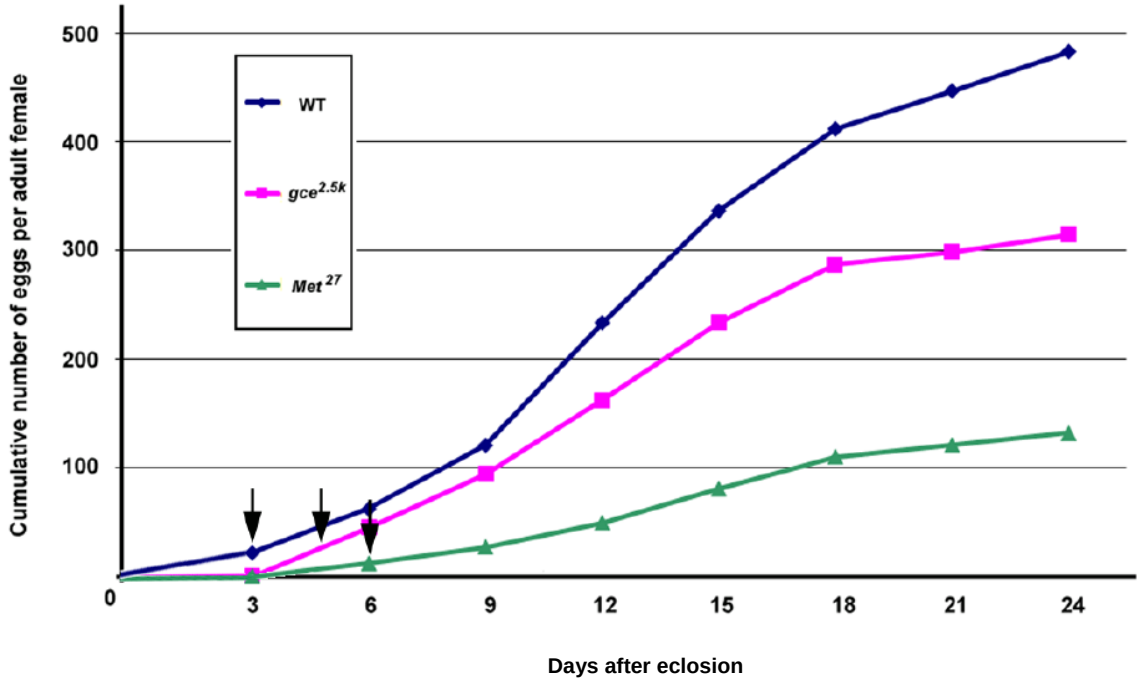

Fig. 2.4. *Met*^{27} and *gce*$^{2.5k}$ mutations affect ovary development and fecundity

Single newly eclosed virgin females of wildtype, *Met*^{27} and *gce*$^{2.5k}$ were crossed with three wildtype males individually. Cumulative numbers of eggs laid by a single female are shown as the mean of ten independent experiments. Arrows point to the average ages when females start to lay eggs.

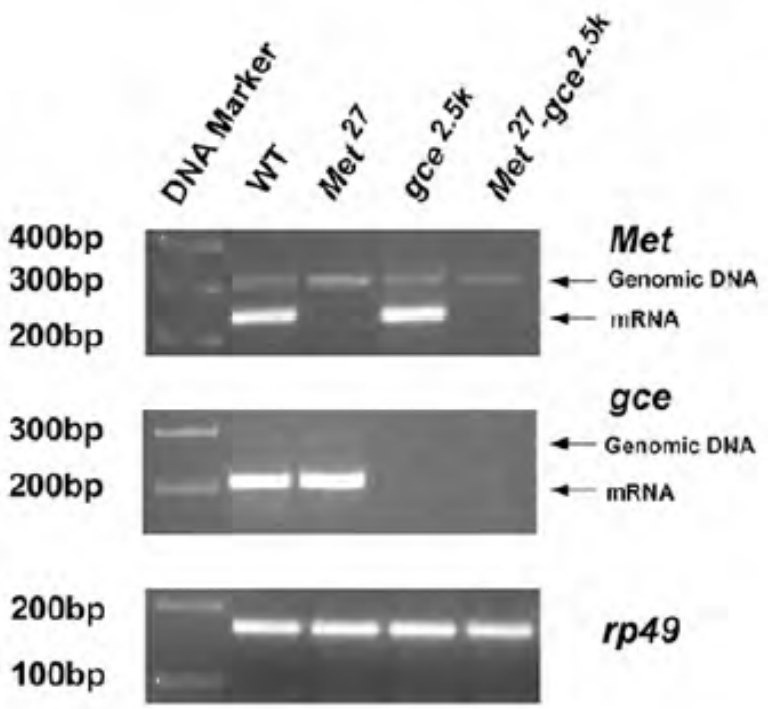

Fig. 2.5. *Met*^{27}-*gce*$^{2.5k}$ double mutations cause prepupal lethality.

DNA agarose gel electrophoresis of reverse transcription PCR products demonstrates that *Met*^{27}-*gce*$^{2.5k}$ double mutations are null for both *Met* and *gce*. Total RNAs were isolated from the 2nd instar larvae of wildtype, *Met*^{27}, *gce*$^{2.5k}$, and *Met*^{27}-*gce*$^{2.5k}$ flies.

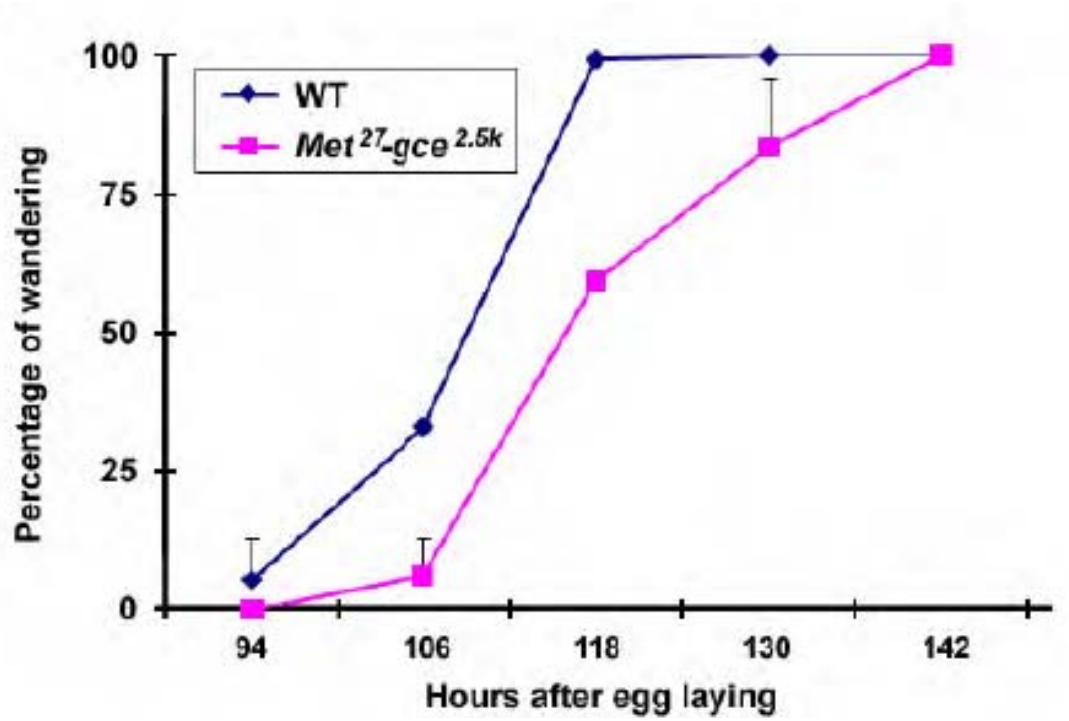

Fig. 2.6. *Met*^{27}*-gce*$^{2.5k}$ double mutations cause prepupal lethality.

One hundred eggs of wildtype and *Met*^{27}*-gce*$^{2.5k}$ were reared on normal food. Cumulative percentages of larvae developing to wandering stage are shown.

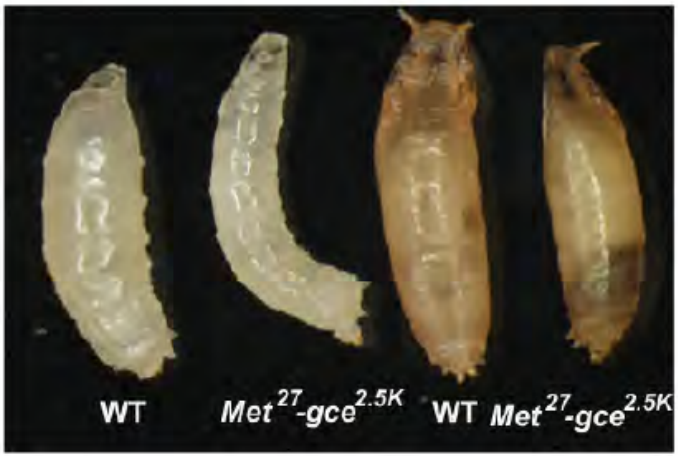

Fig. 2.7. *Met^{27}-gce$^{2.5k}$* double mutations cause prepupal lethality.

Images are of wandering larvae and early pupae of wildtype and *Met^{27}-gce$^{2.5k}$* reared on normal food, showing the reduced body size of *Met^{27}-gce$^{2.5k}$*.

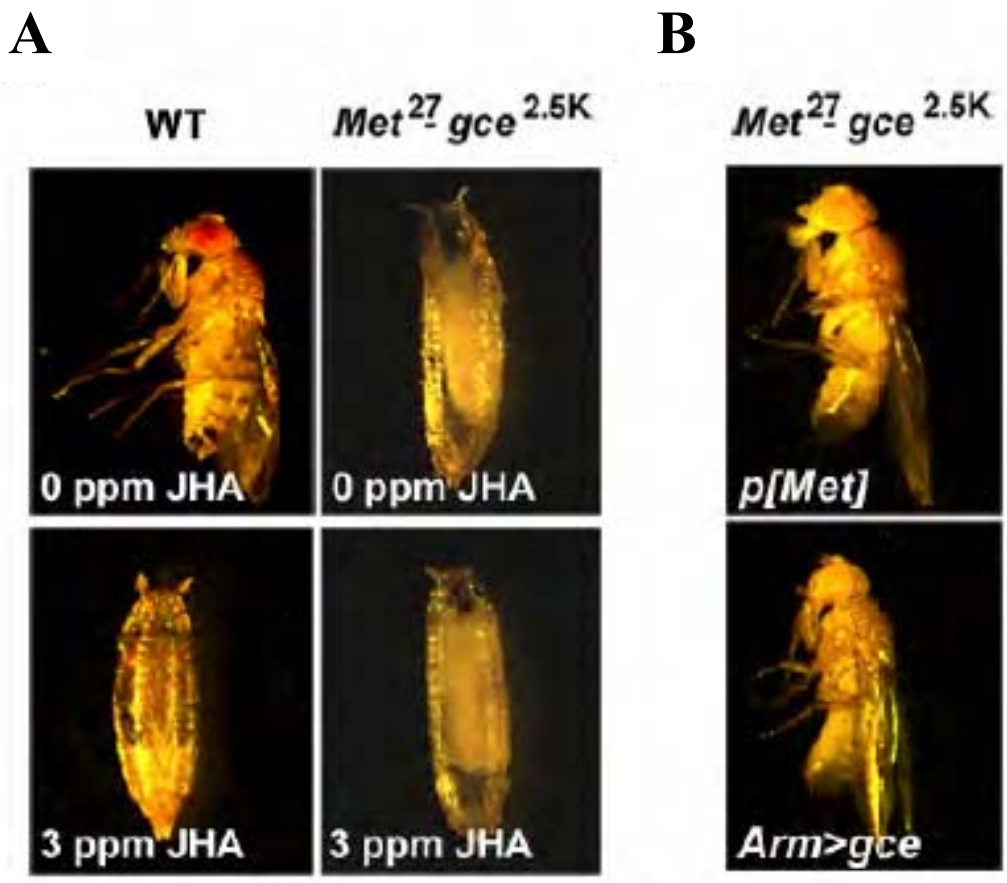

Fig. 2.8. *Met*^{27}-*gce*$^{2.5k}$ double mutations cause prepupal lethality.

(A) Wildtype and *Met*^{27}-*gce*$^{2.5k}$ flies were reared on normal or 1 ppm pyriproxifen-containing food. Images show the final developmental stages of these flies. *Met*^{27}-*gce*$^{2.5k}$ die ~24 hours after pupariation and fail to undergo head eversion. When reared on food containing 3 ppm JHA, all wildtype animals died at the late pupal stage, but the development of *Met*^{27}-*gce*$^{2.5k}$ was not affected by JHA.

(B) The prepupal lethality of *Met*^{27}-*gce*$^{2.5k}$ flies can be fully rescued by transgenic *Met* or *gce*. The genotypes of flies are *Met*^{27}-*gce*$^{2.5k}$*/y; p{Met}/+* (top) and *Met*^{27}-*gce*$^{2.5k}$*/y; arm-GAL4/UAS-gce* (bottom).

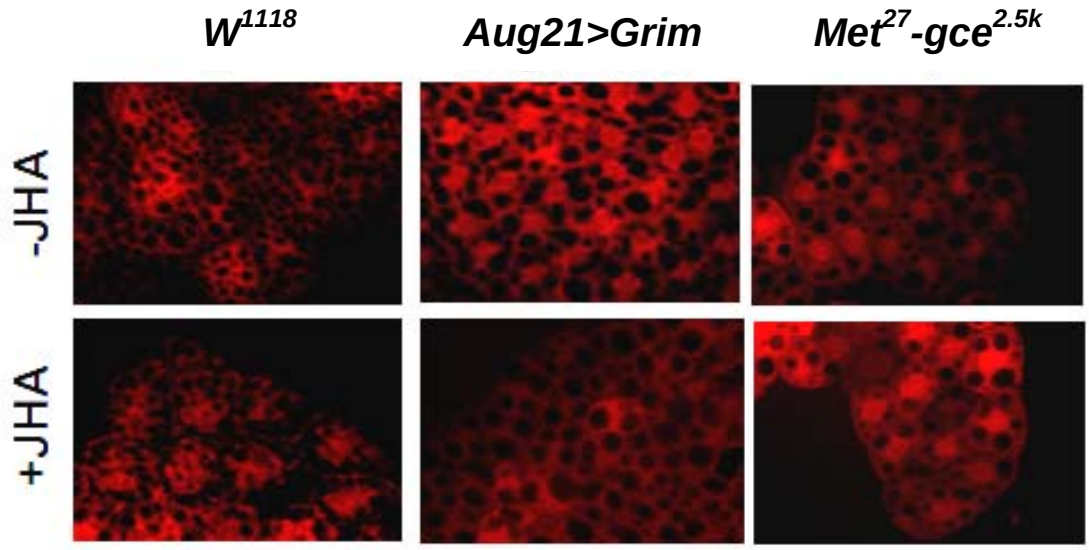

Fig. 2.9. Fat body cells of *Met*^{27}*-gce*$^{2.5k}$ larvae undergo precocious and enhanced caspase-dependent programmed cell death.

Wildtype, *Aug21-GAL4>UAS-grim* (*Aug21>grim*) and *Met*^{27}*-gce*$^{2.5k}$ were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food.

Fat bodies of 2nd instar larvae were stained with the Caspases 3 & 7 Apoptosis Detection Kit (Invitrogen, CA). Apoptotic cells are marked with red.

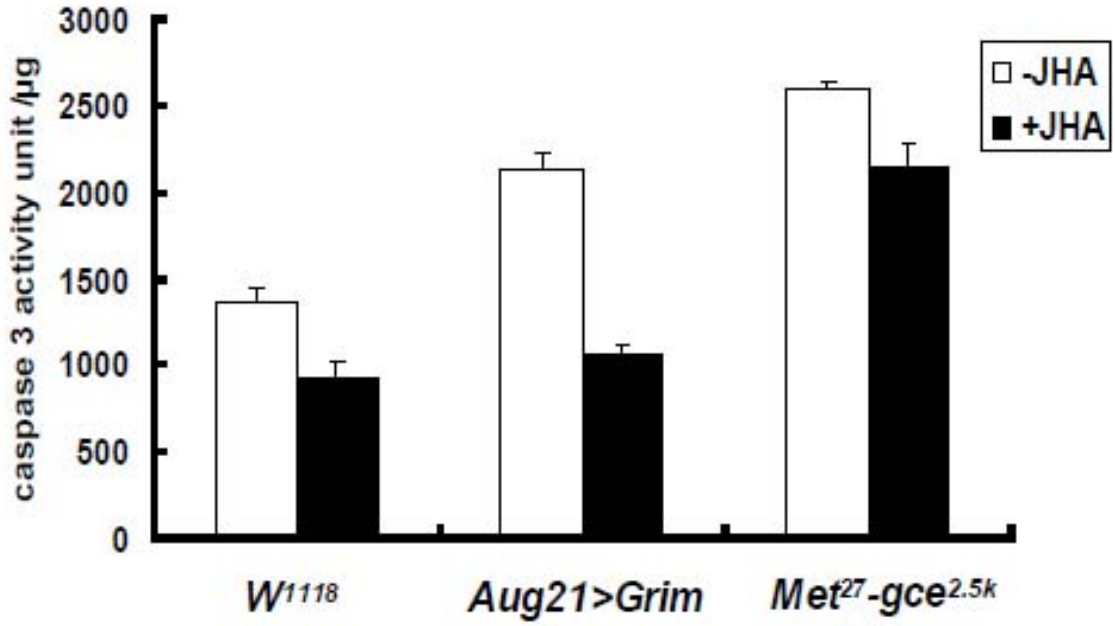

Fig. 2.10. Fat body cells of *Met*^{27}*-gce*$^{2.5k}$ larvae undergo precocious and enhanced caspase-dependent programmed cell death.

Wildtype, *Aug21-GAL4>UAS-grim* (*Aug21>grim*) and *Met*^{27}*-gce*$^{2.5k}$ were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food.

Caspase 3 activity in the fat bodies of 2nd instar larvae was assessed using the Caspase 3 Activity Assay kit (Beyotime, Shanghai, China). Values are the mean of three independent experiments ± standard deviation.

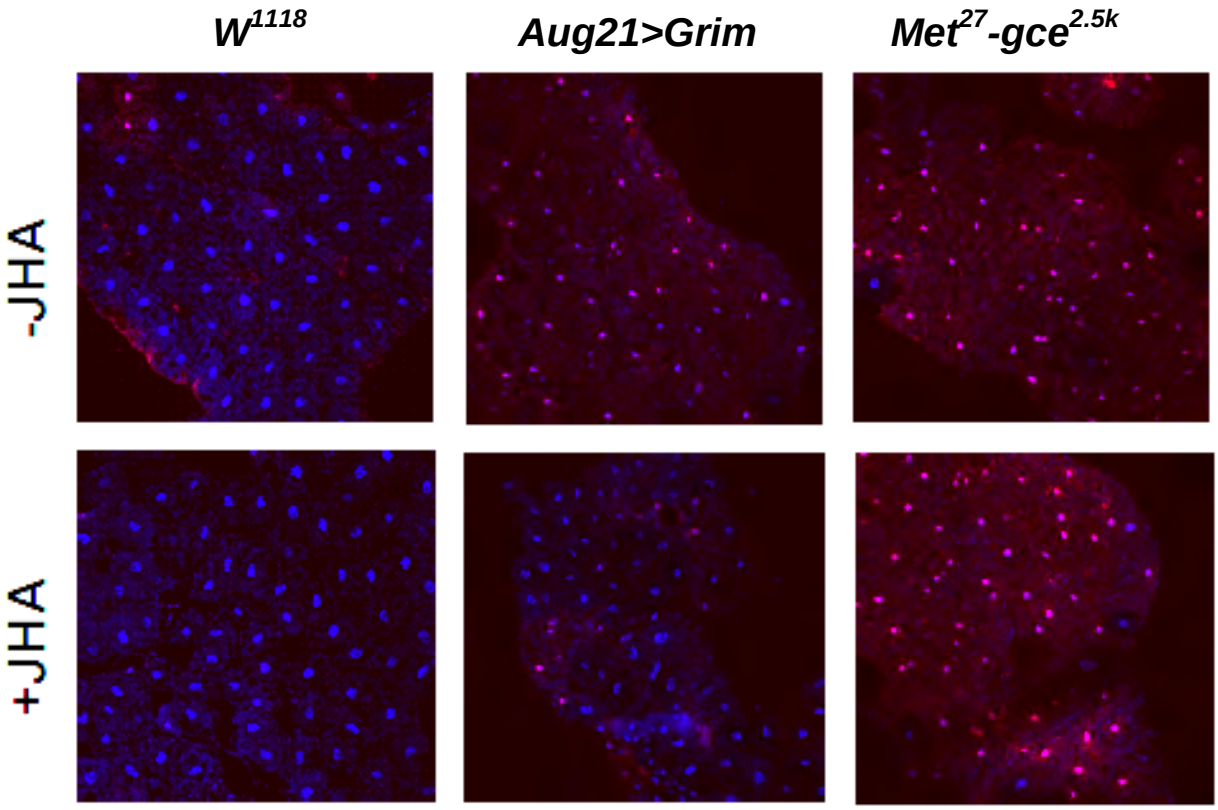

Fig. 2.11. Fat body cells of *Met*^{27}*-gce*$^{2.5k}$ larvae undergo precocious and enhanced caspase-dependent programmed cell death.

Wildtype, *Aug21-GAL4>UAS-grim* (*Aug21>grim*) and *Met*^{27}*-gce*$^{2.5k}$ were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food.

Disrupted plasma membrane was detected by propidium iodide staining (red) and nuclei were labeled by Hoechst 33342 (blue) in the fat bodies of pupae at 4 hours after pupariation.

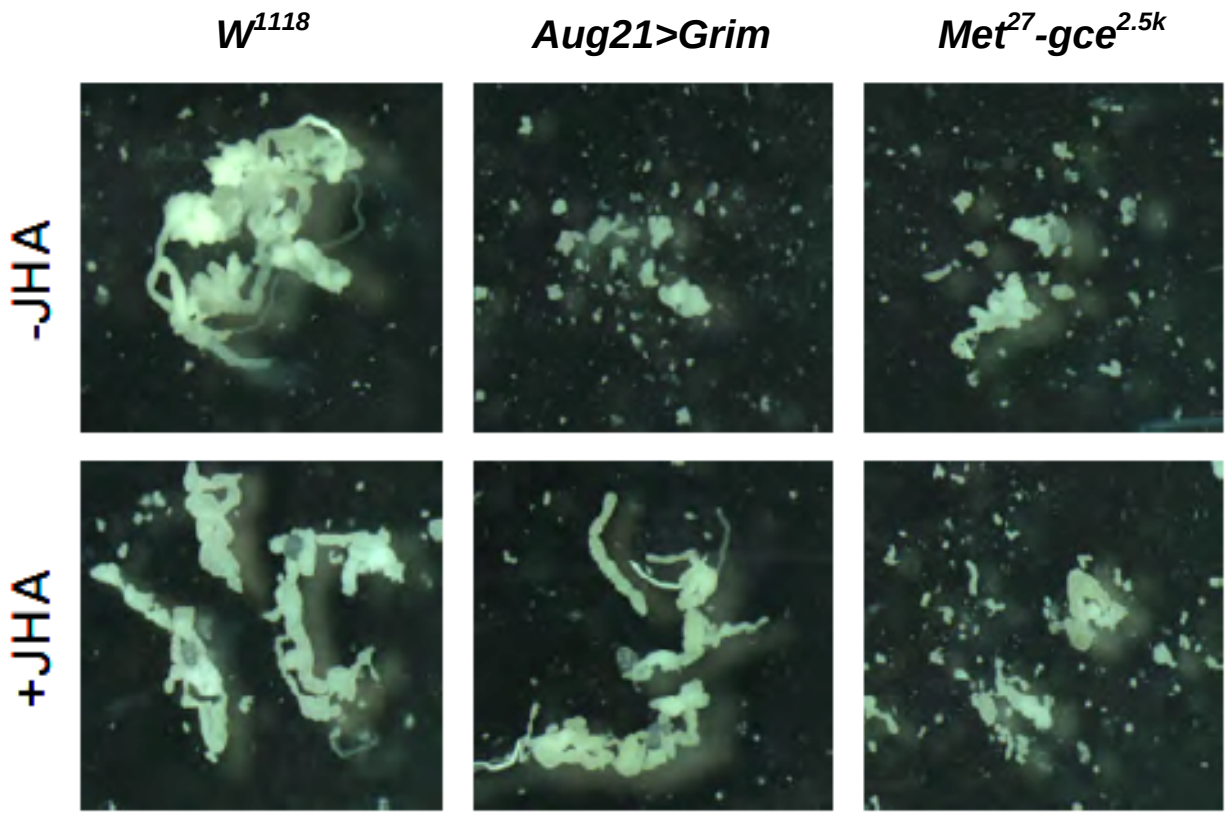

Fig. 2.12. Fat body cells of *Met*^{27}*-gce*$^{2.5k}$ larvae undergo precocious and enhanced caspase-dependent programmed cell death.

Wildtype, *Aug21-GAL4>UAS-grim* (*Aug21>grim*) and *Met*^{27}*-gce*$^{2.5k}$ were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food.

Fat bodies were dissected at 8 hours after pupariation to show precocious cell dissociation in *Met*^{27}*-gce*$^{2.5k}$ and *Aug21>grim* flies.

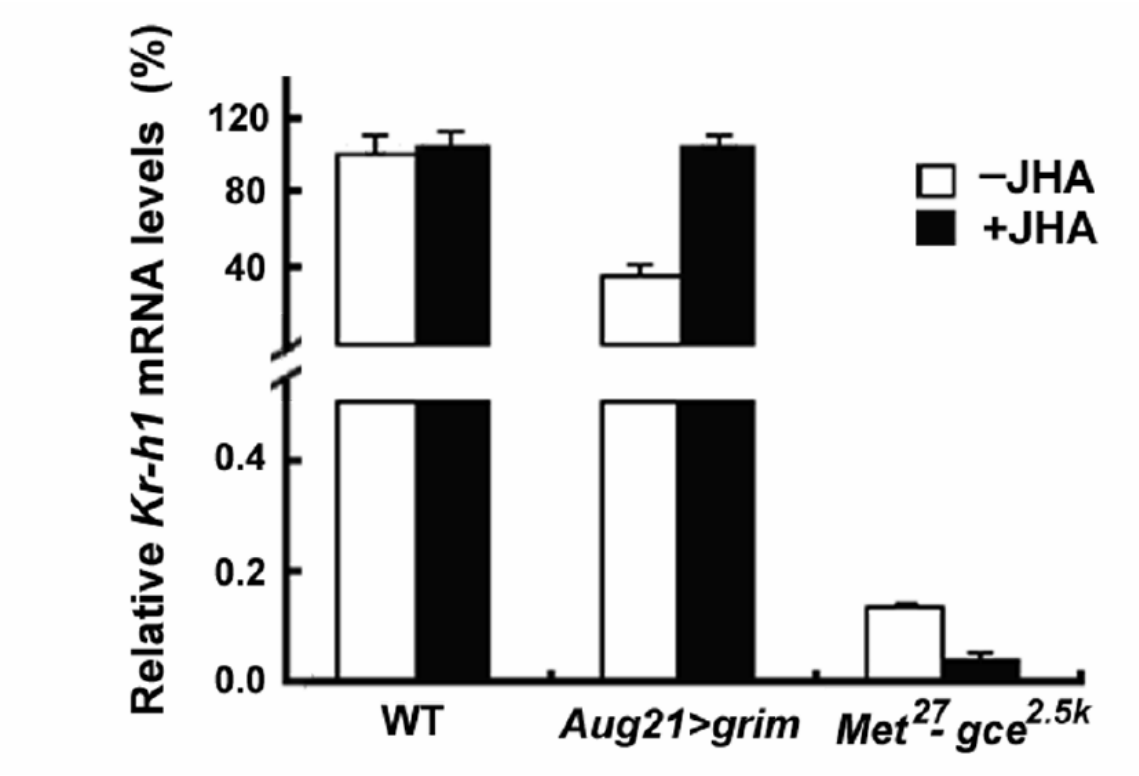

Fig. 2.13. Met and Gce transduce the JH signal to induce *Kr-h1* expression.
Wildtype, *Aug21-GAL4>UAS-grim* (*Aug21>grim*), and Met^{27}-$gce^{2.5k}$ were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Relative *Kr-h1* mRNA levels in the 2nd instar larvae were assessed by quantitative real-time PCR and normalized to *rp49* mRNAs. Values are the means of three independent experiments ±standard deviation.

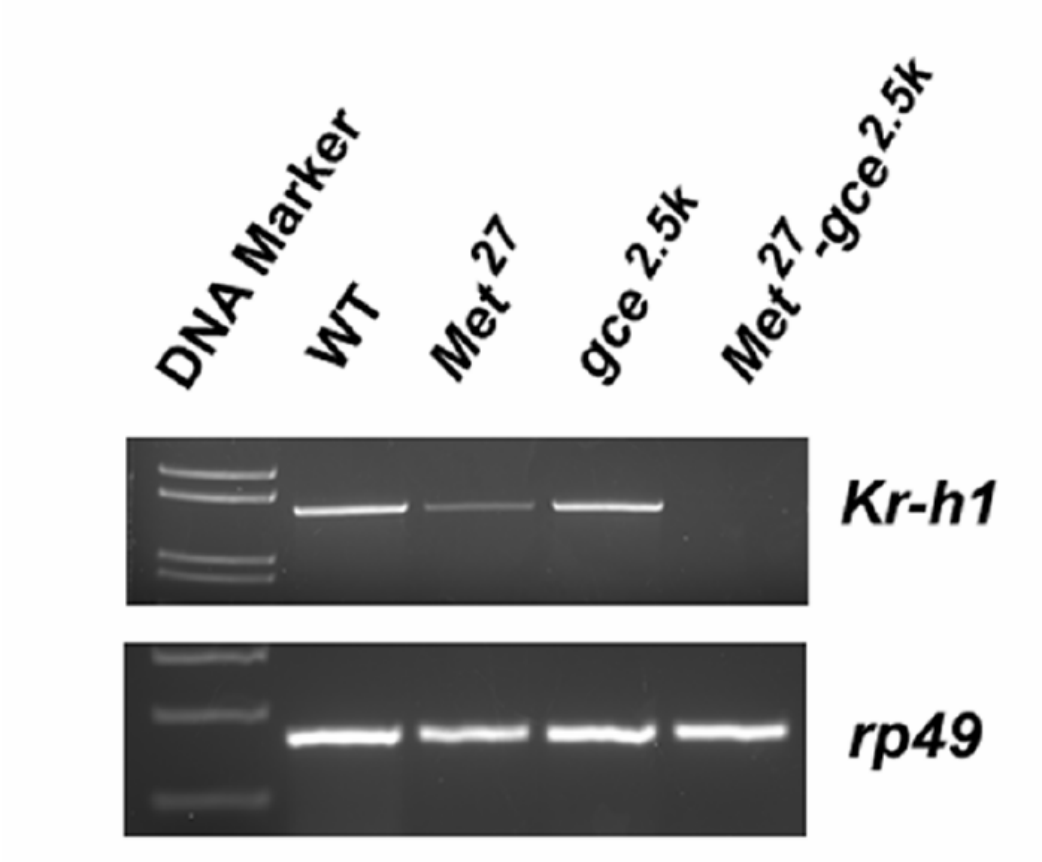

Fig. 2.14. Met and Gce transduce the JH signal to induce *Kr-h1* expression.
Total mRNAs were isolated from wildtype, Met^{27}, $gce^{2.5k}$, and Met^{27}-$gce^{2.5k}$ 2nd instar larvae. Reverse transcription PCR was conducted with 30 cycles. PCR products were analyzed by agarose gel electrophoresis.

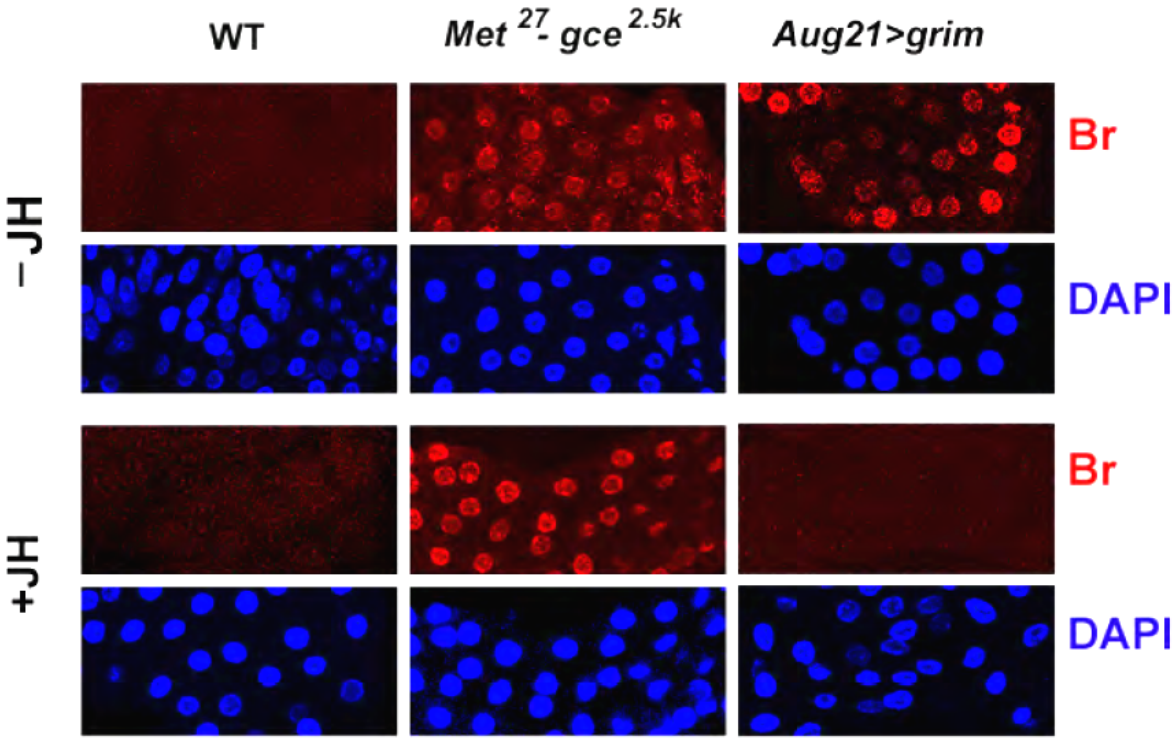

Fig. 2.15. Met and Gce are required for *br* repression in young larvae.

Wild type, *Met*^{27}-*gce*$^{2.5k}$, and *Aug21-GAL4>UAS-grim* (*Aug21>grim*) were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Fat bodies of the 2nd instar larvae were dissected and stained with Br-core antibody (red). Nuclei were labeled with DAPI (blue).

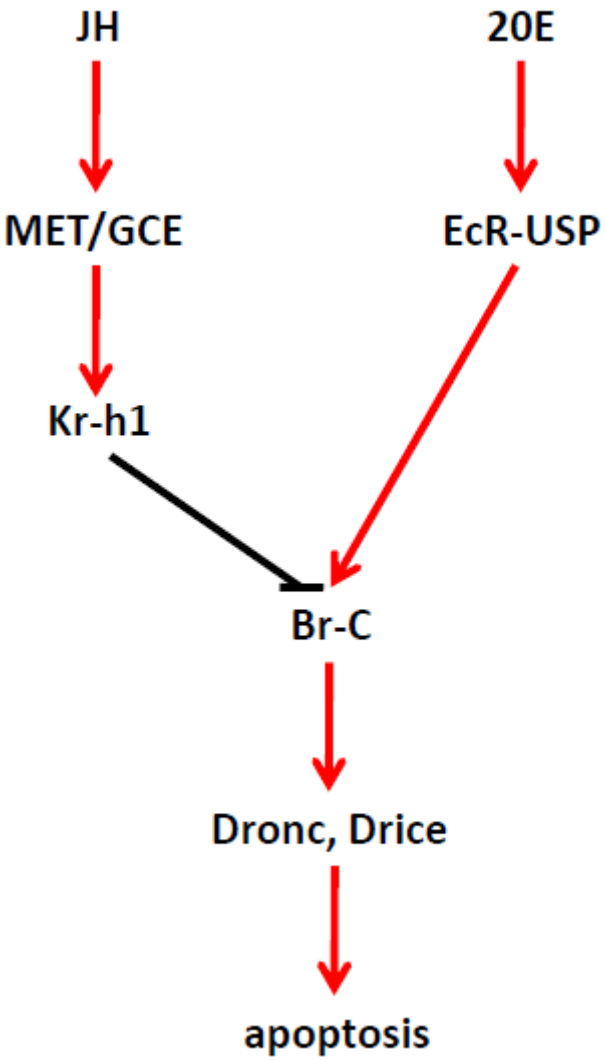

Fig. 2.16. A model for Met and Gce in transducing JH action to prevent 20E-induced programmed cell death.

As described in the text, this model suggests how Met and Gce transduce the JH signal to suppress programmed cell death during larval molts.

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Chapter 3

A Genetic Screen for Genes Involved in Juvenile Hormone Signaling and Biosynthesis in *Drosophila melanogaster*

ABSTRACT

In insects, juvenile hormone (JH) is a key regulator which coordinates with ecdysone (Ec) in regulating growth and metamorphosis. Ec orchestrates the molting process, whereas the nature of the molt is determined by the presence of JH at critical JH-sensitive periods. At the molecular level, Ec and JH co-regulate expression of a small subset of critic genes. The protein products of these genes are usually transcriptional factors which control the expression of large amount of other genes to induce the appropriate biological processes. One example of these critical genes is *broad* (*br*), which was called *Broad-Complex* (*BR-C*) previously. We demonstrated that JH signaling is required to repress *br* expression in the younger *Drosophila* larvae. Accordingly, a genetic screen was designed to dissect JH biosynthesis and signaling pathways based on *br* expression in the 2nd instar larvae.

About 4,400 lethal *p*-insertion or mutant lines were collected from Bloomington Stock Center and tested their impact on *br* expression. 55 mutations were isolated based on the precocious *br* expression in the 2nd instar larvae. Genes associated with these mutations involve various molecular and cellular functions, such as transcription factors, signaling molecules, and enzymes.

From the screen results, three genes or signaling pathways were reported to be involved in regulating JH biosynthesis. They are transcriptional factor *apterous*; insulin-like peptide receptor *InR*, and neuron transmitter *NMAR1*. Interestingly, our screen identified mutations of all three genes as well as JH synthesis enzyme *Fpps*, indicating the high efficiency of this screen. Also our screen identified two main components of TGF-β signaling, *thick vein* (*tkv*) and *mothers against Dpp* (*mad*) to be involved in JH biosynthesis. Further more, we identified those mutations in three Wnt signaling component genes, *Axin* (*Axn*), *supernumerary limbs* (*slmb*), and *naked cuticle* (*nkd*), induced precocious *br* expression.

INTRODUCTION

Juvenile hormone (JH) is a critical hormone that regulates many aspects of insect physiology. One main role of JH is its classic "status quo" action in the regulation of insect development. When 20-hydroxyecdysone (20E) induces molting during early developmental stages, the presence of JH ensures that the molt results in a repeat of the previous stage (Williams, 1961; Riddiford, 1996; 2008; Gilbert et al., 2000). Therefore, JH does not block the 20E-coordinated molting process, but rather directs the action of 20E. During the last two decades, studies on the hormonal regulation of insect development have focused on understanding the molecular basis of 20E, JH, and their interaction.

At the molecular level, 20E binds to its heterodimer receptor, EcR/USP, to directly activate the transcription of a small set of early-response genes that encode transcriptional factors. These genes transduce and amplify the original hormonal

signal by activating a large number of late-response genes that encode tissue-specific effector proteins necessary for insect molts and metamorphosis (Thummel, 2002). One of the 20E-induced early genes, *broad* (*br*), was identified as a key regulator in mediating the cross-talk between the 20E and JH signaling pathways. *Drosophila br* encodes four transcriptional factors that contain a common N-terminal domain and four pairs of different C2H2 DNA-binding zinc finger domains (DiBello et al., 1991; Bayer et al., 1996). The Br proteins directly regulate the transcription of 20E-induced late genes and are essential for the specification of pupal development (Crossgrove et al., 1996; Zhou and Riddiford, 2002). Null *br* mutants can develop normally to the final larval instar but cannot undergo pupal formation (Kiss et al., 1976, 1988). Moreover, ectopic expression of *br* in early 2nd instar larvae induces premature pupal formation (Zhou et al., 2004). Therefore, the Br proteins are necessary and sufficient for the initiation of insect metamorphosis. Consistent with its function, the Br proteins are predominantly expressed during the larval-pupal transition in all of the examined holometabolous insects (Dubrovsky, 2005). Previous studies in *Manduca*, *Bombyx*, and *Tribolium* suggested that the temporal pattern of *br* expression results from the 20E and JH interaction. 20E directly induces *br* expression, which can be prevented by JH in young larvae (Zhou et al., 1998; Reza et al., 2004; Konopova and Jindra, 2008). Here, we demonstrate that JH is also required to repress *br* expression during early larval stages in *Drosophila*.

JH transduces its signal through Methoprene-tolerant (Met), Germ cell-expressed (Gce) and Krüppel-homolog 1 (Kr-h1) and the p160/SRC/NCoA-like molecule (Taiman in *Drosophila* and FISC in *Ades*). The *Drosophila Met* and *gce*

genes encode two functionally redundant bHLH-PAS protein family members, which have been proposed to be components of the elusive JH receptor (Wilson and Ashok, 1998; Baumann et al., 2010; Abdou et al., 2011). Both *Met* and *gce* mutants are viable and resistant to JH analogs (JHA) as well as to natural JH III (Wilson and Fabian, 1986; Abdou et al., 2011). However, *Met-gce* double mutants are prepupal lethal and phenocopies CA-ablation flies (Liu et al., 2009; Riddiford et al., 2010; Abdou et al., 2011). The Met protein binds JH III with high affinity (Shemshedini and Wilson, 1990; Miura et al., 2005). In *Tribolium*, suppression of *Met* activity by injecting double-stranded (ds) *Met* RNA causes precocious metamorphosis (Konopova and Jindra, 2007). Kr-h1 is considered as a JH signaling component working downstream of Met. In both *Drosophila* and *Tribolium*, *Kruppel-homolog1* (*Kr-h1*) mRNA exhibits high levels during the embryonic stage and is continuously expressed in the larvae; then, it disappears during pupal and adult development (Pecasse et al., 2000; Minakuchi et al., 2008, 2009). *Kr-h1* expression can be induced in the abdominal integument by exogenous JH analog (JHA) at pupariation (Minakuchi et al., 2008). Suppression of *Kr-h1* by dsRNA in the early larval instars of *Tribolium* causes precocious *br* expression and premature metamorphosis after one succeeding instar (Minakuchi et al., 2009). Thus, Kr-h1 is necessary for JH to maintain the larval state during a molt by suppressing *br* expression. Studies in *Aedes*, *Drosophila* and *Tribolium* have demonstrated that the p160/SRC/NCoA-like molecule is also required for JH to induce expression of *Kr-h1* and other JH response genes (Li et al., 2011; Zhang et al., 2010). For example, *Ades* FISC forms a functional complex with Met on

the JH response element in the presence of JH and directly activates transcription of JH target genes (Li et al., 2011).

In an attempt to isolate other genes involving JH signaling, we conducted a novel genetic screen

MATERIALS AND METHODS

Fly Strains and Genetics

All fly works were performed based on the standard procedure described in Chapter 2. The *GAL4-PG12* line was a gift from H.-M. Bourbon (Bourbon et al., 2002). All lethal mutant lines used in the genetic screen were obtained from the Bloomington *Drosophila* Stock Center (BDSC).

Generation of *hs-jhe* transgenic flies

To generate *hs-jhe* transgenic flies, total RNA was extracted by TRIzol® Reagent (Invitrogen) as follow:

Homogenize 20 3rd instar larvae in 1 ml TRIzol® Reagent and incubate the sample for 5 min at room temperature. Add 200 µl chloroform, mix with vigorous shaking for 15 seconds and incubate at room temperature for 2-3 min. Centrifuge samples 15 min at 12,000 x g at 4°C. Transfer 800 µl of the colorless upper aqueous phase to a fresh tube. Precipitate the RNA by mixing the sample with 400 µl of isopropanol and incubate at room temperature for 10 min. Then, centrifuge for 10 min at 12000 x g at 4°C. The RNA pellet will be visible on the side of the tube. Remove the supernatant. Wash pellet with 1 ml 75% ethanol by flicking and inverting the tube or vortexing.

Centrifuge the sample at 7500 x g for 5 min at 4°C. Discard the supernatant, air dry, dissolve the RNA pellet in 30 µl RNase free water and store at -80^{o}C.

jhe cDNA was isolated by RT-PCR using SuperScript® III One-Step Reverse Transcriptase Kit with Platinum® Taq DNA (Invitrogen) as follow:

To a 0.2 ml PCR tube, add 25 µl 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM MgSO4), 1 µg template RNA, 1µl Sense primer (10 µM), 1µl Anti-sense primer (10 µM), 2 µl SuperScript® III RT/ PlatinumR Taq Mix, and add RNase free water to 50 µl.

Place the reaction in the preheated thermal cycler programmed as follow:

cDNA synthesis for 1 cycle at 55°C for 30 min – Denaturation for 1 cycle at 94°C for 2 min - PCR amplification for 40 cycles (94°C for 15 s, 60°C for 30 s, 68°C for 1 min) - Final extension for 1 cycle at 68°C for 5 min.

Primer sequences: Forward 5'- ATTCCGCGGCAAatgctacaactgctgcttcttg-3' and reverse 5'- ATTTCTAGAttacttttcgttgagtatatgc-3'. The PCR product was visualized by agarose gel electrophoresis stained with Ethidium Bromide to confirm the right size by comparing with DNA ladder Plus marker (Invitrogen) and recovered using Gel Purification Kit (QIAGEN) according to the manufacture protocol. The purified DNA fragment, which carries Not1 and Xba1 restriction enzyme cut cites that were introduced by PCR primers, was subcloned into *pCaSpeR-hs* plasmid with the same restriction enzymes (Not1 & Xba1).

Transgenic fly lines were generated by *P* element-mediated germline transformation at Rainbow Transgenic Flies, Inc (Camarillo, CA). The *P-element* insertion on the

second chromosome was detected through genetic crosses as described before and kept as homozygous viable transgenic flies carrying two copies of *hs-jhe*.

Heat-shock treatment:

To activate the JH esterase in the transgenic flies, the newly hatched larvae were heat shocked by placing the vials in water path at 38^{o}C for one hour two times a day for two days.

Immunohistochemistry and Microscopy

Larval fat bodies were dissected from the 2nd, early 3rd, or late 3rd instar larvae and stained with the Br- core antibody (25E9.D7, from the DSHB at the University of Iowa) against endogenous Br proteins. Immunohistochemistry was performed as described previously. Florescence signals were captured with Zeiss LSM710 laser scanning confocal microscope (Carl Zeiss) and processed with Adobe Photoshop.

JHA Treatment

The JHA pyriproxyfen (Sigma) was dissolved in 95% ethanol to give a 300 ppm stock solution. JHA-containing fly food was prepared by adding JHA stock solution to the standard cornmeal-molasses-yeast food at 50-55°C to a final concentration of 0.3 ppm.

Western Blotting

Protein extracts isolated from the eggs, 1st, 2nd, early 3rd, and late 3rd instar larvae as well as white pupae, late pupae, adult males, and adult females were analyzed by standard SDS–PAGE and Western blot.

Sample preparations:

Fresh sample from each stage was ground in Laemmli Sample Buffer (Bio-Rad) (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% w/v bromophenol blue) with adding freshly 5% electrophoresis grade β-mercaptoethanol (Sigma), boiled for 5 min, and briefly centrifuged.

Electrophoretic separation procedures:

20 µl of each sample was loaded to 7.5% Tris-HCl 10 well-30 µl comb Ready Gel (Precast Gel Polyacrylamide Electrophoresis; Bio-Rad), 10 µl Prestained dye molecular weight marker was loaded, and 1X Tris/Glycine/SDS Running Buffer was used to separate the samples.

To prepare 1X Running Buffer: Add 45 mL 10X Tris/Glycine/SDS Running Buffer (250 mM Tris, 1.92 M glycine, 0.1% SDS, pH 8.3) to 405 mL distilled water and mix gently.

A constant voltage of 80 V was applied for 2 hrs.

To transfer proteins from the gel to Nitrocellulose Membrane, all the folder layers should be soaked in Transfer Buffer (25mM Tris, 192mM glycine, 20% methanol, pH8.3) prior to assembling the sandwich.

The transfer folder was assembled in a large, baking dish containing enough transfer buffer in the order as described below:

(+) POSITIVE ELECTRODE (ANODE)

- Plastic cassette

- Sponge pad (Scotch-Brite pads)

- Mini Trans-Blot filter papr (Bio-Rad) (or three Sheets Whatman 3MM)

- WestClear Nitrocellulose Membrane pore size 0.2 μm (GenScript).

- Polyacrylamide gel

- Mini Trans-Blot filter papr (Bio-Rad) (or three Sheets Whatman 3MM)

- Sponge pad (Scotch-Brite pads)

- Plastic cassette

(-) NEGATIVE ELECTRODE (CATHODE)

Avoid air bubbles between any of the layers.

Proteins were transferred from the gel to nitrocellulose membrane for 45 min at 80 Volts. Complete transfer can be detected by the presence of the protein markers in the membrane. The membrane then was subjected to Western Blotting analysis by using ONE-HOUR Western™ Detection System (GenScript) according to the manufacture protocol.

The expression of β-tubulin was used as a loading control. Br mouse monoclonal antibody Br-core (25E9.D7) (Emery et al., 1994) and β-tubulin mouse monoclonal antibody (AA12.1) were from the Developmental Studies Hybridoma Bank at the University of Iowa, and they were used as primary antibodies.

The signal was developed by using LumiSensor™ Chemiluminescent HRP Substrate, and the signal was captured by exposing the membrane to Kodak x-ray film.

RESULTS

***GAL4-PG12* recapitulates the *br* expression pattern:** It is well documented that *br* is a molecular marker for pupal commitment and specifies the larval-pupal metamorphosis in a variety of holometabolous insect species (Riddiford et al., 2003). Western blotting using a *Drosophila* Br-core antibody, which recognizes all 4 Br isoforms (Emery et al., 1994), showed that Br proteins were highly expressed in late 3rd instar larvae and pupae. Conversely, no Br proteins were detected from the embryonic stages to early 3rd instar larval stages or in adults. Interestingly, during the larval-pupal metamorphosis, different Br isoforms exhibited distinct expression profiles, with all 4 isoforms (Z1, Z2, Z3, and Z4) expressed from the late 3rd instar to early pupal stages and only 1 or 2 isoforms (Z1 and/or Z3) expressed in the late pupal stage (Fig. 3.1A).

To monitor *br* expression in live organisms, we examined the expression patterns of GAL4 enhancer-trap lines inserted near the *br* gene. One of these lines, *GAL4-PG12*, closely resembled the temporal and spatial expression pattern of the endogenous *br* gene in tissues other than the salivary gland. In 1st, 2nd, and early 3rd instar larval stages of *GAL4-PG12>UAS-mCD8GFP*, GFP expression was only detected in the salivary gland (Fig. 3.1B-D). This expression of *GAL4-PG12* in the salivary gland is a common feature for most GAL4 lines derived from the *P{GawB}* construct, which may carry a position-dependent, unidentified salivary gland enhancer (Brand et al., 1994). However, in late 3rd instar larvae and early pupae, an intensive GFP signal was

observed in most tissues, including epidermis, muscle, and fat body (FB) tissues (Fig. 3.1E and F). Inverse PCR analysis revealed that *GAL4-PG12* carries a *P{GawB}* construct within the first intron of the *br* gene, which caused pupal lethality (Fig. 3.2).

We next compared the expression pattern of *GAL4-PG12>UAS-mCD8GFP* with that of the *br* gene in the larval FB. Neither endogenous Br proteins nor GFP were detectable in the FB of 2nd and early 3rd instar larvae (Fig 3.1G and H). In late 3rd instar larvae, the Br proteins (red) were observed in the FB nuclei in the same cells as mCD8GFP (green), the cell membrane-attached marker driven by *GAL4-PG12* (Fig.3.1I-I"). These results indicate that *GAL4-PG12* can be used to monitor endogenous *br* expression in all tissues except for the salivary gland.

JH represses *br* expression at early larval stages: To determine whether JH represses *br* expression in early *Drosophila* larval instars, we generated a transgenic fly line that harbors *juvenile hormone esterase* (*jhe*) cDNA driven by a heat-shock promoter (*hs-jhe*). JH is a common name for a family of sesquiterpenoid esters of methanol and hydrolysis of the conjugated methyl ester is generally regarded as one of the key pathways for inactivating the hormone (Goodman and Granger 2005). JHE was reported to be the only esterase that hydrolyzes all types of JH in *Drosophila* (Crone et al., 2007). Therefore, we expected that overexpression of *jhe* during early larval stages would reduce the JH titer in the hemolymph.

When treated by heat shock at the 2nd instar larval stage, the *jhe* mRNA level in the *hs-jhe* larvae increased by 2.8-fold compared to that in control larvae (data not shown). At the same time, precocious *br* expression was observed: levels of endogenous Br proteins increased (Fig. 3.3F), as did expression of the *GAL4-PG12>UAS-mCD8GFP* reporter (Fig. 3.3B). However, when *hs-jhe* larvae were reared on food containing 0.1 ppm pyriproxifen, an efficient JH agonist (JHA) that is chemically different from natural JH (Riddiford and Ashburner 1991), precocious *br* expression in the *hs-jhe* larvae was undetectable (Fig. 3.3D and H). Together, these results demonstrate that JH is required to suppress *br* expression during early larval stages in *Drosophila.*

A genetic screen for mutations affecting *br* expression: Because JH represses *br* expression during early larval stages, we reasoned that mutations that reduce the JH titer or disrupt JH action should cause precocious *br* expression in *Drosophila*. Accordingly, we designed and conducted a genetic screen to isolate genes that affect these processes. In these screens, *GAL4-PG12>UAS-mCD8GFP* on the X chromosome was used as a reporter of *br* expression, and lethal mutations or *P*-insertions on the 2nd or 3rd chromosome were made homozygous and screened for precocious *br* expression (Fig. 3.4). Because most of the lethal lines allowed organisms to develop to early larval stages, we were able to examine GFP expression in the 2nd instar under the fluorescent microscope. From 4,400 lethal lines, 55 mutations were isolated based on GFP expression in the 2nd instar larvae. Genes

associated with these mutations encode proteins with various molecular functions, including enzymes, signal transduction molecules, and transcriptional factors.

This genetic screen was efficient in identifying the genes required for JH biosynthesis. It not only isolated genes that are known to be involved in JH biosynthesis, such as *farnesyl diphosphate synthase* (*Fpps*) (Sen et al., 2007), *apterous* (*ap*) (Altaratz et al., 1991), *Insulin receptor* (*InR*) (Tatar et al., 2001, Tu et al., 2005), and N-*methyl*-D-*aspartate receptor 1* (*Nmdar1*) (Chiang et al., 2002), but also revealed that Dpp-mediated TGF-β signaling in the corpus allatum stimulates JH biosynthesis by upregulating transcription of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH synthesis (Huang et al., 2011). The same genetic screen also isolated genes that are involved in JH signaling, such as *Kr-h1*. Another known JH signaling component, Met, was not identified by this screen because the *Met* gene is located to X chromosome. A reverse genetic study showed that precocious *br* expression was also detectable in *Met* mutant larvae (Huang et al., 2011).

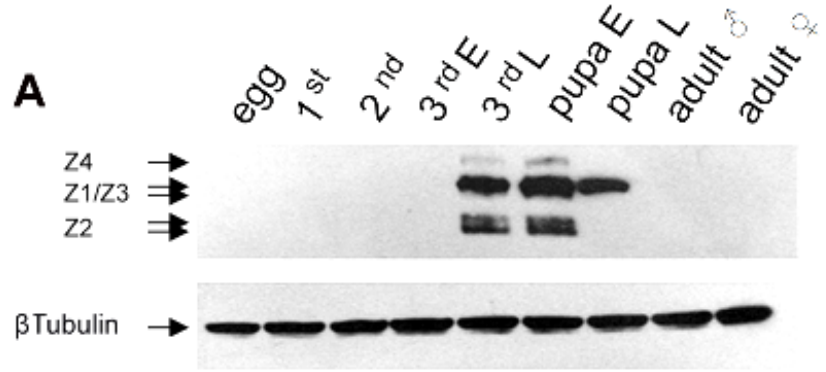
DISCUSSION

JH is required to repress *br* expression during the early larval stages of *Drosophila*

The 'status quo' action of JH in controlling insect metamorphosis is conserved in hemimetabous and most holometabous insects. However, the larval-pupal transition in higher Diptera, such as *Drosophila,* has largely lost its dependence on JH. For

instance, in most insects, the addition of JH in larvae at the last instar causes the formation of supernumerary larvae. However, exogenous JH does not prevent pupariation and pupation in *Drosophila*, and instead only disrupts the development of the adult abdominal cuticle and some internal tissues (Postlethwait 1974, Riddiford and Ashburner 1991). The molecular mechanisms underlying these differential responses to JH are not clear.

Broad is a JH-dependent regulator that specifies pupal development and mediates the 'status quo' action of JH (Zhou and Riddiford 2002). In the relatively basal holometabolous insects, such as beetles and moths, JH is both necessary and sufficient to repress *br* expression during all of the larval stages (Zhou et al., 1998, Reza et al., 2004). Our studies revealed that JH is also required during the early larval stages in the more derived groups of the holometabolous insects, such as *Drosophila*, but it is not sufficient to repress *br* expression at the late 3rd instar. During the early larval stages, overexpression of the JH-degradative enzyme JHE, reduction of JH biosynthesis or disruption of the JH signaling always causes precocious *br* expression. However, exogenous JHA treatment can not repress *br* expression in late 3rd instar larvae (Fig 3.5). This phenomenon accounts for why exogenous JHA treatment can not induce supernumerary larvae in *Drosophila*. The molecular mechanism underlying the developmental stage-specific responses of the *br* gene to JH signaling remains to be clarified.


A
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1st
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Z4
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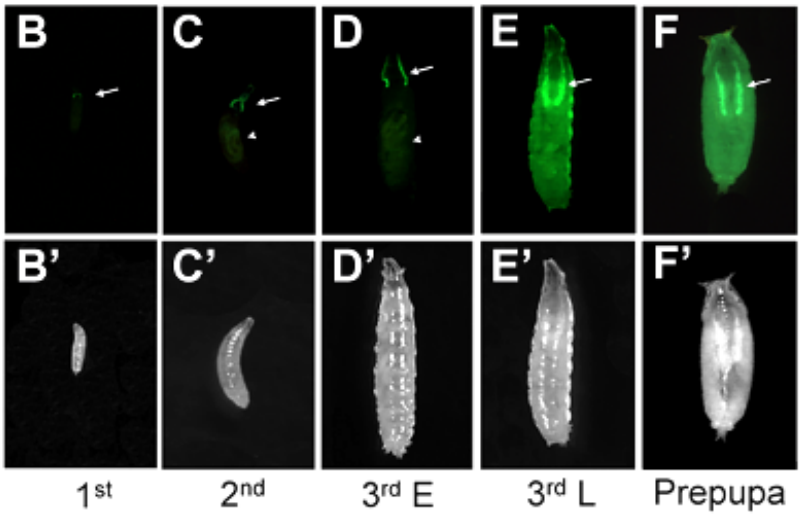
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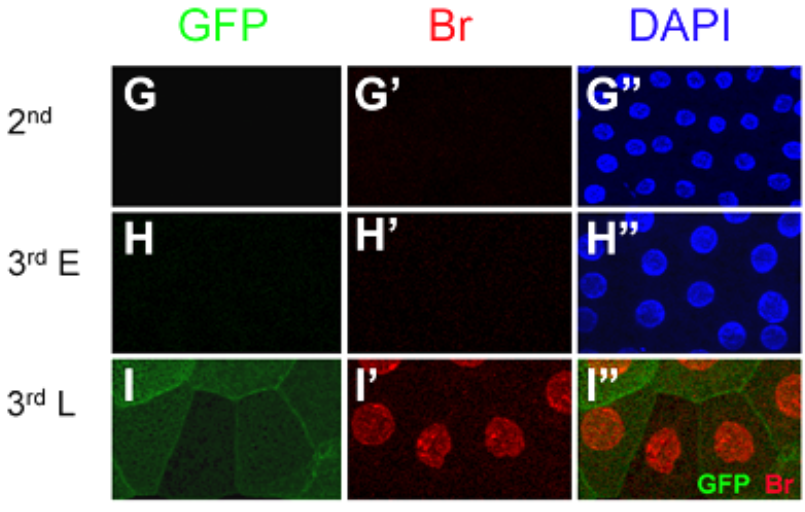
GFP
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Fig 3.1. *GAL4-PG12* resembles endogenous *br* expression patterns

(A) Protein extracts isolated from wild type animals at different developmental stages were separated by SDS-PAGE. Br proteins were assessed by Western blotting using a Br-core antibody. Tubulin-β was used as a loading control. The Br proteins were only detected in the late 3rd instar larval stage to pupal stage. All Br isoforms were expressed in the late 3rd instar larvae and early pupae, but only Z1 and/or Z3 isoforms were expressed in the late pupae.

(B-F) Expression of *GAL4-PG12* was marked by *GAL4-PG12>UAS-mCD8GFP* mCD8GFP, a cell membrane protein. Constitutive expression of *GAL4-PG12* in salivary glands (arrows) and auto-fluorescence of fly food in the midgut (arrowheads) are indicated. In tissues other than those from the salivary gland, *GAL4-PG12/UAS-mCD8GFP* was only expressed in late 3rd instar larval and during early pupal stages (G and H). (B'-F') White light images of the same organisms are shown in [B-F].

(G-I) *GAL4-PG12* expression was monitored by mCD8GFP (green) [G-I]. Endogenous Br proteins were recognized by a Br-core antibody (red) [G'-I'] and nuclei were marked with DAPI (blue) [G"-H"]. Neither endogenous Br nor *GAL4-PG12* were expressed in FB of the 2nd instar or early 3rd instar [G-G" and H-H"], but both were expressed in FB of the late 3rd instar [I-I"]. [I"] is a merged image of [I] and [I'].

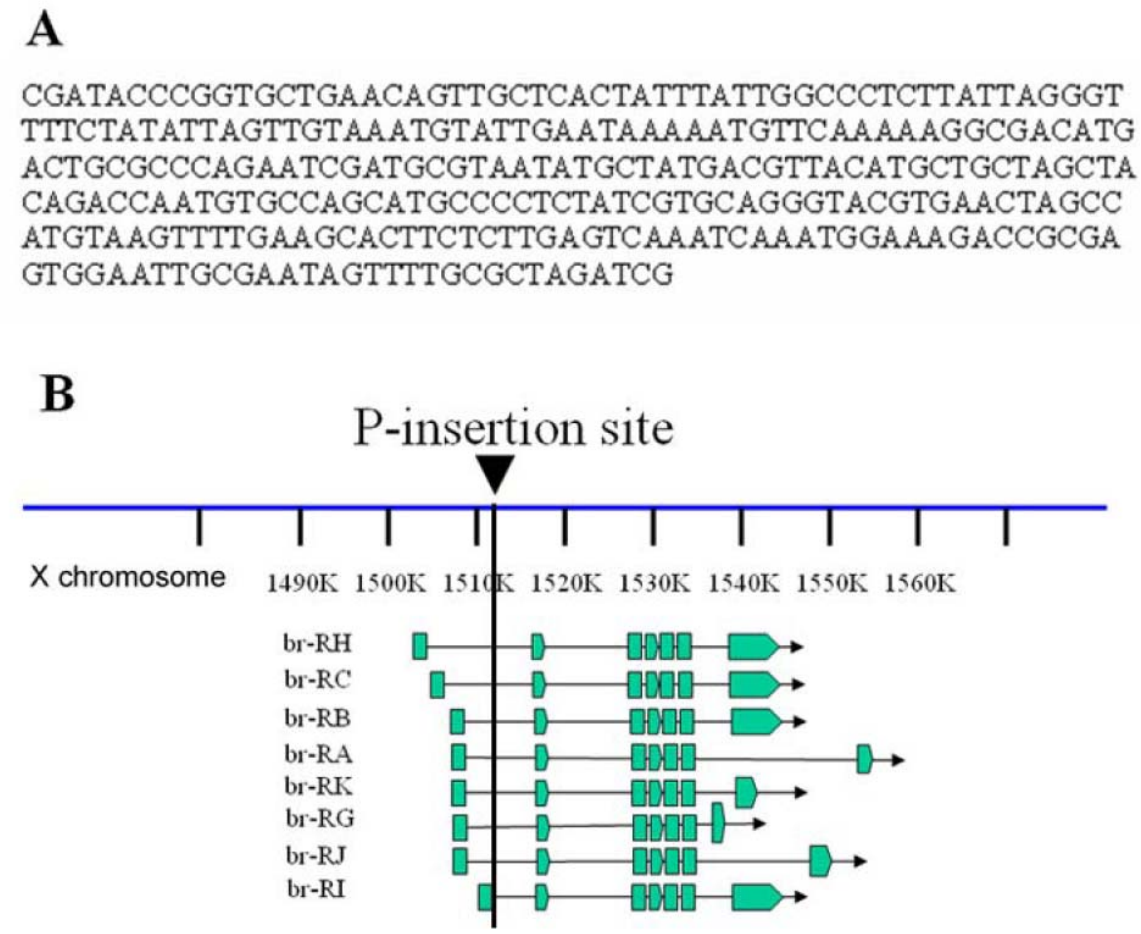

Fig 3.2. *GAL4-PG12* carries a *P*-element insertion in the first intron of *br* gene

(A) The flanking sequence of the *GAL4-PG12 P*-element insertion site identified by inverse PCR analysis.

(B) The insertion site of *GAL4-PG12* was located within the first intron of the *br* gene by comparing the sequence with the *Drosophila* genome.

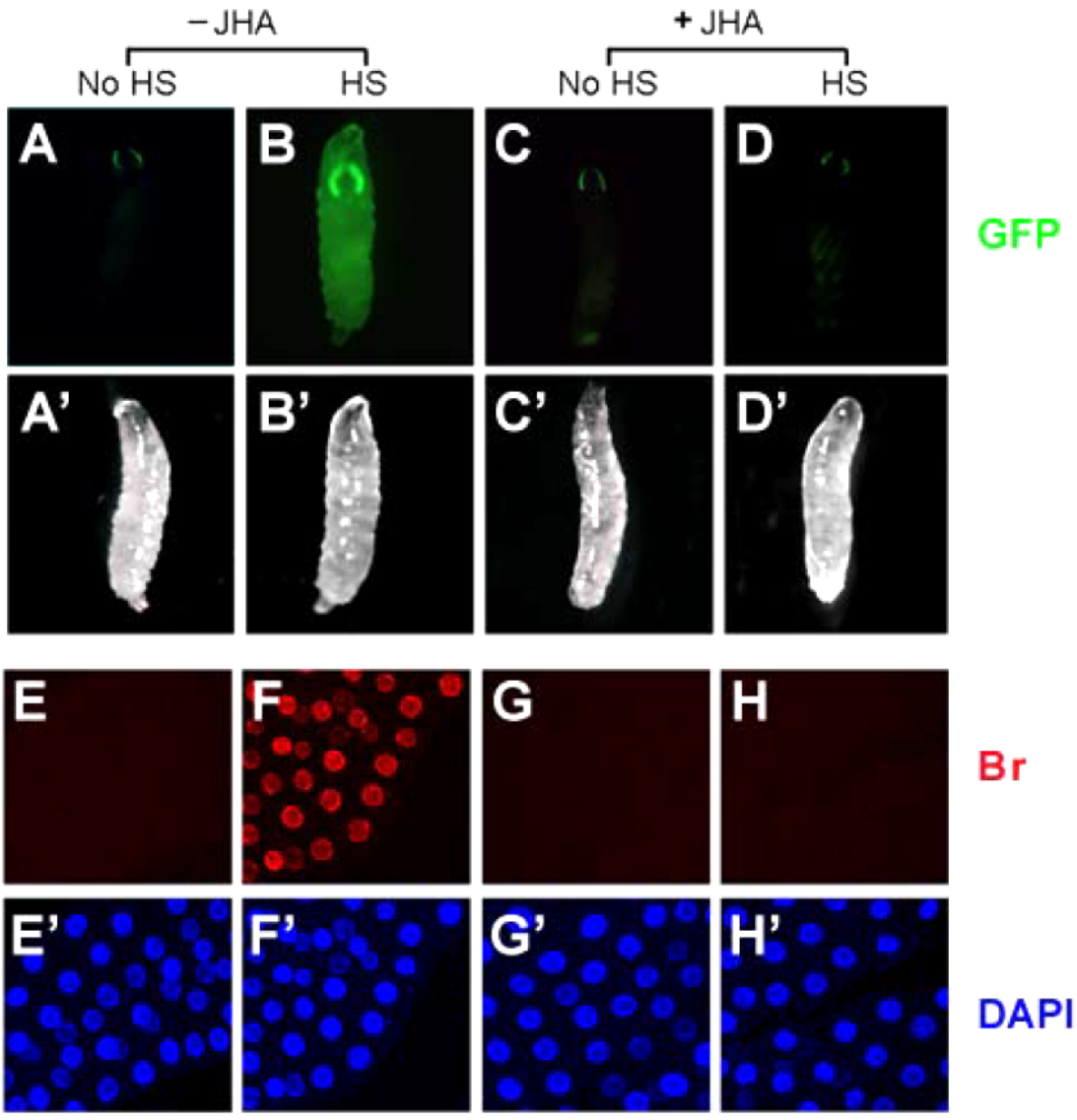

Fig 3.3. Ectopic expression of JHE induces precocious *br* expression in the 2nd instar larvae.

Flies carrying two copies of *hs-jhe* transgenes (*GAL4-PG12, UAS-mCD8GFP/Fm7C; hs-jhe*^{1}*, hs-jhe*^{2}/+) were reared on normal (-JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food and were treated with (HS) or without (no-HS) heat shocking twice a day for 40 min at 37 °C. *Br* expression was monitored by *GAL4-PG12>UAS-mCD8GFP* [A-D] and FB Br-core antibody staining in 2nd instar larvae [E-H]. Precocious *br* expression occurred in 2nd instar larvae that were reared on normal food and treated with heat shocking [B-B' and F-F']. However, this phenotype was blocked by JHA treatment [D-D' and H-H'].

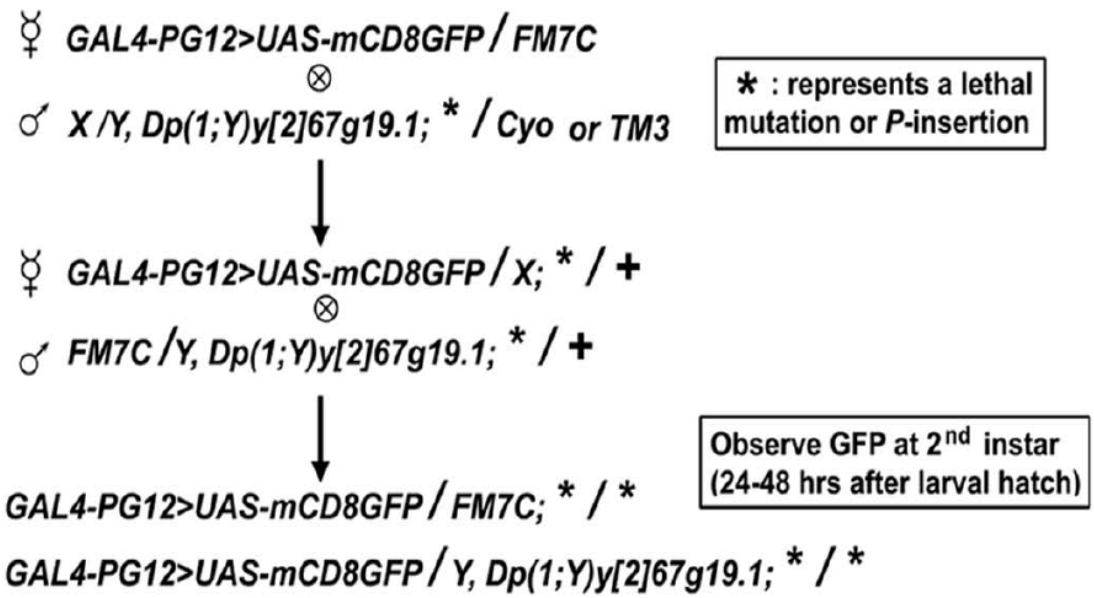

Fig 3.4. A genetic screen identifies genes related JH biosynthesis and signaling pathways

Schematic diagram of genetic crosses for isolating mutations that derepress *br* expression in young larvae. *GAL4-PG12, UAS-mCD8GFP* (X chromosome) was used to monitor *br* expression. The lethal mutation or *P*-insertion on the 2nd or 3rd chromosome is represented by an asterisk (*).

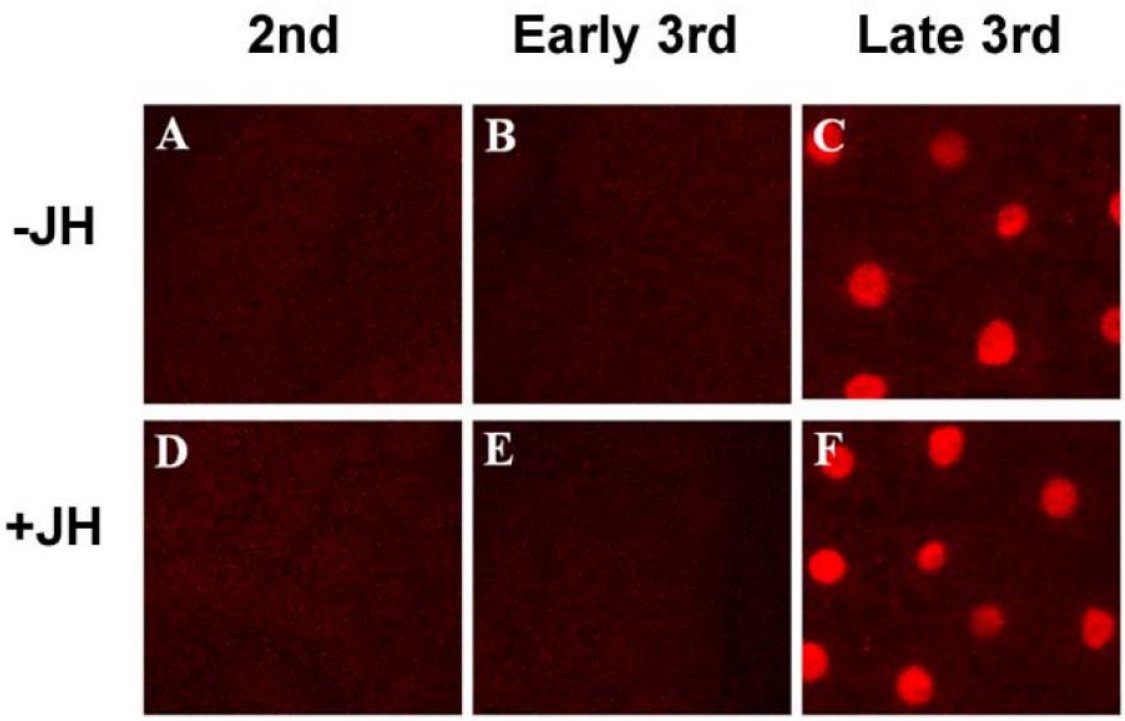

Fig 3.5. Expression of *br* is not suppressed by exogenous JHA during the late 3rd instar larval stage

Wild type flies were reared on normal (-JHA) [A-C] or 0.1 ppm pyriproxifen-containing (+JHA) [D-F] food. Br proteins in the fat bodies of 2nd instar, early 3rd instar and late 3rd instar larvae were recognized by a Br-core antibody (red).

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Chapter 4

DPP-mediated TGF-β Signaling Regulates Juvenile Hormone Biosynthesis by Upregulating the Expression of JH Acid Methyltransferase

ABSTRACT

Juvenile hormone (JH) biosynthesis in the corpus allatum (CA) is regulated by neuropeptides and neurotransmitters produced in the brain. However, little is known about how these neural signals induce changes in JH biosynthesis. Here, we report a novel function of TGF-β signaling in transferring brain signals into transcriptional changes of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH biosynthesis. A *Drosophila* genetic screen identified that Tkv and Mad were required for JH-mediated suppression of *broad* (*br*) expression in the young larvae. Further investigation demonstrated that TGF-β signaling stimulated JH biosynthesis by upregulating *jhamt* expression. Moreover, *dpp* hypomorphic mutants also induced precocious *br* expression. The pupal lethality of these *dpp* mutants was partially rescued by exogenous JH agonist. Finally, *dpp* was specifically expressed in the CA cells of ring glands, and its expression profile in the CA correlated with that of *jhamt* and matched JH levels in the hemolymph. Reduced *dpp* expression was detected in the mutant larvae of *Nmdar1*, a CA-expressed glutamate receptor. Taken together, we conclude that the neurotransmitter glutamate promotes *dpp* expression in the CA, which stimulates JH biosynthesis through Tkv and Mad by upregulating *jhamt* transcription at the early larval stages to prevent premature metamorphosis.

INTRODUCTION

Juvenile hormone (JH) coordinates with 20-hydroxyecdysone (20E) in regulating insect molting and metamorphosis. The molting process is orchestrated by 20E, whereas the nature of the molt is determined by JH during critical JH-sensitive periods. In the presence of JH, 20E induces larva-larva molt, while in the absence of JH, 20E promotes larva-pupa or pupa-adult metamorphosis (Gilbert et al., 2000; Riddiford et al., 2003). The recent progress in our understanding of JH molecular action clarifies the function of Methoprene-tolerant (Met) and Krüppel-homolog 1 (Kr-h1) in transducing JH signaling. The *Drosophila Met* gene encodes a bHLH-PAS protein family member, which is proposed to be a component of the elusive JH receptor (Wilson and Ashok, 1998). Kr-h1 is considered as a JH signaling component which works at downstream of Met (Minakuchi et al., 2008; 2009).

JH biosynthesis is regulated at three closely linked steps. In the first step, developmental, environmental and physiological cues are received by the central nervous system, which determines the appropriate rate of JH synthesis (Riddiford, 1993). In the second step, the brain transfers these signals to mediate JH biosynthesis in an endocrine gland, the corpus allatum (CA). It has long been thought that JH biosynthesis is regulated primarily by two neuropeptides secreted by brain neurosecretory cells: allatotropin (AT) and allatostatin (AST), which stimulates and inhibits JH synthesis, respectively (Stay, 2000; Weaver and Audsley, 2009). Nevertheless, no AT-like neuropeptides or AT receptor genes have been found in the *Drosophila* genome thus far (Nassel 2002; Hauser et al., 2006; Liu et al., 2006;

Yamanaka et al., 2008). Although AST-like neuropeptides exist in *Drosophila* (Lenz et al., 2000), their function of inhibiting JH biosynthesis has not been demonstrated. On the other hand, it has been reported that the brain may directly control JH biosynthesis through neurotransmitters. For example, studies in cockroaches and *Drosophila* revealed that glutamatergic nerves innervate CA cells, and the *N*-methyl-D-aspartate subtype of glutamate receptors (NMDAR) are expressed in both the brain and CA. Additionally, glutamate and NMDA were shown to stimulate JH synthesis *in vitro* (Chiang et al., 2002). In the final step of JH biosynthesis regulation, the brain signals received in the CA should be translated into changes in the expression and/or activity of key regulatory JH biosynthetic enzymes, which directly determine the rate of JH biosynthesis. However, there are major voids in our current understanding of the pathways that lead from brain signals to the activities of JH biosynthetic enzymes.

The evolutionarily conserved TGF-β signaling pathway modulates a wide range of cellular processes, including proliferation, differentiation, migration, apoptosis and cell fate speciation (Kingsley, 1994; Massagué et al., 2000). In addition, studies in *C. elegans* reveal that the TGF-β signaling pathway controls dauer formation through modulation of dafachronic acid synthesis (reviewed in Hu, 2007). Here, we present a novel, gradient-independent function of Dpp, a TGF-β ligand, in controlling JH biosynthesis. Dpp-Tkv-Mad-mediated TGF-β signaling in the CA serves as a bridge to connect brain-derived neurotransmitter signals to the transcriptional changes of JH acid methyltransferase (JHAMT), a key regulatory enzyme of JH biosynthesis (Shinoda and Itoyama, 2003; Sheng et al., 2008).

MATERIALS AND METHODS

Fly Strains and Genetics

All fly strains were grown on standard cornmeal/molasses/agar medium at 25 °C. The mutant alleles for *dpp*, *tkv*, *mad*, *Kr-h1*, and *Nmdar1* used in this study, including dpp^{s11}, dpp^{d5}, tkv^{7}, tkv^{8}, tkv^{k16713}, mad^{1-2}, mad^{8-2}, mad^{k00237}, $mad^{kg00581}$, $Kr\text{-}h1^{k04411}$, $Kr\text{-}h1^{10642}$, $Nmdar1^{05616}$, and $Nmdar1^{DG23512}$, were obtained from the Bloomington *Drosophila* Stock Center. Met^{27} is a gift from T. Wilson. RNAi lines of these genes, including *UAS-dpp RNAi*, *UAS-tkv RNAi*, *UAS-mad RNAi*, *UAS-jhamt RNAi*, and *UAS-Nmdar1 RNAi*, were also obtained from the Bloomington *Drosophila* Stock Center. Other fly lines used in this study include *hs-GAL4*; *Dscam-GAL4* (Wang et al. 2004); *GAL4-Aug21* (Mirth et al. 2005); *UAS-mad* (a gift from S. J. Newfeld); and *UAS-dpp* (Bloomington *Drosophila* Stock Center).

Fly stocks used for the creation of fat body MARCM clones include *FRT40*; *FRT40,* tkv^{8}*/Cyo* (a gift from K. Moses); *FRT40,* mad^{8-2}*/Cyo*; *FRT40,* $Kr\text{-}h1^{10642}$*/Cyo*; *hs-Flp, UAS-mCD8GFP;* and *FRT40, tub-GAL80*.

MARCM analysis:

The MARCM system (Mosaic Analysis with a Repressible Cell Marker) that positively labels mutant cells in the mosaic animals allows genetic analysis of tissue development at unprecedented single-cell resolution (Lee and Luo, 1999). In the MARCM system, yeast GAL80, the suppressor of GAL4 transcription factor, was introduced into GAL4-UAS binary expression system in *Drosophila*. Thus marker

gene under the control of UAS promoter can not be expressed in the cells heterozygous for GAL80. Following FLP/FRT-mediated mitotic recombination, one of the daughter cells becomes GAL80-negative, thus allowing expression of the marker gene specifically in this daughter cell and its progeny. If a mutation is located on the chromosome arm in trans to the chromosome arm containing the GAL80, the homozygous mutant cells will be uniquely labeled. Depending on whether mitotic recombination happens in the fat body or in other tissues, multicellular FB clones or other tissues clones can be generated. Thus, by controlling the timing of heat shock-induced expression of FLPase, the MARCM system allows differentiates label single cell generated at specific development of wild type complex tissue (Jefferis et al., 2002; Lee et al., 1999).

MARCM clones in the fat body were induced by *hs-Flp* through heat shock-independent induction as previously described (Britton et al., 2002).

Construction of *jhamt-GAL4* transgenic flies

To generate *jhamt-GAL4* transgenic flies, a 2 kb *jhamt* promoter was isolated by genomic DNA PCR. Phusion^{TM} High-Fidelity PCR Kit (BioLabs) was used for the PCR reaction according to the manufacture protocol. The 2 kb PCR product was analyzed by agarose gel electrophoresis, purified PCR Purification Kit (Qiagen), fused with GAL4 cDNA, and inserted into *pCaSpeR4*. Transgenic fly lines were generated by *P* element-mediated germline transformation at Rainbow Transgenic Flies, Inc. (Camarillo, CA).

The *jhamt-GAL4* transgenic flies were built through a genetic screening and different crosses to be homozygous viable on the 2nd chromosome.

Immunohistochemistry and Microscopy

Larval fat bodies were dissected from the 2nd or 3rd instar larvae. Immunohistochemistry was performed as described previously. Florescence signals were captured with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) and processed with Adobe Photoshop.

JHA Treatment and JHAMT Activity Assay

The JHA pyriproxifen (Sigma) was dissolved in 95% ethanol to give a 300 ppm stock solution. JHA-containing fly food was prepared by adding JHA stock solution to the standard cornmeal-molasses-yeast food at 50-55°C to a final concentration of 0.1 ppm or as indicated. JHAMT activity in the brain-ring gland complex was measured as previously described (Liu et al., 2009).

Western Blotting and Quantitative Real-time PCR

Protein extracts isolated from 2nd instar larvae were analyzed by standard SDS–PAGE and western blot. The expression of β-tubulin was used as a loading control. Br mouse monoclonal antibody Br-core (25E9.D7) (Emery et al., 1994) and β-tubulin mouse monoclonal antibody (AA12.1) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. The Western blotting analysis was described in details in chapter 3.

Total RNA samples were prepared from the whole body for 2nd instar larvae or ring glands for 3rd instar larvae and pupae. Quantitative real-time PCR was performed using the LightCycler 480 SYBR Green I Master Kit (Roche). The mRNA levels of different genes were normalized to *actin* mRNAs, with 3 replicates for each sample. Primers used are listed in the following Table.

Name	Accession number	Primer F (5'-3')	Primer R (5'-3')
FPPS	NM_058032	TGGCACAAGGTGGAGAACG	CGATTGTCCGCAGGTAGTGA
JHE	NM_079034	AAATCCGCACTACCTGTAATGG	CGGAGTCCATAAAGTATTCGGG
JHAMT	NM_135949	TTTCTTGAGCGAATGCCTGC	AGGAGTCTTGCGAGCATAGGC
actin	NM_167053	GCTGAGCGTGAAATCGTCCG	GGAGTTGTAGGTGGTCTCGTGGA
JHEdup	NM_137241	CTGACGACTATGGTCTTGGAGCA	AACCTTGGCATCTTCCGAGTC
JHEh1	NM_137541	CTTCTTTCCCAAGTCTAACGAG	AGGGCATCCATTTTGTAGCG
JHEh2	NM_137542	GGTTTGTGGACAGCGAGTATGC	TCAGACCACCGTCAGGAAGC
JHEh3	NM_137543	TGGATCATCACTTCCCCGTG	CCGAAGACAGTGACTATGGCG
cyp6g2	NM_136900	CGGATGTGATAGCCACGGTAG	CTTGAATCTAACGAACGGGACC
Famet	NM_137700	CCGAATACGAGGTGCTGTGC	TTCAGTGCGTTGGACATTCG
FARox	NM_132467	GATGTGCTGGTCAACAATGCC	GCCCCAGGATGCTATTGATGAG
FARD	NM_132238	AGCCAAAGCGAACGAATCC	TGATGCGTTGCGGATACAGAT

RESULTS

A genetic screen identifies that Tkv and Mad regulate *br* expression

The *broad* (*br*) gene has been identified as a key regulator in mediating the cross-talk between 20E and JH signaling pathways. Studies in *Manduca* indicated that the expression of *br* is directly induced by 20E, but this induction can be prevented by the presence of JH (Zhou et al., 1998; Zhou and Riddiford, 2002). Therefore, in many tissues, *br* is predominantly expressed during the larval-pupal transition when 20E is high and JH is absent (Huet et al., 1993). To identify genes involved in JH biosynthesis or JH signaling, we developed a *Drosophila* genetic screen to isolate mutations that de-repress *br* expression in the 2nd instar larvae. We reasoned that mutations that block JH biosynthesis or disrupt JH action should reduce JH activity and cause precocious *br* expression.

To monitor *br* expression in live organisms, we examined the expression patterns of GAL4 enhancer-trap lines inserted near the *br* gene. One of these lines, *GAL4-PG12*, closely resembles the temporal and spatial expression pattern of the endogenous *br* gene in tissues other than the salivary gland. In the genetic screen, *GAL4-PG12>UAS-mCD8GFP* on the X chromosome was used as a reporter of *br* expression, and lethal mutations or *P*-insertions on the 2nd or 3rd chromosome were made homozygous and screened for precocious *br* expression. Because most of the lethal lines allowed organisms to develop to early larval stages, we were able to examine GFP expression in the 2nd instar under a fluorescent microscope.

From 4,400 lethal lines, 55 mutations were isolated based on GFP expression in the 2nd instar larvae. Genes associated with these mutations encode proteins with various molecular functions, including enzymes, signal transduction molecules, transcriptional factors, and others. Some of them are known to be involved in JH biosynthesis, such as farnesyl diphosphate synthase (FPPS) (Sen et al., 2007) and NMDAR1 (Chiang et al., 2002).

Among these 55 genes were two main components of TGF-β signaling, *thick vein* (*tkv*) and *mothers against Dpp* (*mad*) (Raftery and Sutherland, 1999). As shown in Fig. 4.1, the expression of *GAL4-PG12>UAS-mCD8GFP* was restricted to salivary glands in the wild type 2nd instar larvae, but ubiquitous expression of *GAL4-PG12>UAS-mCD8GFP* was detected at the same stage of the *tkv*, *mad*, and *Nmdar1* mutant larvae. Consistently, when assessed with Br-core antibody staining, endogenous Br proteins were not detectable in the fat body (FB) of wild type 2nd instar larvae but were observed in the FB nuclei of both tkv^{k16713} and mad^{k00237} mutant larvae (Fig. 4.2A). To further test this finding, we examined other *tkv* and *mad* alleles, including tkv^{7}, tkv^{8}, mad^{1-2}, mad^{8-2}, and $mad^{kg00581}$. Precocious *br* expression was detected in all cases. These results suggest that Tkv- and Mad-mediated TGF-β signaling is required to repress *br* expression at the early larval stages, possibly through regulating JH titer or signaling.

The same genetic screen also isolated genes that are involved in JH signaling, such as *Kr-h1* (Fig. 4.1 and Fig. 4.2A). Another known JH signaling component, Met, was not analyzed by this screen because the *Met* gene is located to X chromosome. A reverse genetic study showed that precocious *br* expression was also detectable in *Met* mutant larvae (Fig. 4.2A).

Exogenous JH agonist prevents precocious *br* expression in *tkv* and *mad* mutants

We next asked whether the effects of *tkv* and *mad* mutations on *br* expression were caused by the decrease in JH titer. Based on this hypothesis, we expected that precocious *br* expression in the *tkv* and *mad* mutant larvae would be blocked by exogenous JH agonist (JHA). Wild type, *tkv*, and *mad* larvae were reared on a diet containing 0.1 ppm pyriproxifen, an efficient JHA (Riddiford and Ashburner, 1991). The Br-core antibody was used to detect Br proteins in the 2nd instar larvae. Immunohistochemical results revealed that the precocious *br* expression was suppressed by exogenous JHA in the FB of *tkv* and *mad* mutants (Fig. 4.2A). However, the precocious *br* expression in *Kr-h1* and *Met* mutants was not suppressed by exogenous JHA, which demonstrates that Met and Kr-h1 function as the JH signaling components in mediating *br* expression (Wilson and Ashok, 1998; Minakuchi et al., 2008; 2009) (Fig. 4.2A).

These observations were further confirmed by western blot analysis (Fig. 4.2B). Thus, we assume that Tkv/Mad-mediated TGF-β signaling maintains JH titers to inhibit *br* expression at the early larval stages, thereby blocking precocious metamorphosis.

Tkv and Mad cell-non-autonomously regulate *br* expression in the FB

Proteins that transduce JH signals should cell-autonomously regulate *br* expression in the JH-affected organs, while proteins that affect JH titer should cell-non-autonomously affect *br* expression. We asked whether the Tkv and Mad proteins were required for the regulation of *br* expression in the FB, a target organ of JH (Liu et al., 2009). A MARCM analysis of FB cells was conducted for tkv^{8}, mad^{8-2}, and $Kr\text{-}h1^{10642}$ mutants. Br protein was assessed with Br-core antibody staining and compared between the homozygous mutant cells (GFP-positive) and the surrounding heterozygous or wild type cells (GFP-negative). As expected, *br* was not expressed in FB of the wild type 2nd instar larvae (Fig. 4.3A-A'), but was expressed in the mutant clones homozygous for *Kr-h1* (Fig. 4.3D-D'). However, *br* expression was not detected in the *tkv* and *mad* mutant FB clones at the same stage (Fig. 4.3B-B' and 2C-C'). This result is different from those shown in Fig. 4.2A, in which *br* was found to be highly expressed in FB cells of the *tkv* and *mad* mutant larvae at the 2nd instar. Therefore, at the early larval stages, the presence of the Tkv and Mad proteins in the FB cells is not required for JH-mediated *br* suppression in these cells. Rather, these proteins function in other tissues to control *br* expression. These results further support the hypothesis that Tkv/Mad-mediated TGF-β signaling inhibits *br* expression at the early larval stages by maintaining JH titers.

Mad functions in the CA to suppress *br* expression in the FB

Because JH titer is mainly determined by JH biosynthesis in the CA, which is controlled by the brain, we tested whether the precocious *br* expression phenotype in the *mad* mutant larvae could be suppressed by expressing *mad* cDNA specifically in the CA or brain. When *hs-GAL4* was used to drive *UAS-mad* ubiquitously in the mad^{k00237} larvae, precocious *br* expression was fully suppressed as expected. By contrast, when we used *Dscam-GAL4*, a pan-neuronal expression driver (Wang et al., 2004), the precocious *br* expression phenotype was not affected. However, when we used *GAL4-Aug21*, a CA-specific GAL4 line (Mirth et al., 2005), precocious *br* expression was completely suppressed (Fig. 4.4A). As the expression of *mad* in the CA was sufficient to suppress *br* expression in FB of the *mad* mutant larvae, we infer that Tkv/Mad-mediated TGF-β signaling promotes JH biosynthesis in the CA at the early larval stages.

Tkv and Mad upregulate *jhamt* transcription and its enzymatic activity

Next, we asked whether TGF-β signaling regulates the expression of genes encoding critical enzymes of JH biosynthesis. We first compared mRNA levels for these genes between wild type and *mad* mutant larvae at the 2nd instar by quantitative real-time PCR. Six enzymes, including JHAMT, farnesyl diphosphate synthase (FPPS), farnesol oxidase (FARox), farnesol dehydrogenase (FARD), cytochrome P450 6g2 (Cyp 6g2), and farnesoic acid O-methyltransferase (Famet), were chosen because they catalyze the key steps of JH biosynthesis and are predominantly expressed in the CA (Belles et al., 2005; Noriega et al., 2006). We found that in the mad^{k00237} larvae,

the mRNA levels of *jhamt* were decreased to less than half of that in wild type. However, no changes were detected for the mRNA levels of the other five enzymes (Fig 4.4B). We also examined the mRNA levels for genes encoding major JH degradative enzymes, including juvenile hormone esterase (JHE), juvenile hormone epoxide hydrolase 1-3 (JHEh1-3), and juvenile hormone esterase duplication (JHEdup) (Goodman and Granger, 2005). Expression of these JH degradative enzymes was not affected in the *mad* mutant larvae (Fig. 4.5).

To test whether the reduced *jhamt* mRNA expression in *tkv* and *mad* mutants resulted in a correspondingly reduced enzymatic activity, we further measured JHAMT activity in the brain-ring gland complex of Tkv- or Mad-deficient larvae. Because *tkv* and *mad* mutants die at early larval stages, we carried out this experiment in the *tkv* and *mad* RNAi larvae. *hs-GAL4*/*UAS-tkv RNAi*, *hs-GAL4*/*UAS-mad RNAi*, and *hs-GAL4*/+ control flies were reared under the normal conditions with or without heat-shock treatment. In the *hs-GAL4*/+ larvae, heat-shock treatment did not affect JHAMT activity. However, JHAMT activity in heat-shocked *hs-GAL4*/*UAS-tkv RNAi* and *hs-GAL4*/*UAS-mad RNAi* larvae was reduced to 40-50% of that in the control (Fig. 4.4C). Additionally, JHAMT activity in non-heat-shocked *hs-GAL4*/*UAS-tkvRNAi* and *hs-GAL4*/*UAS-mad RNAi* larvae was also mildly decreased (~80% of control), likely due to leaky expression of *hs-GAL4*. The lower *jhamt* mRNA levels and JHAMT activity in Tkv- and Mad-deficit larvae indicate that Tkv/Mad-mediated TGF-β signaling in the CA promotes JH biosynthesis by up-regulating *jhamt* expression.

Dpp is the TGF-β ligand in the regulation of JH biosynthesis

The *Drosophila* genome encodes seven TGF-β superfamily members. Three of them, Decapentaplegic (Dpp), Glass bottom boat (Gbb) and Screw (Scw), belong to the BMP subgroup and are suggested to signal through Tkv as the type I receptor (Raftery and Sutherland, 1999). In an attempt to identify TGF-β ligand(s) participating in the regulation of JH synthesis, we found that, like *tkv* and *mad* mutations, hypomorphic *dpp* mutants, dpp^{s11} and dpp^{d5}, also caused precocious *br* expression in the FB (Fig. 4.6A), suggesting that Dpp may be the TGF-β ligand regulating JH biosynthesis in the CA. Noticeably, while both are hypomorphic alleles, dpp^{s11} and dpp^{d5} have rearrangements of the *disk* and *shv* regulatory regions, respectively (Johnston et al., 1990). Likely, both regulatory regions are required for the normal *dpp* expression in the CA.

Next, we asked where the Dpp protein in the CA originates. Dpp is transported via intracellular trafficking initiated by receptor-mediated endocytosis (Entchev et al., 2000). Therefore, we first assessed *dpp* expression in the ring gland and found that *dpp-lacZ* was highly expressed in the CA but not in any other parts of the ring gland (Fig. 4.6B).

Null alleles of *dpp* mutations are embryonic lethal, while hypomorphic alleles, such as dpp^{s11}, were completely pupal lethal. Interestingly, this pupal lethality could be partially rescued by exogenous JHA. When reared on JHA-containing diet, more than

10% of dpp^{s11} larvae developed into adults (Fig. 4.6C). This finding was further supported by a CA-specific *dpp* RNAi assay (Fig. 4.7). These data not only reinforce the importance of Dpp in regulating JH biosynthesis but also implicate that the pupal lethality of dpp^{s11} are partially caused by the reduced *jhamt* expression and JH levels. When *jhamt* was ectopically expressed in the CA, approximately 8% of dpp^{s11} mutants developed to adulthood (Fig. 4.6D). In light of all this evidence, we conclude that Dpp expressed in the CA is the TGF-β ligand of Tkv in stimulating JH biosynthesis.

CA-specific down-regulation of TGF-β signaling induces precocious *br* expression

As mutations in *dpp*, *tkv*, and *mad* induce precocious *br* expression, we asked whether CA-specific knockdown of *dpp*, *tkv*, *mad*, or *jhamt* affects *br* expression. When the expression of *dpp*, *tkv*, *mad*, or *jhamt* was knocked down by CA-specific RNAi, precocious *br* expression was detected in the FBs of 2nd instar larvae in all cases (Fig. 4.8). These results further support that Dpp-Tkv-Mad mediated TGF-β signaling in the CA is required to regulate JH biosynthesis.

Expression of *dpp* in the CA correlates with that of *jhamt*

We have demonstrated that Dpp and its downstream signaling molecules Tkv and Mad are required for normal *jhamt* expression and JH biosynthesis in the CA. It is critical to determine whether TGF-β signaling is an efficient regulation mechanism for *jhamt* transcription. To address this question, we first measured *jhamt* mRNA

levels in the ring glands of Dpp-deficient flies at the wandering larval stage. We found that *jhamt* mRNA levels in the ring glands of dpp^{s11}, dpp^{d5}, and CA-specific *dpp* RNAi larvae were only 10-40% of that in wild type. In contrast, when *dpp* was ectopically expressed in the CA, the mRNA level of *jhamt* increased 4-fold (Fig 4.9A).

We next compared the developmental profiles of *dpp* and *jhamt* expression in the CA of wild type animals. From the late 3rd instar larva to early pupa, only a single high peak of *jhamt* mRNA level was detected in the wandering larval stage, which is consistent both with a previous report (Niwa et al., 2008) and with JH titers in the hemolymph (Riddiford, 1993). As shown in Fig. 4.8B, *jhamt* mRNA level in the ring glands of wandering larvae was ~11-fold higher than that of the larvae 10 hours before the wandering stage. The expression pattern of *dpp* in the CA was similar to that of *jhamt*, but the increase of *dpp* mRNA prior to the wandering larval stage occurred hours earlier than that of *jhamt* mRNA (Fig. 4.9B). Therefore, despite differences in developmental stages of wild type animals or in the Dpp-deficient larvae with distinct genetic backgrounds, *dpp* expression in the CA always correlates with *jhamt* expression *in vivo*. These findings further demonstrate that Dpp-mediated TGF-β signaling plays a crucial role in controlling JH biosynthesis through upregulating *jhamt* expression.

Expression of *dpp* in the CA is controlled by neurotransmitter signals

Finally, we asked whether *dpp* expression in the CA was regulated by brain signals. *Drosophila Nmdar1,* a glutamate receptor, is expressed in the CA and plays a role in regulating JH biosynthesis (Chiang et al., 2002). Our genetic screen also identified that mutations in *Nmdar1* caused precocious *br* expression (Fig. 4.1). As shown in Figure 4.10, at the wandering larval stage, mRNA levels of both *dpp* and *jhamt* in $Nmdar1^{DG23512}$ and $Nmdar1^{05616}$ mutants were reduced to below 30% of those in wild type. In addition, CA-specific knock-down of *Nmdar1* expression also induced precocious *br* expression in the FBs of 2nd instar larvae. These data suggest that *dpp* expression in the CA is directly controlled by neurotransmitter signals from the brain.

DISCUSSION

Roles of TGF-β signaling in insect metamorphosis

The functions of the TGF-β superfamily and other morphogens in regulating insect metamorphosis are rarely reported. In two independent genetic screens, we discovered that *Drosophila* TGF-β signaling controls two different aspects of insect metamorphosis. In a previous study, we found that Baboon (Babo) and dSmad2-mediated TGF-β signaling regulates larval neuron remodeling, which is part of the insect central nervous system metamorphosis induced by 20E during the pupal stage. Further investigation revealed that Babo/dSmad2-mediated TGF-β signaling controls larval neuron remodeling through regulating the expression of *EcR-B1*, a specific isoform of the 20E receptor (Zheng et al., 2003).

In this work, we report several findings. First, *br* is precociously expressed in 2nd instar *tkv* and *mad* mutant larvae (Fig. 4.1). Second, the precocious *br* expression phenotype in *tkv* and *mad* mutant larvae can be suppressed by exogenous JHA (Fig. 4.2). Third, Tkv and Mad repressed *br* expression in a cell-non-autonomous manner (Fig. 4.3). Fourth, the presence of Mad in the CA is sufficient to repress *br* expression in the FB (Fig. 4.4A). Fifth, *jhamt* mRNA levels and JHAMT activity were significantly reduced in the Mad-deficient larvae (Fig. 4.4B and C). These results demonstrate that Tkv and Mad-mediated signaling is required in the CA to activate *jhamt* expression and thus JH biosynthesis, which in turn controls insect metamorphosis.

The *Drosophila* genome encodes two TGF-β type II receptors, Punt (Put) and Wishful thinking (Wit) (Raftery and Sutherland, 1999). Our genetic screen failed to identify a role for either of these receptors in the regulation of JH biosynthesis. Put and Wit are most likely functionally redundant in this biological event, as in the case of TGF-β mediated mushroom body neuron remodeling (Zheng et al., 2004).

Dpp converts brain signals into JH biosynthesis in the CA

Dpp is a key morphogen that controls dorsal/ventral polarity, segmental compartment determination, and imaginal disc patterning. Dpp function usually depends on its gradient distribution (Affolter and Basler, 2007). In an attempt to identify the ligand for Tkv/Mad-mediated TGF-β signaling in the CA, we have discovered a novel,

gradient-independent role for Dpp that controls JH biosynthesis. We demonstrate that Dpp is the ligand of Tkv, which regulates *jhamt* transcription. Loss of Dpp, even RNAi reduction of Dpp in the CA specifically, causes precocious *br* expression at the early larval stages, which phenocopies *tkv* and *mad* mutants (Fig. 4.2A, Fig. 4.4A, and Fig. 4.8). Phenotypes of *dpp*, including precocious *br* expression and lethality, are at least partially rescued by JHA treatment (Fig. 4.6C) or ectopic *jhamt* expression in the CA (Fig. 4.6D). Notably, *dpp-LacZ* is strictly expressed in the CA cells, but not in the other two types of endocrine cells in the ring gland, the prothoracic gland and corpus cardiacum cells (Fig. 4.6B). The developmental expression profile of *dpp* in the CA is always consistent with that of *jhamt* (Fig. 4.9). Finally, *dpp* expression in the CA may be directly controlled by neurotransmitter signals in the brain, which is supported by reduced *dpp* and *jhamt* transcription levels in the *Nmdar1* mutant wandering larvae (Fig. 4.10).

Role of Met/Gce and Kr-h1 in JH action

Several lines of evidence suggest that Met is a critical regulator at or near the top of a JH signaling hierarchy, possibly acting as a JH receptor (Wilson and Ashok, 1998). However, null *Met* mutants of *Drosophila* are completely viable, which is unexpected if Met is a JH receptor. A recent investigation indicated that another *Drosophila* bHLH-PAS protein, Germ cell-expressed (Gce), which has more than 50% homology to Met (Godlewski et al., 2006), may function redundantly to Met in transducing JH signaling (Baumann et al., 2010). Because *Met* is on the X chromosome in the fly genome, it was not covered by our genetic screen. We tested the *br* protein in the FBs

of a *Met* null allele, Met^{27}, at the 2nd instar larval stage and observed precocious *br* expression. Importantly, this precocious *br* expression phenotype could not be suppressed by exogenous JHA (Fig. 4.2A). This result not only supports the previous reports regarding the function of Met in transducing JH signaling but also suggests that the precocious *br* expression is a more sensitive indicator for the reduced JH activity in *Drosophila* compared to precocious metamorphosis, lethality, and other phenotypes.

Kr-h1 was reported to act downstream of Met in mediating JH action. Studies in both *Drosophila* and *Tribolium* reveal that, at the pupal stages, exogenous JHA induces *Kr-h1* expression, which in turn up-regulates *br* expression (Minakuchi et al., 2008; 2009). Our genetic screen successfully identified that Kr-h1 is cell-autonomously required for the suppression of *br* expression at young larval stages (Fig. 4.1; Fig. 4.2 and 4.3). Precocious *br* expression occurred in the FBs of *Kr-h1* mutants and was not suppressed by JHA treatment (Fig. 4.2). Therefore, our studies further suggest that Kr-h1 functions as a JH signaling component in mediating insect metamorphosis. However, our finding shows that, at the larval stages of *Drosophila*, the JH-induced Kr-h1 suppresses, rather than stimulates, *br* expression. This result is consistent with the facts that Kr-h1 functions to prevent *Tribolium* metamorphosis (Minakuchi et al., 2009) and Br is a critical factor to promote pupa formation (Zhou and Riddiford, 2002).

A working model for function of Dpp-mediated TGF-β signaling in controlling insect metamorphosis

Taken together, we find a novel function of Dpp, Tkv, and Mad-mediated TGF-β signaling in controlling insect metamorphosis. As summarized in our model (Fig. 4.11), the brain sends neurotransmitters, such as glutamate, to the CA through neuronal axons. Glutamate interacts with its receptor (NMDAR) on the surface of CA cells to induce *dpp* expression. Dpp protein produced and secreted by CA cells forms a complex with TGF-β type I receptor (Tkv) and type II receptor on the membrane of CA cells, followed by phosphorylation and activation of Tkv. Activated Tkv in turn phosphorylates Mad, which is imported into the nucleus together with co-Smad and stimulates *jhamt* expression. JHAMT in CA cells transforms JH acid into JH, which is released into hemolymph. The presence of JH in young larvae prevents premature metamorphosis through Met/Gce and Kr-h1 by suppressing the expression of *br*, a critical gene in initiating insect metamorphosis.

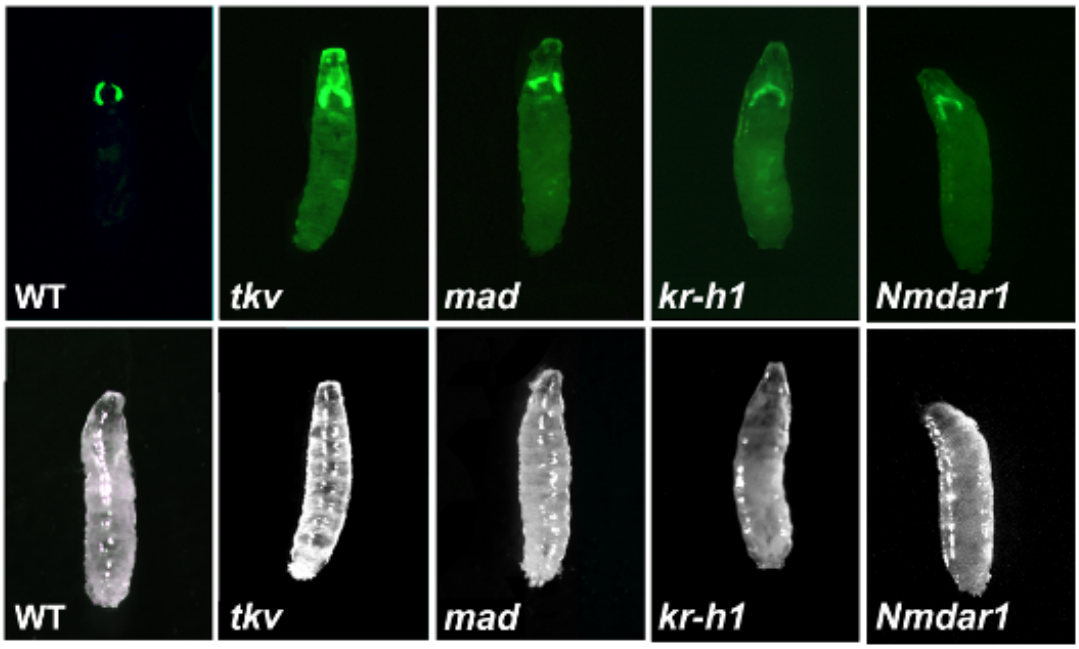


Fig. 4.1. Genetic screen identifies Tkv and Mad as being required for the suppression of *br* expression at early larval stages

(A-E) GFP images showing the expression of *GAL4-PG12>UASmCD8GFP* in 2nd instar larvae. GFP was only expressed in the salivary gland of the wild type (A) larvae but was widely expressed in all tissues of *GAL4-PG12, UAS-mCD8GFP/Fm7C; tkv*^{k1671}*/tkv*^{k16713} (B), *GAL4-PG12, UAS-mCD8GFP/Fm7C; mad*^{k00237}*/mad*^{k00237} (C), *GAL4-PG12, UAS-mCD8GFP/Fm7C; Kr-h1*^{10642}*/Kr-h1*^{10642} (D), and *GAL4-PG12, UAS-mCD8GFP/Fm7C; Nmdar1* ^{DG23512}*/Nmdar1*^{DG23512} (E) larvae. (A'-E') White light images of the same organisms are shown in (A-E).

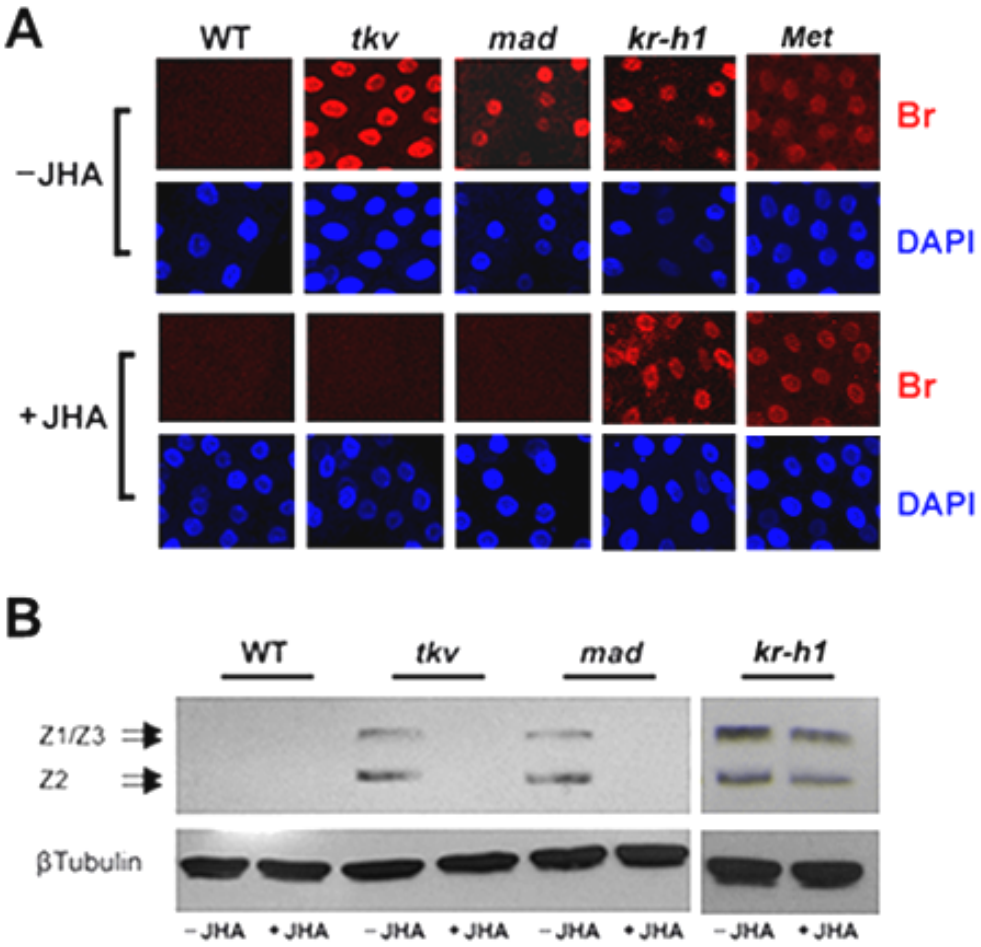


Fig. 4.2. Tkv and Mad repress *br* expression in the FB by maintaining JH levels

(A) Wild type, tkv^{k167}, mad^{k00237}, $Kr\text{-}h1^{10642}$, and Met^{27} flies were reared on normal (-JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Fat bodies of the 2nd instar larvae were stained with Br-core antibody (red). Nuclei were labeled with DAPI (blue). (**B**) Br proteins extracted from wild type, tkv^{k167}, mad^{k00237}, and $Kr\text{-}h1^{10642}$ 2nd instar larvae that were reared on normal (-JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food were assessed by western blotting with Br-core antibody. Tubulin-β was used as a loading control.

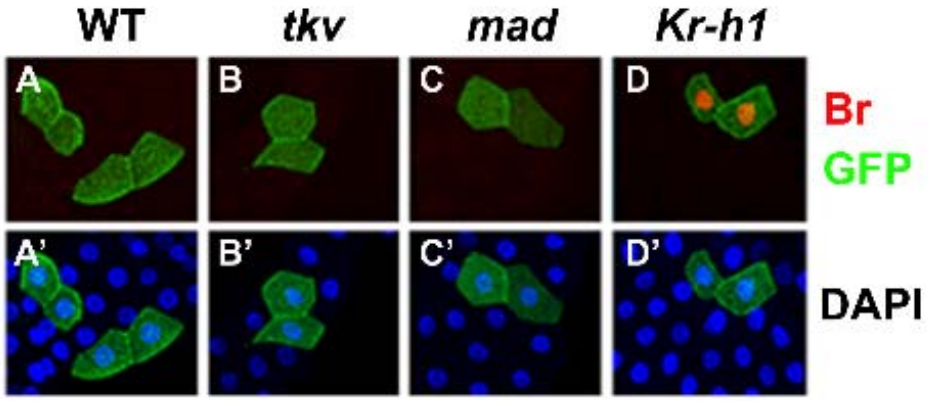


Fig 4.3. Tkv and Mad non-cell-autonomously repress *br* expression in the FB

MARCM analyses were carried out in the FB of wild type (**A-A'**), *tkv* (**B-B'**), *mad* (**C-C'**), and *Kr-h1* (**D-D'**) 2nd instar larvae. Cells homozygous for wild type, tkv^{8}, $mad^{8\text{-}2}$, or $Kr\text{-}h1^{10642}$ were marked by GFP (green). Br proteins were assessed with Br-core antibody (red). DAPI was used to label nuclei (blue).

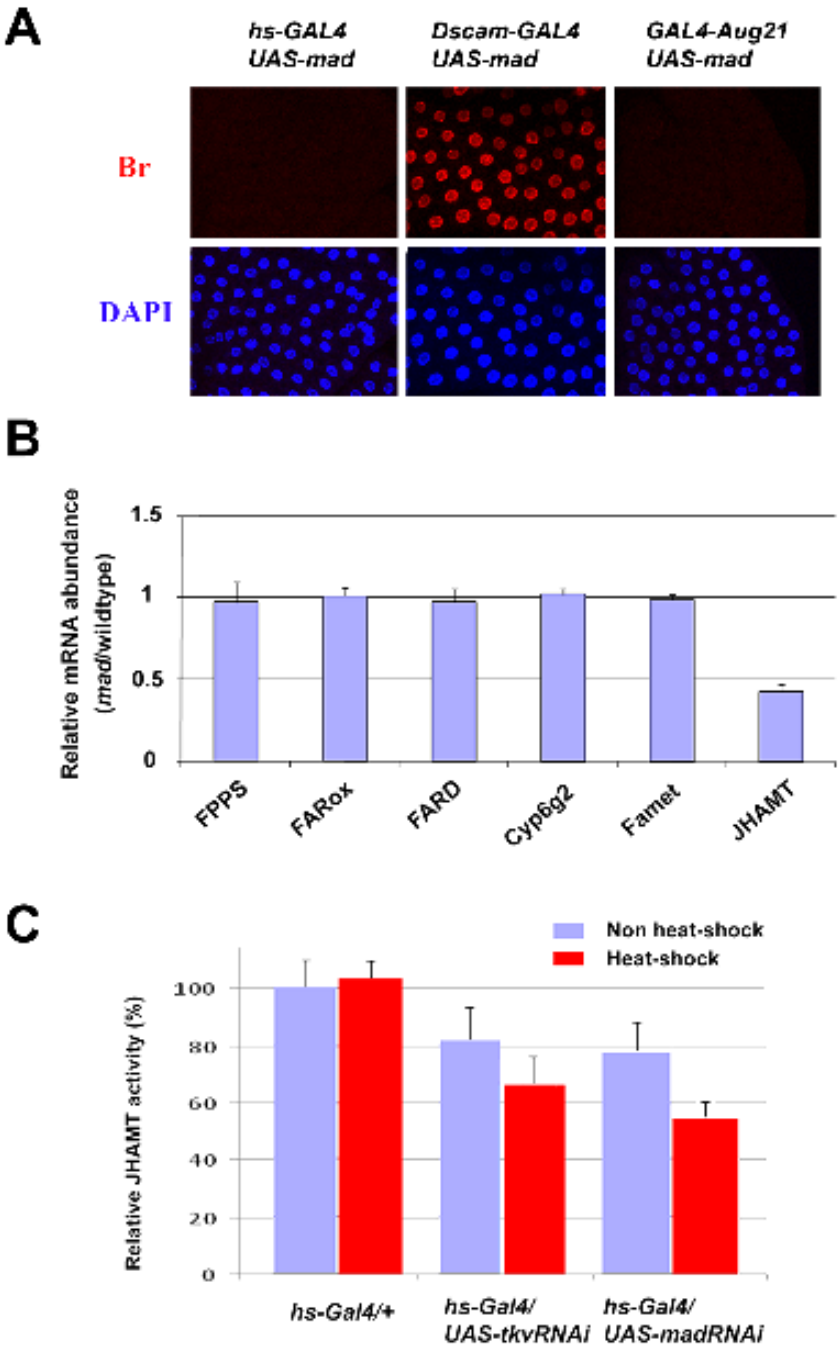

A
hs-GAL4
UAS-mad
Dscam-GAL4
UAS-mad
GAL4-Aug21
UAS-mad
Br
DAPI
B
Relative mRNA abundance
(mad/wildtype)
1.5
1
0.5
0
FPPS
FARox
FARD
Cyp6g2
Famet
JHAMT
C
Non heat-shock
Heat-shock
Relative JHAMT activity (%)
100
80
60
40
20
0
hs-Gal4/+
hs-Gal4/
UAS-tkvRNAi
hs-Gal4/
UAS-madRNAi

Fig 4.4. Tkv and Mad are required in the CA to promote *jhamt* transcription and then repress FB *br* expression

(A) *UAS-mad* was expressed in different tissues of mad^{k00237} mutants using the GAL4 lines that are expressed ubiquitously (*hs-GAL4*), specifically in neurons (*Dscam-GAL4*), or specifically in the CA (*Aug21-GAL4*). FBs of 2nd instar larvae were stained with Br-core antibody (red) and DAPI (blue). (B) mRNA levels of JH biosynthetic enzymes in the wild type and mad^{k00237} 2nd instar larvae were analyzed by quantitative real-time PCR. The ratios of mRNA levels between mad^{k00237} and wild type (means of 3 independent experiments ± standard deviations) larvae are presented. The accession numbers of genes and sequences of the primers are listed in Supplementary Table 1. (**C**) JHAMT activity in the brain-ring gland complexes of wild type, *tkv* RNAi, and *mad* RNAi organisms were measured at the wandering larval stage.

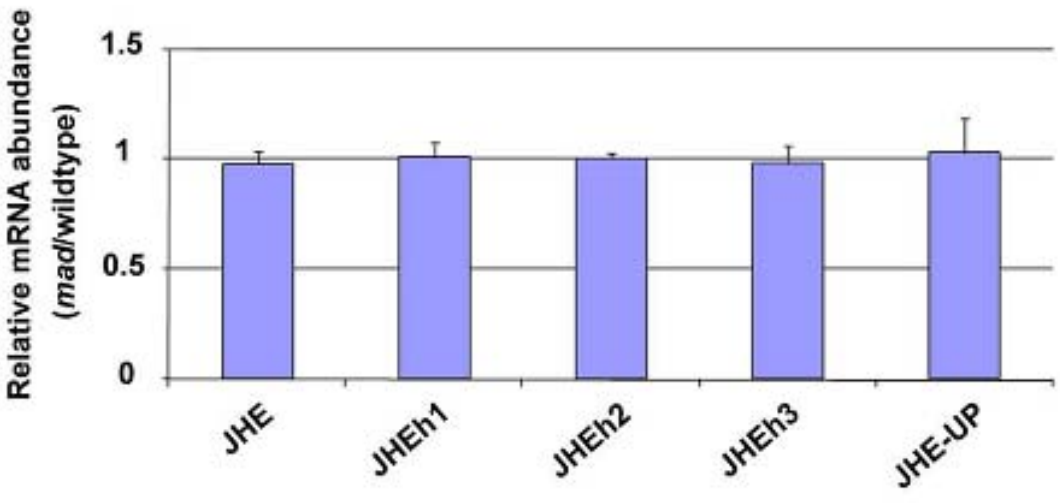


Fig. 4.5. Expression of JH degradative enzymes is not affected in *mad* mutants

The endogenous expression of enzymes related to JH degradation was analyzed by quantitative real-time PCR. Total RNA was prepared from wild-type and mad^{k00237} mutant 2nd instar larvae. Levels of mRNA were normalized to *actin* mRNA. The ratio between *mad* mutant and wild type larvae is presented. The average of three independent experiments is shown. Error bars indicate standard deviations.

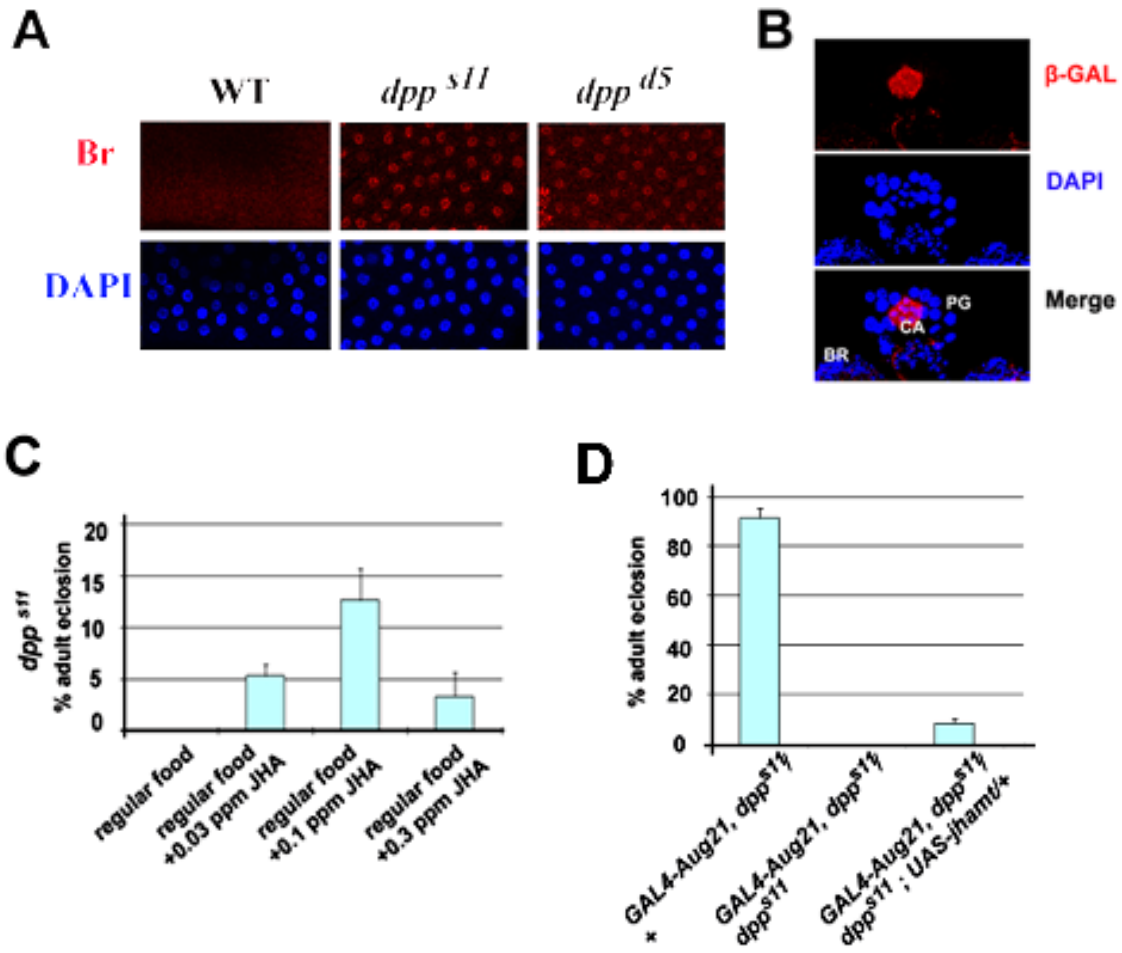
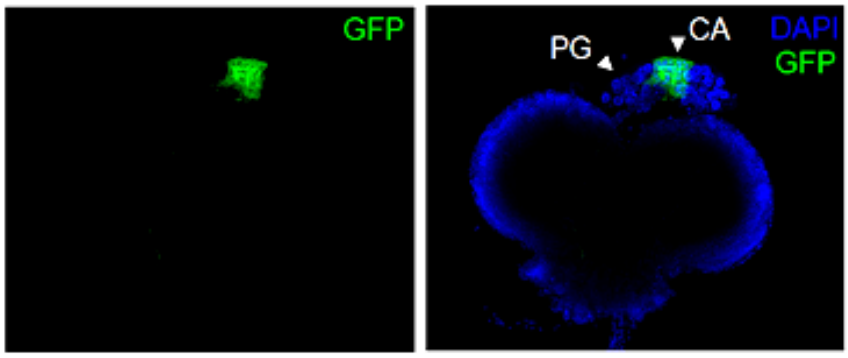


Fig 4.6. Dpp is the ligand of Tkv that mediates JH biosynthesis in the CA.

(A) FBs of wild type, *dpp*^{s11}, and *dpp*^{d5} 2nd instar larvae were stained with Br-core antibody (red) and DAPI (blue). (B) Brain-ring gland complexes of the *dpp-lacZ* transgene at the wandering larval stage were assessed with β-galactosidase antibody staining (red). Nuclei were labeled with DAPI (blue). CA = corpus allatum, PG = prothoracic gland, and BR = brain. (C) One hundred 1st instar larvae of *dpp*^{s11} were reared on normal food or food containing different concentrations of pyriproxifen. The percentages of individuals that develop into adults are shown as the means of 10 replicates ± standard deviations. (D) *GAL4-Aug21*, *dpp*^{s11}*/Cyo, GFP* flies were crossed with (1) +/*Cyo, GFP*, (2) *dpp*^{s1}*/Cyo, GFP*, and (3) *dpp*^{s1}*/Cyo, GFP; UAS-jhamt*. One hundred GFP-negative 1st instar larvae and their progeny were reared on normal fly food at 25 °C. The percentages of individuals that develop into adults are shown as the means of 10 replicates ± standard deviations.

A


GFP
PG
CA
DAPI
GFP

B

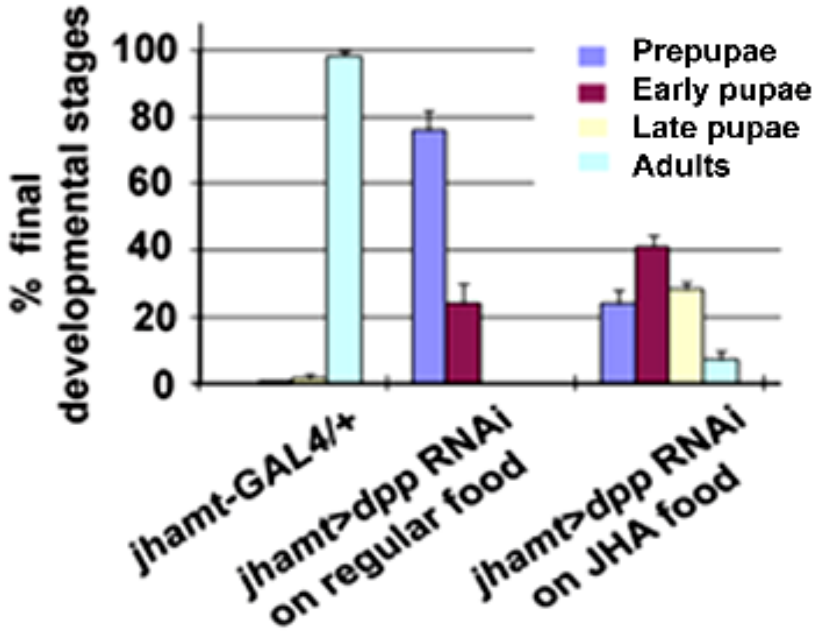

% final developmental stages
100
80
60
40
20
0
Prepupae
Early pupae
Late pupae
Adults
jhamt-GAL4/+
jhamt>dpp RNAi on regular food
jhamt>dpp RNAi on JHA food

Fig. 4.7. The lethality caused by CA-specific *dpp* RNAi is partially rescued by JHA treatment

(A) Brain-ring gland complex showing that the expression of *jhamt-GAL4* is restricted to the CA. *jhamt-GAL4* drives *UAS-mCD8GFP*, which is exclusively expressed in the CA cells. Nuclei were labeled with DAPI (blue). CA = corpus allatum, PG = prothoracic gland. (B) Flies of *jhamt-GAL4>UAS-dpp RNAi*, which are lethal during early-middle pupal stages were reared on regular food or food containing 0.1 ppm pyriproxifen (+JHA food). One hundred 1st instar larvae were reared in each vial. The percentages of individuals developing into each given developmental stage are shown as the means of 10 replicates ± standard deviations. Reared on regular food, most *jhamt-GAL4>UAS-dpp RNAi* flies died at the prepupal stage, and none of them developed into adults. Reared on +JHA food, most of *jhamt-GAL4>UAS-dpp RNAi* flies died at pupal stages, although over 5% of them developed into adults.

Prepupal stage: from pupariation to head eversion

Early pupal stage: from head eversion to yellow eyes

Late pupal stage: from yellow eyes to eclosion

Adult stage: after eclosion.

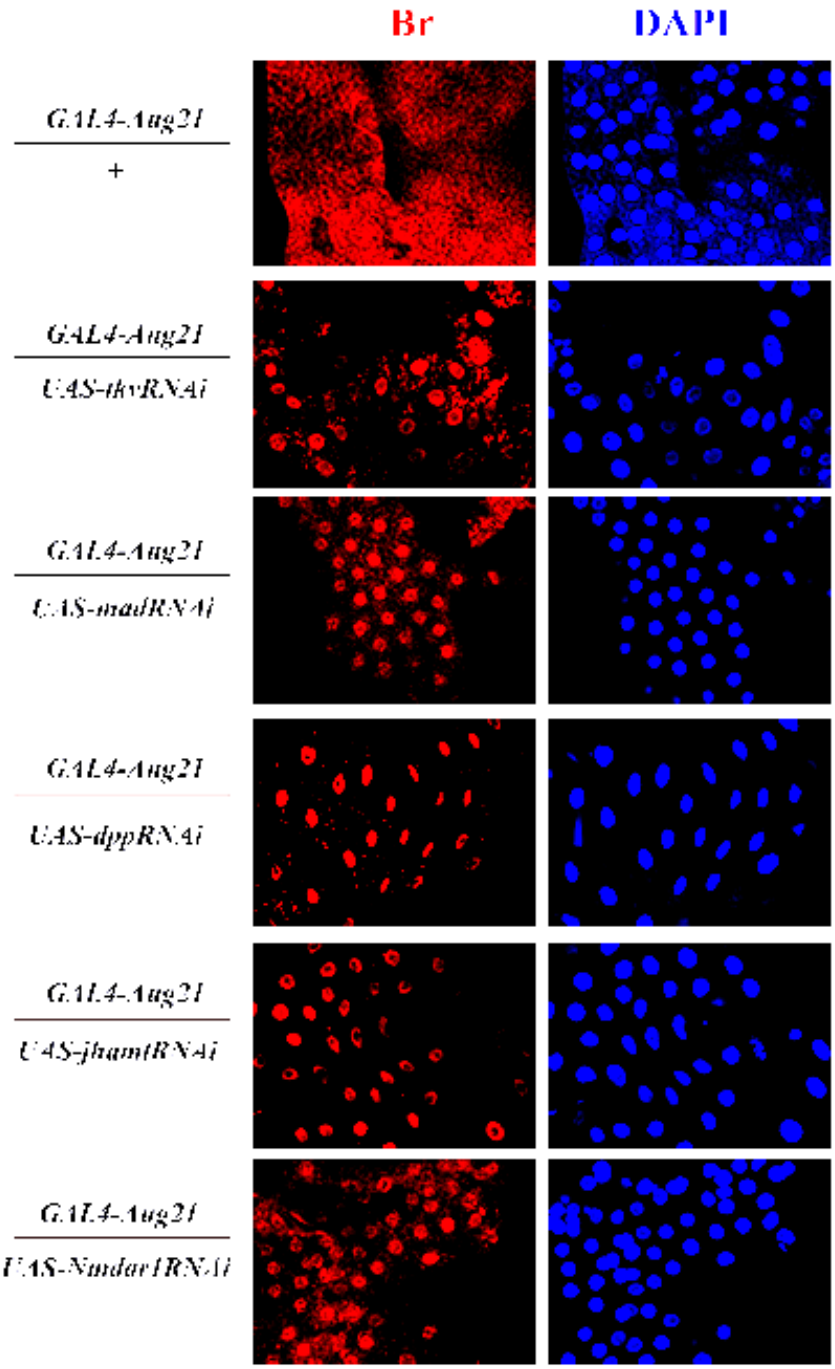


Fig 4.8. CA-specific knockdown of *dpp*, *tkv*, *mad*, *Nmdar1*, or *jhamt* induces precocious *br* expression

GAL4-Aug21 flies were crossed with *UAS-dpp RNAi*, *UAS-tkv RNAi*, *UAS-mad RNAi*, *UAS-Nmdar1 RNAi*, and *UAS-jhamt RNAi*. The FBs of their progeny were dissected at the 2nd instar larval stage and stained with Br-core antibody (red) and DAPI (blue).

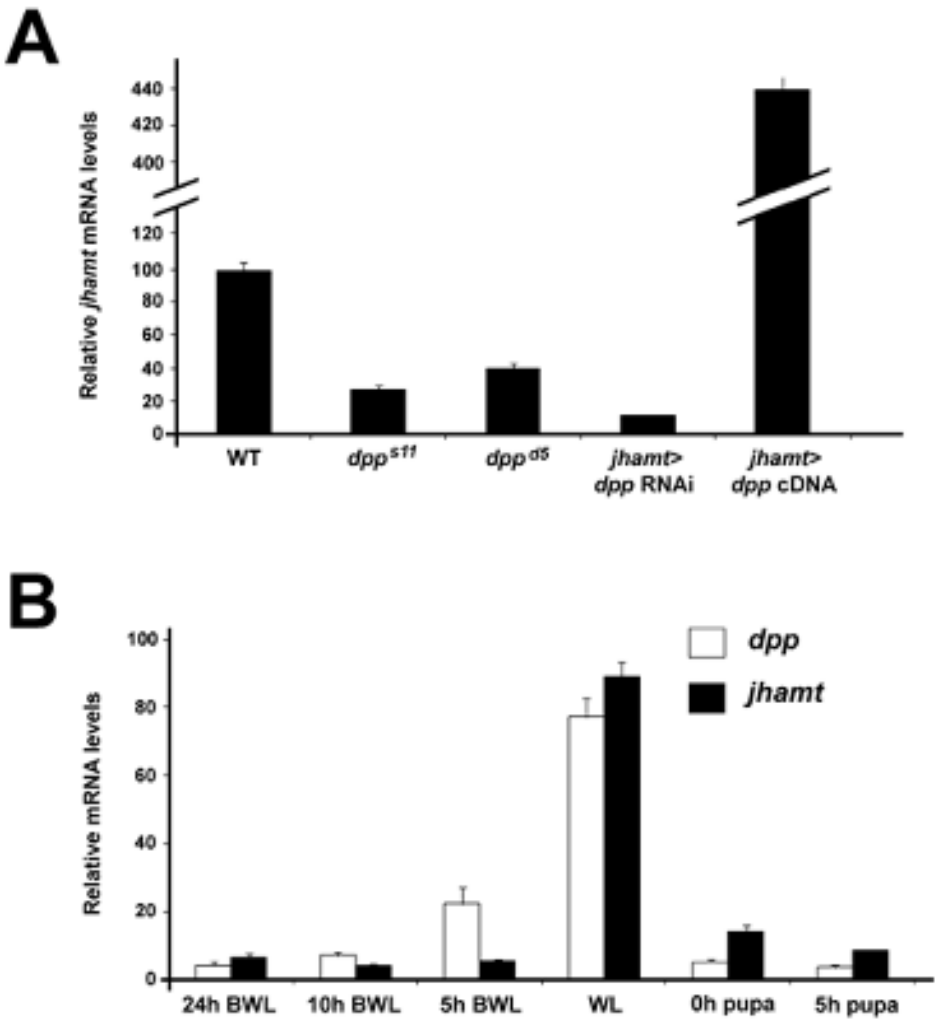


Fig 4.9. Expression of *dpp* in the CA correlates with that of *jhamt*.

(**A**) Relative *jhamt* mRNA levels at the wandering larval stage were compared among flies with different genetic backgrounds, including wild type, dpp^{s11}, dpp^{d5}, *jhamt-GAL4>UAS-dpp RNAi*, and *jhamt-GAL4>UAS-dpp* flies. Total RNA was extracted from the ring gland, and the mRNA levels of *jhamt* were assessed by quantitative real-time PCR. Levels of *jhamt* mRNA were normalized to *actin* mRNA. Values shown are the means of 3 independent experiments ± standard deviations. (B) Relative mRNA levels of *dpp* and *jhamt* in the ring gland were compared among different developmental stages of wild type organisms (Oregon R). Tissue and total RNA preparation, as well as quantitative real-time PCR, are the same as in (A).

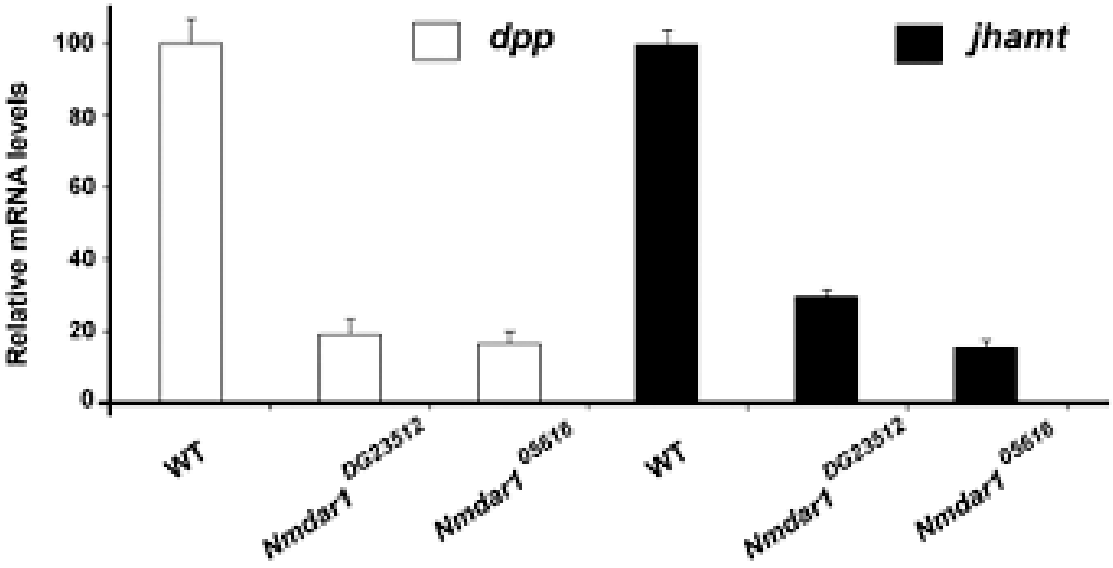


Fig 4.10. The expression of *dpp* and *jhamt* in the CA is reduced in *Nmdar1* mutants

The relative mRNA levels of *dpp* and *jhamt* at the wandering larval stage were compared between wild type and two *Nmdar1* mutant alleles, $Nmdar1^{05616}$ and $Nmdar1^{DG23512}$. Tissue and total RNA preparation, as well as quantitative real-time PCR, were performed as described in Fig. 4.9.

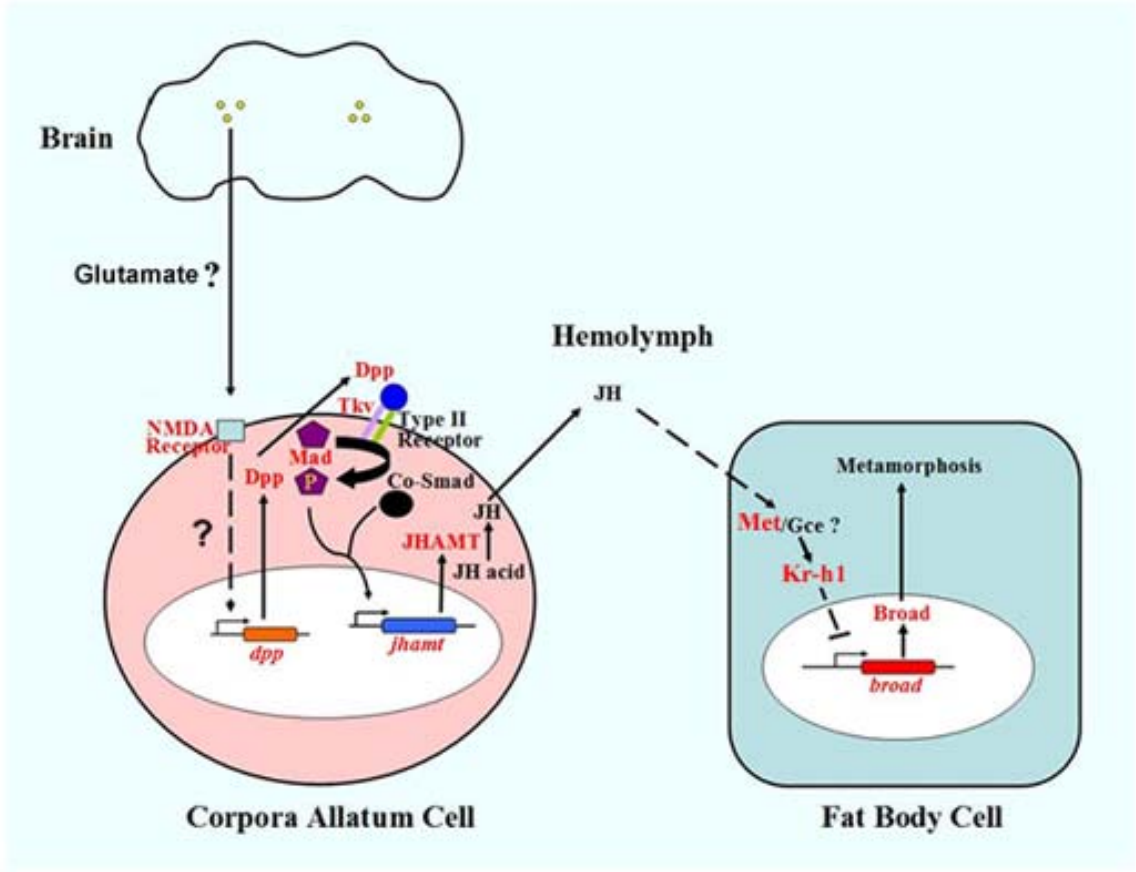


Fig 4.11. A model for the function of TGF-β signaling in controlling JH biosynthesis and insect metamorphosis

Proposed model as described in the text illustrating the function of TGF-β signaling in controlling JH biosynthesis and insect metamorphosis. The genes and proteins involved in this study are highlighted in red.

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Chapter 5

Wnt Signaling Mediates Juvenile Hormone Action through Regulating Expression of *Met* and *gce*

ABSTRACT

Juvenile hormone (JH) plays key roles in controlling insect growth and metamorphosis. However, relatively little is known about the JH signaling pathways. In recent years, evidence has accumulated suggesting that JH modulates the action of 20-hydroxyecdysone (20E) by regulating expression of *broad* (*br*), a 20E early response gene, through Met/Gce and Kr-h1. To identify other genes involved in JH signaling, we designed a novel genetic screen to isolate mutations that derepress JH-mediated *br* suppression at early larval stages. We found that mutations in three Wnt signaling negative regulators, *Axin* (*Axn*), *supernumerary limbs* (*slmb*), and *naked cuticle* (*nkd*), caused precocious *br* expression, which could not be blocked by exogenous JHA. A similar phenotype was observed when *armadillo* (*arm*), the mediator of Wnt signaling, was overexpressed. Quantitative reveres transcriptase PCR revealed that *Met*, *gce* and *Kr-h1*expression was suppressed in the *Axn*, *slmb* and *nkd* mutants as well as in *arm* gain-of-function larvae. Furthermore, ectopic expression of *gce* restored *Kr-h1* expression, but not *Met* expression, in the *arm* gain-of-function larvae. Taken together, we conclude that Wnt signaling cross-talks with JH signaling by suppressing transcription of *Met* and *gce*, genes that encode for putative JH receptors. The reduced JH activity further induces down-regulation of *Kr-h1*expression and eventually derepresses *br* expression in early larval stages.

INTRODUCTION

Juvenile hormone (JH) is a critical hormone that regulates many aspects of insect physiology. A major role of JH is its classic "status quo" action in the regulation of insect development. When 20-hydroxyecdysone (20E) induces molting during early developmental stages, the presence of JH results in a molt that repeats the previous stage (Riddiford 1996, Gilbert et al., 2000). Therefore, JH does not block the 20E-coordinated molting process, but rather directs the action of 20E. During the last two decades, studies on the hormonal regulation of insect development have focused on understanding the molecular basis of 20E, JH, and their interactions.

At the molecular level, 20E binds to its heterodimer receptor, EcR/USP, to directly activate the transcription of a small set of early-response genes that encode transcriptional factors. These genes transduce and amplify the original hormonal signal by activating a large number of late-response genes that encode tissue-specific effector proteins necessary for insect molts and metamorphosis (Thummel 2002). One of the 20E-induced early genes, *broad* (*br*), was identified as a key regulator in mediating the cross-talk between the 20E and JH signaling pathways. *Drosophila br* encodes four transcriptional factors that contain a common N-terminal domain and four pairs of different C2H2 DNA-binding zinc finger domains (DiBello et al., 1991, Bayer et al., 1996). The Br proteins directly regulate the transcription of 20E-induced late genes and are essential for inducing pupal development (Crossgrove 1996, Zhou and Riddiford 2002). Null *br* mutants can develop normally to the final larval instar but cannot form pupa (Kiss et al., 1988). Moreover, ectopic expression of *br* in early

2nd instar larvae induces premature pupal formation (Zhou et al., 2004). Therefore, the Br proteins are necessary and sufficient for the initiation of insect metamorphosis. Consistent with its function, the Br proteins are predominantly expressed during the larval-pupal transition in every holometabolous insect so far examined (Dubrovsky 2005). Previous studies in *Manduca*, *Bombyx*, and *Tribolium* suggested that the temporal pattern of *br* expression results from interaction between 20E and JH. 20E directly induces *br* expression, which can be prevented by JH in young larvae (Zhou et al., 1998, Konopova and Jindra 2008). Here, we demonstrate that JH is also required to repress *br* expression during early larval stages in *Drosophila*.

JH transduces its signal through a pathway including Methoprene-tolerant (Met), Germ cell-expressed (Gce) and Krüppel-homolog 1 (Kr-h1) and the p160/SRC/NCoA-like molecule (Taiman in *Drosophila* and FISC in *Ades*). The *Drosophila Met* and *gce* genes encode two functionally redundant bHLH-PAS protein family members, which have been proposed to be components of the elusive JH receptor (Wilson and Ashok 1998, Abdou et al., 2011). Both *Met* and *gce* mutants are viable and resistant to JH analogs (JHA) as well as to natural JH III (Abdou et al., 2011, Wilson and Fabian 1986). However, *Met-gce* double mutants are prepupal lethal and phenocopies CA-ablation flies (Abdou et al., 2011, Liu et al., 2009, Riddiford et al., 2010). The Met protein binds JH III with high affinity (Shemshedini and Wilson 1990, Miura et al., 2005). In *Tribolium*, suppression of *Met* activity by injecting double-stranded (ds) *Met* RNA causes precocious metamorphosis (Konopova and Jindra 2007). Kr-h1 is considered as a JH signaling component

working downstream of Met. In both *Drosophila* and *Tribolium*, *Kruppel-homolog1* (*Kr-h1*) mRNA is expressed at high levels during the embryonic stage and is continuously expressed in the larvae; then, it disappears during pupal and adult development (Pecasse 2000, Minakuchi et al., 2008). *Kr-h1* expression can be induced in the abdominal integument by exogenous application of the JH analog (JHA) at pupariation (Minakuchi et al., 2008). Suppression of *Kr-h1* by dsRNA in the early larval instars of *Tribolium* causes precocious *br* expression and premature metamorphosis in the next instar (Minakuchi et al., 2009). Thus by suppressing *br* expression, Kr-h1 is necessary for JH to maintain the larval state during a molt. Studies in *Aedes*, *Drosophila* and *Tribolium* have demonstrated that the p160/SRC/NCoA-like molecule is also required for JH to induce expression of *Kr-h1* and other JH response genes (Li et al., 2010, Zhang et al., 2010). For example in the presence of JH, *Ades* FISC forms a functional complex with Met on the JH response element and directly activates transcription of JH target genes (Li et al., 2010).

In an attempt to isolate other genes involved in JH signaling, we conducted a novel genetic screen that identified mutations in three Wnt signaling component genes, *Axin* (*Axn*), *supernumerary limbs* (*slmb*), and *naked cuticle* (*nkd*), which induced precocious *br* expression, mimicking loss of JH activity. The evolutionarily conserved Wnt signaling pathway controls numerous developmental processes (Cadigan and Nusse 1997). The key mediator of the *Drosophila* Wnt pathway is Armadillo (Arm, the homolog of vertebrate β-catenin). When the Wnt signaling ligand, Wingless (Wg), is absent, the destruction complex is active and

phosphorylates Arm, earmarking it for degradation. Upon Wg stimulation, the destruction complex is inactivated; as a result, unphosphorylated Arm accumulates in the cytosol and is targeted to the nucleus to stimulate transcription of Wnt target genes (Bienz 2005). Many players in the Wnt signaling pathway negatively regulate its activity. For example, Axin (Axn) is one of the main components of the destruction complex (Hamada et al., 1999). Supernumerary limbs (Slmb) recognizes phosphorylated Arm and targets it for polyubiqitination and proteasomal destruction (Jian and Struhl 1998). Naked cuticle (Nkd) antagonizes Wnt signaling by inhibiting nuclear import of Arm (Zeng et al., 2000). Our investigations have revealed that the high activity of Wnt signaling in the *Axn*, *slmb*, and *nkd* mutants suppresses the transcription of *Met* and *gce*, genes encoding for putative JH receptors, thus linking Wnt signaling to JH signaling and insect metamorphosis for the first time.

MATERIALS AND METHODS

Fly Strains and Genetics

All *Drosophila* strains were grown on standard cornmeal/molasses/yeast food at 25 °C. *Oregon* R strain was used as wild type. The *GAL4-PG12* line was a gift from H.-M. Bourbon (Bourbon et al., 2002). *UAS-gce* was a gift from T. Wilson (Baumann et al., 2010). The lethal mutant lines of *Axn*, *nkd*, and *slmb* as well as *arm-GAL4* and *UAS-arm*^{S10} were obtained from the Bloomington *Drosophila* Stock Center.

Immunohistochemistry and Microscopy

Immunohistochemistrical analysis of larval fat bodies was performed as previously described in Chapter 2. Florescence signals were captured with a Zeiss LSM510 confocal microscope (Carl Zeiss) and processed with Adobe Photoshop.

JHA Treatment

The JHA pyriproxyfen (Sigma) was dissolved in 95% ethanol to yield a 300 ppm stock solution. The JHA-containing fly food was prepared by adding the JHA stock solution to the standard cornmeal-molasses-yeast food at 50-55°C to a final concentration of 0.1 ppm.

qRT-PCR

Total RNAs were prepared from the 2nd instar larvae using the RNeasy Mini Kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler 480 SYBR Green I Master Kit (Roche). The mRNA levels of different genes were normalized to *rp49* mRNA with 4 replicates for each sample. The primers used in this study are listed in the following Table.

Genes	Purpose	Forward Primers	Reverse Primers
Met	qRT-PCR	5'-GCCAGAACCCTATCAGTTGG-3'	5'-AGCAGACGGTAGCAGCTCTC-3'
gce	qRT-PCR	5'-GATCCGAATCCGATGACTTC-3'	5'-GAATTTGCGGGAACAGAGTC-3'
Kr-h1	qRT-PCR	5'-CTCTGCACGTCAGCGATCTA-3'	5'-AACGTCCGGATTGGGTAGAG-3'
rp49	qRT-PCR	5'-GACAGTATCTGATGCCCAACA-3'	5'-CTTCTTGGAGGAGACGCCGT-3'
Met	RT-PCR	5'-GCAGTGATCTGGAGGAGGAG-3'	5'-ACCGTCTCTGCTGAATCCAC-3'
gce	RT-PCR	5'- CGTCGATCTCGAGGAGGATA -3'	5'-GATCAGCTGCTGTTTGAGCA-3'
Kr-h1	RT-PCR	5'-CGGAGCAGATCCCTATCAGT-3'	5'- AACGTCCGGATTGGGTAGAG -3'
rp49	RT-PCR	5'-GACAGTATCTGATGCCCAACA-3'	5'-CTTCTTGGAGGAGACGCCGT-3'

RESULTS

A genetic screen for mutations affecting *br* expression

Because JH represses *br* expression during early larval stages, we reasoned that mutations that reduce the JH titer or disrupt JH action should cause precocious *br* expression in *Drosophila*. Accordingly, we designed and conducted a genetic screen to isolate genes that affect these processes. In these screens, *GAL4-PG12>UAS-mCD8GFP* on the X chromosome was used as a reporter of *br* expression, and lethal mutations or *P*-insertions on the 2nd or 3rd chromosome were made homozygous and screened for precocious *br* expression (Chapter 3). Because most of the lethal lines allowed organisms to develop to early larval stages, we were able to examine GFP expression in the 2nd instar under the fluorescent microscope. From 4,400 lethal lines, 55 mutations were isolated based on GFP expression in the 2nd instar larvae. Genes associated with these mutations encode proteins with various molecular functions, including enzymes, signal transduction molecules, and transcriptional factors.

This genetic screen was efficient in identifying the genes required for JH biosynthesis. It not only isolated genes that are known to be involved in JH biosynthesis, such as *farnesyl diphosphate synthase* (*Fpps*) (Sen et al., 2007), *apterous* (*ap*) (Altaratz et al., 1991), *Insulin receptor* (*InR*) (Tatar et al., 2001, Tu et al., 2005), and N-*methyl*-D-*aspartate receptor 1* (*Nmdar1*) (Chiang et al., 2002), but also revealed that Dpp-mediated TGF-β signaling in the corpus allatum stimulates JH biosynthesis by upregulating transcription of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH synthesis (Huang et al., 2011). The same genetic screen

also isolated genes that are involved in JH signaling, such as *Kr-h1*. Another known JH signaling component, Met, was not identified by this screen because the *Met* gene is located to the X chromosome. A reverse genetic study showed that precocious *br* expression was also detectable in *Met* mutant larvae (Huang et al., 2011).

Mutations in the negative regulators of Wnt signaling cause precocious *br* expression

Three important components of Wnt signaling, *Axn*, *slmb*, and *nkd* were found among these 55 genes. As shown in Fig. 5.1, expression of *GAL4-PG12>UAS-mCD8GFP* was restricted to salivary glands in the wild type 2nd instar larvae (Fig. 5.1A), but ubiquitous expression of *GAL4-PG12>UAS-mCD8GFP* was detected at the same stage in the *Axn*, *slmb*, and *nkd* mutant larvae (Fig. 5.1B-D). These results suggest that Wnt signaling is required to repress *br* expression during the early larval stages, possibly by regulating either the JH titer or JH signaling.

Exogenous JHA does not prevent precocious *br* expression in *Axn*, *slmb*, and *nkd* mutants

Consistently, precocious *br* expression was observed when we used Br-core antibody staining at the 2nd instar. Endogenous Br proteins were not detectable in the fat body (FB) of the wild type (Fig. 5.2A), but were observed in the FB nuclei of the $Axn^{EY10228}$, $slmb^{EY09052}$, and nkd^{2} larvae (Fig. 5.2B-D). We then examined other *Axn*, *slmb*, and *nkd* alleles, including Axn^{16-21}, $slmb^{00295}$, and nkd^{3}. Precocious *br* expression was detected in all cases.

Next, we asked whether the precocious *br* expression phenotype of the *Axn*, *slmb*, and *nkd* mutants could be blocked by exogenous JHA. Wild type, $Axn^{EY10228}$, $slmb^{EY09052}$, and nkd^{2} larvae were reared on a diet containing 0.1 ppm pyriproxifen. Immunohistochemical results revealed that precocious *br* expression was not suppressed by exogenous JHA in the FB of the *Axn*, *slmb*, and *nkd* mutant larvae (Fig. 5.2F-H).

These results are the opposite of what we observed in mutants that affect JH biosynthesis, such as *tkv* and *mad*, in which the precocious *br* expression was totally suppressed by exogenous JHA (Huang et al., 2011). In contrast, these data are consistent with what we observed in the mutations that affect JH signaling, such as *Kr-h1* and *Met* (Abdou et al., 2011, Huang et al., 2011). Therefore, we suggest that *Axn*, *slmb*, and *nkd* affect *br* expression by modulating JH signaling.

Met, _gce_ and _Kr-h1_expression is suppressed in _Axn_, _slmb_ and _nkd_ mutants

JH functions through Met, Gce and Kr-h1 to suppress *br* expression during the early larval stages (Konopova and Jindra 2008, Abdou et al., 2011, Minakuchi et al., 2008, Minakuchi et al., 2009). We therefore investigated whether Wnt signaling regulates *Met*, *gce* and *Kr-h1*expression. We first used qRT-PCR to compare mRNA levels for *Met*, *gce* and *Kr-h1* between wild type and *Axn*, *slmb* and *nkd* mutants. In the *Axn*, *slmb* and *nkd* mutant 2nd instar larvae, the mRNA levels of *Met*, *gce* and *Kr-h1* were only about 20% of that in wild type at the same stage (Fig. 5.3A). Similarly, when

reverse transcriptional PCR was carried out for 30 cycles, the *Met*, *gce* and *Kr-h1* mRNA levels were also obviously reduced in the *Axn*, *slmb*, and *nkd* mutant 2nd instar larvae (Fig. 5.3B). These results suggest that *Met*, *gce* and *Kr-h1*expression are suppressed in *Axn*, *slmb* and *nkd* mutants, and this results in precocious *br* expression.

Gain-of-function mutation of *arm* activates *br* and suppresses *Met*, *gce* and *Kr-h1* expression

Because Axn, Slmb, and Nkd negatively affect Wnt signaling activity (Hamada et al., 1999, Zeng et al., 2000), increased Wnt signaling activity was expected in the *Axn*, *slmb* and *nkd* mutants. We hypothesized that the high Wnt signaling activity accounted for precocious *br* expression as well as suppression of *Met*, *gce* and *Kr-h1* transcription in the *Axn*, *slmb* and *nkd* larvae. To test this hypothesis, we examined the effects of the *arm* gain-of-function mutation on the expression of *br*, *Met*, *gce* and *Kr-h1* transcription.

Stabilization and accumulation of Arm in the cytosol increases is in the nucleus importation and this activates the transcription of Wnt target genes (Bienz 2005). Arm^{S10} is a constitutively active form of Arm that resist degradation as it carries a 54 amino acid deletion Shaggy phosphorylation sites (Pai et al., 1997). When *UAS-*arm^{S10} that includes the driven by *arm-GAL4* was expressed in the wild type, we detected precocious *br* expression with the Br-core antibody staining fat bodies of 2nd instar larvae (Fig. 5.4B). The qRT-PCR data revealed that mRNA levels of *Met*, *gce* and *Kr-h1* in the *arm-GAL4>UAS-*arm^{S10} 2nd instar larvae were significantly reduced

being less than 20% of that in the wild type (Fig. 5.5). Therefore, the phenotypes of the *arm* gain-of-function mutant are identical to that of *Axn*, *slmb* and *nkd* mutants, supporting the hypothesis that high Wnt signaling activity suppresses *Met*, *gce*, and *Kr-h1* expression and promotes *br* expression.

Wnt signaling indirectly suppresses *Kr-h1* expression by down-regulating *Met* and *gce* expression

Our previous studies revealed that Met and Gce are functionally redundant in transducing JH signaling. The *Met-gce* double mutant can totally eliminate JH-induced *Kr-h1* expression (Abdou et al., 2011). Therefore, we investigated whether Wnt signaling indirectly suppresses *Kr-h1* expression by down-regulating *Met* and *gce*. We co-expressed arm^{S10} and *gce* in wild type flies and examined *br*, *Met*, *gce* and *Kr-h1* expression. When *UAS-*arm^{S10} and *UAS-gce* were driven by *arm-GAL4*, the precocious *br* expression induced by *arm-GAL4>UAS-*arm^{S10} was totally suppressed, as indicated by the absence of Br proteins in the nuclei of 2nd instar larval fat body cells (Fig. 5.4C). In the same organisms, the *gce* mRNA level was increased by > 30-fold, whereas transcription level of *Kr-h1* and Met were ~1.5-fold and ~0.3-fold wild type level (Fig. 5.5). These results demonstrate that ectopic expression of *gce* can block Arm^{S10}-mediated *Kr-h1* suppression, but does not affect Arm^{S10}-mediated *Met* suppression. We conclude that Wnt signaling indirectly regulates *Kr-h1* expression by down-regulating *Met* and *gce*.

Taken together, our genetic screen and further investigations demonstrate that Wnt signaling suppresses transcription of the potential JH receptors *Met* and *gce*, which reduces JH signaling activity as evident by the reduced *Kr-h1*expression and precocious *br* expression. This study reveals that Wnt signaling cross-talks with JH signaling in mediating insect metamorphosis.

DISCUSSION

Interactions between Wnt and JH signaling pathways

As our knowledge of signal transduction increases, we are increasing able to decipher how individual signaling pathways integrate into the broader signaling networks that regulate fundamental biological processes. In vertebrates, Wnt signaling has been found to interact with different hormone signaling pathways to mediate various developmental events. For example, the Wnt/beta-catenin signaling pathway interacts with thyroid hormones in the terminal differentiation of growth plate chondrocytes (Wang et al., 2007) and interacts with estrogen to regulate early gene expression in response to mechanical strain in osteoblastic cells (Kouzmenko et al., 2004, Liedert et al., 2010). In insects, both Wnt and JH signaling are important regulatory pathways, each controlling a wide range of biological processes. Here, we report for the first time that the Wnt signaling pathway interacts with JH in regulating insect development. During the *Drosophila* early larval stages, elevated Wnt signaling activity in the *Axn*, *slmb*, *nkd* mutants and *arm-GAL4>UAS-arm*^{S10} flies represses *Met* and *gce* expression, which down-regulates *Kr-h1* and causes precocious *br*

expression. Ectopic expression of *UAS-gce* in the *arm-GAL4>UAS-arm*^{S10} larvae is sufficient for restoring *Kr-h1* expression and then repressing *br* expression.

Arm is a co-activator that interacts with *Drosophila* TCF homolog Pangolin (Pan), a Wnt-response element-binding protein, to stimulate expression of Wnt signaling target genes (Brunner et al., 1997). In the absence of nuclear Arm, Pan interacts with Groucho, a co-repressor, to repress transcription of Wingless-responsive genes (Cavallo et al., 1998). Upon the presence of nuclear Arm, it binds to Pan, converting it into a transcriptional activator to promote the transcription of Wingless-responsive genes (Brunner et al., 1997). We propose that Wnt signaling indirectly suppresses *Met* and *gce* expression by activating an unknown transcriptional repressor (Fig. 5.6)

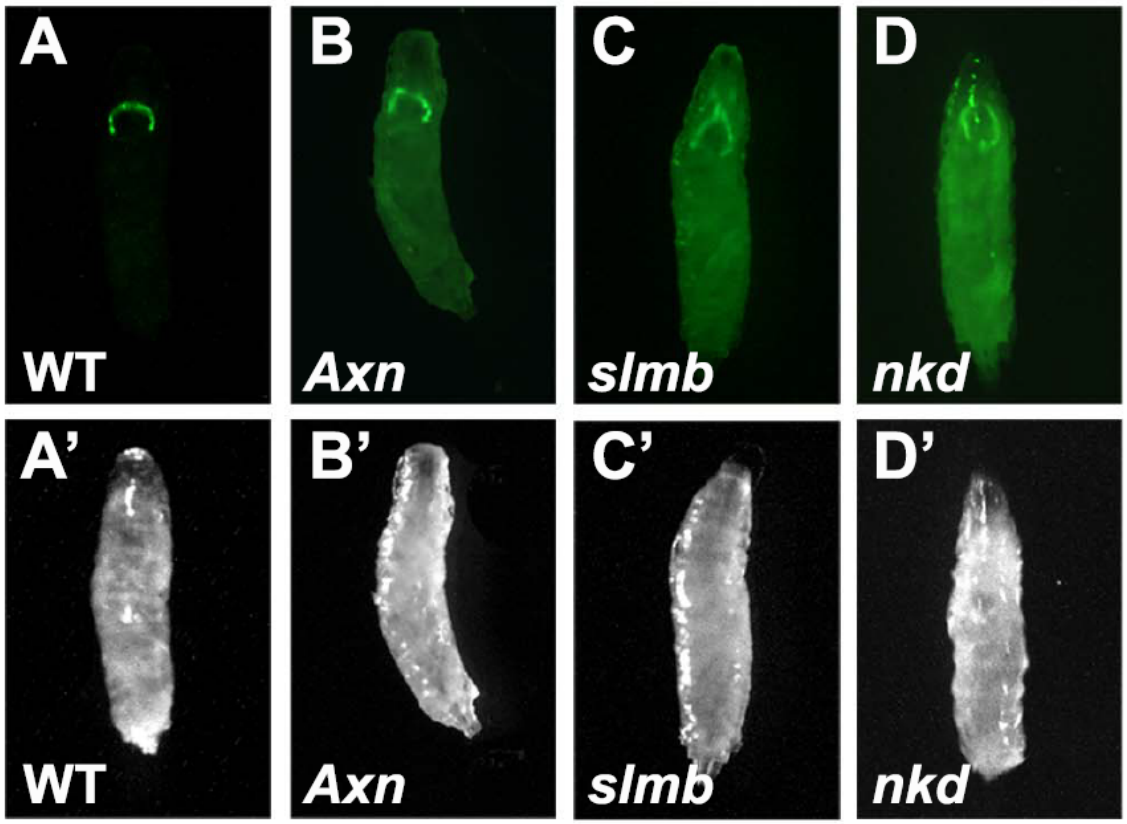

Fig 5.1. A genetic screen identifies that Axn, Slmb and Nkd regulate *br* expression

GFP images show the expression of *GAL4-PG12>UASmCD8GFP* in 2nd instar larvae. GFP was only expressed in the salivary gland of the wild type [A], but widely expressed in all tissues of *Axn* [B], *slmb* [C] and *nkd* [D] mutant larvae. (A'-D') White light images of the same organisms are shown in [A-D].

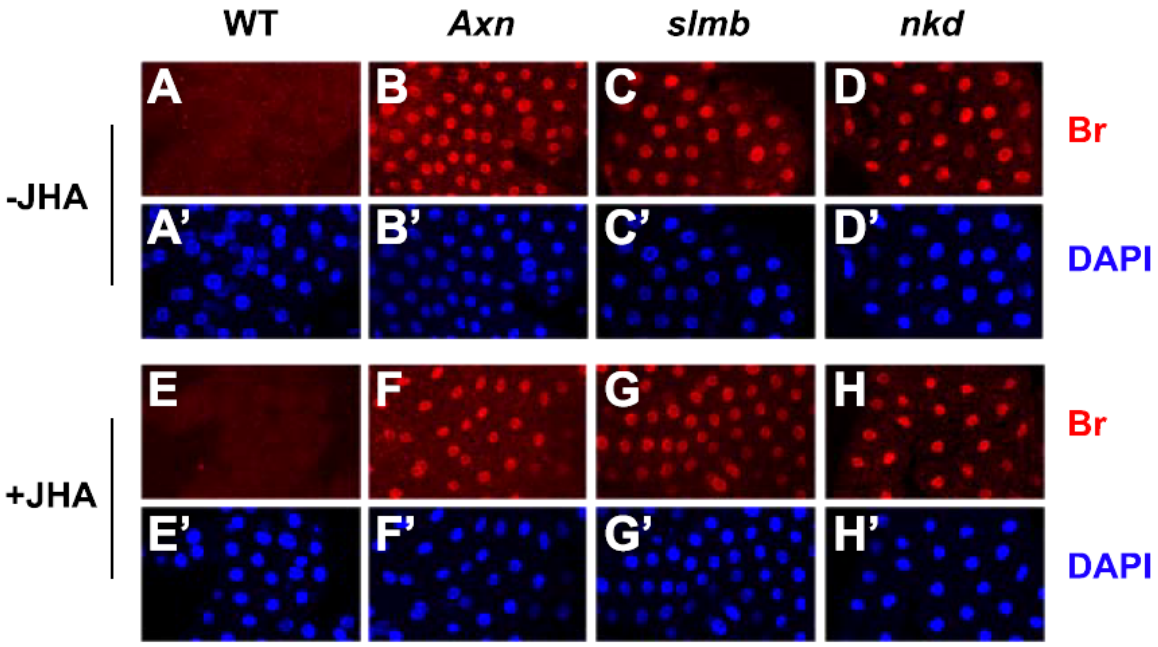

Fig 5.2. Precocious *br* expression in *Axn*, *slmb* and *nkd* mutants is not prevented by JHA

Wild type and the *Axn*, *slmb*, and *nkd* mutants were reared on normal (-JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Fat bodies of the 2nd instar larvae were stained with a Br-core antibody (red), and nuclei were labeled with DAPI (blue).

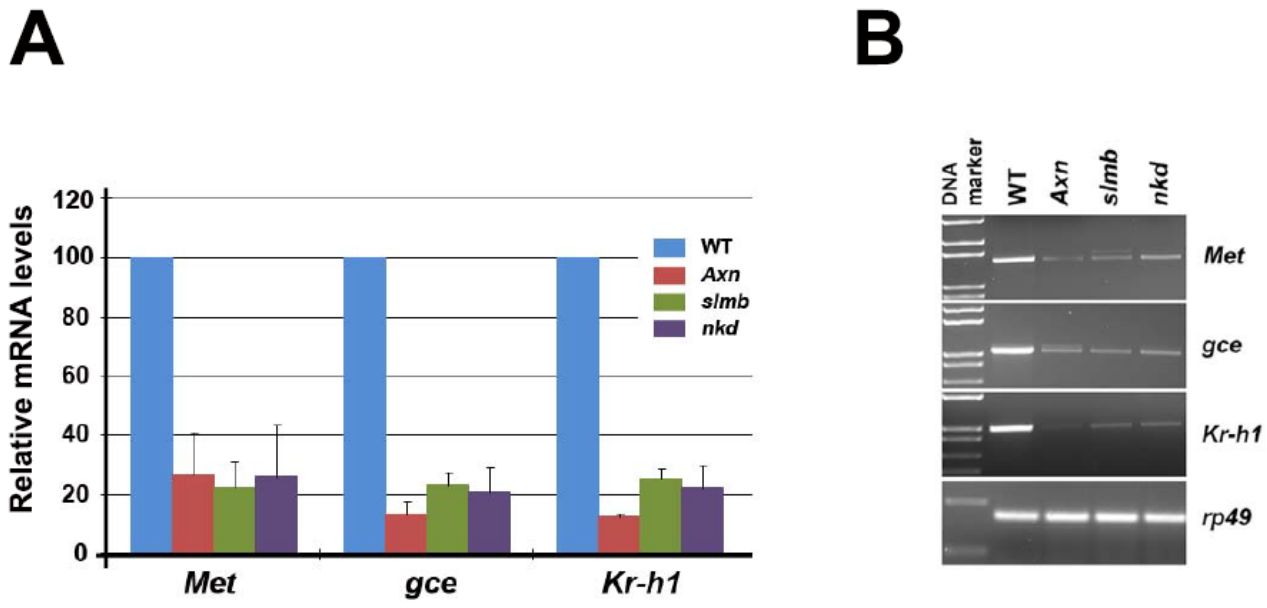

Fig 5.3. Expression of *Met*, *gce* and *Kr-h1* is reduced in the *Axn*, *slmb* and *nkd* mutants.

(C) Total RNAs were extracted from wild type, *Axn*, *slmb* and *nkd* 2nd instar larvae. The mRNA levels of *Met*, *gce* and *Kr-h1* were assessed by quantitative real-time PCR and normalized to *rp49* mRNA. Values shown are the means of 4 independent experiments ± standard deviations.

(D) The same total RNAs described in [A] were used as the templates for a 30-cycle reverse transcriptional PCR. The RT-PCR products were analyzed by DNA agarose gel electrophoresis.

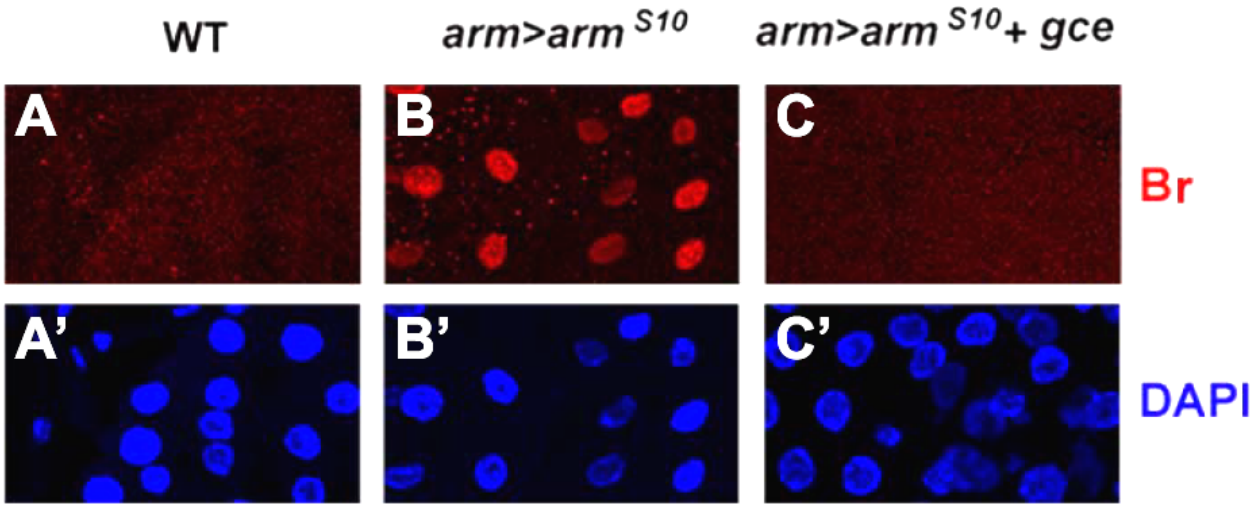

Fig. 5.4. Gain-of-function *arm* mutation induces precocious *br* expression

(A-C) Fat bodies of 2nd instar larvae were stained with a Br-core antibody (red) and DAPI (blue).

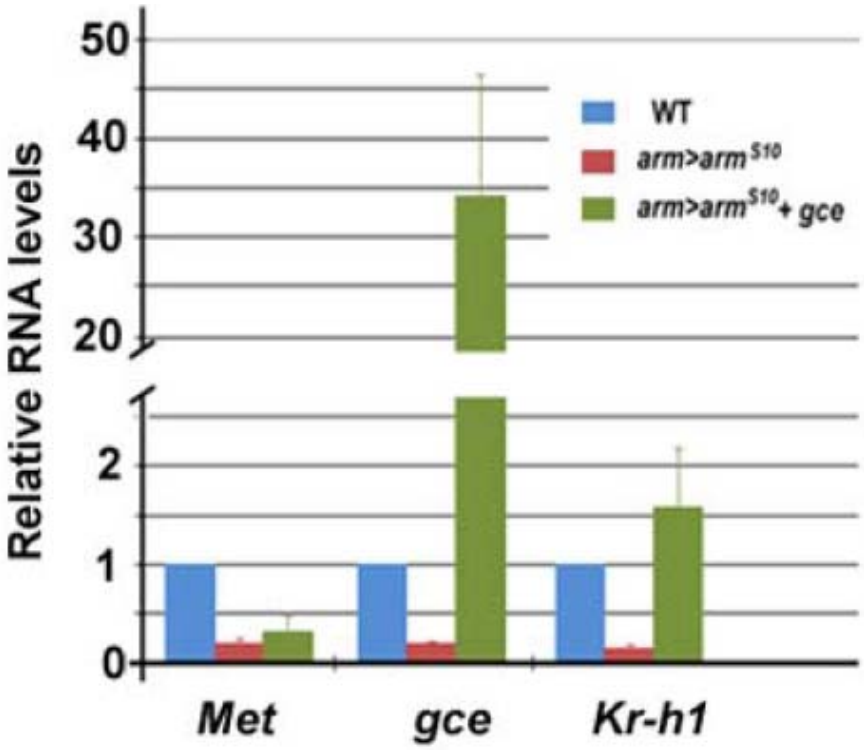

Fig. 5.5. Gain-of-function *arm* mutation suppresses *Met*, *gce* and *Kr-h1* expression
Total RNA was extracted from the 2nd instar larvae. The mRNA levels of *Met*, *gce* and *Kr-h1* were assessed by qRT-PCR and normalized to *rp49* mRNA. Values shown are the means of 4 independent experiments ± standard deviations. Genotypes include: wild type; *arm-GAL4/UAS-arm^{S10}* and *arm-GAL4/UAS-arm^{S10},UAS-gce/+*.

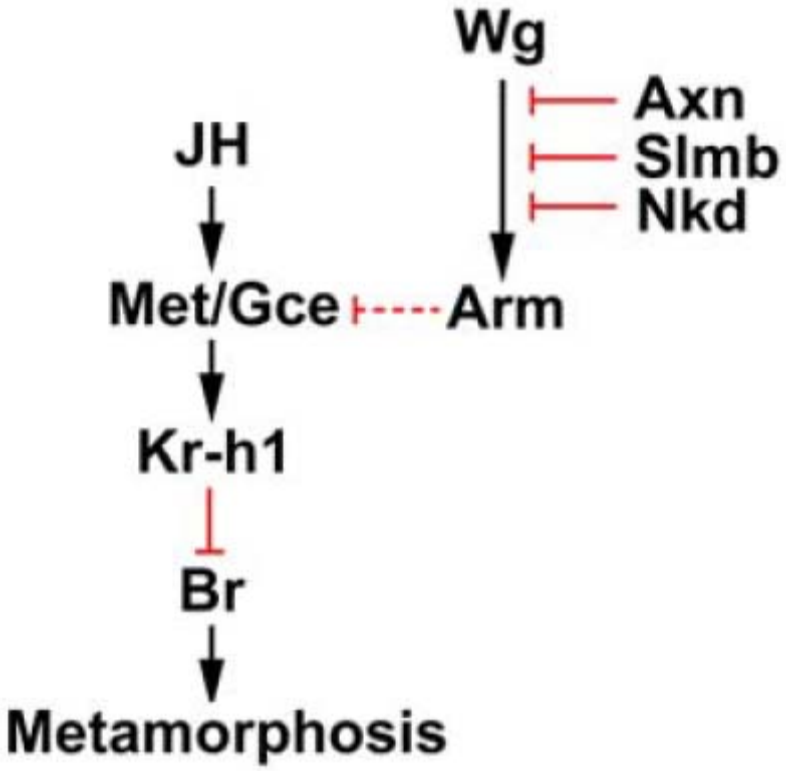

Fig. 5.6. As described in the text, the proposed model illustrates the cross-talk between the Wnt and JH signaling pathways.

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Chapter 6

Conclusions and future perspectives

1- Conclusions:

Chapter 2:

1. Bioinformatic analyses indicated that the *DmMet* and *Dmgce* share high amino acids sequence similarity and high identities in the conserved domains bHLH, PAS-A, and PAS-B.
2. The *gce* null mutant flies, $gce^{2.5K}$, which were generated by imprecise excision, are viable, fertile with low fecundity, and resistant to JH analogs similar to *Met* null mutant flies, Met^{27}. When the two mutations were recombined together, the Met^{27}-$gce^{2.5K}$ double mutant flies died at white pupae during the larval-pupal transition. The expression of *Kr-h1*, which is a JH response gene and functions as inhibitor for 20E-induced *br* expression, is decreased as detected through q-RT-PCR analysis in the young instar larvae (2^{nd} instar) of Met^{27}-$gce^{2.5K}$ animals. Accordingly, *br* expression is precociously activated in the fat bodies of 2^{nd} instar larvae as detected by immunohistochemistry assay using Br-core antibodies.
3. It is well known that the caspase-dependent programmed cell death (PCD) during the larval-pupal transition is induced by Br proteins. In the Met^{27}-$gce^{2.5k}$ double mutants, the precociously expressed Br proteins at 2^{nd} instar larval stages caused the precocious caspase-dependent PCD in the fat body cells, which led to premature and enhanced fat body dissociation.

4. The prepupal lethality, reduced *Kr-h1*expression, precocious *br* expression, and premature caspase-dependent PCD phenotypes of Met^{27}-$gce^{2.5k}$ double mutants were also observed in the JH-deficient animal, *Aug21-GAL4>UAS-grim*, in which the cell death gene *grim* was specifically expressed in the corpora allata to ablate JH production cells. However, when reared on food containing JH analogue (pyriproxyfen), the defective phenotypes and gene expression changes of JH-deficient animals were restored to the wild type, while that of Met^{27}-$gce^{2.5k}$ double mutants were not.

Together, our results demonstrate that *Drosophila* Met and Gce are functionally redundant in transducing the "*status quo*" action of JH. JH induces *Kr-h1* expression through binding with Met and Gce. Kr-h1 inhibits *br* expression and precludes 20E-induced caspase-dependent PCD during larval molts.

Chapter: 3

1. For the better understanding of JH biosynthesis and signaling pathway, we designed a novel genetic screen to identify genes that are involved in either biosynthesis or signaling pathways. To facilitate the screening procedure, we identified a specific Gal4 driver, *PG12-Gal4*, to monitor *br* expression *in vivo*. *PG12-Gal4* is an enhancer trap line that carries a *p{GawB}* insertion in the first intron of *br* gene. We demonstrated that *PG12-Gal4>UAS-mCD8GFP* express with the same temporal and spatial pattern as the endogenous *br* gene. Both *PG12-Gal4>UAS-mCD8GFP* and the endogenous *br* gene were predominantly expressed in the late 3rd instar larvae and pupal stages.

2. The principle of this screening depends on the hypothesis that mutations which reduce JH activity will induce precocious expression of *PG12-Gal4>UAS-mCD8GFP* in the early larval stages. To test this hypothesis, we built a *juvenile hormone esterase* transgenic fly that carries *jhe* cDNA driven by a heat-shock promoter (*hs-jhe*). When *hs-jhe* larvae were treated with heat-shock, the precocious expression of *PG12-Gal4>UAS-mCD8GFP* as well as endogenous *br* gene was detected. This early *br* expression can be fully suppressed by JHA, pyriproxyfen.
3. We screened 4,400 lethal mutation lines and identified 55 genes that may be involved in JH biosynthesis or signaling. Mutations in these genes caused precocious expression of *PG12-Gal4>UAS-mCD8GFP* in the 2nd instar larvae.
4. The 55 genes can be divided into two groups. 35 genes are potentially involved in JH biosynthesis because their phenotypes can be suppressed by JHA treatment. Some of these genes are well known to be required for JH biosynthesis such as *farnesyl diphosphate synthase* (*Fpps*), *apterous* (*ap*), *Insulin receptor* (*InR*), and N-*methyl*-D-*aspartate receptor 1* (*Nmdar1*). The remaining 20 genes are potentially involved in JH signaling pathways including known JH signaling component *Kr-h1*, because their phenotypes can not be suppressed by JHA treatment.

Chapter 4:

1. Our genetic screen identified two TGF-β signaling components, *thick vein* (*tkv*) and *mothers against Dpp* (*Mad*). Mutations in *tkv* and *Mad* induced precocious *br* expression in the 2nd instar larvae.
2. The precocious *br* expression caused by *tkv* and *Mad* mutations can be suppressed by exogenous JHA treatment.
3. MARCM analysis revealed that Tkv and Mad non-cell-autonomously repress *br* expression in the FB. In the fat bodies of 2nd instar larvae, *br* was precociously expressed in the cells of *Kr-h1* mutant MARCM clones, but not in the cells of *tkv* and *Mad* mutant MARCM clones.
4. Expression of *Mad* cDNA specifically in the corpora allata of *Mad* mutants by *Aug21-Gal4>UAS-Mad* blocked the precocious *br* expression phenotypes, suggesting that Mad is required in the corpora allata to regulate JH biosynthesis.
5. qRT-PCR studies revealed that expression levels of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH biosynthesis, in the *Mad*-RNAi and *tkv*-RNAi animals were only about 50% and 60% that of wilt type, respectively.
6. Decapentaplegic (Dpp) is one of seven TGF-β superfamily members in *Drosophila*. Hypomorphic *dpp* mutants also caused precocious *br* expression in the 2nd larva fat body, which could be suppressed by JHA treatment.

7. Hypomorphic *dpp* mutants were completely pupal lethal. Noticeably, this pupal lethality could be partially rescued by JHA treatment and CA-specific expression of *jhamt* cDNA by *Aug21-Gal4>UAS-jhamt*.
8. *dpp* was specifically expressed in the CA cells of ring glands, and its expression profile in the CA correlated with that of *jhamt* and matched JH levels in the hemolymph.
9. Reduced *dpp* expression was detected in the mutant larvae of *Nmdar1*, a CA-expressed glutamate receptor.

In summary, we conclude that the neurotransmitter glutamate promotes *dpp* expression in the CA, which stimulates JH biosynthesis through Tkv and Mad by upregulating *jhamt* transcription at the early larval stages to prevent premature metamorphosis.

Chapter 5:

1. Our genetic screen identified mutations in three Wnt signaling negative regulators, *Axin* (*Axn*), *supernumerary limbs* (*slmb*), and *naked cuticle* (*nkd*), that caused precocious *br* expression, which could not be blocked by exogenous JHA, suggesting that *Axn*, *slmb*, and *nkd* affect *br* expression by affecting JH signaling.
2. qRT-PCR studies discovered that mRNA levels of JH signaling components *Met*, *gce* and *Kr-h1* were reduced in the *Axn*, *slmb* and *nkd* mutants to only about 20% of that in wild type.

3. Because Axn, Slmb, and Nkd negatively affect Wnt signaling activity, increased Wnt signaling activity was expected in the *Axn*, *slmb* and *nkd* mutants, which is represented by the nuclear accumulation of Wnt signaling key mediator, Armadillo (Arm). We found that precocious *br* expression also occurred in the fat bodies of 2nd instar larvae when *UAS-arm*^{S10} (a constitutively active form of Arm) was overexpressed in the *arm-GAL4>UAS-arm*^{S10}. Meanwhile, the mRNA levels of *Met*, *gce* and *Kr-h1* in the *arm-GAL4>UAS-arm*^{S10} larvae were significantly reduced to less than 20% of that in the wild type. Therefore, *arm* gain-of-function phenotypes are identical to that of *Axn*, *slmb* and *nkd* mutants, supporting the notion that high Wnt signaling activity suppresses *Met*, *gce*, and *Kr-h1* expression and promotes *br* expression.
4. When *gce* was co-expressed with *arm*^{S10}, *Kr-h1* mRNA level was restored to ~150% that of wild type, but *Met* mRNA level was still reduced to ~30% that of wild type.

These results suggest that Wnt signaling indirectly regulates *Kr-h1* expression by suppressing transcription of *Met* and *gce*, genes that encode for putative JH receptors, which eventually mediates insect metamorphosis by controlling *br* expression.

2- Future perspectives:

Characterize functions of JH signaling in different tissues by MARCM analysis: Since *Met* was suggested to be the potential JH receptor, many labs have been working to solve the question of why *Met* mutants are viable. Our results demonstrate that *Met* and *gce* are functionally redundant in transducing JH signaling. *Met-gce* double mutant animals are white pupal lethal. MARCM analysis of this double mutant line will be performed to test the functions of Met and Gce in various tissues, such as the fat body, epidermis, midgut, salivary gland, muscle, and nervous system.

MARCM, Mosaic Analysis with a Repressive Cell Marker, is a genetic technique designed to generate and specifically label homozygous mutant clones in a heterozygous background (Lee and Luo, 1999). It is an ideal technique to analyze cell-autonomous function of vital genes in different tissues. Results obtained from the MARCM analysis of *Met-gce* double mutant will reveal functions of Met/Gce, as well as JH signaling, in different tissues. For example, JH is known to be involved in vitellogenesis in adult females and gonads accessory glands in males. However, we do not know whether this involvement is directly within the reproductive organs or indirectly by affecting the other tissues. MARCM analysis will allow us to generate a *Met-gce* mutant ovary or testis in the heterozygous, phenotypically wild type, animals. Preservation of the phenotype will indicate that this influence is cell-autonomous. Loss of the phenotype will indicate that it is cell-non-autonomous

Further clarify functions of other genes isolated by our genetic screen in JH biosynthesis or signaling: Our genetic screen identified 55 genes that are potentially involved in either JH biosynthesis or the JH signaling pathway. Only several of these are known to play roles in JH action. Our current studies have focused on five genes, revealing that two TGF-β signaling pathway components, *tkv* and *mad*, function in the CA to control JH biosynthesis and three Wnt signaling pathway components, *Axn*, *slmb*, and *nkd*, are required for the normal expression of *Met* and *gce*, which encode putative JH receptors. Further investigation will be continuously carried out on the rest of genes.

Based on the results of JHA treatment assay, 35 of the 55 genes are likely required for maintaining JH titer, including known JH biosynthesis enzymes and regulators, such as *Fpps*, *InR*, *ap*, and *NMDAR* (Sen et al., 2007; Tatar et al., 2001; Altaratz et al, 1991; Chiang et al., 2002). 20 other genes are potentially involved in JH signaling, including *Krüppel homolog 1* (*Kr-h1*), one of the known JH signaling components (Minakuchi et al., 2008b). According to their molecular functions and genetic interactions described in the literature, these 20 potential JH signaling components can be divided into five categories: molecules involving Wnt pathway (*axn*, *slmb*, and *nkd*); molecules involving PAR-aPKC system (*par-1*, *pkn,* and *14-3-3ε*); transcriptional factors (*Kr-h1*, *Sin3A*, *Sox15*, and *tsh*); molecules involving Ubiquitin pathways (*ago*, *Pvr*, *cul-4*, *Uba1*, *ubl*, and *CG15141*); and molecules with other or unknown functions (*CG11241*, *CG1600*, and *CG6841*). We will test whether these genes function cell-autonomously in mediating *br* expression by MARCM analysis and whether these genes affect expression of *Met*, *gce*, and *Kr-h1*.

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