Wnt Signaling Cross-Talks with JH Signaling by Suppressing Met and gce Expression

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Abstract

Juvenile hormone (JH) plays key roles in controlling insect growth and metamorphosis. However, relatively little is known about the JH signaling pathways. Until recent years, increasing evidence has suggested 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 Drosophila 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 in Drosophila, 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. qRT-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-h1expression and eventually derepresses br expression in the Drosophila early larval stages.

Citation: Abdou M, Peng C, Huang J, Zyaan O, Wang S, et al. (2011) Wnt Signaling Cross-Talks with JH Signaling by Suppressing Met and gce Expression. PLoS ONE 6(11): e26772. doi:10.1371/journal.pone.0026772

Editor: Immo A. Hansen, New Mexico State University, United States of America

Received July 19, 2011; Accepted October 3, 2011; Published November 8, 2011

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Funding: This work was supported by the National Science Foundation (IOS1021767). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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 [1,2]. 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 [3]. 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 [4,5]. The Br proteins directly regulate the transcription of 20E-induced late genes and are essential for the specification of pupal development [6,7]. Consistent with its function, the Br proteins are predominantly expressed during the larval-pupal transition in all of the examined holometabolous insects [8]. 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 [9–11]. 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 Kruppel-homolog 1 (Kr-h1) and the p160/SRC/NCoA-like molecule (Taiman in Drosophila and FISC in Aedes). 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 [12–14]. Both Met and gce mutants are viable and resistant to JH analogs (JHA) as well as to natural JH III [14,15]. However, Met-gce double mutants are prepupal lethal and phenocopies CA-ablation flies [14,16,17]. The Met protein binds JH III with high affinity [18,19]. In Tribolium, suppression of Met activity by injecting double-stranded (ds) Met RNA causes precocious metamorphosis [20]. 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 larva; then, it disappears during pupal and adult development [21–23]. Kr-h1 expression can be induced in the
abdominal integument by exogenous JH analog (JHA) at pupariation [22]. Suppression of Kr-h1 by dsRNA in the early larval instars of Tribolium causes precocious Br expression and premature metamorphosis after one succeeding instar [23]. 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 [24,25]. For example, Aedes 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 [24].

In an attempt to isolate other genes involving JH signaling, we conducted a novel genetic screen and identified that mutations in three Wnt signaling component genes, Axin (Axn), supernumerary limbs (slmb), and naked cuticle (nkd), induced precocious Br expression, which was similar to a loss of JH activity. The evolutionarily conserved Wnt signaling pathway controls numerous developmental processes [26]. 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 [27]. 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 [28]. Supernumerary limbs (Slmb) recognizes phosphorylated Arm and targets it for polyubiquitination and proteasomal destruction [29]. Naked cuticle (Nkd) antagonizes Wnt signaling by inhibiting nuclear import of Arm [30]. Our investigations reveal 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.

**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 [31]. Western blotting using a Drosophila Br-core antibody, which recognizes all 4 Br isoforms [32], 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 stage 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. 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. 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 [33]. However, in late 3rd instar larvae and early pupae, an intensive GFP signal was observed in the whole organism (Fig. 1E and F). Inverse PCR analysis revealed that GAL4-PG12 carries a P{GawB} construct within the first intron of the br gene (Fig. 2).
We next compared the expression pattern of GAL4-PG12, UAS-mCD8GFP with that of the br gene in the larval fat body (FB). Neither endogenous Br proteins nor GFP were detectable in the FB of 2nd and early 3rd instar larvae (Fig. 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. 1I-I0). These results indicate that GAL4-PG12 can be used to monitor endogenous br expression.

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 [34]. JHE was reported to be the only esterase that hydrolyzes all types of JH in Drosophila [35]. Therefore, we expected that overexpression of jhe during early larval stages would reduce the JH titer in the hemolymph. As the control, heat shock did not induce br expression in the GAL4-PG12, UAS-mCD8GFP 2nd instar larvae (Fig. 3A and F). However, when GAL4-PG12, UAS-mCD8GFP; UAS-jhe 2nd instar larvae were treated by heat shock, precocious br expression was observed: levels of endogenous Br proteins increased (Fig. 3C). Nevertheless, 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 [36], precocious br expression in the hs-jhe larvae was undetectable (Fig. 3E and J). 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. 4A). 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 larvae 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 (Ppfs) [37], apertous (ap) [38], Insulin receptor (InR) [39, 40], and N-methyl-D-aspartate receptor 1 (Nmdar1) [41], 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 [42]. 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 [42].

Mutations in the Wnt signaling negative regulators cause precocious br expression

Three important components of Wnt signaling, Axn, slmb, and nkd were found among these 55 genes. As shown in Fig. 4, expression of GAL4-PG12, UAS-mCD8GFP was restricted to salivary glands in the wild-type 2nd instar larva (Fig. 4B), but ubiquitous expression of GAL4-PG12, UAS-mCD8GFP was detected at the same stage in the Axn, slmb, and nkd mutant larvae (Fig. 4C–E). 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. 5A), but were observed in the FB nuclei of the AxnEY10228, slmbEY09052, and nkdEY09052 larvae (Fig. 5B–D). We then examined other Axn, slmb, and nkd alleles, including Axn16–21, slmbEY09052, and nkd3. 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, AxnEY10228, slmb00295, and nkd2 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. 5F–H).

These results are opposite to 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 [42]. In contrast, these data are consistent with what we observed in the mutations that affect JH signaling, such as Kr-h1 and Met [14,42]. Therefore, we suggest that Axn, slmb, and nkd affect br expression by affecting JH signaling.

Precocious br expression occurs in Axn mutants in a tissue-specific manner

We further detected br expression in different tissues of the wild type and Axn mutant 2nd instar larvae. We found that br expression was detected in some of cells within the brain of wild-type 2nd instar larvae (Fig. 6A). The number and pattern of the br-expressed-cells in the brain of Axn mutant larvae was not drastically changed (Fig. 6D).
Meanwhile, we did not detect precocious \( br \) expression in the midgut of \( Axn \) mutant larvae (Fig. 6C and F), but detect it the fat body. These results indicate that \( Axn \) mutant induces precocious \( br \) expression tissue-specifically.

**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 [11,14,22,23]. We next investigated whether Wnt signaling regulates Met, Gce and Kr-h1 expression. We first compared mRNA levels for Met, Gce and Kr-h1 between wild type and \( Axn, slmb \), and \( nkd \) mutants by qRT-PCR. In the \( Axn, slmb \) and \( nkd \) mutant 2\( \text{nd} \) instar larvae, the mRNA levels of Met, Gce and Kr-h1 were only about 10–30% of that in wild type at the same stage (Fig. 7A–C). 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 2\( \text{nd} \) instar larvae (Fig. 7D). These results suggest that Met, Gce and Kr-h1 expression are suppressed in \( Axn, slmb \) and \( nkd \) mutants, which results in precocious \( br \) expression.

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

Because \( Axn \), \( Slmb \), and \( Nkd \) negatively affect Wnt signaling activity [28–30], increased Wnt signaling activity was expected in the \( Axn, slmb \) and \( nkd \) mutants. We tested the Wnt signaling activity in the \( Axn \) mutant larvae by detecting \( nkd \) expression. *Drosophila nkd* is an inducible antagonist for the Wnt signal. Its expression is
induced by Wnt activity and its product in turn represses Wnt activity [30]. As shown in Fig. 8A, \( nkd \) mRNA level in the \( Axn \) mutant 2\(^{nd} \) instar larvae was more than 2 times that of wild type larvae at the same stage. Therefore, we suggested 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 its nucleus importation, which activates the transcription of Wnt target genes [27]. Arm\(^{S10} \) is a constitutively active form of Arm that carries a 54 amino acid deletion lacking the Shaggy phosphorylation sites and resists degradation [43]. When \( UAS-arm^{S10} \) was driven by \( arm-GAL4 \) to be expressed in the wild type, we detected precocious \( br \) expression with Br-core antibody staining in the fat bodies of 2\(^{nd} \) instar larvae (Fig. 8C). The qRT-PCR data revealed that mRNA levels of \( Met, gce \) and \( Kr-h1 \) in the \( arm-GAL4/UAS-arm^{S10} \) 2\(^{nd} \) instar larvae were significantly reduced to less than 20% of that in the wild type (Fig. 8E). Therefore, the phenotypes of the \( arm \) gain-of-function mutant are identical to that of \( Axn, slmb \) and \( nkd \) mutants, fully supporting 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 [14]. Therefore, we investigated whether Wnt signaling indirectly suppresses \( Kr-h1 \) expression with Br-core antibody staining in the fat bodies of 2\(^{nd} \) instar larvae (Fig. 8C). The qRT-PCR data revealed that mRNA levels of \( Met, gce \) and \( Kr-h1 \) in the \( arm-GAL4/UAS-arm^{S10} \) 2\(^{nd} \) instar larvae were significantly reduced to less than 20% of that in the wild type (Fig. 8E). Therefore, the phenotypes of the \( arm \) gain-of-function mutant are identical to that of \( Axn, slmb \) and \( nkd \) mutants, fully supporting that high Wnt signaling activity suppresses \( Met, gce \), and \( Kr-h1 \) expression and promotes \( br \) expression.

Wnt Signaling Suppresses Met and gce Expression

Figure 5. Precocious \( br \) expression in \( Axn, slmb \) and \( nkd \) mutants is not prevented by JHA. \( Oregon \ R, Axn^{EY10228}, slmb^{00295}, \) and \( nkd^{2} \) mutants were reared on normal (-JHA) or 0.1 ppm pyriproxyfen-containing (+JHA) food. Fat bodies of the 2\(^{nd} \) instar larvae were stained with a Br-core antibody (red). Nuclei were labeled with DAPI (blue). doi:10.1371/journal.pone.0026772.g005

Figure 6. Precocious \( br \) expression occurs in \( Axn \) mutants in a tissue-specific manner. 2\(^{nd} \) instar larvae of \( Oregon \ R \) and \( Axn^{EY10228} \) were dissected and stained with a Br-core antibody (red). Nuclei were labeled with DAPI (blue). Images show central nervous system (CNS) (Fig. 6A and D), fat body (FB) (Fig. 6B and E) and midgut (MG) (Fig. 6C and F). doi:10.1371/journal.pone.0026772.g006
expression by down-regulating Met and gce. We co-expressed armS10 and gce in wild type flies and examined br, Met, gce and Kr-h1 expression. When UAS-armS10 and UAS-gce were driven by arm-GAL4, the precocious br expression induced by arm-GAL4/UAS-armS10 was totally suppressed, indicated by the absence of Br proteins in the nuclei of 2nd instar larval fat body cells (Fig. 8D). In the same organisms, the gce mRNA level was increased by more than 30 times; the Kr-h1 mRNA level was restored to 150% that of the wild type level; and the Met mRNA level was 30% that of wild type level (Fig. 8E). These results demonstrate that ectopic expression of gce can block ArmS10-mediated Kr-h1 suppression, but does not affect ArmS10-mediated Met suppression. We conclude that Wnt signaling indirectly regulates Kr-h1 expression by down-regulating Met and gce (Fig. 8F).

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

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 hemimetabolous and most holometabolous 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 [36,44]. 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 [7]. 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 [9,10]. 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 pathway always causes precocious br expression in the fat body. However, exogenous JHA treatment can not repress br expression in the fat body of late 3rd instar larvae (data not shown). The molecular mechanism underlying the developmental stage-specific responses of the br gene to JH signaling remains to be clarified.

Interactions between Wnt and JH signaling pathways

As our knowledge of signal transduction increases, the next step is to understand 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 [45] and interacts with estrogen to regulate early gene expression in response to mechanical strain in osteoblastic cells [46,47]. 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$^{St10}$ larvae represses Met and gce expression, which down-regulates Kr-h1 and causes precocious br expression in the fat body. Ectopic expression of UAS-gce in the arm-GAL4/UAS-arm$^{St10}$ 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 [48]. In the absence of nuclear Arm, Pan interacts with Groucho, a corepressor, to repress transcription of Wingless-responsive genes [49]. Upon the presence of nuclear Arm, it binds to Pan, converting it into a transcriptional activator to promote the transcription of Wingless-responsive genes [48]. We propose that Wnt signaling indirectly suppresses Met and gce expression by activating an unknown transcriptional repressor.

JH signaling is well known to be a systemic factor that decides juvenile versus adult commitment. Wg is a morphogen that tissue-autonomously promotes proliferation and patterning during organogenesis. Our studies show that ectopically activating Wg signaling, either by mutations of negative regulators or by the ectopic expression of Arm, results in br derepression via loss of Met and Gce. How and why does the localized Wg signaling regulate the global JH signaling during insect development? Our hypothesis is that though JH signaling activity is globally controlled by JH titer in the hemolymph, distinct tissues may response to JH with different sensitivity, which could be regulated by Wnt signaling-mediated Met and gce expression. Actually, we do find that precocious br expression is detectible in the fat body but not

Figure 8. Gain-of-function arm mutation suppresses Met, gce and Kr-h1 expression and induces precocious br expression. (A). Total RNAs were extracted from Oregon R and Axn$^{St10}$ 2nd instar larvae. The mRNA levels of nkd were assessed by quantitative real-time PCR and normalized to rp49 mRNA. Values shown are the means of 4 independent experiments ± standard deviations. (B–D). Fat bodies of 2nd instar larvae were stained with a Br-core antibody (red) and DAPI (blue). (E). 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$^{St10}$ and arm-GAL4/UAS-arm$^{St10}$, UAS-gce/+ . (F). As described in the text, the proposed model illustrates the cross-talk between the Wnt and JH signaling pathways.

doi:10.1371/journal.pone.0026772.g008
midgut of the Ast mutant 2nd instar larvae. This is one line of evidence to support that Wnt signaling regulates Met and gce expression in a tissue-specific manner.

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 [50]. UAS-gce was a gift from T. Wilson [13]. All lethal mutant lines used in the genetic screen as well as arm-GAL4 and UAS-amiRNA were obtained from the Bloomington Drosophila Stock Center. To generate hs-.hs transgenic flies, hs cDNA was isolated by RT-PCR (primer sequences: forward 5'-ATTCGGGGCAATGTT-CAATCGGCGGAGAT-3' and reverse 5'-ATTCTCTGTTAAGATGACTTC-3') and inserted into pCaSpeR-hs. Transgenic fly lines were generated by P element-mediated germline transformation at Rainbow Transgenic Flies, Inc (Camarillo, CA). Heat-shock treatment of hs- hs flies was performed for 45 minutes at 37°C twice a day starting at larva hatching.

Immunohistochemistry and Microscopy

Immunohistochemical analysis of larval fat bodies was performed as previously described [42]. Fluorescence 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.

Western Blotting

Protein extracts isolated from the 2nd instar larvae were analyzed by standard SDS-PAGE and Western blotting. The expression of β-tubulin was used as a loading control. Br mouse monoclonal antibody Br-core (25E9.D7) [32] and β-tubulin mouse monoclonal antibody (AA12.1) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

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 Table 1.

Acknowledgments

We thank Dr. L. Pick for critical discussions and comments on the manuscript. We thank N. B. Randsholt and T. Wilson for fly strains.

Author Contributions

Conceived and designed the experiments: JW SL. Performed the experiments: MAA CP JH OZ SW JW. Analyzed the data: MAA JH SL. Contributed reagents/materials/analysis tools: MAA CP JH. Wrote the paper: JW.

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