Local requirement of the Drosophila insulin binding protein imp-L2 in coordinating developmental progression with nutritional conditions

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ABSTRACT

In Drosophila, growth takes place during the larval stages until the formation of the pupa. Starvation delays puation to allow prolonged feeding, ensuring that the animal reaches an appropriate size to form a fertile adult. Pupariation is induced by a peak of the steroid hormone ecdysone produced by the prothoracic gland (PG) after larvae have reached a certain body mass. Local downregulation of the insulin/insulin-like growth factor signaling (IIS) activity in the PG interferes with ecdysone production, indicating that IIS activity in the PG couples the nutritional state to development. However, the underlying mechanism is not well understood. In this study we show that the secreted Imaginal morphogenesis protein-Late 2 (Imp-L2), a growth inhibitor in Drosophila, is involved in this process. Imp-L2 inhibits the activity of the Drosophila insulin-like peptides by direct binding and is expressed by specific cells in the brain, the ring gland, the gut and the fat body. We demonstrate that Imp-L2 is required to regulate and adapt developmental timing to nutritional conditions by regulating IIS activity in the PG. Increasing Imp-L2 expression at its endogenous sites using an Imp-L2-Gal4 driver delays pupariation, while Imp-L2 mutants exhibit a slight acceleration of development. These effects are strongly enhanced by starvation and are accompanied by massive alterations of ecdysone production resulting most likely from increased Imp-L2 production by neurons directly contacting the PG and not from elevated Imp-L2 levels in the hemolymph. Taken together our results suggest that Imp-L2-expressing neurons sense the nutritional state of Drosophila larvae and coordinate dietary information and ecdysone production to adjust developmental timing under starvation conditions.

Introduction

The evolutionarily well conserved insulin/insulin-like growth factor signaling (IIS) pathway plays important roles in diverse biological processes, such as growth, energy metabolism and development. Since animals are constantly exposed to environmental changes, the ability to adapt quickly to those changes is essential for their survival. Insulin-like growth factors are involved in the adaptation to nutritional restriction by coupling information about nutrient availability with growth and energy metabolism (Edgar, 2006; Wang and Hung, 2006).

In mammals IIS ligands include the insulin-like growth factors -I and -II (IGF-I and -II) controlling growth rates during development, and insulin controlling carbohydrate and lipid metabolism. In Drosophila, eight insulin-like peptides (Dilp1-8) that share homology with vertebrate IGF-I and insulin have been identified as ligands of a unique insulin receptor (InR) (Colombani et al., 2012; Geminard et al., 2006; Rulifson et al., 2002; Brogiolo et al., 2001). Four out of the eight Dilps (Dilp1-2, –4 and –5) are secreted into the hemolymph by two clusters of insulin producing cells (IPCs) located in each hemisphere of the brain (Ikeya et al., 2002; Brogiolo et al., 2001). Binding of Dilps to the insulin receptor in peripheral tissues activates intracellular signal transduction cascades including PI3-kinase and the Target of Rapamycin (TOR), leading to systemic growth activation.
Activity, stability and availability of IGFs are modulated by insulin-like growth factor binding proteins (IGFBPs) (Hwa et al., 1999). Furthermore, additional IGFBP-related proteins, such as the tumor suppressor IGFBP-7, have been identified in mammals (Wajapeyee et al., 2008; Hwa et al., 1999).

In Drosophila, only one Dilp-binding protein, the Imaginal morphogenesis factor-Late2 (Imp-L2), has been identified. Imp-L2, a member of the immunoglobulin superfamily, shares partial sequence homology with IGFBP-7 and binds IGF-I in vitro (Sloth Andersen et al., 2000; Yamanaka et al., 1997; Garbe et al., 1993). More recent studies showed that secreted Imp-L2 binds native Dilp-2 and -5 and thereby controls growth systemically as a negative regulator of IIS activity (Alic et al., 2011; Honegger et al., 2008). Decreasing IIS activity by either repression of InR or ablation of the IPCs leads to growth rate reduction and delayed development (Shingleton et al., 2005; Rulifson et al., 2002). Conversely, loss of Imp-L2 function leads to increased body size and starvation sensitivity (Honegger et al., 2008). However, Imp-L2 shows a complex expression pattern, suggesting that it serves additional local functions.

In Drosophila as in other holometabolous insects, the steroid hormone ecdysone regulates development by controlling the larval/pupal transition and molting (Marchal et al., 2010). Ecdysone production and release is mainly regulated by the prothoracic gland. We observed neurons, in both brain hemispheres and the subesophageal ganglion (SOG) (Fig. 1A) (Honegger et al., 2008). In addition, low levels of Imp-L2 mRNA were detected in the prothoracic gland (PG) portion of the ring gland (Fig. S1A). This diverse expression pattern may underlie tissue-specific roles of Imp-L2, however, studies to elucidate local actions of Imp-L2 have not been carried out yet.

Since ubiquitous overexpression of Imp-L2 with strong drivers like actin-Gal4 leads to lethality (Honegger et al., 2008), we aimed to express Imp-L2 from its endogenous sites and to analyze its function. Thus, we created two different Imp-L2-Gal4 lines, Imp-L2-RA-Gal4 and Imp-L2-RC-Gal4, representing transcript-specific driver lines of Imp-L2. To visualize their expression patterns, we crossed them to flies carrying a CD8-GFP gene under UAS control (UAS-CD8-GFP). CD8-GFP is membrane localized and thus visualizes the projection patterns of the Imp-L2 expressing cells. We stained for Imp-L2 protein expression in the PG. In all larval stages, Imp-L2-RC-Gal4 drives stronger GFP expression than Imp-L2-RA-Gal4 (Fig. 1H). However, we only sporadically detected low Imp-L2 protein levels in the PG even though Imp-L2 mRNA was clearly present (Fig. S1A). Using a GFP reporter construct containing the small second intron of Imp-L2, we discovered that the cis regulatory sequences of Imp-L2-RC reside in this intron and drive GFP expression in the PG (Fig. 1G). Presumably, Imp-L2 protein expression in the PG and the fat body is below detection level, or the protein is secreted into the hemolymph immediately after production. Since the Imp-L2-RA-Gal4 line drives expression in all cells shown to be positive for Imp-L2 protein, it constitutes a powerful tool for further analysis of Imp-L2 function in overexpression experiments.

**Imp-L2 is required to regulate and adapt developmental timing to nutritional conditions**

In Drosophila, nutritional restriction delays pupariation due to an extended feeding phase required to attain critical weight. To test whether Imp-L2, as a negative regulator of IIS activity, is involved in this adaptation process we analyzed the developmental timing of Imp-L2 mutant and Imp-L2-overexpressing larvae under normal and reduced food conditions. Overexpression of Imp-L2 using the Imp-L2-RA-Gal4 line (Imp-L2-RA > Imp-L2) elevates Imp-L2 levels specifically at the endogenous sites of Imp-L2 production. Under normal food conditions neither Imp-L2 overexpression nor Imp-L2 loss of function (LOF) affected developmental timing during the first larval instar (L1). However, the second instar (L2) period was prolonged by six hours in Imp-L2-RA-Gal4/UAS-Imp-L2 animals, whereas Imp-L2 mutant larvae behaved like the control (Fig. 2A). The major effect occurred in the third larval stage (L3), where Imp-L2 mutant larvae pupariated six hours earlier than control larvae, whereas Imp-L2-RA > Imp-L2 larvae were approximately 45 h delayed (Fig. 2B). Notably, these effects were even more striking under starvation conditions (10% yeast), in which Imp-L2 mutant larvae pupariated 22 h earlier and Imp-L2-RA > Imp-L2 72 h later than control larvae (Fig. 2C). Hence, starvation-induced developmental delay is reduced by 64% in Imp-L2 mutants and increased by 75% in Imp-L2-RA > Imp-L2 larvae. On the other hand, survival rates of Imp-L2 mutant larvae are decreased by 60% compared to the control (Fig. 2D). Thus, the lower Imp-L2 levels are, the earlier pupariation takes place under limited food conditions. However, early pupariation comes at the expense of larval survival rates, indicating that Imp-L2 is required for proper adjustment to nutritional changes and is essential for survival under adverse food conditions.
Imp-L2 controls larval growth and behavior by regulating IIS

Given that lowered IIS activity can decrease growth rates and thereby affect the time point at which critical weight is attained, the delayed pupariation is not surprising. Indeed, larval growth rates of Imp-L2-RA\textsuperscript{4} Imp-L2 larvae were decreased, especially in L2, compared to control larvae, while Imp-L2 mutant larvae showed a slightly accelerated growth rate (Fig. 3A and B). To determine whether altered growth rates are exclusively due to metabolic repercussions of changes in systemic IIS activity or if manipulation of Imp-L2 levels also affects behavioral cues, we examined the feeding behavior of early L2 larvae. Imp-L2-RA > Imp-L2 larvae display a reduced food intake and thus grow more slowly, while Imp-L2 mutants feed more and grow faster than the control (Fig. S2A–D). Therefore, food acquisition is also affected by Imp-L2 overexpression; however, it is not clear whether the effect on feeding behavior is based on general changes of IIS activity or whether it can be attributed to increased Imp-L2 production by specific Imp-L2 positive neurons.

We also observed that approximately 48 h after the L3 molt (AL3), Imp-L2 overexpressing larvae reach the weight of wandering L3 control larvae (Fig. 3B). Curiously, Imp-L2-RA > Imp-L2 larvae did not pupariate at this point, but continued wandering around on the food surface without feeding for an additional 42 h, which is most likely responsible for the observed reduction in body mass in this timeframe (Fig. 3B and C–C”). Consistent with the decreased growth rate and food intake, Imp-L2-RA > Imp-L2 female flies showed a 20% reduction in body weight, whereas male body weight was decreased by 40% (Fig. 3F). Furthermore, we demonstrate that both the size as well as the timing effect are Dilp-dependent, since both were fully rescued by co-overexpression of UAS-Dilp2 (Fig. 3E and F). Notably, a decrease of IIS activity within Imp-L2 expressing cells by overexpressing a dominant negative version of PI3K (UAS-dp110DN) using Imp-L2-RA-Gal4 affected neither developmental timing nor final body weight (data not shown and Fig. 3F). Hence, the possibility that the phenotypes of Imp-L2-RA > Imp-L2 larvae are induced by an autocrine effect of Imp-L2 can be excluded.

Taken together these results show that increasing Imp-L2 levels at the endogenous sites of its expression leads to a developmental delay that does not simply result from delayed attainment of critical weight caused by a systemic decrease of IIS activity.
Imp-L2 regulates developmental progression through local regulation of IIS in the prothoracic gland

Since a peak in ecdysone production at the end of the third larval instar is required for larval/pupal transition (Marchal et al., 2010), the developmental delay of Imp-L2 overexpressing larvae could be partially due to decreased ecdysone production by the PG. To test this hypothesis, we measured the levels of 20-hydroxyecdysone (20E), the active metabolite of ecdysone, in Imp-L2 mutants and Imp-L2-RA > Imp-L2 larvae compared to Imp-L2-RA > GFP control larvae by liquid chromatography-mass spectrometry (LCMS). Indeed, at 102 h after egg deposition (AED), the time when control larvae started pupariating, 20E titers of Imp-L2-RA > Imp-L2 larvae were six times lower than in control larvae (Fig. 4A). The 20E levels in Imp-L2 overexpressing larvae were still low one day later at 128 h AED. However, a peak in 20E production took place in the time period between 128 and 150 h AED (Fig. 4A) and 20E titers of Imp-L2-RA > Imp-L2 larvae reached the level that control larvae showed shortly before pupariation. Conversely, Imp-L2 mutant larvae showed a 2.5 fold increase of 20E levels compared to the control at 102 h AED (Fig. 4A).

We tested whether the delayed pupariation could be rescued by feeding Imp-L2-RA > Imp-L2 larvae with 20E (Fig. 4B). PTTH > Grim served as a positive control, since the delay caused by the ablation of PTTH neurons (which decreases ecdysone production) has been shown to be rescued by feeding 20E (Mcbryar et al., 2007). While the delay of PTTH > Grim larvae could be fully rescued (Fig. 4B), 20E feeding only reduced the delay of Imp-L2-RA > Imp-L2 larvae by approximately 26 h (Fig. 4B), suggesting that a large part of the developmental delay is caused by the absence of a 20E peak at the normal time of larval/pupal transition. If Imp-L2 acted exclusively through the PG to trigger a systemic response, one would assume that artificially inducing a 20E peak would be sufficient to rescue the delay. Thus presumably, two separate Imp-L2 dependent mechanisms exist.

Notably, we observed that PGs of Imp-L2-RA > Imp-L2 larvae are greatly decreased in size compared to control PGs (Fig. 4C and D). To test if this size decrease was due to decreased IIS activity in the PG, we monitored PI3K activity in PG cells of Imp-L2-RA > Imp-L2 larvae using the tGPH reporter for IIS activity (Britton et al., 2002). Indeed, IIS activity in the PG cells of Imp-L2-RA > Imp-L2 larvae was strongly decreased compared to the control (Fig. 4C and D). Since altered IIS activity in the PG has been reported to impact on ecdysone production (Caldwell et al., 2005; Mirth et al., 2005), we reasoned that elevated Imp-L2 levels could inhibit IIS activity in the PG, leading to lowered 20E production and thus to a developmental delay. To rule out the possibility that the lowered IIS activity in the PG is due to elevated Imp-L2 levels in the hemolymph and analyze developmental timing. To do so, we used Aug21-Gal4 (expressed specifically in the corpora allata) since it is known to be a strong and clean driver that expresses in an organ specialized for secretion. To verify that hemolymph Imp-L2 levels of Aug21 > Imp-L2 larvae are at least as high as in Imp-L2-RA > Imp-L2 larvae, we performed Western blots on the hemolymph of these larvae. We found that Imp-L2 levels in the hemolymph of these larvae. We found that Aug21 > Imp-L2 larvae possessed significantly higher hemolymph Imp-L2 levels than Imp-L2-RA > Imp-L2 larvae (Fig. 4E). Despite this, IIS activity in PG cells of Aug21 > Imp-L2 was very similar to the control, and PG size also remained unchanged (Fig. S3A,B), indicating that increasing Imp-L2 concentrations in the hemolymph did not affect IIS activity or growth of the PG. However,
Aug21 > Imp-L2 flies displayed a size decrease of 19% in males and of 35% in females (Fig. S4D), and pupariation was delayed by 18 h (Fig. 4F). Since Aug21 > Imp-L2 mutant larvae show a slightly increased growth rate, whereas growth rates of Imp-L2-RA > Imp-L2 larvae were decreased especially in L2. (C–E) Representative pictures of staged larvae fed with blue yeast starting at 92 h AED, taken at different time points during L3. Imp-L2-RA > Imp-L2 larvae are delayed by at least 42 h compared to control and reduce food intake more than 33 h before pupariation. These effects can be rescued by co-overexpression of Dilp-2. (F) Dry body weights of flies overexpressing the following proteins using the Imp-L2-RA-Gal4 driver: Imp-L2, GFP (control), Dp110DN and Imp-L2 co-overexpressed with dilp-2 (n = 100). Males are shown to the left and females to the right. Imp-L2 overexpression causes a size decrease in both males and females. This decrease is rescued by co-overexpression of Dilp-2. Overexpression of Dp110DN has no effect on size. In (A), (B), (D) and (F) Imp-L2-RA > GFP larvae were used as a control since they behaved like wild type (wt) in terms of development and growth. Error bars indicate standard deviations. **P < 0.0001, t-test (two tailed, unpaired).

**Fig. 3.** Imp-L2 controls larval growth through systemic regulation of IIS. (A,B) Growth rates of Imp-L2 mutant, Imp-L2 overexpressing and control larvae in L2 (A) and L3 (B), respectively (n=45). Imp-L2 mutant larvae show a slightly increased growth rate, whereas growth rates of Imp-L2-RA > Imp-L2 larvae were decreased especially in L2. (C–E) Representative pictures of staged larvae fed with blue yeast starting at 92 h AED, taken at different time points during L3. Imp-L2-RA > Imp-L2 larvae are delayed by at least 42 h compared to control and reduce food intake more than 33 h before pupariation. These effects can be rescued by co-overexpression of Dilp-2. (F) Dry body weights of flies overexpressing the following proteins using the Imp-L2-RA-Gal4 driver: Imp-L2, GFP (control), Dp110DN and Imp-L2 co-overexpressed with dilp-2 (n = 100). Males are shown to the left and females to the right. Imp-L2 overexpression causes a size decrease in both males and females. This decrease is rescued by co-overexpression of Dilp-2. Overexpression of Dp110DN has no effect on size. In (A), (B), (D) and (F) Imp-L2-RA > GFP larvae were used as a control since they behaved like wild type (wt) in terms of development and growth. Error bars indicate standard deviations. **P < 0.0001, t-test (two tailed, unpaired).
Since Imp-L2 overexpression using Imp-L2-RA-Gal4 causes a much stronger developmental delay, we suggest that elevated Imp-L2 levels in the neurons targeting the PG, but not covered by any of the chosen Gal4 lines, locally decrease IIS activity and thus ecdysone production. The observation that some Imp-L2 expressing neurons do project to the PG strengthens this hypothesis.

**Discussion**

In this study we demonstrate a so far unknown local function of Imp-L2 in the *Drosophila* larval brain ring gland complex to control developmental timing. By generating an Imp-L2-RA-Gal4 line, we were able to elevate Imp-L2 levels at its endogenous sites. In Imp-L2-RA > Gal4 larvae PG size is reduced, IIS activity is low...
and 20E levels at 102 AED (the time point of wt larval to pupal transition) are greatly decreased compared to the control, resulting in a significant developmental delay. However, feeding the larvae 20E only partly rescued the delay. Since Imp-L2-RA > Imp-L2 larvae also showed decreased growth rates most likely caused by the endocrine growth-inhibiting function of Imp-L2 (Honegger et al., 2008), we concluded that the remaining delay could be attributed to the disability of the larvae to reach critical weight in time, which cannot be rescued by 20E feeding. By exclusion analysis, we suggest that a subset of Imp-L2 expressing neurons that were not covered by any of the Gal4 lines used are most likely responsible for the delayed pupariation. Taken together, these data suggest that Imp-L2-expressing neurons targeting the PG secrete Imp-L2 upon an unknown (probably nutrition dependent) signal, which in turn leads to decreased IIS activity in PG cells and therefore to reduced ecdysone production. In a different manuscript currently under revision, we show that starvation leads to higher IIS activity in Imp-L2 expressing neurons, stimulating Imp-L2 production by these cells. This is consistent with our model that Imp-L2 neurons sense the nutritional state to couple developmental timing with nutrition. However, due to the lack of a tight driver covering the Imp-L2 positive neurons that innervate the PG, we cannot fully exclude the possibility that ecdysone production is regulated by the interplay of different types of Imp-L2 expressing cells.

In higher organisms, the duration of the juvenile stage needs to be variable to ensure the development of a healthy and fertile adult. Environmental stresses, such as adverse nutritional conditions, can delay development until a critical weight is reached. Additional checkpoints ensure that increased growth rates, induced by ideal nutritional conditions, do not lead to a premature passage to the adult stage. In Drosophila, the juvenile growth stage is terminated by pupae formation at the end of the third larval instar. Larval/pupal transition is induced by a pulse of the steroid hormone ecdysone produced by the PG (Marchal et al., 2010). Genetic manipulations of the Drosophila PG revealed the requirements of the IIS, Target of Rapamycin (TOR) and PTTH pathways to control ecdysone production (Colombani et al., 2005; Mirth et al., 2005; Caldwell et al., 2005; Rewitz et al., 2009). Recently, IIS-dependent growth of the PG has been identified as an additional factor controlling ecdysone production. Overexpression of P13K, a positive regulator of IIS, leads to premature, increased ecdysone production resulting in a shortened L3 stage and early pupariation. By contrast, overexpression of negative regulators of IIS in the PG delays pupariation caused by lowered and delayed ecdysone production (Caldwell et al., 2005; Mirth et al., 2005). Reduction of whole organism IIS activity does not change critical weight but delays its attainment (Shingleton et al., 2005). In contrast, ablation of PTTH neurons induces a severe shift in critical weight, suggesting that these neurons play an important role in setting this parameter (Mcbrayer et al., 2007). When larvae reach the critical weight, PTTH is released on the PG and induces transcription of genes involved in ecdysone production (Mcbrayer et al., 2007; Gibbens et al., 2011). However, PTTH expression is not modified upon nutritional restriction, indicating that PTTH signaling does not mediate starvation-induced developmental delay (Layalle et al., 2008). Signaling via TOR, the downstream kinase of IIS (Layalle et al., 2008), links nutritional information to ecdysone production, since starvation induced developmental delay can partially be rescued by upregulating TOR activity in the PG. This suggests that downregulating TOR signaling upon starvation desensitizes the PG for PTTH signals, resulting in delayed ecdysone production (Layalle et al., 2008). In the present study, we show that increased IIS activity in the PG due to Imp-L2 LOF rescues the delay caused by malnutrition to a large extent, indicating that low IIS also renders the PG insensitive to the PTTH signal. Whether the effects of low IIS in the PG are mediated by TOR or whether the two pathways act independently remains to be elucidated.

We present evidence for a number of Imp-L2 expressing neurons to act as possible regulators of IIS activity in the PG. High Imp-L2 levels in the hemolymph can be excluded as possible inhibitors of IIS signaling in the PG, since increasing hemolymph levels of Imp-L2 failed to reduce size and IIS activity of PG cells, but resulted in a size decrease of the whole organism. On the other hand, increasing Imp-L2 levels in Imp-L2 positive neurons targeting the PG causes a massive decrease in PG size and lowers IIS activity within PG cells. These results support the idea that the PG does not receive information about the nutritional state of the organism through the hemolymph but rather from Imp-L2 expressing neurons. Thus, our work reveals a novel local function of the negative growth regulator Imp-L2 in controlling IIS activity and ecdysone production in the PG. This finding reveals a novel mechanism for the spatial regulation of IIS: through locally restricted effects of Imp-L2, diverse tissues can be effectively subjected to different levels of IIS.

Interestingly, the ability of IIS to coordinate growth with development seems to be conserved throughout evolution. In humans, the onset of puberty is linked to the nutritional state, leading to early puberty in well-fed western societies (Kaplowitz et al., 2001). In contrast, juvenile females suffering from type 1 diabetes mellitus display a notable delay in menarche (Kjaer et al., 1992), indicating that decreased IIS also delays maturation in humans. Moreover, in Caenorhabditis elegans, malnutrition during the first larval stage leads to developmental arrest by inducing dauer formation, which is a larval stage best adapted for survival under adverse environmental conditions (Cassada and Russell, 1975). Mutations reducing IIS pathway activity lead to dauer formation independent of the nutritional state (Riddle et al., 1981). Hence, different phyla developed similar strategies to cope with adverse nutritional conditions during the juvenile stage. When IIS activity is below a certain threshold, development is attenuated until sufficient nutrients are available, to ensure the formation of healthy and fertile adults. In Drosophila larval malnutrition leads to delayed pupariation, due to decreased IIS activity in the PG which in turn delays the production of the steroid hormone ecdysone.

Steroid hormones also play an important role in human development. In cases of human hypogonadism, puberty is prolonged, which can lead to abnormally tall adults if not treated with steroid substitutes (Drop et al., 2001). Referring our data to the human system, the putative Imp-L2 homolog IGFBP-7 (also known as IGFBP-rP1) also displays a very diverse protein expression pattern, indicating a specialized function in different organs (Degeorges et al., 2000). Amongst other tissues, IGFBP-7 is expressed in different regions of the human brain (Degeorges et al., 2000), tempting us to speculate that it might act as a local regulator of steroid production as well.

In summary, our data provides novel insights into the coupling of developmental cues to nutritional state. Since IIS and steroid hormones play evolutionarily conserved roles in regulating growth and development, our findings on the local function of the insulin-binding protein Imp-L2 in controlling ecdysone production might be of general interest.

Materials and methods

Fly stocks

The following fly stocks have been used: Imp-L2 def20, Imp-L2-def42, UAS-simp-L2 and UAS-Imp-L2-HA have been described in (Honegger et al., 2008). Imp-L2-Def20/Imp-L2-Def42 larvae were
The galK/Gal4 substitution was carried out on 2-deoxy-galactose (DOG) containing minimal plates with glycerol as the only carbon source to select against the galK cassette since phosphorylation of DOG by galK results in the non-metabolizable and therefore toxic intermediate 2-deoxy-galactose-1-phosphate. Next we integrated the modified Imp-L2 genomic region (sequence between the upstream and the downstream Imp-L2-neighboring genes) of the BAC into a modified [Pacman] vector through recombineering mediated gap repair (Venken et al., 2006). In order to create a Imp-L2 genomic rescue construct containing all regulatory sequences we also introduced a non-modified version of the Imp-L2 locus into the [Pacman] vector. The constructs were injected into embryos of the 86FB fly strain (Bischof et al., 2007).

**Construction of the direct reporter construct**

To design the reporter construct we introduced an hsp70 minimal promoter followed by a GFP sequence into the pattB genomic vector (Bischof et al., 2007). Then the intron between the RB- and the RC-specific exons was cloned in front of the hsp70 minimal promoter. Transgenic flies were established carrying this construct as well as the reporter vector without an insert as a control using the attP/attB system (Bischof et al., 2007.).

Primers used:

- ImpL2-xho-intron3-for CGCTCGAGGTGATGAGTGGGGGAAGT
- ImpL2-xho-Hne-int3-rev CTCGAGCTAGCCGCTGCAAGATCG

**Immunohistochemistry, in situ hybridization and western blotting**

Larval brains, guts and ring glands were fixed in 4% PFA, permeabilized with PBT, and blocked in 2% NDS. Larval fat bodies were fixed in 8% PFA. The antibody against Imp-L2 (a kind gift from Linda Partridge) has been previously described (Alic et al., 2011). For antibody stainings we used the rabbit anti-Imp-L2 1:500. Pictures were taken using a Leica SPE confocal laser scanning microscope. RNA in situ hybridization using digoxigenin-labeled probes was performed as previously described by Brogiolo et al., 2001. The probes against Imp-L2 were derived from s.Imp-L2 in a pBluescript SK+ vector.

**Developmental timing analysis and survival rates**

Fertilized eggs were collected on apple juice agar plates. Previous to the egg collections, female flies were allowed to lay for 1 h in order to remove retained eggs. For the actual collections plates were changed every 2–3 h. To determine the L2/L3 transition, 50 fertilized eggs were placed on a fresh apple agar plate supplemented with yeast. Mouth hooks were counted starting at 60 h AED in 2 h intervals. To determine the timepoint of puparation, fertilized eggs were counted and distributed into vials containing standard cornmeal food supplemented with yeast or starvation food (60–70 eggs per tube). Pupariation was scored three times a day in 3–4 h intervals. For determination of survival rates all adult flies were counted after eclosion. P-values were calculated with t-test (two tailed, unpaired) using Excel, the comparison was to the control (Imp-L2-RA > GFP).
20E feeding

To make 20E food, we used 1 g of mashed cornmeal and mixed it with a 20E stock (10 mg/ml 20E [Sigma] in 95% ethanol) to a final concentration of 0.33 mg/ml 20E and 3% ethanol. For control food, 95% ethanol was added to the mashed cornmeal to a final concentration of 3%. The food was transferred into tubes (1 g food per tube) and 10 L3 larvae were placed into each tube 72 h AED. This experiment has been repeated 4 times.

Determination of body weight

3 days old flies were separated by sex and dried in a heat block at 98 °C for 15 min. A minimum of 30 flies per sex was measured in each experiment. Single fly weight was determined 2 days after drying using a Mettler Toledo MX5 microbalance. For larval body weights, 15 tightly staged larvae (egg deposits in 2 h intervals) were immobilized on ice and weighed individually on a Mettler Toledo MX5 microbalance. Larvae were resynchronized at 68 h after AED. The experiment was repeated 3 times. P-values were calculated with t-test (two tailed, unpaired) using Excel, the comparison was to the control (UAS-GFP).

Feeding assay

Females were allowed to lay eggs for 3 h on standard apple juice agar plates supplemented with yeast. 48 h later, the plates were cleared of all L2 larvae.

All larvae that had molted to L2 within the following 2 h were transferred to a new apple agar plate containing yeast at a maximum density of 20 larvae/plate. 2 h later, the larvae were quickly rolled clean and transferred to a 1% agarose plate covered with a thin layer of blue yeast paste (0.5 g/ml baker's yeast, 0.005 g/ml bromophenol blue sodium salt in water) for 30 min. Then larvae were washed in tap water and imaged from the dorsal and ventral side. Images were collected with a Leica DFC 420C color camera attached to a MZ 16F microscope at 32.

20-hydroxyecdysone measurements

Flies were allowed to lay eggs for two hours. Staged larvae were collected at 78 and 102 h AED (Imp-L2 deficient and control larvae) and at 78, 102, 126 and 150 h AED (Imp-L2-RA > Imp-L2 larvae). For each time point and genotype a minimal number of 100 larvae were collected and stored in −20 °C methanol until homogenization by vortexing in a 50 ml Falcon tube containing 4 mm glass beads. Further, the methanol-solved homogenate was transferred to a glass tube and three times delipidated with one volume hexane. The methanol phase was transferred into a new glass tube and samples were extracted another time with methanol. Pooled extracts were dried in a vacuum centrifuge, resuspended in 30–150 µl methanol and stored at −20 °C until measurement.

To separate 20-hydroxyecdysone we used an Agilent 1290 Infinity liquid chromatography-system (Agilent technologies) with a ZORBAX RRHD SB-C18 column (Agilent technologies, dimensions 2.1 mm × 100 mm, 18 µM). The applied solvents were water with 1 mM ammonium-acetate, and methanol. The gradient started at 5% methanol, which was increased to 10% over 3 min. Afterwards a linear gradient was applied over 17 min to 100% methanol, followed by an isotropic period for 3 min, and a linear gradient down to 10% methanol over 1 min. Finally, the solvent ratio was brought back to the initial conditions (5% methanol) during 1 min.

For detection of 20E, we used an AB Sciex Qtrap 5500 (AB Sciex) in positive mode. Specifically, we generated a multiple reaction monitoring (MRM) method by online fragmentation of a 20E standard in the enhanced product ion (EPI) mode with the following parameters: temperature 600 °C, ion spray voltage 4500 V, collision energy spread 20 + 10 eV. The nebulizer, auxiliary and curtain gas parameters were set to 60, 80 and 10, respectively. For later experiments the following MRM transitions were used with a collision energy of 20 eV and a dwell time of 20 m/sec: 481–371 and 445–371, from which the first transition directly emerges from 20E and the second from an online fragment of 20E. Finally, the signals were integrated with the Analyst software (version 1.5.1, AB Sciex) and normalized to the sample volume and to the concentration of the larval extract. For all genotypes and time points at least biological triplicates were measured.

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Appendix A. Supporting information

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References


