Steroid signaling mediates nutritional regulation of juvenile body growth via IGF-binding protein in *Drosophila*

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Edited by Lynn M. Riddiford, University of Washington, Friday Harbor, WA, and approved April 30, 2018 (received for review October 28, 2017)

Nutritional condition during the juvenile growth period considerably affects final adult size. The insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) nutrient-sensing pathway is known to regulate growth and metabolism in response to nutritional conditions. However, there is limited information on how endocrine pathways communicate nutritional information to different metabolic organs to regulate organismal growth. Here, we show that Imaginal morphogenesis protein-Late 2 (Imp-L2), a *Drosophila* homolog of insulin-like growth factor-binding protein 7 (IGFBP7), plays a key role in the nutritional control of organismal growth. Nutritional restriction during the larval growth period causes undersized adults, which is largely diminished by *Imp-L2* mutation. We delineate a pathway in which nutritional restriction increases levels of the steroid hormone ecdysone, which, in turn, triggers ecdysone signaling-dependent Imp-L2 production from the fat body, a fly adipose organ, thereby attenuating peripheral IIS and body growth. Surprisingly, this endocrine pathway operates independent of the fat-body–TOR internal nutrient sensor, long believed to be the control center for nutrition-dependent growth. Our study reveals a previously unrecognized endocrine circuit mediating nutrition-dependent juvenile growth, which could also potentially be related to the insulin resistance frequently observed in puberty.

juvenile growth | IIS/TOR signaling | steroid hormone | insulin-like growth factor-binding protein | *Drosophila*

The life history of animals exhibits discrete stages, including embryonic development, juvenile growth, sexual maturation, and reproductive adulthood. Among the many environmental and genetic cues influencing juvenile growth, nutrient availability is thought to have major effects (1, 2). Most of the energy resources acquired during this stage are needed to support the exponential increase in body size, such that even short periods of malnutrition during the juvenile growth period have the potential to significantly influence the final adult body size. Nutrient-sensing pathways, such as insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR), have been known to mediate cellular and organismal growth in response to nutritional energy condition (1, 3). The mechanisms by which the IIS and TOR pathways sense energy status and mediate growth in the cell have been thoroughly explored; however, the manner in which these mechanisms are systemically incorporated and regulated at the organismal level during the juvenile growth phase is largely unknown. Development from larva to pupa in *Drosophila* has intriguing parallels to mammalian development from childhood to adulthood. In both cases, growth is largely restricted to the juvenile period and growth cessation in adulthood is accompanied by sexual maturation, as determined by an increase in circulating steroid hormones (2). Recent studies in *Drosophila* have shown that a steroid maturation hormone regulates larval body growth rate and final adult size. Ecdysone, the sole *Drosophila* steroid hormone mediating transitions between distinct developmental states, has been found to influence peripheral IIS and body growth rates; the fat body, the *Drosophila* counterpart of mammalian liver and adipose tissue, is the main target organ of this hormone (4–6). The activity of IIS can be regulated in extracellular spaces by insulin-like growth factor-binding proteins (IGFBPs). By binding to insulin-like growth factors (IGFs), IGFBPs cannot only prolong the half-lives of IGFs but also modulate their availability and activity (7). The *Drosophila* Imaginal morphogenesis protein-Late 2 (Imp-L2) has been regarded as a functional homolog of vertebrate IGFBP7, and Imp-L2 binds to and inactivates *Drosophila* insulin-like peptides (Dilps) (8–10). Recent studies have highlighted the critical implications of Imp-L2 in various physiological and pathophysiological conditions. Flies deficient in Imp-L2 are larger in size and more vulnerable to starvation than wild-type flies (8), whereas increased expression of Imp-L2 extends the fly lifespan (11, 12). Moreover, Imp-L2 expressed in a subset of neurons ensures the proper activity of insulin signaling in the brain and its associated glands (13, 14). Other recent studies have further revealed the role of Imp-L2 in cachexia-like syndromes, where malignant tumors induce organ wasting (15, 16).

In the present study, we reported that Imp-L2 is the key humoral factor mediating the regulation of organismal body growth and IIS activity in response to varying nutritional conditions. In addition to being larger, animals deficient in Imp-L2 are resistant to a decrease in body size under poor nutritional conditions. Imp-L2 expression in fat bodies is up-regulated by malnutrition. Surprisingly, we found that nutrition-dependent regulation of body growth and Imp-L2 expression is mediated by ecdysone signaling rather than the fat-body–TOR internal nutrient sensor, revealing a previously unknown endocrine pathway for organismal nutritional metabolism.

Significance

Nutritional condition during childhood significantly impacts physical growth and height as an adult; hence, elucidating nutritional elements and their underlying mechanisms that influence growth in juveniles has drawn increasing attention. We show that a humoral protein that binds insulin-like growth factor plays a critical role in the nutritional control of juvenile body growth and insulin signaling in *Drosophila*. This protein is found be regulated by a steroid maturation hormone in adipose tissue, and this hormone is further shown to be regulated by the nutritional condition of animals. This study unveils an endocrine pathway for juvenile body growth, and it may also facilitate understanding of the insulin-resistance phenomena often occurring during puberty.

Author contributions: G.J.L. and S.H. designed research; G.J.L., G.H., H.M.Y., J.J.L., S.N., J.L., and S.H. performed research; S.H. contributed new reagents/analytic tools; G.J.L. and S.H. analyzed data; and G.J.L. and S.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718834115/-/DCSupplemental.

Published online May 21, 2018.

Results

Fat-Body–Specific Depletion of Imp-L2 Confers Resistance to Growth Suppression Induced by Poor Nutrition. The previous observation that flies deficient in Imp-L2 show larger body sizes (8) prompted us to investigate which tissues or organs involved in the regulation of body growth express Imp-L2. Examining mRNA expression by quantitative RT-PCR in isolated organs revealed that the Imp-L2 transcript is broadly expressed throughout larval tissues such as the gut, brain, body wall muscle, and fat body, where it is most abundant (*SI Appendix*, Fig. S1). Next, we investigated the effects of Imp-L2 depletion in each of the organs cited above using RNA interference and tissue-specific Gal4 drivers. Knockdown of Imp-L2 in the body wall muscle (*Dmef Gal4*), panneuronal tissues (*Elav Gal4*), or fat body (*Cg Gal4*) all resulted in increased body size to a similar extent (Fig. 1*A* and *SI Appendix*, Fig. S2). During this experiment, we found that the increase in body size resulting from knockdown of Imp-L2 in the fat body was more pronounced when flies were reared on diluted rather than standard fly food (*SI Appendix*, Fig. S3). Since undernutrition in growing larvae leads to significant decreases in final adult size, this finding raised the possibility that Imp-L2 might play a role in the nutritional control of body growth. As expected, wild-type flies showed a sharp decrease in final body size as food richness gradually decreased (Fig. 1*B*). Imp-L2 knockdown flies for either body wall muscle or panneuronal tissues showed similar decreases in adult body size with decreasing food richness. In stark contrast, the body size of flies depleted of Imp-L2 in the fat body was largely unaffected by undernutrition (Fig. 1*B*). Similarly, the resistance to size decrease was seen in Imp-L2 null flies (*Imp-L2*^{Def42}) (Fig. 1*B*). To further confirm this observation, we used a different strategy to restrict nutrient availability. It is known that larvae experiencing starvation after developing to a critical weight (CW) continue to progress with larval development until pupariation without developmental arrest (2, 17). The timing of CW occurs around the early third instar period in normally fed larvae. Thus, we examined the effect of Imp-L2 deletion on the final adult size determined by starvation in larvae from the mid-third instar (~87 h after egg laying) until pupariation. Starvation in third instar larvae drastically decreased the final adult size by about 70% in wild-type control flies (Fig. 1*C*). However, the decrease in body size due to starvation was strongly blunted when Imp-L2 was depleted in the fat body (Fig. 1 *C* and *D*). The blunted effect on body size was also seen in Imp-L2 null flies (Fig. 1*C*). Similar results were also seen when pupal volumes were measured (*SI Appendix*, Fig. S4). These effects, induced by the specific inactivation of Imp-L2 in the fat body, were further confirmed using another fat-body–specific Gal4 driver (*ppl Gal4*) (Fig. 1 *E* and *F*). We also tested the effects of providing fly food in which a specific ingredient was deficient. We found that the yeast-deficient, but not glucose-deficient, food significantly decreased adult body size, a phenomenon that was absent in Imp-2 mutant flies (*SI Appendix*, Fig. S5). This result indicates that the growth effects of diluted food used in this study appeared mainly due to a deficiency in yeast, which is the major source of protein in the fly food. Together, these data suggest that Imp-L2 produced specifically in the larval fat body plays a major role in the nutritional condition-dependent control of organismal growth.

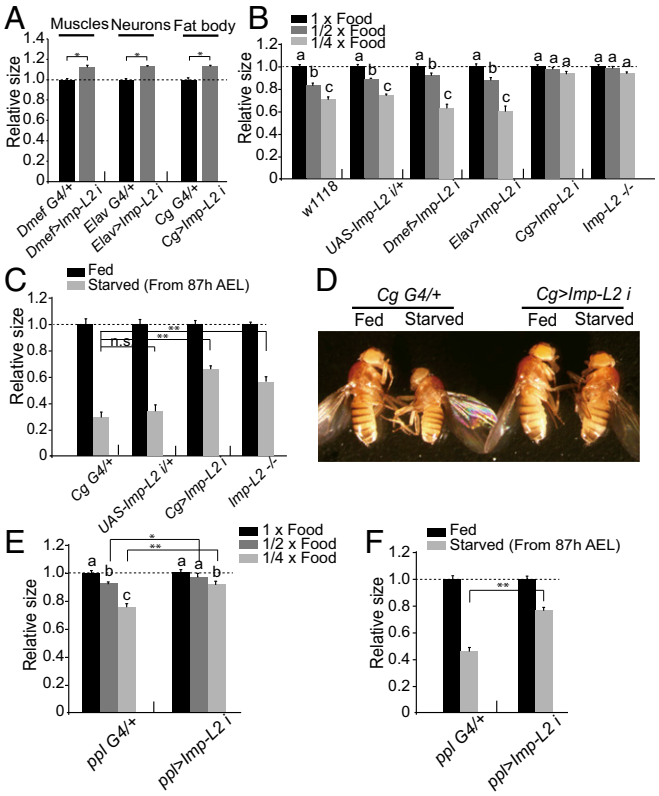

Fig. 1. Fat-body inactivation of Imp-L2 mitigates growth suppression induced by poor nutrition. (*A*) Knockdown of Imp-L2 in muscles (*Dmef Gal4*), neuronal tissues (*Elav Gal4*), or fat bodies (*Cg Gal4*) similarly increases the final adult size. Sizes were normalized by each *Gal4*/$^{+}$ control as 1.0. (*B*) Imp-L2 knockdown in fat bodies alleviates growth suppression caused by undernutrition. Rearing wild-type flies (w^{1118}) on serially diluted foods [1× (no dilution), 1/2×, and 1/4×] gradually decreases final adult size. Imp-L2 knockout in the whole body or depletion in fat bodies rendered animals resistant to size decreases caused by undernutrition, whereas Imp-L2 depletion in other tissues has no such effect. Sizes were normalized by the 1× food condition in each of the genotypes as 1.0. (*C*) Imp-L2 knockdown in fat bodies impedes the decrease in body size induced by starvation from the third larval instar. Sizes were normalized by the fed condition in each of the genotypes as 1.0. AEL, after egg laying. (*D*) Photograph of flies showing that the fat-body–specific knockdown of Imp-L2 renders flies resistant to starvation-induced size decrease. Imp-L2 knockdown using another fat-body–specific Gal4 (*ppl Gal4*) still alleviates growth suppression caused by undernutrition (*E*) or with starvation from the third larval instar (*F*). Sizes were normalized by the 1× food (*E*) or fed (*F*) condition in each of the genotypes as 1.0. In the fly size data shown throughout the figures, female adult flies were measured unless otherwise indicated, and similar phenotypes were also recorded in male flies ($n > 30$ flies per genotype in each of the conditions). $^{**}P < 0.001$; $^{*}P < 0.01$ (two-tailed t test). Different letters indicate significant difference (ANOVA: $P < 0.05$). n.s., not statistically significant. Error bars denote SEM.

Nutritional Regulation of Imp-L2 Expression Occurs Independent of Internal Nutrient Sensor in the Fat Body. The importance of fat-body Imp-L2 in mediating the nutrition-dependent body growth led us to examine whether Imp-L2 expression responded to the richness of the food that larvae feed on. A previous study had reported that 24-h starvation in larvae induces Imp-L2 expression in the fat body (8), and we made a similar observation when mid-third instar larvae were starved for 9 h (Fig. 2 *A* and *B*). Immunohistochemical analyses using antiserum against Imp-L2 revealed tiny dot-like staining patterns for Imp-L2 mainly localized at the periplasmic membrane region of fat-body cells, and the staining signals increased when the larvae were starved (Fig. 2*A*). These patterns of immunostaining were not artifactual because they were not observed in the cells of Imp-L2 null larvae (Fig. 2*A*). Larval fat body in *Drosophila* is thought to act as an internal nutrient sensor that coordinates organismal nutritional metabolism and growth in response to nutrient availability, and the insulin/TOR pathway plays a pivotal role in this (1, 3). Thus, we examined whether Imp-L2 expression is under the control of this pathway. Forkhead box protein O (FOXO) is a transcription factor that crucially mediates the effect of insulin signaling (1), so we tested whether increased Imp-L2 expression with starvation was impaired when *FOXO* was mutated. Interestingly, we found

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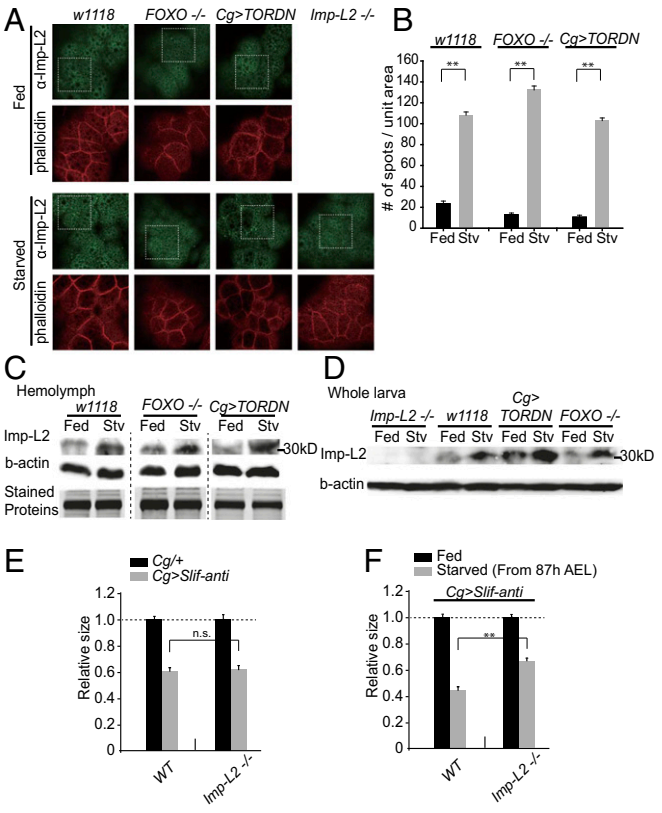

Fig. 2. Starvation-induced expression of Imp-L2 occurs independent of the fat-body nutrient sensor mechanism. (*A*) Immunohistochemical analysis of Imp-L2 expression in cells of larval fat bodies. Tiny spot-like immune signals of Imp-L2, localized mainly at the periplasmic membrane region, are manifested after 9 h of starvation in wild-type (*w1118*) fat-body cells; this phenomenon is also observed in *FOXO* null (*FOXO*^{21rev6A}; *FOXO*$^{-/-}$) or TOR-inhibited (*TOR*^{TED}; *TORDN*) mutant cells. The spot-like immune signals are absent in the cells of Imp-L2 null fat bodies, serving as a negative control. (Magnification, 300×.) Magnified images of outlined regions are shown in *SI Appendix*, Fig. S13. (*B*) Quantification of Imp-2 immune signals in fat-body cells shown in *A*. Western blot analysis reveals an increase in Imp-L2 levels in larval hemolymph (*C*) or in whole-larval bodies (*D*) in response to starvation, despite FOXO or TOR inactivation. β-Actin and the stained PVDF membrane are shown for a loading control. (*E*) Imp-L2 mutation cannot alleviate the size decrease induced by fat-body–specific inactivation of Slif. Sizes were normalized by the *Cg Gal4/*$^{+}$ condition in each of the genotypes as 1.0. (*F*) Starvation further reduces the small body size induced by fat-body–specific inactivation of Slif, which is alleviated by *Imp-L2* mutation. Sizes were normalized by the fed condition in each of the genotypes as 1.0 ($n > 30$ flies per genotype in each of the conditions). WT, wild type. $^{**}P < 0.001$ (two-tailed *t* test). n.s., not statistically significant; Stv, starved. Error bars denote SEM.

that Imp-L2 expression was still induced in the fat body of *FOXO* null larvae (*FOXO*^{21rev6A}) during starvation (Fig. 2 *A* and *B*). Moreover, using the dominant negative transgene of TOR (TORDN; *TOR*^{TED}) (18), we found that Imp-L2 induction by starvation in the fat body still occurred despite TOR inhibition (Fig. 2 *A* and *B*). Because Imp-L2 circulating in the hemolymph is thought to suppress organismal IIS by binding and antagonizing circulating Dilps, the level of circulating Imp-L2 was examined by Western blotting using larval hemolymph. A band corresponding to Imp-L2 was detected at ∼30 kDa (19). Consistent with the results of immunohistochemical analyses, Western blotting confirmed that both hemolymph and whole-body Imp-L2 levels increased in response to poor nutrition, and this was still observed when insulin/TOR signaling was blocked (Fig. 2 *C* and *D*). Increase of hemolymph Imp-L2 upon starvation was further confirmed when other components of insulin/TOR pathways, such as PI3K and Rag GTPase, were mutated (*SI Appendix*, Fig. S6). Moreover, lack of increase of hemolymph Imp-L2 by fat-body–specific depletion of Imp-L2 confirmed that the fat body is the main source of hemolymph Imp-L2 (*SI Appendix*, Fig. S7).

The finding that TOR signaling in the fat body does not appear to mediate Imp-L2 expression in response to nutritional condition led us to examine whether or not this fat-body–TOR nutrient-sensing mechanism involves Imp-L2 in regulating body growth. Fat body down-regulation of *Slimfast* (*Slif*), a gene that encodes a cationic amino acid transporter, has been shown to phenocopy the effects of starvation by suppressing TOR signaling in fat-body cells (3). Surprisingly, in contrast to the resistant response of the size of *Imp-L2* null flies to poor nutritional condition, *Imp-L2* null animals exhibited a rapid decrease in body size in response to fat-body Slif inactivation similar to that seen in wild-type animals (Fig. 2*E*). This result suggests that Imp-L2 does not play a significant role in relaying nutritional information originating from the fat-body–TOR internal nutrient sensor. Furthermore, the small body size induced by Slif inactivation in fat bodies was further rapidly decreased with poor nutrition, but this effect was blunted by the *Imp-L2* mutation (Fig. 2*F* and *SI Appendix*, Fig. S8). These data indicate that additional nutrient-sensing mechanisms such as that mediated by Imp-L2 may work in parallel with the fat-body–TOR in regulating nutrition-dependent growth of the body. Taken together, these findings indicate that Imp-L2 expression is regulated by nutritional condition independent of the internal nutrient sensor of the fat body.

Ecdysone Signaling in the Fat Body Mediates Nutritional Control of Imp-L2 Expression and Body Growth. Imp-L2 was originally isolated as one of the ecdysone-induced genes that encodes a membrane-associated or secreted protein in imaginal disks (20). Thus, we questioned whether Imp-L2 induction by poor nutrition is mediated via ecdysone signaling. To confirm this, we inhibited ecdysone signaling by employing two different genetic manipulations: the transgene of the dominant negative version of the ecdysone receptor (EcRDN; *EcR*^{F645A}) (21) and RNA interference of the ecdysone receptor (EcR i). In contrast to the results of inhibition of insulin/TOR signaling, confocal microscopy of the fat body revealed that inhibition of ecdysone signaling by either of the two genetic manipulations blocked the increase of immunofluorescence signals of Imp-L2 normally associated with poor nutrition (Fig. 3 *A* and *B*); this phenomenon was also confirmed by Western blot analysis using larval hemolymph (Fig. 3*C*). Together, these observations indicate that increased systemic Imp-L2 associated with poor nutrition is mediated by ecdysone signaling, which is stimulated by malnutrition. Then, we examined whether inhibition of ecdysone signaling in the fat body impaired the nutritional control of body size. Indeed, the body size of animals whose fat-body ecdysone signaling was inhibited by either EcRDN or EcR i did not rapidly decrease with undernutrition (Fig. 3*D*), resembling the phenotype of animals with depleted Imp-L2 in the fat body (Fig. 1 *B* and *E*). This blunted body size response induced by fat-body EcR inhibition was also seen under starvation conditions (*SI Appendix*, Fig. S9). Similar results were also seen when pupal volumes were measured (*SI Appendix*, Fig. S4). The effects induced by fat-body EcR inhibition were further confirmed using a different fat-body–specific Gal4 (Fig. 3*E*).

Increase of Circulating Ecdysone upon Poor Nutrition Suppresses Body Growth. We then investigated the influence of poor nutrition on the activity of ecdysone signaling in the fat body by examining the expression of *E74* and *Broad-Complex* (*BR-C*), two of the early-response genes of ecdysone signaling (22). Interestingly, *E74* and *BR-C* transcripts were significantly increased in the fat body when larvae were nutritionally deprived (Fig. 4*A*), indicating that poor nutrition increases the activity of ecdysone signaling in the fat body. This observation led us to investigate whether ecdysone

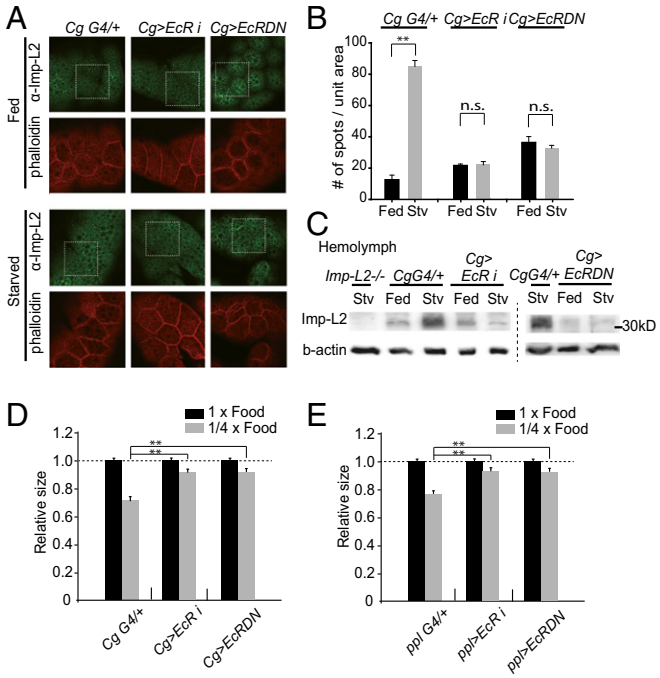

Fig. 3. Fat-body inactivation of ecdysone signaling abolishes the nutritional control of Imp-L2 expression and body size. Inhibition of EcR by RNAi (*EcR i*) or expressing its dominant negative form (*EcRDN*) abrogates the starvation-induced up-regulation of Imp-L2 in fat bodies as determined by immunohistochemical analysis (magnification, 300×) (*A*) and quantification (*B*). Magnified images of outlined regions in *A* are shown in *SI Appendix*, Fig. S13. (*C*) This observation is also confirmed in hemolymph, as determined by Western blotting. (*D*) Fat-body–specific inhibition of EcR using *Cg Gal4* impedes the decrease in body size caused by undernutrition. Sizes were normalized by the 1× food condition in each of the genotypes as 1.0. (*E*) Fat-body–specific inhibition of EcR using *ppl Gal4* impedes the decrease in body size caused by undernutrition. Sizes were normalized by the 1× food condition in each of the genotypes as 1.0 ($n > 30$ flies per genotype in each of the conditions). $^{**}P < 0.001$ (two-tailed *t* test). n.s., not statistically significant; Stv, starved. Error bars denote SEM.

levels in the hemolymph increase when larvae experience poor nutrition. Our results indicated that ecdysone concentration in the hemolymph increases when larvae are starved from the mid-third larval instar on, and that larvae reared with diluted fly food maintain higher hemolymph ecdysone levels throughout the third larval instar than larvae reared with normal fly food (Fig. 4*B*). We then examined whether feeding ecdysone to early third instar larvae could increase Imp-L2 expression similar to that seen in malnutrition. Indeed, ectopic treatment of ecdysone increased the level of Imp-L2 protein both in the fat body and in hemolymph (Fig. 4 *C* and *D*). Collectively, these data suggest that poor nutrition in third instar larvae elevates the levels of circulating ecdysone in the hemolymph, thereby increasing the activity of ecdysone signaling and stimulating Imp-L2 production in the fat body. To further confirm that EcR-dependent induction of Imp-L2 by poor nutrition is triggered by increased ecdysone generation from the prothoracic gland (PG), we utilized mutant *dominant temperature-sensitive 3* (*DTS-3*) flies, which harbor a mutant allele of the *molting defective* gene that encodes a nuclear zinc finger protein required for ecdysone biosynthesis (23). Although *DTS-3* mutants display dominant lethality during larval development owing to low ecdysone titers at restrictive temperatures (29 °C), *DTS-3*$^{/+}$ larvae have been known to develop into adults when reared at 25 °C despite exhibiting several defects associated with reduced ecdysone signaling (23–25). We found that when reared at 25 °C, *DTS-3*$^{/+}$ mutants showed resistance to a decrease in body size in response to poor nutrition (Fig. 4*E*); however, this phenomenon disappeared when the mutants were reared at permissive temperatures (18 °C) (Fig. 4*F*). This result shows that efficient generation of ecdysone through its biosynthetic pathway is critical in the nutritional regulation of *Drosophila* body size.

Inactivation of the Ecdysone/Imp-L2 Axis Abolishes Nutritional Regulation of IIS, but Not of TOR. We determined whether the ecdysone-/mp-L2 axis regulates peripheral IIS activity in response to nutritional condition. The level of the activated form of Akt (phospho-Akt) decreased under the starvation condition in wild-type larval fat body, as has been expected from decreased IIS activity. However, this starvation effect on IIS activity was largely abrogated when Imp-L2 was mutated (Fig. 5 *A* and *B*), which was also similarly seen when EcR activity was blocked (Fig. 5 *C* and *D*). In contrast, the level of the activated form of S6K (phospho-S6K) still decreased under starvation despite Imp-L2 or EcR mutation, indicating that the fat-body–TOR nutrient-sensing mechanism is still working in response to changes in nutritional condition. It is noted that

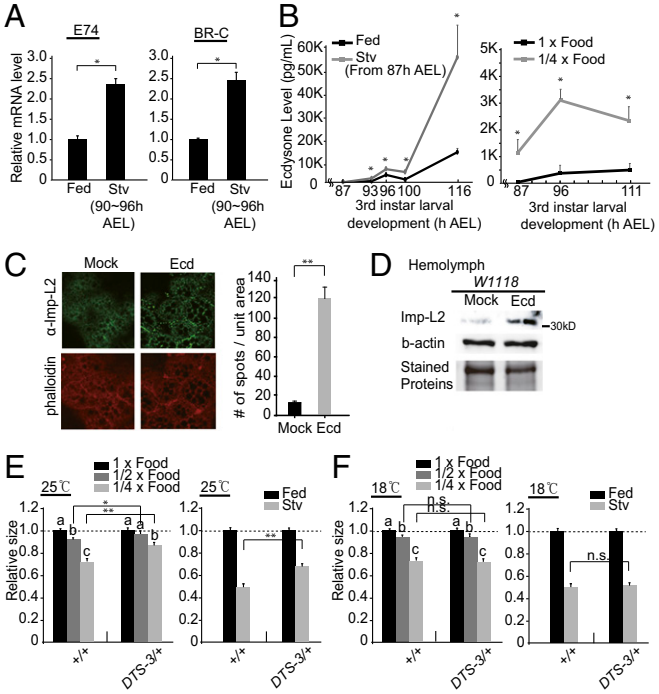

Fig. 4. Increased production of ecdysone by poor nutrition attenuates body growth. (*A*) Six-hour starvation in mid-third instar larvae [90 h after egg laying (AEL)] increases the transcript levels of *E74* (*Left*) and *BR-C* (*Right*), two of the early-response genes of ecdysone signaling, in fat bodies. Four biological replicates (six to nine animals from each genotype per replicate) were used. (*B*) Starvation in mid-third instar larvae (87 h AEL) increases the circulating ecdysone levels in larval hemolymph over time (*Left*), and larvae reared on diluted fly food (1/4×) maintain elevated ecdysone levels in the hemolymph throughout the third larval instar stage (*Right*). Four biological replicates (six to nine animals from each genotype per replicate) were used. Feeding ecdysone (Ecd) to early third instar larvae (~3 h after L2/L3 ecdysis) increases Imp-L2 protein expression in the fat body (magnification, 300×) (*C*) and in hemolymph (*D*), as shown by immunostaining and Western blotting, respectively. (*D*) Immunosignals of Imp-L2 are quantified as in Figs. 2*B* and 3*B*. *DTS-3* mutant flies exhibit resistance to size decrease in response to poor nutrition when reared at nonpermissive temperatures (*E*; 25 °C), but this phenotype disappears when they are reared at permissive temperatures (*F*; 18 °C). Sizes were normalized by the 1× food (*E* and *F*, *Left*) or fed (*E* and *F*, *Right*) condition in each of the genotypes as 1.0. The size was measured in male flies, and similar phenotypes were also recorded in female flies ($n > 30$ flies per genotype in each of the conditions). Different letters indicate significant difference (ANOVA: $P < 0.05$). $^{**}P < 0.001$; $^{*}P < 0.01$ (two-tailed *t* test). n.s., not statistically significant; Stv, starved. Error bars denote SEM.

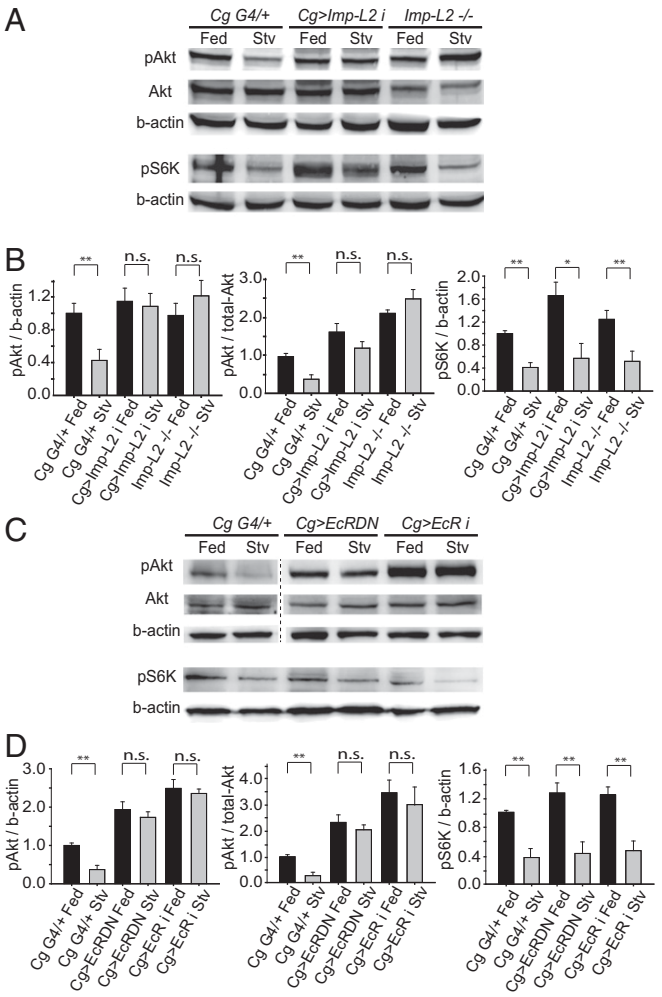

Fig. 5. Inactivation of the ecdysone/Imp-L2 axis abrogates nutritional regulation of IIS activity, but not TOR. (*A*) Representative Western blot image showing that the level of the activated form of Akt [phospho-Akt (pAkt)] decreases under the starvation condition in the wild-type larval fat body, which is not seen when Imp-L2 is inactivated specifically in the fat body or in a whole-animal body. Regulation of the activated form of S6K [phospho-S6K (pS6K)] by starvation is, however, marginally affected by Imp-L2 inactivation. (*B*) Quantification of three biologically replicated results of Western blotting performed as in *A*. (*C*) Representative image of Western blotting showing that the level of pAkt decreases under the starvation condition in the wild-type larval fat body, which is not seen when EcR is inactivated specifically in the fat body by RNAi (*EcR i*) or by expressing its dominant negative form (*EcRDN*). Regulation of pS6K by starvation is, however, marginally affected by EcR inactivation. (*D*) Quantification of three biologically replicated results of Western blotting performed as in *C*. Data are presented as mean ± SEM. $*P < 0.05$; $**P < 0.01$ compared with the respective controls (*t* test). n.s., not statistically significant; Stv, starved.

mutation of Imp-L2 did not appear to significantly affect the secretion of Dilps from insulin-producing cells (IPCs), as determined by Dilp2 accumulation in IPCs upon starvation (*SI Appendix*, Fig. S10). Together, these data suggest that the ecdysone/Imp-L2 axis is responsible for down-regulation of peripheral IIS with poor nutrition, while this axis is dispensable for the regulation of fat-body–TOR.

Discussion

There are numerous reports indicating that nutritional condition during human childhood significantly affects physical growth and height as an adult (26, 27). As such, the nutritional cues influencing growth in juveniles have drawn increasing attention. Although the cellular effects of nutrient-sensing pathways, such as insulin/TOR signaling, have been heavily explored, how these are integrated at the organismal level to contribute to juvenile body growth remains largely unknown. In the present study, we showed that steroid signaling and its regulation of a humoral protein constitute a previously unrecognized circuit that mediates nutrition-dependent organismal growth, which acts in parallel with the fat-body–TOR nutrient sensor. Based on the observation that our varying nutritional conditions change final adult size without significant alteration in pupariation timing (*SI Appendix*, Fig. S11 and discussed below), our current model is proposed as follows: (*i*) Poor nutrition in third instar larvae increases ecdysone generation in the PG, thus elevating basal levels of ecdysone titers in circulation; (*ii*) this increases ecdysone signaling in the fat body, up-regulating Imp-L2 production from the fat body; and (*iii*) finally, increased hemolymph Imp-L2 attenuates peripheral IIS and body growth, eventually leading to smaller sized adults (*SI Appendix*, Fig. S12). It is a well-established theory that TOR signaling in fat body senses systemic levels of amino acids, thereby remotely influencing IPCs to regulate Dilp production; several humoral proteins that mediate this fat-body–IPC communication have been characterized (3, 28–30). Interestingly, we found that inhibition of TOR signaling in the fat body did not impair the regulation of Imp-L2 by nutritional condition, and in contrast to the resistant phenotype to nutrition restriction, *Imp-L2* null mutants exhibited a rapid decrease in body size in response to the inhibition of amino acid transport into the fat body. Thus, it seems unlikely that Imp-L2 is the humoral factor that mediates the communication of nutrient-sensing mechanisms within the fat body. Moreover, the observation that nutritional restriction further decreased the body size of flies whose fat body was unable to sense amino acids suggests that nutritional control of body growth may use multiple parallel endocrine pathways, which likely involve additional nutrient-sensing mechanisms besides the fat-body–TOR. In this situation, the nutritional information processed by yet undetermined nutrient-sensing mechanisms could be conveyed to peripheral tissues via the ecdysone/fat-body/Imp-L2 pathway.

Another interesting observation in our study was that poor nutrition increased circulating ecdysone levels. A recent study showed that starvation that occurred in larvae before reaching CW (rather than after CW as in the present study) decreases circulating ecdysone levels, thereby delaying the time to reaching CW and pupariation (31). Evidence suggests that insulin/TOR signaling in the PG could be responsible for this phenomenon (31, 32). Thus, in the early larval stage before CW, poor nutrition appears to limit ecdysone production from the PG. In contrast, our study showed that after CW, malnutrition in third instar larvae actually increases ecdysone levels in the hemolymph. Although future studies could address these seemingly paradoxical effects of poor nutrition on circulating ecdysone levels with respect to developmental time, our findings might explain the long known but poorly understood observation that malnutrition in *Drosophila* larvae past CW slightly advances the timing of pupariation, a phenomenon also observed among other insects (17, 33, 34) (*SI Appendix*, Fig. S11). In line with this, it might be worth noting that starvation in female adult flies increases ecdysone levels in the hemolymph, leading to apoptosis in egg chamber cells and oogenesis arrest (35). Hence, it appears that changes in circulating ecdysone levels in response to varying nutritional conditions are regulated differentially depending on the developmental phase.

Imp-L2 regulation by ecdysone signaling suggests that Imp-L2 may play a part in the coupled process of juvenile growth and developmental maturation. In addition to dictating developmental transitions, such as larval molting and pupariation, circulating ecdysone has been shown to attenuate peripheral IIS, thereby suppressing larval growth (4–6). Previously proposed mechanisms point to the fat body as being the major target organ

of ecdysone, in which the transcription factor Myc and the microRNA miR-8 act as the downstream effectors for gene expression mediating organismal growth (4, 6). Moreover, after cessation of feeding, Dilp6 is expressed in the fat body by ecdysone, thereby regulating postfeeding body growth (36, 37). Based on the ability of Imp-L2 to suppress body growth and peripheral IIS activity in response to ecdysone signaling, it would be interesting to investigate the mechanistic roles of Imp-L2, in concert with Myc, miR-8, and Dilp8, in maturation signal-induced control of organismal growth.

Intriguingly, it has long been documented that a state of insulin resistance occurs during puberty. Insulin resistance peaks at mid-puberty and declines to nearly prepubertal levels by adulthood, which has been thought to be caused by the elevated serum levels of sex steroids in puberty (38). Thus, our finding that reduced IIS activity by Imp-L2 was associated with increased circulating ecdysone during the juvenile growth period may stimulate further study for investigating the molecular basis of pubertal insulin resistance. Moreover, since the process from juvenile growth to sexual maturation with adult size determination has intriguing parallels with that in humans (1, 2), it would be an interesting future study to determine whether this newly uncovered endocrine circuit involving steroid hormones, metabolic endocrine organs, and IGFBPs works similarly in humans to regulate juvenile body growth in response to various intrinsic and extrinsic cues, such as puberty and nutrition.

Materials and Methods

UAS-EcRDN (*EcRDN^{F645A}*), *UAS-TORDN* (*TOR^{TED}*), *Dmef Gal4*, *Elav Gal4*, UAS-PI3KDN, and *DTS-3* flies were obtained from the Bloomington Drosophila Stock Center. *UAS-EcR RNAi* (stock no. 37058), *UAS-Imp-L2 RNAi* (stock no. 30930), and *UAS-Rag A RNAi* (stock no. 20130) were obtained from the Vienna RNAi Library Center. The *Cg Gal4* and *ppl Gal4* have been described previously (4). The Imp-L2 null fly (*Imp-L2^{Def42}*) has been described previously (8). *UAS-Slif-anti* has been described previously (3). *FOXO^{21rev6A}* is the *FOXO* null allele in which the second site lethal mutation was removed from *FOXO^{21}* (a gift from Marc Tatar, Brown University, Providence, RI). To measure body size, groups of three adult flies, newly eclosed for not more than 5 h, were weighed. All flies were ice-anesthetized before weight measurement. For measurement of pupal volume, digital images of pupae were captured by Toupview software (Touptek) and pupal volume was measured using the formula $4/3\pi\ (L/2)\ (l/2)^2$, where L is length and l is width. Concentrations of 20-hydroxyecdysone were measured using the 20-hydroxyecdysone EIA Kit (Cayman Chemicals) as per the manufacturer's instructions. Ecdysone preparation was performed as previously described elsewhere (31). Hemolymph was pooled from seven to 10 larvae to obtain 1 μL of hemolymph, which was resuspended in EIA buffer (Cayman Chemicals) for the assay. Addition information on the materials and methods used is provided in *SI Appendix*.

ACKNOWLEDGMENTS. We thank Dr. Ernst Hafen, Dr. Marc Tatar, and Dr. Pierre Leopold for their kind gifts of *Imp-L2* null fly, *FOXO* null fly, and *UAS-Slif-anti* fly, respectively. We also thank Dr. Jeanette E. Natzle and Dr. Kweon Yu for sharing Imp-L2 and Dilp2 antibody, respectively. We thank the Bloomington Drosophila Stock Center and the Vienna Drosophila RNAi Center for providing the fly stocks used in this study. This research was supported by the Basic Science Research Program of the National Research Foundation of Korea, which is funded by Ministry of Education, Science, and Technology Grant 2017R1A2A2A05069502 and by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, which is funded by Ministry of Health and Welfare, Republic of Korea Grant HI14C2380.

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