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**Highlights**
- Dietary cholesterol promotes developmental growth and leads to early maturation
- Insulin signaling couples cholesterol intake with systemic growth
- Npc1-TOR couples cholesterol with insulin signaling via glial and fat-tissue relays
- Cholesterol sensing affects a nutritional checkpoint that prevents early maturation

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**In brief**
Cholesterol is necessary for normal physiology, but its overabundance contributes to disease. Texada et al. report that dietary cholesterol activates mTOR in *Drosophila* adipose-/hepatic-like cells and blood-brain-barrier glia, which remotely upregulate systemic insulin-like signaling, a finding that may have implications for health.
Insulin signaling couples growth and early maturation to cholesterol intake in *Drosophila*

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SUMMARY

Nutrition is one of the most important influences on growth and the timing of maturational transitions including mammalian puberty and insect metamorphosis. Childhood obesity is associated with precocious puberty, but the assessment mechanism that links body fat to early maturation is unknown. During development, the intake of nutrients promotes signaling through insulin-like systems that govern the growth of cells and tissues and also regulates the timely production of the steroid hormones that initiate the juvenile-adult transition. We show here that the dietary lipid cholesterol, which is required as a component of cell membranes and as a substrate for steroid biosynthesis, also governs body growth and maturation in *Drosophila* via promoting the expression and release of insulin-like peptides. This nutritional input acts via the nutrient sensor TOR, which is regulated by the Niemann-Pick-type-C1 (Npc1) cholesterol transporter, in the glia of the blood-brain barrier and cells of the adipose tissue to remotely drive systemic insulin signaling and body growth. Furthermore, increasing intracellular cholesterol levels in the steroid-producing prothoracic gland strongly promotes endoreduplication, leading to an accelerated attainment of a nutritional checkpoint that normally ensures that animals do not initiate maturation prematurely. These findings, therefore, show that a Npc1-TOR signaling system couples the sensing of the lipid cholesterol with cellular and systemic growth control and maturational timing, which may help explain both the link between cholesterol and cancer as well as the connection between body fat (obesity) and early puberty.

INTRODUCTION

Animals’ growth and development depend upon nutrient availability. Therefore, specialized cells and tissues have arisen that sense nutritional inputs and adjust growth and developmental programs via systemic hormonal pathways.1 In most eumetazoans, these include the conserved insulin-like peptide and steroid-hormone signaling systems. These become dysfunctional when nutrient levels exceed their physiologically normal range. Overloading of the insulin system leads to obesity, metabolic syndrome, insulin resistance, and other pathophysiologies, and overnutrition also leads to precocious puberty associated with childhood obesity.3

Many animals’ early life is a nonreproductive stage of rapid growth, terminated at some nutritional threshold that signals readiness to become a reproductively fit adult.2 In animals as diverse as humans and insects, this transition is driven by steroid hormones4 – gonadal steroids including testosterone and estrogen trigger mammalian puberty, and insect metamorphosis is initiated by ecdysone, produced in the prothoracic gland (PG). Similar neuroendocrine cascades regulate insect and mammalian steriodogenesis, including the orthologous neuropeptides Allatostatin A/Kisspeptin and Corazonin/gonadotropin-releasing hormone (GnRH)5,6 as well as analogous steroid-feedback circuits.7,8 These axes are clearly linked to the metabolic state of the animal, including attainment of a certain critical size.5 However, the mechanisms of body-size estimation and the effects of nutritional status are not completely understood. Recent work in *Drosophila* suggests that progression to adulthood is gated by a checkpoint system monitoring tissue growth and nutritional status.9 When animals reach a “critical weight” (CW), they become committed to completing their development and maturation, irrespective of further nutritional inputs, whereas animals starved before this checkpoint is satisfied halt their progression to adulthood.10,11 This suggests that the CW checkpoint assesses the animal’s nutritional state, but the specific nutrients required, and the mechanisms by which their levels are sensed, are incompletely defined.

In *Drosophila*, nutritional input drives growth and maturation through the insulin pathway. Nutrient intake, of amino acids in particular, is sensed via the fat body (analogous to mammalian adipose tissue) and the glia of the blood-brain barrier (BBB). These tissues release factors that regulate the expression and release of *Drosophila* insulin-like peptides (ILPs) from the insulin-producing cells (IPCs) within the brain,12,13 which share functional and developmental homologies with mammalian pancreatic beta cells.2 These ILPs promote systemic growth through the conserved insulin-receptor/PI3K/Akt pathway. Insulin also promotes PG ecdysone production, linking nutrition directly to developmental progression.1,14–18
Human puberty-triggering CW appears to be linked to body-fat stores, which may explain the link between childhood obesity and early puberty. Despite this, the mechanism by which adiposity affects puberty initiation is unclear. Furthermore, the role of cholesterol has not been considered, even though adipose tissue is a major cholesterol storage depot, especially in obesity. Sterols such as cholesterol have membrane-structural functions but also play important signaling roles, and sterols are required as substrates for steroid-hormone production. Insects, including Drosophila, have lost the ability to synthesize sterols de novo and thus must acquire them through feeding. Mammals are cholesterol prototrophs, but most intracellular cholesterol still comes from low-density-lipoprotein (LDL)-mediated cellular uptake of dietary cholesterol. In both taxa, consumed sterols are transported in the circulatory system bound within lipoprotein particles (LPPs such as mammalian LDL/HDL), and target tissues take them up through a variety of mechanisms including receptor-mediated endocytosis. LPP-bound sterols are extracted in the lysosome and inserted into the lysosomal membrane by membrane-integral transport proteins. The primary such protein, Niemann-Pick-type C 1 (Npc1), underlies the Niemann-Pick type C lysosomal storage disorder; without Npc1 function, cholesterol accumulates in endosomal-lysosomal compartments, leading to increased intracellular cholesterol signaling. Thus, Npc1 seems to be part of a mechanism by which cells regulate cholesterol signaling.

We wished to determine whether, and the routes by which, cholesterol might regulate Drosophila larval growth. Our findings show that dietary cholesterol dose-dependently promotes growth and accelerates development by increasing insulin signaling. Cholesterol sensing is mediated by the target of rapamycin (TOR) pathway in the cells of the fat body and the glia of the BB, which remotely induce the expression and release of ILPs from the IPCs. Enhancing cholesterol signaling in the PG also promotes TOR activity, drives endoreduplication, and leads to premature attainment of the CW checkpoint. Thus, dietary cholesterol accelerates growth through insulin signaling and leads to early maturation through effects on steroidogenesis, effects which are mediated by promoting TOR activity in sensory tissues.

**RESULTS**

**Dietary cholesterol promotes systemic growth via insulin signaling**

Body growth occurs almost exclusively during larval life in Drosophila, and thus, pupariation fixes animal size. We reared standard laboratory strain w1118 larvae on completely synthetic media containing a range of cholesterol concentrations from 1.2 to 80 µg/ml and measured their pupariation timing and pupal size. Saturated yeast liquid cultures contain ergosterol, the main yeast sterol, at concentrations ranging from ~2 to ~150 µg/ml, and amounts of beta-sitosterol, the main plant sterol, in common fruits fall within roughly 16–320 µg/ml. The concentrations tested here are thus within the ecologically relevant range. Pupal size increased with increasing dietary cholesterol concentrations up to a cholesterol concentration of 25–40 µg/ml (Figure 1A), even though developmental time decreased with dietary cholesterol concentration (Figure 1B), indicating a cholesterol-induced increase in growth rate. Above 25 µg/ml, increasing cholesterol did not further increase size, suggesting saturation of the mechanism(s) involved.

We next wished to determine the underlying systemic basis for the growth-promoting effect of cholesterol. Systemic growth is largely governed by the antagonistic insulin and ecdysone signaling pathways. We therefore asked whether either of these mediates the effects of dietary cholesterol on growth. The ILPs that drive systemic growth are ILP2, ILP3, and ILP5, produced by the IPCs. By promoting body growth, increased ILP activity shortens the time required to reach CW. The effects observed above are consistent with increased insulin-signaling activity; hence, we assessed the necessity of this pathway for cholesterol-induced growth enhancement. Completely synthetic diet is suboptimal, as reflected in the observed prolonged developmental times (Figure 1B), and it is quite different from a natural diet. Therefore, we used a commercial commensal medium (NutriFly, NF hereafter) that is more like an ecologically relevant diet. Furthermore, NF food is lower in sterols than our standard lab diet and can be supplemented with cholesterol to promote growth. We inhibited ILP secretion in the early third-instar (L3) stage by using Ilp2-GAL4 together with Tubulin (Tub)-driven temperature-sensitive GAL80 (together, “Ilp2TS”) to drive expression of the inhibitory potassium channel Kir2.1 in the IPCs. Control animals (Ilp2TS-->+) grew significantly more quickly after transfer to higher-cholesterol diet, whereas increased dietary cholesterol had no effect on the larval weight of Ilp2TS->Kir2.1 animals (Figure 1C; 2-way ANOVA genotype x diet interaction, p = 0.0024). Thus, ILP release is required for the growth-enhancing effects of dietary cholesterol. Importantly, this also demonstrates that cholesterol’s growth-promoting effect does not merely reflect, for example, membrane-structural requirements.

Cholesterol is also the chemical precursor to the steroid hormone ecdysone, which has the effect of reducing the growth of larval tissues. Therefore, cholesterol supplementation during larval life could alter growth through enhanced ecdysone production, although this would be expected to decrease systemic growth. To exclude the involvement of ecdysone, we reduced steroidogenesis through inhibition of PTTH-Torso signaling, using PG-specific phantom-GAL4 (phm>) to drive RNAi against torso, thereby blocking PG activation. Unlike IPC-silenced animals (Figure 1C), animals with reduced ecdysone production (phm-torso-RNAi) exhibited a cholesterol-induced growth increase similar to controls’ (Figure 1D, no significant genotype x diet interaction: p = 0.76). Furthermore, we compared the growth of wild-type (w1118) animals transferred 72 h after egg laying to synthetic diets containing either 1 or 80 µg/ml cholesterol, with and without direct supplementation with 250 µg/ml 20-hydroxyecdysone (20E), the active form of ecdysone. When these animals were weighed 18 h later (90 h after egg laying), we found that added 20E had attenuated larval growth on both cholesterol doses, as expected, and increased cholesterol had induced a similar size increase whether or not 20E was present (Figure S1A, 2-way ANOVA p for interaction: 0.65, nonsignificant). Thus, cholesterol-induced systemic growth increase does not arise through altered ecdysone signaling but does require insulin signaling.
Figure 1. Dietary cholesterol systemically promotes growth through insulin signaling

(A) Larval growth on synthetic medium, reflected in pupal size, increases with increasing dietary cholesterol concentration. (B) Development to the pupal stage on synthetic medium is accelerated with increasing dietary cholesterol concentration. Top: fraction pupariated over time; bottom: time until 50% pupariation; each data point represents a vial of flies.

(C) Cholesterol’s growth-promoting effect requires insulin signaling. Dietary supplementation of lower-sterol NutriFly medium with cholesterol (NutriFly versus NutriFly + 40 µg/ml chol) promotes growth in control animals (Ilp2TS> +), but if the IPCs are silenced through expression of the inhibitory channel Kir2.1 (Ilp2TS>Kir2.1), this growth promotion is blocked (2-way ANOVA p for interaction of genotype and diet, 0.0105). Animals were reared at 18°C on NF medium until 130 h AEL, when half were transferred to NutriFly+40. Animals were then kept at 29°C, and L3 larvae were weighed 24 h later.

(D) The growth-promoting effect of added cholesterol does not arise through effects on ecdysone production. Animals were reared on NF medium until 72 h AEL, when half were transferred to NF+40. L3 larvae were weighed at 104 h AEL. Blocking the production of ecdysone by expressing RNAi against the PTTH receptor Torso in the prothoracic gland (phm>torso-RNAi) had no effect on cholesterol-induced growth (2-way ANOVA p for interaction of genotype and diet, 0.974). See also Figure S1 A.

(E and F) Insulin-gene expression is higher (E), and anti-ILP2 and –ILP5 staining intensity is lower (F), in animals that fed on synthetic medium containing 80 µg/ml cholesterol than in animals that fed on cholesterol-free medium, suggesting increased ILP release on cholesterol-containing medium. Illustrative images are shown below.

(G) Feeding on cholesterol-containing synthetic medium after cholesterol starvation on cholesterol-free synthetic medium appears to acutely induce ILP2 release (within 3 h), as indicated by reduced anti-ILP2 stain in the IPCs.

(H) Hemolymph ELISA against tagged ILP2 indicates correspondingly increased circulating ILP2 after larvae are transferred from cholesterol-free synthetic diet to cholesterol-replete medium.

(I and J) Peripheral insulin-signaling activity is increased by cholesterol refeeding, reflected in anti-phospho-Akt staining normalized to total Akt or to histone H3. Statistics: (A) Welch’s ANOVA with Dunnett’s T3 multiple comparisons. (B and H) Kruskal-Wallis ANOVA with Dunnett’s multiple comparisons. (C and D) Two-way ANOVA with Sidák’s multiple comparisons. (E, F, and G) Mann-Whitney pairwise test. (J) unpaired t test. (A–E, H, and J) Mean ± SEM. (F and G) Median with 95% confidence interval. Significance is noted as ns, p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. See also Figure S1.
Dietary cholesterol promotes the expression and release of ILP2 and ILP5

To test whether cholesterol can induce ILP release acutely, we cholesterol-starved animals for 16 h on synthetic medium and then transferred them to a cholesterol-replete but otherwise identical diet. By the end of 3 h refeeding, IPC ILP2 content had dropped significantly, suggesting acute release of peptide into circulation (Figure 1G). To confirm this, we measured circulating ILP2 levels. When cholesterol-starved animals were transferred to food containing cholesterol, circulating hemolymph ILP2 levels increased slightly after 1 h of cholesterol feeding and significantly after 4 h (Figure 1H). To assess the effect of cholesterol on peripheral insulin signaling, we measured the level of phosphorylation of Akt (pAkt), a readout of intracellular insulin-pathway activity. Cholesterol feeding for 4 h increased whole-animal pAkt levels (Figures 1I and 1J), indicating increased systemic insulin-signaling activity. Together, these findings show that dietary cholesterol promotes insulin expression and increases the release of ILP2 and -5 into the hemolymph, thereby systemically promoting body growth.

Cholesterol signaling promotes systemic growth mainly via glia and fat body

Next, we sought to identify the anatomical routes by which dietary cholesterol influences insulin production and release from the IPCs. Although the IPCs are autonomously sensitive to some nutritional inputs, most nutrition-induced effects on these cells are mediated by sensory mechanisms in peripheral tissues. The fat body is a central nutrient-sensing hub, releasing multi-trophic hormones and nutrients that influence insulin production and release from the gut (Figures S1B–S1E).26,27 Cholesterol accumulates in tissues with loss of Npc1a, but not in Npc1b mutants.40,41 We therefore assessed the effects of knocking Npc1a down in each tissue, because this manipulation should lead to increased levels of intralysosomal cholesterol and thus possibly to increased cholesterol signaling.28 Thus, we aimed to identify tissues in which Npc1a RNAi brings about a body-size increase, thereby pheno-coppying the response to dietary cholesterol supplementation.

Knockdown of Npc1a in the IPCs did not significantly alter developmental timing but did lead to reduced pupal size (Figures 2A and 2A'), suggesting that cholesterol does not positively regulate these cells by direct means. We also further excluded the involvement of ecdysone through manipulating the PTTH-producing neurons (PTTHn). Knockdown of Npc1a in these neurons using Ptth-GAL4 did not significantly alter pupation timing or pupal size, and we confirmed this with a second GAL4 line, Np0423>, that is active in the PTTHn. These data are consistent with cholesterol-induced growth’s being independent of ecdysone (Figures 1D and 1A). We next targeted the entire nervous system with knockdown of Npc1a using R57C10-GAL4 (R57C10>). Pan-neuronal knockdown of Npc1a led to a few hours’ acceleration in pupariation timing (Figure 2B) but did not significantly alter pupal size (Figure 2B') or larval weight (Figure 2B'). The finding of unchanged larval weight makes it seem unlikely that cholesterol signaling regulated by Npc1a in the IPCs, the PTTHn, or other neurons underlies the systemic growth-promoting effects of cholesterol.

We next investigated whether BBB-glial and fat-body cholesterol levels might mediate the effects of dietary cholesterol on insulin signaling and growth, because these tissues relay other types of nutritional information to the IPCs. Perturbation of cholesterol trafficking via Npc1a knockdown in the BBB did not alter developmental timing, but it did lead to a significant increase in pupal size (Figures 2C and 2C'), suggesting an increased rate of growth. Altering cholesterol signaling in the fat body through knockdown of Npc1a led to accelerated pupariation as well as increased pupal size (Figures 2D and 2D'), also consistent with faster growth. No acceleration of pupariation (Figure 2C') or increase in larval or pupal size (Figures 2C' and S2C') were observed in genotypes lacking a GAL4 driver, ruling out the possibility that this effect was due to effects of the RNAi transgene or background alone. Together, these results suggest that Npc1a-regulated cholesterol signaling in glia and fat body increases growth systemically.

To measure possible alterations in larval growth more directly, we measured individual mid-third instar (96–101 h after egg laying; AEL) larval weights in lower-sterol NF cornmeal medium, which we rationalized would provide a more sensitized condition to assess the effects of increased cholesterol signaling. Knockdown of Npc1a in the glia or fat body strongly increased the size of larvae reared on this medium (Figures 2E and 2F), supporting a model in which cholesterol sensing in these tissues, regulated by Npc1a activity, mediates nutritional effects on systemic body growth. Consistent with the notion that this cholesterol sensing mechanism is independent of the nervous system, we did not observe significant phenotypes in larvae reared on lower-sterol medium with knockdown of Npc1b using the mdy-GAL4 driver (Figure S1A’).
Figure 2. Cholesterol-induced growth promotion is mediated by the fat body and the glia of the blood-brain barrier

(A–D) Top panel: pupariation over time on standard lab diet; individual trials are shown as fainter lines, and the average is shown in a darker line. Bottom panel: the time until 50% pupariation; each data point represents a vial of ~40 animals.

(A′–D′) Pupal sizes of animals from several vials of each genotype. In some cases, sexual dimorphism in size is apparent as a bimodal distribution of points. (A′/A′) Npc1a knockdown in the IPCs has no effect on developmental timing but reduces pupal size significantly. (B′/B′) Knockdown of Npc1a throughout the nervous system accelerates pupariation but has no significant effect on pupal size. (C′/C′) Knockdown of Npc1a in the glia of the blood-brain barrier increases pupal size but has no effect on developmental timing. (D′/D′) Knockdown of Npc1a in the fat body both accelerates pupariation and increases pupal size.

(E–J) Individual larvae were harvested from NutriFly medium at 96–101 h AEL and weighed. Sibling genotypes were placed on ice simultaneously to prevent continued growth. (E and G) Npc1a knockdown in the BBB glia with either of two RNAi lines leads to increased larval weight. (F and H) Npc1a knockdown in the fat body with two independent RNAi constructs also strongly increases larval weight. Note that Cg> data are reproduced between these two panels; all data were obtained simultaneously but are presented separately here for visual consistency. Thus, for statistical rigor, an ANOVA was performed, with the same significance results. (I and J) Alternative GAL4 drivers (pan-glial repo> and fat-body ppl>) recapitulate the knockdown phenotypes driven by mdy> and Cg>. See also Figures S2G and S2H for additional confirmation.

(K and L) Npc1a knockdown in the glia of the BBB (K) or the fat body (L) is nonadditive with dietary cholesterol, consistent with their functioning within a single pathway. Left pair: control pupae (GAL4>+ in both tests fed on NutriFly medium supplemented with 40 μg/ml cholesterol (darker data points) are larger than control animals that fed on unsupplemented NutriFly diet (lighter data points). Right pair: knockdown of Npc1a in these tissues (GAL4+Npc1a-RNAi) increases body size on unsupplemented NutriFly, and cholesterol supplementation has no additive effect, suggesting that cholesterol and Npc1a act in the same pathway. Two-way ANOVA indicates strong genotype-by-diet interaction in both cases. Statistics: (E–J), Kruskal-Wallis ANOVA with Dunn’s multiple comparisons. (E–L), two-way ANOVA with Sidák’s multiple comparisons. (A–D, E–L) Mean ± SEM. (A′–D′) Median with 95% confidence interval. Significance is noted as ns, p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. See also Figure S2.
observe any increase in larval size with knockdown of Npc1a in the nervous system (Figure S2B), in the IPCs (using two different GAL4 drivers, Figures S2D and S2E), or in the PTTHn (Figure S2F), further indicating that neuronal cholesterol sensing is not responsible for the systemic growth-enhancing effects of cholesterol. To confirm the systemic body-growth effects of Npc1a-regulated cholesterol sensing in the glia and the fat body, we used a second independent RNAi line (Npc1a<sup>45</sup>) and alternative drivers specific for these tissues. When driven by the initial GAL4 drivers (mdy> and Cg>), this second RNAI construct phenocopied the first knockdown line, strongly increasing larval size and thus minimizing the possibility that the phenotype is due to RNAi off-target effects (Figures 2G and 2H). Next, we used the alternative glial driver, NP2276-GAL4, which like mdy-GAL4 targets the subperineurial glia that mediate nutrient-sensing to regulate Ilp5 expression,<sup>44,45</sup> and the pan-glial driver, repo-GAL4<sup>15,46</sup> to drive RNAi expression. We found that knockdown of Npc1a either in all glia or those of the BBB alone with these drivers resulted in increased larval body size (Figures 2I and S2G). Consistent with the Npc1a knockdown phenotype observed for the fat body using the Cg> driver, the knockdown of Npc1a using either of two additional fat body-specific drivers (ppl-GAL4<sup>47,48</sup> and AkhR-GAL4<sup>15</sup>) significantly increased larval body size, indicating increased growth (Figures 2J and S2H). Together, these results suggest that Npc1a-regulated cholesterol sensing in nutrient-sensing glia of the BBB and in cells of the fat body affects systemic growth.

We next wished to determine the epistatic relationship between Npc1a knockdown and cholesterol availability to define whether this Npc1-mediated effect is cholesterol specific. We raised fat-body and glial Npc1a-knockdown and control animals on NF medium and NF supplemented with 40 μg/ml additional cholesterol. As observed above, cholesterol supplementation strongly increased the size of control pupae (Cg> and mdy>), and Npc1a knockdown (Cg>Npc1a-RNAi and mdy>Npc1a-RNAi) led to a similar increase in the pupal size of animals that fed on unsupplemented medium (Figures 2K and 2L). If these effects were mediated by parallel pathways, one might expect that these size increases would be additive; however, knockdown animals that fed on supplemented medium formed pupae no larger than those seen with each single manipulation (Figures 2K and 2L). Similarly, increased dietary cholesterol accelerated the puation of control animals by several hours but had little effect on the developmental timing of animals with BBB-glia or fat-body Npc1a gene knockdown (Figures S2I and S2J). These effects are consistent with the operation of a single pathway through which cholesterol and Npc1a knockdown promote growth. Taken together, our data suggest that the BBB glia and cells of the fat body sense cholesterol availability through a mechanism affected by Npc1 and regulate body growth accordingly.

### Cholesterol affects insulin signaling via glia and fat body relays

We next characterized the effects of cholesterol-trafficking manipulations on insulin expression and release. We asked whether the increased size caused by Npc1 gene knockdown in BBB glia and the fat body was associated with altered insulin signaling. Inducing fat-body intracellular cholesterol accumulation through Npc1a knockdown led to a significant increase in Ilp5 expression as well as strong reductions of ILP2 and ILP5 staining in the IPCs (Figures 3A and 3A), suggesting increased insulin release. Consistent with this and with the observed enhancement of growth (Figures 2D, 2D’, 2F, 2H, 2J, and S2H), we also found strong reductions in the transcript levels of 4EBP, a negative regulator of growth, and Insulin Receptor (InR) (Figure 3A), indicating increased systemic insulin signaling, which suppresses their expression.<sup>55</sup> We therefore examined whole-body expression levels of known fat-derived insulin-regulating factors and found that of those examined, only Ilp6 displayed altered expression with fat-body Npc1a knockdown (Figure S3). Ilp6 expression in the fat body itself is induced by starvation and ecdysone signaling,<sup>51,52</sup> and thus, the observed increase may not necessarily be involved in relaying cholesterol status from the fat body to the IPCs but may rather be affected by downstream events.

Despite the clear effects on growth of BBB-specific knockdown of Npc1a on normal diet (Figures 2C and 2C’), this manipulation did not significantly alter the expression of genes involved in insulin signaling, and we found no significant changes in ILP2 and ILP5 peptide levels in the IPCs (Figures 3B and 3B’). Given the effects of this perturbation on larval size in animals reared on NF medium (Figures 2E, 2G, 2I, and S2G), we therefore asayed effects of Npc1a knockdown in the BBB glial on the NF lower-storer diet to provide a sensitized condition. In these conditions, glial Npc1a knockdown led to decreased expression of 4EBP, indicating an increase in insulin signaling (Figure 3B”). Consistent with increased insulin signaling indicated by reduced 4EBP, we observed increased whole-body pAkt levels in these animals (Figure 3C). Next, we investigated whether cholesterol affected glial calcium signaling, which has previously been linked to mechanisms by which lipids and other nutrients modulate the activity of the IPCs and other neurons.<sup>53–56</sup> Consistent with glial effects of cholesterol on systemic growth, we found that the signal from CaLexA, a time-integrating transcriptional reporter of intracellular calcium concentration, in the BBB increased with cholesterol feeding (Figures 3D–D’). This suggests that cholesterol promotes glial cell activity that relays information to the IPCs to regulate their activity.

### Nutrient-sensing TOR signaling is activated by dietary cholesterol and is essential for mediating the effects of increased intracellular cholesterol in glia and fat tissue on systemic insulin signaling and body growth

The evolutionarily conserved kinase TOR is the main nutrient sensor that couples intracellular levels of nutrients – amino acids in particular – to systemic growth in <i>Drosophila</i>. In the BBB glia and the fat body, the availability of amino acids is sensed by TOR, which, in turn, remotely controls the expression and release of ILPs from the IPCs.<sup>2,12,13</sup> Cholesterol has recently been shown to promote TOR activation in mammalian cell culture.<sup>26</sup> To resolve the mechanism by which cholesterol promotes systemic growth and enhances insulin signaling, we analyzed (1) whether TOR is activated by cholesterol and (2) whether this activation mediates cholesterol’s growth-promoting effects via remote regulation of insulin. The levels of phosphorylated ribosomal protein S6 (pS6), a downstream target
of TOR and a proxy for its kinase activity, were strongly increased in the larval fat body after dietary cholesterol supplementation, indicating that dietary cholesterol promotes TOR activity (Figure 4A). Next, we analyzed whether Npc1a knockdown mimics cholesterol supplementation and increases pS6 in a TOR-dependent manner. Knockdown of Npc1a using two different fat body–specific drivers strongly enhanced pS6 levels in this tissue, an effect that was completely abrogated by simultaneous knockdown of Tor (Figures 4B/B0 and 4C). These results indicate that cholesterol increase, either derived from dietary supplementation or promoted by Npc1a knockdown, drives TOR activity.

We then asked whether the systemic growth-promoting effect of cholesterol accumulation in fat-body and glial cells is mediated by TOR. To investigate whether cholesterol sensing in the fat body controls systemic body growth through TOR, we tested whether TOR acts downstream of Npc1a to regulate size and timing. We found that fat body–specific knockdown of Tor completely abolished the larval overgrowth (Figure 4D) and accelerated development (Figure 4E) caused by Npc1a knockdown, suggesting that cholesterol sensing in the fat body via TOR promotes systemic body growth. We also ruled out any significant contribution of the RNAi lines alone to these effects (Figures S2 C, S2C0, and S2C00). Next, we found that the increased Ilp5 expression and decreased ILP5 peptide levels within the IPCs, which combined suggest increased ILP5 release, observed in animals with fat-body knockdown of Npc1a were completely rescued by knockdown of Tor (Figures 4F and 4G). Consistent with the insulin-secretion phenotype, knockdown of Npc1a in the fat body led to an elevation in
Figure 4. Cholesterol acts through TOR in the BBB and fat body, which upregulate the insulin-signaling system to promote growth

(A) Animals (w^{1118}) raised on NutriFly medium and transferred at 96 h AEL to NF+40 medium exhibit stronger fat-body pS6 staining after 2 h than similar animals transferred onto fresh NF, indicating that dietary cholesterol promotes fat-body TOR activity.

(B and B') Npc1a knockdown in the fat body driven by Cg-GAL4 (B) or by ppl-GAL4 (B') leads to increased pS6 staining, and this increase is blocked when Tor is simultaneously knocked down, indicating that Npc1a regulates TOR signaling.

(C) Illustrative images of these tissues.

(D) As found earlier, knockdown of Npc1a in the fat with ppl> promotes larval growth; knockdown of Tor reduces larval size, and knockdown of Npc1a in the Tor-RNAi background has no effect on larval size, indicating that the growth-promoting effect of Npc1a knockdown is mediated by TOR.

(E) Consistent with their altered larval growth rates, animals with Npc1a knockdown in the fat pupariate earlier than controls, but RNAi against Npc1a has no effect in a Tor-knockdown background.

(F and G) As seen in Figure 3, Npc1a knockdown in the fat leads to increased expression of Ilp5 (F) and to increased release of this peptide (G). Simultaneous knockdown of Tor blocks these effects.

(H and M) Npc1a-RNAi expression in the fat (H) or BBB (M) leads to increased peripheral insulin signaling, as reflected in increased Akt phosphorylation in whole-larval extracts. Note the assembly of nonadjacent lanes in (H).

(I and N) Consistent with these data, knockdown of Npc1a in the fat (I) or BBB (N) leads to increased larval mass in animals with functional IPCs (without LexAop-Kir2.1), but if insulin release is blocked using Kir2.1, manipulating cholesterol signaling in the fat or BBB is unable to drive an increase in size, indicating that insulin signaling mediates these effects.
whole-larval pAkt levels which was counteracted by knockdown of Tor (Figure 4H). These results suggest that TOR couples cholesterol sensing in the fat body to systemic body growth via remote regulation of ILP secretion from the IPCs. To directly test this hypothesis, we knocked Npc1a down in the fat body while silencing the IPCs by expressing the potassium channel Kir.2.1 in these cells under the control of ilp2-LexA.28 As expected, silencing the IPCs to block ILP release completely abrogated the growth increase induced by knockdown of Npc1a in the fat body, indicating that insulin secretion is required for the growth-promoting effects of cholesterol accumulation in the fat body (Figure 4I).

In contrast with the TOR-dependent fat-body mechanism that controls ILP secretion and affects developmental timing, the TOR-dependent glial nutrient-sensing mechanism promotes growth without altering pupariation timing on normal food.13 Consistent with this previous report, we found that Npc1a or Tor loss in glial cells alters larval size but not developmental timing (Figures 4J and 4K). Furthermore, we found that the systemic overgrowth induced by glia-specific knockdown of Npc1a is completely blocked by simultaneous Tor knockdown, suggesting that lysosomal cholesterol abundance, regulated by Npc1a, acts via TOR in the BBB glia to promote systemic growth. Nutrient-dependent glial TOR promotes growth via a neuronal relay mechanism that primarily upregulates Ilp5 expression in the IPCs.13 Consistent with this, we found that on lower-sterol NF medium, loss of Npc1a in the BBB glia upregulated Ilp5 expression in a TOR-dependent manner (Figure 4L), suggesting that cholesterol accumulation in glia activates TOR and that this promotes systemic growth via upregulation of insulin signaling from the IPCs. Furthermore, knockdown of Tor in the BBB glia rescued the increased levels of pAkt caused by glial Npc1a knockdown (Figure 4M), supporting the role of glial Npc1a-TOR in the regulation of systemic insulin signaling to control body growth. To test this, we investigated whether silencing the secretory activity of the IPCs with Kir.2.1 could rescue the overgrowth caused by glial knockdown of Npc1a. Just as it did with fat-body manipulations, silencing the IPCs completely abrogated the growth increase caused by loss of Npc1a in the BBB glia (Figure 4N). These results suggest that Npc1a loss in nutrient-responsive BBB glia leads to increased intracellular cholesterol that activates TOR, which, in turn, remotely controls Ilp5 expression in the IPCs, promoting body growth. Taken together, our findings suggest that loss of Npc1a in BBB glia and the fat body leads to intracellular cholesterol accumulation, which activates TOR and remotely upregulates IPC insulin production and secretion.

To assess the necessity of TOR activity in these tissues for the growth response to dietary cholesterol, we measured the mass of animals that fed on NF medium and NF supplemented with 40 μg/ml, with and without tissue-specific Tor-RNAi. As expected, added dietary cholesterol led to a significant increase in larval weight in both driver controls, an effect that was strongly attenuated when Tor was knocked down in either the fat body or the BBB glia (Figure 4O), suggesting that normal growth responses to dietary cholesterol require TOR activity in both tissues. Thus, Npc1a-regulated TOR signaling in the BBB glia and the fat body couples cholesterol sensing to systemic growth, and cholesterol-enhanced TOR signaling is required in both of these sensor tissues for growth promotion.

Enhanced cholesterol signaling in the PG alters the nutritional checkpoint that prevents the precocious initiation of maturation

Nutrition influences ecdysone production through insulin and PTTH signaling as well as through TOR activity in the PG itself.15,16,34,59,60 The TOR-mediated mechanism in the PG couples nutritional inputs to endocycling, which in turn is linked to CW.61,62 We therefore asked whether cholesterol levels in the PG might affect these events. To observe the influence of cholesterol on TOR signaling and endoreduplication, we used Tub-GAL80TS; phm> (phmTS>) to induce Npc1a-RNAi in the PG in the mid-second-instar stage (L2). This manipulation, which leads to intracellular cholesterol accumulation,33,40 led to a strong increase in pS6 staining (Figure 5A) and a massive increase in endoreduplication, reflected in increased nuclear size (Figure 5B). The specificity of this effect was confirmed by using a second PG-specific GAL4 line, spok>, to drive expression of a second RNAi line targeting Npc1a (Figure S2K). Both the increased pS6-staining and nuclear-size phenotypes of Npc1a knockdown were rescued completely by simultaneous knockdown of Tor (Figures 5A and 5B), suggesting that TOR drives downstream effects on PG cell growth in response to cholesterol. These results also suggest that cholesterol sensing in this tissue is linked to the mechanism by which larvae estimate CW.

Since cholesterol appears to drive endoreduplication, and endoreduplication drives the irreversible activation of the pupariation-inducing neuroendocrine axis at CW,61,62 we investigated whether increasing PG cholesterol by knockdown of Npc1a in this tissue would reduce CW—that is, if it might cause animals to overestimate their overall body size and therefore pupariate at too small a size. We used the drug-inducible phantom-GeneSwitch-GAL4 (phmGS>) driver to induce Npc1a-RNAi in the PG in mid-L2 by treatment with dietary RU486. We measured PG-cell nuclear size in drug-treated animals versus nontreated controls after 2 or 10 h of feeding after the L2-L3 molt, and after similar animals were starved until 36 h post-molt (schematized in Figure 5C). The body growth of animals with PG-specific Npc1a knockdown was attenuated in larvae beginning at around 12–14 h after the L2-to-L3 transition (Figure 5D), consistent with an increase

(J and K) BBB knockdown of Npc1a leads to increased larval size (J), but no effect on pupariation timing was observed (K). Tor knockdown in the BBB blocks the effects of Npc1a knockdown on larval size (J) but has no effect on timing (K).
(L) The upregulation of Ilp5 by BBB Npc1a knockdown is blocked by simultaneous BBB-specific knockdown of Tor.
(O) Tor knockdown in the BBB or the fat body blocks the effects of dietary cholesterol on growth. Statistics: (A), Mann-Whitney pairwise test; (B, B’, and G), Kruskal-Wallis ANOVA with Dunnnett’s multiple comparisons; (D, I, J, and N), two-way ANOVA with Tukey’s multiple comparisons; (F and L), Welch’s pairwise comparison. (D, E, F, I–O) Mean ± SEM. (A, B, B’, and G) Median with 95% confidence interval. Significance is noted as ns, p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. Scale bars in (A) and (C) represent 30 μm.
in ecdysone production, which inhibits systemic growth. In line with this, we found that temporary drug-induced loss of Npc1a expression in the PG led to increased ecdysone levels measured at a later time point (Figure 5E). Npc1a knockdown in the PG led to a large increase in nuclear size at all time points tested (Figure 5F), indicative of increased endoreduplication. Indeed, PG nuclei were of similar sizes in newly molted L3 animals treated with RU486 (condition “C”) and in nondrug-treated controls that were 10 h older (condition “E”), which had already attained CW as demonstrated by their 100% pupariation rate under starvation (Figure 5G).

Control animals starved before 10 h after the L2-L3 transition never pupariated, whereas all controls starved after this time pupariated (Figure 5G), indicating that animals attain CW after ~10 h L3 feeding, at around 0.8 mg per larva, in agreement with previous findings. However, we found that a significant fraction of drug-treated animals (with previous Npc1a knockdown in the PG) were able to pupariate when starved at early time points (Figure 5G), even immediately following the L2-L3 molt (0–2 h), indicating that they had passed CW, consistent with their increased endoreduplication (Figure 5F) and increased later ecdysone production (Figure 5E). Treated animals as small as 0.5 mg pupariated without further feeding, although they never attained a 100% pupariation rate likely because sufficient Npc1a is important later in the L3 for the trafficking of cholesterol for the peak of ecdysone biosynthesis that triggers pupariation. Nonetheless, our combined results show that Npc1a knockdown in the PG strongly increased endoreduplication and permitted inappropriate pupariation, indicating that it causes premature CW attainment. Larvae starved post-CW commence ecdysone production even in starvation conditions. Consistent with the notion that Npc1a knockdown in the PG causes abrogation or precocious attainment of CW as early as 0–2 h after the L2-L3 molt, we found that these animals exhibited increased ecdysone levels when starved beginning even at this early time point (Figure 5E). Together, this shows that increasing PG cholesterol levels and sensing via Npc1a knockdown causes a strong reduction in the body size required to initiate nutrient-independent maturation by abrogation of the nutritional CW checkpoint.

**DISCUSSION**

Nutrition is one of the most important influences on developmental growth and maturation. Malnutrition or disease can impair growth and delay puberty, whereas obese children enter puberty early. Similarly, Drosophila larvae exposed to poor nutrition, tissue damage, or inflammation delay their development, whereas rich conditions promote rapid growth and maturation. These environmental factors are coupled to the appropriate gating of steroid production via internal checkpoints, one of which is a nutrition-dependent CW required to initiate the maturation process. This suggests that signals reflecting nutritional status and body-fat storage play a key role in activating the neuroendocrine pathways that trigger puberty. Although studies suggest that the adipokine leptin may be involved, the mechanisms linking body fat to puberty are poorly defined, and the potential involvement of lifestyles associated with excessive accumulation of cholesterol, one of the most important lipids, has not been considered. In humans, white adipose tissue is the main site of cholesterol storage and can contain over half the body’s total cholesterol in obesity. Our results show that dietary cholesterol intake promotes systemic body growth through insulin-dependent pathways and that animals raised on high dietary cholesterol initiate maturation earlier. We show that cholesterol is sensed through an Npc1-regulated TOR-mediated mechanism in the fat body and the glial cells of the BBB, which relay information to the IPCs within the brain to promote insulin expression and release, thus coupling growth and maturation with cholesterol status (Figure 5H).

Insect CW likely evolved as a mechanism ensuring that maturation will not occur unless the animal has accumulated adequate nutrient stores to survive the nonfeeding metamorphosis period and has completed sufficient growth to produce an adult of proper size and thus of maximal fitness. Likewise, the link between body fat and maturation in humans probably ensures adequate stores of fat before maturation onset to support pregnancy and reproductive success. In Drosophila, insulin signaling plays a critical role in coordinating steroidogenesis with nutritional conditions. Insulin acts upon the PG and induces a small ecdysone peak early in L3 that is correlated with CW
In combination with nutrition-related signaling mediated via insulin, nutrient availability is also assessed directly in the PG and is coupled to irreversible endoreduplication that permits ecdysone production at CW. Our findings show that accumulation of cholesterol in the PG, induced by the loss of Npc1a, drives a remarkable TOR-dependent increase in endoreduplication and leads to inappropriate attainment of CW. Taken together, our findings indicate that cholesterol sensed by the BBB glia and the fat body promotes growth through insulin signaling and that cholesterol sensed by the PG accelerates maturation through ecdysone signaling.

Loss of Npc1 function in humans leads to the Niemann-Pick lysosomal storage disorder, marked by intracellular cholesterol accumulation. Although neurodegeneration is the hallmark of NPC disease, including in a Drosophila model, alterations in glial, adipose, hepatic, and endocrine systems are also components of NPC syndrome. In humans, Npc1 itself is strongly expressed in glia and in adipose tissues, especially in obese individuals, and variants in Npc1 are associated with obesity, type-2 diabetes, and hepatic lipid dysfunction. Our findings link glial and adipose-tissue cholesterol sensing through Npc1 to systemic growth and metabolic control through effects on insulin signaling. We also find that intracellular cholesterol accumulation driven by Npc1 loss leads to hyperactivation of TOR that drives increases in DNA replication and cell growth. TOR activity is also find that intracellular cholesterol accumulation driven by metabolic control through effects on insulin signaling. We show that cholesterol sensing through Npc1 to systemic growth and a range of cancers. 78

As the coupling of nutrition with growth and maturation is ancient and highly conserved, our work provides a foundation for understanding how cholesterol is coupled to developmental growth and maturation initiation in humans. Our findings link a high concentration of this particular lipid in adipose tissues to the neuroendocrine initiation of maturation, which may explain the critical link between obesity (body fat) and early puberty.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.02.021.

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**AUTHOR CONTRIBUTIONS**

M.J.T. and K.R. designed the study and experiments. M.J.T., K.R., L.H.P., T.K., M.L., and A.M. performed experiments and analyzed data. M.J.T. and K.R. wrote the manuscript. K.R. obtained funding.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


## STAR METHODS

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to the lead contact, Prof. Kim Rewitz (kim.rewitz@bio.ku.dk).

**Materials availability**
*Drosophila* stocks and antibodies created for this work are available from the lead contact without restriction upon request. Other materials should be requested from commercial or academic stock centers or from their respective creators, as detailed in the key resources table.

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**Critical commercial assays**

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Data and code availability

- No data fitting standardized datatypes requiring archival in public databases were generated. Numerical data sets have been deposited at Mendeley and were publicly available at the time of submission. The relevant DOI is listed in the key resources table. Image data will be shared by the lead contact on request.
- No original code was created for this work. The MATLAB script for pupal-size analysis has been published previously, but for the reader’s convenience it has been submitted to a repository and was publicly available at the date of submission. The relevant DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila media and husbandry

For general stock-keeping, flies were kept on medium containing 6% sucrose, 0.8% agar, 3.4% yeast, 8.2% cornmeal, 0.16% Te-gosept antifungal, and 0.48% propionic acid preservative. The synthetic diet used in the experiments of Figures 1A and 1B was that described by Reis et al. This medium appears to be nutritionally poor and is very liquid, so the remaining experiments involving

UAS-Ilp2HF, LexAop-Kir2.1 (University of Nice), and available separately as BDSC stock #37516; this and Ptth-GAL4, UAS-Dicer-2

Ilp2 (an Ilp2 variant, #105405, and one from the GD collection, referred to as ‘’Npc1a-RNAiGD’’, #48030), 86 (an Ilp2> variant, #48030), repo-GAL4 46 (#7415), UAS-calcium-LexA>GFP 47,48 (#58768), and UAS-Tor-RNAi 87 (#34639).

The Vienna Drosophila Resource Center (VDRC) provided two UAS-Npc1a-RNAi lines – one from the KK library, not specially noted in the text, #105405, and one from the GD collection, referred to as “Npc1a-RNAiGD” #42782. The Kyoto stock center provided NP0423> (#103614) and NP2276> (#112853). M. B. O’Connor (University of Minnesota) kindly provided phm-GAL4, 84 phm-GeneSwitch-GAL4, and Ptth-GAL4, 42 Ilp2-GAL4, UAS-GFP carries the Ilp2-GAL4 construct described by Rulifson et al., 31 available separately as BDSC stock #37516; this and Ptth-GAL4, UAS-Dicer-2 were gifts from Pierre Léopold (Institut Curie). UAS-Ilp2HF 35 was a kind gift of S. Park and S. Kim (Stanford). Flies carrying Ilp2-LexA:VP16 58 were kindly provided by N. Romero 5 (University of Nice), and LexAop-Kir2.1 85 was a kind gift of L. Vasconcelos (Champlain) and L. Pallanck (University of Washington). To enable discrimination between larval genotypes, third-chromosome balancers were replaced with TM6B, Tb, and animals with second-chromosome balancers were rebalanced over T(2;3)SM6A-TM6B, Tb (from Bloomington #5687), in which lethal mutual transposition between SM6A and TM6B, Tb causes these chromosomes to appear to co-assort. All fly stocks used are listed in the key resources table.

Fly stocks

Stocks obtained from University of Indiana’s Bloomington Drosophila Stock Center (BDSC) include Cg-GAL4 31 (#7011), moody-GAL4 83 (#90883), pumpless (ppl)-GAL4, 17,46 (#58768), R57C10-GAL4 86 (an nSyb-GAL4 variant, #39171, R96A08-GAL4 86 (an Ilp2> variant, #48030), repo-GAL4 46 (#7415), UAS-calcium-LexA>GFP 47 (“CaLexA”, #66542), and UAS-Tor-RNAi 87 (#34639).

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Animal sex, developmental stage, allocation to statistical groups, and ethical statement

No adult animals were analyzed. Mixed-sex batches of larval and pupal animals were analyzed in this work; sexing of larvae and pupae is rarely performed in Drosophila studies. Any sexual dimorphism in size, growth, and physiology is not analyzed as such; rather it contributes to intra-sample variation like any other variable. No ethical statement is required for studies in Drosophila melanogaster.

METHOD DETAILS

Timed egg-lays

Virgin females of the driver stocks were mated with males of the RNAi lines in egg-lay chambers with apple-juice/agar egg-lay plates supplemented with yeast paste. Eggs were laid between ZT 1 and 5 (i.e., 1 h to 5 h after incubator lights-on) at room temperature. For genotypes including Tub-GAL80, eggs were allowed to develop on the plates for 48 h at 18 °C, and first-instar larvae were collected and transferred to food vials (~30 per vial); transferred larvae were incubated a further 48 h at 18 °C to prevent GAL4 activity during early larval development, prior to incubation at 29 °C. Larvae of other genotypes were collected after 24 hours’ development at 25 °C, transferred to vials (~30 per vial), and incubated at 25 °C.
Developmental timing (pupariation timing)
Timed egg-lays of the appropriate genotypes were made, and animals that had begun to pupate were counted every 2–8 h. The salient feature for the purpose of this measurement was the darkening of the pupal cuticle.

Pupal-size measurement
After all animals in a vial had pupariated, animals were rinsed from the vial with deionized water and arranged on a glass microscope slide. Slides were imaged using a USB camera (Point Grey Grasshopper3, FLIR Systems, Inc.) and software (Point Grey FlyCapture, FLIR Systems, Inc.), and pupal sizes were determined from the images using a script in the MATLAB environment (The MathWorks, Inc.).

Quantitative real-time PCR (qPCR)
Six replicate RNA preparations for each genotype or condition were prepared using the NucleoSpin RNA Plus Mini kit (Macherey-Nagel, #740984.50) kit; each sample contained 3-5 whole late feeding third-instar larvae. Animals were placed in 2-mL Eppendorf tubes containing lysis buffer + 1% beta-mercaptoethanol, and samples were lysed using a bead mill (Qiagen). RNA was purified according to the kit instructions, and cDNA was prepared using the High-Capacity cDNA Synthesis Kit (ThermoFisher, #4368814). QPCR reactions were prepared using RealQ Plus 2x Master Mix Green without ROX (Ampliqon, #A323402) and the gene-specific primers given in the STAR Methods, and runs were performed using a QuantStudio 5 machine (Applied Biosystems).

Western blotting
Three to eight feeding late-third-instar larvae for each sample were lysed in ice-cold 3x SDS loading buffer (Bio-Rad) + 5% beta-mercaptoethanol (60 µl per larva) using a bead mill (Qiagen). Samples for phospho-specific staining included protease and phosphatase inhibitor cocktails (Roche Complete Mini protease inhibitor, Sigma #11836153001, and Roche Complete Ultra phosphatase inhibitor, Sigma #05892970001). Samples were heated at 95 °C for 5 min to denature proteins, and insoluble material was pelleted by centrifugation at top speed for 1 min. Proteins were separated on a precast 4%-20% gradient polyacrylamide gel (Bio-Rad) and transferred to PVDF membrane (Millipore) using a Trans-Blot Turbo (Bio-Rad) dry-transfer apparatus. Membranes were blocked in Odyssey blocking buffer (LI-COR) for 2 h at 4 °C with gentle agitation. Phospho-Akt and histone H3 were detected by incubation with rabbit anti-phospho-Akt (in Figure 1, Cell Signaling Technologies, #4054, was used, diluted 1:1000; this antibody was discontinued by the manufacturer, and Cell Signaling Technologies #4060 was used for the other blots, diluted 1:1000) and rabbit anti-histone-H3 (Abcam #1791, diluted 1:1000) in Odyssey blocking buffer + 0.2% Tween 20 (Sigma). Mouse anti-alpha-Tubulin tubulin (Sigma #T5168, diluted 1:4000) was used in other blots. Membranes were washed 3 times in PBS+0.1% Tween 20, and secondary staining was performed with IRDye 680RD-labeled goat anti-mouse and IRDye 800CW goat anti-rabbit (LI-COR, #925-68070 and #925-32210, 1:10,000 dilution in Odyssey blocking buffer + 0.2% Tween 20). Bands were visualized using an Odyssey Fc gel reader (LI-COR). The blot was stripped using NewBlot IR Stripping Buffer (LI-COR, #928-40028) or Alfa Easar buffer (Thermo Scientific, #J60925) at 37 °C for 30 min and rinsed 3x with PBS + 0.1% Tween 20. Total Akt was detected on the stripped blot with rabbit anti-pan-Akt (Cell Signaling Technologies, #4691).

Anti-DILP5 preparation
Antibodies were raised in rats against the peptide CPNGFNSMFAK, an epitope within the B chain of DILP5, and affinity-purified against the peptide by Genosys, Inc.

Immunostaining, microscopy, and quantification
Tissues were dissected in cold PBS and fixed in fresh 4% paraformaldehyde in PBS at room temperature for 1 h. For antibody staining, samples were quickly rinsed in PBST [PBS + 0.1% Triton X-100 (Sigma)], washed three times for 15 min in PBST, and blocked for at least an hour at 4 °C in PBST + 3% normal goat serum (Sigma). Tissues were incubated overnight at 4 °C with gentle agitation with primary antibodies diluted in PBST and washed three times with PBST. Samples were incubated with secondary antibodies diluted in PBST at 4 °C overnight with gentle agitation. If actin staining was desired, samples were incubated with phalloidin (Alexa Fluor 647 conjugate, ThermoFisher #A22287, or Alexa Fluor 488 conjugate, #A12379), diluted 1:100 in PBST, for 1-4 hours. Samples were washed three times with PBST, and tissues mounted on glass slides or glass-bottomed-dishes (MatTek Life Sciences, #P35G-1.5-10-C) coated with poly-lysine (Sigma, P8920-100ML) in ProLong Glass anti-fade mountant containing NucBlue DNA stain (ThermoFisher #P36985). Mounted tissues were imaged using a Zeiss LSM 900 confocal microscope using a 20x objective (NA 0.8).

All samples used for quantification within a figure were dissected, stained, and imaged simultaneously, using the same fixation and staining preparations and identical hardware settings. For quantification of ILP2/ILP5 staining, Z-stacks were collapsed in FIJI (NIH) using the “sum” method, IPC clusters were manually segmented, and the “raw integrated density” measurement was recorded. The region of interest was moved to an adjacent unstained area of tissue, and the “raw integrated density” of this background was measured and subtracted from the first measurement to give the net signal for each cell cluster.

For the BBB CaLexA assay, linear regions of interest were drawn through the glial layer, perpendicular to the brain surface, in the plane of maximum brain size (in which the glial layer is most perpendicular to the image plane), using the line tool in FIJI; the intensity of CaLexA was measured along these lines, and the peak intensity along each line was recorded.
For quantification of fat-body or PG pS6 levels, a large number of samples were arranged on each slide. Areas of flat, single-cell-thick tissue were selected on each major piece of fat body and were imaged with a 20× objective with 1.5 μm Z spacing. Z-stacks were pre-processed in FIJI by collapsing using the “sum” method. Areas of folded or overlaid tissue were manually segmented away. The channels were split, and the DAPI channel made into a binary mask to permit removal of pS6 signal within the nucleus, which is believed to be spurious. Quantification was automated using CellProfiler 3.1.9: each cell’s cytoplasmic pS6 signal was quantified by segmentation using the nuclear mask and the phalloidin channel. The pS6 signal is measured as the average intensity across each segmented cytoplasm.

Primary antibodies included rabbit anti-ILP2, a kind gift of M. Pankratz (U. Bonn) and E. Hafen (ETH Zurich), diluted 1:1000; rabbit anti-ILP3, a generous gift of Jan Veenstra (University of Bordeaux), diluted 1:500; rabbit anti-pS6 (directed against phospho-Ser233/Ser235/Ser239), diluted 1:500; rat anti-ILP5 (this work), diluted 1:500; mouse anti-GFP (clone 3E6, ThermoFisher #A11120, RRID AB_221568), diluted 1:500; and guinea-pig anti-Shroud, a kind gift of R. Niwa (University of Tsukuba), diluted 1:200. Secondary antibodies were all raised in goats and were cross-adsorbed by the manufacturer to reduce off-target binding; they were obtained from ThermoFisher and diluted 1:500. These included anti-rabbit, Alexa Fluor 488 conjugate (#A32731, RRID AB_2735091); and anti-mouse, Alexa Fluor 488 conjugate (#A32735, RRID AB_250882); anti-rat, Alexa Fluor 555 conjugate (#A48263, no RRID); anti-guinea-pig, Alexa Fluor 647 conjugate (#A21450, RRID AB_2735091); and anti-mouse, Alexa Fluor 488 conjugate (#A32723, RRID AB_2633275).

**DILP2HF ELISA**

Ilp2>ΔDILP2HF animals were reared on normal food until 80 h after egg laying. At this time, they were transferred to cholesterol-free synthetic medium overnight (16 h); after this sterol starvation, animals were transferred onto cholesterol-replete synthetic medium, and hemolymph was sampled at 0, 1, and 4 h. Microelictor batches of hemolymph were heat-treated to prevent coagulation and oxidation, and treated material was stored at -80 °C until use. “F8 MaxiSorp Nunc-Immuno modules” (Thermo Scientific #468667) were coated with anti-FLAG by incubating them overnight with 5 μg/mL mouse anti-FLAG (Sigma #F1804) in 200-mM NaHCO₃ buffer (pH 9.4) at 4 °C. Wells were washed twice with PBS+0.1% Triton X-100 (PBST), blocked with PBST+4% non-fat dry milk for 2 h at room temperature, and washed three more times in PBST. Anti-FLAG:peroxidase (Roche #12013819001) was diluted to 25 ng/ml in PBST + 1% non-fat dry milk. Five microliters of hemolymph was added to 50 μL of this solution, and the mixture was incubated in the coated wells overnight at 4 °C. The wells were emptied and washed six times with PBST. The color reaction was started by adding 100 μL of One-step Ultra TMB ELISA substrate (Thermo Scientific #34028) was added to each well (100 μL); after sufficient color development (~1 min), the reaction was stopped with the addition of 100 μL 2-M sulfuric acid. Absorbance at 450 nm was measured using a PerkinElmer EnSight multimode plate reader.

**Critical-weight determination**

Embryos of phm-GeneSwitchGAL4>Npc1a-RNAi were collected on NutriFly egg-lay plates in a series of two-hour egg lays. At 52–54 h AEL (mid-L2), the medium from each time point was cut in half, and the two halves were joined to half-plates of fresh NF medium. The rejoined halves, roughly 15 ml in total, were painted with 500 μL of a yeast suspension (10% w/v dried S. cerevisiae in water) containing either RU486 at 100 μg/mL (from a 50-mg/ml ethanol stock) or the equivalent volume of ethanol alone; the final concentration of drug in diluted plates, assuming even diffusion, was roughly 3 μg/mL. Animals were incubated at 25 °C for a further 12 h, after which freshly molted L3 animals (determined based on anterior spiracle morphology) were harvested by flotation on 20% sucrose and manually sorting every 2 h and transferred into vials containing NutriFly or NutriFly containing 3 μg/ml RU486. At designated time points, animals were re-harvested from these vials by flotation in 20% sucrose. Batches of animals were prepared for PG staining or for critical-weight assays. For PG staining, larvae were grossly dissected in PBS and fixed in fresh 4% paraformaldehyde in water at room temperature for precisely one hour with rotation. Tissue was rinsed with PBS+0.1% Triton X-100 (PBST) three times for 15 min each and blocked with PBST+3% normal goat serum at 4 °C overnight. Tissue was stained with rabbit anti-Phantom (1:400), a kind gift of Michael O’Connor, at 4 °C overnight with gentle agitation, followed by three washes with PBST. Tissue was incubated at 4 °C overnight with goat anti-rabbit Alexa Fluor 555 (ThermoFisher #A32732, RRID AB_2633281, 1:500; Alexa Fluor 488-conjugated phallolidin (ThermoFisher #12379, 1:100), and DAPI (ThermoFisher #D1306, 1:500). Tissue was rinsed three times with PBST, equilibrated in 50% glycerol/water, and mounted on poly-lysine-coated MatTek imaging dishes in 50% glycerol/water. This liquid was withdrawn and replaced with ProLong Glass solidifying anti-fade mountant (ThermoFisher). After the mountant solidified, PGs were imaged as above. Each data point represents the cross-sectional area of a given nucleus at its greatest diameter, determined in FIJI: for each nucleus, the focal section demonstrating the greatest size was found; the nucleus was manually outlined, and its area was measured. For critical-weight determination, reharvested animals were individually weighed using a Sartorius SE2 ultramicrobalance and transferred to starvation medium (0.6% agarose in water). The number of pupariated animals was recorded periodically. Some animals were also harvested from starvation medium at 36 h after the L2/L3 molt and prepared for PG imaging.

**Ecdysone ELISA**

Animals were raised and harvested as described above for critical-weight determination. Three or four sets of roughly ten animals were harvested at each time point, washed in deionized water, dried on a tissue, weighed, and flash-frozen in a 2-ml Eppendorf tube. Animals were lysed in 300 μl cold methanol with a steel bead in a bead mill (Qiagen). After centrifugation at maximum speed for 10 min, 150 μl of supernatant was transferred into each of two Eppendorf tubes, and the samples were evaporated in a vacuum
centrifuge. Residue in one tube was dissolved in 200 μl ELISA buffer, and this buffer was used to dissolve the second batch of residue. The ELISA was performed using competitive ELISA kit (Bertin Bioreagent Cayman, #501390) according to the manufacturer’s instructions, and final signal was read using a PerkinElmer EnSight multimode plate reader. Each larval sample was measured in sextuplicate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics were computed and graphs were prepared in the Prism software package (GraphPad). Data sets were assessed for normality prior to computation of statistics. The central tendency of normally distributed data is indicated as the mean plus/minus the standard error, and non-normally distributed data are summarized as medians plus/minus 95% confidence interval. More-detailed statistical information, including the specific tests used, is given in each figure legend. P values are represented in all figures as follows: ns, p>0.05; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001.