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Graphical Abstract

Highlights

- A resource for identifying regulators of steroidogenesis and developmental timing
- The fatty acid elongase Sit is involved in cholesterol trafficking and steroidogenesis
- Intracellular cholesterol is sequestered by autophagosomes to mobilize cholesterol
- Cholesterol is regulated by TOR and feedback signaling in steroidogenic cells

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In Brief

Steroid hormones play important roles in development and disease. Danielsen et al. show that TOR and steroid feedback signaling regulate cholesterol substrate levels for steroid production through processes involving the fatty acid elongase Sit and autophagy. This reveals mechanisms regulating steroidogenesis during development with implications for certain diseases, including cancers.
A Drosophila Genome-Wide Screen Identifies Regulators of Steroid Hormone Production and Developmental Timing

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SUMMARY

Steroid hormones control important developmental processes and are linked to many diseases. To systematically identify genes and pathways required for steroid production, we performed a Drosophila genome-wide in vivo RNAi screen and identified 1,906 genes with potential roles in steriodogenesis and developmental timing. Here, we use our screen as a resource to identify mechanisms regulating intracellular levels of cholesterol, a substrate for steriodogenesis. We identify a conserved fatty acid elongase that underlies a mechanism that adjusts cholesterol trafficking and steriodogenesis with nutrition and developmental programs. In addition, we demonstrate the existence of an autophagosomal cholesterol mobilization mechanism and show that activation of this system rescues Niemann-Pick type C1 deficiency that causes a disorder characterized by cholesterol accumulation. These cholesterol-trafficking mechanisms are regulated by TOR and feedback signaling that couples steriodogenesis with growth and ensures proper maturation timing. These results reveal genes regulating steriodogenesis during development that likely modulate disease mechanisms.

INTRODUCTION

Steroid hormone signaling controls important biological functions during development and underlies pathologies of many disorders (Rewitz et al., 2013; Risbridger et al., 2010). During postembryonic development the production and release of steroids from endocrine cells are critical for developmental transitions including growth termination and maturation (Colombani et al., 2015; Garelli et al., 2015; Vallejo et al., 2015). Cells responsible for the production of steroids share similar mechanisms by which cholesterol or other sterol precursors are converted into hormones that are released into circulation to regulate a myriad of physiological and developmental processes. Similarly to mammals, the production of steroids in model organisms such as the fruit fly Drosophila melanogaster is mediated by cytochrome P450 enzymes that convert cholesterol or other suitable sterols to active steroids (Carvalho et al., 2010; Huang et al., 2013). The production of steroids is regulated by multiple signals including insulin, target of rapamycin (TOR), Ras/ERK, transforming growth factor β (TGF-β), and feedback control. In Drosophila, the prothoracic gland (PG) produces the steroid hormone ecdysone from cholesterol and integrates different signals to adjust hormone production in response to nutrient conditions and development (Yamanaka et al., 2015). This ensures that growth is coordinated with activation of steroid production that triggers maturation or metamorphosis to form an adult with a characteristic body size. Many of the signaling pathways that regulate cholesterol trafficking and steroid production are evolutionarily conserved, and dysregulation of these pathways is associated with disease. In humans, cholesterol is derived from two sources, dietary uptake and de novo synthesis, with uptake being the primary source of intracellular cholesterol supply in both endocrine cells and many tumors (Miller and Auchus, 2011; Yue et al., 2014). Intracellular trafficking of low-density lipoprotein (LDL)-derived cholesterol from receptor-mediated endocytosis accounts for ~80% of the cholesterol in mammalian cells. Similarly, Drosophila acquires cholesterol by an LDL-related mechanism, since it lacks the ability to synthesize sterols (Carvalho et al., 2010; Huang et al., 2008). Intracellular trafficking of LDL-derived cholesterol provides a substrate for steroid synthesis. Genetic studies have
shown that trafficking involves Niemann-Pick type C1 (NPC1) disease-associated genes that promote mobilization of LDL-derived cholesterol from late endosomes (Schwend et al., 2011). Mutations that disrupt NPC1 genes cause fatal lipid-storage disease, characterized by accumulation of cholesterol and other lipids in late endosomes/lysosomes, for which there is no cure. Loss of Npc1a, the Drosophila NPC1 homolog, causes insufficient cholesterol delivery to support ecdysone production (Huang et al., 2005). In steroid-related cancers such as prostate cancer, loss of the tumor suppressor PTEN and the subsequent upregulation of the PI3K/AKT/TOR pathway increases cholesterol uptake, leading to an accumulation of cholesterol that drives cancer progression (Yue et al., 2014). Furthermore, studies in mice and Drosophila have linked metabolic disorders and disruption of the conserved insulin-like system to altered steroid signaling and delayed onset of maturation and reproduction (Colombani et al., 2005; Daftary and Gore, 2005; Tennessen and Thummel, 2011).

Our current understanding of steroidogenesis is largely based on cell-culture models, which have limitations since cell lines are unlikely to fully recapitulate physiological processes that occur in endocrine cells in vivo. Therefore, identifying such mechanisms is key to a better understanding of developmental processes and the mechanisms that underlie steroid-related disease. Here, we present a genome-wide in vivo RNAi screen in Drosophila to systematically uncover genes important for steroidogenic tissue function. We identify stuck in traffic (sit), a homolog of a fatty acid elongase linked to prostate cancer, and show that sit is important for cholesterol trafficking in steroidogenic cells. Knockdown of sit results in accumulation of cholesterol-rich lipid droplets, likely due to impaired sphingolipid synthesis, which blocks cholesterol delivery and reduces steroid production. In addition, our data identify an autophagic cholesterol-trafficking system, and we show that inhibition of autophagy leads to accumulation of cholesterol-rich lipid droplets in the PG. We further provide evidence that TOR signaling and steroid feedback coordinate cholesterol uptake and trafficking in PG cells. Our characterization of genes and mechanisms regulating cholesterol levels in endocrine cells provides insight into how steroidogenesis is controlled in a developmental context during the juvenile-adult transition, and molecular clues concerning mechanisms underlying certain cancers and lipid-storage disorders.

RESULTS

A Genome-wide In Vivo RNAi Screen for Genes Involved in Steroidogenesis in Drosophila

To systematically identify genes required for endocrine steroidogenic cell function, we performed a genome-wide in vivo RNAi screen in the steroid-producing PG of Drosophila. For this purpose, we used the Drosophila RNAi library (Dietzl et al., 2007) to reduce expression of 12,504 individual genes (~90% of the protein-coding genes [Matthews et al., 2015]) specifically in the PG cells. The phm-Gal4 (phm->) driver (Ono et al., 2006) was crossed to UAS-controlled transgenic RNAi lines to direct tissue-specific silencing in the PG (Figure 1A). Ecdysone is required for developmental transitions between larval stages, the onset (pupariation), and the completion of metamorphosis. Therefore, impaired production and release of this steroid from the PG causes a gradient of phenotypes ranging from simple delayed development and overgrowth to a more severe developmental arrest at different larval instar stages (Danielsen et al., 2014; Enya et al., 2014; Layalle et al., 2008; Rewitz et al., 2009). Based on developmental defects (Table S1) that range from arrest in the first (L1), second (L2), and third (L3) larval instar to developmental delay (~1 day [minor delay]; ~2 days [delay]; ~3 days [major delay]), we identified 1,906 (15.2%) candidate genes, of which 1,289 have human homologs. Additionally we screened for premature entry into metamorphosis after the second larval instar (L2 prepupa: L2P) and for lethality during the pupal stage (P lethal) (Figures 1B and 1C). The arrest in the different developmental stages likely reflects a failure to produce an ecdysone pulse required to trigger entry into the next stage. Gene hits associated with arrest in L1 or L2, the strongest phenotypes, include genes directly involved in the ecdysone biosynthetic pathway (shroud, phantom, disembodied, shadow, and Cyp6t3). The screen also identified genes associated with cholesterol trafficking (Npc1a, Gste14/Nobo, and snmp1), genes in major signaling pathways such as insulin/TOR (InR, Akt1, raptor, and Tif-1A), PTTH/Torso/Ras (torso and Ras85D) and TGF-β (put and tkv), and transcription factors (vvl, kni, mdl, ouib, br, E75B, EcR, and USP) that are known to regulate ecdysone production in the PG (Caceres et al., 2011; Colombani et al., 2005; Danielsen et al., 2014; Gibbens et al., 2011; Huang et al., 2005; Komura-Kawa et al., 2015; Koyama et al., 2014; Layalle et al., 2008; Mirth et al., 2005; Moeller et al., 2013; Niwa et al., 2010; Niwa and Niwa, 2014; Ou et al., 2011; Rewitz et al., 2009; Talamillo et al., 2013; Zhou et al., 2004). This shows that our screen was successful in identifying genes with known steroidogenic roles, and an additional ~1,800 genes that have not been linked to steroidogenesis, steroidogenic cell function, or general gland viability; indeed, many of these genes have no identified function.

To identify biological processes important for steroidogenic cell activity, we performed functional gene ontology (GO)-term enrichment analysis of the gene hits identified in our screen. We found significant enrichment for multiple cellular processes, such as structure-related processes, cell communication, transport/migration, translation, and cell cycle/apoptosis (Figures 1D and S1; Table S2). Intriguingly, our analysis also revealed significant enrichment of gene functions related to lipid metabolism. Furthermore, many of the most highly expressed genes in the Drosophila ring gland, an endocrine organ largely composed of the PG cells, are related to lipid metabolism (Ou et al., 2016), suggesting a specific role of these genes in steroidogenesis. These include genes involved in uptake and transport of lipids, and regulation of lipid synthesis and modification (Npc1a, S2P, Hmgr, Hmgs, Gste14/Nobo, cubeball, Fatp, and CG5278). Strikingly, reduced expression of these genes in the PG, except for Npc1a and cubeball, results in similar developmental delay phenotypes (Table S1). Lipids are components of cell membranes and control important cellular processes (Wymann and Schneider, 2008), yet their roles in regulating steroidogenesis are largely unknown. We therefore further investigated CG5278/sit, which encodes an uncharacterized fatty acid elongase homolog.
Loss of the Fatty Acid Elongase Homolog \textit{sit} Causes Accumulation of Cholesterol and Impairs Steroidogenic Activity

\textit{sit} encodes a homolog of the human fatty acid elongases \textit{ELOVL1/7} (Figures 2A and S2A), which are specifically linked to breast and prostate cancer (Hilvo et al., 2011; Tamura et al., 2009; Tolkach et al., 2015). ELOVL enzymes are involved in the rate-limiting step in the elongation of very long-chain fatty acids. Expression of \textit{sit} is high in the \textit{Drosophila} ring gland compared with other tissues, suggesting a specific role in steroidogenesis (Figures 2B and S2B). Silencing of \textit{sit} in the PG resulted in a developmental delay, incomplete pupariation, and overgrowth (Figures 2C, 2D, and S2C), consistent with a failure in the production and/or release of ecdysone from the PG that triggers pupariation (Colombani et al., 2012; Rewitz et al., 2009). To confirm that \textit{sit} is required for steroidogenic activity, we measured ecdysone levels in animals with reduced expression of \textit{sit} in the PG. Indeed, reduced expression of \textit{sit} in the PG caused low levels of ecdysone during the L3 stage (Figure 2E). We next generated two mutant lines carrying a deletion of the \textit{sit} coding sequence (Figures S2D and S2E). Animals homozygous for either of two different \textit{sit} deletions (\textit{sit\textsuperscript{D1}} and \textit{sit\textsuperscript{C2}}) or a heteroallelic combination (\textit{sit\textsuperscript{D1/C2}}) showed developmental lethality with the majority of animals dying in larval stages (Figure S2F).

Given that \textit{sit} encodes a fatty acid elongase homolog, we next sought to detect abnormalities in lipid metabolism using labeling-free coherent anti-Stokes Raman scattering (CARS) microscopy, which detects lipids (Nan et al., 2003). Surprisingly, we observed a significant accumulation of lipid droplets in \textit{sit}-deficient PG cells (Figures 2F, S2G, and S2H). By contrast, we found no obvious change in fat body lipid droplets of the \textit{sit} mutants (Figure S2I), consistent with \textit{sit} expression being PG specific, indicating that its primary role is related to endocrine steroidogenic cell function. Steroidogenic cells have a high demand for cholesterol as it is a precursor for steroid synthesis, and intracellular cholesterol typically accumulates in lipid droplets (Miller and Bose, 2011). To examine whether the lipid droplets in the PG detected by CARS contain cholesterol, we used an ex vivo uptake assay with a fluorescent cholesterol analog, NBD-cholesterol (Gimpl, 2010), which showed that lipid droplets detected by CARS in PG cells are cholesterol rich (Figure 2G). Dysfunction of \textit{NPC1} genes is known to cause accumulation of free unesterified cholesterol (Karten et al., 2009). Consistent with this, we observed a strong accumulation of lipid droplets in \textit{Npc1a}-deficient PG cells, similar to the effects caused by loss of \textit{sit} (Figures 2F and S2G). Together, these data suggest that loss of \textit{sit} function is associated with intracellular accumulation of cholesterol-rich droplets, similar to what occurs in \textit{Npc1a}-deficient cells. Next, we conditionally induced global RNAi-mediated knockdown of \textit{sit} and \textit{Npc1a} during the L2 stage to avoid the early developmental arrest caused by early reduction in the expression of these genes, and then measured cholesterol and...
cholesterol ester levels in the L3 larvae. Global reduction of sit or Npc1a indeed resulted in accumulation of free unesterified cholesterol (Figures 2H and S2J).

To investigate whether loss of sit function results in a block of cholesterol delivery for the steroidogenic pathway, we examined the dependence on dietary cholesterol of the PG-specific sit-RNAi phenotype. Previously, it has been shown that the cholesterol limitation that causes the ecdysone deficiency of Npc1a and Gste14/Nobo-deficient PG cells can be rescued by increasing dietary cholesterol (Danielsen et al., 2014; Enya et al., 2014). We confirmed that increasing dietary cholesterol rescues the Npc1a loss-of-function phenotype (Figure S3A). Increasing dietary cholesterol concentrations completely rescued the developmental delay caused by reduced sit expression in the PG cells, indicating that cholesterol is limiting in these cells (Figure S3B). An ex vivo uptake assay confirmed that PG cells lacking sit accumulate NBD-cholesterol (Figure S3C). Taken together, these data suggest that loss of sit causes reduction of intracellular cholesterol transport. Since loss of Npc1a and sit produce similar phenotypes suggestive of altered cholesterol transport, we asked whether overexpression of sit is sufficient to rescue the cholesterol-trafficking defect in Npc1a-deficient cells. Knockdown of Npc1a in the PG causes L1 arrest. Remarkably, we found that overexpression of sit rescues the phenotype caused by lack of Npc1a in the PG (Figure 2I), suggesting that these genes act in an interconnected cholesterol-trafficking pathway, and we therefore named this gene stuck in traffic (sit).

Figure 2. CG5278/sit Is a Conserved Fatty Acid Elongase Homolog Important for Steroid Production that Affects Cholesterol Levels
(A) Sit encodes a protein composed of 295 amino acids (aa) containing a conserved ELO domain similar to the human ELOVL7 fatty acid elongase protein.
(B–D) (B) Expression of sit is high in the ring gland (RG) compared with whole body (WB) 96 hr after egg laying (AEL). Knockdown of sit expression specifically in the PG cells results in (C) delayed and impaired pupariation and (D) an increased pupal size.
(E) RNAi knockdown of sit in PG cells reduces ecdysone levels in the larvae during the L3 stage.
(F) Lipid droplets detected by CARS microscopy in PG cells of L3 larvae with PG-specific RNAi silencing and in sit1D/2C mutants. Scale bars, 10 μm.
(G) Co-localization of lipid droplets detected by CARS microscopy with NBD-cholesterol in the PG of animals with PG-specific silencing of sit. Scale bars, 5 μm.
(H) L3 larvae with ubiquitous RNAi-mediated silencing of Npc1a or sit contain higher levels of cholesterol. The RNAi effect was conditionally induced in L2 larvae 96 hr AEL by shifting larvae from 18°C to 29°C and assayed 2 days later.
(I) Overexpression of an HA-tagged sit (sit-HA) in the PG rescues the developmental arrest phenotype caused by knockdown of Npc1a. Day numbers refer to days AEL. For a detailed description of genotypes see Supplemental Experimental Procedures. 20E, 20-hydroxyecdysone.

Error bars indicate SEM. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figures S2 and S3.
Reduction of sit Phenocopies Reduction in Sphingolipid Levels

We next considered the mechanism that could contribute to the cholesterol-rich droplet accumulation in sit-deficient PG cells. Abnormalities in sphingolipid metabolism are a common feature of many lysosomal storage diseases, including NPC1 disease, and interaction between sphingolipids and cholesterol likely contributes to the pathogenesis (Vanier, 2015). Fatty acid elongases, like Sit, provide the long-chain fatty acid substrate for synthesis of the ceramide precursors for sphingolipids, which are essential structural components of cell membranes (Sassa and Kihara, 2014). The accumulation of cholesterol in sit-deficient cells might therefore be linked to aberrant sphingolipid synthesis. To investigate this possibility, we depleted sphingolipids in the PG by silencing schlank, a gene encoding the single ceramide synthase in Drosophila required for sphingolipid synthesis (Bauer et al., 2009). In our screen, PG-specific loss of schlank was found to cause a phenotype similar to the knockdown of sit, supporting a functional relation between sit and schlank (Table S1). We also observed accumulation of lipid droplets and NBD-cholesterol in schlank-deficient PG cells (Figures 2F, S3C, and S3D). This suggests that sphingolipid depletion causes a developmental phenotype and accumulation of cholesterol similar to the loss of sit, making it possible that Sit-dependent sphingolipid synthesis plays a major role in regulating the trafficking of cholesterol. To investigate whether knockdown of sit results in alterations of ceramide levels, we performed an analysis of ceramides using liquid chromatography–mass spectrometry. Importantly, this confirmed that knockdown of sit causes a reduction in specific ceramide species (Figure S3E), consistent with the notion that its function is required for production of some sphingolipids.

Cholesterol is trafficked through the endosomal-lysosomal pathway. To further examine the effect of sit on cholesterol trafficking, we used a ubiquitously expressed GFP-LAMP marker (Pulipparacharuvil et al., 2005). GFP-LAMP is trafficked through the endosomal-lysosomal pathway where GFP is degraded, but the GFP signal accumulates when this pathway is blocked. Silencing of sit led to a strong accumulation of GFP-LAMP in the PG, indicating a trafficking defect in sit-deficient cells (Figure S3F). To further investigate the effect of sit knockdown on the endosomal pathway, we expressed Rab7-GFP, a late endosomal marker, in combination with sit-RNAi. Loss of sit caused enlargement of Rab7 vesicles in the PG compared with the control, consistent with a defect in the endosomal pathway (Figures S3G and S3H). We also found oversized Rab7 vesicles and accumulation of GFP-LAMP in schlank-deficient PG cells, further supporting the idea that the cholesterol-trafficking defect in cells lacking sit is linked to aberrant sphingolipid synthesis (Figures S3F–S3H).

Cholesterol Uptake and Trafficking Are Correlated with TOR and Ecdysone Signaling

Since steroids are synthesized preferentially from cholesterol, we hypothesized that change in nutritional and developmental cues that control the steroidogenic activity of the PG regulates cholesterol uptake and transport in these endocrine cells to coordinate cholesterol availability with steroidogenic activity. To test this, we determined the expression of sit in dissected ring glands at different time points during the L3 stage. We found a dramatic downregulation of sit mRNA levels during the non-feeding wandering stage 120 hr after egg laying (AEL) (Figure 3A). We therefore asked whether insulin and TOR, the two major nutrient-sensing and growth-promoting signaling pathways that affect ecdysone biosynthesis in the PG (Colombani et al., 2005; Layalle et al., 2008), regulate sit expression. Activating the TOR pathway in the PG by overexpression of Rheb upregulated sit mRNA and protein levels (Figures 3B and 3C). In contrast, activation of insulin signaling by overexpression of the insulin receptor (inR) in the PG had little effect on sit expression, suggesting that sit is regulated preferentially by the TOR pathway.

The decline in sit coincides with the elevated ecdysone signaling during the non-feeding wandering stage that triggers pupariation. Previously, we reported that ecdysone production in the PG is regulated by feedback through the ecdysone receptor (EcR) in the PG during this stage (Moeller et al., 2013). We therefore asked whether EcR acts as a negative regulator of sit and is involved in inhibition of cholesterol uptake and trafficking in the PG during the non-feeding stage. Consistent with this idea, we found a dramatic increase in sit mRNA levels when EcR signaling was blocked in the PG by expression of a dominant-negative form of EcR (EcRDN) (Figures 3C and 3D). Together, these observations suggest that TOR signaling upregulates sit expression during feeding stages in response to nutrient intake, while EcR is a negative regulator of sit, responsible for its downregulation during the non-feeding wandering stage.

Given the strong influence of TOR and EcR on Sit, we investigated whether TOR and ecdysone signaling coordinate cholesterol uptake and transport in the PG by analyzing the expression of genes involved in cholesterol uptake and trafficking. Interestingly, our data show that TOR and also insulin signaling stimulate Npc1a expression in the ring gland, while having no effects on LpR1 and LpR2, which encode LDL-like receptors important for the uptake of neutral lipids such as cholesterol (Parra-Peralbo and Culi, 2011) (Figure 3B). In contrast, we found that EcR is a strong negative regulator of Npc1a, as well as LpR1 and LpR2 (Figure 3D). We next investigated the effects of TOR and EcR signaling on LpR2 protein levels in the PG. While LpR2 localize mostly to the plasma membrane in control PG cells, cells with reduced EcR signaling display increased LpR2 staining as was as in the cytoplasm (Figure 3E). Together, these results suggest that TOR and EcR signaling regulate cholesterol trafficking in opposite directions.

Cholesterol Accumulation Is Driven by TOR and Inhibited by Ecdysone Signaling

Consistent with our data indicating that TOR promotes cholesterol uptake and trafficking, we found that suppressing TOR activity through expression of TSC1 and TSC2 (TSC1/2) (Layalle et al., 2008) in the PG leads to a complete lack of lipid droplets (Figures 4A and S4A). In contrast, we found that inhibition of ecdysone signaling, either by expression of EcRDN or by silencing using EcR-RNAi, caused a strong increase in the number of lipid droplets in the PG, indicating that EcR suppresses cholesterol uptake and trafficking (Figures 4A and S4B). To further determine whether loss of EcR function is sufficient to drive cholesterol influx, we analyzed ex vivo uptake of NBD-cholesterol and found accumulation of NBD-cholesterol in the

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PG when EcR signaling was repressed (Figure 4B). We therefore rationalized that loss of EcR would enhance uptake and delivery of cholesterol and consequently increase ecdysone production in the PG under conditions with high cholesterol. As expected, we found that increasing dietary cholesterol concentrations led to a strong (~15 hr) acceleration of pupariation in animals expressing EcRDN in the PG compared with the control under these conditions (Figure 4C). Animals with reduced EcR signaling in the PG pupariate prematurely ~110 hr AEL on a high cholesterol diet, which shortens the larval growth period and causes ~50% reduction in pupal size (Figure 4D). These data suggest that inhibition of EcR signaling enhances the ability of the PG cells to take up and deliver cholesterol, which provides excess substrate that increases steroidogenesis and causes premature
pupariation. Altogether, these results suggest an essential role for TOR and steroid signaling in the coordination of cellular cholesterol accumulation and mobilization whereby TOR activity promotes cholesterol uptake and trafficking, while EcR activity suppresses it in the PG.

**Cholesterol Trafficking Involves Autophagy and Depends on Nutrient Availability**

Our data show that inhibition of TOR in L2 results in an almost complete lack of lipid droplets in the PG of L3 larvae, which indicates that inhibition of TOR may also promote a process that degrades cholesterol-rich lipid droplets. TOR is a negative regulator of a conserved process known as macroautophagy (hereafter referred to as autophagy), which is an intracellular degradation pathway for cytoplasmic components. Defective autophagy has been associated with NPC1 disease (Sarkar et al., 2013), indicating that autophagy could play a specific role in regulating cholesterol trafficking. First, we asked whether TOR affects autophagy in the PG by analyzing mCherry-positive puncta in larvae expressing UAS-mCherry-Atg8a, a tagged Atg8a protein that labels autophagic vesicles (Chang and Neufeld, 2009). Activation of TOR by overexpression of Rheb decreased Atg8a puncta in the PG, confirming that TOR is a repressor of autophagy (Figures 4E and 4F). To test whether autophagy plays a role in regulating cholesterol trafficking in the PG, we analyzed lipid droplet numbers in Atg8a mutant PG cells as well as in those where essential autophagy genes Atg1, Atg7, and Atg8a were knocked down. Inhibition of autophagy was sufficient to cause massive accumulation of lipid droplets in the PG (Figures 5A and 5B). This suggests that autophagy plays a specific role in
the breakdown of cholesterol-rich droplets in the PG. To test directly whether autophagic vesicles sequester cholesterol-rich droplets, we incubated larval PG cells expressing UAS-mCherry-Atg8a with NBD-cholesterol. Co-localization shows that Atg8a vesicles sequester NBD-cholesterol (Figure 5C), suggesting that autophagy contributes to the mobilization of cholesterol from lipid droplets in the PG.

Because autophagy is a cellular response to starvation, we further investigated whether cholesterol uptake and metabolism is adjusted according to nutrient intake in steroidogenic cells. We examined whether genes involved in cholesterol uptake and trafficking are regulated in response to starvation. Expression of sit was reduced after starvation (Figure 5D). Furthermore, starvation also decreased expression of LpR1 and LpR2. When we examined Sit and LpR2 protein levels by western blotting, we found that both proteins were also reduced in response to starvation (Figure 5E). Taken together, these results indicate that the endocrine cells of the PG coordinate cholesterol uptake, transport, and mobilization in response to nutritional cues to adjust ecdysone production to environmental conditions.

Inhibition of TOR and Induction of Autophagy Provides Rescue of a Drosophila Model of NPC1 Disease

To assess whether TOR inhibition is a potential rational approach to target the deleterious accumulation of cholesterol underlying the pathogenic effects of Npc1a deficiency, we expressed
TSC1/2 in Npc1a-deficient PG cells using the P0206-Gal4 (P0206>) line that drives weaker expression in the PG (Layalle et al., 2008) compared with phm>.

The dramatic lipid accumulation in Npc1a-deficient PG cells was suppressed by TSC1/2 overexpression that inhibits TOR (Figures 6A and S4C). Furthermore, we asked whether activation of autophagic degradation would be a means to rescue the impaired cholesterol metabolism associated with loss of NPC1 activity. Remarkably, we found that ectopic expression of Atg1 and Atg13 (Atg1/13), which is sufficient to induce autophagy (Scott et al., 2007), rescues the phenotype associated with loss of Npc1a in the PG (Figures 6B and 6C). Thus, our data suggest that the inhibition of TOR signaling and the induction of autophagy may form the basis for future strategies aimed at treating NPC1 disease.

DISCUSSION

Here we report a genome-wide in vivo RNAi screen in a Drosophila model, which allows systematic dissection of the genes and pathways that regulate the production of steroids in endocrine cells during development. Importantly, some of the genes that we identified as important for steroidogenesis had no known function until now, but have human homologs that have been associated with diseases in which steroid signaling and cholesterol transport are dysregulated. Our data thus have the potential to uncover genes that play important roles in regulating steroidogenesis during development, which also have general relevance for diseases including some of the most common cancers and NPC1 disease. This is highlighted by our discovery and characterization of sit, an uncharacterized gene (CG5278) encoding a fatty acid elongase homolog. Our data show that sit is involved in a mechanism that controls cellular uptake and trafficking of cholesterol in the PG to produce the steroid pulse that triggers maturation in Drosophila. Elevated expression of ELOVL7, a human homolog of sit, is associated with steroidogenic tissues and prostate cancer progression (Tamura et al., 2009), yet the molecular basis for this relationship remains unclear. Prostate cancer cells acquire the ability to enhance cholesterol uptake, potentially making the cancers more aggressive, but the underlying molecular basis is poorly understood (Peck and Schulze, 2014; Yue et al., 2014). Our data suggest that Sit may play a role in this process.

The exact mechanism by which cholesterol exits endosomes after uptake and moves to other organelles is largely unknown. However, the sterol-sensing NPC proteins have been demonstrated to play a crucial role in this process, and are required for trafficking of cholesterol (Huang et al., 2008; Vanier, 2015). We observed that loss of sit in the PG resulted in lipid droplet accumulation that mimicked the loss of Npc1a function. Furthermore, the loss of sit was accompanied by accumulation of LAMP-GFP and enlarged endosomal vesicles, indicating that Sit is required for vesicle trafficking in the endosomal-lysosomal
pathway. Studies in yeast support our finding that very long-chain fatty acids are required for proper late endosomal trafficking (Obara et al., 2013), but leave open the question as to how silencing of sit leads to endosomal vesicle-trafficking defects and cholesterol accumulation. Our observation that knockdown of sit affects ceramide levels and is phenocopied by silencing of the ceramide synthase schlank, together with the fact that most long-chain fatty acids are found as constituents of sphingolipids (Sassa and Kihara, 2014), suggests that membrane sphingolipid composition is important for endosomal trafficking and movement of cholesterol between organelles, perhaps through alterations of membrane fusion dynamics. Consistent with this view, ceramide stimulates NPC-mediated cholesterol transfer (Abdul-Hammed et al., 2010) and we find that reduced Npc1a function is rescued by overexpression of sit, which suggests a close relationship between Npc1a and sit in cholesterol trafficking. Given that sit is highly expressed in PG cells, which have a high demand for cholesterol, we propose that this fatty acid elongase homolog is required for the trafficking of cholesterol, and thereby provides a molecular context for understanding the association between the dysregulation of its human homolog and certain cancers.

We have previously shown that EcR-mediated feedback control of ecdysone biosynthesis is critical for pupal development in Drosophila (Moeller et al., 2013). Our data show that expression of sit is repressed by EcR, which reduces cellular uptake of cholesterol in the PG. Why does EcR mediate a negative feedback that blocks cholesterol uptake? The most likely explanation is that blocking cholesterol accumulation is required as part of an efficient negative feedback circuit in coordination with downregulation of the ecdysone biosynthetic pathway to generate the temporal steroid pulse that drives developmental progression. Under this view, intracellular cholesterol homeostasis is under tight feedback regulation to control steroid production. Alterations in such feedback mechanisms may cause reprogramming of cholesterol metabolism that allows cells to evade cellular cholesterol homeostatic control in certain cancers. In mammals, cholesterol levels are regulated by liver X receptor (LXR), an ortholog of EcR that protects cells from cholesterol overload (King-Jones and Thummel, 2005; Zhao and Dahlman-Wright, 2010). Our studies suggest that EcR deficiency strongly enhances cholesterol influx, which indicates that EcR is required for homeostatic control to prevent cholesterol overload similar to LXR.

Our data suggest that uptake and trafficking of cholesterol require low EcR signaling in the presence of TOR activity, a condition that occurs during the feeding stage. Previous work has shown that EcR and TOR influence ecdysone biosynthesis in the PG (Layalle et al., 2008; Moeller et al., 2013). Thus, our results suggest that these signaling pathways adjust the uptake and trafficking of cholesterol with dietary intake and developmental cues, thereby coordinating substrate delivery with activity of the ecdysone biosynthetic pathway. According to this view, TOR promotes gland growth and ensures cholesterol uptake during the feeding stage, while EcR represses it during the non-feeding wandering stage. Interestingly, induction of ELOVL7 is mediated by mammalian TOR activity (Purdy et al., 2015), which suggests that the TOR pathway has a conserved role in regulating ELOVL genes and synthesis of long-chain fatty acids that promote the uptake of cholesterol. Altogether, our findings indicate that TOR and EcR are key regulatory mediators of distinct programs that couple intracellular cholesterol homeostasis with steriodogenic activity, nutrition, and developmental progression.

Recent evidence indicates a link between vesicle-trafficking defects in lysosomal storage diseases and abnormalities in autophagy that may contribute to the accumulation of intracellular cholesterol (Walkley and Vanier, 2009). We demonstrate the existence of an autophagosomal mechanism through which cholesterol is trafficked. We find that autophagic Atg8a vesicles sequester cholesterol-rich lipid droplets, indicating a critical function of autophagy in cholesterol metabolism. Our study illustrates that TOR and EcR function as a regulatory switch that adjusts cholesterol uptake and trafficking to nutrient intake and steroid levels (Figure 6D). Based on our observations, we propose that TOR and EcR together regulate cholesterol uptake/trafficking and mobilization/breakdown, the two main processes that determine intracellular cholesterol levels. Our study suggests that these signaling pathways regulate the uptake and trafficking of cholesterol through the endosomal pathway. On the other hand, cholesterol-rich lipid droplets are sequestered by autophagosomes and degraded in a process regulated by TOR to control intracellular cholesterol mobilization in response to the availability of nutrients and cholesterol.

Defective autophagy is associated with many neurodegenerative diseases, including NPC1 disease, which is characterized by excessive cholesterol accumulation (Nixon, 2013; Sarkar et al., 2013). Because cholesterol accumulation underlies the pathology of the disease, we asked whether we could rescue cholesterol accumulation and restore dysfunction in NPC1-deficient cells. Interestingly, inhibition of TOR suppresses cholesterol accumulation caused by Npc1a deficiency in the PG cells. A recent paper has provided initial evidence of a link between NPC1 deficiency and impaired autophagy (Sarkar et al., 2013). Our findings indeed suggest that stimulating autophagy rescues the phenotype associated with Npc1a loss, raising the possibility of targeting TOR and autophagy as a strategy for the treatment of NPC1 disease.

**EXPERIMENTAL PROCEDURES**

**Drosophila Stocks and Transgenes**

Drosophila larvae were raised on standard cornmeal food (Nutri-Fly, Bloomington Formulation) under a 12:12-h light/dark cycle, 50%–70% humidity, and 25°C unless otherwise stated. Transgenic RNAi fly lines for the genome-wide in vivo RNAi screen were obtained from the Vienna Drosophila RNAi Center (VDRC). The RNAi screen was performed using the KK collection (phiC31 inserted at a specific AttP site). In cases where it was not possible to obtain KK lines, GD lines (randomly integrated) were used instead to obtain the best coverage of the genome (Dietzl et al., 2007). Additional stocks used in the study include: tub-Gal80, UAS-CD8-GFP (UAS-GFP), UAS-Rab7-GFP, UAS-EcRZOX from the Bloomington Drosophila Stock Center; tub-GFP-LAMP (Axbor et al., 2009); UAS-TSC1, UAS-TSC2 (UAS-TSC1/2) (Tapon et al., 2001); UAS-Rheb (Patel et al., 2003); UAS-InR29.4 (Mirth et al., 2005); Act-Gal4;Gal80D, a generous gift from Stephen Cohen; P0206-Gal4 (Colombani et al., 2005); Atg8aKO276P, a generous gift from Thomas Neufeld; phm-Gal4 (Ono et al., 2006); v1770 VDRC #60000; UAS-Npc1a-RNAi #24782 VDRC; UAS-Atg1-RNAi #34340 Transgenic RNAi project (TRIP) (Vl et al., 2011), UAS-Atg7-RNAi #2770 TRIP, and UAS-Atg8a-RNAi #26732 TRIP. The sit deletion mutants were generated with two guide RNAs (5′-GTTGGGAG
CAAGAGTCCAA CG-3' and 5'-GTCGACGGAGGAACGAGCA-G3' designed to eliminate the entire coding sequence of CG5278 except the last few nucleotides by using the CRISPR/Cas9 system (Kondo and Ueda, 2013). Oligonucleotides containing these CRISPR target sites were cloned into pBpV-U6.28 vector as described previously. The resulting vector was injected into y<sup>2</sup> ch<sup>1</sup>o<sup>2</sup> v<sup>2</sup> , attP40(nos-Cas9)/CyO embryos by BestGene; the progeny were screened for deletions by genomic PCR using the primers CG5278-forward, 5'-TGGAAAGTGGTGTGCAGCAG-3' and CG5278-reverse, 5'-TTGGTCTCACATGAGTGGTGTCTACC-3', and sequenced. For generation of a UAS-sit-HA line, first the sit (CG5278) coding sequence together with a 3' hemagglutinin tag in the C-terminal end, before the stop codon, was obtained from GeneArt Strings DNA Fragments (LifeTechnologies) and cloned into the pUAS-sit-HA plasmid was sequenced and injected by BestGene into embryos.

**Genome-wide In Vivo RNAi Screen**
Six virgins of phm-Gal4 (phm-Gal4) 3–4 days after eclosion were crossed with four transgenic UAS-RNAi flies from the VDRC collection and allowed to lay eggs on standard food in 25-mm vials for 24 hr. Phenotypes of F<sub>1</sub> offspring were scored at days 11–13 for developmental delay or arrest. Two replicates were collected from each RNAi cross. Each batch of 100 genetic crosses included an isogenic control phm-> (phm- crossed to w<sup>1118B</sup>; the genetic background for the RNAi library from VDRC).

**Imaging of Lipid Droplets Using Coherent Anti-Stokes Raman Scattering Microscopy**
CARSI imaging was performed using a Leica TCS SP8 system with a CARSI laser, picoEmerald (OPA, >600 mW at 780 nm/940 nm, pulse width 5–6 ps, 80 MHz; pump, >750 mW at 1,064 nm, pulse length 7 ps, 80 MHz) and the LAS AF/X software. The lasers were adapted to the symmetrical C-H stretch range by tuning the pump beam to <614 nm while keeping the Stokes beam constant at 1,064.6 nm. The output of both lasers was set to 1.3 W and the scan speed to 400 Hz. Only signals from the epi-CARS and epi-SHG (second harmonic generation) detectors were collected. Images were processed using ImageJ (NIH), and quantification of lipid droplets was performed with CellProfiler (Jones et al., 2008) and manual counting.

**Statistical Analysis**
Error bars in figures indicate SEM, and statistical differences between datasets were calculated using a two-tailed Student’s t-test.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devel.2016.05.015.

**AUTHOR CONTRIBUTIONS**

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