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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 steroidogenesis and developmental timing. Here, we use our screen as a resource to identify mechanisms regulating intracellular levels of cholesterol, a substrate for steroidogenesis. We identify a conserved fatty acid elongase that underlies a mechanism which adjusts cholesterol trafficking and steroidogenesis with nutrition and developmental programs. Additionally, 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

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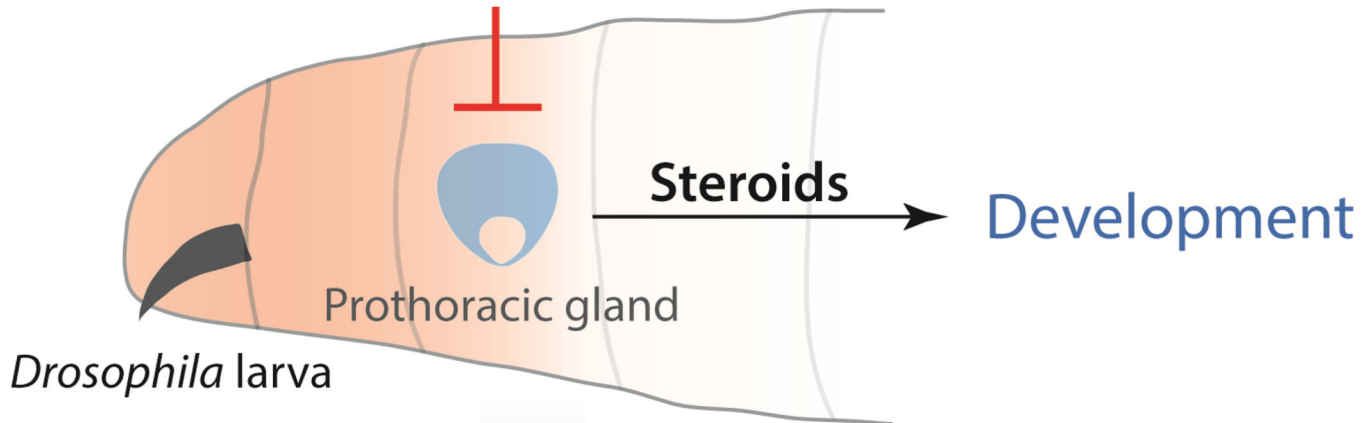
Author Contribution

E.T.D., M.E.M., N.Y., K.K., M.B.O. and K.F.R. conceived the research. E.T.D., M.E.M., N.Y., Q.O., C.S., R.Z., B.P., K.T., J.Z., S.K., C.H.N., E.H., N.J.F., M.J.H., K.A.O., K.K., M.B.O. and K.F.R. performed the experiments. E.T.D., M.E.M., N.Y., Q.O., C.S., C.H.N., E.H., N.J.F., K.K., M.B.O. and K.F.R. analyzed the data. E.T.D., M.E.M., N.Y., N.J.F., K.K., M.B.O. and K.F.R. wrote the manuscript.

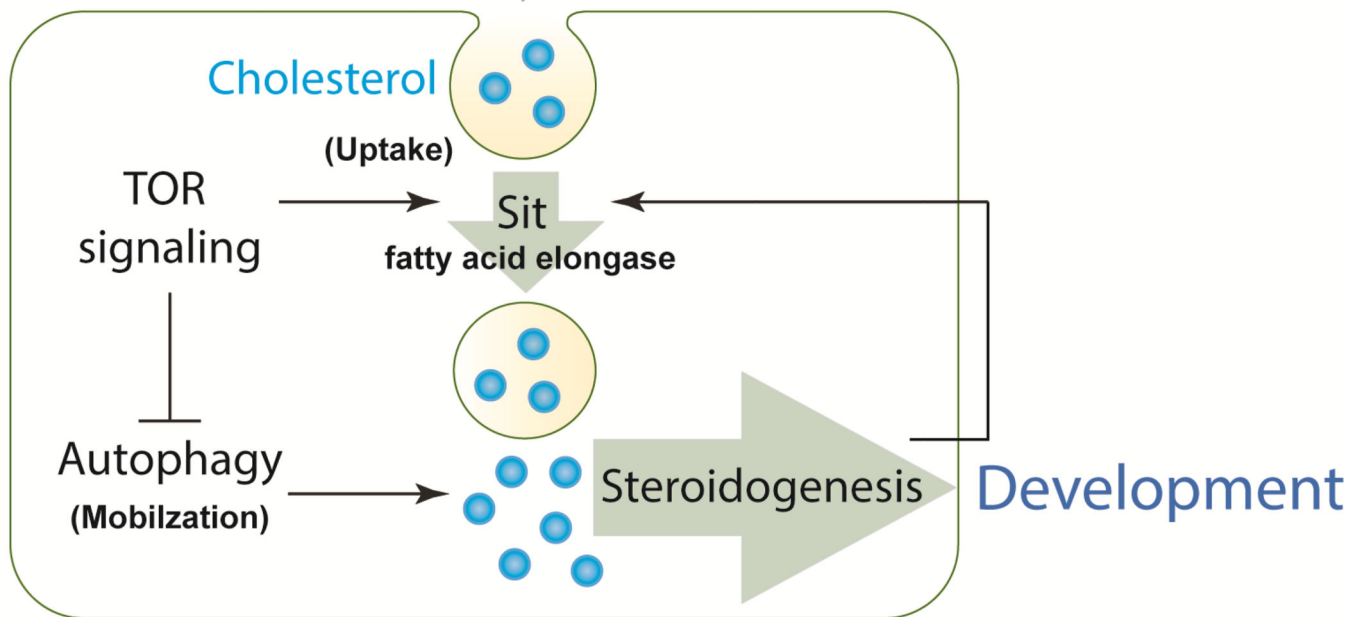
by TOR and feedback signaling that couples steroidogenesis with growth and ensure proper maturation timing. These results reveal genes regulating steroidogenesis during development that likely modulate disease mechanisms.

Graphical Abstract

Genome-wide RNAi screen



Genes and pathways regulating cholesterol trafficking and steroidogenesis



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. Similar 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; Lavrynenko et al., 2015; Rewitz et al., 2013). The production of steroids is regulated by multiple signals including insulin, TOR, Ras/ERK, 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., 2013). 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 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 disorders, 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 it

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. Additionally, 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 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) (Figure 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*, *Tif-1A*), PTTH/Torso/Ras (*torso*, *Ras85D*) and TGF β (*put* and *tkv*), and transcription factors (*vv1*, *kni*, *mld*, *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 either

steroidogenesis, steroidogenic cell function or general gland viability; indeed, many of these genes have no identified function.

In order 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 (Figure 1D, S1 and 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 comprised 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*, *Hmgcr*, *Hmgs*, *GstE14/Nobo*, *cueball*, *Fatp* and *CG5278*). Strikingly, reduced expression of these genes in the PG, except for *Npc1a* and *cueball*, 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/stuck in traffic (sit)*, which encodes an uncharacterized fatty acid elongase homolog.

Loss of the fatty acid elongase homolog *sit* causes accumulation of cholesterol and impairs steroidogenic activity

sit encodes a homolog of the human fatty acid elongases *ELOVL1/7* (Figure 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 *sit* is high in the *Drosophila* ring gland compared to other tissues, suggesting a specific role in steroidogenesis (Figure 2B and S2B). Silencing of *sit* in the PG resulted in a developmental delay, incomplete pupariation and overgrowth (Figure 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 *sit* is required for steroidogenic activity, we measured ecdysone levels in animals with reduced expression of *sit* in the PG. Indeed, reduced expression of *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 *sit* coding sequence (Figure S2D and S2E). Animals homozygous for either of two different *sit* deletions (*sit^{D1}* and *sit^{C2}*) or a heteroallelic combination (*sit^{D1/C2}*) showed developmental lethality with the majority of animals dying in larval stages (Figure S2F).

Given that *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 that detects lipids (Nan et al., 2003). Surprisingly, we observed a significant accumulation of lipid droplets in *sit*-deficient PG cells (Figure 2F, S2G and S2H). By contrast, we found no obvious change in fat body lipid droplets of the *sit* mutants (Figure S2I), consistent with *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 *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 *Npc1a*-deficient PG cells, similar to the effects caused by loss of *sit* (Figure 2F and S2G). Together this suggests that loss-of-*sit* function is associated with intracellular accumulation of cholesterol-rich droplets, similar to what occurs in *Npc1a*-deficient cells. Next, we conditionally induced global RNAi-mediated knockdown of *sit* and *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 (Figure 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 if 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*).

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 is 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 (Figure 2F and 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 trafficking of cholesterol. To investigate whether knockdown of *sit* results in alterations of ceramide levels, we performed an analysis of ceramides using LC-MS. 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 (Pulipparacharuvi 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 to the control, consistent with a defect in endosomal pathway (Figure 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 (Figure 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 regulate 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 hours 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 (Figure 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 delivery for steroidogenesis 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* (*EcR^{DN}*) (Figure 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 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 and also 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 (Figure 4A and S4A). In contrast, we found that inhibition of ecdysone signaling, either by expression of *EcR^{DN}* 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 (Figure 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 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 hours) acceleration of pupariation in animals expressing *EcR^{DN}* in the PG compared to the control under these conditions (Figure 4C). Animals with reduced EcR signaling in the PG pupariate prematurely ~110 hours 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 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 (Figure 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 (Figure 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>)* lines that drives weaker expression in the PG (Layalle et al., 2008) compared to *phm>*. The dramatic lipid accumulation in *Npc1a*-deficient PG cells was suppressed by *TSC1/2* overexpression that inhibits TOR (Figure 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 (Figure 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 where 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 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 endosome 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 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 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 both 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 mTOR 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 uptake of cholesterol. Altogether, our findings indicate that TOR and EcR are key regulatory mediators of distinct programs that couple intracellular cholesterol homeostasis with steroidogenic 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 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 availability of nutrients and cholesterol.

Defective autophagy is associated with many neurodegenerative diseases, including NPC1 disease that is characterized by excessive cholesterol accumulation (Nixon, 2013; Sarkar et al., 2013). Because cholesterol accumulation is underlying 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 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 hour 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 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 as to get the best coverage of the genome (Dietzl et al., 2007). Additional stocks used in the study include: *tub-Gal80^S*, *UAS-CD8-GFP (UAS-GFP)*, *UAS-Rab7-GFP*, *UAS-EcR^{DN}* from the Bloomington *Drosophila* Stock Center (BDSC); *tub-GFP-LAMP* (Akbar et al., 2009); *UAS-TSC1*, *UAS-TSC2 (UAS-TSC1/2)* (Tapon et al., 2001); *UAS-Rheb* (Patel et al., 2003); *UAS-InR^{29.4} (UAS-InR)* (Mirth et al., 2005); *Act-Gal4;Gal80^S* a generous gift from Stephen Cohen; *P0206-Gal4* (Colombani et al., 2005); *Atg8a^{KG0769}* a generous gift from Thomas Neufeld; *phm-Gal4* (Ono et al., 2006); *w¹¹¹⁸* VDRC #60000; *UAS-Npc1a-RNAi* #42782 VDRC; *UAS-Atg1-RNAi* #34340 Transgenic RNAi project (TRIP) (Ni et al., 2011), *UAS-Atg7-RNAi* #27707 TRIP, *UAS-Atg8a-RNAi* #26732 TRIP. The *sit* deletion mutants were generated with two guide RNAs (5'-GTGGGAGCAAGAGTCCAACG-3' and 5'-GCTGCAGCAGGAGAAGCAGA-3') 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 pBFv-U6.2B vector as described previously. The resulting vector was injected into *y² cho² v¹; attP40(nos-Cas9)/CyO* embryos by BestGene Inc (Chino Hills, CA) and the progeny were screened for deletions by genomic PCR using following primers: *CG5278*-forward, 5'-TCGCAAAACTCGTTTCGTTGCGACG-3' and *CG5278*-reverse, 5'-TGGCTCTCAGTGGGTGTTTCTACC-3' and sequenced. To generate a *UAS-sit-HA* line, first the *sit* (*CG5278*) coding sequence together with a 3×HA tag in the C-terminal end, before the stop codon, was obtained from GeneArt Strings DNA Fragments (LifeTechnologies) and cloned into the *pUAST* vector at the *EcoRI* site using In-Fusion cloning (Clontech). The cloned *pUAS-sit-HA* plasmid was sequenced and injected by BestGene into embryos for generation of transgenic *UAS-sit-HA* animals. To generate a transgene carrying a *sit-venus*, the CRISPR/Cas9 system was used to knock-in a *venus* tag on the endogenous *sit* genomic locus. The *venus* was placed immediately downstream of the final coding codon if *sit* by

homologous recombination, to generate a locus encoding a *sit* with a C-terminal *venus*. Details of the targeting vector will be described elsewhere.

Genome-wide *in vivo* RNAi screen

Six virgins of *phm-Gal4* (*phm>*) 3–4 days after eclosion were crossed with four transgenic *UAS-RNAi* males from the VDRC collection and allowed to lay eggs on standard food in 25 mm vials for 24 hours. Phenotypes of F1 offspring were scored at day 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 *w¹¹¹⁸*; the genetic background for the RNAi library from VDRC).

Imaging of lipid droplets using Coherent Anti-Stokes Raman Scattering (CARS) microscopy

Coherent Anti-Stokes Raman Scattering (CARS) imaging was performed using a Leica TCS SP8 system with a CARS laser, picoEmerald (OPO, > 600 mW @ 780 nm to 940 nm, pulse width 5 to 6 ps, 80 MHz; Pump, > 750 mW @ 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 816.4 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 signal from the epi-CARS (E-CARS) and epi-SHG (E-SHG) detectors were collected. Images were processed using ImageJ (NIH), and quantification of lipid droplets were done with CellProfiler (Jones et al., 2008) and manual counting.

Statistical Analysis

Error bars indicate standard error of the mean (SEM) and statistical difference between datasets were calculated using two-tailed Student's t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.

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Highlights

- A resource for identifying regulators of steroidogenesis and developmental timing
- The FA 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

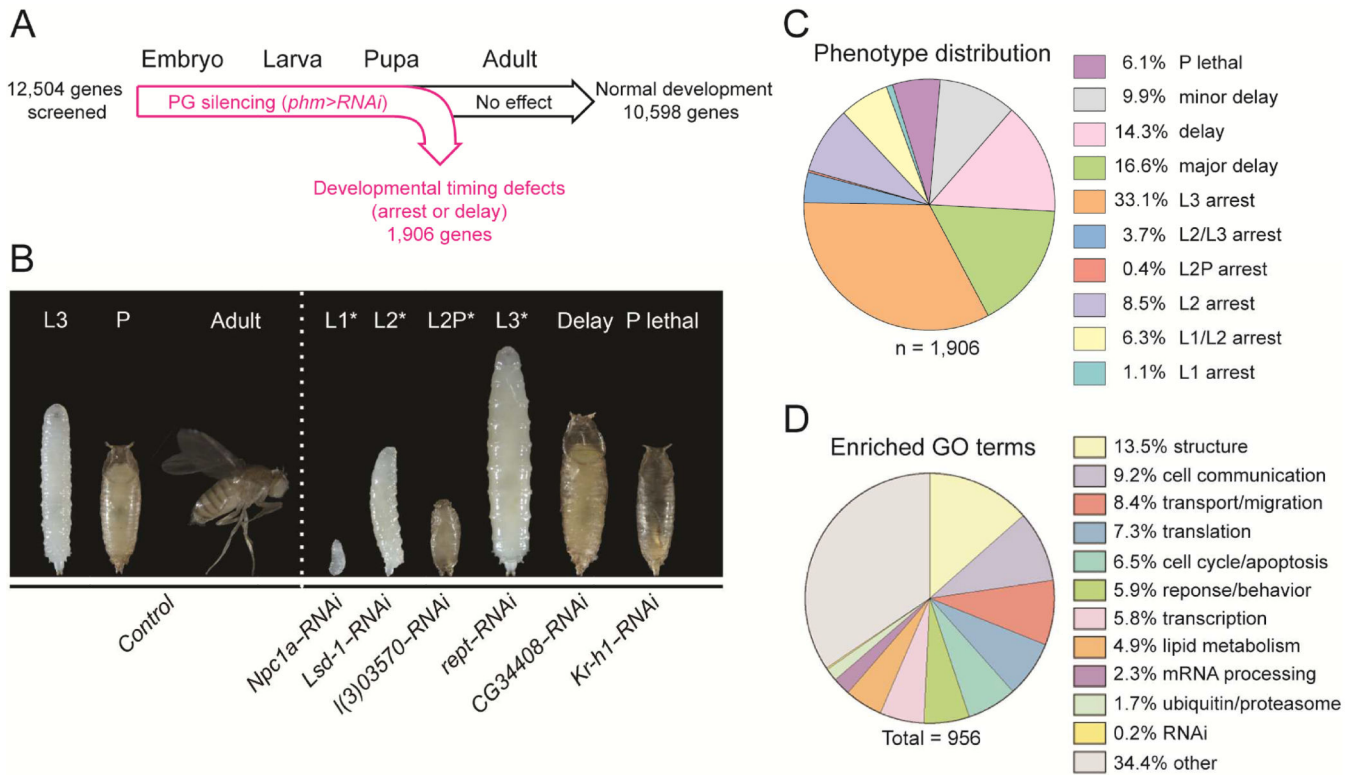


Figure 1. Genome-wide *in vivo* screen for genes involved in steroid hormone production in *Drosophila*

(A) Scheme of the screen design depicting the procedure for prothoracic gland (PG) specific RNAi mediated gene silencing. Virgin females of the PG-specific *phm>* driver line were crossed to a library of transgenic *UAS-RNAi* males to specifically reduce expression of genes in the PG. In total 12,504 RNAi lines, each targeting individual genes, were used. (B) Results from the screen reveal 1,906 candidate genes causing developmental defects including arrest in L1 (L1*), arrest in L2 (L2*), pupariation of L2 larvae (L2P*), arrest in L3 (L3*), developmental delays (delay) and pupal lethality (P lethal), indicating that the genes are important for steroidogenic tissue function. (C) Diagram showing the distribution of the phenotypic categories. (D) Gene ontology (GO) analysis of the gene set showing the top enriched functional categories. Genes were grouped into common functional categories based on GO terms from both *Drosophila* genes and their human orthologs. Numbers indicate total number of GO terms. See also Figure S1, Table S1 and S2.

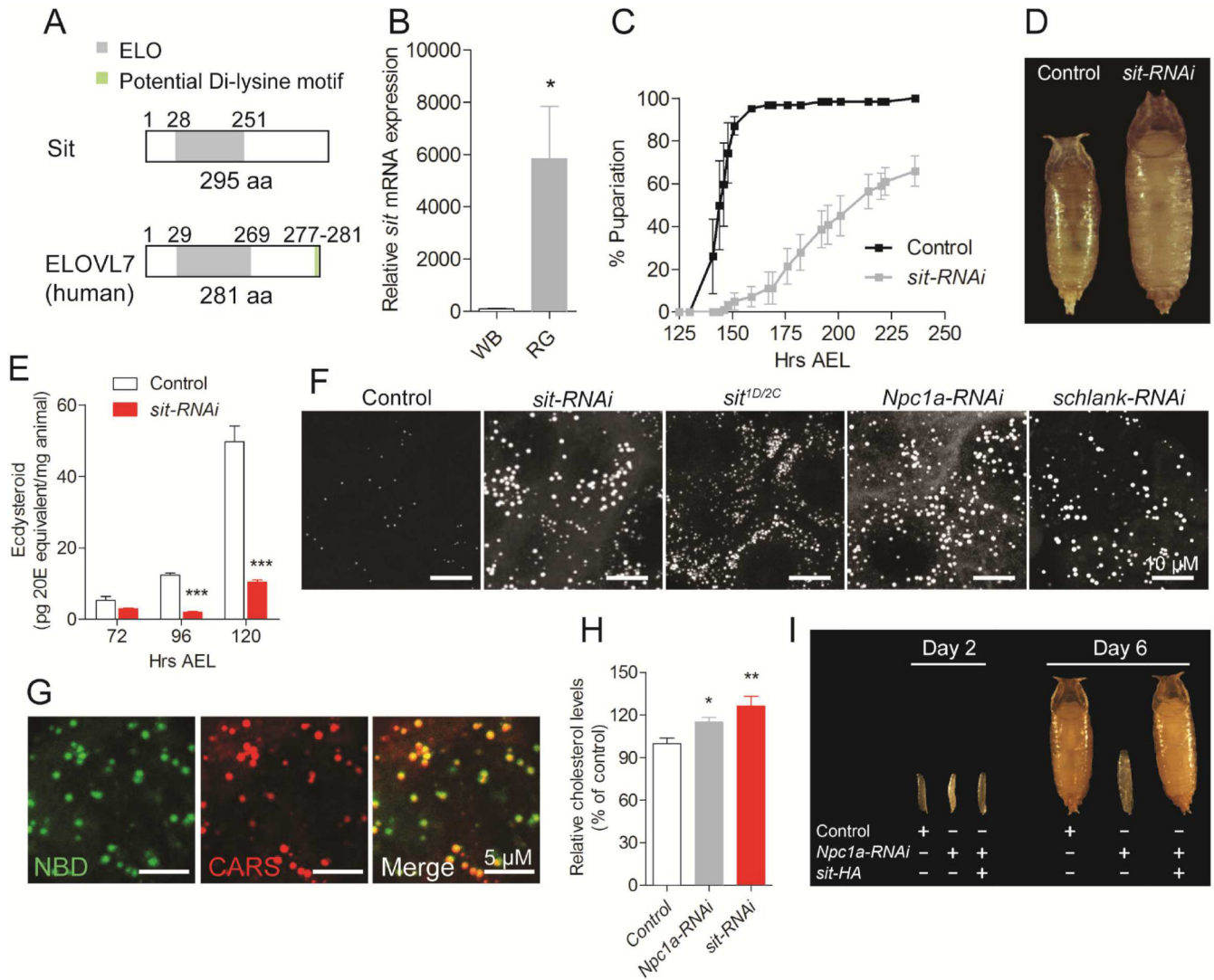


Figure 2. CG5278/stuck in traffic (*sit*) is a conserved fatty acid elongase homolog important for steroid production that affects cholesterol levels

(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) Expression of *sit* is high in the ring gland (RG) compared to whole body (WB) 96 hours after egg laying (AEL). Knock down of *sit* expression specifically in the PG cells results in (C) delayed and impaired pupariation and (D) an increased pupal size. (E) RNAi knock down 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 *sit*^{1D/2C} mutants. (G) Co-localization of lipid droplets detected by CARS microscopy with NBD-cholesterol in the PG of animals with PG-specific silencing of *sit*. (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 hours AEL by shifting larvae from 18°C to 29°C and assayed two days later. (I) Overexpression of an HA-tagged *sit* (*sit*-HA) in the PG rescues the developmental arrest phenotype caused by knock down of *Npc1a*. Day

numbers refer to day AEL. Detailed description of genotypes is given in Supplemental Experimental Procedures. 20E; 20-hydroxyecdysone. Error bars indicate SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). See also Figure S2 and S3.

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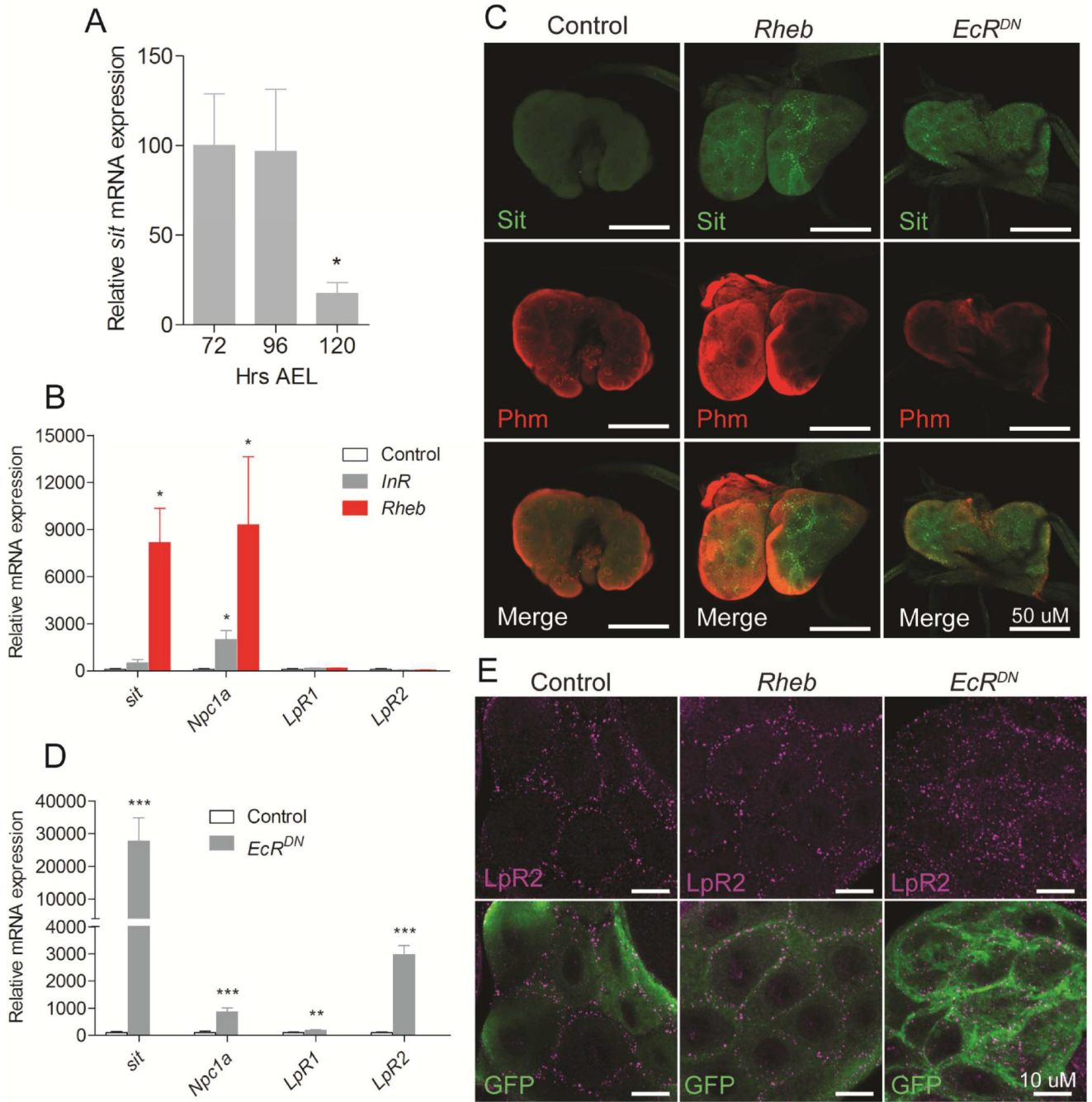


Figure 3. TOR and ecdysone signaling affect cholesterol transport mechanisms in steroid producing tissue

(A) Expression of *sit* in the ring gland decreases from the L3 feeding stages (72 and 96 hours AEL) to the late L3 wandering stage (120 hours AEL). (B) Effect on genes involved in cholesterol trafficking in ring glands with activated TOR signaling by overexpression of *Rheb* or activated insulin signaling by overexpression of the insulin receptor (*InR*) in the PG. (C) Immunolocalization of Sit using a CRISPR/Cas9 generated knock in of a Venus tag on the endogenous genomic *sit* locus (Sit-Venus). Detection of the ecdysone biosynthetic enzyme Phantom (Phm) using anti-Phm (red) and Sit-Venus using anti-GFP shows that Sit

protein levels (green) increases in the PG cells of the ring glands when *Rheb* or *EcR^{DN}* are overexpressed. (D) Effect on genes involved in cholesterol trafficking in ring glands with inhibition of ecdysone feedback regulation by overexpression of a dominant negative form of *EcR* (*EcR^{DN}*) in the PG. (E) LpR2 is localized at the cell membrane in both control PG cells and upon *Rheb* overexpression, while expression *EcR^{DN}* results in increased and scattered LpR2 distribution throughout cytosol. Detailed description of genotypes is given in Supplemental Experimental Procedures. Error bars indicate SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

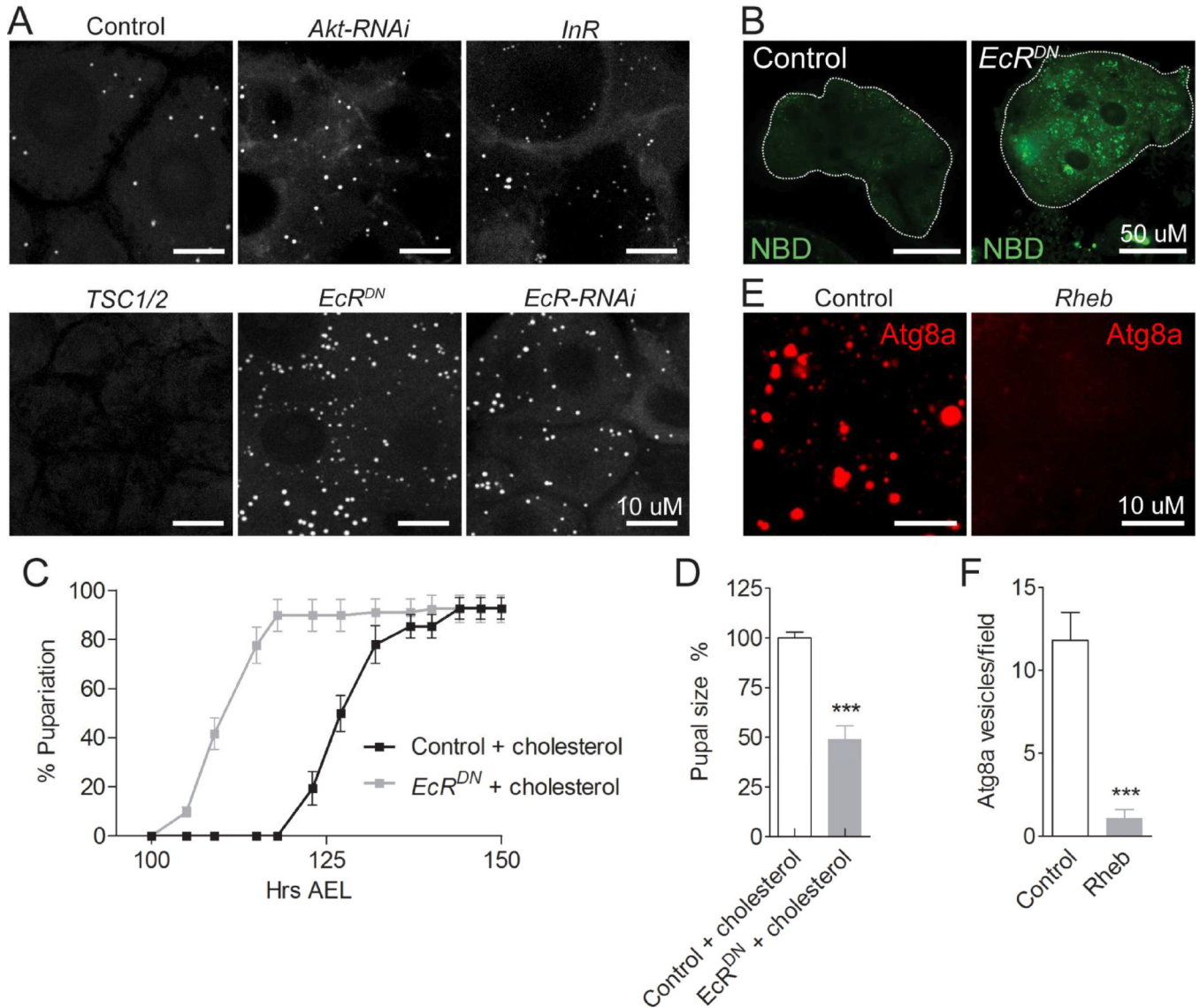


Figure 4. Oposing effects of TOR and EcR on cholesterol and lipid accumulation

(A) CARS images of lipid droplets in PG cells with inhibition of TOR (expression of *TSC1/2*) or ecdysone feedback (expression of *EcR^{DN}* or *EcR-RNAi*), or activation (expression of *InR*) or inhibition (*Akt-RNAi*) of insulin signaling. To allow development to L3, overexpression of *TSC1/2* was induced 96 hours AEL by shifting L2 larvae from 18°C to 29°C and PG cells were imaged two days later. For all other genotypes larvae were reared at 25°C and the PG was assayed 120 hours AEL. (B) *Ex vivo* incubation assay reveals that inhibition of ecdysone feedback by expression of *EcR^{DN}* leads to excessive NBD-cholesterol accumulation in PG cells. Developmental timing of pupariation (C) and pupal size (D) of animals overexpressing *EcR^{DN}* in the PG compared to control animals on a high cholesterol diet (+ cholesterol). (E, F) Effect of TOR on autophagy (Atg8a punctae) in the PG. Detailed description of genotypes is given in Supplemental Experimental Procedures. Error bars indicate SEM (** $P < 0.01$; *** $P < 0.001$). See also Figure S4.

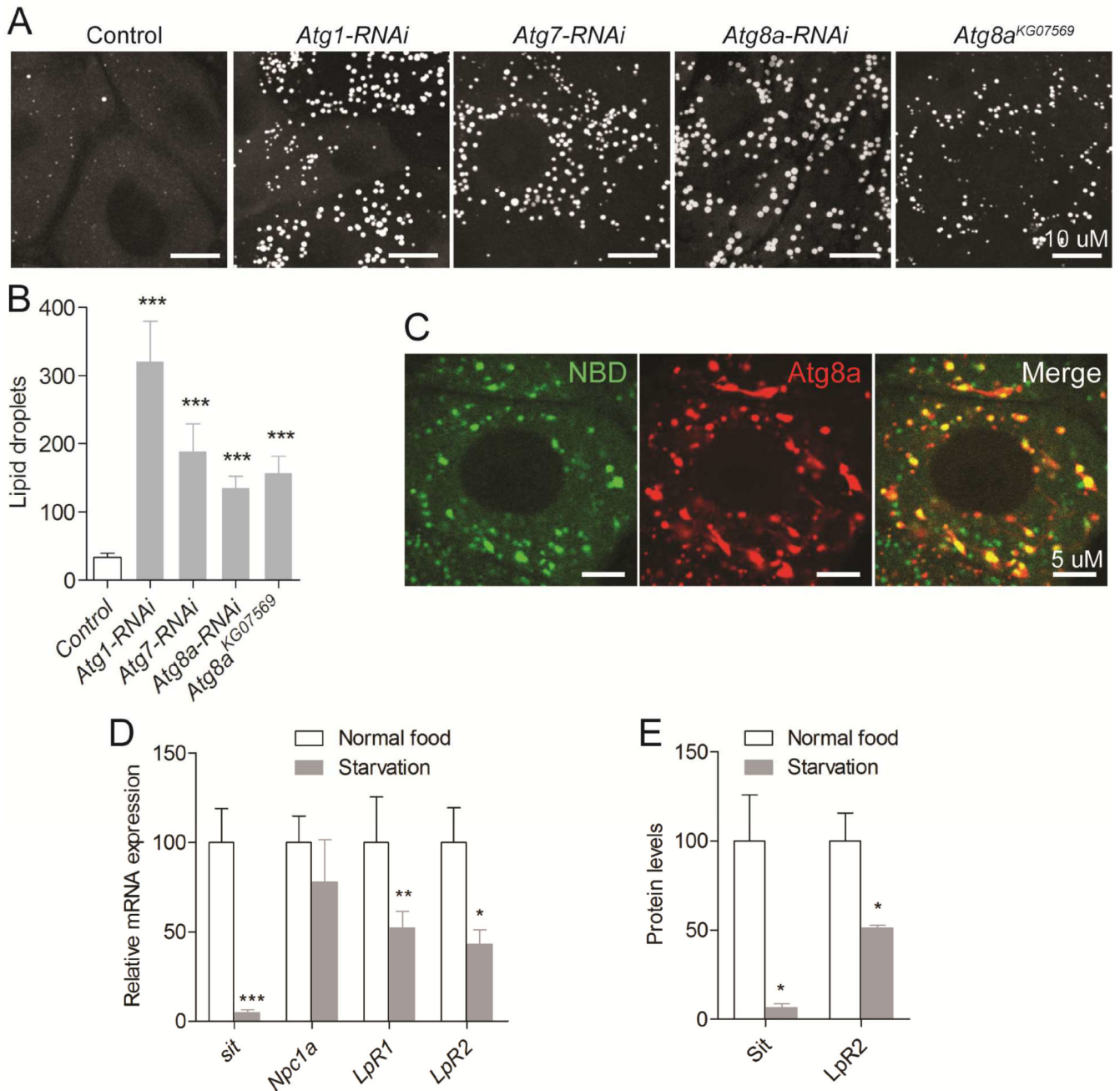


Figure 5. Inhibition of autophagy leads to lipid accumulation, which is coupled to nutrition

(A) Lipid droplets accumulate upon RNAi mediated depletion of *Atg1*, *Atg7*, *Atg8a* in the PG and in the PG of *Atg8a^{KG07569}* mutants, quantified in (B). (C) *Ex vivo* incubation assay shows that NBD-cholesterol (green) co-localize with mCherry-Atg8 positive vesicles (red) in PG cells. mRNA levels (D) and protein levels (E) of genes involved in cholesterol uptake and trafficking in L3 larvae fed on normal food versus L3 larvae starved for 10 hours. Detailed description of genotypes is given in Supplemental Experimental Procedures. Error bars indicate SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

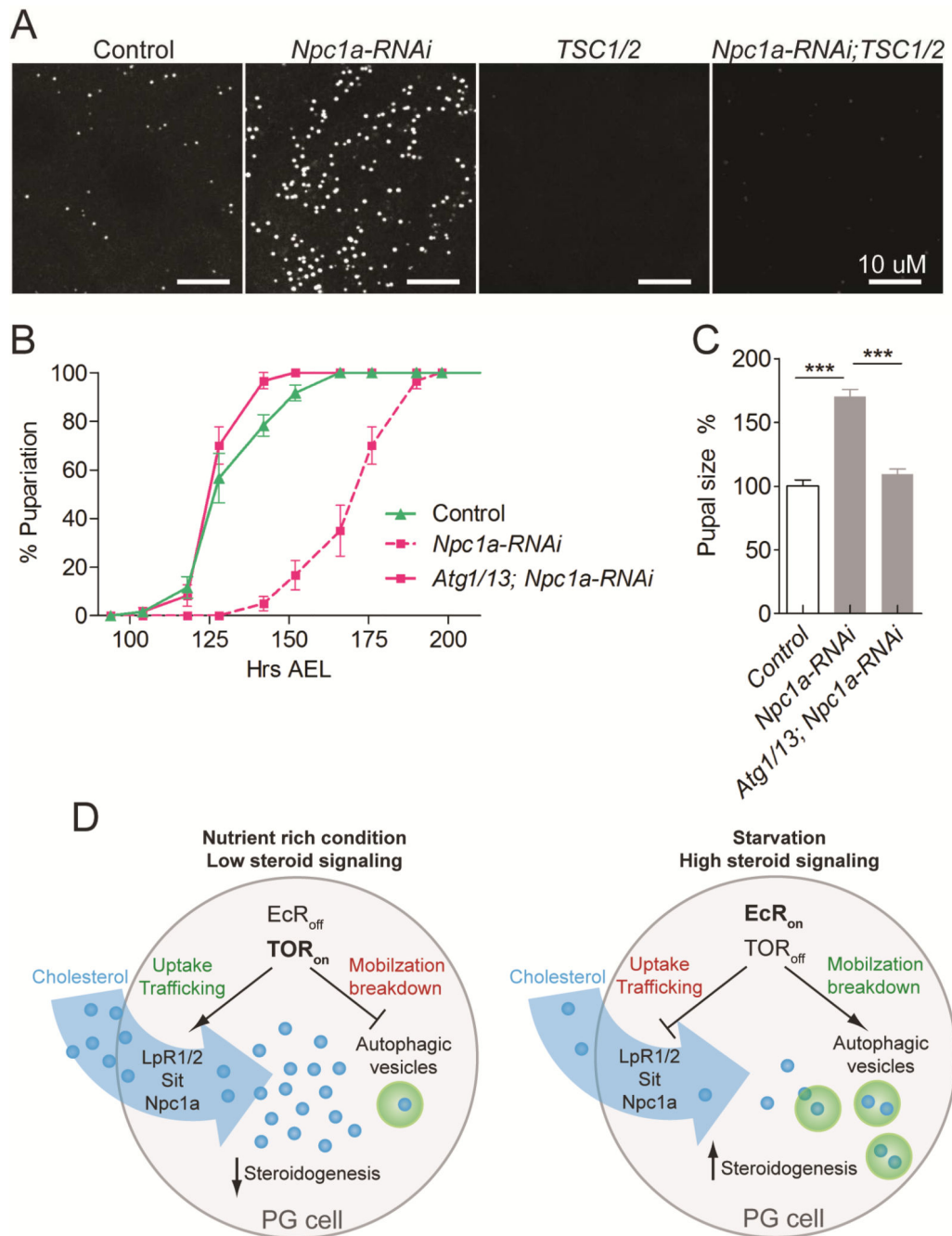


Figure 6. Rescue of *Npc1a*-deficiency phenotypes by inhibition of TOR and activation of autophagy

(A) Inhibition of TOR in PG cells by overexpression of *TSC1/2* suppresses accumulation of lipid droplet due to *Npc1a-RNAi*. (B) Developmental timing of pupariation and (C) pupal size. Stimulation of autophagy by overexpression of *Atg1/13* in the PG rescues the *Npc1a* loss-of-function phenotype, indicating that increased autophagic clearance is sufficient to overcome the block in cholesterol trafficking in *Npc1a* deficient cells. (D) A model for TOR and EcR mediated regulation of cholesterol trafficking mechanisms in the PG cells. Detailed

description of genotypes is given in Supplemental Experimental Procedures. Error bars indicate SEM (***) ($P < 0.001$). See also Figure S4.

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