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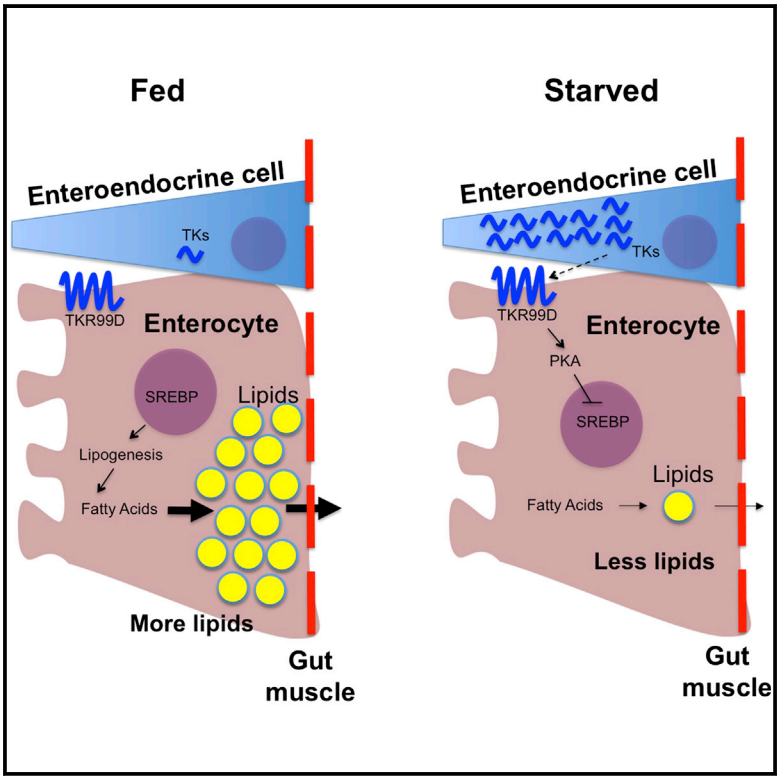
# Control of Lipid Metabolism by Tachykinin in *Drosophila*

## Graphical Abstract




## Highlights

TK EE loss increases intestinal lipid production and systemic lipid storage

Gut TKs control lipid production in ECs

Gut TKs regulate EC lipogenesis via PKA/SREBP signaling

Unlike neuronal TKs, gut TKs do not affect behavioral regulation

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## In Brief

In this study, Song et al. reveal the physiological roles for enteroendocrine cells (EEs) and gut hormones in intestinal lipid metabolism regulation in *Drosophila*. They demonstrate that tachykinin (TK) EEs regulate intestinal lipid production and systemic lipid homeostasis via production of gut TK hormones and modulation of lipogenesis in enterocytes.

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# Control of Lipid Metabolism by Tachykinin in *Drosophila*

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## SUMMARY

**The intestine is a key organ for lipid uptake and distribution, and abnormal intestinal lipid metabolism is associated with obesity and hyperlipidemia. Although multiple regulatory gut hormones secreted from enteroendocrine cells (EEs) regulate systemic lipid homeostasis, such as appetite control and energy balance in adipose tissue, their respective roles regarding lipid metabolism in the intestine are not well understood. We demonstrate that tachykinins (TKs), one of the most abundant secreted peptides expressed in midgut EEs, regulate intestinal lipid production and subsequently control systemic lipid homeostasis in *Drosophila* and that TKs repress lipogenesis in enterocytes (ECs) associated with TKR99D receptor and protein kinase A (PKA) signaling. Interestingly, nutrient deprivation enhances the production of TKs in the midgut. Finally, unlike the physiological roles of TKs produced from the brain, gut-derived TKs do not affect behavior, thus demonstrating that gut TK hormones specifically regulate intestinal lipid metabolism without affecting neuronal functions.**

## INTRODUCTION

Under normal feeding conditions, lipids digested from dietary food are absorbed by enterocytes (ECs) and resynthesized into triglyceride (TG) and packaged into lipoprotein particles that are transported to peripheral tissues for energy supply (Warnakula et al., 2011). Defects in enteric lipid homeostasis have been implicated in obesity, type 2 diabetes, and cardiovascular diseases (Anzai et al., 2009; Warnakula et al., 2011). Thus, characterization of the molecular mechanisms that coordinate lipid uptake, synthesis, and mobilization with lipid homeostasis in the intestine is critical for understanding the basis of lipid metabolic disorders.

Gut hormones secreted from enteroendocrine cells (EEs) play crucial roles in systemic lipid homeostasis, such as the control of appetite and lipid metabolism in peripheral tissues. For example, cholecystokinin (CCK) from I cells, EEs in the mucosal epithelium of the small intestine, reduces food intake through CCK1 receptors on the vagal nerve (Sullivan et al., 2007). Ghrelin from B/D1 cells that are mainly located in the stomach and duodenum reduces lipid mobilization in adipose tissues (Tschöp et al., 2000). Interestingly, glucagon-like peptide-1 (GLP1) secreted from L cells in the ileum and colon suppresses intestinal chylomicron release and postprandial plasma TG levels (Qin et al., 2005), whereas glucagon-like peptide-2 (GLP2), cosecreted with GLP1, stimulates free fatty acid uptake in the intestine and increases plasma TG levels (Hsieh et al., 2009), suggesting that modulation of intestinal lipid metabolism is another important physiological role of gut hormones in maintaining systemic lipid homeostasis. However, probably due to gene redundancy and overlapping functions, loss-of-function studies in the mouse for gut hormones and their receptors have failed to associate them with severe metabolic changes (Mellitzer and Gradwohl, 2011). Thus, how these hormones coordinate lipid metabolism in the intestine is still not clear.

In recent years, *Drosophila* has emerged as a powerful genetic system to study intestinal homeostasis. Although it is simpler than the mammalian gastrointestinal tract, the *Drosophila* gut is similar at both the cellular and molecular levels (Apidianakis and Rahme, 2011). In particular, the adult midgut contains EEs, marked by the transcription factor Prospero (Pros), that express nine major gut prohormones, allatostatin A (AstA), AstB, AstC, neuropeptide F (NPF), short NPF, tachykinin (TK), diuretic hormone 31 (DH31), and CCHamides 1 and 2, which are processed into over 24 mature peptides (Reiher et al., 2011). Previous studies have documented their expression patterns. For example, TK, the most abundant one, is expressed in the anterior, middle, and posterior midgut and encodes six mature peptides (TK1–TK6; Poels et al., 2009; Siviter et al., 2000; Veenstra, 2009; Veenstra et al., 2008). Only in a few cases have the gut-specific functions of these hormones been reported. In vitro treatments have shown that TK1–TK5 can stimulate gut contraction (Siviter et al., 2000), and loss of DH31 EEs or gut hormones in the larval midgut result in impaired peristalsis (LaJeunesse et al., 2010). Other than these examples, the physiological roles of EE hormones in gut lipid metabolism are completely unknown.

In order to analyze the physiological functions of gut hormones, we first characterized a specific Gal4 driver that is expressed in TK EEs. Using this Gal4 driver, we demonstrate that TKs produced by midgut EEs regulate intestinal lipid metabolism by controlling lipid synthesis in ECs.

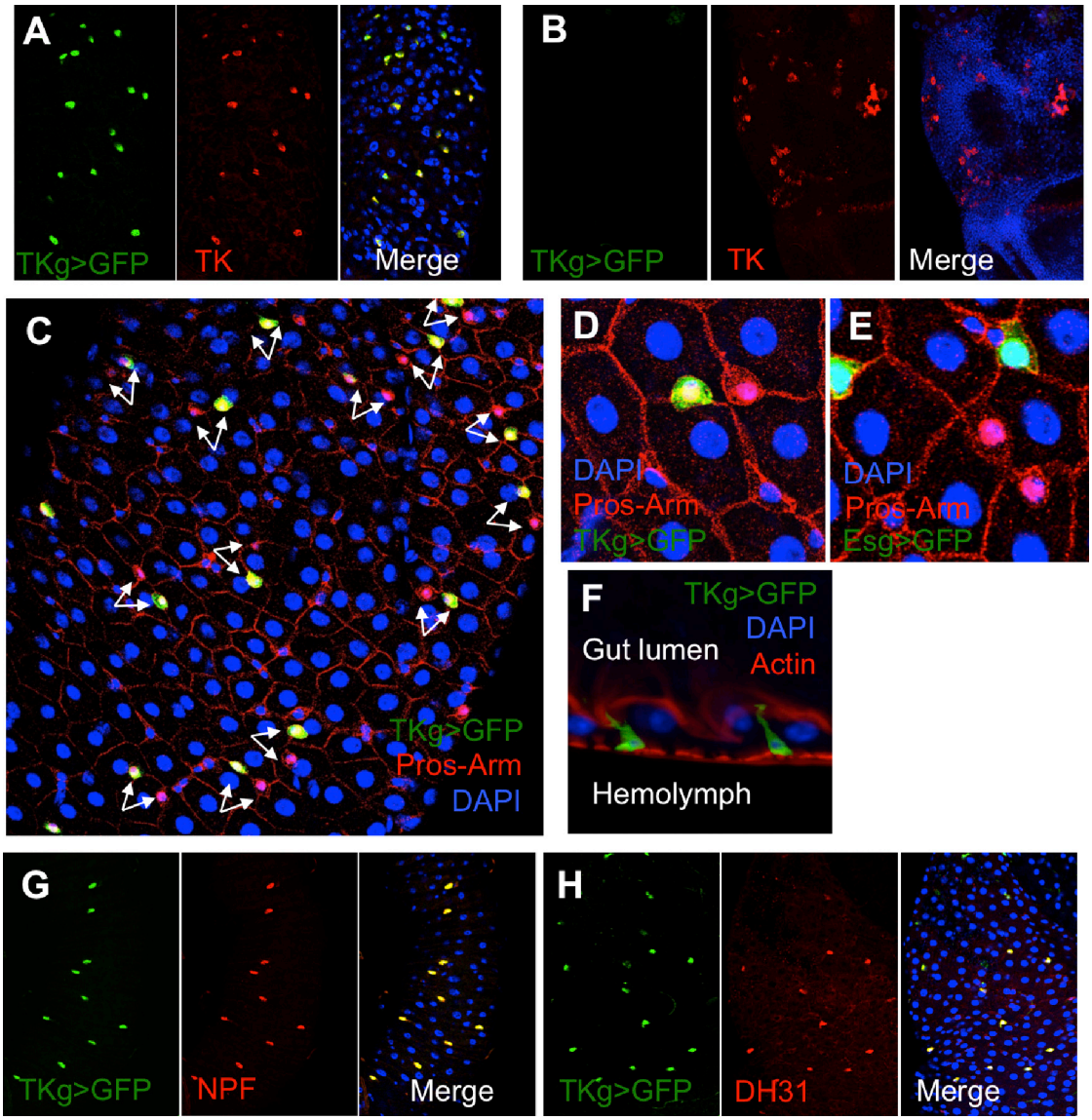


**Figure 1. Characterization of *TKg-Gal4* as a Specific Driver for TK EEs**

(A and B) *TKg-Gal4* specifically targets TK EEs, but not TK brain cells. GFP expression driven by *TKg-Gal4* perfectly colocalizes with TK-positive cells in the gut (A) but is not detectable in TK brain cells (B) (*TKg>GFP* is *UAS-srcGFP*/+; *TKg-Gal4*/+, green; anti-TK, 1:500, red; DAPI, blue).

(C and D) TK-positive cells (TKg > GFP, green) are one of heterologous pair of EEs (anti-Pros, red nuclei).

(E) EEs and ISCs are intermingled among the large ECs. EEs are polygonally shaped, arranged in heterologous pairs, and juxtaposed to two large-nuclear ECs, whereas ISCs are triangularly shaped and reside next to three ECs. ISCs are labeled with GFP (*esg>GFP* is *UAS-GFP*, *esg-Gal4*, green), and EEs are labeled with Prospero (anti-Pros, red nuclei). The cell outlines are labeled with membrane-enriched Armadillo (anti-Arm, red). Nuclei are labeled with DAPI (blue).

(F) Confocal projection image showing that TK-positive cells (TKg > GFP, green) simultaneously contact both the gut lumen and hemolymph. Actin is labeled with phalloidin (red).

(G and H) TK EEs (*TKg>GFP*, green; DAPI, blue) also produce NPF (anti-NPF, red) in the middle midgut (G) and DH31 (anti-DH31, red) in the middle-posterior midgut (H).

## RESULTS

### Identification of a *Gal4* Driver Specifically Targeting TK EEs

Because TKs are expressed both in the CNS and midgut (Asahina et al., 2014; Birse et al., 2011; Reiher et al., 2011; Winther et al., 2006), we first characterized a Gal4 driver that would allow us to perform genetic manipulation in gut TK EEs only. We screened several *TK-Gal4* transgenic lines (see Experimental Procedures) and identified one of them, referred to as "*TK-gut-Gal4* (*TKg-Gal4*)," as driving gene expression solely in TK EEs, but not TK neurons (Figures 1A and 1B).

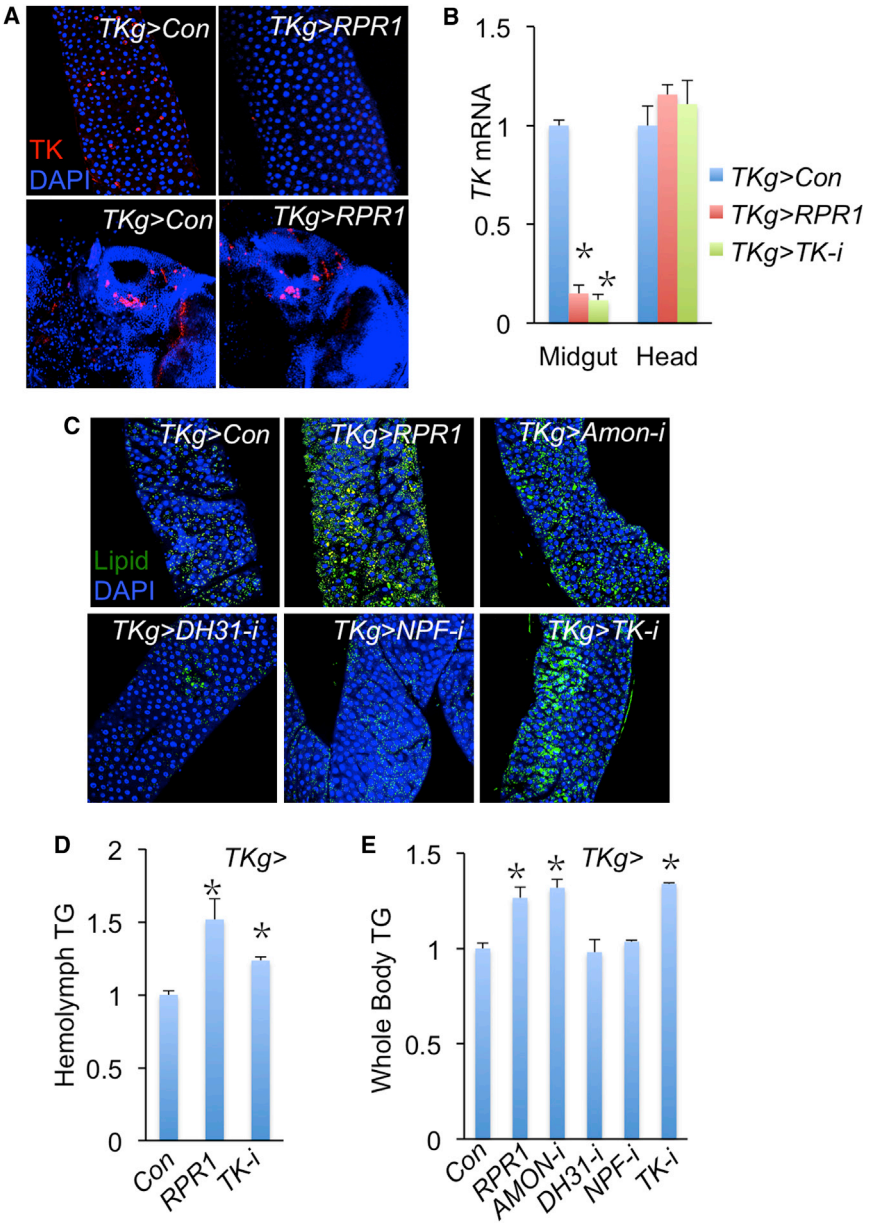


**Figure 2. Gut TKs Affect Intestinal Lipid Metabolism**

(A) *TKg-Gal4* allows specific ablation of TK EEs in the gut (upper), but not TK neurons (lower). Control is *TKg>Con* (*TKg-Gal4/+*), and cell ablation is achieved by expressing *reaper* (*RPR1*) in *TKg>RPR1* (*UAS-rpr1/+*; *TKg-Gal4/+*) animals (anti-TK, 1:500, red; DAPI, blue).

(B) qPCR analysis showing the dramatic decrease of *TK* mRNA in *TKg>RPR1* and *TKg>TK-i* (*UAS-TK-RNAi/TKg-Gal4*) guts (n = 3; 30 guts or 60 heads per group).

(C) Lipid droplet accumulation marked with fluorescent dye Bodipy in the gut (*TKg>Amon-i* is *UAS-Amon-RNAi/+*; *TKg-Gal4/+*. *TKg > DH31-i* is *UAS-DH31-RNAi/+*; *TKg-Gal4/+*. *TKg > NPFi* is *TKg-Gal4/UAS-NPF-RNAi*; n = 3; 30 guts per group).

(D and E) TK EEs ablation or *TK* knockdown in TK EEs increases both circulating TG in hemolymph (D; n = 3; 60 flies per group) and systemic TG storage (E; n = 3; 18 flies per group).

The data are presented as the mean ± SEM.

EEs differ from intestinal stem cells (ISCs) and are present in pairs between two ECs (Figures 1C–1E). TK EEs, which exist as one of the heterologous pairs of EEs (Figures 1C and 1D), are numerous in the anterior, mid, and posterior midgut and have a characteristic shape, simultaneously contacting both hemolymph and the gut lumen (Figure 1F). Additionally, TK EEs also produce NPF in the middle midgut and DH31 in the middle-posterior midgut (Figures 1G and 1H; Veenstra et al., 2008).

## TKs Derived from TK EEs Control Intestinal Lipid Metabolism

To study the physiological role of TK gut hormones, we selectively ablated TK EEs by expressing the apoptotic gene *reaper* (*RPR*) under the control of *TKg-Gal4*. Compared to controls that showed strong TK expression in both the gut and brain (*TKg>Con*), TK expression was lost only in the gut of *TKg>RPR1* flies (Figures 2A and 2B). TK EE ablation also significantly decreased Pros-positive EE number and impaired the paired appearance of EEs in the midgut (Figure S1A). Consistent with TK EE depletion, *NPF* and *DH31* mRNAs and proteins of *TKg>RPR1* flies were dramatically reduced in the gut but remained at normal levels in the CNS (Figures S1B and S1C). However, ablation of TK EEs did not result in significant gut contraction/emptiness defects as analyzed using the blue-dye food assay but was associated with a slight increase in body weight and a slight decrease in food intake (Figures S1D–S1F).

Specific ablation of TK EEs allowed us to examine whether gut peptides affect intestinal lipid metabolism. In wild-type animals, intestinal TG level, the major form of neutral lipid, accounts for only about 1% of the total body TG content (Figure S1G), reflecting the role of the intestine in lipid transport. Moreover, neutral lipid droplets, detected with the neutral lipid Bodipy dye, are most abundant in the ECs located in the anterior and posterior regions of the adult midgut (Figures S1H, S1I, S3D, and S3E). Strikingly, in the absence of TK EEs, we observed a dramatic increase of neutral lipid level in midgut ECs (Figures 2C and S3D; compare to *TKg>Con* control). As midgut lipids are transported throughout the body as energy supplies (Palm et al., 2012; Sieber and Thummel, 2009, 2012), elevation of the intestinal lipid level may be due to an increase in lipid production in the midgut, a decrease in lipid transport, or both. To address this question,

we measured the levels of circulating TG in the hemolymph. *TKg>RPR1* flies showed a 50% increase in TG levels in hemolymph (Figure 2D). Furthermore, whole-body TG (Figure 2E) and neutral lipid levels in the fat body (Figure S3D), a major lipid storage organ, in *TKg>RPR1* were dramatically increased, suggesting that TK EE ablation increases intestinal lipid production and promotes systemic lipid distribution.

To confirm that the elevated lipid production in midgut was due to a deficiency in gut hormones from TK EEs, we suppressed gut hormone process and maturation by knocking down the expression of the proprotein convertase amontillado (AMON) (Reiher et al., 2011). Similar to TK EE ablation, *AMON* knockdown, which has been shown to diminish mature TK production in TK EEs (Reiher et al., 2011), led to increased lipid levels in gut and whole body (Figures 2C and 2E). Further, to identify the hormone(s) involved in lipid metabolic regulation, we knocked down the expression of each of the three gut hormones, TK, NPF, and DH31, in TK EEs (Figures 2B and S1J). Surprisingly, only TK knockdown (*TKg>TK-i*) resulted in an increase of intestinal lipid production, hemolymph TG level, and TG storage (Figures 2C–2E and S3D). Taken together, our observations indicate that TK gut hormones, but not NPF or DH31, regulate intestinal lipid metabolism and subsequently affect systemic lipid storage.

## Brain- and Gut-Derived TKs Play Distinct Physiological Roles

We wondered whether TKs produced from the gut or CNS regulate similar processes. Thus, we compared the phenotypes associated with TK knockdown only in the gut versus both in the brain and gut. RNAi against *TK* driven by *ELAV-Gal4* (*ELAV>TK-i*) diminished TK expression in both the gut and CNS compared to control flies (*ELAV>white-i*; Figures S2A and S2B). These flies showed increased locomotor activity and reduced olfactory responses to certain chemicals (Figures S2C and S2D) as previously reported (Ignell et al., 2009; Winther et al., 2006). However, TK knockdown only in the gut failed to affect locomotor activity or the olfactory response (Figures S2C and S2D). Additionally, unlike the effect observed when brain TKs were depleted (Birse et al., 2011), we did not detect a change in *Drosophila* insulin-like peptide 2 (dILP2) content in insulin-producing cells or body weight (Figures S2E–S2G) when TKs were knocked down only in the gut. These data demonstrate that gut TKs specifically regulate intestinal lipid metabolism and that they are not involved in behavior regulation or secretion of dILPs, functions attributable to TK neuropeptides.

## TKR99D/PKA Regulates Lipid Metabolism in ECs in Response to Gut TKs

To determine how TK regulates lipid production in ECs, we tested whether removal of the TK receptor affects intestinal lipid metabolism. Two different G-protein-coupled TK receptors expressed in gut, TKR99D and TKR86C, have been identified (Birse et al., 2006; Poels et al., 2009). Interestingly, whereas TKR86C is mainly expressed in gut muscles and a few EEs (Poels et al., 2009), TKR99D, as determined using a TKR99D-Gal4 line, appears highly expressed in lipid absorptive ECs (Figure 3A), as well as in a few TK/NPF EEs (Figures S3A and S3B). Strikingly, knockdown of *TKR99D* in ECs by about 60% (Figures 3B and S3C) was sufficient to result in an increase in midgut lipid production and whole-body TG storage (compare *Myo1A>TKR99D-i* to *Myo1A>Con*; Figures 3D, 3E, and S3E).

To test whether gut TKs or TKR99D regulate enteric lipid metabolism through G protein-coupled receptor (GPCR)/cyclic AMP (cAMP)/protein kinase A (PKA) signaling as previously suggested (Birse et al., 2006; Lundquist and Nässel, 1997), we tested the activity of cAMP response element-binding protein (CREB), a direct substrate of PKA, using a CRE-Luciferase reporter (Belvin et al., 1999). TK knockdown (*TKg>TKi*) dramatically decreased CREB transcriptional activity in ECs compared to control (*TKg>white-i*), whereas activation of PKA by feeding flies with forskolin, a PKA agonist, restored CREB transcriptional activity to a normal level (Figure 3C). Furthermore, overexpression of a catalytic form of PKA in ECs (*Myo1A > PKA*, *TKR99D-i*) was sufficient to reverse the increased intestinal lipid production and systemic TG storage (Figures 3D and 3E) associated with *TKR99D* knockdown. Collectively, our results suggest that gut TKs regulate EC lipid metabolism through TKR99D/PKA signaling.

## TKs Suppress Lipogenesis in the Midgut

To identify the lipid-processing pathways regulated by TKs in ECs, we analyzed the mRNA expression profile of genes involved in intestinal lipid metabolism. Interestingly, the intestinal lipases *Magro* (Sieber and Thummel, 2009) and *CG2772*, which regulate dietary lipid digestion in the gut lumen, and the two key enzymes of lipogenesis fatty acid synthase (*FAS*) and acetyl-coenzyme A carboxylase (ACC), which control enteric lipid synthesis (Lim et al., 2011), were all upregulated when TK production was reduced (*TKg > TK-i*; Figure S3F), suggesting that TKs regulate intestinal lipid metabolism via multiple lipid-processing pathways. On the other hand, expression of the lipid transporter *NinaD* involved in lipid absorption (Kiefer et al., 2002), the ER unfolded protein response sensor *IRE1* and *microsomal triglyceride transfer protein*, which regulate lipoprotein particle packaging (Iqbal et al., 2008), and the intestinal lipase *CG31089* that controls dietary lipid digestion remained unaffected (Figure S3F). Notably, mRNA expression of the FoxO target genes 4E binding protein (4EBP) and *insulin receptor* (InR) in the midgut were not affected by removal of TKs (Figure S3F), suggesting that gut TKs do not affect insulin signaling in the midgut.

The upregulation of *FAS* induced by TK deficiency (Figures 4A and S3F) suggested that TKs regulate midgut lipid metabolism, at the least, by modulation of intestinal lipogenesis. To test this hypothesis, flies were fed with $^{14}$C-labeled glucose, and the lipids derived from $^{14}$C-carbon backbones in the gut were measured. *TK>TK-i* flies contained more lipids derived from glucose carbon backbones in the midgut (Figure 4C), suggesting that TK deficiency promotes midgut lipogenesis. Sterol regulatory element-binding protein (SREBP) is a conserved transcription factor for lipogenic genes, like *FAS* (Figure 4B; Kunte et al., 2006) and is negatively modulated by GPCR/cAMP/PKA signaling (Lu and Shyy, 2006). Consistent with this idea, TKR99D/PKA signaling in ECs suppressed *FAS* expression and lipogenesis in the midgut (Figures 4B and 4C).

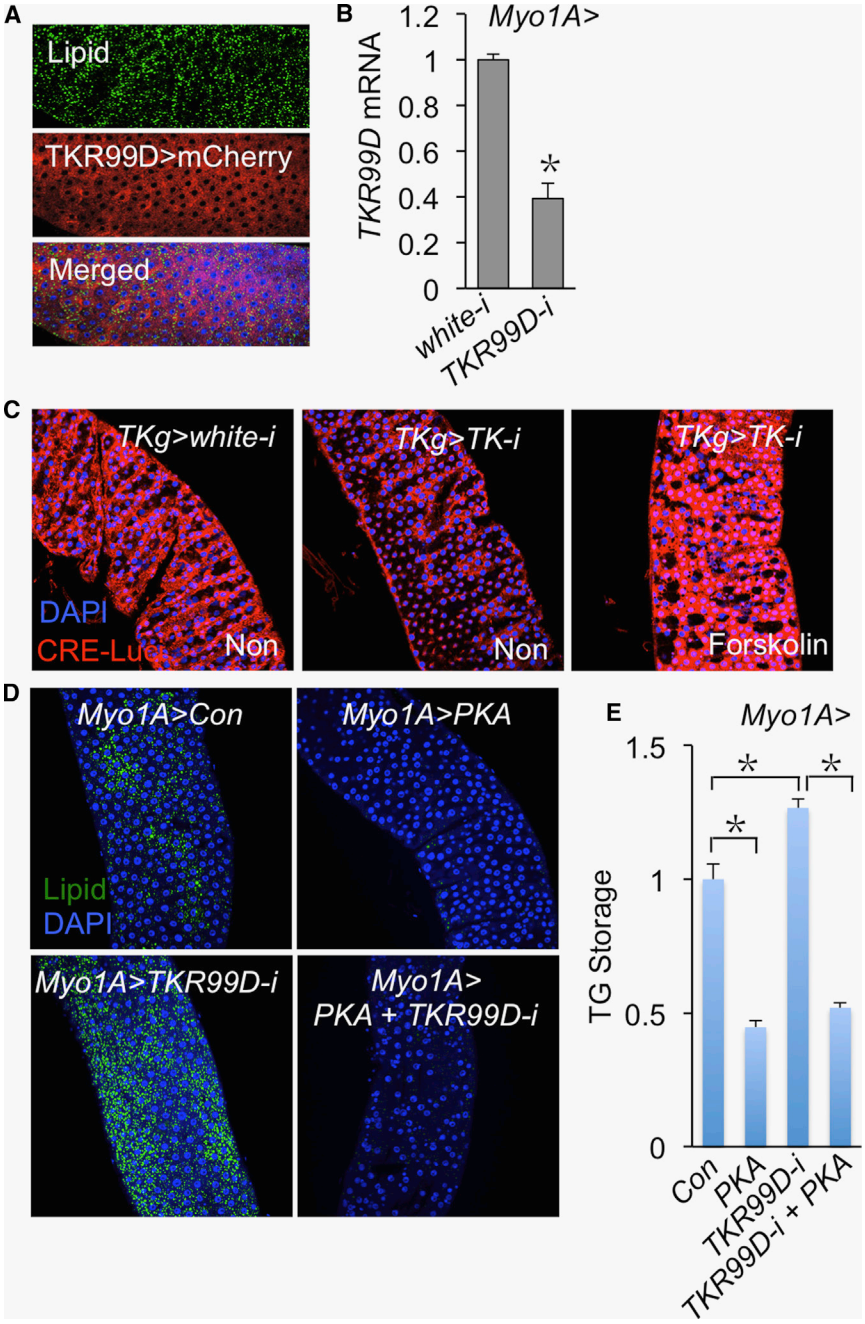


**Figure 3. TKR99D/PKA Signaling Is Essential for EC Lipid Metabolism**

(A) TKR99D is expressed in lipid-absorptive ECs (lipid, green; *TKR99D > mCherry* is *TKR99D-Gal4/UAS-mCherry*, red; DAPI, blue).

(B) qPCR results of *TKR99D* expression in *Myo1A > white-i* (*Myo1A-Gal4/+*; *UAS-white-RNAi/+*) and *Myo1A > TKR99D-i* (*Myo1A-Gal4/+*; *UAS-TKR99D-RNAi/+*) guts (n = 3; 30 guts per group).

(C) CREB transcriptional activity, detected using *CRE-Luci*, is decreased in *TKg > TK-i* guts under normal diet (Non) but restored to normal level when flies are fed with 10 mM Forskolin in normal food (Forskolin).

(D and E) Lipid level in guts (D) and TG storage (E; n = 3; 18 flies per group) of *Myo1A > Con* (*Myo1A-Gal4/+*; *UAS-white-RNAi/+*), *Myo1A > PKA* (*Myo1A-Gal4/+*; *UAS-PKA-C1/+*), *Myo1A > TKR99D-i* (*Myo1A-Gal4/+*; *UAS-TKR99D-RNAi/+*), and *Myo1A > PKA + TKR99D-i* (*Myo1A-Gal4/+*; *UAS-TKR99D-RNAi/UAS-PKA-C1*) animals.

The data are presented as the mean ± SEM.

## Intestinal Lipogenesis Contributes to TK Deficiency-Induced Midgut Lipid Production and Systemic Lipid Storage

To test whether intestinal lipogenesis is sufficient to contribute to changes in midgut lipid production, we expressed an active form of SREBP in ECs (*Myo1A>SREBP*). As predicted, increases of midgut *FAS* mRNA expression, intestinal lipid production, and whole-body TG storage were observed in *Myo1A>SREBP* flies (Figures 4B, 4D, and 4E). Conversely, specific *SREBP* knockdown in ECs (*Myo1A>SREBP-i*) decreased midgut *FAS* expression and body TG storage (Figures 4B and 4G). Thus, intestinal lipogenesis is essential for midgut lipid production and systemic lipid storage.

We further tested whether SREBP-induced lipogenesis is required for TKs regulation of intestinal lipid metabolism. Surprisingly, *SREBP* knockdown in ECs potently blocked the increase of midgut lipid level and systemic TG storage associated with *TKR99D* knockdown (Figures 4F and 4G). Collectively, our results indicate that gut TKs regulate intestinal lipid metabolism through, at least, repression of SREBP-induced lipogenesis.

## The Midgut Produces TKs in Response to Nutrient Availability

Release or production of gut hormones is regulated by diverse physiological conditions in different species. An increase of TKs released from gut into the hemolymph has been observed in the starved locust (Winther and Nässel, 2001). Thus, we tested whether TK levels in the gut are affected by starvation. Flies deprived of food for 24 hr showed a significant increase in TK levels in their midgut (Figure S4A). To test whether increased intracellular TK levels were due to less TK secretion or more TK production, the expression of downstream targets of TK signaling in the midgut were measured. Strikingly, TK/PKA-dependent CREB activity was potently increased (Figure S4B), whereas *FAS* mRNA suppressed by TK signaling was dramatically decreased (Figure S4C), suggesting that starvation enhances TK production in the midgut. Consistent with this idea, diminishing intestinal TK expression partially restored midgut *FAS* expression and lipid production under starvation (Figures S4C and S4D). To determine which nutrient regulates TK

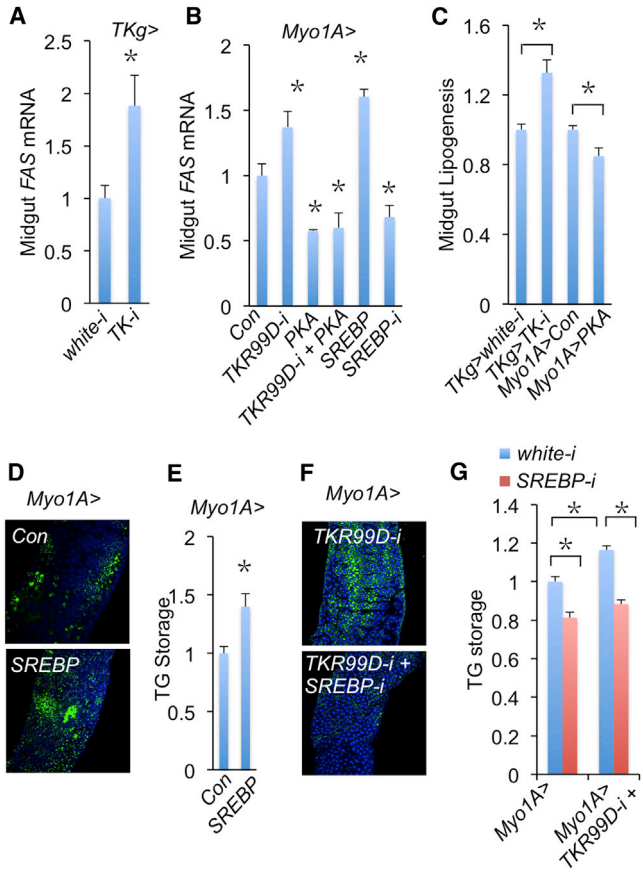


**Figure 4. Gut TKs Suppress Intestinal Lipogenesis**

(A and B) Expression levels of *FAS* in gut analyzed by qPCR (n = 3; 30 guts per group). (A) *FAS* mRNA expression in guts of *TKg>white-i* (*TKg-Gal4/UAS-white-RNAi*) and *TKg > TK-i* (*TKg-Gal4/UAS-TK-RNAi*) flies. (B) *FAS* mRNA expression in the gut of *Myo1A > Con*, *Myo1A > TKR99D-i*, *Myo1A > PKA*, *Myo1A > TKR99D-i + PKA*, *Myo1A > SREBP* (*Myo1A-Gal4/UAS-SREBP*), and *Myo1A > SREBP-i* (*Myo1A-Gal4/+*; *UAS-SREBP-RNAi/+*) flies (n = 3; 30 gut per group).

(C) Midgut lipogenesis was analyzed by $^{14}$C-labeled lipids derived from $^{14}$C-glucose in *TKg > white-i*, *TKg > TK-i*, *Myo1A > Con*, and *Myo1A > PKA* guts (n = 3; 90 guts per group).

(D–G) Intestinal lipid level (D and F) and whole-body TG storage (E and G; n = 3; 18 flies per group) in adult flies. (D and E) *Myo1A>Con* and *Myo1A>SREBP* adult flies. (F and G) *Myo1A > white-i*, *Myo1A > TKR99D-i*, *Myo1A > SREBP-i*, and *Myo1A > TKR99D-i + SREBP-i* (*Myo1A-Gal4/+*; *UAS-TKR99D-RNAi/UAS-SREBP-RNAi*) adult flies.

The data are presented as the mean ± SEM.

production, flies were refed with different ingredients after starvation, such as sucrose, coconut oil, or yeast. Interestingly, only yeast refeeding potently suppressed TK production in midgut (Figure S4E). As amino acids are the major nutrient in yeast, our results suggest that the midgut produces TKs in response to amino acid availability.

To examine whether nutrient status or TKR99D signaling changes in ECs regulate TK production in a feedback manner, we specifically modulated TKR99D signaling in ECs. Knockdown of *TKR99D* or overexpression of *PKA* in ECs showed altered lipid levels (Figures 3D and 3E) but did not affect TK levels in midgut ECs (Figure S4F), suggesting that TK signaling or lipid levels in ECs do not regulate intestinal TK production.

Interestingly, in contrast to mammalian regulation of TK production by infection with a gut pathogen, flies fed with the human pathogen *Pseudomonas aeruginosa 14*, previously shown to cause severe gut defects and gut stem cell proliferation in *Drosophila* (Apidianakis et al., 2009), failed to show any change in intracellular TK levels (Figure S4G). These results suggest that the presence of pathogen does not affect production of gut TKs in *Drosophila*.

## DISCUSSION

Previous studies in mammals have indicated that a few gut secretory hormones, like GLP1 and GLP2, are involved in intestinal lipid metabolism (Qin et al., 2005). However, due to gene and functional redundancy, mammalian genetic models for gut hormones and/or their receptors with severe metabolic defects are not available. Here, we establish that *Drosophila* TKs produced from EEs coordinate midgut lipid metabolic processes. Our studies clarify the roles of TK hormones in intestinal lipogenesis and establish *Drosophila* as a genetic model to study the regulation of lipid metabolism by gut hormones.

Six mature TKs, TK1–TK6, are processed and secreted from TK EEs in both the brain and midgut (Reiher et al., 2011). Using a specific Gal4 driver line, we were able to specifically manipulate gene expression in TK EEs, leading us to demonstrate that loss of gut TKs results in an increase in midgut lipid production. Further, we showed that TKs regulate intestinal lipid metabolism associated with TKR99D, but not TKR86C, which is consistent with the expression of these receptors. Consistent with previous reports that TK/TKR99D signaling regulates cAMP level and PKA activation (Birse et al., 2006; Lundquist and Nässel, 1997), loss of gut TKs is associated with a reduction in PKA activity in ECs, and overexpression of a PKA catalytic subunit was able to reverse the increased intestinal lipid production associated with loss of TKR99D. In addition, the transcription factor SREBP that triggers lipogenesis was controlled by TK/TKR99D/PKA signaling. Taken together, our results suggest that TKs produced from EEs regulate midgut lipid metabolism via TKR99D/PKA signaling and regulation of, at least, SREBP-induced lipogenesis in ECs.

Interestingly, our study reveals that TKs derived from either the brain or gut exhibit distinct functions: TKs derived from gut control intestinal lipid metabolism, whereas TKs derived from brain control behavior. This is reminiscent of the distinct functions of mammalian secreted regulatory peptides, where different spatial expressions or deliveries of peptides like Ghrelin can result in distinct physiological functions (Nakazato et al., 2001; Tschöp et al., 2000). In addition, some prohormones encode multiple mature peptides that can have multiple functions. For example, processing of proglucagon in the pancreas $\alpha$ cells preferentially gives rise to glucagon, which antagonizes the effect of insulin. In intestine L cells, however, proglucagon is mostly processed into GLP1 to promote insulin release (Brubaker and Drucker, 2004). Our studies of TKs exemplify how secreted regulatory peptides derived from different tissues can be associated with fundamentally diverse physiological functions. Clearly, additional studies examining the function of secreted peptides in a cell-type- and

tissue-specific manner are needed to fully appreciate and unravel their complex roles both in flies and mammals.

There is a growing body of studies emphasizing that intestinal lipid metabolism is key to the control of systemic lipid homeostasis. For example, chemicals such as orlistat (Heck et al., 2000), designed to inhibit dietary lipid digestion/absorption in the intestine, efficiently reduce obesity. In addition, mammalian inositol-requiring enzyme 1β deficiency-induced abnormal chylomicron assembly in the small intestine results in hyperlipidemia (Iqbal et al., 2008). Similarly, in *Drosophila*, dysfunction of intestinal lipid digestion/absorption caused by *Magro/LipA* deficiency eventually decreases whole-body lipid storage and starvation resistance (Karpac et al., 2013; Sieber and Thummel, 2009, 2012). Further, intestinal lipid transport, controlled by lipoproteins, is essential for systemic lipid distribution and energy supply in other tissues (Palm et al., 2012; Panáková et al., 2005). Consistent with these observations, we demonstrate that increased midgut lipid synthesis associated with gut TK deficiency is sufficient to elevate systemic lipid storage. Although TK ligands and TK receptors show high homologies between mammals and fruit flies (Birse et al., 2006), whether mammalian TK signaling plays a similar role in intestinal lipid metabolism is largely unknown. Future studies will reveal whether mammalian TK signaling affects intestinal lipid metabolism as in *Drosophila*. If this is the case, it may provide a therapeutic opportunity for the treatment of intestinal lipid metabolic disorder and obesity.

Production and secretion of gut hormones are precisely regulated under various physiological conditions. Similar to previous observations that starvation induces gut TK secretion in other insects (Winther and Nässel, 2001), we found that nutrient deprivation promotes TK production in EEs. Interestingly, feeding of amino-acid-enriched yeast, but not coconut oil or sucrose, potently suppressed gut TK levels, indicating that amino acids may act directly on TK production in EEs. It has been reported that dietary nutrients regulate gut hormone production through certain receptors located on the cell membrane of EEs in mammals (Reimann et al., 2012). Future studies will be necessary to elucidate the detailed mechanism by which nutrients regulate TK production from EEs.

## EXPERIMENTAL PROCEDURES

### *Drosophila* Strains

Expression patterns of different *TK-Gal4* P-element lines that contain the 0.5–2.5 kb fragment upstream of the *TK* gene were examined by crossing to *UAS-srcGFP* flies. One line that showed expression in TK EEs, but not in TK neurons, was referred to as *TK-gut-Gal4* (*TKg-Gal4*) and used for this study. Other lines were obtained from Bloomington *Drosophila* Stock Center, TRiP at Harvard Medical School, and Vienna *Drosophila* Resource Center. See the Supplemental Experimental Procedures for detailed information.

### Immunostaining and Western Blot

Immunostaining of adult midgut and brain and western blot were described previously (Karpowicz et al., 2010; Song et al., 2010). See the Supplemental Experimental Procedures for detailed information.

### TG Measurement

TG measurement was performed as previously described (Song et al., 2010). See the Supplemental Experimental Procedures for detailed information.

### qRT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described (Song et al., 2010). See the Supplemental Experimental Procedures for detailed primer information.

### Midgut Lipogenesis Measurement

Adult flies were fed with 0.2 mCi/ml $^{14}$C-glucose (PerkinElmer) for 3 days. Thirty guts were dissected and homogenized in 200 μl of chloroform/methanol/water (2:1:1) mixture. The lysate was incubated at 37°C for 1 hr before 75 μl chloroform and 75 μl 1 M KCl were added. After centrifugation at 3,000 rpm for 2 min, $^{14}$C-labeled lipids in the chloroform phase were measured by liquid scintillation counting.

### Behavior Assays

Behavior assays were performed as previously described (Winther et al., 2006).

### Statistical Analyses

The data are presented as the mean ± SEM. Student's t tests were used for comparisons between two groups. $p < 0.05$ was considered statistically significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.060.

## AUTHOR CONTRIBUTIONS

W.S. conceived, designed, and performed all experiments. J.A.V. generated *TKg-Gal4* lines and antibodies against TK and DH31 and made constructive comments. W.S. and N.P. discussed results and wrote the manuscript.

## ACKNOWLEDGMENTS

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**Supplemental Information**

# Control of Lipid Metabolism by Tachykinin in *Drosophila*

**Wei Song, Jan A. Veenstra, and Norbert Perrimon**

Figure S1

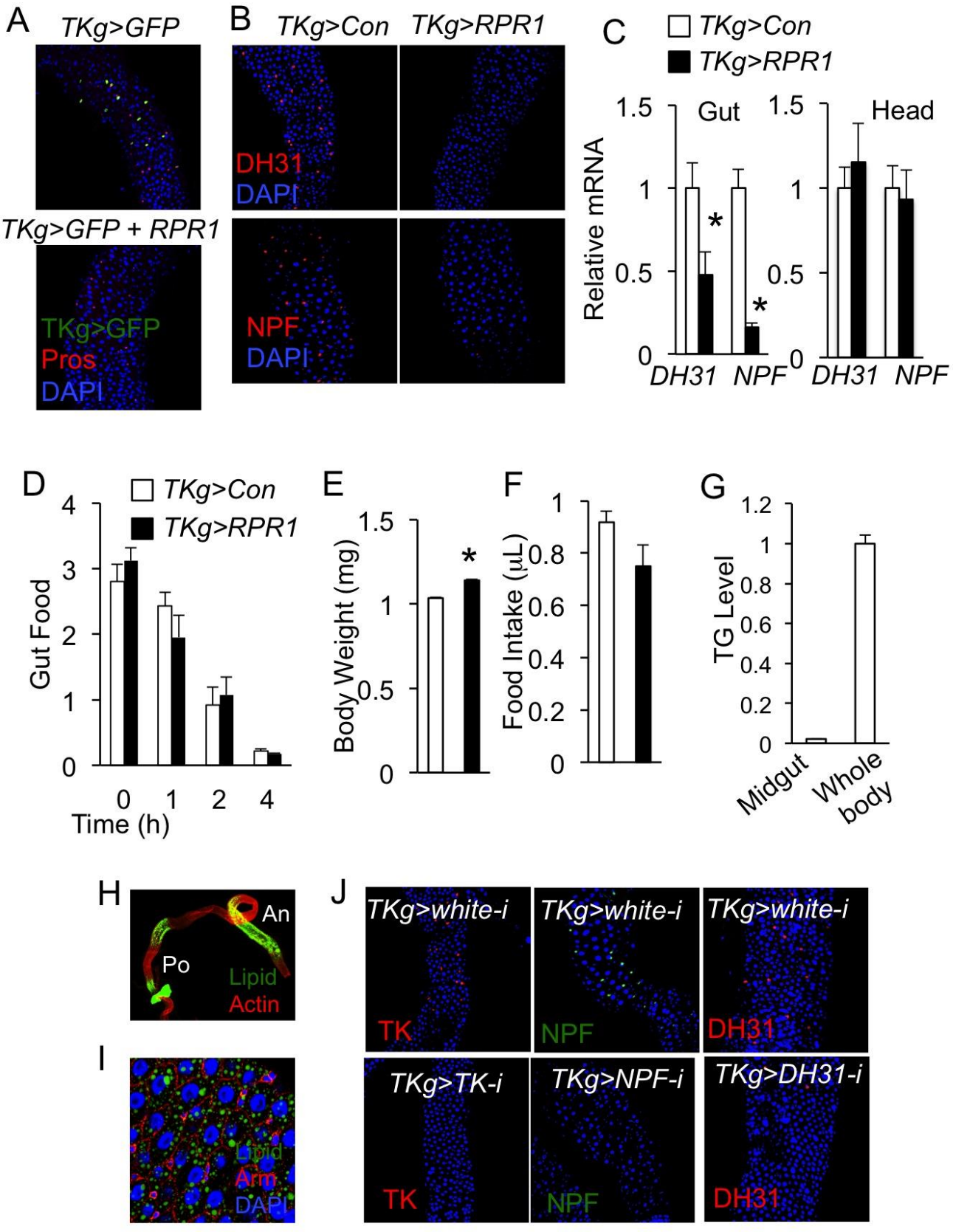

A
TKg>GFP
TKg>GFP + RPR1
TKg>GFP
Pros
DAPI
B
TKg>Con
TKg>RPR1
DH31
DAPI
NPF
DAPI
C
TKg>Con
TKg>RPR1
Relative mRNA
Gut
Head
DH31
NPF
DH31
NPF
D
TKg>Con
TKg>RPR1
Gut Food
Time (h)
E
Body Weight (mg)
F
Food Intake (μL)
G
TG Level
Midgut
Whole body
H
An
Po
Lipid
Actin
I
Lipid
Arm
DAPI
J
TKg>white-i
TK
TKg>white-i
NPF
TKg>white-i
DH31
TKg>TK-i
TK
TKg>NPF-i
NPF
TKg>DH31-i
DH31

**Figure S1. Ablation of TK EEs in midgut, related to Figure 1 and 2**

**A**, TK EEs ablation (*TKg>GFP* + *RPR1*) decreased EEs (anti-Pros, red; TKg>GFP, green) number and impaired EEs paired appearance compared to Control (*TKg>GFP*). **B,** Expression of DH31 (anti-DH31, red, top panels) and NPF (anti-NPF, red, bottom panels) were dramatically decreased in the gut of TK EEs ablated flies (*TKg>RPR1*). **C**, qPCR results indicate that mRNA levels of *DH31* and *NPF* are decreased only in the gut, but not brain, of *TKg>RPR1* (n=3, 30 guts per group). **D,** Gut emptiness of control (*TKg>Con*) and ablated flies (*TKg>RPR1*), as determined by the blue dye containing food that is remained in the gut. **E, F,** body weight (**E**) (n=3, 60 flies per group) was increased slightly, whereas food intake (**F**) (n=3, 30 flies per group) was slightly decreased in *TKg>RPR1*. **G,** TG level in gut and whole body of wild type flies. **H,** Neutral lipid accumulates in the anterior (An) and posterior (Po) regions of the adult midgut as indicated by Bodipy staining. **I,** most lipid droplets (Bodipy, green) are located in ECs with large nuclei (DAPI, blue). Outline of the cells are labeled with membrane-enriched Armadillo (anti-Arm, red). **J,** Specific knockdown of TK (anti-TK, red, left), NPF (anti-NPF, green, middle), or DH31 (anti-DH31, red, right) expression in the midgut. The data are presented as the mean ± SEM.

Figure S2

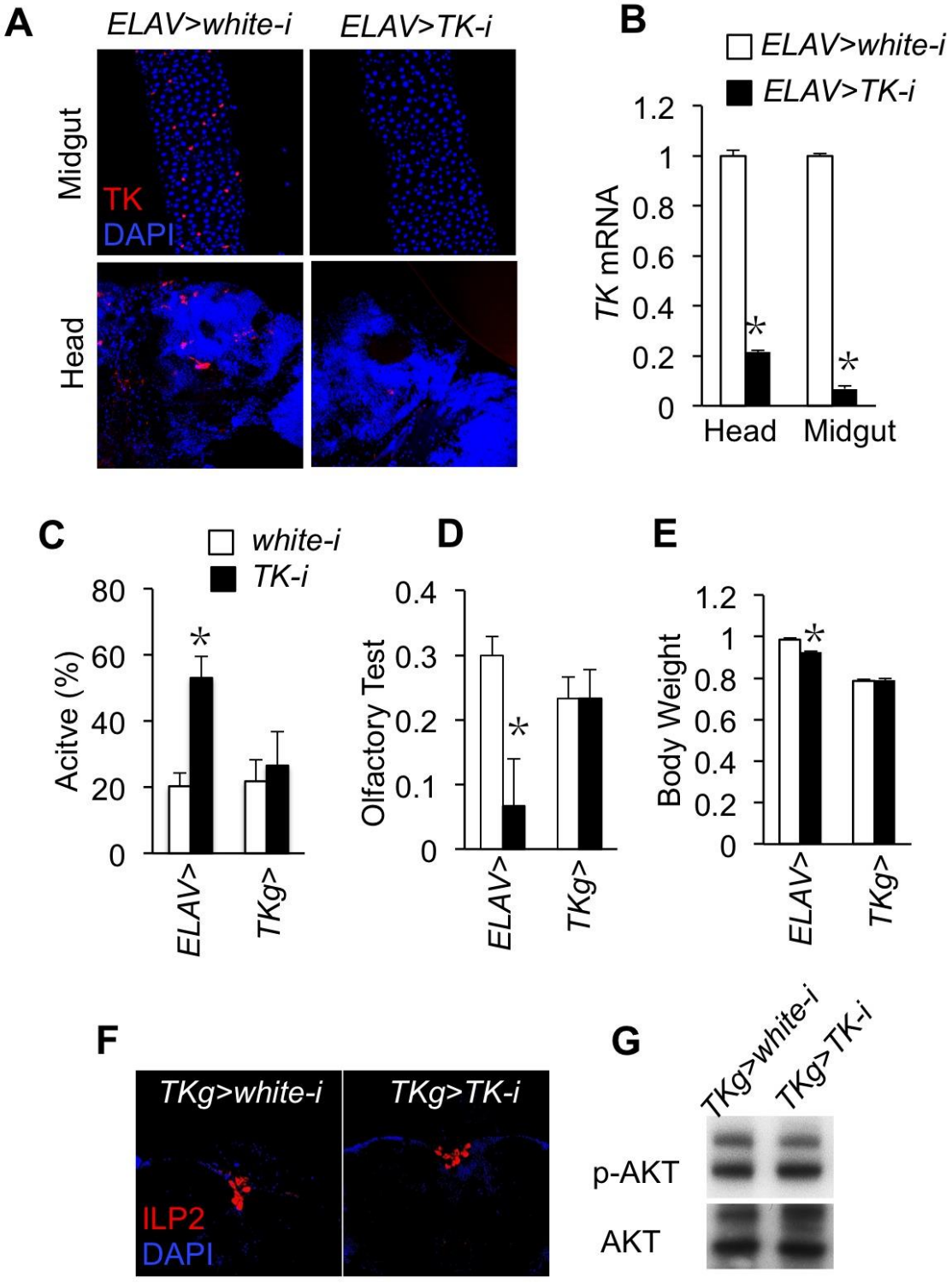
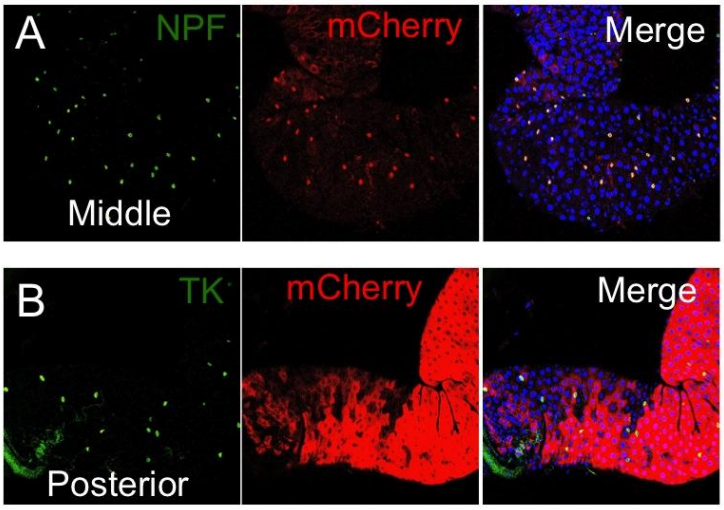
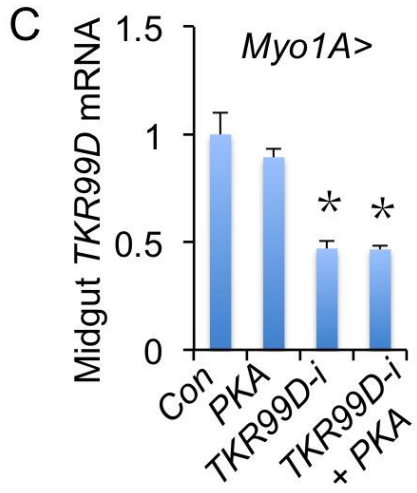

A
ELAV>white-i
ELAV>TK-i
Midgut
Head
TK
DAPI
B
ELAV>white-i
ELAV>TK-i
TK mRNA
1.2
1
0.8
0.6
0.4
0.2
0
Head
Midgut
C
white-i
TK-i
Acitve (%)
80
60
40
20
0
ELAV>
TKg>
D
Olfactory Test
0.4
0.3
0.2
0.1
0
ELAV>
TKg>
E
Body Weight
1.2
1
0.8
0.6
0.4
0.2
0
ELAV>
TKg>
F
TKg>white-i
TKg>TK-i
ILP2
DAPI
G
TKg>white-i
TKg>TK-i
p-AKT
AKT

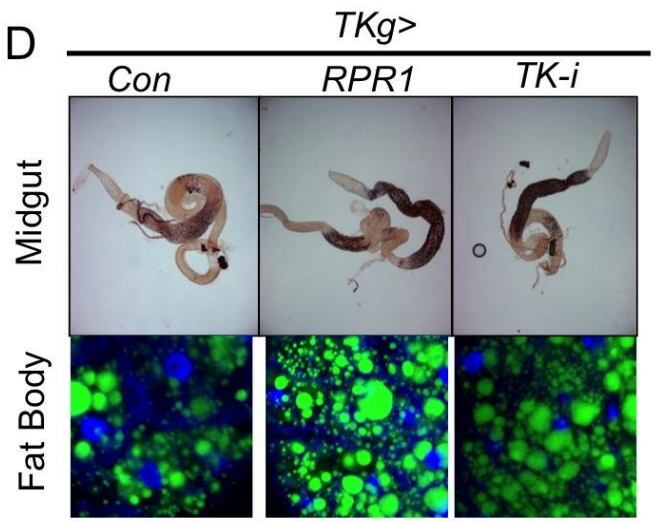
**Figure S2. TKs from brain and EEs have different physiological roles, related to Figure 2**

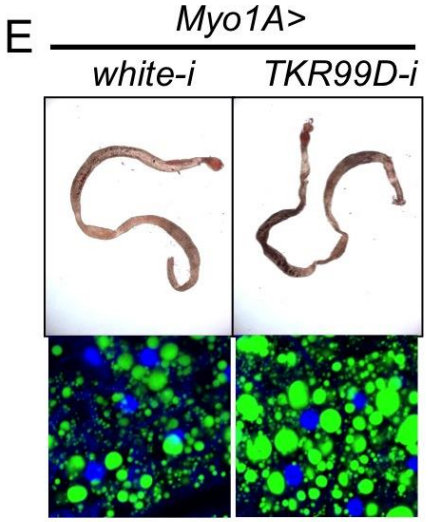
**A,** TK expression (anti-TK, 1:500, red) is absent in both the midgut (upper) and brain (lower) in *ELAV>TK-i* (*ELAV-Gal4/+; UAS-TK-RNAi/+*) animals, compared to control *ELAV>white-i (ELAV-Gal4/+; UAS-white-RNAi/+)*. **B**, qPCR analysis of *TK* mRNA expression in both the midgut and brain revealed a significant decrease in *TK* knockdown (*ELAV>TK-i*) flies (n=3, 60 head or 30 guts per group). **C-E**, Locomotor activity (**C**) (n=3, 45 flies per group), olfactory response to butanol (**D**) (n=3, 60 flies per group), and body weight (**E**) (n=3, 60 flies per group) are affected by TK knockdown in both the brain and midgut (*ELAV>TK-i*), but not affected when TK is knocked down in the midgut only (*TKg>TK-i*). **F, G,** Either brain dILP2 level (**F**) or whole body phosphorylated AKT (**G**) was not affected in *TKg>TK-i* compared to *TKg>white-i* (control) flies. The data are presented as the mean ± SEM.

Figure S3


A
NPF
mCherry
Merge
Middle
B
TK
mCherry
Merge
Posterior


C
Myo1A>
Midgut TKR99D mRNA
1.5
1
0.5
0
*
*
Con
PKA
TKR99D-i
TKR99D-i + PKA


D
TKg>
Con
RPR1
TK-i
Midgut
Fat Body


E
Myo1A>
white-i
TKR99D-i

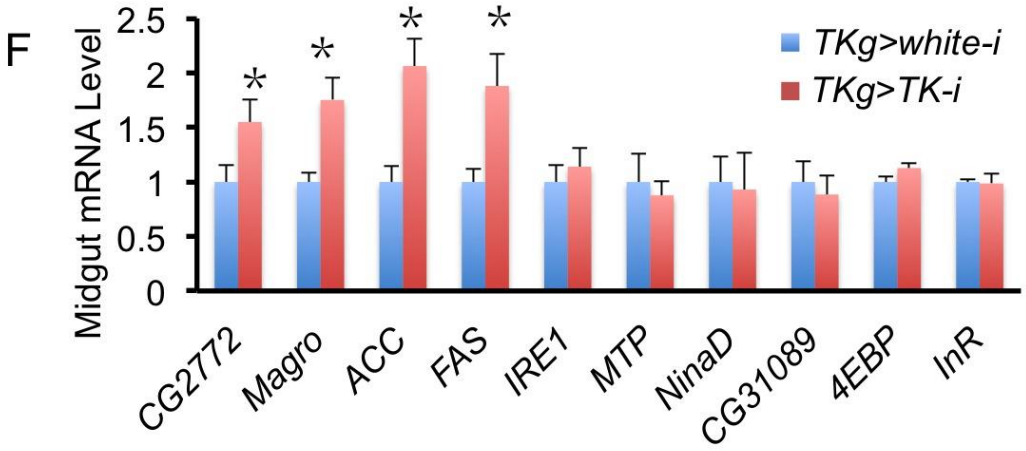

F
Midgut mRNA Level
2.5
2
1.5
1
0.5
0
*
*
*
*
TKg>white-i
TKg>TK-i
CG2772
Magro
ACC
FAS
IRE1
MTP
NinaD
CG31089
4EBP
InR

**Figure S3. TK/TKR99D signaling affects lipid metabolism in ECs, related to Figure 2, 3, and 4**

**A, B,** mCherry expression in *TKR99D>mCherry* co-localizes with NPF/TK EEs in the mid-midgut (**A,** anti-NPF, green) but not in posterior midgut TK EEs (**B,** anti-TK, 1:500, green). **C,** Knockdown of *TKR99D* mRNA expression in midgut (n=3, 30 guts per group). **D, E,** Lipid levels in adult midgut (upper, indicated by Oil Red staining) and fat body (lower, indicated by Bodipy staining). **F,** qPCR results in midguts of *TKg>white-i* and *TKg>TK-i* flies (n=3, 30 guts per group). The data are presented as the mean ± SEM.

Figure S4

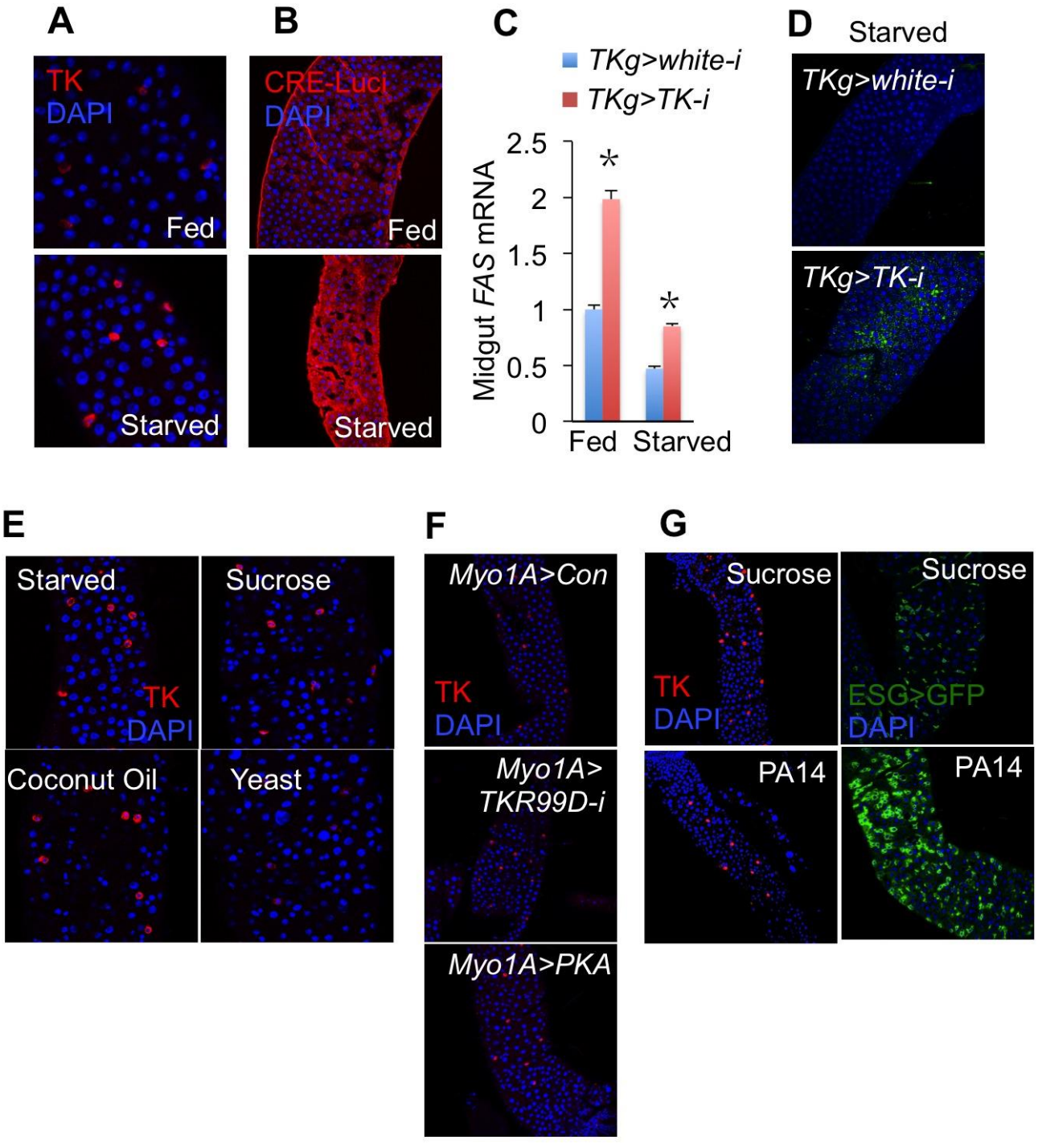

A
TK
DAPI
Fed
Starved
B
CRE-Luci
DAPI
Fed
Starved
C
TKg>white-i
TKg>TK-i
Midgut FAS mRNA
2.5
2
1.5
1
0.5
0
*
*
Fed
Starved
D
Starved
TKg>white-i
TKg>TK-i
E
Starved
TK
DAPI
Sucrose
Coconut Oil
Yeast
F
Myo1A>Con
TK
DAPI
Myo1A>
TKR99D-i
Myo1A>PKA
G
Sucrose
TK
DAPI
Sucrose
ESG>GFP
DAPI
PA14
PA14

**Figure S4. Nutrient deprivation enhances TKs production in TK EEs, related to Figure 2 and 4**

**A, B,** Intracellular TK levels (anti-TK, 1:5000, red) in TK EEs (**A**) and enteric CRE-Luciferase (CRE-Luci) levels (**B**) in wild type fly guts under fed or starved condition. **C, D**, *FAS* mRNA expression levels (**C**) (n=3, 30 guts per group) and lipid levels (**D**) in *TKg>white-i* or *TKg>TK-i* guts under 24h fed or starved condition. **E,** Intracellular TK levels (anti-TK, 1:5000, red) in TK EEs when flies were starved for 24h or starved for 12h and refed with 10% sucrose, 25% coconut oil or yeast paste for 12h. **F,** Intracellular TK levels in TK EEs (anti-TK, 1:5000, red) of *Myo1A>Con*, *Myo1A>TKR99D-i*, and *Myo1A>PKA* flies. **G,** Intracellular TK levels in TK EEs (left, anti-TK, 1:5000, red) and intestinal stem cell (ISC) proliferation (right, esg>GFP, green) of flies treated with 5% sucrose (control) or PA14 (pathogen) for 48h. The data are presented as the mean ± SEM.

## Supplemental Experimental Procedures

***Drosophila* Strains.** *UAS-RPR1* (BL 5823), *UAS-AMON-RNAi* (BL 29010), *UAS-PKA* (BL 35555), *ELAV-Gal4* (BL 8765), *UAS-mCherry* (BL 27392), *UAS-srcGFP* (BL 5432), *UAS-SREBP (BL 8244), UAS-SREBP-RNAi (BL25975)* and $w^{1118}$ were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/). RNAi lines against *white* (JF01545), *TK* (JF01818), *NPF* (JF02555), *and TKR99D* (JF02663) were obtained from the TRiP at Harvard Medical School (http://www.flyrnai.org/TRiP-HOME.html). RNAi line against *DH31* (VDRC50295) was obtained from VDRC (http://stockcenter.vdrc.at/). Other stocks used in this study are: *esg-Gal4* and *myo1A-Gal4* (Karpowicz et al., 2010), *CRE-Luci* (Belvin et al., 1999).

**Immunostaining, lipid staining, microscopy and western blot.** Guts and brains were dissected in PBS and fixed for 15 min in 4% formaldehyde/PBS. After fixation, the samples were washed with 0.2% Triton/PBS and incubated in primary antibodies overnight at 4°C. Secondary antibody stainings were incubated for 1h at room temperature. Tissues were incubated in DAPI for 10 min (1:1000, Invitrogen), washed and mounted in Vectashield (Vector). Primary antibodies used in this study are: rabbit anti-Tachykinin (Veenstra, 2009; Veenstra et al., 2008) (1:500 to detect TK EEs ablation or 1:5000 to detect TKs production), rabbit anti-NPF (a gift from Dr. Ping Shen) (1:1000), rabbit anti-DH31 (Veenstra, 2009; Veenstra et al., 2008) (1:1000), mouse anti-Prospero (Karpowicz et al., 2010) (1:100), mouse anti-Armadillo (Karpowicz et al., 2010) (1:50), rabbit anti-dILP2 (1:5000, gift from Dr. Linda Partridge), rabbit anti-Firefly Luciferase (1:100, Abcam). F-Actin was visualized with Alexa 555-Phallodin (1:1000, Invitrogen). Bodipy 493/503 (1mg/mL, Invitrogen) and Oil Red (0.5%, Sigma) were used for neutral lipid staining for 30 min at room temperature. Regular microscopy was performed on a Zeiss Axioskop 2motplus upright and confocal images were obtained using a Leica TCS SP2 AOBS system. For

Western blot, 8 whole flies were lysed in buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 10 mM Na4P2O7, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Nonidet P-40). Extracts were immunoblotted with indicated antibodies: rabbit anti-AKT (1:1000, Cell Signaling) and rabbit anti-phospho-AKT (S473, 1:1000, Cell Signaling).

**TG measurement.** TG measurement and starvation resistance were performed as previously described (Song et al., 2010). Briefly, 6 flies were anesthetized and homogenized in 500μL 0.1% Triton/PBS, heated at 70°C for 5 min, and centrifuged at 14,000 rpm for 10 min. 10μl supernatant was used to measure TG using Serum TG determination kits (Sigma). Protein amounts were measured using Bradford Reagent (Sigma). TG storage was normalized to protein amount.

**RT-qPCR.** 10 midguts or 20 heads from each genotype were collected on ice. RNA was isolated using Trizol (Invitrogen) and cDNA was transcribed using the iScript cDNA Synthesis Kit (Biorad). qPCR was then performed using iQ SYBR Green Supermix on a CFX96 Real-Time System/C1000 Thermal Cycler (Biorad). Gene expression was normalized to *RPL32*. qPCR primers used are:

*RPL32-F:* *gctaagctgtcgcacaaatg*
*RPL32-R:* *gttcgatccgtaaccgatgt*
*TK-F:* *tacaagcgtgcagctctctc*
*TK-R:* *ctccagatcgctcttcttgc*
*NPF-F:* *gaggcgtccaactccagac*
*NPF-R:* *gctctgtcgccgtagtaggt*
*DH31-F:* *gccaatccaatggaggatac*
*DH31-R:* *gtatgatggtgcgtccaaag*
*TKR99D-F:* *tacttcctgcccatcgtctc*
*TKR99D-R:* *atcatcttcaccacccttcg*
*CG2772-F:* *ggaagtttagctggcacgag*
*CG2772-R:* *gaacccatcacgaagaagga*
*Margo-F:* *acaccgaactgattccgaac*

*Margo-R:* *atccaccattggcaaacatt*
*ACC-F:* *taacaacggagtcacccaca*
*ACC-R:* *caggtcacaaccgatgtacg*
*FAS-F:* *cgtacgacccctctgttgat*
*FAS-R:* *agtgcaagttaccgggaatg*
*IRE1-F:* *cagcctggctaagaacaagg*
*IRE1-R:* *attggtggtggtggtcagat*
*MTP-F:* *gtggatctcaatggcaaggt*
*MTP-R:* *gtgggtgatgctgaaatcct*
*NinaD-F:* *accaaatgcggaatagcaac*
*NinaD-R:* *ggcgtaatgcaaaaattcgt*
*CG31809-F:* *gtcgagcaaatagccgaaag*
*CG31809-R:* *tacgaacctcccagttcagg*
*4EBP-F:* *ctcctggaggcaccaaacttatc*
*4EBP-R:* *ttcccctcagcaagcaactg*
*InR-F:* *acaaaatgtaaaaccttgcaaatcc*
*InR-R:* *gcaggaagccctcgatga*

**Behavior Assays.** The olfactory choice assay has been previously described (Winther et al., 2006). Briefly, 20 flies were introduced in a small chamber where they were allowed to choose between Butanol (1/10, vol/vol, dissolved in water containing 1% Triton-X) and control (water containing 1% Triton-X). The olfactory response index was calculated after 22h as (C-T)/(T+C+NR-D). T is the number of flies in the test vial, C is the number of flies in the control vial, NR is the number of flies remaining in arena, and D is the number of dead flies. For locomotor activity, 20 flies were individually allowed to walk in a narrow transparent tube, and the number of active flies was recorded within 5 min.

## Supplemental References

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