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RESEARCH ARTICLE

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The *Drosophila* E78 nuclear receptor regulates dietary triglyceride uptake and systemic lipid levels

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Abstract

Background: Lipid levels are maintained by balancing lipid uptake, synthesis, and mobilization. Although many studies have focused on the control of lipid synthesis and mobilization, less is known about the regulation of lipid digestion and uptake.

Results: Here we show that the *Drosophila* E78A nuclear receptor plays a central role in intestinal lipid homeostasis through regulation of the CG17192 digestive lipase. *E78A* mutant adults fail to maintain proper systemic lipid levels following eclosion, with this effect largely restricted to the intestine. Transcriptional profiling by RNA-seq revealed a candidate gene for mediating this effect, encoding the predicted adult intestinal lipase CG17192. Intestine-specific disruption of *CG17192* results in reduced lipid levels similar to that seen in *E78A* mutants. In addition, dietary supplementation with free fatty acids, or intestine-specific expression of either *E78A* or *CG17192*, is sufficient to restore lipid levels in *E78A* mutant adults.

Conclusion: These studies support the model that E78A is a central regulator of adult lipid homeostasis through its effects on *CG17192* expression and lipid digestion. This work also provides new insights into the control of intestinal lipid uptake and demonstrate that nuclear receptors can play an important role in these pathways.

KEYWORDS

development, gene regulation, lipid digestion, metabolism, nuclear receptors

1 | INTRODUCTION

Lipid metabolism is a critical part of systemic physiology, with misregulation of these pathways contributing to the increasing prevalence of obesity worldwide. The impact of this disorder on public health has promoted extensive research into the mechanisms that underlie the balance between lipid synthesis (lipogenesis) and lipid breakdown (lipolysis). These studies have provided significant insights into the regulatory mechanisms that maintain lipid homeostasis and demonstrated that these pathways are conserved through evolution, from insects to mammals.[1,2] Systemic lipid levels, however, are also maintained through a third pathway that involves the breakdown and import of dietary fat. Triglycerides obtained through the diet are not readily absorbed by the digestive system. Rather, these fats are first digested in the lumen by secreted lipases, liberating free fatty acids that can then be transported into intestinal enterocytes and used as substrates for reesterification and subsequent

lipid trafficking to peripheral tissues. Digestive lipases include a range of different enzymes, with gastric and pancreatic lipases playing a central role in human lipid digestion, and their orthologs exerting conserved functions in insects.[3-6] Consistent with this, diseases that disrupt pancreatic function have severe effects on nutrient uptake and human health, leading to hypolipidemia and hypocholesterolemia.[7,8] In addition, drugs that target digestive lipases provide a safe and effective treatment for obesity.[9] These drugs never enter the body and form a covalent bond with the conserved lipase active site, inhibiting their activity.[10] As a result, dietary triglycerides remain intact and are excreted from the body. The most widely used gastric lipase inhibitor is Orlistat, which acts in an evolutionarily-conserved manner to promote a lean phenotype in mammals and insects.[1,4,10]

In this study, we describe a role for the E78A nuclear receptor in the transcriptional control of a critical digestive lipase in *Drosophila*. Nuclear receptors comprise a family of ligand-regulated transcription factors that play central roles in development, hormone signaling, and metabolism. They are defined by a conserved zinc finger DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) that can interact with small lipophilic compounds. Although not all nuclear receptors have cognate ligands, those that do can switch their transcriptional activity in response to ligand binding thereby directing a global switch in downstream gene expression. The E78 nuclear receptor is related to the mammalian Rev-Erb and Peroxisome Proliferation Activated Receptor (PPAR) subfamilies.[11,12] Rev-Erb receptors control circadian rhythms and metabolism while PPARs play a central role in lipogenesis and lipid homeostasis.[13,14] Interestingly, the PPARγ agonist pioglitazone can act through E78 in *Drosophila* motor neurons, suggesting that the sequence similarity between these nuclear receptors is of functional significance.[15] In addition, the E78 LBD is active in tissues that are high in lipids, including the fat body, oenocytes, and embryonic yolk, consistent with the role of fatty acids in activating PPAR family members.[16] Taken together, these observations suggest that genetic studies of E78 in *Drosophila* may provide insights into PPAR function and regulation.

E78 was originally isolated and characterized based on its correspondence to a puff in the giant larval salivary gland polytene chromosomes.[17,18] This puff, at cytogenetic location 78C, is part of the transcriptional cascade triggered by the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis.[17,19] E78 encodes two protein isoforms: E78A, which corresponds to the full-length protein consisting of a DBD and LBD, and E78B, which is truncated to include only the LBD and shared C-terminal sequences. An initial study of *E78* mutations showed that the loss of this locus has no effect on development and only minor effects on the puffing response to ecdysone.[17] More recently, *E78* mutants were shown to be female sterile due to defects in germline stem cell niche formation and follicle cell survival during oogenesis.[20]

Our studies here focus on the E78A isoform, which contains both the DBD and LBD and thus can have a direct effect on gene regulation in the animal. Consistent with previous genetic studies, *E78A* mutants develop normally to adulthood. Metabolite assays, however, revealed that these animals have normal lipid levels upon eclosion but display an approximate 30% reduction in whole body triglycerides by 8 days of adulthood. This adult-specific reduction in lipid levels is largely restricted to the intestine suggesting that it might be due to defects in lipid uptake or transport. Expression profiling by RNA-seq identified a previously uncharacterized, predicted intestinal digestive lipase encoded by *CG17192* as one of the most highly reduced transcripts in *E78A* mutant adults. We show that *CG17192* is required in the intestine to maintain systemic lipids levels. Moreover, dietary supplementation with free fatty acids, or intestine-specific expression of either *E78A* or *CG17192*, is sufficient to restore lipid levels in *E78A* mutant adults. These results demonstrate that E78A plays an important role in maintaining adult lipid levels through regulation of the CG17192 digestive lipase in the intestine.

2 | RESULTS

2.1 | Characterization of *E78A* mutants

We generated two *E78A* mutant alleles using CRISPR/Cas9 technology. These alleles contain deletions within the first protein-coding exon resulting in a frameshift, an early stop codon, and a predicted truncated protein that lacks the DBD and LBD (Figure 1A). The presence of the deletions was validated by DNA sequencing of PCR-amplified fragments from homozygous mutant animals (Figure 1B). These mutant alleles were outcrossed to w^{1118} to provide a consistent genetic background, and a transheterozygous combination of both alleles were used for all functional studies ($E78^{\Delta 18}/E78^{\Delta 37}$). From here on we refer to these animals as *E78A* mutants, with controls corresponding to w^{1118} animals. As expected, the levels of *E78A* mRNA in mutant adults are significantly reduced, likely due to nonsense-mediated decay as a result of the premature stop codon (Figure 2A). We conclude that these *E78A* mutations result in a loss-of-function for the locus.

E78A mutants are viable throughout development, with adults displaying normal locomotor responses

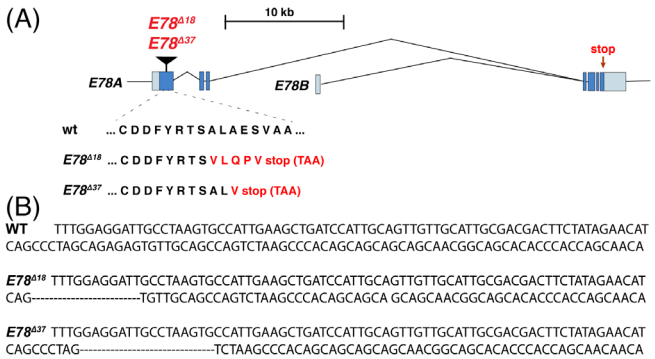


FIGURE 1 *E78A* deletion mutants. A, A schematic representation of the *E78* genomic locus is depicted including protein-coding regions (blue), untranslated regions (light blue), and the stop codon for the *E78A* and *E78B* mRNA isoforms. The *E78A*-specific mutations $E78^{\Delta 18}$ and $E78^{\Delta 37}$ are depicted along with the corresponding protein sequences spanning amino acids 158-173 in wild-type E78A (wt). B, *E78* DNA sequences are depicted from wild-type (WT), $E78^{\Delta 18}$ mutants and $E78^{\Delta 37}$ mutants, with the deleted regions marked by dashes

(Figure 2C). The length of larval development, as assayed by the timing of puparium formation, is normal in mutants (Figure 2D). Similarly, *E78A* mutant adults eclose at the same time as controls (Figure 2E). These observations are consistent with an earlier study that reported no obvious role for the *E78* locus in development.[17] In contrast with the reported sterility of *E78* mutant females,[20] however, *E78A* mutant females are fertile, although this trends toward reduced levels (Figure 2F).

2.2 | *E78A* regulates adult lipid homeostasis

To determine if *E78A* plays a role in maintaining metabolic homeostasis, we measured the major forms of available and stored energy in mutant and control males at different stages of development. Mutants have normal

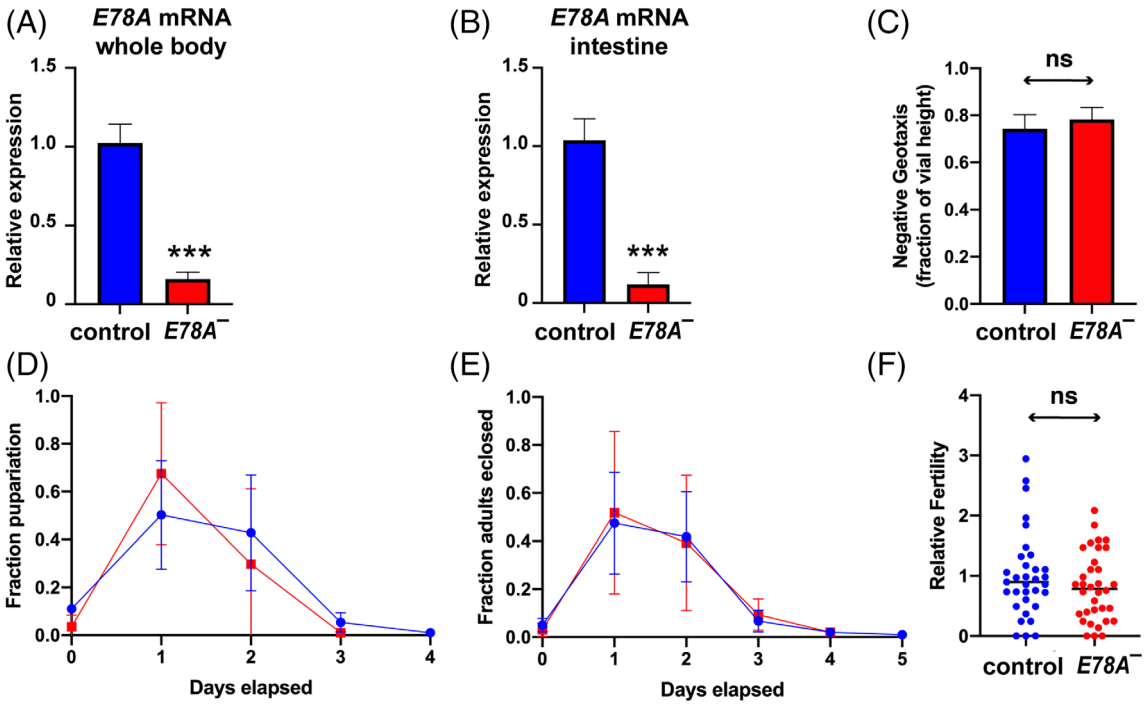


FIGURE 2 *E78A* mutants develop normally. A and B, RT-qPCR was used to assess *E78A* mRNA levels in control (blue) and *E78A* mutant (red) whole animals (A) or dissected adult intestines (B). Transcript levels were normalized to *rp49* and presented relative to control levels. n = 5 independent samples with 7-10 flies per sample. (***) $P \leq .001$. C, Locomotor activity was assayed using the RING assay for controls (blue) and *E78A* mutants (red). n = 9 to 11 independent samples with 8 to 10 animals per sample. D, Developmental timing was assayed by counting the fraction of control (blue) and *E78A* mutant (red) prepupae formed per day after animals began pupariating. n = 52 to 53 independent samples with 10 animals per sample. Data represents the mean ± SD. E, Adult eclosion was assayed by counting the fraction of control (blue) and *E78A* mutant (red) flies that emerged from their pupal cases per day, scored after animals began eclosing. n = 52 to 54 independent samples with 10 flies per sample. Data represents the mean ± SD. F, Fecundity was assayed using single control males crossed to a single control female (blue) or an *E78A* mutant female (red). Following a 24 hours mating period, pairs were cleared and adult progeny were quantified. Data is presented relative to the fertility of control females with the bars representing the median. n = 33 to 34 single mating pairs

metabolite levels at the end of larval development, suggesting that *E78A* does not contribute to nutrient uptake and storage at this stage (Figure 3A-C). This observation is consistent with the normal timing of puparium formation in *E78A* mutants, which is indicative of the proper storage of nutrients for adult development during metamorphosis. Glycogen levels are decreased slightly following metamorphosis, while other metabolites remain unaffected (Figure 3D-F). In contrast, mature mutant males display a ~30% reduction in triglyceride levels (Figure 3G). The reduced glycogen levels seen in newly-eclosed mutants appear to normalize in mature animals, potentially through the dietary uptake of carbohydrates (Figure 3H). Consistent with this, *E78A* mutant adults display a normal feeding rate (Figure 3J). This observation also suggests that the reduced lipids that appear during early adulthood arise from an imbalance in metabolism rather than a defect in nutrient intake. Finally, mature *E78A* mutant females also display a decrease in triglyceride levels, suggesting that similar regulation of lipid metabolism is occurring in both sexes (Figure 3K).

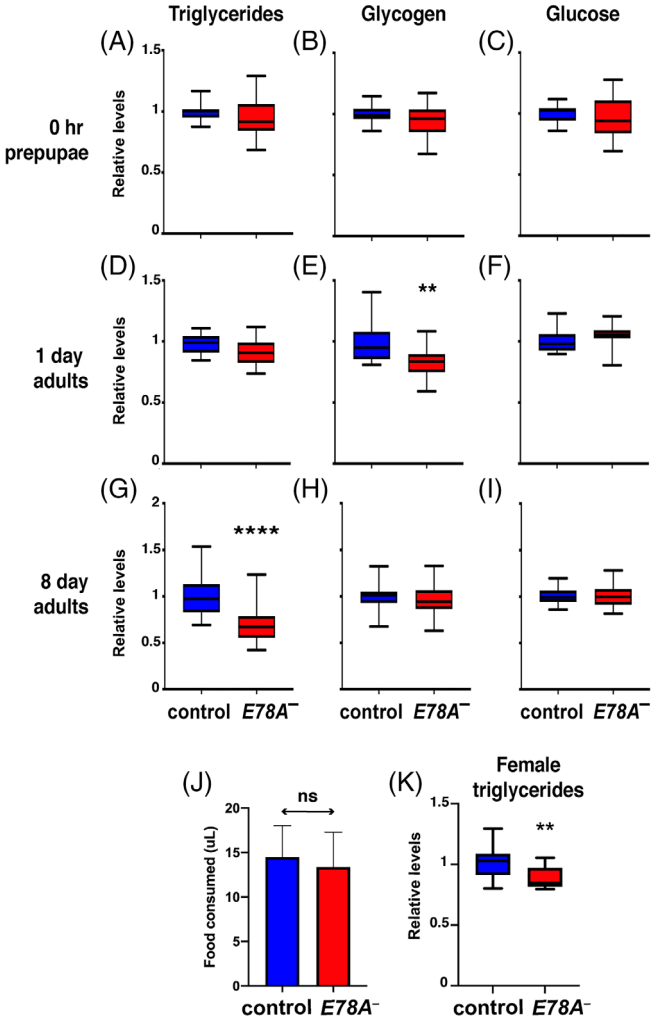


FIGURE 3 *E78A* mutants display reduced lipid levels as mature adults. Triglycerides (A, D, G), glycogen (B, E, H), and glucose (C, F, I) were assayed in male controls (blue) and male *E78A* mutants (red) as white prepupae (A-C), 1-day adults (D-F), and 8-day adults (G-I). Data is presented relative to the amount in controls. n = 10 to 48 independent samples with five animals per sample. $^{**}P \leq .01$; $^{****}P \leq .0001$. Panels with no *P* value depicted have no significant difference between samples. J, Feeding rate was measured using the CAFE assay in controls (blue) and *E78A* mutants (red) over a 24-hour period. Animals at 8 days of adulthood reared on a control diet were assayed. n = 30 to 40 independent samples with 9 to 10 flies per sample. K, Triglycerides were assayed in female controls (blue) and female *E78A* mutants (red) as 8 days mated adults. Data are presented relative to the amount in controls. n = 12 independent samples with five animals per sample. $^{**}P \leq .01$

To localize the lipid defects in *E78A* mutants, we stained dissected tissues from control and mutant animals at 8 days of adulthood using the lipophilic dye BODIPY. Interestingly, this revealed that the majority of reduced lipid stores in *E78A* mutants are localized to the intestine where we consistently see reduced levels of neutral lipid staining (Figure 4A,B). Most mutant fat tissue showed no clear change in lipid levels (Figure 4C,D). However, ~40% of the fat bodies from *E78A* mutants displayed lower levels of BODIPY staining than was seen in controls, which displayed reduced staining in ~20% of the samples, suggesting that there might be a mild effect on this tissue (Figure 4E,F). Taken together, we conclude that *E78A* is required in young adults to accumulate normal lipid stores, and this defect appears to arise primarily from a role for *E78A* in the adult intestine.

2.3 | *E78A* regulates expression of the *CG17192* intestinal lipase gene

As a first step toward defining the molecular mechanisms by which *E78A* regulates adult lipid homeostasis, we performed RNA-seq analysis on controls and *E78A* mutants at 8 days of adulthood. Through this analysis we identified 150 significantly misregulated transcripts, with 85 genes expressed at reduced levels and 65 genes expressed at increased levels in *E78A* mutants (Table S1). The larger number of genes expressed at reduced levels is consistent with the predominant role of nuclear receptors as transcriptional activators and the likelihood that these down-regulated genes include direct targets of E78A. No major gene ontology category emerges from informatic analysis of the genes that change expression in *E78A* mutants. We noticed, however, that several genes that are reduced in expression in mutants are normally expressed in the intestine. To examine this more broadly,

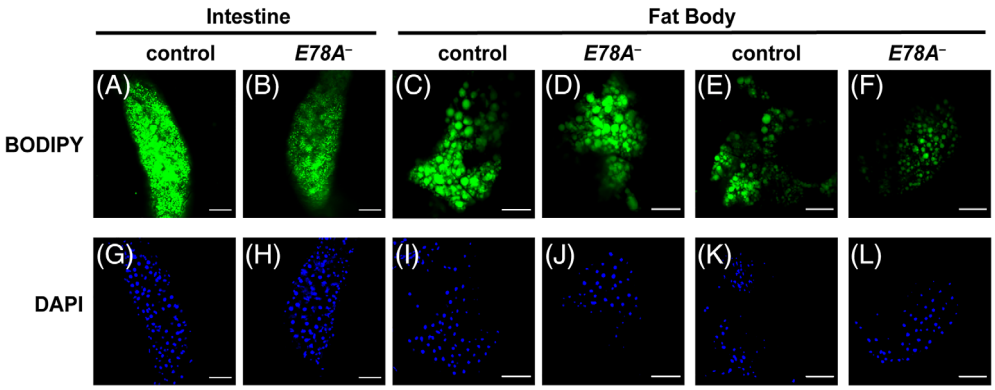


FIGURE 4 *E78A* mutants have reduced intestinal lipid levels. Dissected intestines and fat bodies from controls (A, C, E, G, I, K) and *E78A* mutants (B, D, F, H, J, L) were stained with BODIPY to detect neutral lipids (green, A-F) or DAPI to stain nuclei (blue, G-L). The R2 region of the gut is presented (A, B, G, H). Representative images are shown for the intestines from z stacks of seven controls (A, G) and 15 mutants (B, H). Scale bar, 50 μm. Representative images are shown for the fat bodies from z stacks of 11 controls (C, I, E, K) and 10 mutants (D, J, F, L). Scale bar, 50 μm

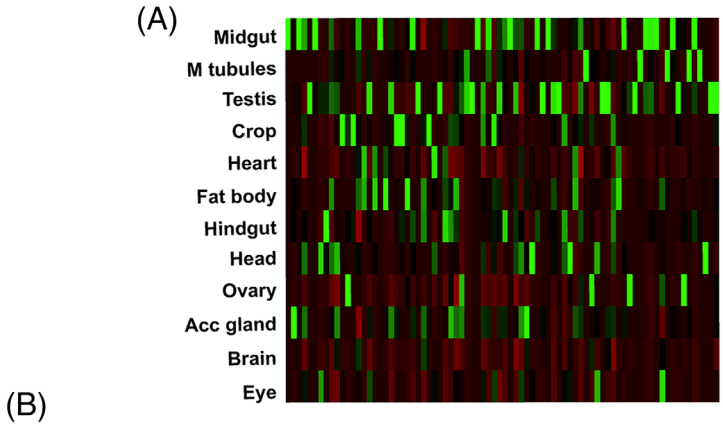
we generated a heat map of the expression levels of *E78A* down-regulated genes in wild-type tissues as reported by FlyAtlas.[21] Interestingly, this revealed that many of these genes are normally expressed in the adult midgut as well as the testis, while expression in other tissues appears more varied (Figure 5A). Consistent with this, *E78A* is expressed in the adult intestine and this expression is reduced in *E78A* mutant animals (Figure 2B). This observation is consistent with the reduced lipid levels in *E78A* mutant intestines and suggests that the receptor plays an important role in adult intestinal function.

To more directly address potential metabolic functions for E78A, we compared our RNA-seq dataset to a list of genes that act in metabolism in *Drosophila*.[22] Interestingly, the third gene that is expressed at reduced levels on this list is a predicted lipase encoded by *CG17192* (Figure 5B). This is the fifteenth most down-regulated gene in *E78A* mutant adults (Table S1). In addition, *CG17192* is specifically and abundantly expressed in the adult intestine, positioning it as a potential key target gene to explain the reduced lipid levels in *E78A* mutants.[21,23] BLAST analysis of the predicted 337 amino acid CG17192 protein product against the human protein database revealed close homology to multiple human gastric and pancreatic lipases. These regions of sequence identity include the classic α/β fold that defines the active site of digestive triacylglycerol lipases.[24,25] This identity raises the interesting possibility that reduced expression of *CG17192* in *E78A* mutants could contribute to their adult-specific lipid defect by reducing their ability to break down and absorb dietary fats.

We used RT-qPCR to examine *CG17192* mRNA levels in *E78A* mutant adults and confirmed that its expression level is significantly reduced in these animals (Figure 5C). Moreover, intestinal-specific depletion of *CG17192* by RNAi in otherwise wild-type animals is sufficient to reduce systemic lipid levels by ~30% (Figure 5D). This phenotype resembles that of *E78A* mutant adults (Figure 3G). It is also consistent with the proposed function of CG17192 as a digestive lipase based on its sequence identities. Taken together, these observations support the model that the intestinal lipase encoded by *CG17192* represents a critical target for *E78A* regulation in young adults, contributing to their ability to generate lipid reserves through the efficient breakdown of dietary fat.

2.4 | Dietary and genetic rescue of the lipid defects in *E78A* mutants

If the reduced lipid levels seen in *E78A* mutants arise from an inability to efficiently digest dietary fat, then this phenotype should be rescued, at least in part, by providing free fatty acids in the diet. This should effectively bypass the need for the breakdown of dietary triglycerides and provide sufficient substrate for triglyceride synthesis within the animal. Accordingly, control and *E78A* mutants were allowed to eclose onto either a control yeast and sugar diet, or the same diet supplemented with free fatty acids (0.5% stearic and oleic acid), and assayed for triglyceride levels at 8 days of adulthood. As expected, triglycerides are significantly reduced in *E78A* mutants on the control diet (Figure 6A, red). Control animals also show an increase in lipid levels when allowed to eclose on the free fatty acid supplemented diet (Figure 6A, light blue). This is consistent with previous studies of diet-induced obesity in *Drosophila*.[26] Importantly, the reduced



(B)

Gene	Function	Metabolic Pathway	log2FoldChange	padj
CG31683	Lecithin:cholesterol/phospholipid:diacylglycerol acyltransferase	Glycerophospholipid Metabolism	-2.2	4.1E-05
CG31809		Short-chain dehydrogenase/reductase SDR	-1.8	1.1E-06
CG17192	Lipase	Lipase	-1.4	1.3E-04
CG9825		Major Facilitator Superfamily Transporter	-1.1	2.4E-02
CG2110	Cyp4ad1	Cytochrome P450	-1.1	2.3E-05
CG10391	Cyp310a1	Cytochrome P450	-1.0	5.1E-04
CG17751		Major Facilitator Superfamily Transporter	-0.9	1.7E-04
CG1944	Cyp4p2	Cytochrome P450	-0.9	4.2E-05
CG15221		Major Facilitator Superfamily Transporter	-0.8	1.2E-03
CG5999	glucuronosyltransferase	Pentose and Glucuronate Interconversions	-0.8	3.9E-03

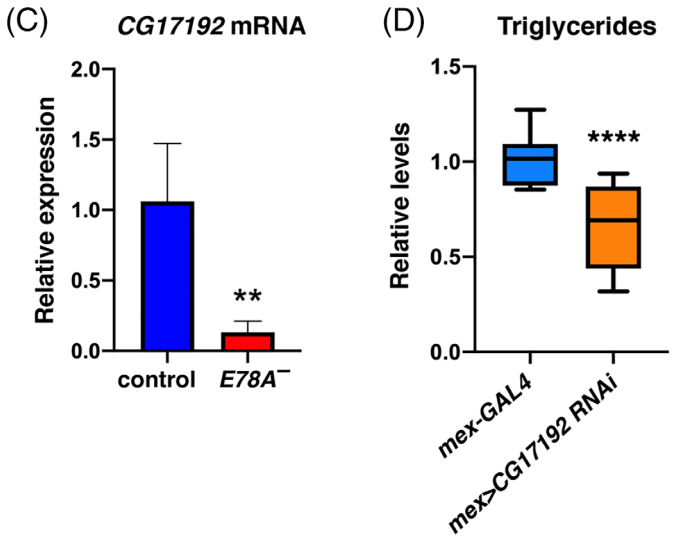


FIGURE 5 *E78A* regulates expression of the intestinal lipase gene *CG17192*. A, Many genes that are abundantly expressed in the midgut of wild-type flies are expressed at reduced levels in *E78A* mutants. A heat map of differential mRNA levels is presented (generated using Heatmapper) with each row representing a specific adult tissue and each column representing an individual gene down-regulated in *E78A* mutants. The color corresponds to the expression level for that gene in wild-type adult tissues as reported by FlyAtlas,[21] with black representing little or no expression, red representing reduced expression, and green representing higher expression. Tissues are ordered from top to bottom by decreasing fold change in gene expression. B, Genes that function in metabolism and are expressed at reduced levels in *E78A* mutants are listed by log2-fold change. C, RT-qPCR measurement of *CG17192* mRNA levels in controls (blue) and *E78A* mutants (red). Transcript levels are normalized to *rp49* mRNA and presented relative to control levels. n = 5 independent samples with 7 to 10 flies per sample. D, Triglyceride levels in *y w mex-GAL4* controls (light blue) and *y w mex > CG17192 RNAi* animals (orange) are depicted. Data are presented relative to the amount in controls. n = 17 to 18 independent samples with five flies per sample. $^{**}P \leq .01$; $^{****}P \leq .0001$

lipids in *E78A* mutants are effectively rescued on this free fatty acid supplemented diet (Figure 6A, light red). This observation is consistent with the model that *E78A* mutants suffer from an inability to establish lipid stores from dietary fat.

The dietary rescue of *E78A* mutants suggests that the reduced expression of the triglyceride lipase CG17192 in these animals contributes to their depleted lipid stores. As a direct test of this model, we expressed *CG17192* specifically in the intestine of *E78A* mutants and assayed the effect on systemic lipid levels. In addition, we tested the tissue-specific role of *E78A* in intestines. Unfortunately, no *UAS-RNAi* constructs are available that allow us to specifically disrupt expression of the *E78A* isoform. We

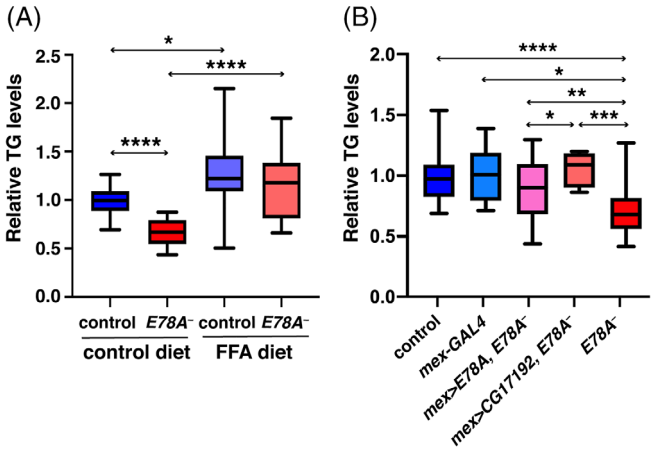


FIGURE 6 Dietary and genetic rescue of lipid levels in *E78A* mutants. A, Triglyceride (TG) levels in controls (blue) and *E78A* mutants (red) reared on a control diet were assayed, as well as controls (light blue) and *E78A* mutants (light red) reared on a diet supplemented with free fatty acids (FFA). Data is presented relative to the amount in controls reared on the control diet. n = 11 to 20 independent samples with five flies per sample. B, Triglyceride (TG) levels were assayed in controls (blue), *mex-GAL4* intestinal driver controls (light blue), animals with intestine-specific expression of *E78A* in an *E78A* mutant background (*mex* > *E78A*, *E78A*$^{-}$, pink), animals with intestine-specific expression of *CG17192* in an *E78A* mutant background (*mex* > *CG17192*, *E78A*$^{-}$, light red), and *E78A* mutants (red). n = 8 to 55 independent samples with five flies per sample. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$

could, however, test the ability of intestine-specific *E78A* expression to rescue the reduced lipid levels in *E78A* mutants.

Both control animals and animals expressing only the intestine-specific *mex-GAL4* driver display normal levels of triglycerides (Figure 6B, blue), while *E78A* mutants are hypolipidemic (Figure 6B, red). In contrast, lipid levels normalize in *E78A* mutants that express either *E78A* (Figure 6B, pink) or *CG17192* (Figure 6B, light red) in the intestine. Taken together with our other studies, these observations indicate that *E78A* plays an important role in the adult intestine in maintaining systemic lipid levels through proper expression of the CG17192 triglyceride lipase.

3 | DISCUSSION

3.1 | E78A acts through the CG17192 digestive lipase to maintain adult lipid levels

Dietary triglycerides cannot be absorbed by the digestive system. Rather, they are hydrolyzed by lipases in the lumen of the stomach and intestine, liberating fatty acids that can then be transported into enterocytes for reesterification and subsequent lipid trafficking. Although digestive lipases have been extensively studied for their enzymatic activities and physiological functions, little is known about how their expression is regulated in the animal. Here we show that a previously uncharacterized *Drosophila* lipase gene, *CG17192*, is required in the intestine to maintain systemic lipid levels, and that its expression is dependent on the E78A nuclear receptor. *E78A* mutants display normal lipid stores when they eclose as young adults, but are unable to maintain their lipid levels during the first week of adulthood (Figure 3). This adult-specific reduction in stored fat is largely restricted to the intestine, suggesting that it arises from a defect in lipid metabolism in this tissue (Figure 4). Consistent with this, the *CG17192* predicted lipase gene is expressed specifically and abundantly in the adult intestine, and is reduced in expression in *E78A* mutant adults (Figure 5C). This adult-specific expression is consistent with the early adult onset of the lean phenotype in *E78A* mutants. Intestine-specific depletion of *CG17192* results in a hypolipidemic phenotype similar to *E78A* mutants (Figure 5D). In addition, restoring either *E78A* or *CG17192* in the intestine of *E78A* mutants is sufficient to rescue their lipid levels (Figure 6B). These results, taken together, support the model that E78A normally maintains proper levels of *CG17192* expression in the adult intestine, facilitating dietary lipid digestion and systemic lipid homeostasis (Figure 7).

Drosophila begin their adult life with almost no lipid in their fat bodies. Rather, they depend on stored triglycerides that are maintained through metamorphosis from larval stages and consumed during the first days of adulthood.[27] Adults begin feeding at this time and depend on dietary sources to establish their proper systemic lipid levels by one week of age.[28] *E78A* and *CG17192* are both required for this adult-specific accumulation of stored fat. This positions these factors as critical parts of this adult-specific developmental switch and indicates that dietary lipids comprise an important source for establishing adult lipid levels.

It is also important to note that almost all of the studies reported here were conducted in males. Females, in contrast, have different levels of metabolic control in order to balance energy homeostasis with the remarkable demands of reproduction. In spite of this altered physiology, however, we observe reduced triglyceride levels in *E78A* mutant females, suggesting that similar levels of regulation are active in both sexes (Figure 3G,K). Future studies could expand upon this effort to determine how *E78A* regulates adult female physiology.

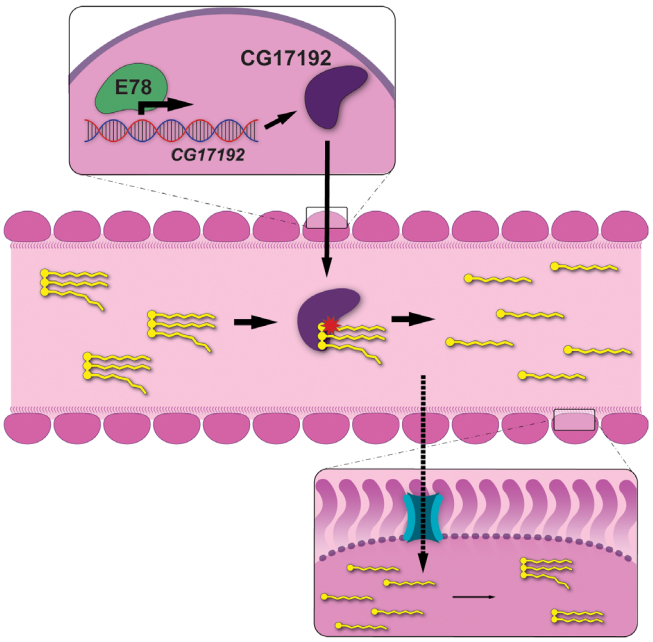


FIGURE 7 Schematic representation of *E78A* regulation of dietary lipid uptake. *E78A* transcriptionally regulates the intestine-specific lipase CG17192, which is secreted into the lumen. CG17192 degrades dietary triglycerides into free fatty acids that can be transported into enterocytes. These free fatty acids are reesterified into diacylglycerides and triglycerides, which can be utilized as energy sources or transported to other tissues in the fly

3.2 | Nuclear receptors regulate digestive lipases

Although little is known about the regulation of digestive lipase gene expression, nuclear receptors have emerged as important players in this process. Studies of the *Drosophila* DHR96 nuclear receptor provide the closest parallel to the results described here. DHR96 maintains normal systemic lipid levels through its regulation of an intestinal digestive lipase encoded by *magro*.[4] This general function also appears to be conserved through evolution. The mammalian DHR96 homolog, LXR, is required for proper pancreatic exocrine secretion, accounting at least in part for the hypolipidemia seen in *LXR* mutant mice.[29] Similarly, the SRC-2 coactivator, which regulates mammalian nuclear receptor function, plays an important role in intestinal fat absorption.[30] Taken together, these studies show that nuclear receptors are important regulators of lipid digestion and absorption.

Interestingly, DHR96 binds cholesterol as a ligand and thus is positioned to act as a dietary fatty acid sensor. The uptake and digestion of dietary lipids could generate increased levels of free fatty acids that could activate DHR96, supporting a feed-forward pathway for triglyceride hydrolysis through the induced expression of *magro*.[4,31] Although no ligand is known for E78A, its LBD is active in embryonic tissues that are rich in lipids.[16] In addition, an agonist for a mammalian homolog of E78A, PPARγ, can act through E78 in *Drosophila* neurons.[15] These observations raise the interesting possibility that E78A might be ligand regulated and could contribute to coupling dietary lipid uptake with nutrient digestion. Moreover, PPARδ and PPARγ are expressed in the intestine and pancreas, supporting a possible role for these nuclear receptors in digestion and nutrient uptake.[32,33] Further studies are needed to determine if the regulatory functions of E78A described here are conserved in mammals. In this regard, however, it is interesting to note that some of the unique properties of rodents might complicate these functional studies. Unlike most mammals, mice rely on lingual lipases for lipid digestion.[34,35] Whether these lingual lipases are subject to the same regulation as gastric and pancreatic lipases remains to be determined.

3.3 | Distinct functions for E78 isoforms

Our studies confirm earlier work showing that *E78* has no significant role during development.[17] However, in contrast to a previous report,[20] we observed no effect of the *E78A* mutation on female fertility (Figure 2F). Our analysis of female fertility in *E78* mutants used different diets and assays from the published work, which could account for some of the disparity between these studies. In addition, that study used a deletion mutant that removes both the *E78A* and *E78B* protein-coding mRNA isoforms, raising the possibility that *E78B*, either alone or in combination with *E78A*, is required for female fertility. The *E78B* isoform encodes a truncated protein with a unique amino-terminal sequence fused to a common carboxy-terminal sequence shared with E78A.[17,18] As a result, *E78B* encodes the LBD, but not the DBD of the E78 nuclear receptor, suggesting that it can exert indirect effects on transcription. In addition, *E78A* and *E78B* appear to be under distinct temporal regulation during development.[18,36] Further studies are required to elucidate the regulatory roles of *E78B* in development and reproduction.

Although this study focuses on the role of *E78A* in regulating *CG17192* and systemic lipid homeostasis, it is important to note that this nuclear receptor appears to control multiple other pathways in the animal (Table S1). No predominant gene ontology category emerged from our transcriptional profiling study of *E78A* mutants. In spite of this, however, a number of target genes provide interesting directions for future research. For example, the gene encoding ETH (ecdysis triggering hormone) is

within the top ten genes that are expressed at reduced levels in *E78A* mutants (Table S1). ETH is a critical neuropeptide hormone that regulates ecdysis in insects, by which they shed their cuticle at the end of each molt.[37,38] This includes the multiple developmental steps involved in adult eclosion.[37,39] Combined with the expression of *E78A* in mid-pupae, this raises the possibility that *E78A* mutants may display defects in this process. In addition, ETH regulates female fertility suggesting that it might contribute to the reduced fecundity of *E78* null mutant females.[20,40] Further studies are required to address the significance of the reduced expression of ETH in *E78A* mutants.

Interestingly, the genes encoding all three members of the ionotropic receptor 75 family are also expressed at reduced levels in *E78A* mutants: *Ir75a*, *Ir75b*, *and Ir75c* (Table S1). These genes are located next to one another in the genome as a tandem array, suggesting that their transcription is coordinately regulated by E78A. These receptors appear to be involved in the sensory detection of acids, with Ir75a acting as an acetic acid receptor in *Drosophila*.[41,42] Further studies could address possible defects in the behavioral response to acidic stimuli in *E78A* mutants.

3.4 | Temporal and spatial regulation of *E78* expression

E78 was originally identified and characterized based on its correspondence to the 78C ecdysone-induced puff in the giant salivary gland polytene chromosomes.[17,18] Consistent with this, *E78B* mRNA is induced directly by the steroid hormone ecdysone.[18] In contrast, little is known about the temporal regulation of *E78A*, although the mRNA is detectable in mature adults (Figure 2A,B). Efforts to raise antibodies directed against the E78A protein were unsuccessful. Further studies are required to determine when and where the distinct *E78* isoforms are expressed during development. This could, in turn, provide new directions for understanding the stage-specific roles of the two *E78* isoforms during *Drosophila* development.

4 | EXPERIMENTAL PROCEDURES

4.1 | *Drosophila* strains and media

Drosophila were reared on a diet containing 8% yeast, 9% sugar, 1% agar, 0.05% $MgSO_4$, and 0.05% $CaCl_2$. Tegosept (10 mL/L) and propionic acid (6 mL/L) were added prior to pouring. Mutations in *E78A* were generated using CRISPR-Cas9 as described[43] with the guide RNA sequence: GGCTGCAACACTCTCTGCTAGGG. Stocks were established using single males, which were then sequenced to confirm the presence of the deletions: the 13 bp $E78^{\Delta 18}$ deletion and the 19 bp $E78^{\Delta 37}$ deletion (Figure 1B). Genetic studies of *E78A* mutants were performed using a transheterozygous combination of the $E78^{\Delta 18}$ (Bloomington 91370) and $E78^{\Delta 37}$ (Bloomington 91371) alleles. These mutant stocks were outcrossed to w^{1118} to provide a consistent genetic background, which was then used as a control for all studies. For the free fatty acid feeding experiment, the diet was supplemented with 5 mg/mL 97% stearic acid (Acros Organic 57-11-4 1 KG) and 5 mg/mL 90% oleic acid (Aldrich Chemistry 364525-1L). Tissue-specific genetic studies were performed using *mex-GAL4* [44], *UAS-CG17192* (FlyORF F002788), and $UAS\text{-}CG17192^{RNAi}$ (Bloomington 56042). The *UAS-E78A* transformant line was generated by inserting an *E78A* cDNA into *pUAST-attB* and integrating that plasmid into an *attP* site on the third chromosome (VK31).[45] The resulting stock was then outcrossed to w^{1118} to establish a common genetic background. Unless otherwise specified, 8-day males were used for all studies, raised on the control yeast/sugar diet and transferred every 2 to 4 days.

4.2 | Developmental timing

Developmental timing was determined by setting up control and *E78A* mutant crosses on egg caps. Egg caps were transferred every 24 hours and 10 embryos were transferred from fresh egg caps to establish a vial. The fraction of total animals that pupariated and eclosed was recorded daily for each vial.

4.3 | Fecundity assays

Fecundity was assayed using single male-female mating pairs. Newly-eclosed flies were separated by sex and reared on the control diet until 5 to 9 days of adulthood. Mating pairs were transferred to fresh vials using a mouth pipette or allowed 24 hours to recover if anesthetized with CO_2. Control and mutant virgins were then crossed to control males. Following a 24 hours mating period, pairs were transferred to fresh vials and the egg lays were quantified at 24 hours. The number of adults that eclosed was quantified and recorded.

4.4 | Locomotor assays

Locomotor activity was measured using the RING assay.[46] Eight to ten flies were transferred to empty vials

with markings every half cm up the side. Motility was measured by tapping animals to the bottom of the vial for 5 seconds then allowing a 5 second recovery period. The average height of all flies over all trials was scored per vial using photos and presented as a fraction of the average height achieved. The assay was repeated for three trials per set.

4.5 | Metabolite assays

Assays for protein, glycogen, glucose, and triglycerides were performed as described.[47] Five adult male flies were placed into microcentrifuge tubes and each sample was homogenized in 100 μL of PBS. Ten microliter was set aside prior to heat-treatment to assay protein levels, the remaining lysate was treated for 10 minutes at 70°C. Triglycerides, glycogen, and glucose levels were normalized to protein and data is presented relative to the metabolite level in controls. Triglyceride assays in females were performed as described using 8 days old mated animals, except that half the amount of extract was used (15 μL) relative to males. The results were normalized to protein levels and are presented relative to the level in controls.

4.6 | Feeding rate assays

The CAFE feeding rate assay was performed as described.[48] Four-well plates were utilized that contained two holes for 20 μL capillary tubes (VWR). Ten adult males were placed into the plates by mouth pipette and the capillary tubes were filled with an 8% yeast 9% sugar liquid solution with a small, black mineral overlay to reduce evaporation and facilitate measurements. A time-lapse camera was used to collect images at 5 minute intervals (Brinno) and assays were run for 24 hours periods. Total consumption of each pair of capillary tubes per plate was quantified, summed, and recorded by the change in position of the mineral dye overlay.

4.7 | BODIPY stains

BODIPY stains were performed on dissected adult intestines and fat bodies. Intestines and abdominal cuticles with the attached adipose tissue were dissected from adult animals in PBS and fixed in 4% formaldehyde for 20 minutes on ice. Following a brief initial wash with PBS, tissues were washed three times for 5 minutes with PBS on ice. BODIPY was added at 1:1000 dilution and samples were covered and incubated for 1 hour at room temperature. Following a brief, initial wash with PBS, tissues were washed three times for 5 minutes with PBS on ice. Fat body tissue was dissected by gently scraping the inner side of the abdominal cuticle with forceps in a drop of Vectashield Vibrance Antifade mounting media with DAPI (Vector Laboratories H-1800-10) on a microscopy slide. Double-sided tape was used as spacers on either side of samples in mounting media containing DAPI. A coverslip was then deposited on top of the dissected tissues before observation by confocal microscopy. Confocal imaging was performed with a Nikon A1 laser scanning confocal microscope, images were processed for brightness and contrast, and assembled using Fiji (ImageJ) and Photoshop CS (Adobe, USA). Representative images are shown in Figure 4A-L, selected from 7 to 15 z stacks for each genotype.

4.8 | RNA-seq transcriptional profiling

RNA was isolated from samples of 15 flies using the Direct-zol RNA Miniprep Kit (Zymo Research). Four to five independent control and mutant samples were submitted for sequencing. Library generation of poly(A) selected RNAs (Illumina RNA TruSeq Stranded mRNA Library Prep kit with oligo dT selection), quality control assays (ScreenTape Assay, Kapa qPCR), and sequencing (Illumina NovaSeq 6000 flow cell using the NovaSeq XP chemistry workflow, 2×51 cycle paired end sequence run) were performed by the High-Throughput Genomics core facility at the University of Utah. The Bioinformatics Core Facility at the University of Utah aligned this dataset to the *Drosophila melanogaster* dm3 genome assembly. We restricted our analysis to identify differentially expressed genes by using a cut-off for significance of Log2 ratio $\pm$ 0.5 and P value $\leq$ .05. RNA-Seq data from this study can be accessed at NCBI GEO (GSE152031).

4.9 | RT-qPCR

RNA was extracted from samples of 10 to 15 animals using a Direct-zol RNA Miniprep Kit (Zymo Research) or NucleoSpin RNA Kit (Macherey-Nagel 740955-50). First-strand cDNA was synthesized using 0.5 μg RNA, Superscript Reverse Transcriptase II (ThermoFisher Scientific, 18064-014), and oligo(dT) primers (Invitrogen, 18418012). qPCR experiments were performed on cDNA as described using an Applied Biosystem Quantstudio 3 device and the SYBR GreenER qPCR SuperMix Universal kit (ThermoFisher Scientific 11762100).[28] ROX Reference Dye was diluted 10 times before use and 0.1 μL was added for a final reaction volume of 20 μL. Fold inductions in transcript level were determined using the ΔΔCt method. Transcript

levels were normalized to *rp49*. The forward and reverse primers used in our qPCR experiments are as follows: *E78A*: CAGCATCACCGGGATTTGGA; GGCTGCAACAC TCTCTGCTA; *CG17192*: GCACTATTAGTAGCGGGTAA TGC; GGACACTTCATTGGAACGGAA; *rp49*: GACGCTT CAAGGGACAGTATCTG; AAACGCGGTTCTGCATGA. Sequences for the *CG17192* primer set were obtained from FlyPrimerBank.[49] qPCR experiments were conducted on five independent *Drosophila* cDNA samples and primers were used after confirming an efficiency between 90% and 110%.

4.10 | Statistical analysis

GraphPad PRISM 6 software was used for graphical representation and statistical analysis throughout. For box and whisker plots, whiskers extend from the minimum and maximum of the dataset, horizontal lines represent the median, and boxes extend from the 25th to 75th percentiles. Statistical comparisons were performed using a Student *T* test with Welch's correction for unequal variances. For multiple comparisons, one way ANOVAs were performed followed by Tukey's multiple comparisons test. Statistical analysis of RNA-seq data is described above.

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AUTHOR CONTRIBUTIONS

Sophia Praggastis: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft; writing-review and editing. **Geanette Lam:** Investigation; methodology. **Michael Horner:** Conceptualization; investigation. **Hyuck-Jin Nam:** Conceptualization; formal analysis; methodology. **Carl Thummel:** Conceptualization; funding acquisition; project administration; resources; supervision; writing-original draft; writing-review and editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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