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Article

The DHR96 Nuclear Receptor Controls Triacylglycerol Homeostasis in *Drosophila*

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SUMMARY

Triacylglycerol (TAG) homeostasis is an integral part of normal physiology and essential for proper energy metabolism. Here we show that the single *Drosophila* ortholog of the PXR and CAR nuclear receptors, DHR96, plays an essential role in TAG homeostasis. *DHR96* mutants are sensitive to starvation, have reduced levels of TAG in the fat body and midgut, and are resistant to diet-induced obesity, while *DHR96* overexpression leads to starvation resistance and increased TAG levels. We show that *DHR96* function is required in the midgut for the breakdown of dietary fat and that it exerts this effect through the *CG5932* gastric lipase, which is essential for TAG homeostasis. This study provides insights into the regulation of dietary fat metabolism in *Drosophila* and demonstrates that the regulation of lipid metabolism is an ancestral function of the PXR/CAR/DHR96 nuclear receptor subfamily.

INTRODUCTION

Fat metabolism is central to the process of energy homeostasis. When nutrients are abundant, dietary fat in the form of triacylglycerol (TAG) is broken down by gastric TAG lipases to release fatty acids. These fatty acids are absorbed by the intestine and used to resynthesize TAG in peripheral tissues. These TAG reserves can be accessed upon nutrient deprivation through the action of specific lipid droplet-associated lipases that release the fatty acids for energy production through mitochondrial fatty acid β-oxidation. Defects in these processes can lead to dramatic changes in TAG levels and a range of physiological disorders, including obesity, diabetes, and cardiovascular disease. The alarming rise in the prevalence of these disorders in human populations has focused attention on understanding the molecular mechanisms that coordinate dietary nutrient uptake with TAG homeostasis. As a result, many regulators of TAG metabolism have been identified, including SREBP, PPAR, and adiponectin. In spite of these advances, however, the molecular mechanisms that coordinate dietary fat uptake, synthesis, storage, and utilization remain poorly understood.

Nuclear receptors (NRs) are ligand-regulated transcription factors that play a central role in metabolic control. They are defined by a conserved zinc-finger DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) that can impart multiple functions, including hormone binding, receptor dimerization, and transactivation. Many NRs are regulated by small lipophilic compounds that include dietary signals and metabolic intermediates, and exert their effects by directing global changes in gene expression that act to maintain metabolic homeostasis. This is exemplified by members of the mammalian PPAR, LXR, and FXR subfamilies, which play critical roles in adipogenesis, lipid metabolism, and cholesterol and bile acid homeostasis, respectively (Chawla et al., 2001; Sonoda et al., 2008).

In this study we use the fruit fly, *Drosophila melanogaster*, as a simple model system to characterize the NR subfamily represented by the Pregnane X Receptor (PXR, NR1I2), Constitutive Androstane Receptor (CAR, NR1I3), and Vitamin D Receptor (VDR, NR1I1) in mammals. Previous studies have defined central roles for these receptors in sensing xenobiotic compounds and directly regulating genes involved in detoxification (Timsit and Negishi, 2007; Willson and Kliewer, 2002). Initial studies showed that the single ancestral *Drosophila* ortholog of this NR subclass, DHR96, has similar functions (King-Jones et al., 2006). A *DHR96* null mutant displays increased sensitivity to the sedative effects of phenobarbital and the pesticide DDT as well as defects in the expression of phenobarbital-regulated genes. These studies, however, revealed other potential roles for the receptor—in particular, unexpected effects on the expression of genes that are predicted to regulate lipid and carbohydrate metabolism (King-Jones et al., 2006). This observation is in line with recent studies that have implicated roles for the mammalian PXR and CAR NRs in metabolic control (Moreau et al., 2008). The molecular mechanism by which they exert this effect, however, remains undefined.

Here we show that *DHR96* null mutants are sensitive to starvation and have reduced levels of TAG, while DHR96 overexpression leads to starvation resistance and elevated TAG levels. A series of studies using metabolic assays, diets, and the drug Orlistat revealed that *DHR96* mutants are defective in their ability to break down dietary lipid. This model was supported by microarray studies, which showed that many genes expressed in the midgut are misregulated in *DHR96* mutants, including highly reduced expression of the gastric lipase gene *CG5932*. We show that *CG5932* is required for proper whole-animal TAG levels, and that selective expression of *CG5932* in the midgut of *DHR96* mutants is sufficient to rescue their lean phenotype. Taken together, these data support a role for the PXR/CAR/DHR96 NR subclass in lipid metabolism and define *DHR96* as a key regulator of dietary TAG breakdown in the *Drosophila* midgut.

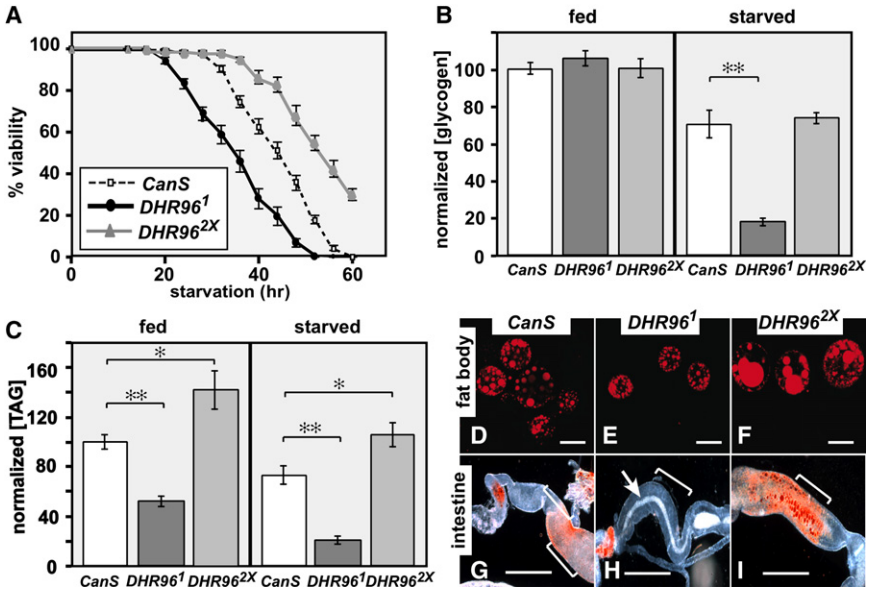

Figure 1. *DHR96* Mutants Are Sensitive to Starvation and Display Decreased Levels of TAG

(A) Mature adult male *Canton-S* (*CanS*) control flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain were subjected to complete starvation, and the number of surviving animals was determined at 4 hr intervals. Results are presented as a percent of the total population, which consisted of 15 groups of 20 animals for each genotype. Similar results were obtained in a second independent experiment as well as from adult flies staged at 1–2 days after eclosion.

(B and C) Total glycogen (B) and TAG (C) levels were measured in *CanS* control flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain under both fed conditions and after a 20 hr starvation. Glycogen and TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%.

(D–F) Nile red staining of dissected fat body cells from 1- to 2-day-old adult male flies shows reduced cell size and lipid droplet size in *DHR96*^{1} mutants (E) and increased lipid droplet size in *DHR96*^{2X} animals (F), relative to *CanS* controls (D).

(G–I) Oil red O staining of dissected adult midguts shows reduced lipid accumulation in the *DHR96*^{1} mutant (H) and enlarged lipid droplets in the *DHR96*^{2X} overexpression strain (I), relative to *CanS* controls (G). The white brackets mark comparable regions of the midgut epithelium. The lumen of the midgut can be seen in the *DHR96*^{1} mutant (arrow). Scale bars are 75 μm (D–F) or 400 μm (G–I). Error bars represent ±SE, *$p < 0.05$ and **$p < 0.001$.

RESULTS

DHR96 Mutants Are Lean and Sensitive to Starvation

Previous studies have shown that *DHR96*^{1} null mutants, grown under normal conditions, are viable and fertile with no apparent developmental defects (King-Jones et al., 2006). To assess potential metabolic roles for *DHR96*, we examined the ability of mutants to survive a period of complete starvation. All studies were performed using *Canton-S* wild-type flies (*CanS*) and *DHR96*^{1} null mutants that had been crossed to *CanS* through nine generations of free recombination. Mature male flies were collected as 5- to 7-day-old adults, transferred to starvation media, and the number of living animals scored every 4 hours. *DHR96*^{1} mutants die at a faster rate than the wild-type controls (Figure 1A). We also tested *DHR96*^{2X} animals, which carry two transgenic copies of *DHR96* along with the endogenous wild-type locus. These flies express approximately 2-fold more DHR96 protein than wild-type animals (M. Horner, personal communication). The *DHR96*^{2X} animals are more resistant to starvation than the wild-type controls (Figure 1A). Taken together, these opposite effects of *DHR96* loss of function and gain of function on the starvation response suggest that this factor plays a central role in maintaining energy homeostasis.

As a first step toward determining the basis for these effects on the starvation response, we measured the major forms of stored energy in the animal, glycogen and TAG levels, in control, *DHR96*^{1}, and *DHR96*^{2X} stocks, under both fed conditions and after 20 hr of starvation. Both the *DHR96*^{1} mutant and *DHR96*^{2X} animals have wild-type levels of glycogen under normal feeding conditions (Figure 1B). The mutant, however, has significantly less glycogen upon starvation than the wild-type control. Interestingly, TAG levels are reduced in both fed and starved *DHR96*^{1} mutants and are elevated in both fed and starved *DHR96*^{2X} animals relative to controls (Figure 1C). An ~2-fold reduction in TAG is also seen with a second *DHR96* null allele and is effectively rescued by a wild-type genomic *DHR96* transgene (see Figure S1 available online). Taken together, these observations suggest that the starvation sensitivity of *DHR96* mutants is caused by a deficit of stored energy in the form of TAG, and implicate a central role for *DHR96* in TAG homeostasis.

To confirm and extend these results, we examined the distribution of neutral lipids in the major sites of fat storage in the animal, the fat body and midgut. Fat body cells were dissected from 1- to 2-day-old control male flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain and stained with Nile red (Figures 1D–1F). *DHR96* mutant fat body cells are approximately half the size of those in controls, with an average ~3-fold reduction in lipid droplet size (Figures S2A and S2B). Conversely, fat body cells from the *DHR96*^{2X} overexpression strain are wild-type in size but have lipid droplets that are, on average, nine times larger than those in control fat cells (Figures S2A and S2B). Similar results were seen when the midguts of control, *DHR96*^{1} mutant, and *DHR96*^{2X} animals were dissected and stained with oil red O (Figures 1G–1I). Whereas low levels of neutral lipids can be detected in the gut epithelium of control flies (Figure 1G), no staining is evident in the gut epithelium of *DHR96*^{1} mutants (Figure 1H), and large lipid droplets are present in the midguts of *DHR96*^{2X} animals (Figure 1I). These results support our measurements of whole-animal TAG levels and indicate that *DHR96* plays a central role in the process of lipid metabolism.

DHR96 Mutants Are Resistant to Diet-Induced Obesity

We next tested whether dietary conditions can alter the TAG levels in *DHR96* mutant flies. Control *CanS* and *DHR96*^{1} mutants

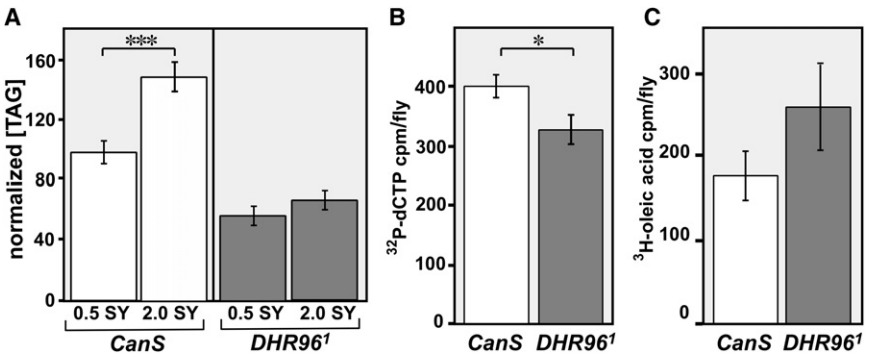

Figure 2. *DHR96* Mutants Are Resistant to Diet-Induced Obesity

(A) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were subjected to either a low-calorie 0.5 SY or high-calorie 2.0 SY diet for 7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%.

(B) Food uptake is slightly reduced in *DHR96* mutants, as determined by scintillation counting of *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants that were maintained on food supplemented with ^{32}P-dCTP.

(C) *DHR96* mutants can efficiently absorb dietary fatty acids, as determined by scintillation counting of *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants that were maintained on food supplemented with ^{3}H-oleic acid ($p = 0.18$).

Error bars represent ±SE, $^{*}p < 0.05$ and $^{***}p < 0.0001$.

were transferred to a previously characterized low-calorie (0.5 SY) or high-calorie (2.0 SY) medium and assayed for specific changes in TAG levels after 1 week (Mair et al., 2005). Control flies maintained on the high-calorie diet display a significant increase in whole-animal TAG levels relative to that seen in flies maintained on the low calorie diet (Figure 2A). Interestingly, *DHR96* mutants grown on either the low-calorie or high-calorie media display a lean phenotype relative to control animals on the restrictive diet. These observations support the proposal that *DHR96* mutants are genetically lean and indicate that they are resistant to diet-induced obesity.

It is possible that a reduced feeding rate could contribute to the lean phenotype in *DHR96* mutants. To test this possibility, both control and *DHR96*^{1} mutant flies were transferred to medium supplemented with a radioactive dCTP tracer, and the amount of retained label was measured after a 12 hr period (Carvalho et al., 2005). *DHR96* mutants display a mild (12%–18%) decrease in the amount of ingested food relative to the controls (Figure 2B). No change in food content, however, was seen upon spectrophotometric measurement of extracts from flies grown on yeast paste supplemented with 0.05% Bromophenol blue (data not shown). Based on these assays, we conclude that a reduction in feeding rate is unlikely to be a major cause of the lean phenotype in *DHR96* mutants. Similarly, there is no significant change in the amount of radiolabeled oleic acid retained in *DHR96* mutants after a 12 hr feeding period, suggesting that they have no major defects in dietary fatty acid uptake (Figure 2C).

DHR96 Mutants Are Resistant to Orlistat Treatment

We next wanted to determine if dietary TAG breakdown contributes to the lean phenotype in *DHR96* mutants. As a first step toward this goal, TAG levels were measured in control and *DHR96*^{1} mutants that were maintained for 5 days on media supplemented with 2 μM Orlistat (Figure 3A). Orlistat (tetrahydrolipstatin) is a widely used over-the-counter weight loss drug (Heck et al., 2000). It acts inside the intestine as a competitive inhibitor of pancreatic and gastric lipases, preventing their interaction with dietary TAG and thus blocking fatty acid release and dietary lipid uptake. Control flies exposed to Orlistat display a significant reduction in fat levels (Figure 3A). A similar effect was seen in mutants for the fly perilipin homolog Lsd-2, which are lean due to reduced levels of fat body TAG, consistent with the conclusion that Orlistat specifically inhibits *Drosophila* gastric lipases (Grönke et al., 2003) (Figure S3A). In contrast, no effect was seen upon exposing *DHR96* mutants to the drug. These observations were confirmed by staining midguts dissected from either untreated or Orlistat-treated control and *DHR96*^{1} mutants

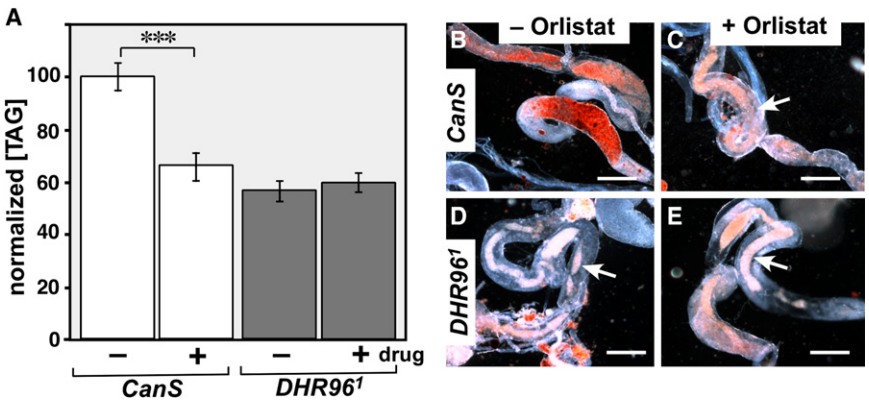

Figure 3. *DHR96* Mutants Are Resistant to Treatment with Orlistat

(A) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were transferred to a diet either with or without 2.0 μM Orlistat for 5–7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%. Error bars represent ±SE, $^{***}p < 0.0001$.

(B–E) Oil red O staining of dissected adult midguts shows that lipid accumulation is significantly reduced in *CanS* flies fed Orlistat (C), relative to controls (B) and undetectable in *DHR96*^{1} mutants maintained on either normal food (D) or food supplemented with Orlistat (E). Low levels of lipids can be detected in the midgut lumen of *DHR96* mutants and *CanS* flies fed Orlistat (arrows). Scale bars are 400 μm.

with oil red O (Figures 3B–3E). Control flies maintained on the high-nutrient diet used for this study display clearly detectable levels of neutral lipids in their gut epithelium, and this level is significantly reduced in the midguts of animals exposed to Orlistat (Figures 3B and 3C). In contrast, *DHR96* mutants display no detectable neutral lipids in their midguts, either in the presence or the absence of drug (Figures 3D and 3E). These results raise the possibility that the lean phenotype in *DHR96* mutants may arise, at least in part, from decreased TAG lipase activity in the midgut.

DHR96 Functions in the Midgut to Control the Breakdown of Dietary TAG

To test the possibility that *DHR96* mutants have a decreased ability to break down dietary fat, lysates were prepared from dissected control *CanS* and *DHR96*^{1} mutant midguts and assayed for TAG lipase activity by examining their ability to cleave a glycerol tributyrate substrate. This experiment showed that lysates from *DHR96* mutants have significantly reduced lipase activity relative to lysates from control animals, suggesting that decreased gastric lipase activity is a primary cause of the lean phenotype in *DHR96* mutants (Figure 4A). To further test this model, we attempted to bypass the need for midgut lipolysis by rescuing the lean phenotype through dietary supplementation with free fatty acids. Control *CanS* and *DHR96*^{1} mutant flies were transferred to a lipid-depleted medium that was either unsupplemented with lipid or supplemented with a mixture of stearic acid and oleic acid (free fatty acids) or a mixture of glycerol tristearate and glycerol trioleate (TAG). Extracts were prepared from these animals after 7 days and assayed for TAG levels (Figure 4B). As expected, *DHR96* mutants maintained on the lipid-depleted medium continue to display the lean phenotype. Interestingly, the same phenotype is seen when *DHR96* mutants are maintained in the presence of supplemented TAG, while the lean phenotype is effectively rescued by dietary supplementation with free fatty acids (Figure 4B). This observation indicates that an inability to break down dietary TAG is a major contributing factor to the lean phenotype seen in *DHR96* mutants. Phospholipid digestion, however, appears to be normal in *DHR96* mutants, as revealed by cleavage of the quenched fluorescent phospholipid PED6, which provides an accurate indicator of gastric phospholipase activity (data not shown) (Farber et al., 2001).

Expression of wild-type *DHR96* effectively rescues the lean phenotype of *DHR96* mutants, indicating that this phenotype can be attributed to a specific defect in *DHR96* function (Figure S1). If, however, *DHR96* regulates the breakdown of dietary fat, then we should be able to rescue the mutant lean phenotype by specifically expressing the wild-type receptor in the midgut of *DHR96* mutants. Consistent with this proposal, specific expression of wild-type *DHR96* in the fat body of *DHR96* mutants does not have a significant effect on the low levels of TAG seen in these animals, while specific expression of wild-type *DHR96* in the midgut of *DHR96* mutants effectively rescues the lean phenotype (Figure 4C). This observation indicates that the reduced levels of TAG seen in *DHR96* mutants arise from defects in midgut function. Taken together with our dietary rescue experiments, we conclude that *DHR96* plays a central role in controlling the breakdown of dietary fat.

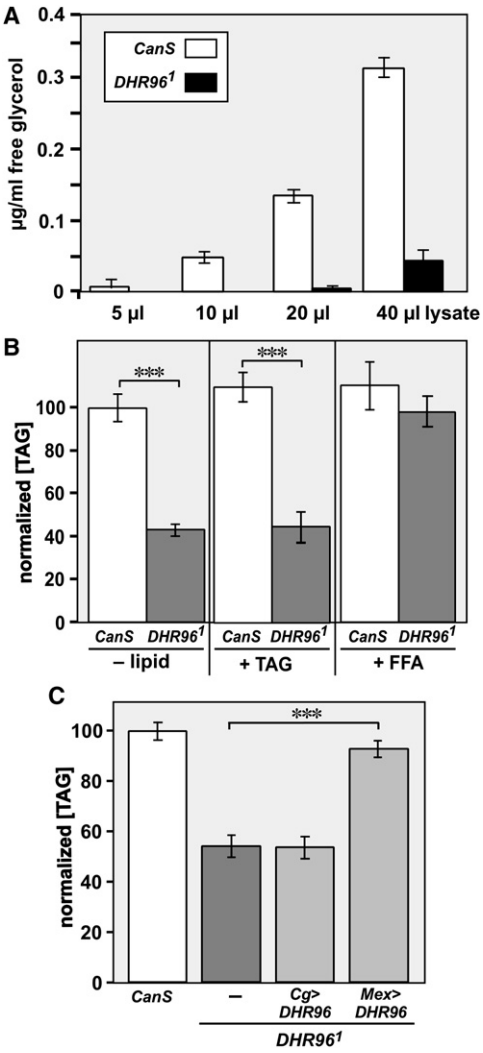

Figure 4. *DHR96* Functions in the Midgut to Control Dietary TAG Breakdown

(A) Midguts dissected from mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were homogenized, and increasing amounts of lysate were tested for TAG lipolytic activity by assaying for the release of glycerol from a glycerol tributyrate emulsion.

(B) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were transferred to lipid extracted 1.0 SY medium alone (−lipid), medium supplemented with TAG (+TAG), or medium supplemented with free fatty acids (+FFA) for 7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a level of 100% in *CanS* flies on the unsupplemented lipid-extracted medium.

(C) Tissue-specific expression of a wild-type *UAS-DHR96* transgene in the midgut of *DHR96* mutants, driven by *Mex-GAL4* (*Mex* > *DHR96*), is sufficient to rescue the lean phenotype. In contrast, little effect is seen when *Cg-GAL4* is used to drive fat body-specific expression of the *UAS-DHR96* transgene (*Cg* > *DHR96*). The presence of the GAL4 drivers alone or the *UAS-DHR96* transgene in the *DHR96* mutant background has no effect on TAG levels (data not shown). Error bars represent ±SE, ***$p < 0.0001$.

DHR96 Regulates Genes Expressed in the Midgut and Involved in Metabolism

Microarray studies were conducted to determine the molecular mechanisms by which *DHR96* regulates lipid homeostasis.

This study revealed that 136 genes are significantly affected by the *DHR96* mutation in fed adult flies, with 94 genes displaying increased levels of expression and 42 genes showing decreased levels of expression (Table S1). A significant number of genes involved in cuticular structure and the peritrophic matrix, which acts as protective layer inside the lumen of the midgut, are upregulated in the mutant, while many genes that encode predicted α-mannosidases and endopeptidases are expressed at lower levels. Genes more directly involved in metabolism are also overrepresented in the list of *DHR96*-regulated genes. These include two genes that encode larval serum proteins: *Lsp1γ* (+2.2-fold in the *DHR96* mutant) and *Lsp2* (+3.1-fold). These proteins are synthesized by the fat body and are thought to provide a source of amino acids to support adult development during metamorphosis (Telfer and Kunkel, 1991). Interestingly, the gene that encodes the larval serum protein receptor, *Fbp1* (+3.1-fold), is also regulated by *DHR96*, suggesting a central role for *DHR96* in LSP function (Burmester et al., 1999). *Mdr50*, which is reduced in its expression in *DHR96* mutants (−1.5-fold), is the fly ortholog of mammalian ATP-binding cassette subfamily B, member 4 (ABCB4), a protein that is primarily expressed in the liver and involved in phospholipid transport. Similarly, the fifth most downregulated gene in *DHR96* mutants, *CG13325*, encodes a protein with a predicted acyltransferase domain that could function in lipid transport. Finally, it is remarkable that the most significantly affected genes in *DHR96* mutants include most of the *Drosophila* orthologs of the Niemann-Pick (NPC) disease genes that play central roles in cholesterol metabolism, including *npc1b* (+2.6-fold up) and five NPC2 family members: *npc2c* (+1.7-fold); *npc2d* (−13-fold); *npc2e* (+21-fold); and two genes that are below our 1.5-fold cutoff in expression level, *npc2g* and *npc2h* (both −1.4-fold).

Closer examination of the *DHR96*-regulated genes reveals that many are expressed exclusively, or most abundantly, in the midgut. Of the 132 genes that are misregulated in *DHR96* mutants, 42 are included in the 2695 midgut-specific genes identified by Li et al. (2008) (32%; p value = 9×10^{-9}). A similar overrepresentation of midgut-expressed genes can be seen by surveying the expression patterns on FlyAtlas (Chintapalli et al., 2007). For example, 12 of the 15 most downregulated genes, and 9 of the 15 most upregulated genes in *DHR96* mutants, are highly expressed in the midgut (Figure S4).

Finally, we found that many of the *DHR96*-regulated genes are located next to one another in the genome. The gene clusters include three genes with predicted α-mannosidase activity, *CG9463* (−3.3-fold), *CG9466* (−5.9-fold), and *CG9468* (−4.3-fold), all of which are among the most highly downregulated genes in the mutant. A divergently transcribed pair of genes with predicted α-mannosidase activity are also misregulated in *DHR96* mutants, *CG5322* (−1.3-fold) and *CG6206* (−1.8-fold), as well as two genes with predicted α-glucosidase activity, *CG14934* (+1.5-fold) and *CG14935* (−1.6-fold), and a cluster of two *Jonah* genes that encode predicted gastric peptidases, *Jon65Ai* and *Jon65Aii* (both −1.7-fold). Two genes with predicted sphingomyelin phosphodiesterase activity, *CG15533* (+2.0-fold) and *CG15534* (+1.3-fold), are regulated by *DHR96*. Moreover, many of the NPC genes that are misregulated in *DHR96* mutants lie within gene clusters. These include *npc2d* (−13-fold), *npc2e* (+21-fold), and *npc2c* (+1.7-fold). An adjacent gene, *fancl*, which has no known function, is downregulated 1.5-fold in *DHR96* mutants. In addition, *npc2g* and *npc2h* are located next to one another in the genome.

DHR96 Regulates the CG5932 Gastric Lipase to Promote TAG Accumulation

Two genes that encode predicted TAG lipases were identified in our microarray study of *DHR96* mutants, *CG5932* and *CG31091* (Table S1). Both genes encode members of the α/β-hydrolase fold lipase family and are homologs of human gastric lipases, with 37% and 33% amino acid identity, respectively. In addition, both genes are expressed almost exclusively in the larval and adult midgut (Chintapalli et al., 2007), raising the possibility that their misexpression may contribute to the reduced ability of *DHR96* mutants to break down dietary TAG. Validation of the effects of the *DHR96* mutation on *CG5932* and *CG31091* expression by northern blot hybridization showed that *CG31091* mRNA levels are very low, consistent with the *CG31091* expression levels reported on FlyAtlas (Chintapalli et al., 2007) (data not shown). In contrast, *CG5932* is abundantly expressed and is downregulated by starvation in wild-type flies (Figure 5A, lanes 1 and 3), and this expression is significantly reduced in fed or starved *DHR96* mutants (Figure 5A, lanes 2 and 4). *CG5932* is also the fourth most highly downregulated gene identified in our microarray study. Chromatin immunoprecipitation of DHR96 protein from wild-type lysates revealed direct binding to sequences immediately upstream from *CG5932* (Figure S5). This binding is not seen at a promoter that is independent of DHR96 regulation or in chromatin immunoprecipitation experiments using lysates made from *DHR96* mutants, suggesting that it represents a specific DNA-protein interaction. Moreover, expression of wild-type *DHR96* in the midgut of *DHR96* mutants is sufficient to restore normal *CG5932* expression (data not shown). Taken together, these observations suggest that *DHR96* maintains TAG homeostasis through direct regulation of *CG5932* expression.

If *CG5932* is essential for TAG homeostasis, then disruption of *CG5932* function should lead to changes in TAG levels. To test this possibility, we used *Act-GAL4* to drive the expression of a *UAS-CG5932* RNAi construct. Whereas flies that carry either the *Act-GAL4* driver alone or the *UAS-CG5932* RNAi construct alone display normal levels of TAG and *CG5932* expression, combining *Act-GAL4* with the *UAS-CG5932* RNAi construct resulted in significant reduction of both whole-animal TAG levels and *CG5932* mRNA accumulation (Figure 5B). Similar results were seen upon driving *UAS-CG5932* RNAi with the midgut-specific *Mex-GAL4* driver (Figure S6A). These animals are also sensitive to starvation, consistent with their lean phenotype (Figure S6B). In addition, purified CG5932 protein has lipolytic activity in vitro, as demonstrated by its ability to break down a glycerol tributyrate substrate (Figure S7). Taken together, these observations indicate that *CG5932* plays a critical role in the midgut to regulate whole-animal TAG homeostasis, most likely through its effect on the breakdown of dietary fat.

If the downregulation of *CG5932* expression in *DHR96* mutants contributes to their inability to break down dietary TAG, then restoring *CG5932* expression specifically in the midgut of these animals should rescue their lean phenotype. To test this possibility, we established transformant lines that

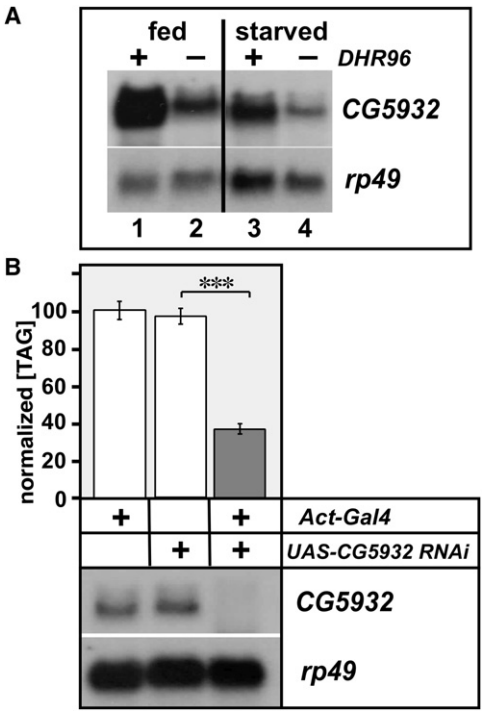

Figure 5. *DHR96* Regulates the *CG5932* Gastric Lipase Gene to Control the Breakdown of Dietary TAG

(A) RNA was isolated from either fed or starved *CanS* control flies (+ *DHR96*) and *DHR96*^{1} mutants (− *DHR96*) and analyzed by northern blot hybridization for *CG5932* transcription. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer.

(B) An *Act-GAL4* driver or the *UAS-CG5932* RNAi transgene alone have no effect on whole-animal TAG levels (above) or *CG5932* mRNA levels (below), as determined by northern blot hybridization. Combining these two transgenes, however, effectively reduces *CG5932* expression and leads to a significant reduction in total TAG levels. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. Error bars represent ±SE, ***p < 0.0001.

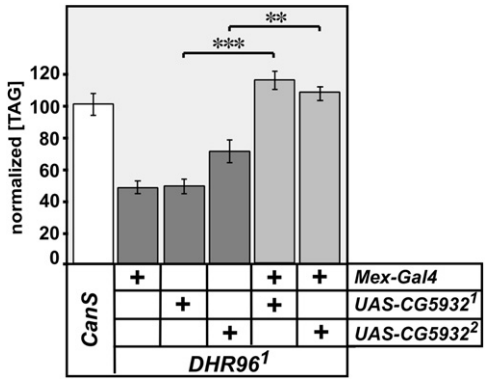

Figure 6. Midgut-Specific Expression of *CG5932* Rescues the Lean Phenotype of *DHR96* Mutants

TAG levels were determined in mature adult male *Canton-S* (*CanS*) control flies or *DHR96*^{1} mutants that carried different transgenes. Either the midgut-specific *Mex-GAL4* driver alone or two different insertions of the *UAS-CG5932* rescue transgene alone (*UAS-CG5932*^{1} or *UAS-CG5932*^{2}) had little or no effect on whole-animal TAG levels. The increased level of TAG seen in the presence of *UAS-CG5932*^{2} alone could be due to background expression from a flanking regulatory element, as is often seen with *P* element insertions. In contrast, combining the *Mex-GAL4* driver with each *UAS-CG5932* rescue transgene restored normal TAG levels. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%. Error bars represent ±SE, **p < 0.001 and ***p < 0.0001.

carry two different insertions of a *UAS-CG5932* transgene. As expected, the presence of either the midgut-specific *Mex-Gal4* driver alone or each *UAS-CG5932* transgene had little or no effect on the reduced TAG levels seen in the *DHR96* mutant (Figure 6). Combining the *Mex-GAL4* driver with either of the *UAS-CG5932* transgenes in a *DHR96* mutant, however, allows the mutant to recover normal levels of TAG (Figure 6). In addition, overexpression of *CG5932* does not stimulate an increase in whole-animal TAG levels, indicating that while *CG5932* is necessary for TAG homeostasis, it is not sufficient to drive TAG accumulation (Figure S8). These results, combined with our observation that the lean phenotype in *DHR96* mutants can be rescued by dietary supplementation with free fatty acids (Figure 4B), and the absence of an effect of Orlistat treatment in *DHR96* mutants (Figure 3A) or *Act* > *CG5932* RNAi animals (Figure S3B), suggests that *DHR96* controls whole-animal TAG levels through its regulation of the CG5932 gastric lipase.

DISCUSSION

Recent studies have implicated roles for mammalian PXR and CAR in controlling lipid metabolism, although little is known regarding the molecular mechanisms by which they exert these effects. Here we show that the single *Drosophila* ortholog of PXR and CAR, DHR96, plays an essential role in maintaining whole-animal TAG levels through the proper breakdown of dietary fat. Our results indicate that DHR96 acts through a previously uncharacterized gastric lipase encoded by *CG5932* to promote dietary lipid uptake and maintain TAG homeostasis.

DHR96 Mutants Are Sensitive to Starvation Due to Decreased Levels of TAG

Although *DHR96* null mutants are viable and fertile, with no morphological defects, they die significantly more rapidly than genetically matched control flies under starvation conditions, while *DHR96* overexpression leads to starvation resistance (Figure 1A). The effects of these genotypes on the major forms of stored energy in the animal, glycogen and TAG, are consistent with their effects on the starvation response. *DHR96* mutants have reduced levels of TAG under both fed and starved conditions, while *DHR96* overexpression leads to increased TAG levels (Figure 1C). Although no effects are seen on whole-animal glycogen levels in fed animals that either lack or overexpress *DHR96*, the mutants consume significantly more glycogen upon starvation than do controls (Figure 1B). This rapid utilization of glycogen stores is most likely due to the decreased energy contribution from TAG. Taken together, these observations suggest that the starvation sensitivity of *DHR96* mutants can be attributed to their lean phenotype, while the starvation resistance of the *DHR96*^{2X} strain is due to their excess energy in the form of TAG. This proposal is supported by the observation that genetically elevating the levels of TAG in *DHR96* mutants by introducing mutations in *bmm* or *AKHR*, which control distinct aspects of fat body TAG lipolysis (Grönke et al., 2007), effectively

rescues their starvation sensitivity (Figure S9). In addition, the opposite effects of *DHR96* loss of function and gain of function on both the starvation response and TAG levels argue that this receptor plays a central role in maintaining whole-animal TAG homeostasis.

DHR96 Functions in the Midgut to Regulate the Uptake of Dietary Nutrients

Several lines of evidence support the conclusion that *DHR96* exerts its primary metabolic functions through the midgut. These include our initial observation that *DHR96* mutants are resistant to treatment with the gastric lipase inhibitor Orlistat (Figure 3) and display reduced levels of midgut lipolytic activity (Figure 4A). In addition, dietary supplementation with free fatty acids, but not TAG, is sufficient to rescue the lean phenotype of *DHR96* mutants, as is midgut-specific expression of wild-type *DHR96* in a *DHR96* mutant background (Figure 4C). We also see a dramatic effect on lipid levels in the midgut, where almost no neutral lipids are detectable in *DHR96* mutants and enlarged lipid droplets are evident in $DHR96^{2X}$ flies maintained on a normal diet (Figures 1H and 1I). Interestingly, while the lumen of the midgut is not evident in control animals, we clearly see material in the lumen of *DHR96* mutant midguts or in control flies that are treated with Orlistat (Figures 1H and 3C–3E). In some cases, this material is stained by oil red O, suggesting that it may represent an increased level of undigested fat in these animals. This phenotype would be similar to that seen in humans who have defects in intestinal lipase activity (Ligumsky et al., 1990). Likewise, an increase in the passage of undigested dietary fat is a complication associated with Orlistat treatment in patients (Heck et al., 2000). Taken together, these observations support the conclusion that *DHR96* acts in the midgut to regulate the breakdown of dietary fat.

An essential role for *DHR96* in the midgut is further supported by our microarray study, which revealed that many *DHR96*-regulated genes are primarily expressed in this tissue. Interestingly, many of these genes have predicted roles related to the breakdown of dietary nutrients (Table S1). These include downregulation of multiple genes with predicted α-mannosidase activity, which is involved in the breakdown of the complex sugars found on glycoproteins. Many genes that encode trypsins and endopeptidases are also expressed at reduced levels in *DHR96* mutants as well as a few genes that encode predicted α-glucosidases, which are involved in the breakdown of dietary carbohydrates. In addition, a number of genes involved in the formation of the peritrophic matrix are more abundantly expressed in *DHR96* mutants. This matrix is comprised of chitin and peritrophic proteins and acts as a protective layer for the epithelial surface of the midgut (Hegedus et al., 2009). The peritrophic matrix also has critical roles in facilitating digestion. Only smaller molecules that arise from the initial digestion of complex nutrients, including peptides, sugars, and lipids, can move through the peritrophic matrix for final digestion and absorption by the midgut epithelium. These events are controlled by the selective partitioning of digestive enzymes to different sides of the peritrophic matrix as well as within the matrix itself. Thus, while midgut morphology appears normal in *DHR96* mutants, the effect of the mutation on the peritrophic matrix could impact nutrient digestion and absorption.

In addition to genes that regulate different aspects of lipid metabolism, our microarray study of *DHR96* mutants identified widespread effects on the expression of *Drosophila* homologs of NPC disease genes. The *npc1b* gene, which encodes an essential cholesterol transporter and the ortholog of mammalian NPC1L1 (Voght et al., 2007), is the tenth most highly upregulated gene in *DHR96* mutants. In addition, five of the eight *Drosophila* NPC2 genes are misregulated in *DHR96* mutants: *npc2c*, *npc2d*, *npc2e*, *npc2g*, and *npc2h* (Huang et al., 2007). These genes encode homologs of mammalian NPC2, which is involved in intracellular cholesterol trafficking (Huang et al., 2007). Remarkably, two of these genes are the most highly up- and downregulated genes identified in the mutant (*npc2e* and *npc2d*, respectively; Table S1). Moreover, many of these *npc* genes are located in clusters, suggesting that they are coregulated by the receptor. Although the function of these *npc2* genes is unknown, their disproportionate representation within the list of *DHR96*-regulated genes implies a critical role for the receptor in regulating cholesterol trafficking. Indeed, dietary cholesterol triggers a widespread transcriptional response in *Drosophila* that is dependent on *DHR96* function (Horner et al., 2009). Moreover, this study showed that *DHR96* mutants display defects in their ability to maintain cholesterol homeostasis when grown on a high-cholesterol diet. Taken together with the results presented here, our studies suggest that *DHR96* plays an essential role in the midgut to coordinate the processes of TAG and cholesterol breakdown, absorption, and trafficking.

DHR96 Regulates *CG5932* to Control the Breakdown of Dietary TAG

Two genes with predicted TAG lipase activity are expressed at lower levels in *DHR96* mutants (Table S1). One of these genes, *CG5932*, is abundantly expressed in the larval and adult midgut, encodes a protein that is highly related to human gastric lipase (LIPF, 37% identity over 358 amino acids), and is essential for maintaining whole-animal TAG levels (Figure 5). In addition, restoring *CG5932* expression in the midguts of *DHR96* mutants is sufficient to rescue their lean phenotype, defining this gene as a critical functional target of the receptor (Figure 6). Interestingly, *CG5932* expression is also regulated by starvation, with reduced expression in the absence of food and increased expression upon refeeding (Figure 5A) (Gershman et al., 2006). This regulation is consistent with an essential role for *CG5932* in the breakdown of dietary fat, where its expression is upregulated when food is present. This response, however, is unaffected in *DHR96* mutants, indicating that it is under independent control, possibly by known regulators of the starvation response such as dFOXO (Gershman et al., 2006).

The identification of *CG5932* as a key functional target of *DHR96* raises the question of how that regulation is achieved. We have tested a range of dietary parameters and candidate ligands using the GAL4-DHR96 ligand sensor but have been unable to identify conditions that activate the DHR96 LBD (Palanker et al., 2006). In addition, the DHR96-binding site remains undefined. DHR96 has a unique P box sequence within its DBD, which determines its DNA-binding specificity. This sequence is only shared by three *C. elegans* NRs, DAF-12, NHR-48, and NHR-8. Consistent with this observation, we found that DHR96 protein fails to bind to most canonical NR-binding

sites, except for weak binding to a palindromic EcR response element (Fisk and Thummel, 1995). One study has shown that DAF-12 displays preferential binding to a direct repeat of two distinct hexanucleotide sequences (AGGACA and AGTGCA), separated by five nucleotides (DR5) (Shostak et al., 2004). Whether DAF-12 contacts these sequences as a homodimer or a heterodimer with another NR, however, remains to be determined. The observation that many *DHR96*-regulated genes are arranged in clusters and DHR96 binds directly to a region upstream from the *CG5932* start site provides an ideal context for defining its DNA-binding specificity as well as determining the molecular mechanisms by which DHR96 coordinates target gene transcription.

DHR96 May Indirectly Regulate Xenobiotic Responses in *Drosophila*

Our studies of *DHR96* raise the interesting possibility that the defects in xenobiotic detoxification seen in *DHR96* mutants may arise, at least in part, from its role in regulating midgut metabolic activity. As noted in our original study, most of the genes that are regulated by phenobarbital in wild-type flies do so independently of *DHR96* (King-Jones et al., 2006). These genes include representatives of the major classes associated with xenobiotic detoxification: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs). Moreover, some phenobarbital-regulated genes that are misregulated in *DHR96* mutants still show a transcriptional response to the drug, although that response is muted. These observations indicate that one or more other factors contribute to the transcriptional response to xenobiotic challenge in *Drosophila*. Interestingly, similar results have been observed in vertebrates, where less then half of the genes that are regulated by xenobiotics are affected in *PXR* and *CAR* mutant mice (Maglich et al., 2002; Ueda et al., 2002). It remains unclear, however, whether this lack of regulation might be due to functional redundancy between these mammalian receptors.

There are several possible mechanisms by which the metabolic functions of *DHR96* could impact xenobiotic responses. First, a recent study of the expression patterns of *Drosophila* P450 genes showed that 34 of 60 genes that could be detected in third-instar larvae are expressed in the midgut or hindgut (Chung et al., 2009). A similar overrepresentation of P450 genes is evident in a microarray study of midgut expressed genes (Li et al., 2008). These observations indicate that, contrary to previous assumptions, the gut, and not the fat body, may be a critical site for xenobiotic detoxification (Chung et al., 2009). If this is true, then the sensitivity of *DHR96* mutants to phenobarbital or DDT treatment may be affected by the effects of this mutation on midgut physiology. This could occur through defects in the peritrophic matrix or through changes in the ability of the midgut to absorb lipophilic xenobiotic compounds such as DDT. An alternative possibility is that the sensitivity of *DHR96* mutants to xenobiotics might be due to their decreased energy stores. Detoxification requires energy expenditure. For example, P450s consume NADPH or NADH for their oxidation of xenobiotics, UGTs consume glucose, and GSTs consume glutathione. Thus, the reduced levels of stored energy in *DHR96* mutants might compromise their ability to properly inactivate toxic compounds. In addition, the reduced lipid stores in *DHR96* mutants might exert an indirect effect on xenobiotic responses by lowering the ability of the animal to sequester toxins in the fat reserves of the animal. Thus, there are multiple pathways by which the midgut-specific metabolic defects associated with the *DHR96* mutation might indirectly affect xenobiotic responses in these animals. Further studies are required to test this possibility and clarify the functional overlaps between the roles of *DHR96* in lipid metabolism and xenobiotic detoxification.

Conserved Roles for the PXR/CAR/DHR96 Nuclear Receptors in Lipid Metabolism

Several recent studies have demonstrated roles for both PXR and CAR in lipid metabolism (Moreau et al., 2008). CAR can repress the transcription of genes encoding carnitine palmitoyltransferase and enoyl-CoA isomerase, key steps in lipid β-oxidation (Ueda et al., 2002). *CAR* mutant mice are also sensitive to starvation, much as we observe for *DHR96* mutants, and lose weight more rapidly than wild-type mice when maintained on a low-calorie diet (Maglich et al., 2004). Transgenic expression of a constitutively active form of PXR in the mouse liver leads to hepatic steatosis along with reduced expression of lipid catabolic genes and increased expression of genes involved in lipid synthesis (Zhou et al., 2006). Importantly, similar effects were observed upon pharmacological activation of PXR using a specific agonist, indicating that the endogenous receptor can contribute to lipid homeostasis (Hoekstra et al., 2009; Nakamura et al., 2007). Most recently, a mutation in *CAR* has been shown to normalize the elevated serum TAG levels seen in *leptin*-deficient mice or in wild-type mice maintained on a high-fat diet (Maglich et al., 2009). Conversely, treatment of wild-type mice with a selective CAR agonist leads to increased serum TAG levels, and this response fails to occur in a *CAR* mutant background. Taken together, these studies indicate that activation of PXR/CAR receptors leads to lipid accumulation while a loss of PXR/CAR activity leads to reduced lipid levels, defining a central role for these NRs in lipid homeostasis. Their role in normal lipid metabolism, however, remains unknown, although it may be masked by functional redundancy between the two receptors. Similarly, the molecular mechanisms by which PXR and CAR can modulate lipid levels remain to be defined.

Our genetic studies of *DHR96* demonstrate that these metabolic activities of PXR and CAR have been conserved through evolution, and represent an essential ancestral function for this NR subfamily. Moreover, the observation that *DHR96* overexpression leads to lipid accumulation and *DHR96* mutants are lean suggests that the molecular mechanisms that underlie these effects are also conserved across species. This conclusion is supported by genetic studies of the *C. elegans* member of this subfamily, DAF-12, which indicate that this receptor is also required for proper levels of stored fat (Gerisch et al., 2001). Our study provides further evidence of a role for this NR subfamily in normal lipid homeostasis and defines the control of dietary fat breakdown as a key step at which this regulation is achieved. In addition, this work provides a foundation for understanding how dietary lipid uptake can impact normal lipid metabolism in *Drosophila* and provides a genetic model for characterizing how dietary factors can lead to lipid metabolic disorders such as obesity.

EXPERIMENTAL PROCEDURES

Fly Stocks

The following stocks were used in this study: *DHR96*^{1} (King-Jones et al., 2006), *Cg-Gal4* (Hennig et al., 2006), *Mex-Gal4* (Phillips and Thomas, 2006), *Act-Gal4/CyO* (Bloomington # 25374), *bmm*^{1} (Grönke et al., 2005), *AKHR*^{2} (Grönke et al., 2007), *Lsd-2*^{KG00149} (Grönke et al., 2003), *w*^{1118}, *DHR96*^{2X} (M. Horner, personal communication), *UAS-DHR96* (M. Horner, personal communication), and *UAS-CG5932* RNAi (National Institute of Genetics stock 5932R-3). Flies were maintained on standard Bloomington Stock Center medium with malt at 25°C. The *UAS-CG5932 P* element construct was made using oligonucleotide primers 5′-ATAGAATTCATGAATCCAATCTTCTGCGC-3′ and 5′- ATACTC GAGCTAGCGACCTTCGTAGGAGT-3′ to amplify the *CG5932* cDNA (GenBank NM_140972) from the DGRC LP10120 cDNA clone by PCR. Following digestion with EcoRI and XhoI, the cDNA was inserted into the corresponding sites of the pUAST vector. This *UAS-CG5932* construct was then integrated into the *w*^{1118} genome.

Metabolic Assays

All studies used 1- to 2-day-old adult male flies that were aged 5–7 days prior to the experiment. Starvation sensitivity assays were conducted by transferring 15 samples of 20 flies of each genotype into vials containing 1% agar. Mortality was assayed every 4 hr as determined by a lack of responsiveness to touch. For glycogen and TAG assays, five to ten mature adult male flies were homogenized in 100–200 μl PBST (PBS, 0.1% Tween 20), heated at 70°C for 5 min to inactivate endogenous enzymes, and the homogenate cleared by centrifugation for 3 min. The supernatant was diluted 1:4 with PBST and assayed for glycogen levels as described (Palanker et al., 2009). TAG assays were conducted as described (Palanker et al., 2009), using the Stanbio triglyceride liquicolor kit (2100-430). The glycogen and TAG levels in each sample were normalized for total protein as determined by a Bradford assay. Both glycogen and TAG data were compiled from six samples collected from each genotype under each condition. All data are presented as normalized to a wild-type level of 100%. All assays were repeated three times, and a representative experiment is presented in each figure. Nile red staining was performed as described by Grönke et al. (2005), using Nile red mounting media (20% glycerol in PBS, with a 1:10,000 dilution of 10% Nile red in DMSO) and imaged on a Leica TCS SP2 confocal microscope using an excitation wavelength of 543 nm and a 600–650 nm emission spectrum. For oil red O stains, midguts from mature adult flies were dissected and fixed in 4% paraformaldehyde/PBS for 20 min. The midguts were washed with distilled water and incubated in 100% propylene glycol for 5 min. Specimens were then incubated at 60°C in oil red O stain (0.5% oil red O in propylene glycol), washed twice with propylene glycol, washed three times with PBS, and mounted in 20% glycerol/PBS for imaging.

Dietary Treatments

All studies used 1- to 2-day-old adult male flies that were aged 5–7 days prior to the experiment. Diet-induced obesity was assayed by transferring flies to either a low-calorie 0.5 SY diet or a high-calorie 2.0 SY diet for 7 days, after which extracts were prepared and assayed for TAG and protein (Mair et al., 2005). Food intake was measured by transferring flies to Bloomington cornmeal, yeast, and molasses medium supplemented with 0.1 μC/ml ^{32}P-dCTP (Perkin-Elmer) for 12 hr, after which they were transferred to normal unlabeled food for 4 hr to remove label that was nonspecifically bound to the outside of the animal (Carvalho et al., 2005). Ten to twelve groups of 20 flies were collected, washed once with 0.1% BSA, and assayed for label retention on a scintillation counter. Fatty acid uptake was determined in a similar manner, using ^{3}H-oleic acid (Moravek Biochemicals) at a final concentration of 3 μC/ml. Treatment with Orlistat was conducted by growing flies on a high-nutrient molasses medium (Bloomington cornmeal, yeast, and molasses media) with or without 2 μM Orlistat for 5–7 days. TAG levels were then assayed as above. All data shown are from two parallel data sets of six samples/stock/condition compiled together and repeated at least three times. Dietary lipid supplementation was performed by transferring mature adult males to lipid-depleted 1.0 SY for 5–7 days. This medium was prepared by extracting the yeast extract and agar components of the SY medium overnight with chloroform, followed by a second 4 hr chloroform extraction. Both components were then allowed to dry for 2–3 days in a fume hood. The lipid-depleted medium was supplemented with either free fatty acids (5 mg/ml stearic acid and 5 mg/ml oleic acid, ChemService) or TAG (5 mg/ml glycerol tristearate and 5 mg/ml glycerol trioleate, ChemService). All data shown are from two collections of six samples collected from each genotype under each condition. Each experiment was repeated at least three times.

Microarrays

Adult male *CanS* and *DHR96*^{1} flies were collected 1–2 days after eclosion and allowed to age for 7 days on normal medium. RNA was extracted from these animals using Trizol (GIBCO) and purified on RNAeasy columns (QIAGEN). All samples were prepared in triplicate to facilitate subsequent statistical analysis. Probe labeling, hybridization to Affymetrix GeneChip *Drosophila* Genome 2.0 Arrays, and scanning were performed by the University of Maryland Microarray Core Facility. Raw data were subjected to quantile normalization using R statistical analysis software, and gene expression changes were determined using SAM 2.0, with a <5% estimated false positive rate and a 1.5-fold cutoff in expression level (Tusher et al., 2001). Comparison between microarray data sets was performed using Microsoft Access.

Statistical Analyses

Statistical significance was calculated using an unpaired two-tailed Student's t test with unequal variance. All quantitative data are reported as the mean ± SEM.

ACCESSION NUMBERS

Microarray data from this study can be accessed at the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) under accession number GSE18576.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and nine figures and can be found with this article online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00336-2.

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