RESEARCH COMMUNICATION

The *Drosophila* DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis

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Cholesterol homeostasis is required to maintain normal cellular function and avoid the deleterious effects of hypercholesterolemia. Here we show that the *Drosophila* DHR96 nuclear receptor binds cholesterol and is required for the coordinate transcriptional response of genes that are regulated by cholesterol and involved in cholesterol uptake, trafficking, and storage. *DHR96* mutants die when grown on low levels of cholesterol and accumulate excess cholesterol when maintained on a high-cholesterol diet. The cholesterol accumulation phenotype can be attributed to misregulation of *npc1b*, an ortholog of the mammalian Niemann-Pick C1-like 1 gene *NPC1L1*, which is essential for dietary cholesterol uptake. These studies define *DHR96* as a central regulator of cholesterol homeostasis.

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Cholesterol is an essential component of cell membranes that influences the permeability and fluidity of the lipid bilayer. Cholesterol also acts as a precursor for steroid hormone biosynthesis and contributes to cell–cell signaling pathways. These critical cellular functions are supported by regulatory mechanisms that maintain normal cholesterol levels and prevent hypercholesterolemia, which is a major risk factor for cardiovascular disease in humans. Cholesterol homeostasis in vertebrates is achieved primarily through de novo synthesis and dietary uptake (Ikonen 2008). Although extensive studies have defined a central role for the sterol regulatory element-binding protein (SREBP) family of transcription factors in controlling cholesterol synthesis (Brown and Goldstein 1997), the mechanisms that regulate dietary cholesterol absorption remain more poorly understood. One central component of this pathway is the Niemann-Pick C1-like 1 gene *NPC1L1*, which encodes a plasma membrane protein that mediates the uptake of dietary cholesterol by the intestine (Wang 2007; Ge et al. 2008). Mouse mutants for *NPC1L1* display significantly reduced levels of cholesterol absorption and are insensitive to treatment with the anti-hypercholesterolemia drug ezetimibe, which acts as a specific NPC1L1 inhibitor (Davis et al. 2008). Another major regulator of cholesterol homeostasis is the liver X receptor α (LXRα) nuclear receptor, which binds cholesterol metabolites and regulates the transcription of genes that control cholesterol transport and metabolism, including *NPC1L1* (Duval et al. 2006; Kalaany and Mangelsdorf 2006; Valasek et al. 2007).

We used the fruit fly, *Drosophila*, as a model system to study the regulation of cholesterol homeostasis. Unlike vertebrates, insects are cholesterol auxotrophs that are unable to synthesize this essential compound (Va'nt Hoog 1936). Little is known, however, about the mechanisms that regulate the uptake of dietary cholesterol in *Drosophila*. A recent study showed that the fly ortholog of *NPC1L1*, *npc1b*, is expressed specifically in the midgut and is essential for dietary cholesterol absorption (Voght et al. 2007). Other *NPC* disease gene homologs in *Drosophila* also contribute to cholesterol homeostasis. The *Drosophila* ortholog of vertebrate *NPC1*, *npc1a*, and two of the eight fly *NPC2* homologs, *npc2a* and *npc2b*, play important roles in intracellular cholesterol trafficking and synthesis of the steroid hormone 20-hydroxyecdysone (20E) (Huang et al. 2005, 2007; Fluegel et al. 2006). Other predicted regulators of cholesterol metabolism in *Drosophila*, however, remain unstudied, and upstream factors that might sense cholesterol levels and control cholesterol homeostasis are undefined.

In this study, we show that the *Drosophila* DHR96 nuclear receptor binds cholesterol, is essential for survival on a low-cholesterol diet, and is required to maintain cholesterol homeostasis when animals are grown on a high-cholesterol diet. We further show that dietary cholesterol regulates the transcription of many genes that are expressed in the midgut and that act in lipid metabolism, and that this transcriptional response fails to occur in *DHR96* mutants. Misregulation of one of these genes, *npc1b*, is sufficient to explain the cholesterol accumulation defect seen in *DHR96* mutants, defining *npc1b* as a critical functional target of the receptor. This study provides a new framework for understanding the molecular mechanisms that regulate cholesterol homeostasis.

Results and Discussion

DHR96 binds cholesterol

Mass spectrometry (MS) was used to identify potential ligands for DHR96. The DHR96 ligand-binding domain (LBD) was overexpressed, purified from insect cells, and subjected to electrospray ionization (ESI) MS, under both denaturing and nondenaturing conditions. The full mass range spectrum of the sample from the denaturing condition had a series of peaks corresponding to the DHR96 LBD, with a measured molecular weight (MW) of 31,831.92 Da, close to the predicted theoretical mass (31,830.84 Da for His_6-$DHR96_{S471\text{-}H723}$) (data not shown). Under nondenaturing conditions, the full mass range scan detected an additional series of peaks corresponding to a MW of 32,218.44 Da. This mass shift indicates that the

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DHR96 LBD copurifies with a molecule of 386.52 Da in a stoichiometry of 1:1, indicative of specific ligand binding.

One of the presumptive DHR96/ligand complex peaks (2685.87 m/z) was then selected for collision-induced dissociation (CID) with a collision voltage of 10 V, resulting in the generation of two ions in the 12^+ charge state (Fig. 1A). One ion represents the mass of the intact DHR96 LBD/ligand complex (2685.87 m/z; 32218.44 Da), while the other represents the unbound DHR96 LBD (2653.66 m/z; 31831.92 Da). Using a higher collision voltage of 50 V completely disrupted the DHR96 LBD/ligand complex to generate a CID spectrum with only an unbound receptor (2653.60 m/z; 31831.2 Da) (Fig. 1B). The charge state of the receptor ion did not change upon loss of the ligand and no peak was observed in the lower mass range, indicating that the ligand is a neutral molecule. These observations, along with the mass shift of 386.52 Da, suggest that the bound molecule may be cholesterol (MW, 386.6 Da).

To further verify the identity of the molecule bound to the DHR96 LBD, purified receptor was extracted with chloroform–methanol and a portion of the extract was derivatized and analyzed by gas chromatography/MS (GC/MS) with electron ionization (EI). A single major peak at 19 min was observed in the GC/MS chromatogram (Fig. 1C). The retention time of this peak was identical to that of a derivatized cholesterol standard (Fig. 1E). Moreover, the major peak of the DHR96 sample on GC/MS produced an EI spectrum (Fig. 1D) that matched a previously described spectrum for derivatized cholesterol (Fig. 1F; Bitsch et al. 2003). Based on these two independent modes of MS—ESI/MS and GC/MS—we conclude that the DHR96 LBD is capable of binding a single molecule of cholesterol. GC/MS analysis of several other *Drosophila* nuclear receptors indicated that this interaction is specific to DHR96 (Supplemental Fig. S1). In addition, although this interaction is stable through three rounds of protein chromatography, the partial dissociation of ligands at 10 V and complete dissociation at 50 V suggests that the bound cholesterol is exchangeable.

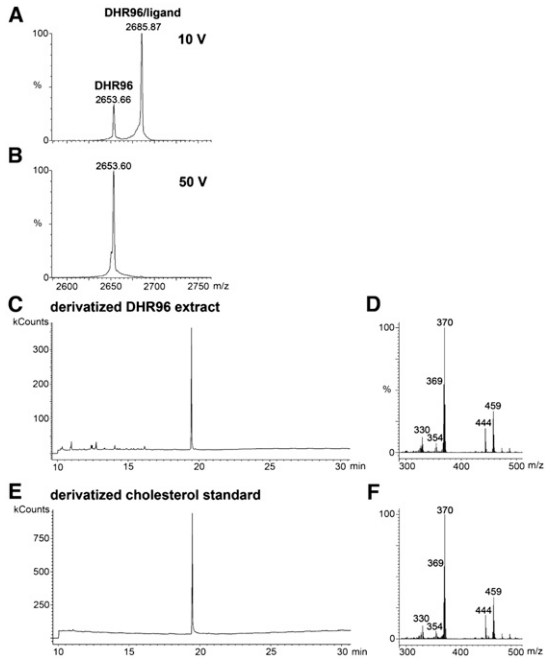

Figure 1. Mass spectrometry identifies cholesterol bound to the DHR96 LBD. (*A*) CID mass spectrum of the DHR96/cholesterol complex under nondenaturing conditions with a collision voltage of 10 V. At this voltage, a portion of the ions representing the 12^+ charge state of the DHR96/ligand complex (2685.87 m/z) fragment to generate ions of the 12^+ charge state of DHR96 (2653.66 m/z), losing the ligand as a free molecule. The charge states of the ions at 2685.87 and 2653.66 m/z were determined from full-range MS scan spectra (data not shown). (*B*) CID mass spectrum of the DHR96/ligand complex with a collision voltage of 50 V caused complete dissociation of the receptor/ligand complex into unbound receptor. (*C–F*) The elution time of the major peak on a gas chromatogram of a derivatized chloroform–methanol extraction of the DHR96 LBD (*C*) matches that of a derivatived cholesterol standard (*E*). The corresponding electron ionization spectrum from the major peak of the DHR96 LBD at 19 min generates major fragmentation ions (*D*) that correspond closely to the major fragmentation ions generated from the major peak of the derivatived cholesterol standard (*F*).

DHR96 *regulates the transcriptional response to cholesterol*

The observation that DHR96 binds cholesterol raises the possibility that it may mediate transcriptional responses to this compound. To test this possibility, control and *DHR96* mutants were grown on low-cholesterol medium in the absence or presence of 0.03% cholesterol, and were subjected to microarray analysis. From this analysis, 117 genes were identified that are up-regulated at least 1.4-fold in response to cholesterol in wild-type larvae, along with 270 genes that are down-regulated (Fig. 2A,B; Supplemental Table S1). This response appears to be rapid, occurring within 1–2 h of cholesterol treatment, and displays similar cholesterol dose response profiles (Supplemental Fig. S2). We also see a response to sitosterol treatment, indicating that it is not specific to cholesterol (Supplemental Fig. S3). Interestingly, a number of the cholesterol-regulated genes are predicted to play central roles in cholesterol metabolism and transport. These include *CG32186*, which encodes a predicted ABCA3-like transporter; *CG6472*, which encodes a lipoprotein lipase (LPL) homolog; and *CG8112*, which encodes acyl-CoA:cholesterol acetyltransferase (ACAT) (Supplemental Table S5). This enzyme plays a critical role in esterifying cholesterol, which is the primary stored form of intracellular cholesterol (Ikonen 2008). Four *Drosophila* homologs of *NPC* genes are also regulated by cholesterol, including *npc1b* and three *NPC2* homologs: *npc2b*, *npc2c*, and *npc2d* (Huang et al. 2007).

Importantly, this transcriptional response is almost entirely dependent on *DHR96* function. Only 13% of the genes that are up-regulated by cholesterol in wild-type larvae (15 genes), and 10% of the genes that are down-regulated (27 genes), display a similar profile of expression in *DHR96* mutants treated with cholesterol (Fig. 2A,B; Supplemental Fig. S4). Moreover, many additional genes become responsive to cholesterol in *DHR96* mutants, with 355 genes up-regulated and 446 genes down-regulated at least 1.4-fold, indicating that *DHR96* normally plays a key role in suppressing this transcriptional program (Supplemental Fig. S4; Supplemental Table S2). These *DHR96*-regulated genes include four of the remaining five *NPC2* family members: *npc2e*, *npc2f*, *npc2g*, and *npc2h* (Fig. 2C; Supplemental Table S5). *DHR96* also regulates *Lip3* (*CG8823*), which encodes a predicted cholesterol ester hydrolase; *CG9663*, which encodes an ABCG1 homolog; *CG11162*, which encodes a sterol-C4-methyl oxidase; and several genes that encode

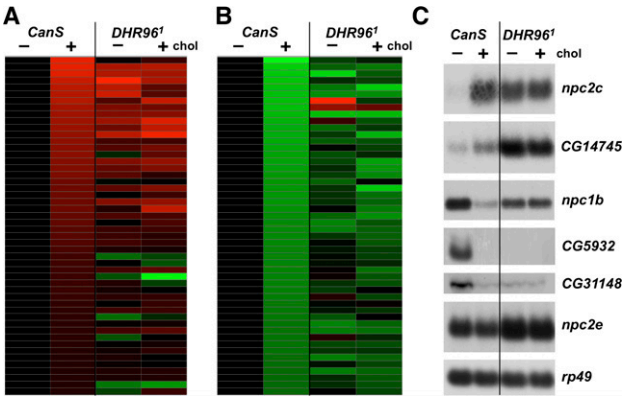

Figure 2. Most cholesterol-regulated genes depend on *DHR96* for their proper expression. (*A*,*B*) Heat maps are depicted representing the top 50 genes that are either up-regulated by cholesterol (*A*) or down-regulated by cholesterol (*B*) in *CanS* wild-type larvae, along with the responses of these same genes in *DHR96*^{1} mutants, as determined by microarray analysis. The heat maps are arranged from *top* to *bottom* by their fold response to cholesterol in wild-type larvae. The expression levels in columns 2–4 of each heat map are normalized to the expression level in column 1 (*CanS* −, cholesterol). Red represents increased transcript levels relative to the transcript level in column 1, while green represents lower transcript levels. (*C*) RNA isolated from *CanS* control larvae and *DHR96*^{1} mutant larvae, grown in either the absence (−) or presence (+) of cholesterol, was analyzed by Northern blot hybridization for expression of *npc2c*, *CG14745*, *npc1b*, *CG5932*, *CG31148*, and *npc2e*. Hybridization to detect *rp49* was used as a control for loading and transfer.

predicted stearoyl-CoA-desaturases. Many genes involved in other aspects of lipid metabolism are also misregulated in *DHR96* mutants (Supplemental Table S5). In addition, *DHR96* itself is down-regulated approximately twofold by cholesterol in wild-type larvae, and this response fails to occur in *DHR96* mutants, suggesting that there is autoregulation. Further examination of the cholesterol-regulated genes in both wild-type and *DHR96* mutant larvae revealed that many of these genes are expressed in the midgut, consistent with the critical role of dietary cholesterol uptake in a cholesterol auxotroph such as *Drosophila* (Supplemental Tables S5, S6).

Northern blot hybridizations confirmed that genes such as *npc2c* and *CG14745* (which encodes a predicted peptidoglycan recognition protein) are induced by cholesterol in wild-type larvae, while genes such as *npc1b*, *CG5932* (which encodes a gastric lipase), and *CG31148* (which encodes a predicted enzyme in sphingolipid metabolism) are repressed by cholesterol (Fig. 2C). Other genes, such as *npc2e*, are not responsive to cholesterol in wild-type larvae. All of these genes, however, are misregulated in *DHR96* mutants.

DHR96 *mutants are unable to survive on a low-cholesterol diet*

The central role of *DHR96* in mediating transcriptional responses to cholesterol suggests that it may contribute to the regulation of cholesterol homeostasis. As an initial test of this possibility, we examined how control and *DHR96*^{1} mutants respond to growth on a low-cholesterol diet. Whereas most wild-type larvae grown on this medium develop through to adulthood, *DHR96* mutants arrest their development primarily as second instar larvae and die within several days (Fig. 3A). Supplementation with a complete nutrient source, yeast, was sufficient to rescue this lethality (Fig. 3B). Efficient rescue was also achieved by supplementing with cholesterol, demonstrating that the lack of this essential nutrient is a cause of the lethality (Fig. 3C). Widespread heat-induced expression of a wild-type *DHR96* transgene, or specific expression of *DHR96* in the midgut, is also sufficient to rescue the lethality of *DHR96* mutants grown on the low-cholesterol diet (Fig. 3D,E). Specific expression of *DHR96* in the fat body of *DHR96* mutants had no effect. Taken together, these observations indicate that *DHR96* mutants are unable to survive under limiting cholesterol conditions, and that this phenotype is due to a specific loss of *DHR96* function in the midgut. Similar results were obtained when control and *DHR96* mutants were raised on a

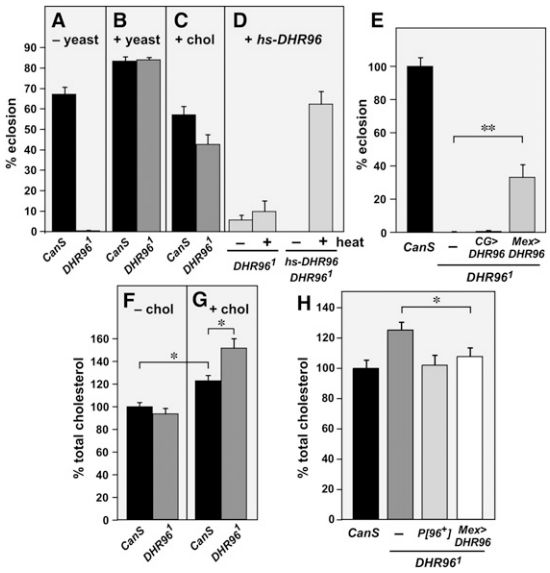

Figure 3. *DHR96* regulates cholesterol homeostasis. (*A–C*) *DHR96* mutants arrest development on a low-cholesterol medium. *CanS* control and *DHR96*^{1} mutant larvae were maintained on a low-cholesterol medium without supplementation (−yeast) (*A*), supplemented with yeast (+yeast) (*B*), or supplemented with 0.03% cholesterol (+chol) (*C*), and scored for the percent of adults that eclosed. (*D*) *DHR96*^{1} mutants carrying a heat-inducible wild-type *DHR96* transgene (+*hs-DHR96*) were grown on the low-cholesterol medium without supplementation in either the absence (−heat) or presence (+heat) of heat treatment, and scored for the percent of adults that eclosed. (*E*) The lethality of *DHR96* mutants maintained on the low-cholesterol medium is rescued by expressing *DHR96* in the midgut. *CanS* control and *DHR96*^{1} mutant larvae, maintained either without any transgenes (−), with *CG-GAL4;UAS-DHR96* (*CG* > *DHR96*; fat body-specific), or with *Mex-GAL4;UAS-DHR96* (*Mex* > *DHR96*; midgut-specific) were grown on the low-cholesterol medium without supplementation and were scored for the percent of adults that eclosed. (*F*,*G*) *DHR96* mutants accumulate cholesterol when grown on a high-cholesterol diet. *CanS* control and *DHR96*^{1} mutant larvae were grown on the low-cholesterol medium either without supplementation (−chol) (*F*) or in the presence of 0.03% cholesterol (+chol) (*G*). Total cholesterol levels were measured in larvae collected 2 d after hatching and normalized for total protein. Data were pooled from two experiments and are presented as normalized to a wild-type (minus added cholesterol) level of 100%. (*H*) *CanS* control and *DHR96*^{1} mutant larvae, maintained either without any transgenes (−), with a wild-type *DHR96* genomic construct (*P[96]*$^{+}$), or with *Mex-GAL4;UAS-DHR96* (*Mex* > *DHR96*) were grown in the presence of 0.03% cholesterol. Total cholesterol levels were measured in larvae collected 2 d after hatching and were normalized for total protein. Data are presented as normalized to a wild-type level of 100%. Error bars are ±SE. (*) $P < 0.05$; (**) $P < 5 \times 10^{-4}$.

chloroform-extracted medium that is deprived of all sterols (data not shown). A dose response study on the low-cholesterol diet revealed that 0.03% cholesterol is the ideal concentration for rescue (Supplemental Fig. S5). This amount is identical to the optimal amount required for *Drosophila* larval survival on a minimal defined medium (Sang 1956). A number of other sterols are able to substitute for cholesterol in these experiments, including 7-dehydrocholesterol, ergosterol, dehydroergosterol, sitosterol, and stigmasterol (data not shown), consistent with their ability to support normal growth of wild-type *Drosophila* (Cooke and Sang 1970). Supplementing the low-cholesterol medium with other lipids, however, including triacylglycerol (TAG) and oleic acid, had no effect, indicating that the rescue is specific to sterols (data not shown).

Several lines of evidence indicate that proper cholesterol metabolism is essential for larval development. *Drosophila* larvae grown in the absence of sterols arrest development at the first or second instar, similar to the lethal phase of *DHR96* mutants grown under low-cholesterol conditions (Cooke and Sang 1970). Similarly, mutants for *npc1a* die as first instar larvae due to defects in intracellular cholesterol trafficking and reduced production of the molting hormone 20E (Huang et al. 2005; Fluegel et al. 2006). Double mutants for *npc2a* and *npc2b* also fail to progress through development and can be rescued by feeding 20E (Huang et al. 2007). *DHR96* mutants grown on a high-cholesterol diet fail to show any clear defects in intracellular cholesterol localization, as detected by filipin staining (Supplemental Fig. S6). In addition, feeding 20E to *DHR96* mutants maintained on the low-cholesterol diet does not rescue their lethality (data not shown). These observations do not support the hypothesis that the lethality in *DHR96* mutants is due to a defect in cholesterol trafficking that affects 20E production. Rather, this phenotype is likely to arise from other defects, such as inefficient cholesterol utilization, changes in cholesterol storage, or disruption of other lipid metabolic pathways.

DHR96 *mutants accumulate cholesterol*

To determine whether *DHR96* mutants display defects in cholesterol homeostasis, we measured total cholesterol levels in control and $DHR96^1$ mutant larvae grown on the low-cholesterol medium, in either the absence or presence of 0.03% cholesterol. *DHR96* mutants have the same level of cholesterol as wild-type larvae when propagated without cholesterol supplementation (Fig. 3F). In the presence of added cholesterol, however, *DHR96* mutants display cholesterol levels that are significantly higher than the 10%–20% increase seen in control larvae (Fig. 3G). This cholesterol accumulation defect can be rescued by either a wild-type genomic *DHR96* transgene or expression of wild-type *DHR96* in the midgut of mutant larvae, indicating that it arises from a specific loss of *DHR96* function in this tissue (Fig. 3H).

An npc1b *mutation rescues the cholesterol accumulation defect in* DHR96 *mutants*

Expression of *npc1b*, which is required for dietary cholesterol absorption, is down-regulated when wild-type larvae are treated with cholesterol (Fig. 2C). The observation that this switch fails to occur in *DHR96* mutants could explain why these animals accumulate excess cholesterol when grown on a high-cholesterol diet. Consistent with this, we found that DHR96 is expressed in the region of the larval midgut, where *npc1b* exerts its functions (Fig. 4A; King-Jones et al. 2006; Voght et al. 2007). This overlap in *DHR96* and *npc1b* expression also appears to be functionally significant because specific expression of wild-type *DHR96* in the midgut of *DHR96* mutants is sufficient to restore appropriate *npc1b* transcriptional repression in response to cholesterol (Fig. 4B). Importantly, the accumulation of cholesterol in *DHR96* mutants is dependent on *npc1b* function because *npc1b;DHR96* double mutants have wild-type levels of cholesterol (Fig. 4C). This is consistent with the normal

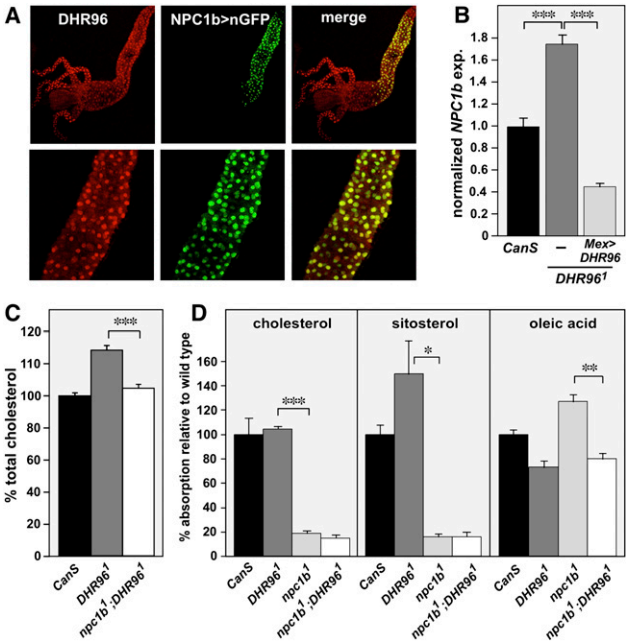

Figure 4. *npc1b* contributes to the cholesterol accumulation phenotype in *DHR96* mutants. (*A*) *DHR96* and *npc1b* are both expressed in the midgut. Midguts were dissected from *NPC1b-GAL4;UAS-nGFP* third instar larvae (NCP1b > nGFP) (Voght et al. 2007) and stained with affinity-purified antibodies to detect DHR96 protein. DHR96 protein is shown in red, and NPC1b expression is shown in green. All images were taken from the same field and focal plane. (*B*) *DHR96* regulates *npc1b* transcription. RNA was extracted from *CanS* control and $DHR96^1$ mutant larvae, maintained in the presence of 0.03% cholesterol either without any transgenes (–) or with *Mex-GAL4;UAS-DHR96* (*Mex* > *DHR96*), and analyzed by quantitative RT–PCR for levels of *npc1b* transcript. *npc1b* expression levels are presented as normalized to the level in *CanS*. (*C*) An *npc1b* mutation rescues the cholesterol accumulation defect in *DHR96* mutants. *CanS* control, $DHR96^1$ mutant larvae, and $npc1b^1;DHR96^1$ double mutants were grown in the presence of 0.03% cholesterol. Total cholesterol levels were measured in larvae collected 2 d after hatching and were normalized for total protein. Data are presented as normalized to a wild-type level of 100%. The cholesterol levels in *CanS* and $npc1b^1;DHR96^1$ double mutants are not significantly different ($P = 0.19$). (*D*) Sterol absorption, but not fatty acid absorption, is blocked by an *npc1b* mutation in a *DHR96* mutant background. *CanS* control, $DHR96^1$ mutants, $npc1b^1$ mutants, and $npc1b^1;DHR96^1$ double mutants were grown on a low-cholesterol medium supplemented with either ^{3}H-cholesterol, ^{3}H-sitosterol, or ^{3}H-oleic acid along with ^{14}C-glucose. Levels of radioactive lipid were normalized to the ^{14}C-glucose and are presented as normalized to a wild-type level of 100%. Error bars are SE. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 1 \times 10^{-4}$.

cholesterol levels seen in *npc1b* mutants at this stage of development, which likely derive from maternal loading of cholesterol during oogenesis (Voght et al. 2007).

We tested whether *DHR96* mutants require *npc1b* function for their ability to absorb dietary cholesterol. Control larvae, $DHR96^1$ mutants, $npc1b^1$ mutants, and $npc1b^1;DHR96^1$ double mutants were grown on the low-cholesterol medium in the presence of ^{3}H-cholesterol. Radioactive glucose was also added to the food in order to normalize the levels of cholesterol absorption and to control for different feeding rates (Voght et al. 2007). Under these conditions, *DHR96* mutants display a similar level of cholesterol uptake as wild-type larvae (Fig. 4D). This result is consistent with the wild-type levels of total cholesterol seen in *DHR96* mutants grown on a low-cholesterol diet (Fig. 3F). In contrast, both *npc1b* mutants and *npc1b;DHR96* double mutants display a dramatic reduction in cholesterol absorption (Fig. 4D). This result agrees with the original study of *npc1b* mutants, and indicates that *DHR96* mutants require NPC1b to take up dietary cholesterol (Voght et al. 2007). A similar result was seen with a key plant sterol, ^{3}H-sitosterol, suggesting that this pathway mediates general sterol absorption (Fig. 4D). Moreover, the *npc1b* mutation has no effect on the ability of *DHR96* mutants to absorb oleic acid, consistent with its specific role in sterol uptake (Fig. 4D). Taken together, these observations indicate that the misregulation of *npc1b* expression in *DHR96* mutants is sufficient to explain their inability to maintain proper cholesterol homeostasis when grown on a high-cholesterol diet.

It is important to note, however, that the cholesterol accumulation defect seen in *DHR96* mutants does not appear to be related to the lethality that is observed when the mutant is grown on a low-cholesterol diet. *DHR96* mutants that arrest their development when maintained on a low-cholesterol diet display normal levels of cholesterol (Fig. 3F). In addition, the *npc1b;DHR96* double mutants are small and die earlier than *npc1b* mutants alone when raised in either the presence or absence of cholesterol, in spite of their wild-type cholesterol levels (M Horner and CS Thummel, unpubl.). Thus, it is likely that the *DHR96* mutants suffer from additional metabolic defects beyond their inability to properly regulate *npc1b* transcription. This conclusion is supported by the widespread effects of the *DHR96* mutation on the expression of genes involved in lipid metabolism and midgut physiology, as well as the expression of DHR96 in tissues outside the midgut (King-Jones et al. 2006). Further studies of *DHR96* mutants should help to uncover its other key metabolic activities.

DHR96 *coordinates the uptake of dietary lipids*

The studies reported here define essential functions for *DHR96* in maintaining cholesterol homeostasis during larval development. A parallel set of studies in our laboratory has also identified nonessential functions for *DHR96* in lipid metabolism during adult stages due to their reduced levels of stored energy in the form of TAG (Sieber and Thummel 2010). Interestingly, microarray analysis of *DHR96* mutant adults raised on normal growth medium revealed widespread effects on the transcription of genes expressed in the midgut, many of which are identical to the *DHR96* regulatory targets described in this study. Among this core set of *DHR96* target genes is *CG5932*, which encodes a gastric lipase that is required for the breakdown of dietary fat. Misregulation of *CG5932* in the midgut is sufficient to explain the defects that we observed in TAG homeostasis in *DHR96* mutants, analogous to the important role of *DHR96* in regulating *npc1b* expression to maintain proper cholesterol homeostasis. Interestingly, *CG5932* transcription is also regulated by cholesterol, raising the possibility that this dietary sterol, acting through *DHR96*, may coordinate dietary TAG breakdown with cholesterol absorption (Fig. 2C). This convergence of our results points to a central role for *DHR96* in midgut physiology and provides a strong foundation to refine our understanding of its critical regulatory functions in maintaining lipid homeostasis.

DHR96 is a nuclear protein, in the presence or absence of cholesterol, consistent with its predicted role as a direct regulator of transcription (Fig. 4A; data not shown). It has a unique P-box sequence within its DNA-binding domain (DBD), which determines its DNA-binding specificity. This sequence is shared by only three *Caenorhabditis elegans* nuclear receptors: DAF-12, NHR-48, and NHR-8. Although a preferred binding site has been identified for DAF-12, it not known whether DAF-12 contacts this sequence as a homodimer or a heterodimer with another nuclear receptor (Shostak et al. 2004). Similarly, it is unclear whether DHR96 binds DNA as a monomer, homodimer, or heterodimer with the *Drosophila* retinoid X receptor (RXR) ortholog, like its mammalian homologs. In addition, the observation that DHR96 target genes display all possible combinations of regulatory responses to cholesterol and the *DHR96* mutation indicates that there is further complexity in this pathway. Studies are under way to define the molecular mechanisms by which DHR96 regulates target gene transcription.

DHR96 represents an ancestral regulator of cholesterol metabolism

Ligands have been identified for only two of the 18 canonical nuclear receptors in *Drosophila*: ecdysone receptor (EcR) and E75 (Koelle et al. 1991; Reinking et al. 2005). This study adds a third such adopted orphan receptor to the list: DHR96. The identification of cholesterol as a DHR96 ligand fits with its membership in the NR1I subfamily of nuclear receptors (Nuclear Receptors Nomenclature Committee 1999). The closest mammalian and *C. elegans* homologs of DHR96—pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D3 receptor (VDR), and DAF-12—are all transcriptionally responsive to cholesterol derivatives. Similar responses to sterol derivatives are seen for members of the next most closely related group of receptors, the NR1H group, which includes LXR, farnesoid X receptor (FXR), and EcR. These observations support the proposal that the NR1H and NR1I groups arose from an ancestral progenitor that acted as a sterol receptor in primitive organisms.

It is important, however, to note that although cholesterol copurifies with the DHR96 LBD, it may not be the natural ligand for this receptor. In our efforts to address this issue, we were unable to detect changes in DHR96 activity in response to exogenous cholesterol, dietary factors, or genetic backgrounds that disrupt cholesterol absorption or intracellular trafficking (data not shown). Further studies are required to determine whether cholesterol or a related metabolite acts as a regulatory ligand to modulate the activation status of DHR96.

Although the sequence of DHR96 is most closely related to mammalian PXR and CAR, it shares significant functional characteristics with its more distant cousin, LXRα. LXRα binds oxysterols and, similar to *DHR96* mutants, *LXRα* mutant mice fail to respond properly to dietary cholesterol and accumulate hepatic cholesterol when maintained on a high-cholesterol diet (Peet et al. 1998). LXRs also play a central role in the transcriptional response to dietary cholesterol in mice, and directly or indirectly control a number of genes that are related to DHR96-regulated genes, including *ABCG1*, *LPL*, *steroyl-CoA desaturase*, *NPC1*, and *NPC2* (Maxwell et al. 2003; Rigamonti et al. 2005; Kalaany and Mangelsdorf 2006). Finally, like DHR96, LXRs repress *NPC1L1* expression, although it is not clear whether this regulation is direct (Duval et al. 2006; Valasek et al. 2007). Taken together, these results indicate that DHR96 and mammalian LXRs act through similar regulatory pathways to control cholesterol homeostasis. This work establishes a new framework for understanding how cholesterol levels are sensed in *Drosophila*, and the molecular mechanisms by which cholesterol homeostasis is maintained.

Materials and methods

Drosophila *stocks*

All studies were performed using *Canton-S* (*CanS*) wild-type flies and *DHR96*^{1}-null mutants that had been crossed to *CanS* for nine generations (King-Jones et al. 2006). The following stocks were used in this study: *npc1a*^{1} (from M. Scott), *npc1b*^{1} and *NPC1b-GAL4* (both from L. Pallanck), *CG-Gal4* (Hennig et al. 2006), and *Mex-Gal4* (Phillips and Thomas 2006). Flies were maintained on standard Bloomington Stock Center medium with malt at 25°C.

Cholesterol assays

Total cholesterol levels were determined using an Amplex Red Cholesterol Assay Kit (Invitrogen). Embryos staged at 0–3 h were collected and allowed to hatch overnight in the absence of food at 25°C. First instar larvae were transferred to low-cholesterol medium with either vehicle alone or 0.03% cholesterol and grown for 2 d at 25°C. After 2 d, 30 larvae per sample were collected and homogenized in 100 μL of 1× buffer included in the kit. The homogenate was centrifuged at 5000 rpm for 5 min and the supernatant was transferred to new tubes. A 50-μL aliquot (15 larvae equivalents) was assayed according to the kit instructions and measured in a Molecular Devices SpectraMax M2 fluorometer. Cholesterol levels were normalized to protein in each homogenate using a Bradford assay (Bio-Rad). Samples were prepared in triplicate, each experiment was repeated at least three times, and the resulting data was pooled. Data was analyzed using an unpaired two-tailed Student's *t*-test with unequal variance. All data are reported as the mean with 1× SEM.

See the Supplemental Material for additional materials and methods.

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References

Bitsch F, Aichholz R, Kallen J, Geisse S, Fournier B, Schlaeppi JM. 2003. Identification of natural ligands of retinoic acid receptor-related orphan receptor α ligand-binding domain expressed in Sf9 cells–a mass spectrometry approach. *Anal Biochem* **323:** 139–149.

Brown MS, Goldstein JL. 1997. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89:** 331–340.

Cooke J, Sang J. 1970. Utilization of sterols by larvae of *Drosophila melanogaster*. *J Insect Physiol* **16:** 801–812.

Davis HR Jr, Basso F, Hoos LM, Tetzloff G, Lally SM, Altmann SW. 2008. Cholesterol homeostasis by the intestine: Lessons from Niemann-Pick C1 Like 1 (NPC1L1). *Atheroscler Suppl* **9:** 77–81.

Duval C, Touche V, Tailleux A, Fruchart JC, Fievet C, Clavey V, Staels B, Lestavel S. 2006. Niemann-Pick C1 like 1 gene expression is down-regulated by LXR activators in the intestine. *Biochem Biophys Res Commun* **340:** 1259–1263.

Fluegel ML, Parker TJ, Pallanck LJ. 2006. Mutations of a *Drosophila* NPC1 gene confer sterol and ecdysone metabolic defects. *Genetics* **172:** 185–196.

Ge L, Wang J, Qi W, Miao HH, Cao J, Qu YX, Li BL, Song BL. 2008. The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell Metab* **7:** 508–519.

Hennig KM, Colombani J, Neufeld TP. 2006. TOR coordinates bulk and targeted endocytosis in the *Drosophila melanogaster* fat body to regulate cell growth. *J Cell Biol* **173:** 963–974.

Huang X, Suyama K, Buchanan J, Zhu AJ, Scott MP. 2005. A *Drosophila* model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis. *Development* **132:** 5115–5124.

Huang X, Warren JT, Buchanan J, Gilbert LI, Scott MP. 2007. *Drosophila* Niemann-Pick type C-2 genes control sterol homeostasis and steroid biosynthesis: A model of human neurodegenerative disease. *Development* **134:** 3733–3742.

Ikonen E. 2008. Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol* **9:** 125–138.

Kalaany N, Mangelsdorf D. 2006. LXRS and FXR: The yin and yang of cholesterol and fat metabolism. *Annu Rev Physiol* **68:** 159–191.

King-Jones K, Horner MA, Lam G, Thummel CS. 2006. The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. *Cell Metab* **4:** 37–48.

Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS. 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67:** 59–77.

Maxwell KN, Soccio RE, Duncan EM, Sehayek E, Breslow JL. 2003. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J Lipid Res* **44:** 2109–2119.

Nuclear Receptors Nomenclature Committee. 1999. A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97:** 161–163.

Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR α. *Cell* **93:** 693–704.

Phillips MD, Thomas GH. 2006. Brush border spectrin is required for early endosome recycling in *Drosophila*. *J Cell Sci* **119:** 1361–1370.

Reinking J, Lam MM, Pardee K, Samson HM, Liu S, Yang P, Williams S, White W, Lajoie G, Edwards A, et al. 2005. The *Drosophila* nuclear receptor E75 contains heme and is gas responsive. *Cell* **122:** 195–207.

Rigamonti E, Helen L, Lestavel S, Mutka AL, Lepore M, Fontaine C, Bouhel MA, Bultel S, Fruchart JC, Ikonen E, et al. 2005. Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. *Circ Res* **97:** 682–689.

Sang J. 1956. The quantitative nutritional requirements of *Drosophila melanogaster*. *J Exp Biol* **33:** 45–72.

Shostak Y, Van Gilst MR, Antebi A, Yamamoto KR. 2004. Identification of *C. elegans* DAF-12-binding sites, response elements, and target genes. *Genes & Dev* **18:** 2529–2544.

Sieber MH, Thummel CS. 2010. The DHR96 nuclear receptor controls triacylglycerol homeostasis in *Drosophila*. *Cell Metab* (in press).

Va'nt Hoog EG. 1936. Aseptic culture of insects in vitamin research (continued). Zt. *Vitaminforsch.* **5:** 118–126.

Valasek MA, Clarke SL, Repa JJ. 2007. Fenofibrate reduces intestinal cholesterol absorption via PPARα-dependent modulation of NPC1L1 expression in mouse. *J Lipid Res* **48:** 2725–2735.

Voght SP, Fluegel ML, Andrews LA, Pallanck LJ. 2007. *Drosophila* NPC1b promotes an early step in sterol absorption from the midgut epithelium. *Cell Metab* **5:** 195–205.

Wang DQ. 2007. Regulation of intestinal cholesterol absorption. *Annu Rev Physiol* **69:** 221–248.