Establishment of a *Drosophila* model of intestinal sterol absorption and trafficking

Stephen P. Voght

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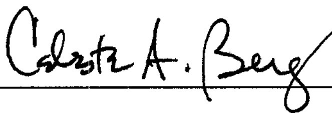

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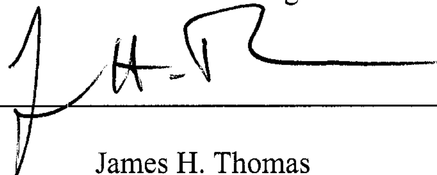

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Abstract

Establishment of a *Drosophila* Model of intestinal sterol absorption and trafficking

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Despite diet being a major source of cholesterol in Western culture, the absorption and downstream trafficking of cholesterol remain poorly understood processes. In humans, proteins of the NPC1 family are essential for these processes. Niemann-Pick Type C1 (NPC1) promotes intracellular sterol trafficking in all tissues, while Niemann-Pick Type C1-Like 1 (NPC1L1) performs as an essential early function in the absorption pathway. However, the specific mechanism by which these proteins function remains unidentified. This dissertation details my efforts to establish a model of dietary sterol absorption and trafficking using the fruit fly *Drosophila melanogaster* through the study of fly homologs of the NPC1 family. Previous work identified the protein NPC1a as the fly homolog of NPC1, while my work primarily focused on studying the functions of the second fly NPC1 family member, NPC1b. Animals lacking NPC1b protein die during larval development. Based on the known roles of the human NPC1 family, I hypothesized that NPC1b functions either specifically in the absorption of dietary sterols or in general intracellular trafficking of sterols. A novel dietary sterol absorption assay revealed that *NPC1b* mutants have dramatically reduced rates of sterol absorption. I found that the essential function of NPC1b is restricted to the midgut, and *NPC1b* mutant midgut

tissues are dramatically devoid of free sterols, indicating that NPC1b must perform an early role in sterol absorption. Together, my results indicate that the essential role for NPC1b lies in the process of dietary sterol absorption. Curiously, in the absence of both NPC1b and NPC1a, dietary sterol absorption rates are comparable to wild-type levels, suggesting that there is a secondary sterol absorption pathway normally inhibited by NPC1a. I found that this secondary pathway is regulated by 20-hydroxyecdysone. These findings suggest that the NPC1 family members are both involved in dietary sterol absorption. A major role of NPC1b is to facilitate absorption of dietary sterols, while NPC1a acts through ecdysone to inhibit a secondary absorption pathway. These findings provide new insights into dietary sterol absorption and trafficking and will allow us to further study the absorption pathway and functions of the NPC1 family.

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Chapter 1: Introduction

Sterols are an essential part of all eukaryotic cells. They perform a variety of critical roles, such as modulating phospholipid membrane fluidity and dynamics, acting as a source for steroid hormone synthesis, and physically modifying some proteins. With sterols performing such important roles in basic biology, dysregulation or disruption of sterol homeostasis has dramatic effects on health. A better understanding of sterol biology is important in an effort to understand the diseases related to this dysregulation [1-6]. In this chapter, I will first review the known cellular functions of sterols, disorders associated with dysregulation of these functions, how sterols are acquired and the proteins known to be involved in these processes. I will focus particular attention on proteins of the Niemann-Pick Type C1 (NPC1) family and the phenotypes associated with loss of these proteins. Lastly, I will review the suitability of *Drosophila melanogaster* for studying sterol biology.

In phospholipid membranes, cholesterol performs several important roles. Intercalation of cholesterol decreases membrane fluidity, and clustered regions of cholesterol within membranes alter their surrounding micro-domain, creating structures known as lipid rafts. These lipid raft regions are thought to be important for proper localization of certain membrane proteins, regulating their interactions with ligands and ability to transduct extracellular signals [7, 8].

Cholesterol is converted into a number of steroid hormones for use in signaling and development. During development, these hormones are used to trigger

events leading to differentiation, migration, apoptosis, and many other cellular processes.

Cholesterol is a required component of the mature Hedgehog developmental morphogen protein. Covalent attachment of a cholesterol moiety to the protein enables it to properly interact with its cognate receptor, Patched. The specific role of the cholesterol molecule in this interaction is unclear, but it is postulated to either limit the diffusion of the otherwise soluble Hedgehog protein by maintaining some interaction with neighboring cell membranes, or to directly affect the ability of Hedgehog to interact with Patched, via the sterol sensing domain found in Patched [8, 9].

Excess cholesterol contributes to a toxic build-up of plaque in artery walls, a condition known as atherosclerosis. Although the mechanism is not entirely clear, an initial deposition of oxidized cholesterol and macrophages on the smooth muscle of artery walls causes an inflammatory response, which leads to the deposition of additional LDL-derived cholesterol. This cholesterol is oxidized, causing further inflammation and the recruitment of macrophages which ingest the cholesterol and become adherent, which results in the build-up of arterial plaques [10]. As plaque levels increase the arteries become more damaged and blood flow becomes restricted. Restricted blood flow leads to increased blood pressure, and often to ischemic shock when blood flow is restricted to a critical level. Atherosclerosis and related heart conditions have become highly prevalent in the Western world, and a primary factor contributing to these disorders is a diet high in cholesterol [11-15].

Defective cholesterol metabolism is associated with several neurodegenerative disorders. In Alzheimer's Disease (AD), two independent findings connect cholesterol to AD incidence. First, the use of statins, a class of drugs that inhibit *de novo* cholesterol synthesis, is associated with a reduced incidence of AD. Second, people who have the ApoE4 allele of Apolipoprotein E, a protein necessary for cholesterol trafficking in brain tissue, have an increased risk of AD, although the specific mechanism is unknown. Although the connection between cholesterol and AD is not fully understood, evidence suggests that plasma membrane cholesterol levels affect enzymatic processes critical for AD progression [1, 6, 16-22]. In AD, the production of a Amyloid-β (Aβ), a neurotoxic cleavage product of amyloid precursor protein, leads to the development of amyloid plaques. The Aβ product is derived from two sequential cleavage events of the precursor protein. The membrane-bound enzyme γ-secretase performs the second cleavage event, and studies have shown that its enzymatic activity is influenced by levels of membrane cholesterol content, with increased levels of cholesterol leading to decreased enzymatic activity [23-25]. Based on this finding, it is hypothesized that the ApoE4 isoform leads to decreased levels of membrane cholesterol in the brain, although the exact mechanism is unknown.

A second, much more rare neurodegenerative disorder, Niemann-Pick Type C Disease (NPCD), also has links to cholesterol homeostasis [26]. In contrast to AD, where cholesterol metabolism indirectly affects proteins that lead to the disease, in NPCD the proteins that cause the disease directly affect cholesterol metabolism,

although it remains disputed whether this function is the cause of neurodegeneration. In NPCD, mutations in one of two proteins, NPC1 or NPC2, result in dysfunctional intracellular cholesterol trafficking and broad neurodegenerative phenotypes leading to lethality, typically in childhood [26]. The function of these proteins is not understood, and studying NPC1 is the focus of my dissertation.

Despite substantial research to understand the important functions of cholesterol in cellular processes, much remains to be discovered before these findings can be used to improve human health. For instance, although the enzymatic pathway of *de novo* cholesterol synthesis is understood and many drugs are available that act on this pathway such as statins, which inhibit HMG-CoA reductase, the rate-limiting step of cholesterol biosynthesis [27], a much more important source of cholesterol in Western society is dietary, and very little is understood about how dietary cholesterol is acquired and trafficked within cells.

My dissertation work developed a model of dietary cholesterol absorption and intracellular trafficking in which the function and interactions of specific proteins on the pathway can be studied. To accomplish this goal, I have studied two insect homologs of the NPC1 protein and used the well-understood genetic tools of *Drosophila melanogaster* to study these proteins and their functions.

The acquisition of dietary cholesterol

A major source of cholesterol acquisition is dietary. A better understanding of the dietary sterol absorption process could lead to development of drugs that inhibit uptake, thereby lowering the amount of circulating cholesterol and decreasing the risk

of diseases such as atherosclerosis. Broadly, dietary and biliary sterols within the intestine are deesterified by pancreatic cholesterol esterase before being solubilized into the hydrophobic centers of micelles consisting of conjugated bile acids, fatty acids and monoglycerides. The micelles facilitate transport across a water layer and to the surface of the luminal cells. Although the micelles themselves do not cross the cell membrane, the cholesterol is transferred and absorbed at the jejunum region of the small intestine, primarily at brush border membranes of enterocytes [28, 29]. Sterol uptake is thought to be mostly non-specific, with plant and fungal sterols being taken up along with cholesterol and other animal sterols. Evidence for this non-specificity comes from individuals with the rare disorder sitosterolemia, who suffer from an inability to efflux unwanted plant sterols back into the intestinal lumen [30]. However, it remains unclear how cholesterol and phytosterols are sorted and separated. Additionally, it is unknown how cholesterol is transported to the endoplasmic reticulum, at which location it is packaged with fatty acids to form lipoproteins called chylomicrons. These particles are then released into the circulatory system by an unknown transport mechanism, and later absorbed by the liver where they are further processed and packaged into low density lipoprotein (LDL) particles, which consist of a lipid and lipoprotein monolayer surrounding a hydrophobic core of esterified cholesterol. LDL particles are released into the bloodstream for use throughout the body.

Proteins involved in dietary sterol acquisition

Despite a large amount of work on the question of how dietary cholesterol is acquired and trafficked, the molecular mechanisms by which cholesterol is absorbed by the intestine remain poorly understood. As mentioned above, individuals suffering from sitosterolemia provided some of the first clues as to proteins involved in these processes. Within the intestinal epithelium, the various absorbed sterols are sorted. Normally most absorbed cholesterol is retained, while the vast majority of plant phytosterols are released back into the intestinal lumen. Studies of individuals with sitosterolemia identified mutations affecting two related proteins, ABCG5 and ABCG8, that lead to this condition [30]. These proteins are members of the ATP-binding cassette (ABC) family of active transport proteins [31], and it is thought that their normal function is to heterodimerize and pump absorbed sterols back into the intestinal lumen (see Figure 1.1 for a diagram of our current understanding of intestinal sterol transport.) Based on these findings, the field hypothesizes that initial sterol absorption is indiscriminate, and a subsequent trafficking step results in the selective retention of useful sterols and excretion of unwanted sterols. However, only a few proteins have been identified that are involved in absorption and subsequent trafficking.

Some proteins that have been identified as being in the trafficking pathway include ABCG5, ABCG8 and scavenger receptor B1 (SR-B1), but murine knockouts in each of these proteins ruled them out as essential for net sterol uptake from the intestine [30, 32-34]. The identification of the drug ezetimibe, a potent inhibitor of dietary cholesterol absorption, gave some of the first indications of the factors

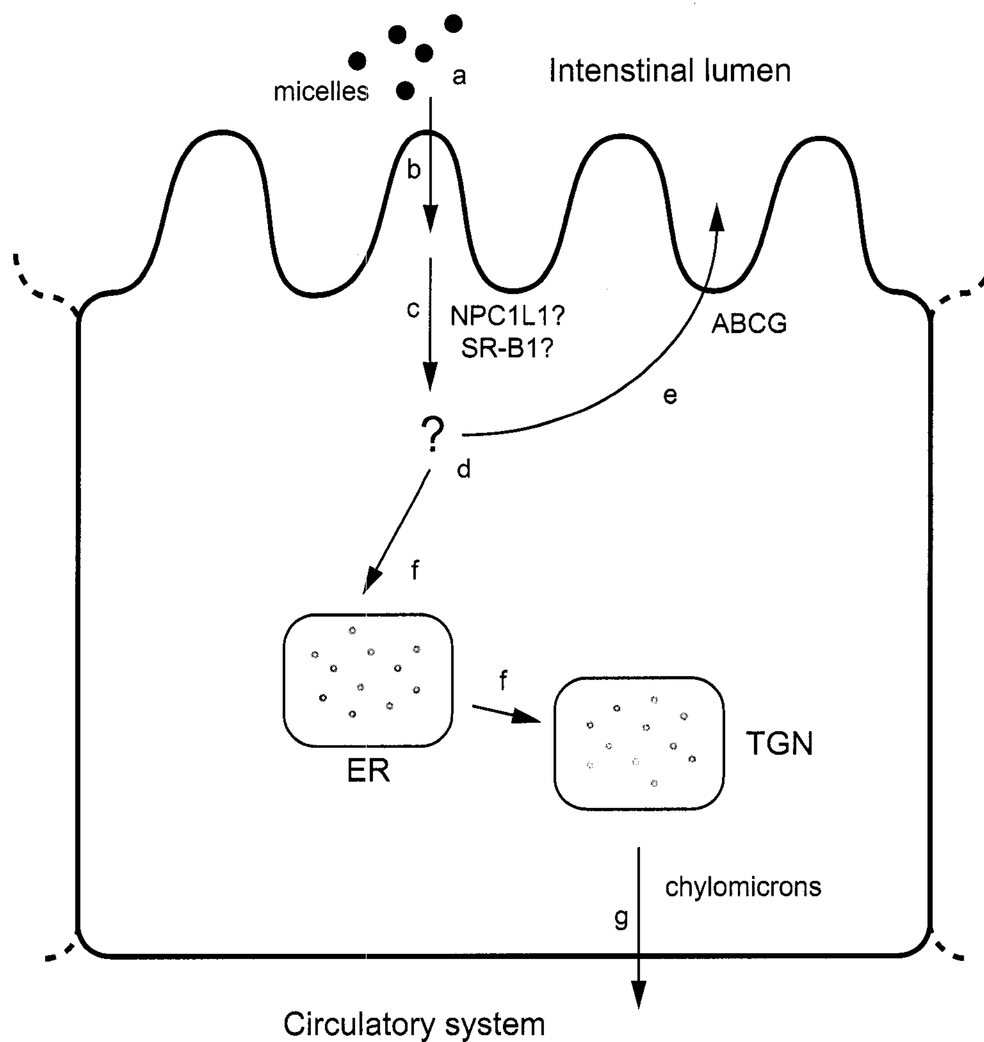


Figure 1.1. Overview of intestinal sterol transport. Dietary sterols are initially associated with fatty acid micelles, which facilitate their transport to the membrane of intestinal epithelial cells (a). There, the sterols become associated with cell membranes through an unknown process (b), and are thought to be absorbed through an NPC1L1- and SR-B1-mediated process (c). Unwanted sterols such as plant phytosterols are sorted away from useful sterols such as cholesterol (d), and transported back to the plasma membrane, where they are actively effluxed back into the intestinal lumen by ABCG-famly proteins (e). Retained sterols are then trafficked to the ER and trans-Golgi network (f), where they are packaged with lipoproteins to form chylomicrons. Chylomicrons are effluxed into the circulatory system, where they then travel to the liver for further processing and utilization (g).

involved in early trafficking processes [35, 36]. During the time I was researching this question in the fruit fly *Drosophila melanogaster*, genetic and pharmacological work was published that indicates that ezetimibe blocks the function of the mammalian Niemann-Pick C1-Like-1 (NPC1L1) protein [37], a closely-related homolog of NPC1, which will be discussed in more detail in the next section. This function was demonstrated by performing radioligand binding assays for ezetimibe using membranes from cells expressing recombinant NPC1L1 and from NPC1L1-deficient mice. These studies demonstrated conclusively that NPC1L1 is a binding target of ezetimibe [37]. Moreover, *NPC1L1*-knockout mice have a dramatically reduced ability to absorb dietary cholesterol and show no additional decrease in absorption rate upon treatment with ezetimibe [38, 39], indicating that NPC1L1 is an essential component of an ezetimibe-sensitive dietary cholesterol absorption pathway. While these findings clearly demonstrate that NPC1L1 promotes dietary cholesterol absorption, the specific mechanism by which it does so remains unclear. For instance additional recent evidence indicates that other factors, such as SR-B1, are involved in the earliest steps of cholesterol absorption at the luminal membrane of the intestine, and that NPC1L1 performs a slightly more downstream role in trafficking. Additionally, ezetimibe also binds to SR-B1, indicating that the drug may be less protein-specific than initially thought, though still specifically targeting the cholesterol absorption pathway [40, 41]. Although these recent findings have contributed to some dispute in the field regarding both the placement of NPC1L1 in the pathway and the actual action (or actions) of ezetimibe, the fact that NPC1L1-

knockout mice have a dramatic dietary sterol absorption defect, and SR-B1-knockout mice do not, confirms that NPC1L1 performs an essential central role in dietary cholesterol uptake. Additionally, although very little is understood yet about NPC1L1, many years of research have gone into studying its closely related homolog NPC1, which should provide insights about its function. I discuss the NPC1 protein family in detail in the next section.

Despite the identification of NPC1L1, SR-B1, ABCG5 and ABCG8 as important components of intestinal cholesterol trafficking, a large number of questions remain about how cholesterol is taken up by the small intestine and transported through intestinal enterocytes.

The NPC1 gene family

As mentioned above, the NPC1L1 protein is a closely related homolog of the NPC1 protein, mutations in which cause Niemann-Pick Type C Disease. The human *NPC1* gene encodes a 1278 amino acid polypeptide that is predicted to contain 13 transmembrane domains, an NPC1 domain, a sterol-sensing domain, a cysteine-rich domain, and a carboxy-terminal LLNF lysosomal targeting sequence (Figure 1.2, [42]). The NPC1 domain contains a leucine-zipper motif near the amino terminus that has high conservation between all the NPC1 family members in different organisms. Its exact function is unknown, but it is thought to mediate protein-protein interactions [43, 44]. The transmembrane and sterol-sensing domains exhibit limited sequence similarity to domains present in the *Drosophila* morphogen receptor *patched*, suggesting the possibility of common functions in these polypeptides [45].

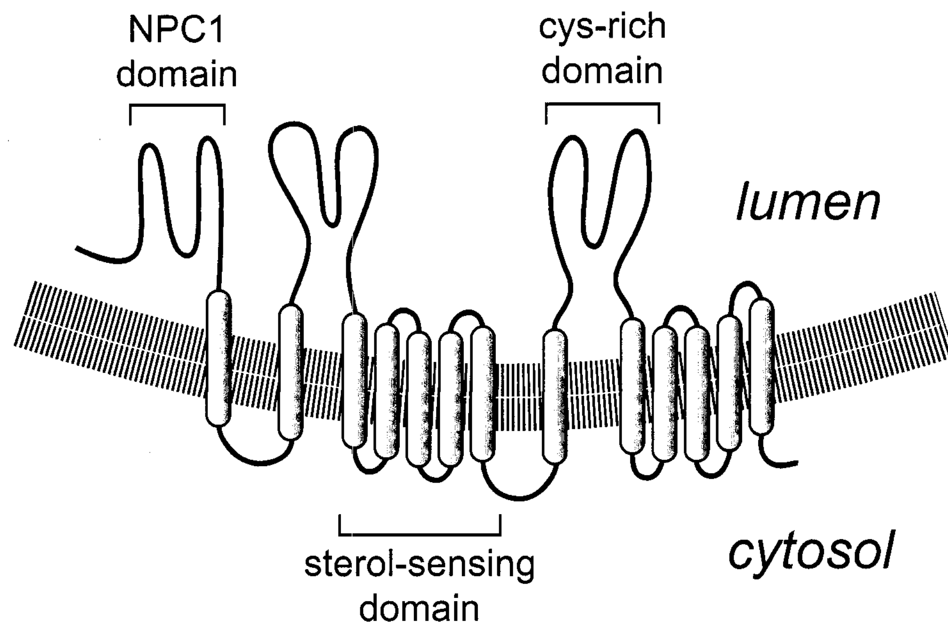


Figure 1.2. General topology of NPC1 family proteins. NPC1 family members are thirteen-pass transmembrane proteins with their amino-terminus on the lumenal side and their carboxy-terminus on the cytosolic side of the membrane into which they are inserted. Three predicted domains are noted in the figure. The NPC1 domain resembles a leucine-zipper and is hypothesized to mediate protein-protein interactions. The sterol sensing domain spans transmembrane domains three through seven and has been characterized in other cholesterol-sensing proteins, where it undergoes conformational changes depending on the sterol content of the membrane it spans. The cysteine-rich domain resembles a ring-finger domain and is also hypothesized to mediate protein-protein interactions. Note that the cytosol/lumen designation is based on the topology of NPC1 within the endosomal and lysosomal membrane as described in [42], and that the specific membrane and orientation of NPC1L1 is still unresolved.

Like the *NPC1* gene product, the *NPC1L1* gene product is also a thirteen-pass transmembrane protein with a putative sterol-sensing domain, NPC1 domain, cysteine-rich domain, broad homology to the Patched morphogen receptor, and structural similarity to the RND family of prokaryotic permeases [45-47]. The *NPC1L1* gene promoter contains two Sterol Regulatory Elements, indicating that expression of the protein is regulated by the amount of cholesterol already present in the cell [39]. The NPC1L1 protein contains a YQRL motif that is thought to function as a trans-Golgi targeting signal. The membrane that NPC1L1 localizes to remains disputed, but one current hypothesis proposes that it translocates between the Golgi and the plasma membrane to perform sterol absorption or transport activities, depending on levels of cholesterol at the plasma membrane [47-50].

The sterol-sensing domain provides some of the best clues as to the function of the NPC1 family of proteins. Many other proteins containing this domain have well-understood roles in cholesterol homeostasis. For instance, the enzyme HMG-CoA-Reductase controls the rate-limiting step of *de novo* cholesterol synthesis. Its sterol-sensing domain detects levels of free cholesterol in the membranes where it is located. When located in cholesterol-rich membranes, it is targeted for degradation, whereas when located in cholesterol-poor membranes, its conformation is such that the active catalytic site is available to perform its reductase reaction, converting 3-hydroxy-3-methyl-glutaryl-CoA into mevalonic acid [51-54]. Another protein with a well-studied sterol sensing domain is the SREBP Cleavage Activating Protein (SCAP), which performs the central regulatory role in cellular cholesterol

homeostasis [55]. This membrane-bound protein localizes to the ER, and when levels of free cholesterol are low in the membrane, it undergoes a conformational change enabling its catalytic domain to cleave Sterol Regulatory Element Binding Protein (SREBP), freeing it from a membrane-bound tether. This active fragment of SREBP acts on sterol regulatory elements throughout the genome, regulating the expression of such proteins as the LDL receptor, NPC1L1, cholesterol esterase, and other cholesterol-related proteins [56]. In all proteins where the sterol-sensing domain has been studied, the domain's function is to undergo a conformational change upon binding to free cholesterol in cellular membranes, suggesting that the sterol-sensing domains in NPC1 proteins perform a similar function, potentially enabling other parts of the protein to interact depending upon levels of cholesterol present [57].

Consistent with the membrane and lysosomal targeting motifs present in *NPC1*, subcellular distribution analysis indicates that the *NPC1* polypeptide localizes with endosomes, lysosomes and the trans-Golgi network [26, 58-61]. These molecular data, together with the phenotypic consequences of mutations in *NPC1*, suggest that the *NPC1* polypeptide functions as a mediator of cholesterol transfer from sterol-rich lysosomal and Golgi membranes to sterol-poor acceptor membranes. The *NPC1* polypeptide could directly participate in these processes or, like *patched*, serve as a signaling molecule to activate other polypeptides involved in these processes. The proposed role of NPC1 in intracellular sterol trafficking will be discussed in more detail in the next section.

Although the structural similarity of NPC1L1 to NPC1 suggests that these proteins play conserved biochemical roles, many important questions concerning the function of NPC1L1 remain unanswered. For example, it remains unclear whether NPC1L1 specifically promotes the absorption of cholesterol, or whether this protein also participates in the absorption of other dietary factors. The identities of key factors that regulate NPC1L1 and that function in concert with NPC1L1 to promote cholesterol absorption in the intestine also remain unknown. Whether NPC1L1 promotes an early step in sterol acquisition and/or, like NPC1, a later step in intracellular trafficking of cholesterol in the intestinal epithelium also remains controversial [48, 50]. Finally, the precise molecular functions of both NPC1L1 and NPC1 remain unknown and the topic of much debate, with hypotheses ranging from them acting as permeases, forming membrane channels to allow the transport of sterols, to flippases, moving sterols from one side of a lipid bilayer to the other, to sensors that simply interact with other functional proteins [62-66].

Intracellular sterol trafficking

Although only a few of the proteins involved in intracellular transport of cholesterol are identified, elegant work has determined the pathway by which plasma cholesterol is acquired and transported by cells [56]. As mentioned previously, LDL particles circulate throughout the bloodstream and serve as the major carrier for plasma cholesterol. The LDL receptor, expressed on the surface of cells, recognizes and binds to ApoB100 apolipoprotein that is present on the surface of LDL particles

[56]. Receptors with bound LDL cluster in clathrin-coated pits on the plasma membrane, and internalization occurs through standard endocytic processes.

Once internalized, the receptor is separated from the LDL particle and recycled back to the cell membrane, while the LDL particle is hydrolyzed, releasing the cholesterol stored within. The early endosome containing the apolipoprotein and cholesterol then matures into a late endosome, and from here the contents are trafficked to the lysosome, where the LDL particle is degraded. Acid lipase de-esterifies the cholesterol, freeing it for use throughout the cell. From here the pathway is less well understood, although studies have shown that some free cholesterol traffics directly to the plasma membrane, while the remainder is sent on to organelles such as the trans-Golgi network, mitochondria and ER through poorly understood vesicular and non-vesicular transport mechanisms [67]. Delivery of cholesterol to the ER is important for proper cellular cholesterol homeostasis, and many well-studied proteins critical for this function reside there. These transmembrane proteins, including HMG-CoA Reductase, SCAP and SREBP family members, react to the levels of free cholesterol in ER membranes and affect downstream steps of cholesterol synthesis and acquisition.

Proteins involved in intracellular sterol trafficking

Although cell biological studies revealed the intracellular routes taken by cholesterol following its initial uptake, the proteins involved in the vesicle-mediated and non-vesicular trafficking of cholesterol remain largely unknown. The vesicular-mediated transport of cholesterol requires Rab proteins, a large family of small G-

proteins involved in general vesicle membrane dynamics and fusion, fission and motor protein localization [68-70]. Although most research on vesicular transport has focused on the secretory pathway and synaptic transmission, cholesterol movement is thought to occur through similar mechanisms and utilize the same general factors including proteins from the NSF, SNAP and SNARE families [71]. Aside from these proteins that are known to perform a role in general vesicular dynamics, only a few proteins are known to be essential for cholesterol transport. Among them are the LDL receptor, and NPC1 and NPC2. I described the LDL receptor and NPC1 in a previous section, and NPC2 is a small, soluble lysosomal protein that has cholesterol-binding properties [72, 73]. Other proteins, such as MLN64, are also involved in intracellular cholesterol transport, but their roles are non-essential [74-76]. It remains unknown if any other proteins are essential in cholesterol trafficking, and how NPC1 and NPC2 facilitate the trafficking process. What is understood is that loss of either NPC1 or NPC2 function leads to a cholesterol-trafficking defect and Niemann-Pick Type C Disease [63].

Niemann-Pick Type C Disease

NPC1 and NPC2 were first implicated in cholesterol trafficking when it was discovered that cells from patients suffering from Niemann-Pick type C disease (NPCD) had a dramatic intracellular cholesterol trafficking phenotype. The disease is rare, affecting approximately 1 in 100,000 births, and is inherited in an autosomal recessive pattern. It is classified as a lysosomal storage disorder, and pathology affects both the viscera and central nervous system [77, 78]. Neurological

phenotypes associated with NPCD include progressive ataxia, dystonia, dysarthria, dementia, and cataplexy, owing to widespread neurodegeneration. Other symptoms include enlarged spleen and liver, although these symptoms do not correlate with severity of the neurological pathology, and in most cases they do not contribute to morbidity. The onset of NPCD neurological phenotypes usually occurs in school-age children, with few surviving into their twenties. However, the NPCD phenotypes are somewhat variable, and onset of symptoms can range from infancy to adulthood [79-82]. Treatment strategies to date are limited to addressing symptoms of the disease and have not had any success in slowing down progression.

At the cellular level, the most pronounced NPCD phenotype is an alteration of intracellular cholesterol homeostasis. Receptor-mediated endocytosis and intracellular transport of cholesterol-rich LDL particles by the LDL receptor appears to proceed normally in NPCD patients up to the lysosome. Cholesterol is de-esterified successfully, but the rate of trafficking from the lysosome to the plasma membrane is severely reduced, and cholesterol accumulates in the lysosome and trans-Golgi cisternae. The induction of cellular cholesterol homeostatic regulatory responses associated with cholesterol enrichment of ER membranes is delayed in cells from NPCD patients, indicating that cholesterol trafficking to the ER is also defective [83, 84]. Together, these data suggest that NPCD is caused by a defect in cholesterol trafficking from the lysosome to the trans-cisternae of the Golgi apparatus, and/or from the trans-Golgi to the ER and plasma membrane. The finding that endocytic organelles from NPCD individuals display altered transport kinetics

reinforces this conclusion [69, 85, 86]. Additionally, there is strong biochemical evidence that cholesterol plays a central role in the disease and in vesicular trafficking. The primary evidence supporting this hypothesis is studies on the NPC2 protein, which has been shown to be a small, soluble cholesterol transport protein [72, 87]. Because loss of this protein results in phenotypes indistinguishable from that of loss of NPC1 function, this finding argues that the central defect in NPCD relates to cholesterol, and that NPC1 also has a direct role in cholesterol trafficking.

Although cholesterol trafficking defects were among the earliest phenotypes observed in cells from NPCD individuals, they are by no means the only defects present. Glycosphingolipid and ceramide levels are often highly elevated in NPCD cells, and these molecules are also trafficked inappropriately in a manner similar to that of the cholesterol defects. They are often found trapped in the same vesicular compartments as cholesterol [78, 88-90], indicating that glycosphingolipid homeostasis may also be affected by NPCD. Additionally, some fat-soluble vitamins, amino acids such as cysteine, and other cellular metabolites such as tyrosinase show mild trafficking defects [91-93], although there are different ideas about what these defects mean. Some investigators argue they are primary defects [62, 94-96] while others argue that they are secondary to other trafficking defects and simply get caught up in mistrafficked vesicles [97, 98].

These dramatic trafficking defects led the field to hypothesize that the build-up of mistrafficked lipids may be toxic to cells [99, 100], and reducing the cholesterol or glycosphingolipid load might alleviate pathology. To address these hypotheses,

researchers engineered mouse knockouts that were mutant for NPC1 and were also unable to either synthesize complex glycosphingolipids (GalNAcT knockout) or take up LDL particles (LDL receptor knockout). Neither mutant combination showed a change in pathology or lethality, despite showing much less severe glycosphingolipid or cholesterol trafficking defects [97, 101, 102]. These results suggest that the pathology of NPCD is more complex, and that cholesterol and glycosphingolipid accumulations might be secondary to the primary problem in NPCD. However, these results do not preclude the possibility that cholesterol is necessary for proper intracellular vesicular transport, and that NPCD lethality is related to this transport defect rather than problems with accumulation of compounds.

Suitability of* Drosophila *for studying cholesterol trafficking

My dissertation work focuses on studying the functions of the *NPC1* gene family by developing a model to study the mechanisms of dietary cholesterol absorption and intracellular sterol trafficking. To accomplish this work, I used a genetic approach in the fruit fly *Drosophila melanogaster*. Although insects lack the ability to synthesize sterols, dietary sterols are required for proper insect development, and previous work suggests that sterol trafficking mechanisms are highly conserved in insects and vertebrates [103]. Although the only use of sterols that has been thoroughly studied in insects is their role in steroid hormone biology, they are also thought to be utilized in membranes, creating sterol-rich and sterol-poor pockets that are presumed to affect processes such as membrane fluidity,

transmembrane protein aggregation, endocytosis and intracellular signaling [104, 105].

Previous study of cholesterol trafficking in *Drosophila* and related invertebrates indicate that these processes are also conserved between flies and mammals. Sterols are trafficked through insect hemolymph by lipoprotein molecules known as lipophorins, which perform a role analogous to that of mammalian HDL and LDL [106]. Like their mammalian counterparts, the lipophorins from locust, cockroach and silkworm come in two forms, known as high- and low-density lipophorin (HDLp and LDLp, respectively) [106-112]. Like mammalian lipoproteins, the insect lipoproteins are composed primarily of apolipoprotein, diacylglycerides and cholesterol, and undergo interconversions upon changes in lipid and apolipoprotein composition [106-110]. Also, like mammalian lipoproteins, the insect lipoproteins bind with high affinity to specific insect plasma membrane endocytic receptors, and this binding can be competed with human lipoproteins [111, 112]. Although lipophorins can be taken up by many insect tissues, an important tissue that requires uptake of dietary cholesterol in *Drosophila* is the larval ring gland, which is the primary steroidogenic organ and the site of synthesis of the *Drosophila* molting hormone ecdysone during the larval stages of development. Growth of *Drosophila* on media lacking cholesterol, or mutations in genes involved in the conversion of cholesterol to ecdysone, result in early larval lethality [105, 113-116]. The mechanism by which internalized cholesterol is trafficked within the ring gland and other insect cells remains unexplored.

Unlike mammals, most insects, including *Drosophila melanogaster,* need to obtain sterols from their diet, as they are unable to synthesize them due to the absence of several key enzymes in the cholesterol synthesis pathway, the earliest being squalene synthase [117]. The source of sterols utilized by each species varies, as does their ability to interconvert sterols between different isoforms and types. For instance, *D. melanogaster* does quite well on ergosterol and cholestanol, while *Aedes egypti*, the yellow fever mosquito, is able to utilize either compound poorly, if at all [105]. Much of the variability between different species is thought to be correlated with their diet and habitat, with broad foragers more able to utilize a wide variety of sterols than very selective species. For example, the species *Drosophila pachea*, which survives specifically on a diet of the fruit from the cactus *Lophacereus schottii*, can not utilize the vast majority of sterols that closely related sibling species can utilize, but instead fares best on the cactus phytosterol schottenol [118]. The specific differences between species in their ability to interconvert various sterols from one form to another remains a mystery, including why sterols that some species survive and proliferate on are toxic to other species. For instance, the corn earworm *Heliothis zea* dies when raised on ergosterol [119], but this sterol is either beneficial or benign to most other species. Although the reasons for these differences in viability on various sterols are likely to be primarily due to enzymatic differences between species, further research is required before any conclusions can be drawn, and the recent divergence of many of these species suggests that sterol metabolism can

rapidly evolve depending upon specialized environmental conditions and available sources.

However, in most cases, although the insects can obtain a variety of sterols from a variety of sources, cholesterol is the preferred sterol absorbed from the diet. Experiments performed in the early 1960s in *Eurycotis floridana*, the common cockroach, demonstrated tissue enrichment of cholesterol versus cholestanol when raised on a diet with a twenty-fold overabundance of the latter sterol [105, 120]. Interestingly, the enrichment does not reflect discrimination in uptake of sterols from the midgut. Similar experiments with other insects have also shown selective enrichment, to the extent that contamination of supposedly sterol-free media with trace amounts of cholesterol has resulted in false data being reported about the survival of insects without sterols [116]. Interestingly, the distribution of cholesterol compared to cholestanol in these and other tracking experiments suggests there are specific structural needs for cholesterol that other sterols cannot fulfill [121, 122]. These needs, however, remain unidentified and poorly understood, and it is unclear if cholesterol is the only sterol capable of fulfilling these needs in all sterol-requiring species.

While much is known about the nutritional requirements, metabolism of and breadth of sterol use in various *Drosophila* species, there remains relatively little known about the mechanisms of absorption and the exact tissue, cellular and subcellular needs for sterols, along with what role they play beyond steroid signaling

and membrane intercalation. Some inference can be made from studies performed in other insect species, notably the tobacco hornworm *Manduca sexta*.

Experiments in *Manduca*, where the animals were fed food containing radiolabeled sterols and then sacrificed at specific time points for analysis, revealed that sterols are initially taken up by the mucosal cells of the midgut. From there, they are trafficked first to the fat body, where they are packaged into lipophorin molecules [123]. The lipophorin is released into the hemolymph, where it travels through the animal and is taken up by other cells that need sterols. Because *Drosophila* has similar anatomy, also featuring mucosal cells, fat body, and lipophorin, I predict that the sterol trafficking pathway acts in a similar manner.

The *Drosophila* genome contains two *NPC1* homologs, designated *NPC1a* and *NPC1b*. We and others have previously demonstrated that NPC1a is ubiquitously expressed and is required for efficient intracellular sterol trafficking in many tissues, including the ring gland, where the major insect steroid hormone ecdysone is synthesized [124, 125]. Loss-of-function mutations in *NPC1a* result in first-instar lethality that can be rescued by feeding exogenous ecdysone or by increased levels of dietary sterols. Adult flies rescued in this manner show a shortened lifespan and neurodegeneration. On a cellular level, NPC1a mutant cells show cholesterol trafficking defects similar to those of mammalian NPC1 mutant cells. Taken together, these findings indicate that NPC1a performs a role homologous to that of mammalian NPC1.

In my dissertation work, I have subjected the *NPC1b* gene to mutational analysis. In the next chapter I discuss how I generated a loss-of-function mutation in *NPC1b*. Additionally, I discuss my findings that demonstrate that NPC1b functions in a role homologous to that of mammalian NPC1L1 in the regulation of dietary sterol uptake from the gut epithelium. I discuss my findings that the gene is essential, with mutants dying during the second-instar larval stage of development. Lastly, I discuss my results that suggest that NPC1b promotes an early step in dietary sterol absorption and may participate in the absorption of other essential dietary factors. In the third chapter, I discuss epistasis between the NPC1a and NPC1b proteins. I also discuss my findings that suggest the existence of an NPC1a- and NPC1b-independent mechanism of sterol absorption mediated by the steroid hormone ecdysone. Finally, in the fourth chapter, I discuss how my findings contribute new knowledge to the field of sterol absorption and trafficking, how my findings fit with several controversies in the field, and some potential experiments to extend the research discussed here. In summary. this work establishes *Drosophila melanogaster* as a model system for studying dietary cholesterol acquisition and establishes tools that will be useful in future research into the pathway.

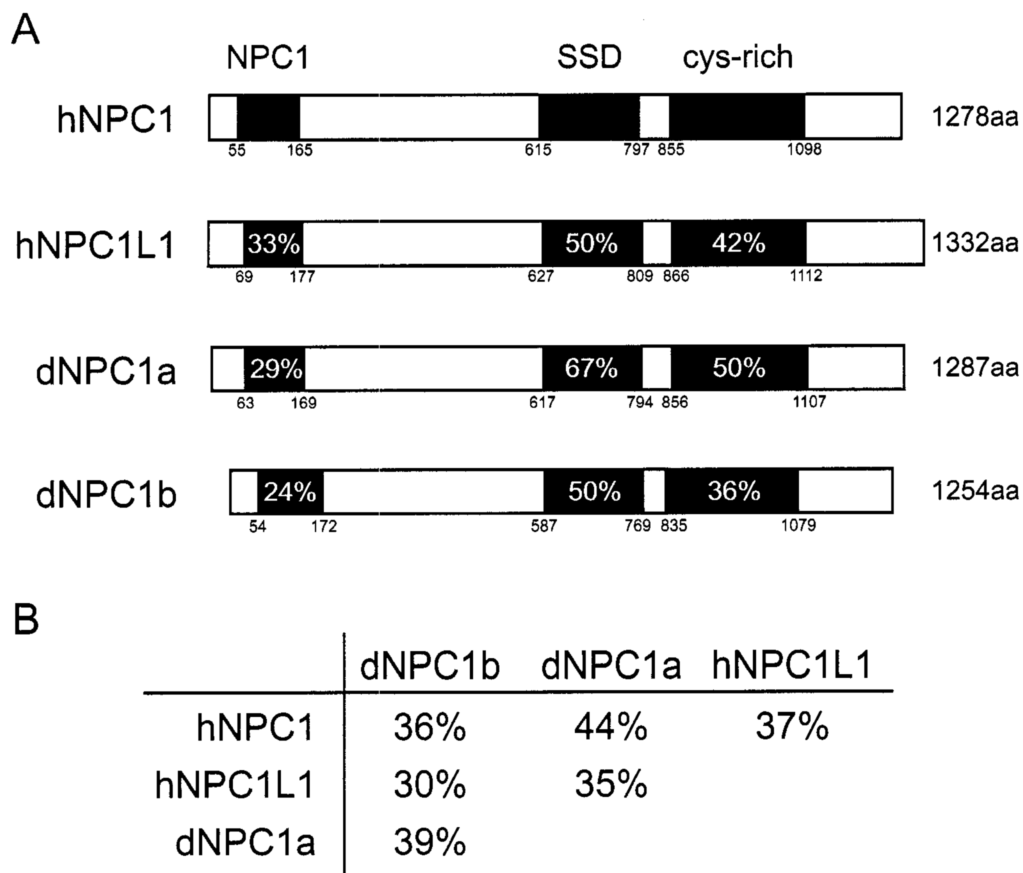
Chapter 2: *Drosophila NPC1b* mutant isolation and characterization

In order to better understand the function of NPC1 and NPC1L1 in sterol trafficking and Niemann-Pick Type C Disease, I set out to isolate loss-of-function mutations in the *Drosophila* NPC1b gene. This chapter describes the identification of two *Drosophila* homologs of NPC1, *NPC1a* and *NPC1b*, summarizes how alleles of *NPC1b* were identified, examines the resulting phenotypes of these alleles, and summarizes initial characterization of the protein. My studies indicate that *NPC1b* is expressed only in the larval and adult midgut. *NPC1b* is an essential gene that is required for proper progression through larval development. Mutations in the gene confer second-instar larval lethality with no overt dysfunctional phenotype. Although my findings suggest that NPC1b is involved in absorption of dietary sterols, *NPC1b* mutant lethality is not affected by supplementation with cholesterol or ecdysone, and total sterol levels in *NPC1b* mutants are not significantly different from wild type levels, implying that *NPC1b* mutants do not die due to a deficiency in sterol metabolism.

Results

Identification of Drosophila **NPC1** *homologs*

Two *Drosophila* homologs of *NPC1*, designated *NPC1a* and *NPC1b*, were identified by Leo Pallanck from BLAST searches of the Berkeley *Drosophila* Genome Project database (Figure 2.1). The *NPC1a* gene (previously designated *NPC1*; [126]) maps to polytene region 31B1 of the second chromosome and encodes a polypeptide of 1,287 amino acids exhibiting 43% amino acid identity to the human



	dNPC1b	dNPC1a	hNPC1L1
hNPC1	36%	44%	37%
hNPC1L1	30%	35%	
dNPC1a	39%		

Figure 2.1. *Drosophila* homologs of NPC1 proteins. A. Scale schematic showing percent identity of specific domains between human NPC1 (hNPC1), human NPC1L1 (hNPC1L1), *Drosophila melanogaster* NPC1a (dNPC1a) and *Drosophila melanogaster* NPC1b (dNPC1b). NPC1 is the NPC1 domain, SSD is the Sterol-Sensing Domain, cys-rich is the cysteine-rich domain. All percentages compare to hNPC1. B. Total percent identity between the above proteins.

NPC1 gene product. The *NPC1b* gene (previously identified as CG12092) maps to polytene region 19E5 of the X-chromosome and is predicted to encode a polypeptide of 1,223 amino acids exhibiting 40% amino acid identity to the human *NPC1* gene product. The *NPC1a* and *NPC1b* gene products also exhibit 36% and 33% identity to the human *NPC1*-like (*NPC1L1*) gene product [47], respectively, indicating that there is not a strict one-to-one orthologous correspondence between the *NPC1a*, *NPC1b* and *NPC1* and *NPC1L1* genes. The next most closely related gene to *NPC1* in the *Drosophila* genome encodes a protein exhibiting less than 30% amino acid identity to the human *NPC1* gene product. Thus, the *NPC1a* and *NPC1b* genes are the best candidates for encoding proteins with functions equivalent to the human *NPC1* gene product in the *Drosophila* genome.

***Corrected annotation of* NPC1b**

Using computational analysis of the NPC1b gene region, I discovered that the gene was incorrectly annotated by the *Drosophila* genome sequencing projects. To make this determination, I aligned DNA sequences from the *NPC1b* region of *Drosophila melanogaster, Drosophila pseudoobscura* and *Anopheles gambiae.* This analysis revealed an area of nucleotide similarity that extended upstream from the predicted *D. melanogaster* start codon. Further, this region of similarity was within the same open reading frame as the predicted start codon, and there was an additional start codon 93 bases upstream of the computationally annotated start codon of NPC1b. To further investigate this region of similarity, the predicted thirty-one amino acid translation of the sequence within this region was compared between

A

```
Dm_NPC1b    ------------------------------------------MIGAHWVNKGDTNPARPL...
Dp_NPC1b    MKGN-----YLWLVGGLMALAVACRAQEEQELGCIWYGQS-HQIGNHWQNLADTDPARAL...
Hs_NPC1     MTAR--------GLALGLLLLLLCPAQVFSQS-CVWYGECGIAYGDKRYNCEYSGPPKPL...
Dm_NPC1a    MSPRSPLRISPFGVHILIAAVLFTLIQSSKQD-CVWYGVCNTNDFSHSQNCPYNGTAKEM...
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B

```
Dm_NPC1b    MK----------VIFATIWLIAGAWSQSAEQLGCIWYGQS-HMIGAHWVNKGDTNPARPL...
Dp_NPC1b    MKGN-----YLWLVGGLMALAVACRAQEEQELGCIWYGQS-HQIGNHWQNLADTDPARAL...
Hs_NPC1     MTAR--------GLALGLLLLLLCPAQVFSQS-CVWYGECGIAYGDKRYNCEYSGPPKPL...
Dm_NPC1a    MSPRSPLRISPFGVHILIAAVLFTLIQSSKQD-CVWYGVCNTNDFSHSQNCPYNGTAKEM...
```

Figure 2.2. Reannotation of *Drosophila melanogaster* NPC1b. Dm: *Drosophila melanogaster*. Dp: *Drosophila pseudoobscura*. Hs: *Homo sapiens*. Bold residues are conserved between all proteins compared. A. Alignment of original annotations computationally derived from sequencing projects and the NIH National Center for Biotechnology Information GenBank database. Only the first few amino acids of each protein are shown, starting with the computationally predicted start codon for each protein. B. Reannotated alignment using an upstream start codon for *Drosophila melanogaster* NPC1b, which is located in the same open reading frame as the computationally predicted start codon used in part A. Note that the five additional conserved residues in the other NPC1 proteins (bold text) are also conserved in this additional sequence for *Drosophila melanogaster* NPC1b.

species, this time also including the insect NPC1a proteins, the *Mus musculus* and human NPC1 and NPC1L1 proteins. This analysis revealed a high degree of similarity between all the sequences, including many perfectly conserved residues (Figure 2.2). Based upon these findings, I concluded that the annotation software incorrectly assigned the start codon for NPC1b, and the gene actually encodes a protein of 1,254 amino acids. I also experimentally verified this conclusion by testing the ability of transgenic constructs that either included or lacked this additional sequence to recapitulate NPC1b function in an NPC1b loss-of-function background.

Identification of* NPC1b *mutants

Because there were no known preexisting mutations in the *NPC1b* gene and Laurie Andrews was unsuccessful in generating *P* element transpositions into the region for transposon-mediated mutagenesis, I utilized a homologous recombination targeting technique [127, 128] to generated a targeted mutation of *NPC1b*.

Jessica Greene and I engineered an *NPC1b* mutant allele by using a gene-targeting construct that included a nonsense mutation at the end of the first exon of *NPC1b* at codon 392 (Y392X), a diagnostic restriction site, and other alterations required for gene-targeting [127]. I used this construct to generate mutations as detailed in the methods section and outlined schematically in Figures 2.3 and 2.5. From a screen of 35,000 flies, I recovered four events where the targeting construct mobilized and moved to another chromosome from its initial location. Of the four events, two mapped to the X chromosome. Targeting was verified experimentally by Southern blotting the *NPC1b* region, where a successful targeting event results in a

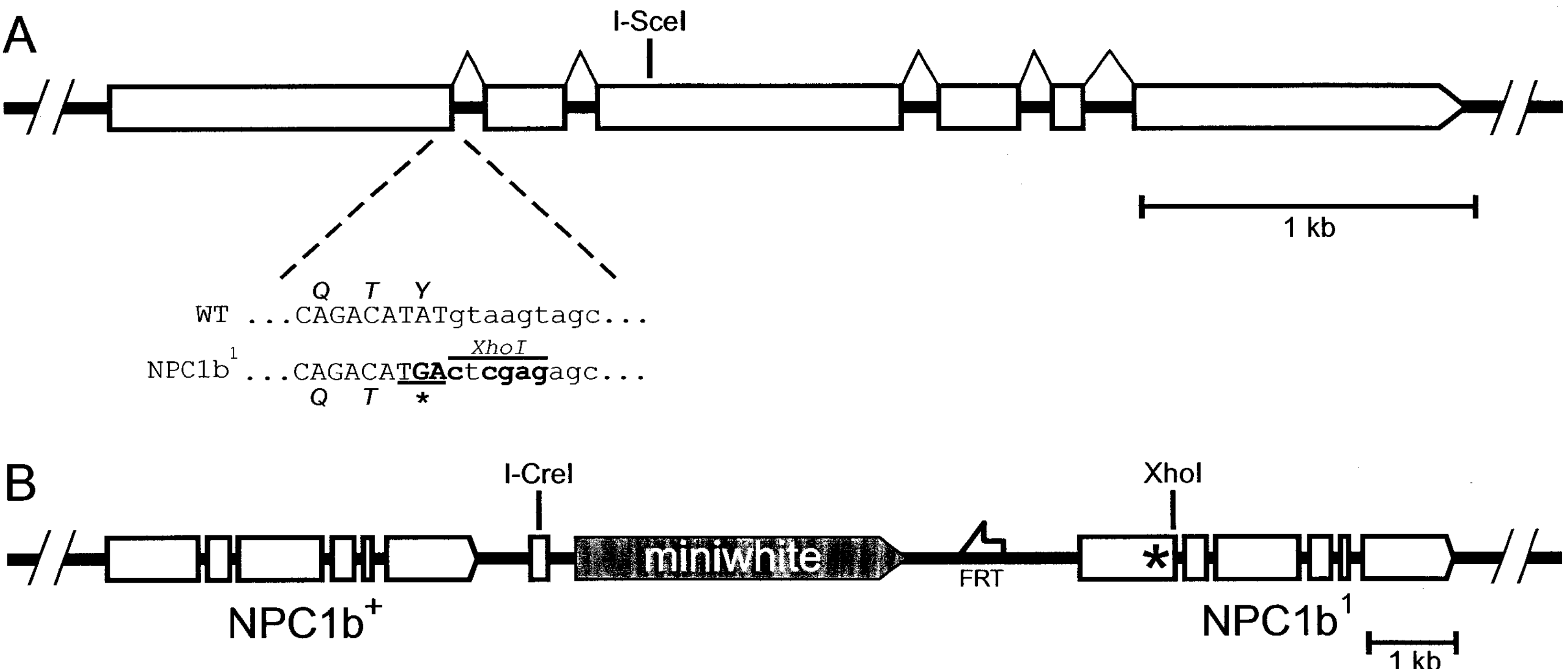


Figure 2.3. Generation of an *NPC1b* null mutation - initial targeting. A. An *NPC1b* genomic fragment was modified to include a stop codon at amino acid position 392, an XhoI diagnostic site, and an I-SceI restriction site. B. After insertion into the *Drosophila* genome, the construct was subsequently excised as a linear fragment via the flanking FRT sites and the I-SceI site. This linear fragment can recombine at the *NPC1b* genomic locus resulting in tandem integration, with the wild type copy of the gene followed by the *miniwhite* transgene, an I-CreI restriction site and the targeted allele.

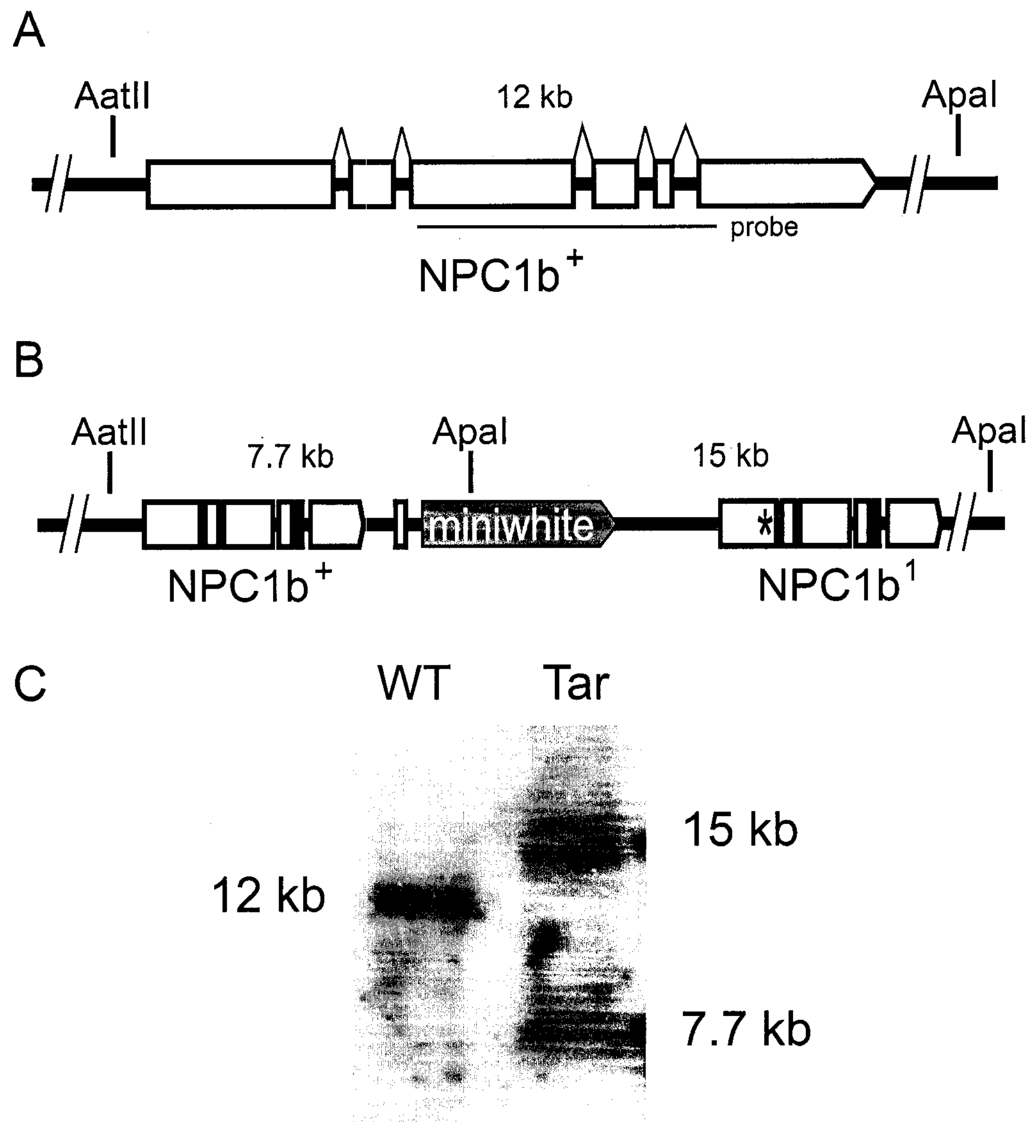


Figure 2.4. Confirmation of successful *NPC1b* targeting construct integration by Southern blot. A. Wild type sequence results in a 12 kb product after digestion with AatII and ApaI. B. Proper targeting integration results in a 7.7kb and 15kb product using the same digestion and probe. C. Representative blot showing wild type sequence (WT) and properly targeted sequence (Tar).

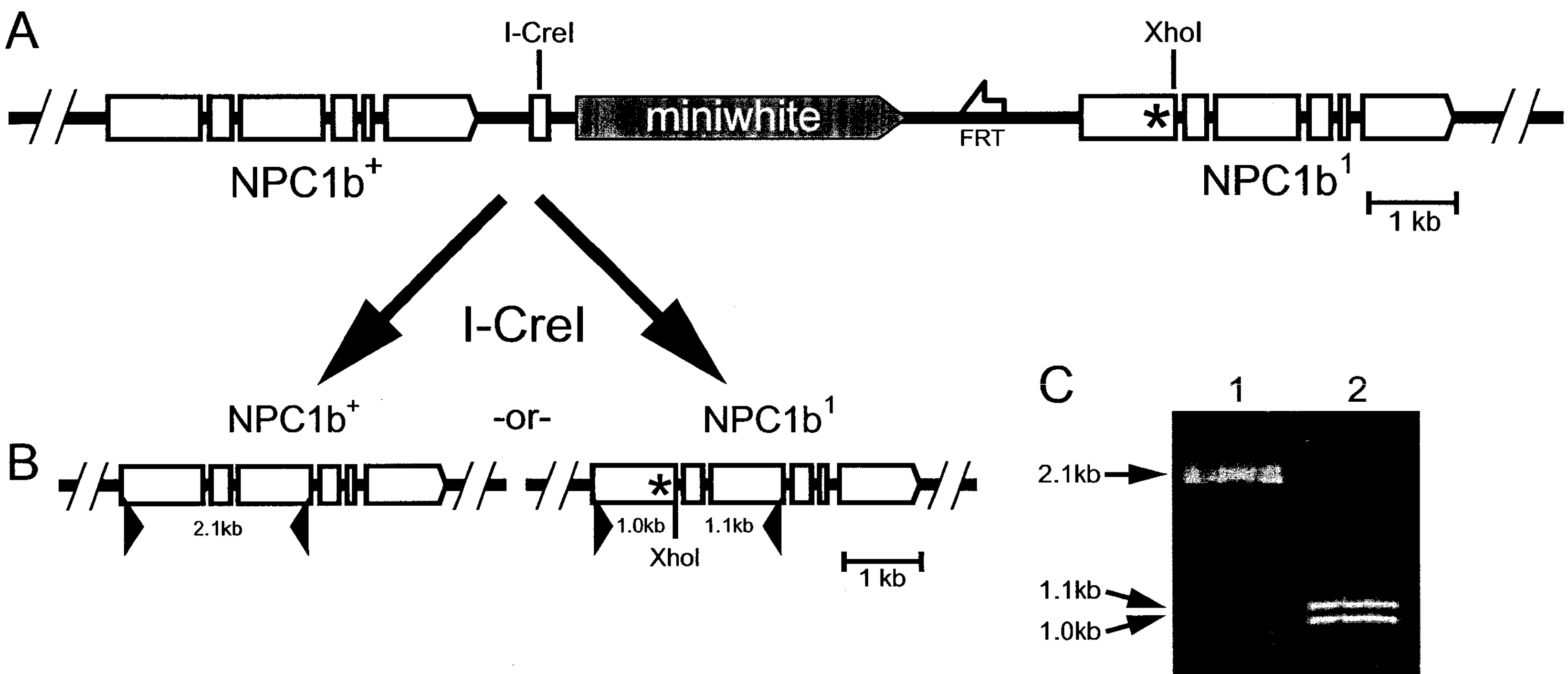
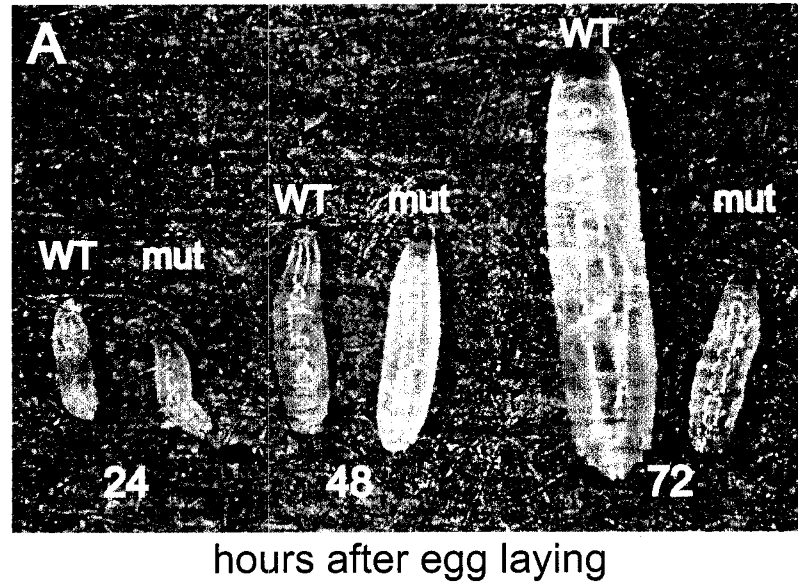


Figure 2.5. Generation of an *NPC1b* null mutation - resolution of tandem duplication. The tandem duplication (A) was resolved to a single copy by generating a double-stranded break at the I-CreI site, followed by recombination-mediated repair, leaving either the wild type *NPC1b* gene or the targeted allele of *NPC1b* with the inactivating mutations (B). C: PCR amplification using primers flanking the inactivating mutation (arrowheads in B), followed by digestion with the XhoI restriction enzyme, results in a 2.1 kb fragment if the wild type gene sequence is present (lane 1) or 1.1 and 1.0 kb digested fragments if the targeted allele is present (lane 2).

two fragments of a different size in the region being probed. This probing revealed that both X-chromosome targeting events consisted of a wild type copy of the *NPC1b* gene and a tandem copy bearing the inactivating mutations (Figure 2.4). Subsequent generation of a double-stranded break between the mutationally altered and wild type copies of the *NPC1b* gene resulted in double-stranded DNA break repair by homologous recombination between the two copies of *NPC1b*. This repair led to the recovery of 33 recombinant lines bearing only the mutant copy of *NPC1b*. Loss of the wild type *NPC1b* allele and retention of the mutation was verified by PCR across the diagnostic restriction site introduced into the targeting construct (Figure 2.5) and was additionally confirmed by DNA sequencing. Initial characterization indicated that all of these lines were molecularly and phenotypically identical, so one line, designated *NPC1b*^{1}, was chosen for detailed characterization.

***Initial characterization of* NPC1b^{1}**

NPC1b is located on the X chromosome, and both hemi- and homozygous *NPC1b*^{1} mutants died at the second-instar larval stage of development, with no difference between male and female larvae. I assayed mutant larvae for standard behavioral responses including tactile response and negative phototaxis, and they showed no obvious deficits in these behaviors. Additionally, mutant larvae showed no gross morphological defects such as developmental defects or necrosis, and were able to survive for at least a week as second-instar larvae, while heterozygous and non-mutant siblings progressed through development normally (Figure 2.6). During the course of making these other observations, I noted that approximately five percent



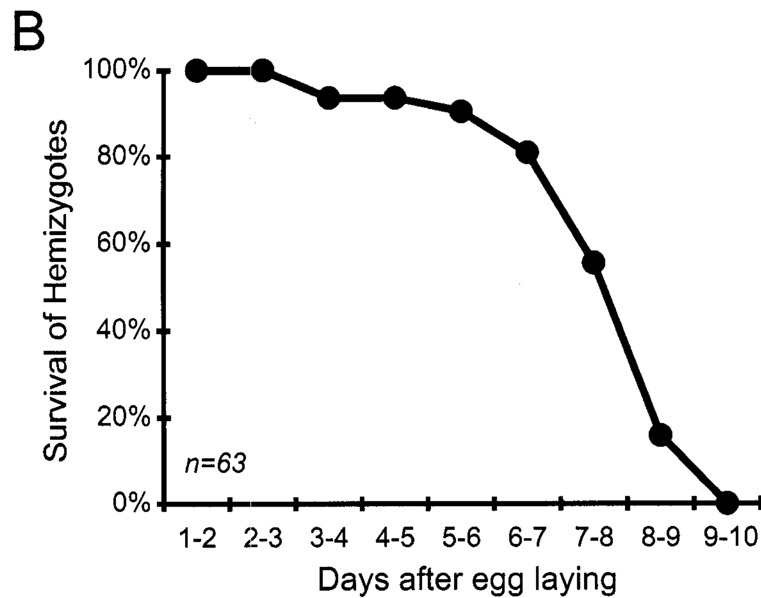


Figure 2.6. *NPC1b* mutants fail to progress through development. A: Comparison of size differences between ***NPC1b***^{1} hemizygous (mut) and isogenic wild type control (WT) larvae. Larvae were genotyped at hatching through use of a GFP-marked balancer chromosome, and photographed at the indicated time points. B: Hemizygous mutant larvae (n=63) were collected at hatching, genotyped through use of a GFP-marked balancer chromosome, and assayed every 24 hours for survival. Though most larvae survive for an extended period of time, they do not develop beyond the second-instar stage.

of hatched mutant larvae arrested at the molt from second- to third-instar, as evidenced by the appearance of a few dead larvae with duplicated mouth hooks and spiracles. To confirm that the recessive lethal phenotype of *NPC1b*^{1} mutants derives specifically from loss of NPC1b function I tested whether the deficiency chromosomes Df(1)C1 and Df(1)A118, which bear deletions that span the *NPC1b* region, were able to complement the *NPC1b*^{1} recessive lethal phenotype. Male flies carrying the duplication chromosome Dp(1;Y)mal^{+} are viable with either of these deficiencies or the *NPC1b*^{1} mutation, enabling the generation of transheterozygous larvae. The *NPC1b*^{1} mutation in *trans* to either of these deficiency chromosomes conferred a phenotype identical to *NPC1b*^{1} hemi- or homozygous mutants.

To further validate the specificity of the *NPC1b*^{1} mutation, I generated a genomic construct consisting of the entire re-annotated predicted coding sequence of the *NPC1b* gene, all introns, approximately 1 kb of downstream sequence, and 860 base pairs of upstream sequence (Figure 2.7). When introduced into an *NPC1b*^{1} mutant background this construct fully rescued the *NPC1b* recessive lethal phenotype to adulthood in expected Mendelian ratios (n>100). The rescued flies were fertile and showed no obvious distinguishable phenotypes or shortened lifespan relative to wild type animals (although this data was not quantified), indicating that this construct contains all of the necessary and essential NPC1b transcriptional elements to produce fertile adult animals.

To determine the specific tissues where NPC1b function is required, I generated two GAL4-responsive *NPC1b* constructs (designated *UAS-NPC1b*)

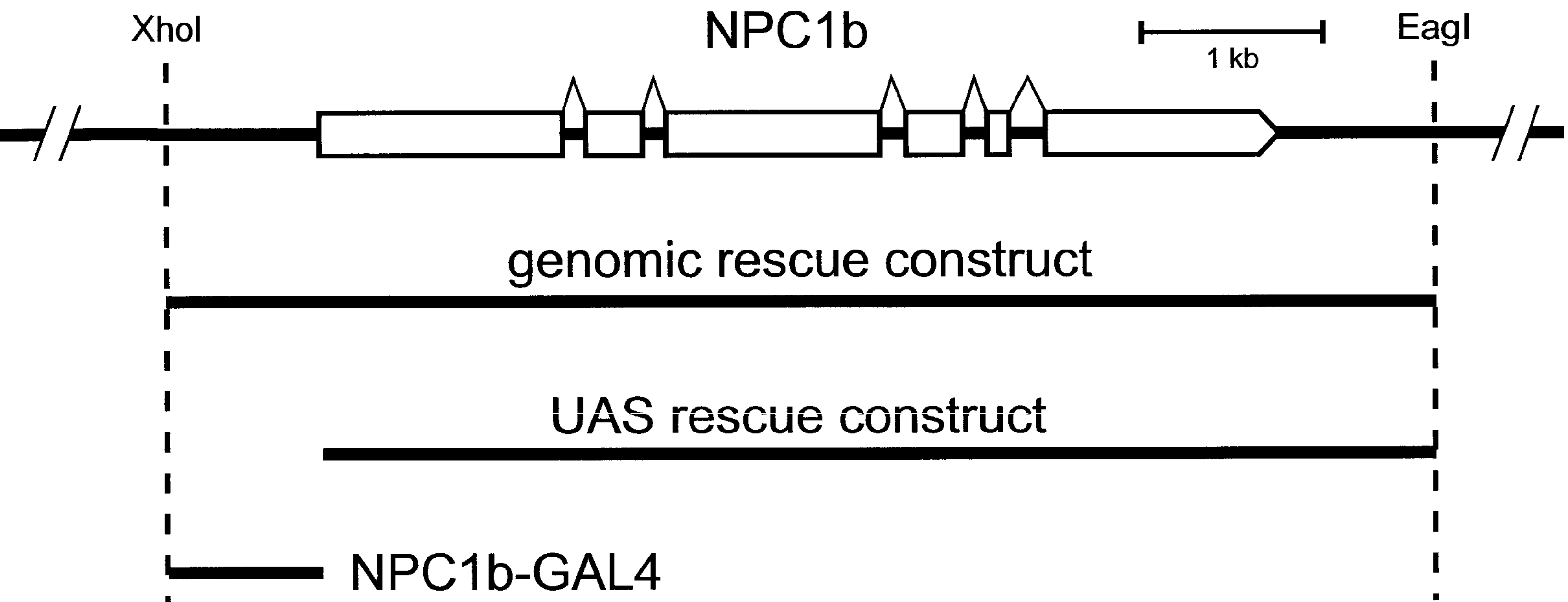


Figure 2.7. Transgenic *NPC1b* constructs. Top: *NPC1b* genomic region. Second from top: XhoI-EagI genomic rescue construct. Second from bottom: UAS-fusion rescue construct. Bottom: *NPC1b-GAL4* promoter-fusion expression construct.

consisting of the *NPC1b* locus lacking all upstream promoter sequences. The first construct, the 1,254 amino acid version of the transgene (designated *UAS-NPC1b*^{1254}), was sufficient to completely rescue the *NPC1b*^{1} mutant phenotype to Mendelian ratios when driven by a GAL4 construct consisting of the NPC1b promoter fused to the GAL4 open reading frame (details outlined below). In contrast, multiple independently-derived lines of the 1,223 amino acid version of the transgene (designated *UAS-NPC1b*^{1223}) were incapable of rescuing the NPC1b^{1} lethal phenotypes when expressed by any promoter. Additionally, I was unable to rescue the lethal phenotype using either transgene in combination with any other promoter-driven GAL4 elements, including elav-GAL4, Mhc-GAL4, and several midgut-GAL4 drivers that are discussed in more detail below, indicating that the NPC1b expression pattern is unique among publicly available GAL4 strains. In all cases, at least 100 transgenic larvae were assayed for each GAL4 element, and none reached the third-instar stage or beyond, although all produced transgenic larvae, indicating that none was lethal when driving expression of an NPC1b transgene. This observation was later verified by testing the expression pattern of additional GAL4 strains that were reported at FlyBase to have expression in tissues similar to that of NPC1b [129]. Of the additional GAL4 strains tested, three were found not to have midgut expression: c805-GAL4 showed hindgut expression, MJ12A-GAL4 showed Malpighian tubule expression, and MJ33A-GAL4 showed foregut and protoventriculus expression. One, 34B-GAL4, showed midgut expression only in the copper cells of the midgut. Two others expressed in non-epithelial midgut tissue: c564-GAL4 showed connective

tissue expression, and 5053a-GAL4 showed neuronal expression. A final strain, c343a-GAL4, showed diffuse expression throughout the foregut, midgut and hindgut, but it was also incapable of rescuing the NPC1b phenotype. Together, these results demonstrate that the phenotypes associated with *NPC1b*^{1} result specifically from loss of NPC1b function. Moreover, my finding that the lethal phase of *NPC1b*^{1} homozygotes is indistinguishable from that of animals bearing the *NPC1b*^{1} allele in *trans* to a deletion of the *NPC1b* region indicates that the *NPC1b*^{1} mutation represents a null allele of the *NPC1b* gene.

***Expression analysis of* NPC1b**

My attempts to determine the expression pattern of NPC1b using *in-situ* hybridization were unsuccessful, possibly due to the NPC1b transcript being expressed at too low of a level to be observed above background staining. However, the discovery that 860 bases of upstream sequence were sufficient to drive expression of a genomic construct in all essential tissues provided an opportunity to use this same sequence to drive a reporter construct consisting of the NPC1b promoter-containing fragment placed upstream of the yeast GAL4 protein coding sequence. Transgenic flies bearing this *NPC1b-GAL4* fusion construct were crossed to flies carrying a *UAS-mCD8-GFP* transgene. This GFP transgene was chosen for its ability to bind cell membranes, providing information about the morphology and cell type where it is being expressed. Larval progeny of this cross that carried both the *NPC1b-GAL4* and *UAS-mCD8-GFP* transgenes expressed GFP in two distinct regions of the midgut, corresponding to compartments m2 and m10-m12 (Figure 2.8;

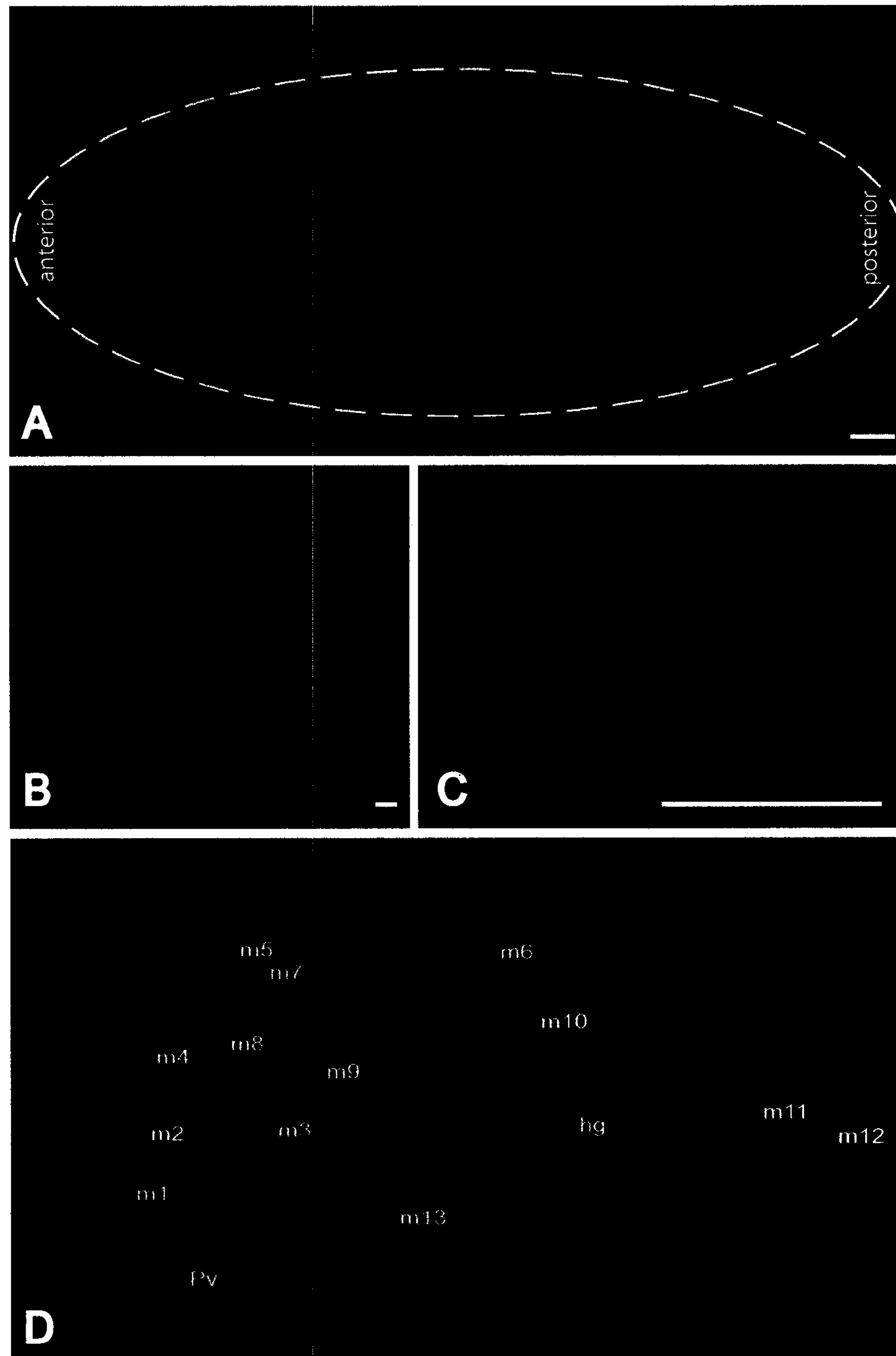


Figure 2.8. Expression profile of *NPC1b-GAL4*. All images are *UAS-mCD8-GFP* under the control of the *NPC1b-GAL4* expression construct. A. Third-instar larvae showing two regions of GFP expression in the midgut. B. Adult midgut tissue, showing a single region of GFP expression. C. Isolated GFP-positive larval midgut tissue, showing the polygonal morphology characteristic of midgut epithelial cells. D. Compartments of the larval midgut, based on [130]. GFP is expressed in compartments m2 and m10-m12.

[130]). GFP expression diminished during pupation, and then reappeared in the adult midgut approximately two days post-eclosion (Figure 2.8B). Higher magnification of the larval midgut revealed that the *NPC1b-GAL4* transgene drives expression specifically in the polygonal cells of the midgut epithelium and not in surrounding muscle tissue (Figure 2.8C). GFP fluorescence was not detected in any tissues outside of the midgut at any stage of development, suggesting that NPC1b expression is restricted to the midgut epithelia throughout the *Drosophila* life cycle. This observation was verified with two independent *NPC1b-GAL4* insertions and two independently derived *UAS-mCD8-GFP* transgenes, all combinations of which gave identical results. Control animals bearing only the *NPC1b-GAL4* or *UAS-mCD8-GFP* transgene failed to produce detectable GFP fluorescence, demonstrating that the *NPC1b* promoter is directly responsible for GFP expression observed in the midgut.

***Identification of additional alleles of* NPC1b**

In an effort to identify additional alleles of *NPC1b*, I tested whether other uncharacterized mutations mapping to the *NPC1b* region could complement the $NPC1b^{1}$ mutation. One mutation, $NPC1b^{R9\text{-}28}$ [131], failed to complement the $NPC1b^{1}$ recessive lethal phenotype. Like $NPC1b^{1}$ mutant larvae, $NPC1b^{R9\text{-}28}$ mutant larvae also die at the second-instar stage when hemi- or homozygous, behaving in a manner indistinguishable from $NPC1b^{1}$. $NPC1b^{1}/NPC1b^{R9\text{-}28}$ transheterozygous mutants also died as second-instar larvae, and showed behavior and lifespan similar to either of their progenitor mutations. Furthermore, the lethality of the $NPC1b^{R9\text{-}28}$ mutation was rescued by both the *NPC1b* genomic rescue construct and the *NPC1b-*

GAL4 transgene in conjunction with the *UAS-NPC1b*^{1254} transgene. Sequencing of the *NPC1b*$^{R9\text{-}28}$ allele revealed a nonsense mutation at amino acid position 289 in the *NPC1b* coding sequence resulting in the mutation W289X, confirming that this mutation is an allele of the *NPC1b* gene. These findings indicate that the *NPC1b*$^{R9\text{-}28}$ allele is, like *NPC1b*^{1}, a null allele of the *NPC1b* gene.

Ruth Steward's lab at Rutgers University provided us with a second mutant, *NPC1b*^{P266}, which they generated and identified based on its failure to complement *NPC1b*$^{R9\text{-}28}$. I verified this finding and confirmed that it also fails to complement *NPC1b*^{1} and deficiencies spanning the region, producing transheterozygous larvae that are indistinguishable from the progenitor mutants. Furthermore, the lethal phenotype of *NPC1b*^{P266} is also fully rescued by all the rescue constructs outlined above. The Steward Lab reports the mutation in *NPC1b*^{P266} as D789V, although this mutation was not independently verified.

Lastly, I performed an EMS mutagenesis screen for new mutations that failed to complement *NPC1b*^{1}. From this screen I identified one mutant, *NPC1b*$^{1116\text{-}13}$, that shows second-instar lethality when transheterozygous with *NPC1b*^{1}, but is embryonic lethal when hemi- or homozygous. Further analysis of this allele indicated that it fails to complement the deficiency chromosome Df(1)T2-14A, which is immediately adjacent to, but does not disrupt *NPC1b*. This finding indicates that the *NPC1b*$^{1116\text{-}13}$ allele is either a small genomic deletion spanning *NPC1b* and other nearby essential genes, or that the chromosome bearing the *NPC1b*$^{1116\text{-}13}$ allele has two tightly linked mutations, one in *NPC1b* and one in a second gene that is essential for embryonic

development that is disrupted by Df(1)T2-14A. Further experimental work was not performed with this allele.

NPC1b *and* NPC1a *cannot substitute for one another*

To determine if lethality could be overcome in either *NPC1a* or *NPC1b* null mutant animals by increased expression of the other gene, I tested whether transgenic expression of NPC1b could substitute for NPC1a and restore sterol trafficking. For this experiment I used the $NPC1a^{57A}$ mutant, which has been characterized and is verified as a null mutation of the *NPC1a* gene [125]. Homozygous *NPC1a* null mutants have widespread defects in intracellular sterol trafficking and die at the first-instar stage of development [124, 125]. However, I was unable to rescue the $NPC1a^{57A}$ recessive lethal phenotype by driving expression of NPC1b using GAL4 drivers that confer rescue with a *UAS-NPC1a* transgene. Similarly, I was unable to rescue the $NPC1b^{1}$ recessive lethal phenotype by driving expression of NPC1a using GAL4 drivers that confer rescue with the $UAS\text{-}NPC1b^{1254}$ transgene. In both cases, transgenic larvae (n>100 for each) did not survive beyond the normal lethal stage of their base mutation.

Dietary supplementation and lethal phase

Insects are unable to synthesize sterols from acetate and thus require a dietary source of sterols for the synthesis of the steroid molting hormone ecdysone, which is required for progression of larval development and metamorphosis. Given the restricted expression of NPC1b to the midgut and the failure of $NPC1b^{1}$ mutants to molt to the third-instar larval stage, I hypothesized that $NPC1b^{1}$ mutants may be

deficient in ecdysone production due to a primary defect in the absorption of a dietary sterol. I performed several experiments to explore this hypothesis. Because the lethal phase of *NPC1a^57A^* mutants is significantly extended by either increased dietary cholesterol content or dietary ecdysone [124, 125], I first tested whether similar treatment of *NPC1b^1^* mutants would also extend their lethal phase. Dietary supplementation with excess cholesterol had no influence on the *NPC1b^1^* lethal phase, even when using feeding paradigms sufficient to rescue *NPC1a^57A^* mutants to the adult stage of development. Moreover, feeding *NPC1b^1^* heterozygous females a diet of high cholesterol in an effort to induce increased cholesterol deposition into developing eggs failed to influence the lethal phase of their *NPC1b^1^* hemizygous offspring (Figure 2.9). Dietary ecdysone also showed no significant effect on lethal phase of mutant animals, although slightly more animals arrested at the second-to-third larval instar transition, exhibiting the double mouth hook and double vertical plate phenotype (Figure 2.9).

Total cholesterol levels are unchanged in* NPC1b^1 *mutants

My inability to detect an effect of dietary cholesterol or ecdysone on the lethal phase of *NPC1b^1^* mutants may reflect an absolute block in sterol absorption in these mutants. To test this hypothesis Laurie Andrews and I compared the sterol abundance of *NPC1b^1^* mutants and wild type controls, but we were unable to detect any significant difference in total sterol levels between these genotypes (Figure 2.10). However, previous work indicates that substantial amounts of sterol are deposited

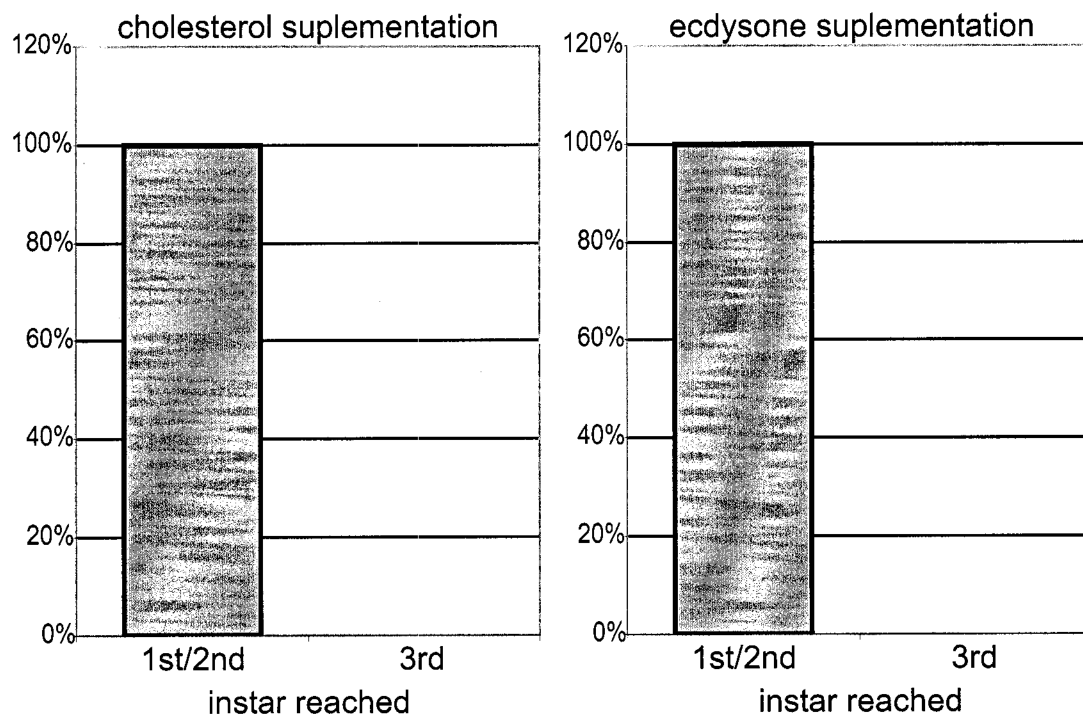


Figure 2.9. Dietary supplementation has no effect on *NPC1b*^{1} mutant larval lifespan. Larvae of the proper genotype were collected shortly after hatching, placed on supplemented media and checked daily for viability and developmental stage. Developmental stage was scored based on presence or absence of third instar spiracles. In no cases were third-instar larvae observed. Number of hatched larvae tested was greater than 100 for each condition.

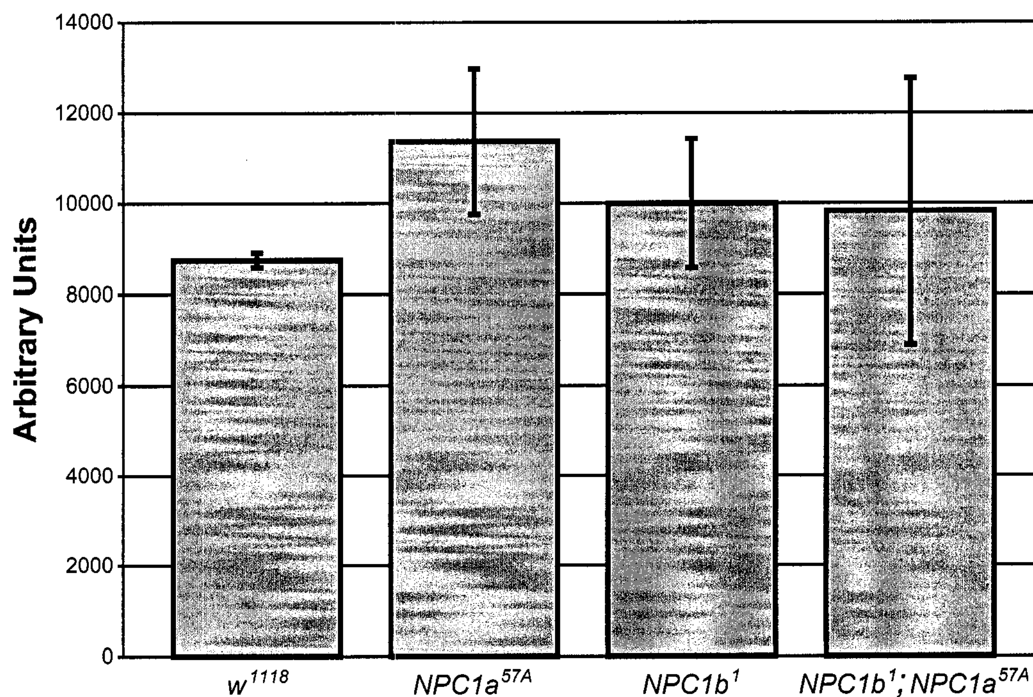


Figure 2.10. Total sterol content of *NPC1b* mutant larvae is not significantly different from wild type control animals. Sterol content in $NPC1b^{1}$ mutants, $NPC1a^{57A}$ mutants, double-mutants and wild type control larvae was assessed using a commercial assay. Although none of these genotypes differed significantly from one another, the total sterol content of $NPC1a^{57A}$ mutants displayed a trend towards elevated sterol content, consistent with previous work [125]. Three samples were measured in duplicate for each genotype; error bars represent standard deviation.

maternally in insects [132], which may mask my ability to detect a defect in dietary sterol absorption in first-instar larvae.

Conclusions

Through the use of a novel gene targeting technique, I was able to generate a loss-of-function null allele of *NPC1b*. This allele revealed an essential requirement for NPC1b during larval development, resulting in second-instar larval lethality when hemi- or homozygous. After identifying this null allele, I also identified multiple additional null alleles in different genetic backgrounds, all showing an identical phenotype.

Rescue experiments revealed a requirement for NPC1b specifically within the larval midgut epithelium, suggesting that NPC1b might perform a role in the absorption or transport of dietary nutrients. Further, the inability of NPC1a to substitute for NPC1b and vice-versa suggests that they perform unique cellular roles, either through different subcellular localization or different functions. Additionally, although NPC1b is also expressed in the adult midgut, it is unclear if there is an essential requirement for NPC1b at this stage, because the tight centromeric linkage of *NPC1b* precluded the use of the Flp/FRT system to generate animals that were specifically mutant in the adult stage but not during larval development.

The double mouth-hook phenotype and molting defect observed in a small percentage of *NPC1b* null mutant larvae indicated that the animals nearly reach the end of the second-instar stage of development before arresting. This observation, combined with the extended second-instar lethal phase of the mutants, is similar to

the defect observed in several known ecdysone synthesis mutants such as *dare* [114], and suggested that defects leading to a deficit in ecdysone levels are to blame for *NPC1b* mutant lethality. I tested the idea that NPC1b function might be necessary for proper ecdysone synthesis by supplementing the diet of *NPC1b* mutant larvae with either 20-hydroxyecdysone or cholesterol. Unlike with known ecdysone synthesis mutants, neither of these supplements is capable of modifying the lethal phase of *NPC1b* mutant larvae, demonstrating that *NPC1b* mutant larvae do not die due to a deficiency in ecdysone production. This implies either that NPC1b is not involved in the ecdysone synthesis pathway, or that NPC1b is also involved in trafficking of other essential compounds in addition to ecdysone precursors.

Based on the finding that NPC1b is exclusively required in the midgut epithelium, it appears that NPC1b plays an important role in dietary transport. However, because there is no effect on the lethal phase of mutant animals when fed exogenous ecdysone or increased dietary cholesterol, it is unclear if cholesterol is the critical factor. This result does not mean that NPC1b does not promote sterol absorption, and similarly the measure of total sterols in wild type versus mutant animals includes a large quantity of maternally supplied sterol and does not directly assess the ability to absorb dietary sterols. To address this question, I proceeded to directly and specifically assess the role of NPC1b in dietary sterol trafficking. I will discuss my findings on this role in Chapter 3.

Methods

Fly strains and culture: All marker mutations and balancer chromosomes are as described and referenced by the FlyBase Consortium [129]. Fly stocks bearing transgenes encoding heat-shock inducible flippase, I-SceI and I-CreI were obtained from Kent Golic (University of Utah). All other fly stocks were obtained from the Bloomington *Drosophila* stock center. Flies were raised on standard cornmeal/molasses food at 25°C unless otherwise noted.

Identification and analysis of *Drosophila NPC1* homologs: Genomic and cDNA sequences encoding the *Drosophila* NPC1a and NPC1b proteins were identified by searching the BDGP database using a human NPC1 polypeptide query sequence (AAD48006). Because no *NPC1b* cDNA sequences are available from public databases, *NPC1b* coding sequences were initially inferred from theoretical translation of the predicted *Drosophila* gene CG12092 (NPC1b) using the BDGP annotation and the online annotation program GenScan (Massachusetts Institute of Technology, [133]). Comparative genomic analysis of corresponding sequences from *Drosophila pseudoobscura* and *Anopheles gambiae* revealed additional homology upstream of the computationally predicted start site for NPC1b. A multiple-protein sequence alignment of the human NPC1 and NPC1L1 (obtained from the GenBank database), *D. melanogaster* NPC1a and NPC1b (obtained from the BDGP and revised by my annotation work), *D. pseudoobscura* NPC1b (obtained from the BDGP), and *A. gambiae* NPC1b (obtained from the BDGP) sequences was created using Vector NTI Advance 9.0 (InforMax). Percent identity between human NPC1, human

NPC1L1 and individual *D. melanogaster* homologs was gathered by calculating pairwise alignments within AlignX.

Generation of a targeted NPC1b allele: An NPC1b mutant was created using a gene-targeting method by engineering a targeting construct with a stop codon mutation at codon 392 of the *NPC1b* gene (CG12092), nucleotide alterations designed to eliminate the adjacent 5' splice site, a diagnostic XhoI restriction site, and other modifications required for gene targeting (Figure 2.3; [127]). The targeting construct includes sequence corresponding to nucleotides 273721-280080 of genomic DNA accession #AE003569. Three separate PCR reactions were performed to amplify this genomic region of *NPC1b*, while also introducing the inactivating mutations and homing endonuclease recognition sites. All fragments were amplified from w^{1118} genomic DNA using the Taq Plus Precision system (Stratagene). One fragment was amplified using primers 5'-AAAAGGTACCGAAGTTGCCGAGCA-3' and 5'-AAAACTGAGTCATGTCTGATTGACGG-3', which generate a 2.1 kb product containing the NPC1b inactivating mutations. Another fragment was amplified using primers 5'-ACACCTCGAGAGCAATAAACTAGGCT-3' and 5'-AAAACTAGTATTACCCTGTTATCCCTACCGGTGGCCAGCAT-3', which generate a 700 bp product containing an I-SceI homing endonuclease recognition site. The third fragment was amplified using primers 5'-AAAACTCGAGACCGGACTAGTGCT-3' and 5'-AAACCGCGGCGGCCGCGATTTTCCTCCAGCTTT-3', which generate a 3.5 kb product. The fragments were joined in sequential steps using unique restriction sites

engineered into the primer sequences, and the proper arrangement was verified by sequencing the junctions. The completed 6.3 kb targeting construct was cloned into a KpnI-NotI digested pTV2 vector (kindly provided by Kent Golic, University of Utah), which supplies a *white*$^+$ marker and FRT sites flanking the targeting construct, and then introduced into the *Drosophila* genome through standard germline transformation techniques [134].

A transgenic line bearing the targeting construct situated on a chromosome with the dominant *Cy* mutation was crossed to flies bearing both flippase and I-SceI transgenes under the control of heat shock promoters. Progeny of this cross were heat shocked at 38°C for one hour 0-3 days after hatching and then grown to adulthood. Expression of flippase in the germline of these flies is expected to excise the targeting construct as a circular fragment, and I-SceI generates a double-stranded break in that fragment to convert the targeting construct to a linear fragment. In rare instances, the linearized targeting construct re-inserts into the genome at the location of the homologous genomic locus, typically generating a tandem duplication of the sequence of interest. Putative targeting events were detected by screening for flies bearing the *white*$^+$ eye color marker that also lack the *Cy* mutation, indicating that the targeting construct mobilized to another chromosome. From among the 35,000 progeny screened, two independent targeting events displaying X-chromosome segregation of the *white*$^+$ marker were identified. Both putative targeting events were subjected to molecular analysis by Southern blot to confirm proper targeting of the X-linked *NPC1b* gene. The probe consisted of a 1.9 kb DNA fragment bounded by a

BamHI restriction site within the third exon and an EcoRI restriction site within the sixth exon, and the sample DNA was digested with the restriction enzymes AatII and ApaI. Wild type sequence spanning the probe fragment is predicted to be 12 kb, while a successful targeting event would produce two probed fragments of 15 kb and 7.7 kb. Both targeting events were confirmed to consist of a tandem arrangement of the mutationally altered and wild type copies of the *NPC1b* gene (Figure 2.4).

To convert the tandem pair of *NPC1b* alleles to a single copy of the mutationally altered *NPC1b* allele, the properly targeted flies were crossed to flies bearing an I-CreI transgene under the control of a heat-shock promoter. Progeny of this cross were heat shocked at 38°C for one hour 0-3 days after hatching and grown to adulthood. Expression of I-CreI is expected to generate a double-stranded break at the I-CreI recognition site in the targeting transgene, and recombinational repair of this double-stranded break can resolve the tandem duplication to a single copy of the *NPC1b* gene. The desired progeny were detected by screening for the absence of the $white^{+}$ eye color marker. From among the 400 progeny screened, 33 mutant lines were isolated and verified by the presence of the diagnostic XhoI restriction site (Figure 2.5). The mutant chromosomes were balanced with an FM7 chromosome carrying a GFP-expressing transgene, which I later utilized in order to select mutant larvae for experiments.

Mutagenesis screen for additional NPC1b alleles: To identify new alleles of NPC1b, young male w^{1118} flies were starved overnight and then fed a sugar solution containing ethyl methanesulfonate (EMS) at a concentration of 25 mM for 24 hours.

After a brief recovery period the flies were then mated *en masse* to attached-X females that also carried a Y chromosome bearing Dp(1;Y)mal^{+}, a duplication that includes the NPC1b region. Male progeny from this cross, which all carry a mutagen-exposed X-chromosome inherited from their fathers, were collected and mated individually to NPC1b^{1}/FM7-GFP virgin females. Offspring of these matings were scored for frequency of the FM7-GFP chromosome. Any matings that exclusively produced offspring carrying the FM7-GFP chromosome were considered to be mutant for NPC1b and were collected for further analysis.

Generation of transgenic constructs: The *NPC1b* genomic rescue construct was generated by cloning a 6.6 kb XhoI-EagI fragment containing the complete *NPC1b* coding sequence and approximately 860 bp of upstream sequence from the BAC clone RPCI-11M14 (BACPAC Resource Center, Oakland, CA) into a modified form of the *Drosophila* vector pUAST lacking UAS motifs. The modified vector was used to ensure there was no spurious UAS-mediated expression of the transgene.

Both *UAS-NPC1b* transgenes were generated from the *NPC1b* genomic rescue transgene. To generate the 1254 amino acid version of the *UAS-NPC1b* transgene, a 5' restriction site was inserted at the new predicted start of *NPC1b* translation by amplifying a 1.1 kb product was amplified from genomic DNA using the primers 5'-ACACCTCGAGCACAACCAAAATGAAAGTGATTTTTGCAAC-3' and 5'-ACGGCCTTTACGAACATCTG-3'. This PCR product was cloned into an XhoI-MluI digested *NPC1b* genomic transgene and confirmed by sequencing the modified region. The entire *NPC1b* coding sequence was then cloned into a KpnI-EagI

digested pUASp *Drosophila* transformation vector [135]. To generate the 1228 amino acid version of the *UAS-NPC1b* transgene, the *NPC1b* genomic transgene was first digested with the restriction enzyme NsiI. The digested end was made blunt by addition of Klenow DNA polymerase, followed by further digestion with EagI. The 5.6kb fragment containing the complete coding sequence was isolated and cloned into a KpnI-EagI digested pUASp *Drosophila* transformation vector that had the KpnI site made blunt by Klenow polymerase. Orientation and proper insertion of both constructs was confirmed by restriction digest prior to introduction into the *Drosophila* genome by standard germline transformation techniques.

Generation of NPC1b-GAL4 promoter construct: The *NPC1b-GAL4* transgene was generated by PCR amplification of an 860 bp fragment immediately upstream of the predicted NPC1b start codon with the primers 5'-CAGGTGGTTGGTATTTCTCAG-3' and 5'-CGCGGATCCTCCGACACTAAAAAAAAAAAAATA-3'. This fragment was then cloned into an XhoI-BamHI digested pG4PB reporter vector [136] (kindly provided by John Carlson, Yale University). The integrity of the construct was verified by sequencing before introduction into *Drosophila* by standard germline transformation techniques.

Cholesterol supplementation assay: *Drosophila* food containing supplementary cholesterol was made by adding cholesterol to standard food preparations after the addition of Nipagin. Cholesterol (Sigma) was used from a 30 mg/ml stock solution in 100% ethanol to produce a final concentration of 200 ng/ml. Larvae were collected

from grape juice agar plates within four hours of hatching and placed in glass vials containing either standard food or food plus cholesterol (n>30 for each of three trials). Experiments were conducted blind and larval development was monitored at 25°C. Rescue was assayed by counting the number of third-instar larvae or later in the vial and comparing to controls.

Ecdysone supplementation assay: *Drosophila* food containing supplementary or 20-hydroxyecdysone (20HE) was made by adding 20HE to standard food preparations after the addition of Nipagin. 20HE (Sigma) was used from a 5 mg/ml stock in 100% ethanol to produce a final concentration of 120 μg/ml. Food was poured into 35 mm Petri dishes (Falcon) and was used immediately. Mutant larvae were collected from grape juice agar plates within four hours of hatching based upon absence of a GFP-marked balancer chromosome, and placed onto supplemented food. All experiments were conducted blind. To mimic the pulses of ecdysone that are necessary for proper development, larvae were transferred periodically between food lacking and food containing 20HE, as described in [125]. The lethal phase was noted.

Sterol Quantification Assay: The Amplex Red cholesterol assay kit (Molecular Probes) was used to assess total sterol content. First-instar larvae were genotyped by use of a GFP-marked balancer chromosome, collected from grape agar plates lacking yeast and placed on yeast paste for 8 hours to feed. Larvae were then collected, rinsed of all yeast, and transferred to apple juice agar lacking yeast for 3 hours. Larvae were then weighed and homogenized in 150 mM NaCl, 50 mM Tris pH 7.5, 2 mM EGTA to make a 100 mg/ml larval homogenate. The homogenate was centrifuged at 5000

rpm for 5 minutes to pellet debris, and the supernatant was assayed according to manufacturer instructions. Fluorescence was measured with a Packard Fluoro-Count fluorometer with a 530/590 nm filter set.

Chapter 3: The role of *Drosophila* NPC1 proteins in dietary sterol absorption

The finding that the essential functions of NPC1b reside in the midgut led me to hypothesize that NPC1b functions in the transport of dietary sterols in a manner similar to that of mammalian NPC1L1, and that loss of NPC1b causes a sterol absorption defect that leads to deficits in downstream pathways such as ecdysone synthesis. In this chapter, I first utilize a novel dietary sterol absorption assay to confirm that NPC1b is involved in an early step of dietary sterol transport. However, this model is complicated by two observations. First, *NPC1b* mutant larvae do not show a deficit in total sterol levels compared to wild type larvae (Figure 2.10). This could be due to the young age of the larvae when assayed, or it might be due to structural, membrane-associated sterols making up a very large percentage of total sterol content. The second complication is the finding that increased levels of dietary sterols or exogenous ecdysone have no effect on the lethal phase of NPC1b mutants, although these observations can be explained in several ways. If NPC1b mutant animals are unable to absorb dietary sterols, then the concentration of sterols in the diet will be irrelevant. Similarly, although NPC1L1 has been shown to facilitate transport of dietary cholesterol, it is possible that it also functions to facilitate transport other compounds. Thus NPC1b might also transport other essential compounds, and replacement of only one downstream product, ecdysone, would be insufficient to compensate for the other absent compounds. The similarities in expression pattern of NPC1b and NPC1L1 also raise the possibility that they act through identical mechanisms to promote dietary sterol absorption. However, the

mechanism that NPC1L1 promotes sterol absorption by remains controversial: One possibility is that NPC1L1 promotes an early event, perhaps at the plasma membrane, to allow the entry of dietary sterols into intestinal epithelial cells [39]; Alternatively, NPC1L1 may play a later role in promoting intracellular trafficking of dietary cholesterol, similar to NPC1 [137]. In this chapter, I investigate some of these hypotheses in order to further elucidate the function of *Drosophila* NPC1b and NPC1a in dietary sterol trafficking.

Through use of the novel dietary cholesterol absorption assay, I found that NPC1b plays a role in dietary sterol trafficking, and based on filipin staining of *NPC1b* mutant midguts, this role appears to be early in the process. Filipin staining of other tissues reveals that NPC1b does not influence sterol trafficking or abundance in other tissues, consistent with its expression pattern and total sterol levels as discussed in Chapter 2. Further, my work reveals a secondary, NPC1b-independent, mechanism of sterol absorption that is activated in *NPC1a* mutants, presumably as a result of decreased ecdysone levels.

Results

Cholesterol absorption is altered in **NPC1b** *and* **NPC1a** *single-mutants*

To address the possibility that maternal depositions of sterols were masking our ability to measure an effect on dietary cholesterol absorption, the lab developed an assay that selectively measures the efficiency of dietary cholesterol absorption in larvae. This assay is performed by collecting larvae shortly after hatching and placing them on food containing ^{3}H-cholesterol and ^{14}C-glucose. Radioactive glucose, which

is acquired through a mechanism distinct from cholesterol [138], was included to allow normalization of cholesterol absorption relative to glucose to control for possible differences in the rate of eating between genotypes. Larvae were given 18 hours to consume labeled food and then transferred to unlabeled food for another 4 hours to purge unabsorbed gut contents. Tracey Parker performed experiments using Coomassie Blue, a dye that cannot be absorbed from the larval midgut, and demonstrated that wild type larvae as well as *NPC1a* and *NPC1b* null mutants completely dispel unabsorbed gut contents within this time period [139]. At the end of this time period, the ^{3}H-cholesterol and ^{14}C-glucose content of larval homogenates was determined. Results of this analysis indicated that the cholesterol/glucose ratio was severely and equally reduced in $NPC1b^{1}$ and $NPC1b^{R9\text{-}28}$ mutants relative to a wild type control (Figure 3.1). By contrast, $NPC1a^{57A}$ null mutants exhibited a significantly increased cholesterol/glucose ratio relative to wild type (Figure 3.1). Because there was no statistically significant difference in glucose uptake between genotypes, and Megan Fluegel performed pulse-chase experiments that indicated there is little or no turnover of cholesterol during the entire time-course of analysis [139], these findings cannot be explained by alterations in glucose absorption or altered metabolism of glucose or cholesterol. Rather, the results are best explained by decreased cholesterol absorption efficiency in *NPC1b* mutants and increased cholesterol absorption efficiency in *NPC1a* mutants.

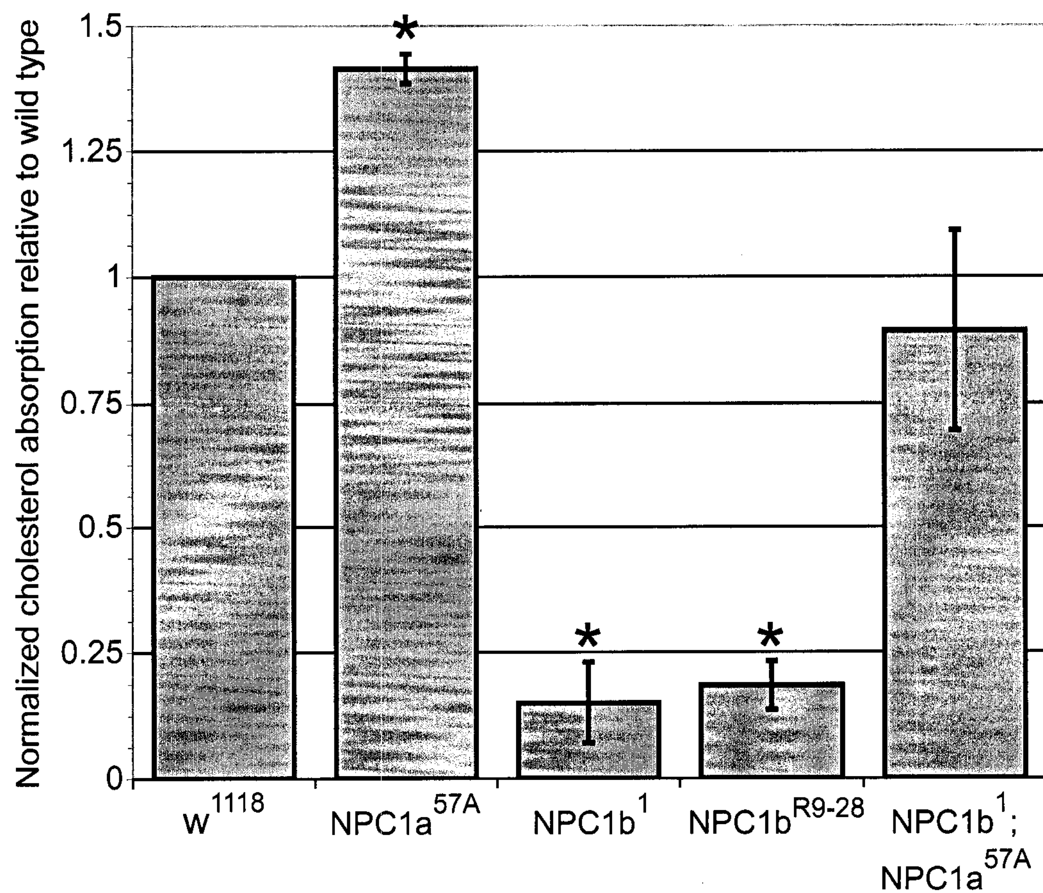


Figure 3.1. Cholesterol absorption is severely impaired in *NPC1b* mutants and increased in *NPC1a* mutants. 3H-cholesterol and 14C-glucose absorption was assessed in first-instar larvae, and the cholesterol absorption values were normalized to glucose absorption. The histogram displays the cholesterol:glucose ratios for each genotype analyzed relative to the cholesterol:glucose ratio of wild type (w^{1118}) larvae. Each value represents the mean and standard deviation of at least three independent experiments to measure cholesterol and glucose absorption. The asterisk (*) indicates a significant difference at $p<0.005$ by Student's t-test compared to wild type.

Cholesterol absorption is normal in* NPC1b^{1}, NPC1a^{57A} *double-mutants

If NPC1b is essential for dietary sterol absorption I would predict that the *NPC1b^{1}* sterol absorption defect would be epistatic to the increased sterol absorption phenotype of *NPC1a^{57A}* mutants. To test this prediction I created *NPC1b^{1}*; *NPC1a^{57A}* double-mutants in order to measure their sterol absorption. *NPC1b^{1}*; *NPC1a^{57A}* double-mutants hatch in Mendelian numbers but die primarily during the first-instar larval stage of development. This lethal phase is indistinguishable from that of *NPC1a^{57A}* mutants and indicates that loss of *NPC1a* is epistatic to loss of *NPC1b* in terms of lethal phase. In contrast to the absorption prediction, *NPC1b^{1}*; *NPC1a^{57A}* double-mutants appear to efficiently absorb sterols and are not significantly different from wild type larvae in this capacity (Figure 3.1). While *NPC1b^{1}*; *NPC1a^{57A}* double-mutants do not absorb sterols as efficiently as *NPC1a^{57A}* single-mutants, these findings indicate that the sterol absorption phenotype of *NPC1a^{57A}* mutants is epistatic to the *NPC1b^{1}* phenotype. Surprisingly, these findings also demonstrate that larvae retain the ability to efficiently absorb sterols in the complete absence of NPC1a and NPC1b.

Exogenous ecdysone modifies the double-mutant absorption profile, but tissue-specific expression of* NPC1a *does not

Given that complete loss of *NPC1a* function leads to the activation of a secondary NPC1a- and NPC1b-independent cholesterol absorption pathway, I predicted that an absence of sterols in an essential tissue was leading to activation of the pathway. If a tissue acts as a cholesterol sensor, it might feed back to activate increased absorption if cholesterol is not being trafficked properly due to mutation of

NPC1a. One potential site of cholesterol sensing is the midgut, where direct sensing of nutrient absorption could take place. In this model, absorbed dietary cholesterol would be transported in an NPC1a-dependent pathway, through which it would be delivered to a sensing organelle that inhibits the secondary absorption pathway. The sensing organelle could be the endoplasmic reticulum, which in mammals serves as the cholesterol sensing organelle via the SCAP and SREBP proteins. A second potential cholesterol sensing tissue is the ring gland, where ecdysone is synthesized. Insufficient cholesterol reaching the steroidogenic sites could result in an attempt to increase sterol levels to compensate. Additionally, ecdysone itself might act as the sensing mechanism.

To test these hypotheses, I generated *NPC1b^{1}; NPC1a^{57A}* double-mutant animals that also carried a GAL4 responsive UAS-NPC1a transgene. These animals were crossed to *NPC1b^{1}; NPC1a^{57A}* double-mutant animals that carried either the NPC1b-GAL4 construct, which drives GAL4 expression exclusively in the midgut, or the P0206-GAL4 construct, which drives GAL4 expression in the ring gland and other tissues [140]. Thus, these animals were mutant for both NPC1b and NPC1a, but had NPC1a function restored specifically in either the midgut or ring gland. Progeny from this cross carrying all the necessary genetic elements were assayed for dietary cholesterol absorption using the same methodology outlined previously.

If any of these genetic modifications of the *NPC1b^{1}; NPC1a^{57A}* double-mutant is sufficient to disable the secondary absorption pathway, I would predict that such animals would show an absorption profile similar to that of *NPC1b^{1}* mutants.

However, expression of NPC1a in midgut or ring gland tissues of otherwise double-mutant animals was insufficient to modify the *NPC1b¹; NPC1a⁵⁷ᴬ* double-mutant absorption profile (Figure 3.2). Although this result implies that the ring gland and midgut do not act as sterol sensing tissues, there are limitations to the experiment. For instance, the P0206-GAL4 driver confers only weak rescue of the $NPC1a^{57A}$ phenotype when driving expression of a UAS-NPC1a transgene [125], and the global disruption of proper sterol trafficking could preclude transgenic modification of the secondary absorption pathway. For example, reintroduction of proper sterol trafficking in the ring gland might not result in increased ecdysone production if sterol trafficking remains perturbed in upstream tissues and sterols are still not being delivered to the ring gland for processing.

To study if ecdysone is responsible for the secondary absorption pathway and in order to bypass any concerns regarding transgene expression and potential organism-wide trafficking defects, I looked at the dietary cholesterol absorption profile of $NPC1b^{1}$; $NPC1a^{57A}$ double-mutant animals that were supplemented with 20-hydroxyecdysone at a level comparable to the amount that provides the most efficient rescue of $NPC1a^{57A}$ single-mutant larvae to the pupal stage [125]. Double-mutant animals raised on ecdysone show an absorption profile similar to $NPC1b^{1}$ mutants, indicating that the secondary absorption pathway in these animals is not activated (Figure 3.3). These findings suggest that lack of ecdysone activates the secondary absorption pathway, although the exact mechanism of this action remains unclear.

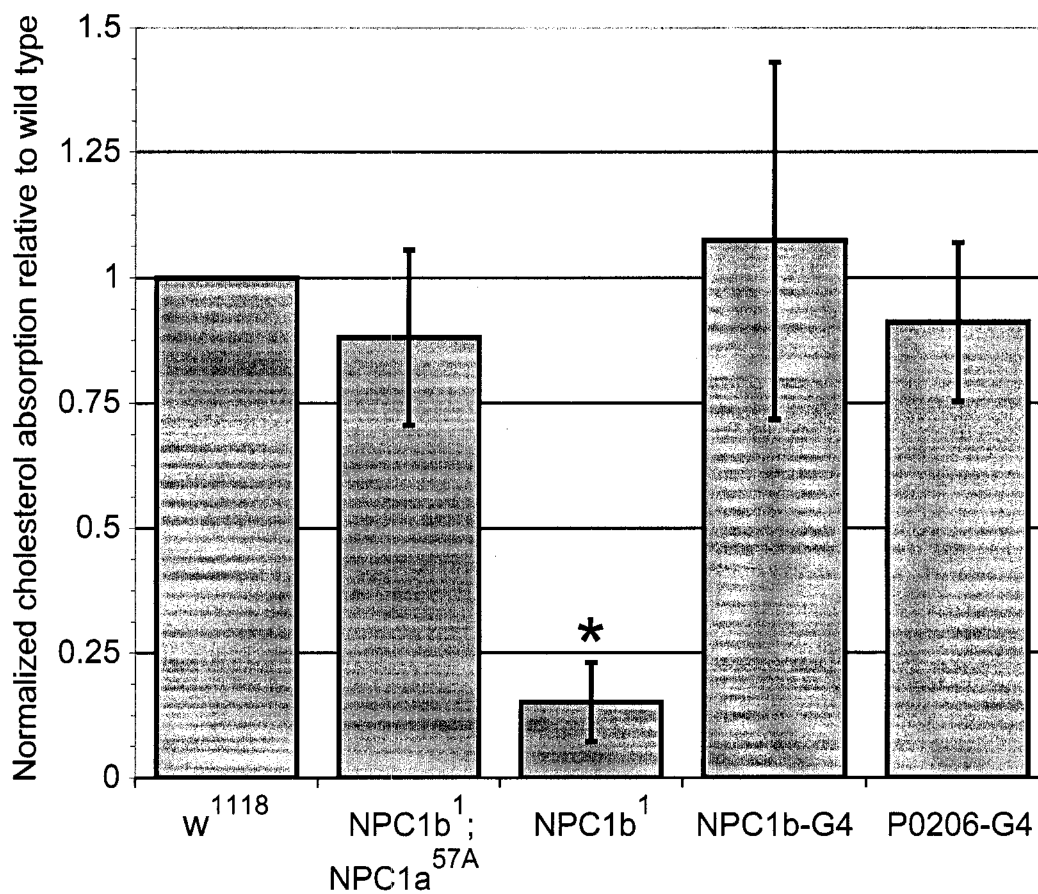


Figure 3.2. ***NPC1b; NPC1a*** **double-mutant cholesterol absorption is not affected by tissue-specific expression of NPC1a.** 3H-cholesterol and 14C-glucose absorption was assessed in first-instar larvae, and the cholesterol absorption values were normalized to glucose absorption. The histogram displays the cholesterol:glucose ratios for each genotype analyzed relative to the cholesterol:glucose ratio of wild type (w^{1118}) larvae. NPC1b-G4 denotes the genotype *NPC1b*^{1}*; NPC1a*^{57A}*, NPC1b-GAL4, UAS-NPC1a*. P0206-G4 denotes the genotype *NPC1b*^{1}*; NPC1a*^{57A}*, P0206-GAL4, UAS-NPC1a*. All other genotypes are exactly as noted. Each value represents the mean and standard deviation of at least three independent experiments to measure cholesterol and glucose absorption. The asterisk (*) indicates a significant difference at $p<0.005$ by Student's t-test compared to wild type.

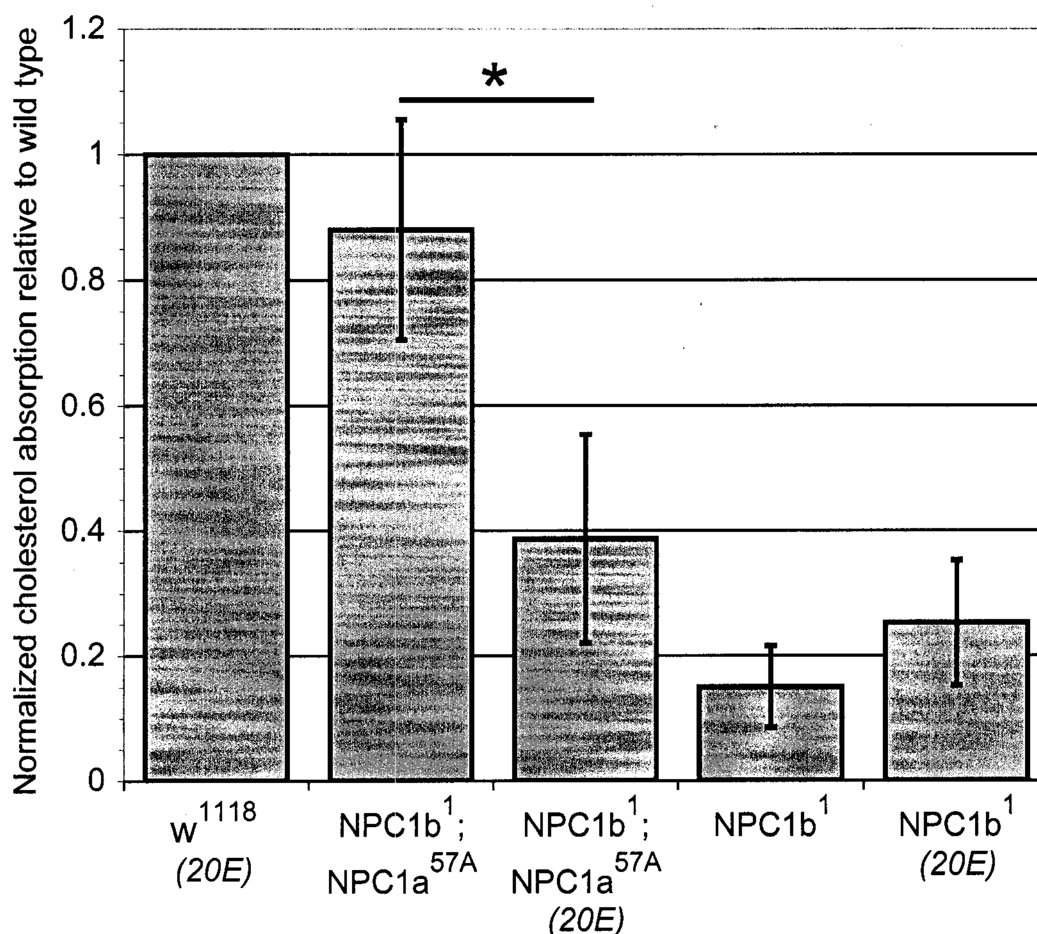


Figure 3.3. ***NPC1b; NPC1a*** **double-mutant cholesterol absorption is significantly altered by the presence of 20-hydroxyecdysone.** 3H-cholesterol and 14C-glucose absorption was assessed in first-instar larvae, and the cholesterol absorption values were normalized to glucose absorption. The histogram displays the cholesterol:glucose ratios for each genotype analyzed relative to the cholesterol:glucose ratio of wild type (w^{1118}) larvae. The *(20E)* notation beneath a genotype denotes that those animals were tested on 20-hydroxyecdysone containing media. Each value represents the mean and standard deviation of at least three independent experiments to measure cholesterol and glucose absorption. The asterisk (*) indicates a significant difference at $p<0.005$ by Student's t-test of an identical genotype on compared to off of 20-hydroxyecdysone-containing media.

NPC1b^{1} *midgut tissues are devoid of free sterols*

In an effort to determine if NPC1b functions in a manner similar to NPC1a in intracellular sterol trafficking, I compared the distribution of sterols in the midgut epithelium of *NPC1a^{57A}* and *NPC1b^{1}* mutants using the fluorescent cholesterol-binding compound filipin (Figure 3.4). Midgut tissues from *NPC1a^{57A}* mutants, *NPC1b^{1}* mutants, and wild type controls were dissected and stained in parallel. As expected from previous work [124], results of this study revealed an accumulation of sterol-rich trafficking organelles in the midgut epithelium of *NPC1a^{57A}* mutants (Figures 3.4E and 3.4F). By contrast, midgut tissues from *NPC1b^{1}* mutants appeared to be largely devoid of filipin staining relative to wild type, and did not display an accumulation of sterol-rich trafficking organelles (Figures 3.4C and 3.4D). Based on this result, I concluded that NPC1b is involved in an early step in dietary sterol absorption.

NPC1b^{1}, NPC1a^{57A} *double-mutant midgut tissues are devoid of free sterols*

Based on the finding that *NPC1b^{1}* mutants appear to have an early deficit in midgut sterol absorption, I predicted that *NPC1b^{1}, NPC1a^{57A}* double-mutant larvae would show a similar phenotype. To test whether mutations in *NPC1b* block the accumulation of sterol-rich trafficking organelles that are observed in the midgut of *NPC1a* mutants, I analyzed the distribution of sterols in *NPC1b^{1}; NPC1a^{57A}* double-mutants. Results of this analysis indicated that midgut tissues from *NPC1b^{1}; NPC1a^{57A}* double-mutants were, like *NPC1b^{1}* mutants, devoid of sterol and failed to accumulate sterol trafficking intermediates (Figures 3.4G and 3.4H). Together, these

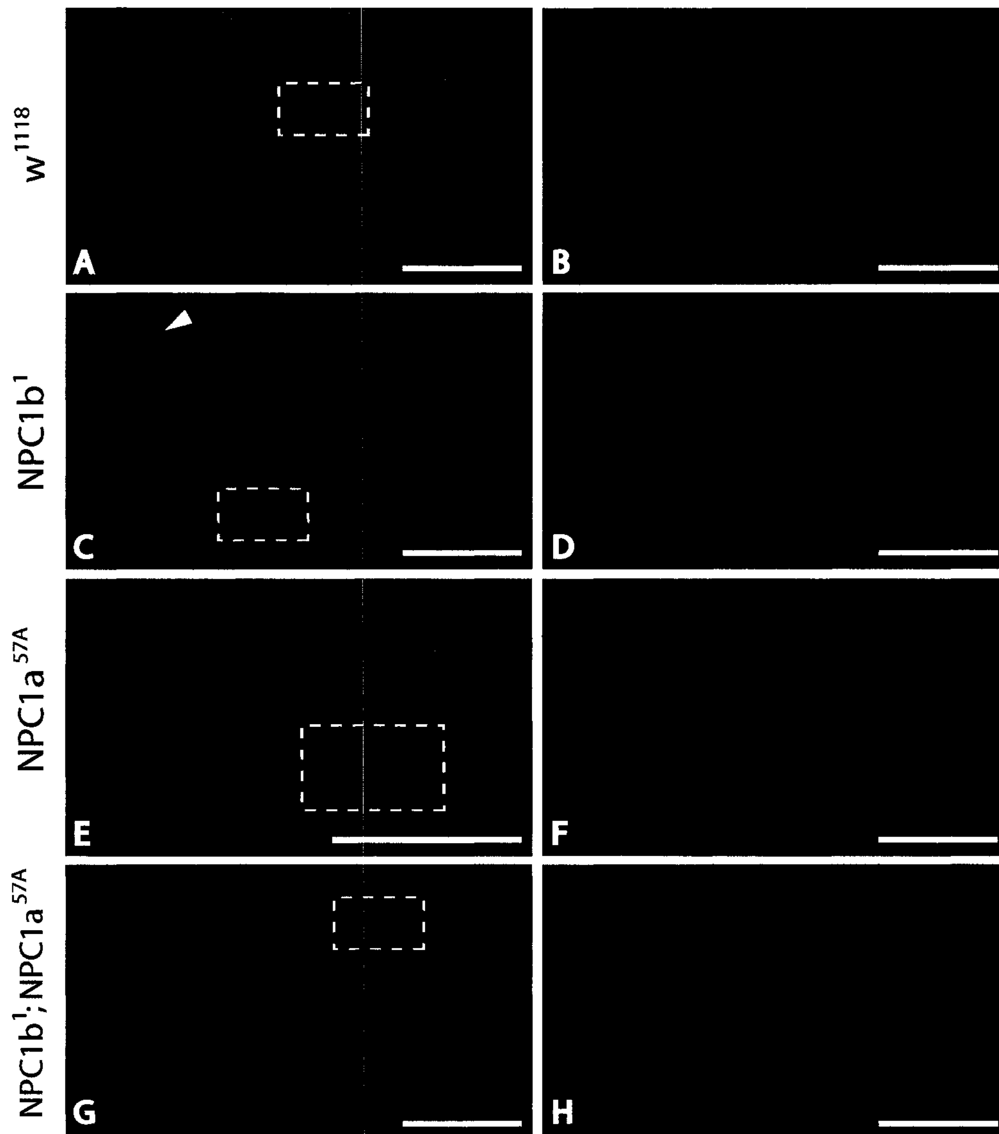


Figure 3.4. *NPC1b* mutants show dramatic absence of midgut sterols. Age-matched wild type (A, B), $NPC1b^{1}$ mutant (C, D), $NPC1a^{57A}$ mutant (E, F), and $NPC1b^{1}$; $NPC1a^{57A}$ double-mutant (G, H) 12-24 hour old larval midguts were stained with filipin in order to assess total sterol levels. Images show protoventriculus, gastric caecae and first two segments of the midgut. Boxes in A, C, E and G denote area of magnification in B, D, F and H, respectively. $NPC1b^{1}$ and $NPC1b^{1}$; $NPC1a^{57A}$ midgut tissues show dramatically less filipin fluorescence than $NPC1a^{57A}$ and wild type controls, with no accumulation of sterol trafficking intermediates. $NPC1a^{57A}$ mutant midgut tissues show sterol trafficking intermediates. Arrowhead in C denotes $NPC1b^{1}$ larval CNS, which shows wild type intensities of filipin staining. Clusters of small punctate spots in C and D are undigested yeast within the midgut lumen. Images were taken at the same exposure settings. Scale bars represent 50 mm in A, C, E and G, and 10 um in B, D, F and G.

findings suggest that the diminished sterol absorption in *NPC1b*^{1} mutants derives from an early defect in sterol absorption rather than a block in intracellular trafficking of cholesterol, and that loss of NPC1b function in the midgut is epistatic to the NPC1a sterol trafficking defect in this tissue.

Non-midgut tissues in* NPC1b^{1} *mutants do not show sterol trafficking defects, but* NPC1b^{1}; NPC1a^{57A} *double-mutant tissues do show trafficking defects

Previous work suggests that intracellular sterol trafficking continues at reduced efficiency in *NPC1a* null mutants in at least some tissues [124, 125]. One possible explanation for this finding is that other factors act redundantly with NPC1a to promote intracellular sterol trafficking. Although use of the *NPC1b-GAL4* reporter construct indicates that NPC1b expression is restricted to the midgut, I cannot exclude the possibility that my system fails to fully recapitulate the NPC1b expression pattern, or that NPC1b expression is altered in an *NPC1a* null mutant. Therefore, to test the hypothesis that NPC1b normally plays a role in intracellular sterol trafficking in peripheral tissues, or is activated to perform this role in an *NPC1a* mutant, I compared the filipin staining pattern in non-midgut tissues from *NPC1a*^{57A} mutants, *NPC1b*^{1} mutants, *NPC1b*^{1}; *NPC1a*^{57A} double-mutants, and wild type controls (Figure 3.5). I did not detect differences in filipin staining in non-midgut tissues from *NPC1b*^{1} mutants and wild type controls (Figure 3.5), indicating that maternally supplied sterols have been trafficked properly in *NPC1b*^{1} mutant animals. Furthermore, the redistribution of sterol into trafficking intermediates that was observed in peripheral tissues of *NPC1a*^{57A} mutants appeared to be indistinguishable from that observed in *NPC1b*^{1}; *NPC1a*^{57A} double-mutants (Figure 3.5).

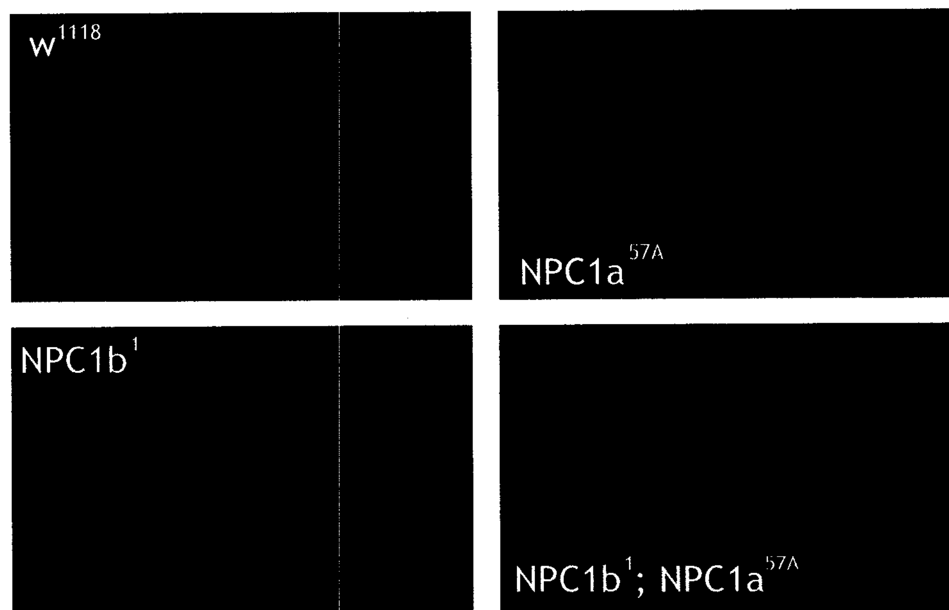


Figure 3.5. Sterol trafficking intermediates accumulate in *NPC1a* mutants and *NPC1b; NPC1a* double-mutants, but not in *NPC1b* mutants. First-instar larval brains were stained with filipin in order to assess cholesterol trafficking. In both wild type (w^{1118}) controls and $NPC1b^{1}$ tissues the sterol distribution appears to be uniform. In $NPC1a^{57A}$ and $NPC1b^{1}$; $NPC1a^{57A}$ larvae, sterols accumulate in a punctate pattern throughout the tissues analyzed.

Together, these findings indicate that NPC1b plays no role in intracellular sterol trafficking in peripheral tissues and that this factor is incapable of substituting for NPC1a.

Conclusions

Although total sterol levels in *NPC1b* mutant animals are not significantly different from wild type (Figure 2.10), the amount of dietary sterol absorbed is significantly decreased in *NPC1b* mutants compared to wild type. This finding implies that the large pool of maternally deposited sterols are properly acquired and utilized in *NPC1b* mutants, but that the source is either exhausted or otherwise unavailable to further advance growth and development beyond the first molt, and dietary sources are required to continue development. Filipin staining of *NPC1b* mutant midgut tissues revealed a dramatic deficit in free cholesterol levels, with no punctate accumulations like those observed in *NPC1a* mutant animals. The lack of midgut sterols suggests that NPC1b acts very early in the absorption pathway, potentially at the lumenal membrane.

Though the sterol absorption assay was primarily developed to explore the function of NPC1b, valuable insights into the regulation and mechanism of sterol absorption were also revealed in the sterol absorption studies of *NPC1a*^{57A} mutants. For example, my results indicate that loss of NPC1a activity results in significantly increased sterol absorption. Surprisingly, the up-regulation of sterol absorption that occurs in *NPC1a*^{57A} mutants appears to be epistatic to the sterol absorption defect of *NPC1b*^{1} mutants, as evidenced by the finding that *NPC1b*^{1}; *NPC1a*^{57A} double-mutants

absorb cholesterol far more efficiently than *NPC1b*^{1} single-mutants. These findings indicate that, while NPC1b normally plays an important role in sterol absorption, there is an NPC1a- and NPC1b-independent mechanism of sterol absorption in *Drosophila* that functions efficiently in the absence of these factors. Despite showing wild type-like levels of sterol absorption, filipin staining of the midgut tissues of double-mutant animals appears very similar to *NPC1b*^{1} mutants, suggesting that the secondary absorption pathway either does not go through the midgut, or that it somehow bypasses membrane enrichment.

The finding that exogenous ecdysone has a significant effect on double-mutant dietary sterol absorption leads to two possible explanations for why tissue-specific expression of NPC1a was unable to modify the absorption rate. One is that the ring gland GAL4 driver is inefficient, and the amount of NPC1a protein in the ring gland was insufficient to traffic enough cholesterol for endogenous ecdysone production. Alternately, other trafficking defects may prevent cholesterol from reaching the ring gland regardless of its ability to be properly trafficked after delivery to the tissue. For instance, if absorbed cholesterol is trapped in the midgut or fat body, then the presence or absence of NPC1a in the ring gland will make no difference in ecdysone production.

The finding that exogenous 20-hydroxyecdysone in the diet of *NPC1b*^{1}, *NPC1a*^{57A} double-mutant animals disables this secondary absorption pathway indicates that either ecdysone or one of its downstream targets acts as a sensor for cholesterol levels within the animal. Loss of NPC1a is sufficient to cause cholesterol

trafficking defects leading to decreased ecdysone production, which must feed back to this independent absorption pathway in an effort to compensate and acquire additional sterol. In *NPC1b[1]* single-mutant animals, sufficient loads of maternal sterol are present to enable ecdysone production during embryonic and first-instar larval development, so there is no perceived shortage of sterols to activate the secondary pathway. Filipin staining data supports this observation, with all the non-midgut tissues observed in *NPC1b[1]* mutant animals appearing very similar to wild type both in staining intensity and in sterol distribution.

Despite the statistically robust results obtained from the sterol absorption assay, there is some currently undetermined variability within it, the resolution of which should improve confidence in it and enable the testing of additional genotypes. Experiments performed on the same day with the same genotype are generally very consistent and well correlated, but trials on separate days with the same genotype are often inconsistent with one another, with the measure of total cholesterol absorbed varying in magnitude. One potential explanation for this inconsistency is the slight variation in the age range of larvae being assayed. Although the assay is sensitive, approximately fifty larvae per plate are required in order for a sufficient quantity to survive through the entire assay, and ideally multiple plates are assayed on the same day to minimize variability. As genotypes become more complex and more chromosomes are involved in producing the proper offspring, obtaining the desired animals becomes more complicated due to them representing a smaller fraction of the total offspring, and often results in a slightly wider range of animal ages in the assay.

This increasing complexity has also prevented the testing of additional transgenic constructs located on other chromosomes for their ability to modify the *NPC1b*^{1}*; NPC1a*^{57A} double-mutant absorption phenotype. To get around this problem, further genetic work would be required to mobilize transgenes onto the chromosomes bearing the *NPC1b*^{1} or *NPC1a*^{57A} alleles. Additionally, other environmental sources of variability such as temperature, moisture of the food, lighting conditions and room humidity could slightly influence results, although those factors should have a similar impact on all genotypes studied in a particular trial. One additional source of concern with the assay is the need to normalize all genotypes to wild type. Performing multiple wild type assays during each trial should compensate for variability.

My analysis of midgut tissues from *NPC1b* mutants supports a model where NPC1b promotes an early role in the absorption of sterols at the plasma membrane. In particular, my findings that the midgut epithelium from *NPC1b* mutants appears to be devoid of intracellular accumulations of sterol in transport organelles and that the midgut epithelium is largely depleted of sterol suggest that NPC1b acts at an early step in sterol absorption, preventing dietary sterols from entering the cell. However, I cannot exclude the possibility that NPC1b performs an additional role in promoting the intracellular trafficking of sterol-enriched transport organelles following its early role in sterol absorption. Such functions and subcellular localizations could be determined by performing live-cell assays using a fluorescently tagged version of the NPC1b protein. Given the similar loss-of-function phenotypes and proposed

biological function of NPC1b and NPC1L1, I propose that NPC1L1 also acts at an early step in sterol absorption within the small intestine.

Methods

Radioactive sterol assay: Food for cholesterol absorption studies was prepared by heating 2.5 g of dextrose and 1.5 g of Nipagin in 30 ml of water until the Nipagin fully dissolved, and by boiling 0.7 g of agar and 1 g of yeast extract in 70 ml of water. These solutions were then combined. 10 μCi [1a,2a(n)-^{3}H]Cholesterol (Amersham) was evaporated in a fume hood to remove toluene, then resuspended in 100% ethanol. 10 μCi [1a,2a(n)-^{3}H]Cholesterol in 100% ethanol and 5 μCi D-[6-^{14}C]Glucose in 3% ethanol (Amersham) were each added to 1 ml of prepared agar along with 100 μl of dilute food coloring. This mixture was then distributed in 200 μl aliquots onto small Petri dishes. Agar plates lacking both radioactivity and food coloring were also prepared. Larvae were collected at room temperature onto wetted 2.5 cm Whatman paper disks from yeasted grape plates 3-5 hours after hatching using GFP fluorescence to identify mutants as necessary.

Collected larvae were transferred en masse to food containing radioactive cholesterol and glucose and incubated for 18 hours at room temperature. Following this incubation period larvae with colored gut contents were then transferred to non-radioactive agar for 4 hours. Larvae that lacked colored gut contents following this incubation period were then placed into microfuge tubes. 100 μl EcoScint A scintillation fluid (National Diagnostics) was added to larval samples that were then homogenized using Kontes pestles. This homogenate was then added to a

scintillation vial containing 5 ml of scintillation fluid and analyzed in a scintillation counter. Cholesterol absorption experiments were repeated at least three times in entirety and the ^{3}H:^{14}C ratio from these experiments were averaged.

Ecdysone-supplemented radiolabeled media was made identically to above, except that 20HE was added to the media just prior to aliquoting onto plates. 20HE was used from a 5mg/ml stock in 100% ethanol to produce a final concentration of 200 μg/ml.

Filipin staining: Larvae were collected from yeasted grape juice-agar plates maintained at 25°C and aged until they were 12-24-hours old. Larval tissues were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 20-30 minutes. After fixation, tissues were washed at least twice with PBS. A 250 μg/ml stock solution of filipin (Sigma) was freshly made daily in DMSO. Tissues were stained with 50 μg/ml filipin in the dark for 30 minutes at room temperature followed by three washes in PBS. Following staining, tissues were placed in 50% glycerol/PBS overnight at 4°C before mounting onto slides with ProLong Gold Anti-Fade reagent (Invitrogen). Stained tissues were analyzed on a Nikon Microphot-FXA microscope using an excitatory wavelength of 360-370 nm, and the filipin signal was detected at wavelengths above 400 nm. Equivalent gain and offset values were used for each tissue type across genotypes.

Chapter 4: Findings and Discussion

The work described in this dissertation was aimed at developing a model of dietary sterol absorption in *Drosophila melanogaster,* in order to better understand the molecular mechanisms responsible for sterol absorption and intracellular trafficking. Although the genetic pathway of sterol trafficking is poorly understood, the general physical pathway of intestinal sterol acquisition and transport has been identified in humans. Dietary and biliary sterols become associated with micelles within the small intestine, and are endocytosed at the brush border membrane of intestinal enterocytes. After endocytosis, sterols are sorted to retain those that are useful to the organism, such as cholesterol, and efflux other sterols, such as phytosterols, back to the intestinal lumen. Retained sterols are esterified and trafficked by vesicular transport to the endoplasmic reticulum, where they become associated with lipoproteins to form chylomicrons. Chylomicrons are then exocytosed and released into the bloodstream [29]. Despite the general knowledge of this pathway, the molecular mechanisms that promote these sorting and trafficking events both within the intestine and in general intracellular transport are mostly unknown, with only a few proteins identified. Most of the factors involved in these processes were first identified because of disease-causing mutations in their corresponding genes. For instance, studies of the disease sitosterolemia led to the identification of the proteins ABCG5 and ABCG8, members of the ABC transporter family that perform an important role in the excretion of absorbed plant phytosterols back into the intestine, as well as in excretion of cholesterol from the liver into the

bile duct [30]. However, although these proteins are involved in the final steps of sterol efflux, the upstream sorting mechanism that delivers sterols to this point remains unidentified. Further, it remains unknown if the molecular mechanism or actual pathway of dietary sterol trafficking within the intestine is similar to that of sterol trafficking in other tissues, both in terms of the actual pathway and the proteins involved, and if any of the few proteins identified as being important in that pathway are also used in the intestinal trafficking and sorting pathway.

Two major breakthroughs in identifying proteins involved in cholesterol absorption and trafficking have involved the NPC1 family of proteins. The first was the discovery of NPC1 mutations as the causative agent of Niemann-Pick Type C Disease, linking the protein to intracellular cholesterol trafficking [45, 141]. The second breakthrough was the recent discovery of the drug ezetimibe, a pharmacological inhibitor of the dietary sterol absorption process, which led to the identification of NPC1L1 as the critical factor for dietary sterol absorption. Studies performed since the start of my research have shown that mammalian NPC1L1 is a target of ezetimibe, and loss-of-function mutations in this protein dramatically decrease the amount of dietary cholesterol that is absorbed [37, 38, 137]. These findings, combined with data suggesting the protein localizes at or near the plasma membrane, led to the conclusion that NPC1L1 must perform a critical role early in the absorption process. However, the mechanism by which it functions and the specific step in the pathway that it acts at remain unknown. My dissertation work has used *Drosophila melanogaster* as a model system to address these questions and

provided the tools that will be necessary for further elucidation of the absorption pathway.

The genomes of humans, mice, flies, and worms each encode a pair of closely related NPC1 homologs. While this finding raises the possibility that the NPC1 paralogs in metazoans partition into specific evolutionarily conserved functional roles, molecular and functional studies have not completely supported this conclusion. For example, worms, flies, mice, and humans each have one NPC1 family member that appears to promote intracellular sterol and lipid trafficking in a broad set of tissues, and another NPC1 family member that is expressed in a more restricted fashion [38, 39, 137, 142]. However, the expression pattern of the more restricted NPC1 family member is not conserved. In particular, murine NPC1L1 is expressed predominantly or exclusively in the intestine where it functions in sterol absorption. By contrast, human NPC1L1 is also highly expressed in liver, suggesting a hepatic function for this protein in addition to its well-documented role in dietary cholesterol absorption [137]. An even greater difference is seen in the expression of *C. elegans* NCR-2, which is restricted to a pair of neuroendocrine cells and the somatic gonad [142, 143]. My dissertation work indicates that *Drosophila* NPC1b is mainly, if not exclusively, required in the midgut, where it plays a major role in the absorption of dietary sterols. These features most closely resemble those of murine NPC1L1, and together with previous work, strongly suggest that *Drosophila* NPC1a and NPC1b provide intracellular sterol trafficking and sterol absorption activities that are equivalent to vertebrate NPC1 and NPC1L1, respectively.

The finding that *Drosophila* NPC1b provides a function that is apparently equivalent to vertebrate NPC1L1 afforded me the opportunity to address conflicting models regarding the role of NPC1L1 in cholesterol absorption. Previous work has generated disagreements on the subcellular distribution of NPC1L1; some studies suggest it localizes to the plasma membrane, others suggest it localizes to internal endosomal compartments, and yet other studies suggest it translocates between compartments as part of a sterol transporter activity [48-50, 64]. These findings raise questions as to whether NPC1L1 promotes an early step in sterol absorption at the plasma membrane and/or a later step in the intracellular trafficking of sterol-rich endocytic trafficking intermediates. My data on NPC1b indicate that it must perform an early role in sterol absorption at or near the plasma membrane, but cannot conclusively rule out an additional later trafficking role.

While our current data suggest that the *Drosophila NPC1a* and *NPC1b* genes provide functions equivalent to the vertebrate *NPC1* and *NPC1L1* genes, respectively, the consequences of mutations in these genes are not equivalent in flies and vertebrates. In particular, mutations in the *Drosophila NPC1* family members result in recessive lethal phenotypes at an early stage of development. In the case of *NPC1a*, this lethality is due to a failure in cholesterol trafficking, leading to decreased production of ecdysone, the steroid hormone responsible for molting and metamorphosis in insects [124, 125]. Null mutations in the vertebrate *NPC1* gene also lead to a failure in cholesterol trafficking and severe phenotypes in youth, but do not lead to defects in endocrine steroidogenesis, as evidenced by the fact that

mutations in the murine *NPC1* gene do not affect the concentrations of several major circulating steroid hormones [144]. These differences likely reflect the fact that insects are sterol auxotrophs [105], whereas vertebrates are able to synthesize sterols from acetate and therefore rely less on dietary cholesterol, and perhaps less on the efficiency of intracellular sterol trafficking. The absolute requirement for sterols in the *Drosophila* diet may also offer an explanation for the recessive lethal phenotype of *Drosophila NPC1b* mutants, relative to the lack of effect of *NPC1L1* mutations on adult viability in vertebrates [145]. However, as discussed more fully below, the effects of *NPC1b* mutations on viability are not fully understood at present and will require further work.

An important question that my current work raises concerns the non-redundant, non-interchangeable nature of the NPC1a and NPC1b proteins. My experimental data indicate that most phenotypes of NPC1a mutants are not observed in NPC1b mutants, including intracellular sterol trafficking defects. The simplest hypothesis is that this difference arises from the expression pattern of the two genes. Because NPC1b is not detectably expressed in any tissues beyond the midgut, loss-of-function mutations do not lead to trafficking defects in those tissues. However, this observation does not account for the lack of trafficking defects observed in midgut tissues, where NPC1b is known to be expressed. Taking this result into consideration, the simplest hypothesis is that this difference derives from differences in subcellular localization of these factors. Alternately, there may be important differences in protein-protein interaction domains, such that they interact with

different cofactors, and they are responsible for the phenotypes observed. Both of these hypotheses could be tested by creating chimeric proteins, fusing specific domains and sequences from NPC1a onto complementary portions of NPC1b to test for the ability of the resulting hybrid protein to rescue the mutant phenotypes of each parental protein knockout. Chimeric proteins should enable the identification of the domains and residues that are critical for the NPC1a-type functions compared to NPC1b-type functions, although more detailed knowledge of the functions of each domain are required to fully interpret any findings. Another experiment in this style would be to test the human NPC1 protein family members for their ability to rescue *Drosophila* mutant phenotypes. A third possibility is that the two proteins have entirely unique functions in sterol trafficking, despite their high degree of sequence similarity. For instance, dietary sterols are esterified close to the time that they are absorbed from the intestinal lumen, so the Sterol Sensing Domain of NPC1L1 might be adapted to facilitate transport of esterified sterols, whereas the intracellular sterols that NPC1 is involved in trafficking have been deesterified prior to encountering the protein. Again in that case, a better understanding of the function of specific protein domains such as the NPC1 domain and Sterol Sensing Domain will be required to distinguish important differences between the proteins.

While my findings indicate that NPC1b promotes an early step in dietary sterol acquisition, several observations suggest that *NPC1b* mutant larvae die for more complicated reasons than sterol deficiency. First, based on a conventional assay to measure total sterol abundance, sterol levels in $NPC1b^{1}$ mutant animals are

comparable to that of wild type larvae. Second, filipin staining of the CNS and Malpighian tubules of *NPC1b¹* mutant animals revealed fluorescence intensities comparable to that of wild type controls. Third, unlike the *NPC1a⁵⁷ᴬ* recessive lethal phenotype, which is strongly influenced by dietary sterols, the *NPC1b¹* mutant lethal phase is not detectably influenced by increased dietary sterol content. Similarly, increased maternal loading of sterols during oogenesis does not shift the lethal phase of *NPC1b¹* mutants, in contrast to results with *NPC1a⁵⁷ᴬ* mutants. Fourth, in contrast to studies of *NPC1a⁵⁷ᴬ* mutants, exogenous ecdysone is unable to alter the lethal phase of *NPC1b¹* mutants. This latter finding cannot be readily explained by a defect in the absorption of this nutrient from the midgut of *NPC1b¹* mutant animals, because previous studies have shown that 20-hydroxyecdysone can be absorbed directly, apparently without need of active transport [146].

Based on the finding that *NPC1b* mutant animals appear to have sufficient cholesterol reserves to make ecdysone and molt once despite being defective in dietary cholesterol acquisition, and that exogenous ecdysone has no influence on their lethal phase, it remains unclear precisely why *NPC1b* mutations are lethal. There is some evidence that a nutrient sensing mechanism might be involved. A large body of literature has demonstrated that *Drosophila* larvae have several mechanisms for detecting nutrient acquisition and determining if sufficient resources have been acquired to continue growth and development, most of which appear to converge on the insulin signaling pathway [147-153]. This so-called "critical weight" sensor determines if the larvae are capable of successfully molting or undergoing

metamorphosis, and if not, delays further development until the deficiency is corrected. From the late second-instar through third-instar stage of development, larvae increase in size dramatically, requiring vast amounts of food to be acquired. This model is supported by the observation that *NPC1b* mutant animals survive for an extended period of time as second-instar larvae, suggesting that they reach a dauer-like phase where they are attempting to endure what they perceive to be poor nutrient conditions due to their inability to acquire sufficient cholesterol. One hypothesis is that due to dietary sterol acquisition being severely disrupted in *NPC1b* mutants, there are insufficient sterols available for cell membrane growth, leading to a structural inability to continue development. Although NPC1b mutant larvae show no significant difference from wild type larvae in total sterol levels (Figure 2.10), it is important to note that the assay used young first-instar larvae, and in later developmental stages there could be important differences in sterol levels due to continuing effects of the *NPC1b* mutant absorption defect. A second hypothesis is that other essential nutrients are mistrafficked in conjunction with cholesterol, resulting in a deficiency in another critical compound. Support for this hypothesis comes from the observation that many compounds are mistrafficked in NPC1 mutant cells, including gangliosides, fatty acids and some amino acids [91-93, 100]. A related hypothesis is that the loss of midgut sterols in *NPC1b* mutants leads to a disruption of membrane dynamics specifically in midgut cells, disturbing the arrangement of other essential transmembrane proteins on the midgut epithelial cell surface. Lipid rafts and sterol-rich membrane moieties are known to be required for

certain transmembrane protein localizations and interactions [7, 8, 154], so disruptions in the arrangement of midgut epithelial membranes could lead to problems in acquisition of other critical dietary nutrients through an indirect mechanism. Antibodies are available for many of these proteins and tagged constructs could be made for others, and a detailed analysis of midgut epithelial membranes should reveal if there are any changes in membrane dynamics or protein localization in mutant cells.

Phenotypes similar to loss of *NPC1b* are observed in mutants of several other nutrient-related and development-related genes. For instance, mutations in the *dare* gene, which controls an early step of ecdysone biosynthesis, lead to late second-instar lethality and occasional mouth-hook phenotypes similar to *NPC1b* [114]. The amino acid transporter *slimfast* is found in the fat body and acts as a nutrient sensor, with loss-of-function mutations leading to a similar larval lethal phenotype [155]. The *Drosophila* SREBP homolog, dSREBP, performs a similar role in fatty acid sensing. Mutations in that gene lead to a deficiency in fatty acids and second-instar lethality that can be compensated for by dietary supplementation of oleate, in a manner similar to cholesterol and *NPC1a* [156]. Furthermore, *dSREBP* expression is necessary only in the larval midgut and fat body, a pattern similar to that of *NPC1b*. However, unlike in the case of mutants of *dare, NPC1a* or *dSREBP*, dietary supplementation of *NPC1b* mutants with fatty acids, ecdysone or cholesterol has no significant effect on the lethal phase.

There are several other possible explanations for the inability of cholesterol supplementation experiments to modify the *NPC1b*^{1} lethal phase. It is possible that unlike in *NPC1a*^{57A} mutants, where sterols are still able to traffic intracellularly at some low rate, the trafficking block in *NPC1b*^{1} mutant animals is more severe, so increased levels of dietary cholesterol are unable to compensate. Although experimental data reveal that NPC1L1 knockout mice retain the ability to absorb some low level of dietary cholesterol [37-39, 137], and my experiments indicate that there is a low level of cholesterol absorption remaining in *NPC1b* mutants, it is unclear if this cholesterol is properly trafficked through the gut, processed and utilized by the organism. Likewise, although it is unclear why increased maternal deposition of cholesterol has no affect on the lifespan of mutant larvae, this finding might be explained by the fact that the primordial midgut develops around the yolk sac early in embryonic development, and NPC1b function might be required for efficient access to those cholesterol stores as well. One way to test these hypotheses would be to inject wild type larval hemolymph into *NPC1b*^{1} mutant larvae, since any lipophorin contained within should bypass the NPC1b-mediated steps of midgut absorption.

An important question raised by the discovery of the secondary absorption pathway is why double-mutant animals die in the first-instar similar to *NPC1a*^{57A} mutant larvae, despite showing wild type levels of sterol absorption. It is possible that although sterols are able to be absorbed efficiently by this secondary mechanism, they may not be processed properly for utilization by the animal without the presence

of functional NPC1a or NPC1b. For instance, the sterols could be esterified and stored in lipid droplets instead of being trafficked to sites of need. This hypothesis could be tested by feeding double-mutant larvae a fluorescent or otherwise tagged analog of cholesterol and tracking its location within the animal in pulse-chase experiments. This experiment has the added potential to identify the location of the secondary absorption pathway. Alternatively, rescue of double-mutant animals may require greater-than wild type rates of sterol absorption in addition to dietary supplementation to sufficiently overcome their trafficking defects. In this model, timed pulses of exogenous 20-hydroxyecdysone in addition to dietary supplementation of cholesterol might be sufficient to modify the lethal phase, although a negative result would be difficult to interpret.

The 20-hydroxyecdysone-mediated modification of the secondary absorption pathway suggests that *Drosophila* have a sensing mechanism for ecdysone production, and the decreased levels of ecdysone found in *NPC1a* mutant animals lead to an up-regulation of sterol absorption. There are several ways this feedback mechanism could function. There may be a sensing mechanism within the ring gland that measures the rate or quantity of ecdysone synthesis, feeding back to the site of secondary absorption by an unknown mechanism in order to increase cholesterol absorption if the synthesis rate is insufficient. Alternately, ecdysone itself could be the feedback sensor and act to inhibit the secondary absorption pathway in the midgut through negative feedback. Ecdysone is known to bind to the Ecdysone Receptor/ultraspiracle heterodimer, which then translocates to the nucleus and acts as

a transcription factor, influencing transcription of a variety of growth and development genes [157]. To test the first hypothesis, larvae mutant for both *NPC1b* and a known component of the ecdysone synthesis pathway, such as *dare*, could be assayed for sterol absorption defects. One would predict that if ecdysone synthesis rates affect the secondary cholesterol absorption pathway, then *NPC1b; dare* double-mutant larvae would have an absorption profile similar to that of *NPC1b; NPC1a* double-mutant larvae. Similarly, *dare* mutants would be predicted to have an elevated absorption rate, similar to *NPC1a* mutants, due to both having ecdysone synthesis defects. Alternately, to test the second hypothesis, *NPC1b*^{1} mutant larvae with a transgene bearing a dominant negative form of the Ecdysone Receptor (DN-EcR) could be assayed for cholesterol absorption rates when driving expression of the transgene in various tissues such as the ring gland and midgut. In this experiment, one would predict that if genes downstream in the ecdysone pathway are responsible for regulating the secondary absorption pathway, the pathway would be activated in mutant animals expressing the transgene in the tissue responsible for secondary absorption due to the DN-EcR transgene mimicking conditions of ecdysone depletion.

Although it remains to be confirmed through experiments utilizing either a dominant negative or mutant form of the Ecdysone Receptor as described in the previous paragraph, it is likely that the secondary absorption pathway is transcriptionally regulated, based on the currently understood function of ecdysone as a ligand for the Ecdysone Receptor transcription factor. In order to identify the genes

involved in the secondary absorption pathway, microarray analysis could be performed. In this experiment, the gene expression profile of double-mutant animals raised on or off of 20-hydroxyecdysone supplemented media would be compared. Candidate proteins showing a difference in expression would be screened for pre-existing mutations, and if any exist they could be placed in an *NPC1b*^{1}; *NPC1a*^{57A} double-mutant background and assayed for their absorption profile similar to the method discussed above.

The disparity between the filipin staining phenotype of *NPC1b*^{1}; *NPC1a*^{57A} double-mutant midguts and their cholesterol absorption profile leads to some hypotheses about how the secondary absorption pathway might act and indicates that the NPC1a- and NPC1b-independent sterol absorption pathway somehow bypasses sterol enrichment of midgut tissues. One possible explanation of this finding is that this secondary absorption pathway esterifies sterols upon import, thus rendering them undetectable by filipin staining. This hypothesis could be tested by treating dissected larval tissues with cholesterol esterase prior to filipin staining, which should deesterify the sterols. Alternatively, the mechanism of the NPC1a- and NPC1b-independent sterol absorption pathway could be fundamentally different from that of the NPC1b-dependent pathway, and does not require or facilitate sterol enrichment of midgut tissues (Figure 4.1). A third possibility is that the NPC1a- and NPC1b-independent sterol absorption pathway may operate in tissues other than the midgut, such as Malpighian tubules. Identification of the NPC1a- and NPC1b-independent sterol absorption apparatus will be required to resolve these matters.

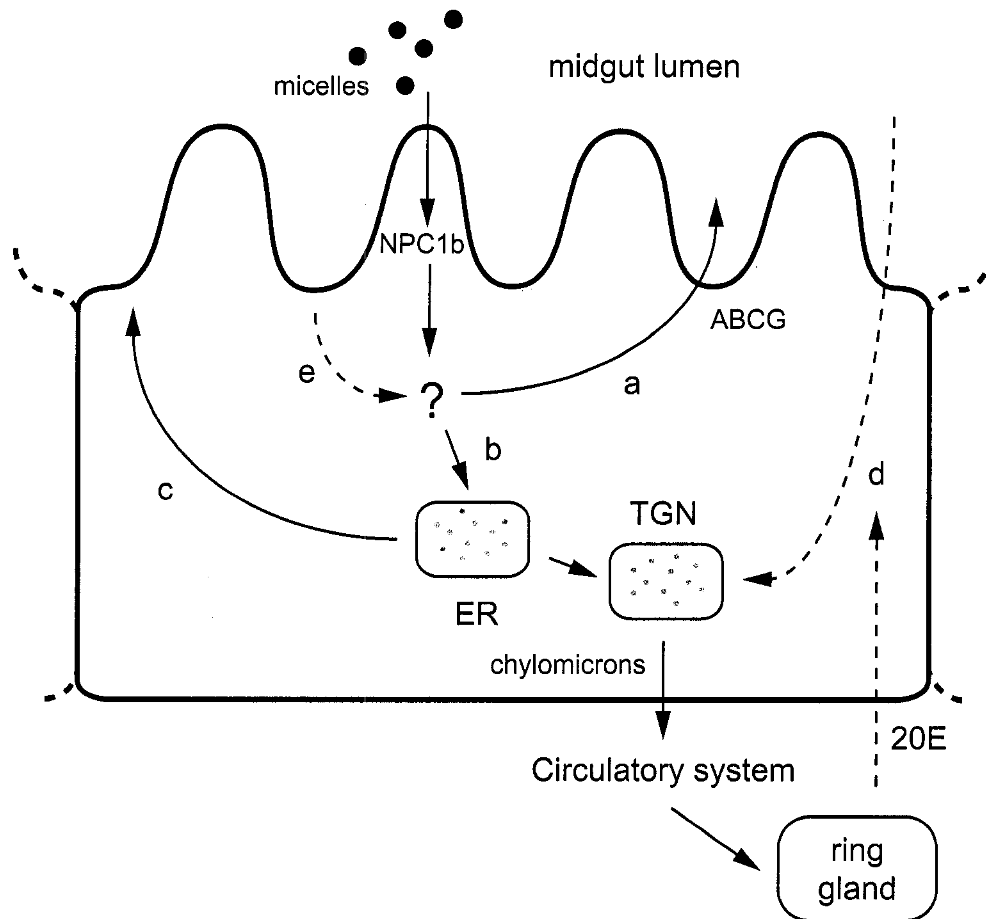


Figure 4.1. Model of Drosophila dietary sterol trafficking pathways. Solid lines denote normal absorption pathway and trafficking, while dashed lines denote hypothetical modes of secondary absorption pathway. Arrows denote overall pathway connections, and are not meant to infer direct steps. Dietary sterols are transported across the midgut lumenal membrane and trafficked via an NPC1b-mediated process to an unknown sorting point (denoted by large question mark). From here, unwanted sterols are transported back to the lumenal membrane for efflux by ABCG proteins (a), while useful sterols are retained and trafficked to the endoplasmic reticulum (ER, (b)). At the ER, some sterols are trafficked to and inserted into the plasma membrane (c) while the remainder are trafficked on to the Trans-Golgi network (TGN) for processing with lipoproteins to form chylomicrons, which are effluxed to the circulatory system for additional processing into lipophorins. At the ring gland, sterols are converted into 20-hydroxyecdysone (20E) which serves as a feedback mechanism to inhibit the secondary absorption pathway. Two models of secondary absorption are shown in the figure. One model hypothesizes that sterols are directly trafficked to the TGN by retrograde transport (d), thereby bypassing the ER and plasma membrane enrichment. A second model hypothesizes that sterols undergo direct transport to the sorting mechanism (e), potentially bypassing important early trafficking steps involving esterification. Several additional models are omitted for simplicity. For instance, NPC1b might also funtion at the step denoted by (a), acting to positively influence retention of useful sterols. Additionally, the secondary absorption mechanism could act in an entirely different tissue such as the Malpighian tubule.

Tools and techniques developed here such as the sterol absorption assay and filipin staining of intracellular trafficking defects will allow us to study other genes potentially involved in both dietary and intracellular sterol trafficking, and to explore the mechanism of regulation of the secondary absorption pathway. For instance, there are many genes implicated in the ecdysone pathway based upon their lethal phenotypes, but that have unclear function and could be more broadly involved in cholesterol transport, such as *ecdysoneless*, *dre4*, and *suppressor of forked* [158-160]. Interestingly, the protein products of some of these genes appear to have DNA binding activities, implying that they could regulate expression of several downstream cholesterol or ecdysone-related genes. Additionally, there are *Drosophila* homologs of many genes implicated in vertebrate cholesterol absorption and transport such as *SR-B1*, *NPC2* and ABC transport family members. The specific role and function of these genes could be determined based on their sterol absorption and filipin staining phenotypes. Genes involved in dietary sterol absorption would be expected to have an impaired absorption phenotype similar to that of NPC1b mutants, while genes involved specifically in intracellular trafficking should have increased absorption rates and intracellular trafficking defects detectable by filipin, similar to that of NPC1a mutants.

A tagged version of NPC1b will resolve several questions about its function. First, it will reveal the subcellular distribution of the protein. Comparing its localization in cholesterol-rich and cholesterol-poor conditions should help clarify its role in cholesterol trafficking. Additionally, proteomic experiments utilizing the

tagged version of NPC1b should identify interacting proteins that can then be characterized using the other tools. Besides any directly-interacting proteins identified with the tagged construct, genetic screens could be developed to identify additional components of the trafficking pathway. In addition to identifying existing mutations in cholesterol trafficking genes through the above-mentioned experiments, these tools provide an opportunity to screen for novel proteins involved in the pathway. Although a large-scale screen using the sterol absorption assay as the screening mechanism is unfeasible, the ability of *NPC1a* mutants to be rescued by increased dietary cholesterol provides both a design and a positive control for a screen to identify new mutants that are also capable of rescue with increased dietary cholesterol. To accomplish this, mutagenized stocks would be raised in parallel on media supplemented with or lacking excess dietary sterol, and mutants which produce viable offspring only on the supplemented media would be candidates for further study. This screen should identify new mutants in the *NPC1a* trafficking pathway, and potentially components of the secondary absorption pathway as well, that can then be characterized for their dietary absorption phenotype.

A final important series of follow-up experiments will be to test these models in a mammalian system to study if the secondary absorption pathway is present there as well. A recent paper examined the possibility that vertebrate NPC1 also performs a role in intestinal sterol transport in a manner similar to that of NPC1a [161]. Their findings indicate that unlike in *Drosophila*, there is no compensatory increase in dietary sterol absorption in NPC1-deficient mice. From this finding the authors

hypothesize that mammals do not possess a secondary absorption pathway. However, there are a few concerns about this hypothesis that were not addressed in their research. Due to the fact that mammals are capable of synthesizing cholesterol *de novo*, any deficiency in absorption or trafficking has been shown to be compensated for by increased synthesis [162-164]. To properly test for the existence of a secondary absorption pathway in mammals, the *de novo* synthesis pathway needs to be inhibited through the use of statins or genetic manipulation, which they did not test in their experiments. Thus, despite their claim that there is no compensatory cholesterol acquisition system present in mammals, NPC1-mutant mammals do actually increase total sterol acquisition with a net result that is very similar to that of *Drosophila*, except that instead of absorbing more dietary sterol they synthesize more to increase the available pool. Based on this poorly designed experiment, evidence for presence or absence of the secondary dietary absorption pathway in mammals remains inconclusive.

In conclusion, my results indicate that the NPC1b protein plays an essential early role in the acquisition of dietary sterols in the midgut epithelium. However, under conditions of defective intracellular sterol trafficking and possibly dietary restriction of sterol, the absorption of sterols increases, apparently through an NPC1a- and NPC1b-independent mechanism. Moreover, my studies raise the possibility that NPC1b also influences the absorption of one or more essential non-sterol nutrients. While my work advances our current understanding of the molecular mechanisms of sterol absorption, it also raises many new questions. In particular, my work raises the

possibility of a mechanism where the efficiency of intracellular trafficking is monitored in insects and conveyed to a novel NPC1-independent sterol absorption apparatus mediated by the level of ecdysone. Our knowledge of the precise mechanism by which NPC1a and NPC1b promote intracellular sterol trafficking and sterol absorption, respectively, and the factors that regulate the expression and function of NPC1a and NPC1b are also far from complete. Further analyses of *Drosophila NPC1a* and *NPC1b* mutants should provide answers to these questions.

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