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The cholesterol trafficking protein NPC1 is required for *Drosophila* spermatogenesis

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ABSTRACT

Niemann–Pick C (NPC) disease is a lethal neurodegenerative disorder affecting cellular sterol trafficking. Besides neurodegeneration, NPC patients also exhibit other pleiotropic conditions, indicating that NPC protein is required for other physiological processes. Previous studies indicated that a sterol shortage that in turn leads to a shortage of steroid hormones (for example, ecdysone in *Drosophila*) is likely to be the cause of NPC disease pathology. We have shown that mutations in *Drosophila npc1*, one of the two NPC disease-related genes, leads to larval lethal and male infertility. Here, we reported that *npc1* mutants are defective in spermatogenesis and in particular in the membrane-remodeling individualization process. Interestingly, we found that ecdysone, the steroid hormone responsible for the larval lethal phenotype in *npc1* mutants, is not required for individualization. However, supplying 7-dehydrocholesterol can partially rescue the male infertility of *npc1* mutants, suggesting that a sterol shortage is responsible for the spermatogenesis defects. In addition, the individualization defects of *npc1* mutants were enhanced at high temperature, suggesting that the sterol shortage may lead to temperature-sensitive defects in the membrane-remodeling process. Together, our study reveals a sterol-dependent, ecdysone-independent mechanism of NPC1 function in *Drosophila* spermatogenesis.

Introduction

Niemann–Pick type C (NPC) disease is an early childhood disease exhibiting progressive neurological degeneration (Mukherjee and Maxfield, 2004). The disease is caused by mutations in either of two genes, *NPC1* (Carstea et al., 1997) or *NPC2* (Naureckiene et al., 2000). At the cellular level, the most notable aspect of the disease is a massive accumulation of cholesterol, glycosphingolipids and other lipids in aberrant organelles. The underlying defect appears to be a failure of normal organelle trafficking and a consequent failure of lipid homeostasis (Liscum and Sturley, 2004; Mukherjee and Maxfield, 2004).

About 95% of cases of human NPC disease are caused by mutations in the *NPC1* gene (Carstea et al., 1997). To explore the molecular mechanisms and discover therapeutic treatments for NPC disease, *NPC1* mutant models have been generated in yeast, worm, fly and mouse (Griffin et al., 2004; Malathi et al., 2004; Sym et al., 2000). In *Drosophila npc1* mutants, sterol accumulation has been found in many tissues similar to the situation in mammalian *NPC* mutants. *Drosophila npc1* mutants show a first-instar larval lethal phenotype which is due to shortage of the steroid hormone ecdysone and can be rescued by tissue-specific expression of *npc1* in the ecdysone producing organ, the ring gland. Moreover, since mutant lethality can be rescued by supplying excess cholesterol or other sterols in the culture medium, a "cholesterol shortage" hypothesis has been proposed to explain NPC disease pathology (Fluegel et al., 2006; Huang et al., 2005). Similarly, in *NPC1* mutant mice, the neurodegeneration phenotype can also be attributed to a shortage of neurosteroid hormones (Griffin et al., 2004). Therefore, the cause of NPC disease pathology may be due to a cholesterol trafficking defect and subsequent shortage of steroid hormones.

Besides neurodegeneration, *NPC* patients and *NPC1* mutant mice exhibit many other abnormalities, for example, hepatosplenomegaly, suggesting that NPC proteins may play important roles in other biological processes (Carstea et al., 1997). It is not known whether these additional defects are due to steroid hormone shortage or other sterol-related mechanisms. Interestingly, NPC2, which is a small secreted lysosomal protein, was previously reported as the major secretory component HE1 of epididymal fluid (Belleannee et al., 2011 Naureckiene et al., 2000; Okamura et al., 1999), implying that NPC2 may have a role in reproduction. In addition, it has been reported that *npc1* mutant mice are male sterile with abnormal sperm morphology (Fan et al., 2006). In a previous study, we found that ecdysone-rescued *Drosophila npc1* mutant males are sterile (Huang et al., 2005), suggesting that the role of NPC1 in male fertility may be evolutionally conserved.

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Here, we investigated the role of *npc1* in *Drosophila* male fertility and showed that *npc1* mutants have spermatogenesis defects and in particular in a process called individualization. During *Drosophila* spermatogenesis, a pair of cyst cells encloses a primary spermatogonial cell and the whole structure is called a cyst. Within a cyst, a primary spermatogonial cell goes through six rounds of division, four mitotic and two meiotic, to form 64 spermatid cells. The spermatids, still connected by cytoplasmic bridges, differentiate synchronously from round spermatids to elongated spermatids, the final shape of long-tail sperms. Individualization is a membrane reorganizing process that occurs after elongation. During individualization, an actin-based complex, the individualization complex (IC), composed of 64 actin cones, forms and progresses caudally along the cyst, remodeling the syncytial membrane to remove excess cytosol and pack each spermatid into its own plasma membrane (Fabrizio et al., 1998; Tokuyasu et al., 1972). Defects in plasma membrane-remodeling, F-actin-based IC movement, or removal of excess cytoplasm can lead to the failure of individualization (Arama et al., 2003; Arama et al., 2007; Farkas et al., 2003; Ghosh-Roy et al., 2005; Huh et al., 2004; Mermall et al., 2005; Noguchi and Miller, 2003; Xu et al., 2002; Zhong and Belote, 2007). In *npc1* mutants, we found that the tightly associated 64-actin cone IC complex is disrupted, along with the incomplete separation of individual spermatids. Furthermore, male fertility was restored by germ cell-specific expression of NPC1, indicating a cell-autonomous function of NPC1.

Consistent with previous findings in the ring gland, *npc1* mutants exhibited a sterol accumulation phenotype in the testes. Supplying 7-dehydrocholesterol (7-dC) partially rescued male sterility in *npc1* mutants, suggesting that sterols play an important role in spermatogenesis in *Drosophila*. However, extensive genetic analysis suggested that the ecdysone pathway is not involved in the individualization process. Interestingly, the individualization defects of *npc1* mutants were enhanced at high temperature and suppressed at low temperature. Together, these results suggest that instead of lacking ecdysone, the sterol shortage in the testis may lead to temperature-sensitive defects in the membrane-remodeling individualization process. In summary, NPC1-regulated sterol trafficking is important for individualization during *Drosophila* spermatogenesis and acts in an ecdysone-independent manner.

Materials and methods

Fly strains and culture

npc1 mutants and *UAS–npc1–EYFP* transgenic lines were reported previously (Huang et al., 2005). All other lines are described in the Flybase and available from the Bloomington Stock Center. Unless otherwise specified, all stocks were cultured on corn medium at 25 °C.

Sterol feeding

For the 7-dC (Sigma) and cholesterol (Sigma) feeding test, 7-dC and cholesterol were diluted with ethanol to a concentration of 10 mg/ml. This stock was added to corn medium to a final concentration of 0.14 mg/g. For the ergosterol (Sigma) and β-sitosterol (Fluka) feeding test, sterols were diluted with isopropanol to give a 5 mg/ml stock and were used at a final concentration of 0.14 mg/g. Equal amounts of ethanol or isopropanol were added to the culture medium as a control. All sterol feeding experiments were performed on ring gland-specific rescued *npc1* mutants.

Fertility test and progeny counting

Virgin females were collected about 5 days before use. Each newborn male fly was crossed with 3 virgin females. All the parents were transferred into new vials at day 9 and removed at day 18. The total number of progeny from the two vials was counted. Only the progeny of fertile *npc1* mutant males was counted in the 7-dC rescue experiment.

Staining and imaging

Phase-contrast and DJ-GFP pictures were taken using a Zeiss microscope. Phalloidin and DAPI staining were executed according to published methods (Arama et al., 2003; Noguchi and Miller, 2003). In brief, samples were dissected in 1× Ringer buffer, fixed in 4% PFA for 20 min, washed twice with PBS, and then stained with phalloidin (1 μg/ml) and DAPI (10 μg/ml) for 2 h. For EcRA staining, animals were dissected in 1× Ringer buffer, fixed in 4% PFA for 20 min followed by 0.3% PBT (PBS + 0.3% Triton X-100) treatment for 30 min, and then stained with EcRA primary antibody (DSHB) at 4 °C overnight. After washed twice with PBS, secondary antibody was added together with phalloidin (1 μg/ml) and DAPI (10 μg/ml) for 2 h at room temperature. Pictures were taken with a Zeiss microscope or Nikon confocal microscope.

Transmission electron microscopy (TEM)

The TEM experiment was performed as described previously (Huang et al., 2005). Briefly, testes from 2–3 day old males were fixed with a solution of 2% glutaraldehyde, and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were post-fixed with 1% OsO4 followed by 1% Uranyl acetate, then dehydrated in an ethanol series and infiltrated with and embedded in EMbed 812 resin (Electron Microscopy Sciences). Ultra-thin sections were cut and stained with 0.08 mM lead citrate trihydrate for 10 min. The sections were examined on a Joel 1230 electron microscope.

Results

npc1 mutants are male sterile

npc1 null mutants are first-instar larval lethal due to insufficient ecdysone biosynthesis (Fluegel et al., 2006; Huang et al., 2005). To systematically investigate the tissue-specific requirements of *npc1*, we rescued mutant lethality with the *UAS–Gal4* system in which the *UAS–npc1–EYFP* transgene can be activated by tissue-specific *Gal4*. It is known that the lethality of *npc1* mutants can be rescued by ring gland-specific expression of *npc1* driven by *2-286 Gal4*; however, most of the surviving adult males are sterile (Huang et al., 2005). We found that, though a few of the rescued males were fertile (13.3%, $N=15$), their progeny numbers were much smaller (5.0 ± 3.1, $N=18$) than wild type (191.3 ± 21.3, $N=8$). To confirm that this sterility is due to *npc1*-deficiency, we examined whether male sterility could be rescued by the expression of NPC1. When an *UAS–npc1–EYFP* transgene was introduced into the *npc1* mutant background under a ubiquitous *tub-Gal4* driver, mutant male fertility was fully rescued. These data suggest that NPC1 is important for male fertility in *Drosophila*.

Furthermore, to examine in which cell types NPC1 is required for fertility, we used several cell type-specific *Gal4* lines in conjunction with ring gland-specific *2-286 Gal4* to examine the rescuing effect. *Hsp83-Gal4* has been used as a germ-line-specific *Gal4* while *C587-gal4* and *ptc-Gal4* are expressed in cyst cells in testes (Arama et al., 2003; Lee et al., 2008). We found that expression of NPC1 under *Hsp83-Gal4* could rescue male sterility, but expression of NPC1 under *C587-Gal4* or *ptc-Gal4* showed no rescue effect on male fertility. These differences in rescue efficiency indicate that NPC1 is required in the germ cells for spermatogenesis.

To explore potential functional mechanisms of NPC1 in male fertility, we examined the squashed testis samples. The testes of *npc1* mutants appear normal under phase-contrast microscopy. The spermatocytes, spermatids and even the elongated spermatids were