NCR-1 and NCR-2, the *C. elegans* homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways

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**Summary**

Mutations in the human NPC1 gene cause most cases of Niemann-Pick type C (NP-C) disease, a fatal autosomal recessive neurodegenerative disorder. NPC1 is implicated in intracellular trafficking of cholesterol and glycolipids, but its exact function remains unclear. The *C. elegans* genome contains two homologs of NPC1, *ncr-1* and *ncr-2*, and an *ncr-2; ncr-1* double deletion mutant forms dauer larvae constitutively (Daf-c). We have analyzed the phenotypes of *ncr* single and double mutants in detail, and determined the *ncr* gene expression patterns. We find that the *ncr* genes function in a hormonal branch of the dauer formation pathway upstream of *daf-9* and *daf-12*, which encode a cytochrome P450 enzyme and a nuclear hormone receptor, respectively. *ncr-1* is expressed broadly in tissues with high levels of cholesterol, whereas expression of *ncr-2* is restricted to a few cells. Both Ncr genes are expressed in the XXX cells, which are implicated in regulating dauer formation via the *daf-9* pathway. Only the *ncr-1* mutant is hypersensitive to cholesterol deprivation and to progesterone, an inhibitor of intracellular cholesterol trafficking. Our results support the hypothesis that *ncr-1* and *ncr-2* are involved in intracellular cholesterol processing in *C. elegans*, and that a sterol-signaling defect is responsible for the Daf-c phenotype of the *ncr-2; ncr-1* mutant.

Key words: Dauer, Niemann-Pick type C disease, Cholesterol, *C. elegans*

**Introduction**

The diverse cellular functions of cholesterol require transport mechanisms that partition it to appropriate subcellular compartments. In mammalian cells, the endosome/lysosome system is crucial for redistributing cholesterol after endocytosis of low-density lipoproteins (Liscum and Munn, 1999). Human NPC1 is thought to be involved in this redistribution process. Defects in NPC1 are the cause of the fatal Niemann-Pick type C1 (NP-C1) disease, an autosomal recessive neurodegenerative disorder (Carstea et al., 1997; Loftus et al., 1997; Vanier and Millat, 2003). Loss of NPC1 function causes accumulation of unesterified cholesterol in the trans-Golgi network (TGN) and abnormal lysosome-like structures (Blanchette-Mackie et al., 1988; Pentchev et al., 1986). The NPC1 gene encodes a membrane glycoprotein with homology to the *Drosophila* morphogen receptor Patched (Ptc) and the bacterial RND family of transporters (Ioannou, 2000). One current model of NPC1 function is that it acts as a molecular pump for retrograde vesicular transport of lysosomal cargoes including cholesterol (Davies et al., 2000).

The *C. elegans* genome contains two homologs of the human NPC1 gene, *ncr-1* and *ncr-2* (NPC1 related genes), formerly known as *npc-1* and *npc-2*, respectively (Sym et al., 2000). Both *ncr* gene products have 31% identity with human NPC1 and contain all the structural domains of NPC1 (Sym et al., 2000), including an N-terminal NPC1 domain and a sterol-sensing domain (SSD) (Chun and Simoni, 1992). *C. elegans* requires cholesterol in the growth medium (Chitwood, 1999), but little is known about the biological pathways mediating the uptake of cholesterol and subsequent processing and distributing steps (Kurzchalia and Ward, 2003). Nonetheless, the sequence conservation between human NPC1 and worm *ncr* genes suggests that an intracellular sterol trafficking pathway might be conserved.

A *C. elegans* model for NP-C1 disease was established by creating deletion mutations in both Ncr genes. Both deletions cause early frameshift mutations, and are thus likely to be null alleles. Although the single *ncr-1* and *ncr-2* mutants have subtle phenotypes, the *ncr-2; ncr-1* double mutant has a strong dauer formation constitutive (Daf-c) phenotype (Sym et al., 2000).

*C. elegans* dauers are alternative third-stage larvae that form in response to stressful environmental cues (Cassada and Russell, 1975; Riddle and Albert, 1997). Mutations in dauer formation (*daf*) genes result in either failure to form dauers in response to dauer inducing stimuli (dauer formation defective, or Daf-d), or constitutive formation of dauer larvae (dauer formation constitutive, or Daf-c). The *daf* genes function in complex parallel signaling pathways, including a cGMP pathway (Ailion and Thomas, 2000; Birnby et al., 2000; Coburn and Bargmann, 1996; Komatsu et al., 1996), a TGF-β...
signaling pathway (Ren et al., 1996; Schackwitz et al., 1996) and an insulin pathway (Kimura et al., 1997; Li et al., 2003; Lin et al., 1997; Ogg et al., 1997). The outputs of these pathways are thought to be integrated by the nuclear hormone receptor DAF-12. Recently, elements of a hormonal pathway that probably acts on DAF-12 have been identified. They include a cytochrome P450 enzyme encoded by daf-9 (Gerisch et al., 2001; Jia et al., 2002) and a PTP (protein tyrosine phosphatase)-like protein encoded by sdf-9 (Ohkura et al., 2003). The DAF-9 protein is hypothesized to catalyze a step in the biosynthesis of a sterol ligand that inhibits the dauer promoting activity of the DAF-12 receptor. Loss-of-function mutants of daf-9 share the pleiotropic developmental phenotypes of class 6 alleles of daf-12, which are thought to abolish ligand binding (Antebi et al., 1998; Antebi et al., 2000). SDF-9 is thought to augment or facilitate DAF-9 function as mutations in sdf-9 function was mapped to the same position in the dauer pathway (Ohkura et al., 2003). Killing the XXX cells (Ohkura et al., 2003) of head cells with possible neuroendocrine characteristics, called XXX cells (Ohkura et al., 2003). Killing the XXX cells induced dauer formation in wild-type animals and this cellular function was mapped to the same position in the dauer pathway as mutations in sdf-9 (Gerisch et al., 2001; Ohkura et al., 2003).

We present evidence that ncr-1 and ncr-2 are involved in processing cholesterol, and that they function upstream of daf-9 and daf-12 in regulating dauer formation. We demonstrate that the expression pattern of ncr-1 largely coincides with the pattern of cholesterol tissue distribution in C. elegans (Matyash et al., 2001; Merris et al., 2003), while the expression of C. elegans (Matyash et al., 2001) is more restricted. Both of the dauer-regulating sterol hormone.

Non-complementation screen for new ncr-1 alleles

ncr-2(nr2023) males were mutagenized by EMS (Sulston and Hodgkin, 1988), and allowed to recover overnight. These males were crossed to ncr-2(nr2023); ncr-1(nr2022) lon-2(e678) or to ncr-2(nr2023); ncr-1(nr2022) lon-2(e678) daf-12(m20). Non-Lon dauers were picked in the F1 and new ncr-1 alleles were made homozygous by selecting F2 animals that segregated no Lon progeny.

Sequencing and cloning cDNAs

The gene structure of ncr-1 was deduced by sequencing the yk39eC DNA clone. The gene structure of ncr-2 was deduced by sequencing RT-PCR products generated by gene specific primers. The 5' end of both genes were determined by sequencing PCR products amplified with SL1 leader sequence and gene specific primers. For ncr-2, the 3' end was determined by sequencing 3' RACE products amplified by gene specific primers and adapter primers (Invitrogen).

Materials and methods

Culture conditions and strains

Strains were cultured using standard methods (Brenner, 1974). Normal NGM agar contained 5 μg/ml added cholesterol, low-sterol NGM plates omitted the cholesterol, and high-sterol NGM plates contained 20 μg/ml to 40 μg/ml added cholesterol. Cholesterol was added as 5 mg/ml or 10 mg/ml stock, dissolved in ethanol. Control plates in the same experiments always contained an equivalent volume of ethanol. Escherichia coli strain OP50 (TJ2) was used as a food source for worms (Ailion and Thomas, 2000). JT10654, the ncr-2; ncr-1 strain we received from Dr Carl Johnson (Sym et al., 2000), contained a background mutation osm-3(sa1206), which apparently masked many of the developmental phenotypes of ncr-2; ncr-1 mutants. JT10800, the ncr-2; ncr-1 strain used here, was rebuilt from the two single mutants and further outcrossed three times. The ncr-1 and ncr-2 strains were also outcrossed three times.

Assays

When continuously propagated, the ncr-2; ncr-1 strain became progressively less healthy. This effect was apparent in poor recovery from the dauer and adult sterility. By contrast, worms that were freshly recovered from starved plates were much healthier and fecund. A similar phenomenon was reported for heterochronic mutants (Antebi et al., 1998; Liu and Ambros, 1991). In order to minimize variability between experiments, all assays involving the ncr-2; ncr-1 strain used worms that were recovered from starved plates or their progeny. For example, in media sterol assays, ncr-2; ncr-1 mutants from starved NGM plates were allowed to recover on the assay plates, and noted as the starting generation (Gen 0). Dauer formation was scored for Gen 1 and Gen 2 progeny. Gen 1 progeny were also scored for dauer recovery, life span and brood size. Control strains in the same assays were treated in the same way.

For dauer formation assays, 6-10 adult hermaphrodites were allowed to lay eggs at room temperature (about 22°C) for 2-6 hours and then incubated at the assay temperature. Dauer and non-dauer animals were scored after 72-80 hours at 20°C, 54-60 hours at 25°C and 44-48 hours at 27°C. Brood size and life span assays were conducted at 20°C as described (Gems et al., 1998), for all strains except ncr-2; ncr-1. The zero time point was the time of the L4 transfer. The number of progeny was scored daily and then summed to get the total brood size. Similar procedures were followed for ncr-2; ncr-1, except that dauers from staged egg-lays were used in the assays instead of L4 larvae. In this case, the zero point was the time of dauer transfer. Samples that died from internal hatching of embryos or from exploded vulva were included in the graphs. Statistical analyses including Student's t-test and the Kruskal-Wallis non-parametric ANOVA test were performed using InStat2.01.

Germline transformation

Germline transformation was carried out as described (Mello et al., 1991), at a concentration of 5-50 ng/ml test DNA with 5-50 ng/ml co-injection marker [myo-2p::gfp; or pBHL98[lin-15+]] and 50-80 ng/ml carrier DNA [pBluescript KS(+)].

Single gene rescue and reporter constructs

A 13 kb KpnI fragment of cosmid F02E8 containing the ncr-1A coding region with 3288 bp of upstream promoter sequence was sufficient to rescue the Daf-c phenotype of daf-9a mutant (see Table S1 in the supplementary material). The gene structure of ncr-2, the 3' end of both genes were determined by sequencing PCR products amplified with SL1 leader sequence and gene specific primers. For ncr-2, the 3' end was determined by sequencing 3' RACE products amplified by gene specific primers and adapter primers (Invitrogen).
plasmid pTJ1664 (ncr-2p::gfp). For plasmid pTJ1728 (ncr-2p::dsRed2), the PsI-Xmal promoter fragment from pTJ1664 was blunt at the PsI site and cloned into the HincII and Xmal sites of pTJ1665.

**Behavioral assays**

Osmotic avoidance assays were performed as described (Culotti and Russell, 1978) by testing whether animals crossed a ring of 8 M glycerol during 10 minute assays. Chemotaxis toward the volatile attractive odorants and NaCl was assayed as described (Bargmann et al., 1991). The brood size and life span of ncr-2; ncr-1 mutants were reduced (Fig. 2B,C) and most ncr-2; ncr-1 adults showed vulval abnormalities (80%, n=49). In less severe cases (33%), the animals had an abnormally protruding vulva, in more severe cases (33%), the uterus everted through the vulval opening. Probably owing to the vulval defects, ncr-2; ncr-1 animals had an egg-laying defective (Egl-d) phenotype: 29% (n=49) eventually died from internal hatching of larvae (Fig. 1G). However, even after excluding worms that died from an erupted vulva or internal hatching of larvae, the ncr-2; ncr-1 mutant still produced fewer progeny (91.2±76.6, n=16) than did the wild type (315.5±51.4, n=40), indicating some additional fertility defect. The cuticle alae of ncr-2; ncr-1 adults sometimes missed segments (28.9%, n=38), a phenotype that suggests defective seam cell development (Fig. 1E). ncr-2; ncr-1 mutants also exhibited a low frequency gonadal migration phenotype (3.1%, n=162).

To summarize, ncr-2; ncr-1 share most of the pleiotropic phenotypes of daf-9 and class 6 daf-12 mutants, including formation of characteristic partial dauers, abnormal vulval morphology and defects in seam cell development and distal tip cell migration. In each case, the phenotype of ncr-2; ncr-1 is less severe than most daf-9 and daf-12 mutants, indicating that ncr-2; ncr-1 causes a similar but less severe underlying defect.

**Results**

**Pleiotropic phenotype of the ncr-2; ncr-1 mutant**

The ncr-2; ncr-1 double mutant animals display developmental phenotypes similar to daf-9 and daf-12 class 6 mutants (Antebi et al., 1998; Gerisch et al., 2001; Jia et al., 2002). Like them, the dauer-like larvae formed by ncr-2; ncr-1 had a pharynx that was partially constricted and continued to pump, the germline continued proliferating, and they were paler and more mobile than normal dauers (Fig. 1A-C). In addition, ncr-2; ncr-1 was also able to form normal dauers in response to starvation or dauer pheromone (data not shown). Under reproductive growth conditions, ncr-2; ncr-1 animals recovered spontaneously from the dauer-like stage and exhibited pleiotropic developmental phenotypes as adults.

Fig. 1. Pleiotropic phenotypes of ncr-2; ncr-1 mutants. (A-C) Characteristic morphology of ncr-2; ncr-1 dauer-like larvae. (A) ncr-2(nr2023); ncr-1(nr2022) dauer-like larva. (B) daf-9(m540) dauer-like larva. (C) daf-7(e1372) dauer larva. Left and right panels show the pharynx and the alae, respectively. In the left panels, the black and white arrowheads indicate the isthmus and the terminal bulb of the pharynx. Compared with the fully constricted pharynx of a daf-7 dauer, which is similar to normal dauers, those of the ncr-2; ncr-1 and daf-9 mutants are only partially constricted. In the right panels, the black arrowheads indicate the dauer lateral alae consisting of morphologically distinct longitudinal ridges. (D-G) Pleiotropic developmental phenotypes of ncr-2; ncr-1 adults. (D) In a young wild-type adult, normal alae consist of three or four parallel longitudinal ridges. (E) Broken alae in an ncr-2; ncr-1 adult. White arrowheads indicate the remaining faint alae up to the point where they are broken. (F) Normal vulval morphology of a wild-type adult. The vulva is a slightly protruding structure, forming the opening through which fertilized eggs are expelled from the uterus. (G) Abnormal vulval morphology of a ncr-2; ncr-1 mutant. The white arrow indicates the vulval protrusion and black arrowheads indicate late stage embryos about to hatch inside the parent. Scale bars: 20 μm.
High media cholesterol rescues ncr-2; ncr-1 mutants

Because the NPC proteins are involved in trafficking sterols in mammals, we tested whether raising the media cholesterol concentration could rescue the ncr-2; ncr-1 mutant. The Daf-c phenotype of the ncr-2; ncr-1 mutant was significantly suppressed by raising the level of cholesterol in the media (Fig. 2A). High cholesterol also promoted recovery from dauer arrest and ameliorated the post-dauer phenotypes, as demonstrated by improved brood size and lengthened average life span (Fig. 2B,C). Increasing media cholesterol had no obvious effect on the dauer formation phenotype of other Daf-c mutants, including daf-9, daf-2 and daf-7 (Fig. 2A), though the recovery of daf-9 dauers was mildly promoted (Jia et al., 2002) (data not shown). These results suggest that cholesterol availability is limiting in ncr-2; ncr-1, but not in the other Daf-c mutants, consistent with an NCR function in delivering sterol substrates to the DAF-9 enzyme.

In order to identify possible substrates and products of the DAF-9 enzyme, we tested the ability of other sterols and steroids to suppress the Daf-c phenotype of the ncr-2; ncr-1 mutant. Because it is abundant in fungi and plants and can substitute for cholesterol to sustain the growth and mutant. Because it is abundant in fungi and plants and can substitute for cholesterol to sustain the growth and reproduction of the worm (Chitwood, 1999), ergosterol is a likely sterol source for C. elegans in the wild. Pregnenolone is the product of the first side-chain cleavage of cholesterol, the rate-limiting step in mammalian steroid biosynthesis (Simpson, 1979). We also tested the insect molting hormone ecdysone and a human steroid hormone progesterone. Only ergosterol moderately rescued the Daf-c phenotype of ncr-2; ncr-1, whereas ecdysone and pregnenolone had no effect (data not shown). Intriguingly, adding progesterone to normal NGM plates retarded the larval development of the ncr-2; ncr-1 mutant and inhibited its recovery from the dauer stage (data not shown). We decided to further examine the effect of progesterone.

ncr-1 mutants are sensitive to progesterone in the growth media

Progesterone can inhibit intracellular cholesterol trafficking in mammalian cells (Butler et al., 1992; Liscum and Munn, 1999). In fibroblast cell cultures, it causes accumulation of unesterified cholesterol in abnormal lysosome-like compartments, the same cellular phenotype that characterizes NP-C1 disease. Multiple steps of cholesterol intracellular trafficking appear to be affected by progesterone, but the exact mechanism and the targets of its action remain unknown.

To determine whether progesterone also disturbs cholesterol trafficking in C. elegans, we examined its effect on wild-type worms. Wild-type worms grown on NGM plates that contained 20 µg/ml progesterone in addition to the normal 5 µg/ml cholesterol developed into fertile adults. However, when progesterone was added to low-sterol NGM plates, pleiotropic phenotypes similar to those caused by cholesterol deprivation (Gerisch et al., 2001; Merris et al., 2003) were observed (Fig. 3). Many animals were defective in gonadal tip cell migration (54%, n=37), with both gonadal arms extending ventrally, failing to make the programmed turns (Fig. 3B). Frequently, sperm were observed in the pseudocoelomic space (39%, n=23), probably owing to a disrupted basement membrane (Fig. 3D). Occasional dauer larvae were also observed.

The ncr-1 single and ncr-2; ncr-1 double mutants were hypersensitive to progesterone treatment (Fig. 4). On low-sterol NGM plates containing 20 µg/ml progesterone, 77% (n=120) of ncr-1 and 85% (n=94) of ncr-2; ncr-1 mutants died
both protein. Similar to the deletion allele, disease mutations are located in this region of the protein. A cluster of human cysteines, C997, of the cysteine-rich loop is a missense mutation in which one of the conserved premature stop codon in exon 13.

We deduced the coding sequence of the protein of 1383 amino acids, corresponding to transcript. Both transcripts would give rise to a PCR products, suggesting that it is the minor transcript was under-represented in the RT-1B and therefore is likely to be non-coding. The combination of sequencing cDNA clones and overlapping RT-PCR were identified; contains an additional 96 bp first exon located 8.7 kb upstream of the first exon of (Fig. 5A). The first exon of ncr-1B contains termination codons and does not contain any additional translational start codons in frame with the downstream protein coding sequence, and therefore is likely to be non-coding. The ncr-1B transcript was under-represented in the RT-PCR products, suggesting that it is the minor transcript. Both transcripts would give rise to a protein of 1383 amino acids, corresponding to the predicted protein product.

Two additional mutations in ncr-1 were isolated from a non-complementation screen. sal420 is a C to T transition which results in a premature stop codon in exon 13. sal421 is a missense mutation in which one of the conserved cysteines, C997, of the cysteine-rich loop is replaced by a tyrosine. A cluster of human disease mutations are located in this region of the protein. Similar to the deletion allele, both sal420 and sal421 confer a strong Daf-c phenotype in combination with ncr-2(nr2023) (Table 1), indicating that they are strong loss-of-function or null mutations.

A single SL1 spliced transcript for ncr-2 was identified from sequencing overlapping RT-PCR products (Fig. 5C). The results confirm the previously predicted gene structure.

Expression patterns of ncr-1 and ncr-2 genes

We used promoter GFP fusions to study the expression patterns of the ncr-1 and ncr-2 genes. Three reporter constructs, ncr-1Ap(s)::gfp, ncr-1Ap(l)::gfp and ncr-1Bp::gfp were made for ncr-1 because of its complex gene structure (Fig. 5B). The results indicate that ncr-1 expression is widespread (Fig. 5B) and largely coincides with the reported distribution pattern of cholesterol in C. elegans, which includes the following tissues: intestine, pharynx, excretory gland cell, nerve ring, spermatheca and germ cells, including both oocytes and sperm (Matyash et al., 2001; Merris et al., 2003). In the following sections, we summarize the observed ncr-1 expression pattern within specific tissues.

Intestine

Both ncr-1Ap(s)::gfp and ncr-1Ap(l)::gfp were strongly expressed throughout the intestine (Fig. 6A), with posterior intestinal expression consistently stronger than anterior expression.

A C. elegans model of Niemann-Pick type C disease

Fig. 3. Progesterone treatment of wild-type worms mimics phenotypes caused by cholesterol deprivation. (A,C) Normaski micrographs of wild-type worms grown on normal plates. (B,D) Normaski micrographs of representative wild-type worms cultured on low-sterol NGM plates containing 20 µg/ml progesterone. The phenotypes of Gen 1 adults are presented (see Materials and methods). (A) Normal gonadal cell migration. (B) Abnormal gonadal cell migration in animals cultured on progesterone plates. The distal tip cell migrated on the ventral side all the way up to the pharynx, failing to make either of the programmed turns. The black arrow indicates the direction of dtc migration. (C) Under normal conditions, the sperm (white arrowheads) are enclosed within spermatheca (sp). (D) Loose sperm in N2 animals cultured on progesterone plates. White arrowheads indicate sperm found within pseudocoelomic space, probably owing to rupture of the basement membrane surrounding the somatic gonad. Scale bars: 20 µm.

Fig. 4. ncr-1 and ncr-2; ncr-2; ncr-1 and ncr-1; ncr-2; ncr-1; ncr-1, ncr-2; ncr-1, strains (Gen 1, see Materials and methods) were cultured on NGM plates with (+) or without (−) 5 µg/ml cholesterol and 20 µg/ml progesterone. The development of wild-type and mutant worms were scored after 72 hours at 20°C. The results from duplicate experiments were pooled together and expressed as a percent value (n>50).
Both ncr-1A(s)::gfp and ncr-1Ap(l)::gfp were strongly expressed in the spermatheca and weakly in the gonadal sheath cells (Fig. 6D). The expression in the somatic gonad could be observed only in adults.

Epidermis

ncr-1Ap(l)::gfp was strongly expressed in the excretory cell (Fig. 6A-C) and rectal epithelial cells (Fig. 6C) from the early L1 larval stage and through all life stages. Seam cell expression was first observed in the late L1 stage (Fig. 6E), while expression in the lateral hypodermis increased during the L3 stage and peaked during the L4 stage (Fig. 6F). Seam cell and hypodermal expression gradually decreased in the adult stage and was hardly visible among older adults (Fig. 6G). ncr-1Ap(s)::gfp was not expressed in the hypodermis under normal growth conditions, though lateral hypodermal but not seam cell expression was dramatically upregulated in starved animals of all developmental stages (Fig. 6J). Similar upregulation of hypodermal expression in response to starvation has been reported for daf-9 (Gerisch and Antebi, 2004). No increase in hypodermal expression was seen in starved ncr-1Ap(l)::gfp animals.

In contrast to the widespread expression of ncr-1, the expression of ncr-2p::gfp was restricted to the XXX cells and the somatic gonad. It was strongly expressed in the proximal gonadal sheath cells and weakly in the spermatheca (Fig. 6L). The XXX expression of ncr-2p::gfp was seen throughout development (Fig. 6M), while the gonadal expression was observed only in adults.

Position of ncr genes in the dauer formation pathway

To determine the position of ncr genes in the dauer pathway, we analyzed the dauer formation phenotypes of daf-d; ncr-2; ncr-1 triple mutants (Table 2). Mutations in osm-6, daf-5 and daf-16 suppress the Daf-c phenotype of mutants in the cGMP, daf-16, and daf-5 pathways.
A C. elegans model of Niemann-Pick type C disease

TGF-β and insulin pathways, respectively (Thomas et al., 1993; Vowels and Thomas, 1992). The Daf-c phenotype of ncr-2; ncr-1 was not suppressed by osm-6 and daf-5 mutations, placing the ncr genes parallel to or downstream of these genes. A severe loss-of-function allele of daf-16, m27, did not suppress the ncr-2; ncr-1 Daf-c phenotype, but the null allele mgDf50 did suppress partially, suggesting that daf-16 might be a minor downstream target of the ncr genes. The loss-of-function allele daf-12(m20), completely suppressed the Daf-c and the pleiotropic post-dauer phenotypes of ncr-2; ncr-1 mutant (Fig. 2B,C), placing the function of the ncr genes upstream of daf-12 and also suggesting that the pleiotropic post-dauer phenotypes of ncr-2; ncr-1 result from unregulated DAF-12 activity. This pathway position for the ncr genes is the same as daf-9 (Gerisch et al., 2001; Jia et al., 2002), suggesting that the ncr genes function in the hormonal branch of the dauer formation pathway.

Overexpression of DAF-9 activity rescues the ncr-2; ncr-1 Daf-c phenotype

We tested the effect of overexpressing DAF-9 activity in the ncr-2; ncr-1 mutant (Table 3). Expressing a wild-type daf-9 fully suppressed the Daf-c phenotype of ncr-2; ncr-1, and

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Table 1. Additional alleles of ncr-1 isolated from a non-complementation screen

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<td>100%</td>
<td>99%</td>
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*The numbers in parentheses show the total numbers of animals scored.

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Fig. 6. Expression patterns of ncr-1 and ncr-2. (A-G) Expression pattern of the ncr-1Ap(l)::gfp construct. (A) Pharynx (pha), excretory cell (exc), and intestine (int) strongly express GFP, in an L1/L2 larva. (B) Enlarged view of the head region in A. Two out of the six IL2 neurons are in focus, indicated by arrowheads. The other head neurons are out of focus or obscured by strong fluorescence from the pharynx. (C) Expression in the excretory canal (ec, arrow) and rectal epithelial cells (arrowhead). (D) GFP is strongly expressed in the spermatheca (sp) of an adult worm, and also weakly expressed in the gonadal sheath and uterus wall. (E-G) Dynamic hypodermal expression of ncr-1Ap(l)::gfp during development. (E) GFP expression is first observed in the lateral hypodermal seam cells during late L1/early L2 stages. Individual seam cells are indicated by white arrowheads. (F) By the L4 stage, the entire hypodermis, consisting of seam cells and the lateral hypodermis, expresses GFP. White arrowheads indicate the edge of the lateral hypodermis. (G) After the L4/adult molt, the hypodermal expression diminishes. (H-J) Expression pattern of ncr-1Ap(s)::gfp fusion. (H) Expression in identified head neurons, including three out of the six IL2 neurons (the other three are out of focus), ASGL, pharyngeal neuron I6 and XXXL cell. (I,J) Starvation induces strong GFP expression in lateral hypodermis, but not in seam cells. Shown here are L2 stage larvae from normal growth conditions (I) and from a plate that has been starved for 12-24 hours (J). (K) The ncr-1Bp::gfp fusion is expressed in about 10 pairs of head neurons, in an L1 stage larva. (L,M) Expression pattern of ncr-2p::gfp. (L) In an adult animal, ncr-2p::gfp is strongly expressed in proximal gonadal sheath cells and weakly expressed in spermatheca. (M) ncr-2p::dsRed2 expression (left) colocalizes with daf-9::gfp (middle) in the XXX cells. ncr-2p::dsRed2 is expressed diffusely throughout the cells, compared with the perinuclear pattern of daf-9::gfp expression. In the merged view (right), daf-9::gfp is pseudocolored yellow to reveal the red color better. Scale bars: 20 μm.
ectopic expression of DAF-9 activity in the hypodermis also significantly suppressed the phenotype. By contrast, overexpressing NCR-1 or NCR-2 activities failed to suppress the dauer formation phenotype of daf-9(m540) (Table S1). These results suggest that NCR proteins function upstream of DAF-9 in the dauer formation pathway.

**Genetic interaction with sdf-9 mutants**

The PTP (protein tyrosine phosphatase)-like protein encoded by sdf-9 is another likely component of the hormonal branch of the dauer formation pathway. At elevated culture temperature, loss-of-function alleles of sdf-9 exhibit an impenetrant Daf-c phenotype: they form characteristic dauer-like larvae that spontaneously recover (Ohkura et al., 2003). Like ncr-2; ncr-1 mutants, sdf-9 mutants are sensitive to cholesterol levels in the culture media, which led us to examine sdf-9 interactions with ncr-1 and ncr-2 (Table 4). At 25°C, all three tested alleles of sdf-9 were strongly Daf-c with the ncr-1 mutation, but not the ncr-2 mutation.

**Neuronal phenotypes of ncr mutants**

Most individuals with NP-C present neurological symptoms of late-infantile or juvenile onset, accompanied by substantial neuronal loss in specific brain regions (Vanier and Millat, 2003). To determine whether the NCR proteins are also required for neuronal function in C. elegans, we evaluated neuronal morphology using cell specific GFP markers or by direct dye filling, and measured neuronal function by quantitative behavioral assays.

A morphological defect was observed in the ASER neuron (gcy-5::gfp) (Yu et al., 1997): during the transient dauer stage, ASER often (69.2%, n=13) had an ectopic posterior process terminating in one or more swellings (Fig. 7A). The defect appeared to be transient because it was absent during the early larval stages, peaked during dauer stage, then regressed as dauers recovered (Fig. 7B). This structural change was not observed in dauers formed by wild-type worms on starved culture media, which led us to examine sdf-9 interactions with ncr-1 and ncr-2 (Table 4). At 25°C, all three tested alleles of sdf-9 were strongly Daf-c with the ncr-1 mutation, but not the ncr-2 mutation.

The structure of many amphid and phasmid neurons can be visualized using lipophilic dyes (Fujisawa et al., 1999; Hedgcock et al., 1985). We examined the morphology of the dye-filled neurons in newly formed ncr-2; ncr-1 dauers. The phasmid neurons were normal in all animals examined (n=47), but a significant proportion of the mutant animals (68%, n=47) contained one or more amphid neurons with a morphological abnormality similar to the ASER neuron. Of the labeled neurons, ASJ seemed to be most severely affected (Table S2).

A similar type of neuron, ASJ, is also affected by dauer arrest. However, this defect was not caused by dauer arrest itself. Moreover, this defect was not observed in ncr-2; ncr-1 mutants, but not in ncr-2 mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dauer-like larva formation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15°C</td>
</tr>
<tr>
<td>Wild type (N2)</td>
<td>0% (699)</td>
</tr>
<tr>
<td>ncr-2(nr2023); ncr-1(nr2022)</td>
<td>91% (447)</td>
</tr>
<tr>
<td>ncr-2; osm-6(p811); ncr-1</td>
<td>98% (581)</td>
</tr>
<tr>
<td>daf-5(e1385); ncr-2; ncr-1</td>
<td>100% (673)</td>
</tr>
<tr>
<td>daf-5(e1386); ncr-2; ncr-1</td>
<td>99% (928)</td>
</tr>
<tr>
<td>daf-16(me27); ncr-2; ncr-1</td>
<td>99% (117)</td>
</tr>
<tr>
<td>daf-16(meD50); ncr-2; ncr-1</td>
<td>77% (753)</td>
</tr>
<tr>
<td>daf-12(m20); ncr-2; ncr-1</td>
<td>0% (456)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses show the total numbers of animals scored.

**Table 3. Suppression of the Daf-c phenotype of ncr-2; ncr-1 by daf-9(+)**

<table>
<thead>
<tr>
<th>Transgene array</th>
<th>Dauer-like larva formation at 20°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 1</td>
<td>0% (182)</td>
</tr>
<tr>
<td>Line 2</td>
<td>0% (68)</td>
</tr>
<tr>
<td>Line 3</td>
<td>0% (84)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses show the total numbers of animals scored.

†+Ex and –Ex refers to individuals that contain or do not contain the trangene arrays, as indicated by the presence or absence of the co-injection marker myo-2::gfp.

**Table 4. Genetic interactions of ncr-1 and ncr-2 with sdf-9**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dauer-like larva formation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>0% (314)</td>
</tr>
<tr>
<td>ncr-1(nr2022)</td>
<td>0% (212)</td>
</tr>
<tr>
<td>ncr-2(nr2023)</td>
<td>0% (221)</td>
</tr>
<tr>
<td>ncr-2; ncr-1</td>
<td>84% (622)</td>
</tr>
<tr>
<td>sdf-9 (ut163)</td>
<td>0% (217)</td>
</tr>
<tr>
<td>sdf-9 (ut169)</td>
<td>1% (181)</td>
</tr>
<tr>
<td>sdf-9 (ut187)</td>
<td>1% (149)</td>
</tr>
<tr>
<td>ut163; ncr-1</td>
<td>2% (769)</td>
</tr>
<tr>
<td>ut169; ncr-1</td>
<td>87% (393)</td>
</tr>
<tr>
<td>ut187; ncr-1</td>
<td>63% (817)</td>
</tr>
<tr>
<td>ncr-2; ut163</td>
<td>0% (549)</td>
</tr>
<tr>
<td>ncr-2; ut169</td>
<td>0% (626)</td>
</tr>
<tr>
<td>ncr-2; ut187</td>
<td>0% (498)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses show the total numbers of animals scored.

other neuron classes during or after the transient dauer stage. We conclude that NCR function is required to maintain the morphological integrity of some but not all neurons during the transient dauer stage and that amphid neurons as a class may be more severely affected than other neurons.

Many amphid neurons function in regulating dauer formation and in chemotaxis response to soluble and volatile environmental stimuli, including a major role for ASE neurons in chemotaxis towards NaCl (Bargmann and Horvitz, 1991). To assess the function of amphid neurons, we measured the avoidance response of ncr-2; ncr-1 adults to high osmotic strength and their chemotaxis response to NaCl, diacetyl,
Fig. 7. Morphological abnormality of the ASER neuron in the ncr-2; ncr-1 mutant. (A) GFP labeled wild-type and mutant ASER neurons. The wild-type ASER neuron (top panel) consists of a cell body (c), a dendritic process (d) to the amphid sensory organ in the nose, and an axon process (a) to the nerve ring. The bottom panel shows the morphology of an ASER neuron of the ncr-2; ncr-1 mutant at the dauer stage. The mutant neuron sends out an ectopic posterior process (epp), often terminating in one or more swellings. (B) The morphological defect of the ASER neuron in ncr-2; ncr-1 mutant is transient and associated with the dauer stage. The percent value of each morphological class is reported.

isoamyl alcohol, and 2, 4, 5-trimethylthiazole (Bargmann et al., 1993; Bargmann and Horvitz, 1991). ncr-2; ncr-1 mutants exhibited robust responses towards all the above stimuli, indicating normal neuronal function. However, the ncr-2; ncr-1 adult animals used in these assays were recovered from starved plates. Ideally, the behaviors should be assessed in animals that have undergone the transient dauer stage, but this was impractical because of complications from the pleiotropic developmental phenotypes. We also measured the same behavioral responses in the ncr single and ncr-2; ncr-1; daf-12 triple mutant adult animals grown under normal conditions, and found that these mutants also had wild-type responses.

Discussion

Genetic position of ncr genes in the dauer formation pathway

Our genetic analysis suggests that ncr-1 and ncr-2 function upstream of daf-9 and daf-12 in a hormonal branch of the dauer formation pathway. First, the ncr-2; ncr-1 double mutant has pleiotropic phenotypes similar to daf-9 and Daf-c alleles of daf-12. Second, epistasis analysis places the ncr-1 and ncr-2 genes upstream of daf-12 and parallel or downstream to the other dauer formation pathways, the same position as daf-9. Finally, overexpressing DAF-9 suppressed the Daf-c phenotype of the ncr-2; ncr-1 mutant, but overexpressing NCR-1 or NCR-2 failed to suppress daf-9(m540) phenotypes, indicating that the ncr genes function upstream of daf-9.

The molecular identities of the ncr and daf-9 genes suggest a model in which NCR proteins are required for redistributing internalized cholesterol to intracellular compartments, including the endoplasmic reticulum (ER). The DAF-9 cytochrome P450 enzyme, which appears to be ER localized (Gerisch et al., 2001; Jia et al., 2002), may depend on sterol substrates delivered by the NCR pathway for the biosynthesis of a DAF-12 ligand (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). In ncr-2; ncr-1 mutants, defective sterol trafficking results in reduced availability of DAF-9 substrates, thus recapitulating the pleiotropic phenotypes of daf-9 and daf-12 class 6 (ligand binding domain) mutants. This model predicts a suboptimal concentration of available cholesterol in the ncr-2; ncr-1 mutant, but a normal or higher available cholesterol concentration in the daf-9 mutant. Rescue of ncr-2; ncr-1 by increased exogenous cholesterol is consistent with this model.

Expression pattern of ncr genes

Two cDNA isoforms of ncr-1 were identified in this study. They differ only in their 5' UTR region caused by an alternative non-coding first-exon located 8.7 kb upstream of the predicted coding region. A sequence homologous to this non-coding exon is located 9.4 kb upstream of the predicted C. briggsae ncr-1 gene in a similar arrangement, suggesting functional significance. The expression pattern of the ncr-1 gene reveals two interesting features. First, the ncr-1 gene expression pattern largely coincides with the tissue distribution of cholesterol in C. elegans (Matyash et al., 2001; Merris et al., 2003), consistent with a function for NCR-1 in cholesterol trafficking. Second, the hypodermal expression of the ncr-1 gene is dynamically regulated by developmental and environmental signals. A similar dynamic regulation of daf-9 expression has been reported (Gerisch and Antebi, 2004; Gerisch et al., 2001).

The expression of ncr-1 and ncr-2 colocalizes with daf-9 and sdf-9 in a pair of neuroendocrine cells called XXX cells (Ohkura et al., 2003). In previous studies, ablating the XXX cells caused the formation of characteristic partial dauers, suggesting that these cells are an important source of the dauer-inhibiting hormone (Ohkura et al., 2003). It was proposed that SDF-9 functions by increasing DAF-9 activity or helping to execute DAF-9 function (Ohkura et al., 2003). We hypothesize that sdf-9 participates in a signal transduction cascade that modulates the output of the daf-9 pathway in concert with intracellular sterol levels. We found that sdf-9 is strongly Daf-c in combination with ncr-1, but not with ncr-2 mutations. This result can be explained by two alternative models. In the first, NCR-1 and NCR-2 function redundantly in the XXX cells in cholesterol trafficking, but NCR-1 plays a more important role than NCR-2. Alternatively, NCR-1 is involved in cholesterol trafficking, whereas NCR-2 functions at a different step in the
dauer signaling pathway, perhaps parallel or downstream to SDF-9 function. The broader expression of NCR-1, as well as its sensitivity towards cholesterol deprivation and progesterone, suggests a major role in cholesterol trafficking. However, further studies, such as determining the subcellular localization of the NCR proteins, are necessary to help resolve the two models.

The DAF-12 ligand

The current study supports the hypothesis that a hormone ligand for DAF-12 is derived from sterol. It has previously been shown that lack of DAF-9 function could not be bypassed by the addition of exogenous pregnenolone (Jia et al., 2002), suggesting that it is not the product of the DAF-9 enzyme. We find that pregnenolone fails to alleviate the phenotypes of the ncr-2; ncr-1 mutant, suggesting that pregnenolone or its derivatives are also unlikely to be the substrates of the DAF-9 enzyme. Pregnenolone is the precursor of vertebrate steroid hormones and the product of the first side-chain cleavage of the P450scc, which catalyzes the complex multi-step reaction of pregnenolone synthesis (Simpson, 1979).

The insect molting hormone ecdysone is structurally different from vertebrate steroid hormones. It contains the sterol multi-ring system and an intact side chain, which is modified by oxidation (mostly hydroxylations) and methylation at multiple positions (Gilbert et al., 2002). As a result, ecdysone is amphipathic, a property that helps its movement across cell boundaries and diffusion through tissues. Although the pathway for ecdysone biosynthesis has not been fully characterized, cytochrome P450 enzymes are likely to catalyze many of the hydroxylation steps (Gilbert et al., 2002). Interestingly, it was recently reported that the levels of hydroxycholesterols are diminished in cell lines lacking NPC1 function, and among different mutant NPC1 lines, hydroxycholesterol levels correlate better with the severity of cellular phenotypes than do ER cholesterol levels (Frolov et al., 2003). It is possible that the hormonal ligand of DAF-12 is structurally more similar to the insect hormone ecdysone, with an intact side chain and modified by hydroxylations, than to the vertebrate steroid hormones.

**C. elegans as a model system to study NPC1 function**

Mammalian pathways for intracellular cholesterol trafficking are probably conserved in *C. elegans*. In addition to ncr-1 and ncr-2, the *C. elegans* genome contains an ortholog of the human NPC2/HE1 gene, the locus responsible for the remaining 5% of cases of NP-C disease (Friedland et al., 2003; Ko et al., 2003; Naureckiene et al., 2000). The *C. elegans* genome also contains homologs of LDL-receptor-like proteins (Grant and Hirsh, 1999; Ishihara et al., 2002; Yochem et al., 2001). Complex glycosphingolipids accumulate in NPC cells in addition to cholesterol (Neufeld et al., 1999; Zhang et al., 2001). It has been hypothesized that the neurological symptoms of NPC disease are caused by sphingolipid accumulation, because they accumulate more extensively than cholesterol in the central nervous system, and because the neuropathological changes in NPC disease resemble those in primary sphingolipid storage disorders (Liu et al., 2000; Zervas et al., 2001b). Although little is known about the metabolism and homeostasis of sphingolipids in *C. elegans*, we find neuronal abnormalities in ncr-2; ncr-1 mutants similar to the ectopic dendritogenesis observed in the mammalian NPC disease models, which is thought to be caused by accumulation of the ganglioside GM2 (Walkley, 1998; Zervas et al., 2001a). In conclusion, *C. elegans* represents a useful model organism for studying conserved pathways of intracellular cholesterol trafficking. Classical genetic approaches and genome-wide screening methods can be employed to identify additional components that interact with the ncr genes and to yield potential therapeutic targets for the treatment of NP-C disease.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/22/5741/DC1

**References**


developmental decision through hormonal signaling in C. elegans. Development 130, 3237-3248.