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Genetic identification of HSD-1, a conserved steroidogenic enzyme that directs larval development in *Caenorhabditis elegans*

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In *C. elegans*, steroid hormones function in conjunction with insulin/IGF-1-like signaling in promoting reproductive development over entry into the diapausal dauer stage. The NCR-1 and -2 (NPC1-related) intracellular cholesterol transporters function redundantly in preventing dauer arrest, presumably by regulating the availability of substrates for steroid hormone synthesis. We have identified *hsd-1* as a new component of this cholesterol trafficking/processing pathway, using an *ncr-1* enhancer screen. HSD-1 is orthologous to 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerases (3β-HSDs), which are key steroidogenic enzymes in vertebrates, and is exclusively expressed in two neuron-like XXX cells that are crucial in preventing dauer arrest, suggesting that it is involved in biosynthesis of dauer-preventing steroid hormones. The *hsd-1* null mutant displays defects in inhibiting dauer arrest: it forms dauers in the deletion mutant backgrounds of *ncr-1* or *daf-28*/insulin; as a single mutant, it is hypersensitive to dauer pheromone. We found that *hsd-1* defects can be rescued by feeding mutant animals with several steroid intermediates that are either downstream of or in parallel to the 3β-HSD function in the dafachronic acid biosynthetic pathway, suggesting that HSD-1 functions as a 3β-HSD. Interestingly, sterols that rescued *hsd-1* defects also bypassed the need for the NCR-1 and/or -2 functions, suggesting that HSD-1-mediated steroid hormone production is an important functional output of the NCR transporters. Finally, we found that the HSD-1-mediated signal activates insulin/IGF-I signaling in a cell non-autonomous fashion, suggesting a novel mechanism for how these two endocrine pathways intersect in directing development.

KEY WORDS: 3β-HSD, Steroid hormone, Dauer, Insulin/IGF-1, NPC1, Niemann-Pick C

INTRODUCTION

Under environmental conditions that are hostile towards growth, *C. elegans* enters a diapausal dauer stage instead of the normal 3rd larval stage. The distinctive morphology and physiology of dauer larvae make them resistant to environmental stresses and thus survive until conditions improve, upon which they resume growth towards reproductive adulthood. Genetic analyses of dauer formation in the worm have revealed several conserved signaling pathways (Hu, 2007). Three types of endocrine signals are known to be involved: steroid hormones (Rottiers and Antebi, 2006), insulin/IGF-1-like peptides (Li et al., 2003; Pierce et al., 2001) and TGFβ-like ligands (Ren et al., 1996; Schackwitz et al., 1996). These signals act in a non-redundant fashion, i.e. active signaling by their respective hormone receptor is required to promote reproductive development and prevent dauer arrest.

The role of steroid-signaling in dauer formation became apparent with the identification of several protein components. These include DAF-12/nuclear hormone receptor (Antebi et al., 2000), two steroid-processing enzymes, DAF-9/cytochrome P450 (Gerisch et al., 2001; Jia et al., 2002) and DAF-36/mono-oxygenase (Rottiers et al., 2006), and the probable intracellular cholesterol transporters NCR-1 and NCR-2 (Li et al., 2004). Recently, several steroids have been identified as DAF-12 ligands that act to prevent dauer arrest (Motola et al., 2006; Held et al., 2006). Δ^4- and Δ^7-dafachronic acids are two such hormones (Motola et al., 2006). Additional hormones may also exist that prevent dauer arrest (Matyash et al., 2004).

A two-branch pathway has been proposed for the biosynthesis of Δ^4- and Δ^7-dafachronic acids from cholesterol (Rottiers et al., 2006). However, the enzymes for several biosynthetic steps remain unknown. Genetic identification of new enzymes will not only help validate/modify the putative dafachronic acid biosynthetic pathway, but also reveal new pathways/networks for the biosynthesis of additional steroid hormones in the worm.

The steroid and insulin/IGF-1-like pathways are likely to converge for coordinated regulation in vivo, as both pathways regulate dauer formation, lifespan and stress resistance (Baumeister et al., 2006; Finch and Ruvkun, 2001). The two pathways also resemble each other in mode of action, as both involve multiple ligands that target one receptor. There are 40 insulin-like peptides in *C. elegans* and several, including DAF-28, have been reported to have functional relationships with DAF-2/insulin-like receptor (Kimura et al., 1997; Pierce et al., 2001; Li et al., 2003). Although the current model is that steroid-signaling functions downstream of or in parallel to insulin-signaling in governing dauer formation (Rottiers and Antebi, 2006), genetic evidence suggests that the two pathways may intersect in a complex manner (Gems et al., 1998). Given that insulin/IGF-1 and steroid pathways also function in concert to regulate development in flies and mice (Tatar et al., 2003), understanding potential crosstalk between the two endocrine pathways in the worm will help reveal conserved mechanisms for hormonal coordination in general.

Nematodes cannot synthesize steroid hormones de novo, but instead modify sterols, such as cholesterol, acquired from their environment (Chitwood, 1999). The *C. elegans* NCR-1 and NCR-2 (Li et al., 2004; Sym et al., 2000) are both orthologs of human

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NPC1, an intracellular cholesterol transporter that results in Niemann-Pick type C (NPC) disease when mutated (Carstea et al., 1997). *ncr-1* and *ncr-2* function redundantly in preventing dauer arrest, as knockout of both but neither alone causes dauer arrest. *ncr-1* is thought to be more important for sterol trafficking as it is more broadly expressed than *ncr-2* (Li et al., 2004). We hypothesized that both NCR proteins are involved in providing cholesterol and/or other sterols for the production of dafachronic acids or related hormones to promote reproductive development.

We identified HSD-1 from an *ncr-1* enhancer screen. HSD-1 is related to 3β-HSD enzymes that are essential for producing active steroid hormones in vertebrates. Based on protein similarity and the role of *hsd-1* in promoting reproductive growth, we hypothesize that HSD-1 is involved in the biosynthesis of Δ^4-dafachronic acid. To determine the function of *hsd-1* and dissect the proposed dafachronic acid-biosynthetic pathway, we performed a series of sterol-feeding experiments on *hsd-1* mutants. The results supported our initial hypothesis. In addition, our sterol-feeding experiments also addressed substrate specificity of the NCR transporters at the organismal level, which we believe will contribute to greater understanding and subsequent treatment of NPC disease. Furthermore, our findings suggest that the expression of *hsd-1* is exclusive to the neuron-like XXX(L/R) cells, and that the HSD-1-mediated steroid signal intersects with the insulin/IGF-I pathway by globally regulating the translocation of the DAF-16/FOXO transcription factor.

MATERIALS AND METHODS

C. elegans strains

C. elegans strains were maintained using standard methods (Sulston and Hodgkin, 1988). The following parental strains were used in this report: N2 (wild type), CB4856, *hsd-1(mg433)* (6× outcrossed), *ncr-2(nr2023)*, *ncr-1(nr2022)*, *daf-36(k114)*, *unc-75(e950)*, *daf-28(tm2308)* (3× outcrossed), *zIs356*[*Pdaf-16*::DAF-16::GFP*; rol-6(su1006)*]. Molecular methods were used to construct strains, in addition to mutant phenotypes.

ncr-1 enhancer screen and *mg433*

An *ncr-1* enhancer screen was designed to identify mutants that phenocopy *ncr-2(nr2023); ncr-1(nr2022)*, which form transient, pale dauers at all temperatures. A synchronized L4 larval population of *ncr-1(nr2022)* (P0) was mutagenized with EMS (Sulston and Hodgkin, 1988). F1 and F2 animals were cultivated at 20°C. Candidate mutants from synchronized F2 animals, which represented the progeny of ~9000 F1s, were divided into 18 pools and then subjected to a 30 minute treatment in 0.5% SDS solution. Pale dauers were then selected from surviving animals and recovered at 20°C. Sixteen mutant isolates were obtained from 10/18 pools, of these *mg433* displayed the highest penetrance of dauer arrest and was selected for further analysis. *unc-75(e950)* was used as a positional marker to introduce a wild-type stretch of chromosome I to the left of *mg433* for crossing out a background mutation.

Constructs for *hsd-1* expression and rescue

The expression pattern of *hsd-1* was determined with *gfp* (Chalfie et al., 1994) fused to the *hsd-1* promoter, which contained 10.5 kb 5′ sequence of *hsd-1*, including the entire *lagr-1* gene. This design was based on a previous prediction in WormBase that these genes form an operon. *Phsd-1*::GFP was generated by in vivo homologous recombination (Aroian et al., 1990) of two injected PCR fragments: the first contains 3.2 kb of promoter and most of *lagr-1*; the second includes the 3′ region of *lagr-1*, the intergenic region between *lagr-1* and the ATG of *hsd-1* fused with *gfp* from pPD95.70, using a PCR-fusion method (Hobert, 2002). *pha-1* DNA was used as the co-injection marker and a *pha-1* temperature-sensitive mutant was used for injection. *Psdf-9/eak-5*::RFP is as described by Hu et al. (Hu et al., 2006).

Two *hsd-1* transgenes were generated to test for rescue of *hsd-1* defects: *Psdf-9::hsd-1* and *Phsd-1::hsd-1*. *Psdf-9::hsd-1* contains the 3.5 kb *sdf-9* promoter fused to the *hsd-1*-coding region and 740 bp downstream sequences. *Phsd-1::hsd-1* contains the 275 bp 5′ sequence of *hsd-1*, the coding region and 180 bp downstream sequence. The co-injection marker was *sur-5::gfp* (Yochem et al., 1998).

Dauer assays, sterol feeding and pheromone treatment

Two different methods were used to synchronize worm populations for dauer assays. The first was hypochlorite treatment of gravid adults as described by Sulston and Hodgkin (Sulston and Hodgkin, 1988). The second was timed egg-laying with seven to eight hermaphrodites per plate, allowing them to lay eggs for 5-6 hours before removing them. The window of time for scoring dauers versus bypassors (L4 or adults) at 25°C is 48-72 hours after egg preparation or egg-laying (65-72 hours for strains containing the *ncr-1* deletion and 48-55 hours for other strains). For dauer assays at 20°C and 15°C, worms were scored 70-76 and 94-100 hours after egg preparation/laying, respectively. The transgenic rescue of *hsd-1; ncr-1* transient dauers was monitored using the developmental timing of synchronized populations, and the percentages of rescued transgenic animals were determined at a time point of 68 hours after egg preparation, when the control *hsd-1; ncr-1* animals were still virtually all dauers and N2 animals were adults.

Agar plates containing different sterols were prepared according to the standard procedure for NGM plates (Sulston and Hodgkin, 1988), except that cholesterol was replaced by different sterols at the equivalent concentration of 12.9 μM (1×). Stock solutions of sterols, dissolved in ethanol, were added into liquid agar media at a high temperature to allow even distribution. Sterols used in this study are as follows: cholesterol, 4-cholesten-3-one, 5-cholesten-3-one, lathosterol, lathosterone, 7-dehydrocholesterol, β-sitosterol/sitosterin and ergosterol (Sigma-Aldrich, St Louis, MO, USA; Steraloids, Newport, RI, USA; Research Plus, Barnegat, NJ, USA).

Crude pheromone extract was obtained as described by Golden and Riddle (Golden and Riddle, 1984). Pheromone extract was diluted with 750 μl of OP50 culture and distributed over the entire surface of a 3 ml NGM plate. Plates were then dried in a tissue culture hood until no liquid was seen on the surface (40-70 minutes). The egg preparation method was used to obtain synchronized populations for the pheromone assay. Bacterial food was always in excess during experiments, which were carried out at 25°C.

Analysis of GFP patterns and imaging

DAF-16::GFP transgenic strains were propagated at 15°C. Five to eight young adults were allowed to lay eggs for 4 hours and develop at 20°C. Animals were scored for GFP subcellular localization under a fluorescent dissecting scope. The fluorescent images in Fig. 1A-C were obtained using a compound scope with 40× optics and Fig. 1D-F using a 16× lens.

RESULTS

Genetic identification of a steroid-processing enzyme that functions in the ncr-1 pathway

In order to identify new components of the cholesterol trafficking/processing pathway that regulates dauer arrest, we performed a genetic screen using the *ncr-1(nr2022)* deletion as a sensitized background. Although *ncr-1* animals have overall wild-type development, they are thought to have attenuated cholesterol transport, based on reported minor phenotypes (Li et al., 2004). We performed an EMS screen for transient dauers resembling *ncr-1; ncr-2* mutant animals. From this screen we isolated *mg433* (for phenotypic analyses, see Table 1) and subsequently mapped it, using the SNP method (Wicks et al., 2001). The analyses of the SNP markers of 55 recombinants on chromosome I located *mg433* to the interval between the cosmid M01E5 and the YAC Y71A12B, which are about 650 kb or 5 cM apart on the physical and genetic maps, respectively. We sequenced three candidates in this interval and found that *mg433* is a mutation in the gene Y6B3B.11. This gene is predicted to encode a protein with extensive similarity to the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase family of enzymes, thus we designated Y6B3B.11 as *hsd-1*. *mg433* has a G to A

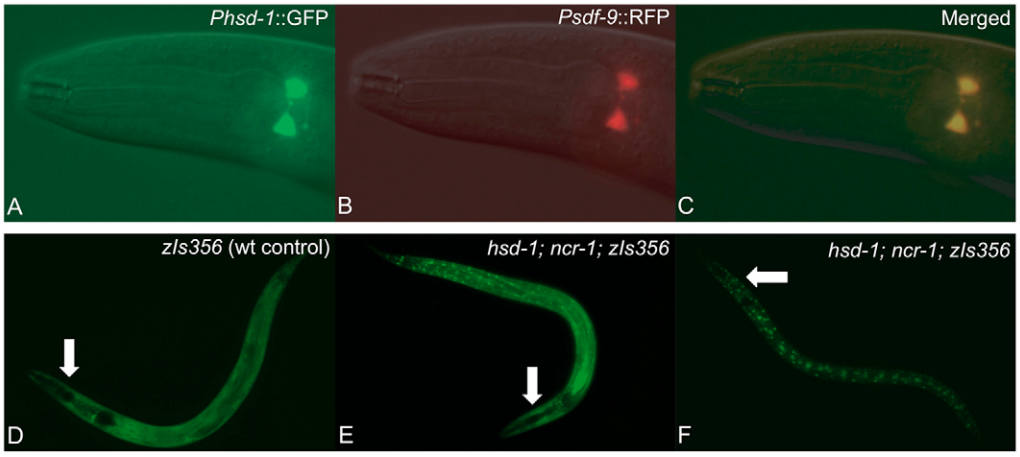

Fig. 1. *hsd-1* expression in the XXX cells globally regulates DAF-16 activity. (A-C) *hsd-1* is exclusively expressed in the paired XXX cells of the head. (**A**) *Phsd-1::*GFP expression. (**B**) *Psdf-9::*RFP expression in the XXX cells. (**C**) Merged image showing that *Phsd-1::*GFP co-localizes with *Psdf-9::*RFP. (D-F) Level of DAF-16::GFP nuclear localization. Anterior of animal indicated by the arrows. (**D**) Diffuse (cytoplasmic) GFP in wild-type control L2 larva. Partially diffuse and punctate (nuclear) GFP (**E**) and almost entirely punctate GFP (**F**) in *hsd-1(mg433); ncr-1(nr2022)* L2d larvae.

transition that results in an early amber stop codon at Trp^{45}, suggesting that *mg433* is a molecular null. Transgenic expression of *hsd-1* fully rescued the constitutive dauer formation phenotype (referred to as dauer arrest throughout this report) of *hsd-1(mg433); ncr-1(nr2022)* (see below), verifying that *hsd-1* is involved in the inhibition of dauer formation and that *mg433* impairs this function.

Using reciprocal BLASTP, we determined that *hsd-1* is an ortholog of the predicted human gene HSPC105; they share 20.5% identity and 32.0% similarity. In addition, among the top hits from the BLASTP of HSD-1 against the human RefSeq protein database are the following 3β-HSDs: NSDHL, HSD3B7 and HSD3B1, which are important for the biosynthesis of cholesterol, bile acids and steroid hormones, respectively. These enzymes are 15.5%, 15.8%, 16.7% identical and 27.0%, 25.7%, 26.5% similar to HSD-1, respectively. The 3β-HSD proteins are fairly short (359-462 amino acids) and share a number of very small conserved motifs across their whole length (data not shown). A major role of these mammalian enzymes is to generate Δ^4-3-keto-sterols and two are implicated in human diseases (Konig et al., 2000; Schwarz et al., 2000). The extensive similarity between HSD-1 and the human 3β-

Table 1. Percentage of animals that undergo dauer arrest in different conditions and recovery times for double mutants

Dauer arrest at different temperatures

	Mean dauer arrest±s.d. (*n*)		
Genotype	25°C	20°C	15°C
hsd-1(mg433); ncr-1(nr2022)	98.3±0.9 (844)	99.9±0.2 (487)	99.4±0.4 (1025)
hsd-1(mg433)	0±0 (864)	–	–
ncr-1(nr2022)	0±0 (317)	0±0 (399)	0±0 (218)
hsd-1(mg433); ncr-1(nr2022); daf-12(RNAi)	1.9±1.3 (807)	–	–
hsd-1(mg433); ncr-2(nr2023)	0±0 (449)	0±0 (247)	–
*hsd-1(mg433); daf-36(k114)**	100±0 (476)	–	–
daf-36(k114)	2.3±2.8 (925)	–	–
hsd-1(mg433); daf-28(tm2308)	89.6±5.2 (1190)	79.8±10.4 (336)	88.2±5.6 (274)
daf-28(tm2308)	28.5±5.3 (2475)	0±0 (584)	0±0 (328)

Dauer arrest induced by pheromone†

	Mean dauer arrest±s.d. (*n*)					
Genotype	0.7 μl	1.4 μl	2.75 μl	5.5 μl	11.0 μl	22.0 μl
N2	–	–	1.6%	2.8%	19.1%	50.5%
hsd-1(mg433)	50.7%	94.9%	98.9%	99.6%	99.7%	100%

Dauer recovery‡

	Mean dauer arrest±s.d. (*n*)			
Genotype	Day 1	Day 2	Day 3	Day 4
hsd-1(mg433); ncr-1(nr2022)	12%	64%	97.8%	100%
ncr-2(nr2023); ncr-1(nr2022)	40%	88%	100%	–

*Synchronized populations of this strain were obtained using the egg/embryo preparation method (see Materials and methods). For all other strains, populations were synchronized using timed egg-lays.
†This batch of pheromone was different to that used in Fig. 5B.
‡Recovery tests were done at 25°C. Dauers were picked 72 hours post egg-lay (day 0) and then monitored daily for recovery to adults. Percentages shown represent cumulative recovery.

Table 2. Rescue of *hsd-1(mg433); ncr-1(nr2022)* dauer arrest with extrachromosomal arrays (20°C, 68 hours post egg-lay)

	% Transgenic adults [mean±s.d. (*n*)]		
Transgene*	Line 1	Line 2	Line 3
No transgene control	0±0 (177)	–	–
Psdf-9::*hsd-1*	99.5±0.8 (413)	98.0±1.7 (604)	95.8±4.0 (666)
Phsd-1::*hsd-1*	95.2±3.7 (202)	–	–

*Both transgenes were co-injected with SUR-5::GFP. Animals were scored as 'transgenic' based on visualization of GFP. We found that worms scored as 'non-transgenic' siblings had low levels of dauer rescue, unlike the no transgene control. P*sdf-9*::*hsd-1* 'non-transgenic' siblings formed 7.1% (*n*=463), 12.7% (*n*=371) and 4.2% (*n*=551) adults in lines 1, 2 and 3, respectively. P*hsd-1*::*hsd-1* 'non-transgenic' siblings formed 0.6% adults (*n*=126). One possibility is that the adult 'non-transgenic' siblings carry transgenes at very low copy numbers that cannot be visualized by GFP.

HSDs suggests that HSD-1 is involved in producing Δ^4-3-keto-steroids such as Δ^4-dafachronic acid. HSD-1 also has two paralogs in *C. elegans*, HSD-2 (ZC8.1) and HSD-3 (ZC449.6).

The XXX cells serve as the endocrine tissue for HSD-1-mediated steroid signaling

Using the *hsd-1* promoter to drive a GFP reporter, we determined that *hsd-1* is exclusively expressed in a pair of neuron-like cells in the head. We surmised that these were the XXX(L/R) cells, because these cells are not only important for preventing dauer arrest (Hu et al., 2006; Ohkura et al., 2003), but are also thought to be involved in steroidogenesis, based on the expression patterns of NCR-1, NCR-2 and DAF-9 (Gerisch and Antebi, 2004; Jia et al., 2002; Li et al., 2004; Mak and Ruvkun, 2004). To confirm this prediction, we examined whether the expression of *Phsd-1::*GFP co-localizes with that of *Psdf-9/eak-5::*RFP. The *sdf-9* gene is expressed only in the XXX cells (Hu et al., 2006; Ohkura et al., 2003) and we observed co-localization of both markers (Fig. 1A-C). Consistent with this, a transgene containing the *hsd-1*-coding sequence driven by the *sdf-9* promoter, rescued the dauer arrest of *hsd-1(mg433); ncr-1(nr2022)* (Table 2). The expression of *Phsd-1::*GFP was initially observed at the pretzel stage of embryogenesis. This XXX cell-specific expression persists through all four larval stages and becomes fainter in adults, which is consistent with the view that the XXX cells are an important endocrine tissue in regulating *C. elegans* larval development.

hsd-1 promotes reproductive growth

hsd-1(mg433); ncr-1(nr2022) animals undergo dauer arrest transiently at all temperatures tested (Table 1), similar to that seen in *ncr-2(nr2023); ncr-1(nr2022)* (Li et al., 2004) and the mutants of DAF-12 that are defective in steroid-ligand binding, such as *rh273* (Antebi et al., 1998). *hsd-1; ncr-1* dauers can resume reproductive growth at all temperatures, including the relatively high 25°C. We compared the dauer recovery rates for *hsd-1; ncr-1* and *ncr-2; ncr-1* at 25°C (Table 1) and found that *hsd-1; ncr-1* dauers appeared to recover slightly slower. We found that *hsd-1*; *ncr-1* dauers recovered poorly and had abnormalities such as protruding-vulvas and 'bagging' (matricide by internal hatching) phenotypes (9/25 animals), similar to *ncr-2; ncr-1* (8/25). Like *ncr-2, ncr-1*, we also found that *hsd-1; ncr-1* has a reduced brood size (Fig. 2A). In the absence of dauer-preventing steroids, such as in *daf-9* or *ncr-2; ncr-1*, the un-liganded form of DAF-12 receptor is known to be required for dauer formation (Rottiers and Antebi, 2006). Similarly, we found that *hsd-1; ncr-1* dauer arrest at 25°C can be suppressed by RNAi of *daf-12* (Table 1). Similar to mutants of other steroid-signaling components, except for null mutants of *daf-12*, *hsd-1; ncr-1* is capable of forming natural dauers under the appropriate physiological conditions. To conclude, in a similar manner to DAF-9, the HSD-1-mediated steroid signal normally promotes reproductive growth.

The *hsd-1(mg433)* single mutant appears to develop normally; however, we observed that it forms dauers more readily than the wild-type N2 under the high population densities present in continuous plate culture. Dauer pheromone is constitutively produced and released by worms, serving as an external signal for judging and regulating population density (Golden and Riddle, 1982; Golden and Riddle, 1984). In order to determine whether the *hsd-1* single mutant is more sensitive to induction by dauer pheromone, we examined the response of *hsd-1* to a crude extract of dauer pheromone. We found that *hsd-1* forms dauers at 98.2% and 56.7%

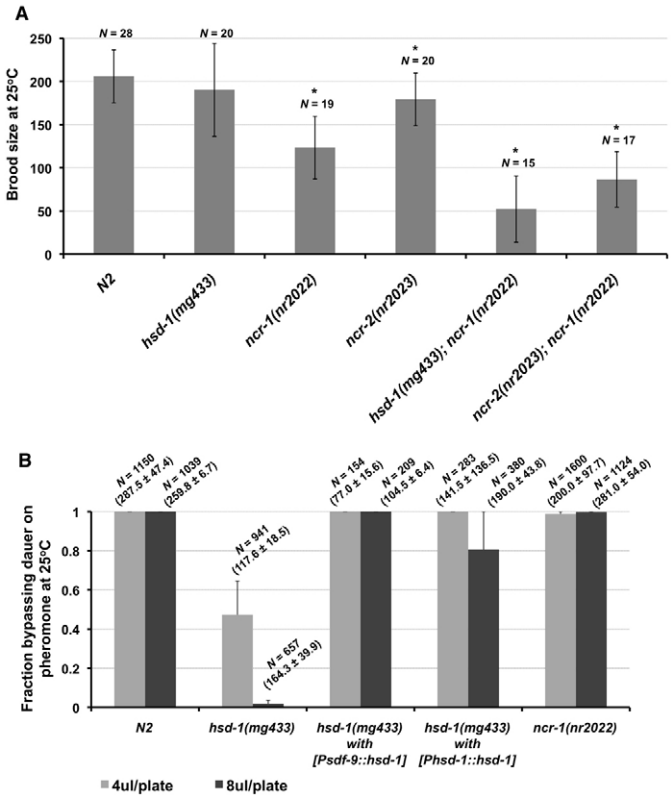

Fig. 2. Phenotypic analyses of *hsd-1*. (**A**) Brood sizes of N2, *hsd-1*, *ncr-1* and *ncr-2* single mutants, as well as *hsd-1*; *ncr-1* and *ncr-2*; *ncr-1* double mutants. The number of parent worms (*n*) is indicated above each column. Error bars: standard deviation (s.d.); asterisks indicate significantly reduced brood sizes compared with N2 ($P<0.01$) by Student's *t*-test. (**B**) Responses of different mutant/transgenic strains to two different concentrations of dauer pheromone extract: 4 μl/plate (light grey) and 8 μl/plate (dark grey). Total numbers scored are above the corresponding columns with the error bars representing the s.d. based on at least three replicates. Number in parentheses indicate average population density (worms/plate) ±s.d.

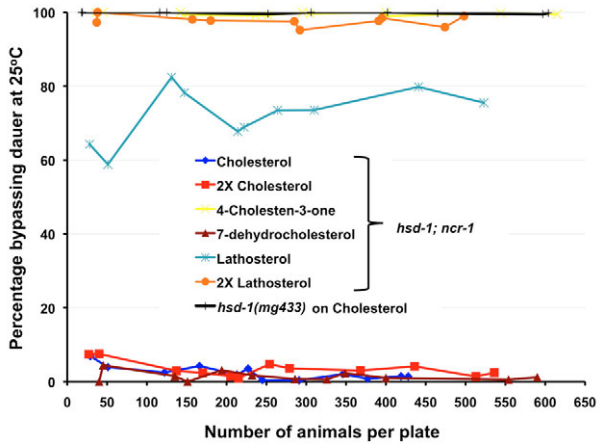

Fig. 3. Population density in the ranges used in this report has minimal influence on dauer formation. In *hsd-1(mg433); ncr-1(nr2022)* worms (colored lines), sterol choice (not worm density) is the major effector of dauer formation at 25°C. *hsd-1(mg433)* (black line) single mutants, grown on standard cholesterol, display almost 100% dauer bypass at all population densities tested.

with 8 and 4 μl of pheromone extract per plate, respectively (Fig. 2B). At the same pheromone concentrations, N2 did not form dauers, indicating that *hsd-1* is hypersensitive to pheromone induction. In fact, it took approximately 30× higher pheromone concentration to induce dauers in N2 than *hsd-1* (Table 1). We also tested whether *ncr-1*, for comparison with *hsd-1*, is hypersensitive to pheromone and found that although it does form some dauers, it is not as sensitive (Fig. 2B). This result suggests that *hsd-1* prevents dauer arrest at population densities lower than those that would normally trigger dauer formation in wild-type worms, which is consistent with a role for *hsd-1* in preventing dauer arrest in the *ncr-1* background. However, in the range of population densities used in this report, *hsd-1* animals rarely form dauers without pheromone (Fig. 3), suggesting that our dauer assays were not influenced by population density.

To investigate possible synergism between *hsd-1(mg433)* and other steroid-pathway null mutants that display only weak dauer arrest phenotypes, we constructed *hsd-1(mg433); ncr-2(nr2023)* and *hsd-1(mg433); daf-36(k114)* double mutants. In contrast to *hsd-1(mg433); ncr-1(nr2022)*, *hsd-1(mg433); ncr-2(nr2023)* animals did not display dauer arrest, consistent with the notion that NCR-1 contributes more to cholesterol trafficking than does NCR-2, and/or suggesting that the functional output of NCR-2 completely relays into that of HSD-1. The putative *daf-36* null allele *k114* displays only a weak dauer arrest phenotype at 25°C (Rottiers et al., 2006). We found that *hsd-1(mg433); daf-36(k114)* displayed 100% dauer arrest at 25°C (Table 1). This result suggests that, compared with HSD-1, DAF-36 functions in a parallel biosynthetic pathway, a different tissue, or both. Rottiers et al. (Rottiers et al., 2006) have previously shown that DAF-36 is expressed in the intestine and may function in the Δ^7-branch (Fig. 4A), which is consistent with the observed synergism.

We also investigated possible synergism between *hsd-1(mg433)* and *daf-28(tm2308)*, a deletion mutant of an insulin. The transient dauer phenotype of the dominant-negative mutant *daf-28(sa191)*

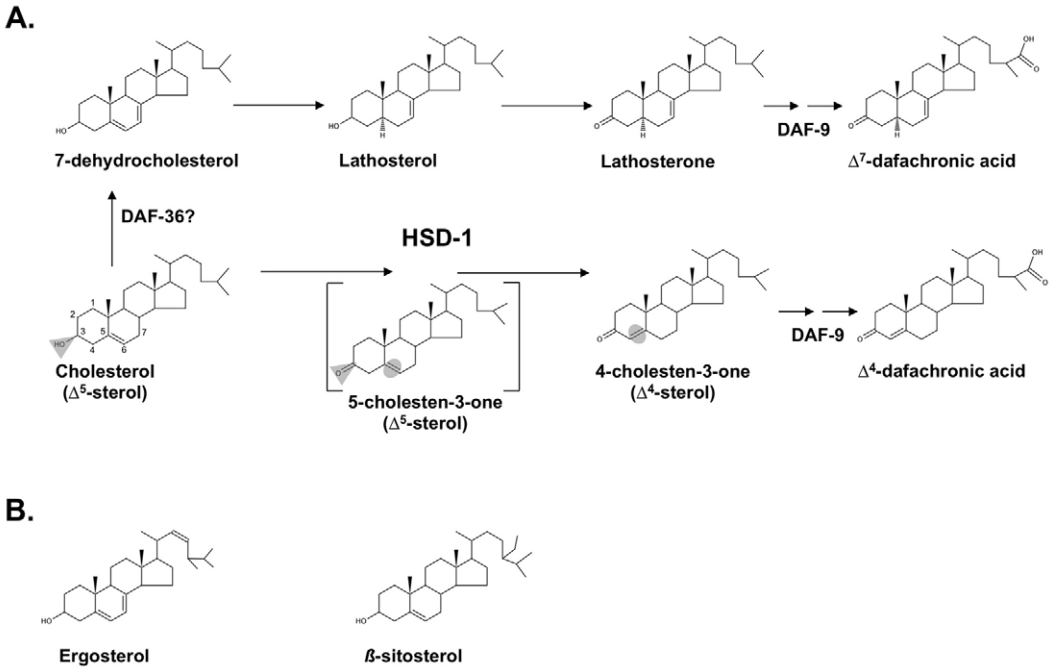

Fig. 4. HSD-1 is involved in Δ^4-dafachronic acid synthesis. (**A**) HSD-1 may function in the proposed biosynthetic pathway of Δ^4- and Δ^7-dafachronic acids [modified, with permission from Rottiers et al. (Rottiers et al., 2006)]. Predicted intermediates for biosynthesis of Δ^7- and Δ^4-dafachronic acids from cholesterol are indicated in the upper and lower branches of the pathway, respectively. The marked positions on the cholesterol structure (1-7) represent the standard numbering of the carbon atoms. 5-cholesten-3-one, in parentheses, is the presumed product, from cholesterol, after the first of the two-step 3β-HSD reactions. Shaded triangles and ovals indicate the sequential changes that HSD-1 is hypothesized to direct: triangles indicate 3β-dehydrogenase activity, ovals indicate isomerase activity. (**B**) The structures of ergosterol and *β*-sitosterol, the major fungal and plant sterols, respectively.

suggests that DAF-28 functions as an agonist of the DAF-2 receptor (Li et al., 2003; Malone and Thomas, 1994). We found that 28.5% of *daf-28(tm2308)* undergo dauer arrest at 25°C (Table 1). Unlike the single mutants of either *hsd-1* or *daf-28*, *hsd-1(mg433); daf-28(tm2308)* animals form 89.6% transient dauers at 25°C (Table 1). This synthetic phenotype suggests that, although the insulin and steroid pathways are non-redundant with respect to each other, a subset of ligands from each pathway can be redundant in preventing dauer arrest. Interestingly, the transient dauers of *hsd-1(mg433); daf-28(tm2308)* have mixed characteristics of dauers induced by either reduced insulin or steroid signaling. They are large dauers, resembling the mutants of the DAF-2 receptor, but also pale and active, resembling *ncr-2; ncr-1* dauers (data not shown).

HSD-1 functions as a 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase in Δ^4-steroid signaling

Biochemical and structural studies on the 3β-HSD family of enzymes, especially on HSD3B1 (Thomas et al., 2003), have shown that they carry out a sequential two-step reaction: (1) the dehydrogenase activity oxidizes the 3β-hydroxyl group of a sterol; (2) the isomerase activity switches the position of the double carbon bond on the sterol ring from Δ^5 to Δ^4 (Fig. 4A). Thus, we hypothesize that HSD-1 functions in the Δ^4-dafachronic acid branch by converting cholesterol to 4-cholesten-3-one (Fig. 4A).

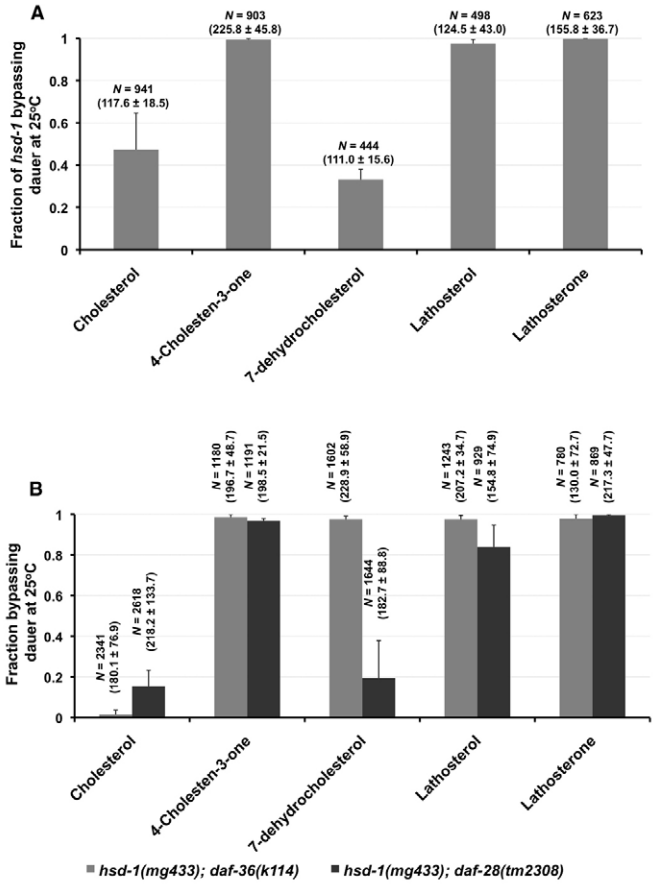

Fig. 5. Effect of feeding different sterols on dauer arrest at 25°C. (**A**) *hsd-1(mg433)* dauers induced by pheromone at 4 μl/plate. (**B**) *hsd-1(mg443); daf-36(k114)* (light-gray bars) and *hsd-1(mg433); daf-28(tm2308)* (dark-gray bars). Total numbers scored are above the corresponding columns with the error bars representing the s.d. based on at least three replicates. Number in parentheses indicate average population density (worms/plate) ±s.d.

In order to test whether dietary supply of 4-cholesten-3-one could compensate for the lack of *hsd-1* activity, we first examined the hypersensitivity of *hsd-1(mg433)* to dauer pheromone when grown on agar plates containing 4-cholesten-3-one instead of cholesterol. We observed substantial rescue of dauer arrest with 4-cholesten-3-one, at two different pheromone concentrations (Fig. 5A). We investigated whether 4-cholesten-3-one could rescue synthetic dauer arrest associated with the double mutants of *hsd-1(mg433)* with *daf-28(tm2308)*, *daf-36(k114)* and *ncr-1(nr2022)*, and we observed robust rescue (Fig. 5B; Fig. 6A,B). As a control, we also tested the predicted product of the first step of the HSD-1 reaction, 5-cholesten-3-one, which contains the C-3 ketone group but still has the Δ^5-ring configuration (Fig. 4A). We found that 5-cholesten-3-one did not rescue *hsd-1; ncr-1* as well as 4-cholesten-3-one (Fig. 6A,B), suggesting that both structural characteristics of 4-cholesten-3-one contribute to the rescue of *hsd-1(mg433)*. The above results are consistent with the hypothesis that HSD-1 converts cholesterol to 4-cholesten-3-one in vivo, or alternatively, HSD-1 modifies a derivative of cholesterol into a Δ^4-3-keto-sterol.

Δ^7-Dafachronic acid, another dauer-preventing hormone, is thought to be produced in parallel to Δ^4-dafachronic acid from cholesterol (Fig. 4A) (Motola et al., 2006). If *hsd-1* is required by the Δ^4-biosynthetic branch, raising the level of Δ^7-signaling by increasing the levels of Δ^7-intermediates may compensate for impaired Δ^4-dafachronic acid biosynthesis. We found lathosterol and lathosterone, two Δ^7-intermediates, can suppress both *hsd-1(mg433)* dauer pheromone hypersensitivity and synthetic dauer arrest in the double mutants of *hsd-1* with *daf-28(tm2308), daf-36(k114)* and *ncr-1(nr2022)* (Fig. 5A,B; Fig. 6A,B). Higher concentrations of lathosterol were required for full suppression of *hsd-1; ncr-1* dauer arrest (Fig. 6A). By contrast, cholesterol was unable to suppress this phenotype at any of the concentrations tested (Fig. 6A). In a titration experiment to compare the efficacy of the sterols in alleviating *hsd-1*-associated dauer phenotypes, we found that, like 4-cholesten-3-one, lathosterone is quite potent at a quarter of the standard 12.9 μM concentration of cholesterol. Lathosterone still displayed rescue at an eighth this concentration (Fig. 6B). The physiological concentration for Δ^4-dafachronic acid is estimated to be 200 nM (Motola et al., 2006), and our experiment showed that lathosterone at a concentration ~8-fold greater than this can be effective. Interestingly, 7-dehydrocholesterol, the first intermediate in the Δ^7-branch, did not suppress *hsd-1*-associated dauer arrest (Fig. 5A,B; Fig. 6A), except in *hsd-1; daf-36*, where the suppression is probably due to the rescue of *daf-36*, as this is the predicted end product of the DAF-36 reaction (Fig. 4A). The above results suggest that some sterol intermediates in the parallel Δ^7-branch can compensate for the lack of HSD-1-mediated Δ^4-dafachronic acid production.

The requirement for the NCR-1 and/or -2 transporter function can be bypassed by dietary supply of certain sterol intermediates

hsd-1(mg433) was identified as an enhancer of *ncr-1(nr2022)*, with the double mutant forming transient dauers when grown on cholesterol-containing media. We considered the possibility that one role of NCR-1 or -2 transporters is to mobilize and display

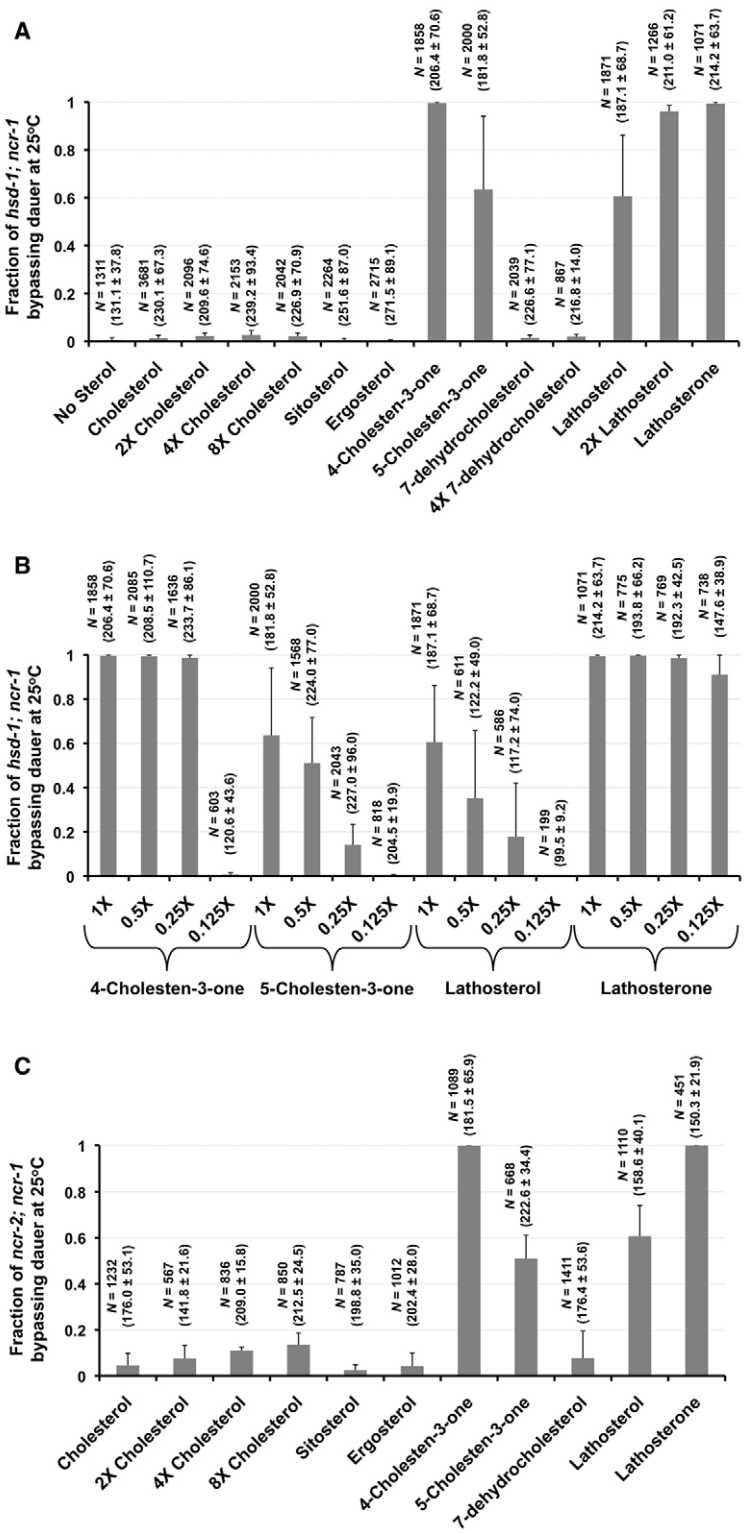

Fig. 6. Effect of feeding sterols at different concentrations on dauer arrest at 25°C. Sterols were at 12.9 μM (1×), the standard concentration for cholesterol in NGM, unless indicated otherwise. (**A**) *hsd-1(mg433); ncr-1(nr2022)* with increasing sterol concentrations. (**B**) *hsd-1(mg433); ncr-1(nr2022)* with decreasing sterol concentrations. (**C**) *ncr-2(nr2023); ncr-1(nr2022).* Total numbers scored are above the corresponding columns with the error bars representing the s.d. based on at least three replicates. Number in parentheses indicate average population density (worms/plate) ±s.d.

cholesterol for HSD-1-mediated 3β-dehydrogenation. According to this hypothesis, sterol intermediates beyond the point of the HSD-1 reaction, or those in the parallel Δ^7-branch, should be able to bypass the requirement for the NCR-1 and NCR-2 transporters in preventing dauer arrest.

We found that dauer arrest in *ncr-2(nr2023); ncr-1(nr2022)* was suppressed when cultivated on the following intermediates: 4-cholesten-3-one, 5-cholesten-3-one, lathosterol and lathosterone (Fig. 6C), suggesting that the NCR-1 and -2 transporters are only required for the transport/mobilization of certain sterols, such as cholesterol and perhaps 7-dehydrocholesterol. Alternative mechanisms may exist for mobilizing other types of sterols, such as lathosterol, for steroidogenesis. We also tested the major plant and fungal sterols, β-sitosterol and ergosterol, respectively (for structures, see Fig. 4B) and found that, like cholesterol, they did not suppress *ncr-2(nr2023); ncr-1(nr2022)*, suggesting that these sterols

Table 3. Percentage of animals displaying a punctate (nuclear) DAF-16::GFP pattern at 20°C

Genotype	% Punctate GFP±s.d. (*n*)*	Development time (stage)†	% Punctate GFP dauers (*n*)‡
Cholesterol			
hsd-1(mg433); ncr-1(nr2022); zIs356	29.0±12.8 (177)	46.5-48.5 hours (L2d)	3.1 (97)
ncr-2(2023); ncr-1(nr2022); zIs356	60.6±5.3 (264)	46.5-48.5 hours (L2d)	0 (160)
zIs356	0±0 (207)	38.5-40.5 hours (L2)	–
zIs356	0±0 (224)	44.5 hours (L2/L3)	–
zIs356	0±0 (142)	45.5 hours (L2/L3)	–
4-cholesten-3-one			
hsd-1(mg433); ncr-1(nr2022); zIs356	0±0 (226)	38.5-40.5 hours (L2)	–
hsd-1(mg433); ncr-1(nr2022); zIs356	1.4±0.2 (135)	46.5-48.5 hours (L2/L3)	–
ncr-2(2023); ncr-1(nr2022); zIs356	0±0 (87)	38.5-40.5 hours (L2)	–
ncr-2(2023); ncr-1(nr2022); zIs356	0±0 (136)	46.5-48.5 hours (L2/L3)	–
zIs356	0±0 (87)	38.5-40.5 hours (L2)	–
zIs356	0±0 (108)	46.5-48.5 hrs (L2/L3)	–

*Most animals have a mixed pattern of punctate and diffuse GFP. In this data set, only 2.7% (*n*=177) of *hsd-1; ncr-1* and 0% (*n*=264) *ncr-2; ncr-1* displayed a complete punctate pattern.
†Time elapsed between the mid-point of the egg-lay and scoring. Approximate developmental stages are indicated in brackets.
‡Scored 70 hours after egg-laying, while animals are still in the dauer stage.

are NCR-1/-2-dependent and thus likely occupy a similar position to cholesterol in the metabolic pathway. β-Sitosterol, ergosterol and 7-dehydrocholesterol are all known to be able to substitute for cholesterol in supporting the growth of wild-type animals (Chitwood, 1999; Hieb and Rothstein, 1968). Given that the sterols that alleviated *hsd-1*-associated defects also appeared to suppress *ncr-2; ncr-1*, HSD-1-mediated hormone production appears to be an important functional output of the NCR transporters.

The NCR-independent sterols block crosstalk between steroid and insulin signaling

In order to detect any potential crosstalk between steroid and insulin/IGF-1-like signaling, we examined the subcellular localization of DAF-16::GFP in *hsd-1(mg433); ncr-1(nr2022)* and *ncr-2(nr2023); ncr-1(nr2022)*. Upon downregulation of DAF-2, DAF-16/FOXO, a transcription factor in the insulin pathway, is activated after translocating from the cytoplasm to the nucleus (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). In wild-type animals, DAF-16::GFP is localized to the cytoplasm, thus displaying a diffuse pattern (Fig. 1D). When DAF-16::GFP is in the nucleus, it displays a punctate pattern. We found that 29% of *hsd-1; ncr-1* and 60.6% of *ncr-2; ncr-1* animals displayed punctate DAF-16::GFP at the L2d stage, a preparatory stage prior to dauer formation (Table 3). The difference between the two double mutants suggests that the downregulation of steroid signaling is more severe in *ncr-2; ncr-1* than in *hsd-1; ncr-1*, although dauer arrest between the two is comparable. We observed variable degrees of DAF-16 nuclear localization amongst individuals of both *hsd-1; ncr-1* and *ncr-2; ncr-1* mutants. For example, Fig. 1E shows a mix of diffuse and punctate DAF-16::GFP, whereas Fig. 1F displays a punctate pattern. Interestingly, we observed that in both double mutants, DAF-16::GFP is often more punctate in the posterior of the worm, as though insulin signaling is more active in the head compared with the tail (Fig. 1E, see Discussion). In addition, we observed that a complete punctate pattern is often found among the double mutants close to or during the molting period prior to dauer formation (Fig. 1F). However, DAF-16::GFP is primarily diffuse amongst dauers (Table 3). These results suggest that the insulin pathway is partially, or transiently, downregulated in both double mutant backgrounds prior to dauer formation.

When *hsd-1(mg433); ncr-1(nr2022)* and *ncr-2(nr2023); ncr-1(nr2022)* animals were grown on plates with either 4-cholesten-3-one (Table 3) or 2× lathosterol (data not shown), we observed diffuse DAF-16::GFP, similar to wild-type controls (Fig. 1D). Thus, rescue of steroid-signaling by 4-cholesten-3-one also results in restoring insulin-pathway activity to wild-type levels in the double mutants.

DISCUSSION

HSD-1 may function as a 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase

From an enhancer screen for mutations that would further impair sterol trafficking/processing mediated by the probable intracellular cholesterol transporter NCR-1 in regulating dauer arrest, we identified HSD-1, which has extensive similarity to mammalian 3β-HSDs. The human HSPC105 protein appears to be the true ortholog of HSD-1, based on reciprocal BLASTP. Although very little is known about HSPC105, several other human 3β-HSDs are important for development or physiology. The major 3β-HSD function is to generate Δ^4-3-keto-sterols, which is thought to be required for the formation of active vertebrate steroid hormones (Payne and Hales, 2004). Although vertebrate steroid hormones lack the long side chain that is present in the dafachronic acids, the presence of a C-3 ketone group appears to be a shared characteristic (Motola et al., 2006). The genetic identification of HSD-1 in *C. elegans* has reinforced this notion.

Our sterol-feeding experiments showed that 4-cholesten-3-one rescued *hsd-1*- associated defects more effectively than 5-cholesten-3-one, suggesting that both structural changes enacted by the dehydrogenase and isomerase activities of 3β-HSD contribute to rescuing *hsd-1*. However, our in vivo experiments did not identify the true or preferred substrate(s) of HSD-1. Conceivably, the substrate(s) of HSD-1 is cholesterol or its derivatives. Our experiments also did not address why 5-cholesten-3-one could rescue the *hsd-1* defects. There are several possible reasons why 5-cholesten-3-one can substantially rescue *hsd-1*; *ncr-1*: (1) this Δ^5-sterol may be converted to Δ^4-sterol by enzymes with ketosteroid isomerase activity, such as GSTs with a similar function to GST A3-3 in humans (Johansson and Mannervik, 2001); (2) this Δ^5-sterol may be processed into a Δ^5-steroid similar to 5-cholesten-3β-ol-(25S)-carboxylic acid, which has dauer-preventing activity (Held

et al., 2006); (3) this Δ^5-sterol may be modified into dauer-preventing hormones that remain unidentified. Therefore, it is possible that the in vivo metabolic pathways/networks are more complex and plastic than the proposed pathway for dafachronic acids, in both protein and sterol/steroid contents. Nevertheless, our results are consistent with the hypothesis that HSD-1 functions as a 3β-HSD in vivo.

hsd-1-mediated Δ^4 steroid signaling in the XXX cells

In *C. elegans*, steroid signaling is thought to be present in multiple tissues. Besides the XXX cells, steroids are believed to be produced in the hypodermis, intestine and somatic gonads (Gerisch et al., 2001; Jia et al., 2002; Mak and Ruvkun; Rottiers et al., 2006). *hsd-1* is the first steroidogenic enzyme that displays expression exclusive to the XXX cells. This expression pattern is not all that surprising: it is known that the XXX cells are where *ncr-2* is predominantly expressed in young larvae (Li et al., 2004), when the dauer decision is made. *hsd-1* and *ncr-2* both enhance *ncr-1*, resulting in dauer arrest and thus may share a similar focus in their functions, which may be to promote the generation of steroids in XXX cells. The molecular identity of HSD-1 and our sterol-feeding results suggest that HSD-1 is likely to function in Δ^4-dafachronic acid biosynthesis (Fig. 4A). Thus, we speculate that the phenotypes associated with the *hsd-1(mg433)* null in this report represent the impairment of Δ^4-signaling in the XXX cells.

hsd-1-mediated Δ^4-signaling in XXX cells contributes to promoting reproductive growth, which is also indicated by the fact that *hsd-1* becomes crucial when the population density reaches a certain level. Dauer pheromone is a surrogate measure of population density. The degree of *hsd-1(mg433)* hypersensitivity to pheromone implies that, without *hsd-1*, a growing worm culture would begin to form dauers at a much lower population density (Fig. 3, Table 1). The involvement of *hsd-1* in promoting growth is also indicated by its synergism with several genes in preventing dauer arrest: *ncr-1*, *daf-28* and *daf-36.* Together, we conclude that HSD-1 becomes crucial in the growth versus dauer decision, when population density is relatively high, cholesterol trafficking is attenuated, insulin-signaling is reduced or a parallel steroid-signaling event is compromised.

The expression of both *hsd-1* and *ncr-2* is limited to the head at the stage when the dauer versus growth decision is made. Given that the formation of dauers requires remodeling of multiple tissues (Cassada and Russell, 1975), including the pharynx and hypodermis, the XXX cell-expressed HSD-1 can apparently function from a distance. Consistent with this prediction, we found that the influence of *hsd-1/ncr-2*-mediated steroid signaling on DAF-16::GFP, which is ubiquitously expressed, appears to affect many cells (Fig. 1E,F). This is consistent with the cell non-autonomous nature of steroid hormone action. Under an epifluorescent dissecting scope, we observed that DAF-16::GFP is often found fully confined to the nucleus in the posterior of the worm, but gradually becomes more cytoplasmic towards the anterior end in both *hsd-1; ncr-1* and *ncr-2; ncr-1* (Fig. 1E). This implies that there is a decreasing gradient of hormones that prevent the downregulation of insulin signaling or DAF-16 nuclear localization, running in an anterior-posterior direction. Alternatively, as a recipient tissue for hormonal signals, posterior cells maybe more prone to downregulation of insulin signaling than their anterior neighbors in *hsd-1; ncr-1* or *ncr-2; ncr-1* animals.

Our finding that DAF-16::GFP undergoes nuclear translocation upon the reduction of steroid signaling suggests a novel mechanism for the cell non-autonomous coordination of these two endocrine pathways in directing development. It has been previously shown that a steroid/lipophilic signal induced by the loss of germline cells extends lifespan by promoting nuclear translocation of DAF-16, indicating that steroid-signaling negatively regulates the insulin pathway (Gerisch et al., 2001; Hsin and Kenyon, 1999; Lin et al., 2001). Our finding suggests a comparable epistatic relationship between the two endocrine pathways in dauer regulation, except that in the latter case, the presence of steroid hormones upregulates insulin signaling. Previously, steroid signaling was thought to function downstream of or in parallel to insulin signaling in regulating development (Rottiers and Antebi, 2006). We believe that our findings are not contradictory to the previous model; instead, it reflects that these two endocrine pathways interact in a complex manner.

Redundancy of steroid signaling in *C. elegans*

Based on comparisons between the null mutant phenotypes, expression patterns and biochemical roles of *daf-9* and *hsd-1*, we predict that *hsd-1* functions in the *daf-9* pathway, but is not the sole input to *daf-9*. *daf-9* worms display a severe dauer arrest phenotype (Gerisch et al., 2001; Jia et al., 2002), whereas *hsd-1* worms only show hypersensitivity to dauer pheromone; *daf-9* is expressed in multiple tissues (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004), whereas *hsd-1* is expressed only in the XXX cells. DAF-9 is involved in biosynthesis of both Δ^7- and Δ^4-dafachronic acids (Motola et al., 2006), whereas HSD-1 is likely only to be involved in the latter. We speculate that HSD-1 provides a C-3 keto-sterol precursor for the production of Δ^4-dafachronic acid by DAF-9 in XXX cells, whereas the role of producing C-3 keto-sterols in other pathways or tissues may be carried out by other 3β-HSDs or sterol dehydrogenases, such as HSD-2 and HSD-3. We speculate that a complex sterol metabolic network involving multiple branches or alternative pathways exists to allow versatile regulation of development in response to different environmental conditions. It is known that brassinolide signaling in plants, which regulates development in response to light, comprises such a network (Thummel and Chory, 2002).

If Δ^7- and Δ^4-dafachronic acids function redundantly in preventing dauer arrest, raising the level of Δ^7-signaling should compensate for the lack of HSD-1-mediated Δ^4-signaling. We found that two predicted Δ^7-intermediates, lathosterol, a 3β-hydroxysterol, and lathosterone, could suppress *hsd-1*-associated defects. Conceptually, suppression is no different from rescue. Therefore, one possible interpretation of this result is that *hsd-1* functions upstream of the generation of lathosterol. However, based on protein similarity, HSD-1 is likely to produce Δ^4-3-keto-sterols (Fig. 4A). Therefore, it is more likely that increased Δ^7-biosynthesis accounts for the ability of lathosterol to suppress *hsd-1* defects. By contrast, feeding of 7-dehydrocholesterol does not compensate for the impairment of Δ^4-signaling (Fig. 5A,B; Fig. 6A), which could result from a rate-limiting step in the trafficking/processing of 7-dehydrocholesterol into later steroid intermediates, as discussed later.

Steroidogenesis, cholesterol intracellular trafficking and Niemann-Pick type C disease

hsd-1 is an enhancer of *ncr-1*, suggesting that steroidogenesis and NCR-mediated cholesterol transport are interdependent. The following are three non-mutually exclusive models that interpret why the *hsd-1* mutant only displays dauer arrest when *ncr-1* is deleted. (1) When both NCR transporters are present, steroid signaling other than *hsd-1*-mediated Δ^4-signaling in XXX cells,

including Δ^7, may be sufficient to prevent dauer arrest; however, when NCR-1 is removed, Δ^7-signaling, for example, may be attenuated due to insufficient sterol substrates, and thus HSD-1 activity becomes crucial. (2) The broadly expressed NCR-1 may be involved in sterol absorption, in addition to intracellular trafficking; thus, a lower level of cholesterol absorption in the *ncr-1* mutant makes HSD-1 activity necessary in preventing dauer arrest. In mice and flies, the NPC1-related proteins NPC1L1 and dNPCb are known to be involved in dietary sterol absorption in the intestine (Altmann et al., 2004; Voght et al., 2007). In worms, although the route for initial sterol absorption remains unknown, the intestinal expression of NCR-1 would be consistent with a possible role in sterol absorption. (3) Perhaps 3β-hydroxyl steroids can prevent dauer arrest when produced in sufficient amounts, which may be permitted by the presence of both NCR transporters. This model is supported by findings showing that a 3β-hydroxyl steroid similar to dafachronic acids, 5-cholesten-3β-ol-(25S)-carboxylic acid, transactivates the DAF-12 receptor in vitro (Held et al., 2006). These models are not mutually exclusive.

NPC1 is thought to mobilize cholesterol out of endo/lysosomes and redistribute it to other cellular compartments through vesicular transport (Ko et al., 2001). Mutations in NPC1 are causal to 95% cases of the fatal neurodegenerative NPC disease (Carstea et al., 1997). NPC1-related transporters potentially are involved in the transport of other sterols/lipids as well. However, little is known about substrate specificity of these transporters in general. Our sterol-feeding studies suggest that intermediates that are either beyond the point of the HSD-1 reaction, such as 4-cholesten-3-one, or the ones that do not need HSD-1 for further processing, such as lathosterol, are independent of the NCR-1 and/or NCR-2 transporters for their trafficking amongst cellular compartments/organelles. Our data further suggest that the function of the NCR transporters is likely to be limited to trafficking common dietary sterols, or those used for storage in vivo. We demonstrated that, besides cholesterol, the NCR transporters are indispensable when worms are fed with either ergosterol or β-sitosterol, which may well be the main dietary sterols available to *C. elegans* in the wild. To control the rate of hormone production accurately, it would be appropriate if restrictive regulation were applied to intracellular trafficking of common dietary sterols, because the intake of these sterols would most probably fluctuate in quantity. We speculate that the NPC1 pathway may serve as one such regulatory 'hurdle'. This potential regulation also appears to apply to 7-dehydrocholesterol, which is known to be a major sterol component of worms (Chitwood, 1999) and thus may be a storage sterol.

The mechanisms resulting in NPC neurodegeneration remain unclear. Although NPC1 was thought to have only general cell biological roles, recent studies in animal NPC models suggest that steroidogenesis could be an important functional output of NPC1-related transporters. Emerging evidence from invertebrate models of NPC disease, including this study, suggests that NCR transporters have a conserved role in supplying sterol substrates for the biosynthesis of dauer-preventing hormones in worms (Li et al., 2004; Motola et al., 2006) and of molting hormones in flies (Fluegel et al., 2006; Huang et al., 2005).

Currently, there are no effective therapeutic treatments for NPC disease. In mice, it has been demonstrated that a combination of the neurosteroid allopregnanolone and a synthetic oxysterol can promote neuronal survival and mildly suppress the lethality of NPC1-deficient mice (Griffin et al., 2004; Langmade et al., 2006). Neurosteroids have thus become a possible direction for development of drugs to treat NPC disease (Mellon et al., 2008). However, the therapeutic effects of the reported treatment in the mouse model are quite mild. As defects in the NPC1 protein impact the production of multiple known neurosteroids (Mellon et al., 2008) and potentially others awaiting identification, an effective treatment may rely on the use of steroid intermediates that are trafficked independently of NPC1 and can still be processed into a diverse range of hormones. Our results showing effective bypassing of the NCR transporters in the worm suggests that a similar steroid intermediate approach may also work in mammals.

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