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The NHR-8 Nuclear Receptor Regulates Cholesterol and Bile Acid Homeostasis in *C. elegans*

Daniel B. Magner[1], **Joshua Wollam**[1], **Yidong Shen**[1], **Caroline Hoppe**[1], **Dongling Li**[1], **Christian Latza**[1], **Veerle Rottiers**[3], **Harald Hutter**[4], and **Adam Antebi**[1,2,3,*]

[1]Max Planck Institute for Biology of Ageing, Gleueler Str. 50a, D-50931 Cologne, Germany

[2]Cologne Excellence Cluster on Cellular Stress Responses in Aging Associated Diseases (CECAD), University of Cologne, D-50674 Cologne, Germany

[3]Department of Molecular and Cellular Biology, Huffington Center on Aging, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

[4]Simon Fraser University, Department of Biological Sciences, 8888 University Drive, Burnaby, BC Canada V5A 1S6

SUMMARY

Hormone-gated nuclear receptors (NRs) are conserved transcriptional regulators of metabolism, reproduction and homeostasis. Here we show that *C. elegans* NHR-8 NR, a homolog of vertebrate Liver-X and Vitamin-D receptors, regulates nematode cholesterol balance, fatty acid desaturation, apolipoprotein production, and bile acid metabolism. Loss of *nhr-8* results in a deficiency in bile acid-like steroids, called the dafachronic acids, which regulate the related DAF-12/NR, thus controlling entry into the long-lived dauer stage through cholesterol availability. Cholesterol supplementation rescues various *nhr-8* phenotypes, including developmental arrest, unsaturated fatty acid deficiency, reduced fertility, and shortened lifespan. Notably, *nhr-8* also interacts with *daf-16/FOXO* to regulate steady-state cholesterol levels, and is synthetically lethal in combination with insulin signaling mutants that promote unregulated growth. Our studies provide important insights into nuclear receptor control of cholesterol balance and metabolism, and their impact on development, reproduction, and aging in the context of larger endocrine networks.

INTRODUCTION

Cholesterol is essential for a diverse range of cellular processes, including hormone signaling, fat metabolism, and membrane structure and dynamics. Dysregulation of cholesterol and lipid homeostasis can have a major impact on development and disease (Kritchevsky and Kritchevsky, 1992; Magkos et al., 2009; Martins et al., 2006; Woollett, 2008). Cholesterol deficiency can result in blunted steroid hormone production, reduced serotonin levels, vitamin deficiencies, and increased mortality, whereas excess cholesterol excess is a risk factor for cardiovascular disease, diabetes, neurodegeneration, and

*Correspondence: AAntebi@age.mpg.de, Tel: +221-4788-9681.

SUPPLEMENTAL INFORMATON

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables.

inflammation (Cai et al., 2004; Iqbal and Hussain, 2009; Martins et al., 2006; Patel et al., 2007; Steegmans et al., 1996; Sturley et al., 2004; Woollett, 2008). Thus understanding cholesterol and lipid homeostasis is critical to illuminating aspects of human health and longevity.

In mammals, a broad transcriptional network controls cholesterol homeostasis (Desvergne et al., 2006; Ikonen, 2006). Several ligand-gated nuclear receptors (NRs) are key transcriptional regulators acting at the stages of cholesterol uptake, storage, transport, metabolism, and excretion (Magner and Antebi, 2008; Mangelsdorf et al., 1995; Wollam and Antebi, 2011). The mammalian liver-X receptors (LXRs) and the farnesoid-X receptor (FXR) are activated by their oxysterol and bile acid ligands, respectively, and regulate in tandem the major flux of dietary cholesterol through conversion of cholesterol to bile acids (Kalaany and Mangelsdorf, 2006; Ory, 2004). Furthermore, LXRs and FXR also regulate genes involved in fatty acid synthesis and desaturation, apolipoprotein genes, and sterol and bile acid transport, thus coordinating both cholesterol and lipid metabolism (Kalaany and Mangelsdorf, 2006).

Studies of cholesterol and lipid homeostasis in the nematode *C. elegans* have provided important insights into how the associated genes modulate health and longevity. In *C. elegans*, cholesterol and lipid homeostasis are regulated by several NRs, including DAF-12, NHR-49, NHR-80, and NHR-25, which function analogously to mammalian NRs. Investigations of these proteins have uncovered previously unknown roles for NRs, particularly with respect to longevity and development (Antebi et al., 2000; Brock et al., 2006; Goudeau et al., 2011; Mullaney et al., 2010; Van Gilst et al., 2005a; Van Gilst et al., 2005b). Among these receptors, DAF-12 is perhaps best understood. DAF-12 is a member of the LXR/FXR NR family, and like its vertebrate relatives can be activated by bile acid-like steroids, in this case called dafachronic acids (DAs). DAF-12 signaling regulates *C. elegans* metabolism, developmental timing, longevity, and the choice between reproductive development and arrest at the dauer diapause, a developmental stage characterized by exceptional stress resistance and longevity (Antebi et al., 2000; Bethke et al., 2009; Gerisch et al., 2007; Motola et al., 2006; Rottiers and Antebi, 2006; Shen et al., 2012; Yamawaki et al., 2010). The dauer decision is governed by a conserved endocrine signaling network coupling nutrient signals to growth regulation and reproductive development (Fielenbach and Antebi, 2008). Molecular genetic experiments in *C. elegans* suggest that in favorable environmental conditions, activation of insulin/IGF-I signaling (IIS), TGF-β, and cGMP pathways together positively regulate DA biosynthesis, thereby stimulating DAF-12 and promoting reproductive growth and normal lifespan. In unfavorable conditions, downregulation of these endocrine pathways results in decreased DA production; unliganded DAF-12 binds the corepressor DIN-1/SHARP to promote entry into the long-lived dauer stage (Fielenbach and Antebi, 2008). Studies of dauer formation and the DA/DAF-12 cascades have provided critical evidence that NR signaling regulates metazoan development and longevity (Gerisch et al., 2007; Hsin and Kenyon, 1999; Motola et al., 2006).

Despite the current knowledge of these endocrine networks, the integration of cholesterol and lipid metabolism within these circuits is poorly understood. Nematodes are unable to synthesize cholesterol and require it in the diet (Kurzchalia and Ward, 2003). As cholesterol is a necessary precursor for DA, the maintenance of cholesterol homeostasis is hypothesized to contribute significantly to DAF-12-mediated processes. Cholesterol deprivation can mimic several *daf-12*-induced phenotypes (Gerisch et al., 2001; Matyash et al., 2004). Nevertheless, the regulatory mechanisms underlying sterol homeostasis in the context of larger endocrine networks remains unclear. Moreover, the role of cholesterol in DA-independent processes is not well understood. We therefore sought to identify regulators of

cholesterol and bile acid homeostasis, with the aim of uncovering their effects on development, reproduction, and aging.

RESULTS

NHR-8 is a Homolog of Sterol-Sensing Nuclear Receptors

The dafachronic acids (DAs) play a critical role in regulating *C. elegans* developmental timing, dauer formation, and life span (Fielenbach and Antebi, 2008). Several hormone biosynthetic genes have been identified, but the nature of the DA production pathway and its regulation are not well understood. To identify genes controlling the regulatory circuits of DAF-12 ligand production, we carried out RNAi enhancer screens on *daf-36*/Rieske oxygenase null mutants, which have partially diminished DA production (Rottiers et al., 2006). We surveyed for enhancement of phenotypes indicating DA deficiency, including constitutive dauer formation (Daf-c) and gonadal migration defects (Mig) in which gonadal path-finding cells fail to turn on schedule. From such screens we identified a number of new hormone biosynthetic genes (Wollam et al., 2012), as well as *nhr-8*.

nhr-8 encodes a nuclear hormone receptor (NR), a class of hormone-gated transcription factors that promote gene expression when bound to their cognate ligands. NHR-8 is related to *C. elegans* DAF-12, *Drosophila* HR96, and the mammalian vitamin-D, constitutive-androstane, liver-X, farnesoid-X, and pregnane-X receptors (VDR, CAR, LXR, FXR, and PXR) (Figure 1A). These sterol-sensing NRs regulate cholesterol, bile acid, and fat metabolism, suggesting that NHR-8 could function similarly. NHR-8 and DAF-12 share significant homology in DNA- and ligand-binding domains (DBD; LBD), and have identical residues in the P-box, a motif in the first zinc finger that functions in DNA recognition (Figure 1C and 1D). Several nematode species, including parasitic *Brugia malayi*, harbor *nhr-8* orthologs, suggesting that its function is conserved. Consistent with a role in transcription, an NHR-8::GFP translational fusion protein under control of its endogenous promoter localized predominately in nuclei of the intestine, the major metabolic and endocrine organ of the worm (Figure 1B and 1E).

Loss of *nhr-8* Induces Phenotypes Consistent with DA Deficiency

To dissect the physiologic function of *nhr-8*, we obtained several deletion mutations and tested their roles in DA-dependent events. Two alleles remove the DBD (*hd117* and *tm1800*) and are predicted null alleles (Figure 1B), whereas another allele, *ok186*, disrupts only the LBD (Lindblom et al., 2001).

Whereas complete DA deficiency, as seen in *daf-9/CYP27A1* null mutants, provokes constitutive dauer formation at all growth temperatures (Gerisch and Antebi, 2004; Motola et al., 2006), mutants with partial DA deficiency, such as *daf-36* nulls, display phenotypes only if placed under stressors such as increased temperature and cholesterol depletion. Consistent with a partial DA deficiency, all three *nhr-8* mutants appeared normal at 20°C (data not shown), but had enhanced dauer formation at 27°C compared to wild-type (N2) animals. The two null alleles showed a higher penetrance than the LBD mutation when grown in normal dietary cholesterol (5 μg/ml) (Figure 2A and 2D). *nhr-8* null mutants also showed increased dauer formation at 25°C upon cholesterol deprivation (0 μg/ml) (Figure 2B). Similar to *daf-36*, *nhr-8* null mutants displayed gonadal Mig phenotypes upon cholesterol deprivation. However, the Mig phenotype was not observed in animals carrying the weaker *ok186* LBD mutation (Figure 2C and 2D), revealing that the LBD is only partially required for NHR-8 function. Mig and Daf-c phenotypes are caused by lesions in *nhr-8*, since they were seen in multiple alleles, and were rescued by an *nhr-8::gfp* transgene (Figure 1E).

If *nhr-8* mutation partly reduces DA production or transport, then combining *nhr-8* with mutations in other genes that perturb these processes should enhance the phenotypes. Indeed, *nhr-8* mutations increased the penetrance of Daf-c defects seen in null mutants for the Niemann-Pick homologs *ncr-1* and *ncr-2*, implicated in sterol transport under 0 μg/ml cholesterol conditions (Figure 2A and 2B) (Li et al., 2004). *nhr-8* mutation also variously enhanced phenotypes in animals with mutations affecting DA biosynthesis, including null mutations in the *daf-36*/Rieske oxygenase, *dhs-16*/SDR, *hsd-1*/3β-HSD, and hypomorphic mutations in *daf-9(k182)/CYP27A1* (Figures 2A–2C and S1A–S1C) at 25°C or when cholesterol was depleted from the diet (0 μg/ml), suggesting a role for *nhr-8* in cholesterol metabolism. Previous studies have shown that Mig and Daf-c phenotypes depend upon the repressive function of unliganded DAF-12 (Ludewig et al., 2004). Accordingly, *daf-12* null mutations suppress the Mig and Daf-c phenotypes of *nhr-8* mutants (Figures 2A–2C). Altogether, our data suggest that *nhr-8* affects sterol processing or transport, which ultimately affects DAF-12 activity.

Interestingly, we also found that *nhr-8* mutants displayed a novel cholesterol-sensitive lethal phenotype. Removing cholesterol from the growth media at high temperatures (0 μg/ml agar plates; 27°C), or from less rich growth media at normal temperatures (0 μg/ml, agarose plates; 20°C) resulted in arrest of *nhr-8* mutants at L1/L2 larval stages. This phenotype was significantly enhanced by *ncr-1* and *ncr-2* mutations, and was not suppressed by *daf-12* mutation or by DA supplementation (Figures 2F, 2G, and S1D). Thus, *nhr-8* plays a unique role in larval development that is influenced by the availability of dietary cholesterol, and can function in a *daf-12*-independent manner.

nhr-8 Phenotypes are Rescued by Sterol Supplementation

Because *nhr-8* mutant animals showed enhanced phenotypes in the absence of dietary cholesterol, we hypothesized that *nhr-8* has a broader role in cholesterol metabolism. We first tested whether *nhr-8* blocks a specific step in the conversion of cholesterol to DA. *C. elegans* produces multiple DAs including Δ^4- and Δ^7-DA, through branched synthetic pathways *via* a series of enzymatic steps. The first committed step in Δ^7-DA production is the conversion of cholesterol to 7-dehydrocholesterol by the Rieske-like oxygenase DAF-36 (Rottiers et al., 2006; Wollam et al., 2011). 7-dehydrocholesterol is thereafter modified stepwise to lathosterol by an unknown reductase, to lathosterone by DHS-16/3-hydrosteroid dehydrogenase (Wollam et al., 2012) and ultimately converted to Δ^7-DA by DAF-9/CYP-27A1 (Motola et al., 2006). Δ^4-DA is thought to be produced from 4-cholestene-3-one, an oxidation product of cholesterol.

To position *nhr-8* within these pathways, we performed a sterol feeding experiment, an assay used previously to pinpoint specific blocks in DA biosynthesis in *daf-36*, *dhs-16*, and *daf-9* mutants (Motola et al., 2006; Rottiers et al., 2006; Wollam et al., 2012). In principle, sterols working downstream of an *nhr-8* block should rescue dauer and larval arrest phenotypes, whereas those working upstream should not. Surprisingly, we found that supplementation with all sterols of the DA biosynthetic pathway, including cholesterol, 7-dehydrocholesterol, lathosterol, lathosterone, 4-cholesten-3-one, as well as DA itself, rescued *nhr-8* Daf-c phenotypes (Figure 2H). By contrast, lophenol, a 4-methyl sterol not implicated in the DA biosynthetic pathways, failed to rescue the *nhr-8* mutant Daf-c phenotype. These results suggest that *nhr-8* might not introduce a specific and complete block at a particular step of DA synthesis, but rather might generally limit the availability of DA and other sterol precursors, which can be rectified by cholesterol loading.

nhr-8 Interacts with the Dauer Endocrine Network

IIS and TGF-β signaling comprise the major endocrine pathways modulating DA production and dauer formation. The *daf-5*/*SNO*/*SKI*, *daf-16*/*FOXO*, and *daf-12*/*FXR* are dauer defective (Daf-d) loci that mediate the transcriptional outputs of TGF-β, IIS, and steroidal pathways, respectively. Previous epistasis experiments revealed that DA biosynthetic genes work downstream of *daf-5* and *daf-16*, but upstream of *daf-12* (Fielenbach and Antebi, 2008). To determine if *nhr-8* is positioned similarly, we made double mutants of *nhr-8* (Daf-c), with *daf-12*, *daf-5*, and *daf-16* (Daf-d), and measured dauer formation. As expected, *nhr-8;daf-12* double mutants failed to form dauers altogether, suggesting that *nhr-8* works upstream of *daf-12* (Figure 3A). *nhr-8(hd117);daf-5(e1386)* animals formed the same fraction of dauers as the *nhr-8(hd117)* mutation alone, suggesting that *nhr-8* works downstream of or parallel to *daf-5*. Surprisingly, *nhr-8;daf-16* double mutants failed to form dauers, a phenotype observed with two different *daf-16* deletion alleles (Figure 3A). A *daf-16(mgDf50)* null allele also suppressed the Mig phenotype of *nhr-8* mutants (Figure 3B). These results place *nhr-8* upstream of or in parallel to *daf-16*. This suggests an unexpected role for *nhr-8* that differs from known DA biosynthetic genes, which typically act downstream of *daf-16*/*FOXO*.

To further explore these observations, we probed the genetic interactions of *nhr-8* with other Daf-d genes in the IIS pathway. We examined *daf-18(e1375)*/*PTEN* null mutation, and *akt-1(mg144)* and *pdk-1(mg142)* gain-of-function mutations, which generally activate IIS and have phenotypes resembling *daf-16* null mutants (Ogg and Ruvkun, 1998; Paradis and Ruvkun, 1998). Unexpectedly, we were unable to recover double mutants of *nhr-8* with all three loci, revealing a synthetic lethal phenotype. Specifically, we observed problems with gametogenesis or embryonic lethality. Thus, *nhr-8* exhibits complex interactions with the IIS pathway, suggesting IIS and *nhr-8* outputs converge on a process essential for viability.

nhr-8 Modulates DAF-16/FOXO and DAF-12/NR Target Gene Expression

The epistasis experiments described above suggest that *nhr-8* outputs influence *daf-16* and *daf-12* activity. If so, then mutations in *nhr-8* might affect the expression of DAF-16 and DAF-12 target genes. Although DAF-16 transcripts were unchanged, we observed that transcript levels of two DAF-16 target genes, *sod-3* (superoxide dismutase) and *dod-3* (downstream of *daf-16*), were elevated in *nhr-8* mutants upon cholesterol deprivation (Figure 3C and 3D), suggesting stimulation of *daf-16* activity. Expression of a *sod-3::gfp* reporter was similarly elevated across all developmental stages of *nhr-8(hd117)* mutants under low cholesterol (Figure 3E and 3F, Figure S2A and S2B). However, elevated *sod-3* and *dod-3* mRNA levels were not completely suppressed by *daf-16* mutation: although *dod-3* mRNA levels were decreased in the absence of *daf-16*, *nhr-8* loss still resulted in a small but significant increase of *dod-3* mRNA in *nhr-8;daf-16* double mutants. Moreover, *daf-16* loss had no effect on *sod-3* mRNA levels in an *nhr-8* mutant background (Figure 3D). This suggests that NHR-8 may antagonize expression of these genes independent from DAF-16's role in their activation.

We next asked whether *nhr-8* affects DAF-12 activity by examining expression of the microRNA *mir-241*, a direct DAF-12 target gene that requires DAF-12 activity for full expression (Bethke et al., 2009). As expected, *mir-241* expression was decreased in *daf-12* and *daf-36* mutant controls relative to wild-type. Likewise, *mir-241* expression was decreased in *nhr-8* mutants, and restored to wild-type levels by DA supplementation (Figure 3G). Mutation of *nhr-8* had no effect on *mir-1*, a constitutively expressed microRNA (Figure S2C). These data further support the idea that *nhr-8* promotes DAF-12/DA signaling.

nhr-8 Regulates Cholesterol Homeostasis

To determine whether *nhr-8* loss influences sterol and bile acid levels, we quantified intermediates in the DA biosynthetic pathways using gas chromatography/tandem mass-spectrometry (GC-MS-MS). In N2 wild-type, cholesterol levels remained constant whether animals were grown on low (0 μg/ml) or normal (5 μg/ml) cholesterol media, indicating that free cholesterol is steadily maintained in wild-type animals (Figure 4B). By contrast, *nhr-8* mutant animals showed a 1.7-fold decrease in endogenous cholesterol when grown in the absence of dietary cholesterol suggesting that NHR-8 maintains endogenous cholesterol levels in response to changes in dietary cholesterol. Interestingly, we also found that *daf-16* mutants had increased cholesterol levels that were further enhanced by *nhr-8* mutation, suggesting a separate role for *daf-16* in regulating cholesterol and complex interactions with *nhr-8*.

nhr-8 is Required for Sterol Transport into Eggs

The reduced cholesterol in *nhr-8* mutants suggested that uptake or transport of cholesterol could be compromised. We fed animals 25-NBD cholesterol, a fluorescent analogue of cholesterol, from hatching, and looked for changes in fluorescence in various tissues during development. Surprisingly, no difference in fluorescence in the intestinal cells of N2 compared to *nhr-8* mutant animals was observed, suggesting that uptake of this analogue is not visibly altered. We did, however, find changes in the germline and specifically the eggs (Figures 4E and 4F). When grown on normal (5 μg/ml) cholesterol media supplemented with 25-NBD cholesterol (1 μg/ml), eggs from both N2 and *nhr-8* mutants had comparable fluorescence (Figure 4E). When grown on low cholesterol (0 μg/ml) media supplemented with 25-NBD cholesterol, eggs from N2 animals showed a strong fluorescent signal (this signal was presumably stronger than under cholesterol replete conditions due to a lack of competition with normal cholesterol). Remarkably, eggs from *nhr-8* mutants grown under low cholesterol conditions had only half the fluorescence as eggs from N2 animals. These data suggest that *nhr-8* affects distribution and transport of cholesterol, more specifically to the germline, and that progeny of *nhr-8* mutants start life with a deficit of endogenous cholesterol.

nhr-8 is Required for Production of Sterol Metabolites and Dafachronic Acid

The first step in Δ^7-DA synthesis is conversion of cholesterol to 7-dehydrocholesterol by the DAF-36/Rieske oxygenase (Figure 4A). In *nhr-8* mutants, we found that 7-dehydrocholesterol levels were dramatically reduced by 73% compared to N2 wild-type when grown in the absence of dietary cholesterol (0 μg/ml) (Figure 4C). Likewise, Δ^7-DA levels were decreased by 78% (Figure 4D). We conclude that *nhr-8* normally facilitates production of 7-dehydrocholesterol and DA. Because *daf-16* mutation suppressed the *nhr-8* mutants' Daf-c phenotypes (Figure 3A), we asked whether *daf-16* mutation affects sterol levels. Loss of *daf-16* alone or in combination with *nhr-8* mutation resulted in a greater than seven-fold increase in the amount of 7-dehydrocholesterol compared to N2 wild-type or *nhr-8* mutant respectively (Figure 4C). Despite this, *daf-16* loss did not restore Δ^7-DA levels in an *nhr-8* mutant background (Figure 4D). Thus, *daf-16* might affect dauer formation through elevation of cholesterol and 7-dehydrocholesterol by promoting production of novel DA metabolites or by functioning in a parallel pathway.

nhr-8 Regulates *daf-36*/Rieske Oxygenase Expression

Given the dramatic decrease of 7-dehydrocholesterol levels in *nhr-8* mutants, we hypothesized that *nhr-8* regulates DAF-36/Rieske oxygenase activity. We measured *daf-36* transcript levels through development by RT-PCR and found significantly reduced *daf-36* mRNA in *nhr-8* mutants compared N2 controls when animals were grown in low cholesterol

conditions (Figures 5A and S3). Interestingly, loss of *daf-16* restored *daf-36* expression levels in *nhr-8* mutants (Figure 5A). Thus, suppression of *nhr-8* induced dauer formation by *daf-16* mutation could result from restored *daf-36* levels. We next measured expression of a DAF-36: :GFP fusion reporter construct (Rottiers et al., 2006) at all stages of development and found that DAF-36: :GFP protein levels were dramatically reduced across all stages (*e.g.*, 8-fold at L3; 13-fold at adult) under low cholesterol conditions in *nhr-8* mutants (Figure 5B and 5C). A similar reduction was also seen with an independent *daf-36: :gfp* reporter construct. Notably, NHR-8 and DAF-36 reside in the intestine, suggesting that *nhr-8* might regulate *daf-36* within this tissue. Moreover, *daf-36: :gfp* overexpression partially suppressed the Daf-c phenotype of *nhr-8* mutants (Figure 5D). We conclude that *nhr-8* is required for normal *daf-36* expression under low cholesterol conditions, and that *daf-36* levels become limiting for dauer formation. Altogether these data suggest that NHR-8 regulates cholesterol and bile acid homeostasis, and promotes the first enzymatic step in the conversion of cholesterol to DA.

nhr-8 Expression Profiles

To obtain an unbiased view of *nhr-8* dependent changes in gene expression, we used microarray analysis to compare mRNA expression of *nhr-8* mutant and WT animals grown in cholesterol deficient conditions at 25°C, harvesting at the L3 stage. We identified 333-up and 232-down regulated genes (≥1.5-fold difference from WT) (Table S1). Differentially expressed genes were enriched in several biological process gene ontology (GO) categories, including fatty acid metabolism, oxidation-reduction, proteolysis, defense response, and determinants of lifespan, which are comprised of genes involved in fatty acid desaturation, lipid transport, lipolysis, sphingolipid metabolism, and proteolysis (Figure S4 and Table S2).

nhr-8 Mutants have Reduced Fatty Acid Desaturase Expression

To better understand the role of *nhr-8* in fatty acid metabolism and desaturation, the most highly enriched GO category with differentially expressed genes identified from microarray analysis, we measured the mRNA levels of several fatty acid metabolism genes by RT-PCR (Figures 6A, 6B, and S5A). We found that the Fatty Acyl CoA desaturases, *fat-5* and *fat-7*, were greatly reduced in *nhr-8* mutants, independent of dietary cholesterol (Figure 6A). These genes encode Δ^9-deasturases, which add a double bond to saturated fatty acids (SFAs) to generate monounsaturated fatty acids (MUFAs). Specifically, FAT-5 converts palmitic acid (C16:0) to palmitoleic acid (C16:1n7), and FAT-7 converts stearic acid (C18:0) to oleic acid (C18:1n9) (Figure 6F). Levels of *fat-6*, also implicated in the conversion of C18:0 to C18:1n9, were unchanged in *nhr-8* mutants as were transcript levels of *nhr-49*, *nhr-80*, *mdt-15*, and *sbp-1*, which encode transcription factors regulating *fat-5* and *fat-7* expression (Figure S5A). These data define *nhr-8* as a new transcription factor regulating Δ^9-deasturase gene expression. We also found that in the absence of dietary cholesterol, *nhr-8* mutants had decreased transcript levels of *elo-1*, a fatty acid elongase, and *fat-2*, a Δ^{12}-deasturase required for the conversion of MUFAs to polyunsaturated fatty acids (PUFAs) (Figure 6B). Specifically, *fat-2* converts oleic acid to linoleic acid (C18:2n6) (Figure 6F). Therefore *nhr-8* generally regulates genes required for elongation and desaturation of fatty acids.

We next asked whether these expression changes are reflected in fatty acid composition. As predicted, under low-cholesterol conditions, *nhr-8* animals had increased SFAs, and decreased MUFAs and PUFAs as measured by gas chromatography (Figure 6C and 6D). These phenotypes were cholesterol dependent, as no changes were seen upon cholesterol provision (5 μg/ml). In particular, levels of both C16:0 and C18:0 SFAs doubled in *nhr-8* mutants relative to N2 wild-type, with an overall 2.3-fold increase of SFAs. MUFA levels were correspondingly decreased 2.3-fold overall (Figure 6D), though oleic acid levels were maintained despite decreased *fat-7* expression. We speculate that this might be due to a

compensatory mechanism. Finally, all PUFAs were decreased in *nhr-8* mutants relative to wild-type in low cholesterol conditions, resulting in an overall 6.6-fold reduction. These alterations in fatty acid content were delayed in *daf-12;nhr-8* double mutants at L3. By adult both *nhr-8* and *daf-12;nhr-8* double mutants had comparably dramatic changes in SFA and PUFA levels indicating that the effect of *nhr-8* on fat composition is largely independent of *daf-12* (Figures S5B and S5C). Thus, *nhr-8* is required for desaturation and maintenance of normal fatty acid composition in response to reduced dietary cholesterol.

We also investigated whether triacylglyceride (TAG) levels were changed in *nhr-8* mutants. Although TAGs tended to increase slightly with increased cholesterol, *nhr-8* mutants did not show a significant difference in TAG levels compared to wild-type (Figure 6E).

nhr-8 Mutants have Reduced Fertility and Longevity

In mammals, chylomicrons and low-density lipoproteins (LDLs) transport sterols and lipids between tissues. Their formation and proper trafficking requires apolipoproteins (*e.g.*, ApoE), which act as both structural components and ligands for cell-surface receptors, such as the LDL receptor. The two major apolipoproteins in *C. elegans* are vitellogenins encoded by *vit-1* and *vit-2*, and were among the most significantly enriched down-regulated genes as determined by microarray analysis. By RT-PCR, *vit-1* and *vit-2* expression was reduced by ~5-fold in *nhr-8* mutants (Figure 7A). Similarly, a *vit-2::gfp* reporter showed decreased expression throughout development (*e.g.*, 7.8-fold at L3; 3.8-fold at adult) in *nhr*-8 mutants, which was restored upon cholesterol provision (Figure 7B). Additionally, VIT-2::GFP fluorescence in the eggs of *nhr-8* mutants was only 20% of wild-type when fed a low cholesterol diet, correlating well with the observed decrease of 25-NBD cholesterol transport to the eggs (Figures S6A and 4F).

Since cholesterol depletion and vitellogenin deficiencies reduce fertility in wild-type animals (Shim et al., 2002), we examined the effect of *nhr-8* loss on brood size. In the absence of dietary cholesterol (0 μg/ml), both N2 wild-type and *nhr-8* animals had reduced fertility, with *nhr-8* mutants showing a 50% reduction in progeny production compared to N2 wild-type (Figure 7C). Supplementation with cholesterol (5 μg/ml) increased fertility in both backgrounds, with *nhr-8* animals showing greater improvement. High amounts of dietary cholesterol (25 μg/ml) significantly improved the fertility of *nhr-8* animals to 80% of wild-type.

We then asked whether *nhr-8* could influence lifespan. Consistent with previous findings (Lee, Paik 2009), we found that N2 animals grown in the absence of dietary cholesterol had a 34% decrease in median lifespan compared to cholesterol replete conditions. *nhr-8* mutants had a 28–35% decrease in mean lifespan compared to N2 at all cholesterol concentrations (0, 5, and 25 μg/ml). Thus, *nhr-8* mutation shortens mean lifespan independent of available dietary cholesterol. However, increasing the amount of dietary cholesterol rescued the maximum lifespan of *nhr-8* mutants. Whereas *nhr-8* mutants grown in the absence of cholesterol (0 μg/ml) showed a 26% decrease in maximum lifespan, animals grown in high dietary cholesterol (25 μg/ml) restored maximal lifespan back to wild-type (Figures 7D and 7E). Conditions that induce dauer formation often correlate with increased longevity, Because *nhr-8* loss stimulated dauer formation in the absence of dietary cholesterol at 25°C, we also asked whether lifespan was increased under these conditions. On the contrary, *nhr-8* mutants were also short lived when grown in the absence of dietary cholesterol at 25°C (Figure S6B). Additionally, we found that the life-shortening effect of *daf-12* and *nhr-8* mutations were additive, suggesting independent effects on lifespan (Figure S6C).

DISCUSSION

Cholesterol and its metabolites play critical roles in animal health and disease. Here we show that the *C. elegans* nuclear receptor NHR-8, a homolog of the mammalian LXR and FXR nuclear receptors, plays an important role in cholesterol and bile acid steroid homeostasis, fatty acid metabolism, and displays novel interactions with components of insulin/IGF signaling, which affect development, reproduction, and lifespan.

Several lines of evidence demonstrate that NHR-8 regulates production of the bile acid-like dafachronic acids (DA). First, mutants display phenotypes typical of DA deficiency, including gonadal outgrowth defects in low-cholesterol conditions and constitutive entry into the dauer stage. Accordingly, mutants have decreased levels of DA and its precursors, including the *daf-36* product 7-dehydrocholesterol. Expression of *daf-36*/Rieske oxygenase is correspondingly reduced under low-cholesterol conditions. *nhr-8* mutants also show decreased expression of *mir-241*, a *let-7*-related microRNA and direct target of DAF-12. Many *nhr-8* phenotypes are rescued by the addition of dietary cholesterol, suggesting that *nhr-8* impacts cholesterol availability, transport, and/or metabolism.

nhr-8 mutants also display unique phenotypes that distinguish it from other DA-hormone biosynthetic mutants. Most strikingly, *nhr-8* mutants have a different pattern of genetic epistasis with respect to the *daf-16*/FOXO transcription factor. Whereas most hormone biosynthetic genes act downstream of *daf-16* with regards to dauer formation and gonadal migration, *nhr-8* acts upstream or in parallel to *daf-16*. In particular, mutation of *daf-16*/FOXO suppresses *nhr-8* Daf-c phenotypes, suggesting that *nhr-8* normally promotes insulin signaling or inhibits *daf-16*/FOXO activity. The DAF-16/FOXO target genes, *sod-3* and *dod-3*, are upregulated in *nhr-8* mutants grown under low-cholesterol conditions. Surprisingly, this induction is largely independent of *daf-16*, revealing that *nhr-8* normally represses their expression or modulates transcription factor(s) other than *daf-16* required for their expression. Alternatively, *daf-16* may suppress *nhr-8* mutant phenotypes by elevating cholesterol and 7-dehydrocholesterol levels. The elevation of sterols in *daf-16* null mutants may indicate a broader role of FOXO in regulating cholesterol metabolism in metazoans. *nhr-8* mutants also exhibit striking synthetic lethality with several mutations that elevate IIS, including gain-of-function mutations in *akt-1* and *pdk-1*, as well as loss-of-function mutations in *daf-18*/*PTEN*. These findings indicate that NHR-8 and IIS converge on a critical physiologic process, potentially related to growth, metabolism, or cholesterol availability. Future work should elucidate the nature of this interaction.

nhr-8 mutants also display cholesterol-dependent but DA-independent phenotypes. Animals arrest during larval development under low-cholesterol conditions at elevated temperatures or on agarose plates. This phenotype is rescued by cholesterol supplementation but not by DA, suggesting possible cholesterol-dependent roles in the regulation of growth, molting, or fertility. Loss of *nhr-8* results in misregulation of endogenous cholesterol levels, resulting in phenotypes that can be compensated for with exogenous substrate. *nhr-8* mutants have phenotypes, such as reduced brood size and life span, which are ameliorated by further cholesterol supplementation. These observations suggest that NHR-8 has a key role in maintaining cholesterol homeostasis, presumably regulating uptake, excretion, or partitioning of cholesterol. Presumably the ability to rescue various phenotypes with cholesterol reflects bulk cholesterol loading or transport mechanisms that remain intact in *nhr-8* mutants. These mechanisms appear to be somewhat independent of Niemann-Pick homologs since *nhr-8;ncr-1;ncr-2* triple mutant phenotypes are also rescued by cholesterol excess. A role for *nhr-8* in partitioning of cholesterol is supported by the observed reduction of 25-NBD cholesterol and VIT-2: :GFP in the eggs of *nhr-8* mutants compared to wild-type animals grown under low-cholesterol conditions. *nhr-8* additionally regulates vitellogenins,

which are implicated in transport of cholesterol and lipids. Apparently, NHR-8 may bear similar responsibilities in regulating aspects of cholesterol, bile acid, and fatty acid metabolism as LXR in mammals and HR96 in fruit flies (Horner et al., 2009; Kalaany and Mangelsdorf, 2006).

Previous work on *nhr-8* suggests a role in xenobiotic metabolism with an LBD-deficient mutant showing sensitivity to chloroquine and colchicine (Lindblom et al., 2001). In this view, *nhr-8* may function like PXR, the vertebrate xenobiotic receptor. Unlike PXR, we could not detect a function for these xenobiotics in transactivating the NHR-8 receptor in human cell culture (D.B. Magner, A. Antebi, unpublished). *nhr-8* mutant toxin sensitivity could be secondary to another process. Given the role of *nhr-8* in cholesterol metabolism, and its homology to other sterol-sensing NRs, we speculate that its ligand(s) are sterol derivatives. LXR binds to oxysterols, including 24(S),25-epoxycholesterol and 22(R)-, 24(S)-, and 27-hydroxycholesterol, but these molecules did not activate NHR-8 in cell culture. The related *Drosophila* HR96 ortholog binds cholesterol (Horner et al., 2009), but this molecule also does not appear to directly regulate NHR-8 transcriptional activity (D.B. Magner, A. Antebi, unpublished). A future challenge will be to identify ligands and direct target genes of NHR-8 and to further elucidate the transcriptional networks for cholesterol, lipid, and bile acid homeostasis.

Invertebrate models of NR signaling have yielded important insights into functions as diverse as control of stem cell biology, development, reproduction, metabolism, and longevity. In *C. elegans*, the FXR homolog DAF-12 is a key regulator of developmental timing circuits, dauer formation, and longevity induced by loss of germline stem cells. Both NHR-80, an HNF4-like homolog, and NHR-49, a PPAR-α homolog, regulate the *SCD-1*-like Δ^9-desaturases, which synthesize MUFAs (Brock et al., 2006; Van Gilst et al., 2005a), and also influence life span. NHR-80 is required for longevity induced in germlineless animals, and NHR-49 is required for long-term survival under starvation in the so-called adult reproductive diapause (Angelo and Van Gilst, 2009; Goudeau et al., 2011). Our findings here show that NHR-8, like these other *C. elegans* NRs, is also required to achieve maximal lifespan. *nhr-8* mutants are short lived, but supplementation with cholesterol results in suppression of late-life mortality. The basis of this mortality is not completely understood, but speculatively arises from metabolic dyshomeostasis, lipotoxicity, or cholesterol misregulation. Several regulatory targets of *nhr-8*, including the steroyl-CoA fatty acid desaturases, have been implicated in lifespan regulation (Goudeau et al., 2011; Van Gilst et al., 2005a). Evidently, distinct metabolic states can cause morbidity, or enhance health and lifespan. Further dissecting NR regulation of cholesterol and lipid homeostasis in the context of larger endocrine networks should reveal how these processes alter disease states, health, and longevity.

EXPERIMENTAL PROCEDURES

C. elegans Growth Conditions

Worms were grown on NGM agar plates, supplemented with chromatography grade cholesterol (>99% pure, Sigma C8667) at 5 μg/ml (normal cholesterol) or 25 μg/ml (high cholesterol), and seeded with *Escherichia coli* bacteria OP50 (Brenner, 1974). Cholesterol was omitted under 0 μg/ml (low cholesterol) growth conditions.

Construction of *gfp* Tagged *nhr-8*

Primers 5’-gcggccgcaccaagtgcaggattacgatga-3’ and 5’-ggtaccatcgatggagaagacgaagg-3’ were used to amplify a 3123 bp promoter region and cloned into L3781 (Fire Vector Kit, 1997) between *Kpn*I and *Not*I sites. Primers 5’-gctagcgacgagtctcgaaggtctgc-3’ and 5’-

actagtctccatctttctgcccttga-3' were used to amplify a 3095-bp fragment containing the *nhr-8* coding sequence and cloned between *Spe*I and *Nhe*I sites.

qRT-PCR

Worms were synchronized by egg laying and collected in TRIzol (Invitrogen) at the L3 stage. mRNA or microRNA was prepared using an RNeasy or miRNeasy Mini kit (QIAGEN), respectively. cDNA was generated with iScript (Bio-Rad). microRNA was reversed transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems (ABI)). qRT-PCR was performed with Power SYBR® Green (ABI) on a 7900HT Fast Real-Time PCR System (ABI). *ama-1* or snoRNA U18 were used as internal controls for mRNA or microRNA, respectively. Primer sequences are listed in Table S3.

GFP Expression Profiling

For each analyzed strain, 25 to 35 day 1-adult transgenic animals were placed on 10 cm plates seeded with *E. coli* strain OP50 and left to proliferate for 3 days. The resulting mixed-stage population was washed from the plate, flow sorted, and GFP intensity was analyzed using a COPAS Biosort (Union Biometrica). Chronograms were generated using in-house software utilizing a described method (Dupuy et al., 2007).

Lifespan Analysis

Lifespan assays were performed as described previously. Exploded and egg-laying defective animals were censored from analysis (Gerisch et al., 2001).

Microarray and Expression Analysis

Seven independent synchronized populations of N2 and *nhr-8(hd117)* animals were grown from eggs on low cholesterol plates at 25°C until mid-L3 larval stage and collected for microarray RNA expression analysis. RNA levels were measured using Agilent *C. elegans* 44K arrays (WormBase Release W188) and processed at the NIH Neuroscience Microarray Consortium (4 sets) or the Baylor College of Medicine Microarray Core facility (3 sets). Expression data was read in the statistical programming environment R (Team, 2012) and processed for background correction, quantile normalization and summarized for genes that differed in expression ≥1.5-fold (N2 vs *nhr-8*) and a p-value ≤0.01 using the Limma package (Gentleman et al., 2005). All microarray gene expression data reported in this study are available from the NCBI Gene Expression Omnibus (GEO), accession number GSE48675.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- *nhr-8* loss results in misregulated cholesterol, bile acid, and fat metabolism
- Production of dafachronic acid, a ligand for DAF-12, is reduced in *nhr-8* mutants
- *nhr-8* interacts with *daf-16/FOXO* to regulate cholesterol balance
- Cholesterol feeding rescues defects of development, reproduction, and lifespan

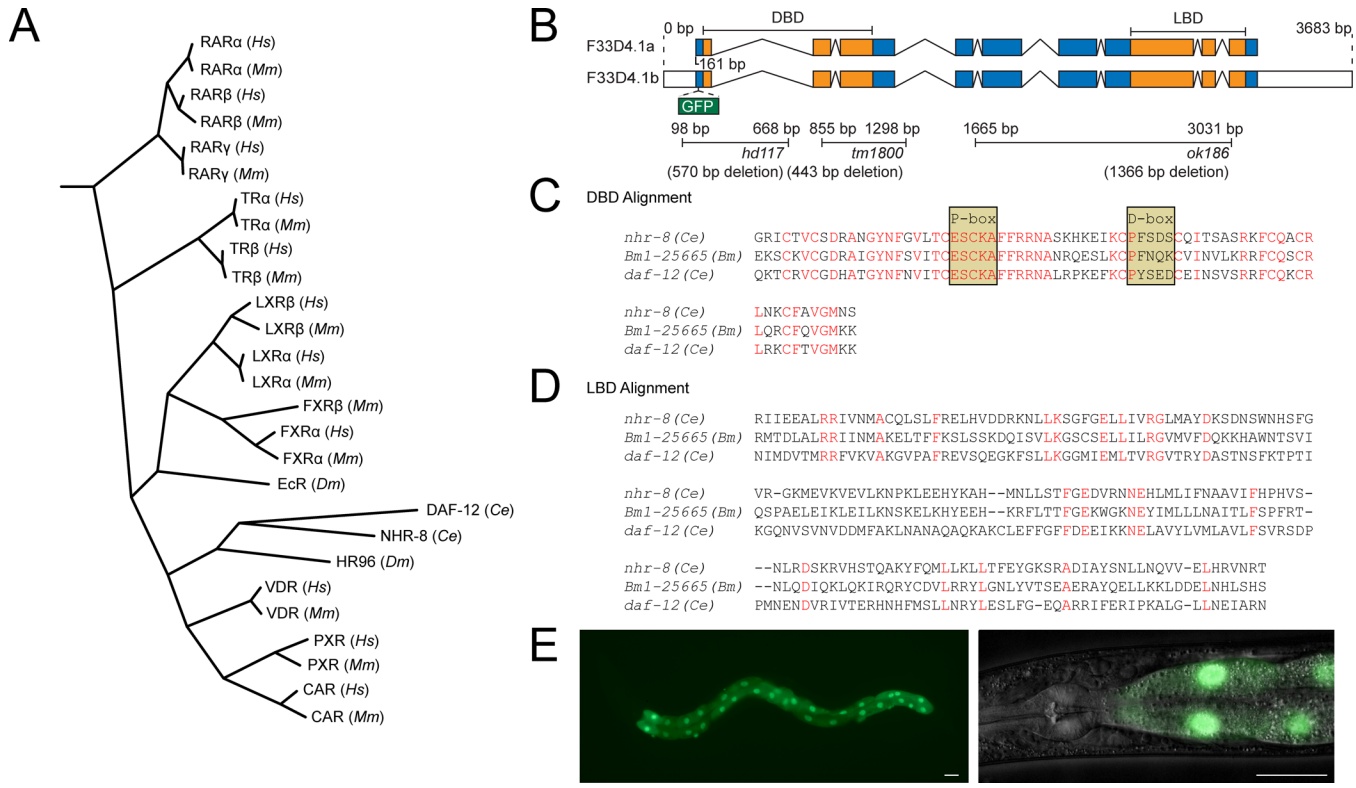

Figure 1. NHR-8 Structure and Expression

(A) Phylogenetic relationship of NHR-8 to other mammalian, *Drosophila*, and *C. elegans* nuclear receptors based on amino acid sequence of the entire proteins as determined by maximum likelihood from aligned sequences. **(B)** Location of the DNA-binding domain (DBD), ligand-binding domain (LBD), and deletion coordinates of alleles in both transcripts of *nhr-8* (F33D4.1a and F33D4.1b) **(C and D)** Amino acid sequence alignment of the DBDs and LBDs of *C. elegans* NHR-8 and DAF-12 and the NHR-8 homolog from the pathogenic nematode *Brugia malayi*. **(E)** NHR-8 fused to GFP is expressed in the intestinal cells, and localized primarily in the nucleus. Animals shown are at the L3 larval stage. Abbreviations: *Caenorhabditis elegans* (*Ce*), *Drosophila melanogaster* (*Dm*), *Homo sapiens* (*Hs*), *Mus musculus* (*Mm*), *Brugia malayi* (*Bm*). Scale bar = 20 μm.

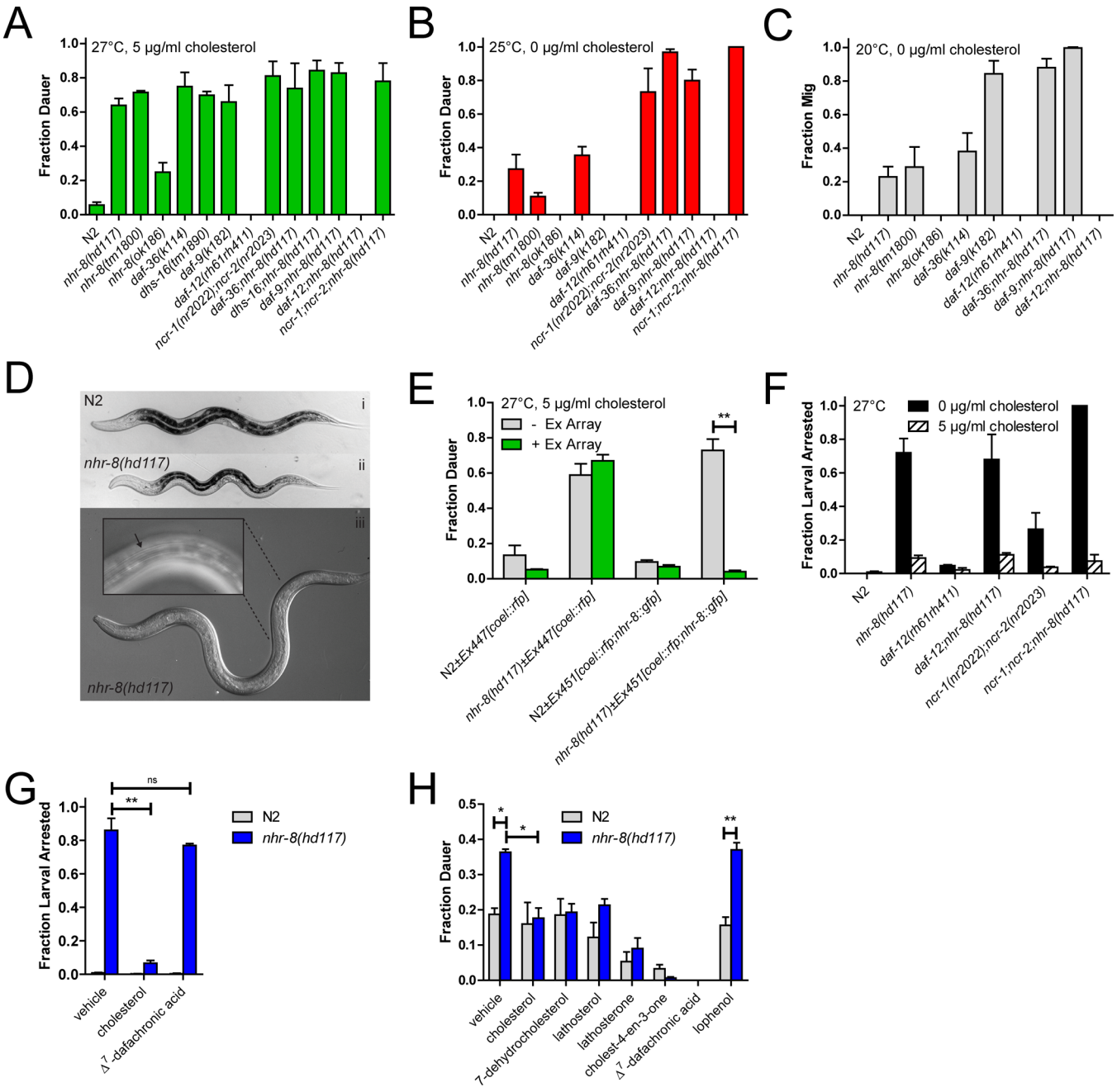

Figure 2. *nhr-8* Animals Display Phenotypes of Dafachronic Acid Deficiency

(A and B) Animals were grown from eggs at 25°C or 27°C on plates containing either 0 µg/ml or 5 µg/ml of pure cholesterol. The fractions of animals that formed dauer were scored. **(C)** Fraction of animals with gonad migration defects. **(D)** Representative pictures of (i) wild-type (N2) animals with normal gonad migration, (ii) *nhr-8(hd117)* animals with a gonad migration defect (Mig), and (iii) an *nhr-8(hd117)* dauer (inset shows dauer alae). **(E)** A complementing extrachromosomal *nhr-8::gfp* expression construct (Figure 1B, E) is sufficient to rescue Daf-c phenotypes of *nhr-8(hd117)* animals. **(F)** *nhr-8* animals display early L1/L2 larval arrest upon cholesterol deprivation at 27°C, independent of *daf-12*. Animals were grown from eggs at 27°C on plates containing either 0 µg/ml or 5 µg/ml of pure cholesterol. **(G)** L1/L2 larval arrested *nhr-8* animals are rescued by addition of dietary

cholesterol, but not DA. Animals grown at 27°C on plates supplemented with 250 nM of the indicated compound (~1 μg of each compound added to the surface of plate). **(H)** Dauer rescue of *nhr-8* animals by excess cholesterol, sterols, and DA. Animals grown at 27°C on plates containing 5 μg/ml cholesterol plus 250 nM of each compound (~1 μg of each compound added to the surface of the plate). *$p<0.05$, **$p<0.01$. All values from $n\geq3$ independent experiments show as mean ± SEM. See also Figure S1.

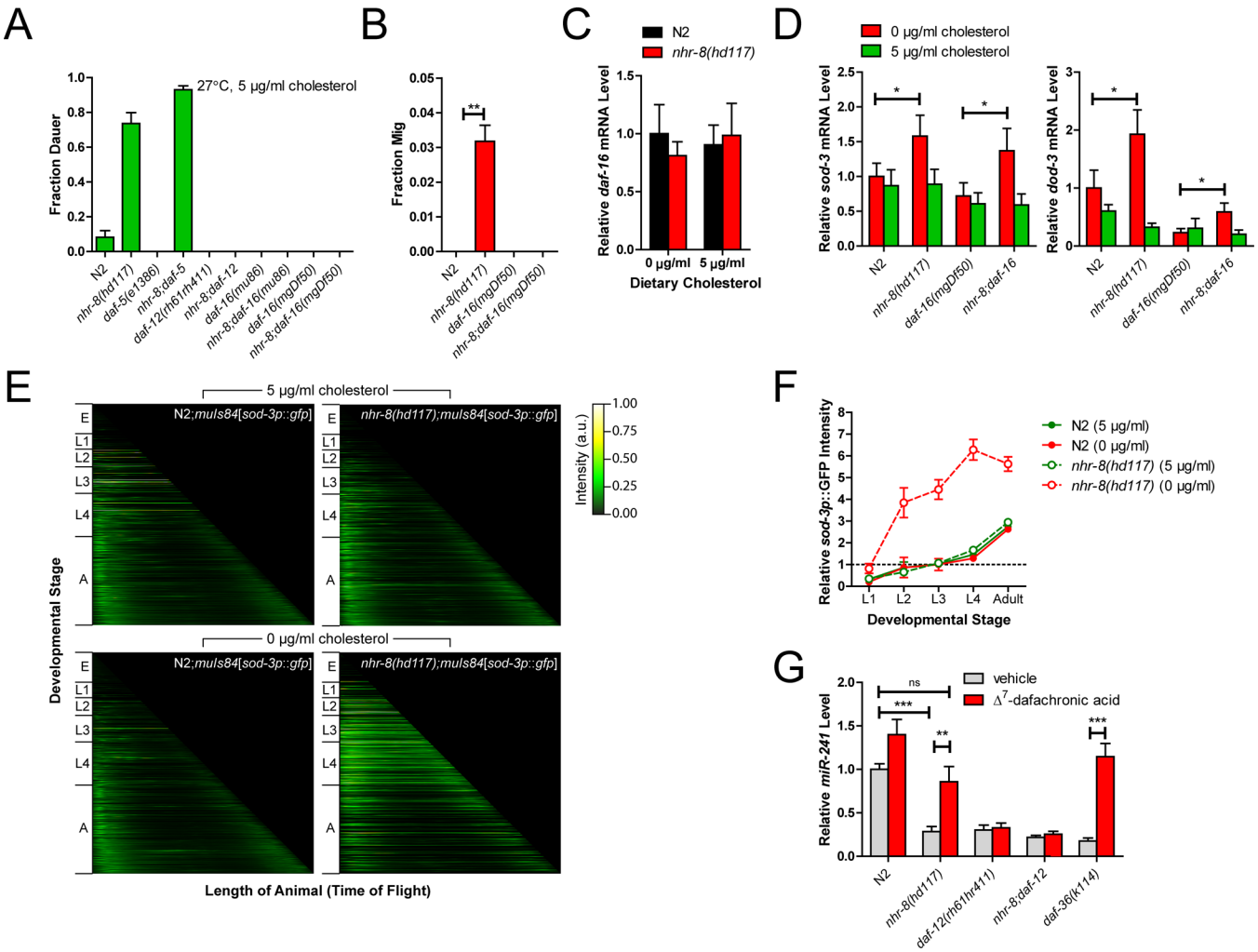

Figure 3. Genetic Epistasis Places *nhr-8* Upstream of *daf-12*/NR and *daf-16*/FOXO

(A) Dauer formation in *nhr-8* mutants is suppressed by mutations of *daf-12* and two alleles of *daf-16*/FOXO, but not *daf-5*/SNO. **(B)** Gonad migration defects (Mig) of *nhr-8* animals are suppressed by a *daf-16* null mutation. **(C)** *daf-16* expression is not changed in *nhr-8(hd117)* mutants. **(D)** In the absence of dietary cholesterol, *sod-3* and *dod-3* are upregulated in *nhr-8* mutants. **(E)** Chronogram representation of the spaciotemporal expression of *sod-3P::gfp* from egg to adult of animals grown at 25°C. *sod-3P::gfp* is upregulated in *nhr-8* mutants in the absence of dietary cholesterol across all developmental stages. Shown are averaged profiles of identical sized animals and oriented left to right as head to tail. GFP intensities and body length (measured as time of flight) were determined using a COPAS Biosorter. Developmental stages are indicated on the *y*-axis and verified by microscopy. The color scale indicates the expression level of a given position on the chronogram relative to all chronograms. **(F)** Quantification of GFP intensity from chronograms in (E). **(G)** DAF-12 microRNA target, *miR-241*, is down-regulated in *nhr-8* animals. Expression is rescued by supplementation with the DAF-12 ligand Δ^7-DA (100 nM). *miR-241* expression also require *daf-12* and *daf-36*. *p<0.05, **p<0.01, ***p<0.001, ns=not significant. Mean±SEM. See also Figure S2.

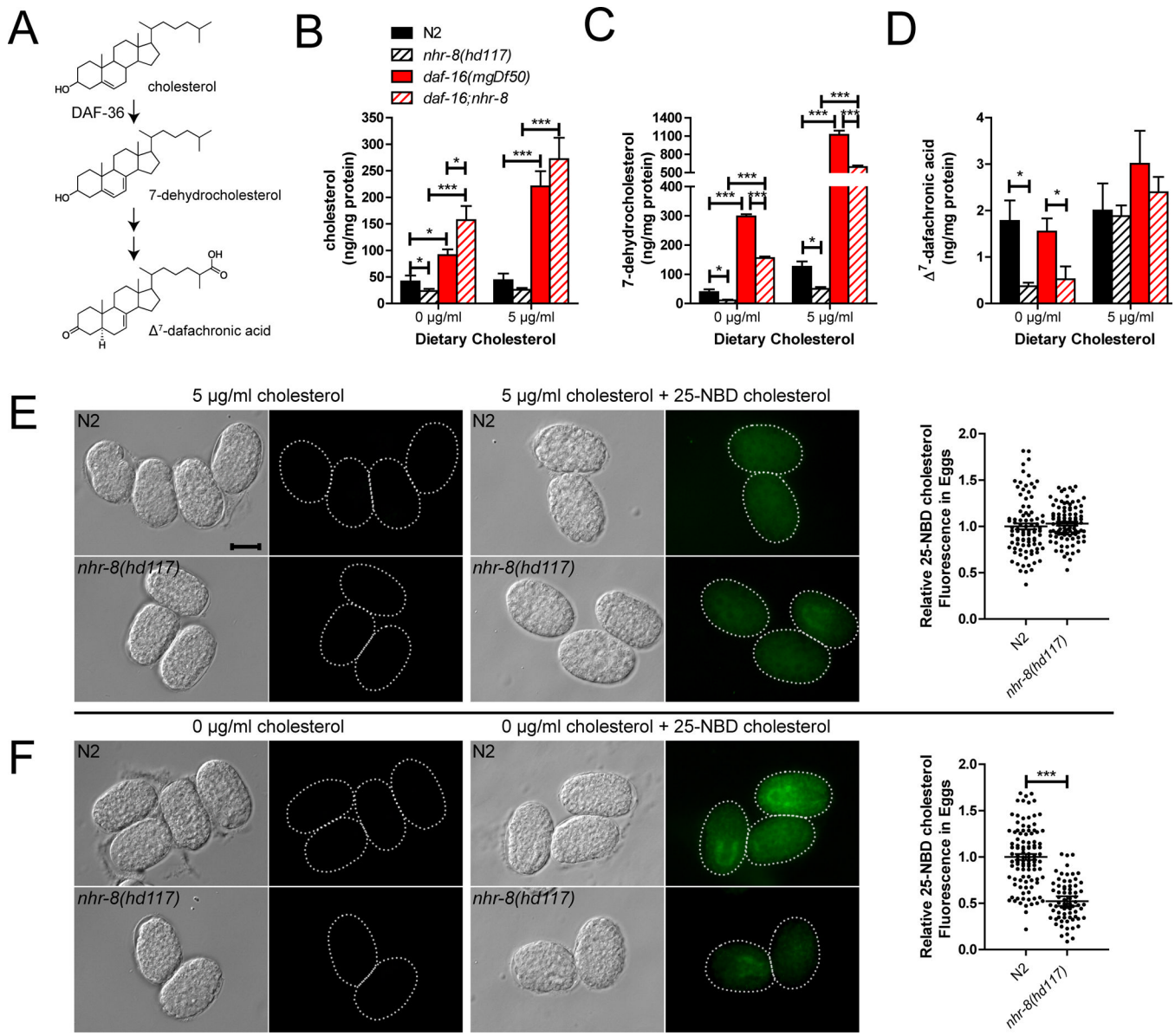

Figure 4. Loss of *nhr-8* Influences Sterol Metabolism

(A) Schematic of Δ7-DA biosynthesis from dietary cholesterol. **(B)** Endogenous free cholesterol levels are reduced in *nhr-8* animals grown in the absence of dietary cholesterol. Mutation of *daf-16* generally increases cholesterol levels. **(C)** Production of 7-dehydrocholesterol requires *nhr-8*. Mutation of *daf-16* increases 7-dehydrocholesterol production to greater than wild-type levels independently of *nhr-8*. **(D)** Δ^7-DA levels are lower in *nhr-8* animals in the absence of dietary cholesterol. Mutation of *daf-16* does not rescue Δ^7-DA production. **(E and F)** Micrographs and quantification of the fluorescence intensity of individual eggs from animals fed 25-NBD cholesterol (1 μg/ml). In the presence of 5 μg/ml cholesterol, N2 and *nhr-8* mutants deposit the same amount of 25-NBD cholesterol in their eggs. In the absence of dietary cholesterol, *nhr-8* mutants deposit half the amount of 25-NBD cholesterol in their eggs as N2. *p<0.05, **p<0.01. Mean±SEM. Values from n≥5 (B–D) or n=3 (F) independent experiments. Scale bar = 20 μm.

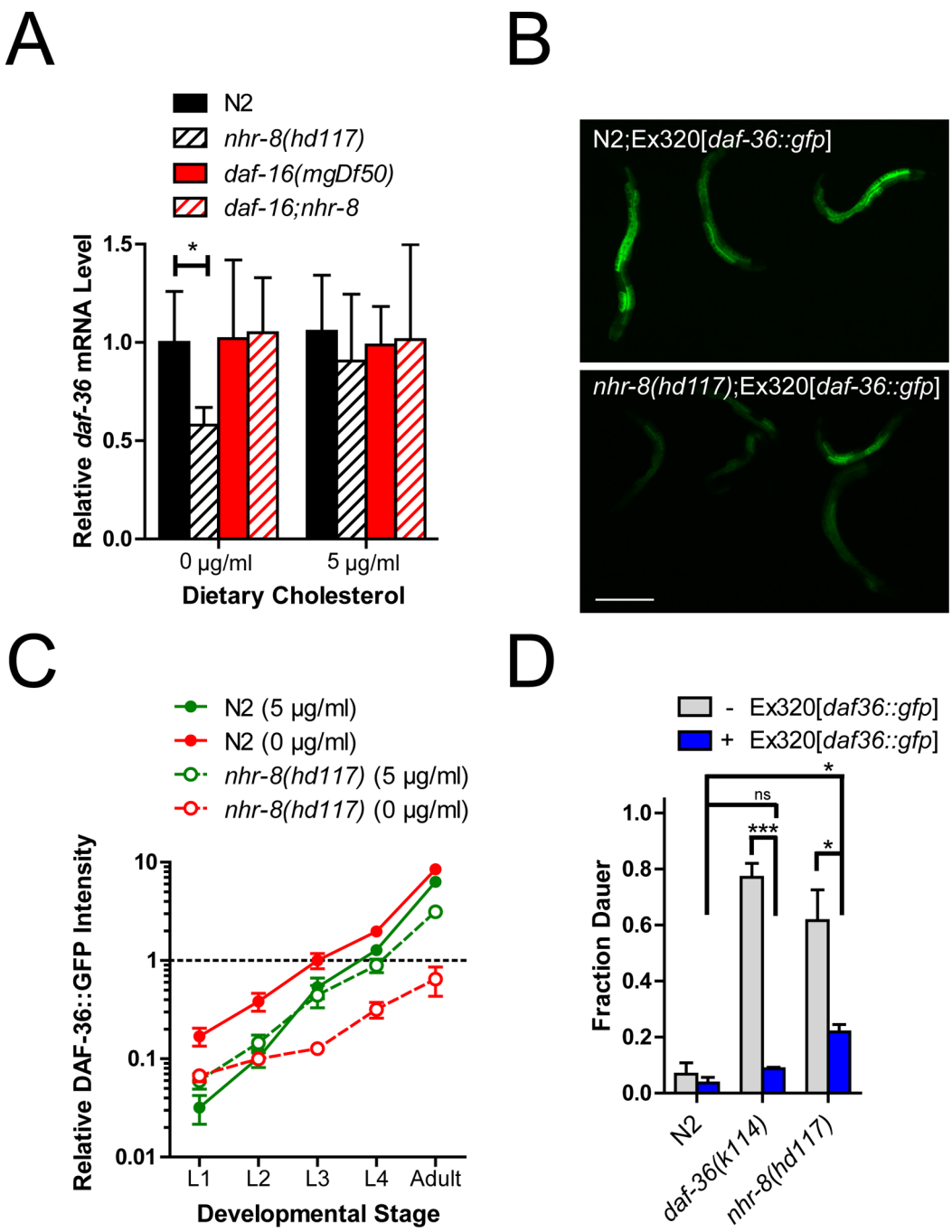

Figure 5. *nhr-8* Affects Expression of *daf-36*

(A) *daf-36* mRNA expression is down-regulated in *nhr-8* at L3. **(B)** Micrographs showing reduced expression of DAF-36: :GFP protein in *nhr-8* animals at L3. Bar = 100 µm. **(C)** DAF-36: :GFP expression is reduced throughout development in *nhr-8* animals grown in the absence of dietary cholesterol. Data were quantified from GFP intensities of several thousand animals using a COPAS biosorter (similar to Figure 3E) **(D)** Overexpression of *daf-36* partially rescues dauer formation of *nhr-8* animals. *p<0.05, ***p<0.001, ns=not significant. Mean±SEM. See also Figure S3.

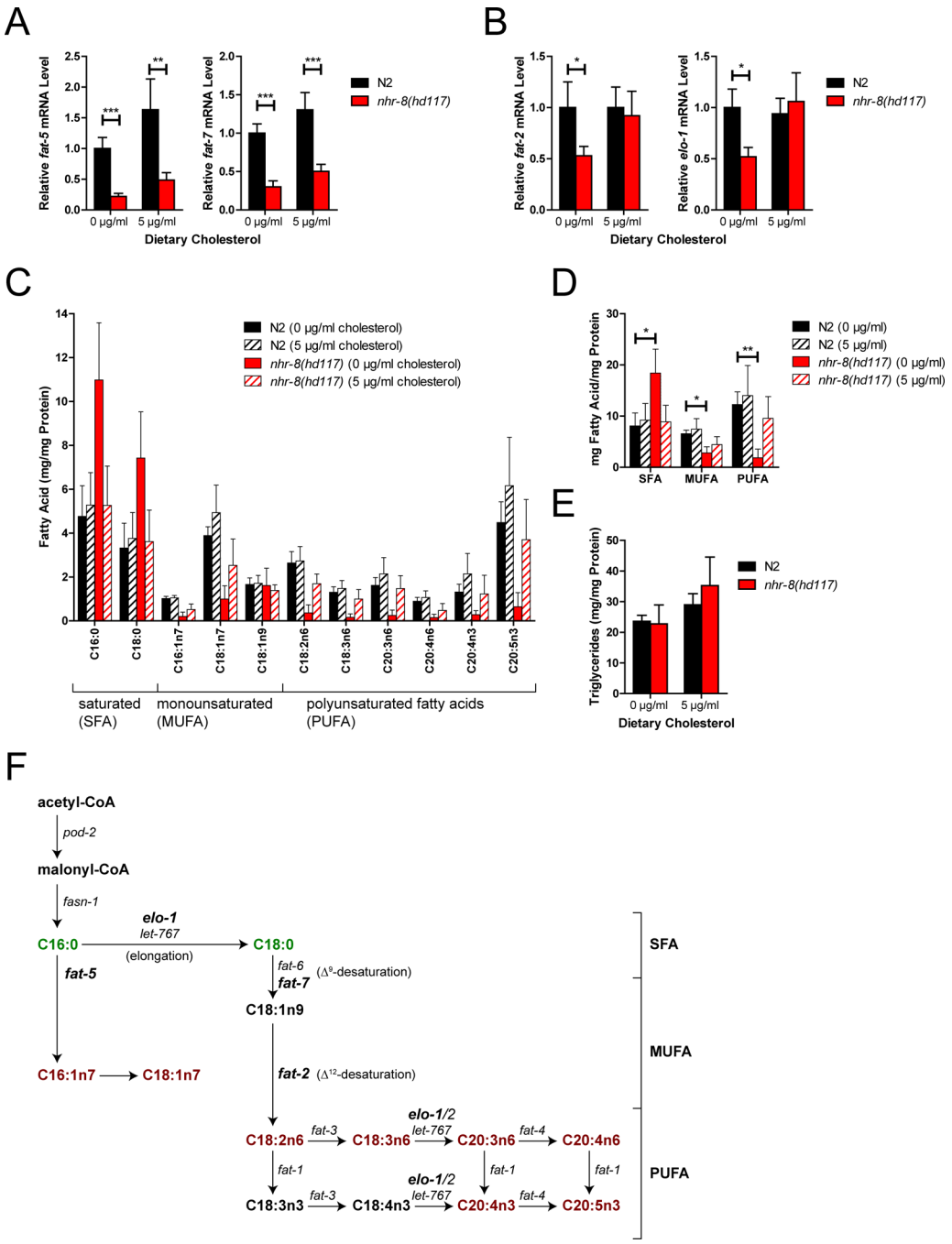

Figure 6. *nhr-8* Affects Fatty Acid Metabolism

(A) *fat-5* and *fat-7* transcript levels are decreased in *nhr-8* animals, independent of dietary cholesterol **(B)** *fat-2* and *elo-1* transcript levels are decreased in *nhr-8* when grown in the absence of dietary cholesterol **(C)** Fatty acid profiles as determined by GC analysis show an increase in saturated and a decrease in mono and polyunsaturated fatty acids in *nhr-8* animals at the L3 stage of development in 0 µg/ml cholesterol. Comparable changes were also seen in day 1 adult animals. **(D)** Quantification of the different classes of fatty acids summed from (C). **(E)** Triglyceride levels in *nhr-8* animals are similar to wild-type. **(F)** A model of fatty acid synthesis. Genes implicated in various steps shown in italics. Bold type

indicates those that are downregulated in *nhr-8* in the absence of dietary cholesterol. Fatty acids indicated in green are increased, whereas those in red are decreased in *nhr-8* animals. Data shown are from L3 animals grown at 25°C. *p<0.05, **p<0.01, ***p<0.001. Mean ±SEM. See also Figure S5.

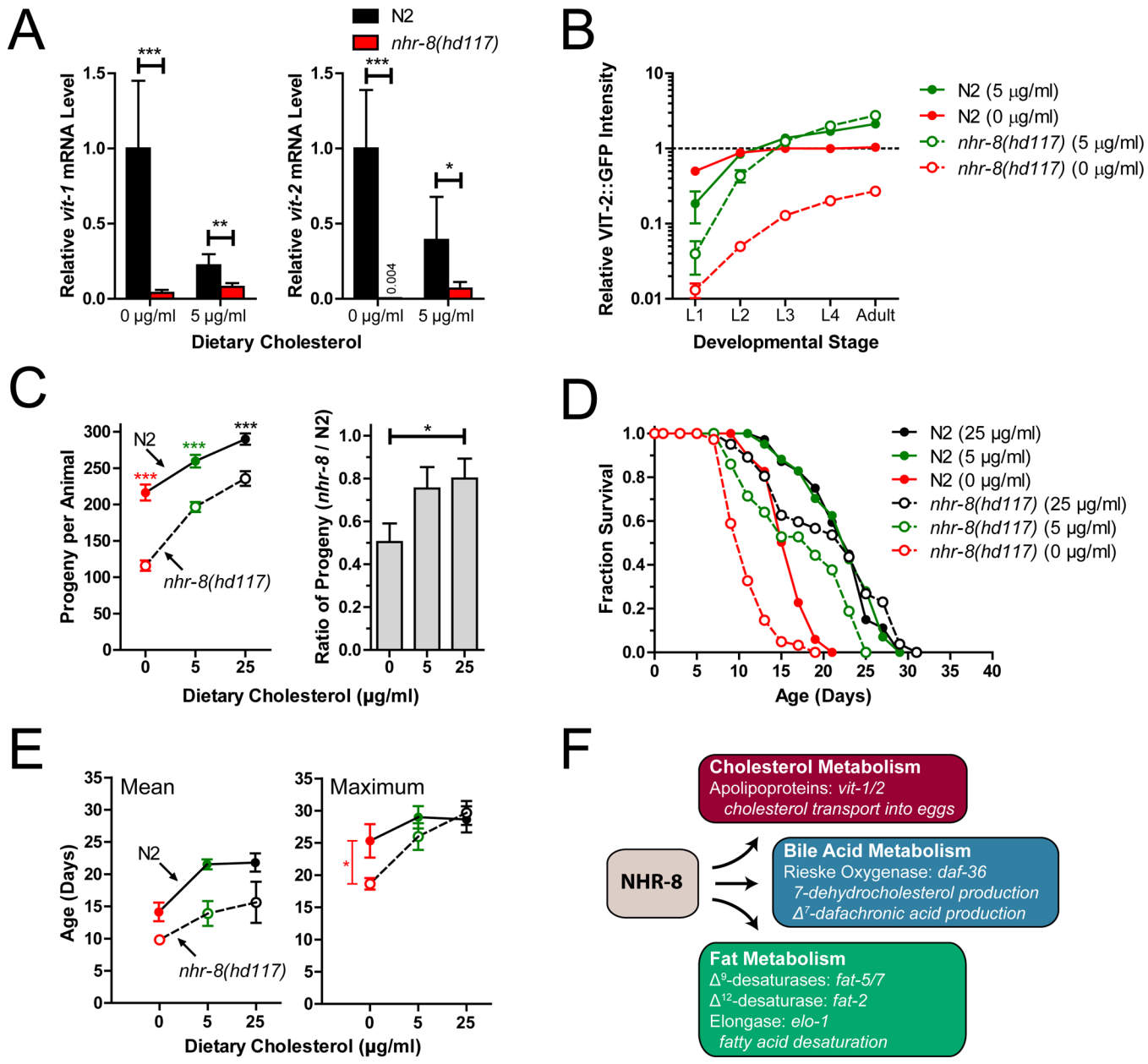

Figure 7. *nhr-8* Brood Size and Lifespan and are Cholesterol Dependent

(A) Transcript levels of the Apolipoprotein-like vitellogenins, *vit-1* and *vit-2*, are decreased in *nhr-8* animals. **(B)** VIT-2: :GFP expression is reduced throughout development in *nhr-8* animals grown in the absence of dietary cholesterol. Data were quantified from GFP intensities of several thousand animals using a COPAS Biosort (similar to Figure 3E). **(C)** *nhr-8* animals have reduced brood size. Supplementation with high amounts of cholesterol significantly improves fecundity of *nhr-8* animals relative to wild-type grown under the same conditions. **(D)** *nhr-8* mutant animals show reduced lifespans compared to N2. Excess dietary cholesterol rescues the maximum, but not the mean *nhr-8* lifespan. Representative lifespan assay of N2 and *nhr-8* animals grown from day 1 adult under low (0 µg/ml), normal (5 µg/ml), or high (25 µg/ml) amounts of dietary cholesterol. Development from egg to adult of all animals was in the presence of 5 µg/ml dietary cholesterol. **(E)** Quantification of mean and maximum lifespan of wild-type and *nhr-8* animals mutants supplemented with 0, 5, or

25 μg/ml dietary cholesterol. (F) Schematic representation of the metabolic processes regulated by NHR-8. n≥3 experiments. *p<0.05, **p<0.01, ***p<0.001. Mean±SEM. See also Figure S6.