Steroid hormones play essential roles in a wide variety of biological processes in multicellular organisms. The principal steroid hormones in nematodes and arthropods are dafachronic acids and ecdysteroids, respectively, both of which are synthesized from cholesterol as an indispensable precursor. The first critical catalytic step in the biosynthesis of these ecdysozoan steroids is the conversion of cholesterol to 7-dehydrocholesterol. However, the enzymes responsible for cholesterol 7,8-dehydrogenation remain unclear at the molecular level. Here we report that the Rieske oxygenase DAF-36/Neverland (Nvd) is a cholesterol 7,8-dehydrogenase. The daf-36/nvd genes are evolutionarily conserved, not only in nematodes and insects but also in deuterostome species that do not produce dafachronic acids or ecdysteroids, including the sea urchin *Hemicentrotus pulcherrimus*, the sea squirt *Ciona intestinalis*, the fish *Danio rerio*, and the frog *Xenopus laevis*. An in *vitro* enzymatic assay system reveals that all DAF-36/Nvd proteins cloned so far have the ability to convert cholesterol to 7-dehydrocholesterol. Moreover, the lethality of loss of *nvd* function in the fruit fly *Drosophila melanogaster* is rescued by the expression of *daf-36/nvd* genes from the nematode *Caenorhabditis elegans*, the insect *Bombyx mori*, or the vertebrates *D. rerio* and *X. laevis*. These data suggest that *daf-36/nvd* genes are functionally orthologous across the bilaterian phylogeny. We propose that the *daf-36/nvd* family of proteins is a novel conserved player in cholesterol metabolism across the animal phyla.

Steroid hormones are crucial for development, growth, and homeostasis in multicellular organisms. Cholesterol and other sterol(s) serve as indispensable precursors for the biosynthesis of steroid hormones, and the conversion of cholesterol to the next specific intermediate is a crucial biochemical step across species (1–4). In the biosynthesis of vertebrate steroid hormones, cholesterol is commonly converted to pregnenolone by side-chain cleavage catalyzed by the cytochrome P450 monooxygenase P450scCYP11A1 (2). The step catalyzed by CYP11A1 is the key rate-limiting step in the synthesis of all vertebrate steroids, and it thus is controlled by numerous physiological responses throughout the life cycle (2, 5).

Cholesterol is also required for steroid hormone biosynthesis in protostomes, including nematodes and arthropods (1, 6). However, the molecular mechanisms of cholesterol metabolism in these ecdysozoan animals have not been fully elucidated. The principal steroid hormones in nematodes and arthropods are dafachronic acids and ecdysteroids, respectively, both of which play pivotal roles in the regulation of developmental timing, reproduction, and longevity (1, 7, 8). In the biosynthesis of both dafachronic acids and ecdysteroids, which is different from the biosynthesis of vertebrate steroid hormones, cholesterol is converted to 7-dehydrocholesterol (7dC) by dehydrogenation of the carbons at positions 7 and 8 (Fig. 1A) (9). Nematodes and arthropods lost the ability to synthesize sterol *de novo* and must take in exogenous cholesterol or plant sterols from their diet (1, 6). Therefore, the conversion of cholesterol to 7dC is the first crucial step of steroid hormone bio-

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* The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Tables S1 and S2, and Figs. S1–S4.
* The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB607950–AB607954.
* This work was supported in part by the Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government; by grants from the Agricultural Chemical Research Foundation and the Japan Association for Marine Biology (to R. N.); and by Grant-in-Aid for Scientific Research 19380034 from the Japan Society for the Promotion of Science (JSPS) and a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences (to H. K.).
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synthesis in these ecdysozoans. However, no enzyme responsible for cholesterol 7,8-dehydrogenase has yet been identified at the molecular level.

The DAF-36/Neverland (Nvd) proteins are strong candidates for harboring cholesterol 7,8-dehydrogenase activity (10, 11). Both of the nematode Caenorhabditis elegans daf-36 mutants and the fruit fly Drosophila melanogaster nvd loss of function animals exhibit defects in their steroid hormone production. The developmental abnormalities caused by loss of either daf-36 or nvd function are rescued by the topical application of 7dC, but not cholesterol, strongly indicating that daf-36/nvd is involved in the cholesterol 7,8-dehydrogenation in both nematodes and arthropods. The DAF-36/Nvd proteins possess a Rieske [2Fe-2S] motif (CXFaB,CXbH) and the non-heme iron binding motif (D/E)XaD,XbXcHg,H) are colored in magenta and cyan, respectively. Percentages indicate amino acid identities between Nvd-Bm and the other proteins of the Rieske domain and a highly conserved domain in the C-terminal region after the Rieske domain. Also indicated are the total numbers of residues for the individual proteins.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Vector Construction—Details of the molecular cloning of nvd-Xl, nvd-Dr, nvd-Ci-1, nvd-Ci-2, and nvd-Hp are described in the supplemental material. We deposited the DNA and amino acid sequences of the nvd orthologs in GenBank trade mark (accession numbers AB607950–AB607954). To generate the constructs expressing HA-tagged daf-36/nvd genes under the control of a GAL4/UAS system (15), each ORF region was ligated into the puAST vector with sequences coding three tandem 3× HA tags at the C terminus (16). The generation of the nvd point mutant constructs is described in the supplemental material.

Cell Culture and the in Vitro Enzymatic Activity Assay System—Cholesterol and 7dc were purchased from Sigma. Culturing and transfection of S2 cells were performed as previously described (16). UAS vectors were transfected with the Actin5C-GAL4 construct (a gift from Yasushi Hiromi). Two days after the transfection of S2 cells with the vectors in a 60-mm culture dish, the medium was replaced with fresh medium (2 ml) containing 50 μM cholesterol with 0.9% 2-hydroxypropyl β-cyclodextrin (Wako) (17). After a 24-h incubation, the medium and cells were collected and mixed with an equal volume of ethyl acetate. Then an aliquot of supernatant (1.2 ml) was collected, desiccated, and redissolved in 200 μl of methanol. Reverse phase high pressure liquid chromatography (RP-HPLC) analysis was performed using a 2695 Separations Module apparatus (Waters) equipped with a 996 Photodiode Array Detector (Waters) or a 2996 Photodiode Array Detector (Waters). The RP column used in this study was the Senshu Pak PEGASIL ODS column (4.6 × 250 mm). The conditions were as follows: solvent, 100% methanol; flow rate, 1 ml/min; detection, UV absorption at 281 nm.

Derivatization of Steroids and HPLC-Electrospray Ionization Liquid Chromatography-Tandem Mass Spectrometry (ESI-MS/MS)—Derivatization of steroids using Girard P reagent was performed essentially as described (18). The HPLC fraction corresponding to 7dC was collected, evaporated to dryness, and dissolved in a mixture of 10 μl of isopropyl alcohol, 2 μl of 1 mg/ml cholesterol oxidase (ToYoBo), and 200 μl of 50 mM KH2PO4 (pH 7.0). The mixture was incubated in the dark at room temperature for more than 2 h. The reaction was stopped with 400 μl of methanol and then mixed with 30 μl of Girard P reagent (Tokyo Kasei Kogyo) and 30 μl of glacial acetic acid. The mixture was left overnight in the dark at room temperature. LC-ESI-MS/MS analyses were performed on an Agilent HPLC system equipped with a Qstar system (Applied Biosystems) and a PEGASIL ODS column (2 × 100 mm, 3 μm; Senshu Scientific). The flow rate was 0.2 ml/min, and the injection volume was 500 μl (~80% of the total reaction volume). After sample injection in 70% ethanol, the flow from the HPLC was discarded for the first 10 min of the run to remove salts and excess derivatization reagents. The Qstar was then reconnected, and the following gradient was applied: 0–10 min, methanol 70%; 10–30 min, methanol 70–95%; 30–40 min,
methanol 95%. All MS spectra were acquired in TOF-MS mode with the ion spray voltage adjusted to 5500 V and the curtain gas 30 V in the positive ion mode. MS/MS spectra of m/z 516.35 were acquired in product ion scan mode with the following parameters: MS range, 50–550; Q1 resolution, Unit; collision energy, 50 eV.

Immunostaining and Western Blotting—The immunostaining procedure for S2 cells was described previously (16). The anti-HA monoclonal antibody 16B12 (1:200 dilution; Covance) and anti-mouse Alexa 568 (1:200 dilution; Molecular Probes) were used for staining. UAS-mSpitz-GFP (a gift from Ben-Zion Shilo) was used as a microsomal marker (19). The anti-mouse β-actin monoclonal antibody C4 (1:50,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for Western blotting.

Fly Strains and Rescue Experiments—D. melanogaster flies were reared on standard agar-cornmeal medium. RNAi to knock down nvd-Dm with 2-286-GAL4 (a gift from Carl S. Thummel) and UAS-nvd-Dm-IR-1 transgenic flies were described previously (11). Transgenic flies carrying HA-tagged nvd-Bm, daf-36, nvd-Dr, and nvd-Xl genes were established using standard protocols. See also supplemental Table S2 and its legend for more details.

RESULTS

Insect and Nematode DAF-36/Nvd Convert Cholesterol to 7dC—To examine whether DAF-36/Nvd proteins convert cholesterol to 7dC, we adopted an in vitro enzymatic assay system using cultured D. melanogaster S2 cells that has been well utilized to characterize the catalytic roles of the ecdysteroidogenic P450s (20, 21). S2 cells expressing either the silkworm Bombyx mori nvd (nvd-Bm) (11) or the C. elegans daf-36 (10) gene were incubated with cholesterol, followed by analysis of the cell extracts and culture medium by RP-HPLC. A single HPLC peak was observed at a retention time identical to that of standard 7dC (Fig. 2, A and B), whereas no peak or only a much smaller peak was observed in the GFP-transfected control S2 cells (Fig. 2C). Over a 24-h incubation of S2 cells expressing nvd-Bm or daf-36 with 50 μM cholesterol, ~0.9 or 2.6% of the cholesterol was converted to 7dC, respectively (supplemental Table S1). For an unknown reason, we did not obtain any detectable protein expression of Nvd-Dm in the S2 cells under our experimental conditions. However, HPLC peaks corresponding to 7dC were observed by incubating cholesterol with S2 cells expressing nvd genes from other drosophilids: Drosophila mojavesensis and Drosophila acanthopera.

The identity of the DAF-36/Nvd metabolite was confirmed by HPLC-ESI-MS/MS following derivatization with Girard P reagent (supplemental Fig. S1A), which is widely used for analyses of sterols (18, 22). Derivatized 7dC standard gave a molecular ion at m/z 516 that generates fragment ions of m/z 437, 409, 151, and 137 from two major transitions (Fig. 2D and supplemental Fig. S1B). The derivatized Nvd-Bm cholesterol metabolite generated fragments with mass spectra identical to those of the derivatized 7dC standard (Fig. 2E). These results indicate that both nematode and insect DAF-36/Nvd have cholesterol 7,8-dehydrogenase activity, consistent with the genetic predictions of previous reports (10, 11).

Microsomal Localization of DAF-36/Nvd—A previous study reported that the insect cholesterol 7,8-dehydrogenation reaction occurs in microsomes (23). Consistent with this observation, the subcellular distribution of wild type Nvd-Bm with epitope tags (Fig. 3A) was mostly in the peripheral region of microsomes in S2 cells (Fig. 3B). Specifically, signals of wild type Nvd-Bm were detected in the periphery of 92% of the punctate microsomes observed in 51 puncta of 10 S2 cells. In contrast, Nvd-Bm protein lacking the N-terminal region, which contains a computationally predicted transmembrane domain (ΔN-Nvd-Bm; Fig. 3A), was more uniformly distributed in S2 cells (Fig. 3B). We observed that only 31% of microsomes overlapped signals of ΔN-Nvd-Bm in 54 microsomal puncta of 10 S2 cells. Moreover, we also found that the cholesterol catalytic activity of S2 cells expressing ΔN-Nvd-Bm was almost completely abolished compared to that of S2 cells expressing the wild type form (Fig. 3D), suggesting that the microsomal localization of

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DAF-36/Nvd via the N-terminal region is important for its enzymatic activity.

The Conserved Motifs of DAF-36/Nvd Are Crucial for Its Enzymatic Activity—We further investigated the contribution of the conserved protein motifs of DAF-36/Nvd to its catalytic activity. In the bacterial Rieske oxygenases, the Rieske \([2\text{Fe}-2\text{S}]\) motif and the non-heme iron binding motif are involved in electron transfer and are critical for their catalytic function (24). We introduced site-directed mutations of the conserved amino acids to alanine in either the Rieske \([2\text{Fe}-2\text{S}]\) motif or the non-heme iron binding motif (Fig. 3C). The single residue replacements H190A and H282A each totally abolished the cholesterol 7,8-dehydrogenase activity of Nvd-Bm (Fig. 3E). These results demonstrate that the canonical conserved motifs of the Rieske oxygenase family are essential for the cholesterol 7,8-dehydrogenase activity of DAF-36/Nvd.

Enzymatic Activity of DAF-36/Nvd Is Required for D. melanogaster Development—To assess the importance of DAF-36/Nvd enzymatic activity during development, we examined whether expression of the catalytically inactive form of Nvd could rescue the lethality of transgenic \(\text{nvd-Dm}^{\text{RNAi}}\) \(D.\) melanogaster animals (11). When \(\text{nvd}^\text{+}\) was silenced specifically in the prothoracic gland, which is the endocrine organ that synthesizes ecdysteroids during insect larval development (21), the \(\text{nvd-Dm}^{\text{RNAi}}\) animals died at the first larval instar stage (11) (Table 1 and supplemental Table S2). Wild type Nvd-Bm rescued the lethality of the \(\text{nvd-Dm}^{\text{RNAi}}\) animals, whereas Nvd-Bm-H190A and Nvd-Bm-H282A did not (Table 1 and supplemental Table S2). In a wild type background, expression of either wild type Nvd-Bm or the catalytically inactive Nvd-Bm in the prothoracic gland had no visible effect on \(D.\) melanogaster development (Table 1 and supplemental Table S2). These results support the hypothesis that the catalytic activity of DAF-36/Nvd is required for its \textit{in vivo} roles.

**FIGURE 3. Role of the conserved domains of Nvd-Bm and DAF-36**. A, schematic representation of wild type Nvd-Bm (WT; top) and mutated Nvd-Bm protein lacking the N-terminal region (amino acids 1–75), including the transmembrane domain (amino acids 52–74) (\(\Delta\text{N};\) bottom). B, immunostaining of S2 cells transfected with HA-tagged WT or \(\Delta\text{N}-\text{Nvd-Bm}\). Arrowheads, positions of punctate microsomes labeled with mSpi-GFP. Scale bar, 5 \(\mu\text{m}\). C, sequence alignments of the Rieske motif (top) and the non-heme iron binding motif (bottom). Open red squares indicate the conserved residues across species. Black squares indicate the amino acid residues that were mutated in this study. D–G, cholesterol 7,8-dehydrogenase activities of Nvd-Bm-\(\Delta\text{N}\) (D), Nvd-Bm-H190A, Nvd-Bm-H282A (E), DAF-36-C122A, DAF-36-D234A (F), and Nvd-XI-C178A (G). Nvd-Bm-H190A, DAF-36-C122A, and Nvd-XI-C178A represent the alanine substitutions in the Rieske motif of Nvd-Bm, DAF-36, and Nvd-XI, respectively. Nvd-Bm-H282A and DAF-36-D234A represent the alanine substitution in the iron-binding motif of Nvd-Bm and DAF-36, respectively. S2 cells were transfected with the WT or mutant construct and then incubated with 50 \(\mu\text{M}\) cholesterol for 24 h. The average amount of 7dehydrocholesterol formation during incubation with the WT protein was set as 1. Error bars, S.E. Three (D, F, and G) or five (E) independent experiments were performed. S2 cells expressing \(\text{GFP}\) were used as a negative control. Western blots show the amount of DAF-36/Nvd proteins tagged with HA and the amount of actin protein in the S2 cells. The Nvd-Bm-\(\Delta\text{N}\) and DAF-36 constructs gave rise to multiple protein bands for unknown reasons.
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**TABLE 1**

The lethality of *nvd-Dm* RNAi animals with the expression of *daf-36/nvd*

The number of viable *nvd-Dm* RNAi adults was scored using two or three independent transgenic lines. Values in parentheses indicate the number of viable control non-RNAi progeny that carried the GAL4 and UAS-daf-36/nvd transgenes from the parental strains in the same experimental batches. See supplemental Table S2 for more details.

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Number of adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 (349)</td>
</tr>
<tr>
<td>nvd-Bm</td>
<td>366 (348)</td>
</tr>
<tr>
<td>nvd-Bm-H190A</td>
<td>0 (410)</td>
</tr>
<tr>
<td>nvd-Bm-H282A</td>
<td>0 (517)</td>
</tr>
<tr>
<td>daf-36</td>
<td>313 (449)</td>
</tr>
<tr>
<td>nvd-Dr</td>
<td>361 (341)</td>
</tr>
<tr>
<td>nvd-Xl</td>
<td>293 (309)</td>
</tr>
</tbody>
</table>

*daf-36/nvd* Genes Are Structurally Conserved across Animal Phyla—A BLAST search using available genome information showed that one or two predicted orthologs of *daf-36/nvd* are encoded in each genome of several deuterostome species as well as in nematodes and insects (supplemental Fig. S2). cDNAs of putative orthologs of *daf-36/nvd* were successfully amplified by PCR using reverse transcribed templates prepared from embryos of the sea urchin *Hemicentrotus pulcherrimus* (*nvd-Hp*), the sea squirt *Ciona intestinalis* (*nvd-Ci-1* and *nvd-Ci-2*), the fish *Danio rerio* (*nvd-Dr*), and the frog *Xenopus laevis* (*nvd-Xl*) (Fig. 1B; see supplemental material). By determining the cDNA sequences, we confirmed that all of the deduced proteins (*Nvd-Hp, Nvd-Ci-1, Nvd-Ci2, Nvd-Dr, and Nvd-Xl*) possess the characteristic domains of DAF-36/Nvd, including the Rieske [2Fe-2S] domain, the non-heme iron domain, and the putative transmembrane domain (Fig. 1B and supplemental Fig. S3). These data suggest that *daf-36/nvd* genes are structurally conserved and are expressed in *vivo* in both protostomes and deuterostomes.

*daf-36/nvd* Genes Are Functionally Orthologous between Protostomes and Deuterostomes—To examine whether deuterostome DAF-36/Nvd proteins also convert cholesterol to 7dC, we transfected S2 cells with plasmids expressing each of the deuterostome genes. In every case, an HPLC peak at a retention time identical to the 7dC standard was observed (Fig. 4, A and B, and supplemental Fig. S4). The conversion efficiencies from cholesterol to 7dC by S2 cells expressing these deuterostome *daf-36/nvd* genes were ~0.2–2.0% from 50 μM cholesterol over a 24-h period, which are comparable with the conversion efficiencies of Nvd-Bm and DAF-36 (Fig. 4C and supplemental Table S1). The enzymatic activity of deuterostome DAF-36/Nvd also depends on the Rieske [2Fe-2S] motif because a Nvd-Xl protein with an amino acid substitution in the motif lost its enzymatic activity (Fig. 3G).

We next investigated whether *daf-36/nvd* genes from species other than *D. melanogaster* can substitute for *nvd-Dm* function during development. Indeed, expression of either *C. elegans* *daf-36, nvd-Dr,* or *nvd-Xl* allowed *nvd-Dm* RNAi animals to complete their development and grow into adult stages (Table 1 and supplemental Table S2). Taken together, our results suggest that the *daf-36/nvd* genes are functionally orthologous between protostomes and deuterostomes.

**DISCUSSION**

In this study, we have shown that the Rieske oxygenase DAF-36/Nvd is the enzyme responsible for cholesterol 7,8-dehydrogenation, which is the first crucial step in the production of steroid hormones in nematodes and arthropods. We have also found that the protein structures and enzymatic activities of DAF-36/Nvd are conserved not only in protostomes but also in deuterostomes, for which the biosynthesis of dafachronic acids and ecdysteroids have not been confirmed. Importantly, *daf-36/nvd* genes from *C. elegans* and vertebrates can substitute for *D. melanogaster* *nvd* function in *vivo*. We therefore propose that the DAF-36/Nvd family of proteins is a novel evolutionarily conserved player in cholesterol metabolism.

It has long been assumed that cholesterol 7,8-dehydrogenation in insects is mediated by a P450 enzyme based on experiments using pharmacological inhibitors (1, 23). Although our findings in conjunction with previous data (10, 11) strongly suggest that DAF-36/Nvd is the true identity of the cholesterol...
7,8-dehydrogenase, this does not exclude the possible existence of such a P450. The activation mechanism of Rieske oxygenases by electrons has been proposed to be analogous to that of the P450s (25). Therefore, it would be intriguing to examine whether the P450 inhibitors also hamper the enzymatic activity of DAF-36/Nvd.

Compared with the conversion rate (~20–80%) of previously reported ecysteroidogenic P450s in S2 cells (21), our study yielded lower conversion (maximally 2.6%) of the substrate. This may partly reflect the insolubility of cholesterol. In our assay, cholesterol was provided in a stable water-soluble vehicle using cyclodextrin (see “Experimental Procedures”), and the enzyme-substrate complex might thus be formed in an irregular way. An alternative possibility is that DAF-36/Nvd requires an unknown cofactor to exhibit its maximal enzymatic activity. In bacteria, the Rieske oxygenases work with one or two partner proteins that transport electrons (12). For example, 3-ketosteroid 9α-hydroxylation is catalyzed by a complex of the Rieske oxygenase KshA and the Rieske oxygenase reductase KshB (13, 14). It is therefore likely that there is an unidentified innate reducing partner of Nvd that is not present or is little present in S2 cells.

Our study has demonstrated that several deuterostome DAF-36/Nvd exhibit the cholesterol 7,8-dehydrogenase activity. It is uncertain, however, whether the reaction of cholesterol to 7dC occurs physiologically in these animals (26). In vertebrates, the reduction of 7dC to cholesterol, which is the reverse of the reaction mediated by DAF-36/Nvd, is instead known to be the critical step in the cholesterol biosynthesis pathway (27). It has been demonstrated that the 7dC reductase (DHCR7) is essential for vertebrate development (28, 29) and that deficiency in DHCR7 in humans leads to a metabolic and developmental disorder known as Smith-Lemli-Opitz syndrome (27). We speculate that daf-36/nvd is involved in cholesterol metabolism and homeostasis in deuterostomes by acting in concert with DHCR7 to maintain a balance between the amounts of cholesterol and 7dC (Fig. 4D). To test this hypothesis, detailed expression analyses and knockdown experiments of deuterostome daf-36/nvd are now under way. In addition, the biochemical properties of DAF-36/Nvd, especially its substrate specificity, should also be elucidated.

It should be noted that no orthologs of daf-36/nvd have been found in mammalian genomes (Fig. 4D). In parallel, no structural or functional orthologs of DHCR7 have been identified in the genome information available for insects (30), and a reducing reaction from 7dC to cholesterol is undetectable in C. elegans (31) (Fig. 4D). Curiously, the genome of the more primitive animal, sea anemone Nematostella vectensis (32), encodes both proteins highly similar to DAF-36/Nvd (Protein ID 183599; supplemental Fig. S2) and DHCR7 (Protein ID 229921). Thus, an interesting hypothesis is that during evolution, ecystozoa lost the catalytic reaction from 7dC to cholesterol and mammals lost that from cholesterol to 7dC. Alternatively, ecystozoa and mammals might be able to catalyze the interconversion of 7dC and cholesterol by other unique unidentified enzymes that have been evolved independently from the acquisition of daf-36/nvd and DHCR7. Our findings and further studies will shed light on the evolution of cholesterol metabolism and steroid hormone biosynthesis.

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The Cholesterol 7,8-Dehydrogenase DAF-36/Neverland