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Identification of Ligands for DAF-12 that Govern Dauer Formation and Reproduction in *C. elegans*

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SUMMARY

In response to environmental and dietary cues, the *C. elegans* orphan nuclear receptor, DAF-12, regulates dauer diapause, reproductive development, fat metabolism, and life span. Despite strong evidence for hormonal control, the identification of the DAF-12 ligand has remained elusive. In this work, we identified two distinct 3-keto-cholestenoic acid metabolites of DAF-9, a cytochrome P450 involved in hormone production, that function as ligands for DAF-12. At nanomolar concentrations, these steroidal ligands (called dafachronic acids) bind and transactivate DAF-12 and rescue the hormone deficiency of *daf-9* mutants. Interestingly, DAF-9 has a biochemical activity similar to mammalian CYP27A1 catalyzing addition of a terminal acid to the side chain of sterol metabolites. Together, these results define the first steroid hormones in nematodes as ligands for an invertebrate orphan nuclear receptor and demonstrate that steroidal regulation of reproduction, from biology to molecular mechanism, is conserved from worms to humans.

INTRODUCTION

The genome of *C. elegans* is predicted to contain 284 nuclear receptors (Gissendanner et al., 2004; Sluder and Maina, 2001). Forward and reverse genetic studies have uncovered roles for these receptors in diverse physiological processes such as development, reproduction, and metabolism. However, all nuclear receptors in worms remain orphans, since ligands regulating their function have not been identified (Sluder and Maina, 2001; Van Gilst et al., 2005a; Van Gilst et al., 2005b).

In contrast to other *C. elegans* nuclear receptors, a considerable amount of genetic evidence supports the existence of a steroid-like ligand for the orphan receptor, DAF-12. *daf-12* belongs to a group of over 30 genes, collectively called daf (*da*uer *f*ormation) genes, which transduce environmental signals that influence the choice between alternative developmental programs of dauer diapause or reproductive development (Antebi et al., 2000; Riddle and Albert, 1997). Dauer diapause occurs when second-larval-stage animals (L2) delay further reproductive development under conditions of diminishing food or overcrowding and instead form the nonfeeding, nonreproductive, and long-lived dauer larva (Riddle and Albert, 1997). Under more favorable conditions, dauer larvae resume feeding and reproductive growth. Mutations in Daf genes generally produce a dauer-constitutive phenotype (Daf-c) or a dauer-defective phenotype (Daf-d). Daf-c mutants always arrest as dauers, while Daf-d mutants bypass dauer, regardless of environmental signals. Loss of *daf-12* results in Daf-d as well as L3 stage heterochronic phenotypes, indicating that *daf-12* is required for dauer formation and for proper developmental timing in the reproductive state (Antebi et al., 1998; Antebi et al., 2000).

Analysis of Daf genes has revealed that favorable environments activate insulin/IGF-1 and TGFβ signaling pathways within the organism that converge on DAF-12 to inhibit its dauer-promoting function and activate its reproductive function (Kimura et al., 1997; Ren et al., 1996; Schackwitz et al., 1996). Acting cell nonautonomously, these pathways are believed to directly or indirectly stimulate the production of a DAF-12 ligand by the cytochrome P450, DAF-9 (Gerisch et al., 2001; Jia et al., 2002). Evidence for this model stems from the findings that insulin-like receptor (*daf-2*), TGFβ (*daf-7*), and cytochrome P450 (*daf-9*) signaling mutants are Daf-c and act upstream of *daf-12* (Vowels and Thomas, 1992; Thomas et al., 1993;

Gerisch et al., 2001). In addition, Daf-c mutants of *daf-12* have been isolated that map to a single residue (R564) in the putative ligand binding domain of DAF-12 and are predicted to perturb ligand binding (Antebi et al., 2000). Phenotypically, these mutants arrest as partial dauers but recover and resemble weak *daf-9* alleles that exhibit gonadal migration (Mig) defects (Gerisch et al., 2001; Jia et al., 2002). Thus, the predicted loss of hormone production in *daf-9* null worms or loss of hormone binding by *daf-12* Daf-c worms results in the failure to inhibit dauer-promoting functions and activate L3 stage reproductive functions of DAF-12.

Several lines of evidence suggest that DAF-12 ligands may be derived from cholesterol and function as either endocrine or paracrine hormones. First, *C. elegans* lacks the ability to synthesize cholesterol (Chitwood, 1999), and cholesterol deprivation produces Mig and Daf-c phenotypes (Gerisch et al., 2001; Jia et al., 2002; Matyash et al. 2004). Second, worms missing both homologs (*ncr-1*, *ncr-2*) of the human Niemann-Pick type C1 gene, a membrane glycoprotein implicated in cholesterol transport, arrest constitutively as dauers (Li et al., 2004). Furthermore, recent evidence shows lipid extracts from wild-type worms can rescue *daf-9* phenotypes (Gill et al., 2004). Finally, unlike DAF-12, DAF-9 functions non-cell autonomously and is restricted to a few cell types such as XXX cells, which are believed to be neuroendocrine cells and a main source of DAF-12 ligands (Ohkura et al., 2003). Although these findings support the hypothesis that a sterol-derived hormone promotes reproductive development in *C. elegans*, the identities of DAF-9-derived hormonal ligands that activate DAF-12 have remained elusive.

In this study, we identified a group of 3-keto-sterols that serve as DAF-9 substrates and are metabolized through successive oxidation to produce DAF-12 ligands. These ligands rescue the Daf-c phenotypes displayed by mutations in *daf-9*, *daf-2*, *daf-7*, and *ncr-1;ncr-2*. Finally, fractionation of *C. elegans* lipid extracts shows that these metabolites exist in vivo but are absent in *daf-9* null worms. These data provide unequivocal evidence for the existence of nematode hormones and demonstrate the evolutionary conservation of broad aspects of endocrine steroid signaling from worms to human.

RESULTS

Identification of 3-Keto-Steroids as DAF-12 Activators

DAF-12 ligand screening was performed using a chimeric GAL4-DAF-12 cotransfection assay in HEK293 cells. Assays were performed in the presence or absence of cotransfected DAF-9, based on genetic evidence suggesting its requirement for synthesis of the DAF-12 ligand (Gerisch and Antebi, 2004; Gerisch et al., 2001; Gill et al., 2004; Jia et al., 2002; Mak and Ruvkin, 2004). Our initial compound screen included bile acids, steroids, and lipophilic compounds that are known ligands for PXR, VDR, and LXR, the closest vertebrate homologs of DAF-12 (Antebi et al., 2000; Mooijaart et al., 2005). This screen identified 3-keto-lithocholic acid (3K-LCA, Figure 1A) as a weak activator of DAF-12 independent of DAF-9 (Figure 1B). Lithocholic acid (LCA), which differs from 3K-LCA by an α-hydroxyl group at C-3, did not exhibit activity on its own or in the presence of cotransfected DAF-9 (Figure 1B). These results suggested that a C-3 ketone was required for DAF-12 activation by 3K-LCA. No other bile acids or steroids tested activated DAF-12, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), 6-keto-lithocholic acid (6K-LCA), 7-keto-lithocholic acid (7K-LCA), progesterone, pregnenolone, testosterone, estradiol, corticosterone, 1,25-dihydroxyvitamin D3, and 20-hydroxyecdysone (Figure 1B and data not shown).

The above data suggested that endogenous 3-keto-sterols from *C. elegans* are candidate DAF-12 ligands. Lathosterol and its 4-methyl-derivative, lophenol, are cholesterol metabolites that have distinct effects on the nematode life cycle (Chitwood et al., 1983; Matyash et al., 2004; Merris et al., 2003). When given as the sole dietary sterol, lathosterol supported full reproductive growth (Merris et al., 2003), while worms grown only in the presence of lophenol constitutively entered dauer diapause (Matyash et al., 2004). These studies suggest lathosterol but not lophenol may be a direct precursor to the DAF-12 ligand. Therefore, we tested lathosterol and lophenol along with their respective 3-keto derivatives, lathosterone and lophenone, for activity in the cotransfection assay. Upon cotransfection with DAF-9, activation of DAF-12 was markedly increased by lathosterone (433-fold) and lophenone (103-fold), but not by their respective 3β-hydroxy derivatives (Figure 1C). In addition, 4-cholesten-3-one, a natural oxidation product of cholesterol, activated DAF-12 (109-fold) in the presence of DAF-9 (Figure 1C). Unlike 3K-LCA, activation of DAF-12 by lathosterone, lophenone, and 4-cholesten-3-one required the presence of DAF-9. Structurally, 3K-LCA differs from these 3-keto-sterols in the length and oxidation state of the side chain and in the saturation of the sterol nucleus (Figure 1A). These results suggest that DAF-9 converts 3-keto-sterols, possibly through side chain oxidation, into DAF-12 activators.

DAF-9 Derivatives of 3-Keto-Sterols Rescue *daf-9* Null Worms

To determine whether the 3-keto-sterol metabolites were biologically relevant we tested their ability to rescue the Daf-c and Mig phenotypes of *daf-9* null animals (Albert and Riddle, 1988; Gerisch et al., 2001; Jia et al., 2002). Individual 3-keto sterols or their respective 3β-hydroxy sterols were incubated with *Sf9* cell microsomes containing DAF-9 and the human P450 oxidoreductase (hOR), which we refer to in all future experiments as DAF-9 microsomes. As a control, microsomes from cells expressing hOR alone were used. For rescue experiments we utilized *daf-9(dh6)dhEx24* worms, which carry an unstable extrachromosomal array of *daf-9*(+) linked to the nuclear marker *sur-5::gfp* (Gerisch et al., 2001). The progeny of these animals, which contain a mixture of both *daf-9*(+)

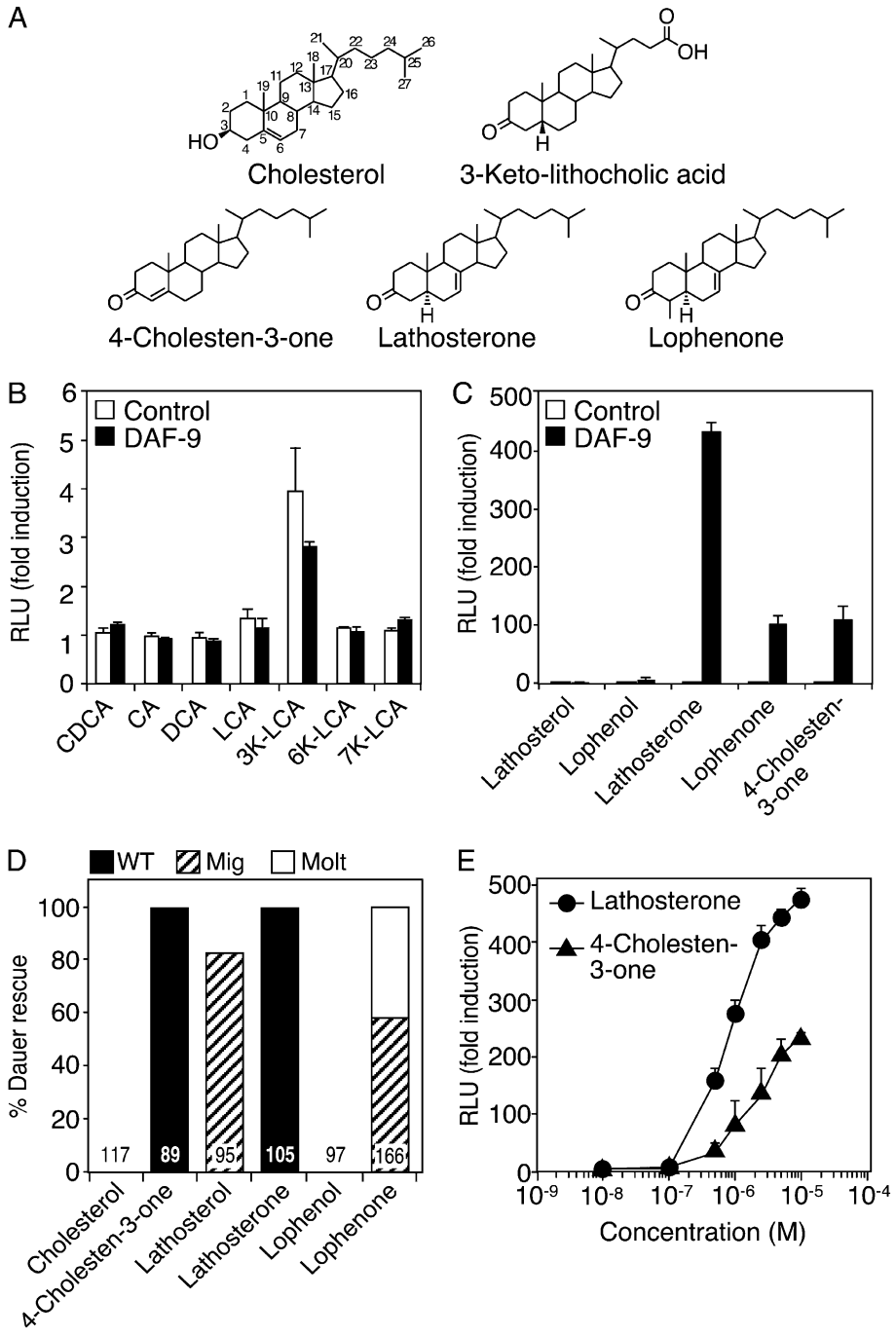

Figure 1. Activation of DAF-12 by 3-Keto-Sterols Requires DAF-9

(A) Structures of candidate DAF-12 ligand precursors relative to cholesterol and 3-keto-lithocholic acid.

(B and C) Activation of DAF-12 by 10 μM bile acids (B) or *C. elegans* sterols (C) and their 3-keto derivatives in the presence of DAF-9 (black bars) or an empty CMX control (white bars) vector. In (B), cotransfection of the intestinal bile acid transporter (IBAT) expression plasmid was used to facilitate bile acid uptake.

(D) Rescue of *daf-9(dh6)* null worms by sterols after incubation with DAF-9 microsomes. Results are reported as percentage of animals rescued from dauer as wild-type gravid adults (wt), Mig adults, or molt-defective larvae. Numbers in each bar refer to worms tested.

(E) Dose response of DAF-12 activation to indicated sterols in cells cotransfected with DAF-9.

Abbreviations: chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic (DCA) acid, lithocholic acid (LCA), 3-keto-lithocholic acid (3K-LCA), 6-keto-lithocholic acid (6K-LCA), 7-keto-lithocholic acid (7K-LCA). Reporter gene activity is expressed as fold induction of relative light units (RLU) compared to ethanol control (n = 3 ± SD).

and *daf-9*(−) worms, were treated with the microsomal extracts for 48 hr. *daf-9*(−) worms were then isolated and scored 24 hr later for presence of the dauer phenotype. Extracts from DAF-9 microsomes incubated with either 4-cholesten-3-one or lathosterone resulted in 100% rescue of the Daf-c and Mig phenotypes in *daf-9*(−) animals (Figure 1D). Remarkably, these animals were indistinguishable from wild-type adults. Indeed, they bypassed dauer, exhibited normal gonadal migration, and produced all Daf-c progeny upon passage to plates lacking microsomal extracts. Rescue by 4-cholesten-3-one and lathosterone required conversion by DAF-9, since their incubation with control microsomes did not rescue any *daf-9* phenotypes (data not shown). Extracts from DAF-9 microsomes incubated with lathosterol (which does not contain a 3-keto group) partially rescued (83%) the Daf-c phenotype, but these animals were Mig and sterile (Figure 1D). This effect also required DAF-9 and was not seen using control microsomes (data not shown). Finally, although incubation of lophenone with DAF-9 microsomes resulted in rescue of Daf-c, none of these animals were normal (58% were Mig; 42% failed to enter dauer, had molting defects, or were dead; Figure 1D). This effect was dependent on DAF-9 and the C-3 ketone in lophenone, since no effect was seen using lophenol or control microsomes. Also, no effects were seen using cholesterol as a substrate. Altogether, these assays revealed that DAF-9 microsomes convert 4-cholesten-3-one and lathosterone into activities that completely rescued *daf-9* null animals.

Identification of DAF-9 Metabolites of 4-Cholesten-3-One

The data above suggested that DAF-9 has enzymatic activity that converts 4-cholesten-3-one and lathosterone into DAF-12 ligands. Although the absolute potency of these DAF-9 products could not be determined using the rescue assay, dose-response curves from the cotransfection assay revealed that lathosterone metabolites were either significantly more potent or more abundantly produced than 4-cholesten-3-one metabolites (Figure 1E). To identify these DAF-9 products, we utilized liquid chromatography/mass spectrometry (LC/MS). The 3-keto-Δ^4-enone structure present in 4-cholesten-3-one has significant UV absorbance at 240 nm, permitting detection of the metabolites. Incubation of 4-cholesten-3-one with DAF-9 microsomes yielded two new peaks at 240 nm that were not present in the control microsomes (Figure 2A). These products eluted at 4.0 (peak 1) and 5.5 (peak 2) minutes on a reverse phase C_{18} column, raising the possibility that they were oxidized derivatives of 4-cholesten-3-one, which elutes at 12.5 min (Figure 2A). Since lathosterone is not UV active, its DAF-9 metabolites were scanned in negative-ion mode, revealing two peaks that were not present in the control microsome reactions. DAF-9 products of lathosterone eluted much earlier than lathosterone at 4.0 and 4.2 min (peaks 3 and 4), analogous to the pattern seen for 4-cholesten-3-one (Figure 2B and data not shown). Fractions from DAF-9 microsomal reactions subjected to reverse-phase HPLC were also tested for DAF-12 activation and rescue of *daf-9* null animals (Figures 2C–2F). Fractions corresponding to peaks 1–4 (i.e., the 4-cholesten-3-one and lathosterone derivatives) activated DAF-12 several hundred-fold in the absence of cotransfected DAF-9 (Figures 2C and 2D) and rescued *daf-9* null animals (Figures 2E and 2F). Again, the lathosterone derivatives were stronger at activating DAF-12 and rescuing *daf-9*, suggesting these compounds may be more abundant, potent, or efficacious.

Next, we utilized LC/MS as a first step in the identification of the activities in peaks 1–4. Based on the molecular weight of 4-cholesten-3-one ($[M + H]^+$ m/z = 385) and the retention times and mass spectra of the new compounds, peak 2 is consistent with a monohydroxylated derivative of 4-cholesten-3-one ($[M + H]^+$ m/z = 401) and peak 1 is consistent with a carboxylic acid derivative ($[M + H]^+$ m/z = 415) (Figure 2G). Further evidence that peak 1 represented a carboxylic acid derivative was found after a negative-ion scan in which a unique peak at 4.0 min was found only in the DAF-9 microsomes with a base peak at m/z 413 (Figure 2G). Peak 4, which was scanned in negative ion mode, yielded similar mass spectra to peak 1, consistent with the conclusion that peak 4 is the carboxylic acid derivative of lathosterone (Figure 2H). Finally, peak 3 contained one DAF-9-specific product at m/z 415 (Figure 2H). Although the identity of this peak remains unknown, the activity we observed in fractions 4 and 5 (from Figures 2B and 2D) tracks predominantly with peak 4 and not peak 3 (see Figure S1 in the Supplemental Data available with this article online).

DAF-9 Is a 3-Keto-Sterol-26-Monooxygenase

The finding that 3K-LCA was a weak activator of DAF-12 suggested that the position of oxidation of DAF-9 metabolites was on the side chain. The commercial availability of monohydroxylated derivatives of cholesterol permitted us to focus on defining the site of oxidation on the 4-cholesten-3-one metabolites of DAF-9. A panel of side chain oxidized 4-cholesten-3-one derivatives was generated by converting 5-cholesten-3β-ol (ring A, 3β-hydroxy-Δ^5) oxysterols (20(S)-OH-, 22(R)-OH-, 22(S)-OH-, 24-OH-, 25-OH-, (25R),26-OH-, and (25S),26-OH-cholesterol) into their respective 4-cholesten-3-one (ring B, 3-keto-Δ^4) oxysterols using cholesterol oxidase (Figure 3A). When tested in the cotransfection assay without DAF-9, two diastereomers of 26-hydroxy-4-cholesten-3-one ((25S),26-hydroxy-4-cholesten-3-one and (25R),26-hydroxy-4-cholesten-3-one) were strongly active (Figure 3B). In contrast, the ring A oxysterols were inactive, confirming the idea that DAF-12 ligands require a 3-keto group. Chromatographic separation of the ring B oxysterols and comparison to the 4-cholesten-3-one metabolites of DAF-9 resolved peak 2 (Figure 2A) into two peaks that coeluted with the diastereomers of 26-hydroxy-4-cholesten-3-one (Figure 3C). These results revealed that DAF-9 is a nonstereoselective 4-cholesten-3-one 26-hydroxylase.

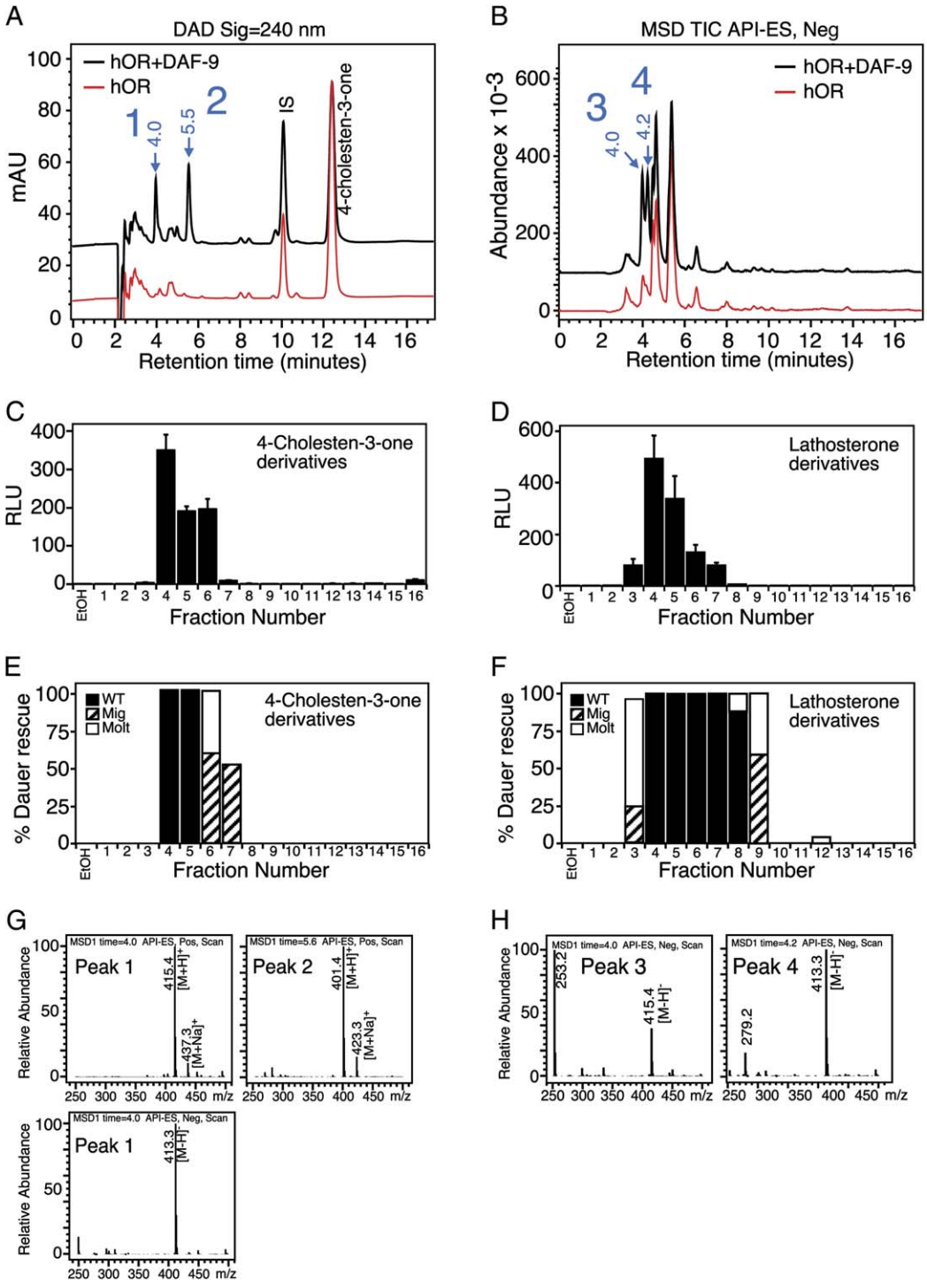

Figure 2. Identification of Carboxylated Metabolites of 4-Cholesten-3-One and Lathosterone as DAF-12 Agonists

(A) Representative UV chromatogram of 4-cholesten-3-one and (B) reconstructed total-ion-current chromatogram of lathosterone after incubation of 100 μM substrate with DAF-9 (black line) or control (red line) microsomes. Product peaks unique to DAF-9 and their retention times are shown in blue. IS, internal standard of 1,4-cholestadien-3-one.

(C–F) HPLC fractions from ten pooled DAF-9 microsomes incubated with 4-cholesten-3-one or lathosterone were tested for GAL4-DAF-12 activity in the absence of DAF-9 (C and D; n = 3 ± SD) or for rescue of *daf-9* null phenotypes (E and F). Fractions correspond to 1 min intervals of retention times in (A and B). Transfections and rescue assays are described in Figure 1 legend. Average number of worms tested in (E) and (F) were 75 and 125, respectively. (G and H) Mass spectra of DAF-9 metabolites of 4-cholesten-3-one (peaks 1 and 2) and lathosterone (peaks 3 and 4) scanned from *m/z* 250–500.

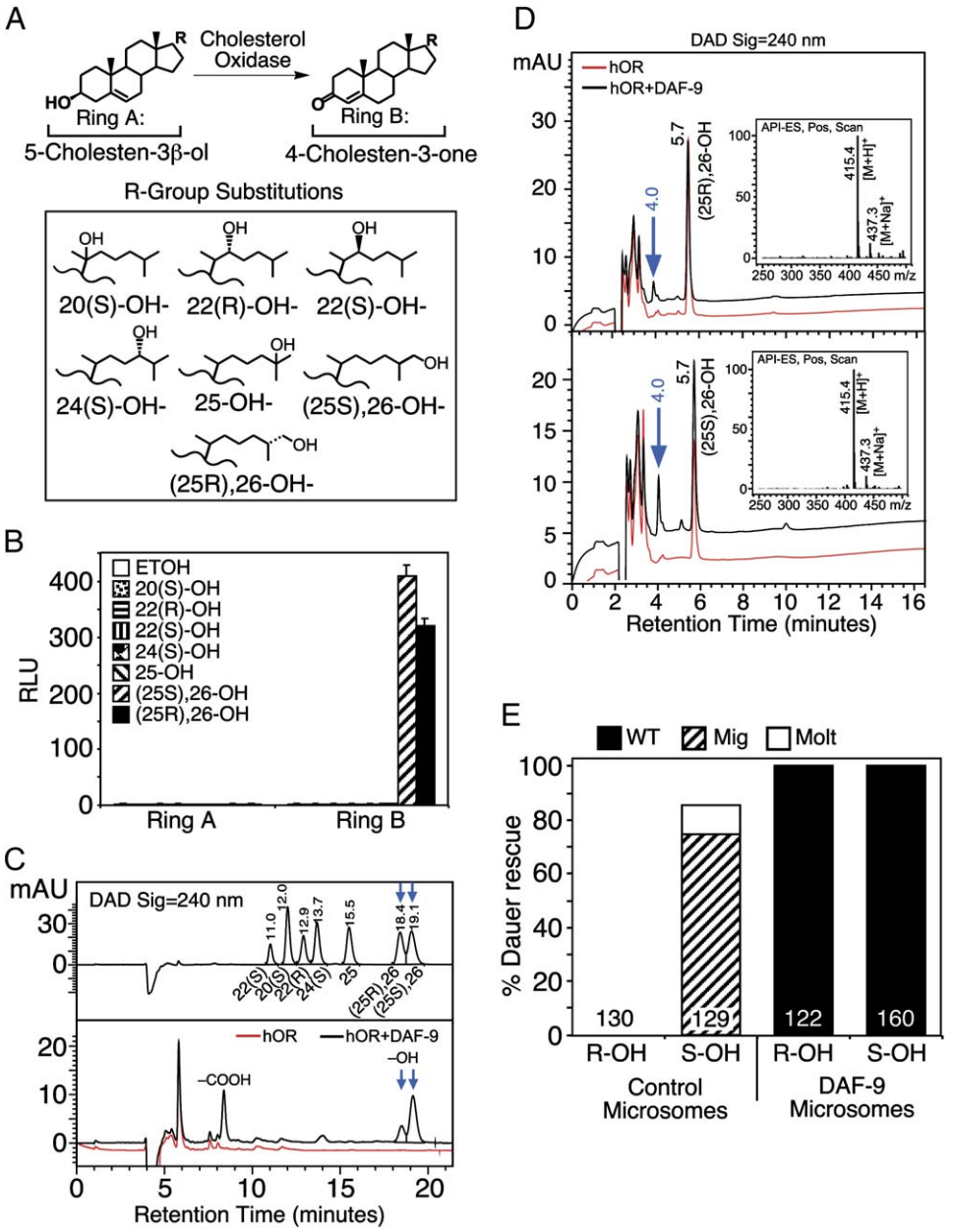

Figure 3. DAF-9 Is a 3-Keto-Sterol C-26 Monooxygenase

(A) Side chain substitutions of 5-cholesten-3β-ol (ring A) and 4-cholesten-3-one (ring B) derivatives.

(B) DAF-9-independent activation of GAL4-DAF-12 in HEK293 cells after incubation with the indicated sterols (10 μM for all sterols except 22(R)-hydroxy-4-cholesten-3-one, which was 4 μM). n = 3 ± SD.

(C) UV chromatogram of 4-cholesten-3-one oxysterols (top panel) compared to DAF-9 (black line) or control (red line) microsomes incubated with 100 μM 4-cholesten-3-one (bottom panel). Blue arrows indicate coeluting sterols. The carboxylic acid and alcohol metabolites of DAF-9 are indicated.

(D) UV chromatogram of DAF-9 (black line) and control (red line) microsomes after incubation with 100 μM (25R),26-hydroxy-4-cholesten-3-one (top panel) or (25S),26-hydroxy-4-cholesten-3-one (bottom panel). Arrows indicate products unique to DAF-9 microsomes. Mass spectra of DAF-9 metabolites (insets) were obtained in positive ion scan mode.

(E) Microsome reactions from (D) were diluted 8-fold and tested for *daf-9* rescue as in Figure 1D. Numbers indicate worms included in each experiment.

Given that DAF-9 microsomes produced both hydroxylated and carboxylated metabolites of 4-cholesten-3-one, we investigated whether DAF-9 oxidizes 4-cholesten-3-one at C-26 to produce both diastereomers of 26-hydroxy-4-cholesten-3-one and their corresponding carboxylic acids. Indeed, incubation of either (25S) or (25R),26-hydroxy-4-cholesten-3-one with DAF-9 microsomes resulted in the production of a single UV active peak at 4 min and

m/z 415 in positive-ion mode (Figure 3D). This retention time and mass spectral property were identical to the carboxylated metabolite found after incubation 4-cholesten-3-one with DAF-9 microsomes (Figure 2A, peak 1) and were not detected in control microsomal reactions (Figure 3D). In this context, DAF-9 substrate specificity required a 3-keto-Δ^4 structure, since the 3β-hydroxy-Δ^5-sterols, (25R) and (25S),26-hydroxycholesterol were not oxidized (data not shown). Finally, incubation of either sterol (25R),26-hydroxy-4-cholesten-3-one (R-OH) or (25S),26-hydroxy-4-cholesten-3-one (S-OH) with DAF-9 microsomes resulted in complete rescue of Daf-c and Mig phenotypes in 100% of animals tested (Figure 3E). In contrast, extracts from control microsomes incubated with the (25R)-sterol had no effect, while the (25S)-sterol caused an incomplete rescue (10% molt defects, 75% sterile Mig adults) resembling the activity in peak 2 of Figure 2A (Figure 3E). These results demonstrated that DAF-9 converts 4-cholesten-3-one into *daf-9* rescuing activities through successive oxidations at C-26, resulting in the production of carboxylic acid metabolites.

DAF-9 and Mammalian CYP27A1 Are Functional Orthologs

Like DAF-9, successive oxidation of sterol substrates at C-26 has been demonstrated for the mammalian cytochrome P450, CYP27A1 (Cali and Russell, 1991). Notably, in vitro studies have shown that CYP27A1 utilizes 4-cholesten-3-one more efficiently than cholesterol (Norlin et al., 2003). Therefore, we tested the ability of CYP27A1 to metabolize 4-cholesten-3-one into a DAF-12 activator. Cotransfection of HEK293 cells with GAL4-DAF-12, human or mouse CYP27A1, and bovine adrenodoxin resulted in strong activation of GAL4-DAF-12 (Figure S2). In contrast, DAF-12 was not activated by 4-cholesten-3-one in the presence of bovine adrenodoxin alone. Interestingly, cotransfection of CYP27A1 in the presence of 25μM lathosterone (which is a very good DAF-9 substrate) had no effect. Therefore, DAF-9 and CYP27A1 have similar enzymatic activities and overlapping, but distinct, substrate specificities.

3-Keto-4-Cholestenoic Acid Is a Hormonal Activator of DAF-12

To confirm C-26 carboxylic acids of 4-cholesten-3-one (i.e., 3-keto-4-cholestenoic acid) as the more potent DAF-9 metabolites, we synthesized their diastereomers (Figure 4A; Table S1) and tested their ability to transactivate DAF-12. The synthetic compounds exhibited chromatographic and mass spectral properties identical to the acidic metabolites obtained from DAF-9 microsomes (Figure S3). When tested in the cotransfection assay, DAF-12 responded to all four steroids with the following rank order of potencies: (25S),26-3-keto-4-cholestenoic acid (EC_{50} = 100 nM); (25R),26-3-keto-4-cholestenoic acid ($EC_{50} \geq 1$ μM); (25S),26-hydroxy-4-cholesten-3-one ($EC_{50} \geq 1$ μM); (25R),26-hydroxy-4-cholesten-3-one ($EC_{50} \geq 2$ μM) (Figure 4B). Activation by (25S),26-3-keto-4-cholestenoic acid was specific to DAF-12 and not observed with other *C. elegans*, *Drosophila*, and human nuclear receptors (Figure S4). In addition, the DAF-12 ligand binding domain mutants (R564C, R564H) were dramatically attenuated in their response to (25S),26-3-keto-4-cholestenoic acid (Figure S4) and did not respond to (25R),26-3-keto-4-cholestenoic acid (data not shown).

Consistent with its function as a DAF-12 hormonal ligand, (25S),26-3-keto-4-cholestenoic acid rescued Daf-c and Mig *daf-9* phenotypes (Figures 4C and 4D). At hormone concentrations of 250 nM, *daf-9* animals were indistinguishable from wild-type: they bypassed dauer diapause to become reproductive adults (Figures 4C and 4D) and reproduced like wild-type (~300 offspring; n = 5 worms). Rescued animals also had normal gonads, lacked dauer alae, and displayed normal pharyngeal expansion (Figure 4C). Without hormone, these animals produced all dauer progeny, confirming their *daf-9* genotype and demonstrating a lack of maternal rescue (data not shown). Similarly, the Mig phenotype of the weak allele *daf-9(rh50)* was reversed at 250 nM hormone (Figure 4E). At intermediate concentrations (50–100 nM), a proportion of null mutants exhibited Mig and molting defects, suggesting these phenotypes arise from a reduction in hormone levels (Figure 4D and data not shown). The 25R diastereomer of 3-keto-4-cholestenoic acid also rescued *daf-9* phenotypes, albeit at 5- to 10-fold higher concentrations (Figure 4D). Consistent with the data above (Figure 3E), (25R),26-hydroxy-4-cholesten-3-one had no effect on *daf-9* nulls, even at concentrations as high as 33 μM, while (25S),26-hydroxy-4-cholesten-3-one caused 93% of worms (n > 60) to bypass dauer but still exhibit Mig and/or molting defects and sterility (data not shown).

As predicted by cotransfection assays (Figure S4), *daf-12* LBD mutants were compromised in their ability to respond to (25S),26-3-keto-4-cholestenoic acid (Figure 4E). Hormone had no effect on *daf-12(rh61)*, which truncates the ligand binding domain and results in strong Mig phenotypes (Figure 4E). Another mutant, *daf-12(rh273)* contains a missense lesion at a predicted ligand contact site and gives Daf-c, Mig, and molting defects. Interestingly, at ≥ 250 nM (25S),26-3-keto-4-cholestenoic acid, Daf-c but not Mig or molt phenotypes were rescued (Figures 4E and 4F and data not shown), implying this mutation decreases ligand binding affinity. Together, these results revealed that 3-keto-4-cholestenoic acid can function as a *C. elegans* hormone that inhibits dauer formation and promotes reproductive development.

3-Keto-4-Cholestenoic Acid Acts Downstream of Insulin, TGFβ, and Cholesterol Lysosomal Transport Pathways

To further investigate the biological activity of 3-keto-4-cholestenoic acid, we tested its ability to rescue the dauer constitutive phenotypes of worms carrying mutations in the insulin-like receptor (*daf-2*), TGFβ (*daf-7*), and the Niemann-Pick type C1 homologs (*ncr-1;ncr-2*) positioned upstream of *daf-9* and *daf-12*. Accordingly, (25S),26-3-keto-4-cholestenoic acid completely rescued the Daf-c

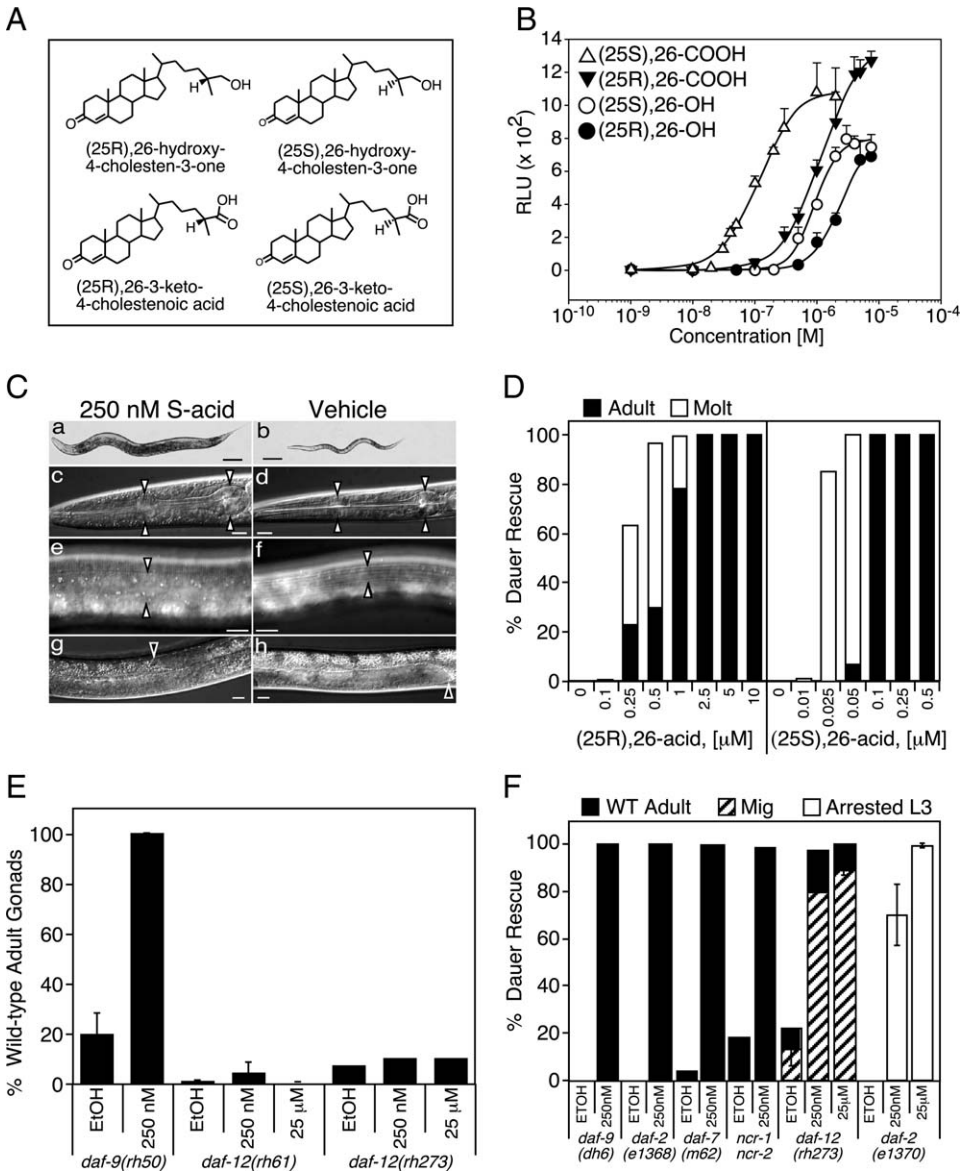

Figure 4. 3-Keto-4-Cholestenoic Acid Is a Hormonal Ligand of DAF-12

(A) Structures of 4-cholesten-3-one metabolites of DAF-9

(B) Dose response of GAL4-DAF-12 activation to 4-cholesten-3-one metabolites in HEK293 cells (n = 3 ± SD).

(C) DIC microscopy of *daf-9(dh6)* (a-f) and *daf-9(rh50)* (g and h) mutants treated with or without 250 nM (25S),26-3-keto-4-cholestenoic acid. (a) Rescued adult, (b) partial dauer, (c) head of rescued L3 larva, (d) head of partial dauer, (e) cuticle of rescued L3 larva, (f) dauer alae, (g) reflexed gonad of L3 larva, (h) unreflexed gonad of L3 larva.

(D) Response of *daf-9(dh6)* nulls treated with (25S),26-3-keto-4-cholestenoic acid or (25R),26-3-keto-4-cholestenoic acid. Results expressed as percentage of worms rescued from dauer after 3 days at 20°C. Worms were scored as adults or molt-defective larvae.

(E) Rescue of Mig phenotypes by (25S),26-3-keto-4-cholestenoic acid. Results expressed as percentage of reflexed gonadal arms scored after 3 days at 20°C (n > 60 from at least two independent experiments ± SD).

(F) Rescue of dauer phenotypes by (25S),26-3-keto-4-cholestenoic acid. Also shown by different shading are the percentage of dauer-rescued worms that exhibited wild-type adult (black bar) or Mig (striped bar) gonads or an arrested L3 phenotype (white bar). Dauer rescue was scored after 2 days at 25°C (*daf-2* and *daf-7*) or 3 days at 20°C (*daf-9*, *daf-12*, and *ncr-1;ncr-2*). n > 200 from at least two independent experiments ± SD.

phenotypes of all these mutants (Figure 4F). However, the stronger *daf-2* mutant (*e1370*) circumvented dauer morphogenesis but remained developmentally arrested as dark L3-like larvae, consistent with the similar phenotype seen in *daf-2;daf-12* double mutants (Vowels and Thomas, 1992). These results imply that insulin/IGF signaling must impinge upon the pathway both upstream, downstream, or parallel to hormone production.

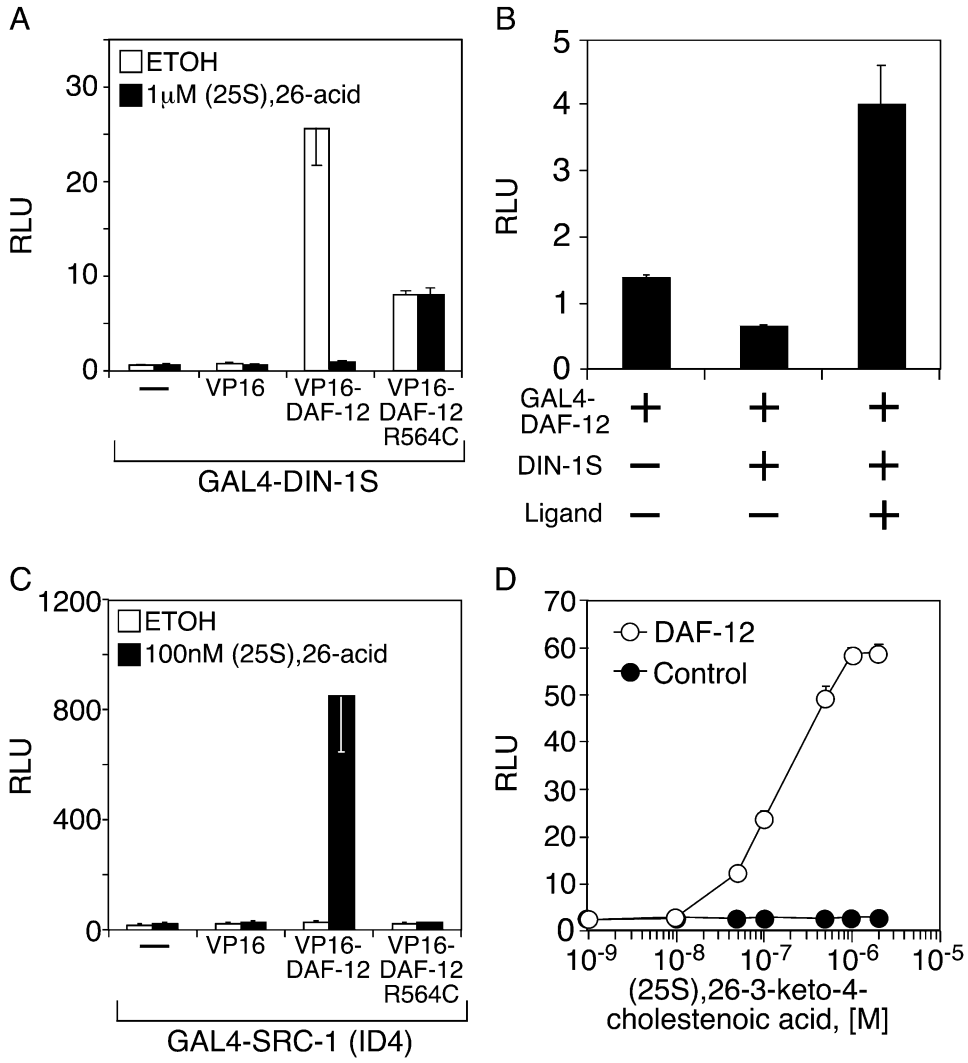

Figure 5. 25(S),26-3-Keto-4-Cholestenoic Acid Functions as a Classical Nuclear-Receptor Ligand

(A) Ligand-dependent interaction of DAF12 with DIN-1S by mammalian two-hybrid analysis.

(B) Effect of DIN-1S on DAF-12 basal activation with (+) or without (—) 100 nM ligand. Cells were transfected with 45 ng/well DIN-1S and 15 ng/well GAL4-DAF-12.

(C) Ligand-dependent interaction of DAF12 with SRC-1(ID4) by mammalian two-hybrid analysis.

(D) Ligand-dependent activation of full-length DAF-12 on a *lit-1* kinase reporter gene.

Empty CMX or CMX-VP16 vectors were used as controls in (A) and (C). Ligand, (25S),26-3-keto-4-cholestenoic acid. RLU, relative light units. n = 3 ± SD.

3-Keto-Cholestenoic Acids Bind DAF-12 as Bona Fide Ligands

A hallmark of nuclear receptor agonists is their ability to diametrically regulate interactions with corepressors and coactivators. To test this, we utilized a mammalian GAL4-coregulator/VP16-receptor two-hybrid assay in HEK293 cells. In the absence of ligand, DIN-1S, a putative DAF-12 corepressor (Ludewig et al., 2004), interacted with DAF-12 as predicted (Figure 5A). Addition of 1 μM (25S),26-3-keto-4-cholestenoic acid completely abolished this interaction, supporting the conclusion that the hormone disrupts dauer-promoting complexes involving DAF-12 and DIN-1S. Hormone did not disrupt the interaction between DIN-1S and a DAF-12-R564C mutant. We also observed that DIN-1S-dependent repression of DAF-12 basal activity could be reversed by addition of 100 nM (25S),26-3-keto-4-cholestenoic acid (Figure 5B). In a similar coactivator interaction assay, (25S),26-3-keto-4-cholestenoic acid induced the interaction between the fourth receptor interaction domain (ID4) of the mammalian coactivator protein SRC-1 and DAF-12, but not mutant DAF-12-R564C (Figure 5C). We also tested transactivation of full-length DAF-12 on a luciferase reporter plasmid containing the DAF-12 binding sites of *lit-1* kinase,

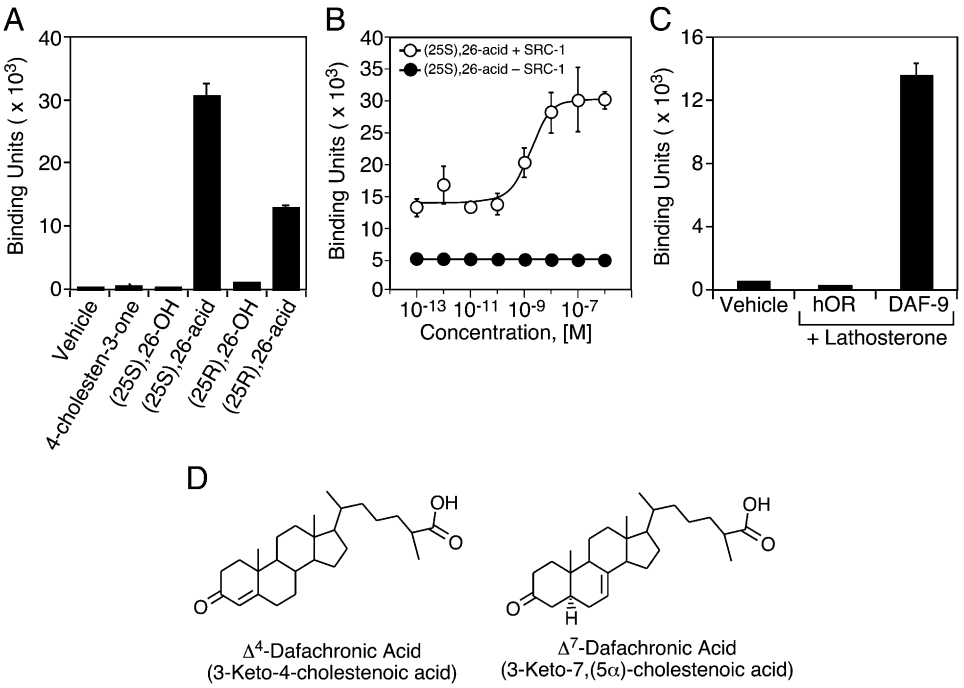

Figure 6. DAF-9 Metabolites of 4-Cholesten-3-One and Lathosterone Bind DAF-12 as High Affinity Ligands

(A–C) AlphaScreen assay for ligand-dependent coactivator recruitment to the DAF-12 ligand binding domain. Reactions were performed in the presence of the indicated sterols (1 μM) (A), increasing concentrations of (25S),26-3-keto-4-cholestenoic acid (B), or a 1:5000 dilution of DAF-9 or control microsomes incubated with 100 μM lathosterone (C). Results expressed as arbitrary binding units from triplicate assays (±SD).

(D) Structures of DAF-12 ligands.

a proposed DAF-12 target gene (Shostak et al., 2004). In the presence of (25S),26-3-keto-4-cholestenoic acid, DAF-12 transactivated the *lit-1* kinase reporter plasmid in a dose-dependent manner (EC_{50} = 100 nM; Figure 5D).

Finally, to determine the in vitro ligand binding properties of DAF-12, we employed an AlphaScreen assay, which detects ligand-dependent interactions between receptors and coactivator peptides (Xu et al., 2002). At 1 μM, the (25S) and (25R),26-3-keto-4-cholestenoic acids produced 58-fold and 24-fold increases in binding units, respectively, compared to vehicle control (Figure 6A). In contrast, the precursors, 4-cholesten-3-one and (25S),26-hydroxy-4-cholesten-3-one, showed no significant binding. Weak binding (2-fold) was detected for the less-active diastereomer, (25R),26-hydroxy-4-cholesten-3-one. Saturation binding kinetics revealed that (25S),26-3-keto-4-cholestenoic acid binds DAF-12 with high affinity (EC_{50} = 1 nM; Figure 6B). A similar analysis showed (25R),26-3-keto-4-cholestenoic acid binds DAF-12 but with >10-fold lower affinity (data not shown). Although the synthetic version of the lathosterone carboxylic acid (3-keto-7,(5α)-cholestenoic acid) is not available, strong ligand binding activity was detected in DAF-9 microsomes that were incubated with lathosterone (Figure 6C) and contain the carboxylic acid metabolite (Figure 2). From these results, we conclude that both the 3-keto-4-cholestenoic acid and 3-keto-7,(5α)-cholestenoic acid hormones (Figure 6D) mediate their effects in vivo through direct binding to DAF-12.

3-Keto-Cholestenoic Acids Are Endogenous, DAF-9-Dependent Hormones

A key prediction to the hypothesis that the 3-keto-cholestenoic acids are endogenous DAF-12 ligands is that they should be present in wild-type but not *daf-9* null worms. To that end, crude lipid extracts from both wild-type and *daf-9* null (*daf-9(e1406);daf-12(m20)*) animals were generated as described in the Experimental Procedures and analyzed for GAL4-DAF-12 activity in HEK293 cells. Wild-type worm extracts had strong DAF-12 activity, while as expected no activity was detected from *daf-9* null animals (Figure S5). Wild-type and *daf-9* extracts were then dissolved in methanol, fractionated by reverse-phase HPLC, and tested again for activity. DAF-12 activity was found only in HPLC fractions 4 and 5 from wild-type lipids but not *daf-9* null lipids (Figure 7A). Analysis of fractions 4 and 5 by selective ion monitoring (SIM) LC/MS showed that the activity was specifically associated with a peak at *m/z* 413 that was not detected in the inactive fractions from *daf-9* null animals (Figure 7B). The peak at *m/z* 413 was identical in mass and HPLC retention time to 3-keto-7,(5α)-cholestenoic acid (the carboxylic acid derivative of lathosterone) that was produced by DAF-9 (Figure 2B). The estimated endogenous concentration of this activity is ~200 nM (see Experimental Procedures for details), which is well within the predicted limit for activation of DAF-12. Although further purification was required to detect the 3-keto-4-cholestenoic acid (see

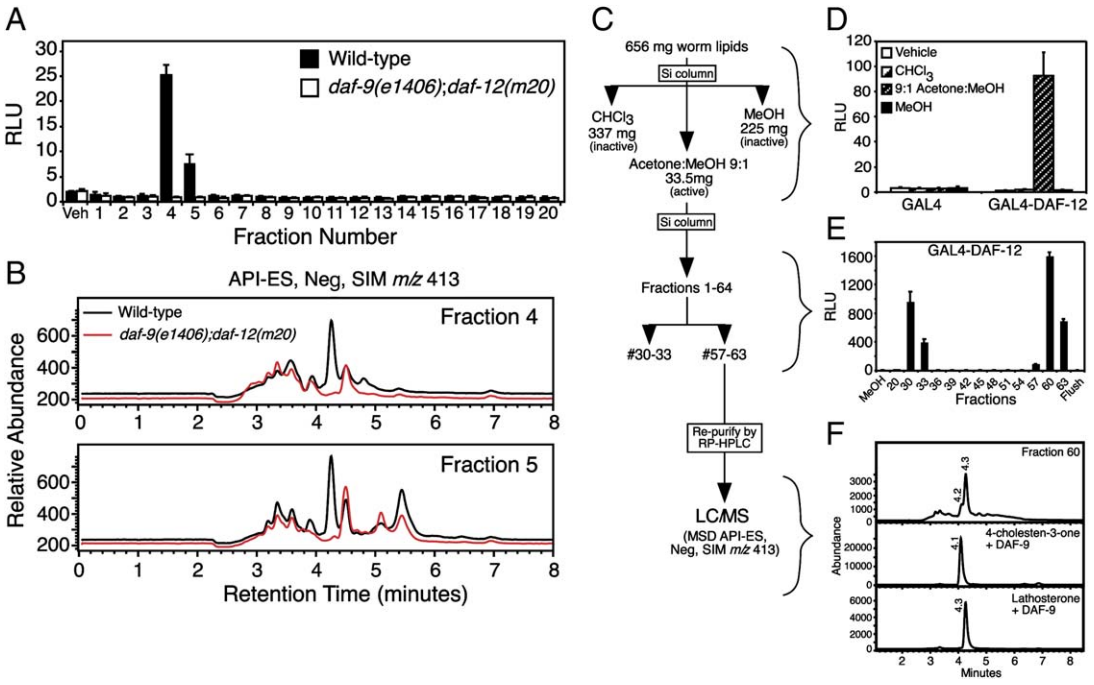

Figure 7. 3-Keto-Cholestenoic Acids Are Endogenous Hormones

(A) DAF-12 activation by HPLC fractions from crude lipid extracts from wild-type and *daf-9(e1406);daf-12(m20)* worms.

(B) LC/MS chromatograms of the HPLC fractions 4 and 5 in (A).

(C) Strategy used to purify endogenous DAF-12 agonists from *C. elegans* lipid extracts.

(D) DAF-12 activation by fractionated lipid extracts.

(E) DAF-12 activation by silica column fractions of lipids eluted with acetone:methanol.

(F) LC/MS analysis of pooled and repurified fractions 57–64 in negative SIM mode (*m/z* 413) compared with DAF-9 metabolites of 4-cholesten-3-one (middle panel) and lathosterone (bottom panel).

RLU, relative light units; n = 3 ± SD.

below), the fact that *daf-9* null animals have no detectable DAF-12 activity in any of the HPLC fractions indicates *daf-9* null worms lack significant amounts of either of the DAF-12 ligands, including 3-keto-4-cholestenoic acid.

To provide further evidence for the presence of the 3-keto-cholestenoic acid ligands in vivo, we purified lipid extracts from L3- to L4-staged worms. Crude extracts were fractionated by silica column chromatography (Figure 7C), and the DAF-12 activating fraction was determined to be in the acetone:methanol eluate (Figure 7D). Subsequent fractionation of this activity revealed the presence of two distinct DAF-12 activity peaks at fractions 30–33 and 57–63 (Figure 7E). Although the level of activity in fraction 30–33 was too low for further analysis, its chromatographic properties were consistent with the alcohol derivatives of 4-cholesten-3-one. However, after pooling and repurification by HPLC of fractions 57–63 enough material was obtained to identify the carboxylic acid derivatives by LC/MS (Figure 7F, upper panel). SIM mode identified a peak at *m/z* 413 in negative-ion mode with a retention time similar to the 3-keto-4-cholestenoic acid metabolite of DAF-9 (Figure 7F, middle panel). This signal correlated with DAF-12 activity, as it was not present in neighboring fractions that lacked activity (data not shown). As expected, a second peak of higher abundance was also detected that comigrated with the lathosterone metabolite, 3-keto-7,(5α)-cholestenoic acid (Figure 7F, bottom panel). Although the concentration of 3-keto-4-cholestenoic acid could not be determined with accuracy, its relative abundance in the LC/MS indicates the in vivo concentration is less than the 3-keto-7,(5α)-cholestenoic acid. These pooled fractions rescued the Daf-c and Mig phenotypes in 100% of *daf-9* null worms tested (n > 300). Taken together, these results provide further evidence that both of the 3-keto-cholestenoic acids (Figure 6D) are endogenous hormonal ligands of DAF-12.

DISCUSSION

Discovery of Ligands for a *C. elegans* Orphan Nuclear Receptor

In this paper, we detail the identification of 3-keto-4-cholestenoic acid and 3-keto-7,(5α)-cholestenoic acid as endogenous, hormonal ligands for DAF-12. Both compounds are 3-keto, C-26 oxidized derivatives of cholesterol that differ in the position of an unsaturated double bond at C-4 or C-7 of the steroid nucleus. Given their ability to govern dauer formation and heterochronic developmental

pathways, we propose to name these hormones Δ^4-dafachronic acid and Δ^7-dafachronic acid (Figure 6D). Consistent with other nuclear-receptor ligands, the stereochemistry of the DAF-12 ligand is an important determinant for binding. Thus, the (25S),26-carboxylic acid is ~10-fold more potent than the (25R),26-carboxylic acid as a DAF-12 ligand. Although most of the studies presented here focused on Δ^4-dafachronic acid (i.e., 3-keto-4-cholestenoic acid), we provide strong evidence to suggest that the C-26 carboxylic acid metabolite of lathosterone (i.e., Δ^7-dafachronic acid) is also a bona fide DAF-12 ligand. Like Δ^4-dafachronic acid, the Δ^7-dafachronic acid was shown to bind and transactivate DAF-12, rescue *daf-9* worms, and was present in the most active DAF-9 metabolites and lipid extracts from wild-type (but not *daf-9* null) worms at physiologically relevant concentrations. In fact, the activity and concentration of Δ^7-dafachronic acid suggest that it is more abundant and efficacious in vivo than Δ^4-dafachronic acid. Further evidence of the potency of Δ^7-dafachronic acid as a DAF-12 ligand awaits its de novo chemical synthesis. Taken together, the dafachronic acids identified here represent the first ligands for a nematode nuclear receptor and are the first steroid hormones identified in *C. elegans*.

An interesting aspect of this work is the finding that multiple DAF-12 ligands may exist. The ligands identified by our approach were shown to exist in the most active lipid fractions, but the possibility remains that other chemically similar ligands may exist in these fractions as well. Our work provides evidence for at least two ligands, raising the intriguing possibility that each hormone may govern overlapping but distinct transcriptional networks. Thus far, the dafachronic acids have similar activities in preventing dauer formation and promoting gonadal migration. However, it remains possible that these or other ligands differentially affect other DAF-12-dependent functions, such as life span. For example, DAF-9 and DAF-12 have been shown paradoxically to be required for both inhibition and extension of life span (Gerisch et al., 2001; Jia et al., 2002; Larsen et al., 1995; Hsin and Kenyon, 1999; Gems et al., 1998). Future work is being directed toward determining whether the two dafachronic acids may be responsible for these opposing activities or whether other discriminating ligands also exist. In addition, these ligands should provide useful tools for characterizing DAF-12 target genes and the biology of this hormone-receptor system.

Evolutionary Conservation of Steroid Hormone Endocrinology

Since its discovery, DAF-9 has been postulated to play a key role in the synthesis of a cholesterol-derived hormone that serves as a DAF-12 ligand and controls dauer formation and reproductive development in *C. elegans* in an endocrine fashion (Gerisch et al., 2001; Jia et al., 2002; Gerisch and Antebi, 2004; Mak and Ruvkin, 2004). The work presented here provides the first direct evidence for the involvement of DAF-9 in a steroidogenic pathway that is conserved from worms to mammals. As with mammalian steroidogenic enzymes, DAF-9 appears to act at a key position in the pathway where it generates nuclear-receptor ligands. The selectivity of DAF-9 for 3-keto-sterols implies that enzymes analogous to mammalian 3β-hydroxysteroid dehydrogenases (3β-HSD), which are essential for production of all active steroid hormones in vertebrates, as well as the short-chain oxidoreductases (SCOR), participate upstream of the production of these DAF-9 substrates. Interestingly, a search of the *C. elegans* genome reveals four genes (Y6B3B.11, ZC8.1, C32D5.12, ZC449.6) homologous to human 3β-HSD family members and 84 genes homologous to the SCOR family. Already, a recent finding by our group suggests a unique Rieske-like oxygenase, *daf-36*, is involved in the conversion of cholesterol into 7-dehydrocholesterol, a potential precursor of Δ^7-dafachronic acid (Rottiers et al., 2006). Finally, we note that DAF-9 is a functional ortholog of mammalian CYP27A1, a cytochrome P450 that yields sterol-derived ligands for the bile acid receptor, FXR (Russell, 2003). These findings support the notion that oxidation of sterols to generate signaling molecules coincided with nuclear-receptor ligand binding and was an acquired trait that occurred throughout evolution.

Multiple Endocrine Networks Control Hormone Production

Genetic analysis of the dauer formation pathway has led to the identification of a network of genes that couples environmental signals to the selection of alternative developmental pathways of dauer arrest or reproduction. In favorable environments, these networks utilize peptide hormones such as insulin and TGFβ to positively influence hormone production in *C. elegans*. Although the exact mechanisms have not been elucidated, our results demonstrate that this regulation may lie at the level of hormone production as Δ^4-dafachronic acid potently rescued the dauer arrest phenotypes of mutations in *daf-2* and *daf-7*. Surprisingly, strong *daf-2* mutants were rescued from dauer but could not fully execute reproductive development. This result implies an additional requirement for insulin signaling in reproductive development that may lie parallel to hormone action. Finally, we show that Δ^4-dafachronic acid rescues the Daf-c phenotype of the *ncr-1;ncr-2* mutant, confirming previous findings that the products of these genes likely regulate hormone signaling upstream of *daf-9* at the level of substrate availability (Li et al., 2004). It will be interesting to see whether similar networks link dietary cues to reproduction in vertebrates.

Perspectives

As demonstrated by this work, a distinct advantage to working in *C. elegans* as a model system is the ability to combine the predictive power of genetics with biochemistry to elucidate physiologic pathways. Given the previous success of nuclear receptor pharmacology in vertebrates, this work also raises the possibility of targeting nuclear receptors in parasitic nematodes as a strategy for controlling their growth. The discovery of dafachronic acids as

nematode hormones and the adoption of the orphan nuclear receptor DAF-12 set an important precedent for further work aimed at identifying ligands for many of the other *C. elegans* orphan receptors. For now, that's one down and 283 to go….

EXPERIMENTAL PROCEDURES

Chemical Reagents

4-cholesten-3-one, lathosterol, 20S-hydroxycholesterol, 22S-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 3-keto-lithocholic acid, 7-keto-lithocholic acid, 12-keto-lithocholic acid were purchased from Steraloids, Inc. Lophenol, (25S),26-hydroxycholesterol, and (25R),26-hydroxycholesterol were purchased from Research Plus. Deuterated chenodeoxycholic acid (CDCA-d4) was from C/D/N Isotopes. Unless otherwise noted, all other reagents were purchased from Sigma.

Sterol Synthesis

3-Keto-Δ^4-oxysterol derivatives were generated with cholesterol oxidase and catalase as described (Zhang et al., 2001). Lathosterone and lophenone were generated by reacting 2.5 molar equivalents of Dess-Martin reagent with one equivalent of each sterol at 25C, and then purified by silica chromatography with 95:5 hexane:ethylacetate. To oxidize the alcohols into C-26 acids, Jones reagent (0.14 ml, 0.15 mmol) was added dropwise to a stirred solution of each alcohol (12 mg, 0.03 mmol) in acetone (4 ml) at 0°C. After stirring for 1 hr, the reaction was quenched with isopropanol, and the product was extracted with diethyl ether. The organic phase was washed with saturated $NaHCO_3$, dried over solid Na_2SO_4, filtered, and concentrated in vacuo. Crude extracts were chromatographed on silica gel, and the product (10 mg, 90% yield) eluted with 40% ethyl acetate in hexane. All structures were confirmed by MS, UV spectra, and ^{13}C- (data not shown) and ^1H-NMR (Table S1).

Nematode and Bacterial Strains

Worms were grown on NGM agar with OP50 bacteria at 20°C unless noted otherwise (Brenner, 1974). Strains used were as follows: *daf-9(dh6) dhEx24* (containing the cosmid T13C5 and pTG96 [*sur-5::gfp*]), *daf-9(rh50)*, *daf-12(rh273)*, and *daf-12(rh61)*; *daf-2(e1368)*, *daf-2(e1370)*, *daf-7(m62)*, and N2 (from the CGC); *ncr-1(nr2023)ncr-2(nr2022)* (from J.H. Thomas); and *daf-9(e1406);daf-12(m20)* (from D. Riddle).

Plasmids

Mammalian expression plasmids were cloned into CMX vectors (Umesono et al., 1991; Willy et al., 1995). NHR-8 (NM_171382, wormbase F33D4.1a), NHR-23 (C01H6.5b), DAF-9 (NM_171699, wormbase T13C5.1b), and DIN-1S (wormbase F07A11.6d) were obtained by RT-PCR from mixed stage or L2-/L3-staged animals. Other cDNAs used were DAF-12 (from K. Yamamoto); CMV-hCYP27A1, CMV-mCYP27A1, CMV-adrenodoxin (from D. Russell); human P450 oxidoreductase (Open Biosystems). GAL4 DNA binding domain fusions were generated with amino acid sequences 184–754 aa (DAF-12), 92–561 aa (NHR-8), 78–361 aa (NHR-23), 2–567 aa (DIN-1S). The VP16 activation domain was fused to residues 2–754 of DAF-12 to make VP16-DAF-12. DAF-12 mutants were generated by site-directed mutagenesis. The 4.2 genomic fragment from pODLO_82 (from K. Yamamoto) was inserted into the reporter plasmid tk-luc to make lit-1K-tk-luc. DAF-9 and hOR baculovirus expression plasmids were created using the pFastBac Dual system (Invitrogen).

Cell Culture and Cotransfection Assay

Cotransfections in HEK293 cells were performed in 96-well plates as described (Makishima et al., 1999) using 50 ng of luciferase reporter, 20 ng CMX-β-galactosidase reporter, 15 ng of CMX receptor expression plasmid, and control plasmid to maintain 150 ng/well. Candidate ligands were added at 4000-fold dilution 8 hr posttransfection. Luciferase activities were normalized to the β-galactosidase control. Data represent the mean ± SD of triplicate assays.

Preparation of DAF-9 and Control Microsomes from Sf9 Cells

Sf9 ($2x10^6$ cells/ ml) were cultured in SF-900 SFM and infected with baculovirus (MOI = 2–4) in medium containing 0.5μg/ml hemin chloride, 100μM δ-amino-levulinic acid, and 100μM ferric citrate. Cells were harvested 60 hr post-infection and microsomes prepared as described (Hood et al., 1996).

DAF-9 Microsomal Incubations

Microsomes containing DAF-9/hOR or hOR alone (as a control) were generated from *Sf9* cells, thawed on ice, and brought to 0.5 mg/ml in 0.1 M potassium phosphate buffer containing a NADPH regenerating system (50 U/ml DL-isocitrate dehydrogenase, 0.1 M isocitrate and 0.1 M $MgCl_2$). Substrates were added at 100 μM in 0.5 ml total volume, preincubated 3 min at 37C, and then reacted with 1 mM NADPH for 16 hr. Reactions were processed by extracting twice with 2 ml methyl-tert-butyl-ether, combining the top layers and drying under nitrogen. In some experiments, 0.5 μg of 1,4-cholestadiene-3-one was added as an internal standard for the extraction.

Rescue Assays

Microsomal extracts were resuspended in 50 μl methanol, mixed with 5× concentration of HB101 bacterial paste, vacuum dried, resuspended in 100 μl 5× concentrated HB101, and plated on 3 cm plates containing 4 ml NG agar. For rescue, ~200 embryos from a 4–8 hr egg laying were transferred onto the dried bacterial lawn. Mixtures of *daf-9*(+),*gfp*(+) and *daf-9*(−),*gfp*(−) embryos were placed on agar plates containing a mixture of bacteria and extracts from either DAF-9 or control microsomal reactions. GFP expressing worms were removed after 48 hr and the remaining *daf-9*(−),*gfp*(−) animals were scored for dauer arrest 24 hr later. For rescue experiments using pure steroids, 10 μl of compounds were mixed with 5× (90 μl) concentrated OP50 bacteria and plated. Final concentrations refer to the amount plated in agar (3–4 ml/plate). Strains tested were grown reproductively onto regular NG agar for two generations at 20C.

C. elegans Lipid Extracts

Worms were grown on twenty 10 cm NGM plates seeded with HB101 bacteria. Gravid adults were bleached and the resulting embryos incubated in 2.8 liter Fernbach flasks containing 100–350 ml S medium supplemented with 5 μg/ml Nystatin, 50 μg/ml streptomycin sulfate overnight to allow synchronization of L1s (Stiernagel, 1999). Two to three successive rounds of growth (with 1%–2% HB101) and lysis of gravid adults were performed until ~20-100 million synchronized L1 larvae were obtained. In Figures 7A and 7B, growth of wild-type and *daf-9;daf-12* was performed in Fernbach flasks at 22.5C for 48 hr until just prior to dorsal turn of distal tip cells. In Figures 7C–7F, final growth to the L3/L4 stage was performed in a 15 l New Brunswick BioFloIV fermentor, with a working volume of 10.5 l at 20C with agitation (100 RPM, 25% O_2 saturation). Worms were harvested and bacteria and debris were removed by sucrose flotation, then frozen in liquid nitrogen and stored at −80C. Thawed worms were lyophilized for measurement of dry weight, resuspended in 0.1M NaCl, and homogenized using an Emulsi-flex C-5 homogenizer (Avestin). Total lipids (plus 1 μg CDCA-d4/10^7 worms) were extracted with 2:1 chloroform:methanol. The resulting chloroform layer was back-extracted with two-thirds volume of water. The final chloroform layer was dried with Na_2SO_4, filtered through Whatman filter paper and concentrated in vacuo. The resulting extract (~100 mg/10^7 worms) was resuspended in chloroform, adsorbed to a silica column, and lipids eluted in three fractions with 100 ml chloroform, 200 ml 9:1 acetone:methanol, and 100 ml methanol/100 mg extract (Figure 7C). The 9:1 acetone:methanol extract was further fractionated by silica chromatography using

chloroform and increasing concentrations of methanol to 100%. Fractions were dried under nitrogen and tested for DAF-12 activation.

LC/MS Analysis

Samples were analyzed by LC/MS using a DAD in tandem with an MSD single quadropole instrument (Agilent Technologies) with API-ES in both positive and negative ion modes. Samples were dissolved in methanol and loaded onto a precolumn (Zorbax C_8, 4.6 × 12.5 mm, 5 μm, Agilent) at 4 ml/min for 1 min with 30:70 methanol/water, both containing 5 mM NH_4Ac, and then back flushed onto the analytical column at 0.4 ml/min (Zorbax C_{18}, 4.6 × 50 mm, 5 μm, Agilent). The mobile phase consisted of methanol (A) and methanol/acetonitrile/water (60:20:20) (B), both containing 5 mM (NH_4Ac). The following gradient was run for a total of 20 min: 0–6.5 min, 75% to 100% (A); 6.5–18 min, 100% (A); 18.1–20 min, 75% (A). MS parameters were as follows: gas temperature 350°C, nebulizer pressure 30 psig, drying gas (nitrogen) 12 l/min, VCap (positive and negative) 4000V, fragmentor voltage 150V (positive ions) or 200V (negative ions). For experiments in scan mode, mass ranges between *m/z* 250–500 were used. Using SIM (in positive-ion mode), signals for $[M + H]^+$ ions were observed for 4-cholesten-3-one (*m/z* 385, retention time [RT] 12.5 min), lathosterone (*m/z* 384, RT 14.0 min), 1,4-cholestadien-3-one (*m/z* 383, RT 10.2 min), (25R/S),26-hydroxy-4-cholesten-3-one (*m/z* 401, RT 5.7 min), (25R/S),26-3-keto-4-cholestenoic acid (*m/z* 425, RT 4.0 min). SIM LC/MS in negative-ion mode gave signals for $[M - H]^-$ ions of (25R/S),26-3-keto-4-cholestenoic acid (*m/z* 413, RT 4.0 min) and CDCA-d_4 (*m/z* 395, RT 3.9 min). Positive and negative ions were monitored simultaneously in SIM mode. Separation of 4-cholesten-3-one oxysterols was achieved as described (Uomori et al., 1987).

Calculation of Endogenous DAF-12 Ligand Concentration

The efficiency of lipid extraction using the CDCA-d_4 internal standard was 71% for wild-type and 85% for *daf-9(e1406);daf-12(m20)* worms. Crude lipid extracts (792 mg from 50 × 10^6 wild-type worms, 230 mg from 19 × 10^6 *daf-9* null worms) were suspended in methanol at 100 mg/ml, filtered through a PVDF membrane, diluted 10-fold and injected for LC/MS analysis as described above. The calculated concentration of the *m/z* 413 peak was 81 ng/ml (208 nM/worm) based on an external calibration curve using 3-keto-4-cholestenoic acid and an L4-stage worm volume of 1.5 nl.

Binding Assay

DAF-12 ligand binding domain (aa 507–753) was expressed in BL21(DE3) cells as a 6× His-GST fusion protein using pET24a (Novagen). Ligand binding was determined by AlphaScreen assays from Perkin-Elmer (Xu et al., 2002) with 40 nM receptor and 40 nM of biotinylated SRC1-4 (QKPTSGPQTPQAQQKSLLQQLLTE) peptide in the presence of 5 μg/ml donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 50 mM CHAPS, and 0.1 mg/ml bovine serum albumin at pH 7.4. EC_{50} binding values were determined from nonlinear least square fit of the data based on an average of three experiments.

Supplemental Data

Supplemental Data include five figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/124/6/1209/DC1/.

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REFERENCES

Albert, P.S., and Riddle, D.L. (1988). Mutants of Caenorhabditis elegans that form dauer-like larvae. Dev. Biol. *126*, 270–293.

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans. Development *125*, 1191–1205.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes Dev. *14*, 1512–1527.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Cali, J.J., and Russell, D.W. (1991). Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. J. Biol. Chem. *266*, 7774–7778.

Chitwood, D.J. (1999). Biochemistry and function of nematode steroids. Crit. Rev. Biochem. Mol. Biol. *34*, 273–284.

Chitwood, D.J., Lusby, W.R., Lozano, R., Thompson, M.J., and Svoboda, J.A. (1983). Novel nuclear methylation of sterols by the nematode Caenorhabditis elegans. Steroids *42*, 311–319.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., and Riddle, D.L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics *150*, 129–155.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development *131*, 1765–1776.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. Dev. Cell *1*, 841–851.

Gill, M.S., Held, J.M., Fisher, A.L., Gibson, B.W., and Lithgow, G.J. (2004). Lipophilic regulator of a developmental switch in Caenorhabditis elegans. Aging Cell *3*, 413–421.

Gissendanner, C.R., Crossgrove, K., Kraus, K.A., Maina, C.V., and Sluder, A.E. (2004). Expression and function of conserved nuclear receptor genes in Caenorhabditis elegans. Dev. Biol. *266*, 399–416.

Hood, S.R., Shah, G., and Jones, P. (1996). Expression of cytochromes P450 in a baculovirus system. In Methods in Molecular Biology, I.R. Phillips and E.A. Shephard, eds. (Totowa, NJ: Humana Press), pp. 203–217.

Hsin, H., and Kenyon, C. (1999). Signals from the reproductive system regulate the life span of C. elegans. Nature *399*, 362–366.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development *129*, 221–231.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science *277*, 942–946.

Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics *139*, 1567–1583.

Li, J., Brown, G., Ailion, M., Lee, S., and Thomas, J.H. (2004). NCR-1 and NCR-2, the C. elegans homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. Development *131*, 5741–5752.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes Dev. *18*, 2120–2133.

Mak, H.Y., and Ruvkin, G. (2004). Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development *131*, 1777–1786.

Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J., and Shan, B. (1999). Identification of a nuclear receptor for bile acids. Science *284*, 1362–1365.

Matyash, V., Entchev, E.V., Mende, F., Wilsch-Brauninger, M., Thiele, C., Schmidt, A.W., Knolker, H.J., Ward, S., and Kurzchalia, T.V. (2004). Sterol-derived hormone(s) controls entry into diapause in Caenorhabditis elegans by consecutive activation of DAF-12 and DAF-16. PLoS Biol. *2*, e280 10.1371/journal.pbio.0020280.

Merris, M., Wadsworth, W.G., Khamrai, U., Bittman, R., Chitwood, D.J., and Lenard, J. (2003). Sterol effects and sites of sterol accumulation in Caenorhabditis elegans: developmental requirement for 4alpha-methyl sterols. J. Lipid Res. *44*, 172–181.

Mooijaart, S.P., Brandt, B.W., Baldal, E.A., Pijpe, J., Kuningas, M., Beekman, M., Zwaan, B.J., Slagboom, P.E., Westendorp, R.G., and van Heemst, D. (2005). C. elegans DAF-12, nuclear hormone receptors and human longevity and disease at old age. Ageing Res. Rev. *4*, 351–371.

Norlin, M., von Bahr, S., Bjorkhem, I., and Wikvall, K. (2003). On the substrate specificity of human CYP27A1: implications for bile acid and cholestanol formation. J. Lipid Res. *44*, 1515–1522.

Ohkura, K., Suzuki, N., Ishihara, T., and Katsura, I. (2003). SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in C. elegans. Development *130*, 3237–3248.

Ren, P., Lim, C.S., Johnsen, R., Albert, P.S., Pilgrim, D., and Riddle, D.L. (1996). Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science *274*, 1389–1391.

Riddle, D.L., and Albert, P.S. (1997). Genetic and environmental regulation of dauer larva development. In C. elegans II, D.L. Riddle, B. Meyer, J. Priess, and T. Blumenthal, eds. (Colds Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 739–768.

Rottiers, V., Motola, D.L., Gerisch, B., Cummins, C.L., Nishiwaki, K., Mangelsdorf, D.J., and Antebi, A. (2006). Hormonal control of *C. elegans* dauer formation and life span by a Rieske-like oxygenase. Dev. Cell, in press.

Russell, D.W. (2003). The enzymes, regulation, and genetics of bile acid synthesis. Annu. Rev. Biochem. *72*, 137–174.

Schackwitz, W.S., Inoue, T., and Thomas, J.H. (1996). Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron *17*, 719–728.

Shostak, Y., Van Gilst, M.R., Antebi, A., and Yamamoto, K.R. (2004). Identification of C. elegans DAF-12-binding sites, response elements, and target genes. Genes Dev. *18*, 2529–2544.

Sluder, A.E., and Maina, C.V. (2001). Nuclear receptors in nematodes: themes and variations. Trends Genet. *17*, 206–213.

Stiernagel, T. (1999). Maintenance of C. elegans. In C. elegans: A. Practical Approach, I.A. Hope, ed. (New York: Oxford University Press), pp. 51–67.

Thomas, J.H., Birnby, D.A., and Vowels, J.J. (1993). Evidence for parallel processing of sensory information controlling dauer formation in Caenorhabditis elegans. Genetics *134*, 1105–1117.

Umesono, K., Murakami, K.K., Thompson, C.C., and Evans, R.M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell *65*, 1255–1266.

Uomori, A., Seo, S., Sato, T., Yoshimura, Y., and Takeda, K. (1987). Synthesis of (25R)-[26-$^{2}H_{1}$]Cholesterol and ^{1}H N.m.r., and H.p.l.c. Resolution of (25R)- and (25S)-26-Hydroxycholesterol. J. Chem. Soc. Perkin Trans. I *1*, 1713–1718.

Van Gilst, M.R., Hadjivassiliou, H., Jolly, A., and Yamamoto, K.R. (2005a). Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in C. elegans. PLoS Biol. *3*, e53 10.1371/journal.pbio.0030053.

Van Gilst, M.R., Hadjivassiliou, H., and Yamamoto, K.R. (2005b). From the cover: a Caenorhabditis elegans nutrient response system partially dependent on nuclear receptor NHR-49. Proc. Natl. Acad. Sci. USA *102*, 13496–13501.

Vowels, J.J., and Thomas, J.H. (1992). Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. Genetics *130*, 105–123.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Mangelsdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev. *9*, 1033–1045.

Xu, H.E., Stanley, T.B., Montana, V.G., Lambert, M.H., Shearer, B.G., Cobb, J.E., McKee, D.D., Galardi, C.M., Plunket, K.D., Nolte, R.T., et al. (2002). Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. Nature *415*, 813–817.

Zhang, Z., Li, D., Blanchard, D.E., Lear, S.R., Erickson, S.K., and Spencer, T.A. (2001). Key regulatory oxysterols in liver: analysis as delta4–3-ketone derivatives by HPLC and response to physiological perturbations. J. Lipid Res. *42*, 649–658.