IDENTIFICATION OF LIGANDS FOR THE ORPHAN NUCLEAR RECEPTOR DAF-12

THAT GOVERN DAUER FORMATION AND REPRODUCTION IN *C. ELEGANS*

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For Barrie, Sol, Lauren, Gabrielle, and Tingting

For their endless support and encouragement.

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by

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The orphan nuclear hormone receptor, DAF-12, plays a central role in the physiology of free-living nematode, *C. elegans*. DAF-12 is best known for its role in regulating dauer formation, a non-reproductive larval state entered in harsh environments and marked by developmental arrest, stress resistance, and extended life-span. Genetic screens for genes controlling dauer formation have identified conserved endocrine signaling pathways that

converge on DAF-12 to influence the choice between dauer formation and reproductive development. Detailed genetic analysis of these signaling pathways suggests that they promote reproductive development in favorable environments by influencing the production of a ligand for DAF-12 by the cytochrome p450, DAF-9. Despite abundant evidence for hormonal control, the identity of the DAF-12 ligand has remained elusive.

Using a cell-based ligand screening assay I initially identified a group of 3-keto-containing sterols that potently activated DAF-12 in a DAF-9-dependent manner. Subsequent analysis using a variety of techniques showed that DAF-9 catalyzes the non-stereo-selective addition of a carboxylic acid to the terminal side-chain methyl groups of 3-keto-sterols, producing 3-keto-cholestenoic acids. In collaboration with the laboratory of Dr. Eric Xu, we demonstrated that 3-keto-cholestenoic acids, referred to as dafachronic acids, directly bind to DAF-12 as bona fide ligands. In collaboration with Dr. Adam Antebi we found that these ligands also potently rescued the phenotypes resulting from mutations in *daf-9* or its upstream activating genes. Dafachronic acids are also shown to be endogenous hormones, as they could be detected in DAF-12 activating lipid fractions from wild-type but not *daf-9* null worms. Taken together, this work defines 3-keto-cholestenoic acids as the first hormonal ligands for an invertebrate orphan nuclear receptor and the first endogenous steroid hormones in nematodes. In addition, these findings demonstrate that steroid hormone control of reproduction is conserved from worms to humans. Finally, given the existence of DAF-12 homologs in parasitic nematodes, this work raises the possibility of targeting DAF-12 in parasitic nematodes as a means for controlling their growth.

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3K-LCA	3Keto-Lithocholic Acid
3β-HSD	3-beta-hydroxysteroid dehydrogenase
6K-LCA	6-keto-Lithocholic Acid
7K-LCA	7-Keto-Lithocholic Acid
AceyDAF-12	A. cyelanicum DAF-12
AF-2	Activation Function 2
Apo-RXR	Unliganded RXR
AR	Androgen Receptor
BMP	Bone Morphogenic Protein
C	Carboxy
CA	Cholic Acid
CAR	Constitutive Androstane Receptor
CDCA	Chenodeoxy Cholic Acid
CeDAF-12	C. elegans DAF-12
CeNHRs	*C. elegans* Nuclear Hormone Receptors
$CHCl_3$	Chloroform
cGMP	Cyclic Guanosine Monophosphate
daf	dauer formation
Dafachronic acid	(25S),26-3keto-cholestenoic acic
Daf-c	dauer formation constitutive
Daf-d	Dauer formation defective
DBD	DNA binding domain
DCA	Deoxycholic Acid
DR	Direct Repeat
EcR	Ecdysone Receptor
ER	Estrogen Receptor

ER	Everted Repeat
ESTs	Expressed Sequence Tags
EtOH	Ethanol
FXR	Farnesoid X Receptor
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
HNF4	Hepatocyte Nuclear Factor 4
HSP	Heast Shock Protein
ID	Interaction Domain
IR	Inverted Repeat
LBD	Ligand Binding Domain
LC/MS	Liquid Chromatography Mass Spectrometry
LCA	Lithocholic Acid
LXR	Liver X Receptor
m/z	Mass/Charge
MeOH	Methanol
Mig	Migration defect of gonads
N	Amino
N-CoR	Nuclear receptor co-repressor
NHRs	Nuclear Hormone Receptors
PI3K	Phosphatydialinositol-3-OH-Kinase
PPARs	Peroxisome Proliferator-activated Receptors
PR	Progesterone Receptor
RAR	Retinoic Acid Receptor
RLU	Relative Light Unit
RNAi	RNA interference
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RTH	Resistance to Thyroid Hormone Syndrome
RXR	Retinoid X Receptor

SCOR	Short-chain oxidoreductases
Sf9	Spodoptera frugiperda 9
SID/RD	SMRT interaction domain/repressor domain
SIM	Selective Ion Monitoring
SMAD	Sma and Mad Related Proteins
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
TGFβ	Transforming Growth Factor Beta
TRα, β	Thyroid Receptor alpha, beta
TZDs	Thialazodinediones
USP	Ultraspiracle
VDR	Vitamin D Receptor
woc	without children
WT	Wild Type
20E	20-hydroxyecdysone
Δ^4-dafachronic acid	(25S),26-3-keto-4-cholestenoic acid
Δ^7-dafachronic acid	(25S),26-3-keto-7(5α)-cholestenoic acid
3K-LCA	3Keto-Lithocholic Acid

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO NUCLEAR HORMONE RECEPTORS

Nuclear hormone receptors are a class of transcription factors found throughout metazoans that regulate gene expression in a ligand-dependent fashion. The founding members of this protein family were originally identified biochemically as soluble receptors for steroid hormones, such as estrogen (ER), testosterone (AR), or progesterone (PR). Later, the identification of cDNAs for the steroid hormone receptors revealed striking similarity in domain architecture, which ultimately facilitated the subsequent cloning of the "orphan" receptors and led to the realization that the steroid receptors were part of a large and diverse superfamily. The orphan members of the family were so called because they lacked known regulatory ligands; however, some of these receptors were soon found to bind to known steroid and endocrine hormones such as vitamin D (VDR), thyroid hormone (TR), retinoic acid (RAR), and the insect molting hormone 20-hydroxyecdysone (EcR). Given the important roles of the steroid receptors in normal development, reproduction, and other aspects of human physiology and insect metamorphosis, the identification of ligands for the remaining orphan receptors has become a continual interest of several labs. The first success in this area was marked by the identification of 9-cis retinoic acid as the endogenous ligand for the retinoid x receptor, RXR, as well as the finding that RXR serves as a common heterodimer partner for a number of the orphan receptors (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1990).

The discovery of the nuclear receptor superfamily marked the birth of molecular endocrinology and with it the era of "Reverse Endocrinology" in which receptors were used to identify previously unknown ligands and their respective signaling pathways. The impact of the discoveries in this field over the years to human biology cannot be overstated as a large body of work has led to the identification of new ligands for many orphan receptors and with them the discovery of new and diverse physiologic pathways. For example, ligand discovery has provided new insights into the molecular basis of cholesterol, bile acid, and triglyceride metabolism, as well as foreign compound detoxification. Finally, and most importantly, ligand discovery has had significant impact on many aspects of human health--nuclear receptor ligands are successfully used in the treatment of cancer, diabetes, and inflammation-- and has fueled the search for new and more effective drugs to treat a variety of pathologic states. In the following sections I will briefly review nuclear receptor structure and mechanism of action, and describe the different classes of vertebrate receptors known today.

A Conserved Structure and Mechanism of Action

The structure of the nuclear receptor provides it with a unique ability to transduce small molecule messengers into programs of gene expression. At the sequence level, the nuclear hormone receptors (NHRs) are easily recognized by a variable amino (N)-terminal region, a highly conserved DNA-binding domain (DBD) composed of two zinc-fingers, and a less conserved, although structurally similar, carboxy (C)-terminal ligand-binding domain (LBD) (Mangelsdorf and Evans, 1995, Figure 1-1,). Through their DNA-binding domains, nuclear receptors bind to specific arrangements of a core

hexanucleotide binding site or response element within the promoter/regulatory regions of their target genes. The canonical response element is a half site (AGGTCA) oriented alone, in a tandem direct repeat (DR), everted repeat (ER), or inverted repeat (IR) (Figure 1-1B). This varied orientation provides specificity needed for each class of receptor to regulate a specific set of target genes and carry out its function.--heterodimers (DR, ER, IR), homodimers (IR,DR), or monomers (single half site).

The ligand binding domain (LBD) is connected to the DBD by a flexible linker or hinge region and plays a critical role in signal transduction mediated by the nuclear receptor. The three-dimensional structure of the LBD has been revealed by X-ray crystallography and is characterized as a three-layered, anti-parallel sandwich consisting of 12-alpha helices that together create an internal ligand binding pocket. Small changes in the LBD sequence as well in the LBD pocket size allow each nuclear receptor to specifically bind a given ligand or range of ligands while maintaining a conserved mechanism of transcriptional activation. Within helix 12 is a conserved region of the LBD that is required for ligand dependent activation and is known as the activation function 2 (AF-2). Early structural studies on the ligand-bound thyroid hormone receptor alpha (TRα) and un-liganded RXRα (apo-RXRα) provided insight into the molecular mechanism through which the AF-2 in helix 12 functioned to mediate the transcriptional switch that occurs upon ligand binding (Bourguet et al., 1995; Wagner et al., 1995). In contrast to the apo-RXRα structure, ligand binding to TRα revealed a considerable change in the conformation of helix 12, which is known to be the site of a C-terminal activation function or AF-2 domain. In apo-RXRα helix 12 is positioned

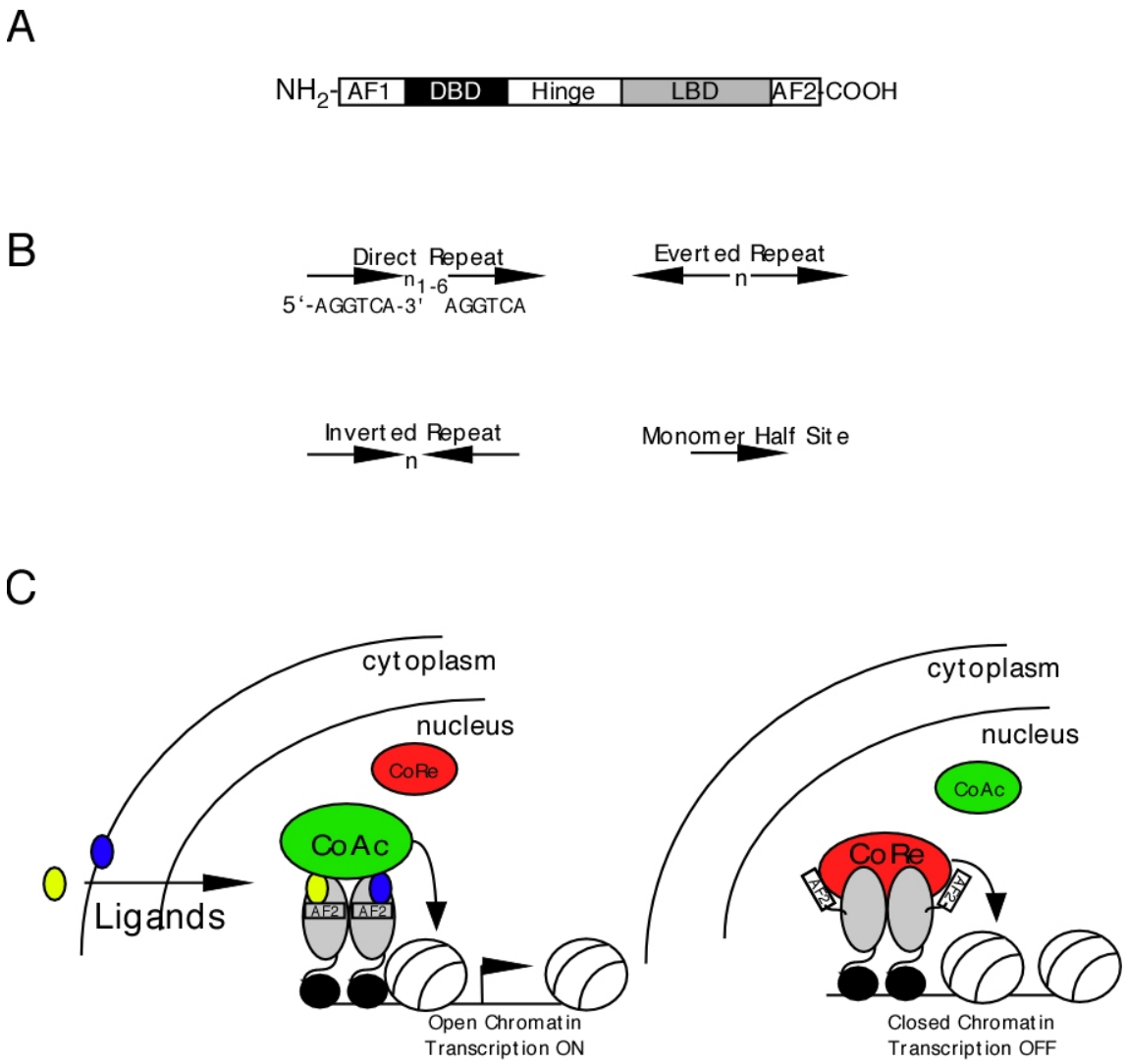

Figure 1-1 Structure of Nuclear Receptors and Mechanism of Action

(A) Nuclear receptors have a conserved structure consisting of an N-terminal activation function (AF-1), DNA binding domain (DBD), ligand binding domain (LBD), and a C-terminal activation function (AF-2). (B) Nuclear receptors bind to response elements containing the core hexad sequence, AGGTCA. Specificity is conferred by hexad orientation as direct, everted, or inverted repeats. In addition to orientation, the number of nucleotides separating these repeats (n) also provides specificity. (C) Nuclear receptors are ligand activated transcription factors. (Left side) Lipophilic ligands traverse the membranes of the cell and bind to their cognate receptors. Heterodimers, illustrated here, are constitutively bound to DNA. Ligand binding triggers a conformational change in the AF-2 domain that favors binding of co-activator (CoAc) complexes and destabilizes binding of co-repressor complexes (CoRe). Histone acetyl-transferase activities within the CoAc complexes modify the chromatin into an open state to facilitate gene expression. (Right side) In the absence of ligand, the AF-2 domain is in an inactive confirmation allowing binding of CoRe complexes. CoRe complexes modify chromatin into a closed state, thereby shutting off transcription. The cartoon in C is an adaptation from Andy Shulman's thesis.

away from the LBD core at a 45° angle, but in ligand-bound TRα it is found to fold back into the receptor to form part of the ligand binding pocket. A similar rearrangement of the AF-2 was later seen in the structure of RXRα bound to its natural ligand, 9-cis retinoic acid (Egea et al., 2000).

Transcriptional Regulation by Co-repressors and Co-activators

Since the original structures of RXRα and TRα were reported, structures for more than 20 other nuclear receptor LBDs have been solved and it is now widely accepted that the AF-2 structural rearrangement represents a common theme among liganded nuclear receptors. Molecular, biochemical, and structural studies have further elucidated the molecular basis of ligand-dependent transcriptional activation (Glass and Rosenfeld, 2000). Ligand-induced rearrangement of the AF-2 into the LBD generates a charge clamp between a conserved glutamate in the AF-2 domain and a conserved lysine in helix 3. This creates a grove through which hydrophobic regions (containing LXXLL motifs) found within several transcriptional co-activators, such as the p160/SRC-1 family, are recruited and stabilized (Figure 1-1C). These and other identified co-activator proteins are contained within complexes that consist of chromatin remodeling enzymes required for transcription. However, in the absence of ligand, or when bound to antagonists, nuclear receptors adopt a confirmation that pushes the AF-2 away from the LBD core, providing a docking site for co-repressors such as SMRT (silencing mediator or retinoic acid and thyroid hormone receptor) and N-CoR (nuclear receptor co-repressor), which recruit enzymes that remove histone modifications leading to repression of basal gene expression (Figure 1-1C). Binding of co-repressors, similar to

that of co-activators, involves interactions of nuclear receptors with conserved hydrophobic motifs albeit with the sequence LXXXIXXXL. Binding, however, is mutually exclusive, as co-repressor and co-activator binding sites on receptors overlap (Li et al., 2003b).

1.2 VERTEBRATE NUCLEAR HORMONE RECEPTORS

Reverse Endocrinology: Modern Ligand Hunting

The discovery of the orphan nuclear receptors brought the total number of human receptors to 48. This unexpected number raised a series of important questions: do any of the orphans have ligands, what are their ligands, and how do these orphan receptors fit into the bigger picture—what was their physiologic role? Several major technical advances, namely the co-transfection assay and domain swapping, allowed investigators to begin exploring the biological roles of these receptors. In a process called "Reverse Endocrinology" only the sequence was needed to being identifying its ligand. No prior knowledge of a given receptor's function was necessary. This strategy has been called reverse endocrinology because it is the reverse of classic endocrinology, which used known ligands, such as the steroid hormones, to identify their respective receptor. Domain swapping took advantage of the modularity of the nuclear receptor such that a DBD from a receptor with a known target gene could be fused to the LBD of an orphan of interest. This strategy, together with the ease of the co-transfection assay, allowed the detection of ligands without prior knowledge of the receptor's target genes or physiologic role. Ligands were identified by screening known drugs, steroids, or lipid metabolites

whose sites of action or production co-localized with the expression of a given receptor. Other approaches involved high-throughput screening of synthetic compound libraries to identify small molecule tools that could be used to probe receptor function. With an agonist or endogenous ligand in-hand, the biological pathways influenced by each receptor/agonist pair were then elucidated by identifying target genes using a variety of techniques. The identity of the ligand and target genes suggested the physiologic role of each receptor, which was then tested by using animal models and cell-based assays. Finally, these results were confirmed through targeted deletion of a given receptor.

Adopted Orphan Receptors

Reverse endocrinology has since led to discovery of ligands for several of the orphan receptors, and hence their "adoption". The "adopted orphans" include receptors for oxysterols, the liver x receptors (LXRs); bile acids, the farnesoid x receptor (FXR); fatty acids, prostaglandins, and the insulin sensitizing Thiazoladinediones (TZDs), the peroxisome proliferator-activated receptors (PPARs); and finally, a diverse array of xenobiotic compounds, pregnane x receptor and constitutive androstane receptor (PXR and CAR). All of these receptors have a common obligate heterodimer partner, RXR, through which they each bind to direct, inverted, or everted repeats with varying nucleotide spacings. These receptors are localized to the nucleus and bound to DNA in the absence of ligand. Studies that have characterized the ligands of these receptors and the physiologic pathways they regulate have highlighted a general theme among this group of receptors: they function as dietary and endogenous lipid sensors that act to maintain the lipids at non-toxic levels. This understanding comes from the fact that these

receptors bind their cognate ligands with low affinity (>1-10μM) and in response activate cellular metabolic programs that result in the transcriptional activation of cytochrome p450s, ATP-cassette transporters, and lipid binding proteins, among others, that metabolize, export, or store these lipids (Chawla et al., 2001). Interestingly, lipid homeostasis may be a general property of nuclear receptors as detoxification of secondary bile acids has recently been added to the list of functions of the vitamin D receptor (Makishima et al., 2002). It binds to the toxic secondary bile acid, lithocholic acid (LCA) and up-regulates detoxifying enzymes in the intestine that lead to its elimination.

Members of the human nuclear receptor superfamily can now be generally organized into one of three groups that reflects the diversity that is seen in ligand binding potential, mode of DNA binding and dimerization, and physiologic role (Table 1). The adopted orphan receptors discussed above belong to one of these groups and the two remaining include the endocrine and orphan receptors.

Table 1: Functional Organization of Vertebrate Nuclear Receptors

Endocrine	Ligands	Adopted Orphan	Ligands	Orphan
AR	Testosterone, DHT	LXR	Oxysterols	SF-1/LRH-1
ER	Estradiol	FXR	Bile acids	Nurr1/Nor1//NGFI-B
GR	Cortisol	PXR	Xenobiotics	DAX/SHP
PR	Progesterone	CAR	Xenobiotics	Rev-erbs
MR	Aldosterone	PPARα	PUFAs, Fibrates	HNF4
		PPARγ	TZDs	GCNF
VDR	1,25-dihydroxy vitamin D3, Lithocholic Acid	PPARδ		TR2 TR4
TR	T3	RXR	9-cis Retinoic Acid	PNR
RAR	All trans retinoic acid			ERRα
				COUP TFs
				RORs

Endocrine Receptors

As stated previously, the steroid hormone receptors are the founding members of the nuclear receptor superfamily, and they include the androgen (AR), estrogen (ER), glucocorticoid (GR), mineralocorticoid (Merris et al.) and progesterone (PR) receptors. Based on their known biological functions and distinct modes of dimerization and DNA binding they are classified as endocrine receptors. The endocrine receptors bind steroid hormones with high-affinity and selectivity and the levels of these ligands are precisely maintained under feedback control by the hypothalamic-pituitary axis. The endocrine receptors respond to their ligands by regulating important aspects of human biology including sexual differentiation, parturition, salt balance, and glucose metabolism. Their mode of action is similar to that of other nuclear receptors but they are distinct because they homodimerize. While ERs bind to response elements configured as inverted repeats of the canonical hexanucleotide sequence, AGGTCA, separated by three nucleotides, other endocrine receptors bind to AGAACA. In addition, steroid receptors (except ER) are bound to heat shock proteins in the cytosol and are released into the nucleus upon ligand binding.

The thyroid hormone (TR), vitamin D (VDR), and retinoic acid receptors (RAR) are also found within the endocrine receptor groups as is the insect ecdysone receptor (EcR)(see section 1.3). Interestingly, these four receptors share properties unique to the endocrine group and the adopted orphan group. They bind ligand with high affinity, similar to the steroid receptors, but like the adopted orphan receptors, they heterodimerize with RXR. Similar to the steroid receptors, both EcR and RAR regulate aspects of

development and morphogenesis, respectively. Production of ligands for TR, VDR, EcR, and RAR are under tight endocrine regulation, similar to the hypothalamic-pituitary control of steroid hormone production. However, ligands for these receptors all require exogenous components, such as dietary components or exposure to sunlight, making them also similar to the adopted orphans.

Orphan Receptors

Finally, the last group of receptors has largely remained orphan receptors. These receptors form homodimers that bind to direct repeats, as characterized by Hepatocyte Nuclear Factor 4 (HNF4), or are monomeric receptors, such as Liver Receptor Homolog 1 (LRH-1) and Steroidogenic Factor 1 (SF-1), that bind to hexanucleotide half-sites containing 5'extensions important for binding specificity**.** These receptors are known to be constitutively active or function as potent trans-repressors of other receptors (Mangelsdorf and Evans, 1995). Understanding the function of these proteins has been considerably challenging, and it is thought that most may in fact not be regulated by ligands. However, alternative approaches, such as structural studies, have provided insights into their ligand binding properties. In these studies, X-ray crystallography revealed the presence of fatty acids in the LBD of HNF4 and phospholipids in the LBDs of hSF-1 and hLRH-1 (Dhe-Paganon et al., 2002; Krylova et al., 2005; Li et al., 2005; Wang et al., 2005; Wisely et al., 2002). However, these lipids may not serve as regulatory ligands but instead may function as lipid co-factors that help stabilize active confirmations of the LBD AF-2 (Forman, 2005 ; Li et al, 2003). Finally, other orphan receptors completely lack a DBD, such as Small Heterodimer Partner (SHP) and DAX.

These receptors are examples of trans-repressors of other nuclear receptors, however, the mechanisms through which these receptors function is not well understood.

1.3 *DROSOPHILA* NUCLEAR HORMONE RECEPTORS

Ecdysone Receptor and the Ecdysone-Cascade

Study of nuclear receptors in the fruit fly, *Drosophila melanogaster,* has provided us with essentially all of our knowledge about the function of nuclear receptors in invertebrates. The genome of *Drosophila melanogaster* contains 21 nuclear hormone receptors; however, ligands are only known to exist for the ecdysone receptor (EcR). EcR is the ortholog of human FXR and LXR and it heterodimerizes with the fly RXR homolog, ultraspiracle (USP), to form the receptor for 20-hydroxyecdysone (20E) (Thummel, 1995). Together, the 20E/USP/EcR receptor complex is responsible for temporal control of *Drosophila* postembryonic development and metamorphosis. Interestingly, it was in Drosophila that steroid hormones (20E) were found to affect changes at the level of chromatin (Thummel, 1995). It is now understood that discrete pulses of 20E direct each of the major developmental transitions in the life cycle of *Drosophila*. Early pulses of 20E trigger larval molts, while later high-titer pulses lead to pupal formation and eventual metamorphosis. The transcriptional output of 20E pulses induces the expression of a number of transcription factors including six *Drosophila* nuclear hormone receptors: *DHR3*, *DHR4*, *DHR39*, *E75*, *E78,* and *ftz-f1 (King-Jones and Thummel, 2005).* The resulting cascade of these nuclear receptors and other transcription

factors specifies the appropriate responses from one pulse of 20E to the next and thus ensures proper development.

All of the *Drosophila* nuclear receptors are represented by conserved phylogenetic subfamilies found in humans; however, many members within the vertebrate subfamilies are missing in *Drosophila*. These include members of the aforementioned endocrine receptors, including the classic steroid receptors (AR, PR. MR, GR. ER), as well as TR, RAR, and PPAR. The endocrine receptors are presumed by some to be the most ancient of the nuclear receptors and may have been lost in arthropods. The fact that flies have maintained the heterodimer partner paradigm suggests that RXR is evolutionarily ancient as well. Remarkably, the list comprising the receptors missing in the fly is approximately two-thirds of the known ligand-regulated vertebrate nuclear receptors. This raises the question of whether the remaining 19 orphan receptors in *Drosophila* will have ligands and whether any of them have subsumed roles similar to the missing receptors. Within the remaining orphan receptors, one group, containing DHR51, DHR83, tailless (tll), dissatisfaction (dsf), and seven up (svp), has conserved roles in neuronal development (King-Jones and Thummel, 2005). The second group includes DHR38, DHR78, DHR96, HNF4, and ERR. Since mutations in DHR96, HNF4, or ERR have not been isolated little is known of their function in the fly. There is some speculation that DHR96 may serve as a xenobiotic receptor given its close phylogenetic relationship with vertebrate PXR, CAR, and VDR; however there is no direct evidence to support this idea.

Ligand Hunting in *Drosophila*

Considerable effort has been made to identify ligands for *Drosophila* orphan receptor using the techniques of reverse endocrinology; however these attempts have been largely unsuccessful (Baker et al., 2000). For example, the *Drosophila* hemolymph contains many bioactive intermediates in the synthesis of 20E that are postulated to be potential nuclear receptor ligands, especially for receptors involved in development and metamorphosis (Baker et al., 2000). However, there are no reports that these compounds can act to directly regulate a *Drosophila* orphan nuclear receptor. Following the lead of research on vertebrate orphan receptors, structural approaches are now being taken in an effort to gain insight into the ligand binding properties of *Drosophila* orphan receptors. These approaches include crystallization, as well as over-expression followed by liquid chromatography and mass spectrometry (LC/MS), to analyze the ligand binding pocket for the presence of co-purifying small molecules that provide clues to the identity of natural ligands. Some success with these approaches has recently come from studies on two receptors, DHR38 and E75:

DHR38

DHR38 is the *Drosophila* homolog of the vertebrate NGFI-B-type orphan receptor. These receptors bind to DNA as monomers; however, DHR38 can form heterodimers with RXR and USP and thus be activated by the RXR ligand, 9-cis-retinoic acid. Mutations in *dhr38* produce defects in adult cuticles near the end of metamorphosis placing DHR38 downstream of the ecdysone cascade (Kozlova et al., 1998). Interestingly, DHR-38 can be activated by a number of ecdysteroids, but only when

bound by activated USP (VP16-USP) or by RXR in the presence of its ligand, 9-cis retinoic acid (Baker et al., 2003). This represents the first example of a nuclear receptor requiring pre-activation of its RXR or USP heterodimer partner for its own activity, a finding that suggests USP may have a ligand. However, the real puzzle has come from structural studies that show DHR38's ligand-binding pocket is too small to bind ligands and that the receptor lacks a conventional co-activator binding surface (Baker et al., 2003). Efforts have failed to show direct binding of ecdysteroids to DHR-38 or an interaction with known co-activators. Thus, DHR38 mediates an atypical ecdysteroid signaling pathway that may involve a novel co-activation mechanism. The identification of a co-activator that interacts with DHR-38 in response to ecdysteroids, as well as the ligand that pre-activates USP, are challenges for the future. Finally, given that DHR38 is homologous to the mammalian NGFI-B receptors, which are known to be involved in dopaminergic neuron survival and differentiation, these results are highly provocative as they hint at the possible existence of a new mode of receptor activation.

E75

The *Drosophila* orphan receptor, E75, is one of the early ecdysone-inducible gene products that participate in larval molting and morphogenesis. E75 is required for ecdysone production so it also acts upstream of the ecdysone-induced cascade. Its mechanism of action is thought to involve, in part, antagonism of DNA binding by another receptor, DHR3. New insight into E75 function has recently come from the observation that a red chromophore co-purified with the receptor after it was expressed and purified from bacteria (Reinking et al., 2005). The bright red color of the

chromophore led the authors to guess that it was heme, a hypothesis they later confirmed by a variety of biophysical measurements, including mass spectrometry. Interestingly, heme levels in the expression system dramatically affect expression levels of E75, presumably through an effect on its folding and stability, and no apo-E75 was ever detected. This implies that heme is essential for E75 protein folding and accumulation and suggests that E75 is a heme sensor, accumulating, and regulating gene expression in response to heme levels. Furthermore, the redox state of the Fe(II) in the associated heme can be reversibly modulated by the redox state of the cell and this affects the ability of E75 to antagonize DHR3. In a reduced form E75-heme binds a DHR3 AF2 peptide but not when heme is oxidized. Interestingly, binding of E75-heme, in its reduced state, to DHR3 is inhibited by NO and CO binding. The data from this work suggests that E75 may function as a heme sensor as well as a sensor of the cellular redox state. The modulation of E75 activity by NO and/or CO binding also suggests that E75 mediates intracellular signaling by these gases, which behave as putative ligands. Finally, the identification of heme as a cofactor for E75 provides several interesting links between the function of E75 and its involvement in the synthesis of ecdysone, which is dependent on CYP P450s that utilize heme, and the regulation of molting and morphogenesis. Further links can be made to the circadian regulation of molting and morphogenesis: E75 is an invertebrate homolog of Rev-Erbα, an established regulator of the circadian rhythm in mammals that functions to inhibit the DHR3 homomlog, RORα. Circadian cycles are themselves known to be modulated by diatomic gas production. It will be interesting to see whether Rev-Erbα also binds heme and is modulated by redox and diatomic gases.

1.4 *C. ELEGANS* NULCEAR HORMONE RECEPTORS

Since its completion in 1998, the sequence of the *C. elegans* genome has revealed the existence of 284 nuclear hormone receptors (CeNHRs). This represents the largest family of nuclear receptors identified to-date; however, in contrast to our great understanding of many aspects of *C. elegans* biology comparatively little is known about the biological processes governed by this large family of nematode receptors and all receptors in *C. elegans* have no known ligands. The number of CeNHRs represents a greater than 5-fold expansion over those found in humans (48) or *Drosophila* (21). The magnitude of expansion is further illustrated when one considers that as an adult, *C. elegans* is made up of only 959 cells. Why are there so many CeNHRs? Phylogenetic analysis of this family shows that only 15 appear to have close homologs in vertebrates or insects, while the remaining 269 appear to be considerably divergent members. The majority of these receptors appear to be expressed genes based on the finding of expressed sequence tags (ESTs) for 79 of the predicted 284 genes (Sluder and Maina, 2001). The divergent CeNHRs may represent new subfamilies found only in nematodes or instead represent considerably divergant groups within one or more of the major subfamilies. Recent phylogenetic analysis of 76 unique CeNHR cDNA sequences, which focused on the ligand binding domain (LBD) and the DNA binding domain (DBD), suggests the later to be true as analysis reveals that most CeNHRs have arisen from an "explosive burst" of nuclear receptor duplication of an ancestral HNF4 receptor (Robinson-Rechavi et al., 2005).

Function of Conserved CeNHRs

The 15 conserved receptors haven been grouped into 5 out of the 6 broadly conserved subfamilies found in metazoans (Table (Sluder and Maina, 2001). Surprisingly, the majority of known vertebrate and insect ligand-responsive receptors are absent from *C. elegans*. For example, the steroid receptor subfamily members are completely missing from *C. elegans.* As in *Drosophila*, no receptors homologous to the thyroid hormone (TR), peroxisome proliferators-activated (PPAR), or retinoic acid receptors (RAR) have been identified in *C. elegans*. In addition, *C. elegans* lacks a clear homolog for RXR and despite being classified as a member of the Ecdysoza (a molting organism), *C. elegans* also lacks a clear ortholog of the Ecdysone receptor (EcR). Thus, it appears that CeNHRs have undergone considerable expansion and divergence.

So what then are the functions of CeNHRs and are any of them regulated by ligands? The divergence of receptors in the *C. elegans* is most striking when one considers the LBD alone. LBDs of CeNHR appear to have undergone rapid evolution and as a result no receptor LBD in *C. elegans* has > 33% amino acid identity to that of its vertebrate or insect counterpart (Gissendanner et al., 2004). This lack of sequence identity makes predicting the ligands based on their sequence similarity to other receptors difficult (if not impossible) and suggests that the large number of diverse CeNHRs may have evolved to bind to an equally diverse array of small molecule ligands. Surprisingly, little effort has been made to apply techniques of reverse endocrinology to the discovery of ligands for nematode nuclear receptors. Such efforts may be hindered by the fact that

little is known about the role that lipids play as signaling molecules in *C. elegans* physiology.

So far, forward and reverse genetic studies have revealed functions for all of the conserved CeNHRS (Table 2). Some of these roles have highlighted the involvement of several *C. elegans* nuclear receptors in conserved processes, whereas others have biological functions specific to the nematode.

Molting

Two receptors in particular, NHR-23 and NHR-25, have both been found through RNA interference (RNAi) to be involved in molting, as well as epidermal cell differentiation, a function that is conserved in their closest respective *Drosopihla* relatives, DHR3 and FTZ-F1 (Asahina et al., 2000; Gissendanner and Sluder, 2000, see above; Kostrouchova et al., 2001). The mRNA expression of *nhr-23* and *nhr-25* oscillates in a manner very similar to the pattern of expression seen for their *Drosophila* orthologs (Gissendanner et al., 2004). The human homologs of NHR-23, the RORs, are involved in cerebellar differentiation and also in the circadian clock. Thus, these receptors appear to regulate aspects of biological clocks in all three species. The human homologs of NHR-25, SF-1 and LRH-1, are responsible for steroidogenesis and cholesterol and bile acid metabolism, respectively. RNAi of *nhr-67*, a homolog of vertebrate TLX receptor, revealed it may also play a role in larval molting (Gissendanner et al., 2004). Additionally, *nhr-41* may play a role in molting as its mRNA expression oscillates with the molt cycle despite lacking an RNAi phenotype. While NHR-23 and

Table 2: Function of Conserved *C. elegans* Nuclear Receptors and their Homologs

C. elegans NHRs	Function	D. melan. NHRs	Function	H. sapiens /M. Mus. NHRs	Function
DAF-12	Dauer formation Lipid Metab. L3-L4 Devel. Life Span	DHR96	Unknown	PXR (DBD) CAR VDR LXR (FL)	Xenobiotic Detox. Calcium Homeo/Bile acid detoxification. Cholesterol Homeo,BileAcid Synthesis,
NHR-8	Xenobiotic Metabolism				
NHR-48	Unknown				
SEX-1	Sex determination	E78	Molting	Reverb	Circadian clock, transcriptional repression
NHR-23	Dauer,Molting Epidermal Differ.	DHR3	Embryonic Development, Molting, Metam.	ROR α/β/γ	Cerebellar differ./Circadian Clock/Thymop.
NHR-25	Molting Epidermal Differ,	FTZ-F1 Alpha/beta	Segmentation/Molting,Metamorphosis	SF-1/LRH-1	Gonado-, Steroidogenesis/Cholesterol Homeo/Bile Acid Synthesis
NHR-49[2]	Fatty acid oxidation Starvation Response	dmHNF4	Unknown	HNF4	Lipid glucose
UNC-55	Neuronal differentiation	Seven-up	Photoreceptor fate	Coup TF1 Coup TF2	Neuronal and Heart Development,
FAX-1	Neuronal development	dmFAX-1	Unknown	PNR	Photoreceptor fate
NHR-91[1]	No obvious function	DHR4	Unknown	GCNF	Germ cell differentiation, embryogenesis
NHR-85[1]	Egg laying, molting?	E75	Molting, heme, gas, redox sensor	Rev-erb	Circadian rhythm
NHR-67[1]	Molting, growth, vulval formation	Tailless	A/P patterning, neurogenesis	TLX	Forebrain development, neural stem cell maintenance
NHR-41[1]	Dauer molt or formation	DHR78	Molting. metamorphosis	TR2/TR4	Unknown
NHR-6[1]	Ovulation	DHR38	Adult cuticle formation	Nurr1 NGFI-B/Nurr77	Dopamine neuron differentiation Apoptosis, immediate early response

[1]Function of *C. elegans* nuclear receptor was determined by RNAi screen [2]Although related to HNF4 (33% identity in LBD) may function more like PPARα. This table was borrowed and adapted with permission from A. Antebi (Antebi, 2006). (DBD) (FL) comparison based on DBD or full length sequence. Receptors in bold are orthologs of NRs that function in molting and metamorphosis in insects

NHR-25 function as conserved members of the molting process in the nematode, it remains unclear whether these receptors are part of a steroid hormone-induced cascade of transcription factors that operates to control ecdysis. Interestingly, a handful of studies implicate a role for cholesterol in nematode molting, as cholesterol starvation and mutations affecting proteins implicated in sterol transport and metabolism result in molting defects (Kuervers et al., 2003; Matyash et al., 2004; Merris et al., 2003; Yochem et al., 1999). The particular steroid metabolite and its receptor remain to be identified.

Neuronal Development

Two receptors found to be involved in neuronal differentiation in *C. elegans* (FAX-1 and UNC-55) are members of the conserved subfamily containing the vertebrate COUP-TFs and PNR nuclear receptors, which are also involved in neuronal development. ODR-7 (not listed in table 2), is a considerably divergent receptor that lacks a LBD and plays a role in sensory neurons where it has been found to control the olfactory neuron identity (Colosimo et al., 2003; Sengupta et al., 1994).

Xenobiotic Detoxification

NHR-8 is related to the vertebrate xenobiotic receptors PXR and CAR and is expressed in the intestine. Surprisingly, mutations in NHR-8 confer sensitivity to two toxins chloroquine and colchicine, suggesting a role for NHR-8 in xenobiotic detoxification (Lindblom et al., 2001). However, there are no reports that it binds directly to these compounds or that it regulates xenobiotic detoxification genes in the worm, such as the cytochrome P450 family.

Metabolism

Despite lacking NHRs orthologous to the major lipid sensing vertebrate PPARs, *C. elegans* appears to have maintained the function of lipid sensing and control over fat metabolism in a divergent receptor, NHR-49, that arose from expansion of an ancestor that also gave rise to the hepatocyte nuclear factor 4 family of receptors (HNF4s). Putative null mutations in *nhr-49* shorten life-span of animals and cause accumulation of fat in the intestine as well as changes in fatty acid composition (increased stearic acid:oleic acid ratio). The observed phenotypes appear to be primarily linked to deficiencies in the expression of genes involved in fatty acid beta oxidation and desaturation (Van Gilst et al., 2005a). Whereas reductions in *asc-2* and *ech-1* are sufficient to produce the high fat phenotype, reduced expression of a stearyl-coA desasturase, *fat-7,* is responsible for changes in fatty acid composition and life-span. Paradoxically, *fat-7* RNAi reduces fat content independent of *nhr-49* suggesting that *fat-7* is involved in a feedback mechanism that inhibits control of fatty acid oxidation by NHR-49.

Further insight into the function of NHR-49 has come from studies analyzing gene expression in response to fasting. Fasting results in predictable increases in the expression of genes involved in fatty acid oxidation (Van Gilst et al., 2005b). In particular, the proposed NHR-49 target gene, *acs-2,* is rapidly up-regulated in an NHR-49 dependent manner. However, *fat-7* is turned off by fasting resulting in increased levels of stearic acid. This presumably shuts off the feed-back inhibition of fatty acid oxidation

that is normally seen in fed conditions and allows fat to be consumed for energy and while maintaining levels of saturated fatty acids.

Taken together, these findings reveal several important parallels with the regulation of fat oxidation and composition in vertebrates by the PPARs, namely PPARα (Desvergne and Wahli, 1999). The involvement of NHR-49 in lipid metabolism reveals the first description of a fat metabolism regulatory network governed by a nuclear receptor in invertebrates. In addition, the work suggests that members of the HNF4 family in vertebrates may have roles in fatty acid metabolism that remain uncharacterized. Finally, it is tempting to think that NHR-49 might be regulated by a lipid ligand such as a fatty acid because it is known that both human HNF4 and the PPARs bind to a different types of fatty acids (Dhe-Paganon et al., 2002; Kliewer et al., 1997; Wisely et al., 2002). Regulation by fatty acids would position NHR-49 to act as a sensor of dietary lipids and metabolites and allow it to coordinate dietary availability of nutrients with the regulation of fatty acid oxidation and composition. However, it remains to be determined if NHR-49 is directly regulated by a fatty acid ligand.

Dauer Formation

Perhaps the most well characterized member of the nuclear receptor family in *C. elegans* is DAF-12. Although related to NHR-8, DAF-12 does not appear to regulate xenobiotic detoxification. Instead, DAF-12 is one of over 30 daf genes (dauer formation genes) that act together to sense and integrate environmental signals and respond by choosing between continuous reproductive development or the formation of the dauer larva, which is an alternative L3 larval state specialized for survival. Detailed analysis of

dauer formation and the daf genes has provided considerable evidence for endocrine-like control of *C. elegans* reproductive development and strongly indicates that the choice between dauer formation and reproductive development is governed by the production of steroid hormones that signal through DAF-12. RNAi studies have also identified a role for NHR-41, NHR-85, NHR-23, and NHR-25 in dauer formation. NHR-41 and NHR-23 appear to be required for proper tissue remodeling while NHR-85 and NHR-25 may play a role in the dauer molt (Gissendanner et al., 2004). The analysis of the dauer formation pathway, the daf genes, and the regulatory networks they form are the subject of the remaining portions of this introduction.

1.5 THE DAUER DECISION IN THE *C. ELEGANS* LIFE CYCLE

In environments deemed favorable for reproduction, *C. elegans* develops continuously from an embryo to an adult through four larval stages, marked L1-L4, that are each characterized by a molt in which new cuticle is secreted and the old cuticle is shed. However, in unfavorable environments, such as conditions of diminishing food, high temperature, or overcrowding, second larval stage animals (L2) delay further reproductive development and instead form the non-feeding, non-reproductive, and long-lived dauer larva (Riddle and Albert, 1997). Dauer larvae undergo considerable morphological and behavioral changes that specialize this larval stage for survival. Amazingly, dauer larvae exhibit 4-8 fold extensions in life-span compared to the normal 2 week life-span found in non-dauer animals. Upon entry into more favorable environments dauers resume feeding and continue normal reproductive development. Over the past 30 years extensive efforts have been made to characterize the

environmental signals that influence the dauer decision as well as the genetic regulatory networks that integrate these signals into an organism-wide developmental response. As mentioned, over 30 genes, collectively called daf (dauer formation) genes, have been identified which transduce environmental signals of population density, food, and temperature into the choice between alternative developmental programs of dauer diapause and reproductive development. Extensive genetic analysis of the daf genes has ordered these genes into a complex regulatory pathway. The molecular identification of the majority of these genes demonstrates that conserved signaling pathways act in an endocrine-like fashion to mediate organism-wide developmental responses. As will be described below, these networks identify numerous hormonal pathways, both steroidal and non-steroidal, in the control of dauer formation and reproduction.

Environmental Signals: Food, Pheromone, Temperature

Three primary environmental signals that influence the formation and recovery of dauer larvae have been identified as food, a constitutively secreted dauer inducing pheromone, and temperature (Golden and Riddle, 1982; Golden and Riddle, 1984). The molecular identity of the food signal remains to be determined but early studies have found it both within the bacterial food source (E. coli) and yeast extract. The dauer pheromone is constitutively secreted and serves as an external measure of population density (Golden and Riddle, 1982). A bioassay testing the ability of dauers to recover in the presence of varying amounts of both factors found that these two signals compete with one another such that the relative proportion of the two signals, not their actual concentrations, determines the worm's response. The third signal, temperature,

modulates the response to the levels of food and pheromone such that the percentage of animals induced to form dauers in the presence of a known amount of food and pheromone increases as temperatures are raised above 20°C. The dauer pheromone, or daumone, was recently identified as a single fatty acid derivative through large scale purification and direct chemical synthesis (Jeong et al., 2005).

Temporal Action of Food, Pheromone, and Temperature

The precise temporal action of the signals that influence the dauer decision has been determined by shifting animals from non-dauer inducing conditions to dauer inducing conditions at various times throughout development. These studies have revealed that dauer formation can only occur at the L2 molt (Golden and Riddle, 1984). In addition, commitment to the non-dauer developmental pathway occurs sometime shortly before the L1 molt, although the commitment is not absolute. Final commitment to dauer formation is made a few hours prior to the L2 molt. Similar studies with temperature shifts revealed that up-shifts in temperature (from 15 to 25°C) increase the percentage of dauer formation if they occur prior to the L1 molt. As expected, down-shifts prior to the L1 molt reduce the effect of high temperature. Together, these results demonstrate that dauer formation is a developmental decision that results from the integration of environmental signals of food, pheromone, and temperature. In the next section I will describe some of the morphological and behavioral changes that accompany dauer formation. It will hopefully become apparent that the decision to form a dauer or a reproductive animal entails organism-wide developmental commitments that strongly implicate endocrine-like control.

Structural and Behavioral Adaptations in Dauers

Dauer larvae undergo considerable changes morphologically and behaviorally that specialize this larval stage for survival (Riddle and Albert, 1997). Some of these changes can be easily seen with a dissecting microscope. On an agar plate, dauers appear much thinner and darker than corresponding non-dauer L3 stage animals. The thin appearance of these animals occurs as a result of radial shrinking of the hypodermis, the sheath of cells that surrounds the animals and gives rise to the cuticle. Radial shrinkage follows the dauer specific L2 molt, which occurs after a period of delay that is called L2d or the pre-dauer stage. The L2d stage is the earliest stage at which one can distinguish between animals committed to non-dauer development and those that are not (Golden and Riddle, 1984). At the L2d stage the most visible difference is the darkening of the intestine, a characteristic that is maintained in the fully formed dauer larva. Decreased motility is also associated with dauer larvae, however, dauer larvae are easily aroused upon prodding, and they undergo rapid movements.

At an ultra-structural level the dauer cuticle appears as a thick outer cortex also containing a striated inner layer. On the exterior the dauer cuticle has characteristic lateral ridges or alae. A characteristic of dauer larva attributable to these changes in cuticle is resistance to treatment with 1% SDS. In addition to changes in cuticle, many other tissues are remodeled during dauer formation. Specific segments of the pharynx including the terminal bulb and isthmus become smaller and pharyngeal pumping is suppressed. Gonadal migrations, which normally progress at each molt, cease after the dauer larval molt. Finally, the positions of chemosensory neurons and supports cells in

the major sensory organ, the amphid, are altered in the dauer larvae. One interesting behavioral change that may also assist dauer larva in finding new environments is nictation. I have personally witnessed this on moldy plates. Dauer larvae climb the fungal hyphae that extend from the surface of the agar plate and then stand on their tails and wave their bodies in the air. These morphological and behavioral changes combine together with metabolic adaptations to allow survival in harsh environments and dispersal to more favorable ones.

Genetics of Dauer Formation

Mutations in daf genes 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 cues. For the purposes of this discussion, the Daf genes will be placed into three groups (Table 3). The first group of genes is involved in the production and detection of the dauer pheromone and is considered here collectively as the chemosensory genes. The second group, the second messenger and growth factor signaling genes, are downstream of the chemosensory genes and include components of a cyclic Guanosine Monophospate (cGMP) signaling pathway as well as two parallel pathways involving insulin/Insulin-like Growth Factor-1 (IGF-1) and Transforming Growth Factor β (TGFβ) signaling. Together these genes function in favorable environments to promote reproductive development and are inhibited by pheromone and signals from the chemosensory genes in unfavorable environments. The third group, the steroid signaling genes, lies at the convergence of the second messenger and growth factor pathways and is of particular interest as it encodes a member of the

Table 3. Dauer Formation Genes

Function	Phenotype	Gene name	Molecular Identity
Group 1: Chemosensory			
Pheromone			
	Daf-d	*daf-22*	Uncloned
	Daf-d	*daf-6/ptr-7*	Patched Related Protein with sterol sensing domain
Cilia structure genes			
	Daf-d	*che-2*	WD40 protein
		che-3	Dynein heavy chain (HC) 1b
		che-11	large unknown protein (IFT)
		daf-10	IFTP with WD WAA motifs
		osm-1	Intraflagellar transport (IFT) raft protein
		osm-3	Motor kinesin HC
		osm-5	TPR repeat protein (IFTP)
		osm-6	IFTP
Group2: Second Messenger and Growth Factor			
cGMP			
	Daf-c	*daf-11*	Guanylyl cyclase
		daf-21	Heat shock protein 90
TGFβ			
	Daf-c	*daf-1*	type I receptor
		daf-4	type II receptor
		daf-7	TGFβ peptide
		daf-8	SMAD-like
		daf-14	SMAD-like
	Daf-d	*daf-3*	SMAD
		daf-5	Sno/Ski
insulin/IGF-1			
	Daf-c	*daf-2*	insulin Receptor
		daf-28	insulin-like Peptide
		age-1	PI3-Kinase
	Daf-d	*daf-16*	Forkhead Transcription Factor
		daf-18	PTEN Lipid Phosphatase
Group 3: Steroid Hormone			
	Daf-c	*daf-9*	Cytochrome P450
	Daf-d and Daf-c	*daf-12*	Nuclear Hormone Receptor
	Daf-d	*din1-s*	SHARP Co-repressor

cytochrome p450, nuclear hormone receptor, and nuclear receptor co-repressor families. A genetic model for the control of dauer formation by these genes has been determined through analyzing the epistatic relationships between double mutants of Daf-d and Daf-c mutants in the pathway, as well as by creating double mutants between Daf-c mutants. In following section I describe the genes in the dauer formation pathway. The reader may find it helpful to refer to Figure 1-2.

Chemosensory genes

As previously described, the dauer pheromone is a critical determinant of the dauer decision The first gene in the dauer formation pathway, *daf-22*, is thought to be involved in the secretion of the dauer pheromone as these mutants do not form dauers in dauer-inducing conditions but can form dauers in the presence of the crude dauer pheromone extract (Golden and Riddle, 1985). The levels of the dauer pheromone compared to dauer-inhibiting signals must be properly detected and ultimately conveyed to the rest of the tissues in the organism. Not surprisingly, the majority of the Daf-d mutants isolated include a total of 9 genes identified on the basis of defects in chemosensation and the common morphological finding of defects in the formation of the major sensory organ of the worm, the amphid sensilla (genes are listed in Table 3, Figure 1-2). Mutations in these so called cilia structure genes produce a Daf-d phenotype, but these mutants can form dauers when combined with downstream Daf-c mutants or when specific sensory neurons in their amphid sensilla are killed with a laser (see below).

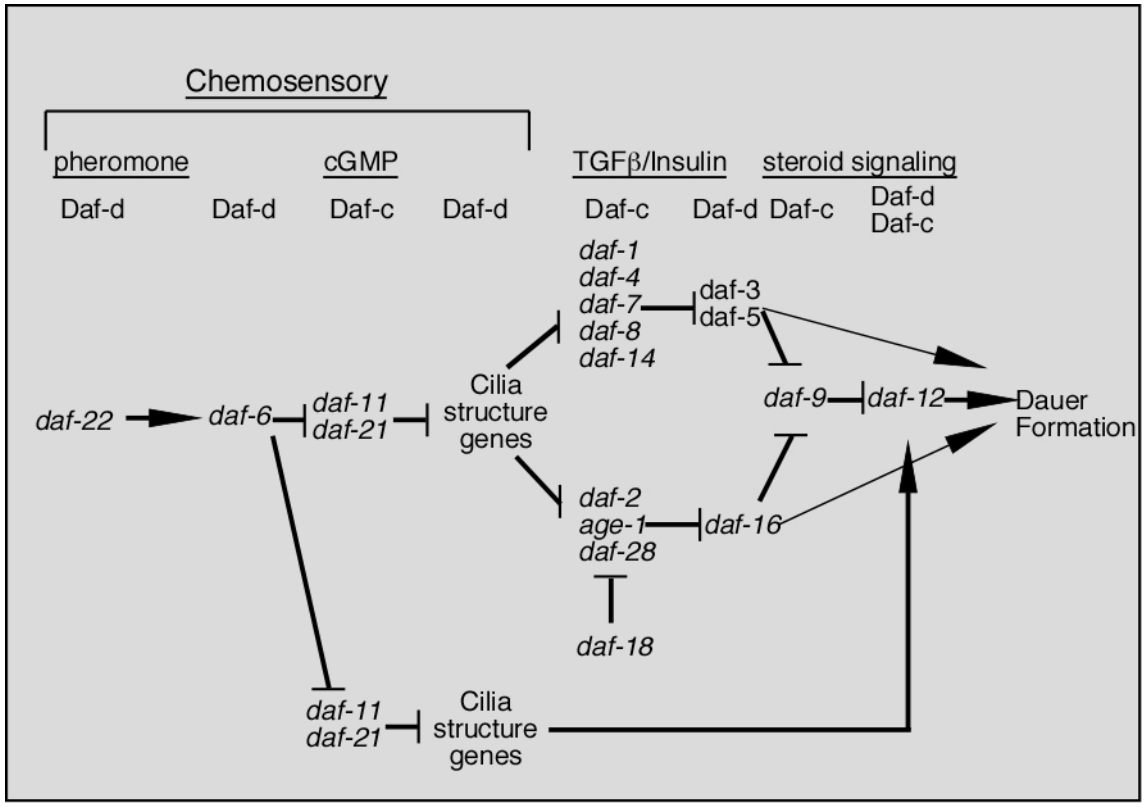

Figure 1-2. Genetic Map of the Dauer Formation Pathway

The dauer pheromone is secreted by *daf-22* and signals through *daf-6* and chemosensory neurons in the amphid to promote dauer formation. Dauer pheromone shuts down cGMP signaling (*daf-11*, *daf-21*), which is required for insulin and TGFβ production in ASI neurons, resulting in de-repression of dauer formation. *daf-11* and *daf-21* also act in parallel to insulin and TGFβ because double mutants are syn-Daf-c. In nondauer-inducing conditions, cGMP is produced in ASI and ASJ neurons causing activation of signal transduction pathways involving insulin, and TGFβ, which function in ASI, ADF, and ASG neurons to repress dauer formation. *daf-12* is positioned at the convergence of all three signaling pathways because it is required for dauer formation in dauer-inducing conditions and suppresses all Daf-c phenotypes in upstream Daf-c mutants. *daf-9* is downstream of insulin and TGFβ and acts to inhibit *daf-12* dauer promoting function. *daf-3*, *daf-5*, and *daf-16* inhibit *daf-9* activity and act in parallel to *daf-12* for some aspects of dauer formation: *daf-16* is required for pharyngeal remodeling, *daf-3* and *daf-5* are required for L1-L2d progression. Phenotypes for each mutant or group of mutants is indicated as dauer constitutive (Daf-c) or dauer defective (Daf-c.). Genetic pathways adapted from (Thomas, 1993; Thomas et al., 1993).

Of the 12 sensory neurons in the amphid, 8 are non-motile ciliated neurons that are exposed to the external environment. Experiments with laser microsurgery have confirmed roles for three of these eight ciliated neurons (ASI, ADF, ASG) in inhibiting dauer formation and one neuron (ASJ) in promoting dauer formation and recovery. Under nondauer-inducing conditions, ablation of both ASI and ADF results in a Daf-c phenotype; however, animals recover after 1 day unless ASJ is also ablated (Bargmann and Horvitz, 1991). This result suggests that ASI and ADF neurons operate under non-dauer inducing conditions to select reproduction, while ASJ promotes recovery from dauer. Interestingly, ablation of ASJ alone prevents dauer formation in the presence of exogenous pheromone, also suggesting a role for ASJ in promoting dauer formation. Thus, mutations affecting the amphid cilia prevent the dauer pheromone from activating the ASJ neuron and inhibiting the ASI and ADF neurons and lead to a Daf-d phenotype.

Finally, each unit of the amphid sensillum exists in a pore that is continuous with the cuticle and formed by a socket and sheath cell. Mutation of one of the cilia structure genes, *daf-6,* causes morphological defects in the amphid sheath cell resulting in occlusion of the amphid pore, and unlike *daf-22* worms, *daf-6* worms do not respond to dauer pheromone. This chemical epistasis places *daf-6* downstream of *daf-22*. The chemosensory genes are placed downstream of *daf-6* based on their proposed site of action in the ASI, ADF, and ASJ neurons. Taken together, the above data have produced a model for chemosensory control of dauer formation. In unfavorable conditions dauer pheromone is produced and/or secreted by the *daf-22* gene product. The pheromone gains access to the sensory cilia via the amphid socket formed by the amphid socket and

sheath cells, via *daf-6*(+), where it de-represses a dauer-inhibiting action of the ASI and ADF neurons and activates a dauer-inducing function of ASJ. This model nicely explains why under non-dauer inducing conditions ablation of ASI, ADF, and ASG neurons causes dauer arrest.

Second messenger and growth factor signaling

Genetic experiments have identified three groups of Daf-c genes that differ in their epistatic relationships to the recently described chemosensory genes. These groups of genes are believed to act in parallel or together to inhibit dauer formation through antagonism of downstream effector genes whose wild type (WT) activity is required for dauer formation.

cGMP Signaling

The first set of genes includes *daf-11* and *daf-21,* which differ from the remaining two groups in that their Daf-c phenotypes require the chemosensory genes (Thomas, 1993; Thomas et al., 1993; Vowels and Thomas, 1992). In addition, *daf-11* and *daf-21* also require the ASJ neuron for their Daf-c phenotype, which suggests that mutations in *daf-11* and *daf-21*activate the dauer promoting function of ASJ. These results place *daf-11* and *daf-21* at an early step in the chemosensory response to pheromone. The *daf-11* gene encodes a transmembrane guanylyl cyclase implicating a role for the second messenger cGMP in the control of dauer formation (Birnby et al., 2000). This is strongly supported by the finding that the *daf-11* Daf-c phenotype, as well as WT dauers induced by pheromone, are rescued by a membrane permeable cGMP analogue, 8-bromo-cGMP.

In addition, *daf-11* is expressed in ASI and ASJ neurons, which, as previously mentioned, are required for the Daf-c phenotype of *daf-11 and daf-21*. *daf-21* encodes a *C. elegans* homolog of Heat Shock Protein 90 (HSP-90) (Birnby et al., 2000). Interestingly, it too is rescued from dauer formation by 8-bromo-cGMP, but its direct role in cGMP production is unknown. A current model incorporating the above data suggests that in dauer inducing conditions, dauer pheromone acts on ASI and ASJ neurons to inhibit the production of cGMP by *daf-11* and *daf-21,* possibly through a G-protein coupled phosphodiesterase. In nondauer-inducing conditions *daf-11* and *daf-21* produce cGMP that may activate the cyclic nucleotide gated ion channel, TAX-4, as *tax-4* mutants are weakly Daf-c and cannot be rescued by 8-bromo-cGMP (Birnby et al., 2000). Although these two genes act at an early step of chemosensation they have been included in this section as they participate in signal transduction pathways responsible for coupling pheromone sensation to developmental switching. The next two groups of Daf-c genes differ from *daf-11* and *daf-21* because they do not require ASJ or the chemosensory genes for their Daf-c phenotypes. As such, they have been positioned downstream of the chemosensory genes but also lie parallel to *daf-11* and *daf-21* due to strong synthetic Daf-c phenotypes seen in double mutants (Thomas et al., 1993).

TGFβ Signaling

daf-1, *daf-4*, *daf-7*, *daf-8*, and *daf-14* mutants are Daf-c and are at the same position in the dauer pathway as the ASI and ADF neurons because mutants form dauers after ASJ is ablated while ASI and ADF are left intact. This suggests that dauer pheromone inhibits ASI and ADF neurons by regulating the function of this group of

Daf-c genes. Collectively, these genes make up a TGFβ signaling pathway that includes a ligand (*daf-7*), its receptor (*daf-1* and *daf-4*), and downstream effector proteins (*daf-8* and *daf-14*). The *daf-7* gene encodes a novel TGFβ member with 28% identity to human TGFβ and 34% identity with human bone morphogenic protein-4 (BMP-4) in its ligand domain (Ren et al., 1996; Schackwitz et al., 1996). As predicted by genetic studies, *daf-7* is expressed in ASI neurons and its expression is negatively regulated by dauer pheromone and high temperature and positively regulated by food in recovering dauer animals (Ren et al., 1996; Schackwitz et al., 1996). In addition, *daf-11* positively influences *daf-7* expression in nondauer-inducing conditions as it was re-isolated in screens for mutations that affect *daf-7* expression in neurons (Murakami et al., 2001). Northern analysis also shows that its expression is maximal at the L1 stage, which correlates with the period of non-dauer commitment. Although there is no direct biochemical evidence, the *daf-7* receptor in *C. elegans* is presumed to be encoded by the products of the *daf-1* and *daf-4* genes, which encode TGFβ-type I and type II trans-membrane serine/threonine receptor kinases, respectively (Estevez et al., 1993; Georgi et al., 1990). In the vertebrate system, type II receptors bind ligand and recruit the type I receptor to form a heterodimeric complex. The type I receptor is subsequently phosphorylated by the type II receptor, which activates the type I receptor kinase domain resulting in phosphorylation of downstream effector Smad proteins (Shi and Massague, 2003). The Smads are known to translocate to the nucleus and bind DNA to activate transcription.

In *C. elegans*, *daf-8* and *daf-14* genes encode divergent Smad proteins that contain similar sites for phosphorylation but they do not contain the conserved amino-terminal regions responsible for binding DNA. Instead of binding DNA, these proteins are thought to antagonize the activity of two downstream genes (*daf-3* and *daf-5*) (Inoue and Thomas, 2000; Riddle and Albert, 1997). The *daf-3* and *daf-5* genes are Daf-d and have been placed downstream of the *daf-7*/TGFβ signaling pathway because they are required for the Daf-c phenotype of *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* (Vowels and Thomas, 1992) as well as the Daf-c phenotype that results from killing ASI and ADF neurons (Bargmann and Horvitz, 1991). The *daf-3* gene encodes a more conserved Smad protein that retains DNA binding capacity (Patterson et al., 1997; Thatcher et al., 1999). The *daf-5* gene encodes a Sno/Ski oncoprotein recently found to serve as a cofactor for *daf-3 (*da Graca et al., 2004*)*. The current model of TGFβ signaling in the control of dauer formation is based on the epistatic relationships between these proteins and structural conservation with their respective vertebrate counterparts. In response to non-dauer-inducing conditions, ASI neurons release TGFβ and ligand binding by *daf-1* and *daf-4* leads to phosphorylation and activation of *daf-8* and *daf-14.* These receptor Smads then transduce the signal by antagonizing *daf-3* and *daf-5*. In the absence of TGFβ (i.e. *daf-7* mutants or dauer-inducing conditions) *daf-3* and *daf-5* act unopposed in the nucleus to regulate gene expression programs required for dauer formation. These studies have revealed that the TGFβ pathway in *C. elegans* differs considerably from that in vertebrates because *daf-3* (SMAD) can function in the absence of a receptor to mediate dauer formation.

Iinsulin/IGF-1 Signaling

Finally, the third pathway that functions in parallel of the cGMP and TGFβ pathways was originally found to involve three genes *daf-2*, *daf-23/age-1*, and *daf-16*. Strong alleles of *daf-2* and *age-1* were found to have a non-conditional Daf-c phenotype while *daf-16* was Daf-d and strongly epistatic to *daf-2* and *age-1 (*Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Riddle et al., 1981; Vowels and Thomas, 1992*)*. Interestingly, weak alleles of *daf-2* and *age-1* cause profound increases in longevity. The effect of this pathway on life-span will be returned to in the discussion section. Early genetic epistasis experiments suggested that in favorable conditions *daf-2* and *age-1* acted to antagonize the function of *daf-16* and thus select continuous reproductive growth as well as short life-span. This genetic pathway nicely predicted the biochemical functions now known to exist for these proteins. It is now known that these genes make up a conserved insulin/IGF-1 signaling pathway. The pathway is mediated by *daf-2*, the *C. elegans* homolog of the insulin-like/IGF-1 receptor family that acts through downstream genes to antagonize DAF-16, a fork-head transcription factor (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Interestingly, the exact ligand for DAF-2 is unknown as the *C. elegans* genome encodes more than 30 insulin-like peptides. However, a dominant mutation in one of these insulin-like proteins, DAF-28, has confirmed a role for insulin-like proteins in the control of dauer formation (Li et al., 2003a). Like *daf-7*, *daf-28* is expressed in chemosensory neurons and is transcriptionally down-regulated under dauer inducing conditions and positively regulated by *daf-11* in nondauer-inducing conditions. The downstream signaling components through which *daf-2* functions include: a

conserved catalytic subunit of phosphatydalinositol-3-OH-kinase (PI3K) encoded by *age-1*, two AKT/Protein kinase-B homologs (*akt-1* and *akt-2),* and the AKT-kinase, PDK-1. This pathway also includes a negative regulator of *daf-2* signaling, a homolog of the lipid phosphatase PTEN, ecoded by *daf-18* (Ogg and Ruvkun, 1998). In the *C. elegans* insulin-like/IGF-1 pathway DAF-16 is believed to be the primary downstream target of the DAF-2 receptor as no suppressors of *daf-2* mutants have been identified other than *daf-16* and *daf-18*. DAF-16's transcriptional activity is regulated through protein localization via several conserved AKT phosphorylation sites. Under non-dauer inducing conditions DAF-2 is active and causes the phosphorylation and nuclear exclusion of DAF-16. Antagonism of *daf-16* by *daf-2* signaling is highly analogous to the antagonism of *daf-3* by *daf-1* and *daf-4* signaling in the TGFβ pathway. The insulin-like/IGF-1 pathway acts in parallel to the *daf-7*/TGFβ pathway as null mutations in *daf-16* do not rescue the Daf-c phenotype of *daf-7* mutants, although dauers that form have incomplete remodeling of the pharynx and intestine, which suggests that some features of dauers require *daf-16*. The parallel nature of these pathways has been further shown by the failure of a *daf-3* Daf-d mutant to rescue the Daf-c phenotype of a strong mutation in *daf-2,* e1370 (Ogg et al., 1997).

Evidence for Secondary Hormonal Signaling Pathways

As described above, three parallel signaling pathways and operate in sensory neurons to inhibit dauer formation by promoting the production of the ligands for the TGFβ and insulin-like/IGF-1 pathways. Together, these pathways operate in nondauer-inducing conditions by releasing growth factors that initiate downstream signal

transduction cascades that lead to the antagonism of two important transcriptional regulators, DAF-3(SMAD) and DAF-16 (FOXO). Two models can be proposed to account for the global affects of TGFβ and insulin signaling in dauer formation. The first model suggests that secreted ligands act directly in affected tissues to initiate the dauer remodeling. This is supported to some extent in the case of TGFβ signaling, as both the receptor (DAF-4) and downstream transcription factors (DAF-3 and DAF-14) are expressed in multiple tissues, particularly in tissues remodeled during dauer formation such as the pharynx, intestine, and hypodermis (Inoue and Thomas, 2000; Patterson et al., 1997). However, in the insulin-like/IGF-1 signaling pathway, although DAF-16 is expressed in multiple tissues it is not expressed in all remodeled tissues. In particular, DAF-16 is not expressed in the pharynx despite the fact that it has been shown to be required for pharynx remodeling during dauer formation in response to pheromone and in *daf-7* dauers (Ogg et al., 1997; Vowels and Thomas, 1992). The lack of DAF-16 expression in the pharynx thus raises the second possibility that TGFβ and insulin-like/IGF-1 components act indirectly via a secondary, broad acting signal that is produced as a result of direct action in a select group of cells. Examination of cell autonomy of TGFβ and insulin-like/IGF-1 components has addressed this issue.

Cell non-autonomy of TGFβ and insulin-like/IGF-1 Receptors

Mosaic analysis and tissue specific expression of the receptor components of the TGFβ and insulin-like/IGF-1 pathways (DAF-2 and DAF-4, respectively) in mutant backgrounds have demonstrated that the activity of both genes may not be required in all

tissues, but instead activity in a select number of cells is sufficient for specifying the fate of all other cells (Apfeld and Kenyon, 1998; Inoue and Thomas, 2000). Tissue specific expression indicates that neurons are the targets for these pathways because expression of *daf-2* or *daf-4* in neurons results in complete rescue of Daf-c phenotypes in all tissues of mutant animals. This suggests that *daf-2* and *daf-4* control dauer formation in cell non-autonomous fashion and that another level of signaling downstream of TGFβ and insulin-like/IGF-1 signaling exists that helps to coordinate the developmental fates of all cells. Additional evidence in support of this comes from the finding that activation of *daf-3* and *daf-16* is not the only output of pheromone, as *daf-3* mutants form normal dauers in pheromone while *daf-16* mutants form partial dauers.

1.6 STEROID HORMONE SIGNALING GOVERNS DAUER FORMATION AND REPRODUCTION

DAF-12, an Orphan Nuclear Hormone Receptor

Considerable insight into the cell non-autonomous function of the TGFβ and insulin-like/IGF signaling pathways has come from the finding that these parallel pathways converge on the nuclear hormone receptor, DAF-12, to inhibit dauer formation (Antebi et al., 1998; Antebi et al., 2000; Gerisch et al., 2001). Epistasis experiments have shown that *daf-12* lies at the convergence of TGFβ, insulin-like/IGF-1, and cGMP signaling pathways because the putative null allele, *daf-12(m20)*, is Daf-d and suppresses dauer formation in all Daf-c genes in the pathway (Vowels and Thomas, 1992). However, one exception was the finding that double mutants of *daf-12(m20)* and the temperature

sensitive Daf-c allele *daf-2(e1370)* arrested at the L1 or L2 stage but did not form dauers; implying that one or both gene products are required in processes independent of their role in dauer formation, such as progression to the L3 stage (described below). In addition, *daf-12* nulls do not form dauers in the presence of dauer pheromone. These results suggest that under dauer-inducing conditions *daf-12* is required for all aspects of dauer formation and that in favorable environments insulin/IGF-1 and TGFβ signaling inhibit this activity and instruct L3 developmental programs.

daf-12 is unique among the daf genes because unlike all other dauer pathway mutants, Daf-c mutations in *daf-12* have also been found. These mutations specifically affect R564 in the ligand binding domain of DAF-12 and result in arginine to cystiene or arginine to histidine mutations (alleles *daf-12(rh274)* and *daf-12(rh273),* respectively). These animals have penetrant Daf-c phenotypes and exhibit heterochronic phenotypes in L3 stage gonadal migrations (Mig). Heterochronic phenotypes are defined as the failure of cells to select their stage-appropriate developmental programs and mutations in "heterochronic" genes can cause advances or delays in cellular programs. In normal L3 development the two gonad arms extend anterior and posterior along the length of the worm and make a dorsal turn; however, unlike those of normal L3s the gonads of *daf-12(rh273)* and *daf-12(rh274)* fail to make the dorsal turn. Failure of the dorsal turn is described as a Mig phenotype. Analysis of several other *daf-12* alleles reveals that mutations in specific domains of the receptor produce predictable phenotypes. With the exception of *daf-12(rh61),* all mutations that affect conserved residues in the DBD produce Daf-d phenotype with impenetrant heterochronic phenotypes in seams cells but

normal gonadal migrations, likely due to the inability of the DAF-12 to bind DNA. These phenotypes are similar to those of two null mutations, *daf-12(m20)* and *daf-12(rh61 rh411)*. *daf-12(rh61)* is a truncation in the LBD domain after helix 5 that produces a Daf-d phenotype and penetrant heterochronic delays. Phenotypically, mutations in the LBD of DAF-12 appear stronger than mutations in the DBD and suggest that DAF-12 may regulate dauer formation through its ability to bind DNA and repress gene expression and that inhibition of dauer formation and advancement of L3 programs of development requires ligand binding and activation.

Cloning of *daf-12* by Antebi et al (2000) led to the important discovery that *daf-12* encodes a conserved member of the nuclear receptor superfamily and that its expression overlaps particularly with tissues affected during dauer formation (Antebi et al., 2000). Within its conserved DBD, DAF-12 is most related to the vertebrate pregnane x (PXR), vitamin D, and constitutive androstane receptors (CAR); however, its LBD is quite divergent from these receptors and exhibits closer homology to *Drosophila* DHR96. A more recent sequence analysis using the full length DAF-12 sequence shows it is more similar to the vertebrate Liver X receptors, which act as cholesterol sensors by binding oxidized sterols (oxysterols) and activating expression of genes involved in their metabolism to bile acids as well as storage and export (Chawla et al., 2001; Mooijaart et al., 2005). The identification of *daf-12* as a conserved member of the nuclear receptor superfamily hints at the possible regulation of dauer formation by a lipophilic hormone, the production of which could regulated by upstream signaling through the TGFβ, insulin/IGF-1 and cGMP pathways. Therefore, in unfavorable environments, DAF-12

represses reproduction and promotes dauer formation, while in favorable environments, insulin/IGF-1 and TGFβ signaling promotes the production of a ligand for DAF-12 that antagonizes the dauer promoting function of DAF-12 and activates its reproductive function. This model explains why mutations in the LBD of DAF-12 result in Daf-c and heterochronic phenotypes while complete loss of DAF-12 or mutations in the DBD result in Daf-d phenotypes. The impenetrant heterochronic delays associated with Daf-d phenotypes may reflect de-repression of target genes due to combined loss of DNA binding, co-repressor recruitment, and transcriptional activation by ligand.

DAF-9, a Cytochrome P450

The molecular identity of *daf-12* and analysis of mutant alleles were the just the first pieces of evidence indicating that a lipophilic hormone might serve as one of the secondary signals downstream of the TGFβ and insulin-like/IGF-1 pathways by acting as a DAF-12 ligand. The final link between the upstream neuroendocrine signaling pathways and DAF-12 function was provided by the molecular identification of the Daf-c gene, *daf-9*, which was found to encode a conserved cytochrome p450 (Gerisch et al., 2001; Jia et al., 2002). Null mutations in *daf-9* result in unconditional dauer arrest; animals form partial dauers (incomplete pharyngeal remodeling and germ cell arrest) and a small percentage spontaneously recovers to sterile adults. *daf-9* lies upstream of *daf-12* and likely at the convergence of insulin/IGF-1 and TFGβ signaling pathways because like Daf-c mutations in the insulin/IGF-1 and TGFβ signaling pathway, *daf-9* phenotypes are suppressed by null mutations in *daf-12.* These findings implicate *daf-9* in the production of a hormonal ligand for DAF-12 that ultimately dictates the activity of *daf-12*.

A number of important observations, besides the molecular identities of *daf-9* and *daf-12*, support the idea that dauer formation and reproduction is under the control of a DAF-9-produced hormonal ligand for DAF-12. Further evidence comes from phenotypic similarities between *daf-9* and *daf-12* mutants. As mentioned previously, mutations in *daf-12* produces both Daf-d and Daf-c phenotypes. The Daf-c mutation in *daf-12(rh273)* produces partially remodeled dauers at all temperatures but they recover to fertile adults with Mig phenotypes. These mutations affect a predicted ligand contact site in the LBD of DAF-12. Additionally, the hypomorphic allele *daf-12(rh284),* also affected in the LBD, has normal dauer regulation but a penetrant Mig phenotype. The severity in the phenotypes between these two *daf-12* mutants may likely be a result of a difference in ligand affinity or response. This is indirectly supported by the finding that the *daf-9* nulls are Daf-c and resemble the *daf-12*(rh273) allele, while a weaker mutation, *daf-9(rh50),* results in a low or no penetrance Daf-c phenotype but a penetrant Mig phenotype, similar to *daf-12(rh284)*. A simple hypothesis proposed by Gerisch et al (2001) is that Daf-c phenotypes of *daf-9* and *daf-12* result from loss of hormone production and binding and/or response, respectively, and that less hormone is required to overcome dauer than is required to progress through L3 reproductive development events. As previously mentioned, mutations in the LBD of DAF-12 produce stronger phenotypes than complete loss of *daf-12* or mutation of the DBD. Some nuclear receptors, such as TRβ, actively repress gene expression in the absence of ligand due to binding of co-repressors, whereas ligand binding causes both de-repression of basal gene expression as well as activation. A resistance to thyroid hormone syndrome (Merris et al.) in humans occurs as a result of

mutations in the LBD of TRβ that abolish its transactivation by hormone, but maintain its ability to repress target genes (Yoh et al., 1997). By analogy with RTH, Daf-c phenotypes and heterochronic delays may result from uncoupling of co-repressor dissociation and hormone binding. In the absence of ligand binding (or a response to it) (i.e. *daf-12(rh273)* or *daf-12(rh284)*) or loss of ligand production (*daf-7*, *daf-2*, *daf-9*) DAF-12 remains bound to co-repressors and promotes dauer formation and/or inhibits L3 stage developmental programs. This idea fits nicely with the genetic model whereby *daf-9* inhibits *daf-12* with respect to dauer formation (Figure 1-2).

DIN-1S, a Putative DAF-12 Co-repressor

Yeast two hybrid analysis using the LBD domain of DAF-12 recently identified the DAF-12 interacting protein, DIN-1S (Ludewig et al., 2004). Molecular, genetic, and cellular analysis of DIN-1S function strongly suggests that it functions as a nuclear co-repressor of DAF-12. First, molecular analysis revealed that DIN-1S contains a conserved C-terminal domain called SID/RD, for SMRT interaction domain/repressor domain. This domain is typical of a class of large nuclear proteins found in flies, mice and humans. The human homolog, SHARP (SMRT/HDAC1 Associated Repressor Protein), is a nuclear receptor co-repressor protein that binds SMRT/NcoR co-repressors through the SID/RD domain. A larger isoform of DIN-1S (DIN-1L) lacks a single exon required for interaction with DAF-12 and RT-PCR shows that it is likely transcribed from a promoter separate from the short isoform. Furthermore, the DAF-12 interacting domain (RID) contains several hydrophobic motifs (LXXLL and the like) resembling those found in co-repressor proteins. Second, RNAi of DIN-1S or mutations that disrupt the RID

completely suppress the heterochronic defects seen in *daf-12(rh61)*, *daf-12(rh274),* and *daf-9(rh50),* which are mutations that presumably disrupt ligand binding and production. In addition, RNAi of DIN-1S caused Daf-d phenotypes and suppressed the Daf-c phenotypes of temperature sensitive mutations in *daf-2*, *daf-7*, *daf-11*, and also in the null allele of *daf-9*. Third, DIN-1S is ubiquitously expressed and localized to the nucleus of all tissues remodeled during dauer formation. Taken together, these findings show that that DIN-1S interacts genetically with un-liganded DAF-12, which is consistent with a role for DIN-1S as a co-repressor of DAF-12. In this view, DIN-1S binds DAF-12 in the un-liganded state to select dauer formation, whereas ligand binding leads to disassociation of the DAF-12:DIN-1S complex favoring selection of reproductive growth and proper developmental timing.

Endocrine-like Expression Patterns and Action

The expression pattern of *daf-9* relative to *daf-12* and *din-1s* strongly suggests that they function together in an endocrine-like fashion. Whereas *daf-12 and din-1s* are ubiquitously expressed in phenotypically affected tissues, *daf-9* is specifically localized to two neuron-like head cells in the worm (XXX cells), the hypodermis, and spermatheca (Antebi et al., 2000; Gerisch et al., 2001; Ludewig et al., 2004). The restricted expression pattern of DAF-9, compared to DAF-12 and DIN-1S suggests that it can function in a cell non-autonomous fashion. Indeed, expression of *daf-9* under a heterologous promoter in the hypodermis is sufficient to rescue all phenotypes of *daf-9(dh6)* and *daf-9(rh50) (Gerisch and Antebi, 2004)*. Over-expression of *daf-9* under the same promoter is also sufficient to rescue the Daf-c phenotype of *daf-12(rh273)*, but not the Mig phenotype of

daf-12(rh273) or *daf-12(rh61)* (truncation of the LBD), which again suggests that more ligand is needed to bypass L3 reproductive delays than is needed for inhibition of dauer formation. Finally, mutations in *daf-7* and *daf-2* may result in limiting amounts of *daf-9* activity because over-expression of *daf-9* rescued the Daf-c phenotype of *daf-7* but only partially rescued that of *daf-2* (Gerisch and Antebi, 2004).

The expression of *daf-9* in the hypodermis is also under feedback control by DAF-12, which is reminiscent of endocrine-like regulation. A green fluorescent protein (GFP) reporter fused to the *daf-9* promoter and cDNA (containing intron-1) is expressed in XXX cells, hypodermis, and spermatheca in WT animals. Its expression is up-regulated in *daf-9(rh50)* and in the hypodermis of WT animals exposed to mild stress, but shut off in *daf-9(dh6)* or in high stress (low food, high temperature, and high pheromone) (Gerisch and Antebi, 2004). Hypodermal *daf-9* expression and regulation by environmental inputs require *daf-12* because they are lost in *daf-12(rh61rh411)* nulls.

Taken together, these studies provide compelling evidence for involvement of *daf-9* in the production of a hormonal ligand for DAF-12. Given that *daf-9* action is cell non-autonomous and rate limiting in *daf-2* and *daf-7* mutants, *daf-9* is a likely candidate for the source of the secondary endocrine output responsible for cell non-autonomous function of insulin/IGF-1 and TGFβ receptors. More recently, further proof of a secondary lipophilic hormone hypothesis has come from the finding that crude lipid extracts prepared from WT or *daf-12(m20)* worms, but not *daf-9* null animals, can rescue the Daf-c phenotypes of *daf-2*, *daf-7*, and *daf-9* animals (Gill et al., 2004). Partial

purification of the activity suggests that it is a small hydrophobic molecule with some hydrophilic properties.

A Role for Cholesterol in Dauer Formation and Reproduction

Most nuclear receptors bind to small lipophilic molecules such as fatty acids or steroid hormones. DAF-9 is related to CYP2 family members, which are steroid and fatty acid hydroxlyases. Interestingly, *C. elegans* requires sterols in its diet for normal growth and reproduction because it lacks the ability to synthesize cholesterol from acyclic precursors, (Chitwood, 1999). Cholesterol deprivation of WT worms phenocopies Mig phenotypes of *daf-9* worms and significantly enhances the Daf-c and Mig phenotypes of hypomorphic alleles of both *daf-9(rh50)* and *daf-12(rh284)* at 20°C, which normally appear only at low penatrance at 25°C (Gerisch et al., 2001). Similar results were obtained from an independently isolated hypomorphic allele *daf-9(m540)* (Jia et al., 2002). These data strongly suggest that DAF-9 produces a steroid hormone ligand for DAF-12. Further evidence implicating a role for cholesterol in dauer formation comes from the finding that mutations in both *ncr-1* and *ncr-2*, two *C. elegans* homologs of the human Niemen-Pick Type C (NPC) gene involved in the lysosomal transport of cholesterol, produce a Daf-c phenotype and act upstream of *daf-9* (Li et al., 2004).

Analysis of the tissue distribution and growth and developmental requirements for cholesterol in *C. elegans* indicates that cholesterol is used in signaling rather than in general maintenance of membrane integrity. In *C. elegans*, unlike in mammalian systems, cholesterol appears to concentrate in only a limited number of cells (Entchev and

Kurzchalia, 2005; Matyash et al., 2004; Matyash et al., 2001). In addition only very small amounts of cholesterol (~20nM) are required to support normal reproduction (Matyash et al., 2004; Merris et al., 2003). It should be mentioned, however, that cholesterol visualization studies relied on the naturally fluorescent sterol, dehydroergosterol, and a polyene antibiotic (filipin) that becomes fluorescent upon forming a complex with sterols containing a 3β-hydroxyl group. Thus, any metabolites of these sterols that did not retain the structural properties required for visualization would not be detected. Despite this, these studies have demonstrated that sterols are required in small amounts and they are not widely distributed as would be expected were they to play a more general role in *C. elegans* membrane integrity.

Taken together these data provide compelling evidence for a model in which DAF-9 produces a cholesterol-derived ligand for DAF-12 that inhibits dauer formation and promotes reproductive development. However, as will be described below, no oxidation products of cholesterol such as known steroid, endocrine, or insect molting hormones have yet been detected in *C. elegans*.

Cholesterol Metabolism

Although the identity of the cholesterol derived DAF-12 ligand remains elusive investigations into the metabolism of cholesterol in nematodes provide some very important clues. Biochemical analysis of the cholesterol in *C. elegans* and other nematodes has led to a description of its major metabolites in the worm and a proposed route for its metabolism (Figure 1-3) (Chitwood, 1999). *C. elegans* has is the ability to de-saturate cholesterol at the 7 position, producing 7-dehydrocholesterol, as well as to

carry out 5α-reduction, which produces lathosterol. Notably, this route of cholesterol metabolism in *C. elegans* occurs in a direction opposite to the route of cholesterol synthesis in humans. *C. elegans* also exhibits a surprising ability to methylate the sterol nucleus at C-4 producing the Δ^7-4α-methylsterol, lophenol. Finally, *C. elegans* has the ability to isomerize double bonds, such as producing the $\Delta^{8(14)}$-4α-methylsterol, lophenol 8(14). Finally, *C. elegans* can be propogated on cholestanol, and they do not convert it to cholesterol (Lozano et al., 1987). These results suggest that *C. elgans* lacks the ability to desaturate the double bond at C-5 and argues that *C. elegans* may have a specific requirement for Δ^7 sterols. Despite an exhaustive screen for sterol metabolites in *C. elegans* no other transformations to the sterol side chain or nucleus, such as oxidation, were detected (Chitwood, 1999). The apparent absence of oxidation products in *C. elegans* is surprising given the fact that *C. elegans* possess numerous enzymes known to be involved in sterol metabolism in humans, such as DAF-9 and ~79 other members of the cytochrome p450 family.

The specific functional requirement met by each of the sterol metabolites described above has been investigated by supplying the individual sterol as the sole dietary sterol and then examining its effect on growth and development. In axenic

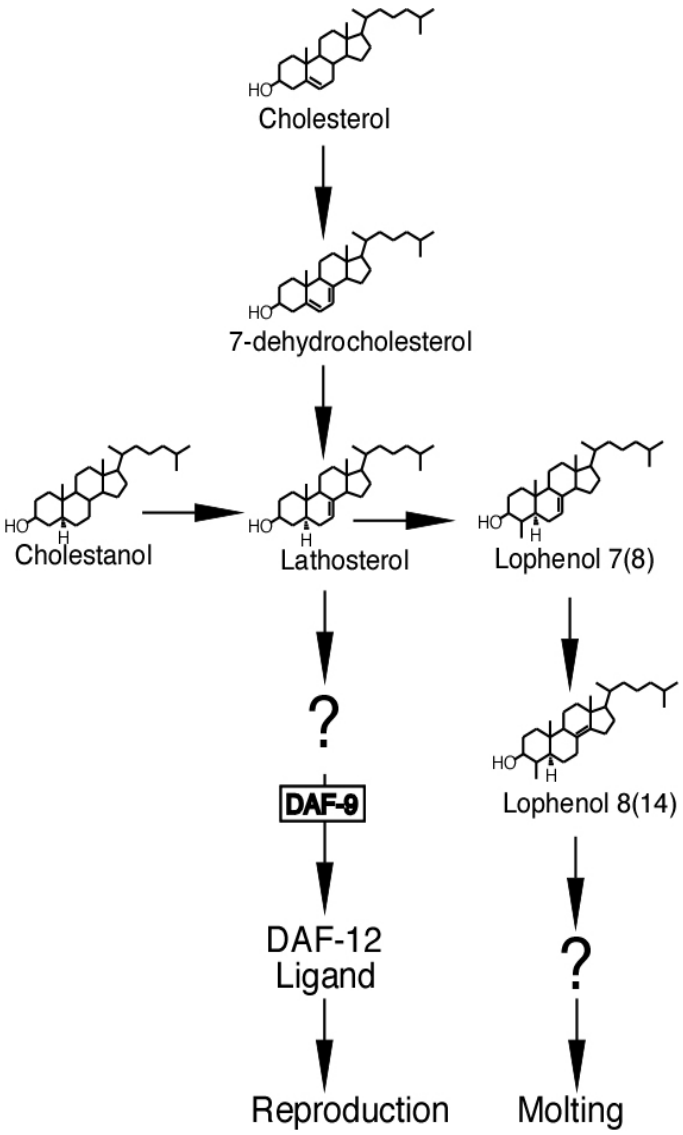

Figure 1-3 Metabolism and Function of Cholesterol in *C. elegans*

C. elegans is a cholesterol auxotroph and requires sterols in its diet for at least two processes, reproductive development and molting. *C. elegans* metabolizes dietary sterols, such as cholesterol, into 4-methylsterols (lophenol) and desmethylsterols. Either of the major des-methylsterols in *C. elegans,* 7-dehydrocholesterol and lathosterol, supports normal reproductive growth and molting, whereas lophenol supports molting to dauer in the absence of other sterols. Lathosterol is considered to be a precursor to the DAF-12 ligand and lophenol a precursor to a molting hormone. (Chitwood 1999; Matayash et al 2004)

culture, *C. elegans* has no absolute requirement for cholesterol or 7-dehydocholesterol because growth on lathosterol is sufficient for reproductive development (Lozano et al., 1987). Lophenol and its 8(14) analog do not support normal growth and reproduction. When *E. coli* is used as the food source and stringent conditions are used to remove trace amounts of sterols from agar WT animals will arrest in the second generation at the L2 molt as partial dauer animals that exhibit a normal dauer cuticle surrounded by an L2 cuticle. Under these conditions, when WT animals are fed any one of the major 4-desmethylsterols (Figure 1-3) animals develop continuously to reproductive adulthood. However, when lophenol is supplied as the sole dietary sterol, animals complete their molt to dauer and do not recover to reproductive adulthood (Matyash et al., 2004). The dauer cuticle phenotype requires *daf-12* because *daf-12* nulls arrest as L2s but lack dauer alae. Interestingly, when lophenol is provided as the sole dietary sterol, *daf-12* animals grow to reproductive adults. These results suggest that lathosterol and not lophenol is a likely precursor to the DAF-12 ligand because it inhibits dauer formation and supports reproductive development of WT animals. In addition, since lophenol is sufficient to support molting and reproductive growth of *daf-12* null animals, which have no requirement for a DAF-12 ligand, lophenol or another 4-methyl-sterol is likely to be a precursor to a *C. elegans* molting hormone (Figure 1-3).

1.7. STATEMENT OF PURPOSE

The evidence that a steroid hormone governs the dauer decision through modulating the activity of the orphan nuclear hormone receptor DAF-12 is compelling.

Despite the elegant genetic studies that have defined the role of DAF-9 in the production of this hormone no known steroids or steroid hormones have been identified that can rescue the defects of *daf-9* animals. However, these studies have relied primarily on in vivo screening assays that are low throughput and do not fully exploit the knowledge that has been gained through the analysis of the dauer formation pathway. The genetics tells us that DAF-9 acts at a key step in the pathway. Exactly where in the biosynthesis of the DAF-12 ligand it is positioned is unclear, but since it is the only sterol-metabolizing enzyme identified to-date with a role in dauer formation it is likely to act at a crucial or even rate-limiting step. Therefore, using the limited knowledge of sterol metabolism in *C. elegans* and the important role of DAF-9 in the dauer pathway I established a ligand screening system to identify either synthetic or endogenous sterol activators of DAF-12. The lack of cell lines derived from *C. elegans* presented a potential problem, as there was no guarantee that DAF-12 would function in a mammalian, insect, or otherwise unrelated cell line. Despite this fear, I chose the mammalian one-hybrid co-transfection assay in HEK293 cells, as it has been successfully used in the past to identify ligands for a number of nuclear receptors, including invertebrate receptors. Many features of this system, including its relatively high-throughput format and sensitivity, made it an attractive system. I decided to take two approaches to identify DAF-12 ligands. The first approach involved a focused screen of the known sterol metabolites found in *C. elegans* along with known insect and vertebrate steroids and endocrine hormones. The second approach was a more classical biochemical screen of lipid extracts from large-scale

synchronously grown liquid cultures of L3-L4 stage WT animals. Both approaches would rely on the co-transfection assay and in the end complemented one another nicely.

By establishing this screening assay I hoped to address a number of important questions. What is the DAF-12 ligand? What are the sterol molecules that function in the nematode to mediate dauer formation and reproductive development? What is the biochemical activity of DAF-9? How does it regulate the function of DAF-12? What are the target genes of DAF-12? How does DAF-12 regulate dauer formation in one environmental context and regulate reproductive development in another? I knew that the identification of ligands for DAF-12, whether these ligand be endogenous or not, would provide answers to a few if not all of these questions. However, what I did not expect was that my discoveries would soon give rise (in my mind) to a number of new ideas and questions that could have far reaching impact on humans afflicted with diseases caused by parasitic nematodes. In the next chapter I describe my efforts that culminated in the identification of two endogenous ligands for DAF-12. Much of this work could not have been accomplished if it were not for the help of a number of important collaborators, whom were colleagues as well as friends. In the remaining chapters I briefly describe some of the questions that have recently become important to me and my efforts to address them.

CHAPTER 2

IDENTIFICATION OF LIGANDS FOR DAF-12 THAT GOVERN DAUER FORMATION AND REPRODUCTION IN *C. ELEGANS*

2.1 CANDIDATE SCREENING IDENTIFIES 3-KETO-LITHOCHOLIC ACID AS A WEAK DAF-12 AGONIST

To screen for DAF-12 ligands a chimeric GAL4-DAF-12 co-transfection assay in HEK293 cells was performed and all screens were performed in the presence or absence of co-transfected 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 Ruvkun, 2004). The 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 2-1A) as a weak activator of DAF-12 independent of DAF-9 (Figure 2-1B). Lithocholic acid (LCA, Figure 2-1A), 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 co-transfected DAF-9 (Figure 2-1B). 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,

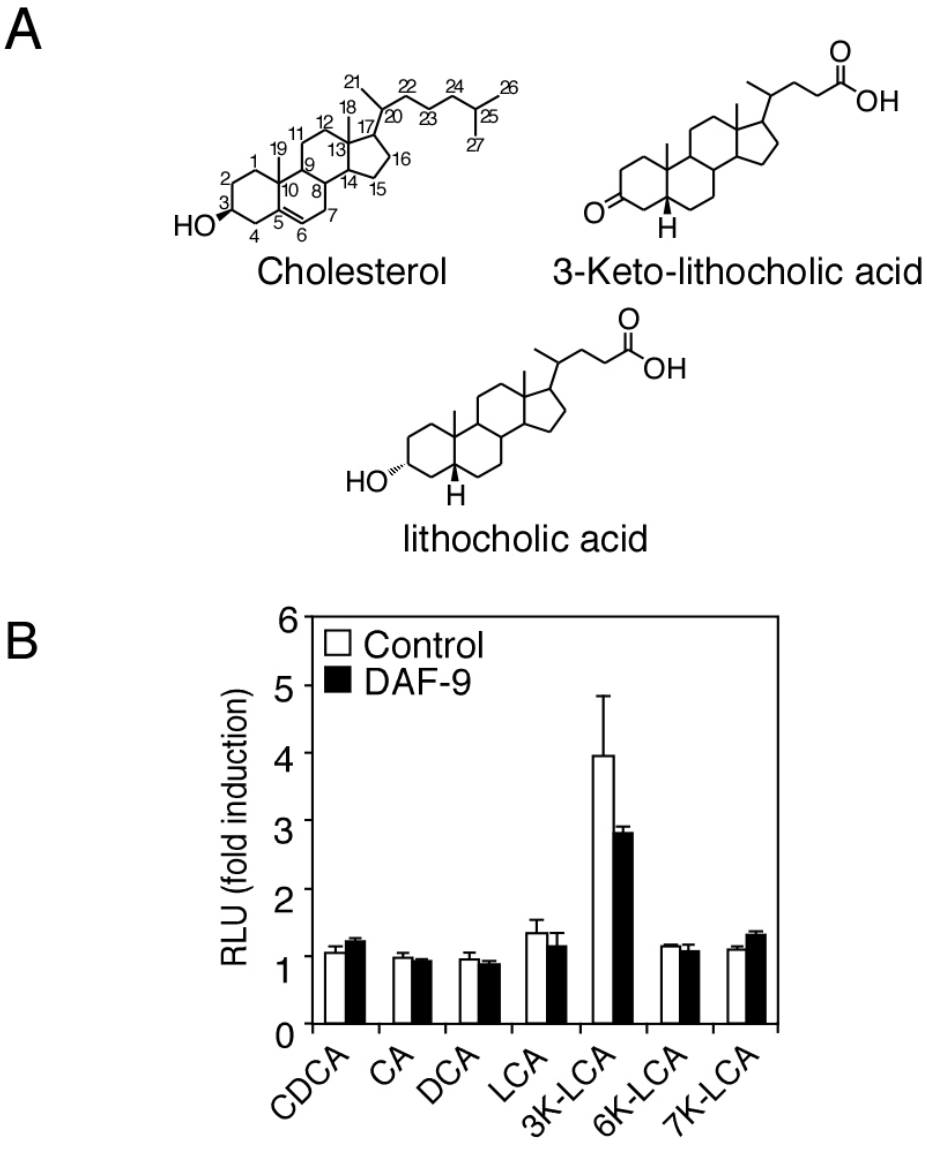

Figure 2-1 3-Keto-Lithocholic Acid is a Weak Agonist of DAF-12

(A) Structure of cholesterol relative to 3-keto-lithocholic acid and lithocholic acid (B) Activation of DAF-12 by 10μM bile acids in the presence of DAF-9 (black bars) or an empty CMX control (white bars) vector. In (B) co-transfection of the intestinal bile acid transporter (IBAT) expression plasmid was used to facilitate bile acid uptake. Reporter gene activity is expressed as fold induction of relative light units (RLU) compared to ethanol control (n = 3 ± SD).

estradiol, corticosterone, 1,25-dihydroxyvitamin D3, and 20-hydroxyecdysone (Figure 2-1B, see Appendix A for a complete list of the compounds that were tested).

2.2 IDENTIFICATION OF 3-KETO STEROLS AS DAF-9-DEPENDENT ACTIVATORS OF DAF-12

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 (Figure 1-3). Therefore, I tested lathosterol and lophenol along with their respective 3-keto derivatives, lathosterone and lophenone, for activity in the co-transfection assay (Figure 2-2A). Upon co-transfection 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 2-2B). In addition, 4-cholesten-3-one, which is a biosynthetic and auto-oxidation product of cholesterol (Parish et al., 1991), activated DAF-12 (109-fold) in the presence of DAF-9 (Figure 2-2B). 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 2-2A). These results suggest that DAF-9 converts 3-keto-sterols, possibly through side-

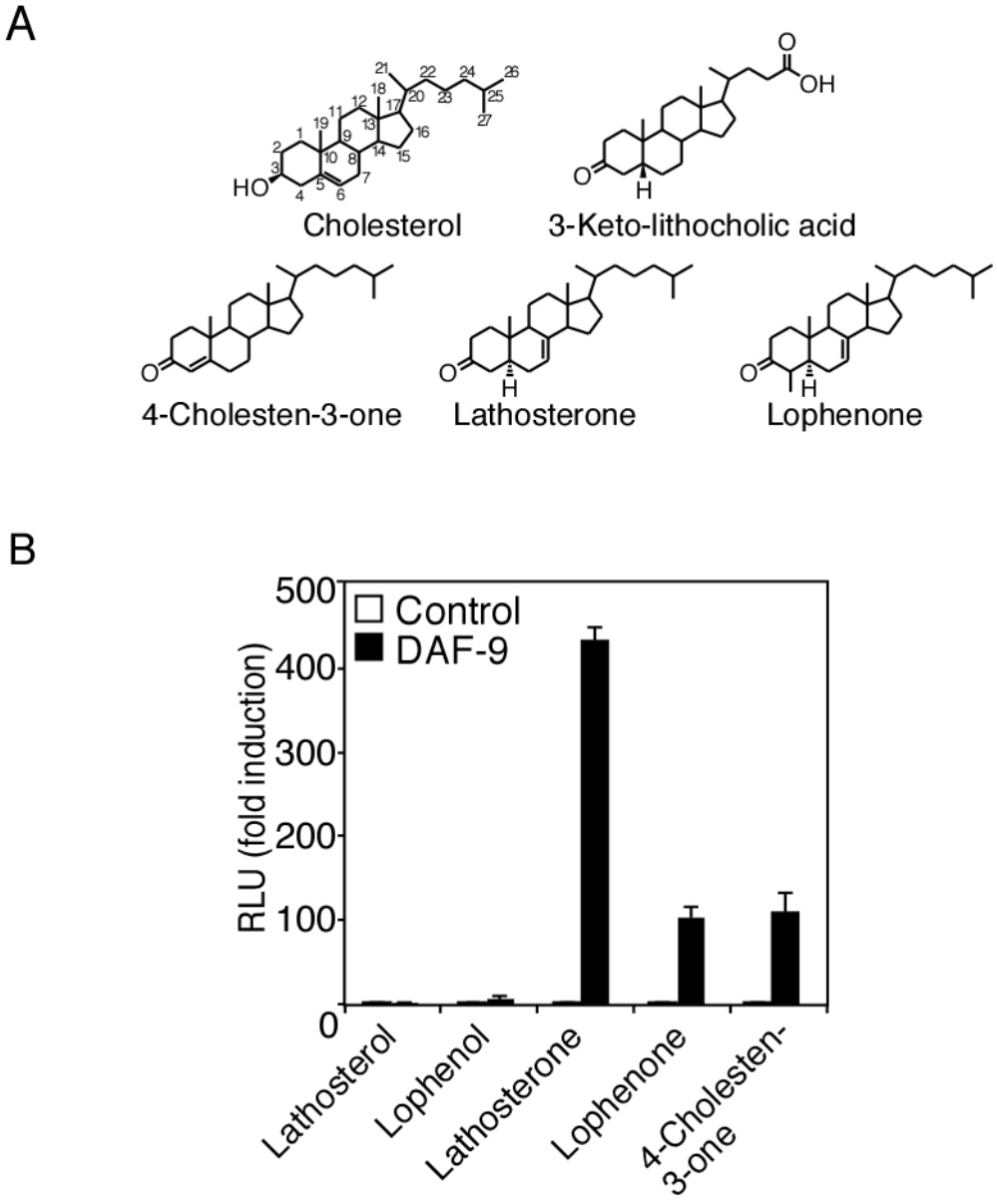

Figure 2-2 Activation of DAF-12 by 3-Keto-sterols Requires DAF-9

(A) Structures of candidate DAF-12 ligand precursors relative to cholesterol and 3-keto-lithocholic acid. (B) Activation of DAF-12 by 10μM *C. elegans* sterols and their 3-keto derivatives in the presence of DAF-9 (black bars) or an empty CMX control (white bars) vector. Reporter gene activity is expressed as fold induction of relative light units (RLU) compared to ethanol control (n = 3 ± SD).

chain oxidation, into DAF-12 activators.

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

To determine whether the 3-keto-sterol metabolites were biologically relevant I 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 and their respective 3β-hydroxy sterols were incubated with *Spodoptera frugiperda* (*Sf-9)* cell microsomes containing DAF-9 and the human P450 oxidoreductase (hOR), which I refer to in all future experiments as DAF-9 microsomes. As a control, microsomes from cells expressing hOR alone were used. For rescue experiments, I used *daf-9(dh6) dhEx24 [daf-9(+):sur-5::GFP]* worms that were obtained from the Antebi lab. These worms 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*(+) and *daf-9*(-) worms, were treated with the microsomal extracts for 48h. *daf-9*(-) worms were then isolated and scored 24h 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 2-3A). 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

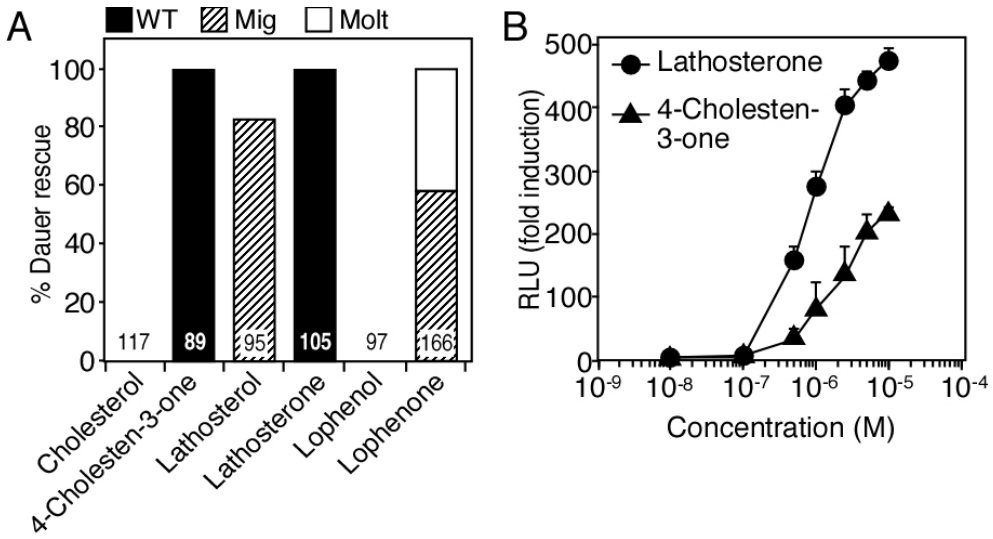

Figure 2-3. 3-Keto-sterol Metabolites of DAF-9 Rescue *daf-9* Null Worms.

(A) Rescue of *daf-9(dh6)* 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. (B) Dose response of DAF-12 activation to indicated sterols in cells co-transfected with DAF-9. Reporter gene activity is expressed as fold induction of relative light units (RLU) compared to ethanol control (n = 3 ± SD).

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 2-3A). 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 2-3A). 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.

Separate DAF-9 microsomes were generated that utilized the *C. elegans* homolog of the human P450 oxidoreductase, which I call wOR. The CGC gene name is K10D2.6 or *emb-8*. These microsomes were found to produce lower amounts of DAF-9 metabolites (by LC/MS analysis) and so they were not utilized for rescue assays. I do not know if there is a strict requirement for an oxidoreductase in these DAF-9 microsomes because DAF-9 was never expressed in their absence. However, as described above all activities observed were DAF-9-dependent.

Lathosterone and 4-Cholesten-3-one Support Reproductive Development

The above data suggested that lathosterone and 4-cholesten-3-one serve as substrates for DAF-9 that are metabolized into activities that completely rescue the

phenotype of *daf-9* animals. However, a weak activity was detected for the 3-keto-4-methyl sterol lophenone. This result was surprising as the 3β-hydroxy derivative, lophenol, showed no activity in the assay and has been previously found to be incapable of supporting reproductive development. To rule out the possibility that lophenone can function as a DAF-9 substrate in vivo, I compared the ability of lophenone, 4-cholesten-3-one, and lathosterone to support reproductive development on their own or in the presence of 13uM lophenol. Normally, when WT worms are deprived of all sterols they arrest as partial dauers that fail to complete the molt. Providing lophenol as their sole dietary sterol allows completion of the dauer molt whereas cholesterol, 7-dehydrocholesterol, or lathosterol inhibits developmental arrest and promotes reproductive development in the presence or absence of lophenol (Matyash et al., 2004). At concentrations as low as 25nM, I found that lathosterone and 4-cholesten-3-one supported full reproductive development (Figure 2-4). Interestingly, addition of 13μM lophenol slightly reduced the effect at the lowest dose of lathosterone and 4-cholesten-3-one tested (data not shown). In contrast, growth on lophenone alone, at concentrations as high as 13μM, resulted in almost complete dauer arrest. Identical results were seen in the presence of 13μM lophenol (data not shown). Taken together, these results demonstrated that lophenone is not an in vivo substrate of DAF-9 because it does not support reproductive growth on its own or in the presence of lophenol, whereas 4-cholesten-3-one and lathosterone do. Although a DAF-9-dependent activity of lophenone was seen in the co-transfection assay and in vitro microsomal rescue assays, methylation at C-4 may preclude sterols from entering the DAF-9 pathway in vivo.

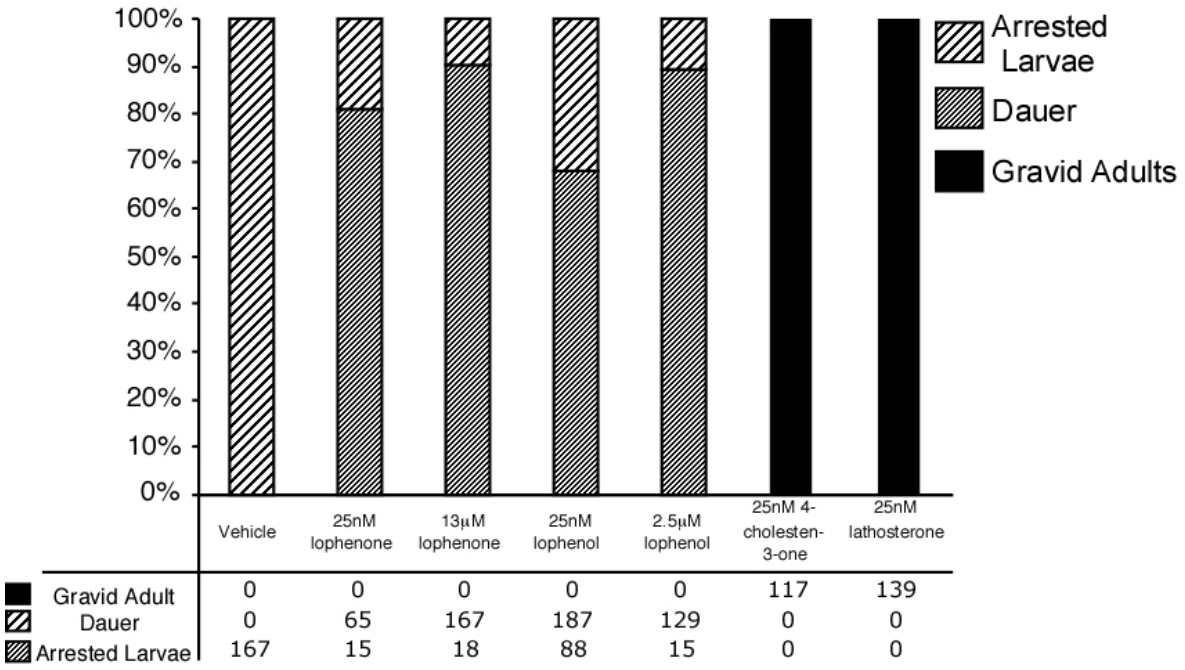

Figure 2-4. 4-Cholesten-3-one and Lathosterone Support Reproductive Growth of *C. elegans*

The progeny of cholesterol deprived WT worms were grown on cholesterol free plates in the presence of bacteria mixed with the indicated concentrations of the above sterols. Worms that arrested as L2/dauer like animals are indicated as arrested larvae. The percentage each group contributed to the entire population is shown on the y-axis. Number of worms in each category is shown in the table; data are from one experiment.

2.3 IDENTIFICATION OF DAF-9 METABOLITES OF 4-CHOLESTEN-3-ONE AND LATHOSTERONE

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 co-transfection assay revealed that lathosterone metabolites were either significantly more potent or more abundantly produced than 4-cholesten-3-one metabolites (Figure 2-3B). Liquid chromatography/mass spectrometry (LC/MS) was next used to identify these DAF-9 products. (Note: All LC/MS work was performed by Dr. Carolyn L. Cummins). 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 2-5A). 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 13 min (Figure 2-5A). 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 minutes (peaks 3 and 4), analogous to the pattern seen for 4-cholesten-3-one (Figure 2-5B; data not shown). Fractions from DAF-9 microsomal reactions subjected to reverse phase high performance liquid chromatography (RP-HPLC) were also tested for DAF-12 activation and rescue of *daf-9* null animals (Figure 2-5C-F). Fractions corresponding to peaks 1-4 (i.e., the 4-cholesten-3-one and

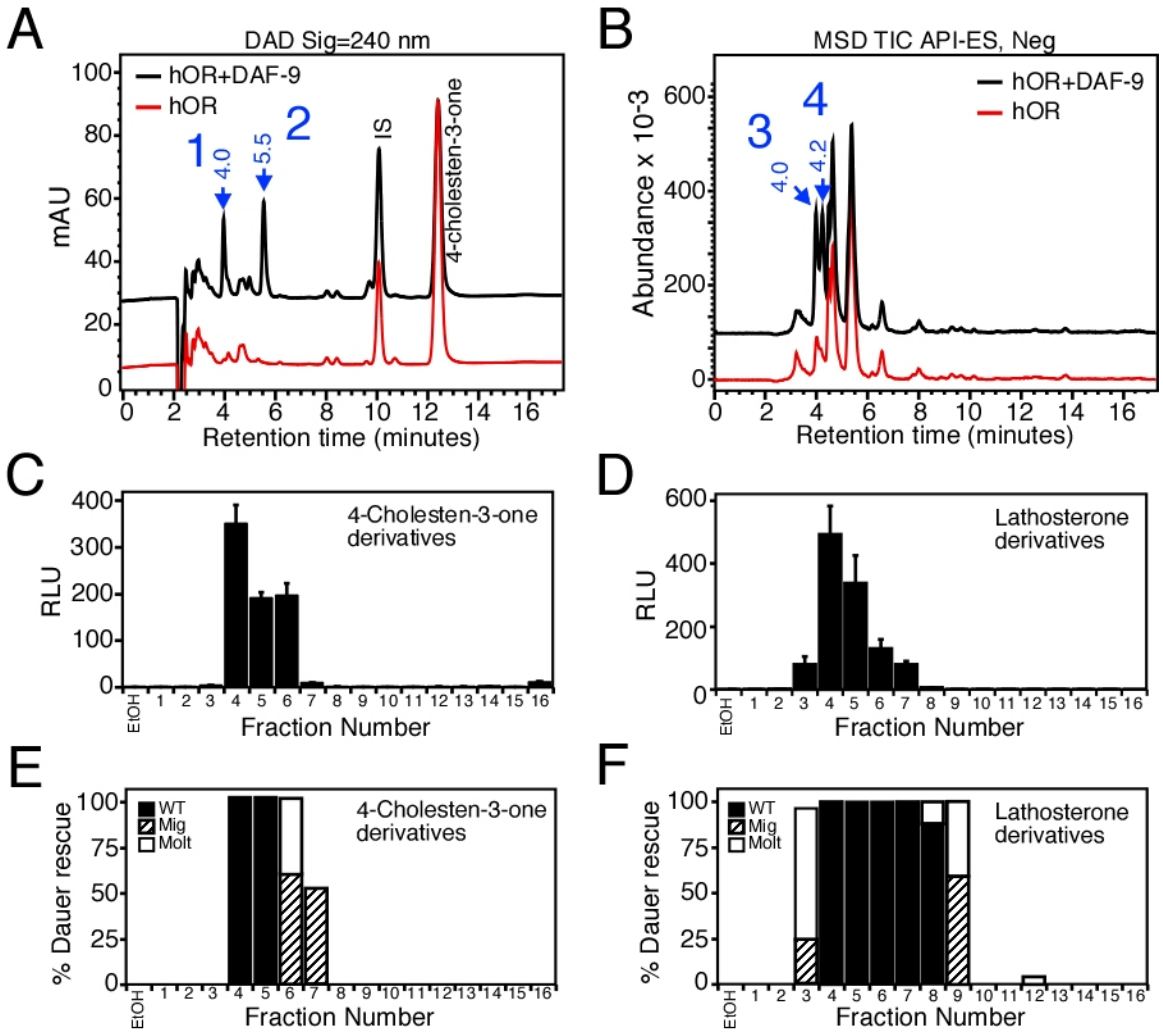

Figure 2-5. Chromatographic Properties and Activities of DAF-9 Metabolites of 4-Cholesten-3-one and Lathosterone

(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 10 pooled DAF-9 microsome reactions incubated with 4-cholesten-3-one or lathosterone were tested for GAL4-DAF-12 activity in the absence of DAF-9 (C and D), or for rescue of *daf-9* null phenotypes (E and F). Fractions correspond to 1 min intervals of retention times in (A and B). Rescue assays are described in Figure 2-3 legend. Average number of worms tested in (E) and (F) were 75 and 125, respectively.

lathosterone derivatives) activated DAF-12 several hundred-fold in the absence of co-transfected DAF-9 (Figure 2-5C and D) and rescued *daf-9* null animals (Figure 2-5E and F). 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, LC/MS was used 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 mono-hydroxylated 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 2-6A). 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 2-6A). 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 2-6B). Finally, peak 3 contained one DAF-9 specific product at *m/z* 415 (Figure 2-6B. Although the identity of this peak remains unknown, the activity observed in fractions 4 and 5 (from Figure 2-5 D) tracks predominantly with peak 4 and not peak 3 (data not shown).

DAF-9 is a 3-Keto-Sterol-C26-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. Because monohydroxylated derivatives of cholesterol were commercially available I was able to focus on defining the site of oxidation on the 4-cholesten-3-one metabolites of DAF-9. A

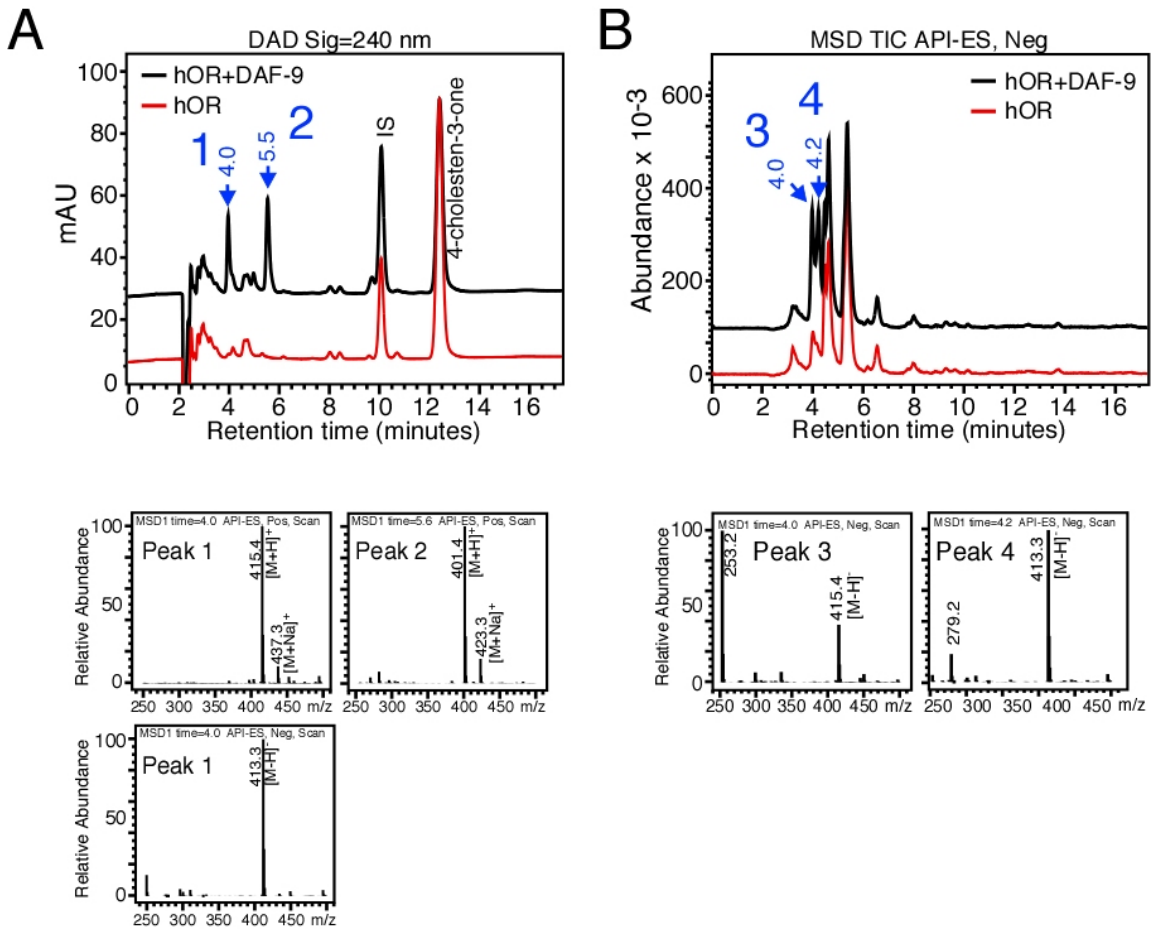

Figure 2-6, Identification of Carboxylated Metabolites of 4-Cholesten-3-one and Lathosterone as DAF-12 Agonists

(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. Mass spectra of DAF-9 metabolites of 4-cholesten-3-one (peaks 1-2) and lathosterone (peaks 3-4) scanned from m/z 250-500 are shown at the bottom.

panel of side-chain oxidized 4-cholesten-3-one derivatives was generated by converting 5-cholesten-3β-ol (Ring A, 3β -hydroxy-Δ5) 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-Δ4) oxysterols using cholesterol oxidase (Figure 2-7A). When tested in the co-transfection 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 2-7B). 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 2-5A) into two peaks that co-eluted with the diastereomers of 26-hydroxy-4-cholesten-3-one (Figure 2-7C). These results revealed that DAF-9 is a non-stereoselective 4-cholesten-3-one 26-hydroxylase.

Given that DAF-9 microsomes produced both hydroxylated and carboxylated metabolites of 4-cholesten-3-one, I 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 with 4 min retention time and mass spectra at *m/z* 415 in positive ion mode (Figure 2-8A). This retention time and mass spectral property were identical to the carboxylated metabolite found after incubation of 4-cholesten-3-one with DAF-9 microsomes (Figure 2-5A, peak 1) and were not detected in control microsomal reactions (Figure 2-8A).

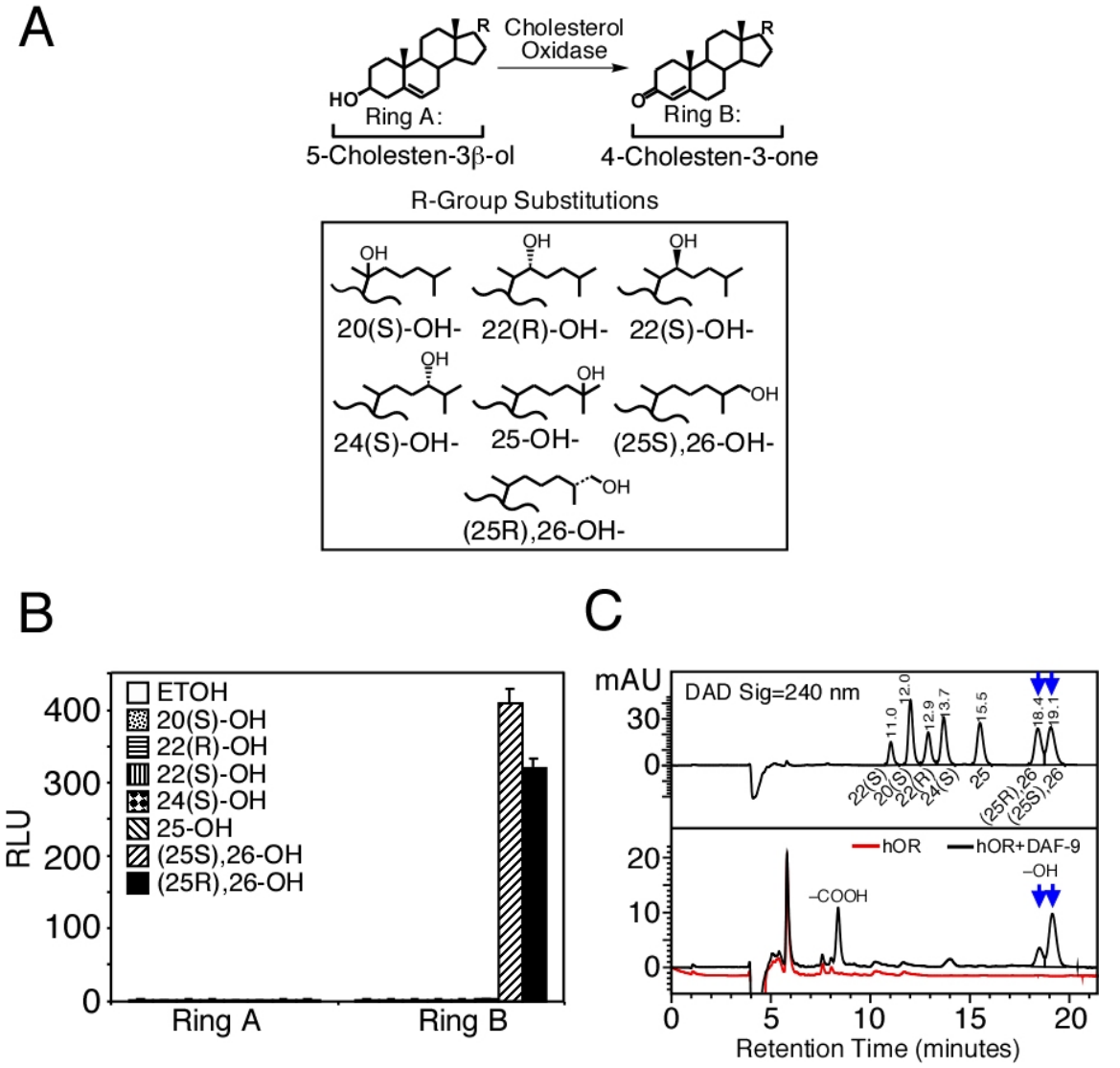

Figure 2-7. DAF-9 is 3-Keto-Sterol C-26 Monooxygenase

(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 (10μM for all sterols except 22(R)-hydroxy-4-cholesten-3-one, which was 4μM). (C) UV chromatogram of 4-cholesten-3-one oxysterols (top panel) compared to DAF-9 (black line) or control (red line) microsomes incubated with 100μM 4-cholesten-3-one (bottom panel). Blue arrows indicate co-eluting sterols. The carboxylic acid and alcohol metabolites of DAF-9 are indicated.

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 2-8B). 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 2-5A (Figure 2-8B). 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). CYP27A1 metabolizes cholesterol into (25R),26-hydroxy-cholesterol and then catalyzes a subsequent oxidation at C-26, using its own product as a substrate, to produce cholestenoic acid. 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. Co-transfection of HEK293 cells with GAL4-DAF-12, human or mouse CYP27A1, and bovine adrenodoxin resulted in strong activation of GAL4-DAF-12 (Figure 2-9). In contrast, DAF-12 was not activated by 4-cholesten-3-one in the presence of bovine

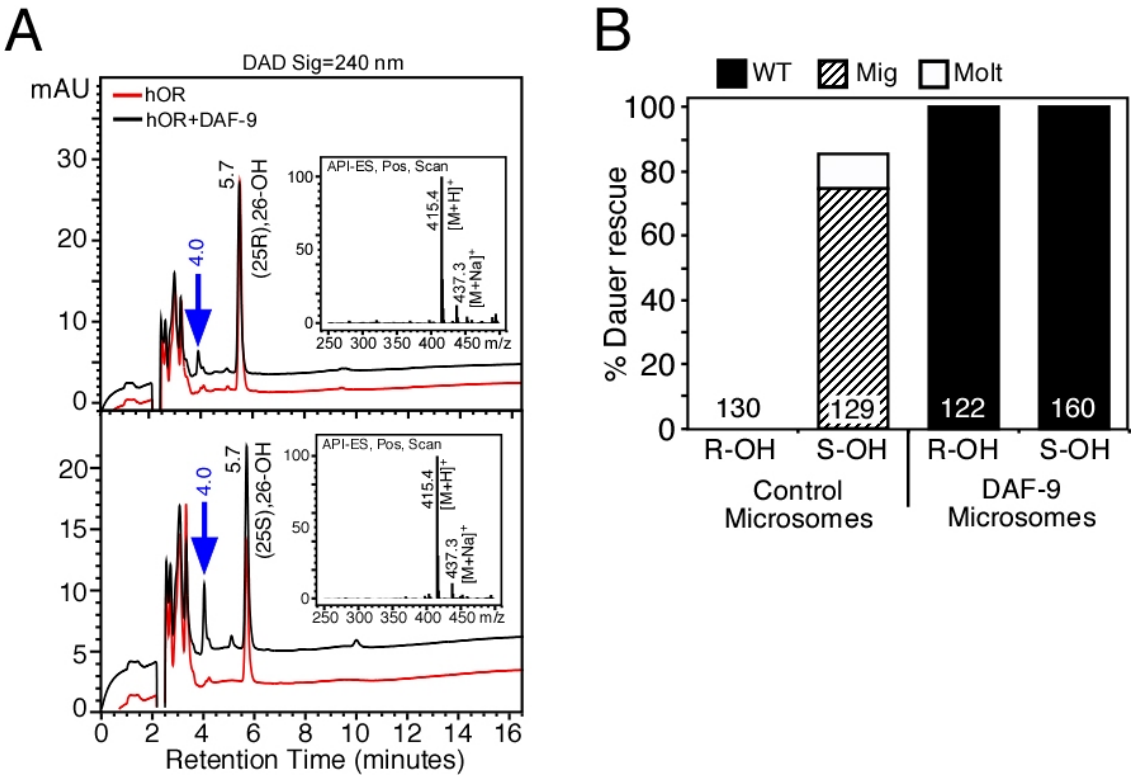

Figure 2-8. DAF-9 Produces Carboxylated Metabolites Through Successive Oxidations at C-26

(A) UV chromatogram of DAF-9 (black line) and control (red line) microsomes after incubation with 100μM (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. (B) Microsome reactions from (A) were diluted 8-fold and tested for *daf-9* rescue as in Figure 2-3. Numbers indicate worms included in each experiment.

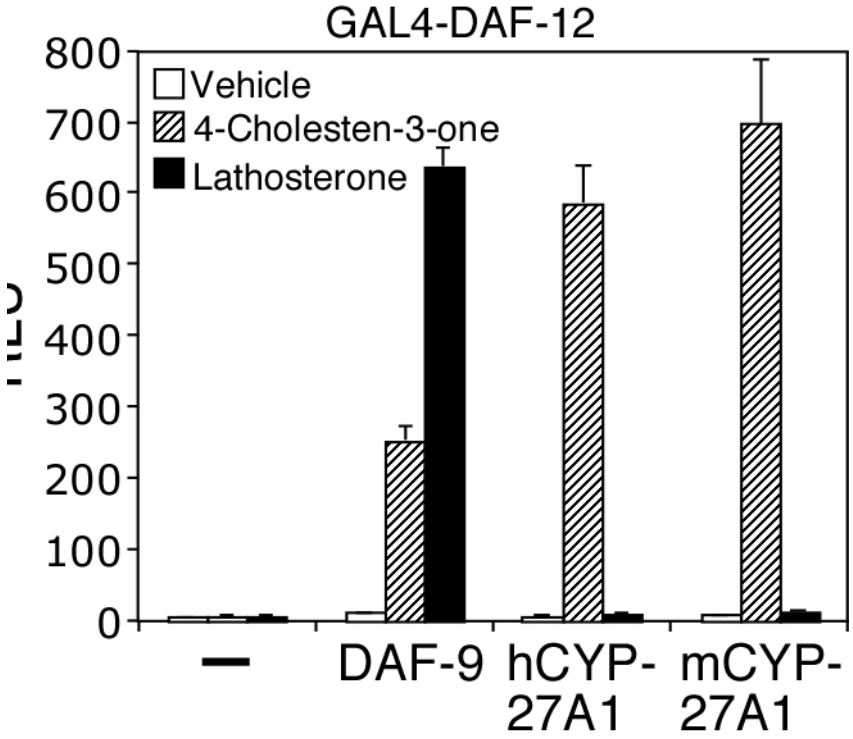

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Figure 2-9. DAF-9 is a Functional Ortholog of Mammalian CYP27A

HEK293 cells were co-transfected with GAL4-DAF-12, bovine adrenodoxin, a GAL4 luciferase reporter gene, and the indicated P450 expression plasmids or empty CMX expression vector (–). 4-Cholesten-3-one and lathosterone were added at 25μM and activation was compared to vehicle control (n = 3 ± SD). RLU, relative light units.

adrenodoxin alone. Interestingly, co-transfection 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.

2.4 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 2-10A , Appendix B) 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 2-10B). When tested in the co-transfection assay, DAF-12 responded to all 4 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 2-10C). Activation by (25S),26-3-keto-4-cholestenoic acid required a 3-keto group, as DAF-12 showed a considerably weaker dose response to its 3β-hydroxy-Δ5 sterol derivative, cholestenoic acid (Figure 2-10D). This result is in agreement with the finding that DAF-12 is potently activated by 3-keto-sterols but not 3β-hydroxy-sterols. 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 2-11). In addition, the DAF-12 ligand binding domain mutants (R564C, R564H) were dramatically attenuated

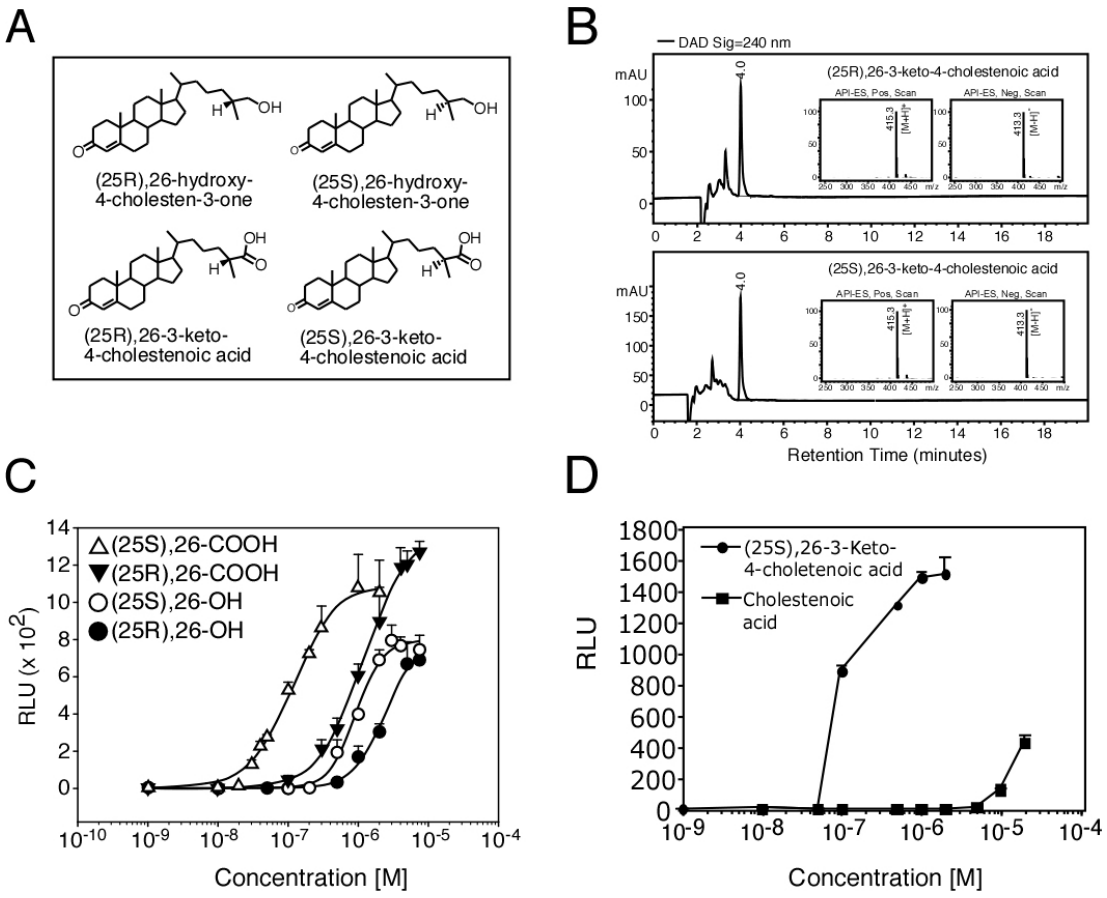


Figure 2-10. 3-Keto-4-Cholestenoic Acids Potently Activate DAF-12

(A) Structures of 4-cholesten-3-one metabolites of DAF-9 (B) LC/MS analysis of authentic standards for (25R),26-3-keto-4-cholestenoic acid (upper panel) and (25S),26-3-keto-4-cholestenoic acid(lower panel) are shown. Insets show mass spectra obtained in both positive (left) and negative (right) ion mode.(C) Dose response of GAL4-DAF-12 activation to 4-cholesten-3-one metabolites in HEK293 cells. (D) Dose response of GAL4-DAF-12 activation to (25S),26-3-keto-4-cholestenoic acid and cholestenoic acid ((25S),26-3β-hydroxy-5-cholestenoic acid).

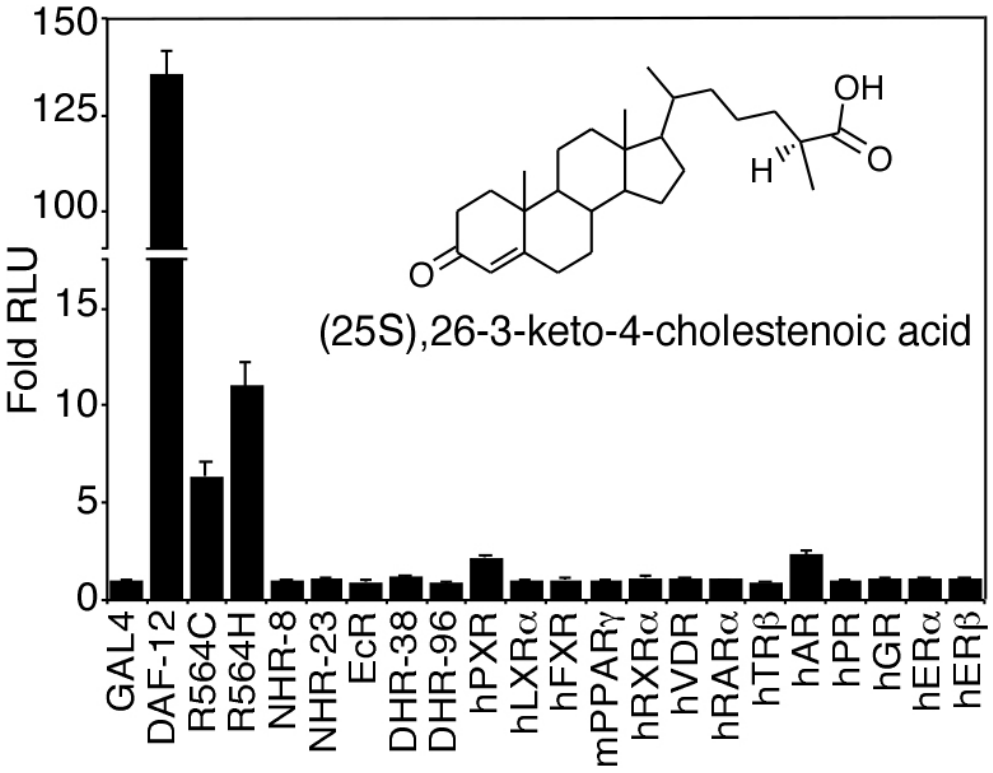


Figure 2-11. Specificity of Nuclear Receptor Activation by (25S),26-3-Keto-4-Cholestenoic Acid

Expression plasmids for GAL4-DAF-12, GAL4-DAF-12-R564C, GAL4-DAF-12-R564H, and a panel of vertebrate and invertebrate GAL4-nuclear receptors were tested for activation by 5μM (25S),26-3-keto-4-cholestenoic acid in HEK293 cells. Results for each condition were obtained from triplicate assay (±SD) and expressed as fold induction relative to ETOH vehicle. RLU, relative light units.

in their response to (25S)-26-3-keto-4-cholestenoic acid (Figure 2-11) and did not respond to (25R),26-3-keto-4-cholestenoic acid (data not shown).

3-Keto-4-Cholestenoic Acids Rescue *daf-9* Null Phenotypes

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 (Figure 2-12A and B). At hormone concentrations of 250 nM, *daf-9* animals were indistinguishable from wild-type: they bypassed dauer diapause to become reproductive adults (Figure 2-12A and B), 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 2-12A). 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 2-12A and C). 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 2-12B and data not shown). The 25R diastereomer of 3-keto-4-cholestenoic acid also rescued *daf-9* phenotypes, albeit at 5-10-fold higher concentrations (Figure 2-12B). Consistent with the data above (Figure 2-8B), (25R),26-hydroxy-4-cholesten-3-one had no effect on *daf-9* nulls, even at concentrations as high as 33μM, 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).

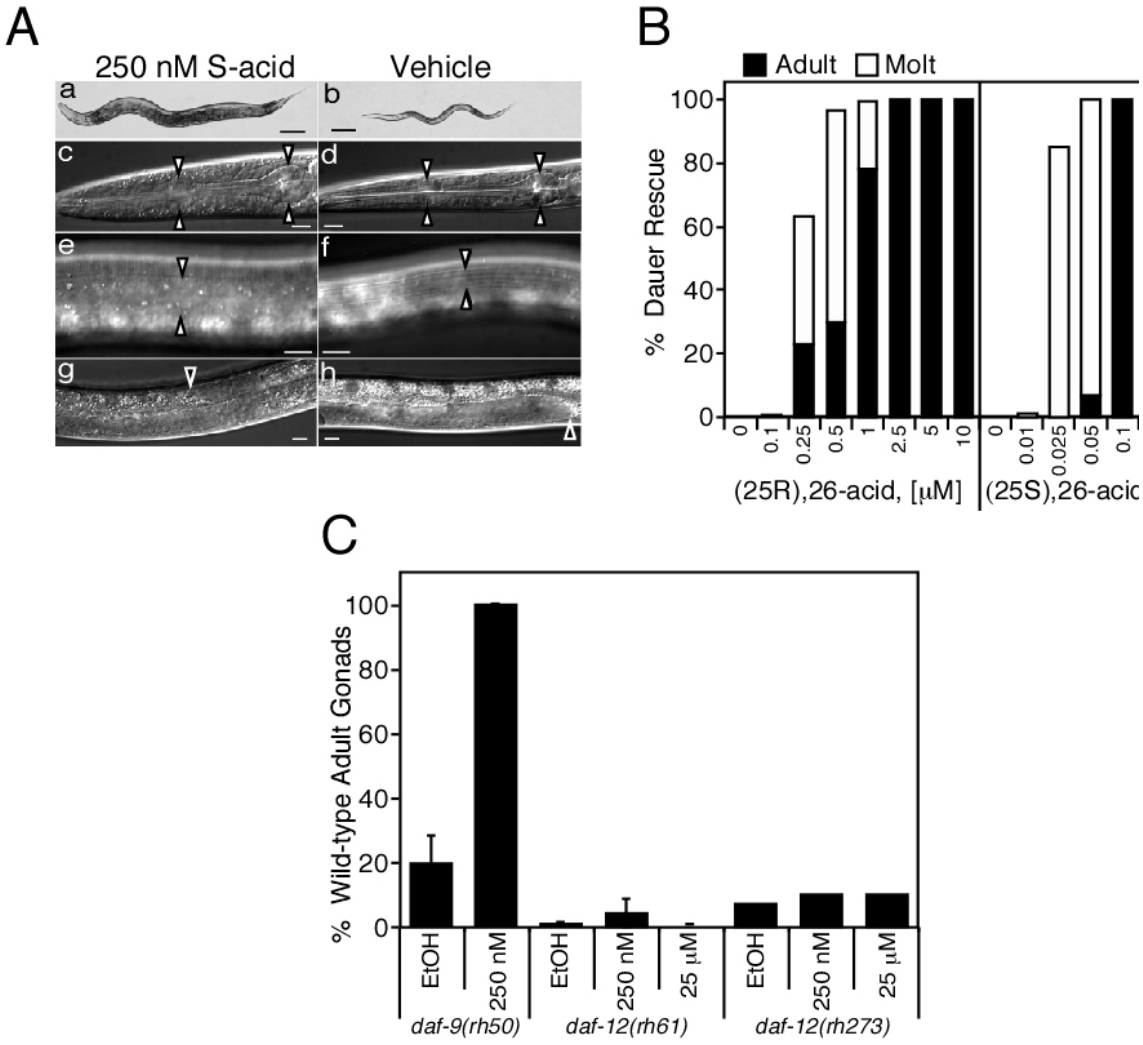


Figure 2-12. 3-Keto-4-cholestenoic Acid Is a Hormonal Ligand of DAF-12

(A) DIC microscopy of *daf-9(dh6)* (a-f) and *daf-9(rh50)* (g-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) un-reflexed gonad of L3 larva. (B) 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 3d at 20°C. Worms were scored as adults or molt-defective larvae. (C) Rescue of Mig phenotypes by (25S),26-3-keto-4-cholestenoic acid. Results expressed as percentage of reflexed gonadal arms scored after 3d at 20°C (n > 60 from 2 experiments). Rescue data and DIC images provided by Dr. Veerle Rottiers and Dr. Adam Antebi.

As predicted by co-transfection assays (Figure 2-11), *daf-12* LBD mutants were compromised in their ability to respond to (25S),26-3-keto-4-cholestenoic acid (Figure 2-12C). Hormone had no effect on *daf-12(rh61),* which truncates the ligand binding domain and results in strong Mig phenotypes (Figure 2-12C). 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 (Figure 2-12C and 2-13, 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 Transport Pathways

To further investigate the biological activity of 3-keto-4-cholestenoic acid we tested its the 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* (Figure 1-2). Accordingly, (25S),26-3-keto-4-cholestenoic acid completely rescued the Daf-c phenotypes of all these mutants (Figure 2-13). 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-1 signaling

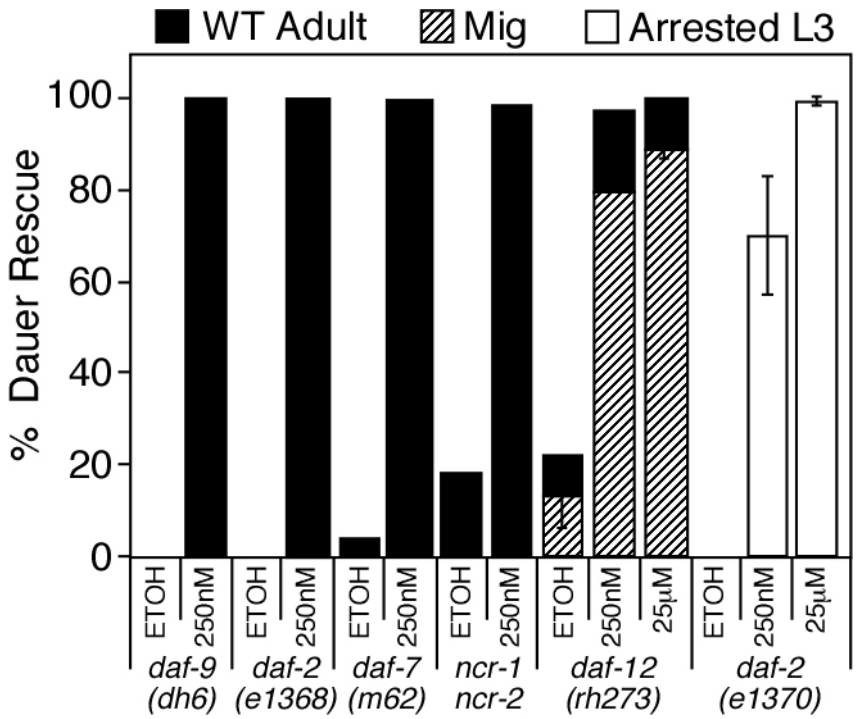


Figure 2-13. (25S),26-3-Keto-4-Cholestenoic Acid Acts Downstream of insulin, TGFβ, and Cholesterol Transport Pathways

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 2d at 25°C (*daf-2* and *daf-7*), or 3d at 20°C (*daf-9*, *daf-12* and *ncr-1;ncr-2*). n > 200 from 2 independent experiments. Rescue data provided by Dr. Veerle Rottiers and Dr. Adam Antebi..

must impinge upon the pathway both upstream, downstream, or parallel to hormone production.

2.5 3-KETO-CHOLESTENOIC ACIDS BIND DAF-12 AS *BONA FIDE* LIGANDS

3-Keto-4-Cholestenoic Acid Affects DAF-12 Coregulator Recruitment

A hallmark of nuclear receptor agonists is their ability to diametrically regulate interactions with co-repressors and co-activators. 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 co-repressor (Chapter 1.6) (Ludewig et al., 2004), interacted with DAF-12 as predicted (Figure 2-14A). Addition of 1μM (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. I also observed that DIN-1S-dependent repression of DAF-12 basal activity could be reversed by addition of 100nM (25S),26-3-keto-4-cholestenoic acid (Figure 2-14B). In a similar co-activator interaction assay, (25S),26-3-keto-4-cholestenoic acid induced the interaction between the fourth receptor interaction domain (ID4) of the mammalian co-activator protein SRC-1 and DAF-12, but not mutant DAF-12-R564C (Figure 2-14C). I also tested transactivation of full-length DAF-12 on a luciferase reporter plasmid containing the DAF-12 binding sites of LIT-1 kinase, a proposed DAF-12 target gene (Shostak et al., 2004). In the presence of (25S),26-3-keto-4-cholestenoic acid, DAF-12

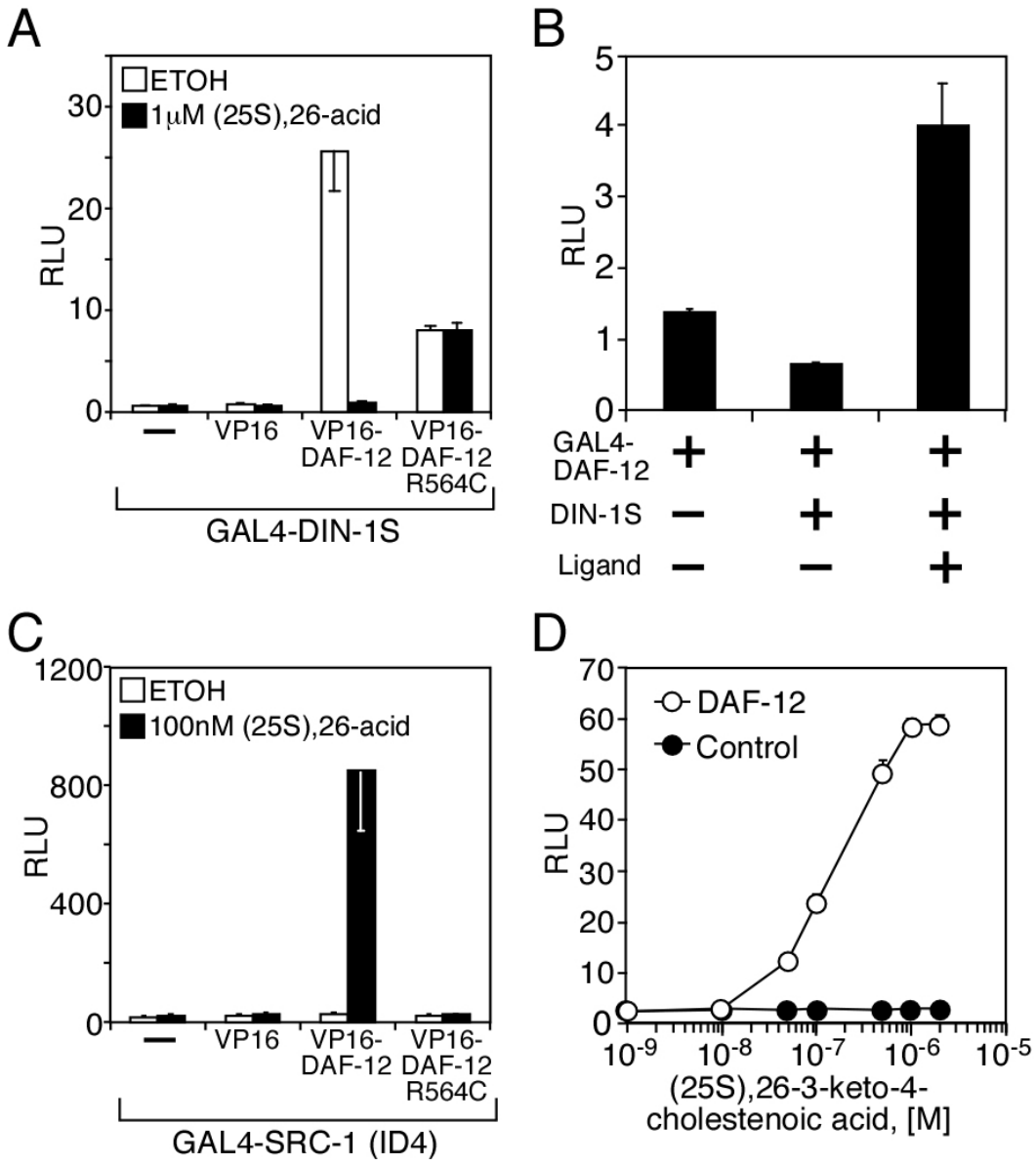


Figure 2-14. (25S),26-3-Keto-4-cholestenoic Acid Functions as a Classical Nuclear Receptor Ligand

(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 45ng/well DIN-1S and 15ng/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.

transactivated the LIT-1 kinase reporter plasmid in a dose-dependent manner (EC_{50} = 100nM) (Figure 2-14D).

3-Keto-Cholestenoic Acids Bind DAF-12

Finally, to determine the *in vitro* ligand binding properties of DAF-12, an Alpha Screen assay, which detects ligand-dependent interactions between receptors and co-activator peptides, was carried out in collaboration with the Eric Xu laboratory (Xu et al., 2002). At 1μM, 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 2-15A). 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} =1nM) (Figure 2-15B). 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 2-15C) and contain the carboxylic acid metabolite (Figure 2-5). From these results I concluded that both the 3-keto-4-cholestenoic acid and 3-keto-7,(5α)-cholestenoic acid hormones (Figure 2-15D) mediate their effects *in vivo* through direct binding to DAF-12. Since these compounds bind DAF-12 and rescue the

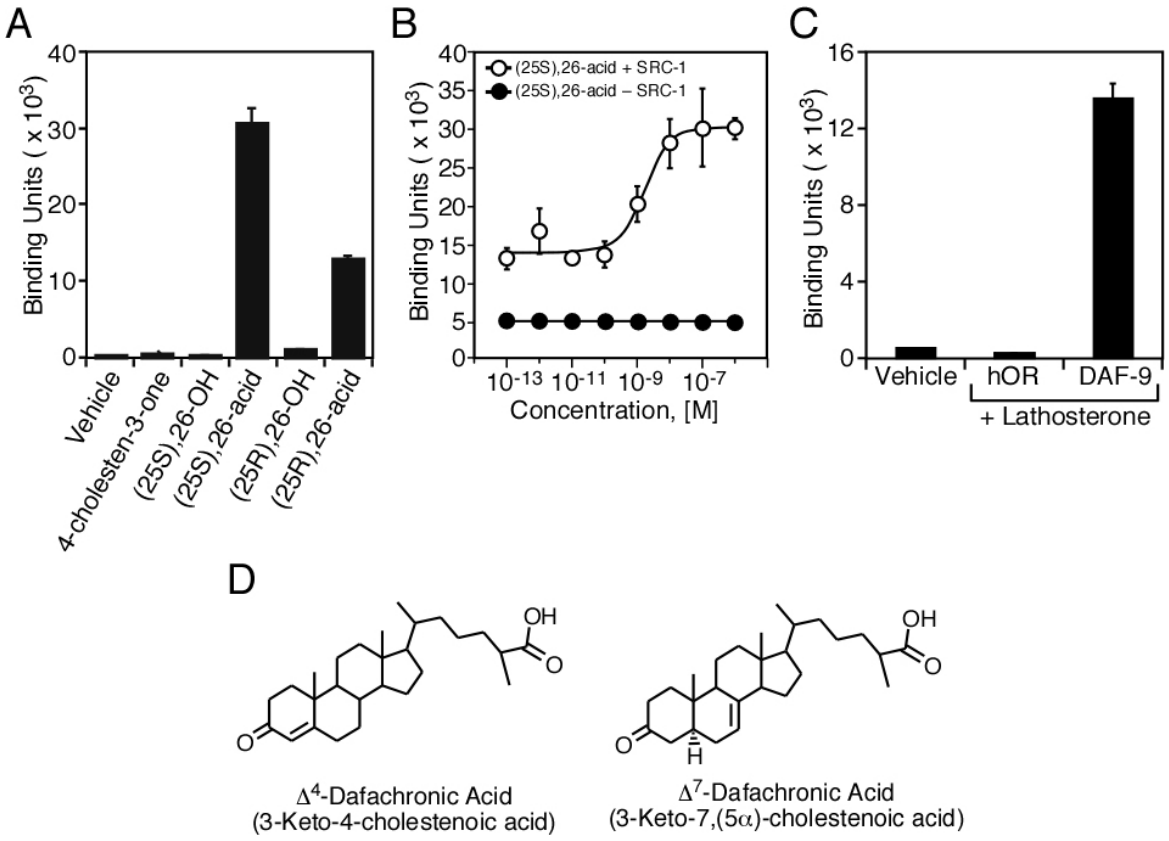


Figure 2-15. DAF-9 Metabolites of 4-Cholesten-3-one and Lathosterone Bind DAF-12 as High Affinity Ligands

(A-C) Alpha Screen assay for ligand-dependent co-activator recruitment to the DAF-12 ligand binding domain. Reactions were performed in the presence of the indicated sterols (1μM). (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 100μM lathosterone. Results expressed as arbitrary binding units from triplicate assays (±SD). (D) Structures of DAF-12 ligands. Ligand binding data provided by Dr. Eric Xu and colleagues.

dauer and heterochronic defects of *daf-9* null worms we call them Δ^4 and Δ^7 dafachronic acid, respectively.

2.6 3-KETO-CHOLESTENOIC ACIDS ARE ENDOGENOUS, DAF-9 DEPENDENT HORMONES

A key prediction of 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 Methods 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 2-16A and B). Wild-type and *daf-9* extracts were then dissolved in methanol, fractionated by RP-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 2-17A). Fractions 4 and 5 were analyzed for the presence of 3-keto-cholestenoic acids by selective ion monitoring (SIM) LC/MS. DAF-12 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 2-17B). 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 2-5B). The estimated endogenous concentration of this activity is ~200 nM (see Methods 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 below), the fact that *daf-9* null animals have no detectable DAF-12 activity in any of the HPLC

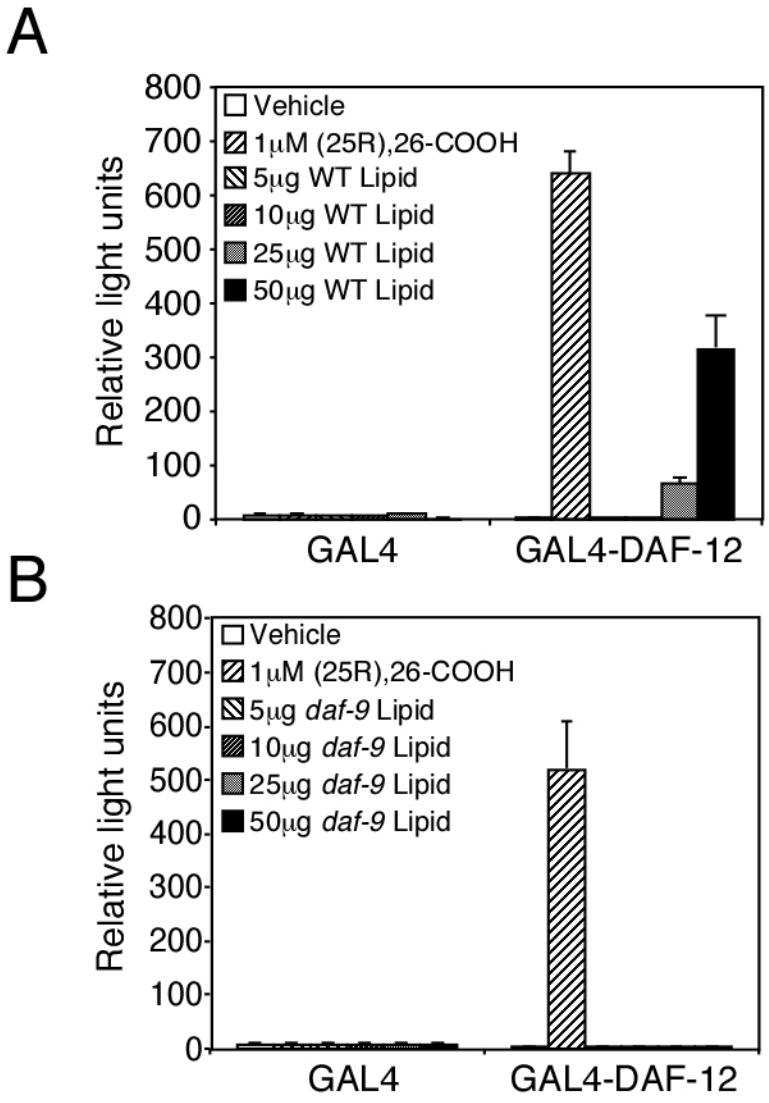


Figure 2-16. Lipid Extracts from Wild-Type but Not *daf-9* Null Worms Activate DAF-12

Increasing concentrations of lipid extracts from wild-type (A) or *daf-9* null (B) worms were tested for DAF-12 activation in the GAL4 co-transfection assay in HEK293 cells. As a positive control, 1μM (25R),26-3-keto-4-cholestenoic acid was used to activate DAF-12.

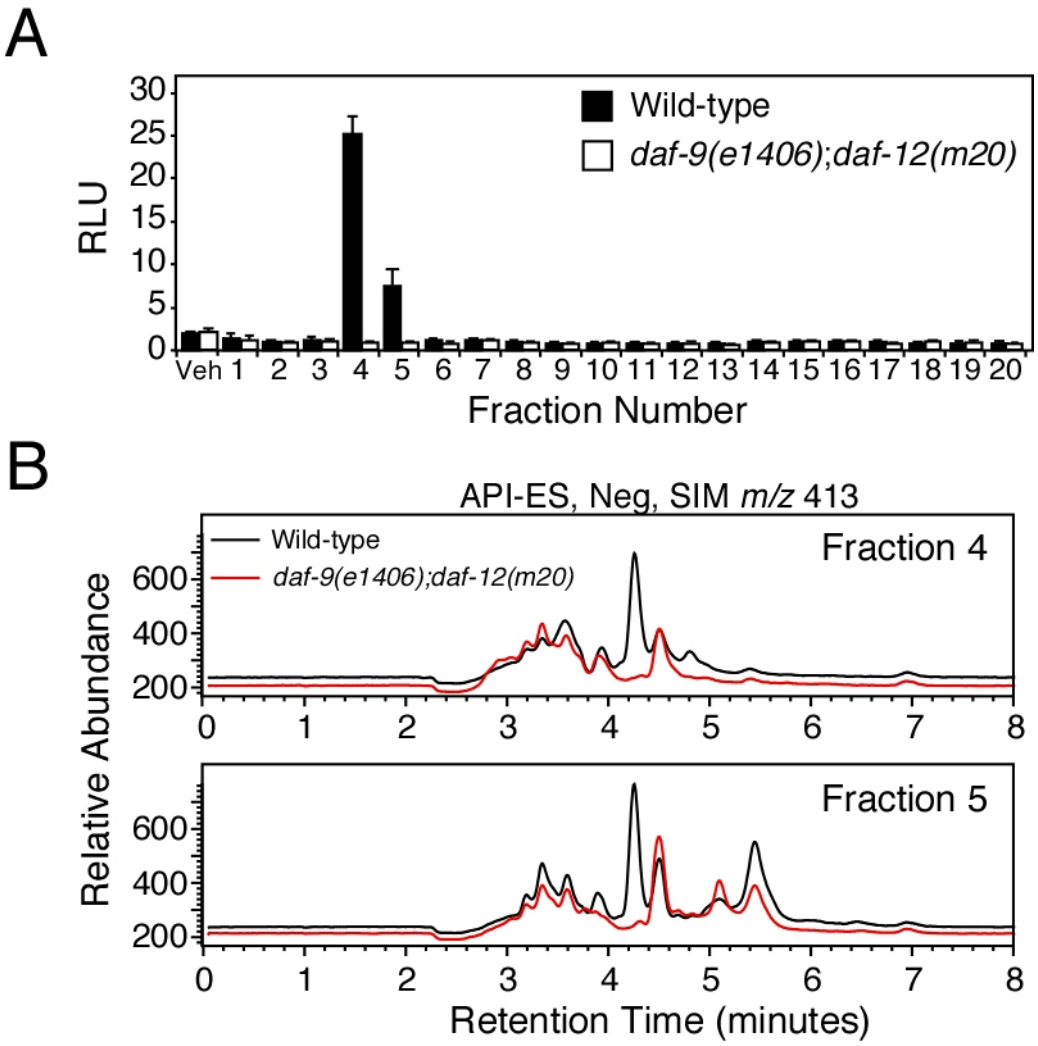


Figure 2-17. 3-Keto-7(5α)-Cholestenoic Acid is an Endogenous Hormone

(A) Activation of DAF-12 by HPLC fractions obtained 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). Lipids were analyzed in selective ion monitoring mode (negative) for the presence of *m/z* 413

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-L4 staged worms. Crude extracts were fractionated by silica column chromatography (Figure 2-18A) and the DAF-12 activating fraction was determined to be in the acetone:methanol eluate (Figure 2-18B). Subsequent fractionation of this activity revealed the presence of two distinct DAF-12 activity peaks at fractions 30-33 and 57-63 (Figure 2-18C). 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 re-purification by HPLC of fractions 57-63 enough material was obtained to identify the carboxylic acid derivatives by LC/MS (Figure 2-18D, 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 2-18D, 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 co-migrated with the lathosterone metabolite, 3-keto-7,(5α)-cholestenoic acid (Figure 2-18D, 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 ($n > 300$).

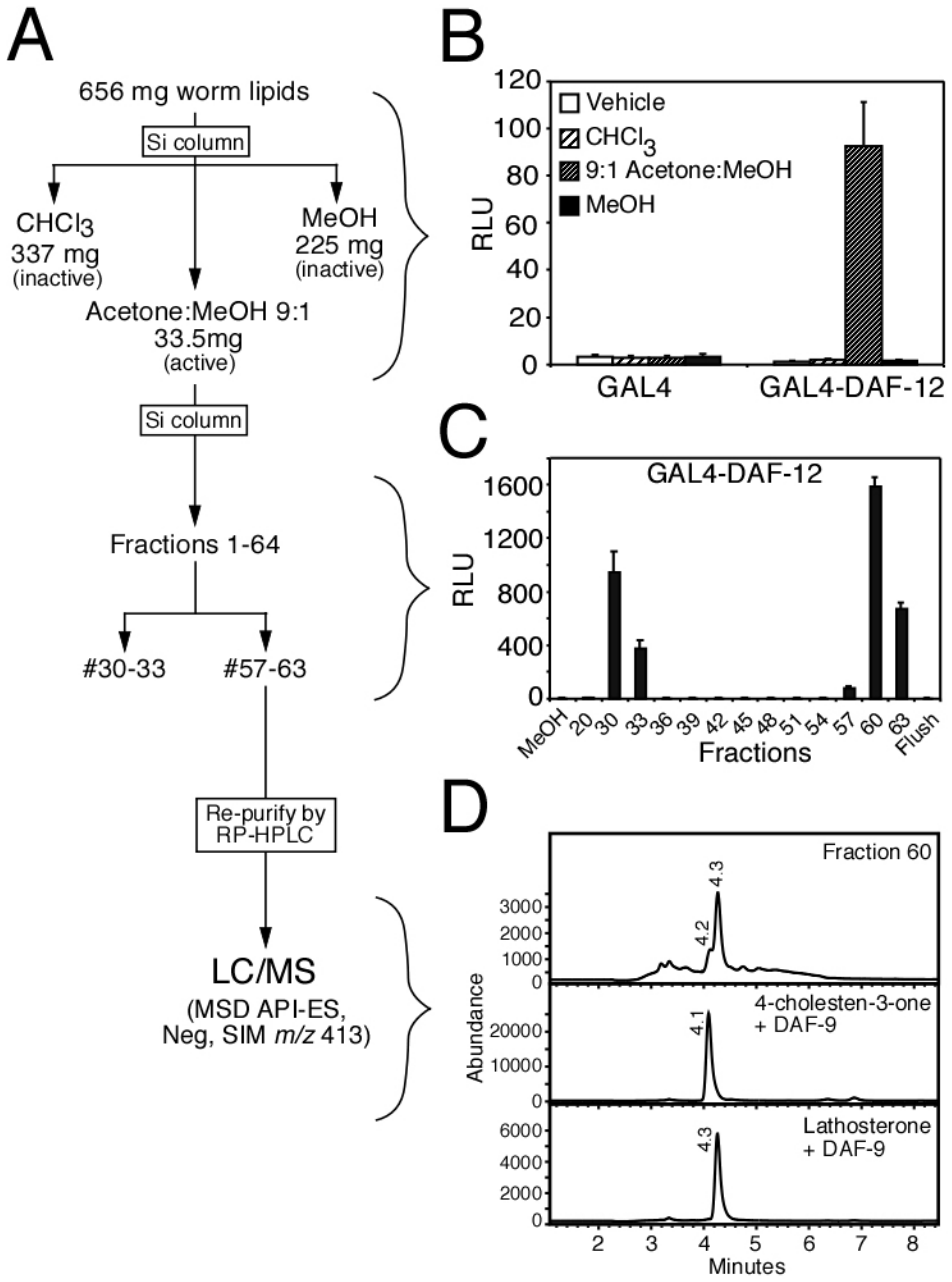


Figure 2-18. 3-Keto-Cholestenoic Acids are Endogenous Hormones

(A) Strategy used to purify endogenous DAF-12 agonists from *C. elegans* lipid extracts. (B) DAF-12 activation by fractionated lipid extracts. (C) DAF-12 activation by silica column fractions of lipids eluted with acetone:methanol. (D) LC/MS analysis of pooled and re-purified 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).

Taken together, these results provide further evidence that both of the 3-keto-cholestenoic acids (Figure 2-15D) are endogenous hormonal ligands of DAF-12.

Based on the above analysis, 3-keto-cholestenoic acids co-eluted with the most polar activity recovered in fractions 57-64 (Figure 2-18C). However, it remained possible that additional activities co-eluted with 3-keto-cholestenoic acids in this particular RP-HPLC solvent system because they were poorly resolved. Therefore, further characterization of the endogenous DAF-12 activities was carried out by subjecting semi-purified WT lipid fractions containing DAF-12 activity to separation under a new RP-HPLC solvent system. A method was employed that could separate compounds of varying side-chain length and polarity thus providing better resolution of the DAF-12 activities found endogenously. Four compounds were used to develop this separation assay and included 20-Hydroxy-Ecdysone (20E), Chenodeoxycholic acid (CDCA), Δ^4-dafachronic acid, and (25S),26-hydroxy-4-cholesten-3-one (Figure 2-19A and B). Fractions containing DAF-12 activity were re-pooled and further separated by RP-HPLC as shown in Figure 2-19B. One minute fractions were collected and assayed in the DAF-12 co-transfection assay. Despite further separation, all activity co-migrated with the authentic standard for Δ^4-dafachronic acid (Figure 19-C). These results suggest that the DAF-12 activity in WT lipids has chromatographic properties that are more similar to Δ^4-dafachronic acid than to the poly-hydroxylated insect molting hormone 20E or the C-24 bile acid, CDCA.

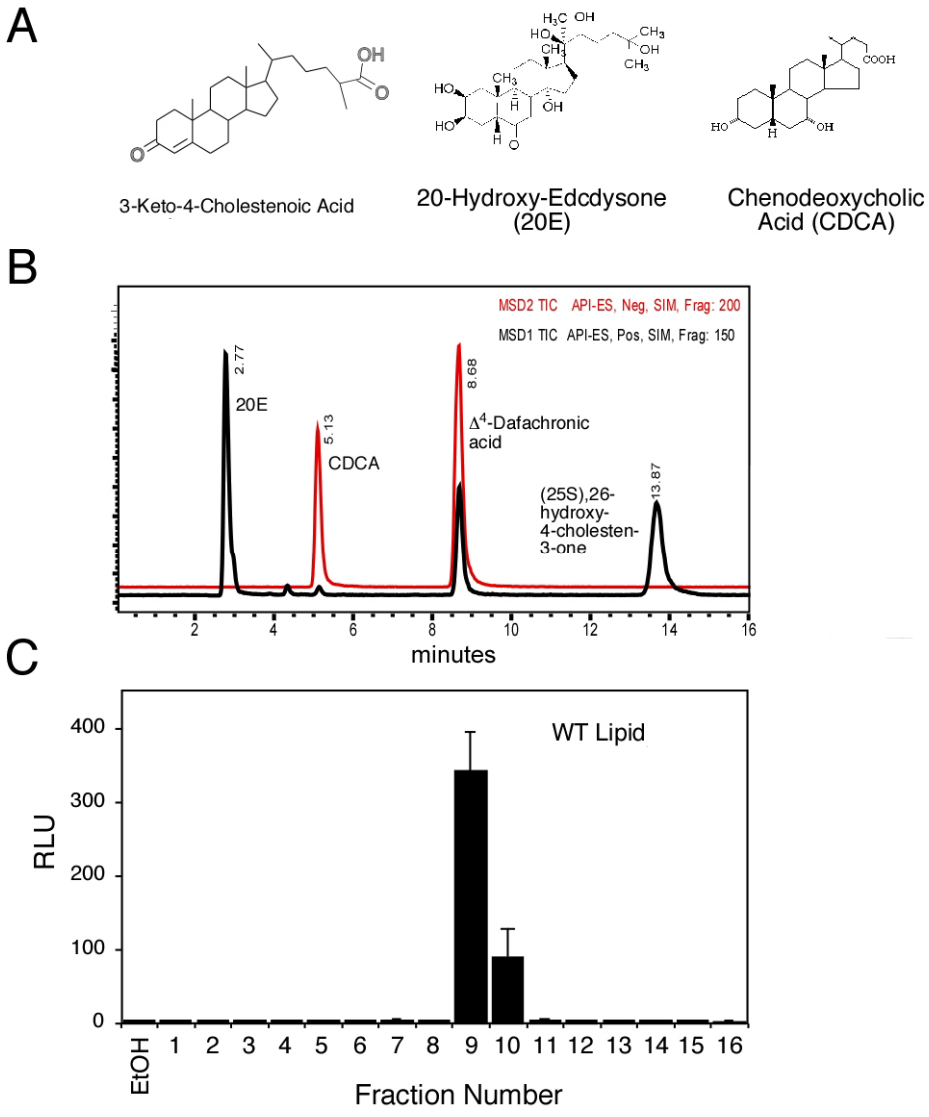


Figure 2-19. Higher Resolution HPLC Separation of Endogenous DAF-12 Activity

(A) Structures of 20-hydroxyecdysone and chenodeoxycholic acid compared to 3-keto-4-cholestenoic acid (26-hydrox-4-cholesten-3-one not shown). (B) RP-HPLC Separation of compounds in (A). Total Ion Chromatogram for compounds is shown by LC/MS in negative (red) or positive (black) ion SIM mode. (C) 250µg from WT lipids (Fraction II) was separated by RP-HPLC as in B and 1 minute fractions were collected and tested for DAF-12 activation. RLU, relative light units (n=3, +/- SD) Carolyn Cummins developed the RP-HPLC system in A and carried out the separation and fraction collection.

CHAPTER 3

CHARACTERIZATION OF DAF-36, A NOVEL RIESKE OXYGENASE

3.1 INTRODUCTION

In the previous chapter, I provided strong evidence that DAF-9 metabolizes the 3-keto-sterol 4-cholesten-3-one and lathosterone into 3-keto-cholestenoic acids, which function as high affinity endogenous ligands for DAF-12. DAF-9 acts at a key position in the synthesis of DAF-12 ligands by catalyzing successive oxidation at C-26 in the sterol side-chain of 3-keto-sterol metabolites of cholesterol. Presumably, additional biosynthetic enzymes exist in *C. elegans* that act upstream of DAF-9 and are responsible for metabolizing cholesterol into DAF-9 substrates (Figure 3-1). The chemical structure of the known cholesterol metabolites gives clues as to their possible enzymatic activities and order of action. These activities may include a cholesterol Δ^7-deasturase, a 7-dehydrocholesterol 5α-reductase, and possibly a lathosterol 3β–hydroxysteroid dehydrogenase or SCOR (Figure 3-1). However, the enzymes involved in this pathway have yet to be identified.

In collaboration with the laboratory of Dr. Adam Antebi, the work I described in Chapter 2 has helped to elucidate a role for a newly discovered enzyme in the pathway

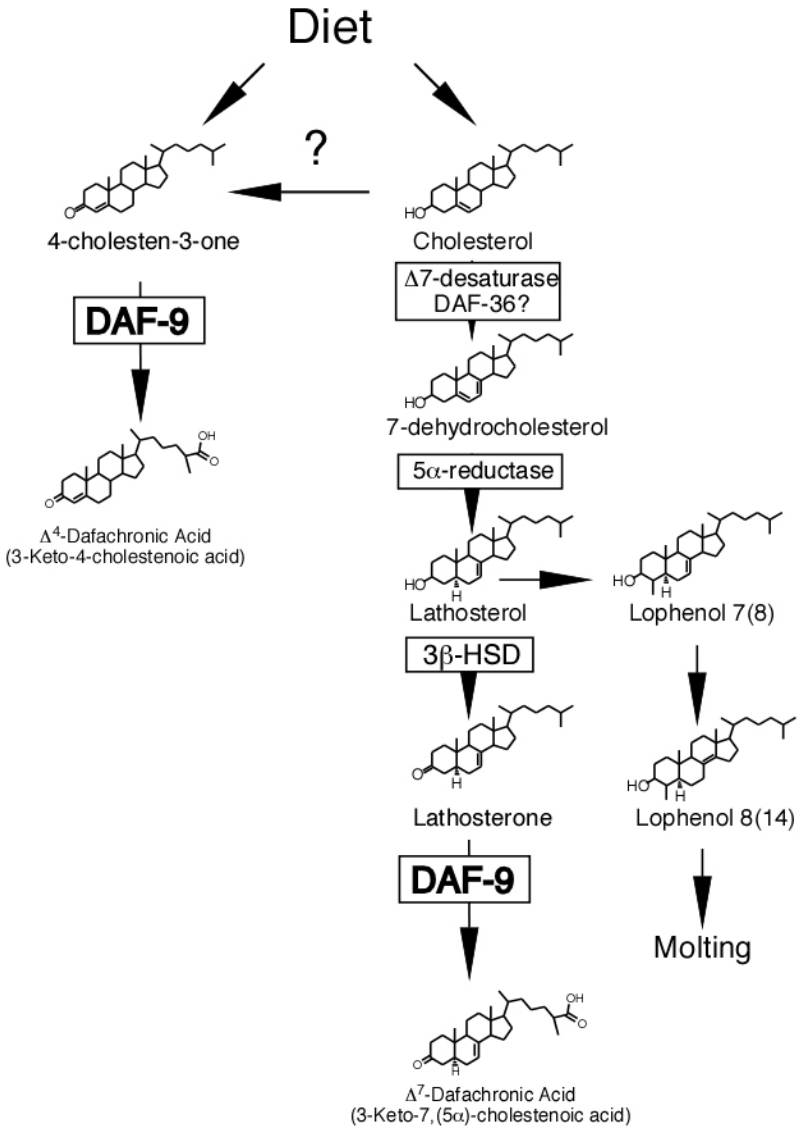


Figure 3-1. An Updated Model of Cholesterol Metabolism in *C. elegans*

This pathway takes into account the newly identified DAF-9 substrates and metabolites. DAF-9 remains the only enzyme known to be involved in cholesterol metabolism in *C. elegans*. The putative enzymatic activities for the remaining enzymes may give clues to their molecular identities. The source of 4-cholesten-3-one is not clear at this time.

encoded by *daf-36*. *daf-36* was identified by Dr. Veerle Rottiers, a post-doctoral fellow in the Antebi lab, in genetic screens for mutations affecting the hormone synthesis pathway. Mutations in *daf-36* result in Daf-c and Mig phenotypes at 27°C, and cholesterol deprivation uncovered these phenotypes at permissive temperatures (Rottiers et al., 2006). In addition, *daf-12* suppresses *daf-36*, while a *daf-36* allele strongly enhances a weak *daf-9* allele. These results indicated that *daf-36* is positioned either upstream or parallel to *daf-9* in the synthesis of DAF-12 ligands. Molecular cloning of *daf-36* revealed that it encodes a protein homologous to bacterial and plant Rieske-like oxygenases (Rottiers et al., 2006). Rieske-like oxygenases catalyze a variety of chemical reactions and are a class of enzymes containing both a Rieske FeS coordination center, involved in redox reactions, and a nonheme iron binding domain, involved in oxygen binding and catalysis (Que, 2000).

Since Rieske oxygenases are not known to participate in steroid hormone synthesis it was difficult at first sight to place *daf-36* in the dafachronic acid biosynthetic pathway. However, chemical epistasis experiments were carried and provided significant insight into the possible function of this gene. When *daf-36* mutants were grown under permissive temperatures they bypassed dauer; however, lipid extracts from these worms were unable to rescue the dauer phenotype of *daf-9* null animals, whereas lipid extract from similarly grown WT animals did. These findings indicated *daf-36* mutants had a block in the synthesis of DAF-12 ligands. Adding back any of the known precursors to dafachronic acids revealed that the block was positioned between cholesterol and 7-dehydrocholesterol, as *daf-36* phenotypes could be rescued by supplementing the diet

with 7-dehydrocholesterol, lathosterol, or DAF-9 substrates, but not cholesterol (Rottiers et al., 2006). Taken together, these results strongly suggested that *daf-36* acts at an early step in the synthesis of dafachronic acids, possibly acting as the putative cholesterol Δ^7-desaturase in the conversion of cholesterol to 7-dehydrocholseterol (Figure 3-1). Despite this strong genetic evidence the direct biochemical activity DAF-36 remains unknown. In the following section I describe my efforts to determine the biochemical activity of DAF-36.

3.2 *DAF-36* MUTANTS LACK 7-DEHYDROCHOLESTEROL AND Δ^7-DAFACHRONIC ACID

To confirm the involvement of DAF-36 in the synthesis of 7-dehydrocholesterol I used LC/MS to compare the levels of 7-dehydrocholesterol between WT and *daf-36* worms that were grown under permissive temperatures. In permissive temperatures *daf-36* worms grow continuously to reproductive adulthood as do their WT counterparts. Whereas WT worms possessed detectable levels of 7-dehydrocholesterol, no 7-dehydrocholseterol could be detected in *daf-36* mutants (Figure 3-2A). In addition, the cholesterol content of *daf-36* mutant worms appeared to be elevated two-fold compared to WT worms.

The cholesterol metabolism pathway in *C. elegans* (Figure 3-1) indicates that 7-dehydrocholesterol serves as an important precursor to Δ^7-sterols in *C. elegans,* including

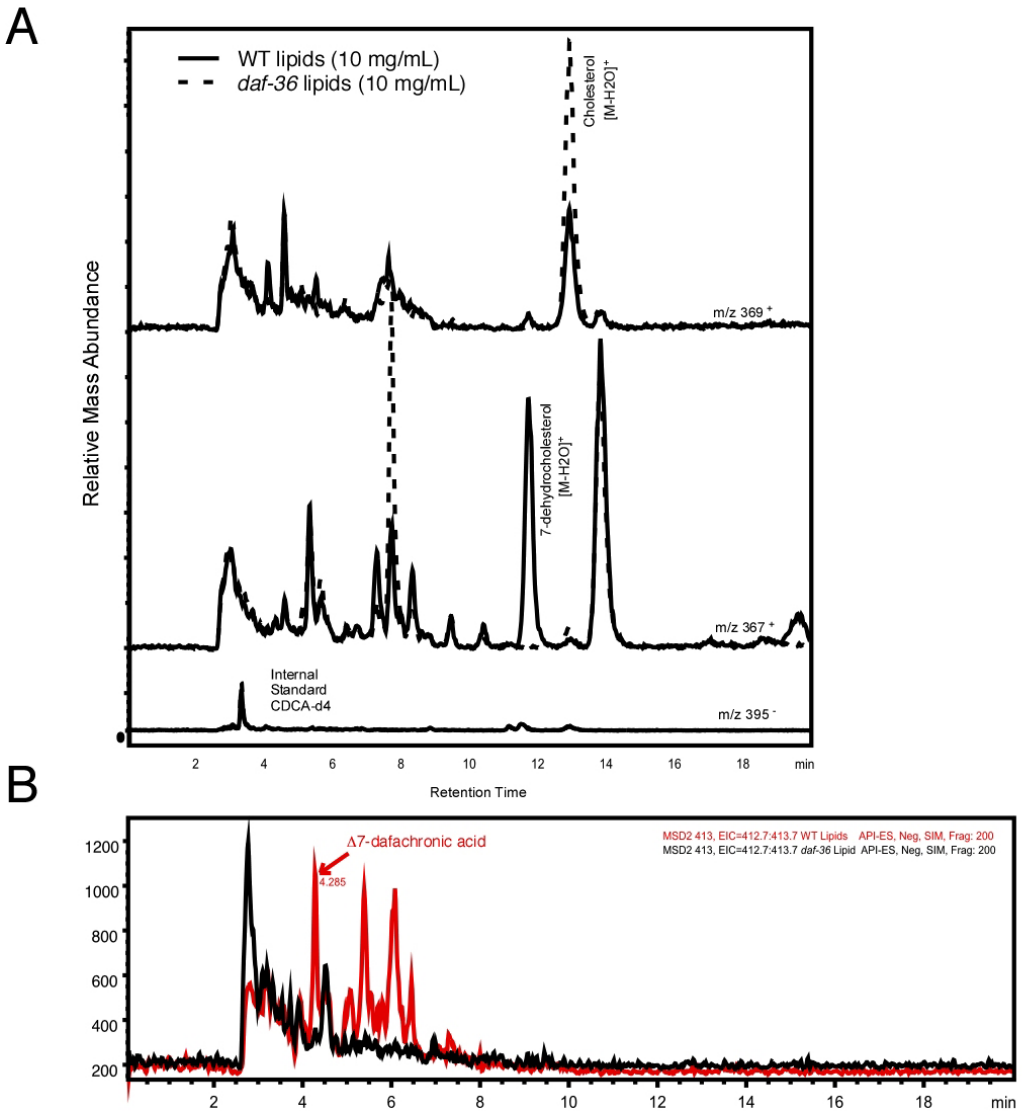


Figure 3-2. Analysis of Δ^7-Sterols in Lipids from WT and *daf-36* Worms

Detection of cholesterol (*m/z* 369+), 7-dehydrocholesterol (*m/z* 367+), and d4-CDCA (*m/z* 395-), which served as an internal standard for extraction efficiency, in lipids from WT (solid line) and *daf-36* (dashed line) worms. The total ion current (TIC) traces for WT and *daf-36* lipids are overlayed to compare relative intensity of ions in the two samples. (B) Detection of of dafachronic acids (*m/z* 413-) in lipids from WT(red) and *daf-36* (black) worms. Extraction efficiency was equal between samples.

lathosterol, lophenol, lathosterone, and Δ^7-dafachronic acid. Mutations that block synthesis of 7-dehydrocholesterol should also block synthesis of these endogenous downstream Δ^7-sterols. Therefore, I analyzed lipid extracts from WT and *daf-36* mutants for presence of each of these sterols by LC/MS. The only Δ^7-sterol that could be detected in WT lipids, other than 7-dehydrocholesterol, was Δ^7-dafachronic acid. This may be due to poor ionization of neutral sterols and the possibility that immediate precursors to Δ^7-dafachronic acid exist at very low levels. In contrast to WT worms, *daf-36* worms contained no detectable Δ^7-dafachronic acid (Figure 3-2B). This confirms the previous conclusion as to why *daf-36* lipids do not rescue *daf-9* phenotypes (Rottiers et al 2006). Taken together, these findings show that loss of *daf-36* results in an inability to produce 7-dehdrocholesterol and its downstream metabolite, Δ^7-dafachronic acid.

3.3 EXISTENCE OF PARALLEL PATHWAYS

daf-36 mutants arrest as dauers at high temperatures but can be grown continuously under permissive temperatures (Rottiers et al., 2006). Despite the ability to bypass dauer and exhibit normal molting, *daf-36* worms still exhibited defects in the production of 7–dehydrocholesterol and Δ^7-dafachronic acid (Figure 3.2A and B). This indicates that pathways independent of *daf-36* exist for the production of DAF-12 ligands that may be distinct from those described in the previous chapter. To detect ligands produced by this pathway I compared the ability of lipid extracts from *daf-36* and WT worms grown under permissive temperatures to activate DAF-12 in the co-transfection system. Crude lipids

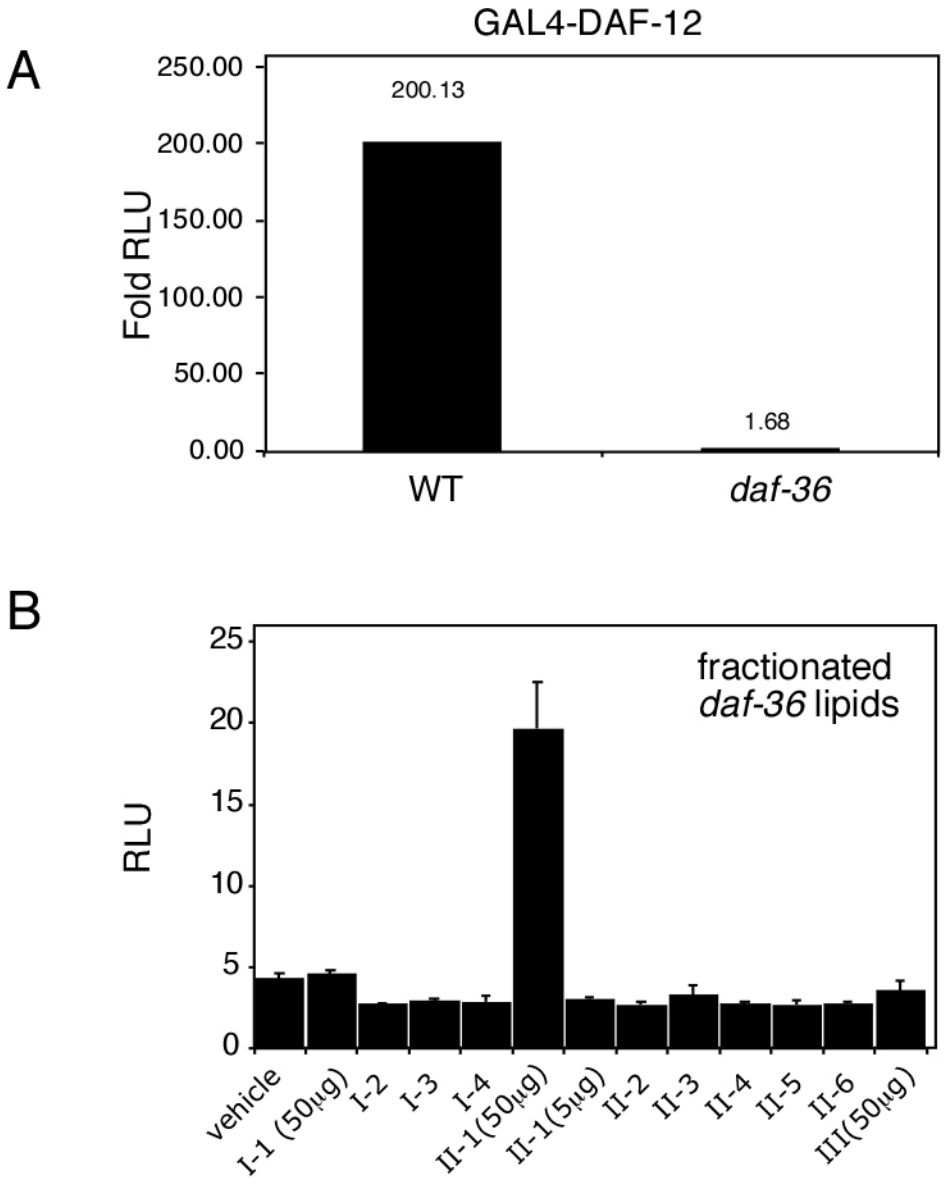


Figure 3-3. *daf-36* Lipids Contain a DAF-12 Activity

(A) Activation of GAL4-DAF-12 by crude lipid extracts from WT and *daf-36* worms. 50μg of lipid delivered per well. (B) Activation of GAL4-DAF-12 by *daf-36* lipids after fractionation. 5-50μg of lipid delivered per well (n=3 +/- SD). Values are expressed as Fold RLU (fold induction relative to vehicle) in A and RLU in B. I=chloroform, II=9:1 acetone:methanol, III:methanol.

from WT animals possessed a strong DAF-12 activity (200 Fold RLU, Figure 3-3A). In contrast, no activity was detected in *daf-36* lipids. Because it remained possible that the DAF-12 activity in *daf-36* worms was below the limit of detection, I fractionated crude *daf-36* lipids by silica column chromatography (as in Figure 2-18A) in order to increase their specific activity. Surprisingly, fractionation of *daf-36* lipids revealed a weak DAF-12 activity in the first fraction eluted by 9:1 acetone:MeOH, which is the solvent that eluted a WT DAF-12 activity (Figure 3-3B and Figure 2-18). In agreement with previous findings (Figure 3-2), LC/MS analysis of this active fraction failed to detect Δ^7-dafachronic acid or Δ^4-dafachonic acid (*m/z* 413-) (data not shown). The internal standard, d4-CDCA, was ruled out as a possible activator because it was present--and more abundant--in fractions that showed no activity. The identity of the active component(s) remains to be determined.

3.4 EXPRESSION OF DAF-36 IN A *SF-9* CELLS IS ASSOCIATED WITH THE PRODUCTION OF 7-DEHYDROCHOLESTEROL

The above data suggested that *daf-36* activity is required at an early step in the synthesis of 7-dehydrocholesterol. To determine whether DAF-36 was necessary and sufficient for production of 7-dehydrocholesteroI I overexpressed it in *Sf-9* cells using a baculovirus expression system. A virus carrying only recombined multiple-cloning site sequences was used as a control. Several observations indicate that DAF-36 is a membrane bound protein: (1) DAF-36 contains a 30 amino acid hydrophobic leader sequence that is predicted to localize it to the endoplasmic reticulum, (2) GFP-tagging in

C. elegans localized it within the cytoplasm in a reticular pattern that concentrated around the nucleus (Rottiers et al., 2006) and (3) expression of an C-terminal His6x-fusion protein in *Sf-9* cells resulted in concentration of protein in the microsomal fraction (data not shown). Therefore, I tested DAF-36 and control microsomal fractions for their ability to metabolize cholesterol into 7-dehydrocholesterol. Since the ciliate *Tetrahymena thermpohila* possesses a microsomal cholesterol Δ^{7}-desaturase activity that is stimulated by 6mM ATP and requires NADPH or NADH and cytochrome *b*5 for activity I tested the effect of these co-factors on activity (Nusblat et al., 2005; Valcarce et al., 2000). DAF-36 and control microsomes were incubated at 37°C in the presence or absence of exogenously added cholesterol and with various combinations of the above co-factors. Lipids were extracted from independent reactions at either t=0 or t=16 hours and subjected to LC/MS analysis. Interestingly, equal amounts of a unique metabolite exhibiting a UV-absorption spectrum and chromatographic and mass spectral properties identical to the authentic standard for 7-dehydrocholesterol could be detected in all DAF-36 microsome reactions, incubated with or without exogenously added cholesterol, but not in control microsomes. Its abundance did not change with time and was unaffected by addition of cofactors (Figure 3-4 and data not shown). Further analysis revealed that cholesterol derived from the culture medium was converted into 7-dehydrocholesterol in a DAF-36-dependent manner during the expression process. Although these results clearly demonstrate that DAF-36 is required for the production of 7-dehydrocholesterol future work is needed to determine if DAF-36 is sufficient.

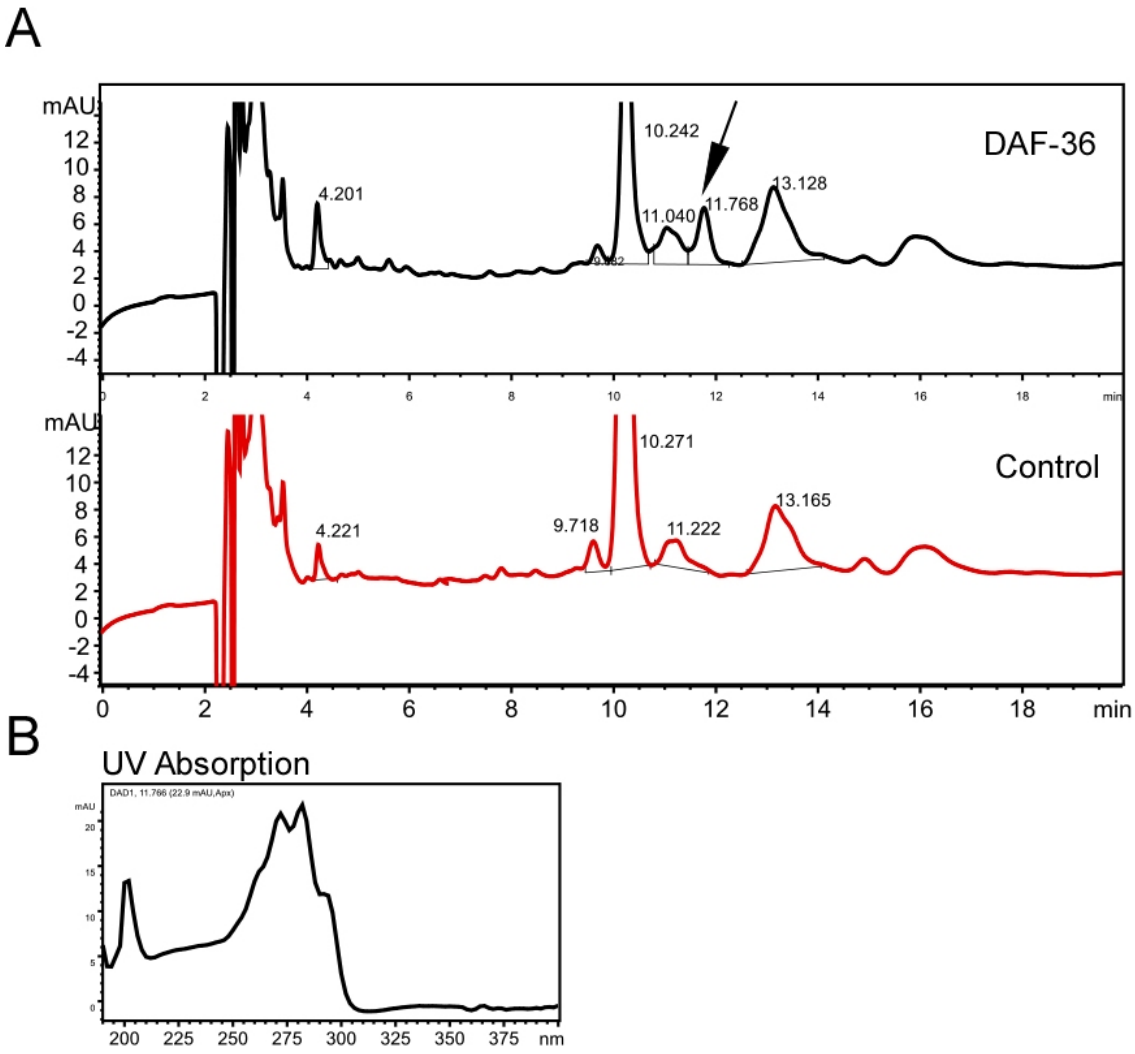


Figure 3-4. Detection of 7-Dehydrocholesterol in DAF-36 *Sf-9* Cell Microsomes

(A) UV trace (240 nm) of lipids extracted from DAF-36 (top panel, black trace) and control (bottom panel, red trace) microsomes at t=0. A peak unique to DAF-36 microsomes is indicated by the arrow. (B) UV spectrum of DAF-36 peak at 11.768 minutes. Mass spectrum of the peak at 11.768 was obtained at *m/z* 367+ (data not shown)

CHAPTER 4

IDENTIFICATION OF A HOOKWORM DAF-12 HOMOLOG AND ITS ACTIVATION BY BILE ACID PRECURSORS

4.1 Introduction

While *C. elegans* is a harmless free-living nematode, many other nematode species have evolved parasitic life-styles with plant and animal hosts. Parasitic nematodes are major burdens to both the developed and developing world: plant-parasitic nematodes cause crop damage while animal-parasitic nematodes cause substantial loss of live-stock and debilitating human infections (Colley et al., 2001). The most notable parasites are hookworms, ascaris, and whipworm, which together cause intestinal infections in > 1 billion people each, and the filarial nematodes that cause elephantiasis and African river blindness (>100 million infections) (Weller and Nutmann). Given the socio-economic and health related burdens imposed by parasitic nematodes there is considerable interest in understanding the cellular and molecular mechanisms that regulate parasitism in nematode.

The study of *C. elegans,* particularly dauer formation (Chapter 1.5), has provided interesting insights into processes that might control parasitic nematode development. This has largely come from the recognition that many similarities exist between the infectious stage of parasitic nematodes, such as hookworm, and the dauer stage of *C.*

elegans (Hotez et al., 1993). For example, like dauers, the infectious stage of parasitic nematodes is a developmentally arrested third stage larva (L3) that is morphologically specialized for survival. As in *C. elegans,* development to adulthood is activated upon encountering a specific environmental signal. In the case of the hookworm L3, larvae gain entry through the skin and resume development to reproductive adulthood in response to an unknown host-derived signal. These similarities have led to the suggestion that dauer formation in *C. elegans*, specifically recovery from dauer, may serve as a model for understanding the infectious process of hookworm and other parasitic nematodes (Hotez et al., 1993).

Several EST-sequencing projects involving parasitic nematodes are underway and the availability of sequences from the *C. elegans* genome has helped to identify parasitic nematode genes that are homologs of *C. elegans daf* genes. A homolog for *C. elegans* DAF-7 has been identified in *Ancylostoma caninum* (*A. caninum*, dog hookworm) and homologs for DAF-12 and DAF-16 have been identified from *Strongyloides stercoralis* (intestinal nematode) (Brand et al., 2005; Massey et al., 2003; Siddiqui et al., 2000). Determining whether these genes play a role in regulating L3 activation remains a significant challenge because functional genomic tools, such as RNAi and targeted mutagenesis, do not exist. This is largely due to a lack of in vitro systems for culturing parasitic nematodes apart from their hosts.

One approach that holds promise is the use of *C. elegans* as a heterologous transformation system to study the function of homologous daf genes identified in parasitic nematodes. The *S. stercoralis* DAF-16 homolog, FKTF-1b, was shown to be

sufficient to substitute for the function of *C. elegans daf-16,* as it supported dauer formation in a *daf-2* background when expressed from a *daf-16* promoter (Massey et al., 2006). These results suggest that DAF-16 homlogs in Strongyloides and other parasites may play an important role in L3 developmental arrest. Further evidence that L3 activation in parasitic nematodes uses pathways analogous to those used for the recovery from dauer formation in *C. elegans* dauer formation has come from pharmacological manipulation of insulin and cGMP signaling in *A. caninum* (Brand and Hawdon, 2004; Hawdon and Datu, 2003). These studies have relied on an in vitro activation assay of hookworm L3 larvae. Normally, in the presence of host serum fractions and methylated glutathione, L3 stage *A. caninum* will resume feeding, although they do not initiate molts to reproductive development (Hawdon et al., 1996; Hawdon and Schad, 1993). 8-bromo-cGMP induced feeding in L3s in the absence of serum, suggesting that cGMP signaling is downstream of the serum signal (Hawdon and Datu, 2003). Furthermore, activation by serum or cGMP is inhibited in the presence of the PI3-kinase inhibitor, LY294002 (Brand and Hawdon, 2004). These results demonstrate that cGMP and insulin signaling in hookworms influence L3 activation in response to host derived signals and suggest that the pathways for recovery from dauer arrest are conserved in hookworms.

Although the molecular evidence for parallels between dauer formation and the infectious stage of parasitic nematodes is not overwhelming, it is tempting to speculate that a DAF-12 homolog in a parasitic nematodes regulates the switch to parasitism in much the same way as it governs recovery from dauer in *C. elegans*. Specifically, DAF-12 may repress the transition to parasitism until a host-derived signal turns on the

production of cGMP, which is followed by release of an insulin-like protein, and subsequent production of dafachronic acid-like DAF-12 ligands. An attractive, although not mutually exclusive, alternative hypothesis is that the host derived signal or a component of it might function directly as the DAF-12 ligand. In the remaining section of this chapter I briefly describe the cloning of a hookworm DAF-12 homolog and its activation by dafachronic acids and other related compounds.

4.2 CLONING OF DAF-12 LBDS FROM *ANCYLOSOTOMA CEYLANICUM* AND *STRONGYLOIDES STERCORALIS*

The sequence of the *C. elegans* DAF-12 LBD was blasted against NCBI databases containing EST-sequences produced by a number of nematode sequencing centers. Three likely DAF-12 homologs were identified among these sequences. One of these was a clone (kp26e06, Washington Univ. St Louis) that matched the published sequence for *S. stercoralis* DAF-12 (Siddiqui et al., 2000). A second was a clone (pk90d07, Washington Univ. St Louis) obtained from an infective L3 of the human hookworm, *Ancylostoma ceylanicum* and a third (pb33f08, Washington Univ St. Louis) was from the dog hookworm, *A. caninum*. All clones appeared to be C-terminal cDNA fragments as they did not contain 5'UTRs or upstream stop sites. Of these three clones only *S. stercoralis* and *A. ceylanicum* were recovered from received plasmids.

A sequence alignment of the *C. elegans* DAF-12 LBD (CeDAF-12) with that from *S. stercoralis* (SsterDAF-12) and *A. ceylanicum* (AceyDAF-12) revealed that AceyDAF-12 had higher overall sequence identity to CeDAF12 (58%) than did SsterDAF-12 (43%) , which correlates with their phylogeny (Figure 4-1A). Strongest

conservation between CeDAF12 and AceyDAF-12 was seen in helices 1-5 (71% identical, 91% similar). Notably, two residues in the LBD of CeDAF-12 that result in Daf-c and heterochronic defects when mutated, R564 and P746, are conserved in both AceyDAF-12 and SsterDAF-12 (Figure 4-1A).

4.3 ACTIVATION OF HOOKWORM DAF-12 BY CHOLESTENOIC ACIDS

To determine if these parasitic nematode homologs of DAF-12 can be activated by dafachronic acids I generated GAL4-fusions of AceyDAF-12 and SsterDAF-12 LBD and tested them for activation in the co-transfection system (Figure 4-1B). A dose response of AceyDAF-12 to activation by Δ^4-dafachronic acid revealed that AceyDAF-12 has a low dynamic range of activation compared to CeDAF-12 (compare Figure 2-10C to Figure 4-2C). AceyDAF-12 is maximally activated only 7-8 fold at 1μM (a saturating dose for CeDAF-12) and has an approximate Ec50 of >500nM which is 5-times higher than CeDAF-12 (see Figure 4-2C). Saturating doses (relative to CeDAF-12) of each known CeDAF-12 agonist modestly activated GAL4-AceyDAF-12 (Figure 4-1B). SterDAF-12 was not activated by Δ^4-dafachronic acid (data not shown). Δ^7-dafachronic acid was tested indirectly using co-transfected DAF-9 and lathosterone as a substrate and was found to also weakly activate AceyDAF-12 compared to CeDAF-12 (3 fold vs 200 fold). These data demonstrate that hookworm DAF-12 can be activated by dafachronic acids, albeit with less efficacy and potency compared to CeDAF-12.

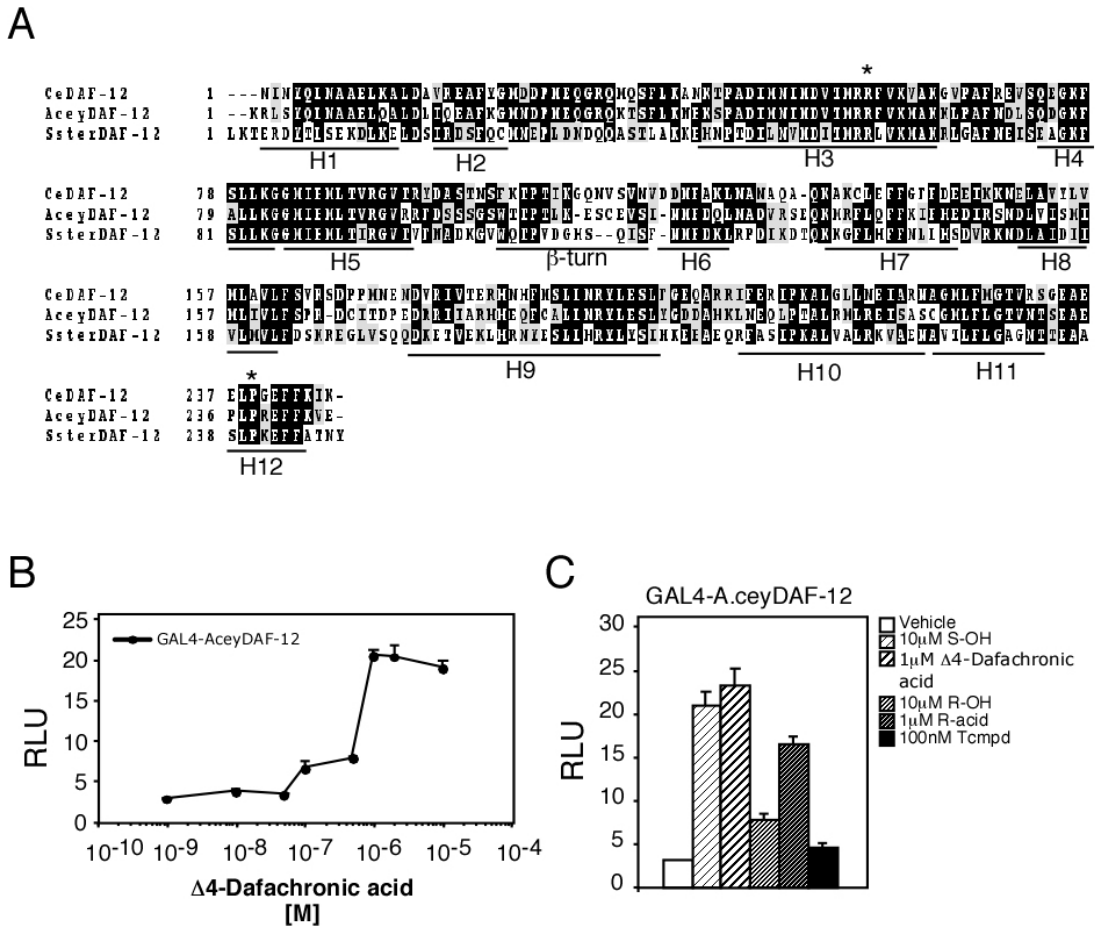


Figure 4-1. Activation of a Hookworm DAF-12 Homolog by Dafachronic Acid

(A) Sequence alignment of *C. elegans* DAF-12 (CeDAF-12) with its homologs from the human hookworm, *Ancylostoma ceylanicum*, and *Strongyloides stercoralis*. Asterisks indicate mutations in CeDAF-12 that produce Daf-c and Mig phenotypes. (B) Dose response of hookworm DAF-12 to Δ^4-dafachronic acid. (C) Activation of hookworm DAF-12 by CeDAF-12 agonists. S-OH: (25S),26-hydroxy-4-cholesten-3-one, R-OH: (25R),26-hydroxy-4-cholesten-3-one, R-Acid: (25R)-3-keto-4-cholestenoic acid, Tcmpd: T-compund, synthetic LXR agonist

To test the alternative hypothesis that a component of human serum serves as hookworm DAF-12 ligand I tested the human bile acid precursor, cholestenoic acid. Cholestenoic acid was previously described in Chapter 2 as the product of CYP27A1, a functional ortholog of DAF-9 that synthesizes bile acid ligand for FXR. It weakly activated CeDAF-12, likely owing to the absence of a 3-keto group (Figure 2-10D). Coincidentally, cholestenoic acid primarily originates from lung macrophages, and the lung is a organ through which infectious stage hookworms migrate and molt to L4 (Anderson, 2000; Babiker et al., 1999). Along with its 3-keto-Δ4- and 3β-OH-Δ5-7α-hydroxylated derivative, cholestenoic acid circulates in human blood at a concentration of 200-250nM (Axelson et al., 1988). Dose response curves of AceyDAF-12 and CeDAF-12 were compared using both diastereomers of cholestenoic acid (Figure 4-2A and B). In both cases, AceyDAF-12 was more potently activated by cholestenoic acid than CeDAF-12. Saturation of AceyDAF-12 response was achieved for 25S-cholestenoic acid and had an Ec50 >500nM, which is 2-fold higher than its physiological concentration human serum. In contrast, saturation of CeDAF-12 was not obtained at a concentration as high as 20μM (data not shown). Stereochemistry at C-26 in cholestenoic acid was important for activation of both receptors, as the 25R-diastereomer of cholestenoic was less potent than the 25S-diastereomer (Figure 4-2B). SsterDAF-12 was not activated by either of the cholestenoic acids (data not shown).

Finally, bile acids were tested for their ability to activate AceyDAF-12 since they are found in the lumen of the small intestine along with adult-stage hookworms. While

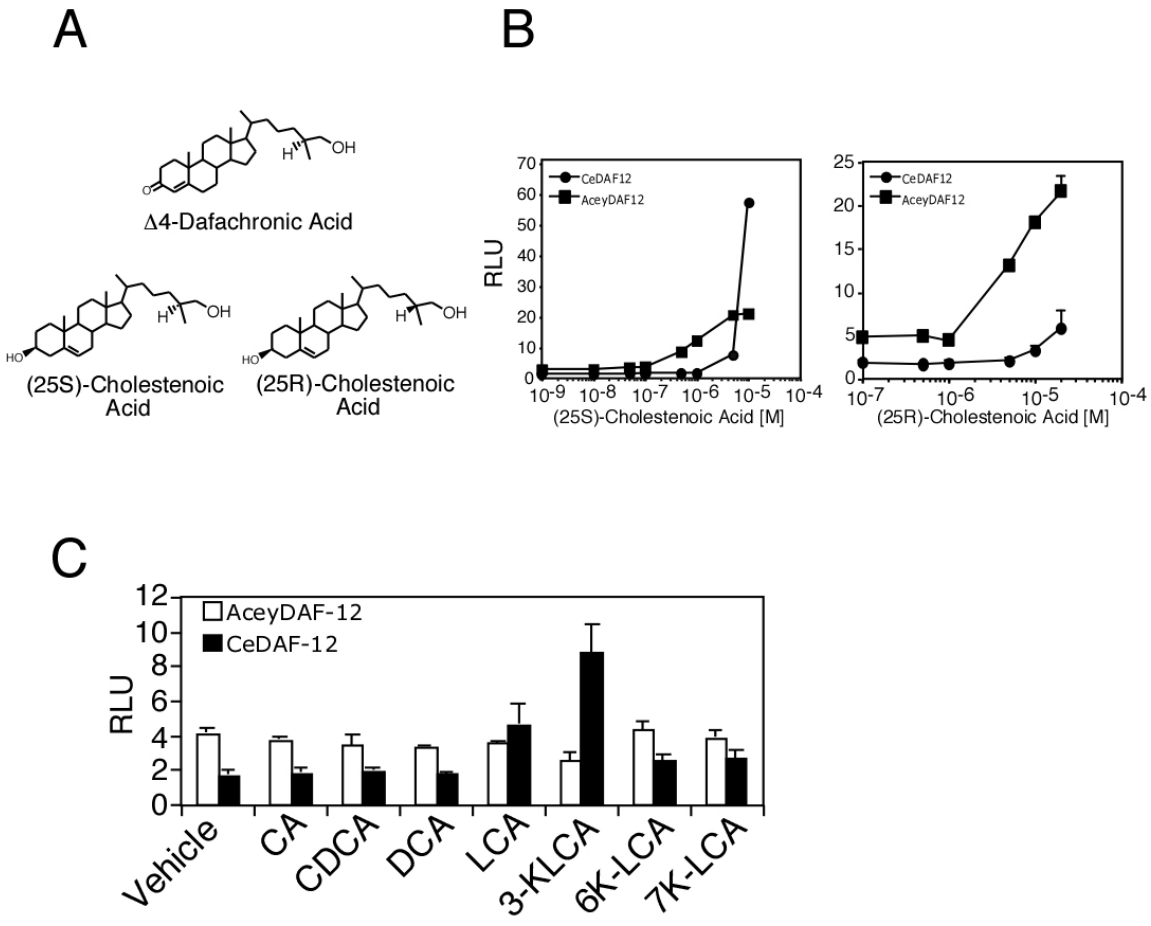


Figure 4-2. Activation of *A. ceylanicum* DAF-12 by Cholestenoic acids

(A) Structures of the *C. elegans* DAF-12 ligand, Δ^4-dafachronic acid, compared to cholestenoic acids. (B) Dose response of DAF-12 from *C. elegans (Ce)* and *A. ceylanicum (Acey)* to (25S)-cholestenoic acid (left panel) or (25R)-cholestenoic acid (right panel). (C) Activation of DAF-12 by 50μM bile acids. mIBAT cotransfected to facilitate uptake.

3K-LCA activated CeDAF-12, it did not activate AceyDAF-12 nor did any other bile acid tested (Figure 4-2C).

CHAPTER 5

DISCUSSION AND PERSPECTIVES

5.1 DISCOVERY OF LIGANDS FOR A *C. ELEGANS* ORPHAN NUCLEAR RECEPTOR

In chapter 2, I detailed 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. Another chemical feature that distinguishes these two ligand is that 3-keto-7,(5α)-cholestenoic acid is a 5α-hydrogenated sterol. However, this chemical distinction results in a similar planar arrangement of the sterol ring. Given their ability to govern dauer formation and heterochronic developmental pathways, I propose to name these hormones Δ^4-dafachronic acid and Δ^7-dafachronic acid (Figure 2-15D). Consistent with other nuclear receptor ligands, the stereochemistry of the DAF-12 ligand is an important determinant for binding. With respect to 3-keto-4-cholestenoic acid, 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), I 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 and its production by DAF-9 awaits its *de novo* chemical synthesis. In addition, it remains to be determined whether DAF-9 can synthesize both diastereomers of 3-keto-7,(5α)-cholestenoic acid, as was seen for 3-keto-4-cholestenoic acid (Figure 2-8A). Taken together, the dafachronic acids identified here represent the first ligands for a nematode nuclear receptor and 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 this approach were shown to exist in the most active lipid fractions from WT animals. Although the existence of other chemically similar ligands cannot be ruled out I found that improving the separation of lipid extracts by RP-HPLC did not resolve new peaks of activity (Figure 2-19). The results presented in chapter 2 provide evidence for at least two ligands, raising the intriguing possibility that each hormone may govern overlapping but distinct transcriptional networks. Arguing against this is the finding that the dafachronic acids have similar structure and similar activities in preventing dauer formation and promoting gonadal migration. However, it remains possible that these ligands differentially affect other DAF-12-dependent functions, such as lifespan. For example, DAF-9 and DAF-12 have been shown paradoxically to be required for both inhibition and extension of lifespan (Gerisch et al.,

2001; Hsin and Kenyon, 1999; Jia et al., 2002). Future work is being directed toward determining the role these ligands play in regulating lifespan and whether the two dafachronic acids may be responsible for these opposing activities, or whether other discriminating ligands also exist.

5.2 THE BIRTH OF REVERSE ENDOCRINOLOGY IN *C. ELEGANS*

The identification of dafachronic acids as endogenous ligands for DAF-12 will make it possible to answer a number of questions pertaining to the function of DAF-12 in the regulation of dauer formation and reproduction. For example, what are the target genes of DAF-12 that it regulates to control the two developmental programs of dauer formation and reproductive development? Reverse endocrinology has had much success in delineating genetic regulatory networks controlled by vertebrate nuclear receptors (Chapter 1). The identification of the dafachronic acids is an important step forward and brings *C. elegans* nuclear receptors firmly into the era of Reverse Endocrinology. With the ligand in hand we many now be able to find answers to these answers by utilizing micro-array expression analysis of mutants in both *daf-9* and *daf-12*. Genes that are regulated in a DAF-12 and ligand dependent manner can be tested in heterologous systems for direct activation by DAF-12 and dafachronic acids. The role these genes play can then be analyzed by looking at their temporal expression patterns and the phenotypic consequences of their over-expression and RNAi-directed knockdown. Together, these approaches can be powerfully combined to help elucidate the transcriptional regulatory networks governed by DAF-12 and dafachronic acids. Other interesting areas of future exploration include the identification of co-regulators utilized by DAF-12. We already

know of one such co-regulator, DIN-1S, and our results nicely confirm the findings from genetic studies that suggested it acts as a co-repressor of DAF-12 (Figure 2-14). However, still outstanding are the identities of co-activators. Dafachronic acids will facilitate the identification and characterization of these proteins.

5.3 NEW INSIGHTS INTO CHOLESTEROL METABOLSIM AND FUNCTION IN *C. ELEGANS*

The previously accepted model of cholesterol metabolism in *C. elegans* was established based on the identification of the major sterol metabolites in *C. elegans* and the functional requirements met by each sterol. This work importantly established that the major cholesterol metabolite in *C. elegans* is 7-dehdrocholesterol, which is converted into the Δ^7 sterol, lathosterol and its 4-methyl derivative, lophenol (Chitwood 1999). Sterol deprivation studies in which sterols were provided as the sole dietary sterol indicated that lathosterol supports normal growth and reproduction and thus may be a precursor to a DAF-12 ligand. In contrast, lophenol does not support reproductive development and instead of functioning as a precursor to a DAF-12 ligand may play a role in molting. The identification of the Δ^7 non-methyl-sterol, lathosterone, as a DAF-9 substrate that is metabolized into the DAF-12 ligand, 3-keto- Δ^7 -cholestenoic acid , confirms the idea that non-methylated Δ^7 sterol metabolites of lathosterol function as DAF-12 ligands.

The identification of lathosterone, a 3-keto derivative of the endogenous worm sterol lathosterol, as a DAF-9 substrate 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), may participate in the production of lathosterone (Figure 3-1). 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. I have cloned two of these genes (ZC8.1 and C32D5.12) but was unable to detect activities for them in a co-transfection assay (data not shown). Although lathosterone has never been reported as an endogenous cholesterol metabolite in *C. elegans* the finding that Δ^7-dafachronic acid is an endogenous DAF-9-dependent hormone indirectly supports the idea that this lathosterone exists in *C. elegans*. Its ability to support normal reproductive growth and development (Figure 2-4) is further evidence that it can function as an important dietary sterol in *C. elegans*. Regardless, the studies presented here should initiate new investigations into cholesterol metabolism in *C. elegans* and provided definitive evidence for the existence of lathosterone.

The work described in Chapter 3 has helped to elucidate the role for the Rieske oxygenase, DAF-36, in the *C. elegans* steroid hormone biosynthetic pathway. *daf-36* mutants lacked 7-dehydrocholesterol and Δ^7-dafachronic acid. Furthermore, its expression in *Sf-9* cells was associated with the production of 7-dehydrocholesterol from endogenous cholesterol in these cells. This supports the notion DAF-36 acts in a cholesterol Δ^7-desaturase reaction, converting cholesterol into 7-dehydrocholesterol. (Rottiers et al., 2006, see Chapter 3). However, it remains to be determined how DAF-36 participates in this reaction. Purification of DAF-36 and reconstitution of its activity in vitro will be required to determine if DAF-36 directly catalyzes the Δ^7-desaturation

reaction or instead requires additional enzymes and co-factors common to *C. elegans* and *Sf-9* cells.

Based on the current model of cholesterol metabolism in *C. elegans,* DAF-36 should be required for production of both the Δ^{7}-dafachronic acid and the putative 4-methylsterol molting hormone. However, this is hard to reconcile with the weak phenotypes exhibited by *daf-36* mutants. As shown in Chapter 3, when *daf-36* mutants are grown at permissive temperatures they bypass dauer despite the fact that lipid analyses demonstrated an absence of 7-dehyrocholesterol, a precursor to both Δ^{7}-dafachronic acid and the 4-methyl-sterol, lophenol. The absence of dauer and molting phenotypes at permissive temperatures may be due to contaminating sterols that bypass the *daf-36* block or due to up-regulation of parallel pathways for ligand production. Activation of DAF-12 by an unidentified activity in *daf-36* lipids, discovered after fractionation, lends support to these hypotheses. Interestingly, similar "phantom" activities have been observed in the *Drosophila* without children mutant (*woc*) (Warren et al., 2001). These mutants also fail to synthesize 7-dehydrocholesterol, which is an important precursor to 20E synthesis in the fly, yet they manage to make two molts before failing to enter metamorphosis. It will be interesting in the future to see whether the activity in *daf-36* worms originates from a parallel pathway involving metabolism of cholesterol or instead from background sterols that bypass *daf-36*. Finally, the identification of DAF-9 substrates, dafachronic acids, and the Rieske oxygenase, DAF-36, furthers our understanding of the function and metabolism of cholesterol in *C. elegans.*

5.4 AN UPDATED AND ALTERNATIVE MODEL FOR CHOLESTEROL METABOLISM IN *C. ELEGANS*

An updated model for cholesterol metabolism in *C. elegans* was presented in Figure 3-1. This model adds lathosterone downstream of lathosterol and illustrates that it is the long-sought substrate that is converted by DAF-9 into the DAF-12 ligand. 4-cholesten-3-one is also metabolized by DAF-9 into a DAF-12 ligand (Δ^4-dafachronic acid), however it is not clear if 4-cholesten-3-one originates from the diet through auto-oxidation of cholesterol or via a cholesterol 3β-HSD with Δ^4-Δ^5 isomerase activity. When *C. elegans* is provided 5α-cholestanol it does not produce cholesterol (Chitwood, 1999), suggesting that *C. elegans* does not contain a Δ^5-desaturase and that there is likely to be no absolute requirement for the 4-cholesten-3-one pathway. Finally, DAF-36 can be placed at the top of the pathway, carrying out the Δ^7-desaturation reaction on cholesterol to produce 7-dehydrocholesterol. Lathosterol may be produced from 7-dehydrocholesterol via a 5α-reductase step. Lathosterol is the most downstream metabolite of cholesterol that is sufficient to support growth and reproduction--in fact no absolute requirement exists for sterol upstream of it (Chapter 1.5). This is likely due to its unique position in the pathway as a precursor to Δ^7-dafachronic acid and the methyl-sterols important for molting. Lathosterol is then converted to the DAF-9 substrate, lathosterone, possibly by a 3β-HSD. However, in mammals these enzymes typically act on Δ^5-sterols during steroidogenesis. Therefore, an alternative model that takes this into

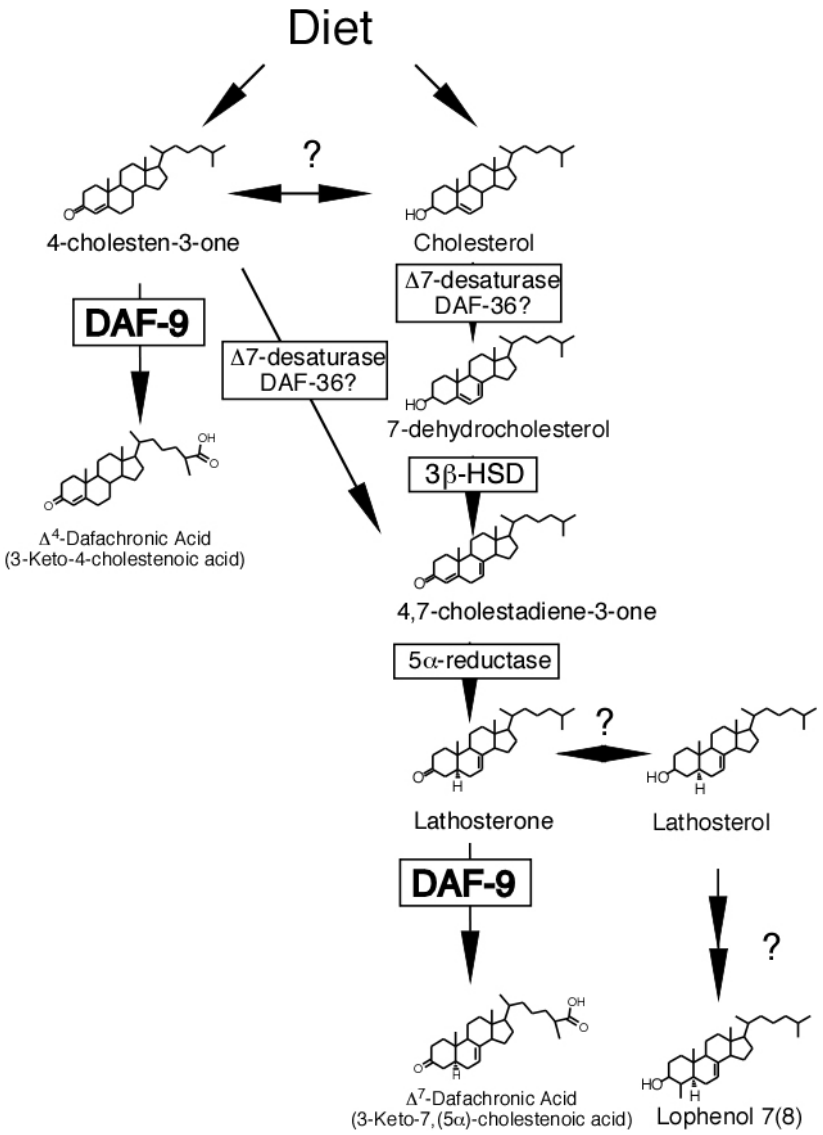


Figure 5-1. An Alternative Model for Cholesterol Metabolism in *C. elegans*

This pathway differs from the pathway in Figure 3-1 in that the 4,7-cholestadiene-3-one intermediate is incorporated downstream of 7-dehydrocholesterol. A 3β-HSD is more likely to act at this step in the pathway because they typically carry out Δ^5-Δ^4 isomerization. Additionally, 5α-reductase in humans reduces 3-keto-Δ^4 steroids. It is not clear if lathosterone is upstream or downstream of lathosterol.

account is presented in Figure 5-1. In this model the 3β-HSD acts at an earlier step, converting 7-dehdrocholesterol into the 3-keto- Δ^7-sterol 4,7-cholestadiene-3-one. This is subsequently 5α-reduced to lathosterone. Lathosterol may derive via a reduction step that is reversible, since lathosterol promotes reproductive development in *C. elegans* (Chapter 1.5). In this model I also suggest an explanation for how 4-cholesten-3-one can support reproductive growth and development in *C. elegans*. It is either converted back to cholesterol or metabolized by DAF-36 into 4,7-cholestadiene-3-one. This alternative model is highly speculative but I have included it here as a framework for testing the enzymatic activity and specificity of DAF-36 and any other enzymes that might be later identified in the dauer formation pathway.

5.5 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 endocrine hormone that serves as a DAF-12 ligand to control organism-wide developmental commitments to dauer formation or reproductive development in *C. elegans* (Gerisch et al., 2001; Jia et al., 2002). 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 is expressed in a select set of tissues where it acts at a key position in the pathway to generate nuclear receptor ligands that act in an endocrine fashion. 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). Although dafachronic

acids are bile acid-like steroids they likely do not function as detergents because they are found at levels too low to do so. Instead, they have been adopted by *C. elegans* for use as signaling molecules in the control of dauer formation and reproductive development. 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, chemosensory neurons secreted peptide hormones such as insulin and TGFβ to positively influence steroid hormone production in *C. elegans* (Chapter 1, Section 1.5). Although the mechanism through which insulin and TGFβ control hormone production is not known, the ability to rescue dauer arrest phenotypes of *daf-2* and *daf-7* mutants with Δ^4-dafachronic acid (Figure 2-13) demonstrates that this regulation may lie at the level of *daf-9* activity, which is in line with previous genetic analyses. 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 be independent of its influence over hormone production. 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).

The work by Rottiers et al (2006) demonstrated that *daf-36* is encodes an enzyme that acts early in the hormone biosynthetic pathway. Interestingly, *daf-36* is expressed predominantly in the intestine, and its expression is influenced by insulin signaling. Thus, different tissues may act coordinately in the production of DAF-12 ligands such that each tissue casts a 'vote' in the decision to become a reproductive adult. For example, chemosensory neurons provide information about food availability and population density to influence peptide hormone production, while the intestine provides information about the nutritional status—including cholesterol levels—of the worm and conveys this information to DAF-9 expressing tissues by carrying out intestine specific steps in hormone production. Interesting parallels can be seen between reproductive development in worms and humans. In women, the onset of the reproductive age is associated with menarche (menstruation) and is strongly associated with nutritional status. Body fat in humans, the equivalent of the worm intestine, may produce peripheral signals that act in the nervous system to influence the timing of sexual maturation and production of reproductive steroid hormones.

5.6 TARGETING NEMATODE NUCLEAR RECEPTORS

Given the previous success of nuclear receptor pharmacology in vertebrates, the identification of ligands for a nematode nuclear receptor raises the possibility of targeting nuclear receptors in parasitic nematodes as a strategy for controlling their growth. In chapter 4, I cloned the LBD of a hookworm DAF-12 homolog and showed that it could be activated by dafachronic acids as well as cholestenoic acids, which are products of the human enzyme CYP27A1. In addition, activation of hookworm DAF-12 by cholestenoic

acid was specific, as it did not activate a DAF-12 homolog from *S. stercoralis*. This is not surprising when one considers that *A. ceylanicum* and other hookworm species are more closely related to *C. elegans* than is *S. stercoralis* (Blaxter et al., 1998).

An interesting aspect of this work was the finding that hookworm DAF-12 was more potently activated by the human bile acid precursor, cholestenoic acid, than was *C. elegans* DAF-12. It is tempting to speculate that the hookworm DAF-12 ligand may derive from the serum of its host. However, it is also possible that hookworms rely on a host signal to initiate a signaling cascade leading to the production of an endogenous DAF-12 ligand. The identification of a DAF-9 hookworm homolog may help answer this question, but so far no hookworm EST in public databases can be found that is homologous to DAF-9. A more directed approach will be necessary to determine if one exists. In any case, given that AceyDAF-12 is activated by dafachronic acids and cholestenoic acids it will be important in the future to see how these agonists affect the activation of hookworm L3 larvae and their transition to parasitism.

Future work aimed at identifying more potent agonists and antagonists of these hookworm receptors may lead to the development of new drugs to combat the debilitating infections caused by hookworms. In addition to targeting DAF-12, the enzymes involved in production of the endogenous ligand might serve as acceptable targets as well. DAF-9 and DAF-36 are excellent targets, if they are conserved in parasitic nematodes, because they are not found in humans. Similarly, the enzyme or enzymes responsible for the production of the molting hormone derived from lophenol is

another target, as methylation of the sterol nucleus does not occur in humans. The identity of this enzyme(s) remains to be determined (Figure 5.1).

Finally, the discovery of dafachronic acids as nematode hormones and the adoption of the orphan nuclear receptor DAF-12 sets an important precedent for further work aimed at identifying ligands for many of the other *C. elegans* orphan receptors. As of today, many questions still remain unanswered: For example, what is the ligand of NHR-49? Is it a fatty acid? Also, what is the identity of the steroid hormone that controls molting in *C. elegans*? Is its receptor a CeNHR, such as NHR-25 or NHR-23? The work described in this thesis will hopefully prove to be a useful framework for future students interested in identifying ligands for these and other interesting receptors. For now, that's one down and 283 to go…

CHAPTER 6

MATERIALS AND METHODS

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, and 12-keto-lithocholic acid were purchased from Steraloids, Inc. (Newport, RI). Lophenol, (25S),26-hydroxycholesterol, and (25R),26-hydroxycholesterol were purchased from Research Plus (Manasquan, NJ). Deuterated chenodeoxycholic acid (CDCA-d4) was from C/D/N Isotopes (Pointe Claire, Quebec). Unless otherwise noted, all other reagents were purchased from Sigma-Aldrich.

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 0^0C. After stirring for 1 h, the reaction was quenched with isopropanol, and the product 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 ^{1}H-NMR (Table 4). NMR data was provided by Dr. Richard J. Auchus and Dr. Kamalesh Sharma.

Nematode and Bacterial Strains

Worms were grown on NGM agar with OP50 bacteria at 20°C unless noted otherwise (Brenner, 1974). Strains used were: *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 184-754aa (DAF-12), 92-561aa (NHR-8), 78-361aa (NHR-23), 2-567aa (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 50ng of luciferase reporter, 20ng CMX-b-galactosidase reporter, 15 ng of CMX receptor expression plasmid, and control plasmid to maintain 150ng/well. Candidate ligands were added at 4000-fold dilution 8h post-transfection. Luciferase activities were normalized to the β-galactosidase control. Data represent the mean ± SD of triplicate assays.

Tests for Reproductive Growth by Individual Sterols

Cholesterol free plates were prepared by omitting peptone and replacing BactoAgar with sterol free agarose. Background sterols from agarose (Low EEO, Fisher) were removed by chloroform extraction. 25g agarose was mixed with 250ml chloroform and shaken vigorously in a 2L flask. Agarose was then dried over Whatman filter paper. This procedure was repeated 3 times. DA837 *E.coli* was grown for 12 hours or overnight in DMEM buffered with 200mM HEPES pH7.4. The bacterial pellet was then washed 2x with M9 buffer and concentrated 5x in M9. WT animals were grown for one generation on 10cm cholesterol-free plates containing washed DA837 and embryos from gravid adults were harvested by hypochlorite lysis. Recovered embryos were placed on 3cm plates containing DA837 mixed with the indicated sterols. Sterols were added to 100µl of 5x DA837 as a 1:10 dilution of a concentrated EtOH stock. Final concentrations were

estimated as if the sterol was delivered over the entire volume of the plate. Growth was monitored until worms arrested or became gravid WT adults.

Preparation of DAF-9 and control microsomes from *Sf9* cells

Sf-9 cells ($2x10^6$ / ml) were cultured in SF-900 SFM and infected with baculovirus (MOI = 2-4) in medium containing 0.5mg/ml hemin chloride, 100mM d-amino-levulinic acid, and 100mM ferric citrate. Cells were harvested 60 h 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 0.1M 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 mM in 0.5 ml total volume, preincubated 3 min at 37C, and then reacted with 1 mM NADPH for 16h. 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 mg of 1,4-cholestadiene-3-one was added as an internal standard for the extraction.

Rescue Assays

Microsomal extracts were resuspended in 50ml methanol, mixed with 5x concentration of HB101 bacterial paste, vacuum dried, resuspended in 100µl 5x concentrated HB101, and plated on 3cm plates containing 4ml NG agar. For rescue, ~200 embryos from a 4-8 hour 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 hrs and the remaining *daf-9*(-),*gfp*(-) animals were scored for dauer arrest 24h later. For rescue experiments using pure steroids, 10µl of compounds were mixed with 5X (100µl) concentrated OP50 bacteria and plated. Final concentrations refer to the amount plated in agar (3-4ml/plate). Strains tested were grown reproductively onto regular NG agar for 2 generations at 20C. Rescue data in Figure X was provided by Dr. Veerle Rottiers in the laboratory of Dr. Adam Antebi.

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 L Fernbach flasks containing 100-350ml S-medium supplemented with 5mg/ml Nystatin, 50 mg/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 Figure 2-17 growth of wild-type and *daf-9; daf-12* was performed in Fernbach flasks at 22.5C for 48h until just prior to the dorsal turn of distal tip cells. In Figure 2-18 final growth to the L3/L4 stage was performed in a 15L New Brunswick BiofloIV fermentor, with a working volume of 10.5L at 20C with agitation (100 RPM , 25% O_2 saturation). In figure 3-2 *daf-36* and WT worms were grown as described (Rottiers et al., 2006). All worms were harvested and bacteria and debris were removed by sucrose flotation, then the worms were 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, Ottawa, Canada). Total lipids 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 (~100mg/10^7 worms) was re-suspended in chloroform, adsorbed to a silica column, and lipids eluted in three fractions with 100ml chloroform, 200ml 9:1 acetone:methanol, and 100ml methanol/100mg extract (Figure 2-18 and Figure 3-3). 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

Dr. Carolyn L. Cummins, a post-doctoral fellow in the laboratory of Dr. David J. Mangesldorf, performed all LC/MS work. Samples were analyzed by LC/MS using a Diode array detector (DAD) in tandem with a Mass Spec Detector (MSD) single quadropole instrument (Agilent Technologies, Palo Alto, CA) with API-ES in both positive and negative ion modes. Samples were dissolved in methanol and loaded onto a pre-column (Zorbax C_8, 4.6 x 12.5 mm, 5 mm, 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 x 50 mm, 5 mm, 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). For separation of 20E, CDCA, Δ^4-dafachronic acid, and 26-hydroxy-4-cholesten-3-one the following conditions were used: solvents A: MeOH, B: MeOH:ACN:H2O (60:20:20). Start at 0min 100%B, 6.5 min 50% B, hold 50% B until 18 minutes then at 18.1 return to 100%B..

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 x 10^6 wild-type worms, 230 mg from 19 x 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 (208nM/worm) based on an external calibration curve using 3-keto-4-cholestenoic acid and an L4-stage worm volume of 1.5nl.

Binding Assay

DAF-12 ligand binding domain (aa 507-753) was expressed in BL21(DE3) cells as a 6xHis-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 5mg/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. These assays were performed by Kelly Suino-Powell and Yong Li in the laboratory of Dr. Eric Xu.

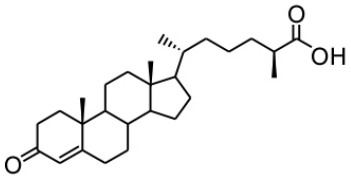
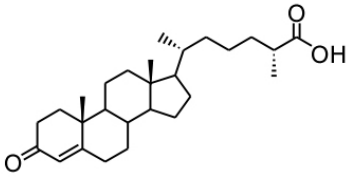
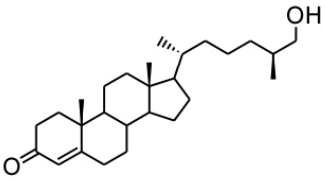
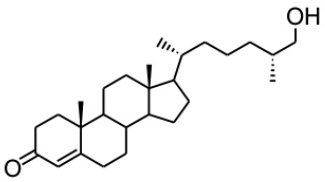
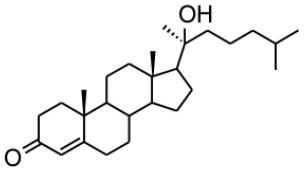
APPENDIX A: LIST OF COMPOUNDS TESTED

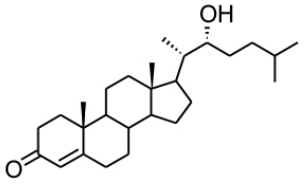
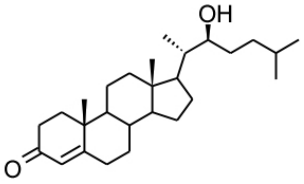
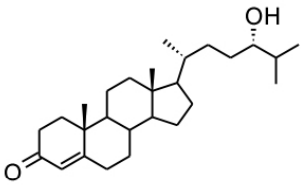
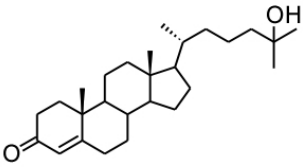
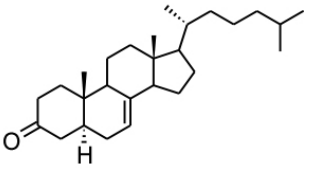
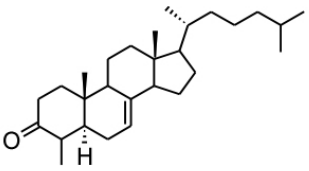
Compound	Concentration μM	Fold Activity (if > than 0, or not in Cell paper)
4-cholesten-3-one	50	DAF9: 135
Pregnenelone	10	
Progesterone	10	
20β -OH-Pregenelone	10	DAF9: 0.6
21-OH-Pregnenelone	10	
17,20-OH-Pregenelone	10	
3,7,12-trihydroxycholestanol	10	
4β -OH-cholesterol	10	
Androstenone	10	
Androstenol	10	
Adrostendione	10	
Corticosterone	10	
7α-OH-4-cholesten-3-one	10	
5α-cholestan-3-one	25	
5β -cholestan-3-one	25	
20-OH-cholesterol	10	
22R-OH-cholesterol	10	
24S-OH-cholesterol	10	
25-OH-cholesterol	10	
27(25R,26)-OH-cholesterol	10	
7β -OH-4-cholesten-3-one	25	
7α -OH-4-cholesten-3-one	25	
6β--Oh-4-cholesten-3-one	25	
4,22-cholestadiene-24b-ethyl	25	
1,4-cholestadiene-3-one	25	
4,6-cholestadiene-3-one	25	DAF9: 29
4,7-cholestaidene-3-one		
1,25-OH2-D3	0.1	
VD3	10	
VD2	10	
25OH-VD3	0.1	
7-dehyrdocholesterol	10	
5β-cholanic acid -3-one	25	<5
5α-cholanic acid 3,6-dione	25	<5
5β-cholanic acid 3,6-dione	25	<5
5β-cholanic aid 3,7-dione	25	
5β-cholanic acid 3,12-dione	25	<5
CDCA	30-50	

Compound	Concentration μM	Fold Activity (if greater than 0, or not in Cell paper)
CA	30-50	
LCA	30-50	
DCA	30-50	
3K-LCA	30-50	5-7
6K-LCA	30-50	
7K-LCA	30-50	
12K-LCA	30-50	
22R-OH-4-cholesten-3-one	4-10	
20-OH-4-cholesten-3-one	4-10	
24S-OH-4-cholesten-3-one	10	
25-OH-4-cholesten-3-one	10	
25R,26-OH-4-cholesten-3-one	10	
25S,26-OH-4-cholesten-3-one	10	
(25R)-3,16-keto-4-cholestenoic acid (Javitz cmpd)	10	
24,25-epoxy-4-cholesten-3-one	10	2
24,25-epoxy-cholesterol	10	

APPENDIX B: STEROID NMR DATA

All NMR Data kindly provided by Dr. Richard J. Auchus and Dr. Kamalesh Sharma.

Compound	Structure	^{1}H NMR (400 MHz)
(25S),26-3-keto-4-cholestenoic acid		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.69 ppm (s, 3H) H19, δ = 1.16 ppm (s, 3H) H21, δ = 0.89 ppm (d, 3H, *J* = 6.8 Hz) H27, δ = 1.17 ppm (d, 3H, *J* = 6.4 Hz) No detectable impurities
(25R),26-3-keto-4-cholestenoic acid		H4, δ = 5.72 ppm (s, 1H) H18, δ = 0.69 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 0.89 ppm (d, 3H, *J* = 6.8 Hz) H27, δ = 1.17 ppm (d, 3H, *J* = 6.4 Hz) >95% pure
(25S),26-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H26a, δ = 3.41 ppm (dd, 1H, *J* = 10.4, 5.6 Hz) H26b, δ = 3.50 ppm (dd, 1H, *J* = 10.4, 6.4 Hz) H18, δ = 0.70 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 0.90 ppm (d, 3H, *J* = 6.4 Hz) H27, δ = 0.91 ppm (d, 3H, *J* = 6.4 Hz) No detectable impurities
(25R),26-hydroxy-4-cholesten-3-one		H4, δ = 5.73 ppm (s, 1H) H26a, δ = 3.43 ppm (dd, 1H, *J* = 10.4, 5.6 Hz) H26b, δ = 3.52 ppm (dd, 1H, *J* = 10.4, 6.4 Hz) H18, δ = 0.71 ppm (s, 3H) H19, δ = 1.19 ppm (s, 3H) H21, δ = 0.92 ppm (d, 3H, *J* = 6.8 Hz) H27, δ = 0.93 ppm (d, 3H, *J* = 6.4 Hz) No detectable impurities
20(S)-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.85 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 1.26 ppm (s, 3H) H26, H27, δ = 0.87 ppm (d, 6H, *J* = 6.4 Hz) >95% pure

Compound	Structure	^{1}H NMR (400 MHz)
22(R)-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.72 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 0.91 ppm (d, 3H, *J* = 6.4 Hz) H22, δ = 3.59 ppm (br d, 1H, *J* = 8 Hz) H26, H27 δ = 0.88 ppm (d, 3H, *J* = 6.4 Hz); δ = 0.89 ppm (d, 3H, *J* = 6.4 Hz) 3-5% Δ5 (~95% pure)
22(S)-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.71 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H22, δ = 3.61 ppm (br m, 1H) H21, H26, H27 δ = 0.88 ppm (br d, 9H, *J* = 6.8 Hz) No detectable impurities
24(S)-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.70 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 0.88 ppm (d, 3H, *J* = 6.8 Hz) H24, δ = 3.30 ppm (br m, 1H) H26, H27, δ = 0.91 ppm (d, 3H, *J* = 6.8 Hz); δ = 0.92 ppm (d, 3H, *J* = 6.8 Hz) No detectable impurities
25-hydroxy-4-cholesten-3-one		H4, δ = 5.71 ppm (s, 1H) H18, δ = 0.69 ppm (s, 3H) H19, δ = 1.17 ppm (s, 3H) H21, δ = 0.91 ppm (d, 3H, *J* = 6.4 Hz) H26, H27, δ = 1.20 ppm (br s, 6H) No detectable impurities
lathosterone		H7, δ = 5.17 ppm (dd, 1H, *J* = 4.4, 1.6 Hz) H18, δ = 0.55 ppm (s, 3H) H19, δ = 1.00 ppm (s, 3H) H21, δ = 0.91 ppm (d, 3H, *J* = 6.8 Hz) H26, H27, δ = 0.85 ppm (d, 3H, *J* = 6.8 Hz); δ = 0.86 ppm (d, 3H, *J* = 6.4 Hz); No detectable impurities
lophenone		H4α, δ = 0.99 ppm (d, 3H, *J* = 6.4 Hz) H7, δ = 5.19 ppm (dd, 1H, *J* = 6.0, 1.6 Hz) H18, δ = 0.55 ppm (s, 3H) H19, δ = 1.07 ppm (s, 3H) H21, δ = 0.91 ppm (d, 3H, *J* = 6.8 Hz) H26, H27, δ = 0.85 ppm (d, 3H, *J* = 6.8 Hz); δ = 0.86 ppm (d, 3H, *J* = 6.8 Hz); No detectable impurities

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