A FRAMEWORK OF STEROID METABOLISM AND NUCLEAR RECEPTOR INTERACTIONS IN NEMATODES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2019

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Cornell University [2019]

ABSTRACT

The primary mechanism for organisms to react to their rapidly changing environments is by alteration of gene expression profiles. An organism is capable of doing so, via small molecules that integrate extracellular cues and relay them intracellularly through cognate receptor binding. Hence small molecules act as principle mediators of organismal development and studying their regulation and interactions with other biomolecules including DNA and coregulatory proteins can provide critical insights into organismal physiology. The work presented in this dissertation combines liquid-chromatography – mass spectroscopy (LC-MS) based metabolomics with protein biochemical studies to explore the small molecule – receptor biology in the nematode *C. elegans*.

In *C. elegans*, steroidal small molecule ligands called dafachronic acids (DAs) regulate reproductive development and lifespan through interaction with the conserved nuclear receptor DAF-12. While some of the key enzymes in DA biosynthesis pathway are well studied, many aspects of DA receptor interactions, their biosynthesis and metabolism remain to be uncovered. In the first part, we characterize DAF-40 as the critical DA-oxidoreductase that uses known DAs as precursors in the biosynthesis of highly unstable, yet highly potent DAF-12 ligands to mediate

reproductive development. It uncovers the complex pool of chemically labile DAF-12 ligands and adds a novel steroid metabolome regulatory node. Next, we investigate the intersection of DA/DAF-12 signaling with DAF-12 receptor homologs - NHR-8 and NHR-48. We complement protein biochemistry studies with metabolomics and reveal the dimeric interactions between these receptors and correspondingly their role in coregulating the lipid metabolome. Lastly, while the pivotal roles of the DA family of steroids have been acknowledged, a systematic exploration of the structural and hence functional diversity of steroids has not been achieved in *C. elegans* or any other animal model system. In the final chapter, we implement a ^{13}C stable isotope-based metabolomics approach that uncovers the striking structural diversity in steroidal small molecules. We reason the use of these novel steroids as a starting point to mine for other nuclear receptor ligands in nematodes.

As a whole, this dissertation delves into the deeply interconnected steroid and lipid metabolism network that controls nematode physiology via nuclear receptor interactions. With the findings presented here, we aim to complement the extensive genomics and proteomics in nematodes with a comprehensive structural and functional characterization of the metabolome.

BIOGRAPHICAL SKETCH

Pooja Gudibanda hails from Mumbai, India where she completed her primary schooling at Fr. Agnel Multipurpose School in Vashi, Navi Mumbai. As a high school student, she was captivated by the structure and complexity of the human genome. Keen to understand the basics of life and evolution, in 2009 she went on to earn her Bachelor's degree in Biotechnology from Ramnarain Ruia College, under the University of Mumbai as one of the few recipients of the Government Open merit scholarship, the University's highest honor. With a strong desire to continue pursuing her scientific interests from an institution dedicated to inter-disciplinary research, in 2010 she started a Master's degree in Biotechnology at the State University of New York, at Buffalo on a J. N. Tata and K.C. Mahindra scholarship. During this time, she worked with Professor Richard W. Browne and identified cholesterol and its associated lipoproteins as biomarkers to predict embryo quality and clinical reproductive outcomes. In 2012 she was awarded the Mark Diamond Research fund for her thesis work and was nominated for the 'Whos Who among students in American Universities' award. Wanting to harness the combined power of molecular biology and analytical chemistry tools to answer complex biological questions, Pooja then came to Cornell University to pursue her Ph.D. in Biochemistry with Professor Frank C. Schroeder. Here she explored the complex interaction network between small molecule signaling steroids and nuclear receptor proteins that ultimately govern nematode development. Alongside, she served as an active board member of the Cornell India Association for 3 years and enjoyed bringing together the diverse student population that Cornell hosts. As a part of her graduate school journey, Pooja has enjoyed taking on challenging projects and hopes to contribute towards unlocking the mysteries of life.

Somewhere between living and dreaming, lies this journey

ACKNOWLEDGEMENTS

Special Committee Chair and Advisor: Professor Frank C. Schroeder

Special Committee: Professor Brian Crane and Professor Sylvia S. Lee

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Core Facilities: Gary Horvath (BTI), Biotechnology Resource Center

Organizations: Department of Molecular Biology and Genetics, Field of Biochemistry, and The Boyce Thompson Institute for Plant Research, Cornell University

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PREFACE

INTRODUCTION

Biological systems function through a collection of complex and highly organized intra-and inter-molecular interactions that determine the functioning of the system and together form the basis of life. Molecular interactions occur between four levels – the genome, transcriptome, proteome and metabolome. While the genome is built of a rather finite combinations of nucleotide building blocks and stays largely static between different tissues within an organism; the transcriptome and the proteome are subject to more modifications and spatio-temporal regulations in response to environmental stimuli. On the other hand, the metabolome is highly dynamic, structurally unpredictable and differs not just between tissues or cells, but often times between cellular compartments too. The small molecule metabolome is constantly fine-tuned in response to environmental stimuli. Further, the metabolome is constantly interacting with the proteome. Small molecules can directly modulate the activity of enzymes, allowing cells to respond to the environment in timescales on the order of seconds. Small molecules can also directly bind to and modulate transcription factors, that in turn control gene expression profiles. Orchestrated by these small molecules, the organism in constantly engaged in a feedback regulatory loop, enabling it to respond and adapt to the changing environmental conditions **(Figure P.1a)**. Thus, small molecules do not just serve as biological intermediates and building blocks for cells, but they play critical regulatory roles. These complex, yet highly specific interaction systems create a deeply interconnected metabolic web. Studying such a complex system can be challenging. But, with the characterization of even a single class of metabolite, immense information can be

obtained - especially on the enzymes that act on them, the regulatory nodes they govern, and eventually the metabolic diseases based on the disruption of their biosynthetic paths.

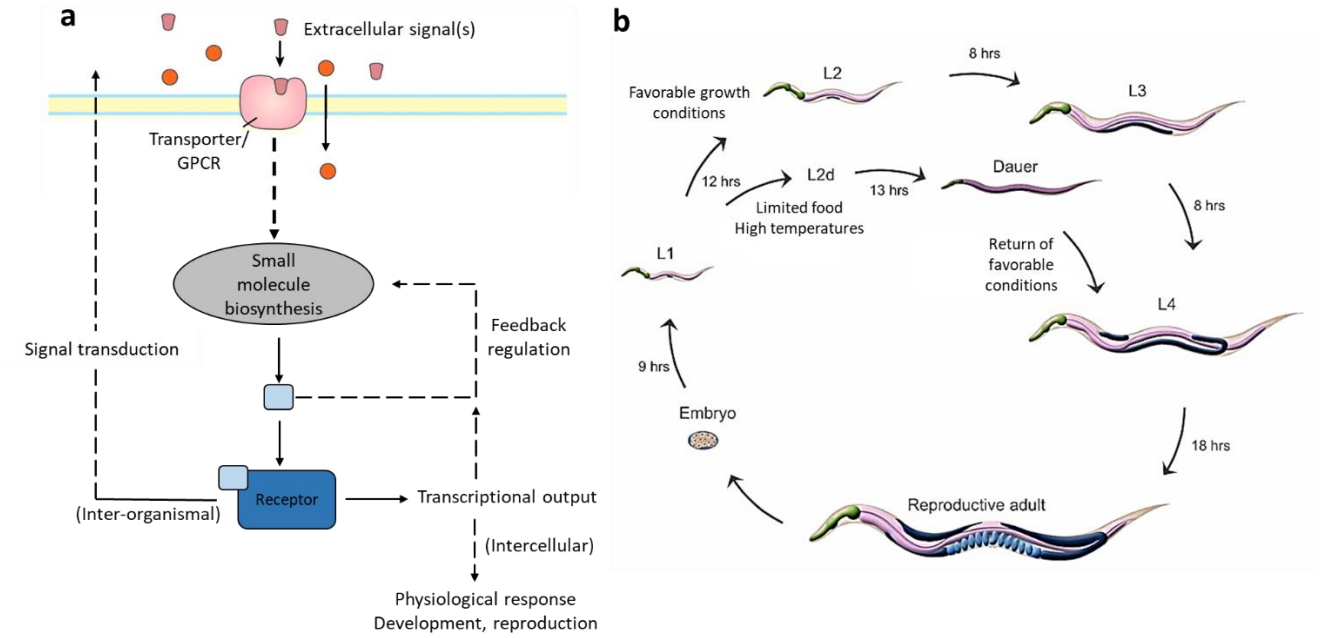

Figure P.1: (a) Simplified schematic of a small molecule mediated signaling. **(b)** Life cycle of nematode *Caenorhabditis elegans* with the dauer branch (Adapted from WormAtlas).

Nematodes as a model organism for development and aging studies

To answer the challenging questions on complex molecular interactions, it is necessary to focus our investigations on rather simpler, but representative systems. In this regard, the nematode *Caenorhabditis elegans* owing to its short life cycle, genetic tractability and well-characterized genome serves as an important model organism[1, 2]. Despite being small enough for high throughput operations, *C. elegans* is highly differentiated. Being a eukaryote, many physiologically relevant cellular and molecular pathways in *C. elegans* especially those towards health and aging are homologous to higher animals (including humans), making it a prime model

to study conserved signaling mechanisms of gene regulation and function[3]. As a result, findings from *C. elegans* biology have had considerable impact on furthering our understanding of metabolism, immunity and metabolic disease regulation[4-7].

Notably, most of the research aimed at understanding adult lifespan and aging has focused on understanding the unique and alternative developmental stage called 'dauer diapause'. Under favorable growth conditions, such as adequate food supply and temperatures, the worm develops *ex utero* from an egg through four larval stages to reach reproductive maturity **(Figure P.1b)** [8, 9]. This cycle typically takes 3 days after which the adult can live for 2-3 weeks under optimal conditions. However, under unfavorable growth conditions, *C. elegans* exit at the L2 larval stage and enter into a non-feeding, non-reproductive and developmentally arrested state, called dauer diapause, which enables the worm to survive for several months, beyond its normal lifespan of 2-3 weeks[9]. Dauer is a highly resistant stage characterized by quiescent transcription, complete morphological remodeling and reduced pharyngeal pumping[10, 11]. The most fascinating part of this developmental plasticity is its reversibility, where in upon return of favorable conditions, dauers can resume reproductive development, making the dauer stage as being "ageless"[12].

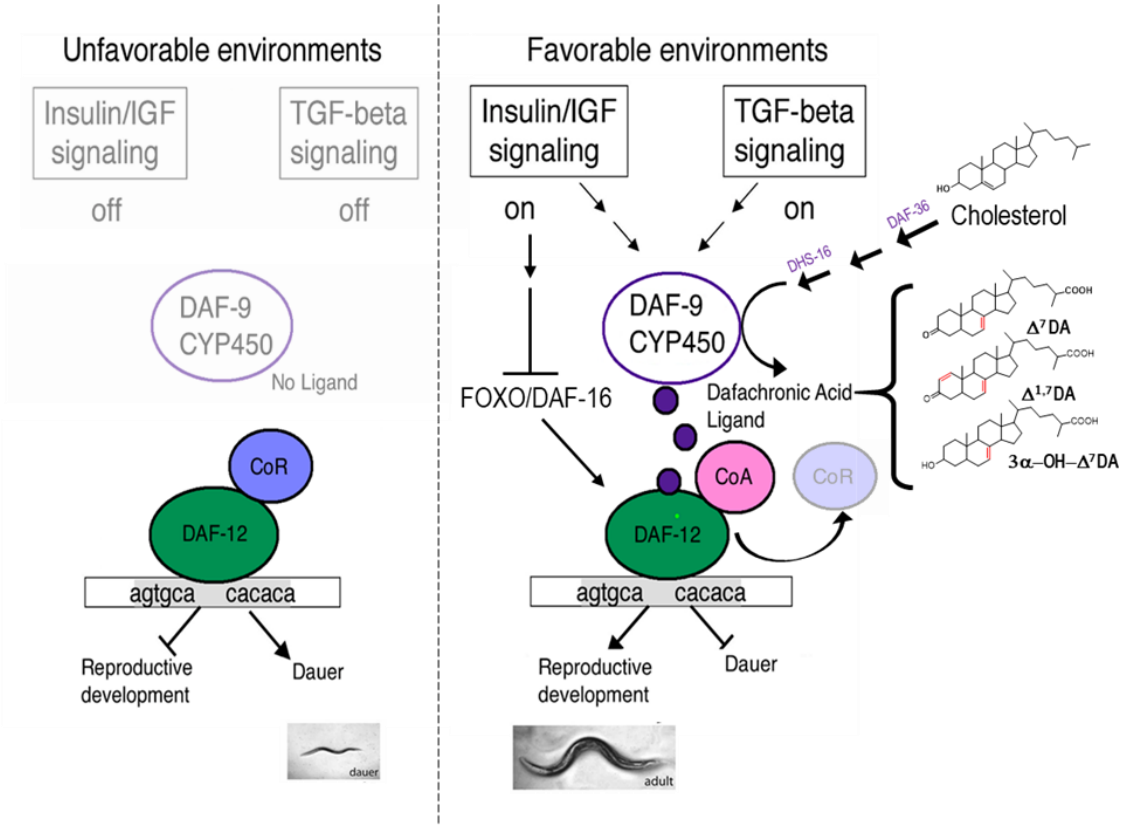

Figure P.2: Dauer versus reproductive development regulatory network in *C. elegans*. Adapted from A. Antebi, Wormbook [13].

Genetic screens on dauer-defective and dauer-constitutive mutants have identified about 30 DAF (DAuer Formation) genes that directly control dauer formation and, subsequent studies have revealed a significant role of these DAF genes in the overall regulation of lifespan and development[14]. Additionally, the repertoire of small molecule signals critical to such a dramatic developmental re-programming have been explored before and almost all these signals converge at the nuclear receptor DAF-12 (a homolog of the human vitamin D and liver X receptors) making it a central regulator of development versus dauer decision[13, 15, 16]. In a highly simplified representation **(Figure P.2)**, favorable growth and environmental cues (such as abundant food supply and growth temperatures) are perceived by chemosensory neurons that activate the

insulin/IGF-1 (*daf-2*) and TGFβ (*daf*-7) signaling pathways in the worm that then converge on the biosynthetic enzyme cascade (including *daf-36* and *daf-9*) that synthesize dafachronic acids (DAs), the bile acid-like endogenous ligands of DAF-12[13]. Under unfavorable conditions however, dauer pheromone biosynthesis is switched on, and the insulin-like and TGF-β signaling is repressed, in which case the unliganded DAF-12 (bound to its corepressor DIN-1) promotes dauer[17]. Thus, DAF-12 with its associated regulatory proteins, works as a ligand-regulated molecular switch to specify the two distinct life history traits, reproductive development versus dauer diapause. Interestingly, conserved orthologs of *C. elegans* DAF genes, particularly those within the nuclear receptor family of proteins, such as PPARs and ERs, appear to regulate metabolism and aging in higher organisms as well [18-22].

PREVIEW OF CHAPTERS

Endogenous small molecule ligands integrate environmental cues and regulate development via binding to their cognate receptors. Hence, uncovering the structural diversity and biosynthetic origins of these ligands can enhance to our understanding of how this bouquet of ligands contributes to receptor biology. In *C. elegans*, dafachronic acids (DAs) regulate reproductive development, lifespan and dauer through interactions with the conserved nuclear receptor DAF-12. While the key enzymes in this DA biosynthesis pathway are well studied, the extent of this pathway holds intense interest. In the first chapter, we characterize DAF-40 as a novel DA-oxidoreductase that uses the known DAs as precursors and synthesizes highly labile, yet potent DAF-12 ligands that regulate reproductive development. It elucidates that there exists a more

complex pool of chemically labile DAF-12 ligands while adding a novel steroid metabolome regulatory node.

While DAF-12 is classically known as the central switch in developmental decisions, recent work from the Schroeder lab and others has revealed overlapping functions of receptors NHR-8 - in mediating population-density dependent acceleration of development, along with the dafachronic acid ligands of DAF-12, and, of NHR-48 - in developmental acceleration of hermaphrodites in response to male-secretory signals, along with corepressor DIN-1. However, the mechanisms by which these receptors regulate different phenotypes have remained largely speculative. Towards a better understanding of these observations, in the second chapter, using protein biochemistry tools we reveal the dimer complex formations between DAF-12, NHR-8 and NHR-48, as well as the shared function of corepressor DIN-1. Correspondingly, using untargeted metabolomics we show the downstream co-regulation of the lipid metabolome by these receptor complexes and uncover EMB-8 as a likely biosynthetic enzyme component coregulated by these NRs.

Lastly, while the pivotal roles of the DA family of steroids has been studied, a systematic exploration of the structural and hence functional diversity of steroidal ligands has been lacking in *C. elegans*. In the final chapter, we implement a ^{13}C stable isotope-based metabolomics approach to uncover the striking structural diversity in steroidal small molecules, and reason the use of these novel steroids as a starting point to mine for other NR ligands in nematodes.

As a whole, this dissertation delves into the deeply interconnected steroid and lipid metabolism network that exerts critical inputs into nematode physiology via NHR interactions. With these

findings, we aim to complement the extensive genomics and proteomics in nematodes with a comprehensive structural and functional characterization of the metabolome.

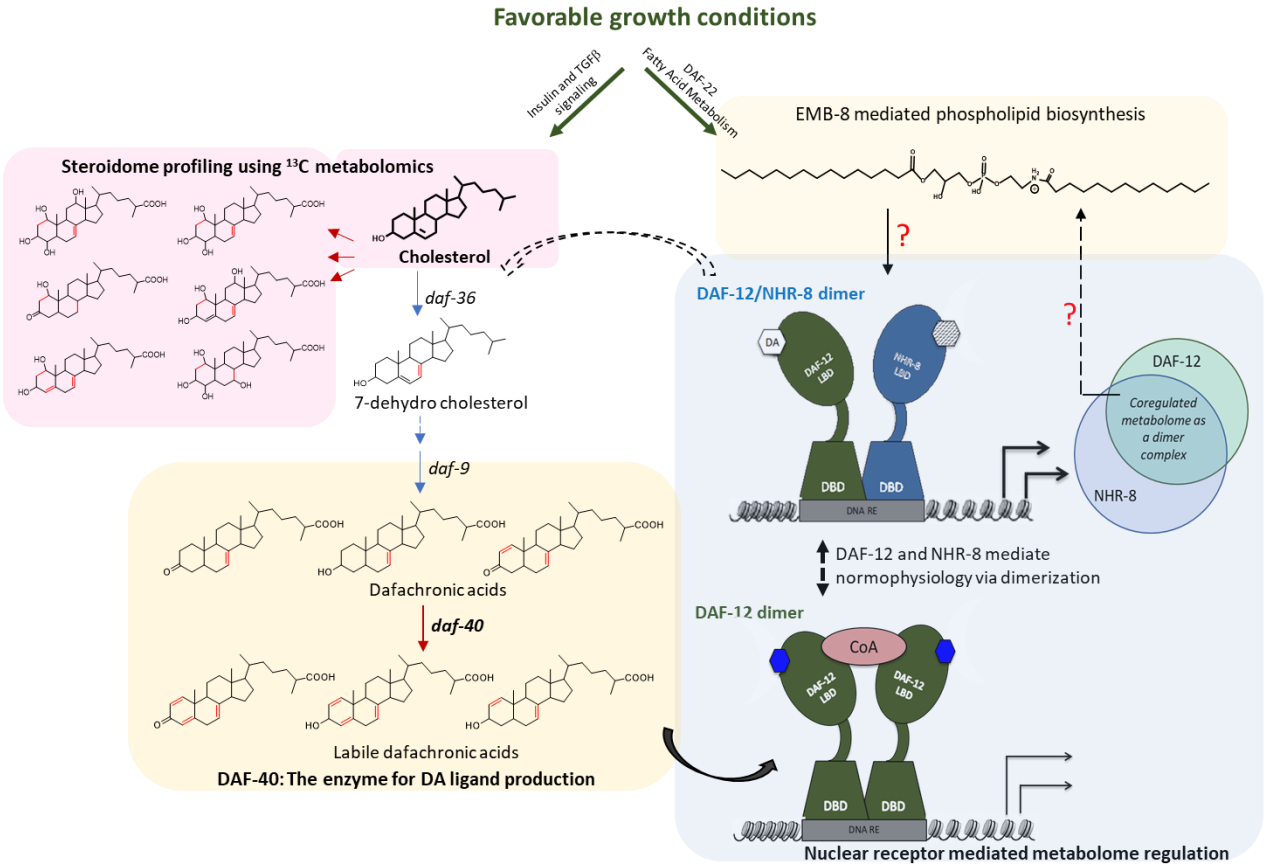

Figure P.3: Dissertation overview: The work presented here reveals the deeply interconnected steroid and lipid metabolism network that exerts critical inputs into nematode physiology via nuclear receptor interactions.

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CHAPTER 1

STEROID HORMONE PRODUCTION AND REGULATION BY DAF-40: A NOVEL CYTOCHROME P450 IN *C. ELEGANS*

ABSTRACT

Endogenous small molecule ligands integrate environmental cues and regulate development via binding to their cognate receptors. Hence, uncovering the structural diversity and biosynthetic origins of these ligands can enhance our understanding of how this *bouquet* of ligands contributes to receptor biology. In *C. elegans*, dafachronic acids (DAs) regulate reproductive development, lifespan and dauer through interactions with the conserved nuclear receptor DAF-12. While some of the key enzymes in this DA biosynthesis pathway are well studied, the extent of this pathway holds intense interest.

In genetic enhancer screens to identify new genes involved in DA regulation, *daf-40* (a cytochrome P450 homolog of mammalian thromboxane A2 synthase) was identified for its role in dauer formation. Mutants display phenotypes characteristic of DA-deficiency, however, *daf-40* dauers are only partially rescued by high doses of known DAs. Corresponding metabolome analysis of *daf-40* mutants did not show absence, but rather a mild upregulation of two of the known DAs, suggesting these could be shunt metabolites that build-up due to the lack of DAF-40-mediated conversion to the *real* DAF-12 ligand. While *in vitro* biochemical assays validate DAF-40 as a DA-oxidoreductase, they also demonstrate its potential to perform unique redox chemistry on the A-ring of cholesterol. Coupled with the observation that synthetic hydroxylated

and unsaturated derivates of DAs show strong rescue of *daf-40* dauers, it appears that such derivatives are the putative products of DAF-40 but are not readily detected *in vivo* either due to their unstable/labile nature or transient production. While this study demonstrates that DAF-40 can shift the threshold for sensitivity of known DAs, it suggests that there exists a more complex pool of chemically labile DAF-12 ligands and reveals a novel node in the *C. elegans* steroid metabolome regulatory network. Our results further suggest the possibility that currently known mammalian receptor ligands also have yet unsuspected derivatives with higher potency or unique functions.

INTRODUCTION

Small molecule metabolites represent a complex component of biochemical activities in an organism, the characterization of which can immensely further our understanding of diverse biological processes. Among these molecules, several have recently emerged as modulators of longevity in multiple organisms, including mono- and polyunsaturated fatty acids, spermidine, endocannabanoids, pyruvate and trehalose[1-5]. In the nematode *C. elegans,* lifespan-extending metabolites include the dafachronic acids (DAs), the first bile acid-like steroids discovered to regulate lifespan[6, 7]. DAs serve as ligands for the steroid receptor DAF-12 (a homolog of mammalian vitamin D and liver x receptors), which promote larval developmental progression and adult longevity through microRNA-mediated pathways[8-10].

In the dafachronic acid (DA) biosynthetic pathway, dietary cholesterol is metabolized through a series of enzymatic reactions to produce several different DAF-12 ligands that control its activity and thereby *C. elegans* lifespan[6, 7, 11-13]. Central to DA biosynthesis are enzymes DAF-36 - the Rieske-like oxygenase that acts as the rate limiting enzyme converting cholesterol to 7-dehydrocholesterol, and DAF-9 – the cytochrome P450 that adds the terminal carboxylic acid group **(Figure 1.1)**[7, 13]. As currently known, the family of DAs consists of three endogenously present DAF-12 ligands, Δ^7 DA, $\Delta^{1,7}$ DA and 3α-hydroxy-Δ^7 DA, the synthesis of which require the cytochrome P450 DAF-9 [11].

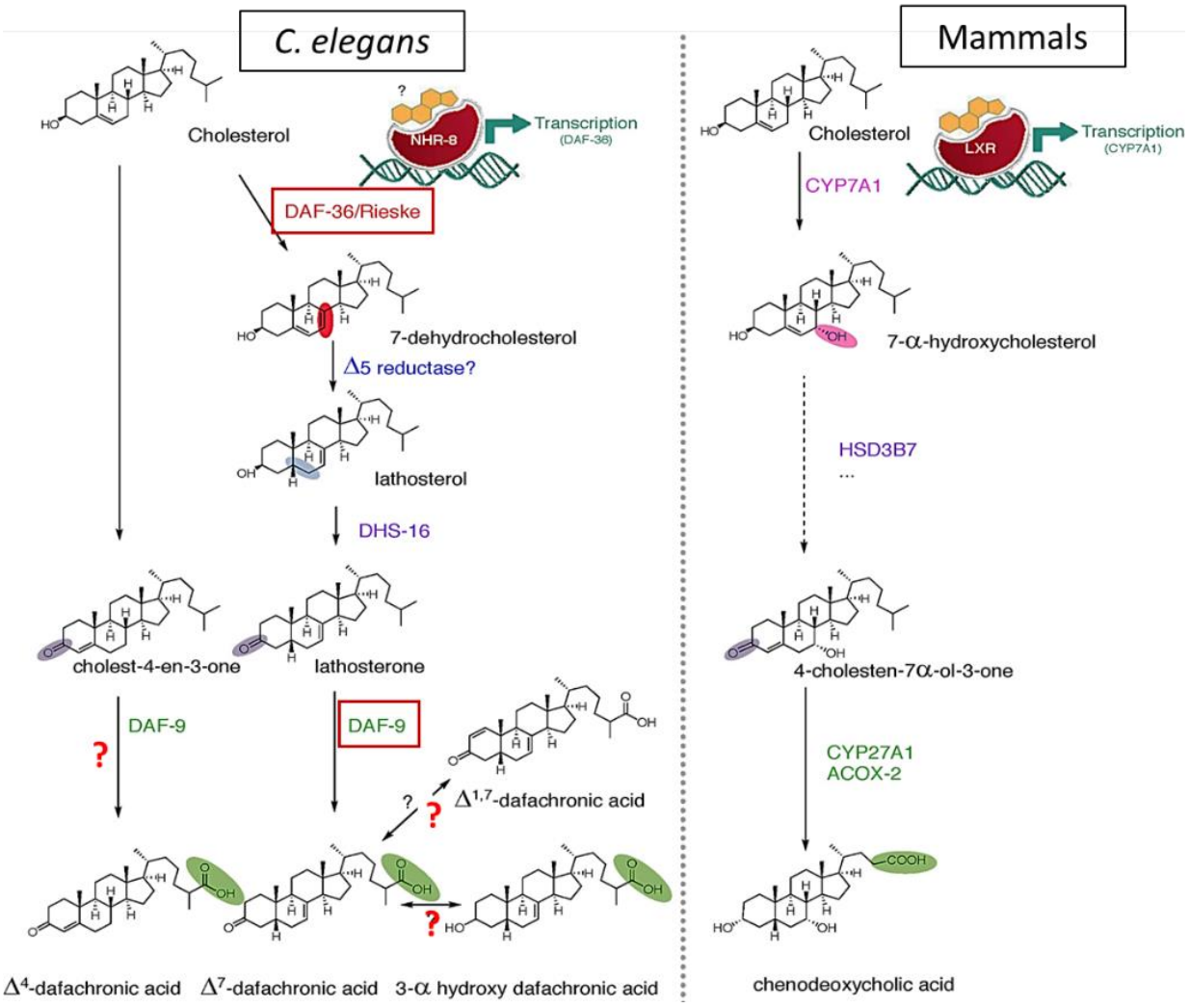

Figure 1.1: Current understanding of dafachronic acid (DA) biosynthesis. Adapted from Antebi A., Nuclear Hormone Receptors in *C. elegans,* Wormbook [14].

In response to environmental conditions, dauer pathway components including TGF-β and Insulin/IGF-1 signaling (IIS) converge on steroid hormone production to regulate DAF-12, which mediates the choice between dauer arrest and reproductive development[14]. Unliganded DAF-12 specifies dauer while the DA-bound, liganded receptor bypasses dauer and permits reproductive development[15]. The rather recent identification of $\Delta^{1,7}$ DA and 3α-hydroxy-Δ^{7} DA suggested that there likely exists a larger pool of steroidal ligands than previously recognized. Additionally,

biosynthetic studies revealed identities of key enzymes involved in several parts of the DA biosynthesis pathway, and in part, indicated that previous biosynthesis models must be revised [11]. Knowledge of DAF-12 ligand biosynthesis is essential for understanding *C. elegans* lifespan and development. Additionally, the ligands identified thus far, most likely represent a small subset of a larger, more complex set of signaling ligands. Correspondingly, identification of DA biosynthetic enzymes would provide the deeper and much needed understanding of important regulatory nodes controlling DAF-12 activity.

RESULTS

As an entry point for uncovering enzyme components of the DAF-12 ligand biosynthesis, our collaborators in the Antebi lab (*Max Plank Institute for Biology of Aging, Cologne, Germany*) conducted genetic enhancer screens of the *daf-36(k114)* null allele, in which DA ligand production is reduced but not abolished. As in previously reported genome-wide RNAi screens, the Antebi lab scored for the appearance of gonadal migration (Mig) and dauer constitutive (Daf-c) phenotypes at the normally permissive temperature of 25 °C, which reflect loss of ligand-mediated DAF-12 activation[13]. From such screens, several loci that affect DA production, including the short-chain dehydrogenase/reductase *dhs-16*, the cytochrome P450 reductase *emb-8* (Revisited in chapter 2), and the nuclear receptor *nhr-8* (Revisited in chapter 2) were found. Additionally, they identified the cytochrome P450, T10B9.7/*cyp-13a2*, now named *daf-40*, for its role in *C. elegans* dauer formation **(Figure 1.2a)**. CYP450s function in various essential processes, including carbon assimilation, biosynthesis of steroids, bile acids and fatty acids, as

well as xenobiotic detoxification. DAF-40 is one of a family of 9 related CYP450 genes of the cyp-13 subgroup found in *C. elegans*. The closest vertebrate relative of DAF-40, thromboxane A2 synthase, is ~30% identical and orthologous by reciprocal blast. Thromboxane A2 synthase produces the arachidonic acid derivative thromboxane A2, a mediator of platelet aggregation and vasoconstriction, and is implicated in numerous disorders including thrombosis, atherosclerosis, and allergic reactions[16].

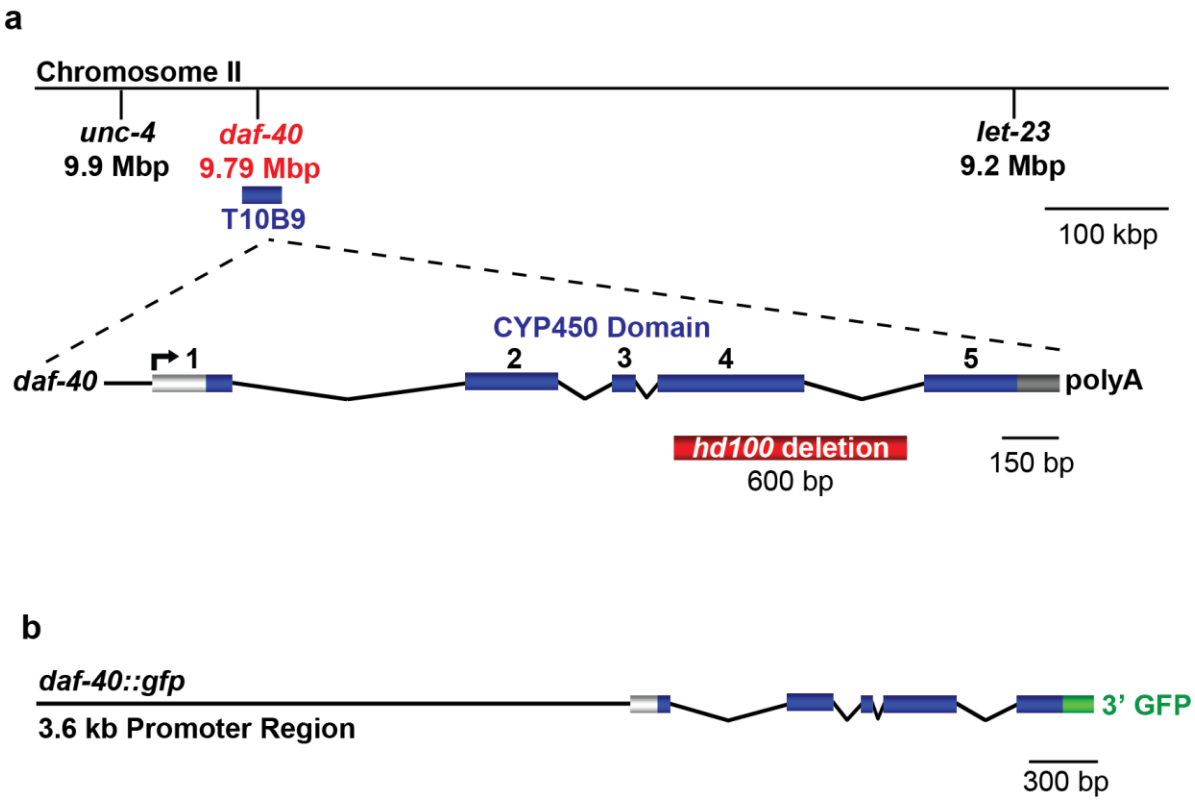

Figure 1.2: Gene structure of the *daf-40* locus. (a) (A) The genomic region of the *daf-40* locus on Chromosome II is shown. Cosmid T10B9 (blue) contains the *daf-40* sequence (T10B9.7). The *hd100* allele (red) is a 600 bp deletion removing the fourth and largest exon in the cytochrome P450 domain and following intron and is a predicted null allele. **(b)** The structure of the C-terminal *daf-40::gfp* fusion construct used in most expression analyses. Figure courtsey, J. Wollam, Antebi lab.

To ascertain a possible role of *daf-40* in DAF-12 ligand biosynthesis, the Antebi lab obtained a deletion mutant with allele *hd100*, that deletes 600 bp and truncates the predicted protein out of frame, removing most of the cytochrome P450 domain, and is a predicted null allele **(Figure 1.2a)**. After outcrossing, *daf-40(hd100)* mutants were Daf-c at the elevated temperature of 27°C and displayed gonadal migration defects upon cholesterol deprivation, phenotypes consistent with reduced DAF-12 ligand production **(Figure 1.3a)**.

To investigate the functional relationship of *daf-40* in the dauer signaling pathways, the Antebi lab performed genetic epistasis experiments combining dauer constitutive Daf-c and dauer defective Daf-d loci **(Figure 1.3b)**. *daf-40* mutation leads to Daf-c phenotypes at 27°C, whereas mutations in the IIS mediator *daf-16/FOXO* transcription factor, TGF-β signaling co-repressor *daf-5/SNO-SKI*, and *daf-12/FXR* lead to the opposite Daf-d phenotypes. To order them into pathways, they constructed double mutants and scored for dauer formation at 27°C. Whereas *daf-40* Daf-c phenotypes were independent of *daf-16*/*FOXO* and *daf-5*/*SNO-SKI* mutation, they were dependent on *daf-12*/FXR mutation. Thus *daf-40* lies downstream of *daf-16* and *daf-5,* but upstream of *daf-12*, similar to other genes involved in DA production. In accord with decreased ligand production, DAF-12 transcriptional activity was reduced in *daf-40* mutants, as measured by diminished expression of the DAF-12/FXR target genes, the *let-7* related microRNAs *mir-84* and *mir-241* involved in L2/L3 transitions **(Figure 1.3c)**. Similarly, loss of *daf-40* resulted in upregulation of hypodermal *daf-9::gfp* **(Figure 1.3d)**. Overall, these data support a role of DAF-40 in affecting DA synthesis.

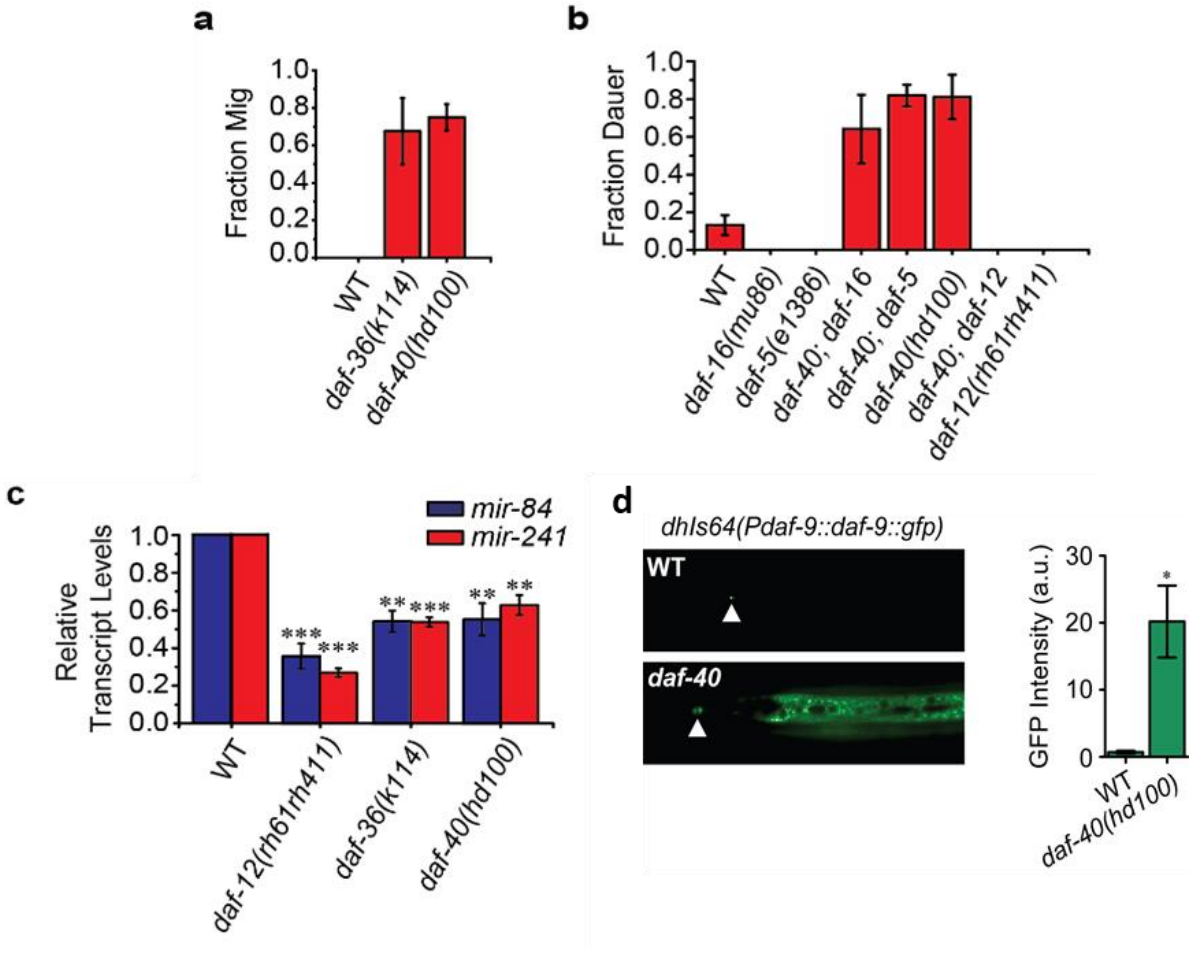

Figure 1.3: *daf-40* phenotypes place *daf-40* downstream of *daf-16* and *daf-5,* and upstream of *daf-12*. **(a)** *daf-40(hd100)* mutants show gonadal Mig phenotypes on NGM without added cholesterol (N=3, mean ± SEM). **(b)** *daf-40* null animals are Daf-c at 27°C and are suppressed by Daf-d *daf-12* mutations, but not by *daf-16/FOXO* or *daf-5/Sno-Ski.* This places *daf-40* downstream of *daf-16* and *daf-5,* and upstream of *daf-12* (N=3, mean ± SEM), similar to other genes involved in DA production. **(c)** qRT-PCR analysis of DAF-12 target genes *mir-84* and *mir-241* reveals reduced expression in *daf-40* mutants (at L3 stage, at 20°C) (N=6, ***P*<0.001, ****P*<0.0001), in accord with decreased DAF-12 ligand production and DAF-12 activation. **(d)** Loss of *daf-40* leads to elevated expression of DAF-9::GFP in the hypodermis of L3 stage animals, (shown quantitatively on the right), consistent with a role of *daf*-40 in influencing DA production and/or homeostasis through a feedback mechanism (N=3, mean± SEM, **P*<0.05). Data courtesy, J. Wollam, Antebi lab.

Further, the Antebi lab established transgenic worms expressing *daf-40::gfp* driven by its endogenous promoter from extrachromosomal arrays **(Figure 1.2b)**. This construct was functional, since it rescued *daf-40(hd100)* Daf-c phenotypes. DAF-40::GFP resided in several different tissues, including epidermal seam cells, muscle, posterior pharynx, intestine, and several unidentified neurons. Notably, DAF-40::GFP expression partly overlaps with DAF-36/Rieske (*e.g.* intestine, seam) and DHS-16/SDR (*e.g.* pharynx), but not with DAF-9/CYP27A1. Thus, hormone biosynthesis occurs in both overlapping and non-overlapping tissues, suggesting cell non-autonomous pathways. Further, analysis of *daf-40::gfp* expression in dauer signaling mutants revealed regulation by IIS, TGF-β, and steroid hormone signaling pathways similar to those made for other components of DA biosynthesis[12, 13].

DAF-12 acts to modulate lifespan through both the control of the dauer decision and in response to signals from the gonad. Animals lacking germline stem cells live 50-60% longer than wild type animals, termed the gonadal longevity pathway[17, 18]. Prolonged lifespan depends on the presence of the somatic gonad, which is thought to be a source of DA. Indeed, several DA biosynthetic genes including *daf-36*/*Rieske*, *dhs-16*/SDR, and *daf-9*/CYP450, as well as the transcription factors *daf-12*/FXR and *daf-16/*FOXO are required for longevity when germline precursors are removed[17-19] . Additionally, DAF-16::GFP localizes to intestinal nuclei in response to germline ablation, in a manner dependent upon DA signaling[8]. *daf-36* expression as well as Δ^7-DA levels become elevated in germline-less mutant animals[13]. DAF-12 ligands thus promote increased lifespan in the gonadal longevity pathway. The Antebi lab observed that longevity was significantly reduced in *daf-40* germline-less animals compared to wild type, but not abolished,

as *daf-40* germline-less animals still lived longer than mock-ablated *daf-40* animals. Together these suggest the partial requirement of *daf-40* in gonadal longevity.

The production of DAs is thought to occur through at least two pathway branches, both starting from dietary cholesterol. In one pathway, the DAF-12 ligands are likely produced via 7-dehydrocholesterol, lathosterol, and lathosterone and then onto the final products via DAF-9, which introduces the carboxy moiety[6, 11]. In a second, parallel pathway, the Δ^7-double bond is likely introduced by the dehydrogenase HSD-1[20] . To understand where daf-40 acts in DA biosynthesis, the Antebi lab performed sterol supplementation experiments, assaying phenotypic rescue with predicted intermediates in the pathway. Compounds lying downstream or parallel to the daf-40 biosynthetic block should rescue, whereas those lying upstream should not, a strategy used successfully to place *daf-36*, *dhs-16* and *daf-9* in DA pathways. Surprisingly, all compounds partially rescued daf-40 Daf-c phenotypes, although only the DAs themselves fully rescued **(Figure 1.4).** Similarly, sterol rescue experiments carried out using double mutants with *daf-36*, *dhs-16*, and *daf-9* showed the same rescue pattern as the single mutant, and not that of *daf-40*. This spectrum of graded rescue by all known sterols differs significantly from most other known DA biosynthetic mutants, suggesting a role of daf-40 either upstream or parallel to known components, in a novel position or pathway.

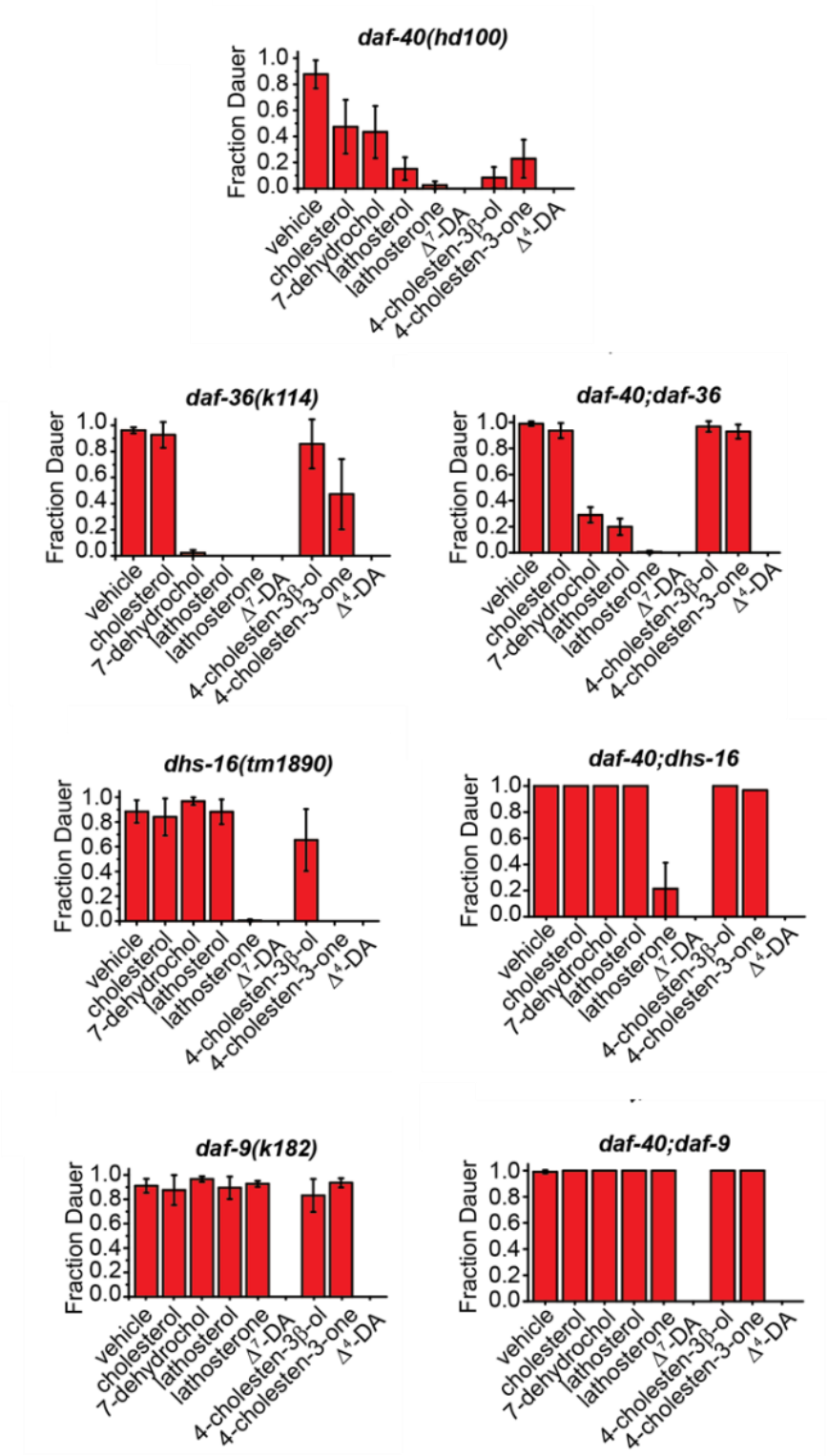

Figure 1.4: Sterol supplementation reveals a novel role of *daf-40* in DA biosynthetic pathway. Rescue of *daf-40(hd100)* Daf-c mutant phenotype at 27°C with 33μM of sterols. All compounds tested partially rescue *daf-40*, although only the known DAs completely rescue. Data courtesy, J. Wollam, Antebi lab.

To better understand *daf-40*'s role in DA production the Antebi lab performed dose response experiments for rescue of Daf-c phenotypes. Intriguingly, despite its less severe Daf-c phenotypes, *daf-40* mutants required 5-10-fold higher concentrations of Δ^7 for rescue compared to *daf-9* null animals. Moreover, daf-*40;daf-9* double mutants were even less sensitive than either single mutant, suggesting the genes could work in parallel pathways. Dose response experiments with the other two DAs gave similar results **(Figure 1.5).** By contrast, *daf-36, daf-9*, and *daf-36;daf-9* double mutants yielded identical dose response curves to Δ^7 DA, supporting the idea that they work in the same pathway. As *daf-40* expression is downregulated by reduced insulin and TGF-β signaling, we wondered whether mutations in these pathways display a similar loss of sensitivity to DAs. Indeed, they found that mutations in daf-2/insulin receptor and daf-7/TGF-β were also less responsive to rescue by Δ^7-DA. However, *daf-40;daf-2* double mutants required even higher concentrations than either single mutant, suggesting they work independently. Thus, loss of daf-40 or reduction of IIS and TGF-β signaling raise the threshold of DA required for bypassing dauer and activating DAF-12/FXR activity.

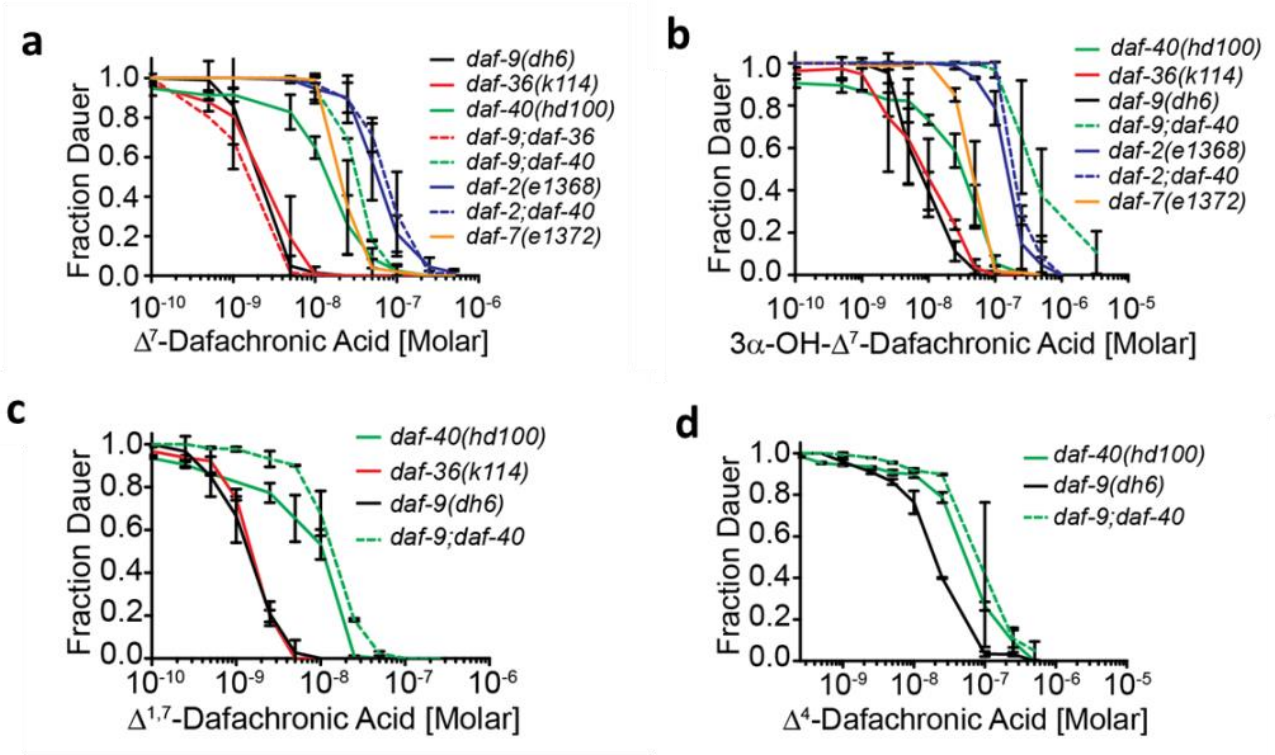

Figure 1.5: *daf-40* mutants are less responsive to DA rescue than *daf-9.* (a) The less severe Daf-c phenotype, *daf-40(hd100)* require 5-10 fold higher concentrations of Δ^7-dafachronic acid (Δ^7-DA) to rescue dauer formation at 27°C compared to *daf-9(dh6)* mutants. *daf-9;daf-40* are even less responsive than either single mutants. *daf-36(k114)* and *daf-36;daf-9* double mutants are similar to *daf-9* single mutants, consistent with *daf-36* acting upstream of *daf-9.* (N=3, mean ± SEM). **(b)** 3α-hydroxy-Δ^7-dafachronic acid shows slightly reduced ability to rescue *daf-9* and *daf-40* dauers as concentrations higher than those of Δ^7-DA are required for dauer rescue, and *daf-9;daf-40* double mutants show an even pronounced response. **(c, d)** Dose response experiments with $\Delta^{1,7}$- and Δ^4 - dafachronic acid show a trend similar to Δ^7-dafachronic acid, as higher concentrations are required to rescue *daf-40(hd100)* dauer formation at 27°C compared to *daf-9(dh6)* mutants. Data courtesy, J. Wollam, Antebi lab.

To understand how loss of *daf-40* affects known sterol intermediates in the DA pathways, we examined their sterol profile by GC/MS/MS. Surprisingly little or no difference was seen in levels of known precursors of Δ^7-DA synthesis, including cholesterol, 7-dehydrocholesterol, lathosterol or lathosterone **(Figure 1.6a)**. Next, we used high-resolution LC/MS to quantify DA levels in *daf-*

40 mutants. Interestingly, Δ^7 – and 3α-hydroxy-Δ^7-DA levels were slightly higher in *daf-40(hd100)* than in wildtype **(Figure 1.6b)**. Both Δ^7 – and 3α-hydroxy-Δ^7-DA levels returned to almost wildtype in animals expressing an integrated *dhIs839(daf-40::gfp)* overexpression construct **(Figure 1.6c).** If the known DAs were direct enzymatic products of DAF-40, we would expect to see reduced levels of these DAs. Instead, the increased levels of known DAs in *daf-40* mutant suggest that these DAs represent shunt metabolites that build up in the absence of *daf-40.* It is important to note that, our steroid extraction and LC-MS based detection protocol did not allow reproducible measurement of $\Delta^{1,7}$DA, likely due to the compound's susceptibility to nucleophilic attack.

Given that the levels of measured DA precursors remained unchanged, but DAs accumulate in *daf-40* mutants we hypothesized that *daf-40* acts on DAs (the products of *daf-9*) in a novel enzymatic route. To determine the biochemical activity of DAF-40, we expressed the gene in HEK-293T cells and assayed activities from isolated microsomes. Indeed, in we observed that DAF-40 could interconvert multiple substrates including Δ^7 , 3α-hydroxy-Δ^7 , and $\Delta^{1,7}$ DA **(Figure 1.7)**. Particularly, using 3α-OH-Δ^7 as a substrate, we observed the DAF-40 dependent conversion to Δ^7, $\Delta^{1,7}$ as well 3α-OH-$\Delta^{1,7}$ as verified with corresponding synthetic standards. Additionally, we observed that 3β-hydroxy-$\Delta^{1,7}$ remained largely unreacted, indicating that DAF-40 acts in a highly substrate-specific manner. Assays using $\Delta^{1,4,7}$ DA did not result in any detectable product and unreacted $\Delta^{1,4,7}$ could not be recovered from the assay, likely due its high susceptibility to nucleophilic attack. Together, these results confirm that DAF-40 acts as a DA-oxidoreductase that interconverts the hydroxy- and keto-versions of the known DAs and can introduce additional unsaturation in the A ring.

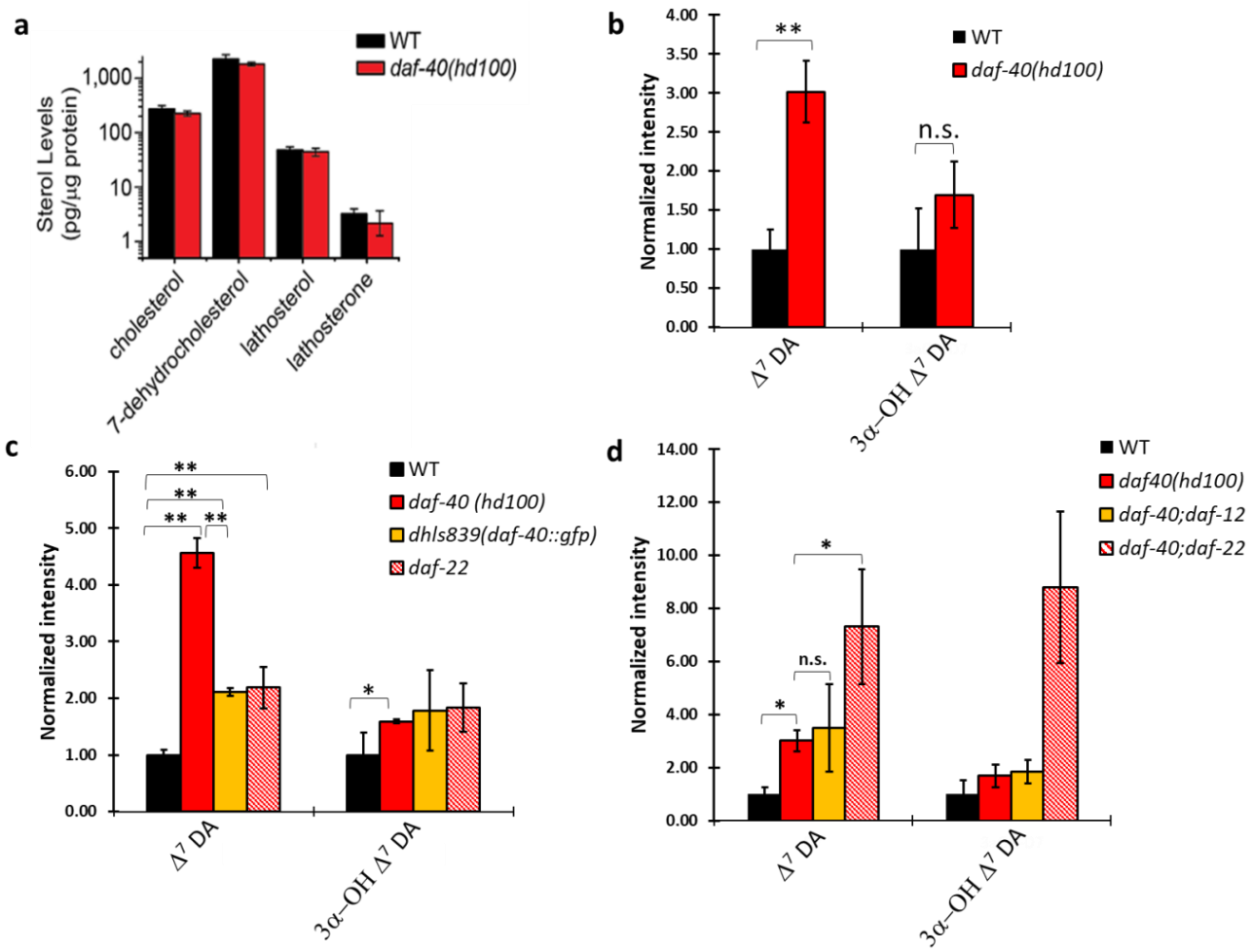

Figure 1.6 : Mass spectrometry-based analysis suggests dafachronic acids accumulate as shunt metabolites in *daf-40* mutants (a) GC/MS/MS sterol analysis reveals that levels of known DA precursors remain largely unchanged in *daf-40(hd100)* compared to N2 worms. **(b)** High resolution LC-MS analysis reveals a significant increase in the levels of Δ^7- and 3α-hydroxy-Δ^7-dafachronic acid in *daf-40(hd100)* worms (N=3 ± SEM). **(c)** Animals expressing an integrated *dhIs839(daf-40::gfp)* overexpression construct show reduced levels of Δ^7-dafachronic acid compared to *daf-40* mutants (N=2 ± SEM). **(d)** *daf-40;daf-22* double mutants show higher DA production than either single mutant. *daf-12* has no significant effect of DA levels. (N=3 ± SEM).

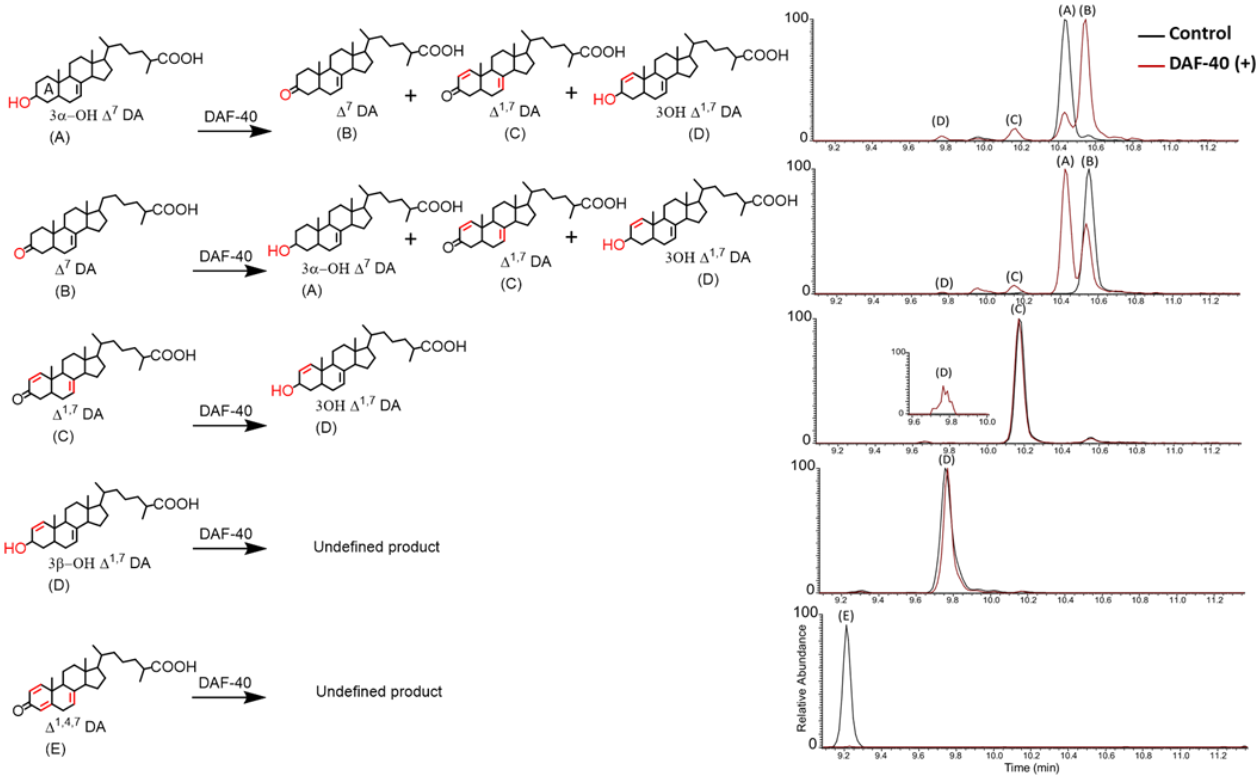

Figure 1.7: DAF-40 acts as DA oxidoreductase. DAF-40 mediated enzymatic functions (on the left), and corresponding LC/MS chromatograms (on the right) of microsome extracts expressing DAF-40 (DAF-40(+), red traces) or controls (no microsomes, black traces) incubated with different DAs as substrates. We note that that 3β-hydroxy-$\Delta^{1,7}$ remained largely unreacted and using $\Delta^{1,4,7}$ DA as substrate (E) did not result in any detectable product and despite intense efforts unreacted $\Delta^{1,4,7}$ could not be recovered from the assay, likely due its high susceptibility to nucleophilic attack.

To determine whether hydroxylated and/or more highly unsaturated DAs (such as 3-hydroxy-$\Delta^{1,7}$ DA) could be the true products of DAF-40 and hence potent DAF-12 ligands, the Antebi lab performed *daf-40(hd100)* dauer rescue experiments with synthetic 3-hydroxy-$\Delta^{1,7}$ DA. Indeed, we observed that 3-hydroxy-$\Delta^{1,7}$ DA rescued *daf-40* dauers much more effectively than any of the other DAs **(Figure 1.8a)**. Correspondingly, we observed that these DA-derivatives, particularly $\Delta^{1,4,7}$, 3-hydroxy-$\Delta^{1,7}$ DA and $\Delta^{1,7}$ DA strongly activated DAF-12 in cell culture based luciferase

assays **(Figure 1.8b)**. We therefore conclude that 3-hydroxy-$\Delta^{1,7}$ DA, and similar unsaturated DA-derivates are the true enzymatic products of DAF-12 and act as potent DAF-12 ligands.

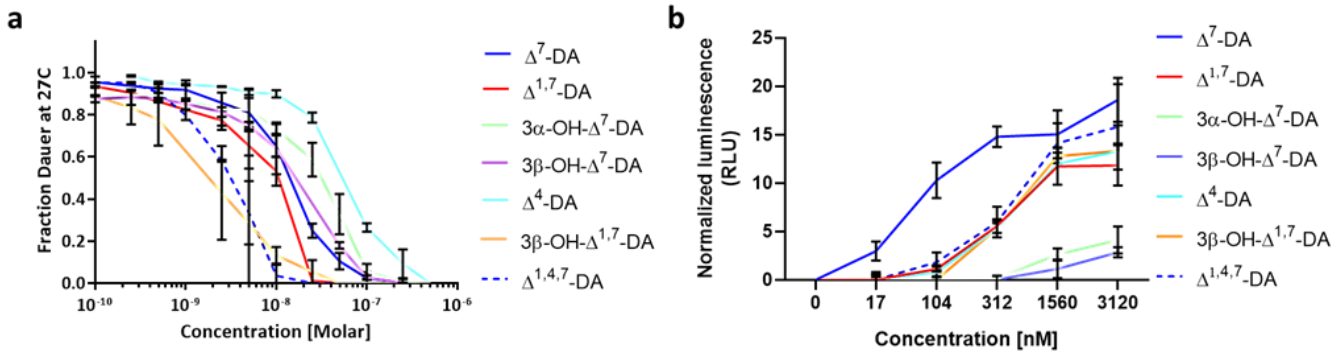

Figure 1.8: Highly unsaturated DA derivatives rescue *daf-40(hd100)* dauers with high potency. **(a)** Dose response curves of dauer rescue on *daf-40(hd100),* using synthetic unsaturated DA-derivatives show more potent rescue by these DA-derivatives than by known Δ^7, $\Delta^{1,7}$ and 3α-hydroxy-Δ^7 DA. Data coutesey J. Wollam, Antebi lab. **(b)** Cell culture based luciferase assays show DAF-12 activation by DAs and DA-derivatives. N=3 ± S.D.

DISCUSSION

Lipophilic hormones working through nuclear receptors control multiple aspects of metazoan biology including metabolism, reproduction and longevity. The production and regulation of structurally distinct variants presents a unique way to fine-tune receptor-mediated functions and alter physiological outputs. Utilizing a multidisciplinary approach, here we characterized the cytochrome P450 DAF-40 as a novel node of DAF-12 ligand biosynthesis that ultimately influences development and lifespan, while revealing the complexity of nematode bile acid metabolism. Several lines of evidence indicate that *daf-40* affects DA synthesis, and thereby DAF-12 activity and lifespan. Mutant animals displayed phenotypes characteristic of DA deficiency including Daf-c and gonadal Mig phenotypes, altered regulation of hypodermal *daf-9* expression, and reduced

lifespan extension in the absence of the germline. In addition, genetic epistasis experiments placed *daf-40* at a position consistent with DA pathways, acting downstream of insulin/IGF-I and TGF-β signaling components and upstream of receptor *daf-12*. We found *daf-40::gfp* expression to be regulated by components of both upstream pathways as well as *daf-12*, similar to other DA components. Importantly, the phenotypes of *daf-40* mutant animals were completely rescued by DAs. The known DAF-12 ligands, but at significantly higher concentrations relative to previously studied mutants of the DA pathway.

Although clearly involved in modulating DAF-12 activity, *daf-40* exhibits several unique features suggestive of a novel role in the previously proposed DA biosynthetic pathway. In contrast to most genes involved in DA production, *daf-40* mutant phenotypes were partially rescued by all known and proposed DA precursors, including cholesterol. In this regard it does not easily fit into the predicted model of DA synthesis **(Figure 1.1)** and suggests a novel role. Interestingly, *nhr-8* mutants have a similar behavior but give only weak synergy with *daf-40*, suggesting possible overlapping pathways. By contrast, mutations with other DA pathway components greatly enhanced *daf-40* phenotypes but were epistatic regarding rescue by DA precursors. Remarkably, we also saw that *daf-40* null animals were substantially less responsive to all known DAF-12 ligands than *daf-9* mutants. *daf-40;daf-9* double mutants were even less responsive than either single mutant, suggesting a synergy in role of *daf-40* in biosynthesis or regulatory mechanisms.

The other striking finding that *daf-40* loss altered the dose response curve to DAF-12 ligands, suggesting its absence resets the threshold required to bypass dauer and promote reproductive development, increasing the amount by 5-10 fold. Reduced IIS or TGF-β signaling also shifted this threshold, and reduced IIS decreased *daf-40::gfp* expression. That environmental and signaling

inputs shift thresholds for reproductive commitments could provide an important response mechanism lending plasticity to metazoan maturation.

However, it was unclear how *daf-40* resets this threshold, but several scenarios could be proposed: *daf-40* might influence sterol uptake or transport, alter nuclear co-regulator levels or activity, produce other unidentified and functionally distinct DAF-12 ligands, or affect metabolism of a DA inhibitor. Perturbation of sterol transport seems unlikely given that steady state levels of cholesterol and most other DA precursors were unchanged in *daf-40* mutants. Nor does mutation of daf-16/FOXO or daf-5/SNO-SKI transcription factors alter the dose response of *daf-40*. What appears most likely is that *daf-40* provides an alternate route of DAF-12 ligand production **(Figure 1.9)**.

Our findings strongly support this latter model. First, *daf-40* mutants showed no changes in the abundances of early precursors in the DA biosynthetic pathway, whereas Δ^7 and 3α–hydroxy-Δ^7 DA levels were higher than in wildtype. Additionally, these elevated DA levels returned to wildtype in an integrated overexpression strain. This together suggested that increased DA levels could represent shunt metabolites that build up in *daf-40* mutants due to lack of DAF-40 mediated conversion into the "real", more potent ligand. Indeed, in *in-vitro* assays we found that DAF-40 acts as a DA-oxidoreductase. We demonstrated that DAF-40 perform redox chemistry specifically on the A ring of cholesterol, and accordingly can introduce unsaturations and hydroxylations to producing additional DA-variants. Significantly, we observed the DAF-40 dependent production of 3-hydroxy-$\Delta^{1,7}$ DA from both Δ^7 DA and 3-hydroxy-Δ^7 DA. 3α–hydroxy-$\Delta^{1,7}$ DA rescues *daf-40* dauers much more effectively than Δ^7 DA, -$\Delta^{1,7}$ DA, or 3-hydroxy-Δ^7 DA.

This demonstrates that DAF-40 functions in late-stage modification DAs into more active derivatives and that *daf-9* is not the last step in DAF-12 ligand synthesis as previously believed.

The unstable nature of the DA-derivatives that most effectively rescue *daf-40* mutants - 3α–hydroxy-$\Delta^{1,7}$ DA and $\Delta^{1,4,7}$ DA has important implications for the role of DAF-40 in DA homeostasis. After validating the capability of our LC-MS based method to detect $\Delta^{1,7}$ DA, we observed that $\Delta^{1,7}$ DA and more so $\Delta^{1,4,7}$ DA do not survive our extraction conditions and hence could not be detected in our worm samples due to decomposition, either during extraction or during LC-MS. Correspondingly, untargeted metabolome analysis of *daf-40* mutants did not pick up any significantly absent or downregulated metabolites. However, our results strongly suggest that metabolites such as 3α–hydroxy-$\Delta^{1,7}$ DA and other possible highly oxidized and thus unstable DA-derivatives represent the products of DAF-40 and thus the most potent endogenous ligands of DAF-12.

It seems likely that the DAF-12 ligand's chemical lability serves a biological purpose: their transient nature exists to ensure rapid on/off switching and fine tuning of receptor-mediated expression profiles. Additionally, it is possible that ligands with different half-lives are produced in a tissue- and/or developmental-stage specific manner. While *daf-36* is expressed in the intestines[13], *dhs-16* in the hypodermis[12] and *daf-9* in both hypodermis and XXX cells[7], which indicates that DA biosynthesis proceeds in a distributed and cell-non-autonomous manner.

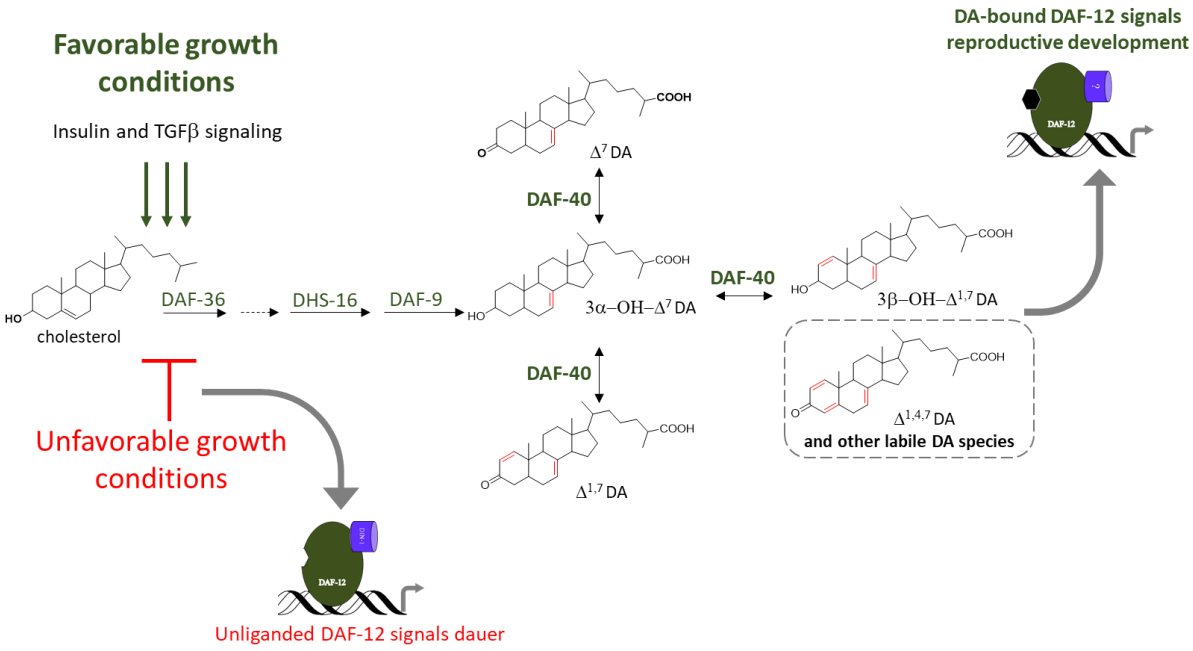

Figure 1.9: Model representing DAF-40 dependent synthesis of a complex pool of labile, yet potent DAF-12 ligands. While the roles of DAF-36, HSD-16 and DAF-9 towards dafachronic acid (DA) biosynthesis has previously been studied, this study explores the role of DAF-40 as a DA oxidoreductase representing a novel node in developmental regulation. DAF-40 uses known DAs as substrates in the synthesis of highly labile, functionalized DA-derivatives that act as potent DAF-12 ligands that together mediate reproductive development.

Conceivably, different cocktails of DA-like molecules could elicit differential activities, much as mixtures of various ascarosides do [21]. Our characterization of the ability of several novel DAs to rescue the Daf-c has not yet uncovered strict functional differences, although differences in potency between the ligands is apparent. Further investigations into gene expression profiles of animals after treatment with different DAs should provide insight into functional differences, if they exist. But acquiring a better understanding of how these multiple DAs are synthesized is a start to reveal such regulation.

Small molecules are principle mediators of essential processes across phyla, including signal transduction and metabolism, as well as chemical attraction and defense. This study provides a

glimpse of the utility of multidimensional approaches, combining genetic and metabolomic techniques to dissect small molecule signaling pathways. Moving forward, comprehensive analysis of the steroid metabolome of worms and other organisms will be essential to unravel novel signaling activities. Given the conserved nature of metabolic pathways and metabolites, it seems conceivable that currently known mammalian receptor ligands too have yet unknown derivatives with higher potency or specific functions.

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CHAPTER 2

NUCLEAR RECEPTOR MEDIATED METABOLIC AND REGULATORY NETWORK IN NEMATODES

ABSTRACT

Nuclear receptors (NRs) are evolutionarily conserved, ligand-modulated transcription factors that interact with coregulatory proteins to control development and metabolism. In *C. elegans*, the vitamin-D and liver-X receptor homolog DAF-12 functions as the central switch to mediate both larval development and adult lifespan. In our current understanding of DAF-12 function, the interaction of DAF-12 with its co-repressor DIN-1 promotes entry into the developmentally arrested dauer state while endogenous steroidal ligands of DAF-12, the dafachronic acids (DAs), promote reproductive development and longevity via members of the *let-7* microRNA family. But recent work from our lab and others have indicated intersecting roles of DAF-12 with its *C. elegans* paralog receptors - NHR-8 (in mediating population-density dependent acceleration of development, along with DAs) and NHR-48 (in developmental acceleration of hermaphrodites in response to male-secretory signals, along with DIN-1). However, the mechanisms by which these receptors regulate different phenotypes remain unclear.

In this study, through a combination of cell-culture based NR activation assays and *in vitro* protein interactions we first demonstrate that the binding of DAs to DAF-12 prevents association with corepressor DIN-1 but does not induce dissociation of already formed DAF-12-DIN-1 complex. These findings validate how DA ligands orchestrate the ordered recruitment of coregulatory complexes to mediate receptor function. We further show that DIN-1 acts as a shared

corepressor between DAF-12, NHR-8 and NHR-48 via its conserved interaction domain. Additionally, we demonstrate that DAF-12 forms homodimers, and heterodimers with NHR-8 and NHR-48 via its DNA-binding domain, in a DA-independent manner. Through an untargeted LC-MS-based analysis, we correspondingly reveal co-regulation of a subset of lipids by these receptors. Together based on the findings of this study, we propose a model in which DNA-guided NR dimerization and interactions with ligands and different coregulators, resulting in different transcriptional outcomes that underlie observed NR-dependent phenotypes in *C. elegans*.

INTRODUCTION

Nuclear receptors (NRs) are members of the largest superfamily of evolutionarily conserved ligand-modulated transcription factors[1, 2]. Sequence homology and phylogenetic studies have identified 48 human NRs and as many as 284 in the nematode *C. elegans* that regulate critical processes including cell proliferation, differentiation, development, homeostasis, and metabolism[3]. In response to endocrine and dietary signals, NRs mediate diverse metabolic pathways and often the same NR under different cellular and environmental conditions can target multiple downstream effectors. Such complex NR signaling systems create a deeply interconnected metabolic network that enables the organism to modulate and fine-tune its response to the constantly changing stimuli.

Owing to its short life span, genetic tractability, and well-characterized genome, the nematode *C. elegans* represents an excellent model organism to study conserved signaling mechanisms of gene regulation and function[4]. Despite being small enough for high throughput operations, *C. elegans* is highly differentiated. Being a eukaryote, many physiologically relevant cellular and molecular pathways in *C. elegans* are homologous to those in higher animals (including humans), making it a prime model for the study of NR biology, especially for those pathways pertaining to growth, metabolism, and ultimately lifespan. Notably, most of the research aimed at understanding adult lifespan and aging has focused on a unique feature of the nematode development plasticity between reproductive growth and the 'dauer' stage[5-9]. Under unfavorable growth conditions (such as inadequate food supply or temperature), *C. elegans* enter into a non-feeding, non-reproductive and developmentally arrested state, called dauer diapause, which

enables the worm to survive for several months, as opposed to its normal lifespan of 2-3 weeks. Genetic screens on dauer-defective and dauer-constitutive mutants have identified about three dozen DAF (DAuer Formation) genes that directly control dauer formation and, subsequent studies have revealed a significant role of these DAF genes in the overall regulation of lifespan and development [10]. Interestingly, conserved orthologs of *C. elegans* DAF genes, particularly those within the NR family of proteins, such as PPARs and ERs, appear to regulate metabolism and aging also in higher organisms[11, 12].

In *C. elegans*, the most well studied NR that modulates development and dauer is DAF-12. DAF-12 signaling also influences fat metabolism, developmental timing, and adult longevity[13-17]. DAF-12 is the *C. elegans* homolog of vertebrate vitamin D and liver-X receptors[18]. A body of work on the dauer pathway reveals DAF-12 as the central, ligand-dependent switch between adult lifespan and larval development in *C. elegans* (**Figure 2.1)**[16, 18-21]. In a simplified model, favorable growth and environmental cues (such as abundant food supply and growth temperatures) are perceived by chemosensory neurons that activate the insulin/IGF-1 (*daf-2*) and TGFb(*daf*-7) signaling pathways in the worm that then converge on a set of biosynthetic enzymes that synthesize dafachronic acids (DAs), the bile acid-like endogenous ligands of DAF-12[19, 22-24]. Under unfavorable conditions however, repression of insulin-like and TGF-β signaling results in repression of the DA biosynthetic pathway, and the unliganded DAF-12 promotes dauer[25]. Thus, DAF-12 with its associated nodes, works as ligand-regulated molecular switch to specify two different life history traits, reproductive development vs. the dauer diapause.

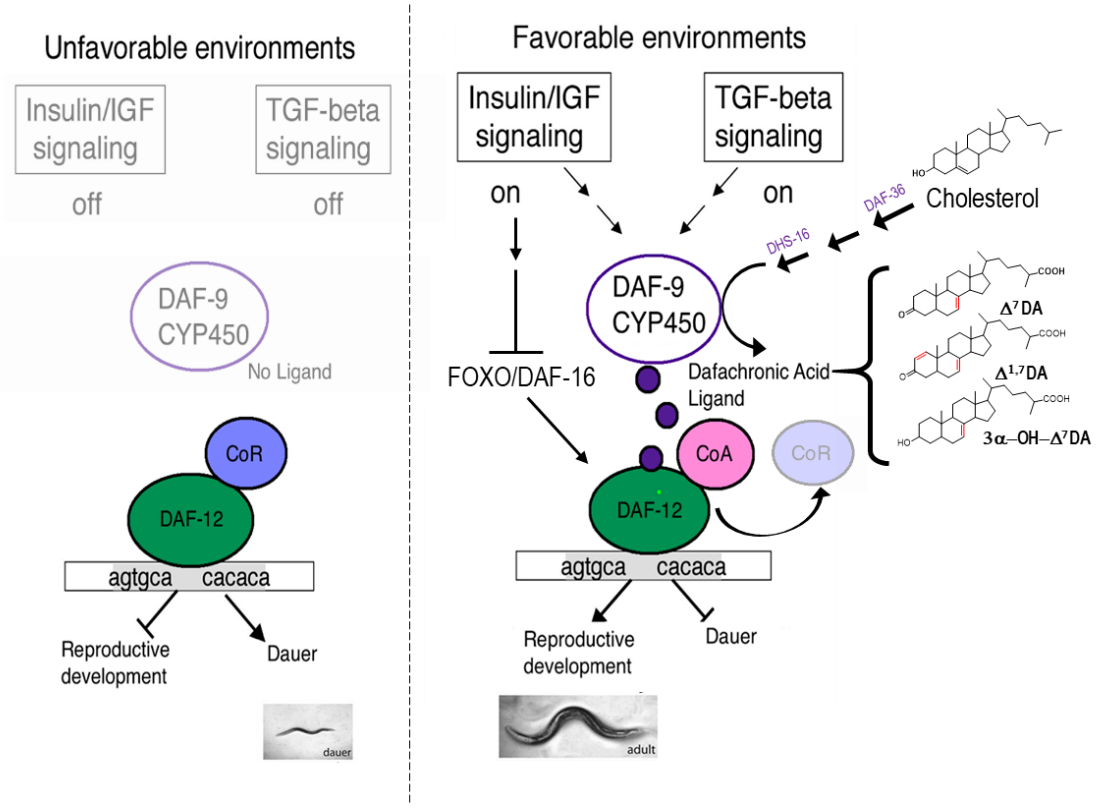

Figure 2.1: Dauer vs reproductive development regulatory network. In favorable environments, Insulin/IGF-I and TGF-beta peptide signal converge on the nuclear receptor branch of the dauer pathways. Dietary cholesterol is used as a precursor by steroid ligand biosynthetic enzymes (including DAF-36, DHS-16-1 and DAF-9), triggering synthesis of dafachronic acid (DA) ligands. In the presence of DAs, NR/DAF-12 assembles the presumed coactivator complex that guides expression of genes involved in reproductive development, fat metabolism, and accelerated aging. In unfavorable environments, DA biosynthesis is suppressed. Unliganded DAF-12 then binds to its corepressor DIN-1, promoting gene expression patterns associated with dauer diapause, delayed development, fat storage, and retarded aging. Adapted from A. Antebi, Wormbook.

DAF-12 is believed to be localized constitutively in the nucleus and bears high sequence similarities to mammalian NRs at both the DNA and ligand binding domains **(Figure 2.2)**[2]. The N-terminal half of DAF-12 contains the DNA binding domain (DBD) comprising of two Cys4 zinc finger motifs that bind specific DNA response elements (REs) located in the regulatory regions of its target genes[18-20]. DAF-12 control via these REs is exerted by its miRNA targets. Three miRNA

promoters of the conserved *let-7* family – *mir241p, mir84p* and *mir48p* serve as targets of ligand activated DAF-12[13]. Detailed analysis of DAF-12 REs reveal that the RE sequences for maximum DAF-12 transcriptional activation contain at least two 'half-sites' that together make a complete response element site **(Figure 2.2)[26].** Each DAF-12 half-site is a hexamer, exactly or closely related to the sequence AGTTCA or AGTGCA. Interestingly, this feature of multiple half site REs is conserved with mammalian VDR (Vitamin D receptor) and LXR (Liver X receptor) REs[27-29]. Mutation(s) within the half site sequence or alterations in the spacing between two half-sites greatly affects DAF-12 binding and activity, demonstrating the importance of DNA binding and RE-specify in DAF-12 activity[26]. The significance of the RE half-sites is also illustrated by analyses of the ligand mediated dimerization of mammalian VDR[30-32]. VDR is a monomer in solution, however its binding to specific REs leads to the formation of homodimers. Additionally, the presence of its ligand, vitamin D3, shifts the DNA-bound VDR homodimer towards a VDR-RXR (retinoid X receptor) heterodimer, such that each receptor monomer now occupies a single half site and binds to its respective ligand. This example of VDR – RXR heterodimerization provides a classical example for how a NR can bind to DNA constitutively in the absence of a ligand as well as regulate transcription in a fully ligand-dependent manner via co-operative binding to DNA. Correspondingly, the homology of DAF-12 (and its RE elements) to mammalian NRs has always suggested that DAF-12 may act as a homo- or heterodimer[26].

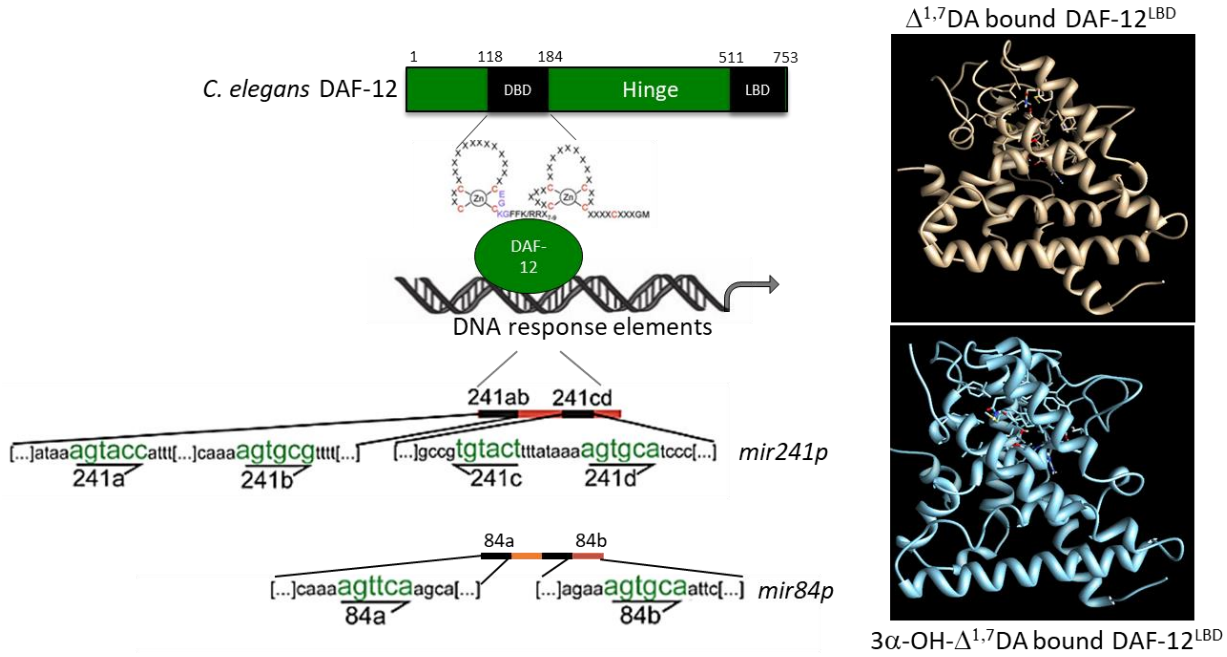

Figure 2.2: *C. elegans* DAF-12 nuclear receptor architecture. The *N*-terminal half of DAF-12 contains the DNA binding domain (DBD) comprising of two Cys4 zinc finger motifs that bind specific *miR* DNA response elements, shown here *mir241p* and *mir84p* (structure courtesy A. Bethke, *Science* 2009) . The *C*-terminal half of DAF-12 contains the dafachronic acid ligand binding domain (LBD). Shown in the insert is modeled *ce*DAF-12 LBD bound to either $\Delta^{1.7}$ DA or 3α-OH-Δ^{7} DA each of which significantly alter the domain structure. Modeling is based on crystal structure of *Strongyloides stercoralis* DAF-12 LBD, by Joshua Judkins, Schroeder lab.

While the *N* terminal DBD controls DNA binding specificity, the core of NR signaling "intelligence" lies in the *C*-terminal half which is the ligand binding domain (LBD) **(Figure 2.2)**[33, 34]. The LBD consists of a series of 12 alpha helices that create a hydrophobic pocket that can sequester a hydrophobic, small molecule ligand. In addition, it features conserved trans-activation domains to interact with coregulator proteins. Playing a central role in DAF-12-dependnet dauer formation, DIN-1 was characterized as a corepressor of DAF-12 that bears high homology to the SHARP family of mammalian corepressors[25]. Although DIN-1 exists in two isoforms, only DIN-1s (the short isoform, also known as DIN-1e) is known to interact with DAF-12. The interaction

between DIN-1 and DAF-12 occurs between the conserved SID/RD (repressor domain) at the C terminal end of DIN-1 to the hydrophobic alpha-helices in the LBD of DAF-12 and is DA dependent. And hence, under unfavorable environmental conditions, ligand production is ceased, leaving the DAF-12 unliganded to complex with its corepressor DIN-1 to specify programs of dauer diapause and delayed development[25]. However, under favorable conditions, when DA ligand biosynthesis is switched on, DAF-12 dissociates from its co-repressor DIN-1 and likely assembles a coactivator complex that directs genes expression towards reproductive development aging by recruiting chromatin modifying machinery to favor downstream gene transcription towards development. **(Figure 2.1)**. Although coactivators for DAF-12 have not been identified, the mammalian coactivator SRC-1 (a member of the bHLH-PAS family) binds and activates DAF-12 through a conserved LxxLL recognition motif[21]. It remains unclear whether in the absence of a ligand, the DIN-1-DAF-12 complex represses reproductive genes, activates dauer-specific genes or does both. Taken together, our current understanding of DAF-12 function to some extent explains the diversity of *daf-12* mutant phenotypes: DBD-mutants fail to form dauer larvae (dauer defective), whereas some LBD mutants are constitutively dauer active (dauer constitutive)[20, 35].

The center of the DAF-12 functional diversity and complexity largely is dictated by ligand binding. Hence identifying the true, endogenous NR ligands is central to understanding NR biology. Using NMR-spectroscopy based metabolome analysis, the Schroeder lab previously identified three dafachronic acids - Δ^7, $\Delta^{1,7}$ and 3α-OH- Δ^7 DA were identified as *bona* fide endogenous ligands of DAF-12 that promoted recruitment of mammalian coactivator SRC-1 to DAF-12, rescued *daf-9*

dauers and promoted reproductive development (**Figure 2.1**)[15, 21]. This also unambiguously confirmed the absence of Δ^4 DA endogenously.

While in *C. elegans* biology DAF-12 has been shown to play a central role in orchestrating development and reproduction, recent studies from our and other labs have shed light on how the two DAF-12 paralogs in *C. elegans* - NHR-8 and NHR-48 - intersect with DAF-12. NHR-8 was initially characterized for its role in cholesterol and fatty acid homeostasis[36, 37]. NHR-8 also transcriptionally regulates *daf-36*, the first and rate-limiting enzyme in the DA biosynthesis pathway and hence can indirectly regulate DA biosynthesis[37]. Subsequent work elucidated a role of NHR-8 in dietary restriction (DR) mediated longevity[38]. This study reported an increase in DAF-9 activity and Δ^7 DA levels under DR. Under these conditions, while Δ^7 DA activity was independent of DAF-12, it required NHR-8 (and mTOR signaling). This study, however, did not provide direct any evidence towards direct binding or activation of NHR-8 by DAs but rather established how steroid signaling links into physiology and lifespan via several NHRs, via pathways that are at best partially understood.

More recently, work from the Schroeder lab has demonstrated that under high population density conditions, *C. elegans* hermaphrodites develop faster than isolated worms[39]. This phenotype, referred to as Pdda – population density-dependent acceleration of development - is induced by a yet unknown, endogenously produced, secretory signal (**Figure 2.3**). In a recent body of work (A. H. Ludewig, 2018, *submitted*), the Schroeder lab identified nacq#1 (an N-acyl glutamine) as a male-enriched metabolite involved Pdda and in lifespan shortening of hermaphrodites. While this developmental acceleration by nacq#1 is dependent on *daf-12*, the phenotype was also abolished in *nhr*-8 and *nhr-48* mutants, suggesting it required all three NRs.

Additionally, nacq#1 also required DIN-1 and promoted dauer exit. While the role of DIN-1 – DAF-12 interaction has so far been known to function in dauer arrest, the requirement of DIN-1 in nacq#1 mediated dauer exit may suggest additional roles for DIN-1.

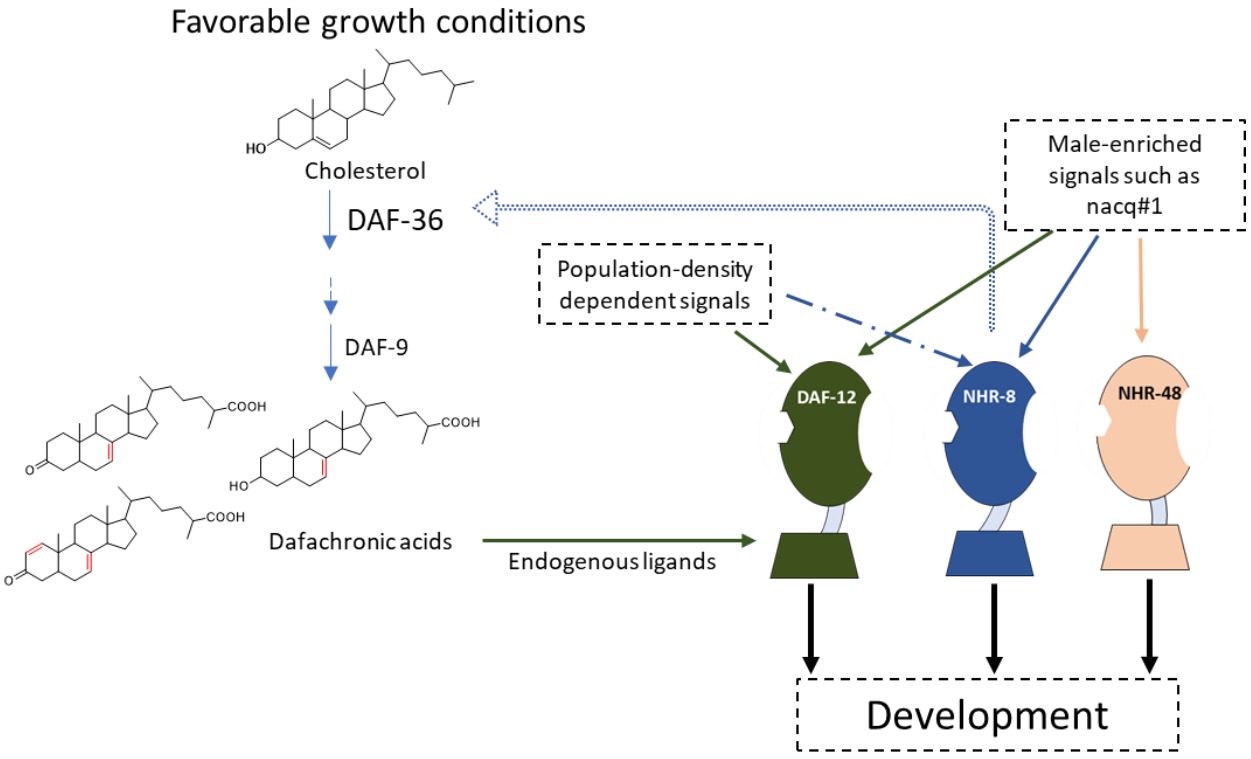

Figure 2.3: Deeply interconnected *C. elegans* developmental network regulated by receptors DAF-12, NHR-8 and NHR-48. Several distinct small molecule signal – receptor interactions regulate reproductive development. Dafachronic acids (DAs) produced from cholesterol under favorable growth conditions activate DAF-12. NHR-8 in turn promotes DA/DAF-12 signaling by regulating DAF-36 - the rate limiting enzyme of DA production. Under population density dependent conditions, developmental timing is controlled by NHR-8 and DAF-12, while developmental acceleration in response to male-enriched secretory signals requires NHR-8, DAF-12 and NHR-48.

On one hand as the complexity in NR-mediated signaling by DAF-12, NHR-8 and NHR-48 reveals itself, the duality of coregulator protein functions adds a second level of complexity. Our understanding on the role of NRs and coregulatory proteins is rapidly evolving. The studies

mentioned above collectively portrait how deeply interconnected the network of NRs is and how each component is required towards orchestrating nematode normophysiology. Developing hypotheses for explaining these complex NHR-dependent phenotypes could benefit from understanding how these receptors interact with each other and how they interact with metabolism. To answer these questions, the study presented in this chapter coupled *in vivo* NR activation assays with *in vitro* protein binding analyses to uncover the direct binding between key receptors of *C. elegans* development - DAF-12, NHR-8 and NHR-48, along with their shared interactions with corepressor DIN-1. Correspondingly, this study also demonstrates co-regulation of lipid metabolism by these NRs through an untargeted metabolomics approach. Together, this study builds a model in which DNA-guided NR dimerization mediates interactions with different ligands and coregulators, such that each assembly facilitates different transcriptional outcomes towards the different phenotypes observed.

RESULTS

Several studies from our lab and others have explored the intersecting roles of the DAs and receptors DAF-12, NHR-8 and NHR-48 towards *C. elegans* development. To determine whether DAs also activate NHR-8 and NHR-48, we used HEK-cell culture based luciferase assays (**Figure 2.4a**). Briefly, for the luciferase assays, HEK293T cells were co-transfected with *C. elegans* NR expression constructs of *daf-12*, *nhr-8* and/or *nhr-48*, miR promoter target of DAF-12 (*miR-241p* or *84p*) fused to a downstream luciferase gene and, in some cases, the *C. elegans* co-repressor *din-1e*. The assay detects the luminescence output from an active NR complex,

which in the case of DAF-12 relies on recruitment of the mammalian coactivator SRC-1 upon binding of DAF-12 to DA. All luminescence output is normalized to β-galactosidase activity that serves as a transfection control. As a validation that our assay works, we observe strong DAF-12 activation by 100 nM Δ^7 DA when compared to the ethanol negative control (**Figure 2.4b**). This activation in largely abolished in the presence of corepressor DIN-1e, indicating that, under the assay conditions, binding to DIN-1e prevents activation. Shown here are data using the *mir-241p* target of DAF-12, however, this DIN-1e mediated repression in DAF-12 activity was also observed for *mir-84p* as well as ligands $\Delta^{1,7}$ and 3α-OH Δ^7 DA. We also validated DAF-12 nuclear localization by western blotting (**Figure 2.5)**.

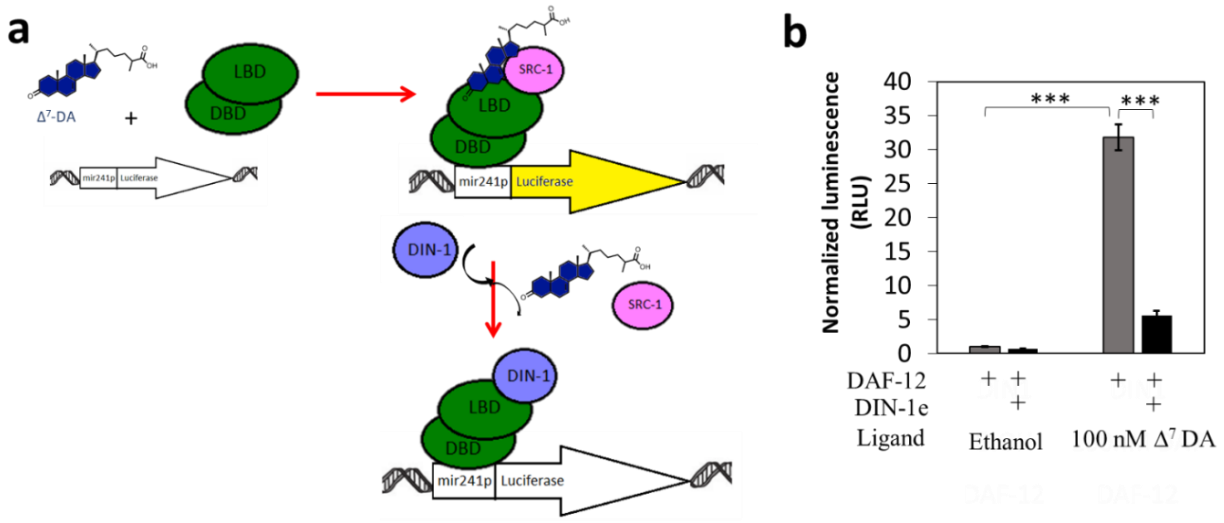

Figure 2.4: Cell culture based luciferase assays validate dafachronic acid - dependent DAF-12 activation. (**a**) Schematic of HEK293T – based luciferase assay for NR activity monitoring. (**b**) Δ^7 DA strongly induces DAF-12 activation (shown here for *mir-241p* target), which is largely abolished in the presence of corepressor DIN-1e. Luminescence is normalized to β-galactosidase activity and represented as normalized to negative control (DAF-12, no DIN-1e, no ligand). n = 4, ± S.D.,*** $p < 0.001$, Student's t-test.

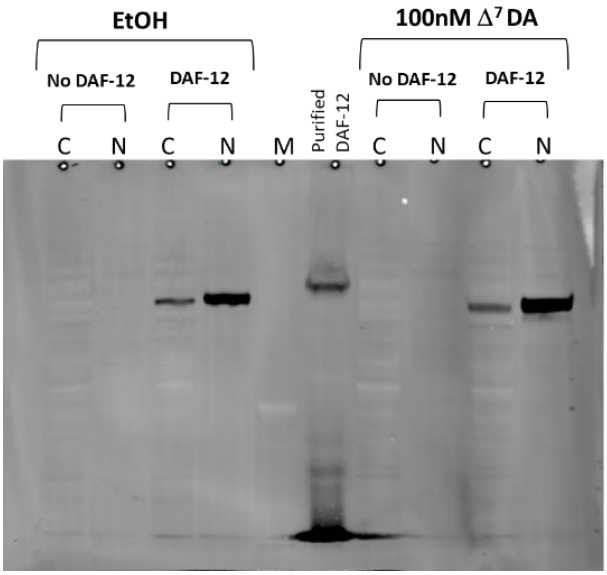

Figure 2.5: Dafachronic acid ligand-independent nuclear localization of DAF-12. *N*-terminal flag-tagged full-length DAF-12 was transiently expressed in HEK-293T cells, with either ethanol (EtOH, negative control) or 100 nM Δ^7 DA to assess nuclear vs cytoplasmic localization by α-flag western blotting. DAF-12 is enriched in the nucleus and this nuclear localization is Δ^7 DA-ligand independent.

We then asked if DAs induce activation of NHR-8 and NHR-48. At the outset, we assumed that mammalian SRC-1 could serve as a general coactivator for these NHRs (as it did to DAF-12[15]), and that NHR-8 and NHR-48 could bind to *mir-241p* and/or *mir-84p* owing to the high homology observed in their DNA-binding domains compared to DAF-12. However, when FL-NHR-8 was transfected with multiple *miR::luciferase* constructs (including *let-7p*), we saw no reproducible DA-mediated activation (**Figure 2.6a**). We then presumed that this lack of inactivation could be due to lack of NHR-8 DBD binding to DAF-12's *miR* promoter targets. We hence created a chimeric NHR-8 construct consisting of DAF-12 DBD$^{1-499}$ and the NHR-8$^{91-560}$ LBD (which includes the hinge, previously reported to play a role in the activity of several mammalian NRs) to ensure binding to DAF-12 *miRs*. While this construct stably expressed in cell culture **(Figure A2.1)**, DAs still did not

induce any activation, with several *miR* reporters tested, with and without DIN-1e (**Figure 2.6b**). We do however note that FL-NHR-8 showed higher background activation (without DA) with *miR-84p* and *miR-48p*, which was slightly reduced by 100 nM DAs. However, further analysis with other *C. elegans* and mammalian NRs indicate that this activation may be due to the promiscuous nature of the *miR-84p* and *miR-48p* RE sequence and is not NHR-8-specific. We observed similar lack of activation for full length NHR-48, as well as chimeric NHR-48 (consisting of DAF-12 DBD$^{1-499}$ and the NHR-48$^{163-817}$ LBD) with several *miR* reporters tested (not shown here).

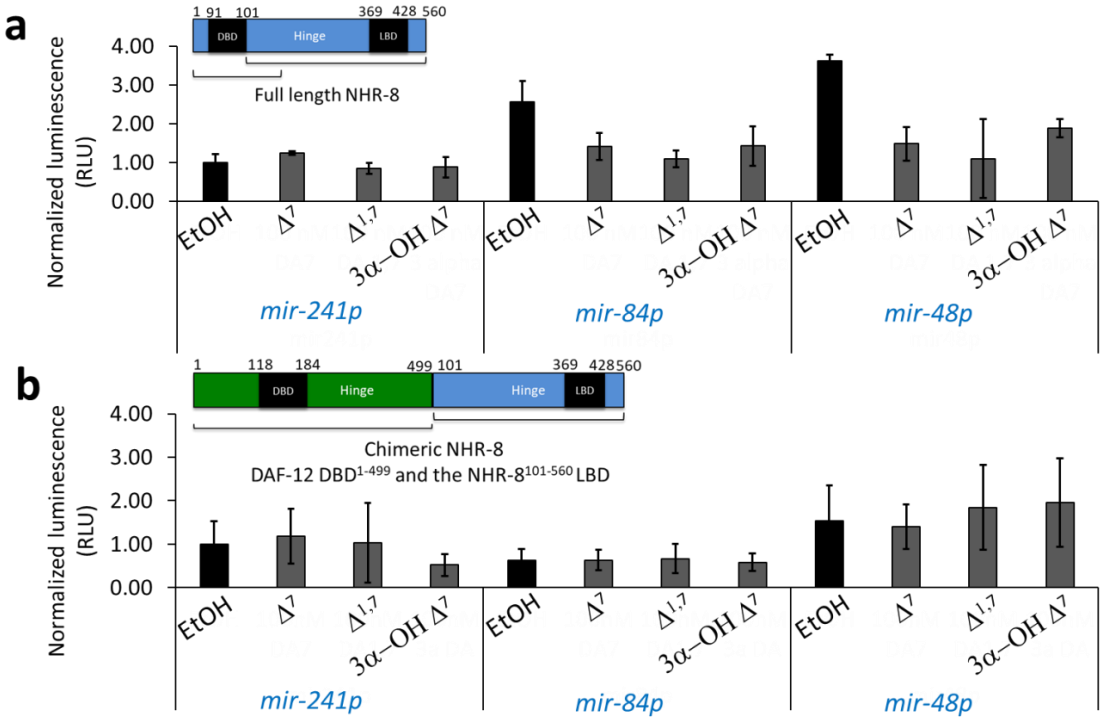

Figure 2.6: Dafachronic acids do not induce NHR-8 receptor activation in luciferase assays (**a**) 100 nM dafachronic acids (Δ^7, $\Delta^{1,7}$ and 3α-OH Δ^7 DA) do not activate full length *C. elegans* NHR-8 under multiple *miR* response elements. n=4, ± S.D. (**b**) 100 nM dafachronic acids (Δ^7, $\Delta^{1,7}$ and 3α-OH Δ^7 DA) do not activate long-hinge, chimeric NHR-8 (consisting of DAF-12 DBD$^{1-499}$ fused to NHR-8$^{91-560}$ LBD) under multiple *miR* response elements. n=4, ± S.D.

As dafachronic acids did not show significant NHR-8 or NHR-48 activation under our *in vivo* assay conditions, we sought to test wild-type *C. elegans* metabolome fractions for small molecule activators of these receptors. In preparation for this untargeted NR – ligand screen, metabolome extracts obtained from large scale, mixed stage, liquid cultures of wild-type (N2) *C. elegans* strains were fractioned using an automated, highly reproducible chromatography system (detailed previously). The resulting individual fractions (80 in total) were combined into pools (18 in total) of these were individually assessed for their effect on NHR-8 activation in luciferase assays. Chimeric NHR-8 construct showed no significant activation by any of the metabolome pools (**Figure 2.7a).** The reduced DAF-12 activation by pool 18 seen here is due to the high amounts of salts present in this pool, that negatively affect HEK cell viability. Through independent mass-spectrometry based analysis, pool 9 was identified to contain Δ^7 DA, along with $\Delta^{1,7}$ DA as a minor component. Correspondingly, pool 9 significantly activated DAF-12 (**Figure 2.7b)** comparable to 100 nM synthetic Δ^7 DA. This validates the potential of our metabolome fractionation process to identify ligand-dependent NR activation.

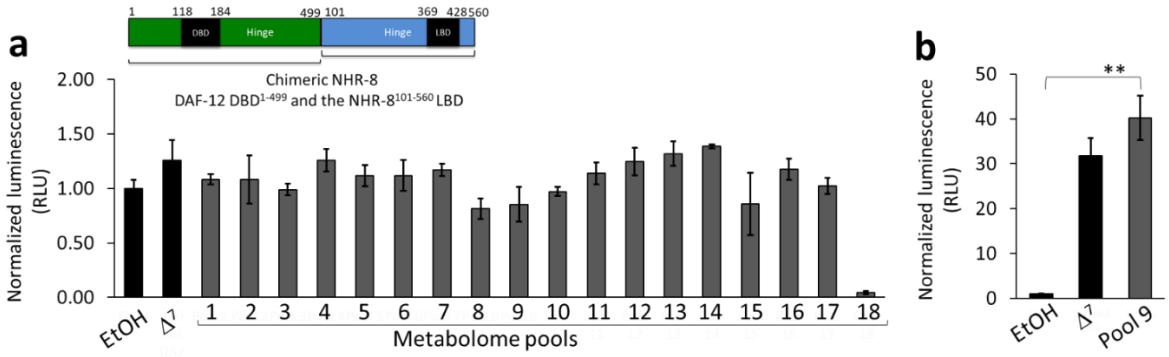

Figure 2.7: Untargeted screening of wildtype *C. elegans* metabolome fractions for potential NHR-8 activator(s) (a) None of the metabolome pools significantly affected NHR-8 activity. The reduced DAF-12 activation by pool 18 seen here is due to the high amounts of salts present in this pool, that negatively affect HEK cell viability. n=4, ± S.D. **(b)** Wildtype metabolome pool 9, primarily containing Δ^7 DA, significantly activated DAF-12 at levels comparable to 100 nM synthetic Δ^7 DA. n=4, ± S.D., **p<0.01, Student's ttest.

Phenotypes such as Pdda and nacq#1-mediated hermaphrodite development acceleration suggested a shared role of DIN-1 in association with DAF-12, NHR-8 and NHR-48. While DIN-1 is classically known as the corepressor of DAF-12, we asked whether DIN-1 can also interact with NHR-8 and NHR-48. Indeed, in luciferase assays DIN-1 co-transfection with NHR-8 and NHR-48 significantly repressed background activation (**Figure 2.8**). Additionally, this repression was DIN-1 specific, since co-transfection with deletion mutant DIN-1$^{\Delta 145–567}$ did not show such repression. Taken together, these results suggest that while DAs do not activate NHR-8 or NHR-48, these receptors likely interact with the corepressor DIN-1 similar to DAF-12.

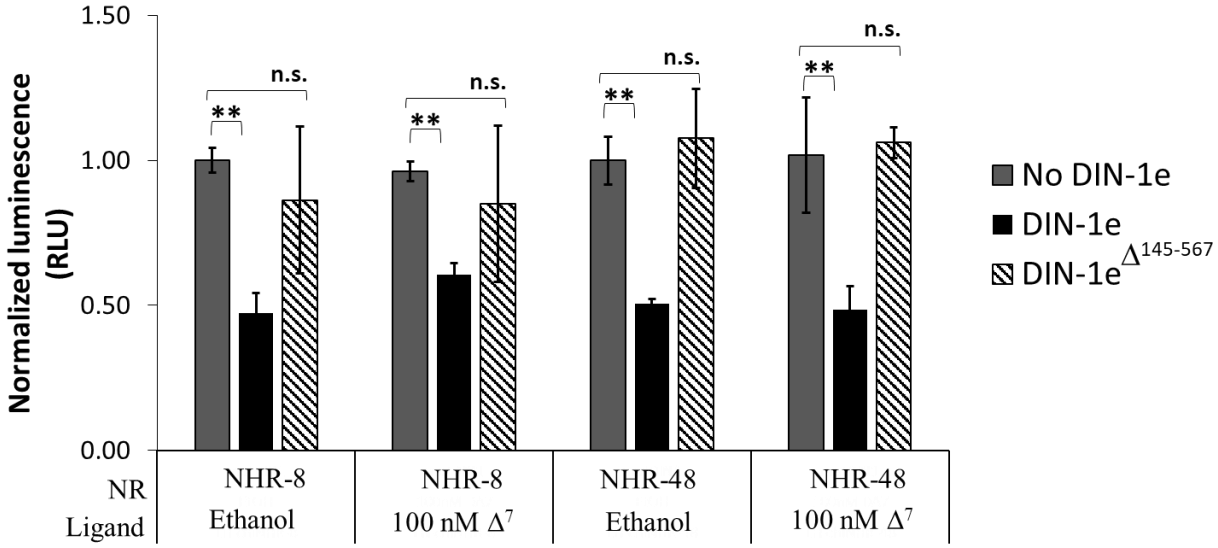

Figure 2.8: NHR-8 and NHR-48 repression by DIN-1e. DIN-1e co-transfected represses background activation of chimeric NHR-8 and NHR-48 in a DA-ligand independent manner. This repression is lost in mutant DIN-1e $^{\Delta 145-567}$. n=4, ± S.D., **p<0.01, Student's ttest.

Interpreting results from cell-culture based ligand assays such as the luciferase assay above, one should consider that any small molecule added to this system can be further modified by endogenous cellular enzymes, thereby interfering with detection of true ligand activity. Additionally, the NR-activation assays presented above rely on recruitment of appropriate mammalian coactivator complex(es) that can effectively activate *C. elegans* receptors. While SRC-1 filled that role the case of DAF-12, the failure to identify any metabolome fractions that activates NHR-8 or NHR-48 could be due to mammalian SRC-1 not binding to these receptors, which may require specialized, yet unknown *C. elegans* proteins for their function. Understanding these shortcomings of *in vivo* assays, we moved on to *in vitro*, cell-free protein interaction assays. The AlphaScreen™ and AlphaLisa™ (by Perkin Elmer) are bead-based proximity assays used for assessing direct binding of small molecule ligands to receptors. AlphaScreen has previously been

used in the successful characterization of Δ^7, $\Delta^{1,7}$ and 3α-OH Δ^7 dafachronic acids as high affinity ligands of DAF-12 [15, 21].

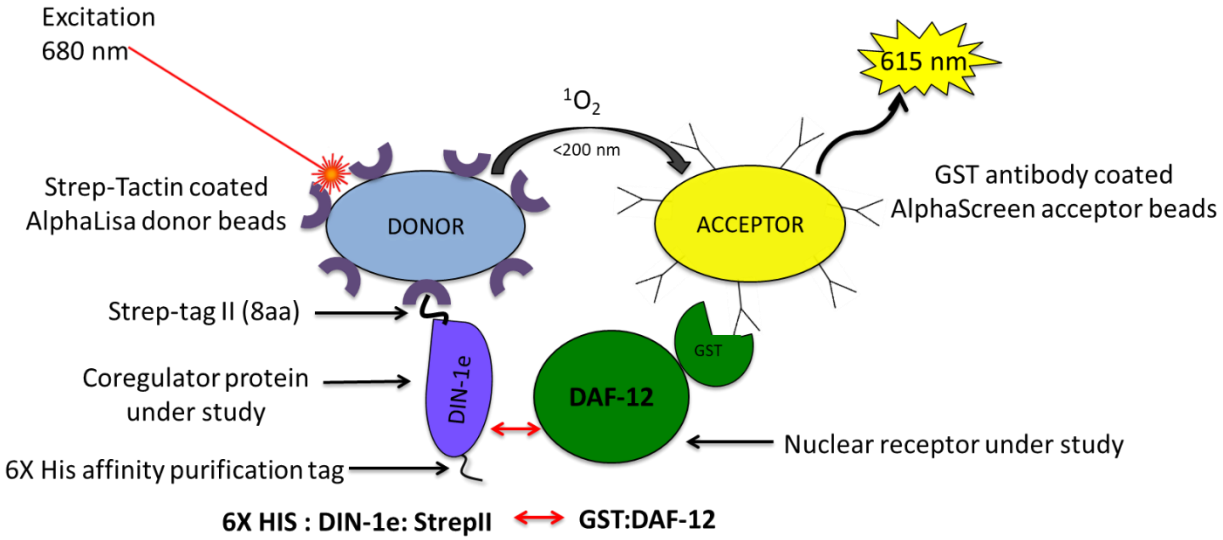

Figure 2.9: Schematic of the AlphaScreen™ assay for studying ligand modulated nuclear receptor and coregulatory protein interactions. When a true biological interaction (such as that between a nuclear receptor and its coregulatory protein) brings the beads together, a cascade of chemical reactions acts to produce a greatly amplified signal. On laser excitation, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with the Acceptor bead that subsequently emits luminescence. In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result, only a very low background signal is produced. The AlphaScreen assay provides a highly versatile, sensitive and miniaturizable means to efficiently perform assay development and high throughput screens with low costs.

We first evaluated the interaction between DAF-12 and DIN-1. For this, *N*-terminal GST-tagged DAF-12$^{281-753}$ (hinge + LBD) and C-terminal Strep-tag II tagged full length DIN-1e were purified from Sf9 insect cells, using the GST- and an *N*-terminal 6x-His tags for affinity purification respectively **(Figure A2.2)**. Each of the proteins were bound to their respective AlphaLisa or Alphascreen beads (PerkimElmer) as shown in **Figure 2.9** and luminescence post incubation was

analyzed. As seen in **Figure 2.10a**, DAF-12 binds to DIN-1e in a concentration dependent manner, validating the ligand-independent interaction between these proteins. Additionally, the specificity of this interaction is demonstrated by no binding between DAF-12 and GFP (green trace) or just with DIN-1e by itself (blue trace). Since this is the first report of insect-cell based purification of these recombinant *C. elegans* proteins, our observations on DAF-12, DIN-1 binding specificity validates that these proteins appropriately fold when expressed in insect cells, allowing to study their activity under *in vitro* conditions. Further, we tested the effect of DA ligands on this interaction. As shown in **Figure 2.10b**, when DAF-$12^{281\text{-}753}$ (10 nM) was pre-incubated with varying concentrations of DA ligands, it prevented association with DIN-1e (10 nM), as observed by the reduction in luminescence signal with increasing DA concentration. While each of the three DAs had slightly varying potencies in this function, at 100 nM each DA almost completely prevented association of DAF-12 and DIN-1. We confirmed that this action was specific to DAs, as no such effect was not observed with cholesterol or 7-ketocholesterol. Additionally, we observed that to a pre-formed DAF-12 – DIN-1e interaction, DAs had no effect. Together this demonstrates that the binding of DAs to DAF-12 prevents association with DIN-1 but does not induce dissociation of already formed DAF-12-DIN-1 complex.

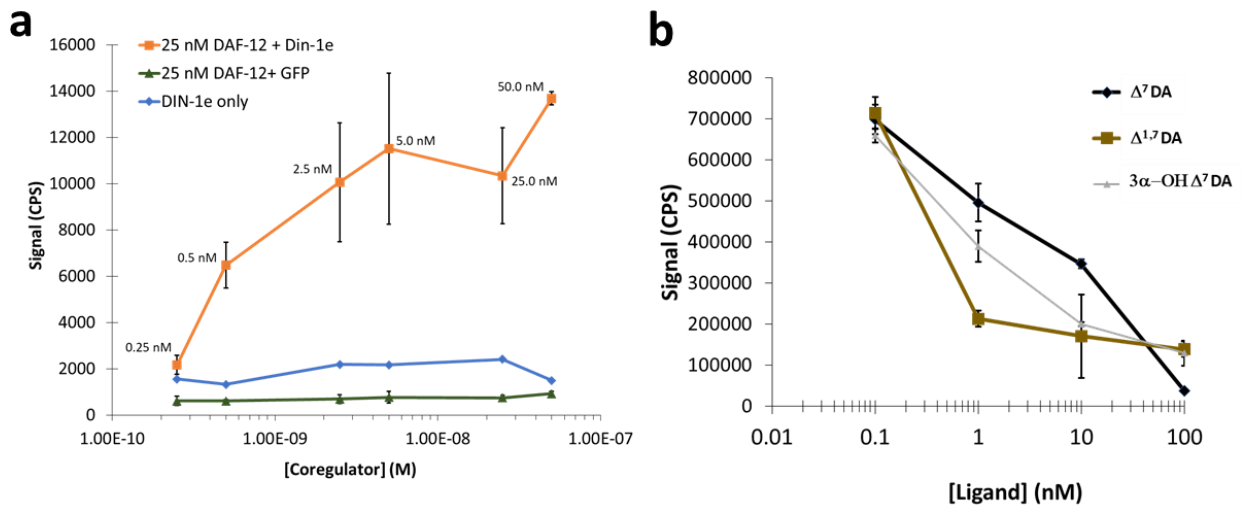

Figure 2.10: Dafachronic acids prevent association of corepressor DIN-1e to DAF-12. (a) In the AlphaScreen assay, 25 nM of DAF-12$^{281-753}$ strongly binds to DIN-1e in a concentration dependent manner, independent of dafachronic acids (orange trace). No binding is observed between 25 nM DAF-12 and GFP (negative control, green trace) or with DIN-1e by itself (blue trace). n=3, ± S.D. **(b)** Dafachronic acids (Δ^7, $\Delta^{1,7}$ and 3α-OH Δ^7 DA) pre-incubated with DAF-12 (10 nM used here), prevent association with DIN-1 e (10 nM) in a concentration dependent manner. n=3, ± S.D.

The SHARP family of corepressors bind unliganded mammalian receptors RAR and PPARδ through its RID (Receptor Interaction Domain), and several motifs resembling the hydrophobic corepressor helix (L/IXXI/VI) are predicted to mediate this interaction. DIN-1 isoforms contain variants of this motif (L/IXXI/V/L) (**Figure 2.11b)**, as well as LXXLL motifs found in several nuclear coregulators, though the overall identity in the RID is weak. Hence, we tested the requirement of this domain in mediating interactions with DAF-12 by creating truncated DIN-1e mutants (**Figure 2.11a)**. Through AlphaScreen (**Figure 2.11c)** and western blotting (**Figure 2.11d)** we confirmed the requirement of RID for DA-independent association with DAF-12.

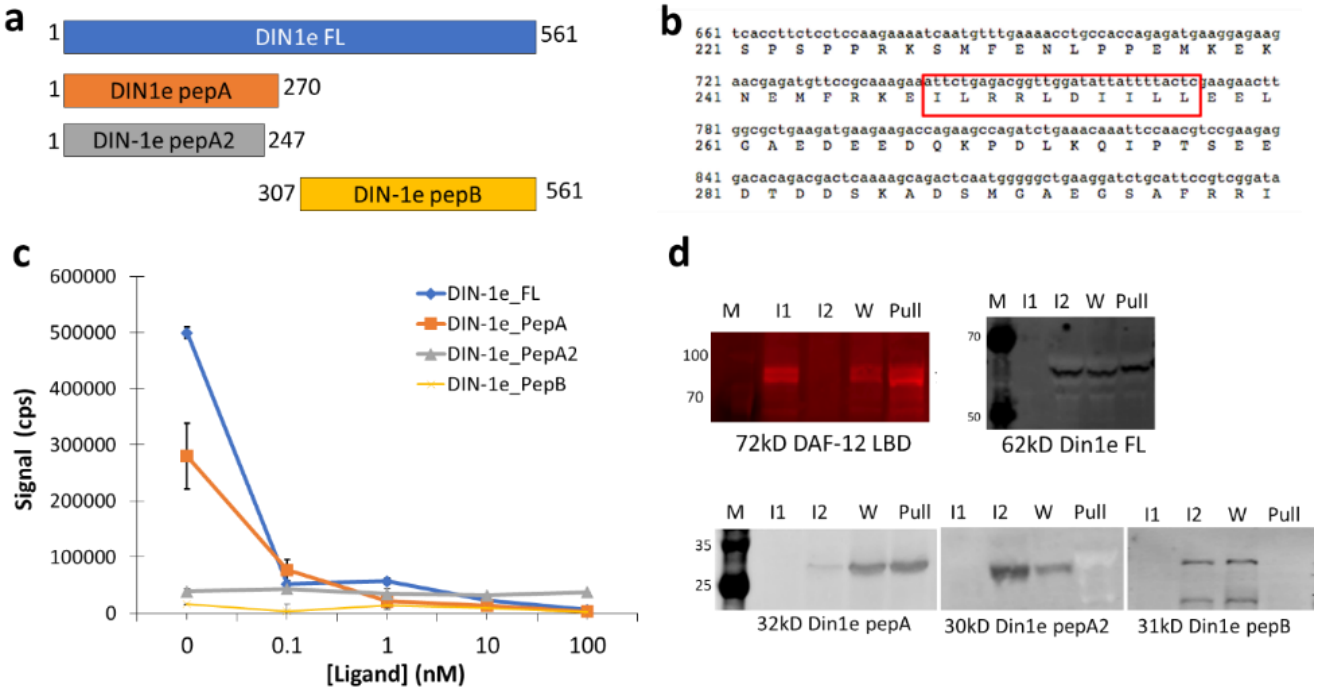

Figure 2.11: DIN-1e requires the conserved receptor interaction domain (RID) for interactions with DAF-12. (a) Schematic of truncated DIN-1e variants purified to test the role of RID in DAF-12 – DIN-1e interactions. **(b)** RID in DIN-1e that is a variant of a conserved repressor motif (L/IXXI/V/L) in mammalian regulatory proteins. **(c)** AlphaScreen with different truncated DIN-1e variants (pep A, A2 and B) vs full length (FL) Din-1e. Only FL and 'pepA' Din-1e that contain the RID show DA-independent binding to DAF12. These interactions are lost upon incubation with increasing levels of Δ^7 DA ligand. n=3, ± S.D. **(d)** Protein pull down captures detected by western blotting confirm ligand-independent interactions of DAF-12 and DIN-1e. (M) Spectra BR marker, (I1) Input 1: Sf9 cell lysate expressing DAF-12, (I2) Input 2: Sf9 cell lysate expressing FL-Din-1e or truncated DIN-1e variants, (W) Pull-down wash, (Pull) Pull-down captures showing binding of DAF-12 to FL-DIN-1 and pepA only.

Pdda and nacq#1-mediated hermaphrodite developmental acceleration indicated a shared role of DIN-1 in association with DAF-12, NHR-8 and NHR-48, which is also suggested by luciferase based assays in which DIN-1e repressed background activation by these receptors. Hence, we sought to test the direct binding between DIN-1 and NHR-8 and NHR-48 through the AlphaScreen. As seen in **Figure 2.12**, DIN-1 directly binds to receptors NHR-8 and NHR-48,

comparable to its interaction with DAF-12. While DAs prevent the association between DIN-1 and DAF-12, DAs had no effect on the interactions between DIN-1 and NHR-48. In the case of NHR-8, addition of DAs may have a weak effect that did not reach significance.

In the case of mammalian NRs (including VDRs, LXRs and RXRs) it has been shown how the architecture of the DNA binding region (containing multiple direct repeats of the binding sequence) can greatly influence dimerization capability of the ligand-bound NRs so as to facilitate co-operativity and fine-tuning of high affinity interactions. Indeed, regions of DAF-12 (primarily within the DBD) bear high sequence homology to the VDR dimerization interfaces. Hence, we tested the dimerization potential of DAF-12 as well as NHR-8 and NHR-48 through the AlphaScreen. Interestingly, we observed that DAF-12 could homodimerize (**Figure 2.13a)**, while neither NHR-8 nor NHR-48 showed any evidence for formation of homodimers. Additionally, we found that DAF-12 heterodimerizes with NHR-8 or NHR-48, while no heterodimerization was observed between NHR-8 and NHR-48. Additionally, by creating either DBD-only or LBD-only containing versions of DAF-12 and NHR-8, we mapped the domains required for this dimerization **(Figure 2.13b)**. The DBD^{1-501} of DAF-12 is necessary and sufficient for its homodimerization and is also required for heterodimerization with the DBD^{1-108} of NHR-8. Both DAF-12 homodimerization and heterodimerization with NHR-8 and NHR-48 were DA-independent under the tested conditions.

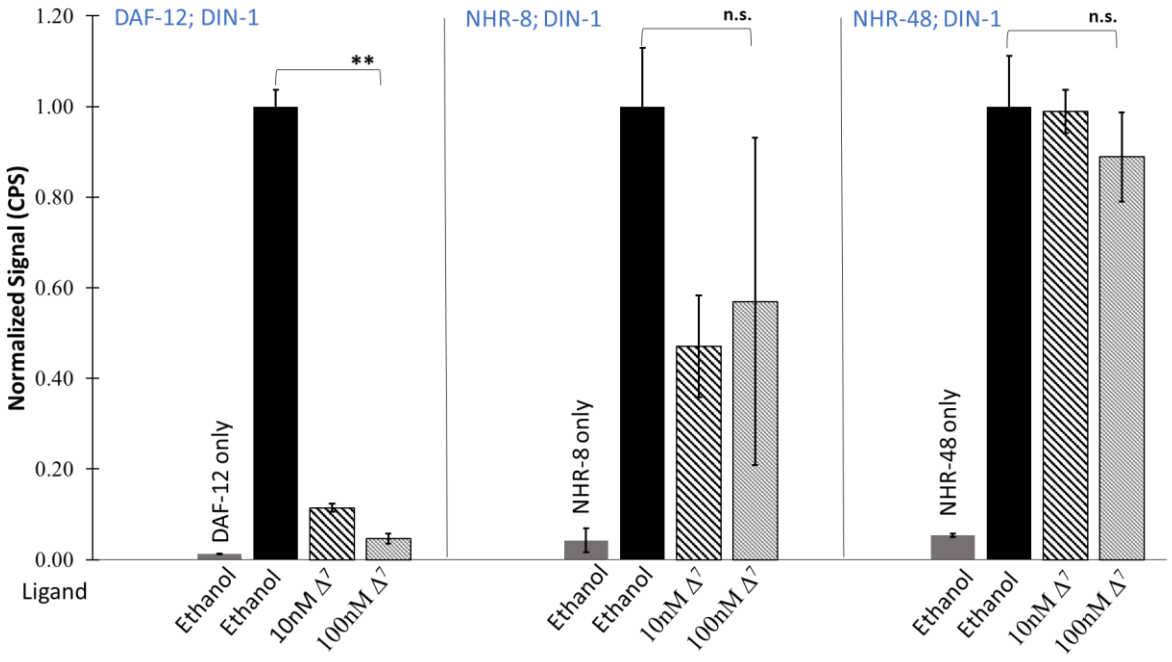

Figure 2.12: Corepressor DIN-1e directly binds to receptors NHR-8 and NHR-48. In AlphaScreen assays, DIN-1e directly binds full-length NHR-8 and NHR-48 receptors. While Δ^7 DA prevents the interaction of DIN-1e and DAF-12 (shown here at 10 nM and 100 nM Δ^7 DA), it had no significant effect on the interactions between DIN-1 and NHR-8 or NHR-48. n=3, ± S.D. **p<0.01, p value for NHR-8;DIN-1 with ethanol vs with 100 nM Δ^7 DA = 0.088, Student's ttest.

As a combination our cell culture based receptor activation assays and *in vitro* protein interaction assays, it appears that DAF-12 interacts with both NHR-8 and NHR-48 through the formation of heterodimeric complexes. If these receptors do form a receptor interaction network, they most likely also co-regulate metabolism through downstream transcriptional output. To investigate the role of receptors DAF-12, NHR-8 and NHR-48 in regulating metabolism, we grew liquid cultures of mutants *daf-12(rh411;rh61), nhr-8(ok186)* and *nhr-48(ok178),* along with wild-type (N2) in triplicates and analyzed their endo-metabolomes through an untargeted LC-MS based metabolomics approach. Data analysis was performed using the MetaboSeek software suite (Developed by M. Helf, Schroeder lab). All features showing 10-fold or more misregulation (up or

downregulation, when compared to wildtype N2), are presented in **Figure 2.14a**. We observed that a large number of downregulated features were shared between *daf-12* and *nhr-8* mutants. Preliminary analysis based on formulae prediction and MS/MS fragmentation patterns revealed that >80% of these features belong to the phospholipid family of compounds. Representative structures of the lipids completely abolished in *daf-12* and *nhr-8* mutants are shown in **Figure 2.14b**. While these results are in good agreement with previous transcriptomics data that identified misregulation in fatty acid biosynthetic genes in these individual *nhr* mutants, it further suggests the likelihood of a heterodimeric DAF-12-NHR-8 complex co-regulating downstream lipid composition.

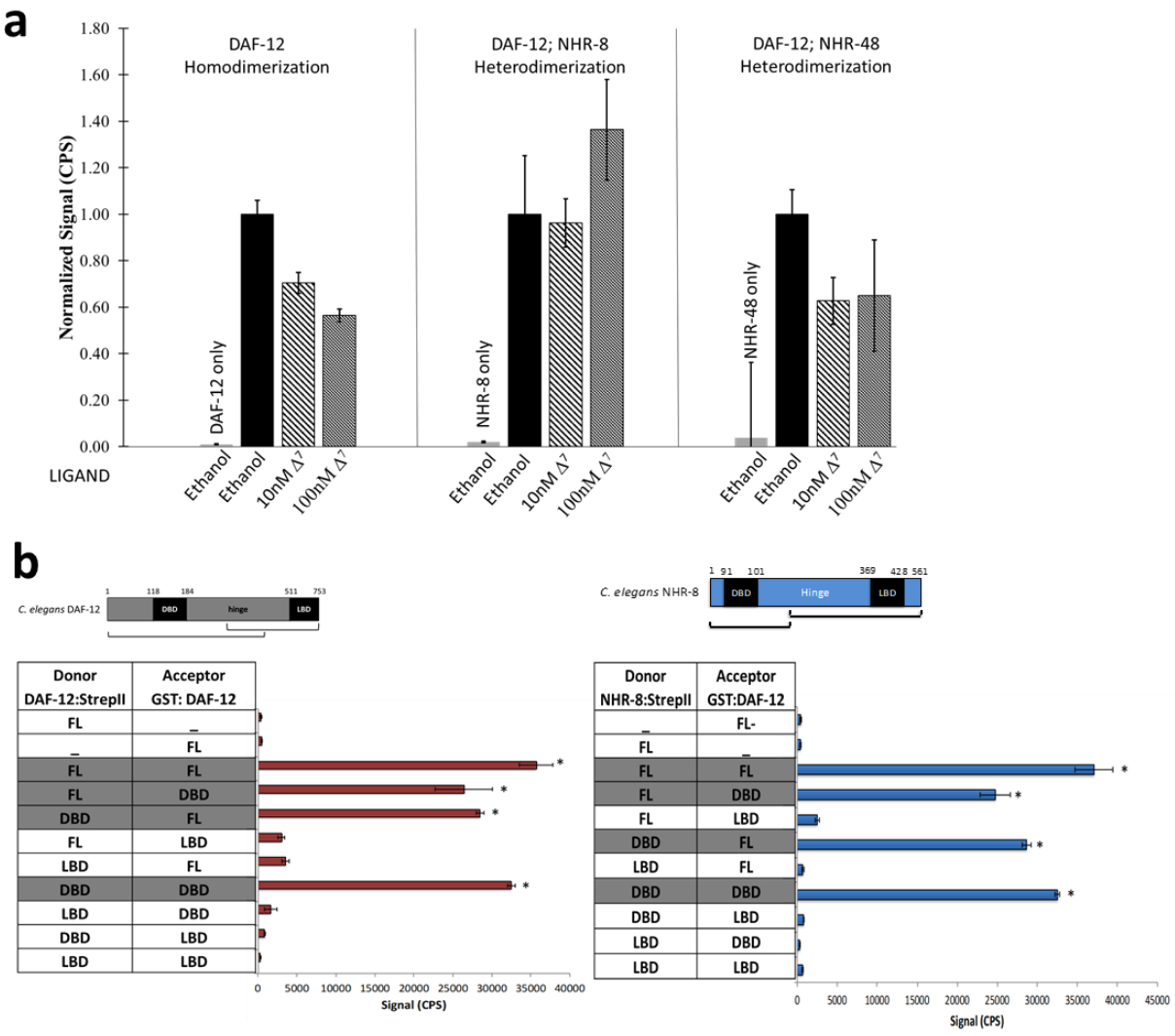

Figure 2.13: DAF-12 forms homodimers, and heterodimers with NHR-8 and NHR-48, in a DA-independent manner. (a) AlphaScreen assay demonstrating homodimerization of full-length DAF-12 and heterodimerization of full-length DAF-12 with either NHR-8 or NHR-48, in a Δ^7 DA-independent manner (shown here at 10 nM and 100 nM Δ^7 DA). n=3, ± S.D. **(b) T**he DBD of DAF-12 (marked in upper panels) is necessary and sufficient to mediate homodimerization of DAf-12 and heterodimerization with NHR-8. n=3, ± S.D.

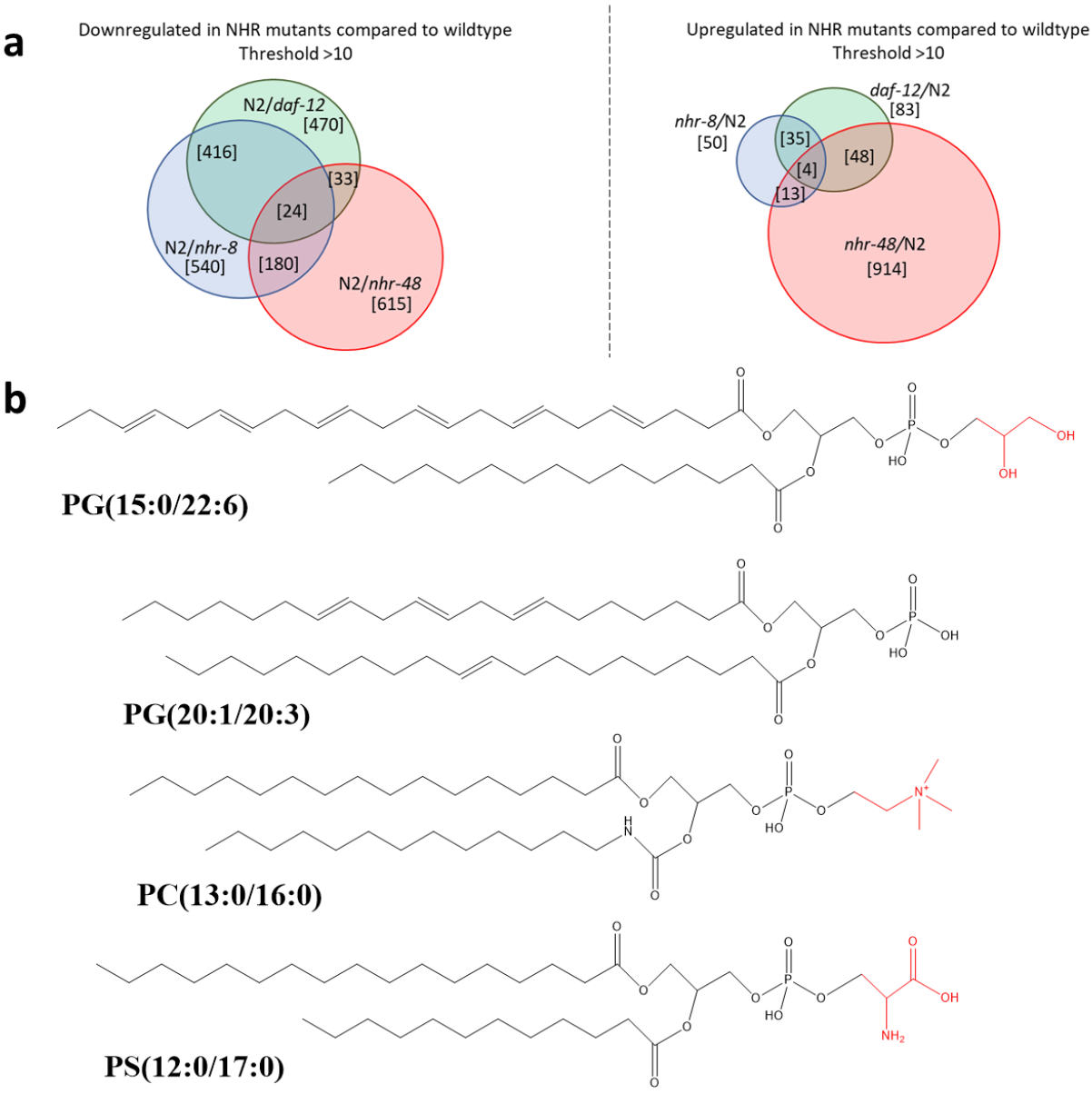

Figure 2.14: Metabolome coregulation by *C. elegans* NRs DAF-12, NHR-8 and NHR-48. (a) Endometabolomes of NHR mutants *daf-12 (rh411;rh61), nhr-8(ok186)* and *nhr-48(ok178)* were analyzed in an untargeted LC-MS based approach, in both ESI- and ESI+ modes to reveal features down regulated (left side) or up-regulated (right side) by at least 10 fold, in three independent replicates. Numbers in parenthesis denote total number of misregulated features and those features shared by mutants are denoted at the intersection. **(b)** Examples of phosphatidylglycerols (PG), phosphatidylserine (PS) and phosphatidylcholine (PC) class of lipids co-downregulated by *daf-12* and *nhr-8* mutants based on preliminary MS analysis and formulae prediction.

A previous report suggested EMB-8 (the ortholog of human P450 oxidoreductase POR) was critical to egg shell formation, as well as regulating the thermosensitive fusion of fat-storing lipid droplets in a DAF-12 dependent manner in *C. elegans*[40, 41]. Correspondingly *emb-8* mutants showed severe egg laying defects and accumulation of lipid droplets. This study concluded that EMB-8 (together with CYP-37A1[42]) synthesized a lipophilic hormone, that was not a DA, but one that represses the fusion-promoting function of DAF-12. Intrigued by this report, we investigated the enzymatic role of *emb-8* using our untargeted metabolome analysis pipeline. *emb-8(hc69)* mutants showed complete absence of a large family (~20) of glycerophosphoethanolamines **(Figure 2.15a)**. This family of compounds was not misregulated in *daf-9(dh6)* or *daf-40(hd100)* – two *C. elegans* cytochrome P450s, eliminating the possibility of a rather generic defect of P450 enzymatic activity **(Figure A2.3)**. Upon further LC-MS/MS analysis we identified that these compounds have a rather unusual structures, with predominantly almost exclusively saturated C17 – C20 acyl and C14 – C20 unsaturated acyl amines attached to a glycerophosphate backbone **(Figure 2.15b, Table A2.1)**. Further, these compounds were almost completely abolished in *daf-12(rh411;rh61)* and *nhr-8(ok186)*, but not in *nhr-48(ok178)* **(Figure 2.15c)**. Together, this family of phospholipids is particularly interesting because *(1)* they may represent the likely enzymatic products of a yet uncharacterized *C. elegans* CYP450 or another oxidase partner that interacts with EMB-8, *(2)* this family of phospholipids represents a novel aspect of *daf-12* and *nhr-8* lipid metabolism and likely sets an example of lipid metabolites coregulated by an NHR-8 – DAF-12 dimer and *(3)* this family of phospholipids may underlie the reported egg shell formation defects in *emb-8* mutants.

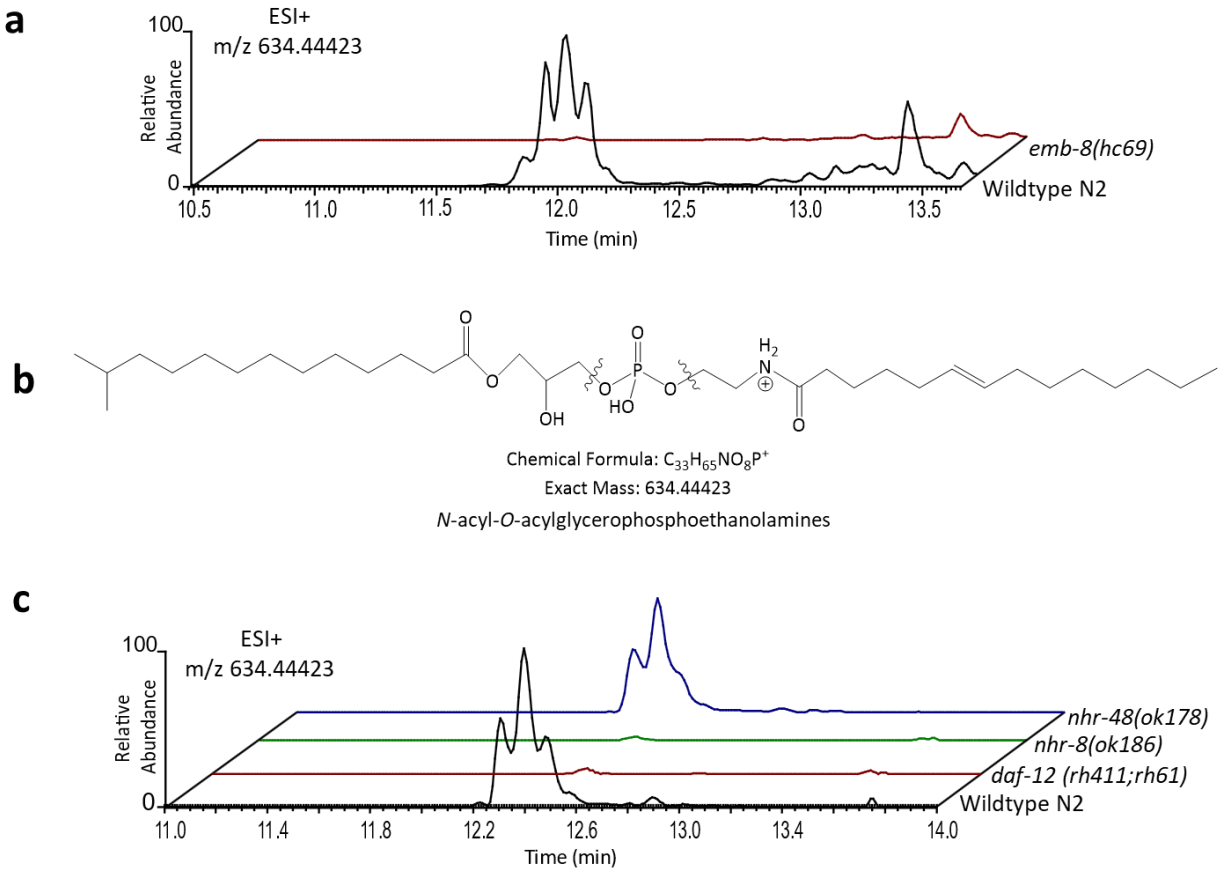

Figure 2.15: Characterization of a novel class of phosphoethanolamines synthesized by *emb-8* and regulated by both *daf-12 and nhr-8*. (a) Phosphoethanolamine family of compounds (specifically shown here for m/z 634.44423, in ESI+) absent in *emb-8(hc69)* mutants. **(b)** LC-MS-MS fragmentation pattern reveals the unique structure for the *N*-acyl-*O*-acylglycerophosphoethanolamine family of metabolites misregulated in *emb-8(hc69)*. Specifically shown here is the tentative structure for m/z 634.44423 (in ESI+). **(c)** The same phospholipid family identified in **(a)** and **(b)** is absent in *daf-12* and *nhr-8* mutants, but not in *nhr-48* (specifically shown here for m/z 634.44423, in ESI+).

DISCUSSION

Nematodes like *C. elegans* can survive the harshest of environmental conditions by adopting an alternative developmental program and entering into the dauer state. The entry into or exit from dauer in *C. elegans* is a carefully weighted decision[9]. Such critical metabolic and physiological

changes are choreographed by small molecule ligands that modulate changes in gene expression via NR signaling. Thus, evaluating the fundamental mechanisms of NR function provide insights on how simple environmental changes are translated into organism-wide physiological effects.

A body of work has established that DAF-12 is a central mediator in the decision between reproductive development versus dauer[16, 20]. But more recent work has revealed the complex and overlapping functions of receptors NHR-8 - in mediating population-density dependent acceleration of development - and, NHR-48 - in developmental acceleration of hermaphrodites in response to male-secretory signals, along with intersecting roles of corepressor DIN-1 and dafachronic acid ligands[39]. However, the mechanisms by which these receptors regulate different phenotypes have remained largely speculative.

The study presented here used a combination of cell-based receptor activation, *in vitro* protein interaction assays and mass-spectrometry based untargeted metabolomics to reveal the deeply interconnected network of nuclear receptor and coregulatory protein interactions that together control *C. elegans* metabolism. Using the rather well studied DA-DAF-12 system, we first established how DA steroidal ligands control nuclear receptor interactions with coregulatory proteins that then alter gene expression (**Figure 2.16a)**. In the absence of DAs, DAF-12 constitutively binds corepressor DIN-1e causing downstream gene repression. On the contrary, binding of DAs to DAF-12 prevents association with DIN-1e, but rather favors binding with a coactivator that then activates downstream transcription. We next recognized that DIN-1e acts as a shared corepressor between DAF-12, NHR-8 and NHR-48. DIN-1e could directly bind NHR-8 as well as NHR-48 in a DA-independent manner to repress downstream gene transcription. These interactions of DIN-1 provide compelling evidence for a model where different NR-DIN-1

interactions have different outcomes such as, DAF-12 – DIN-1 interaction is required for dauer entry, and NHR-8 and/or NHR-48 - DIN-1 association could promote dauer-exit and developmental acceleration as observed in hermaphrodite response to nacq#1.

We next explored how *C. elegans* receptors interact with each other to reveal DA-independent DAF-12 homodimerization as well as heterodimerization with NHR-8 and NHR-48. In recognizing the importance of domain-domain connections in receptor complexes, our analysis also makes clear that individual DNA-binding domains are sufficient to provide nearly full binding affinity. Such DBD-mediated dimeric complexes could occupy individual half-sites within the *miR* family of DAF-12 targets (or other unknown targets) to co-regulate gene expression. Such dimer formation also explains how DAs can be "perceived" by NHR-8 while in a complex with DAF-12, without direct binding *per se*. To this end, we observed no direct activation or binding of DAs to NHR-8 or NHR-48. We believe that the lack of information on the *C. elegans* coactivators and DNA binding regions of NHR-8 and NHR-48 could have prevented an active receptor-complex formation, and hence in conditions that activate DAF-12, we observed no activation for NHR-8 or NHR-48 through metabolome fraction screening.

Supporting *in vitro* observations of DAF-12, NHR-8 and NHR-48 receptor dimers, we also observed a large overlap between the lipid metabolome misregulated by *daf-12*, *nhr*-8 and *nhr-48* mutants. Particularly, the large family of phospholipids abolished in both *daf-12* and *nhr-8* provides an example for the cooperative roles of these receptors. It demonstrates how a DAF-12 - NHR-8 dimer, likely occupying neighboring regions of common gene targets, can coregulate the metabolome composition. An example of one such common NR target appears to be *emb-8.* The unique family of phospholipids identified as the enzymatic products of *emb-8* (a yet

uncharacterized *C. elegans* cytochrome P450 reductase) was regulated by both *nhr-8* and *daf-12*[40]. While these lipids seem vital to egg shell composition and development, their co-regulation by *nhr-8* and *daf-12* provides insights on additional roles of these NRs. One possibility of how this may work is that *emb-8* is a direct downstream gene target of *daf-12* and/or *nhr-8.* But an intriguing alternative is that *emb-8* regulates *nhr-8* (and in part *daf*-12) activity via supply of ligand(s) or activator(s), and these receptors in turn set a signaling circuit to regulate ligand biosynthesis **(Figure 2.16b)**. Further studies clarifying either of these two possibilities (primarily through transcriptomic analysis) would further our understanding of NR dimer mediated activities.

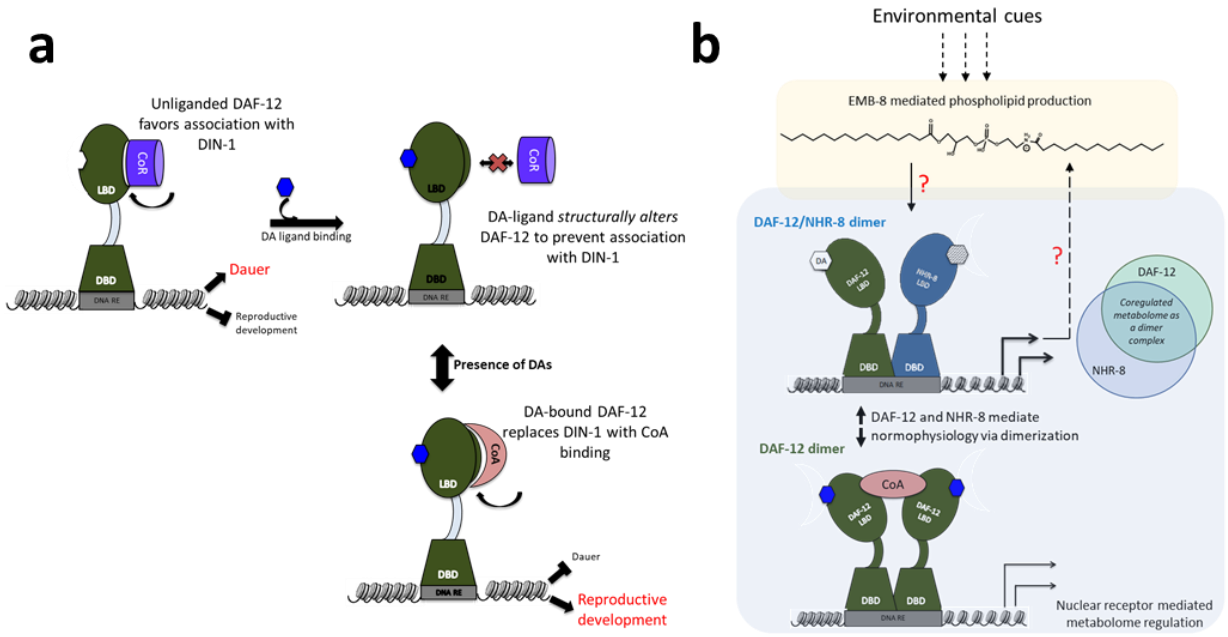

Figure 2.16: Schematic representing the nuclear receptors - coregulatory protein - small molecule metabolite network uncovered in this study. (a) Dafachronic acid (DA) ligands alters *C. elegans* receptor DAF-12 *structurally* to mediate interactions with coregulatory proteins, setting an example of ligand mediated ordered recruitment of different coregulatory complexes to nuclear receptors. **(b)** In *C. elegans,* nuclear receptor DAF-12 and NHR-8 heterodimers mediate organism-wide changes in lipid metabolism (likely via EMB-8) to regulate overall normophysiology.

Overall this study supports the combinatorial roles *C. elegans* receptors DAF-12, NHR-8 and NHR-48. By combining protein biochemical studies and metabolomics, it explores both receptor-protein interactions and their role in metabolome regulation towards *C. elegans* development. Studies on mammalian receptor architecture, endogenous ligands and interactions with coregulatory complexes have together provided keen insights into molecular mechanisms that modulate organismal physiology and with this study we aim to further similar efforts in *C. elegans*.

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CHAPTER 3

PROFILING THE STEROIDOME OF NEMATODES *C. ELEGANS* AND *P. PACIFICUS*

ABSTRACT

Steroids serve critical roles in regulating cellular and organismal physiology, including membrane fluidity, sexual characteristics, reproduction, and metabolism. While known steroidal metabolites demonstrate structural and functional diversity across animal kingdoms, a systematic curation to understand the true extent of this chemical and functional diversity is lacking. Among model organisms, the nematode *C. elegans*, being a cholesterol auxotroph, presents a unique system to comprehensively investigate the steroidome. In *C. elegans,* one of the most tightly gated decisions - continuing through reproductive development versus entering the dormant dauer stage - is governed by a steroidal family of compounds called dafachronic acids (DAs) that function via nuclear receptor DAF-12. While the pivotal roles of this family of steroids in *C. elegans* have been acknowledged, a systematic exploration of the structural and hence functional diversity of steroids beyond that of DAs has not been achieved in nematodes. Additionally, several closely-related nuclear receptors, particularly NHR-8 and NHR-48, play intersecting roles with DAF-12 in mediating cholesterol availability, metabolism and lifespan, yet remain orphan receptors (with no known endogenous ligand). Hence, to comprehensively profile the steroidome in nematodes *C. elegans* and *P. pacificus* in an unbiased, untargeted manner, we implemented a liquid-chromatography-high-resolution mass spectrometry - (LC-HR-MS) based approach employing ^{13}C-stable isotope labeling. With this method, we validate the cholesterol dependent

de novo production of the known dafachronic acids. Further, after removal of chromatographic adducts and OP50-derived metabolites, our results reveal 80 yet unknown endogenous sterols. Of these 48 were classified as novel endogenous sterols and 32 as likely sterol esters. 5 of the novel sterols are conserved between *C. elegans* and *P. pacificus.* At least 40% (20/48) of these sterols are likely sterol acids, and, interestingly, while their production is dependent on *daf-36* (the rate-limiting enzyme in the DA biosynthesis pathway), it is independent of *daf-9*, the canonical cytochrome P450 for DA production. Additionally, since these newly identified sterol acids are not sourced directly from bacteria, it exposes the complexity of steroid tailoring enzymes in nematodes. Given the strikingly diverse steroidome uncovered through this study and the yet unknown endogenous ligands for several NHRs in *C. elegans* we reason using these metabolites as a starting point to mine for NR ligands in nematodes.

INTRODUCTION

For growth and survival, all organisms need to constantly perceive their environment and adapt to any deviations from favorable growth conditions such as changes in food availability, population density or temperature. Most animals relay these changing environmental cues intracellularly through specific small molecule ligand activated nuclear receptor (NR) signaling cascades. Thus, NRs can perform critical roles in development, homeostasis and metabolism and often a single NR can perform distinct functions in response to different dietary or endocrine signals by building complex, interconnected, ligand-gated networks**.**

Several NR signaling studies have elucidated that many endogenous NR modulators are either fatty acids (as PPAR ligands and phospholipids as SF-1 ligands[1]), steroid hormones (e.g. estrogens and glucocorticoids as ER and GR ligands respectively), or other cholesterol-derived metabolites (e.g. oxysterols and bile acids as LXR and FXR ligands, respectively)[2-6]. While the diversity within the fatty acid family of molecules has been explored, steroid and cholesterol-based ligands remain less well characterized. In fact, the four-membered hydrocarbon ring system of steroids generates a large structural and thus functional diversity across animal kingdoms. In different animal and plant kingdoms, steroids serve a myriad of functions ranging from regulating membrane fluidity and permeability (e.g. phytosterols sitosterol and stigmasterol in plants[7, 8]), as molting hormones (e.g. ecdysones in insects[9]) and as hormones regulating reproduction and sexual development (e.g. progesterone and androgens in mammals[4]). While the pool of steroids appears large, their structural distinctions are often minute, usually around the side chain of the cyclic ring system, and/or the presence and location of substituents **(Figure 3.1)**.

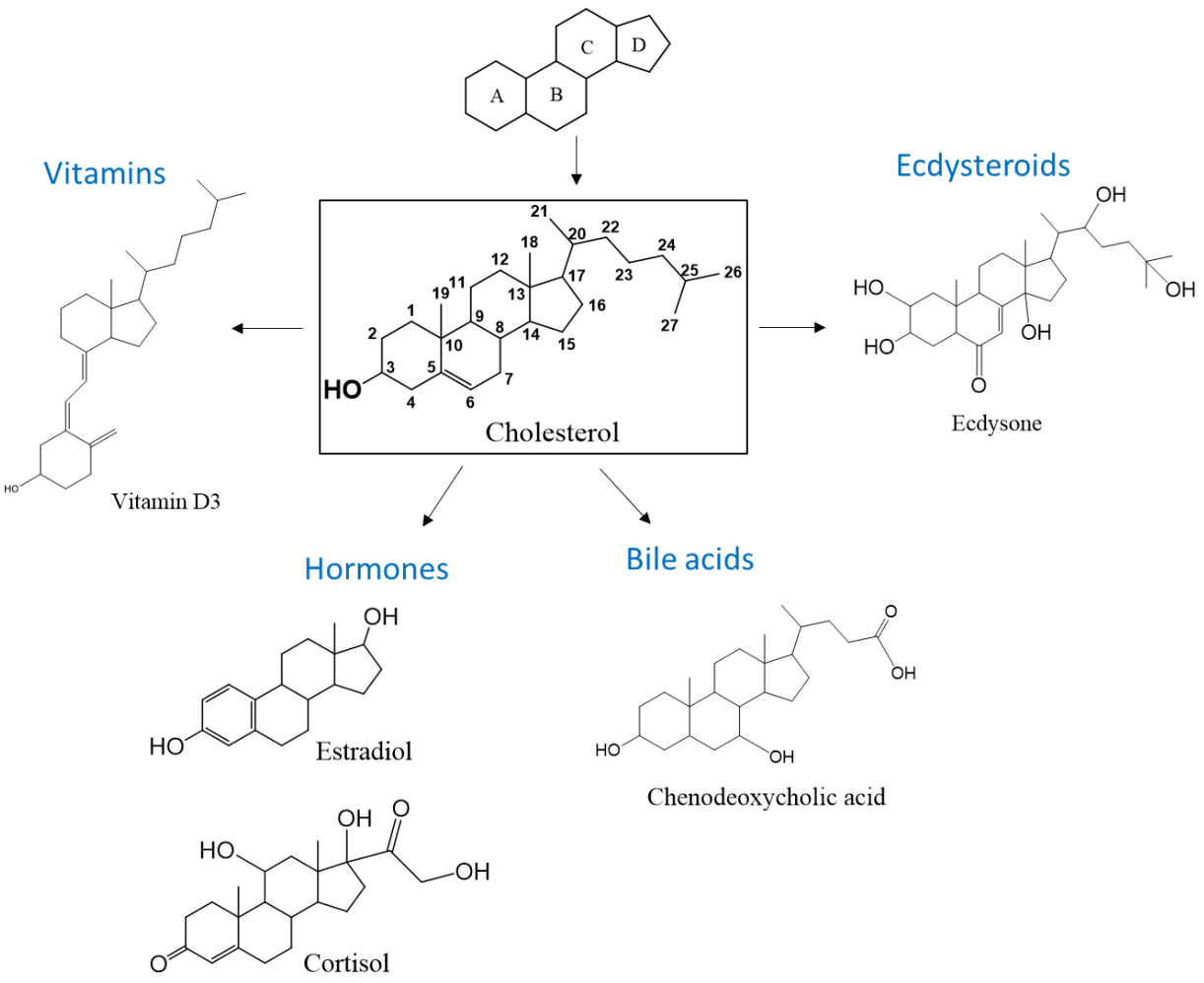

Figure 3.1: Structural and functional diversity of known steroids across animal kingdoms.

Sequence homology and phylogenetic studies have uncovered 48 NRs in humans and 284 in the nematode *C. elegans*[10]. However, the identification of their endogenous small molecule ligands has proven challenging, leaving many NRs orphaned. This is primarily because steroidal signaling molecules constitute only minor (and often transiently produced) components of a highly complex metabolome, which when coupled with limited detection sensitivity of mass spectrometry-based approaches makes ligand identification difficult[11,12]. To circumvent these challenges, most studies resort to targeted small molecule screens either based on a pathway of

interest or through a selection of synthetic compounds. As a result, a systematic curation uncovering the structural and functional diversity of steroidal small molecules has been neglected.

Apart from uncovering the structural diversity they present; steroid ligand identification will provide essential tools towards understanding NR biology itself. Along with regulating downstream gene transcription activities, NRs often maintain homeostasis of the ligands themselves[13,14]. Hence the identification of their steroidal ligands can act as a predictive markers of the metabolic pathways they regulate. A classic example of this mechanism is how bile acids and cholesterol bind FXRs and LXRs respectively, and these NRs in turn regulate their ligand homeostasis[15]. Thus, uncovering steroidal diversity holds great potential in associating intrinsic functions to the receptor itself.

For an unbiased exploration of the steroidal diversity, the nematode *C. elegans* presents itself as a unique model system. A major biochemical difference between vertebrates and nematodes is that, nematodes (such as the model organisms *C. elegans* and *P. pacificus*) are incapable of *de novo* sterol biosynthesis and hence demonstrate a nutritional requirement for sterols[16-19]. Despite being cholesterol auxotrophs, in *C. elegans* the steroidal family of compounds called dafachronic acids (DAs) regulate reproductive development and lifespan through interactions with an evolutionarily conserved nuclear receptor DAF-12 (a homolog of the vertebrate vitamin D and liver X receptors)[20-22]. Under cholesterol deprived conditions, *C.* elegans constitutively enter dauer instead of proceeding to the third larval stage. However, upon favorable environmental growth conditions (including adequate food availability), dietary cholesterol is utilized for biosynthesis of at least three structurally distinct DAs that are essential in reaching

reproductive adulthood[23,24]. Genetic analyses of dauer signaling pathways have revealed high homology between the DA biosynthetic cascade and mammalian bile acid synthesis (outlined in **Figure P.2**).

While the receptor DAF-12 (regulated by its endogenous DA steroidal ligands) acts as a central switch in mediating development and lifespan, two other closely related receptor homologs, specifically NHR-8 (a homolog of vertebrate LXR and FXR) and NHR-48 (a homolog of vertebrate VDR) are key to *C. elegans* cholesterol metabolism and regulation. *nhr-8* mutant phenotypes suggest that *nhr-8* impacts cholesterol availability, transport and/or metabolism[25,26]. Additionally, under dietary restriction conditions, enhanced *C. elegans* lifespan has been attributed to the involvement of NHR-8 and DAs (specifically Δ^7 DA), without the requirement of DAF-12 or *daf-9* (the canonical cytochrome P450 in DA production)[27]. While the known DAs do not directly activate NHR-8 under tested *in vitro* or *in vivo* assays (Author's work presented in Chapter 2), it remains to be elucidated how DAs are perceived by NHR-8 (tentative model suggested by work in Chapter 2) and what the true endogenous ligand(s) of NHR-8 are. Interestingly under low cholesterol and elevated temperatures, *nhr-8* mutants arrest, and the arrested larvae can be rescued by cholesterol supplementation, but not by DA[26]. This suggests that the NHR-8 ligand is most-likely not DA or DA-derived but in fact is cholesterol-derived. Furthermore, *nhr-48* mutant phenotypes also suggest its role in reproductive development and considering its homology to DAF-12 and NHR-8, has been postulated to function alongside DAF-12 and NHR-8 in regulating steroidal metabolism in *C. elegans.* Yet, both receptors NHR-8 and NHR-48 remain orphan.

The obligate requirement of cholesterol by nematodes, coupled with a largely uncharacterized cholesterol-derived metabolome, and the yet unclear roles of several closely linked orphan NHRs in mediating cholesterol metabolism collectively necessitate a comprehensive profiling of the steroidome in nematodes. Hence this study presents a profile of the nematodes steroidome by using an untargeted ^{13}C - stable isotope pipeline. Given the strikingly diverse steroidome uncovered through this study and the yet unknown endogenous ligands for several NHRs in *C. elegans* we reason using these metabolites as a starting point to mine for NHR ligands in nematodes.

RESULTS

C. elegans are cholesterol auxotrophs, and hence supplementation with cholesterol is strictly required for normal development. This nutritional dependency creates a unique opportunity to use *C. elegans* as a model organism to study the steroid metabolome using stable-isotope labeling. To obtain a comprehensive overview of the cholesterol derived metabolome (the 'steroidome"), we analyzed two nematode species, *C. elegans* and its satellite model *P. pacificus.* Briefly, mixed stage liquid cultures were grown with 5 mg/L of either ^{12}C-cholesterol (as done during routine culture conditions) or with 5 mg/L of $^{13}C_2$-cholesterol, for 24 h to 144 h, alongside bacteria OP50-only controls. For both species we analyzed the *endo*-metabolomes, derived from extraction of the worm bodies, and the *exo*-metabolomes, obtained from extraction of the supernatant media. These samples were analyzed by high resolution LC-MS in both negative and positive electrospray ionization modes, followed by XCMS-based feature detection and

comparative analysis using the MetaboSeek software suite (Developed by M. Help, Schroeder lab, http://metaboseek.com, *in prep.*[28, 29]). All features presenting a mass shift of +2.0067 (within 5 ppm accuracy), within a retention time difference of 1 sec, and incorporating at least 35% of the $^{13}C_2$ label were curated as cholesterol-derived features **(Figure 3.2a)**.

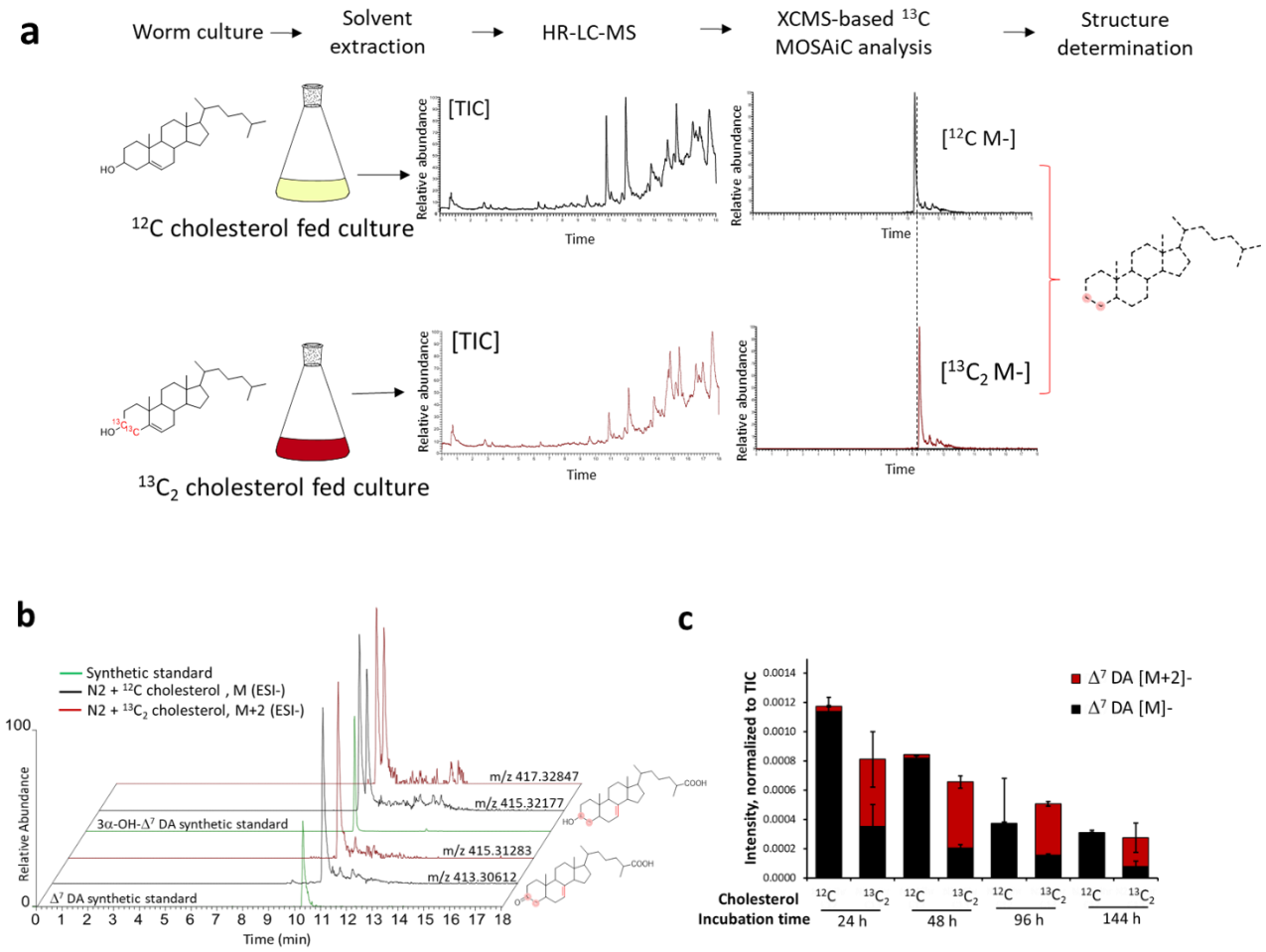

Figure 3.2: Validation of nematode steroidome using unbiased ^{13}C metabolomics pipeline (a) Schematic of ^{13}C stable isotope-based metabolomics pipeline used in this study to systematically profile cholesterol-derived metabolites in nematodes. **(b)** Representative EICs validating incorporation of $^{13}C_2$-cholesterol into *de novo* synthesis of Δ^7 (m/z 413.30612) and 3α and β-hydroxy Δ^7 (m/z 415.32177) dafachronic acids in *C. elegans* in ESI negative mode. **(c)** Cholesterol incorporation into *de novo* synthesis of Δ^7 dafachronic acid over 144h of $^{13}C_2$ cholesterol feeding (Per time point n=2, error bar ± SE).

In *C. elegans*, dafachronic acids (DAs) regulate reproductive development, lifespan and dauer through interactions with the nuclear receptor DAF-12[20, 30]. Under favorable growth conditions, dafachronic acids are synthesized enzymatically using cholesterol as a precursor, with key enzymes being its rate-limiting enzyme DAF-36 converting cholesterol to 7-dehydrocholesterol and DAF-9 adding the terminal carboxylic acid group[21, 31]. Hence, to validate the efficiency of our ^{13}C-stable isotope metabolomics pipeline we first analyzed cholesterol incorporation into the known dafachronic acids. Each of the known DAs (Δ^7-, $\Delta^{1,7}$- and 3α/β-OH-Δ^7-DA) showed significant incorporation of the labeled cholesterol, validating their *de novo* synthesis from cholesterol and their detection through our LC-MS method **(Figure 3.2b)**. Additionally, we noted that over the course of incubation from 24 h to 144 h, the percentage incorporation of the $^{13}C_2$ cholesterol label only mildly increased from 56% (at 24 h) to 72% (at 144h) for Δ^7 DA **(Figure 3.2c).** While this suggests the high turnover rate and the dynamic production of DAs**,** the unlabeled portion likely represents metabolites directly taken up from the egg or trace amounts of contaminant sterols and not actively synthesized by the worm. Over the 144 h period however the overall DA production significantly decreased by 67% (likely due to accumulation of dauer pheromones such as induced by increased culture density). Hence as a compromise between the percentage cholesterol incorporated and abundance of steroidal small molecules (using Δ^7 DA as a proxy), the data presented hereon are from the 48 h time point analysis at which Δ^7 DA showed almost 70% $^{13}C_2$ label incorporation **(Figure 3.2c)**.

Combining the *endo*- and *exo*-metabolome LC-HRMS data, we detected a total of 450 features in *C. elegans* that fit all of our selection criteria **(Table 3.1, Figure 3.3a)**. After manual quality control check, we then removed any features that merely represent natural ^{13}C abundance, LC/MS

adducts of known DAs, bacterial OP50 derived compounds from bacterial control experiments and any known or previously studied sterols **(Figure 3.4)**. After this clean-up, we detected 156 new sterols (48 in ESI negative ionization modes and 108 in ESI positive) that are synthesized *de novo* from cholesterol in *C. elegans.* Further, we noted that under our chromatography conditions, 48 of these compounds in ESI negative ionization modes clustered within a narrow m/z range of 400-550 and likely constitute the family of endogenously produced sterols in *C. elegans* while 32 of these had higher masses, were more non-polar and likely represent sterols with lipid side chains **(Figure 3.3b)**. Most new sterols identified in ESI positive were either unsaturated derivatives of 7-ketocholesterol or sterol esters. This observation also implies that the cholesterol taken up from food source is retained largely within the steroid family of small molecules and under our growth conditions intersects with very few other pathways (if at all). Additionally, our unbiased metabolomics also identified cholesterol-dependent *de novo* biosynthesis of DAs in *P. pacificus*, suggesting conservation in biosynthetic machinery of steroidal compounds between *C. elegans* and *P. pacificus.* However, analysis of *P. pacificus* revealed only 10 cholesterol-derived compounds, 5 of these (including 3 DAs) were sterols found in *C. elegans* as well **(Figure 3.3a)**.

Among the 48 newly identified sterols in ESI negative mode, we assigned more than 20 features as sterol acids based on their ionization in the negative mode (also referred to as Unknown Sterol Acids or USAs). Several of these sterol acids occur as isomers that possibly differ in placement of double bonds and hydroxy groups **(Figure 3.5)**. Hence, at large, 8 new groups of sterol acids each with 2 to 6 isomers were identified. Using MS/MS we could in part confirm the polyhydroxy

nature of these sterol acids through their subsequent loss of water **(Figure 3.6)**. However further characterization via NMR would be desired to assign exact structures.

Table 3.1: Overview of features detected in $^{13}C_2$-labled cholesterol feeding experiments.

Sample type	Features in ESI-	Features in ESI+
C. elegans $^{13}C_2$ cholesterol fed pellet	173 (with adducts)	277 (with adducts)
C. elegans $^{13}C_2$ cholesterol fed media	226 (with adducts)	207 (with adducts)
P.pacificus $^{13}C_2$ cholesterol fed pellet	10	103
P.pacificus $^{13}C_2$ cholesterol fed media	11	53
Op50 $^{13}C_2$ cholesterol fed pellet	1	0
Op50 $^{13}C_2$ cholesterol fed media	n.d.	n.d.
Op50 $^{13}C_3$ Δ^7DA fed pellet	7	1
Op50 $^{13}C_3$ Δ^7DA fed media	n.d.	n.d.
DA synthetic standards	9	6

*****n.d. not determined

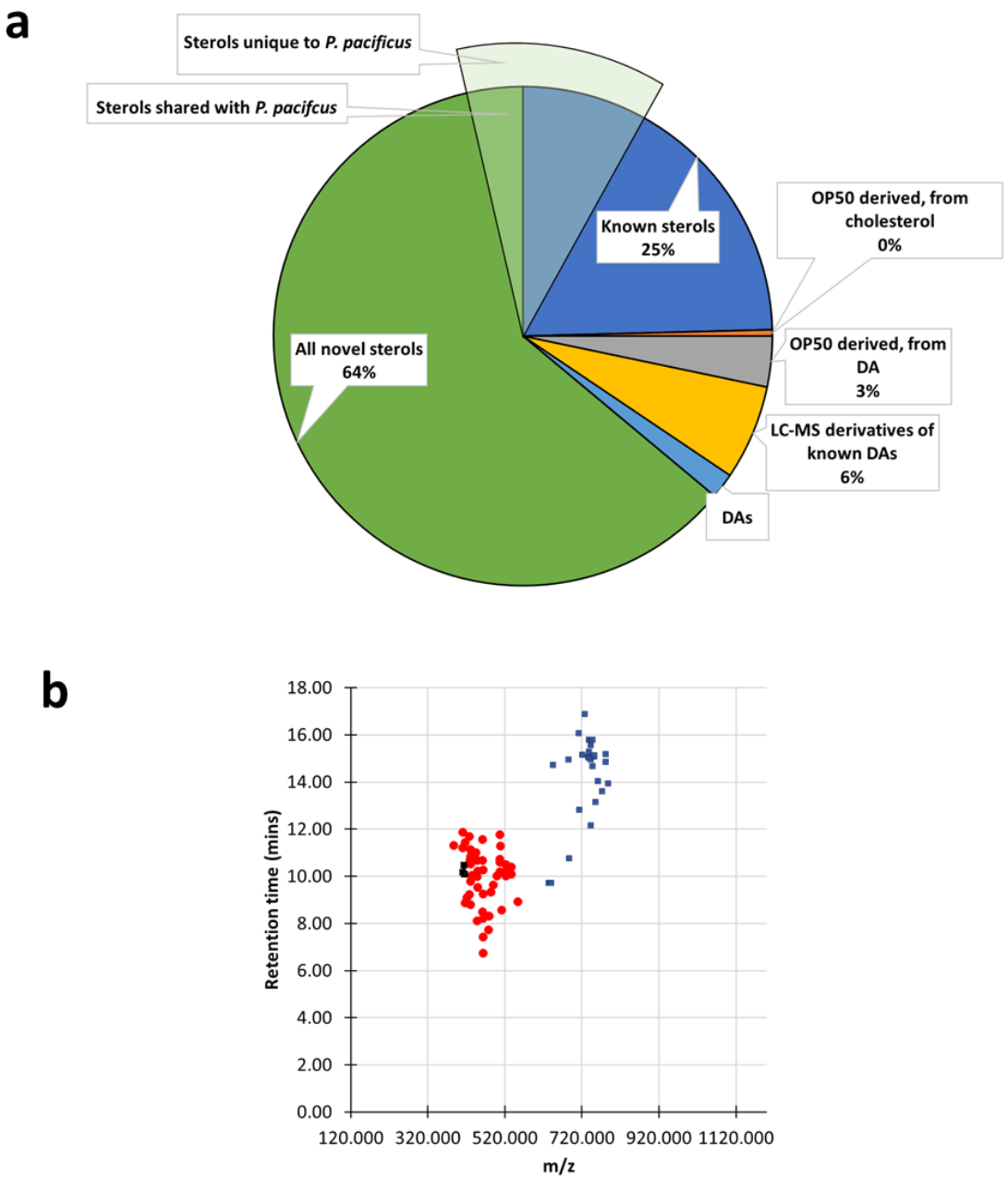

Figure 3.3: Data overview of unbiased characterization of nematode steroidome (a) Combining endo- and exo-metabolome data, a total of 196 features derived from cholesterol incorporation were detected. After removal of mass spectrometric adducts and bacteria OP50-derived features, 80 features remained, of which 48 were classified as novel endogenous sterols and 32 as likely sterols with lipid chains. The previously known *C. elegans* DAs as well as three of the newly identified sterols were common between *C. elegans* and *P. pacificus*. **(b)** m/z and retention time based clustering of known dafachronic acids (in black), 48 new sterols (in red), and 32 likely sterols esters with lipid side chains (in blue).

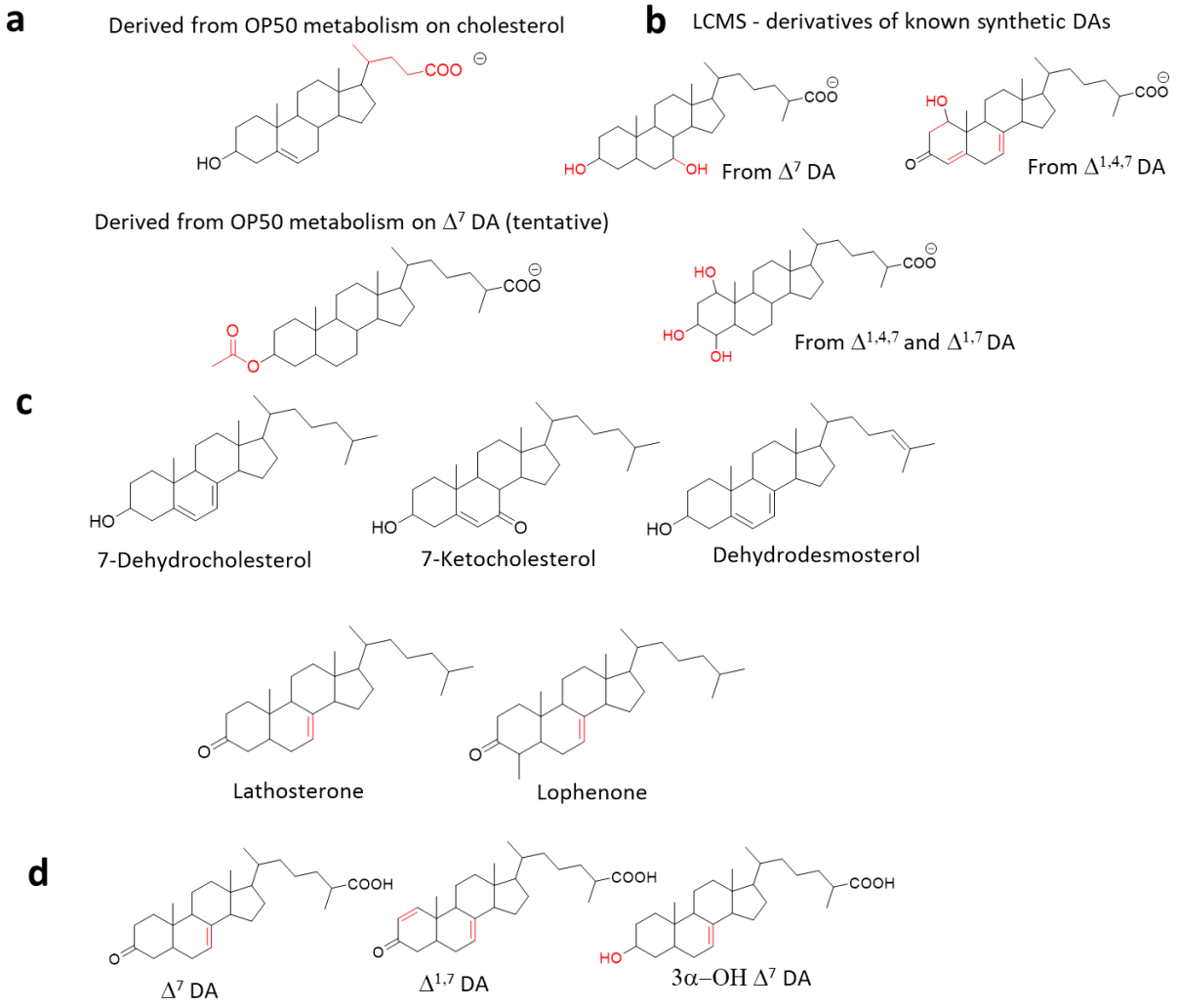

Figure 3.4: Bacteria derived, and known sterols identified in this work. **(a)** Cholesterol and DA-derivatives obtained from OP50 bacterial metabolism. **(b)** LC-MS derivatives of known synthetic DAs. **(c)** Structures of known cholesterol derivatives identified in ESI+ mode. **(d)** Structures of previously characterized dafachronic acids identified in ESI- mode.

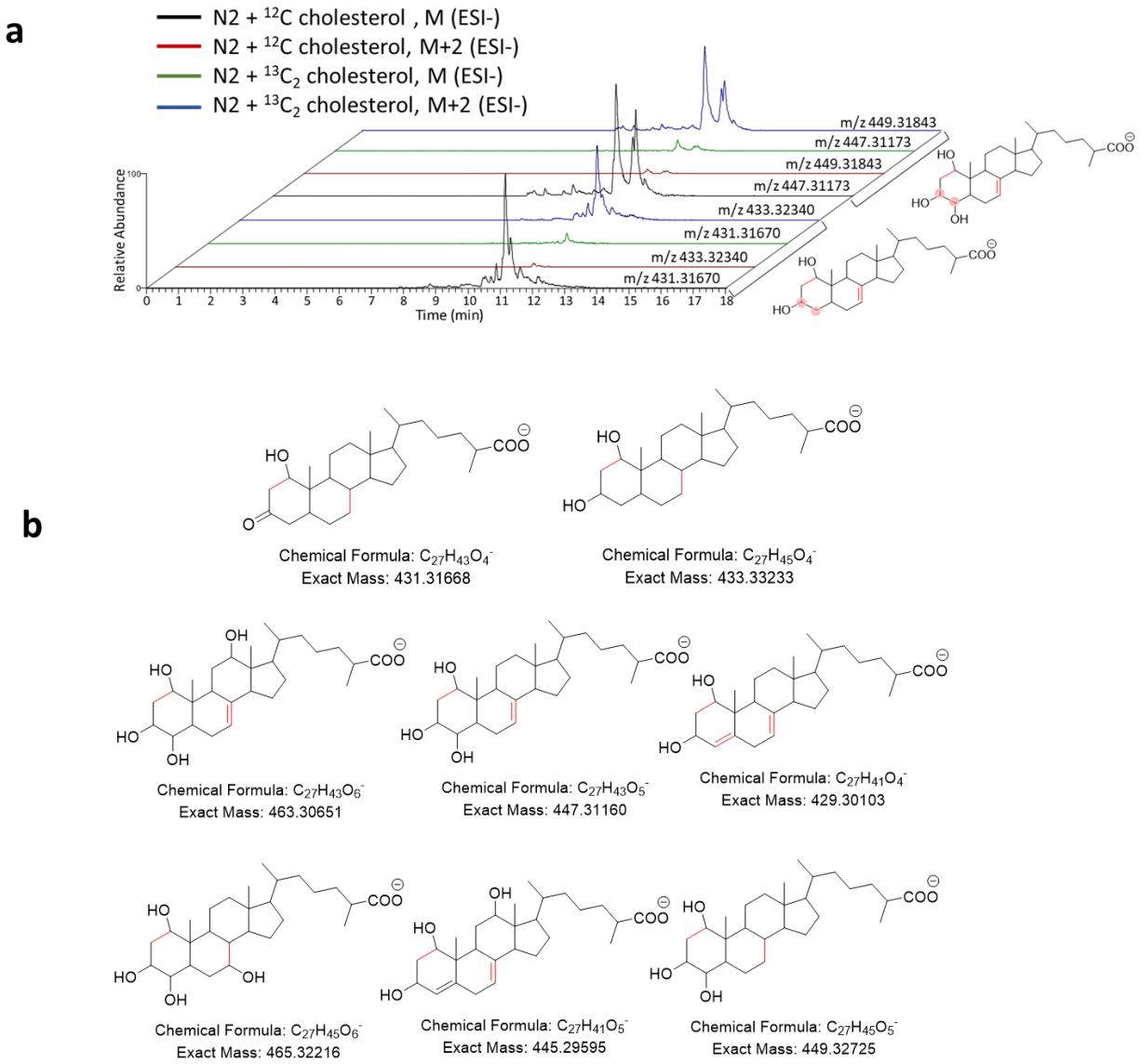

Figure 3.5: Examples of proposed structures of newly identified sterol acids. (a) Representative chromatograms and proposed structures of new sterol acids 1,3-dihydroxy Δ^7 (m/z 431.31670) and 1,3,4-trihydroxy Δ^7 (m/z 447.31173) identified in ESI-. Multiple peaks for the same m/z value likely represent isomers. **(d)** Proposed structures of mono/poly-hydroxy sterol acids found in both *C. elegans* and *P. pacificus*. Shown structures are proposed on the basis of MS/MS analysis and should be considered tentative, with regard to placement of hydroxy groups and double bonds.

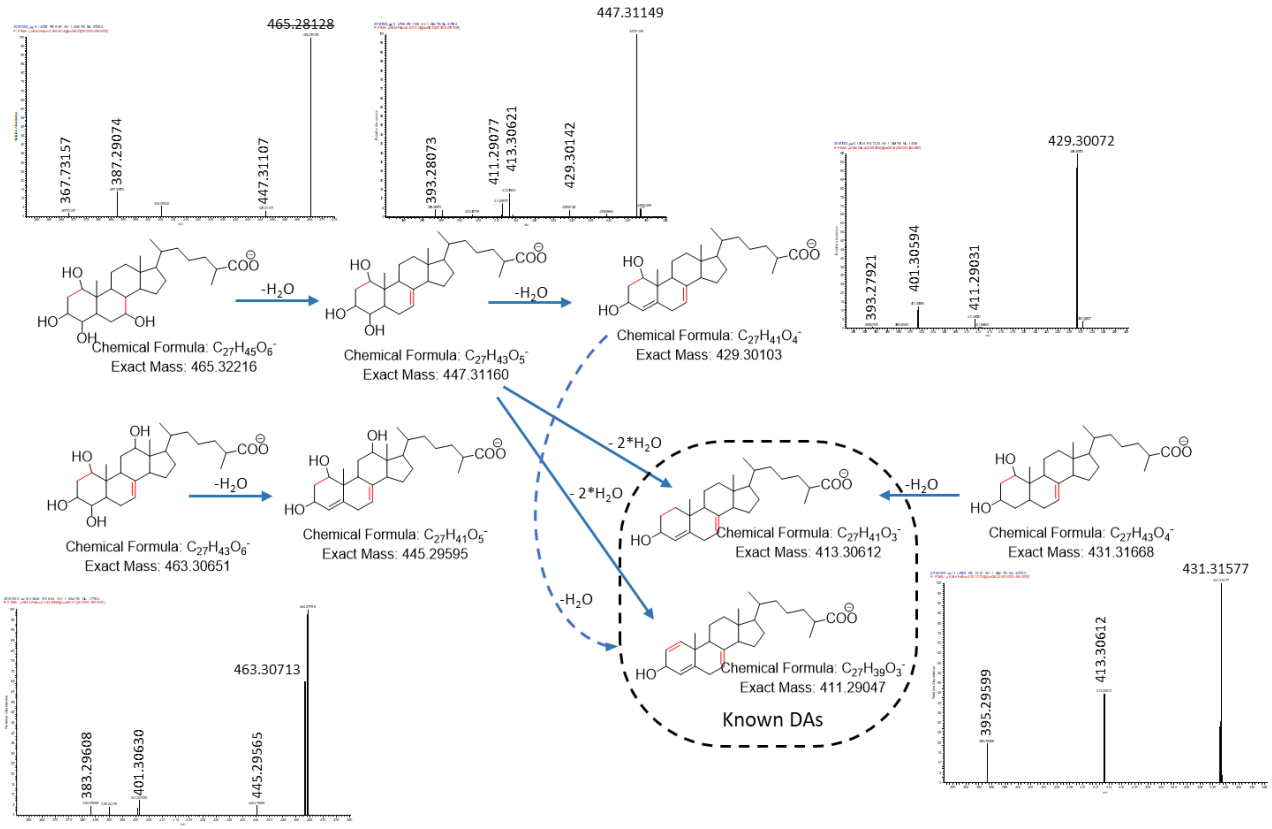

Figure 3.6: MS/MS fragmentation of newly identified sterol acids. MS/MS fragmentation in ESI- showing multiple water losses, indicating polyhydroxylation of the steroid core in the newly detected sterol acids.

In *C. elegans, daf*-9 is strictly required for DA production as it catalyzes the last step of their biosynthesis, introducing the carboxylic acid moiety in their side chain[32]. We asked if the *C. elegans* sterol acids (USAs) identified above are synthesized using the DA biosynthetic machinery and are hence downstream products of DAs. Since *daf-9(dh6)* mutant worms enter dauer unless exogenously supplemented with DAs, feeding *daf-9(dh6)* mutants with labeled DA enables controlled interrogations of pathways and metabolites specifically derived from DAs.

With the aim to analyze yet unknown DA-derivatives as well as to check if the newly identified sterol acids were derived from DAs, we synthesized $^{13}C_3$ labeled Δ^7 DA and tracked its label incorporation in *daf-9(dh6)* mutant worms. Prior to analysis, *daf-9(dh6)* worms were grown for 4 generations on physiologically relevant concentrations of (100 nM) $^{13}C_3$ labeled Δ^7 DA to ensure near complete replacement of natural isotope ^{12}C Δ^7 DA from previous culture conditions **(Figure 3.7a)**. We also confirmed that $^{13}C_3$ labeled Δ^7 DA rescues *daf-9(dh6)* dauers as effectively as ^{12}C Δ^7 DA, at concentrations as low as 10 nM. Additionally, $^{13}C_3$ labeled and ^{12}C Δ^7 DA have comparable efficacies in activating DAF-12 under *in vitro* conditions. Tracking the $^{13}C_3$ label in the $^{13}C_3$ - Δ^7 DA fed *daf-9(dh6)* worms, we only observed production of 3α-OH Δ^7 DA. All other labeled compounds identified were more non polar than Δ^7 DA itself **(Figure 3.7b)**. Based on MS fragmentation patterns these are likely sterol esters with lipid side chains **(Figure 3.7d)**. These features were identified in both $^{13}C_2$ cholesterol fed N2 worms and $^{13}C_3$ Δ^7 DA fed *daf-9* worms.

Surprisingly, we observed that none of the newly identified sterol acids incorporated the Δ^7 DA label suggesting that these compounds are not derived from Δ^7 DA in *daf-9* worms **(Figure 3.7c)**. Furthermore, these sterol acids were present in *daf-9(dh6)* mutant worms at levels comparable to wildtype N2, indicating that their biosynthesis is not dependent on *daf-9* and thus distinct from the dafachronic acid family of sterol acids, all of which are strictly *daf-9* dependent. Our results pose the intriguing possibility that the newly identified family of sterol acids are synthetized through a yet unknown route and are tailored by yet unidentified CYPs that function as sterol side chain oxidases.

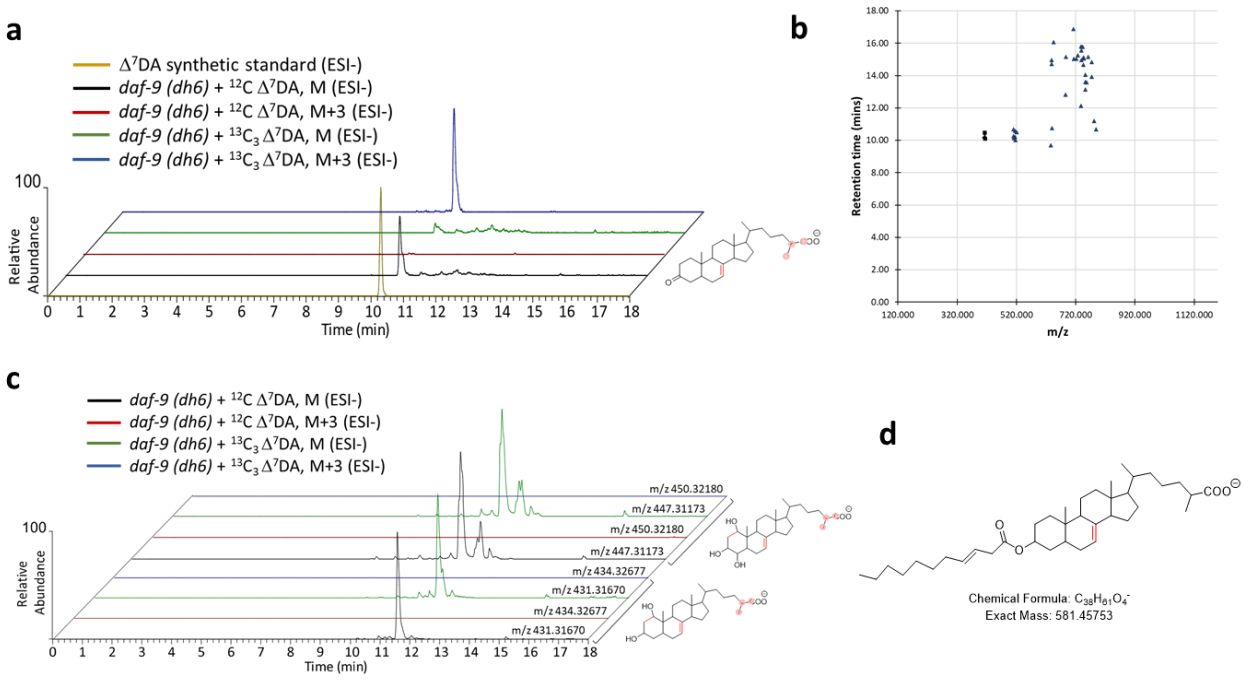

Figure 3.7: *daf*-9/dafachronic acid – independent biosynthesis of newly identified sterol acids **(a)** *daf-9(dh6)* mutant worms cultured for four generations in $^{13}C_3$ labeled Δ^7 DA show near complete replacement of ^{12}C labeled Δ^7 DA. **(b)** m/z and retention time based clustering of known dafachronic acids (in black circles), likely sterols with lipid side chains (in blue triangles) in $^{13}C_3$ labeled Δ^7 DA fed *daf-9(dh6)*. **(c)** *daf-9(dh6)* mutant worms produce unlabeled sterol acids and hence these sterol acids are not derived from $^{13}C_3$ Δ^7 DA. **(d)** Tentative structure for sterol-ester derived from Δ^7 DA, shown here specifically m/z 581.45753 in ESI-.

The enzyme DAF-36 serves as the first and rate limiting step in DA biosynthesis converting cholesterol to 7-dehydrocholesterol. Since the USAs were not *daf-9* dependent, but were derived from cholesterol, we next analyzed USAs in *daf-36(k114)* mutants, along with *hsd-1(mg345)* (the 3β-hydroxysteroid dehydrogenase ortholog known for its ambiguous involvement in DAF-12 ligand production[40]) and *strm-1(tm1781)* (Sterol-A-Ring Methylase, known to produce lophenol from lathosterol and lathosterone). In comparative metabolome analysis of these mutants, we validated that 7-dehydrocholesterol and dafachronic acids was absent in *daf-36(k114)*. While, in our samples lophenol was not detected, lophenone and a likely methyl-hydroxy sterol was absent

in *strm-1(tm1781)* mutants. These 2 metabolites were also absent in *daf-36(k114)* mutants confirming that *strm-1* works downstream of *daf-36*. Interestingly, all USAs identified previously were completely absent in *daf-36(k114)* mutants, confirming that indeed these are cholesterol-derived sterols and likely use DAF-36 towards their production **(Figure 3.8)**.

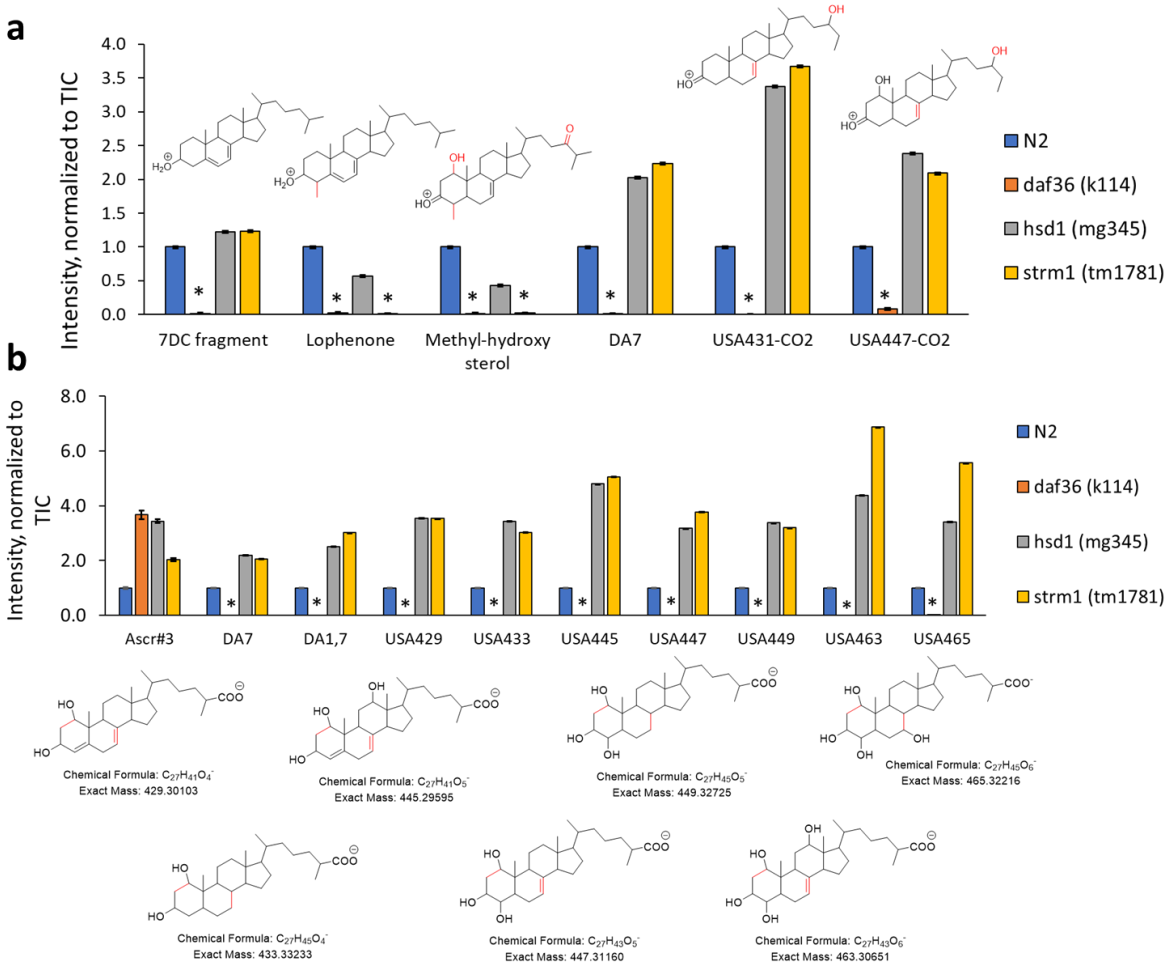

Figure 3.8: Characterization of sterols and sterol acids (USAs) in DA biosynthetic mutants. (a) As previously described 7DC is absent in *daf-36* mutants. Lophenone and tentative methyl-hydroxy sterol are absent in *daf-36* and *strm-1* mutants. Decarboxylation products of sterol acids (USAs) are absent in *daf-36* mutants. Data represent m/z as detected in ESI+ mode. **(b)** Dafachronic acids and all identified sterol acids (USAs) are absent in *daf-36* mutants. Data represent m/z as detected in ESI- mode. N=3, error bar ± S.D.

DISCUSSION

Steroidal ligands play a central role in regulating nuclear receptor transcription activity and thereby its signaling functions towards organismal development, reproduction and metabolism[17, 22,33]. However, the low abundance of such steroidal signaling molecules coupled with the challenges in spectrometry based detection methods has severely restricted the systematic curation of the steroid metabolome across animal kingdoms. But *C. elegans* being a cholesterol auxotroph makes a unique model organism to explore the steroidal structural diversity and in turn further our understanding of nuclear receptor biology.

In this study, we profiled the cholesterol-derived metabolome of nematodes *C. elegans* and *P. pacificus* using a ^{13}C-stable isotope based untargeted metabolomics approach. Our results reveal an unsuspecting diversity in the nematode steroidome than previously assumed. After removal of chromatographic adducts and bacteria OP50-derived metabolites, our results revealed several yet unknown endogenous sterols, with limited conservtaion between *C. elegans* and *P. pacificus.* We must note that the molecules identified through this study may only represent a fraction of the steroidome owing to challenges in extraction and LC-MS ionization efficiencies, along with the likely transient synthesis and rapid turnover of these signaling molecules. While few published works have expanded on the autooxidation properties of cholesterol[34, 35], our own observations on how several synthetic DA mixtures non-enzymatically formed oxysterol-like DA derivatives hints on the overall reactive and unstable nature of these molecules.

We found that most features identified belong within the steroidal family of compounds, suggesting limited intersection between cholesterol metabolism and other primary metabolism pathways. Strikingly, almost 40% of the sterol-features identified are likely sterol acids (USAs),

closely resembling the dafachronic acid family of DAF-12 ligands but differing from them minutely by unsaturations and/or hydroxy groups. We further identified that these USAs were dependent on *daf-36 but* were not derived from dafachronic acids and that their biosynthesis was *daf-9* (the canonical cytochrome P450 in DA production) independent. These finding questions our perception of the role(s) of *daf-9*. In our current understanding, *daf*-9 is strictly required for DA production and DAF-12 mediated development, reproduction and lifespan[32]. While *daf-9* mutants fail to reproduce or survive due to the absence of DAs, when exogenously supplemented with small amounts of DAs (low nano molar range) the mutants function seamlessly as reproductive adults, making *daf*-9 a gate keeper of cholesterol metabolism and overall organismal development. This by itself is intriguing on the role(s) of cholesterol and its non-DA metabolites? Our finding that there exists a rather large pool of other such steroidal, non DA metabolites, adds to questions on their utility in orchestrating *C. elegans* biology. Additionally, in future work it is of prime importance to clarify the true enzymatic nature of USA production as opposed to mere autooxidation of the highly reactive 7-dehydrocholesterol by performing similar feeding studies with synthetic 7-dehydrochoelstrol.

The identification of polyhydroxy-sterols and sterol acids in *C. elegans* is reminiscent of mammalian oxysterols and bile acids[6, 36]. Indeed, in mammals bile acids play major roles in solubilization of lipids and as ligands in nuclear receptor mediated signaling (eg: bile acids CDCA and CA serve as natural ligands of FXRs)[37, 38]. Several studies have demonstrated that the biosynthesis of a full complement of mammalian bile acids requires at least 17 different enzymes of the cytochrome P450 (CYP) family, each of which is under tight regulatory control and is spread across several cellular compartments[36, 39]. Since the sterols and sterol acids identified in this study

are endogenously produced and are largely independent of bacterial metabolism, its exposes the complexity of steroid tailoring enzymes present in nematodes. Additional NMR-based characterizations will help confirm the structures of these molecules and assign potential enzymes needed for their biosynthesis as well.

Several genetic analyses demonstrate intersecting roles of NHR-8 (the DAF-12 paralog) in cholesterol and fatty acid metabolism and *daf-9* independent lifespan[26, 27, 41]. While our and other labs have observed no NHR-8 or NHR-48 activation by DAs leaving these receptors orphan, the sterol acids identified in this study may serve as starting points to mine for these nuclear receptor ligands in nematodes.

Further, while the overall *P. pacificus* steroidome showed lesser steroidal diversity than *C. elegans*, we identified *de* novo biosynthesis of dafachronic acids in *P. pacificus* demonstrating conservation in the chemical structures of these DAF-12 ligands[42]. We further see that DAs are excreted in physiologically relevant concentrations by both *C. elegans* and *P. pacificus*, raising the possibility of inter-species regulation of development by DAs and other sterols[43]. Interestingly, our labeled Δ^7 DA reveled conversion to 3α-OHΔ^7 DA. But apart from this DA, all other DA-derived compounds appear to be sterol esters. Based on mammalian cholesterol ester studies, it appears that nematodes could convert DAs into such relatively inert storage forms and package into lipid droplets for further metabolism[44, 45].

Overall, we believe that the ^{13}C-based untargeted metabolomics approach presented here demonstrates its value in exploring the vast metabolome and that its application here in

nematode steroidome profiling opens new avenues towards exploring the structural and functional small molecule diversity.

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OUTLOOK

Organisms constantly adapt to their changing environments through nuclear receptor mediated signaling cascades. Small molecules relay exogenous cues endogenously via cognate nuclear receptor binding. This further alters gene expression profiles and ultimately an organism's behavior. Such precise and organized biomolecular interactions together form a deeply interconnected web of cellular metabolism and ultimately organismal physiology. A robust and representative model organism to study such a convoluted network of interactions is the nematode *C. elegans,* owing to its homology to receptor signaling cascades in higher animals and genetic tractability. This dissertation work combines tools of protein biochemistry with small molecule metabolomics to explore nuclear receptor interactions and small molecule mediated signaling in nematodes. Together it unravels the deeply interconnected and fine-tuned nature of small molecule ligand - receptor interactions that are central to nematode development.

We begin by characterizing a key enzyme in steroidal ligand production in *C. elegans* in *Chapter 1*. It helps us appreciate how the pool of steroidal ligands in *C. elegans* is complex and highly labile. Our findings allude to the chemical nature of such small molecule signals that likely serve the biological purpose of ensuring rapid on/off and fine tuning of receptor-mediated expression profiles. It stresses on how future studies to explore the tissue- and/or developmental - stage specific synthesis of these ligands would be central towards our understanding on these finely-tuned signaling systems. Further investigations into gene expression profiles of animals after treatment with different ligands will provide insights into functional differences between ligands, but this work acquired a deeper understanding of how these multiple ligands are synthesized as a start to such revelations.

Understanding that there exists a much larger ligand pool than previously assumed, we next deepened our understanding on ligand mediated nuclear receptor and coregulatory protein interactions in *Chapter 2*. Our findings support the combinatorial roles of a triad of receptors that together mediate nematode development through dimer formations and reveal an example family of phospholipids co-regulated by such receptor complexes. The collective information presented in this chapter on receptor biology, coregulatory protein interactions and their roles in metabolome regulation is a leap towards us understanding signaling mechanisms in higher organisms. However, the next challenges involve the search for the endogenous ligands of these nuclear receptors. Only 50% of mammalian receptors have known ligands but 50% of these have eventually been useful drug targets. Nematodes like *C. elegans*, with almost 280 orphan receptors, present a vast, untapped mine of unidentified small molecule signaling metabolites. Moving forward, endogenous ligand discovery studies would make *C. elegans* a more powerful model to study aging, metabolism and development.

The currently known endogenous ligands for mammalian receptors consist of a wide range of chemical structures, such as bile acids, phospholipids, steroid hormones, retinoids, and vitamins. Further, it has been suggested that the same receptor may have distinct endogenous ligands to enable altering its functions in distinct cell types or cellular conditions. This not only necessities a systematic exploration of small molecule ligands organism-wide, but also perhaps in a distinct cellular compartments. Yet, the characterization of endogenous ligands has remained largely incomplete owing to multiple reasons. Primarily, ligands constitute only minor components of highly complex metabolomes, and hence low ligand abundance coupled with limited detection sensitivity makes identification difficult and inaccurate. Secondly, failure to consider that an

exogenous compound can be metabolized intracellularly to form the actual ligand capable of activating the receptor has resulted in questionable claims on the "endogenous" nature of identified ligands in the past. Lastly, the bias created in the class of ligands being screened through traditional candidate-based approaches, has severely constrained our chances of identifying novel classes of ligand molecules. Hence, to date, orphan nuclear receptors represent a diverse yet largely untapped resource to uncover biological regulatory systems via identification of their endogenous ligands.

Realizing the structural and hence likely functional diversity in nematode signaling steroids in *Chapter 1*, and their potential roles as endogenous receptor ligands in *Chapter 2*, we implemented an unbiased metabolomics approach to comprehensively profile the nematode "steroidome" in *Chapter 3*. To our surprise, the *C. elegans* steroidome is strikingly diverse, presenting minute structural differences and yet unidentified biosynthetic routes. We believe using these novel compounds as targets for future receptor ligand searches will speed-up endogenous ligand discovery. Additionally, understanding their syntheses and regulation will prove central to our understanding on the myriad of steroidal functions they perform.

For any perturbation introduced to a given biological system, there is a corresponding molecular signature. The high-resolution mass spectrometry based untargeted metabolomics pipeline described in *Chapter 3* of this dissertation has demonstrated its potential in exploring such signatures. The extent of structural and functional information obtained for steroidal compounds that make up less than 0.1% of the nematodes stands testament to its applications. We believe that this combinatorial metabolomics and protein biochemistry pipeline should eagerly be

harnessed to study various organisms, from bacteria to humans, to understand the roles of biogenic signaling molecules at organismal, tissue-specific, cellular and sub-cellular levels.

Organismal biology is highly complex and the nematode *C. elegans* is no exception. However, unlike other organisms, *C. elegans* has once again proven to be a unique system to study conserved signaling mechanisms of development and aging. In unraveling some aspects of *C. elegans* biology through this dissertation, we also unravel how much more there is to explore and hope future discoveries will benefit from the findings presented here.

APPENDIX A

SUPPLEMENTAL INFORMATION FOR CHAPTER 1

Materials and methods –

Mutants used here were created by J. Wollam, A. Antebi lab. Data shown in Figures 1.3, 1.4, 1.5, 1.6a and 1.8a were performed by J. Wollam, A. Antebi lab and described elsewhere.

All synthetic dafachronic acids used here were synthesized by Russell Burkhardt (Schroeder lab) and will be reported in detail elsewhere.

C. elegans strains

Worms were grown on NGM agar seeded with the *E. coli* bacteria OP50 at 20°C on NGM containing cholesterol at a 5 µg/mL concentration. The following genotypes were used: N2, *daf-36(k114)*, *dhs-16(tm1890), daf-40(hd100), daf-9(dh6), daf-12(rh61rh411)*, *hsd-1(mg433), daf-2(e1368)*, *daf-2(e1370)*, *daf-7(e1372), daf-5(e1386), daf-16(mu86)*, *daf-16(mgDf50), daf-22(m130), daf-40(hd100);daf-12(rh61rh411), daf-40(hd100);daf-16(mgDf50), daf-40(hd100);daf-5(e1386), daf-40(hd100);daf-36(k114), daf-40(hd100);dhs-16(tm1890), daf-40(hd100);daf-9(k182), daf-40(hd100);daf-9(dh6), daf-40(hd100);daf-2(e1368), daf-40(hd100);daf-2(e1370), daf-40(hd100);daf-22(m130), dhIs64(Pdaf-9::daf-9::gfp), daf-40(hd100);dhIs64(Pdaf-9::daf-9::gfp), dhEx460(Pdaf-40::daf-40::gfp;coel::rfp), dhIs839(Pdaf-40::daf-40::gfp;coel::rfp).*

Metabolome extraction

Worms were grown on 10 cm NGM agar plates seeded with OP50 bacteria at 20°C. Gravid adults were bleached and the resulting embryos transferred to 3-5 fresh 10 cm plates. After two to three successive rounds of growth, worms from 3 individual plates were washed into 25ml liquid culture, in S-complete media, grown at 20°C, 220rpm. Worms were fed on days 1, 3 and 5 with concentrated OP50 and harvested as mixed culture on the 7th day. Media and worm pellets were analyzed separately. For pellet (endometabolome) analysis, pellets were dried by lyophilization, crushed in 1.5 ml Eppendorf tubes with 3 small metal balls in a tissue grinder (Cryoblock, Model 1660) that was cooled with liquid nitrogen. Samples were subjected to disruption at 1100 RPM for three rounds of 2 X 30 sec with re-cooling in liquid nitrogen between rounds. Crushed material was transferred to 4 mL glass vials with 9:1 ethyacetate:ethanol solvent for overnight extraction at room temperature with a stir bar. Following day, the extract was separated from insoluble material by centrifugation, dried in a Speedvac Concentrator (Thermo Scientific) and resuspended in 100% ethanol for MS analysis. For media (exometabolome) analysis, media was dried by lyophilization, followed by two consecutive rounds of extraction – first with 100% ethanol and then with 9:1 ethyacetate:ethanol solvent for 12 hours each. Both extracts were combined, dried in a Speedvac Concentrator and resuspended in 100% ethanol for MS analysis. All extracts were stored at -20°C.

Mass spectrometry analysis

Reverse-phase chromatography was performed using a Dionex Ultimate 3000 Series LC system (HPG-3400 RS High Pressure pump, TCC-3000RS column compartment, WPS-3000TRS autosampler, DAD-3000 Diode Array Detector) controlled by Chromeleon Software (Thermo Fisher Scientific) and coupled to Orbitrap Q-Exactive mass spectrometer controlled by the Xcalibur software (Thermo Fisher Scientific). A Kinetex EVO C18 150mm x 2.1mm 1.7um at 40^{o}C and the flow rate 0.5 mL/min of mobiles phases A (H_2O, 0.1% Ammonium Acetate) and B (100% AcN) was used for separation of target molecular features. The gradient starting condition was 0%B for 0.5 min, rising to 100% B in 13.5 min, was held for 4 min, and followed by 3 min re-equilibration at the starting condition. A heated electrospray ionization source (HESI-II) in negative mode was used for the ionization of dafachronic acids with parameters of spray voltage at 3 kV, capillary temperature at 320^{o}C, sheath gas and auxiliary gas flow at 70 and 2 arbitrary units respectively, and probe heater temperature 300^{o}C. For post column ionization: D (H_2O 1% ammonium solution) directly to post column. The data were acquired in m/z range of 200-800, 140,000 FWHM resolution, AGC target 3e6, maximum injection time of 500 ms. Synthetic dafachronic acids were used as standards to monitor mass accuracy, instrument sensitivity and relative concentrations in biological samples. All dafachronic acids were detected as $[M-H]^-$ ions in ESI negative ionization mode. Quantification of dafachronic acids in mutants are represented as normalized intensity in corresponding wildtype samples.

Isolation of microsomes

HEK293T cells were grown in 10 cm plates with DMEM + 10% FBS + 1% penn-strep and transfected with pCMV::FLAG vector only or FLAG-tagged DAF-40 using Lipofectamine 3000 (Thermo Fisher Scientifc). Expression of DAF-40 was verified by immunoblot, using anti-FLAG antibodies (Sigma). For isolation of microsomes cells were harvested (90-100% confluent) using a sterile scraper, into 50 mL PBS and pelleted by centrifuging 10 min at 1000 x g, room temperature, using tabletop centrifuge. Supernatant was decanted and and cell pellets were resuspended in 8 mL ice-cold sucrose homogenization medium containing freshly added protease inhibitor cocktail. Cells were then disrupted in a Dounce homogenizer. Nuclei, cell debris, and unbroken cells were pelted by low-speed centrifugation, two times for 5 min at 600 x g, 4^{o}C. Supernatant was then centrifuged for 10 min at 10,300 x g, 4^{o}C. Microsomes were pelleted by ultracentrifuging supernatant (in pre-weighed tubes) for 60 min at 100,000 x g, 4^{o}C, using swinging bucket rotor. Microsome containing pellet was resuspended in 0.1 M KPO_4 buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol, and stored at -80°C. Protein content by determined by Bradford Assay.

Microsomal incubations

Microsomes were thawed on ice and brought to 50 µg/mL in 0.1 M KPO_4 buffer, pH 7.4, containing 1 mM EDTA, 48 pmol purified recombinant human cytochrome P450 reductase, and 1 µM hematin. Synthetic dafachronic acid substrates were added at 10-100 µM in 0.25 mL total volume, pre-incubated for 5 minutes at 37°C, and reacted with NADPH regeneration system for 16 hours at 37°C. Ethanol only reactions were used as negative controls. Reactions were

processed by extracting twice with 2 mL MTBE, combining the top layers and drying under argon in glass vials and stored at -20°C. Reactions were resuspended in 100% ethanol just prior to mass spectrometry based analysis.

Luciferase assay for DAF-12 activation

To determine dafachronic acid-mediated DAF-12 transcriptional activation, N-terminal flag tagged expression construct of full length DAF-12 isoform A3 in a pCMV vector backbone was used. HEK 293T cells (grown and passaged in DMEM + 10% FBS + 1% penn-strep) were seeded at 0.8 X 10^6 cells per well (with DMEM + 10% ssFBS + 1% penn-strep media) in 96-well plates and transfected (per well) with 30 ng flag-tagged DAF-12 expression vector, 30 ng of *mir-241p* driven luciferase reporter, and 5 ng β-galactosidase expression vector (used as transfection normalization control) using the calcium phosphate precipitate method. Ethanol (negative control) or ethanolic solutions of synthetic dafachronic acids were added 8 hr post transfection, and the luciferase and β -galactosidase activities were measured by a Synergy 2 BioTek luminometer with Gen5 1.11 software 16 hr post compound addition. Data are represented as luciferase RLU, normalized to b-galactosidase activity. Data shown is represented as average of individual replicates.

Statistical analysis

Results are presented as mean ± SD or SEM, as indicated. *P* values were calculated using GraphPad Prism software or Excel by student's t-test or ANOVA as noted.

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER 2

Materials and methods –

All synthetic dafachronic acids used here were synthesized by Russell Burkhardt (Schroeder lab) and will be reported in detail elsewhere.

Luciferase assays for receptor activation

To determine dafachronic acid mediated NHR transcriptional activation, N-terminal flag tagged chimeric expression constructs of NHR-8 and NHR-48, consisting of DAF-12 DBD$^{1-499}$ and the respective LBD (NHR-8$^{91-560}$, NHR-48$^{163-817}$) in a pCMV vector backbone were created by Gibson assembly. Expression was checked by anti-flag tag Western blotting (Sigma). HEK 293T cells (grown and passaged in DMEM + 10% FBS + 1% penn-strep) were seeded at 0.8 X 10^6 cells (in DMEM + 10% ssFBS + 1% penn-strep media) per well in 96-well plates and transfected (per well) with 30 ng flag-tagged NHR expression vector, 30 ng of flag-tagged DIN-1e, 30 ng of *mir-241p* (or *mire-84p, mir*-48p) driven luciferase reporter, and 5 ng β-galactosidase expression vector (used as transfection normalization control) using the calcium phosphate precipitate method. Ethanol (negative control) or ethanolic solutions of synthetic dafachronic acids were added 8 hr post transfection at a final concentration of 100 nM (unless otherwise specified), and the luciferase and β -galactosidase activities were measured by a Synergy 2 BioTek luminometer with Gen5 1.11 software 16 hr post compound addition. Data are represented as luciferase RLU, normalized to β-galactosidase activity. Data shown is represented as average of atleast four individual replicates.

Preparation of nuclear extract for DAF-12 localization studies

HEK293T cells (at 50-60% confluency, in DMEM + 10% FBS + 1% penn-strep media, in 10 cm plates) were transfected with 10 μg FLAG-tagged full-length DAF-12 (in pCMV vector backbone) using jetPRIME (Polyplus transfections) protocol. 16 hours post transfection, ethanolic solution of Δ^7 dafachronic acid (or 100% ethanol as negative control) was added at 100 nM final concentration (total ethanol volume <0.1%) and 3 days post transfection cells were harvested by pelleting at 4°C. Nuclear extracts were prepared based on Schreiber et al., 1990 with a few changes in buffer composition. Briefly, cells were washed with PBS, dislodged by scrapping and pelted by centrifugation and resuspended in cell lysis buffer A (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 2mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and 0.5% NP-40, in water) and allowed to swell on ice, with intermittent mixing. Tubes were vortexed to disrupt cell membrane, and centrifuged at 12,000 g at 4°C for 10 min. The supernatant was stored as cytoplasmic extract at -80°C. The pelleted nuclei were washed in buffer A and resuspended in nuclear extraction buffer B (25 mM Tris-HCl, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 25% sucrose, in water) and incubated on ice for 30 min. Nuclear extract was collected by centrifugation at 12,000 g for 15 min at 4°C. Protein concentration was estimated by Bradford's' method and equal quantities of

extract were loaded per well. Western blot analysis was performed with anti-Flag antibody (Sigma) and anti-H3 histone (Abcam).

Preparation of metabolome fractions

Wild type (N2) worms from four 10 cm NGM agar plates were washed using M9-medium into 100 mL S-complete medium pre-culture where they were grown for four days at 22 °C, at 220rpm. Concentrated bacteria derived from 1 L of E. coli OP50 culture was added at days 1 and 3. Subsequently, on day 4 the pre-culture was divided equally into sixteen 500 mL cultures, each containing 100 mL of S-complete medium, which was then grown for an additional 5 days at 22 °C, at 220rpm and fed with concentrated bacteria on each day. The cultures were harvested on day 5 and centrifuged at 20,000g for 6 min, at 4°C to separate the supernatant media and worm pellets. At harvest, liquid cultures contained approximately 60% L1-L3 worms.
The worm pellets were then lyophilized to dryness and stored at -20°C. The lyophilized residue was crushed to a fine powder using a mortar and pestle with dry ice. The powder was then extracted twice with 250 mL of 9:1 ethylacetate:ethanol mixture for 12 h each. The resulting yellow-brown suspension was filtered, and the filtrate was evaporated *in vacuo* at room temperature to produce the worm pellet metabolome extract used for chromatographic fractionation.
Fractionation was performed as detailed earlier in P. Mahanti, N. Bose, et al., *Cell Met* 2014. Briefly, 8 g of Celite, prewashed with ethyl acetate, was added a solution of worm pellet metabolome extract from 16 cultures 9as detailed above). After evaporation of the solvent, the Celite was dry-loaded into an empty 25 g RediSep Rf loading cartridge. Fractionation was performed using a Teledyne ISCO CombiFlash system over a RediSepRf GOLD 40 g HP Silica Column using a normal phase dichloromethane-methanol solvent system, starting with 6 min of 100% dichloromethane, followed by a linear increase of methanol content up to 10% at 24 min, followed by another linear increase of methanol content up to 40% at 40 min, followed by another linear increase of methanol content up to 95% at 45 min which was then continued to 48 min. 81 fractions (~25 mL each) generated from the CombiFlash run were individually evaporated in vacuo.
For combining these fractions into select number of pools, each individual fraction was resuspended 2 ml of 100% ethanol, and 15% of this (=300 μl) was added to its respective pool. The 18 pools were made by combining as follows – pool 1 (fraction 4-5), pool 2 (fraction 6-7), pool 3 (fraction 8-10), pool 4 (fraction 11-13), pool 5 (fraction 14-16), pool 6 (fraction 17-19), pool 7 (fraction 20-22), pool 8 (fraction 23-25), pool 9 (fraction 26-28), pool 10 (fraction 29-30), pool 11 (fraction 33-36), pool 12 (fraction 37-40), pool 13 (fraction 41-44), pool 14 (fraction 45-48), pool 15 (fraction 49-53), pool 16 (fraction 54-59), pool 17 (fraction 60-68) and pool 18 (fraction 69-81). Each pool was then individually dried, resuspended in 100 μl 100% ethanol, and used for bioactivity assays (as in Figure 2.7).

Expression and purification of receptors DAF-12, NHR-8 and NHR-48 and DIN-1e

Following receptor domains were cloned into pDEST-20 vector backbone (containing an N-terminal GST tag) by Gateway recombination, and in pFastBac vector backbone (containing N terminal 6X-His tag and C-terminal Strep-II tag) by restriction enzyme cloning – FL-DAF-12, DAF-

12^{1-501} (DBD + hinge), DAF-$12^{281-753}$ (hinge + LBD), FL-NHR-8, NHR-8^{1-108} (DBD + hinge), NHR-$8^{101-560}$ (hinge + LBD), FL-NHR-48, NHR-48^{1-163} (DBD + hinge), NHR-$48^{163-817}$ (hinge + LBD), FL-DIN1e, DIN-$1e^{1-270}$, DIN-$1e^{1-247}$ and DIN-$1e^{307-561}$. Corresponding bacmids were isolated from DH10Bac cells. For P1 virus production, Sf9 cells were transfected with bacmids using Cellfectin-II reagent (Thermo Scientific) in Grace's insect cell media according to manufacturer's protocol. Cells were monitored for infection signs and typically 3-5 days post transfection, protein expression was tested by immunoblotting. Corresponding P1 – P3 baculovirus titer stocks were used for subsequent large-scale infections and purifications, typically at MOI (multiplicity of infection) of 10 or more.

For GST-tagged protein purifications, infected Sf9 cells were harvested at day 5 in ice-cold PBS, and pelleted at 800 g, for 10 min at 4°C. Cell lysates were prepared by sonication in cell extraction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Tween-20, 1 mM DTT, Roche EDTA-free protease inhibitor cocktail and Benzonase, in water). The lysate was cleared by centrifugation at 20,000 g, for 30 min, at 4°C. This lysate was incubated with pre-equilibrated Pierce™ Glutatione Magnetic beads in Binding buffer (125 mM Tris, 150 mM NaCl, pH 8.0) for 4 h, at 4°C. GST-tagged proteins were eluted in freshly made Binding buffer with 50 mM reduced glutathione, concentrated and buffer exchanged with an Amicon centrifugal filter to storage buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 25% glycerol), snap-frozen in liquid nitrogen and stored at -80°C. Purified proteins were analyzed by SDS-PAGE and anti-GST immunoblot.

For 6X-His-tagged protein purifications, protein purification was carried out at 4 °C by resuspending the pellet in lysis buffer (100 mM phosphate buffer, pH 7.5, 10 mM imidazole, 10% glycerol, 1 mM PMSF, 0.1 mg/ml lysozyme and 0.1 mg/ml Benzonase, in water) and sonicated. Lysed cells were centrifuged at 20,000g for 30 min and loaded onto TALON affinity resin (Clontech) pre-equilibrated with lysis buffer and incubated for 4 h at 4°C with rotation. Resin was loaded onto a column and washed with lysis buffer (without lysosome or Benzonase). His-tagged proteins were eluted in lysis buffer with 250 mM imidazole. Only FPLC fractions containing pure protein was pooled, concentrated and buffer exchanged with an Amicon centrifugal filter to storage buffer (100 mM phosphate buffer, pH 7.5, 25% glycerol) snap-frozen in liquid nitrogen and stored at -80°C. Purified proteins were analyzed by SDS-PAGE and anti-His, anti-Strep-II immunoblot.

AlphaScreen assay for direct binding of receptors and dafachronic acid ligands

Purified proteins (as detailed above) were used for AlphaScreen assays. For unliganded assays, 10-100 nM of each protein was added per well of a 384-well Optiplate (PerkinElmer), followed by 1 hour incubation, at room temperature, in absolute dark, with an adhesive seal to prevent evaporation and with mild shaking. Then, per well, 10 μl Acceptor beads (Anti-GST AlphaLISA ® Acceptor beads, PerkinElmer) and 10 μl Donor beads (Strep-Tactin® AlphaLISA® Donor beads, PerkinElmer) (in that order, to a final concentration of 20 μg/mL each) were added while on ice and set for a second incubation for 1 hour, at room temperature, in absolute dark, with an adhesive seal and with mild shaking. The plates were then read using a Synergy 2 Biotek LC luminometer using the manufacturer's Alphascreen detection protocol. In experiments

containing dafachronic acid ligands, ethanolic solutions (0.1% is ideal) of the ligand were used, with 100% ethanol as negative control.

Metabolome analysis

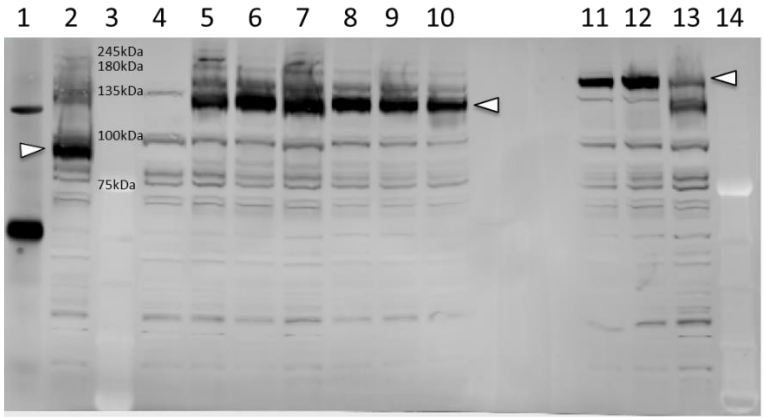
Worms were grown on NGM agar seeded with the *E. coli* bacteria OP50 at 20°C on NGM containing cholesterol at a 5 µg/mL concentration. The following genotypes were used: N2 (wild type), *daf-12(rh411;rh61), nhr-8(ok186), nhr-48(ok178)* and *emb-8(hc69)*. *emb-8(hc69)* being a temperature sensitive mutant, was grown and maintained at 16°C and eggs from bleach-prep were hatched at 16°C for 2 days.

For metabolome analysis, at least 25 mL of each strain was grown in triplicates. Endometabolomes were processed and analyzed by mass spectrometry as detailed in **Appendix A**. Samples were analyzed in both ESI ionization modes. Untargeted data analysis was performed using the MetaboSeek software suite (Developed by M. Helf, Schroeder lab). For data analysis, all features with a minimum intensity of 5e4 showing a 10-fold or more misregulation (up or down regulation when compared to wildtype N2) were included. For *emb-8(hc69)* mutant analysis, a minimum N2 intensity of 1e5, with N2 minimum fold over rest intensity threshold of of 100 was used.

Statistical analysis

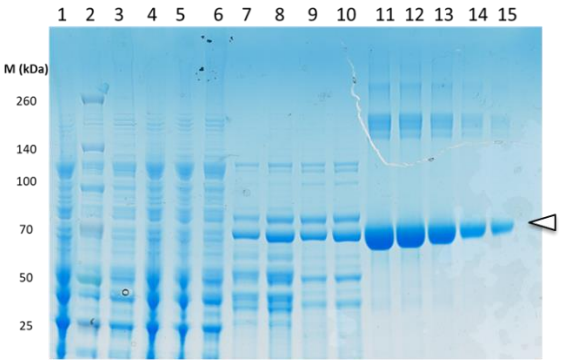
Results are presented as mean ± SD or SEM, as indicated. *P* values were calculated using GraphPad Prism software or Excel by student's t-test or ANOVA as noted.

Supplemental figures –



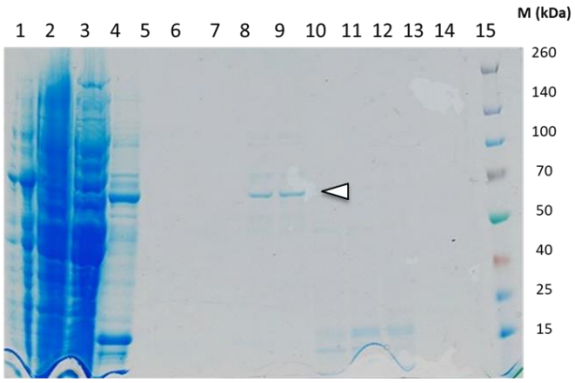
1. Flagged-tagged protein control
2. FL-DAF-12
3. BLUEstain™ Protein ladder, GoldBio
4. Cells only control
5. LH-chi NHR-8 #9
6. LH-chi NHR-8 #10
7. LH-chi NHR-8 #11
8. LH-chi NHR-8 #14
9. LH-chi NHR-8 #17
10. LH-chi NHR-8 #18
11. LH-chi NHR-48 #26
12. LH-chi NHR-48 #27
13. LH-chi NHR-48 #32
14. BLUEstain™ Protein ladder, GoldBio

Figure A2.1: Expression of chimeric NHR-8 and NHR-48 constructs in HEK293T cells. N-terminal flag tagged long-hinge (LH) chimeric (chi) expression constructs of NHR-8 and NHR-48, consisting of DAF-12 DBD$^{1-499}$ and the respective LBD (NHR-8$^{91-560}$, NHR-48$^{163-817}$) in a pCMV vector backbone were tranfected in HEK293T cells and expression in whole cell lysate was checked by anti-flag immunoblot. Expected molecular weights for FL-DAF-12, LH-chi-NHR-8 and LH-chi-NHR-48 are 85 kDa, 110kDa and 132kDa respectively.



1. Insoluble fraction (0.1%)
2. Marker + cells @ 2 days
3. Cells @ 3 days
4. Total soluble (0.1%)
5. Unbound (1%, concentrated)
6. Wash 2 (1% concentrated)

7 -10. Gradient elution with 50mM reduced Glutathione
11-15. Concentrated protein at 10ug/7ug/5ug/2.5ug/1.25ug loading (~ 0.1ug/ul = 1.39uM)



1. Raw Sf9 cell lysate (-ve control)
2. Insoluble fraction
3. Total soluble
4. 250 mM Imidazole elution fractions (X4, pooled)
5. FPLC fraction # 3
6. FPLC fraction # 4
7. FPLC fraction # 5
8. **FPLC fraction # 15**
9. **FPLC fraction # 16**
10. FPLC fraction # 20
11. FPLC fraction # 21
12. FPLC fraction # 22
13. FPLC fraction # 30
14. FPLC fraction # 31
15. Spectra BR marker

Figure A2.2: Purification of DAF-12 and DIN-1e from Sf9 cells. Representative SDS-PAGE gels of of GST::DAF-12 and 6X-His::DIN-1e::StepII purification from Sf9 cells from 25 mL Sf9 cell culture.

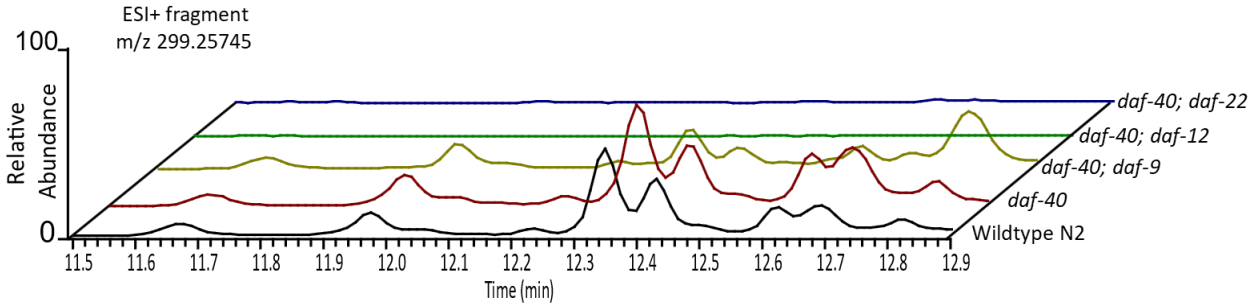


Figure A2.3: *emb-8* regulated glycerophosphoethanolamines are independent of *daf-40* and *daf-9*. e*mb-8* glycerophosphoethanolamines (Shown here is the $C_{18}H_{35}O_3$ fragment, [M+H]+ 299.25745) that is not misregulated by mutations in two *C. elegans* cytochrome P450s - *daf-9(dh6)* or *daf-40(hd100)*, eliminating the possibility of a rather generic defect of P450 enzymatic activity. This fragment (and other e*mb-8* glycerophosphoethanolamines fragments) are however absent in *daf-40; daf-12* and *daf-40;daf-22* double mutants.

Table A2.1: Predominant C17 – C20 acyl and C14 – C20 acyl amine fragments of glycerophosphoethanolamines absent or downregulated in *emb-8(hc69)* mutants.

[M+H]+	rt_mins	ESIpos_Features DOWN/ABSENT in *emb8* mutants	
226.21593	12.40	C14 H28 O N	C14:2
238.21592	12.38	C15 H28 O N	C15:3
240.23148	12.27	C15 H30 O N	C15:2
252.23148	12.81	C16 H30 O N	C16:3
254.24705	13.01	C16 H32 O N	C16:2
266.24704	13.09	C17 H32 O N	C17:3
268.26254	13.39	C17 H34 O N	C17:2
285.24158	12.29	C17 H33 O3	Ether lipid?
278.24716	12.82	C18 H32 O N	C18:4
280.26287	12.58	C18 H34 O N	C18:3
299.25745	11.93	C18 H35 O3	Ether lipid?
294.27816	13.65	C19 H36 O N	C19:3
311.25719	12.28	C19 H35 O3	Ether lipid?
313.27282	13.09	C19 H37 O3	Ether lipid?
306.27809	13.49	C20 H36 O N	C20:3
327.28866	12.40	C20 H39 O3	Ether lipid?

APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER 3

Materials and methods –

All synthetic dafachronic acids used here were synthesized by Russell Burkhardt (Schroeder lab) and will be reported in detail elsewhere.

Worm strains and routine culturing

Strains used in this study are – wild type *C. elegans* (N2), wild type *P. pacificus* (RS2333), *daf-9(dh6)*, *daf-36(k114), hsd-1(mg345),* and *strm-1(tm1781)*.

Routinely, worms were grown on NGM agar plates containing cholesterol at a 5 mg/L concentration, seeded with the *E. coli* bacteria OP50 at 20°C. For *daf-9(dh6)* culture, OP50 was mixed with Δ^7 dafachronic acid at 100 nM final concentration (in 100% ethanol, <0.1% per plate v/v) and seeded onto plates.

Preparation of $^{13}C_2$ labeled cholesterol fed worms

Prior to experiment N2 and RS2333 worms were grown on NGM agar seeded with the *E. coli* bacteria OP50 at 20°C on NGM containing cholesterol at a 5 mg/L concentration for 3 days. Mixed cultures of N2 and RS2333 worms from 3 independent 10 cm agar plates were washed into 3 independent 25 ml pre cultures in S-complete liquid media, containing 5 mg/L cholesterol and fed with concentrated OP50 *ad lib.* Pre cultures were grown at 20°C, 220 rpm. After 4 days, 250,000 mixed stage worms from each pre culture were seeded into two 100 mL S-complete media prepared without any cholesterol. To one, unlabeled ^{12}C cholesterol (Sigma) was added, while to the other labeled $^{13}C_2$ cholesterol (Cholesterol-3,4-$^{13}C_2$- Sigma >99% HPLC purity, Product number 488585) was added, as 100% ethanolic solutions, 5mg/L each. Triplicate pairs of labeled and unlabeled 100 mL cultures were set up at 20°C, 220 rpm and fed *ad lib*. At time points of 24 h, 48 h, 96 h and 144 h, 25ml of the culture was removed and prepared for mass spectrometry analysis.

Preparation of $^{13}C_3$ labeled Δ^7 dafachronic acid (DA) fed daf-9 (dh6) worms

daf-9(dh6) worms were grown for at least 4 generations on NGM agar plates (prepared without any cholesterol) and seeded with concentrated OP50 mixed with either synthetic, unlabeled Δ^7 DA or $^{13}C_3$ labeled DA (25,26,27 $^{13}C_3$ Δ^7 DA, synthesized by R. Burkhardt, Schroeder lab, >90% HPLC purity) at 100 nM final concentration. After 4 generations, mixed stage worms from individual plates were washed into 25 mL liquid cultures made with S-complete media (without any cholesterol) and supplemented with 100 nM (final concentration) of respective labeled or unlabeled Δ^7 DA and concentrated OP50. Cultures were grown at 20°C, 220 rpm for 3 days before harvesting for mass spectrometry analysis.

Metabolome analysis

Endometabolomes (worm pellets) and exometabolomes (worm media) were processed and analyzed by mass spectrometry as detailed in **Appendix A**. Samples were analyzed in both ESI positive and negative ionization modes. All chromatograms were analyzed using XCMS-based MetaboSeek pipeline. Cholesterol-derived features were identified based on +2.00670 mass shift between ^{12}C and $^{13}C_2$ cholesterol fed samples. Δ^7 DA -derived features were identified based on +3.010065 mass shift between ^{12}C and $^{13}C3$ Δ^7 DA fed samples. All features incorporating more than 35% of the label were curated. Alongside the worm metabolomes, OP50 bacteria cultured in S-complete with $^{13}C_2$ cholesterol and $^{13}C_3$- Δ7-DA were evaluated for bacterial incorporation and metabolism of cholesterol and Δ^7 DA respectively. Synthetic standards and dafachronic acids were used to monitor mass accuracy, instrument sensitivity and relative concentrations in biological samples.

Comparative metabolome analysis of DA biosynthetic mutants

25 ml mixed stage liquid cultures of N2, *daf-36(k114), hsd-1(mg345),* and *strm-1(tm1781)* mutants were grown in triplicates, at 20°C, 220 rpm, for 3 days. Endometabolome samples were prepared for mass spectrometry analysis as detailed in Appendix A. For data analysis, MetaboSeek was used, and all features with a minimum intensity of 5e4 showing a 10-fold or more down regulation when compared to wildtype N2 were included.

Statistical analysis

Results are presented as mean ± SD or SEM, as indicated. *P* values were calculated using GraphPad Prism software or Excel by student's t-test or ANOVA as noted.