



The 'nuclear option' revisited: Confirmation of *Ss-daf-12* function and therapeutic potential in *Strongyloides stercoralis* and other parasitic nematode infections

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ABSTRACT

Mechanisms governing morphogenesis and development of infectious third-stage larvae (L3i) of parasitic nematodes have been likened to those regulating dauer development in *Caenorhabditis elegans*. Dauer regulatory signal transduction comprises initial G protein-coupled receptor (GPCR) signaling in chemosensory neurons of the amphidial complex that regulates parallel insulin- and TGF β -like signaling in the tissues. Insulin- and TGF β -like signals converge to co-regulate steroid signaling through the nuclear receptor (NR) DAF-12. Discovery of the steroid ligands of DAF-12 opened a new avenue of small molecule physiology in *C. elegans*. These signaling pathways are conserved in parasitic nematodes and an increasing body of evidence supports their function in formation and developmental regulation of L3i during the infectious process in soil transmitted species. This review presents these lines of evidence for G protein-coupled receptor (GPCR), insulin- and TGF β -like signaling in brief and focuses primarily on signaling through parasite orthologs of DAF-12. We discuss in some depth the deployment of sensitive analytical techniques to identify $\Delta 7$ -dafachronic acid as the natural ligand of DAF-12 homologs in *Strongyloides stercoralis* and *Haemonchus contortus* and of targeted mutagenesis by CRISPR/Cas9 to assign dauer-like regulatory function to the NR *Ss*-DAF-12, its coactivator *Ss*-DIP-1 and the key ligand biosynthetic enzyme *Ss*-CYP-22a9. Finally, we present published evidence of the potential of *Ss*-DAF-12 signaling as a chemotherapeutic target in human strongyloidiasis.

1. Introduction

For almost three decades prior to this review, parasitologists have recognized striking similarities between the biological characteristics of infectious third-stage larvae (L3i) of parasitic nematodes of medical and agricultural importance and dauer third-stage larvae of the free-living nematode *Caenorhabditis elegans* [1–3]. These forms have similar structural modifications (Fig. 1 A, B). They are both radially constricted in body form compared to the preceding first (L1) and second (L2) larval stages. The cuticles of dauers and L3i that are acquired from soil or vegetation are similarly remodeled to resist chemical and physical degradation. Behaviorally, dauers and L3i are both non-feeding stages

and are both actively motile, seeking out new microenvironments that are uncrowded and have ample bacterial food in the case of *C. elegans* and susceptible hosts in pre-parasitic L3i. Dauer larvae of *C. elegans* and pre-parasitic L3i are in a state of developmental arrest that is a fixed characteristic of L3i in most parasitic nematodes [3] but facultative in *C. elegans* (Fig. 1 A) and in the post-parasitic generation of *Strongyloides* spp. (Fig. 1 B). Both forms exit their states of developmental arrest conditionally, dauers upon entering microhabitats with sufficient microbial food and low population density of conspecifics (Fig. 1 A) and L3i upon infecting a susceptible host (Fig. 1 B).

The genetic, molecular and cellular mechanisms governing dauer larval development in *C. elegans* have been the subject of intense study

Abbreviations: $\Delta 7$ -DA, $\Delta 7$ -dafachronic acid; FL, free-living; ILS, insulin-like signaling; ILP, insulin-like peptide; L3i, infective third-stage larva; L3a, auto-infective third-stage larva; L3 +, developmentally activated post-parasitic third-stage larva; NR, nuclear receptor; NSG, NOD scid gamma mouse; PP, post-parasitic; PFL, post free-living; siRNA, small inhibitory RNA.

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and are increasingly well understood. Commitment to dauer or continuous development takes place in first-stage larvae, based on their processing of chemical cues in the form of food and a mix of ascaroside pheromones that reflect the population density of conspecific worms in their microhabitat, respectively (Fig. 1 A). This dauer checkpoint is

regulated by G protein-coupled receptor (GPCR) pathways in chemo- and thermosensory neurons of the amphidial complex that transduce environmental chemical cues. GPCR signaling regulates release of insulin-like peptide (ILP) ligands that signal through parallel insulin-like signaling (ILS) and TGF β -like signaling pathways to regulate tissue

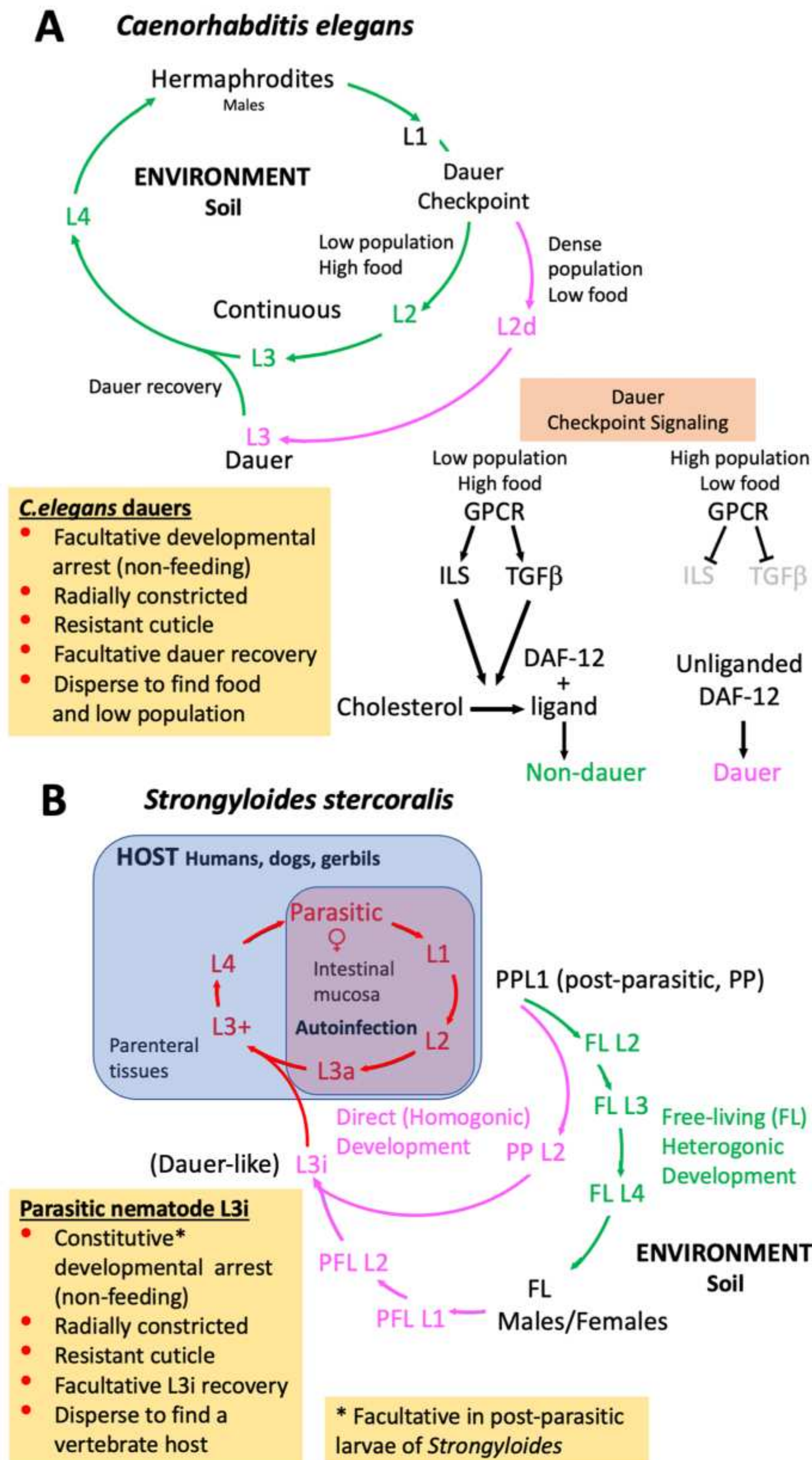


Fig. 1. Morphological and developmental similarities of dauer larvae of *C. elegans* and infective third-stage larvae (L3i) of *S. stercoralis* and many other species of parasitic nematodes. A Developmental switching of first-stage larval *C. elegans* L1 between continuous development through repeated generations of reproductive hermaphrodites and males and development to a specialized arrested, or dauer, form of the L3 [6]. Morphologic, developmental and behavioral characteristics of *C. elegans* dauers (yellow box) are striking in their similarity to analogous characteristics infectious L3 (L3i) of parasitic nematodes [3,9,86]. Population density, indicated by levels of constitutively released ascaroside pheromones, and abundance of microbial food in the microenvironment are sensed by G-protein coupled receptors in neurons of the amphidial complex. The resulting neuronal outputs govern the developmental switch by up- or down-regulating synthesis of insulin- and TGF β -like peptides. These peptides act through receptor kinases to regulate nuclear localization of transcription factors that establish patterns of either dauer or non-dauer development (salmon colored box) [6]. When parallel insulin-like (ILS) and TGF β -like pathways are activated under conditions favoring continuous development, their signals converge on a steroid nuclear receptor (NR) signaling pathway involving the DAF-12 NR and its dafachronic acid ligands. Biosynthesis of dafachronic acids is upregulated by ILS and TGF β -like signaling and liganded DAF-12 effects recovery of dauers and continuous reproductive development [4]. Absent these upstream signals, biosynthesis of dafachronic acids is downregulated and unliganded DAF-12 promotes dauer arrest. B *Strongyloides* spp. differ from other parasitic nematodes like hookworms, ascarid round worms and trichostrongyles in having post parasitic L1 that can switch, at a decision point similar to the dauer checkpoint in *C. elegans*, between direct development to the dauer-like L3i (homogonic development), which is similar to fixed patterns of development in hookworms and trichostrongyles, and development through one or more generations of free-living (heterogonic) development [87–89]. L3i of *Strongyloides* spp. and many other species of soil transmitted parasitic nematodes share common properties with *C. elegans* dauers. They arrest their development, albeit constitutively in all but *Strongyloides* and related species (asterisk), and are non-feeding, radially constricted forms with cuticles resistant to chemical and physical degradation. They also reactivate development facultatively upon invading a permissive host (yellow box). Reactivated L3i (L3+) develop within the host (blue box) to parasitic adults within the host gut. All parasitic adult *Strongyloides* are female and produce eggs parthenogenetically. Eggs of most *Strongyloides* species are passed in the feces, but in immunocompetent hosts, *S. stercoralis* L1 hatch within the host intestine, and most are passed in the feces [90]. A proportion of these *S. stercoralis* L1 develop precociously in the host gut to autoinfective L3 (L3a), which penetrate the gut wall and enter the parenteral tissues to initiate sequential generations of autoinfection. This autoinfection, which is well regulated in immunocompetent hosts, serves to replace senescent females and support chronic infection [91]. Immunocompromised hosts allow autoinfection to progress to disseminated hyperinfection resulting in geometric growth of intestinal worm burdens and potentially fatal damage to lungs and other organs during migration by overwhelming numbers of autoinfective larvae [91].

remodeling during dauer morphogenesis. Outputs from these two pathways, or lack thereof, govern biosynthesis of dafachronic acids (DAs), the steroid ligands of the DAF-12 nuclear receptor (NR) [4–8]. Active signaling through ILS and the TGF β -like pathways promotes biosynthesis of dafachronic acids, and ligand-bound DAF-12 effects continuous reproductive development and dauer recovery and confers normal (relatively short) lifespan to adult *C. elegans* (Fig. 1 A). Conversely, when ILS and TGF β are shut down, biosynthesis of dafachronic acids ceases and unliganded DAF-12 effects dauer larval arrest and lifespan extension in *C. elegans* (Fig. 1 A) [6]. Molecular signaling pathways analogous to those regulating dauer arrest and lifespan in *C. elegans* have been discovered in parasitic nematodes from diverse phylogenetic clades [9]. The title of the present review derives from Dr. Richard Martin's published commentary entitled "Nuclear option prevents hyperinfection in the *Strongyloides* worm war" [10] on the first report indicating the potential of signaling through the DAF-12 NR as a therapeutic target [11].

2. DAF-12 signaling in *C. elegans* provides a framework for hypotheses about hormonal regulation of development during the infectious processes of soil transmitted parasitic nematodes

The NR encoded in *daf-12* regulates dauer, in response to signaling through G-protein coupled receptor (GPCR) and parallel insulin- and TGF β -like pathways. Mutations in *daf-12* are epistatic to mutations in genes encoding elements in the insulin-like and TGF β -like dauer regulatory signaling pathways, supporting that DAF-12 signaling operates downstream of these pathways [6,7]. DAF-12 function is modulated by a co-repressor encoded by *din-1* [12]. Mutations in *daf-12* confer a variety of phenotypes depending on their position within the molecule, but most mutants are dauer-defective [7]. At the time of its discovery, the predicted structure of DAF-12 supported its function as a steroid nuclear receptor and prior to discovery of the ligand, genes encoding enzymes likely involved in its biosynthesis came to light. These included the cytochrome P450 encoded in *daf-9* [13], the Reiske-like oxygenase encoded in *daf-36* and the 3-hydroxysteroid dehydrogenase encoded in *dhs-16* [14]. Consistent with a function of ligand-bound DAF-12 in promoting continuous reproductive development, loss of function mutations in genes encoding these biosynthetic enzymes confer dauer constitutive phenotypes and extended lifespan in *C. elegans* [13–15]. Under environmental conditions such as crowding and limiting food, the co-repressor encoded in *din-1* interacts with unliganded DAF-12 to direct larval development towards dauer arrest and lifespan extension [12]. Despite strong evidence of hormonal control of dauer development, and significant homology of the predicted DAF-12 protein to nuclear receptors (NR) of steroids [7], this developmental regulatory NR remained an orphan until discovery of its dafachronic acid ligands in 2006 [4]. It is noteworthy that administration of exogenous dafachronic acids suppresses dauer constitutive mutations in both the insulin-like and TGF β -like pathways supporting genetic epistasis studies that operate upstream of the DAF-12 pathway to regulate larval development and lifespan in *C. elegans* [4]. This discovery was accompanied by confirmation of the action of *daf-9*, *daf-36* and *dhs-16* products in biosynthesis of the primary ligand Δ^7 -dafachronic acid and by recognition of other dafachronic acids that could signal through DAF-12 [4,16]. Altogether, "deorphanizing" DAF-12, and subsequent discoveries in *C. elegans* opened the field of endocrinology in this model organism and marked the first instance of a pathway regulating dauer arrest and lifespan in that worm that could be manipulated by small molecules administered to living worms. This fundamental advancement led to the hypothesis that DAF-12 signaling is conserved in soil transmitted parasitic nematodes and that administered dafachronic acids or their analogs constitute a novel intervention in diseases of humans and animals caused by these parasites [17].

3. Certain dauer regulatory signaling mechanisms in *C. elegans* are conserved in parasitic nematodes

Molecular signaling pathways analogous to those regulating dauer arrest and lifespan in *C. elegans* have been discovered in parasitic nematodes from diverse phylogenetic clades [9,18]. Genes encoding homologs of intermediates in the dauer regulatory GPCR pathway in *C. elegans* are conserved in *Strongyloides stercoralis* [19,20], and administration of the membrane permeable analog, 8-bromo-cGMP, promotes resumption of feeding by L3i of this parasite, an event deemed comparable to resumption of feeding by L3 of *C. elegans* during dauer recovery [21]. Similarly, this cell permeable cGMP analog promotes resumption of feeding when administered to cultured L3i of the hookworm *Ancylostoma caninum* [22]. By contrast, 8-bromo-cGMP does not promote resumption of feeding by cultured L3i of the hookworm-related parasite *Nippostrongylus braziliensis* [23]. This finding leaves open the possibility that sensory cues mediating developmental reactivation of *N. brasiliensis* L3i are processed by mechanisms differing from the GPCR mediated signaling functions in *C. elegans* and the other parasitic nematodes scrutinized thus far.

A significant body of evidence supports conservation of the dauer regulatory function of ILS as a mechanism regulating morphogenesis and development of parasitic L3i during the infectious process [9,24]. Homologs of *C. elegans* *daf-2*, which encodes a dauer regulatory insulin-like receptor kinase, have been discovered in *S. stercoralis* [20, 25] and in *Haemonchus contortus* [26]. Likewise, *S. stercoralis* [20,27] and *H. contortus* [28] also have homologs of *C. elegans* *age-1* and *aap-1*, which encode catalytic and regulatory subunits, respectively, of a phosphoinositol 3 kinase (PI3K) that signals downstream of *Ss-daf-2*. Several lines of evidence support a developmental function for *age-1* homologs in parasitic L3i. In *S. stercoralis*, a transcriptional reporter comprising the coding sequence of green fluorescent protein (*gfp*) under the *Ss-age-1* promoter is expressed in a subset of amphidial neurons corresponding to neuronal sites of *age-1* expression in *C. elegans* [27]. Furthermore, administering compound LY294002, an inhibitor of PI3K function, blocks resumption of feeding by L3i of *Ancylostoma caninum*, *A. ceylanicum* [29] and *S. stercoralis* [27] cultured under host-like culture conditions. The gene *daf-16* encodes a forkhead transcription factor that governs transduction of dauer regulatory ILS signals to cell nuclei in *C. elegans*. Under conditions promoting continuous reproductive development, active ILS serves to keep DAF-16 in the cell cytoplasm, but under dauer inducing conditions shutdown of ILS releases DAF-16 to translocate to the nucleus where it binds to specific response elements in the genome to activate a pattern of gene expression that promotes formation of dauer L3 and extends lifespan. Homologs of *daf-16* have been detected in a range of parasitic nematodes including the hookworms *A. caninum* and *A. ceylanicum* [30], *S. stercoralis* [31], and the trichostrongyle of ruminants *Haemonchus contortus* [32]. In-depth study has revealed sequences within the genome of *A. caninum* that constitute response elements of *Ac-daf-16* located in some 22 genes [33]. Screening of the entire *A. caninum* genome revealed putative *Ac-daf-16* target genes *Ac-snr-3*, encoding a core small ribonucleoprotein, and *Ac-lpp-1* that encodes a lipid phosphate phosphohydrolase. Temporal expression patterns of these genes support functions in development of fourth-stage larvae of the parasite [34]. Neurotransmission through muscarinic acetylcholine receptors stimulates recovery of *C. elegans* dauers and developmental reactivation of *A. caninum* L3i [35]. Specifically, the muscarinic agonist oxytremorine stimulates these developmental events and atropine ablates the effect. It is noteworthy in the present context that mutations in ILS elements block the effect of oxytremorine on *C. elegans* dauer recovery but mutations in TGF β signaling elements do not [35], underscoring the importance of ILS in mediating the stimulatory effects of this class of cholinergic neurotransmission on dauer recovery, and perhaps by extension, on reactivation of parasitic nematode L3i. By contrast, TGF β signaling does not appear to mediate these stimulatory effects.

Functional genomic platforms either do not exist for parasitic nematodes or have only recently been developed. Consequently, early functional studies assessed the ability of parasitic nematode genes to complement loss-of-function mutations in their *C. elegans* orthologs as a measure of functional homology. Such heterologous complementation studies have yielded varying results for parasite homologs of *daf-16*. *Ss-daf-16* from *S. stercoralis* fully complements a null mutation (*mu86*) in *C. elegans* [36] as does one of the two isoforms of *Hc-daf-16* from *H. contortus* [32]. Notably, a dominant interfering transgene comprising the coding sequence of *Ss-daf-16* with mutations conferring constitutive nuclear localization and loss of transactivating function prevented normal morphogenesis and developmental arrest of L3i when over expressed in progeny of free-living female *S. stercoralis* [37]. This marked the first direct support for conservation of function in *Ss-daf-16*. Likewise, *Ac-daf-16* from the hookworm *A. caninum* complements the *mu86* null mutation in *C. elegans daf-16* as does a mutant form of the gene with phospho-null mutations in its AKT phosphorylation sites [38]. These findings, and those of similar studies involving heterologous complementation, support that products of some parasite genes, structural homologs of *daf-16* in this case, have biochemical properties that allow them to perform functions of interest within *C. elegans*, but they fall short of proving conservation of basic *daf-16* function in hookworms, *Strongyloides* or other parasites. This cautionary interpretation is bolstered by the surprising result that the AKT phospho-null mutant form of *Ac-daf-16* failed to prevent dauer recovery by *C. elegans daf-2* (*e1370*)/*daf-16*(*mu86*) double mutants placed at a lower, permissive temperature [38]. Equally surprising was that the recombinant AKT phosphorylation site mutant *Ac-DAF-16* exited the nucleus upon recovery of *C. elegans* dauers, opening the possibility that nuclear localization of the hookworm transcription factor may be mediated by mechanisms that differ from those operating in *C. elegans* [38]. In sum, with few exceptions, evidence from experiments conducted prior to 2017 that involved administering small molecule effectors or inhibitors and heterologous complementation of mutations in *C. elegans* by putative parasite homologs support, but do not prove structural and functional conservation of GPCR signaling and ILS pathways in the dauer like process of morphogenesis and development of L3i during the infectious processes of parasitic nematodes.

As stated above, signaling through a TGF β -like pathway acts in parallel to ILS to regulate biosynthesis of the dafachronic acids ligands of DAF-12, which in turn directly regulates the switch between continuous and dauer development in *C. elegans* [6]. Briefly, loss-of-function mutations in *daf-7*, which encodes a TGF β -like cytokine [39], in either *daf-1* or *daf-4*, which encode the components of the heterodimeric TGF β -like receptor [40,41] and in receptor and co-SMAD proteins encoded in *daf-5* and *daf-3* [42,43] confer a dauer-constitutive phenotype in *C. elegans*. These genetic studies support that active TGF β -like signaling is necessary for continuous reproductive development in *C. elegans*, and that downregulation of this signaling confers dauer arrest. Homologs of *daf-7* are conserved in a number of parasitic nematodes, including *Strongyloides ratti* [44], *S. stercoralis* [20,45], *Parastrongyloides trichosuri* [44], *Ancylostoma caninum* [46,47], *Haemonchus contortus*, *Nippostrongylus braziliensis*, *Heligmosomoides polygyrus*, *Teladorsagia circumcincta* [48,49], *Brugia malayi* and *B. pahangi* [50–52]. Without exception, transcripts encoding *daf-7* homologs in parasitic nematodes are found at highest levels in developmentally arrested L3i stages of soil transmitted nematodes or in similarly arrested microfilariae of the filarids. This, the failure of the *daf-7* homolog of *P. trichosuri* to complement a *daf-7* mutation in *C. elegans* [44] and the non-dependence on TGF β signaling of muscarinic agonist-stimulated dauer recovery [35], led to the prevailing hypothesis that TGF β -like cytokines from parasitic nematodes are not involved in dauer-like regulation of infective larval development in parasitic nematodes but rather have evolved immunomodulatory functions that blunt protective responses by the host [9,44,53,54]. However, genes encoding the full complement of TGF β -like signaling elements have been discovered in *H. contortus* [49,55–58]. Attendant functional

genomic experiments involving transcriptional silencing by RNAi, siRNA or specific chemical inhibition revealed that functions of the TGF β homolog *Hc-tgh-2*, the Type I and II TGF β -like receptors encoded in *Hc-tgfr1* and *Hc-tgfr2*, and the co- and receptor Smads encoded in *Hc-daf-3* and *Hc-daf-8*, respectively, are required for normal resumption of development by *H. contortus* L3i under host-like in vitro culture conditions [49,55–57]. These findings support that TGF β -like signaling does, in fact, regulate resumption of development by infective *H. contortus* larvae during the infectious process. It is noteworthy that transcript levels for the *daf-7* homolog *Hc-tgh-2* in *H. contortus* peak in the L3i as do *daf-7* homologs in other parasitic nematodes. These results call for re-examination of the function of TGF β -like signaling in other parasitic nematodes, especially in *Strongyloides* spp. and related parasites where more direct functional genomic methods are available.

4. Conserved DAF-12 signaling in soil transmitted parasitic nematodes: parallels with and departures from the *C. elegans* paradigm

Following closely on the discovery of dafachronic acids as the ligands of DAF-12 in *C. elegans* was an alliance of pharmacologists and *C. elegans* biologists with parasitologists focusing on the developmental aspects of the infectious process in soil transmitted parasitic nematodes and on the growing body of evidence supporting dauer-like signaling as the regulator of this development. Initial studies resulting from this collaboration demonstrated that *daf-12* homologs are present in the genomes of the threadworm *S. stercoralis* (*Ss-daf-12*) and of the hookworm *A. caninum* (*Ac-daf-12*) [17,20]. Pharmacological experiments involving a cell-based assay of DAF-12 ligation revealed that the known ligands of *C. elegans* DAF-12, $\Delta 4$ - and $\Delta 7$ -dafachronic acids, can also bind and activate the parasite nuclear receptors *Ac-DAF-12* and *Ss-DAF-12* [17]. In the cases of both parasites, $\Delta 4$ -dafachronic acid was a less potent agonist of DAF-12 homologs than $\Delta 7$ -dafachronic acid. Crystallographic study of *Ss-DAF-12* ligand binding domain with bound $\Delta 7$ -dafachronic acid ($\Delta 7$ -DA) combined with site-directed mutagenesis revealed amino acid residues crucial for ligand binding [17]. These pharmacological and structural findings are borne out by the profound biological activity of $\Delta 7$ -DA administered to cultured larvae of *S. stercoralis* and *A. caninum*. Exogenous $\Delta 7$ -DA acts in dose-dependent fashion to substitute for host-like signals as cues for resumption of development by cultured L3i *S. stercoralis* (Fig. 2 A) [17,21,59], *A. caninum* [17], *N. brasiliensis* [60] and *Dirofilaria immitis* [61]. Administration of $\Delta 7$ -DA to larval progeny of free-living *S. stercoralis* males and females, which develop exclusively to L3i under normal conditions, completely suppresses morphogenesis of L3i in this population, largely with lethal results, but promotes development of second-generation free-living L4 and adult females in the few survivors [17,59]. $\Delta 7$ -DA is even more potent in suppressing L3i and promoting development of second-generation free-living females of *Strongyloides papillosus*, a parasite of ruminant animals. Whereas development of such second-generation free-living forms in the presence of $\Delta 7$ -DA was abortive in nature in the case of *S. stercoralis*, post free-living *S. papillosus* under treatment with $\Delta 7$ -DA develop fully to sexually competent free-living females [62]. Post-parasitic first-stage larvae (L1) of *Strongyloides* spp. that are voided in the host feces undergo a dauer-like checkpoint where innate genetic factors and environmental cues (primarily temperature) combine to govern a quantitative shift between either direct (homogonic) development to the L3i or heterogonic development to a generation of free-living males and females [63]. A strain of *S. stercoralis* (UPD) in the authors' laboratories that tends toward heterogonic development at ambient temperatures (20 °C) shifts its development to direct or homogonic development to L3i when temperatures are elevated to approximate host body temperature (37 °C). Administering $\Delta 7$ -DA during this homogonic shift overrides the elevated temperature cue and restores heterogonic development to a significant proportion of worms [59], supporting the hypothesis that signaling through *Ss-DAF-12* regulates dauer-like developmental switching in

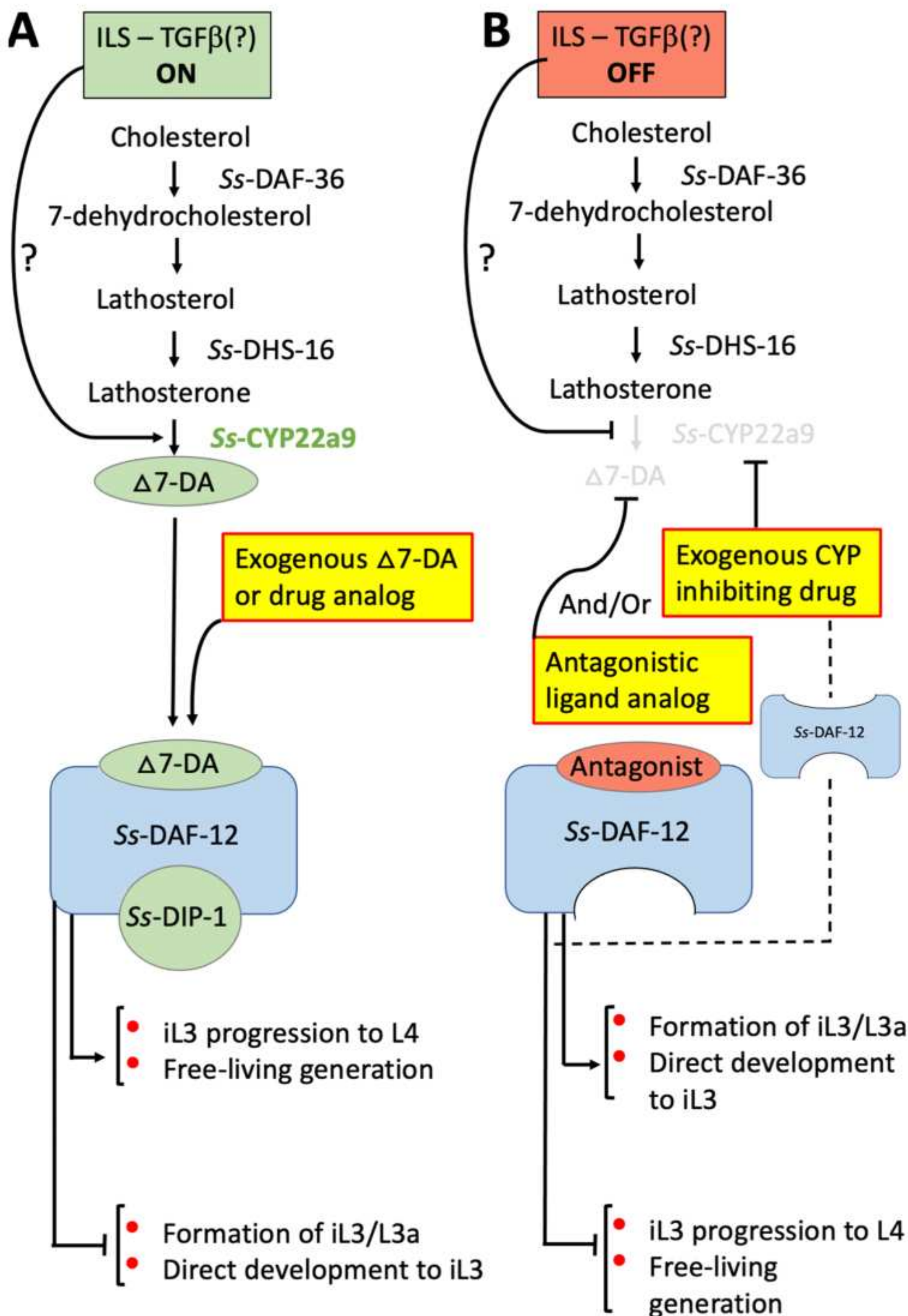


Fig. 2. Ss-DAF-12 transduces signals from upstream pathways to directly regulate development of infective and autoinfective third-stage larvae as well as switching between parasitic and free-living life cycle alternatives in *Strongyloides stercoralis*. **A.** When insulin- (ILS) and possibly TGFβ-like signaling are activated or “on” (green box), the cytochrome P450 Ss-CYP-22a9 is upregulated (green font) to catalyze conversion of lathosterone to the Ss-DAF-12 ligand Δ7-dafachronic acid (Δ7-DA, green oval) [70]. Liganded Ss-DAF-12 binds to its co-activator Ss-DIP-1 and promotes developmental activation of infective third-stage larvae (L3i) [67]. When bound to ligand and Ss-DIP-1, Ss-DAF-12 also switches developmental by post-parasitic first stage larvae towards development to free-living adults and suppresses formation of both L3i and autoinfective L3 (L3a) [17,59,67]. Administering Δ7-DA or a drug-like analog (red/yellow box) would constitute a therapy to block formation of L3i and L3a [11,17,70]. **B.** When ILS and TGFβ-like signaling are off (red box), Ss-CYP-22a9 is not upregulated to synthesize Δ7-DA (gray font). The Ss-DIP-1 coactivator is not bound in the absence of ligand, and Ss-DAF-12 alone promotes formation of L3i and L3a, directs development of post-parasitic L1 to L3i and suppresses activation of L3i and development of post-parasitic L1 to free-living adults. Question marks (?) denote signaling mechanisms hypothesized based on findings in *Caenorhabditis elegans* and the parasite *Haemonchus contortus* but not yet supported in *S. stercoralis*. These include the regulatory function of TGFβ-like signaling and regulation of Ss-CYP-22a9 by the upstream pathways. A CYP-inhibiting drug or an antagonistic analog of Δ7-DA (red/yellow boxes, red oval) are potential therapies to prevent development of L3i in the host.

S. stercoralis L1, driving it towards free-living (heterogonic) development and suppressing direct development to the L3i (Fig. 2 A). Beyond studies in *Strongyloides* spp. and *Ancylostoma* spp., proof of principle for developmental regulatory DAF-12 signaling in *H. contortus* has been demonstrated comprehensively [64,65].

Although the preponderance of experimental evidence in *S. stercoralis* supports conservation of GPCR, ILS and steroid-NR signaling mechanisms from *C. elegans* as regulators of morphogenesis and development of L3i, there are two lines of evidence that suggest an alteration of this scheme during the evolution of parasitism in Clade IV nematodes at least. This relates to early changes in levels of transcripts encoding insulin-like peptides (ILPs) during resumption of feeding by L3i of *S. stercoralis* in the absence of host-like temperature cues but in the

presence of exogenous small molecule activators of GPCR or DAF-12 NR signaling. Administering 8-bromo-cGMP to cultured L3i incites an increase in transcripts encoding the putatively agonistic ILPs Ss-ILP-5 and Ss-ILP-6 concomitant with stimulating resumption of feeding [21]. This finding is consistent with the hypothesis that GPCR signaling acts upstream to promote ILS-mediated resumption of development during the infectious process in *S. stercoralis*. Surprisingly, however, administered Δ7-DA also upregulates transcripts encoding the same putatively agonistic ILPs, opening the possibility of a positive feedback of ligand-dependent Ss-DAF-12 signals to GPCR signaling to enhance ILS. The physiological implications of this finding are as yet unclear. Another surprising result from in vitro studies of *S. stercoralis* L3i relates to the ordering of ILS and Ss-DAF-12 signaling in the mechanism of

developmental regulation in L3i. Consistent with earlier findings supporting a requirement for insulin-dependent PI3K signaling in regulating developmental activation of parasitic nematode L3i [66], the small molecule PI3K inhibitor LY294002 acts in dose-dependent fashion to prevent resumption of feeding by *S. stercoralis* L3i cultured under host-like conditions [27]. The suppressed developmental reactivation of L3i imposed by LY294002 corresponds to the dauer-constitutive (daf-c) phenotype associated with loss-of-function mutations in the *age-1*, which encodes the catalytic subunit of an insulin-dependent dauer regulatory PI3K in *C. elegans*. Exogenous $\Delta 7$ -DA suppresses daf-c mutations in signaling intermediates of the ILS and TGF β pathways, underscoring that DAF-12 signaling acts downstream of both in regulating dauer. Surprisingly, $\Delta 7$ -DA administered at 400 nM, the maximally effective concentration for stimulating resumption of feeding by *S. stercoralis* L3i, fails to rescue suppressed resumption of feeding by L3i cultured in the sub-maximal concentration (100 nM) of LY294002 [21]. Both of these findings, upregulation of agonistic ILP transcripts and failure to rescue developmental suppression by the PI3K inhibitor LY294002, call into question the strict linear ordering of GPCR, ILS and Ss-DAF-12 signaling in mechanisms governing morphogenesis and development in *S. stercoralis* L3i.

Another salient difference between DAF-12 signaling in *C. elegans* and *S. stercoralis* is the presence and function of co-regulatory subunits for this NR. Under dauer-inducing conditions, *C. elegans* DAF-12, absent ligand, acts in concert with the co-repressor DIN-1S to effect dauer arrest. Surprisingly, *S. stercoralis* has no recognizable DIN-1S ortholog. On the contrary, *S. stercoralis* expresses a *Strongyloides*-specific co-activator dubbed Ss-DIP-1 (DAF-12 interacting protein-1) concurrently and in an overlapping anatomical pattern with DAF-12 [67]. Ss-DIP-1 complexes with ligand-bound Ss-DAF-12 and, as will be discussed presently, enables its stimulation of developmental re-activation by L3i under host-like conditions. Ss-DIP-1 does not bind to Ss-DAF-12 in the absence of ligand. (Fig. 2 A) [67].

5. Deployment of contemporary analytical and functional genomic tools combine to confirm the function of endogenous DAF-12 signaling in *S. stercoralis* and *H. contortus*

Genomic and transcriptomic investigations revealed genes encoding homologs of DAF-12 and some attendant enzymes of the biosynthetic pathway leading to synthesis of DAF-12 ligands in *Ancylostoma* spp and *Strongyloides* spp. [17,20,68]. However, until 2019, there had been no biochemical confirmation of endogenous DAF-12 ligands in parasitic nematodes, and prior to that time, findings in support of a function for DAF-12 signaling were based on experiments in which synthetic $\Delta 7$ - or $\Delta 4$ -dafachronic acids were applied to developing parasite larvae or used in cell-based assays incorporating recombinant DAF-12 homologs from either *S. stercoralis* or *A. caninum* [17,21,59,62,69]. More recently, however, advanced LC/MS analyses have revealed endogenous DAF-12 ligands in *H. contortus* [65] and *S. stercoralis* [70].

Levels of $\Delta 7$ -DA in *S. stercoralis* vary widely among the developmental stages of the parasite, with presence of the ligand coinciding with continuous larval development in the heterogonic or free-living phase of development and with the resumption of development by L3i to the postparasitic L3 + upon entry into the host (Fig. 3) [70]. Absence of the ligand coincides with larval development to the dauer-like L3i in the homogonic phase of post-parasitic development and in post free-living larval stages (Fig. 3) [70]. These observations support that liganded Ss-DAF-12 suppresses morphogenesis of L3i and drives continuous or resumed larval development and that unliganded Ss-DAF-12 promotes morphogenesis of L3i and shifts development away from the free-living cycle. The absence of $\Delta 7$ -DA in free-living adult *S. stercoralis* (Fig. 3) [70] is consistent with the uniform developmental fate of their progeny to arrest as L3i. The finding that $\Delta 7$ -DA levels peak in the parasitic female of *S. stercoralis* (Fig. 3) [70] was surprising in that this represents a terminal stage in the parasitic generation of this parasite's development.

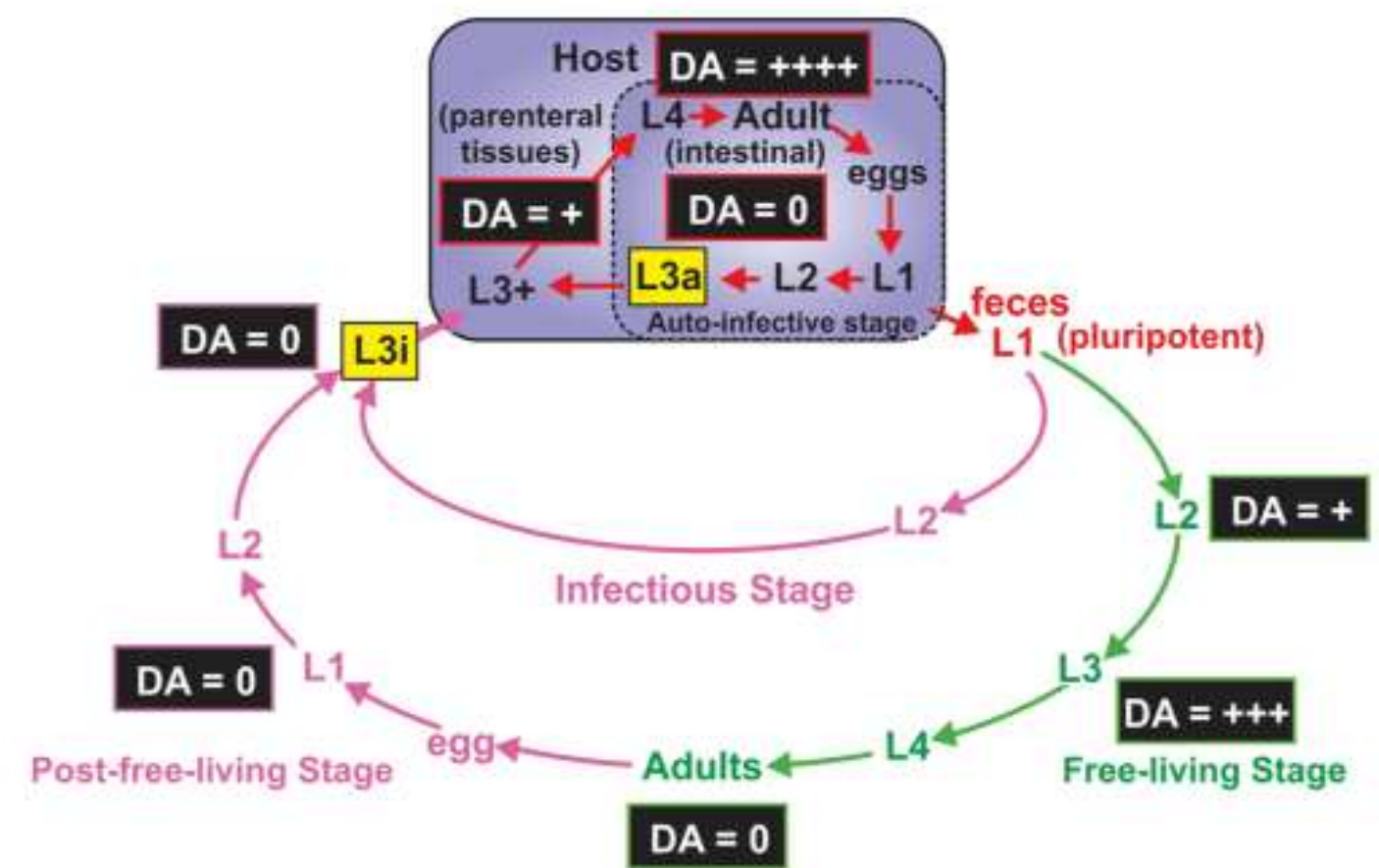


Fig. 3. Relative levels of $\Delta 7$ -dafachronic acid ($\Delta 7$ -DA) in stages and phases of the *Strongyloides stercoralis* life cycle. Presence of this Ss-DAF-12 ligand correlates with free-living (heterogonic) larval development, with the start of parasitic development in host tissues by infective (L3i) and autoinfective (L3a) third-stage larvae and with the attainment of reproductive adulthood by parasitic female worms [70]. High titers of the ligand occur in free-living larval stages, the reactivated L3 +, and the highest of all in parasitic females. $\Delta 7$ -DA is undetectable in both L3i and L3a and in larval stages leading to the L3i via the direct or homogonic route or to the post free-living generation or to the L3a via the autoinfective route. Trends in $\Delta 7$ -DA titers in the environmental stages of *S. stercoralis* are consistent with ligand-bound Ss-DAF-12 promoting continuous larval development in the free-living phase and with unliganded Ss-DAF-12 promoting larval development to the dauer-like L3i. For parasitic stages of *S. stercoralis*, mounting titers of $\Delta 7$ -DA accompany developmental progression of L3i and L3a to L3 + within the tissues. Experimental evidence supports that the increase in titer in L3i entering the host is necessary for resumption of development by these arrested stages [21]. While L3a are morphologically similar to L3i, it is unknown whether they undergo a period of developmental arrest that is terminated by ligation of Ss-DAF-12 upon their invading the somatic tissues of the host. High titers of $\Delta 7$ -DA in parasitic females were unexpected given the terminal differentiation of this stage. The high $\Delta 7$ -DA titers may function to establish the strong propensity for free-living over direct parasitic development exhibited by the UPD strain of *S. stercoralis* used in the supporting studies [59].

It is notable, however, that the UPD strain of *S. stercoralis* used in this study [70], has a strong tendency (approximately 95%) towards free-living development in its post-parasitic generation. It is possible that the high levels of $\Delta 7$ -DA that occur in parasitic females of this strain represent a maternal factor that establishes the heterogonic fate in their progeny.

Activity of the cytochrome P450 (CYP) encoded in *daf-9* is required for the last step in biosynthesis of dafachronic acids in *C. elegans*. It is noteworthy that discovery of endogenous ligands in both *S. stercoralis* and *H. contortus* were predicted or confirmed by the discoveries that chemical inhibitors of cytochrome P450s, ketoconazole and dafadine, suppress developmental reactivation by cultured L3i of *S. stercoralis* (Fig. 2B) and *H. contortus*, respectively, and that in both cases, this suppression can be rescued by administering exogenous dafachronic acid [59,65].

Limited capacity to directly interrogate gene function in parasitic nematodes has until recently, hampered efforts to prove that homologs of dauer signaling pathways in *C. elegans* undertake similar functions in regulating larval development in these important pathogens. RNA interference, which has been harnessed so successfully to suppress specific gene transcripts and infer their function in *C. elegans*, has been problematic in animal parasitic nematodes giving highly variable results with different target genes, based apparently on tissue sites of transcript expression [71–73]. Nevertheless, with deployment of small interfering RNAs (siRNAs) and improved methods for their delivery, it has been possible to achieve effective knockdown of specific transcripts in parasitic nematodes and thus infer their functions [74]. It is noteworthy that

confirmation of dauer-like function of the TGF β -like pathway in *H. contortus* was achieved using target-specific siRNA's transduced by lipofection [49, 55–57]. In the present context it is also notable that siRNA-mediated transcriptional silencing of *Sr*-DAF-12 in *Strongyloides ratti* strongly suppressed development of post free-living larvae to the L3i, a phenotype similar to that conferred by exogenous treatment of *S. stercoralis* larvae at this stage with exogenous Δ 7-DA [75].

Owing to their capacity to undertake one or more generations of free-living development, members of the genus *Strongyloides* are amenable to DNA transformation using methods adapted from those for gonadal microinjection used in *C. elegans* [49, 55–57, 76–80]. These methods have allowed CRISPR/Cas9 to be deployed for targeted mutagenesis in *Strongyloides* spp. to verify gene function and potentially, to knock single copies of transgenes into precise genomic loci [81,82]. These methods have already found application in defining a requirement for the neuronal channel component *Ss*-TAX-4 in mediating thermosensory behaviors in *S. stercoralis* L3i [83–85]. In the present context, studies involving transgenesis and CRISPR/Cas9 have confirmed the functions of *Ss*-DAF-12 and its newly discovered co-activator, *Ss*-DIP-1 in normal morphogenesis of L3i in *S. stercoralis* [67]. These methods have also been deployed to assign functions to genes encoding enzymes for biosynthesis of endogenous dafachronic acids in this parasite [70].

CRISPR/Cas9-mediated insertional mutagenesis and disruption of *Ss*-daf-12 was achieved by knocking in a GFP reporter cassette, including a terminator, within a region of the gene encoding the ligand-dependent activation function (AF-2) domain of the NR. This resulted in strong suppression of L3i formation in the post free-living generation of *S. stercoralis* [67]. This was lethal in the majority of knockout larvae, but, significantly, the small proportion of survivors included some morphologically normal second-generation free-living females along with numerous abortively developing forms. This suppression of L3i formation by *Ss*-DAF-12 knockout is consistent with phenotypes resulting from administration of Δ 7-DA to populations of post free-living *S. stercoralis* [17,59] or from transcriptional silencing of *Sr*-DAF-12 in *S. ratti* [75]. The occurrence of morphologically normal second-generation free-living females, albeit at a low frequency, may appear to be at odds with the requirement for liganded DAF-12 for continuous development by *C. elegans* and free-living development by *Strongyloides*. This apparent contradiction, that both gain and complete loss of DAF-12 function result in similar phenotypes, can be explained by the fact that DAF-12, like many ligand-dependent nuclear receptors, functions as both an activator (in the presence of ligand) and a repressor (in the absence of ligand). In the unliganded state DAF-12 represses the developmental and metabolic pathway leading to maturity, which is why in the absence of dafachronic acid the worms arrest at dauer (or L3i) (Fig. 1). Deleting DAF-12, removes the repression, resulting in transcriptional activation. These worms are now dauer-defective, which is similar to ligand activation in WT worms. Our work has demonstrated that this is likely the evolutionary driver for the conservation of DAF-12 in parasites, as it is necessary for formation of infectious (L3i) worms.

Knockout of *Ss*-dip-1 by a similar strategy of CRISPR/cas9-mediated insertional mutagenesis resulted in suppressed developmental reactivation of *S. stercoralis* L3i in a permissive culture medium, rescue of L3i lethality in post free-living larvae treated with Δ 7-DA and suppressed transcription of the *Ss*-DAF-12 target genes *acbp-1*, *acs-1* and *F28H7.3.1*, thus confirming that *Ss*-DIP-1 is necessary for physiological and gene regulatory functions of ligand bound *Ss*-DAF-12 [75].

The advent of targeted mutagenesis by CRISPR/Cas9 in *Strongyloides* spp., along with metabolic studies involving isotope labeling, has also allowed us to confirm function of known orthologs of genes essential for biosynthesis of DAF-12 ligands in *C. elegans*. Genomic [68] and transcriptomic [20] data confirm the presence of *S. stercoralis* orthologs of the Rieske oxygenase encoded in *daf-36* and the 3-hydroxysterol dehydrogenase encoded in *dhs-16* in *C. elegans*. Cells transfected with transgenes encoding these *S. stercoralis* orthologs, convert cholesterol to 7-dehydrocholesterol and lathosterol to lathosterone, respectively,

confirming their conserved biochemical functions [70]. In contrast to *Ss*-daf-36 and *Ss*-dhs-16, no ortholog of *C. elegans* *daf-9*, which encodes the CYP catalyzing the final step in biosynthesis of Δ 7-DA, could be identified among the 26 predicted CYPs in *S. stercoralis* based on sequence similarity or developmental expression patterns [20]. However, studies involving a modification of the cell-based assay used to ascertain binding of Δ 7-DA by *Ss*-DAF-12 [17] revealed that only one of the candidate CYPs encoded in the *S. stercoralis* genome, *Ss*-CYP22a9, catalyzed conversion of precursor lathosterone to Δ 7-DA [70], thus identifying it as the functional ortholog of *C. elegans* DAF-9 and the potential regulatory target of upstream signaling through the GPCR, insulin-like and, possibly, the TGF β signaling pathways (Fig. 2 A, B). Supporting this is the fact that disrupting *Ss*-cyp-22a9 by CRISPR/Cas9 blocks 8-Br cGMP-stimulated resumption of development by cultured L3i of *S. stercoralis* [70]. This also supports that unliganded *Ss*-DAF-12 drives formation of L3i and autoinfective L3 (L3a) and suppresses L3i progression to L4 and switching of post-parasitic L1 to heterogonic or free-living development (Fig. 2B). It is likely that ketoconazole suppresses developmental reactivation in L3i [59] by inhibiting *Ss*-CYP22a9 (Fig. 2B), suggesting this crucial regulatory enzyme as a novel therapeutic target in strongyloidiasis of humans and domestic animals. The fact that dafadine, a specific inhibitor of DAF-9 in *C. elegans*, suppresses developmental progression by *H. contortus* L3i in a permissive culture system also underscores the potential of dafachronic acid biosynthesis as a therapeutic target in this parasite of high agricultural significance [65].

6. DAF-12 signaling as a therapeutic target in uncomplicated and disseminated strongyloidiasis

Ss-DAF-12 signaling in *S. stercoralis* is subject to regulation by exogenous small molecules at two points at least, the ligation state of *Ss*-DAF-12 itself and the biosynthesis of its natural ligand, Δ 7-DA, from its precursor lathosterone. Targeting the ligation state of *Ss*-DAF-12 has been the focus of work to date, which has demonstrated that Δ 7-DA administered in the drinking water of NSG mice with glucocorticoid-induced disseminated *S. stercoralis* hyperinfection significantly reduces burdens of autoinfective third-stage larvae (L3a) in the animals' tissues compared vehicle-fed controls [11]. This finding is consistent with suppression of L3i morphogenesis by Δ 7-DA in cultured *S. stercoralis* larvae in both the post-parasitic and post free-living generations [17, 59]. Studies in a gerbil model that recapitulates both uncomplicated and hyperinfective *S. stercoralis* infection, also support the therapeutic potential of Δ 7-DA or its analogs. Feeding 50 μ M Δ 7-DA in drinking water, reduces mortality among gerbils with glucocorticoid-induced hyperinfection more potently than the current drug of choice, ivermectin at 300 μ g/kg, and a combination of ivermectin and Δ 7-DA at the stated doses reduces mortality among these gerbils most potently of all [70]. These trends in relative efficacies of Δ 7-DA or ivermectin alone or the two compounds combined were also noted in their capacities to suppress L1-L3a in the intestinal lumen, L3a in the host tissues and adult worms in the intestinal mucosa. The most striking finding in this chemotherapeutic study was that while 50 μ M Δ 7-DA has little or no capacity to render the gerbils completely free of *S. stercoralis* infection, and ivermectin at 300 μ g/kg has less than 10% efficacy in this regard, a combination of the two compounds at these doses can effect a complete cure in 80% of gerbils with disseminated *S. stercoralis* hyperinfection [70]. These findings strongly support further investigation of *Ss*-DAF-12 signaling as an intervention in human strongyloidiasis, in its potentially debilitating uncomplicated form and in its potentially fatal disseminated hyperinfective form.

7. Future directions in research on DAF-12 signaling in parasitic nematodes

An increasingly large body of evidence supports a developmental

regulatory function for DAF-12 signaling in the infectious processes of diverse parasitic nematodes. There is also support for regulation by GPCR, and insulin-like signaling pathways homologous to those regulating ligation of the DAF-12 NR in *C. elegans*. A similar role for TGF β -like signaling in parasitic nematodes has been discounted previously but has gained new support recently from work in *H. contortus*. Beyond this, however, evidence for ordered regulation by the three pathways is either lacking or at odds with their ordering in *C. elegans*. A crucial question to be addressed in this regard is whether homologs of the insulin-regulated transcription factor DAF-16 or of TGF β -regulated SMAD proteins affect the expression or activity of functional homologs of the DAF-9 CYP in *C. elegans*. Also, the functional significance of apparent feedback regulation of insulin-like peptide expression by $\Delta 7$ -DA in *S. stercoralis* deserves further study as does the failure of $\Delta 7$ -DA to rescue the developmental phenotype associated with chemical inhibition of the PI3 kinase *Ss*-AGE-1. Another question is the evolutionary significance of loss of *din-1*, which encodes a co-repressor with DAF-12 in *C. elegans* and gain of *Ss*-*dip-1*, which encodes a co-activator with *Ss*-DAF-12 in *S. stercoralis*. The fact that while dauer development is a facultative process in *C. elegans* and likely in many other free-living nematodes, dauer-like formation of L3i in obligately parasitic nematodes is more or less fixed. These findings suggest that while the paradigm of the nuclear receptor DAF-12 signaling pathway has been a crucial evolutionary development in many if not all nematodes, individual species have adopted unique, niche-specific ways to elaborate this nuclear option and thereby contribute greatly to the evolutionary success of the phylum.

Perhaps the greatest imperative to come from this body of work is to further study the chemotherapeutic potential of targeting DAF-12 signaling in parasitic nematodes. The natural ligand of *Ss*-DAF-12, $\Delta 7$ -DA, has demonstrated significant activity against both uncomplicated and disseminated hyperinfective strongyloidiasis in two rodent models. However, this natural ligand lacks many of the pharmacokinetic properties of an effective drug, most significantly persistence. Medicinal chemistry to optimize drug-like characteristics of dafachronic acids is a crucial area ripe for study. The additive, even synergistic, effects of combined ivermectin and $\Delta 7$ -DA therapy are also top priorities in developing this novel intervention.

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CRedit authorship contribution statement

James B. Lok: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. **Steven A. Kliever:** Conceptualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **David J. Mangelsdorf:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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