Strongyloides stercoralis age-1: A Potential Regulator of Infective Larval Development in a Parasitic Nematode

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Abstract

Infective third-stage larvae (L3i) of the human parasite Strongyloides stercoralis share many morphological, developmental, and behavioral attributes with Caenorhabditis elegans dauer larvae. The ‘dauer hypothesis’ predicts that the same molecular genetic mechanisms control both dauer larval development in C. elegans and L3i morphogenesis in S. stercoralis. In C. elegans, the phosphatidylinositol-3 (PI3) kinase catalytic subunit AGE-1 functions in the insulin/IGF-1 signaling (IIS) pathway to regulate formation of dauer larvae. Here we identify and characterize Ss-age-1, the S. stercoralis homolog of the gene encoding C. elegans AGE-1. Our analysis of the Ss-age-1 genomic region revealed three exons encoding a predicted protein of 1,209 amino acids, which clustered with C. elegans AGE-1 in phylogenetic analysis. We examined temporal patterns of expression in the S. stercoralis life cycle by reverse transcription quantitative PCR and observed low levels of Ss-age-1 transcripts in all stages. To compare anatomical patterns of expression between the two species, we used Ss-age-1 or Ce-age-1 promoter:enhanced green fluorescent protein reporter constructs expressed in transgenic animals for each species. We observed conservation of expression in amphidial neurons, which play a critical role in developmental regulation of both dauer larvae and L3i. Application of the PI3 kinase inhibitor LY294002 suppressed L3i in vitro activation in a dose-dependent fashion, with 100 μM resulting in a 90% decrease (odds ratio: 0.10, 95% confidence interval: 0.08–0.13) in the odds of resumption of feeding for treated L3i in comparison to the control. Together, these data support the hypothesis that Ss-age-1 regulates the development of S. stercoralis L3i via an IIS pathway in a manner similar to that observed in C. elegans dauer larvae. Understanding the mechanisms by which infective larvae are formed and activated may lead to novel control measures and treatments for strongyloidiasis and other soil-transmitted helminthiases.

Introduction

Helminth infections represent a vast global burden of disease, with parasitic nematodes infecting more than one billion people [1,2]. The infectious form of many parasitic nematodes, including the medically relevant species which cause strongyloidiasis, filariasis, and hookworm disease [3], is the third-stage larva (L3i). L3i developmentally arrest, sometimes for months, before resuming development upon encountering a host [3,4]. Despite their potential as new therapeutic targets, the signaling proteins and pathways controlling developmental arrest and activation of L3i are unknown.

Our lab has employed Strongyloides stercoralis, a parasitic nematode infecting nearly 100 million people [2,5], as a model to study molecular mechanisms in parasitic nematodes, and we have developed methods for transgenesis that are unavailable in other species [6–8]. Study of S. stercoralis is facilitated by a life cycle that includes both parasitic and free-living generations, allowing us to probe factors driving L3i development in an otherwise “obligately parasitic” group of organisms [8,9]. In S. stercoralis, L3i form conditionally in the post-parasitic generation (homogenic development), along with free-living males and females (heterogenic development), and constitutively in the post-free-living generation [10]. This life cycle provides a unique opportunity to interrogate the mechanisms governing L3i formation and activation.

A similar developmentally arrested stage, the dauer larva, is formed by the free-living nematode Caenorhabditis elegans in response to unfavorable conditions [11,12]. Interestingly, C. elegans dauer larvae and S. stercoralis L3i arrest at the same third larval stage and share similar characteristics of morphology, extended lifespan, stress-resistance, and cessation of feeding [13,14]. Dauer larvae and L3i resume development soon after encountering favorable environmental conditions or the definitive host, respectively [10,15]. The “dauer hypothesis” proposes that the molecular mechanisms governing L3i developmental arrest and recovery in S. stercoralis and other parasitic nematodes are similar to those regulating dauer formation and recovery in C. elegans [12,14,16,17].

In C. elegans, the insulin/IGF-1 signaling (IIS) pathway plays a critical role in dauer formation and recovery. When environmental conditions are limiting, IIS signaling is suppressed, resulting in
the forkhead transcription factor [35], we endeavored to clone and isolate Ce-AGE-1 with the UPD strain, into each well of a 96 well tissue culture plate. Freshly isolated from the feces of a dog infected with the UPD strain as a line with limited genetic variability. To derive this line, virgin free-living females were prepared by placing one adult female and larval progeny. The free living generation is unknown. However, members of the IIS pathway have been cloned from several parasitic nematodes [32–34], including *S. stercoralis*, as well as the first stage larva, freshly isolated from the feces of a dog infected with *S. stercoralis*. The genomic sequence of *S. stercoralis* is of the 96 wells contained an adult female and larval progeny, demonstrating the importance of IIS for regulating dauer development [31]. Members of the IIS pathway have been cloned from several parasitic nematodes [32–34], including *S. stercoralis*, but it remains unclear whether they comprise a pathway controlling the developmental arrest and activation of L3i. Recent studies in our lab have demonstrated that the *S. stercoralis* DAF-16 homolog is required for normal arrest of L3i under conditions of decreased IIS [6]; however, it is unknown whether the IIS pathway also regulates developmental activation of L3i in the host through increased IIS. Since *Ce*-daf-2 is the main mediator of increased IIS signaling from the *Ce*-daf-2 insulin-like receptor to the downstream *Ce*-daf-16 forkhead transcription factor [35], we endeavored to clone and characterize the *S. stercoralis* homolog of *Ce*-age-1. Here we present striking similarities between *Ce*-age-1 and *Ss*-age-1 as well as the first evidence of IIS regulating L3i activation in *S. stercoralis*.

Materials and Methods

Ethics Statement

No human subjects were used in these studies. The *S. stercoralis* UPD strain and PV001 line were maintained in prednisolone-treated beagles in accordance with protocols 702342, 801905, and 802593 approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Experimental infections of *S. stercoralis* were conducted in Mongolian gerbils under the same IACUC-approved protocols, and animals were sacrificed by CO2 asphyxia in accordance with standards established by the American Veterinary Medical Association. All IACUC protocols, as well as routine husbandry care of the animals, were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

*S. stercoralis* and *C. elegans* Strains and Maintenance

The *S. stercoralis* UPD strain was maintained and cultured as previously described [8,36,37]. UPD strain free-living adults for DNA transformation and RNA extraction were isolated via the Baermann technique from two-day-old charcoal coprophagous incubated at 22°C. UPD strain L3i for genomic DNA and in vitro activation were isolated via the Baermann technique from seven-day-old charcoal coprophagous incubated at 25°C.

The *Strongyloides* isofemale line PV001 was derived from the UPD strain as a line with limited genetic variability. To derive this line, virgin free-living females were prepared by placing one first-stage larva, freshly isolated from the feces of a dog infected with the UPD strain, into each well of a 96 well tissue culture plate. The plate had been prepared by placing 50 μl of 1% agar (Lonza, Basel, Switzerland) into each well and then adding a small (several mg) piece of normal dog feces to the surface. After three days, two of the 96 wells contained an adult female and larval progeny. The mechanism by which progeny arise in the absence of mating in the free living generation is unknown. However, *S. stercoralis* parasitic females are presumed to reproduce by mitotic parthenogenesis [38,39], and it is possible that this phenomenon occurs at a low frequency among free-living females as well. At six days post plating, 34 L3i were removed from one of the positive wells and used to infect a gerbil. The gerbil was given 2 mg methylprednisolone acetate (SQ) at the time of infection and weekly thereafter [37]. L3i recovered from coprophagous made from this gerbil’s feces were used to infect another gerbil and the L3i recovered from coprophagous of this second gerbil’s feces were used to infect the dog from which feces were isolated for use in the RT-qPCR study.

C. elegans N2 worms were maintained by standard methods [40]. The CY246 strain, age-1(mg44); sqt-1(e15)/mmt1 dpy-16(e128); unc-52(e444) II, a gift from Catherine Wolkow, was maintained by picking phenotypically WT (wild-type) worms. The *age-1(mg44); sqt-1(e15) F1 progeny in this strain, which are marked in co with the *sqt-1* roller phenotype and exhibit maternal rescue, produce F2 progeny that conformally form roller dauers. The BC10837 strain [41], ppy-5(e907) I, a gift from Catherine Wolkow, was maintained by picking phenotypically WT worms. The *age-1(mg44); sqt-1(e15) F1 progeny in this strain, which are marked in co with the *sqt-1* roller phenotype and exhibit maternal rescue, produce F2 progeny that conformally form roller dauers. The BC10837 strain [41], ppy-5(e907) I, a gift from Catherine Wolkow, was maintained by picking phenotypically WT worms.

DNA and cDNA Preparation

Genomic DNA from the *S. stercoralis* UPD strain was isolated from L3i by phenol/chloroform extraction [7]. UDP strain RNA for cDNA synthesis was isolated from free-living adults by TRIZol reagent extraction (Life Technologies, Grand Island, New York, USA); total cDNA was synthesized using SuperScript III reverse transcriptase and an oligo dT primer (Life Technologies) following the manufacturer’s protocol. cDNA with ligated adapters for 5’ and 3’ RACE was prepared using the GeneRacer kit (Life Technologies).

For *C. elegans* genomic DNA preparations, starved plates of N2 worms were washed and DNA extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA). RNA for cDNA synthesis was isolated from mixed-stage plates of N2 worms and cDNA synthesized by the same methods as for *S. stercoralis*.

Cloning of *S. stercoralis* Genes

The genomic sequence of *Ss*-age-1 was determined using degenerate primers to amplify a coding sequence homologous to a *Parastrongyloides trichosuri* EST (GenBank: B1742945), which was identified by BLAST searches of the nematode EST database (http://nematode.net/NN3_frontpage. gen?navbar_selection = nemagenecsubm_selection = nemablast) with the *Ce*-AGE-1 sequence (GenBank: AAC47459). Repeated cycles of inverse PCR and established methods were used to determine the complete coding sequence as well as sequences of the 5’ and 3’ regions of *Ss*-age-1 (GenBank: JQ777308) [32,42,43]. Both 5’ and 3’ RACE were performed using the GeneRacer protocol (Life Technologies) and Phusion polymerase (New England Biolabs, Ipswich, Massachusetts, USA). 5’ RACE used the *Ss*-age-1-17R outer primer and *Ss*-age-1-20R nested primer; 3’ RACE used the *Ss*-age-1-20F outer primer and *Ss*-age-1-11F nested primer (Table S1). Genomic DNA and RACE PCR products were cloned using the Zero Blunt TOPO PCR cloning kit (Life Technologies) and sequenced in triplicate. The full-length *Ss*-age-1 cDNA was amplified from total cDNA with Phusion polymerase (New England Biolabs) using the *Ss*-age-1-24FattB1 and *Ss*-age-1-24RattB2 primers, while 1.3 kb of 5’ region was amplified using the *Ss*-age-1-23FattB4 and *Ss*-age-1-23RattB1r primers (Table S1). The 1,327 bp 5’ region (pPV430) and 3,630 bp *Ss*-age-1 cDNA (pPV451) were cloned into pDONR
Phylogenetic Analysis

A protein alignment of PI3 kinase catalytic subunits was generated using Clustal X2 [45] and a BLOSUM62 matrix. A neighbor-joining tree was constructed using MEGA version 4.0 [46] with bootstrapping scores (1000 iterations) shown for robust clades (>50% cutoff). A global protein alignment between Ca-AGE-1 and Ss-AGE-1 was performed using EMBOSS Needle and a BLOSUM62 matrix [47] and was annotated for coding exons to determine conservation of introns between Ss-AGE-1 and Ca-AGE-1. Accession numbers were: Strongyloides stercoralis (GenBank: JQ772018), Strongyloides ratti (Dataset S1), Parasstrongyloides trichosuri AGE-1 (GenBank: ADN44511), Brugia malayi P3K (NCBI: XP_001902593), Caenorhabditis briggsae AGE-1 (NCBI: XP_002631094), Caenorhabditis elegans AGE-1 (GenBank: AAC74459), Homo sapiens P3KCA (GenBank: AA13604), Drosophila melanogaster P3K92E (NCBI: NP_659092), Drosophila melanogaster P3K59F (NCBI: NP_477133), Homo sapiens P3K3C3 (NCBI: NP_002638), Caenorhabditis elegans VPS-34 (NCBI: NP_491741), Sarcocystis cerevisiae VPS34 (NCBI: NP_013341), Drosophila melanogaster P3K68D (NCBI: NP_524028), Caenorhabditis elegans F39B1.1 (NCBI: NP_510529), and Homo sapiens P3K3CA2A (NCBI: NP_002636).

A protein alignment of PI3 kinase accessory/regulatory subunits using Clustal W and a BLOSUM matrix was generated using Geneious version 5.5.6 [48] and was annotated for coding exons to determine conservation of introns between Ss-aap-1 and Ce-aap-1. A global protein alignment between Ce-AAP-1 and Ss-AAP-1 was performed using EMBOSS Needle and a BLOSUM62 matrix [47]. Accession numbers were: Strongyloides stercoralis AAP-1 (GenBank: JQ781500), Strongyloides ratti AAP-1 (Dataset S1), Caenorhabditis elegans AAP-1 (NCBI: NM_059121), Trichinella spiralis AAP-1 (GenBank: EFV56516), Brugia malayi AAP-1 (GenBank: EDP31759), Ascaris suum AAP-1 (GenBank: ADY59992), Drosophila melanogaster P3K-2b (GenBank: CAA73100), and Homo sapiens P3K-p85α (UniProtKB: P27986).

RT-qPCR

Developmental stages of S. stercoralis line PV001 were isolated for RT-qPCR as previously described [32], washed, and rendered free of fine particle debris by migration through agarose [49] into BU buffer [40]. See Supplemental Methods for detailed protocol (Text S1). Worms were snap-frozen in TRIzol reagent (Life Technologies) in liquid nitrogen; total RNA was extracted using the manufacturer’s protocol. Purified RNA was quantified using the Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, California, USA) and only samples with an RNA integrity number greater than 8.0 were used.

Gene specific primer sets, which only amplified splice cDNA, included Ss-1RT-3F and -R, Ss-ac2RT-2F and -R, and Ss-gpdirRT-2F and -R (Table S1) [7,50,51]. Primer sets were calibrated using a five dilution series of total RNA and efficiencies calculated using standard methods [52]. Gene specific RT-qPCR was performed on three biological replicates, each in duplicate, using the Brilliant II SYBR Green QRT-PCR kit (Agilent Technologies) with 50 ng of total RNA on an Applied Biosystems 7500 (Life Technologies) instrument with the following parameters: RT step of 30 min at 50°C; 10 min at 95°C; and 40 cycles of 30 sec at 95°C and 60 sec at 60°C (detection step); followed by a melting temperature curve. Controls omitting template or reverse transcriptase were also included.

Results were analyzed by computing the mean Ct value for each replicate. Abundances were calculated using the slope of the calibration curve and normalizing to an arbitrarily determined mean of 10 copies of Ss-age-1 in free-living females [51,52]. Log transformed values, ±1 SEM, were plotted in Prism version 5.03 (GraphPad Software, Inc., La Jolla, California, USA).

Plasmid Construction

Plasmids containing Ce-age-1 promoter and egfp reporter constructs were assembled by overlap extension PCR. The construct Ce-age-1p:egfp::Ce-tbb-2t, contained in plasmid pPV452, was assembled in two steps. The 866 bp of Ce-age-1 5′ region was PCR amplified using C. elegans genomic DNA as a template. The 870 bp egfp coding region, including introns, and 333 bp of the Ce-tbb-2 3′ region (terminator) were amplified using pJA257 as template (Addgene, Cambridge, Massachusetts, USA). These PCR products were fused by overlap extension PCR using the overlapping primers Ce-age-1p::EGFP-F and -R (Table S1). The product was cloned into pUC19 (New England Biolabs) and the insert fully sequenced.

The construct Ce-age-1p:Ce-age-1(102bp)::egfp::Ce-age-1t, contained in plasmid pPV455, was assembled by a similar scheme. The 919 bp segment of Ce-age-1 5′ region, including the first 102 bp of the Ce-age-1 coding sequence, was PCR amplified using C. elegans genomic DNA as a template. The 870 bp egfp coding sequence, with introns, was PCR amplified using pJA257 as a template. A 1,071 bp segment of Ce-age-1 3′ region (terminator) PCR amplified using genomic DNA as a template. These three segments were joined using the overlapping primers Ex10837-EGFP-F and -R and EGFP-Ceage1t-F and -R (Table S1), the product T/A cloned into the pCR-TOPO- XL vector (Life Technologies), and the insert fully sequenced.

The construct Ce-age-1p:Ce-age-1(5,549bp)::Ce-unc-54t, contained in plasmid pPV454, was assembled by a similar process. An 873 bp segment of Ce-age-1 5′ region was amplified from genomic DNA and fused to 2,376 bp of the 5′ region of Ce-age-1, amplified from cDNA, using the overlapping primers Ceage1p-cDNA-F and -R (Table S1), the product T/A cloned into the pCR-TOPO XL vector (Life Technologies), and the insert fully sequenced.
The construct \( \text{Ss-age-1}\)-\( \text{p::mCherry::Ce-unc-54t} \) (pCFJ90) co-injection marker at 2 ng/\( \mu \)l was used as a control. For all other conditions, greater than 90% of L3i were active at 24 hours. Data for five experiments were plotted, +1 SEM, in Prism version 5.03 (GraphPad Software, Inc.). The relationship between condition and resumption of feeding was modeled using a generalized linear mixed-effects model and a logistic regression model, with condition as either a categorical predictor or a linear predictor. A logistic regression model with condition as a categorical predictor best described the data and was used to calculate the odds ratios, 95% confidence intervals, and p-values for each condition with respect to the DMSO control. All statistical models were fit using R version 2.14.1 [57].

**Results and Discussion**

**Identification of \( \text{S. stercoralis} \) Age-1**

To identify the \( \text{S. stercoralis} \) homolog of \( \text{Ca-age-1} \), we performed BLAST searches of the nematode expressed sequence tag (EST) database and found an EST with homology to \( \text{Ca-age-1} \) in \( \text{Parastrongyloides trichiura} \), a closely related parasitic nematode. We designed degenerate primers and amplified an \( \text{S. stercoralis} \) sequence from genomic DNA. Using successive rounds of inverse PCR, we elucidated the 3,999 base-pair (bp) genomic coding region for \( \text{Ss-age-1} \) along with 1.3 kilobase (kb) of the upstream sequence and 1.8 kb of the downstream sequence (GenBank: JQ729018). To determine the 5’ and 3’ ends of the \( \text{Ss-age-1} \) coding sequence, we performed rapid amplification of cDNA ends (RACE) using adapter-ligated cDNA. Subsequently, we cloned the 3,630 bp \( \text{Ss-age-1} \) coding sequence from cDNA derived from free-living adults (Figure 1A) and inferred a predicted peptide of 1,209 amino acids (Figure 1B). The locations of the three introns in \( \text{Ss-age-1} \) were not conserved in \( \text{Ca-age-1} \).

Since \( \text{S. stercoralis} \) and \( \text{C. elegans} \) are members of nematode clades IV and V, respectively, their common ancestry is much more distant than their morphologic similarity suggests [58]. Therefore, we performed phylogenetic analysis to determine whether the predicted \( \text{Ss-Age-1} \) protein grouped with \( \text{Ca-Age-1} \). We obtained catalytic subunit sequences from public databases for class I, II, and III PI3Ks found in a variety of metazoan organisms [59]. The predicted proteins were aligned, and a neighbor-joining phylogenetic tree was constructed (Figure 1C). In this analysis, \( \text{Ss-Age-1} \) grouped with other class I PI3 kinase catalytic subunits, including \( \text{Ca-Age-1} \). A global protein alignment of \( \text{Ss-Age-1} \) and \( \text{Ca-Age-1} \) demonstrated an overall 90.6% amino acid identity and 49.6% similarity. A search of the conserved domain database also revealed conservation of the five PI3 kinase catalytic subunit...
domains in Ss-age-1 [44,59]. Therefore, we conclude that Ss-age-1 is indeed the homolog of Ce-age-1.

Identification of S. stercoralis AAP-1

Functional PI3 kinases include both a catalytic subunit as well as an accessory/regulatory subunit, each encoded by a separate gene [59]. The PI3 kinase accessory/regulatory subunit in C. elegans has been identified as Ce-AAP-1 [28]. To identify the accessory/regulatory subunit for Ss-Age-1, we BLAST-searched the Strongyloides ratti genome database for the homolog of Ce-AAP-1. We identified a putative S. ratti AAP-1 homolog and used degenerate primers to amplify a genomic region encoding Ss-AAP-1 from S. stercoralis genomic DNA.

Using the same protocol as for Ss-age-1, we performed 5’ RACE and identified the coding sequence of Ss-aap-1 (GenBank: JQ781500). The location of the single intron in Ss-aap-1 was not conserved in Ce-aap-1. Protein alignment with other PI3 kinase accessory/regulatory subunits revealed close similarity to Ce-AAP-1 as well as conservation of both SH2 domains, which mediate binding to phosphorylated tyrosine residues on the activated insulin-like receptor [28,60] (Figure S1). A global protein alignment of Ss-AAP-1 and Ce-AAP-1 demonstrated an overall 14.9% amino acid identity and 28.9% similarity. This analysis led us to conclude that the components of a functional PI3 kinase, consisting of an Ss-Age-1 catalytic subunit and an Ss-AAP-1 accessory/regulatory subunit, are present in S. stercoralis.

Ss-Age-1 is Expressed Throughout the S. stercoralis Life Cycle

In C. elegans, it is hypothesized that regulation of IIS is controlled transcriptionally at the level of the insulin-like peptides, while the cytoplasmic signaling components, including AGE-1, are constitutively expressed. Low, but constitutive, expression of Ce-age-1 has been noted in microarray analyses in both the various C. elegans developmental stages [41] and dauer recovery [61]. A recent study confirmed these findings through a careful quantitative examination of all C. elegans insulin-like peptides, daf-2, age-1, and daf-16 transcripts in a variety of developmentally arrested and reproducively developing stages [62].

To determine whether Ss-age-1 is transcriptionally regulated or constitutively expressed over the course of the S. stercoralis life cycle, we performed reverse transcription quantitative PCR (RT-
qPCR) for six developmental stages. Transcript abundance was calculated for Ss-age-1, as well as two reference genes, act-2 (Ss-
act-2) and glyceraldehyde 3-phosphate dehydrogenase (Ss-gapdh), using 50 ng of total RNA isolated from three biological replicates of each developmental stage. We calculated transcript abundances, which were normalized to an arbitrarily determined mean of 10 copies of Ss-age-1 in free-living females and log transformed. We observed Ss-age-1 expression at low, but consistent, levels in comparison to both Ss-act-2 and Ss-gapdh for all life stages examined (Figure 2). Although expression of Ss-age-1 was at its nadir in free-living females, the biological relevance of this difference is questionable, since similar levels of variability between developmental stages were also observed for Ss-act-2 and Ss-gapdh. Overall, these data suggest that, like Ce-age-1, Ss-age-1 is expressed at a low level throughout the course of the S. stercoralis life cycle. This is consistent with the hypothesis that IIS signaling through AGE-1 is regulated post-translationally and not at the transcriptional level.

Ce-Age-1 and Ss-Age-1 have Similar Anatomical Expression Patterns

In C. elegans, expression of a Ce-age-1 cDNA from either a pan-neuronal or intestinal promoter is sufficient to rescue the constitutive dauer arrest phenotype of strong Ce-age-1 mutant alleles [63]. Thus, it is thought that IIS signaling in amphidial neurons and/or intestinal cells is important for regulating dauer arrest and activation. To determine whether anatomical expression of Ss-age-1 was similarly regulated, we compared expression of transcriptional reporters under the control of putative promoters for Ce-age-1 and Ss-age-1.

We first sought to determine the tissues in which Ce-age-1 is expressed to serve as a basis for comparison to Ss-age-1. Previous work has reported expression of a Ce-age-1 transcriptional reporter in amphidial neurons and the intestine, although the sole publicly available image of the BC10837 strain is limited to the head region of the worm [41,64]. To corroborate and expand these findings, we obtained the BC10837 strain and imaged first-stage larvae (L1) and adult hermaphrodites. In order to replicate the findings from the BC10837 strain, we constructed two transgenic C. elegans lines, in the wild-type N2 background, that expressed enhanced green fluorescent protein (EGFP) driven by a Ce-age-1 promoter with a Ce-tbb-2 3' sequence from extra-chromosomal arrays. To determine whether regulatory sequences for Ce-age-1 expression were present in the first part of the coding sequence or the 3' region, three additional lines were transformed with a construct that fused the first 102 bp of Ce-age-1 coding sequence to egfp, driven by the Ce-age-1 promoter and using the native Ce-age-1 3' sequence.

In all six lines, we observed strong EGFP fluorescence in two pairs of amphidial neurons and their dendritic processes, a pair of inter-neurons or support cells anterior to the nerve ring, and the sphincter connecting the pharynx to the intestine (Figure 3A–D).

We also noted variable expression in the hypodermis and the intestine between lines, with some lines having moderate expression in these tissues and others having little or no expression. Weak expression in a phasmidial neuron was observed in a minority of worms in each line. This led us to conclude that the main regulatory elements controlling anatomical expression of Ce-age-1 are located in the putative promoter region and not in the 5' or the 3' coding sequence or 3' region.

Intestinal fluorescence was often weak and punctate in all transgenic lines except BC10837, an observation consistent with auto-fluorescence or limited expression. Since intestinal expression in C. elegans is controlled by the ELT-2 transcription factor [63,66], we searched for extended TGATAA motifs in the 5' region of Ce-age-1. A single non-consensus motif at −289 bp was identified within 2 kb upstream of the Ce-age-1 translation start site, potentially supporting our observation of weak intestinal expression. However, this observation does not preclude the possibility of distant enhancer regions that result in strong intestinal expression of Ce-age-1 from its native locus in vivo.

To test the functionality of the Ce-age-1 promoter region, we made a construct fusing the Ce-age-1 promoter to a full-length Ce-age-1 cDNA. We found that this construct was sufficient to fully rescue constitutive dauer arrest in two lines derived from the CY246 strain that carries the Ce-age-1(mg44) putative null allele. We were able to maintain these transgenic lines for more than 10 generations, indicating complete complementation. Thus, we concluded that we had identified the major tissues in which Ce-age-1 is expressed, providing a consistent basis for comparison with Ss-age-1.

To determine the anatomical expression pattern of Ss-age-1, we transformed parental free-living S. stercoralis females with a construct fusing 1.3 kb of Ss-age-1 5' region to egfp, along with an Ss-act-2::mRFPmars co-injection marker, as previously described [7]. Injected females were paired with S. stercoralis males, and their post-free-living L1 progeny were screened for fluorescence. Of the more than 1,000 F1 larvae screened, 55 transgenic larvae expressing EGFP were observed. Each transgenic L1 was imaged at 400x magnification and scored for fluorescence in several tissues (Table 1). Strong EGFP expression was most frequently observed in the anterior intestine, gonadal primordium, amphidial/head neurons, and phasmidial/tail neurons (Figure 4A–D). Consistent with the observed strong intestinal expression, a search of the 1.3 kb region upstream of the Ss-age-1 translational start site for ELT-2 recognition motifs [66] revealed two consensus TGATAA motifs 136 bp and 630 bp upstream of the start site.

Since specific amphidial neurons are important in the arrest and recovery of both C. elegans dauer larvae [67,68] and S. stercoralis L3i [55,69,70], we sought to identify the amphidial neurons in which
Ce-age-1 and Ss-age-1 are expressed. Examination of transgenic C. elegans L1 and adult hermaphrodites revealed expression of Ce-age-1 in the AWC and ASJ amphidial neuron pairs (Figure 3C–D). AWC neurons are important in thermotaxis [71] as well as chemotaxis to volatile odorants [72,73]. ASJ neurons play an important role in regulating dauer formation [68] as well as dauer recovery [25,67]. Additionally, ASJ neurons are sites of expression of daf-28 [23] and ins-6 [25], both encoding agonistic insulin-like peptides.

A similar examination of transgenic S. stercoralis post-free-living L1 was limited by the number of worms expressing the construct in amphidial neurons. However, using neuronal maps for both Caenorhabditis spp. [74] and P. trichosuri [75], we were able to locate expression in an amphidial neuron pair that we regard as the positional homolog of the AWC amphidial neuron pair in C. elegans (Figure 4C–D). This does not preclude the possibility of Ss-age-1 expression in other amphidial neurons, including ASJ; however, the current limitations of transgenesis in S. stercoralis prevented us from pursuing this in the present study.

We conclude that although the anatomical expression patterns of Ce-age-1 and Ss-age-1 are not completely consistent, expression in important sites of IIS regulation, including amphidial neurons, are conserved between the two species (Figure 3C and Figure 4C). Conservation of expression in these tissues, which sense and direct responses to environmental cues in both species, is consistent with the hypothesis that the function of AGE-1 in directing dauer and L3i development is conserved between C. elegans and S. stercoralis. Furthermore, the C. elegans intestine is an important endocrine tissue [76] and expresses antagonistic insulin-like ligands [22]. Strong expression of Ss-age-1 in the intestine (Figure 4A and Table 1) suggests that the intestine may also be a site of IIS regulation during L3i development in S. stercoralis.

**Table 1. Sites of Ss-age-1 expression in transgenic S. stercoralis post-free-living first-stage larvae.**

|           | Intestine | Gonadal Primorium | Head | Tail | Sheath/Socket | Pharynx | Other Cell Body | Total |
|-----------|-----------|-------------------|------|------|---------------|---------|----------------|-------|
| Expt 1 n, (%) | 11 (73%) | 11 (73%) | 8 (53%) | 4 (27%) | 5 (33%) | 6 (40%) | 6 (40%) | 3 (20%) | 15     |
| Expt 2 n, (%) | 15 (79%) | 7 (37%) | 4 (21%) | 8 (42%) | 7 (37%) | 4 (21%) | 2 (11%) | 19     |
| Expt 3 n, (%) | 15 (71%) | 12 (57%) | 5 (24%) | 5 (24%) | 5 (24%) | 6 (29%) | 6 (29%) | 2 (10%) | 21     |
| Total n, (%)  | 41 (75%) | 30 (55%) | 20 (36%) | 12 (22%) | 18 (33%) | 19 (35%) | 16 (29%) | 7 (13%) | 55     |

doi:10.1371/journal.pone.0038587.t001

Figure 3. **Ce-age-1 is expressed in amphidial neurons and other tissues.** Fluorescence (A,C) and DIC (B,D) images of transgenic C. elegans first-stage larvae expressing Ce-age-1p::Ce-age-1(102bp)::egfp::Ce-age-1t from an extra-chromosomal array. (A,B) Strong expression of the EGFP reporter was present in amphidial neurons (a), a neuron or support cell anterior to the nerve ring (c), and the sphincter connecting the pharynx to the intestine (s). Weak expression was present in the intestine (i), hypodermis (h), and a phasmidial neuron (p). (C,D) EGFP reporter expression was present in the amphidial neurons AWC (short arrow) and ASJ (long arrow). Cell bodies of the amphidial neurons align just lateral to the black lines in panel D [74]. Scale bars = 100 μm.

doi:10.1371/journal.pone.0038587.g003
PI3 Kinase Inhibition Blocks *S. stercoralis* L3i Activation

Previous work in *C. elegans* has demonstrated that pharmacological inhibition of IIS, using the PI3 kinase inhibitor LY294002, leads to dauer arrest under well-fed conditions [77]. This work was extended to clade V parasitic nematodes, the same clade as *C. elegans*, by Brand and Hawdon, who demonstrated that LY294002 could inhibit feeding of *Ancylostoma caninum* and *A. ceylanicum* L3i [78]. LY294002 has a similar effect on larval development in another clade V nematode, *Nippostrongylus brasiliensis* [79]. However, it has never been demonstrated that inhibition of IIS signaling prevents resumption of feeding by infective larvae in any other clade of parasitic nematode in phylum Nematoda, in which animal parasitism is thought to have evolved independently at least four times [58].

To determine whether IIS regulates activation of clade IV *S. stercoralis* L3i under host-like conditions, we performed an *in vitro* L3i activation assay [55,56]. Host-like conditions included incubation in DMEM, supplemented with 10% canine serum and 12.5 mM reduced glutathione, for 24 hours at 37°C and 5% CO₂. Resumption of feeding, a hallmark of activation, was assessed by the ingestion of FITC dye into the pharynx.

We assessed the percentage of L3i feeding in three concentrations of LY294002, 10–100 μM, and compared this to a DMSO (carrier) positive control. We observed a significant, dose-dependent reduction in L3i activation in response to LY294002 (Figure 5). Logistic regression analysis revealed a 90% decrease (odds ratio: 0.10, 95% confidence interval: 0.08–0.13) in the odds of resumption of feeding for L3i at 100 μM, a 67% decrease (odds ratio: 0.33, 95% confidence interval: 0.28–0.40) in the odds of

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Figure 4. *Ss-age-1* is expressed in amphidial neurons, the intestine, and other tissues. Fluorescence (A,C) and DIC (B,D) images of transgenic *S. stercoralis* post-free-living first-stage larvae expressing *Ss-age-1p::egfp::Ss-era-1t* from an extra-chromosomal array. (A,B) Expression of the EGFP reporter was present in the intestine (i), gonadal primordium (g), amphidial/head neuron (a), hypodermis (h), and phasmidial/tail neuron (p). (C,D) Expression of the EGFP reporter was present in an amphidial neuron (long arrow), with positional homology to AWC in *C. elegans*. The other cell body of the amphidial neuron pair is out of the plane of focus (short arrow). Cell bodies of the amphidial neurons align just lateral to the black lines in panel D [74]. Scale bars = 100 μm.

doi:10.1371/journal.pone.0038587.g004
resumption of feeding for L3i at 50 μM, and a 25% decrease (odds ratio: 0.75, 95% confidence interval: 0.63-0.89) in the odds of resumption of feeding for L3i at 10 μM, all in comparison to the DMSO positive control. All odds ratios were significant at conventional statistical probabilities (p<0.001). At 100 μM of the PI3 kinase inhibitor, resumption of feeding by L3i was nearly inhibited to the level observed in the M9 buffer negative control. These data support the hypothesis that IIS signaling through the PI3 kinase, composed of Ss-age-1 and Ss-aap-1, is necessary for resumption of L3i development in S. stercoralis upon encountering a host.

To our knowledge, these data demonstrate, for the first time, that PI3 kinase function and IIS is important in L3i activation in nematodes outside of clade V. This suggests that IIS may regulate activation of L3i in other parasitic nematode species upon encountering their respective hosts. Future studies may provide additional insight into the role of IIS in modulating L3i activation.

Implications for the ‘Dauer Hypothesis’ and Future Directions

We have previously demonstrated that the S. stercoralis ortholog of the Ce-DAF-16 forkhead transcription factor is required for L3i arrest [6]. This is consistent with the function of Ce-DAF-16, which translocates to the nucleus when IIS is diminished under dauer-promoting conditions [30]. In this study, we have presented evidence that the S. stercoralis homolog of Ce-AGE-1 regulates L3i activation. This is also consistent with the function of Ce-AGE-1, which mediates an increase in IIS when dauer larvae encounter favorable conditions [30] and a 25% decrease (odds ratio: 0.75, 95% confidence interval: 0.63-0.89) upon encountering a host. Thus, we find the “dauer hypothesis” to be a relevant and informative framework for investigating the mechanisms governing L3i development in the parasitic nematode S. stercoralis.

Future progress towards defining the role of Ss-age-1 in the development of S. stercoralis L3i will require new or enhanced functional genomic tools. Knock-down of the Ss-age-1 message using RNA interference, which has previously been of limited utility in many parasitic nematodes including S. stercoralis [81–83], could provide an excellent means of assessing the role of Ss-age-1 throughout the life cycle. Previously, gene function in parasitic nematodes has also been inferred from heterologous complementation of mutations in C. elegans, an approach we have taken to assess the function of Ss-daf-16 [84]. However, using established methods [34,84], we have been unable to express Ss-age-1 sequences in the C. elegans CY246 strain to ascertain heterologous complementation. Future studies may reveal the mechanism that accounts for this lack of expression and allow us to pursue this line of experimentation.

Improvements in S. stercoralis transgenesis would also provide several more definitive means of assessing the function of Ss-age-1. Currently, we are able to observe effects of transgene expression in only small numbers of transiently transformed S. stercoralis in the post-free-living generation [6]. Inducible expression of dominant loss- or gain-of-function constructs targeting Ss-age-1 or other IIS components, preferably in stable transgenic lines, would allow us to interrogate functions of specific genes and to assess the epistatic relationships between components of the IIS pathway in regulating development of S. stercoralis L3i. Similar constructs could also be used to determine the role of Ss-age-1 in homogonic versus heterogonic development of S. stercoralis post-parasitic L1. Our studies to elucidate the role of Ss-age-1 in post-parasitic development using the PI3 kinase inhibitor LY294002 have thus far been uninterpretable due, we believe, to our inability to procure developmentally uncommitted L1 [70]. As we establish more robust methods, including the ability to generate stable transgenic lines and undertake conditional transgene expression, we will address additional questions regarding the role of Ss-age-1 and of IIS generally in S. stercoralis development. A greater understanding of the role of IIS in L3i development may lead to novel control strategies as well as new treatments for strongyloidiasis and other diseases caused by parasitic nematodes.

Supporting Information

Figure S1 SS-AAP-1 is a PI3 kinase accessory/regulatory subunit. Protein alignment of PI3 kinase accessory/regulatory subunits. The two Scr homology 2 (SH2) domains (grey bars) have the highest conservation of residues (black highlights) between all sequences. Abbreviations: Strongylidae stercoralis (Sr), Strongylidae ratti (Sr), Brugia malayi (Bm), Ascaris suum (As), Trichinella spiralis (Ts), Caenorhabditis elegans (Ce), Homo sapiens (Hs), and Drosophila melanogaster (Dm). Accession numbers listed in Methods. (TIF)

Table S1 Primer names and sequences. (DOC)

Dataset S1 Predicted protein sequences for Strongyloides ratti age-1 and aap-1 homologs. (DOC)

Text S1 Supplemental Methods - isolation of S. stercoralis developmental stages. (DOC)
Acknowledgments

Thanks to Cathy Wolkow for her generous gift of the CY246 strain and Yuichi Iino for his generous gift of the Pilpy-30-age-1 plasmid. A special thanks to He Zhu for assistance in identification of amphidial neurons in C. elegans and S. stercoralis. Thanks to Brianna Bradford for assistance with the RNA isolation protocol and Susan Roth for work on the Si-age-1 inverse PCR. A special thanks to Andrew Xinghu Lee, Najitu Ranjit, and Beth Maguire for assistance with isolation of parasitic females. A special thanks to Robert Greenberg and Kristina Lewis for critical reading of the manuscript.

Nematode strains N2 and BC10837 used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

Author Contributions

Conceived and designed the experiments: JDS HCM JBL. Performed the experiments: JDS. Analyzed the data: JDS SDG. Contributed reagents/materials/analysis tools: JDS HCM TJD SGDL. Wrote the paper: JDS.

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