Morphogenesis of *Strongyloides stercoralis* Infective Larvae Requires the DAF-16 Ortholog FKTF-1

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**Abstract**

Based on metabolic and morphological similarities between infective third-stage larvae of parasitic nematodes and dauer larvae of *Caenorhabditis elegans*, it is hypothesized that similar genetic mechanisms control the development of these forms. In the parasite *Strongyloides stercoralis*, FKTF-1 is an ortholog of DAF-16, a forkhead transcription factor that regulates dauer larval development in *C. elegans*. Using transgenesis, we investigated the role of FKTF-1 in *S. stercoralis*’ infective larval development. In first-stage larvae, GFP-tagged recombinant FKTF-1b localizes to the pharynx and hypodermis, tissues remodeled in infective larvae. Activating and inactivating mutations at predicted AKT phosphorylation sites on FKTF-1b give constitutive cytoplasmic and nuclear localization of the protein, respectively, indicating that its post-translational regulation is similar to other FOXO-class transcription factors. Mutant constructs designed to interfere with endogenous FKTF-1b function altered the intestinal and pharyngeal development of the larva and resulted in some transgenic larvae failing to arrest in the infective stage. Our findings indicate that FKTF-1b is required for proper morphogenesis of *S. stercoralis* infective larvae and support the overall hypothesis of similar regulation of dauer development in *C. elegans* and the formation of infective larvae in parasitic nematodes.

**Introduction**

Parasitism among nematodes appears to have arisen multiple times throughout evolution [1]. However, the exact mechanism by which nematodes developed parasitic life histories is unknown. Altering gene regulation through variation in conserved signaling systems, is a potential mechanism by which a free-living species might develop characteristics required for parasitism [2]. Insulin-like signaling regulates metabolism and lifespan in a variety of organisms including nematodes, insects and mammals [3,4]. In *Caenorhabditis elegans*, this signaling pathway mediates entry into the dauer larval diapause by negatively regulating DAF-16, a forkhead transcription factor that regulates dauer larval development in *C. elegans* [5]. Biological requirements of *C. elegans* dauer larvae include increased resistance to stress and a metabolism altered to allow the animal to persist, potentially for months, in unfavorable environments [6]. Infective larvae of parasitic nematodes, such as *S. stercoralis*, have similar requirements for survival prior to host finding. The ‘dauer hypothesis’ recognizes the common physiological characteristics of dauer larvae and parasitic infective larvae, and proposes that the same molecular genetic mechanisms control the morphogenesis of both forms [7].

The life cycles of *Strongyloides* and *Parastrongyloides* spp., unusual among the parasitic nematodes, alternate between free-living and parasitic generations [8,9]. First-stage larval progeny of parasitic *S. stercoralis* females typically develop into free-living adults unless triggered by genetic, environmental or host-associated conditions to develop directly into infective third-stage larvae (L3i) [10]. Progeny of the free-living generation of *S. stercoralis* are uniformly fated to become L5i that invade the host and develop into parasitic females. Previous work identified the FOXO encoding gene *fktf-1* (forkhead transcription factor-1) as the ortholog of *C. elegans* daf-16 in *S. stercoralis* [11]. In heterologous rescue experiments, a transgene construct designed to express FKTF-1b (isoform b) partially restored DAF-16 function to *C. elegans* daf-2;daf-16 double mutants [12] rescuing the dauer development phenotype. These data indicate that *fktf-1b* encodes a working forkhead transcription factor that can function in insulin-like signaling to regulate L3 development in *C. elegans*.

The more relevant question of whether FKTF-1b regulates infective larval development in *S. stercoralis* itself can now be addressed using new methods for transgenesis in this parasite [13,14]. In the present study, we transformed free-living adult female *S. stercoralis* with constructs encoding a 2.6 kb *fktf-1b* promoter controlling expression of GFP::FKTF-1b fusion proteins. We then examined first-stage larval progeny of these female worms for anatomical and intra-cellular localization of GFP::FKTF-1b fusion proteins. We next examined the anatomical expression patterns of *fktf-1b*. First, we asked whether the localization of *fktf-1b* expression in *S. stercoralis* mimics that of *C. elegans* daf-16, which is expressed primarily in the pharynx and body neurons [5]. First-stage *S.
Author Summary

Parasitic nematodes are an important threat to public health in much of the world. Understanding how these worms find and invade their hosts may lead to improved therapies. The infectious forms of many parasitic nematodes developmentally arrest as infective third-stage larvae that require hosts to reactivate. Development of these larvae has been compared to that of the diapausing dauer larvae of Caenorhabditis elegans. Our lab studies the development of the human nematode parasite Strongyloides stercoralis. We identified S. stercoralis’ FKTF-1 as an ortholog of DAF-16, a forkhead transcription factor controlling dauer larval development in C. elegans. Transgenes were introduced into S. stercoralis to investigate the possibility that FKTF-1 regulates development of its infective larvae. We discovered that recombinant FKTF-1b tagged with GFP localizes to specific tissues remodeled in infective larvae. Furthermore, mutant forms of FKTF-1b designed to interfere with endogenous FKTF-1b function resulted in incomplete development of the infective larval structures and prevented some transgenic larvae from arresting in the infective stage. Indicating that FKTF-1b is required for the proper development of Strongyloides stercoralis infective larvae, our findings support the hypothesis of similar controls over parasitic and free-living nematode development and pave the way for further comparative studies.

S. stercoralis larvae expressed fktf-1b:gfp::fktf-1b (Figure S1) predominantly in the procorpus of the pharynx (Figure 1A, and Figure 1B, arrow) and the hypoderms (Figure 1C and 1D). These expression patterns continued into the L3i (Figure 1E and 1F). In S. stercoralis, remodeling of the short, trilobed rhabditiform pharynx of the L1 into the long, cylindrical filariform pharynx of the L3i is a hallmark of the transition to infectivity [15]. The rhabditiform pharynx, found in all free-living stages, has three main components: the procorpus, the isthmus and the terminal bulb. The procorpus of the pharynx is the muscular region anterior to the procorpus, the isthmus and the terminal bulb. The procorpus of the pharynx is the muscular region anterior to the narrow isthmus and is primarily responsible for food intake [15]. The pharynx of the non-feeding L3i, is not contractile and has no readily identifiable lobes [15]. Interestingly, expression of the fktf-1b reporter construct in the filariform pharynx was restricted to a band (Figure 1F arrow) analogous to the procorpus of a rhabditiform pharynx. The hypodermal cell layer is responsible for secretion of the cuticle in a stage specific manner [16]. The infective larval cuticle must not only protect the L3i, it must also allow the L3i to sense the presence of a host and secrete molecules facilitating invasion. Although the expression patterns of the fusion protein in L1 varied somewhat (Figure 1G), the fact that the predominant sites of expression were the pharyngeal procorpus and the hypodermis bolsters confidence that the endogenous fktf-1b promoter is active in these tissues in wild-type larvae. This pattern of expression is consistent with a role for FKTF-1b in the development of structures characteristic of infective larvae.

FKTF-1b is regulated via phosphorylation

Insulin-like signaling negatively regulates the function of forkhead transcription factors, including DAF-16, via phosphorylation of serines or threonines at specific sites by Akt/PKB kinases [17]. To ascertain similar post-translational regulation of FKTF-1b, we transformed S. stercoralis with vectors encoding mutant versions of GFP::FKTF-1b that were predicted to behave as either constitutively phosphorylated or non-phosphorylated forms of the protein. Substitution of charged residues, either aspartic or glutamic acids, for serines at Akt/PKB phosphorylation sites in the forkhead domain of human FOXOs is sufficient for disruption of DNA binding by these proteins and for their export from the nucleus [18,19]. Homologous ‘phospho-mimetic’ mutations in predicted Akt/PKB sites of FKTF-1b also resulted in constitutive export of the fusion protein GFP::FKTF-1b(S238E/T240E) (encoded by pPV244, Figure S1) from nuclei of hypodermal cells in transgenic S. stercoralis L1 (Figure 2A and 2B and 2G). Likewise, disruption of all four predicted Akt/PKB sites in FKTF-1b by substitution of the neutral amino acid alanine for critical serine or threonine residues (see Figure 1. Anatomical expression patterns of fktf-1b:gfp::fktf-1b. DIC and fluorescence images of transgenic S. stercoralis larvae. Each DIC image is a separate individual. All scale bars = 10 μm. (A,B) Transgenic first-stage larvae with GFP expression in the procorpus (arrow) of the pharynx. (C,D) Expression of the GFP::FKTF-1b(wt) transgene in the hypodermis of an L1. (E,F) Transgenic L3i expressing the GFP::FKTF-1b(wt) fusion protein in the hypodermis and a narrow band in the pharynx (arrow). (G) Sites of gfp expression under the direction of the fktf-1b promoter in 125 transgenic first-stage larvae from five or more experiments expressing gfp reporters under the direction of fktf-1b promoter. Due to variations in transformation rates between experiments, all transgenic larvae were pooled to quantify expression patterns. χ² test, P = 2.9E-14. doi:10.1371/journal.ppat.1000370.g001
pPV243, Figure S1) resulted in strongly enhanced nuclear localization of the 'phospho-null' fusion protein GFP::FKTF-1b(4A) (Figure 2E–2G). These data indicate that FKTF-1b's intracellular localization, and thereby its access to genomic response elements, is regulated by phosphorylation in a similar manner to DAF-16 and other FOXO-class transcription factors.

Dominant interfering transgenes cause altered intestinal morphology in L1s

Its anatomical localization and intra-cellular trafficking support the hypothesis that FKTF-1b is an ortholog of DAF-16 and that through it, insulin-like signaling regulates *S. stercoralis* larval development. More conclusive testing of this hypothesis requires experimental manipulation of gene function and evaluation of phenotypic outcomes. Thus far, *S. stercoralis*, like many other parasitic nematodes, has proven insensitive to targeted gene silencing via RNAi [20]. Therefore, we opted for an approach based on transgenesis in which we express altered forms of FKTF-1b designed to interfere with the function of the endogenous transcription factor. Two such mutant proteins, encoded by plasmids pPV251 and pPV298, respectively (Figure S1), are tagged with GFP and carry the four 'phospho-null' mutations described above, causing them to be sequestered in the nucleus

Figure 2. Intra-cellular localization of GFP::FKTF-1b phosphorylation mutants. DIC and fluorescence images of representative hypodermal cells of transgenic *S. stercoralis* L1s. In all images, the nucleus is identified with an arrow. All scale bars = 10 μm. (A,B) L1 expressing pPV244(S238E/T240E), the "phospho-mimetic" GFP::FKTF-1b with fluorescence in the cytoplasm. (C,D) L1 expressing pPV234, GFP::FKTF-1b(wt), with fluorescence in both the cytoplasm and the nucleus. (E,F) L1 with strong nuclear localization of the FKTF-1b(4A) fusion protein. (G) Percentages of hypodermal cells in transgenic larvae with intra-cellular localization of GFP classified as "cytoplasmic", "cytoplasmic/nuclear", or "nuclear". Results include transgenic larvae from at least two separate microinjection experiments per transgene. Phospho-mimetic: n = 47 GFP+ cells in 11 larvae. GFP::FKTF-1b: n = 29 GFP+ cells in 8 larvae. Phospho-null: n = 37 GFP+ cells in 8 larvae. χ² test P = 1.23E-10.
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where they presumably out-compete native FKTF-1b for response elements in the genome. In addition, both mutant proteins are truncated within the C-terminal domain immediately downstream of the fourth regulatory phosphorylation site, ablating key transactivator binding motifs. In one of the dominant interfering proteins, encoded by pPV251 and dubbed GFP::FKTF-1b(dominant-repressor), the truncated C-terminal domain is fused to the repressor domain of Ce-PIE-1, a protein responsible for the transcriptional repression characterizing the germline precursor of

C. elegans [21]. In the other mutant protein, encoded by pPV298 and dubbed GFP::FKTF-1b(dominant-negative), the truncated C-terminal domain is not linked to Ce-PIE-1.

Upon hatching, larvae expressing either of the dominant interfering constructs were shorter but virtually identical in form to larvae expressing GFP-tagged wild-type FKTF-1b at similar levels (Figure S2), indicating that the S. stercoralis transcription factor, like DAF-16 in C. elegans [22], does not play a significant role in embryonic development. By contrast, at 24 hours, S. stercoralis L1 expressing either of the dominant interfering mutants of FKTF-1b exhibited phenotypic changes in the form and apparent function of their intestinal cells, with these being most evident in larvae expressing the dominant-repressor construct. These phenotypes ranged from flattening of the normally apically rounded intestinal cells and a decrease in the number of cytoplasmic storage granules in the presence of GFP::FKTF-1b(dominant-negative) (compare Figure 3J to Figure 3H and Figure 3I) to an almost complete loss of intestinal cell architecture and of cytoplasmic storage granules in the presence of the GFP::FKTF-1b(dominant-repressor). Perhaps due to compromised intestinal cell function, S. stercoralis L1 expressing GFP::FKTF-1b(dominant-repressor) exhibited significant (P<0.01) growth retardation at 24 hours (Figure 3N). Opposite to the severity of the associated phenotypes, none of the larvae expressing GFP::FKTF-1b(dominant-repressor) survived beyond the L1. The fact that S. stercoralis L1 expressing comparable levels of wild-type FKTF-1 tagged with GFP (Figure 3B, 3E, and 3I) were morphologically similar to untransformed larvae (Figure 3A and 3H) argues against the observed phenotypes being due to non-specific effects of recombinant protein expression. Therefore, it is clear from these findings that FKTF-1 is necessary for normal development of intestinal cells in pre-infective larvae of S. stercoralis and specifically for accumulation of storage granules, which may contain reserves necessary for survival of the L3i.

Dominant interfering transgenes cause aberrant morphogenesis in S. stercoralis L3

With regard to the dauer hypothesis and the role of insulin signaling in infective larval development, the most significant results in the present study were the morphogenetic changes seen in L3 expressing the GFP::FKTF-1b(dominant-negative) transgene. Under the null hypothesis, all of our transgenic larvae should develop to L3i. While L3i expressing the wild-type GFP::FKTF-1b fusion protein were morphologically identical to their non-transgenic counterparts (Figure 4A and 4B, compare to Figure S3A), L3 expressing the dominant-negative transgene (Figure 4C and 4D) exhibited some indications of bypassing developmental arrest and failing to undergo the pharyngeal and intestinal remodeling characteristic of L3i. Three of the 11 transgenic L3 appeared to initiate an aberrant molt to the fourth stage as evidenced by the presence of a pointed tail inside a notched L3i cuticle cast (Figure 4E and 4F compared to wild-type Figure 4G). The notched tail is characteristic of infective larvae and is created by pairs of ‘L3i-specific’ alae [15]. Another L3 expressing the dominant-negative construct exhibited an elongated rhabditiform pharynx complete with a grinder-like structure (Figure 4H and 4I) instead of the expected filariform pharynx (Figure S3B and Figure S3C). Incomplete remodeling of the rhabditiform pharynx and initiation of a supernumerary molt in culture are consistent with expression of the interfering FKTF-1b transgenes in the pharyngeal procorpus and the hypodermis. Initiation of ecdysis by L3 in combination with retention of some rhabditiform pharyngeal characteristics as we observed suggests that worms expressing GFP::FKTF-1b(dominant-negative) were developing in the direction of a second-generation free-living L4. While such a form occurs in some strongyloidoid species (e.g. Strongyloides rhabdoides, Panstrongylus trihouni), it does not exist in the natural life cycle of S. stercoralis [8,23].

Five of the 11 transgenic L3 expressing the dominant-negative construct exhibited changes consistent with a failure to remodel the free-living intestine into the darkened, radially constricted intestine of the L3i. In some cases, the L3 intestine retained bacteria (Figure 4E) and in others, it failed to constrict and close (Figure 4I to the left of the black triangle). The incompletely remodeled intestine seen in the transgenic L3 is consistent with the defects in intestinal structure seen in the L1. Together, these data indicate that FKTF-1b is required for the proper remodeling of the pharynx and the intestine of a free-living larva into structures characteristic of the infective larva.

Our findings support the ‘dauer hypothesis’ [7] by showing that the forkhead transcription factor FKTF-1b, presumably under the control of insulin-like signaling, regulates infective larval development in Strongyloides stercoralis in a manner similar to the dauer regulatory functions of DAF-16 in Caenorhabditis elegans. Furthermore, in this study, we have demonstrated the utility of transgenesis in S. stercoralis for investigation not only of temporal and spatial patterns of gene expression, but also of endogenous gene function. This work opens new avenues of inquiry into the genes involved in the shift between free-living and parasitic states in Strongyloides stercoralis and ultimately into the evolution of parasitism in nematodes generally.

Materials and Methods

Parasite maintenance and culture

The UPD strain of Strongyloides stercoralis was maintained in immuno-suppressed dogs and cultured as described [24]. Free-living adult S. stercoralis were isolated from two-day-old coprocultures via Baermann funnels. The worms were washed twice with sterile deionized water to reduce carryover of fecal bacteria and plated on Nematode Growth Medium (NGM) agar plates seeded with Escherichia coli OP50. All cultures of S. stercoralis were incubated at 22°C unless otherwise noted.

Transgene construction

**gfp reporters.** In C. elegans, the promoter for splice form daf-16b exists in the large intron between exons 4 and 5 [25]. The flt-1 genomic sequence also includes a large, approximately 6 kb, intron between the end of the flt-1a-specific exons (exons 1–3) and the beginning of the flt-1b-specific exon 4 [11]. We used 2.6 kb sequence directly 5’ of the flt-1b-specific ATG codon plus the first 90 bp of the coding sequence as the flt-1B promoter element. Using the primers SflmBrdfl and SbrdPrsfl2 (Table S1), we amplified the putative flt-1B promoter from plasmid pPV57. The PCR product was cloned into pAqJ01, a promoter-less vector containing the gfp coding sequence fused to the Strongyloides era-1 3’ UTR, with HindIII and PstI restriction enzymes using standard techniques. The resulting flt-1B::gfp::era-1 reporter construct was designated pPV232 (Figure S1).

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Figure 3. *S. stercoralis* transgenic L1s 24 hours post-hatch. Representative images of *S. stercoralis* wild-type and transgenic larvae at 24 hours post-hatch. Each DIC image is a separate individual. All scale bars = 10 μm. By 24 hours, the wild-type (A) and GFP::FKTF-1b(wt) (B) larvae have grown to comparable lengths with similar numbers of intestinal granules. (C) GFP::FKTF-1b(dom-neg)–expressing larvae are a similar length but show...
To create the fusion protein GFP::FKTF-1b, the original construct pPV232 was altered via site-directed mutagenesis (QuikChange Site-Directed Mutagenesis, Stratagene, La Jolla, California, USA), to remove the terminator for the gfp coding region and to introduce the unique restriction site BspEI using the primers GFPnoTER-BspEF and R (Table S1). The clone with the appropriate mutation and the new restriction site was designated pPV233 (βfktf-1b::gfp (no ter)::era-1). The cDNA sequence for βfktf-1b had been cloned previously into a plasmid vector pPV207. The coding region of βfktf-1b was amplified from pPV207 using the primers: BspE-FkBF (Table S1) and FkAvHR (Table S1) and inserted into pPV233 using the restriction enzymes BspEI and AvrII via standard techniques. The clone containing the full-length βfktf-1b promoter with gfp fused N-terminal to and in frame with the βfktf-1b coding region was designated pPV234 (βfktf-1b::gfp::βfktf-1b::era-1) (Figure S1).

Phosphorylation mutant constructs. Phosphorylation-null and phosphorylation-mimetic constructs were created from pPV234 using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis, Stratagene, La Jolla, California, USA). All phosphorylation-null mutagenesis reactions were carried out on the cDNA sequence of βfktf-1b in the pCR-BluntII-TOPO cloning vector (Invitrogen, Carlsbad, California, USA) to improve the efficiency of the reactions. The phosphorylation-null construct was created in three stages, each stage mutating the key regulatory residues in one of the three functional domains. Primers FK-1bPst1F2 (Table S1) and FK-1bPst1R (Table S1) mutated threonine 22 to alanine in the N-terminal domain. Primers SD16Pst1F2 and SD16Pst1R (Table S1) mutated both serine 218 and threonine 240 to alanine. Primers FkCPsite3KOR2 and FkCPsite3KOR2 (Table S1) mutated the phosphorylation site serine 317 to alanine in the C-terminal transactivation domain. Once all four sites were mutated, the wild-type βfktf-1b coding region was removed from the pPV234 vector, and the phosphorylation-null βfktf-1b(4A) coding region was cloned in using BspEI and AvrII sites using standard techniques. The phosphorylation-null construct was designated pPV243 (βfktf-1b::gfp::βfktf-1b(4A)::era-1) (Figure S1).

Phosphorylation-mimetic primers FkB S238E&T240E F and FkB S238E&T240E R (Table S1) mutated serine 238 and threonine 240, key residues in the forkhead domain, to glutamic acid to mimic the negative charge of phosphorylated residues. The phosphorylation-mimetic construct was designated pPV244 (βfktf-1b::gfp::βfktf-1b(S238E&T240E)::era-1) (Figure S1).

Dominant-interfering constructs. The phosphorylation-null construct, GFP::FKTF-1b(4A), which sequesters in the nucleus, still contains functional forkhead (DNA binding) domain and C-terminal transactivation domains and thus should out compete endogenous FKTF-1b for DNA binding sites. Truncation of the C-terminal domain in the phosphory-null GFP::FKTF-1b, should create a protein constitutively bound to DNA but unable to activate transcription. Subsequently, replacing the endogenous transactivation domain of FKTF-1b with a repressor domain should create a protein actively repressing transcription specific to FKTF-1b’s DNA binding domain.

The transactivation domain was truncated using an introduced ClaI site immediately downstream of the regulatory phosphorylation site S317. The phospho-null βfktf-1b(4A) in the pCR-BluntII-TOPO vector (Invitrogen, Carlsbad, California, USA) was again used for mutagenesis using the primers FKTImutClaF and FKmutTrcClaR2 (Table S1) to introduce the ClaI site in the desired location. The plasmid was cut using BspEI and ClaI to release the βfktf-1b(4A, truncated) coding region (BspEI-βfktf-1b(4A, trunc)-ClaI).

The coding region for the C. elegans gene pie-1 was amplified from RNA prepared from a pool of C. elegans N2 worms using the primers Ce-pie-1ATGF and Ce-pie-1StopR (Table S1) and cloned into a pCR-BluntII-TOPO cloning vector (Invitrogen, Carlsbad, California, USA). The active repressor domain of PIE-1 consists of 81 amino acids at the C-terminal (CTD) [21]. Using the primers Clal-pieCTDF and Ce-pie-IutraVR (Table S1) the repressor domain alone was PCR amplified using PfuTurbo (Stratagene, La Jolla, California, USA). The resulting PCR product was digested with Clal and AvrII for cloning, (Clal-pieCTD-AvrII).

pPV234 was digested with BspEI and AvrII to remove the wild-type βfktf-1b coding region. The ligation reaction included the digested pPV234 vector, the BspEI-βfktf-1b(4A, trunc)-Clal and the Clal-pie-1CTD-AvrII simultaneously. The resulting construct, pPV251 (βfktf-1b::gfp::βfktf-1b(4A, trunc)::pie-1CTD::era-1), incorporated gfp upstream of the mutated and truncated βfktf-1b region, which became fused to the pie-1CTD (Figure S1). The dominant-negative construct, pPV298 (βfktf-1b::gfp::βfktf-1b(4A, trunc)::era-1) (Figure S1), was created by simply removing the pie-1CTD from pPV251 via restriction enzyme digest with Clal and AvrII. The digested ‘sticky ends’ were filled in with PfuTurbo (Stratagene, La Jolla, California, USA) using standard methods and blunt-end ligated.

Transformation of S. stercoralis

Adult female S. stercoralis were transformed with transgene encoding plasmids via intra-gonadal microinjection using standard protocols [24,26]. Coding plasmids were injected at a concentration of 10–100 ng/ml with non-coding plasmids being used as necessary to make up the total DNA concentration to 100 ng/ml. Following injection, worms were transferred to clean NGM OP50 plates with an excess of males and incubated at 22°C. For general expression patterns, plates were scored at 24 hour intervals for adult survival and frequency of transgene expression among F1 progeny. For specific time points, adults were transferred to clean NGM OP50 plates at three to five hour intervals to obtain egg cohorts. All plates with eggs were checked at hourly intervals for the presence of transgenic progeny. When the time of hatch was known, transgenic larvae were transferred to clean plates marked with the time point and examined after the
found that C. elegans transformed with the same gfp::fktf-1b coding sequence under the control of the daf-16 promoter exhibit GFP fluorescence and express a full length transcript encoding the fusion protein (data not shown). Current methods only allow us to observe transgene expression in F1 generation following transformation.

DIC and Fluorescent Microscopy

Transgenic larvae were identified based on GFP fluorescence using an Olympus SZX12 stereomicroscope with coaxial epifluorescence. For more detailed examination of particular tissues and individual cells, larvae were immobilized on 4% Agar Noble (Sigma, St. Louis, Missouri, USA) pads in 10 mM (L1) or 20 mM (L3) levamisole and observed using an Olympus BX60 compound microscope equipped with Nomarski Differential Interference Contrast (DIC) optics and epifluorescence (Olympus America Inc., Center Valley, Pennsylvania, USA). Specimens were imaged with a Spot RT Digital Color camera and images were processed using either the Spot Advanced image analysis software package (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA) or Adobe Photoshop 7.0. All image-processing algorithms (e.g. brightness and contrast adjustments) were applied in a linear fashion to the entire image.

Measurements

Worm lengths were measured using the ImageJ program available from the National Institutes of Health [http://rsb.info.nih.gov/ij/] [27]. Calibrations were done by determining the distance of 10 μm on a micrometer in pixels and then setting the scale in the program. All measurements were done in duplicate using the freehand line option, taking the average of the results for analysis.

Statistical Analysis

As categorical data, expression patterns, localization and phenotypes, were analyzed using χ² tests. Analysis of the expression patterns of the fktf-1b promoter constructs was based on the null hypothesis that the expression patterns were not specific to the hypodermis and the pharynx. Categories of intracellular GFP localization were analyzed based on the null hypothesis that the phosphorylation status of the FKTF-1b protein had no effect on its localization. In order to compare the mean lengths of wild-type and transgenic larvae, we used the Mann-Whitney test, which makes no assumptions as to the population distribution of the observations.

Supporting Information

Figure S1 Diagrams of fktf-1b constructs used to transform S. stercoralis. pPV232 encoding the fktf-1b::gfp transcriptional reporter. pPV234, the Gfp::FKTF-1b(wt) fusion protein expression vector. pPV243 (Gfp::FKTF-1b(4A)) has all four canonical Akt/PKB phosphorylation sites mutated to alanine. pPV244 (Gfp::FKTF-1b(S238E/T240E)) has the phosphorylation sites in the forkhead domain changed to the phospho-mimetic glutamic acid. pPV251 (Gfp::FKTF-1b(dom-rep)) and pPV298 (Gfp::FKTF-1b(dom-neg)) both contain the four alanine mutations. pPV251 encodes a chimeric fktf-1b with the repressor domain from Ce-pie-1 replacing the endogenous transactivation domain. pPV298 is truncated just downstream of the fourth regulatory phosphorylation site and thus lacks either a transactivation or a repressor domain.

Found at: doi:10.1371/journal.ppat.1000370.s001 (0.63 MB TIF)
Figure S2  Transgenic *S. stercoralis* L3i at 1 hour post-hatch. DIC and fluorescence images of *S. stercoralis* larvae. Each DIC image is a separate individual. All scale bars = 10 μm. (A–D) 1-hour-old transgenic hatchlings exhibit similar morphology to the non-transgenic hatchlings. E-G The fusion protein transgenes have similar levels of expression throughout the larvae. (H–K) Intestinal cells of the 1 hour-post-hatch larvae were examined for presence or absence of granules using the primordial gonad (arrow) as a landmark. All larvae, wild-type and transgenic, show healthy looking cells with little granularity at this early timepoint. The similar morphologies of the larvae at 1 hour post-hatch indicate apparently normal embryogenesis of transgenic larvae. Found at: doi:10.1371/journal.ppat.1000370.s002 (3.25 MB TIF)

Figure S3 Examples of *Strongyloides stercoralis* wild-type L3i. (A) DIC image of wild-type L3i showing filariform pharynx, pharynx-intestinal junction (black triangle), and constricted, dark intestine. Scale bar = 20 μm. (B) Anterior half of L3i pharynx showing constricted cylindrical structure characteristic of the filariform pharynx. Scale bar = 10 μm. (C) Pharynx-intestinal junction (black triangle) of an L3i. Note the lack of a grinder-like structure at the base of the pharynx and the closed intestine to the left of the junction. Scale bar = 10 μm. Each image is a separate individual. Found at: doi:10.1371/journal.ppat.1000370.s003 (3.95 MB TIF)

Table S1  Primer sequences used in construct creation. Found at: doi:10.1371/journal.ppat.1000370.s004 (0.06 MB DOC)

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