Identification and characterization of an R-Smad homologue (Hco-DAF-8) from Haemonchus contortus

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Abstract

Background: Smad proteins are essential cellular mediators within the transforming growth factor-β (TGF-β) superfamily. They directly transmit incoming signals from the cell surface receptors to the nucleus. In spite of their functional importance, almost nothing is known about Smad proteins in parasitic nematodes including Haemonchus contortus, an important blood-sucking nematode of small ruminants.

Methods: Based on genomic and transcriptome data for H. contortus and using bioinformatics methods, a Smad homologue (called Hco-daf-8) was inferred from H. contortus and the structural characteristics of this gene and its encoded protein Hco-DAF-8 established. Using real-time PCR and immunofluorescence assays, temporal transcriptional and spatial expression profiles of Hco-daf-8 were studied. Gene rescue in Caenorhabditis elegans was then applied to assess the function of Hco-daf-8 and a specific inhibitor of human Smad3 (called SIS3) was employed to evaluate the roles of Hco-DAF-8 in H. contortus development.

Results: The features of Hco-DAF-8 (502 amino acids), including conserved R-Smad domains and residues of the L3-loop that determine pathway specificity, are consistent with a TGF-β type I receptor-activated R-Smad. The Hco-daf-8 gene was transcribed in all developmental stages of H. contortus studied, with a higher level of transcription in the fourth-stage larval (L4) females and the highest level in adult males. Hco-DAF-8 was expressed in the platymyarian muscular cells, intestine and reproductive system of adult stages. Gene rescue experiments showed that Hco-daf-8 was able to partially rescue gene function in a daf-8 deficient mutant strain of C. elegans, leading to a resumption of normal development. In H. contortus, SIS3 was shown to affect H. contortus development from the exsheathed third-stage larvae (L3s) to L4s in vitro.

Conclusions: These findings suggest that Hco-DAF-8, encoded by the gene Hco-daf-8, is an important cellular mediator of H. contortus development via the TGF-β signalling pathway. They provide a basis for future explorations of Hco-DAF-8 and associated pathways in H. contortus and other important parasitic nematodes.

Keywords: Haemonchus contortus, R-Smads, TGF-β signalling pathway, Development, Gene rescue, Specific inhibitor of human Smad3 (SIS3)

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Background
The transforming growth factor-β (TGF-β) signalling pathway regulates the growth, development and differentiation of cells in diverse organisms including humans, mice, flies and worms [1, 2]. In this pathway, signalling is initiated when the ligand induces the assembly of a heteromeric complex of type II and type I receptors. Upon activation of type I receptors, signalling from cell-surface receptors to the nucleus is mediated by Smad proteins.

Based on their functions, Smad proteins are classified into three subclasses, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads) [3]. R-Smads are direct substrates of type I receptors, represented by TGF-β/Activin-activated R-Smads and BMP-activated R-Smads. The former molecules are specifically activated by activin/nodal and TGF-β type I receptors, such as Hs-Smads 2 and 3; the latter molecules are activated by BMP (bone morphogenetic protein) type I receptors, including Hs-Smads 1, 5 and 8 [4]. Following phosphorylation by a type I receptor, R-Smads interact with Co-Smads to form a complex, which is then translocated into the nucleus to regulate the transcription of target genes. In these processes, R-Smads usually play a central role in maintaining specificity in the TGF-β signalling pathway by functioning both as a substrate of the type I receptor and as an usher for Co-Smad [5]. In addition, R-Smads are regulated by inhibitory Smads, which prevent the phosphorylation and/or nuclear translocation of R-Smads [6].

In Caenorhabditis elegans, the TGF-β signalling pathway (daf-7 pathway) functions to regulate dauer formation via the R-Smads Cel-DAF-8 and Cel-DAF-14 [7]. In favourable environments, the daf-7 signal is received by a heterotetrameric receptor composed of two DAF-1 type I receptor and two DAF-4 type II receptor subunits on the cell surface. The DAF-4 type II receptor first phosphorylates the DAF-1 type I receptor, which then propagates the signal through phosphorylation of the R-Smad proteins Cel-DAF-8 and Cel-DAF-14. Activated R-Smads complexes are translocated into the nucleus and inhibit the functions of DAF-3/Co-Smad and DAF-5, regulating the transcription of genes involved in normal larval development [8]. Under harsh environmental conditions the daf-7 pathway is inactive, allowing downstream genes such as daf-3 and daf-5 to promote dauer formation [9]. Cel-DAF-3 can repress the transcription of Cel-DAF-8, which drives a negative feedback loop of this pathway [10]. In C. elegans, R-Smads Cel-DAF-8 and Cel-DAF-14 are co-expressed in a subset of neurons, the intestine and excretory cells, and, interestingly, have partial functional redundancy of one another [10, 11].

In the parasitic nematode H. contortus, recent studies identified some members of the TGF-β signalling pathway including TGF-β type I receptor [12], TGF-β type II receptor [13] and Co-Smad [14] and evaluated their important roles in larval development from the exsheathed third-stage larvae (L3s) to L4s in vitro. As there is no information available on these R-Smads for parasitic nematodes of clade V [15], we took the opportunity of exploring the structure and function of the homologue of Cel-DAF-8 in H. contortus (barber’s pole worm), one of the economically most important parasites of small ruminants worldwide [16].

Herein, a TGF-β-activated R-Smad gene from H. contortus, called Hco-daf-8 was isolated and characterised. The transcription profile of the Hco-daf-8 gene in distinct developmental stages of H. contortus and protein localisation in adults were investigated. Gene rescue experiment showed that Hco-daf-8 was able to partially rescue the dauer phenotype of daf-8-deficient mutant strain of C. elegans, and a Smad3-specific inhibitor SIS3 was shown to affect H. contortus development from L3s to L4s in vitro.

Methods
Caenorhabditis elegans and H. contortus strains, and their maintenance
Caenorhabditis elegans strains were purchased from the Caenorhabditis Genetic Center (CGC) (University of Minnesota, USA) and cultured with Escherichia coli OP50 as a food source on nematode growth medium (NGM) using standard procedures [17], unless otherwise stated. Worm strains used were N2 wild-type and a daf-8 mutant (CB1393), the latter of which is temperature-sensitive and has a dauer phenotype at 25 °C. Haemonchus contortus (Haecon 5 strain) employed here was maintained by serial passages in experimental goats (raised helminth-free; 14 weeks of age); kids were infected orally with 7000 third-stage (infective) larvae (iL3s). Eggs isolated from the faeces by sucrose flotation [18] were cultured in tissue culture flasks at 28 °C in a nutritive medium (0.1 ml/ml of culture of 1× Earle’s balanced salt solution (EBSS, Sigma-Aldrich) and 0.5% (w/v) of yeast extract) [19]. First-stage larvae (L1s), second-stage larvae (L2s) and iL3s were obtained from eggs cultured at 28 °C (constant) for 1, 4 and 7 days, respectively [20]. Fourth-stage larvae (L4s; both sexes) and adults (both sexes) were collected from the abomasum of infected goats, euthanased 8 and 30 days, respectively, following oral infection with iL3s [20]. Males and females of L4s and adults were separated as described previously [21].

DNA and RNA preparation
Genomic DNA was extracted from mixed developmental stages of C. elegans using an EasyPure Genomic DNA kit (TransGen Biotech, Beijing, China). Genomic
DNA was also isolated from adult *H. contortus* (both sexes) by mini-column (Wizard® Clean-Up; Promega, Beijing, China) purification following small-scale SDS/proteinase K digestion [22]. DNA samples were stored at −20 °C. Total RNA was isolated using the TRIPure reagent (Aidlab, Beijing, China) as recommended by the manufacturer. RNA quality and yields were verified by electrophoretic analysis and spectrophotometry (NanoDrop Technologies, Wilmington, USA), respectively. First-strand cDNA synthesis was performed using the extracted RNA and PrimeScript™ RT reagent kit with DNA Eraser (Takara, Beijing, China). RNA and cDNA samples were stored at −80 °C.

**Identification of the *C. elegans* DAF-8 homologue in *H. contortus***

The *Cel-daf-8* gene sequence was obtained from WormBase (WS271; code R05D11.1), and an homologue of this gene was identified by searching the *C. elegans* protein sequence against the non-redundant (nr) protein database using the protein BLAST from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). Homologues of the *Cel-DAF-8* protein were inferred from the *H. contortus* genomes (PRJEB506 and PRJNA205202) using BLAST/BLAT (https://wormbase.org/tools/blast_blat) [23].

**Isolation of *Hco-daf-8* genomic sequence and coding DNA sequence (CDS)**

Using genomic and transcriptomic data sets for *H. contortus* [16, 24], the sequences of the *Hco-daf-8* coding region and full-length genomic region were retrieved (GenBank: HF958885.1). The coding region of *Hco-daf-8* was PCR-amplified from cDNA with primer pair *Hco-daf-8*-F and *Hco-daf-8*-R (Additional file 1: Table S1) using the following cycling conditions: 98 °C for 5 min; then 98 °C for 10 s, 55 °C for 5 s, 72 °C for 90 s for 35 cycles; and a final extension step at 72 °C for 5 min. The full-length genomic sequence of *Hco-daf-8* was PCR-amplified from *H. contortus* genomic DNA using the same primer pair and conditions as described above, except that the extension time was 3 min, instead of 90 s. These two PCR products were individually inserted into pTOPO-Blunt vector (CV17; Aidlab) and directly sequenced in both directions (via TsingKe Biological Technology, Wuhan, China).

**Bioinformatic and phylogenetic analyses**

Nucleotide (nt) sequences and amino acid (aa) sequences were assembled and aligned using the programs BLASTx and Clustal W [25]. Briefly, the sequence of *Hco-daf-8* was compared with sequences in non-redundant databases using the BLASTx from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the identity of cloned genes. Each cDNA sequence was conceptually translated into predicted amino acid sequence using DNASTar software (http://www.dnastar.com). To determine the sequence characteristics of *Hco-DAF-8*, amino acid sequence comparison was performed. The predicted amino acid sequence of *Hco-DAF-8* was aligned with two R-Smads (*Cel-DAF-8* and *Cel-DAF-14*) involved in the DAF-7 signallng pathway in *C. elegans* and a panel of reference sequences: TGF-β/Activin-activated R-Smads (*Hs-Smad2, Hs-Smad3* of *Homo sapiens*; *Dm-Smox of Drosophila melanogaster*; and *Dar-MAD of Danio rerio*) and BMP-activated R-Smads (*Hs-Smad1, Hs-Smad5* of *H. sapiens*; *Dm-MAD of D. melanogaster*; and *Mm-MAD1 of Mus musculus*) (Additional file 1: Table S2) using the program Clustal W [25]. Graphic view was conducted using BioEdit software (https://bioedit.software.informer.com). Sequence identity was assessed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Protein motifs of *Hco-DAF-8* were identified by scanning the databases Pfam (www.sanger.ac.uk/Software/Pfam) and PROSITE (http://prosite.expasy.org/). Exon and intron boundaries in DNA sequences were inferred using the software MAFFT (https://mafft.cbrc.jp/alignment/server/) and the “GT-AG” rule [26]. Nuclear localisation signal (NLS) sequence was predicted using the program cNLS Mapper [27].

The alignment of *Hco-DAF-8* with selected references including *Cel-DAF-8* and its homologues (*Cbmdaf-8* and *Cjp-DAF-8*), *Cel-DAF-14* and its homologues (*Cbr-DAF-14* and *Cjp-DAF-14*), *Cel-SMA-2* and its homologues (*Cbr-SMA-2, Ovo-SMA-2, Hco-SMA-2, Cel-SMA-3* and *Cbr-SMA-3*, as well as four R-Smads from *Homo sapiens* and Smox from *D. melanogaster*) were subjected to phylogenetic analysis. An inhibitory-Smad protein of *D. melanogaster* (*Dm-DAD*) was used as an outgroup (Additional file 1: Table S2). Phylogenetic analyses were conducted using the neighbour-joining (NJ), minimum evaluation (ME) and maximum likelihood (ML) methods employing the Jones–Taylor–Thornton (JTT) model [28]. Confidence limits were assessed using a bootstrap procedure, with 1000 bootstrap replications for NJ and default settings (in MEGA7; [28]) for the other methods. A 50% cut-off value was implemented for the consensus tree.

**Assessing transcript abundance using real-time PCR**

Transcription of *Hco-daf-8* was examined in each of the six developmental stages (eggs, L1s, L2s, iL3s, L4s and adults) and both sexes (males and females) of *H. contortus* (Haecon-5 strain) by real-time PCR using the
primers rtHco-daf-8-F and rtHco-daf-8-R (Additional file 1: Table S1). RNA was isolated separately from eggs, L1s, L2s and il3s (10,000 of each stage) and female L4, male L4 (five of each), female adults or male adults (three of each) using TRIPure TriZol (Aidlab) according to the manufacturer’s protocol. An equal amount (1 μg) of RNA from each stage was used to synthesise the first-strand cDNA by random primer using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara), respectively. The RT-PCR (10 μl) was performed using the TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, No. RR820A) in a thermal cycler (ALL A7; Bio-Rad, Berkeley, USA) under the following protocol: 50 °C for 2 min and 95 °C for 30 s for the first cycle, followed by 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s for 40 cycles. Each sample was tested in triplicate, employing a β-tubulin 8–9 gene (GenBank: M76493) as a reference gene (using specific primers Tubulin-F and Tubulin-R; Additional file 1: Table S1) [29]. The mean quantification cycle (Cq) values were subjected to compare the relative quantities with egg (egg = 1) using the 2−ΔΔCq method [30]. This assay was repeated three times. Statistical analysis was carried out using one-way ANOVA in GraphPad Prism 6 (https://www.graphpad.com/support/faqid/1745/). P-values were calculated using the Tukey’s post-hoc test; values of < 0.05 were considered statistically significant.

Production of polyclonal antibody against recombinant Hco-DAF-8 and immunoblot analysis

The truncated cDNA fragment of Hco-DAF-8 coding for 251 aa was PCR-amplified using the primer pair rtHco-DAF-8-F/R (Additional file 1: Table S1) and then cloned into the prokaryotic expression vector pGEX-4T, to construct the prokaryotic expression plasmid pGEX-4T-Hco-DAF-8. The insert was sequenced, and the recombinant plasmid was then transferred to BL21 (DE3) cells. Recombinant Hco-DAF-8 (rHco-DAF-8) expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C overnight. Expressed proteins were purified from culture supernatants using the glutathione sepharose™ 4B column system (GE Healthcare, Pittsburgh USA) and then used to immunise rabbits to produce anti-rHco-DAF-8 polyclonal antibody. Briefly, recombinant protein rHco-DAF-8 (500 μg) was administered three times subcutaneously 14 days apart and once intravenously (100 μg) 6 days prior to euthanasia using pentobarbital. Serum was collected and affinity purified over a Protein A-sepharose column (Thermo Fisher Scientific, Waltham, USA) and quantified [31]. Pre-immune rabbit serum (negative control) was processed in the same manner. All sera were stored at −80 °C.

Affinity-purified antibody against rHco-DAF-8 was used for immunofluorescence and immunoblot analyses. For immunoblot, proteins extracted from adult male or female H. contortus worms extracted from adult male or female H. contortus worms using a Total Protein Extraction Kit (BestBio, Shanghai, China) were electrophoresed in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to an Immobilon™-PSQ Transfer Membrane (Merck Millipore Ltd., Darmstadt, Germany). After being blocked with 1% (w/v) BSA (BioFROXX, Guangzhou, China) in phosphate-buffered saline (PBS, pH 7.4) containing 20% Tween-20 (PBST) for 6 h at 4 °C, the membranes were incubated in the primary antibody against rHco-DAF-8 (diluted 1:1000 in PBST) overnight at 4 °C, followed by 5 min × 6 washes in PBST and subsequent incubation with the goat anti-rabbit IgG secondary antibody conjugated with horse radish peroxidase (Beyotime Biotechnology, Shanghai, China) diluted 1:1000 in PBST for 2 h at 37 °C. After five washes of the membranes, immunodetection was carried out by chemiluminescence (WesternBright ECL kit, cat. no. K-12045-D10; Aibio, Shanghai, China) as recommended, and imaging was conducted using the ChemiDoc XRS+ system (Bio-Rad).

Immunofluorescent assay to evaluate protein expression in H. contortus

Purified polyclonal antibody against rHco-DAF-8 was used to detect the expression of native Hco-DAF-8 in parasite sections. Briefly, freshly collected H. contortus adults were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4 °C, as described previously [32]. Subsequently, the worms were washed, dehydrated, immersed in paraffin wax and cut into 4 μm-thick sections. Sections were processed and incubated in 3% hydrogen peroxide for 10 min at room temperature (24 °C) to quench endogenous peroxidase activity. The sections were then pre-blocked with bovine serum albumin (BSA) for 20 min at 37 °C, before probing overnight with the anti-rHco-DAF-8 antibody (1:100 in PBST) at 4 °C. Negative control sections were probed with the pre-immune rabbit serum (1:100 dilution). Following three washes (5 min each), the sections were subjected to incubation with the Alexa Fluor® 594 goat anti-rabbit IgG antibody ReadyProbes® reagent (R37117; Thermo Fisher Scientific) at a 1:3000 dilution for 50 min at 37 °C. Unbound secondary serum was removed before an incubation with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at 24 °C. Subsequently, worm sections were washed and mounted in mounting medium [33]. Fluorescence was detected using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Construction of plasmids and gene rescue in C. elegans

To conduct the gene rescue experiment in C. elegans, two plasmids including a test plasmid Cel-daf-8p::Hco-daf-8::gfp and a positive control plasmid Cel-daf-8p::Cel-daf-8::gfp...
were constructed (Additional file 1: Table S1, Additional file 2: Figure S1). In brief, the predicted promoter of Cel-daf-8 was PCR-amplified from the genomic DNA of C. elegans using a forward primer Cel-daf-8p-jy-F (Additional file 1: Table S1) containing a linker sequence (21 bp) identical to a partial sequence of pPV199 and a reverse primer Cel-daf-8p-jy-R (Additional file 1: Table S1) employing the following cycling conditions: 98 °C for 5 min; then 98 °C for 10 s, 55 °C for 5 s, 72 °C for 3 min for 35 cycles; followed by 72 °C for 5 min. The full length coding sequence (CDS) of Hco-daf-8 was PCR-amplified using the primer pair Hco-daf-8-jy-F/Hco-daf-8-jy-R (Additional file 1: Table S1) from H. contortus cDNA under the following protocol: 98 °C for 5 min; then 98 °C for 10 s, 55 °C for 5 s, 72 °C for 3 min for 35 cycles; followed by 72 °C for 5 min. The vector pPV199 was linearised by Agel and BamHI. Then three fragments were ligated together to generate a test rescue plasmid Cel-daf-8p::Cel-daf-8::gfp using CloneExpress® Multis One Step Cloning Kit (Vazyme, Nanjing, China). Similarly, the CDS of Cel-daf-8 was amplified from the cDNA of C. elegans using the primer pair Cel-daf-8-jy-F/Cel-daf-8-jy-R (Additional file 1: Table S1) and then ligated with the amplified promoter sequence of Cel-daf-8 and the digested pPV199 fragment to produce the positive control plasmid Cel-daf-8p:Cel-daf-8::gfp for the rescue assay. The sequences of the inserts of the two plasmids were each verified by sequencing.

The rescue assay was conducted as described previously [20]. Rescue plasmid (50 ng/μl) was micro-injected into the adult germline of daf-8 (CB1393) mutant worms, together with the pRF-4 (50 ng/μl) as a marker for transformation. The transgenic strain, selected based on its green fluorescence/roller phenotype, was assessed for rescue. For each transgenic line, 15 green fluorescent protein (GFP)-positive, gravid hermaphrodites were transferred to an NGM plate containing OP50 and allowed to lay eggs at 16 °C for 3 h. Then, adults were removed, and eggs were kept at 25 °C for 3 days. Adult and dauer worms with green fluorescence were counted. Dauer was assessed based on the radial constriction of the body and a lack of pharyngeal pumping [7]. The percentage of worms rescued was calculated based on the number of adults with GFP divided by total number of GFP-positive worms. Each phenotypic assay was repeated at least three times per transgenic line. GFP expression of the rescued worms was examined using a compound epifluorescence microscope (BX51; Olympus) with Nomarski differential interference contrast (DIC) optics equipped with a digital camera.

Inhibitor assay
The specific inhibitor of human Smad3 (SIS3) was purchased from MedChemExpress (HY-13013; Shanghai, China). SIS3 was stored as a solution in dimethyl sulfoxide (DMSO) at a final concentration of 50 mM; this solution was used after being diluted to 0.5 mM with Luria Bertani medium containing 2.5 μg/ml amphotericin, 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco, New York, USA) (LB*, [34]) for each assay. Seven groups were set up, including a LB* group, negative control group (0 μM SIS3) with maximum DMSO (2.0%), and five experimental groups with different inhibitor concentrations ranging from 2 μM to 50 μM (5 steps). Infective L3s were exsheathed with 0.15% sodium hypochlorite for 30 min at 38 °C, followed by 6 washes in PBS and 3 washes in LB*. Exsheathed L3s (xL3s) were incubated in 100 μl LB* at a concentration of 500–1000 larvae/ml. Inhibitor was added to xL3s at a final concentration of 2 μM to 50 μM in five different experimental groups in triplicate, and larvae were cultured for 7 days at 37 °C under 20% (v/v) CO₂. The percentages of xL3s that developed to L4s was counted using a microscope (cf. [35]). Experiments were repeated three times.

Results
Structural features and sequence analysis of Hco-DAF-8
The full-length genomic DNA sequences of Hco-daf-8 and Cel-daf-8 were each amplified and sequenced. The sequences of Hco-daf-8 and Cel-daf-8 were identical to HF958885.1 and NC_003279.8 (GenBank databases), respectively. Although similar in gene length (~3000 bp long) and CDS length (~1500 bp) to Cel-daf-8, Hco-daf-8 has 12 exons (81–221 bp) and 11 introns (50–630 bp) compared with 6 exons (93–477 bp) and 5 introns (123–560 bp) in Cel-daf-8 (Additional file 3: Figure S2).

The full-length open reading frame of Hco-daf-8 was 1509 bp in length, encoding Hco-DAF-8 of 502 aa. The alignment of amino acid sequences indicated that all selected R-Smads have a Mad homology 1 (MH1) domain (pink) at the N-terminus, which binds DNA and a Mad homology 2 domain (MH2, blue) at the C-terminus, which participates in inter-molecular interactions and regulates transcription [36]. In the MH1 domain, a nuclear localisation signal (NLS) sequence LAKRLK was identified (Fig. 1). Between the MH1 domain and the MH2 domain is a proline-rich ligation region containing six SP motifs (Fig. 1), which function as target sites for phosphorylation by intracellular serine/threonine kinases [37]. Within the MH2 domain, three of the five subtype-specific residues (black arrowhead in Fig. 1) in the MH2 domain of Hs-Smads 2 and 3, which are important for the interaction with Smad anchor for receptor activation (SARA) [38], were identified in Hco-DAF-8 (Fig. 1). In addition, also within the MH2 domain, two of the four conserved large, non-polar residues (open circle) in a characteristic spacing of nuclear export signal (NES) in the MH2 domain [39] were present.
Two residues (R and T, red arrowhead in Fig. 1) in the conserved L3 loop within the MH2 domain, which determine the specific interaction between R-Smads and TGF-β type I receptors [40, 41], were also identified (Fig. 1), whereas BMP-activated R-Smads have two different residues (H and D) (Fig. 1). At the extreme carboxyl terminus of \( Hco \)-DAF-8, there is a variant SSXT (X represents any amino acid) motif (SSFT) which is similar to that of \( Cel \)-DAF-8 (SSRT) (Fig. 1), but distinct from the typical SSX5 motif of R-Smad, having been demonstrated to be required for the phosphorylation by TGF-β type I receptors [1]. The amino acid sequence comparison showed that \( Hco \)-DAF-8 has higher identities to the TGF-β/Activin-activated R-Smads including \( Hs \)-Smad3 (47.7%) and \( Hs \)-Smad2 (46.9%) than to the BMP-activated R-Smads including \( Hs \)-Smad1 (42.9%) and \( Hs \)-Smad5.
(43.0%) (Fig. 1). Taken together, these findings suggest that Hco-DAF-8 is a member of R-Smads subfamily and has more sequence features in common with TGF-β/Activin-activated R-Smads [4].

**Genetic relationships of Hco-DAF-8 with Smads from other species**

There are four R-Smads in *C. elegans*, including Cel-DAF-8 and Cel-DAF-14 which are involved in DAF-7 signalling pathway and Cel-SMA-2 and Cel-SMA-3 which are involved in the DBL-1 (Dpp and BMP-like-1) signalling pathway. Herein, the aa sequence of Hco-DAF-8 was aligned with these four R-Smads and eight homologues of nematodes (*Cjp*-DAF-8, *Cbn*-DAF-8, *Ovo*-SMA-2, *Hco*-SMA-2, *Cbr*-SMA-2, *Cbr*-DAF-14 and *Cjp*-DAF-14) as well as five R-Smads from *H. sapiens* and *D. melanogaster* (*Hs*-Smad3, *Hs*-smad2, *Hs*-Smad5, *Hs*-smad1 and *Dm*-Smox) for subsequent phylogenetic analysis. Consistent tree topologies were obtained for three analyses (NJ, MP and ML). The results revealed that Hco-DAF-8 clustered with three DAF-8s from *Caenorhabditis* species with a nodal support of 89%. In addition, this cluster grouped with three TGF-β/Activin-activated R-Smads of *H. sapiens* and *D. melanogaster* with a nodal support of 52%. On the other hand, four nematode SMA-2s formed a cluster with strong (100%) nodal support. Additionally, two BMP/Dpp-activated R-Smads from *H. sapiens* (*Hs*-Smad1 and *Hs*-Smad5) and two SMA-3s from *Caenorhabditis* species (*Cel*-SMA-3 and *Cbr*-SMA-3) grouped together, with absolute nodal support. The five small clusters (with bootstrap supports ranging from 89 to 100%) grouped together with 95% support to the exclusion of the cluster formed by three DAF-14 (*Cbr*-DAF-14, *Cjp*-DAF-14 and *Cel*-DAF-14) (Fig. 2).

**Transcription of hco-daf-8 in different developmental stages of H. contortus**

Real-time PCR analysis revealed that *Hco-daf-8* was transcribed in all eight developmental stages/sexes of *H. contortus*, with the lowest levels in the free-living stages and the highest level in the adult male stage, followed by the L4 female stage. The transcript abundance in adult males was markedly higher than that of any other stage (*F* (7, 16) = 77.48, *P* < 0.0001). In addition, there was a significant difference between L4 female and each egg (*P* = 0.03), L1 (*P* = 0.04) and L2 (*P* = 0.04) stages. No significant difference was observed between other stages (Fig. 3).

![Fig. 2](image-url) Phylagenetic relationships of *Haemonchus contortus* hco-DAF-8 with other R-Smads from selected species. The phylagenetic tree was constructed using amino acid sequences of hco-DAF-8 and the R-Smad homologues from species, including six nematodes (*Caenorhabditis brenneri*, *C. briggsae*, *C. elegans*, *C. japonica* and *Onchocerca volvulus*), one arthropod (*Drosophila melanogaster*) and one chordate (*Homo sapiens*). The tree was constructed using the neighbour-joining (NJ) method, employing the *Dm*-DAD from *D. melanogaster* as an outgroup. Nodal support values are shown above or below the branches. Accession numbers are listed next to the R-Smad designation. Information on sequences used in the analysis is listed in Additional file 1: Table S2.
plasmid Cel-daf-8p::Cel-daf-8::gfp (positive control) or Cel-daf-8p::Hco-daf-8::gfp (test plasmid) into the Cel-daf-8 mutant strain. The results showed that Cel-daf-8p::Hco-daf-8::gfp was expressed in all life stages, and GFP expression was mainly detected in the intestine and nervous system (Fig. 5a–f), consistent with the expression patterns in worms transformed with Cel-daf-8p::Cel-daf-8::gfp (Fig. 5a–f). In C. elegans control-transgenic lines transformed with Cel-daf-8p::Cel-daf-8::gfp plasmid, ~90% (89.5 ± 4.0%) of the offspring expressing GFP developed to adults (Fig. 5n, p). In contrast, the transgenic lines transformed with Cel-daf-8p::Hco-daf-8::gfp plasmid, partial (57 ± 6.4%) offspring developed to adults (Fig. 5o, p). The pharynx in rescued worms resumed pumping, the vulva projected and the uterus contained eggs, while ‘unrescued’ worms remained as dauer after the same period of development (Fig. 5m). There were significant differences in the percentages of developed adult worms in Ce-daf-8 transgenic line and Hc-daf-8 transgenic line compared with the untreated groups ($F_{(2, 9)} = 65.58, P < 0.0001$ and $P = 0.0002$, respectively) (Fig. 5p).

SIS3 inhibits the development of H. contortus from xL3s to L4s

To further assess the functionality of Hco-DAF-8 as an R-Smad protein, the inhibitor assay using R-Smad inhibitor SIS3 was conducted in vitro. The xL3s of H. contortus developed to L4s in vitro under normal culture conditions. Compared with xL3s, the buccal capsule of L4s was fully developed and functional [42, 43]. Results showed that SIS3 significantly inhibited L4 development at five concentrations from as low as 2 µM to 50 µM (5 µM, 10 µM, 20 µM and 50 µM) ($F_{(6, 21)} = 60.45, P < 0.001$). The inhibitory effect was dose-dependent, with an increased inhibition in the concentration range of 2 µM to 10 µM, reaching a maximum inhibitory effect at 10 µM and beyond (Fig. 6). There were no significant differences among groups 10 µM, 20 µM and 50 µM ($F_{(2, 9)} = 0.21$).

Discussion

In the present study, an R-Smad protein coding gene (Hco-daf-8) and its predicted product (Hco-DAF-8) of H. contortus were identified and characterised both structurally and functionally. Hco-DAF-8 has characteristics consistent with TGF-β/Activin-activated R-Smads, especially the two distinctive amino acids (R$^{462}$ and T$^{465}$) in the L3 loop determining the specific interaction between R-Smads and TGF-β type I receptors [40, 41]. In addition, phylogenetic analysis showed that Hco-DAF-8 is closely related to DAF-8 homologues of Caenorhabditis species and grouped with the TGF-β/Activin-activated R-Smads. These results suggest that Hco-DAF-8 is a member of
TGF-β/Activin-activated R-Smads that usually transmit the TGF-β signals.

To understand at which time points Hco-DAF-8 functions in *H. contortus*, the transcription of its encoding gene *Hco-daf-8* in different developmental stages of *H. contortus* was assessed by real-time PCR. Hco-daf-8 was transcribed in all developmental stages studied, with the lowest transcription in the egg, L1 and L2 (free-living) stages, higher transcription in L4 females, and the highest level in adult males. In contrast, *C. elegans* daf-8 transcript level peaked in the early larval stages (egg and L1 stages), but then declined to low levels in L2 to adult stages [44]. This finding indicates that Cel-daf-8 might play a role in mediating developmental switch (entry into}

![Fig. 4](image-url) The localization of Hco-DAF-8 in *Haemonchus contortus* adults by immunofluorescence. a–c Localization of Hco-DAF-8 in *H. contortus* adult females: in the platymyarian muscle cells (msc) under cuticle, in intestine (i) and some eggs (e). g–i Localization of Hco-DAF-8 in adult males: in the platymyarian muscle cells (msc) under cuticle, in intestine (i), seminal vesicle (vs) and cement gland (gc). Ovaries (ov), cuticle (cu) and uterus (ut) are also indicated. No fluorescence labelling was observed in negative controls probed with serum representing the pre-bleed prior to immunization (d–f, j–l). DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) is used to label the nucleus. Scale-bars: 100 μm.
dauer or normal L3 stage) in *C. elegans*, while *Hco-daf-8* may influence the continuous development of *H. contortus* from iL3s as larval arrest is facultative in *C. elegans*, but constitutive in the parasitic nematodes [45, 46]. Recent findings from RNAi-based studies also confirm functional roles for genes encoding TGF-β type I receptor (*Hc-tgfbr1*) [12], TGF-β type II receptor (*Hc-tgfbr2*) [13] and co-Smad (*Hc-daf-3*) [14] in the development from the free-living to the parasitic stages of *H. contortus*.

The high transcript level of *Hco-DAF-8* and notable protein expression in the male reproductive system may indicate an important role of this molecule in male *H. contortus*. In *C. elegans*, Cel-DAF-8 was proven to negatively regulate the germline proliferative zone when it was expressed in the distal tip cell [10]. Another study [47] showed that a reduction of the TGF-β Smad/Mab signalling pathway in *C. elegans* extends the reproductive span by maintaining oocyte and germline quality. In addition, in the flatworm *Schistosoma mansoni*, it has been shown that TGF-β signalling pathway is crucial for the embryo development and egg production [48, 49]. We speculate...
that Hco-DAF-8 is involved in parasite growth and development as well as germline quality maintenance and reproduction. The expression of eggs in the female uterus indicates a possible effect of this molecule on the embryogenesis and development. Interestingly, the high level of transcription in adult males is also seen for Hc-tgftr2 [13]; both Hco-DAF-8 and Hc-TGFTR2 are highly expressed in the reproductive system. The similar transcriptional and expression patterns of these two molecules suggest that the Hco-DAF-8 may function together with Hc-TGFTR2 in reproductive development in H. contortus.

Besides the expression in the reproductive system, Hco-DAF-8 was also highly expressed in the intestinal cytoplasm of H. contortus adult worms, indicating that this protein might affect the digestive system and/or be immunogenic, given this worm’s gut is a source of effective vaccine molecules [50]. Its expression in the platymyrian muscle cells under cuticle suggests that Hco-DAF-8 or TGF-β signalling pathway may relate to cuticle formation, muscle movement, the transmission of external signals from host and ionic homeostasis [51]. Collectively, these findings might indicate an important role for Hco-DAF-8 in growth and development and/or as a regulator of transcription in response to developmental cues.

To verify whether the function of Hco-DAF-8 is similar to that of Cel-daf-8, gene rescue was performed. Results showed that Hco-DAF-8 can be expressed in the specific tissues consistent with Cel-daf-8 and can rescue the dauer phenotype of Cel-daf-8 mutant strain but with less efficiency. This is possibly due to differences in the target gene sequence and the transgene expression efficiency. Other reasons could be species-specific differences in gene expression that might have been reflected in sub-optimal expression/function of H. contortus elements in C. elegans. Moreover, Cel-DAF-8 encoded by Cel-daf-8 interacts with some other endogenous proteins like Cel-DAF-14 [10], although it is possible that the binding of Hco-DAF-8 to endogenous Cel-DAF-14 is not as strong as to Cel-daf-8 in C. elegans. Nevertheless, the effective rescue indicated that the biochemical properties of Hco-DAF-8 are similar enough to those of Cel-DAF-8, such that it can function as Cel-DAF-8 when expressed at a corresponding location and time in C. elegans.

It is well known that Cel-daf-8 regulates dauer formation via TGF-β signalling. Mutant C. elegans rescued by Hco-DAF-8 can restore TGF-β signalling to complete normal development. To answer as to whether Hco-DAF-8 participates in this signalling pathway and plays key roles in the development of H. contortus L3 (analogous to dauer in C. elegans), we used a Smad3-specific inhibitor (SIS3) to assess TGF-β signalling pathway function. SIS3 was first reported as a potent and selective inhibitor of Smad3 in this pathway of H. sapiens and affected neither the phosphorylation of Smad2 nor the phosphorylation of other signalling pathways [52]. Due to its important functions in suppressing cancer growth, invasion and metastasis, and in preventing the cancer death, SIS3 was described in a patent as a novel and effective anti-cancer drug by way of regulating cellular signal transduction mediated by TGF-β/Smad3 in the USA [53]. Moreover, recently, SIS3 was applied to the parasitic flatworm Echinococcus granulosus, resulting in a marked effect on the key components of the Smad signalling pathway, inhibiting the growth and survival of protoscoleces [54]. These published findings stimulated our interest in assessing this inhibitor on H. contortus in vitro. The results revealed that SIS3 inhibited the development of L3 larvae in a dose-dependent manner, suggesting a role for the Smad3 homologue in larval development in H. contortus, like other members of the TGF-β signalling pathway studied previously in this parasite [12–14]. Sequence analysis showed that Hco-DAF-8 has a higher similarity with Hs-Smad3 than the other Smads in H. sapiens at the amino acid level, suggesting that SIS3 specifically inhibits the function of Hco-DAF-8 in H. contortus. Future work might conduct structure activity relationship (SAR) studies of SIS3 analogs to establish whether they might show promise as a new nematocide for H. contortus and related worms.

In C. elegans, Cel-DAF-8 can inhibit the Co-Smad Cel-DAF-3 or vice versa. Herein, overlapping expression can be detected in the platymyrian muscle cells under the cuticle in both sexes of adult worms or in the cement gland in the adult male between Hco-DAF-8 and Hco-Daf-14 [14], suggesting some common roles in these tissues. Considering that chemical inhibition resulted in the retarded larval development in vitro, we speculate that R-Smads positively regulate the downstream Co-Smads in the cuticle, which sense the host environment and promote transition from the free-living stage (L3s) to the parasitic stage (L4s) in H. contortus.

Conclusions

We identified a gene encoding an R-Smad protein (Hco-DAF-8) in H. contortus. Hco-DAF-8 is an R-Smad with inferred functional domains typical of the Smad protein family. This protein is widely expressed and appears to control the development of xL3s to L4s in H. contortus. The highest transcription level and high protein expression in the reproductive organs of male adults suggest that Hco-DAF-8 has a role in male reproduction. SIS3 inhibited the development from the xL3 to the L4 stage, suggesting a key role in developmental transition. Taken together, the findings of this study demonstrate that Hco-DAF-8 is a key molecule in the TGF-β signalling pathway of H. contortus and regulates development and/or reproduction in this worm.
Additional file 1: Table S1. Oligonucleotide primers used in the present study. Table S2. Sequences used for phylogenetic and alignment analyses.

Additional file 2: Figure S1. Schematic diagram explaining the process of constructing gene rescuing plasmids by homologous recombination. The C. elegans Cel-daf-8 promoter region was PCR-amplified using primers F1/R1. In addition, the coding region (CDS) of Cel-daf-8 or H. contortus Hco-daf-8 was amplified using primers F2/R2 or F3/R3, respectively.

Additional file 3: Figure S2. Gene structures of R-Smad homologues from H. contortus (Hco-daf-8) and C. elegans (Cel-daf-8). Black boxes represent exons and the numbers above display the lengths of exons. Lines between the exons represent introns, and the numbers below indicate the lengths of introns.

Additional file 4: Figure S3. Expression and purification of recombinant Hco-DAF-8 protein of H. contortus and immunoblot analysis. a: Puriﬁcation of recombinant protein Hco-DAF-8. Lane M: protein marker; Lane 1: expressed products of pGEX-4T-Hco-DAF-8 (94–344) non-induced; Lane 2: expressed products of pGEX-4T-Hco-DAF-8 (94–344) induced; Lane 3: expressed products of pGEX-4T-Hco-DAF-8 empty vector induced; Lane 4: puriﬁed pGEX-4T-Hco-DAF-8 (94–344) protein. b: Immunoblot analysis of natural Hco-DAF-8 protein. Lane M: protein marker; native Hco-DAF-8 protein was detected in adult male and female using anti-Hco-DAF-8 anti-serum (antiserum), using a pre-immune serum as a control.

Abbreviations
TGF-β: transforming growth factor-β; R-Smad: receptor-regulated Smad; SIS3: specific inhibitor of human Smads; Co-Smads: common-partner Smads; I-Smads: inhibitory Smads; NGM: nematode growth medium; EBSS: Earle's balanced salt solution; CDS: coding DNA sequence; hco-DAF-8: recombinant Hco-DAF-8; IPTG: isopropyl β-D-thiogalactopyranoside; SDS: sodium dodecyl sulfate; PBS: phosphate-buffered saline; PFA: paraformaldehyde; DAPI: 4′, 6-diamidino-2-phenylindole; GFP: green fluorescent protein; DMDS: dimethyl sulfoxide; SRA: Smad-anchoring for receptor activation; NLS: nuclear localisation signal; NES: nuclear export signal; DBL-1: Dpp and BMP-like-1; SAR: structure activity relationship.

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Authors’ contributions
MH conceived and designed the project. FFL performed most experiments and prepared the first draft of the manuscript. RBG redrafted the manuscript, and RBG and MH edited and revised it. WDD, FFL and LH performed the real-time PCRs. FL, JNS, CXZ and CQW were responsible for sample collection and RNA extractions. RF commented on project design. All authors read and approved the final manuscript.

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Availability of data and materials
Data supporting the conclusions of this article are included within the article and its additional files. The amino acid sequence of Hco-DAF-8 is available on GenBank under the accession number CDJ83310.1

Ethics approval and consent to participate
All of the experimental animals used in this project were treated in strict accordance with the recommendations in the Guide for the Regulation for the Administration of Affairs Concerning Experimental Animals of the People’s Republic of China. The protocol was approved by the Ethics Committee of Huazhong Agriculture University (permit HZAUUGO-2016-007).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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