A Transcription Factor DAF5 Functions in *Haemonchus Contortus* Development

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Research Article

Keywords: DAF5, Development, Interaction, RNAi, siRNA, Transcription factor

Posted Date: August 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-768609/v1>

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Abstract

Background: *Daf5* (Dauer abnormal formation gene), located in the downstream of DAF-7 signalling pathway, mainly functions in dauer formation and reproductive processes in the free-living nematode *Caenorhabditis elegans*. Although its structure and function have been studied clearly in *C. elegans*, it was totally unknown in *Haemonchus contortus*, a socio-economically important parasitic nematode of gastric ruminants.

Methods: Here, we identified and characterized a homologue of *Daf5*, *Hcdaf5* and its inferred product (*HcDAF5*) in *H. contortus*. Using an integrated molecular approach, we studied the transcriptional profiles of *Hcdaf5* and the anatomical expression of *HcDAF5* in *H. contortus*. RNA interference (RNAi) was performed to explore its function in transition from the exsheathed third-stage larvae (xL3) to the fourth-stage larvae (L4) *in vitro*. Interaction of *HcDAF5* and *HcDAF3* (a co-SMAD) was also detected by bimolecular fluorescence complementation system (BiFc) *in vitro*.

Results: Here, we showed that *HcDAF5* is a member of the Sno/Ski superfamily. *Hcdaf5* was transcribed in all developmental stages of *H. contortus*, with a significant up-regulation in L3. Immunohistochemistry localized native *HcDAF5* to the reproductive organs, cuticle and intestine. RNAi revealed specific siRNAs (small interfering RNA) could retard the xL3 development. In addition, the interaction between *HcDAF5* and *HcDAF3* indicated the SDS box region of *HcDAF5* is dispensable for the binding of *HcDAF5* to *HcDAF3* and the region in *HcDAF3* that binds to *HcDAF5* is MH2 domain.

Conclusion: In summary, these findings show that *Hcdaf5* functions in developmental processes of *H. contortus*, and this is the first characterization of *daf-5* gene in parasitic nematodes.

Background

Transcription factors function in basal transcription by binding to particular DNA sequences in gene regulatory regions to control their transcription. Based on their functions, transcription factors are commonly classified into families of activation and repression. Although the majority of transcription factors act in a positive manner, a number of transcription factors exert an inhibiting effect on transcription [1].

For TGF- β signalling pathway in vertebrate, activation of receptors causes downstream R-Smads phosphorylation, which allows the latter to form an active Smad transcriptional complex with co-Smads. The function of this complex is affected positively or negatively through interaction with cofactor, coactivator, and corepressor molecules, which ultimately regulates cellular processes such as proliferation, differentiation and apoptosis [2]. As downstream components of TGF- β signalling pathway, Ski proto-oncoprotein family of corepressors, including c-Ski and SnoN, play important roles in tightly controlled repression of Smad-mediated transcriptional activation, and antagonize TGF- β signalling by binding to SMAD transcriptional complex, preventing their interaction with co-activators, and recruiting

co-repressors [3]. Besides, the transcription of Sno/Ski is regulated by Smads. Thus, degrading or accumulation of Sno/Ski protein occurs depending on the context of TGF- β signalling [4].

Nevertheless, as a transcription factor homologue of vertebrate Ski/Sno of the free-living nematode *Caenorhabditis elegans*, DAF5, which functions in TGF- β signalling pathway (DAF-7 signaling pathway) for dauer regulation and egg laying of this nematode, is not a conventional Sno/Ski protein [5]. Shortly, the DAF-7 signal is transduced by R-Smads. These molecules when activated, function as a transcriptional complex and inhibit the functions of DAF5. DAF5 acts as a co-factor of co-Smad (DAF3), rather than an antagonist of the DAF3, and they act as a complex and are antagonized by upstream R-Smads components [5].

In spite that the functions of Sno/Ski and DAF5 have been clearly identified in vertebrates and *C. elegans*, respectively, it was totally unknown in parasitic nematodes. Elucidating the functions of DAF5 in parasitic nematode will help us understand how this molecule and the DAF-7 signalling pathway work in the parasites. Herein, a homologue of *daf5* from *Haemonchus contortus*, a blood-sucking gastric parasitic nematode of small ruminants, was identified. Its temporal transcriptional profiles in different developmental stages and special expression patterns in adult worms were explored by real-time PCR and immunohistochemistry, respectively. In addition, siRNAs were used to knockdown *Hcdaf5* in xL3 by soaking. Furthermore, BiFc experiment was also performed to demonstrate the interaction between *HcDAF5* and *HcDAF3*.

Methods

Worm maintenance

The *H. contortus* Hacon-5 strain was maintained by goats, which were infected orally with 5,000 L3 (Di et al., 2020). Free-living stages including eggs, first-stage larvae (L1), second-stage larvae (L2) and L3s were harvested from faeces of infected goats. Parasitic stages (L4s and adults) were collected from the abomasa of infected goats euthanized at 8 and 30 d, respectively, and these two developmental stages were washed extensively in 0.85% sodium chloride, and male and female worms were separated carefully under microscopy prior to storage at -80°C.

Nucleic acids and protein preparation and gene cloning

Total RNA was extracted from individual developmental stages of *H. contortus* using TRIzol (Simgen, China). All RNA samples were treated with DNase I to remove gDNA, and then integrity and yields were verified by electrophoresis and spectrophotometric analysis. Complementary DNA (cDNA) was synthesized from RNA (1 μ g) using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan) for coding sequence (CDS) amplification and real-time PCR. RNA was stored at -80°C and DNA was stored at -20°C until use.

The whole-worm protein was prepared by grinding adult worms in phosphatase inhibitor and protein lysate (Roche Molecular Biochemicals, Switzerland). Proteins were fractionated into soluble and insoluble material after centrifugation at 10000 *g* for 3 min at 4°C. Then the former soluble material was stored at -80°C after protease inhibitor (Thermo, USA) added until subjected to the following Western blot experiment.

Using transcriptomic and genomic datasets of *H. contortus*, the sequences of full-length cDNA and gDNA as well as the intergenic region between the start codon and the stop codon of the upstream gene of *Hcdaf5* were retrieved. The CDS was amplified from cDNA with primer pair Hc-daf-5-cF/Hc-daf-5-cR (Additional file 1, Table S1) using the following protocol: 98°C/10 min; followed by 98°C/10 s, 55°C/5 s, 72°C/2 min for 35 cycles; and then 72°C/10 min. PCR product was inserted into pMD-19T and sequenced in both directions directly (via TsingKe Biological Technology, China).

Bioinformatic analyses

The coding sequences of *Hcdaf5* were conceptually translated into predicted amino acids using DNA star software (<http://www.dnastar.com/>). The predicted amino acid sequence of *HcDAF5* was compared with sequences in non-redundant protein databases using BLASTp from the National Center for Biotechnology Information (NCBI) to confirm the homologous sequences.

The structural domains (Dach boxes and SDS) of *HcDAF5* were aligned with its homologues from 11 species (*Ancylostoma ceylanicum*, *Brugia malayi*, *Caenorhabditis briggsae*, *C. elegans*, *Danio rerio*, *Drosophila melanogaster*, *Equus caballus*, *Homo sapiens*, *Mus musculus*, *Nippostrongylus brasiliensis*, *Toxocara canis*) (Additional file 1, Table S2) using the program Bioedit. For *HcDAF5*, the coiled coil region is predicted by Expasy (https://embnet.vital-it.ch/software/COILS_form.html).

The homologues from eight species (*C. briggsae*, *C. elegans*, *D. rerio*, *D. melanogaster*, *H. sapiens*, *M. musculus*, *N. brasiliensis*, *T. canis*) (Additional file 1, Table S2) were aligned and used for phylogenetic analyses using the neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. Confidence limits were assessed using a bootstrap procedure employing 1000 pseudo-replicates in MEGA [6].

Transcriptional analyses of *Hcdaf5* in different stages of *H. contortus* by real-time PCR

Real-time PCR was carried out using specific primers Hc-daf-5-qF/Hc-daf-5-qR (Additional file 1, Table S1) to determine the mRNA levels in different developmental stages of *H. contortus* including eggs, L1s, L2s, L3s and both sexes of L4s and adult worms. Total RNA of each stage was isolated with TRIzol reagents according to the manufacturer's instruction and treated with DNase I to remove gDNA before synthesis of cDNA. The real-time PCR reaction condition included: one cycle at 50°C/2 min, 95°C/30 s; 40 cycles at 95°C/15 s, 60°C/15 s, 72°C/20 s; followed by a cycle at 60 °C/1 min, 95 °C/15 s, 60 °C/15 s. Each sample was tested in triplicate, employing a *β-tubulin* of *H. contortus* (GenBank: M76493) as a reference gene (using specific primers Hc-tubulin-qF and Hc-tubulin-qR; Additional file 1, Table S1), and average threshold (Ct) was taken to compare the relative quantities with Am (Am = 1) using $2^{-\Delta\Delta C_t}$ method [7].

This assay was repeated three times. Statistical analysis was carried out using one-way ANOVA in GraphPad Prism 6. *P*-values were calculated using the Turkey's *post-hoc* test; values of < 0.05 were considered statistically significant.

Production of polyclonal antibody against recombinant HcDAF5 and immunoblot analysis

A pair of specific primers Hc-daf-5-pF/Hc-daf-5-pR (Additional file 1, Table S1) containing double restriction sites were designed according to the CDS of *Hcdaf5* and used in PCR to amplify the CDS of *Hcdaf5*. Then amplified sequence was cloned into the prokaryotic expression vector to create the expression plasmid pET-28a-Hc-daf-5. Then this plasmid was transformed into BL21 (DE3) cells of *Escherichia coli*, followed by 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) induction at 16°C for 12 h to produce recombinant rHcDAF5. The recombinant rHcDAF5 was purified using a Ni Sepharose column system (GE, USA) according to the manufacturer's protocol. Then purified rHcDAF5 was used in immunizing New Zealand white rabbit to produce anti-HcDAF5 polyclonal antibody. The titer and specificity of anti-HcDAF5 polyclonal antibody were determined by Enzyme-linked immunosorbent assay (ELISA) and Western blot.

Western blot was performed as follows. Proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon®-PSQ membrane (Merck Millipore Ltd). The immunoblot membranes were blocked with blocking buffer (1% (w/v) BSA (BioFROXX, China) in PBS, 20% Tween-20; PBST) for 6 h at 4°C, washed 3 times with PBST and incubated with the HcDAF5 antiserum (1:1000 in PBST) overnight at 4°C. Samples were washed 6x in PBST and subsequently incubated with an HRP-conjugated goat anti-rabbit antibody (1:1000, Beyotime Biotechnology, China) for 2 h at 37°C, followed by 5x additional washes. Immunodetection was performed by chemiluminescence (WesternBright ECL kit; K-12045-D10, China), and images were acquired by ChemiDoc XRS + system (Bio-Rad, USA).

Immunofluorescent assay to evaluate protein expression in *H. contortus*

Fresh male and female adults of *H. contortus* were fixed in 4% paraformaldehyde at 4°C for 24 h and then were consecutively dehydrated in an ethanol series (75% for 4 h, 85% for 2 h, 90% for 2 h, 95% for 1 h, and 100% for 30 min twice). Then dehydrated worms were incubated in xylene: absolute ethanol (1:1) solution for 5 min and xylene for 10 min, then embedded in paraffin. Paraffin sections of 4 μ m were subjected to immunofluorescence staining. For immunofluorescent assay, slices were treated with EDTA buffer at 100°C for 10 min. After blocking with 5% BSA for 20 min, anti-HcDAF5 polyclonal antibody and goat anti-rabbit IgG diluted 1:100 was sequentially added and incubated at 4°C overnight and 37°C for 50 min. Then sections were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at 37°C in the dark place. After washed by PBS thoroughly, sections were observed under fluorescence microscopy (Olympus CX-21, Japan).

RNA interference in *H. contortus* by soaking in siRNA

The CDS of *Hcdaf5* was used to design the siRNA sequences with the siRNA Design Tools program, and siRNA oligos (Additional file1, Table S3) were synthesized chemically by Shanghai GenePharma Co. Ltd.

For RNAi experiment, L3s were exsheathed and washed five times in 0.9% NaCl solution followed by centrifuged at 600 *g* with DEPC-treated water for further three times. For each silencing assay, 50 µl of nematode suspension (about 5,000 larvae) was placed into a 96-well plate. 10 µl Lipofectamine 2000 (Thermo Fisher, USA) were incubated with 5 µl EBSS (pH 5.2) containing 2.5 µg/µl amphotericin, 100 µg/µl streptomycin and 100 IU/ml of penicillin (Gibco, USA) at 25°C for 5 min. The RNasin (0.2 U) and siRNA solution were added and incubated for 15 min. Three siRNAs of *Hc-daf-5* were mixed with equal amounts, and the final concentration of each siRNA was 1 µM, while final concentration of negative control siRNA was 3 µM, and nuclease-free water was set as blank control.

The knock down experiments were carried out as previously described [8]. In brief, three groups each consisting of 5,000 xL3s kept in 80 µl EBSS (pH 5.2) supplemented with the respective siRNA were soaked for 72 h. Approximately, 100 larvae of each group were transferred to fresh EBSS culture medium for another 5 d to assess their development. The remaining larvae were subjected to RNA extraction.

All RNA extractions were performed with the Trizol according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara, China). The same amount of cDNA for each sample was applied to a 10 µl reaction. The *18S* gene of *H. contortus* was served as the endogenous control using the primer set of Hc-18S-qF/Hc-18S-qR (Additional file 1, Table S1). Primers (Hc-daf-5-qF/Hc-daf-5-qR) used in detecting transcriptional profiles were also used here for detection the transcriptional changes of *Hcdaf5* in worms treated with siRNA. Amplification efficiency of the primers was tested by a standard curve assay and linear regression analysis showed similar slopes for all tested primers. The real-time PCR was performed on an ABI 7100 thermal cycler (Applied Biosystems, Germany) using the following conditions: one cycle at 50°C/2 min, 95°C/30 s; 40 cycles at 95°C/15 s, 60°C/15 s, 72°C/20 s; followed by a cycle at 60°C/1 min, 95°C/15 s, 60°C/15 s. Fold change expression of *Hcdaf5* after RNAi calculated by 2- $\Delta\Delta C_t$ method. $\Delta\Delta C_t = [(C_t^{RNAi, Hcdaf5}) - (C_t^{RNAi, Hc18S})] - [(C_t^{Blank, Hcdaf5}) - (C_t^{Blank, Hc18S})]$ [9].

Assessing the interaction between HcDAF5 and HcDAF3 by BiFC

The BHK21 cells were grown and maintained in DMEM (Dulbecco's modified eagle medium) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The plasmid pbJun-HA-KN151 and pbFos-Myc-LC151 were used as original plasmids for plasmid construction. In brief, the CDS fragment of *Hcdaf3* was amplified with primer sets of Hc-daf-3-fF/Hc-daf-3-fR (additional file 1, Table S1) and cloned into pbJun-HA-KN151 between NheI and XhoI sites to replace bJun, generating the p*HcDAF3*-HA-KN151 plasmid, while the fragment of *Hcdaf5* gene was amplified with primer sets of Hc-daf-5-fF/Hc-daf-5-fR (additional file 1, Table S1) and cloned into pbFos-Myc-LC151 between NheI and PvuI sites to replace *bFos*, generating p*HcDAF5*-Myc-LC151 plasmid. BHK21 cells were seeded onto 6-well plates and grown to 70 ~ 80% confluency for transfection. To examine the interactions between *HcDAF3*

and *HcDAF5*, the plasmids *pHcDAF3*-HA-KN151 and *pHcDAF5*-Myc-LC151 were co-transfected using Lipofectamine 2000 (Thermo Fisher, USA). Then cells were incubated at 37°C with 5% CO₂ for further 20 h, subsequently fluorescent was detected within a range from 580 to 680 nm and imaged.

In addition to the full-length constructs of *HcDAF3*, a DNA fragment encoding the *HcDAF3*-MH2 domain region (645 bp; 484–697 aa) was amplified with primer sets of *Hc-daf-3*-MH2-F/*Hc-daf-3*-fR (additional file 1, Table S1), sequenced and cloned into *pHcDAF3*-MH2-HA-KN151. Similarly, a DNA fragment encoding the *HcDAF5*-SDS domain region (900 bp; 1–300 aa) was also amplified with primer sets of *Hc-daf-5*-fF/*Hc-daf-5*-SDS-R (additional file 1, Table S1), sequenced and cloned into *pHcDAF5*-SDS-Myc-LC151. Plasmids *pbFos*-Myc-LC151 and *pbJun*-HA-KN151, which express bJun and bFos, respectively, were co-transfected as positive control, while *pHA*-KN151 and *pHcDAF5*-SDS-Myc-LC151 were performed as negative control.

Results

Sequence and phylogenetic analyses of *HcDAF5*

The *Hcdaf5* CDS is 1629 bp in length (GenBank accession no. MK159305), predicted to encode a protein (*HcDAF5*) of 542 amino acids. The full-length genomic sequence of *Hcdaf5* is 8535 bp long containing 11 exons (73–207 bp) and 10 introns (57–3071 bp). Compared with *Cedaf5* [5], *Hcdaf5* has more exons and introns.

A blast of the *HcDAF5* protein sequences against NCBI GenBank databases reveals similarities to the SKI, SnoN, Skate and Icy in *H. Sapiens* and Snowski, Iceskate in *D. melanogaster* (Table 2) [10, 11]. The predicted protein sequence of *HcDAF5* was then aligned with homologues from five nematodes and five other metazoans. Multiple sequence comparison revealed the highest identity of *HcDAF5* to SKI of *N. brasiliensis* (58.3%), but low identities ranging from 18.7–28.7% to its homologues of different metazoan species, including *B. malayi* (Snowski, 22.7%), *C. briggsae* (DAF5, 21.8%), *C. elegans* (DAF5, 23.0%), *D. rerio* (SKI, 24.1%), *D. melanogaster* (Iceskate, 20.3%; Snowski, 20.9%), *E. caballus* (SKI, 22.9%), *H. sapiens* (Icy, 18.7%; Skate, 21.1%; SKI, 23.1%; SnoN, 20.4%), *M. musculus* (SKI, 21.9%) and *T. canis* (SKI, 28.7%).

The alignment of these sequences also indicated that *HcDAF5* has two functional domains including a Dach box (Dachshund-homology domain) and a SDS domain (Smad binding domain) (Fig. 1A). Dach box is a domain shared by the Sno/Ski, Skate/Icy and Dachshund superfamily. Dachshund is a transcription regulator conserved throughout bilateria. Alignment of Dachshund-homology domain among selected species (Fig. 1B, above) revealed that Dach box of *HcDAF5* has higher similarity to that of *NbSKI* than to *CeDAF5* and *CbDAF5*. In *H. contortus*, DAF5 clearly has an SDS box in Smad binding domain, which is not found in any other proteins in *H. contortus* (Fig. 1A). SDS box of *HcDAF5* has higher similarity to that of the Snowski group than to that of the Iceskate group. In human SKI, the SDS box and about 20 amino acids on either side constitute the minimal region required for Smad4 binding, thus this region was also used for alignment (Fig. 1B below). All members of this family have a conserved zinc

finger in the SDS domain. SDS domain of *HcDAF5* has higher similarity to that of parasitic nematodes (such as *B. malayi*) than to that of *C. elegans* (Fig. 1B, below). Same as *CeDAF5*, *HcDAF5* also has a predicted coiled coil structure at the C terminus (Fig. 1A).

The results of phylogenetic analysis showed that the topologies of the MP, ML and NJ trees were concordant. *HcDAF5* grouped with *Nb-SKI* with 100% bootstrap support, they then grouped with DAF5 from *C. elegans* and *C. briggsae* with 92% bootstrap support. This DAF5 cluster grouped with the Ski/Sno and Ice/Skate from *H. sapiens*, *M. musculus*, *D. rerio*, *D. melanogaster* and *T. canis*, with 85% bootstrap support. Dachshund subfamily from *C. elegans*, *D. melanogaster*, *D. rerio*, *H. sapiens* and *M. musculus* constitutes an independent cluster with 99% bootstrap support (Fig. 1C).

Transcription of *Hcdaf5* and expression of *HcDAF5* in *H. contortus*

The transcriptional profile of *Hcdaf5* was explored in eight developmental stages and sexes including egg, L1, L2, L3, L4 female, L4 male, adult female and adult male throughout the life cycle of *H. contortus*. *Hcdaf5* was transcribed at detectable level in all tested stages and sexes with a significant up-regulation in L3 ($F(7, 16) = 12.82$, $P < 0.01$) (Fig. 2A).

Truncated *HcDAF5* (108–297 aa) was expressed in *E. coli* BL21 (DE3) with a size of 27.5 kDa (Fig. 2B). The polyclonal antibody produced against r*HcDAF5* was used in Western-blot and recognized a band of around 60 kDa in total protein extracted from adult worms while no band was recognized by the negative serum (Fig. 2C). In the further immunofluorescent assay, this anti- r*HcDAF5* polyclonal antibody detected the endogenous *HcDAF5* expression in cuticle and intestine of both female and male adult worms as well as in ovary of female adult worms and cement gland of male adult worms. No fluorescence was observed in worm sections probed with the negative serum (Fig. 2D).

Effect of specific siRNA on xL3 development of *H. contortus*

As observed in previous studies, the development of *H. contortus* from xL3 to L4 underwent six stages of morphological changes, including stage A to stage F [12, 13]. Here in RNAi assay, the soaking of xL3s in *Hcdaf5* specific siRNA for 72 h resulted in a significant reduction in *Hcdaf5* transcript abundance whereas no reduction in *Hcdaf5* transcript abundance was detected in irrelevant siRNA treated group or untreated group ($F(2, 6) = 10.25$, $P = 0.0066$ and $P = 0.0060$, respectively) (Fig. 3A). In addition, the proportion of xL3 larvae that developed to stage B and beyond in *Hcdaf5* siRNA treated group was significantly lower than that in the irrelevant siRNA treated group and the untreated group ($F(2, 6) = 34.36$, $P = 0.0095$ and $P = 0.0014$, respectively) (Fig. 3B).

HcDAF5* interacts with Smad *HcDAF3

To detect the interaction between *HcDAF5* and *HcDAF3* in live mammalian cells, biomolecular fluorescence complementation assay (BiFC) was performed. As the positive control, cells co-transfected with pbJun-HA-KN151 and pbFos-Myc-LC151 shows bright red fluorescence, consistent with a previous report (Fig. 3C) [14]. No red fluorescence was observed in cells co-transfected with pHA-KN151 and

pHcDAF5-SDS-Myc-LC151, just the same as the blank. In two experimental groups, red fluorescence was observed in cells co-transfected with pHcDAF3-HA-KN151 and pHcDAF5-SDS-Myc-LC151 as well as in cells co-transfected with pHcDAF3-MH2-HA-KN151 and pHcDAF5-SDS-Myc-LC151. While former group shows bright fluorescence, latter group shows punctate red fluorescence in nucleus. However, cells co-transfected with pHcDAF3-HA-KN151 and pHcDAF5-Myc-LC151 or pHcDAF3-MH2-HA-KN151 and pHcDAF5-Myc-LC151 shows no red fluorescence (not shown).

Discussion

In the present study, a homologue of *Cedaf5*, a transcription factor encoding gene, *Hcdaf5*, was identified and characterized in *H. contortus*. Sequence analysis revealed that the predicted protein HcDAF5 contains a dach box, a SDS domain and a coiled coil motif. The later motif constitutes 3% of all protein residues, and functions by mediating oligomerization of a large number of protein subunits. It can be involved in signal-transducing events or act as a molecular recognition system [15]. The conservation of coiled coil motif between HcDAF5 and CeDAF5 indicates the potential function of HcDAF5 in transcriptional regulation [15].

Recent research has shown that insulin-like signalling and dafachronic acid (DA) signalling play a conserved role in larval development in both free-living and parasitic species [16, 17, 18, 19]. In contrast, TGF- β signalling does not seem to be conserved [20]. In *C. elegans*, *Cedaf5* is transcribed in all developmental stages except for dauer larvae, peaks in L1, and decreases thereafter (<https://wormbase.org>). However, in *Strongyloides stercoralis*, the transcription of *Ssdaf-5* was higher in L3i (infective third-stage larvae) [21]. In the present study, the transcription of *Hcdaf5* was low in the free-living stages but peaked in the infective L3 (L3), which is different from *Cedaf5*, but similar to *Ssdaf5*. In addition, the transcription pattern of *Hcdaf5* is consistent with that of genes in DAF-7 signalling pathway of *H. contortus*, including *Hcdaf3* and *Hctgfbr2* [22, 23]. The difference in transcriptional profiles of *daf5* between *C. elegans* and parasitic nematodes (including *H. contortus* in Clade V and *S. stercoralis* in Clade IV here), together with previous studies [23], indicates divergent functions of TGF- β signalling between the free-living and parasitic species, suggesting that parasitic nematodes have used this existing pathway for different purposes in evolution.

Considering the high transcription level of *Hcdaf5* in L3, RNAi mediated by siRNA was performed to explore its role in transition from the free-living stage (xL3) to the parasitic stage (L4). Here, silencing of *Hcdaf5* decreased the developmental rate of xL3, as specific siRNA-treated group showed less worms developed, for which buccal development failed to initiate, same as the *Hcdaf3* and *Hcakt1* [23, 24], suggesting that *Hcdaf5* functions in the development of xL3 to L4.

In the present study, HcDAF5 native protein was expressed prominently in the reproductive organs, which is consistent with HcDAF3 [23]. Together with the consideration that CeDAF5 is required for egg laying [5], we propose that HcDAF5 may also function in embryonic development and spermatogenesis. In addition to the gonad organs, HcDAF5 was also expressed in the cuticle and intestine of adult worms. In *H.*

contortus, the cuticle enabling the worms resistant to harmful substances [25]. Taken together, *HcDAF5* may also be associated with embryonic development and environmental resistance processes.

Phylogenetic analysis in this study revealed the relatively close relationship between DAF-5s of the free-living nematodes and parasitic species. In *C. elegans*, DAF-5 functions as a co-factor of DAF-3 (co-Smad), rather than an antagonist. However, its homologues functions as an antagonist of co-Smad in vertebrates [3]. The relatively close relationship between *HcDAF5* and *CeDAF5* suggested that *HcDAF5* probably also functions as a co-factor of *HcDAF3*. In order to testify this hypothesis, bimolecular fluorescence complementation system was used here for verifying the interaction between *HcDAF5* and *HcDAF3*. Results showed that the region downstream of the SDS box in *HcDAF5* is dispensable for *HcDAF3* binding, and MH2 domain of *HcDAF3* is sufficient for its interaction with *HcDAF5*. This is consistence with the *C. elegans* and vertebrates [5], which suggests that *HcDAF3* and *HcDAF5* interact and function as a transcriptional regulatory complex to further regulate the downstream transcription.

Conclusion

In conclusion, we investigated a transcription factor, *Hcdaf5* in *H. contortus*. This gene was transcribed throughout the life cycle of this parasitic nematode with the highest level at L3 stage and the native *HcDAF5* was expressed in adult reproductive organs, cuticle and intestine. In addition, knockdown of *Hcdaf5* retarded larval development *in vitro*. Moreover, the interaction between *HcDAF5* and *HcDAF3* was also verified. Taken together, these results provide evidence that *Hcdaf5* play important roles in the development of *H. contortus*.

Declarations

Acknowledgements

This study was supported by the National Key Basic Research Program (973 program) of China (grant no. 2015CB150300), the National Natural Science Foundation (grant no. 31872462) and the Natural Science Foundation of Hubei Province (grant no. 2017CFA020) to MH.

Authors' contributions

MH conceived the project. WDD carried out laboratory work. FFL, LH, CQW and CXZ performed the bioinformatics data analyses. LL, LSY and JC managed sheep hosts and *H. contortus* isolates. WDD wrote the manuscript, MH received the manuscript and contributed to the final submission. All authors read and approved the final manuscript.

Funding

The authors thank the National Key Basic Research Program (973 program) of China (grant no. 2015CB150300), the National Natural Science Foundation (grant no. 31872462) and the Natural Science Foundation of Hubei Province (grant no. 2017CFA020).

Availability of data and materials

The data supporting the conclusions of this article are provided within the article.

Ethics approval and consent to participate

Helminth-free goats were maintained in accordance with protocols approved by the Scientific Ethic Committee of Huazhong Agricultural University (Permit code: HZAUGO-2016-007) and Animal Ethics Guidelines from the People's Republic of China.

Consent for publication

Not applicable.

Competing interests Not applicable.

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Figures

Figure 1

HcDAF5 displays conserved features with homologues of Sno/Ski proteins. **A.** Primary structures of HcDAF5 and CeDAF5 with signature domains and motifs. **B.** Amino acid sequence alignment of Dach boxes (above) and SDS (below) of Sno/Ski proteins from *Haemonchus contortus* and other species. The triangle symbols below the SDS box show conserved zinc chelating residues. Accession Numbers and abbreviations: Hs, *Homo sapiens* (DACH1-NP_723968.1); Dm, *Drosophila melanogaster* (DACH1-NP_723968.1); Mm, *Mus musculus* (DACH1-NP_001033699.1); Ce, *Caenorhabditis elegans* (DACH1-NP_001021129.1); Dr, *Danio rerio* (DACH1-XP_009304051.1); Hs, (Icy-XP_292349; Skate-XP_064560); Dm, (Iceskate-NP_651946.1); Hs, (SnoN-NP_005405.2; SKI-NP_003027); Dm, (Snowski-ABV53643.1); Ec, *Equus caballus* (SKI-NP_001075287.1); Mm, (SKI-NP035515.2); Dr, (SKI-NP_571013.1); Tc, *Toxocara canis* (SKI-KHN86433.1); Bm, *Brugia malayi* (Snowski-AAQ93809.1); Ac, *Ancylostoma ceylanicum* (SKI-EYB97253.1); Nb, *Nippostrongylus brasiliensis* (SKI-found in Wormbase as NBR_0001757401); Cb, *Caenorhabditis briggsae* (DAF5-XP_002631643.1); Ce, *Caenorhabditis elegans* (DAF5-NP_496941.1). **C.** A rooted neighbour joining tree showing the relationships of *Haemonchus contortus* DAF5 to 17 homologues from other eight organisms. The tree was calculated using the Jones-Taylor-Thornton model in the MEGA program v.6.0. Bootstrap values above or below the branches (1000 iterations) are shown for robust clades (>50%). These eight species include four nematodes (*Caenorhabditis briggsae*: DAF5; *Caenorhabditis elegans*: DAF5, DAC1, MAB31; *Nippostrongylus brasiliensis*: SKI; *Toxocara canis*: SKI), one insect (*Drosophila melanogaster*: Snowski, Iceskate, Dachshund), one fish (*Danio rerio*: SKI, Dachshund), and two mammals (*Homo sapiens*: SKI, SnoN, Icy, Skate, DACH1; *Mus musculus*: SKI, Dachshund). The accession numbers of 17 molecules are given to the right of each molecule's name. CeMAB31 was used as the outgroup.

Figure 2

Spatiotemporal expression of HcDAF5 in *Haemonchus contortus*. A. Transcriptional levels of *Hcdaf5* in different developmental stages of *H. contortus*. Transcript abundance was detected and compared among eight developmental stages including eggs (E), the first-stage larvae (L1), the second-stage larvae (L2), the third-stage larvae (L3), the fourth-stage female (L4f), the fourth-stage male (L4m), adult female (Af) and adult male (Am) of *H. contortus*. All gene transcription levels were normalized to that of the β -

tubulin gene. Error bars indicate SEM from three independent experiments. Significant difference between stages was indicated by ** ($P < 0.01$). B, C. SDS-PAGE (B) and Western blot (C) analysis of recombinant HcDAF5. Lane M: standard protein molecular weight marker. For B, Expression of HcDAF5 was induced with 1 mM IPTG. Lane 1: recombinant HcDAF5 expressed in *E. coli* before induction. Lane 2: recombinant HcDAF5 expressed after induction. Lane 3: purified recombinant HcDAF5. C. Western-blot analysis. Lane 1: rabbit antiserum containing anti-rHcDAF5 antibody. Lane 2: pro-immunization rabbit serum (as control). D. Localization of HcDAF5 in adult worms. Images in line 1 and line 2 were taken under UV light corresponding to GFP and DAPI; merged images are given in line 3. Scale-bars: 100 μm . cu (cuticle), ov (ovary), int (intestine), cem (cement gland).

Figure 3

HcDAF5 is involved in differentiation of xL3 to L4 and interacts with HcDAF3 in *Haemonchus contortus*. A, B. The effects of RNAi on the development of *H. contortus* xL3 in vitro. A. The transcriptional changes of HcdaF5 in *H. contortus* after RNAi, detected by real-time PCR. For transcription assay, P values versus HcdaF5 siRNA treated group, are irrelevant siRNA group, $p=0.0066$; blank, $p=0.0060$. B. L3 developmental rate in vitro for 5 days following RNAi. For L3 developmental rate, P values versus HcdaF5 siRNA treated group, are irrelevant siRNA group, $p=0.0095$; blank, $p=0.0014$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. C. Bimolecular fluorescence complementation (BiFC) assessing the interaction between HcDAF3 and HcDAF5 in live mammalian cells. BHK21 cells were cotransfected with plasmids and images were acquired 20 h after transfection. Scale-bars: 100 μm . Full length HcDAF3 interact with HcDAF5 SDS domain (HcDAF3-HA-KN151 and HcDAF5-SDS-Myc-LC151), while HcDAF3 MH2 domain also interact with HcDAF5 SDS domain (HcDAF3-MH2-HA-KN151 and HcDAF5-SDS-Myc-LC151). Positive control (bJun-

HA-KN151 and bFos-Myc-LC151) and negative control (HA-KN151 and HcDAF5-SDSMyc-LC151) are also shown.

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