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ORIGINAL ARTICLE

RNA interference can be used to disrupt gene function in tardigrades

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Abstract How morphological diversity arises is a key question in evolutionary developmental biology. As a long-term approach to address this question, we are developing the water bear *Hypsibius dujardini* (Phylum Tardigrada) as a model system. We expect that using a close relative of two well-studied models, *Drosophila* (Phylum Arthropoda) and *Caenorhabditis elegans* (Phylum Nematoda), will facilitate identifying genetic pathways relevant to understanding the evolution of development. Tardigrades are also valuable research subjects for investigating how organisms and biological materials can survive extreme conditions. Methods to disrupt gene activity are essential to each of these efforts, but no such method yet exists for the Phylum Tardigrada. We developed a protocol to disrupt tardigrade gene functions by double-stranded RNA-mediated RNA interference (RNAi). We showed that targeting tardigrade homologs of essential developmental genes by RNAi produced embryonic lethality, whereas targeting green fluorescent protein did not. Disruption of gene functions appears to be relatively specific by two criteria: targeting distinct genes resulted in distinct phenotypes that were consistent with predicted gene functions and by RT-PCR, RNAi reduced the level of a target mRNA and not a control mRNA. These studies represent the first evidence that gene functions can be disrupted by RNAi in the phylum Tardigrada. Our results form a platform for dissecting tardigrade gene functions for understanding the evolution of developmental mechanisms and survival in extreme environments.

Keywords *Hypsibius dujardini* · Tardigrade · RNA interference · Evo-devo · Extreme environments

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Introduction

A key question in evolutionary developmental biology is how morphological diversity arises by alterations to developmental patterning mechanisms. Uncovering the molecular changes that contribute to the phenotypic variety found in animals is facilitated by comparing developmental processes in closely related organisms (Simpson 2002). As a long-term approach to address the question of the origin and diversification of body plans, we are developing a close relative of both *Drosophila* (Phylum Arthropoda) and *Caenorhabditis elegans* (Phylum Nematoda), the water bear *Hypsibius dujardini* (Phylum Tardigrada), as a model system (Fig. 1a, b). These three phyla are members of the Ecdysozoa, one of two protostome superclades (Aguinaldo et al. 1997). Recent molecular evidence supports the clustering of arthropods, tardigrades, and onychophorans in a Panarthropoda superphylum of segmented animals with paired appendages (Rota-Stabelli et al. 2010; Campbell et al. 2011; Giribet and Edgecombe 2012). Whether tardigrades are more closely related to arthropods and onychophorans or to nematodes remains controversial (Giribet and Edgecombe 2012). Although *Drosophila* and *C. elegans* are well-studied model systems, very little is known

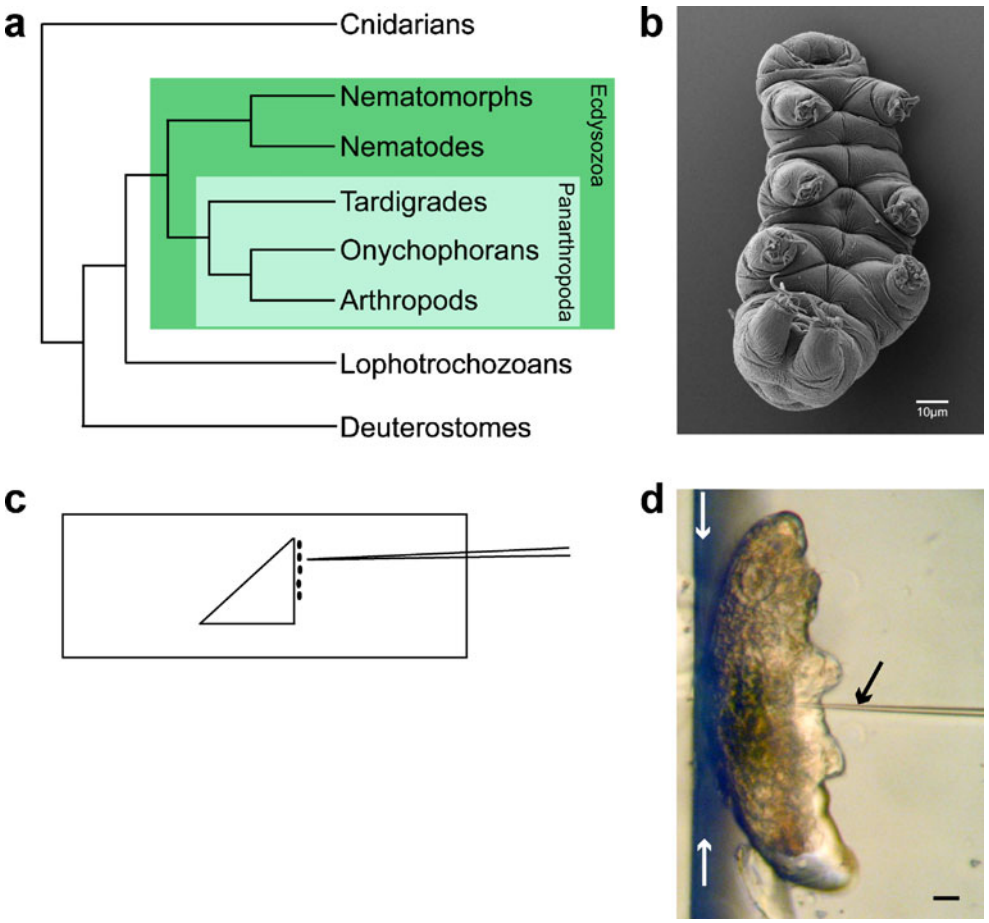

Fig. 1 Phylogenetic position and anatomy of *H. dujardini*. **a** Evolutionary position of tardigrades, with relationships to selected taxa shown. Within Ecdysozoa, arthropods, onychophorans, and tardigrades are proposed to form a Panarthropoda sub-clade. Some studies place tardigrades as a sister clade to the nematodes and nematomorphs. Drawn from Dunn et al. (2008), Telford et al. (2008), Hejnol et al. (2009), Rota-Stabelli et al. (2010), and Campbell et al. (2011). **b** Scanning electron micrograph of an *H. dujardini* adult, ventral view, ~150 μm long. *Scale bar*=10 μM. **c** Schematic drawing of injection slide showing coverslip corner (*triangle*) affixed to glass slide. Up to five tardigrades (*black ovals*) were positioned *lengthwise* against the straight edge of the coverslip, and an injection needle was positioned *perpendicularly* to each animal. **d** Lateral view of adult with needle (*black arrow*) inserted into midsection. The coverslip edge (*white arrows*) is positioned *vertically to the left* of the animal. The head of this animal is at the *top* of the image. *Scale bar*=10 μM

about the functions of developmental genes in other Ecdysozoan phyla. Tardigrades share many features with both arthropods and nematodes, suggesting that some of the techniques for studying development in these phyla might be transferable to tardigrades. We expect that using a close relative of two model systems, in which many orthologous genes are predicted to have similar functions, will mean that a large number of developmental genes and developmental processes will be valuable in understanding the evolution of development.

The tardigrade species *H. dujardini* has several favorable characteristics for evo-devo studies. This species can be maintained in the laboratory in cultures containing spring water and green algae as a food source (Ammermann 1967; Gabriel et al. 2007). Embryos are optically clear, facilitating the study of development by live microscopy. While males have been described in the wild, *H. dujardini* raised in culture to date are parthenogenetic; all embryos are diploid females (Ammermann 1967; Ramazzotti and Maucci 1983). Female reproduction is tightly linked to molting, and broods of 1–10 embryos are deposited into the shed exoskeleton or exuvium (Bertolani 1983; Ramazzotti and Maucci 1983; Gabriel et al. 2007). Embryos hatch about 4 days after deposition; the complete life cycle is relatively brief with a generation time of 13–14 days at room temperature (Gabriel et al. 2007). *H. dujardini* has a compact genome of about 70 Mb, and genome sequencing is in progress (Gabriel et al. 2007). Our lab has developed protocols for cryopreservation, live microscopy, and immunostaining to facilitate studies of *H. dujardini* (Gabriel and Goldstein 2007; Gabriel et al. 2007). Several tardigrade species have been shown to have the unique ability to withstand extreme conditions by cryptobiosis (Wright 2001; Guidetti et al. 2011; Wełnicz et al. 2011). Evidence suggests that *H. dujardini* can survive dessication, although the mechanisms underlying dessication

tolerance are not well understood in this species (Wright 1989, 2001). Other tardigrade species were the first multicellular organisms to survive exposure to outer space (Jönsson et al. 2008; Rebecchi et al. 2009). These traits have led to a growing interest in tardigrades in research investigating how organisms and biological materials can survive extreme conditions (Jönsson 2007; Møbjerg et al. 2011).

To address the issues above, it is essential to be able to disrupt gene function in *H. dujardini*. However, no such method exists for Phylum Tardigrada. RNA interference (RNAi) is a robust and well-conserved mechanism for gene silencing in many organisms, including nematodes and arthropods (Hannon 2002; Cerutti and Casas-Mollano 2006). In *C. elegans*, RNAi is systemic and heritable; injection of double-stranded RNA (dsRNA) into the intestine or germline of adults can disrupt gene activity in injected animals and in their progeny (Fire et al. 1998).

We have developed a protocol for RNA interference in *H. dujardini* by microinjection of dsRNA. We targeted six *H. dujardini* genes identified by their homology to genes with known functions in development in other organisms. We demonstrate that injection of dsRNA resulted in sequence-specific gene silencing, and therefore, is an effective method to dissect gene function in a species that may be valuable for understanding the evolution of developmental mechanisms, as well as survival in extreme environments.

Materials and methods

Tardigrade culture

H. dujardini cultures were maintained at room temperature in spring water and fed green algae in 60 or 150 mm glass Petri dishes as described (Gabriel et al. 2007).

Preparation of genomic DNA

To separate tardigrades from their algae food, we took advantage of the phototactic behavior of *H. dujardini* (B.G., unpublished observation). Cultures in 150-mm dishes were placed near a full-spectrum light source for several hours. Tardigrades accumulated on one side of the plate and were periodically transferred to a clean glass dish. Collected tardigrades were allowed to starve overnight, and their water changed several more times to reduce algal contamination. Tardigrades were transferred to low-retention microcentrifuge tubes (Fisher) and allowed to settle by gravity. The supernatant was removed and the tardigrade pellets were stored at −80 °C. Four 150-mm dishes typically yielded approximately 30 μg of compacted tardigrades.

Genomic DNA was prepared from approximately 30 μg of mixed-stage tardigrades using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's protocol. After elution, genomic DNA was concentrated by ethanol precipitation and resuspended in nuclease-free water or Tris–Cl solution.

Preparation of cDNA

Mixed-stage embryos ($n>50$) were collected and transferred to a microcentrifuge tube. Embryos were centrifuged and the supernatant removed. Crushed pieces of glass coverslips were added to the tube and embryos were vortexed in 100 μl of TRIzol Reagent (Invitrogen) at room temperature for 2 h. One-half volume of chloroform was added to the tube to extract RNA. Extracted RNA was concentrated by ethanol precipitation and resuspended in nuclease-free water. Genomic DNA was removed by treatment with DNaseI, Amp Grade (Invitrogen) as directed. Oligo-dT-primed first-strand cDNA synthesis was performed using the SuperScript III kit (Invitrogen) following manufacturer's recommendations.

Cloning

Sequences cloned for this study are listed in Table 1; the closest *Drosophila melanogaster* and *C. elegans* genes identified by BLAST for each sequence are listed in Online Resource 1. Sequences were aligned using ClustalW (Thompson et al. 1994; Online Resource 2).

Gene-specific primers were used to amplify the target sequence from genomic DNA or cDNA. Primer sequences are listed in Online Resource 3. PCR products were cloned into the pGEM-T-Easy vector (Promega), and several clones were sequenced for each gene.

Each gene was amplified in a second PCR reaction using gene-specific primers with a T7 polymerase promoter site added to the 5′ end of each primer. The final PCR product was cleaned using the PCR Purification Kit (Qiagen) as directed. DNA was eluted in Tris–Cl.

Plasmid pJT06 was created by PCR amplification of GFP from a *pie-1*:*GFP* expression vector (Strome et al. 2001) using sequence-specific primers with *Xho*I or *Xba*I adapters. The GFP PCR product was cloned into the *Xho*I/*Xba*I sites of vector L4440 (Fire Lab Vector Kit, Addgene). Vector-specific primers were used to amplify GFP and flanking T7 polymerase promoter sites by PCR (Online Resource 3).

RNA interference

For each gene, dsRNA was synthesized from 1 μg of T7-tagged DNA using the T7 RiboMax Express RNAi System (Promega) as directed. Following ethanol precipitation, dsRNA was resuspended in nuclease-free water. Working stocks were prepared at 1 μg/μl concentration, except for the dsRNA specific to the 5′ and 3′ halves of *Hd-mag-1*/*mago nashi*, which were prepared at 1.5 μg/μl concentration.

Table 1 *H. dujardini* sequences cloned in this study

GenBank accession no.	Gene name	Predicted protein	Predicted functions
CK326228	*Hd-act-1*	Actin	Cell division, cytoskeleton (Pollard and Cooper 2009)
CD449832	*Hd-dfd-1*	Deformed/HoxB4	Segment identity (Regulski et al. 1987)
CO508038	*Hd-dlc-1*	Cytoplasmic dynein light chain	Cell division, nuclear migration, germ cell proliferation (Karki and Holzbaur 1999; Dorsett and Schedl 2009)
CF544286	*Hd-ftt-1*	14-3-3ζ	Germline development, cell polarity (Benton et al. 2002; Morton et al. 2002)
CK326599	*Hd-mag-1*	Mago nashi	Germline development, sex determination, embryo elongation (Boswell et al. 1991; Li et al. 2000)
CF075689	*Hd-myo-1*	Muscle myosin class II heavy chain	Locomotion (Epstein et al. 1974)

Injection needles were pulled from borosilicate glass capillaries (World Precision Instruments) using a Flaming/Brown Micropipette Puller (Sutter Instrument Co.). Needles were back-filled with dsRNA immediately prior to injections.

Approximately 20–30 adult females were transferred to a deep depression slide and rinsed several times with spring water. To anesthetize animals, the water was replaced with Levamisole (Sigma-Aldrich) dissolved in spring water to a final concentration of 5 mM. Animals became largely immobile within 15 min. Animals remained viable in anesthetic for up to 1 h.

Injection slides were prepared by securing a right triangle cut from the corner of a 22×22-mm 1 1/2 glass coverslip onto a standard glass microscope slide. Halocarbon oil (Halocarbon Products Inc.) was overlaid on the coverslip, and up to five tardigrades were transferred to the slide. Tardigrades were braced against a straight edge of the coverslip (Fig. 1c, d).

Injections were performed using a Picosptrizer II (Parker-Hamilton Corp.) and Three-Axis Oil Micromanipulator (Narshige MMO-203), mounted on a Nikon Eclipse TE300 inverted microscope equipped with a 20× Plan Fluor objective. The Picospritzer was set to deliver 100-ms pulses of air to the injection needle. Needle tips were broken by gently stroking the tips against the edge of the triangular coverslip piece. In each case, the needle was inserted into the gonad or intestine and successful injection was confirmed by swelling of the gonad or intestine during injection. To ensure that tardigrades can tolerate microinjection, adult females were injected with a phenol red solution diluted 1:100 in spring water. Of 10 adults injected, 8 recovered and produced offspring, suggesting that microinjection is not deleterious to tardigrades. In these 8 adults, phenol red collected at the center of the site of injection (either the gonad or intestine) and eventually dissipated.

Injected tardigrades were transferred to a depression slide containing spring water and allowed to recover for about 30 min. Injected animals were then transferred to single wells of sterile, non-treated 96-well plates (Grenier bio) to which 100 μl of spring water and 5 μl of algae were added. After each round of egg laying, adults were transferred to new wells to more easily monitor progeny. Injected animals and their progeny were monitored for 30 days. For injected females, we recorded the total number of broods deposited, the number of embryos per brood, and the time between broods. Since embryos hatch about 4.5 days after deposition, we noted any embryos that failed to hatch after 5 days, and where possible, examined these embryos by DIC microscopy (see below). Embryos that hatched were followed until they began reproducing. For all animals, we also recorded their lifespan and monitored for any apparent changes in morphology or behavior.

Microscopy

Live embryos were prepared for microscopy by either of two methods. In the first method, embryos were transferred to a drop of bottled spring water on epoxy-coated three-well slides (Cel-Line/Thermo Scientific), supported by glass microspheres (diameter 37.36±0.39 μm, Whitehouse Scientific). In the second method, embryos were mounted on freshly prepared agar pads (2 % agarose in spring water). Coverslips were sealed with molten Vaseline. 4D differential interference contrast (DIC) microscopy was carried out on a Nikon Eclipse 800 microscope mounted with a Diagnostic Instruments SPOT2 camera. Images were acquired at 1 μm optical sections and analyzed with Metamorph v 6.3r7 (Molecular Devices).

Single-embryo cDNA synthesis and RT-PCR

Embryos were collected upon deposition into the exuvium by the molting female. This collection strategy permitted us to stage the embryos since embryos have either initiated or completed the first mitosis at this time. In the cases where

more than one embryo was deposited into the exuvium, embryos were separated from each other using a 26 G 1/2 needle (Becton-Dickinson). Embryos were mounted for imaging as described above to assess their phenotypes. To synthesize cDNA from single embryos, we modified a protocol developed for *C. elegans* embryos (Robertson et al. 2004). Single *H. dujardini* embryos were transferred from the microscope slide to the cap of a thin-walled, 0.2 ml PCR tube. The water was replaced with 0.2 μl of cDNA synthesis buffer prepared from the reagents included in the SuperScript III First-Strand cDNA Synthesis kit (Invitrogen), supplemented with the detergent Triton-X-100 (Sigma) [1× RT Buffer, 5 mM $MgCl_2$, 10 mM DTT, 2.5 μM oligo(dT), 0.5 mM dNTPs, 40U RNase OUT, 0.5 % Triton-X-100]. The embryo was briefly frozen on dry ice, then crushed using the tip of a 26 G 1/2 needle. An additional 1.8 μl of cDNA synthesis buffer was added to the cap, and the extract was collected by brief centrifugation. The extract was frozen on dry ice. For first-strand cDNA synthesis, 17 μl of cDNA synthesis buffer was added to each extract and incubated at 65 °C for 5 min. After this step, the SuperScript III protocol was followed according to manufacturer's instructions.

For RT-PCR, 3 μl of each first-strand cDNA product was used as the template, using primers specific to *β-tubulin* (*Hd-tbb-1*) or mago nashi [*Hd-mag-1*(*FL*)] (Online Resource 3). Thermal cycling conditions were as follows: 94 °C for 30 s (1 cycle), 94 °C for 30 s, 59 °C for 30 s, 72 °C for 45 s (35 cycles), and 72 °C for 10 min (1 cycle). All products were separated by gel electrophoresis, stained with ethidium bromide, and visualized by UV light. Gel images were captured digitally and the pixel values of each band were measured in ImageJ (National Institutes of Health). The ratio of *β-tubulin* to *mago nashi* pixel intensities was calculated for both wild-type and *Hd-mag-1*(*RNAi*) embryos, and statistical significance assessed by the Student's *t* test (unpaired, two-tailed).

Results

Molecular cloning of *H. dujardini* genes

Some organisms respond to the introduction of dsRNAs in sequence non-specific manners (Stark et al. 1998). We reasoned that for an organism for which few molecular tools existed to date, we could first gauge the specificity of RNAi using a biological indicator—by determining the extent to which disrupting genes with predicted functions would result in distinct and predictable phenotypes. To first identify *H. dujardini* genes with predicted roles in development, we compared by BLAST *H. dujardini* ESTs inGenBank to the *C. elegans* and *D. melanogaster* genomes, ordering results by *E* values. From the resulting lists, we selected six *H. dujardini* genes for which loss-of-function phenotypes might be predicted based on functions of homologs in *C. elegans* and/or *Drosophila* (Table 1, Online Resource 1). The proteins encoded by these six genes include putative homologs of actin, Deformed/HoxB4, dynein light chain, 14-3-3 protein, Mago nashi, and muscle myosin. The genes were cloned by PCR amplification from genomic DNA and cDNA using gene-specific primers. While the *H. dujardini* ESTs were prepared from mixed-stage animals, we confirmed by RT-PCR that all six genes appeared to be expressed embryonically (data not shown).

Two gene products appeared to contain complete coding sequences, as determined by presence of a predicted full-length ORF and by alignment to *D. melanogaster* and *C. elegans* sequences (Online Resource 2). *Hd-dlc-1* encodes a full-length protein of 89 aa with significant identity to a dynein light chain. Alignment of sequences from genomic DNA and cDNA shows that the *Hd-dlc-1* locus contains two exons separated by a single intron (Online Resource 4a). *Hd-mag-1* encodes a protein of 147 aa with significant identity to Mago nashi. A single exon of 444 bp defines the *Hd-mag-1* locus; there are no introns (Online Resource 4b). The remaining gene products each contained at least 400 bp of coding sequence.

RNAi by microinjection of dsRNA

To disrupt gene function in *H. dujardini*, we adapted a protocol for microinjection of dsRNA into *C. elegans* (Fire et al. 1998), but anesthetizing animals and mounting them in a custom injection chamber (see the "Materials and methods" section). To assess the efficacy of RNAi, for each dsRNA injected, we monitored injected adults and their progeny for 30 days following injections. Microinjection of dsRNA did not appear to be deleterious to the viability of adult females as 86 % of injected females recovered (n=203). Of the females that recovered, 60 % laid at least one brood of embryos (n=175). We monitored injected females and their progeny for several parameters, including brood size, brood frequency, and viability. For five of six genes, embryonic lethality was observed in progeny of females injected with dsRNA (Fig. 2a; Online Resource 5). For example, 86 % of all progeny of females injected with *Hd-act-1*/*actin* dsRNA failed to hatch (n=22), while 16 % of *Hd-mag-1*/*mago nashi*(*399 bp*)(*RNAi*) embryos did not hatch (n=207). Conversely, injection of adults with water or dsRNA targeting green fluorescent protein (GFP) had no effect on embryo viability or development (Fig. 2a, Online Resource 5). In all experiments, progeny that hatched and progressed through adulthood had no obvious defects in morphology, behavior, or fecundity.

In addition to effects on embryo viability, injection of gene-specific dsRNA also appeared to affect the fecundity

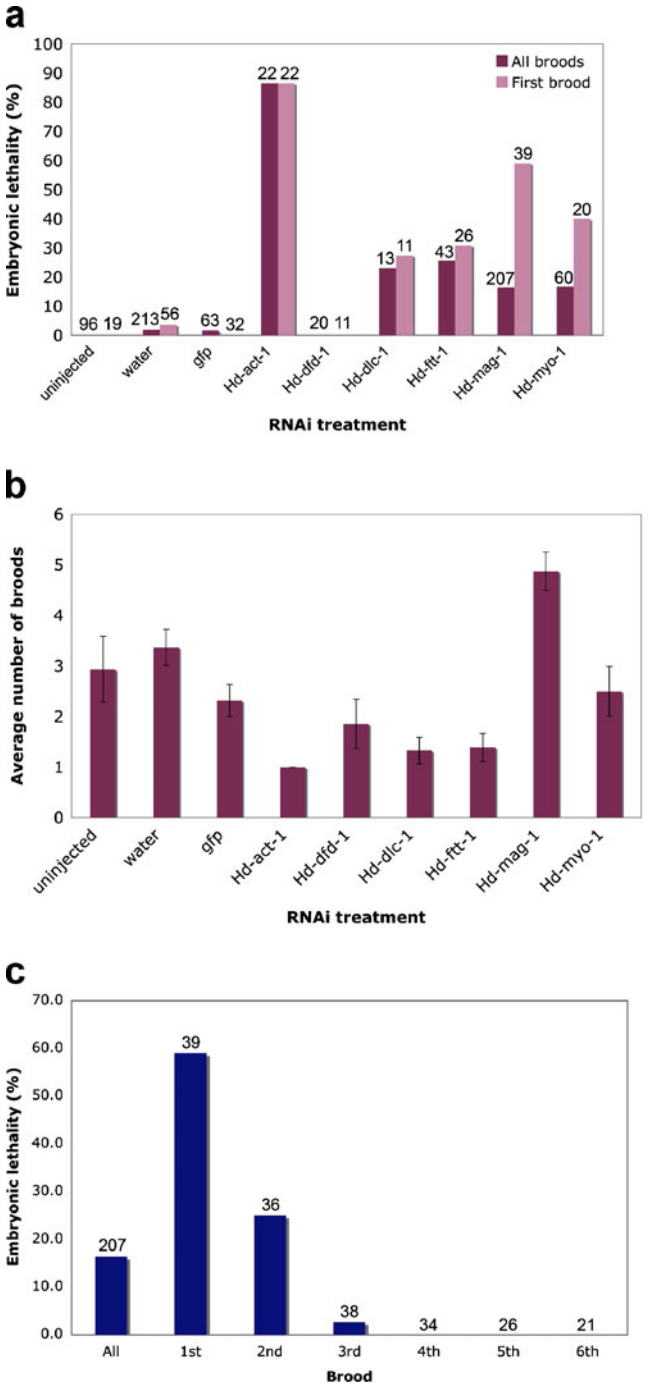

Fig. 2 RNAi of selected targets affects embryo viability and adult fecundity. **a** Percent embryonic lethality observed for progeny of females injected with dsRNA compared to progeny from uninjected control animals. For each condition, data are shown for progeny from all broods (*dark pink bars*) and for progeny from the first brood only (*light pink bars*). **b** Average number of broods for injected females. *Error bars* indicate 95 % confidence interval. **c** Embryonic lethality in *Hd-mag-1*/*mago nashi*(*RNAi*) progeny decreased with each successive brood. *Numbers over each bar* indicate the total number of embryos observed in each brood

of injected adults. While water-injected *H. dujardini* females produced an average of 3.4 broods within 30 days of injection, significant reductions in brood numbers were observed in females injected with dsRNA targeting *Hd-act-1*/*actin*, *Hd-ftt-1*/*14-3-3*, or *Hd-dlc-1*/*dynein light chain* (Fig. 2b, Online Resource 5). Surprisingly, females injected with *Hd-mag-1* dsRNA had a significant increase in the number of broods; no such effect on fecundity has been described for loss of *mago nashi* function in other organisms.

H. dujardini females laid eggs in discrete broods approximately 4 days apart throughout their reproductive lifespan (mean=4.4±1.5 SD, range 3–9 days, n=42 broods). Females produced an average of two embryos per brood (mean=2.2±0.9 SD, n=92 embryos). In the course of analyzing the RNAi experiments, we observed that the percentage of embryonic lethality decreased significantly with each successive brood. For example, in *Hd-mag-1*(*RNAi*) experiments, the majority of progeny laid in the first brood did not hatch (59 %, n=39), whereas only 25 % of progeny laid in the second brood did not hatch (n=36; Fig. 2c). Within the first brood, the highest proportion of lethality was observed in embryos laid from 3 to 9 days after injection (Online Resource 6). After the third brood, all progeny hatched and developed into adults. These results suggest that the penetrance of phenotypes following injection of dsRNA is time sensitive.

We sought to determine whether the efficacy of RNAi depended on the template from which dsRNA was prepared. While the presence of introns in dsRNA does not negatively affect the efficacy of RNAi in *C. elegans* (Fire et al. 1998), results from our study were inconclusive. For example, dsRNA specific to *Hd-myo-1*/*myosin* was prepared from both genomic DNA and cDNA. Injection of *Hd-myo-1* dsRNA prepared from genomic DNA, containing one predicted intron, had no effect on embryo viability (100 % hatched; n=82), while 16.7 % of progeny injected with *Hd-myo-1* dsRNA prepared from cDNA failed to hatch (Fig. 2a; Online Resource 5). Conversely, injection of *Hd-act-1* dsRNA prepared from genomic DNA, which contains two predicted introns, had a strong effect on animal development (Fig. 2a; Online Resource 5). It will be of interest to determine the minimum length of contiguous coding sequence in dsRNA necessary to effectively silence target genes in tardigrades.

Sequence-specific disruption of gene function

To assess the specificity of injected dsRNA for its target gene, we compared embryonic development in progeny of injected females with phenotypes that we expected based on each gene's predicted function. *H. dujardini* embryos develop in a stereotyped pattern (Gabriel et al. 2007). By ~20 h after egg deposition, wild-type embryos begin to elongate along the anterior-posterior axis, and markers of tissue differentiation, such as birefringent granules produced by the

intestine, first appear (Fig. 3a). After 4 days, just prior to hatching, well-developed structures are present in embryos, including the pharynx and intestine (Fig. 3b). Below, we describe phenotypes associated with disruption of four genes: *Hd-act-1*/*actin*, *Hd-dlc-1*/*dynein light chain*, *Hd-ftt-1*/*14-3-3*, and *Hd-mag-1*/*mago nashi*.

The protein actin has highly conserved roles in cell division, cell movement, and other processes (Pollard and Cooper 2009). Based on actin's fundamental role in cytokinesis, we predicted that *Hd-act-1*(*RNAi*) embryos would display defects in cell division, resulting in multinucleated cells. Of 17 *Hd-act-1*(*RNAi*) embryos examined by DIC microscopy, 10 were arrested early in development, with multinucleated cells (Fig. 3d). Filming early embryogenesis confirmed that cytokinesis was disrupted (Online Resource 7). Six of 17 embryos appeared to express birefringent gut granules, a marker of intestinal tissue differentiation (Gabriel and Goldstein 2007; Gabriel et al. 2007), but failed to elongate along the anterior-posterior axis. One embryo appeared to complete morphogenesis but failed to hatch. These results are consistent with a role for actin in cytokinesis and other cell processes. Interestingly, all females injected with *Hd-act-1* dsRNA that laid one brood died within several days, failing to produce additional broods ($n=14$). This observation suggests that depletion of *Hd-act-1* affected not only embryogenesis in progeny, but also the physiology of injected adults. Injection of short interfering RNAs (siRNAs) targeting actin had no effect on animal viability or development (data not shown), suggesting that longer dsRNA may be necessary to trigger the RNAi.

Dynein light chain is a subunit of cytoplasmic dynein, a complex of microtubule-associated proteins with roles in cell division, nuclear migration, intracellular transport, and germ cell proliferation (Karki and Holzbaur 1999; Dorsett and Schedl 2009). Among *Hd-dlc-1*(*RNAi*) embryos, 3 of 11 embryos failed to hatch. These embryos initiated, but did not complete morphogenesis. Each embryo appeared to contain a partial pharynx, and lacked birefringent granules seen in intestines in wild-type embryos (Fig. 3f). These three embryos were produced by different mothers, supporting the conclusion that this phenotype is likely to be specific to loss of function of *Hd-dlc-1*.

14-3-3 proteins are involved in protein–protein binding and have conserved roles in cell polarity and germline development (Benton et al. 2002; Morton et al. 2002). Targeting a 14-3-3-encoding gene resulted in 26 % of embryos failing to hatch ($n-39$). Phenotypes were highly variable. Interestingly, *Hd-ftt-1*(*RNAi*) appeared to affect the fecundity of injected females. While water-injected *H. dujardini* females produced an average of 3.4 broods (±2.1

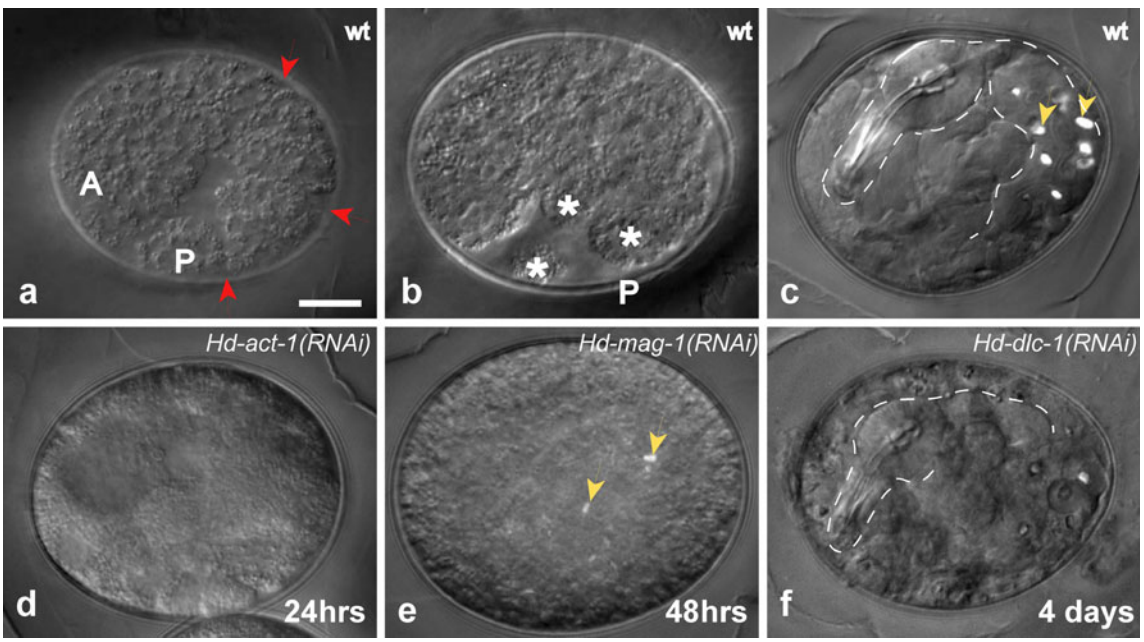

Fig. 3 RNAi results in target-specific depletion of gene function. **a**–**c** Representative images of wild-type embryos. **a** Stage 13 embryo (~24 h after egg laying), showing elongation along the anterior–posterior axis, with ectodermal segmentation (*red arrows*). **b** Same embryo as in **a**, at late stage 15 (~48 h after egg laying), showing three developing limb buds (*asterisks*). Intestinal birefringent granules are visible in a higher focal plane. **c** Stage 19 embryo (~4 days after egg laying), prior to hatching. The pharynx and intestine are outlined (*white dotted lines*). *Yellow arrows* in this panel and in panel (**e**) mark birefringent granules, a marker of intestine differentiation. Note that this embryo is different from the one shown in **a**, **b**. **d** *Hd-act-1*(*RNAi*) embryo, ~24 h after egg laying. **e** *Hd-mag-1*(*RNAi*) embryo, ~48 h after egg laying. The embryo has not elongated along the anterior–posterior axis, but birefringent granules are visible. **f** *Hd-dlc-1*(*RNAi*) embryo, ~4 days after egg laying. The pharynx and part of the intestine are outlined (*white dotted lines*). The intestine appears to lack birefringent granules. *A*=anterior; *P*=posterior. *Scale bar*=10 μm. Embryos were staged as described in Gabriel et al. (2007)

SD) within 30 days of injection, females injected with *Hd-ftt-1*/*14-3-3* dsRNA produced an average of only 1.4 broods (±0.9 SD; Fig. 2b, Online Resource 5). These data are consistent with a possible role for 14-3-3 protein in tardigrade germline development.

Mago nashi is a highly conserved protein with multiple roles in development, particularly in morphology. In *D. melanogaster*, *mago nashi* function is required for specification of posterior segments and for maintenance of the germline (Boswell et al. 1991). In *C. elegans*, *mago nashi* is required both for embryo elongation along the anterior-posterior axis, and for sex determination (Li et al. 2000). Of 28 *Hd-mag-1*(*RNAi*) embryos that failed to hatch, 14 produced birefringent gut granules, a marker of intestinal tissue differentiation, but failed to elongate along the anterior-posterior axis (Fig. 3e). This phenotype is consistent with elongation defects observed in *C. elegans mag-1* mutant embryos (Li et al. 2000). Of the remaining embryos, 12 arrested at an earlier stage and 2 completed morphogenesis but failed to hatch. Among progeny that hatched and developed to adulthood, there were no obvious defects in morphology, germline development, or fecundity. As a further test of the specificity of *Hd-mag-1*(*RNAi*), we generated dsRNA against two shorter regions of *Hd-mag-1*. Injection of either *mago-5′* dsRNA or *mago-3′* dsRNA (Online Resource 6) produced phenotypes in progeny similar to injection of *Hd-mag-1*(*399 bp*) dsRNA (Online Resource 5). These results further support our conclusion that *Hd-mag-1* dsRNA targets *Hd-mag-1* specifically.

The observation that each dsRNA produced distinct and reproducible phenotypes suggested to us that the cognate genes were specifically targeted. To further test this conclusion, we used RT-PCR to confirm that injection of dsRNA resulted in depletion of a target mRNA. Seven wild-type and seven *Hd-mag-1*(*RNAi*) embryos were collected 48 h after the first mitosis, and cDNA was synthesized from single embryos (see the "Materials and methods" section). Using the first-strand cDNA as a template, *Hd-tbb-1*/*β-tubulin* and *Hd-mag-1* were amplified by PCR and separated by gel electrophoresis. Comparison of the relative ratios of *Hd-mag-1* to *Hd-tbb-1* amplified from both wild-type and *Hd-mag-1*(*RNAi*) embryos demonstrated that expression of *Hd-mag-1* was significantly reduced in *Hd-mag-1*(*RNAi*) embryos (unpaired, two-tailed *t* test, $P<0.05$; Fig. 4b, c). We conclude that injection of dsRNA resulted in specific depletion of a target mRNA.

Discussion

RNAi is a potent method for disrupting and assessing gene function in many organisms (Cerutti and Casas-Mollano 2006). Long dsRNAs can elicit sequence non-specific responses in some organisms, triggering, for example, interferon responses in mammalian cells (Stark et al. 1998). We have found that dsRNA-mediated RNAi can be used to disrupt gene functions in the tardigrade *H. dujardini*. We predict that the development of a method for RNAi in this species will be valuable for investigating the evolution of developmental mechanisms as well as survival in extreme environments.

The penetrance of RNAi appears to be time sensitive, with progeny laid in the earliest broods most affected. With the exception of *Hd-act-1*(*RNAi*), in all cases where embryonic lethality was observed in progeny of dsRNA-injected females, the percentage of embryonic lethality was higher in the first brood than in subsequent broods (Fig. 2a, c). Several reasons may explain this observation. First, eggs take up a significant portion of the adult's body volume, and in egg laying, adults lose a significant percentage of their volume (Ramazzotti and Maucci 1983; Rebecchi and Bertolani 1994). It is possible that this loss of volume flushes out dsRNA cleavage products, diluting the effect of RNAi for the next brood. There may also be a general mechanism promoting turnover of dsRNA, resulting in time-dependent loss of dsRNA (Bosher and Labouesse 2000; Tuschl 2001). Finally, the site of injection may be important. In organisms such as *C. elegans*, the site of dsRNA injection does not affect the efficacy of RNAi (Fire et al. 1998), although in some cases, injection into the gonad produced a stronger phenotype (Ahringer 2006). We observed RNAi phenotypes in progeny of females injected in either the intestine or the ovary, and future experiments will determine the importance of the site of injection in RNAi penetrance. It will be interesting to determine which of these explanations is correct toward further optimizing the RNAi protocol.

The long-term goal of this study was to facilitate utilization of tardigrades' close relationship to arthropods and nematodes toward understanding the evolution of development. For example, the position of tardigrades as a panarthropod phylum makes them a potentially valuable outgroup for determining ancestral states of arthropod ancestors, alongside the other panarthropod phylum, the onychophorans, in which no gene disruption technique yet exists (Janssen et al. 2010). We focused on genes known to affect development in other organisms. Homeobox (Hox) genes have highly conserved roles in morphogenesis in multiple systems. In the *H. dujardini* EST database, we found a putative homolog of Deformed/HoxB4, which is required for normal head morphology in *D. melanogaster* (Regulski et al. 1987). However, injection of dsRNA targeting *Hd-dfd-1*/*deformed* had no effect on embryo viability or development neither were any morphological abnormalities observed in hatched progeny. This result may suggest that *Hd-dfd-1* mRNA was not sufficiently depleted, that *Hd-*

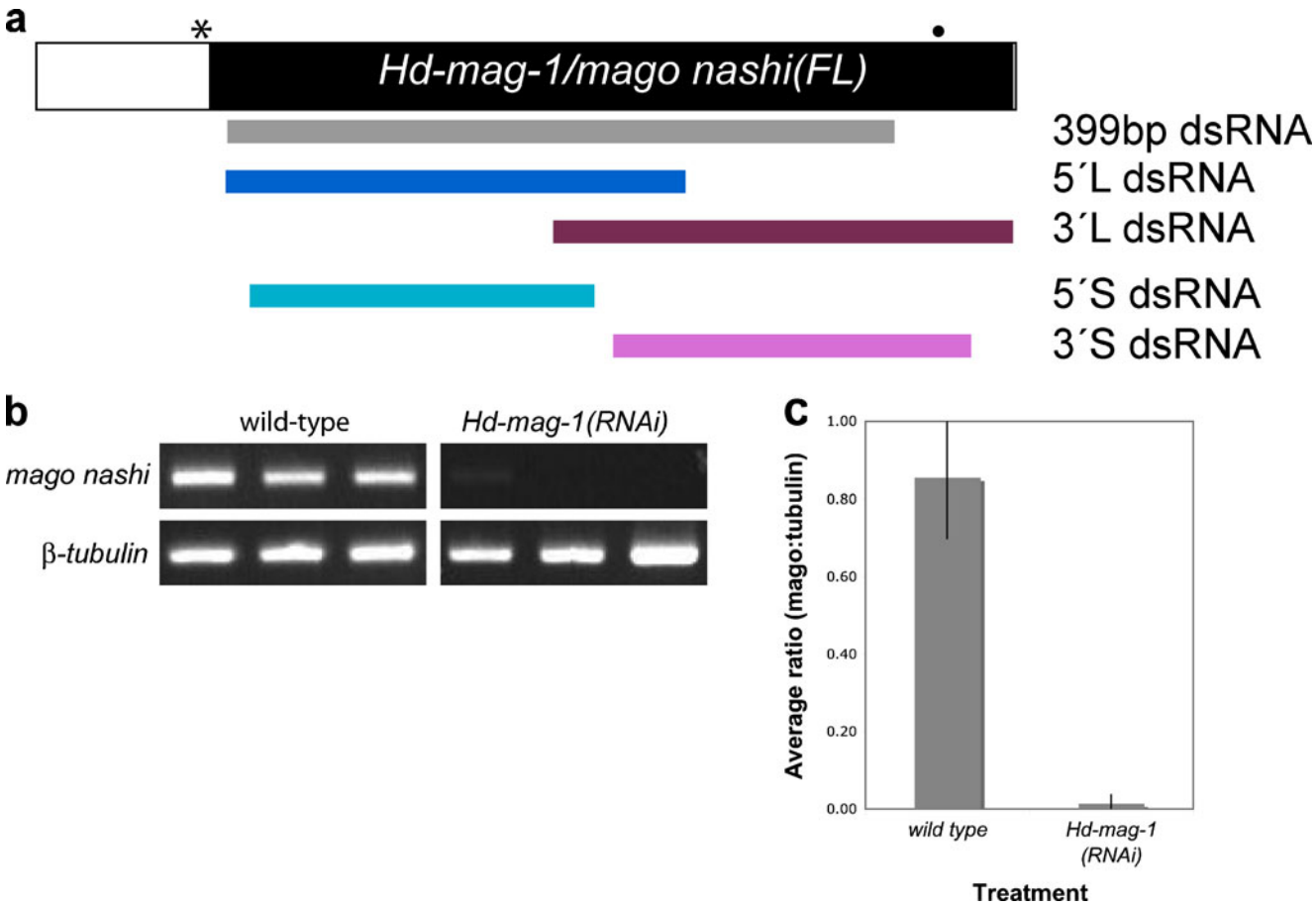

Fig. 4 RNAi leads to decrease in *Hd-mag-1* expression level. **a** Schematic of the *Hd-mag-1* coding sequence, showing the *Hd-mag-1*(*FL*) product amplified by RT-PCR (*black bar*). The positions of the start (ATG) and stop (TGA) codons are marked with an *asterisk* and *filled circle*, respectively. *Below the box* the sequences used as templates for dsRNA synthesis are indicated: *Hd-mag-1* (399 bp; *gray bar*), *Hd-mag-1* (*5'L*; *blue bar*), *Hd-mag-1* (*3'L*; *purple bar*), *Hd-mag-1* (*5'S*; *cyan bar*), and *Hd-mag-1* (*3'S*; *magenta bar*). **b** Relative expression of *Hd-tbb-1*/*β*-tubulin and *Hd-mag-1*/*mago nashi* amplified by RT-PCR in three wild-type and three *Hd-mag-1*(*RNAi*) embryos. **c** Histogram comparing ratios of amplified *Hd-mag-1* and *Hd-tbb-1* levels in the wild-type and *Hd-mag-1*(*RNAi)* embryos shown in **b**. Averages were calculated from pixel intensities measured for each band. *Error bars* indicate standard deviation

dfd-1 does not function in *H. dujardini* development, or that it acts redundantly with another gene. *Hd-dfd-1* appears sufficiently conserved to suggest that it is likely to have a function in *H. dujardini*. As the *H. dujardini* genome sequence becomes available, it will be interesting to identify other homeobox genes and to assess their roles in the development of the *H. dujardini* body plan.

In addition to homeobox genes, we focused on Mago nashi, an ancient and highly conserved protein found in many diverse phyla (Micklem et al. 1997; Newmark et al. 1997; Wiens et al. 2006). In many organisms, Mago nashi has roles in body axis specification, and in reproduction, either by regulating germline development or by involvement in the sex determination pathway (Boswell et al. 1991; Newmark and Boswell 1994; Newmark et al. 1997; Li et al. 2000; Wiens et al. 2006; Parma et al. 2007). We did not observe any deleterious effects on germline development in adults injected with *Hd-mag-1* dsRNA or in their viable progeny. This result may suggest that Mago nashi is not required for germline development in *H. dujardini*, that depletion was insufficient to reveal a role in germline development requiring only low levels of mRNA, or that the embryonic phenotype masks any later developmental role. Phenotypic analysis of *Hd-mag-1*(*RNAi*) embryos revealed that the majority of affected embryos failed to elongate along the anterior–posterior axis, but were able to initiate differentiation of certain tissues, including the intestine. This phenotype is distinct from that observed in *D. melanogster mago* mutant embryos, which lack abdominal segments and posterior pole cells (germline precursor cells), indicative of defects in establishing the anterior–posterior axis of oocytes (Boswell et al. 1991). Instead, the *Hd-mag-1* (*RNAi*) phenotype resembles that observed in *C. elegans* embryos lacking Mago nashi function, which fail to complete elongation (Li et al. 2000). This conserved role in embryonic elongation suggests that Mago nashi may have

played a role in elongation in ancestral Ecdysozoans. Alternatively, this role may have evolved in tardigrades and nematodes independently, perhaps via distinct processes required for elongation. Determining the downstream effectors of *mago nashi* in both *H. dujardini* and *C. elegans* would provide valuable insight into the evolution of Mago nashi function.

In conclusion, we have demonstrated that RNAi is an effective method to disrupt gene function in *H. dujardini*. This method represents the first protocol for disrupting gene function in any member of Phylum Tardigrada. Our initial analysis of developmentally important genes, such as *Hd-act-1*/*actin*, *Hd-dlc-1*/*dynein light chain*, *Hd-ftt-1*/*14-3-3*, and *Hd-mag-1*/*mago nashi*, suggests that the functions of the proteins encoded by these genes can be dissected by reverse genetics approaches. We expect that the ability to disrupt gene functions in *H. dujardini* may be valuable for studies on the evolution of development and on survival in extreme environments.

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