Fish and Shellfish Immunology 93 (2019) 1056–1066

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Two DExD/H-box helicases, DDX3 and DHX9, identified in rainbow trout are able to bind dsRNA

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ARTICLE INFO

Keywords:
DeXD/H box helicase
Rainbow trout
Nucleic acid sensors

ABSTRACT

In mammals, the multifunctional DExH/D-box helicases, DDX3 and DHX9, are nucleic acid sensors with a role in antiviral immunity; their role in innate immunity in fish is not yet understood. In the present study, full-length DDX3 and DHX9 coding sequences were identified in rainbow trout (*Oncorhynchus mykiss)*. Bioinformatic analysis demonstrated both deduced proteins were similar to those of other species, with ~80% identity to other fish species and ~70–75% identity to mammals, and both protein sequences had conserved domains found amongst all species. Phylogenetic analysis revealed clustering of DDX3 and DHX9 with corresponding proteins from other fish. Cellular localization of overexpressed DDX3 and DHX9 was performed using GFP-tagged proteins, and endogenous DDX3 localization was measured using immunocytochemistry. In the rainbow trout gonadal cell line, RTG-2, DHX9 localized mostly to the nucleus, while DDX3 was found mainly in the cytoplasm. Tissue distribution from healthy juvenile rainbow trout revealed ubiquitous constitutive expression, highest levels of DDX3 expression were seen in the liver and DHX9 levels were fairly consistent among all tissues tested. Stimulation of RTG-2 cells revealed that DDX3 and DHX9 transcripts were both significantly upregulated by treatment with the dsRNA molecule, poly I:C. A pull-down assay suggested both proteins were able to bind dsRNA. In addition to their roles in RNA metabolism, the conserved common domains found between the rainbow trout proteins and other species having defined antiviral roles, combined with the ability for the proteins to bind to dsRNA, suggest these proteins may play an important role in fish innate antiviral immunity. Future studies on both DDX3 and DHX9 function will contribute to a better understanding of teleost immunity.

1. Introduction

The DExD/H-box family of helicases includes a diverse array of proteins with multifunctional roles. Each DExD/H-box helicase contains a helicase motif II (DEAD, DEAH, DExD or DExH), as well as at least eight conserved motifs involved in adenosine triphosphate (ATP) binding and hydrolysis, nucleic acid binding, and RNA unwinding [1]. The DExD/H domain is well conserved from viruses and bacteria to mammals [2]. DExD/H-box helicases are involved in nearly all aspects of RNA-related cell processes including pre-mRNA splicing, mRNA export, and translation [3,4]. In addition to roles in RNA metabolism, some DExD/H-box helicases play a role in the innate antiviral response, acting as sensors for viral nucleic acids and/or affecting downstream signaling events [5–7]. Sensing viral nucleic acids is a key factor in host recognition of a viral infection; nucleic acids with unique defining features act as pathogen-associated molecular patterns (PAMPs) that are recognized by host-derived pattern recognition receptors (PRRs). Long dsRNA produced during viral infection is one example of a nucleic acid PAMP, length is a distinguishing feature to separate viral dsRNA from host dsRNA. PRRs can be found on the cell surface (ex. class A scavenger receptors), in the endosome (ex. toll-like receptors (TLR) TLR9 or TLR3), or in the cytoplasm, (ex. RIG-I like receptors (RLRs) including RIG-I and MDA5 [8]. MDA5 and RIG-I are DExD/H-box helicases that act as dsRNA sensors and play a pivotal role in activating signaling cascades, which culminate in the production of type I interferons (IFN) and the establishment of an antiviral state [9]. More recently, many other members of the DExD/H-box helicase family are now being recognized for their roles in nucleic acid sensing and mediating immune pathways [10].

The present study focused on two DExD/H-box helicases that are not part of the RLR family, DHX9 and DDX3, in rainbow trout (*Oncorhynchus mykiss)*. DDX3 plays an important role in nearly all

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https://doi.org/10.1016/j.fsi.2019.07.054
Received 27 March 2019; Received in revised form 15 July 2019; Accepted 18 July 2019
Available online 21 July 2019

Table 1
Primers, forward (F) and reverse (R), used for sequencing, cloning, and RT-qPCR for DDX3 and DHX9 in rainbow trout. Primer sequences are given in 5′-3′ orientation, product size in base pairs (bp) and annealing temperatures (T_a) are reported. Where applicable: extra nucleotides for the GC clamp or to keep a protein in frame are shown in bold, a human influenza hemagglutinin (HA) protein tag sequence is italicized, restriction sites are underlined and reported in the primer name, and the GSG linker sequence is in bold italics.

Primer	Sequence 5′-3′	Size (bp)	T_a (°C)
Sequencing			
DDX3	F - ATGAGTCATGTGGCCGTCG	2136	50
Full-length	R - TTAGTTGCCCCACCAGTCCA		
DHX9	F1-EcoRI - **GCGGCG**GAATTC**A**GCGGACATCAAGAACTTCCTGTAT	990	50
Frag.	R1 - AGTACTTGGATCAGGTGGCG	1257	50
	F2 - GCTTTTGAGGTGAATGTGGTGGA	1798	50
	R2 - TCTGGGGATCTGAGAGTGGA		
	F3 - ACACCTGGAGATGAACCCAC		
	R3-ApaI - **GCGGGC**GGGCCCCTAATATCCCTGGCCCCCTCCATA		
DHX9 Full- length	F - ATGGCGGACATCAAGAACTTCCTGTAT	3846	55
	R - CTAATATCCCTGGCCCCCTCCATA		
Cloning			
pCDNA3.1-DDX3-HA	F-EcoRI - **CGGCCG**GAATTCATGAGTCATGTGGCCGTCG	2216	50
	R-XhoI -**GCGCCC**CTCGAG*TTAGTTGCCCCACCAGTCCA**GGTAGTGGT**TACCCATACGATGTTCCAGATTACGCT*		
pCDNA3.1-DHX9-HA	F-EcoRI - **GCGGCG**GAATTCATGGCGGACATCAAGAACTTCCTGTAT	3926	50
	R-XbaI -**GCGCCC**TCTAGACTAATATCCCTGGCCCCCTCCATA***GGTAGTGGT**TACCCATACGATGTTCCAGATTACGCT***		
peGFP-C1- DDX3	F-XhoI - **CGGCCG**CTCGAG***C**GAGTCATGTGGCCGTCG	2158	50
	R-EcoRI - **GGGCCC**GAATTCTTAGTTGCCCCACCAGTCCA		
peGFP-C1- DHX9	F - see DHX9 Frag F1	3868	50
	R - see DHX9 Frag R3		
RT-qTPCR			
DDX3 qPCR	F - GAAACCAAGAAGGGAGCGGA	70	55
	R - GGATACTGGTGCAGGCGTAA		
DHX9 qPCR	F - GGGTATTTGAGCCTGTGCCT	197	55
	R - CTTCCTCTGCTCCAGGTTGG		
MDA5 qPCR	F - GGTGTCCTGATGGCTGTGAA	109	55
	R - CCAATGTCTCCTGCTCTGGG		

aspects of RNA metabolism [11]. In mammals DDX3 has two forms, DDX3X and DDX3Y, the genes encoding these proteins are found on the X and Y chromosomes respectively [12]. DDX3X is widely expressed and has many roles including cell homeostasis, innate immunity, and viral replication; DDX3Y protein is only expressed in the male germline and plays a role in spermatogenesis [13]. For host innate immunity, DDX3X increases type I IFN production by acting as a viral dsRNA sensor that associates with the adaptor protein IFN-β promoter stimulator 1 (IPS-1) to induce IFNs. DDX3X is also a transcriptional regulator that binds to the IFN-β promoter and enhances transcription [14–16]. DDX3X reduces influenza A replication via its role in stress granule formation [17]. In contrast to an antiviral function, DDX3X is also used by viruses to promote viral replication, as has been reported for hepatitis C virus, West Nile virus, and human immunodeficiency virus-1 (HIV-1; [18–20].

DHX9 (also known as Nuclear DNA helicase II (NDH II) or RNA helicase A (RHA)) has RNA, DNA, and triple-helical DNA structure helicase activity [21–23]. In mammals, DHX9 binds dsRNA and signals through IPS-1 to induce type I IFN [7,24]. DHX9 is also a substrate for phosphorylation by the dsRNA-receptor protein kinase R (PKR; [25]. As with DDX3, in addition to its antiviral activity used by the host, many viruses actively recruit DHX9 to enhance their replication. These viruses include: HIV-1, hepatitis C virus, cytomegalovirus, adenovirus, hepatitis E, influenza A, classical swine fever virus, and foot and mouth disease virus [22].

Very few studies have explored DHX9 or DDX3 in teleost fish, therefore, the nomenclature for DDX3, either X,Y, or other, has yet to be defined; as such, rainbow trout DDX3 will be referred to without further nomenclature in this study. DHX9 was identified in channel catfish (*Ictalurus punctatus;* [26] and goldfish (*Carassius auratus;* [27], neither study addressed a role for DHX9 in immunity. DDX3 has been identified in goldfish [27], olive (Japanese) flounder (*Paralichthys olivaceus*; [28], orange-spotted grouper (*Epinephelus coioides*; [29], and zebrafish (*Danio rerio*; [30]. In the olive flounder two DDX3 genes were identified from the transcriptome, DDX3a and DDX3b [28]. Both genes were expressed in different proportions in a wide variety of tissues, and there were sex-dependent differences in expression, however unlike in mammals these variants are not sex-linked [28]. Zebrafish DDX3 had differential expression in fish treated with heat-killed *Escherichia coli* [30]. DDX3 from *Epithelioma Papulosum Cyprini* (EPC; a fathead minnow cell line), is a binding partner for the nonvirion (NV) proteins from two novirhabdoviruses, suggesting DDX3 plays an important role in either enhancing innate immunity or promoting virus replication in fish [31]. Additionally, overexpressed grouper DDX3 protected cells against grouper nervous necrosis virus but did not affect Singapore grouper iridovirus replication and was involved in enhancing IFN-related antiviral pathway components [29].

In this study, sequences for DDX3 and DHX9 from rainbow trout were identified and bioinformatically analyzed. Endogenous expression levels of both transcripts were measured in both cell lines and tissues, and the effect of stimulation with the synthetic dsRNA, polyinosinic: polycytidylic acid (poly I:C) was measured in RTG-2 cells. The ability for DDX3 and DHX9 to bind to dsRNA was explored using *in vitro* transcribed dsRNA.

2. Materials and methods

2.1. Cell culture

The rainbow trout gonad (RTG-2), rainbow trout gill (RTgill-W1), rainbow trout gut (RTgutGC), and *epithelioma papulosum cyprini* (EPC) cell lines were obtained from N. Bols (University of Waterloo; [32–35]. Cell lines were grown in 75 cm^2 plastic tissue culture flasks (BD Falcon, Bedford, MA, USA) at 20 °C (rainbow trout cells) and 25 °C (EPC) in Leibovitz's L-15 media (HyClone, Logan, UT, USA) supplemented with 10% v/v fetal bovine serum (FBS; Fisher Scientific, Fair Lawn, NJ, USA), and 1% v/v penicillin/streptomycin (P/S; 10 mg/mL streptomycin and 10000U/mL penicillin; Fisher Scientific).

2.2. CDS identification

Primers for the full-length coding sequences (CDS) of rainbow trout DDX3 and DHX9 were designed. DDX3 primers were designed from an unnamed rainbow trout protein product (CDQ76520.1) which demonstrated similarity to olive flounder DDX3 variant 1 (AKS43549.1). The DHX9 primers were based on a predicted Atlantic salmon (*Salmo salar*) DHX9 sequence (XM_014190662.1). DDX3 was amplified from start to stop codon using the predicted primers and DHX9 was sequenced in three overlapping fragments, Table 1. RNA was extracted from RTG-2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and DNase treated using the TURBO DNA-free kit (Fisher Scientific). cDNA was synthesized as per manufacturers' instructions using iScript Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA, USA), 1 μg of RNA/20 μL reaction. PCR reactions were performed using Phusion High-Fidelity 2x master mix (ThermoFisher Scientific), 0.5 μM forward primer, 0.5 μM reverse primer, 2 μL of cDNA and nuclease-free water (Fisher Scientific) to a total volume of 20 μL. The following protocol was completed in a T100 Bio-Rad thermocycler: 98 °C - 30s, 29 cycles of 98 °C - 10s, T_a (Table 1) - 10s, 72 °C - 1min 30s, followed by a final extension at 72 °C - 5min. DDX3 was cloned directly into the peGFP-C1 vector for sequencing of the full-length product; DHX9 fragments had A-overhangs added with GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) as per pGEM T-easy instructions, and were subsequently cloned into pGEM T-easy (Promega) for sequencing prior to full-length cloning into expression vectors.

2.3. Full-length DHX9 amplification

To PCR amplify the entire DHX9 sequence a method adapted from Ref. [36] was used. First three overlapping fragments were amplified as described above, Table 1. The products were gel purified using the QiaQuick gel extraction kit (Qiagen, Hilden, Germany). A 100 μL PCR reaction (reaction A) containing Phusion High-Fidelity 2x master mix and 100 ng of each purified product were combined and the following protocol was completed in a T100 Bio-Rad thermocycler: 98 °C - 1min followed by 10 cycles of 98 °C - 10s, 55 °C - 10s, 72 °C – 1min 30s. A second 100 μl reaction (reaction B) containing Phusion High-Fidelity 2x master mix was created and included 3 μl of unpurified reaction A and 0.5 μM of DHX9 full-length forward and reverse primers with no tags, Table 1. The following protocol was completed: 98 °C–1 min, 34 cycles of 98 °C - 10s, 55 °C - 10s, 72 °C – 2min, followed by 72 °C – 5min. The product was then purified using the GeneJET PCR Purification kit (Fisher Scientific) and restriction sites were added using the pEGFP-C1-DHX9 primers from Table 1 under the conditions for reaction B. The sequences have been deposited into the NCBI database, DDX3: MG976579 and DHX9: MG976580.

2.4. Bioinformatics

Other potential variants of DDX3 and DHX9 were identified by blasting the full-length CDS with the Rainbow trout genome at http://www.genoscope.cns.fr/trout/ [37]. DDX3 and DHX9 nucleotide sequences were translated into deduced amino acid sequences using ExPASy; subsequent analysis was performed on these sequences [38]. Conserved motifs found within the DDX and DHX families were identified based on the report by Ref. [26]. The amino acid sequences were compared to other published sequences using EMBOSS Needle pairwise sequence alignment and the percent similarity and identity are reported. Amino acid sequences were aligned using ClustalW (within MEGA7) and MEGA7 was used to create a Neighbor-joining tree using the p-distance method [39–41]. A bootstrap analysis was done for 1000 replicates, the percentage of replicate trees that create the presented clusters is shown above each branch [42]. Conserved domains were identified using the NCBI conserved domain search and figures were created using the IBS illustrator program [43,44]. All accession numbers can be found in Table 2.

2.5. RT-qPCR

2.5.1. RNA extraction

Tissue samples were collected from three juvenile rainbow trout, ranging in weight between 75 g and 210 g. Tissue was added to TRIzol reagent (50 mg tissue/mL TRIzol; Invitrogen) and homogenized with a manual homogenizer. For endogenous expression rainbow trout cells were plated at 1×10^6 cells in a 6-well plate (BD Falcon), after overnight attachment TRIzol was added to the monolayer. RNA was isolated using TRIzol reagent (Invitrogen) and treated using the TURBO DNA-free kit (Fisher Scientific). For stimulation studies, RTG-2 cells were plated at 8×10^5 cells in a 6-well plate (BD Falcon) and allowed to attach overnight. Cells were treated with 50 μg/mL poly I:C (resuspended in PBS at 10 mg/mL; Sigma-Aldrich) in regular growth media, or media alone, for 4 h or 24 h. RNA was extracted using the Bio-Rad Aurum RNA extraction kit, including an on-column DNase I digestion (Bio-Rad).

2.5.2. cDNA synthesis and qPCR reactions

RNA from tissues and cell lines was reverse transcribed to cDNA using iScript reverse transcriptase as per manufacturers' instructions at 1 μg of RNA/20 μL reaction. qPCR reactions contained: 2 μl of 10^{-1} diluted cDNA, 1X SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.2 μM forward primer, 0.2 μM reverse primer, and nuclease-free water (Fisher Scientific) to a total volume of 10 μL, primers listed in Table 1. Triplicate technical replicates were performed. qPCR reactions were performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad). The program used for all qPCR reactions was: 98 °C - 2 min, 40 cycles of 98 °C - 5s, 55 °C - 10s, followed by a melting curve completed from 65 °C to 95 °C with a read every 5s. Product specificity was determined through single PCR melting peaks, and a no-reverse transcriptase (NRT) control reaction was also included for each sample. Data were analyzed using the $\Delta\Delta C_t$ method; gene expression was normalized to the housekeeping gene (β-actin). For tissue and cell culture endogenous expression samples were presented as relative to the gonadal tissue sample or RTG-2 cells. For poly I:C stimulation trials the data are presented as relative to an unstimulated control. Data represent three individual fish or three independent replicates and were statistically analyzed with GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, CA USA, www.graphpad.com). A one-way ANOVA with Tukey's multiple comparison test was used to check for significant differences between data points, a p value < 0.05 was considered significant.

2.6. Expression vectors and cellular localization

The two plasmids used in this study were peGFP-C1 (Clontech, Mountain View, California, USA) and pCDNA3.1 (+) (Invitrogen). Cloned inserts were amplified using Phusion High-Fidelity 2x master mix as described in 5.3.2 and 5.3.3 and gel purified using the QiaQuick gel extraction kit (Qiagen). The insert and vector were digested with the two restriction enzymes (FastDigest Thermo Fisher) corresponding to the added restriction sites on the insert, for 1 h at 37 °C, Table 1. A 5:1 ratio of insert to 50 ng of vector were ligated with NEB T4 ligase, overnight at 4 °C (New England Biolabs, Ipswich MA, USA). Plasmids was transformed into JM109 competent *E. coli* cells (Promega). Positive colonies were cultured overnight in Luria broth containing 100 μg/mL of kanamycin or ampicillin (peGFP-C1 or pCDNA3.1 respectively) and plasmids were purified using the GenElute Plasmid Preparation kit (Sigma-Aldrich). For cellular localization, 3×10^5 RTG-2 cells were plated in 12-well plates (BD Falcon) on glass coverslips. Plasmids were transfected into RTG-2 cells using Fugene6 (Promega) at a ratio of 1 μg plasmid to 3 μL of Fugene6. At 48 h post-transfection cells were fixed using 10% neutral buffered formalin, nuclei were counterstained with

Table 2
Sequence similarity of rainbow trout DDX3 and DHX9 proteins to other animal species. Emboss Needle pairwise alignment was used to compare the deduced rainbow trout protein sequences to published (A) DDX3 and (B) DHX9 sequences available on NCBI. % identity indicates the percentage of identical matches over reported aligned region, % similarity is the percentage of identical matches as well as similar amino acids (those awarded a positive value from the alignment matrix).

DDX3

Species	Accession	% Identity	% Similarity
Rainbow trout *Oncorhynchus mykiss*	CDQ76520.1 unnamed protein product	98.0	98.0
Olive flounder 2 *Paralichthys olivaceus*	AKS43550.1	81.0	87.0
Fathead Minnow *Pimephales promelas*	CZQ42530.1	80.4	85.6
Olive flounder 1 *Paralichthys olivaceus*	AKS43549.1	79.7	83.4
African clawed frog *Xenopus laevis*	NP_001080283.1	73.4	80.9
Western clawed frog *Xenopus tropicalis*	NP_989196.1	73.8	80.7
Domestic cow *Bos taurus*	NM_001192962.1	70.7	78.1
Human *Homo sapiens*	AAC34298.1	70.4	77.9
House mouse *Mus musculus*	NP_034158.1	70.3	77.7
Golden hamster *Mesocricetus auratus*	NM_001281387.1	70.2	77.6
Brown rat *Rattus norvegicus*	NM_001167665.1	68.5	76.0

DHX9

Species	Accession	% Identity	% Similarity
Zebrafish *Danio rerio*	NP_001188373.1	80.3	89.2
Japanese pufferfish *Takifugu rubripes*	BAV72136.1	78.3	87.0
African clawed frog *Xenopus laevis*	NP_001087383.1	75.7	85.4
Domestic cow *Bos taurus*	NP_776461.1	74.6	83.5
Human *Homo sapiens*	NM_001357.4	74.8	84.0
Rainbow trout *Oncorhynchus mykiss*	CDQ56455.1 unnamed protein product	73.1	73.3
Brown rat *Rattus norvegicus*	NP_001100654.1	71.4	80.1
House mouse *Mus musculus*	NP_031868.2	70.4	79.1
American alligator *Alligator mississippiensis*	KYO24241.1	56.1	62.8

10 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Fisher Scientific), and coverslips were mounted on glass slides with SlowFade Gold mounting medium (Fisher Scientific). Images were captured using an inverted fluorescence microscope, Nikon Eclipse TiE with Qi1 camera.

2.7. Immunofluorescence

RTG-2 cells were plated at 2×10^5 cells in a 12-well plate (BD Falcon) on glass coverslips and cells were allowed to attach overnight. Cells were rinsed with PBS before fixation with 10% neutral buffered formalin and permeabilized in 0.1%v/v Triton-X-100 in PBS. After a 1 h blocking period in blocking buffer (3% w/v BSA, 3% v/v goat serum, 0.02%v/v Tween-20, in PBS) the primary rabbit *anti*-DDX3 antibody (CusaBio, CSB-PA002106) was applied at a 1:100 dilution in blocking buffer for 1 h. A goat anti-rabbit Fluorescein isothiocyanate (FITC) secondary antibody (Santa Cruz; SC2012) was applied at a 1:200 dilution in blocking buffer for 1 h. Nuclei were counterstained with 10 μg/mL DAPI (Fisher Scientific) and coverslips were mounted on glass slides with SlowFade Gold mounting medium (Fisher Scientific). Images were captured using an inverted fluorescence microscope, Nikon Eclipse TiE with Qi1 camera. A secondary only control was used to account for background fluorescence. There have been no reported antibodies that cross-react with teleost DHX9. Cross-reactivity trials with one commercially available DHX9 antibody (A300-854A) were attempted and were unable to detect rainbow trout DHX9; the antigen of most commercial antibodies surveyed did not encompass a conserved area in the rainbow trout protein and therefore were not experimentally tested. DHX9 localization was measured using GFP-tagged protein overexpression and expression was limited to RT-qPCR studies for transcript expression.

2.8. Western blotting

Tissue samples from three juvenile rainbow trout, between 75 g and 210 g, were taken and added directly to lysis buffer. Prior to protein extraction RTG-2, RTgill-W1, and RTgutGC were plated at 1×10^6 cells in a 6-well tissue culture plate and allowed to attach overnight (BD Falcon). Proteins were extracted from tissue or cells lines using radioimmunoprecipitation assay (RIPA) buffer (50 mg tissue/mL or 1×10^6 cells/250 μL; 25 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100). Before use 2 μL/mL of protease inhibitor cocktail (Sigma-Aldrich) was added to RIPA buffer. Proteins were quantified using Bio-Rad Quick Start Bradford Protein Assay (Bio-Rad) and 20 μg of protein samples were run on a 10% acrylamide SDS-PAGE gel alongside 5 μL of PageRuler Prestained Protein Ladder (Thermo Scientific), after which proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using the *Trans*-Blot Turbo system (Bio-Rad) and the mixed molecular weight program (1.3A; up to 25 V for 7min). Blots were blocked for 1 h in 5% skim milk powder in tris buffered saline with 0.1% Tween 20 (TBS-T). Blots were probed with a 1:2000 dilution of rabbit *anti*-DDX3 primary antibody (CusaBio, CSB-PA002106) or rabbit *anti*-β-actin (Sigma-Aldrich, A2066) for 1 h at room temperature. Blots were incubated in a 1:4000 dilution of goat anti-rabbit HRP-conjugated secondary antibody (Bio-Rad, 172–1019) for 1 h at room temperature. Protein was detected on blots using the chemiluminescent Clarity Western ECL Substrate (Bio-Rad) and images were captured using a VersaDoc Imager (Bio-Rad).

2.9. Pull-down assay

EPC cells were seeded at 2×10^6 cells in a 25 cm^2 flask (BD Falcon). After overnight incubation cells were transfected with 4 μg of plasmids for expression of HA-tagged protein and 12 μl of Fugene6 transfection reagent in L-15 with 10% FBS and no antibiotics. 72 h post-transfection cells were lysed using 500 μl of lysis buffer containing 50 mM Tris, 150 mM NaCl, pH 7.2 and 1%v/v Triton-X-100. Before use 2 μl/mL of protease inhibitor cocktail (Sigma-Aldrich) was added to lysis buffer. The cell lysate was mixed with 10 μl of anti-HA magnetic beads (Bimake, Houston, TX, USA) that had been equilibrated in tris-buffered saline (TBS). 1 μg of *in vitro* transcribed dsRNA, a 200bp molecule of viral sequence (previously described in Ref. [45], was added to the cell mixture and the cell/protein/dsRNA mix was rotated overnight at 4 °C. Beads were washed with TBS until a NanoLite Spectrophotometer (Fisher Scientific) read 0 at absorbance 260 nm. The beads were boiled in 20 μL of 1x DNA gel loading dye (Fisher Scientific) and an immunoblot was performed using the J2 monoclonal antibody for dsRNA, as previously described [66]. The immunoblot included 1 μg of dsRNA

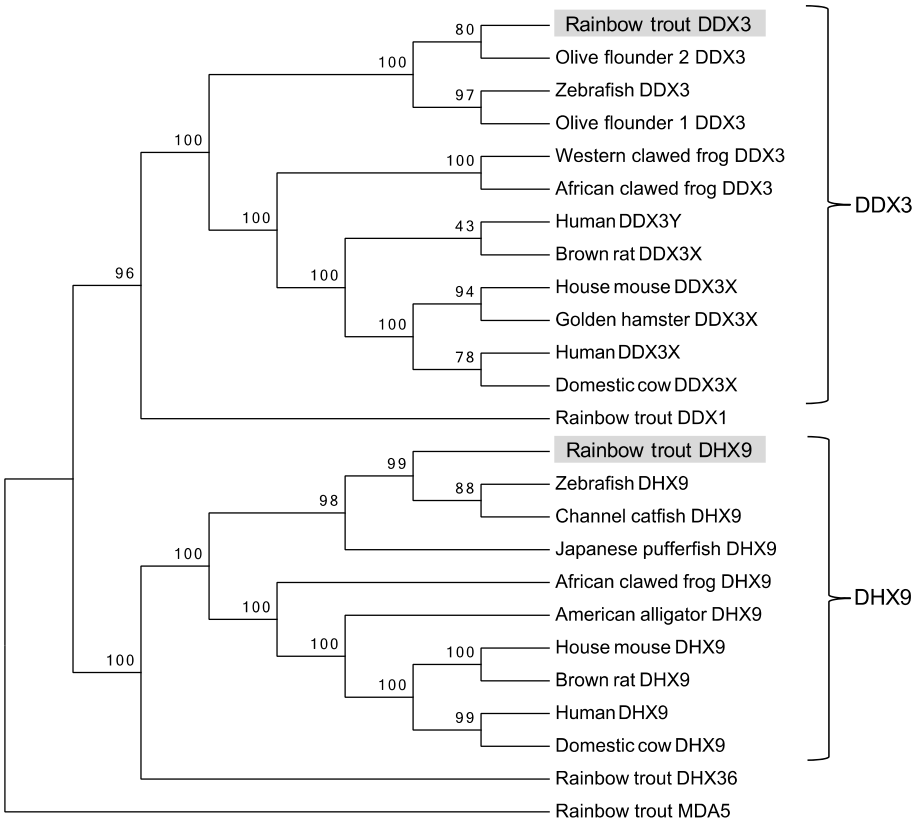

Fig. 1. Rainbow trout DDX3 and DHX9 cluster closely with respective proteins from other fish species. A Neighbor-joining tree was constructed based on the deduced protein sequence of rainbow trout DDX3 and DHX9 and published protein sequences from NCBI. Analysis was performed using Mega 7 after alignment with ClustalW. 1000 bootstrap replicates were tested and the percentage of replicates trees where the shown taxa clustered together is reported above each branch. Accession numbers are reported in Table 2 with the exception of rainbow trout MDA5 (NP_001182108.1), rainbow trout DHX36 (XP_021444178.1), rainbow trout DDX1 (XP_021456817.1), human DDX3Y (NP_004651.2).

ladder (New England BioLabs) and 20 ng of the *in vitro* transcribed dsRNA as controls.

2.10. Key resources table

Resource	Source	Identifier
Antibodies		
anti-HA magnetic beads	Bimake	
goat anti-rabbit Fluorescein isothiocyanate (FITC) secondary	Santa Cruz, SC2012	
goat anti-rabbit HRP-conjugated secondary	Bio-Rad, 172-1019	
J2 monoclonal	Cedarlane	
rabbit *anti*-DDX3 primary	CusaBio, CSB-PA002106	
rabbit *anti*-β- actin	Sigma-Aldrich, A2066	
CellLine		
EPC cells	Dr. Niels Bols	
RTG-2	Dr. Niels Bols	
RTgill-W1	Dr. Niels Bols	
RTgutGC	Dr. Niels Bols	
Chemical		
Aurum RNA extraction kit	BioRad	
iScript reverse transcriptase	BioRad	
peGFP-C1	Clontech	
SsoFast Evagreen Supermix	BioRad	
Trizol	Invitrogen	
TURBO DNA-free kit	Fisher Scientific	

3. Results

3.1. Rainbow trout DDX3 and DHX9 sequence similarity and phylogeny

Full-length coding sequences for a single DDX3 and DHX9 were identified from rainbow trout cells. The deduced amino acid sequence of DDX3 aligned with 98% similarity to an unnamed rainbow trout protein product in the NCBI database (CDQ76520.1) and 87% similarity to the olive flounder 2 protein (DDX3b), and out of surveyed sequences had the lowest similarity to brown rat (*Rattus norvegicus*), Table 2. DHX9 had an 89.2% similarity to zebrafish and was least similar to the American alligator (*Alligator mississippiensis*), Table 2. As salmonids have undergone two additional whole genome duplications compared to other bony vertebrates [37] it is very likely that there are more than one copy of each of these proteins encoded within the rainbow trout genome. Indeed the olive flounder sequence appears to have two copies of DDX3 (DDX3a and DDX3b; [28], therefore, the identity of possible gene paralogues were identified from the sequenced rainbow trout genome. When aligned to the rainbow trout genome, DDX3 aligned with 100% identity to scaffold 876. Four other partial alignments had a high percent identity, but no alignment covered more than 60% of the protein, Supplementary Table 1. DHX9 showed a 99.2% identity with a sequence on scaffold 6, this sequence had a 92% coverage. These possible paralogues appear to be fragments and were not pursued further in the present study; however, they would be interesting to investigate in future work. A Neighbor-joining tree was used to infer evolutionary history; both rainbow trout DDX3 and DHX9 clustered closely with other fish species, unsurprising given the high sequence similarity, Fig. 1. The fish species formed a clade separate from the other non-fish species for each protein. The rainbow trout DDX3 clustered more closely to the second variant of olive flounder DDX3 (DDX3b), whereas the other fish species examined clustered with the first olive flounder variant, DDX3a. Rainbow trout DDX3 and DHX9 did not cluster with any of the other known DExH-box proteins from rainbow trout, including: DDX1, DHX36, and MDA5.

3.2. Conserved domains and motifs in rainbow trout DDX3 and DHX9

The conserved sequence motifs common to DEAD-box or DExH-box helicases were identified. All DDX3 sequences, including rainbow trout, contained nine conserved motifs (Q, I, Ia, Ib, II (DEAD), III, IV, V and VI), while the DHX9 sequences, including the rainbow trout sequence, contained eight conserved motifs (Q, Ia, Ib, II (DEIH), III, IV, V and VI),

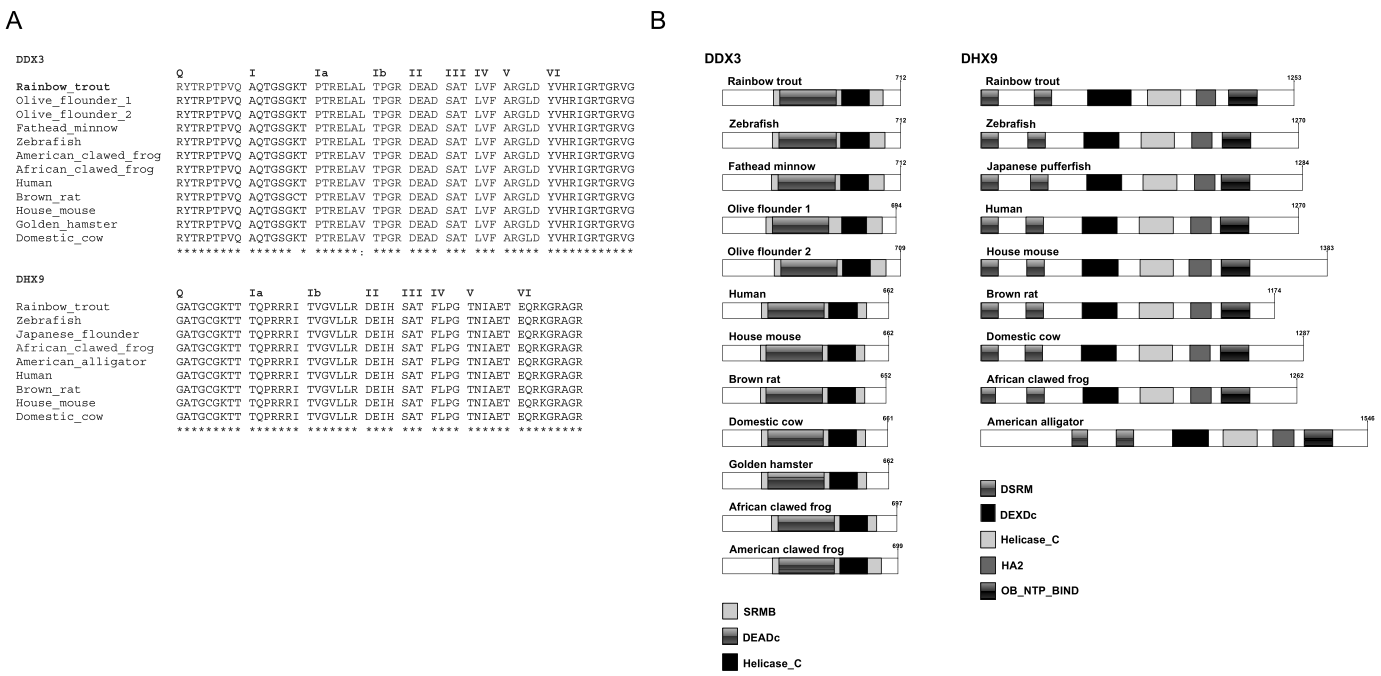

Fig. 2. Rainbow trout DDX3 and DHX9 proteins have conserved motifs and domains. **(A)** Nine and eight conserved motifs were identified in DDX3 and DHX9 rainbow trout protein sequences respectively, as well as in the amino acid sequences of other species. Amino acids that are identical between all species are marked with an asterisk. **(B)** Conserved domains were identified in the DDX3 and DHX9 proteins using the NCBI conserved domain search and figures were created using the IBS illustrator program. Proteins and domains are drawn to scale with the total length in amino acids is reported at the end of each protein. Accession numbers can be found in Table 2. The DDX3 protein domains identified were: the SRMB domain (COG0513), the superfamily II DNA and RNA helicases; the DEAD_C domain (cd00268) a helicase domain found in members of the DEAD-box family; and the Helicase_C domain, a helicase conserved C-terminal domain, only found in DEAD/H helicases. The DHX9 protein domains identified were: a DEXDc domain (cd00046), found in members of the DEAD-like helicases superfamily; the Helicase_C domain (pfam00271); an HA2 domain (SM00847), a helicase-associated domain; two DSRM domains (Smart00358), double-stranded RNA binding motifs, and an OB_NTP_BIND domain (pfam07717), an oligonucleotide/oligosaccharide-binding fold.

Fig. 2A [26]. These motifs were conserved between all animal species included for analysis. NCBI's conserved domain search software was used to identify conserved domains in the rainbow trout DDX3 and DHX9 protein sequences, Fig. 2B. The DDX3 proteins contain: a SRMB domain (COG0513), the superfamily II DNA and RNA helicases; the DEAD_C domain (cd00268) a helicase domain found in members of the DEAD-box family; and the Helicase_C domain, a helicase conserved C-terminal domain, only found in DEAD/H helicases that contains motif III involved in ATP hydrolysis and RNA binding. DHX9 proteins contain: a DEXDc domain (cd00046), found in members of the DEAD-like helicases superfamily and contains an ATP binding site, a putative Mg^{++} binding site; the Helicase_C domain (pfam00271); an HA2 domain (SM00847), a helicase-associated domain found in a diverse set of RNA helicases; two DSRM domains (Smart00358), double-stranded RNA binding motifs, and an OB_NTP_BIND domain (pfam07717), an oligonucleotide/oligosaccharide-binding-fold found in the C-terminus of DEAD-box helicases, generally associated with the HA2 domain.

3.3. Differential cellular localization of DDX3 and DHX9 in rainbow trout cells

Cellular localization of DDX3 and DHX9 was first measured using ectopically expressed proteins. The intracellular location of GFP-tagged DDX3 and DHX9 proteins was monitored in RTG-2 cells using fluorescence microscopy, Fig. 3 peGFP-C1-DDX3 localized to the cytoplasm, exhibiting a consistent punctate structure, Fig. 3A. Interestingly, when the cellular localization of endogenous DDX3 was measured using immunocytochemistry (ICC; Fig. 4) this punctate staining was not reproduced. Using ICC, DDX3 was still cytoplasmic, but with a more diffuse and even staining pattern. peGFP-C1-DHX9 localized mainly to the nucleus, with some cells showing low levels of green fluorescence in the cytoplasm, Fig. 3B. Some nuclei exhibited brightly fluorescent punctate structures, while all nuclei exhibited fluorescent exclusion from the nucleoli. Nucleoli were identified by localized DAPI staining exclusion [46], as indicated by arrowheads, Fig. 3B.

3.4. Ubiquitous, constitutive expression patterns for DDX3 and DHX9 in rainbow trout tissues and cell lines

DDX3 and DHX9 expression patterns were measured in tissues extracted from juvenile rainbow trout and from three rainbow trout cell lines. Transcripts for DDX3 and DHX9 were found in the ten tissues tested, Fig. 5A. Brain and heart showed levels that were elevated higher than gut and liver showed transcript levels higher still, Fig. 5A. Head kidney, spleen, thymus, gill, gonad, and muscle showed levels of DDX3 that were not significantly different than gut. While not quantitative, at the protein level gonad, brain and liver showed a trend towards higher amounts of protein being expressed, Fig. 5C. The three common rainbow trout cell lines, RTG-2, RTgutGC, and RTgill-W1 expressed DDX3 transcripts, with no significant difference between the cell lines, Fig. 5B. These three cell lines were chosen because they represent three different tissues of origin and behave differently in response to both poly I:C and viral infection [45,47]. Additionally, they represent two different cell types, fibroblastic and two epithelial cell lines respectively. In cell lines at the protein level RTG-2, RTgill-W1, and RTgutGC showed similar levels of DDX3, Fig. 5C. DHX9 transcripts were also found in all ten tissues. There were no significant differences between any tissues, however gut showed the highest amounts of transcript, with considerable variability, and gonad had the lowest levels of expression, Fig. 5A. In the three cell lines tested there was no significant difference between the cell lines, although there was a slightly increased level of transcript in RTgill-W1 compared to RTG-2 and RTgutGC, Fig. 5B.

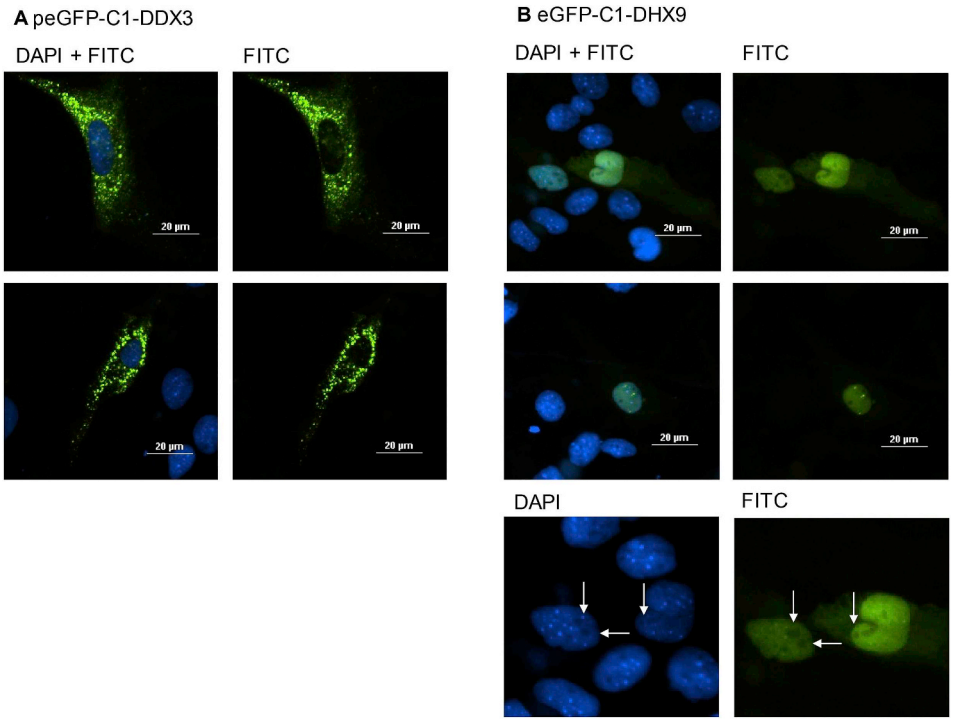

Fig. 3. In rainbow trout cells, ectopic expression of DDX3 and DHX9 reveals that DDX3 localizes in punctate cytoplasmic structures and DHX9 localizes to the nucleus with nucleolus exclusion. Rainbow trout **(A)** DDX3 and **(B)** DHX9 sequences in the pEGFP-C1 expression vector were overexpressed in RTG-2 cells (GFP; green) and nuclei were counterstained with DAPI (blue). Two different fields of view are shown for each protein. The bottom image of **(B)** is a close-up image of a DHX9-expressing cell with white arrowheads indicating areas of DAPI exclusion in the nucleus, indicating nucleoli, and the corresponding areas of low-FITC expression. Magnification 400× for both A and B. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Upregulation of DDX3 and DHX9 transcripts by poly I:C stimulation

The effect of dsRNA-stimulation on DDX3 and DHX9 expression levels were measured in RTG-2 cells stimulated with 50 μg/mL of poly I:C for 4 h and 24 h, Fig. 6. MDA5, a dsRNA sensor that has previously been shown to increase in expression following poly I:C treatment in RTG-2 cells, was included as a positive control [48]. While there was a modest and non-significant increase in transcript levels of all three sensors at 4 h post-treatment, by 24 h transcripts of all three helicases were significantly upregulated.

3.6. DDX3 and DHX9 are capable of binding dsRNA

The ability of rainbow trout DHX9 and DDX3 to bind dsRNA was measured using pull-down assays. HA-tagged DHX9 and DDX3 proteins were overexpressed in EPC cells and cell lysates were mixed with *in vitro* transcribed dsRNA of 200bp in length. The 200bp molecule was used instead of poly I:C as the defined length produces a very clear band on an immunoblot, unlike the smear produced by poly I:C [45]. The HA-proteins were pulled-down using magnetic anti-HA beads and dsRNA was detected in the resulting mixture by immunoblot analysis

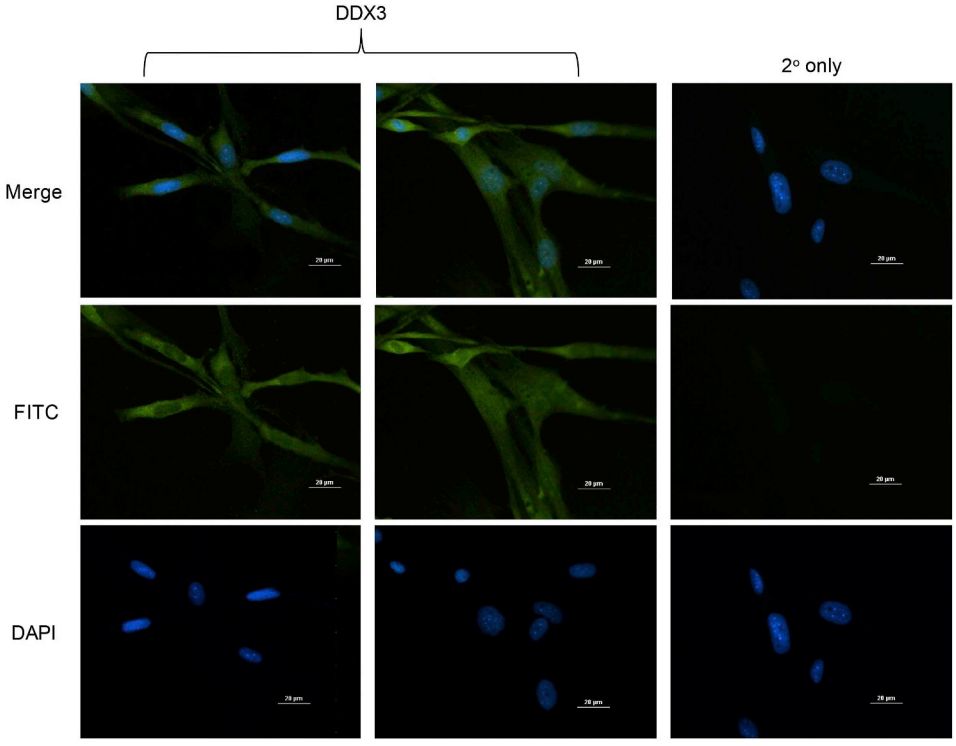

Fig. 4. Immunocytochemistry for endogenous DDX3 shows localization is cytoplasmic but not punctate. To look at endogenous protein expression an *anti*-DDX3 antibody was used to detect DDX3 (FITC; green) in unstimulated RTG-2 cells. Nuclei were counterstained with DAPI (blue). A secondary (2°) only control showed no background FITC signal. Magnification 200X. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

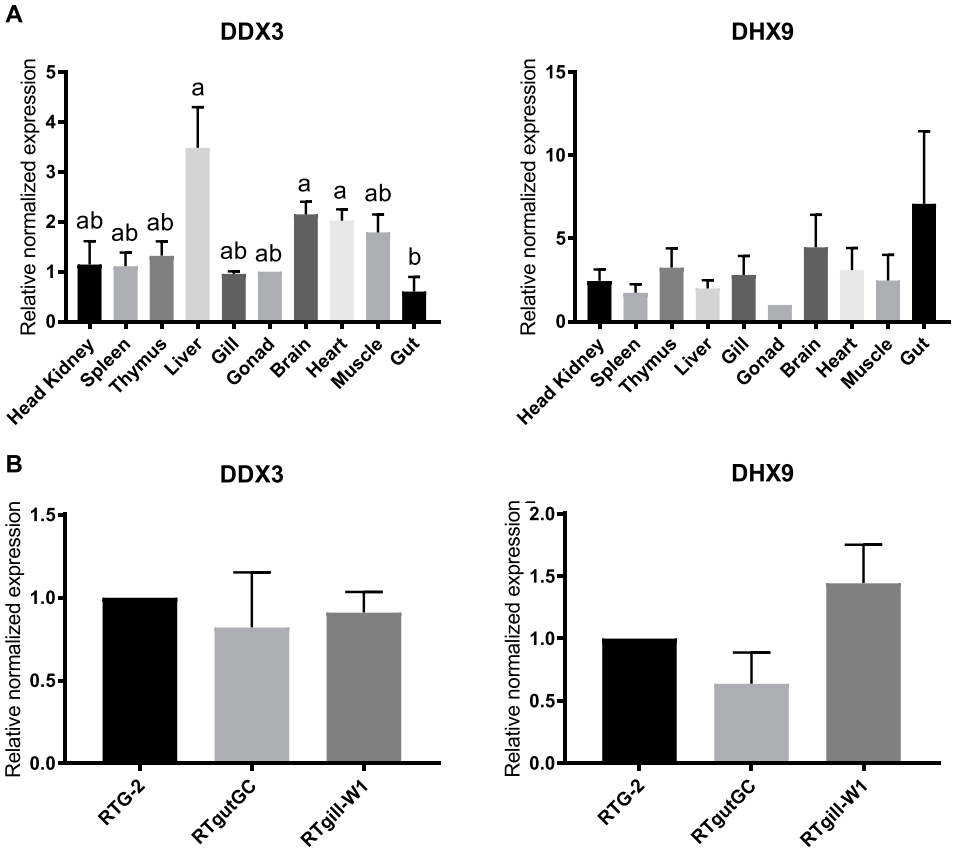

Fig. 5. DDX3 transcript and protein, and DHX9 transcripts are found across rainbow trout tissues and cell lines. Juvenile rainbow trout tissues were collected and **(A)** RT-qPCR was performed to measure DDX3 and DHX9 transcript levels across tissues. (**B**) DDX3 and DHX9 transcripts in three rainbow trout cell lines, RTG-2, RTgutGC, and RTgill-W, were measured using RT-qPCR. **(A)** and **(B)** Data were analyzed using a $\Delta\Delta C_t$ method, gene expression was normalized to the housekeeping gene (β-actin), and presented as relative expression to the gonadal tissue or RTG-2 cells. N = 3, data are presented with the standard error of the mean (SEM). Statistical analysis was performed on log2 transformed data and data were analyzed using a one-way ANOVA and Tukey's multiple comparison test, a p value < 0.05 was considered significant. Data points denoted with the same letter were not significantly different from each other, if no letters are present there were no significant differences. **(C)** DDX3 protein expression was measured by Western blot analysis using an *anti*-DDX3 antibody, *anti*-β-actin was used as an internal control; data are representative of three individual fish.

using the J2 *anti*-dsRNA antibody. Cell lysate containing DDX3 and DHX9 was able to pull-down dsRNA but the control cell lysate showed no binding to dsRNA, Fig. 7. When compared to the dsRNA-only control the dsRNA from the pull-down was a slightly larger size, possibly due to interference from proteins in the cell lysate mixture.

4. Discussion

In mammals, DDX3 and DHX9 play important roles in cell homeostasis and innate immunity, however currently there is little knowledge about these helicases in fish. When a phylogenetic analysis was performed on the DDX3 and DHX9 deduced protein sequences, the

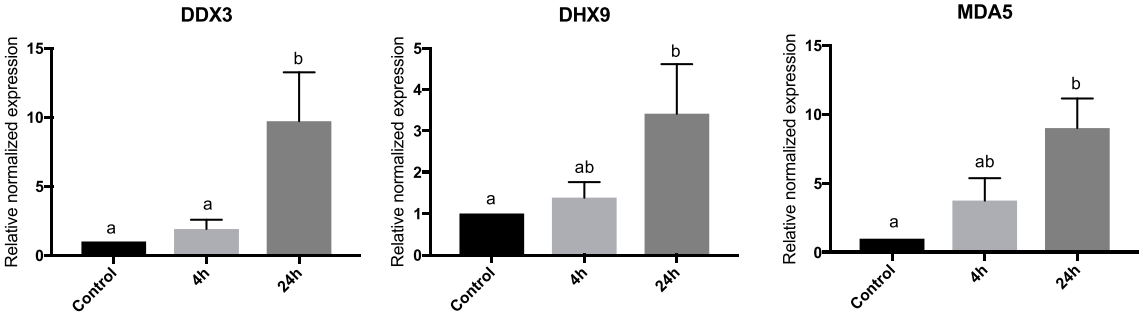

Fig. 6. dsRNA (poly I:C) stimulation upregulates DDX3, DX9, and MDA5 transcript expression in RTG-2 cells. RTG-2 cells were stimulated extracellularly with 50 μg/mL of poly I:C for 4 h and 24 h. DHX9, DDX3, and MDA5 transcripts were measured using RT-qPCR. Data were analyzed using a $\Delta\Delta C_t$ method; gene expression was normalized to the housekeeping gene (β-actin) and then presented as relative to the unstimulated control. Data represent three individual replicates and are presented with the standard error of the mean (SEM). Data were log2 transformed prior to statistical analysis using a one-way ANOVA with a Tukey's multiple comparison test, and a p value < 0.05 was considered significant. Data points labelled with the same letter do not have a statistically different average.

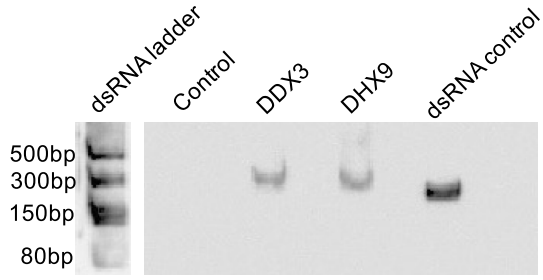

Fig. 7. Rainbow trout DDX3 and DHX9 bind dsRNA. HA-tagged DDX3 and DHX9 were overexpressed in EPC cells and cell lysate was collected. Cell lysate was mixed with a 200bp *in vitro* transcribed dsRNA molecule. Protein/nucleic acid complexes were pulled down using magnetic anti-HA beads. The resulting pull-down mixture was analyzed for dsRNA using an immunoblot with the J2 antibody, an *anti*-dsRNA antibody. A control was included, cell lysate isolated from untransfected cells. The dsRNA control lane represents 20 ng of *in vitro* transcribed dsRNA loaded onto the gel. This is a representative blot from three independent experiments.

clustering was as expected based on species relationships; the DDX3 and DHX9 sequences clustered separately from each other, and the fish sequences grouped with other fish over amphibians or mammals. This is congruent with previous studies of DDX3 in grouper and olive flounder, both of which had similar topology in that fish clustered in a separate clade then other species; olive flounder express a DDX3a and DDX3b and rainbow trout clustered with the DDX3b variant [28,29]. The domains and motifs identified in both rainbow trout DDX3 and DHX9 were conserved across known animal species sequences; indeed, rainbow trout DDX3 and DHX9 contained all the same motifs and domains as the other animal species analyzed. The domains identified with DDX3 and DHX9 are mainly involved with the proteins' role as helicases; DHX9 has two dsRNA binding motifs that are common to all species and have been described in channel catfish as well; these dsRNA binding motifs in mammals are necessary for dsRNA binding [7,26]. It was therefore unsurprising that DHX9 was able to bind to dsRNA, as demonstrated using a pull-down technique. DDX3 does not have a dsRNA binding motif per se; however, its ability to bind the dsRNA molecule poly I:C has been reported in mammals and the ability to bind to *in vitro* transcribed dsRNA was seen in this study [49]. Instead of using a dsRNA binding motif, DDX3 may be binding dsRNA via its helicase C domain, as was the case for two different DExD/H helicases that sense dsRNA, DHX33 and DHX15 [50,51]. Whether this is the case for DDX3 remains to be elucidated.

DDX3 localized to the cytoplasm of RTG-2 cells, which is consistent with mammalian DDX3 as well as grouper DDX3 [29,52]. This is congruent with DDX3's role as a cytoplasmic sensor for dsRNA, capable of interacting with cytoplasmic signaling proteins such as IPS-1 (Oshiumi et al., 2010A; Oshiumi et al., 2010B). It should be noted that DDX3 does not always localize to the cytoplasm, early studies in HeLa cells found DDX3 located predominantly in the nucleus, and treatment with leptomycin B, an inhibitor of CRM1-mediated nuclear transport, showed accumulation of previously cytoplasmic DDX3 in the nucleus; as such DDX3 is considered a nucleo-cytoplasmic protein [11,14,53,54].

It is interesting that overexpressed DDX3 formed punctate staining while endogenous DDX3 did not. This finding is similar to mammalian GFP-tagged DDX3, where the overexpressed DDX3-GFP localized to stress granules in mammalian cells [55]. It should be noted that the markers of stress granules in mammals, poly (A) binding protein (PABP1 [56]; and T cell intracellular antigen-1(TIA-1; [57], did not colocalize with DDX3-GFP containing granules in the present overexpression study in RTG-2 cells, as determined by immunocytochemistry (data not shown). Therefore, the identity of the punctate structures containing overexpressed DDX3-GFP in rainbow trout remains unknown. GFP-tagged DDX3 in grouper also demonstrated cytoplasmic localization, however there were fewer punctate structures and more diffuse staining than what was observed in rainbow trout cell lines although the same vector was used in both studies (peGFP-C1; [29].

peGFP-C1-DHX9 in RTG-2 cells localized almost exclusively to the nucleus in a diffuse pattern with nucleolus exclusion. There were some cells that demonstrated faint expression in the cytoplasm. In mammalian cells DHX9 expression is generally nuclear, one exception is plasmacytoid dendritic cells where DHX9 was found in the cytosol [5,58]. This is congruent with DHX9's ability to act as a transcriptional regulator [2]. peGFP-C1-DHX9 expression in RTG-2 was similar to HEK293T cells and HeLa cells where DHX9 localized to the nucleus and was excluded from the nucleolus [58–60]. This is in opposition to DHX9 expressed in the human bone osteosarcoma cell line U2OS where GFP-DHX9 localized to the nucleolus [61]. This difference could possibly be due to the different cell types used for expression studies. While DHX9 is largely a nuclear protein, it has been shown in mammals that DHX9 is able to bind to cytoplasmic IPS-1, along with RIG-I and MDA5 (cytoplasmic proteins) in myeloid dendritic cells, thus at least in some cell types or at low levels DHX9 must be cytoplasmic [7].

Expression of both DDX3 and DHX9 was constitutive across all tissues and cell lines tested, which was also observed in olive flounder and grouper tissue panels [28,29]. This ubiquitous expression profile would be one indication DDX3 in rainbow trout is more similar to DDX3X in mammals, as DDX3X has a broad distribution [13]. This ubiquitous expression profile is to be expected as both helicases play roles in maintaining cell homeostasis [3,4]. Interestingly, in the olive flounder differences were seen between sexes of the fish, with higher levels of both DDX3 variants being present in the ovary compared to the testis, and differences in both levels of expression and relative variant expression seen in some tissues [28]. The rainbow trout used in the present study had immature gonads and were not separated by gender, potentially a contributing factor to the variation seen between individuals [28]. DDX3 transcript expression patterns were similar between rainbow trout and grouper, where liver demonstrated the highest levels of DDX3 transcript expression, brain and heart were moderate and gut demonstrated the lowest levels of expression [29]. Liver and brain also express high levels of DDX3 in olive flounder [28].

DDX3 and DHX9 exhibited increased expression at the transcript level following dsRNA treatment. MDA5, a DExH/D-box helicase and dsRNA sensor, which had been previously shown to be upregulated following dsRNA treatment was used as a positive control [48]. This is consistent with grouper DDX3, which was upregulated 5-fold 24 h following poly I:C transfection [29]. This suggests that DDX3 in fish might be inducible by type I IFN, which is contrary to mammals, where dsRNA (poly I:C) does not appear to upregulate DDX3 protein [62]; Oshiumi et al., 2010A). The mammalian DHX9 promoter sequence contains an interferon-sensitive response element suggesting it is an ISG [25]. Mouse and human DDX60, another DExD/H-box helicase family member, were both upregulated in response to recombinant IFN [63]. It should be noted that dsRNA treatment is not synonymous with IFN treatment and that dsRNA can induce genes independently of IFNs [64,65]. In addition, it cannot be concluded that the observed upregulation was due to the interferon response alone. The DeXD/H-box helicase family plays a role in all aspects of RNA metabolism, and dsRNA treatment could affect other aspects of RNA metabolism that could indirectly cause upregulation of these proteins.

Functional antiviral experiments were unsuccessful in this study; neither protein provided protection against VHSV-IVb when each protein was ectopically expressed in RTG-2 cells (data not shown). It is not clear whether this was due to a lack of antiviral function of DDX3 or because the overexpressed protein did not appear to behave as the native protein, as was seen in the punctate structure (Fig. 3A). The authors hypothesize that DHX9 did not have a high enough transfection efficiency to protect a cell monolayer; the large size of the plasmid resulted in a minimal transfection efficiency. Further studies are necessary to understand DDX3 and DHX9's induction mechanisms.

The present study has demonstrated that rainbow trout express both DDX3 and DHX9. These DExD/H-box helicase family members are structurally similar to their respective proteins in other animal species. In agreement with their roles in cell homeostasis, their expression patterns are ubiquitous and constitutive; however, they are inducible following innate immune stimulation with dsRNA. Finally, this study suggests a role for these proteins as sensors of dsRNA. The present study represents the initial groundwork needed to understand these important yet lesser studied innate immune proteins in fish.

Acknowledgements

The authors wish to thank Dr. Brian Dixon for providing the rainbow trout, Dr. Dawn Bowdish for graciously providing the plasmids used in this study, Jondavid de Jong for his scientific support, Danyel Evseev for the technical information and Dr. Aaron Frenette for his expertise in fish dissections. An NSERC Discovery grant and Early Research Award awarded to SDO funded this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.054.

References

[1] A. Fullam, M. Schröder, DExD/H-box RNA helicases as mediators of anti-viral innate immunity and essential host factors for viral replication, Biochim. Biophys. Acta Gene Regul. Mech. 1829 (8) (2013) 854–865.
[2] F.V. Fuller-Pace, DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation, Nucleic Acids Res. 34 (15) (2006) 4206–4215.
[3] P. Linder, E. Jankowsky, From unwinding to clamping—the DEAD box RNA helicase family, Nat. Rev. Mol. Cell Biol. 12 (8) (2011) 505–516.
[4] W.Y. Tarn, T.H. Chang, The current understanding of Ded1p/DDX3 homologs from yeast to human, RNA Biol. 6 (1) (2009) 17–20.
[5] T. Kim, S. Pazhoor, M. Bao, Z. Zhang, S. Hanabuchi, V. Facchinetti, L. Bover, J. Plumas, L. Chaperot, J. Qin, Y.J. Liu, Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells, Proc. Natl. Acad. Sci. 107 (34) (2010) 15181–15186.
[6] M. Miyashita, H. Oshiumi, M. Matsumoto, T. Seya, DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling, Mol. Cell. Biol. 31 (18) (2011) 3802–3819.
[7] Z. Zhang, B. Yuan, N. Lu, V. Facchinetti, Y.J. Liu, DHX9 pairs with IPS-1 to sense double-stranded RNA in myeloid dendritic cells, J. Immunol. 187 (9) (2011) 4501–4508.
[8] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (6) (2010) 805–820.
[9] M. Yoneyama, M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.M. Loo, M. Gale, S. Akira, S. Yonehara, Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity, J. Immunol. 175 (5) (2005) 2851–2858.
[10] N. Sugimoto, H. Mitoma, T. Kim, S. Hanabuchi, Y.J. Liu, Helicase proteins DHX29 and RIG-I cosense cytosolic nucleic acids in the human airway system, Proc. Natl. Acad. Sci. 111 (21) (2014) 7747–7752.
[11] M. Schröder, Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation, Biochem. Pharmacol. 79 (3) (2010) 297–306.
[12] F. Valiente-Echeverría, M.A. Hermoso, R. Soto-Rifo, RNA helicase DDX3: at the crossroad of viral replication and antiviral immunity, Rev. Med. Virol. 25 (5) (2015) 286–299.
[13] H.J. Ditton, J. Zimmer, C. Kamp, E. Rajpert-De Meyts, P.H. Vogt, The AZFa gene DBY (DDX3Y) is widely transcribed but the protein is limited to the male germ cells by translation control, Hum. Mol. Genet. 13 (19) (2004) 2333–2341.
[14] M. Schröder, M. Baran, A.G. Bowie, Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKε-mediated IRF activation, EMBO J. 27 (15) (2008) 2147–2157.
[15] H. Oshiumi, K. Sakai, M. Matsumoto, T. Seya, DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-β-inducing potential, Eur. J. Immunol. 40 (4) (2010) 940–948.
[16] D. Soulat, T. Bürckstümmer, S. Westermayer, A. Goncalves, A. Bauch, A. Stefanovic, O. Hantschel, K.L. Bennett, T. Decker, G. Superti-Furga, The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response, EMBO J. 27 (15) (2008) 2135–2146.
[17] S.N.T. Raman, G. Liu, H.M. Pyo, Y.C. Cui, F. Xu, L.E. Ayalew, S.K. Tikoo, Y. Zhou, DDX3 interacts with influenza A Virus NS1 and NP proteins and exerts antiviral function through regulation of stress granule formation, J. Virol. 90 (7) (2016) 3661–3675.
[18] Y. Ariumi, M. Kuroki, K.I. Abe, H. Dansako, M. Ikeda, T. Wakita, N. Kato, DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication, J. Virol. 81 (24) (2007) 13922–13926.
[19] H.S. Chahar, S. Chen, N. Manjunath, P-body components LSM1, GW182, DDX3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication, Virology 436 (1) (2013) 1–7.
[20] M. Radi, F. Falchi, A. Garbelli, A. Samuele, V. Bernardo, S. Paolucci, F. Baldanti, S. Schenone, F. Manetti, G. Maga, M. Botta, Discovery of the first small molecule inhibitor of human DDX3 specifically designed to target the RNA binding site: towards the next generation HIV-1 inhibitors, Bioorg. Med. Chem. Lett 22 (5) (2012) 2094–2098.
[21] A. Jain, A. Bacolla, P. Chakraborty, F. Grosse, K.M. Vasquez, Human DHX9 helicase unwinds triple-helical DNA structures, Biochemistry 49 (2010) 6992–6999.
[22] T. Lee, J. Pelletier, The biology of DHX9 and its potential as a therapeutic target, Oncotarget 7 (27) (2016) 42716–42739.
[23] S. Zhang, F. Grosse, Nuclear DNA helicase II unwinds both DNA and RNA, Biochemistry 33 (1994) 3906–3912.
[24] C.J. Desmet, K.J. Ishii, Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination, Nat. Rev. Immunol. 12 (7) (2012) 479–491.
[25] A.J. Sadler, O. Latchoumanin, D. Hawkes, J. Mak, B.R. Williams, An antiviral response directed by PKR phosphorylation of the RNA helicase A, PLoS Pathog. 5 (2) (2009) e1000311.
[26] C. Tian, S. Tan, L. Bao, Q. Zeng, S. Liu, Y. Yang, X. Zhong, Z. Liu, DExD/H- box RNA helicase genes are differentially expressed between males and females during the critical period of male sex differentiation in channel catfish, Comp. Biochem. Physiol. Genom. Proteonom. 22 (2017) 109–119.
[27] H.C. Silva de Assis, D.B. Simmons, J.M. Zamora, W.E. Lado, A.M. Al-Ansari, J.P. Sherry, J.M. Blais, C.D. Metcalfe, V.L. Trudeau, Estrogen-like effects in male goldfish co-exposed to fluoxetine and 17 alpha-ethinylestradiol, Environ. Sci. Technol. 47 (10) (2013) 5372–5382.
[28] Z. Wang, W. Liu, H. Song, H. Wang, J. Liu, H. Zhao, X. Du, Q. Zhang, Comparative evolution of duplicated Ddx3 genes in teleosts: insights from Japanese flounder, *Paralichthys olivaceus*, G3: Genes| Genomes| Genetics 5 (8) (2015) 1765–1773.
[29] J. Liu, X. Huang, Y. Yu, J. Zhang, S. Ni, Y. Hu, Y. Huang, Q. Qin, Fish DDX3X exerts antiviral function against grouper nervous necrosis virus infection, Fish Shellfish Immunol. 71 (2017) 95–104.
[30] A. Pradhan, H. Khalaf, S.A. Ochsner, R. Sreenivasan, J. Koskinen, M. Karlsson, J. Karlsson, N.J. McKenna, L. Orbán, P.E. Olsson, Activation of NF-κB protein prevents the transition from juvenile ovary to testis and promotes ovarian development in zebrafish, J. Biol. Chem. 287 (45) (2012) 37926–37938.
[31] S. Biacchesi, E. Mérour, D. Chevret, A. Lamoureux, J. Bernard, M. Brémont, NV proteins of fish novirhabdovirus recruit cellular PPM1Bb protein phosphatase and antagonize RIG-I-mediated IFN induction, Sci. Rep. 7 (2017) 44025.
[32] N.C. Bols, A. Barlian, M. Chirino-Trejo, S.J. Caldwell, P. Goegan, L.E.J. Lee, Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills, J. Fish Dis. 17 (6) (1994) 601–611.
[33] N. Fijan, D. Sulimanović, M. Bearzotti, D. Muzinić, L.O. Zwillenberg, S. Chilmonczyk, J.F. Vautherot, P. De Kinkelin, 2, Some Properties of the Epithelioma Papulosum Cyprini (EPC) Cell Line from Carp Cyprinus carpio vol 134, Annales de l'Institut pasteur/Virologie, 1983, pp. 207–220.
[34] A. Kawano, C. Haiduk, K. Schirmer, R. Hanner, L.E.J. Lee, B. Dixon, N.C. Bols, Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide, Aquacult. Nutr. 17 (2) (2011) e241–e252.
[35] K. Wolf, M.C. Quimby, Established eurythermic line of fish cells in vitro, Science 135 (3508) (1962) 1065–1066.
[36] N.A. Shevchuk, A.V. Bryksin, Y.A. Nusinovich, F.C. Cabello, M. Sutherland, S. Ladisch, Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously, Nucleic Acids Res. 32 (2) (2004) e19-e19.
[37] C. Berthelot, F. Brunet, D. Chalopin, A. Juanchich, M. Bernard, B. Noël, P. Bento, C. Da Silva, K. Labadie, A. Alberti, J.M. Aury, The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates, Nat. Commun. 5 (2014) 3657.
[38] P. Artimo, M. Jonnalagedda, K. Arnold, D. Baratin, G. Csardi, E. De Castro, S. Duvaud, V. Flegel, A. Fortier, E. Gasteiger, A. Grosdidier, ExPASy: SIB bioinformatics resource portal, Nucleic Acids Res. 40 (W1) (2012) W597–W603.
[39] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[40] M. Nei, S. Kumar, Molecular Evolution and Phylogenetics, Oxford University Press, New York, 2000.
[41] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[42] J. Felsenstein, Confidence limits on phylogenies: an approach using the boostrap, Evolution 39 (1985) 783–791.
[43] W. Liu, Y. Xie, J. Ma, X. Luo, P. Nie, Z. Zuo, U. Lahrmann, Q. Zhao, Y. Zheng, Y. Zhao, Y. Xue, IBS: an illustrator for the presentation and visualization of biological sequences, Bioinformatics 31 (20) (2015) 3359–3361.
[44] A. Marchler-Bauer, M.K. Derbyshire, N.R. Gonzales, S. Lu, F. Chitsaz, L.Y. Geer, R.C. Geer, J. He, M. Gwadz, D.I. Hurwitz, C.J. Lanczycki, CDD: NCBI's conserved domain database, Nucleic Acids Res. 43 (D1) (2014) D222–D226.
[45] S.J. Poynter, S.J. DeWitte-Orr, Length-dependent innate antiviral effects of double-stranded RNA in the rainbow trout (*Oncorhynchus mykiss*) cell line, RTG-2, Fish Shellfish Immunol. 46 (2) (2015) 557–565.
[46] V. Sirri, S. Urcuqui-Inchima, P. Roussel, D. Hernandez-Verdun, Nucleolus: the fascinating nuclear body, Histochem. Cell Biol. 129 (1) (2008) 13–31.
[47] S.J. Poynter, J. Weleff, A.B. Soares, S.J. DeWitte-Orr, Class-A scavenger receptor function and expression in the rainbow trout (Oncorhynchus mykiss) epithelial cell lines RTgutGC and RTgill-W1, Fish Shellfish Immunol. 44 (1) (2015) 138–146.

[48] M. Chang, B. Collet, P. Nie, K. Lester, S. Campbell, C.J. Secombes, J. Zou, Expression and functional characterization of the RIG-I-like receptors MDA5 and LGP2 in Rainbow trout (*Oncorhynchus mykiss*), J. Virol. 85 (16) (2011) 8403–8412.
[49] H. Oshiumi, M. Ikeda, M. Matsumoto, A. Watanabe, O. Takeuchi, S. Akira, N. Kato, K. Shimotohno, T. Seya, Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1-mediated IFN–beta induction, PLoS One 5 (12) (2010) e14258.
[50] Y. Liu, N. Lu, B. Yuan, L. Weng, F. Wang, Y.J. Liu, Z. Zhang, The interaction between the helicase DHX3 and IPS-1 as a novel pathway to sense double-stranded RNA and RNA viruses in myeloid dendritic cells, Cell. Mol. Immunol. 11 (1) (2014) 49–57.
[51] H. Lu, N. Lu, L. Weng, B. Yuan, Y.J. Liu, Z. Zhang, DHX15 senses double- stranded RNA in myeloid dendritic cells, J. Immunol. 193 (3) (2014) 1364–1372.
[52] C.S. Lee, A.P. Dias, M. Jedrychowski, A.H. Patel, J.L. Hsu, R. Reed, Human DDX3 functions in translation and interacts with the translation initiation factor eIF3, Nucleic Acids Res. 36 (14) (2008) 4708–4718.
[53] A.M. Owsianka, A.H. Patel, Hepatitis C virus core protein interacts with a human DEAD box protein DDX3, Virology 257 (2) (1999) 330–340.
[54] A. Ranji, K. Boris-Lawrie, RNA helicases: emerging roles in viral replication and the host innate response, RNA Biol. 7 (6) (2010) 775–787.
[55] M.C. Lai, Y.H.W. Lee, W.Y. Tarn, The DEAD-Box RNA helicase DDX3 associates with export messenger ribonucleoproteins as well as Tip-associated protein and participates in translational control, Mol. Biol. Cell 19 (9) (2008) 3847–3858.
[56] J.R. Buchan, R. Parker, Eukaryotic stress granules: the ins and outs of translation, Mol. Cell 36 (6) (2009) 932–941.
[57] N. Gilks, N. Kedersha, M. Ayodele, L. Shen, G. Stoecklin, L.M. Dember, P. Anderson, Stress granle assembly is mediated by prion-like aggregation of TIA-1, Mol. Biol. Cell 15 (12) (2004) 5383–5398.
[58] H. Fujita, T. Ohshima, T. Oishi, S. Aratani, R. Fujii, A. Fukamizu, T. Nakajima, Relevance of nuclear localization and functions of RNA helicase A, Int. J. Mol. Med. 15 (2005) 555–560.
[59] J.S. Capitanio, B. Montpetit, R.W. Wozniak, Human Nup98 regulates the localization and activity of DExH/D-box helicase DHX9, eLife 6 (2017) e18825.
[60] B. Fuchsova, P. Hozak, The localization of nuclear DNA helicase II in different nuclear compartments is linked to transcription, Exp. Cell Res. 279 (2) (2002) 260–270.
[61] M. Huang, B.S. Mitchell, Guanine nucleotide depletion mediates translocation of nucleolar proteins, including RNA helicase A (DHX-9), Nucleosides Nucleotides Nucleic Acids 27 (6–7) (2008) 704–711.
[62] K.M. Sparrer, M.U. Gack, Intracellular detection of viral nucleic acids, Curr. Opin. Microbiol. 26 (2015) 1–9.
[63] D. Goubau, A.G. der Veen, P. Chakravarty, R. Lin, N. Rogers, J. Rehwinkel, S. Deddouche, I. Rosewell, J. Hiscott, C. Reis e Sousa, Mouse superkiller-2-like helicase DDX60 is dispensable for type I IFN induction and immunity to multiple viruses, Eur. J. Immunol. 45 (12) (2015) 3386–3403.
[64] C. Daly, N.C. Reich, Double-stranded RNA activates novel factors that bind to the interferon-stimulated response element, Mol. Cell. Biol. 13 (6) (1993) 3756–3764.
[65] N. Reich, R. Pine, D.A. Levy, J.E. Darnell, Transcription of interferon-stimulated genes is induced by adenovirus particles but is suppressed by E1A gene products, J. Virol. 62 (1) (1988) 114–119.
[66] S.J. Poynter, S.J. DeWitte-Orr, Visualizing virus-derived dsRNA using antibody-independent and-dependent methods, Innate Antiviral Immunity, Humana Press, New York, NY, 2017, pp. 103–118.