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Full Length Article

Identification and comparative study of melanoma differentiation-associated gene 5 homologues of triploid hybrid fish and its parents

Jun Xiao [1], Huijuan Zhong [1], Jun Yan, Zhenghao Li, Shaojun Liu, Hao Feng [*]

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China

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ABSTRACT

Sterile triploid fish (3n = 150), derived from the hybridization between red crucian carp (*Carassius auratus* red var., ♀, 2n = 100) and allotetraploid (♂, 4n = 200), exhibits the improved disease resistance compared with its parents, but the current knowledge of the immunity of triploid fish is limited. Here, we report the identification and characterization of melanoma differentiation-associated gene 5 (MDA5) homologues from red crucian carp, triploid fish and allotetraploid. In this study, one red crucian carp MDA5 transcript (2nMDA5), two triploid fish MDA5 transcripts (3nMDA5-a and 3nMDA5-b) and two allotetraploid fish MDA5 transcripts (4nMDA5-a and 4nMDA5-b) have been cloned and identified separately. Immunofluorescence staining assay displayed that these MDA5 proteins were cytoplasmic proteins. RT-qPCR assay showed that, in response to spring viremia of carp virus (SVCV) and poly (I:C) stimuli, the increase of 3nMDA5 mRNA level was obviously higher than those of 2nMDA5 and 4nMDA5. Interestingly, the reporter assay and plaque assay revealed collectively that 3nMDA5-b, a shorter splicing form of MDA5, exhibited the strongest IFN promoter-inducing ability and antiviral activity. Additionally, when co-expressed with 3nMAVS, 3nMDA5-b induced a considerably higher level of IFN promoter activation than 3nMDA5-a; and the interactions between 3nMAVS/3nMDA5-a and 3nMAVS/3nMDA5-b were verified by co-IP assay. Taken together, our findings support the conclusion that in triploid fish, 3nMDA5-b mediates a robust antiviral signaling in host innate immune response.

1. Introduction

Hybridization and polyploidization are two significant ways to promote the species evolution and the creation of new species (Comai, 2005; Wang et al., 2019). Interspecific hybridization has occurred in approximately a quarter of plants and about 10% of animals (Abbott et al., 2013). Fish are among the most diverse vertebrate species on the planet, in which interspecific hybridization is also widespread (Alves et al., 2001). The fertile allotetraploid (AT; 4n = 200) were previously achieved by crossing female red crucian carp (*Carassius auratus* red var.) with male common carp (*Cyprinus carpio* L.) (Liu et al., 2001; Liu, 2010). Furthermore, through hybridization between allotetraploid (♂) and red crucian carp (♀), the sterile triploid progenies (3n = 150) were developed in large scale (Chen et al., 2009). Triploid fish outperforms its parents in terms of growth rate and disease resistance. However, the mechanism of its improved disease resistance remains poorly understood.

Disease resistance is intimately related to host immunity (Polinski et al., 2021). In comparison to higher vertebrates, the adaptive immune system of fish is rather underdeveloped; nevertheless, fish have developed sophisticated innate immune system that plays a predominant role in the defense against the invasion of pathogenic microbes (Lazarte et al., 2019). To recognize the different kinds of non-self components, fish have evolved a set of pattern recognition receptors (PPRs) like its mammalian counterparts, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors and cytosolic DNA sensors (Akira et al., 2006; Chen et al., 2017). Among them, RLRs are the key intracellular PRRs detecting viral RNA, which consist of three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Yoneyama et al., 2005). Upon binding to the ligands, RIG-I and MDA5 can interact with their downstream

* Corresponding author.
E-mail address: fenghao@hunnu.edu.cn (H. Feng).
[1] These authors contribute equally to this paper.

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adaptor protein mitochondrial antiviral signaling protein (MAVS) through the CARD domains (Seth et al., 2005). MAVS then recruits TBK1 and IKKε, which facilitate the phosphorylation of IRF3/7, and finally induce the production of type I interferons (IFNs) and other pro-inflammatory cytokines (Zou and Secombes, 2011).

MDA5 and RIG-I share similar domain structure, including two caspase recruitment domains (CARDs) at the N-terminus, a DEAD/DEAH box helicase domain (DEXDc), a helicase C-terminal domain (HELICc) and a C-terminal regulatory domain (RD) (Reikine et al., 2014). Human MDA5 was first reported in 2002 (Kang et al., 2002). Since then, teleost MDA5 has been studied in several species, such as rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), orange spotted grouper (*Epinephelus coioides*), grass carp (*Ctenopharyngodon idella*) and black carp (*Mylopharyngodon piceus*) (Su et al., 2010; Chang et al., 2011a; Zou et al., 2014; Huang et al., 2016; Liu et al., 2017). According to these research, fish MDA5, like its mammalian counterparts, plays critical roles in host antiviral and antibacterial innate immune processes. However, the regulatory and functional mechanism of teleost MDA5 still needs further elucidation.

To get a better understanding of the innate immunity of hybrid fish, we have cloned and identified the MDA5 homologues from red crucian carp, triploid fish and allotetraploid in this study. One transcript of MDA5 was identified in red crucian carp (named 2nMDA5). Two transcripts of MDA5 were found in triploid fish and tetraploid fish, which were named as 3nMDA5-a, 3nMDA5-b, 4nMDA5-a and 4nMDA5-b accordingly. Reporter assay and plaque assay demonstrated that these MDA5 homologues exhibited the different interferon promoter induction ability and antiviral activity. Interestingly, 3nMDA5-b, the shorter variants, mediated the strongest antiviral signaling than others, which implied that it plays a crucial role in the innate immune response of triploid fish.

2. Materials and methods

2.1. Cells and plasmids

HEK293T cells, *Epithelioma Papulosum Cyprinid* (EPC) cells, 2nFC, 3nFC and 4nFC cells were cultured as decribed previously (Xiao et al., 2018). Fish cells were grown at 25 °C in DMEM (containing 10% FBS (Gibco) and appropriate concentration of penicillin and streptomycin); human cells were cultured in DMEM (Gibco) at 37 °C. The transfection experiment employed the polyethylenimine (PEI) (Polysciences) reagent. The following plasmids were stored in the lab: pcDNA5/FRT/TO, pRL-TK, Luci-DrIFNφ1/3 (for zebrafish IFNφ1 or IFNφ3 promoter activity study respectively) and eIFN (for fathead minnow IFN promoter activity analysis).

2.2. RNA extraction, molecular cloning and plasmid construction

Total RNA was extracted using TRIzol Reagent (Invitrogen) from 2nFC, 3nFC or 4nFC, and the RNA was treated with RNase-free DNase I according to the manufacturer's protocol. The first-strand cDNA was synthesized by using the Revert Aid First Strand Synthesis Kit (Thermo). Based on the transcriptome data from 2nFC, 3nFC and 4nFC, the specific primers were designed to amplify the coding sequence (CDS) of MDA5 (Table 1). For eukaryotic expression, the recombinant plasmids including pcDNA5/FRT/TO-Flag-2nMDA5, pcDNA5/FRT/TO-Flag-3nMDA5-a, pcDNA5/FRT/TO-Flag-3nMDA5-b, pcDNA5/FRT/TO-Flag-4nMDA5-a and pcDNA5/FRT/TO-Flag-4nMDA5-b were constructed, respectively.

2.3. Virus produce and titration

SVCV (strain: SVCV 741) was propagated in EPC cells at 25 °C in the presence of 2% FBS. Virus titer was measured independently on EPC cells using plaque assay. In brief, a 10-fold serially diluted viral supernatant was introduced to EPC cells in a 24-well plate and incubated at 25 °C for 2 h. After incubation, the supernatant was replaced with new DMEM containing 2% FBS and 0.75 percent methylcellulose (Sigma). Plaques were counted on the third day post-infection.

2.4. Real-time quantitative PCR (qPCR)

MDA5 mRNA levels in 2nFC, 3nFC and 4nFC cells post SVCV infection or poly (I:C) (Sigma-Aldrich) were measured using reverse transcription and qPCR (RT-qPCR). The primers for detecting MDA5

Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
CDS		
MDA5-F	ATGAGCTGCGATMAGGAC	For 2/3/4nMDA5 CDS cloning
MDA5-R	TTAKTCAGTTTCCATGTCTTCTTCT	
Expression vector		
2n/3n/4nMDA5-F	ACTGACGGTACCATGAGCTGCGATMAGGAC	For expression vector construction
2n/3n/4nMDA5-R	ACTGACCTCGAGTTAKTCAGTTTCCATGTCTTCTTCT	
RT-qPCR		
β-actin-F	TGCTATGTGGCTCTTGACT	in vitro RT-qPCR
β-actin-R	AGGTCCTTACGGATGTCG	
q-MDA5-F	AGCCATGAGCCAGCAGAGAG	
q-MDA5-R	TCTTGATCACGTCTCCAAAC	
q-SVCV-N-F	GGGTCTTTACAGAGTGGG	
q-SVCV-N-R	TTTGTGAGTTGCCGTTAC	
q-SVCV-G-F	GATGACTGGGAGTTAGATGGC	
q-SVCV-G-R	ATGAGGGATAATATCGGCTTG	
q-EPC IFN-F	ATGAAAACTCAAATGTGGACGTA	
q-EPC IFN-R	GATAGTTTCCACCCATTTCCTTAA	
q-EPC viperin-F	GCAAAGCGAGGGTTACGAC	
q-EPC viperin-R	CTGCCATTACTAACGATGCTGAC	
q-EPC PKR-F	ACCTGAAGCCTCCAAACATA	
q-EPC PKR-R	GCATTCGCTCATCATTGTC	
q-EPC Mx1-F	TGGAGGAACCTGCCTTAAATAC	
q-EPC Mx1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
q-EPC ISG15-F	TGATGCAAATGAGACCGTAGAT	
q-EPC ISG15-R	CAGTTGTCTGCCGTTGTAAATC	
q-EPC actin-F	AAGGAGAAGCTCTGCTATGTGGCT	
q-EPC actin-R	AAGGTGGTCTCATGGATACCGCAA	

expression were designed based on the conserved sequences of 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a and 4nMDA5-b; and the β-actin was performed as internal control (Table 1). The RT-qPCR procedure was as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, followed by dissociation curve program. The threshold cycle (CT) value was calculated using 7500 Real-Time PCR System (ABI). The relative expression ratios of target genes (treated vs control) were determined using $2^{-\triangle\triangle CT}$ method.

2.5. Immunoblotting

HEK293T (1.5×10^6 cells/well) in 6-well plate were separately transfected with plasmids expressing 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a or 4nMDA5-b. Cells were harvested at 48 h post-transfection (hpt) and lysed for IB assay, as previously reported (Xiao et al., 2019). In brief, 9% SDS-PAGE was used to isolate WCL, and the transferred membrane was probed with mouse monoclonal anti-Flag Ab (1:3000; Sigma), followed by incubation with goat-anti-mouse IgG (1:30000; Sigma). BCIP/NBT was used to expose target proteins.

2.6. Immunofluorescence microscopy

In a 24-well plate, EPC cells were transfected with plasmids expressing 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a or 4nMDA5-b. Twenty-four hours later, the transfected cells were fixed with 4% (v/v) paraformaldehyde then permeabilized with Triton X-100 (0.2% in PBS). The nuclei were stained with DAPI (Sigma). The subcellular localization of target protein was determined using monoclonal anti-Flag Ab (1:300, Sigma) and Alexa 488-conjugated secondary Ab (1:1000, Invitrogen).

2.7. Dual-luciferase reporter assay

In a 24-well plate, EPC cells were transfected with plasmids expressing MDA5 or the empty vector at indicated concentration, as well as 25 ng pRL-TK, 200 ng Luci-DrIFNφ1 or Luci-DrIFNφ3 plasmids. The cells were harvested at 24 hpt and utilized to assay the activity of firefly luciferase and renilla luciferase according to procedure.

2.8. Co-immunoprecipitation (Co-IP)

To detect the interaction between 3nMDA5-a (3nMDA5-b) and 3nMAVS, HEK293T cells were transfected with plasmids expressing HA-tagged 3nMAVS together with Flag-tagged 3nMDA5-a or 3nMDA5-b. At 48 hpt, the cells were collected, treated with lysis buffer (containing protease inhibitor) and ultrasonicated. Samples were incubated with anti-Flag agarose affinity gel. After washed with IP buffer. The whole cell lysate and the immunoprecipitated proteins were then analyzed by Western blot analysis using anti-Flag or anti-HA Abs.

2.9. Statistics analysis

The data of RT-qPCR, luciferase reporter assay and viral titration were generated from three independent experiments. Significant differences analysis was carried out by using two-tailed student's t-test. Asterisks represent significant differences: $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Molecular cloning and sequence analysis of MDA5 homologues

Using the first strand cDNA of 2nFC (3nFC or 4nFC) as templates, we have amplified one MDA5 transcript from red crucian carp, two transcripts from triploid hybrid and two transcripts from allotetraploid, which named as 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a and 4nMDA5-b, respectively (GenBank accession number: MW266114, MW266115, MW266116, MW266117 and MZ568801). These MDA5 transcripts were different in ORF length and amino acid sequence (Supplementary Fig. 1). It was found that the 2nMDA5, 3nMDA5-a, 4nMDA5-a and 4nMDA5-b, like their mammalian counterparts, contained conserved domains, including two adjacent N-terminal CARD domains, a DEXDc domain, a HELICc domain and a C-terminal RD domain. However, 3nMDA5-b lacks partial CDS sequence (71 bp), which leads to the premature termination of its translation. As a result, a small part of HELICc domain and the entire RD domain are absent in the 3nMDA5-b protein (Fig. 1A and B). Besides, the spatial structure of 3nMDA5-b protein evidently lacks its C-terminal region when compared with the structure of 3nMDA5-a (Fig. 1C). To learn more about MDA5 evolution, we compared the amino acid sequences of MDA5 homologues of different species (Table 2) and conducted a phylogenetic analysis (Fig. 1D). The findings revealed that the chosen MDA5 proteins were divided into two groups: fish and mammals. In the fish group, 3nMDA5-b and 4nMDA5-a were clustered with common carp (*Cyprinus carpio*) MDA5, while 2nMDA5, 3nMDA5-a and 4nMDA5-b were clustered together.

3.2. MDA5 expression in host cells in response to different stimuli

To study the profile of MDA5 transcription during the innate immune response, 2nFC, 3nFC and 4nFC were infected with SVCV (0.01 MOI) or treated with poly (I:C) (25 μg/ml), and MDA5 mRNA levels were determined by RT-qPCR. After SVCV infection, the mRNA level of 2nMDA5 increased significantly at 24 h post-infection (hpi) and reached the peak at 48 hpi (32-fold) (Fig. 2A). The 3nMDA5 transcription significantly increased from 12 hpi and peaked at 48 hpi (52.5-fold) (Fig. 2B). However, the transcription level of 4nMDA5 did not increase obviously during the first 24 h, it reached to 10.4-fold that of the control until 48 hpi (Fig. 2C). Similar phenotypes were observed in poly (I:C)-treated cells. MDA5 mRNA levels in 2nFC, 3nFC and 4nFC increased and peaked at 48 h, while the increase of 3nMDA5 transcription remained significantly higher than that of 2nMDA5 and 4nMDA5 within 48 hpi (Fig. 2D∼F).

3.3. Protein expression and intracellular distribution of MDA5 homologues

To investigate the protein expression of MDA5, HEK293T cells were transfected with Flag-tagged 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a, 4nMDA5-b or the empty vector, then the expression of these proteins was examined by Western blot analysis. 3nMDA5-b, lacking the C-terminal region, migrated ∼95 kDa in the western blotting, whereas the other four full-length MDA5 proteins showed the similar molecular weight of ∼120 kDa, and also showed higher expression levels than that of 3nMDA5-b (Fig. 3A).

To further determine the intracellular distribution of MDA5 of red crucian carp, triploid hybrid and allotetraploid, EPC cells were transfected with the plasmids mentioned above, and used for IF staining assay. In the result of confocal laser scanning microscopy, the expressing region of all the MDA5 proteins (green) were distributed in the cytoplasm of EPC cells, indicating that MDA5 was a majorly cytosolic protein, which was similar to their mammalian counterparts (Fig. 3B).

3.4. IFN-inducing activities and antiviral activities of MDA5

In order to investigate whether the MDA5 of red crucian carp, triploid hybrid and allotetraploid were involved in the induction of IFN, EPC cells were transfected with MDA5-expressing plasmids at different doses (50, 100 and 200 ng) together with IFN promoter plasmids, and used for dual luciferase reporter assay. The results showed that the transcription of DrIFNφ1, DrIFNφ3 and eIFN promoter could be activated by overexpression of all the five MDA5 homologues. However, it is interesting that 3nMDA5-b exhibited the strongest induction activity among them

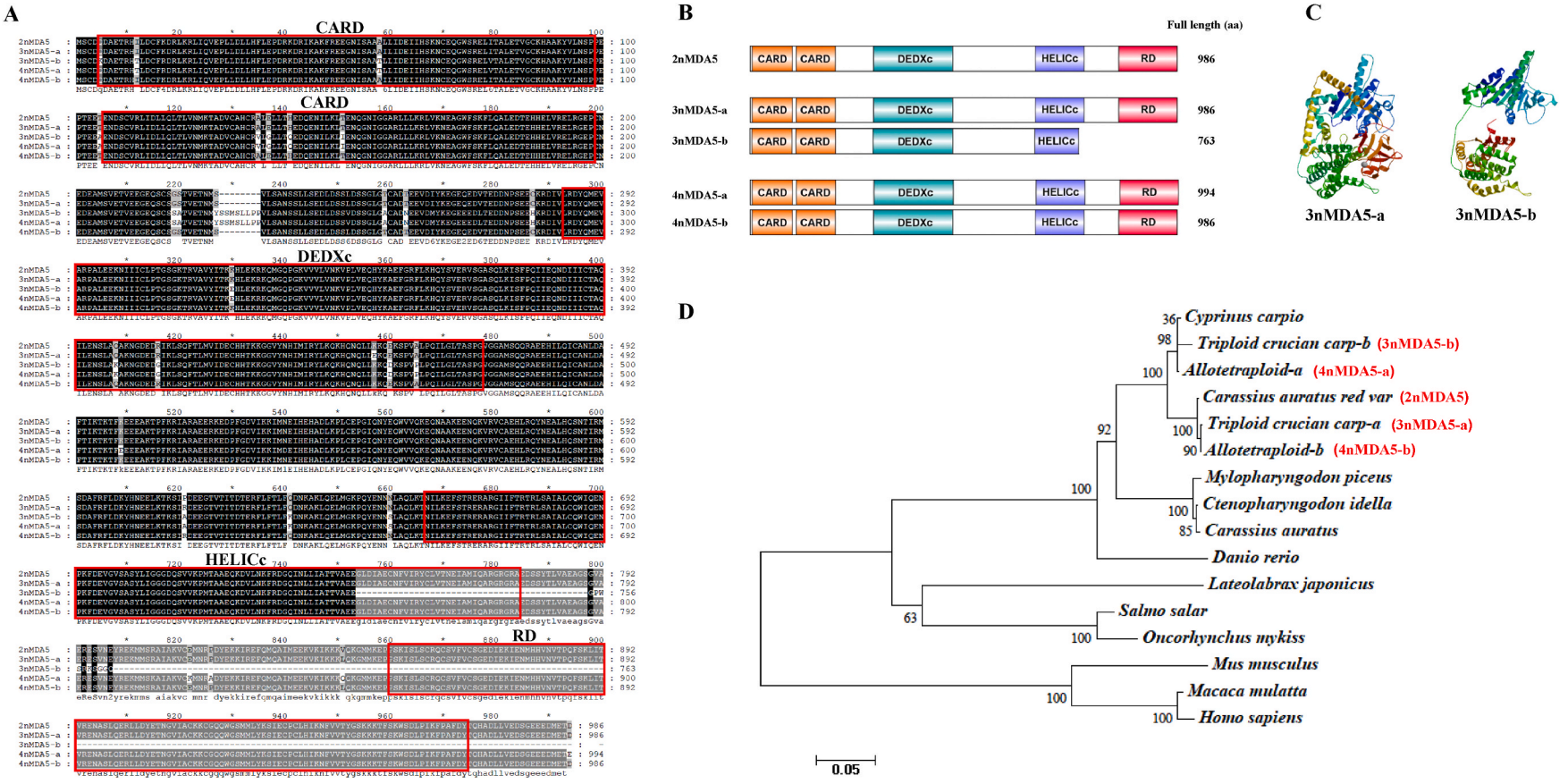

Fig. 1. Amino acid sequence analysis of MDA5 of red crucian carp, triploid fish and allotetraploid. (A) Amino acid sequence alignment of 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a and 4nMDA5-b. (B) Schematic diagram of 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a and 4nMDA5-b proteins. The number on the right side of the diagram represents the size of each MDA5 protein (in amino acids) (C) Prediction of 3D protein structures of 3nMDA5-a and 3nMDA5-b by using I-TASSER online server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). (D) Phylogenetic tree analysis of MDA5 homologues from different vertebrates, including red crucian carp (MW266114), triploid fish (MW266115 and MW266116), allotetraploid (MW266117 and MZ568801), *Cyprinus carpio* (KM374815), *Mylopharyngodon piceus* (KX871189), *Ctenopharyngodon idella* (FJ542045), *Carassius auratus* (JF970226), *Danio rerio* (NM_001308563), *Lateolabrax japonicus* (KU317137), *Salmo salar* (KU376486), *Oncorhynchus mykiss* (FN396357), *Mus musculus* (NM_027835.3), *Macaca mulatta* (DQ875603.1) and *Homo sapiens* (AF095844.1). The phylogenetic tree was conducted by using the neighbor-joining method.

Table 2
Comparison of vertebrate MDA5 homologues (%).

Species	2nMDA5		3nMDA5-a		3nMDA5-b		4nMDA5-a		4nMDA5-b	
	Identity	Similarity	Identity	Similarity	Identity	Similarity	Identity	Similarity	Identity	Similarity
2nMDA5	100	100	99.6	99.9	72.2	73.6	95.8	97.5	99.7	99.9
3nMDA5-a	99.6	99.9	100	100	72.0	73.6	95.6	97.5	99.9	100
3nMDA5-b	72.2	73.6	72.0	73.6	100	100	75.6	76.0	72.1	73.6
4nMDA5-a	95.8	97.5	95.6	97.5	75.6	76.0	100	100	95.7	97.5
4nMDA5-b	99.7	99.9	99.9	100	72.1	73.6	95.7	97.5	100	100
Gold fish MDA5	85.8	91.3	85.5	91.2	64.5	68.8	86.5	91.0	91.4	85.5
Common carp MDA5	94.5	96.7	94.4	96.7	75.5	76.0	98.1	99.0	96.8	94.4
Grass carp MDA5	80.5	86.2	80.2	86.1	66.1	70.3	81.4	86.0	80.3	86.1
Black carp MDA5	85.8	91.2	85.5	91.1	64.6	68.6	86.5	90.9	85.6	91.1
Zebrafish MDA5	82.0	89.1	81.7	89.0	61.9	67.2	83.8	89.3	81.8	89.0
Atlantic salmon MDA5	62.8	75.2	62.5	75.1	46.9	55.2	63.3	74.6	62.6	75.1
Japanese seaperch MDA5	59.5	72.3	59.4	72.3	43.6	53.5	59.1	72.0	59.5	72.3
Rainbow trout MDA5	61.9	74.9	61.7	74.8	45.9	54.8	62.5	74.2	61.8	74.8
House mouse MDA5	47.0	62.4	46.8	62.6	35.6	47.1	47.1	62.2	46.9	62.6
Rhesus Macaque MDA5	48.5	64.0	48.3	64.2	37.1	49.4	49.2	64.3	48.4	64.2
Human MDA5	48.0	63.5	47.9	63.6	36.9	49.0	48.8	63.8	48.0	63.6

The MDA5 protein IDs in the table corresponded to those in Fig. 1.

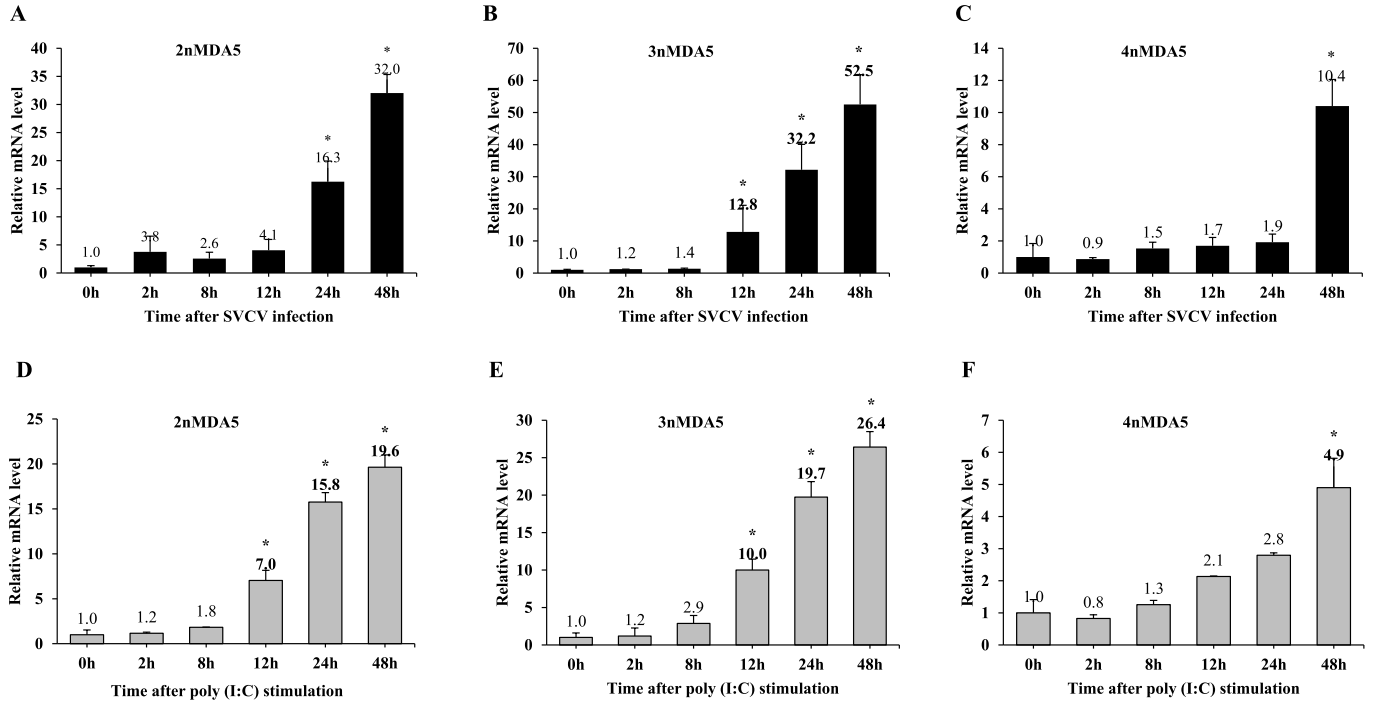

Fig. 2. The mRNA profiles of MDA5 of red crucian carp, triploid fish and allotetraploid in response to different stimuli. 2nFC, 3nFC and 4nFC cells in 12-well plates were infected with SVCV at MOI 0.003 or treated with poly (I:C) (25 μg/ml) and harvested independently at 0, 2, 8, 12, 24 and 48 h post treatment for total RNA extraction and subsequential RT-qPCR. Data represent the means (±SEM) of three independent experiments.

(Fig. 4A–C). In addition, poly (I:C) stimulation could further enhance the induced zebrafish IFNφ1 promoter activation by MDA5s; and under this condition, 3nMDA5-b still exhibited the strongest induction effect (Fig. 4D).

Moreover, to determine and compare the functional roles of the five MDA5 transcripts in the antiviral innate immunity, EPC cells were transfected with the plasmids expressing MDA5 indicated above, followed by the SVCV infection at various MOIs (0.001, 0.01 and 0.1). The results of crystal violet staining and plaque assay showed that, overexpression of MDA5 in EPC cells enhanced their antiviral activities against SVCV, compared with the control cells. Among them, 3nMDA5-b-expressing cells showed the biggest reduction of cytopathic effects (CPEs) and the lowest viral titer (Fig. 5A&B). Furthermore, the RT-qPCR assays revealed that, 3nMDA5-b significantly inhibited the transcription level of viral genes (SVCV-N and SVCV-G) compared with 3nMDA5-a (Fig. 5C). Meanwhile, the mRNA levels of antiviral cytokines, including IFN, viperin, PKR, Mx1 and ISG15, in 3nMDA5-b-expressing cells were also obviously higher than those in 3nMDA5-a-expressing cells (Fig. 5D). These results collectively suggested that overexpression of MDA5 of red crucian carp, triploid hybrid and allotetraploid offered the protection for the EPC cells against SVCV infection, and 3nMDA5-b mediated the strongest antiviral activity.

3.5. *The relationship between triploid fish MDA5 and MAVS*

MAVS is the adaptor protein of MDA5 and plays an indispensable role in downstream signal transduction. To elucidate the relationship between 3nMDA5-a/3nMDA5-b and 3nMAVS, the co-IP assay between

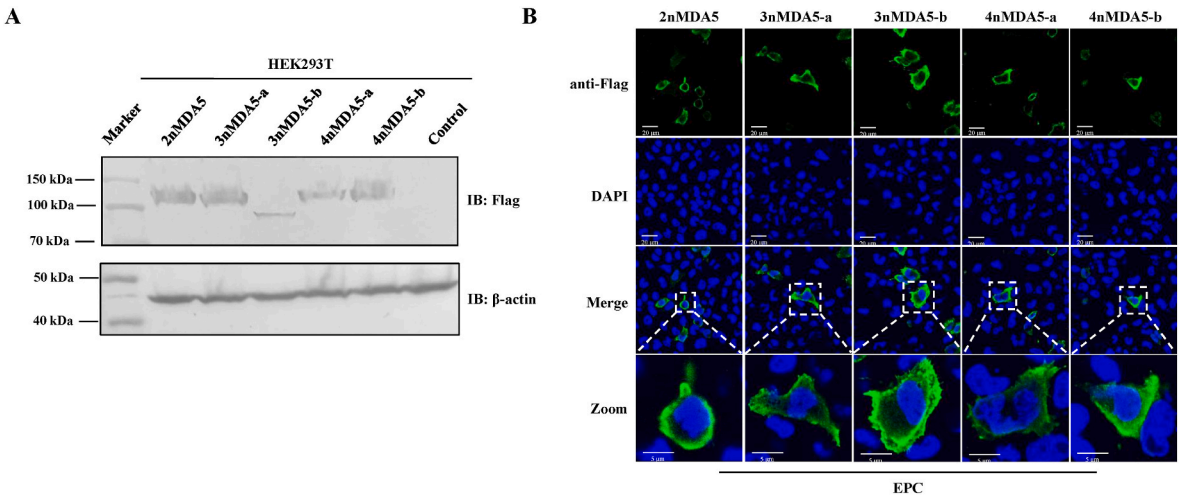

Fig. 3. Protein expression and subcellular distribution of MDA5. (A) HEK293T cells were transfected with plasmids expressing Flag-2nMDA5, Flag-3nMDA5-a, Flag-3nMDA5-b, Flag-4nMDA5-a, Flag-4nMDA5-b or empty vector. Cells were harvested at 48 hpt and used for immunoblotting. Control: cells transfected with the empty vector. (B) EPC cells in 24-well plate were transfected with plasmids expressing MDA5 as indicated above, and used for IF staining. The scale represents 5 μm and 20 μm, respectively.

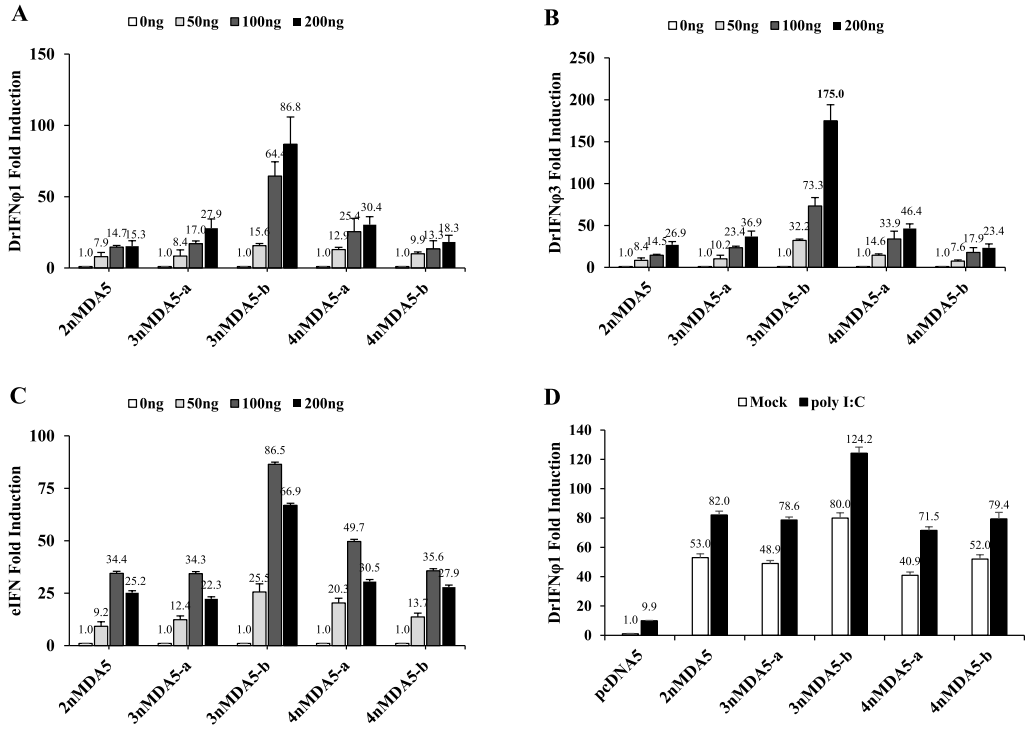

Fig. 4. IFN-inducing activities of MDA5 from red crucian carp, triploid fish and allotetraploid. (A~C) EPC cells in 24-well plates were transfected with plasmids expressing 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a, 4nMDA5-b or the empty vector at the indicated amounts (50, 100 and 200 ng), together with 25 ng pRL-TK and 200 ng Luci-DrIFNφ1 (A) or 200 ng Luci-DrIFNφ3 (B) or 200 ng eIFN (C). The cells were harvested at 24 hpt and used for luciferase reporter assays. (D) EPC cells in 24-well plates were transfected with 50 ng plasmids expressing MDA5 indicated above, together with 25 ng pRL-TK and 200 ng Luci-DrIFNφ1. At 24 hpt, cells were transfected again with poly (I:C) (1 μg/ml) and the cells were collected for luciferase reporter assays at 24 h post poly (I:C) stimulation.

3nMAVS and 3nMDA5-a (or 3nMDA5-b) was recruited, in which the HEK293T cells were co-transfected with plasmids expressing HA-3nMAVS and Flag-3nMDA5-a (or Flag-3nMDA5-b). HA-3nMAVS was detected in the proteins immunoprecipitated by Flag-3nMDA5-a/Flag-3nMDA5-b, meaning that 3nMAVS could interact with 3nMDA5-a and 3nMDA5-b (Fig. 6A). To gain further insight into the role of 3nMDA5-a and 3nMDA5-b in MAVS/IFN signaling, 3nMDA5-a and 3nMDA5-b were introduced into EPC cells alone or together with 3nMAVS, and the transfected cells were utilized for luciferase reporter assay. As shown in Fig. 6B&C, the DrIFNφ3 and eIFN promoter induction folds in EPC cells co-expressing 3nMDA5-a and 3nMAVS, or 3nMDA5-b and 3nMAVS were higher than those in EPC cells expressing 3nMDA5-a, 3nMDA5-b or 3nMAVS alone. However, the synergic effect of 3nMDA5-b and 3nMAVS on IFN production was obviously stronger than that of 3nMDA5-a and 3nMAVS.

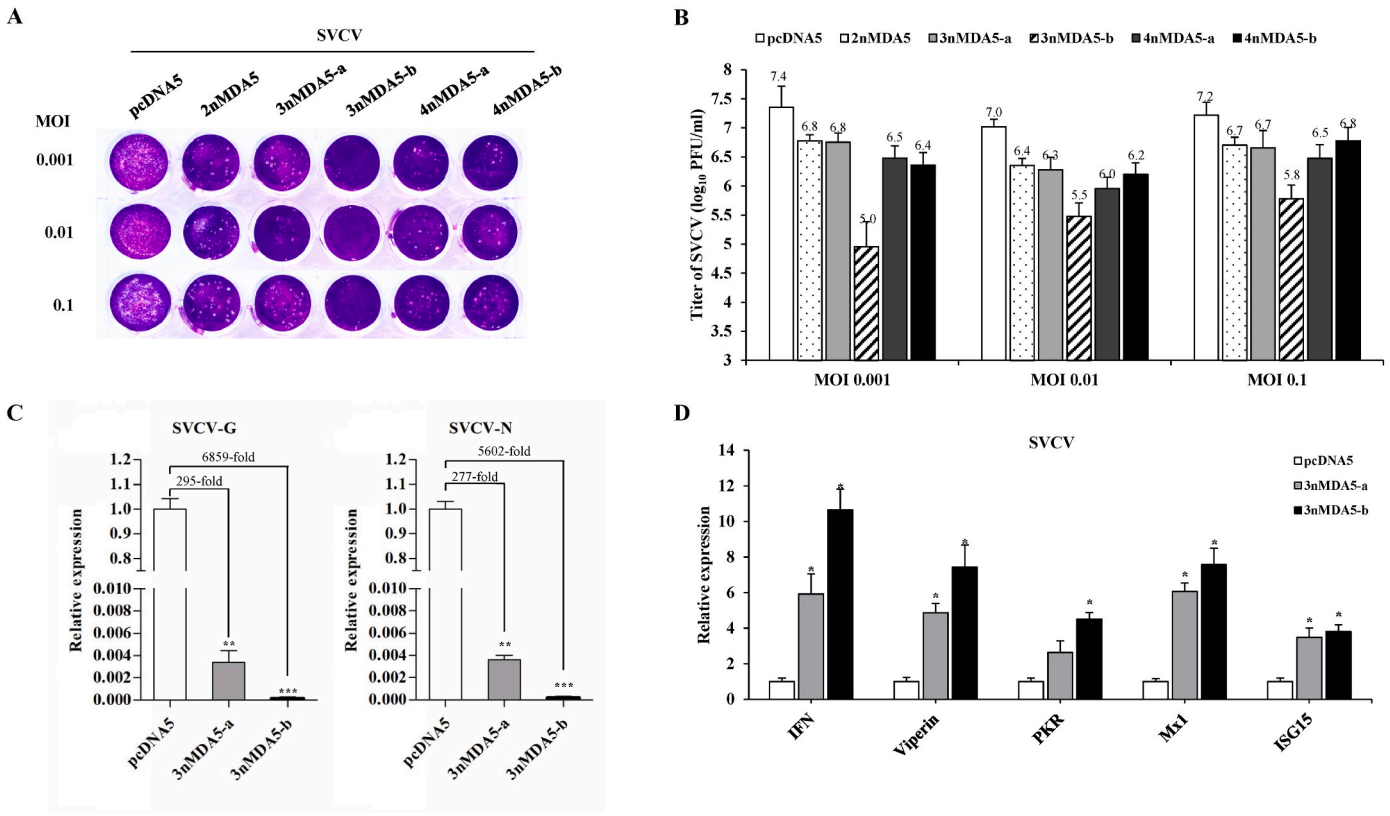

Fig. 5. Antiviral activities of MDA5 from red crucian carp, triploid fish and allotetraploid. (A&B) EPC cells in 24-well plate were separately transfected with the 500 ng plasmids expressing 2nMDA5, 3nMDA5-a, 3nMDA5-b, 4nMDA5-a, 4nMDA5-b or the empty vector. At 24 hpt, cells were subjected to SVCV infection at different MOIs (0.001, 0.01 and 0.1). The cells were fixed and stained with crystal violet solution (A) and the virus titer in supernatant were measured through plaque assay at 48 hpt (B). (C&D) EPC cells in 6-well were transfected with plasmids expressing 3nMDA5-a, 3nMDA5-b or empty vector (2 μg/well), and infected with SVCV at MOI 0.01 24 hpt. Twenty-four hours later, cells were collected and utilize for RT-qPCR to detect SVCV genes (C) and cytokine genes (D). The relative expression is represented as fold induction relative to the expression level in control cells (set to 1). 2nMDA5: pcDNA5/FRT/TO-Flag-2nMDA5; 3nMDA5-a: pcDNA5/FRT/TO-Flag-3nMDA5-a; 3nMDA5-b: pcDNA5/FRT/TO-Flag-3nMDA5-b; 4nMDA5-a: pcDNA5/FRT/TO-Flag-4nMDA5-a; 4nMDA5-b: pcDNA5/FRT/TO-Flag-4nMDA5-b; pcDNA5: pcDNA5/FRT/TO.

4. Discussion

Disease is becoming a major obstruction restricting the healthy development of aquaculture industry. One of the effective ways to solve this problem is genetic improvement of the aquaculture species through hybridization and selection breeding (Wang et al., 2019). According to this strategy, triploid crucian carp was generated by interspecies crossing female red crucian carp with male allotetraploid, and it is noteworthy that the triploid offspring obtained the trait of enhanced disease resistance. The heterosis of triploid fish may derived from a variety of causes, including the changes in gene expression patterns (Yan et al., 2016, 2017). The earlier transcriptome analysis of the cultured cells of the hybrid fish (3nFC) suggested that the RLR signaling pathway of the triploid fish might contribute importantly to its enhanced antiviral activity (Xiao et al., 2018). Later on, the study on MAVS, the key antiviral adaptor protein in the RLR signaling pathway, has revealed that 3nMAVS possessed the much more potential to trigger the antiviral signaling than 2nMAVS and 4nMAVS (Xiao et al., 2019). These findings motivate us to further investigate and compare the functional involvement of other RLR family members of triploid fish and its parents in host innate antiviral immune response.

Two transcripts of MDA5 were detected respectively in triploid fish and allotetraploid, but intriguingly, a shorter splicing form (3nMDA5-b) was found in triploid fish. According to the sequence alignment, a deletion of 71 bp occurred in the coding region of 3nMDA5-b, resulting in the premature end of translation of this triploid MDA5 homologue. Due to the limited genomic DNA information of tripoloid fish, the precise splicing site of 3nMDA5-b was unknown. In fact, alternative splicing is a frequent regulatory process that may create several types of mRNA from a single encoding gene, and the functions of the proteins generated by these mRNAs are often distinct (Chang et al., 2011a). Many human genes, particularly those associated to immunity, are subjected to alternative splicing (Wang et al., 2008). In teleost, a number of studies have revealed that, alternative splicing of PRRs genes also plays significant roles in host innate immune response. For example, NLRs were found to undergo alternative splicing: two rainbow trout NOD2 splicing transcripts showed different activities to induce IFN and other cytokines; and zebrafish NLRC5 isoforms showed discrepancy function in antiviral and antibacterial immune responses (Chang et al., 2011b). In addition, splicing isoforms were also reported in RLRs: zebrafish RIG-I had four different transcripts, among them, RIG-Ia serves as enhancer in RIG-Ib-mediated signaling (Zou et al., 2015); rainbow trout LGP2a and zebrafish LGP2 are positive regulators in antiviral signaling, but rainbow trout LGP2b and zebrafish LGP2v1/v2 play the opposite role (Chang et al., 2011a; Zhang et al., 2018); Zou first reported MDA5a and its shorter splicing form (MDA5b) in zebrafish, and discovered that MDA5b could augment MDA5a/MAVS-mediated IFN signaling (Zou et al., 2014). Therefore, the above studies suggest that the alternative splicing of fish pattern recognition receptors is significant in the process of fish innate immune response.

The current study found that, MDA5 homologues of triploid fish and its parents were all up-regulated in host cells after SVCV infection and poly (I:C) stimulation, suggesting that they were engaged in the antiviral innate immunity (Fig. 2). The luciferase reporter assay and antiviral

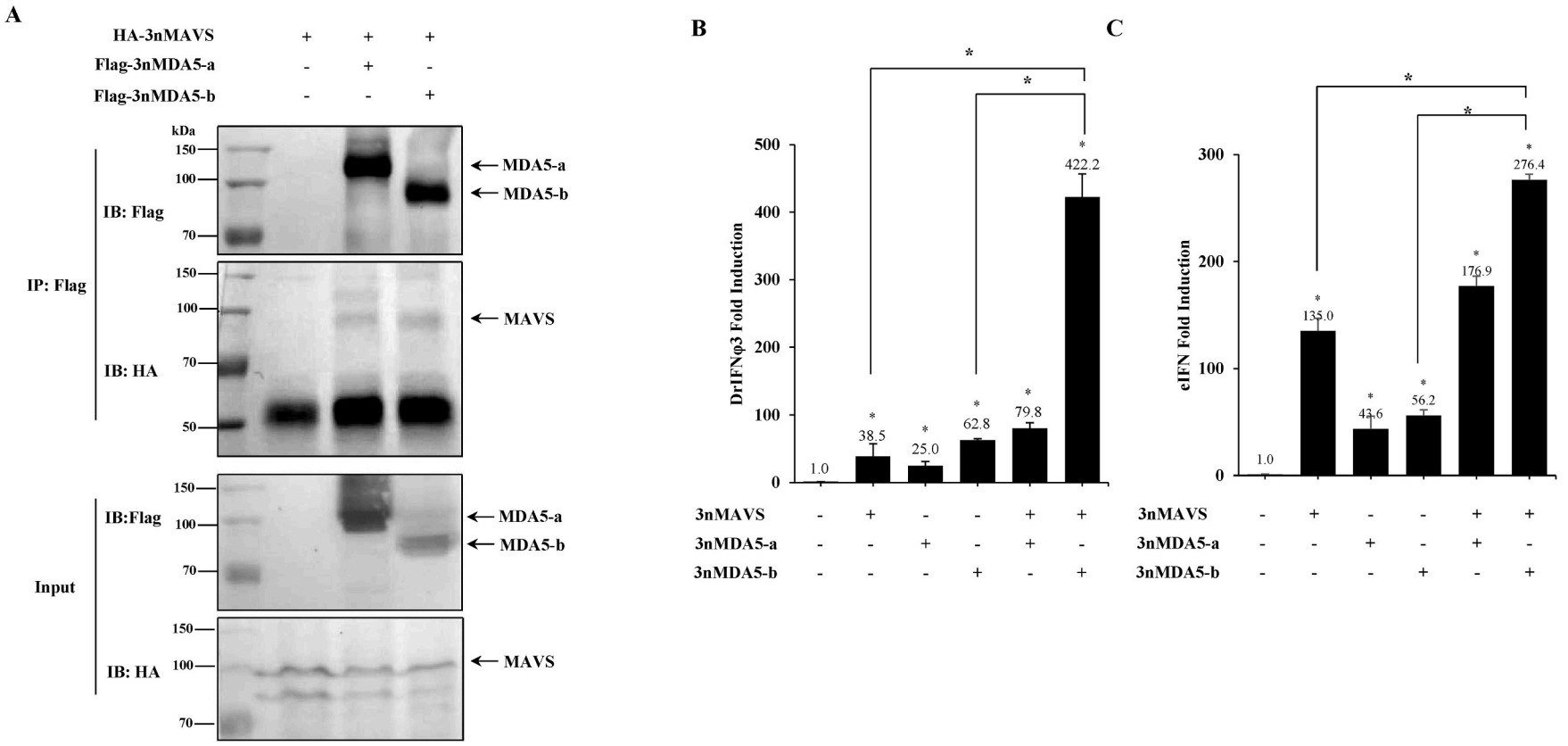

Fig. 6. The interaction between 3nMDA5-a/3nMDA5-b and 3nMAVS. (A) HEK293T cells in 10 cm dishes were transfected with expression plasmids for Flag-3nMDA5-a, Flag-3nMDA5-b and/or HA-3nMAVS (7.5 μg each), then used for co-IP assay according the methods. IB:immunoblot; IP: immunoprecipitation. (B&C) EPC cells in 24-well plates were transfected with plasmids expressing 3nMDA5-a, 3nMDA5-b, 3nMAVS or empty vector (200 ng/well) as indicated, together with 25 ng pRL-TK and 200 ng Luci-DrIFNφ3 (B) or 200 ng eIFN (C). Cells were harvested at 24 hpt and used for luciferase reporter assays.

assay showed that overexpression of the MDA5 homologues in EPC cells could induce IFN promoter transcription and enhance the antiviral activity of the cells against SVCV (Figs. 4 and 5). Interestingly, 3nMDA5-b exhibited the stronger antiviral activity than other full-length MDA5s, which was different from the previous result in zebrafish, in which zebrafish MDA5b (shorter splicing form) showed the lower effect on IFN signaling than zebrafish MDA5a (Zou et al., 2014). By comparing the structural domains of 3nMDA5-b and zebrafish MDA5b, we discovered that, although both of them are truncated isoforms with a deletion of C-terminal region, zebrafish MDA5-b loses most of the HELICc domain, whereas 3nMDA5-b lacks just a small part. Another study in zebrafish reported that, overexpression of HELICc domain alone or together with NOD1 could increase the expression of IFN, IRF3 or RSAD2, implying that HELICc of MDA5 plays an essential role in downstream signaling (Wu et al., 2020). Therefore, it is speculated that the majority of the HELICc domain that 3nMDA5-b preserved may function crucially in its enhanced antiviral activity.

In general, this manuscript highlights that MDA5 of red crucian carp, triploid fish and allotetraploid play significant roles in host antiviral innate immune response, but 3nMDA5-b, a shorter variant, mediates the strongest antiviral signaling among them, which provides the new insight on the RLR signaling of triploid fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104294.

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