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CARD and TM of MAVS of black carp play the key role in its self-association and antiviral ability

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ABSTRACT

Mitochondrial antiviral signaling protein (MAVS) is an adaptor protein of the innate immune system of higher vertebrate. In this paper, the transcription profile of black carp MAVS (bcMAVS) in host cells in response to spring viremia of carp virus (SVCV) and grass carp reovirus (GCRV) infection was identified. EPC cells expressing bcMAVS possessed obviously enhanced antiviral activity against both SVCV and GCRV. Immunofluorescence (IF) staining data demonstrated that bcMAVS molecules were redistributed and formed aggregates on the mitochondria of EPC cells after virus infection. Co-immunoprecipitation (co-IP) assay in HEK293T cells demonstrated that bcMAVS proteins bound to each other, which suggested that this fish protein owned self-association *in vivo*. IF assay identified that the transmembrane (TM) domain of bcMAVS was crucial for its mitochondrial localization. Co-IP assays among bcMAVS mutants demonstrated that both *N*-terminal caspase recruitment domain (CARD) and TM domain were indispensible for dimerization of bcMAVS. It was interesting that Truncated-bcMAVS possessed much enhanced interferon-inducing activity and antiviral ability than wild type bcMAVS, which only contains CARD and TM. All the data generated in this study support the idea that oligomerization of bcMAVS on mitochondrion is crucial for the antiviral ability of bcMAVS, which is depend on both CARD and TM domain of this fish MAVS orthologue.

1. Introduction

Fishes possess both innate and adaptive immune systems; however, fishes depend on innate immunity much more than adaptive immunity to protect themselves against environmental disadvantages such as pathogen microbes invasion [1–3]. Like their mammalian counterparts, fish innate immune systems detect viral components utilizing pattern recognition receptors (PRRs), which include toll-like receptors (TLRs), retinoic acidinducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs), *C*-type lectins (CTLs), AIM2-like receptors (ALRs) and OAS-like receptors in mammals [4–9]. RLRs function as cytosolic RNA virus sensors and are activated after recognizing viral components, which subsequently trigger the downstream MAVS through its exposed CARD domain [10].

MAVS, also known as IPS-1/VISA/Cardif, is the downstream adaptor protein in both RIG-I and MDA5 signaling, which has been proved to play a key role in the host innate immune response against RNA virus [11–14]. Human MAVS is composed of an *N*-terminal CARD domain, a proline-rich region and a *C*-terminal transmembrane (TM) domain. The CARD domain of MAVS interacts with the CARD domain of RIG-I or MDA5 after viral recognition, which shares some homology with the first CARD domain in of both MDA5 and RIG-I. Deletion of the CARD domain of MAVS abolished its ability to activate the IFN promoter [11]. As a mitochondrial outer membrane protein, MAVS targets to the mitochondria through its TM domain. It is reported that mitochondrial localization is important for the function of human MAVS in transcriptional activation of IFN, removal of TM domain by artificial means disrupted its signaling function [11]. Previous works have reported that human MAVS self-association, which mediated by TM domain, is crucial for its antiviral innate immune signaling [15,16]. Another

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study have confirmed that human MAVS form self-perpetuating fiber-like polymers through CARD domain during viral infection, which can efficiently convert endogenous MAVS into functional aggregates and then highly potent in activating IRF3 [17]. Although these studies were not consistent with each other in the determining domain of MAVS dimerization, they shared the similar viewpoints that the dimerization of MAVS play a essential role in its antiviral ability.

Since the functional importance of dimerization of human MAVS was identified, we wanted to find out whether fish MAVS performed similar self-interaction in its antiviral signaling. Here we demonstrate that overexpression of bcMAVS in EPC cells led to strong antiviral state against RNA viruses. Similar to human MAVS, bcMAVS aggregate formation in response to RNA viruses infection in cells was observed, which suggested this fish antiviral protein owned self-association *in vivo*. Besides, CARD and TM domains are required to this transmembrane-dependent dimerization, because deletion either of the two domains prevents the dimerization and antiviral ability of bcMAVS. This is the first report that both CARD and TM domain of fish MAVS are crucial for its dimerization and antiviral ability in host innate immune response against viral invasion.

2. Materials and methods

2.1. Cells and plasmids

HEK293T (293T) cells, epithelioma papulosum cyprini (EPC) cells, *C. idella* kidney (CIK) cells and *Mylopharyngodon piceus* fin (MPF) cells were kept in the lab [18]. All the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin. Transfection was done as previously described [19], calcium phosphate was used for 293T transfection and Fugene 6 (Promega) was used for EPC transfection.

pcDNA5/FRT/TO-HA, pcDNA5/FRT/TO-Flag, pcDNA5/FRT/TO-HA-bcMAVS, EGFP-bcMAVS, pRL-TK and Luci-eIFN (for fathead minnow interferon promoter activity analysis) were kept in the lab [20]. The bcMAVS mutants, including Truncated-bcMAVS, ΔCARD-bcMAVS and ΔTM-bcMAVS, were generated by PCR and inserted into pcDNA5/FRT/TO-HA or pcDNA5/FRT/TO-Flag between *Kpn*I and *Xho*I restriction sites separately.

2.2. Virus produce and titration

SVCV and GCRV were kept in the lab [21]. SVCV and GCRV were propagated in EPC and CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque forming assay on EPC cells separately as previously described [22]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) was added. Plaques were counted at day 3 post-infection.

2.3. Quantitative real-time PCR

MPF cells were seeded in 6-well plate (2×10^6 cells/well) 16 h before viral infection. MPF cells were infected with SVCV or GCRV at a multiplicity of infection (MOI) of 0.01, 0.1or 1 independently. The MPF cells were harvested at different time points post SVCV or GCRV infection (0 h, 2 h, 8 h, 24 h, 48 h, 72 h) separately and used for RNA isolation. The relative bcMAVS mRNA level in the MPF cells was determined by quantitative real-time PCR (q-PCR), in which β-actin was used as an internal parameter. The primers for q-PCR were listed in Table 1. The q-PCR program was: 1 cycle of 50°C/2min, 1 cycle of 95°C/10min, 40 cycles of 95°C/15s, 60°C/1min, followed by dissociation curve analysis (60°C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

2.4. Immunoblotting

HEK293T cells were transfected with pcDNA5/FRT/TO-Flag-bcMAVS, pcDNA5/FRT/TO-Flag-Truncated-bcMAVS, pcDNA5/FRT/TO-Flag-ΔCARD-bcMAVS, or pcDNA5/FRT/TO-Flag-ΔTM-bcMAVS separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [23]. Briefly, the proteins in whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membrane was probed with anti-Flag antibody (1:4000; Sigma) and proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.5. Co-immunoprecipitation (Co-IP)

HEK293T cells in 10 cm petri dish were co-transfected with pcDNA5/FRT/TO-HA-bcMAVS and pcDNA5/FRT/TO-Flag-bcMAVS or pcDNA5/FRT/TO-Flag-Trucated-bcMAVS, pcDNA5/FRT/TO-Flag-ΔCARD-bcMAVS, pcDNA5/FRT/TO-Flag-ΔTM-bcMAVS separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoprecipitation (IP) assay as previously described [23]. The whole cell lysate of the transfected cells was incubated with protein A/G agarose beads at 4 °C for 2 h. HA-conjugated or Flag-conjugated protein A/G agarose beads were added in the supernatant after pre-cleaning and incubated with the supernatant media at 4 °C for 4 h. The HA-conjugated or Flag-conjugated protein A/G agarose beads were boiled in 2 × smaple buffer after 3 times of wash and the eluted proteins were used for IB as above.

2.6. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-eIFN and plasmid expressing bcMAVS or its mutants. For each transfection, the total amount of plasmids was balanced with the empty vector. The cells were harvested and lysed on ice at 24 h post transfection. The centrifuged supernatant was used to measure firefly luciferase and renilla luciferase activities according to the instruction of the manufacturer (Promega) as previously.

2.7. Immunofluorescence microscopy

EPC cells were transfected with pcDNA5/FRT/TO-HA-bcMAVS, pcDNA5/FRT/TO-HA-Truncated-bcMAVS, pcDNA5/FRT/TO-HA-ΔCARD-bcMAVS, pcDNA5/FRT/TO-HA-ΔTM-bcMAVS or the empty vector separately. The transfected EPC cells were fixed with 4% (v/v) paraformaldehyde at 36 h post-transfection. The cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immune-fluorescent staining as previously described [22]. Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:300 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:1000; DAPIwas used for nucleus staining; Mitotracker Deep Red FM (M22426, Invitrogen) was used to stain the mitochondria according the manual of the company.

Table 1
Primers used in the study.

Primer name	Sequence (5'→3')	Primer information
bcMAVS q-PCR-F	ATGACAGGATCAGGGGAAT	q-PCR
bcMAVS q-PCR-R	ATGTTGGAAGGGGGAGTTG	
β-actin-F	TGGGCACTGCTGCTTCCT	q-PCR
β-actin-R	TGTCCGTCAGGCAGCTCAT	
bcMAVS-F1	ACTGACGGTACCATGTCATTGACACGTGAAC	bcMAVS mutants
bcMAVS-R1	ACTGACCTCGAGTTATCGATAGCCCTCTTCTCT	
bcMAVS-F2	ACTGACGGTACCATGTATGACAGGATCAGGGGAATC	
bcMAVS-R2	ACTGACCTCGAGTTAATGCTTTAGCTTCCAGGC	
bcMAVS-F3	CCCAGACCTGCACCTATCAACTACCGAGAG	
bcMAVS-R3	CTCTCGGTAGTTGATAGGTGCAGGTCTGGG	

3. Results

3.1. bcMAVS is an anti-viral protein against SVCV and GCRV

To characterize elaborated bcMAVS mRNA expression profile during the host innate immune response, MPF cells were infected with SVCV or GCRV at different MOIs and harvested for q-PCR at different time points after infection. In general, after SVCV infection, the mRNA transcription of bcMAVS variated right after viral invasion and the trends were same in all groups infected at different MOIs (1, 0.1 or 0.01). The mRNA transcription of bcMAVS was up regulated to the highest level at 48 hpi (2.1 folds) and decreased to the original level at 72 hpi, which might because that most cells were dead at that time point (Fig. 1A and B). For the group of GCRV infection, mRNA transcription of bcMAVS in MPF cells was increased right after viral invasion and the trends were similar in all groups infected at different MOIs (1, 0.1 or 0.01). The mRNA transcription levels of bcMAVS of 1MOI, 0.1MOI and 0.01 MOI were all significantly up regulated at 48 hpi and increased to the highest level at 72 hpi (Fig. 1C and D). However, the mRNA transcription level of bcMAVS of 0.1MOI was increased to a high level (15.6 folds) at 8hpi and decreased to a low level (2.7 folds) at 24 hpi, which was different from those of groups of 0.01MOI and 1 MOI.

Overexpression of MAVS resulted in an enormous antiviral state in higher vertebrate and fish cells [24], which triggered us to examine whether bcMAVS could induce antiviral effects *in vivo*. To test this, EPC cells were transfected with pcDNA5/FRT/TO-HA-bcMAVS or the empty vector separately and infected with SVCV or GCRV at 24 h post infection. For SVCV infection, both cytopathic effect (CPE) and viral titer in the supernatant media of the EPC cells overexpressing bcMAVS were obviously decreased, which was determined by crystal violet staining and classic plaque assay separately (Fig. 2A and B). The data in GCRV infection group was similar to that of the SVCV group, both CPE and viral titer in the supernatant media of the EPC cells transfected with pcDNA5/FRT/

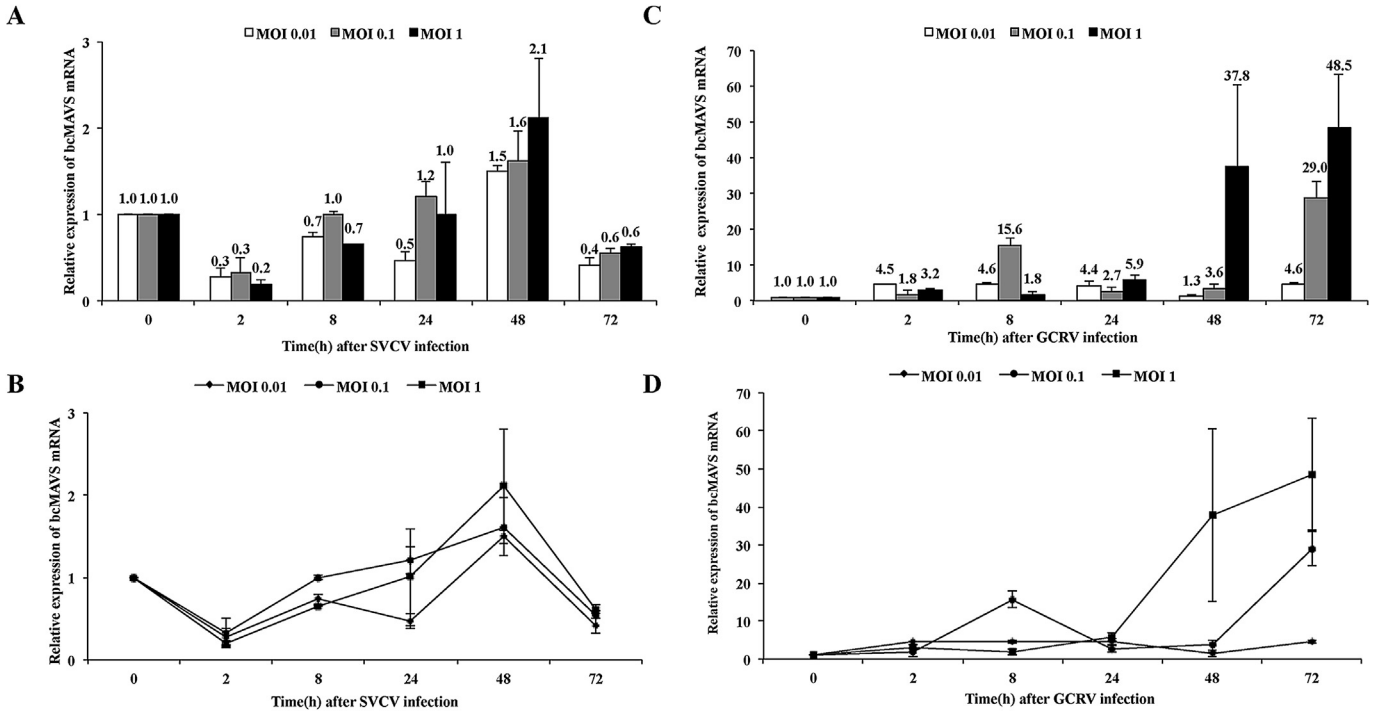

Fig. 1. bcMAVS transcription in MPF cells varies after SVCV and GCRV infection. MPF cells were infected with SVCV or GCRV at indicated MOIs and harvested at different time points post infection. bcMAVS mRNA transcription was determined by q-PCR as described in methods. (A, C): The bar chart of relative bcMAVS mRNA transcription levels in MPF cells. (B, D): The trend chart of relative bcMAVS mRNA transcription levels in MPF cells. Error bars denote standard deviation and data represent three independent experiments. The numbers above the error bars stand for average relative bcMAVS mRNA levels.

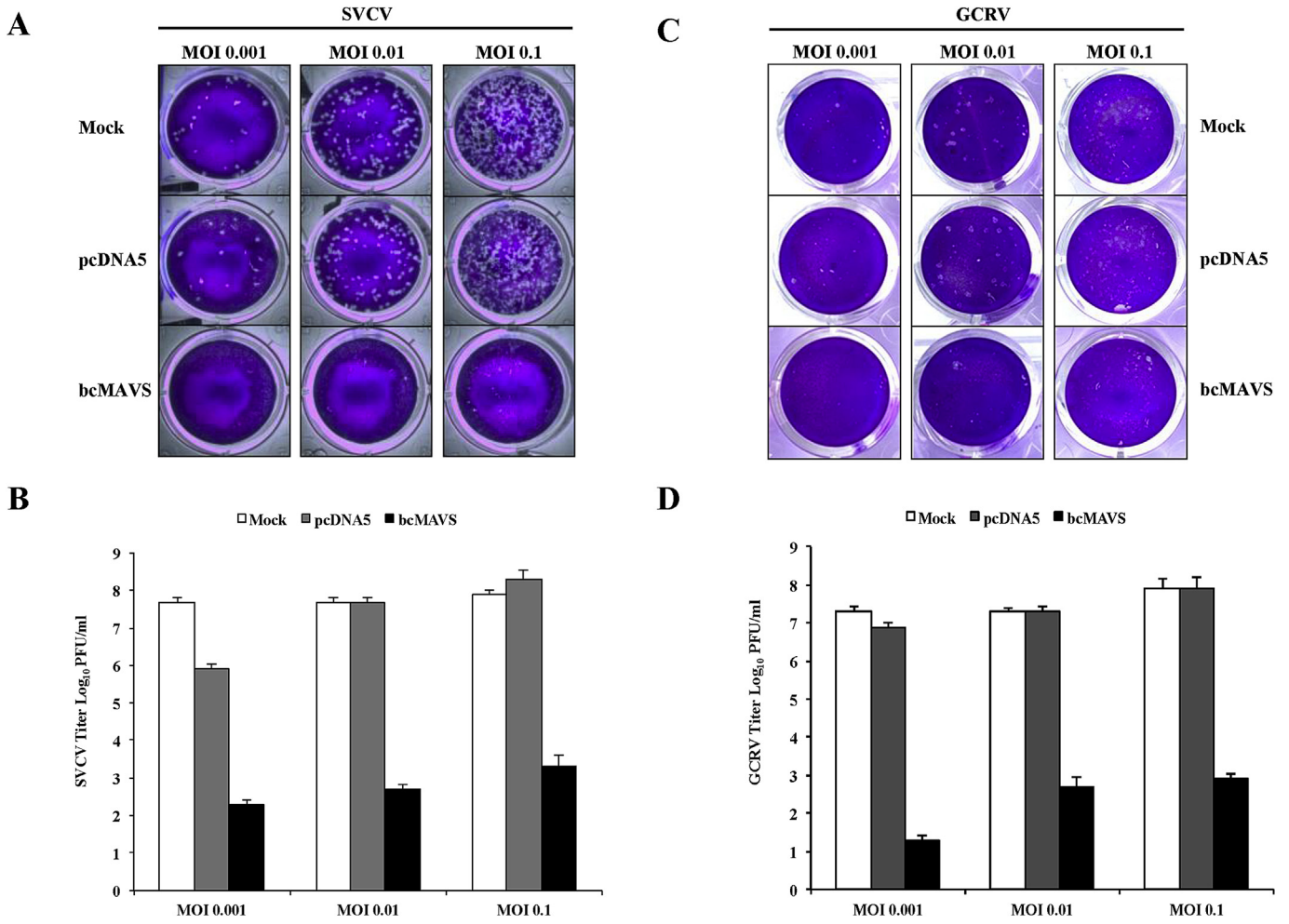

Fig. 2. bcMAVS is an anti-viral protein against SVCV and GCRV. EPC cells seeded in 24-well plates were transfected with 250 ng of pcDNA5/FRT/TO-HA-bcMAVS, the empty vector separately. The cells were infected with SVCV or GCRV at indicated MOIs at 24 h post-transfection and the culture supernatants were collected at 72 h post infection (hpi). The cell monolayers were stained with crystal violet (A, C) and the viral titers of the collected culture supernatants were determined by plaque assays on EPC cells (B, D). Error bars denote standard deviation and data represent three independent experiments; Mock: EPC cells without transfection; pcDNA5: pcDNA5/FRT/TO; bcMAVS: pcDNA5/FRT/TO-HA-bcMAVS.

TO-HA-bcMAVS were obviously decreased (Fig. 2C and D). These data demonstrated clearly that exogenous of bcMAVS remarkably improved the antiviral ability of EPC against SVCV and GCRV, which suggested that this fish RLR menber functioned importantly in the antiviral innate immunity of black carp.

3.2. bcMAVS redistributed after viral infection

Redistribution of human MAVS to form oligomers is essential for this adaptor protein to activate downstream signaling [15,17,25]. To investigate if fish MAVS orthologue redistributes or not during viral invasion, EPC cells were transfected with plasmid expressing bcMAVS, which was fused with EGFP to make it visible for bcMAVS potential distribution. The immunofluorescence microscopy result showed that the intracellular distribution of EGFP-bcMAVS overlapped with the staining pattern of the mitochondrial marker (Mitotracker) in the absence of virus infection (Fig. 3, upper panel). Obviously, after infection with SVCV and GCRV, EGFP-bcMAVS appeared to form clusters that partially overlapped with Mitotracker, suggesting that bcMAVS form aggregates in response to SVCV or GCRV infection which similar to that of human MAVS (Fig. 3, middle panel&lower panel) [17].

3.3. bcMAVS owned self-interaction

Our observation that bcMAVS proteins form aggregates after virus infection suggests that bcMAVS might possess self-association. To better understand the molecular mechanism underlying the antiviral action of bcMAVS, we carried out a co-immunoprecipitation (co-IP) assay in HEK293T cells to examine the bcMAVS self-association. Whole cell lysates of HEK293T cells transiently transfected with plasmids expressing HA-bcMAVS and/or Flag-bcMAVS were precipitated with anti-HA antibody and precipitates were analyzed by immunoblot with anti-Flag antibody. The result showed that Flag-bcMAVS was precipitated by anti-HA antibody in the presence of HA-bcMAVS (Fig. 4A). Reciprocally, HA-bcMAVS was also precipitated by anti-Flag antibody in the presence of Flag-bcMAVS (Fig. 4B). The co-IP assays results demonstrate that bcMAVS possess self-association *in vivo*.

3.4. TM domain was crucial for mitochondrial localization of bcMAVS

To identify functional regions of bcMAVS mediating its localization, dimerization and signaling, plasmids expressing Truncated-bcMAVS, ΔCARD-bcMAVS or ΔTM-bcMAVS were generated

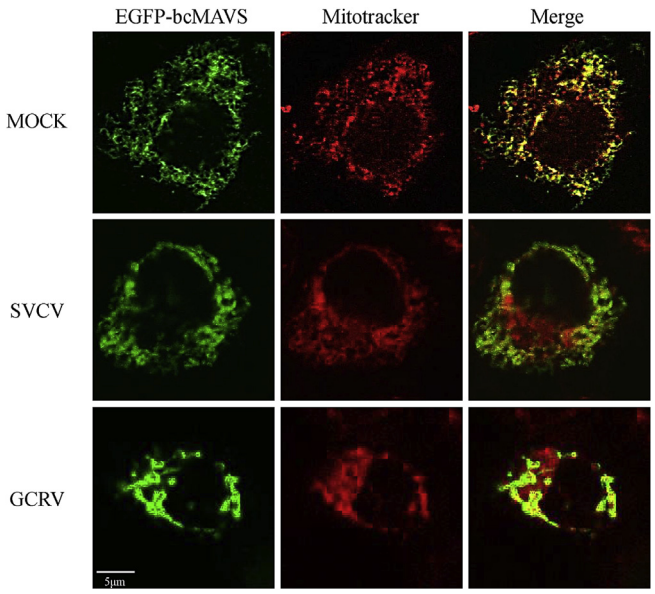

Fig. 3. Viral infection induces the redistribution of bcMAVS. Plasmids expressing bcMAVS fused with an EGFP tag (EGFP-bcMAVS) was transfected into EPC cells. At 24 h post-transfection, cells were uninfected (MOCK) or infected with SVCV or GCRV for 12 h, stained with Mitotracker, and then visualized by confocal fluorescent microscope. The bar stands for 5 μm.

separately. Truncated-bcMAVS was composed of only CARD and TM, CARD domain was deleted in ΔCARD-bcMAVS and ΔTM-bcMAVS was bcMAVS truncated without TM domain (Fig. 5A). Immunoblotting (IB) data demonstrated that wild type bcMAVS and its mutants were well expressed in HEK293T cells (Fig. 5B). An apparent band of ~120 KDa was detected by anti-Flag antibody in the lane of wild type bcMAVS, which was bigger than the predicted molecular weight (~62.4 KDa) [22]. The smaller band of bcMAVS might be the degraded form of this fish protein. Meanwhile, an obvious band of ~110 KDa in the lane of ΔTM-bcMAVS and a clear band of ~90 KDa in the lane of ΔCARD-bcMAVS were detected though their molecular weights were much bigger than the predicted. An obvious band (~20 KDa) was detected in the lane of Truncated-bcMAVS, which was consistent with its predicted molecular weight (Fig. 5B). It is speculated that the increased molecular weight of bcMAVS and its mutants was because of its proline-rich domain, which was not exist in the Truncated-bcMAVS [26].

As its mammalian homologue, bcMAVS locates on the mitochondria of host cells, which suggests that bcMAVS might function on the mitochondria as an adaptor after pathogen recognition [22]. Subcellular localizations of bcMAVS and its mutants were examined by immunofluorescent (IF) staining, which was aimed to determine the crucial domain for mitochondrial localization of this protein. bcMAVS, Truncated-bcMAVS and ΔCARD-bcMAVS all presented the same subcellular location with those of Mitotracker, which demonstrated that both Truncated-bcMAVS and ΔCARD-bcMAVS located on the mitochondria. However, the subcellular location of ΔTM-bcMAVS was not matched that of mitochondria, which distributed in cytosol and on cell membrane similar to that of skeleton protein (Fig. 5C). The IF data demonstrated that TM

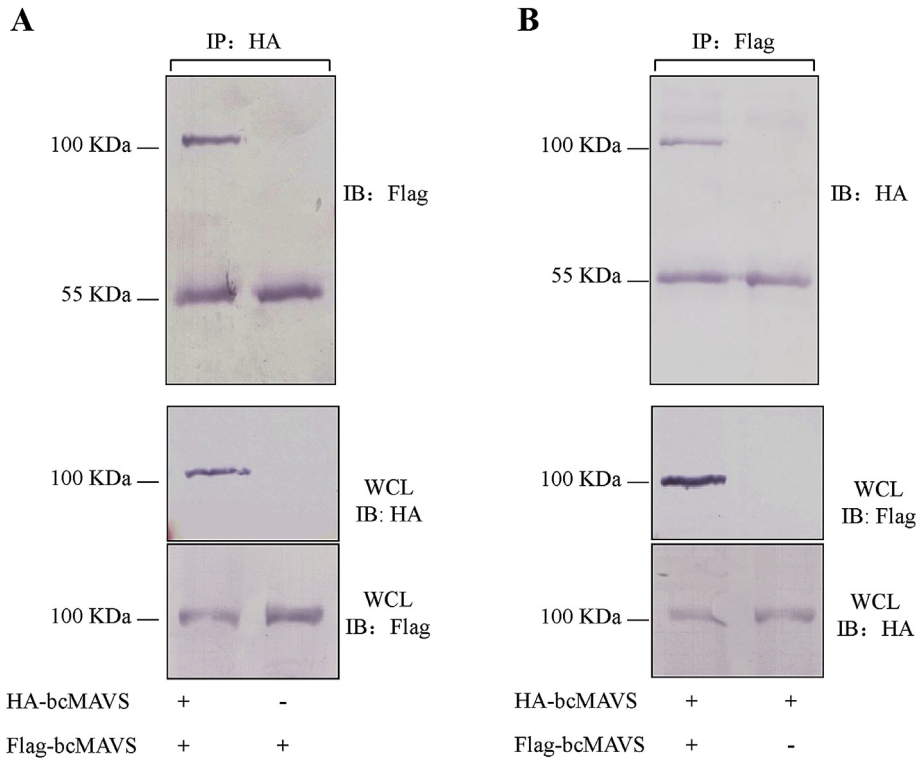

Fig. 4. bcMAVS proteins interact with each other. (A) HEK293T cells were transfected with plasmids containing HA-bcMAVS and Flag-bcMAVS. Proteins precipitated with anti-HA antibody were resolved by SDS-PAGE and analyzed by immunoblot with anti-Flag antibody. Whole cell lysates (WCLs) were analyzed by immunoblot (IB) with anti-HA (middle panel) and anti-Flag (lower panel) antibodies. (B) Proteins precipitated with anti-Flag antibody were analyzed by immunoblot with anti-HA antibody. WCLs were analyzed by immunoblot with anti-Flag (middle panel) and anti-HA (lower panel) antibodies. IP: immunoprecipitation; Flag-bcMAVS: pcDNA5/FRT/TO-Flag-bcMAVS; HA-bcMAVS: pcDNA5/FRT/TO-HA-bcMAVS.

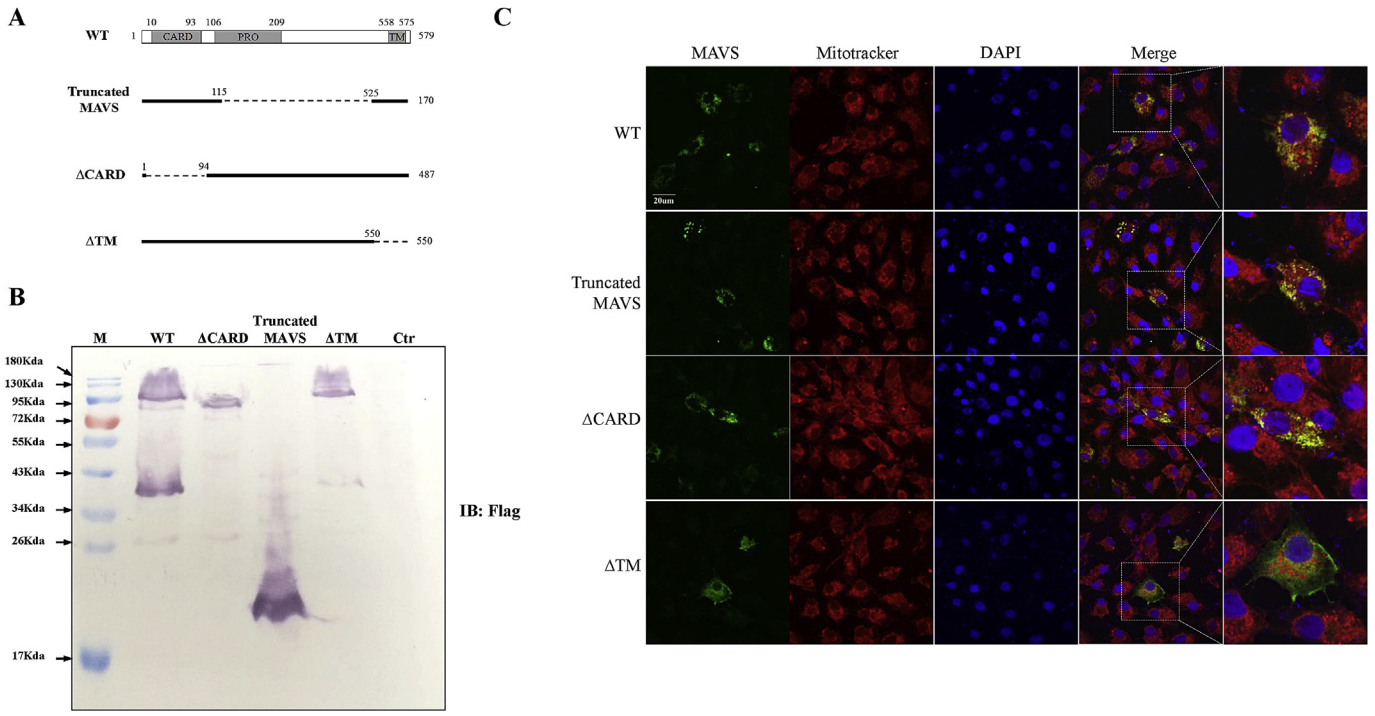

Fig. 5. TM domain is crucial for mitochondrial localization of bcMAVS. (A) Schematic presentation of bcMAVS and its mutants. WT: wild type bcMAVS; Truncated MAVS: bcMAVS lacking residues 116 to 524; ΔCARD: ΔCARD-bcMAVS, lacking the CARD domain (residues 2 to 93); ΔTM: ΔTM-bcMAVS, lacking the mitochondrial membrane targeting sequence (residues 551 to 579). (B) Protein expression of bcMAVS and its mutants in HEK293T cells Ctr: control, 293T cells transfected with empty vector. (C) EPC cells were transfected with plasmids expressing bcMAVS or its mutants separately. The transfected cells were used for IF staining. MAVS (green) indicating bcMAVS or its mutants; Mitotracker (red) indicating the mitochondria; DAPI (blue) indicating the nuclei; the bar stands for 20 μm. WT: pcDNA5/FRT/TO-HA-bcMAVS; Truncated MAVS: pcDNA5/FRT/TO-HA-Trucated-bcMAVS; ΔCARD: pcDNA5/FRT/TO-HA- ΔCARD-bcMAVS; ΔTM: pcDNA5/FRT/TO-HA-ΔTM-bcMAVS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

domain of bcMAVS, like its counterpart domain of mammalian MAVS, was indispensable for its locating to mitochondria.

3.5. Both CARD and TM of bcMAVS are essential for its self-interaction

To test which domain of bcMAVS determining its self-association, we examined the abilities of ΔCARD-bcMAVS, Truncated-bcMAVS and ΔTM-bcMAVS to bind with wild type bcMAVS. Co-IP data showed that Truncated-bcMAVS was able to interact with wild type bcMAVS. Unlike Truncated-bcMAVS, ΔCARD-bcMAVS and ΔTM-bcMAVS failed to bind with wild type bcMAVS (Fig. 6). This result suggests that both CARD domain and TM domain are required for bcMAVS proteins to interact with each other. The data that Truncated-bcMAVS could bind to each other support the idea that CARD and TM domain of bcMAVS are sufficient for its self-association. Thus, these data collectively support that bcMAVS could form oligomer and that both CARD domain and TM domain are indispensable and sufficient for its self-interaction.

3.6. Both CARD and TM of bcMAVS are crucial for its IFN induction ability and antiviral ability

MAVS dirmerization was previously proposed to be linked to its activation of downstream transcription factors [15,16]. Our above results identified the ability of bcMAVS to form dimer, functional studies were performed to investigate the relationship between bcMAVS dimerization and its IFN promoter induction ability. Truncated-bcMAVS significantly induced the activation of fathead minnow IFN (eIFN), however, ΔCARD-bcMAVS and ΔTM-bcMAVS showed little effect on the activation of eIFN. It is surprising that the eIFN fold induction by Truncated-bcMAVS (512.6—578.6) was much higher than that (63.3—117.6) by wild type bcMAVS (Fig. 7A). Additionally, the protein expression of bcMAVS and its mutants in EPC cells was also detected by western blotting using anti-HA antibody, which confirmed the successful expression of bcMAVS and its mutants in EPC cells (Fig. 7B). Combined our above data, it is speculate that the ΔCARD-bcMAVS, which localized on mitochondria but deficient in dimerization ability, was unable to activate downstream signaling. While the wild type bcMAVS and Truncated-bcMAVS, which had mitochondrial localization and dimerization ability, enabled activation of the IFN reporter.

The above data showed that both CARD and TM of bcMAVS were crucial for its oligomerization, activation of IFN and TM of bcMAVS was indispensable for its localization to mitochondria (Figs. 5C, 6 and 7), which implied that CARD and TM were indispensible for its antiviral ability. To test this, EPC cells were transfected with plasmids expressing bcMAVS, Truncated-bcMAVS, ΔCARD-bcMAVS or ΔTM-bcMAVS separately and used for SVCV or GCRV infection independently. To our surprise, Truncated-bcMAVS, which only containing the CARD and TM, showed even much stronger antiviral ability against both SVCV and GCRV comparing that of wild type bcMAVS (Fig. 8). It is nothing surprising that ΔCARD-bcMAVS or ΔTM-bcMAVS possessed no antiviral ability against these two RNA virus (Fig. 8), since these two bcMAVS mutants had no effect on activating IFN produce (Fig. 7A). All our data demonstrates that "self-association of bcMAVS on mitochondria" is essential for this fish antiviral protein against RNA virus invasion, e.g. SVCV and GCRV, which is similar to that of human MAVS.

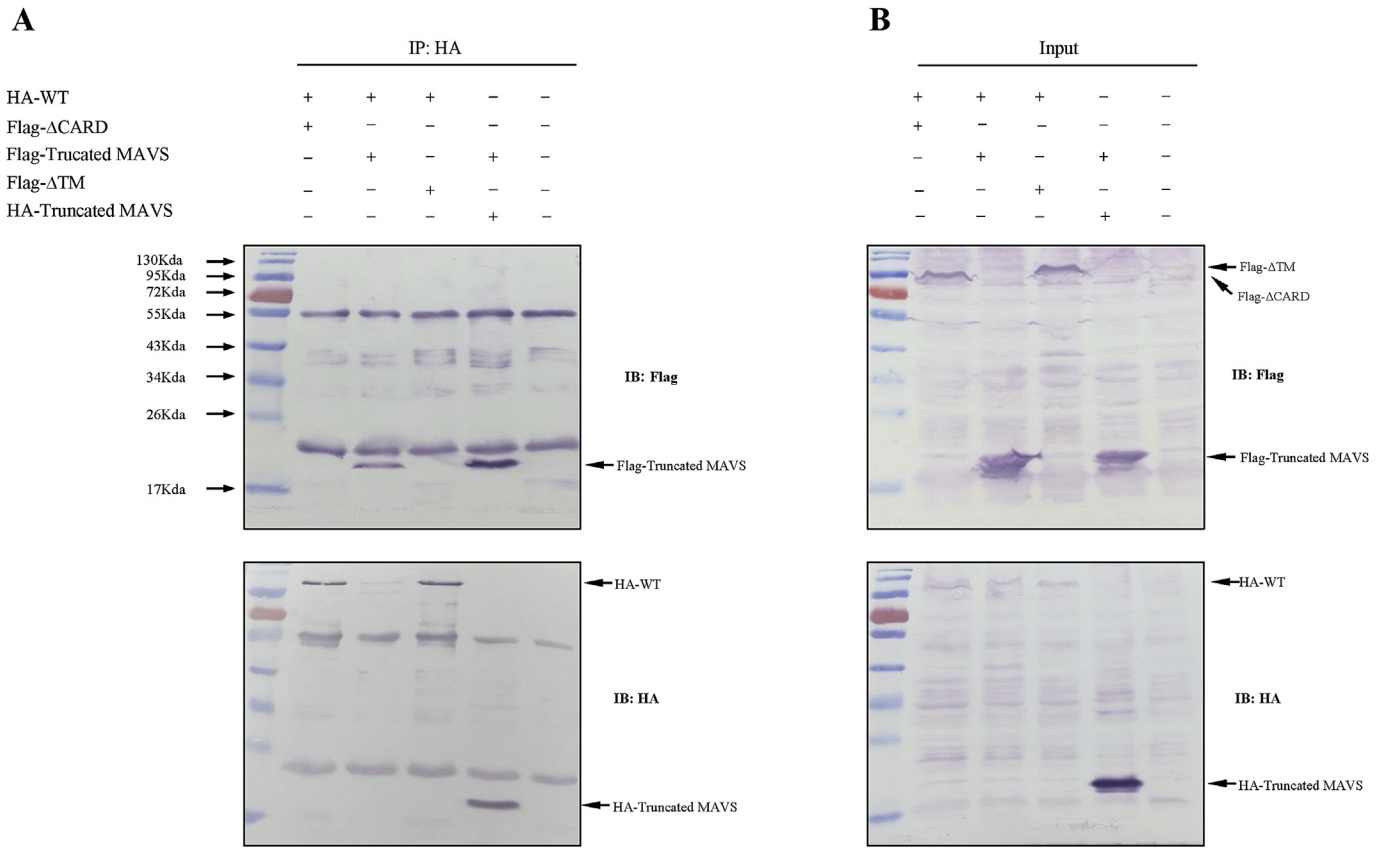

Fig. 6. Both CARD domain and TM domain are indispensable for the dimerization of bcMAVS. (A) HEK293T cells were transfected with the indicated bcMAVS or its mutants constructs. Co-IP was performed using an anti-HA antibody followed by immunoblotting with an anti-Flag antibody or anti-HA antibody. (B) Equal input (5%) showed the expression of transfected protein in whole-cell lysates. Control: 293T cells without transfection.

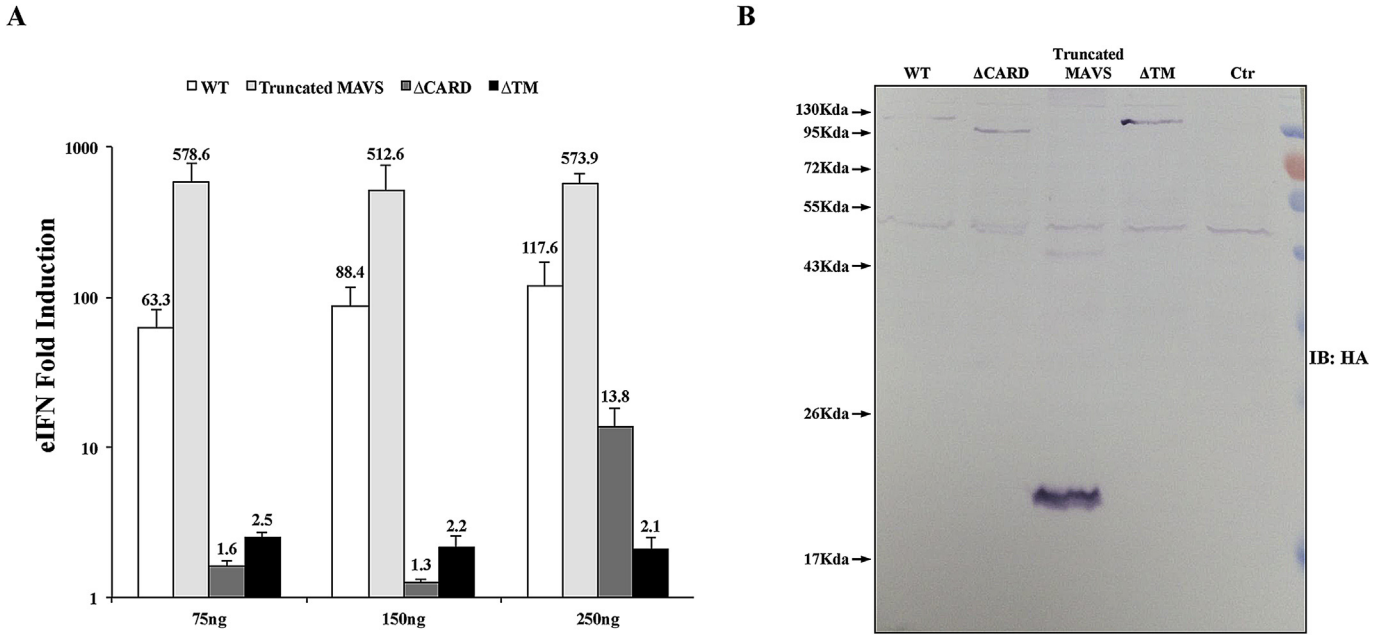

Fig. 7. Truncated MAVS owns strong IFN-inducing ability. (A) EPC cells in 24-well plate were co-transfected with 25 ng pRL-TK, 250 ng Luci-eIFN and plasmids expressing bcMAVS or its mutants with indicated amount. The relative eIFN expression levels were determined by reporter assay as described in methods. The error bars represent the standard deviation and data represent three independent experiments. (B) Protein expression of bcMAVS and its mutants in EPC cells by western blotting. EPC cells in 6 well plate were transfected with plasmids expressing bcMAVS or its mutants respectively. WT:pcDNA5/FRT/TO-HA-bcMAVS; Truncated MAVS: pcDNA5/FRT/TO- HA-Trucated-bcMAVS; ΔCARD: pcDNA5/FRT/TO-HA-ΔCARD-bcMAVS; ΔTM: pcDNA5/FRT/TO-HA-ΔTM-bcMAVS.

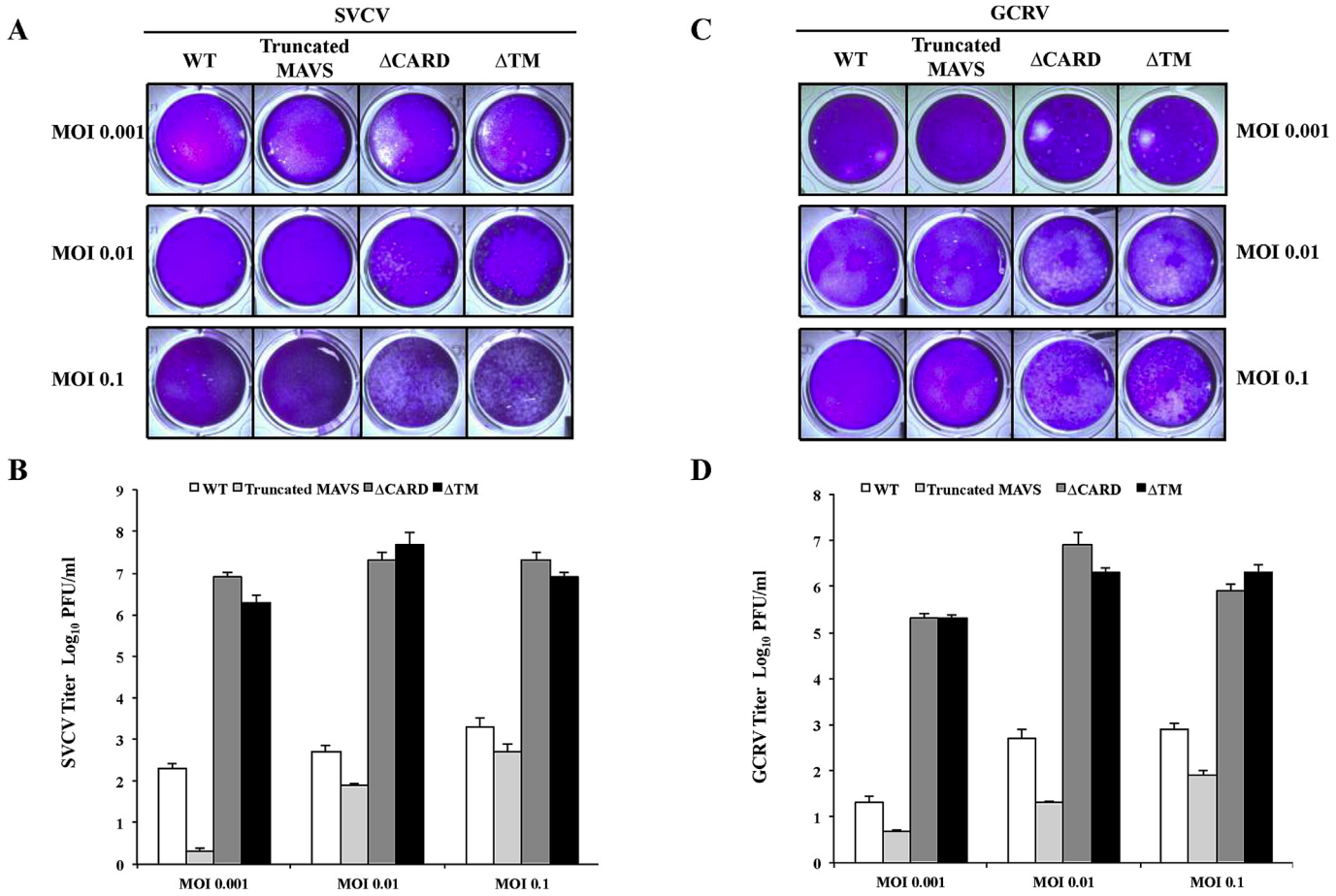

Fig. 8. Truncated MAVS owns strong antiviral ability. EPC cells in 24-wells plate were transfected with 250 ng of plasmids expressing bcMAVS or its mutants separately. The cells were infected with SVCV or GCRV at indicated MOIs at 24 h post-transfection and the culture supernatants were collected at 72 hpi. The cell monolayers were stained with crystal violet (A, C) and the viral titers of the collected culture supernatants were determined by plaque assays on EPC cells (B, D). Error bars denote standard deviation and data represent three independent experiments. WT: pcDNA5/FRT/TO-HA-bcMAVS; Truncated MAVS: pcDNA5/FRT/TO-HA-Trucated-bcMAVS; ΔCARD: pcDNA5/FRT/TO-HA-ΔCARD-bcMAVS; ΔTM: pcDNA5/FRT/TO-HA-ΔTM-bcMAVS.

4. Discussion

Since MAVS was first found as an adaptor protein of RLR pathway, this antiviral protein has been extensively studied [27–29]. MAVS is downstream of RIG-I and MDA5, which are key sensors for RNA virus detection in human cells. Thus RIG-I/MAVS signaling is considered a key system in human innate immunity against RNA viral invasion; however, MAVS of salmon (*Salmo salar*) showed strong antiviral ability against both DNA and RNA virus [24]. Human MAVS recruits multiple ubiquitin E3 ligases to trigger downstream pathway and phosphorylation of MAVS is an essential and conserved mechanism to recruit IRF3 to trigger type I IFNs transcription [28]. Studies on fish MAVS has been carried out among model fish and industrial important species in recent years. Similar to their mammalian counterpart, fish MAVS locates on the mitochondria and activates type I IFNs transcription when it has been activated by upstream virus sensor, such as RIG-I. However, the mechanism behind how this fish antiviral protein is activated and how it activates downstream signaling still needs to be further explored.

In this paper, the mRNA transcription of bcMAVS in MPF cells after SVCV or GCRV infection was detected by q-PCR, which suggested the activated time point of bcMAVS in host innate immune response against these two RNA virus (Fig. 1). It is interesting that bcMAVS mRNA transcription was decreased to the lowest level in all SVCV infected groups right after viral invasion (2 hpi) (Fig. 1), which implied that invaded SVCV utilized some unknown mechanism to avoid or block host innate immunity in the early time post infection. In the group of SVCV infection, the mRNA expression of bcMAVS in host cells was obviously up-regulated from 24 hpi; however, the mRNA transcription of bcMAVS in the group of GCRV was obviously up-regulated from 48 hpi. The data implied that SVCV and GCRV utilized different mechanism to invade MPF cells and initiated host RLR/MAVS signaling antiviral pathway. It is speculated that the RLR/MAVS signaling pathway of MPF cells reposed much quickly to SVCV infection than it did to GCRV infection.

We elucidated the antiviral ability of bcMAVS against two RNA virus, SVCV and GCRV. Both SVCV and GCRV could infect MPF cells effectively and propagate in these black carp derived cells with high yielding, however, the low transfection efficiency of MPF cells made EPC cells to be chosen for bcMAVS transfection and antiviral test, which was a model cell line for SVCV and GCRV related study [30]. The phenomena that over-expression of bcMAVS in EPC cells led to the enhanced antiviral ability against both SVCV and GCRV should be explained by that abundant exogenous bcMAVS activated host IFNs transcription and initiated host antiviral innate immune response besides the endogenous EPC MAVS (Fig. 8).

It has been reported that human MAVS harbors binding motifs for several TRAF proteins, including TRAF2, TRAF5 and TRAF6,

which were crucial in IRF3 activation in antiviral immune responses and mutations all of these TRAF-binding sites abolished IRF3 activation [28]. However, by analyzing the amino acid sequence of Truncated-bcMAVS, none of these TRAF-binding sites above was found in it, which indicated Truncated-bcMAVS itself might unable to activate IFN induction. So it is interesting why Truncated-bcMAVS showed much stronger IFN induction ability and antiviral ability than wild type bcMAVS did (Figs. 7 and 8). The initial report showed that human MAVS mutants only containing CARD fused to TM domain is sufficient to transducer signaling by its transient overexpression, which is consistent with our results. However, when it was expressed in endogenous MAVS knock down cells, the IFN indction was abrogated [11]. It is implied that the high IFN induction and antiviral ability of EPC by Truncated-bcMAVS might correlate with that it act through endogenous MAVS of host cells (the MAVS of EPC cells) to activate downstream signaling. Moreover, the protein expression level (Figs. 5B and 7B) and protein-protein interaction intensity (Fig. 6) of Truncated-bcMAVS in HEK293T cells was much higher than those of wild-type bcMAVS, which suggested that Truncated-bcMAVS own stronger ability to “recruit” endogenous MAVS to form oligomers, and then made Truncated-bcMAVS performed stronger ability to activate downstream signaling.

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