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Black carp STING functions importantly in innate immune defense against RNA virus

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A R T I C L E   I N F O

A B S T R A C T

Stimulator of interferon genes (STING) is a central and multifaceted mediator in the innate immune response of higher vertebrates. To explore its role in teleost fish, the STING homolog of black carp (*Mylopharyngodon piceus*) (bcSTING) has been cloned and characterized in this paper. bcSTING transcription in *Mylopharyngodon piceus* fin (MPF) cells increased remarkably in response to GCRV and SVCV infection, or poly (I:C) stimulation. bcSTING migrated around 42 KDa in immunoblot assay and was identified as a cytosolic protein locating on ER majorly through immunofluorescence staining. Under condition of SVCV/GCRV infection or poly (I:C) stimulation, the subcellular distribution of bcSTING majorly displayed on mitochondria, which overlapped with that of bcMAVS. HA-bcSTING instead of bcSTING-HA presented strong IFN-inducing activity in reporter assay and antiviral ability against both SVCV and GCRV in plaque assay. Site mutation of serine (S) on C-terminus of bcSTING demonstrated that both S371 and S379 were crucial for its mediated signaling. Taken together, our study support the conclusion that bcSTING plays an important role in host innate immune defense against RNA virus such as SVCV and GCRV, in which its C-terminus functions crucially.

1. Introduction

Innate immunity of vertebrates provides the first line of host defense against pathogenic microbes, in which host cells utilize a variety of germline encoded pattern recognition receptors (PRRs) to detect the invading pathogens [1—4]. Function differently, PRRs are involved in cell activation in response to diverse microbial pathogen-associated molecular patterns (PAMPs) [5,6], which include the toll-like receptors (TLRs), retinoic acid inducible gene (RIG)-I-like receptors (RLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs), C-type lectins (CTLs), AIM2-like receptors (ALRs) and OAS-like receptors [7—10]. After recognizing the components of invading pathogens, PRRs initiate the production of type I interferons (IFNs) through downstream signaling, which trigger the transcription of a broad spectrum of interferon stimulated genes (ISGs) and activate the innate immune response [11—14].

STING (also called MITA/ERIS/MPYS), an originally identified adaptor protein in the innate immune response to cytosolic DNA, was found to be an adaptor in the innate immune signaling triggered by cytosolic RNA [15]. In mammals, STING is a transmembrane protein localized on endoplasmic reticulum (ER), mitochondria, and mitochondrial associated membrane (MAM), which is required for effective type I IFNs production in response to certain viral infection such as vesicular stomatitis virus (VSV) and Sendai virus (SeV) [16,17]. This adaptor protein was identified to function downstream of MAVS and upstream of TBK1/IRF3 in the RIG-I initiated IFN production during VSV and SeV infection [18,19]. Recent evidence indicated that STING associated with dsDNA directly besides the role as a signaling adaptor to initiate IFN response, such as cyclic dinucleotides (CDNs) secreted by bacteria (e.g., cyclic di-GMP, cyclic di-AMP and cyclic GAMP) [20—22].

Teleost fish possess both innate and adaptive immune system, however, teleost fish depend more on innate immunity to survive from the disadvantage environment and pathogen invasion [23,24]. Although the innate immune system of fish has been extensively studied in recent years, the detailed role of teleost STING during viral infection still remains largely unknown. To date, STING homologs have been cloned and characterized in several species such as crucian carp (*Carassius auratus* L.), zebrafish (*Danio rerio*), grass

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carp (*Ctenopharyngodon idella*), grouper (*Epinephelus coioides*) and common carp (*Cyprinus carpio*) [25–29]. Evidence supported that teleost fish possessed a functional RLR pathway in which MAVS and STING are downstream signaling molecules of RIG-I [26]. Crucian carp STING contributed to the antiviral response mediated by RIG-I, in which the conserved RIG-I/STING/TBK1/IRF3/7/IFN signaling cascade was addressed [25].

Black carp (*Mylopharyngodon Piceus*) is among the "four famous domestic fishes" for over a thousand years in China and this economically important species is subjected to bulk of pathogenic microorganisms such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), which are among the major RNA viruses threatening the fresh water industry of China. However, the innate immune system of black carp remains largely unknown. In our previous study, MAVS, the adaptor protein of RLR signaling, was cloned and characterized from black carp (bcMAVS), which functioned against both SVCV and GCRV in host innate immune response [30]. To further explore the RLR signaling of black carp, bcSTING has been cloned and characterized in this paper, which was found involved in host innate immune response initiated by GCRV and SVCV. bcSTING presented strong IFN-inducing ability, which correlated with its antiviral activity against both SVCV and GCRV. After SVCV/GCRV infection or co-expressed with bcMAVS, the subcellular distribution of bcSTING was majorly on mitochondria, which matched that of bcMAVS firmly. Thus, it is speculated that bcSTING is recruited into and functioned importantly in bcMAVS mediated antiviral signaling against invading RNA virus, such as SVCV and GCRV.

2. Materials and methods

2.1. Cells and plasmids

HEK293T (293T), HeLa, MPF, *C. idella kidney* (CIK) and *epithelioma papulosum cyprini* (EPC) cells were kept in the lab [31]. HEK293T and HeLa were cultured at 37 °C; CIK, EPC and MPF were cultured at 25 °C. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Transfection was done as previously described, calcium phosphate was used for 293T transfection, Fugene®6 (Promega) was used for EPC transfection and Lipofectamine®2000 was used for HeLa transfection.

pcDNA5/FRT/TO (Invitrogen), pcDNA5/FRT/TO-HA, EGFP-bcMAVS, pRL-TK, Luci-zIFN1 and Luci-zIFN3 (for zebrafish IFN1 and IFN3 promoter activity analysis accordingly) were kept in the lab [32]. The recombinant vector pcDNA5/FRT/TO-HA-bcSTING and pcDNA5/FRT/TO-bcSTING-HA were constructed by cloning the open reading frame (ORF) of bcSTING fused with an HA tag at its N-terminus or C-terminus into pcDNA5/FRT/TO, respectively. The unphosphorylated mutants of bcSTING (S371A/S379A/SSAA) were generated by site mutation based on pcDNA5/FRT/TO-HA-bcSTING as previously [33], in which serine (S) of residue 371 or/and 379 were altered to alanine separately.

2.2. Cloning the cDNA of bcSTING

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcSTING based on the sequences of STING of *C. idella* (KF494194.1), *C. auratus* (JF970229.1), *C. carpio* (KP205544.1) and *D. rerio* (HE856619.1). Total RNA from the spleen of black carp was extracted by using RNAiso Plus (TaKaRa) according to the manufacturer's instructions. The RNA was used to generate full-length cDNA by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Rapid amplification of cDNA ends (RACE) was performed to obtain 3′UTR of bcSTING cDNA by using 3′Full RACE kite separately (TaKaRa). The cDNA of bcSTING was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV and GCRV were kept in the lab and propagated in EPC or

Table 1
Primers used in the study.

| Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
STING-5′	GTCCCCAGAGCGCGCAGCAGG	1153
STING-3′	AATAATCRGTRGTCTCCAC	Gene cloning
3′RACE		
STING-S1	AACCTGAAGAGGAGGACACT	3′ UTR 1st PCR
STING-S2	AGCCACCAAATGAAATCCATCC	3′ UTR 2nd PCR
UPM-longer	CTAATACGACTCACTATAGGGCAAGC	
	AGTGGTATCAACGCAGAGT	
UPM-Short primer	CTAATACGACTCACTATAGGGC	
ORF		
STING-F	ATGTSWGGTGTGATSGGAGAGGAC	1197
STING-R	CTACTTGTAATAATCAGTAGTCTCCACTG	ORF cloning-reverse
Expression construct		
STING-N-F	ACTGACGGTACCATGTGTGGTGTGATCGGA	
STING-N-R	ACTGACCTCGAGCTACTTGTAATAATCA	HA-STING
STING-C-F	ACTGACGGTACCGCCACCATGTGTGGTGTGAT	
STING-C-R	ACTGACCTCGAGCTTGTAATAATCAGT	STING-HA
Mutants		
S371A-F	AGACCCAATGGCCAGTGAACC	
S371A-R	GGTTCACTGGCCATTGGGTCT	S371A
S379A-F	TCATGTTCGCCCCACCCCAGT	
S379A-R	ACTGGGGTGGGGCGAACATGA	S379A
SSAA-F	CAATGGCCAGTGAACCCACCCTCATGTTCGCCCCAC	
SSAA-R	GTGGGGCGAACATGAGGGTGGGTTCACTGGCCATTG	SSAA
q-PCR		
bc-actin-Q-F	TGGGCACCGCTGCTTCCT	
bc-actin-Q-R	TGTCCGTCAGGCAGCTCAT	In/Ex vivo q-PCR
STING-SQ-F	TTCATTCAAGGCAAAGG	
STING-SQ-R	GAAACGGCACCAAACAT	In vivo q-PCR
STING-Q-F	TGTTTGGTGGCGTTTC	
STING-Q-R	GAGCCACATTCATCTTCTTC	Ex vivo q-PCR

A

B

C
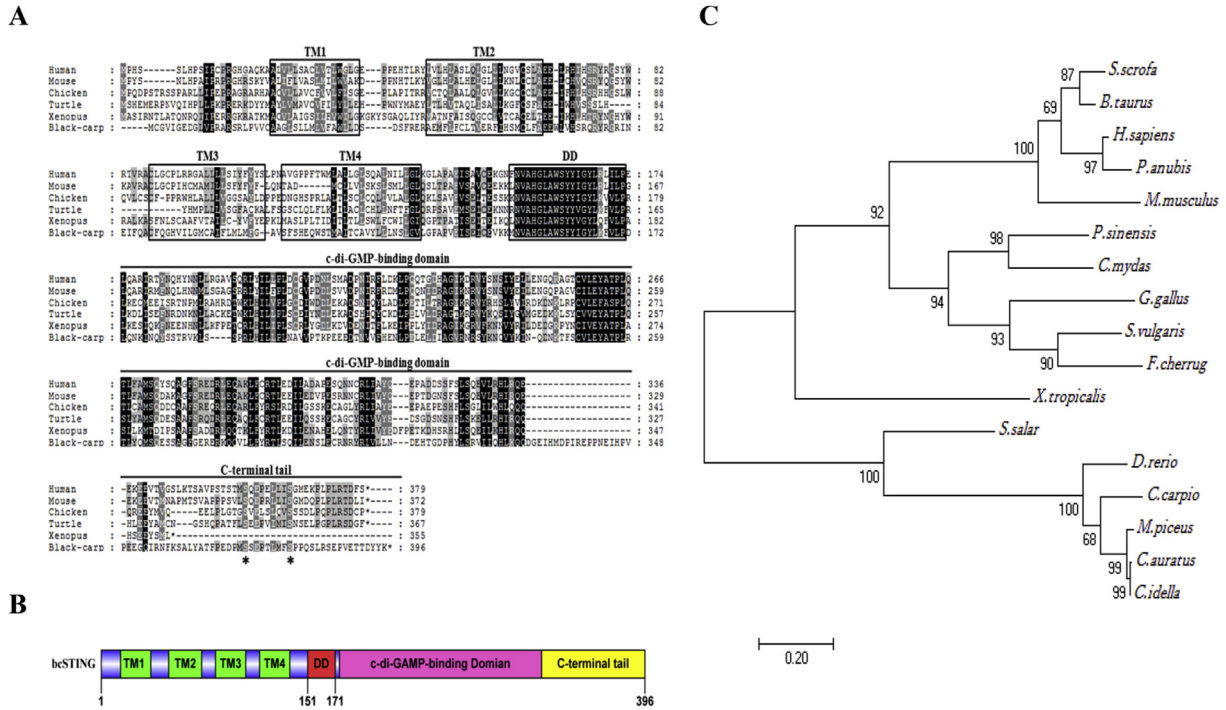
Fig. 1. Sequence analysis of STING.
(A) The predicted bcSTING protein contains 396 amino acids. The transmembrane domains (TMs) and dimerization domain (DD) are indicated with rectangle. The c-di-GMP-binding domain and C-terminal tail domain are labeled above the sequences, and the conserved serine residues (human S358 and S366) are indicated with asterisks. The protein domains were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.emble -heidelberg.de).
(B) Diagrams the functional domains of bcSTING.
(C) Phylogenetic tree of vertebrate STING. Maximum likelihood phylogenetic tree was generated from vertebrate STING of different species by using MEGA 7.0 program, which included (GenBank accession number, unless indicated otherwise): grass carp (*C. idella*; KF494194.1), gold fish (*C. auratus*; JF970229.1), common carp (*C. carpio*; KP205544.1), zebrafish (*D. rerio*; HE856619.1), pig (*S. scrofa*; NM_001142838.1), mice (*M. musculus*; KR154221.1), chicken (*G. gallus*; KP893157.1), chinese softshell turtle (*P. sinensis*; XM_014578148.1), human (*H. sapiens*; NM_198282.3), baboon (*P. Anubis*; XM_003900183.3), green turtle (*C. mydas*; XM_007059190.1), common starling (*S. vulgaris*; XM_014887664.1), atlantic salmon (*S. salar*; XM_014213010.1), bufonid (*X. tropicalis*; NM_001112974.1), cow (*B. Taurus*; XM_015472257.1), saker (*F. cherrug*; XM_014276466.1). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

Table 2
Comparison of bcSTING with other vertebrate STING (%).

Species	Full-length sequence of protein	
	Identity	Similarity
M.piceus	100.0	100.0
C.idella	95.7	96.7
C.auratus	95.5	96.5
C.carpio	79.0	84.9
D.rerio	73.8	81.5
S.scrofa	46.2	62.3
M.musculus	31.9	48.8
P.sinensis	32.2	48.6
H.sapiens	32.2	48.3
P.anubis	32.5	47.9
C.mydas	29.4	47.1
S.vulgaris	31.7	46.9
S.salar	30.8	45.5
X.tropicalis	28.4	45.2
G.gallus	30.0	43.4
F.cherrug	27.1	41.5
B.taurus	17.9	25.6

Fig. 2. Tissue-specific transcription of bcSTING.
Black carp of six months (weight of 120 g) were injected intraperitoneally with SVCV (2.43 × 10^6 pfu/fish), GCRV (2.52 × 10^6 pfu/fish) or sterile PBS (as healthy control) separately and cultured at 25 °C. The injected black carp were sacrificed at 33 h post injection and total RNA was isolated from the indicated tissues independently. Three injected (PBS, GCRV or SVCV) fishes were collected for one group; total RNA samples of each tissue were combined in each group and used for cDNA synthesis. The transcription of bcSTING after virus challenge or in healthy condition (PBS injection) was determined by q-PCR separately. The numbers above the error bars stand for average bcSTING mRNA level. The relative expression bcSTING was normalized to the expression of β-actin.

CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque assay on EPC cells as previously described. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was replaced with DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post-infection.
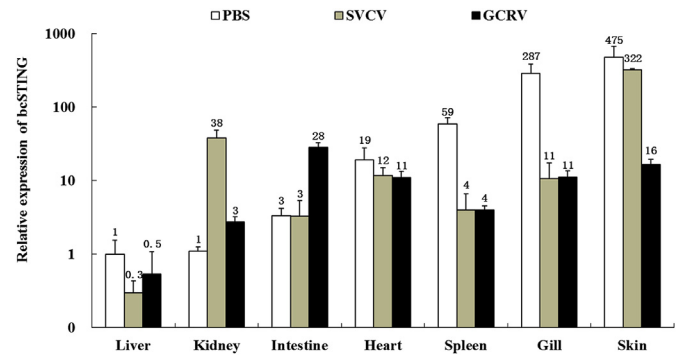
2.4. Quantitative real-timePCR

The relative bcSTING mRNA level in the selected tissues of black

carp or MPF cells was determined by quantitative real-time PCR (q-PCR). The primers for bcSTING and β-actin (as internal control) were listed in Table 1. The 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 cycle threshold (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System (ABI) 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.

2.5. Immunoblotting

HEK293T cells were transfected with HA-bcSTING, bcSTING-HA, HA-bcSTING mutants (S371A/S379A/SSAA) or the empty vector separately. The transfected cells (2×10^6 cells/well) were harvested at 48 h post transfection and lysed for immunoblot assay as previously described [34]. Briefly, whole cell lysates were isolated by 12% SDS-PAGE and the transferred membrane was probed with mouse monoclonal anti-HA antibody (1:3000; Sigma). Target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

For bcSTING dimerization detection, HEK293T cells expressing HA-bcSTING or bcSTING-HA were lysed on ice for 15 min in lysis buffer [1% Nonidet P40, 10 mM PIPES-KOH buffer (pH 7.0), 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 10% glycerol and a mixture of protease inhibitors] and the whole cell lysates were analyzed by 12% SDS-PAGE at 4 °C and applied to immunoblot assay as above.

2.6. Immunofluorescence microscopy

HeLa cells or EPC cells were transfected with HA-bcSTING, EGPF-bcMAVS or the empty vector separately. The transfected cells were fixed with 4% (v/v) paraformaldehyde at 36 h post transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immune-fluorescent staining as previously described [34]. Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:300; Rabbit polyclonal anti-PDI (ER marker/Abcam) was probed at the ratio of 1:600 and Alexa 488-conjugated secondary
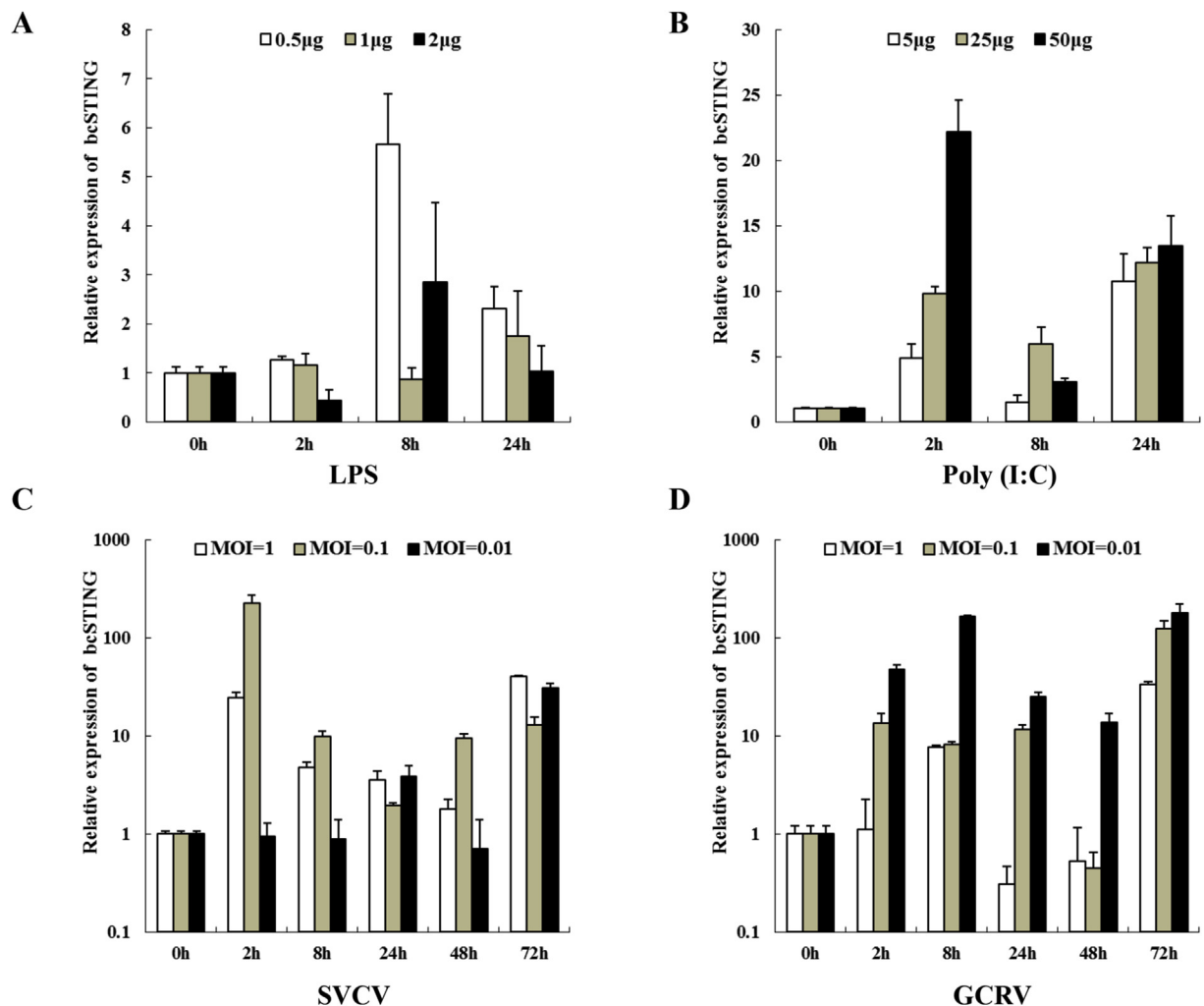

Fig. 3. bcSTING transcription in MPF cells in response to different stimuli.
(A)&(B) MPF cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before stimulation. The cells were treated with LPS or poly (I:C) at the indicated concentration separately and harvested for q-PCR independently at the indicated time points post stimulation.
(C)&(D) MPF cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before viral infection. The cells were infected with SVCV or GCRV at indicated MOI separately and harvested for q-PCR independently at the indicated time points post infection.
The relative expression of bcSTING was normalized to the expression of β-actin.

antibody (Invitrogen) was probed at the ratio of 1:1000; DAPI was used to stain the nucleus; Mitotracker Deep Red FM (M22426, Invitrogen) was used to stain the mitochondria according the manual of the company.

2.7. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with HA-bcSTING or bcSTING-HA, pRL-TK (25ng/well) and Luci-zIFN1 or Luci-zIFN3 (200ng/well). For each transfection, the total amount of plasmid (425ng/well) 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 [34].

2.8. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$. The data were analyzed by two-tailed Student's t-test.

3. Results

3.1. Molecular cloning of the cDNA of bcSTING

STING was the central component in pathogen derived DNA and RNA sensing [35]. To get insight into the role of STING of black carp in the innate immunity, the cDNA of STING orthologue was cloned from the spleen of black carp (KY681061.1). The open reading frame (ORF) of bcSTING consists of 1191 nucleotides and the predicted bcSTING protein contains 396 amino acids (Supplementary Fig. 1). Amino acid alignment showed that bcSTING contained four predicted transmembrane domains (TMs) in the N terminal, a C-terminal domain (CTD) consisting of a dimerization domain (DD), c-di-GMP-binding domain (CBD) and a C-terminal tail (CTT). In addition, serine residues S358 and S366 in human STING, which were essential for TBK1-mediated phosphorylation of STING and STING-dependent IRF3 activation, were also conserved in bcSTING (Fig. 1 A&B).

To gain insight into STING evolution, protein sequence of bcSTING was subjected to multiple alignments with STING from different species. Phylogenetic analysis of STING proteins from the selected species demonstrated that these homologues could be divided into five groups, consisting of mammalia, aves, reptilia, amphibia and fish branches (Fig. 1C). bcSTING shared high protein sequence similarity with cyprinid fishes such as grass carp (96.7%),
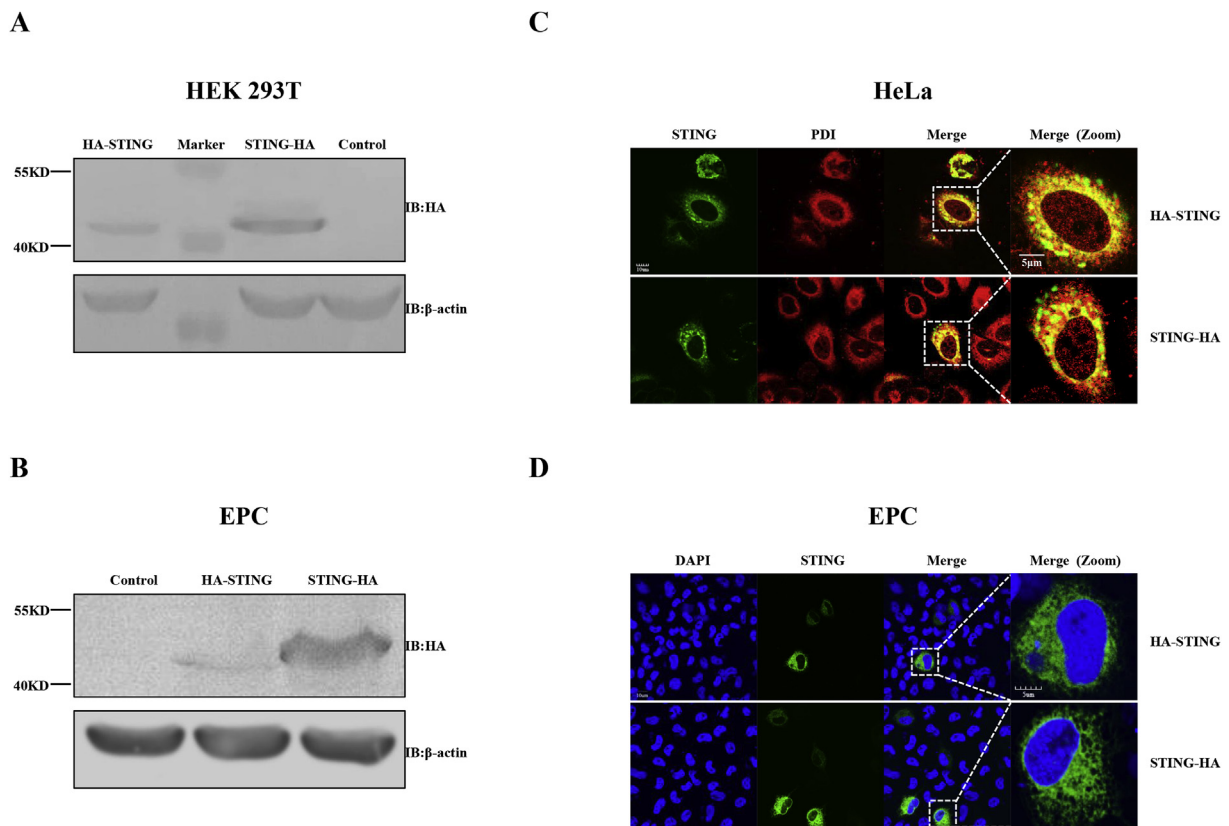

Fig. 4. Protein expression and subcellular distribution of bcSTING.
(A)&(B) HEK293T and EPC cells (in 6-well plate) were transfected with HA-bcSTING, bcSTING-HA or the empty vector (as control) separately. The transfected cells were harvested and lysed at 48 h post transfection. Whole cell lysate was used for western blot in which bcSTING was detected by anti-HA antibody. IB: immunoblot; HA-STING: pcDNA5-FRT-TO-HA-bcSTING; STING-HA: pcDNA5-FRT-TO-bcSTING-HA.
(C)&(D) EPC and HeLa cells were transfected with bcSTING or the empty vector separately; the transfected cells were fixed at 36 h post transfection and used for immunofluorescence staining according to the methods. STING (green) indicates intracellular expression of bcSTING, DAPI (blue) indicates nucleus of EPC (D); PDI (red) indicates ER of HeLa. HA-STING: pcDNA5-FRT-TO- HA-bcSTING; STING-HA: pcDNA5-FRT-TO-bcSTING-HA; the bars stand for the scale of 10 μm and 5 μm accordingly. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crucian carp (96.5%), common carp (84.9%) and zebrafish (81.5%) (Table 2).

3.2. Tissue distribution of bcSTING mRNA

The expression of bcSTING in various tissues before/after viral infection was examined by q-PCR, which included liver, skin, heart, intestine, spleen, gill and kidney. The transcription of bcSTING was constitutively detected in all the selected tissues of black carp under healthy condition, however, the bcSTING mRNA level in skin and gill was much higher than that in other tissues (Fig. 2). It was interesting that bcSTING mRNA increased in intestine and kidney but decreased in liver, skin, heart, spleen and gill of black carp in response to GCRV infection. For SVCV infected group, bcSTING mRNA increased in kidney but decreased in in liver, skin, heart, spleen, gill, and not varied much in intestine (Fig. 2).

3.3. bcSTING expression in MPF cells in response to different stimuli

To obtain the transcription profile of bcSTING in host cells, MPF cells were treated with poly (I:C) or LPS at different concentrations, or infected with SVCV or GCRV at different MOIs. The cells were harvested at indicated time point post stimulation and bcSTING

mRNA level was examined through q-PCR. bcSTING mRNA level in MPF cells was not obviously undulated after LPS treatment, which suggested that higher dose of LPS and longer time treatment needed to be taken into consideration to see if bcSTING may be induced by non-nucleic acid stimulations or not (Fig. 3A). However, bcSTING mRNA was significantly induced immediately after poly (I:C) treatment with the peak value at 2 h, then the transcription level was recovered at 8 h and up-regulated once again at 24 h (Fig. 3B). Similar results to that of poly (I:C) group were observed in both SVCV and GCRV infected MPF cells. bcSTING mRNA level was significantly increased immediately after viral infection and recovered; then increased again during the first 72 h post viral infection (Fig. 3C&D). These data indicated clearly that bcSTING was recruited in host antiviral innate immune response against these two RNA viruses.

3.4. Protein expression and intracellular distribution of bcSTING

The immunoblot data in HEK293T cells demonstrated that bcSTING had been well expressed in mammalian system. This fish protein migrated around ~42 KDa in immunoblotting and the expression level of bcSTING-HA was much higher than that of HA-bcSTING, which matched its predicted molecular weight
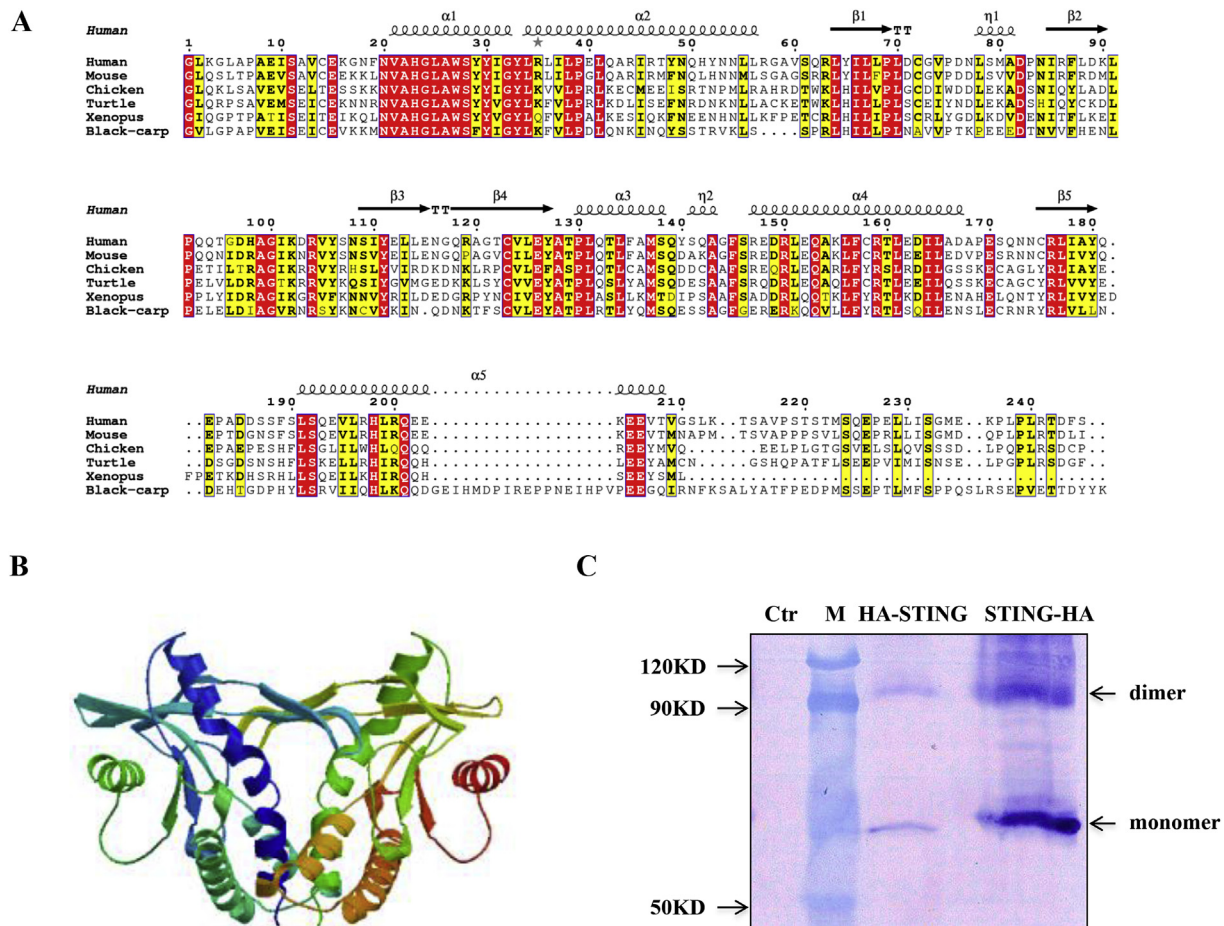

Fig. 5. Homology modelling and dimer formation of bcSTING.
(A) Alignment of the CTD sequences of STING from human (NM_198282.3), mouse (KR154221.1), chicken (KP893157.1), turtle (XM_014578148.1), xenopus (NM_001112974.1) and black carp. Schematic representations of the secondary structure elements of unliganded CTD are shown at the top of the sequences. The α helix is depicted by coil and the β strand is indicated by arrow. ClustalW conducted alignment and the figure was generated by ESPript.
(B) 3-D homology model of the CTD of bcSTING was generated by the SWISS-MODEL program (template: 4ksy.1. A). The SWISS-MODEL template library was searched with Blast and HHBlits for evolutionary related structures matching the target sequence of bcSTING. (http://swissmodel.expasy.org/).
(C) HEK293T cells (in 6-well plate) were transfected with HA-bcSTING, bcSTING-HA or the empty vector (as control) separately. The transfected cells were harvested at 48 h post transfection and used for dimerization detection as described in methods.

(Fig. 4A&B).

To determine the intracellular distribution of bcSTING, both EPC cells and HeLa cells were transfected with HA-bcSTING or bcSTING-HA; and used for immunofluorescence staining (IF), in which bcSTING was detected by anti-HA antibody and nucleus were stained with DAPI. The IF data of both EPC and HeLa cells showed clearly that bcSTING expression region (green) surrounded tightly the nucleus (blue), which demonstrated that bcSTING was a cytosolic protein. In the IF data of HeLa cells, the PDI stained region (red) overlapped most bcSTING-expressing area (green), which demonstrated that bcSTING majorly located on ER (Fig. 4C&D).

Amino acid sequence analysis showed that CTD of STING, especially its dimerization domain (DD, 153–173 of human STING) was conserved in all the species examined, which included human, mouse, chicken, turtle, xenopus, and black carp (Fig. 1A). Dimerization of human STING was crucial for the activation of this adaptor protein, which was happened between CDNs-binding domains of STING (CBD, residues153 to 340) and formed a butterfly-shaped dimer [36]. 3-D homology model of the CTD domain of bcSTING was generated in this study and the butterfly-shaped dimer of bcSTING was similar to that of human STING (Fig. 5 A&B). In the bcSTING dimerization detection assay, specific bands for dimer and monomer were detected through immunoblot assay in the whole cell lysate of 293T cells transfected with bcSTING, in which the lagged migration of bcSTING (>50 KDa for the monomer) was derived from the non-denaturing treatment (Fig. 5C). These data demonstrated clearly that bcSTING could form the hydrophobic dimer like its mammalian counterpart.

3.5. Induced IFN expression by bcSTING

In the innate immune response of higher vertebrates, activated STING induced the expression of type I IFNs and IFN-stimulated-genes (ISGs) through activation of IRF-3/7 [17]. To investigate whether bcSTING induced IFN expression, EPC cells were co-transfected with HA-bcSTING/bcSTING-HA and Luci-zIFN1/Luci-zIFN3 and applied to reporter assay. Overexpression of HA-bcSTING in EPC cells induced both zebrafish IFN1 and IFN3 promoter activity in a dose dependent manner. However, bcSTING-HA showed little effect on the induction of either zebrafish IFN1 or IFN3 promoter activity (Fig. 6A&B). The data of luciferase reporter assay demonstrated clearly that HA-bcSTING instead of bcSTING-HA triggered downstream signaling and induced IFN expression in tissue culture, which implied that C-terminal of bcSTING was crucial for its mediated signaling. Actually, our q-PCR data showed that the expression of bcSTING in MPF cells induced the obviously enhanced transcription of bcIFNb, bcIFNaR, bcMx1 and bcRSAD2 (Supplementary Fig. 2).

3.6. Antiviral activity of bcSTING against SVCV and GCRV

Overexpression of human and mouse STING significantly induced type I IFN and ISGs, and conversely, deficiency in STING expression impaired the antiviral response and increased susceptibility to pathogen invasion [37,38]. To elucidate the function of bcSTING in the host innate antiviral immune response, EPC cells were transfected with HA-bcSTING, bcSTING-HA or the empty vector separately at 24 h before SVCV/GCRV infection. For SVCV infection, both cytopathic effect (CPE) ratio and viral titer in the supernatant media of the EPC cells expressing HA-bcSTING were obviously decreased, which was determined by crystal violet staining and classic plaque assay separately. However, both CPE ratio and viral titer in the supernatant media of the EPC cells expressing bcSTING-HA were similar to that of control cells (Fig. 7A). Similar results were observed in GCRV infection group, viral replication was obviously inhibited by HA-bcSTING but not by
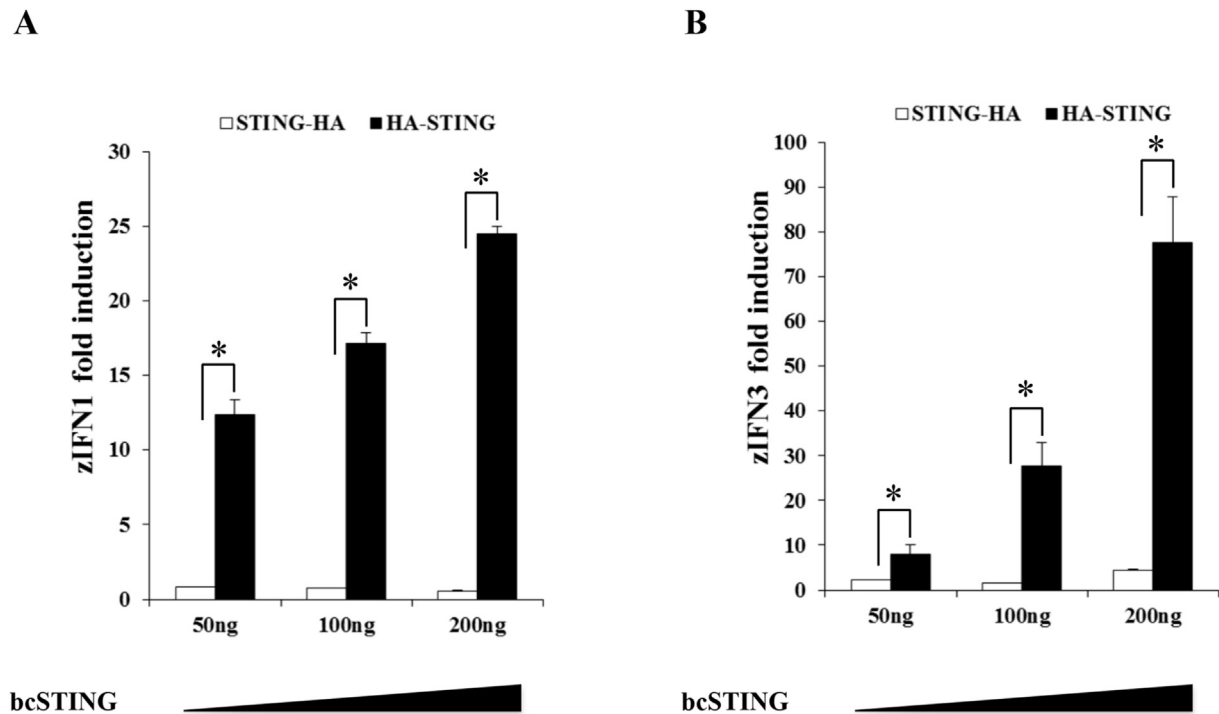
A

B

Fig. 6. Induced IFN promoter activity by bcSTING.
EPC cells were co-transfected with HA-bcSTING or bcSTING-HA, pRL-TK, Luci-zIFN1 or Luci-zIFN3. The transfected cells were harvested and used for luciferase reporter assay at 48 h post transfection. Ctr: the cells transfected with empty vector (pcDNA5/FRT/TO-HA); HA-STING: pcDNA5-FRT-TO-HA-bcSTING; STING-HA: pcDNA5-FRT-TO-bcSTING-HA.
The fold induction relative to the IFN promoter activity was normalized to control cells transfected with empty vector.
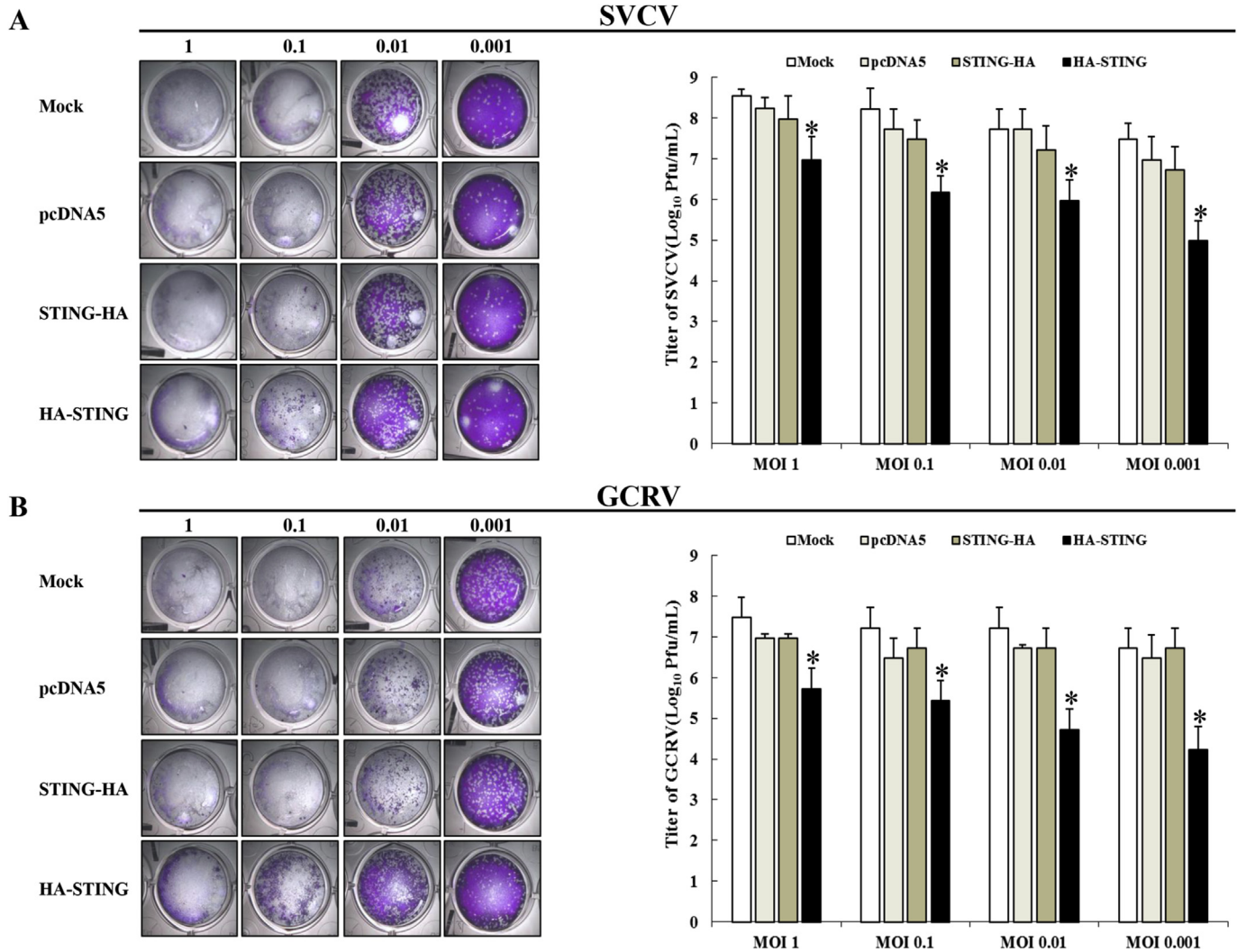
Fig. 7. EPC cells expressing bcSTING showed enhanced antiviral ability.
EPC cells in 24-well plate were transfected with 250 ng of HA-bcSTING, bcSTING-HA or the empty vector separately and infected with SVCV (**A**) or GCRV (**B**) at the indicated MOI at 24 h post transfection. The cell monolayers were stained with crystal violet (left panel) and the virus titers in the supernatant media were examined by plaque assay at 48 h post-infection (right panel). Mock: EPC cells without transfection; pcDNA5: EPC cells transfected with pcDNA5/FRT/TO-HA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bcSTING-HA (Fig. 7B). These data demonstrated clearly that exogenous bcSTING remarkably improved the antiviral ability of EPC against both SVCV and GCRV, which indicated that bcSTING functioned importantly in the antiviral innate immune response of black carp. Accordingly to the data of reporter assay, it was suggested that C-terminal of bcSTING was crucial for its antiviral activity.

3.7. Redistributed subcellular location of bcSTING by viral infection

In mammalian innate immune system, the ability of RIG-I and MAVS to induce IFN-b was diminished in STING-deficient cells, whereas the ability of STING to induce IFN-b was not affected in MAVS-deficient cells, indicating that STING functions downstream of MAVS. Mechanistically, STING interacts with MAVS, most likely at mitochondria or MAM [39]. To explore the relation between bcMAVS and bcSTING, EPC cells were transfected with bcSTING and applied to immunofluorescence staining under the condition of viral infection. bcSTING distribution partially overlapped with the area stained by Mito-tracker. However, the intracellular location of bcSTING matched the mitochondria region much well at 12 h after SVCV and GCRV infection and the similar result was seen in the poly (I:C) stimulated cells (Fig. 8), which demonstrated that viral infection led to the bcSTING transferring from ER to mitochondria. When co-expressing with bcMAVS, the intracellular location of bcSTING matched that of bcMAVS firmly whatever with or without viral infection (Fig. 9). Thus, it was speculated that bcSTING transferred to mitochondria or MAM after viral infection; where it associated with bcMAVS directly or un-directly.

Both S371 and S379 were crucial in bcSTING-induced IFN production.

HA-bcSTING instead of bcSTING-HA showed strong IFN-inducing activity suggested that C-terminus of this fish protein was crucial for it's signaling (Fig. 6). In mammal, S358 and S366 of STING were crucial for the TBK1-mediated phosphorylation of STING and for STING-dependent IRF3 activation separately [40]. Sequence alignment suggested that both S371 and S379 of bcSTING were conserved among STINGs from human, mouse, chicken, turtle
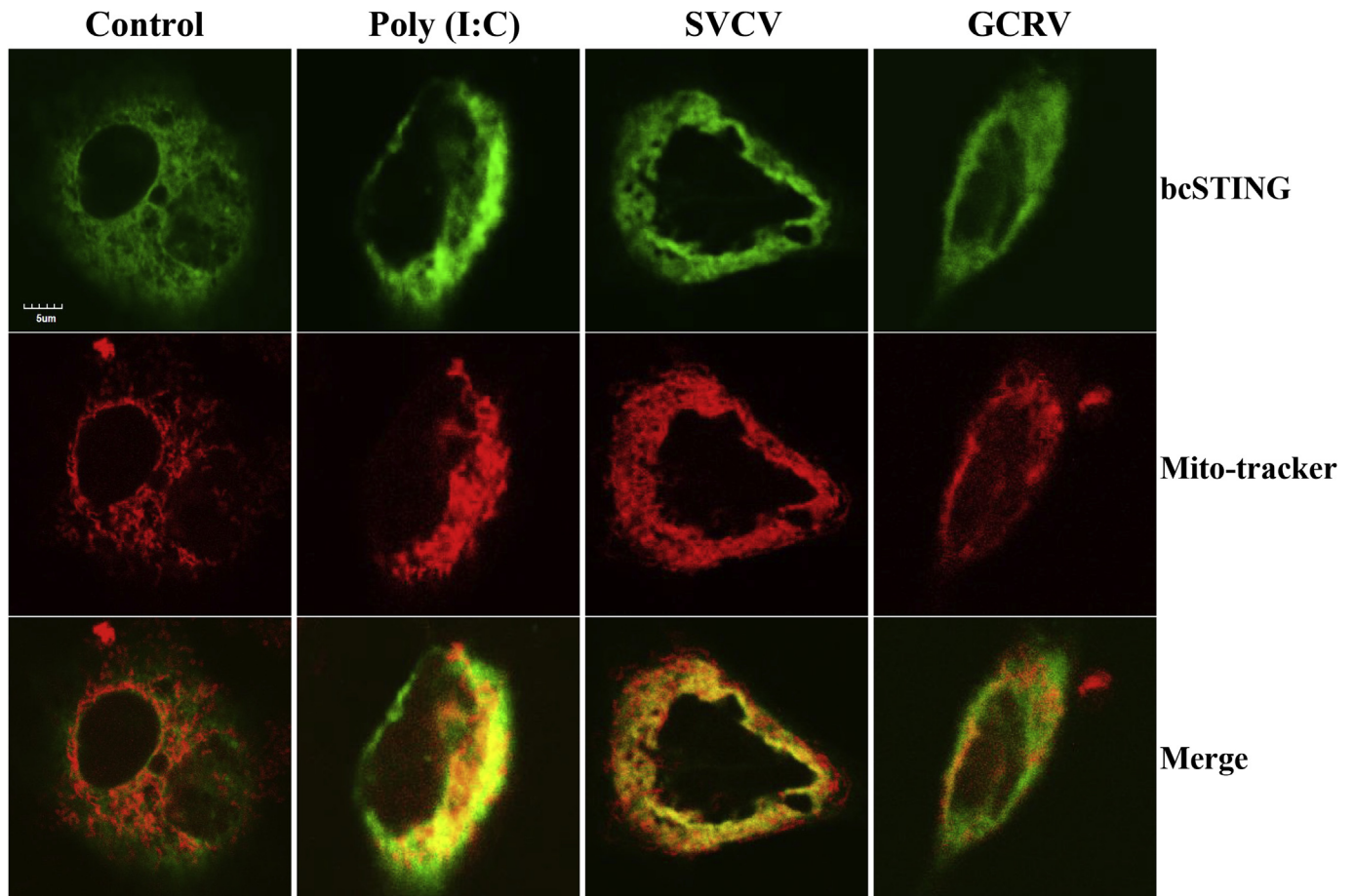
Fig. 8. Viral infection induced the redistribution of bcSTING.
EPC were transfected with bcSTING and the transfected cells were challenged with poly (I:C) (25μg/ml), SVCV or GCRV (1MOI) at 24 h post transfection. The cells were fixed at 36 h post transfection and used for immunofluorescence staining according to the methods. Control, EPC cells without challenge; bcSTING (green) indicates intracellular distribution of bcSTING; Mito-track indicates mitochondria of EPC; the bar stands for 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and xenopus (Fig. 1), which triggered us to test the roles of these two serine residues. Reporter assay data showed that single mutation of each serine residue (S371A or S379A) dramatically decreased the IFN-inducing ability of bcSTING, and mutation of both serine residues (SSAA) almost eliminated this ability (Fig. 10), which demonstrated clearly that both S371 and S379 were crucial for bcSTING-mediated signaling.

4. Discussion

DNA molecules in the cytosol are also danger signals for the innate immune system, such as exogenous DNA derived from invading pathogens and endogenous inappropriately aggregated self-DNA [41]. STING was identified as a critical mediator of the innate immune response to cytosolic DNA ligands by several groups independently [17,20—22]. Compared to the universal requirement for STING in cytosolic DNA-triggered signaling, mammalian STING seems to be involved in innate immune responses against RNA viruses in a virus- and cell type-specific manner [16,17]. Knockdown of STING impaired Sendai virus (SeV)- and vesicular stomatitis virus (VSV)-induced type I IFN production in human transformed cell lines, such as HEK293 and HeLa cells. In teleost, crucian carp STING was reported to mediate RIG-I/MDA5-activated antiviral signaling

against RNA virus [25]. Upon viral infection, STING transmits the RIG-I/MAVS-mediated signals to induce strong IFN responses to virus, demonstrating the interplay between DNA and RNA sensing, with STING identified as the central component in this crosstalk [42]. Similarly, expression of STING of crucian carp, grass carp and grouper *ex vivo* significantly inhibited virus replication through activating IRF3/7-dependent IFN expression [25—28]. To better understand the function of STING in teleost, the STING homologue was identified and characterized from black carp in this paper. And its expression profiles and downstream signaling cascades were examined in order to understand its potential roles in innate immunity.

Protein structural analysis implied that bcSTING was a membrane-bound protein with five N-terminal TM domains, however, the last TM domain was actually a DD which has recently shown to be a cytosolic and dimerization domain in human (Fig. 1) [36]. TM domains of STING are not only essential for its localization and dimerization, but also important in its interaction with MAVS to active IRF3 and induce IFNs [37,38]. The DD was the most conserved region in all species examined in this paper, suggesting an important and conserved role for this region in the function of bcSTING (Figs. 1 and 5). bcSTING shared only 48.3% identity to that of human STING (Table 2), however, the serine residues in the C-
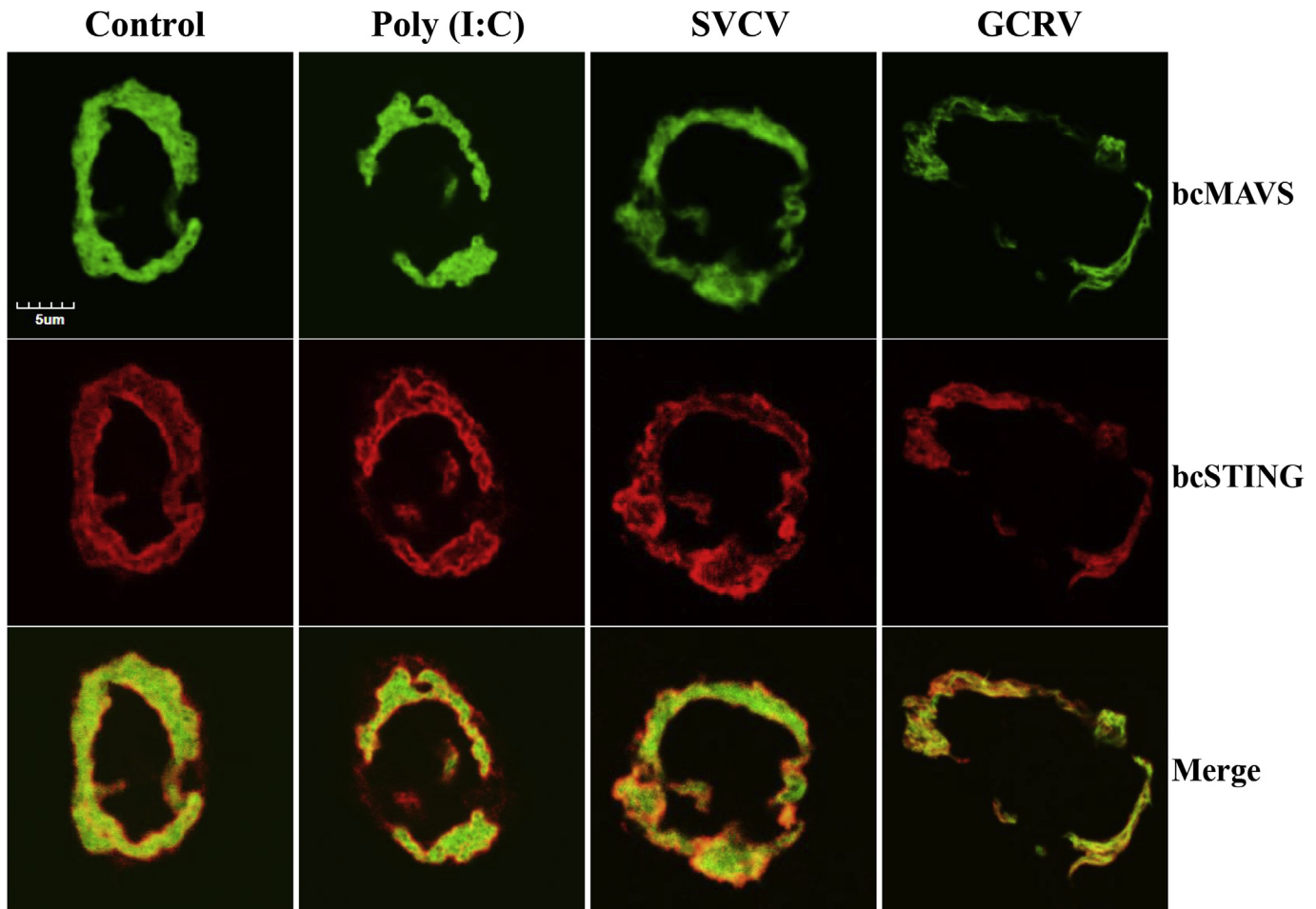
Fig. 9. Co-expressed bcSTING and bcMAVS presented identical subcellular distribution.
EPC were co-transfected with bcSTING and bcMAVS; then the transfected cells were challenged with poly (I:C) (25μg/ml), SVCV or GCRV (1MOI) at 24 h post transfection. The cells were fixed at 36 h post transfection and used for immunofluorescence staining according to the methods. Control, EPC cells without challenge; bcMAVS (green) indicates intracellular distribution of bcMAVS; bcSTING (red) indicates intracellular distribution of bcSTING; the bar stands for 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

terminal tail of bcSTING (S371 and S379) were conserved as well as human STING (S358 and S366) (Fig. 1A). Site mutation and reporter assay demonstrated that both S371 and S379 of bcSTING were crucial for its mediated signaling, which suggested the importance of the phosphorylation of these two serine residues (Fig. 10). It was interesting that bcSTING-HA presented similar IFN-inducing activity to that of HA-bcSTING-SSAA (Figs. 6 and 10). It is speculated that the C-terminal HA tag dampened the phosphorylation of S371 and S379 of bcSTING [40].

The transcription of bcSTING was constitutively detected in all the selected tissues of black carp under healthy condition (Fig. 2), which was similar to that of STING of human and murine [37,43]. After stimulation by poly (I:C), SVCV or GCRV, bcSTING mRNA level was significantly elevated at early stage, then down-regulated to the control level, while up-regulated at late phrase (Fig. 3), which suggested that the early inhibition of bcSTING may be a regulatory mechanism to prevent harmful excessive activation like that of human STING, since inappropriate or excessive activation of STING led to autoimmune disease [44,45].

When expressed in fish cells (EPC) and mammalian cells (HeLa), bcSTING scattered "completely" in the cytoplasm (Fig. 4 C&D),

which matched the distribution of ER. However, viral infection and poly (I:C) stimulation made the subcellular distribution of bcSTING "condensed ", which more matched the region labeled by Mitotracker (Fig. 8). This phenotype suggested that activated endogenous MAVS of EPC cells "attracted" bcSTING (also endogenous STING) to mitochondria after viral infection or poly (I:C) stimulation. This phenotype was more convincing when both bcSTING and bcMAVS were co-expressed in EPC cells, which demonstrated abundant exogenous bcMAVS (compared with endogenous MAVS of EPC cells) presented much more potential ability to "attract" bcSTING to mitochondrial with or without viral infection (Fig. 9). Our lab is working on the mechanism behind the association between bcSTING and bcMAVS.

There are a series of reports about the structure of human and mouse STING; however, only clear structures of its functional domains were presented since full-length STING could not be expressed as a soluble protein in E. coli. [36]. For human STING, CDNs-binding domain (CBD) exhibited a α1/β fold and formed a butterfly-shaped dimer [39]. CDNs binding was associated with an open-to-closed conformational change corresponding to open-inactivated and close-activated states [46]. Our data showed
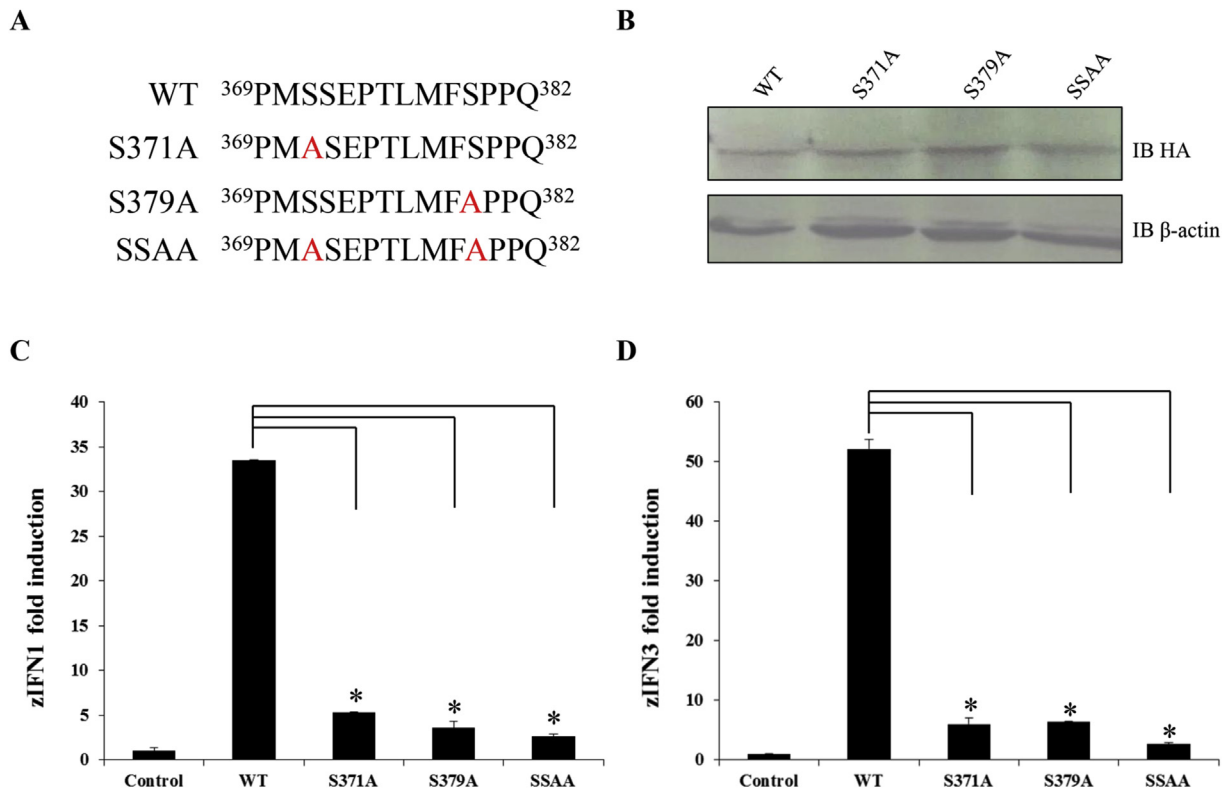
A

WT ^{369}PMSSEPTLMFSPPQ^{382}

S371A ^{369}PM**A**SEPTLMFSPPQ^{382}

S379A ^{369}PMSSEPTLMF**A**PPQ^{382}

SSAA ^{369}PM**A**SEPTLMF**A**PPQ^{382}

B

C

D

Fig. 10. Both S371 and S379 were crucial for bcSTING mediated signaling.
(A) The scheme map of the mutants of bcSTING. **(B)** EPC cells (in 6-well plate) were transfected with HA-bcSTING or its mutants separately. The transfected cells were harvested for immunoblot assay at 48 h post transfection. IB: immunoblot. **(C)&(D)** EPC cells were co-transfected with HA-bcSTING or its mutants, pRL-TK, Luci-zIFN1 or Luci-zIFN3. The transfected cells were harvested and used for luciferase reporter assay at 48 h post transfection. Control: the cells transfected with empty vector; WT: wild type bcSTING; S371A: bcSTING-S371A; S379A: bcSTING-S379A; SSAA: bcSTING-SSAA.

clearly that bcSTING also formed a hydrophobic dimer similar to that of its mammalian counterpart, which was reported in teleost fish first time (Fig. 6).

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.08.037.

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