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Black carp PRMT6 inhibits TBK1-IRF3/7 signaling during the antiviral innate immune activation

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ABSTRACT

Protein arginine methylation is a prevalent posttranslational modification and protein arginine methyltransferases 6 (PRMT6) has been identified as a suppressor of TBK1/IRF3 in human and mammals. To explore the role of PRMT6 in teleost fish, PRMT6 homologue of black carp (*Mylopharyngodon piceus*) has been cloned and characterized in this study. Black carp PRMT6 (bcPRMT6) transcription in host cells varies in response to different stimuli and bcPRMT6 migrates around 43 kDa in the immunoblot assay. Like its mammalian counterpart, bcPRMT6 has been identified to distribute majorly in the nucleus through the immunofluorescent staining assay. bcPRMT6 shows little interferon (IFN) promoter-inducing activity in the reporter assay and bcPRMT6 shows no antiviral activity against either grass carp reovirus (GCRV) or spring viremia of carp virus (SVCV) in plaque assay. When co-expressed with bcPRMT6, the IFN promoter-inducing abilities of black carp TBK1 (bcTBK1) and IRF3/7 (bcIRF3/7) are fiercely attenuated. Accordingly, bcTBK1-mediated antiviral activity in EPC cells is obviously dampened by bcPRMT6. The interaction between bcPRMT6 and bcIRF3/7 has been identified by co-immunoprecipitation assay; however, no direct association between bcPRMT6 and bcTBK1 has been detected. Taken together, our data elucidates for the first time in teleost fish that PRMT6 suppresses TBK1-IRF3/7 signaling during host antiviral innate immune activation.

1. Introduction

Vertebrates utilize their immune system to survive from the adverse environments such as virus infection, which can be classified into innate immune system and adaptive immune system [1]. The initial sensing of the invading pathogenic microbes is mediated by innate pattern recognition receptors (PRRs), which detect the molecular components of microorganisms known as pathogen-associated molecular patterns (PAMPs) [2,3]. In human and mammals, once viral nucleic acids have been recognized by cytosolic RIG-I-like receptors (RLRs), different adaptor proteins, such as mitochondria antiviral-signaling protein (MAVS) or stimulator of interferon genes (STING), are promptly recruited to activate TANK-binding kinase 1 (TBK1) through down-stream signaling. Activated TBK1 then phosphorylates interferon regulatory factor 3/7 (IRF3/7) and triggers its nuclear translocation, which initiates the production of type I interferon (IFN) and finally triggers host innate immune activation [4,5].

Protein arginine methylation is a prevalent posttranslational modification, which is implicated in a bulk of biological processes, such RNA processing, DNA repair and signal transduction [6,7]. Nine mammalian protein arginine methyltransferase (PRMT) members have been identified in humans till now, which all contain a conserved core region that includes a methyltransferase (MTase) domain, a β-barrel, and a dimerization arm [8–10]. Protein arginine methyltransferase 6 (PRMT6) was first identified as a nuclear enzyme displaying unique substrate specificity [11]. Subsequential study has realized that PRMT6 plays a role as repressor in transcriptional process. For instance, PRMT6 suppresses the transcription of p21, which is a cyclin-dependent kinase (CDK) inhibitor [12]. PRMT6 is also found to be involved in virus-related diseases in human, in which overexpression of PRMT6 reduces *trans*-activator of transcription (Tat) transactivation of HIV-1 and viral replication [13]. Recent evidence has indicated that PRMT6 deficiency enhances the antiviral immune response by promoting the activation of IRF3 and the production of type-I interferon, which provides new insight into the regulation of TBK1–IRF3 signaling and characterizes PRMT6 as a key regulator of antiviral innate immunity [14].

Compared with its mammalian counterpart, the role of teleost PRMT6 remains largely unknown. Hitherto, PRMT6 homologues have

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
CDS		
bcPRMT6-F	ATGGCAAACTTGGGGAAA	1068bp
bcPRMT6-R	CTATTTAACTTCTAAATACTGATCAG	bcPRMT6 CDS cloning
Expression construct		
HA-bcPRMT6-F	ACTGACGCTAGCATGGCAAACTTGGGGAAA	
HA-bcPRMT6-R	ACTGACGGGCCCCTATTTAACTTCTAAATACTGATCAG	FRT-To-HA-bcPRMT6
bcPRMT6-HA-F	ACTGACAAGCTTGCCACCATGGCAAACTTGGGGAAA	
bcPRMT6-HA-R	ACTGACGCGGCCGCAGTTTAACTTCTAAATACTGATCAG	FRT-To-bcPRMT6-HA
q-PCR		
bc Q actin-F	TGGGCACCGCTGCTTCCT	
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	q-PCR
bc-QPRMT6-F1	TGATTGTCAGCGAGTGGA	
bc-QPRMT6-R1	TATGGGAGCGATGTAAAG	q-PCR

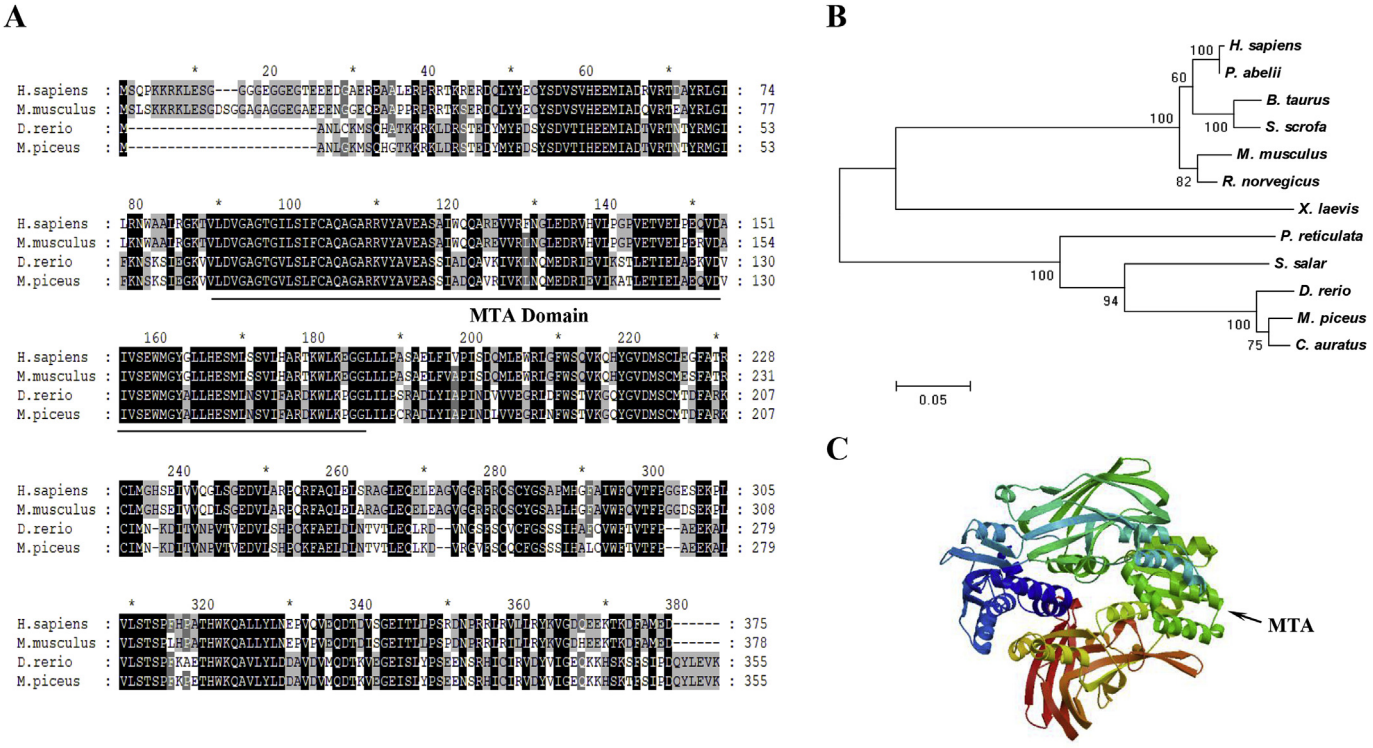

Fig. 1. Evolution of vertebrate PRMT6.
A. Comparisons of bcPRMT6 with other vertebrate PRMT6 proteins by using MEGA 6.0 program and GeneDoc program, which including: *H. sapiens* (AAH73866.1), *M. musculus* (AAH66221.1), *D. rerio* (NP_001157460.2), and *M. piceus* (MK713969). The protein domains were predicted by CDS (Conserved Domain Search) of NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), and Simple Modular Architecture Research Tool (SMART) (http://smart.emble-heidelberg.de).
B. By using MEGA 5.0 program, the maximum likelihood phylogenetic tree was generated from vertebrate PRMT6 of different species, which include (GenBank accession number): *C. auratus* (XP_026093589.1), *D. rerio*:(NP_001157460.2), *S. salar* (XP_014012802.1), *P. reticulata* (XP_008436573), *M. musculus* (AAH66221.1), *B. taurus* (NP_001014962.1), *H. sapiens* (AAH73866.1), *X. laevis* (NP_001087520.1), *S. scrofa* (NP_001177112.1), *R. norvegicus* (NP_001099936.1), and *P. abelii*: (XP_002832593.4). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.
C. The predicted protein structure of bcPRMT6 (by SWISS-MODEL; https://www.swissmodel.expasy.org/).

been cloned and characterized only in several species, such as puffer fish (*Fugu rubripes*), zebrafish (*Danio rerio*) and Japanese flounder (*Paralichthys olivaceus*) [15]. Study of PRMT6 of zebrafish and puffer fish demonstrates that the characteristic one coding exon in PRMT6 is conserved from fish to human and zebrafish PRMT6 is expressed in brain but rare in other tissues [16]. Another study indicates that PRMT6 is essential for early zebrafish development [17].

Black carp (*Mylopharyngodon piceus*) is one of the "Four Domesticated Fish" in China. This economically important species is susceptible to bulk of pathogenic microorganisms including grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), which are two major RNA viruses threatening Chinese fresh water industry [18]. In our previous study, bcTBK1 and bcIRF3/7 have been cloned and characterized. And similar to their mammalian counterparts, bcTBK1 and bcIRF3/7 play the crucial roles in the host antiviral immune responses against GCRV and SVCV [19,20]. To elucidate the function of PRMT6 in black carp during the innate immune response against GCRV and SVCV, PRMT6 of black carp (bcPRMT6) has been cloned and characterized in this paper. This fish PRMT6, like its mammalian counterpart, is expressed in the nucleus. bcPRMT6 interacts with bcIRF3/7 and negatively regulates TBK1-IRF3/7 signaling during host innate immune response against GCRV and SVCV, which is similar to

Table 2
Comparison of bcPRMT6 with other vertebrate PRMT6 (%).

Species	Full-length sequence	
	Similarity	Identity
Mylopharyngodon. piceus	100	100
Mus. musculus	66.6	50.8
Homo. sapiens	67.6	51.4
Rattus. norvegicus	67.9	50.9
Sus. scrofa	67.1	50.9
Bos. taurus	66.8	50.1
Pongo abelii	67.9	51.7
Xenopus laevis	72.5	55.9
Poecilia reticulata	84.9	70.7
Carassius auratus	96.6	95.5
Salmo salar	88.1	79.5
Danio. rerio	96.3	94.4

PRMT6 of human and mammals.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK), and *Mylopharyngodon piceus* kidney (MPK) cells were kept in the lab [21]. HEK293T cells were cultured at 37 °C with 5% CO_2; EPC, CIK and MPK cells were cultured at 26 °C with 5% CO_2. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was done as previously described, calcium phosphate was used for HEK293T transfection; Lipomax (SUDGEN) was used for EPC cells transfection [22].

pcDNA5/FRT/TO (Invitrogen, USA), pcDNA5/FRT/TO-HA-bcTBK1, pcDNA5/FRT/TO-HA-bcIRF3, pcDNA5/FRT/TO-HA-bcIRF7, pcDNA5/FRT/TO-bcTBK1-Flag, pcDNA5/FRT/TO-Flag-bcIRF3, pcDNA5/FRT/TO-Flag-bcIRF7, pRL-TK, Luci-DrIFNϕ1/3 (for zebrafish IFNϕ1/3 promoter activity analysis accordingly) and Luci-bcIFNa (for black carp IFNa promoter activity analysis) were kept in the lab [23]. The recombinant expression vector pcDNA5/FRT/TO-HA-bcPRMT6 and pcDNA5/FRT/TO-bcPRMT6-HA were constructed by cloning the open reading frame (ORF) of bcPRMT6 fused with a HA tag at its N-terminus/C-terminus into pcDNA5/FRT/TO, respectively.

2.2. Virus produce and titration

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab and propagated in EPC or CIK cells separately at 26 °C in the presence of 2% fetal bovine serum. EPC or CIK cells were infected with SVCV or GCRV accordingly; the cells and the supernatant media were

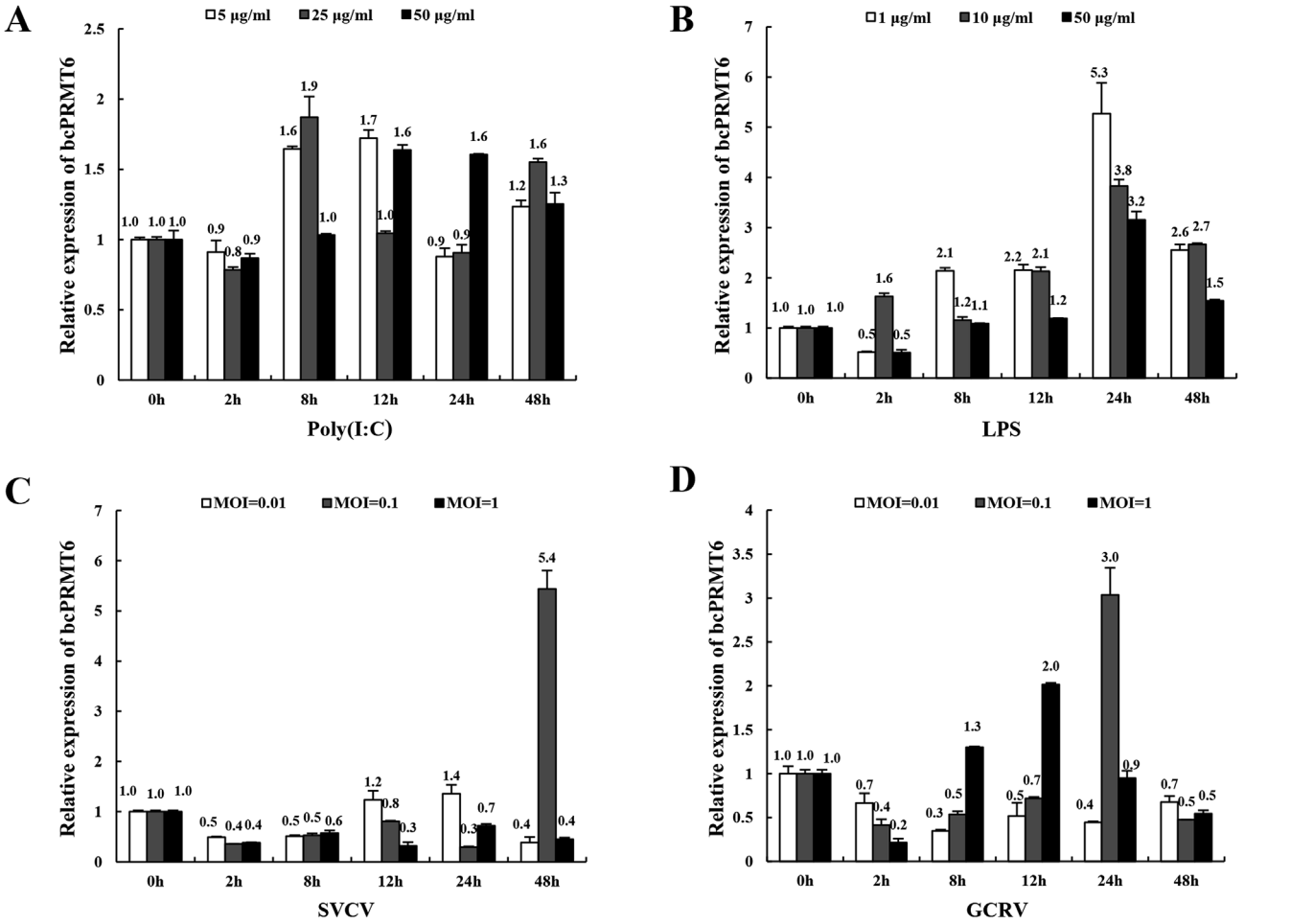

Fig. 2. Expression of bcPRMT6 in response to different stimuli.
MPK cells in 6-well plate (2×10^6 cells/well) were treated with poly (I:C) (A) or LPS (B) at the indicated concentration separately; or infected with SVCV (C) or GCRV (D) at the indicated MOI separately. The cells were harvested at the indicated time points post stimulation separately and used for RNA isolation. The relative bcPRMT6 mRNA level was examined by q-PCR. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$. Two-tailed Student's *t*-test was used for the statistical analysis with the GraphPad Prism 4.0 software (GraphPad Prism, USA).

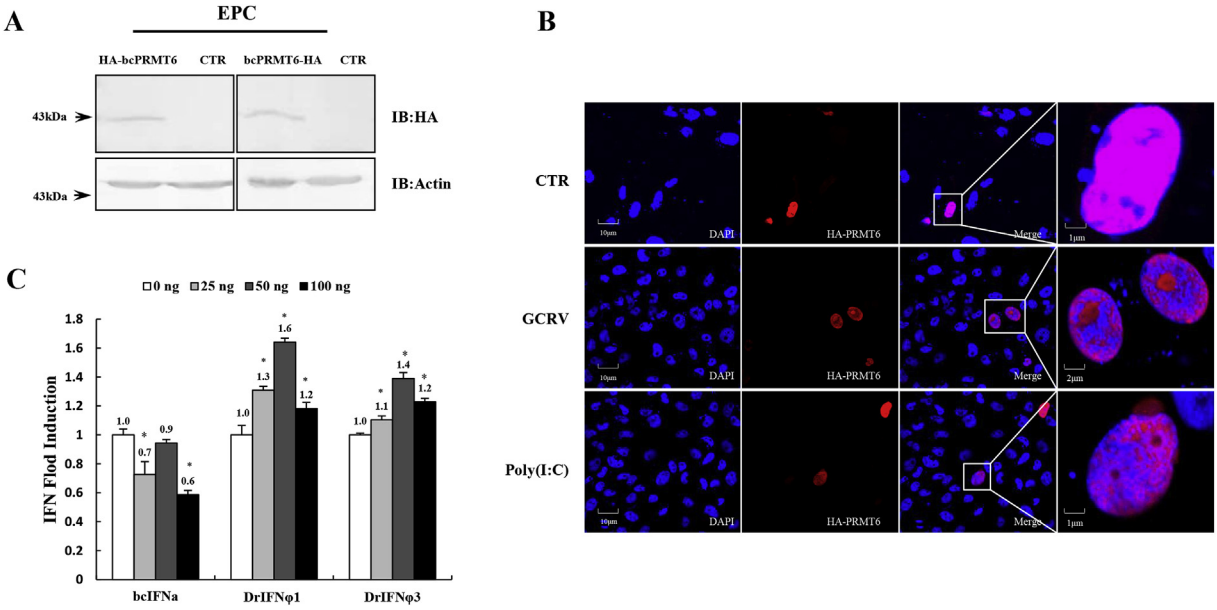

Fig. 3. Protein expression, subcellular distribution and IFN-inducing ability of bcPRMT6.
(A): Immunoblot assay of bcPRMT6 in EPC cells. CTR: the control cells transfected with empty vector, HA-bcPRMT6: pcDNA5/FRT/TO-HA-bcPRMT6, bcPRMT6-HA: pcDNA5/FRT/TO-bcPRMT6-HA, IB: immunoblot.
(B): Immunofluorescence staining of bcPRMT6 in EPC cells. HA-bcPRMT6: pcDNA5/FRT/TO-HA-bcPRMT6; CTR: EPC cells without treatment; poly (I:C): EPC cells treated with poly (I:C) (25 μg/ml) for 2 h; GCRV: EPC cells treated with GCRV (MOI = 1) for 2 h. The bar stands for the scale of 1 μm and 10 μm accordingly.
(C): IFN promoter activity induced by bcPRMT6. HA-bcPRMT6: pcDNA5/FRT/TO-HA-bcPRMT6; bcIFNa: black carp IFNa, DrIFNϕ1: zebrafish IFN1; DrIFNϕ3: zebrafish IFN3. The numbers above the error bars stand for the average IFN fold induction. 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.

collected together when the cytopathic effect (CPE) was about 50% and stored at −80 °C. After freezing and thawing for three times, the mixture was used for virus titer mensuration. Virus titers were determined by plaque assay on EPC cells as previously described [24]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 26 °C. The supernatant was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) after incubation. Plaques were counted at day 3 post infection.

2.3. LPS and poly (I:C) treatment

MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before treatment. Poly (I:C) (Sigma, USA) was used for synthetic dsRNA stimulation, which was heated to 55 °C (in PBS) for 5 min and cooled at room temperature before use. MPK cells were replaced with fresh media containing poly (I:C) at the final concentration of 5 μg/ml, 25 μg/ml, or 50 μg/ml and harvested at different time points (2 h, 8 h, 12 h, 24 h, and 48 h) post treatment. For LPS (Sigma, USA) treatment, MPK cells in 6-well plate (2×10^6 cells/well) were treated with LPS (1 μg/ml, 10 μg/ml, and 50 μg/ml) separately and harvested at different time points (2 h, 8 h, 12 h, 24 h, and 48 h) post stimulation as above.

2.4. Quantitative real-time PCR

The relative bcPRMT6 mRNA level in the MPK cells was determined by quantitative real-time PCR (q-PCR). The primers for bcPRMT6 and β-actin (as internal control) were listed in Table 1. The q-PCR program was: 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 Applied Biosystems Fast 7500 Real-Time PCR System (ABI, USA) and exported into a Microsoft Excel spreadsheet for subsequent data analysis where the relative expression ratios of target gene in treated groups versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method [21].

2.5. Luciferase reporter assay

EPC cells in 24-well plate (4×10^5 cells/well) were co-transfected with expression plasmids as required, pRL-TK, and Luci-DrIFNϕ1/3 (or Luci-bcIFN). For each transfection, the total amount of DNA was balanced with the empty vector. The cells were harvested and lysed at 24 h post transfection. The centrifuged supernatant was used to measure the activities of firefly luciferase and renilla luciferase according to the instruction of the manufacturer (Promega, USA) as described previously [25].

2.6. Immunoblotting

EPC cells or HEK293T cells in 6-well plate (2×10^6 cells/well) were transfected with pcDNA5/FRT/TO-HA-bcPRMT6, pcDNA5/FRT/TO-bcPRMT6-HA or the empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [23]. Briefly, the whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membranes were probed with mouse monoclonal *anti*-HA antibody (1:3000; Sigma, USA), which were followed by the incubation with goat-*anti*-mouse IgG (1:30000; Sigma, USA). The target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, USA).

2.7. Immunofluorescence microscopy

EPC cells in 24-well plate were transfected with plasmids expressing bcPRMT6 or the empty vector separately. The transfected cells were fixed with 4% (v/v) paraformaldehyde at 24 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 [21]. Rabbit monoclonal *anti*-HA antibody (Sigma, USA) was probed at the ratio of 1:500; Alexa 594-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:1000; DAPI was used for nucleus

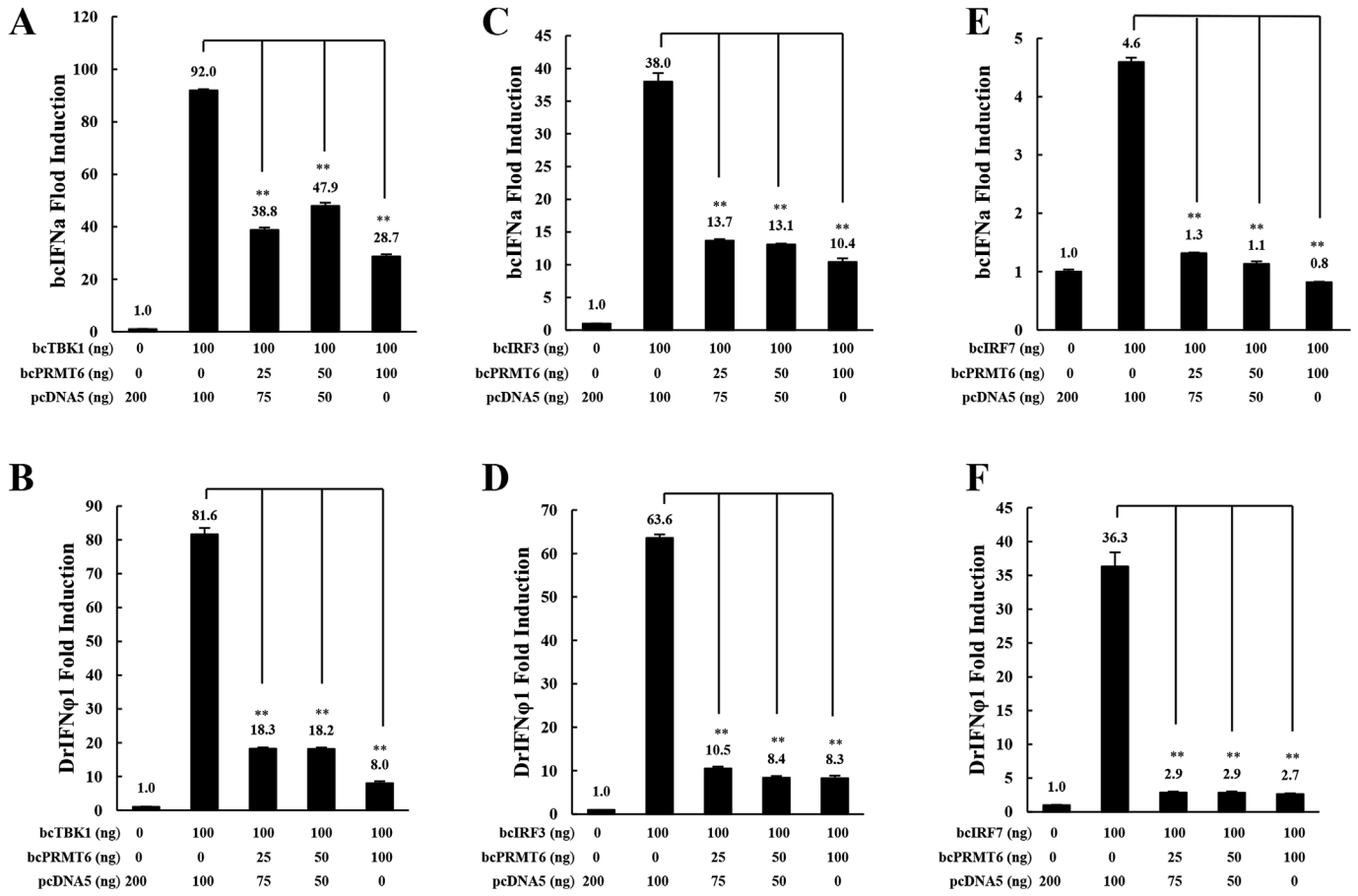

Fig. 4. IFN-inducing activity of bcTBK1 and bcIRF3/7 was down-regulated by bcPRMT6.
EPC cells in 24-well plate were co-transfected with bcPRMT6, bcTBK1 or bcIRF3/7, pRL-TK, Luci-zIFNϕ1 or Luci-bcIFNa, and the cells were harvested at 24 h post transfection and used for reporter assay. For each transfection, the total amount of DNA was balanced with the empty vector. (A, C and E) Black carp IFNa promoter activity induction. (B, D and F) Zebrafish IFNϕ1 promoter activity induction. bcPRMT6: pcDNA5/FRT/TO-HA-bcPRMT6, bcTBK1: pcDNA5/FRT/TO-HA-bcTBK1, bcIRF3: pcDNA5/FRT/TO-HA-bcIRF3, bcIR7: pcDNA5/FRT/TO-HA-bcIRF7, pcDNA5: pcDNA5/FRT/TO empty vector. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$ and (**) stands for $p < 0.01$. The data were analyzed by two-tailed Student's t-test.

staining.

2.8. Co-immunoprecipitation (Co-IP)

HEK293T cells in 10 cm plate were co-transfected with pcDNA5/FRT/TO- HA-bcPRMT6 and/or pcDNA5/FRT/TO-bcTBK1-Flag (pcDNA5/FRT/TO- Flag-bcIRF3 or pcDNA5/FRT/TO-Flag-bcIRF7). The transfected cells were harvested at 48 h post-transfection and lysed for immunoprecipitation (IP) assay as previously described [26]. The whole cell lysates of the transfected cells was incubated with protein A/G agarose beads at 4 °C for 2 h. Flag-conjugated protein A/G agarose beads were added in the supernatant after pre-clearing and incubated with the supernatant media at 4 °C for 4 h. Flag-conjugated protein A/G agarose beads were boiled in 6 x sample buffer after 3–5 times of wash and the eluted proteins were used for IB as above.

3. Results

3.1. Molecular cloning and sequence analysis of bcPRMT6

To learn the role of bcPRMT6 in teleost, the cDNA of PRMT6 was cloned from the liver of black carp and the coding sequence of bcPRMT6 consists of 1068 nucleotides (NCBI accession number: MK713969). Initial sequence analysis of bcPRMT6 predicts that bcPRMT6 contains 355 amino acid residues, consisting of a catalytic core sequence common to other PRMTs, named methyltransferase (MTA) domain (65aa-161aa), which also are conserved among PRMT6 proteins from human (*H. sapiens*), mouse (*M. musculus*) and zebrafish. The data demonstrates that PRMT6 is a conserved protein in vertebrates, especially its MTA domain (Fig. 1A and C). bcPRMT6 has a calculated molecular weight of 39.95 kDa and an isoelectric point of 5.39 (http://web.expasy.org/protparam/). To gain insight into bcPRMT6 evolution, amino acid sequence of bcPRMT6 has been subjected to multiple alignments with those of PRMT6 proteins from different species. The result showed that bcPRMT6 shares high amino acid sequence similarity with zebrafish PRMT6 (96.3%) and goldfish (*C. auratus*) PRMT6 (96.6%) (Table 2). Phylogenetic analysis of PRMT6 proteins from the selected species demonstrates that these PRMT6 homologue proteins could be divided into three groups, consisting of mammals, amphibians and fish branches (Fig. 1B). Obviously, bcPRMT6 is clustered with zebrafish and goldfish PRMT6 tightly, which correlates with the close genetic relationship of these two species.

3.2. bcPRMT6 expression in vitro in response to different stimuli

To learn bcPRMT6 mRNA profile during host innate immune response, MPK cells were subject to different stimuli and bcPRMT6 transcription was examined by qPCR. In poly (I:C) treated MPK cells, bcPRMT6 mRNA was not increased obviously after treatment at all doses, and the highest level was up to 1.9-fold (25 μg/ml; 8 h point) to

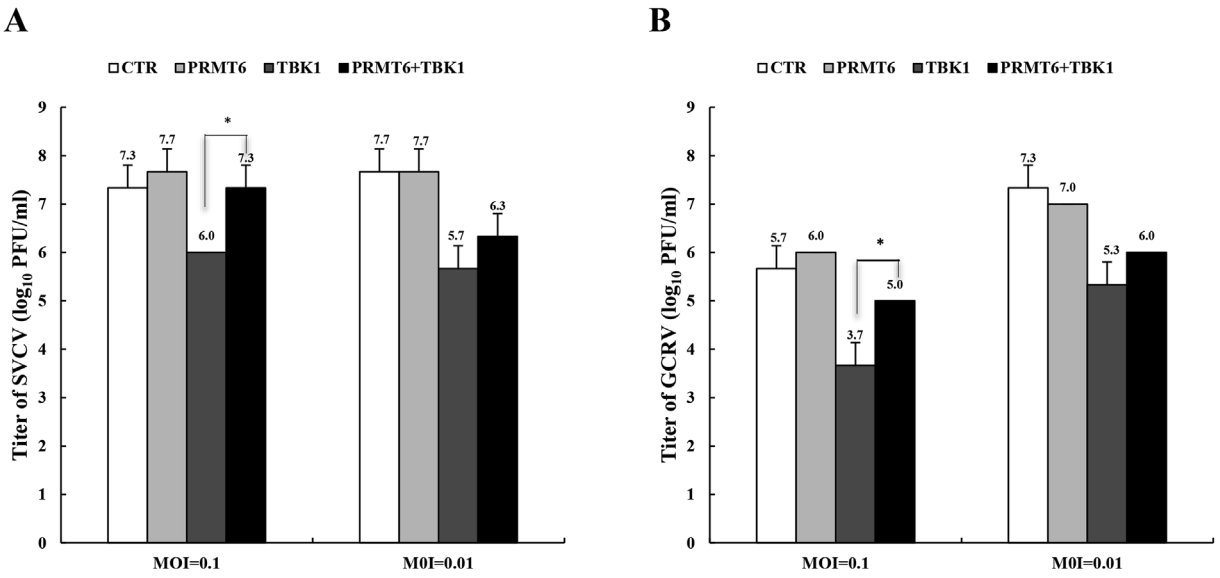

Fig. 5. Down-regulated antiviral activity of bcTBK1 by bcPRMT6.
EPC cells in 24-well plate were transfected with bcPRMT6 and/or bcTBK1; and then infected with GCRV or SVCV at 24 h post transfection. The virus titers in the supernatant media were determined by plaque assay at 24 h post infection. pcDNA5: pcDNA5/FRT/TO; PRMT6: pcDNA5/FRT/TO-HA-PRMT6; TBK1: pcDNA5/FRT/TO-HA-bcTBK1. Error bar represents 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.

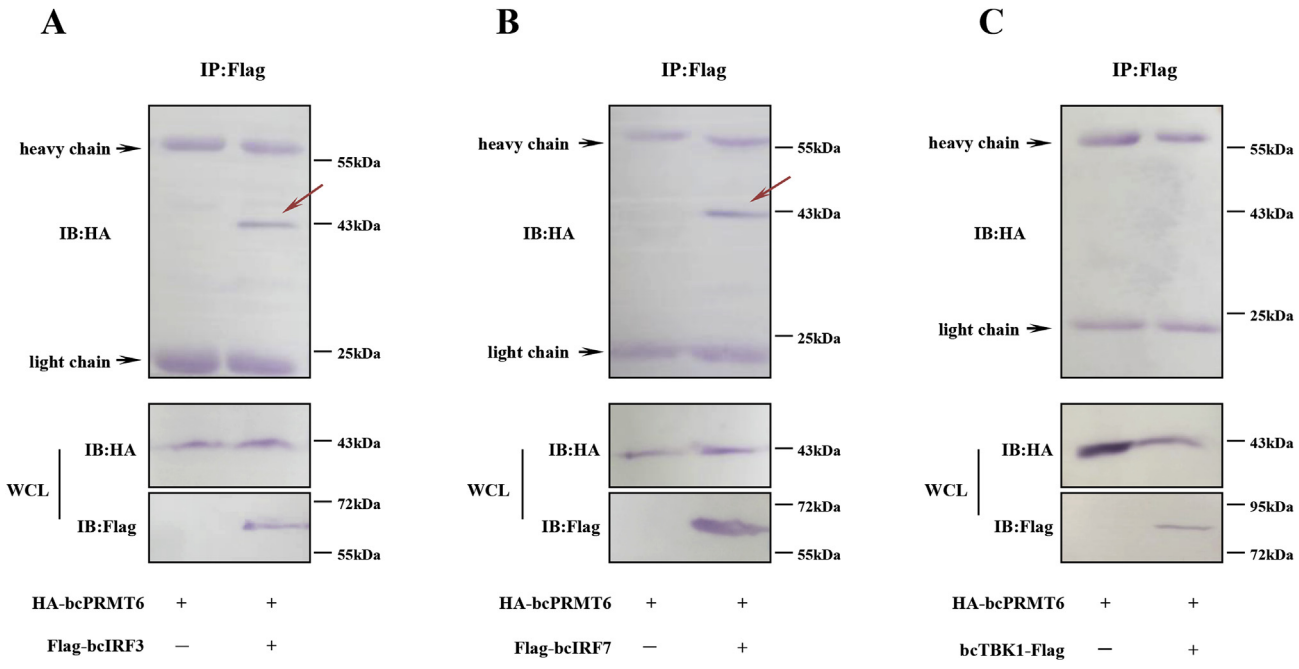

Fig. 6. The interaction between bcPRMT6 and bcIRF3/7.
A) Co-IP between bcPRMT6 and bcIRF3.
B) Co-IP between bcPRMT6 and bcIRF7.
C) Co-IP between bcPRMT6 and bcTBK1.
IB: immunoblot; IP: immunoprecipitation; WCL: whole cell lysate; HA-bcPRMT6: pcDNA5/FRT/TO-HA-bcPRMT6; bcTBK1-Flag: pcDNA5/FRT/TO-bcTBK1-Flag; Flag-bcIRF3:pcDNA5/FRT/TO- Flag-bcIRF3; Flag-bcIRF7:pcDNA5/FRT/TO-Flag-bcIRF7. The heavy chain and light chain were indicated with black arrow.

that of control (Fig. 2A). In LPS treated MPK cells, the transcription of bcPRMT6 increased right after stimulation except a decrease at 2 h point of 1 μg/ml and 50 μg/ml treatment, it was interesting that bcPRMT6 mRNA level reached the peak at 24 h for all doses within 48 h post infection (hpi) (Fig. 2B). In SVCV infected MPK cells, bcPRMT6 mRNA level varied within 48 hpi. In the group of 0.01 MOI, bcPRMT6 mRNA level was decreased right after infection and increased at 12 hpi, then decreased again at 48 hpi. In the group of 0.1 MOI, bcPRMT6 mRNA level was decreased right after infection and obviously increased at 48 hpi. In the group of 1 MOI, bcPRMT6 mRNA level was decreased right after infection and kept lower than that of control within 48 hpi (Fig. 2C). In GCRV infected MPK cells, bcPRMT6 mRNA level in the group of 0.1 MOI and 1 MOI was decreased right after infection (2 hpi) and was increased from 8 hpi, then was decreased again. However, bcPRMT6 mRNA level in the group of 0.01 MOI was deceased right after infection and kept lower than the control within 48 hpi (Fig. 2D).

These data indicated that bcPRMT6 was involved into host innate immune response initiated by virus and bacteria, however, might be recruited into different mechanisms.

3.3. Protein expression and subcellular distribution of bcPRMT6

EPC cells were transfected with plasmids expressing bcPRMT6 and used for immunoblotting (IB) assay to investigate the protein expression of bcPRMT6, in which mouse *anti*-HA antibody were used to detect the exogenous bcPRMT6. In the IB assay of EPC cells, a specific band of ~43 kDa was detected in the whole cell lysate of EPC cells expressing HA-bcPRMT6 or bcPRMT6-HA (Fig. 3A), which matched the predicted molecular weight of bcPRMT6. To determine the subcellular location of bcPRMT6, EPC cells were transfected with plasmids expressing bcPRMT6 and used for immunofluorescence staining (IF). In the results of IF assay, the red color representing bcPRMT6 expressing region overlapped with the blue fluorescence (DAPI), which illustrated that bcPRMT6 distributed majorly in nucleus. It has been shown that the distribution of bcPRMT6 in EPC cells was not impacted by the treatment of poly (I:C) or the infection of GCRV (Fig. 3B).

3.4. IFN signaling regulated by bcPRMT6

In mammals, PRMT6 attenuates the antiviral innate immunity by blocking TBK1/IRF3 signaling [14]. To investigate the effect of bcPRMT6 on IFN signaling, EPC cells were transfected with plasmids expressing bcPRMT6 and used for the dual luciferase reporter assay. The data showed that bcPRMT6 had feeble ability to activate the transcription of bcIFNa and DrIFNϕ1/3 promoter (Fig. 3C). To further explore the role of bcPRMT6 in fish RLR/IFN signaling, EPC cells were co-transfected with bcPRMT6, bcTBK1, bcIRF3 and bcIRF7, which are the key components of RLR/IFN signaling of black carp and possess IFN-inducing activity in host innate immune activation [27]. The reporter assay data showed that the induced transcription of both bcIFNa promoter and DrIFNϕ3 promoter by bcTBK1 was fiercely reduced by bcPRMT6 (Fig. 4A and B). Similar results were observed in EPC cells co-expressing bcIRF3/7 and bcPRMT6, in which bcIRF3/7 activated IFN promoter transcription was obviously reduced by bcPRMT6. (Fig. 4C, D, E & F). The data implies that this fish PRMT6 functions as a suppressor of TBK1/IRF signaling like its mammalian counterpart.

3.5. Down-regulated antiviral activity of bcTBK1 by bcPRMT6

Our previous study has identified that overexpressed bcTBK1 enhanced the antiviral activity of EPC cells against both SVCV and GCRV [20]. To characterize the role of bcPRMT6 in bcTBK1-mediated antiviral activity, EPC cells were transfected with bcTBK1 and/or bcPRMT6 and subjected to GCRV or SVCV infection separately. The viral titers of GCRV and SVCV in the media of EPC cells expressing bcTBK1 alone were obviously lower than those of EPC cells expressing both bcTBK1 and bcPRMT6 (Fig. 5), which clearly demonstrated that bcPRMT6 negatively regulated bcTBK1-mediated antiviral activity during the innate immune activation. In mammalian RLR signaling, PRMT6 blocks the assembly of the TBK1–IRF3 signaling complex by binding and sequestering IRF3 and then attenuates the antiviral innate immunity [14]. To further elucidate the position of bcPRMT6 in black carp RLR signaling, IF staining and co-IP assay were used to characterize the interaction between bcPRMT6 and black carp RLR components, including bcTBK1, bcIRF3 and bcIRF7. Co-IP data has identified the direct interaction between bcPRMT6 and bcIRF3/7. (Fig. 6A and B, the red arrow indicated). However, no direct association was detected between bcPRMT6 and bcTBK1 through co-IP (Fig. 6C). Thus, the data implied that bcPRMT6 functioned through similar mechanism to that of mammalian PRMT6, which further suggested the conservation of innate immunity from fish to mammals.

4. Discussion

Mammalian RLR signaling is fine-tuned through the post-translational modification of its key components, such as phosphorylation, ubiquitination, acetylation, sumoylation and methylation [28–30]. Arginine methylation plays critical roles in the establishment and maintenance of the lymphoid and myeloid lineages and in inflammatory responses [31]. Protein arginine methyltransferases (PRMTs) are a group of enzymes that can methylate arginine residues on histones and other proteins, which function importantly in various physiology process and are classified as type I–IV according to the methylarginine products [32–36].

Since the first time that PRMT6 was cloned and characterized in 2002, many studies have been reported about this PRMT member. As a type I PRMT, PRMT6 is primarily thought to play the important roles in the regulation of transcription. Additional studies have also shown that PRMT6 functions as a co-activator of NF-κB and this PRMT member has been reported to be overexpressed in bladder and lung cancer cells [37,38]. It has been reported that $PRMT^{-/-}$ mouse is viable, but mouse embryonic fibroblast (MEF)/$PRMT^{-/-}$ undergoes rapid cellular senescence. However, the role of PRMT6 in teleost remains largely unknown. It is interesting that the transcription level of PRMT6 was not obvious changed by VSV and HSV-1 infection in human A594 cells; however, VSV and HSV-1 infection markedly increased PRMT6 protein level [14,39]. In this study, PRMT6 of black carp has been identified and characterized, which is aimed to elucidate the function of PRMT6 in host antiviral innate immunity. Sequence analysis demonstrates that bcPRMT6 amino acid sequence possesses highly similarity with those of human and mouse, especially its MTA domain, which implies the conserved function of PRMT6 in vertebrates.

In human and mammals, the TBK1–IRF3/7 signaling is activated by the adaptor antiviral proteins including MAVS and STING, which plays the crucial role in IFN induction during host innate immune activation [40,41]. Luciferase reporter assay data has indicated that the induced IFN-β promoter transcription by TBK1 and IRF3 was significantly inhibited in the presence of PRMT6. This inhibition by PRMT6 was because this PRMT member attenuated IRF3 phosphorylation and presented more obviously after virus stimulation [14]. Similar phenomenon was seen in this study, in which the induced transcription of both bcIFNa promoter and DrIFNϕ3 promoter by bcTBK1 was fiercely reduced by bcPRMT6 in reporter assay (Fig. 4), and bcTBK1-mediated antiviral activity in EPC cells was obviously inhibited by bcPRMT6 (Fig. 5). In the condition of VSV and HSV-1 infection, PRMT6 inhibits antiviral innate immunity by sequestering IRF3, thereby blocking TBK1-IRF3 signaling, which is independent of PRMT6 methyltransferase activity [14]. However, whether the mechanism behind the inhibition of bcTBK1-mediated antiviral signaling by bcPRMT6 is the same to that of human and mammal needs to be further investigated. The IF data showed that bcPRMT6 was majorly expressed in the nucleus and the data of co-IP demonstrated that bcPRMT6 interacted with bcIRF3/7 but not with bcTBK1. Thus, it is speculated that this fish PRMT6 homologue sequesters bcIRF3/7 after the nuclear translocation of these two transcription factors and blocks the association between bcIRF3/7 and IFNs.

In our previous study, the suppressor of IKKε (SIKE) of black carp (bcSIKE) has been identified to interact with bcTBK1 and inhibit bcTBK1-mediated antiviral signaling during host innate immune activation. The association between bcSIKE and bcTBK1 has been identified; however, no interaction between bcSIKE and bcIRF3/7 has been detected [26]. In this study, bcPRMT6 interacts with bcIRF3/7 but not bcTBK1, and inhibits bcTBK1-bcIRF3/7 signaling. Thus, black carp TBK1-IRF3/7 antiviral signaling is negatively regulated at different steps, which leads to the finely tuned innate immunity of this fish.

The proteins IDs of PRMT6 in the table are the same as those in Fig. 1B.

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