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USP14 negatively regulates IFN signaling by dampening K63-linked ubiquitination of TBK1 in black carp

Can Yang [a], Juanjuan Shu [a], Xiao Yang [a], Yujia Miao [a], Ji Liu [a], Jun Li [b], Jun Xiao [a], Weiguang Kong [c], Zhen Xu [c], Hao Feng [a,*]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China
[b] Key Laboratory of Hunan Province for Study and Utilization of Ethnic Medicinal Plant Resources, College of Biological and Food Engineering, Huaihua University, Huaihua, 418008, China
[c] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China

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ABSTRACT

USP14 regulates the immune related pathways by deubiquitinating the signaling molecules in mammals. In teleost, USP14 is also reported to inhibit the antiviral immune response through TBK1, but its regulatory mechanism remains obscure. To elucidate the role of USP14 in the RLR/IFN antiviral pathway in teleost, the homolog USP14 (bcUSP14) of black carp (*Mylopharyngodon piceus*) has been cloned and characterize in this paper. bcUSP14 contains 490 amino acids (aa), and the sequence is well conserved among in vertebrates. Overexpression of bcUSP14 in EPC cells attenuated SVCV-induced transcription activity of IFN promoters and enhanced SVCV replication. Knockdown of bcUSP14 in MPK cells led to the increased transcription of IFNs and decreased SVCV replication, suggesting the improved antiviral activity of the host cells. The interaction between bcUSP14 and bcTBK1 was identified by both co-immunoprecipitation and immunofluorescent staining. Co-expressed bcUSP14 obviously inhibited bcTBK1-induced IFN production and antiviral activity in EPC cells. K63-linked polyubiquitination of bcTBK1 was dampened by co-expressed bcUSP14, and bcTBK1-mediated phosphorylation and nuclear translocation of IRF3 were also inhibited by this deubiquitinase. Thus, all the data demonstrated that USP14 interacts with and inhibits TBK1 through deubiquitinating TBK1 in black carp.

1. Introduction

Pattern recognition receptors (PRRs)-dependent innate immunity, as the host's first line of defense against foreign invasion, protects host against foreign microorganisms by regulating the production of interferon (IFN) and inflammatory factor [1]. Among them, IFNs are a series of inducible cytokines that can be induced by signaling pathways such as RLRs and TLRs to be expressed after viral infection of the host, thereby activating antiviral effectors to inhibit viral activity [2]. In RLRs/TLRs signaling transduction, IRF3 and IRF7 are critical for inducing IFN expression [3,4]. Upon activated by phosphorylation medicated by upstream kinase, these proteins migrate towards the nucleus and activate transcription of type I IFNs.

TANK binding kinase (TBK1) is a key kinase for IRF3 and IRF7, and its functions have been extensively concerned. The essential role of TBK1 was originally identified in the innate immune response against pathogens, as it regulates the production of type I interferons, including IFN-α and IFN-β [5]. Since then, TBK1 has been extensively studied as a key signaling kinase in the regulation of innate immune responses. TBK1 seems to assume an indispensable role in the activation of IRF3. In cells where TBK1 has been knocked down, activation of IRF3 is inadequate, and the IRF3-IFN antiviral signaling axis is not efficiently activated [6]. Further studies have demonstrated TBK1's involvement in a range of processes, including metabolism, proliferation, development, pathogen-induced inflammation/immunity, and its possible role in regulating various human diseases, such as type II diabetes (T2D), obesity, neurodegenerative diseases, and a variety of cancers [7].

Abnormal production of type I IFNs is harmful to the host. Therefore, preventing the deleterious overproduction of type I IFNs is crucial for host to sustain self-homeostasis in immune response [8]. As one of the key kinases for IRF3/IRF7 activation, the activity of TBK1 is tight controlled by multiple ways to maintain immune homeostasis. Post-translational modifications play a key role in regulating TBK1 activity, particularly through phosphorylation and ubiquitination [9].

* Corresponding author.
E-mail address: fenghao@hunnu.edu.cn (H. Feng).

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Information
CDS		
bcUSP14-F	ATGCCCGTATTTACAGTG	For bcUSP14
bcUSP14-R	CGTAGACACTGCACTGT	CDS cloning
Expression vector		
bcUSP14-F	ACTGACGGGCCCATGCCCGTATTTACAGTG	For expression
bcUSP14-R	ACTGACGGTACCCGTAGACACTGCACTGT	Vector construction
qRT-PCR		
q-bcUSP14-F	GAAGTTTGATGCGGTGGAG	*ex vivo* qRT-PCR
q-bcUSP14-R	CGTAGACACTGCACTGTTG	
q-bcIFNa-F	AAGGTGGAGGACCAGGTGAAGTTT	
q-bcIFNa-R	GACTCCTTATGTGATGGCTTGTGG	
q-bcIFNb-F	GACCACGTTTCCATATCTTT	
q-bcIFNb-R	CATTTTTTCTTCATCCCACT	
q-bcactin-F	TGGGCACCGCTGCTTCCT	
q-bcactin-R	TGTCCGTCAGGCAGCTCAT	
q-SVCV-*M*-F	CGACCGCGCCAGTATTGATGGATAC	
q-SVCV-M-R	ACAAGGCCGACCCGTCAACAGAG	
q-SVCV-N-F	GGGTCTTTACAGAGTGGG	
q-SVCV-N-R	TTTGTGAGTTGCCGTTAC	
q-SVCV-P-F	AACAGGTATCGACTATGGAAGAGC	
q-SVCV-P-R	GATTCCTCTTCCCAATTGACTGTC	
q-SVCV-G-F	GATGACTGGGAGTTAGATGGC	
q-SVCV-G-R	ATGAGGGATAATATCGGCTTG	
q-epcViperin-F	ATGAAAACTCAAATGTGGACGTA	
q-epcViperin-R	GATAGTTTCCACCCATTTCCTTAA	
q-epcIFN-F	ATGAAAACTCAAATGTGGACGTA	
q-epcIFN-R	ATGAAAACTCAAATGTGGACGTA	
q-epcMx1-F	TGGAGGAACCTGCCTTAAATAC	
q-epcMx1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
q-epcISG15-F	TGATGCAAATGAGACCGTAGAT	
q-epcpcISG15-R	CAGTTGTCTGCCGTTGTAAATC	
q-epcActin-F	AAGGAGAAGCTCTGCTATGTGGCT	
q-epcActin-R	AAGGTGGTCTCATGGATACCGCAA	
shRNA		
bcUSP14-shRNA-1-F	CCGGGGGAAAGGAGAAGTTTGATGCCTCGAGGCATCAAACTTCTCCTTTCCC TTTTTG	PLKO.1-shbcUSP14 construction
bcUSP14-shRNA-1-R	AATTCAAAAAGGGAAAGGAGAAGTTTGATGCCTCGAGGCATCAAACTTCTCCTTTCCC	
bcUSP14-shRNA-2-F	CCGGATGCGGGTCCTTCAGCAGAAACTCGAGTTTCTGCTGAAGGACCCGCATTTTTTG	
bcUSP14-shRNA-2-R	AATTCAAAAAATGCGGGTCCTTCAGCAGAAACTCGAGTTTCTGCTGAAGGACCCGCAT	
bcUSP14-shRNA-3-F	CCGGGCATACCTCACGGTTCAAATGCTCGAGCATTTGAACCGTGAGGTATGCTTTTTG	
bcUSP14-shRNA-3-R	AATTCAAAAAGCATACCTCACGGTTCAAATGCTCGAGCATTTGAACCGTGAGGTATGC	

Numerous viruses target TBK1 to evade the immune response by disrupting its phosphorylation. For instance, the Dengue Virus inhibits RIG-I/MAVS mediated IFN signaling and antiviral activity by blocking the phosphorylation of TBK1 [10]. PPM1B, a protein phosphatase 1B, physiologically binds to TBK1 and phosphorylates it after viral infection, resulting in the cessation of TBK1-mediated IRF3 activation [11]. Similar to that, the activity of TBK1 is also regulated by ubiquitination modification [12]. The E3 ubiquitin ligases RNF128 [13], MIB1, MIB2 [14] and Nrdp1 [15] have been found to positively regulate TBK1 by promoting its K63-linked polyubiquitination. The E3 ubiquitin ligase DTX4 and TRAF-interacting protein (TRIP) have been shown to restrict TBK1 activation and antiviral response by promoting proteasomal degradation of TBK1 [16,17], and the deubiquitinating enzymes CYLD [18] and USP2b [19] inhibit TBK1 activation by cleaving K63-linked ubiquitin chains from TBK1. In teleost fish, TBK1 homologues also have significant function in antiviral signaling. The regulation and the activity of TBK1 heavily relies on its ubiquitination modification. In grass carp, low dosage of GCRV infection restrained TBK1 activation by repressing TBK1 K63-linked ubiquitination and facilitating its K48-linked ubiquitination [20]. However, the E3 ubiquitin ligase and deubiquitinases responsible regulating the polyubiquitination for TBK1 has not been identified in teleost.

Up to now, more than one hundred deubiquitinases have been reported and they have been classified into seven groups, of which the USP family is the largest. USP14, which was identified in 2001 as being involved in proteasomal activity, has been reported to be involved in the regulation of multiple cancers as a tumor-promoting factor [21,22]. At the same time, USP14 has been suggested to negatively regulate immune responses and promote viral replication [23]. In bony fish, USP14 has been cloned and characterized in orange spotted grouper (*Epinephelus coioides*) and Chinese perch (*Siniperca chuatsi*). The grouper USP14 has been showed to attenuate interferon antiviral signaling by inhibiting TBK1 and MDA5 [24,25]. However, the mechanism behind the negatively regulatory role of fish USP14 in antiviral immune response need further study.

In this study, USP14 was cloned and was investigated in black carp, and demonstrated its inhibition of IFN antiviral signaling by eliminating the K63-linked polyubiquitination modification of TBK1, which in turn attenuates the phosphorylation modification and nuclear translocation of IRF3. Our study provides a new perspective for the regulatory mechanism of USP14 in the antiviral immune response.

2. Material and methods

2.1. Cells, virus and plasmids

HEK 293T cells, HeLa cells, *Epithelioma papulosum* cyprini (EPC) and *Mylopharyngodon piceus* kidney (MPK) cells were maintained in the laboratory. HEK 293T cells were cultured at 37 °C, and EPC, MPK cells were cultured at 26 °C, all with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10 % FBS. HEK 293T and EPC cells were transfected with PEI (Yeasen, 40816ES02), while to improve the transfection efficiency, Lipomax (Sudgen, 32,012) was used to transfect MPK cells according to the manufacturers' instructions.

Spring viremia of carp virus (SVCV/strain: SVCV741) were amplified in EPC cells, while virus titers were determined by plaque assay on EPC

cells as previously described [26]. Briefly, EPC cells were infected SVCV at corresponding MOI, then the media were collected to use for plaque assay at 24 h post infected. The collected media was then 10-fold serially diluted and added into EPC cells in 48-well plates. After incubated for 1 h, the media was replaced by fresh DMEM containing 2 % FBS and 0.75 % methylcellulose (Sigma, USA). Finally, the plaques were counted after incubating for 72 h.

pcDNA5/FRT/TO-Flag, pcDNA5/FRT/TO-Flag-TBK1, pEGFP-N1 and pEGFP-N1-TBK1, pRL-TK, Luci-bcIFNa (for black carp IFNa promoter activity analysis) were kept in the lab. The knockdown vector targeting bcUSP14 was constructed by insert the shRNAs into PLKO.1, which was designed according to the protocol on the website (http://rnaidesigner.thermofisher.com/rnaiexpress).

2.2. Cloning and expression vector construction of bcUSP14

Based on the data in the black carp full-length transcriptome, specific primers (Table 1) were designed to clone the coding regions (CDS) of bcUSP14 from the cDNA obtained from liver of black carp. To create a recombinant expression vector, the coding sequence of bcUSP14 was linked to the FRT/TO-pcDNA5 vector. This vector is suitable for both transient and stable expression in eukaryotic cells.

2.3. LPS、poly I:C treatment and SVCV infection

MPK cells were treated with different working concentrations of LPS or poly I:C for different treatment durations (0, 2, 8, 12, 24 and 48 h). LPS was added directly to the medium at concentrations of 5 or 50 μg/ml, respectively. Poly I:C was heated at 55 °C for 5 min and then cooled to room temperature before use at a concentration of 5 or 25 μg/ml. And 0.01 or 0.1 MOI SVCV was used to infect MPK cells for 0, 2, 8, 12, 24 and 48 h, severally.

2.4. Sequence analysis and phylogenetic analysis

The exon and intron sequences of the USP14 gene were obtained from the black carp genomic sequencing (data unpublished). The amino acid sequences of USP14 homologues were aligned using Gene Doc. The phylogenetic tree of USP14 was constructed by maximum likelihood method (ML, bootstrap 1000) using MEGA 7.0. Based on homology modelling, Swiss Model was used to construct 3D structure prediction of USP14. Theoretical isoelectric point (pI) and molecular weight (Mw) were calculated using Compute Mw/pI software.

2.5. Subcellular localization

Immunofluorescence (IF) assay is used to probe subcellular localization. Briefly, the transfected cells were fixed with 4 % paraformaldehyde at 24 hpt then permeabilized with Triton X-100 (0.2 % in PBS). And it was then blocked with 10 % goat serum (solarbio SL038). Cells were then incubated with anti-Flag antibody (Abmart, M20008) or anti-HA antibody (Abmart, M20003) for 1 h. Nuclei were then stained by adding 5 μl DAPI on microscopic slides [26]. The Olympus confocal microscope was used for fluorescence observation of stained cells.

2.6. qRT-PCR

Total RNA was isolated for qRT-PCR analysis to measure mRNA abundance of the indicated genes. The relative mRNA expression levels of indicated genes derived from EPC and MPK cells were normalized to the corresponding β-actin. The indicated gene-specific primers were listed in S. Table 1. The relative expression ratio of the target gene was calculated by $2^{-\Delta\Delta CT}$ method.

Table 2
Comparison of bcUSP14 with other USP14 congeners.

Species	Accession ID	Similarity (%)	Identity (%)
Mylopharyngodon piceus	OR224865	100	100
Ctenopharyngodon idella	XP_051717123.1	99.0	98.2
Pimephales promelas	XP_039513257.1	98.2	96.9
Danio rerio	NP_956267.1	96.5	93.3
Astyanax mexicanus	XP_022521247.1	94.9	90.2
Scomber japonicus	XP_053185846.1	94.9	89.6
Denticeps clupeoides	XP_028833134.1	94.1	89.8
Esox lucius	XP_010884751.1	95.3	90.6
Takifugu rubripes	XP_003975373.1	92.6	88.1
Oncorhynchus keta	XP_035644295.1	93.9	87.9
Boleophthalmus pectinirostris	XP_033838533.1	94.3	87.9
Electrophorus electricus	XP_026883163.2	93.1	87.1
Cynoglossus semilaevis	XP_008332677.1	93.9	87.7
Tachysurus fulvidraco	XP_026990020.1	93.9	86.9
Anas platyrhynchos	EOB08802.1	92.3	83.1
Bos taurus	NP_001068657.1	91.6	82.7
Felis catus	XP_003995106.1	91.9	82.3
Rattus norvegicus	NP_001008302.1	91.6	81.8
Capra hircus	XP_017894972.1	91.4	82.7
Mus musculus	NP_067497.2	91.4	82.1
Notechis scutatus	XP_026526098.1	91.5	82.1
Gallus gallus	XP_040521491.1	92.4	82.2
Zootoca vivipara	XP_034980971.1	91.3	82.9
Hemicordylus capensis	XP_053099822.1	91.9	83.1
Ailuropoda melanoleuca	XP_002922668.1	92.1	82.3
Homo sapiens	NP_005142.1	91.9	82.1
Xenopus tropicalis	NP_001005641.1	91.8	81.5
Saimiri boliviensis	XP_003924840.1	91.6	81.7
Ornithorhynchus anatinus	XP_028925242.1	91.4	82.1
Sus scrofa	XP_020951818.1	91.8	83.1
Geospiza fortis	XP_014167483.1	91.6	82.8
Falco cherrug	XP_055559446.1	91.6	82.5
Columba livia	XP_013226076.1	91.6	82.5

2.7. Immunoblotting (IB) and co-immunoprecipitation (co-IP)

The transfected EPC or HEK 293T cells were harvested and lysed for immunoblot (IB) assay as previously described [27]. Briefly, whole cell lysates transferred to polyvinylidene difluoride (PVDF) membrane after being separated by 10 % SDS-PAGE. The membranes were probed with anti HA/Flag monoclonal primary antibody and followed by the incubation with secondary antibody. Target proteins were visualized in membrane with BCIP/NBT Alkaline Phosphatase Color Development Kit.

HEK 293T cells seeded in 100 mm cell culture dishes were transfected with the plasmids. The transfected cells were harvested at 48 h post-transfection and lysed for immunoprecipitation (IP) assay as previously described. The cellular debris was removed by centrifugation at 10,000×*g* for 5 min at 4 °C. The supernatant was pre-cleared with Protein Agarose A/G beads for 1 h and incubated overnight with anti-Flag/HA agarose beads on a rocker platform at 4 °C. These samples were boiled in SDS sample buffer after 5 times of wash and the eluted proteins were used for IB as above.

2.8. Luciferase reporter assays

EPC cells in 24-well plate were transfected with indicated expressing plasmids, pRL-TK, Luci-bcIFNa or Luci-EpcIFN. Lucifer assays were performed using the dual luciferase reporter assay according to the instruction of the manufacturer (Promega). Firefly luciferase activities were normalized on the basis of Renilla luciferase activities. bcIFNa belongs to the IFN-a subgroup of type I interferons in bony fish, and the Luci-bcIFNa promoter was constructed by the group ourself [28,29].

2.9. Statistics analysis

All data existed in three replicate experiments and statistical

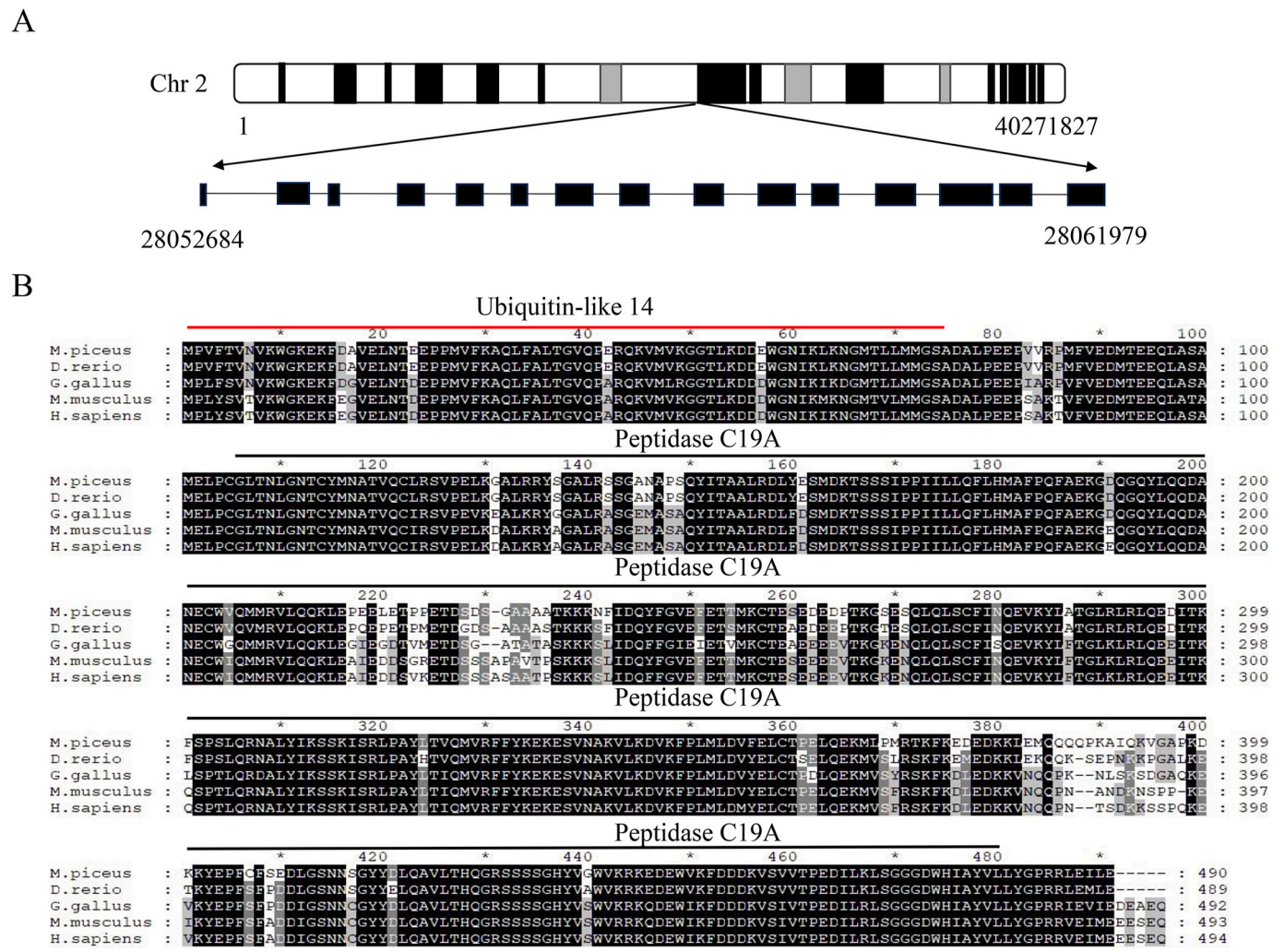

Fig. 1. Homology comparison and phylogenetic analysis.
A: Chromosomal distribution and exon information of the bcUSP14. B: Amino acid sequences were compared between USP14 homologues with human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*) and black carp (*Mylopharyngodon piceus*). The bcUSP14 structural domains were obtained by prediction from the NCBI Conserved Domain Database. C: SWISS-Model was used for the structure prediction. D: Evolutionary trees of USP14 were constructed by maximum likelihood method using MEGA6 software.

analyses were performed using two-tailed *t-tests* for GraphPad Prism 8.0. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ mean significant. "Ns" indicates $p > 0.05$, not statistically significant.

3. Result

3.1. Sequence analysis of bcUSP14

Black carp genomic information shows that the bcUSP14 gene is located on chromosome 2 within the range of 28,052,684–28,061,979 bp, which contains 15 exons and 14 introns. bcUSP14 has a CDS of 1471 bp that encodes 490 amino acids (Fig. 1A). Comparative analysis of the amino acid sequence demonstrates remarkable conservation of bcUSP14 with homologous genes in other species, containing conserved Ubiquitin-like 14 (1–76 aa) and Peptidase C19A structural domain (106–481 aa) (Fig. 1B). The tertiary structure pattern diagram of bcUSP14 was obtained by Swiss model homology modeling, and the results showed that its tertiary structure was highly conserved with humanUSP14 (Fig. 1C). Phylogenetic analysis showed that bcUSP14 belongs to the fish branch and is evolutionarily closest to *Pimephales promelas* and *Cetenopharyngodon idella* USP14 (Fig. 1D & Table 2).

3.2. Expression profiles of bcUSP14 in MPK cells

LPS and Poly I:C were used to treat MPK cells to investigate changes in the expression level of USP14 in response to different stimulants. The mRNA level of USP14 in both LPS treatment groups (5 μg/ml and 25 μg/ml) increased to a maximum after 2 h of treatment, and then decreased to a plateau, which was slightly higher than the expression level of USP14 at 0 h of treatment (Fig. 2A). In contrast, the mRNA level of USP14 was significantly down-regulated at 2 h in both Poly I:C treated groups (5 and 25 μg/ml) (Fig. 2B). In the low-dose group, USP14 expression level continued to increase until it peaked at 12 h and then gradually declined. In the high dose group, the expression level of USP14 increased to a maximum at 8 h and was maintained thereafter. When infected with SVSV, the transcript levels of USP14 showed significant down-regulation at different times of 0.01 MOI infection. The mRNA levels of USP14 were lower than those of the untreated group after different times of 0.1 MOI infection, except for a transient up-regulation at 2 h of infection (Fig. 2C).

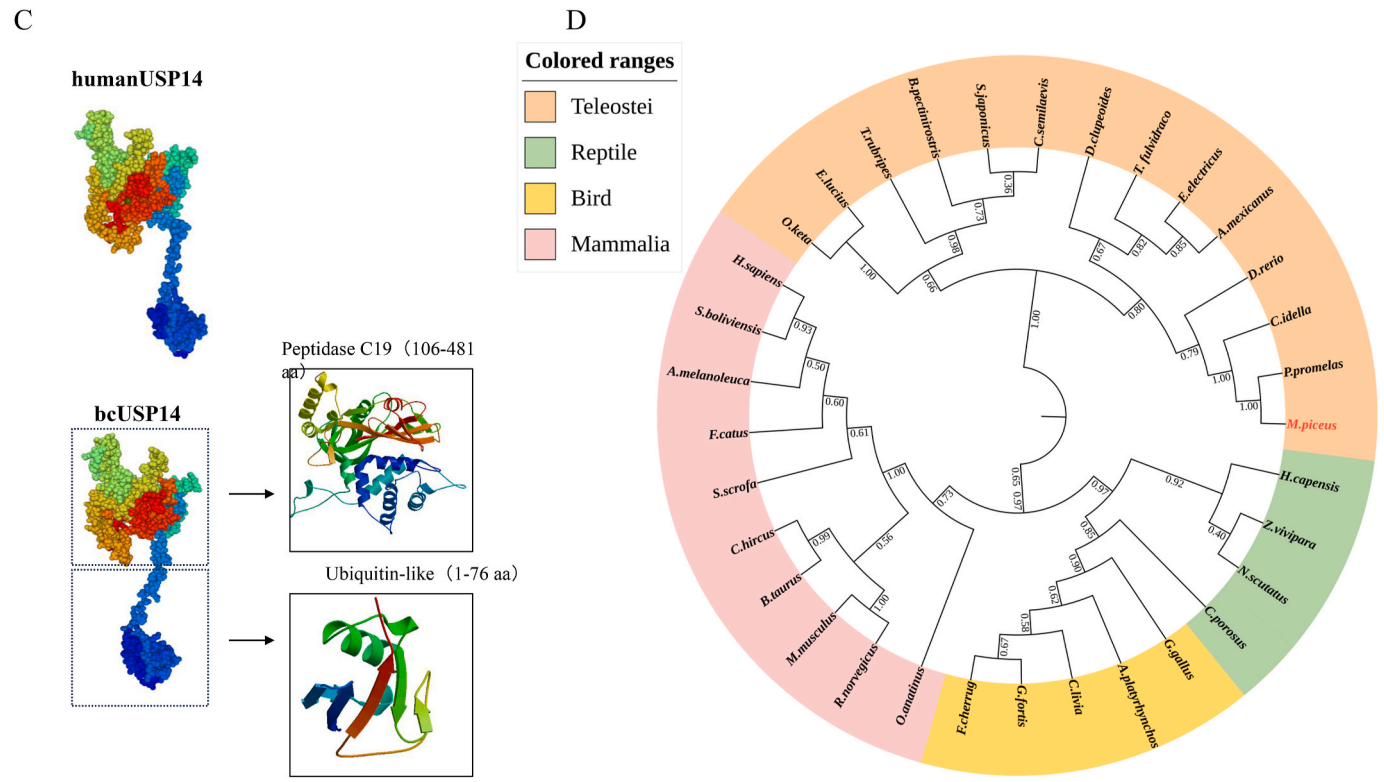

Fig. 1. (*continued*).

3.3. Expression and intracellular localization of bcUSP14

HEK 293T and EPC cells were transfected with bcUSP14 recombinant expression vector and bcUSP14 expression was detected by immunoblotting assay, and mouse-derived anti-Flag antibody was used to detect the recombinant protein. The results were the same in both HEK 293T and EPC cells, with a clear band at around 60 KDa in the USP14 over-expression group while no band in the control group (Fig. 3A). To determine the subcellular distribution of bcUSP14, EPC and HeLa cells were transfected with plasmid expressing bcUSP14 and employed immunofluorescence assay to study the subcellular distribution of bcUSP14. The results showed that bcUSP14 was distributed in both cytoplasm and nucleus (Fig. 3B).

3.4. bcUSP14 attenuates IFN antiviral signaling

To investigate the role of bcUSP14 in IFN antiviral signaling, the reporter gene system of the bcIFNa promoter was used for functional exploration of bcUSP14. The results showed that overexpression of bcUSP14 in uninfected EPC cells resulted in statistically insignificant changes in the activity of the bcIFNa promoter, whereas bcUSP14 significantly attenuated virus-induced activation of the bcIFNa promoter when SVCV was infected for 24 h (Fig. 4A). And the regulated epcIFN promoter is similarly (Fig. 4B). For USP14-expressing EPC cells, both the SVCV titer in the supernatant media and the cytopathic effect (CPE) of the cell monolayer were obviously higher than those of the control cells (Fig. 4C&D). In addition, the mRNA levels of *IFN* and *ISG15* were decreased in USP14-expressing cells, and the mRNA levels of SVCV *G*, *N*, *P* genes were increased in USP14-expressing cells accordingly (Fig. 4E). Therefore, the above results suggest that overexpression of bcUSP14 may promote viral replication by inhibiting IFN signaling. Further, three shRNAs (sh1/sh2/sh3) targeting bcUSP14 was constructed to validate the function of bcUSP14. The immunoblotting experiments showed that sh3 had the highest knockdown efficiency (Fig. 5A). Overexpression of sh3 in MPK cells led to a decrease in viral titers in cell supernatants (Fig. 5B). In addition, the qPCR assay showed that sh3 was capable of lowering the mRNA level of USP14 in the MPK of host cells by approximately 50 %. This, in turn, resulted in an increasing in the mRNA level of IFNa and IFNb (Fig. 5C). Furthermore, the knockdown of bcUSP14 significantly decreased the G/N/P expression of SVCV protein in MPK cells after SVCV infection (Fig. 5D). Taken together, these data suggest that bcUSP14 attenuates IFN antiviral signaling.

3.5. bcUSP14 inhibits TBK1-mediated interferon antiviral signaling

TBK1 is a key regulator of IFN antiviral signaling [30]. Reporter assays showed that bcUSP14 significantly inhibited TBK1-mediated activation of the epcIFN and bcIFNa promoters (Fig. 6A&B). Further experiments showed that bcUSP14 dose-dependently attenuated bcTBK1-mediated bcIFNa promoter activity (Fig. 6C). This inhibitory effect was maintained during SVCV infection (Fig. 6D). Overexpression of bcUSP14 in EPC cells significantly inhibited TBK1-induced mRNA expression levels of IFN and interferon-downstream ISGs (viperin, MX1 and ISG15) (Fig. 7A). These data suggest that bcUSP14 inhibits the TBK1 mediated IFN signaling. In addition, overexpression of bcTBK1 significantly reduced viral titers in cell supernatants after SVCV infection, whereas co-expression of bcUSP14 obviously attenuated this effect (Fig. 7B). The crystal violet assay also presented that USP14 inhibited the antiviral effects of TBK1 (Fig. 7D). Similarly, the reduced level of SVCV protein mRNA expression by bcTBK1 in EPC cells was somewhat rescued by co-expression of bcUSP14 (Fig. 7C). These data suggest that bcUSP14 significantly inhibits bcTBK1-mediated IFN antiviral signaling.

3.6. bcUSP14 attenuates K63-linked polyubiquitination of TBK1

In order to further investigate the mechanism by which bcUSP14 inhibits TBK1-mediated antiviral signaling, the existence of an interaction between bcUSP14 and bcTBK1 was probed by co-IP experiments. The results showed that the band representing bcUSP14 was found in the HA-bcTBK1 precipitated protein, which suggests that bcUSP14 interacts

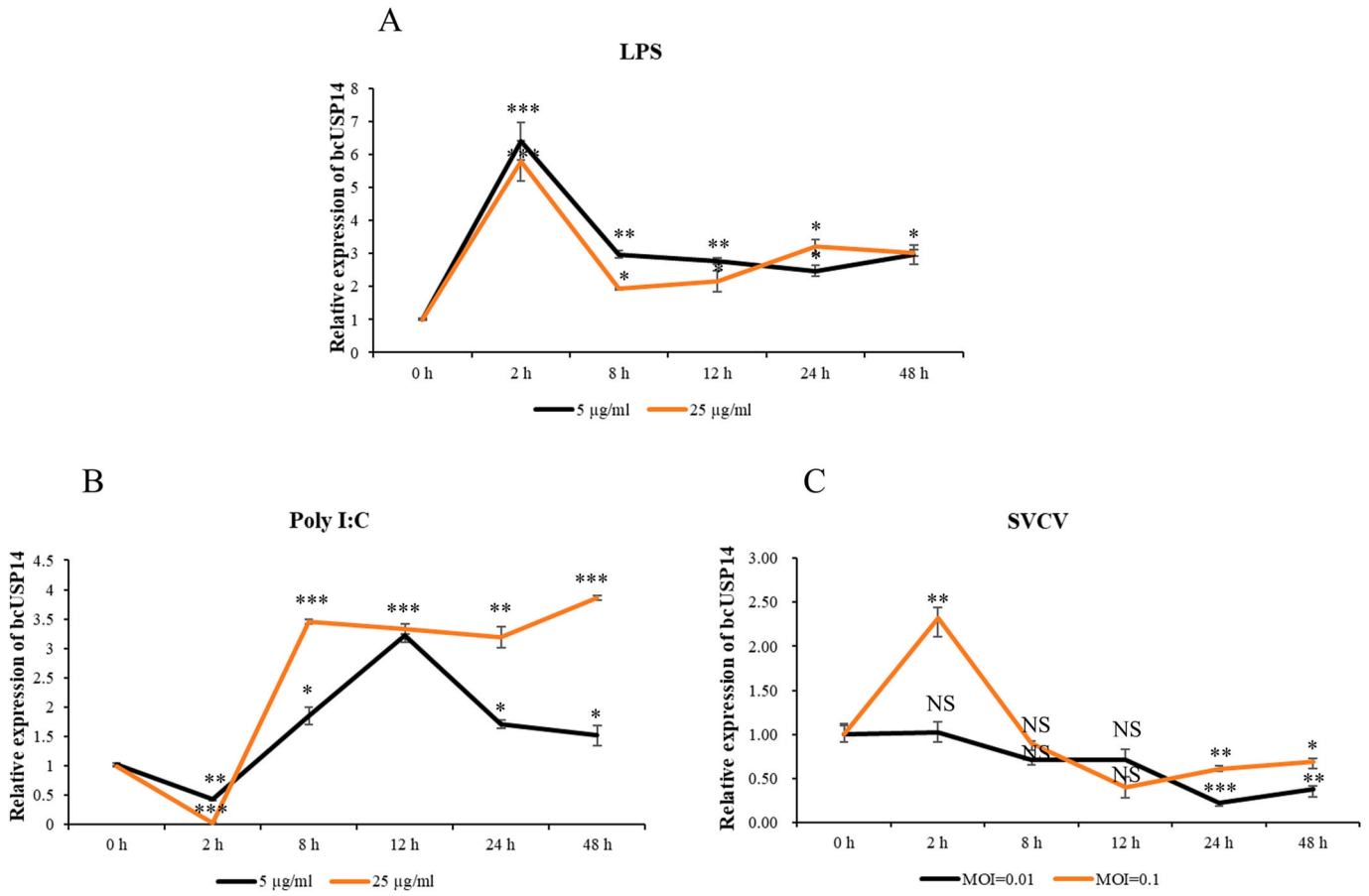

Fig. 2. expression patterns of bcUSP14 in ex *vivo*
MPK cells cultured overnight in six-well plates were treated with the doses indicated for LPS (A) and Poly I:C (B) or the MOI indicated for SVCV (C) respectively and harvested for qPCR assays at the time points indicated.

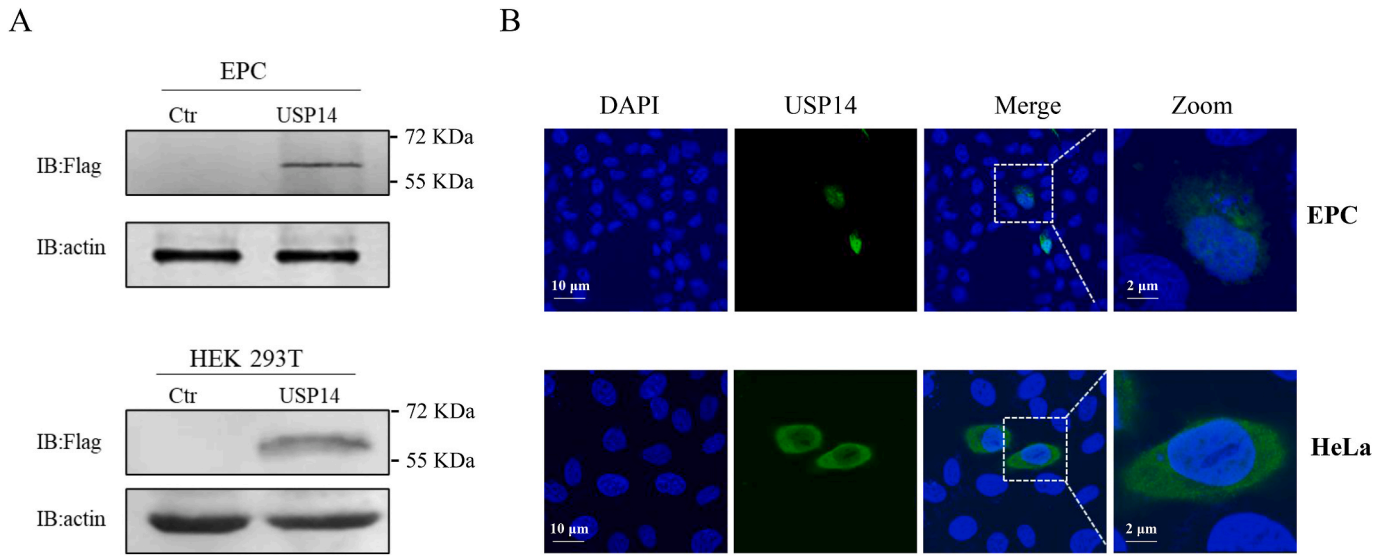

Fig. 3. Protein expression and intracellular localization of bcUSP14
A: The recombinant vector Flag-bcUSP14 was overexpressed in EPC and HEK 293T cells, respectively, and the cells were harvested for immunoblotting assay at 48 h post-transfection (hpt). B: EPC and HEK 293T cells were transfected with the recombinant vector Flag-bcUSP14, and the cells were harvested for immunofluorescence at 24 hpt.

with bcTBK1 (Fig. 8A). Furthermore, the IF results showed that bcUSP14 was present in both the cytoplasm and nucleus, while its co-localization with bcTBK1 was only observed in the cytoplasm, suggesting an interaction between bcUSP14 and bcTBK1 (Fig. 8B).

To further elucidate the down-regulation of bcTBK1 by bcUSP14, we co-transfected EGFP-bcTBK1 plasmid, Flag-bcUSP14 plasmid with

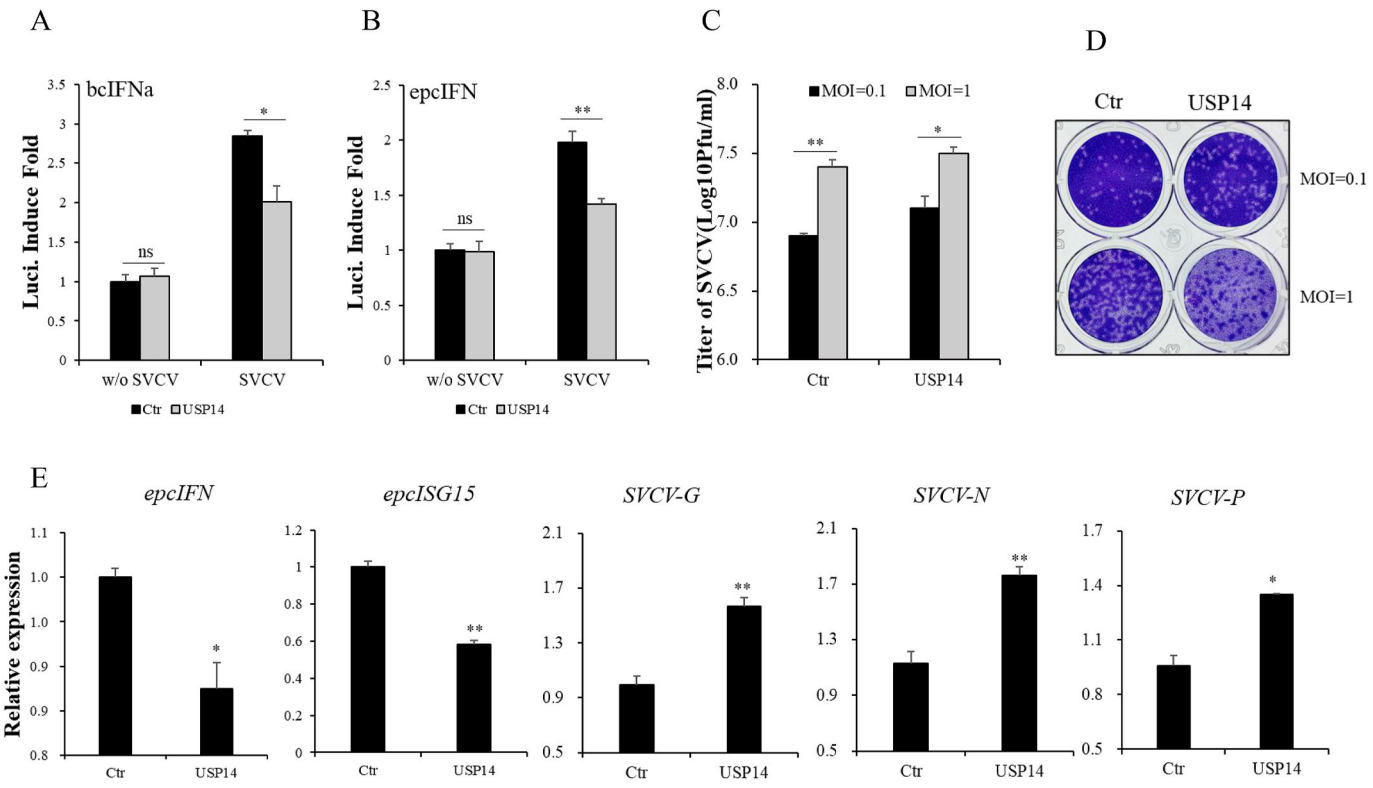

Fig. 4. bcUSP14 attenuates IFN signaling

A&B: EPC cells were transfected with bcUSP14 vector and reporter gene vectors, infected with SVCV (MOI = 0.1) at 24 hpt and harvested for reporter gene assays at the indicated time of infection. The transfected EPC cells infected with SVCV (MOI = 0.1&1) at 24 hpt. The supernatant was used for or titering of SVCV (D) and the cell harvested for qPCR assay (E) at 24 h post-infection (hpi).

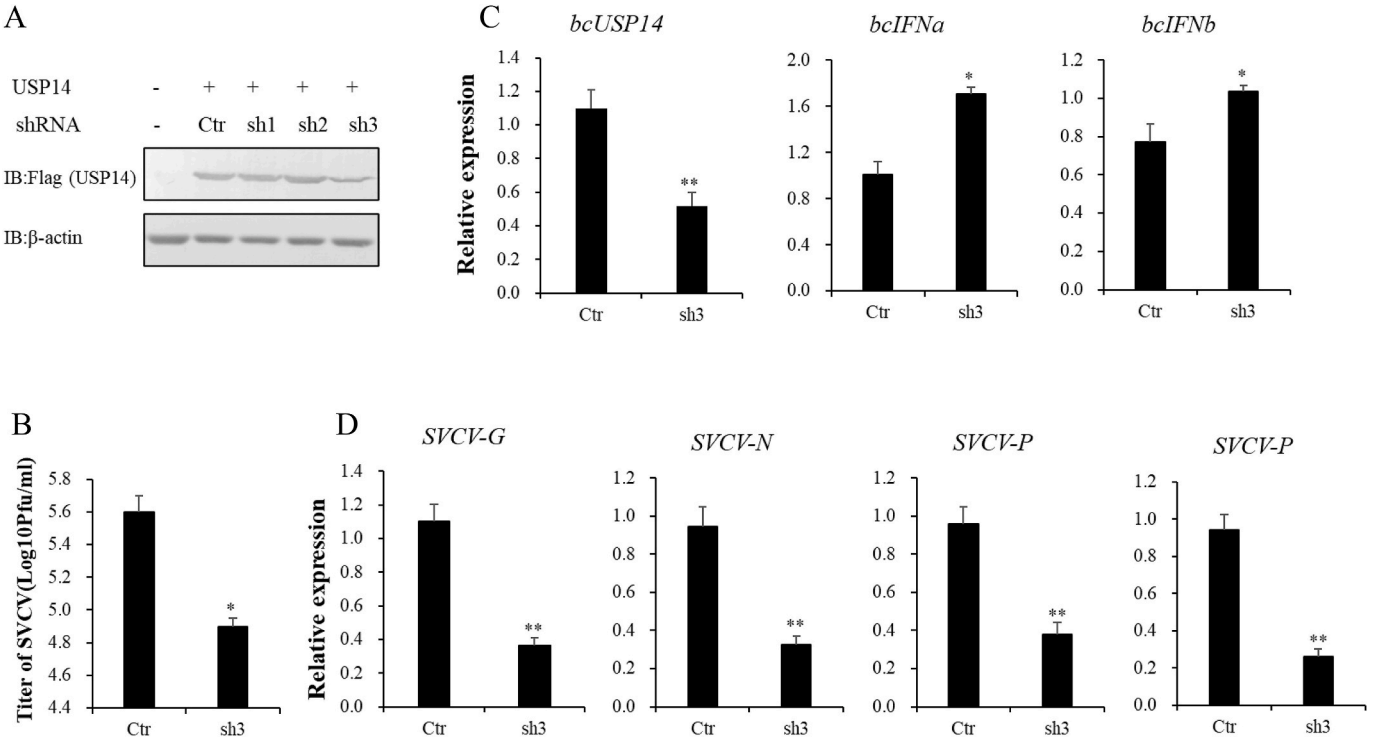

Fig. 5. Knockdown bcUSP14 accelerates IFN antiviral axis.

A: HEK 293T cells were co-transfected with sh-USP14-1/2/3 (sh1/2/3) and Flag-bcUSP14 and harvested for WB to detect knockdown efficiency. MPK cells were transfected with sh3, infected with SVCV (MOI = 0.1) at 24 hpt the supernatant and cells were harvested for titration (B) and qPCR assay at 24 hpi (C&D), respectively.

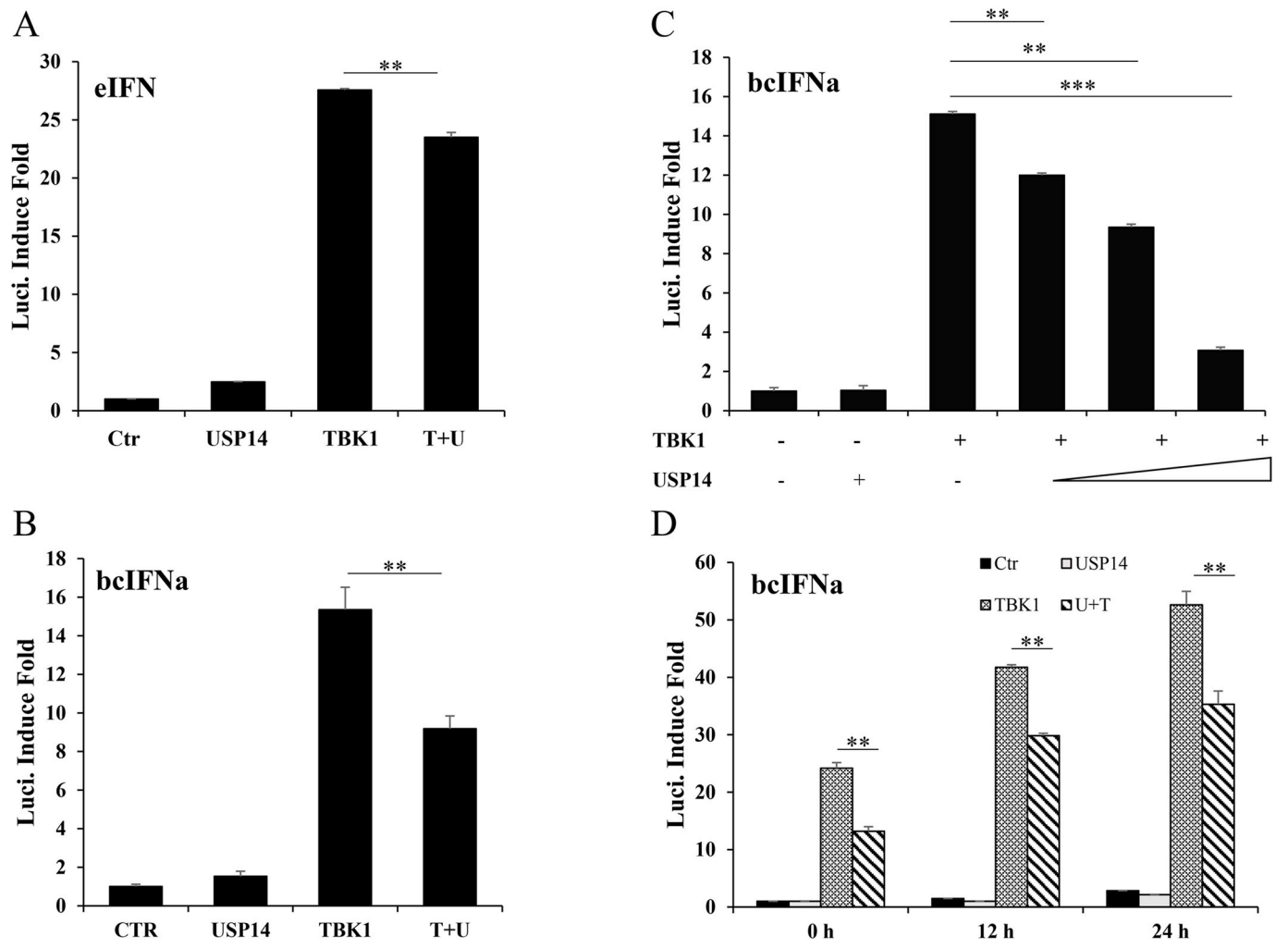

Fig. 6. bcUSP14 hinders TBK1 mediated induction of IFN.
The indicated plasmids and reporter gene plasmids were overexpressed in EPC cells and used for reporter gene detection at 24 hpt. epcIFN (A) and black carp IFNa (B) promoter activity induced by indicated genes were detected. C: TBK1-induced bcIFNa activity was detected at different bcUSP14/bcTBK1 ratio (USP14:TBK1 = 1:2; 1:1; 2:1, respectively). D: Transfected cells were infected with SVCV at 24 h post-transfection with MOI = 0.1 for the times indicated then used for reporter gene activity assay.

ubiquitin plasmid in HEK 293T cells and detected the ubiquitylation level of bcTBK1 by immunoblotting assay. The results showed that bcUSP14 substantially reduced the total ubiquitination level of bcTBK1, resulting in approximately 20 % down-regulation. The K63-linked polyubiquitination level of bcTBK1 was decreased by approximately 25 % through the action of bcUSP14 (Fig. 8C). Additionally, bcUSP14 attenuates TBK-mediated nuclear translocation and phosphorylation modification of bcIRF3 (Fig. 8D&E). These results indicate that bcUSP14 interacts with bcTBK1 and removes its K63-linked polyubiquitination modification, which in turn inhibits the antiviral signaling that is mediated by bcTBK1.

4. Discussion

USP14 is a member of the deubiquitinase family that plays a significant role in regulating tumorigenesis and inhibiting interferon production [31]. In this study, we have successfully cloned a USP14 homolog in black carp, which exhibits a relatively conserved amino acid sequence, structural domain and tertiary space structure in vertebrate. Our work demonstrates that, similar to its mammalian homolog, bcUSP14 has a negative impact on interferon signaling that is activated by viruses [32]. Viral infection post overexpression of bcUSP14 notably decreased activation of the interferon promoter and facilitated viral replication (Fig. 4A–D), whereas knockdown of bcUSP14 enhanced IFN signaling but inhibit SVCV replication in host cells (Fig. 5). This implies that bcUSP14 deters the activation of host IFN signaling, thus promoting viral replication.

In mammals, the activation of TBK1 is critically important for IFN induction. The regulation of TBK1 activity is crucial and varied. MAVS facilitates the activation of IFN signaling through recruitment TBK1/IKKε by TRAFs [33]. TANK, NAP1 and other scaffolds protein also mediate the assembly of the TBK1/IKKε complex, which in turn promotes the activation of TBK1-IFN signaling [34,35]. And E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1 [13]. However, inhibition of over-activated TBK1 is a part of the control of immune homeostasis. There are several methods to impede TBK1 activity, including constraining TBK1 binding to downstream substrates like IRF3, hindering *S*-glutathionylation of TBK1 that is associated with TBK1 activation, breaking down TBK1 via the ubiquitinated proteasomal pathway, and inhibiting the K63 linkage of TBK1 by multimeric ubiquitination. USP19 promotes the degradation of TBK1 through chaperone-mediated autophagy [36]. On the other hand, a significant proportion of viruses also inhibit TBK1 to achieve immune escape. RNF138 inhibits K63-linked polyubiquitination of TBK1 to inhibit its activity, and this inhibition can be exploited by African Swine Fever Virus pI215L to facilitate

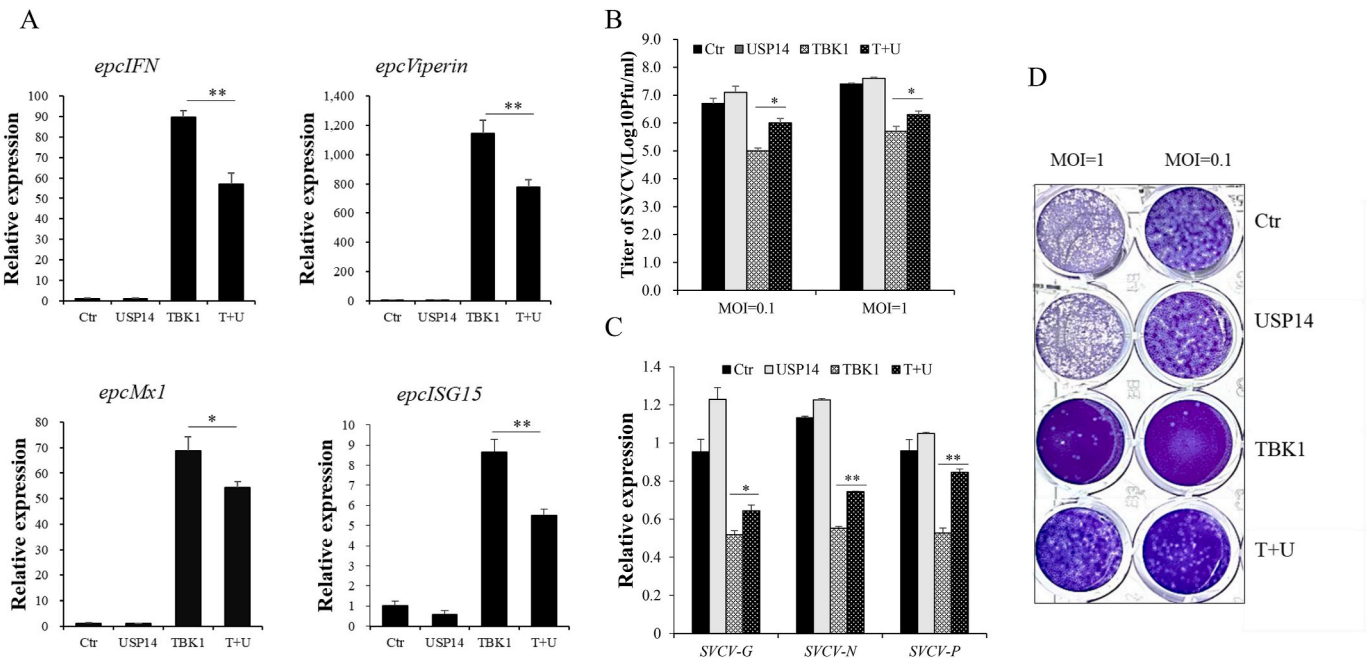

Fig. 7. bcUSP14 attenuated bcTBK1-mediated antiviral ability

A: EPC cells were transfected indicated plasmids and were harvested for qPCR assay to detect the relative mRNA levels of *IFN*, *viperin*, *Mx1* and *ISG15*. EPC cells were overexpressed with the indicated plasmids and infected with SVCV (MOI = 0.1&1) 24 h after transfection. The supernatants of cells from both treatments were used for titer assay (B), while cells from the MOI = 0.1 infected group were used for qPCR to detect the mRNA level of SVCV-G/N/P at 24 hpi (C). Other infected cells were used in virus plaque assay (D).

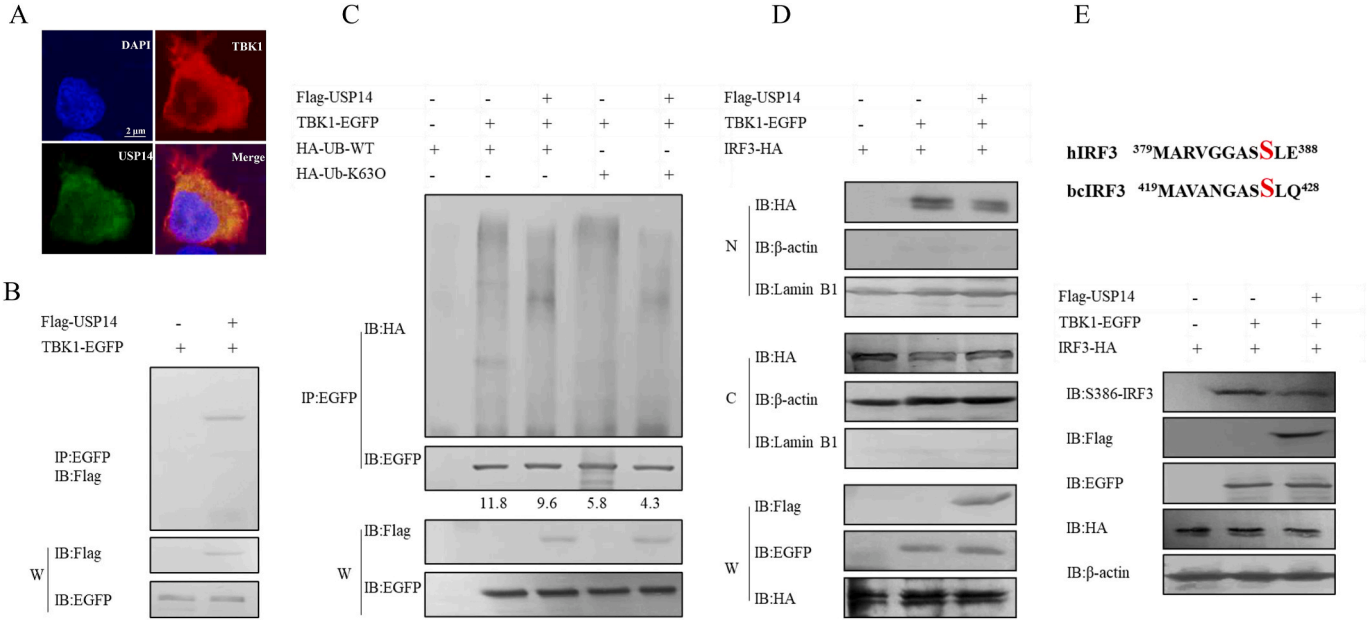

Fig. 8. bcUSP14 impairs the K63-linked ubiquitination of bcTBK1

A: HEK 293T cells were transfected with TBK1-EGFP and/or Flag-bcUSP14 and harvested for co-IP at 48 hpt. B: EPC cells were co-transfected with HA-bcTBK1 and Flag-bcUSP14 and harvested for IF assay at 24 hpt. C: HEK 293T cells were co-expressed indicated vector and the cells were harvested to determine the ubiquitination levels of bcTBK1. Indicated vector were transfected in HEK 293T cells and the transfected cells were harvested at 48 h post-transfection and used for nuclear location (D) and phosphorylation (E) of bcIRF3 separately. For nuclear and cytoplasmic protein extraction, the cells were briefly lysed in 200 μL of lysate containing 0.5 % NP40, 10 mM tris-HCl, 150 mM NaCl and protease inhibitors for 20 min at 4 °C. Following centrifugation at 700×*g*, the resulting supernatant was the cytoplasmic component, and the precipitate was the nuclear component. N: Nucleus; C: cytoplasm; W: whole cell lysate. S386-IRF3: phosphorylation of hIRF3 Ser386 (Abmart, TU402422).

autochthonous replication [37]. Membrane proteins of SARS-CoV-2 impede the production of type I interferon through ubiquitin-mediated degradation of TBK1 [38]. In teleost, some viruses have also taken to inhibiting TBK-IFN signaling to achieve immune escape. Zebrafish MARCH8 inhibited the IFN response by targeting TBK1 degradation, whereas Ring Finger Protein 34 promoted Nervous Necrosis Virus

immune escape by targeting both TBK1 and IRF3 degradation [39–41]. DDX15, on the other hand, both promoted TBK1 degradation and inhibited TBK1-TRAF3 complex formation to inhibit IFN production, which leads to stronger virus replication [42]. In grouper, it is proposed that USP14 may negatively regulated the antiviral signaling by targeting TBK1 [24]. In this study, it is showed that bcUSP14 restricted interferon induction by targeting TBK1, which leads to promotion of viral replication.

Ubiquitination-mediated cellular events are typically cleaved of specific ubiquitin linkages or more generally removed from ubiquitination modifications by deubiquitinating enzymes once they have fulfilled their purpose [43]. In mammals, deubiquitinating enzymes are crucial in maintaining the equilibrium of the ubiquitin system, but they also have the ability to regulate signaling pathways [44]. In recent years, numerous studies have indicated that USPs regulate the activity of TBK1 via removing its ubiquitination modification. USP2b deubiquitinates K63-linked polyubiquitination chains of TBK1 to terminate TBK1 activation and negatively regulate IFN-β signaling and the antiviral immune response [19]. USP38 selectively removes K33-linked polyubiquitin from TBK1 at K670, allowing subsequent K48-linked ubiquitination at the same position mediated by DTX4 and TRIP [45]. However, the mechanism how deubiquitination of TBK1 regulates the downstream signaling remains unclear. In this study, the results show that bcUSP14 interacts with TBK1, inhibiting its K63-linked ubiquitination modification. In addition, inhibition of TBK1-mediated nuclear translocation and phosphorylation modification of IRF3 are observed, which impedes the IFN antiviral response. Considering in mammalian, two hallmarks of TBK1 activated downstream events are the phosphorylation of IRF3 S386 and the nuclear translocation of IRF3 [46]. It is reasonable to propose that USP14 inhibits K63-linked polyubiquitination of TBK1, resulting in reduced phosphorylation and nuclear migration of IRF3. Ultimately, this leads to weakened IFN antiviral signaling. However, the detailed mechanism still needs further study.

CRediT authorship contribution statement

Can Yang: Investigation, Writing – original draft. **Juanjuan Shu:** Investigation, Writing – original draft. **Xiao Yang:** Investigation, Formal analysis. **Yujia Miao:** Investigation. **Ji Liu:** Investigation, Writing – original draft. **Jun Li:** Investigation, Formal analysis. **Jun Xiao:** Data curation, Formal analysis, writing. **Weiguang Kong:** Data curation, Formal analysis, writing. **Zhen Xu:** Project administration, Data curation. **Hao Feng:** Conceptualization, Formal analysis, Project administration, writing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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