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GCRV hijacks TBK1 to evade IRF7-mediated antiviral immune responses in grass carp *Ctenopharyngodon idella*

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ABSTRACT

TANK-binding kinase 1 (TBK1) is an important kinase that regulates the activation of interferon regulatory factor 3/7 (IRF3/7) to induce type I interferon (IFN–I) production in antiviral immune responses. However, in long-term virus-host crosstalk, viruses have evolved elaborate strategies to evade host immune defense mechanisms. In the present study, we found that grass carp (*Ctenopharyngodon idella*) reovirus (GCRV) hijacks TBK1 to escape IRF7-IFN-Is signaling activation. In brief, GCRV inhibited TBK1 activation by restaining K63-linked ubiquitination of TBK1 and promoting its K48-linked ubiquitination. This regulation resulted in that under low titer of GCRV infection, TBK1 overexpression specifically supressed promoter activity and phosphorylation of IRF7 and induction of downstream IFN1and IFN3. qRT-PCR data uncovered that TBK1 negatively regulated IRF7, IFN1 and IFN3 transcription levels under low viral titer infection. Along with enhancement of GCRV titers, TBK1 swiched its function to up-regulate IRF7, IFN1 and IFN3 mRNA levels. Accordingly, TBK1 promoted GCRV replication at low infected titer, but inhibited GCRV replication at high infected titer. All these results revealed a viral evasion strategy that GCRV utilizes TBK1 to block cellular IFN responses at low titers or early stages in fish species, which will lay a foundation for further researching on host-virus interactions and developing novel antiviral strategies in lower vertebrates.

1. Introduction

Type I interferon (IFN–I) plays a fundamental role in response to viral infection [1–4]. During the process of antiviral immunity activation, viral nucleic acids, including DNA and RNA are firstly recognized by pattern-recognition receptors (PRRs) [5,6]. Among the PRRs, RIG-I-like receptors (RLRs), Toll-like receptors (TLRs) and cyclic GMP-AMP synthase (cGAS) are mainly receptors that recognize viral nucleic acids [7–10]. Upon binding to nucleic acids, those PRRs recruit and activate adaptor proteins IFN-β promoter stimulator 1 (IPS-1, also known as MAVS/VISA) TIR-domain-containing adaptor inducing interferon-β (TRIF), and mediator of IRF3 activation (MITA) [5,7,8,11,12]. Active IPS-1 and TRIF recruit TRAF family ubiquitin E3 ligases to promote polyubiquitination of IKK and TBK1, while MITA directly recruits and activates TBK1. The recruited TBK1 and IKK in turn phosphorylate IPS-1, TRIF and MITA. Then, those phosphorylated adaptor proteins binds to IFN regulatory factor 3 (IRF3), and IRF3 is efficiently phosphorylated by TBK1 [13]. Phosphorylated IRF3 forms dimer and enters into the nucleus. Activated TBK1 also induces phosphorylation and nuclear translocations of IRF7 [14]. In nucleus, IRF3, IRF7 together with other transcription factors bind to the promoters of type I interferons and ultimately evoke the production of IFN-I, IFN-stimulated genes (ISGs) and inflammatory cytokines [15].

TBK1 is a non-canonical IκB kinase (IKK) that plays essential roles in interferon production and innate antiviral immunity [5,6]. In mammals, TBK1 consists of three domains which are an N-terminal serine/threonine kinase domain (KD), an ubiquitin-like domain (ULD), and a C-terminal domain (CTD) (also known as two C-terminal coiled coil domains) [15,16]. The KD domain is responsible for its catalytic activity [15]; the ULD domain has a variety of functions, such as sulfur transfer, signal transduction, mediating the protein-protein interactions, substrate presentation and the control of kinase activation [15,17]; the CTD domain can associate with optineurin (OPTN) to amplify the mitophagy process [18]. Structurally, TBK1 is similar to IKKε, IKKα and IKKβ. TBK1 and IKKε can phosphorylate IKKα/β, and decrease the activity of canonical IKK complex. Meanwhile, IKKα/β can phosphorylate and activate TBK1 and IKKε [6]. TBK1 also induces IκB degradation and NF-κB activity through IKKβ [6]. To maintain immune homeostasis, activation of TBK1 is tightly regulated by phosphorylation and ubiquitination, kinase activity modulation and prevention of

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Table 1
Primer sequences for constructing expression vectors of TBK1 and its domains in this study.

Plasmid name	Primer name	Primer sequence (5′–3′)
TBK1-HA	TF984	**ACTG**ggtaccGCCATGATGGAGAGTACGGCGAACTAC
	TR985	**ACTG**gggcccTCA*AGCGTAGTCTGGGACGTCGTATGGGTA*TCTCATCCGATCCACGGTC
TBK1-KD-Flag	TF984	**ACTG**ggtaccGCCATGATGGAGAGTACGGCGAACTAC
	TR20	**ACTG**gggcccTCA*CTTATCGTCGTCATCCTTGTAATC*GATATCGCTGGTCTCGGCAA
TBK1-KU-Flag	TF984	**ACTG**ggtaccGCCATGATGGAGAGTACGGCGAACTAC
	TR21	**ACTG**gggcccTCA*CTTATCGTCGTCATCCTTGTAATC*GATGGGATTTTCTCTGGAGGTC
TBK1-CTD-Flag	TF22	**ACTG**ggtaccGCCATGATGCTGCTCAGCAGAGACC
	TR923	**ACTG**gggcccTCA*CTTATCGTCGTCATCCTTGTAATC*TCTCATCCGATCCACGGTC

Note: The nucleotides in overstriking represent protective bases, and in lowercase indicate the sites of restricted enzymes. Those in italic stand for HA or Flag tag sequence.

functional TBK1-containing complexes formation [6,15]. Auto-phosphorylation of TBK1 at Ser172 is essential for its activation. Meanwhile, some phosphatases such as SH2-containing inositol-5′-phosphatase 1 (SHIP-1), protein phosphatase Mg^{2+}/Mn^{2+} dependent 1B (PPM1B) and glucocorticoid hormones can induce phosphorylation or dephosphorylation of TBK1 to regulate the IFN-β production [6]. Additionally, ubiquitination and deubiquitination can mediate TBK1 activity and degradation [6]. Some E3 ubiquitin ligases, such as TNF receptor-associated factor 3 (TRAF3), neuregulin receptor degradation protein-1 (Nrdp1), mind bomb 1 and 2 (MIB1 and MIB2) can activate TBK1 by promoting its K63-linked polyubiquitination [19,20]. While some deubiquitinases, for instance deubiquitinating enzyme cylindromatosis (CYLD), A20, RING finger protein 11 (RNF11) can disrupt K63-linked polyubiquitination to terminate TBK1-mediated signaling transduction [21–23]. Besides modulation of TBK1 K63-linked polyubiquitination, some E3 ligases specifically target K48-linked ubiquitination of TBK1 to promote its degradation through proteasome pathway [24–26].

TBK1 has been cloned and investigated in many fish species, such as grass carp (*Ctenopharyngodon idella*) [27], common carp (*Cyprinus carpio* L.) [28], Atlantic cod (*Gadus morhua* L.) [29], large yellow croaker (*Larimichthys crocea*) [30], zebrafish (*Danio rerio*) [31], and black carp (*Mylopharyngodon Piceus*). In grass carp, TBK1 overexpression induces the upregulations of IFN1 and Mx1 upon grass carp reovirus (GCRV) infection [27]. Grass carp TBK1 interacts with IRF7 and triggers the induction of IFN and ISGs in response to LPS stimulation [32]. Overexpression of common carp TBK1 results in a robust activation and upregulation of IRF3, IFN and Mx1 [28]. In zebrafish, full-length TBK1 significantly activates the IFNs signaling pathway in response to spring viremia of carp virus (SVCV) infection, whereas the TBK1 isoforms inhibit the activation of antiviral immune responses [31]. Black carp shows strongly antiviral activity in response to GCRV and SVCV infection [33]. Taken together, all those studies proof that TBK1 plays essential roles in fish innate immunity.

Grass carp is one of the most economically important freshwater fish species in China. But hemorrhagic disease caused by GCRV seriously affects the grass carp cultivation industry [34]. In this study, we found that TBK1 overexpression significantly inhibited the promoter activities of IRF7, IFN1 and IFN3, but not IRF3 post low titers of GCRV infection. Meanwhile, TBK1 overexpression suppressed IRF7 protein and IRF7 phosphorylation at late stage of GCRV infection. Furthermore, GCRV restrained TBK1 activation by repressing TBK1 K63-linked ubiquitination and facilitating its K48-linked ubiquitination, which resulted in that TBK1 inhibited IRF7-mediated IFN-I signal activation and promoted GCRV replication under low dose viral infection, but enhanced IFN production and restricted GCRV replication upon high dose viral infection. Those results uncovered a viral evasion strategy that GCRV escaped host IFN system by hijacking TBK1 to block downstream activation of IRF7-IFN-Is signaling pathway. This study lays a foundation for further investigating the crosstalk between host innate immunity and viral infection in fish species.

2. Materials and methods

2.1. Cell and virus infection

C. idella kidney (CIK) cell line was obtained from China Center for Type Culture Collection and cultured in medium DMEM (Sigma, USA). Fathead minnow (FHM) cell line was preserved in our lab and maintained in M199 (Gibco). Both CIK and FHM cells were supplemented with 10% inactivated fetal bovine serum, 100 U/ml of penicillin and 100 U/ml of streptomycin sulfate, and maintained at 28 °C with 5% CO_2.

For virus infection, CIK cells were plated for 24 h in advance and then infected with GCRV 097 stain (Type II GCRV) with different multiplicity of infection (MOI). After 2 h, the virus inoculum was removed, the cells were washed with PBS, and further incubated with new medium.

2.2. Plasmid constructions and transfections

pCMV-CMV-EGFP was used as original plasmid [35] for constructing the following expression plasmids: TBK1-HA, TBK1-KD-Flag, TBK1-KU-Flag and TBK1-CTD-Flag. GenBank accession number of grass carp TBK1 cDNA is JN704345. The ORF or domains of TBK1 were amplified from grass carp spleen tissue cDNA and then inserted behind the first CMV promoter. The Flag or HA tags were introduced by the reverse primers, and the primers were listed in Table 1. The luciferase reporter vectors of IRF3, IRF7, IFN1 and IFN3 were constructed in our previous report [36].

For transfections, CIK cells were plated in cell culture plates with different sizes for 24 h, and transfection was performed with FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's instructions [36].

2.3. Dual luciferase reporter assays

FHM cells were seeded in 24-well plates for 24 h and cotransfected with the indicated luciferase reporter plasmids, overexpression plasmids and pRL-TK vector. At 16 h post-transfection, the cells were infected with GCRV or treated with PBS for 24 h. Then the cells were washed, collected and lysed for dual-luciferase reporter assay according to the manufacturer's instructions (Promega) [36]. Luciferase activity was measured by Multiscan Spectrum (PerkinElmer). The results were obtained from three independent experiments.

2.4. Western blotting (WB) analyses and ubiquitination assays

CIK cells were transfected with TBK1-HA or empty vector in 6-well plates. Twenty-four hours later, the transfected cells were infected with GCRV for 12 h, 24 h, 48 h and 72 h. Then the cells were collected and lysed for WB analysis according to our previous description [36]. The following antibodies were used: anti-Flag (mouse monoclonal, 1:1000)

(Abcam), anti-HA (mouse monoclonal, 1:1000) (Abcam), anti-β-Tubulin (rabbit polyclonal, 1:5000) (Abcam). Rabbit polyclonal antiserum of IRF3 (1:1000) was kindly provided by Prof. Yibing Zhang and rabbit polyclonal antiserum of IRF7 (1:1000) was prepared in our laboratory.

Ubiquitination assay was performed as our previously described [36]. In brief, FHM cells were cotransfected with TBK1-KD-Flag and HA-Ub-K48O, TBK1-KD-Flag and HA-Ub-K63O, TBK1-KU-Flag and HA-Ub-K48O, TBK1-KU-Flag and HA-Ub-K63O, TBK1-CTD-Flag and HA-Ub-K48O, TBK1-CTD-Flag and HA-Ub-K63O, respectively. Twenty-four hours later, the cells were collected and subjected to IP with HA Ab, IB with Flag Ab.

2.5. qRT-PCR

CIK cells transfected TBK1-HA and empty vector were infected with GCRV for 3 h, 6 h, 12 h, 24 h and 48 h. All the cells were collected and subjected to total mRNA isolation using RNAiso Plus (TaKaRa) according to manufacturer's instructions. cDNA reverse transcription was performed as our previous report [35]. qRT-PCR was established in Roche LightCycler® 480 system to quantify the mRNA expressions of related genes. EF1α was employed as an internal control gene [37]. qRT-PCR amplification system and statistical analysis were carried out as previous study [35]. The specific primers for qRT-PCR of each gene were described in our previous publication [36]. The results were obtained from three independent experiments.

3. Results

3.1. TBK1 inhibits promoter activities of IRF7, IFN1 and IFN3 post GCRV infection at low titers

In mammals, TBK1 is crucial to receive signals from TRIF, MAVS and STING, and further induces IRF3/7 phosphorylation and IFN-I production [5]. Here, we firstly examined the influence of grass carp TBK1 overexpression on the promoter activity of IRF3 and IRF7 in response to GCRV infection with MOI = 0.5. Interestingly, TBK1 overexpression significantly inhibited the promoter activity of IRF7 but not IRF3 (Fig. 1A). Further result showed that the inhibition of IRF7 promoter activity was significantly enhanced along with the increase of TBK1 plasmid doses (Fig. 1B), which indicated that TBK1-induced inhibition of IRF7 promoter has a dose-dependent manner. In teleost, IFN1 and IFN3 have been reported to play important roles in response to viral infection [38–40]. Previous study reported that zebrafish IRF7 predominantly stimulates IFN3 [38]. So we examined the influence of TBK1 on IFN1 and IFN3 promoter activities. As shown in Fig. 1C, TBK1 overexpression remarkably inhibited the promoter activities of IFN1 and IFN3. Those data underlined that TBK1 may serve as a suppressor to IRF7-IFN-Is signaling pathway upon GCRV infection under certain condition.

3.2. TBK1 and LGP2 synergistically inhibit the promoter activity of IRF7

Our previous study proved that grass carp LGP2 (laboratory of genetics and physiology 2) overexpression inhibits the activation of IRF7 promoter [36]. Whether TBK1-induced IRF7 inhibition is correlated with LGP2? To clarify this hypothesis, we expressed TBK1 with or without LGP2 to analyze the promoter activation of IRF7. Dual-luciferase reporter assays uncovered that co-expression of LGP2 and TBK1 evoked more significant inhibition of IRF7 promoter activity than that induced by TBK1 or LGP2 individual overexpression upon GCRV infection at low titers (Fig. 2). This result showed that TBK1 and LGP2 can synergistically inhibit promoter activity of IRF7 in response to GCRV infection.

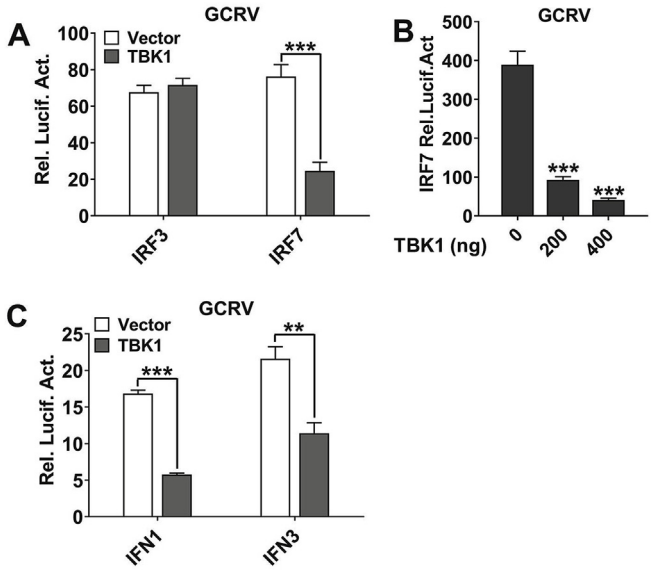

Fig. 1. TBK1 overexpression inhibits the promoter activities of IRF7, IFN1 and IFN3 post GCRV infection at low titers. (**A**) TBK1 overexpression inhibits the promoter activity of IRF7, but not IRF3. FHM cells were cotransfected with 400 ng of TBK1-HA overexpression plasmid, 40 ng pRL-TK, and 400 ng report vector IRF3pro-luc or IRF7pro-luc in 24-well plates. Control groups were transfected with 400 ng empty vector, 40 ng pRL-TK and 400 ng of the corresponding report vectors. After 16 h post-transfection, the cells were infected with GCRV, and dual-luciferase report assays were conducted at 24 h post GCRV infection. Error bars indicate standard deviations (SDs) (n = 4). (**B**) TBK1 suppresses the promoter activities of IRF7 in a dose-dependent manner. FHM cells were seeded in 24-well plates for 24 h, and transfected with 400 ng IRF7pro-luc, 40 ng pRL-TK and TBK1-HA (0 ng, 200 ng and 400 ng) together with decreasing amounts of empty vector (400 ng, 200 ng and 0 ng). Then the cells were infected with GCRV for 24 h. Dual-luciferase report assays were performed as above description. (**C**) TBK1 suppresses the promoter activities of IFN1 and IFN3. FHM cells were cotransfected with 400 ng of TBK1-HA overexpression plasmid or empty vector, 40 ng pRL-TK, and 400 ng report vector IFN1pro-luc or IFN3pro-luc in 24-well plates. At 16 h post-transfection, the cells were infected with GCRV at 24 h, and dual-luciferase report assays were conducted as above description. Asterisks indicate significant differences between TBK1-HA and empty vector transfected groups at the same time points (**$P < 0.01$; ***$P < 0.001$).

3.3. TBK1 overexpression inhibits phosphorylation of IRF7 under low titers of GCRV infection

Phosphorylation is critical for IRF3 and IRF7 activation and subsequent IFN-I production [36,38]. To explore the influence of TBK1 overexpression on IRF3 and IRF7 activations, protein levels of IRF3 and IRF7 were examined with or without TBK1 overexpression in response to GCRV infection with MOI = 0.5. From 0 h to 24 h post GCRV infection, TBK1 overexpression hardly changed the total protein and phosphorylation levels of IRF7 (Fig. 3A–C). However, TBK1 overexpression significantly restrained them at 48 h and 72 h after GCRV infection (Fig. 3A–C). As for IRF3, TBK1 overexpression just up-regulated the total protein level at 12 h and 24 h, but had no influence on the phosphorylation level post GCRV infection (Fig. 3A, D and E). These results proved that TBK1 can specifically inhibit the activation of IRF7 but not IRF3 in response to GCRV infection. Interestingly, under normal condition, some TBK1-HA specific bands with bigger molecular weights (MW) than basal TBK1 were detected by HA antibody. Upon GCRV infection, the expressions of these bands were increased at 12 h and 24 h, but diminished at later time points (at 48 h and 72 h) (Fig. 3A). Meanwhile, TBK1 basic level was decreased at late phase post GCRV infection. This finding underlined that TBK1 undergoes some kinds of posttranslational modification (PTM), and those modifications is

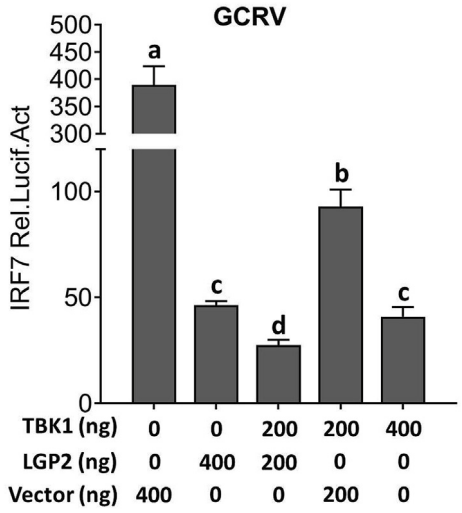

Fig. 2. TBK1 and LGP2 synergistically suppress the promoter activities of IRF7. FHM cells were cotransfected 400 ng IRF7pro-luc, 40 ng pRL-TK, and the indicated amounts of TBK1-HA, LGP2-Flag and empty vector for 16 h. Then, the cells were infected with GCRV for 24 h, and dual-luciferase report assays were conducted. Error bars indicate SDs (n = 4). Lowercases indicate significant differences.

involved in the process of GCRV infection.

3.4. Ubiquitination is involved in regulation of TBK1 activation

TBK1 activation is regulated in a variety of ways, in which protein ubiquitination and phosphorylation are two crucial PTMs that mediate TBK1 [5]. To identify the PTM of TBK1 mentioned in Fig. 3A, ubiquitination and phosphorylation of TBK1 were investigated at protein levels. In TBK1-HA overexpression cells, no phosphorylation bands were detected by IP with anti-HA Ab and IB with anti-pSer, anti-pThr, anti-pTyr Abs according to our previous description (data no shown) [36]. This result proved that the detected modification of TBK1 in Fig. 3A is not phosphorylation. Interestingly, protein level of TBK1-Flag was significantly enhanced upon proteasome inhibitor MG132 treatment (Fig. 4A), which means that ubiquitination is involved in TBK1 activation. Subsequently, expression plasmids of TBK1 different truncations fusing Flag were constructed (Fig. 4B). Under basal condition, only PTM bands were detected in TBK1-CTD, but not in TBK1-KD and TBK1-KU transfected cells (Fig. 4C), and the PTM bands in TBK1-CTD domain were similar with those detected in full-length TBK1 (Fig. 3A), which suggested that the PTM detected in Fig. 3A mainly occurred in the CTD domain of TBK1. To identify the ubiquitination types of TBK1, expression plasmids of TBK1 different domains were cotransfected with HA-Ub-K48O or HA-Ub-K63O plasmids, respectively. As shown in Fig. 4D, both K48- and K63-linked ubiquitination were detected in TBK1-KU domain but not in TBK1-KD domain under basal condition. Meanwhile, significant K63-linked ubiquitination and weak K48-linked ubiquitination were occurred in TBK1-CTD domain (Fig. 4D). Upon MG132 treatment, the protein level of TBK1-CTD was slightly enhanced but had no significant difference (Fig. 4E), which indicated that TBK1-CTD domain mainly undergoes K63-linked ubiquitination under basal condition. Therefore, the PTM mentioned in Fig. 3A is K63-linked ubiquitination which was enhanced at the early stage but inhibited at the late stage post GCRV infection. K48-linked ubiquitination in TBK1-KU domain may result in TBK1 degradation via proteasome pathway.

3.5. TBK1 suppresses IRF7-mediated IFN signaling in response to low titers of GCRV infection

In zebrafish, full-length TBK1 positively regulates IFN-Is signaling pathway in response to SVCV infection [15]. However, our results uncovered the negative role of TBK1 in IRF7-mediated signaling post GCRV infection. To address the issue, TBK1 overexpression cells were infected by GCRV with different viral infection doses (MOI = 0.5, 1.0, 2.0), and mRNA levels of IRF7, IFN1 and IFN3 were measured by qRT-PCR. Empty vector transfected cells were used as control. Because high doses of GCRV infection caused all the cells death, we collected the samples at 3 h, 6 h, 24 h, 48 h post low dose of GCRV infection (MOI = 0.5), at 3 h, 6 h, 12 h and 24 h with MOI of 1.0, and at 3 h, 6 h and 12 h after high dose of GCRV infection (MOI = 2.0). As shown in Fig. 5A upper, TBK1 overexpression significantly inhibited mRNA levels of IRF7 at 6 h and 48 h post GCRV infection with low infected dose (MOI = 0.5). With GCRV MOI = 1.0, TBK1 overexpression still suppressed the transcription level of IRF7 at 6 h, but significantly enhanced the expression of IRF7 at 24 h (Fig. 5A middle). Furthermore, TBK1 overexpression significantly upregulated the expression of IRF7 at 12 h (Fig. 5A below). Consistently, at low dose of GCRV (MOI = 0.5) infection, TBK1 overexpression significantly inhibited the transcription levels of IFN1 at 12 h, 24 h and 48 h, and down-regulated mRNA levels of IFN3 at 24 h and 48 h, respectively (Fig. 5B and C, upper). With GCRV MOI = 1.0, TBK1 overexpression enhanced the expression levels of IFN1 and IFN3 at 24 h (Fig. 5B and C, middle). With high dose of GCRV infection (MOI = 2.0), TBK restrained the expression levels of IFN1 and IFN3 at 3 h, while promoted mRNA levels at 12 h (Fig. 5B and C, below). Collectively, all these results implied that TBK1 function is dependent on the doses of GCRV infection. In detail, TBK1 inhibited IRF7-mediated IFN-Is activation in response to GCRV with low MOI. Along with the increasing of GCRV infected doses, TBK1 switches it function as a positive regulator to antiviral signaling. Meanwhile, the more infected GCRV quantity, the more rapid functional switch of TBK1 from negative to positive regulation.

TBK1 promotes GCRV replication in low titers of GCRV infection but inhibits GCRV replication in high titers of GCRV infection.

To investigate the impact of TBK1 overexpression on GCRV replication in cells infected with GCRV at different viral doses, transcription level of GCRV VP4 gene was examined by qRT-PCR in the samples described above. As shown in Fig. 6, TBK1 overexpression significantly up-regulated the expression levels of VP4 at 12 h, 24 h and 48 h post low titers (MOI = 0.5) of GCRV infection (Fig. 6A). When the dose of GCRV increased to MOI = 1.0, there was no significant difference of VP4 expression between TBK1 overexpression and empty vector transfected cells (Fig. 6B). Upon high dose of GCRV (MOI = 2.0) infection, TBK1 overexpression switched the function to inhibit VP4 expression (Fig. 6C). All these results suggested that TBK1 promotes viral replication in cells infected with low dose of GCRV, but inhibits viral replication in cells infected with high dose of GCRV.

4. Discussion

IFN-I and IFN-stimulated genes (ISGs) are the first line of host to defense against virus infection [41]. Upon virus infection, viral specific signatures are recognized by PRRs, and further induce PRR downstream signaling cascades. TBK1 is an important checkpoint that receives signals from TLR pathway, RLR pathway and cGAMP synthase (cGAS) pathway [42–44], which is essential for IFN-I production in antiviral immune responses [5]. Up to now, numerous reports certify the vital antiviral function of TBK1 in response to virus infection not only in mammal, but also in fish species [15,45–47]. Our previous study demonstrated that TBK1 overexpression enhanced the transcription levels of IRF3, IRF7 and IFN1 in response to GCRV infection with MOI = 1 [27], which means that TBK1 is essential for GCRV-induced immune activation. Interestingly, there is a curious phenomenon during GCRV infection: when infected with GCRV with MOI = 1, CIK cells firstly undergoes cytopathic effect (CPE), then more serious CPE, and final death. However, when reducing GCRV titers to MOI = 0.5, CPE is induced at early stage, and then fades away along with the time

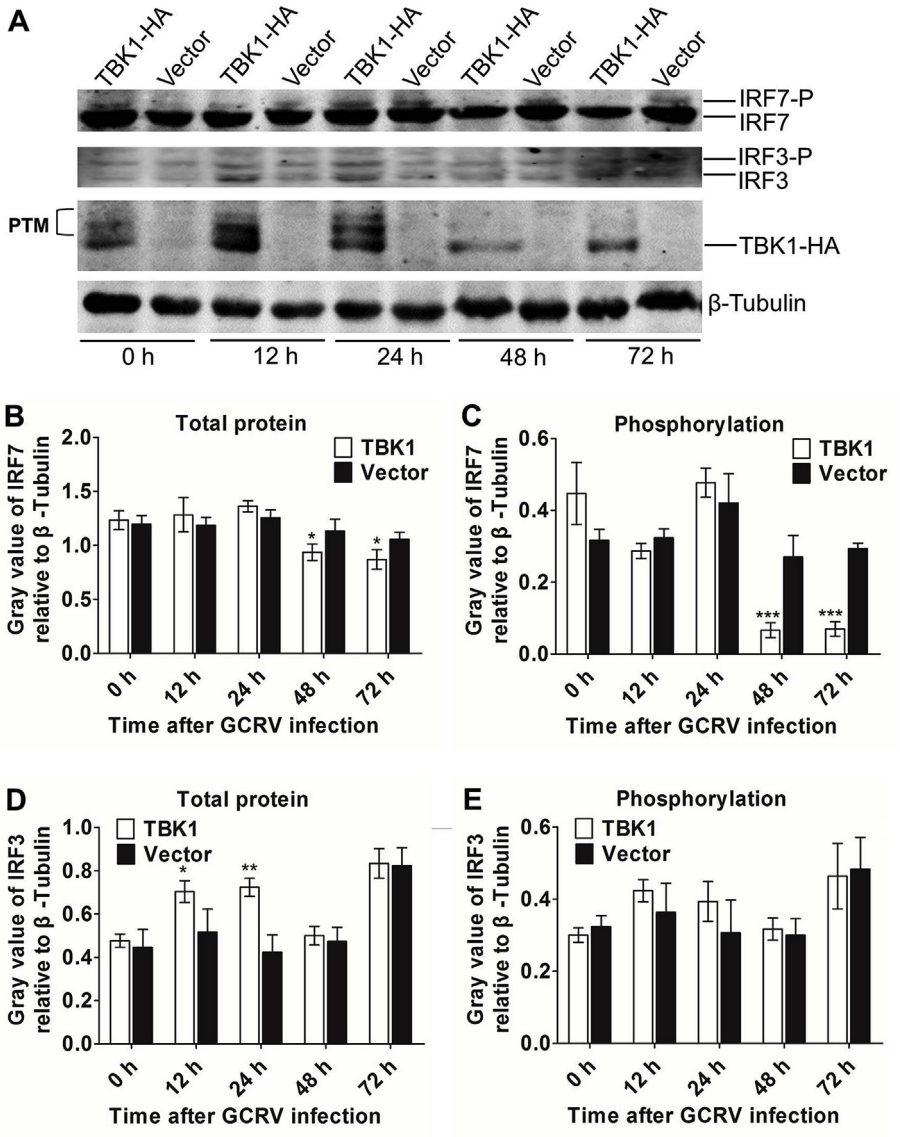

Fig. 3. Influence of TBK1 overexpression on protein levels of IRF3 and IRF7 post GCRV infection. (**A**) CIK cells were seeded in 6-well plates for 24 h, and transfected with 2000 ng TBK1-HA or empty vector for 16 h. Then the cells were infected with GCRV for 12 h, 24 h, 48 h and 72 h. Whole cell lysate was subjected to WB analysis with anti-IRF3, anti-IRF7, anti-HA and anti-β-Tubulin Abs, respectively. (**B-E**) The relative protein expression levels of IRF3 and IRF7 were quantified by using ImageJ software. Error bars indicate SDs (n = 3). Asterisks indicate significant differences from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

extension. Finally, the infected cells tend to grow well at late stage. This phenomenon raises a question that whether GCRV with various titers induce immune activation of different pattern? Considering the essential role in delivering signals from different PRRs, TBK1 was selected to verify this hypothesis. Interestingly, TBK1 overexpression inhibited phosphorylation of IRF7 and promoter activities of IRF7, IFN1 and IFN3 in response to low titers of GCRV infection. Those data underline a negative role of TBK1 in response to GCRV with low concentration infection. However, along with the increasing of viral titers, TBK1 switches it function from an inhibitor to a positive regulator for IRF7-IFN-Is signaling. Accordingly, we can speculate that low GCRV titers are insufficient to trigger the activation of host antiviral immune system. Under this condition, a portion of virion can successfully escape the detection from PRRs. In cytoplasm, GCRV hijacks TBK1 to silence cytoplasmic PRR activation and IFN induction which is beneficial for viral replication. Under this condition, GCRV on one hand tends to inhibit TBK1 K63-linked ubiquitination, and on the other hand induces degradation of TBK1 by promoting K48-linked ubiquitination. For lack of signaling delivered from upstream PRRs, TBK1 is unable to induce IRF7 activation. Along with the increase of virus quantity, PRRs are activated, which further positively regulate K63-linked ubiquitination of TBK1. Correspondingly, GCRV-induced inhibition of TBK1 is eliminated, and TBK1's antiviral function is recovered. Actually, this is not the first report on viral concentration-induced function switch of antiviral immune genes. In zebrafish, SVCV induces antithetic effects of LGP2 under different viral titers [40]. The difference is that zebrafish LGP2 functions positively at the beginning (low viral replication) of SVCV infection, and negatively in the late phase (high viral replication). Although LGP2 is highly conserved between zebrafish and grass carp, our previous study uncovered its negative role in response to GCRV infection [36]. We also found that TBK1 and LGP2 can synergistically inhibit promoter activity of IRF7 in response to GCRV infection, which means that negative function of TBK1 is associated with LGP2. More data are needed to clarify the regulation of LGP2 to TBK1. All those data underline the complexity of fish innate immune system.

During co-evolution of viruses and hosts, hosts have developed a robust IFN system to resist viral infection, while viruses have evolved multiple ways to dampen the host IFN response by interfering or

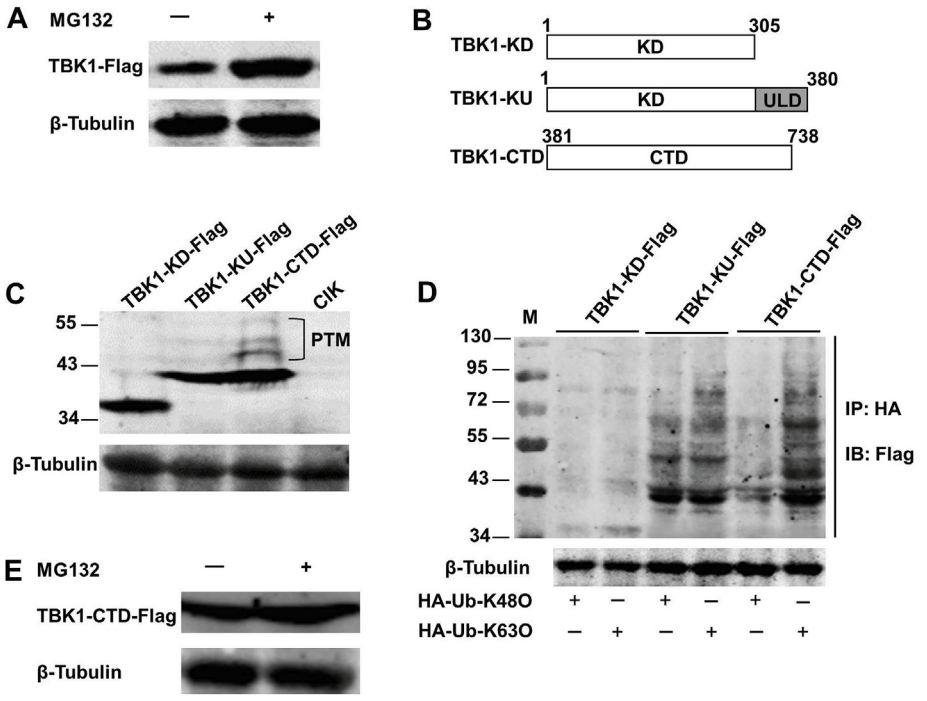

Fig. 4. Identify the ubiquitination of TBK1. (**A**) Protein stability analyses of TBK1-Flag in the absence or presence of MG132. CIK cells were transfected TBK1-Flag for 24 h, and then treated with MG132 for 8 h. The cell lysate was subjected to WB analysis with anti-Flag and β-Tubulin Abs. (**B**) Schematic representations of three truncations used in this study. (**C**) Examining the expressions of exogenous TBK1-KD, TBK1-KU and TBK1-CTD in CIK cells. CIK cells were transfected with TBK1-KD-Flag, TBK1-KU-Flag and TBK1-CTD-Flag in 6-well plates. Twenty-four hours later, the cell lysate was subject to WB analysis with anti-Flag and anti-β-Tubulin Abs. (**D**) Ubiquitination analyses of different TBK1 constructions. FHM cells were cotransfected with TBK1-KD-Flag and HA-Ub-K48O, TBK1-KD-Flag and HA-Ub-K63O, TBK1-KU-Flag and HA-Ub-K48O, TBK1-KU-Flag and HA-Ub-K63O, TBK1-CTD-Flag and HA-Ub-K48O, TBK1-CTD-Flag and HA-Ub-K63O, respectively. Twenty-four hours later, the cells were collected and subjected to IP with HA Ab, IB with Flag Ab. (**E**) Analyzing the protein stability of TBK1-CTD domain in response to MG132 treatment. CIK cells were transfected TBK1-CTD-Flag in 6-well plates for 24 h, and then treated with MG132 for 8 h. WB analysis was conducted with anti-Flag and β-Tubulin Abs respectively.

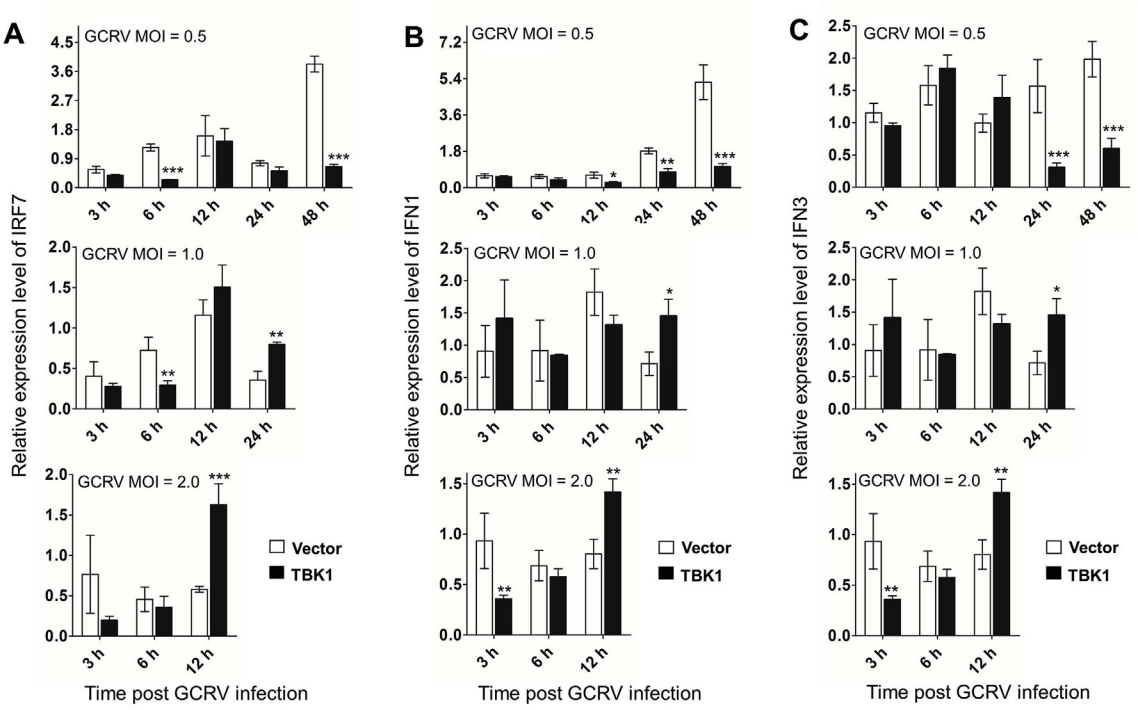

Fig. 5. Influences of TBK1 overexpression on IRF7, IFN1 and IFN3 mRNA levels in response to GCRV infection with different viral titters. CIK cells were transfected with TBK1-HA or empty vector in 12-well plates. At 16 post transfection, cells were infected with GCRV at MOI = 0.5, 1.0, and 2.0, respectively. The genes expression levels of IRF7 (**A**), IFN1 (**B**) and IFN3 (**C**) were measured at the indicated time points post GCRV infection. EF1α was utilized as an internal control gene. Asterisks (*) indicate significant differences between TBK1-Flag and empty vector transfected cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Error bars indicate SDs (n = 4).

evading specific host regulators [48]. Present study found a phenomenon that GCRV antagonizes host immune system to benefit its own replication by hijacking TBK1 to repress IRF7-mediated IFN-Is signaling under low infected dose. We also preliminarily uncovered a probable mechanism that GCRV inhibited TBK1 activation via regulating its ubiquitination. Up to date, GCRV genome encodes in total 7 structural proteins and 6 nonstructural proteins, but none of those proteins possess E3 ligases or E3 ligases-like functions [34,49]. So the regulation of TBK1 ubiquitination mediated by GCRV is indirect. Some cellular E3 ligases must be utilized by GCRV to antagonize TBK1 activation. In zebrafish, major vault protein (MVP) recruits and degrades full-length TBK1 through lysosome but not proteasome dependent pathway to inhibit IFN production [47]. Meanwhile, another two alternatively spliced isoforms of zebrafish TBK1, termed TBK1_tv1 and TBK1_tv2 are

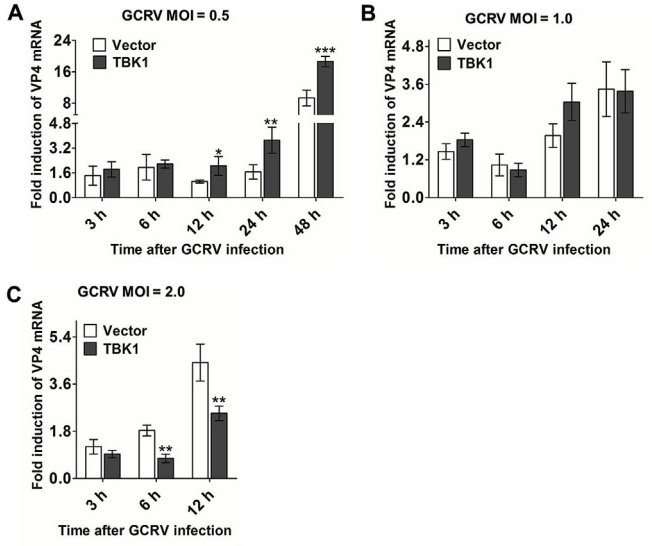

Fig. 6. Influences of TBK1 overexpression on GCRV replication. CIK cells were transfected with TBK1-HA or empty vector. mRNA levels of GCRV VP4 gene was examined at the indicated time points post GCRV infection with MOI = 0.5 (**A**), MOI = 1.0 (**B**) and MOI = 2.0 (**C**). EF1α was utilized as an internal control gene. Asterisks (*) indicate significant differences between TBK1-Flag and empty vector transfected cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Error bars indicate SDs (n = 4).

found to negatively regulate TBK1-IRF3 antiviral pathway [15]. All the two findings emphasize the strategies of hosts to maintain immune homeostasis via regulating TBK1 activation. Our data supported an evasion mechanism of GCRV to define host immune system. However, more studies are needed to identify which viral protein(s) and cellular E3 ligase(s) participate in this process.

In conclusion, present study uncovered an evasion strategy of GCRV to escape cellular antiviral immune responses, by which GCRV restrains TBK1 activation via inhibiting K63-linked ubiquitination and promoting K48-linked ubiquitination of TBK1 to antagonize host IFNs responses post GCRV infection at low titers or early stages. Under this circumstance, TBK1 overexpression could not promote but inhibit IRF7 activation and downstream IFN-Is induction. This finding provides a novel insight into the viral evasion mechanism in low vertebrates, which lays a foundation for further researches in the context of host-virus interactions.

Disclosures

The authors have no competing interests.

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References

[1] C. Langevin, E. Aleksejeva, G. Passoni, N. Palha, J.P. Levraud, P. Boudinot, The antiviral innate immune response in fish: evolution and conservation of the IFN system, J. Mol. Biol. 425 (2013) 4904–4920.
[2] H.J. Moon, J.S. Lee, Y.K. Choi, J.Y. Park, M.R. Talactac, M.Y. Chowdhury, H. Poo, M.H. Sung, J.H. Lee, J.U. Jung, C.J. Kim, Induction of type I interferon by high-molecular poly-gamma-glutamate protects B6.A2G-Mx1 mice against influenza A virus, Antivir. Res. 94 (2012) 98–102.
[3] L. Unterholzner, R.P. Sumner, M. Baran, H. Ren, D.S. Mansur, N.M. Bourke, F. Randow, G.L. Smith, A.G. Bowie, Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7, PLoS Pathog. 7 (2011) e1002247.
[4] Y.B. Zhang, J.F. Gui, Molecular regulation of interferon antiviral response in fish, Dev. Comp. Immunol. 38 (2012) 193–202.
[5] G. Song, B. Liu, Z. Li, H. Wu, P. Wang, K. Zhao, G. Jiang, L. Zhang, C. Gao, E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1, Nat. Immunol. 17 (2016) 1342–1351.
[6] W. Zhao, Negative regulation of TBK1-mediated antiviral immunity, FEBS Lett. 587 (2013) 542–548.
[7] A.L. Blasius, B. Beutler, Intracellular toll-like receptors, Immunity 32 (2010) 305–315.
[8] D. Goubau, S. Deddouche, E.S.C. Reis, Cytosolic sensing of viruses, Immunity 38 (2013) 855–869.
[9] S. Reikine, J.B. Nguyen, Y. Modis, Pattern recognition and signaling mechanisms of RIG-I and MDA5, Front. Immunol. 5 (2014) 342.
[10] S.R. Margolis, S.C. Wilson, R.E. Vance, Evolutionary origins of cGAS-STING signaling, Trends Immunol. 38 (2017) 733–743.
[11] H. Ishikawa, G.N. Barber, STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling, Nature 455 (2008) 674–678.
[12] Y. Ran, H.B. Shu, Y.Y. Wang, MITA/STING: a central and multifaceted mediator in innate immune response, Cytokine Growth Factor Rev. 25 (2014) 631–639.
[13] S. Liu, X. Cai, J. Wu, Q. Cong, X. Chen, T. Li, F. Du, J. Ren, Y.T. Wu, N.V. Grishin, Z.J. Chen, Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation, Science 347 (2015) aaa2630.
[14] Q. Liang, B. Fu, F. Wu, X. Li, Y. Yuan, F. Zhu, ORF45 of Kaposi's sarcoma-associated herpesvirus inhibits phosphorylation of interferon regulatory factor 7 by IKKepsilon and TBK1 as an alternative substrate, J. Virol. 86 (2012) 10162–10172.
[15] Y. Hu, J. Zhang, X. Wu, L. Cao, P. Nie, M. Chang, TANK-binding kinase 1 (TBK1) isoforms negatively regulate type I interferon induction by inhibiting TBK1-IRF3 interaction and IRF3 phosphorylation, Front. Immunol. 9 (2018) 84.
[16] M. Lin, Z. Zhao, Z. Yang, Q. Meng, P. Tan, W. Xie, Y. Qin, R.F. Wang, J. Cui, USP38 inhibits type I interferon signaling by editing TBK1 ubiquitination through NLRP4 signalosome, Mol. Cell 64 (2016) 267–281.
[17] J. Li, J. Li, A. Miyahira, J. Sun, Y. Liu, G. Cheng, H. Liang, Crystal structure of the ubiquitin-like domain of human TBK1, Protein Cell 3 (2012) 383–391.
[18] F. Li, X. Xie, Y. Wang, J. Liu, X. Cheng, Y. Guo, Y. Gong, S. Hu, L. Pan, Structural insights into the interaction and disease mechanism of neurodegenerative disease-associated optineurin and TBK1 proteins, Nat. Commun. 7 (2016) 12708.
[19] S. Li, L. Wang, M. Berman, Y.Y. Kong, M.E. Dorf, Mapping a dynamic innate immunity protein interaction network regulating type I interferon production, Immunity 35 (2011) 426–440.
[20] C. Wang, T. Chen, J. Zhang, M. Yang, N. Li, X. Xu, X. Cao, The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon, Nat. Immunol. 10 (2009) 744–752.
[21] M. Zhang, X. Wu, A.J. Lee, W. Jin, M. Chang, A. Wright, T. Imaizumi, S.C. Sun, Regulation of IkappaB kinase-related kinases and antiviral responses by tumor suppressor CYLD, J. Biol. Chem. 283 (2008) 18621–18626.
[22] K. Parvatiyar, G.N. Barber, E.W. Harhaj, TAX1BP1 and A20 inhibit antiviral signaling by targeting TBK1-IKKi kinases, J. Biol. Chem. 285 (2010) 14999–15009.
[23] S. Charoenthongtrakul, L. Gao, K. Parvatiyar, D. Lee, E.W. Harhaj, RING finger protein 11 targets TBK1/IKKi kinases to inhibit antiviral signaling, PLoS One 8 (2013) e53717.
[24] J. Cui, Y. Li, L. Zhu, D. Liu, S. Zhou, H.Y. Wang, R.F. Wang, NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4, Nat. Immunol. 13 (2012) 387–395.
[25] M. Zhang, L. Wang, X. Zhao, K. Zhao, H. Meng, W. Zhao, C. Gao, TRAF-interacting protein (TRIP) negatively regulates IFN-beta production and antiviral response by promoting proteasomal degradation of TANK-binding kinase 1, J. Exp. Med. 209 (2012) 1703–1711.
[26] L. Galluzzi, O. Kepp, G. Kroemer, Autophagy and innate immunity ally against bacterial invasion, EMBO J. 30 (2011) 3213–3214.
[27] X. Feng, J. Su, C. Yang, N. Yan, Y. Rao, X. Chen, Molecular characterizations of grass carp (*Ctenopharyngodon idella*) TBK1 gene and its roles in regulating IFN-I pathway, Dev. Comp. Immunol. 45 (2014) 278–290.
[28] F. Sun, Y.B. Zhang, T.K. Liu, J. Shi, B. Wang, J.F. Gui, Fish MITA serves as a mediator for distinct fish IFN gene activation dependent on IRF3 or IRF7, J. Immunol. 187 (2011) 2531–2539.
[29] H. Chi, Z. Zhang, J. Bogwald, W. Zhan, R.A. Dalmo, Cloning, expression analysis and promoter structure of TBK1 (TANK-binding kinase 1) in Atlantic cod (*Gadus morhua L.*), Fish Shellfish Immunol. 30 (2011) 1055–1063.
[30] D.L. Zhang, D.H. Yu, J. Chen, S. Fan, Z.Y. Wang, Expression profiles and interaction suggest TBK1 can be regulated by Nrdp1 in response to immune stimulation in large yellow croaker *Larimichthys crocea*, Fish Shellfish Immunol. 46 (2015) 745–752.
[31] L. Zhang, W.Q. Chen, Y.W. Hu, X.M. Wu, P. Nie, M.X. Chang, TBK1-like transcript negatively regulates the production of IFN and IFN-stimulated genes through RLRs-MAVS-TBK1 pathway, Fish Shellfish Immunol. 54 (2016) 135–143.
[32] N. Yu, X. Xu, G. Qi, D. Liu, X. Chen, X. Ran, Z. Jiang, Y. Li, H. Mao, C. Hu, Ctenopharyngodon idella TBK1 activates innate immune response via IRF7, Fish Shellfish Immunol. 80 (2018) 521–527.
[33] C. Yan, J. Xiao, J. Li, H. Chen, J. Liu, C. Wang, C. Feng, H. Feng, TBK1 of black carp plays an important role in host innate immune response against SVCV and GCRV, Fish Shellfish Immunol. 69 (2017) 108–118.
[34] Y. Rao, J. Su, Insights into the antiviral immunity against grass carp (*Ctenopharyngodon idella*) reovirus (GCRV) in grass carp, J. Immunol. Res. 2015 (2015) 670437.
[35] Y. Rao, J. Su, C. Yang, L. Peng, X. Feng, Q. Li, Characterizations of two grass carp *Ctenopharyngodon idella* HMGB2 genes and potential roles in innate immunity, Dev. Comp. Immunol. 41 (2013) 164–177.

[36] Y. Rao, Q. Wan, C. Yang, J. Su, Grass carp laboratory of genetics and physiology 2 serves as a negative regulator in retinoic acid-inducible gene I- and melanoma differentiation-associated gene 5-mediated antiviral signaling in resting state and early stage of grass carp reovirus infection, Front. Immunol. 8 (2017) 352.
[37] J. Su, R. Zhang, J. Dong, C. Yang, Evaluation of internal control genes for qRT-PCR normalization in tissues and cell culture for antiviral studies of grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 30 (2011) 830–835.
[38] H. Feng, Q. Zhang, Y. Zhang, Z. Li, J. Zhang, Y. Xiong, M. Wu, J. Gui, Zebrafish IRF1, IRF3, and IRF7 differentially regulate IFNPhi1 and IFNPhi3 expression through assembly of homo- or heteroprotein complexes, J. Immunol. 197 (2016) 1893–1904.
[39] Q. Wan, C. Yang, Y. Rao, Z. Liao, J. Su, MDA5 induces a stronger interferon response than RIG-I to GCRV infection through a mechanism involving the phosphorylation and dimerization of IRF3 and IRF7 in CIK cells, Front. Immunol. 8 (2017) 189.
[40] Q.M. Zhang, X. Zhao, Z. Li, M. Wu, J.F. Gui, Y.B. Zhang, Alternative splicing transcripts of zebrafish LGP2 gene differentially contribute to IFN antiviral response, J. Immunol. 200 (2018) 688–703.
[41] P. Feng, A. Moses, K. Fruh, Evasion of adaptive and innate immune response mechanisms by gamma-herpesviruses, Curr. Opin. Virol. 3 (2013) 285–295.
[42] L. Sun, J. Wu, F. Du, X. Chen, J. C.Z, Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway, Science 339 (2013) 786–791.
[43] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (2010) 805–820.
[44] J. Zhang, J. Zhao, S. Xu, J. Li, S. He, Y. Zeng, L. Xie, N. Xie, T. Liu, K. Lee, G.J. Seo, L. Chen, A.C. Stabell, Z. Xia, S.L. Sawyer, J. Jung, C. Huang, P. Feng, Species-specific deamidation of cGAS by herpes simplex virus UL37 protein facilitates viral replication, Cell Host Microbe 24 (2018) 234–248.
[45] M. Du, J. Liu, X. Chen, Y. Xie, C. Yuan, Y. Xiang, B. Sun, K. Lan, M. Chen, S.J. James, Y. Zhang, J. Zhong, H. Xiao, Casein kinase II controls TBK1/IRF3 activation in IFN response against viral infection, J. Immunol. 194 (2015) 4477–4488.
[46] R. Fang, Q. Jiang, X. Zhou, C. Wang, Y. Guan, J. Tao, J. Xi, J.M. Feng, Z. Jiang, MAVS activates TBK1 and IKKepsilon through TRAFs in NEMO dependent and independent manner, PLoS Pathog. 13 (2017) e1006720.
[47] S. Li, L. Lu, Z. Li, C. Zhang, X. Zhou, Y. Zhou, Y. Zhang, Zebrafish MVP recruits and degrades TBK1 to suppress IFN production, J. Immunol. 202 (2019) 559–566.
[48] A. Garcia-Sastre, Ten strategies of interferon evasion by viruses, Cell Host Microbe 22 (2017) 176–184.
[49] F. Yu, L. Wang, W. Li, L. Lu, Identification of a novel membrane-associated protein from the S7 segment of grass carp reovirus, J. Gen. Virol. 100 (2019) 369–379.