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Overexpression of CiIKKβ enhances CIK cell viability against ER stress

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ABSTRACT

Recently, studies have shown that IκB kinase β (IKKβ), a critical kinase in the nucleus factor kappa-B (NF-κB) pathway, participates in inflammatory responses associated with unfolded protein response (UPR) and plays an important role in ER stress-induced cell death. The unfolded protein response (UPR), which is a regulatory system to restore cellular homeostasis in the endoplasmic reticulum (ER), such as oxidative stress, bacterial infection, and virus invasion. The UPR pathways have been reported to be involved in immune responses in mammals, including the classical NF-κB pathway. However, the molecular mechanism of their crosstalk remains to be elucidated. Previously, we demonstrated that IKKβ also has some conserved functions between fish and human, as grass carp (*Ctenopharyngodon idella*) IKKβ (CiIKKβ) can activate NF-κB pathway. In this study, we found that CiIKKβ level in nucleus was elevated under ER stress and CiIKKβ can interact with grass carp X-box-binding protein 1 (CiXBP1S), a key transcription factor in UPR. Consistently, fluorescent histochemical analysis of grass carp kidney (CIK) cells indicated that CiIKKβ and CiXBP1S colocalized under ER stress. Furthermore, overexpression of CiIKKβ in CIK cells enhanced ER stress tolerance by regulating UPR signaling and resulted in the significant increase of cell viability.

1. Introduction

While organisms need effective immune responses to protect themselves from pathogens [1], they also require immune regulations to control inflammatory responses and prevent autoimmune diseases [2]. NF-κB is a master transcriptional regulator of such inflammatory cascade, and IκB kinase β (IKKβ) is essential for NF-κB activation. Once IKKβ is activated, the NF-κB inhibitory protein IκBα is degraded, and NF-κB is translocated to the nucleus, mediating transcription of the downstream target genes. In addition, IKKβ can interact with many other immune-related proteins, such as forkhead transcription factor FOXO3a [3], insulin Receptor Substrate 1 (IRS1) [4] and docking protein 1 (DOK1) [5]. Therefore, IKKβ performs important functions in cellular immune responses.

Various stressors and stimuli, including pathogens [6], virus [7], reactive oxygen species (ROS) [8,9], and nutrients [10] are considered to be immune response inducers, and they can also disrupt homeostasis of endoplasmic reticulum (ER), a continuous membrane organelle in eukaryotic cells with multiple functions such as protein synthesis, fold, modification, and transport. Alterations in ER homeostasis can cause the accumulation of misfolded proteins, induces ER stress [11], and triggers the unfolded protein response (UPR), an adaptive response designed to restore protein-folding homeostasis. There are three ER stress sensors that can initiate the UPR: inositol-requiring 1 (IRE1), PKR-like ER kinase (PERK), and transcription factor 6 (ATF6) [12]. In the IRE1 pathway, the activated IRE1-α allows translation of a gene encoding a major regulator of ER homeostasis XBP1S [13]. Then, XBP1S is translocated into the cell nucleus and triggers the expression of ER chaperons, therefore enhancing the stability of homeostasis.

Immune responses are often accompanied with UPR, and many studies demonstrated that the UPR and innate immune pathways are interconnected. Immune cells need to rapidly increase protein production against immunogens and avoid ER stress-induced apoptosis [14,15]. For instance, UPR is activated during the differentiation of B lymphocytes to prepare for antibody production and secretion [16]. Liu et al. (2016) also showed that IKKβ activity in the liver of obese mice significantly enhanced expression of XBP1S. Those researches indicated a close relationship between immune response and ER homeostasis.

Recently, an increasing number of genes associated with the NF-κB pathway have been identified in fish, such as nucleotide-binding

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oligomerization domain-containing protein 1 (NOD1) [17], retinoic acid-inducible gene I (RIG-I) [18], and thioester-containing proteins (TEPs) genes [19]. Although there are several reports on fish UPR pathway [20–22], the molecular mechanism of the crosstalk between the NF-κB and UPR pathways is still unclear. To better understand the relationship between IKKβ and ER stress in fish, we performed subcellular localization and co-immunoprecipitation (Co-IP) assays in grass carp (*Ctenopharyngodon idella*), and the results indicated the interaction of CiIKKβ and CiXBP1S under ER stress. Further analyses showed that the overexpression of CiIKKβ enhanced the tolerance to tunicamycin (TM)-induced ER stress. Therefore, fish IKKβ is suggested to play an important role in cellular UPR via interaction with XBP1S. These results provide a novel insight between immune responses and ER stress.

2. Materials and methods

2.1. Primers and plasmids

The primers were designed by Oligo 7 software and synthesized by Sangon Biotech Company (Shanghai, China). Grass carp (*C. idella*) IKKβ (CiIKKβ) open reading frames (ORFs) were cloned into pCMV-C-mCherry (Beyotime) and pCMV-Flag (Beyotime) plasmids, respectively. Grass carp (*C. idella*) XBP1S (CiXBP1S) open reading frames (ORFs) were cloned into pEGFP-C1 (Addgene) and pCMV-HA (Beyotime) plasmids, respectively. All primers used in plasmids construction and RT-qPCR process were provided in Table 1.

2.2. Cell culture, transfection and RNA interference

HEK-293T cells (ATCC) were cultured in DMEM medium (Corning) with 10% FBS (Biological Industries) at 37 °C suitable incubator with 5% CO_2 in the air atmosphere. *C. idella* kidney (CIK) cells (preserved in our lab) were maintained at 28 °C with M199 medium (Corning) plus 10% FBS (Biological Industries).

Cells were transfected with the plasmids using FuGENE 6 Transfection Reagent Kit (Promega) according to the manufacturer's instructions. To induce ER stress, a final concentration of 2.5 μg/ml tunicamycin (TM) (SIGMA) was added to the transfected cells. siRNA sequence against *CiXBP1S* gene and the negative control RNA oligonucleotides were designed and synthesized by Shanghai GenePharma. siRNA-mediated knockdown assays were performed as previously described [24].

Table 1
Sequences and applications of primers used in this study.

Primer name	Primer Sequence (5′-3′)	Application
ikkβ-Flag-F	CGGGATCCCGATGAGCCGTCCGCCCTCC	Plasmid
ikkβ-Flag-R	CGGAATTCCGAGCCAGATCCTGCCCTCC	Constructs
ikkβ-mCheery-F	CGGGATCCCGATGAGCCGTCCGCCCTCC	
ikkβ-mCheery-R	CGGAATTCCGAGCCAGATCCTGCCCTCC	
XBP1S-HA-F	CGGGATCCCGATGGTCGTAGTTACAGCTG	
XBP1S-HA-R	CGGAATTCCGGACACTAATCAGTTGGGGAA	
XBP1S-EGFP-F	GGAATTCCATGGTCGTAGTTACAGCTG	
XBP1S-EGFP-R	CGGGGTACCGACACTAATCAGTTGGGGAA	
XBP1S-RT-F	TTCTGAGTCCGCAGCAGGTG	Real-time PCR
XBP1S-RT-R	GTTCTGGGTCAAGGATGTCC	
PERK-RT-F	TCGTCCGCAAGTTCTTCCA	
PERK-RT-R	CGCCTCAAAAACGACACCA	
DDIT3-RT-F	CCGAGTTTCTGGATGTGT	
DDIT3-RT-F	GAGGTGTGCTCCGAATC	
β-actin-F	CACTGTGCCCATCTACGAG	
β-actin-R	CCATCTCCTGCTCGAAGTC	
siXBP1S-F	CCAUGUGCUUCAUAGUUAUTT	Knockdown
siXBP1S-R	AUAACUAUGAAGCACAUGGTT	XBP1S

2.3. Confocal microscope imaging

Cells were seeded in a Nunc™ Glass Bottom Dish (Thermo Scientific). Confocal fluorescent images of live cells were taken with a Leica SPE. Agron and Diode Pumped Solid State (DPSS) were used with sequential lasers excitation at 488 nm and 535 nm, respectively. Both green and red fluorescent signals were collected from each cell. Images processing and quantification of fluorescence intensity were performed by LAS AF software (Leica).

2.4. Sample protein extract and Co-Immunoprecipitation

Cell nucleus and cytoplasmic proteins were extracted using Nucleus-Cytosol Extraction Kit (Biotime) following the manufacturer's protocol. Co-immunoprecipitation of 293T cell proteins were performed essentially as previously reported [23]. The cells were transfected with 5 μg plasmids in a 10-cm culture dish. Following 48 h incubation, the cells were washed three times with PBS and incubated in 1 mL RIPA lysis buffer containing protease inhibitors at 4 °C for 30 min. Then the cell lysate was transferred to a microcentrifuge tube and centrifugated at 12,000g for 10 min. Cell debris was discarded, and the lysate was transferred to a sterile tube gently rocking for 12 h at 4 °C with EZview™ Red ANTI-FLAG® M2 or Anti-Ha Affinity Gel (SIGMA). Beads were washed 4–6 times with PBS, and then denatured and eluted by boiling in Laemmli buffer containing β-mercaptoethanol.

The following western blotting was performed according to the previously described procedure [24]. Rabbit Monoclonal ANTI-FLAG Clone SIGI-25 (F2555, SIGMA), Anti-HA antibody produced in rabbit (SAB4300603, SIGMA) and secondary antibody goat anti-Rabbit IgG (A6154, SIGMA) were used for the analysis. Histone (ab94817, Abcam) and GAPDH (ab8245, Abcam) were used to as loading controls for the nucleus and cytoplasmic extracts, respectively.

2.5. Cell viability assays

TransDetect® Cell Counting Kit (TransGen Biotech) was used to assess cell proliferation. Then, 10% in total volume of CCK solutions was added to each well and incubated at 28 °C for 2 h. A total of 100 μl from each well was transferred to 96-well microtiter plate. The absorbance was measured at 450 nm using an iMark™ microplate reader (Bio-Rad).

2.6. Real-time qPCR

Total RNA was extracted by TRIzol™ Reagent (Invitrogen) according to its instruction. cDNA was synthesized with RevertAid™ H Minus Reverse Transcriptase (RT) Kit (Thermo Scientific) by following conditions: incubation at 25 °C for 10 min followed by 42 °C for 60 min, and termination of the reaction by heating at 70 °C for 10 min. Gene expression levels were quantified by CFX Connect Real-Time System (Bio-Rad) with SYBR Green Real-Time PCR Master Mix (Takara) and normalized to *β-actin*.

3. Results

3.1. Colocalization of CiXBP1S and CiIKKβ in vitro

To determine the celullar localization of CiXBP1S and CiIKKβ, CIK cells were transfected with pEGFP-C1 recombinant plasmid (expressing CiXBP1S-GFP fusion protein) and pCMV-C-mCherry recombinant plasmid (expressing CiIKKβ-mCherry fusion protein). In normal condition, GFP and mCherry signals partially overlapped, which indicates that CiIKKβ and CiXBP1S do not always colocalize. However, the two proteins showed a higher overlap pattern strictly in the nucleus following the treatment with TM. Consistently, the quantified fluorescence intensity indicates colocalization of the two proteins under ER stress (Fig. 1A), which may suggest interaction between them.

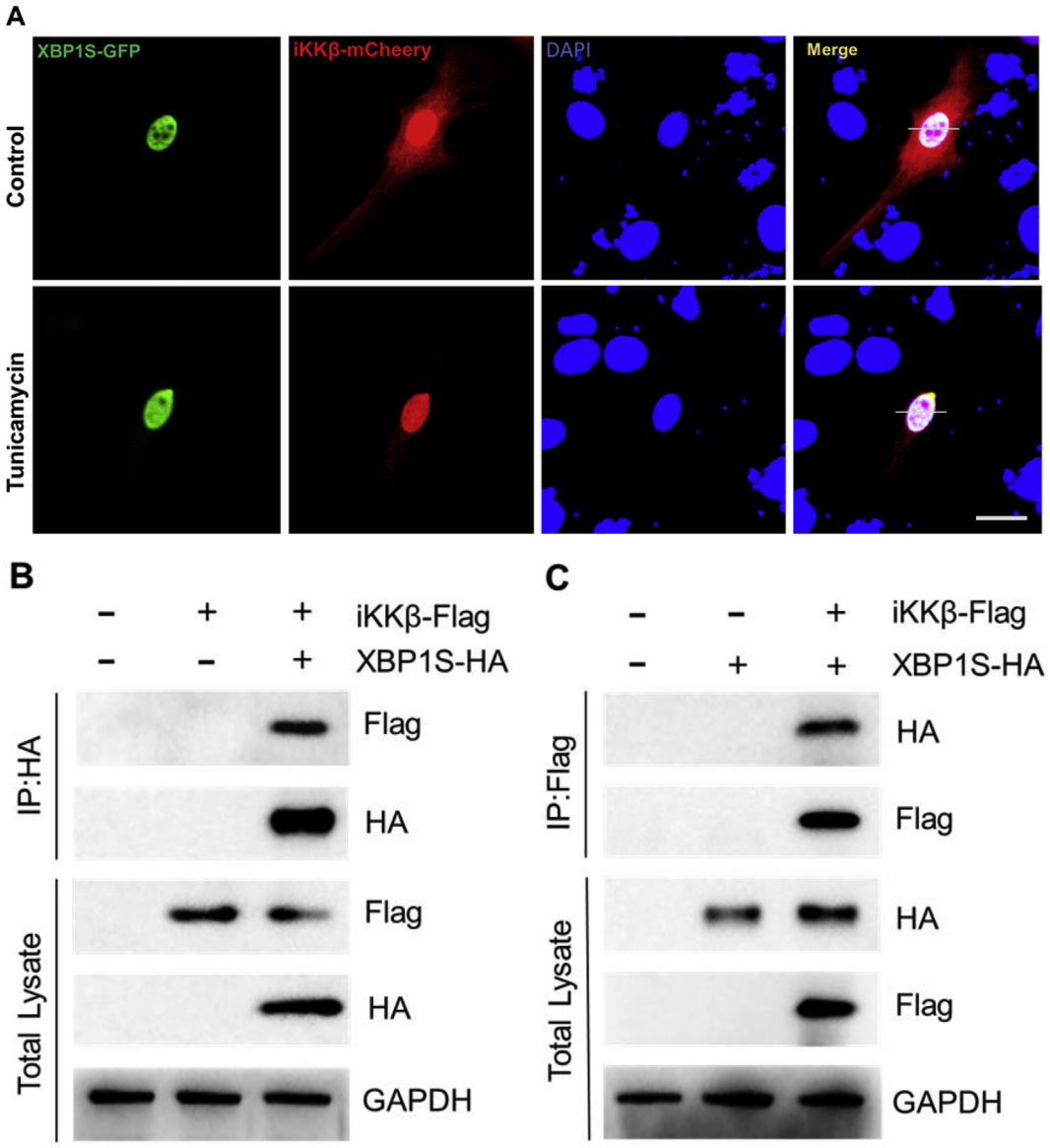

Fig. 1. Interactions between CiIKKβ and CiXBP1S. Intracellular colocalization of CiXBP1S and CiIKKβ (A). CIK cells expressing CiXBP1S-GFP and CiIKKβ-mCheery fusion proteins were observed before and after 36 h of TM treatment. Immunoblotting for HA-tagged XBP1S and flag-tagged IKKβ after immunoprecipitation of HA-tagged IKKβ proteins (B) and flag-tagged CiXBP1S proteins (C) from HEK293T cells. Total cell lysates before immunoprecipitation (Input) and immunopreciptates (IP) were analyzed by immunobloting with the indicated antibodies.

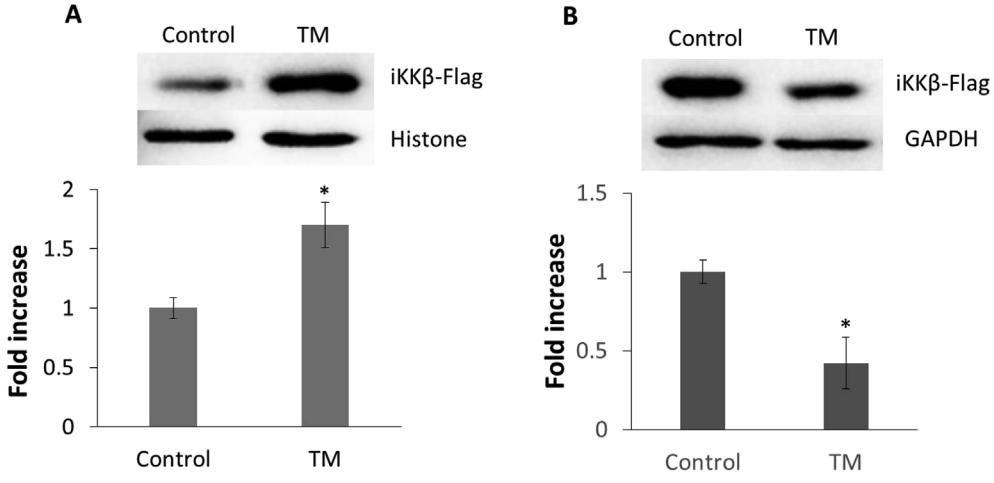

Fig. 2. Nucleus and cytoplasm distribution of IKKβ after TM treatment. Protein was extracted from CIK cells transfected with CiXBP1S-HA and CiIKKβ-Flag plasmids after 24 h of TM treatment. The distribution of nucleus IKKβ increased after TM treated (A) while that of IKKβ decreased in the cytoplasm compare with control (B). Control values were set to 1; values are mean ± SD of three experiments. *p < 0.05 compared with control.

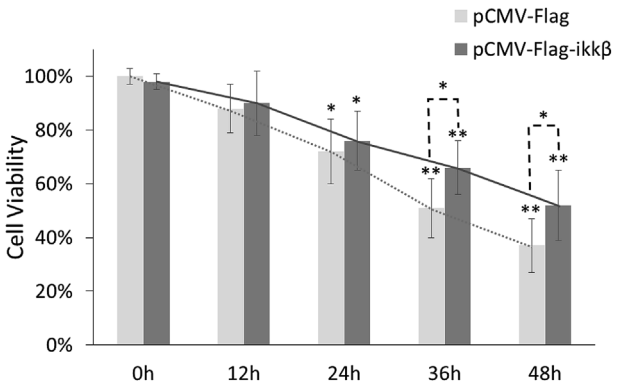

Fig. 3. The viability of IKKβ overexpression CIK cells after TM treated. CIK cells were treated with 2.5 μg/mL TM for different time periods (0, 12 h, 24 h, 36 h, 48 h) after pCMV-Flag-IKKβ plasmid transfection. The viability of IKKβ overexpressing cells is significantly enhanced compared with control after 36 h. Values are mean ± SD of three experiments. *p < 0.05 compared with control. **p < 0.01 compared with control.

3.2. Interaction of CiXBP1S and CiIKKβ

To verify the interaction between CiXBP1S and CiIKKβ, we constructed pCMV-Flag-IKKβ, pCMV-HA-XBP1S plasmids and co-transfected them into 293T cells. In co-immunoprecipitation, IKKβ-Flag protein was successfully co-precipitated with XBP1S-HA by HA affinity Gel (Fig. 1B). Similarly, XBP1S-HA protein was successfully co-precipitated with IKKβ-Flag by Flag affinity Gel (Fig. 1C). The result indicates that CiIKKβ and CiXBP1S interact with each other *in vitro*.

3.3. Intracellular distribution of CiIKKβ protein

To determine the intracellular distribution of CiKKβ protein, its expression was quantified in the nucleus and cytoplasmic protein extracts of 293T cells by western blotting. The nucleus distribution of CiIKKβ was increased by 1.7 fold after treatment with TM (Fig. 2A). Meanwhile,CiIKKβ in the cytoplasm was decreased by 0.42 fold compared with the control (Fig. 2B).

3.4. CiIKKβ contributed to cell against ER stress

When CIK cells were treated with TM, the viability of cells transfected with pCMV-Flag-IKKβ showed a significant difference compared to that of control cells transfected with pCMV-Flag. At 36 h post-transfection, the viability of cell transfected with CiIKKβ was 66% while the control viability was 51%. At 48 h post-transfection, 52% of the cells transfected with CiIKKβ still remained viable while only 37% of the control was viable (Fig. 3), indicating that the overexpression of CiIKKβ enhances cell tolerance to ER stress.

3.5. Overexpression of CiIKKβ influenced transcription of UPR target genes

To further investigate the effect of fish IKKβ in ER stress, we studied the gene expression level of Ci*XBP1S*, Ci*PERK,* and Ci*DDIT3* under TM treatment in CIK cells transfected with pCMV-Flag-IKKβ. In the Ci*IKKβ*-overexpressing cells, Ci*XBP1S* transcription level was also markedly enhanced in response to TM treatment (Fig. 4A). In contrast, the expression level of Ci*PERK* was significatily decreased at 12 h, 24 h, 36 h and 48 h post-treatment (Fig. 4B). The expression of pro-apoptotic transcription factor *DDIT3* showed a significant decrease at all the time points, and the change was pronounced especially at 36 h and 48 h post-treatment compared with the control (Fig. 4C).

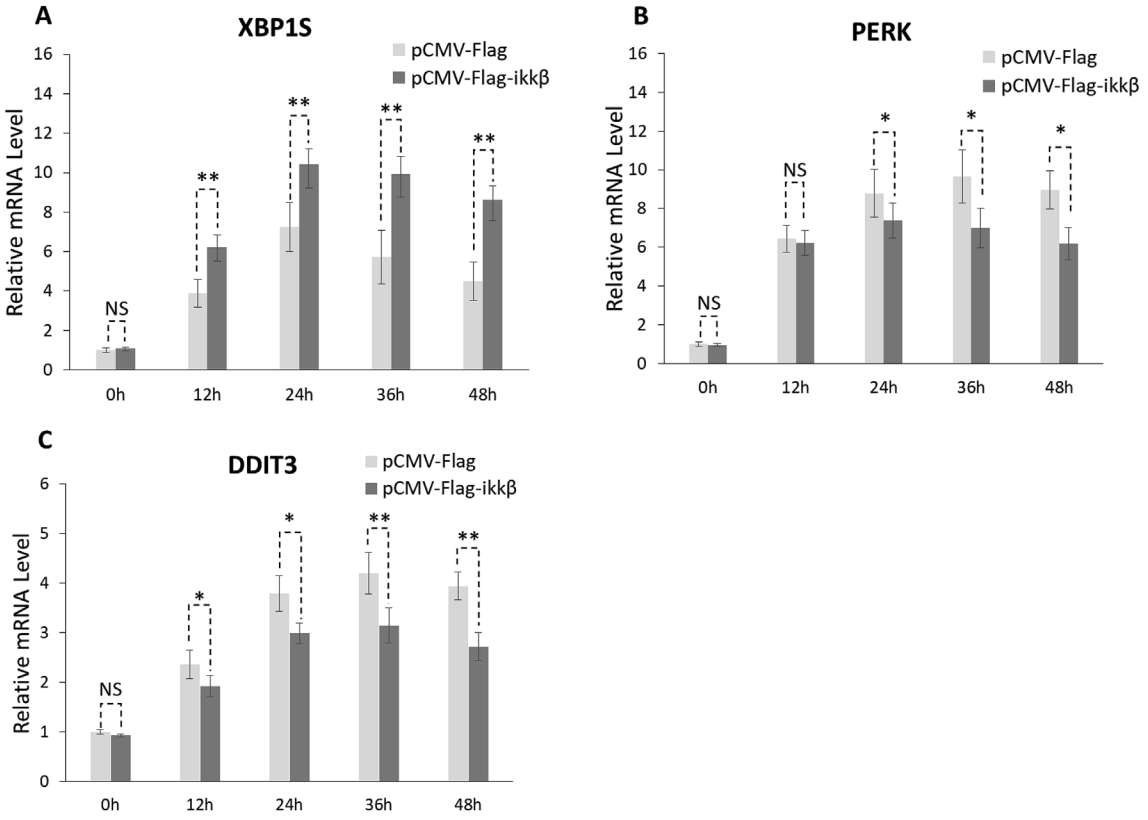

Fig. 4. Gene expression levels of XBP1S, PERK, and DDIT3 in IKKβ overexpressing CIK cells following TM treatment for 0, 12, 24, 36, 48 h. The gene levels of (A) XBP1S, (B) PERK and (C) DDIT3 are all normalized to β-actin. Values are mean ± SD of three experiments. *p < 0.05 compared with control. **p < 0.01 compared with control.

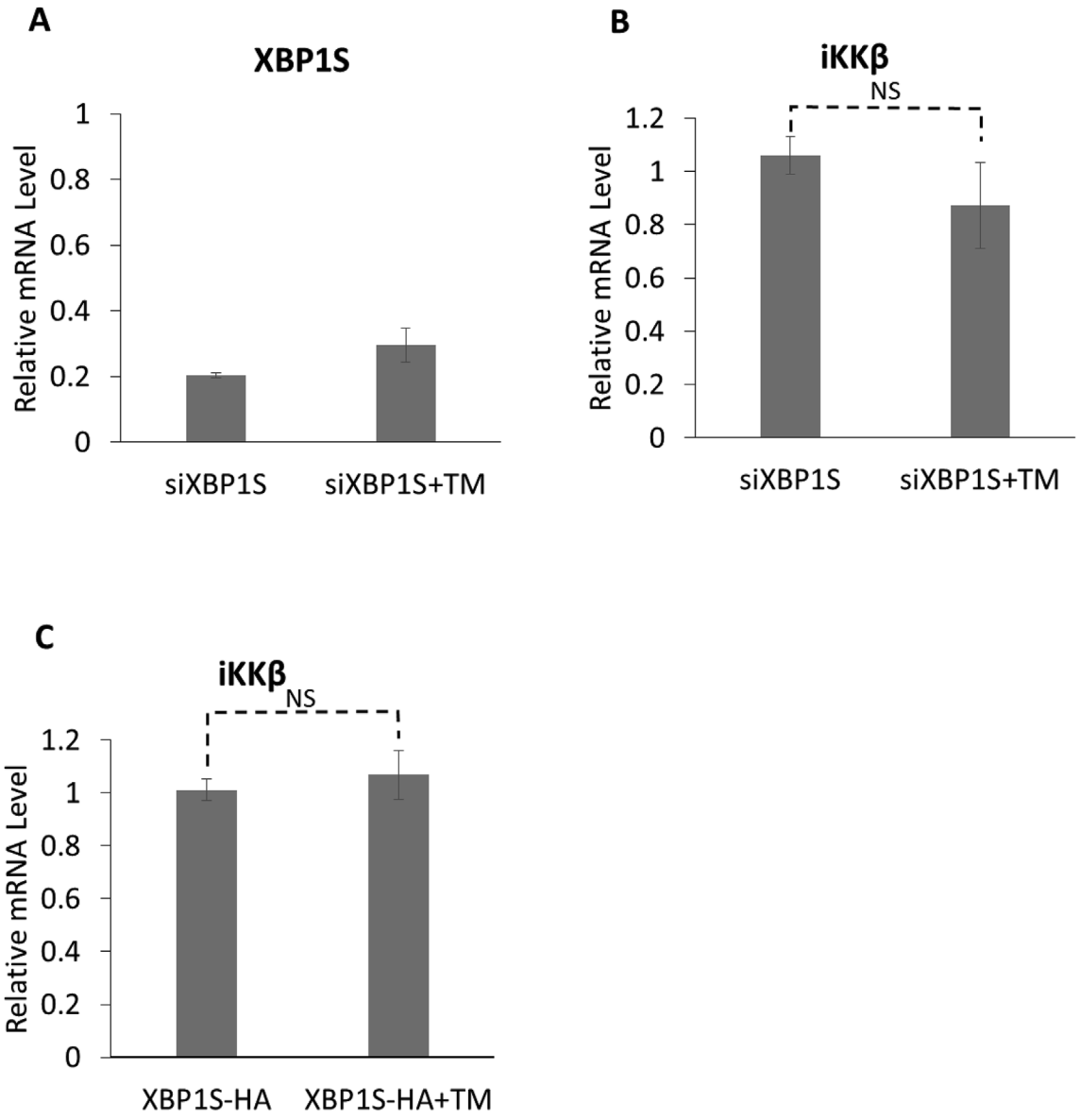

Fig. 5. Gene expression levels of IKKβ after XBP1S knockdown or overexpression. The CIK cellular transcription level of XBP1S (A) and IKKβ (B) were quantified in XBP1S knockdown cells following 24 h TM treatment. The CIK cellular transcription level of IKKβ (C) was quantified in XBP1S overexpressing cells following 24 h TM treatment. The expression levels were normalized to control (wild type CIK cells); values are mean ± SD of three experiments.

3.6. CiXBP1S did not affect the CiIKKβ transcription level

In order to assess the impact of CiXBP1S on CiIKKβ transcription, we knocked down and also overexpressed Ci*XBP1S* in fish cells by siRNA-mediated silencing and transfection with the XBP1S-HA plasmid, respectively. As shown in Fig. 5, the transcription level of Ci*IKKβ* did not show any significant difference in either knockdown (Fig. 5B) or overexpression group (Fig. 5C).

4. Discussion

Apart from the canonical NF-κB activation pathway, NF-κB is also activated in certain ER stress conditions induced by UV [25], hydrogen peroxide (H_2O_2) [26], TM and TG [27–29]. In addition, during development in *Caenorhabditis elegans*, XBP1 has a protective role in a cytotoxic immune response [14]. Here, in fish, we first discovered XBP1S co-localized with IKKβ in cells (Fig. 1A). Furthermore, our following experiments revealed that the colocalization of CiIKKβ and CiXBP1S increased in cells under ER stress. Together, these studies highly suggest a close relationship between CiXBP1 and CiIKKβ.

A role of XBP1 in antigen presentation [30] and inflammation [31,32] were also reported in zebrafish (*Barchydanio rerio*). Furthermore, recent studies indicated that XBP1 splicing pathway could be activated by ISAV infection in salmon (*Oncorhynchus*) [33]. Therefore, fish XBP1 is closely related to their immune responses. As described earlier, IKKβ leads to the activation of NF-κB signal pathway, which initiates the transcription of a series of inflammation-related genes and ER-stress signaling pathway genes, including *XBP1*. In mammal, it has been reported that XBP1S and IKKβ interaction can increase XBP1S activity and reduce ER stress. However, the molecular mechanism of the IKKβ-XBP1S crosstalk remains uncertain. To figure out the effect of fish IKKβ under ER stress, we overexpressed CiIKKβ protein in CIK cells. The results showed that it significantly increased cell viability after 24 h of transfection (Fig. 3). In addition, we found that it significantly increased the expression of *XBP1S* and decreased the expression of *Ci-PERK* and *DDIT3* (Fig. 4). These results indicate that CiIKKβ activates the IRE1 pathway in the cell and reduced ER stress. In UPR pathway, PERK phosphorylated eIF2a, resulting in translation of ATF4 [34]. The

transcription activator target DDIT3 is usually considered as a pro-apoptotic signaling [35]. In particular, the down-regulation of DDIT3 expression in cells decreases pro-apoptotic activity. On the other hand, the decrease of PERK expression and its pathway prevents the shift from pro-survival to pro-apoptotic signaling [34,36], thereby preventing cell death. In conclusion, our study showed that IKKβ promotes cell survival through the interaction with XBP1S to activate the IRE1 pathway. Our results are consistent with a recent study that shows XBP1S activity elevation by IKKβ to reduce ER stress in mice [37].

Liu et al. (2016) found that the formation of XBP1S-IKKβ complex does not change the IKKβ mRNA transcription level but makes IKKβ protein more stable, allowing it to function longer. Similarly in this study, the transcription level of *CiIKKβ* had no significant difference regardless of knockdown or overexpression of XBP1S, indicating that XBP1S does not affect IKKβ gene expression. It will be interesting to investigate whether CiXBP1S increases the stability of CiIKKβ in future studies.

In summary, our results elucidated a crosslink between IKKβ and XBP1S in a teleost. As a “master switch” of NF-κB signal pathway, IKKβ can partially reduce ER stress-induced CIK cell death. It provided a novel view of the relationship between innate immunity and ER stress and will contribute to the development of therapies to modulate cellular stress and innate immunity. Further investigation will reveal more details about the mechanisms of fish IKKβ involvement in ER stress.

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