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Full Length Article

Grass carp (*Ctenopharyngodon idellus*) Cdc25a down-regulates IFN 1 expression by reducing TBK1 phosphorylation

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ABSTRACT

In vertebrates, TANK Binding Kinase 1 (TBK1) plays an important role in innate immunity, mainly because it can mediate production of interferon to resist the invasion of pathogens. In mammals, cell division cycle-25a (Cdc25a) is a member of the Cdc25 family of cell division cycle proteins. It is a phosphatase that plays an important role in cell cycle regulation by dephosphorylating its substrate proteins. Currently, many phosphatases are reported to play a role in innate immunity. This is because the phosphatases can shut down or reduce immune signaling pathways by down-regulating phosphorylation signals. However, there are no reports on fish Cdc25a in innate immunity. In this paper, we conducted a preliminary study on the involvement of grass carp Cdc25a in innate immunity. First, we cloned the full-length cDNA of grass carp *Cdc25a* (*CiCdc25a*), and found that it shares the highest genetic relationship with that of *Anabarilius grahami* through phylogenetic tree comparison. In grass carp tissues and CIK cells, the expression of *CiCdc25a* mRNA was up-regulated under poly (I:C) stimulation. Therefore, *CiCdc25a* can respond to poly (I:C). The subcellular localization results showed that CiCdc25a is distributed both in the cytoplasm and nucleus. We also found that CiCdc25a can down-regulate the expression of IFN 1 with or without poly (I:C) stimulation. In other words, the down-regulation of IFN1 by CiCdc25a is independent of poly (I:C) stimulation. Further functional studies have shown that the inhibition of IFN1 expression by CiCdc25a may be related to decrease of TBK1 activity. We also confirmed that the phosphorylation of TBK1 at Ser^{172} is essential for production of IFN 1. In short, CiCdc25a can interact with TBK1 and subsequently inhibits the phosphorylation of TBK1, thereby weakens TBK1 activity. These results indicated that grass carp Cdc25a down-regulates IFN 1 expression by reducing TBK1 phosphorylation.

1. Introduction

Innate immunity has a variety of functions in many ways, such as in heart protection (Zuurbier et al., 2019), in the prevention of type 1 diabetes (Needell and Zipris, 2017), in cancer therapy (Demaria et al., 2019), in the treatment of neuropsychiatric disorders (Salam et al., 2018) and in antiviral immunity. In the antiviral response, pattern recognition receptors (PRRs) can recognize viral nucleic acid and trigger the corresponding immune response through a certain signal pathway (Guo et al., 2020; Ng et al., 2012; Paludan and Bowie, 2013).

IFN response is an important part of innate immunity and an important line of defense for the host against viral infections. In mammals, IFN has been identified as three types, namely IFN type I (mainly including IFNα/β), IFN type II (IFNγ) and IFN type III (IFNλ1/2/3). Of which, type I and type III IFN participate in the immune response of virus infection through the same JAK-STAT signaling pathway (Sadler and Williams, 2008). Different from mammals, fish IFN contains two groups, i.e. IFN-I and IFN-II (only exists in a few species of fish) (Zou and Secombes, 2011). Fish type I IFN is divided into IFN1, IFN2, IFN3, IFN4 (Liao et al., 2016; Zou et al., 2007).

TBK1 is a noncanonical IκB kinase (IKK)-related kinase. The role of TBK1, such as in immunity, autophagy, neuroinflammation, cancer, etc, has been extensively studied (Ahmad et al., 2016; Durand et al., 2018; Kawai and Akira, 2007; Weidberg and Elazar, 2011). As we all know, TBK1 plays an important role in the immune response. It has been reported in mammals that the phosphorylated TBK1 promotes IFN

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

Primer name	Primer sequence(5′-3′)	Application
Cdc25a-ORF-F	ATGGATTTAGATATTGTTCCAGGC	ORF
Cdc25a-ORF-R	TTAAAGTTTTTTGAGACGGCTG	
Cdc25a5′ RACE1	TAGAGTTCAGGGTAATGGAGGT	RACE-PCR
Cdc25a5′ RACE2	CACCCTTAATATGGCCGCCCTCA	
Cdc25a3′ RACE1	ACACGATGATGACGATGATGG	
Cdc25a3′ RACE2	CGCCTGTTATTCGCTGCCGTC	
pEGFP-Cdc25a-F	CCAAGCTTCGATGGATTTAGATATTGTTCCAGGC	Construct plasmid
pEGFP-Cdc25a-R	GGGGTACCTTAAAGTTTTTTGAGACGGCTG	
pCMV-FLAG-Cdc25a-F	CGGAATTCAATGGATTTAGATATTGTTCCAGGC	
pCMV-FLAG-Cdc25a-R	GGGGTACCTTAAAGTTTTTTGAGACGGCTG	
si-Cdc25a	CCAUUACCCUGAACUCUAUTT AUAGAGUUCAGGGUAAUGGTT	siRNA
si-TBK1	GCCAUAUAACGAAGAACAATT UUGUUCUUCGUUAUAUGGCTT	
negative control	UUCUCCGAACGUGUCACGUTT	
β-Actin-F	CCTTCTTGGGTATGGAGTCTTG	Real-time PCR
β-Actin-R	AGAGTATTTACGCTCAGGTGGG	
RT-Cdc25a-F	TGGCTGCGAAAACAAAGAGA	
RT-Cdc25a-R	GGATGATGTAGGGGAAAGCATT	
RT-TBK1-F	GAGACATCAAGCCAGGGAAC	
RT-TBK1-R	AAAACGTGACTCCGATGCTC	
RT-IFNI-F	GTCAATGCTCTGCTTGCGAAT	
RT-IFNI-R	CAAGAAACTTCACCTGGTCCT	

production. Such as Glycogen Synthase Kinase 3β (Gsk3β) can mediate the production of IFN by enhancing the phosphorylation of TBK1; Raf kinase inhibitory protein (RKIP) can form a positive feedback loop with TBK1 to enhance the expression of IFN (Gu et al., 2016; Lei et al., 2010). Correspondingly, the inhibition of TBK1 phosphorylation or dephosphorylation of TBK1 will decrease the expression of IFN. For example, protein phosphatase 1B (PPM1B) down-regulates IFN by dephosphorylating TBK1; Protein phosphatase 4 (PP4) negatively regulates IFN expression by inactivating TBK1 (Biacchesi et al., 2017; Zhan et al., 2015). In short, the negative regulation of immune response is very important by the dephosphorylation of TBK1.

Cdc25a, also known as cell division cycle-25a, is a member of Cdc25 phosphatase family. Cdc25 is present in many eukaryotes, but not found in plants (Boudolf et al., 2006). Cdc25a has been reported more frequently for its role in controlling cell division into S and M phase. Nowadays, it is found that many cancers are related to Cdc25a, such as silencing Cdc25a inhibits the proliferation of liver cancer cell (Chen et al., 2020a); MicroRNA-99a-5p targets Cdc25a to inhibit breast cancer cell proliferation (Chen et al., 2020a). Cdc25a also regulates immune response by regulating the proliferation of immune cells (Khaled et al., 2005; Kittipatarin et al., 2010).

The first fish Cdc25a was cloned in zebrafish (Nogare et al., 2007). Fish Cdc25a is closely related to cell proliferation and hypocotyl extension (Liu et al., 2017). The restricted expression of Cdc25a is extremely important for muscle differentiation (Bouldin et al., 2014). However, there is no report on the function of fish Cdc25a in innate immunity. In this study, we found that grass carp Cdc25a can participate in innate immunity mainly by regulating the phosphorylation of TBK1.

2. Materials and methods

2.1. Grass carp

Grass carp (mean weight about 20 g) were provided by Nanchang Shenlong Fisheries Development (Jiangxi, China). Before the experiments, grass carp were fed in a fish tank with an aerator for more than two weeks.

2.2. Cell culture and poly (I:C)

Grass carp kidney cells (CIK) and grass carp overy (CO) cells were gifted by Professor Pin Nie (School of Marine Science and Engineering, Qingdao Agricultural University, China). CIK and CO cells were cultured in M199 medium containing 10% serum and 1% Penicillin-Streptomycin Liquid at a constant temperature of 28 °C. CO cells need to be cultured in an environment with 5% CO_2. Human embyonic kindy 293T cells (HEK 293T) were purchased from ATCC and cultured in DMEM medium containing 10% serum and 1% Penicillin-Streptomycin Liquid at 37 °C in an incubator containing 5% CO_2. poly (I:C), a double-stranded RNA virus analogue, was purchased from Sigma (USA).

2.3. Vectors, stains and antibody

The pEASY-T1, p3 × FLAG-myc-CMV-24, pEGFP-C1 and pCDNA3.1 were purchased from Transgen (China), Sigma (USA), Promega (USA) and Invitrogen (USA), respectively. *Escherichia Coli* DH5α as the competent cells were bought from Promega (USA). Anti-FLAG antibody and anti-GFP antibody were purchased from Sigma (USA) and Abmart (Shanghai, China), respectively. Anti-TBK1 was purchased from ABPECTA (SuZhou, China). Phospho-TBK1/NAK (Ser172)(D52C2) XP® Rabbit mAb (CST, USA) was used to detect the phosphorylation of TBK1. In addition, CiGAPDH and CiIFN1 antibodies were kept in our laboratory. Goat anti-mouse and anti-rabbit antibodies were purchased from AlpaLife (Shenzhen, China).

2.4. Cloning of CiCdc25a and CiTBK1

Total RNA was obtained from CIK cells using total RNA extraction kit (Tiangen, China). SMART cDNA was obtained from Super Script III reverse polymerase (Invitrogen). Then, PCR was performed to amplify the sequence of *CiCdc25a* with specific primers. In order to obtain the full-length coding sequence (CDS) of *CiCdc25a*, Rapid Amplification of cDNA Ends (RACE) PCR was used. PCR products of *CiCdc25a* were ligated into pEASY-T1 and sequenced. After verifying the cDNA sequence of *CiCdc25a*, the peptide was confirmed by the online software ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). NCBI blast server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for multiple alignments of Cdc25a from different species. The sequence was translated into an amino acid sequence by DNAMAN. Clustal X 1.83 was used for multiple sequence alignment. The phylogenetic tree was inferred by Neighbor-Joining algorithm implemented in MEGA software version 6.0. *CiTBK1* sequence was kept by our laboratory (Yu et al., 2018).

2.5. Plasmid construction

The ORFs of *CiCdc25a* and *CiTBK1* were separately inserted into pEGFP -C1 and pCMV-FLAG named Cdc25a-GFP, TBK1-GFP, Cdc25a-FLAG, TBK1-FLAG for subcellular localization/colocalization and immunoprecipitation assays. All the recombinant plasmids had been sent to Sangon Biotech for first generation sequencing, and the primers were listed in Table 1.

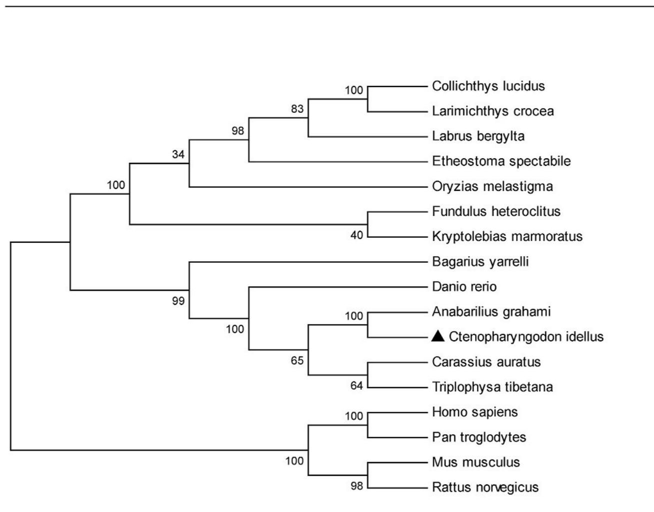

Fig. 1. Analysis of characteristics and phylogeny of Cdc25a
The amino acid sequences of Cdc25a from 17 species were aligned based on ClustalX. The tree was generated by an N-J algorithm using the Mega 4.0 program. *C. idellus* Cdc25a was marked with "▲".

Table 2
GenBank accession number of the Cdc25a sequence used for phylogenetic analysis.

Species	Accession NO.
Anabarilius grahami	ROK15824.1
Danio rerio	ACH53118.1
Carassius auratus	XP_026134605.1
Triplophysa tibetana	KAA0717783.1
Collichthys lucidus	TKS87239.1
Fundulus heteroclitus	XP_012727593.1
Etheostoma spectabile	XP_032366976.1
Oryzias melastigma	XP_024132606.1
Bagarius yarrelli	TSW48767.1
Labrus bergylta	XP_020514285.1
Kryptolebias marmoratus	XP_017288902.1
Larimichthys crocea	XP_010752619.2
Homo sapiens	AAM77917.1
Mus musculus	AAA85580.1
Rattus norvegicus	BAA03761.1
Pan troglodytes	XP_009443662.1
Ctenopharyngodon idellus	QMX42368

2.6. Transfection and RNA interference–mediated gene-knockdown assays

CIK and CO cells were all transfected with LipoMax (SUDGEN, China)/Lipo 2000 (Thermo Fisher Scientific, USA). The plasmid or poly (I:C) was mixed with the transfection reagent. After 15 min of incubation at room temperature, the mixed solution was transfered into CIK cells or CO cells. According to the instructions in Calcium Phosphate Cell Transfection Kit (Beyotime, China), 293T cells were transfected according to the operation steps. The recombinant plasmid was added to calcium chloride first, then the solution were transferred into BBA reagent and mixed. After 15 min at room temperature for incubation, the complexes were added onto the dish. Small interfering RNAs (siRNA) against *CiCdc25a* and *CiTBK1* were purchased from GenePharma (ShangHai, China). HiPerFect Transfection Reagent (QIAGEN, Germany) was used to transfect siRNA interference reagent, and the experimental procedures were carried out according to the instructions. siRNA was first added to serum-free M199 medium, and then the transfection reagent was added. The complex was mixed at room temperature and incubated for 10 min, finally added drop-wise onto the cells.

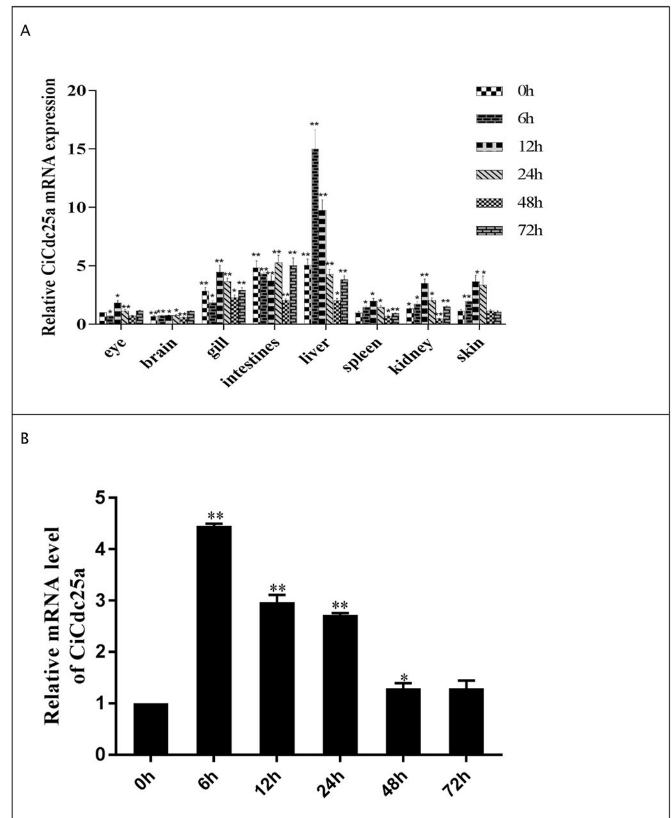

Fig. 2. Expression of *CiCdc25a* in grass carp tissues and cells
(A): Healthy grass carp was injected with poly (I:C). The stimulation time periods were 0h, 6h, 12h, 24h, 48h, 72h, respectively. The expression of *CiCdc25a* was detected in various tissues (kidney, skin, spleen, liver, brain, intestine, eyes, and gills) of grass carp. Eye (treatment for 0 h) was as the calibrator. (B): Transfection of 2 μg poly (I:C) into CIK cells, the mRNA expression of *CiCdc25a* in different time periods. The expression level is relative to the corresponding expression level of β-actin (*$p < 0.05$; **$p < 0.01$).

2.7. Quantitative real-time PCR analysis

Poly (I:C) was injected into the abdominal cavity of grass carp for 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, respectively. RNA was extracted from skin, brain, intestine, gills, kidney, liver, spleen and eyes, respectively. CIK cells connected to the six-well plate were approximately 80% covered until cells adhered. CIK cells were transfected with 2 μg poly (I:C) (1 μg/μl) for 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, respectively.

Total RNA of cells and grass carp tissues were extracted by TIANGEN (Beijing, China). The extraction steps followed the instructions provided by TIANGEN. cDNAs were synthesized using the Prime Script RT reagent kit (TaKaRa). TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (TAKARA) was used to analyzed the mRNA expression levels of specific genes. Quantitative real-time PCR was used to detect the mRNA expression of *CiIFN1* and *CiCdc25a* with *β-actin* as the internal control on CFX Connect TM Real-Time System (Bio-Rad, Hercules, USA). Amplification reactions were performed in triplicate in 20 μl. The composition of reaction solution is as follows: 10 μl SYBR premix Ex Taq, 7.2 μl ddH$_2$O, 2 × 0.4 μl primer and 2 μl cDNA sample. PCR reaction conditions were 1 cycle of 94 °C/5 min; 40 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s. The primers used in Q-PCR reaction were listed in Table 1.

2.8. Subcellular localization analysis and immunofluorescence

When CIK cells grew to 70%–80% in Glass Bottom Cell Culture Dish (NEXT), 2 μg pEGFP-C1, 2 μg TBK1-GFP, 2 μg Cdc25a-GFP were respectively transfected into the cells. 24 h later, the medium was

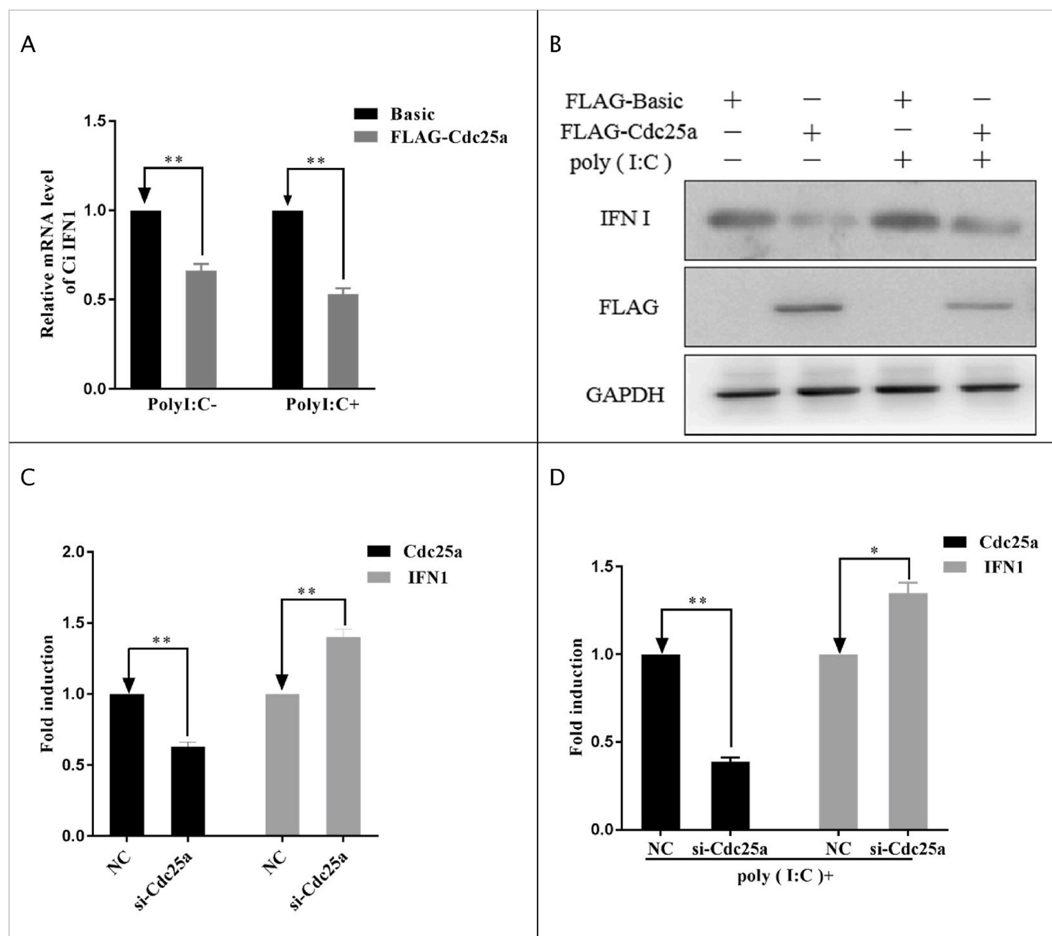

Fig. 3. CiCdc25a down-regulates IFN expression
(A) and (B): CIK and CO cells were transfected with FLAG-basic and FLAG-Cdc25 respectively. After 12 h of transfection, the cells were stimulated. After another 12 h, total RNA was extracted. (C) and (D): si-Cdc25a and NC were transfectced into CIK cells respectively, and poly (I:C) stimulation was applied after the cells grew for 12h. After another 12 h, the total RNA was extracted. RT-PCR was used to detect the mRNA expression of *CiCdc25a* and *CiIFN1*. Western blot was used to detect the expression of IFN1. Q-PCR experiments were normalized to *β-actin*, GAPDH level was monitored as a loading control.

removed and washed three times with PBS. The cells were fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature, and the nuclei were stained with DAPI (0.1 g/ml) and observed under a confocal microscope. Immunofluorescence was consistent with the cell growth state required for subcellular localization. 1 μg Cdc25a-FLAG and 1 μg TBK1-GFP were transfected. 24 h after transfection, the culture medium was discarded, and the petri dish was washed 3 times with PBS. Then the cells were fixed with 4% (v/v) paraformaldehyde at room temperature for 20 min. CIK cells were treated with 3% BSA at room temperature for 1 h, and then incubated with primary antibody overnight. Essentially, CIK cells were observed under a confocal microscope after incubated the secondary antibody.

2.9. Immunoblotting assays

CO cells grew to 70%–80% in the dish and then transfected with 2 μg Cdc25a-FLAG, 2 μg pCMV-FLAG empty vetors respectively. After 12 h of transfection, 2 μg poly (I:C) stimulation was performed. After the cells grew for another 12 h, the cells were processed. In the absence of stimulation, 24 h after transfection, the next step of the experiment was carried out directly. All culture medium was aspirated, CO cells were washed with PBS and completely lysed using 1 ml NP40 lysis solution (1 mM/L PMSF, 1 μg/ml Leupeptin and 1 μg/ml Aprotinin) on ice for 30 min. Then the lysis of CO cells were collected, and the supernatant protein was obtained by centrifugation at 12000 g and 4 °C for 15 min. Enhanced BCA Protein Assay Kit (Beyotime) was used to determine the concentration of lysate supernatant. GAPDH level was monitored as a loading control.

The remaining protein was resuspended in 5 × SDS loading bufferand and then boiled for 5 min. The processed cell protein suspension was separated on polyacrylamide SDS-PAGE (Bio-Rad, USA) and transferred to nitrocellulose membrane (Millipore, USA). The membrane was washed three times in TBS solution and blocked in 5% skimmed milk powder solution at room temperature for 1 h. The corresponding antibody was incubated with target protein overnight at 4 °C, and then incubated with the secondary antibody coupled to horseradish peroxidase. Finally, chemiluminescence imaging system (CLINX, China) was used for detection.

2.10. Co-immunoprecipitation assays

Co-IP was used to analyze the interaction between grass carp TBK1 and Cdc25a. When 293T cells grew to 70%–80%, 3 μg Cdc25a-FLAG and 3 μg TBK1-GFP were transfected. 24 h after transfection, the protein was cleaved and collected as described. 80 μl cell lysate was pipetted into a 1.5 ml centrifuge tube, and then 20 μl 5 × SDS loading buffer was added. The mixture was boiled for 5 min after being mixed as an imput stored at - 20 °C. The rest of untreated protein were incubated with anti-Flag agarose (Sigma) or KT anti-GFP (A) (AlpaLife) at 4 °C for 2 h. Next, the beads were washed 3 times with PBS, and then 50 μl 2 × SDS sample

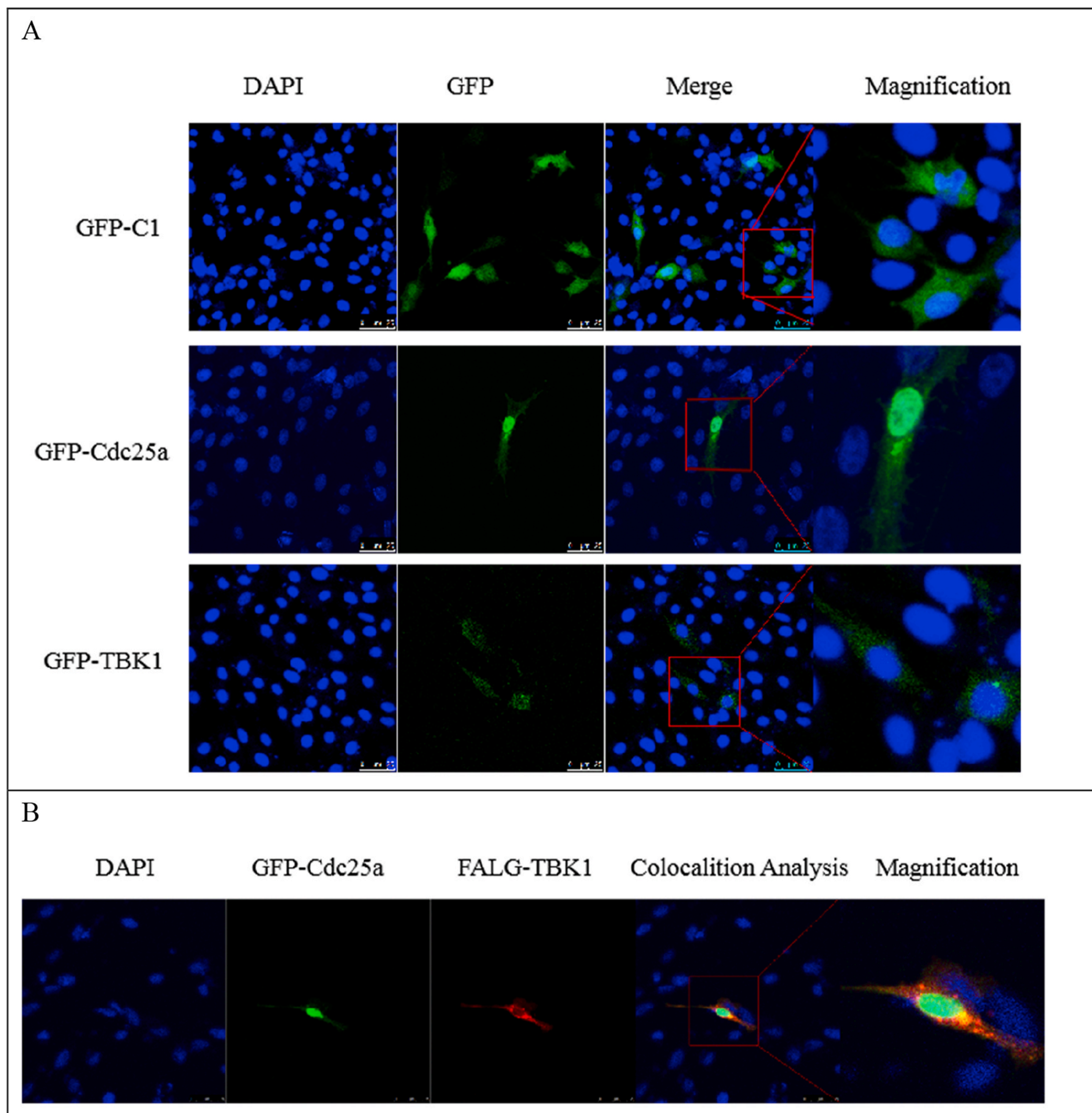

Fig. 4. Subcellular localization of CiCdc25a and CiTBK1
(A): pEGFP-C1,Cdc25a-GFP and TBK1-GFP were respectively transfected into CIK cells which grown on a microscope dish. After a series of treatments, the cells were fixed and observed with a confocal microscope. pEGFP-C1 was used as a blank control. (B): Cdc25a-GFP and TBK1-FLAG were co-transfected into CIK cells. After 24 h, the cells were processed and observed under a confocal microscope.

buffer was added for elution. Finally, the collected mixture was boiled at 95 °C for 10 min. The precipitate was detected by immunoblotting with the indicated antibody. GFP-basic and FLAG-basic were used as the control.

2.11. *Phosphorylation analysis*

When CO cells grow to 70%–80% in the six-well plate, pCMV-GFP and Cdc25a-GFP were respectively transfected into the cells. 12 h later, CO cells were stimulated by transfection with poly (I:C). After another 12 h the protein was extracted. The composition of the lysate is the same as that described above, except that the Phosphatase Inhibitor Cocktail (100 ×) was added. The subsequent experimental operation is the same as that of immunoprecipitation. TBK1 antibody and phosphorylation antibody were used to detect the corresponding protein in cells. GAPDH level was monitored as a loading control.

2.12. *Data analysis*

All of the experiments were repeated three times. All data were statistically analyzed with Prism v6.0 (Graphpad Software). The data was expressed as mean ± SEM through unpaired two-tailed Student's t-test. The statistical significance of the data was evaluated by ANOVA. *P* value less than 0.05 is statistically significant.

3. Results

3.1. *Analysis of characteristics and phylogeny of CiCdc25a*

After RACE experiment, the full-length cDNA sequence of *CiCdc25a* was cloned. *CiCdc25a* sequence contains 2985 bp, consisting of a 5′UTR (294 bp), a ecoding sequence (1695 bp), and a 3′UTR (996 bp). In order to compare the evolutionary relationship among grass carp Cdc25a and other species, NCBI blast server was used to find some published Cdc25a protein sequences from different species. The results showed that the

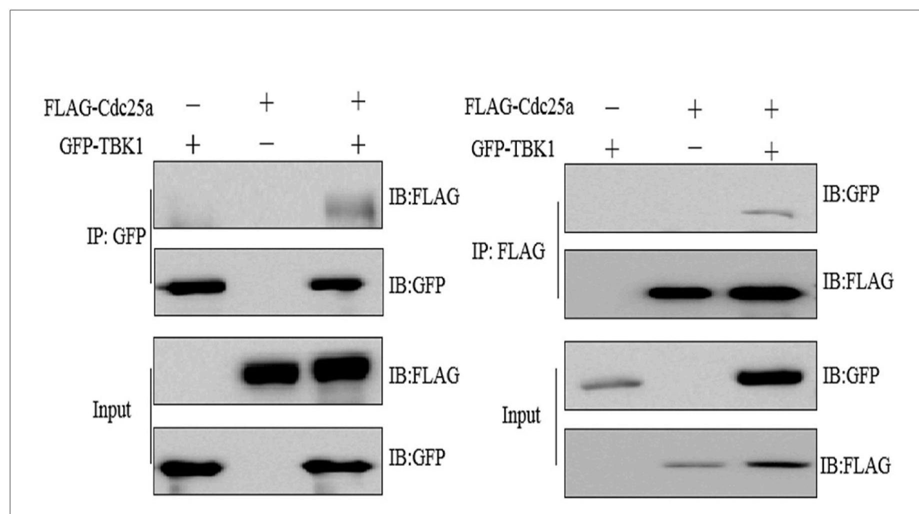

Fig. 5. Interaction of CiCdc25a and CiTBK1
Cdc25a-FLAG and TBK1-GFP were co-transfected into 293 cells. After 24 h, the medium was removed and washed with PBS. The lysate mixture was used to incubated with anti-FLAG agarose beads or anti-GFP agarose beads, and finally detected by Anti-FLAG and Anti-GFP.

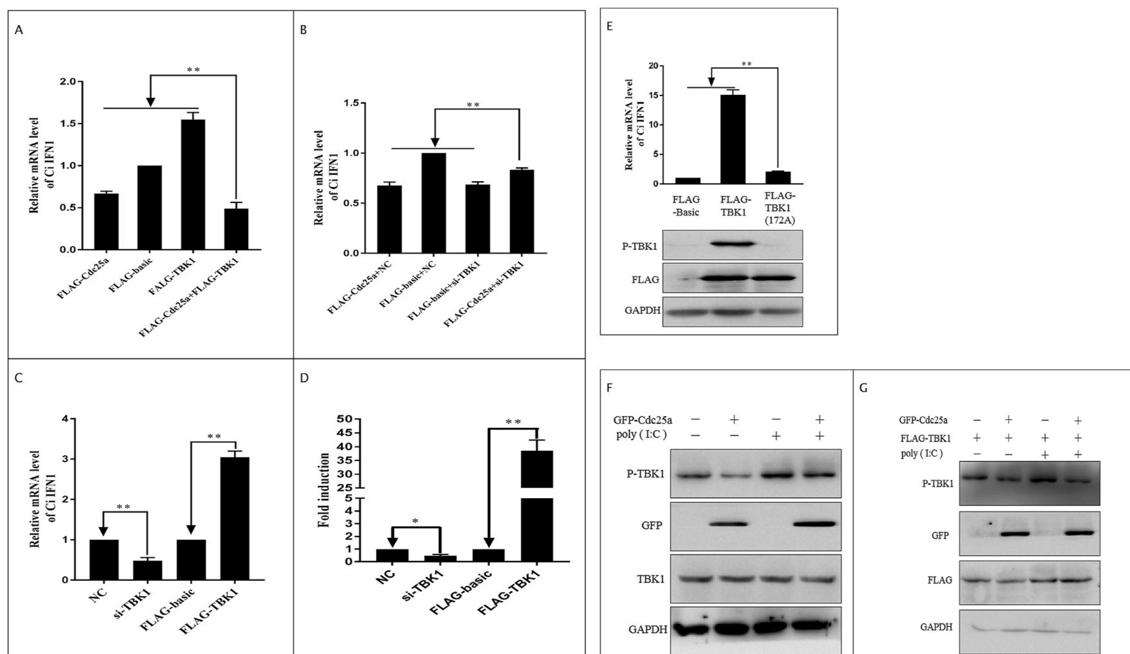

Fig. 6. CiCdc25a down-regulates IFN1 expression through dephosphorylation of CiTBK1
(A): FLAG-TBK1, FLAG-Cdc25a, FLAG-TBK1+FLAG-Cdc25a were respectively transfected into CIK cells grown in six-well plates, and total RNA was extracted at 24 h post transfection. (B): NC and si-TBK1 were transfected into CIK cells. 12 h later, FLAG-basic and FLAG-Cdc25a were transfected into CIK cells respectively. After another 24 h, total RNA was extracted. (C) and (D): NC, si-TBK1, FLAG-basic, FLAG-Cdc25a were transfected into CIK cells respectively, and total RNA was extracted at 24 h post transfection. (E): FLAG-basic, FLAG-TBK1, FLAG-TBK1 (172A) were transfected into CIK or CO cells respectively, and total RNA or protein was extracted at 24 h post transfection. (F): GFP-basic and GFP-Cdc25a were separately transfected into CO cells separately. 12h after transfection, CO cells were stimulated by poly (I:C). After another 12 h, cells were lysed with lysis buffer to obtain protein. Phospho-TBK1/NAK (Ser172) antibody was used to detect the phosphorylation of Ser^{172} of CiTBK1. (G): 1 μg FLAG-TBK1+1 μg GFP-basic and 1 μg FLAG-TBK1+1 μg GFP-Cdc25a were transfected into EPC cells respectively, and poly (I:C) stimulation was applied 12 h later. The protein was extracted after another 12 h qPCR was used to detect the mRNA expression of *IFN1*, *TBK1*, and *Cdc25a*. Phospho-TBK1/NAK (Ser172) antibody was used to detect the phosphorylation of Ser^{172} of TBK1. Q-PCR experiments were normalized to *β-actin*, GAPDH level was monitored as a loading control.

evolutionary relationship between grass carp (*C. Idellus)* Cdc25a and Kanglang white minnow *(Anabarilius grahami*) Cdc25a is the closest compared with other fish (Fig. 1). Genbank accession number are shown in Table 2.

3.2. *Expression of CiCdc25a in grass carp tissues and cells*

After intraperitoneal injection of poly (I:C) for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h, qPCR was used to determine the expression level of *CiCdc25a* mRNA in eight tissues (gill, brain, skin, liver, kidney, spleen, intestine and eye) of grass carp. The results showed that *CiCdc25a* was up-

regulated in all grass carp tissues, especially in liver. The expression level of *Cicdc25a* began to rise at 6 h post poly (I:C) stimulation and then recovered at 72 h (Fig. 2A).

Consistently, CIK cells were transfected with poly (I:C) for 0 h, 6 h, 12 h, 24 h, 48 h, 72 h. The expression level of *CiCdc25a* was increased from 0 h to 6 h and reached the maximum at 6 h, and then declined. Around 48 h, the expression of *CiCdc25a* almost returned to the initial level (Fig. 2B).

3.3. CiCdc25a down-regulates IFN 1 expression

To explore the regulation effect of CiCdc25a on IFN 1 expression, we transfected pCMV-FLAG-Cdc25a into CIK cells. When CiCdc25a was overexpressed in CIK cells, IFN 1 expression was decreased. Similarly, When the CIK cells were stimulated by poly (I:C), IFN 1 expression was also reduced (Fig. 3A). The immunoblotting experiment in CO cells also showed the consistent results (Fig. 3B). In order to explain the down-regulation of IFN1 by CiCdc25a in more detail, we used the interference reagent of CiCdc25a to verify this result. The final results showed that, when CiCdc25a was interfered, the expression level of IFN 1 will increase correspondingly (Fig. 3C, 3D).

3.4. Subcellular localization of CiCdc25a

pEGFP-Cdc25a, pEGFP-TBK1 and pEGFP-C1 were respectively transfected into CIK cells. 24 h later, the cells were fixed. Under the confocal microscope, we observed that CiTBK1 was mainly distributed in cytoplasm, while CiCdc25a was distributed in both cytoplasm and the nucleus (Fig. 4A). When pEGFP-Cdc25a and pCMV-FLAG-TBK1 were co-transfected into CIK cells, we found that CiCdc25a (green) and CiTBK1 (red) were accumulated in cytoplasm (yellow) (Fig. 4B). This implies that CiTBK1 and CiCdc25a may combine with each other.

3.5. The interaction of CiCdc25a and CiTBK1

We already know the distribution of CiTBK1 and CiCdc25a in cells. In subsequent study, we constructed co-IP to verify that CiCdc25a and CiTBK1 can interact. After pCMV-FLAG-Cdc25a and pEGFP-TBK1 were transfected into 293T cells, the proteins were collected for Western blotting. According to the co-IP results, we proposed that CiCdc25a and CiTBK1 have the potential to combine with each other (Fig. 5).

3.6. CiCdc25a down-regulates IFN1 expression through dephosphorylation of CiTBK1

FLAG-Cdc25a and FLAG-TBK1 were transferred to CIK cells together. Through qPCR experiments, we found that the mRNA expression level of IFN 1 was reduced (Fig. 6A). Later, we interfered with TBK1 and over-expressed CiCdc25a. At this time, we found that the down-regulation of IFN1 did not change significantly (Fig. 6B). On the contrary, over-expression of TBK1 increased the expression of IFN 1; the expression of IFN 1 was decreased after interference with TBK1 (Fig. 6C, 6D). The phosphorylation of TBK1 at Ser^{172} was extremely important for the expression of IFN 1 (Fig. 6E). Therefore, we believe that CiCdc25a down-regulates the expression of IFN1 through TBK1.

Further, we explored how CiCdc25a regulates the expression of IFN 1 through TBK1. When CiCdc25a was overexpressed in CO cells, the phosphorylation level of TBK1 was decreased. When poly (I:C) was used to stimulate CO cells, the phosphorylation leve of $TBK1^{Ser172}$ was also reduced (Fig. 6F). We performed the verification on EPC cells, the same results were obtained (Fig. 6G).

4. Discussion

Poly (I:C) is a synthetic dsRNA and acts as a kind of virus analog. In mammals, poly (I:C) is mainly recognized by TLR3 and RLRs. RIRs-TBK1-IFN and TLR3-TBK1-IFN pathways also exist in fish (Langevin et al., 2013). In the two signal pathways, TBK1 is a hub protein.

There are many reports that phosphatases are involved in immune response. Such as, SH2-containing inositol 5′-phosphatase (SHIP)-1 regulates the activity and localization of TBK1, thereby inhibiting IFN expression (Gabhann et al., 2010); phosphatase and tensin homolog deleted on chromosome ten (PTEN) enhances IFN expression by promoting IRF3 activation and nuclear translocation (Cao et al., 2018).

Virus infection puts pressure on cells, which leads to cell cycle disorder. Cdc25a can respond to the stimulation of poly (I:C)(Fig. 2). As a joint protein that regulates cell cycle, Cdc25a also plays a role during viral infection. When Adenovirus infects cells, E1A proteins up-regulate the expression of Cdc25a, which causes the cell to enter S phase of division (Spitkovsky et al., 1996). Human T-cell leukemia virus type 1 Tax inhibits the kinase activity of CHK1, prevents the degradation of Cdc25a and delays S phase delay caused by DNA damage (Park et al., 2004). In the DNA damage response process caused by ATR-CHK1 signal, CHK1 plays a potential role in IRF3 regulating innate immunity (Chen et al., 2020b). Cdc25a can dephosphorylate its substrate proteins. For example, Cdc25a dephosphorylates cdk1 and cdk2 so that the cell can enter S phase of mitosis (Donzelli and Draetta, 2003); Cdc25a promotes DNA synthesis by the dephosphorylation of cdk2 (Tomko et al., 2009); The activation of MARCH3 depends on its dephosphorylation induced by Cdc25a, thereby inhibiting IL-1RI-mediated signal transduction (Lin et al., 2018). Cdc25a negatively regulates IFN expression by inhibiting the RLR signaling pathway (Qi et al., 2018). Therefore, Cdc25a participates in innate immunity, cell cycle and DNA damage.

In the existing research, Cdc25a can shuttle between the nucleus and cytoplasm (Kallstrom et al., 2005). In the nucleus, Cdc25a can be phosphorylated and activated to regulate cell division cycle (Hoffmann et al., 1994; Sanchez et al., 1997). In the cytoplasm, it is also active and promotes cell anti-apoptosis (Conklin et al., 1995; Leisser et al., 2004). In this paper, we found that CiCdc25a exists both in the nucleus and cytoplasm (Fig. 4A).

It has been reported that phosphorylation of TBK1 at Ser^{172} determines the kinase activity (Kishore et al., 2002; Ma et al., 2012). The activated TBK1 can activate IRF3/IRF7 and increase the production of IFN (Chau et al., 2008). we found that phosphorylation of TBK1 at Ser^{172} is particularly important for the production of IFN1 (Fig. 6E).

In fact, Cdc25a and TBK1 can interact with each other (Figs. 4B and 5). Similarly, Cdc25a and TBK1 have a certain relationship in regulating the expression of IFN1. In mammals, Cdc25a down-regulates the expression of IFN by dephosphorylation of TBK1 (Qi et al., 2018). Our research are similar to Qi's research, but CiCdc25a inhibiting the phosphorylation of TBK1 is independent of poly (I:C) stimulation (Fig. 6F, 6G). This interesting phenomenon needs to be further studied. In the next experiment, we need to study the molecular mechanism of CiCdc25a dephosphorylating TBK1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104014.

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