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Grass carp (*Ctenopharyngodon idella*) DYRK2 modulates cell apoptosis through phosphorylating p53

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ABSTRACT

In mammals, DYRK2 increases p53 phosphorylation level by interacting with it and then promotes cell apoptosis. However, the function of fish DYRK2 has not yet been elucidated. In this paper, we cloned and identified the coding sequence (CDS) of a grass carp DYRK2 (*CiDYRK2*) which is 1773 bp in length and encodes 590 amino acids. SMART predictive analysis showed that CiDYRK2 possesses a serine/threonine kinase domain. Subsequently, we used the dsRNA analog polyinosinic-polycytidylic acid (poly (I:C) and Grass carp reovirus (GCRV) to stimulate grass carp and CIK cells for different times and found that *CiDYRK2* mRNA was significantly up-regulated both in fish tissues and cells. To explore the function of CiDYRK2, we carried out overexpression and knockdown experiments of CiDYRK2 in CIK cells. Real-time quantitative PCR (Q-PCR), TdT-mediated dUTP nick end labeling (TUNEL) assay and flow cytometry were used to detect the ratio of BAX/BCL-2 mRNA, the number of TUNEL positive cells, the proportion of Annexin V-positive cells respectively. The results showed that CiDYRK2 significantly up-regulated BAX/Bcl-2 mRNA ratio and increased the number of TUNEL-positive cells, as well as the proportion of Annexin V-positive cells. On the contrary, knock-down of CiDYRK2 significantly down-regulated BAX/Bcl-2 mRNA ratio in the cells. Therefore, CiDYRK2 promoted cell apoptosis. To study the molecular mechanism by which CiDYRK2 promoting cell apoptosis, subcellular localization and immunoprecipitation experiments were used to study the relationship between grass carp DYRK2 and the pro-apoptotic protein p53. The results showed that CiDYRK2 and Cip53 were located and co-localized in the nucleus. Co-immunoprecipitation experiment also showed that CiDYRK2 and Cip53 can bind with each other. We further found that DYRK2 can increase the phosphorylation level of p53. In a word, our results showed that grass carp DYRK2 induces cell apoptosis by increasing the phosphorylation level of p53.

1. Introduction

Apoptosis is a kind of programmed cell death that is closely related to innate immunity [1]. Many studies have shown that apoptosis in fish and mammals is similar in some aspects [2,3]. In mammals, p53 is a transcription factor that can regulate DNA repair, apoptosis and so on [4,5]. p53 can be phosphorylated, acetylated, methylated by p53 reactive target proteins, like p53AIP1 [6]. The phosphorylated p53 at Ser46 is an activated state and can promote apoptosis [7].

Belonging to the CMGC group, the dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase (DYRK) is an evolutionarily conserved family [8]. DYRK consists of three subfamilies, which are DYRK subfamily, homeodomain-interacting kinase (HIPK) subfamily and pre-messenger RNA-processing protein 4 kinase (PRP4K) subfamily [9]. DYRK kinase is "dual specificity" kinase that can phosphorylate tyrosine (Y) and serine/threonine (S/T) [10]. It plays the function in brain development, proliferation and so on [11]. DYRK subfamily is formed by two kinds of classes, class I (DYRK1A and DYRK1B) and class II (DYRK2, DYRK3, DYRK4A and DYRK4B). Among them, DYRK2 is a conserved enzyme in evolution [12,13]. DYRK2 interacts with and phosphorylates TBK1, subsequently leads to the ubiquitination of TBK1 and decreases expression of IFN [14]. Furthermore, DYRK2 regulates cell division, proteotoxic stress, cell cycle, tumor progression, cardiomyocyte growth, apoptosis and glycogen synthesis [15–21]. Phosphorylating p53 in the nucleus and promoting apoptosis is DYRK2 most classic function [22].

Zebrafish DYRK2 was first cloned and identified in fish, which is related to muscle fibers [23] and the proper positioning of neurons

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

Primer name	Primer sequence (5′-3′)	Application
DYRK2-CDS-F	ATGTTAACTAAGAAACCCTGCGCTG	CDS
DYRK2-CDS-R	GTGTTGCCAAAATTAGTCAGCTGA	
CiDYRK2-RT-F	CTGCTACTGCTGTCTACCCGA	RT-PCR
CiDYRK2-RT-R	CCGTGAGAGAGTTGCTGTTCC	
IFN-RT-F	GTCAATGCTCTGCTTGCGAAT	
IFN-RT-R	CAAGAAACTTCACCTGGTCCT	
β-actin-F	CACTGTGCCCATCTACGA	
β-actin-R	CCATCTCCTGCTCGAAGTC	
Bax-RT-F	CTCATCAGGGTGGTAAGACAT	
Bax-RT-R	CCTATCACCAATCACTTTAATG	
Bcl2-RT-F	GATACCGCAAGATTCCATACCC	
Bcl2-RT-R	TCCTTTCTATCTCGTCTCCAG	
si-DYRK2	CCACAUCACCCGUCACAUUTT	siRNA
Negative Control	UUCUCCGAACGUGUCACGUTT	
DYRK2-pcDNA3.1-F	CGGAATTCATGTTAACTAAGAAACCCTGCGCTG	vector construction
DYRK2-pcDNA3.1-R	TTGCGGCCGCTCAGCTGACTAATTTTGGCAACAC	
DYRK2-pCMV-FLAG-F	TTGCGGCCGCGATGTTAACTAAGAAACCCTGCGCTG	
DYRK2-pCMV-FLAG-R	CGGAATTCTCAGCTGACTAATTTTGGCAACAC	
DYRK2-GFP-F	CCCTCGAGCTATGTTAACTAAGAAACCCTGCGCTG	

during neurulation [24]. In recent years, more and more functions of fish DYRK2 have been reported successively. However, the role of fish DYRK2 in apoptosis has not been studied. Here, we authenticated grass carp DYRK2 and explored the relationship between DYRK2 and apoptosis. We found that grass carp DYRK2 induces cell apoptosis by increasing the phosphorylation level of p53.

2. Materials and methods

2.1. Grass carp and cells

Grass carp (weight 20–30g) were from Nanchang Shenlong Fisheries Development (China) and fed in a tank with adequate oxygen for two weeks or even longer. Grass carp kidney (CIK) cells and grass carp ovary cells (CO) were cultured at 28 °C in medium 199 added with 10% FBS (VivaCell, Shanghai, China), 100 U/ml penicillin, and 100 mg/ml streptomycin. In addition, Co cells were cultured with 5.0% CO_2 to maintain pH of surrounding.

2.2. Cloning and constructing grass carp DYRK2

The open reading frame (ORF) of *CiDYRK2* was cloned according to the known *Danio rerio* DYRK2 (*DrDYRK2*) (BC139631.1). The phylogenetic tree of DYRK2 was built by MEGA 7.0.26 and Clustalx1.83. The amino acid sequence of CiDYRK2 and the similarity percentage of all sequences were analyzed by DNAMAN. The conserved domain and tertiary structure were predicted by SMART and SWISS-MODEL respectively.

The ORF of *CiDYRK2* was constructed into pEASY-T1 (Trans Gen, China), pcDNA3.1 (Beyotime, China), pEGFP-C1 (Invitrogen USA) and p3 × FLAG-myc-CMV-24 (Sigma, USA) respectively to facilitate subcellular localization and Co-IP.

2.3. The expression level of CiDYRK2

Poly(I:C) (Sigma, USA) (500 μl per 100 g) or GCRV097 (50 μl) was injected into the cultured experimental grass carp by intraperitoneal injection, and the injection intervals were 0 h, 6 h, 12 h, 24 h and 48 h, respectively. GCRV 097 is kindly provided by Dr. Jianguo Su (Huazhong Agricultural University, Wuhan, China). Virus titer is 10^{-8} TCID50 [25]. The total RNA from intestines, liver, brain, gills, kidney, eyes were acquired by RNA simple total RNA kit (Tiangen Biotech, China). RNA was reversed into cDNA by Prime Script RT reagent Kit (Takara, Japan) and analyzed by Q-PCR. The condition is 94 °C/5min; 40 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s.

2.4. Apoptosis

CIK cells were cultured in six-well plates (Nest Biotech, China) for 12h and then transfected with 1.5 μg pcDNA3.1-DYRK2 or 1.5 μg pcDNA3.1-basic. Meanwhile, 5 μl siDYRK2 or 5 μl negative control (NC) was transiently co-transfected into the cells using 4 μl LipoRNAi. After 24 h, the apoptosis-related genes like BAX and Bcl-2 were detected.

CIK cells cultivated in 20 mm confocal dishes were transfected with 1 μg pcDNA3.1-DYRK2 or 1 μg pcDNA3.1-basic. Twenty four hours later, the cells were washed two times by phosphate buffer saline (PBS) (pH = 7.4), then fixed by 4% paraformaldehyde. Thirty minutes later, washed the cells two times by PBS (pH = 7.4) again. Thirdly, the dishes were added into 0.3% Triton X-100 for 5min. Lastly, the samples were dyed by mixture of 5 μl TDT and 45 μl fluorescent labeling solution and were tested on the microscope.

Transfer CIK cells from 25 cm^2 culture dish to 25 mm Petri flask for overnight cultivation. Then 2 μg pcDNA3.1-DYRK2 and 2 μg pcDNA3.1-basic were transiently transfected into cells using Lip8000 transfection reagent (Sudgen Biotech, China) for 24 h. The cells were collected by pancreatin without ethylenediamine tetraacetic acid (EDTA) and washed two times by PBS. The cell pellets were obtained by centrifuge (300 g, 3 min), the next step was in accordance with the instruction of the Annexin V, FITC Apoptosis Detection Kit (Dojindo, Japan, AD10). The final result was analyzed by FlowJo vX.0.7 software.

2.5. Subcellular localization assays

We added 1 μg GFP- CiDYRK2 and 1 μg GFP-basic into 20 mm confocal flasks with cell volume of 70%–80% respectively. The cells were washed and fixed with 4% paraformaldehyde for 15 min. Then we added 200 μl DAPI (1 μg/ml) (BBI Biotech CAN) into cells to stain cells. Lastly, the cells were washed with PBS 3 times. The results were observed by laser confocal microscopy.

2.6. Co-immunoprecipitation

CO cells were cultured in 10 cm^2 cell-cultured dish and then transfected with 2.5 μg Flag-CiDYRK2 and 2.5 μg GFP-Cip53. After transfection for 36h, whole protein of the cells was extracted. We took 100 μl cell lysate as the input, and the rest cell lysates were incubated with 40 μl anti-GFP beads (Alpalife Biotech, China) at 4 °C for 2 h. Another 1800 μl cell lysates were severally incubated with 4 μl Flag antibody (Sigma, USA) or 4 μl IgG antibody (Beyotime Biotechnology, China) for 1h. Then, they were incubated with 40 μl Anti-Mouse IP beads (Alpalife Biotech, China) for 1 h, respectively. The protein was separated by 10%

Table 2
GenBank accession numbers of the sequences used for phylogenetic analysis.

Species	Accession NO.
Anabarilius graham	ROI15395.1
Danio rerio	NP_001038298.1
Triplophysatibetana	KAA0720935.1
Carassius auratus	BAN05303.1
Oncorhynchustshawytscha	XP_042169661.1
Oncorhynchus kisuth	XP_031671123.1
Salvelinus namaycush	XP_038842281.1
Denticeps clupeoides	XP_028811370.1
Carassius auratus	XP_026090519.1
Tachysurusfulvidrac	XP_027007376.1
Pteropus giganteus	XP_039721881.1
Nannospalax galili	XP_008845183.1
Orycteropus aferafer	XP_007955573.1
Orcinus orca	XP_004276654.1
Balaenoptera musculus	XP_036721166.1
Delphinapterus leucas	XP_022427305.1
Homo sapiens	AAH06375.1
Mus musculus	NP_001014412.1
Rattus norvegicus	NP_001101570.1
Pan troglodytes	JAA43175.1
Ctenopharyngodon idellus	MZ504993

SDS-PAGE gel, and then transferred onto NC membranes and incubated by Flag and GFP antibodies (Abmart, USA). Imaging system (CLINX, China) detected the interaction of grass carp DYRK2 and p53.

2.7. Phosphorylation analysis

CO cells were transfected with 2.5 μg pcDNA3.1-basic + 2.5 μg GFP-Cip53 or 2.5 μg pcDNA3.1-DYRK2 + 2.5 μg GFP-Cip53. When transfected 24h, whole protein was extracted by NP-40 pyrolysis with phosphatase inhibitor liquid (100 ×). Take 100 μl cell lysate as input. The input was incubated with GFP antibody and GAPDH antibody respectively (preserved in our lab). The rest of supernatant was incubated with anti-GFP beads. The nonspecific bands were washed with CO-IP buffer. Phosphorylation level of grass carp p53 was tested by western-blot with phosphor-Ser/Thr/Tyr antibody (Invitrogen USA). GAPDH was as the internal.

2.8. Statistical analysis

Q-PCR data were analyzed by GraphPad Prism software version 8.0 (Graph Pad Software, San Diego, CA, USA). The data were accessed with students' T-test and one-way ANOVA test. Statistical significance was represented by a P-value (*$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$).

3. Results

3.1. Phylogenetic tree and characteristic analysis of CiDYRK2

The ORF of *CiDYRK2* (MZ504993) is 1733bp in length and encodes 590 amino acids. The sequence and application of primers were shown in Table 1. Grass carp DYRK2 shares a close relationship with *Anabarilius graham* DYRK2 (Fig.SA). DYRK2 from grass carp, humans, zebrafish and

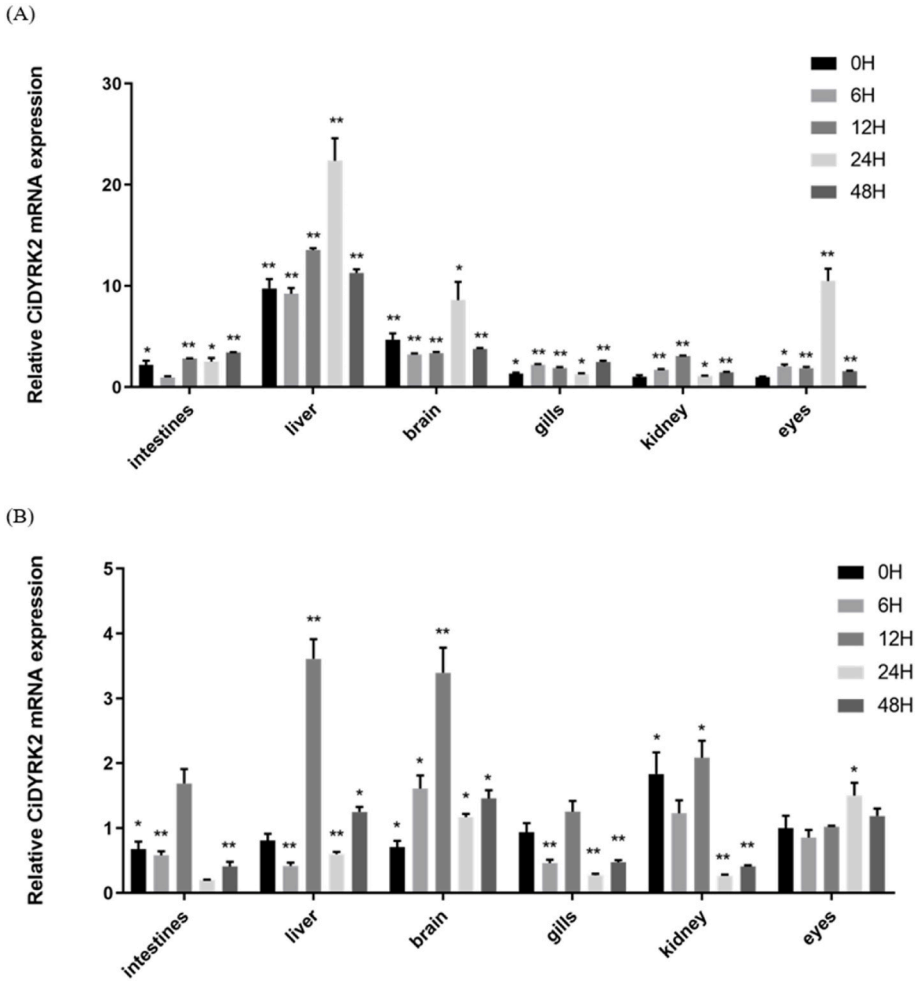

Fig. 1. The expression of *CiDYRK2* was up-regulated by poly (I:C) and GCRV
Poly(I:C) or GCRV was injected into the cultured experimental grass carp by intraperitoneal injection, and the injection intervals was 0 h, 6 h, 12 h, 24 h and 48 h, respectively. We extracted total RNA from tissues and performed Q-PCR to detect the expression of DYRK2. Q-PCR data were analyzed by GraphPad Prism software, and then accessed with students' T-test and one-way ANOVA test. In this study, each group of experiments consisted of at least three independent replicates.

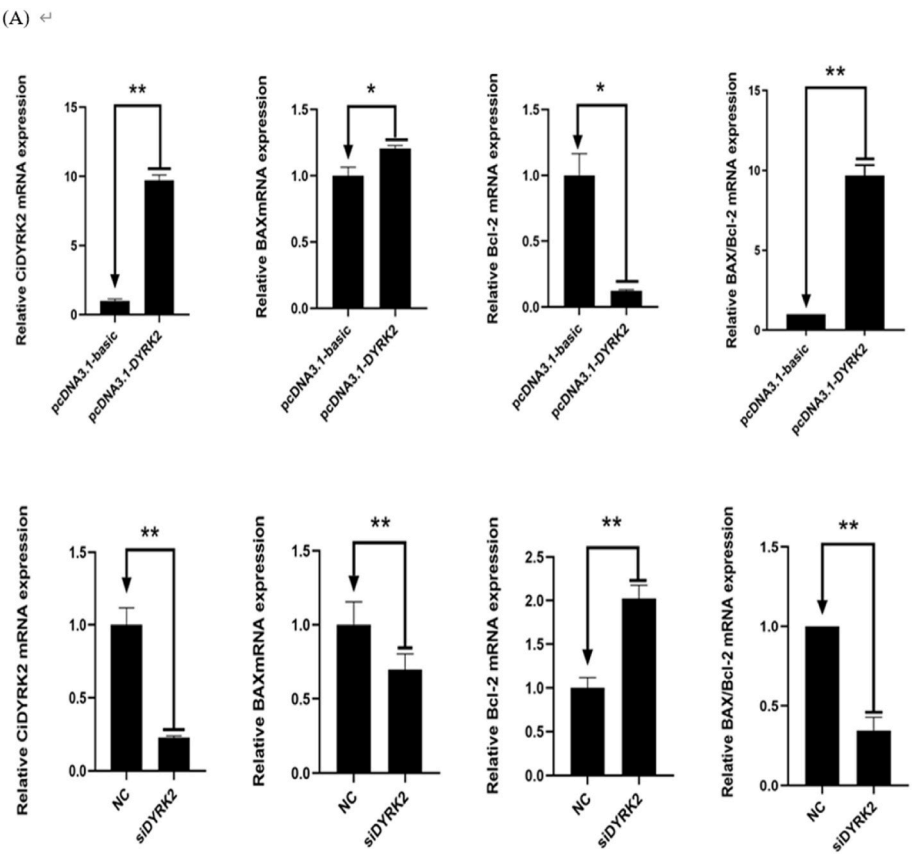

Fig. 2. CiDYRK2 promotes apoptosis

(A) CIK cells were transfected with pcDNA3.1-basic, pcDNA3.1-DYRK2, NC and siDYRK2, respectively. Total cellular RNA was extracted and the ratio of BAX/Bcl2 was detected by Q-PCR. (B) CIK cells were transfected with pcDNA3.1-basic or pcDNA3.1-DYRK2, respectively, and cell apoptosis was detected by TUNEL kit. (C) We transfected pcDNA3.1-basic or pcDNA3.1-DYRK2 into CIK cells. After staining with the Annexin V, FITC Apoptosis Detection kit, cell apoptosis was detected by flow cytometry.

lilang white fish was relatively conservative, especially the similarity with that of lilang white fish was 99% (Fig.SB). The conserved domain of CiDYRK2 is serine/threonine protein kinases domain, which acts as catalysis. The domain contains 314 amino acids from 211 to 524 (Fig. SC). The relevant parameters of the model with the best predicted tertiary structure results are as follows that the seq identity is 90.66%, the GMQE is 0.58 (Fig.SD). GenBank accession numbers of DYRK2 from different species are shown in Table 2. The results of amino acid sequence analysis and informatics website prediction indicated that grass carp DYRK2 have not the same phosphorylation sites as human.

3.2. The expression of CiDYRK2 was up-regulated by poly (I:C) and GCRV

Poly (I:C) stimulation can significantly up-regulated the expression of *CiDYRK2* in grass carp tissues. After stimulation, DYRK2 transcripts were increased and almost peaked at 12h or 24 h in different tissues (Fig. 1A). GCRV stimulation can up-regulate the expression level of *CiDYRK2* in grass carp tissues too. Its expression was upregulated in different tissues and almost peaked at 12h generally (Fig. 1B). The expression of the gene in eye at 0 h was as the control.

3.3. CiDYRK2 promotes apoptosis

The ratio of apoptosis-related genes Bax/Bcl-2 mRNA was increased when we overexpressed DYRK2 in CIK cells. However, the ratio was decreased when we knocked down DYRK2 in CIK cells (Fig. 2A). It can be seen also from TUNEL experiment when DYRK2 was overexpressed, the apoptotic cells were increased significantly (Fig. 2B). Finally, according to the results of flow cytometry experiments, when DYRK2 was overexpressed, the proportion of Annexin V-positive cells was increased (Fig. 2C). So, we can conclude that CiDYRK2 promotes apoptosis.

3.4. Subcellular localization of CiDYRK2

We analyzed the distribution of grass carp DYRK2 in CIK cells. The result showed that CiDYRK2 was mainly located in the cytoplasm, and a litter in the nucleus (Fig. 3).

3.5. Interaction of CiDYRK2 and Cip53

CO cells were co-transfected with 2.5 μg Flag-DYRK2 and 2.5 μg GFP-p53. Co-immunoprecipitation assays revealed that CiDYRK2 can interact with Cip53 (Fig. 4).

3.6. CiDYRK2 promotes Cip53 phosphorylation

We transfected 2.5 μg pcDNA3.1-basic + 2.5 μg GFP-p53 or 2.5 μg pcDNA3.1-DYRK2 + 2.5 μg GFP-p53 into CO cells. When CiDYRK2 was overexpressed in CO cells, the degree of phosphorylation of Cip53 was increased (Fig. 5).

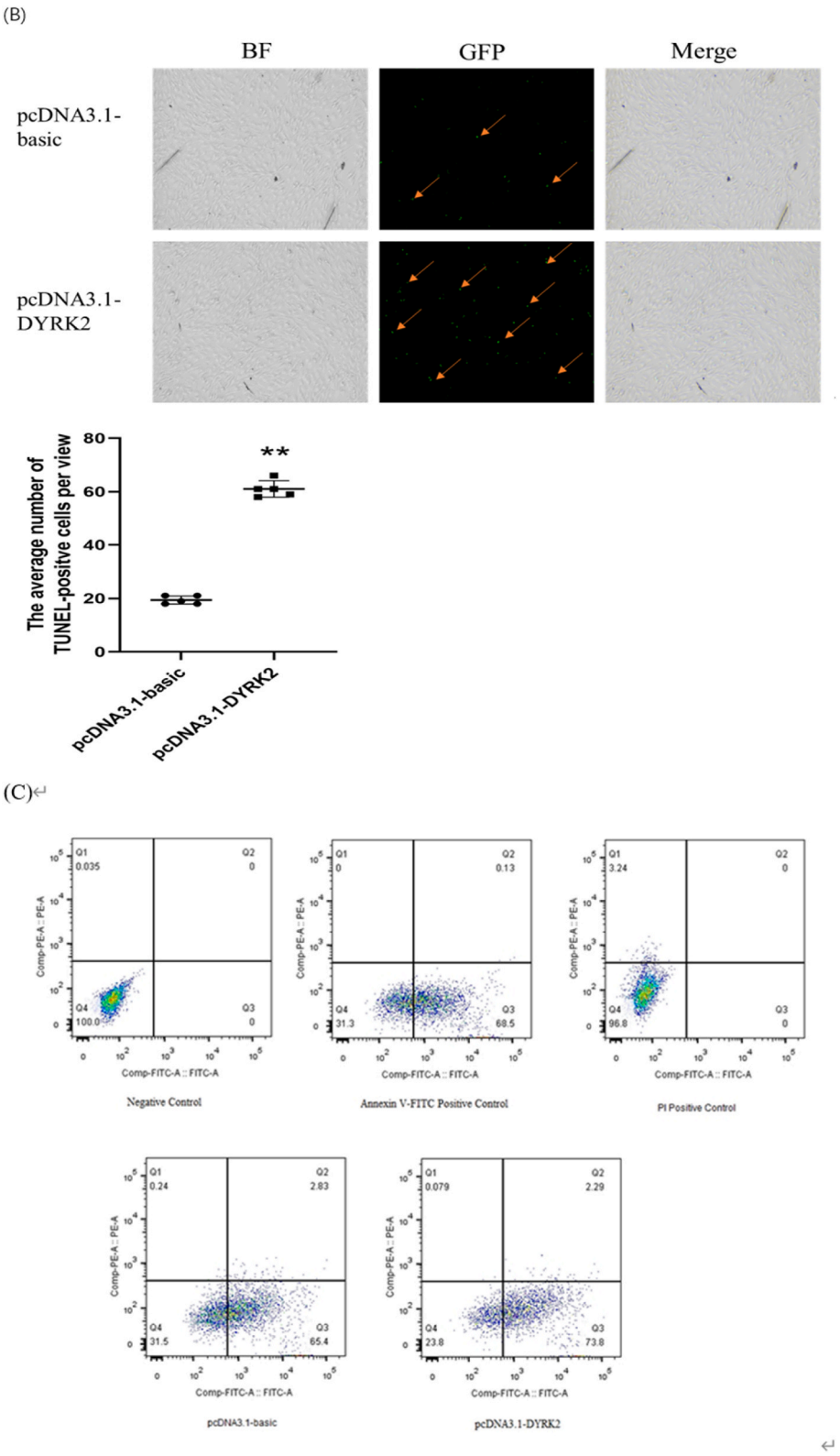

Fig. 2. (*continued*).

4. Discussion

Grass carp DYRK2 was first cloned and analyzed in this paper. CiDYRK2 has a close evolutionary relationship with that of *Anabarilius grahami* (Fig. SA). We can see that DYRK2 is very conservative from fish to mammals (Fig. SB).

DYRK2 has been found in human fetal brain cDNA library [26]. In mammals, CiDYRK2 may play a role in events associated with brain and liver function, such as neurodevelopment and liver cancer. Yogosawa et al. (2019) found that the downregulation of DYRK2 enhances proliferation and cell cycle in liver cancer cells [27]. DYRK2-GSK3β complex can phosphorylate NDEL1 to regulate neuronal morphogenesis [28].

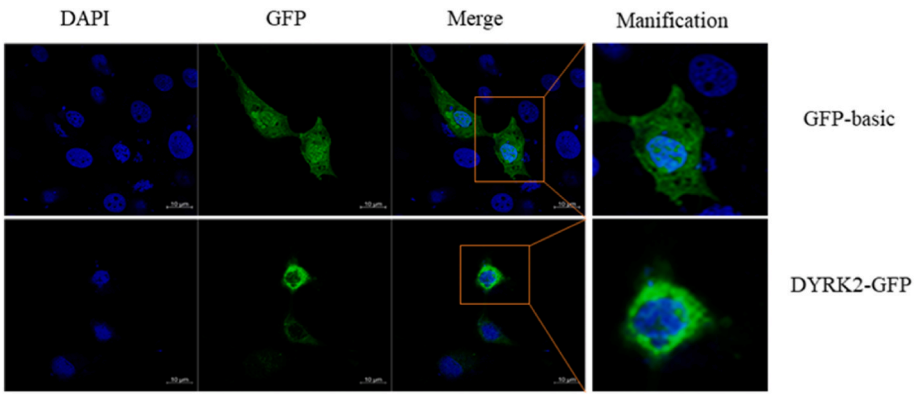

Fig. 3. Subcellular localization of CiDYRK2
GFP-DYRK2 or GFP-basic was transfected into CIK cells, respectively. GFP-basic was used as a control. We observed its subcellular location by confocal laser microscopy (green excitation light at 488 nm for the GFP vector). The scale bar is 10 μm.

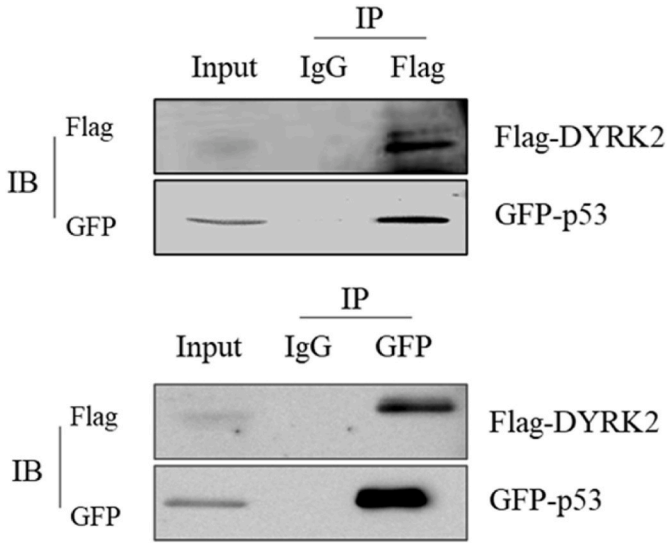

Fig. 4. Interaction of CiDYRK2 and Cip53
CO cells were transfected with Flag-DYRK2 and GFP-p53. After 36h, the whole protein was extracted. 100 μl of lysate was used as input. Cell lysates were maintained with anti-mouse IP beads conjugated with Flag/GFP antibodies, and co-immunoprecipitation was used to detect the interaction of CiDYRK2 and Cip53 via anti-Flag and anti-GFP.

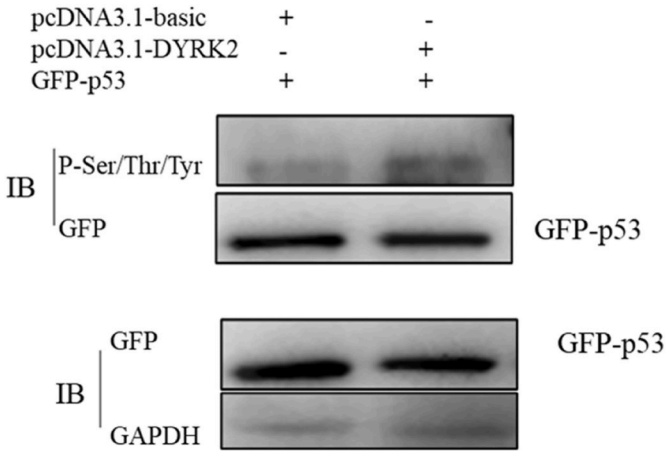

Fig. 5. CiDYRK2 enhances phosphorylation of Cip53
PcDNA3.1-basic and GFP-p53 or pcDNA3.1-DYRK2 and GFP-p53 were transfected into CO cells. Twenty four hours later, the whole protein was extracted, and 100 μl were taken from different dishes as input. The rest of protein was incubated with anti-GFP beads. Finally, the input was incubated with anti-GFP and GAPDH, and GFP beads were incubated with anti-GFP and anti-P-Ser/Thr/Tyr. GAPDH was used as an internal reference.

Likewise, in zebrafish, Tanaka et al. (2012) displayed DYRK2 can phosphorylate Dpysl2 (CRMP2) and Dpysl3 (CRMP4) to help the proper location of neurons during neurulation [24]. In this paper, we used GCRV and poly(I:C) to stimulate grass carp and found that the expression of CiDYRK2 in tissues was significantly increased. The results also showed that the expression of CiDYRK2 was higher in brain and liver than other tissues (Fig. 1A and B), indicating that the function of DYRK2 is relatively conservative.

These results also indicated that fish DYRK2 can participate in a series of reactions induced by viruses. In mammals, DYRK2 responds to the stimulation of genotoxic stress like exposure to ADR [20]. Viruses can induce apoptosis, so we hypothesized that fish DYRK2 is closely associated with apoptosis. In mammals, when DYRK2 was silenced, the apoptosis induced by ADR was decreased, on the contrary, when DYRK2 was overexpressed, the number of Annexin V-positive cells were increased [22,29]. CiDYRK2 increased the ratio of BAX/Bcl-2, the number of apoptosis cells and the proportion of Annexin V-positive cells was increased (Fig. 2A, B, C), which suggested grass carp DYRK2 may have the similar function to mammalian DYRK2.

Mammalian DYRK2 predominantly exists in the cytoplasm, another small part in the nucleus [30]. Shmueli et al. (2007) thought that DYRK2 regulates apoptosis by p53 [31]. So how does DYRK2 get into the nucleus to bind with p53? DYRK2 can be phosphorylated by ATM at Thr-33 and Ser-369, thereby dissociates the complex between MDM2 and DYRK2 and avoids the degradation of DYRK2. At the same time, ATM can promote the entry of DYRK2 into the nucleus, thereby strengthens the binding and phosphorylation of p53. Finally, it promotes apoptosis [32].

The previous study showed that grass carp p53 is located in the nucleus [33]. CiDYRK2 was mainly located in the cytoplasm, and a litter in the nucleus (Fig. 3). Therefore, they have a material basis for combining. Subsequently, co-immunoprecipitation result showed that they have the physical interaction between CiDYRK2 and Cip53 (Fig. 4). Our research showed that grass carp DYRK2 increased the phosphorylation level of p53 (Fig. 5).

In mammals, DYRK2 regulates p53 via Ser46 phosphorylation to promote apoptosis [31]. The molecular mechanism of the specific phosphorylation site of p53 in fish remains to be further studied.

In general, we found the important role of CiDYRK2 in apoptosis. The results revealed that grass carp DYRK2 can respond to poly (I: C) or

GCRV stimulation and regulate apoptosis by increasing the phosphorylation level of p53.

CRediT authorship contribution statement

Shanshan Zeng: Formal analysis, Writing – original draft. **Meifeng Li:** Formal analysis. **Xining Cheng:** Formal analysis. **Shina Lu:** Formal analysis. **Zhiqing Feng:** Formal analysis. **Zeyin Jiang:** Formal analysis. **Zhichao Sun:** Data curation. **Xiaowen Xu:** Data curation. **Huiling Mao:** Writing – review & editing. **Chengyu Hu:** Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.06.065.

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