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Research article

Identification and functional characterization of the transcription factor coding *Dp1* gene in large yellow croaker *Pseudosciaena crocea*

Xiaohui Cai [a,1], YanQing Huang [b,1], Honglin Chen [a,c], Qiancheng Qi [a], Meijuan Xu [a], Peng Xu [a], Xinzhong Wu [a,b,*]

[a] Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Beibu Gulf University, Qinzhou, 536011, China
[b] Zhejiang University, College of Animal Sciences, Hangzhou, 310058, China
[c] Guangxi University, College of Life Science and Technology, Nanning, 530000, China

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ABSTRACT

The transcription factor Dp1, as a binding partner, often forms a dimerization complex with typical E2F to play a central role in regulating gene expression during G1/S cell cycle progression. In this study, a full-length *dp1* cDNA (*Pcdp1*) was successfully cloned and characterized from the large yellow croaker *Pseudosciaena crocea*. The nucleotidic sequence of *Pcdp1* is 1,427 bp long with an open reading frame (ORF) of 1,239 bp encoding a putative protein of 412 amino acids, a 5′-untranslated region of 116 bp and a 3′-untranslated region of 70 bp. Prediction of protein domains showed that PcDp1 contains a DNA-binding domain (DBD) with a DEF box, a dimerization domain and an acidic region at *C terminus* with transcription activity. Homology comparisons indicated that PcDp1 shared the highest sequence identity of 98.55% with *Oreochromis niloticus* dp1, followed by 88.72% identity with *Danio rerio* dp1 and a relatively low identity of 78.91–80.55% with its mammalian and amphibian counterparts. The mRNA of *Pcdp1* showed ubiquitously expression in all analyzed tissues, with the highest level of expression in the body kidney. Moderate expression levels of *Pcdp1* was found in several immune-related tissues including the gills, head kidney and liver, indicating that PcDp1 might play an important role in osmotic pressure regulation and immune response of the large yellow croaker. The subcellular localization of PcDp1 revealed that it is mainly distributed in the cytoplasm both in COS-7 and parenchymal cells of the spleen, head kidney and kidney tissues. Furthermore, the recombinant PcDp1 exhibited DNA-binding activity to E2F site *in vitro*. In conclusion, these results indicated that PcDp1 may participate in immune regulation and provide a foundation for further study of the regulatory mechanism of Dp1 in teleosts.

1. Introduction

Dp1 belongs to the DP subfamily of the E2F/DP transcription factor family, which was originally identified by sequencing purified protein bound to an E2F DNA-binding site (Girling et al., 1993). Besides the *dp1* gene, the E2F/DP transcription factor family also contains eight E2F family members (typical E2F1-6 and atypical E2F7-8) and two DP family members (Dp2/3 and Dp4) (Lammens et al., 2009; Milton et al., 2006; Ramirez-Parra et al., 2007). Among them, the typical E2F and DP sub-family members all possess both DNA binding and dimerization domains. However, the DP sub-family members, unlike typical E2Fs, have neither transactivation nor regulatory domains of pocket proteins (Ramirez-Parra et al., 2018). Therefore, DP sub-family members often form heterodimers with typical E2F to enhance their DNA binding and transactivation activities (Bandara et al., 1993; Helin et al., 1993; Krek et al., 1993). Huber et al. (1993) found that a dramatic (100- to 1,000-fold) increase in specific DNA binding activity of E2Fs was observed on mixing the E2Fs and Dp1 components together. It is well established that the targeted genes of the E2F/DP heterodimer are involved in cell-cycle progression, DNA replication, apoptosis, tumor suppression, extra-embryonic development and embryonic survival, muscle growth, centriole duplication, anteroposterior neural patterning and so on (Hitchens and Robbins, 2003; van den Heuvel and Dyson, 2008; Kohn et al., 2003; Wang et al., 2001; Komori et al., 2018; Zappia and Frolov, 2016; Miller et al., 2016; Kim et al., 2012). On the other hand, more and more studies have shown that Dp1 can also regulate specific genes

* Corresponding author.
E-mail address: wuxzqinzhou@163.com (X. Wu).
[1] Co-first authors.

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independently of E2F. For example, Dp1 independently from E2F exerts both positive and negative roles in Wnt/β-catemin signaling during anteroposterior neural patterning in Xenopus embryos (Kim et al., 2012). Dp1 could also directly bind to the promoter of the tumor suppressor ARF to cause cell cycle arrest in G_1 (Datta et al., 2005). Those studies showed that Dp1 plays a diverse roles in cellular functions both dependent upon or independent of E2F.

The large yellow croaker (*Pseudosciaena crocea* Richardson, 1864) is one of the most important economical resources cultured on the coasts of continental East Asia, especially in the Fujian and Zhejiang provinces of China (Chen et al., 2018). According to FAO statistics, the global production of large yellow croaker reached 269,300 tons in 2016, mainly produced by China (99%) and South Korea (1%). In recent years, with the rapid culture industry development of the large yellow croaker, serious epidemics caused by virus, bacteria and parasitic infections in fry to marketable size fish, are becoming more severe and resulting in great economic losses (Chen et al., 2003; Wang et al., 2010; Zuo et al., 2012). Especially in 2009, the parasite *Cryptocaryon irritans* caused the death of about 4,000 tons of marketable-sized fish in the Fujian Province of China, which resulted in direct economic loss of over 60 million RMB (Liao et al., 2011). Although genome sequencing (Wu et al., 2014) and identification of partially immune-related genes, such as Toll-like receptor 3 and 22, interferon gamma, MyD88, interleukin-8, antimicrobial peptide NK-lysin (Huang et al., 2011; Xiao et al., 2011; Yang et al., 2017; Yao et al., 2009; Zhou et al., 2018, 2019; Mu et al., 2014), have revealed the molecular composition and partial function of immune system, little information is available on the basal immune response and physiological processes on the large yellow croaker. In order to isolate the differentially expressed genes involved in physiological and pathological processes, the suppression subtractive hybridization (SSH) of head kidney of the large yellow croaker with PG as the immunostimulant was constructed in our laboratory (Huang, 2009). It is noteworthy that one transcription factor (*dp1*, *DRTF1 polypeptide 1*), known to be a binding partner of the E2F transcription factor, was found to be differentially expressed in this SSH library. And up to now, there has been no reports about the function of Dp1 in teleosts.

In this study, the full-length cDNA of *dp1* in *Pseudosciaena crocea* (*Pcdp1*) was cloned and characterized. Its expression and subcellular localization in immune-related tissues and its parenchymal cells were investigated. Furthermore, the DNA binding activity of PcDp1 with was analyzed by an EMSA assay. This study will lay a foundation for further study of immune regulation and function of Dp1 in teleosts.

2. Materials and methods

2.1. Experimental fish and sampling

Healthy large yellow croakers, average body weight 150 g, were obtained from a marine cage culture farm at 20 ± 1 °C at Xiangshan harbour, Ningbo City, Zhejiang Province, China.

To amplify *Pcdp1* full-length sequence and measure its mRNA tissue distribution in healthy fish, the following samples including spleen, body kidney, head kidney, liver, heart, intestine, gill and muscle were collected from three fish, frozen immediately in liquid nitrogen, and stored at -80 °C until use. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC, China).

2.2. Cloning the full-length cDNA of Pcdp1

Total RNA was extracted from spleen, body kidney, head kidney, liver, heart, intestine, gill and muscle of healthy samples by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The first strand cDNA (5′- and 3′-ready cDNA) was synthesized from 2 μg mixed RNA (mixture composed with equal amount of spleen, kidney, head kidney, liver, heart, intestine, gill and muscle) by using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). Rapid amplification of cDNA ends (RACE) was performed to clone the full-length cDNA of *Pcdp1* with specific primers (Table 1) using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The PCR was performed using a touchdown PCR procedure: 5 cycles of denaturation at 94 °C for 30 s, 72 °C for 3 min; 5 cycles of denaturation at 94 °C for 30 s, 70 °C for 3 s, and 72 °C for 3 min; 25 cycles of denaturation at 94 °C for 30 s, 69 °C for 3 s, and 72 °C for 3 min. PCR products were analyzed on a 1.5% agarose gel and purified with a QIAquick PCR Purification Kit (Qiagen, USA). The purified PCR products were ligated into a pMD19-T Vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5α cells. The positive clones were sequenced by Sangon Biotech (Shanghai) Co. Ltd. The full length cDNA sequences were obtained by assembling the 5′- and 3′-end corresponding sequences using the software DNAMAN V6 (Lynnon Biosoft, USA).

2.3. Sequence and phylogenetic analysis

The homology searches of the full length cDNA sequence of *Pcdp1* gene were performed using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program, and the open reading frames (ORFs) and amino acid sequences were identified and predicted by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The molecular weight and pI of PcDp1 were calculated with the Expasy compute pI/MW tool (https://web.expasy.org/protparam//), and the signal peptides of PcDp1 were predicted by SignaIP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The functional conserved domains of PcDp1 were predicted using the CDD program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignments were performed using Clustal X 2.0 program and visualized using GeneDoc software. Phylogenetic analyses were performed using the Neighbor-Joining method in the MEGA 7.0 with a minimum of 1000 bootstraps replications.

2.4. Tissue distribution of Pcdp1 in healthy large yellow croaker

Transcription tissue distributions of *Pcdp1* in healthy large yellow croaker were analyzed by using real-time PCR. The total RNA from tissues mentioned in section 2.2 was extracted as template to synthesize the first strand cDNA by using the EasyScript One-Step cDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China). Real-time PCR was performed with the specific primers (RT-DP1-F and -R) designed based on the full-length sequence of *Pcdp1* by using QuantStudio™ 6 Flex Real-Time PCR System, and the *β-actin* gene was used as an internal control in all qRT-PCR experiments (Table 1). Each reaction contains 1 μL of reverse and forward primers, 10 μL of SYBR Green Mix (TransGen, Beijing, China), and 2 μL of 1:8 diluted cDNA and RNase-free water to a final reaction volume of 20 μL. The program was performed as follows: 1 cycle of 10 min at 95 °C, 40 cycles of 10 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C. The relative expression level of *Pcdp1* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each experiment was performed in triplicate.

2.5. Expression and purification of recombinant PcDp1 protein

To investigate the biological function of PcDp1, the full-length sequence was amplified with specific primers (E-Dp1-F and –R with *Bam* HI and *Hind* III restriction sites) (Table 1), and then inserted into the expression vector pET-28a. Then, the recombinant plasmid pET-His-PcDp1 was transformed into *E. coli* BL21 (DE3) competent cells

Table 1. Primers used in this study.

Primers	Sequences (5'—3')	Purpose
5 PcDp1-R	CCAATCCATTTGATTTCTTTCTT	5' RACE
3 PcDp1-F	CTGGATGAAGGTGTGTGAGAAGGTGC	3' RACE
RT-Dp1-F	TCGACTGCAGCATCTCAAAC	Real-time PCR
RT-Dp1-R	TTACTGATGGGACCCTTTGC	Real-time PCR
β-actin-F	GCGACCTCACAGACTACCTC	Real-time PCR
β-actin-R	GTAGGTGGTCTCGTGGAT	Real-time PCR
E-Dp1-F	CATGGATCCATGGCTAAAGATGCTGGTCTG	Protein expression
E-Dp1-R	TCGAAGCTTGTGTTCATCGTCGTTCTCGT	Protein expression
G-Dp1-F	CCGCTCGAGATGGCTAAAGATGCTGGTCTG	EGFP reporter assay
G-Dp1-R	GCGTCGACGTGTTCATCGTCGTTCTCGT	EGFP reporter assay
WE2Fsite-F	GAGATGACGTAG**TTTTCGCGC**GAAACTAG	EMSA
WE2Fsite-R	CTAGTTTC**GCGCGAAAA**CTACGTCATCTC	EMSA
ME2Fsite-F	GAGATGACGTAG**TTTTCG*AT*C**GAAACTAG	EMSA
ME2Fsite-R	CTAGTTTC**G*AT*CGAAAA**CTACGTCATCTC	EMSA

Bam HI, *Hind* III, *Xho* I and *Sal* I sites are underlined. E2F site are bold.

(Tiangen, Beijing, China). Recombinant PcDp1 protein (rPcDp1) was expressed by different concentrations (0.2, 0.4, 0.8 and 1 mM) IPTG induction at 37 °C for 8 h and SDS-PAGE was performed for expression analysis of recombinant protein. The culture without IPTG was used as a negative control. The rPcDp1 was purified by Ni-NTA nitrilotriacetic acid (Ni-NTA) affinity chromatography using the Ni-NTA purification Kit (Novagen, USA) according to manufacturer's protocol. The purified proteins were quantified using the Bradford protein quantification Kit (Tiangen, China), and analyzed by SDS-PAGE.

2.6. Preparation of polyclonal antibody of PcDp1 and Western blot analysis

To acquire polyclonal antibodies of PcDp1, two male New Zealand rabbits with body weight of 2 kg were immunized with purified rPcDp1 protein according to previous studies with some modification (Yin et al., 2019). Briefly, rabbits were vaccinated subcutaneously (ten locations) with 500 μg of rPcDp1 emulsified with complete Freund's adjuvant (FCA) at 2 week intervals. The following immunization was performed three times with 300, 300 and 500 μg of rPcDp1 and incomplete Freund's adjuvant (FIA), respectively. The carotid artery blood was collected prior to immunization and 5 days after individual immunization and the antibody titer was analyzed by ELISA (Yang et al., 2011). A large amount of blood was collected from the immunized rabbits and tipped at 4 °C for 24 h to harvest the antiserum and stored at -20 °C.

In Western blot analysis, the rPcDp1 was separated by SDS-PAGE and electrophoretically transferred onto PVDF membrane. The membrane was blocked by incubation in 5% skim milk powder solution at 37 °C for 2 h. After washing in TBS and PBST (PBS with 0.1% (w/v) Tween 20) for five times (5 min each time) respectively, the membrane was incubated with 1:500 (v/v) prepared rPcDp1 antibody (pre-immune serum was used as negative control) at room temperature for 1.5 h and washed with PBST for five times, and then incubated with 1:5,000 (v/v) horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. After a final five times of washing, the membrane was incubated in TMB substrate (Solarbio, China) in dark for 5–10 min until the strips appear and the staining was stopped by washing in distilled water. Then, images of the strips were photographed using a gel imaging systems camera (Sagecreation, China). after drying in the air.

2.7. Subcellular localization of PcDp1

The full-length ORF of *Pcdp1* was PCR-amplified using gene-specific primers G-Dp1-F and -R containing *Xho* I and *Sal* I sites (Table 1) and cloned in pEGFP-N1 vector (a gift from Jichang Jian from Fisheries College of Guangdong Ocean University) to generate the recombinant expression plasmid Dp1-GFP with green fluorescent protein. COS-7 cells were plated onto 14-mm diameter Poly-D-lysine-coated coverslips (Shanghai Solarbio Bioscience & Technology Co., Ltd., Shanghai, China) placed inside 24-well plates. Twenty-four hours after seeding, cells were transfected with GFP fusion plasmids or pEGFP-N1 empty vectors. Forty-eight hours after transfection, cells on coverslips were washed with 1×PBS three times and fixed in paraformaldehyde for 30 min followed by DAPI staining. Images were taken by the confocal fluorescence microscope (Olympus, FV10i-Oil, Tokyo, Japan).

Subsequently, to analyse tissue localization of PcDp1 in the normal large yellow croaker, immunohistochemical tests were applied as follows on sections of spleen, head kidney, kidney and gill tissue fixed for 24 h in 10% neutral buffered formalin. Firstly, the sections were embedded in paraffin wax and cut into 5 mm sections. Then, the sections were immersed in xylene, dehydrated in graded ethanol, retrieved in boiled water and incubated in 0.01 M Sodium citrate buffer for 30 min. After blocking endogenous peroxidase activity and non-specific staining by sequentially treating sections with 3% H_2O_2/PBS and 1.5% BSA/PBS, sections were incubated with polyclonal antibody of PcDp1 (1:5,000) prepared as in section 2.7 as the first antibody at 4 °C for 24 h. Then, the sections were washed in 1× PBS buffer for three times and incubated for 2 h with an HRP-labeled goat anti-rabbit IgG (1:2,000, Beijing Dingguo Biological Product Co, Ltd, China). Peroxidase activity was visualized by incubation of the sections at room temperature with DAB kit (Beijing Dingguo Biological Product Co, Ltd, China). After redyeing with hematoxylin and differentiating in 0.1% acid alcohol, sections were dehydrated, mounted, observed and photographed using a light microscope.

2.8. The DNA-binding activity of PcDp1

The DNA-binding activity of PcDp1 was analyzed by using electrophoretic mobility shift assay (EMSA). The specific wild probe sequences (WE2Fsite-F and -R) and mutant probe sequences (ME2Fsite-F and -R) of PcDp1 were synthesized and are listed in Table 1. The complementary sequence probe was incubated at 95 °C for 10 min in a 1:1 ratio, and then cooled slowly to 15–25 °C to form the two-stranded DNA probes. The two double-stranded DNA probes were labeled with biotin according to the protocols described in DIG Oligonucleotide 3′ End Labeling Kit (Roche Diagnostics, Germany). In the EMSA, biotin-labeled DNA probes were successively incubated with the purified rPcDp1 protein at room temperature for 15 min and on ice for 25 min in the presence of binding buffer from EMSA/gel-shift kit (Roche Diagnostics, Germany). For competition experiment, the purified rPcDp1 protein was re-incubated with the wild probes (twice) for 30 min before addition of the biotin-labeled probes. After the binding reactions, DNA-protein complexes were resolved by electrophoresis in 6% native acrylamide gel and transferred to a positively charged nylon membrane (Osmonics, USA). The membrane was immediately cross-linked with UV-light for 10 min. Then the membrane was fixed in a film cassette and was visualized by exposure to X-ray film in a dark room at room temperature for 15–25 min.

3. Results

3.1. Sequence characterization and phylogenetic analysis

The full-length cDNA of *Pcdp1* (GenBank accession number: DQ821446) is 1,427 bp long with an open reading frame (ORF) of 1,239 bp encoding a putative protein of 412 amino acids, a 5′-untranslated region of 116 bp, and a 3′-untranslated region of 70 bp including a putative polyadenylation consensus signal (AATAAA) (Figure 1). No signal peptide was found in the deduced protein and the calculated molecular mass of the deduced mature PcDp1 was 45.7 kDa. The evaluation of PcDp1 acidity and alkalinity showed that it had a theoretical isoelectric point of 6.21, and the N-terminal of PcDp1 protein was composed of mostly basic amino acids and the C-terminal was composed of mostly

acidic amino acids (Figure 1). Prediction of protein domains showed that PcDp1 consisted of a DNA-binding domain (DBD) (121–199 aa) which contained a DEF box (173–199 aa), and a dimerization domain (206–344 aa) (Figure 1). Furthermore, multiple alignment of PcDp1 with typical E2F from large yellow croaker showed a highly conserved RRXYD motif located in the DEF box (Fig. S1B).

Homology comparisons indicated that PcDp1 shared the highest sequence identity of 98.55% with *Oreochromis niloticus* Dp1, followed by 88.72% identity with *Danio rerio* Dp1 and a relative low identity of 78.91–80.55% with its mammalian and amphibian counterparts (Fig. S1A). To determine the evolutionary relationship between PcDp1 and that of other species, a phylogenetic tree was constructed using the neighbor-joining method with a bootstrap of 1,000 replications. As shown in Figure 2, all the Dp1 proteins used in this study were grouped into the Aves, Reptile, Mammalian, Amphibian and Teleostei clade. *Pseudosciaena crocea* Dp1 was clustered with *Oreochromis niloticus* and *Takifugu rubripes* Dp1 together, and separating from *Danio rerio* Dp1.

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                                                                                                    ggccaacaacaacagccgtcgaagaa
acaggacagtgtgcactcgggtctaaatagtgacaaatagaagaacattttctttttaacctgaagctgacttctccggtcctttggatc
ATGGCTAAAGATGCTGGTCTGATTGAAACAAATGGAGAGCTGAAGGTTTTCATCGATCAGAATCTAAGCCCAAGCAAAGGTGTCCTATCT
 M  A  K  D  A  G  L  I  E  T  N  G  E  L  K  V  F  I  D  Q  N  L  S  P  S  K  G  V  L  S
TTGGTTACTGTCCAGCCTACAGCTGCCCCCGTAGCCAAACAGCTACTACCCAAAACTCTTGGGCCATCCAATGTGAACATTGCTGCACAC
 L  V  T  V  Q  P  T  A  A  P  V  A  K  Q  L  L  P  K  T  L  G  P  S  N  V  N  I  A  A  H

ATGCAACATGTGCCACACATGGTGATTGGAACACCCCAGAGGCCCACGGTATCGAATACCATTTTGGTAAACAGTCCACACACACCCAGC
 M  Q  H  V  P  H  M  V  I  G  T  P  Q  R  P  T  V  S  N  T  I  L  V  N  S  P  H  T  P  S

TCGCAGTTCCTCACTCAGAGTCAACCATCTGATGCCTCTCCGTGGTCATCAGGGAAACGTGGCAAGAAAGGGGAAAAGAATGGAAAAGGC
 S  Q  F  L  T  Q  S  Q  P  S  D  A  S  P  W  S  S  G  K  R  G  K  K  G  E  K  N  G  K  G

TTGAGGCATTTCTCCATGAAGGTGTGTGAGAAGGTGCAGAAGAAAGGAGTAACCACCTACAATGAAGTGGCAGACGAACTGGTCGCAGAA
 L  R  H  F  S  M  K  V  C  E  K  V  Q  K  K  G  V  T  T  Y  N  E  V  A  D  E  L  V  A  E

TTCAGCTCTTCAGACAACCACATGTCACCCAATGACTCGCACGTGTACGACCAGAAGAACATTAGGCGGCGTGTGTACGACGCACTTAAC
 F  S  S  S  D  N  H  M  S  P  N  D  S  H  V  Y  D  Q  K  N  I  R  R  R  V  Y  D  A  L  N

GTGCTCATGGCCATGAACATTATCTCTAAAGAGAAGAAAGAAATCAAATGGATTGGCCTTCCCACCAACTCAGCGCAAGAGTGCCAAAAC
 V  L  M  A  M  N  I  I  S  K  E  K  K  E  I  K  W  I  G  L  P  T  N  S  A  Q  E  C  Q  N

CTCGAGGTGGAGAGACAAAGGCGGCTAGAGAGGATTAAGCAGAAACAGTCACAGCTTCAGGAGCTCATACTGCAGCAAATAGCTTTTAAG
 L  E  V  E  R  Q  R  R  L  E  R  I  K  Q  K  Q  S  Q  L  Q  E  L  I  L  Q  Q  I  A  F  K

AACCTGGTTCAGCGGAACAGACAGACAGAGCAGCAGGCAAACAGACCTCCACCTCCAAACTCCATCATCCACCTTCCCTTCATTATTGTC
 N  L  V  Q  R  N  R  Q  T  E  Q  Q  A  N  R  P  P  P  P  N  S  I  I  H  L  P  F  I  I  V

AACACCAGCAAGAAAACTGTCATCGACTGCAGCATCTCAAACGACAAGTTCGAGTATTTGTTCAACTTTGACAGCATGTTTGAGATCCAC
 N  T  S  K  K  T  V  I  D  C  S  I  S  N  D  K  F  E  Y  L  F  N  F  D  S  M  F  E  I  H

GATGACATCGAGGTGCTGAAGCGCATGGGCATGGCCTGTGGCTTGGAGGTGGGAAAGTGTTCTCCAGAGGATCTGAAGGTCGCACGTAGC
 D  D  I  E  V  L  K  R  M  G  M  A  C  G  L  E  V  G  K  C  S  P  E  D  L  K  V  A  R  S

CTGGTGCCCAAAGCTCTGGAGCCGTACGTTATAGAGATGGCAAAGGGTCCCATCAGTAACGTCTACATCACTGGAGGGTCCTCTGCCAAC
 L  V  P  K  A  L  E  P  Y  V  I  E  M  A  K  G  P  I  S  N  V  Y  I  T  G  G  S  S  A  N

GGAGCCCGTTACCCTGCAAGTGACGGCTGCACCGACGGCACCATGGCGTCCAGCTCGAATGACTCTCACTACAGCGGCTCTCGCGTGGAG
 G  A  R  Y  P  A  S  D  G  C  T  D  G  T  M  A  S  S  S  N  D  S  H  Y  S  G  S  R  V  E

ACTCCAGTGTCTTATATGGGGGACGACGACGACGAGGACGAGTACGACGAGAACGACGATGAAGACTAAatctgtctacgcctcgccact
 T  P  V  S  Y  M  G  D  D  D  D  E  D  E  Y  D  E  N  D  D  E  D  *

ccaacaagcacagtccttcaataaaaaaaaaaaaaaaaaaaaaaaaaagt
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Figure 1. The full-length cDNA and deduced amino acid sequences of *Pcdp1*. The coding region and untranslated regions are indicated in uppercase and lowercase letters, respectively. The initiation codon (ATG) is shaded, and the stop codon (TAA) is indicated by an asterisk. The polyadenylation signal (AATAAAA) are indicated by the wavy line. The DNA binding domain is bolded, the dimerization domain is underlined, and the DEF box is boxed. Leucine-rich nuclear export signals (NES) are shaded with blue. The acidic region is marked in grey.

3.2. Tissue distribution of Pcdp1 in healthy large yellow croaker

To determine the tissue distribution of *Pcdp1*, real-time PCR was used to analyse the expression levels of *Pcdp1* in healthy large yellow croaker tissues. The results showed that *Pcdp1* is ubiquitously expressed in all analyzed tissues, with the highest level of expression in body kidney, followed by heart, gill, brain, head kidney and liver, weakly in the spleen, and negligibly in the intestine (Figure 3).

3.3. The recombinant protein and polyclonal antibody of PcDp1

The recombinant plasmid pET-His-PcDp1 was transformed into *E. coli* strain BL21 (DE3) and induced by IPTG. As showed in Fig. S1A, bands of approximately 47 kDa were observed after inducing at different concentration (0.2–1 mM) IPTG, in accordance with the predicted molecular mass. To confirm the expression of recombinant protein, affinity chromatography was used to purify the rPcDp1 and Western blot analysis was further performed by using the His-tag monoclonal antibody. The result revealed that a single band of PcDp1 was obtained from the total bacterial protein, and a specific band reacted with His-tag monoclonal antibody, indicating that rPcDp1 protein was expressed (Fig. S2B).

The specificity of anti-PcDp1 antibody was also performed by Western blot, and a high specificity reaction band was observed (Fig. S2B, lane 4). No reaction band appeared when the pre-immune serum was used as negative control (Fig. S2B, lane 3).

3.4. Subcellular localization of PcDp1

Using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) programs and NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/), PcDp1 protein was predicted to have one leucine-rich nuclear export signal (LQELIL, 229–234 aa) (Figure 1). To determine if the large yellow croaker Dp1 was localized to the cytoplasm, we performed an EGFP reporter assay in COS-7 cells and used immunohistochemical section observation in tissues of healthy large yellow croaker. Firstly, COS-7 cells were transfected with plasmid constructs expressing an EGFP-tagged Dp1 protein, and the empty pEGFP-N1 plasmid was used as the negative. As shown in Figure 4A, the PcDp1 was exclusively localized to the cytoplasm of the transfected cells, the same as pEGFP-N1. Subsequently, we further analyzed the tissue localization of PcDp1 in spleen, head kidney, kidney and gills of healthy large yellow croaker by immunohistochemical method using a polyclonal antibody of PcDp1 with the pre-immune rabbit serum as control groups. The results showed that all tissues analyzed in this study observed DAB positive particles in polyclonal antibody of PcDp1 groups, but not in control groups (Figure 4B). It was revealed that PcDp1 is localized predominantly in cytoplasm of parenchymal cells, which is consistent with the result of EGFP-tagged Dp1 protein in COS-7 cell.

3.5. DNA binding activity of PcDp1

To analyse the DNA binding activity of PcDp1 to the E2F site *in vitro*, an EMSA assay was performed. Based on the specific DNA-binding sequence of typical E2F/DP, the oligonucleotide probes of E2F site was synthesized (Table 1) and incubated with purified rPcDp1 *in vitro*. The result showed that PcDp1 can directly bind to the wild DNA sequence of E2F site (Figure 5, lane 2). When the mutant probes were added, the reaction band did not change showing the same result as the wild probes (Figure 5, lane 3), while the reaction band became weaker with the addition of the competition probes (Figure 5, lane 4). The results revealed that recombinant PcDp1 can specifically bind to the DNA sequence of the E2F site, indicating that it was a functionally active

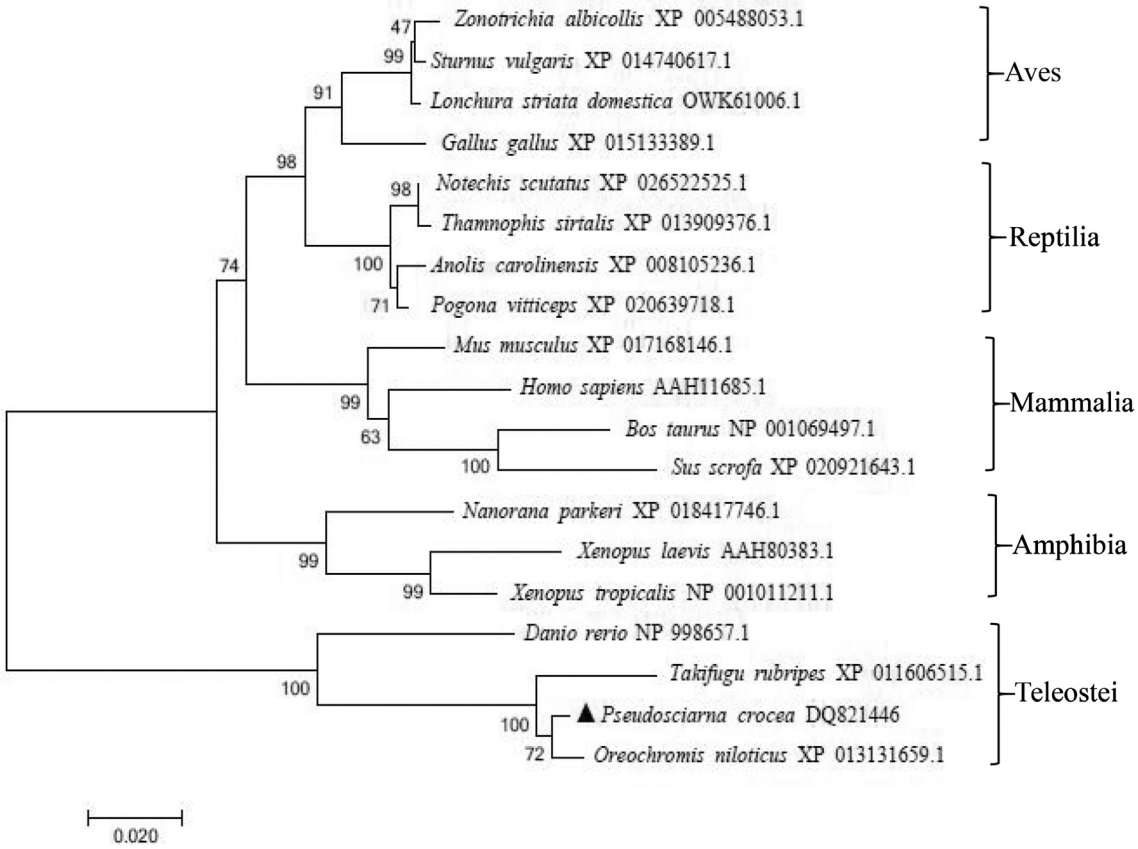

Figure 2. Phylogenetic tree analysis of the full-length amino acid sequences of Dp1 from various species. A phylogenetic tree is constructed by neighbor-joining method using MEGA 7.0 with the bootstrap values of 1,000 replicates. Based on the protein sequence, the relations of different organisms are shown by dendrogram graphically. The scale bar and the branch lengths in terms of genetic distance is denoted below the tree. PcDp1 was marked with a triangle.

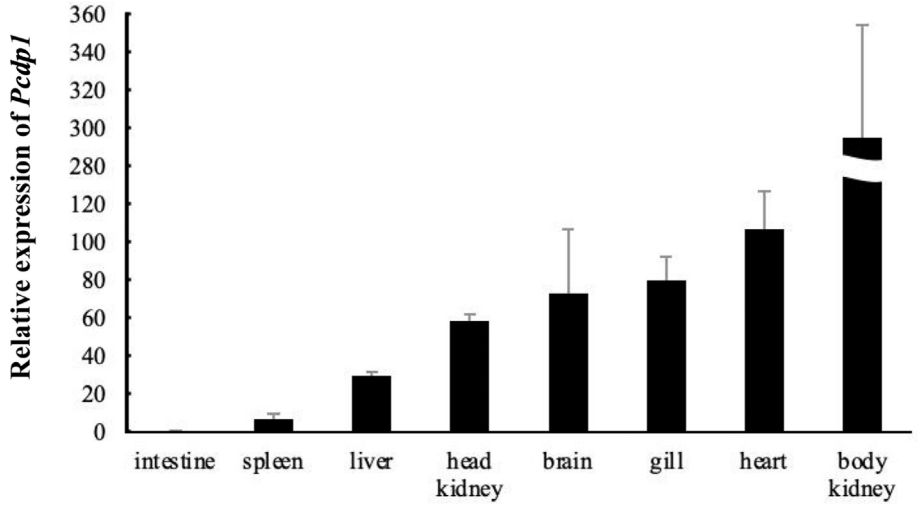

Figure 3. Tissue distribution of *Pcdp1* in healthy large yellow croaker. The ratio refers to the gene expression in different tissues relative to that in intestine, and target gene expression is normalized to β-actin. Each vertical bar represents the Mean±standard deviation (n = 3).

protein, which lays the foundation for further study of its role in transcription regulation.

4. Discussion

The transcription factor Dp1, as a binding partner, often forms a dimerization complex with E2F to play a central role in regulating gene expression during G1/S cell cycle progression (Milton et al., 2006; Ramirez-Parra et al., 2018; Bandara et al., 1993; Helin et al., 1993; Krek et al., 1993; Huber et al., 1993). In this study, a full-length *dp1* cDNA (*Pcdp1*) was successfully cloned and characterized from the large yellow croaker *P. crocea*. Protein domains prediction analysis revealed that PcDp1 possesses two conserved structures characteristic of DP subfamily proteins, including one DNA-binding domain with a DEF box and one dimerization domain (Figure 1). Among them, the DNA-binding domain contributes to dimerization by forming the winged-helix fold (Zheng et al., 1999), and the DEF box, especially the RRXYD motif located in the C-terminal region of the DNA binding domain, is necessary for the formation of a DP/E2F DNA binding heterodimer (Bandara et al., 1993) due to its sequence similarity with the typical E2F family members (Ohtani and Nevins, 1994). Multiple sequence alignment showed that the full-length PcDp1 shared a very high sequence identity with other species, and the DEF box sequence displayed 100% similarity with other teleosts, mammals and amphibians (Figure 3) and showed a highly conserved RRXYD motif located in the DEF box comparing with E2F1-6 of larger yellow croaker (Fig. S1B), suggesting that Dp1 protein were conserved in evolution and thus might have similar function of Dp1 among these species. Notably, like the other known DP proteins, PcDp1 has an acidic region at the *C terminus* (Fig. S1A). While it is generally believed that Dp1 facilitates the typical E2Fs binding to target DNA and does not possess a transactivation domain. Okuda et al. (2016) confirmed that the acidic region of human Dp1 could act as a transactivation domain to regulate the transcriptional activity of the E2F1/Dp1 heterodimer by anchoring its Phe403 into a pocket in the plekstrin homology (PH) domain of p62 subunit of TFIIH. However, we found that the Phe403 of human Dp1 has been replaced by Tyr405 in the acidic region of large yellow croaker, and Glu395 of human Dp1 is missing in that of large yellow croaker (Fig. S1A), whether that will affect the transcription activity of the acidic region of PcDp1 needs further investigation.

Accumulated evidence suggests that *dp1* is ubiquitously expressed at high levels in tissues and in cell lines (Wu et al., 1995; Chang et al., 2004; Gopalkrishnan et al., 1996), which is consistent with the idea that Dp1 fulfills a central function in the development and physiology of many tissues. For example, Dp1 is required for extraembryonic development, and *dp1* null mice die at approximately 10 days of gestation because of abnormalities in trophectoderm-derived tissues (Kohn et al., 2003). In addition, the high expression of *dp1* in the ectoderm and all epidermal layers during embryogenesis determines its necessity for Keratinocyte growth and epidermal stratification (Yasui et al., 2003). Over-expressed *dp1* was analyzed in hepatocellular carcinomas, indicating it can promote growth of the tumor cells and show significant correlation with large tumor size (≥5cm) (Yasui et al., 2003). In this study, *Pcdp1* was also ubiquitously expressed in all analyzed tissues, with the highest level of expression in body kidney, followed by heart, gill, brain, head kidney and liver, weakly in the spleen and intestine (Figure 2). It is noteworthy that the mRNA expression level of *Pcdp1* is about 305 fold higher in body kidney than in intestine. Furthermore, the moderate expression level of *Pcdp1* were mainly analyzed in several immune-related tissues, including gill, head kidney and liver. Given these findings, we speculated that PcDp1 might play an important role in osmotic pressure regulation and immune response of large yellow croaker.

Subsequently, the subcellular localization of PcDp1 was observed by the EGFP reporter assay in COS-7 cells and immunohistochemical section observation in tissues of healthy large yellow croaker. The results showed that PcDp1 is mainly distributed in the cytoplasm both in COS-7 cell and the parenchymal cells of large yellow croaker spleen, head kidney and kidney tissues. However, in the early neurula *Xenopus* embryo, Dp1 was not only distributed throughout the cell cytoplasm in the cranial region, but also displayed nuclear localization in the trunk region of dorsal explants (Kim et al., 2012). More importantly, as the heterodimer partner, the distribution of human Dp1 was determined by the specific E2F/DP heterodimers (Magae et al., 1996). In transfected Chinese hamster and human cells, cytoplasmic human Dp1 would accumulate in the nucleus when it was coexpressed with E2F1, E2F2, or E2F3. Similar results were observed by Datta et al. (2005). The tumor suppressor protein ARF could also relocate Dp1 from the cytoplasm to the nucleus to participate in the regulation of the cell cycle. The differential nucleocytoplasmic localizations of Dp1 might contribute to its central function in the development and physiology of many tissues. For example, nuclear Dp1 is only sufficient to the activation of Wnt/β-catenin signaling during posterior neural development whereas cytoplasmic Dp1 is important for the inhibition of signaling during anterior neural development (Kim et al., 2012). In addition, it has been confirmed that nuclear heterodimer of E2F1/Dp1, E2F2/Dp1, or E2F3/Dp1 and the cytoplasmic heterodimer of E2F4/Dp1, E2F5/Dp1 had been categorized as activators and repressors according to their regulation on the target gene transcription during the cell cycle (Müller et al., 1997). Thus, the cytoplasmic localization of PcDp1 might be due to the fact that the analyzed tissues in this study are highly

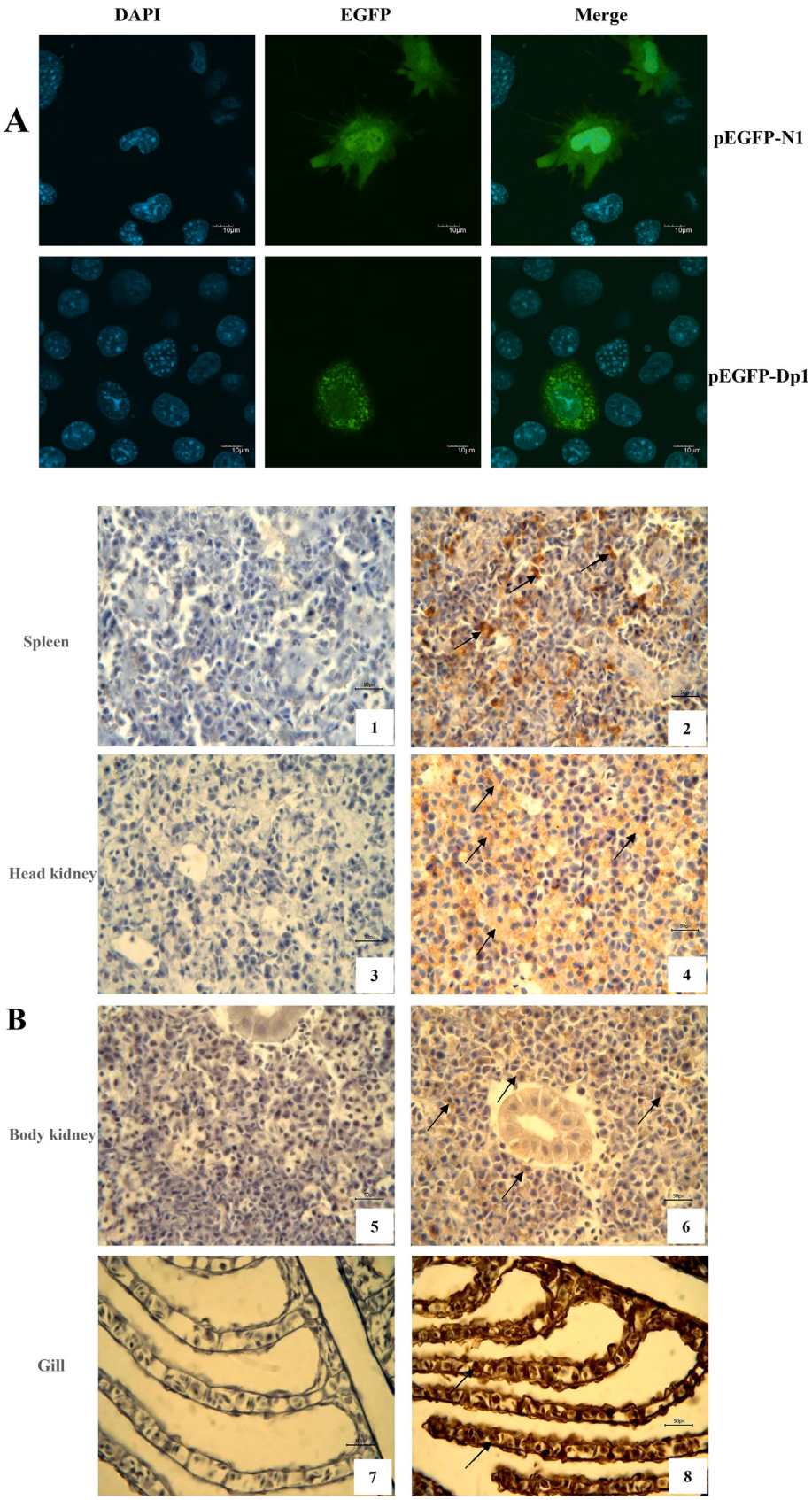

Figure 4. Subcellular localization of PcDp1. (A) Subcellular colocalization of PcDp1 protein analyzed by a GFP reporter assay. COS-7 cells were transfected with GFP reporter constructs expressing an EGFP-tagged Dp1. Empty pEGFP-N1 vector was used as a control. Nuclear DNA was stained with DAPI and cells were analyzed with a fluorescence confocal microscope. Scale bars, 10 μm. (B) Immunohistochemical localization (chocolate brown) of PcDp1 in tissues of health large yellow croaker. The specimens were fixed in 10% buffered formalin, paraffin embedded and sectioned. After antigen retrieval, sections were stained with rabbit polyclonal PcDp1 antisera or control sera, followed by HRP-coupled secondary Ab and visualized by the DAB method. Sections were visualized at 10×100 magnification. The specificity of the PcDp1 staining was confirmed by negative staining patterns observed with the normal rabbit serum staining. Note: 1,2-sections of spleen; 3,4-sections of head kidney; 5,6-sections of kidney; 7,8-sections of gills; 1,3,5,7-control sections; 2,4,6,8-positive sections. Arrows indicate areas of positive reaction in cytoplasm.

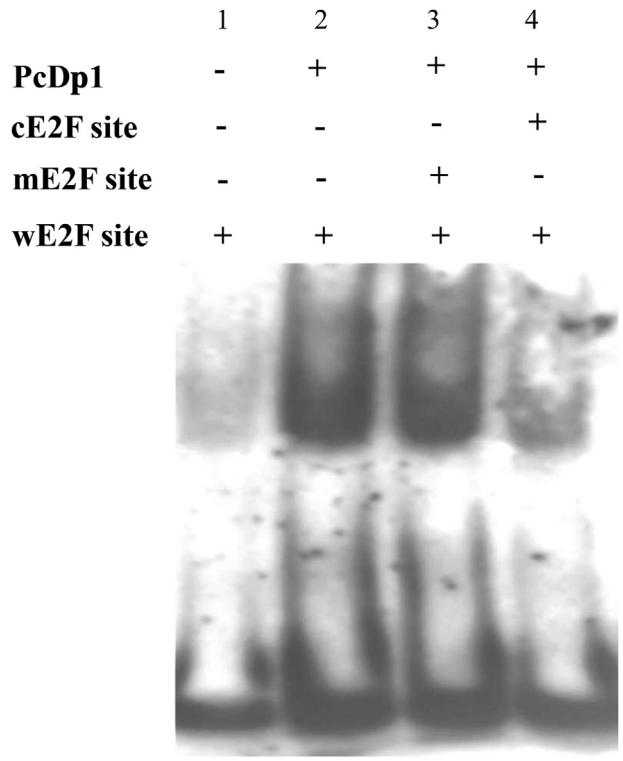

Figure 5. DNA-binding analysis of recombinant PcDp1 protein by EMSA. The purified recombinant PcDp1 protein was incubated with the DIG-labeled probe containing wild-type E2F binding site (wE2F), mutant binding E2F site (mE2F), or competition binding E2F site (cE2F). Complexes were separated on an 8% native PAGE and transferred onto a nylon+ membrane. Lane 1: negative group (without PcDp1 protein); Lane 2: wild E2F site group (PcDp1 protein with wild biotinylated E2F site); Lane 3: mutant E2F site group (PcDp1 protein with wild and mutant biotinylated E2F site); Lane 4: competition E2F site group (PcDp1 protein with wild and competition biotinylated E2F site).

differentiated organs and the COS-7 cells were analyzed after transfection at 2 days when the cells were in quiescence.

As the cell cycle-regulated transcription factor, the DP proteins in complex with typical E2Fs have been reported to regulate G1/S phase transition by binding to the a specific promoter sequence of target gene, termed as E2F site (5′- TTTc/gGCGCc/g-3′) (Lees et al., 1993; Buck et al., 1995; Zhang and Chellappan, 1995). On the other hand, previous studies have also confirmed that Dp1 itself can bind specific DNA sequences, although its binding activity is far lower than the heterodimer E2F/DP (Girling et al., 1993). Furthermore, Dp1 on its own was found to directly associate with other proteins or participate in cell pathways. For example, p53 can also compete for binding to Dp1 with E2F1, which results in decreased E2F/DP DNA binding (Sorensen et al., 1996). Moreover, inactivation of Dp1 could result in mRNA induction for *p53* and p21Waf1/Cip, thereby augmenting senescence in human somatic cells (Ohdaira et al., 2012). In the present study, the purified recombinant PcDp1 could directly bind wild E2F site (5′-GAGATGACGTAG**TTTTCGCGC**GAAACTAT-3′), but not mutant E2F site (5′-GAGATGACGTAG**TTTTCG*ATC***GAAACTAT-3′) *in vitro*, implying that PcDp1 on its own could act as DNA-binding protein to recognize the core sequence TTTTCGCGC in wild E2F site. However, which gene promoter region contains E2F site sequence of large yellow croaker and whether it can be regulated by PcDp1? Maybe the genome of large yellow croaker *P. crocea* in the GenBank database will help provide further insight into this problem.

In conclusion, a large yellow croaker *DRTF1 polypeptide 1* (*Pcdp1*) was identified and characterized from large yellow croaker *P. crocea*. The amino acid sequence of PcDp1 possessed a DNA-binding domain (DBD) which contains a DEF box, a dimerization domain, and an acidic region at the *C terminus* with transcription activity. *Pcdp1* was ubiquitously expressed in all analyzed tissues, with the highest level of expression in body kidney. The subcellular localization of PcDp1 showed that it is mainly distributed in the cytoplasm both in COS-7 cell and the parenchymal cells of large yellow croaker spleen, head kidney and kidney tissues. The recombinant PcDp1 exhibited binding activity to E2F site in vitro. These results are significant in further understanding of the regulatory mechanism of Dp1 in teleosts.

Declarations

Author contribution statement

Xinzhong Wu: Conceived and designed the experiments.

Xiaohui Cai: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yanqing Huang: Performed the experiments; Analyzed and interpreted the data.

Honglin Chen, Qiancheng Qi, Meijuan Xu, Peng Xu: Performed the experiments.

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Data availability statement

Data associated with this study has been deposited at GenBank under the accession number DQ821446.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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