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FOXO1A promotes neuropeptide FF transcription subsequently regulating the expression of feeding-related genes in spotted sea bass (*Lateolabrax maculatus*)

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ABSTRACT

FOXOs belong to the forkhead transcription factor superfamily, several of which are suggested to be involved in the control of food intake. Previously, we proved that the neuropeptide FF (NPFF) peptide was involved in feeding regulation in spotted sea bass. In the present study, seven members of the *foxo* family were identified in the whole genome of spotted sea bass. The distributions of these genes in different tissues were analyzed by qRT-PCR. Variations in the *foxo1a* and *npff* expression profiles during short-term starvation showed similar expression patterns. The colocalization of *foxo1a* and *npff* in the telencephalon, hypothalamus, stomach and intestine further provided evidence that *foxo1a* may act directly to promote the transcription of *npff*. Thirteen predicted FOXO1 binding sites were found in the 5′ upstream region of *npff*. Luciferase assay results showed that FOXO1A was able to activate *npff* transcriptional responses by directly binding DNA response elements, and the key regulatory areas and sites of FOXO1A on the *npff* promoter were confirmed by deletion and site-directed mutagenesis analyses. These findings may help to elucidate the role of FOXO1 in the regulation of feeding processes in teleosts.

1. Introduction

The transcription factor FOXO (FOXO1, FOXO3, FOXO4 and FOXO6), homologous to *Caenorhabditis elegans* protein DAF-16, which belongs to the O ('other') class of the FOX superfamily (Barthel et al., 2005; Kaestner et al., 2000), is involved in various signaling pathways and controls a wide range of biochemical processes, including cellular differentiation, tumor suppression, metabolism, cell cycle arrest, cell death, and protection from stress (Barthel et al., 2005; Accili and Arden, 2004; Brunet and Greer, 2005). Under starvation or low glucose conditions, *foxo1* is activated to alter metabolism in a way that ensures glucose homeostasis (Kousteni, 2012). FOXO1 was also found to affect the fasting response through changes in the central nervous system, thereby influencing both behavior and metabolism (Ren et al., 2012). In the structure of FOXOs, the third α-helix (H3) plays the most important role in the DNA-binding specificity of a winged helix/forkhead protein (Clark et al., 1993). In addition, both winged loops also make important interactions with DNA (Clark et al., 1993; Boura et al., 2007). As transcription factors, FOXO1 regulates the transcription of genes by binding to its response element. To date, three different DNA elements bound to the FOXO1 crystal structure have been described in many studies. The insulin response element (IRE) with the consensus sequence of TT(G/A)TTTTG was the first recognition element reported for FOXO1 (Guo et al., 1999; Tang et al., 1999) and the Daf-16 family binding element (DBE) with the consensus sequence of TT(G/A)TTTAC, which is bound by FOXO1 more strongly (Furuyama et al., 2000). In addition, FOXO1 DBD bound to a higher affinity DBE sequence containing the same 8-base consensus sequence as DBE DNA, while the 3′ flanking sequence is two bases longer and has been replaced with the 5′-ATTTTG-3′ sequence (Brent et al., 2008).

FOXO1, originally named FKHR, or forkhead in rhabdomyosarcomas, and its close paralogs (*Foxo3*, *Foxo4* and *Foxo6*) were thought to have some degree of functional diversification during development (Hosaka et al., 2004; Arden, 2007). In mouse, *POMC* and *Agrp* genes were suggested to be direct targets of FOXO1 in the arcuate nucleus of the hypothalamus (ARH) (Kitamura et al., 2006; Kim et al., 2006). Furthermore, FOXO1 was able to regulate AGRP neurons in the hypothalamus and the sensitivity of leptin (Plum et al., 2009; Cao et al., 2011). *Foxo1* was also highly expressed in many other hypothalamic nuclei, including the dorsomedial nucleus of the hypothalamus (DMH) and the ventral medial nucleus of the hypothalamus (VMH) (Kitamura et al., 2006; Kim et al., 2006), which has long been of interest as a site

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regulating body weight (Dhillon et al., 2006; Bingham et al., 2008). Taken together, these findings indicate that FOXO1 is the key regulator of energy balance and glucose homeostasis. However, in teleosts, the target genes mediating the effects of FOXO1 have not been determined.

Neuropeptide FF (NPFF), originally isolated from the bovine brain (Yang et al., 1985), is highly detected in the central nervous system (CNS), especially in the hypothalamus, posterior pituitary, medulla and spinal cord (Kivipelto et al., 2010; Panula et al., 1996). *NPFF* neurons are located in the feeding regulation area of the dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH) (Jhamandas et al., 2001). In vertebrates, NPFF can regulate many physiological functions, such as the analgesic effect of morphine (Yang et al., 1985), body temperature (Zajaca and Desprata, 1997), blood pressure (Allard et al., 1995), gastrointestinal motility (Decker et al., 1997), epilepsy (Mollereau et al., 2002) and insulin secretion (Minault et al., 1994). More evidence has proven that NPFF can maintain energy homeostasis by effectively reducing food intake and increasing water intake in rats (Murase et al., 1996; Sunter et al., 2001).

Previously, we identified the *npff* and three receptor genes from spotted sea bass and localized the *npff* to the brain. Using static incubation, we also proved that the conserved NPFF peptide may play a stimulating role in feeding control of spotted sea bass (Li et al. 2019). In the present study, by detecting the expression pattern of both *foxo*s and *npff* during starvation and colocalization of *foxo1a* and *npff* to the same cells in different areas in the brain, stomach and intestine, we hypothesized that *foxo1a* could act directly to promote the transcription of *npff*. Employing a luciferase assay, we further confirmed the binding site of FOXO1a to the promoter of *npff*.

2. Materials and methods

2.1. Animals and short-term starvation

One hundred healthy spotted sea bass weighing 100.0 ± 5.0 g were kept in the cement pool for two weeks before the experiments. Fish received a commercial diet twice per day. Samples for short-term starvation were collected at 0, 1, 6, 12, 24, 48 and 72 h following a meal, with 0 h serving as the control group. Three fish were randomly sampled at different time courses and decapitated after anesthetization with MS-222 (200 mg/L). Brains, intestines, and stomachs were quickly dissected, frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. To determine the expression profile of *foxo*s, telencephalon, hypothalamus, cerebellum, midbrain, medulla, pituitary, stomach, intestine, gonad and muscle samples were collected from three adult spotted sea bass. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China.

2.2. Identification and sequence analyses of foxo genes in spotted sea bass

To identify *foxo* genes in spotted sea bass, the reference genome (PRJNA407434) and transcriptomic database (SRR4409341, SRR4409397) were searched by TBLASTN using sequences of *Foxo* genes from human (*Homo sapiens*) and zebrafish (*Danio rerio*) retrieved from GenBank (NCBI) as queries with a cutoff E-value of 1e-5. Then, a unique set of sequences was retained after removing the repeated entries for further analysis. Open reading frames (ORFs) of spotted sea bass *foxos* were predicted by ORF Finder (https://www.ncbi.nlm.nih.gov/gorf/gorf.html) and further validated by BLASTP against the NCBI nonredundant protein database (nr).

To further confirm the *foxo*s gene sequence, gene cloning and sequencing were performed. All primers (Table 1) for ORF cloning were designed using Premier 5 software. For PCR, 2 × Taq PCR Master Mix (Tiangen, China) was used according to the manufacturer's instructions. The purification product was subcloned into the PEASY-T1 vector (Tiangen, China), and seven different individual positive clones were confirmed by sequencing (Beijing Genomics Institute, China).

Phylogenetic analysis was conducted using the amino acid sequences of *foxo* genes from spotted sea bass (*Lateolabrax maculatus*) and several representative vertebrates retrieved from NCBI, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), takifugu rubripes (*takifugu rubripes*), large yellow croaker (*Larimichthys crocea*), Atlantic salmon (*Salmo salar*), Atlantic herring (*Clupea harengus*) and spotted gar (*Lepisosteus oculatus*). Multiple amino acid sequences were aligned by the Clustal W2 program (https://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters. The phylogenetic tree was constructed by MEGA 6 software using the neighbor-joining (NJ) method with 1000 bootstrap replicates.

The physicochemical properties and structure of FOXO proteins were analyzed by bioinformatic methods. The chemicophysical properties of predicted FOXO proteins, including the number of amino acids, molecular weight (MW, kDa) and theoretical isoelectric point (pI), were calculated by the ProtParam tool (http://web.expasy.org/protparam/). The conserved domains were identified and predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/). The conserved motifs of FOXO proteins were observed with MEME Suite 5.0.4 (http://alternate.meme-suite.org/). The gene structures of *foxo*s were visualized using TBtools software (https://github.com/CJ-Chen/TBtools). Furthermore, the three-dimensional protein structures of spotted sea bass *foxos* were systematically constructed by Swiss Model software (http://swissmodel.expasy.org/), and all structure figures were prepared with PyMol software.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the collected samples of spotted sea bass using TRIzol Reagent (Invitrogen, USA). The total RNA concentration and purity were measured using a UV spectrophotometer (ChampGel 5000, China). A total of 1 μg of total RNA was used as a template for reverse transcription using a two-step method with a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan). The cDNA samples were subsequently used for the determination of *foxo1a*, *foxo1b*, *foxo3a*, *foxo3b*, *foxo4*, *foxo6a*, *foxo6b* and *npff* gene expression by qRT-PCR. All gene-specific primers used in this study are listed in Table 1. qRT-PCR was performed using TB Green™ II Premix Ex Taq™ GC (Perfect Real Time) (Takara, Japan) with a reaction mixture containing 10 μL of TB Green™ Premix (2 ×), 2 μL of template, 6.8 μL of sterilized distilled water, 0.4 μL of ROX and 0.4 μL of forward and reverse primer. The template was amplified at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s and extension at 72 °C for 30 s. The *18S* rRNA gene was used as the internal reference for qRT-PCR normalization.

2.4. Dual-fluorescence in situ hybridization of npff and foxo1a

To confirm the direct regulation of FOXO1A on *npff* expression, dual-fluorescence *in situ* hybridization was performed according to our previous research (Qi et al., 2017). Briefly, brain, intestine and stomach tissues were collected and fixed in 4% paraformaldehyde/PBS for 24 h. For dual-fluorescence *in situ* hybridization, we used a mixture of digoxigenin-labeled *npff* probe and fluorescein-labeled *foxo1a* probe. After the hybridization and posthybridization steps, the sections were washed and blocked with blocking reagent (Roche Diagnostics, Mannheim, Germany). Slides were incubated for 1 h with a horseradish peroxidase-conjugated anti-fluorescein antibody or anti-digoxigenin (diluted 1:500 with blocking reagent; Roche Diagnostics) for *foxo1a* or *npff* detection, respectively. Sections were incubated with tyramide kits with Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen, Carlsbad, CA) after two rinses with PBS for 5 min. Between two different antibody incubations, sections were incubated with an overdose of H_2O_2 for 20 min to inactivate all the horseradish peroxidase (0.3% H_2O_2 in PBS) to avoid the influence of the first detection. The detection reaction was

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)
Primers for qRT-PCR	
npff-qRT-F	CTGCGGTGGTGACTCTTCTGG
npff-qRT-R	ATCCTCCGACATTACCTGCCC
foxo1a-qRT-F	ACAGCAAGTTCACCAAGAGCAGAG
foxo1a-qRT-R	CGAGAGCAAGTTCAGGTTGTCCAG
foxo1b-qRT-F	GCTGGAGGAAGGCTACGAGGAG
foxo1b-qRT-R	GGAGGAGGAGGAGGAGGAGGAG
foxo3a-qRT-F	TTCTTCTAACGGTGGTGGCAATGG
foxo3a-qRT-R	CAGTCGCAGCAGCATCACCTC
foxo3b-qRT-F	TGAGGTGCCTGACGACGACTC
foxo3b-qRT-R	GTGGTCTGCTTGTTCTCCTGGATG
foxo4-qRT-F	TGGACAGGAGGACGACGACAAC
foxo4-qRT-R	TGATGGAAGGAGGAGAAGCTGGAG
foxo6a-qRT-F	AAGGCGACAGCAACAGTTCAGC
foxo6a-qRT-R	CAGTGTGGAGGCAGAGGAGGAG
foxo6b-qRT-F	GCCTCGCTCACAACGGTCAC
foxo6b-qRT-F	ATGTCCAGGTCAGCAGGTAGTCTC
Primers for ORF cloning	
foxo1a-ORF-F	ATGTCATATGCAGACCTC
foxo1a-ORF-R	CTACCCTGACACCCAGCTGT
foxo1b-ORF-F	ATGGCCGAGGCGCCGCCGCC
foxo1b-ORF-R	CTAGCCTGACACCCAGCTGT
foxo3a-ORF-F	ATGATGGCCGAGGCTCCGCT
foxo3a-ORF-R	TCAGCCGGGTACCCGGCTCT
foxo3b-ORF-F	ATGGCTGAGGCGCCGCGCGAC
foxo3b-ORF-R	TCAGCTTGGGACCCAGCTGT
foxo4-ORF-F	ATGGAGGATTCGTCGGTG
foxo4-ORF-R	TTAGCTGGGCACCCAGCTG
foxo6a-ORF-F	ATGCTGATGATGATGAAGAAC
foxo6a-ORF-R	TGGTGGTTGTTGTGGTAGTAG
foxo6b-ORF-F	ATGTTGATGATGGAGGACGAC
foxo6b-ORF-R	TCAGCCAGGCACCCAGCTCTG
Primers for in situ hybridization probe preparation	
npff-ish-F	CGCATTTAGGTGACACTATAGAAGCGCTGCGGTGGTGACTCTTCTGG
npff-ish-R	CGTAATACGACTCACTATAGGGAGACACGACATTACCTGCCCTCT
foxo1a-ish-F	CGCATTTAGGTGACACTATAGAAGCGACAGCAAGTTCACCAAGAGCAGAG
foxo1a-ish-R	CCGTAATACGACTCACTATAGGGAGACAAGGGTGTTCATTCCTGCTT
Primers for construction of report plasmids	
npff-2074-F	CGGGGTACCTTAGAGGTGCTGGTAGATG
npff-2074-R	CCGCTCGAGTGTGAAGAGCCTGACTGA
npff-1874-F	CGGGGTACCTTGAAACACCCCTGAATGC
npff-1718-R	CGGGGTACCTTGTATTAGACTATACAGG
npff-302-F	CGGGGTACCTGGTTAAGTCTCATTTTAG
npff-56-F	CGGGGTACCATCTCCCTCGGCCTTGGTC
Foxo1a-ORF-F	CCCAAGCTTATGTCATATGCAGACCTCATAACC
Foxo1a-ORF-R	TGCTCTAGACTACCCTGACACCCAGCT
Primers for site-directed mutagenesis	
npff-302MU1-F	GAGCCGAGCCAAGTTGGACGGTGTAATC
npff-302MU1-R	GATTACACCGTCCAACTTGGCTCGGCTC
npff-302MU2-F	AGGTGTAATCGGATGGACGAAATCTCC
npff-302MU2-R	GGAGATTTCGTCCATCCGATTACACCT
Reference gene	
18s-F	GGGTCCGAAGCGTTTACT
18s-R	TCACCTCTAGCGGCACAA

stopped by washing in PBS containing 0.5 mM EDTA. Sections were coverslipped with ProLong Gold antifade mountant with DAPI (Invitrogen, Carlsbad, CA). Fluorescence was observed under a Leica TCS SP2 laser-scanning confocal microscope (Leica, Wetzlar, Germany).

2.5. Cloning and sequence analysis of the promoter of the spotted sea bass *npff* gene

The 5′-flanking regions of *npff* were predicted from the whole genome sequence database and confirmed by PCR using Fastpfu DNA polymerase (TransGen, China). The cycling protocol was initial denaturation at 95 °C for 2 min; 35 cycles of denaturation for 20 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C; and a final elongation of 5 min at 72 °C. The 2074-bp fragment was obtained from a pair of primers containing two restriction enzyme cutting sites, namely, KpnI and XhoI, and the PCR product was digested with KpnI and XhoI restriction endonucleases (New England Biolabs, USA). The digested products were purified and then subcloned into the KpnI and XhoI sites of the pGL3-basic vector (Promega, USA). The constructs were used as templates to generate a series of deletion mutant constructs of the *npff* promoters. For the ORF of *foxo1a*, primers containing restriction sites of HindIII and XbaI were used to construct expression plasmids through ligation to

Table 2
Characteristics of *foxo* genes identified in spotted sea bass.

Gene name	Gene ID	Cds-length (bp)	Protein Length (aa)	Exon	Molecular Weight (kDa)	pI	Accession number
foxo1a	evm.model.scaffold_16.177	1524	507	3	54.146	6.55	MN296401
foxo1b	evm.model.scaffold_26.48	2025	674	4	73.944	6.81	MN296402
foxo3a	evm.model.scaffold_1.362	2016	671	4	70.320	5.02	MN296403
foxo3b	evm.model.scaffold_79.91	1860	619	2	65.426	5.06	MN296404
foxo4	evm.model.scaffold_109.37	1998	665	3	69.958	5.66	MN296405
foxo6a	evm.model.scaffold_95.33	1974	657	2	71.362	8.72	MN296406
foxo6b	evm.model.scaffold_24.180	2193	730	2	78.228	6.87	MN296407

Table 3
Copy number of *foxo* genes among a variety of vertebrate species.

Gene name	Human	Mouse	Chicken	Zebrafish	Torafugu	Large yellow croaker	Japanese medaka	Rainbow trout	spotted gar	Spotted Sea bass
Foxo1	1	1	1	2	1	1	1	1	1	2
Foxo3	1	1	1	3	1	1	1	1	1	2
Foxo4	1	1	1	1	1	1	0	1	0	1
Foxo6	1	1	1	2	1	1	1	1	1	2
Total	4	4	4	8	4	4	3	4	3	7

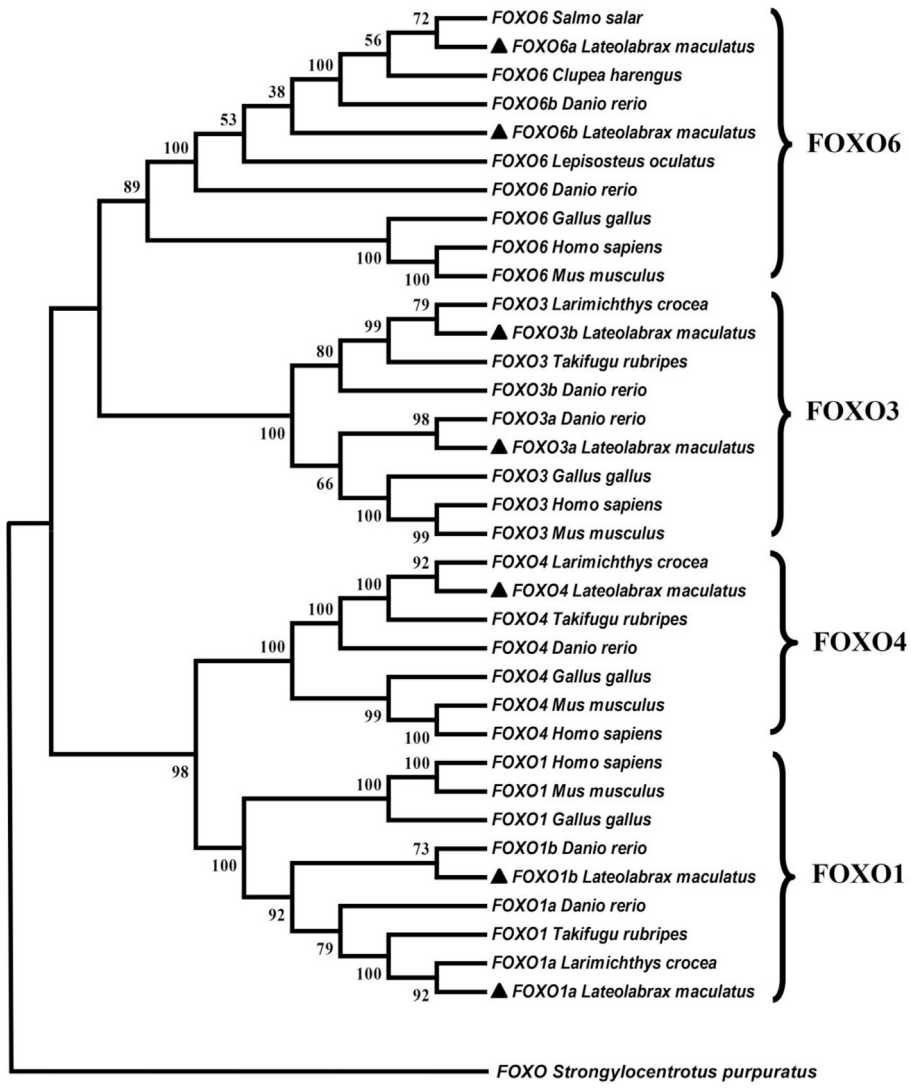

Fig. 1. Phylogenetic analysis of spotted sea bass *foxo* genes. The phylogenetic tree was constructed by the amino acid sequences from several representative mammals and teleosts with 1000 bootstrap replications in MEGA 6. The *foxo* genes of spotted sea bass are labeled with black triangles. The phylogenetic tree was divided into four separate clades (FOXO1, FOXO3, FOXO4 and FOXO6) with covered lines.

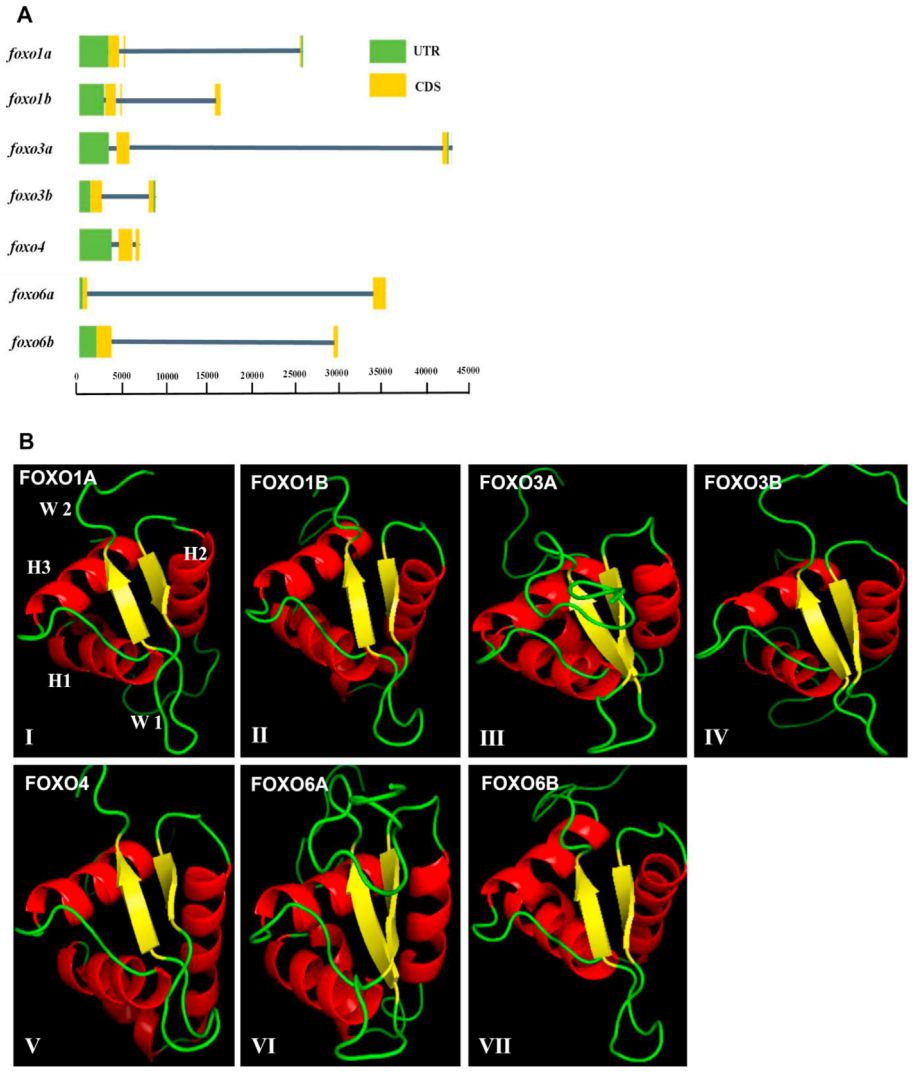

Fig. 2. Gene and tertiary structures of spotted sea bass *foxo* genes. (A) Gene structures; the yellow boxes, black lines and green boxes indicate exons, introns and untranslated regions (UTRs), respectively; (B) Tertiary structures. The wings are denoted by W1 and W2; the helixes are named H1, H2 and H3. All structure figures were prepared with PyMol.

a similarly restricted pcDNA3.1(+) vector (Invitrogen, UK). All constructs were verified by sequencing, and all plasmid DNA used in the present study was prepared using the EndoFree Mini Plasmid Kit II (Tiangen, China). The primer sequences used are listed in Table 1.

2.6. Cell culture, transfection and dual-luciferase reporter assays

Prior to transfection, 293T cells were maintained at 37 °C in DMEM (SparkJade, China) supplemented with 10% fetal bovine serum (FBS) (BioInd, Israel). Sixteen hours before transfection, 1×10^5 cells/well were seeded into 24-well tissue culture plates. Then, 1000 ng of *npff* promoter construct of different lengths in pGL3-basic, 1000 ng of pcDNA3.1-*foxo1a*, and 100 ng of pRL-TK (to normalize transfection efficiency) containing Renilla luciferase were transiently cotransfected into the cells in 750 ml of serum-free medium using XfectTM Polymer (Takara, Japan). Four hours after transfection, the medium was aspirated and replaced with complete growth medium. After 48 h, cells were collected, and luciferase activity assays were carried out using a Dual-Luciferase kit (Promega, USA). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as the fold increase relative to the activity of the promoterless pGL3-basic vector.

2.7. Site-directed mutagenesis

Mutations of putative FOXO1 binding sites of the *npff* promoter were carried out by PCR using Fastpfu DNA polymerase (TransGen, China), and the primer sequences are listed in Table 1. The primers containing two restriction enzyme cutting sites and the desired point mutations were used to carry out PCR amplification of the fragments upstream (primers npff-2074-F/npff-302MU1-R or npff-2074-F/npff-302MU2-R) and downstream (primers npff-302MU1-F/npff-2074-R or npff-302MU2-F/npff-2074-R) of the point mutations. The conditions of the first round of PCR were denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s with a final extension step at 72 °C for 5 min. The amplified fragments were purified using the TIANgel Midi Purification Kit (Tiangen, China), placed in the same tube and amplified in a second PCR step using primers npff-2074-F/npff-2074-R or npff-302-F/npff-2074-R only (Table 1). The second round PCR was first performed at elongation for 10 min at 72 °C. Primers were added at this point, and PCR continued for an additional 35 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s with a final extension step at 72 °C for 5 min. The fragments of npff-2074MU1, npff-2074MU2, npff-302MU1 and npff-302MU2 were gel electrophoresed, isolated and extracted using the TIANgel Midi Purification Kit (Tiangen, China). Purified fragments were digested with the appropriate

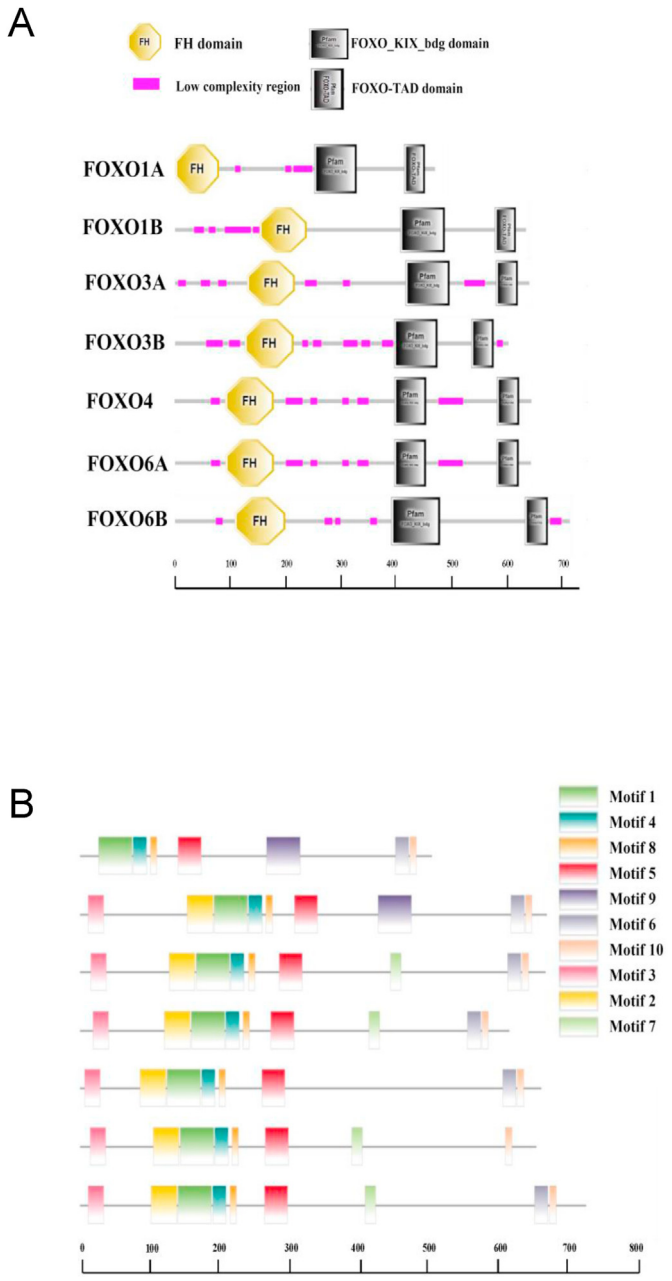

Fig. 3. Homeodomain and motif analyses of spotted sea bass Foxo proteins. The domain of each Foxo protein was analyzed by the SMART analysis service. Ten typical motifs in the Foxo proteins were obtained by the MEME database. The width regions of each motif were permitted between 6 and 50 amino acids. Different color blocks represent different motifs. The bottom line and the numbers indicate the length of Foxo amino acid sequences.

restriction endonucleases and inserted into a pGL3-basic vector. The resultant plasmids containing the inserted fragments were identical to the original DNA sequence, except for the engineered point mutations. The mutated constructs were confirmed by sequencing.

2.8. Statistical analysis

All data are shown as the mean ± SEM. Statistical analysis was carried out using SPSS software version 20.0. One-way ANOVA followed by Duncan's multiple range test and Fisher's least significance difference (LSD) test was used to identify significant differences. Any difference with a P value < 0.05 was considered to be significant.

3. Results

3.1. Identification, sequence analysis and phylogenetic analysis of foxo genes in spotted sea bass

Seven *foxo* genes were identified from the spotted sea bass genome database, including *foxo1a*, *foxo1b*, *foxo3a*, *foxo3b*, *foxo4*, *foxo6a* and *foxo6b*. All *foxo* cDNA sequences were submitted to GenBank, and their accession numbers and protein characteristics are presented in Table 2. Copy numbers of the *foxo* genes in the spotted sea bass genome were compared with model animals (human, mouse, chicken and zebrafish) and several teleost species (Table 3). Human, mouse and chicken had 4 *Foxo* members (*Foxo1*, *Foxo3*, *Foxo4*, *Foxo6*), and each *Foxo* gene contained only one copy. In contrast, multiple copies were identified for several *foxo* members in zebrafish and spotted sea bass; meanwhile, each *foxo* gene was only identified as one copy in the other representative teleosts that we investigated. In addition, *foxo4* was not found in Japanese medaka or spotted gar.

Phylogenetic analyses (Fig. 1) revealed that the spotted sea bass *foxos* remain largely conserved compared with other vertebrates and clustered with their respective counterparts, as expected, and 4 clades were generated (FOXO1, FOXO3, FOXO4, and FOXO6). As a result, the phylogenetic analysis further confirmed the annotation of spotted sea bass *foxo* genes. In general, *foxo* genes were relatively conserved during evolutionary history.

3.2. Gene structure and tertiary structure analysis

To obtain further insights into the structural diversity of the *foxo* genes in spotted sea bass, exon-intron analysis and 3D protein structure analysis were performed. In Fig. 2A, *foxo1a* and *foxo4* contained 3 exons, *foxo1b* and *foxo3a* had four exons, but *foxo3b*, *foxo6a* and *foxo6b* had two exons each. Spotted sea bass FOXO-predicted proteins revealed conserved tertiary structures with three helices and two wing loops typical of the FOXO family in equivalent positions and with similar predicted folding (Fig. 2B).

3.3. Conserved domain and motif analysis

In general, the protein domain is associated with protein function; therefore, the conserved domains and motifs of spotted sea bass *foxos* were analyzed. The conserved homeodomains of FORKHEAD (FH, or winged helix), FOXO_KIX_bdg and FOXO-TAD were detected on each of the FOXO proteins, and few component divergences were present across different members of FOXOs (Fig. 3 A). To further interpret the structural and functional diversity of FOXO proteins, 10 conserved motifs were identified (Fig. 3 B). The results showed that motif 1, motif 4, motif 5, motif 8 and motif 10 can be detected in all FOXO members. All the proteins except FOXO6A showed motif 6. Furthermore, motif 3 and motif 9 were found only in FOXO1A and FOXO1B, whereas motif 2 and motif 3 were absent in FOXO1A in comparison to other FOXO members.

3.4. Tissue expression analysis of foxos

We then investigated the expression patterns of *foxos* in spotted sea bass using qRT-PCR. The *foxo* mRNAs were constitutively expressed in all brain regions, intestine, stomach, muscle and gonad, but differences were observed among these genes. The highest expression levels of *foxo1a*, *foxo3a*, *foxo3b*, *foxo4* and *foxo6b* mRNAs in the central nervous system (CNS) were observed in the cerebellum. *foxo1b* and *foxo6a* showed relatively high expression levels in all brain regions (Fig. 4).

In peripheral tissues, *foxo1a* mRNA showed relatively high expression levels in muscle followed by intestine, stomach and gonad (Fig. 4A). Furthermore, *foxo1b* (Fig. 4B) in the intestine and gonad, *foxo3a* (Fig. 4C) and *foxo6b* (Fig. 4G) in the gonad, *foxo3b* (Fig. 4D) and *foxo4* (Fig. 4E) in the muscle and gonad, and *foxo6a* (Fig. 4F) in the

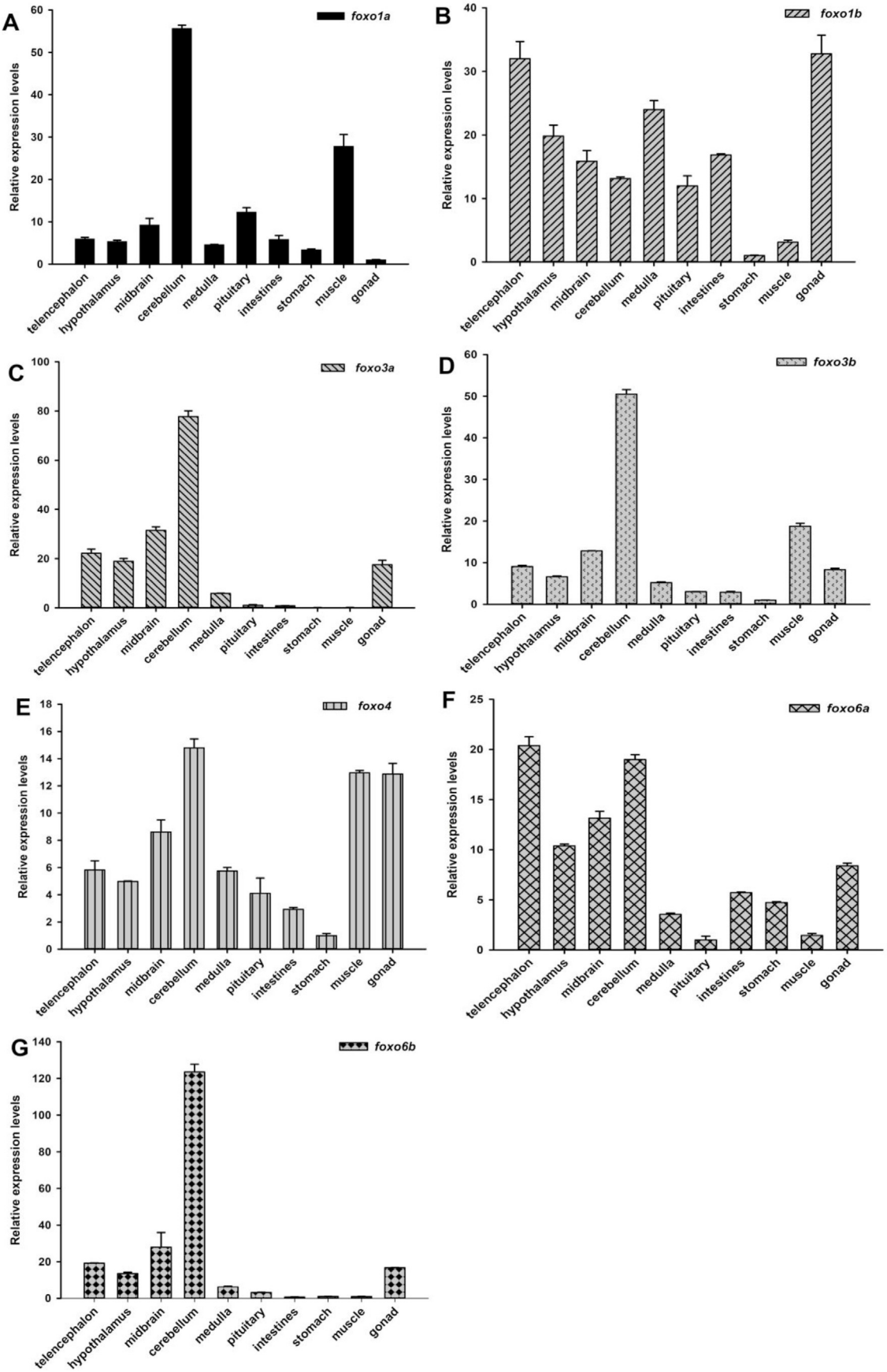

Fig. 4. Tissue expression of *foxo1a* (A), *foxo1b* (B), *foxo3a* (C), *foxo3b* (D), *foxo4* (E), *foxo6a* (F) and *foxo6b* (G) in spotted sea bass. The results are expressed as the means ± SEM (n = 3).

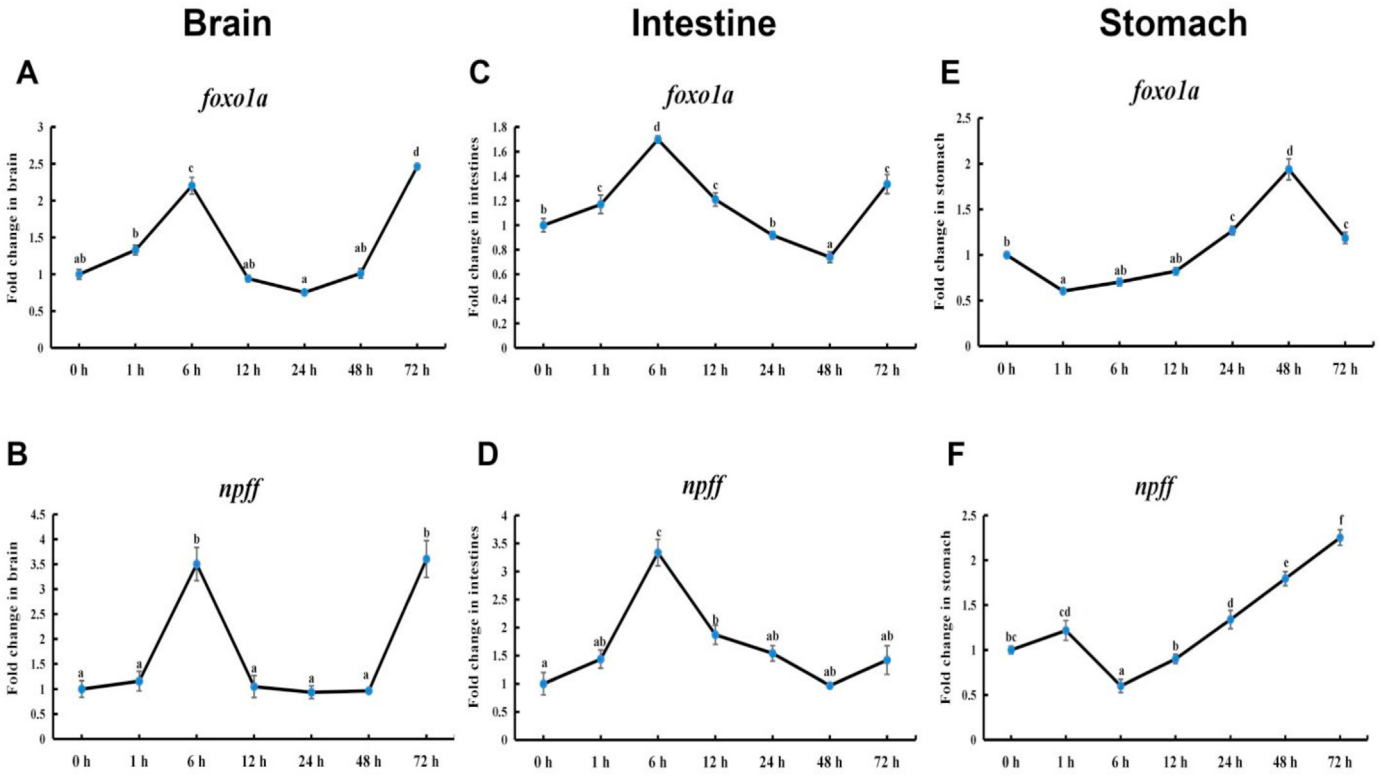

Fig. 5. Effect of different fasting times on *foxo1a* and *npff* expression in the brain (A, B), intestine (C, D) and stomach (E, F). The y-axis expressed the scale of relative expression levels, and the x-axis provided the period of fasting time. Significant differences were noted by different letters in each gene ($P < 0.05$).

gonad, intestine and stomach were relatively high.

3.5. Expression of foxo and npff genes after short-term starvation

The expression of *foxo* and *npff* genes in the brain, intestine and stomach of spotted sea bass during short-term starvation treatment was examined at 0 h, 1 h, 6 h, 12 h, 24 h, 48 h, and 72 h. The expression of *foxo1a* and *npff* showed similar trends in the brain, stomach and intestine during starvation. The highest expression levels were observed at 6 h followed by a decline until 48 h in the brain. Subsequently, an abrupt increase in expression was observed at 72 h (Fig. 5A and B). The patterns of expression of *foxo1a* and *npff* in the intestine were similar to those in the brain, except that *npff* expression did not increase dramatically after 48 h (Fig. 5C and D). In stomachs, the expression levels of *foxo1a* and *npff* were highest at 48 h and 72 h, respectively. However, the expression levels were significantly lower than those in the control group ($P < 0.05$) at 1 h and 6 h (Fig. 5E and F). However, other *foxo*s and *npff* did not show similar trends after starvation in different organs (Fig. S1).

3.6. Colocalization of npff and foxo1a mRNA in spotted sea bass brain, intestine and stomach

To investigate the regulation of *npff* expression in the brain and digestive system by FOXO1A, we then performed dual-fluorescence *in situ* hybridization assays. Regular *in situ* hybridization was performed to confirm that the probes were suitable for dual-fluorescence *in situ* hybridization (data not shown) before the dual-fluorescence experiments. Colocalization of *npff* and *foxo1a* mRNA in spotted sea bass brain areas, stomach and intestine are shown in Fig. 6. The results showed that *foxo1a* mRNA colocalized with *npff* neurons in the ventralis telencephali pars ventralis (Vv) (Fig. 6A1-4), the nucleus ventromedialis thalami (NVM) and the nucleus anterior tuberis (NAT) (Fig. 6B1-4); however, no colocalization signals were found in the optic tectum (OTec) (Fig. 6C1-4). In the stomach and intestine, foxo1a signals were also found in npff-expressing cells in gastric and intestinal gland and epithelial cells (Fig. 6D and E).

3.6.1. Identification of functional FOXO1A binding sites in the upstream region of the npff gene

To understand the transcriptional regulatory mechanism of the *npff* gene in spotted sea bass, 2074 bp of the 5′ upstream region of *npff* were isolated. Upstream sequence analysis revealed the presence of 13 predicted FOXO1 binding sites and a TATA box (Fig. S2). The functionalities of the putative promoter region of the spotted sea bass *npff* gene were tested by transient transfection in cultured cells by a reporter gene assay. Basal promoter activities were detected for the *npff* gene promoter (Fig. S3A). FOXO1A significantly increased the promoter activity of *npff* (Fig. S3B). These results indicated that FOXO1A plays a crucial role in promoting the expression of the *npff* gene in spotted sea bass. Based on these results, to assess the functionality of FOXO1A-responsive regions, a series of PCR-based deletion constructs were cloned into the pGL3-basic vector and transfected into 293T cell lines. Deletion of the *npff* promoter to position −1718 (pnpff-1718/Luc) did not abolish the promoter activity. Deletion of the *npff* promoter at −302 (pnpff-302/Luc) still exhibited promoter activities, but such activity was eliminated when the promoter between −302 and −56 (pnpff-56/Luc) was deleted (Fig. 7A). Therefore, two key FOXO1A binding sites between positions −302 and −56 were further analyzed by site-directed mutagenesis in 2074 bp and 302 bp constructs, respectively. As shown in Fig. 7B, mutation of the first FOXO1A site (pnpff-2074MU1/Luc or pnpff-302MU1/Luc) eliminated *npff* promoter activity. On the other hand, *npff* promoter activity was decreased when the second FOXO1A site (pnpff-2074MU2/Luc or pnpff-302MU2/Luc) was mutated in different length constructs. Hence, two FOXO1A binding sites between positions −302 and −56 are critical for the transcriptional regulation of *npff* in spotted sea bass.

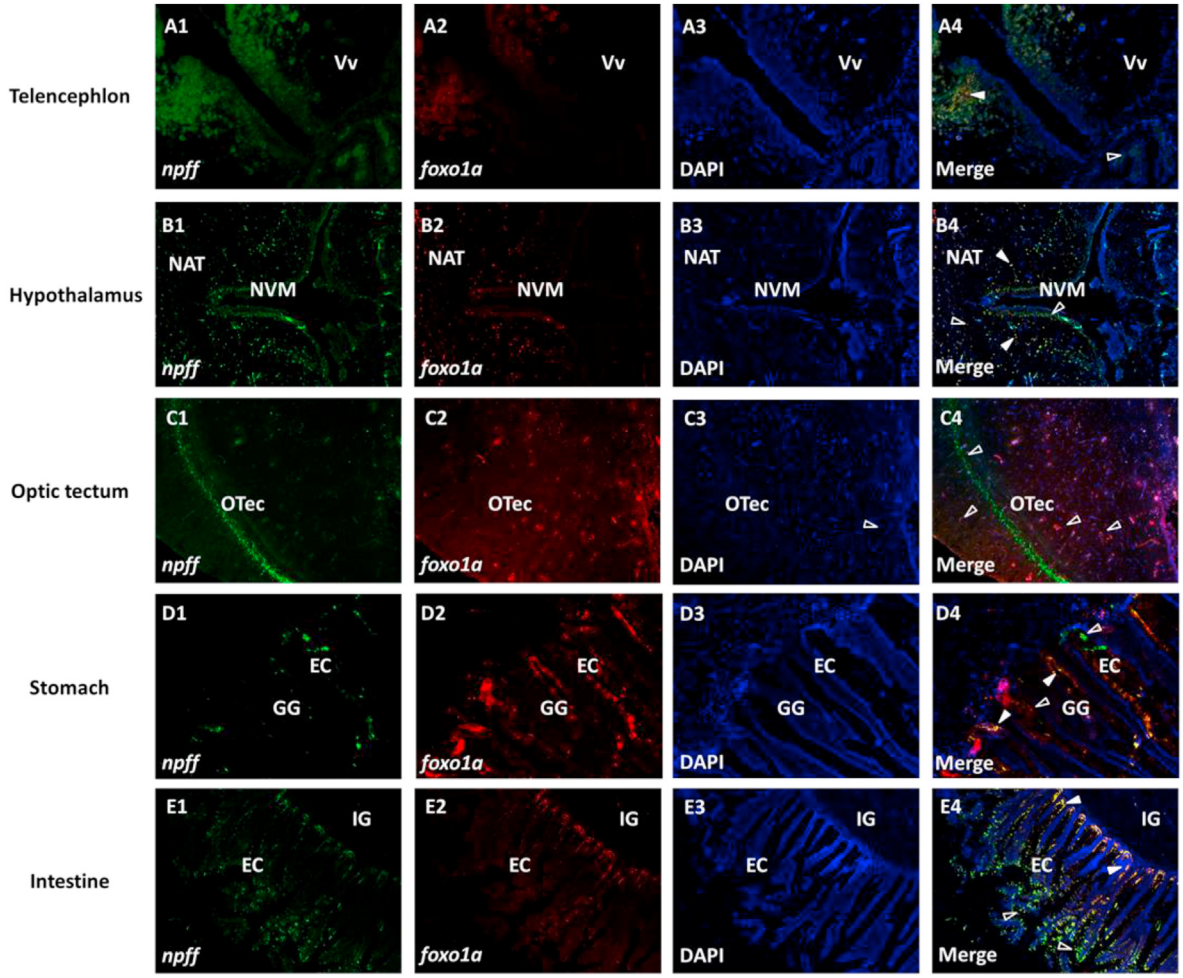

Fig. 6. Cellular colocalization of *npff* and *foxo1a* mRNA in the spotted sea bass telencephalon, hypothalamus, stomach and intestine. The *npff* (green)- and foxo1a (red)-expressing cells were colocalized in the telencephalon (A), hypothalamus (B), stomach (D) and intestine (E) but not in the optic tectum (C). Arrowheads indicate *npff* cells coexpressing *foxo1a*. Open arrowheads indicate *npff*- or foxo1a-expressing cells alone. Abbreviations: Vv, ventralis telencephali pars ventralis; OTec, optic tectum; NAT, nucleus anterior tuberis; NVM, nucleus ventromedialis thalami; GS, gastric serosa; IG, intestinal gland; EC, epithelial cell.

4. Discussion

FOXOs have major roles in cellular differentiation, tumor suppression, metabolism, cell cycle arrest, cell death, and protection from stress (Barthel et al., 2005; Accili and Arden, 2004; Brunet and Greer, 2005). Despite their importance, the *foxo* gene family has not been systematically studied in spotted sea bass, an important type of commercial fish in Asia. In the present study, 7 *foxo* genes were identified, and their phylogenetic relationships, gene and 3D structures, conserved domains and motifs, and expression profiles in spotted sea bass were determined. Analysis of the copy numbers in representative vertebrates helped to elucidate the evolution of *foxo* genes. The number of *foxo* genes varies slightly among different species. Zebrafish and spotted sea bass have more *foxo* gene copies than other vertebrates. This result may be caused by teleost-specific whole genome duplication (Berthelot et al., 2014; Olivier et al., 2004).

The gene expression patterns could provide important clues for exploring gene function. In humans and mice, each *Foxo* gene showed different expression patterns in tissues (Furuyama et al., 2000; Anderson et al., 1998; Iii and Webster, 2001). In the present study, *foxo*s were expressed in all tissues examined with different expression patterns. *foxo*s showed relatively high expression levels in the central nervous system (CNS), such as the cerebellum, hypothalamus, telencephalon and midbrain. In the peripheral tissues, *foxo1a* mRNA showed the highest expression levels in spotted sea bass muscle, which may be consistent with its vital role in muscle cells (Furuyama et al., 2003; Bastie et al., 2005) followed by the intestine, stomach and gonad. Furthermore, *foxo1b* in the intestine and gonad, *foxo3a* and *foxo6b* in the gonad, *foxo3b* and *foxo4* in the muscle and gonad, and *foxo6a* in the gonad, intestine and stomach were relatively higher levels of expression compared with the rest of the tested genes. Different expression patterns of the *foxo* genes indicate variation in their potential physiological roles.

Increasing evidence has shown that the NPFF peptide can regulate feeding-related processes by acting on the hypothalamic nucleus in several vertebrates (Murase et al., 1996; Sunter et al., 2001; Kavaliers et al., 1985). The FOXO1 factor exerts further control of food intake and energy balance by coordinating neuropeptide production in the hypothalamic feeding center (Kitamura et al., 2006; Kim et al., 2006; Ki Woo et al., 2012). Moreover, FOXO1 has been proven to be the transcriptional chief of staff of energy metabolism (Kousteni, 2012). As transcription factors, FOXO1 performs its functions via the transcription regulation of genes. Accordingly, we suspected that FOXO1 was able to transcriptionally regulate *npff* to stimulate feeding. In spotted sea bass, the NPFF peptide promoted feeding-related processes in our previous study (Li et al., 2019). In the present study, similar expression patterns of *npff* and *foxo1a* in the same tissue were observed during fasting; however, the expression of other *foxo* genes did not show such variation trends after starvation in different organs (Fig. S1). This finding may provide preliminary evidence for the transcriptional regulation of

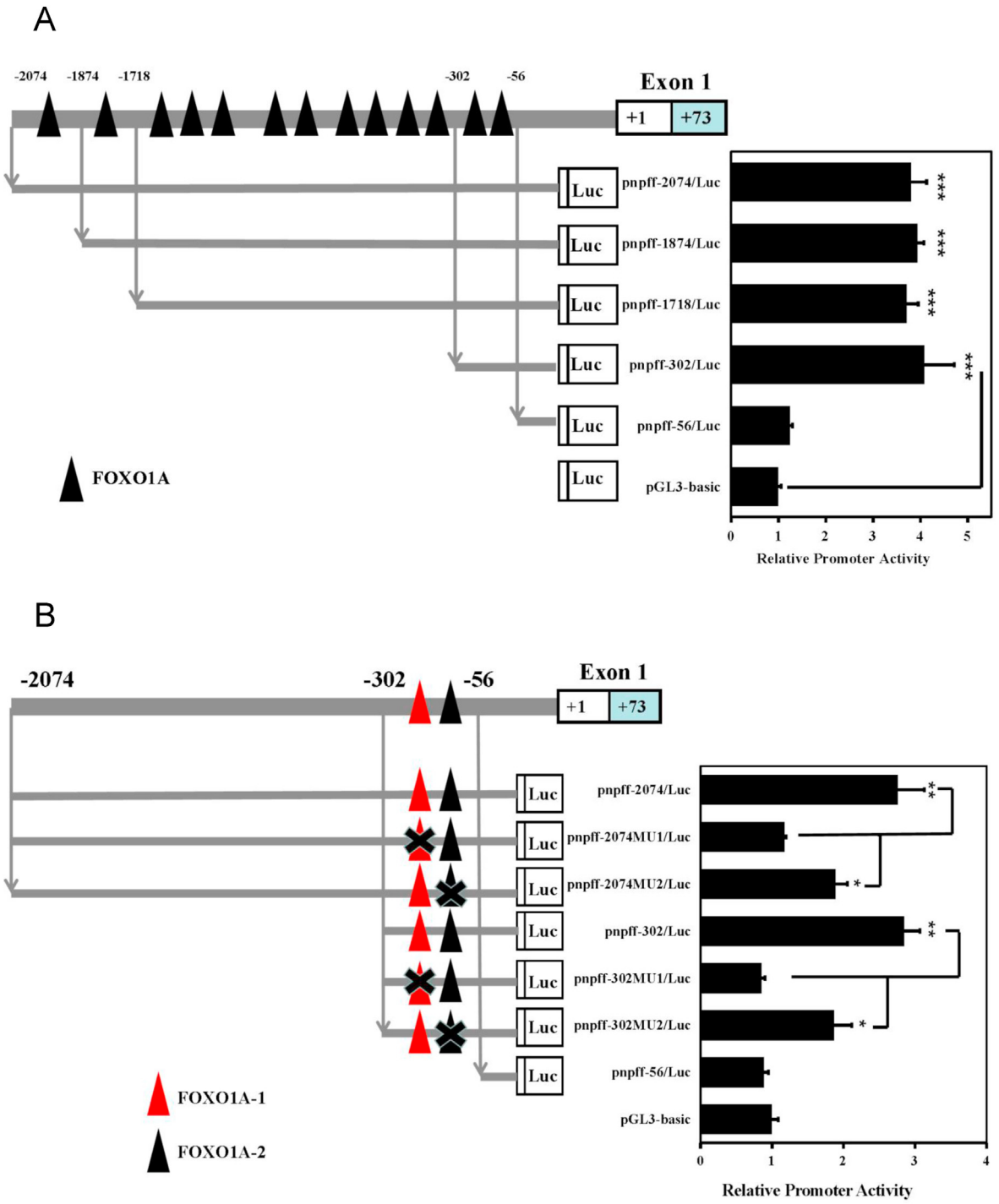

Fig. 7. Deletion and mutation analysis of the spotted sea bass *npff* promoter. Left panel: schematic representation of the 5′-deletion constructs and the positions of the putative FOXO1 binding sites (A) and mutation sites marked in red (B). Cells were harvested, and the relative promoter activities (right panel) of *npff* were determined by measuring firefly and Renilla luciferase activities and normalized with the empty pGL3-basic vector. Bars represent mean values ± SEM, *P < 0.05; **P < 0.01; and ***P < 0.001 compared with the corresponding control. The results were from at least three independent experiments.

FOXO1A on the expression of *npff* in spotted sea bass.

Unlike in mice, the localization and functional contributions of *foxo1* have not been studied in teleosts. In the mouse brain, *Foxo1* mRNA was also highly expressed in many hypothalamic nuclei, including the dorsomedial nucleus of the hypothalamus (DMH) and the ventral medial nucleus of the hypothalamus (VMH) (Kitamura et al., 2006; Kim et al., 2006), which is similar to those observed in spotted sea bass. These reports have also demonstrated that FOXO1 regulates food intake through the hypothalamic insulin and leptin signaling pathways (Kitamura et al., 2006; Kim et al., 2006). Accordingly, the similar localization of the *foxo1* gene in hypothalamic neurons between mouse and spotted sea bass indicates its conserved function in feeding regulation. On the other hand, spotted sea bass *foxo1a* was highly expressed in the epithelial cells (ECs) of the intestinal villi (IV) and intestinal gland (IG), while it was highly expressed in epithelial cells (ECs) and the gastric gland (GG) in the stomach. These regions are critical for regulating ingestion and gastrointestinal motility by releasing such hormones as Motilin, Grelin, Cholecystokinin and

Somatostatin (Chung et al., 1992; Strader and Woods, 2005). In addition, the latest report suggested that intestinal insulin/IGF1 signaling through FOXO1 regulates epithelial integrity (Ostermann, 2019). This evidence proves the potential action of *foxo1a* in the digestive system. Moreover, by dual-fluorescence *in situ* hybridization, we first provide evidence that *foxo1a* mRNA was expressed in *npff* neurons of spotted sea bass brain and gastrointestinal system, especially in the NVM and NAT of the hypothalamus, which are related to feeding regulation (Dhillon et al., 2006; José Miguel and Richard Ector, 2003; Cerdá-Reverter et al., 2000). Accordingly, *foxo1a* and *npff* colocalized in the same cell of the main feeding regulation regions to achieve direct transcriptional regulation.

To further confirm the transcriptional regulation of *foxo1a* on *npff*, luciferase assays were performed in the present study. Thirteen FOXO1 binding sites were predicted in the 2074 bp sequence of the 5′ upstream region of *npff*. These predicted FOXO1 binding sites possess a core sequence of "TGTT(T/G)" in the forward strand or "(A/C)AACA" in the reverse strand, which is similar to that in mammals (Guo et al., 1999; Tang et al., 1999; Furuyama et al., 2000; Brent et al., 2008). A series of deletion and mutation construct experiments involving the FOXO1 factor confirmed its action to regulate the *npff* gene. The results showed that two FOXO1 binding sites between positions −302 and −56 are critical for the transcriptional regulation of *npff* in spotted sea bass. Our present findings demonstrated that FOXO1A is a strong transcriptional activator of the *npff* promoter in spotted sea bass.

In summary, for the first time, we performed a genome-wide identification of the *foxo* genes in spotted sea bass and analyzed them. Many differences in conserved motifs and expression profiles between *foxo1a* and other *foxo* genes indicate variation in their potential physiological roles. Under short-term starvation, only *foxo1a* showed a similar expression pattern to *npff*, which may provide preliminary evidence for the transcriptional regulation of FOXO1A on the *npff* gene in spotted sea bass. The colocalization of *foxo1a* and *npff* mRNAs in the brain, intestine and stomach provided further evidence for the potential involvement of FOXO1A in the transcriptional regulation of the *npff* gene. Finally, the present study demonstrated for the first time that the spotted sea bass FOXO1A can regulate *npff* transcriptional responses dependent on direct DNA binding and confirmed the key regulatory areas and sites of FOXO1A on the *npff* promoter by deletion and site-directed mutagenesis analyses. Our findings may help to elucidate the role of FOXO1 in the regulation of feeding processes in vertebrates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110871o

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