# Novel Insights for PI3KC3 in Mediating Lipid Accumulation in Yellow Catfish *Pelteobagrus Fulvidraco*

**Mei-Qin Zhuo** ( zmq@whpu.edu.cn )
Wuhan Polytechnic University https://orcid.org/0000-0002-7877-9136
**Jun Chen**
Wuhan Polytechnic University
**Mei-Li Wu**
Wuhan Polytechnic University
**Wen-biao Wang**
Wuhan Polytechnic University

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# Abstract

In this study, the transcriptional regulation of PI3KC3 by three transcript factors (PPARγ, PPARα and STAT3) and the potential role of PI3KC3 in mediating lipid accumulation were determined in yellow catfish *Pelteobagrus fulvidraco*. The 5'-deletion assay, overexpression assay, site-mutation assay and electrophoretic mobility shift assay suggested that PPARα, PPARγ and STAT3 negatively regulated the promoter activity of *pi3kc3*. Moreover, the transcriptional inactivation of *pi3kc3* was directly mediated by PPARα and PPARγ under fatty acid (FA) treatment. Using primary hepatocytes from yellow catfish, FA incubation significantly increased triacylglyceride (TG), NEFA content, the mRNA level of *pparα*, *pparγ*, *stat3* and *dnmt3b,* the protein level of PPARα, PPARγ and STAT3, and the methylation level of *pi3kc3*, but significantly reduced the mRNA and protein level of PI3KC3. Our findings offer new insights into the mechanisms for transcriptional regulation of PI3KC3 and for PI3KC3-mediated lipid accumulation in fish.


# Introduction

Phosphatidylinositol-3 kinase (PI3K) are the key signalling molecules, which controlling many cellular processes including cell growth, proliferation, differentiation, survival, intracellular trafficking, and nutrient metabolism (Foster et al. 2003; Liu et al. 2006; kok et al. 2009;). Our previous study indicated that PI3K pathway was involved in regulating lipid metabolism in yellow catfish *Pelteobagrus fulvidraco* (Zhuo et al. 2015; 2018), but the relationship between PI3KC3 and lipid metabolism has not been investigated. PI3KC3 belongs to the type III PI3K families, and its cDNA sequence and core promoter have been cloned from yellow catfish in our previous study (Zhuo et al. 2017; 2018). However, the underlying transcriptional mechanism and the function of PI3KC3 were still limited to known.

The expression of gene was regulated by the interaction of transcription factors with promoter elements. Signal transducers and activators of transcription proteins (STATs), a family of latent cytoplasmic transcription factors, which participated in gene regulation. STAT3, a member of STAT family, which modulated the expression of many target genes involved in lipid metabolism (Wu et al. 2016; 2018). Peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ) are the two important transcription factors that modulated the expression of many target genes involved in lipid metabolism (Zheng et al. 2015a, b). Several previous studies have suggested that PI3K pathway activated PPARα or PPARγ, and plays an important role in the regulation of cellular lipid metabolism (Zhuo et al. 2015; 2018; Yang et al. 2018). However, limited studies were reported whether the transcription of PI3K was regulated by the transcription factors related to lipid metabolism, such as STAT3, PPARα, and PPARγ.

The methylation of DNA belongs to one of the most important epigenetic mechanisms, which represses gene expression by recruiting proteins or by preventing the binding of the transcription factors to DNA sequences (Nagase and Ghosh, 2008; Moore et al. 2013). DNA methylation is primarily modulated by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B (Nagase and Ghosh, 2008). Many studies also suggested that aberrant DNA methylation were correlated with disorders and dysregulation of lipid accumulation. Nutritional factors including dietary high-fat or fatty acid supplement

could modify specific gene transcription through the alteration of DNA methylation status (Ge et al. 2014; Marco et al. 2014; Kim et al. 2015; Zhang et al. 2017; Li et al. 2018; Parsanathan et al. 2019; Hunter et al. 2019). DNA methylation often happened on the CpG islands within the promoter region of gene. Interestingly, two CpG islands were predicted on the promoter of *pi3kc3*, which attracted our great interest to study whether DNA methylation was involved in the PI3KC3 of yellow catfish.

Yellow catfish, an omnivorous freshwater fish, is widely distributed in the inland freshwater waters in China. Gong et al. (2018) published its genomic sequences, which provides good basis for exploring the regulatory mechanism of lipid metabolism. Moreover, our previous study suggested that PI3K pathways were involved in regulating lipid metabolism. To further investigate the function of individual PI3K members and the regulatory mechanism of PI3KC3-mediated lipid accumulation in yellow catfish. In this study, the transcriptional regulation of *pi3kc3* by three transcript factors (PPARγ, PPARα, and STAT3) was studied. Our results suggested that the promoter activity of *pi*3kc3 were negatively regulated by PPARα, PPARγ and STAT3, and the transcriptional inactivation of *pi3kc3* was directly mediated by PPARα and PPARγ under FA treatment. Meantime, by using primary hepatocytes from yellow catfish, we found that FA incubation disturbed the methylation and gene expression of *pi3kc3*, and PI3KC3 plays a negative role in FA-induced lipid accumulation in the hepatocytes of yellow catfish. Our study elucidated innovative insights into the regulatory mechanism of PI3KC3 in fish.

# Materials And Methods

Experimental animals and reagents

Yellow catfish (body weight: 22.5 ± 4.4 g) were obtained from a local commercial farm. HEK293T cell lines were purchased from the Cell Resource Center in the Fishery College of Huazhong Agricultural University. Dulbecco's Modified Eagles Medium (DMEM), 0.25% trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen, USA. Dimethyl sulphoxide (DMSO), penicillin, palmitic acid, oleic acid, streptomycin, trypan blue and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). We ensured that the experiments were performed in accordance with the experimental protocols of Wuhan Polytechnic University (WHPU) and were approved by the ethics committee of WHPU.

Experimental Treatment

Two experiments were carried out. Exp. 1 was conducted to study the transcriptional regulation of *pi3kc3* promoter. Exp. 2 was conducted to determine the potential role of PI3KC3 in influencing lipid accumulation in the hepatocytes from yellow catfish under FA incubation.

Exp. 1: Transcriptional regulation assay of *pi3kc3* promoter

Promoter cloning and plasmids construction

The genomic DNAs were extracted from the liver of yellow catfish by using a commercial DNA extracted kit (Omega, Norcross, GA, USA). The promoter sequence of *pi3kc3* was obtained by RT-PCR according to

the genome of yellow catfish (Gong et al. 2018). The primers for *pi3kc3* promoter cloning were presented in Table S1. For generating the luciferase reporter construct, we subcloned different plasmids with *pi3kc3* promoter into pGl3-Basic vectors (Promega, USA) by using SacI and HindIII restriction sites. On the basic of the distance from its TSS, we named the plasmid as pGl3-1781/+59 of *pi3kc3* promoter. Then, we used the template of pGl3-1781/+59 vector to produce the plasmids pGl3-1361/+59, pGl3-848/+59, and pGl3-381/+59 of *pi3kc3* vectors. We used ClonExpress II One Step Cloning Kit (Vazyme, Piscataway, NJ, USA) to ligate all of the products. We performed the PCR via the TaKaRa PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Tokyo, Japan). Finally, we sequenced all these plasmids in the Tsingke company (Wuhan, China). The primers for the plasmids construction were presented in Table S2. In addition, The overexpression plasmids of PPARα, PPARγ and STAT3 were obtained from our previous studies (Lv et al. 2021).

Sequence analysis

We used BLAST network service at the NCBI (http://blast.ncbi.nlm.nih.gov/) to compare the nucleotide sequences with DNA sequences from the GenBank database. Several online softwares, such as the MatInspector database (http://www.genomatix.de/), the JASPAR database (http://jaspar.genereg.net/) and the TFSEARCH database (http://www.cbrc.jp/research/db/TFSEARCH.html), were utilized to analyze the potential transcription factor binding sites (TFBS). The CpG islands were predicted by the online tool MethPrimer (http://www.urogene.org/methprimer/index1.html) with parameters as follows: window 100, shift 1, observed CpG/expected CpG ≥ 0.60 and GC % ≥40.

Plasmid transfections and assays of luciferase activities

HEK293T cells were cultured in DMEM medium with the 10% fetal bovine serum (FBS) (Gibco, C lsbad, CA, USA) in an incubator (5% $CO_2$ and 37°C). Prior to the transfection, HEK293T cells were seeded at a density of $1.2\times10^5$ in 24-well plate. They were cultured until the 70-80% confluence. Lipofectamine™2000 (Invitrogen) was utilized to transfect all these plasmids into HEK293T cells, based on the manufacture's protocol. The 500 ng overexpression plasmids, 400 ng reporter plasmids and 20 ng pRL-TK (the internal control with a Renilla luciferase reporter vector), were co-transfected into HEK293T cells. After 4 h, we replaced the transfection medium by 10% FBS-DMEM or 10% FBS-DMEM + 0.6 mM FA. FA was added as a mixture of palmitic acid and oleic acid at a ratio of 1:1. The form and the concentration of FA were selected according to our pilot trial and the publications of the in vitro studies (Wu et al. 2019; Wu et al. 2020; Chen et al 2020a; Song et al 2020). Then, after 24 h incubation, cells were collected to determine the promoter activity, based on the manufacturer's instruction of the Dual-luciferase Reporter Assay System (Promega). The relative luciferase activities were obtained by calculating the ratio of Firefly luciferase activity to Renilla luciferase activity. We conducted all these experiments in triplicates.

Site-mutation analysis of binding sites on the *pi3kc3* promoter

To identify the corresponding binding sites on the regions of *pi3kc3 promoter*, we used QuickChange II Site-Directed Mutagenesis Kit (Vazyme, Piscataway, NJ, USA) to perform site-directed mutagenesis

analysis. Several mutations were performed at the sites of -1621/-1611 bp, -1603/-1594 bp, -922/-907 bp, -1083/-1076 bp, and -245/-230 bp of *pi3kc3* promoter; The primers used for mutagenesis were shown in Table S3. The DNA sequencing was utilized to confirm these mutations. Then, the Lipofectamine 2000 reagent (Invitrogen) was utilized to co-transfect the plasmids into HEK293T cells. After 4 h transfection, the medium was substituted with 10% FBS-DMEM or 10% FBS-DMEM + 0.6 mM FA. After 24-h incubation, we harvested the cells to determine the luciferase activities, based on the procedures mentioned above.

Electrophoretic mobility-shift assay (EMSA)

The EMSA was conducted to confirm the functional PPARα, PPARγ and STAT3 binding sties on the *pi3kc3* promoter according to our and other recent publications (Xu et al. 2017; Zhuo et al. 2018; Chen et al. 2020b). Nuclear and cytoplasmic extracts were extracted according to the method of Read et al. (1993). Protein contents were determined by the BCA method (Smith et al. 1985). The oligonucleotide probes were synthesized in the Tsingke company (Wuhan, China). Nuclear extracts (10 μg) were incubated for 30 min at the room temperature by using the binding buffer (20 mM HEPES, pH7.9, 1 mM MgCl2, 0.5 mM DTT, 4% Ficoll, 110 mM KCl, 0.2 μg Poly(dI-dC)). Then, the biotin-labeled double-stranded oligo nucleotides (Table S4) were added. The reaction continued for 30 min and then the electrophoresis was performed on 6% native polyacrylamide gels. For the competitive binding analysis, a 100-fold excess of unlabeled double-stranded DNA oligo with mutant binding site (Table S4) was added with the corresponding labeled one.

Exp2. FA incubation with hepatocytes of yellow catfish

Hepatocytes were isolated from yellow catfish according to our previous studies and were cultured in M199 medium containing 1 mmol/L L-glutamine, 5% (v/v) FBS, penicillin (100 IU/mL) and streptomycin (100 g/mL) in a humidified atmosphere with 5% $CO_2$ at 28°C (Zhuo et al. 2018). Hepatocytes were counted using a hemocytometer based on the trypan blue exclusion method and only more than 95% cell viability were used for the present experiment. Hepatocytes were plated onto 25 $cm^2$ flasks at the density of $10^6$ cells/mL, and then they were incubated with PBS (control) and 0.6 mM FA. Each treatment was performed in triplicate and three independent experiments were carried out. After 48 h, the hepatocytes were gathered for the following analysis.

TG, NEFA, and lipid drops assay

TG and nonesterified fatty acid (NEFA) concentrations were determined with commercial kits (Nanjing Jian Cheng Bioengineering Institute, China), according to the manufacturer's instructions. Bodipy 493/503 staining were used to assess the changes of intracellular lipid drops (LDs). Briefly, hepatocytes were cultured in 12-well plates and treated with the corresponding treatments for the required period, and then they were washed twice with PBS. After that they were incubated with 5 mg/ml Bodipy 493/503 (D3922; Thermo Fisher Scientific Waltham, MA, USA) for 30 min, followed by 3 PBS washes. Then the hepatocytes were observed with a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) to visualize the intensity of fluorescence. The green dots were defined as lipid drops, which

were quantified with a CytoFlex flow cytometer (Beckman Coulter, Brea, CA, USA). Data analysis was performed with FlowJo v.10 software (Ashland, OR, USA).

mRNA level determination by real-time Q-PCR

Total RNA was isolated using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction. cDNA was then reverse-transcribed from normalized RNA using oligo (dT) primers and M-MLV reverse transcriptase (TaKaRa, Dalian, China). The mRNA levels of (*ppara*, *ppary, stat3, dnmt1, dnmt3a, dnmt3b*, and *pi3kc3*) were examined by quantitative PCR (Q-PCR). Q-PCR assays were performed in a quantitative thermal cycler (MyiQ™ 2 TwoColor Quantitative PCR Detection System, BIO-RAD, USA) with a 20 μL reaction volume containing 10 μL SYBR Premix Ex Taq™ II (TaKaRa, Japan), 1 μL of diluted cDNA (10-fold), 10 mM each of forward and reverse primers (0.4 μL), and 8.2 μL $H_2O$. Primers are given in Table S5. The Q-PCR parameters consisted of initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 57°C for 30 s and 72°C for 30 s. All reactions were performed in duplicates and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. The melting curve was generated for every PCR product to confirm the specificity of the assays. A set of seven common housekeeping genes (*β-actin, 18s-rrna, gapdh, rpl7, hprt, ubce*, and *tuba*) were selected from the literature (Vandesompele et al. 2002) in order to test their transcription stability. Two most stable control genes (*gapdh* and *18srrna*, M=0.35) were selected by using geNorm software. The relative expression levels were calculated with the “delta–delta Ct” method (Pfaffl 2001), and normalized in terms of the geometric mean of two genes by geNorm.

Analysis of protein expression by western blot

Western blotting was performed according to the previous study (Wu et al. 2020). Hepatocytes were lysed in RIPA buffer (Sigma, USA). Equal amounts of protein were separated on 12% SDS-PAGE, transferred onto PVDF membranes, and then blocked with 8% (w/v) dry milk. After that, the membranes were incubated with primary antibodies as follows: rabbit anti-PPARα (15540-1-AP, Proteintech, USA), rabbit anti-PPARγ (16643-1-AP, Proteintech, USA), rabbit anti-STAT3 (10253-2-AP, Proteintech, USA), rabbit anti-PI3KC3 (AbClone, A12295, USA), and anti-GAPDH (10494-1-AP; Proteintech, USA) overnight at 4°C. Then, HRP-conjugated anti-rabbit secondary antibody (CST, USA) was used to probe with. Finally, the protein bands were visualized with enhanced chemiluminescent (ECL) and quantified by Image J software.

Methylation analysis of CpG islands of *pi3kc3* promoter

Genomic DNA from hepatocyte was extracted using AxyPrep DNA Kit (Axygen Biotechnology, Hangzhou, China) according to the manufacturer's instructions. The genomic DNA extracted above was modified according to the manufacturer's protocol using the DNA Methylation Gold Kit (Zymo research, Orange, CA). Two CpG islands on the *pi3kc3* promoter were predicted. The bisulfite modified DNA was amplified by nest polymerase chain reaction (PCR) with two BSP (bisulfite sequencing PCR) specific primer pairs (list in Table S6), under the following conditions: 95°C denaturation for 3 min; 30 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 40 s; and 72°C extension for 5 min. The PCR products were gel purified and

were subjected to cloning into a PMD 19-T Vector (TaKaRa, Dalian, China). After the cloning, total of 20 clones from each treatment were randomly selected for DNA sequencing. The methylation level was analysis by the online website (http://quma.cdb.riken.jp/).

# Statistical analysis

We used SPSS 19.0 software for all these statistical analysis. All of these data were expressed as means ± SEM (standard errors of means). Before statistical analysis, we evaluated all data for normality using the Kolmogorov-Smirnov test. In order to test the homogeneity of variances, we performed Bartlett's test. We analyzed data with Duncan's multiple or Student's t-test where appropriate. Difference was considered significant at $p < 0.05$.

# Results

Sequence analysis of *pi3kc3* promoter

In the present study, we successfully obtained -1781 bp of *pi3kc3* promoter from yellow catfish. On the *pi3kc3* promoter, one PPARα binding site (at -245/-228 bp), one PPARγ binding site (at -922/-904 bp), and three STAT3 binding sites (at -1603/-1593 bp, -1621/-1610 bp, -741/-731 bp) were predicted, respectively (Fig. 1A, B). In addition, two CpG islands were also predicted on the promoter of *pi3kc3* (Fig. 1C).

5'-deletion analysis

Deletion the sequences from -1781 bp to -1361 bp, -1361 bp to -848 bp, and -848 bp to –381 bp significantly increased the relative luciferase activities of *pi3kc3* promoter, indicating that the -1781/-1361 bp, -1361/-848 bp, and -848/-381 bp regions negatively controlled *pi3kc3* promoter activity (Fig. 2A).

Overexpression of PPARα markedly reduced the activities of pGl3-1781/+59, pGl3-1361/+59, pGl3-848/+59, and pGl3-381/+59 of *pi3kc3* plasmids (Fig. 2B). Overexpression of PPARγ greatly decreased the activities of pGl3-1781/+59 and pGl3-1361/+59 plasmids, and presented no significant influences on the activities of pGl3-848/+59 and pGl3-381/+59 plasmids (Fig. 2C). Overexpression of STAT3 significantly reduced the promoter activities of pGl3-1781/+59, pGl3-1361/+59 and pGl3-848/+59 plasmids, and showed no influence on the promoter activity of pGl3-381/+59 plasmid (Fig. 2D).

Site-mutation analysis

To further elucidate whether the regions of *pi3kc3* promoter possessed PPARα, PPARγ and STAT3 response elements, site-directed mutation were performed. Overexpressed PPARα markedly reduced the promoter activity of the wide-type *pi3kc3* plasmid, and its inhibitory effect was completely abolished when the -245/-230 bp PPARα site was mutated, suggesting that the -245/-230 bp PPARα site negatively controlled *pi3kc3* transcription (Fig. 3A).

Overexpressed PPARγ inhibited the promoter activity of the wide-type *pi3kc3* plasmid. However, mutation the -922/-908 bp PPARγ site recovered this inhibitory effect by PPARγ, indicating that the -922/-908 bp PPARγ site might inhibit *pi3kc3* transcription (Fig. 3B).

Overexpressed STAT3 inhibited the promoter activity of the wide-type *pi3kc3* plasmid. However, this inhibitory effect was completely abolished by the mutation of (-741/-731 bp) STAT3 site, but not the mutation of -1621/-1611 bp and -1603/-1593 bp STAT3 sites, suggesting that only the -741/-731 bp STAT3 site down-regulated *pi3kc3* transcription (Fig. 3C).

EMSA analysis of binding sequence of transcription factors

Next, we examined whether PPARα, PPARγ and STAT3 functionally bind with their corresponding regions of *pi3kc3* promoter. For the PPARα binding assay, the 100-fold unlabeled PPARα binding sequence competed for the binding when we used biotin-labeled PPARα binding sequence (-245/-220 bp of *pi3kc3* promoter) as the probe, while the 100-fold unlabeled mutated PPARα binding sequence markedly reduced this competition, indicating that PPARα binding sequence was functionally bound by PPARα (Fig. 4A). Similar results are also found for the PPARγ binding sequence (-922/-900 bp of *pi3kc3* promoter, Fig. 4B) and STAT3 binding sequence (-743/-720 bp of *pi3kc3* promoter, Fig. 4C), suggesting that both PPARγ and STAT3 were also functionally bound by *pi3kc3* promoter.

Effect of FA incubation on the promoter activity of *pi3kc3* promoter

To investigate the response of promoters induced by FA, we used 0.6 mM FA to incubate HEK293T for 24 h. FA incubation significantly reduced the luciferase activities of pGl3-1781/+59, pGl3-1361/+59, pGl3-848/+59 and pGl3-381/+59 of *pi3kc3* plasmids (Fig. 5A). To further determine whether FA-induced the decreasing of *pi3kc3* promoter activity could be mediated by PPARα, PPARγ and STAT3 elements, we conducted the site-directed mutation at their corresponding sites of *pi3kc3* promoter and used FA to incubate the cells. Compared to the wild type pGl3-1781/+59 vector of *pi3kc3* promoter, FA-induced transcriptional inactivation of *pi3kc3* was subdued after the mutation of PPARα and PPARγ elements, but not after the mutation of STAT3 element (Fig. 5B).

Effect of FA incubation on lipid accumulation in the hepatocytes from yellow catfish

Compared to the control group, FA incubation significantly increased the TG content, NEFA content and lipid drops in the hepatocytes from yellow catfish (Fig. 6).

Effect of FA incubation on gene and protein expression.

For the mRNA expression, compared to the control group, FA incubation notably up-regulated the mRNA of *ppara, pparγ, stat3* and *dnmt3b*, but down-regulated the mRNA level of *pi3kc3*, and showed no effect on the mRNA level of *dnmt1* and *dnmt3a* in the hepatocytes from yellow catfish (Fig. 7A).

For the protein expression, compared to the control group, FA incubation significantly increased the protein level of PPARα, PPARγ and STAT3, but decreased the protein level of PI3KC3 (Fig. 7B).

Effect of FA incubation on the methylation of *pi3kc3* promoter

The putative result of first CpG island in *pi3kc3* promoter and the amplification sequence by nest polymerase chain reaction (PCR) with two BSP specific primer pairs (Fig. 8A). Sequencing chtomatogram analysis of partial bisulfite-modified DNA after amplification was shown in the Fig. 8B. Compared to the control group, FA incubation increased the methylation level of *pi3kc3* promoter at -1290, -1263, -1250, -1217, -1197 and -1129 CpG sites (Fig. 8C).

# Discussion

At present, the underlying transcriptional mechanism of PI3KC3 was still little known in fish. Our previous study have isolated partial promoter sequence of *pi3kc3* from yellow catfish and have founded that FOXO1 positively regulated the transcription of *pi3kc3* (Zhuo et al. 2018). However, whether the transcription of *pi3kc3* could be regulated by the transcript factors of STAT3, PPARα and PPARγ was not studied due to the short length of *pi3kc3* promoter sequence we have obtained. Fortunately, Gong et al. (2018) successful assembly the genome sequences of yellow catfish, which help us able to amplify the long length sequence of *pi3kc3* promoter. On the other hand, the methylation of CpG island on the promoter is also important for the transcription of gene. Since two CpG islands were predicted on the promoter of *pi3kc3*, we are interesting to study whether DNA methylation modify the promoter of *pi3kc3.* Furthermore, our previous study indicated that PI3K pathway was involved in regulating lipid metabolism in yellow catfish (Zhuo et al. 2015; 2018), but the role of PI3KC3 in regulating lipid metabolism in yellow catfish remains largely unknown.

In order to identify the role of *pi3kc3* in regulating lipid metabolism, it's very important to explore the transcriptional regulation of *pi3kc3* by transcript factors related to lipid metabolism. PPARα and PPARγ are the two important nuclear transcription factors that regulate lipid metabolism (Zheng et al. 2015a; b). Yang et al. (2018) reported that PIK3R3 mediated hepatic lipid homeostasis through PPARα. Several other studies also revealed that PPARα and PPARγ positively regulated the expression of PI3K regulatory subunit in mammals (Rieusset et al. 1999; 2001a; b). However, to our best known, the regulations of PPARα and PPARγ on the expression of PI3K catalytic subunit were never been reported. In this study, we founded that overexpression of PPARα and PPARγ significantly decreased the transcriptional activity of *pi3kc3*, and subsequent site mutation and EMSA assay demonstrated that PPARγ and PPARα directly mediated transcriptional activity of *pi3kc3*, implying that PPARγ and PPARα negatively regulated the transcriptional activity of *pi3kc3*. STAT3 belongs to a family of transcriptional regulator, which modulates the expression of many target genes related to lipid metabolism (Wu et al. 2018). It has been reported that PI3K and STAT3 were interdependent in many cellular process (Vogt and Hart 2011; Hart et al. 2011; Chu et al. 2014). In this study, we found that overexpression of STAT3 reduced the transcriptional activity of *pi3kc3*, and subsequent site mutation and EMSA assay demonstrated that STAT3 directly mediated

the transcriptional activity of *pi3kc3*, implying that STAT3 also negatively regulated the transcriptional activity of *pi3kc3*. Conversely, Abell et al. (2005) reported that STAT3 positively regulated the expression of PI3K regulatory subunit in mammary gland tissue. Taken together, our study found that PPARα, PPARγ and STAT3 negatively regulated the transcriptional activity of *pi3kc3* from yellow catfish.

Fatty acid is the directly factor that regulated intracellular lipid level. In the present study, we found that FA incubation markedly increased lipid and NEFA level in hepatocytes from yellow catfish, in agreement with other studies (Wu et al. 2019; Wu et al. 2020; Chen et al. 2020; Song et al. 2020). In addition, FA incubation significantly reduced the mRNA and the protein level of PI3KC3, but increased the mRNA and the protein level of PPARα and PPARγ. Similarly, Zhong et al. (2019) also reported that fatty acid stimulated lipid droplets formation and PPARα expression in HepG2 cells. Our studies also demonstrated that FA-induced transcriptional inactivation of *pi3kc3* was subdued after the mutation of PPARα and PPARγ elements, implying that FA decreased the expression of *pi3kc3* directly through PPARα and PPARγ in yellow catfish. Moreover, our study also revealed that FA-induced hepatocellular TG accumulation coincided with the decreasing expression of PI3KC3. However, other study and our previous study have suggested that there was a positively relationship between PI3K activity and TG accumulation (Zhuo et al. 2015; Wang and Sul 1998). Thus, we speculated that the decreasing expression of PI3KC3 may result in the increasing of PI3KC3 activity. For example, other studies pointed out that increasing the expression of PI3KCa and PI3KCb resulted in the decreasing of theirs activities in human (Pankow et al. 2006). Meantime, we also found that FA incubation up-regulated the mRNA level and protein level of STAT3. However, FA-induced transcriptional inactivation of *pi3kc3* was not changed after the mutation of STAT3 element, indicating that STAT3-PI3KC3 is probable not the prioritized binding under FA treatment.

On the other hand, DNA methylation has a specific effect on gene expression, methylation of the CpG islands on the promoter region of gene directly represses gene expression (Bird 2002; Chan 2007). Recently, It has been reported that PI3K pathway regulated DNA methylation in several specific gene loci (Yang et al. 2019). Barberio et al. (2019) also reported that the methylation and expression of the gene in the upstream or downstream of PI3K signaling pathway are altered in obesity. However, to our best knowledge, the methylation status of PI3K itself has yet not been investigated. In this study, for the first time, we found that FA induced the hypermethylation of *pi3kc3*, but reduced its mRNA expression. Similarly, other previous studies also pointed out that methylation of CpG islands impaired transcription factor binding to its targets and accordingly led to silence of gene expression (Siegfried and Simon 2010; Moore et al. 2013). Moreover, we found FA induced the hypermethylation of *pi3kc3* promoter along with the up-regulation of mRNA expression of *dnmt3b*. Our previous study also found that high-fat induced the expression of *dnmt3b*, but not *dnmt1* and *dnmt3a* in the ovary of yellow catfish (Zhuo et al. 2019). Some other studies also pointed out that high fat or fatty acid supplement induced the global and gene-specific DNA methylation along with increased the expression of the *dnmts* in mice both in vivo and in vitro (Kim et al. 2015; Hunter et al. 2019; Parrillo et al. 2016). Together, these results indicated that DNA methylation participated in FA-induced the expression of *pi3kc3* expression in the hepatocytes of yellow catfish.

In summary, we identified that three transcription factors (PPARα, PPARγ and STAT3) negatively regulated the transcription activity of *pi3kc3* promoter. The transcriptional inactivation of *pi3kc3* was directly mediated by PPARα and PPARγ under FA treatment. Furthermore, for the first time, we found that FA-induced the expression of PI3KC3 was regulated by DNA methylation, and PI3KC3 plays a negative role in FA-induced lipid accumulation in the hepatocytes of yellow catfish.

# Abbreviations

FA, fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthineguanine phosphoribosyltransferase; RPL7, ribosomal protein L7; TG, triacylglyceride; TUBA, tubulin alpha chain; UBCE, ubiquitin-conjugating enzyme; STAT3, Signal transducers and activators of transcription proteins 3; PPAR, Peroxisome proliferator-activated receptor; PI3K, Phosphatidylinositol-3 kinase;

# Declarations

## Author Declarations

### Funding

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### Conflict of Interest

The authors disclosed no conflict of interest.

### Ethics declarations

All these animal experiments followed the guideline of the Animal Experimentation Ethics Committee of Wuhan Polytechnic University (WHPU) and were approved by Ethics Committee of WHPU.

### Consent to participate

All of the authors consent to participate the study above.

### Consent for publication

The manuscript submitted to Fish Physiology and Biochemistry is under the permission of all authors.

### Data availability

Not applicable.

### Code availability

Not applicable.

### Authors' contributions

MQ Zhuo designed the experiments; MQ Zhuo and J Chen performed the experiments, and analyzed the samples with the help of ML Wu and WB Wang; J Chen and ML Wu analyzed the data; J Chen and MQ Zhuo wrote the manuscript. MQ Zhuo revised the manuscript. All the authors approved the manuscript.

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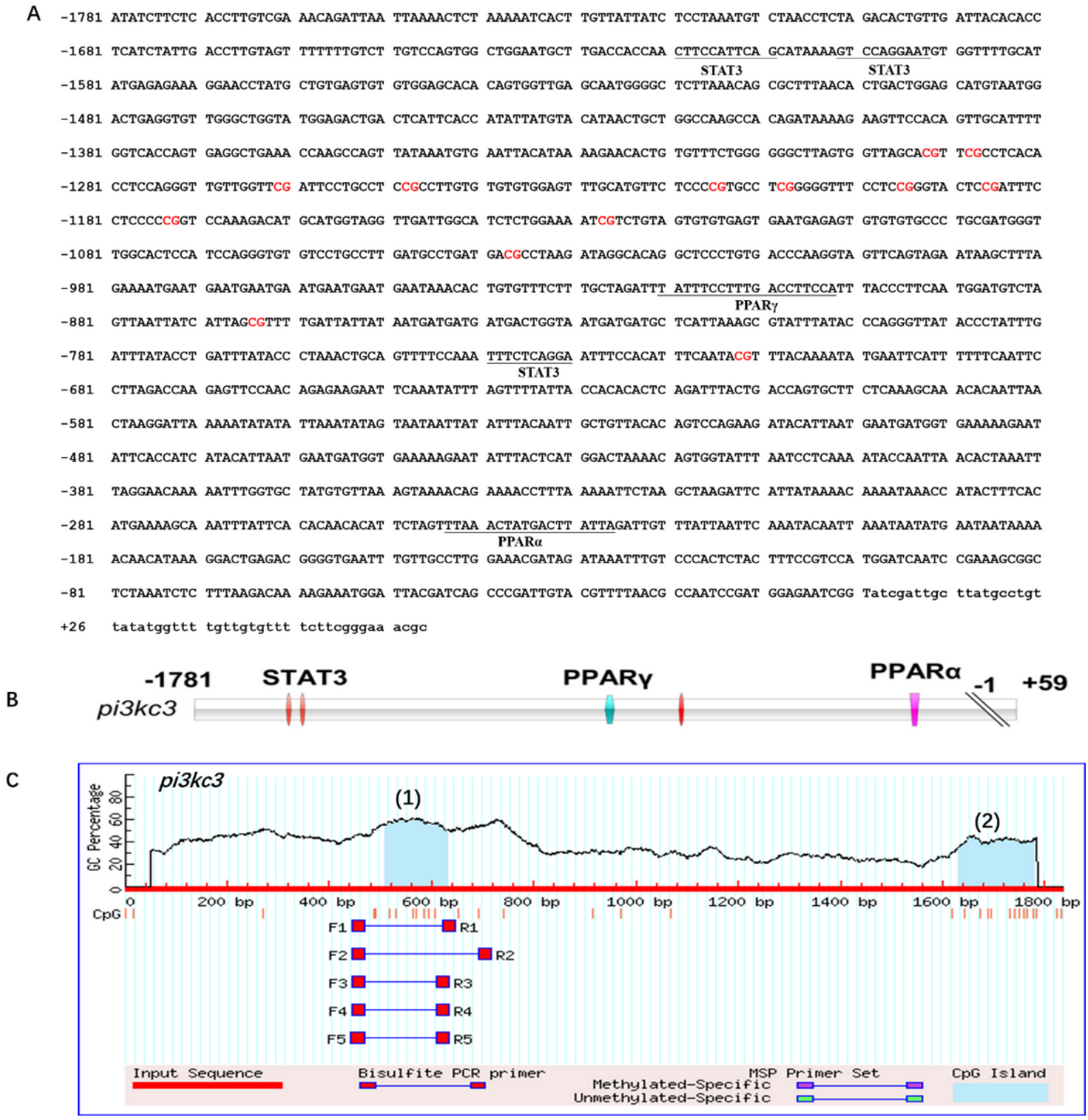
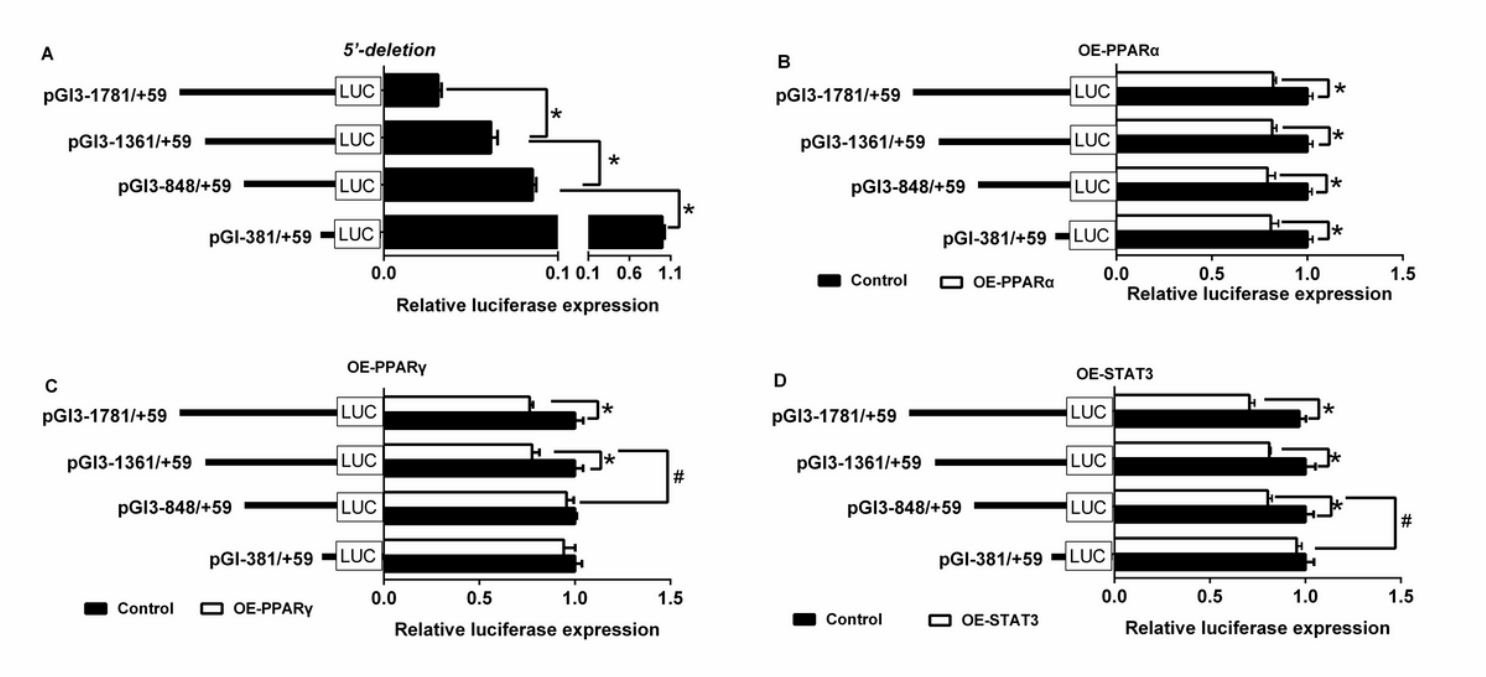
# Figures




## Figure 1

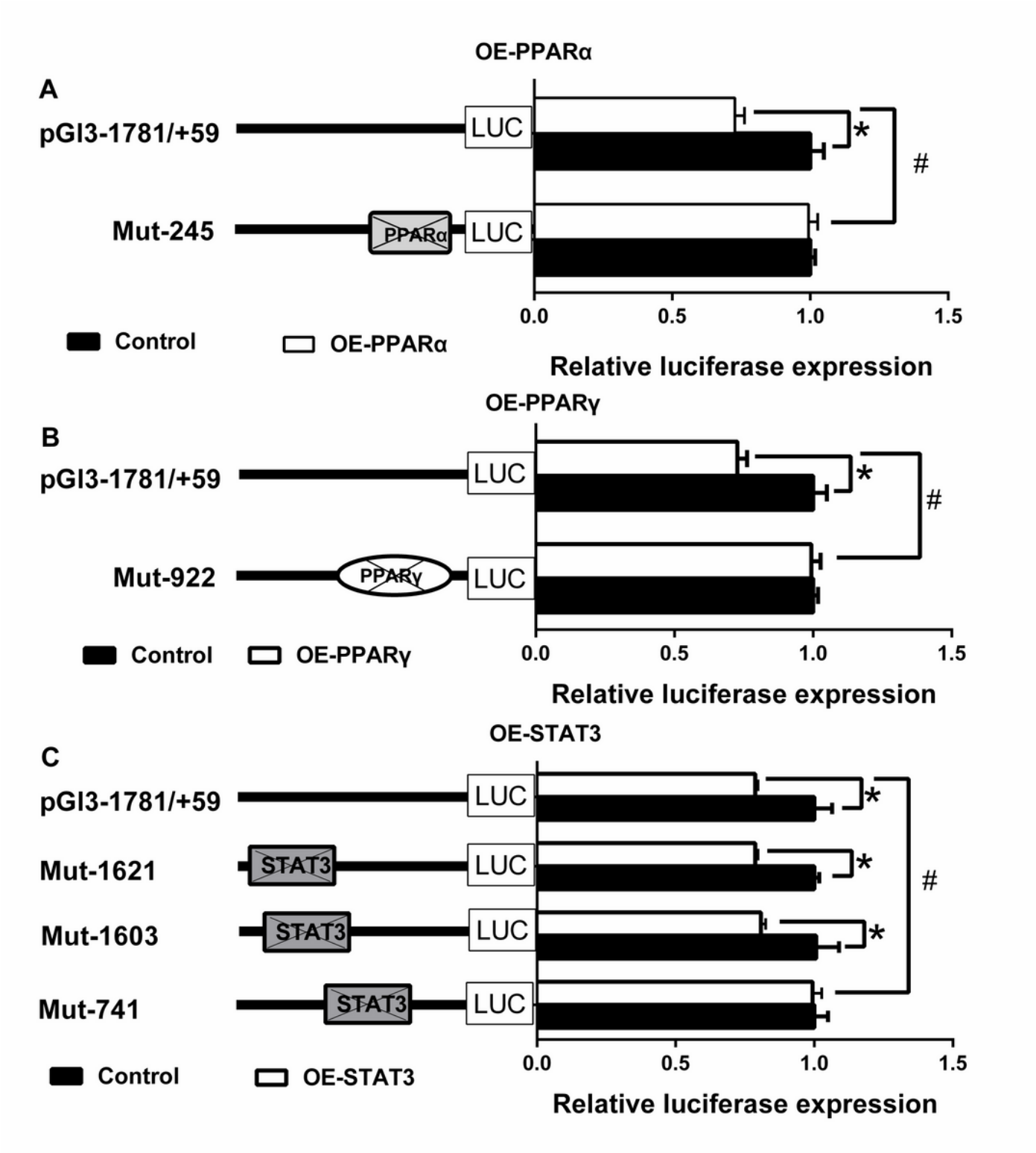
Sequences analysis of *pi3kc3* promoter. (A) Nucleotide sequences of the 5'-flanking region of *pi3kc3*, numbers are relative to the transcription start site (+1). The upstream sequences of transcription start site are in the capital letters, while the downstream sequences of transcription start site are in lowercase letters. The putative transcription factor binding sites are underlined. (B). The schematic diagram of

*pi3kc3* premotor structure; (C) The prediction results of CpG islands on *pi3kc3* promoter. The blue area indicate the region of CpG island.




## Figure 2

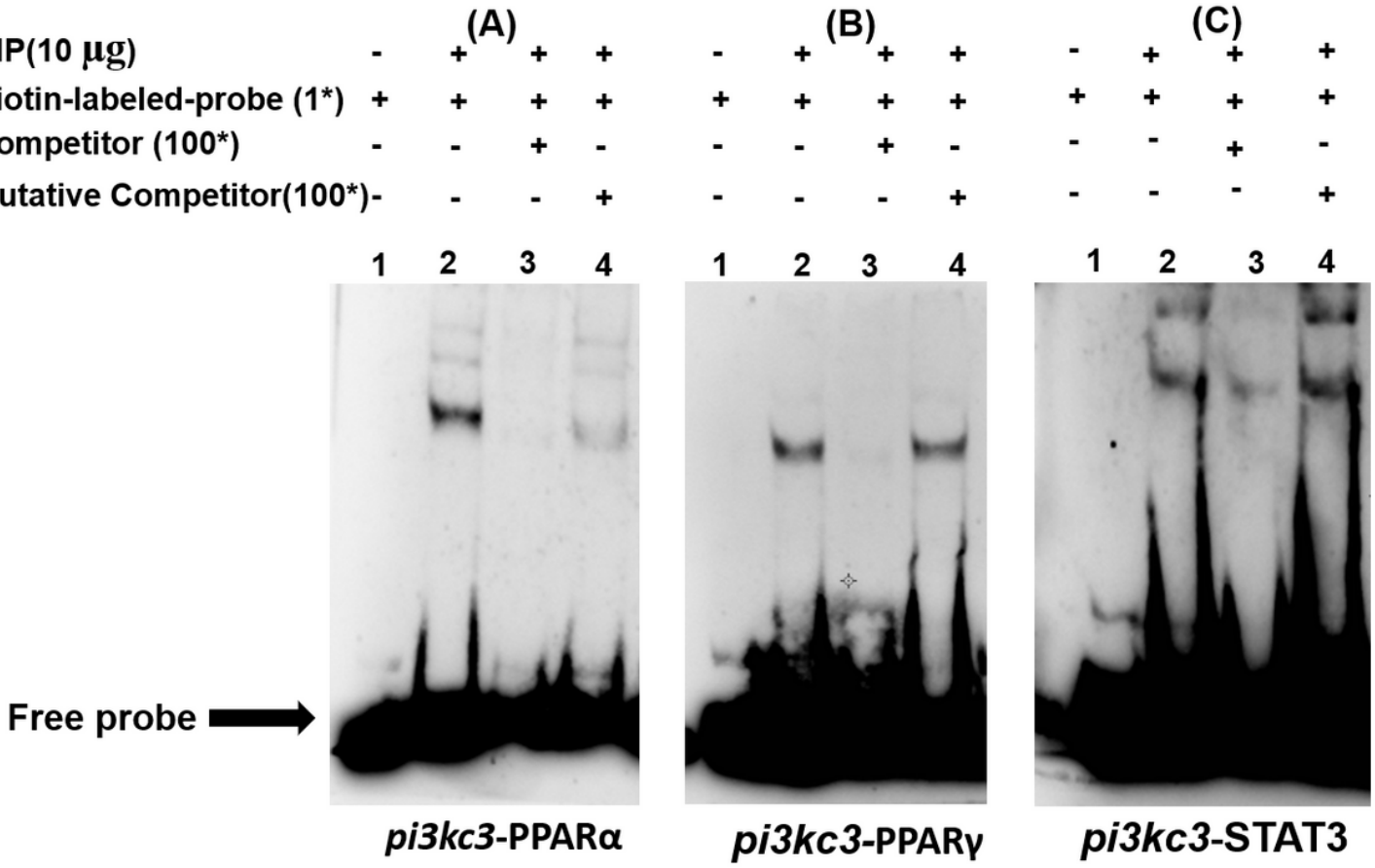
5'-deletion analysis of the *pi3kc3* promoter from yellow catfish. (A) A series of plasmids containing 5' unidirectional deletions of the *pi3kc3* promoter regions (pGl3-1781, -1361, -848 and -381) fused in frame to the luciferase gene were transfected into HEK293T cells; (B) Overexpression (OE) of PPARα analysis of 5' unidirectional deletion assays of the *pi3kc3 promoter* of yellow catfish; (C) Overexpression of PPARα analysis of 5' unidirectional deletion assays of the *pi3kc3 promoter* of yellow catfish; (D). Values are presented as mean ± SEM (n=3). Asterisk (*) indicates significant differences in relative luciferase activities between the Overexpression PPARα, PPARγ and STAT3 group and the control ($p$ 0.05). Hash symbol (#) indicates significant difference between the same OE groups with different deletion regions ($p < 0.05$).




## Figure 3

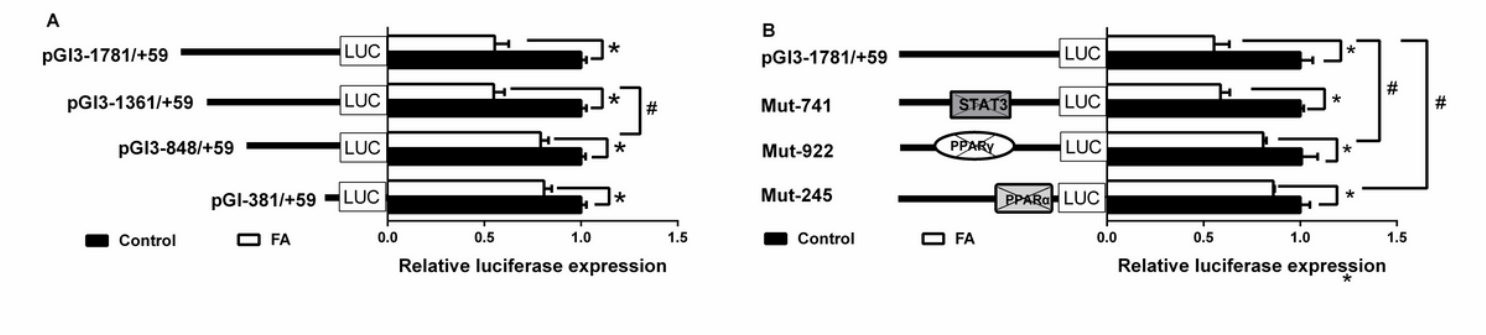
Assays of *pi3kc3* promoter activities by mutagenesis on predicted PPARα, PPARγ and STAT3 binding sites. (A) Site mutagenesis of PPARα site (-245/-230 bp) on pGl3-1781/+59 promoter; (B) Site mutagenesis of PPARγ site (-922/-907 bp) on pGl3-1781/+59 promoter; (C) Site mutagenesis of STAT3 sites (-741/-731 bp, -1621/-1611 bp and -1603/-1593 bp) on pGl3-1781/+59 promoter. Values are presented as mean ±SEM (n=3). Asterisk (*) indicates significant differences between PPARα, PPARγ and

STAT3 overexpression and the control ($p$ 0.05). Hash symbol (#) indicates significant differences between two plasmids under the same treatment ($p < 0.05$).




## Figure 4

EMSA analysis of predicted PPARα, PPARγ and STAT3 binding on *pi3kc3 promoter*. (A) -245/-220 bp PPARα binding site; (B) -922/-900 bp PPARγ binding site; (C) -743/-720 bp STAT3 binding site.




## Figure 5

Effect of FA incubation on the promoter activity of *pi3kc3*. (A) Effect of FA incubation on the promoter activity of 5'-deletion *pi3kc3* promoter; (B) Effect of FA incubation on the promoter activities of *pi3kc3*

promoter after PPARα, PPARγ and STAT3 mutagenesis. Values are presented as mean ±SEM (n=3). Asterisk (*) indicates significant differences between FA treatment and the control ($p$ 0.05). Hash symbol (#) indicates significant differences between two plasmids under the same treatment ($p < 0.05$).

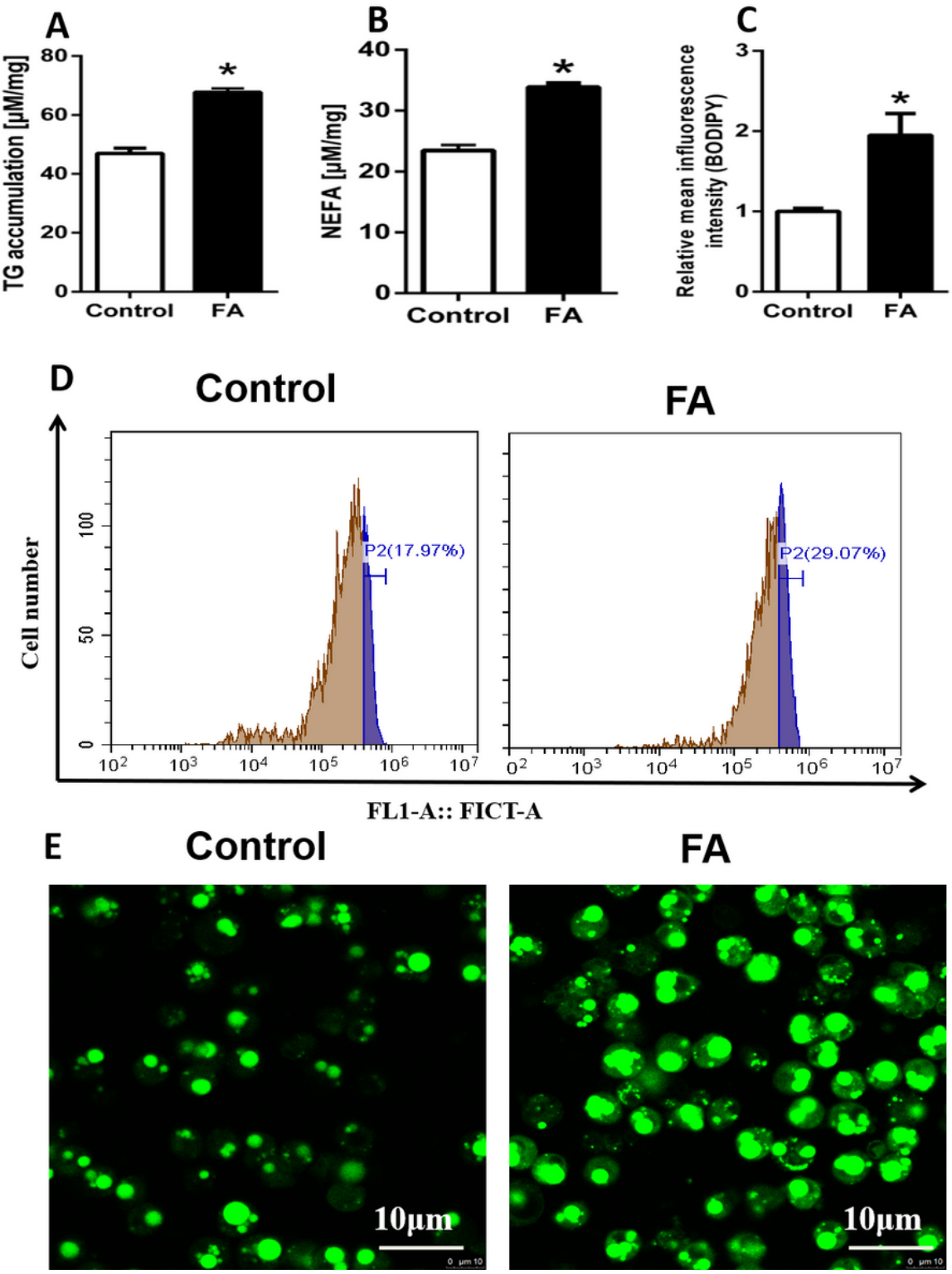
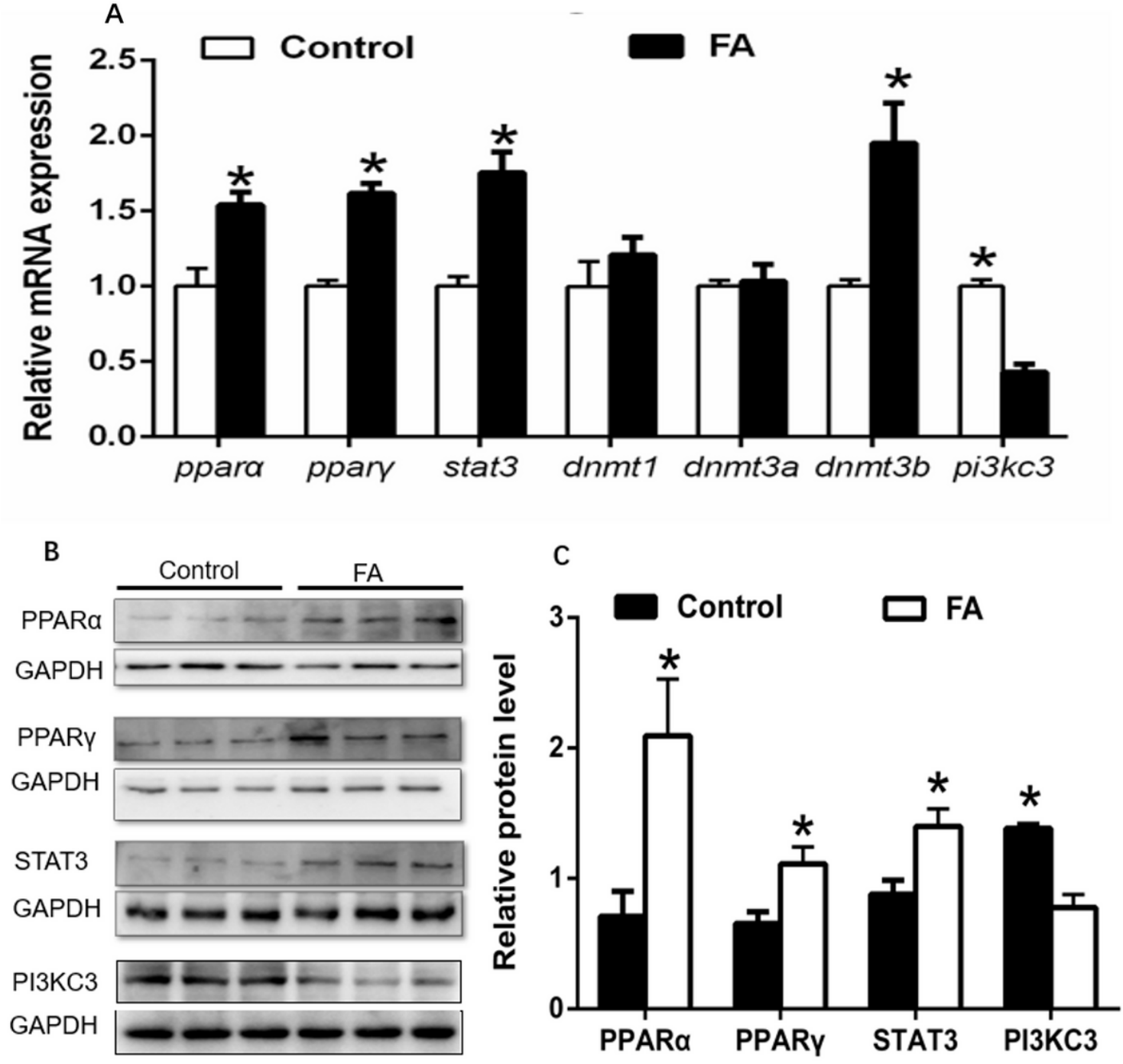



Figure 6

Effect of FA incubation on the TG content, NEFA content, and lipid content on the hepatocytes from yellow catfish. Asterisk (*) indicates significant differences between FA treatment and the control ($p$ < 0.05).




## Figure 7

Effect of FA incubation on the genes expression and the protein level of PI3KC3. (A) Effect of FA incubation on the mRNA level of *ppara, ppary, stat3, dnmts* and *pi3kc3*. (B) Effect of FA incubation on the

protein level of PI3KC3. Asterisk (*) indicates significant differences between FA treatment and the control ($p$ 0.05)

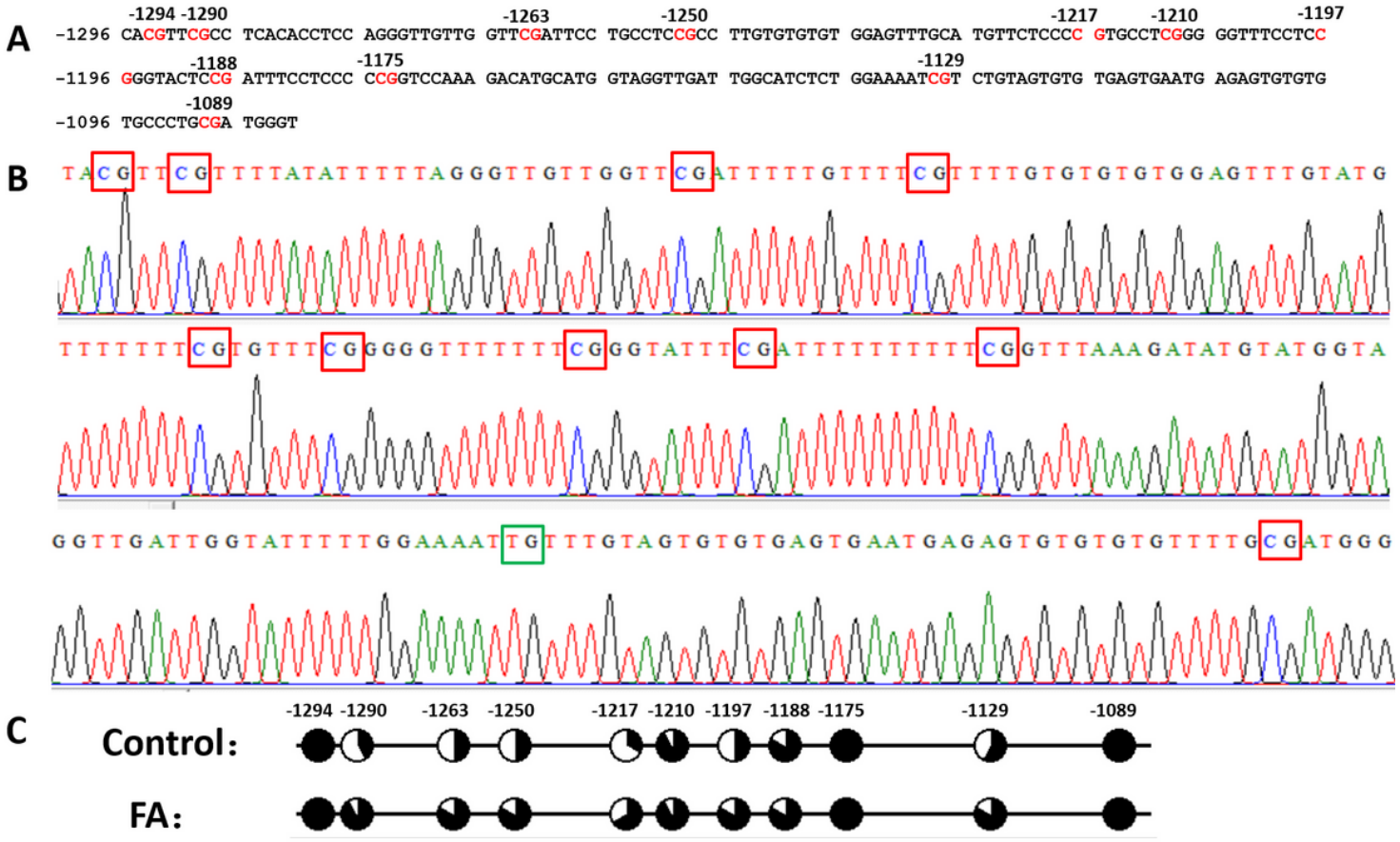



**Figure 8**

Effect of FA incubation on the methylation level of *pi3kc3*. (A) The putative result of first CpG islands in *pi3kc3* promoter and the amplification sequence by nest polymerase chain reaction (PCR) with two BSP specific primer pairs; (B) Sequencing chtomatogram analysis of partial bisulfite-modified DNA after amplification. The red rectangles indicate methylated CpG locus; (C) Methylation status of individual CpG dinucleotides of *pi3kc3* in the hepatocytes of yellow catfish after FA incubation. Different percentages of methylation are represented by the proportion of black area in the circle;

# Supplementary Files

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- Supplementarytables.docx