Novel insights for PI3KC3 in mediating lipid accumulation in yellow catfish *Pelteobagrus fulvidraco*

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Abstract In this study, the transcriptional regulation of PI3KC3 by three transcription 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) content, non-esterified fatty acid (NEFA) content, and lipid drops (LDs) content, the mRNA level of *ppara*, *pparg*, *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.

Keywords PI3KC3 · Transcriptional regulation · Methylation · Lipid accumulation · Fish

Abbreviations
- FA: Fatty acid
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- HPRT: Hypoxanthineguanine phosphoribosyltransferase
- LDs: Lipid drops
- NEFA: Non-esterified fatty acid
- Mut: Mutagenesis
- NP: Nuclear protein
- PPAR: Peroxisome proliferator-activated receptor
- OE: Overexpression
- PI3K: Phosphatidylinositol-3 kinase
- RPL7: Ribosomal protein L7
- STAT3: Signal transducers and activators of transcription proteins 3
- TG: Triacylglyceride
- TUBA: Tubulin alpha chain
- UBCE: Ubiquitin-conjugating enzyme

Introduction

Phosphatidylinositol-3 kinases (PI3Ks) are the key signaling molecules, which control 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). We only found that FOXO1 positively regulated the transcription of *pi3kc3* due to the short length of *pi3kc3* promoter sequence we have obtained (Zhuo et al. 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) belong to a family of latent cytoplasmic transcription factors, which participate in gene regulation. STAT3 is a member of STAT family, which modulates 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 modulate 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 and PPARγ, and played an important role in the regulation of cellular lipid metabolism (Zhuo et al. 2015; 2018; Yang et al. 2018). However, limited studies 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 mediated by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B (Nagase and Ghosh, 2008). Many studies suggested that aberrant DNA methylation was 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. 2013; 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 the gene. Interestingly, two CpG islands were predicted on the promoter of *pi3kc3*, which attracting 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 provided good basis for exploring the regulatory mechanism of lipid metabolism and for amplifying the long length sequence of *pi3kc3* promoter. Moreover, our previous study suggested that PI3K pathways were involved in regulating lipid metabolism. HEK293T is a good cell line, with very high transfection efficiency during the transfection, which has been widely used to study the function of fish promoters (Xu et al. 2017; Chen et al. 2020a, b; LV et al. 2021). To further investigate the function of PI3KC3 promoter and the regulatory mechanism of PI3KC3 in mediating lipid accumulation in yellow catfish. In this study, the transcriptional regulation of *pi3kc3* by three transcript factors (PPARγ, PPARα, and STAT3) was studied by using HEK293T. Our results suggested that the promoter activity of *pi3kc3* was 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*. Our study elucidated innovative insights into the regulatory mechanism of PI3KC3 in fish.

### Materials and methods

**Experimental animals and reagents**

To eliminate gender-differentiated response, the mixed sex yellow catfish (body weight: 22.5 ± 4.4 g, male: female = 1:1) 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 Eagle’s Medium (DMEM), 0.25% trypsin–EDTA, and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen, USA. Dimethyl...
sulfoxide (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 six yellow catfish (male: female = 1:1) by using a commercial DNA extracted kit (Omega, Norcross, GA, USA). The promoter sequence of pi3kc3 was obtained by RT-PCR (reverse transcription-polymerase chain reaction) according to the genome of yellow catfish (Gong et al. 2018). The primers for pi3kc3 promoter cloning are 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 basis of the distance from its TSS, we named the plasmid as pGl3-1781/+59 of pi3kc3 promoter. Then, we used pGl3-1781/+59 vector as the template 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 are 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% FBS (Gibco, Carlsbad, CA, USA) in an incubator (5% CO2 and 37 °C). Prior to the transfection, HEK293T cells were seeded at a density of 1.2×10^5 in 24-well plate. They were cultured until the 70–80% confluence. Lipo-lectamine™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, 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 and three independent experiments were carried out.
Site-mutation analysis of binding sites on the \textit{pi3kc3} promoter

To identify the corresponding binding sites on the regions of \textit{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 \textit{pi3kc3} promoter. The primers used for mutagenesis are 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. We conducted all these experiments in triplicates and three independent experiments were carried out.

Electrophoretic mobility shift assay (EMSA)

The EMSA was conducted to confirm the functional PPAR\(\alpha\), PPAR\(\gamma\), and STAT3 binding sites on the \textit{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 \(\mu\)g) were incubated for 30 min at room temperature by using the binding buffer (20 mM HEPES, pH7.9, 1 mM MgCl\(_2\), 0.5 mM DTT, 4% Ficoll, 110 mM KCl, 0.2 \(\mu\)g 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 unlabelled 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 (each independent experiment six fish were used, male: female = 1:1) 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.

Triacylglyceride (TG), non-esterified fatty acid (NEFA), and lipid drops (LDs) assay

TG and NEFA contents were determined with commercial kits (Nanjing Jian Cheng Bioengineering Institute, China), according to the manufacturer’s instructions. Bodipy 493/503 staining was used to assess the changes of intracellular 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 LDs, 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 quantitative PCR (RT-qPCR)

Total RNA was isolated using Trizol reagent (TaKaRa, Dalian, China) according to the manu-
facturer’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 \textit{pparα}, \textit{pparγ}, \textit{stat3}, \textit{dnmt1}, \textit{dnmt3a}, \textit{dnmt3b}, and \textit{pi3kc3} were examined by RT-qPCR. RT-qPCR 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 (tenfold), 10 mM each of forward and reverse primers (0.4 μL), and 8.2 μL H2O. Primers are given in Table S5. The qPCR 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 (\textit{β-actin}, \textit{18s rrna}, \textit{gapdh}, \textit{rpl7}, \textit{hprt}, \textit{ubce}, and \textit{tuba}) were selected from the literature (Vandesompele et al. 2002) in order to test their transcription stability. Two most stable control genes (\textit{gapdh} and \textit{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 (15,540–1-AP, Proteintech, USA), rabbit anti-PPARγ (16,643–1-AP, Proteintech, USA), rabbit anti-STAT3 (10,253–2-AP, Proteintech, USA), rabbit anti-PI3KC3 (AbClone, A12295, USA), and anti-GAPDH (10,494–1-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 island of \textit{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 \textit{pi3kc3} promoter were predicted. The bisulphite-modified DNA was amplified by nest PCR with two BSP (bisulphite 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. Sequencing chromatogram analysis of partial bisulphite-modified DNA after amplification is shown in Fig. S1. The methylation level was analyzed by the online website (http://quma.cdb.riken.jp/).

Statistical analysis

We used SPSS 19.0 software for all these statistical analyses. 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 \textit{pi3kc3} promoter

In the present study, we successfully obtained -1781 bp of \textit{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. S2A,B). In addition, two CpG islands were also predicted on the promoter of pi3kc3 (Fig. S2C).

5'-Deletion analysis

Compared to the full sequence of the pi3kc3 promoter, deletion of the sequences from -1781 to -1361 bp, -1361 to -848 bp, and -848 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. 1A).

Compared to the control group (overexpression of pcDNA3.1), 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. 1B). Compared to the control group (overexpression of pcDNA3.1), 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. 1C). Compared to the control group

![Fig. 1](https://example.com/figure1)

**Fig. 1** 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 of PPARα analysis of 5' unidirectional deletion assays of the pi3kc3 promoter of yellow catfish; C overexpression of PPARγ analysis of 5' unidirectional deletion assays of the pi3kc3 promoter of yellow catfish; D overexpression of STAT3 analysis of 5' unidirectional deletion assays of the pi3kc3 promoter of yellow catfish. Values are presented as mean ± SEM (n = 3). Asterisk (*) indicates significant difference in relative luciferase activities between the overexpression of the PPARα, PPARγ, and STAT3 group and the control (p < 0.05). Hash symbol (#) indicates significant difference between the same overexpression group with different deletion regions (p < 0.05). OE is the abbreviation of overexpression.
(overexpression of pcDNA3.1), overexpression of STAT3 significantly reduced the promoter activities of pGI3-1781/+59, pGI3-1361/+59, and pGI3-848/+59 plasmids, and showed no influence on the promoter activity of pGI3-381/+59 plasmid (Fig. 1D).

Site-mutation analysis

To further elucidate whether the regions of pi3kc3 promoter possessed PPARα, PPARγ, and STAT3 response elements, site-directed mutations 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. 2A).

Overexpressed PPARγ inhibited the promoter activity of the wide-type pi3kc3 plasmid. However, mutation of 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. 2B).

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. 2C).

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. 3A). Similar results are also found for the PPARγ binding sequence (-922/-900 bp of pi3kc3 promoter, Fig. 3B) and STAT3 binding sequence (-743/-720 bp of pi3kc3 promoter, Fig. 3C), 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. Compared with the control group, FA incubation significantly reduced the luciferase activities of pGI3-1781/+59, pGI3-1361/+59, pGI3-848/+59, and pGI3-381/+59 of pi3kc3 plasmids (Fig. 4A). 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 pGI3-1781/+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. 4B).

Fig. 3 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. NP is the abbreviation of nuclear protein. The symbols “+” or “−” in the top row indicate the presence or absence of nuclear protein extract, probes, competitors, and mutative competitors.

Fig. 4 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 activity of pi3kc3 promoter after PPARα, PPARγ, and STAT3 mutagenesis. Values are presented as mean ± SEM (n = 3). Asterisk (*) indicates significant difference between the FA treatment and the control (p < 0.05). Hash symbol (#) indicates significant difference between two plasmids under the same treatment (p < 0.05).
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 LDs content in the hepatocytes from yellow catfish (Fig. 5).

Effect of FA incubation on gene expression, protein expression, and methylation level

For the mRNA expression assay, compared to the control group, FA incubation notably up-regulated the mRNA level of \textit{ppara}, \textit{pparg}, \textit{stat3}, and \textit{dnmt3b}, but down-regulated the mRNA level of \textit{pi3kc3}, and showed no effect on the mRNA level of \textit{dnmt1} and \textit{dnmt3a} in the hepatocytes from yellow catfish (Fig. 6A).

For the protein expression assay, 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. 6B,C).

For the methylation level assay, compared to the control group, FA incubation increased the methylation level of \textit{pi3kc3} promoter at -1290, -1263, -1250, -1217, -1197, and -1129 CpG sites (Fig. 6D).

\textbf{Fig. 5} Effect of FA incubation on the TG content (A), NEFA content (B), and LDs content (C) in the hepatocytes from yellow catfish. D The presence of Bodipy 493/503–stained LDs was assayed by flow cytometry. E Representative confocal microscopy image of hepatocytes stained with Bodipy 493/503. Asterisk (*) indicates significant difference between the FA treatment and the control ($p < 0.05$).
Discussion

At present, the underlying transcriptional mechanism of PI3KC3 and the role of PI3KC3 in regulating lipid metabolism in yellow catfish remain largely unknown. In order to identify the role of pi3kc3 in regulating lipid metabolism, it is very important to explore the transcriptional regulation of pi3kc3 by transcription 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 PI3K catalytic 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 regulatory subunit have never been reported. In this study, we found 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.

Fig. 6 Effect of FA incubation on gene expression, protein expression, and methylation level in the hepatocytes from yellow catfish. A Effect of FA incubation on the mRNA levels of pparα, pparγ, stat3, dnmts, and pi3kc3. B, C Effect of FA incubation on the protein levels of PPARα, PPARγ, STAT3, and PI3KC3. D 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. Asterisk (*) indicates significant difference between the FA treatment and the control (p < 0.05)
genes related to lipid metabolism (Wu et al. 2018). It has been reported that PI3K and STAT3 were interdependent in many cellular processes (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 direct 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, 2020; Chen et al. 2020a, b; Song et al. 2020). In addition, FA incubation significantly reduced the mRNA and the protein levels of PI3KC3, but increased the mRNA and the protein levels 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 revealed that FA-induced hepatocellular TG accumulation coincided with the decreasing expression of PI3KC3. However, previous studies have suggested that there was a positive 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 PI3KCα and PI3KCβ resulted in the decreasing of their 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 probably not the prioritized binding under FA treatment.

On the other hand, DNA methylation has a specific effect on gene expression, and 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 were 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 that 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 the increased 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 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 transcriptional 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 expression of PI3KC3 was regulated by DNA methylation in the hepatocytes of yellow catfish.

**Author contribution** 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; and J Chen and MQ Zhuo wrote the manuscript. MQ Zhuo revised the manuscript. All the authors approved the manuscript.
Funding This work was supported by the National Natural Science Foundation of China (Grant No. 31902381).

Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval 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.

Conflict of interest The authors declare no competing interests.

References

Abell K, Bilancia A, Clarkson RW, Tiffen PG, Altparmakov AI, Burdon TG, Watson CJ (2005) Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition. Nat Cell Biol 7:392–398

Barberio MD, Nadler EP, Sevilla S, Lu R, Harmon B, Hubal MJ (2019) Comparison of visceral adipose tissue DNA methylation and gene expression profiles in female adolescents with obesity. Diabetol Metab Syndr 11:98

Bird A (2002) DNA methylation patterns and epigenetic memory. Gene Dev 16:6–21

Chan TM, Leung KS, Lee KH (2007) TFBS identification based on genetic algorithm with combined representations and adaptive post-processing. Bioinformatics 24:341–349

Chen GH, Lv W, Xu YH, Wei XL, Xu YC, Luo Z (2020) Functional analysis of MTF-1 and MT promoters and their transcriptional response to zinc (Zn) and copper (Cu) in yellow catfish Pelteobagrus fulvidraco. Chemosphere 246:125–792

Chen GH, Wu K, Zhao T, Ling SC, Liu W, Luo Z (2020) MiR-144 mediates high fat-induced changes of cholesterol metabolism via direct regulation of c/ebpα in the liver and isolated hepatocytes of yellow catfish. J Nutr 150:464–474

Chu SC, Chen PN, Hsieh YS, Yu CH, Lin MH, Lin YH, Kuo DY (2014) Involvement of hypothalamic PI3K-STAT3 signalling in regulating appetite suppression mediated by amphetamine. Br J Pharmacol 171:322–333

Foster FM, Traer CJ, Abraham SM, Fry MJ (2003) The phosphoinositide (PI) 3-kinase family. J Cell Sci 116:3037–3040

Ge ZJ, Luo SM, Lin F, Liang QX, Huang L, Wei YC, Sun QY (2013) DNA methylation in oocytes and liver of female mice and their offspring: effects of high-fat-diet–induced obesity. Environ Health Persp 122:159–164

Gong G Dan C Xiao S Guo W Huang P Xiong Y Chen N 2018 Chromosomal-level assembly of yellow catfish genome using third generation DNA sequencing and Hi-C analysis Giga Science 7 gyi120

Hart JR, Liao L, Yates JR3rd, Vogt PK (2011) Essential role of Stat3 in PI3K-induced oncogenic transformation. Proc Natl Acad Sci USA 108:13247–13252

Hunter DJ, James L, Hussey B, Wadley AJ, Lindley MR, Mastana SS (2019) Impact of aerobic exercise and fatty acid supplementation on global and gene-specific DNA methylation. Epigenetics 14:294–309

Kim AY, Park YJ, Pan X, Shin KC, Kwak SH, Bassas AF, Sal-lam RM, Park KS, Alfeld AA, Xu A, Kim JB (2015) Obesity-induced DNA hypermethylation of the adiponectin gene mediates insulin resistance. Nat Commun 6:7585

Kok K, Geering BA, Vanhaesebroeck B (2009) Regulation of phosphoinositide 3 kinase expression in health and disease. Trends Biochem Sci 34(115):127

Li W, Tang R, Ma F, Ouyang S, Liu Z, Wu J (2018) Folic acid supplementation alters the DNA methylation profile and improves insulin resistance in high-fat-diet-fed mice. J Nutr Biochem 59:76–83

Liu L, Song X, He D, Komma C, Kita A, Virbasius JV, Zhou GW (2006) Crystal structure of the C2 domain of class II phosphatidylinositol 3-kinase C2α. J Biol Chem 281:4254–4260

Lv WH, Chen GH, Zhuo MQ, Xu YH, Xu YC, Tan XY (2021) Functional analysis of steroidogenic factor 1 (sf-1) and 17α-hydroxylation/lyase (cyp17α) promoters in yellow catfish Pelteobagrus fulvidraco. Int J Mol Sci 22:195

Marco A, Kisliouk T, Tabachnik T, Meiri N, Weller A (2014) Overweight and CpG methylation of the Pome promoter in offspring of high fat diet fed dams are not “reprogrammed” by regular chow diet in rats. FASEB J 28:4148–4157

Moore LD, Le T, Fan G (2013) DNA methylation and its basic function. Neuropsychopharmacology 38:23–38

Nagase H, Ghosh S (2008) Epigenetics: differential DNA methylation in mammalian somatic tissues. FEBS J 275:1617–1623

Pankow S, Bamberger C, Klippel A, Werner S (2006) Regulation of epidermal homeostasis and repair by phosphoinositide 3-kinase. J Cell Sci 119:4033–4046

Parrillo L, Costa V, Raciti GA, Longo M, Spinelli R, Esposito R, Nigro C, Vastolo V, Desiderio A, Zatterale F, Ciccodi cola A, Formisano P, Miele C, Beguinot F (2016) Hoxa5 undergoes dynamic DNA methylation and transcriptional repression in the adipose tissue of mice exposed to high-fat diet. Int J Obesity 40:929–937

Parsanathan R, Jain SK (2019) Glutathione deficiency induces epigenetic alterations of vitamin D metabolism genes in the livers of high-fat-diet-fed obese mice. Sci Rep 9:14784

Pfafl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29:45–45

Read MA, Cordle SR, Veach RA, Carlisle CD, Hawiger J (1993) Cell-free pool of CD14 mediates activation of transcription factor NF-kappa B by lipopolysaccharide in human endothelial cells. Proc Natl Acad Sci USA 90:9887–9891
Rieusset J, Auwerx J, Vidal H (1999) Regulation of gene expression by activation of the peroxisome proliferator-activated receptor g with Rosiglitazone (BRL 49653) in human adipoocytes. Biochem Biophys Res Commun 265:265–271

Rieusset J, Chambrier C, Bouzakri K, Dusserre E, Auwerx J, Riou JP, Vidal H (2001) The expression of the p85alpha subunit of phosphatidylinositol 3-kinase is induced by activation of the peroxisome proliferator-activated receptor gamma in human adipoocytes. Diabetologia 44:544–554

Rieusset J, Roques M, Bouzakri K, Chevillotte E, Vidal H (2001) Regulation of p85phosphatidylinositol3-kinase expression by peroxisome proliferator-activated receptors (PPARs) in human muscle cells. FEBS Lett 502:98–100

Siegfried Z, Simon I (2010) DNA methylation and gene expression. Wiley Interdiscip Rev Syst Biol Med 2:362–371

Smith PK, Krohn RJ, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinechonic acid. Anal Biochem 150:76–85

Song YF, Hogstrand C, Ling SC, Chen GH, Luo Z (2020) Creb Pgc1alpha pathway modulates the interaction between lipid droplets and mitochondria and influences high fat diet-induced changes of lipid metabolism in the liver and isolated hepatocytes of yellow catfish. J Nutr Biochem 80:108364

Vandesompele J, De-Preeter K, Pattyn F, Poppe B, Van-Roy N, De-Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:R0034

Vogt PK, Hart JR (2011) PI3K and STAT3. New Alliance Cancer Discov 1:481–486

Wang D, Sul HS (1998) Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway involvement of protein kinase B/Akt. J Biol Chem 273:25420–25426

Wu K, Tan XY, Xu YH, Chen GH, Zhuo MQ (2018) Functional analysis of promoters of genes in lipid metabolism and their transcriptional response to STAT3 under leptin signals. Genes 9:334

Wu K, Tan XY, Xu YH, Chen QL, Pan YX (2016) JAK and STAT members of yellow catfish Pelteobagrus fulvidraco and their roles in leptin affecting lipid metabolism. Gen Comp Endocrinol 226:14–26

Wu K, Zhao T, Hogstrand C, Xu YC, Ling SC, Chen GH, Luo Z (2020) FXR-mediated inhibition of autophagy contributes to FA-induced TG accumulation and accordingly reduces FA-induced lipotoxicity. Cell Commun Signal 18(1):47

Wu LX, Wei CC, Yang SB, Zao T, Luo Z (2019) Effects of fat and fatty acids on the formation of autolysosomes in the livers from yellow catfish Pelteobagrus Fulvidraco. Genes 10:10

Xu YH, Luo Z, Wu K, Fan YF, You WJ, Zhang LH (2017) Structure and functional analysis of promoters from two liver isoforms of CPT I in grass carp Ctenopharyngodon Idella. Int J Mol Sci 18:11

Yang Q, Jiang W, Hou P (2019) Emerging role of PI3K/AKT in tumor-related epigenetic regulation. Semin Cancer Biol 59:112–124

Yang X Fu Y Hu F Luo X Hu J Wang G 2018 PIK3R3 regulates PPARx expression to stimulate fatty acid β-oxidation and decrease hepatosteatosis. Exp Mol Med 50 e431

Zhang P, Chu T, Dedousis N, Mantell BS, Sipula I, Li L, Argmann C (2017) DNA methylation alters transcriptional rates of differentially expressed genes and contributes to pathophysiology in mice fed a high fat diet. Mol Metab 6:327–339

Zheng JL, Zhuo MQ, Luo Z, Pan YX, Song YF, Huang C, Zhu QL, Hu W, Chen QL (2015) Peroxisome proliferator-activated receptor gamma (PPARG) in yellow catfish Pelteobagrus fulvidraco: molecular characterization, mRNA expression and transcriptional regulation by insulin in vivo and in vitro. Gen Comp Endocrinol 212:51–62

Zheng JL, Zhuo MQ, Luo Z, Song YF, Pan YX, Huang C, Hu W, Chen QL (2015) Peroxisome proliferator-activated receptor alpha1 in yellow catfish Pelteobagrus fulvidraco: molecular characterization, mRNA tissue expression and transcriptional regulation by insulin in vivo and in vitro. Comp Biochem Physiol B Biochem Mol Biol 183:58–66

Zhong W, Fan B, Cong H, Wang T, Gu J (2019) Oleic acid-induced perilipin 5 expression and lipid droplets formation are regulated by the PI3K/PPARx pathway in HepG2 cells. Appl Physiol Nutr Metab 44(8):840–848

Zhuo MQ, Luo Z, Pan YX, Wu K, Fan YF, Zhang LH, Song YF (2015) Effects of insulin and its related signaling pathways on lipid metabolism in the yellow catfish Pelteobagrus fulvidraco. J Exp Biol 218:3083–3090

Zhuo MQ, Luo Z, Xu YH, L DD, Pan YX, Wu K, (2018) Functional analysis of promoters from three subtypes of the pi3k family and their roles in the regulation of lipid metabolism by insulin in yellow catfish Pelteobagrus fulvidraco. Int J Mol Sci 19:265

Zhuo MQ, Pan YX, Wu K, Xu YH, Luo Z (2017) Characterization and mechanism of phosphoinositide 3-kinases (PI3Ks) members in insulin-induced changes of protein metabolism in yellow catfish Pelteobagrus fulvidraco. Gen Comp Endocrinol 247:34–45

Zhuo MQ, Yang SB, Ling S, Luo Z (2019) Effects of dietary lipid on lipid metabolism, methylation and expression of PI3KCa in the ovary of yellow catfish (Pelteobagrus fulvidraco). J Fish China 43:149–158

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