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Short Communication

# Molecular characterization and expression analysis of PPP1R3C in hypoxia-tolerant Indian catfish, *Clarias batrachus* (Linnaeus, 1758) under hypoxia

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ABSTRACT

Hypoxia is an important environmental stressor that leads to rapid adaptive changes in metabolic organization. However, the molecular mechanisms of hypoxia tolerance in fish remain largely unknown. The present work was focused on understanding the molecular mechanisms and signaling pathways that may lead to tolerance of *Clarias batrachus* to hypoxic stress. Protein phosphatase 1 regulatory subunit 3C (PPP1R3C) is a new hypoxia-inducible factor (HIF) targeted gene and is regulated by HIF-1 under hypoxic conditions. Overexpression of PPP1R3C increases glycogen accumulation through activation of several enzymes and processes. In this study, for the first time, full length cDNA of PPP1R3C from *C. batrachus* was characterized and its expression pattern in the brain, liver, muscle and spleen under short (progressive hypoxia; PH, 1 h, 6 h and 12 h) and long-term (natural) hypoxic conditions was investigated. The complete cDNA of PPP1R3C was of 1499 bp, encoding 285 amino acid residues. The identified protein had a protein phosphatase 1 binding motif and a carbohydrate binding domain, thought to be involved in the regulation of glycogen metabolism. Short-term hypoxia exposure caused significant increase in PPP1R3C transcripts in the liver (6 h; 6.96 fold and 12 h; 3.91 fold) and muscle (progressive hypoxia; 3.46 fold), while, after long-term hypoxia exposure, significant up-regulation in the liver (7.77 fold) and spleen (6.59 fold) tissues was observed. No significant differences were observed in the brain for any time periods. Thus PPP1R3C may play an important role in the tolerance of *C. batrachus* to hypoxia.



## 1. Introduction

In many aquatic ecosystems, environmental hypoxia, due to low dissolved oxygen, is a naturally occurring phenomenon. Many hypoxia tolerant species adapt to these low oxygen levels by activating a signaling cascade driven by the transcription factor, hypoxia-inducible factor (HIF), which is well characterized in mammalian systems, but has only recently been identified in fish (Chen et al., 2012; Rahman and Thomas, 2007; Rytkonen et al., 2007; R.J. Shen et al., 2010). The HIFs play a major role in species adaptation to hypoxia by regulating the expression of numerous genes involved in angiogenesis, glycolysis, apoptosis, metastasis, proliferation and differentiation, while the majority of the gene products involved in glycolysis are up-regulated (Pelletier et al., 2012). HIF mediates the uptake of glucose as well as induces the transcription of glucose transporters (Chen et al., 2001). Recently, it was identified that apart from regulating the glycolysis, glycogenolysis is emerging as a new metabolic survival pathway and some reports have described the glycogen accumulation in cells under hypoxia conditions (Pescador et al., 2010; Vigoda et al., 2003).

G.M. Shen et al. (2010) reported that in human MCF-7 cells, under hypoxia, the protein phosphatase 1 regulatory subunit 3C gene (PPP1R3C) was regulated by HIF-1, which activated glycogen synthase and limited the glycogen breakdown. It was identified as a new HIF-targeted gene (Ortiz-Barahona et al., 2010) which acts as a glycogen-targeting subunit for protein phosphatase-1 (PP1) and regulates its activity. PP1 in turn plays a key role in the regulation of glycogen metabolism, through the dephosphorylation of glycogen synthase, phosphorylase and phosphorylase kinase (Armstrong et al., 1997). In humans, PPP1R3C is most highly expressed in the skeletal muscle and liver, but the mRNA was also present in a wide variety of other tissues (Doherty et al., 1996). However till date, there are no reports in fishes describing the role of PPP1R3C under any physiological conditions.

The Indian catfish *Clarias batrachus* (commonly known as "mangur") is an air-breathing teleost, endemic to the Indian subcontinent (Chonder, 1999). The fish inhabits in ponds, lakes, swamps and various habitats, which include those deficient in dissolved oxygen. The fish can survive these habitats of low dissolved oxygen for long periods which can be lethal for other organisms (Mohindra et al., 2013; Saha and Ratha, 2007). These features make *C. batrachus* a useful model to

*Abbreviations:* rRNA, ribosomal RNA; UTR, untranslated region; ORF, open reading frame; qRT-PCR, quantitative real-time PCR; HIF, hypoxia inducible factor; PTG, protein targeting to glycogen; CBD21, carbohydrate binding domain type 21; MEGA4, Molecular Evolutionary Genetics Analysis version 4; polyA$^+$, poly adenylational; pI, isoelectric point; MW, molecular weight; kDa, kilodalton.

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study the mechanism of hypoxia tolerance. The objective of the present study was to elucidate the role of PPP1R3C for hypoxia tolerance in C. *batrachus* by its characterization and expression in different tissues, under short and long-term hypoxia.

## 2. Materials and methods

### 2.1. Animals and hypoxic conditions

Live fish (30–80 g, 16–20 cm) were collected from commercial catches and were brought to the laboratory for acclimatization. Every care was taken not to cause distress to the fish during transportation. Fish were acclimatized at normoxia (5.00 ± 0.1 mg/l, dissolved oxygen), at least for a month in tanks of 100 liter capacity filled with 25 l of water at 22 ± 3 °C. Processed feed of goat liver or flesh and soybean powder was provided to feed them once a day. Feeding was stopped 48 h before the start of experiment. The hypoxic treatments to fish were as given in Tripathi et al. (2013). Briefly, fish were kept in water with the decline in dissolved oxygen concentration by fish's own respiration (progressive hypoxia, referred to as PH) up to 0.98 ± 0.1 mg/l of dissolved oxygen (DO), which was maintained by aeration for 1, 6 and 12 h (short term). The fish in the control group were kept in a similar way under normoxia (≈5.00 ± 0.1 mg/l, DO) for same time periods. Prior to sample collection, fish were rapidly euthanized with MS222 to ameliorate suffering. Brain, muscle, liver and spleen samples were collected and snap frozen in liquid $N_2$ until further analysis. The tissue samples were also collected from fish exposed to hypoxia in natural habitat (long term) during summer periods and corresponding lab acclimatized control fish, following the same protocol. The protocols followed were approved by IAEC.

### 2.2. Identification of PPP1R3C transcript through SSH library construction

Total RNA was extracted from the tissue samples using the NucleoSpin RNA II kit (Macherey-Nagel, Germany), according to the manufacturer protocol. cDNA was synthesized from 1 μg total RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen) and SSH library (Super SMART cDNA Subtraction kit, Clontech) was constructed from control and hypoxia challenged fish. Clones of *C. batrachus* screened from the SSH library were sequenced and EST sequences were analyzed for the PPP1R3C transcripts expressed under hypoxia exposure.

### 2.3. Characterization of full length cDNA and structural analysis of PPP1R3C

Full length cDNA of PPP1R3C was identified by the similarity search and GeneScan tool (http://genes.mit.edu/GENSCAN.html). The protein-coding region was determined by selecting the longest amino acid sequence terminated before the polyadenylation signal and the deduced amino acid sequences were subjected to homology analysis using BLASTP program against protein database searches at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Physiochemical properties of PPP1R3C were identified through ProtParam and ScanProsite tools on the ExPASy Server (http://www.expasy.org), while functional domains were identified by SMART tool (smart.embl-heidelberg.de/). Subcellular localization was predicted through PSORT and iPSORT (Bannai et al., 2002; Nakai and Kanehisa, 1992, http://psort.nibb.ac.jp/). NetPhos 2.0 was used for consensus phosphorylation (serine, threonine or tyrosine) site detection (Blom et al., 1999, www.cbs.dtu.dk/services/NetPhos/). Secondary structure was predicted by PDBsum analysis at European Molecular Biology Laboratory (EMBL) server. Homology model was constructed by SWISS-MODEL Workspace Version 8.05 (Arnold et al., 2006) based on template, protein phosphatase 1, regulatory (inhibitor) subunit 3B of *Homo sapiens* (Tomizawa et al., unpublished data, 2eefA) from fragment CBM_21 domain. Quality of protein model was estimated by QMEAN6 (Benkert et al., 2009) and PROCHECK tools (Laskowski et al., 1993).

### 2.4. Multiple sequence alignment and phylogenetic analysis

The deduced amino acid sequences of PPP1R3C from *Danio rerio* (PPP1R3Ca; NP_001002376.1, PPP1R3Cb; NP_957128.1), *Xenopus* (*Silurana*) *tropicalis* (NP_001106579.1), *Xenopus laevis* (NP_001085902.1), *H. sapiens* (EAW50107.1), *Mus musculus* (EDL41774.1), *Pan troglodytes* (JAA37775.1) and *Bos taurus* (AAI20043.1) (Table 1) were obtained from the Entrez database (http://www.ncbi.nlm.nih.gov/Entrez/) and aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2). The multiple sequence alignments were adjusted manually corresponding to different motifs and domains. Domain characterization of PPP1R3C was according to *H. sapiens* protein (EAW50107.1), as presented in the UniProt database (http://www.uniprot.org/). An unrooted phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with bootstrap (2000 replicates; 15565 seed). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). All positions containing alignment gaps and missing data were eliminated by pairwise deletion. Phylogenetic analysis was conducted in MEGA4 (Tamura et al., 2007).

### 2.5. Quantitative real-time PCR (qRT-PCR) and statistical analysis

Total RNA was extracted from the tissue samples using the NucleoSpin RNA II kit (Macherey-Nagel, Germany), according to the manufacturer's protocol. cDNA was synthesized from 1 μg total RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, USA) from control and hypoxia challenged fish.

The expression of PPP1R3C (GenBank accession no. KC633817) in *C. batrachus* was determined by qRT-PCR in brain, liver, muscle and spleen tissues and its expression was normalized to ribosomal protein l30 (*rpl30*), elongation factor-1α (*elf1α*), 28S rRNA (*28S*) and Tubulin (*TUB*) expression, selected as suitable tissue specific reference genes under hypoxia (Mohindra et al., 2013). Primers (Supplementary Table 1) were designed using the PrimerQuestSM (Integrated DNA Technologies). Primer efficiencies (E) were calculated from 10-fold dilution series to make standard curves from which E was calculated according to the formula $E = 10^{-1}/\text{slope}$. The specificity of the primer sets used was confirmed by the presence of a single band of correct size on gel electrophoresis in addition to the presence of a single peak in the dissociation curve analysis. All qRT-PCR reactions were performed in 20 μl total reaction volume (18 μl master mix and 2 μl undiluted cDNA made from 1 μg RNA/PCR product). The master mix contained 7.2 μl of $H_2O$, 0.8 μl of each primer (0.4 μM final concentration) and 10.0 μl of the SYBR Green Mix (Roche Applied Science, Laval, PQ, Canada). The following cycling conditions were used: (1) denaturation, 5 min at 95 °C; (2) amplification repeated 40 times, 10 s at 95 °C, 10 s at 55 °C, and 15 s at 72 °C with ramp rate of 4.4 °C/s, 2.2 °C/s, and 4.4 °C/s, respectively; (3) melting curve analysis, 5 s at 95 °C and 1 min at 65 °C with ramp rate of 4.4 and 2.2 °C/s, respectively, then up to 95 °C at a rate of

**Table 1**
PPP1R3C protein sequences from *Clarias batrachus* and other species used for domain identification, sequence alignment and phylogenetic analysis.

| S. no. | Organism | Protein | Accession number |
|---|---|---|---|
| 1 | *Clarias batrachus* | PPP1R3C | KC633817 (present study) |
| 2 | *Danio rerio* | PPP1R3Cb | NP_957128.1 |
| 3 | *Danio rerio* | PPP1R3Ca | NP_001002376.1 |
| 4 | *Homo sapiens* | PPP1R3C | EAW50107.1 |
| 5 | *Mus musculus* | PPP1R3C | EDL41774.1 |
| 6 | *Pan troglodytes* | PPP1R3C | JAA37775.1 |
| 7 | *Sus scrofa* | PPP1R3C | ABA00738.1 |
| 8 | *Bos taurus* | PPP1R3C | AAI20043.1 |
| 9 | *Xenopus* (*Silurana*) *tropicalis* | PPP1R3C | NP_001106579.1 |
| 10 | *Xenopus laevis* | PPP1R3C | NP_001085902.1 |

0.1 °C/s; (4) cooling, 10 s at 40 °C with ramp rate of 2.2 °C/s. For each treatment and control, 3 individuals were analyzed in duplicate and reactions were performed in a Light Cycler 480 (Roche Applied Science) system. Crossing point (Ct) values were obtained by employing the second derivative maximum method. Crossing point values were compared and converted to fold differences by the relative quantification method using the relative expression software tool (REST) 384 v. 2 (Pfaffl et al., 2002). p values below 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Characterization of C. batrachus PPP1R3C: cDNA transcript and protein

The full-length cDNA of PPP1R3C (GenBank accession no. KC633817) consisted of 1499 bp, with 131 bp 5′ UTR, a 510 bp 3′UTR and an open reading frame (ORF) of 858 bp. The 3′ UTR contained a putative polyadenylation signal (aataaa) and a polyA$^+$ tail. The ORF encoded a polypeptide of 285 amino acid residues (Supplementary Fig. 1). The results of primary structure analysis suggested a putative pI of 9.17, MW of 326.9 kDa.

ScanProsite predicted two protein kinase C, five Casein kinase II, two N-glycosylation and two N-myristoylation post translational modification sites (Table 2). Analysis by InterProScan and SMART tools showed the presence of CBM21 (carbohydrate binding type-21) domain from amino acids 133–241 (Fig. 1A). PSORT and iPSORT analyses showed that PPP1R3C protein is mainly localized in the nucleus (64%). NetPhos 2.0 predicted the presence of phosphorylation site for serine (8 sites), threonine (3 sites) and tyrosine (2 sites) (Fig. 1B). Secondary structure (amino acids 112 to 247) of PPP1R3C consisted of 3 sheets, 4 beta hairpins, 1 beta bulge, 10 strands, 1 helix, 17 beta turns and 3 gamma turns (Fig. 2A). The Ramachandran plot of psi and phi backbone conformational angles for each residue in the homology model (Fig. 2B) of PPP1R3C showed that 73.4% residues were in the most favored regions and 26.6% residues were in the additionally allowed regions, while no residues were in the generously allowed and disallowed regions (Figs. 2C,D).

An alignment of the deduced amino acid sequences of PPP1R3C with fishes, amphibians and mammalian proteins is shown in Fig. 3. Multiple sequence alignment analysis revealed that tetramer PP1-binding motif (KVXF) was present in fishes and amphibians, while in mammals, this motif was represented by conserved RVVF amino acids. The phylogenetic tree clearly revealed two lineages of PPP1R3C and that of *C. batrachus* was found to be clustered with PPP1R3Ca isoform of *D. rerio* (Fig. 4).

### 3.2. Tissue-specific expression under normoxia

The qRT-PCR results showed that in *C. batrachus*, PPP1R3C mRNAs were constitutively expressed in the all the tissues tested (brain, liver, muscle and spleen), under normoxic condition. Among the examined tissues the transcript level of PPP1R3C was highest in the liver ($7.5 \times 10^{-2} \pm 1.4 \times 10^{-2}$ ng) followed by muscle ($2.3 \times 10^{-3} \pm 3.3 \times 10^{-4}$ ng) and spleen ($1.5 \times 10^{-3} \pm 1.1 \times 10^{-3}$ ng), while in the brain its expression was the least ($9.8 \times 10^{-5} \pm 1.3 \times 10^{-5}$ ng).

**Table 2**
ScanProsite prediction of post translational modification sites found in the PPP1R3C protein of *Clarias batrachus*.

| S. No. | Post translational modification sites | Amino acid position | Sequence |
|---|---|---|---|
| 1 | Protein kinase C phosphorylation | 24–26 | SYK |
| | | 156–158 | TQK |
| 2 | Casein kinase II phosphorylation | 24–27 | SYKD |
| | | 90–93 | SPCE |
| | | 228–231 | THWD |
| | | 242–245 | TTHD |
| | | 243–246 | THDE |
| 3 | N-glycosylation | 52–55 | NTSL |
| | | 161–164 | NVSF |
| 4 | N-myristoylation | 80–85 | GMSLTA |
| | | 193–198 | GCRDTD |

### 3.3. After short and long-term hypoxia

Following progressive hypoxia (PH) till 0.98 ± 0.1 mg/l DO, the PPP1R3C transcript level was significantly up-regulated (3.46 fold) in the muscle (Fig. 5A), while after 6 and 12 h of hypoxia exposure, it was significantly up-regulated in the liver (6.96 fold and 3.91 fold, respectively) (Fig. 5B). However, no significant difference was found in the brain and spleen (Fig. 5C) during short term hypoxia exposures. After long-term (natural) hypoxic conditions, its expression was significantly up-regulated in the liver (7.77 fold) (Fig. 5B) and spleen tissues (6.59 fold) (Fig. 5C), while, that of in the muscle and brain showed no significant change.

## 4. Discussion

### 4.1. Characterization of PPP1R3C: cDNA and protein

The present study is the first report on the molecular constructions and transcriptional responses of PPP1R3C from fish. The PPP1R3C protein of *C. batrachus* showed high homology to other fish (100% to *D. rerio*, 84% to *Oreochromis niloticus*), amphibians (87% to *X. laevis*) and mammalians (84% *H. sapiens* and *M. musculus*) species. The presence of potential phosphorylation sites in the PPP1R3C protein of *C. batrachus* indicated that the protein might be phosphorylated by protein kinase C or casein kinase II. These protein phosphorylation events are an essential contributor to the delay between the signal and the negative feedback (Harms et al., 2004).

The presence of N-terminal glycosylation and myristoylation in PPP1R3c may indicate its role in stimulating the signal transduction pathways. Myristoylation of a protein plays a vital role in membrane targeting and signal transduction in responses to environmental stress, especially in plants (Podell and Gribskov, 2004). It affects both the rate and intensity of receptor activation-dependent changes detected during the dynamic process of protein signaling. Glycosylation helps in the proper folding of protein to facilitate their delivery to the correct destination and to mediate cell attachment or stimulate signal transduction pathways (Wormald et al., 2002). PPP1R3C or protein targeting to glycogen (PTG) is one of four targeting proteins (other three being PPP1R3, PPP1R4 and PPP1R6) that binds to PP1 and glycogen, targeting the phosphatase to the glycogen particle (Fong et al., 2000). The binding of PPP1R3C to PP1 and glycogen is mediated by the PP1 binding motif, which is located in the N-terminal region and contains the consensus (K/R)VXF motif (Zhao and Lee, 1997). In mammals, this motif is highly conserved (RVVF), while in *C. batrachus*, *D. rerio* and amphibians, it is represented by conserved KVXF motif, which is very likely a common site of interaction with the phosphatase (Fong et al., 2000).

The PPP1R3C protein of *C. batrachus* also contains a carbohydrate binding domain type 21 (CBD21), which is located at C-terminus. The CBD is critical for association with glycogen both in vitro and in vivo and localizes the phosphatase to specific subcellular compartments that correspond to the expression pattern of glycogen processing enzyme, glycogen synthase (Wang et al., 2002). In this domain, amino acid sequences were highly conserved, however unique substitutions, specific to fishes, were observed, the importance of which are needed to be further explored.

The *H. sapiens* PPP1R3B "2eef" (NP_001188258.1) was found to be the closest homology model available in protein data bank and its solution structure consists of CBM21 domain of 156 amino acids. Secondary structure prediction for the PPP1R3C protein of *C. batrachus* from the CBM21 domain indicates that β-strands would be expected to occur in positions equivalent to known β-strand locations in the CBM21 domain of PPP1R3B from *H. sapiens*. The homology model of

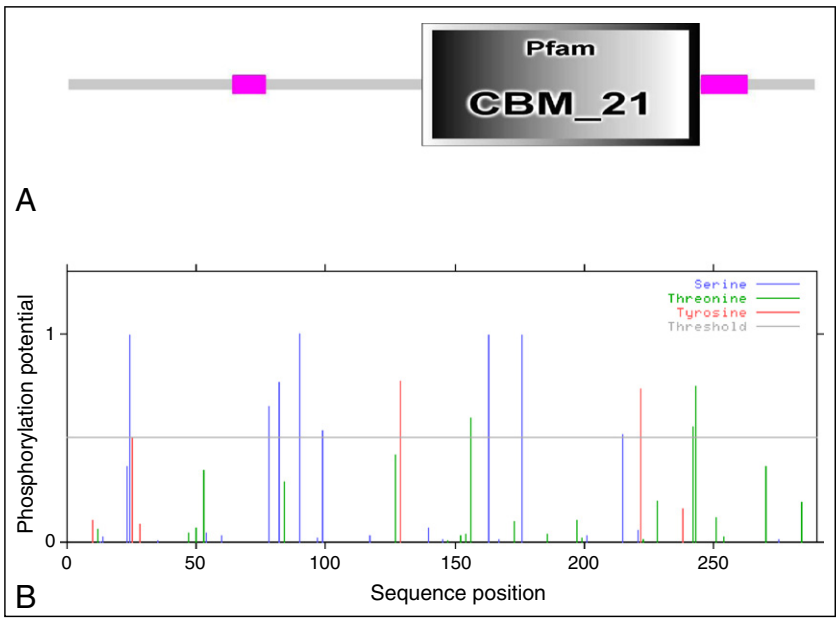



**Fig. 1.** (A) Carbohydrate binding type-21 domain prediction in the PPP1R3C protein of *Clarias batrachus*. (B) Prediction of serine, threonine and tyrosine phosphorylation sites in the PPP1R3C protein. X-axis represents the position of sequence while, Y-axis represents the phosphorylation potential (cutoff 0.5).

PPP1R3C from *C. batrachus* was constructed from amino acids 112 to 247 which consist of complete CBM21 domain. Ramachandran plot of psi and phi backbone conformational angles for each residue in the homology model of PPP1R3C assessment revealed a normal distribution of amino acid residues with respect to regions in the Ramachandran plot. These findings, together with the secondary structure prediction, strongly support the idea that the three-dimensional structures of the CBM21 domain of PPP1R3C of *C. batrachus* and PPP1R3B of *H. sapiens* were highly similar and suggest that the two proteins may have similar function and can be grouped into similar CBM clan. Among the two

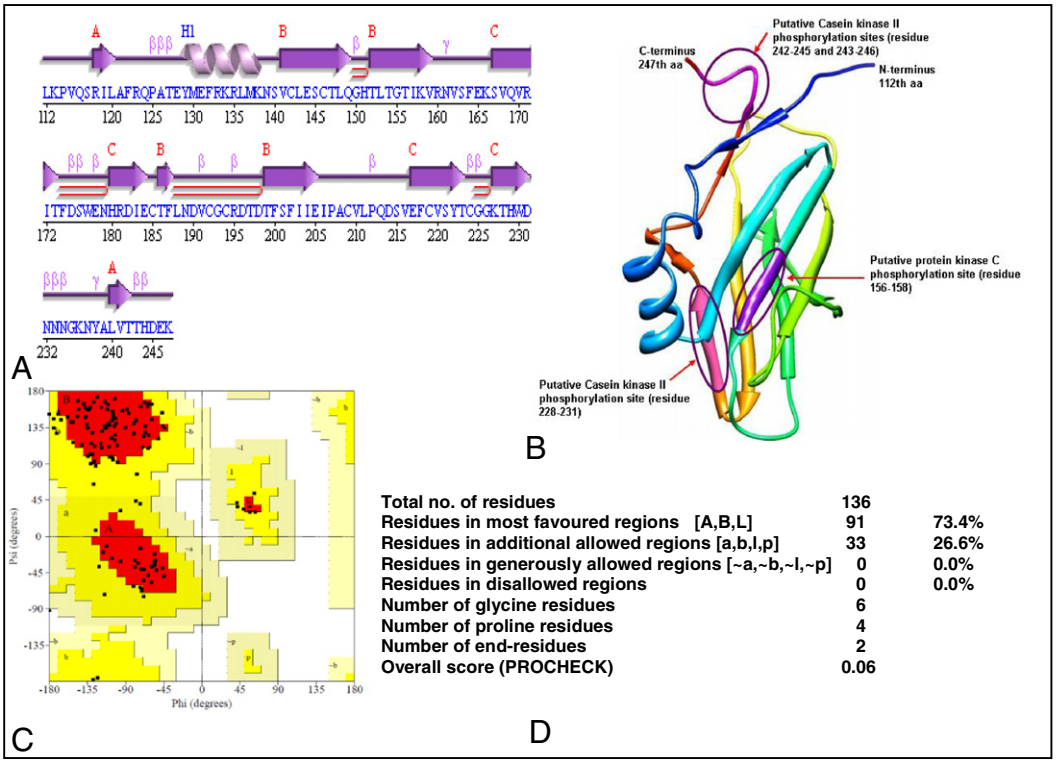



**Fig. 2.** (A) Secondary structure prediction of PPP1R3C (from amino acid residues 112 to 247) where, A, B, and C in red represents strands by their sheets; β, beta turn; γ, gamma turn; ⟹, beta hairpin and H1, helices. (B) Schematic view of the PPP1R3C homology model showing the putative phosphorylation sites (purple; protein kinase C and magenta; casein kinase II), enclosed in circles. (C) Conformational map (Ramachandran plot) of the psi and phi backbone angles for each residue in the modeled PPP1R3C, where "most favored" (colored in red), "generously allowed" (yellow), "additionally allowed" (beige), and "disallowed" (white) regions are described. (D) Ramachandran plot statistics for PPP1R3C homology model. The overall PROCHECK score of >— 0.50 is recommended and investigation is needed for a score <— 1.0.

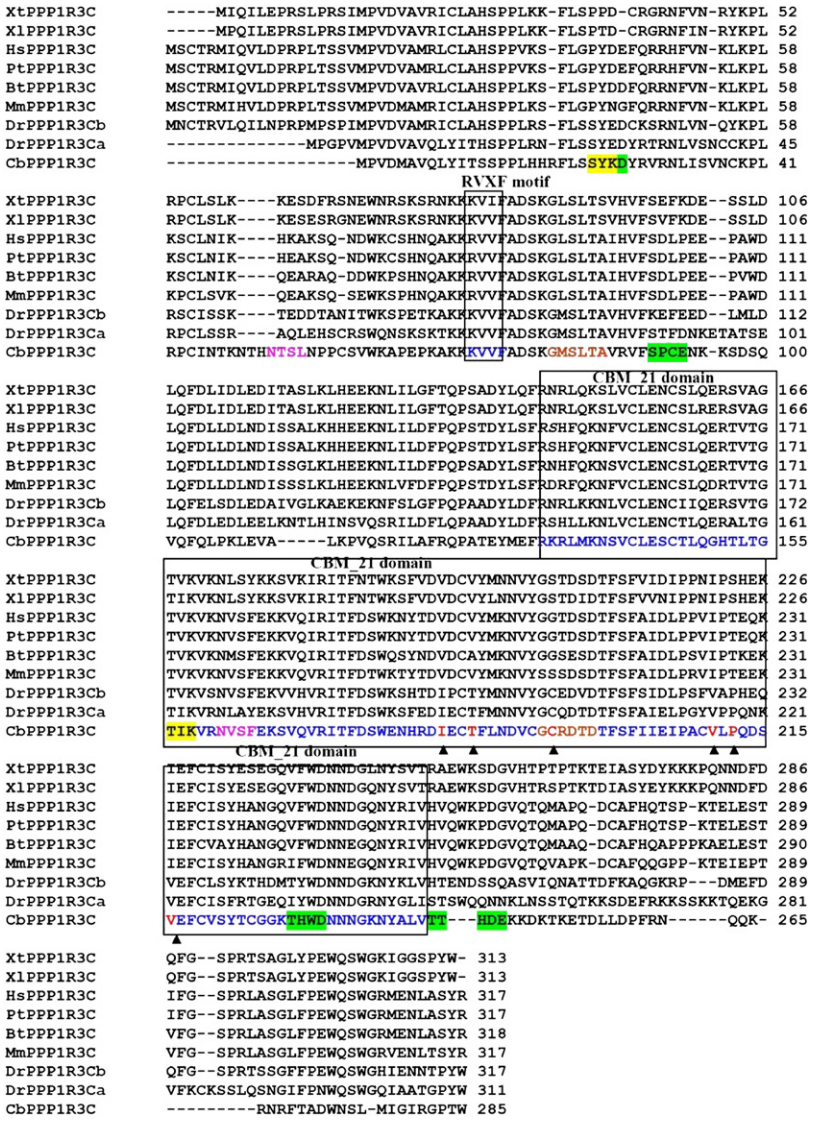



**Fig. 3.** Multiple alignment of the deduced amino acid sequences of *Clarias batrachus* (CbPPP1R3C), *Danio rerio* (DrPPP1R3Ca and DrPPP1R3Cb), *Xenopus (Silurana) tropicalis* (XtPPP1R3C), *Xenopus laevis* (XlPPP1R3C), *Homo sapiens* (HsPPP1R3C), *Mus musculus* (MmPPP1R3C), *Pan troglodytes* (PtPPP1R3C) and *Bos taurus* (BtPPP1R3C) PPP1R3C protein sequences. Domain (CBM_21) and motif (RVXF) of typical characterization of PPP1R3C were marked on the alignment (blue colored, boxed). The domain characterization of individual subunit was according to *Homo sapiens* PPP1R3C (EAW50107.1), as presented in uniprot database. Symbol '▲ (red colored residues)' denotes unique substitutions in the fishes PPP1R3C protein. Putative protein kinase C (yellow highlight, SYK, residues 24 – 26; TQK, residues 156-158), casein kinase II phosphorylation (green highlight, SYKD, 24-27; SPCE, 90-93; THWD, 228-231; TTHD, 242-245; THDE, 243-246), N-glycosylation (purple, NTSL, 52-55; NVSF, 161-164) and N-myristoylation (brown, GMSLTA, 80-85; GCRDTD, 193-198) sites are shown.

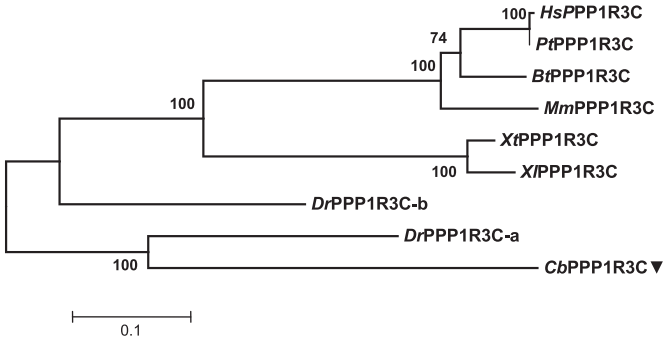


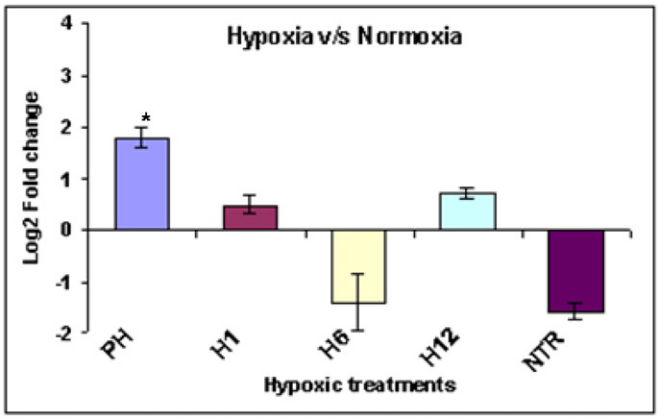

**Fig. 4.** Phylogenetic tree of *Clarias batrachus* PPP1R3C (CbPPP1R3C) with *Danio rerio* (DrPPP1R3Ca and b), *Xenopus* (*Silurana*) *tropicalis* (XtPPP1R3C), *Xenopus levis* (XlPPP1R3C), *Mus musculus* (MmPPP1R3C), *Bos torus* (BtPPP1R3C), *Pan troglodytes* (PtPPP1R3C) and *Homo sapiens* (HsPPP1R3C). (Bootstrap values are given in percent and the scale bar indicates 0.1 substitutions per site). PPP1R3C members included are from Table 1.

isoforms reported in fishes, PPP1R3C of *C. batrachus*, which was found to be differentially expressed under hypoxia, was closer to PPP1R3C-a isoform.

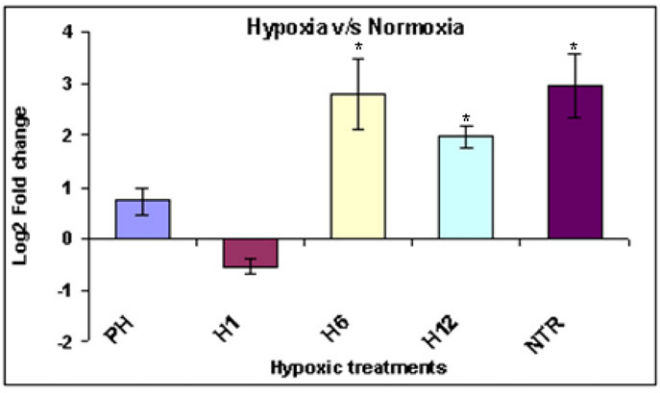
### 4.2. Expression of PPP1R3C: normoxia v/s hypoxia

PPP1R3C has been suggested to be critical for glycogen metabolism, possibly functioning as a molecular scaffold and its overexpression markedly increased basal and insulin-stimulated glycogen synthesis (Printen et al., 1997). In this study, PPP1R3C was found to be ubiquitously expressed under normoxic condition in examined tissues of *C. batrachus*. However, its expression was higher in insulin sensitive tissues (liver and muscle) than in the brain and spleen.

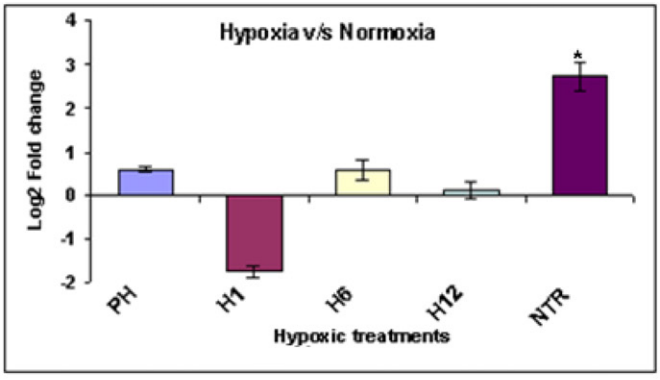
Glycogen accumulation has been proposed as a metabolic survival pathway where hypoxia plays a role in glycogen metabolism (Pescador et al., 2010; Vigoda et al., 2003) and glycogen synthase 1 (GYS1) through HIF-1 was implicated in hypoxia-induced glycogen metabolism together




A) Muscle




B) Liver




C) Spleen

**Fig. 5.** Differential expression of PPP1R3C mRNA estimated by qRT-PCR, after short-term (PH, 1, 6 and 12 h) and long-term (NTR) hypoxia exposures in *Clarias batrachus* (A, B, C; in muscle, liver and spleen, respectively). Y-axis represents the log2 ratio of PPP1R3C expression, mean ± SE (N = 3, in duplicate) as fold change. X-axis represents hypoxic treatments as PH, progressive hypoxia up to 0.98 ± 0.1 mg/l, dissolved oxygen, H1, H6 and H12 (hypoxic time period, 1, 6 and 12 h at 0.98 ± 0.1 mg/l, dissolved oxygen) and NTR, natural hypoxia exposure. Significant differences ($p < 0.05$) in the expression levels of PPP1R3C in comparison to normoxic control group are indicated by asterisks (*) above bars.

with up-regulation of the enzymes UTP: glucose-1-phosphate uridylyltransferase (UGP2) and 1,4-α glucan branching (GBE1). Similarly, it has been reported that under hypoxic condition in human MCF-7 cells, the PPP1R3C gene also regulated by HIF-1, activated glycogen synthase, and limited glycogen breakdown through a reduction in the glycogen phosphorylase activity. These two actions lead to glycogen accumulation (G.M. Shen et al., 2010).

In our earlier study, *C. batrachus* was found to be an oxy-conformer, where it relies on aerobic metabolism and maintained a hypometabolic state for survival under periods of hypoxia (Tripathi et al., 2013). In general, during the food deprivation and under hypoxia, fish species deploy different mechanisms to mobilize glycogen. The snakehead (*Ophiocephalus maculatus*) employs complete glycogenolysis (Woo and Cheung, 1980) whereas salmonids partially or almost completely protect endogenous carbohydrates, while mobilizing lipids (van Raaij et al., 1996). American eel (*Anguilla anguilla*) conserve glycogen, relying heavily on protein and/or lipid catabolism to maintain metabolic energy equilibrium (Larsson and Lewander, 1973). However, in the present study, the mechanism to mobilize glycogen by *C. batrachus* under hypoxia is not known. However in *C. batrachus*, decrease in serum glucose level (Tripathi et al., 2013) and increased expression of HIF-1 in the liver (Mohindra et al., unpublished data, manuscript under revision) were observed during short periods of hypoxia. Thus, the significant increase in mRNA expression of PPP1R3C in the liver and muscle of *C. batrachus* may suggest its possible involvement in the process of HIF-dependent glycogen storage, although further studies are required to ascertain resultant up-regulation of its protein activity. However, increase in PPP1R3C transcripts in the spleen, not specialized for metabolic function, observed under the present study also needs further investigation to elucidate its possible roles.

The present study characterized PPP1R3C in a hypoxia-tolerant freshwater Indian catfish, *C. batrachus* and the expression pattern of PPP1R3C during short and long term hypoxic conditions presents evidence for its potential involvement in hypoxia tolerance.

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## Conflicts of interest

The authors report no conflicts of interest.

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