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# Cloning and Expressions of Chop in Loach (Misgurnus Anguillicaudatus) and Its Response to Hydrogen Peroxide (H2O2) Stress

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# Abstract

C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (*chop*) which plays an important role in endoplasmic reticulum stress-induced apoptosis, was investigated here by RACE and qPCR in an aquaculture animal for the first time. The full-length cDNA sequences of loach (*Misgurnus anguillicaudatus*) *chop* were 2533bp, encoding 266 amino acids. The expression levels of loach *chop* changed obviously during different early life stages, and the highest appeared at the 8-cell stage. Among different tissues, loach *chop* predominantly expressed in gill, spleen and gonad. Then, we performed a hydrogen peroxide ($H_2O_2$, a common-used disinfectant) stress trial to explore the role of loach *chop*, with three different concentrations (0 μM, 50 μM and 100 μM) of $H_2O_2$. Half of the loaches from the 100 μM group were dead, while all loaches from the other two treatment groups were alive. The activities of CAT, SOD and GPX in loach gill, liver and spleen decreased with extended stress time and increased $H_2O_2$ concentration. The expression levels of gill *chop* in loaches from the 100 μM group were significantly higher than those from the other two treatment groups between 12 and 24 hours of exposure. *atf4* and *bax*, two proapoptotic genes, were significantly up-regulated in gills of loaches from the 100 μM group compared to the other two groups 18 hours and 24 hours after treatment, while *bcl2*, an antiapoptotic gene, presented an opposite trend. These results indicated a close relationship between $H_2O_2$ stress and fish apoptosis, and loach *chop* played an important role in $H_2O_2$ stress response.


# 1. Introduction

C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (*chop*), also known as the growth arrest and DNA damage-inducible gene 153 (GADD153), is considered as a master proapoptotic molecule of endoplasmic reticulum (ER) stress-induced apoptosis (Li et al. 2014). Although ubiquitously expressing at very low levels under normal conditions (Ariyama et al. 2008), *chop* is a highly stress-inducible expressed gene, markedly following the ER stress (Zhang et al. 1999; Cheng et al. 2009; Pirot et al. 2007). Cells respond to ER stress by activating a series of signaling pathways, collectively termed the unfolded protein response (UPR) pathway (Walter and Ron 2011). It has been proved that three branches of the UPR pathway, namely inositol requiring enzym1 (IRE1), prospective evaluation of radial keratotomy (PERK) and activating transcription factor 6 (ATF6) branch, trigger apoptosis when the ER stress is severe, prolonged, or chronic unmitigated (Zhang et al. 2016; Deegan et al. 2015; Shang ang Lehrman 2004). The apoptotic program is activated by the elevated expression of massive kinds of transcription factors (Rutkowskiet al. 2006), and *chop* is the key one.

More recently, it is reported that *chop* is mainly up-regulated in the PERK branch when organisms are exposed to the ER stress (Cao et al. 2012). PERK is also known as eukaryotic translation initiation factor 2α (eIF2α) kinase 3 (Mounir et al. 2011). It can up-regulate the translations of some specific genes, like activation of transcription factor-4 (*atf4*) (B'chir et al. 2013), by phosphorylating its substrate eIF2α. *atf4* is a member of C/EBP family, and it elevates expression of its key downstream target gene *chop* when stress cannot be alleviated, resulting in the onset of apoptosis (Su and Kilberg 2008). In the PERK branch,

*chop* is suggested to facilitate apoptosis through repressing the transcriptional expression of the antiapoptotic gene B-cell lymphoma-2 (*bcl2*) in response to ER stress (McCullough et al. 2001). Another widely accepted mechanism of *chop*-induced apoptosis is the induced overexpression of proapoptotic gene BCL2-associated X (*bax*), which may function as an executioner in ER stress-mediated apoptosis (Zou et al. 2013). *chop*-mediated apoptosis enhanced by ER stress plays a significant role in the pathophysiology of many mammalian diseases (Gopalan et al. 2013; Oyadomari et al. 2002; Lindholmi et al. 2006; Thorp et al. 2009; Gotoh et al. 2010; Lee et al. 2012), affecting the survival of organisms. So far, many studies on functions of *chop* have been carried out in mammals. However, no information on the cloning and roles of *chop* in aquaculture animals is available.

The water environment changes are easy to cause ER stress responses in aquaculture animals (Chen et al. 2015; Zhu et al. 2013; Ji 2014), which have many adverse effects (Cripps and Bergheim 2000), including poor growth, greater incidence of disease, increased mortality and low production (Barton and Iwama 1991). At present, due to the impact of the 2019-nCoV epidemic, hydrogen peroxide ($H_2O_2$) has been widely used as an environmental disinfectant (https://www.sohu.com/a/375631984_120113054). Part of the $H_2O_2$ residual might enter the aquaculture environment, resulting in changes of environment parameters. In order to understand the impact of $H_2O_2$ on aquaculture animals, loaches (*Misgurnus anguillicaudatus*) are used in this study. The loach, belonging to the family Cobitidae, is one of the most commercially momentous cultured species in several Eastern Asian countries including Korea, Japan and China. In this study, we first cloned the *chop* gene in loaches, and then monitored its expression profiles in different tissues and early life stages. In addition, a $H_2O_2$ stress experiment was performed to study the stress response in aquaculture fish in order to explore the role of *chop*. The results will further our understanding of the $H_2O_2$ stress responses and the role of *chop* in aquaculture animals.

## 2. Materials And Methods

# 2.1. Fish and sample collections

Loaches were obtained from Baishazhou fish market (Wuhan, China). All loaches were subjected to a ploidy analyzer (Partec, Germany) to determine their ploidy level. Diploid loaches were used and divided into four groups. The first group (15g ± 0.2) was used for cloning *chop*. Three female and three male adult loaches of the second group (15g ± 0.2) were used for determining expression levels of *chop* in ten tissues including liver, gill, brain, spleen, heart, intestine, muscle, gonad, kidney and skin, and each tissue served as an experimental unit (n = 3). A pair of parents from the third group (20g ± 0.2) was used for reproduction and embryos were collected at different early life stages, including oosperm, 2-cell stage, 8-cell stage, 32-cell stage, blastula stage, gastrula stage, neurula stage, tail-bud forming stage, muscle effect stage, heart-beating stage, and hatching stage. All samples were frozen at -80°C prior to RNA extraction. The fourth group (5g ± 0.2) contained 270 individuals, was used for $H_2O_2$ stress experiment.

# 2.2. Cloning the full-length cDNA of *chop* in loach

Total RNA was isolated from the liver tissues by using RNA isoPlus (TaKaRa, Japan). Quantities and qualities of isolated RNAs were measured by electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Following the method of the SMART™ RACE cDNA amplification kit (Clontech, USA), the RNA obtained was completely reverse-transcribed into cDNA, which was next used to clone the gene.

A pair of universal amplified primers was designed (Table S1), depending on the multiple sequences alignments of numerous species including zebrafish (*Barchydanio rerio var*), common carp (*Cyprinus carpio*), roughskin sculpin (*Trachidermus fasciatus*), yellow cartfish (*Pseudobagrus fulvidraco*), etc. The PCR program was set as follow: initial denaturing for 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 55°C and 18 s at 72°C, and extra elongation for 5 min at 72°C. The PCR products were collected with 2% agarose (Sangon, China) and then purified with a TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa). After purification, the DNA fragments were ligated into PMD19 (TaKaRa) and the randomly selected positive transformants were opted and sequenced (Invitrogen, China).

## 2.3. Sequence and phylogenetic analysis

Similarity analysis of all the sequences was conducted by BLAST program at the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of loach *chop* were translated into amino acid sequences by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Multiple alignments of the deduced amino acid sequences were conducted by BioEdit software. Other vertebrate *chop* amino acid sequences for alignments and constructing phylogenetic tree were obtained from NCBI and the identities of these sequences were blasted by Blastp (NCBI). The phylogenetic tree of amino acid sequence of *chop* was constructed by MEGA6.0 program using the neighbor-joining method (NJ, bootstrap method: 1000 replications, Arizona State University, USA). The evolutionary distance between loach *chop* and other *chop* sequences was calculated by using p-distance and gaps were removed by pairwise detection, using default parameters.

## 2.4. $H_2O_2$ stress experiment

270 loaches were stocked in nine 30 L white tanks (10 L water volume and 25 ± 0.5°C water temperature) at a stocking density of 30 fishes/tank. Loaches were respectively treated with 0 μM, 50 μM and 100 μM $H_2O_2$ with triplicate for 24 h. During the experiment, there was no feeding and human interference to prevent introducing other stressors. The number of dead individuals in each tank was recorded every 6 hours. The data were input into GraphPad Prism 6.0 and the survival rate curves were generated.

Gill, liver and spleen tissues were collected from three loaches per tank at 6-, 12-, 18-, and 24-hour $H_2O_2$ stress exposure. All tissues were frozen in liquid nitrogen and stored at -80°C for determining catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities. In addition, gills were also used for detecting expression levels of *chop* and its related genes (*atf4*, *bcl2* and *bax*).

## 2.5. Real-time quantitative PCR (qPCR)

qPCR was performed using a Mini opticon real-time detector (BIO-RAD, Hercules, CA USA). Specific primers of the target genes and reference genes (*β-actin* and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase)) are summarized in Table S1. The relative expressions of the target genes were calculated with the comparative Ct method ($2^{(-\Delta\Delta Ct)}$). All the procedures were based on the methods from our laboratory described by Cui et al. (2018).

## 2.6. Determination of antioxidant enzyme activities

The activities of SOD, GPX and CAT were estimated by using the commercially available kits (Nanjing Jiancheng Bioengineering Institute, China). The examinations of the three antioxidant enzyme activities were carried out according to the manuals of the kits.

## 2.7. Statistical analysis

The data were expressed as the means ± SD (standard deviation) or SE (standard error). One-way analysis of variance (ANOVA) was conducted by using Tukey's *post hoc* test in SPSS statistical package version 25.0 (SPSS Inc., USA). $P < 0.05$ was significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

# 3. Results

## 3.1. Gene cloning of *chop* and its sequence analysis in loach

The full-length of loach *chop* cDNA was 2533bp, containing 801bp open reading frame (ORF) encoding 266 amino acids, a 217bp 5'-untranslated region (UTR) and a 1519bp 3'-untranslated region.

Deduced amino acid sequences of loach *chop* shared high similarity with those of many other species. The putative results indicated that several completely conserved residues might be functionally important (Fig. 1a). The resulting phylogenetic tree of *chop* amino acid sequences demonstrated that the *chop* in loach was grouped into distinct clade similar to higher vertebrate species, and the deduced amino acid sequences of *chop* in loach displayed high similarity to those in *Danio rerio* and *C. carpio* (Fig. 1b).

## 3.2. Spatial and temporal expression profiles of *chop* in loach

The expression levels of loach *chop* in different tissues were detected (Fig. 2a). The highest expression of *chop* was found in gill, the second-highest in spleen and the third-highest in gonad. The expression levels of loach *chop* in different early life stages were observed as well (Fig. 2b). The expression level at 8-cell stage was the highest, then in sequence were muscle effect stage and blastula stage ($P < 0.05$). There were no significant differences in *chop* expression level among oosperm, 2 cell-, 32 cell-, gastrula-, neurula-, tail-bud forming-, heart-beating-, and hatching-stage.

## 3.3. $H_2O_2$ stress experiment

### 3.3.1. Survival

At 12-hour of $H_2O_2$ stress, the loaches in the 100 μM group began to die. The percentages of death at 12-, 18- and 24-hour of the 100 μM group were respectively 10%, 25%, and 15%. After 24 hour $H_2O_2$ stress, half of the loaches from the 100 μM group were dead, while all loaches from the other two groups (0 μM and 50 μM) were alive (Fig.S1).

### 3.3.2. The antioxidant enzyme activities

As shown in Fig. 3a, at each time point of $H_2O_2$ stress, gill CAT activities of loaches from the 100 μM group were significantly lower than those from the 0 μM and 50 μM groups. Between 12 and 24 hours of $H_2O_2$ stress, loaches from the 50 μM and 100 μM groups presented significantly lower hepatic CAT activities, compared with the control group (Fig. 3b). Figure 3c showed that spleen CAT activities in the 100 μM group were the lowest. In addition, the trends of SOD and GPX activities were similar to the CAT activity trend (Fig. 3d-3i).

### 3.3.3. Expression levels of *chop* and its related genes in gill

Expression levels of *chop* and its related genes *atf4*, *bcl2* and *bax* in gills of loaches from the 0 μM, 50 μM and 100 μM groups were analyzed (Fig. 4). The expression levels of *chop* from the 50 μM and 100 μM groups were significantly higher than those from the 0 μM group at 12-, 18- and 24- hour of $H_2O_2$ stress (Fig. 4a). The expression level of *chop* from the 100 μM group reached the highest at 24-hour of $H_2O_2$ stress, 10 times of that compared to the 0 μM group.

There were no significant differences in the expression levels of *atf4* and *bax* (two proapoptotic genes) among the three groups (0 μM, 50 μM and 100 μM) at the 6-hour and 12-hour of $H_2O_2$ stress (Fig. 4b,4d). Similar to *chop*, the expression levels of *atf4* and *bax* from the 100 μM group were the highest at the 24-hour of $H_2O_2$ stress. However, Fig. 4c showed that the expression profile of *bcl2* was opposite to those of *chop*, *atf4* and *bax*.

## 4. Discussion

This study cloned *chop* gene from an aquaculture animal (namely *M. anguillicaudatus*) for the first time. The multiple alignments and phylogenetic tree of the deduced amino acid sequences of *chop* genes showed that the loach *chop* kept relatively conservative sequences and structural homology compared to other vertebrates, suggesting *chop* functions are stable during vertebrate evolution (Luethy et al. 1990; Lee et al. 2011). The expression profiles of loach *chop* were determined here as well. The results showed that loach *chop* was highly expressed during certain embryogenesis periods, consistent with Xenopus (Iijima et al. 2003). In addition, the tissue expression analysis showed that *chop* predominantly expressed

in the gill and spleen of loach. It has been previously reported that gills and spleens are parts of the immune system, responding to the organism stress (Dautremepuits et al. 2009; Kocabas et al. 2002). The elevated loach *chop* expression in gills and spleens may indicate its immunological functions related to stress response.

At present, due to the impact of the 2019-nCoV epidemic, part of the $H_2O_2$ residual might enter the aquaculture environment. In order to explore the effects of $H_2O_2$ stress in fish, we treated the loaches with different concentrations of $H_2O_2$. Our results confirmed that activities of antioxidant enzymes in the loaches had a highly negative correlation with the prolongation of $H_2O_2$ stress and the increase of $H_2O_2$ concentrations, which were similar to the results of other stress studies. For example, when oxidative stress occurred, the activities of SOD and CAT in gills of zebrafish significantly dropped (Jin et al. 2015). Analogous results were also found in mice liver and spleen (Meng et al. 2004; Matsumoto et al. 2002). In addition, oxidative stress reduced GPX activities in gills and livers in black porgy (Chen et al. 2009), as well as in spleens of zebrafish (Komoike and Matsuoka 2013). In summary, $H_2O_2$ appears to induce stress responses in the loach due to decreased antioxidant enzymes expressions.

In the $H_2O_2$ stress experiment, we analyzed the expression levels of *chop* and its related genes *atf4*, *bcl2* and *bax* in gills of the loach. Our study evidenced that the expression level of *atf4* in the loach surged under high concentration $H_2O_2$ stress. The expression of loach *chop* significantly up-regulated with the increase of concentrations and the prolongation of $H_2O_2$ stress. Correspondingly, the more severe the inhibition of *bcl2* expression the more the acceleration of *bax* expression. These results were consistent with previous studies. When the apoptotic pathway is turned on, under the regulation of a series of upstream factors, the mRNA transcription of *atf4* increased (Cripps and Bergheim 2000). Afterwards, expression of its key downstream transcriptional target *chop* elevated (Barton and Iwama 1991), resulting in the repression of the antiapoptotic gene *bcl2* and the overexpression of proapoptotic gene *bax* (Luethy et al. 1990). A reasonable conclusion is that *chop* may promote apoptosis in the loach under $H_2O_2$ stress. However, the specific functions of *chop* warrants further studies.

In conclusion, we identified and characterized full-length cDNAs of loach *chop* for the first time. Expression profiles of *chop* in different tissues and different early life stages suggested its potential immunological functions in loach. The variety of *chop* expression levels in loaches treated with various concentrations of $H_2O_2$ indicated that loach *chop* expression was sensitive to $H_2O_2$ stress and that the stress induced apoptosis in loach. Finally, this study provides critical information for further investigation of *chop* functions in fish under $H_2O_2$ stress.

# Declarations

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### Conflicts of interest

The authors declare that they have no conflict of interest.

### Ethical approval

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to minimize suffering of the loaches.

### Availability of data and material

Not applicable.

### Code availability

Not applicable.

### Authors' contributions

Xiaojuan Cao and Zhiying Jia designed the experiment. Hui Li, Minxin Kang, Shouxiang Sun and Jian Gao carried out the experiments. Hui Li and Minxin Kang analyzed the data. The article was written by Hui Li with input from all authors. Xiaojuan Cao contributed to the revision of the manuscript. All authors reviewed and approved the paper.

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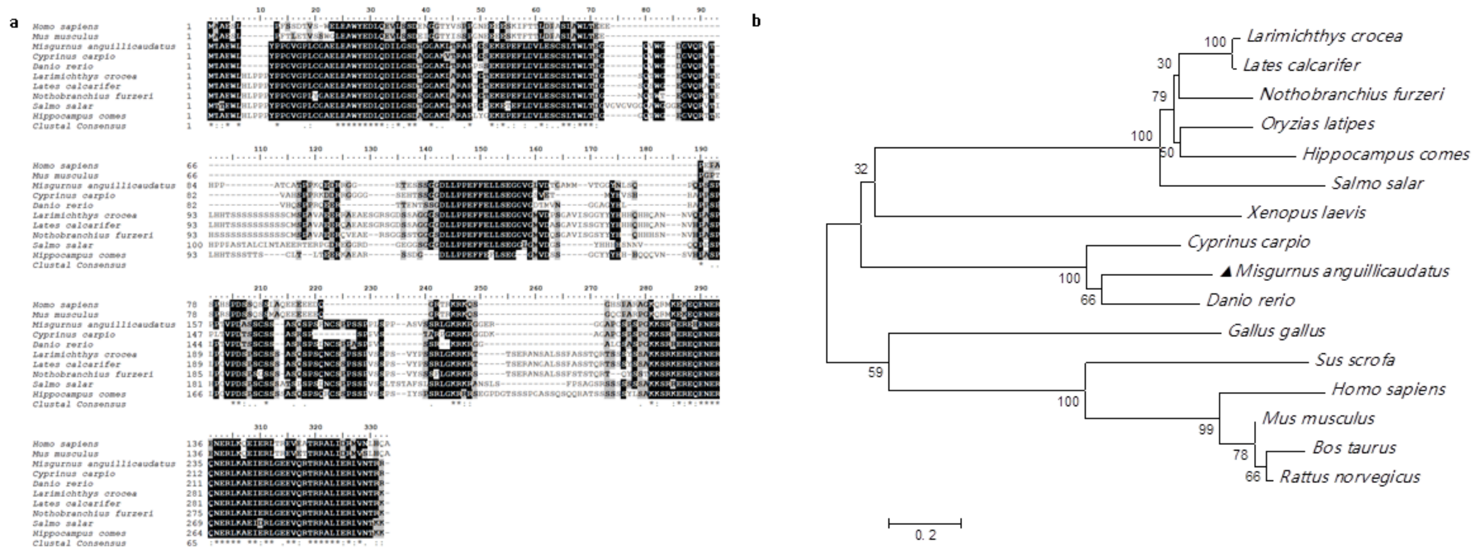
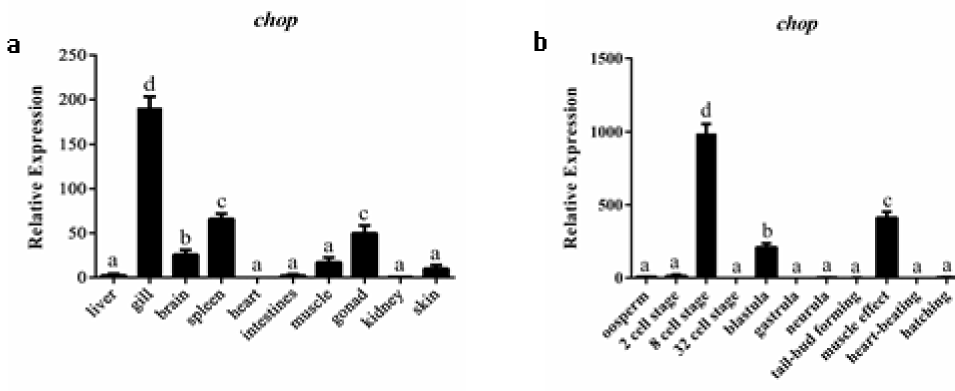
# Figures



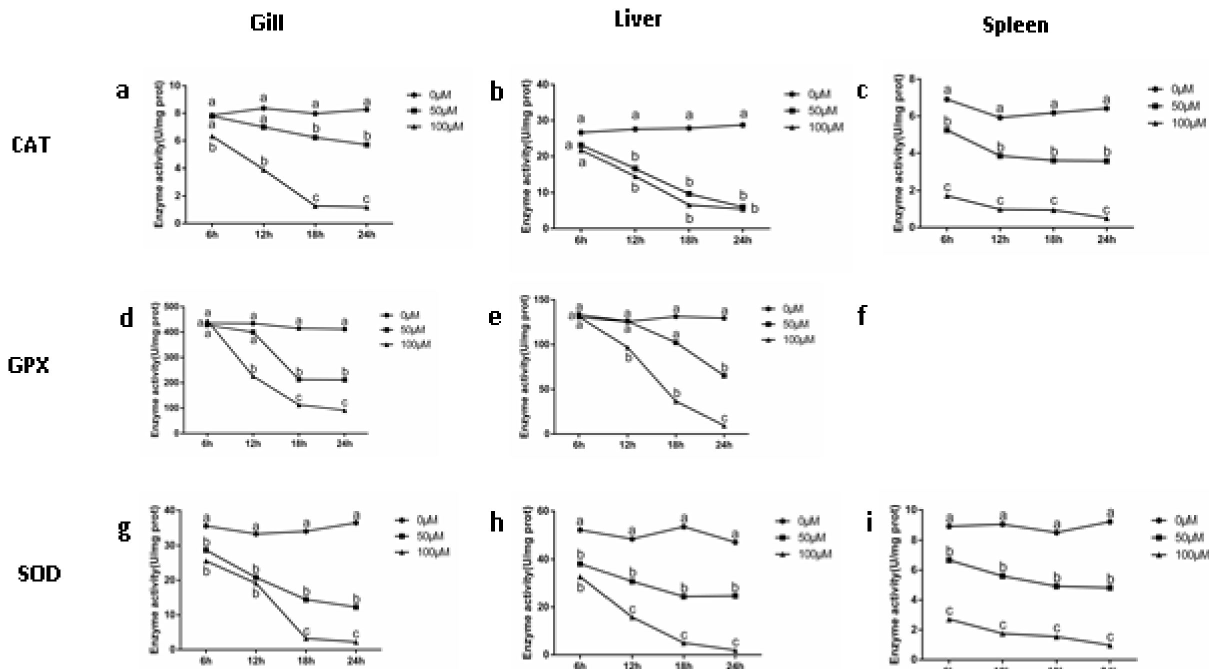

## Figure 1

Alignment and phylogenetic tree of the deduced amino acid sequences of chop in Misgurnus anguillicaudatus and other species. (a) Alignment of the deduced amino acid sequences of chop. The same or similar amino acids are respectively highlighted by black and gray. Black is used to labeled amino acids with a similarity more than 75% and light gray is to those with a similarity merely more than 50%. (b) Phylogenetic tree of the deduced amino acid sequences of chop. The horizontal branch length is proportional to the amino acid substitution rate at each position (The scale is 0.2). The genbank accession numbers for animals involved in the figure, except Misgurnus anguillicaudatus, are set out as follows: Homo sapiens (AAB22646.1), Mus musculus (BAE20435.1), Cyprinus carpio (KTF75003.1), Danio rerio (XP_005166228.1), Larimichthys crocea (XP_010730414.2), Lates calcarifer (XP_018534443.1), Nothobranchius furzeri (XP_015814628.1), Salmo salar (XP_014021417.1), Hippocampus comes (XP_019716650.1), Bos taurus (NP_001071631.1), Gallus gallus (AJA72779.1), Oryzias latipes (BAL14286.1), Rattus norvegicus (NP_001103456.1), Sus scrofa (NP_001138317.1), Xenopus laevis (NP_001082635.1).



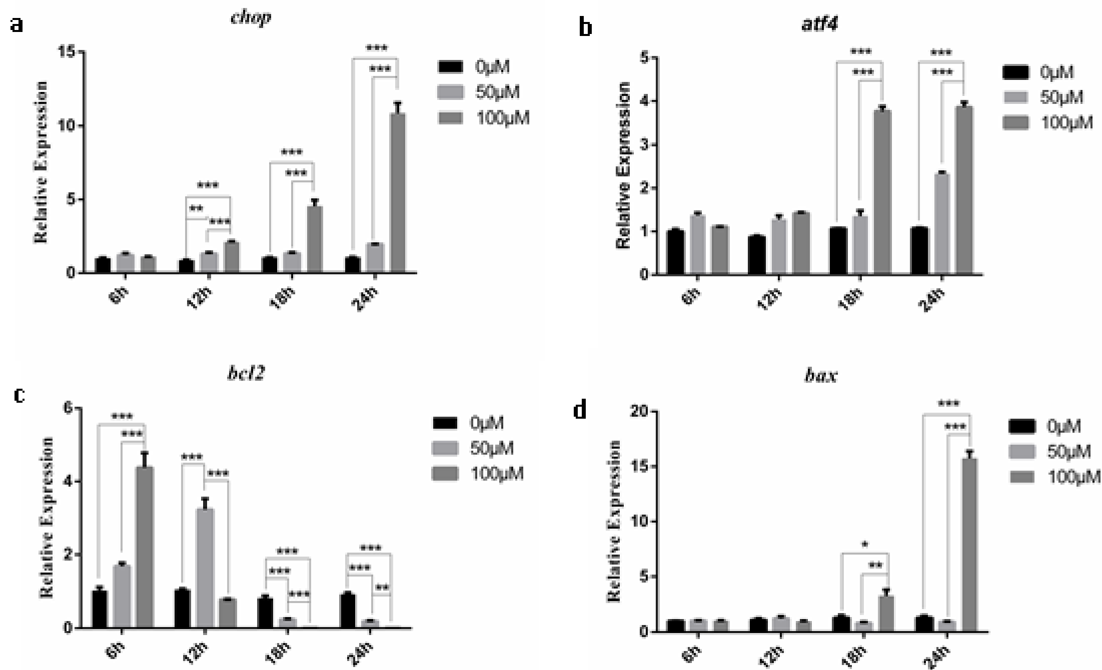

## Figure 2

Expression levels of chop in ten different tissues (namely, liver, gill, brain, spleen, heart, intestine, muscle, gonad, kidney and skin) (a) and in eleven different early life stages (namely, oosperm, 2 cell stage, 8 cell stage, 32 cell stage, blastula stage, gsatrula stage, neurula stage, tail-bud forming stage, muscle effect stage, heart-beating stage and hatching stage) (b) of Misgurnus anguillicaudatus, determined by real-time quantitative PCR. Significant differences are represented by different small letters.




## Figure 3

Determinations of three antioxidant enzyme activities in Misgurnus anguillicaudatus under three different concentrations (0μM, 50μM and 100μM) of H2O2 stress. Catalase (CAT) activities in gills (a), livers (b) and spleens (c) of the loaches at the 6-, 12-, 18- and 24-hour of H2O2 stress; Glutathione peroxidase (GPX) activities in gills (d), livers (e) and spleens (f) of the loaches at the 6-, 12-, 18- and 24-hour of H2O2 stress; Superoxide dismutase (SOD) activities in gills (g), livers (h) and spleens (i) of the loaches at the 6-, 12-, 18- and 24-hour of H2O2 stress. Significant differences for each antioxidant enzyme activity at each time point among the three different stress groups are represented by different small letters.




### Figure 4

Expression levels of chop (a) and its related genes (atf4 (b), bcl2 (c) and bax (d)) in gills of Misgurnus anguillicaudatus under three different concentrations (0μM, 50μM and 100μM) of $H_2O_2$ stress. The values are expressed as the means ± SEs. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. chop, C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene; atf4, activation of transcription factor-4; bcl2, B-cell lymphoma-2; bax, BCL2-associated X.

# Supplementary Files

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- 5supplementaltableandfigure.doc