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# Fish SUMO3 functions as a critical antiviral molecule against iridovirus and nodavirus

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ABSTRACT

Protein SUMOylation (SUMO is small ubiquitin-related modifier) is a dynamic process that is strictly regulated under physiological and pathological conditions. We previously cloned and characterized two SUMO homologue genes (EcSUMO1 and EcSUMO2) from orange-spotted grouper (*Epinephelus coioides*). In the present study, the SUMO3 homologue from *E. coioides* (EcSUMO3) was cloned and its possible roles in fish immunity were analyzed. The open reading frame of EcSUMO3 contains 285 base pairs encoding a 94 amino acid protein with a predicted molecular mass of 10.73 kDa. The protein sequence of EcSUMO3 revealed similar domains with mammals, including the UBQ (ubiquitin-like proteins) domain, the hydrophobic surface, the Ulp1-Smt3 interaction sites, a VKTE motif and the C-terminal Gly residues. EcSUMO3 shares 46.83% and 89.58% identity with EcSUMO1 and EcSUMO2, respectively, and it shares 94%, 98%, and 98% identity with SUMO3 from *Oreochromis niloticus*, *Danio rerio*, and *Homo sapiens*, respectively. Quantitative real-time polymerase chain reaction analysis indicated that EcSUMO3 was constitutively expressed in all of the analyzed tissues in healthy grouper. EcSUMO3 expression levels were remarkably ($p < 0.01$) up-regulated in grouper spleen (GS) cells in response to stimulation with red-spotted grouper nervous necrosis virus (RGNNV) and Singapore grouper iridovirus (SGIV). EcSUMO3 was distributed in both the cytoplasm and nucleus in GS cells. EcSUMO3 enhanced SGIV and RGNNV replication during viral infection *in vitro*. These results are important for better understanding of the SUMO pathway in fish and provide insights into the regulatory mechanism of viral infection in *E. coioides* under farmed conditions.

## 1. Introduction

SUMOylation (SUMO is small ubiquitin-like modifier) is a post-translational modification in which SUMOs are covalently conjugated to target proteins [1]. The mammalian SUMO family mainly includes three isoforms: SUMO1, SUMO2, and SUMO3. The SUMO2 and SUMO3 genes share only approximately 46% sequence similarity to SUMO1, but they share 96% similarity to each other [2–4]. SUMOylation requires the sequential function of activating, conjugating, and ligating enzymes, and it is a process mechanistically similar to conjugation pathways of other ubiquitin-like proteins. SUMOylation is a dynamic and reversible process, as SUMO-conjugated proteins are deconjugated by sentrin/SUMO-specific proteases [5].

The SUMO gene has been identified in many eukaryotes. Only a single SUMO-encoding gene has been reported in yeast and invertebrates. However, vertebrate and plant genomes contain four SUMO genes that encode SUMO paralogs 1–4 [6]. In many species, SUMO proteins are highly conserved and are important in many eukaryotic cell processes [7]. In contrast with SUMO1, SUMO2 and SUMO3 harbor a consensus SUMOylation site at their N-terminal region and distribute to similar subcellular locations. In addition, SUMO1 is commonly conjugated to substrates, whereas SUMO2 and SUMO3 appear to be unconjugated until rapidly conjugated in response to a variety of cellular stresses [2,4,8–10].

The orange-spotted grouper, *Epinephelus coioides*, is a valuable and popular marine fish, and it is one of the major mariculture species in China. However, in recent years, rapid development of marine farming activities and outbreaks of viral diseases have negatively affected the grouper aquaculture industry, causing heavy economic losses. Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous

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**Table 1**
Primers used in this study.

| Name | Sequence (5′–3′) |
|---|---|
| F1 | ATGTCAGAGGAAAAGCCAAAGGAG |
| R1 | TTAGCAGTGCCCGCCTGTCTGCTGC |
| F2 | GGGTCGGTGGTCCAGTTCAAAATCA |
| R2 | CAGCTGTGCAGGTGTATCCGTCTCA |
| F3 | CTCAAGCTTCGATGTCAGAGGAAAAGCCAAAGGAGG |
| R3 | GCGGATCCGCAGTGCCCGCCTGTCTGCTGCTGG |
| F4 | CGGGATCCATGTCAGAGGAAAAGCCAAAGGAGG |
| R4 | CGCTCGAGTTAGCAGTGCCCGCCTGTCTGCTGC |
| β-actin-F | TACGAGCTGCCTGACGGACA |
| β-actin-R | GGCTGTGATCTCCTTCTGCA |
| MCP-RT-F | GCACGCTTCTCTCACCTTCA |
| MCP-RT-R | AACGGCAACGGGAGCACTA |
| ICP-18-RT-F | ATCGGATCTACGTGGTTGG |
| ICP-18-RT-R | CCGTCGTCGGTGTCTATTC |
| VP19-RT-F | TCCAAGGGAGAAACTGTAAG |
| dRp-RT-F | GTGTCCGGAGAGGTTAAGGATG |
| RdRp-RT-R | CTTGAATTGATCAACGGTGAACA |
| CP-RT-F | CAACTGACAACGATCACACCTTC |
| CP-RT-R | CAATCGAACACTCCAGCGACA |

necrosis virus (RGNNV) are the most important viruses [11–14]. SGIV, which belongs to the Iridoviridae family, was isolated from diseased groupers [12,13]. RGNNV belongs to the genus *Betanodavirus*, whose members are the causative agents of a highly lethal disease in larval grouper [14]. To better understand the virus–host interaction, we previously constructed two cDNA libraries from spleens of grouper infected with SGIV and identified many SUMOylation proteins [15–17]. SUMO1 and SUMO2 from *E. coioides* (EcSUMO1 and EcSUMO2) contain the long flexible N-terminus, which is a unique feature of SUMO. EcSUMO1 and EcSUMO2 enhanced SGIV and RGNNV replication during viral infection *in vitro* [16]. The Aos1 and Uba2 homologues (EcAos1 and EcUba2) from grouper were also cloned and their possible roles in fish immunity were analyzed. EcAos1 and EcUba2 had antiviral effects during RGNNV infection [17].

In the present study, the SUMO3 gene of *E. coioides* (EcSUMO3) was cloned, and its expression at the transcript level in SGIV- and RGNNV-infected grouper spleen (GS) cells was analyzed. The intracellular localization of EcSUMO3 and its effects on virus replication also were studied. Results of this study provide a better understanding of the innate immune mechanisms in the virus response of grouper.

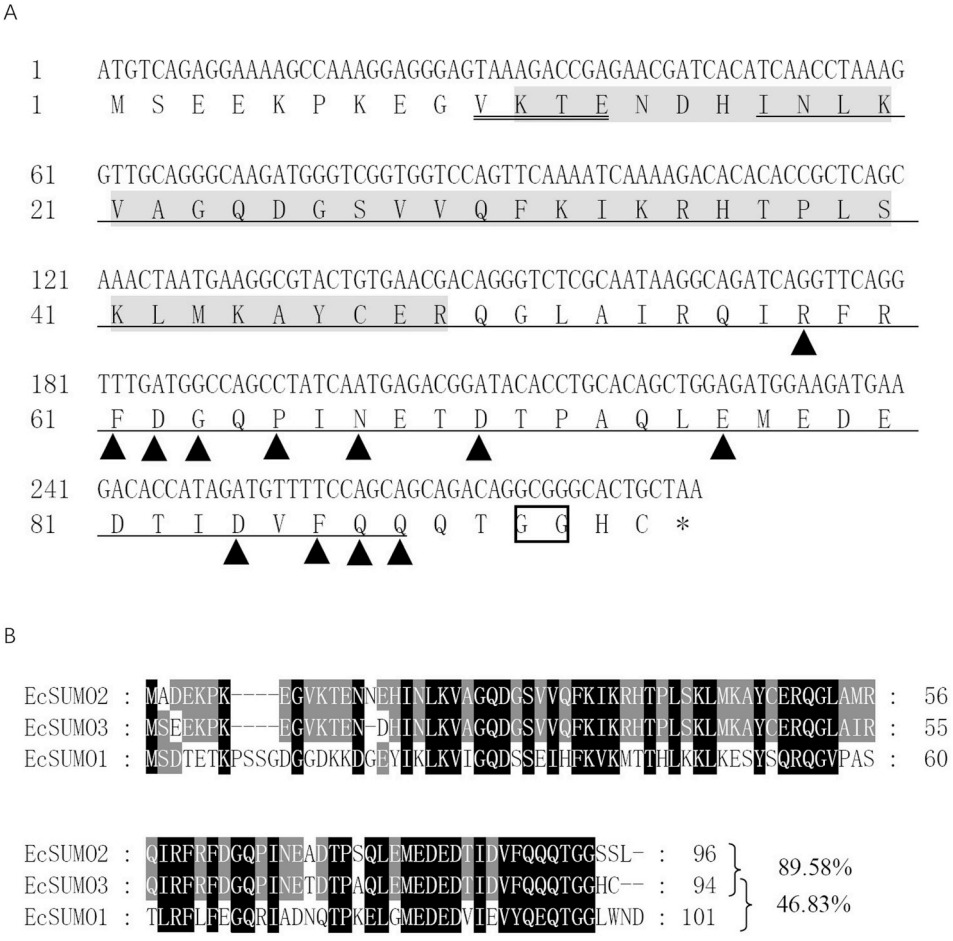
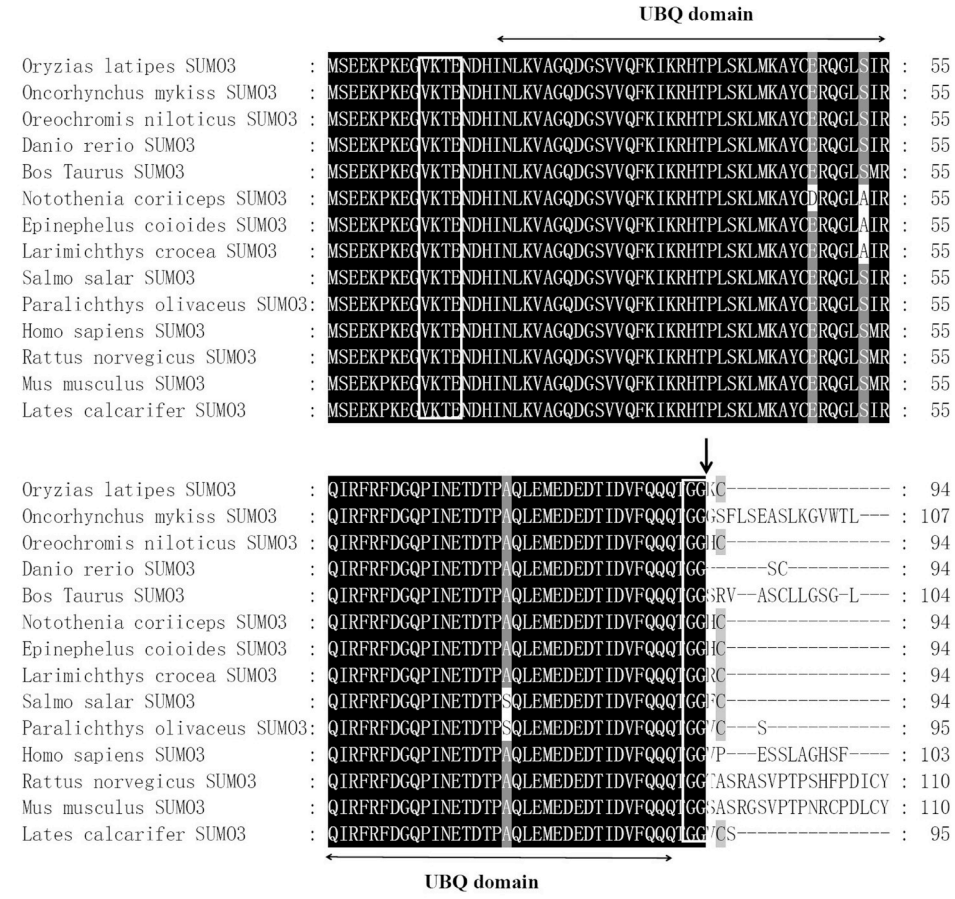



**Fig. 1.** Molecular cloning of grouper SUMO3. (A) Nucleotide and deduced amino acid sequences of EcSUMO3. A ubiquitin homologue (UBQ) domain is underlined. The VKXE motif are double underlined. The hydrophobic surface is shaded. Residues below symbols (▲) are identified as the Ulp1-Smt3 interaction sites. The conserved C-terminal Gly residues are boxed. (B) Multiple sequence alignments of EcSUMO1, EcSUMO2 and EcSUMO3. (C) Multiple sequence alignments of SUMO3s. The full-length amino acid sequences of SUMO3s from typical organisms were aligned using the Clustal X 2.0 program. Arrow represents the UBQ domain. The VKXE motif are boxed. The conserved C-terminal Gly residues were boxed, and the predicted cleavage site is marked by an arrowhead. *Oreochromis niloticus* (XP_003442976.1), *Notothenia coriiceps* (XP_010784790.1), *Larimichthys crocea* (XP_010744946.1), *Danio rerio* (NP_001002677.2), *Oryzias latipes* (NP_001165521.1), *Lates calcarifer* (XP_018557983.1), *Salmo salar* (NP_001158768.1), *Paralichthys olivaceus* (XP_019963648.1), *Oncorhynchus mykiss* (ACO08645.1), *Homo sapiens* (NP_008867.2), *Bos taurus* (NP_001069917.1), *Rattus norvegicus* (NP_001019466.1), *Mus musculus* (NP_064313.1), and *Epinephelus coioides*. (D) A phylogenetic tree was constructed based on alignment of amino acid sequences using the NJ method within MEGA 5.0 and with 1000 bootstrap replications. The bootstrap values are indicated at the nodes of the tree. *E. coioides* is boxed with solid lines.

C

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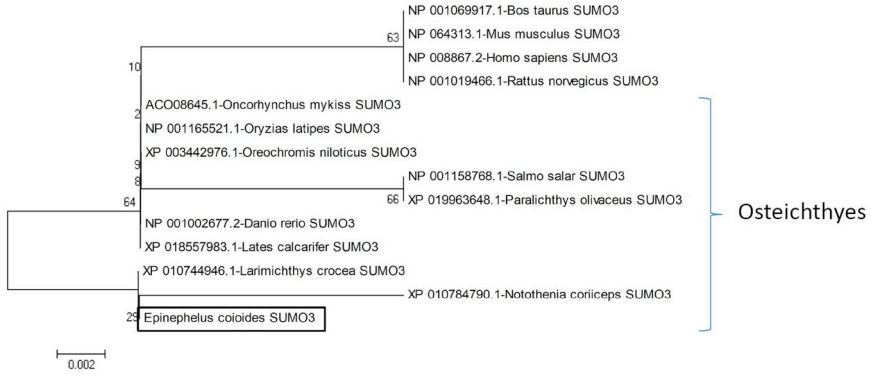


**Fig. 1.** (*continued*)

## 2. Materials and methods

### 2.1. Tissue collection, RNA isolation, and cDNA synthesis

Juvenile *E. coioides* (20–30 g) were purchased from a mariculture farm located in Yantai City, Shandong Province, China. After acclimating in aerated flow-through seawater for 7 days, these fish were used for the challenge experiments. A series of tissue samples, including liver, spleen, kidney, brain, intestine, heart, skin, muscle, stomach, gill, fin, and head kidney, were dissected from the euthanized fish and immediately frozen in liquid nitrogen followed by storage at −80 °C until used for RNA analysis and cDNA synthesis [17].

Total RNA was isolated from different tissues of grouper using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The quality of total RNA was assessed by electrophoresis on 1% agarose gel. Total RNA was reverse transcribed to synthesize first-strand cDNA using the ReverTra Ace kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

### 2.2. Cell lines and viruses

GS cells were propagated using recommended methods in Leibovitz's L15 culture medium with 10% fetal calf serum at 28 °C [18]. Propagation of SGIV and RGNNV was performed as described previously [18,19]. The viral titers of SGIV and RGNNV were $10^5$ $TCID_{50}$/ml, and GS cells were infected the viruses as previously described. Cells were collected for quantitative real-time polymerase chain reaction (qRT-PCR) at 0, 3, 6, 12, 24, and 36 h.

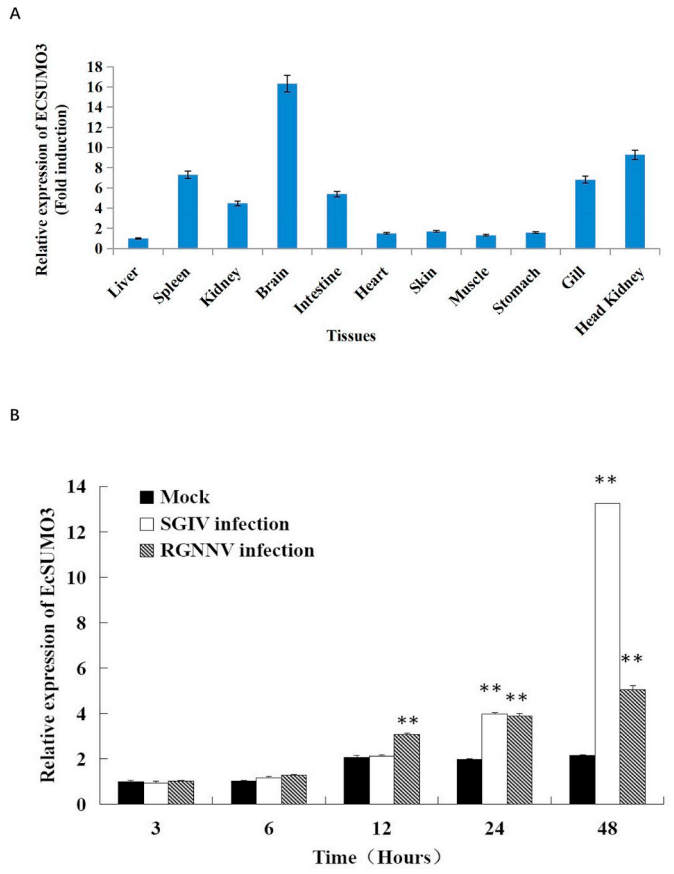


**Fig. 2.** (A) Tissue distribution of EcSUMO3 transcripts. Liver, spleen, kidney, brain, intestine, heart, skin, muscle, stomach, gill, and head kidney tissues were harvested from healthy *E. coioides* to extract total RNA for a tissue distribution analysis. (B) Expression of EcSUMO3 in GS cells at different time points after infection with SGIV and RGNNV. β-actin was used as the internal control. Asterisks (*) mark significant differences between experimental and control groups ($p < 0.05$). Error bars indicate standard error ($n = 3$).

### 2.3. Cloning of EcSUMO3 and bioinformatic analysis

The sequence of open reading frame (ORF) of EcSUMO3 was obtained by transcriptomic sequencing of grouper spleen [15], and confirmed by replication with PCR using specific primers F1 and R1 (Table 1). The full-length cDNA sequence of sumo3 was edited and analyzed using the program EDITSEQ (DNAstar) to search for the open reading frame (ORF). It was then translated into amino acid sequence using standard genetic codes. Sequence alignments and percentage of amino acid conservation were assessed by Clustal-W multiple alignment algorithm. Domains were analyzed with the online CDD tool at NCBI (http://www.ncbi.nlm.nih.gov/) and the SMART program (http://smart.embl-heidelberg.de/). The phylogenetic trees were generated through neighbor-joining (NJ) method with MEGA 5.0.

### 2.4. qRT-PCR

Gene-specific primers F2 and R2 were used to verify the specificity of qRT-PCR. β-actin-F and β-actin-R were used as primers for the β-actin internal control to verify successful transcription and to calibrate the cDNA template for corresponding samples. qRT-PCR amplification was performed on an ABI Quant studio 5 device (Applied Biosystems, Foster City, CA, USA) using the 2 × SYBR Green Real-time PCR Mix (TOYOBO) as previously described.

### 2.5. Cellular localization analysis

Specific primers F3 and R3 were used to amplify the DNA fragments encoding the mature peptide of EcSUMO3. The target PCR products were digested with *EcoR* I and *BamH* I (Takara, Tokyo, Japan) and then subcloned into the *EcoR* I/*BamH*I sites of expression vector pEGFP-C1. Intracellular localization of EcSUMO3 was performed following a previously described method [17]. All glass slides were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

### 2.6. Generation of stable cell lines overexpressing EcSUMO3

Specific primers F4 and R4 were used to amplify the DNA fragments encoding the mature peptide of EcSUMO3. The expression vector pcDNA3.1-3 × HA was constructed. Stable clones of GS cells expressing EcSUMO3 were generated following a previously described method [16]. The resulting lines were named GS/pcDNA3.1-3 × HA and GS/pcDNA3.1-EcSUMO3, respectively.

### 2.7. SGIV and RGNNV quantification

To quantify SGIV and RGNNV in GS cells, viral replication was detected using qRT-PCR in stable cell lines overexpressing EcSUMO3. qRT-PCR was performed in a ABI Quant studio 5 (Applied Biosystems, USA). The expression level of viral genes (SGIV-MCP, SGIV-ICP18, SGIV-VP19, RGNNV-RdRp, and RGNNV-CP) were all detected as previously described method [17]. The relative expression ratio of the selected gene versus β-Actin was calculated using $2^{-\Delta\Delta CT}$ method. Reactions of SYBR Green were performed in a 10 μl volume containing 5 μl 2 × SYBR® Premix Ex Taq™, 0.3 μl each forward and reverse primer (10 μM), and 3.4 μl water and 1 μl cDNA. All experiments were performed in triplicate and the cycling parameters were designed according to the instructions. One-way analysis of variance (ANOVA) was used to evaluate the variability between treatment groups, the significance level was set at $p < 0.05$.

### 2.8. Dual-luciferase reporter assays

To evaluate the promoter activity regulated by EcSUMO3, the luciferase reporter plasmids interferon-stimulated response element (ISRE)-Luc, interferon 3 (IFN3)-Luc, and nuclear factor (NF)-κB (Clontech, Mountain View, CA, USA) were used for co-transfection. Briefly, GS cells were transiently co-transfected with the luciferase plasmids and EcSUMO3 expression vectors using Lipofectamine 2000 reagent. The pRL-SV40 Renilla luciferase vector was used as the internal control. Luciferase activity of total cell lysates was measured using a luciferase reporter assay system (Promega).

### 2.9. Statistical analyses

Student's t-tests were performed using Microsoft Excel to compare the means of two samples. Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

## 3. Results

### 3.1. Sequence and phylogenetic analysis of EcSUMO3

Based on the EST sequence, the ORF of grouper SUMO3 (named EcSUMO3) was obtained using the PCR approach. The ORF of EcSUMO3 contains 285 base pairs encoding a 94 amino acid protein with a predicted molecular mass of 10.73 kDa. No signal peptide and transmembrane helices were found in the deduced amino acid sequence of EcSUMO3. The protein sequence of EcSUMO3 revealed similar domains with mammals, including the UBQ (ubiquitin-like proteins)

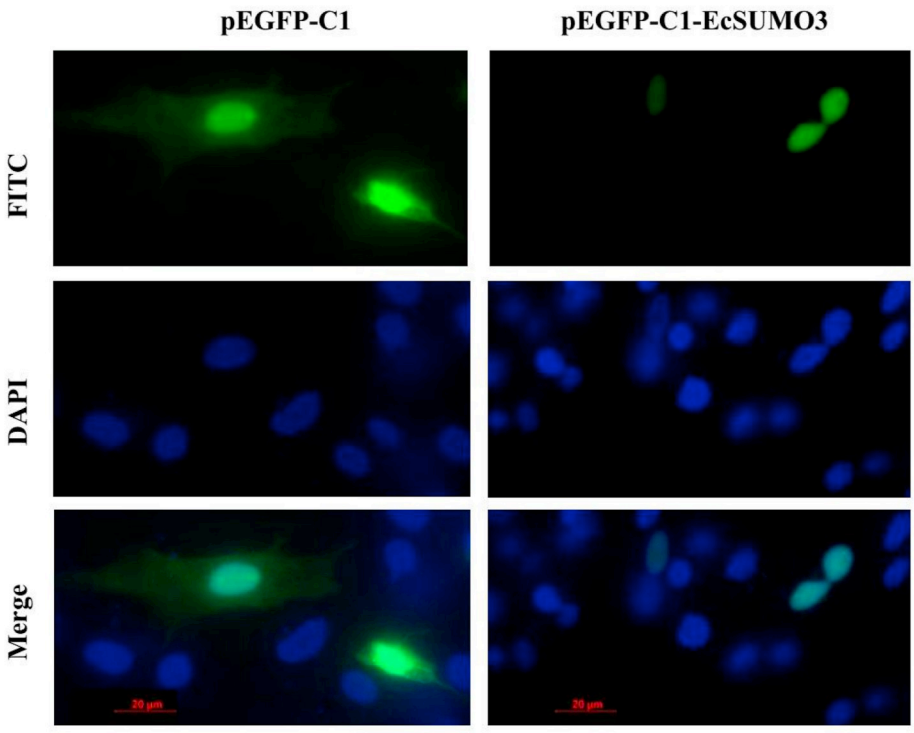


**Fig. 3.** Cellular localization of EcSUNO3. GS cells ($1 \times 10^6$ per well) were seeded onto coverslips in 24-well plates the day before transfection. Cells were transfected with the pEGFP-C1 and pEGFP-C1-EcSUMO3 plasmids using Lipofectamine 2000. At 24 h post-transfection, cells on the coverslips were washed with phosphate buffered saline and fixed with 4% paraformaldehyde for 1 h at 4 °C, then the coverslips were blocked using 2% bovine serum albumin at room temperature for 30 min and stained with DAPI for 10 min.

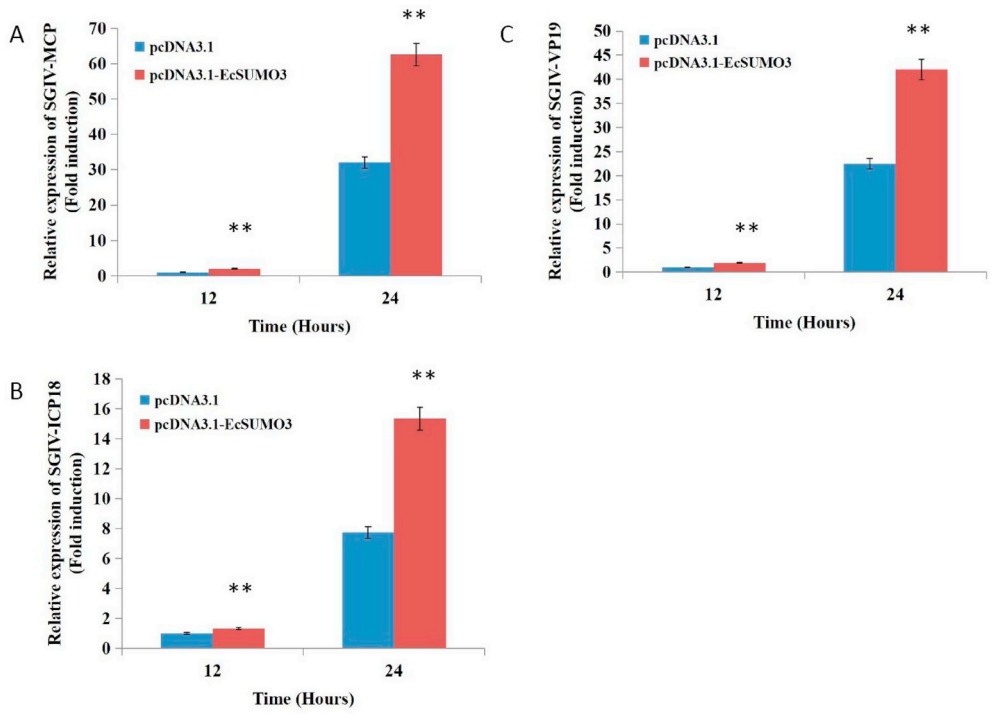


**Fig. 4.** Activities of EcSUMO3 in SGIV infection and replication *in vitro*. Expression levels of (A) MCP, (B) ICP18, and (C) VP18 mRNA in stable cell lines of GS/pcDNA3.1 and GS/pcDNA3.1-EcSUMO3 during SGIV infection. Cell lysates were used for RNA extraction at 12 and 24 h post-infection. Relative expression of MCP, ICP18, and VP18 mRNA was assessed by RT-qPCR and normalized to the 18S reference gene. Data are represented as mean ± standard deviation (n = 3). *, $p < 0.05$; **, $p < 0.01$.

domain, the hydrophobic surface, the Ulp1-Smt3 interaction sites, a VKTE motif and the C-terminal Gly residues. The ubiquitin homologue domain was at residues 17–88 (Fig. 1A). BLAST analysis revealed that EcSUMO3 shares 46.83% and 89.58% identity with EcSUMO1 and EcSUMO2, respectively (Fig. 1B). EcSUMO3 shares 98% identity with SUMO3s from *Danio rerio*, and *Homo sapiens*, respectively. SUMO3 share the conserved C-terminal diglycine cleavage/attachment from *Saccharaomyces cerevisiae* to *H. sapiens* (Fig. 1C). Multiple sequence alignments were carried out using Clustal W multiple-alignment software. Phylogenetic trees were made using the NJ method with 1000 bootstraps. EcSUMO3 clustered into the Osteichthyes branches (Fig. 1D).

### 3.2. Expression patterns of EcSUMO3

qRT-PCR was employed to examine the tissue-specific expression of EcSUMO3 mRNA with β-actin as the internal control. In healthy grouper, the mRNA transcript of EcSUMO3 was constitutively expressed in a wide range of tissues with different expression levels. The highest expression was detected in the brain (Fig. 2A). The time-course analysis

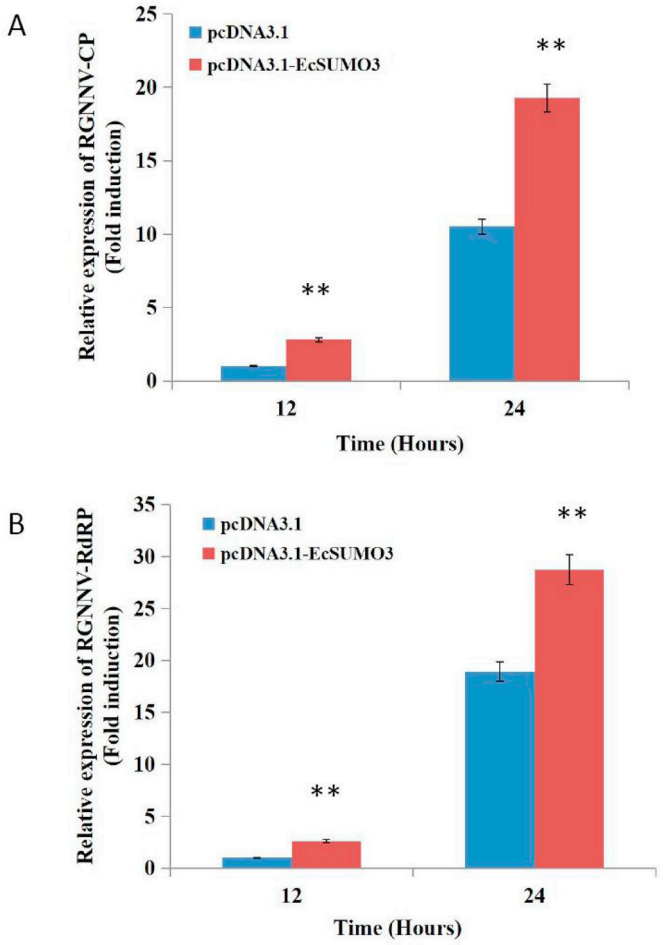


**Fig. 5.** Activities of EcSUMO3 in RGNNV infection and replication *in vitro*. Expression levels of (A) CP and (B) RdRp mRNA in stable cell lines of GS/pcDNA3.1 and GS/pcDNA3.1-EcSUMO3 during RGNNV infection. Cell lysates were used for RNA extraction at 12 and 24 h post-infection. Relative expression of CP and RdRp mRNA was assessed by RT-qPCR and normalized to the 18S reference gene. Data are represented as mean ± standard deviation (n = 3). *, $p < 0.05$; **, $p < 0.01$.

revealed 6.2-fold increased EcSUMO3 expression in GS cells after 36 h of infection with SGIV (Fig. 2B). The expression of EcSUMO3 was also up-regulated significantly ($p < 0.01$) in GS cells after RGNNV infection; it reached the maximal level of a 2.4-fold increase at 36 h compared with the blank group (Fig. 2B).

### 3.3. Intracellular localization of EcSUMO3

To demonstrate the intracellular localization of EcSUMO3, pEGFP-EcSUMO3 was transfected into grouper cells, and the fluorescence was observed under fluorescence microscopy. As shown in Fig. 3, green fluorescence revealed that EcSUMO3 was localized in both the cytoplasm and nucleus of EcSUMO3 transfected grouper cells (Fig. 3). In pEGFP-C1 transfected cells, fluorescence was also distributed throughout both the cytoplasm and nucleus.

### 3.4. EcSUMO3 overexpression increased virus replication

To assess the effects of EcSUMO3 on the replication of SGIV and RGNNV, the transcription kinetics of SGIV and RGNNV genes were measured by qRT-PCR. The expression levels of MCP, ICP18, and VP49 of SGIV were higher in GS/pcDNA3.1-EcSUMO3 than in GS/pcDNA3.1 cell at 12 and 24 h (Fig. 4A, B, C). The expression levels of CP and RdRp of RGNNV were also higher in GS/pcDNA3.1-EcSUMO3 than in GS/pcDNA3.1 cell at both time points (Fig. 5A and B). These data suggest that over-expressed EcSUMO3 enhanced SGIV and RGNNV replication during viral infection *in vitro*.

### 3.5. Overexpression of EcSUMO3 regulated IFN and ISRE promoter activity

To further explore the roles of EcSUMO3 during fish virus infection, reporter genes in typical antiviral pathways, including interferon-stimulated response element (ISRE), type I interferon (IFN) and NF-κB were measured with plasmids ISRE-Luc, INF-Luc and NF-κB-Luc. As shown in Fig. 6, EcSUMO3 overexpression increased the IFN promoter activity and ISRE promoter activity (Fig. 6A and B). While EcSUMO3 could not regulate NF-κB promoter activity (Fig. 6C).

## 4. Discussion

Protein SUMOylation plays an important part in cellular functions and has been linked to changes in DNA repair, intracellular trafficking, cell signaling, and stress responses [20–24]. Although a growing number of studies have identified new SUMOylated proteins and related biological events [25–28], the regulation of SUMOylation remains a challenging topic [4,29]. After being expressed and processed, SUMOs, especially SUMO2/3, can remain free or can be conjugated to targets [30,31]. SUMO has been cloned and characterized in *H. sapiens* and a range of model species [16,32–34]; however, little is known about SUMOs of groupers. To the best of our knowledge, it is the first report of cloning and investigating the expression profiles of the grouper SUMO3 gene.

In this study, we identified one SUMO3 homologue gene in *E. coioides*. EcSUMO3 contains a ubiquitin homologue domain, which is a unique feature of SUMOs. Previous studies reported that the cleavage site of SUMO lies between a Gly–Gly motif and the C terminal amino acids, which is the critical region for SUMO protein maturation and conjugation [16]. EcSUMO3 also has the Gly–Gly motif in its C-terminus, implying that its maturation process is similar to those of other SUMO and ubiquitin-like modifiers [16]. EcSUMO3 also possess a VKTE motif, which is consistent with the SUMOylation consensus YKXE (Y represents a hydrophobic amino acid, X means any amino acid), and this consensus sequence is functional for possible polymerization [35].

The sumoylation pathway is conserved from yeast to human. In mammalian cells, different SUMO paralogs appear to share common conjugation properties, but also have some specificities, such as subcellular distribution and substrate preferences. For instance, it is reported that, within cells, there is a larger pool of free, non-conjugated SUMO2/3 than that of SUMO1 [23]. The distribution of the SUMO paralogs within cells also seems to be different. SUMO1 is found within the nucleoli, the nuclear envelope and cytoplasmic foci, whereas SUMO2/3 are dominant on chromosomes. In our previous studies, EcSUMO1 and EcSUMO2 were distributed in both cytoplasm and nucleus in GS cells [16]. In the present study, the intracellular localization of EcSUMO3 was also examined. EcSUMO3 was distributed in both the cytoplasm and nucleus in the GS cells, which is similar to that of EcSUMO1/2 and human SUMO1/2/3 [16,36]. These results indicate that grouper SUMOs may have a function similar to that of human SUMOs.

In this work, we examined the tissue distribution of EcSUMO3 as a preliminary step to shed some light on their physiological roles. The expression of human SUMO1 and SUMO2/3 is ubiquitous. In healthy grouper, the expression of EcSUMO3 was constitutively expressed in a wide range of tissues with different expression levels, which is consistent with the pattern for EcSUMO1 and EcSUMO2 [16], implying that they participated in general SUMOylation reactions occurring at basal levels in all tissues. Moreover, our study indicated that EcSUMO3 showed relatively high mRNA levels in brain, gill, spleen, head kidney, kidney and intestine, and lowest mRNA levels in liver, muscle, skin, heart, and stomach. High mRNA expression of sumo1 and sumo2 in gills

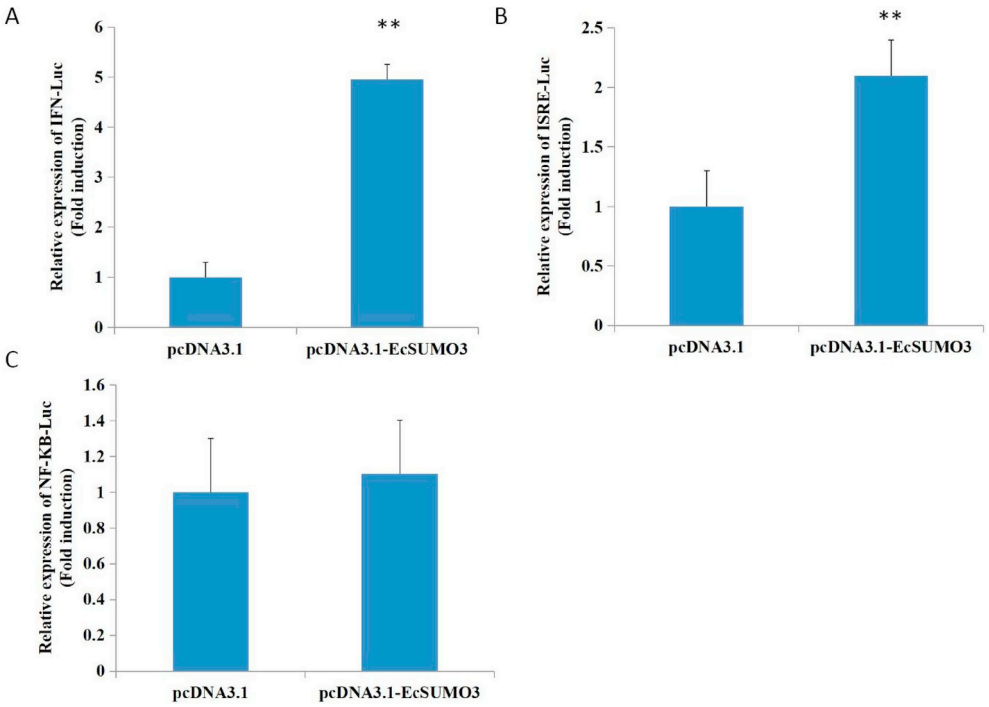


**Fig. 6.** Overexpression of EcSUMO3 increased IFN promoter and ISRE promoter activity. GS cells were co-transfected with IFN-Luc/ISRE-Luc/NF-κB-Luc and EcSUMO3. After 24 h, promoter activity was measured using the luciferase reporter gene assay.

also had been reported in grouper [16]. The gills were primary sites where fish encounter environmental stresses [37]. Therefore, we can reasonably infer that the high expression level of these genes in the gills might be a sensor for environmental stress in fish that stimulates the SUMOylation and deSUMOylation responses.

SUMOylation, the post-translational conjugation of the Small Ubiquitin-like MOdifier (SUMO) to a target protein, regulates a wide array of cellular processes and plays important roles for numerous viruses during infection [38]. In WSSV infection experiment, SUMO and UBC9 from Chinese shrimp *Fenneropenaeus chinensis* (named as FcSUMO and FcUBC9) were up-regulated post infection, and the up-regulation extent of these two genes in hemocytes was much higher than that in ovary [39]. Similarly, the mRNA expressions of FcSUMO and FcUBC9 were significant up-regulated post WSSV infection in the hepatopancreas and intestine [40]. These results demonstrate that SUMOylation plays an important role in immune response of hemocytes in shrimp to viral infection. To characterize how the EcSUMO3 gene responds to virus infection, the time-course of EcSUMO3 expression in GS cells after SGIV and RGNNV challenges was evaluated in this study. SGIV, a novel ranavirus belonging to the family *Iridoviridae*, was first isolated from the brown-spotted grouper, causing serious systemic diseases and can result in more than 90% mortality in grouper in fish farms or challenge experiments [12]. RGNNV, belonging to the family *Nodaviridae*, genus Betanodavirus, and possesses a bipartite, positive sense, single-stranded RNA genome, is the causative agent of a highly lethal disease in larval grouper [14]. In SGIV and RGNNV infection experiments, EcSUMO3 were up-regulated post infection in GS cells. Similarly, EcSUMO1 and EcSUMO2 were identified as a remarkably ($P < 0.01$) up-regulated protein responding to poly(I:C) and SGIV stimulation in head kidney of grouper [16]. These results indicate that SUMOylation may play important roles in immune response of fish to viral infection.

Detection of nucleic acids by innate immune sensors triggers the production of type I interferons (IFNs) [41]. While IFNs are essential for host defense against viral infection, dysregulated production of IFNs underlies numerous autoinflammatory diseases. The loss of sumoylation results in a potent, spontaneous IFN response. In previous study, an unanticipated mechanism of IFN regulation and a noncanonical pathway that potently activates the type I IFN response were identified. SUMO2 and SUMO3 are redundant and essential negative regulators of a spontaneous IFN response in human cells [41]. In this study, EcSUMO3 enhanced SGIV and RGNNV replication during viral infection *in vitro*. To further explore the roles of EcSUMO3 during virus infection in fish, reporter genes in typical antiviral pathways were measured. EcSUMO3 only increased the IFN promoter activity and ISRE promoter activity, indicating that EcSUMO3 may be involved in the IFN signaling pathways in fish. Which transcription factors are required for EcSUMO3 to activate the IFN signaling pathway needs further study.

In conclusion, EcSUMO3 was cloned and characterized in this study. EcSUMO3 is evolutionarily conserved and its expression is responsive to viral infections. EcSUMO3 enhanced the replication of SGIV and RGNNV during viral infection *in vitro*. These results illustrate that SUMO3s of fish and mammals are evolutionarily conservative in function and that SUMO3 expression is responsive to viral infection.

## Acknowledgements

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