Fish and Shellfish Immunology 94 (2019) 113–121

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular cloning, expression and functional analysis of Atg16L1 from orange-spotted grouper (*Epinephelus coioides*)

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ARTICLE INFO

Keywords:
Grouper
Autophagy
Atg16L1
SGIV
RGNNV

ABSTRACT

Autophagy related gene 16 (Atg16), which encodes a core protein for autophagosome formation, participates in autophagy activity, the ubiquitin proteasome system and inflammatory response in mammals. In this study, we cloned and characterized an Atg16 homolog from orange-spotted grouper (*Epinephelus coioides*) (EcAtg16L1). EcAtg16L1 encodes a 656-amino acid polypeptide, which shares 94.22% and 72.65% homology with large yellow croakers (*Larimichthys crocea*) and humans (*Homo sapiens*), respectively. EcAtg16L1 contains a conserved Atg16 domain and a WD-repeat-containing domain. Subcellular localization showed that EcAtg16L1 was distributed in the cytoplasm of grouper cells with a dot-like pattern. EcAtg16L1 overexpression promoted Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous necrosis virus (RGNNV) replication, as evidenced by the increase in viral gene transcription and viral coat protein. Furthermore, EcAtg16L1 overexpression negatively regulated interferon (IFN)-related molecules and proinflammatory cytokines, and decreased IFN, IFN-stimulated response element, and nuclear factor κB promoter activities. Taken together, aside from its function in autophagosome formation, EcAtg16L1 also plays role in promoting SGIV and RGNNV replication and the pro-viral effect might involve its down regulation to interferon and inflammatory responses.

1. Introduction

Autophagy is a ubiquitous phenomenon in eukaryotes. In addition to being involved in adaptation to nutrient deprivation, autophagy plays a role in regulating inflammation, defending against invading pathogens, reducing apoptosis, and maintaining cell homeostasis [1,2]. Autophagy starts with the formation of membrane vesicles called autophagosomes, which subsequently fuse with lysosomes and break down the sequestered cargo, including damaged organelles and invading pathogens [3]. The process is precisely regulated by a series of autophagy related genes (Atg*s*). Among them, 15 Atgs have been confirmed as “core” members because they are necessary for autophagosome formation [4]. Microtubule-associated protein 1 light chain 3 (LC3/Atg8) is the marker protein of autophagy, as PE conjugation of LC3 (LC3 lipidation, LC3-II) locates on the autophagosome membrane. The Atg12-Atg5 conjugate facilitates and recruits LC3-II to the isolation autophagosome membrane [5]. Atg16L (Atg16-like protein, a homolog of Atg16 in yeast) or Atg16L1 is responsible for guiding Atg12-Atg5 and LC3-II correctly to target the autophagosome membrane [6–8]. In addition, Atg16L1 has been reported to play crucial roles in other physiological or pathological conditions, including mediating cross-talk between autophagy and the ubiquitin proteasome system, regulating inflammatory transcriptional responses, and mediating bacterial infection [9,10].

Groupers (*Epinephelus* spp.) are popular commercial species in China and Southeast Asia. Unfortunately, the marine fishes always suffer from viruses infection. Among them, Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous necrosis virus (RGNNV) are two representative pathogens which have caused more than 90% mortality in grouper and seabass [11,12]. In recent years, numerous interferon (IFN)-related molecules of grouper have been cloned, and their roles on viruses replication have been elucidated [13–18]. Upon challenge with RGNNV, the transcript of grouper interferon regulatory factor 3 (IRF3) and interferon stimulated genes 15 (ISG15) were significantly up-regulated, and the overexpression of IRF3 and ISG15 inhibited RGNNV replication but showed no significant effect on SGIV [13,14]. However,

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https://doi.org/10.1016/j.fsi.2019.09.004
Received 17 June 2019; Received in revised form 16 August 2019; Accepted 2 September 2019
Available online 03 September 2019

grouper IRF7 was up-regulated in SGIV-infected cells and decreased the virus replication [19]. The overexpression of grouper EcMDA5 in *vitro* obviously decreased virus multiplication of RGNNV and SGIV [15]. In study on grouper Atgs, the latest research shows that grouper Atg5 promotes SGIV and RGNNV replication by negatively regulating the antiviral immune response [20]. While the function of grouper Atg16L1 remains uncertain. In mammals, a great deal work focused on the Atg16L1 deficiency enhanced the resistance to intestinal bacterial infection in mice and humans, revealing the importance of Atg16L1 in the suppression of intestinal inflammation [9,21–24]. There is relatively few research about the effect of Atg16L1 on virus replication. Atg16L1 increased in hepatitis B virus (HBV)-hepatocellular carcinoma cells. But silencing Atg16L1 has no significant effect on HBV copy number titer [25].

In this study, we cloned and characterized a core autophagy gene (Atg16L1) from orange-spotted grouper (*Epinephelus coioides*) (EcAtg16L1). The subcellular localization of EcAtg16L1 was investigated in transfected grouper spleen (GS) cells, and the effects of EcAtg16L1 overexpression on SGIV and RGNNV replication were evaluated. Additionally, the roles of EcAtg16L1 in autophagy, interferon and inflammatory responses were investigated. The results of this study provide a better understanding of the autophagy-dependent or -independent functions of EcAtg16L1.

2. Materials and methods

2.1. Fishes, cells, and virus

Orange-spotted groupers (50–60 g) used in this study were purchased from a farm located in Hainan Province, China. The fishes, with no symptoms of disease, were kept in our laboratory as described previously [26]. The grouper spleen (GS) cell line was established in our laboratory and maintained at 28 °C in Leibovitz's L15 medium supplemented with 10% fetal bovine serum [27]. Virus stocks of RGNNV and SGIV were separated and propagated, as described previously [11,12].

2.2. Cloning of EcAtg16L1 and bioinformatic analysis

To obtain the open reading frame of EcAtg16L1, the primers listed in Table 1 were designed for cloning based on the expressed sequence tag sequences of EcAtg16L1 from the grouper transcriptome [28]. The sequenced EcAtg16L1 was analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast), and the conserved domain was analyzed using the SMART program (http://smart.emblheidelberg.de/). Amino acid alignments were carried out using MEGA5.0 software and edited using the GeneDoc program. The phylogenetic analysis was carried out using the boot-strapped neighbor joining method in MEGA 5.0.

2.3. Expression patterns of EcAtg16L1 in grouper

Three asymptomatic groupers were used in this study. Total RNA from 12 tissues (liver, spleen, brain, head kidney, kidney, gill, heart, muscle, intestine, fin, skin, and stomach) were individually extracted, as well as DNA from spleen. Because the brain and spleen are respectively the target organs of RGNNV and SGIV, the cDNA from brain and DNA from spleen were detected by PCR using the primers for RGNNV and SGIV, respectively. Virus-free cDNA were used as templates for tissue distribution. The relative expression level of EcAtg16L1 was detected by quantitative real-time PCR (qRT-PCR), as described in a subsequent section.

To determine the expression profiles of EcAtg16L1 in response to virus infection, GS cells were infected with SGIV or RGNNV at a multiplicity of infection (MOI) of 2.0. At 3, 6, 9, 12, 24, 30, and 36 h post infection (h.p.i.), each group of GS cells from three wells of 24-well plates were collected for RNA extraction and further qRT-PCR analysis.

Table 1
Primers used in this study.

Name	Sequence (5′–3′)
EcAtg16L1-F	ATGGCGGAGCGGCGGG
EcAtg16L1-R	TCACATGTCCGACCACAG
C1-EcAtg16L1-F	GGGGTACCATGGCGGAGCGGCGGG
C1-EcAtg16L1-R	CGGGATCCTCACATGTCCGACCACAG
HA-EcAtg16L1-F	GGGGTACCATGGCGGAGCGGCGGG
HA-EcAtg16L1-R	CGGAATTCTTCACATGTCCGACCACAG
EcAtg16L1-RT-F	GAACAGCCAACTCCTTCAGCACG
EcAtg16L1-RT-R	TCCTTTAGGCTCACAGCGACCAG
β-actin-RT-F	TACGAGCTGCCTGACGGACA
β-actin-RT-R	GGCTGTGATCTCCTTCTGCA
EcIRF3-RT-F	ATGGTTTAGATGTGGGGGTGTCGGG
EcIRF3-RT-R	GAGGCAGAAGAACAGGGAGCACGGA
EcIRF7-RT-F	CAACACCGGATACAACCAAG
EcIRF7-RT-R	GTTCTCAACTGCTACATAGGGC
EcISG15-RT-F	CCTATGACATCAAAGCTGACGAGAC
EcISG15-RT-R	GTGCTGTTGGCAGTGACGTTGTAGT
EcMDA5-RT-F	ACCTGGCTCTCAGAATTACGAACA
EcMDA5-RT-R	TCTGCTCCTGGTGGTATTCGTTC
EcMXI-RT-F	CGAAAGTACCGTGGACGAGAA
EcMXI-RT-R	TGTTTGATCTGCTCCTTGACCAT
EcLGP2-RT-F	TGGTGGTACGCTATGGACTGC
EcLGP2-RT-R	TTGTAGCTCAGTTATCTTTGTGCGA
IFP35-RT-F	TTCAGATGAGGAGTTCTCTCTTGTG
IFP35-RT-R	TCATATCGGTGCTCGTCTACTTTCA
MyD88-RT-F	AGCTGGAGCAGACGGAGTG
MyD88-RT-F	GAGGCTGAGAGCAAACTTGGTC
EcTNF-α-RT-F	GTGTCCTGCTGTTTGCTTGGTA
EcTNF-α-RT-R	CAGTGTCCGACTTGATTAGTGCTT
EcIL-1β-RT-F	AACCTCATCATCGCCACACA
EcIL-1β-RT-R	AGTTGCCTCACAACCGAACAC
EcIL8-RT-F	GCCGTCAGTGAAGGGAGTCTAG
EcIL8-RT-R	ATCGCAGTGGGAGTTTGCA
MCP-RT-F	GCACGCTTCTCTCACCTTCA
MCP-RT-R	AACGGCAACGGGAGCACTA
ICP18-RT-F	ATCGGATCTACGTGGTTGG
ICP18-RT-R	CCGTCGTCGGTGTCTATTC
VP19-RT-F	TCCAAGGGAGAAACTGTAAG
VP19-RT-R	GGGGTAAGCGTGAAGACT
LITAF-RT-F	GATGCTGCCGTGTGAACTG
LITAF-RT-R	GCACATCCTTGGTGGTGTTG
CP-RT-F	CAACTGACAACGATCACACCTTC
CP-RT-R	CAATCGAACACTCCAGCGACA
RdRp-RT-F	GTGTCCGGAGAGGTTAAGGATG
RdRp-RT-R	CTTGAATTGATCAACGGTGAACA

2.4. Plasmid construction and cell transfection

To clarify the subcellular location of EcAtg16L1 in *vitro*, EcAtg16L1 was subcloned into pEGFP-C1. To analysis the function of EcAtg16L1, the gene was cloned into pcDNA3.1-3 × HA. The primers used are listed in Table 1. The recombinant plasmid (named C1-EcAtg16L1 or HA-EcAtg16L1, respectively) was subsequently confirmed by DNA sequencing.

Cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. Briefly, GS cells were seeded into 24-well plates or glass bottom cell culture dishes at 70–80% confluence, then plasmid and Lipofectamine 2000 were, respectively, diluted in Opti-MEM (Gibco, USA) in two separate sterile tubes. After incubation for 5 min at room temperature, the Lipofectamine 2000 and diluted plasmid were mixed gently and thoroughly. The mixture was further incubated for 25 min at room temperature before being added dropwise to the cells. After 6 h, fresh normal medium was used to replace the incubation medium for further culture.

2.5. Fluorescence microscopy

To examine the subcellular localization of EcAtg16L1, pEGFP-C1 and C1-EcAtg16L1 plasmids were transfected into GS cells as described

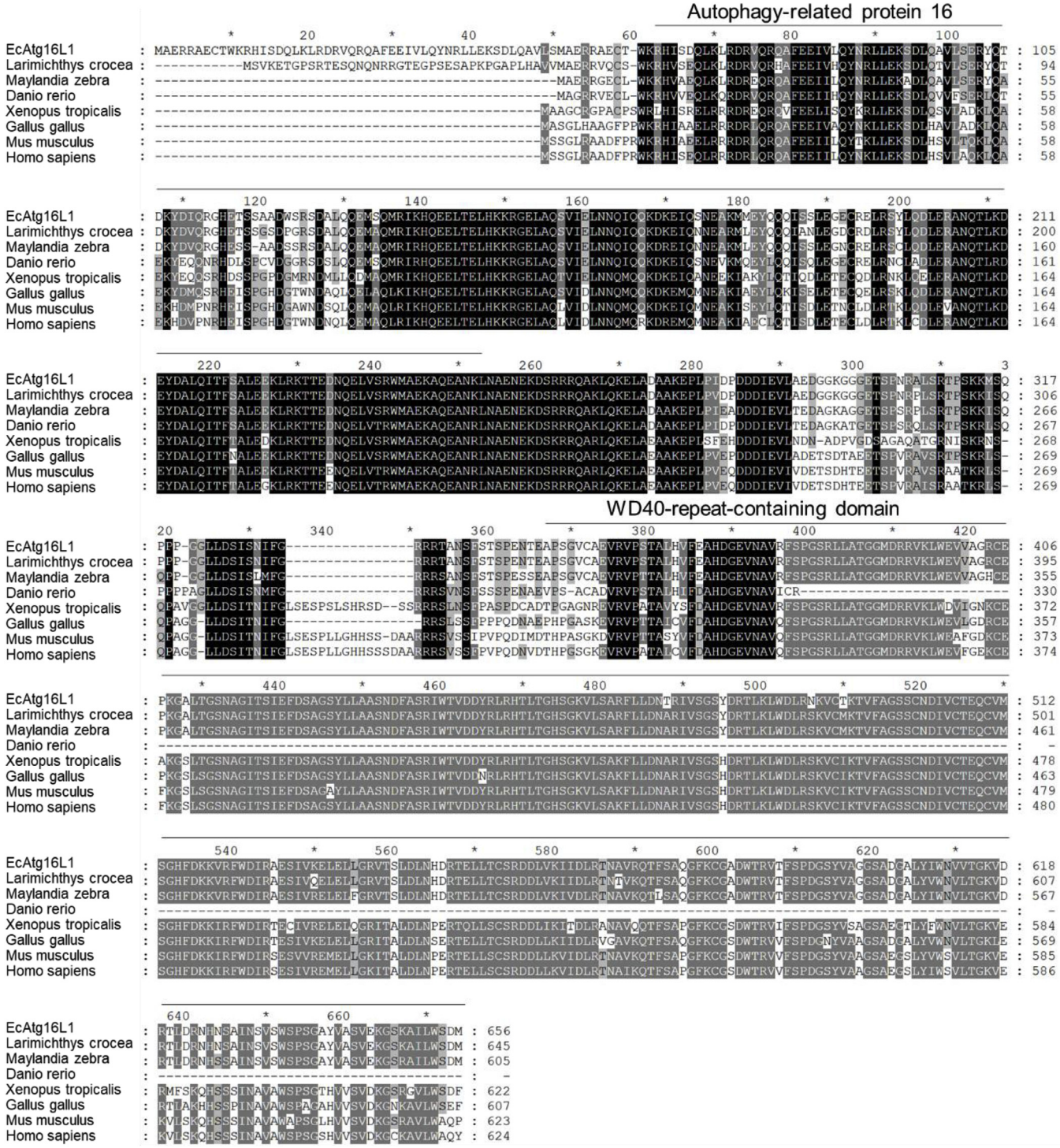

Fig. 1. Multiple sequence alignments of EcAtg16L1 amino acid sequences with that in other species. The putative conserved domain is indicated.

above. To examine the effect of EcAtg16L1 overexpression on autophagy, pcDNA3.1-3 × HA or HA-EcAtg16L1 was co-transfected with C1-LC3 (preserved in our laboratory). Cells were fixed with polyformaldehyde at 24 h post transfection and then stained with 4,6-diamidino-2-phenylindole (DAPI). After washing with phosphate buffered saline (PBS), cells were observed under a fluorescence microscope.

2.6. Virus infection assay

To evaluate the effects of EcAtg16L1 on virus replication, GS cells overexpressing pcDNA3.1-3 × HA or HA-EcAtg16L1 were infected with SGIV (MOI = 2) or RGNNV (MOI = 2). At the indicated time points, cell morphology was observed under a phase contrast microscope. Virus-infected cells were harvested for RNA and protein extraction.

2.7. RNA extraction and qRT-PCR for gene expression analysis

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA), and reverse transcription was carried out with ReverTra Ace (Toyobo, Osaka, Japan), according the manufacturer's instructions. qRT-PCR was performed using SYBR® Green Realtime PCR Master Mix (Toyobo) in an Applied Biosystems

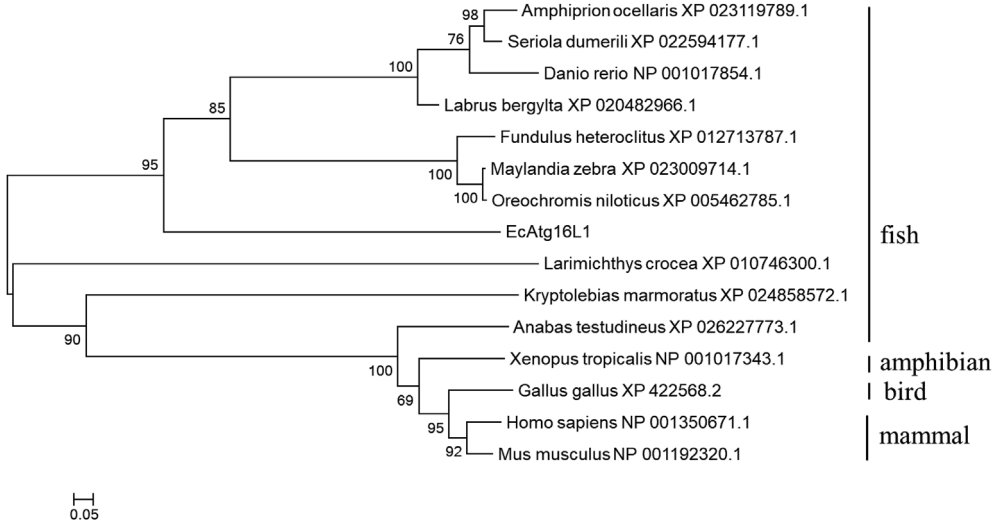

Fig. 2. Phylogenetic tree of Atg16L1 proteins. The accession number for each species is listed to the right of the species name. Numbers at the nodes denote the bootstrap values of 1000 replicates. The scale bar represents 0.05 change per site.

QuantStudio 5 Real Time Detection System (Thermo). Table 1 lists all primers used for qRT-PCR. The primers for EcAtg16L1 were designed in this study. The primers for the transcription of host IFN signaling molecules (IRF3, IRF7, ISG15, MDA5, LGP2, IFP35, MXI, MyD88), proinflammatory factors (IL-1β, IL-8, TNF-α), SGIV genes (MCP, ICP18, VP19, LITAF), and RGNNV genes (CP, RdRp) were designed previously [13–15,19,26]. Each assay was carried out in triplicate with the following cycling conditions: activation at 95 °C for 1 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The expression levels of target genes were normalized to β-actin and calculated using the $2^{-\triangle\triangle CT}$ method. The data are represented as mean ± standard error of the mean.

2.8. Western blot analysis

Cells were collected, and the pellets was lysed in RIPA lysis buffer. Proteins were separated by suitable SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 5% skim milk, membranes were incubated with the indicated primary antibody for 2 h at room temperature. The primary antibodies used in this study were anti-HA (Sigma), anti-LC3B (Abcam), anti-actin (Proteintech), anti-SGIV MCP, and anti-RGNNV CP (prepared in our laboratory). After washing with PBST (PBS + 0.1% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse IgG. After washing three times with PBST, immunoreactive proteins were visualized by chemiluminescence using Thermo Scientific Pierce Western Blot ECL Plus (Thermo).

2.9. Dual-luciferase reporter gene assays

To evaluate whether EcAtg16L1 overexpression affects IFN promoter activity, luciferase plasmids, including IFN-Luc, IFN-stimulated response element (ISRE)-Luc, and nuclear factor κB (NF-κB)-Luc, were co-transfected with pcDNA3.1-3 × HA or HA-EcAtg16L1, respectively. Meanwhile, pRL-SV40 Renilla luciferase vector (Promega) was co-transfected as an internal control. At 36 h post transfection, cells were harvested to detect the luciferase activities using the Dual-Luciferase® Reporter Assay System (Promega) on a Varioskan Flash (Thermo).

2.10. Statistical analyses

Statistical analyses were carried out using SPSS version 16.0. One-way analysis of variance was used to evaluate the differences between pcDNA3.1-3 × HA and HA-EcAtg16L1 in relative expression of genes, as well as the relative promoter activity of reporter genes. The differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Characterization of EcAtg16L1

The full-length open reading frame of EcAtg16L1 was obtained using PCR amplification. Sequence analysis indicated that EcAtg16L1 encodes a 656-amino acid protein that shares 94.22% and 72.65% identity with large yellow croakers (*Larimichthys crocea*) and humans (*Homo sapiens*), respectively. Amino acid alignment analysis showed that EcAtg16L1 contains the Atg16 domain (63–253 aa) and WD-repeat-containing domain (367–656 aa), which are conserved from fishes to humans (Fig. 1). Phylogenetic analysis indicated that EcAtg16L1 was most closely related to that of *L. crocea* (Fig. 2).

3.2. Expression patterns of EcAtg16L1

Using qRT-PCR, the expression patterns of EcAtg16L1 in different tissues and under virus stimulation were detected. EcAtg16L1 was distributed in all tissues, but predominantly in the spleen, kidney, and skin (Fig. 3A). In response to SGIV infection, the transcription levels of EcAtg16L1 decreased after 6 h.p.i. However, in RGNNV infected cells, the transcription levels of EcAtg16L1 increased compared with the non-infected cells at the same time.

3.3. EcAtg16L1 encoded a cytoplasmic protein

The plasmids pEGFP-C1 and C1-EcAtg16L1 were transfected into GS cells, then the fluorescence was observed under a fluorescence microscope. As shown as in Fig. 4, The green fluorescence was distributed in both cytoplasm and nucleus in pEGFP-C1 transfected cells, whereas in C1-EcAtg16L1 transfected cells, the green fluorescence was observed in the cytoplasm in the form of clusters (Fig. 4). The results suggested that EcAtg16L1 might be a cytoplasmic protein.

3.4. EcAtg16L1 overexpression facilitated SGIV and RGNNV replication in vitro

GS cells overexpressing HA-EcAtg16L1 or the empty vector (Fig. 5A) were infected with SGIV or RGNNV, then viral replication was

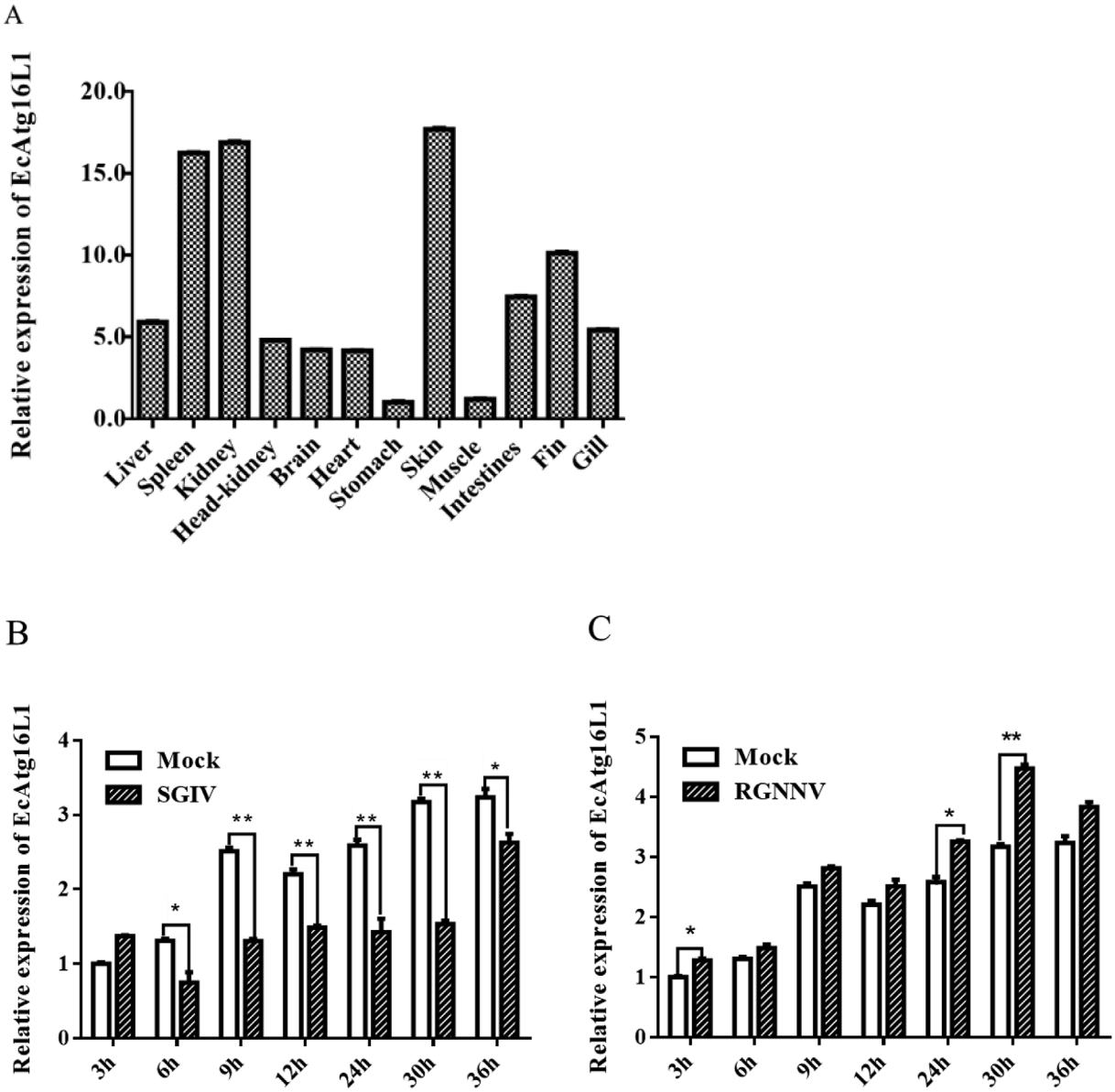

Fig. 3. Expression pattern of EcAtg16L1 determined by qPCR analysis. (A) Expression levels of EcAtg16L1 in different tissues from asymptomatic groupers. Expression levels of EcAtg16L1 in GS cells at different time points post SGIV (B) or RGNNV (C) infection. The β-actin gene was used as the internal control. An asterisk denotes a significant difference (*$p < 0.05$, **$p < 0.01$).

investigated. At 12 and 24 h.p.i., quantitative analysis indicated that overexpression of EcAtg16L1 significantly increased the transcription of RGNNV CP and RdRp genes (Fig. 5B). The transcription levels of SGIV MCP, ICP18, VP19, and LITAF significantly increased in EcAtg16L1-overexpressing cells compared with control cells (Fig. 5C). We also investigated the level of viral protein in infected GS cells at 18 and 24 h.p.i. Consistently, the RGNNV CP protein level and SGIV MCP protein level were increased in EcAtg16L1 overexpressing cells (Fig. 5D and E).

3.5. EcAtg16L1 overexpression enhanced autophagy activity

Autophagy is characterized by the formation of autophagosomes. LC3-II, which is located in the autophagosome, is used as a faithful marker of the occurrence of autophagy. Thus, the role of EcAtg16L1 in classical autophagy was examined by investigating GFP-LC3 dot formation and LC3-I/LC3-II conversion. At 24 h after co-transfection, the GFP-LC3 reporting system showed that significant fluorescent dots were present in EcAtg16L1-overexpressing cells compared with the empty vector overexpressing cells (Fig. 6A). Meanwhile, endogenic LC3 was detected by Western blot after separate transfection with pcDNA3.1-3 × HA or HA-EcAtg16L1. A notable increase in LC3-II was visualized in EcAtg16L1-overexpressing cells, indicating that EcAtg16L1 overexpression enhanced autophagy activity in GS cells (Fig. 6B).

3.6. EcAtg16L1 overexpression negatively regulated IFN signaling molecules and proinflammatory factors

To further explore why EcAtg16L1 overexpression facilitated virus replication, the effects of EcAtg16L1 on IFN signaling molecules and proinflammatory factors were evaluated using qRT-PCR. At 24 h after transfection, the expression levels of IFN signaling molecules (IRF3, IRF7, ISG15, MDA5, LGP2, IFP35, MXI, MyD88) were all significantly decreased in EcAtg16L1-overexpressing cells compared with the control vector transfected cells (Fig. 7). Moreover, the expression of IL-1β and IL-8 were significantly decreased in EcAtg16L1-overexpressing cells,

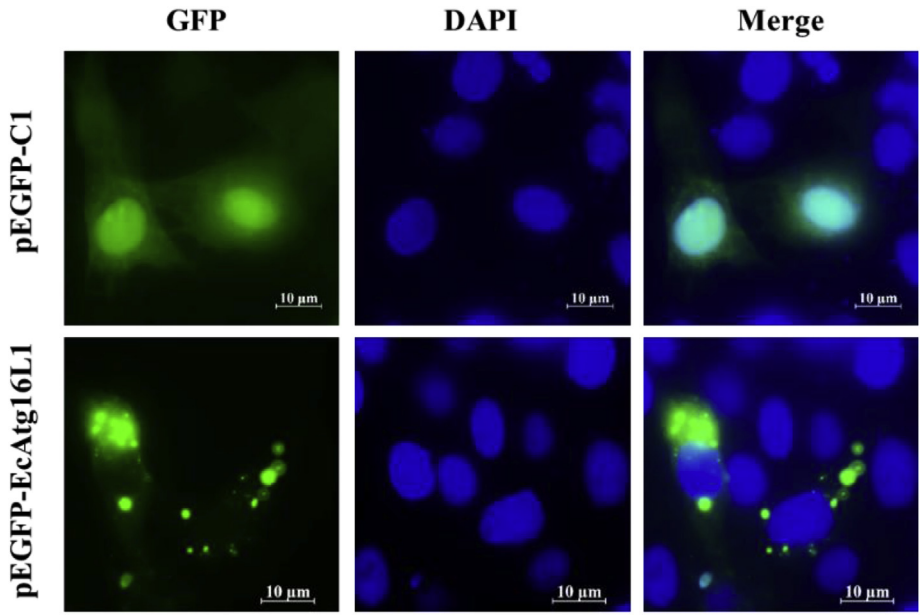

Fig. 4. Subcellular localization of EcAtg16L1 in grouper cells. GS cells were transfected with plasmids of pEGFP-C1 or C1- EcAtg16L1 and then stained with DAPI. Samples were observed under a fluorescence microscope.

whereas no significant changes in the transcription of TNF-α was detected compared with control cells (Fig. 8). These results suggested that EcAtg16L1 overexpression might negatively regulate the IFN and inflammatory transcriptional responses to a certain extent.

3.7. EcAtg16L1 overexpression decreased ISRE, IFN, and NF-κB promoter activity

Using the dual-luciferase reporter assay system, we evaluated the promoter activity of IFN, ISRE, and NF-κB after co-transfection with EcAtg16L1 or the empty vector. EcAtg16L1 overexpression significantly reduced the relative luciferase activity of ISRE, IFN, and NF-κB promoters compared with the cells transfected with the empty vector (Fig. 9). This result suggests that EcAtg16L1 decreased the IFN and NF-κB promoter activity and negatively regulated gene transcription.

4. Discussion

In complex multicellular organisms, Atg proteins participate in other processes besides their autophagy-dependent roles [1,29,30]. Atg16L, a core protein for autophagosome formation, also participates in innate immunity, inflammatory responses, and response to bacterial infection in mammals [9,31]. However, the role of Atg16L1 in fishes is still unknown. To better understand the function of fish Atg16L1, we cloned and characterized grouper Atg16L1 (EcAtg16L1) and investigated its roles during SGIV and RGNNV infection.

Bioinformatics analysis revealed that EcAtg16L1 shares 94.22% identity with the Atg16L1 from *L. crocea*. In yeast, Atg16 contains an N-terminal AFIM (Atg5-interacting motif) and a C-terminal coiled-coil domain, which collectively are referred to as the Atg16 domain, but it lacks the WD40 repeats that are regarded as a distinct ubiquitin-binding domain [8,32]. Amino acid alignment analysis showed that EcAtg16L1 contains the characteristic Atg16 domain and WD40 repeats domain, which suggests that WD40 repeats in Atg16 are conserved from fishes to mammals. The results of qPCR analysis showed that EcAtg16L1 was distributed in all tested tissues of groupers, which confirms the premise that autophagy is a fundamental physiological activity.

Proper subcellular localization is critical for proteins to function [33]. Atg16L1 was found to be a cytosolic protein in mammals [34,35]. The results of our study indicated that EcAtg16L1 distributed in the cytoplasm in transfected GS cells, exhibiting a dot aggregation pattern. It has been reported that Atg16 was required for LC3 positive puncta (LC3-II) in *Dictyostelium* cells [36] and LC3 lipidation was impaired in Atg16 mutant drosophila larvae [37]. The human Atg16L specified the site of LC3 lipidation for membrane biogenesis [7]. The dot aggregation pattern of EcAtg16L1 may be related to its function of guiding LC3 to correctly locate on the autophagosome membrane. When EcAtg16L1 was co-transfected with EcLC3 to detect the fluorescent punctate pattern of LC3 and the conversion of LC3-I to LC3-II, we found that EcAtg16L1 overexpression increased the level of LC3 lipidation. This result suggested that EcAtg16L1 might have an autophagy-dependent function similar to that found in other eukaryotes.

Autophagy can be triggered by a variety of factors, including starvation, reactive oxygen, and microbial invasion [38–40]. As a core autophagy gene, EcAtg16L1 should respond to virus stimulation. Thus, we detected the expression of EcAtg16L1 under stimulation by SGIV and RGNNV. Transcription levels of EcAtg16L1 was increased in RGNNV infected cells, but decreased in SGIV infected cells compared with non-infected cells. These results were similar to those of previous studies of EcAtg5 [20], suggesting that RGNNV might induce autophagy activity, but SGIV might inhibit it in GS cells. However, the exact relationship between autophagy and the two grouper viruses required further investigation. The altered expression level of EcAtg16L1 in response to SGIV and RGNNV suggested that EcAtg16L1 might play a role in virus replication. Therefore, we investigated virus proliferation in EcAtg16L1 overexpressing cells. The results showed that EcAtg16L1 overexpression facilitated SGIV and RGNNV replication, as evidenced by the increased transcription levels of viral genes and the increased levels of viral proteins. Considering the different expression level of EcAtg16L1 in response to SGIV and RGNNV, we inferred that EcAtg16L1 might promote replication of both viruses with an autophagy-independent rather than the only autophagy-dependent role.

Numerous studies have reported that Atg16L1 is an essential component in regulation of the immune response [22,24,31,41]. In the present study, we examined the expression of IFN signaling molecules and proinflammatory cytokines in EcAtg16L1 overexpressing cells, and then the concurrently transfected cells in other wells were infected with SGIV or RGNNV. The results showed that EcAtg16L1 overexpression decreased the expression levels of selected IFN signaling molecules (IRF3, IRF7, ISG15, MDA5, LGP2, IFP35, MXI, MyD88) and pro-

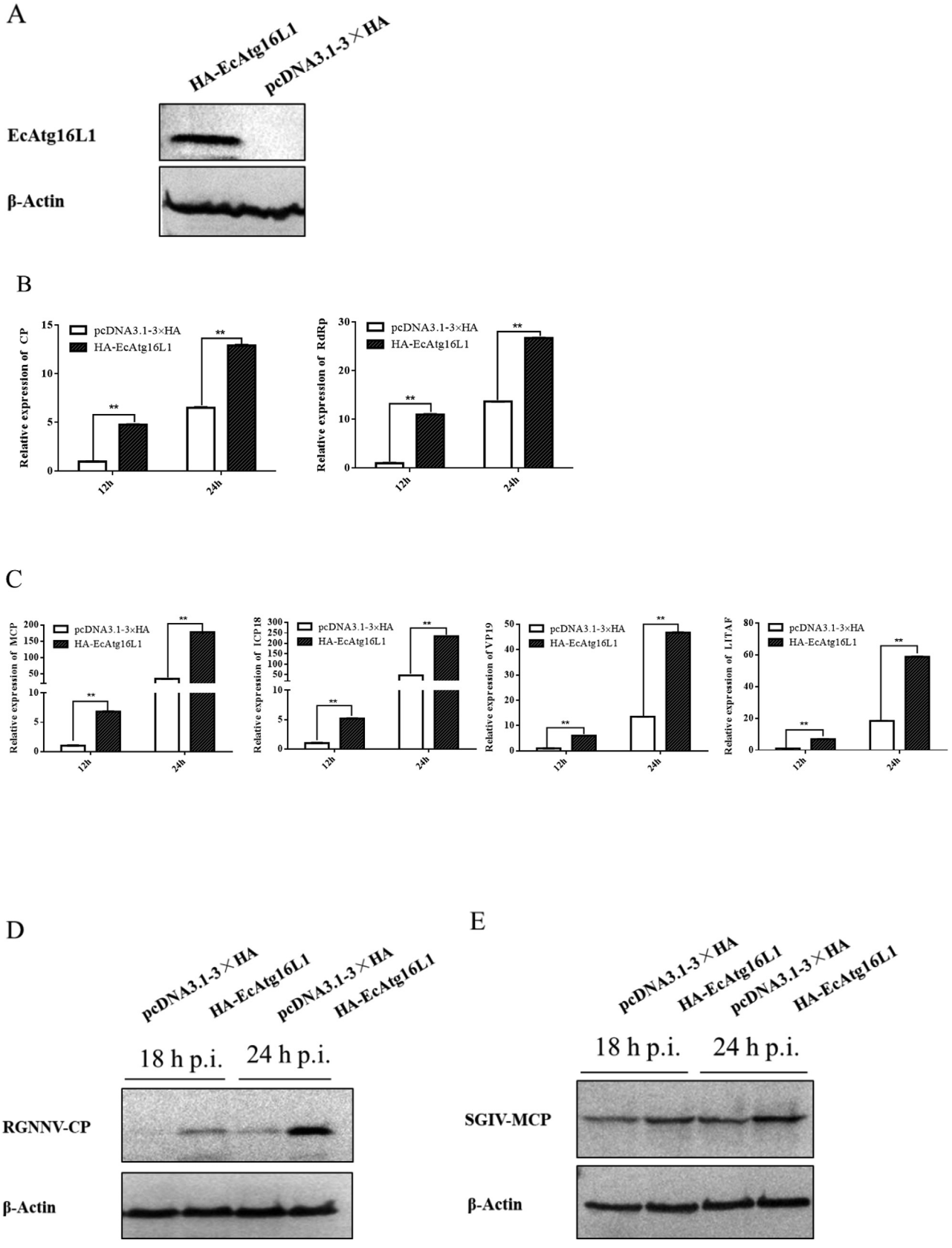

Fig. 5. Effect of EcAtg16L1 overexpression on virus replication. (A) Detection of recombinant protein in pcDNA3.1-3 × HA or HA-EcAtg16L1 transfected cells by Western blot. β-actin was used as the internal control. Expression levels of RGNNV (B) or SGIV (C) genes in transfected GS cells determined by qPCR analysis. The β-actin gene was used as the internal control. An asterisk denotes a significant difference (**$p < 0.01$). Detection of RGNNV-CP (D) or SGIV-MCP (E) protein in transfected cells by Western blot. β-actin was used as the internal control.

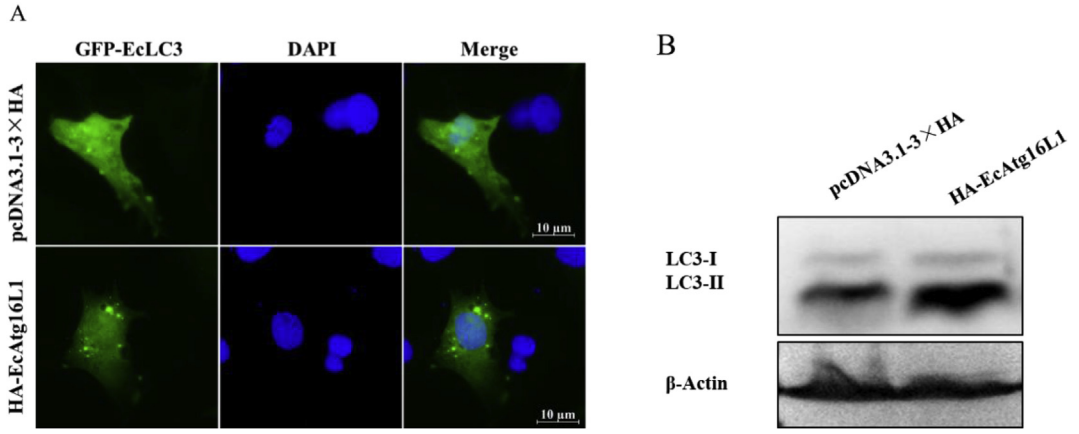

Fig. 6. Effects of EcAtg16L1 overexpression on the autophagy marker LC3. (A) Observation of LC3 fluorescence aggregation. Cells were co-transfected with pcDNA3.1-3 × HA/HA-Atg16L1 and C1-LC3 plasmid. After being stained with DAPI, samples were observed under a fluorescence microscope. (B) Detection of the conversion from LC3-I to LC3-II in pcDNA3.1-3 × HA or HA-EcAtg16L1 transfected GS cells by Western blot. β-actin was used as the internal control.

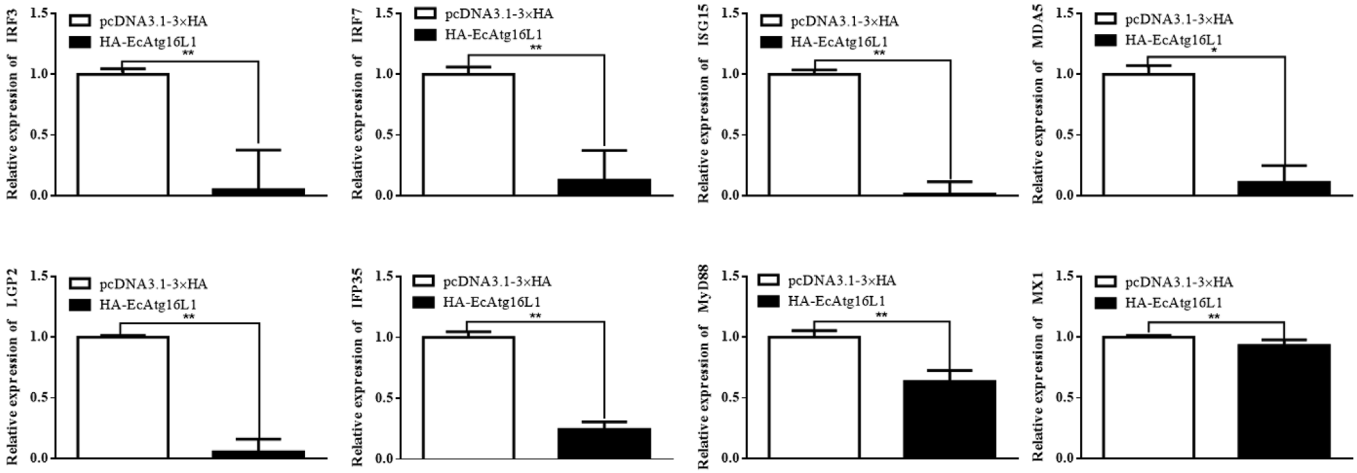

Fig. 7. Effects of EcAtg16L1 overexpression on IFN-signaling molecules. The relative expression levels of IRF3, IRF7, ISG15, MDA5, LGP2, IFP35, MXI, and MyD88 were detected in transfected cells by qPCR. An asterisk denotes a significant difference (*p < 0.05, **p < 0.01).

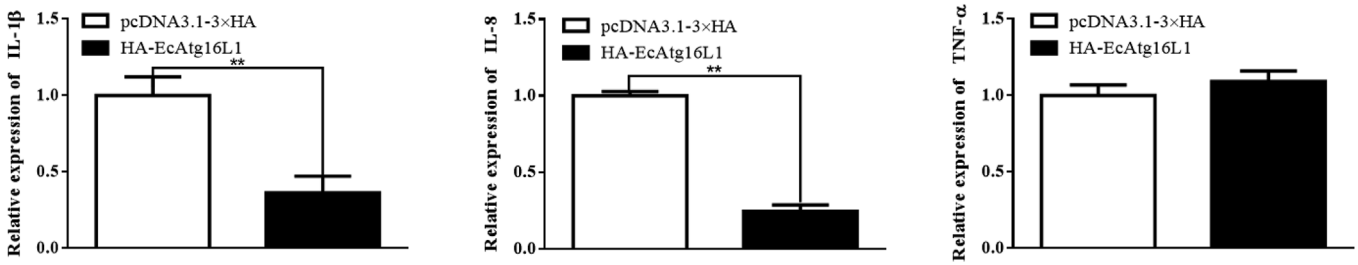

Fig. 8. Effects of EcAtg16L1 overexpression on proinflammatory cytokines. The relative expression levels of IL-1β, IL-8, and TNF-α were detected in transfected cells by qPCR. An asterisk denotes a significant difference (**p < 0.01).

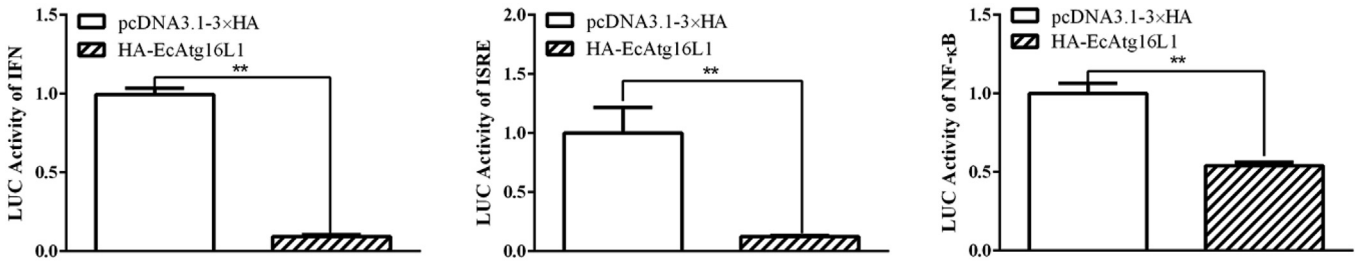

Fig. 9. Effects of EcAtg16L1 overexpression on IFN and NF-κB promoter activity. GS cells were co-transfected with IFN-Luc/ISRE-Luc/NF-κB-Luc and HA-EcAtg16L1/pcDNA3.1-3 × HA, then the promoter activity was detected using the luciferase reporter gene assay. An asterisk denotes a significant difference (**p < 0.01).

inflammatory factors (IL-1β, IL-8). Further analysis showed that EcAtg16L1 overexpression reduced IFN, ISRE, and NF-κB promoter activities. Atg16L1 has been commonly presented as a mutation associated with the inflammatory response. For instance, Atg16L1 deficient macrophages induced IFN-β-dependent activation of caspase-1, leading to the increased production of IL-1β [24]. The loss of human Atg16L1 showed an increased IL-8 response to invasive *Shigella* infection [42] Another study suggested that human Atg16L1 suppressed IL-1β signaling by down-regulating p62 levels [43]. Based on these results, we speculated that EcAtg16L1 has similar functions with Atg16L1 from other eukaryotes in regulating inflammatory responses. EcAtg16L1 overexpression promoted SGIV and RGNNV replication and the proviral effect might involve its down regulation to interferon and inflammatory responses. However, the specific mechanism requires further study.

In summary, an Atg16 homolog from orange-spotted grouper (EcAtg16L1) was cloned and characterized. The expression level of EcAtg16L1 differed in response to SGIV and RGNNV. Furthermore, EcAtg16L1 encoded a cytoplasmic protein with a dot aggregation pattern. Aside from its function in autophagosome formation, EcAtg16L1 also played important role in down regulating immune and inflammatory responses and promoting virus replication.

Acknowledgements

This work was supported by grants from the National Key R&D Program of China (2018YFD0900501 and 2018YFC0311302), China Agricultural Research System (CARS-47-G16), National Natural Science Foundation of China (31572643 and 31772882), Open Fund of Key Laboratory of Experimental Marine Biology, Chinese Academy of Sciences (No.KF2018NO3), Science and Technology Planning Project of Guangdong Province, China (2015TQ01N118), and National High Technology Development Program of China (863) (2014AA093507).

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