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Grouper interferon-induced transmembrane protein 3 (IFITM3) inhibits the infectivity of iridovirus and nodavirus by restricting viral entry

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A R T I C L E   I N F O

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A B S T R A C T

Interferon-induced transmembrane proteins (IFITMs) have been identified as important host restriction factors in mammals for the control of infection by multiple viruses. However, the antiviral functions of IFITMs against fish viruses remain largely uncertain. In this study, the IFITM3 homolog from orange spotted grouper (EcIFITM3) was cloned and its roles in grouper virus infection were investigated. The full-length cDNA of EcIFITM3 was 737 bp, which was composed of a 16 bp 5′-UTR, a 274 bp 3′-UTR, and a 447 bp ORF. EcIFITM3 encodes a 148-amino-acid polypeptide, which contains five domains, i.e., the N-terminal domain (aa 1–65), TM1 (aa 66–90), the cytoplasmic domain (aa 91–110), TM2 (aa 111–140), and the C-terminal domain (aa 141–148), and shares 78% and 47% identity with IFITM3 of gilthead seabream (Sparus aurata) and human (Homo sapiens), respectively. EcIFITM3 mRNA was detected in 12 tissues of healthy groupers, with the highest expression levels in the head kidney. Additionally, the in vitro mRNA levels of EcIFITM3 were significantly upregulated by infection with Singapore grouper iridovirus (SGIV) or red spotted grouper nervous necrosis virus (RGNNV), or treatment with polyinosinic-polycytidylic acid (poly I:C) or lipopolysaccharide (LPS). Subcellular localization analysis showed that EcIFITM3 was mainly distributed in the cell membrane of grouper cells. In vitro, the ectopic expression of EcIFITM3 inhibited SGIV and RGNNV infection, as demonstrated by the reduced severity of the cytopathic effect, decreased virus production, and low levels of viral mRNA and proteins. Consistently, knockdown of EcIFITM3 by small interfering RNAs (siRNAs) enhanced SGIV and RGNNV replication. EcIFITM3 overexpression and knockdown experiments both suggested that EcIFITM3 inhibits the infection of SGIV and RGNNV by restricting viral entry.

1. Introduction

The host innate immune response, in which interferon (IFN) plays an important role by inducing the expression of numerous IFN-stimulated genes (ISGs) through signaling cascades, is the first line of defense against pathogens [1–3]. During virus-host interactions, IFN signaling is abolished by viruses to complete their life cycle [4,5]. IFN-induced transmembrane proteins (IFITMs), which are a family of ISGs, restrict the entry of viruses, possibly by altering the properties of cellular endosomal membranes; thus, inhibiting viral fusion with the membranes [6–9]. IFITMs are conserved across different vertebrate species. They contain two variable transmembrane domains (TMs), a conserved short cytoplasmic loop, and highly variable N- and C-termini [7,9]. In humans, five functional IFITMs (IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10) have been identified and characterized. IFITM1, IFITM2, and IFITM3, which are highly expressed upon stimulation with both type I and type II IFN, have antiviral functions against a broad variety of viruses. To date, the antiviral activity of IFITM5 and IFITM10 remains unclear [10–13].

It has been revealed that IFITM3 is involved in the immune defense against not only numerous enveloped RNA viruses, including human immunodeficiency virus 1 (HIV-1) [14], hepatitis C virus (HCV) [15], Zika virus (ZIKV) [16], dengue virus (DENV) [17], influenza A virus (IAV) [18], West Nile virus (WNV) [19], severe acute respiratory
syndrome coronavirus (SARS-CoV) [20], avian Tembusu virus (ATMUV) [21], and a non-enveloped RNA virus-reovirus [22], but also several DNA viruses, including African swine fever virus (ASFV) [23], herpes simplex virus type 1 (HSV-1) [24], and vaccinia virus (VACV) [25]. However, IFITM3 has little or no effect on several other viruses, including lymphocytic choriomeningitis virus (LCMV), Lassa virus (LASV), murine leukemia virus (MLV), adenovirus type 5 (Ad5), human cytomegalovirus (HCMV), and human papillomavirus-16 (HPV-16) [26]. Although progress has been made in our understanding of the antiviral functions of mammalian IFITM3, little is known about its roles in fish [27].

Groupers, Epinephelus spp., are economically important cultured fish species in South China and Southeast Asian countries, which frequently suffer from viral diseases caused by Singapore grouper iridovirus (SGIV) and red spotted grouper nervous necrosis virus (RGNNV) [28,29]. SGIV is an enveloped double-stranded large cytoplasmic DNA virus belonging to the genus Ranavirus, family Iridoviridae, and encodes 162 open reading frames (ORFs) [28,30,31]. RGNNV, a non-enveloped small RNA virus, belongs to the genus Betanodavirus, family Nodaviridae. The RGNNV genome is composed of two single-stranded positive-sense RNAs, RNA1 (3.1 kb) and RNA2 (1.4 kb), which encode RNA-dependent RNA polymerase (RdRp) and capsid protein (CP), respectively [32]. Recently, great progress has been made not only with respect to the mechanism of SGIV and RGNNV pathogenesis [33–36], but also regarding the grouper immune evasion strategies, such as the action of IFN and ISGs [37–43]. However, no IFITM family members have been characterized in groupers.

In the present study, we cloned the IFITM3 homolog from orange spotted grouper (EcIFITM3) and examined the expression of EcIFITM3 under different stimuli. Moreover, we detected the subcellular localization of EcIFITM3 and analyzed the antiviral roles of EcIFITM3 against grouper viruses. Our results provide new insights into the roles of fish IFITM3 in the response to infection with RNA and DNA viruses.

2. Materials and methods

2.1. Cells and virus

Grouper spleen (GS) cells were cultured in Leibovitz's L15 medium with 10% fetal bovine serum (FBS; Gibco, USA) at 28 °C [44]. The SGIV and RGNNV stocks were propagated in GS and grouper brain cells, respectively, and were maintained at −80 °C [28,45].

2.2. Gene cloning and plasmid construction

Based on the expressed sequence tag (EST) sequences of EcIFITM3 from the GS transcriptome [37], the full-length cDNA of EcIFITM3 was obtained by rapid amplification of cDNA ends (RACE) PCR using the SMARTer® RACE 5′/3′ Kit (Clontech). The sequence of EcIFITM3 was verified by DNA sequencing and analyzed by BLAST program. The conserved domains were predicted using InterPro (http://www.ebi.ac.uk/interpro). Multiple amino acid sequence alignment was performed with ClustalX 1.83 software and the phylogenetic tree was constructed by MEGA 6.0 software using the neighbor joining method.

To study the localization and function of EcIFITM3 in vitro, the full length ORF of EcIFITM3 was cloned into pcDNA3.1-3 × HA and pEGFP-C1. The recombinant plasmids (pEGFP-EcIFITM3 and HA-EcIFITM3) were confirmed by DNA sequencing. All primers used for

Table 1

| Primer names | Sequence (5′-3′) |
|--------------|-----------------|
| IFITM3-AP1   | CAGGACCTGGCTATGATTG |
| IFITM3-AP2   | GCTGACACCTGATGTCC |
| IFITM3-SP1   | AGGGCTCCAGGACCAT |
| IFITM3-SP2   | GGATGGGCGGCTTATAGC |
| EcIFITM3-3HA-KpnI-F | GGGGTACCATGAGATCCTGCACACCTTC |
| EcIFITM3-3HA-Xhol-R | CCGCTCGAGTCAGATTAGGGGG |
| EcIFITM3-C1-KpnI-R | CCCGTCACTGACATTTAGGAG |
| siRNA1-EcIFITM3 | TACAGTGAGCAGGAGAAGAT |
| siRNA2-EcIFITM3 | TACAGTGAGCAGGAGAAGAT |
| siRNA3-EcIFITM3 | TACAGTGAGCAGGAGAAGAT |
| EcIFITM3-RT-F | AGCCCTTCCTCACAATACTCA |
| EcIFITM3-RT-R | GCTGAACTCATGATGTC |
| Actin-RT-F | TACGAGCTGCTGAGGAGAC |
| Actin-RT-R | GGGCTGGTATCCTCAGTCA |
| SGIV-MCP-RT-F | GCACTGGTCTCCACACAC |
| SGIV-MCP-RT-R | AAACGCGAACGGGACGAT |
| SGIV-VPIC-RT-F | TCGAGGGGAAACGCTGAG |
| SGIV-VPIC-RT-R | GGGTCAGGGGTCGGAAG |
| RGNNV-CP-RT-F | CAACGTCAGAAAGATGACAC |
| RGNNV-CP-RT-R | CAATCGAAGACCCAGCAGA |
| RGNNV-RdRp-RT-F | GTGGCCAGGAGTATAGGAT |
| RGNNV-RdRp-RT-R | CTGATTGGTTCAAAGGTCAG |

Fig. 1. The sequence of EcIFITM3. The nucleotides and amino acids are numbered along the margin.
plasmid construction are listed in Table 1.

2.3. Expression profiles of EcIFITM3

Orange spotted groupers (E. Coioides) (50–60 g) were purchased from a marine farm in Wenchang city, Hainan Province, China, and kept in a laboratory recirculating seawater system before use. To clarify the tissue distribution pattern of EcIFITM3, we extracted total RNA from 12 tissues of healthy groupers, i.e., fin, skin, intestine, stomach, gill, heart, muscle, brain, spleen, liver, head kidney, and kidney, and the relative EcIFITM3 mRNA levels were measured by quantitative real-time PCR (qPCR).

To analyze the roles of EcIFITM3 in innate immunity, GS cells were infected with different viruses and treated with pathogen-associated molecular pattern (PAMP) molecules to determine the expression changes of EcIFITM3. In detail, GS cells were infected with SGIV or RGNNV at a multiplicity of infection (MOI) of 2.0 and harvested at 3, 6, 18, 24, or 36 h post-infection (h.p.i.) for qPCR analysis. Moreover, GS cells were transfected with 200 ng polyinosinic-polycytidylic acid (poly(I:C)) or treated with 4 μg/ml lipopolysaccharide (LPS) and harvested at 4, 8, 12, 24, or 36 h for qPCR analysis.

2.4. Cellular localization of EcIFITM3

To explore the subcellular localization of EcIFITM3, GS cells were seeded on microscopic coverslips (25 × 25 mm) in 6-well plates, and

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Fig. 2. Identification of EcIFITM3. (A) Alignment of the EcIFITM3 amino acid sequence with other IFITM3 proteins. The accession numbers are the following: Sparus aurata, XP_030281037.1; Lates calcarifer, XP_018531428.1; Larimichthys crocea, NP_001290318.1; Gallus gallus, NP_001336990.1; Xenopus tropicalis, NP_001015758.1; Mus musculus, NP_079654.1; Homo sapiens, NP_066362.2. (B) Phylogenetic tree of IFITM3, constructed using MEGA 6.0 software. Numbers at the nodes denote the bootstrap values from 1000 replicates. The scale represents the number of substitutions per 1000 bases.
transfected with pEGFP-C1 or pEGFP-EcIFITM3 using Lipofectamine 2000 (Invitrogen). GS cells were fixed with 4% paraformaldehyde (PFA) at 48 h post-transfection and stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min. Cells were imaged by fluorescence microscopy (Zeiss, Germany).

2.5. Virus replication assay

For the overexpression assay, GS cells were transfected with pcDNA3.1-3 × HA or HA-EcIFITM3 for 24 h, infected with SGIV or RGNNV (MOI = 2), and harvested at 12 h.p.i. and 24 h.p.i. for qPCR or western blot analysis. Whole cell lysates of SGIV-infected cells were used for the 50% tissue culture infective dose (TCID50) assay of virus titers.

For the knockdown assay, small interfering RNAs (siRNAs) were designed for EcIFITM3 knockdown (Table 1). GS cells were transfected with si-EcIFITM3 or negative control (NC) at 160 nM for 24 h and infected with SGIV or RGNNV (MOI = 2) for 24 h. The infected cells were harvested for qPCR analysis.

2.6. RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted using the SV Total RNA Isolation Kit (Promega) and cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo) following the manufacturer’s protocol. qPCR was performed on an Applied Biosystems QuantStudio 5 Real Time Detection System (Thermo Fisher, USA) using 2 × SYBR Green Real-time PCR Mix (Toyobo) with the following PCR conditions: 95 °C for 1 min for activation, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The qPCR primers are listed in Table 1. Relative mRNA levels were calculated with the 2−ΔΔCt method with β-actin as the internal control. Data are presented as mean ± standard deviation (SD) of three independent experiments.

2.7. Western blot assays

GS cells infected with virus were harvested and lysed with RIPA buffer (Pierce). The total protein concentration was measured using a BCA protein assay kit (Kaiji) according to the manufacturer’s instructions. Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were
incubated with anti-RGNNV CP or anti-β-tubulin at a dilution of 1:1500 for 2 h, washed, and incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG or sheep anti-mouse IgG at a dilution of 1:5000 for 2 h. After washing, protein bands were visualized with an enhanced HRP-DAB Substrate Chromogenic Kit (Tiangen). Signal intensities were quantified using ImageJ software, with β-tubulin as an internal reference.

2.8. Virus entry assay

To analyze the effects of EcIFITM3 on virus entry, we quantitatively analyzed SGIV particles entering GS cells by confocal laser scanning microscopy (CLSM) (Zeiss, Germany) and the cytoplasmic mRNA levels of viral genes by qPCR. Briefly, GS cells cultured in glass-bottom cell culture dishes were transfected with pEGFP-C1 or pEGFP-EcIFITM3, and were infected with Cy5-labeled purified SGIV at 24 h post-transfection. At 1 h.p.i., cells were fixed with 4% PFA overnight and observed by CLSM. In each sample, 30 cells were randomly selected to analyze the SGIV virus particles in the cells. Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments [46,47]. In addition, GS cells were seeded in 24-well plates, transfected with HA-EcIFITM3 or siRNA-EcIFITM3 for 24 h, and infected with SGIV or RGNNV for 1 h. Then the cells were washed three times with cold serum-free medium to remove unbound virus particles and cultured at 28°C for another 4 h (SGIV) or 1 h (RGNNV). Cells were collected at indicated time points for RNA extraction and qPCR analysis [46].

2.9. Statistical analysis

Differences between groups were analyzed by one-way ANOVA using SPSS version 20. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Gene clone and sequence characterization of EcIFITM3

Using the EST sequence, the full-length cDNA sequence of EcIFITM3 was amplified from the GS transcriptome by RACE. EcIFITM3 contains five domains, i.e., a variable N-terminal domain (NTD) (aa 1–65), a conserved transmembrane domain (TM1) (aa 66–90), a conserved cytoplasmic domain (aa 91–110), a variable transmembrane domain (TM2) (aa 111–140), and a highly variable C-terminal domain (CTD) (aa 141–148) (Fig. 2A). The phylogenetic analysis indicated that EcIFITM3 from grouper and other fish were clustered into one group, which was separated from that of bird, amphibian, and mammalian IFITMs.

3.2. Tissue distribution and expression profiles of EcIFITM3

The EcIFITM3 mRNA levels in various tissues from healthy groupers were analyzed by qPCR. EcIFITM3 was distributed in all detected tissues, with the highest expression levels detected in the head kidney (Fig. 3A). In addition, the expression patterns of EcIFITM3 in GS cells challenged with RGNNV or SGIV, or poly(I:C) or LPS treatment were examined by qPCR. As shown in Fig. 3B, the expression levels of EcIFITM3 during SGIV infection increased from 24 h.p.i. and reached up to 91-fold higher levels than in mock-infected cells at 30 h.p.i. After incubation with anti-RGNNV CP or anti-β-tubulin at a dilution of 1:1500 for 2 h, washed, and incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG or sheep anti-mouse IgG at a dilution of 1:5000 for 2 h.
Fig. 5. Overexpression of EcIFITM3 inhibited SGIV and RGNNV replication. (A) The mRNA and protein levels of GS cells transfected with pcDNA3.1-3 × HA or HA-EcIFITM3, as analyzed by qPCR and western blot. (B) The severity of the CPE induced by RGNNV and SGIV at 24 h.p.i. White arrows indicate the cell rounding and aggregation evoked by SGIV infection, black arrows show that vacuole formation was induced by RGNNV infection. (C, E) mRNA levels of viral genes in transfected GS cells. GS cells transfected with pcDNA3.1-3 × HA or HA-EcIFITM3 were infected with SGIV or RGNNV (MOI = 2.0) for 24 h, and collected at 12 and 24 h.p.i. to measure the mRNA levels of (C) SGIV MCP and VP19 and (E) RGNNV CP and RdRp by qPCR. (D) The virus production of SGIV in infected GS cells. GS cells infected with SGIV were collected at 12 and 24 h.p.i. to measure the virus titer by TCID50 assay. (F) The protein levels of RGNNV CP in transfected GS cells were analyzed by western blot (n = 3, mean ± SD). *P < 0.05.
infection with RGNNV, EcIFITM3 mRNA levels in GS cells were upregulated from 18 h.p.i. and reached up to 61-fold higher levels than in mock-infected cells at 30 h.p.i. (Fig. 3C). Poly(I:C) and LPS also induced the expression of EcIFITM3 (Fig. 3D and E). Therefore, EcIFITM3 might play a key role in the response to virus infection.

3.3. Subcellular localization of EcIFITM3

To explore the localization of EcIFITM3 in GS cells, cells were transfected with pEGFP-C1 or pEGFP-EcIFITM3 for 48 h and stained with DAPI. As shown in Fig. 4, the green fluorescence was distributed throughout the cytoplasm and nucleus in pEGFP-C1 transfected cells, whereas in pEGFP-EcIFITM3 transfected cells, it was observed mainly in the cell membrane and in little cytoplasmic dot-like aggregations.

3.4. EcIFITM3 overexpression inhibits SGIV and RGNNV replication

The effects of EcIFITM3 on SGIV infection were evaluated from different aspects, including the progression of the cytopathic effect (CPE) and the quantitation of the viral mRNA levels, viral protein level, and viral yield. Firstly, the mRNA and protein levels of EcIFITM3 in GC cells transfected with pcDNA3.1-3 × HA or HA-EcIFITM3 were determined by qPCR and western blot. As shown in Fig. 5A, EcIFITM3 (from the recombinant plasmid HA-EcIFITM3) was successfully overexpressed in GS cells. EcIFITM3 overexpression obviously weakened the severity of the CPE induced by SGIV and RGNNV (Fig. 5B). Consistently, the qPCR results showed that the mRNA levels of SGIV MCP and VP19 (Fig. 5C), and RGNNV RdRp and CP (Fig. 5E) were significantly decreased upon EcIFITM3 overexpression. The virus titer assay results indicated that the production of SGIV virus in EcIFITM3 overexpressing cells was significantly reduced compared with control vector cells (Fig. 5D). Western blot analysis showed that the protein levels of RGNNV CP in EcIFITM3-overexpressing cells were significantly reduced compared with control vector cells (Fig. 5F).

3.5. Knockdown of EcIFITM3 promotes SGIV and RGNNV replication

We also investigated whether knockdown of EcIFITM3 promotes SGIV and RGNNV replication. Firstly, the interference efficiency of si-EcIFITM3 was examined by qPCR. As shown in Fig. 6A, the expression of EcIFITM3 was significantly decreased in siRNA1- or siRNA3-transfected cells compared with the cells transfected with NC siRNA. SiRNA1 was used in subsequent experiments. Next, GS cells transfected with siRNA1 for 24 h were infected with SGIV or RGNNV and harvested at 24 h.p.i. to examine the mRNA levels of viral genes. SiRNA-EcIFITM3 significantly increased the mRNA levels of SGIV MCP and VP19 (Fig. 6B), and RGNNV RdRp and CP genes (Fig. 6C). These results indicate EcIFITM3 reduces SGIV and RGNNV replication.

3.6. EcIFITM3 impedes the entry step of SGIV and RGNNV

To investigate the effects of EcIFITM3 on the early stage of SGIV and RGNNV infection, we determined the mRNA levels of viral genes by qPCR and the number of Cy5-labeled SGIV particles in the cytoplasm by CLSM. The number of red fluorescence-labeled SGIV particles in EcIFITM3 overexpressing cells was significantly reduced by 51% compared with the control vector cells (Fig. 7A and B). Overexpression of EcIFITM3 decreased the mRNA levels of SGIV MCP and RGNNV CP in the cytoplasm (Fig. 7C). Knockdown of EcIFITM3 significantly increased the mRNA levels of SGIV MCP and RGNNV CP (Fig. 7D). These results indicate that EcIFITM3 inhibits virus replication by blocking SGIV and RGNNV entry into host cells.

4. Discussion

IFITM3, a small transmembrane protein that is expressed upon IFN stimulation, has been demonstrated to play crucial roles in the early stage of virus infection [6–9]. However, it remains unclear whether the antiviral functions of fish IFITM3 are conserved from lower vertebrates to mammals. Here, we cloned a novel IFITM3 gene from grouper (EcIFITM3) and examined its roles during SGIV and RGNNV infection.

Although sequence analysis showed that EcIFITM3 only shares 47% sequence identity with human IFITM3, it also contains five domains, i.e., the NTD, TM1, a cytoplasmic domain, TM2, and the CTD, similar to IFITM3 of birds and mammals [15,21]. The expression levels of IFITMs are very low in most cells and tissues, but can be significantly increased by IFN, DNA viruses, RNA viruses, poly(I:C), or LPS [21,48–51]. Consistent with previous studies, we found that the mRNA levels of

Fig. 6. Knockdown of EcIFITM3 by siRNA promotes fish virus replication in vitro. (A) EcIFITM3 mRNA levels in GS cells transfected with NC or specific siRNAs against EcIFITM3. (B, C) qPCR analysis of the mRNA levels of (B) SGIV MCP and VP19 and (C) RGNNV CP and RdRp (n = 3, mean ± SD). *P < 0.05.
EcIFITM3 significantly increased upon infection with SGIV or RGNNV, or stimulation with poly(I:C) or LPS, suggesting that EcIFITM3 plays an important role in the innate immune response. A previous study has shown that IFITM3 is mainly localized in the membrane structures in HEK293T and HeLa cells [25]. Consistently, we found EcIFITM3 is mainly localized in the cell membrane and in little cytoplasmic dot-like aggregations. In hepatocytes, IFITM3 was found to localize in specific compartments in the cytoplasm [15]. Therefore, it was speculated that the subcellular localization of IFITMs may be cell type-dependent [15].

A growing number of studies have revealed that IFITM3 has broad-spectrum antiviral activity against DNA and RNA viruses. For example, IFITM3 overexpression reduced the number of copies of the ASFV genome and decreased the expression of the early protein p30 and the late protein p72 [23]. Similarly, overexpression of chicken or duck IFITM3 could restrict ATMUV infection, as reflected by lower TCID$_{50}$ titers and a decrease in viral gene expression, while knockdown of IFITM3 significantly promoted ATMUV replication [21]. Moreover, IFITM3 knockout in a mouse model increased the replication of influenza virus in the lungs, spleen, and heart [52]. In accordance with these previous studies, overexpression of EcIFITM3 significantly reduced CPE severity upon SGIV and RGNNV infection, reduced SGIV production, and inhibited the mRNA and protein expression of viral genes, while knockdown of EcIFITM3 significantly promoted the replication of SGIV and RGNNV, suggesting that EcIFITM3 suppresses viral gene expression, and thereby restricts DNA and RNA virus infection in fish.

Although the mechanisms underlying the effects of IFITM3 on virus entry remain unclear, studies have shown that IFITM3 resides in late endosomal and lysosomal structures, in which they prevent viral fusion with cell membranes [6]. Moreover, most of the viruses inhibited by IFITM3 enter cells in a pH- or cathepsin-dependent manner [9]. For example, overexpression of IFITM3 restricted low pH-dependent VACV entry, but not low pH-independent virus entry [25]. In addition, a previous study in our laboratory has shown that SGIV enters GS cells in a pH-dependent manner [47]. Consistently, we found that EcIFITM3 restricts SGIV and RGNNV entry. However, the exact mechanisms by
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