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# The antiviral mechanism of viperin and its splice variant in spring viremia of carp virus infected fathead minnow cells

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ABSTRACT

Viperin is known to play an important role in innate immune and its antiviral mechanisms are well demonstrated in mammals. Fish Viperin mediates antiviral activity against several viruses. However, little has been done to the underlying mechanism. Here, we discovered a novel Viperin splice variant named Viperin_sv1 from viral-infected FHM cells. Spring varimia of carp virus (SVCV) was able to increase the mRNA levels of both Viperin and Viperin_sv1, while poly(I:C) only has effect on Viperin. Viperin functions as an antiviral protein at 24 h post-SVCV infection, but the antiviral activity dramatically declined at late infection stages. However, Viperin_sv1 inhibited SVCV replication significantly at all the tested time. Viperin_sv1, but not Viperin can facilitate the production of type I IFN and IFN stimulate genes (ISGs) through activation of RIG-1, IRF3 and IRF7 signaling cascades. On the other hand, SVCV down-regulated Viperin_sv1 at the protein level through the proteasome pathway to keep itself away from the immune system monitoring. Taken together, these findings provide new insights into the regulation of Viperin from the posttranscriptional modification perspective and the role of splicing variant Viperin_sv1 in virus-host interaction.

## 1. Introduction

Viral infection can induce the expression of type I interferons (IFNs) depending on recognition of pattern recognition receptor (PRR). These PRRs recruit downstream adaptor proteins including TRIF [1] and MAVS [2], which further activate TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase (IKK), leading to the activation of transcription factors IRF3/IRF7 and nuclear factor kappa-B (NF-κB) to enhance the expression of IFN [3–5].

Interferon represents the first line of host defense against viral infection and hundreds of genes are regulated by IFN for establishing a broadly effective antiviral state [6]. Viperin, also known as Rsad2, Vig1, or Cig5, is characterized as an antiviral protein which was first identified in fibroblasts infected with human cytomegalovirus (HCV) [7,8]. Viperin is highly conserved in evolution and composed of three distinctive regions: N-terminal variable transmembrane domain, followed by the radical SAM domain containing an Iron-sulfur (Fe−S) cluster binding motif, and a C-terminal region [9,10]. The N-terminal amphipathic-helix mediates localization of Viperin to the cytosolic face of the endoplasmic reticulum and inhibits protein secretion [11]. The internal S-adenosyl methionine domain of Viperin was essential for its antiviral activity to HIV-1 [12]. The C-terminal domain of Viperin exerts the major anti-HCV effect [13].

Viperin is known to play an antiviral role against a number of mammalian viruses, including influenza virus [14], sindbis virus [15], hepatitis C virus (HCV) [13], human immunodeficiency virus [12], chikungunya virus [16], dengue virus [17], porcine reproductive and respiratory syndrome virus (PRRSV) [18], rabies virus [19], and Zika virus (ZIKV) [20]. Fish are known to have viperin homologs, which was firstly identified in *Oncorhynchus mykiss* [21]. The expression of *Siniperca chuatsi* viperin was detected in almost all the organs investigated after stimulation with virus or Poly I:C [22]. Constitutive expression of red drum *Sciaenops ocellatus* viperin (SoVip) mRNA was relatively high in blood, muscle, brain, spleen, and liver, and SoVip involved in host immune response during bacterial infection [23]. Rock bream *Oplegnathus fasciatus* viperin (OfVip) induced by megalocytivirus in kidney, liver, and spleen, and it exhibited an important role in the reduction of tissues viral loads [24]. *Crucian carp* viperin was activated by two types

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**Table 1**
Primer sequences used in this study.

| Primer name | Accession number | Sequence (5'–3') | Application |
|---|---|---|---|
| Viperin-F | XM_019115998.1 | ATGTTGACATCAAACCAAGTTGG | Amplification |
| Viperin-R | | TCACCAGTCCAGTTTCATATCAG | |
| pcDNA4-Viperin-F | XM_019115998.1 | CGGAATTCATGTTGACATCAAACCAAGTTG | Eukaryotic expression |
| pcDNA4-Viperin-R | | CCGCTCGAGCCAGTCCAGTTTCATATC | |
| pcDNA3.1-Viperin-F | XM_019115998.1 | CGGAATTCATGTTGACATCAAACCAAG | Eukaryotic expression |
| pcDNA3.1-Viperin-R | | CCCTCGAGTCACTTATCGTCGTCATCCTTGTAATCCCAGTCCAGTTTCATA | |
| Viperin-ASF | XM_019115998.1 | CAAGACTTCCTGGACCGC | Alternative splicing verification |
| Viperin-ASR | | CACACATATTTCCCCCCT | |
| zViperin-ASF | NM_001025556.1 | CAAGACTTCCTGGAGCGC | Alternative splicing verification |
| zViperin-ASR | | CACACATATTTTCCTCCT | |
| TBP-F | AF503449.1 | TTACCCACCAGCAGTTTAG | Quantitative real-time PCR |
| TBP-R | | ACCTTGGCACCTGTGAGTA | |
| EF1α-F | FJ915061.1 | TGTGCTGTGCTGATTGTTGC | Quantitative real-time PCR |
| EF1α-R | | CGCTGACTTCCTTGGTGATT | |
| qSVCV-G-F | KR012468.1 | CGACCTGGATTAGACTTG | Quantitative real-time PCR |
| qSVCV-G-R | | AATGTTCCGTTTCTCACT | |
| qIFN1-F | FN178457.1 | AACGCAGCACAATGGAAC | Quantitative real-time PCR |
| qIFN1-R | | TGATGGATGGTGGTATCG | |
| qPKR-F | JX516101.1 | CCAACATCGTCCGCTACTACTC | Quantitative real-time PCR |
| qPKR-R | | GCGTGTCTCCCTCACAAAG | |
| qMxA-F | NM_182942.4 | AGACCATCCTCATTTCAGCAAACTCT | Quantitative real-time PCR |
| qMxA-R | | CAATCTTTTTGTTGAATGAATCCCCTG | |
| qRIG-I—F | FN178456.1 | TGCTGGACCGGATGTGTTATCT | Quantitative real-time PCR |
| qRIG-I-R | | TGGTGATCGATGGTTCGATTCT | |
| qIRF3-F | HE856621.1 | GTTTAGAGGGACAATTAACTGGACTA | Quantitative real-time PCR |
| qIRF3-R | | GAGGGTCCACTCTTTGAAAATG | |
| qIRF7-F | KF844251.1 | CCATTCATTGCTGACATCTACAGT | Quantitative real-time PCR |
| qIRF7-R | | GTTCGTCTCAAAGTTGCTCCTC | |
| qNF-κB-F | XM_019115296.1 | GGAGAGGAGGTCTATCTGCTATG | Quantitative real-time PCR |
| qNF-κB-R | | GGCTTCTGGAGGTTCTGGTC | |

of fish IFNs and poly(I:C), and exerted a conserved antiviral function against infection of grass carp reovirus (GCRV) [25].

Spring viremia carp virus is a negative-sense ssRNA virus and responsible for high mortality in common carp (*Cyprinus carpio*) [26]. This rhabdovirus encodes five viral proteins including nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and viral RNA-dependent RNA polymerase (L) [27]. Present study has shown that vaccination approaches can play an essential role in combating SVCV infection [28]. The IFN system helps the host to defense against viral invasion. However, viruses have evolved a number of elaborate strategies to avoid being eliminated. Recent report have shown that the N protein of SVCV is able to inhibit IFN1 production by degrading the mitochondrial antiviral signaling protein (MAVS) to initiate virus immune evasion [29], the P protein of SVCV negatively regulates the fish interferon response by inhibiting TBK1 kinase activity [30], the glycoprotein of SVCV is able to activate the autophagy pathway, promoting the survival of SVCV-infected cells by eliminating damaged mitochondrial DNA generated, and thus enhancing viral production [31].

Recently, increasing research has been devoted to explore the alternative splicing in innate immunity. Alternative splicing of IFN genes can produce a functional intracellular IFN (iIFN), and variants of the two IFNR receptor chains (IFNAR1 and IFNAR2) are also present and may act as a novel defense to combat viral infection in trout [32]. Laboratory of Genetics and Physiology 2 (LGP2), the third member of the RLR family, plays different roles in regulation of IFN signaling with its splicing variant LGP2v1 and LGP2v2 [33]. The cooperation of MAVS variants and RIG-I could induce IFNs production efficiently and contribute to the antiviral response [34]. However, many alternatively spliced molecules from immunologically relevant genes remain unknown. In this study, Viperin and its splice variant Viperin_sv1 are identified in SVCV-infected fathead minnow (FHM) cells and their antiviral function and the potential involvement in production of type I IFN are also elucidated.

## 2. Materials and methods

### 2.1. Cells and virus

Fathead minnow (FHM) cells (ATCC: CCL-42) were cultured in medium 199 (M199, Hyclone, USA). These growth media were supplemented with 10% fetal bovine serum (FBS, Natocor, Argentina). The FHM cells were incubated at 28 °C with 3% $CO_2$. Spring viraemia of carp virus (SVCV, ATCC: VR-1390) was propagated in the FHM cells. Cells at exponential growth phase were infected with SVCV at a multiplicity of infection (MOI) of 0.1 or 0.05, and viral production was maintained with the medium supplemented with 5% FBS after 1 h viral adsorption. Virus productions were measured using a 50% tissue culture infective dose ($TCID_{50}$/mL) assay and calculated by the method of Reed and Muench [35]. Inactivation of SVCV was conducted by ultraviolet rays as described previously [31].

### 2.2. SVCV infection and tissues harvest of zebrafish

Adult wild-type (WT) AB strain zebrafish were purchased from China Zebrafish Resource Centre (CZRC). Zebrafish were maintained under temperature 17 °C initially, and divided into two groups. To ascertain whether the alternative splicing of Viperin were induced by SVCV in vivo, test group of zebrafish was inoculated with 10 μL/fish of SVCV at the dose of $1 \times 10^5$ $TCID_{50}$ Units. Control group of the fish was injected with 10 μL/fish of culture medium M199 correspondingly. The cDNA of zebrafish spleen was harvested at days 1 and 2 post injection. The injection experiments were performed with anesthesia by MS-222, and all procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### 2.3. Molecular cloning and sequence analysis

Total RNA from FHM cells that infected with SVCV or mock-infected

with PBS was extracted using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol. One μg of total RNA was used for the reverse transcription reaction using PrimeScript TMRT reagent Kit containing a gDNA Eraser (TaKaRa) for removing any contaminant DNA. Recovered cDNA was stored at −20 °C until used. PCR was carried out using 2x High-Fidelity Master Mix (Tsing Ke, China) with primers of Viperin-F and Viperin-R shown in Table 1 for the cloning of Viperin according to the manufacturer's instruction.

The Viperin gene sequence was confirmed by NCBI BLAST analyses (http://www.ncbi.nlm.nih.gov/blast) with its homology sequences. The ClustalW and GeneDoc program was used for multiple sequence alignments between FHM Viperin, FHM Viperin_sv1, and Viperin from Homo sapiens (NP_542388), House mouse (CAJ18585.1), Gallus gallus (ACA83729), Danio rerio (NP_001020727), Branchiostoma (EEN65148). Structural model of FHM Viperin and Viperin_sv1 were predicted on the Phyre2 server as homology modeling method. All structural images were prepared with PyMOL and superposition of 3D structures was done with Align. The secondary structure analysis of sequence was performed on the ESPript server.

### 2.4. Silver staining of PCR products

Based on alignments of Viperin and its splice variant, primers Viperin-ASF/Viperin-ASR or zViperin-ASF/zViperin-ASR that shown in Table 1 were used for amplify partial sequence of Viperin containing alternative splicing region. PCR products were separated by using 12% non-denaturing polyacrylamide gels. The electrophoresis was run with 1 × TBE buffer (100 mM Tris-HCl, 83 mM boric acid, and 1 mM EDTA-$Na_2$ at pH 8.0) at 220 V for 90 min. Silver staining of PCR products in polyacrylamide gels was performed using DNA Silver Stain Kit (KeyGEN BioTECH, China) according to the manufacturer's protocol.

### 2.5. Plasmids and transfections

For eukaryotic expression, the coding region of Viperin and Viperin_sv1 was cloned with primers of pcDNA4-Viperin-F/pcDNA4-Viperin-R. The pcDNA4-Viperin and pcDNA4-Viperin_sv1 plasmids were generated by inserting Viperin or Viperin_sv1 cDNA into the pcDNA4-His vector respectively. Plasmids pcDNA3.1-Viperin and pcDNA3.1-Viperin_sv1 with Flag-tag were constructed with primers of pcDNA3.1-Viperin-F and pcDNA3.1-Viperin-R for immune fluorescence assays. All plasmids were verified by sequencing analysis. The primers including the restriction enzyme cutting sites that used for plasmid construction were listed in Table 1.

For transient transfection, FHM cells were seeded in 6-well plates or 10 $cm^2$ dishes with 70–90% confluence. Approximately 24 h later, transfection was performed with FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's instructions.

### 2.6. Indirect immunofluorescence assay and western blotting assay

To observe the effects of Viperin and Viperin_sv1 on SVCV visually, FHM cells were transfected with pcDNA3.1-Viperin, pcDNA3.1-Viperin_sv1 or empty vector pcDNA3.1 as control. Cells were fixed with methanol at 24 h post SVCV infection at a MOI of 0.1, and then incubated with mixture of rabbit anti-Flag antibodies (ABclonal, 1:500 dilution) and SVCV-N monoclonal antibody [36] as first antibodies for 1 h, and then incubated mixture of FITC-conjugated goat anti-rabbit antibodies (ABclonal, 1:2000 dilution) and Cy3-conjugated goat anti-mouse antibodies (ABclonal, 1:2000 dilution) as second antibody for 45 min, and then cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under confocal laser scanning microscopy (Leica TCS SP8, Japan).

For western blotting assay, cells with different treatments were lysed directly with 5 × SDS loading buffer and boiled. Samples were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membranes were blocked 1 h with freshly prepared TBSA (3% BSA in TBST buffer). Blots were probed with anti-His, anti-β-actin (ABclonal, 1:5000 dilution), SVCV-G [37], and SVCV-M [38] primary antibody, followed by HRP conjugated secondary antibody (ABclonal, 1:5000 dilution). Protein bands were visualized with ECL chemical luminescence substrate (Advansta).

### 2.7. Quantitative real-time PCR

To detect the relative mRNA level of genes in different treatments, cells were collected for RNA extraction. SYBR Green qPCR SuperMix (TaKaRa, China) was used in a Roche LightCycler® 480II Real Time Detection System (Roche, German). The reaction volume and the cycling conditions were the same as described previously [39]. All samples were analyzed in triplicate and the expression values were normalized to TBP or EF1α. Primers used for qRT-PCR analysis were designed with Primer 5.0 based on target sequences and listed in Table 1.

### 2.8. Statistics analysis

All statistical analyses were performed using Graphpad Prism5.0 (GraphPad Software, CA, USA). Data are expressed as means ± standard deviations (SDs) of at least three independent experiments. The P values were calculated by ANOVA or two-way ANOVA, and $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. SVCV induces viperin and its splice variant Viperin_sv1

In the process of amplifying Viperin gene from SVCV-infected FHM cells, two PCR products 1071bp and 1038 bp were cloned respectively. Analysis of the sequences indicated that two PCR products were the different transcripts of Viperin and generated through alternatively splicing pattern. Aligning with zebrafish Viperin, which composed of 6 exons and 5 introns, the transcripts containing the whole exons was named Viperin and transcripts that lack the exon 5 was named Viperin_sv1 (Fig. 1A).

The expression of Viperin and Viperin_sv1 was analyzed in response to SVCV and poly(I:C) (Sigma) to confirm the relationship between SVCV and Viperin alternative splicing. FHM cells seeded in 6-well plates and infected with SVCV at a MOI of 0.1, or transfected with poly (I:C) (2 μg/mL) for 24 h qRT-PCR analysis showed that total Viperin was significantly stimulated upon poly(I:C) transfection or SVCV infection compared to mock control (Fig. 1B). The PCR products of Viperin partial sequence (corresponding to 814–1040 bp of Viperin, that containing alternative splicing area) were detected by 12% non-denaturing polyacrylamide gels and the bands were identified by sequencing. It showed that mRNA expression of Viperin was stronger than Viperin_sv1 and both of them were upregulated by SVCV stimulation. Only Viperin was upregulated in poly(I:C) transfection cells (Fig. 1C, upper). For in vivo assay, the splicing variant of Viperin was also induced by SVCV in zebrafish spleen (Fig. 1C, below), which is consistent with the prediction of transcriptome analysis [39].

The multiple sequence alignment showed that the C-terminal region of Viperin was highly conserved except of FHM Viperin_sv1 and branchiostoma Viperin, both of which have 11 aa deletion that marked with the box (Fig. 1D). The comparison of the structural model of Viperin and Viperin_sv1 showed that the 11aa which Viperin_sv1 lack was close to the catalytic region (Fig.S1).

### 3.2. Overexpression of Viperin and Viperin_sv1 impairs SVCV replication in vitro

Viperin is known to play an antiviral role against various

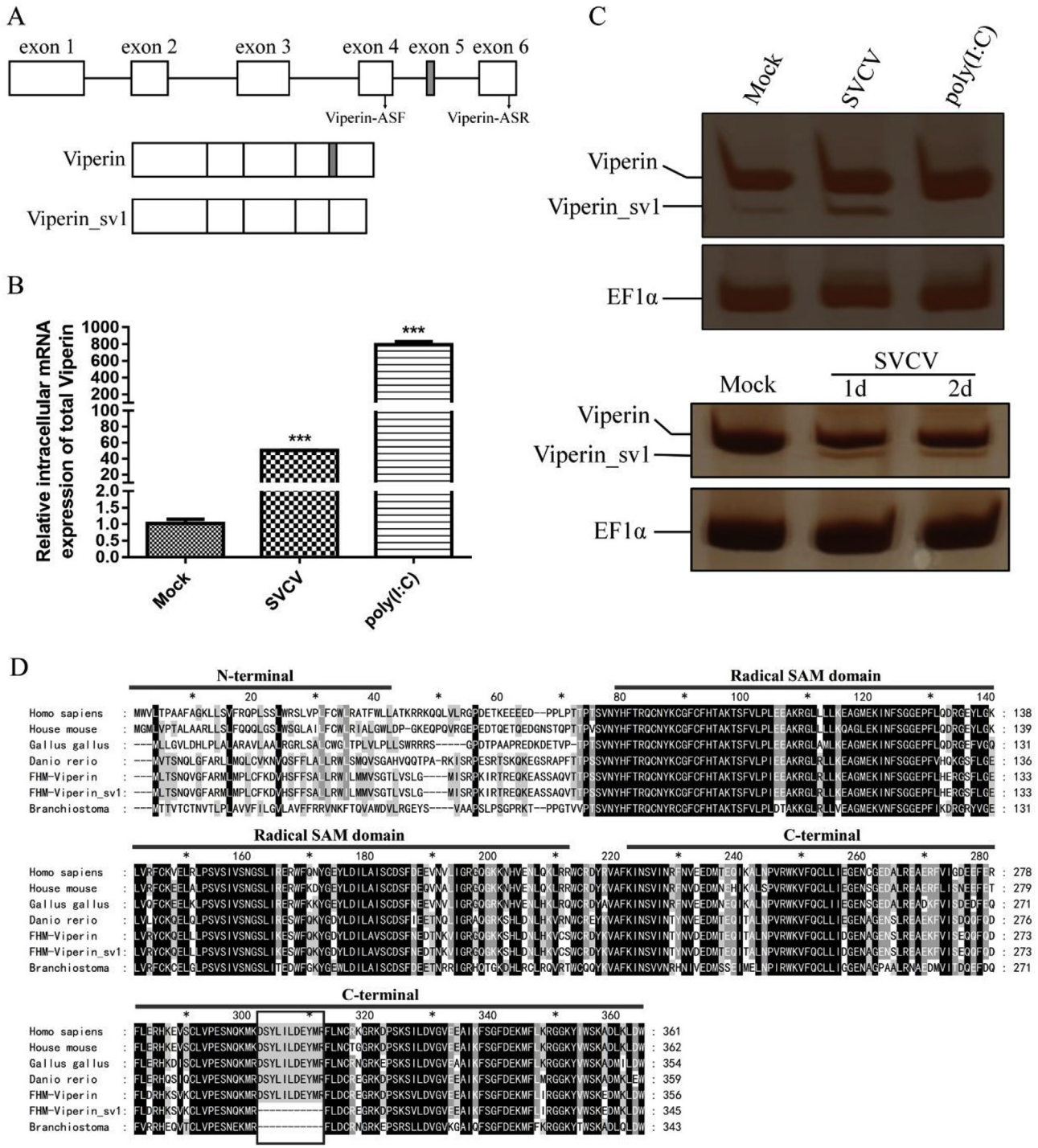


**Fig. 1.** Sequence and expression characteristics of Viperin and its splice variant. (A) Schematic diagrams of exon-intron arrangements of Viperin gene and generation of two transcriptions. Exons are indicated as rectangle boxes, and the introns as straight lines. The arrow represents the position of the primers for alternative splicing verification. (B) Expression analysis of total Viperin response to SVCV or poly(I:C). The relative mRNA level of total Viperin was determined by qRT-PCR and normalized to the expression of TBP. Error bars represent SD obtained by measuring each sample in triplicate (***, $p < 0.001$). (C) Semi-quantitative RT-PCR analysis of the partial mRNAs of Viperin and Viperin_sv1 in FHM cells (upper) and zebrafish spleen (below). EF1α was used as the internal control. All the data are representative of at least three independent experiments. (D) Amino acid alignment of Viperin. Three functional domains of Viperin across different species were marked with a solid bold line. The amino acid deletion of Viperin_sv1 was marked with box.

mammalian viruses and aquatic animal viruses. Viperin could also be coopted by HCMV to facilitate its infection process [40]. To determine the roles of Viperin and its splice variant in the process of SVCV replication, Viperin and Viperin_sv1 were overexpressed in FHM cells. Indirect immunofluorescence assay was performed to determine the effects of Viperin and Viperin_sv1 on SVCV at 24 h post-infection. As shown in Fig. 2A, both Viperin and Viperin_sv1 were able to inhibit SVCV infection and the inhibitory effect of Viperin_sv1 was stronger than Viperin.

To confirm the results, FHM cells were transfected with pcDNA4-Viperin, pcDNA4-Viperin_sv1 or empty vector pcDNA4 as control. At 24 h post transfection, cells were infected with SVCV at a MOI of 0.05

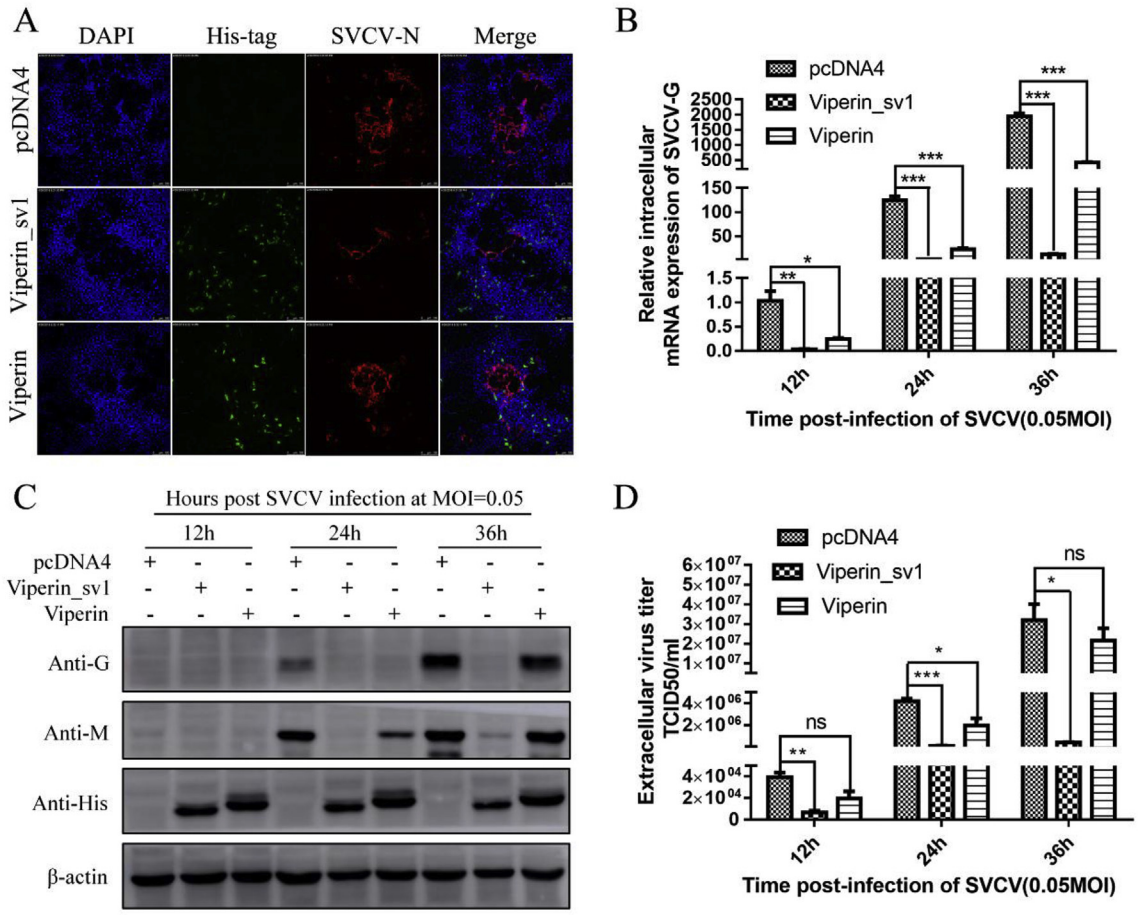


**Fig. 2.** The effect of Viperin and Viperin_sv1 on SVCV replication. (A) The effect of Viperin and Viperin_sv1 on SVCV infection directly. Representative images were photographed under confocal laser scanning microscopy. (B–D) FHM cells transfected with plasmids including Viperin, Viperin_sv1 or empty vector were infected with SVCV post 24 h and samples were prepared at the indicated times post viral infection. (B) mRNA levels of SVCV. qRT-PCR were used and then normalized to EF1α. Error bars represent SD obtained by measuring each sample in triplicate (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). (C) Western blot analysis using anti-G, anti-M, anti-His and anti-actin antibodies. +, present; -, absent. (D) The SVCV titers in the supernatants measured using $TCID_{50}$. The pcDNA4 transfected group was used for control. Error bars represent SD obtained by measuring each sample in triplicate.

and samples were collected at 12 h, 24 h, and 36 h post infection. qRT-PCR was used to evaluate the mRNA level of SVCV-G gene. The results showed that the transcription of SVCV-G gene was significantly decreased in Viperin and Viperin_sv1 overexpressed cells at 12 h, 24 h and 36 h post-infection compared with the pcDNA4 group and the effect of Viperin_sv1 is more persistent than Viperin (Fig. 2B). Viral proteins of SVCV were detected by Western blot assay using SVCV G and M monoclonal antibody. Expression level of SVCV M and G proteins was significantly reduced by Viperin_sv1 at 24 h and 36 h. Both M and G proteins of SVCV were reduced by Viperin at 24 h, while the reduction receded at 36 h (Fig. 2C). Virus titer of the supernatant from the three groups were determined by $TCID_{50}$ assay. Virus titer was reduced significantly over time for all points in Viperin_sv1 overexpressing cells compared with pcDNA4-transfected cells. Overexpression of Viperin decreased SVCV production at 24 h post-infection, but influenced viral titer slightly at 12 h and 36 h post-infection (Fig. 2D).

### 3.3. Splice variant Viperin_sv1, not viperin facilitates the production of type I IFN and ISGs

A previous report indicated that Viperin acts as a regulator in innate immune and promote the production of type I IFN [40]. To determine whether fish Viperin and Viperin_sv1 can regulate the expression of IFN1, FHM cells were transfected with pcDNA4-Viperin, pcDNA4-Viperin_sv1 or pcDNA4, and qRT-PCR was used to evaluate the transcripts levels of IFN-1 at 6 h, 12 h, 24 h and 36 h post-transfection. Interestingly, Viperin_sv1 but not Viperin upregulated the transcription of cellular IFN-1 genes and two ISGs (MxA and PKR) dramatically at 24 h and 36 h post-transfection (Fig. 3). These results may imply that the splice variant Viperin_sv1 is involved in the regulation of the IFN pathway to inhibit viral replication.

### 3.4. Effects of viperin splice variant on the expression of RIG-1, IRF3, IRF7 and NF-κB

Fish RLR signaling cascades were reported to activate IFN expression [41]. We then assessed whether Viperin_sv1 regulates the production of type I IFN through RLR signaling cascades. FHM cells were used to transfect with pcDNA4-Viperin, pcDNA4-Viperin_sv1 or pcDNA4 plasmids. Transcripts levels of RIG-1, IRF3 and IRF7 were monitored by qRT-PCR at 6 h, 12 h, 24 h and 36 h post-transfection. Consistently, splice variant Viperin_sv1, not Viperin increased the expressions of RIG-1, IRF3 and IRF7 significantly at 24 h and 36 h post-transfection (Fig. 4A–C). Previous research showed that NF-κB, the key regulators of inflammatory and immune response, was modulated by Viperin in early development of Th2 cells [42]. We also estimated the transcription of NF-κB in recombinant plasmid transfected cells. There was no significant difference in the transcription of NF-κB between the three groups (Fig. 4D). This may predict that Viperin_sv1 upregulates the production of type I IFN through the activation of RIG-1, IRF3, IRF7

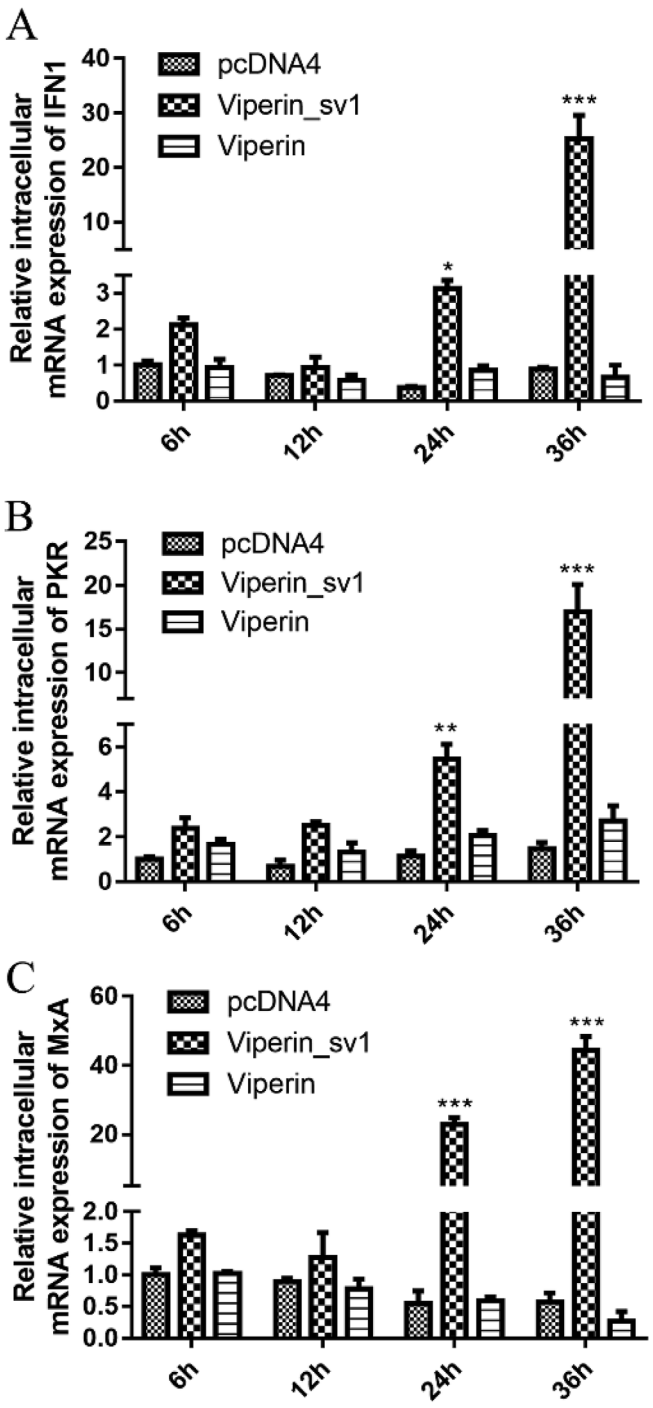


**Fig. 3.** Viperin_sv1 upregulated the expression of IFN1 and ISGs. Transcripts of IFN1 (A), MxA (B) and PKR (C) were detected using qRT-PCR at different time post recombinant plasmids transfection. EF1α was used as the internal control. Error bars represent SD obtained by measuring each sample in triplicate (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

signaling cascades.

### *3.5. SVCV decrease the protein level of Viperin_sv1 to counteract its antiviral effect in FHM cells*

Since overexpression of Viperin and Viperin_sv1 impairs SVCV replication, we investigated the regulation of SVCV to Viperin and Viperin_sv1 at the protein level. FHM cells were transfected with the recombinant plasmids and then treated with SVCV (0.05 MOI), UV inactivated SVCV or mock-infected with PBS at 12 h post transfection. The results showed that SVCV inhibited the expression of Viperin_sv1 significantly but had no notable effect on Viperin. The protein level of Viperin and Viperin_sv1 was not changed obviously in SVCV inactivated group compared with the PBS group (Fig. 5A). These results imply that Viperin_sv1 is more susceptible to SVCV than Viperin.

Previous studies demonstrated that Viperin protein was degraded in JEV-infected cells through a proteasome-dependent mechanism [15]. To investigate whether the decrease of Viperin_sv1 is regulated through a proteasome-dependent pathway, the Viperin_sv1 transfected cells were infected with SVCV at MOI of 0.1 and followed treatment with 10 μM MG132 (Selleckchem) at 6 h, 8 h and 10 h before samples harvest. Western blot analysis showed that the protein of Viperin_sv1 was inhibited by SVCV significantly compared with mock group, but obviously recovered by proteasome inhibitor MG132. Antiviral activity of Viperin_sv1 also got improved gradually in cells treated with MG132 at different time point before harvest (Fig. 5B). These results show that SVCV can regulate Viperin_sv1 for protein degradation through a proteasome-dependent pathway to counteract its antiviral effect.

## 4. Discussion

During viral infection, the expression of multiple host factors including Viperin could increase to enhancing host response to some viruses. Monkey Viperin (mViperin) suppresses PRRSV replication by blocking the early steps of PRRSV entry, genome replication, and translation [18]. Viperin inhibited influenza A virus release by impairing the formation of cholesterol-enriched plasma membrane microdomains, or lipid rafts [14]. In BSR cells (a clone of baby hamster kidney cells), Viperin reduced cholesterol and sphingomyelin on the cellular membrane, that suppressed rabies virus (RABV) budding and release [19]. Overexpression of crucian carp Viperin in cultured fish cells conferred significant protection against infection of GCRV [25]. In this study, a novel splice variant of Viperin named Viperin_sv1 was identified in SVCV infected FHM cells (Fig. 1A). Viperin is up-regulated by both IFN-dependent and IFN-independent pathways in mammals [8,43]. Previous studies have shown that the induction of Viperin is mediated via IRF-1 during infection of VSV [43]. Human cytomegalovirus infection can activate the type I IFN signaling pathway, which enhances Viperin transcription [44]. Crucian carp Viperin is also induced at mRNA level by fish IFNs and IFN stimuli such as poly(I:C) [25]. Here we have shown that SVCV is able to increase Viperin and Viperin_sv1 mRNA expression in FHM cells, while poly(I:C) can increase Viperin but not Viperin_sv1 expression (Fig. 1C). This may suggest that Viperin_sv1 is up-regulated by SVCV through IFN-independent pathways which argues for more validation and confirmation in future. A comparison of fathead minnow Viperin and Viperin_sv1 with other species revealed that the splice variant that lacks exon 5 was also existed in Branchiostoma. This implies that splice variant Viperin_sv1 may play a certain role in these marine species. Therefore, the aim of this study is to identify the function of fish Viperin and its splicing variant Viperin_sv1 in SVCV infection.

Recent studies have shown that C-terminal domain of Viperin plays an important role in the antiviral response. Using a panel of Viperin mutants, the importance of the carboxy-terminal domain of Viperin was highlighted for anti-viral activity against ZIKV [20]. Viperin could interact with human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33) via its C-terminal domain, and further disturbs the interactions of hVAP-33 with nonstructural protein 5A (NS5A) of HCV to inhibit HCV replication [13]. C-terminal end of Viperin also has anti-viral actions in resisting dengue virus type-2 (DENV-2) [17]. Splicing variant Viperin_sv1 lack the exon 5 at C-terminal domain. Overexpression of Viperin_sv1 inhibited virus replication significantly at different time points in FHM cells during the course of SVCV infection. Viperin-transfected FHM cells showed an anti-viral effect in the early stage of SVCV infection, while the antivirus effect was

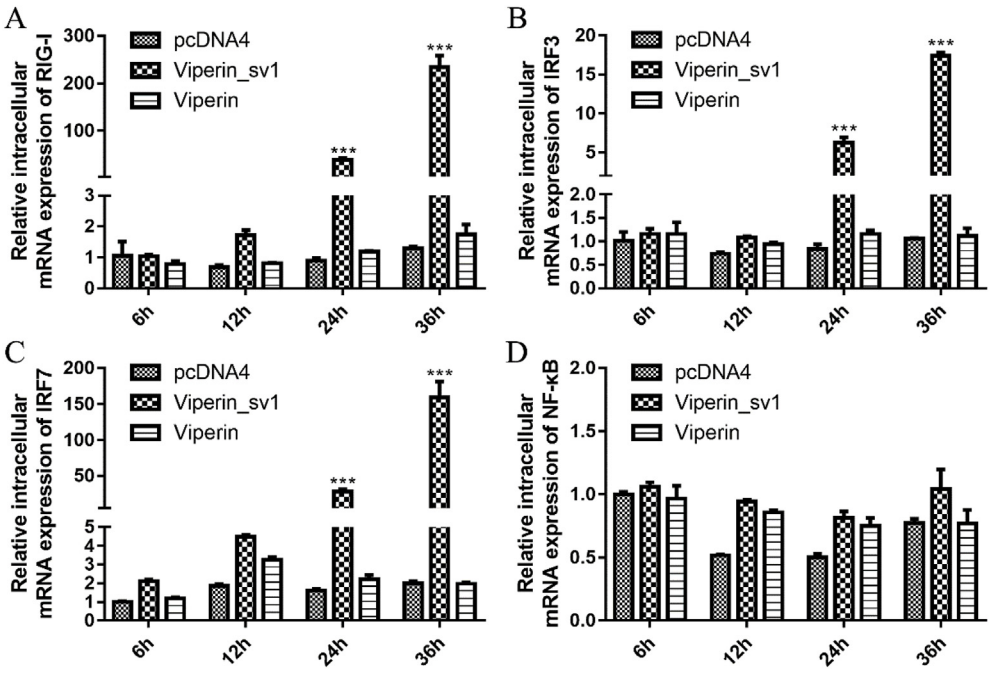


**Fig. 4.** Viperin_sv1 rather than Viperin could facilitate IRF3 and IRF7 signal activation. (A–D) Effect of Viperin and Viperin_sv1 on signaling molecules that induce type I interferon. Transcripts of RIG-1 (A), IRF3 (B), IRF7 (C), NF-κB (D) were detected using qRT-PCR. EF1α was used as the internal control. Error bars represent SD obtained by measuring each sample in triplicate (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

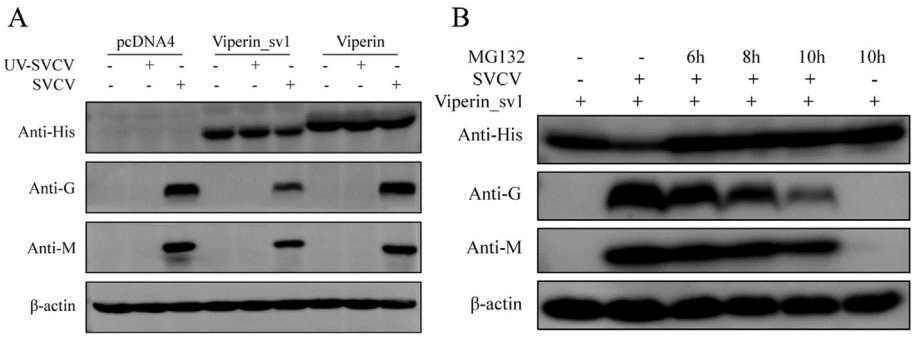


**Fig. 5.** The regulation of SVCV on Viperin and Viperin_sv1 at the protein level. (A) The protein levels of Viperin_sv1 regulated by SVCV. Western blot assay was proceeded using anti-His, anti-G, anti-M and anti-actin antibodies. (B) The protein levels and antiviral effect of Viperin_sv1 affected by MG132. FHM cells transfected with Viperin_sv1 and infected with SVCV as the description above. Cells were treated with MG132 at 6 h, 8 h and 10 h before harvest and western blot analysis was followed with anti-His, anti-G, anti-M and anti-actin antibodies.

substantially reduced at 36 h post viral infection (Fig. 2). This may imply that Viperin_sv1 has more persistent effect than Viperin on antiviral responses.

A previous study indicated that Viperin involved in the regulation of innate immune to affect virus infection [40]. Unlike mammals, our results show that fish Viperin_sv1 but not Viperin is able to induce IFN1 and ISGs including MxA and PKR in FHM cells (Fig. 3). It was reported that Viperin interacted with the signal mediators IRAK1 and TRAF6 to induce the nuclear translocation of transcription factor IRF7 and promoted the production of type I IFN in mice Plasmacytoid Dendritic cells [40]. In this study, fish Viperin_sv1 rather than Viperin induced type I IFN expression by activating RIG-1, IRF3 and IRF7 signaling cascade. Generally, amino acid deficiency leads to gene function changed. Crystal structures of mouse viperin (residues 45–362) complexed with S-adenosylhomocysteine (SAH) and [4Fe−4S] cluster show that there are three α-helices (α7, α8, and α9) in the C-terminal extension [45]. The 11 amino acids which Viperin_sv1 lack were between α8 and α9. When Viperin_sv1 lacked the 11 aa (result from exon 5 deletion), the subsequent α9 may closer to the active site to perform certain functions. This allows to conclude that through exon 5 lacking Viperin_sv1 altered its structure, which is important to activate the RLR signaling cascade. However, the specific regulatory mechanisms need further study in future. Viperin plays dual roles: a relatively direct suppression of viral replication and a facilitation of TLR7-and TLR9-mediated production of type I IFN [40]. This may infer that the full-length Viperin plays a more direct role in suppression of viral replication, while its splice variant Viperin_sv1 acts as a regulator in PRR-mediated innate immune.

It is well known that immunologically relevant genes play an important role in antivirus response. On the other hand, viruses also have evolved elaborate strategies to disable host's immune system for their invasion and survival. Infection of sendai virus, VSV, and herpes simplex virus 1 (HSV-1) could induce upregulation of INKIT, thereby restricting innate antiviral signaling [46]. Human CMV can exploit Viperin to assist its infection in trophoblast cells [47]. Even though JEV can highly induce Viperin transcription, it reduced Viperin at the protein level by the proteasome pathway to counteract its antiviral effect [15]. In this study, we demonstrated that SVCV could reduce Viperin_sv1 protein but has no significant effect on Viperin. Protease inhibitor MG132, a membrane-permeable proteasome inhibitor, protected Viperin_sv1 from protein degradation and rescued its antiviral activity against SVCV (Fig. 5). These results indicate that the degradation of Viperin_sv1 is regulated by SVCV for evaded innate immune response through proteasome pathway. More in-depth studies are needed to confirm this observation and to understand the related mechanism in future.

In summary, this study revealed that SVCV infection could increase expression of alternative splice variant of viperin in fish cells. Splicing variant Viperin_sv1 could significantly inhibit SVCV replication. Viperin inhibits SVCV at early stages of virus infection, but the suppression was quickly reduced along SVCV replication. Viperin_sv1 rather than Viperin can facilitate the production of IFN1 and ISGs through the activation of RIG-1, IRF3 and IRF7 signaling cascades. SVCV

degraded Viperin_sv1 protein and attenuated its antiviral effect through the proteasome pathway, while it had no significant effect on Viperin. Our study has provided new insights into the role of splicing variant Viperin_sv1 in the regulation of host interferon defense system and revealed a new strategy of SVCV to evade host innate immunity in lower vertebrates.

## Conflicts of interest

The authors declare to have no conflict of interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.012.

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