Gig1, a novel antiviral effector involved in fish interferon response

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A B S T R A C T

Vertebrate interferon (IFN) response defenses against viral infection through the induction of hundreds of IFN-stimulated genes (ISGs). Most ISGs are conserved across vertebrates; however, little is known about the species-specific ISGs. In this study, we reported that grass carp reovirus (GCRV)-induced gene 1 (Gig1), previously screened as a virus-induced gene from UV-inactivated GCRV-infected crucian carp (*Carassius auratus*) blastulae embryonic (CAB) cells, was a typical fish ISG, which was significantly induced by intracellular poly(I:C) through retinoic acid-inducible gene I (RIG-I)-like receptors-triggered IFN signaling pathway. Transient or stable overexpression of Gig1 prevented GCRV replication efficiently in cultured fish cells. Strikingly, Gig1 homologs were found exclusively in fish species forming a novel gene family. These results illustrate that there exists a Gig1 gene family unique to fish species and the founding gene mediates a novel fish IFN antiviral pathway.

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Introduction

In mammals, the mechanism underlying type I interferon (IFN) response has been well-characterized. Once upon infection, viral proteins and nucleic acids are detected by host pattern recognition receptors (PRRs) including retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) (Beutler et al., 2007). Such recognition activates signaling cascades resulting in the induction of type I IFNs, which subsequently establishes the so-called "host antiviral state" in both autocrine and paracrine fashions by inducing the expression of hundreds of interferon stimulated genes (ISGs) (Sadler and Williams, 2008; Schoggins and Rice, 2011). Thus, the cellular effectors that limit the spread of viral infection are the products of ISGs. Whereas a few ISGs have been characterized with respect to antiviral potential, most of them are of unknown or incompletely understood function (Schoggins and Rice, 2011). Recent studies have revealed more targeted antiviral action of function-characterized ISGs in a tissue-, cell- and virus-specific manner (Fensterl et al., 2012, 2008; Schoggins et al., 2011; Szretter et al., 2011).

Since the discovery of IFN more than 50 years ago, the IFN antiviral system has been believed to exist across vertebrates. This notion is strengthened by the recent findings that the lower vertebrate fish possess *IFN* genes and many conserved ISGs (Gui and Zhu, 2012; Zhang and Gui, 2012). Similar to mammals, emerging studies showed that fish RLRs family members RIG-I, MDA5 can trigger *IFN* gene expression by recruitment of MITA (mediator of IRF3 activation), which subsequently activates the TBK1-IRF3-IFN signaling pathway (Chang et al., 2011; Sun et al., 2011). Fish MAVS (mitochondrial antiviral signaling protein) seems to be involved in IFN induction through the RLR signaling pathway (Biacchesi et al., 2009, 2012). The resultant fish IFNs exert antiviral effects through upregulation of the downstream ISGs likely by the Janus kinase (Jak)-signal transducer and activator of the transcription (Stat) pathway (Shi et al., 2012; Skjesol et al., 2010; Yu et al., 2010). In support of these observations, fish ISGs promoters harbor a DNA sequence, termed IFN-stimulated response element (ISRE), which is responsible for IFN induction (Altmann et al., 2004; Jiang et al., 2009; Li et al., 2012b; Liu et al., 2011; Sun et al., 2010); two fish ISGs, *Mx* and *PKR*, similar to their mammalian homologs (Sadler and Williams, 2008), share abilities of restricting virus replication (Larsen et al., 2004; Liu et al., 2011; Zhu et al., 2008). Besides these ISGs conserved in all vertebrates, there exist IFN-inducible antiviral components unique to distinct vertebrate lineages. For example, human schlafen 11, belonging to a gene family that is limited to mammalian organisms, has recently been identified as an IFN-inducible antiviral effector that suppresses HIV protein synthesis by means of codon-bias discrimination (Li et al., 2012a); mouse schlafen 2 and human schlafen 5 induce a growth-suppressive effects of IFNs in malignant cells (Katsoulidis et al., 2009, 2010), highlighting the physiological significance of mammals-specific ISGs in IFN response.

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In the past decades, great endeavors to identify virus-induced genes in fish found that some are not homologous to any mammalian genes. These genes include *PKR-like/PKZ* (protein kinase containing Z-DNA binding domains) (Hu et al., 2004), *Gig1* and *Gig2* (grass carp reovirus (GCRV)-induced gene 1 and 2) (Zhang and Gui, 2004), which are firstly retrieved from UV-inactivated GCRV-infected crucian carp (*Carassius auratus*) blastulae embryonic (CAB) cells (Zhang et al., 2003), and later found in other fish species (Bergan et al., 2008; Krasnov et al., 2011; Martin et al., 2007; Rothenburg et al., 2008, 2005). Further studies have revealed that *PKZ* encodes a novel fish IFN-induced eIF2α kinase working in concert with *PKR* to block virus infection through phosphorylation of cellular eIF2α (Liu et al., 2011), and that *Gig2* gene is specific to both fish and amphibians (Zhang et al., 2013) and displays an ability to inhibit viral replication in cultured fish cells (Li et al., 2012b). However, the expression regulation and biological function of fish *Gig1* remains to be investigated.

In the present study, we further identified crucian carp *Gig1* as a typical ISG with antiviral function. *Gig1* harbored an ISRE-containing promoter contributing to the induction by IFN and poly(I:C). Poly(I:C) transfection of fish cells activated the RLR signaling pathway to induce the production of IFN protein, which in turn upregulated the expression of Gig1 by the Jak-Stat pathway.

We found that fish Gig1 protein suppressed GCRV replication in cultured fish cells, as further verified by real-time PCR analyses showing dramatically decreased amplification of GCRV genome. Finally, *Gig1* homologs existed exclusively in fish lineages forming a gene family with divergence in protein sequences and expression properties. This study identified a novel gene family unique to fish species, and provided evidence for antiviral roles of the founding gene *Gig1* in fish IFN response.

Results

Fish Gig1 is upregulated in cultured cells by poly(I:C) and IFN

Gig1 was first identified as a virus-induced gene in UV-inactivated GCRV-infected CAB cells (Zhang and Gui, 2004; Zhang et al., 2003). Similarly, *Gig1* was transcriptionally induced in a time-dependent manner by UV-inactivated GCRV and extracellular poly(I:C) (Fig. 1A and B). To further investigate the expression of Gig1 at the protein level, we expressed a recombinant Gig1 protein (rGig1) by the prokaryotical expression system, and subsequently obtained a polyclonal anti-Gig1 serum
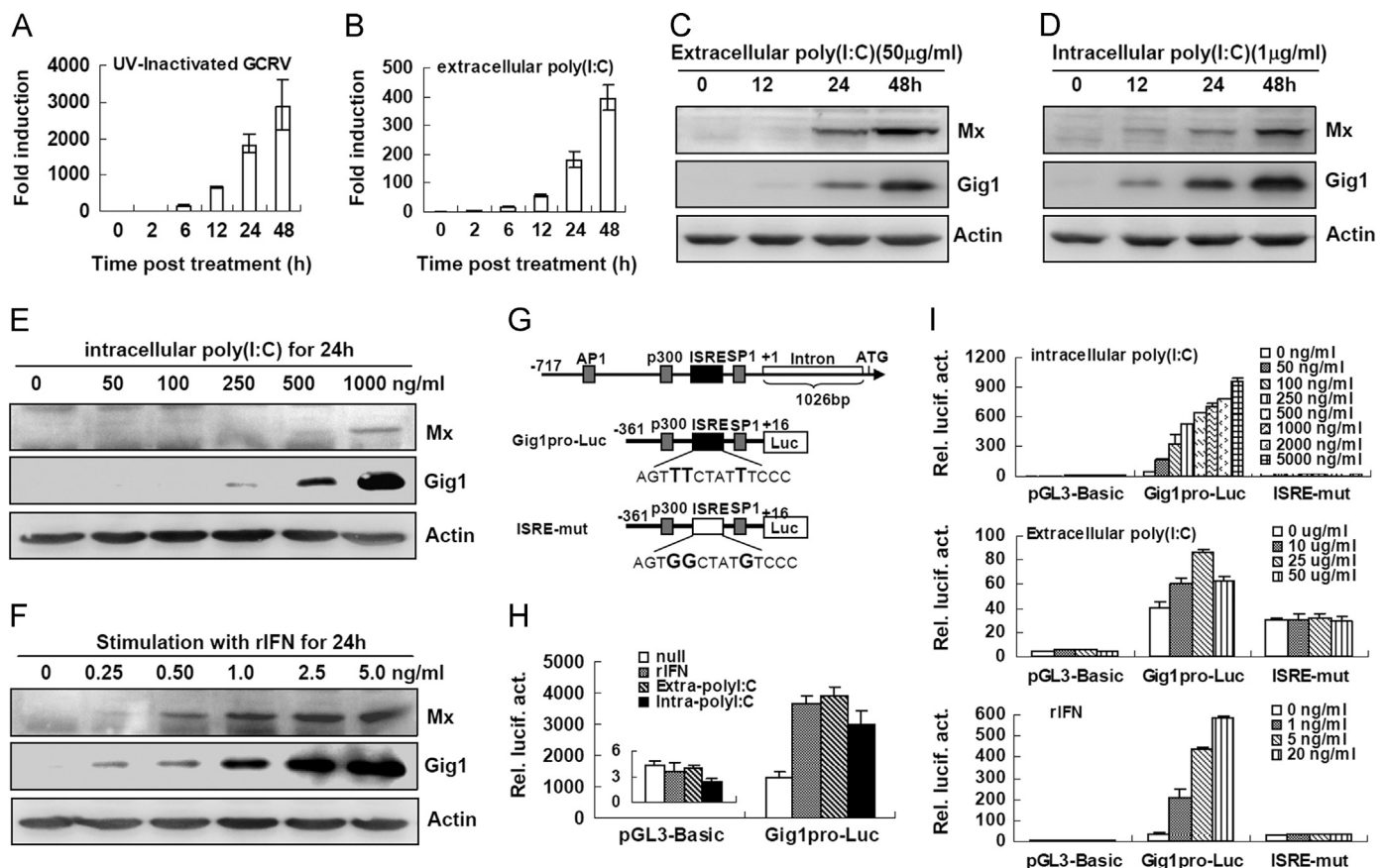
Fig. 1. Induction of crucian carp Gig1 in CAB cells by poly(I:C) and IFN. (A–F) CAB cells in each well of 24-well plates were treated for different times with UV-inactivated GCRV (1×10^9 TCID$_{50}$/ml exposed to UV) (A), extracellular poly(I:C) (50 μg/ml) (B and C), or transfected with 1 μg/ml of poly(I:C)(intracellular poly(I:C)) (D), or transfected for 24 h with different doses of intracellular poly(I:C) (E) and recombinant crucian carp IFN (rIFN) (F). At the indicated time points, cells were collected for detection of *Gig1* mRNA by real-time PCR (A and B), or of Gig1 protein by western blot analyses (C–H). (G) Schematic of *Gig1* promoter and Gig1 promoter-driven luciferase constructs. (H) Induction of *Gig1* promoter by IFN and poly(I:C). CAB cells seeded in 24-well plates overnight were cotransfected with 0.25 μg Gig1pro-Luc or control vector pGL3-basic in 500 μl medium. 0.025 μg pRL-TK was included to normalize the expression level. 24 h later, the transfected cells were washed three times to remove transfection mixture, and then treated again with 10 ng/ml rIFN, 50 μg/ml extracellular poly(I:C), or transfected again with 1 μg/ml poly(I:C) as intracellular poly(I:C), or left untreated as a control (null) for an additional 24 h, followed by detection of luciferase activity. (I) CAB cells seeded in 24-well plates were transfected as the same in H with pGL3-basic, Gig1pro-Luc and ISRE-mut, respectively. 24 h later, the transfected cells were washed three times, and then transfected again with different doses of poly(I:C) as intracellular poly(I:C), or treated directly with different doses of poly(I:C) as extracellular poly(I:C) or rIFN for an additional 24 h. Luciferase assays were performed in triplicates and repeated at least three times. Western blot results were representative of at least two different experiments.
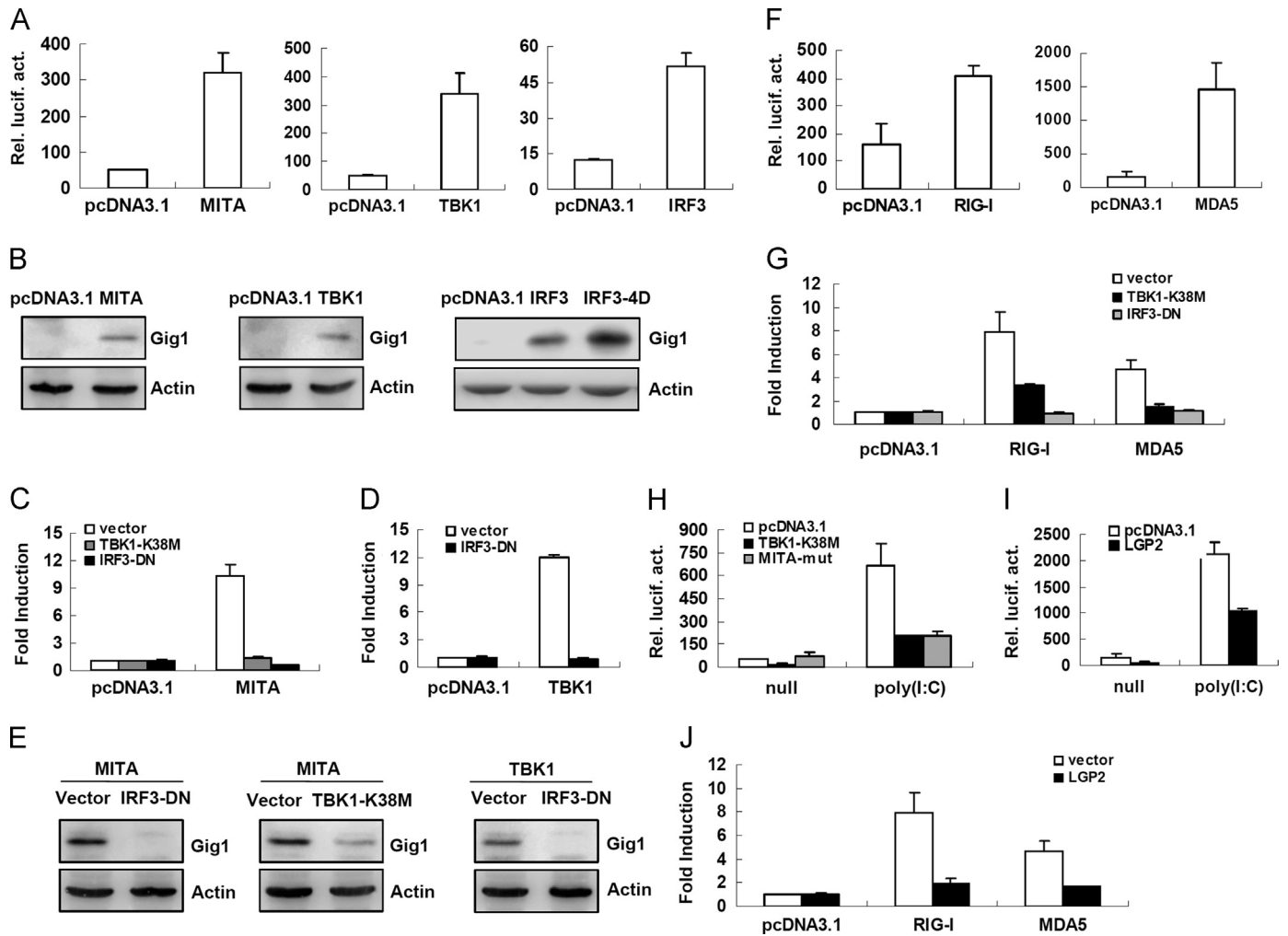
Fig. 2. Activation of Gig1 production through RLR signaling pathway. (A) CAB cells seeded in 24-well plates overnight were transfected with a total of 0.525 μg of the indicated constructs (MITA, TBK1, IRF3, or pcDNA3.1 as control), Gig1pro-Luc, and pRL-TK as an internal control, at a ratio of 10:10:1. At 48 h post-transfection, cells were harvested for detection of luciferase activity. (B) CAB cells seeded in 6-well plates overnight were transfected as the same in A with the indicated plasmids (1.6 μg per well). 48 h later, the transfected cells were sampled to detect Gig1 protein by western blot analysis. (C and D) CAB cells seeded in 24-well plates were cotransfected with 0.25 μg MITA or TBK1, 0.25 μg of the indicated expression mutants, and 0.25 μg Gig1pro-Luc. 0.025 μg pRL-TK was included to normalize the expression level. 48 h later, cells were harvested for measurement of luciferase activity. (E) CAB cells seeded in 6-well plates were cotransfected as in C and D with MITA or TBK1, and the mutants TBK1-K38M or IRF3-DN (0.8 μg each), respectively. At 48 h post-transfection, Gig1 protein was detected by western blotting. (F) Activation of Gig1 promoter by RIG-I and MDA5. CAB cells seeded in 24-well plates were transfected as in A with the indicated constructs. (G) RIG-I/MDA5-mediated activation of Gig1 promoter was inhibited by the dominant negative mutants of TBK1 and IRF3. The reporter experiments were performed as in C and D with the indicated constructs. (H-I) Poly(I:C)-stimulated activation of Gig1 promoter was impaired by the mutants of MITA and TBK1 (H), and LGP2 (I). CAB cells seeded in 24-well plates overnight were cotransfected as in A with the indicated constructs. At 24 h post transfection, the cells were transfected again with poly(I:C) (200 ng/ml) for an additional 24 h. (J) RIG-I/MDA5-mediated activation of Gig1 promoter was negatively regulated by LGP2. The reporter assay was carried out as in C and D. Luciferase assays were performed in triplicates and repeated at least three times. Western blot results were representative of at least two different experiments.

by immunization of rabbits with the purified rGig1 (see Fig. S1A and S1B in the Supplemental materials). The specificity of anti-Gig1 serum was confirmed by further assays, where this antiserum could recognize an expected protein of about 23 kDa in either poly (I:C)-treated cells or Gig1-overexpressed cells, but neither pre-immunized rabbit serum nor anti-Gig1 serum pre-absorbed with rGig1 could detect the same protein band (Fig. S1B).

Western blots showed that the Gig1 protein was induced by either transfection of poly(I:C) (intracellular poly(I:C)) or extra-cellular ploy(I:C), with a rapider induction by intracellular poly(I:C) (Fig. 1C and D). This was consistent with a previous finding that intracellular poly(I:C) was more effective to induce fish IFN activity than extracellular poly(I:C) (Yu et al., 2010). Consistently, Mx, a hallmark protein of IFN response, was also robustly induced. Both intracellular poly(I:C) and recombinant crucian carp IFN (rIFN) stimulated Gig1 expression in a dose-dependent manner (Fig. 1E

and F). These results indicated that fish Gig1 is upregulated in response to poly(I:C) and rIFN.

Fish Gig1 gene harbors an ISRE motif-containing promoter

To further characterize the expression regulation of crucian carp *Gig1*, a genomic DNA containing *Gig1* gene was cloned by genome walking (see Fig. S2 in the Supplemental materials). An intron of 1026 bp was found within the 5′UTR of crucian carp *Gig1* gene, and multiple transcription factor binding sites were predicated within 5′-flanking regulatory region, including a typical ISRE motif between −72 and −84 relative to the transcriptional start site (Fig. 1G, Fig. S2). Accordingly, a ∼360-bp DNA fragment containing the ISRE motif was identified as a minimal promote to generate a promoter-driven luciferase reporter construct (Gig1pro-Luc) and simultaneously, a mutant construct (ISRE-mut) was made by replacement of thymine
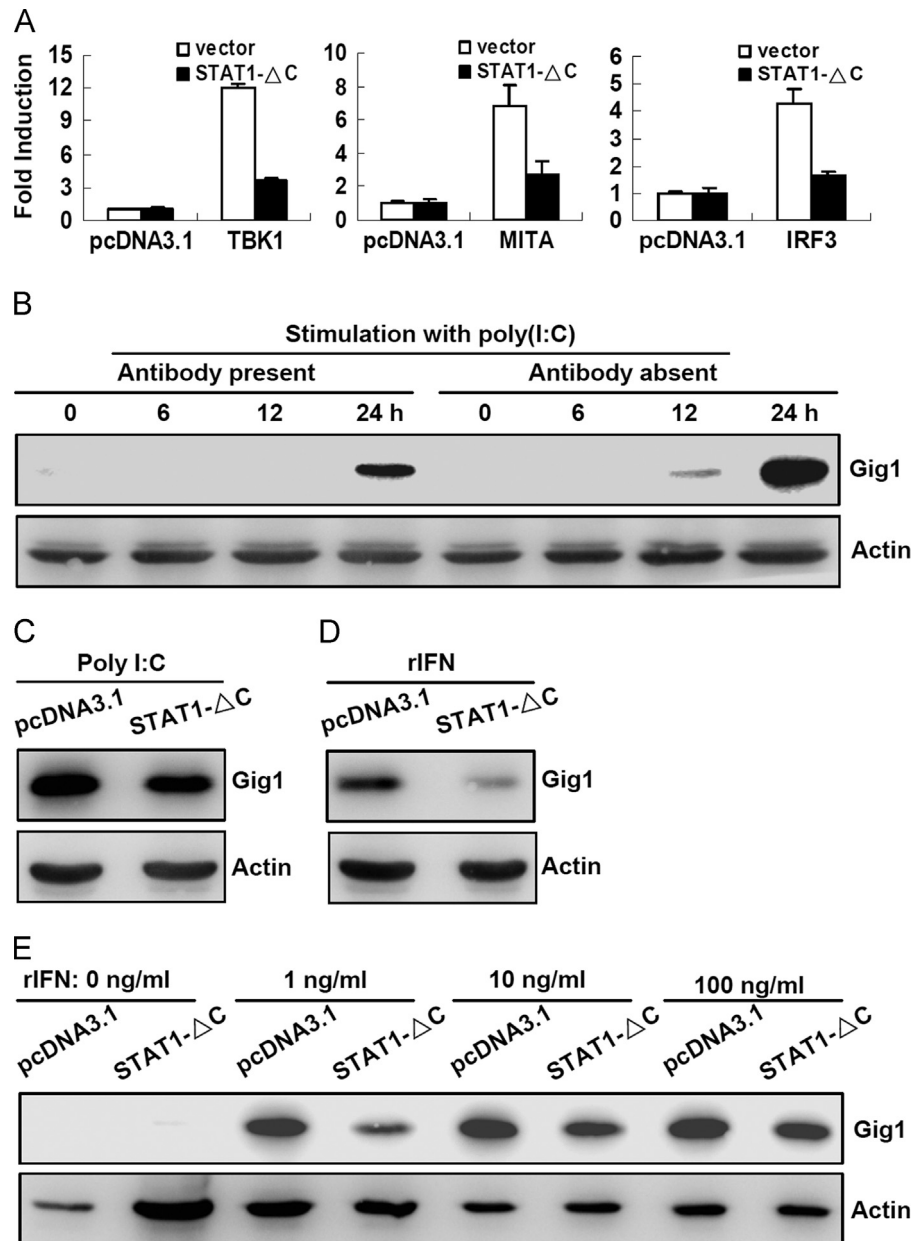
Fig. 3. Requirement of IFN-Stat1 pathway in intracellular poly(I:C)-induced Gig1 activation. (A) Activation of Gig1 promoter by MITA, TBK1 and IRF3 was impeded by Stat1-ΔC. The experiments were carried out as in Fig. 2C and D. (B) Intracellular poly(I:C)-induced Gig1 was blocked by anti-IFN serum. CAB cells seeded in 6-well plates were transfected with 1 μg/ml poly(I:C) in the presence or absence of polyclonal anti-IFN serum (100 ng/ml). At the indicated time points, cells were collected for detection of Gig1 protein by western blotting. (C–E) Requirement of Stat1 for Gig1 induction by transfected poly(I:C) and rIFN. Two stable transformants that were stably transfected with STAT1-ΔC or pcDNA3.1 were seeded in 6-well plates overnight, and treated with 1 μg/ml intracellular poly(I:C) (C) or 5 ng/ml rIFN (D) or different doses of rIFN (1, 10 and 100 ng/ml) (E). 10 h later, the cells were harvested for detection of Gig1 protein. Luciferase assays were performed in triplicates and repeated at least three times. Western blot results were representative of at least two different experiments.

(T) with guanine (G) within the ISRE motif of Gig1pro-Luc (Fig. 1G). Luciferase activity assays showed that Gig1pro-Luc was significantly activated by rIFN and IFN stimuli poly(I:C), respectively (Fig. 1H). Whereas intracellular poly(I:C), extracellular poly(I:C) and rIFN activated the Gig1 promoter in a dose-dependent manner, such stimuli did not stimulate any activities of ISRE-mut compared to basal activities of Gig1pro-Luc (Fig. 1I). These results indicated that the ISRE motif is necessary for induction of Gig1 by rIFN and poly(I:C).

*Fish Gig1 is induced by RLR-mediated signaling*

To determine whether the fish RLR signaling pathway was involved in the expression regulation of Gig1, we initially investigated the roles of some pivotal RLR signaling factors by

cotransfection of CAB cells with Gig1pro-Luc and MITA, TBK1 or IRF3. Overexpression of MITA, TBK1 or IRF3 gave a significant activation of Gig1pro-Luc by 4~6-fold increase against that of pcDNA3.1 (Fig. 2A), and induced a strong increase in Gig1 protein (Fig. 2B). In contrast, overexpression of dominant negative forms of fish TBK1 and IRF3, named TBK1-K38M and IRF3-DN (Sun et al., 2010), blocked MITA-mediated activation of Gig1pro-Luc (Fig. 2C); TBK1-mediated activation was severely impaired by overexpression of IRF3-DN (Fig. 2D). Similarly, MITA-induced Gig1 protein was diminished in CAB cells when either TBK1-K38M or IRF3-DN was overexpressed, and TBK1-induced protein was also decreased when overexpression of IRF3-DN (Fig. 2E). These results confirmed that MITA-TBK1-IRF3 signaling is involved in regulation of fish Gig1.

In subsequent assays, transfection of CAB cells with either RIG-I or MDA5 led to a significant activation of fish *Gig1* promoter (Fig. 2F); however, such activation was severely inhibited by overexpression of the dominant-negative mutants of TBK1 or IRF3(TBK1-K38M or IRF3-DN), respectively (Fig. 2G). Considering that the fish RLR signaling pathway can be activated by intracellular poly(I:C) (Sun et al., 2011), we next investigated the roles of these RLR signaling factors in fish Gig1 expression under intracellular poly(I:C). As anticipated, overexpression of either TBK1-K38M or MITA-mut diminished intracellular poly(I:C)-induced activity of Gig1pro-Luc (Fig. 2H). A similar result was obtained when transfection of LGP2 (Fig. 2I), a third RLR family member that showed inhibitory effects during poly(I:C)-triggered IFN response (Sun et al., 2011). Transfection of LGP2 also diminished RIG-I- or MDA5-mediated activation of the *Gig1* promoter (Fig. 2J). Therefore, intracellular poly(I:C) upregulated fish Gig1 through the RLR signaling pathway.

Fish Gig1 is a typical ISG

Fish Stat1 is a pivotal component in the IFN-activated Jak-Stat signaling pathway (Yu et al., 2010; Zhang and Gui, 2012). We further investigated the role of Stat1 in intracellular poly(I:C)-induced expression of fish Gig1. Initially, luciferase assays showed that overexpression of CAB cells with a Stat1 mutant (Stat1-ΔC), which lacks the C-terminal transcriptional activation domain displaying a dominant negative effect (Yu et al., 2010), severely impeded MITA-induced activation of Gig1pro-Luc (Fig. 3A). TBK1- or IRF3-induced activation of Gig1pro-Luc was also significantly impaired by simultaneous overexpression of Stat1-ΔC (Fig. 3A), indicating that the Stat1 pathway was involved in fish RLR-mediated expression of Gig1. Subsequently, Gig1 proteins were detected in CAB cells transfected with poly(I:C) in the presence or absence of anti-IFN antibody. A time course analyses showed that poly(I:C) induction of Gig1 was retarded in antibody-treated cells
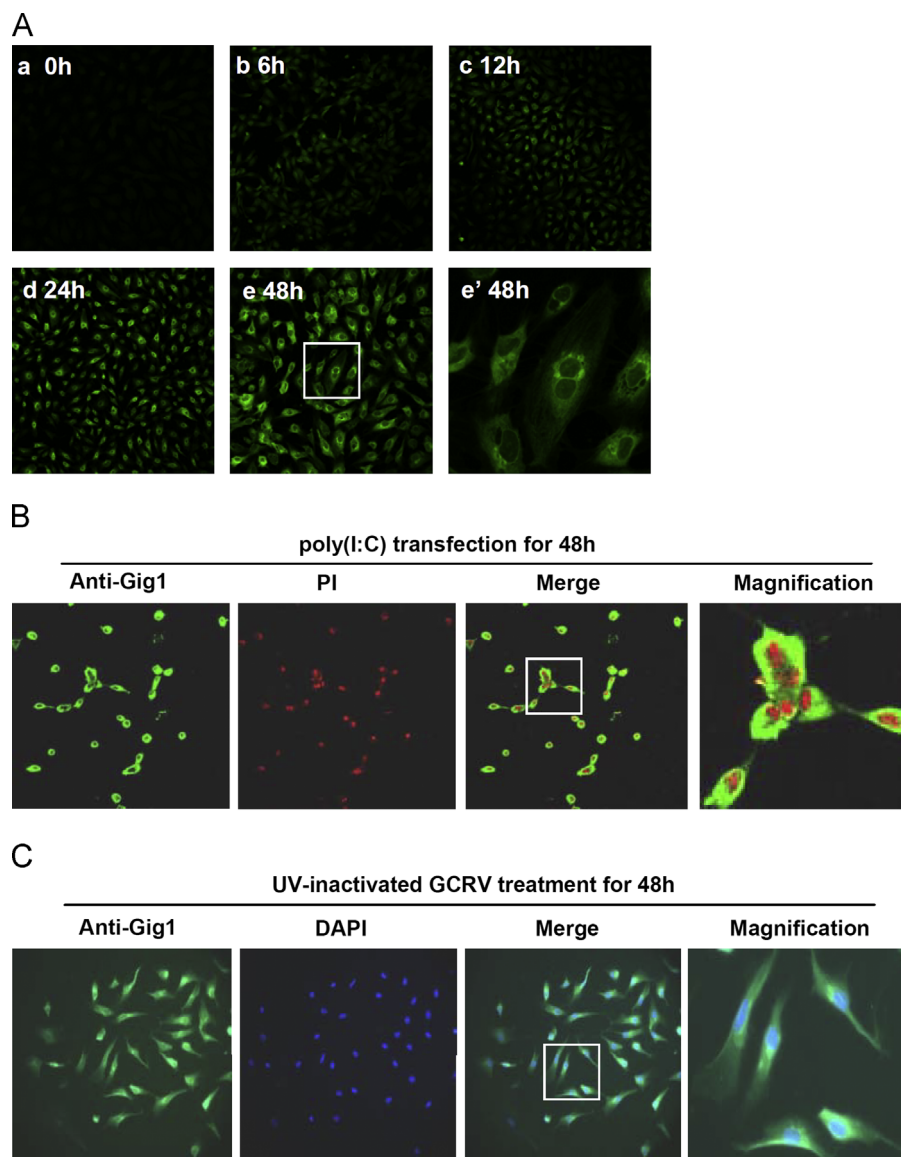
Fig. 4. Cytoplasmic distribution of Gig1 protein. (A) A time course observation of Gig1 protein in poly(I:C)-transfected cells. CAB cells plated onto glass coverslips in 6-well plates overnight were transfected with 0.5 μg poly(I:C) in 1 ml medium. At the indicated time points, the cells were fixed for immunofluoresence analysis by polyclonal anti-Gig1 antiserum. e′ represented the magnification of the framed area in e. (B and C) CAB cells plated onto glass coverslips in 6-well plates overnight were transfected with 0.5 μg/ml poly(I:C) (B) or treated with UV-inactivated GCRV (1×10^9 TCID$_{50}$/ml exposed to UV) (C) for 48 h and then fixed for immunofluoresence analysis by polyclonal anti-Gig1 antiserum. The nuclei were stained by PI (red) in B and DAPI (blue) in C. All results were representative of two independent experiments.
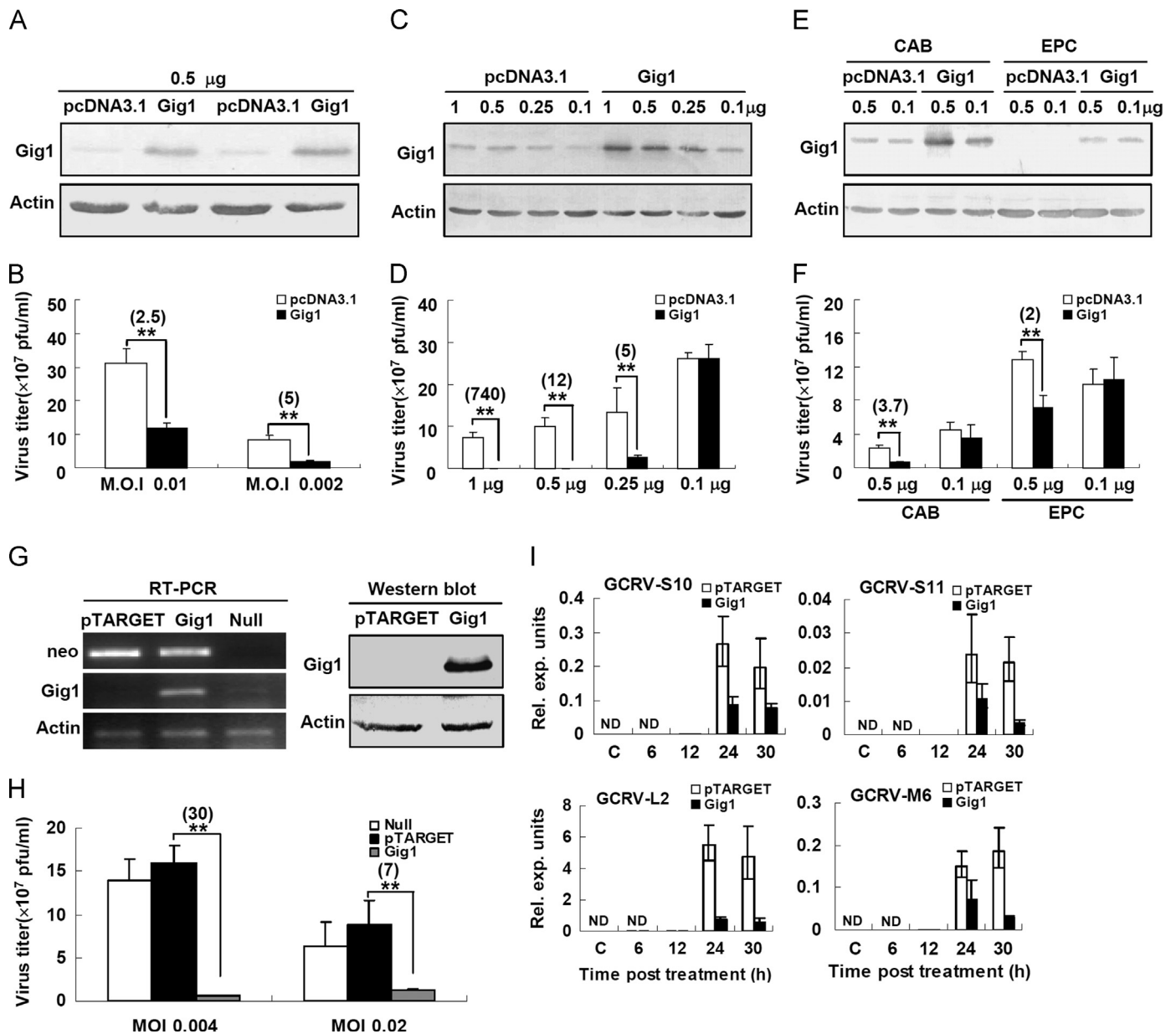
Fig. 5. Antiviral effects of Gig1 protein. (A and B) CAB cells seeded in 24-well plates were transfected with 0.5 μg Gig1 construct or 0.5 μg pcDNA3.1 as control. At 30 h post transfection, one group of cells was sampled for detection of Gig1 protein by western blotting (A), and the other group of cells was further challenged with GCRV at M.O.I of 0.01 or 0.002 (B). An additional 48 h later, the GCRV-infected cells were frozen and thawed three times, and then the supernatants were collected for determination of virus yields by plaque forming-unit assays (B). (C and D) CAB cells seeded in 24-well plates were transfected with different amounts of Gig1 (0.1, 0.25, 0.5 or 1 μg per well) or pcDNA3.1 as control. At 30 h post transfection, one group of cells was sampled for detection of Gig1 protein by western blotting (C). The other group of cells was subsequently challenged with GCRV at M.O.I of 0.002 for an additional 48 h, and then the supernatants were collected for determination of virus yields by plaque forming-unit assays (D). (E and F) CAB or EPC cells seeded in 24-well plates were transfected with the indicated amounts of Gig1 or pcDNA3.1. At 30 h post transfection, one group of cells was sampled for detection of Gig1 protein by western blotting (E). The other group of cells was further challenged with GCRV at M.O.I of 0.005 for an additional 48 h, and then the supernatants were collected for determination of virus yields by plaque forming-unit assays (F). (G-I) GCRV replication was inhibited in stably Gig1-transfected CAB cells. RT-PCR and western blot analysis were used to detect the expression of Gig1 in CAB cells that had been stably transfected with pTARGET-Gig1 or empty construct pTARGET (G). Both CAB transfomants were infected with GCRV at M.O.I of 0.004 or 0.02. 48 h later, the cells was frozen and thawed three times, and the supernatants were collected for determination of virus yields (H). Or in the indicated times, the cells were sampled for detection of four segment dsRNAs of GCRV genome by real-time PCR analysis (I). The results above were representative of at least two independent experiments. **$p < 0.01$ indicates significant differences. Numbers in brackets above the columns indicated fold induction against the corresponding control.

as relative to mock-treated cells (Fig. 3B). For example, although Gig1 protein was undetectable at 12 h in antibody-treated cells, a robust induction was seen in mock-treated cells (Fig. 3B), demonstrating that intracellular poly(I:C)-induction of Gig1 protein required production of fish IFNs as intermediates, which is consistent with the previous results that poly(I:C)- or GCRV-induced *Gig1* transcription was blocked by cycloheximide (Zhang and Gui, 2004; Zhang et al., 2007). Finally, compared to transfection of CAB cells with empty vector pcDNA3.1, transfection of Stat1-ΔC resulted

in a diminished level of Gig1 protein induced by intracellular poly(I:C) (Fig. 3C), and by rIFN (Fig. 3D), even in rIFN dose-dependent manner (Fig. 3E). These results indicated that intracellular poly(I:C) induces fish Gig1 expression dependent on IFN production.

Fish Gig1 is a cytosolic protein

The expression dynamics of fish Gig1 was next investigated by immunofluorescence assays. Compared to no appreciable signal in

normal CAB cells (Fig. 4A-a), a strong fluorescent signal was detected in poly(I:C)-transfected cells, showing that Gig1 protein was detectable at 6 h post transfection (Fig. 4A-b), and gradually induced to a greater extent over time (Fig. 4A-c–A-e). At 48 h post transfection, Gig1 protein was induced in almost all transfected cells (Fig. 4A-e), and the green fluorescent signal was seen exclusively in the cytoplasm (Fig. 4A-e′). To further probe subcellular localization of the Gig1 protein, PI (propidium iodide) was used to stain the nuclei of poly(I:C)-transfected cells differentiating from the cyptoplasm. It showed that the red signal by PI staining did not overlap with the green fluorenscence by anti-Gig1 serum (Fig. 4B). A similar result was observed in CAB cells treated with UV-inactivated GCRV (Fig. 4C). Finally, western blot analyses of the partitioned nucleus and cytoplasm of UV-inactivated GCRV-treated CAB cells showed that Gig1 protein accumulated in the cytoplasm but not in the nuclei (see Fig. S3 in the Supplemental materials). These results indicated that the poly(I:C)-induced or GCRV-induced Gig1 protein resides in the cytoplasm.

Fish Gig1 displays an ability of inhibiting virus infection

The biological function of fish Gig1 was investigated. Initially, CAB cells were transiently transfected with Gig1 or empty vector as a control followed by infection with GCRV. Western blotting confirmed successful transfection of fish Gig1 (Fig. 5A). A 48 h-infection with GCRV resulted in a broad CPE in control cells, whereas a delayed CPE was observed in Gig1-overexpressed cells, with a better effect when challenge GCRV was at lower multiplicity of infection (M.O.I.) (0.002 of M.O.I versus 0.01 of M.O.I) (see Fig. S4A in the Supplemental materials). In CAB cells challenged with GCRV at 0.01 of M.O.I, a virus titer of 1.2×10^8 pfu/ml was detected in Gig1-overexpressed cells, which was about a 2.5-fold reduction relative to control cells (3.1×10^8 pfu/ml; Fig. 5B). When cells were challenged with GCRV at 0.002 of M.O.I, a more obvious protection was observed, with about 5-fold reduction of virus load relative to control cells (1.7×10^7 pfu/ml versus 8.4×10^7 pfu/ml; Fig. 5B). Additionally, fish Gig1 inhibited GCRV replication in a dose-dependent manner, with about 740-fold reduction of virus titer relative to control cells when 1 μg/ml of constructs were transfected followed by challenge of GCRV at 0.002 of M.O.I (Fig. 5C and D, Fig. S4B). Transfection of 0.5 μg/ml Gig1 was more efficient than that of 0.1 μg/ml, at which Gig1 did not protect the CAB cells against GCRV infection at 0.002 of M.O.I (Fig. 5D; Fig. S4B). In a similar experiment where the challenged GCRV was

at 0.005 of M.O.I, an appreciable inhibition of GCRV replication was also observed when EPC cells were transfected with 0.5 μg/ml of Gig1 (about 2-fold reduction of virus titer against control cells), although no clear inhibition was seen when transfection with 0.1 μg/ml of Gig1 (Fig. 5E and F; Fig. S4C).

Subsequently, two transformants of CAB cells that had been stably transfected with either pTARGET-Gig1 or empty vector pTARGET were obtained. RT-PCR and western blot detection confirmed constitutive expression of ectopic genes (Fig. 5G). Infection with GCRV at 0.004 of M.O.I resulted in about 30-fold reduction of virus titer in Gig1-overexpressed transformant compared to control transformant; about 7-fold difference was observed when GCRV challenged cells at 0.02 of M.O.I (Fig. 5H). To further assess the inhibitory role of Gig1, real-time PCR was used to measure a possible reduction of genome dsRNA (vRNA) of intracellular GCRV. GCRV genome contains 11 dsRNA segments which are divided into three categories based on their sizes: L (large), M (medium) and S (small) (Cheng et al., 2008). We determined the expression of 4 segments: S10, S11, L2, and M6. At 6 h and 12 h post infection, no vRNA or a very weak expression was detected in both GCRV-infected transformants (Fig. 5I). At 24 h and 30 h post infection, vRNAs were easily detected; however, the expression level was significantly lower in the Gig1 transformant than that in the control transformant (Fig. 5I), indicating that the reduction of vRNA transcription was attributable to Gig1. Collectively, these results implied that fish Gig1 is capable of inhibiting GCRV replication.

Gig1 homologs form a fish-specific gene family

Besides crucian carp *Gig1* gene, homology search of EST databases and genome databases identified 18 other homologous genes from 9 ray-finned fish species (Table 1). Using the identified 19 *Gig1* genes as queries, we found 95 sequence hits in the genome databases of 8 ray-finned fish species and 1 lobe-finned fish (coelacanth *Latimeria chalumnae*); however, no additionally annotated genes were found (see Table S2 in Supplemental materials). No homologous sequences were found from all available genome databases of invertebrates, amphibians, reptiles, birds and mammals, particularly from the genome of elephant shark (*C. milii*), a cartilaginous fish species (Venkatesh et al., 2007). Strikingly, there are 8 *Gig1* genes in zebrafish (*D. rerio*) and 3 in tilapia (*O. niloticus*) (Table 1). Therefore, there should be a *Gig1* gene family specific to fish lineages.

Table 1
Gig1 homogloue genes identified from EST databases.

Species	Gene name	Type	Accession number	Protein size
Crucian carp *C. auratus*	*CaGig1*	cDNA	AAP49828.1	194
Grass carp *C. idella*	*CiGig1*	cDNA	ADE73874.1	194
Zebrafish *D. rerio*	*DrGig1A*	Chr 1 and EST	XP_003197778.1	182
	DrGig1B	Chr 1 and EST	XP_687069.2	213
	DrGig1C	Chr 1	XP_002660606.2	221
	DrGig1D	Chr 1	XP_003197780.1	179
	DrGig1E	Chr 1 and cDNA	XP_001336849.1	237
	DrGig1F	Chr 1	XP_002660711.1	306
	DrGig1G	Chr 2 and EST	XP_001343562.1	600
	DrGig1H	Chr 16 and EST	XP_001922525.1	164
Atlantic salmon *S. salar*	*SsGig1*	cDNA	ACH85344.1	224
Nile tilapia *O. niloticus*	*OnGig1A*	Scaffold00136 and EST	XP_003453550.1	175
	OnGig1B	Scaffold00257 and EST	XP_003457740.1	345
	OnGig1C	Scaffold00125 and EST	XP_003452907.1	235
Turbot *S. maximus*	*SmGig1*	cDNA	ABJ98621.1	140
Medaka *O. latipes*	*OlGig1*	NW_004089626.1 and EST	XP_004084664.1	363
Tongue sole *C. semilaevis*	*CsGig1*	cDNA	AFR33114.1	507
Rainbow trout *O. mykiss*	*OmGig1*	cDNA	AF483541_1	171
Grouper *E. coioides*	*EcGig1*	partial cDNA	AEA39726.1	69 (partial)
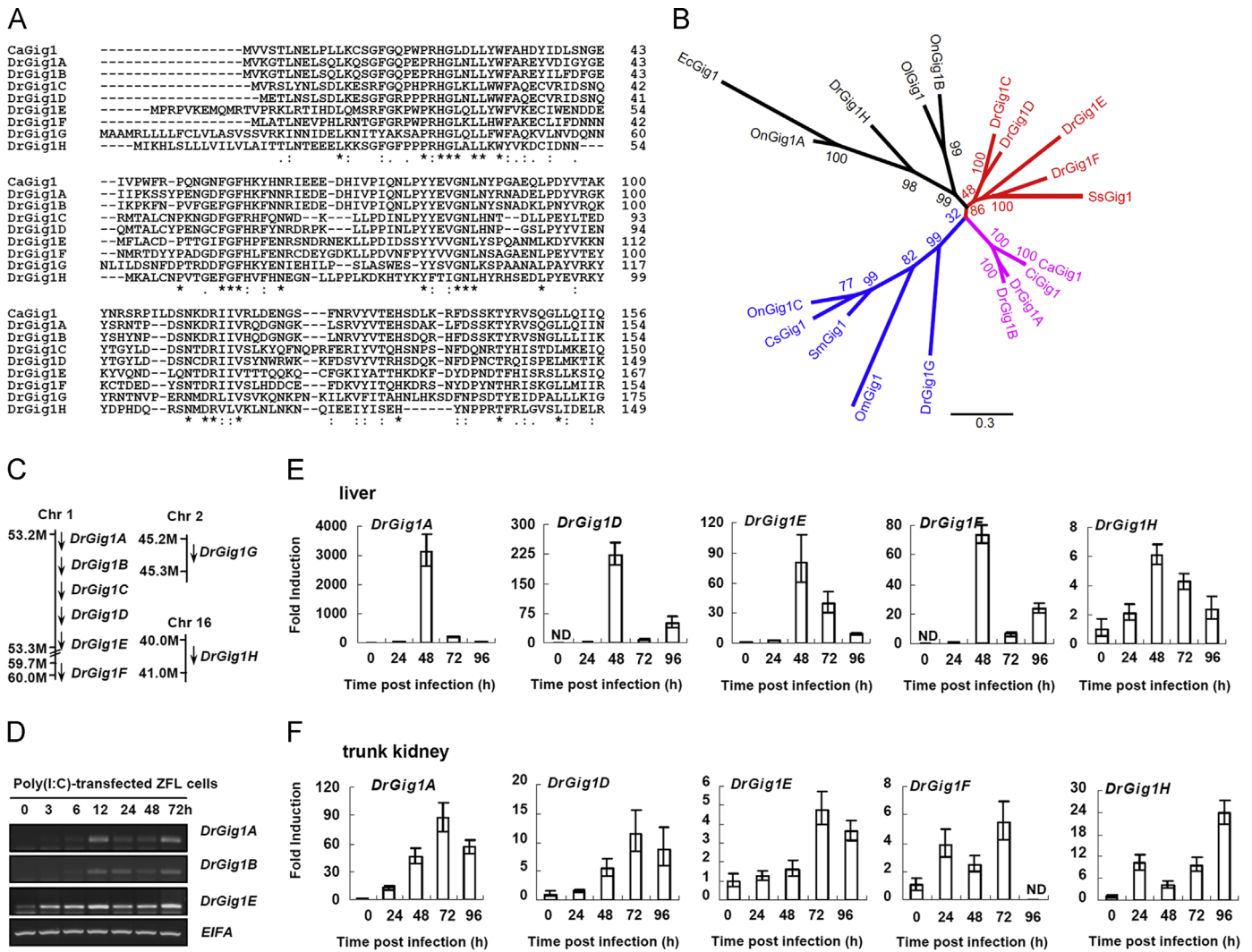
Fig. 6. Characterization of zebrafish *Gig1* gene family. (A) Multiple sequence alignments of crucian carp Gig1 and 8 zebrafish Gig1 homologs by Clustal W. Only the N-terminal regions of Gig1 protein alignments were shown due to no significant homology in their C-terminus. Identical (*) and similar (: or.) amino acid residues were marked. (B) Phylogenetic tree of 19 Gig1 proteins known in fish. A neighbor-joining tree was constructed based on the analysis of the conserved N-terminus sequences using Clustal W and Neighbor-Joining method in Geneious software. The accession numbers were shown in Table 1. The bootstrap value is 1000. (C) Schematic representation of gene loci of zebrafish Gig1 family genes. (D) Expression profile of zebrafish *Gig1* genes in cultured cells. ZFL cells seeded in 35 mm dishes overnight were transfected with 2 μg poly(I:C). Then the cells were harvested at the indicated time points to detect zebrafish *Gig1* transcripts by RT-PCR. (E and F) Expression profile of zebrafish *Gig1* genes in tissues. Adult zebrafish was intraperitoneal injected with SVCV at $1 \times 10^8 TCID_{50}/20\ \mu l$ per fish. At each indicated time, 3 fishes were dissected and several tissues, such as liver (E) and trunk kidney (F), were extracted to detect the transcriptional expression levels of 8 zebrafish *Gig1* genes.

These putative 19 fish Gig1 proteins vary in sizes from 140 to 600 amino acids (Table 1), and share sequence homology mainly in the N-terminal region (the first ∼150 amino acids) (Fig. 6A and data not shown). Search of NCBI's conserved domain database using each Gig1 protein sequence did not find any known domains, even in the conserved N-terminus. Phylogenetic analysis showed that these 19 proteins were clustered into four major subgroups (Fig. 6B), which corresponded to chromosome location of 8 zebrafish Gig1 members that are distributed in 3 different chromosomes (Fig. 6C). 6 members in zebrafish chromosome 1 were grouped into two clades (one including *DrGig1A* and *DrGig1B*, and the other including *DrGig1C*, *DrGig1D*, *DrGig1E*, *DrGig1F*), but *DrGig1G* in chromosome 2 and *DrGig1H* in chromosome 1, together with the other fish *Gig1* members, formed another two clades, respectively (Fig. 6B and C). Similarly, 3 tilapia members were clustered into two subgroups, respectively (Fig. 6B). These results illustrated protein sequence divergence of *Gig1* family members during radiation of fish species.

To determine whether there was function divergence of *Gig1* family genes, the expression of 8 zebrafish *Gig1* genes was investigated. Only *DrGig1E* was constitutively expressed in cultured ZFL cells;

poly(I:C) transfection resulted in a dramatically transcriptional upregulation of *DrGig1A*, *DrGig1B* and *DrGig1E* but not of the other 5 genes (Fig. 6D). Detection of zebrafish tissues revealed a differentially constitutive expression of 8 genes. Although both *DrGig1A* and *DrGig1B* were orthologous to crucian carp *Gig1* (Fig. 6B), *DrGig1A* other than *DrGig1B* was constitutively expressed in zebrafish liver and trunk kidney (Fig. 6E and F). In response to SVCV, only five genes including *DrGig1A*, *DrGig1D*, *DrGig1E*, *DrGig1F* and *DrGig1H* were transcriptionally induced in liver and trunk kidney (Fig. 6E and F) and also in head kidney (Fig. S5A). The transcripts of *DrGig1C* and *DrGig1G* were not detected in liver and trunk kidney, but *DrGig1G* mRNA was detectable in SVCV-infected head kidney (Fig. S5B). These results indicated a cell- and tissue-specific regulation of 8 zebrafish *Gig1* family genes.

Discussion

In the present study, we identified fish *Gig1* as a typical ISG by a line of evidence. First, IFN directly induces the expression of

crucian carp Gig1 through the Stat1 pathway. Second, crucian carp *Gig1* bears a typical ISG promoter, the ISRE motif of which contributes directly to IFN induction. Third, both extracellular poly(I:C) and intracellular poly(I:C) are able to upregulate Gig1 expression, which is consistent with the finding that they both are able to induce fish IFN activities (Yu et al., 2010). Finally, intracellular poly(I:C) induction of Gig1 is mediated by RLR signaling and dependent on the production of IFN as intermediates. Based on the recent studies (Matsuo et al., 2008; Zhang and Gui, 2012), extracellular poly(I:C) induction of Gig1 is likely mediated through the TLR pathway although this is needed for determination. Therefore, once upon infection, fish Gig1 is upregulated to exert biological actions in IFN antiviral response.

Obviously, fish Gig1 represents a novel ISG with antiviral effects, since it encodes a protein with no similar sequences to other proteins and no domains to be known so far. Generally, overexpression strategy is effective to test antiviral ability of ISGs in mammals (Everitt et al., 2012; Raychoudhuri et al., 2011; Schoggins et al., 2011) and also in fish (Biacchesi et al., 2009; Larsen et al., 2004; Liu et al., 2011; Zhu et al., 2008). According to this strategy, two expression vectors (pcDNA3.1 and pTARGET) were used to overexpress Gig1 by either transient transfection or stable transfection, and similar results were observed (Fig. 5). Dose-dependent antiviral effects were seen in both fish cell lines compared to overexpression of empty vectors. Therefore, there is no doubt that fish Gig1 functions as an antiviral effector downstream of IFN signaling, even though we failed to design antisense RNA to test whether blockade of Gig1 expression enables fish cells highly susceptible to GCRV infection (data not shown). Notably, fish Gig1 antiviral effects seemed to become weak when cells were challenged with much higher titer of GCRV (Fig. 5A and B) or when the amount of transfected Gig1 was not enough (Fig. 5C and D). This is likely because IFN-mediated protection often requires a combinatorial action of multiple ISGs (Carlton-Smith and Elliott, 2012; Raychoudhuri et al., 2011; Schoggins et al., 2011). In some cases, a single ISG is essential to defend the host against virus attack after it is upregulated up to some extent, such as the *IFITM* gene alone against influenza virus in human and mouse (Everitt et al., 2012). Fish IFNs also have to be upregulated to some extent before they can efficiently defend cells against viral challenge (Yu et al., 2010). Whereas it is noted that antiviral trend is observed in dose-dependent assays showing that overexpression of fish Gig1 can defend cells against virus infection (Fig. 5B and D), sometimes it is hard to get very consistent fold reduction of virus replication under similar conditions. For example, two similar assays showed 12- and 5-fold reduction of GCRV titers in Gig1-overexpressed CAB cells compared to control cells under GCRV challenge at 0.002 of M.O.I. (Fig. 5B and D). The difference is likely due to the fact that the obvious antiviral effect of Gig1 needs high expression; however, transfection efficiency in fish cells is not stable and much lower than that in mammalian cells, which might make it hard to get a consistent fold reduction of virus replication in different transfection experiments.

Indeed, crucian carp *Gig1* mRNA seemed to be the most abundant virus-induced transcript during screening of a suppressed subtractive cDNA library (Zhang and Gui, 2004; Zhang et al., 2003), highlighting the pivotal role of *Gig1* in IFN antiviral response. The data in the present study further showed that crucian carp Gig1 protein was almost undetectable in normal fish cells, but was dramatically upregulated by either IFN or IFN stimuli, to a higher level than a well-characterized IFN-inducible protein Mx (Fig. 1C–F), whose induction is always thought as the hallmark of IFN response in mammals and fish (Altmann et al., 2004; Sadler and Williams, 2008; Schoggins and Rice, 2011). In addition, immunofluoresence microscopy revealed a rapid induction of Gig1 protein in response to poly(I:C) transfection in CAB cells, starting earlier than 6 h post transfection (Fig. 4). The rapid and abundant expression might be helpful for Gig1 to quickly exert its antiviral effects when host cells respond to virus infection.

A question raised in this study is how fish Gig1 controls GCRV infection. A possibility is that Gig1 works as a signal factor to augment IFN response, since we can not exclude a possibility that fish Gig1 exerts a regulatory function in a cell type- or tissue-specific manner, like viperin, which not only mediates an IFN-activated antiviral pathway but also regulates type I IFN expression in plasmacytoid dendritic cells (pDCs) by TLR7/9 pathway (Saitoh et al., 2011). Another useful clue comes from the subcellular localization analysis, showing cytoplasmic distribution of fish Gig1 in the absence or presence of poly(I:C) treatment or UV-inactivated GCRV infection (Fig. 4). This means that fish Gig1 should play its antiviral role in the cytoplasm. GCRV is a tentative member in the genus Aquareovirus of family Reoviridae (Cheng et al., 2008), and thus its replication and assembly are thought to occur in the cytoplasm of infected cells (Kobayashi et al., 2009). Nonetheless, we do not know which one of the essential steps of GCRV life cycle is targeted by fish Gig1. Considering that ISGs can modulate any step of the viral life cycle, including entry, uncoating, genome replication, particle assembly and egress (Schoggins and Rice, 2011), the observation that lower levels of GCRV genome dsRNA are detected in Gig1-overexpressed cells indicates that fish Gig1 might function at a step that at least precedes assembly of GCRV particles. Although further studies are needed to clarify which step is exactly targeted, it is possible that fish Gig1 influences GCRV protein synthesis, because most viral RNA produced in a reovirus-infected cell is the product of secondary (progeny SVP) gene expression that is dependent upon viral protein synthesis (Ooms et al., 2012).

Another important finding in the present study is the identification of a novel gene family that consists of genes homologous to *Gig1*, which is found only in teleost fish including ray-finned fish species and lobe-finned fish species but not in cartilaginous fish. Gig1 family members vary in protein size but share ~150 N-terminal amino acids stretch conserved to some extent (Fig. 6A); therefore, the N-terminus might not only be unique to this gene family but also essential for their biological function. Notably, some members with the similar protein size display high level of homology through their whole protein sequences, such as crucian carp Gig1 and zebrafish Gig1A and Gig1B. This conservation gives a predication that they might exhibit a conserved expression pattern and function. In fact, zebrafish *Gig1A*, similar to carp *Gig1*, was induced significantly not only in cultured cells but also in most tissues (Fig. 6D–F, Fig. S5). However, zebrafish *Gig1B* was expressed in cultured fish cells but not in selected tissues including liver, head and trunk kidney, and zebrafish *Gig1C* was not detected in normal and virus-infected cells or tissues. Therefore, *Gig1* family genes should have diverged in their biological function during evolution. Given that all 8 zebrafish genes retained the same antiviral function, the different expression patterns still suggest a cell- or tissue-specificity of biological effects. Similar findings are also found in mammals. For example, mammalian IFIT family members exhibit different tissues-induction patterns and function (Fensterl et al., 2008; Terenzi et al., 2006); IFIT2 protects mouse against VSV in brain but not in other organs, and even in the brain, it targets neurons but not the other cell types (Fensterl et al., 2012); viperin contributes to restrict WNV replication mainly in the central nervous system of mouse (Szretter et al., 2011). These results suggest the mechanism complexity of IFN antiviral action.

The results presented here identify a novel antiviral effector involved in fish IFN response. The substantial data on induction regulation support the notion that fish have developed conserved RLR signaling pathways to trigger IFN expression and subsequently

to activate a given ISG expression counteracting viral invasion. During review of our manuscript, Sun et al. showed that a grass carp Gig1 homolog is able to defend cultured CIK cells against GCRV infection (Sun et al., 2013). However, the authors failed to detect IFN-induced transcription of grass carp Gig1 in CIK cells in the presence or absence of cyclohemide, which is inconsistent with our results. Although we do not know the reason, previously also we did similar experiments showing that cycloheximide treatment blocked the induction of crucian carp Gig1 by both poly(I:C) and GCRV but not by IFN (Zhang and Gui, 2004; Zhang et al., 2007). The data in the present study further support a notion that crucian carp *Gig1* is a typical ISG. To our knowledge, *Gig1* is the first fish ISG which is well-characterized in expression regulation. The specificity of *Gig1* to fish species illustrates that fish IFN antiviral response has self-specific characteristics. It is intriguing why this gene family is found only in some fish species, or why and how this gene family is lost in reptiles, birds and mammals if this gene family actually existed in a vertebrate ancestor. Further studies on function clarification of *Gig1* gene family will aid in answering this question and also in understanding evolution of the IFN antiviral system in vertebrates.

Materials and methods

Plasmids and antibodies

Two wild type *Gig1* constructs, pcDNA3.1-Gig1 and pTARGET-Gig1, were generated by cloning the ORF of crucian carp *Gig1* (GeneBank accession no. AY293927) into *Nhe* I/*Not* I sites of pcDNA3.1(+) (Invitrogen) or inserting the cDNA fragment of *Gig1* (nucleotides 9-1051) into pTARGET (Promega). The empty vector pTARGET was obtained from pTARGET-Gig1 by digestion of *EcoR* I followed by self-ligation. Other constructs including RIG-I, Mda5, LGP2, MITA, TBK1, IRF3, MITA-mut, TBK1-K38M, IRF3-DN and Stat1-ΔC were previously described (Sun et al., 2011; Yu et al., 2010). Promoter-driven luciferase construct Gig1pro-Luc was made by cloning a 377-base-pair stretch of 5′-flanking regulatory region of *Gig1* (between −361 and +34) in front of a luciferase gene in pGL3-Basic (Promega). ISRE-mut was modified from Gig1pro-Luc by overlapping extension PCR with mutation altered primers. A prokaryotic expression construct was made by cloning *Gig1* ORF into pGEX-KG. The purified recombinant Gig1 fusion protein was made to generate rabbit polyclonal antibodies according to previous reports (Jiang et al., 2009; Yu et al., 2010). The recombinant crucian carp IFN (rIFN) and anti-IFN serum were described in a previous study (Yu et al., 2010).

Cells, virus

Crucian carp (*Carassius auratus*) blastulae embryonic (CAB) cells and epithelioma papulosum cyprinid (EPC) cells were maintained at 28 °C in medium 199 supplemented with 10% fetal calf serum (FCS). Zebrafish (*Danio rerio*) liver (ZFL) cells were cultured according to a previous study (Li et al., 2012b). Two stable transformant mixtures were obtained by transfection of CAB cells with either pTARGET-Gig1 or empty vector pTARGET, followed by selection with 400 μg/ml Geneticin (G418, Amersco). Stat1-ΔC-overexpressed transformants were previously described (Sun et al., 2010). Grass carp reovirus (GCRV), a dsRNA virus, and spring viremia of carp virus (SVCV), a negative ssRNA virus, were propagated and titered by TCID$_{50}$ assays or by plaque-forming unit assays, in CAB cells and EPC cells, respectively.

Induction, transfection, and luciferase assays

For induction, cells seeded overnight in 6- or 24-well plates were treated directly with UV-inactivated GCRV, poly(I:C), rIFN, or transfected with poly(I:C). Transfection by Lipofectamine 2000 (Invitrogen) and luciferase assays were performed according to our previous studies (Sun et al., 2010, 2011). All experiments were performed in triplicates and repeated at least three times.

Fish infection assays

Zebrafish were maintained with a controlled light cycle of 14 h light/10 h dark at 28 °C. Adult zebrafish were injected intraperitoneally with SVCV (1×10^8 TCID$_{50}$/20 μl each). At every indicated time point, a group of zebrafish ($N=3$) were anesthetized. Several tissues, including liver, head kidney and trunk kidney, were sampled and homogenized for RT-PCR detection of indicated gene transcripts.

Immunofluorescence microscopy and western blotting

Immunofluorescence analysis was used to analyze the expression and subcellular localization of endogenous Gig1 protein. Briefly, CAB cells plated onto glass coverslips in 6-well plates overnight were treated by UV-inactivated GCRV (1×10^9 TCID$_{50}$/ml exposed to UV) or transfected with 0.5 μg/ml poly(I:C). 48 h later, cells were fixed with 4% paraformaldehyde solution in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, blocked with 10% goat serum for 1 h, followed by incubation with primary antibody dissolved in 10% goat serum for 2 h. After washed three times in 1% goat serum, cells were incubated with secondary antibody for 2 h in dark, subsequently washed three times in 1% goat serum. The nuclei were stained by PI or DAPI. The slides were viewed under a confocal microscope (Leica).

Western blot analysis was performed according to our previous studies (Sun et al., 2010, 2011). The anti-Gig1 antiserum was diluted at 1:1000 for western blot analysis and 1:200 for immunofluorescence. Other antibodies used in this study were: anti-actin (Santa Cruz Biotechnology) at 1:1000; HRP-conjugated goat anti-rabbit IgG at 1:5000 and HRP-conjugated goat anti-mouse IgG at 1:2000 (Pierce); FITC-conjugated goat anti-rabbit antibody (Pierce) at 1:100.

Antiviral effect evaluation

Cytopathetic effect (CPE) reduction assays and virus yield reduction assays were used to analyze the antiviral effects of Gig1 according to our previous reports (Yu et al., 2010). Briefly, CAB cells or EPC cells seeded in 24-well plates were transfected by wild type Gig1 vectors followed by GCRV challenge. At the indicated times, the supernatant aliquots were harvested for detection of virus titers by plaque-forming unit assays, and the cell monolayer was fixed for CPE observation.

Real time-PCR was included to detect amplification of GCRV genome according to previous studies (Li et al., 2012a). Total RNAs were extracted by the SV Total RNA Isolation System (Promega). First-strand cDNA was synthesized using random primers and M-MLV reverse transcriptase (Promega). Real-time PCR was done on a Bio-Rad CFX96™ Real-Time system with iQ™ SYBR® Green Supermix (Bio-Rad). All samples were analyzed in triplicate and relative RNA levels were calculated after normalization to β-actin. The primers were designed against the different segments of GCRV, including S10 (GenBank accession no. AF403396), S11 (AF403397), capsid protein VP2 in L2 (AF284502) and an outer capsid protein VP5 gene in M6 (AF239175). All primers used in this study are shown in Supplementary Table S1.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.10.029.

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