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# Fish MITA Serves as a Mediator for Distinct Fish IFN Gene Activation Dependent on IRF3 or IRF7

Fan Sun, Yi-Bing Zhang, Ting-Kai Liu, Jun Shi, Bing Wang, and Jian-Fang Gui

**In mammals, cytosolic sensors retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) activate multiple signaling cascades initiating IFN- $\alpha/\beta$  expression. IFN regulatory factor 3 (IRF3) is required for the activation of IFN- $\beta$ , which, in turn, primes the expression of most IFN- $\alpha$  genes by IFN-induced IRF7 through the STAT1 pathway. In fish, RIG-I overexpression inhibits virus infection by induction of IFN response; however, the subtle signaling cascade mechanism remains to be identified. In this study, we clone an ortholog of MITA, a recently identified adaptor responsible for RLR pathway, from crucian carp (*Carassius auratus* L.), and demonstrate its ability to suppress viral replication through IRF3/7-dependent IFN response. The pivotal signaling molecules of RLR pathway, including RIG-I, melanoma differentiation-associated gene 5, laboratory of genetics and physiology 2, and TANK-binding kinase 1, are also cloned and characterized, confirming that the RLR-mediated IFN activation is conserved from fish to mammals. Further characterization of distinct IFN gene activation reveals that zebrafish IFN1 and IFN3 are induced by the MITA pathway but are dependent on distinct transcription factors. Whereas fish IFN genes cannot be classified into IFN- $\alpha$  or IFN- $\beta$ , zebrafish IFN1 is primarily regulated by IRF3, thereby resembling that of IFN- $\beta$ , and zebrafish IFN3 is regulated by IRF7, thereby resembling those of IFN- $\alpha$ s. In contrast with mammalian IFN- $\alpha/\beta$ , zebrafish IFN1 and IFN3 are induced by the basally expressed IRF3 or IRF7, both of which are upregulated by IFN and virus infection. Collectively, these data suggest that IFN genes in fish and mammals have evolved independently to acquire a similar mechanism triggering their expression. *The Journal of Immunology*, 2011, 187: 2531–2539.**

**I**n mammals, the activation of type I IFNs (primarily including IFN- $\alpha/\beta$ ) are initiated through the recognition of viral products by host pattern recognition receptors including retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and TLRs (1). Distinct from TLRs that sense virus motifs present at the cell surface or within the endosomal compartment in immune cell lineages, RLRs recognize cytosolic viral component in most cell types. The RLR family comprises three cytoplasmic receptors: RIG-I, mel-

anoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (2–4). Both RIG-I and MDA5 harbor two N-terminal caspase recruiting domains (CARDs), a central DexD/H-box RNA helicase domain (HD), and a C-terminal regulatory domain (RD), but LGP2 does not contain CARDs within the N terminus (3). In response to RNA virus infection, the N-terminal CARDs of RIG-I and MDA5 interact with the CARD of mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, and Cardif) that subsequently recruits and activates cytosolic protein kinases TANK-binding kinase 1 (TBK1) and canonical IKK complex (IKK $\alpha/\beta/\gamma$ ), which enable the transcription factors IFN regulatory factor 3 (IRF3) and NF- $\kappa$ B together to translocate into the nucleus, turning on the transcription of IFN- $\beta$  (1). According to the current model (5–7), IFN- $\beta$  is the early-phase IFN that is regulated by IRF3, and most IFN- $\alpha$  genes are late-phase IFNs, which are transcriptionally activated by IFN-induced IRF7 through the STAT1 pathway.

Recently, mediator of IRF3 activation (MITA; also known as STING, ERIS, and MYPS), an endoplasmic reticulum (ER) resident transmembrane protein, is identified as an adaptor to link the signaling transduction between MAVS and downstream cytosolic kinase TBK1 (8–10). In response to vesicular stomatitis virus and Sendai virus, MITA triggers RIG-I-mediated IFN- $\beta$  induction downstream of MAVS and upstream of TBK1 and IRF3 (8, 9). Consistently, MITA-deficient mice severely impair MAVS-mediated IFN response and are exquisitely sensitive to virus infection (9). However, loss of MITA does not affect IFN- $\beta$  induction in response to synthetic dsRNA polyinosinic:polycytidylic acid [poly(I:C)] and encephalomyocarditis virus, which elicit an MDA5-dependent response (9, 11). In addition to the role in response to RNA viruses, MITA also links cytosolic DNA-mediated signaling to TBK1 and IRF3 activation, leading to initiation of IFN- $\beta$  (9, 11). Therefore, MITA is likely the junction adaptor molecule that integrates both DNA and RNA signaling pathways in the cytosol.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CAB, crucian carp (*Carassius auratus* L.) blastulae embryonic cell; CARD, caspase recruiting domain; CPE, cytopathic effect; EPC, epithelioma papulosum cyprini; ER, endoplasmic reticulum; GCRV, grass carp reovirus; HD, helicase domain; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE/IRF-E, IFN-stimulated regulatory element/IRF-binding element; LGP2, laboratory of genetics and physiology 2; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; MITA, mediator of IRF3 activation; ORF, open reading frame; poly(I:C), polyinosinic:polycytidylic acid; RD, regulatory domain; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor; SVCV, spring viremia of carp virus; TBK1, TANK-binding kinase 1; TM, transmembrane motif.

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The past several years have witnessed tremendous advances in understanding of fish IFN response. Similar to mammals, fish appear to possess the functional TLR pathway, because overexpression of either fugu TLR3 or TLR22 activates IFN expression (12). This notion is supported by the findings that fish TBK1 interacts with the N terminus of TICAM1 (13), and that both fish IRF3 and IRF7 are necessary for expression of IFN-stimulated genes (ISGs) by extracellular poly(I:C) (14). Fish genome also contains pivotal signaling molecules involved in the RLR signaling pathway, including three RLR family members RIG-I, MDA5, and LGP2 (15), MAVS (16–18), and TBK1 (13). Overexpression of either fish RIG-I or MAVS induces powerful antiviral activity, possibly because of their abilities to induce the expression of IFN and ISGs (16). However, little is known about whether fish MITA participates in RLR signaling pathway, and the role of fish TBK1 in IFN production remains elusive. In addition, fish virus-induced IFNs, now classified into group I and group II IFNs (19–23), act through distinct receptors that are different from mammalian type I IFN receptors (22), and interestingly, some of them are able to regulate the expression of themselves (21, 24), by the conserved STAT1 pathway (23). These striking differences pose the question whether both groups of fish virus-induced IFN genes are induced through similar or distinct signaling transduction pathways.

In this study, we report a characterization of a fish MITA ortholog from crucian carp (*Carassius auratus* L.) and demonstrate its ability to suppress viral replication through activating IRF3/7-dependent IFN response. Further characterization of some pivotal signaling molecules including three RLR family members and TBK1 shows that fish MITA functions in the RIG-I/MDA5-mediated IFN pathway and upstream of TBK1 and IRF3, demonstrating that the RLR-mediated IFN activation is conservative from fish to mammals. Finally, we provide evidence that, whereas fish virus-induced IFN genes are not the true orthologs of mammalian IFN- $\alpha/\beta$  (19–23), they are regulated in a way similar to IFN- $\alpha/\beta$ , through the MITA pathway but dependent on distinct transcription factors. These findings provide essential perspective into the evolutionary differentiation of fish MITA-mediated IFN activation.

## Materials and Methods

### Cells, transfection, and viruses

Crucian carp (*Carassius auratus* L.) blastulae embryonic cells (CABs) and epithelioma papulosum cyprini (EPC) cells were cultured at 28°C in medium 199 supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. COS7 cells were maintained at 37°C, 5.0% CO<sub>2</sub> in DMEM supplemented with 10% FCS. Transfection was performed according to a previous report (14). In brief, CABs seeded in 6-well plates overnight were transfected with the mixture containing 1.6 µg plasmids and 4 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 1 ml FCS-free 199 medium per well. At 6 h posttransfection, the mixture was replaced with 2 ml fresh medium. Grass carp reovirus (GCRV), a dsRNA virus, and spring viremia of carp virus (SVCV), a negative ssRNA virus, were propagated in CABs and EPCs, respectively, according to previous reports (25). Virus titers were measured using the 50% tissue culture infection dose method. The recombinant crucian carp IFN was produced by a prokaryotic expression system according to a previous study (23).

### Gene cloning and plasmids

Expression sequence tags of crucian carp MITA, TBK1, RIG-I, MDA5, and LGP2 were retrieved from a suppressed subtractive cDNA library (25, 26). RACE-PCR was used to clone the full-length cDNAs. For eukaryotic expression, full-length and N-terminal truncated open reading frame (ORF) of MITA were inserted into pCMV-Tag4B or pcDNA3.1/myc-His(-) A vectors (Invitrogen, Carlsbad, CA). The other ORFs (crucian carp RIG-I, MDA5, and TBK1, zebrafish IRF3 and IRF7) were subcloned into pcDNA3.1/myc-His(-) A vector (Invitrogen). The ORF of LGP2 was subcloned into pRK-Flag vector, and the two LGP2 mutants LGP2-RD and LGP2-HD were made by insertion of the RD (aa 461–680) and the HD (aa 1–507) of LGP2 into pRK-Flag, respectively. The TBK1 mutant (TBK1-

K38M) was generated by designing altered primers with mutation by splicing by overlapping extension PCR. For subcellular localization, the whole ORF of MITA was inserted into pEGFP-N3 vector (Clontech, Mountain View, CA). The indicated 5'-flanking regulatory sequences of zebrafish virus-induced IFNs were cloned and inserted into pGL3-Basic luciferase reporter vector (Promega, Madison, WI) to analyze the promoter activity. All constructs were verified by sequencing analysis. Crucian carp IRF3, IRF3-DN, IRF7, IRF7-DN, STAT1- $\Delta$ C, and crucian carp IFNpro-Luc were previously described (14). Human NF- $\kappa$ B luciferase reporter plasmid was purchased from Clontech (Mountain View, CA). The primers including the restricted enzyme cutting sites used for constructs are listed in Supplemental Table I.

### Fluorescence microscopy and luciferase activity assay

For fluorescence microscopy, CABs were plated overnight on microscopic coverglass in six-well plates. After being washed three times by FCS-free medium, cells were cotransfected with pDsRed2-ER and pEGFP-MITA or empty vector at a ratio of 1:1 using Lipofectamine 2000 (Invitrogen). After 24 h posttransfection, the transfected cells were rinsed twice with PBS followed by fixation with 4% (v/v) paraformaldehyde for 15 min at room temperature. Then cells were examined under a confocal microscope (Leica).

For luciferase activity assays, CABs seeded in 24-well plates overnight were cotransfected with various constructs at a ratio of 10:10:1 (expression vectors of RIG-I/MDA5/LGP2/TBK1/IRF3/IRF7, IFNpro-Luc, pRL-TK) using Lipofectamine 2000 (Invitrogen). At 48 h posttransfection, the cells were harvested and lysed according to the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were measured by a Junior LB9509 luminometer (Berthold, Pforzheim, Germany) and normalized to the amounts of Renilla Luciferase activities. The results were representative of more than three independent experiments, each performed in triplicate.

### Antiviral effect evaluation

CABs or EPC cells seeded in 24-well plates were transfected with 0.5 µg wild type MITA construct or empty vector (pcDNA3.1). Twenty-four hours later, the transfected cells were washed twice and then infected with 1000 TCID<sub>50</sub> of GCRV or SVCV per well, respectively. At 48 h postinfection, the supernatant aliquots were harvested for detection of virus titers according to previous reports (23, 25–27). The infected cells were washed with PBS, fixed by 30% formaldehyde for 30 min, stained by 1% (w/v) crystal violet for 30 min, and observed for cytopathic effect (CPE).

### Abs, coimmunoprecipitation, and Western blotting

Mouse mAbs against Flag and Myc were purchased from Sigma. Mouse polyclonal anti-actin was from Santa Cruz Biotechnology. Rabbit polyclonal anti-crucian carp IRF3 antiserum and mouse polyclonal anti-crucian carp Mx1 antiserum were made by immunization of animals with prokaryotic expressed proteins of IRF3 and Mx1, according to previous reports (14).

For Western blotting, equal amounts of protein extracts were separated on 12 or 15% SDS-PAGE gels and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked in freshly prepared TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature, incubated with primary Ab in TBST buffer containing 1% milk overnight at 4°C, washed three times with TBST, each for 10 min, and then incubated with secondary Ab for 1 h at room temperature. After another three 10-min washes with TBST buffer, the membrane was stained with ECL system.

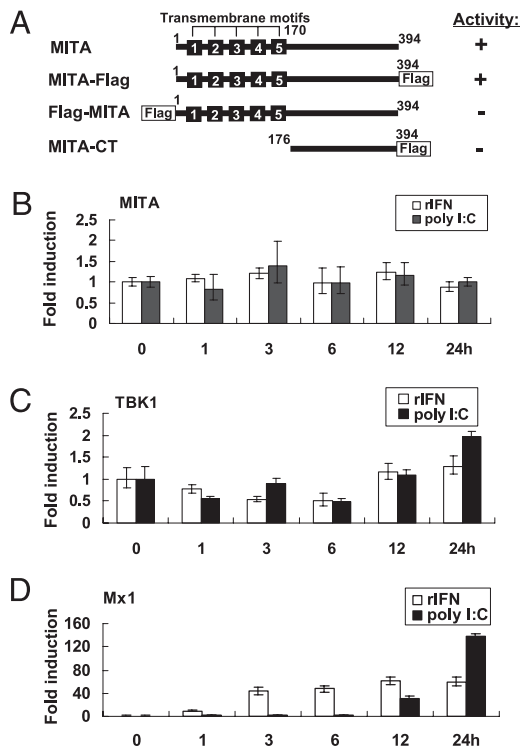
For coimmunoprecipitation experiments, COS7 cells seeded in 10-cm dishes were transfected with a total of 10 µg indicated plasmids. At 24 h posttransfection, the medium was removed carefully, and cell monolayer was washed twice with 10 ml ice-cold PBS. Then cells were lysed in 1 ml radioimmunoprecipitation buffer containing protease inhibitors at 4°C for 30 min on a rocker platform. The cells were collected by cell scraper, and the cellular debris was removed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was transferred to a fresh tube and incubated with anti-Flag (or anti-myc) agarose conjugate (Sigma) overnight at 4°C. The beads were washed with ice-cold PBS four times and eluted with 40 µl 2× SDS sample buffer by boiling for 5 min at 95°C. The precipitates were detected by immunoblotting with indicated Abs.

## Results

### Identification of fish MITA and TBK1 orthologs in crucian carp

By searching a subtractive cDNA library made from UV-inactivated, GCRV-infected CABs (25, 26), we retrieved expression sequence tags homologous to mammalian MITA. RACE-PCR was used to

obtain the full-length cDNA of crucian carp MITA consisting of 1416 bp with an ORF encoding a 394-aa protein (GenBank accession no. JF970229). Similar to mammalian orthologs (8, 9), fish MITA harbors five predicted transmembrane motifs (TMs) and a putative signal cleavage motif at the corresponding regions within its N terminus (Fig. 1A, Supplemental Fig. 1A). Multiple sequence alignment reveals that TM5 is most conserved (Supplemental Fig. 1A). Crucian carp MITA shows the highest sequence identity and similarity to zebrafish MITA (76 and 84%, respectively), which is supported by a phylogenetic analysis (Supplemental Fig. 1B). Similar methods were introduced to clone and identify crucian carp TBK1 (GenBank accession no. JF970228). Sequence analysis indicates that all vertebrate TBK1 proteins are highly conserved (76% identity and 89% similarity between fish and human TBK1; Supplemental Fig. 1C). Moreover, fish MITA and TBK1 exhibited the similar expression properties to mammalian orthologs, being constitutively expressed in fish cells and not induced by either rIFN treatment or poly(I:C) transfection (Fig. 1B, 1C). In contrast, fish Mx1 was significantly upregulated under the same conditions (Fig. 1D).



**FIGURE 1.** Identification of crucian carp MITA and TBK1 orthologs. *A*, Schematic representation of crucian carp MITA variants used in this study. The five putative TMs are indicated within its N terminus. Attachment of a Flag tag to the C terminus of MITA (MITA-Flag) does not impair its activity on IFN promoter, whereas the activity is lost when MITA is tagged at the N terminus (Flag-MITA), similar to MITA-CT that deletes the N-terminal region of wild type MITA. The activities of these vectors are illustrated in Fig. 6. *B–D*, Real-time PCR detection of mRNA level of MITA, TBK1, and Mx1 on stimulation. CABs seeded in six-well plates were treated with 5 ng/ml rIFN (white bars) or transfected with 2  $\mu$ g/ml poly(I:C) (black bars) and were sampled at various times. The transcriptional expression of MITA (*B*), TBK1 (*C*), and Mx1 (*D*) was detected by quantitative RT-PCR. The relative expression was normalized to the expression of  $\beta$ -actin and represented as fold induction relative to the expression level in control cells that was set to 1. Error bars represent SDs obtained by measuring each sample in triplicate.

### Fish MITA inhibits virus replication through activation of IFN response

To determine the physiological function of fish MITA, we initially investigated the subcellular localization of fish MITA by transfection of CABs with two constructs, pEGFP-MITA and pDsRed2-ER, that encode the fusion protein MITA-EGFP and KDEL-DsRed2, respectively. Confocal microscopy examination revealed colocalization of MITA-EGFP and DsRed2-ER, indicating that fish MITA is an ER protein (Fig. 2A, upper panel). In contrast, cells transfected with pEGFP-N3 and pDsRed2-ER did not give an overlapping image (Fig. 2A, lower panel).

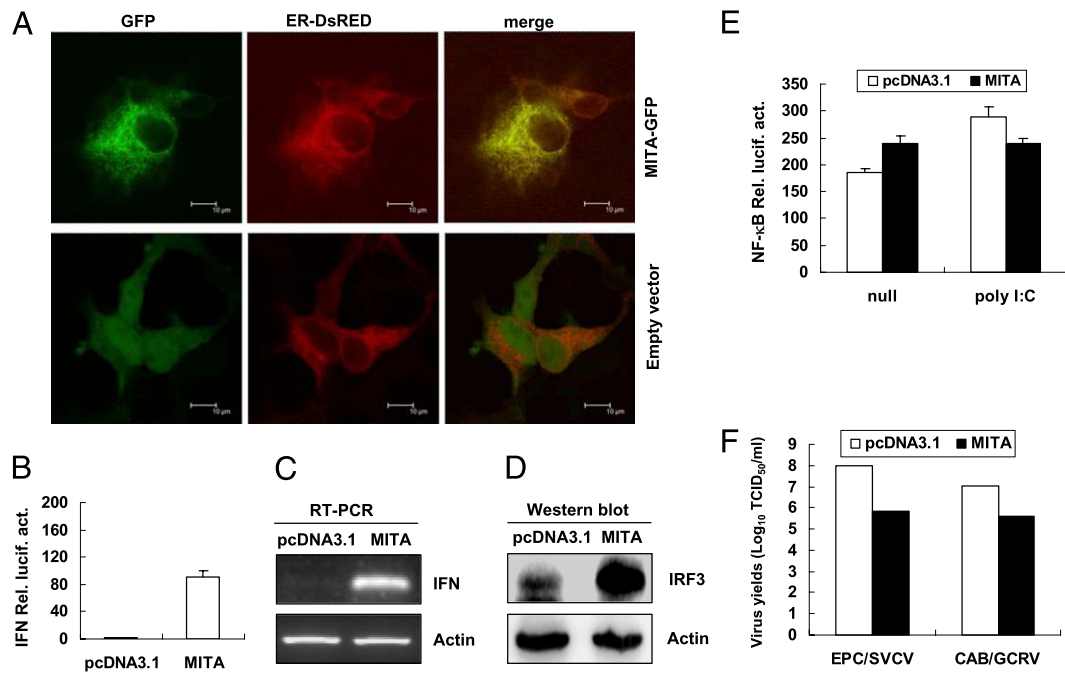
Subsequently, luciferase assays were used to evaluate the expression of IFN by cotransfection of CABs with wild type MITA construct and IFNpro-Luc, a crucian carp IFN mini promoter-driven luciferase construct (14). Overexpression of MITA gave a strong activation of IFNpro-Luc by up to 50-fold against that of pcDNA3.1 (Fig. 2B) and induced a significant increase in fish IFN mRNA (Fig. 2C), as well as in fish IRF3 protein (Fig. 2D), an indicator of fish IFN-inducible proteins (14), demonstrating that fish MITA is capable of activating fish IFN response. However, overexpression of wild type MITA did not induce the activation of NF- $\kappa$ B even in treatment with poly(I:C) (Fig. 2E).

Finally, the role of fish MITA in cellular antiviral response against virus infection was further examined. EPC cells were transfected with wild type MITA or empty vector as a control followed by infection with SVCV. A 48-h infection with SVCV resulted in broad CPE in control cells, whereas no obvious CPE was observed in MITA-transfected cells (data not shown). Consistently, a virus titer of  $6.8 \times 10^5$  TCID<sub>50</sub>/ml was detected in the supernatant from MITA-overexpressing cells, which was a >140-fold reduction relative to control cells ( $10^8$  TCID<sub>50</sub>/ml; Fig. 2F). In a similar experiment, infection with GCRV revealed up to a 27-fold reduction of virus titer in MITA-transfected cells (Fig. 2F).

### Fish MITA activates IFN response via MITA-TBK1-IRF3 signaling pathway

In mammals, TBK1 and IRF3 are involved in MITA-mediated IFN signaling (8, 9). To understand the mechanism underlying fish MITA-mediated IFN response, in initial experiments, we transfected CABs with constructs expressing fish TBK1 and IRF3, respectively, and examined the expression of IFN and two IFN-inducible proteins Mx1 and IRF3. As anticipated, overexpression of either fish TBK1 or IRF3 resulted in a robust activation of IFN promoter (Fig. 3A), an increase in IFN mRNA, as well as IRF3 and Mx1 proteins (Fig. 3B, 3C).

In subsequent assays, the dominant negative forms of fish TBK1 and IRF3, named TBK1-K38M and IRF3-DN (14, 28), were constructed and used to delineate their roles in fish virus-induced IFN signaling. Consistently, fish MITA-mediated IFN promoter activity was severely impaired in CABs when cotransfected with either TBK1-K38M or IRF3-DN (Fig. 4A), and overexpression of the construct IRF3-DN blocked TBK1-induced activation of fish IFN promoter (Fig. 4B), indicating that fish MITA activates IFN response via a MITA-TBK1-IRF3 signaling pathway. These results were further supported by the findings that transfection of CABs with either TBK1-K38M or IRF3-DN abrogated MITA-induced expression of IRF3 protein (Fig. 4C), and that transfection with IRF3-DN significantly blocked TBK1-induced expression of IRF3 (Fig. 4D). Moreover, cotransfection of STAT1- $\Delta$ C, a dominant negative mutant of STAT1 (23), did not weaken but led to even more enhanced IFN promoter-driven luciferase activity than that in control cells (Fig. 4E). These results together indicated that MITA



**FIGURE 2.** MITA facilitates the activation of IFN response against viral infection. *A*, Subcellular localization of fish MITA. CABs seeded on microscopy coverglass in six-well plates were cotransfected with 0.8  $\mu$ g pDsRed2-ER and 0.8  $\mu$ g MITA-GFP (*upper panel*) or empty vector (*lower panel*). At 24 h posttransfection, the cells on microscopy coverglass were fixed and examined using a confocal microscopy. The yellow staining in the overlay image (*right*) indicates that MITA is localized to ER. *B*, Induction of IFN promoter by overexpression of MITA. CABs seeded in 24-well plates were cotransfected with 0.25  $\mu$ g IFNpro-Luc and 0.25  $\mu$ g MITA. A total of 0.025  $\mu$ g pRL-TK was included to normalize the expression level. At 48 h posttransfection, the transfected cells were harvested for detection of luciferase activity. *C* and *D*, Induction of IFN and ISG by overexpression of MITA. CABs seeded in six-well plates were transfected for 48 h with 1.6  $\mu$ g MITA or empty vector pcDNA3.1 as control. RT-PCR was used to detect endogenous IFN mRNA (*C*), and Western blot was used to detect the expression of IRF3 protein (*D*). *E*, Effect of MITA on NF- $\kappa$ B activation. CABs seeded in 24-well plates were transfected as in *B* with indicated plasmids. Twenty-four hours later, the cells were transfected with 0.5  $\mu$ g poly(I:C) per well. Then the cells were harvested for detection of luciferase activity after a 24-h stimulation. *F*, Inhibition of virus replication by overexpression of MITA. EPCs or CABs seeded in 24-well plates were transfected with 0.5  $\mu$ g MITA or empty vector. At 24 h posttransfection, the transfected cells were infected with SVCV for EPCs or GCRV for CABs (1000 TCID<sub>50</sub>/ml per well). Another 48 h later, the supernatants were harvested for measurement of virus titers by standard TCID<sub>50</sub> method. The results were the representative of two independent experiments. Error bars in *B* and *E* represent SDs obtained by measuring each sample in triplicate.

regulates the expression of IFN through the MITA-TBK1-IRF3 cascade but independently of STAT1 pathway.

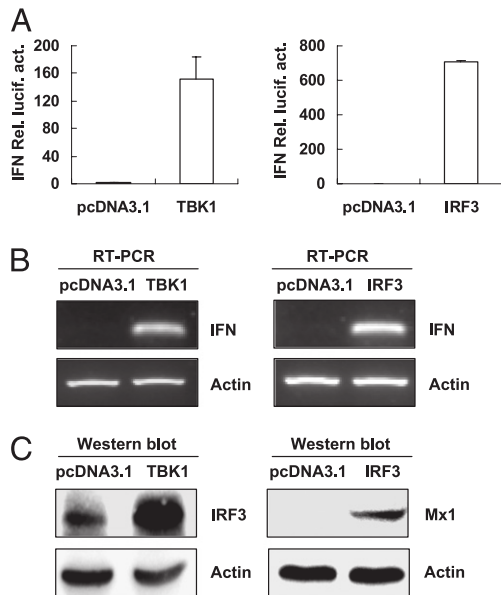
Finally, the interaction of fish MITA with TBK1 or IRF3 was determined by coimmunoprecipitation with Tag Abs. In COS7 cells that were cotransfected with MITA-Flag and TBK1-myc, anti-Flag Ab-immunoprecipitated protein complex was also recognized by anti-myc Ab (Fig. 5A, *left panel*). In a reverse assay, anti-myc Ab-immunoprecipitated complex was recognized by anti-Flag Ab (Fig. 5A, *right panel*). These results indicated that fish MITA was associated with TBK1 in transfected cells. Similarly, fish MITA was confirmed to be associated with IRF3 (Fig. 5B), and fish TBK1 also interacted with IRF3 (Fig. 5C). These data suggested that fish MITA, TBK1, and IRF3 might form a complex to signal IFN response.

#### *Fish MITA regulates IFN expression downstream of RIG-I and MDA5*

We next determined whether fish MITA participates in dsRNA-mediated IFN response. Similar to our previous report (14), transfection of poly(I:C) [intracellular poly(I:C)] efficiently induced fish IFN promoter activity (Fig. 6A). Overexpression of fish MITA promoted poly(I:C)-induced luciferase activity of IFNpro-luc (~4-fold against empty vector; Fig. 6A). Consistently, overexpression of Flag-MITA abrogated poly(I:C)-induced IFN promoter activity (4-fold reduction against empty vector; Fig. 6A). In this experiment, the construct Flag-MITA, tagged with Flag at the N terminus (Fig. 1A), displayed a dominant negative effect, whereas MITA-Flag remained the same activity as the wild type MITA (data not shown), which was similar to mammalian MITAs (9).

To further delineate the role of fish MITA in cytosolic dsRNA-triggered IFN signaling, we cloned and identified three cytoplasmic dsRNA sensor genes, including RIG-I, MDA5, and LGP2 (GenBank accession no. JF970225, JF970226, and JF970227; Supplemental Fig. 2). Transfection of CABs with wild type constructs, either RIG-I or MDA5, led to a strong activation of fish IFN promoter (>30-fold against empty vector; Fig. 6B). In contrast with MITA, attachment of a Flag epitope to N terminus of both RIG-I and MDA5 did not impair their abilities to activate fish IFN promoter (Fig. 6B). Subsequently, overexpression of Flag-MITA impeded the ability of fish RIG-I or MDA5 to stimulate IFNpro-Luc (Fig. 6C). To confirm the blockade effect of Flag-MITA, transfection of the other dominant negative mutant of MITA (MITA-CT), which was devoid of its N terminus (Fig. 1A), obtained a similar result (Fig. 6C). Further experiments demonstrated that the activation of IFN promoter by RIG-I and MDA5 was severely inhibited by the dominant-negative mutants of TBK1 or IRF3 (TBK1-K38M or IRF3-DN; Fig. 6D). These results indicated that MITA signals IFN response downstream of RIG-I and MDA5 in response to intracellular poly(I:C).

Finally, the role of fish LGP2 in cytosolic dsRNA-triggered IFN response was investigated. As anticipated, overexpression of LGP2 diminished transfected poly(I:C)-induced activity of IFNpro-luc (Fig. 6E). This inhibitory ability seemed to be retained by the RD, but not by the HD of fish LGP2, because transfection of deletion construct LGP2-RD, but not LGP2-HD, displayed the inhibitory effect (Fig. 6E). Similarly, RIG-I- or MDA5-induced



**FIGURE 3.** Activation of IFN response by fish TBK1 and IRF3. *A*, Induction of IFN promoter by TBK1 (*left panel*) and IRF3 (*right panel*). CABs seeded in 24-well plates were cotransfected with 0.25  $\mu$ g IFNpro-Luc and 0.25  $\mu$ g TBK1 or IRF3. A total of 0.025  $\mu$ g pRL-TK was included to normalize the expression level. At 48 h posttransfection, the transfected cells were harvested for detection of luciferase activity. Error bars represent SDs obtained by measuring each sample in triplicate. *B* and *C*, Induction of IFN and ISG by TBK1 (*left panel*) and IRF3 (*right panel*). CABs seeded in six-well plates were transfected for 48 h with 1.6  $\mu$ g TBK1 (*left panel*) and IRF3 (*right panel*). Simultaneously, empty vector pcDNA3.1 was transfected as control. RT-PCR was used to detect endogenous IFN mRNA (*B*), and Western blot was used to detect the expression of IRF3 protein and Mx1 protein (*C*).

IFN promoter activity was diminished in LGP2-overexpressing cells (>18- or 6-fold reduction as relative to controls; Fig. 6*F*). These results indicated that fish LGP2 functions as a negative regulator of both RIG-I and MDA5 in cytosolic dsRNA-induced IFN signaling.

#### Fish MITA activates distinct fish virus-induced IFN signaling

The existence of two groups of virus-induced IFN genes in fish prompted us to investigate whether they were activated by MITA-mediated signaling. To address this question, we searched the *Danio rerio* genome for four IFN genes. Characterization of ~2-kb 5'-flanking regulatory sequences upstream of all four zebrafish IFN genes revealed multiple transcription factor binding sites. The putative IFN-stimulated regulatory element/IRF-binding element (ISRE/IRF-E) motifs, which were bound to IRFs for gene transcription (14), were found in all four zebrafish IFN promoters, although they were located in different regions as relative to the transcription start site (Supplemental Fig. 3). Among these promoters, zebrafish IFN1 promoter is most similar to crucian carp IFN promoter, which contains a NF- $\kappa$ B binding site flanked by two typical ISRE/IRF-E motifs (14, 23). Actually, zebrafish IFN1 is most homologous to crucian carp IFN, both of which formed a clade together with zebrafish IFN4 in a phylogenetic tree analysis (23). We cloned the minimal promoter sequence containing the proximal putative ISRE/IRF-E motifs in front of the luciferase gene to make four luciferase constructs (Fig. 7*A*). The promoters from zebrafish IFN1 and IFN3 were much more effectively activated by transfected poly(I:C) than crucian carp IFN promoter; however, no stimulatory activity was observed for the generated promoters of zebrafish IFN2 and IFN4 (Fig. 7*B*). Therefore,

zebrafish IFN1 and IFN3 promoters were further analyzed as representatives of both groups' fish IFN genes.

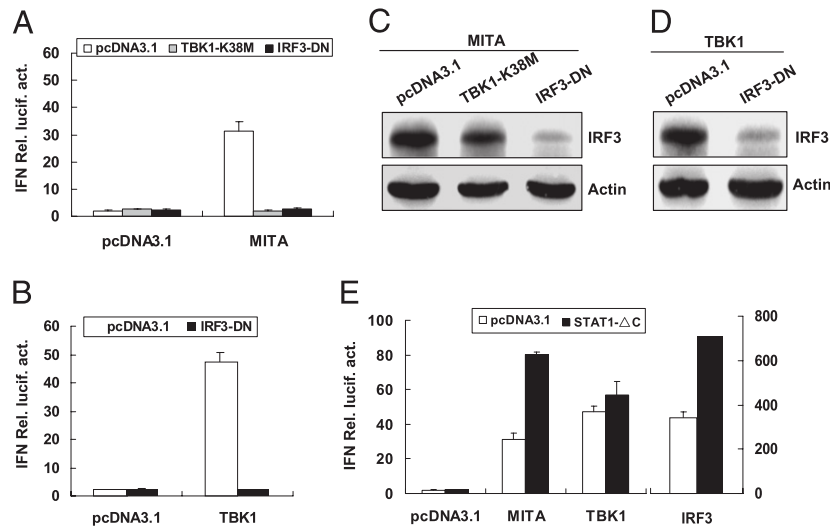
The role of signaling molecules including RIG-I, MAD5, MITA, TBK1, and IRF3 on both IFN promoters was examined. Similar to crucian carp IFN promoter, overexpression of respective signaling molecules except IRF3 significantly induced luciferase activities of both DrIFN1pro-luc and DrIFN3pro-luc, and a greater induction was observed for DrIFN3pro-luc (Fig. 7*C*). However, under the condition of overexpression of IRF3 alone, zebrafish IFN1 promoter, but not zebrafish IFN3 promoter, was effectively activated (~50-fold induction versus 1.3-fold against empty vector; Fig. 7*C*). In subsequent assays, overexpression of TBK1 was sufficient to activate the luciferase activity of both DrIFN1pro-luc and DrIFN3pro-luc, but such activation was severely inhibited by cotransfection of either IRF3-DN or IRF7-DN alone, but not by cotransfection of STAT1- $\Delta$ C (Fig. 7*D*), indicating that TBK1-activated expression of two zebrafish IFN genes was mediated by IRF3/IRF7 but independent of Stat1 pathway.

The cooperative effect of fish IRF3 and IRF7 on two zebrafish IFN promoters was next examined. Whereas zebrafish IFN1 promoter, similar to crucian carp IFN promoter, was induced by IRF7 alone at a weaker level than by IRF3 alone (~11-fold induction versus 56-fold against empty vector), IRF7, but not IRF3, resulted in weak activation of zebrafish IFN3 promoter (~7.5-fold induction versus 1.5-fold against empty vector; Fig. 7*E*). Simultaneous transfection of both IRF3 and IRF7 significantly induced luciferase activity of DrIFN1pro-luc (1.6-fold increase against IRF3 alone, 8-fold increase against IRF7 alone), but no obvious cooperative stimulation was observed for DrIFN3pro-luc (Fig. 7*E*). A similar result was observed with transfection of zebrafish IRF3 and IRF7 (Fig. 7*F*).

## Discussion

Recent identification of fish virus-induced IFN genes suggests that fish possess an IFN antiviral response similar to mammals (19–24); however, the subtle signaling transduction pathway is still unknown. Previously, we identified many genes involved in fish IFN response (25–27). Further, we demonstrated that fish IFN exerts antiviral function through the STAT1 pathway (23), and that IRF3-dependent IFN response is conserved in fish (14). In this study, we confirmed that fish has developed a conserved RLR-triggered IFN response, which is mediated by the MITA-TBK1-IRF3 pathway. Strikingly, although fish IFNs are not true homologs of mammalian type I IFNs (19–23), their expression is activated by a similar regulatory mechanism, which has occurred independently in fish and mammals probably by a process of convergent evolution.

In mammals, on viral infection, MITA recruits TBK1 and IRF3 to form a complex triggering IFN- $\beta$  activation (8–10). The following observations strongly suggest that the MITA-mediated IFN signaling is conserved from fish to mammals. First, fish MITA possesses the conservative protein structure consisting of one signal peptide and five N-terminal transmembrane motifs, and similar to human MITA (9, 10), it is constitutively expressed in ER and not induced by IFN and poly(I:C). Second, overexpression of fish MITA leads to a significant upregulation of IFN and ISGs, and concomitantly induces a strong antiviral state against SVCV in EPCs and GCRV in CABs. Third, further characterization of crucian carp TBK1 reveals its ability to induce IFN response, as evidenced by the finding that it is associated with fish MITA and IRF3. Finally, the MITA-induced IFN response is severely impaired by blocking the function of cellular TBK1 or IRF3 by transfection of dominant negative mutants TBK1-K38M and IRF3-DN, respectively, whereas the induction of IFN and ISGs by fish TBK1 is just blocked by IRF3-DN. A previous study showed that overexpression of Atlantic salmon RIG-I N terminus confers full protection on fish cells



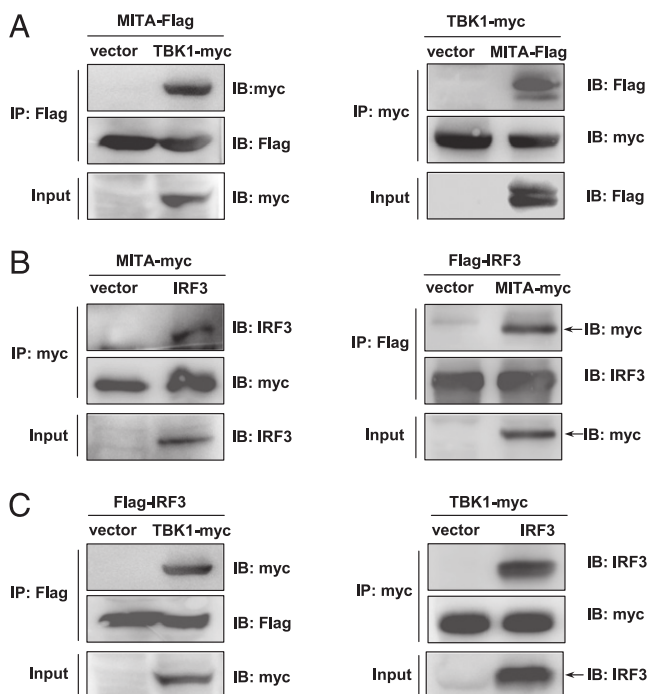
**FIGURE 4.** Activation of IFN response by MITA through MITA-TBK1-IRF3 signaling cascade. *A*, Induction of IFN promoter by MITA is inhibited by TBK1 and IRF3 mutants. CABs seeded in 24-well plates were cotransfected with 0.25  $\mu$ g IFNpro-Luc, 0.25  $\mu$ g MITA, and 0.25  $\mu$ g TBK1-K38M or IRF3-DN or empty vector as control. A total of 0.025  $\mu$ g pRL-TK was included to standardize the expression level. Forty-eight hours later, the transfected cells were harvested for detection of luciferase activity. *B*, Induction of IFN promoter by TBK1 is inhibited by IRF3 mutant. Reporter assays were performed as in *A* by cotransfection with TBK1 and IRF3-DN. *C*, Induction of IRF3 protein by MITA is impaired by TBK1 and IRF3 mutants. CABs seeded in six-well plates were cotransfected with 0.8  $\mu$ g MITA and the indicated mutant TBK1-K38M or IRF3-DN or empty vector as control (0.8  $\mu$ g each). At 48 h posttransfection, the whole-cell lysates were prepared for detection of IRF3 protein by Western blot analysis. *D*, Induction of ISG by TBK1 is impaired by IRF3 mutant. The experiments were performed similarly as in *C* by cotransfection with TBK1 and IRF3-DN or empty vector (0.8  $\mu$ g each). *E*, Induction of IFN promoter by MITA is not blocked by STAT1- $\Delta$ C. Reporter assays were performed as in *A* by transfection with the indicated plasmids (0.25  $\mu$ g each). Error bars in *A*, *B*, and *E* represent SDs obtained by measuring each sample in triplicate.

against RNA virus infection by a strong induction of both IFN and ISGs (16). We extend this notion by luciferase reporter assays in which each of dominant negative mutants for fish MITA, TBK1, and

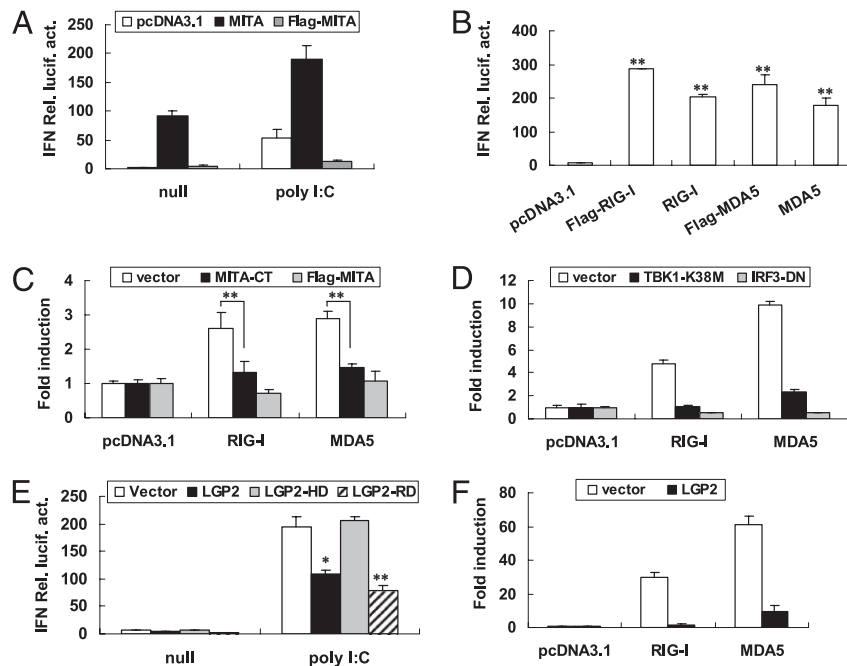
IRF3 is able to effectively abrogate fish RIG-I- or MDA5-induced IFN promoter activity. Consistently, the dominant negative mutant of MITA is able to suppress transfected poly(I:C)-induced IFN expression in fish cells. Therefore, our data together provide evidence that fish possess a conserved RIG-I/MDA5-MITA-TBK1-IRF3-IFN signaling cascade in response to intracellular dsRNA and virus infection.

It must be noted that there are some controversial observations for MITA signaling. We observed that transfection of either MDA5 or its ligand poly(I:C) evoked fish IFN response, and such induction was blocked in fish cells when cotransfected with the dominant negative mutant of MITA (Fig. 6). This finding is consistent with a previous study that RNA interference knockdown of human MITA in 293T cells impaired IFN- $\beta$  induction by intracellular poly(I:C) (8). However, in the other study, using deficient mice, Sting/MITA did not appear to be required for response to poly(I:C) (9, 11). We also did not observe the activation of NF- $\kappa$ B in fish cells by fish MITA overexpression, even followed by stimulation with poly(I:C) (Fig. 2E), which is in agreement with the finding from Zhong et al. (8), but not from Ishikawa and Barber (9) or Sun et al. (10). In addition, reporter assays in this study demonstrated that, consistent with some previous reports (3, 4), crucian carp LGP2 functioned as a negative regulator of RIG-I and MDA5 (Fig. 6). However, LGP2-deficient mice were highly susceptible to encephalomyocarditis virus infection (29), and a recent study showed that Japanese flounder LGP2 positively regulated IFN response (30). Despite these differences, the data presented in this article substantiate that fish MITA serves as a critical mediator for fish IFN response.

One striking finding in this study is that the MITA pathway is also involved in the activation of distinct fish IFN genes. Zebrafish genome contains four virus-induced IFN genes that are divided into two groups: group I IFNs with two cysteines including zebrafish IFN1 and IFN4, and group II IFNs with four cysteines including IFN2 and IFN3 (22). Crucian carp IFN is most homologous to zebrafish IFN1, thus belonging to group I (23). Similar to the



**FIGURE 5.** The interaction among MITA, TBK1, and IRF3. COS7 cells seeded in 10-cm dishes were cotransfected with 5  $\mu$ g MITA-Flag and 5  $\mu$ g TBK1-myc (*A*), 5  $\mu$ g MITA-myc and 5  $\mu$ g wild type IRF3 or Flag-IRF3 (*B*), or 5  $\mu$ g Flag-IRF3 and 5  $\mu$ g TBK1-myc (*C*). Empty vector pcDNA3.1 was transfected in parallel as control. Twenty-four hours later, cell lysates were immunoprecipitated with anti-Flag Ab (*A*, *C*, left panels; *B*, right panel) or anti-myc Ab (*A*, *C*, right panels; *B*, left panel). Then the immunoprecipitates were analyzed by Western blotting with the corresponding Tag Abs, except that IRF3 was detected by IRF3 Ab.



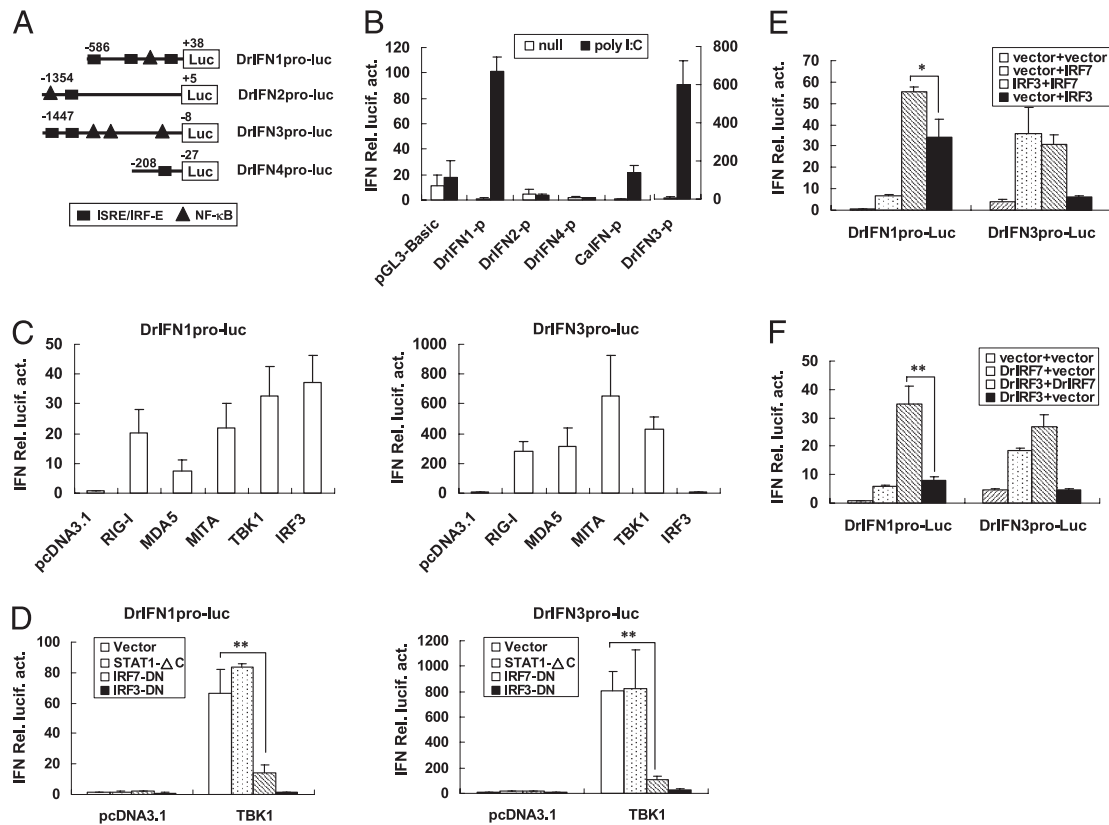
**FIGURE 6.** MITA mediates RIG-I/MDA5-activated IFN signal pathway. *A*, The involvement of MITA in poly(I:C)-induced IFN activation. CABs seeded in 24-well plates overnight were transfected with 0.25  $\mu$ g IFNpro-Luc and 0.25  $\mu$ g wild type MITA or Flag-MITA or empty vector pcDNA3.1 as control. A total of 0.025  $\mu$ g pRL-TK was introduced as an internal control. At 24 h posttransfection, the cells were transfected again with or without poly(I:C) (1  $\mu$ g/ml). Another 24 h later, the cells were harvested for detection of luciferase activity. *B*, Overexpression of RIG-I and MDA5 activates IFN promoter. CABs seeded in 24-well plates were transfected as in *A* with the indicated plasmids. Forty-eight hours later, the transfected cells were harvested for detection of luciferase activity. *C* and *D*, Induction of IFN promoter by RIG-I and MDA5 is impaired by two MITA mutants (*C*) or by TBK1 and IRF3 mutants (*D*). CABs seeded in 24-well plates were transfected as in *A* with the indicated plasmids. Forty-eight hours later, the transfected cells were harvested for detection of luciferase activity. *E*, Poly(I:C)-induced IFN activation is inhibited by LGP2. Reporter assays were performed as in *A* by transfection with the indicated plasmids. *F*, LGP2 inhibits both RIG-I- and MDA5-mediated activation of IFN promoter. CABs seeded in 24-well plates were transfected as in *A* with the indicated plasmids. Forty-eight hours later, the transfected cells were harvested for detection of luciferase activity. Error bars represent SDs obtained by measuring each sample in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

result from crucian carp IFN, ectopic expression of the components of RLR pathways induces promoter activities of zebrafish IFN1 and IFN3, demonstrating that RIG-I or MDA5 activates two zebrafish IFN genes dependent on the MITA pathway. Intriguingly, distinct transcriptional factors are required for the activation of both zebrafish IFN genes. In mammals, RLR pathway activates the expression of type I IFNs (IFN- $\alpha/\beta$ ) in most cells by an ingenious mechanism (2, 3). In this model, TBK1 is recruited to activate IRF3 and IRF7. The ubiquitously and constitutively expressed IRF3 is exclusively required for rapid expression of IFN- $\beta$ , the early-phase IFN gene. The late-phase IFN genes, including most IFN- $\alpha$ , instead are regulated by IRF7 that is induced by the IFNs produced at an early time of virus infection (5–7). Consistent with this notion, TBK1 stimulates IFN- $\alpha$  expression efficiently in the presence of IRF7 (31, 32). In addition, IRF7 is essential for IFN- $\beta$  expression, possibly with IRF3 forming a heterodimer (7, 32). In this study, whereas fish IRF3 and IRF7 are critical for the activation of zebrafish IFN1 and IFN3 (Fig. 7*D*), individual activation and the synergistic effect by both IRF3 and IRF7 are obviously observed only for zebrafish IFN1, and IRF7 rather than IRF3 displays an ability to activate zebrafish IFN3 (Fig. 7*E*). Combined with the recent finding that crucian carp IFN gene is activated primarily by fish IRF3 and weakly by IRF7 (14), these data indicate that zebrafish IFN1 and crucian carp IFN are regulated by the MITA pathway dependent on IRF3 and IRF7, thereby resembling that of the IFN- $\beta$  gene, whereas zebrafish IFN3 is mainly controlled by IRF7, similar to those of IFN- $\alpha$  genes. Notably, although transfection of IRF3 alone fails to activate zebrafish IFN3 promoter (Fig. 7*C*), TBK1-induced activation

of zebrafish IFN3 is significantly abrogated by transfection of IRF3-DN (Fig. 7*D*). We speculate that such inhibitory effect might result from the interaction of IRF3-DN and endogenous IRF7 or TBK1. In the future, *in vivo* studies are helpful to clarify the distinct roles of IRF3 and IRF7 on regulation of various fish IFN genes.

Previous studies showed that there are differential expression patterns of both groups of rainbow trout IFNs (19), and that three rainbow trout IFN genes belonging to group I display a similar kinetics after stimulation with poly(I:C) (21). In Atlantic salmon, a total of 11 IFN genes have been identified (20). Strikingly, both salmon IFN $\alpha$ 1 and IFN $\alpha$ 2 belonging to group I seem to mimic IFN- $\beta$  based on the poly(I:C)-induced expression patterns, whereas four salmon IFN $\beta$  genes belonging to group II are upregulated by the imidazoquinoline S-27609, a TLR7 ligand, in head kidney and leukocytes (20). These findings indicate that fish virus-induced IFNs are regulated in a way similar to mammalian type I IFNs (20). It is well-known that fish IFN genes and mammalian type I IFN genes have evolved independently to form a large family, respectively, although they originate from one common ancestor (19). The fish-specific genome duplication and species-specific gene duplication result in different copies of IFN genes in different fish species, from 1 in pufferfish and 11 in Atlantic salmon (19–21, 23). Consistently, the divergence of mammalian IFN- $\alpha/\beta$  occurs after separation of mammals and birds (19). Compared with vertebrate IFN genes, the components of RLR pathway, including RIG-I, MDA5, TBK1, and IRF family, display clear orthologous relationships between fish and mammals (13–16), indicating that they diverge earlier than vertebrate IFN genes and pre-exist as





**FIGURE 7.** MITA activates distinct fish IFNs via IRF3 or IRF7. *A*, Schematic representation of zebrafish IFN promoter-driving luciferase constructs. *B*, Characterization of zebrafish IFN promoters. CABs seeded in 24-well plates overnight were transfected with the indicated luciferase constructs (0.25  $\mu$ g each); 0.025  $\mu$ g pRL-TK was introduced to normalize the expression level. At 24 h posttransfection, the transfected cells were transfected for 24 h with 1  $\mu$ g/ml poly(I:C) and harvested for luciferase activity detection. *C*, The components of the RLR pathway activate zebrafish IFN1 (left panel) and IFN3 promoters (right panel). CABs seeded in 24-well plates were cotransfected with 0.25  $\mu$ g zebrafish IFNpro-Luc and 0.25  $\mu$ g individual constructs from the component of the RLR pathway. A total of 0.025  $\mu$ g pRL-TK was included to normalize the expression level. At 48 h posttransfection, the transfected cells were harvested for detection of luciferase activity. *D*, Induction of zebrafish IFN promoters is dependent of IRF3/IRF7, but not STAT1. Reporter assays were performed as in *A* by transfection with the indicated plasmids. *E*, Cooperative effect of crucian carp IRF3 and IRF7 on zebrafish IFN activation. CABs seeded in 24-well plates overnight were cotransfected with 0.25  $\mu$ g zebrafish IFN promoter-driving luciferase plasmid and the indicated expression construct (0.5  $\mu$ g total at a ratio 1:1); 0.025  $\mu$ g pRL-TK was introduced as an internal control. Forty-eight hours later, the cells were harvested for detection of luciferase activity. *F*, Cooperative effect of zebrafish IRF3 and IRF7 on zebrafish IFN activation. Reporter assays were performed similarly as in *E*. Error bars represent SDs obtained by measuring each sample in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ .

orthologs in the common ancestor of both fish and mammals. Based on these findings, it is likely that, although the species-specific duplication of IFN genes occurs independently in fish and mammals, if expressed as multiple copies, they have convergently evolved a similar regulatory mechanism that adopts the pre-existing signaling molecules to control IFN response. This notion is further supported by recent studies that mammalian type III IFNs are induced by a panel of viruses and dsRNA in similar patterns to type I IFNs (33, 34), although the involved pathways are not entirely identical (35). Interestingly, IFN- $\lambda$ 1 is regulated by IRF3 and IRF7, thus resembling IFN- $\beta$ , and IFN- $\lambda$ 2/3 is regulated mainly by IRF7, thus resembling IFN- $\alpha$  (34).

However, it is obvious that fish IFN regulation is not identical to that of mammalian type I IFNs. For example, TBK1-mediated activation of crucian carp IFN gene and both zebrafish IFN genes is not abrogated by overexpression of the dominant negative mutant of STAT1 (STAT1- $\Delta$ C), indicating that fish IFN genes are upregulated directly by the basally expressed transcription factors IRF3 and IRF7, and that zebrafish IFN1 and IFN3 appear not to be induced as early or later phase IFNs. However, besides the constitutive expression level, both fish IRF3 and IRF7 are induced by IFN and IFN stimuli through the STAT1 pathway (14, 23), and importantly, fish IRF3 is phosphorylated by IFN (14). Therefore, it

is likely that fish MITA- or TBK1-mediated IFN response might be augmented by both the IFN-induced IRF3 and IRF7. If this is the case, fish IFNs may possess a unique positive feedback loop for their regulation. In the first wave of induction, multiple fish IFN genes are activated by basally expressed IRF3 and IRF7. The ongoing produced IFNs induce the expression of IRF3 and IRF7 through the STAT1 pathway and also activate them. The activated IRF3 and IRF7 in turn participate in the second wave of IFN induction. This mechanism differs from that in mammals, where IFN- $\beta$  is first induced by the constitutively expressed IRF3 and then primes the expression of the late-phase IFN- $\alpha$  by the induced IRF7 through the STAT1 pathway (6, 36). Another interesting finding in fish is that recombinant zebrafish IFN upregulates the expression of itself and other IFN genes (24), as seen also in rainbow trout (21) and crucian carp (23). The autoregulation of crucian carp IFN seems to be mediated through the STAT1 pathway (23). Conversely, mammalian type I IFN cannot be induced by themselves (36, 37), whereas IFN- $\beta$  is required for most IFN- $\alpha$  expression (5, 6). Interestingly, mammalian type III IFNs display the expression trait similar to that of fish IFNs, because they are induced directly by type I IFNs and also themselves (34, 37, 38).

Collectively, the data described in this study demonstrated that the MITA pathway is essential for fish IFN gene activation, and that

the RLR-activated IFN signaling cascade is conserved from fish to mammals. Although fish IFN genes evolve independently of mammalian type I IFNs, there is a similar evolutionary differentiation of distinct IFN gene activation in fish and mammals. It is plausible that the conserved signaling molecules in these species leads to occurrence of a common regulatory mechanism responsible for the activation of differentiated IFN genes including type III IFNs, which might result from long-term responsiveness to selective pressures from virus infection. Despite the resemblance, fish IFNs are not induced entirely identical to type I or type III IFNs. Because of failure to acquire active promoters of zebrafish IFN2 and IFN4, we do not know whether both IFN genes are regulated in the same fashion to zebrafish IFN1 and IFN3, respectively. Preliminary studies showed that the regulatory sequences of these genes are not conserved and the transcription factor binding sites differ (Supplemental Fig. 3). In addition, fish group II IFNs have been found only in zebrafish, rainbow trout, and Atlantic salmon to date (19, 21). It is not clear how these fish species, without group II IFNs or with one IFN gene, efficiently defense against viral infection. Considering that fish IFN genes have four introns, which may add another posttranscriptional regulatory event into the biology of these IFNs and make it more complex to regulate their expression, further investigation is required to identify the positive or negative regulator of RLR pathway and to understand the fine-tuning of gene regulatory events involved in the expression of fish IFN genes.

## Disclosures

The authors have no financial conflicts of interest.

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