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Zebrafish ISG15 Exerts a Strong Antiviral Activity against RNA and DNA Viruses and Regulates the Interferon Response

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ISG15, a 15-kDa interferon-induced protein that participates in antiviral defenses of mammals, is highly conserved among vertebrates. In fish, as in mammals, viral infection and interferon treatment induce *isg15* expression. The two ubiquitin-like domains of ISG15 and the presence of a consensus LRLRGG sequence in the C-terminal region, which is required for the covalent conjugation to a substrate protein, are also conserved in fish. Our data demonstrate that overexpression of zebrafish ISG15 (zf-ISG15) in EPC cells is sufficient to inhibit viral infection by RNA viruses belonging to the genera *Novirhabdovirus* and *Birnavirus* and by DNA viruses of the genus *Iridovirus*. In coexpression experiments with IHNV proteins, we demonstrate specific ISGylation of phosphoprotein and nonvirion protein. Mutation of the glycine residues in the consensus LRLRGG motif abolishes zf-ISG15 conjugation to these proteins and the cellular protection against viral infection, thus connecting ISGylation and ISG15-dependent viral restriction. Additionally, zf-ISG15 overexpression triggers induction of the *rig-I* and *viperin* genes as well as, to a lesser extent, the IFN gene. Overall, our data demonstrate the antiviral effect of a fish ISG15 protein, revealing the conservation among vertebrates of an ISGylation mechanism likely directed against viruses. Furthermore, our findings indicate that zf-ISG15 affects the IFN system at several levels, and its study shall shed further light on the evolution of the complex regulation of the innate antiviral response in vertebrate cells.

Upregulation of type I interferons (IFNs) represents the main pathway of the antiviral innate immune response of vertebrates. Upon infection, detection of viral compounds quickly triggers signaling pathways that lead to interferon induction (1). The main sensor families for RNA viruses are the cytoplasmic retinoic acid-inducible gene I-like receptors (RLRs) and the membrane-anchored Toll-like receptors (TLRs). The cytoplasmic RLR helicases include RIG-I (retinoic acid-inducible gene I), which mainly targets short 5' triphosphate viral RNA (2), and MDA5 (melanoma differentiation-associated gene 5), which detects viral double-stranded RNA (dsRNA) (3). Ligand binding to these receptors in the cytoplasm induces their multimerization, which activates the helicase activity. This leads to the recruitment of the mitochondrial adaptor MAVS, activating downstream IFN regulatory factor 3 (IRF3)/IRF7 pathways and, finally, inducing IFN production. In contrast, TLRs are expressed at the cell surface or on endosome membranes. After binding their target, the intracytoplasmic Toll/interleukin-1 receptor (TIR) domain of TLRs involved in RNA virus sensing recruits adaptor proteins like MyD88 and TIRAP and triggers NF- κ B- and IRF3-dependent type I IFN production. Secreted type I IFNs induce an antiviral state in exposed cells that express IFN receptors. IFN molecules do not directly block the viral infection, but via a signaling pathway involving JAK kinases and STAT transcription factors, they induce more than 200 interferon-stimulated genes (ISGs) (4, 5). A significant antiviral activity has been demonstrated for a number of ISGs, including Mx, VIG-1/VIPERIN, ISG15, OAS, PKR, RNase L, IFITMs, and TRIMs (6, 7). However, even for those ISGs, the detailed antiviral mechanisms are far from being completely understood. Additionally, recent reports have indicated that several ISGs, in addition to their effector antiviral functions, participate in the regulation of IFN gene expression (8–10).

One of the most expressed ISGs is *isg15* (IFN-stimulated gene

15), which was first identified as encoding a 15-kDa protein induced in the early phase of the IFN response in bovine and human cells (11, 12). In these species, the protein is synthesized as a precursor of 17 kDa, trimmed in the C-terminal end to generate the active isoform (13). In contrast, the ISG15 protein encoded by sheep and cow or fish species is directly synthesized as an active isoform. The gene cloned and sequenced from human revealed a strong sequence homology with ubiquitin, allowing ISG15 recognition by antiubiquitin antibodies (14). Like ubiquitin, cytosolic ISG15 may be free or covalently conjugated to other proteins (15). It was soon discovered that free ISG15 can also be secreted by human monocytes and lymphocytes (16). It then acts as a cytokine that promotes IFN production by T cells (17) and activates NK cell proliferation (18, 19). Later, the conjugation of ISG15 to target proteins—a process called ISGylation—appeared to be analogous to the classical ubiquitination. This protein modification is operated by enzymes E1 (Ube1L) and E2 (UBC8) and various E3 enzymes (Herc5, HHARI, or TRIM25), whose expression is dependent on type I IFNs (20–22). The presence of an LRLRGG motif at the C terminus of ISG15 is required for ISGylation. Whereas ubiquitin conjugation has been linked to well-described signaling pathways, the cellular functions of ISGylation are still largely undefined. Large-scale analyses revealed that ISGylation targets pro-

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teins involved in distinct cellular pathways, including IFN signaling as well as RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeleton organization, and regulation of stress responses and translation (23). Recent studies have supported a role of ISGylation in the modulation of targeted protein activity and/or interaction with cellular partners (24–26).

Since its discovery in the 1980s, ISG15 has been involved in different mechanisms of viral inhibition targeting a large spectrum of RNA and DNA viruses: Sindbis virus (27–29) influenza A and B viruses (29–33), Ebola virus (34, 35), HIV (34, 36–38), Chikungunya virus (39), hepatitis C virus (40, 41), herpes simplex virus 1 (29), murine gamma herpesvirus 68 (29), and vaccinia virus (42). Mechanisms of inhibition are diverse and interfere with virus infection at several levels of the viral cycle. In the case of HIV, Okumura et al. demonstrated that ISG15 targets virus release by inhibiting the ubiquitination of GAG, which is required for assembly and budding (37). This inhibition occurred by preventing the GAG-TSG101 interaction, in the absence of detectable conjugation of ISG15 to GAG or TSG101 (37). The same group also reported Ebola virus inhibition through ISG15 interaction with the NEDD4 ubiquitin ligase protein preventing VP40 virus-like particle budding (35). Furthermore, Sindbis virus as well as influenza A and B virus infections trigger ISGylation of viral and cellular proteins, which likely leads to different antiviral processes (28, 31, 32, 43). Hence, while the importance of ISG15 in the early response to different viruses is firmly established, many aspects of the ISG15 antiviral activity remain to be clarified. Importantly, viruses have developed strategies to counteract the antiviral effect of ISG15 by inhibition of ISGylation (42, 43) or induction of de-ISGylation activity (44–47). Overall, these data demonstrated that ISG15 plays an important role in viral inhibition mechanisms in mammals.

The IFN system is unknown in invertebrates but appears to be well conserved among vertebrates. Bony fish possess a variable number of virus-induced IFNs, known as phi IFNs (IFN- ϕ) (48, 49), that share their three-dimensional structure with mammalian type I IFNs (50). Several important downstream effectors of the mammalian IFN system, including PKR, MX, and VIG-1/VIPERIN but also ISG15, have virus-inducible orthologues in fish (48), suggesting that these proteins are involved in an ancient core network of antiviral pathways assembled around the IFNs when they emerged in the first vertebrates. Hence, the characterization of antiviral mechanisms associated with such conserved ISGs should provide hints to provide an understanding of the origin and the fundamental structure of the vertebrate IFN system.

In fish, well-conserved *isg15* sequences have been described in several species. An *isg15*-like sequence was first identified in rainbow trout leukocytes incubated with the novirhabdovirus viral hemorrhagic septicemia virus (VHSV) and named *vig-3* for VHSV-induced gene 3 (51). As in mammals, this protein contains the critical C-terminal diglycine motif required for ISGylation, thus suggesting that it could have functions similar to the function observed for mammalian ISG15. *isg15* homologues were then described in different fish species, including zebrafish (*Danio rerio*), pufferfish (*Fugu rubripes*), catfish (*Ictalurus punctatus*) (52), crucian carp (*Carassius auratus*) (53), black rockfish (*Sebastes schlegeli*) (54), Japanese flounder (*Paralichthys olivaceus*) (55), and red drum (*Sciaenops ocellatus*) (56), as well as Atlantic salmon (*Salmo salar*) (57), Atlantic cod (*Gadus morhua*) (58, 59), and, more recently, tongue sole (*Cynoglossus semilaevis*) (60). In several

fish species, including sole, red drum, and flounder, the ISG15 protein contained a supplemental C-terminal extension behind the LRLRGG motif, which is still present in the active secreted sole ISG15 cytokine-like factor (60). The promoters of the fish *isg15* genes contain typical IFN-sensitive response elements (ISREs), and the corresponding transcripts have been found to be upregulated upon different viral or bacterial infections as well as upon poly(I-C) treatment (53, 55–57). Additionally, ISG15 was able to interact with cellular proteins and with a viral protein (57) in Atlantic salmon cells infected by infectious salmon anemia virus (ISAV).

In this work, we demonstrate that zebrafish ISG15 (zf-ISG15) is a restriction factor of a number of RNA and DNA viruses. zf-ISG15 overexpression leads to ISGylation of both neosynthesized cellular and viral proteins and mediates cellular protection in an ISGylation-dependent manner. Additionally, we show that zf-ISG15 significantly modulates the expression of several key genes of the IFN pathway.

MATERIALS AND METHODS

Zebrafish infection. All animals were handled in strict accordance with good animal practice, as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and by the Regional Paris South Ethics committee. All animal work was approved by the direction of the Veterinary Services of Versailles (authorization number 78-28). Zebrafish (strain AB) were intraperitoneally injected with 10^5 PFU of IHNV (strain 25-70) and sampled at 48 h postinfection. Alternatively, fish were infected by immersion with spring viremia of carp virus (SVCV; 2.4×10^5 PFU/ml) and sampled at 72 h postinfection.

Antibodies and reagents. Primary antibodies used for Western blot analyses were the following: anti-hemagglutinin (anti-HA) monoclonal antibody was purchased from Cell Signaling, anti-green fluorescent protein (anti-GFP) polyclonal antibody and anti-V5 monoclonal antibody were from Molecular Probes, and monoclonal anti- α -tubulin antibody was from Sigma. Viral proteins were detected with rabbit antiserum raised against purified infectious hematopoietic necrosis virus (IHNV).

Cloning of *isg15* and *trim25*. Starting from the translated sequence of the trout *vig-3* cDNA that we cloned previously (51), we retrieved by TBLASTN zebrafish expressed sequence tag (EST) sequences (Unigene identifier Dr. 114892), all of which matched a unique *isg15* homologue located on chromosome 5 in the zebrafish zv9 genome assembly. Subsequent TBLASTN searches performed on the zebrafish genome, using as queries human or fish *isg15* sequences, failed to reveal other *isg15* paralogues; subsequent hits corresponded to ubiquitin genes. Primers zfISG15HA_fw and zfISG15_rv (Table 1) were designed on the basis of this sequence, with the forward primer containing the sequence for HA to generate a construct with an HA tag at the N terminus. We amplified the mature form of zebrafish *isg15* from a cDNA from the gut of an adult zebrafish injected with bacterial extract. This sequence was cloned into the mammalian expression vector pCR3.1 (Invitrogen) with an HA tag, yielding the pHA-zf-ISG15 plasmid. Synthesis of the ISG15 LRLRAA mutant from zf-ISG15 was performed by a PCR amplification reaction using primer zfISG15HA_fw and a reverse primer encoding the mutated sequence, zfISG15_LRAA_rv (Table 1). The TRIM25-coding region was amplified using primers zfTRIM25-Attb1 and zfTRIM25Attb2nostop (Table 1), cloned into the entry vector of the Gateway cloning system (Invitrogen), and then transferred into the vector pDEST6.2-V5 to be expressed with a V5 tag fused to the C terminus.

Cells and viruses. Cells of the *Epithelioma papulosum cyprini* cell line (EPC cells) were maintained in Glasgow's modified Eagle's medium (GMEM)–25 mM HEPES (Eurobio) supplemented with 10% fetal bovine serum (FBS; Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM l-glutamine (PAA), and antibiotics (100 μ g/ml penicillin [Biovalley], 100

TABLE 1 Primers

Primer	Sequence (5'→3')
zfISG15HA_fw	ATGTACCCATACGATGTTCCAGATTACGCTGGATC GGGATCGATGCAGCTGACTGTAAAACCTGC
zfISG15_rv	TTATCTCCTCGTAGACGGAGAG
zfISG15 _{LRAA} -rv	CGAACCCCTTTACGCCGCTCGTAGACGGAG
zfTRIM25-Attb1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAA GGAGATAGAACCATGGCGGAACAAATGTCTCTG
zfTRIM25Attb2nostop	GGGACCACTTTGTACAAGAAAGCTGGGTCTTAT TGAGCTTGCACAGAGAG
RIG-I-fw	TGCTGGACCGGATGTGTTATCT
RIG-I-rv	TGGTGATCGATGGTTTCGATTCT
VIG-1-fw	AGCGAGGCTTACGACTTCTG
VIG-1-rv	GCACCAACTCTCCAGAAAA
ZfISG15-fw	AACTCGGTGACGATGCAGC
ZfISG15-rv	TGGGCACGTTGAAGTACTGA
IFNPh1-fw	ATGAAAACCTCAAATGTGGACGTA
IFNPh1-rv	GATAGTTTCCACCCATTTCCITTA
E1f- α _fw	GCTGATCGTTGGAGTCAACA
E1f- α _rv	ACAGACTTGACCTCAGTGGT

$\mu\text{g/ml}$ streptomycin [Biovalley]). Transfection experiments, virus production, and titration were performed in EPC cells. The novirhabdoviruses IHNV 32-87 and VHSV 07-71 were produced at 14°C on EPC cells in GMEM supplemented with 2% fetal bovine serum, 5% tryptose, and 2 mM L-glutamine. Heat-adapted variants IHNV 25-70 and VHSV 25-111 were similarly propagated in EPC cells at 24°C, and the iridovirus epizootic hematopoietic necrosis virus (EHNV) was propagated at 20°C. The birnavirus infectious pancreatic necrosis virus (IPNV) VR299 was propagated on blue fin 2 (BF-2) cells at 14°C. Viruses were titrated on EPC cells by plaque assay as previously described (61). The cytopathic effect was evaluated at 72 h postinfection after cell fixation with 10% Formol, prior to coloration in 2% crystal violet.

Transfection. EPC cells were transfected using nucleofection (nucleo-transfected) with a Nucleofector kit T (Lonza) following the manufacturer's recommendations. Briefly, 4×10^6 EPC cells were plated in P6 wells. On the day after that, cells were trypsinized and resuspended in 100 μl of Nucleofector solution with 3 μg of DNA. After nucleotransfection, the cells were resuspended in a 6-well plate and left for 3 days at 24°C. Cells were then lysed for analyses or split at 1 million per 24-well plate for 24 h before viral infections.

Immunoprecipitations and Western blotting. Transfected cells were lysed in lysis buffer (50 mM Tris, pH 8, 300 mM NaCl, 0.5% Triton X-100) supplied with protease inhibitor cocktail (Roche). Viral proteins were immunoprecipitated with Dynabeads-protein G (Invitrogen) following the manufacturer's recommendations. Briefly, 50 μl of Dynabeads was incubated with polyclonal anti-GFP antibody (Roche), anti-phosphoprotein (anti-P) of IHNV monoclonal antibody, or anti-V5 monoclonal antibody (Invitrogen) for 1 h at room temperature. After washes, Dynabeads were incubated with cell extracts overnight at 4°C. The immunoprecipitated complex was then washed in lysis buffer, resuspended in Laemmli buffer, denatured at 90°C for 5 min before SDS-PAGE on Mini-Protean TGX gels (Bio-Rad), and transferred onto a nitrocellulose membrane using semidry transfer (Bio-Rad). Protein immunodetection was performed by incubation with anti-HA antibody (cell signaling), horseradish peroxidase (HRP)-coupled anti-HA antibody (Roche), HRP-coupled anti-GFP antibody (Roche), or anti-V5 antibody (Invitrogen) before treatment with anti-mouse or anti-rabbit HRP-conjugated antibodies (P.A.R.I.S.) and enhanced chemiluminescence detection (GE Healthcare). Protein expression was evaluated by Western blotting of 20 μg of proteins and normalized by α -tubulin detection. Alternatively, zebrafish TRIM25 (zf-TRIM25) ISGylation was determined by immunoprecipitation with agarose-immobilized anti-HA antibody (Bethyl Laboratories) prior to zf-TRIM25 immunodetection with anti-V5 or anti-HA antibodies and an antimouse-HRP conjugate as secondary antibody.

In vitro infections. At 96 h posttransfection (hpt), EPC cell monolayers were infected with the different viruses. Briefly, cells were incubated with viral inoculum at the indicated multiplicity of infection (MOI) in

GMEM–2% FBS for 1 h at 14°C. After removal of the inoculum, cells were incubated in GMEM–2% FBS at the optimal temperature of infection. Cell supernatant was taken after 24, 48, and 72 h of infection for virus titration experiments, before fixation for cytopathic effect analysis at 72 h postinfection (hpi).

Real-time qPCR. Extraction of total RNA from 4 million EPC cells was performed by use of the TRIzol reagent (Invitrogen) at 72 hpt. RNA was purified using an RNeasy minikit (Qiagen) according to the manufacturer's instructions and treated with DNase. A reverse transcription experiment was performed on 1 μg of total RNA using 125 ng of random hexamer primers (Roche) in a SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Gene expression was measured by real-time PCR with a Realplex² Mastercycler instrument (Eppendorf) using Power SYBR green PCR master mix (Applied Biosystems). Each reaction mixture contained 5 μl of primers (300 nM each), 5 μl of cDNA (diluted 1/10), and 10 μl of the PCR master mix. Samples were first incubated for 2 min at 50°C and for 10 min at 95°C and then subjected to 40 amplification cycles (95°C for 15 and 60°C for 1 min), followed by 15 s at 95°C, 15 s at 60°C, 20 min from 60°C to 95°C, and finally, 15 s at 95°C, to establish the melting curve for the PCR products. Gene expression was computed according to the ABI Prism 7700 user bulletin (Applied Biosystems) and normalized to the β -actin expression level. Primers used for quantitative PCR (qPCR) were previously described (61).

RESULTS

zf-ISG15 is involved in cell protein ISGylation. A unique *isg15* gene encoding a 157-amino-acid (aa) protein with two ubiquitin-like domains also present in other vertebrates was found in the zebrafish genome and in zebrafish ESTs (Fig. 1A). A conserved LRLRGG motif was found at the C-terminal end of the protein, which contains the diglycine motif involved in isopeptide formation with target proteins in the so-called ISGylation process. The C-terminal extension that has to be cleaved for protein activation in humans and mice was missing in the zebrafish. Phylogenetic analysis of zf-ISG15 confirmed that it represents the orthologue of mammalian ISG15 (52, 53, 57, 59) (data not shown), with all ISG15 proteins clustering in a branch distinct from ubiquitin sequences. *zf-isg15* transcript expression was assessed in the adult zebrafish upon infection by the vesiculovirus SVCV or by the novirhabdovirus IHNV (strain 25-70). Both viruses induced a strong *zf-isg15* induction in the gut (17- and 7-fold, respectively; Fig. 1B) compared to the level of expression in the noninfected state. Similar inductions were observed in the pronephros (data not shown).

We then characterized the expression of zf-ISG15 and its conjugation after transfection of an expression plasmid encoding the HA-tagged protein (pHA-zf-ISG15) in cells of a fish epithelial cell line (EPC cells). In Western blot analysis performed with anti-HA antibody, HA-zf-ISG15 appeared to be a major band of the expected size (19.2 kDa) in cellular extracts at 72 h posttransfection (Fig. 1C, lane 2). Additional bands were also revealed at higher molecular masses ranging from 36 to more than 130 kDa; these presumably corresponded to ISGylated proteins specifically detected in transfected cells. As for ubiquitin and other ubiquitin-like proteins, a C-terminal GG motif is required for ISG15 conjugation to target proteins. Hence, we generated a double mutant of zf-ISG15 carrying mutations of the C-terminal GG to AA residues (zf-ISG15_{LRAA}), which abrogate ISG15 conjugation to cellular proteins in mammals (28). Accordingly, transfection of a plasmid encoding this dominant negative mutant (pHA-zf-ISG15_{LRAA}) resulted in the detection of only the 19-kDa band corresponding to unconjugated ISG15 (Fig. 1C, lane 3).

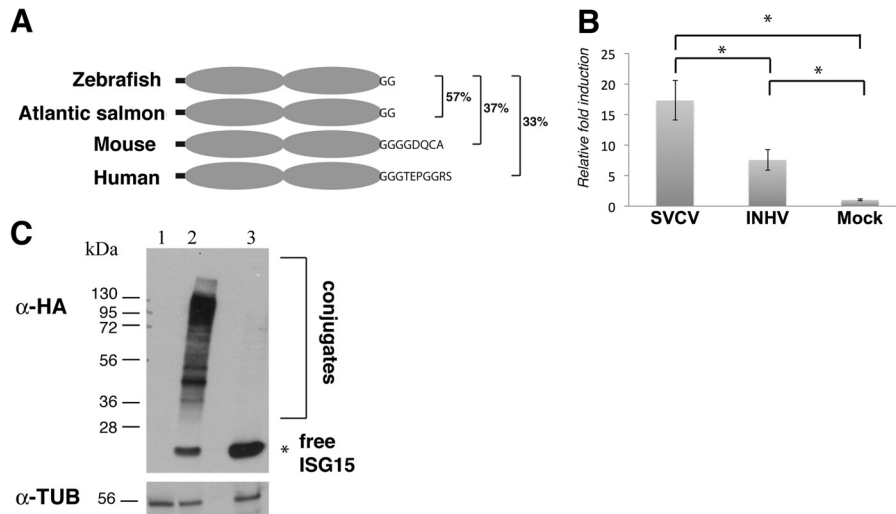


FIG 1 Characterization of zf-ISG15 expression *in vivo* and *in vitro*. (A) zf-ISG15 contains two ubiquitin-like domains (UBL) and a C-terminal peptide with a diglycine motif directly involved in ISG15 conjugation to the target proteins. The level of protein sequence identity between ISG15 from different species is indicated on the right. (B) zf-isg15 is induced upon viral infection. Adult zebrafish were infected by IHNV 25-70 or SVCV, and zf-isg15 gene expression in the gut of infected or mock-infected animals was measured by qPCR. Transcript copy numbers were normalized to the level of β -actin expression (measured ratio of mRNA of interest/ β -actin mRNA). The means and SDs of three experiments are shown. Significant differences determined using Welch's unpaired *t* test are indicated (*, $P = 0.05$). (C) HA-zf-ISG15 (the free and conjugated isoforms are indicated on the right) was detected in cell lysates of pHA-zf-ISG15-transfected EPC cells (lane 2) after anti-HA immunoblotting, while no signal was detected from mock-transfected cells (lane 1). In contrast, HA-zf-ISG15_{LRAA} mutant expression was restricted to free isoforms (lane 3). α -TUB, α -tubulin.

Taken together, these experiments show that an active ISGylation process of cellular proteins occurs in EPC cells overexpressing zf-ISG15 and involves the canonical LRGG C-terminal motif. Thus, our data indicate that the overexpression of zf-ISG15 protein is sufficient to trigger ISGylation of cellular proteins. Interestingly, in our system, the overexpression of zf-ISG15 was sufficient to trigger conjugation, which classically requires the additional overexpression of the E1, E2, and E3 enzymes, as used in mammalian cellular systems for *in vitro* ISGylation.

zf-ISG15 has antiviral activity against viruses from different families. ISG15 is well conserved and highly inducible by viral infection from fish to mammals, which suggests a conserved antiviral function across vertebrates.

We first examined whether zf-ISG15 overexpression could mediate antiviral activity against a number of RNA and DNA viruses. The list included four novirhabdoviruses: two strains of IHNV, clinical isolate IHNV 32-87 and the heat-adapted strain IHNV 25-70, and two strains of VHSV, wild-type isolate VHSV 07-71 and the heat-adapted strain VHSV 25-111. A double-stranded RNA virus and a DNA virus were also tested: IPNV, a birnavirus, and EHNV, an iridovirus. EPC cells were transfected with pHA-zf-ISG15, pHA-zf-ISG15_{LRAA}, or the empty vector for 96 h before viral infections at an MOI of 1. An inhibitory effect was determined by titration of virus from the supernatant of infected cells from 0 to 72 h postinfection, as shown in Fig. 2A. Titration curves did not reveal strong virus replication before 4 or 8 hpi. Later on, virus titers increased up to 24 hpi, before reaching a plateau with lower replication rates up to the end of the experiment.

HA-zf-ISG15 overexpression significantly inhibited IHNV growth from 3,200-fold for IHNV 32-87 to 2,200-fold for heat-adapted IHNV 25-70 at 72 hpi compared to that for noninfected and mock-transfected cells at 72 hpi. The effect on IPNV infection was even stronger, with a reduction of 4,500-fold. HA-zf-ISG15

also inhibited VHSV from 18-fold for VHSV 07-71 to 170-fold for heat-adapted VHSV 25-111 during the same period. The inhibition was a specific effect, since overexpression of the ISG15_{LRAA} mutant or GFP did not trigger any inhibition (Fig. 2A). Interestingly, a significant ISG15 antiviral effect was also observed on the DNA virus EHNV, with a reduction of 690-fold compared to the effect on nontransfected or mock-transfected cells. The numbers cited above refer to titers measured at 72 hpi, but for all viruses, attenuation of viral replication in zf-ISG15-transfected cells was already visible at 24 hpi. The efficiency of viral inhibition was dose dependent, as it was reduced with increasing MOI (data not shown).

Thus, these observations demonstrate a general antiviral effect of HA-zf-ISG15 against DNA and RNA viruses which was not observed upon HA-zf-ISG15_{LRAA} mutant expression, suggesting that it was exerted through functional ISGylation.

Western blot analysis of cell extracts performed at 24 hpi with IHNV 25-70 revealed the presence of viral proteins (Fig. 2B, lane 1), while no signal could be detected upon overexpression of zf-ISG15 (lane 3).

In parallel with the virus titration and Western blot analyses, we also evaluated the cytopathic effect of viral infection during the time course of the experiment, presented as a crystal violet coloration of cell monolayers at 72 hpi (Fig. 2C). Infection of control EPC cells with IHNV 25-70 led to a complete destruction of the cell monolayer after 72 hpi, as shown in the leftmost column of Fig. 2C. In contrast, we observed only a transient cytopathic effect at 24 hpi upon overexpression of HA-zf-ISG15, as demonstrated by crystal violet staining of the cell monolayer (Fig. 2C, IHNV 25-70). Of note, overexpression of HA-zf-ISG15 also conferred resistance to infection performed with purified virus, excluding a potential role of cytokines present in the original virus inoculum (data not shown). In accordance with the viral titrations, we also

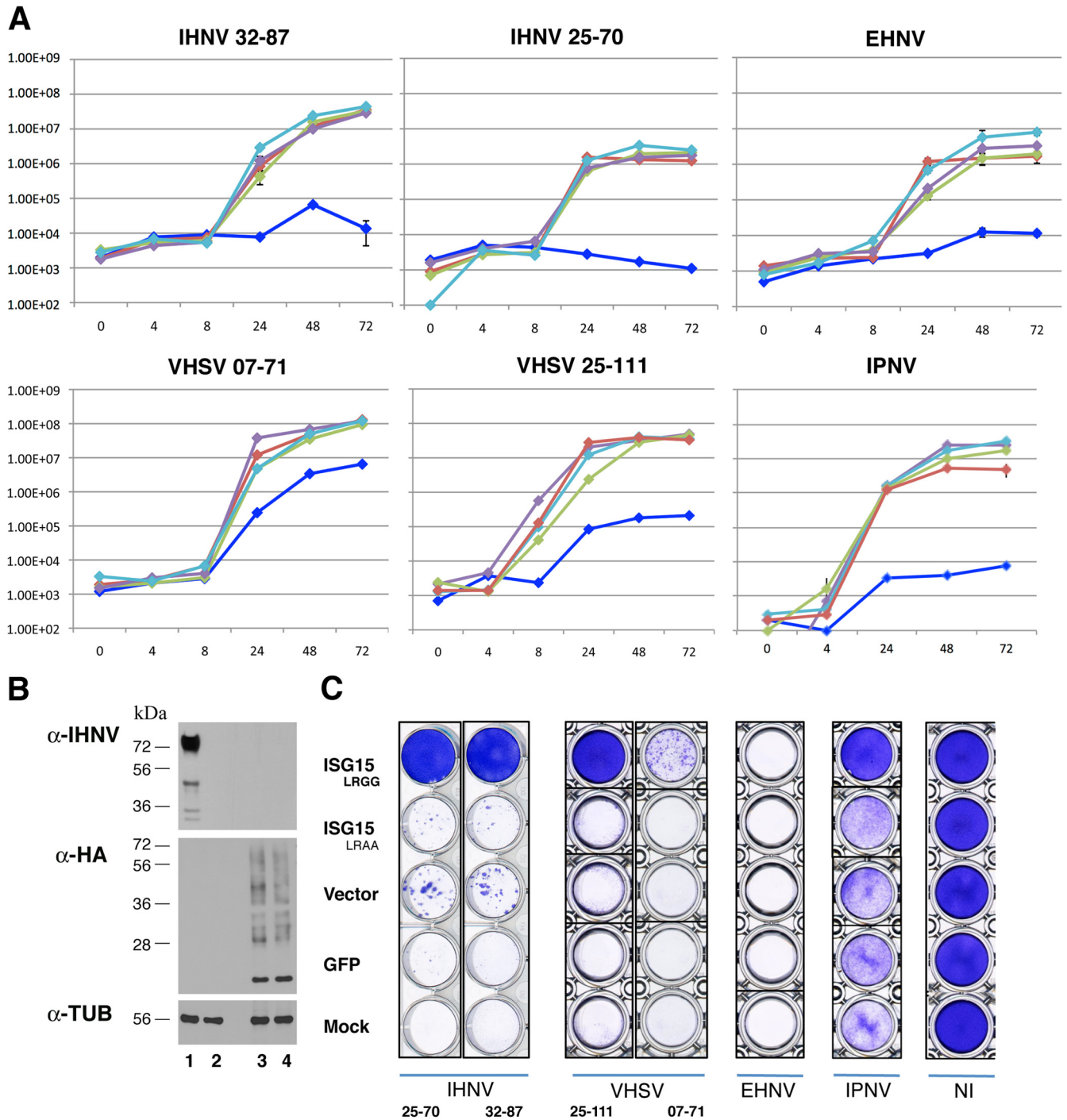


FIG 2 Inhibition of different RNA and DNA viruses by zf-ISG15 overexpression. (A) Kinetics of viral growth measured by viral titration from 0 to 72 hpi in the supernatant of EPC cells transfected with pHA-zf-ISG15_{LRGG} (dark blue), pHA-zf-ISG15_{LRAA} (red), GFP (green), or an empty vector (violet) or mock transfected (blue) and infected with the indicated RNA and DNA viruses at an MOI of 1. The means and SDs of three independent experiments are shown; the error bars are not always visible when the differences are very small. The x axis shows the times (hours) posttransfection, and the y axis shows the virus titers (PFU). (B) Western blot analysis of viral protein expression under zf-ISG15 overexpression. EPC cells were transfected with zf-ISG15 or empty vector before infection with IHNV 25-70 at an MOI of 1 for 24 h. Viral protein expression was evaluated by immunoblotting using anti-IHNV polyclonal antibody (α -IHNV), while ISG15 expression was detected by using anti-HA antibody (α -HA). α -Tubulin was used as a loading control. Lanes: 1, empty vector plus IHNV 25-70; 2, empty vector; 3, HA-zf-ISG15 plus IHNV 25-70; 4, HA-zf-ISG15. (C) Cytopathic effects of different viruses at 72 hpi on a lawn of EPC cells at an MOI 1, assessed by staining of viable cells by crystal violet. Cells had previously been transfected with pHA-zf-ISG15 (ISG15_{LRGG}), pHA-zf-ISG15_{LRAA} (ISG15_{LRAA}), GFP, or an empty vector (vector) or were mock transfected (mock). NI, noninfected cells presented as a control.

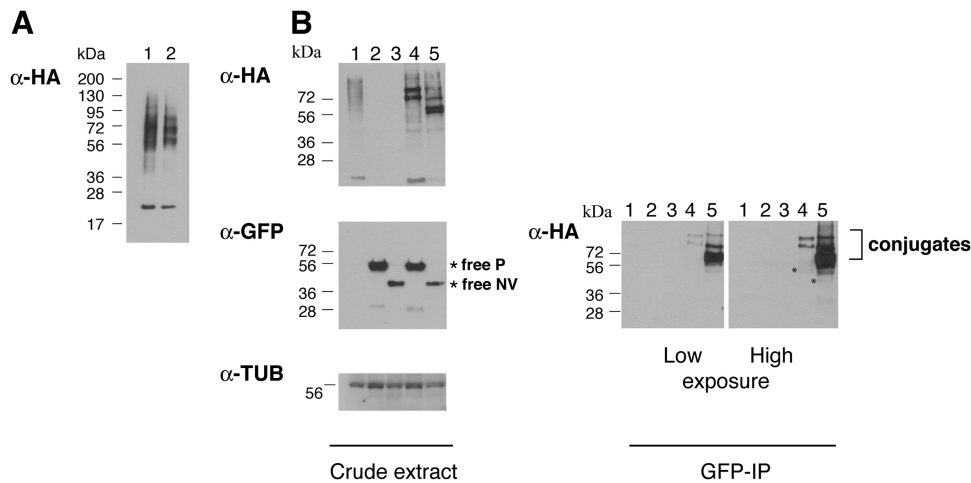


FIG 3 ISG15 conjugation targets viral proteins. (A) ISGylated proteins were immunoprecipitated with agarose-immobilized anti-HA antibody before anti-HA immunodetection from EPC cells overexpressing HA-zf-ISG15 infected (lane 1) or not (lane 2) with IHNV 25-70. (B) ISG15 binding to IHNV proteins was evaluated in EPC cell extracts at 72 hpt with expression plasmid pHA-zf-ISG15 alone or combined with IHNV NV-GFP or P-GFP. The expression level of the tagged proteins was determined from total cell lysates (crude extracts) by Western blotting using anti-HA antibody (α -HA), anti-GFP antibody (α -GFP), and α -tubulin (α -TUB) as a gel loading control. Native P and NV proteins are indicated by asterisks on the right. ISGylation of the viral proteins was then visualized by anti-HA immunodetection of proteins coimmunoprecipitated with GFP antibody (GFP-IP) after two different exposure times. Lanes: 1, pHA-zf-ISG15; 2, P-GFP; 3, NV-GFP; 4, pHA-zf-ISG15 plus P-GFP; 5, pHA-zf-ISG15 plus NV-GFP (lane 5). *, molecular masses of native viral proteins. ISG15 conjugates are indicated on the right.

observed that ISG15 overexpression prevents the cytopathic effect caused by other RNA viruses (IHNV 32-87, IHNV 25-70, VHSV 25-111, VHSV 07-71, or IPNV), while we could not detect any inhibition after overexpression of mutant ISG15 or GFP (Fig. 2C).

As EHNV infection triggers reduction of cell attachment to the culture plate, the infected cell monolayer was lost during the experiment. This was not reflecting extensive cell death, since we could still detect viral production up to 72 hpi (Fig. 2A). Despite significant viral inhibition, this loss of adherence was also observed with HA-zf-ISG15-transfected cells.

Hence, zf-ISG15 possesses powerful antiviral activity against fish novirhabdoviruses and a birnavirus. Interestingly, a similar effect was observed with a fish iridovirus, suggesting a general inhibition mechanism interfering with RNA and DNA viruses.

ISG15 overexpression triggers ISGylation of IHNV proteins regulating the IFN response. To determine whether viral proteins can be targeted for ISGylation in infected cells, we first infected HA-zf-ISG15-overexpressing cells with IHNV 25-70. Cell lysates were obtained at 24 hpi, and ISGylated proteins were immunoprecipitated through use of the HA tag. Figure 3A does not show major differences in the patterns of the ISGylated proteins between IHNV-infected cells (lane 1) and noninfected cells (lane 2), suggesting that IHNV infection does not lead to a critical modification of the repertoire of proteins targeted by ISG15. Unfortunately, the inhibition of viral growth observed upon ISG15 overexpression strongly reduced viral protein expression as soon as 24 hpi (Fig. 2B), and Western blot analysis with polyclonal antibody raised against IHNV failed to detect sufficient expression of the structural viral proteins, thus excluding the possibility that ISGylated viral proteins would be detected.

To increase the sensitivity of the assay, we reexamined the ISGylation of IHNV proteins in coexpression experiments. To this aim, EPC cells were cotransfected with pHA-zf-ISG15 and with expression plasmids for IHNV proteins fused to GFP. The nonvi-

ron protein (NV) and phosphoprotein (P) were selected because they are involved in mechanisms that counteract the IFN response in IHNV or related viruses (62–69) and would therefore represent worthy targets for ISGylation. Proper protein expression was first verified in doubly transfected cells (HA-zf-ISG15 plus GFP-tagged viral protein) by Western blotting using anti-GFP antibody (Fig. 3B, α -GFP in crude extract panel; expected molecular masses, 54.45 kDa for P-GFP and 41.91 kDa for NV-GFP), prior to secondary staining with anti-HA antibody to detect free or conjugated HA-zf-ISG15 (Fig. 3B, α -HA in crude extract panel).

ISGylation of neosynthesized viral proteins was determined by Western blotting of GFP-immunoprecipitated proteins, using anti-HA antibodies for staining (Fig. 3B, α -HA in the GFP-IP [immunoprecipitation] panel). At 72 hpt, HA-zf-ISG15 conjugates of either the GFP-NV or GFP-P proteins could be immunoprecipitated from cell lysates by anti-GFP antibody. The HA immunoreactive species (ISG15 conjugates) migrated at higher molecular masses than the native proteins (marked with asterisks) detected in cell extracts by GFP antibody, suggesting conjugation of one or several ISG15 residues on NV-GFP (expected molecular masses, 61.11, 80.31, and 99.51 kDa for the mono-, bi-, and tri-ISGylated forms, respectively) and P-GFP (expected molecular masses, 73.65 and 92.85 kDa for the mono- and di-ISGylated forms, respectively), potentially indicating the existence of mono-, bi-, and tri-ISGylated forms of the viral proteins.

Altogether, these results demonstrate conjugation of ISG15 with GFP-NV and GFP-P proteins of IHNV proteins that are involved in the subversion of the IFN response, suggesting that NV and P of IHNV may constitute new target proteins for ISG15.

The LRAA motif is required for ISGylation of cellular and viral proteins. In order to determine the HA-zf-ISG15 conjugation to cellular target proteins in our cell system, we focused on the tripartite motif protein TRIM25. In mammals, TRIM25 has been shown to be an E3 ligase that regulates the activation of the anti-

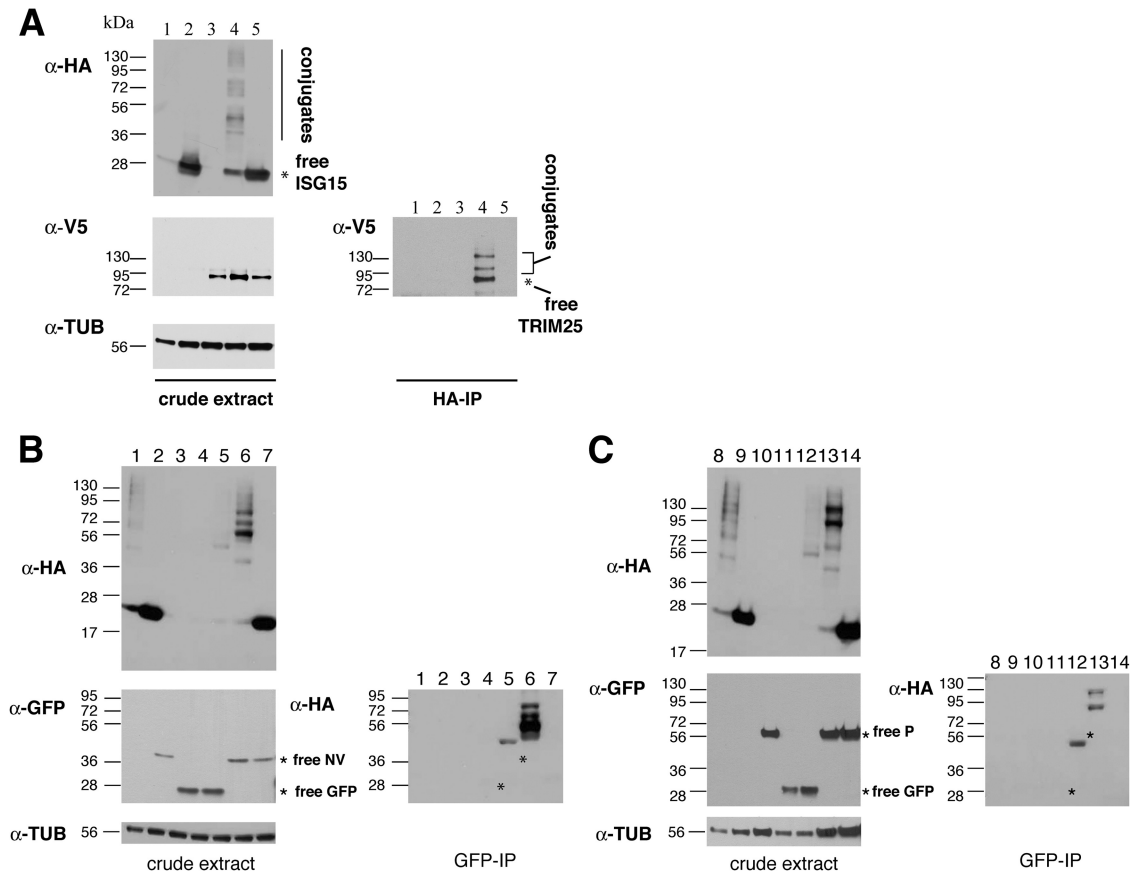


FIG 4 The LRAA motif is required for ISGylation of cellular and viral proteins. (A) Coimmunoprecipitation of HA-zf-ISG15 and zf-TRIM25-V5 with agarose-immobilized anti-HA antibody (HA-IP), prior to Western blot detection of immunoprecipitated proteins using anti-HA (α -HA) antibody. The level of expression of tagged proteins in cell lysate at 72 hpt (crude extract) was evaluated by Western blotting using anti-HA or anti-V5 antibodies and normalized with α -tubulin. *, position of native HA-ISG15 or TRIM25-V5 protein. ISGylated conjugates are indicated on the right. Lanes: 1, HA-zf-ISG15; 2, HA-zf-ISG15_{LRAA}; 3, zf-TRIM25-V5; 4, HA-zf-ISG15 plus zf-TRIM25-V5; 5, HA-zf-ISG15_{LRAA} plus zf-TRIM25-V5. ISG15_{LRAA} does not interact with the IHNV NV (B) or P (C) protein. ISGylated proteins were immunodetected with anti-HA antibody after immunoprecipitation of EPC cell extracts with anti-GFP antibody (GFP-IP). Evaluation of the level of expression of tagged proteins was performed by Western blotting on total cell lysate (crude extracts) using anti-GFP (α -GFP) and anti-HA (α -HA) antibodies, and normalization was performed with α -tubulin detection. (B) pHA-zf-ISG15 (lane 1), pHA-zf-ISG15_{LRAA} (lane 2), NV-GFP (lane 3), GFP (lane 4), pHA-zf-ISG15 plus GFP (lane 5), pHA-zf-ISG15 plus NV-GFP (lane 6), pHA-zf-ISG15_{LRAA} plus NV-GFP (lane 7); (C) pHA-zf-ISG15 (lane 8), pHA-zf-ISG15_{LRAA} (lane 9), P-GFP (lane 10), GFP (lane 11), pHA-zf-ISG15 plus GFP (lane 12), pHA-zf-ISG15 plus P-GFP (lane 13), pHA-zf-ISG15_{LRAA} plus P-GFP (lane 14). *, molecular masses of native GFP or viral proteins.

viral protein RIG-I by catalyzing the conjugation of ubiquitin and ISG15 molecules to RIG-I (6, 70). Two *trim25* paralogues can be found in the zebrafish genome, but only one of the two proteins (here named zf-TRIM25) displays all the domains of the human protein, thus being its likely functional orthologue (71). EPC cells were transfected with pHA-zf-ISG15 alone or in combination with a plasmid encoding the zebrafish TRIM25 tagged with the V5 peptide (pzf-TRIM25-V5). Interaction between HA-zf-ISG15 and zf-TRIM25-V5 was determined by Western blotting of the cellular proteins coimmunoprecipitated with HA-zf-ISG15. As shown in Fig. 4A (HA-IP panel, lane 4), zf-TRIM25-V5 (molecular mass, 75.7 kDa) was coimmunoprecipitated with HA-zf-ISG15 upon protein coexpression, while zf-TRIM25-V5 was not precipitated in the absence of HA-zf-ISG15 expression (lane 3) or upon coexpression with the conjugation-defective mutant of ISG15 (lane 5). Finally, the presence of higher bands in the HA-zf-ISG15 immunoprecipitation products was also revealed by anti-V5 Western blotting at predicted molecular masses of 95 and 114 kDa, potentially corresponding to a mono- or bi-ISGylated form of

TRIM25-V5 (lane 4). In lane 4, a band migrating at the size of the native protein was also detected; since TRIM proteins homomultimerize through their coiled-coil region (72), this band suggested the formation of oligomers containing both ISGylated and unmodified TRIM25.

In keeping with this, EPC cells were cotransfected with plasmids encoding either GFP alone or GFP-tagged NV or P viral proteins, together with pHA-zf-ISG15 or the mutant pHA-zf-ISG15_{LRAA}. Proper protein expression was verified by anti-HA and anti-GFP Western blotting (Fig. 4B and C, crude extract panels). ISGylation of viral proteins was determined by HA immunodetection of GFP-immunoprecipitated proteins, as described above. As shown in Fig. 4B and C (compare lanes 6 and 7 and lanes 13 and 14), NV and P protein immunodetection with anti-HA antibody was abrogated when ISG15 was replaced by the mutant, confirming that the GG motif is required for HA-zf-ISG15 conjugation to these viral proteins. Our results also revealed that GFP was mono-ISGylated and then migrated as a band with an apparent molecular mass of 45 kDa. This observation is in accordance

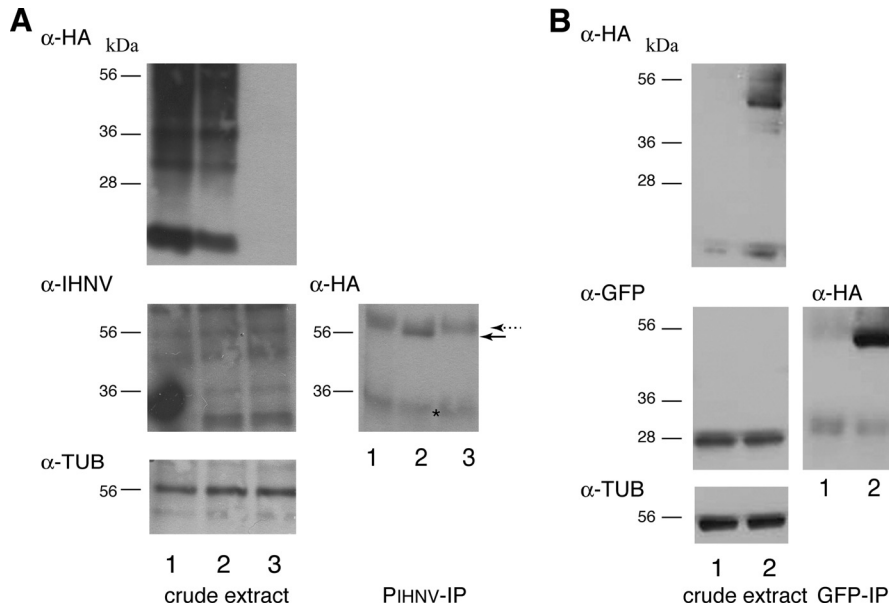


FIG 5 Anti-P immunoprecipitation supports ISGylation of the INHV P protein. ISGylated proteins were immunodetected with anti-HA antibody after immunoprecipitation of EPC cell extracts with anti-IHNV P antibody ($P_{\text{IHNV-IP}}$). Evaluation of the level of P protein expression was performed by Western blotting on total cell lysate (crude extracts) using anti-IHNV (α -IHNV) and anti-HA (α -HA) antibodies, and normalization was performed with α -tubulin detection. Lanes: 1, pHA-zf-ISG15; 2, pHA-zf-ISG15 plus INHV P; 3, INHV P. *, molecular mass of native P; plain arrow, mono-ISGylated conjugates of the P protein; dotted arrow, Ig heavy chain.

with the concept of ISGylation of *de novo*-synthesized proteins proposed by Durfee et al. (73). Since, in the same assay, GFP-NV and GFP-P were found to be tri- and bi-ISGylated, respectively, it can be deduced that the NV moiety can be conjugated with up to two ISG15 subunits, while P has one ISGylation site. To confirm the ISGylation of viral proteins (and the absence of GFP tag poly-ISGylation), the assay was reproduced upon cotransfection of pHA-zf-ISG15 and an expression plasmid of a nontagged INHV P protein, using an anti-P antibody (NV was not included due to the lack of a specific antibody). Accordingly, the pattern revealed by the anti-HA antibody from the proteins precipitated using the anti-P antibody was consistent with (mono-) ISGylation of the P protein (Fig. 5, $P_{\text{IHNV-IP}}$ panel, lane 2). Nevertheless, the lower immunoprecipitation efficiency observed with anti-P antibody did not allow us to detect the bi-ISGylated P form previously described using anti-GFP antibody. The NV protein was not included due to the lack of a specific antibody.

Altogether, these results establish that the P and NV proteins of IHNV, presumably involved in the subversion of the IFN response, constitute targets for ISGylation, consistent with the antiviral role of zebrafish ISG15 established previously.

ISGylation regulates the expression of components of the RIG-I/IFN/ISG pathways. Since ISGylation represents one of the regulatory mechanisms of the RIG-I pathway for IFN induction (8), we next investigated the impact of ISG15 overexpression on the expression of the viral sensor *rig-I*, which is functionally regulated by ISGylation. We also studied *vig-1/viperin*, an ISG highly conserved between fish and mammals also known as *rsad2* (74), and the housekeeping gene *Efl- α* as a control. Overexpression of HA-zf-ISG15 in EPC cells led to a robust induction of *vig-1/viperin* (>30-fold, normalized to the level of β -actin expression) relative to the level of expression measured in cells transfected with the empty vector (Fig. 6). At the same time, *rig-I* mRNA was

also strongly induced (17-fold) (Fig. 6), indicating that *isg15* may regulate the RIG-I pathway at several levels, since in mammals, ISG15 conjugation to RIG-I also mediates a negative-feedback loop on the activity of the IFN promoter (8). The induction of *rig-I* and *vig-1/viperin* either could be mediated by an intermediate upregulation of the IFN- ϕ gene or could occur via a direct interferon-independent pathway, as previously shown for *vig-1/viperin* in fish and in human (10, 74); in that regard, we detected a modest upregulation of IFN- ϕ gene transcripts. The requirement for IFN as an intermediate is often tested for by the addition of cyclohex-

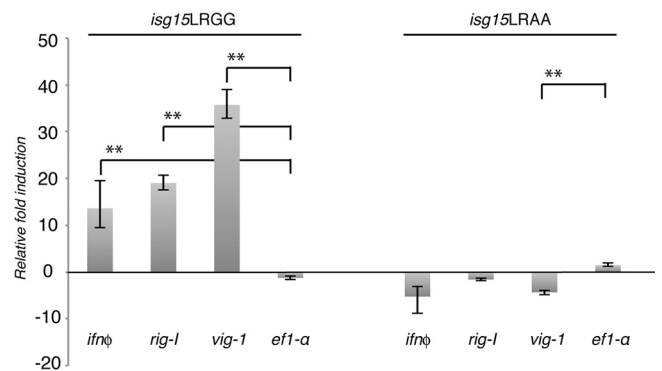


FIG 6 ISG15 modulates expression of the IFN- ϕ gene, *vig-1/viperin*, and *rig-I*. Expression of the IFN- ϕ gene, *vig-1/viperin*, *rig-I*, and *Efl- α* , in EPC cells overexpressing HA-zf-ISG15_{LRGG} or HA-zf-ISG15_{LRAA} was measured by qPCR at 72 hpt. IFN- ϕ gene, *vig-1/viperin*, *rig-I*, and *Efl- α* transcript copy numbers were normalized to the level of β -actin expression (measured ratio of mRNA of interest/ β -actin mRNA). Means and SDs of the fold induction or repression are shown for triplicates of a representative experiment. Significant differences were determined using Welch's unpaired *t* test performed on $\Delta\Delta C_T$ values (where C_T is the threshold cycle) (**, $P = 0.01$).

imide (CHX), a potent inhibitor of protein synthesis, but this strategy could not be applied to pHA-zf-ISG15-transfected cells, as it would also block HA-zf-ISG15 expression. However, when EPC cells were transfected by poly(I-C), in the presence or in the absence of CHX, we observed a significant upregulation of *vig-1/viperin* and *rig-I* transcripts, indicating that the expression of IFN proteins is dispensable and other pathways may substitute for it (data not shown). The expression of *vig1/viperin*, *rig-1*, and IFN gene transcripts was normalized to the expression of the β -actin gene; as an additional control for the upregulation of these genes, we measured the level of expression of the housekeeping gene *Efl- α* , which did not show notable modification upon ISG15 overexpression. Finally, we investigated the implication of ISGylation in *rig-I*, IFN- ϕ gene, and *vig-1/viperin* overexpression. We therefore transfected EPC cells with pHA-zf-ISG15 or pHA-zf-ISG15_{LRAA} and quantified IFN- ϕ , *rig-I*, and *vig-1/viperin* mRNAs at 72 hpt. Strikingly, when EPC cells were transfected with pHA-zf-ISG15_{LRAA} instead of pHA-zf-ISG15, we observed a modest downregulation of IFN- ϕ gene and *vig-1/viperin* transcripts and no induction of *rig-I* mRNA (Fig. 6), indicating a complex involvement of ISG15 in the transcriptional regulation of the IFN system.

Overall, these experiments revealed an additional level of regulation of the IFN pathway by ISG15. ISG15 overexpression can affect the expression of key molecules of the IFN system at the mRNA level, but not necessarily via the IFN itself. Moreover, these regulatory mechanisms likely involve ISGylated factors that are required to maintain the basal level of IFN gene and *vig-1/viperin* transcripts.

DISCUSSION

In this work, we investigated the basis of the antiviral activity mediated by the ubiquitin-like ISG15 protein from zebrafish. We showed that expression of *zf-isg15* is induced by virus infection in adult zebrafish. We further demonstrated that *zf-ISG15* overexpression in EPC cells triggers ISGylation of cellular proteins and ensures protection against different RNA and DNA viruses. This protection required functional ISGylation, since overexpression of a mutated ISG15 devoid of the conserved C-terminal diglycine motif failed to restrict virus production in the transfected cells. In addition, we analyzed the antiviral function at the molecular level, showing that the NV and P proteins of IHNV are targets for ISGylation. Moreover, our data indicate that ISG15 modulates the expression of *rig-I*, IFN- ϕ , and *vig-1/viperin* mRNAs, suggesting a key role in the regulation of the antiviral response at several levels.

Transient overexpression of zebrafish ISG15 in cyprinid EPC cells allowed us to detect ISG15 conjugates within cellular proteins, indicating a functional ISGylation process in fish cells. Surprisingly, ISGylation occurred in the absence of overexpression of the E1, E2, and E3 enzymes, which is usually required for *in vitro* ISG15 binding and conjugation to the targeted proteins. This suggests that these proteins are expressed at a significant level in EPC cells and are functionally conserved between zebrafish and fathead minnow (*Pimephales promelas*), another cyprinid species from which EPC cells originated. ISG15 overexpression was sufficient to confer robust protection against novirhabdoviruses. The inhibition was clearly shown by titration experiments as soon as 8 hpi with IHNV and VHSV (using wild-type or heat-adapted strains for both novirhabdoviruses). The level of viral proteins detected by Western blotting was drastically reduced at 10 hpi and unde-

tectable after 24 hpi in cell lysates of IHNV 32-87-infected cells when ISG15-transfected cells were compared to mock-transfected cells (data not shown). Immunolabeling performed on infected cells at 72 hpi showed that IHNV antigen was, in fact, expressed only in cells with nuclear condensation, while cells expressing ISG15 did not present IHNV staining or apoptotic nuclei. Altogether, these data indicate a strong antiviral effect mediated by ISG15 against different novirhabdovirus strains occurring in the early steps of the viral cell cycle.

We next expanded our observations to other viruses. EPC cells overexpressing ISG15 were resistant to the birnavirus IPNV (a dsRNA virus) and to the iridovirus EHN (a DNA virus) but not to the vesiculovirus SVCV (data not shown). This is in good accordance with the viral restriction mediated by mammalian ISG15 against DNA and RNA viruses (for a review, see reference 75) but excluding the vesiculovirus vesicular stomatitis virus (76) as well as the arenavirus lymphocytic choriomeningitis virus (76, 77). Thus, it seems that ISG15 exerts broad antiviral activity but that its activity may be counteracted by certain groups of viruses, such as members of the genus *Vesiculovirus*.

In humans, the free isoform of ISG15 secreted from different cell types upon interferon treatment has been shown to play the role of an antiviral cytokine (15–18). Having established the antiviral function of *zf-ISG15* when overexpressed in EPC cells, we attempted to detect an effect of secreted ISG15 on viral growth, as recently observed in fish for the tongue sole ISG15 after iridovirus infection (60). Although we could detect some ISG15 protein in the supernatant of *zf-ISG15*-transfected cells, the pretreatment of EPC cells with such a conditioned culture medium containing soluble ISG15 was not sufficient to confer protection against IHNV 25-70 infection. This lack of protection could be due to the low level of ISG15 expression in the supernatant or to improper maturation when secreted by EPC cells; in the absence of signal peptide, ISG15 should be secreted via a nonconventional pathway, which may be deficient in nonhematopoietic cells. This observation is in contrast to the findings of recent studies indicating a major cytokine function of ISG15 in different species (60, 78, 79). Interestingly, Bogunovic et al. (78) showed that extracellular ISG15 promotes IFN- γ production, modulating mycobacterial infections but having no effect on viral infections in human. In a flat fish, Wang et al. described a cytokine effect of ISG15, protecting head kidney lymphocytes of tongue sole against megalocytovirus infection (60). In both cases, this effect apparently did not require a C-terminal LRG motif and, hence, functional ISGylation. The exposure to recombinant ISG15 protein, rather, induced strong overexpression of chemokines, inflammatory cytokines, and TLR9 receptor, highlighting its role as a modulator of the immune response. In our model, *zf-ISG15* mediated antiviral activity via a distinct pathway requiring ISGylation but not the cytokine-like activity of secreted ISG15.

We further investigated the ISGylation of viral proteins, since interaction with ISG15 has been proven for proteins from HIV (80), influenza A virus (32), hepatitis C virus (41), and ISAV (57). As ISG15 induced a strong decrease of the expression of viral proteins during the first steps of infection, we were not able to detect their ISGylation under these conditions. In contrast, the nonviral protein (NV) and the phosphoprotein (P) of IHNV were found to interact with ISG15 in EPC cells in cotransfection experiments, thus appearing to be new viral targets for ISGylation. Additionally, the ISGylation of NV and P may have a significant effect

on the IFN pathway, since these proteins have been shown to mediate anti-interferon mechanisms in rhabdoviruses (65–72). Interestingly, the mutation of the ISG15 diglycine motif at the C terminus led to a loss of function of the protein, with abrogation of both ISGylation of viral proteins and loss of protection against IHNV infection, as previously described for Sindbis virus with human ISG15 (28). Thus, our results indicate that efficient ISGylation activity is required for the zf-ISG15 effect against IHNV, VSHV, IPNV, and EHN, as demonstrated for HIV-1 and Sindbis virus (28, 37). However, the viral proteins targeted by this process were not clearly identified. Altogether, these observations suggest that ISG15 may contribute to counteracting the anti-interferon activity of rhabdovirus NV and P proteins. It should be noted, however, that this interaction may also underscore a strategy used by the virus to quench the host cell antiviral response. The two views are not incompatible. Furthermore, the outcome may be dictated by the relative abundance of ISG15 and P and NV proteins. Further studies will be required to dissect this pathway and assess its importance in the innate antiviral response.

ISG15 plays a complex role in the IFN response. ISG15 is one of the genes that are highly induced by early IFN production in response to viruses and poly(I-C). In fact, not only viral infections but also bacterial infections lead to overexpression of ISG15 through various pathways (20). Besides IFN expression, protein kinase R can act as a viral sensor and induce a number of IRF3-dependent genes, including *isg15* (40). *isg15* was also found to belong to the subset of ISGs that are directly inducible by IRF1 (5) and that contribute to another alternative IFN and ISG induction pathway (81). In the same time, ISG15 is a negative regulator of the RIG-I/MAVS pathway, as it controls cellular levels of RIG-I protein through protein ISGylation (8). In fish, it has recently been established that not only IFN- ϕ but also IFN- γ induces *isg15* expression (82). Strikingly, our results indicate that ISG15 overexpression in EPC cells rapidly triggers a strong induction of *vig-1/viperin* and *rig-I* transcription. This fast upregulation gave rise to questions about the induction pathway of these ISGs. In fact, ISG15 induced the IFN- ϕ mRNA itself, although to a relatively modest extent. A kinetic analysis from 24 to 72 hpt did not reveal an early peak of IFN- ϕ , explaining the subsequent *viperin* and *rig-I* overexpression (data not shown). Interestingly, a significant induction of *viperin* and *rig-I* mRNAs was observed upon poly(I-C) transfection in the presence of cycloheximide, demonstrating the existence of an IFN-independent pathway for *vig-1/viperin* and *rig-I* induction. Unfortunately, our transfection system did not allow us to use cycloheximide to test the relative implication of the IFN-mediated and IFN-independent pathways in the induction mediated by the ISG15 expression. Altogether, our results indicate that the overexpression of ISG15 may induce IFN-dependent and/or IFN-independent upregulation of ISGs such as *vig-1/viperin* and *rig-I*. Since VIG-1/VIPERIN itself is an efficient inducer of type I IFNs (10), our findings suggest that ISG15 is involved in a positive regulatory loop of the antiviral response. Furthermore, while ISG15 negatively controls the RIG-I/MAVS signaling pathway (8) through modulation of ubiquitination (62), our results indicate that it induces the expression of *rig-I* at the mRNA level.

Overall, our data show that ISG15 plays a key role in the antiviral response across vertebrate species. We confirmed the antiviral activity of zf-ISG15 against a number of fish viruses and showed that it requires functional ISGylation activity but not

ISG15 secretion. We also identified fish novirhabdovirus proteins NV and P with potential anti-IFN properties to be ISG15 conjugation targets. In mammals, ISGylation typically acts against viral infection through targeting of a wide array of constitutively expressed proteins, thus extending the list of cellular functions affected by IFN- α/β . Our findings indicate that zebrafish ISG15 also upregulates key elements of the IFN system and appears to be involved in a positive amplification loop of the antiviral innate immunity. Future studies shall clarify the relative importance of the different antiviral pathways in which ISG15 is involved in vertebrate cells and the relative contributions of IFN-dependent and IFN-independent pathways in ISG15 activity.

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