Zebrafish ISG15 exerts a strong anti-viral activity against RNA and DNA viruses and regulates the interferon response.

C. Langevin¹, L.M. van der Aa², A. Houel¹, C. Torhy¹, V. Briolat³,4, A. Lunazzi¹,
A. Harmache¹,*, M. Bremont¹, J.-P. Levraud³,4 and P. Boudinot¹, #

¹INRA, Virologie et Immunologie Moléculaires, 78352 Jouy-en-Josas, France
²Wageningen University, Cell Biology and Immunology Group, Dept of Animal Sciences, P.O. Box 338, 6700 AH, Wageningen, The Netherlands
³Institut Pasteur, Unité Macrophages et Développement de l'Immunité, rue du Docteur Roux, F-75015 Paris, France
⁴CNRS, URA 2578 F-75015 Paris, France
* Present address: INRA, Unité Infectiologie et Santé Publique, 37380 Nouzilly, France
# Corresponding author:
Pierre Boudinot, Virologie et Immunologie Moléculaires, INRA, 78352 Jouy-en-Josas FRANCE

Phone 0033 1 34652585
Fax 0033 1 34652591
Email: pierre.boudinot@jouy.inra.fr
Abstract

ISG15, a 15kDa interferon-induced protein that participates in anti-viral 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 over-expression of zebrafish ISG15 (zf-ISG15) in EPC cells is sufficient to inhibit viral infection by RNA viruses belonging to Novirhabdovirus and Birnavirus genus and by DNA viruses of the Iridovirus genus. In co-expression experiments with IHNV proteins, we demonstrate specific ISGylation of P and NV proteins. 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 over-expression triggers induction of rig-I and viperin genes as well as ifn to a lesser extent. Overall, our data demonstrate the anti-viral 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 anti-viral response in vertebrate cells.
Introduction

Up-regulation 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 (RLR) and the membrane anchored Toll-like receptors (TLR). 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) that detects viral 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 IRF3/7 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/IL-1 receptor (TIR) domain of TLRs involved in RNA virus sensing recruits adaptor proteins like MyD88 and TIRAP and trigger NFκB- and IRF3-dependent type I IFN production. Secreted type I IFNs induce an anti-viral state in exposed cells that express IFN receptors. IFN molecules do not block directly 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 anti-viral activity has been demonstrated for a number of ISGs, including Mx, VIG-1/viperin, ISG15, OAS, PKR, RNAseL, IFITMs and TRIMs (6,7). However, even for those ISGs the detailed anti-viral mechanisms are far from being completely understood. Additionally, recent reports have indicated that several ISGs, in addition to their effector anti-viral functions, participate to the regulation of ifn expression (8-10).
One of the most expressed ISGs is Isg15 (IFN-stimulated gene 15), which was first identified as encoding a 15kDa-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 17kDa, 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 anti-ubiquitin 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), then acting 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 E1 (Ube1L), E2 (UBC8) and various E3 enzymes (Herc5, HHARI or TRIM25), whose expression is dependent on type I IFNs (20-22). The presence of a LRLRGG motif at the C-terminus of ISG15 is required for ISGylation. Whereas ubiquitin conjugation has been linked to well-described signaling pathways, cellular functions of ISGylation are still largely undefined. Large-scale analyses revealed that ISGylation targets proteins 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 eighties, ISG15 has been involved in different mechanisms of viral inhibition targeting a large spectrum of RNA and DNA viruses: Sindbis (27-29) influenza A, B (29-33), Ebola (34,35), HIV (34,36-38), Chikungunya (39), hepatitis C (40,41), herpes simplex
type I (29), murine gamma herpes virus 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 the 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 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 VLP budding (35). Furthermore Sindbis, as well as influenza A and B infections trigger ISGylation of viral and cellular proteins, which likely leads to different anti-viral 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 anti-viral activity remain to be clarified. Importantly, viruses have developed strategies to counteract the anti-viral effect of ISG15 by inhibition of ISGylation (42,43) or induction of deISGylation 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 IFNφ (48,49), that share their 3D structure with mammalian type I IFNs (50). Several important downstream effectors of the mammalian IFN system including PKR, MX, 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 anti-viral pathways assembled around the IFNs when they emerged in the first vertebrates. Hence, the characterization of anti-viral mechanisms associated with such conserved ISGs should provide hints to understand 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 norovirhabdovirus VHSV and named vig-3 for VHSV induced gene-3 (51). As in mammals, this protein contained the critical C-terminal di-glycine motif required for ISGylation thus suggesting that it could have similar functions to that 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), blackrock fish (Sebastes schlegeli) (54), Japanese flounder (Paralichthys olivaceus) (55), 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 (ISRE), and the corresponding transcripts have been found up-regulated 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 anaemia 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 over-expression 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.
Material 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 i.p. injected with $10^5$ pfu of IHNV (strain 25-70) and sampled 48 hours post infection. Alternatively, fish were infected by immersion with SVCV ($2.4 \times 10^5$ pfu/ml) and sampled 72 hours post infection.

Antibodies and reagents

Primary antibodies used for Western Blot analyses were the following: anti-HA monoclonal antibody was purchased from Cell Signaling, 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 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 EST sequences (Unigene Dr.114892), all matching 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 based on this sequence, with the forward primer containing the sequence for HA to generate a construct with a HA-tag at the N-terminus. We amplified the mature form of zebrafish isg15 from a bacterial extract-injected adult zebrafish gut cDNA. This sequence was cloned into the mammalian expression vector pCR3.1 (Invitrogen) with a HA tag, yielding the pHA-zf-ISG15 plasmid. Synthesis of ISG15 LRLRAA mutant was performed from zf-ISG15 by PCR amplification reaction using zfISG15HA_fw and a reverse primer encoding the mutated sequence zfISG15_LRAA-rv (see Table 1). TRIM25 coding region was amplified using primers zfTRIM25-Attb1 and zfTRIM25_Attb2nostop (Table 1), cloned into the entry vector of the Gateway cloning system (Invitrogen) then transferred to the vector pDEST6.2-V5 to be expressed with a V5 tag fused to the C terminus.

Cells and viruses

EPC (Epithelioma papulosum cyprini) cell line was maintained in Glasgow’s modified Eagle’s medium-HEPES 25mM medium (Eurobio) supplemented with 10% fetal bovine serum (FBS, Eurobio), 1% tryptose phosphate broth (Eurobio), 2mM L-glutamine (PAA) and antibiotics 100µg/mL Penicillin (Biovalley), 100µg/mL Streptomycin (Biovalley). Transfection experiments, viral production and titration were performed in EPC cells. The novirhabdovirus, Infectious Hematopoietic Necrosis Virus 32-87 (IHNV) and Viral Hemorrhagic Septicemia Virus 07-71 (VHSV) were produced at 14°C on EPC in GMEM media supplemented with 2% fetal bovine serum, 5% tryptose and 2mM L-glutamine. Heat adapted variants IHNV 25-70 and VHSV 25-111 were similarly propagated in EPC at 24°C and the iridovirus, Epizootic Haematopoietic Necrosis Virus (EHNV) at 20°C. The birnavirus, Infectious
Pancreatic Necrosis Virus (IPNV) VR299 was propagated on BF cells at 14°C. Viruses were titrated on EPC by plaque assay as previously described (61). Cytopathic effect was evaluated 72 hours post-infection after cell fixation with 10% formol prior to coloration in 2% cristal violet.

Transfection

EPC cells were nucleotransfected with the nucleofector kit T (Lonza) following the manufacturer’s recommendations. Briefly, 4 \(10^6\) EPC cells were plated in P6 wells. The day after, cells were trypsinized, resuspended in 100µL of nucleofector solution with 3µg of DNA. After nucleotransfection, the cells were resuspended in a P6 well and left for 3 days at 24°C. Cells were then lysed for analyses or split at 1 million per P24 well for 24hrs before viral infections.

Immunoprecipitations and Western Blot

Transfected cells were lysed in lysis buffer (50mM Tris pH8, 300mM 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, 50µL of dynabeads were incubated with polyclonal anti-GFP antibody (Roche), anti-P of IHNV monoclonal antibody or anti-V5 monoclonal antibody (Invitrogen) for 1hr at RT. After washes, dynabeads were incubated with cell extracts overnight at 4°C. Immunoprecipitated complex was then washed in lysis buffer, resuspended in Laemli Buffer, denatured at 90°C for 5min before SDS-PAGE on Mini-Protean TGX gels (Biorad) and transferred onto nitrocellulose membrane using semi-dry transfer (Biorad). Protein immunodetection was performed by incubation with anti-HA antibody (cell signaling), 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 ECL detection (GE Healthcare). Protein expression was evaluated by Western blot analysis of 20µg of proteins and normalized by α-tubulin detection. Alternatively, 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 anti-mouse HRP conjugated as secondary antibody.

**In vitro Infections**

Ninety-six hours post transfection (hpt), EPC cell monolayers were infected with the different viruses. Briefly, cells were incubated with viral inoculum at the indicated MOI in GMEM 2% FBS for 1 hour 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 72hrs of infection for virus titration experiments, before fixation for cytopathic effect analysis at 72hpi.

**Real time Q-PCR**

Total RNA extraction was performed by TRIZOL (Invitrogen) from 4 million EPC cells at 72 hpt. RNA was purified using the RNeasy mini kit (QIAGEN) according to the manufacturer’s instructions and treated with DNAse. Reverse transcription experiment was performed on 1µg of total RNA using 125ng 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 Realplex® Mastercycler Instrument (Eppendorf) using Power SYBR® Green PCR Mastermix (Applied Biosystems). Each sample is composed by 5µL...
of primers (300nM each), 5µL of cDNA (diluted 1/10) and 10µL of PCR Mastermix. Samples were first incubated for 2 minutes at 50°C and for 10 minutes at 95°C, then subjected to 40 amplification cycles (95°C for 15 and 60°C for 1 minute), followed by 15 seconds at 95°C, 15 seconds at 60°C, 20 minutes from 60°C to 95°C and finally 15 seconds at 95°C, to establish the melting curve of PCR products. Gene expressions were computed according to the ABI Prism 7700 user bulletin (Applied biosystems) and normalized to the beta-actin expression level. Primers used for Q-PCR were previously described (61).

**Results**

zfISG15 is involved in cell and viral protein ISGylation

An unique isg15 gene was found in the zebrafish genome and in zebrafish ESTs, encoding a 157 amino-acid (aa) protein with two ubiquitin-like domains also present in other vertebrates (Figure 1A). A conserved LRLRGG motif was found at the C-terminal end of the protein, which contains the di-glycine motif involved in the isopeptide formation with target proteins in the so-called ISGylation process. The C-terminal extension that has to be cleaved for protein activation in human and mice was missing in the zebrafish sequence. Phylogenetic analysis of zf-ISG15 confirmed that it represents the orthologue of mammalian ISG15 ((52,53,57,59) and data not shown), all ISG15 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, Figure 1B) as compared to non-infected 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 a fish epithelial cell line (EPC). In Western blot analysis performed with anti-HA antibody, HA-zf-ISG15 appeared as a major band at the expected size (19.2kDa) in cellular extracts at 72 hours post transfection (Figure 1C, lane 2). Additional bands were also revealed at higher molecular weights ranging from 36 to more than 130kDa, presumably corresponding 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-terminus GG to AA residues (zf-ISG15_{LRAA}), which abrogates 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 (Figure 1C, lane 3).

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

Zf-ISG15 has anti-viral 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 over-expression could mediate an antiviral activity against a number of RNA and DNA viruses. The list included four novirhabdoviruses: two strains of Infectious Hematopoietic Necrosis Virus (IHNV), the clinical isolate IHNV 32-87 and the heat adapted strain IHNV 25-70, and two strains of Viral Hemorrhagic Septicemia Virus (VHSV): the wild type isolate VHSV 07-71 and the heat adapted strain VSHV 25-111. A double-stranded RNA and a DNA virus were also tested: the Infectious Pancreatic Necrosis Virus (IPNV), a birnavirus, and the Epizootic Haematopoietic Necrosis Virus (EHNV), an iridovirus. EPC cells were transfected with pHA-zf-ISG15, pHA-zf-ISG15_{LRAA} or the empty vector for 96 hours before viral infections at MOI 1. Inhibitory effect was determined by virus titration from the supernatant of infected cells from 0 to 72 hours post-infection as shown in Figure 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 over-expression significantly inhibited IHNV growth from 3200 fold for IHNV 32-87 to 2200 fold for heat adapted IHNV 25-70 – at 72hpi - as compared to non-infected and mock transfected cells at 72hpi. The effect was even stronger on IPNV virus infection with a reduction of 4500 fold. HA-zf-ISG15 also inhibited VSHV from 18 fold for VSHV 07-71 to 170 fold for heat adapted VSHV 25-111 during the same period. The inhibition was a specific effect since over-expression of the ISG15_{LRAA} mutant or GFP did not trigger any inhibition (Figure 2A). Interestingly, a significant ISG15 anti-viral effect was also observed on the DNA EHV virus with a reduction of 690 fold as compared to non-transfected or mock-transfected cells. The numbers above refer to titers measured at 72 hpi, but for all viruses, while 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 demonstrated a general anti-viral effect of HA-zf-ISG15 against DNA and RNA viruses, which was not observed upon HA-zf-ISG15LRAA mutant expression, suggesting that it was exerted through functional ISGylation.

Western Blot analysis of cell extracts performed 24hpi with IHNV 25.70 revealed the presence of viral proteins (Figure 2B, lane 1), while no signal could be detected upon overexpression of zf-ISG15 (lane 3).

In parallel of the virus titration and Western blot analyzes, we also evaluated the cytopathic effect of viral infection during the time course of the experiment presented as a crystal violet coloration of cell monolayer at 72hpi (Figure 2C). Infection of control EPC cells with IHNV 25-70 led to a complete destruction of the cell monolayer after 72hpi as shown on the left column of Figure 2C. In contrast, we only observed a transient cytopathic effect at 24hpi upon over-expression of HA-zf-ISG15 as demonstrated by crystal violet staining of the cell monolayer (Figure 2C, IHNV 25-70). Of note, over-expression 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 observed that ISG15 overexpression prevents 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 over-expression of mutant ISG15 or GFP (Figure 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 still could detect viral production up to 72hpi (Figure 2A). Despite significant viral inhibition, this loss of adherence was also observed with HA-zf-ISG15-transfected cells.

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

**ISG15 over-expression 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 IHNV25-70. Cell lysates were obtained 24hpi and ISGylated proteins were immunoprecipitated through the HA tag. Figure 3A does not show major differences of the pattern of ISGylated proteins between IHNV infected cells (lane 1) and non-infected 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 over-expression strongly reduced viral protein expression as soon as 24hpi (Figure 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 to detect ISGylated viral proteins.

To increase the sensitivity of the assay, we re-examined ISGylation of IHNV proteins in co-expression experiments. To this aim, EPC cells were co-transfected with pHA-zf-ISG15 and with expression plasmids for IHNV proteins fused to GFP. The non-virion 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. First, proper protein expression was verified in doubly transfected cells (HA-zf-IS15 plus GFP-tagged viral protein) by Western blot using anti-GFP antibody (Figure 3B, α-GFP in crude extract panel; expected MW for P-GFP: 54.45 kDa, and for NV-GFP: 41.91 kDa) prior to second staining with anti-HA antibody to detect free or conjugated HA-zf-ISG15 (Figure 3B, α-HA in crude extract panel).

ISGylation of neo-synthesized viral proteins was determined by Western blot analysis of GFP-immunoprecipitated proteins, using anti-HA antibodies for staining (Figure 3B, α-HA in GFP-IP panel). Seventy-two hours post transfection, HA-zf-ISG15 conjugates of either GFP-NV or GFP- P proteins could be immunoprecipitated from cell lysates by anti-GFP antibody. The HA immuno-reactive species (ISG15 conjugates) migrated at higher molecular weights that native proteins (marked with asterisk) detected in cell extracts by GFP antibody suggesting conjugation of one or several ISG15 residues on NV-GFP (expected MW of 61.11; 80.31 and 99.51kDa for the mono-, bi and tri-ISGylated forms, respectively) and P-GFP (with expected MW of 73.65 and 92.85kDa for the mono- and di-ISGylated forms) potentially indicating mono, bi and tri ISGylated form of the viral proteins, respectively.

Altogether, these results demonstrate conjugation of ISG15 with GFP-NV and P proteins of IHNV viral 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-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 (hereafter 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 blot analysis of the cellular proteins co-immunoprecipitated with HA-zf-ISG15. As shown in Figure 4A (α-HA IP panel, lane 4), zf-TRIM25-V5 (MW 75.7 kDa) was co-immunoprecipitated with HA-zf-ISG15 upon protein co-expression while zf-TRIM25-V5 was not precipitated in the absence of HA-zf-ISG15 expression (lane 3) or upon co-expression 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 blot at the predicted MW of 95 and 114 kDa, potentially corresponding to a mono or bi-ISGylated form of TRIM25-V5 (lane 4). In this lane, a band migrating at the size of the native protein was also detected; since TRIM proteins homo-multimerize 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 co-transfected 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 blot (Figure 4 B and C, crude extracts panels). ISGylation of viral proteins was determined by HA immunodetection of GFP immunoprecipitated proteins as described above. As shown in Figure 4 B and C (comparing lanes 6-7 and 13-14), NV and P protein immunodetection with
anti-HA antibody was abrogated when ISG15 was replaced by the mutant, confirming that
the GG motif was required for HA-zf-ISG15 conjugation to these viral proteins. Our results
also revealed that GFP was mono-ISGylated, then migrating as a band with an apparent
molecular weight of 45kDa. This observation is in accordance 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 are found to be tri- or 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 polyISGylation),
the assay was reproduced upon co-transfection of p-HA-zf-ISG15 and an expression plasmid
of a non-tagged IHNV 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 a (mono) ISGylation
of the P protein (Figure 5, α-PiHV IP panel, lane2). Nevertheless, the lower
immunoprecipitation efficiency observed with anti-P antibody did not allow us to detect 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 over-expression 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 ef-1α as a control. Over-expression of HA-zf-ISG15 in EPC cells led to a robust induction of vig-1/viperin (>30 fold, normalized to β-actin expression) relatively to expression measured in cells transfected with the empty vector (Figure 6). In the same time, the rig-I mRNA was also strongly induced (17 fold) (Figure 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 IFN promoter (8). The induction of rig-I and vig-1/viperin could be either mediated by an intermediate up-regulation of ifnφ, 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 up-regulation of ifnφ transcripts. The requirement for IFN as an intermediate is often tested by the addition of cycloheximide (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 presence or in absence of CHX, we observed a significant up-regulation 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-I and IFN transcripts was normalized to the expression of β-actin; as an additional control for the up-regulation of these genes, we measured the expression level of the housekeeping gene Efl-α, which did not show notable modification upon ISG15 over-expression. Finally, we investigated the implication of ISGylation in the rig-I, IFNφ and vig-1/viperin over-expression. We therefore transfected EPC cells with pHα-zf-ISG15 or pHα-zf-ISG15LRAA and quantified ifnφ, rig-I, vig-1/viperin mRNAs 72 hpt. Strikingly, when EPC cells
were transfected with pHA-zf-ISG15LRAA instead of pHA-zf-ISG15, we observed a modest
down-regulation of ifnφ and vig-1/viperin transcripts, and no induction of rig-I mRNA (Figure
6), indicating a complex involvement of ISG15 in the transcriptional regulation of the IFN
system.

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

Discussion

In this work, we investigated the basis of the anti-viral activity mediated by the
ubiquitin-like ISG15 protein from zebrafish. We showed that expression of zf-isg15 is
induced in adult zebrafish by virus infection. We further demonstrated that zf-ISG15 over-
expression in EPC cell line triggers ISGylation of cellular proteins and ensured protection
against different RNA and DNA viruses. This protection required functional ISGylation since
over-expression of a mutated ISG15 devoid of the conserved C-terminal di-glycine motif
failed to restrict viral production in the transfected cells. In addition, we analyzed the anti-
viral function at the molecular level, showing that NV and P proteins of IHNV are targets for
ISGylation. Moreover, our data indicate that ISG15 modulates the expression of rig-I, ifn
and vig-1/viperin mRNAs, suggesting a key role in the regulation of the anti-viral response at
several levels.
Transient over-expression 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 over-expression 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 over-expression was sufficient to confer a robust protection against novirhabdoviruses. The inhibition was clearly showed by titration experiments a soon as 8 hpi with IHNV and VHSV (using WT or heat adapted strains for both novirhabdoviruses). The level of viral proteins detected by Western blot was drastically reduced at 10 hpi and undetectable after 24hpi in cell lysates of IHNV 32-87 infected cells comparing ISG15- to mock- transfected cells (data not shown).

Immunolabelling performed on infected cells 72hpi showed that IHNV antigen was in fact only expressed in cells with nuclear condensation while cells expressing ISG15 did not present IHNV staining or apoptotic nuclei. Altogether, these data indicate a strong anti-viral 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 over-expressing ISG15 were resistant to the birnavirus IPNV (dsRNA virus) and to the iridovirus EHNV (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 review (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 a broad
antiviral activity, but that it may be counteracted by certain groups of viruses such as Vesiculovirus.

In humans, the free isoform of ISG15 secreted upon interferon treatment from different cell types has been shown to play a role of anti-viral cytokine (15-18). Having established the anti-viral function of zf-ISG15 when over-expressed in EPC cells, we attempted to detect an effect of secreted ISG15 on the 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 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 expression level of ISG15 in the supernatant or to an improper maturation when secreted by EPC cells; in absence of signal peptide ISG15 should be secreted via a non-conventional pathway, which may be deficient in non-hematopoietic cells. This observation is in contrast with recent studies indicating a major cytokine-function of ISG15 in different species (60,78,79). Interestingly, Bogunovic et al. showed that extracellular ISG15 promotes IFN-γ production, modulating mycobacterial infections but having no incidence on viral infections in human. In a flat fish, Wang et. al described a cytokinic effect of ISG15 protecting HK lymphocytes of Tongue sole against megalocytovirus infection (60). In both cases, this effect apparently did not require a C terminal LRGG motif, hence functional ISGylation. The exposure to recombinant ISG15 protein rather induces a strong over-expression of chemokines, inflammatory cytokine and TLR9 receptor highlighting a role of modulator of the immune response. In our model, zf-ISG15 mediated an anti-viral activity via a distinct pathway requiring ISGylation, but not a cytokine-like activity of secreted ISG15.
We further investigated ISGylation of viral proteins, since interaction with ISG15 has been proven for proteins from HIV (80), influenza A (32), hepatitis C (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 non-virion protein (NV) and the phosphoprotein (P) of IHNV were found to interact with ISG15 in EPC cells in co-transfection experiments, thus appearing as new viral targets for ISGylation. Additionally, the ISGylation of NV and P may have 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 C terminus di-glycine motif led to a loss of function of the protein, with abrogation of both ISGylation of viral proteins and loss of protection against IHNV viral infection, as previously described for Sindbis virus with human ISG15 (28). Thus, our results indicate that an efficient ISGylation activity is required for the zf-ISG15 effect against IHNV, VSHV, IPNV and EHNV, 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 counteract the anti-interferon activity of NV and P rhabdovirus 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 – both views not being incompatible, with an outcome 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 anti-viral response.

ISG15 plays a complex role in the IFN response. ISG15 is one of the genes that are highly induced by the early IFN production in response to viruses and poly(I:C). In fact, viral but also bacterial infections lead to over-expression of ISG15 through various pathways (20). Besides IFN expression, PKR protein can act as a viral sensor and induce a number of IRF3-
dependant genes including isg15 (40). Isg15 was also found to belong to the subset of ISGs directly inducible by IRF1 (5), which 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 been recently established that not only IFNφs but also IFN-γ induce isg15 expression (82).

Strikingly, our results indicate that ISG15 over-expression in EPC cells rapidly triggers a strong induction of vig-1/viperin and rig-I transcription. This fast up-regulation gave rise to questions about the induction pathway of these ISGs. In fact, ISG15 induced the IFNφ mRNA itself, although to a relatively modest extent. A kinetic analysis from 24 to 72hpt did not reveal an early peak of IFNφ explaining the subsequent viperin and rig-I over-expression (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 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 over-expression of ISG15 may induce IFN dependent and/or independent up-regulation 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 controls negatively 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 anti-viral response across vertebrate species. We confirmed the anti-viral activity of zf-ISG15 against a number of fish viruses, and show that it requires functional ISGylation activity but not ISG15 secretion. We also identified fish novirhabdovirus proteins NV and P with potential anti-IFN properties, as 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-alpha/beta. Our findings indicate that zebrafish ISG15 also up-regulate 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.

Acknowledgements

This work was supported by the Institut National de la Recherche Agronomique, by the Wageningen University and by the Institut Pasteur. It was partly funded by the ANR grant “Zebraflam” (ANR-10-MIDI-009). We thank N. Palmic for excellent technical help with zebrafish infections.
Figure legends

Figure 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 di-glycine motif directly involved in ISG15 conjugation to the target proteins. 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 measured in the gut by qPCR in infected or mock infected animals. Transcript copy numbers were normalized to ß-actin expression (measured ratio of mRNA of interest/ß-actin mRNA). The mean and SD of three experiments is shown; stars indicate significant differences using Welch's unpaired t test (*: p=0,05) (C) HA-zf-ISG15 (free and conjugated isoforms indicated on the right side of the figure) is detected in cell lysate of p-HA-zf-ISG15 transfected EPC (lane2) after anti-HA immunoblot, while no signal is detected from mock transfected cells (lane1). In contrast, the HA-zf-ISG15_{LRAA} mutant expression is restricted to free isoforms (lane3).

Figure 2. Inhibition of different RNA and DNA viruses by Zf-ISG15 over-expression.

(A) Kinetic of viral growth measured by viral titration from 0 to 72hpi in the supernatant of EPC cells transfected with pHA-zf-ISG15_{LRGG} (dark blue), p-HA-zf-ISG15_{LRAA} (red), GFP (green), with an empty vector (violet) or mock transfected (blue) and infected with RNA and DNA viruses indicated on the figure, at MOI 1. The mean and SD of three independent experiments are shown; the error bars are not always visible when the differences are very small. (B) Western Blot analysis of viral proteins expression under zf-ISG15 overexpression. EPC cells were transfected with zf-ISG15 or empty vector before infection with IHNV 25-70.
MOI 1 for 24 hours. Viral protein expression was evaluated by immunoblot using anti-IHNV polyclonal antibody (α-IHNV), while ISG15 expression was detected by anti-HA antibody (α-HA). Tubulin was used as loading control (α-tub): Empty vector+IHNV25-70 (lane1), Empty vector (lane2), HA-zf-ISG15+IHNV25-70 (lane3), HA-zf-ISG15 (lane4). (C) Cytopathic effects of different virus 72hpi on a lawn of EPC cells at a MOI 1, assessed by staining of viable cells by crystal violet. Cells had been previously transfected with pHA-zf-ISG15 (ISG15LRGG), pHA-zf-ISG15LRAA (ISG15LRAA), GFP with an empty vector (vector) or mock transfected (mock). Non-infected cells are presented as a control (NI).

Figure 3. ISG15 conjugation targets viral proteins.

(A) ISGylated proteins were immunoprecipitated with agarose immobilized anti-HA antibody before anti-HA immunodetection from EPC cells over-expressing 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 extract 72hpt with expression plasmid pHA-zf-ISG15 alone or combined with IHNV NV-GFP or P-GFP. Expression level of the tagged proteins was determined from total cell lysates (crude extracts) by Western blot 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 were indicated by asterisks on the right side of the figure. ISGylation of the viral proteins was then visualised by anti-HA immunodetection (α-HA) of proteins co-immunoprecipitated with GFP antibody (GFP-IP) after two different exposure times. p-HA-zf-ISG15 (lane1), P-GFP (lane2), NV-GFP (lane3), p-HA-zf-ISG15+P-GFP (lane4) and p-HA-zf-ISG15+NV-GFP (lane5). Asterisks indicated native viral proteins molecular weights, while ISG15 conjugates were indicated on the right side of the figure.

Figure 4. The LRAA motif is required for ISGylation of cellular and viral proteins
Co-immunoprecipitation of HA-zf-ISG15 and zf-TRIM25-V5 with agarose immobilized anti-HA antibody (α-HA-IP), prior to Western blot detection of immunoprecipitated proteins using anti-HA (α-HA) antibody. The level of expression of tagged proteins was evaluated in cell lysate 72hpt (crude extract) by Western blot using anti-HA or anti-V5 antibodies and normalized with α-tubulin. A asterisks indicates the position of native TRIM25-V5 protein, while ISGylated conjugates are indicated on the right side of the figure. HA-zf-ISG15 (lane1), HA-zf-ISG15_{LRAA} (lane2), zf-TRIM25-V5 (lane3), HA-zf-ISG15+ zf-TRIM25-V5 (lane4), HA-zf-ISG15_{LRAA}+ zf-TRIM25-V5 (lane5). ISG15_{LRAA} does not interact with NV-IHNV (B) and P-IHNV (C) viral proteins. ISGylated proteins were immunodetected with anti-HA antibody after immunoprecipitation of EPC cell extracts with anti-GFP antibody (GFP-IP). Evaluation of the expression level of tagged proteins was performed by Western blot analysis on total cell lysate (crude extracts) using anti-GFP (α-GFP) and anti-HA antibodies (α-HA) and normalization was performed with tubulin detection (α-tub). (A) p-HA-zf-ISG15 (lane1), p-HA-zf-ISG15_{LRAA} (lane2), NV-GFP (lane3), GFP (lane4), p-HA-zf-ISG15+GFP (lane5), p-HA-zf-ISG15+ NV-GFP (lane6), p-HA-zf-ISG15_{LRAA}+ NV-GFP (lane7); (B) p-HA-zf-ISG15 (lane8), p-HA-zf-ISG15_{LRAA} (lane9), P-GFP (lane10), GFP (lane11), p-HA-zf-ISG15+GFP (lane12), p-HA-zf-ISG15_{LRAA}+ NV-GFP (lane13), P-GFP (lane14). An asterisk indicated native viral proteins molecular weights, while ISG15 conjugates were indicated on the right side of the figure.

Figure 5. The anti-P immunoprecipitation supports an ISGylation of the INHV P protein.

ISGylated proteins were immunodetected with anti-HA antibody after immunoprecipitation of EPC cell extracts with anti-P antibody (P_{INHV}-IP). Evaluation of the expression level of the P protein was performed by Western blot analysis on total cell lysate (crude extracts) using anti-HA (α-HA) antibody. The level of expression of tagged proteins was evaluated in cell lysate 72hpt (crude extract) by Western blot using anti-HA or anti-V5 antibodies and normalized with α-tubulin. A asterisks indicates the position of native TRIM25-V5 protein, while ISGylated conjugates are indicated on the right side of the figure. HA-zf-ISG15 (lane1), HA-zf-ISG15_{LRAA} (lane2), zf-TRIM25-V5 (lane3), HA-zf-ISG15+ zf-TRIM25-V5 (lane4), HA-zf-ISG15_{LRAA}+ zf-TRIM25-V5 (lane5). ISG15_{LRAA} does not interact with NV-IHNV (B) and P-IHNV (C) viral proteins. ISGylated proteins were immunodetected with anti-HA antibody after immunoprecipitation of EPC cell extracts with anti-GFP antibody (GFP-IP). Evaluation of the expression level of tagged proteins was performed by Western blot analysis on total cell lysate (crude extracts) using anti-GFP (α-GFP) and anti-HA antibodies (α-HA) and normalization was performed with tubulin detection (α-tub). (A) p-HA-zf-ISG15 (lane1), p-HA-zf-ISG15_{LRAA} (lane2), NV-GFP (lane3), GFP (lane4), p-HA-zf-ISG15+GFP (lane5), p-HA-zf-ISG15+ NV-GFP (lane6), p-HA-zf-ISG15_{LRAA}+ NV-GFP (lane7); (B) p-HA-zf-ISG15 (lane8), p-HA-zf-ISG15_{LRAA} (lane9), P-GFP (lane10), GFP (lane11), p-HA-zf-ISG15+GFP (lane12), p-HA-zf-ISG15_{LRAA}+ NV-GFP (lane13), P-GFP (lane14). An asterisk indicated native viral proteins molecular weights, while ISG15 conjugates were indicated on the right side of the figure.
Figure 6. ISG15 modulates the expression of \textit{ifn\(\phi\) vig-1/viperin} and \textit{rig-1}.

Expression of \textit{ifn\(\phi\) vig-1/viperin}, \textit{rig-1} and \textit{elf} in EPC cells over-expressing HA-zf-ISG15_{LRGG} or HA-zf-ISG15_{LRAA} was measured by qPCR 72hpt. \textit{ifn\(\phi\) vig-1/viperin}, \textit{rig-1} and \textit{Ef1-\alpha} transcript copy numbers were normalized on the \textit{\beta}-actin expression (measured ratio of mRNA of interest/\textit{\beta}-actin mRNA). Mean and SD of induction or repression fold are shown, for triplicates of a representative experiment; stars indicate significant differences using Welch’s unpaired t test performed on \(\Delta\Delta\text{Ct}\) values (**: \(p=0.01\)).
References


Figure 2.

A

IHNV 32-87 | IHNV 25-70 | EHNV

VHSV 07-71 | VHSV 25-111 | IPNV

B

\( \alpha \)-IHNV

\( \alpha \)-HA

\( \alpha \)-TUB

C

ISG15

ISG15

Vector

GFP

Mock

IHNV 25-70 | IHNV 32-87

VHSV 25-111 | VHSV 07-71

EHNV | IPNV | NI
Figure 5.
Figure 6.
Table 1. Primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-&gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>zfISG15HA-fw</td>
<td>ATUTACCCCATACGATGTTCCAGATATTACGCTGGAATCGATCGATCGACGCTGACTGTAAGAACTGAC</td>
</tr>
<tr>
<td>zfISG15-fv</td>
<td>TTAATCTCTCTGTAAGACCAGAGGAGGAG</td>
</tr>
<tr>
<td>zfISG15LRAA-rv</td>
<td>CGAACCCCTTTTACCCCGCTCTGTAAGACCAGAG</td>
</tr>
<tr>
<td>zfTRIM25-Attb1</td>
<td>GGGGACAAATTTTTGTAACAAAAGCGAGCTTCCCGAAAGAGATAGACGACGCTGACTGTAAGAACTGAC</td>
</tr>
<tr>
<td>zfTRIM25Attb1nostop</td>
<td>GGGGACAGTTTTTACACAAGAACGAGCTTCCCGAAAGAGATAGACGACGCTGACTGTAAGAACTGAC</td>
</tr>
<tr>
<td>RIG-I-fw</td>
<td>TGCCTGGACCCGATUTGTATATC</td>
</tr>
<tr>
<td>RIG-I-rv</td>
<td>TGCTGATCGATGTTGTTGATTCT</td>
</tr>
<tr>
<td>VIG-1-fw</td>
<td>AGCCGAAATCCCTCCAGAAAA</td>
</tr>
<tr>
<td>VIG-1-rv</td>
<td>GCAAAACTCTCCCGACAAAG</td>
</tr>
<tr>
<td>ZFISG15-fw</td>
<td>AACTCGGGCAGGACGAGGACG</td>
</tr>
<tr>
<td>ZFISG15-rv</td>
<td>TCGCAACTTTGAAAGTACTA</td>
</tr>
<tr>
<td>IFNPhi1-fw</td>
<td>ATGAAACACCTCAAAGUGUGAGACA</td>
</tr>
<tr>
<td>IFNPhi1-rv</td>
<td>CAGATTCCACCCATTTCTAAA</td>
</tr>
<tr>
<td>Eif-α-fw</td>
<td>GCTGATCGTTGAAAGTACA</td>
</tr>
<tr>
<td>Eif-α-rv</td>
<td>ACCAATTTGCACTTTGCTG</td>
</tr>
</tbody>
</table>