Differential antiviral activity of European sea bass interferon-stimulated 15 protein (ISG15) against RGNNV and SJNNV betanodaviruses

Patricia Moreno, Daniel Alvarez-Torres, Esther Garcia-Rosado, Juan J Borrego, M Carmen Alonso*

Universidad de Málaga, Departamento de Microbiología, Facultad de Ciencias, 29071 Málaga, Spain

Running title: Anti-betanodavirus activity of European sea bass ISG15

*Correspondence

Keywords: Transfection, betanodavirus, European sea bass, ISG15, antiviral activity, immunomodulation

ABSTRACT

ISG15 is an antiviral protein acting intracellularly, by conjugation to viral or cellular proteins, or extracellularly, as cytokine. In this work, an *in vitro* system, consisting of E-11 cells over-expressing European sea bass ISG15 (Dl_ISG15_E11 cells), has been developed to evaluate the European sea bass ISG15 protein activity against RGNNV and SJNNV isolates. Regarding RGNNV, RNA2 copy number and viral titres were similar in E-11 and Dl_ISG15_E11 cells, and the cellular survival analyses demonstrated that Dl_ISG15_E11 cells were not protected against this virus. In contrast, ISG15 compromises SJNNV replication, since a reduction of the SJNNV genome synthesis has been recorded. The ISG15 anti-SJNNV activity was confirmed by viral titration and survival assays. In addition, a role of the intracellular ISG15 in modulating the transcription of endogenous genes has being recorded, with *tlr3* gene being knocked out and *e3* gene being up-regulated in RGNNV-inoculated Dl_ISG15_E11 cells. Sea bass ISG15 has also been detected extracellularly, and its activity has been evaluated by co-culture. The survival rate of RGNNV-inoculated E-11 cells increased from 25% to 46% when they were co-cultured with ISG15-producing cells. Similarly, the survival rate of SJNNV-inoculated E-11 cells increased from 27% to 51% in co-culture with ISG15-producing cells. To our knowledge, this is the first description of a differential antiviral activity of an ISG15 protein against two betanodavirus species, and the first evaluation of the cytokine-like activity of a fish ISG15 protein on non-immune cells.

1. Introduction

Type I interferon (IFN I) is a cytokine involved in the antiviral innate immune system of vertebrates [1–3]. After viral infection, cells secrete IFN I, which is detected by neighbouring cells, inducing the transcription of numerous interferon-stimulated genes (ISGs), including the interferon-stimulated gene 15 (*isg15*). The *isg15*-encoding protein (ISG15) is a 15-kDa protein composed of two ubiquitin-like domains (UBL), connected by a short linker sequence, and a conserved C-terminal RLRGG motif, which is required for conjugation to viral or cellular proteins, in a process called ISGylation.

ISGylation occurs through a pathway similar to that of ubiquitination [4], resulting in the modification of some characteristics of the conjugated proteins, such as location, stability and activity [5]. In mammals, hundreds of proteins, involved in all stages of cellular biology, have been described as ISGylation-target proteins [6–8]. Regarding viral infections, ISG15 has been described to limit virus release by conjugating cellular proteins involved in ligase activity and secretory pathways, such as Nedd4, TSG101 and CHMP5, in Ebola virus, influenza virus and HIV-1 virus infections, respectively [9–11], or conjugating viral proteins, such as influenza NS1 and NP proteins [12,13].

ISG15 proteins have also been found extracellularly, acting as cytokine [14,15]. Thus, this protein has been detected in IFN I-treated human leukocyte and monocyte cultures [14,16], promoting IFN-γ production by immune system cells, mainly natural killers (NK) and lymphocytes T [14,17–19].

Viral nervous necrosis is a widely spread neuropathological disease that affects European sea bass (*Dicentrarchus labrax*), specially at larval and juvenile stages [20–24]. Main signals of this disease are lesions in nervous tissues, causing alterations in swimming and floatability, anorexia and visual anomalies. The etiological agent is the

nervous necrosis virus (NNV, *Betanodavirus* genus), a single-stranded, positive-sense RNA virus, with a bipartite genome composed of RNA1 and RNA2 segments, coding the viral polymerase and the capsid protein, respectively [25]. Based on the nucleotide variability in the RNA2 segment, betanodaviruses have been clustered into four species (former genotypes) [26,27]; however, only red-spotted grouper- (RGNNV) and striped jack- (SJNNV) nervous necrosis virus have been detected in sea bass, which is susceptible to both viral species, although causing very different mortality rates, as it has been demonstrated by experimental infections [28,29].

In fish, *isg15* is one of the earliest and most highly-expressed ISGs following viral infections [30–34]. Recently, Moreno et al. [35] have described the structure of European sea bass *isg15*, which is similar to that described for other fish *isg15* genes, being composed of one 510-bp long open reading frame (ORF), with an intron located in the 5'-untranslated region (UTR). The *in vivo* transcription of this gene after RGNNV and SJNNV inoculation has also been evaluated, demonstrating that both viral species are strong *isg15* inductors in sea bass brain and head kidney [35,36].

In addition, studies on the antiviral activity of ISG15 proteins from several fish species, such as grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*), have revealed activity against several fish viruses, such as grouper nervous necrosis virus (GGNV) [37], infectious hematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and epizootic haematopoietic necrosis virus (EHNV) [38].

The aim of the present study has been to study the role of the European sea bass ISG15 protein against betanodavirus infections. As a first step, the *isg15* ORF has been cloned into an expression vector in order to develop an *in vitro* system suitable for evaluating the antiviral activity of this protein.

2. Materials and methods

2.1. Viruses and viral propagation

Viral isolates SpDl_IAusc965.09 (RGNNV) [35] and SJ93Nag (SJNNV) [39] were propagated and titrated on the E-11 cell line [40].

E-11 cells were grown in Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 unit/ml penicillin and 10 mg/ml streptomycin (Sigma) (growth medium) at 25 ^{o}C until confluence. Once inoculated, these cells were maintained in L-15 supplemented with 2% FBS, 100 units/ml penicillin and 10 mg/ml streptomycin (maintenance medium) at 25 ^{o}C (RGNNV) or 20 ^{o}C (SJNNV), until cytopathic effect (CPE) development. Viral suspensions were titrated on E-11 cells following the 50% tissue culture infective dose method ($TCID_{50}$) [41].

2.2. Establishment of a cell line constitutively expressing sea bass ISG15 protein (Dl_ISG15)

Sea bass *isg15* cDNA was obtained from juvenile specimens (n = 3) (6 g, average weight) intraperitoneally injected with polyinosinic:polycytidylic acid (poly I:C, CalBiochem, 1 mg/fish). At 8 h post-infection (p.i.), fish were killed by anaesthetic overdose (MS-222, Sigma), and head kidneys were frozen in liquid nitrogen until used. Head kidneys were individually homogenized, and total RNA was extracted with the TRI reagent solution (Sigma). After RNA treatment with DNase I Recombinant from

bovine pancreas (Roche), cDNA was synthesized using 1 μg of RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Sea bass *isg15* was amplified using the pair of primers DlISG15 NotI-F (5'-GATCACAAGGGCGGCCGCATGATGGATATAACC-3') and DlISG15 XhoI-R (5'-TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG-3'), and the Universe High-Fidelity Hot Start DNA polymerase (Biotools), as described by Moreno et al. [35]. Amplified products were cloned in the pGemT easy vector (Promega) and subsequently sub-cloned in the expression vector pcDNA™4/HisMax B (Invitrogen), containing the cytomegalovirus (CMV) promoter, a polyhistidine metal-binding tag, and the Zeocin™ resistance gene. Purified plasmids (labelled as Dl_ISG15_pcDNA) were stored at -20 ^{o}C until cell transfection.

Monolayers of E-11 cells grown on 6-well plates were transfected using the Lipofectamine® 2000 Transfection Reagent (Invitrogen), according to Iwamoto et al. [42]. Transfected cells were sub-cultured in 25-cm^2 flasks and selected with zeocine (1250 μg/ml, Invitrogen) to obtain the Dl_ISG15_E11 cell line.

2.3. Characterization of the Dl_ISG15_E11 cell line

Analyses of cellular growth kinetics, ISG15 immuno-detection, and *isg15* gene transcription have been performed.

For the cellular growth analysis, multiplication of Dl_ISG15_E-11 and E-11 cells has been comparatively evaluated. Both cell lines were seeded (1.4x10^5 cells/ml) on 24-well plates, and maintained in growth medium at 25 ^{o}C. Cells from three wells were collected at 2, 3, 4, 5, 7 and 8 days to be counted, in triplicate, using the Z1 coulter (Beckman Coulter). This assay was performed in duplicate.

144 The Dl_ISG15 protein has been detected by western blotting. Supernatants and cells
145 from both cell lines, grown in 75-cm^2 flasks, were separately collected and processed to
146 detect extracellular and intracellular recombinant Dl_ISG15 protein.
147 Extracellular Dl_ISG15 was purified and concentrated with an immuno-precipitation
148 kit (Roche), using A/G agarose beads, and the HisG Epitope Tag Monoclonal Antibody
149 (Invitrogen, Thermo), according to commercial guidelines.
150 For intracellular Dl_ISG15 detection, cells were washed with PBS, sonicated at 4 ^{o}C
151 for 10 min, and centrifuged at 1500 x*g* at 4 ^{o}C for 5 min. Proteins in the resulting
152 supernatant were concentrated using the centrifugal filter Amicon® Ultra-15 (Merck),
153 with a molecular weight cut-off of 3000, at 5000 x*g* for 30 min. The resulting sample
154 was analysed by western blot.
155 For western blotting, proteins separated by 12.5% SDS-PAGE electrophoresis were
156 transferred to an Immobilon membrane (Millipore) [43]. The HisG Epitope Tag
157 Monoclonal Antibody (1/1000 in 5% skimmed-powder milk-PBS-Tween20) was used
158 to detect the 6xHis-tagged recombinant ISG15 protein. The monoclonal Anti-β-Actin
159 Antibody (Sigma, 1/5000 in 5% skimmed-powder milk-PBS-T) was used as cellular
160 control. The secondary antibody used was HRP-conjugated goat anti-mouse IgG
161 (Sigma, 1/20000 in 5% skimmed-powder milk-PBS-T). The specific band was
162 visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo)
163 and the ChemiDoc™ Imaging Systems (Bio-Rad).
164 The effect of viral infection on sea bass *isg15* transcription has been evaluated by
165 relative RT real-time PCR in presence or in absence of RGNNV or SJNNV. Each virus
166 was inoculated at 0.1 multiplicity of infection (MOI) on Dl_ISG15_E-11 cells grown on
167 24-well plates. Cells from three wells were harvested at 24 h p.i., and total RNA was
168 extracted with the E.Z.N.A. total RNA Kit I. After treatment with DNase I Recombinant

169 from bovine pancreas, RNA (1 μg) was reverse-transcribed with the Transcriptor First
170 Strand cDNA Synthesis Kit. Amplifications of *isg15* were performed following the
171 methodology described by Moreno et al. [35], using the specific primers shown in Table
172 1, and *beta-actin* as endogenous reference gene. Relative values were calculated by the
173 $2^{-\Delta Ct}$ method. This assay was performed in duplicate.
174
175 *2.4. Anti-betanodavirus activity of intracellular sea bass ISG15 protein*
176
177 Viral replication on Dl_ISG15_E11 and E-11 cells has been comparatively analysed
178 by virus quantification and cellular survival assays.
179 For virus quantification analyses, Dl_ISG15_E11 and E-11 cells grown on 24-well
180 plates were inoculated with RGNNV or SJNNV at 0.1 MOI. At 0, 24, 48 and 72 h p.i.,
181 supernatants and cells from three wells were separately collected for viral titration, or
182 viral genome quantification, respectively. This assay was performed in duplicate.
183 Viral genome was quantified by RT absolute real-time PCR. Cellular RNA and
184 cDNA were obtained as previously described. All the amplifications were conducted
185 with the LightCycler 96 Thermocycler in 20-µl mixtures containing cDNA generated
186 from 50 ng of RNA, 1x Fast Start Essential DNA Green Master Mix (Roche) and 10
187 pmol specific primers (Table 1). Amplification conditions were 95 ^{o}C for 10 min, and
188 45 cycles at 95 ^{o}C for 10 s, 60 ^{o}C for 10 s and 72 ^{o}C for 10 s. Melting curves were
189 obtained at 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s. Serial dilutions of the pJET
190 vector (Thermo) containing the RGNNV RNA2 sequence, and the pCR™4-TOPO® TA
191 vector (Invitrogen) containing the SJNNV RNA2 sequence [44] were used to generate
192 reference standard curves.

In order to determine cellular survival rate after virus infection, Dl_ISG15_E11 and E-11 cells grown on 24-well plates were inoculated with RGNNV or SJNNV at 0.1 MOI. Cells from three wells were collected at 0, 48 and 72 h p.i. to be counted in triplicate (Z1 coulter, Beckman Coulter). Non-inoculated cells, negative control, were processed in the same way.

2.5. Immunomodulation activity of intracellular sea bass ISG15 protein

The possible modulation of the transcription of mx dynamin like GTPase (*mx*), toll-like receptor 3 (*tlr3*) and ubiquitin ligase E3 (*e3*) genes in Dl_ISG15_E11 cells has also been evaluated. Monolayers of E-11 and Dl_ISG15_E11 cells seeded on 24-well plates were inoculated with RGNNV or SJNNV at 0.01 MOI. RNA and cDNA were obtained as previously described.

All amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix, using cDNA generated from 50 ng of RNA and the primers shown in Table 1. Amplifications consisted of 95 ^{o}C for 10 min followed by 45 cycles at 95 ^{o}C for 10 s, 52 ^{o}C for 10 s and 72 ^{o}C for 10 s. Melting curves were obtained at 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s. *Beta-actin* was the endogenous reference gene used (Table 1). Relative fold change values were calculated by the Pfaffl method [45].

2.6. Anti-betanodavirus activity of extracellular sea bass ISG15 protein

These analyses have been conducted by co-culture of E-11 and Dl_ISG15_E11 cells, as well as by E-11 incubation with ISG15-containing medium.

The co-culture protocol used [46] allows the culture of two different cell lines (donor and target cells) in droplets within the same well on a 6-well plate. The possible protection of Dl_ISG15_E11 cells over inoculated E-11 cells was measured by crystal violet staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction quantification.

Droplets (150 µl) of donor (Dl_ISG15_E11) and target (E-11) cells, containing $9x10^4$ cells, were separately seeded in the same well, and they were incubated in a humid chamber at 25 ^oC overnight for cell attachment. A negative control, in which two droplets of E-11 cells were seeded, was also set up. After that time, droplets were removed, and a volume of 2 ml of L-15 growth medium was added. These cells were incubated at 25 ^oC for 24 h before virus inoculation (10^4 $TCID_{50}$/ml). Inoculated cells were incubated at optimal virus replication temperature until fully CPE appearance. A negative control, non-inoculated cells, was also included.

Once CPEs were observed, cells in two wells were fixed and stained with 1% crystal violet in 22% formaldehyde (Sigma) at room temperature for 10 min. After several washes with PBS at room temperature for 5 min, droplets were photographed and pictures were analysed with the ImageJ (NIH) software.

Since crystal violet staining is a semi-quantitative technique, a modification of the above explained protocol, based on MTT reduction, has been developed. In this modified protocol, cells in droplets were attached on glass coverslips (Nunc™ Thermanox™ Coverslips, Thermo), which were placed into 6-well plates for virus inoculation and the subsequent incubation, as described previously. Once fully CPEs were observed, coverslips were moved to a 24-well plate for MTT reduction development [47]. Optical density (OD) was quantified at 550 nm using the Whittaker Microplate Reader 2001 (Anthos Labtec).

The role of the secreted Dl_ISG15 protein in protecting E-11 cells against betanodavirus has been further investigated by culturing these cells with medium from Dl_ISG15_E11 cells. This medium was obtained from sea bass ISG15-producing and non-producing cells cultured at 25 ^{o}C for 72 h. All the experiments have been conducted with these batches of ISG15-containing and ISG15-free media.

E-11 cells grown on 96-well plates were inoculated with RGNNV (10^4 $TCID_{50}$/ml). After 1-h incubation at 25 ^{o}C, the viral suspension was removed and replaced by L-15 maintenance medium mixed with medium from ISG15-producing cells (1/1). A control group, consisting of inoculated cells incubated with medium from non-producing E-11 cells (in the same proportion), was also included. Plates were incubated until CPE appearance, and the protective effect was then visualized by MTT addition in triplicate.

2.7. Statistical analyses

Data were analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Normality distribution was verified by the Shapiro-Wilk test, and the t-student was the statistical tests used. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Characterization of the Dl_ISG15_E11 cell line

Growth curve analysis (Fig. 1) showed no significant differences ($P > 0.05$) between the growth performance of Dl_ISG15_E11 and control E-11 cells, since cell concentration was the same for both cell types at all sampling times. Maximum cellular

concentration was recorded after 7 days of incubation, with $3.9x10^5$ and $3.6x10^5$ cells/ml for E-11 and Dl_ISG15_E11, respectively.

In addition, the recombinant Dl_ISG15 protein has been detected by western blot extracellularly and intracellularly (Fig. 2). The extracellular detection of Dl_ISG15 is depicted in Figure 2A, showing a band of the expected size after analysing medium from Dl_ISG15-producing cells immuno-precipitated with an anti-His monoclonal antibody (lane 4). However, no bands were observed by processing crude Dl_ISG15-producing cell medium (lane 2). Moreover, ISG15 was not observed when control E-11 medium was processed in the same way (lane 3).

In order to clarify if extracellular Dl_ISG15 protein is secreted or is a consequence of cellular lysis, the presence of beta-actin in the extracellular medium was evaluated (Fig. 2B). Beta-actin was detected in concentrated extracts of Dl_ISG15_E11 (lane 7) and E-11 (lane 8) cells, which constitutes a positive control for the beta-actin detection. Lanes 9 and 10 show absence of beta-actin in the extracellular fraction of Dl_ISG15_E11 and E-11 cells, respectively, suggesting absence of cellular proteins in the medium. Intracellular Dl_ISG15 detection is represented in Figure 2C, showing the presence of this protein only within Dl_ISG15_E11 cells (lane 13). E-11 cells have been analysed as negative control (lane 12).

Finally, the level of *isg15* mRNA in absence and in presence of viral infection has been analysed. Figure 3 evidences no significant differences ($P > 0.05$) in *isg15* transcription due to viral infection, with mean relative values of 0.96, 0.90 and 0.63 for non-infected, RGNNV- and SJNNV-infected cells, respectively. Viral multiplication in these cells has been confirmed by RT absolute PCR (data non-shown).

3.2. Anti-betanodavirus activity of the intracellular Dl_ISG15 protein

Viral multiplication in Dl_ISG15_E11 and E-11 cells has been determined by quantification of viral genome within the cells, and by titration of infective viral particles in the supernatant. In addition, in a parallel assay, survival rates of inoculated cells have also been determined.

The results of quantitative analysis of viral genome are shown in Figure 4A. The copy number of RGNNV RNA2 was similar in both cell types at all sampling times ($P > 0.05$). However, the number of SJNNV RNA2 copies was lower in Dl_ISG15-producing cells at 48 h ($P = 0.004$) and 72 h ($P = 0.01$) p.i., recording mean values of $3.2x10^{10}$ and $2.4x10^{9}$ RNA2 copies/µg RNA at 48 h p.i. in E-11 and Dl_ISG15_E11 cells, respectively.

The virus titration yielded similar results (Fig. 4B). Thus, viral titres recorded in supernatants of RGNNV-inoculated Dl_ISG15_E11 and E-11 cells were similar ($P > 0.05$) at all sampling times analysed, with maximum titres at 72 h p.i. ($9.9x10^{5}$ and $6.4x10^{5}$ $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11 cells, respectively). However, SJNNV titre in supernatants of Dl_ISG15_E11 cells ($2.2x10^{3}$ $TCID_{50}$/ml) was significantly lower than the titre obtained in E-11 supernatants ($1.2x10^{4}$ $TCID_{50}$/ml) at 48 h p.i. ($P = 0.01$). The maximum SJNNV titre was observed at 72 h p.i., with mean values of $1.9x10^{5}$ and $8.9x10^{4}$ $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11 cells, respectively (Fig. 4B).

In order to complete this analysis, a survival assay of inoculated E-11 and Dl_ISG15_E11 cells has been performed (Fig. 4C). Regarding RGNNV-inoculated cells, results showed similar survival percentages for both cell lines ($P > 0.05$) at all sampling times, with the minimum mean value (ca. 80%) at 72 h p.i. However, the percentage of cells surviving SJNNV infection was significantly higher for cells

318 expressing the sea bass ISG15 protein at 48 and 72 h p.i. (P = 0.01 and P = 0.005,
319 respectively). Thus, the survival rate of Dl_ISG15_E11 cells was 100% at 48 h p.i. and
320 95% at 72 h p.i., whereas the survival percentage recorded for E-11 cells was 83% at 48
321 h p.i., and 85% at 72 h p.i.
322
323 *3.3. Modulation of endogenous immunogene transcription*
324
325 The transcription of *mx, tlr3* and *e3* has been evaluated in sea bass ISG15-producing
326 cells both in presence and in absence of viruses.
327 In non-inoculated Dl_ISG15_E11 cells (Fig. 5A), *e3*, *tlr3* and *mx* genes were down-
328 regulated (in relation to the transcription of these genes in E-11 cells), showing relative
329 mean fold change values below 1 (0.17, 0.1 and 0.4 for *e3*, *tlr3* and *mx* respectively).
330 After RGNNV inoculation, *mx* transcription (Fig. 5B) was triggered only at 6 h p.i. in
331 E-11 cells (3.19 mean fold change value). Regarding SJNNV-inoculated cells,
332 significant transcription was recorded at 3 and 6 h p.i. in E-11 cells (2.6 and 4.9 mean
333 fold change values, respectively) and only at 6 h p.i. in Dl_ISG15_E11 cells (6.9 mean
334 fold change value), not being observed significant differences between both cell lines (P
335 > 0.05).
336 The transcription of *tlr3* (Fig. 5C) was significantly induced in E-11 cells inoculated
337 with RGNNV at all sampling times, with the maximum transcription fold change value
338 (12.6) at 6 h p.i. (P = 0.005), whereas this transcription was knocked out in RGNNV-
339 infected Dl_ISG15_E11 cells. However, in SJNNV-infected cells, *tlr3* transcription was
340 induced at 6 h p.i. in Dl_ISG15_E11 cells, without significant differences between both
341 cell lines (P > 0.05).

342 Finally, *e3* transcription is induced in Dl_ISG15_E11 cells infected with RGNNV at
343 all sampling times (Fig. 5D), with the maximum transcription value at 24 h p.i. (4.6),
344 being significantly higher ($P = 0.001$) than the induction value recorded in E-11 cells at
345 the same sampling time (non-induced, mean fold change < 1.5). In SJNNV-infected
346 groups, *e3* transcription was also observed in Dl_ISG15_E11 cells, at 3 and 24 h p.i.,
347 although without significant differences between the cell lines analysed ($P > 0.05$).
348
349 *3.4. Anti-betanodavirus activity of the extracellular Dl_ISG15 protein*
350
351 The role of the sea bass ISG15 protein as secreted cytokine has been evaluated by
352 co-culture of E-11 and Dl_ISG15_E11 cells, and by E-11 incubation with ISG15-
353 containing medium obtained from Dl_ISG15-producing cells.
354 Results obtained by co-culture (Fig. 6A, 6B) showed putative protective effect of
355 the Dl_ISG15 protein to co-cultured E-11 cells inoculated with either, RGNNV or
356 SJNNV. Figure 6A shows the results obtained estimating the level of surviving E-11
357 cells by crystal violet staining. Staining intensity of the target E-11 cells was maximal in
358 non-inoculated cells. After RGNNV infection, there were no surviving cells for control
359 inoculated cells (E-11/E-11), where ISG15-producing cells were not seeded, whereas
360 the staining intensity was intermediate for E-11 cells sharing the medium with ISG15-
361 producing cells (Dl_ISG15_E-11/E-11). The same pattern was obtained when SJNNV-
362 inoculated cells were analysed. These results were confirmed by the MTT assay (Fig.
363 6B). Thus, the survival rate of RGNNV-inoculated cells increased significantly, from
364 25% to 46% ($P = 0.01$), when these cells shared medium with ISG15-producing cells. A
365 similar result was obtained after SJNNV inoculation. In this experimental group,
366 survival rate of E-11 cells co-cultured with ISG15-producing cells was 51%, whereas

this value dropped to 27% (P = 0.01) when they were co-cultured with non-producing E-11 cells.

In addition, RGNNV-inoculated E-11 cells were incubated with ISG15-containing medium mixed with fresh medium (1/1). Cellular survival was determined by MTT reduction, showing a significant increase (P = 0.0005) of cellular survival, which was 33% for control cells (incubated with ISG15-free medium) and increased up to 99% in presence of sea bass ISG15 protein (Fig. 6C).

4. Discussion

Recent *in vivo* studies have demonstrated an important up-regulation of European sea bass *isg15* transcription after RGNNV or SJNNV infection [35,36], which may suggest an important role of this protein against betanodavirus infections in this fish species. For this reason, the present study is focused on evaluating the Dl_ISG15 activity against betanodaviruses. To fulfil this aim, the Dl_ISG15_E11 cell line has been developed.

Experimental systems based on the constitutive expression of exogenous ISG15 proteins have been previously applied, being the transient transfection the modality most frequently used [9,12,32,38,48]. The development of a permanently transfected cell line performed in the present study provides a stable tool, which has allowed to conduct different assays under steady and homogeneous conditions.

The suitability of this cell line to determine the Dl_ISG15 anti-betanodavirus activity has been evaluated by conducting studies on cellular growth kinetics and *isg15* gene transcription and expression. The growth performance of Dl_ISG15-transfected and non-transfected E-11 cells is similar (Fig. 1), which indicates that the expression of the exogenous gene does not affect important cellular functions and, therefore, putative

differences in viral replication recorded in both cell types are not consequence of the exogenous Dl_ISG15 protein expression, but derived from the antiviral activity of this protein itself. In addition, *isg15* transcription is stable, not being affected by the presence of viruses.

The recombinant Dl_ISG15 protein has been detected by western blot after processing Dl_ISG15_E11 cellular extracts (Fig. 2C), showing the presence of a single 20-25 kDa band, corresponding to the 15-kDa ISG15 protein plus the protein tags. Previous studies have also reported similar molecular weights for recombinant ISG15 proteins expressed in cellular systems [38,49,50]. Moreover, this protein has been located in the cytoplasm of the producing cells by immunofluorescence (data non-shown). In addition, a single band with the same molecular weight has also been detected in the medium of these cells (Fig. 2A), even though this protein does not have any signal peptide sequence. This extracellular ISG15 protein seems to be secreted, as it is suggested by the absence of beta-actin in the extracellular fraction, even after its concentration by immuno-precipitation (Fig. 2B), which may rule out cell disruption as possible cause for ISG15 presence in the medium, as previously suggested by Wang et al. [51]. ISG15 has previously been detected as protein secreted by immune cells, such as IFN I-treated human leukocytes and monocytes [14,16], although the mechanism underlying ISG15 secretion is unknown [4]. In fish, ISG15 has been detected extracellularly in cultures of red drum (*Sciaenops ocellatus*) and tongue sole (*Cynoglossus semilaevis*) IFN I-activated head kidney lymphocytes [49,51]. Furthermore, other cell types, such as epithelial-derived cell lines, fibroblasts and neutrophils, have also been described as ISG15-releasing cells in vertebrates [15]. In fact, Langevin et al. [38] reported extracellular zebrafish ISG15 in transfected epithelioma papulosum cyprini (EPC) cell cultures. All these previous investigations

support our results, in which a fibroblastic cell line (E-11 cells, derived from whole fry snakehead fish, *Channa striata*) secretes Dl_ISG15. Taken together, these results show that Dl_ISG15_E11 cell line is an adequate tool to determine the antiviral activity of the intracellular and extracellular Dl_ISG15 protein.

The intracellular activity of Dl_ISG15 has been evaluated by comparison of viral replication in ISG15-producing and non-producing cells (Fig. 4). The number of RGNNV RNA2 copies was similar in both cellular types at all times analysed, indicating that Dl_ISG15 does not affect viral genome synthesis. Although some ISG15 proteins have been reported to act at a post-transcriptional level, by blocking virion assembly or viral budding [9,50], this possibility has been ruled out in the present study, since no significant reduction in viral titre due to the Dl_ISG15 protein expression has been observed (Fig. 4B). Furthermore, these results were confirmed by analysing survival rates, which demonstrated that Dl_ISG15-producing cells were not protected against RGNNV infection (Fig. 4C). All these results confirmed the lack of intracellular anti-RGNNV activity of the Dl_ISG15 protein.

On the contrary, sea bass ISG15 compromises SJNNV replication, since a reduction of the SJNNV genome synthesis was recorded (Fig. 4A). This result has been confirmed by viral titration and surviving assays (Fig. 4B, 4C), concluding that intracellular sea bass ISG15 exerts an antiviral role against SJNNV infection.

This differential antiviral activity of Dl_ISG15 against both viral species suggests that this protein may play different roles in the course of RGNNV and SJNNV infections. The lack of ISG15 activity against some fish viruses (both DNA and RNA viruses) has been previously evidenced. In addition, Langevin et al. [38] failed in the detection of zebrafish ISG15 activity against spring viremia of carp virus (SVCV), a

441 rhabdovirus, although this ISG15 protein inhibits the replication of a wide range of fish
442 viruses (both RNA and DNA), even other member of the *Rhabdoviridae* family.

443 Previous studies conducted with similar *in vitro* systems, although using transiently
444 transfected cells, have shown that exogenous ISG15 proteins may regulate the
445 transcription of endogenous genes, which may be involved in cellular processes, such as
446 osteoclastogenesis [52], or antiviral activity [38]. For this reason, this study has
447 addressed the transcriptional analyses of several genes in Dl_ISG15_E11 cells, such as
448 *mx*, *tlr3*, and *e3*. These analyses showed that the over-expression of the exogenous
449 ISG15 protein did not trigger the transcription of the endogenous IFN I system
450 (estimated by *mx* transcription), unlike it has been reported for zebrafish ISG15
451 expressed in transiently-transfected EPC cells [38]; however, it would be interesting to
452 extend the analysis to other IFN I system-related genes.

453 After SJNNV inoculation, the transcription of these genes was similar ($P > 0.05$) in
454 Dl_ISG15_E11 and E-11 cells at all times analysed. On the contrary, *tlr3* and *e3*
455 transcription was significantly different ($P < 0.05$) in both types of cells inoculated with
456 RGNNV (Fig. 5). Thus, a clear knock out of *tlr3* transcription has been detected in
457 ISG15-producing cells. This gene encodes for a receptor responsible for the IFN I
458 system activation, and its low transcription may suggest that the presence of sea bass
459 ISG15 could be preventing the IFN I system activation and, therefore, could be
460 protecting the virus from the innate immune system, which could partially account for
461 the lack of intracellular anti-RGNNV activity recorded in this study. Although ISG15
462 has generally been considered as an antiviral effector, some studies have described that
463 this protein can favour viral infections. Thus, human ISG15 has been shown to have a
464 pro-viral role in the course of hepatitis C virus infection [53,54], and Chen et al. [55]
465 demonstrated that protein ISGylation promotes hepatitis C production. In fact,

466 experiments conducted with fibroblastic cells have suggested that human ISG15 may be
467 a negative regulator of the IFN system [56], as it may be happening in E-11 cells over-
468 expressing the sea bass ISG15 protein infected with RGNNV.

469 The transcription of the endogenous *e3* gene was also altered by the presence of the
470 sea bass ISG15 protein in RGNNV-infected cells, with Dl_ISG15_E11 cells showing a
471 significantly higher level of *e3* transcripts than E-11 cells at 24 h p.i. (Fig. 5D). E3 are
472 ligases that may be involved in ubiquitination and ISGylation. In higher vertebrates, a
473 variable number of E3 ligases have been recorded in different host-species, showing
474 different protein specificity, and therefore, regulating different pathways, as it has been
475 reported for human cells [56]. The lack of this information regarding our experimental
476 system (E-11 cells) makes difficult to deduce the functional implications of the up-
477 regulation of this gene transcription; however, it corroborates sea bass ISG15
478 immunomodulation in RGNNV-infected cells acting at different levels, and suggests the
479 presence of a high number of cellular proteins functionally altered in Dl_ISG15_E11
480 cells infected with RGNNV. Therefore, Dl_ISG15 immunomodulation is different in
481 RGNNV- and SJNNV-infected cells, which shows that both viral species interact in a
482 different way with the host immune system. This result may be consequence of different
483 RGNNV and SJNNV adaptation to host. In fact, a recent study has recorded differences
484 in the transcription of several immunogenes after the infection with IPNV isolates
485 belonging to different genogroups, which has also been reported in the present study,
486 suggesting that genetic similarity in fish viruses does not necessarily mean functional
487 similarity [57].

488 In this study, sea bass ISG15 has been detected extracellularly, and its antiviral
489 properties as signal molecule have been evaluated. To our knowledge, there is only one
490 previous study reporting ISG15 in medium of a fish cell line. Thus, Langevin et al. [38]

491 detected zebrafish ISG15 in the medium of transiently transfected EPC cells over-
492 expressing this protein. However, no antiviral activity of this secreted ISG15 was
493 recorded, which was suggested to be due to a concentration problem. In this regard, it is
494 important to highlight the advantage of using a permanently transfected cell line, which
495 assures that all cells are expressing the exogenous protein and, therefore, the
496 concentration of this protein in the medium is increased.

497 The co-culture assays have evidenced that ISG15-producing cells exert protection
498 over E-11 cells infected with either RGNNV or SJNNV, which is unlikely to be due to
499 the activation of the IFN I system in donor cells, since *mx* transcription has only been
500 detected in SJNNV-inoculated cells, and without significant differences between E-11
501 and Dl_ISG15_E11 cells. This hypothesis has been confirmed by incubating RGNNV-
502 inoculated E-11 cells with medium from Dl_ISG15_E11 cells, which resulted in a
503 significant increase of surviving cells, from 33% to 99% (Fig. 6C). This medium was
504 collected from non-inoculated transfected cells, which have been demonstrated not to
505 have induced any of the genes tested, including *mx,* and, therefore, their medium would
506 not contain cytokines derived from the IFN I system. However, to confirm this
507 hypothesis, it would be necessary to extend the transcription study to cover a wider
508 range of cytokine-coding genes.

509 In conclusion, in this study an *in vitro* experimental system (Dl_ISG15_E11), which
510 has been demonstrated to be a valuable tool to characterize the anti-betanodavirus
511 properties of the sea bass ISG15 protein, has been developed. The antiviral action of the
512 intracellular sea bass ISG15 protein against SJNNV has been stablished by
513 demonstrating reduction of viral genome and infective viral particles, as well as by
514 showing increased cellular surviving. In addition, the transcription of several
515 endogenous genes has been analysed, revealing modulation of *tlr3* and *e3* transcription

in Dl_ISG15-producing cells in the course of RGNNV infection. Finally, the Dl_ISG15-containing medium conferred protection against RGNNV and SJNNV infections. These results suggest and promote the use of the intracellular and extracellular sea bass ISG15 protein in future investigations as an antiviral, immuno-modulator and protective agent against betanodaviruses.

Acknowledgements

This study has been supported by the project AGL2017-84644-R (MINECO/AEI/FEDER, UE). P. Moreno was supported by a fellowship from Ministerio de Educación, Cultura y Deporte (FPU12/00265, Spanish Government).

References

[1] E.M. Coccia, A. Battistini, Early IFN type I response: learning from microbial evasion strategies, Semin. Immunol. 27 (2015) 85–101. doi:10.1016/j.smim.2015.03.005.

[2] F.C. Lin, H.A. Young, Interferons: success in anti-viral immunotherapy, Cytokine Growth Factor Rev. 25 (2014) 369–376. doi:10.1016/j.cytogfr.2014.07.015.

[3] J.S. Yoo, H. Kato, T. Fujita, Sensing viral invasion by RIG-I like receptors, Curr. Opin. Microbiol. 20 (2014) 131–138. doi:10.1016/j.mib.2014.05.011.

[4] D.J. Morales, D.J. Lenschow, The antiviral activities of ISG15, J. Mol. Biol. 425 (2013) 4995–5008. doi:10.1016/j.jmb.2013.09.041.

[5] D. Zhang, D.-E. Zhang, Interferon-stimulated gene 15 and the protein ISGylation system, J. Interf. Cytokine Res. 31 (2011) 119–130. doi:10.1089/jir.2010.0110.

[6] N.V. Giannakopoulos, J.K. Luo, V. Papov, W. Zou, D.J. Lenschow, B.S. Jacobs, E.C. Borden, J. Li, H.W. Virgin, D.E. Zhang, Proteomic identification of proteins conjugated to ISG15 in mouse and human cells, Biochem. Biophys. Res. Commun. 336 (2005) 496–506. doi:10.1016/j.bbrc.2005.08.132.

[7] C. Villarroya-Beltri, S. Guerra, F. Sanchez-Madrid, ISGylation – a key to lock the cell gates for preventing the spread of threats, J. Cell Sci. 130 (2017) 2961–2969. doi:10.1242/jcs.205468.

[8] C. Zhao, C. Denison, J.M. Huibregtse, S. Gygi, R.M. Krug, Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways, Proc. Natl. Acad. Sci. 102 (2005) 10200–10205. doi:10.1073/pnas.0504754102.

[9] A. Okumura, P.M. Pitha, R.N. Harty, ISG15 inhibits Ebola VP40 VLP budding

554 in an L-domain-dependent manner by blocking Nedd4 ligase activity, Proc. Natl.
555 Acad. Sci. 105 (2008) 3974–3979. doi:10.1073/pnas.0710629105.
556 [10] A. Pincetic, Z. Kuang, E.J. Seo, J. Leis, The interferon-induced gene ISG15
557 blocks retrovirus release from cells late in the budding process, J. Virol. 84
558 (2010) 4725–4736. doi:10.1128/JVI.02478-09.
559 [11] S. Sanyal, J. Ashour, T. Maruyama, A.F. Altenburg, J. Jose, A. Bilate, A.M.
560 Avalos, L. Kundrat, A. Garcia-Sastre, H.L. Ploegh, Golgi for glycoprotein
561 trafficking during influenza virus infection, 14 (2014) 510–521.
562 doi:10.1016/j.chom.2013.10.011.Type-I.
563 [12] C. Zhao, T.-Y. Hsiang, R.-L. Kuo, R.M. Krug, ISG15 conjugation system targets
564 the viral NS1 protein in influenza A virus-infected cells, Proc. Natl. Acad. Sci.
565 107 (2010) 2253–2258. doi:10.1073/pnas.0909144107.
566 [13] C. Zhao, H. Sridharan, R. Chen, D.P. Baker, S. Wang, R.M. Krug, Influenza B
567 virus non-structural protein 1 counteracts ISG15 antiviral activity by sequestering
568 ISGylated viral proteins, Nat. Commun. 7 (2016) 1–12.
569 doi:10.1038/ncomms12754.
570 [14] J.D. Cunha, E. Knight, A.L. Haast, R.L. Truitt, E.C. Borden, Immunoregulatory
571 properties of ISG15, an interferon-induced cytokine, Immunology 93 (1996)
572 211–215. doi:10.1073/pnas.93.1.211.
573 [15] C.D. Swaim, A.F. Scott, L.A. Canadeo, J.M. Huibregtse, Extracellular ISG15
574 signals cytokine secretion through the LFA-1 integrin receptor, Mol. Cell. 68
575 (2017) 581–590.e5. doi:10.1016/j.molcel.2017.10.003.
576 [16] E. Knight, B. Cordova, IFN-induced 15-kDa protein is released from human
577 lymphocytes and monocytes, J. Immunol. 146 (1991) 2280–2284.
578 [17] D. Bogunovic, M. Byun, L. a Durfee, A. Abhyankar, D. Mansouri, S. Salem, I.

579 Radovanovic, A.V. Grant, P. Adimi, N. Mansouri, S. Okada, V.L. Bryant, X.
580 Kong, A. Kreins, M.M. Velez, B. Boisson, S. Khalilzadeh, U. Ozcelik, I.A.
581 Darazam, J.W. Schoggins, C.M. Rice, S. Al-, M. Behr, G. Vogt, A. Puel, J.
582 Bustamante, Mycobacterial disease and impaired IFN-g immunity in humans
583 with inherited IGS15 deficiency, Science 337 (2012) 1684–1688.
584 [18] D. Bogunovic, S. Boisson-Dupuis, J.L. Casanova, ISG15: leading a double life as
585 a secreted molecule, Exp. Mol. Med. 45 (2013). doi:10.1038/emm.2013.36.
586 [19] Y. Xiang, W. Liu, P. Jia, Y. Li, Y. Jin, L. Chen, J. Zhang, K. Jia, M. Yi,
587 Molecular characterization and expression analysis of interferon-gamma in black
588 seabream *Acanthopagrus schlegelii*, Fish Shellfish Immunol. 70 (2017) 140–148.
589 doi:10.1016/j.fsi.2017.08.046.
590 [20] L. Bigarre, J. Cabon, M. Baud, M. Heimann, A. Body, F. Lieffrig, J. Castric,
591 Outbreak of betanodavirus infection in tilapia, *Oreochromis niloticus* (L.), in
592 fresh water, J. Fish Dis. 32 (2009) 667–673. doi:10.1111/j.1365-
593 2761.2009.01037.x.
594 [21] Q.K. Doan, M. Vandeputte, B. Chatain, T. Morin, F. Allal, Viral encephalopathy
595 and retinopathy in aquaculture: a review, J. Fish Dis. 40 (2017) 717–742.
596 doi:10.1111/jfd.12541.
597 [22] V. Panzarin, A. Fusaro, I. Monne, E. Cappellozza, P. Patarnello, G. Bovo, I.
598 Capua, E.C. Holmes, G. Cattoli, Molecular epidemiology and evolutionary
599 dynamics of betanodavirus in Southern Europe, Infect. Genet. Evol. 12 (2012)
600 63–70. doi:10.1016/j.meegid.2011.10.007.
601 [23] R. Thiery, J.C. Raymond, J. Castric, Natural outbreak of viral encephalopathy
602 and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested
603 reverse transcriptase-polymerase chain reaction, in: Virus Res., 1999: pp. 11–17.

604 doi:10.1016/S0168-1702(99)00053-2.
605 [24] N. Vendramin, F. Padros, T. Pretto, E. Cappellozza, V. Panzarin, G. Bovo, A.
606 Toffan, C. Terregino, Viral encephalopathy and retinopathy outbreak in
607 restocking facilities of the endangered freshwater species, *Salaria fluviatilis*
608 (Asso), J. Fish Dis. 35 (2012) 867–871. doi:10.1111/j.1365-2761.2012.01429.x.
609 [25] K.I. Mori, T. Nakai, K. Muroga, M. Arimoto, K. Mushiake, I. Furusawa,
610 Properties of a new virus belonging to *Nodaviridae* found in larval striped jack
611 (*Pseudocaranx dentex*) with nervous necrosis, Virology 187 (1992) 368–371.
612 doi:10.1016/0042-6822(92)90329-N.
613 [26] T. Nishizawa, M. Furuhashi, T. Nagai, T. Nakai, K. Muroga, Genomic
614 classification of fish nodaviruses by molecular phylogenetic analysis of the coat
615 protein gene, Appl. Environ. Microbiol. 63 (1997) 1633–1636.
616 [27] F. Pascoli, A. Guazzo, A. Buratin, M. Toson, F. Buonocore, G. Scapigliati, A.
617 Toffan, Lack of *in vivo* cross-protection of two different betanodavirus species
618 RGNNV and SJNNV in European sea bass *Dicentrachus labrax*, Fish Shellfish
619 Immunol. (2017). *in press.* doi:10.1016/j.fsi.2017.10.033.
620 [28] S. Souto, B. Lopez-Jimena, M.C. Alonso, E. Garcia-Rosado, I. Bandin,
621 Experimental susceptibility of European sea bass and Senegalese sole to different
622 betanodavirus isolates, Vet. Microbiol. 177 (2015) 53–61.
623 doi:10.1016/j.vetmic.2015.02.030.
624 [29] N. Vendramin, A. Toffan, M. Mancin, E. Cappellozza, V. Panzarin, G. Bovo, G.
625 Cattoli, I. Capua, C. Terregino, Comparative pathogenicity study of ten different
626 betanodavirus strains in experimentally infected European sea bass,
627 *Dicentrarchus labrax* (L.), J. Fish Dis. 37 (2014) 371–383.
628 doi:10.1111/jfd.12117.

[30] D. Alvarez-Torres, A.M. Podadera, M.C. Alonso, I. Bandin, J. Bejar, E. Garcia-Rosado, Molecular characterization and expression analyses of the *Solea senegalensis* interferon-stimulated gene 15 (*isg15*) following NNV infections, Fish Shellfish Immunol. 66 (2017) 423–432. doi:10.1016/j.fsi.2017.05.040.

[31] D. Alvarez-Torres, V. Gomez-Abellan, M. Arizcun, E. Garcia-Rosado, J. Bejar, M.P. Sepulcre, Identification of an interferon-stimulated gene, *isg15*, involved in host immune defense against viral infections in gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 73 (2018) 220–227. doi:10.1016/j.fsi.2017.12.027.

[32] C. Furnes, Ø. Kileng, C.H. Rinaldo, M. Seppola, I. Jensen, B. Robertsen, Atlantic cod (*Gadus morhua* L.) possesses three homologues of ISG15 with different expression kinetics and conjugation properties, Dev. Comp. Immunol. 33 (2009) 1239–1246. doi:10.1016/j.dci.2009.07.005.

[33] J.Y. Lin, G. Bin Hu, D.H. Liu, S. Li, Q.M. Liu, S.C. Zhang, Molecular cloning and expression analysis of interferon-stimulated gene 15 (ISG15) in turbot, *Scophthalmus maximus*, Fish Shellfish Immunol. 45 (2015) 895–900. doi:10.1016/j.fsi.2015.05.050.

[34] Y.B. Zhang, Y.L. Wang, J.F. Gui, Identification and characterization of two homologues of interferon-stimulated gene ISG15 in crucian carp, Fish Shellfish Immunol. 23 (2007) 52–61. doi:10.1016/j.fsi.2006.09.004.

[35] P. Moreno, E. Garcia-Rosado, J.J. Borrego, M.C. Alonso, Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene, Fish Shellfish Immunol. 55 (2016) 642–646. doi:10.1016/j.fsi.2016.06.043.

[36] P. Moreno, B. Lopez-Jimena, E. Randelli, G. Scapigliati, F. Buonocore, E. Garcia-Rosado, J.J. Borrego, M.C. Alonso, Immuno-related gene transcription

654 and antibody response in nodavirus (RGNNV and SJNNV)-infected European
655 sea bass (*Dicentrarchus labrax* L.), Fish Shellfish Immunol. 78 (2018) 270–278.
656 doi:10.1016/j.fsi.2018.04.054.
657 [37] X. Huang, Y. Huang, J. Cai, S. Wei, Z. Ouyang, Q. Qin, Molecular cloning,
658 expression and functional analysis of ISG15 in orange-spotted grouper,
659 *Epinephelus coioides*, Fish Shellfish Immunol. 34 (2013) 1094–1102.
660 doi:10.1016/j.fsi.2013.01.010.
661 [38] C. Langevin, L.M. van der Aa, A. Houel, C. Torhy, V. Briolat, A. Lunazzi, A.
662 Harmache, M. Bremont, J.-P. Levraud, P. Boudinot, Zebrafish ISG15 exerts a
663 strong antiviral activity against RNA and DNA viruses and regulates the
664 interferon response, J. Virol. 87 (2013) 10025–10036. doi:10.1128/JVI.01294-12.
665 [39] T. Iwamoto, T. Mori, K. Arimoto, M. Nakai, High permissivity of the fish cell
666 line SSN-1 for piscine nodaviruses, Dis. Aquat. Organ. 39 (1999) 37–47.
667 doi:10.3354/dao039037.
668 [40] T. Iwamoto, T. Nakai, K. Mori, M. Arimoto, I. Furusawa, Cloning of the fish cell
669 line SSN-1 for piscine nodaviruses, Dis. Aquat. Organ. 43 (2000) 81–89.
670 doi:10.3354/dao043081.
671 [41] H. Reed, L.J. Muench, A simple method of estimating fifty percent endpoints,
672 Am. J. Trop. Med. Hyg. 27 (1938) 493–497.
673 [42] T. Iwamoto, K. Mise, K.I. Mori, M. Arimoto, T. Nakai, T. Okuno, Establishment
674 of an infectious RNA transcription system for striped jack nervous necrosis virus,
675 the type species of the betanodaviruses, J. Gen. Virol. 82 (2001) 2653–2662.
676 doi:10.1099/0022-1317-82-11-2653.
677 [43] T. Mahmood, P.C. Yang, Western blot: technique, theory, and trouble shooting,
678 N. Am. J. Med. Sci. 4 (2012) 429–434. doi:10.4103/1947-2714.100998.

679 [44] B. Lopez-Jimena, E. Garcia-Rosado, C. Infante, D. Castro, J.J. Borrego, M.C.
680 Alonso, Effect of the coexistence on the replication of striped jack nervous
681 necrosis virus (SJNNV) and red-spotted grouper nervous necrosis virus
682 (RGNNV) using an *in vitro* approach, J. Appl. Ichthyol. 30 (2014) 916–922.
683 doi:10.1111/jai.12473.
684 [45] M. Pfaffl, Quantification strategies in real-time PCR Michael W. Pfaffl, in: A-Z
685 Quant. PCR, 2004: pp. 87–112. doi:http://dx.doi.org/10.1007/s10551-011-0963-
686 1.
687 [46] R. Parreño, S. Torres, L. Almagro, M. Bello-Perez, A. Estepa, L. Perez,
688 Induction of viral interference by IPNV-carrier cells on target cells: a cell co-
689 culture study, Fish Shellfish Immunol. 58 (2016) 483–489.
690 doi:10.1016/j.fsi.2016.09.052.
691 [47] J.C. Stockert, A. Blazquez-Castro, M. Cañete, R.W. Horobin, A. Villanueva,
692 MTT assay for cell viability: intracellular localization of the formazan product is
693 in lipid droplets, Acta Histochem. 114 (2012) 785–796.
694 doi:10.1016/j.acthis.2012.01.006.
695 [48] T. Hishiki, Q. Han, K.I. Arimoto, K. Shimotohno, T. Igarashi, S.G. Vasudevan,
696 Y. Suzuki, N. Yamamoto, Interferon-mediated ISG15 conjugation restricts
697 dengue virus 2 replication, Biochem. Biophys. Res. Commun. 448 (2014) 95–
698 100. doi:10.1016/j.bbrc.2014.04.081.
699 [49] C. Sheng Liu, Y. Sun, M. Zhang, L. Sun, Identification and analysis of a
700 *Sciaenops ocellatus* ISG15 homologue that is involved in host immune defense
701 against bacterial infection, Fish Shellfish Immunol. 29 (2010) 167–174.
702 doi:10.1016/j.fsi.2010.03.012.
703 [50] T.P. Røkenes, R. Larsen, B. Robertsen, Atlantic salmon ISG15: expression and

conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections, Mol. Immunol. 44 (2007) 950–959. doi:10.1016/j.molimm.2006.03.016.

[51] W. Wang, M. Zhang, Z. zhong Xiao, L. Sun, *Cynoglossus semilaevis* ISG15: a secreted cytokine-like protein that stimulates antiviral immune response in a LRGG motif-dependent manner, PLoS One. 7 (2012) 1–11. doi:10.1371/journal.pone.0044884.

[52] T. Takeuchi, G. Shimakawa, M. Tamura, H. Yokosawa, Y. Arata, ISG15 regulates RANKL-induced osteoclastogenic differentiation of RAW264 cells, Biol. Pharm. Bull. 38 (2015) 482–486. doi:10.1248/bpb.b14-00410.

[53] R. Broering, X. Zhang, S. Kottilil, M. Trippler, M. Jiang, M. Lu, G. Gerken, J.F. Schlaak, The interferon-stimulated gene 15 functions as a proviral factor for the hepatitis C virus and as a regulator of the IFN response, Gut. 59 (2010) 1111–1119. doi:10.1136/gut.2009.195545.

[54] P.K. Chua, M.F. McCown, S. Rajyaguru, S. Kular, R. Varma, J. Symons, S.S. Chiu, N. Cammack, I. Najera, Modulation of alpha interferon anti-hepatitis C virus activity by ISG15, J. Gen. Virol. 90 (2009) 2929–2939. doi:10.1099/vir.0.013128-0.

[55] L. Chen, J. Sun, L. Meng, J. Heathcote, A.M. Edwards, I.D. McGilvray, ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production *in vitro*: implications for chronic infection and response to treatment, J. Gen. Virol. 91 (2010) 382–388. doi:10.1099/vir.0.015388-0.

[56] M. Hermann, D. Bogunovic, In sickness and in health, Trends Immunol. 38 (2017) 79–93. doi:10.1038/sj.bdj.4811811.

[57] A.A.A. Gamil, Ø. Evensen, S. Mutoloki, Infection profiles of selected

aquabirnavirus isolates in CHSE cells, PLoS One. 10 (2015) 1–14. doi:10.1371/journal.pone.0134173.

[58] L. Poisa-Beiro, S. Dios, A. Montes, R. Aranguren, A. Figueras, B. Novoa, Nodavirus increases the expression of Mx and inflammatory cytokines in fish brain, Mol. Immunol. 45 (2008) 218–225.

[59] W. Chen, L. Yi, S. Feng, X. Liu, M. Asim, Y. Zhou, J. Lan, S. Jiang, J. Tu, L. Lin, Transcriptomic profiles of striped snakehead fish cells (SSN-1) infected with red-spotted grouper nervous necrosis virus (RGNNV) with an emphasis on apoptosis pathway, Fish Shellfish Immunol. 60 (2017) 346–354.

740 **Figure Legends**

741

742 **Fig. 1.** Growth curves of Dl_ISG15_E11 and E-11 cells. The t-student test was used to
743 compare cell concentration within each sampling time. Values of $P < 0.05$ were
744 considered significant. Results are mean ± standard deviation (SD) (n = 3).

745

746 **Fig. 2.** Detection of the recombinant Dl_ISG15 protein by western blot. Lanes 1, 6 and
747 11: precision plus protein standard marker (Bio-Rad). (A) Extracellular Dl_ISG15. Lane
748 2: non-concentrated Dl_ISG15_E11 medium. Lane 3: immuno-precipitated E-11
749 medium. Lane 4: immuno-precipitated Dl_ISG15_E11 medium. Lane 5: positive
750 control. (B) Intracellular and extracellular beta-actin. Lane 7: Dl_ISG15_E11 cell
751 extracts. Lane 8: E-11 cell extracts. Lane 9: immuno-precipitated Dl_ISG15_E11
752 medium. Lane 10: immuno-precipitated E-11 medium. (C) Intracellular Dl_ISG15.
753 Lane 12: E-11 cell extracts. Lane 13: Dl_ISG15_E11 cell extracts.

754

755 **Fig. 3.** Sea bass *isg15* mRNA quantification in non-inoculated, RGNNV- and SJNNV-
756 inoculated Dl_ISG15_E11 cells analysed at 24 h p.i. Data were statistically analysed
757 with the t-student test. Values of $P < 0.05$ were considered significant. Results are mean
758 ± SD (n = 3).

759

760 **Fig. 4.** Intracellular Dl_ISG15 antiviral activity. (A) Viral RNA2 copies in E-11 and
761 Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV. (B) Infective viral particles in
762 supernatants of E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV. (C)
763 Survival percentage of E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or
764 SJNNV. Data were statistically analysed with the t-student test. Asterisks indicate

765 significant differences between cell types within each sampling time: *P = 0.01; **P =
766 0.005; ***P = 0.004. Results are mean ± SD (n = 3).
767
768 **Fig. 5.** Transcription analyses of endogenous genes. (A) *e3*, *tlr3* and *mx* transcription in
769 non-inoculated Dl_ISG15_E11 cells. (B, C, D) *mx*, *tlr3* and *e3* transcription in
770 RGNNV- and SJNNV-inoculated Dl_ISG15_E11 cells. The cut-off value considered
771 for induction was 1.5. Data were statistically analysed with the t-student test. Asterisks
772 indicate significant differences between cell types within each sampling time: *P =
773 0.01; **P = 0.005; ***P = 0.001. Results are mean ± SD (n = 3).
774
775 **Fig. 6** Extracellular Dl_ISG15 antiviral activity. (A) Co-culture in droplets: cellular
776 survival after RGNNV or SJNNV inoculation visualized by crystal violet staining. (B)
777 Co-culture in droplets: cellular survival after RGNNV or SJNNV infection developed
778 by MTT reduction. (C) Incubation with ISG15-containing medium: survival percentage
779 of RGNNV-inoculated E-11 cells incubated with Dl_ISG15-containing medium (white)
780 or Dl_ISG15-free medium (black). The t-student test was the statistical analysis
781 performed. Asterisks indicate significant differences: *P = 0.01; **P = 0.0005. Results
782 are mean ± SD (n = 3).

Table 1.

Primers used in this study.

Target gene	Sequence (5'-3')	Amplicon size (bp)	Reference
RGNNV RNA2	ACCGTCCGCTGTCTATTGACTA CAGATGCCCCAGCGAAACC	126	[35]
SJNNV RNA2	GACACCACCGCTCCAATTACTAC ACGAAATCCAGTGTAACCGTTGT	75	[44]
Sea bass *isg15*	CGACTCAAAGCCTCTCTGCTACT CGTTTCTGACGAACACCTGGAT	100	[35]
E-11 *tlr3*	TGCAACACTCCACTGACTTACTTTAA AGGACAGCTGTGCTAAGTATATAA	115	This study
E-11 *e3*	TGCACTTGCAAGGCTGTCA CTCCTAGGATACTTGCATAGAAGACAAC	100	This study
E-11 *mx*	GGGGTCAGAAGGAGATCACA ATGATGCACCAGCTCAAGTG	150	[58]
E-11 *beta-actin*	CACTGTGCCCATCTACGAG	200	[59]

Figure1

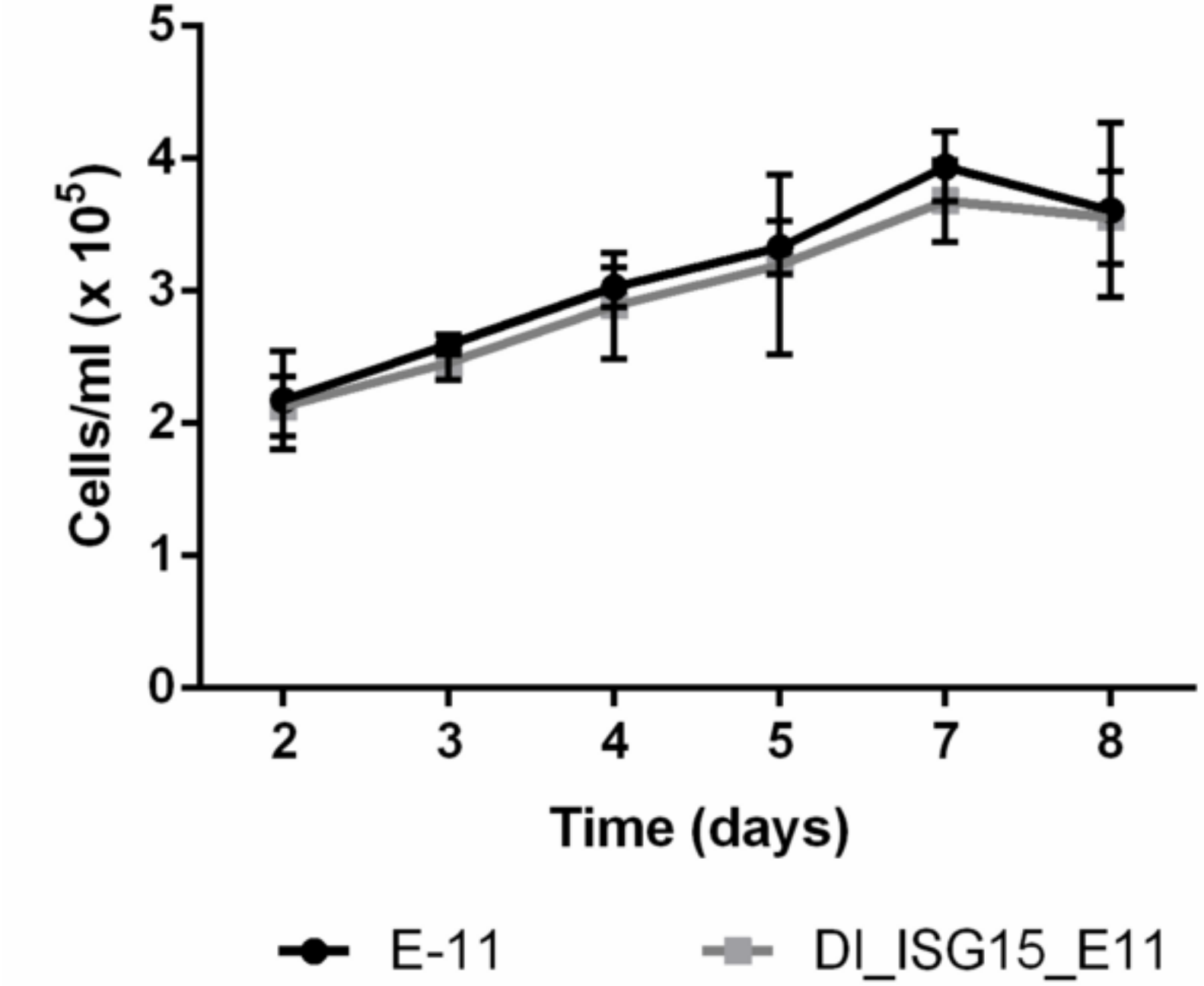


Figure2

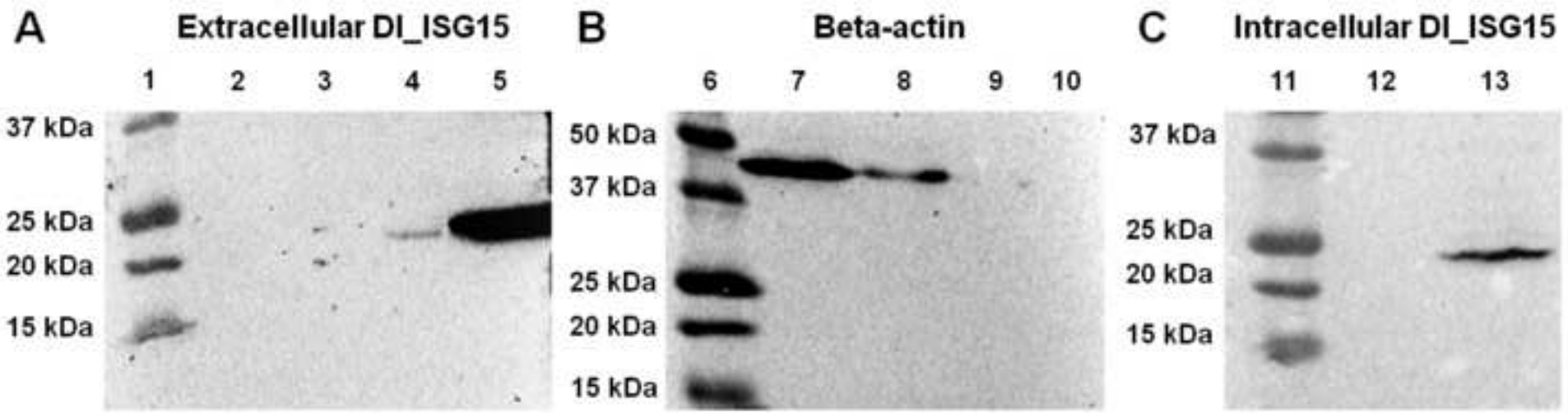

A
Extracellular DI_ISG15
1
2
3
4
5
37 kDa
25 kDa
20 kDa
15 kDa
B
Beta-actin
6
7
8
9
10
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
C
Intracellular DI_ISG15
11
12
13
37 kDa
25 kDa
20 kDa
15 kDa

Figure3

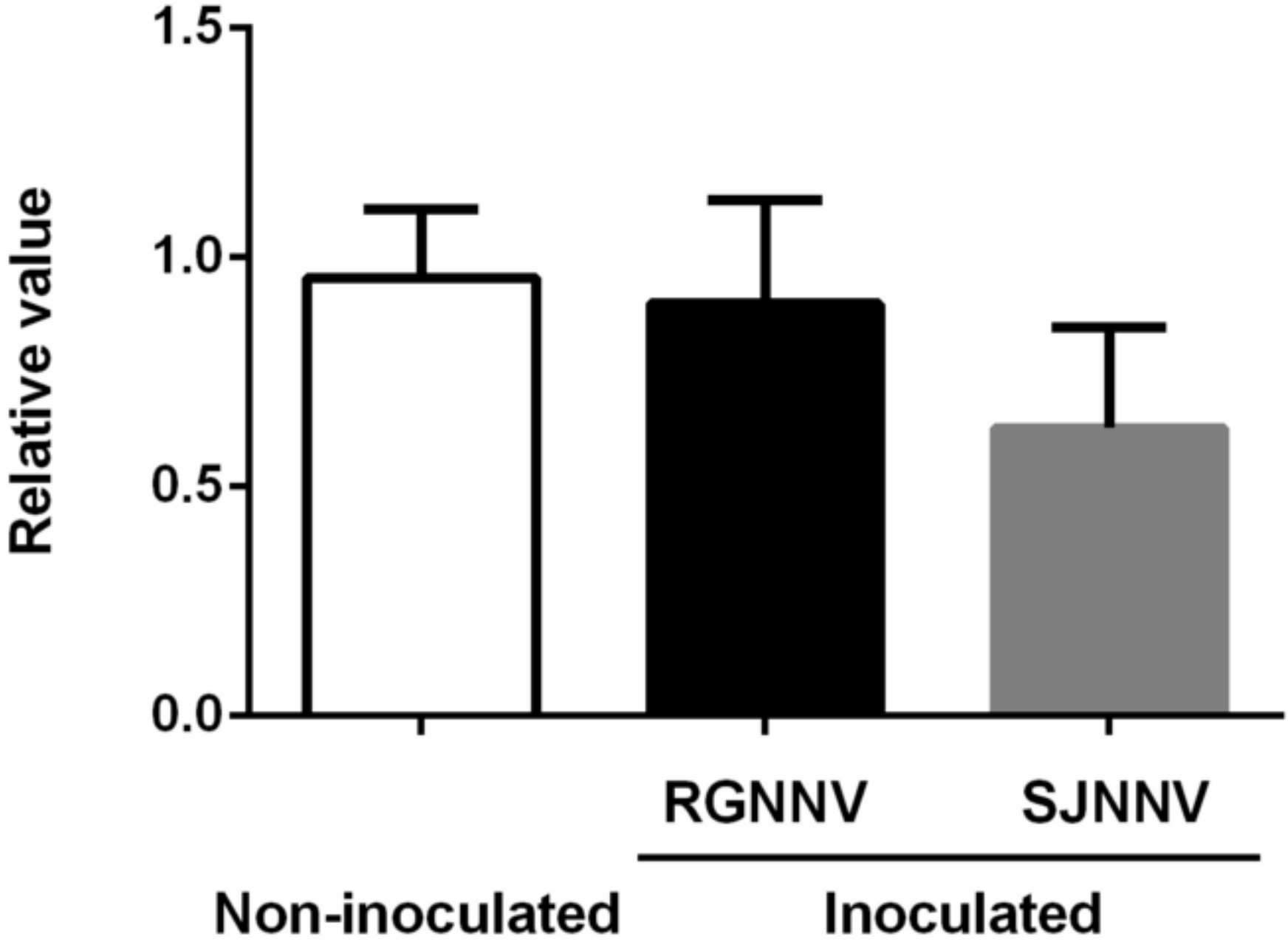

1.5
1.0
0.5
0.0
Relative value
RGNNV
SJNNV
Non-inoculated
Inoculated

Figure4

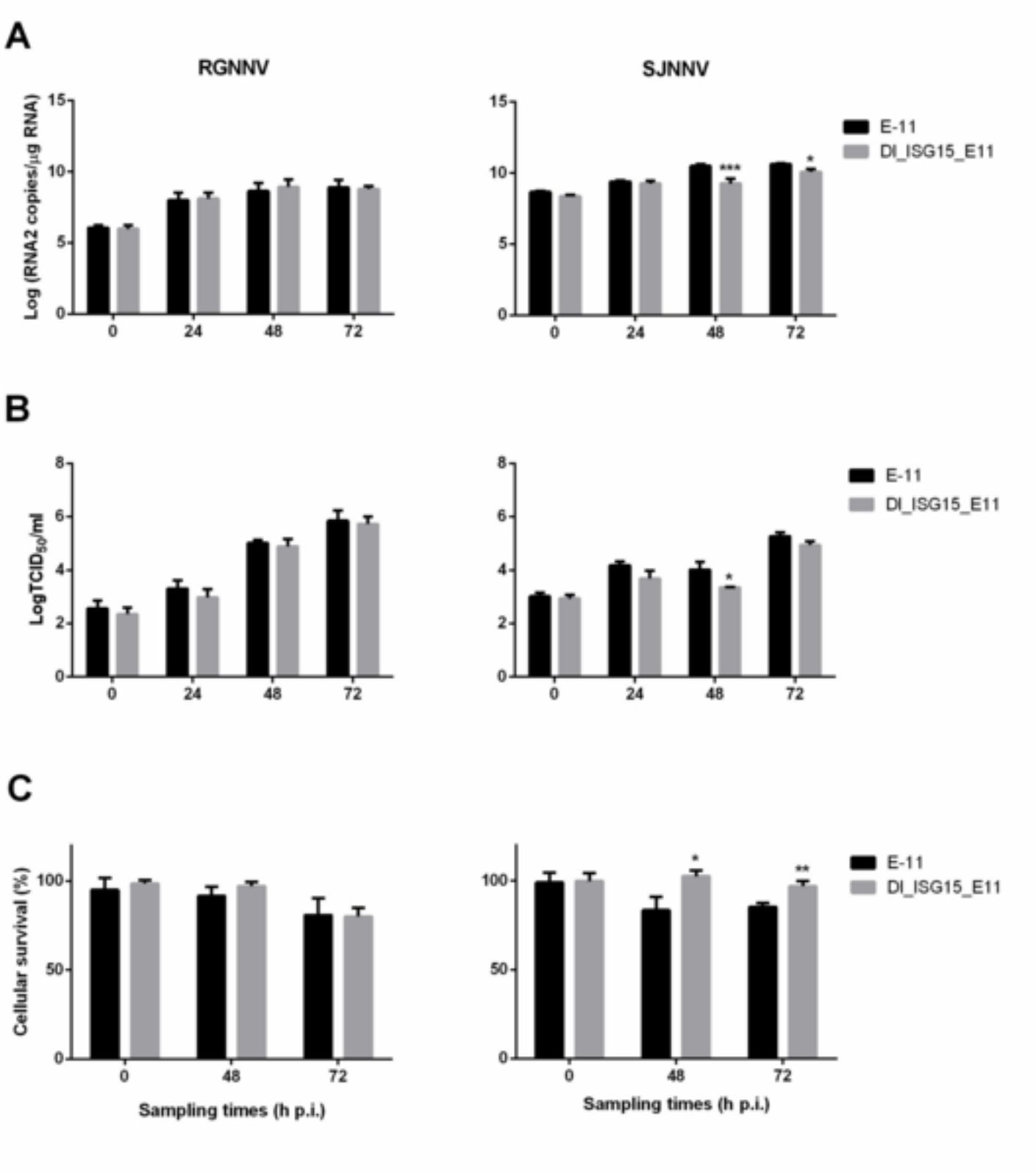

A
RGNNV
SJNNV
Log (RNA2 copies/μg RNA)
E-11
DI_ISG15_E11
***
*
B
LogTCID50/ml
E-11
DI_ISG15_E11
*
C
Cellular survival (%)
E-11
DI_ISG15_E11
*
**
Sampling times (h p.i.)
Sampling times (h p.i.)

Figure5

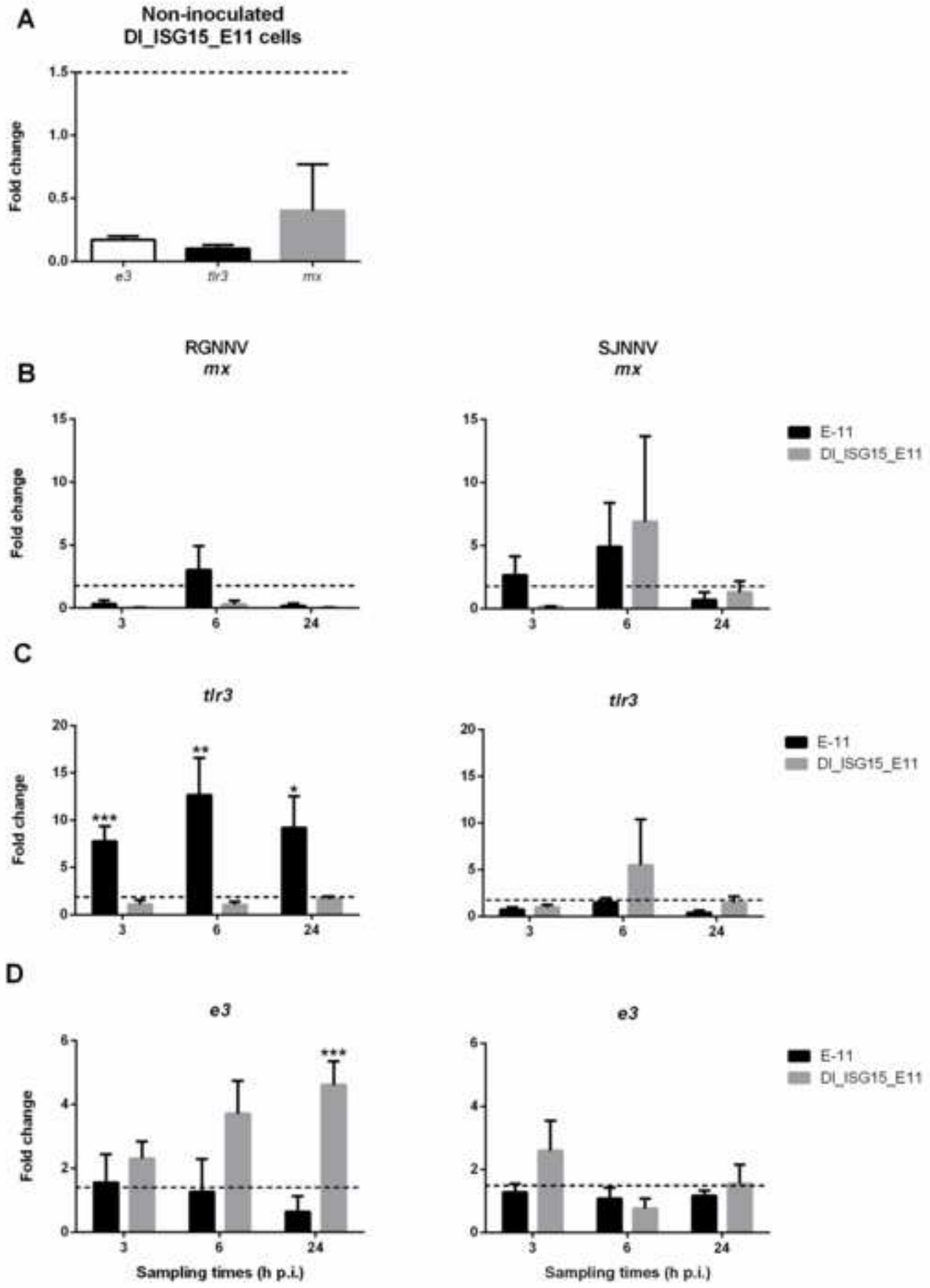
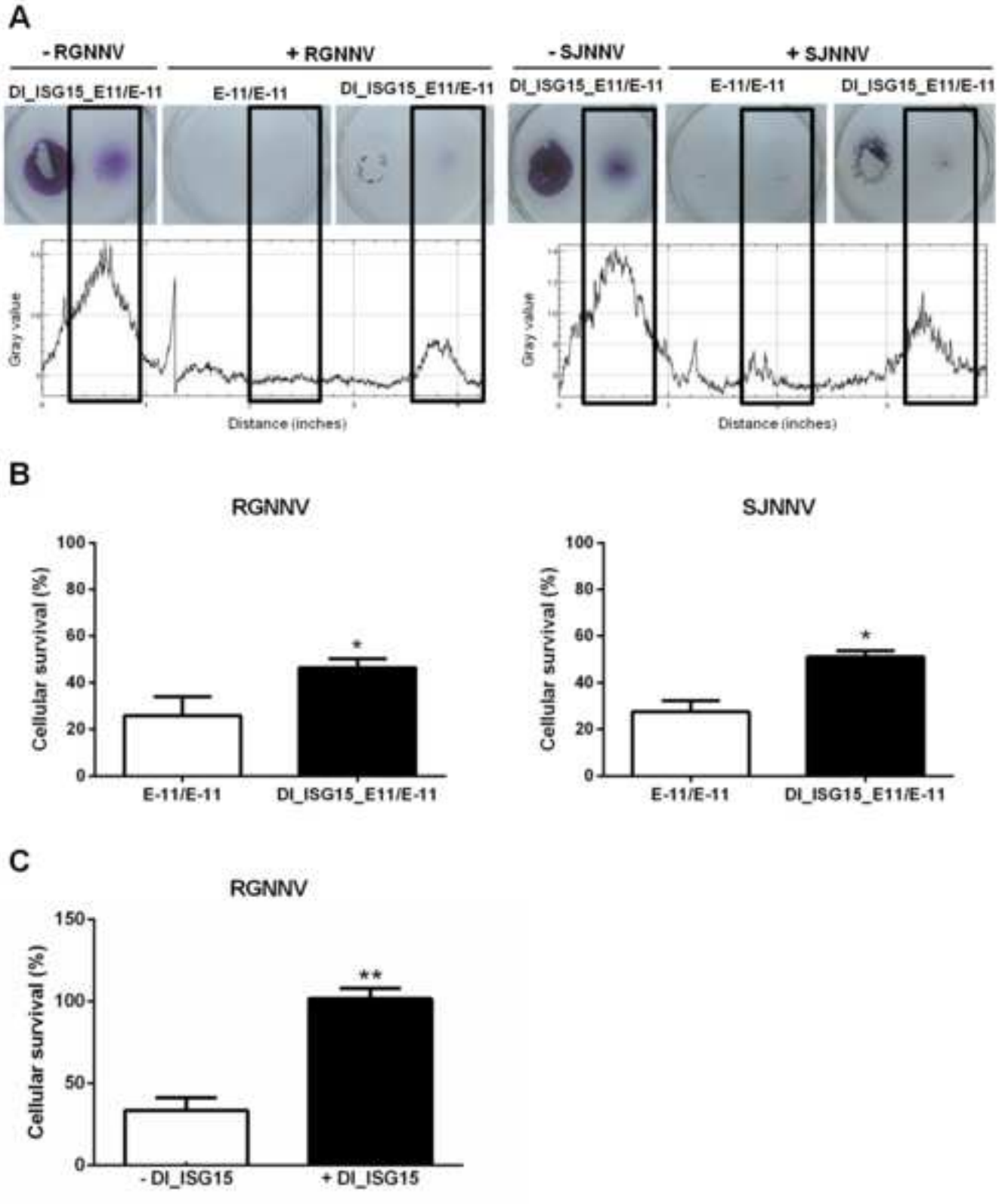

A
Non-inoculated
DI_ISG15_E11 cells
Fold change
1.5
1.0
0.5
0.0
e3
tlr3
mx
B
RGNNV
mx
SJNNV
mx
Fold change
15
10
5
0
3
6
24
E-11
DI_ISG15_E11
C
tlr3
tlr3
Fold change
20
15
10
5
0
***
**
*
3
6
24
E-11
DI_ISG15_E11
D
e3
e3
Fold change
6
4
2
0
***
3
6
24
Sampling times (h p.i.)
Sampling times (h p.i.)
E-11
DI_ISG15_E11

Figure6


A
- RGNNV
+ RGNNV
- SJNNV
+ SJNNV
DI_ISG15_E11/E-11
E-11/E-11
DI_ISG15_E11/E-11
DI_ISG15_E11/E-11
E-11/E-11
DI_ISG15_E11/E-11
Gray value
Distance (inches)
Gray value
Distance (inches)
B
RGNNV
SJNNV
Cellular survival (%)
100
80
60
40
20
0
*
E-11/E-11
DI_ISG15_E11/E-11
Cellular survival (%)
100
80
60
40
20
0
*
E-11/E-11
DI_ISG15_E11/E-11
C
RGNNV
Cellular survival (%)
150
100
50
0
**
- DI_ISG15
+ DI_ISG15