Viral Resistance and IFN Signaling in STAT2 Knockout Fish Cells

Carola E. Dehler,* Katherine Lester,† Giulia Della Pelle,‡ Luc Jouneau,‡ Armel Houel,‡ Catherine Collins,ERVER Tatiana Dovgan,*‡ Radek Machat,‡ Jun Zou,*‡ Pierre Boudinot,‡ Samuel A. M. Martin,* and Bertrand Collet†,‡

IFN belong to a group of cytokines specialized in the immunity to viruses. Upon viral infection, type I IFN is produced and alters the transcriptome of responding cells through induction of a set of IFN stimulated genes (ISGs) with regulatory or antiviral function, resulting in a cellular antiviral state. Fish genomes have both type I IFN and type II IFN (IFN-γ), but no type III (λ) IFN has been identified. Their receptors are not simple counterparts of the mammalian type I/II IFN receptors, because alternative chains are used in type I IFN receptors. The mechanisms of the downstream signaling remain partly undefined. In mammals, members of the signal transducer and activator of family of transcription factors are responsible for the transmission of the signal from cytokine receptors, and STAT2 is required for type I but not type II IFN signaling. In fish, its role in IFN signaling in fish remains unclear. We isolated a Chinook salmon (Oncorhynchus tshawytscha) cell line, GS2, with a stat2 gene knocked out by CRISPR/Cas9 genome editing. In this cell line, the induction of ISGs by stimulation with a recombinant type I IFN is completely obliterated as evidenced by comparative RNA-seq analysis of the transcriptome of GS2 and its parental counterpart, EC. Despite a complete absence of ISGs induction, the GS2 cell line has a remarkable ability to resist to viral infections. Therefore, other STAT2-independent pathways may be induced by the viral infection, illustrating the robustness and redundancy of the innate antiviral defenses in fish.

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Typical genes coding for type I and type II, but not type III, IFN have been identified in fish genomes. In fact, IFN and many ISG genes have shown high rates of change during evolutionary time in fish as in tetrapods, and the number of paralogs can be greatly expanded or reduced to a single gene. Fish type I IFN genes were prone to extensive diversification, especially in Salmonids (16), as is also observed in mammalian genomes (17). Importantly, fish homologs of the key signaling factors downstream of IFNR, such as STAT1/2 and IRF9, have been found, indicating that the general structure of the pathways is probably similar in fish and mammals. However, several important differences between fish and mammalian’s IFN system hamper a straightforward comparison: 1) in fish, many signaling factors have several paralogs for a unique counterpart in human or in the mouse, which can lead to sub-functionalization. Thus, the multiple fish Jak paralogs are not employed equally for the transmission of the IFN-γ signal (18), 2) Fish type I IFN are divided in two main classes using different receptors, which have some features in common with components of the receptors for mammalian type III IFN (18–20), and (3) fish IFN-γ1 and IFN-γ2 do not share the same receptor either (18). Hence, the precise role of the different signaling factors downstream of IFN receptors remains unresolved in fish.

STAT proteins are no exceptions to this. They have been described as an ancient and highly conserved family, with most members already defined in the common ancestor of fish and mammals (15). Also, stat1 and stat2 genes are induced by viral infections in different fish species, suggesting that they are implicated in the antiviral response as their homologs in mammals. However, STATs show varying levels of paralogue retention in salmonids, with four stat1 copies for only a single stat2 copy. The respective roles of the different STAT1 and of STAT2 in IFN signaling is therefore still undefined.

In the current study, we produced a salmonid cell line in which stat2 has been disrupted using a CRISPR/cas9 based approach. We used these cells to demonstrate that STAT2 is necessary for the severe disruption of the type I IFN induced by the RNA virus viral hemorrhagic septicemia virus (VHSV). However, the severe disruption of the type I IFN induced by the lack of functional STAT2 was not associated with viral hypersensitivity and fast, dramatic cell destruction.

Materials and Methods

Isolation of the GS2 cell line

For generation of the stat2 knockdown cell line, a Chinook salmon (Oncorhynchus tsawytscha) embryo (CHSE) cell line that was previously modified to stably express both a monomeric enhanced green fluorescence protein (mEGFP) and Cas9 (CHSE-EC) was chosen as the starting point of this study, further referred to as EC (21). The whole genomes of two different Oncorhynchus species (Chinook salmon O. tsawytscha and the rainbow trout O. mykiss) were examined for the presence and copy numbers of the stat2 gene, which consistently showed as a single copy located on chromosome 2 and 17 of the Chinook salmon and rainbow trout genome, respectively (22; Fig. 1A, 1B). A small fragment containing the start of the stat2 open reading frame (ORF) was amplified from genomic DNA purified from the EC cell line using the primers STAT2F and STAT2R, purified and sequenced (see Table I). Two single guide RNAs (sgRNAs) located in the first 50 nt of the stat2 ORF (Fig. 1C) were produced by a combination of PCR and in vitro transcription. To identify potential off-targets of the guide RNA, both sgRNA1 and sgRNA2 sequences were used to search against nucleotide sequences using Blastn limited to highly similar sequences (megablast) and restricted to entries associated with the family “Oncorhynchus” (taxid: 9016). No off-target genes with 100% coverage and 100% identity were identified for either sgRNA1 or sgRNA2, suggesting high specificity of the designed guide RNAs. To generate the sgRNA template, amplification of a 120 nt blunt-ended PCR product was carried out using the Q5 Taq polymerase (New England Biolabs). Each sgRNA template was amplified in five 50-μl reactions each with 25 μl 2× Q5 master mix, 400 nM DR274F forward primer (Table I), 40 nM STAT2R1-2 reverse primer, and 20 nM template STAT2T1-2 (see Table I). Cycling was as follows: 98°C for 30 s then 35 cycles of 98°C for 5 s, 60°C for 10 s, 72°C for 10 s, and a final extension of 72°C 30 s. The five reactions were pooled and purified using QiAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions and eluted in 50 μl water. The sgRNAs were synthesized using the RiboMAX Express T7 kit (Promega) purified using TRizol (Thermo Fisher Scientific) according to the manufacturers’ instructions. The sgRNAs were resuspended in RNAse- and DNase-free water and quantified by nanodrop prior to transfection. The quality and purity of the RNA was verified on agarose-ÈBr gel before or after RNAse A treatment (Qiagen).

For transfection, the two stat2 sgRNAs were mixed with the sgRNA targeting mEGFP and used to introduce EC cells; 100 ng of each of the three sgRNA (two targeting stat2 and one egfp) per 10 μl of cell suspension was used as described previously (21). Transfected cells were plated onto a 75 cm² flask and passed weekly for 3 wk. Nonfluorescent single transpnsized cells were then individualized by FACs onto a 48-well tissue culture plate by a BD Influx BSLII Sorter (Iain Fraser Cytometry Centre, University of Aberdeen) and propagated in 1 ml culture medium for 1 mo. Four nonfluorescent clones (i.e., with mutated EGFP) were propagated in 25 cm² flasks and characterized further.

Genomic DNA was purified by the HMW DNA kit and magnet (Qiagen) according to the manufacturer’s instructions. A 306-bp segment containing the targeted site was amplified by PCR from the genomic DNA using primers STAT2F and STAT2R (Table I). The PCR product was purified using QiAquick PCR purification kit (Qiagen) and directly sequenced using the same amplification primers (Sequencing Service, University of Dundee).

All cells were grown at 22°C in EMEM medium supplemented with 500 μg/ml G418 (Sigma-Aldrich), 30 μg/ml Hygromycin (Thermo Fisher scientific), and 10% FBS (Nalgene).

Characterization of EC and GS2 cell lines by quantitative RT-PCR

For the initial characterization of the response to IFN, EC and GS2 cells were seeded into six-well plates (Greiner Bio-One). Wells were left unstimulated or stimulated with 250 ng/ml recombinant rainbow trout type I IFN [IFNA2; AJ582754; (23)] or 250 ng/ml rainbow trout type II IFN [IFNG1; CAE82300; (23)] for 30 h. Recombinant IFNA2 and IFNG1 were produced as described previously (16, 23, respectively). Brefly, the recombinant type I and II IFN were H8S cloned-tagged versions of the rainbow trout IFNA2 and IFNG1, respectively, produced under native condition in Echerichia coli and purified under native conditions using NTA columns. Following stimulation, cells were lysed by addition of 1 ml of TRizol (Thermo Fisher Scientific), and the total RNA was purified according to the manufacturer’s instructions. cDNA was synthesized from 500 ng of total RNA using the ABI MultiScribe reverse transcriptase with random hexamers priming according to the manu-
In a second experiment, GS2 cells were co-transfected as described above with a transfection control plasmid (pRFP-KDEL), pmx-EGFP expressing EGFP under the control of the rainbow trout MtX1 gene promoter obtained by Sacl/Xhol-subcloning from pGGL3-Neo-Basic-pomMtX1 (24; Addgene no. 30536) into pGL4.22-Pur vector (Promega), pS2, or a control plasmid pCDNA3.1-Hyg (pCont; Thermo Fisher Scientific). Transfected cells were plated on a 6-dish multiwell slide chamber and stimulated with rIFNA2 for 40 h at 20˚C, and the EGFP and GFP fluorescence were visualized on a Zeiss fluorescent microscope.

**Viral infection**

The viral isolates used were salmon pancreatic disease virus (SPDV) isolate F07-220, HSV1 (25), and EHV2 (25, 26).

For assessment of viral infection by cytopathogenic effect, EC and GS2 cells were seeded on the same 96-well plate (200 µl cell suspension per well). Four wells were left uninfected, and for each cell line a series of four wells were infected with serial dilutions (1–100,000) of inoculum. The viral titer was identical for all three viral isolates and estimated to 10^5 PFU/ml, corresponding to a multiplicity of infection (m.o.i.) of ~0.01 for the lowest dilution. The procedure was carried out in three sets of plates and harvested after 3, 5, and 7 days post infection (dpi). The quantification of cytopathogenic effect was performed as described previously (25). Briefly, dilutions of inoculum were incubated for different durations, the cells were fixed, stained with crystal violet, and rinsed, and the OD at 450 nm was read. The percentage of viral cytopathology was calculated as 100*(ODv−ODc)/ODc, with ODc as OD of uninfected cells and ODv as OD of infected cells.

The release of viral particles over time was measured by infecting the EC and GS2 cell lines grown in 25 cm^2 flasks at the same density with EHV2 (3.1 × 10^3 PFU/ml) at m.o.i. of 0.3 or HSV1 (1.1 × 10^4 PFU/ml) at m.o.i. of 0.1 and collecting supernatant after 1, 2, and 5 days. Titers of infectious virions were measured by plaque assay on monolayers of Epithelioma papulosum cyprini cells (EPC; AFCC CRL-2872). Collected supernatants were serially diluted in duplicates for the plaque assay. The infection was performed at 14˚C under a layer of methylcellulose (0.75% final concentration) for 3 days after an adsorption step at 14˚C for 1 hour in liquid phase. The plaques were fixed with formaldehyde (10%), stained using crystal violet (1% final dilution), and photographed.

**Transcriptome deep sequencing analysis**

IFN stimulation. For IFNA2 stimulations, two 75-cm^2 flasks each for EC and GS2 were grown to 100% confluence as described above. The cells were then rinsed with Dulbecco’s PBS twice and detached with 4 ml of trypsin per flask. The two replicate flasks for each cell type where combined in a falcon tube with 2 ml of Eagle’s MEM (EMEM) medium (10% FBS) and suspended. One milliliter of each cell mix was seeded in a 6-dish well at 1 × 10^6 cells/plate (0.75% final concentration) for 3 days after an adsorption step at 14˚C for 1 hour in liquid phase. The cells were fixed with formaldehyde (10%), stained using crystal violet (1% final dilution), and photographed.

**Functional annotation analysis.** For each rainbow trout gene (assembly Omyk_1.0, NCBI), the longest protein model was extracted and used as a bait for blastp analysis against the human proteome (Ab initio, Ensembl GRC37.8). The best blast hit annotations were collected (e value <0.01).

**Statistical analyses.** QPCR data analysis was carried out as described previously (23). The comparisons between experimental groups in the gene expression levels were carried out by t test on the log-transformed levels of expression relative to eif4a gene, followed by Benjamini correction for multiple comparison. The comparison between viral cytopathology levels were carried out in a similar way after logit transformation. All variables were considered heteroscedastic.

**Results**

The genome of Chinook salmon contains a single stat2 gene

TBlastn analysis of the genome assembly of *O. tshawytscha* identified a unique sequence highly similar to human *stat2* gene, corresponding to the locus LOC112217577 on chromosome 2. This gene encodes a protein (814 AA, XP_024233747) that is 42% similar to the human STAT2 (851 AA). Single *stat2* genes were found in the other fish genomes, including other salmonids belonging to the genus Oncorhynchus and Salmo. These included Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*), coho salmon (*O. kisutch*), and Chinook salmon (*O. tshawytscha*), from which derives the EC line, expressing nCas9n and mEGFP constitutively (21). Only in Atlantic salmon (*O. salar*) have two *stat2* sequences been reported (32), but only one gene is present in the current genome assembly. Hence, only one copy of *stat2* has been retained in salmonids after the two whole genome duplications that occurred during the early evolution of teleosts and salmonids, respectively. All *stat2* sequences from fish and tetrapods cluster in a well-supported branch of the phylogenetic tree (Fig. 1A) that recapitulates the tree of species, suggesting that *stat2* sequences constitute a well-supported group of orthologs. Other fish *stat* sequences are not found in this branch and group with their human respective counterparts, indicating that the *stat* family had already diversified in the common ancestor to fishes and mammals in a similar way as *irf* or *sox* gene families (15). In keeping with this, Chinook salmon and rainbow trout *stat2* genes belong to syntenic groups partly conserved in zebrafish and human, which confirms the orthology observed by the tree (Fig. 1B). Of note, no more additional *stat2*-like sequences could be found in salmonids from the genus Oncorhynchus, either from Whole Genome Shotgun or from Expressed Sequence Tags databases, strongly suggesting that the genome of this genus, indeed contain only one copy of this gene.

**Production of GS2, a double mutant stat2−/− EC cell line**

Following *stat2*- and egfp-sgRNA transfections, four non-fluorescent clones (i.e., mutants in egfp) were chosen for characterization,
The expression of a number of key ISGs (mx, irf1, ddx58, pkr, and ifna3) were measured by real-time QPCR following stimulation with IFNA2 (Fig. 2A) or IFNG1 (Fig. 2B). After stimulation with the type I IFN IFNA2, a typical response of these typical ISGs was observed in the EC cells but was abolished in the GS2 cells, as shown by the lack of significant induction of mx, irf1, ddx58, and pkr genes (Fig. 2A). After stimulation with type II IFN (IFNG1), the induction profile in EC cells was different, with no significant induction of mx gene but a very potent and significant induction of irf1 gene (Fig. 2B). Interestingly, ifna3 was also significantly induced by IFNG1, in addition to ddx58 and pkr (Fig. 2B). In GS2 cells, the induction level of all tested genes but mx remained significant, but was somewhat reduced compared with EC, suggesting a possible role of STAT2 in their expression. Importantly, the induction of irf1 remained very high in GS2, with an FC of 26.8. Stimulated levels of irf1 induction were not significantly different between EC and GS2. Overall, our data indicate that the induction of typical ISGs by type I IFN (IFNA2) in EC cells is abolished in GS2 cells, whereas the response to type II IFN (IFNG1) remains significant in both cell lines.

Additionally, using a stat1a−/− stat2a−/− cell line (GS1A) obtained using the same procedure than GS2 (Supplemental Fig. 1), we showed that these mutations abolished all upregulation of mx, irf1, ddx58, and ifna3 genes either by IFNA2 or by IFNG1 (Fig. 2C, 2D). Importantly, although irf1 was significantly induced (1462 ± 269-fold; p < 0.01) in the EC cell line or in the GS2 line, it was not upregulated in GS1A cells (0.5 ± 0.1-fold; p = 0.42; Fig. 2D), indicating that the response to type II IFN was also affected in this case.

These conclusions based on gene expression in GS2 cells 30 h poststimulation with IFNA2 or IFNG1 were confirmed on a time course of induction 6, 24, and 48 h after addition of rIFNA2 or IFNG1 (Fig. 2E–G). Only irf1—but not mx nor ifna3—was significantly induced in the GS2 cell line after IFNG1 stimulation for 6, 24, or 48 h, corroborating the abolishment of IFNA2 but not IFNG1 signaling in the stat2−/− cells and the irf1 status as a marker for type II IFN activity.

To verify that the modifications in IFN signaling are due to the mutation in stat-2, we transfected GS2 cells with a stat2 expression plasmid and tested the effect of 30 h IFNA2 stimulation. As shown in Fig. 3A, the induction was 3.6- and 2.2-fold for mx and irf1 genes, respectively, showing a significant restoration of Stat2 function. This was verified at the cellular level by the activation of a reporter system in transfected cells when STAT2 was provided by an expression plasmid after stimulation with rIFNA2. In transfected cells (positive for RFP), GFP was higher than the autofluorescence background only when GS2 cells were cotransfected with the pS2 plasmid expressing STAT2, demonstrating the restoration of the IFNA2 signaling pathway (Fig. 3B).
Whole transcriptome analysis shows that the response to type I IFN (IFNA2) is almost fully abolished in GS2 cells

To achieve a global overview of the transcriptional response between EC and GS2 cells, the whole transcriptome was analyzed by Illumina deep sequencing (RNA-seq) with or without stimulation for 24 h with IFNA2. The dataset has been submitted to NCBI under the BioProject 495492. The RNA-seq mapping showed 30,536 unique loci when aligned to the rainbow trout genome; of these, 27,972 were annotated as producing a functional protein. Expression changes of the 40 genes most induced by IFNA2 in EC are represented as a heatmap in Fig. 4, with 35 of them (88%) present in the Interferome database (33). None of these genes were found significantly induced in GS2 (Fig. 4), indicating a general breakdown of the ISG response to IFNA2. Genes upregulated by IFNA2 with an FC > 5 in EC, but not in GS2, included several typical and well-conserved ISG such as vtcn1, mx3, ifis5, helz2, stat1b1, cd9, and ifis27 (4, 15). A full table of genes significantly modulated by IFNA2 is shown in Supplemental Table I. In total, 34 genes were significantly induced more than 2.5-fold in the parental cells (EC) (51 genes more than 2-fold) when stimulated with IFNA2, whereas only a single gene was downregulated <0.4-fold (Supplemental Fig. 2, Supplemental Table I). In contrast, only two genes were significantly increased >2.5-fold in GS2 cells upon IFNA2 stimulation (44 genes more than 2-fold), with four genes decreased in expression <0.4-fold (16 <0.5-fold). A logFC/logFC representation of the differential response of EC and GS2 cells is shown in Supplemental Fig. 2A, 2B, highlighting the lack of highly induced genes in GS2. To further characterize the responses of EC and GS2 cells, we used both gene ontology (biological process) enrichment analysis and KEGG pathway analysis to identify the main functional groups that were enriched upon IFNA2 stimulation (Supplemental Fig. 2C). Results from this analysis showed a clear difference between the cell lines. The enrichment of biological processes in EC cells was significant for the Gene Ontology (GO) identifier “defense response to virus” (Benjamini corrected e value <5%), whereas the GO enriched biological process in GS2 was “Platelet aggregation” with the KEGG pathway “Regulation of actin cytoskeleton,” which did not evoke a link with the IFN-based antiviral response.

Interestingly, there were 224 (up) 178 (down) genes significantly altered between nonstimulated EC and nonstimulated GS2 cells (Supplemental Table II), suggesting that changes are induced by the Stat2 knockdown; however, these were not significantly enriched for GO functional terms related to IFN or antiviral activity.

The level of expression of stat1 and stat2 as measured by deep sequencing showed that only the stat1b1 paralogue and stat2 were found upregulated in EC, and neither in GS2, corresponding to the 6th and 37th genes in Fig. 4, respectively.

For further examination of the RNA-seq data, confirmation of the expression of the genes encoded by the stable transfected plasmids was made. The transcripts count mapped to the Cas9 expression plasmid was 42,387 and 18,437 for EC and GS2, respectively. The counts of transcripts mapped to mEGFP expression plasmid was 192,847 and 13 for EC and GS2, respectively, indicating a 99.99% reduction in the abundance of mEGFP transcripts in the GS2 cells related to EC. The transcript count for elf1a, which we used as house-keeping gene for QPCR, was 72,016 and 76,357 for EC and GS2, respectively. Interestingly, the mEGFP transcript count was the highest recorded value for any of the 30,535 mapped transcripts in any of the samples analyzed and may be a contributor to the transcriptional activity differences between the EC and GS2 control cells.

Moderate modification of the sensitivity of GS2 cells to viral infection

For SPDV, EHNV, and VHSV, the cytopathic effect was significantly higher in GS2 than in EC cells; for SPDV this was observed at 5 and 7 dpi, for EHNV at 3 and 5 dpi, and VHSV was only increased at 3 dpi (Fig. 5A–C). At 7 dpi, the maximum increase in percentage of cytopathology in GS2 was for VHSV, with 33.9 ± 17.5% in EC and 43.5 ± 21.9% in GS2, but the difference was not significant. At 7 dpi, the only consistent significant, but minimal, increase was for SPDV, for which the percentage cytopathology was 81.2 ± 1.5% in EC and 83.4 ± 0.7% in GS2.

Table II. Primers used for QPCR gene expression

| Gene   | Sequence 5′-3′ Forward | Sequence 5′-3′ Reverse | Accession Number       |
|--------|------------------------|------------------------|------------------------|
| mx     | 5′-GTGTTCAGACCCACTGCGAGC-3′ | 5′-CGATCGACCCATGAGATCTGA-3′ | xm1 XM_024415949; xm3 XM_024415945 |
| pkr    | 5′-AAAAACCTCCATCTCGAGAGG-3′ | 5′-GACAGACAGCCATGTCAGCC-3′ | XM_024425247           |
| dha58  | 5′-CGGTCGACCAATCTGTTTGGTG-3′ | 5′-CGCGAGGGAACTTCGACCC-3′ | XM_024390155           |
| ifs1   | 5′-CTCTCACCACGAGGCTGATTC-3′ | 5′-GGGTCTGGTCCAGTGCTGCTC-3′ | XM_024432485           |
| ifns2  | 5′-ACTGTGAGCTTCCGATGATCG-3′ | 5′-CGAGATGGTGCTGATGCTGTC-3′ | XM_024434108           |
| stat2  | 5′-CCCACTCCCTGACTGCTGCTG-3′ | 5′-GACTACCCCTGGCCTGCTGCTG-3′ | XM_024377979           |
| elf1a  | 5′-CCCTCAGGGATGTTTAAA-3′ | 5′-CACCCGCCCCGAGGCCAC-3′ | XM_024441752           |
The titrations of EHNV at 2 dpi and VHSV at 5 dpi from EC and GS2 supernatants are shown in Fig. 5D, 5E, respectively. Clear signs of EHNV infection with visible plaques were seen for the GS2 cell line up to dilution 10^4–10^5, but no signs were visible for the EC cell line supernatant at any dilution (Fig. 5D). Similar results were seen with supernatant collected at dpi 1 and 2 (data Table III. Summary of the levels of expression and induction of the stat1 and stat2 genes as measured by RNA-seq in EC and GS2 following stimulation with IFNA2

| Accession Number (GeneID NCBI) | O. mykiss | O. tshawytscha | EC ifn, EC control | GS2 ifn, GS2 control | EC control, GS2 control |
|---------------------------------|----------|----------------|-------------------|--------------------|------------------------|
| stat 1a1 NP_001118179 (GeneID 100136755\(^a\)) | LOC11226655\(^b\) | LOC112253950\(^b\) | NS | NS | NS |
| stat 1a2 XP_021463912 (GeneID 100137016\(^a\)) | LOC112253897\(^b\) | LOC112253778\(^b\) | NS | NS | NS |
| stat 1b1 XP_021443486 (LOC110501544) | LOC112244573 | LOC112244573 | <0.0001 (10.6) | NS | NS |
| stat 1b2 XP_021452654 (LOC110520020) | LOC112235369 | LOC112235369 | <0.0001 (2.1) | NS | NS |
| stat 2 XP_02144956 (LOC110494323) | LOC112217577 | LOC112217577 | <0.0001 (1.8) | NS | NS |

\(^a\) Provisional RefSeq status as of 13th March 2019.
\(^b\) Duplication due to assembly errors in the O. tshawytscha genome as of March 13, 2019.

FIGURE 2. Gene expression in EC and GS2 cell line after IFNA2 or IFNG1 stimulation. Expression levels of ISGs mx, irf1, dhx58, and ifna3 ISGs measured by QPCR in EC and GS2 cell lines after 30 h induction with recombinant rainbow trout type I (IFNA2) (A) or type II (IFNG1) (B) IFN. Expression levels of ISGs mx, irf1, dhx58, and ifna3 ISGs measured by QPCR in EC and GS1A cell lines after 30 h induction with recombinant rainbow trout type 1 (IFNA2) (C) or type II (IFNG1) (D) IFN. The GS1A cell line has been obtained using a similar approach than GS2 (Supplemental Fig. 1) * indicates level of significance of the fold increase versus corresponding control. Kinetics of induction of mx (E), irf1 (F), and ifna3 (G) genes in EC or GS2 cells after 6, 24, or 48 h stimulation with IFNA2 (IFNA) or IFNG1 (IFN-\(\gamma\)). Data represent average FC (n = 3) relative to the corresponding unstimulated control.
not shown). No clear differences could be observed in the viral titer of supernatant collected after VHSV infection with signs of infection in both cell lines (Fig. 5E).

Discussion

Type I IFN are central to the host defense against viruses and are believed to activate via a conserved Jak/Stat signaling pathway. As part of our effort to unravel the detailed IFN signaling mechanisms in cells from a genome-duplicated lower vertebrate, we demonstrate for the first time, to our knowledge, that Stat2 is an obligatory player in type I IFN signaling, confirming that this pathway is conserved between teleosts and mammals.

Workflow for salmonid knockout cell lines

The present work demonstrates a robust approach to generate a knockout somatic salmonid fish cell line with a high efficiency by using a fluorescent reporter transgene as a screening cotarget (21). The parental cell line, EC, constitutively expressing a functional nuclear nCas9n and monomeric mEGFP, is transiently transfected with a mix of sgRNAs designed against the gene of interest (stat2 in this study) and against megfp, mEGFP-deficient single cells are isolated by FACS and left to propagate into clonal cell lines. Because of the slow growth of fish cells, the timeline for the isolation of such a cell line was ~3 mo from the initial sgRNA transfection to the isolation of clonal cell lines, with sufficient material to sequence and PCR for multiplex gene editing. Besides the successful multiplex gene editing, it is remarkable that all three clones isolated were carrying homozygote biallelic mutations on the stat2 locus similarly to results obtained in previous studies in mouse embryonic stem cells (34, 35) or in embryos of other fish species or in anagathans [Zebrafish (36); Lamprey (37); Medaka (38); Salmon (39)]. In somatic cells, the exact mechanism by which Cas9 induces homozygote biallelic mutation is currently unknown.

It is important to have in mind that the cotargeted locus, megfp, in the EC cell line is a transgene with an uncharacterized site(s) of genomic integration.

The IFNA2 inducibility was partially restored by transfection of GS2 cells with a STAT2 expression plasmid. The rescue of stat2 function was carried out by transfection with a reporter system, the GS2 rescue could be visualized at the cellular level when STAT2 was expressed and IFN present.

The current assembly of the Atlantic salmon S. salar genome also contains a single copy of the stat2 gene. From the RNA-seq data, only one stat2 transcript could be detected in the EC cell line with or without IFNA2 induction, confirming the in silico analyses. In contrast, there were four paralogs of stat1. Two copies were closely related to the zebrafish stat1a gene that was not induced by Chikungunya infection, and two copies corresponding to stat1b, which was significantly upregulated (15). In the current study, out of the four mEGFP-deficient clones isolated, and three were mutated in the targeted gene stat2, with one of them being a null mutant. Besides the successful multiplex gene editing, it is remarkable that all three clones isolated were carrying homozygote biallelic mutations on the stat2 locus similarly to results obtained in previous studies in mouse embryonic stem cells (34, 35) or in embryos of other fish species or in anagathans [Zebrafish (36); Lamprey (37); Medaka (38); Salmon (39)]. In somatic cells, the exact mechanism by which Cas9 induces homozygote biallelic mutation is currently unknown.

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Strong nonsense-mediated mRNA decay for egfp but not stat2 in GS2 cell line

The expression level of the stat2 mRNA was not affected by the null mutation, suggesting that its transcript is resistant to the nonsense-mediated mRNA decay (NMD). This was expected, as in the corresponding mutated stat2 mRNA the premature termination codon is close to the 5′ untranslated region and would escape NMD destruction (40, 41). In contrast, there was a 99.99% decrease in the megfp transcript abundance in the GS2 relative to EC cells. This result is surprising, as the mEGFP transgene is intronless (21) and as such, according to some NMD models, is thought to be immune to the NMD pathway (42). In this context, the marked difference in the NMD sensitivity of the stat2 and egfp transcripts in GS2 cells is interesting and may suggest an alternative NMD model (43) or a peculiar NMD mechanism in fish, as highlighted in immune cells producing Ig and TCR transcripts (44).

Stat2 knockout affects type I but not type II IFN signaling pathway

The initial characterization of the type I IFN–induction phenotype by QPCR after incubation with recombinant type I IFN showed a general disruption of ISGs induction in the GS2 cell line. This was confirmed by the overall transcriptome analysis by RNA-seq. In a human fibrosarcoma cell line, IRF 1 (irf1) has been found to be induced substantially only by type II IFN (45), and a strong modulation of this transcript is now considered as a hallmark of Stat1 dimer signaling/GAS-specific ISG (46). Our results showed...
that fish irf1 gene was very strongly induced by IFNG1 but not by IFNA2 in the parental cell line EC, which supported that irf1 is a good marker of the type II IFN response as in mammals. In GS2 cell lines, irf1 gene was strongly induced as in the parental cell line, indicating that irf1 induction does not rely on stat2 signaling. Similar results were reported in primary embryonic fibroblast cells isolated from stat2 knockout mice (47). In contrast, the GS1A cell line with deficient stat1a1 and stat1a2 genes had both IFNA2 and IFNG1 pathways disrupted. This was particularly clear with irf1, strongly induced after stimulation with IFNG1 in EC but not in GS1A (Fig. 2C, 2D), confirming that the signaling for IFNA2 is impacted by Stat1A proteins.

pkr, mx, or dhx58 are type I IFN–specific ISGs (48), and their induction by IFNA2 was completely abolished in the GS2 cell line in comparison with the parental EC cell line. Similarly, primary kidney cells isolated from stat2 knockout hamsters were unable to induce pkr after incubation with IFN-α (49).

These results demonstrate that in salmonid cells, Stat2 is involved essentially in the signaling pathway of type I IFN in agreement with the canonical mammalian model for the IFN signaling (50), whereas it is not required for type II IFN signaling. Thus, our results support a model in which fish type II IFN signals by Stat1:Stat1 dimers, whereas type I IFN signals by the ISGF3, a Stat2:Stat1:IRF9 heterotrimer (2). Although we observed a moderate induction of dhx58 and pkr by rIFNG1 in EC cells, it was likely an indirect effect due to the modest upregulation of type I IFN by this treatment. The abolition of these effects in GS2 would therefore be consistent with the canonical model mentioned above.

Similar results were published in mammalian stat2 knockout models such as primary culture of peritoneal macrophages harvested from stat2 knockout mice (47) or the human U6A cell line (51). In NB4 cells (human acute promyelocytic leukemia cells), irf1 gene is induced more intensively by type II than type I IFN (52) and follows the same pattern of expression than in EC cells.

**Viral sensitivity**

Although the IFNA2 signaling pathways were completely abolished, the GS2 cell line exhibited notable elevation in the percentage viral cytopathology that was observed at the early stage of infection following infection with SPDV, EHNV, or VHSV at 15°C. This variation in the kinetics of infection between EC and GS2 cell lines may not be more remarkable than differences that would be observed between two clonal cell lines from the same origin (53, 54). However, if the viral cytopathology was mainly linked to the induction of Stat2-dependent ISGs, we would have observed a more pronounced alteration of the effect of viral infection in GS2 cells in comparison with EC cells.

The amount viral particles produced at early stage of infection with EHNV, a DNA virus, is higher in GS2 than in EC and is coherent with an increase of cytopathology with this virus. However, the results are different with VHSV, a negative

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**FIGURE 5.** Percentage of viral cytopathogenicity after 3-, 5-, or 7-d infection and viral titer in EC and GS2 cell lines. Viral cytopathology measured postinfection with EHNV (A), SPDV (B), and VHSV (C). Visualization of the viral titer determined on EPC cells in supernatant from EC or GS2 cells infected with EHNV for 2 d (D) or VHSV for 5 d (E).
ssRNA virus, whereby GS2 cells produce less viral particles. Replication of EHNV was found to be very sensitive to the level of ISG15 in cyprinid cells (55) and only mildly to the presence of Mx1 protein in salmonid cells (25). The absence of induction of these genes in GS2 may therefore explain, although partially, the increased viral cytopathy and shedding in the cell line.

This contrasted with results in mice where infection with vesicular stomatitis virus of primary or immortalized fibroblast cultures isolated from stat2 knockout mice exhibited viral titers increased up 80- and 20-fold relative to wild type, respectively (47).

From the RNA-seq data, following stimulation with IFNA2, a discrete number of ISGs were induced significantly in both EC and GS2 cell lines. As an example, two of them, guanine nucleotide-binding protein G subunit α-like (GNAI) and glutathione peroxidase 1 (GPX-1), were induced over 2-fold in both cell lines after stimulation with IFNA2. The latter have been associated with viral sensitivity (56) and may explain, at least partially, the GS2 viral resistance. Further functional characterization of such genes may reveal important roles in the Stat2-independent antiviral activity.

Possible role of IRF1-regulated genes in antiviral resistance at the cellular level

The induction of ISGs via alternative pathway(s) can be possible without the involvement of either Stat1 or Stat2 as described in West Nile virus EG101–infected mouse cells (57). These results are in agreement a previous study (58) whereby surviving patients with deficient type I IFN pathway caused by Stat2 null phenotype are remarkably healthy with no evident impairment in their innate immunity. However, other reports may suggest different consequences of disappearance of Stat2 in other species. Approximately 40% of hamsters lacking Stat2 succumbs to Zika virus (59); however, in this study, there was no side-by-side comparison with the wild type.

In human, Schoggins et al. (4) reported that IRF1 inhibited many viruses, including hepatitis C virus, HIV-1, yellow fever virus, West Nile virus, Venezuelan equine encephalitis virus, and chikungunya virus, even in a stat1−/− background, indicating that this transcription factor triggers a particular antiviral pathway. In fact, IRF1 overexpression in Stat1−/− fibroblasts upregulated the expression of many well-known ISGs and effector proteins such as Mx1, IFIT-6, -27, -30, -35, and 44, IFIT1 and 3, and ISG15, IRF9, SAMD9, USP18, and ISG20 but not type I IFN. Thus, the repertoire of IRF1 induced genes only partly overlaps the typical set of ISGs induced via IFNR and Jak/Stat.

Overall, our QPCR and whole transcriptome data suggest that the irf1 expression in GS2 is not sufficient to rescue a detectable induction of ISGs. Thus, a number of fish orthologs of ISGs inducible by IRF1 overexpression in human Stat1−/− fibroblasts were induced by trout IFNA2 in EC but not in GS2. These genes included mx1, ifi27, ifi64, gbpl, lgals3BP, parp14, usp18, samd9l, dhx58, ifi55, and cx3hav1. In fact, no gene significantly induced by type I IFN in GS2 was orthologous to a member of the IRF1-stimulated gene list reported previously (60). Overall, these results indicate that the faint (and nonsignificant) irf1 induction observed in GS2 after type I IFN stimulation does not complement the disruption of the Stat2 dependent signaling.

We have demonstrated that the function of Stat2 in salmonid cells follows the canonical signaling pathway described in higher vertebrates. However, the effect of the Stat2 loss of function on the ability to resist to a viral infection depends on the type of virus. Further transcription studies on the GS2 cell line upon early infection with different categories of viruses are required to identify genes responsible for viral resistance.

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Disclosures

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