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Infectious salmon anaemia virus (ISAV) isolates induce distinct gene expression responses in the Atlantic salmon (*Salmo salar*) macrophage/dendritic-like cell line TO, assessed using genomic techniques

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ABSTRACT

Infectious salmon anaemia virus (ISAV) is a marine orthomyxovirus of significant interest not only as a cause of a fatal disease of farmed Atlantic salmon resulting in severe economic losses to the aquaculture industry, but also as the only poikilothermic orthomyxovirus. ISAV targets vascular endothelial cells and macrophages, and is known to influence the expression of both innate and adaptive immune response relevant genes. ISAV isolates from different geographic regions have been shown to vary considerably in their pathogenicity for Atlantic salmon. This study aimed to characterize the Atlantic salmon TO macrophage/dendritic-like cell responses to infection with a selection of ISAV isolates of different genotypes and pathogenicity phenotypes. The first TO infection trial used ISAV isolates NBISA01 and RPC/NB-04-085-1 of high and low pathogenicity, respectively, and global gene expression analyses were carried out using ~16,000 gene (16K) salmonid cDNA microarrays to compare RNA samples extracted from TO cells harvested 24 and 72 h post-infection versus time-matched uninfected controls. Overall, the microarray experiment showed that RPC/NB-04-085-1-infected cells had a higher total number of reproducibly dysregulated genes (88 genes: the sum of genes greater than 2-fold up- or down-regulated in all four replicate microarrays of a given comparison) than the NBISA01-infected cells (10 genes) for the combined sampling points (i.e. 24 and 72 h). This microarray experiment identified several salmon genes that were differentially regulated by NBISA01 and RPC/NB-04-085-1, and which may be useful as molecular biomarkers of ISAV infection. An initial quantitative reverse transcription-polymerase chain reaction (QRT-PCR) study involving 25 microarray-identified genes confirmed the differences in the level of dysregulation of host transcripts between the two ISAV isolates (i.e. NBISA01 and RPC/NB-04-085-1). A second TO infection trial was run using a selection of four clinical ISAV isolates (Norway-810/9/99, a high pathogenicity isolate of European genotype; RPC/NB-04-085-1, a low pathogenicity isolate of European genotype; NBISA01, a high pathogenicity isolate of North American genotype; and RPC/NB-01-0593-1, an intermediate pathogenicity isolate of North American genotype), and UV-inactivated RPC/NB-04-085-1, with sampling at 24, 36, 48, 72, 96, and 120 h post-infection. The microarray-identified, QRT-PCR validated suite of 24 molecular biomarkers of response to ISAV were used in a second QRT-PCR experiment to assess the TO cell gene expression responses to the four ISAV isolates at all six time points in the infection. The QRT-PCR data showed that RPC/NB-04-085-1 caused the highest fold changes of most immune-relevant genes [such as interferon-inducible protein Gig1, Mx1 protein, interferon-induced protein with tetratricopeptide repeats 5, Radical S-adenosyl methionine domain-containing protein (viperin), and several genes involved in the ISGylation pathway], followed by Norway-810/9/99. NBISA01 and RPC/NB-01-0593-01 (both of North American genotype) showed low fold up-regulation of transcripts that were highly induced by RPC/NB-04-085-1 isolate. These findings show that ISAV isolates have strain-specific variations in their ability to induce immune response genes.

1. Introduction

Infectious salmon anaemia (ISA) virus (ISAV) is a significant fish pathogen that continues to cause severe economic losses to the salmon-farming industry in an increasing number of countries (Kibenge et al., 2004). The clinical disease, ISA, in marine-farmed

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Atlantic salmon (*Salmo salar*) is characterized by variable mortality ranging from 0% to 50% (Thorud and Djupvik, 1988). Most recently and for the first time in the Southern hemisphere, the virus was found to be responsible for the on-going massive disease outbreaks in farmed Atlantic salmon in Chile (Godoy et al., 2008).

ISAV is a segmented, negative-sense, single-stranded (ss) RNA virus of the family *Orthomyxoviridae*, together with the influenza viruses (Kawaoka et al., 2005). Characterization of the ISAV genome is largely complete, and it is now clearly evident that the gene order and putative functional identities of the ISAV proteins are significantly different from those of other orthomyxoviruses (Aspehaug et al., 2005; Kibenge et al., 2007a). ISAV RNA segments 1 through 6 encode one protein each in the following order: PB2, PB1, nucleoprotein (NP), PA, fusion (F) protein, haemagglutinin-esterase (HE) protein. It was originally thought that ISAV segments 7 and 8 encoded two proteins each (Ritchie et al., 2002; Biering et al., 2002). However, using immunoprecipitation assays with rabbit antiserum to ISAV, it was possible to demonstrate the existence of three proteins translated from segment 7 (Seg7 ORF1, and two alternatively spliced products – ORF1/2 and ORF1/3 – based on removal of introns from the ORF1 transcript) (Kibenge et al., 2007b). The Seg7 ORF1/3 protein is a new protein of unknown status and function, and consists of the first 22 amino acids of the Seg7 ORF1 product with a 257 nucleotide intron spliced out so that the translation continues in the +3 reading frame for a total of 81 amino acids with a predicted molecular weight of 9.5 kDa (observed, 11 kDa) (Kibenge et al., 2007a). The Seg7 ORF1 product, one of the most variable of the ISAV genes characterized to date (Ritchie et al., 2002), was recently reported as an interferon-signaling antagonist but its mechanism of action was not established (McBeath et al., 2006; Garcia-Rosado et al., 2008). Genomic segment 8 uses a bicistronic coding strategy with one protein identified as the virus matrix protein (Falk et al., 2004), and the second protein (s8ORF2 product, Garcia-Rosado et al., 2008) as a structural protein having a predominantly nuclear localization and type I interferon (IFN) antagonizing activity (Garcia-Rosado et al., 2008).

The response against a viral infection starts with the innate immune response, which is triggered by the recognition of the pathogen presence by germ-line encoded pattern recognition receptors, such as the Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002). Host immune cells respond by phagocytosis, secretion of cytokines (type I IFNs and proinflammatory cytokines: IL-1, IL-2, TNF-α) and chemokines, as well as by direct killing of infected cells (Janeway et al., 2005). One of the well-studied innate immune responses to virus infection is the secretion of type I IFNs (IFN-α/β). Most virus-infected cells use the cytoplasmic RNA helicases RIG-I and Mda5, or TLRs, to sense viral nucleic acids. Binding of viral replication nucleic acid intermediates to RIG-I, Mda5, or TLR results in a coordinated activation of the transcription factors NF-κB, interferon regulatory factor 3 (IRF-3) and IRF-9 (Randall and Goodbourn, 2008). These transcription factors in turn regulate the expression of hundreds of genes such as IFNs and IFN-stimulated genes, and proinflammatory cytokines and chemokines, which are involved in the orchestration of the adaptive immune response (Katze et al., 2008, Haller et al., 2006). As virus replication proceeds, antigen presenting cells display the antigens bound to MHC class I molecules to the cell surface for detection by cytotoxic T lymphocytes. In addition, antigens will be processed for production of specific antibodies that are important in protection against viral infections (Janeway et al., 2005).

Atlantic salmon cells/hosts infected with pathogenic ISAV isolates have been shown to up-regulate the expression of innate (IFN-α, Mx, ISG-15, PKR) as well as adaptive (MHC class I genes) immune response genes (Jørgensen et al., 2007; Kileng et al., 2007; McBeath et al., 2007; Workenhe et al., 2008a). However, none of these studies considered the variability of host responses to ISAV isolates that differ in their virulence properties and pathogenicities for fish hosts (Kibenge et al., 2006, 2007b). It is known that the mechanisms by which human influenza A virus antagonizes the IFN-α/β response are virus-strain specific (Kochs et al., 2007; Hayman et al., 2006). Moreover, ISAV isolates NBISA01 and RPC/NB-04-085-1 of differing pathogenicities and geographic locations (Kibenge et al., 2006) when used to infect fish erythrocytes showed different endocytosis, replication, and host immune responses (Workenhe et al., 2008a). This suggests the existence of differences in the molecular aspects of pathogenesis of this disease and the host responses to distinct ISAV isolates from different geographic regions.

The responses of host cells to different viral isolates may be assessed using DNA microarrays, a widely used approach for high-throughput analysis of gene expression. To date, microarray-based analyses of host response to ISAV have been limited only to highly pathogenic virus isolates, such as European ISAV isolate 12 (Mjaaland et al., 2005) and ISAV Glesvaer/2/90 (Dannevig et al., 1995). The Glesvaer/2/90 isolate induces high mortalities in rainbow trout (*Oncorhynchus mykiss*) even with waterborne infection (Biacchesi et al., 2007). Moreover, these investigations utilized a salmonid microarray containing only 1800 different cDNAs selected for their putative immune response and inflammation-related functions (SFA2.0 immunochip, GEO GPL6154) (Jørgensen et al., 2008; Schiøtz et al., 2008). The microarray platform used in the present study to identify Atlantic salmon genes differentially expressed in the macrophage/dendritic-like cell line TO infected with different strains of ISAV was the *c*onsortium for *G*enomics *R*esearch on *A*ll Salmonids *P*roject (cGRASP) ~16,000 gene (16K) salmonid microarray (GEO GPL2716) (von Schalburg et al., 2005). The microarray experiment identified candidate host molecular biomarkers of ISAV response, 25 of which were QRT-PCR validated using the same virus isolates (NBISA01 and RPC/NB-04-085-1) and time points post-infection (24 and 72 h) that were used in the microarray experiment. A second TO infection trial was run using four ISAV isolates (Norway-810/9/99, a high pathogenicity isolate of European genotype; RPC/NB-04-085-1, a low pathogenicity isolate of European genotype; NBISA01, a high pathogenicity isolate of North American genotype; and RPC/NB-01-0593-1, an intermediate pathogenicity isolate of North American genotype), and UV-inactivated RPC/NB-04-085-1, with sampling at 24, 36, 48, 72, 96, and 120 h post-infection. The microarray-identified, QRT-PCR validated suite of 24 molecular biomarkers of response to ISAV was used in a second QRT-PCR experiment to assess the TO cell gene expression responses to the four ISAV isolates at all six time points in the infection. The objective of this second infection trial and QRT-PCR experiment was to study the potential influence of pathogen genotype versus pathogenicity on host antiviral gene expression responses.

2. Materials and methods

2.1. Viruses

Four ISAV isolates of differing genotypes and pathogenicity phenotypes were used. NBISA01 is a highly pathogenic isolate belonging to the North American genotype, whereas RPC/NB-04-085-1 is a low pathogenicity isolate of the European genotype found in Eastern Canada and its HE protein places it in a unique, highly polymorphic region (HPR) group (Kibenge et al., 2006). Norway-810/9/99 is a high pathogenicity isolate of the European genotype whereas RPC/NB-01-0593-1 is an intermediate pathogenicity isolate of the North American genotype. NBISA01 and RPC/NB-01-0593-1 belong to HPR21 group while Norway-810/9/99 belongs to HPR15. In a challenge experimental study using Atlantic

salmon, the NBISA01 and Norway-810/9/99 strains were shown to induce high cumulative mortalities of ≥90%; RPC/NB-01-0593-1 showed intermediate cumulative mortality of 50% whereas RPC/NB 04-085-1 was shown to induce low cumulative mortality of 18.2% (Kibenge et al., 2006). The viruses were propagated in TO cells and the lysates were titrated in TO cells as previously described (Kibenge et al., 2001). All of the virus isolates used in the present study had been passaged less than 6 times in TO cells; continuous passage of a tick-transmitted orthomyxovirus, *Thogotovirus*, in cell culture is known to lead to loss of the ability to control IFN induction (Hagmaier et al., 2003) as a result of a mutation introduced by stuttering of the viral RNA polymerase during replication (Zheng et al., 1999).

2.2. UV Inactivation of ISAV

UV inactivation of ISAV strain RPC/NB-04-085-1 was carried out with a germicidal UV lamp (G30T8 with 30 W and 36 in. length, and a UV intensity of 125 μW/cm^2 at 1 m from the lamp) suspended in a biological safety cabinet (Class II A/B3 BSC, Thermo Forma) as described in Workenhe et al. (2008a). Complete UV inactivation of the virus was confirmed by titration in TO cell monolayers (Kibenge et al., 2001) before use in the TO cell infection experiments.

2.3. Virus infection of TO cells

2.3.1. Infection Trial 1 (used for the microarray experiment and the first QRT-PCR validation experiment)

The TO cell line (Wergeland and Jakobsen, 2001) was grown in Hanks' minimum essential medium (HMEM) (Bio Whittaker, Waterville, MD) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids and 50 μg/ml gentamicin. Cells were incubated at room temperature (24 °C) and the monolayers were used after 24 h. The virus stocks of RPC/NB-04-085-1 ($10^{5.75}$ $TCID_{50}$/ml), NBISA01 ($10^{8.75}$ $TCID_{50}$/ml) propagated in TO cells were diluted to $10^{5.0}$ $TCID_{50}$/ml using HMEM with 2 mM L-glutamine, 1% non-essential amino acids and 50 mg gentamicin. One milliliter of the diluted virus was applied to each well of a six-well plate. The virus was allowed to adsorb for 2 h at room temperature before addition of 1 ml of HMEM with 5% FBS, 2 mM L-glutamine, 1% non-essential amino acids and 50 μg/ml gentamicin. Infected cells were incubated at 16 °C until sampling, which was done at 24 and 72 h post-infection by freezing the plate at −80 °C, until RNA extraction and QRT-PCR analysis. Microarray analysis was carried out on the 24- and 72-h infected and time-matched uninfected controls.

2.3.2. Infection Trial 2 (used for the second QRT-PCR experiment)

The TO cell line was grown as for Infection Trial 1. The virus stocks of Norway-810/9/99 ($10^{6.5}$ $TCID_{50}$/ml), RPC/NB-04-085-1, NBISA01, and RPC/NB-01-0593-1 ($10^{8.75}$ $TCID_{50}$/ml) propagated in TO cells were diluted to $10^{5.0}$ $TCID_{50}$/ml and virus inoculation and adsorption to cell monolayers in six-well plates were performed as for Infection Trial 1. Infected cells were incubated at 16 °C until sampling, which was done at 24, 36, 48, 72, 96, 120 h post-infection by freezing the plate at −80 °C, until RNA extraction and QRT-PCR analysis.

2.4. RNA extraction and DNAse I treatment

Total RNA from the TO cell monolayers was extracted by lysing the TO cell monolayers using 1.25 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA). After incubation of the lysate at room temperature for 10 min, 300 μl of chloroform was added to the mixture and thoroughly mixed before centrifugation for 15 min at 12,000 rpm at 4 °C. The subsequent RNA extraction steps were carried out by transferring the aqueous phase into RNeasy Mini Spin Columns of Qiagen RNeasy Mini kit (Mississauga, ON, Canada). The extracted RNA was DNAse I treated using the Qiagen RNase-free DNase I (Mississauga, ON, Canada) following the manufacturer's procedures. DNAse I-treated RNA samples were column-purified using the RNeasy MinElute Cleanup kit (Qiagen, Mississauga, ON, Canada). The integrity of the RNA samples was checked by running them in 1% agarose/ethidium bromide gels and the purity of the RNA was checked using the A260/A230 and A260/A280 ratios calculated from UV spectrophotometer readings.

2.5. Array hybridization

The microarray experiments were designed to comply with the Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma et al., 2001). Experiments were conducted using the cGRASP 16K array version 2.1 (von Schalburg et al., 2005). Microarray construction and fabrication were previously described (von Schalburg et al., 2005). Column-purified, DNAse I-treated RNA extracted from Infection Trial 1 TO cells harvested at 24 and 72 h

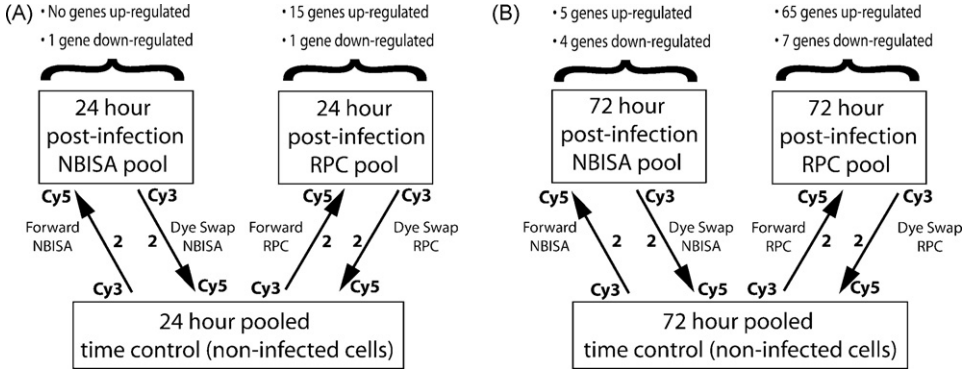


Fig. 1. Overview of microarray experimental design and results. (A) 24 h study. (B) 72 h study. Arrows between samples represent microarrays with the base of the arrow on the Cy3-labeled sample and the arrow-head on the Cy5-labeled sample. The number of replicate microarrays used in each comparison is shown next to the arrows. Number of genes (above each bracket) reported as reproducibly dysregulated (i.e. up- or down-regulated) had fluorescence signal above threshold (i.e. average background corrected, Lowess normalized signal of all *Arabidopsis* features in the dominant channel plus 2 standard deviations, see Section 2) in at least three out of four replicate microarrays and exhibited higher than or equal to 2-fold change in expression in infected relative to non-infected TO cells in all four replicate microarrays of a given comparison (e.g. 24 h NBISA versus 24 h Control) including two dye-swaps. For the comparison of samples collected 24 h post-infection with NBISA, there were no up-regulated genes that met the above criteria and therefore we report no reproducibly up-regulated genes for that comparison. The pooling strategy is described in detail on Section 2. NBISA = TO cells infected with the high pathogenicity NBISA01 ISAV isolate, RPC = TO cells infected with the low pathogenicity RPC/NB-04-085-1 ISAV isolate. Detailed results are in online Supplemental Tables S2a–S2h, and in Fig. 2.

Table 1
Reproducibly informative[a] genes dysregulated in TO cells at 24 h post-infection with the low pathogenic ISAV isolate RPC/NB-04-085-1.

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[h]	QPCR mean (SD) fold change[i]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			RPC[j]	NBISA[k]
Up-regulated genes							
CA055102	Transmembrane protein 106B [*Salmo salar*]	68/139 (48%)	1e-43	Integral to membrane (CC)[g]	52.00 (49.23)	Not done	Not done
CA061046	Unknown	na[m]	na	na	47.24 (22.64)	Not done	Not done
CA057633	Clone B143 VHSV-induced mRNA, partial sequence (*Oncorhynchus mykiss*)[b]	63/84 (75%)	3e-53		28.63 (11.10)	Not done	Not done
CA051350	Interferon-induced protein with tetratricopeptide repeats 5 [*Salmo salar*]	141/141 (100%)	1e-75	Binding (MF)[f]	26.88 (6.91)	17.27 (1.77)[o]	−1.75 (0.17)
CA062838	Unknown	na	na	na	13.53 (4.62)	Not done	Not done
CB516446	Mx1 protein [*Salmo salar*]	202/202 (100%)	2e-105	GTP binding (MF), GTPase activity (MF)	10.94 (6.91)	19.76 (3.09)[o]	−2.51 (0.28)
CA044879	Galectin-9 [*Salmo salar*]	101/101 (100%)	6e-55	Sugar binding (MF)[e]	6.01 (1.23)	Not done	Not done
CA055205	Tripartite motif-containing protein 16 [*Salmo salar*]	84/125 (67%)	2e-39	nf[n]	4.83 (0.53)	Not done	Not done
CA038906	Tripartite motif-containing protein 16 [*Salmo salar*]	68/78 (88%)	9e-30	Zinc ion binding (MF)[g], cytoplasm (CC)[g]	4.22 (0.51)	2.28 (0.32)[o]	−1.77 (0.14)
CA060690	Tripartite motif-containing protein 25 [*Salmo salar*]	238/238 (100%)	2e-134	nf	4.04 (0.36)	1.99 (0.19)[o]	0.78 (0.07)
CA042663	Tripartite motif-containing protein 25 [*Salmo salar*]	170/172 (98%)	8e-92	nf	3.95 (0.59)	1.44 (0.18)[o]	−1.78 (0.13)
CA054622	VHSV-induced protein-10 [*Oncorhynchus mykiss*][l]	24/31 (77%)	8e-14	Nucleus (CC)[f], regulation of transcription, DNA dependent (BP)[f]	3.86 (0.91)	Not done	Not done
CA040401	Tripartite motif-containing protein 16 [*Salmo salar*]	119/147 (80%)	6e-62	nf	3.67 (0.64)	Not done	Not done
CA052520	Galectin-9 [*Salmo salar*][l]	95/95 (100%)	1e-49	Sugar binding (MF)[e]	2.76 (0.25)	Not done	Not done
CA044359	Cytochrome P450 1A1 [*Salmo salar*]	104/105 (99%)	1e-56	Oxidoreductase activity (MF), oxidation reduction (BP)	2.40 (0.20)	Not done	Not done
Down-regulated genes							
CA059691	Unknown	na	na	na	−4.95 (0.74)	−2.34 (0.10)	−2.90 (0.36)

[a] 2-fold or greater up-/down-regulated in ISAV infected TO cells relative to non-infected time-matched controls in all four replicate microarrays. For less stringent lists (≥2-fold up- or down-regulated in ISA infected TO cells relative to non-infected time-matched controls in any three out of four replicate arrays), see online Supplemental Tables S2a and S2b.
[b] Denotes a BLASTN hit gene identification that was used in cases where no significant BLASTX hit was found or BLASTN hit was more significant than a low significance BLASTX hit (i.e. BLASTN hit $<10^{-10}$ and BLASTX hit $\geq 10^{-5}$).
[c] The BLASTX or BLASTN hit with the lowest *E*-value and a gene name (e.g. not predicted or hypothetical) is shown. BLAST statistics were collected on the 10th of December of 2008 and reflect the entries on the nr protein database up to that date. %ID = percent identity of amino acid residues or nucleotides over length of alignment (length align.).
[d] Functional annotation associated with the salmonid cDNA's best BLAST hit or an annotated putative orthologue from *Danio rerio* ([e]), *Homo sapiens* ([f]) or *Mus musculus* ([g]). Gene ontology (GO) categories: biological process (BP), molecular function (MF) and cellular component (CC).
[h] Microarray fold change was calculated as the average of the BCLN signal ratios (R) between RPC infected samples and control samples from all four replicate microarrays, including two dye-swaps. Standard errors (SE) for microarray data reflect technical rather than biological variability, since mean fold change values were derived from four technical replicate microarrays comparing the same pools of samples. Where necessary, fold down-regulation was calculated as the inverse of fold up-regulation.
[i] QRT-PCR fold change values were calculated as described in Section 2 and refer to the first QPCR study that surveyed gene expression only in NBISA01 and RPC/NB-04-085-1-infected TO cells. Standard deviations (SD) for QRT-PCR data reflect technical and biological variability. Down-regulation was calculated when necessary as the inverse of relative fold up-regulation obtained using the Pfaffl (2001) equation and is denoted by a "–" symbol.
[j] RPC = low pathogenicity ISA isolate (RPC/NB-04-085-1).
[k] NBISA = high pathogenicity ISA isolate (NBISA01).
[l] Synonyms for gene names obtained from a BLASTX hit of *Salmo salar* with similar *E*-value or from Swiss-Prot Knowledgebase based on the putative orthologue with functional annotation: VHSV-induced protein-10 – poly [ADP-ribose] polymerase 14, PARP-14, B aggressive lymphoma protein 2; Galectin-9 – novel protein similar to vertebrate galectins.
[m] na: not-applicable.
[n] nf: no functional annotation found for best BLAST hit or any putative orthologues.
[o] Statistically significant differences between the two virus isolates ($p < 0.05$).

post-infection, and time-matched non-infected TO cells, were used for target synthesis using the Array 900 Detection Kit (Genisphere, Hatfield, PA) and Superscript II (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Fig. 1 represents a schematic of the microarray experimental design. Briefly, each viral isolate infected cell sample (i.e. NBISA01 and RPC/NB-04-085-1) was compared to its time-matched, non-infected control using four replicate microarrays that included two dye-swaps. The 16K arrays, obtained from the consortium for Genomic Research on All Salmonids Project (cGRASP) (http://web.uvic.ca/grasp/), were all from the same printing batch (EB017). Arrays were washed prior to hybridization 2 times for 10 min in 0.1% SDS (Ambion, Austin, TX) at room temperature and 5 times for 1 min in nuclease-free water (Gibco-Invitrogen, Carlsbad, CA), immersed in ~95 °C nuclease-free water for 3 min to denature the double stranded cDNA, centrifuged dry (2000 rpm for 5 min at room temperature) in loosely-capped 50 ml conical tubes (BD Falcon, Franklin Lakes, NJ), and stored at 50 °C in a hybridization oven until hybridizations were performed. For each target synthesized, 1 μg of column-purified DNAse I-treated total RNA was reverse-transcribed for 2 h and 30 min at 42 °C using 200 units of Superscript II and the provided 5× Superscript II buffer (Invitrogen, Carlsbad, CA) with specific primers for the different Cy3 and Cy5 Capture Reagent anchors (Genisphere, Hatfield, PA). Complementary DNA (cDNA) from the different treatments, each containing an anchor for one of the Cy dye labeled Capture Reagents (Genisphere, Hatfield, PA) were pooled (e.g. NBISA01-infected TO cell cDNA with a Cy5 anchor was pooled with Control non-infected TO cell cDNA containing a Cy3 anchor) and mixed with 2× formamide-based hybridization buffer containing bovine serum albumin (BSA) (Vial 7 in the Array 900 Kit) and LNA dT as blocking agents following the manufacturer's protocol. All array washes were performed using sterile 50 ml conical tubes (BD Falcon, Franklin Lakes, NJ) and all wash solutions were made with nuclease-free water (Gibco-Invitrogen, Carlsbad, CA). All hybridizations were performed using HybriSlips cover-slips (Grace Biolabs/Sigma Co, St. Louis, MO) and Corning microarray hybridization chambers in a water bath at 50 °C. Targets were hybridized to the arrays overnight (~16 h) at 50 °C. After this period cover-slips were floated off of the arrays in pre-warmed (50 °C) 2× SSC/0.2%SDS (Ambion, Austin, TX) and washed for 15 min in the same solution with gentle agitation, followed by a 15-min wash in 2× SSC (room temperature with gentle agitation) and a 15-min wash in 0.2× SSC (room temperature with gentle agitation). After the washes, slides were centrifuged dry as before and stored in a hybridization oven at 50 °C. The Capture Reagents (Array 900 Kit, Genisphere) containing the fluorescent dyes were combined and mixed with 2× formamide-based hybridization buffer and nuclease-free water following the manufacturer's instructions. The Capture Reagent hybridizations were carried out as described previously for 4 h at 50 °C and slides were washed and dried as before. The arrays were scanned immediately at 10 μm resolution using a PerkinElmer ScanArray Express. The Cy3 and Cy5 cyanine fluorophores were excited at 543 and 633 nm, respectively, at 90% laser power and photomultiplier tube (PMT) settings of 70 for the Cy3 channel and 67 for the Cy5 channel for all microarrays involved in the study.

2.6. Microarray image analysis

Fluorescence intensity data were extracted from TIFF images using ImaGene 5.6.1 software (BioDiscovery, El Segundo, CA). All grids were manually aligned with the spots and the spot sizes were automatically adjusted using ImaGene. Spatial effects (e.g. dust particles) were manually flagged. Quality statistics (e.g. average signal-to-background ratios) were calculated in Excel, and background correction, Lowess normalization, and analysis of background corrected Lowess normalized (BCLN) data (e.g. fold

Table 2
Reproducibly informative[a] genes dysregulated in TO cells at 24 h post-infection with the highly pathogenic ISAV isolate NBISA01.

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[f]	QPCR mean (SD) fold change[g]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			NBISA[h]	RPC[i]
Down-regulated genes							
CA046558	ATP synthetase subunit g, mitochondrial [*Salmo salar*]	100/103 (97%)	3e-51	Mitochondrial membrane (CC)[e], ATP synthesis coupled proton transport (BP)[e]	−2.79 (0.39)	−2.07 (0.32)	−1.63 (0.30)

[a] 2-fold or greater up-/down-regulated in ISAV infected TO cells relative to non-infected time-matched controls in all four replicate microarrays. For less stringent lists (≥2-fold up- or down-regulated in ISA infected TO cells relative to non-infected time-matched controls in any three out of four replicate arrays), see online Supplemental Tables S2c and S2d.
[b,c] Refer to footnotes in Table 1.
[d] Functional annotation associated with the salmonid cDNA's best BLAST hit or an annotated putative orthologue from *Danio rerio* ([e]) gene ontology (GO) categories: biological process (BP), molecular function (MF) and cellular component (CC).
[f] Microarray fold change values were calculated as the average of the background corrected Lowess normalized (BCLN) signal ratios (R) between NBISA infected samples and control samples from all four replicate microarrays, including two dye-swaps. Where necessary, fold down-regulation was calculated as the inverse of fold up-regulation. Standard errors (SE) for microarray data reflect technical rather than biological variability, since mean fold change values were derived from four technical replicate microarrays comparing the same pools of samples.
[g] QRT-PCR fold change values were calculated as described in Section 2 and refer to the first QPCR study that surveyed gene expression only in NBISA01 and RPC/NB-04-085-1-infected TO cells. Standard deviations (SD) for QRT-PCR data reflect technical and biological variability. Down-regulation was calculated when necessary as the inverse of relative fold up-regulation obtained using the Pfaffl (2001) equation and is denoted by a "−" symbol.
[h] RPC = low pathogenicity ISA isolate (RPC/NB-04-085-1).
[i] NBISA = high pathogenicity ISA isolate (NBISA01).

Table 3
Reproducibly informative[a] genes dysregulated in TO cells at 72 h post-infection with the low pathogenic ISAV isolate RPC/NB-04-085-1.

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[h]	QPCR mean (SD) fold change[i]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			RPC[j]	NBISA[k]
Up-regulated genes							
CA038906	Tripartite motif-containing protein 16 [*Salmo salar*]	69/78 (88%)	9e-30	Intracellular (CC)[e], protein binding (MF)[e]	169.43 (157.98)	Not done	Not done
CA061919	Galectin-9 [*Salmo salar*]	59/60 (98%)	5e-29	Sugar binding (MF)[e]	167.22 (142.61)	Not done	Not done
CK990445	Unknown	na[m]	na	na	138.79 (101.25)	Not done	Not done
CA061238	Serum paraoxonase/arylesterase 2 (pon2)[b]	46/51 (90%)	1e-06	nf[n]	55.78 (12.68)	Not done	Not done
CB499972	Ubiquitin-like protein 1 [*Salmo salar*]	155/156 (99%)	2e-82	Protein binding (MF)[g]	40.41 (22.70)	9.21 (2.20)[o]	−1.15 (0.30)
CA064176	Ubiquitin-like protein 1 [*Salmo salar*]	115/117 (98%)	4e-59	Protein binding (MF)[g]	37.44 (12.11)	Not done	Not done
CA044879	Galectin-9 [*Salmo salar*]	101/101 (100%)	6e-55	Sugar binding (MF)[e]	37.09 (13.93)	Not done	Not done
CA051350	Interferon-induced protein with tetratricopeptide repeats 5 [*Salmo salar*]	141/141 (100%)	1e-75	Binding (MF)[g]	30.24 (8.70)	100.28 (10.33)[o]	−1.75 (0.17)
CK990223	Unknown	na	na	na	29.74 (11.47)	Not done	Not done
CB516446	Mx1 protein [*Salmo salar*]	202/202 (100%)	2e-105	GTP binding (MF), GTPase activity (MF)	29.55 (8.03)	217.22 (31.28)[o]	1.13 (0.12)
CA057098	Barrier-to-autointegration factor [*Salmo salar*]	77/97 (79%)	8e-38	DNA binding (MF)[e]	27.94 (6.64)	Not done	Not done
CA056962	Similar to ubiquitin specific protease 18 [*Danio rerio*]	25/41 (60%)	1e-06	Ubiquitin thiolesterase activity (MF), ubiquitin-dependent protein catabolic process (BP)	25.41 (14.87)	18.62 (0.72)[o]	−1.10 (0.06)
CA054858	Arginine/serine-rich coiled-coil 2 [*Danio rerio*]	59/71 (83%)	5e-25	nf	24.09 (10.60)	Not done	Not done
CA058263	Radical S-adenosyl methionine domain-containing protein 2[l] (synonym: viperin) [*Salmo salar*]	91/91 (100%)	8e-47	Endoplasmic reticulum (CC), catalytic activity (MF), response to virus (BP)	21.41 (10.89	794.82 (114.05)[o]	1.19 (0.05)
CA054694	Clone B225 VHSV-induced mRNA, partial sequence [*Oncorhynchus mykiss*][b]	363/416 (87%)	2e-147	nf	20.98 (5.67)	1.92 (0.20)[o]	−1.58 (0.35)
CA061046	Unknown	na	na	na	17.71 (9.67)	Not done	Not done
CA052560	Similar to Probable E3 ubiquitin-protein ligase HERC4 (HECT domain and RCC1-like domain-containing protein 4) [*Danio rerio*]	65/107 (60%)	4e-31	Intracellular (CC)[f], ubiquitin-protein ligase activity (MF), ubiquitin cycle (BP)[f]	16.61 (3.59)	383.86 (95.02)[o]	−2.09 (0.23)
CA054622	VHSV-induced protein-10 [*Oncorhynchus mykiss*][l]	24/31 (77%)	8e-14	Nucleus (CC)[f], regulation of transcription, DNA dependent (BP)[f]	16.05 (6.14)	Not done	Not done
CA040010	Clone ssal-rgf-540-239 Tripartite motif-containing protein 16 putative mRNA, complete cds [*Salmo salar*][b]	653/666 (98%)	0.0	nf	14.41 (4.79)	Not done	Not done
CA050625	Similar to interferon-inducible protein Gig1 [*Danio rerio*]	39/95 (41%)	2e-11	nf	14.24 (2.53)	786.54 (79.69)[o]	2.85 (0.95)
CA062838	Unknown	na	na	na	13.56 (4.32)	Not done	Not done
CA042663	Tripartite motif-containing protein 25 [*Salmo salar*]	170/172 (98%)	1e-91	nf	13.25 (1.64)	Not done	Not done
CA056844	VHSV-induced protein [*Oncorhynchus mykiss*]	104/109 (95%)	5e-55	Receptor activity (MF)[g], ribonuclease activity (MF)[g], RNA binding (MF)[g]	13.24 (2.50)	4682.93 (705.22)[o]	3.34 (0.75)
CA056191	Neurogranin, TIP41-like protein (TIP41), MHC class II antigen beta chain (Sasa-DBB), MHC class II antigen alpha chain (Sasa-DBA), leucine rich repeat containing 35-like protein, and alpha-tectorin-like protein genes, complete cds [*Salmo salar*][b]	133/148 (89%)	1e-46	nf	12.97 (4.99)	Not done	Not done

Table 3 (*Continued*)

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[h]	QPCR mean (SD) fold change[i]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			RPC[j]	NBISA[k]
CA058271	Interferon-inducible protein Gig2-like [*Salmo salar*]	106/131 (80%)	2e-55	nf	12.97 (3.70)	Not done	Not done
CB494193	VHSV-induced protein-3 [*Oncorhynchus mykiss*][l]	122/122 (100%)	5e-63	Protein modification process (BP)	12.79 (4.53)	Not done	Not done
CA057633	Clone B143 VHSV-induced mRNA, partial sequence [*Oncorhynchus mykiss*][b]	358/396 (90%)	9e-152	nf	12.61 (3.72)	Not done	Not done
CA052004	Unknown	na	na	na	10.14 (3.39)	Not done	Not done
CA053164	Sacsin (synonym: spastic ataxia of Charlevoix-Saguenay) [*Mus musculus*]	43/116 (37%)	7e-14	ATP binding (MF), heat shock protein binding (MF)	9.55 (3.32)	40.24 (7.08)[o]	−1.68 (0.42)
CB515563	CD9 antigen [*Salmo salar*]	234/235 (99%)	234/235 (99%)	Integral to membrane (CC)	9.45 (2.06)	421.02 (73.55)[o]	−1.18 (0.24)
CA052520	Galectin-9 [*Salmo salar*][o]	95/95 (100%)	1e-49	Sugar binding (MF)[j]	9.08 (2.28)	Not done	Not done
CA064247	Clone ssal-rgf-516-278 CD9 antigen putative mRNA, complete cds [*Salmo salar*][b]	712/715 (99%)	0.0	nf	8.91 (2.22)	Not done	Not done
CB516202	Clone 261P24 interferon-inducible GTPase_b and interferon-inducible GTPase_a genes, complete cds; and TCR-gamma constant region locus [*Salmo salar*][b]	296/393 (75%)	7e-70		8.77 (2.69)	Not done	Not done
CA050461	Unknown	na	na	na	8.77 (4.21)	Not done	Not done
CA055205	Tripartite motif-containing protein 16 [*Salmo salar*]	84/125 (67%)	2e-39	nf	8.08 (1.47)	Not done	Not done
CA057036	Clone ssal-rgf-540-239 Tripartite motif-containing protein 16 putative mRNA, complete cds [*Salmo salar*][b]	377/388 (97%)	0.0	nf	7.99 (2.54)	Not done	Not done
CA060690	Tripartite motif-containing protein 25 [*Salmo salar*]	238/238 (100%)	2e-134	nf	7.82 (3.88)	Not done	Not done
CB517062	Clone ssal-rgf-540-239 Tripartite motif-containing protein 16 putative mRNA, complete cds [*Salmo salar*][b]	316/345 (91%)	2e-134	nf	7.61 (2.62)	Not done	Not done
CA040505	VHSV-induced protein-10 mRNA, complete cds [*Oncorhynchus mykiss*][b]	493/581 (84%)	0.0	nf	6.77 (2.66)	7.53 (1.51)[o]	−1.44 (0.38)
CB498971	Clone ssal-rgf-531-067 Tripartite motif-containing protein 16 putative mRNA, complete cds [*Salmo salar*][b]	708/715 (99%)	0.0	nf	6.59 (2.52)	Not done	Not done
CB517430	Unknown	na	na	na	6.16 (1.47)	Not done	Not done
CA044358	Galectin-9 [*Salmo salar*]	83/83 (100%)	6e-42	Sugar binding (MF)	6.08 (2.87)	Not done	Not done
CB515011	Galectin-3-binding protein precursor [*Salmo salar*]	198/198 (100%)	4e-86	Protein binding (MF)[e], scavenger receptor activity (MF)[e]	5.76 (1.05)	Not done	Not done
CB516789	Clone ssal-rgf-504-007 Ornithine decarboxylase 1 putative mRNA, complete cds [*Salmo salar*][b]	618/620 (99%)	0.0	nf	5.74 (3.25)	Not done	Not done
CA049872	Clone ssal-rgf-531-067 Tripartite motif-containing protein 16 putative mRNA, complete cds [*Salmo salar*][b]	578/589 (98%)	0.0	nf	5.67 (1.39)	Not done	Not done
CA043257	MHC class 1b antigen [*Oncorhynchus mykiss*]	190/227 (83%)	6e-110	MHC class I protein complex (MF), antigen processing and presentation (BP), immune response (BP)	5.66 (1.70)	4.08 (0.40)[o]	−1.08 (0.07)
CB500108	60S ribosomal protein L35 [*Salmo salar*]	123/123 (100%)	2e-49	Ribosome (CC)[e], structural constituent of ribosome (MF)[e], negative regulation of cell cycle (BP)[e]	5.48 (1.61)	Not done	Not done
CB511792	Unknown	na	na	na	5.45 (2.14)	Not done	Not done
CA040401	Tripartite motif-containing protein 16 [*Salmo salar*]	119/147 (80%)	6e-62	nf	5.40 (2.24)	Not done	Not done
CB512373	Barrier-to-autointegration factor [*Salmo salar*]	52/68 (76%)	1e-21	DNA binding (MF)[e]	5.28 (1.84)	Not done	Not done

Table 3 (*Continued*)

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[h]	QPCR mean (SD) fold change[i]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			RPC[j]	NBISA[k]
CA059978	Pre-B cell enhancing factor [*Cyprinus carpio*][l]	134/154 (87%)	4e-74	Cytoplasm (CC)[g], nicotinamide phospho-ribosyltrasfersase activity (MF)[g], NAD biosynthetic process (BP)[g]	5.14 (1.14)	5.07 (0.51)[o]	−1.33 (0.10)
CA044387	MHC class I (UBA) mRNA, UBA*0301 allele, complete cds [*Salmo salar*][b]	685/698 (98%)	0.0	nf	4.61 (2.30)	Not done	Not done
CB499584[q]	Importin subunit alpha-2 [*Salmo salar*][l]	203/213 (95%)	9e-101	Nuclear pore (CC)[e], protein transport activity (MF)[e], protein import into the nucleus (BP)[e]	4.54 (0.80)	2.00 (0.13)	−1.13 (0.05)
CB511632	Proteasome subunit beta type-9 [*Salmo salar*]	177/177 (100%)	1e-95	Proteasome core complex (CC), threonine-type endopeptidase activity (MF), immune response (BP), ubiquitin-dependent protein catabolic process (BP)	4.52 (1.11)	Not done	Not done
CB499451	CD9 protein (LOC100136380), mRNA [*Salmo salar*][b]	677/677 (100%)	0.0	nf	4.40 (1.17)	Not done	Not done
CA051735	Unknown	na	na	na	4.15 (0.58)	Not done	Not done
CA041367	Clone ssal-evf-572-211 beta-2-microglobulin precursor putative mRNA, complete cds [*Salmo salar*][b]	428/430 (99%)	0.0	nf	4.07 (1.55)	Not done	Not done
CB497413	Reproduction regulator 2 [*Epinephelus coioides*]	44/110 (40%)	8e-14	nf	3.98 (1.08)	Not done	Not done
CB500763	Clone ssal-rgb2-510-275 beta-2-microglobulin precursor putative mRNA, complete cds [*Salmo salar*][b]	548/552 (99%)	0.0	nf	3.69 (2.04)	1.98 (0.21)	1.20 (0.15)
CA051372	Proteasome subunit beta type-9 [*Salmo salar*]	145/145 (100%)	4e-77	Proteasome core complex (CC), threonine-typeendopeptidase activity (MF), immune response (BP), ubiquitin-dependent protein catabolic process (BP)	3.55 (1.15)	Not done	Not done
CA064302	Proteasome subunit beta type-9 [*Salmo salar*]	139/139 (100%)	7e-74	Proteasome core complex (CC), threonine-type endopeptidase activity (MF), immune response (BP), ubiquitin-dependent protein catabolic process (BP)	3.42 (0.84)	Not done	Not done
CB511439	Endoplasmic reticulum to nucleus signaling 2 [*Xenopus tropicalis*]	41/58 (70%)	9e-18	Endoribonuclease activity, producing 5′-phosphomonoesters (MF), protein amino acid phosphorylation (BP)	3.18 (0.81)	Not done	Not done
CA060011	Barrier-to-autointegration factor [*Salmo salar*]	96/97 (98%)	5e-50	DNA binding (MF)[e]	3.04 (0.80)	Not done	Not done
CB509315	Unknown	na	na	na	2.70 (0.63)	Not done	Not done
CB501070	Clone ssal-rgf-528-084 Cytochrome P450 1A1 putative mRNA, complete cds [*Salmo salar*][b]	704/704 (100%)	0.0	nf	2.69 (0.59)	Not done	Not done
Down-regulated genes							
CA059375	mRNA for putative ISG12 (3) protein (isg12 (3) gene) [*Salmo salar*][b]	603/604 (99%)	0.0	nf	−5.48 (1.58)	Not done	Not done
CA058274	Ribosomal protein L15 [*Salmo salar*]	204/204 (100%)	2e-108	Ribosome (CC), structural constituent of ribosome (MF), translation (BP)	−4.59 (2.11)	Not done	Not done
CB511186	Retinoic acid receptor gamma b (Rargb) gene, partial cds [*Salmo salar*][b]	471/472 (99%)	0.0	nf	−3.33 (0.62)	Not done	Not done
CB514092	Glutamine synthetase [*Salmo salar*]	27/27 (100%)	6e-08	Glutamate-ammonialigase activity (MF), glutamine biosyntheticprocess (BP)	−3.19 (0.64)	−1.40 (0.07)[o]	−1.27 (0.20)

Table 3 (*Continued*)

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[h]	QPCR mean (SD) fold change[i]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			RPC[j]	NBISA[k]
CA037505	Snrpb2 protein [*Danio rerio*]	39/41 (95%)	9e-14	Nulceotide binding (MF)[e]	−3.02 (0.58)	Not done	Not done
CB515569	MYG1 protein [*Bos taurus*]	132/212	1e-73	nf	−2.68 (0.13)	Not done	Not done
CB512171	Tryptophanyl-tRNA synthetase [*Danio rerio*]	85/103 (82%)	8e-44	Cytoplasm (CC), tryptophan-tRNA ligase activity (MF), trytophanyl-tRNA aminoacylation (BP)	−2.62 (0.69)	−1.13 (0.025)[o]	−1.23 (0.10)

[a]2-fold or greater up-/down-regulated in ISAV infected TO cells relative to non-infected time-matched controls in all four replicate arrays. For a less stringent list refer to online Supplemental Tables S2e and S2f (2-fold or greater up-or down-regulation in ISA infected TO cells relative to non-infected time-matched controls in any three out of four replicate arrays).
[b,c]Refer to footnotes in Table 1
[d]Functional annotation associated with the cod cDNA's best BLAST hit or an annotated putative orthologue from *Danio rerio* ([e]), *Homo sapiens* ([f]) or *Mus musculus* ([g]). Gene ontology (GO) categories: biological process (BP), molecular function (MF) and cellular component (CC).
[h]Microarray fold change was calculated as the average of the BCLN signal ratios (R) between RPC infected samples and control samples from all four replicate microarrays, including two dye-swaps. Where necessary, fold down-regulation was calculated as the inverse of fold up-regulation. Standard errors (SE) for microarray data reflect technical rather than biological variability, since mean fold change values were derived from four technical replicate microarrays comparing the same pools of samples.
[i,j,k]Refer to footnotes in Table 1.
[l]Synonyms for gene names obtained from an BLASTX hit of *Salmo salar* with similar *E*-value or from Swiss-Prot based on the putative orthologue with functional annotation: Radical S-adenosyl methionine domain-containing protein 2 – virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible, Viperin; VHSV-induced protein-10 – poly [ADP-ribose] polymerase 14, PARP-14, B aggressive lymphoma protein 2; VHSV-induced protein – novel protein, possible orthologue of human peroxisomal proliferator-activated receptor A interacting complex 285 PRIC285; VHSV-induced protein-3 – ubiquitin-like protein 1; Pre-B cell enhancing factor – nicotinamide phosphoribosyltransferase, Visfatin; Importin subunit alpha-2 – Karyopherin alpha-2; Snrpb2 protein – signal peptide peptidase-like 2A.
[m,n,o]Refer to footnotes in Table 1.
[q]Primers for this gene were designed based on the sequence of the informative feature of same annotation from the 24 h experiment (i.e. CA043335). BLASTX analysis of these two ESTs reveal that they share the same top BLASTX hit and therefore are likely to represent the same transcript.

Table 4
Reproducibly informative[a] genes dysregulated in TO cells at 72 h post-infection with the highly pathogenic ISAV isolate NBISA01.

Microarray feature representative EST accession number	BLASTX or BLASTN ([b]) Identification[c] of informative microarray features			Functional annotation[d]	Microarray mean (SE) fold change[f]	QPCR mean (SD) fold change[g]	
	Gene name [*Species* of best BLAST hit]	Length align. (%ID)	*E*-value			NBISA[h]	RPC[i]
Up-regulated genes							
CA037733	RNase 2 [*Oncorhynchus masou formosanus*]	91/132 (68%)	2e-46	Nucleic acid binding (MF)	4.10 (0.58)	Not done	Not done
CB489043	beta-2-microglobulin (β2-m) mRNA [*Oncorhynchus mykiss*][b]	410/419 (97%)	0.0	nf[l]	3.87 (1.14)	1.98 (0.21)	1.20 (0.)
CA050381	Unknown	na[k]	na	na	3.72 (0.83)	Not done	Not done
CA056199	Spermidine/spermine N1-acetyltransferase [*Danio rerio*]	149/170 (87%)	5e-84	N-acetyltranferase activity (MF), metabolic process (BP)	3.47 (0.74)	2.48 (0.49)	2.36 (0.42)
CA043660	Nuclear receptor subfamily 0 group B member 2 (nr0b2) [*Salmo salar*][b]	532/533 (99%)	0.0	nf	3.19 (0.97)	Not done	Not done
Down-regulated genes							
CA060050	Homeobox protein HoxB13ab (HoxB13ab) [*Salmo salar*][b]	421/471 (89%)	2e-171	nf	−5.46 (2.43)	−1.08 (0.08)	−1.27 (0.15)
CA062564	Unknown	na	na	na	−5.15 (2.18)	Not done	Not done
CA060971	Signal peptide peptidase-like 2A [*Gallus gallus*]	39/60 (65%)	5e-15	Integral to membrane (CC)[e], aspartic-type endopeptidase activity (MF)[e]	−3.57 (1.10)	Not done	Not done
CB492839	Altantic salmon ependymin (SS-II) gene, complete cds [*Salmo salar*]	369/428 (86%)	2e-139	nf	−2.44 (0.17)	Not done	Not done

[a]2-fold or greater up-/down-regulated in ISAV infected TO cells relative to non-infected time-matched controls in all four replicate arrays. For a less stringent list refer to online Supplemental Tables S2g and S2h (2-fold or greater up-regulation in ISA infected TO cells relative to non-infected time-matched controls in any three out of four replicate arrays).
[b,c]Refer to footnotes in Table 1.
[d]Functional annotation associated with the salmonid cDNA's best BLAST hit or an annotated putative orthologue from *Homo sapiens* ([e]). Gene ontology (GO) categories: biological process (BP), molecular function (MF) and cellular component (CC).
[f]Microarray fold change values were calculated as the average of the background corrected Lowess normalized (BCLN) signal ratios (R) between NBISA01-infected samples and control samples from all four replicate microarrays, including two dye-swaps. Where necessary, fold down-regulation was calculated as the inverse of fold up-regulation. Standard errors (SE) for microarray data reflect technical rather than biological variability, since mean fold change values were derived from four technical replicate microarrays comparing the same pools of samples.
[g,h,i]Refer to footnotes on Table 2.
[k]na: not-applicable.
[l]nf: no functional annotation found for best BLAST hit or any putative orthologues.

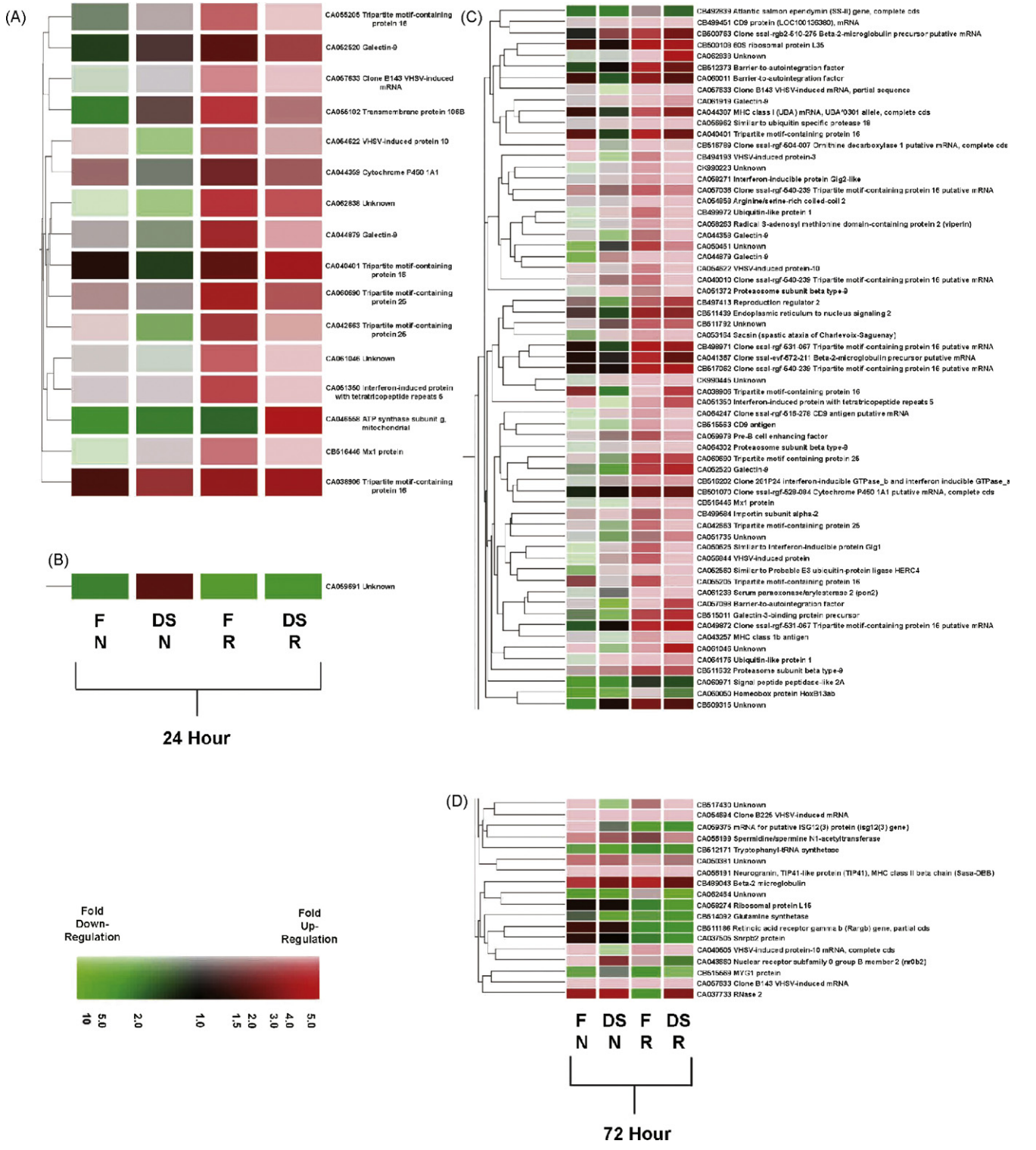


Fig. 2. Hierarchical clustering analysis of reproducibly dysregulated (i.e. more than 2-fold dysregulated in all four replicate arrays of a given comparison) genes after infection of TO cells with NBISA01 (N) and RPC/NB-04-085-1 (R) isolates of ISAV, visualized as gene trees with heat maps. Mean background corrected, Lowess normalized infected/non-infected signal ratios for the two microarrays in each "forward" comparison (F: Cy3-labeled non-infected and Cy5-labeled infected; see Fig. 1) and the two microarrays in each dye-swap (DS: Cy3-labeled infected and Cy5-labeled non-infected; see Fig. 1) are included. Genes shown were ≥2-fold up- or down-regulated in all four microarrays of a given comparison. A and B contain the 17 genes that were reproducibly dysregulated at the 24 h time point (see Fig. 1A); the majority of these informative genes were up-regulated by RPC/NB-04-085-1 ISAV isolate. C and D contain the 81 genes that were reproducibly dysregulated at the 72 h time point (see Fig. 1B); the majority of these informative genes were up-regulated by RPC/NB-04-085-1 ISAV isolate. (bold A) A region of the 24 h microarray experiment containing genes that were mostly up-regulated by RPC/NB-04-085-1 ISAV isolate. (bold B) A region of the 24 h microarray experiment containing one gene that was down-regulated by RPC/NB-04-085-1 ISAV isolate. (bold C) A region of the 72 h microarray experiment containing genes that were mostly up-regulated by RPC/NB-04-085-1 ISAV isolate. (bold D) A region of the 72 h microarray experiment gene tree with no clear trends.

change calculations) were performed in GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA). Transcripts with consistent ≥2-fold difference in expression between RNA samples in all four slides of a given comparison (e.g. up-regulated in NBISA01-infected TO cells relative to non-infected control TO cells at the 72 h time point) were identified in GeneSpring using Venn diagrams to determine the intersection of all possible combinations between the four technical replicates including dye-swaps. Data were then thresholded to assess quality based on fluorescence signal strength using the BCLN signal of all 24 *Arabidopsis* cDNA features present in the 16K array (von Schalburg et al., 2005). Thresholds for each channel in each array were calculated as the average BCLN signal of all *Arabidopsis* features plus two standard deviations (von Schalburg et al., 2005). Reported dysregulated genes in Tables 1–4 and Fig. 2A–D were identified in the highest stringency gene lists (i.e. 2-fold or higher dysregulation in all four arrays including two dye-swaps) while those presented in Supplemental Table S2 were identified in lower stringency gene lists (i.e. 2-fold or higher dysregulation in any three out four arrays including one dye-swap). Genes in either list had BCLN signal in the dominant channel (i.e. the channel with the highest signal in a given comparison) above threshold in at least three of the four technical replicate microarrays.

2.7. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

The first QRT-PCR study, using Infection Trial 1 samples, was run to confirm the microarray results (i.e. the dysregulation of a selection of 25 microarray-identified genes in response to RPC/NB-04-085-1 and NBISA01 at 24 and 72 h time points). Twenty-three out of 25 microarray-identified genes for QRT-PCR were selected from the high stringency reproducibly dysregulated genes list based on level of up-regulation, and immune-relevant functional annotations associated with informative microarray features. The exception was importin subunit alpha-2 (CA043335) that was selected from a lower stringency gene list (i.e. genes dysregulated in any three out of four replicates microarrays including one dye-swap). However, other features with the same annotation were present in the high stringency gene lists (e.g. CB499584). Moreover, the selection was also intended to represent both up-regulated and down-regulated genes. The second QRT-PCR study, using Infection Trial 2 samples, was designed to survey the expression of the 24 microarray-identified, QRT-PCR confirmed ISAV-responsive genes in TO cells exposed to four different ISAV isolates and sampled at six time points post-infection (24, 36, 48, 72, 96, and 120 h). For the QRT-PCR validation of the microarray observations, the Infection Trial 1 RNA samples of the three biological replicates (i.e. from three different cell culture wells infected with a given ISAV isolate of interest at a particular time point post-infection) were used individually (i.e. RNA samples were not pooled) for cDNA synthesis. For studying the expression of candidate ISAV-responsive genes with QRT-PCR, the Infection Trial 2 RNA samples of the three biological replicates were pooled and samples were run in triplicate for QRT-PCR. First strand cDNA synthesis was done using the Transcriptor reverse transcriptase first strand cDNA synthesis kit (Roche). The cDNA synthesis used 300 ng of total RNA and a master mix consisting of 4 μl of 5× RT reaction buffer, 2 μl of dNTP mix (200 μM), 2 μl of random hexamer (600 μM), 0.5 μl RNase inhibitor (40 U/μl), 0.5 μl of transcriptor reverse transcriptase (20 U/μl), and nuclease-free water to adjust the 20 μl volume. The RT step was carried out as described by Workenhe et al. (2008a). PCR primers for the genes of interest (GOI) were designed using either Primer3 (http://frodo.wi.mit.edu/) or Primer Express (Applied Biosystems, Foster City, CA) and are listed in Supplemental Table S1. For determining amplification efficiency of each primer set, standard curves were generated using six serial 5-fold dilutions of cDNA run in triplicates. Efficiencies were calculated as described in Pfaffl (2001). The LightCycler LC480 and LC480 SYBR Green master mix (Roche, Indianapolis, IN) were used for validating the microarray-identified dysregulated genes. The 20 μl QRT-PCR reaction consisted of 10 μl SYBR Green I (2×), 7 μl nuclease-free water, and 0.5 μM of the forward and reverse primers. The PCR cycling consisted of a hot-start of 95 °C for 10 min, followed by an amplification programme of 10 s at the annealing temperatures (56–60 °C depending on primer pair) (Supplemental Table S1), 15 s at 72 °C and detection at 80 °C for 2 s. The Ct values were analyzed using the LC480 software (version 1.5), and technical replicates showing more than 0.5 Ct values difference between two out of three triplicates were discarded as outliers (Nolan et al., 2006). For detailed information regarding QRT-PCR data refer to Supplemental Tables S3a–S3x. The stability of expression of the 18S ribosomal RNA, the normalizer gene, was strictly validated. The Ct values were then analyzed using the Pfaffl method (Pfaffl, 2001) to get the relative fold ratio as described in the Pfaffl equation (shown below). The Pfaffl method is based on efficiency correction of the Ct value difference using the PCR efficiencies of the target and the normalizer gene primer pairs. Relative fold increases of the transcripts were calculated compared to uninfected control samples.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct[\text{target(uninfected control}-\text{ISAV infected})]}}{(E_{18S})^{\Delta Ct[18S(\text{uninfected control}-\text{ISAV infected})]}}$$

The amplification efficiencies of the PCR primers used for validation of the microarray data are shown in Supplemental Table S1.

To test for statistical significance between the observed mean differences of relative fold induction between the two virus isolates (NBISA01, and RPC/NB-04-085-1) of Infection Trial 1, Microsoft Excel was used for two tailed *t*-tests assuming normal distribution of the data and after running an *F*-test for homogeneity of variance. All of the statistically significant mean differences mentioned in the results indicate statistically significant higher fold induction by RPC/NB-04-085-1 compared to NBISA01 isolate. Quantitation of ISAV copy number equivalents and determination of ISAV titer from Ct value data were performed as described by Workenhe et al. (2008b).

3. Results

3.1. Microarray analysis of global gene expression after infection with different ISAV isolates

In order to characterize the transcriptome changes of TO cells in response to different ISAV isolates, cells infected with RPC/NB-04-085-1 or NBISA01 (Infection Trial 1) and time-matched uninfected controls were harvested at 24 and 72 h post-infection for RNA extraction and used for microarray and the first round of QRT-PCR analyses. These time points were chosen in order to span critical periods of infection for both the highly pathogenic NBISA01 (which was anticipated to start prior to 24 h) and the low pathogenicity RPC/NB-04-085-1 (which was anticipated to start after 24 h but before 48 h). In the microarray analysis, transcripts of ISAV infected cells that showed ≥2-fold increase or decrease in background corrected Lowess normalized (BCLN) fluorescent signal ratio between the two channels representing infected and the time-matched, non-infected control samples (i.e. expression) in all four technical replicate microarrays (including two dye-swaps), and that passed the threshold signal in at least three out of four replicates (see Section 2), were considered reproducibly dysregulated (Fig. 1 and Tables 1–4). The 24 h RPC/NB-04-085-1-infected TO cells had 15 reproducibly up-regulated genes and 1 reproducibly down-regulated gene (Table 1). The 24 h NBISA01-infected TO cells had no reproducibly up-regulated genes and one reproducibly down-

regulated gene (Table 2). The 72 h RPC/NB-04-085-1-infected TO cells had 65 reproducibly up-regulated genes and 7 reproducibly down-regulated genes (Table 3). The 72 h NBISA01-infected cells had five reproducibly up-regulated genes and four reproducibly down-regulated genes (Table 4). The BLASTX/BLASTN (Altschul et al., 1997) hits and functional annotations for these reproducibly dysregulated genes are listed in Tables 1–4. For both virus strains and time points, less stringent informative gene lists (i.e. ≥2-fold dysregulation in any three out of four replicate microarrays) are available in Supplemental Tables S2a–S2h.

Only the absolutely reproducible microarray results (i.e. from Tables 1–4, containing genes that were informative in all four technical replicate slides of a given comparison) are presented hereafter. This section does not consider genes in the less stringent gene lists (i.e. reproducibly dysregulated in any three out of four replicate microarrays, including one dye-swap) contained in Supplemental Tables S2a–S2h. At the 24 and/or the 72 h sampling time points several immune-relevant genes were reproducibly up-regulated (i.e. 2-fold or higher up-regulation in all four arrays of a given comparisons, including two dye-swaps) in RPC/NB-04-085-1-infected TO cells when compared to the time-matched non-infected controls (Tables 1 and 3). These included Mx1 protein (24 and 72 h) (informative microarray feature representative EST GenBank dbEST accession number: CB516446), interferon-inducible protein Gig1 (72 h) (CA050625) and Gig2-like (72 h) (CA058271), interferon-induced protein with tetratricopeptide repeats 5 (24 and 72 h) (CA051350), galectin-9 (24 and 72 h) (e.g. CA044879), galectin-3-binding protein precursor (72 h) (CB515011), and several putative members of the *TRI*partite *M*otif (TRIM) protein family (24 and 72 h) (e.g. CA055205). Moreover, 14 of the 15 genes that were up-regulated by RPC/NB-04-085-1 at 24 h were also up-regulated by the same strain at 72 h (Fig. 2A–D).

A first round QRT-PCR study was carried out on selected genes from the reproducibly dysregulated gene lists (Tables 1–4), and fold changes caused by infection with either NBISA01 or RPC/NB-04-085-1 are presented in Tables 1–4 alongside the microarray gene expression data. The QRT-PCR validation generally showed similar direction of gene expression response (up- or down-regulation) as seen in the microarray experiment. However, the fold change values were different between the microarray and the QRT-PCR, potentially due to differences in the sensitivity of the techniques, different methods of data analysis, and the use of separate sets of RNA samples for the microarray and the QRT-PCR experiments. Seventeen microarray-identified genes up-regulated by RPC/NB-04-085-1 (24 and/or 72 h) [Mx1 protein (CB516446), TRIM 16 protein (CA038906), TRIM 25 protein [CA042663 and CA060690, likely representing the same cDNA since they share 99% identity over 550 aligned nucleotides (*E*-value of 0.0) and 99% identity over 180 aligned predicted amino acids (*E*-value of $4e^{-114}$).], interferon-induced protein with tetratricopeptide repeats 5 (CA051350), viperin (Radical S-adenosyl methionine domain-containing protein 2 – CA058263), beta-2-microglobulin precursor (CB489043), ubiquitin specific protease 18-like (CA056962), pre-B cell enhancing factor (CA059978), MHC class Ib antigen (CA043257), ubiquitin-like protein 1 (CB499972), Clone B225 VHSV-induced mRNA, VHSV-induced protein-10, and VHSV-induced protein (CA054694, CA040505 and CA056844, respectively), E3 ubiquitin-protein ligase HERC4-like (CA052560), interferon-induced protein Gig1-like (CA050625), Sacsin (CA053164), and CD9 antigen (CB515563)] were all consistently found to be up-regulated by QRT-PCR analysis of RPC/NB-04-085-1 samples (Tables 1–3). A microarray-identified gene up-regulated by the NBISA01 strain of ISAV (72 h) [spermidine/spermine N1-acetyltransferase (CA056199)] was confirmed as up-regulated using QRT-PCR of NBISA01 samples (Table 4). Four microarray-identified genes down-regulated by RPC/NB-04-085-1 [importin subunit alpha-2 (CA043335; greater than 2-fold down-regulated in three out of four replicate microarrays, see Supplemental Table S2b), one unknown gene (CA059691), tryptophanyl-tRNA synthetase (CB512171) and glutamine synthetase (CB514092)] were also down-regulated in the QRT-PCR analysis of RNA samples. Two microarray-identified genes down-regulated by NBISA01 [ATP synthetase subunit g, mitochondrial (CA046558) and homeobox protein HoxB13ab (CA060050)] were down-regulated in NBISA01-infected TO cells, by 24 and 72 h; respectively, and the same direction of fold change was observed by QRT-PCR validation. A different microarray feature (CB499584) with similar gene annotation of importin subunit alpha-2 was shown to be up-regulated in 72 h RPC/NB-04-085-1-infected TO cells by the microarray study.

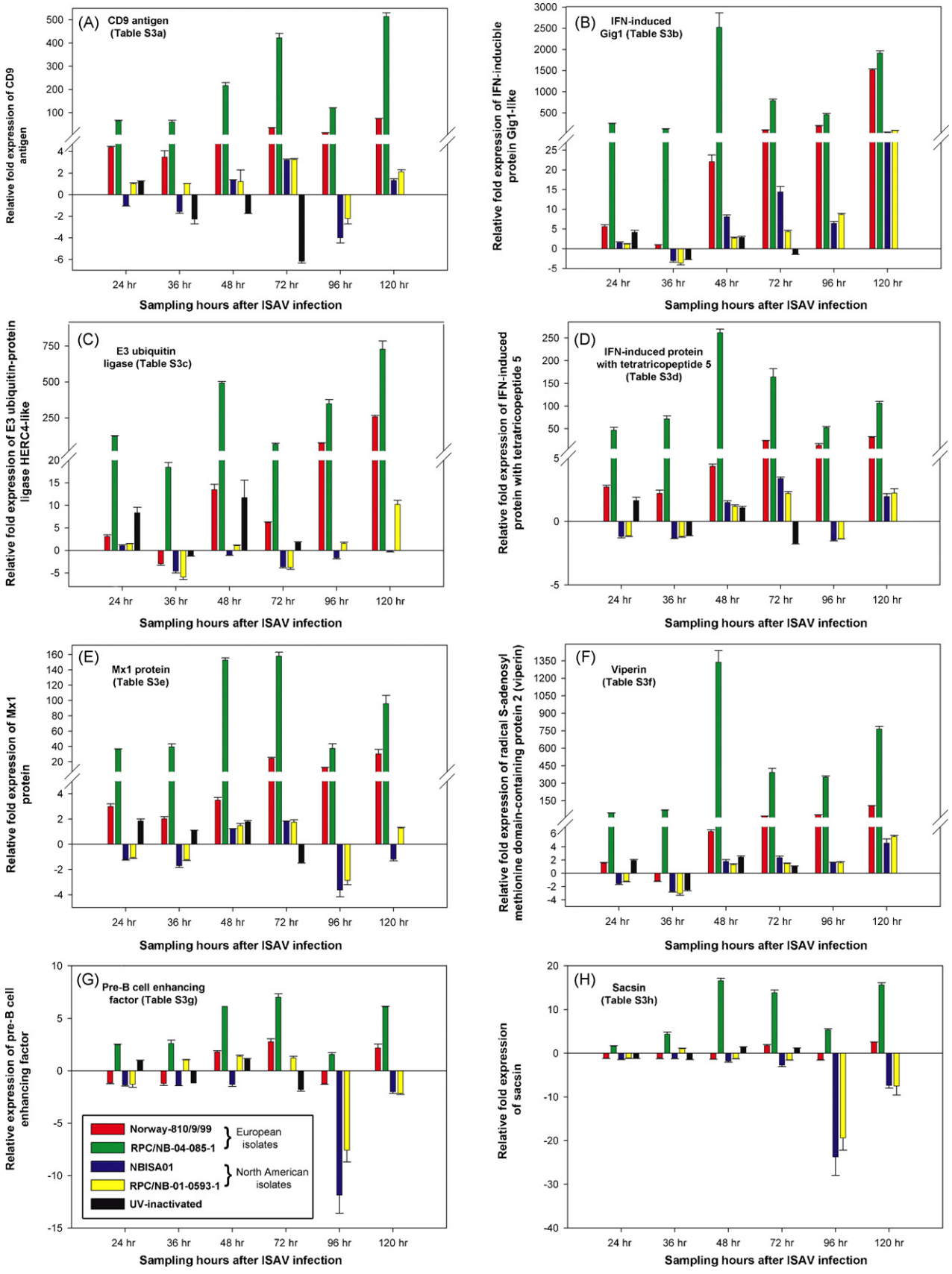
Using the QRT-PCR validation data *t*-tests were carried out to identify mean fold changes that show statistically significant differences between the two virus isolates used in Infection Trial 1. Out of all the QRT-PCR validated genes, Mx1, three TRIM proteins, beta-2-microglobulin, viperin, MHC class 1b antigen, Ubiquitin specific protease 8, pre-B cell enhancing factor, VHSV-induced protein-10, interferon-induced protein with tetratricopeptide repeats 5, Clone B225 VHSV-induced mRNA, E3 ubiquitin-protein ligase, VHSV-induced protein, interferon-induced Gig1, Sacsin, and CD9 antigen had statistically significant higher mean induction in RPC/NB-04-085-1 compared to NBISA01 ($p < 0.05$).

To ensure that the microarray and QRT-PCR validation-observed transcript dysregulations correlated to ISAV replication in TO cells, QRT-PCR with absolute quantitation of ISAV segment 8 transcripts was performed to estimate ISAV copy number equivalents/ng of total RNA. Both RPC/NB-04-085-1 and NBISA01-infected TO cells showed a decrease in Ct value and therefore increase in infectious titer and ISAV copy number equivalents/ng of total RNA extracted from samples obtained at 24–72 h (Supplemental Table S3).

3.2. Expression of QRT-PCR validated genes of interest in response to four different ISAV isolates

The microarray analysis and QRT-PCR validation data of Infection Trial 1 (presented in Tables 1–4) confirmed that the two ISAV isolates NBISA01 (high pathogenicity isolate of North American genotype) and RPC/NB-04-085-1 (low pathogenicity isolate of European genotype) induce differing immune responses. It was not clear if the differences were associated with genotypic or phe-

Fig. 3. Relative fold expression of the microarray-identified and QRT-PCR validated genes in response to infection of TO cells with ISAV isolates Norway-810/9/99 (red), RPC/NB-04-085-1 (green), NBISA01 (blue) and RPC/NB-01-0593-1 (yellow) and UV-inactivated RPC/NB-04-085-1 (black). Relative fold change of each sampling point are PCR efficiency-corrected fold changes calibrated to their time-matched uninfected controls and normalized to the expression level of 18S ribosomal RNA. For each target gene [A – CD9 antigen, B – IFN-inducible protein Gig1-like, C – E3 ubiquitin-protein ligase HERC4-like, D – IFN induced protein with tetratricopeptide 5, E – Mx1 protein, F – Radical S-adenosyl methionine domain-containing protein (viperin), G – pre-B cell enhancing factor, H – Sacsin (spastic ataxia of Charlevoix-Saguenay), I – tripartite motif-containing protein 16, J – ubiquitin-like protein 1-like, K – ubiquitin specific protease 18-like, L – VHSV-induced protein-10 mRNA, M – beta-2-microglobulin, N – importin subunit alpha-2, O – MHC class 1b antigen, P – spermidine/spermine N1-acetyltransferase, Q – tripartite motif-containing protein 25 (CA042663), R – tripartite motif-containing protein 25 (CA060690), S – VHSV-induced mRNA, T – ATP synthetase subunit g, U – glutamine synthetase, V – tryptophanyl-tRNA synthetase, W – unknown gene (CA059691), X – ISAV segment 8]. RNA extracted from a pool of three different cell culture wells infected with a given ISAV stain and sampled at the different time points was used for cDNA synthesis. Gene of interest expression was quantified in 96-well plates with triplicate technical replicates. Error bars are standard deviations of the QRT-PCR data and reflect technical rather than biological variability, since mean fold change values were derived from three technical replicates comparing the same pools of RNA samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)


(A) CD9 antigen (Table S3a)
Relative fold expression of CD9 antigen
(B) IFN-induced Gig1 (Table S3b)
Relative fold expression of IFN-inducible protein Gig1-like
(C) E3 ubiquitin ligase (Table S3c)
Relative fold expression of E3 ubiquitin-protein ligase HERC4-like
(D) IFN-induced protein with tetratricopeptide 5 (Table S3d)
Relative fold expression of IFN-induced protein with tetratricopeptide 5
(E) Mx1 protein (Table S3e)
Relative fold expression of Mx1 protein
(F) Viperin (Table S3f)
Relative fold expression of radical S-adenosyl methionine domain-containing protein 2 (viperin)
(G) Pre-B cell enhancing factor (Table S3g)
Relative expression of pre-B cell enhancing factor
(H) Sacsin (Table S3h)
Relative fold expression of sacsin
24 hr
36 hr
48 hr
72 hr
96 hr
120 hr
Sampling hours after ISAV infection
Norway-810/9/99
RPC/NB-04-085-1
European isolates
NBISA01
RPC/NB-01-0593-1
North American isolates
UV-inactivated

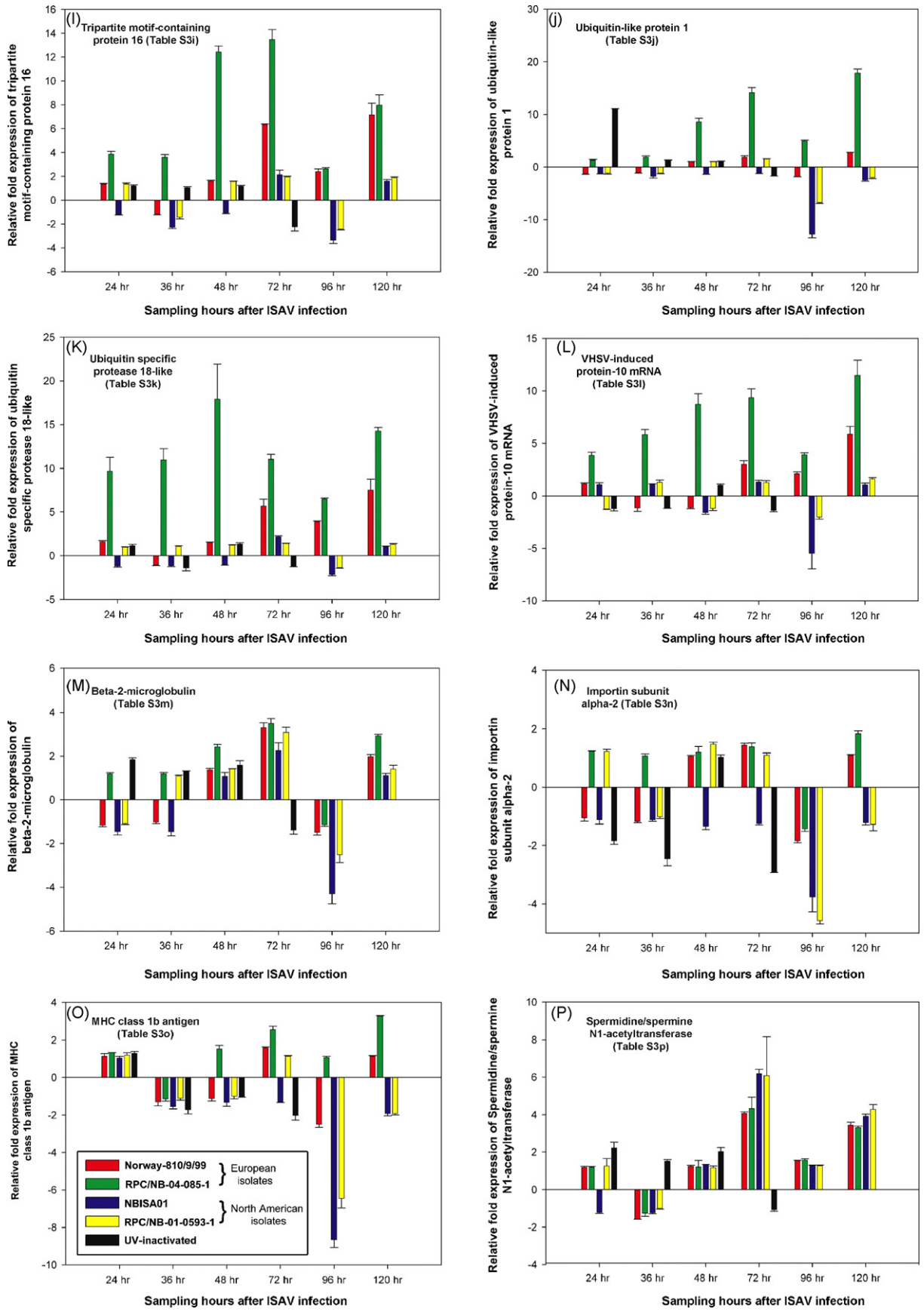


Fig. 3. (*Continued*)

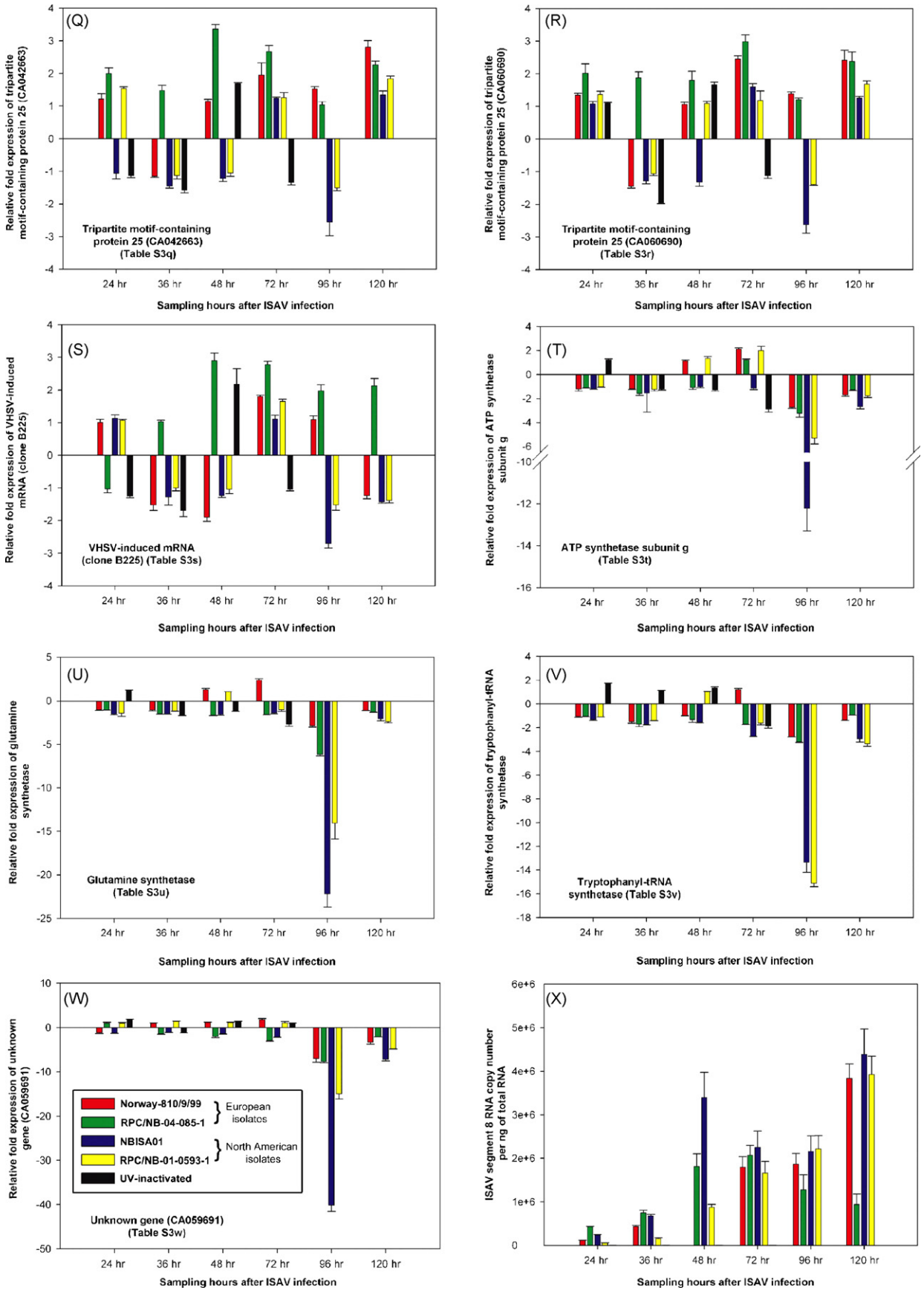


Fig. 3. (*Continued*).

notypic differences of the isolates. To further clarify the basis for the differences in dysregulation, Infection Trial 2 was set up to analyze expression of the 24 QRT-PCR validated genes in TO cells infected with NBISA01, RPC/NB-04-085-1, Norway-810/9/99 (high pathogenicity isolate of European genotype), RPC/NB-01-0593-1 (intermediate pathogenicity isolate of North American genotype), and UV-inactivated RPC/NB-04-085-1 (used as a control for inoculum effects in gene expression) and samples collected at 24, 36, 48, 72, 96, and 120 h post-infection. The data showing the relative fold expression of all the genes studied in Infection Trial 2 are shown in Fig. 3A–W. The UV-inactivated virus infected TO cells showed cell death and the whole monolayer was lost by 96 h; as a result data for 96 and 120 h UV-inactivated virus is missing for all the genes. Moreover, we did QRT-PCR for 25 genes listed in Supplemental Table S1; however, technical replicates showing more than 0.5 Ct value difference between two out of three triplicates were discarded. VHSV-induced protein (CA056844) had a lot of sampling points discarded; as a result QRT-PCR for this transcript is not presented in this section.

In general the QRT-PCR data of the 24 genes can be classified into four patterns of regulation. The first group of genes consists of transcripts that showed a greater than 20 relative fold up-regulation in at least two sampling time points in RPC/NB-04-085-1-infected TO cells (Fig. 3A–F). This group of six genes were highly induced by the ISAV of European genotype (RPC/NB-04-085-1 and Norway-810/9/99), and showed relatively low up-regulation in response to ISAV of North American genotype (NBISA01 and RPC/NB-01-0593-1) and it consists of CD9 antigen (CB515563) (Fig. 3A), interferon-inducible protein Gig1-like (CA050625) (Fig. 3B), E3 ubiquitin-protein ligase HERC4-like (CA052560) (Fig. 3C), Interferon-induced protein with tetratricopeptide repeats 5 (CA051350) (Fig. 3D), Mx1 protein (CB516446) (Fig. 3E), and Radical S-adenosyl methionine domain-containing protein (viperin) (CA058263) (Fig. 3F). The second group of genes consists of six transcripts that were moderately up-regulated by RPC/NB-04-085-1 (i.e. showed a relative fold up-regulation of 5–20 in at least three sampling points); the same ISAV isolate (RPC/NB-04-085-1) caused the highest fold up-regulation in all sampling points compared to the other three ISAV isolates (Fig. 3G–L). This group consists of pre-B cell enhancing factor (CA059978) (Fig. 3G), Sascin (spastic ataxia of Charlevoix-Saguenay) (CA053164) (Fig. 3H), Tripartite motif-containing protein 16 (CA038906) (Fig. 3I), ubiquitin-like protein 1 (CB499972) (Fig. 3J), ubiquitin specific protease 18 (CA056962) (Fig. 3K) and VHSV-induced protein-10 mRNA (CA040505) (Fig. 3L). The third group consists of seven genes that showed very low up-regulation (i.e. fold change of less than 5 in at least 5 sampling points) in RPC/NB-04-085-1-infected cells, and mixed up-/down-regulation in response to the other three ISAV isolates (Fig. 3M–S). This group consists of beta-2-microglobulin (β2-m) (CA489043) (Fig. 3M), importin subunit alpha-2 (CA043335) (Fig. 3N), MHC class Ib antigen (CA043257) (Fig. 3O), spermidine/spermine N1-acetyltransferase (CA056199) (Fig. 3P), tripartite motif-containing protein 25 (CA060690) (Fig. 3Q), tripartite motif-containing protein 25 (CA042663) (Fig. 3R), VHSV-induced mRNA (CA054694) (Fig. 3S). The fourth group of genes consists of four genes that were identified as down-regulated in the microarray analysis and QRT-PCR in response to both of the ISAV isolates used in Infection Trial 1, and showed similar QRT–PCR expression profiles in Infection Trial 2 samples in response to most of the virus isolates at most of the sampling time points (Fig. 3T–W). This group includes ATP synthetase subunit g (CA046558) (Fig. 3T), glutamine synthetase (CB514092) (Fig. 3U), tryptophanyl-tRNA synthetase (CB512171) (Fig. 3V), and an unknown gene (CA059691) (Fig. 3W).

The expression of ISAV segment 8 (Fig. 3X) showed three profiles: (1) Norway-810/9/99 and RPC/NB-01-0593-1 had an increasing trend of ISAV segment 8 copy number/ng total RNA until the last sampling point, (2) RPC/NB-04-085-1 showed an increasing trend until the 72 h time point and a declining level thereafter until the last sampling point and (3) NBISA01 showed a bimodal-trend with initial increasing level that peaked at 48 h and then declined by 72 and 96 h and again peaked to its maximum abundance by 120 h (Fig. 3X).

The time series expression of the microarray reproducibly up-regulated genes in Infection Trial 2 samples was similar to the QRT-PCR validation data using samples from Infection Trial 1. Similarly most of the microarray reproducibly down-regulated genes showed similar trend of expression at most of the sampling points in the same expression study. However, homeobox protein HoxB13ab (CA060050) (Supplemental Fig. 1) which was identified as down-regulated in NBISA01-infected cells by 72 h upon microarray analysis and QRT-PCR validation using samples from Infection Trial 1 did not show down-regulation by NBISA01 at 72 h in the time series expression study (Infection Trial 2). The observed difference in timing of down-regulation could be due to the fact that these are 2 different exposure experiments (see Supplemental Fig. 1).

3.3. Sequence identity of ISAV proteins for the four different ISAV isolates

Pairwise comparisons of the five proteins of the four ISAV isolates used in this study were carried out (Supplemental Tables S4a–S4e) using BLASTN and TBLASTX for nucleotide and predicted amino acid alignments, respectively. The pairwise sequence comparison of PB2 (Supplemental Table S4a), and ISAV fusion protein (Supplemental Table S4b) show that NBISA01 and RPC/NB-01-0593-1 protein and nucleotide sequences had the highest identity to each other whereas Norway-810/9/99 and RPC/NB-04-085-1 have the highest identity to each other, which is consistent with the genotypes of the isolates. The protein sequence of NBISA01 haemagglutinin-esterase protein (Supplemental Table S4c) shows higher identity to RPC/NB-04-085-1 (of European genotype found in North America) than Norway-810/9/99 (of European genotype found in Europe). The nucleotide sequence of segment 7 (Supplemental Table S4d) shows higher identity between NBISA01 and Norway-810/9/99 (both, high pathogenicity isolates), than between isolates belonging to the same genotype. In contrast, the nucleotide sequence of segment 8 (Supplemental Table S4e) shows higher identity between NBISA01 and RPC/NB-04-085-1 (of North American and European genotype, respectively) than between isolates belonging to the same genotype.

4. Discussion

ISAV isolates have been shown to vary considerably in their cytopathogenicity in Atlantic salmon cells and their pathogenicity for Atlantic salmon hosts (Kibenge et al., 2006, 2007b). Previous observations of *in vivo* challenge experiments indicate that a combination of ISAV virulence and host susceptibility determines the outcome of ISAV infections in fish (Kibenge et al., 2006; Ritchie et al., 2009). As a host susceptibility factor, the immune response is a key player. There have been relatively few studies investigating the interaction of ISAV and the Atlantic salmon immune system. Some of these studies indicate that ISAV up-regulates the expression of both the innate and adaptive immune response genes of Atlantic salmon (Jørgensen et al., 2008; Schiøtz et al., 2008) and/or is resistant to host antiviral responses (Kileng et al., 2007). Because none of the previous studies factored in the differences in ISAV strains, there is a paucity of information on the level and extent of dysregulation of immune response relevant genes in response to different

ISAV genotypes and pathogenicity phenotypes. The current study analyzed the general transcriptome response of Atlantic salmon TO cell line infected by ISAV isolates of high and low pathogenicities (i.e. NBISA01 and RPC/NB-04-085-1, respectively) and studied the expression of selected 24 genes in response to four ISAV isolates originating from different regions and belonging to North American (NBISA01 and RPC/NB-01-0593-1) and European (RPC/NB-04-085-1 and Norway-810/9/99) genotypes. Such studies are essential to further our understanding of virus–host cell interactions and the capacity of ISAV isolates to modulate the immune system; this knowledge is necessary as a basis for the identification of virus virulence molecular markers (i.e. expression profiles and sequence variations that correlate with virus resistance), as well as potential targets for therapeutic interventions to reduce the level of disease.

The IFN induced antiviral Mx1 protein (CB516446) has been shown to protect Atlantic salmon cells from IPNV infection (Larsen et al., 2004) but had been previously reported to be unresponsive to ISAV infection (Jensen and Robertsen, 2002). This study showed that Mx transcripts were highly up-regulated by the European genotype isolates (Norway-810/9/99 and RPC-04-085-1) at all sampling points of Infection trial 2 and down-regulated or lowly up-regulated in response to the North American genotype isolates (NBISA01 and RPC/NB-01-0593-1).

The microarray data showed four up-regulated transcripts with high similarity to TRIM proteins. TRIM proteins are characterized by the presence of tripartite motifs, which consist of a RING domain, one or two B-box motifs and a coiled coil region (Borden et al., 1995; Reymond et al., 2001; Towers, 2007). Genes belonging to this family are implicated in a variety of processes such as development and cell growth, and are involved in several human diseases (Sardiello et al., 2008). TRIM5α has been implicated as a major factor restricting HIV-1 replication during the early phase of infection (Brass et al., 2008). Moreover, there is increasing evidence suggesting that TRIM19, also known as promyelocytic leukemia (PML), may have antiviral activity. Antiviral activity of PML bodies towards several viruses has been reported (Everett and Chelbi-Alix, 2007). Particularly, TRIM25 can modify itself and other proteins by conjugating ISG15 (Zou and Zhang, 2006). TRIM25 interacts with the N-terminal CARDs of RIG-I to promote the K-(63)-linked ubiquitination, which is critical for the interaction of RIG-I with its downstream signaling partner MAVS/VISA/IPS-1/Cardif (Gack et al., 2007). A large set of closely related genes and transcripts that contain three motifs typical of TRIM proteins have been identified in several teleost fish species, and they have been shown to be specifically induced by viruses and poly (I:C) (van der Aa et al., 2009). In Atlantic salmon, previous microarray work has shown up-regulation of TRIM protein-like transcripts in ASK-2 cells and Atlantic salmon organs (Jørgensen et al., 2008; Schiøtz et al., 2008). In the present study, three of the four microarray-identified ISAV-responsive TRIM protein-like transcripts were validated by QRT-PCR, and showed significant differences between the two ISAV isolates used in Infection Trial 1. Moreover, multiple different features annotated as TRIM16 (e.g. CA038906) and 25 (e.g. CA042663) were reproducibly up-regulated by infection with the less virulent strain (RPC/NB-04-085-1) and therefore the microarray results also provided internal validation of the importance of this protein family in the Atlantic salmon response to viruses. In the serial time expression study involving four different ISAV strains and a UV-inactivated ISAV control (Infection Trial 2), all three QPCR studied TRIM-like transcripts (i.e. one TRIM16-like annotated feature and two TRIM25-like features that are likely to represent the same cDNA, see Section 2 for details) showed up-regulation by RPC/NB-04-085-1 (between ~2- and ~10-fold) followed by Norway-810/9/99 but presented no clear trend with regard to response to NBISA01 and RPC/NB-01-0593-1.

Viperin (CA058263) was one of the highly up-regulated (i.e. ~100 or higher fold up-regulation with maximum fold induction of over 1000) (Fig. 3F) transcripts in RPC/NB-04-085-1-infected cells and to a lesser extent at 48–120 h in Norway-810/9/99 infected TO cells. It presented an early (i.e. 24 and 36 h) down-regulation and low later (i.e. 48–120 h) up-regulation in response to NBISA01 and RPC/NB-01-0593-1. Viperin is an evolutionarily conserved protein that is highly inducible by both type I and II IFNs (Chin and Cresswell, 2001). Many viruses induce the expression of viperin suggesting a role in antiviral response. The product of this gene has been shown to inhibit the release of influenza virus during budding in the virus replication cycle, as a result of viperin-induced disruption of lipid-raft micro-domains that play a role in the replication cycle of many viruses (Wang et al., 2007). Viperin in rainbow trout (vig1) is a rhabdovirus-induced antiviral gene (Boudinot et al., 1999), and it is up-regulated in Atlantic cod (*Gadus morhua*) in response to the viral mimic dsRNA [poly (I:C)] (Rise et al., 2008).

MHC class I molecules control the response of cytolytic lymphocytes to virus-infected cells (Janeway et al., 2005). Beta-2-microglobulin, an MHC class I molecule, expression levels have previously been shown to be up-regulated in response to ISAV infection (Jørgensen et al., 2007, 2008; Schiøtz et al., 2008). The second MHC I related gene up-regulated in response to RPC/NB-04-085-1 infection was MHC class 1b antigen (CA043257). Both genes did not show remarkable patterns of induction in response to different ISAV isolates.

In addition to regulation of intracellular protein levels, protein ubiquitination regulates many aspects of the innate immune response, including signal transduction (for example activation of NF-κB) and functions of the adaptive immune response, such as initiating tolerance (Liu et al., 2005). The IFN-regulated ubiquitin-like protein response (ISGylation) is mediated by ISGs. ISG15 is a key player in the ISGylation process and the expressed protein has two ubiquitin-like domains (Ritchie and Zhang, 2004). ISG15 is conjugated by a thiolester bond to cysteine residues of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase enzyme (E3), before being transferred to lysine residues of protein substrates (Welchman et al., 2005). Many ISG15 putative targets have important roles in the type I IFN response, including JAK1, STAT1, RIG-I, MxA, PKR, and RNaseL (Zhao et al., 2005). Similar to ubiquitination, ISGylation is a reversible process, and several enzymes that catalyze the process have been identified (Sadler and Williams, 2008). Ubiquitin protease 18 is a de-ISGylating protease that removes ISG15 from proteins to which it was conjugated (Malakhov et al., 2002). The microarray data and QRT-PCR validation showed that Ubiquitin specific protease 18-like (CA056962), and E3 ubiquitin ligase-like (CA052560) transcripts were highly inducible in TO cells infected with the RPC/NB-04-085-1 isolate compared to NBISA01. Similarly, the microarray data shows that several features annotated as ubiquitin-like protein 1 (e.g. CB499972) were up-regulated 72 h after infection with the less virulent strain of ISAV (RPC/NB-04-085-1). Moreover, this protein could be related to ISG15 since one of its best BLASTX hits is a salmonid ISG-15-like transcript (AAX98145 – 127 identities over 156 aligned amino acids for an identity of 81%; *E*-value = 1e-65). Differential up-regulation of ubiquitin E3 ligase, ubiquitin specific protease 18 and ubiquitin-like proteins at all sampling points in response to different ISAV isolates (Fig. 3C, J and K) suggests differing activity of ISGylation and de-ISGylation (at the transcriptional level) upon infection with different ISAV strains. These observations also support that, similar to mammals, ubiquitination in fish is essential in regulating the innate immune response.

Pre-B-cell colony enhancing factor is an inflammatory mediator that is highly conserved in bacteria (Martin et al., 2001), fish (Fujiki et al., 2000), and mammals (McGlothlin et al., 2005). Pre-B-cell colony enhancing factor was induced by lectins and prevented apoptosis of neutrophils (Luk et al., 2008). Earlier microarray work using ISAV showed up-regulation of the pre-B-cell colony enhanc-

ing factor-like gene (Jørgensen et al., 2008). In the current study, pre-B-cell colony enhancing factor (CA059978) transcripts were up-regulated by RPC/NB-04-085-1 at all sampling points while showing lower up-regulation or down-regulation in response to other isolates.

Interferon-induced protein containing multiple tetratricopeptide repeats domains are highly IFN-inducible genes (Sadler and Williams, 2008). Members of the group include ISG-56 (also known as IFIT-1 or GARG-16), ISG-54 (also known as IFIT-2 or GARG-39), and ISG-49 (also known as IFIT-3 or GARG-49) (de Veer et al., 1998). In mammalian cells, ISG-49, ISG-54, and ISG-56 are rapidly induced by both type I and type II IFNs (Smith and Herschman, 1996; Terenzi et al., 2005). The expression of ISG-54 and ISG-56 is also induced directly by the SeV as well as by dsRNA (Terenzi et al., 2005). The human orthologues of ISG-54 and ISG-56 are also induced by IFN, dsRNA, and a range of viruses, including SeV, encephalomyocarditis virus, and cytomegalovirus (Guo et al., 2000; Preston et al., 2001). Murine ISG-56 and ISG-54 inhibited protein synthesis *in vitro* by binding to the "c", but not the "e", subunit of the translation initiation factor, eIF-3 (Terenzi et al., 2005). The deduced sequence of virus induced gene 4 (Vig 4) in trout is homologous to the ISG-56 family of mammals (Robertsen, 2008). The time series expression study of the interferon-induced protein with tetratricopeptide repeats 5 (CA051350) showed higher mean relative fold induction by RPC/NB-04-085-1 and Norway-810/9/99 when compared to NBISA01 and RPC/NB-01-0593-1-infected cells.

Crucian carp *Carassius auratus* blastulae (CAB) cells stimulated with UV-inactivated grass carp hemorrhagic septicemia virus has been shown to up-regulate the transcription of two novel interferon stimulated genes, Gig1 and Gig2 (Zhang and Gui, 2004). Atlantic salmon TO cells infected with RPC/NB-04-085-1 showed up-regulation of Gig1 (CA050625) and Gig2 like (CA058271) transcripts. QRT-PCR validation of Gig1 showed very high up-regulation (2523.61-fold) in response to RPC/NB-04-085-1, and a much lesser up-regulation in NBISA01 (41.22-fold). Interestingly, RPC/NB-04-085-1 caused high up-regulation of this transcript in all time-points in a bimodal fashion, with highest fold up-regulation at 48 and 120 h. High up-regulation is not induced by Norway-810/9/99 until 48 h and it steadily increases up to 120 h, not showing the bimodal profile induced by RPC/NB-04-085-1. On the other hand the North-American genotype isolates (i.e. NBISA01 and RPC/NB-01-0593-1) did not show high up-regulation of this transcript up until the last time point (i.e. 120 h).

Accurate translation of the genetic information into proteins is a complex process requiring essential cellular players such as the ribosome, messenger RNAs, aminoacylated tRNAs, and a host of additional protein and RNA factors. Aminoacyl-tRNA synthetases join amino acids with their cognate transfer RNAs in a high-fidelity reaction (Park et al., 2004). In eukaryotic cells specific aminoacyl-tRNA synthetases play roles in amino acid biosynthesis, cell cycle control, RNA splicing, and export of tRNAs from nucleus to cytoplasm (Martinis et al., 1999; Francklyn et al., 2002). A primary antiviral response of host cells to virus infection involves activation of PKR in response to viral dsRNA and thereby inhibition of viral and cellular protein synthesis (Schneider and Mohr, 2003). The observation of down-regulated tryptophanyl-tRNA synthetase (CB512171) by RPC/NB-04-085-1-infected cells in infection trial 1 and its down-regulation at most of the sampling points in response to the four virus isolates used in Infection Trial 2 suggest that TO cells down-regulate tryptophanyl-tRNA synthetase as part of the host induced protein synthesis shut off. This is consistent with the observed up-regulation of interferon-induced protein with tetratricopeptide repeats 5, which has been shown to inhibit protein synthesis (Terenzi et al., 2005). On the contrary, mammalian tryptophanyl-tRNA synthetase is the only aminoacyl-tRNA synthetase that has been shown to be up-regulated in response to interferon-γ treatment (Craven et al., 2004) and ISAV infection has been shown to up-regulate IFN-γ (Jørgensen et al., 2007). This suggests that fish tryptophanyl-tRNA synthetase may be not IFN-γ responsive.

Among enzymes involved in metabolism, ATP synthetase subunit g (CA046558) and glutamine synthetase (CB511492) were down-regulated by both NBISA01 and RPC/NB-04-085-1 in infection trial 1 samples by 72 and 24 h, respectively. Microarray studies using white spot syndrome virus of shrimp (Wang et al., 2006) and West Nile virus (Koh and Ng, 2005) also showed down-regulation of genes involved in energy synthesis such as ATP synthetase, and cytochrome *C* oxidase. Glutamine synthetase is a multifunctional enzyme involved in amino acid balance, protein metabolism, nucleotide biosynthesis, neurotransmitter metabolism, as well as ammonia detoxification (Watford, 2000). Glutamine synthetase has been shown to be down-regulated in cultured embryonic chick neural retina cells by the addition of chick interferon preparation (Matsuno et al., 1976). The down-regulation of glutamine synthetase caused by ISAV may also be a part of the host induced protein synthesis shut off and/or be associated with virus-induced IFN production as evidenced from the IFN-stimulated Mx1 up-regulation.

In general, the induction pattern of the immune responsive genes correlates with the replication of the different ISAV strains as evidenced from the ISAV segment 8 RNA copy numbers. Moreover, UV-inactivated RPC/NB-04-085-1 showed low fold induction or down-regulation of the immune responsive genes when compared to the live RPC/NB-04-085-1, suggesting virus replication as the requirement for induction of immune responsive genes. The expression of most up-regulated genes in response to RPC/NB-04-085-1 and Norway-810/9/99 appears to have more than one phase of induction. This can be explained by the fact that most of the up-regulated genes are IFN inducible; the expression of type I IFNs in response to poly (I:C) had three sequential waves (Démoulins et al., 2009).

Genes belonging to groups 3 and 4 (with the exception of spermidine/spermine N1-acetyltransferase (CA056199)), and genes such as CD9 (CB515563), Mx1 protein, pre-B cell enhancing factor, sascin (CA053164), TRIM16, ubiquitin-like protein 1 in groups 1 and 2 showed down-regulation at 96 h post-infection caused by both NBISA01 and RPC/NB-01-0593-1 ISAV isolates. This might be associated with virus induced cytopathic effect that might have affected the host transcriptional machinery resulting in severe down-regulation of genes.

The high correlation of induction pattern of immune response genes and RPC/NB-04-085-1 segment 8 copy number suggests that the virus induces most of the immune responsive genes to a very high level and possibly the protein products of those genes restricts the replication of the virus as evidenced by the declining viral copy numbers starting from 72 h post-infection. Infection Trial 2 (the second QRT-PCR expression study) revealed differences in host responses to different ISAV isolates; specifically most of the genes that were highly up-regulated (group 1 genes in Section 3.2) showed strain-specific differences in that the isolates of European genotype were potent inducers while isolates of North American genotypes showed low fold up-regulation. Most of the group 1 genes are virus responsive genes that have been shown to be IFN inducible. However, the molecular basis for these differences in the level and extent of dysregulation of immune response relevant genes cannot be presently explained.

5. Conclusions

In conclusion, the microarray and QRT-PCR results suggest that ISAV isolates interact with the immune system of Atlantic salmon

in different ways. The data show that ISAV isolates have virus strain-specific differences in their capacity to induce innate as well as adaptive immune response genes. The responses do not seem to relate with the level of ISAV pathogenicity in that both high pathogenicity isolate Norway-810/9/99 and low pathogenicity isolate – RPC/NB-04-085-1 caused high fold induction of immune-relevant genes. Some of the observed variations could be related to intrinsic variations in virus virulence mechanisms that avoid the immune response, and others could be due to the differences in virus antagonistic proteins that counteract the host immune system, which is consistent with the fact that that RNA viruses evade the host response at different levels such as interference with antigen presentation, inhibition of cytokine action, modulation of chemokine activity, modulation of apoptosis, and manipulation of humoral immunity (Mahalingam et al., 2002). The present study used field ISAV isolates that have uncontrolled variations in the virulence factors. Further characterization of immune response antagonizing virulence factors of ISAV will require use of recombinant viruses generated by reverse genetics tools that would allow varying one ISAV virulence factor at a time without changing the other viral proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.06.015.

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