Induction of Atlantic salmon type I interferon and antagonism by infectious pancreatic necrosis virus

Silje Lauksund
A dissertation for the degree of Philosophiae Doctor – January 2014
Acknowledgements

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Finally, I would like to thank my closest family, Hogne, Balder and August. Thank you for your encouragement and enthusiasm. I could not have done this without your support.

Tromsø, 2013
Silje Lauksund
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## Abbreviations

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<thead>
<tr>
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<tbody>
<tr>
<td>As</td>
<td>Atlantic salmon</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>Nuclear factor kappa-B kinase subunit</td>
</tr>
<tr>
<td>IKKε</td>
<td>IκB kinase ε</td>
</tr>
<tr>
<td>IPNV</td>
<td>Infectious pancreatic necrosis virus</td>
</tr>
<tr>
<td>IPS1</td>
<td>Interferon-beta promoter inducing protein one</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IRF-E</td>
<td>IκB-binding element</td>
</tr>
<tr>
<td>ISAV</td>
<td>Infectious salmon anemia virus</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NAP1</td>
<td>NAK-associated protein 1</td>
</tr>
<tr>
<td>NEMO</td>
<td>NFκB essential modulator</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear transcription factor kappaB</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PKZ</td>
<td>Protein kinase Z</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RIP1</td>
<td>receptor interacting protein 1</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>SINTBAD</td>
<td>similar to NAP1 TBK1 adaptor</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of IFN genes</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF-family member associated NFkB activator</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase I</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR1 associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor (TNF) receptor-associated factor</td>
</tr>
<tr>
<td>NOD2</td>
<td>nucleotide-binding oligomerization domain (NOD)-like receptor 2</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IFN regulatory factors</td>
</tr>
<tr>
<td>DHX</td>
<td>DExD/H box helicases</td>
</tr>
</tbody>
</table>
List of papers

Paper I
Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions.
Veronica Bergan, Silje Steinsvik, Hao Xu, Øyvind Kileng and Børre Robertsen (2006)

Paper II
Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-kappaB and localizes to mitochondria.
Silje Lauksund, Tina Svingerud, Veronica Bergan and Børre Robertsen (2009)
*Developmental and Comparative Immunology.* 33: 1196–1204.

Paper III
Infectious Pancreatic Necrosis Virus Proteins VP2, VP3, VP4 and VP5 Antagonize IFNa1 Promoter Activation while VP1 induces IFNa1.
Silje Lauksund, Linn Greiner-Tollersrud and Børre Robertsen.
Manuscript (December 2013).


**Introduction**

Atlantic salmon, *Salmo salar*, is the predominant species in the Norwegian aquaculture industry. Generating a biomass of 1.2 million tons of slaughtered salmon in 2012, Norway is one of the largest producers of salmon in the world (1). Despite the large amounts produced, production loss is high, especially in the seawater phase. A major part of the losses can be attributed to various viral diseases. Viral infections in the fish farming industry are of economical concern, but also a fish welfare issue. The most common viral diseases of salmon in Norway are caused by infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), salmon pancreas disease virus (SPDV), piscine reovirus (PRV) and piscine myocarditis virus (PMCV).

Vaccines are available for IPNV, SPDV and ISAV, but at present vaccination against these viruses do not give satisfactory protection. To develop more efficient vaccines and other methods to fight viral diseases of Atlantic salmon, there is a need for a better understanding of antiviral immune mechanisms in this species. Interferons (IFNs) play a crucial role in innate immune responses against virus infections in mammals whereby host cells synthesize and secrete IFNs upon recognition of viral nucleic acids. IFNs protect other cells from further viral infection by inducing antiviral proteins that inhibit viral replication and stimulate adaptive immune responses against the infecting virus. The importance of the IFN system in immunity is highlighted by the fact that most vertebrate viruses encode antagonistic proteins that inhibit transcription of IFN or IFN-stimulated genes (2, 3).
This thesis has focused on the mechanism involved in how viruses activate transcription of IFN in Atlantic salmon, and how IPNV antagonize IFN transcription. To give an introduction to this work, I will first give a brief description of the role of IFN in the innate antiviral immunity. Then the different receptors and signaling pathways that the cell utilizes for recognition of viruses to induce type I IFN are reviewed. Some selected antiviral proteins induced by IFN are described. I will also give a description of infectious pancreatic necrosis virus in more detail.

**Immunity**

The immune system plays a key role in protecting animals against invading pathogens. In vertebrates it consists of two different yet intermingled branches, the innate and the adaptive immune system. Whilst the innate immune system operates in the immediate frontline in the combat against invading pathogens, the adaptive immune system gives a later and more targeted response (4). Innate immunity is conferring a broad response consisting of physical barriers, cellular mechanisms and antiviral proteins generally believed to lack specific memory. The adaptive immunity consists of a targeted response mediated by antibody producing B-cells and cytotoxic T-cells adapted to the specific pathogen and with an acquired specific memory function. The focus in this thesis will be on the innate immune system.
**Innate immunity**

Evolved early in the metazoan lineage, innate immunity can be considered to be the first line of defense against invading pathogens. Invertebrates for instance lack the adaptive branch of the immune system, and are believed to rely solely on innate immunity.

Dependent on the ability of the organism to recognize foreign molecular patterns, the innate immune system comprise a wide range of germ line-encoded non-rearrangable pattern-recognition receptors (PRR), antimicrobial molecules and immune cells. The innate immune system does not only combat the infection itself, but is also a link to activation of adaptive immunity in vertebrates. The recognition of pathogen associated molecular patterns (PAMPs) by the PRR initiates effector molecules inside the cells as well as secretion of cytokines. Cytokines are a group of secreted proteins that regulate important cellular functions. Some cytokines such as IFNs function as alarm molecules, warning other cells of the ongoing infection. Cytokines are also involved in regulation of the adaptive immune system by promoting B- and T-cell differentiation, and thereby provide a link between innate and adaptive immunity (5).

**The interferon system**

IFNs are a group of cytokines involved in activation of immune cells, up-regulation of antigen presentation to T-cells and in inducing an antiviral state in uninfected cells to protect against infection. IFNs can be subdivided into three types, type I, type II and type III, defined by their differences in structure, receptor usage and biological activity. Type I IFNs and the more recently discovered type III IFNs both have antiviral properties and play a major role in the innate immunity against viruses. Mammalian type I IFNs consists
of several classes, with the IFNa and IFNb as the predominant IFNs in the first line of defence against viruses. There are 13 subtypes of IFNa (α), and a single IFNb (β) protein. Also IFNκ (κ), IFNω (ω), IFNε (ε), IFNδ (δ) and IFNτ (τ) are present (6). Type III IFNs encompass IFNλ (λ). Type II IFN is identical to IFNg (γ) and is mainly implicated in the adaptive immune response, where it is produced by T-cells and activate macrophages (7, 8). Type I IFNs were the first cytokines that were identified, and are named after their ability to interfere with the replication of invading viral pathogens (9). Type I interferons can also enhance the development of adaptive immune responses (10-13). Mammalian type I IFNs are single-exon genes, the type II is a four exon – one intron gene, and type III IFNs are five exon – four intron genes. The type I, type II and type III IFNs signal through distinct receptor complexes, composed of IFNAR1/IFNAR2, IFNL1/IL-10R2 and IFNGR1/IFNGR2 respectively (6).

Type I and type II IFNs have been identified in several fish species, but to date type III IFNs have not been found in fish. The first type I IFN genes from fish were cloned in 2003, from Atlantic salmon, zebrafish (Danio rerio) and green spotted puffer (Tetraodon nigroviridis) (14-16). The type I IFN genes in fish are of particular interest because fish, like mammals, have multiple linked copies of IFNs in the genome. But unlike the one-exon type I IFN genes found in mammals, reptiles and birds, the fish type I IFNs contain four introns. This makes them interesting not just in functional studies but also from an evolutionary viewpoint. There is also a question whether this apparent redundancy of IFNs and also of IFN-inducing pathways, and the great sequence
differences despite similar functions in as divergent species as fish and mammals, reflects co-evolution between host and virus.

Mammalian type I IFN members are not true orthologues of fish type I IFN members, as the expansion of the mammalian IFN genes is believed to have occurred after the divergence of birds and mammals (17). The mammalian type I IFN gene also lack introns in contrast to the fish type I IFN genes which typically contain five exons and four introns. Analyses of amino-acid sequences and structure of fish IFNs, and resolution of the crystal structure of two zebrafish IFNs supports their classification as type I IFNs and not type III IFNs (14, 15, 18-20). The question thus arises whether the fish IFNs have similar properties as the mammalian IFNs.

**Type I IFNs**

Cells infected with viruses synthesize and secrete type I IFNs, warning other cells of the ongoing infection. When IFNs bind IFN receptors on cells, a signaling cascade called the JAK-STAT pathway is activated, resulting in transcription of hundreds of IFN-stimulated genes (ISGs). Some of these genes encode proteins with potent antiviral functions (21). An overview of the IFN response is shown in figure 1.
Figure 1. Overview of the IFN response. The IFN response circuit is a two-step signaling process where the first step is recognition of RNA from an invading virus by the pattern-recognition receptors RIG-I/MDA5 and TLR3. This results in activation of transcription factors that leads to the transcription, translation and secretion of IFN. The next step is for the secreted IFNs to bind IFN receptors on the same or on other cells. The JAK/STAT signaling pathway is activated, resulting in transcription and translation of a range of IFN-stimulated genes (ISGs), some encoding antiviral proteins. The antiviral proteins inhibit viral propagation by various means, creating an antiviral state.
The type I IFN family in humans consists of IFNα, IFNβ, IFNε, IFNκ and IFNω. 13 IFNα subtypes, and one each of the other have been identified (6). In addition to the human IFNs, other mammalian type I IFNs have been described that only exist in some species. The main type I IFNs involved in the response to viral infection in humans are IFNα and IFNβ. Even though these different IFNs bind the same receptor, the physiological responses induced are distinct. It has recently been shown that the binding affinity of the separate type I IFNs to the receptor determines the biological activity (22). The type I IFN genes can also be divided in genes belonging to an early phase that does not require ongoing protein synthesis, and a later phase with a delayed response. IFNβ belongs to the early phase IFNs, whilst most of IFNαs belong to the later phase (23).

In fish, type I IFN can be classified into two groups, one with IFNs containing two conserved cysteine residues (2C IFNs), and another with four conserved cysteine residues (4C IFNs). The 2C IFNs can be further subdivided into IFNa and IFNd, and the 4C IFNs can be divided into subgroup IFNb and IFNc (24, 25). So far, 13 type I IFN genes have been identified in Atlantic salmon. The 4C IFNs have only been reported in Atlantic salmon, rainbow trout (Onchorhynchus mykiss) and zebrafish, and Atlantic salmon is at present the only species with all four subgroups of type I IFN has been found. Interestingly, in contrast to mammalian type I IFNs that utilize the same receptor, zebrafish type I IFNs signals through different receptors depending on whether they are 2C or 4C IFNs (19).
Because the type I IFNs of mammals and fish have evolved independently, the pathways leading to induction of IFN transcription, and the cell types producing them could also have differences. In a recently published study by Svingerud et al on IFNs from Atlantic salmon, the 2C IFNa and 4C IFNc show similar antiviral activities and ability to induce antiviral genes (26). The 4C IFNb has some antiviral activity, but markedly lower than IFNa and IFNc. No antiviral activity could be detected for the 2C IFNd. When comparing expression of the IFNs by the IFN inducers poly(I:C) and R848, poly(I:C) is a strong inducer of IFNa in cell lines, whilst the other IFNs showed little response. This indicates that IFNa is the main IFN subtype induced through the RLR pathway (27, 28). R848 on the other hand induced high transcript levels of IFNb and IFNc, and low levels of IFNa in the lymphoid organs head kidney and spleen. The imidazoquinoline R848 is known to induce IFNs through TLR7 (29, 30). IFNd show constitutive expression in cells and organs that is unaffected by poly(I:C) and R848 treatment. The findings were supported by in situ hybridization studies, that showed poly(I:C) to induce IFNa and IFNc in a variety of cells and organs, whilst R848 induced co-expression of IFNb and IFNc in distinct cells in head kidney and spleen. These cells are reminiscent of the plasmacytoid dendritic cells in mammals that are specialized IFN-superproducers (26).
**Downstream signaling - The JAK-STAT pathway**

In mammals, the type I IFN receptor is expressed in most cells (31). This gives the type I IFNs the capacity to alert the cells of the presence of pathogens, so that an antiviral response can be mounted. After IFNs have bound to the IFN receptor, the JAK-STAT pathway is activated. It culminates with the formation of a transcription factor complex consisting of STAT1, STAT2 and IRF9, forming the IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus, where it binds to IFN-stimulated response elements (ISRE) in the promoters of ISGs, initiating the transcription of antiviral genes (7, 32). An overview of the JAK-STAT pathway is shown in figure 2.
Figure 2. Type I IFN signaling through the JAK/STAT pathway. Upon binding of type I IFN to the IFN receptor heterodimer, TYK2 and JAK1 phosphorylates the receptor. In turn, STAT1 and STAT2 are phosphorylated and associate with IRF9, forming the ISGF3 complex. ISGF3 translocates to the nucleus, where it binds ISRE-elements in the promoter regions of ISGs, initiating gene transcription. Some ISGs encode antiviral proteins.

Sensing of pathogens, and signaling pathways of type I IFN induction

The main viral PAMPs recognized by the cell are viral nucleic acids. PRRs that can recognize viral nucleic acids can be divided into extracytoplasmic and intracytoplasmic receptors. The extracytoplasmic receptors consist of several members of Toll-like receptors (TLRs). The intracytoplasmic receptors include the RNA sensing receptors
retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5), protein kinase R (PKR), and a range of DNA sensing receptors including DAI and AIM2. Together, these PRRs form a network of signaling pathways that converge in activation of nuclear factor kappa B (NFκB) and interferon regulatory factor (IRF) activation (33).

**Extracytoplasmic pathways for sensing viruses**

In total, 13 TLRs have been identified in mammals (34, 35). All TLRs share an N-terminal extracytoplasmic domain with leucine-rich repeats (LRR) which is responsible for pathogen sensing, a transmembrane (TM) domain, and cytoplasmic Toll/IL-1 receptor (TIR) domain responsible for downstream signaling through TIR-domain containing adaptor proteins. The extracytoplasmic domains are diverse and can recognize a wide variety of pathogens by interacting with specific PAMPs associated with them.

TLR2, 3, 7, 8 and 9 mediate IFN induction in response to viral infections (table 1). TLR2 resides in the plasma membrane, and is mainly known for its ability to detect bacterial cell wall components. TLR3, 7, 8 and 9 all mainly reside in the endosomal compartment and are specialized in detection of different nucleic acids. TLRs mainly function in specialized cells of the innate immune system to detect viral nucleic acids, such as macrophages and plasmacytoid dendritic cells (pDC). They are largely dispensable for most other cell types, which rely on the cytoplasmic sensors of pathogens (36, 37).
Table 1  Extracytoplasmic sensors of viral nucleic acids

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Ligand</th>
<th>(Signal mediators)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td></td>
<td>Myd88</td>
</tr>
<tr>
<td>TLR3</td>
<td>(short*) dsRNA</td>
<td>TRIF</td>
</tr>
<tr>
<td>TLR7/8</td>
<td>ssRNA</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA</td>
<td>Myd88</td>
</tr>
<tr>
<td>TLR22</td>
<td>(long*) dsRNA</td>
<td>TRIF</td>
</tr>
</tbody>
</table>

*fish

TLR3 was the first PRR identified for sensing viral dsRNA (28). It is primarily located in endosomes, although it can also be found in the plasma membrane of epithelial cells and fibroblasts (38). TLR7 and TLR8 can both sense viral ssRNA in endosomes (39, 40). TLR9 senses unmethylated CpG DNA motives in endosomes (41). Binding of viral nucleic acids to TLR7, 8 and 9 activates IRF7 by signaling through the adaptor protein Myd88. TLR2 is best known for sensing bacterial cell wall components, and does not induce an antiviral response in most cells. However, it can detect vaccinia virus infection in inflammatory monocytes of bone marrow, and like TLR7, 8 and 9 it can induce type I IFN by signaling through Myd88 (42).

In addition to the orthologues of the mammalian TLRs, fish also contain several TLRs not found in the mammalian genome (43). One of the fish specific TLRs, TLR22, is a dsRNA sensor that like TLR3 can recognize the synthetic dsRNA poly(I:C) (44). In mammals TLR3 can be found in either the endosomes or the plasma membrane, in fish it
appears that TLR3 is located in the endosomes while TLR22 is located on the plasma membrane.

**Cytoplasmic pathways for sensing viruses**

A wide range of cytoplasmic PRR for nucleic acids exist (Table 2). Cytoplasmic PRR for detecting viral nucleic acids include the RIG-I like receptors (RLR) RIG-I and MDA5. Both RIG-I and MDA5 recognize viral RNA in the cytoplasm. MDA5 recognizes long dsRNA, and RIG-I recognizes 5’-three-phosphate (5’3P) containing uncapped ssRNA and short dsRNA (45). Most self-RNAs are capped at the 5’ end, while many ssRNA viruses lack this capping. The versatility of these receptors enables them to recognize RNA from a wide variety of both single stranded and double stranded RNA viruses, and also some DNA viruses (table 2). The detection of DNA viruses involves the DNA-dependent RNA polymerase III, which can convert cytoplasmic B-form dsDNA (poly dA:dT) to 5’3P-RNA that is detected by RIG-I (46, 47).
Table 2  
Cytoplasmic sensors of nucleic acids

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Ligand</th>
<th>Signal mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIG-I</td>
<td>5’3p ssRNA, short Poly I: C</td>
<td>IPS-1</td>
</tr>
<tr>
<td>MDA5</td>
<td>Long Poly I: C</td>
<td>IPS-1</td>
</tr>
<tr>
<td>LGP2</td>
<td>dsRNA</td>
<td>IPS-1</td>
</tr>
<tr>
<td>NOD2</td>
<td>ssRNA</td>
<td>IPS-1</td>
</tr>
<tr>
<td>RNA pol III</td>
<td>Poly(dA:dT)</td>
<td>IPS-1</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA</td>
<td>eIF-2α</td>
</tr>
<tr>
<td>AIM2</td>
<td>dsDNA</td>
<td>Inflammasome</td>
</tr>
<tr>
<td>DAI/DLM-1/ZBP1</td>
<td>DNA (all conformations)</td>
<td></td>
</tr>
<tr>
<td>LRRFIP1</td>
<td>dsRNA, dsDNA</td>
<td>B-catenin</td>
</tr>
<tr>
<td>DHX9</td>
<td>dsDNA</td>
<td>Myd88</td>
</tr>
<tr>
<td>DHX36</td>
<td>dsDNA</td>
<td>Myd88</td>
</tr>
<tr>
<td>IFI16/p204</td>
<td>dsDNA</td>
<td>STING</td>
</tr>
</tbody>
</table>

Although TLRs and RLRs are thought to be the main PRRs for detection of viral nucleic acids, several DNA sensors with the capacity of inducing gene transcription of type I IFN have recently been identified. So far NOD2, DAI, DHX9, DHX36 and the AIM2-like receptors IFI16 and p204 are recognized as sensors mediating induction of type I IFN transcription (48-53). Induction of type I IFN in response to cytoplasmic DNA requires the signal mediator STING (54).
Signal transduction in the RLR pathway

Upon recognition of viral RNA in most cell types, the RLRs RIG-I and MDA5 are the main sensors of inducers of IFN (55, 56). The construction of RIG-I knockout mice demonstrated that fibroblasts, conventional dendritic cells and epithelial cells are dependent on the RLR pathway for stimulating IFN production (57). However, plasmacytoid dendritic cells (pDC), known as superproducers of IFNα, prefer to use TLR mediated signaling over RLR (58).

RIG-I and MDA5 both contain two N-terminal caspase activation and recruitment (CARD) domains that are essential for their signaling activity (59). They also contain an internal DExD/H-box helicase domain, with an ATPase activity that is necessary for signaling (60, 61). RIG-I has a C-terminal repressor domain not found in MDA5 (62). The third member of the RLR family, LGP2, has a helicase domain and repressor domain but lack the tandem CARD domains at the N-terminal end. The role of LGP2 in RLR signaling is disputed. It was initially identified as a negative regulator of RIG-I and MDA5 mediated signaling, proposed to act through sequestration of RNA (63, 64). The view on LGP2 as a negative regulator has later been nuanced, as studies revealed that LGP2 was required for RIG-I and MDA5 mediated antiviral responses (65, 66).
The RLR proteins can be divided into three basic domains. The tandem CARD domains in the N-terminal end, the central helicase domain with ATPase activity and the C-terminal regulatory region. The regulatory region of RIG-I and LGP2 contains a repressor domain. LGP2 lacks the CARD-domains found in the other RLRs. IPS-1 has a single CARD domain that physically interacts with the CARD-domains of activated RIG-I or MDA5.

While inactive, the RIG-I helicase acts as a monomer. Upon binding of viral RNA to the helicase domain, the molecule undergoes a conformational change that promotes dimerization. This conformational change is dependent on the ATPase activity of the helicase domain. As a dimer, RIG-I is believed to bind directly to IFNβ-promoter stimulator 1 (IPS-1) through their shared CARD-domain (figure 3).

IPS-1 is also known as mitochondrial antiviral signaling protein (MAVS), virus-induced signaling adaptor (VISA) or CARD adaptor inducing IFNβ (Cardif) (67-70). IPS-1 contains an N-terminal CARD domain, a proline rich region and a C-terminal transmembrane (TM) domain. The TM domain anchors the protein in the mitochondrial membrane, the mitochondrial-associated ER membrane or the peroxisomes associated with the mitochondrial-associated ER (68, 71, 72). The transmembrane domain is, like the CARD-domain, vital for a functional signal transduction through IPS-1. Upon
interaction with an activated RIG-I or MDA5, IPS-1 oligomerizes and IPS-1 acts as a scaffold for a multiprotein complex. This complex leads to a coordinated activation of IRF3/7 and NFκB (figure 4).

**Figure 4. Activation of IFNβ transcription through the RLR pathway.** Detection of foreign RNA by the RLRs MDA5 or RIG-I in the cytoplasmic compartment. The RLRs interact with the mitochondria-bound molecule IPS-1. This activates a signaling cascade resulting in activation of the transcription factors IRF3/IRF7 and NFκB, which moves from the cytoplasm to the nucleus. The blue boxes represent the steps in the signal transduction after activation of IPS-1. In the nucleus, the transcription factors associate with their respective binding sites in the IFN promoter, leading to transcription of the IFN gene. When the IFN transcripts have been translated to proteins, the IFNs are released from the cell.
**NFκB activation by IPS-1**

In activation of NFκB by IPS-1, IPS-1 binds TRADD which interacts with RIP1 in a complex with FADD through their shared death-domain (DD) (67, 73, 74). This complex is further associated with TRAF2 and/or TRAF6 (69). FADD interacts with caspase8 and caspase10, which are cleaved upon stimulation to act as positive stimulators (75). The signal is further transmitted through a duplex consisting of TANK and NEMO (also known as IKKγ) (76, 77). The TANK/NEMO is found in both the NFκB- and the IRF-activating branch of IPS-1 signaling. In NFκB-activation, TANK/NEMO initiates the activation of IKKα and IKKβ. This in turn phosphorylates the inhibitory IkB subunit of the NFκB complex, releasing active NFκB that translocate to the nucleus (78).

**IRF activation by IPS-1**

The IRFs are a family of transcription factors consisting of nine members in mammals, IRF1 – IRF9 (79, 80). An additional member not found in mammals, IRF10, has been identified in chicken (81). The IRFs consist of a conserved N-terminal DNA-binding domain (DBD) that can recognize the IRF-binding element (IRF-E) binding site in the IFN promoters. The consensus sequence for the IRF-E, G(A)AAA^{G/C}_{T/C}GAAA^{G/C}_{T/C}, overlaps with the consensus sequence for ISRE, ^{A/G}_{C}NGAAANNGAAGACT, which binds IRF9 in ISG promoters (82, 83). Orthologues of all IRF members have been found in fish genomes (84-86). In fish, IRF1, IRF3 and IRF7 have been shown to be able to activate expression of IFNs and ISGs (87-90).
In activation of IRFs, IPS-1 binds TRAF3, and TRAF3 binds UXT-V1 (91, 92). This then interacts with TANK/NEMO, NAP1 and SINTBAD. Both NAP1 and SINTBAD can interact with both IKKe and TBK1 (93, 94). TBK1/IKKe are essential for phosphorylation of the transcription factors IRF3 and IRF7. The phosphorylation of IRFs by TBK1/IKKe promotes dimerization and translocation of the IRF dimer into the nucleus (95).

Another key adaptor molecule acting as a scaffold for downstream signaling from RIG-I is STING (also known as MITA, ERIS and MPYS) (96-99). STING is only involved in RNA sensing downstream of RIG-I, not MDA5, whilst IPS-1 is involved in both RIG-I and MDA5 signaling (96). STING predominantly resides in the endoplasmatic reticulum (ER), but is also found in the mitochondrial membrane and even the plasma membrane (99).

**Positive and negative regulators of the RLR pathway**

Prolonged or excessive activation of the RLR pathway will have deleterious effects on the host tissues, and in concurrence with this the induction of IFN by RLR is subject to stringent control by a wide range of regulators. Among these regulations we find modifications by protein-protein interactions and post-translational modifications, such as ubiquitination, ISGylation and SUMOylation (100-105). The previously discussed RLR LGP2 is one of the modifiers acting through protein-protein interactions. Other regulators acting through protein-protein interactions include splice variants of both IPS-1 and RIG-I, the NLRX1 protein, Tetraspanin6, and dihydroacetone kinase (106-110).
**The RLR signaling pathway - What about fish?**

Recently a number of studies in fish have identified functional orthologues of the central members of the RLR-signaling pathway. RIG-I, MDA5 and LGP2 have all been identified in fish, RIG-I in salmon, trout and cyprinids, MDA5 in trout, cyprinids and flounder, LGP2 in trout, cyprinids and flounder (111-116). The gene sequence for NOD2 has been identified in several fish species (117). The zebrafish sequence of the negative regulator NLRX1 predicts that it will localize to the mitochondria, like its mammalian orthologue (118). Another negative regulator, a PIAS4 homologue, PIAS4a has been identified in zebrafish, where it acts as an inhibitor of IPS-1-mediated type I IFN induction (119). In mammals, the proteins belonging to the PIAS family are known as regulators of the STAT-factors in JAK/STAT signaling downstream of IFN induction, and the zebrafish PIAS4a is the first PIAS molecule to be identified to be involved in negative regulation of IPS-1 mediated signaling (120). The mammalian PIAS2β has been proposed to positively regulate MDA5 by SUMOylation (102).

Orthologues of all IRF members have been found in fish genomes (84-86). In fish, IRF1, IRF3 and IRF7 have been shown to be able to activate expression of IFNs and ISGs (87-90). STING has been identified in carp, where it can mediate antiviral activity through induction of IRF3/7 (116).
Antiviral proteins induced by IFN

Hundreds of different ISGs have been identified in humans by microarray and gene knockout studies (121, 122). Knock-out studies demonstrated the redundancy of the ISGs, as multiple knock-out mice were still able to establish a functional antiviral response. Taken together, the antiviral proteins induced by IFN are collectively able to inhibit virtually every step of the viral replication cycle (123). The different IFNs have their own ISG-profile, and some ISGs can even be induced directly by viruses without the presence of IFNs (124). Some ISGs are affecting a wide range of viruses, whilst others have a more targeted effect (125). Some of the best characterized antiviral ISGs that have also been found in fish are PKR, myxovirus resistance (Mx) proteins and ISG15. Also antiviral proteins specific to fish have been revealed, one of which is the protein kinase Z (PKZ).

Mx proteins have broad antiviral activity against RNA viruses, and also confer protection against some DNA viruses. They were named after the ability to provide resistance against the orthomyxoviruses (126). Mx proteins are relatively well conserved among vertebrates. The Mx protein is a GTPase that can be present both in the nucleus and in the cytoplasm. The antiviral mechanism of Mx is not completely understood, and it is possible that the mechanism varies between different species. Mx from fish was first cloned from the common perch (127). In Atlantic salmon, three Mx encoding cDNAs have been identified (128). Salmon Mx has a cytoplasmic localization, and it has been shown that Atlantic salmon Mx1 has potent antiviral activity against IPNV (129).
ISG15 is an ubiquitin-like protein that is heavily induced upon type I IFN stimulation in mammals (130, 131). ISG15 is conjugated to target proteins by a process called ISGylation. ISGylation is a post-translational process that can modify the stability, function or localization of the targeted protein. The antiviral effect of ISG15 seems in many cases to be through competition with ubiquitin, where the ISGylation prevents ubiquitination of the target proteins. This is the case for the Gag protein from HIV-1 and for the VP40 protein of Ebola virus (132, 133). Also other viruses, like Sindbis virus and influenza B virus, are strongly inhibited by ISG15, but the specific target protein is not known (134, 135). In Atlantic salmon, ISG15 has been shown to be overexpressed after infectious salmon anemia virus (ISAV) infection, Salmon anemia virus (SAV) infection, and by dsRNA treatment (136, 137). Atlantic salmon ISG15 is able to interact with intracellular proteins and also with an ISAV protein.

PKR is a dsRNA-dependent protein kinase involved in many cellular processes, including cell proliferation, cell growth, apoptosis and tumor suppression (138). Human PKR is constitutively expressed in most cells, and is activated by autophosphorylation when it interacts with dsRNA (139, 140). The best-characterized function of PKR is its ability to phosphorylate the eukaryotic initiation factor 2 (eIF-2α) (141). PKR has two regulatory dsRNA binding domains (dsRBD), and a kinase domain. Upon activation, PKR induces eIF-2α, resulting in a potent inhibition of protein synthesis (142). A wide range of viruses have developed different mechanisms to inhibit the activation of PKR upon infection (143-145). Several PKR-encoding genes have been identified in different fish species, where the number of dsRBD varies from one to three (146). It has been
shown that overexpression of PKR from flounder increases eIF-2\(\alpha\) phosphorylation, and has antiviral effect (147).

A PKR-like protein has also been identified in fish, but instead of the dsRBD found in PKR, the protein contains two Z-DNA-binding (Z\(\alpha\)) domains (148-151). Atlantic salmon PKZ has been shown to be able to phosphorylate eIF-2\(\alpha\), indicating a similar role as the PKR (150). Gene conservation analysis indicates that fish PKR is more related to fish PKZ than to mammalian PKR (149). So far, PKZ has only been identified in fish species.

**Viral evasion of IFN immune responses**

The mechanisms used by viruses to circumvent the IFN response can be divided into several categories. One of the mechanisms used is inhibition of cellular gene expression. This can be obtained by inhibition of transcription, RNA processing and inhibition of transcription and translation (152). Viruses specifically target pathways involved in IFN response, by viral proteins known as IFN antagonists. IFN antagonism most often targets the induction of IRFs, the JAK-STAT pathway or the PKR pathway. The antagonism can happen at any level of the signaling pathways, and some viral products can even inhibit several of the pathways, for instance respiratory syncytial virus NS1 and NS2 inhibit both IRF3 and STAT activation (123).

Another strategy employed by viruses is to limit the viral PAMPs available for the host to recognize. The virus can also have a replication strategy that is insensitive to the
IFN effect. Multifunctional viral proteins with multiple antagonistic effects on the IFN system are not uncommon. A single viral protein can even target several distinctly different components of the same pathway. And a virus can display antagonistic effects on more than one of the pathways in the IFN immune response.

Taken together, viruses have many potential targets in the IFN inductive pathway, and a single viral protein can have many functions in evading the immune response. In order for the host not to be overwhelmed by the viral smartness, the host needs to employ advanced strategies to combat the viral infection. The IFNs and the antiviral proteins induced by the IFN response have evolved as pivotal components in the viral combatting machinery of the host. The constant strain of viral infections have allowed for a co-evolution of both viral and host genes in a constant standoff between the virus trying to evade destruction by the immune system, and the hosts attempt to terminate the viral infection.
Figure 5. Members of the RLR signaling pathway that are targeted by viral IFN antagonistic proteins. The blue boxes groups viruses that have proteins antagonizing the same part of the RLR pathway. For an expansion of the abbreviations used for the viruses, see table 3.
### Table 3  Antagonism of type I IFN induction by the RLR pathway

<table>
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Antagonism on induction of type I IFN – the RLR pathway

Table 3 lists viruses with antagonistic effects on the RLR pathway. The viral proteins utilize a range of different strategies in preventing the mounting of a successful IFN response. Paramyxovirus protein V binds MDA5, thereby inhibiting interaction with IPS-1, whilst the poliovirus promotes degradation of MDA5 (153, 154). When it comes to RIG-I, four viruses seem to hide or protect its viral RNA genome from being detected by RIG-I (156, 158). Influenza A NS1 inhibits RIG-I from being activated by ubiquitination. The poliovirus 3Cpro protein cleaves RIG-I, whilst the respiratory syncytial virus NS2 and the New World arenavirus Z protein both bind RIG-I (155, 157, 159, 160).

Antagonism on IPS-1 was first identified shortly after the identification of IPS-1, when the cleavage of IPS-1 by the viral protease NS3/4A was shown to suppress the induction of IFNβ (161). The viral NS3/4A protease of the GB virus B, a virus related to hepatitis C virus, is also able to cleave IPS-1 (162). Also the protease precursor 3ABC from hepatitis A virus cleaves IPS-1 (163). Hepatitis B virus HBx protein also targets IPS-1. HBx promotes ubiquitination of IPS-1, targeting it for proteasomal degradation (164).

Many different viruses target the IRFs, either directly or indirectly. The SARS coronavirus M protein, the NY-1 hantavirus Gn protein and the hepatitis C virus NS3 all binds TBK1 or other members of the TBK1 signaling complex. This prevents TBK1 from phosphorylation of the IRFs (166-168). Both the Borna disease virus with its P protein and the Kaposi sarcoma-associated herpesvirus with ORF45 provide viral proteins as alternative substrates for phosphorylation by the TBK1/IKKε complex, limiting
phosphorylation of IRFs (171, 178). Viral proteins from three different viruses have been shown to bind IRF7, the Epstein-Barr virus BZLF-1, the bovine herpesvirus 1 bICP0 and the Ebolavirus VP35. In addition to inhibiting IRF7, bICP0 can also degrade IRF3. The VP35 inhibits IRF7 by exploiting the host SUMOylation machinery, by interacting with IRF7, a host SUMO E2 enzyme and E3 ligase together, it enhances SUMOylation of IRF7 (176). The inhibition of IRF7 by VP35 is specific for the RLR pathway, and has no effect on the TLR mediated induction of IFN (177). Human papillomavirus 16 E6 protein and Epstein-Barr virus BGLF4 inhibits IFN production by binding IRF3 (170, 173). The Murine hepatitis virus NS3 protein has deubiquitinase activity, and functions by deubiquitinating IRF3, preventing it from being able to enter the nucleus (169).

The targeting of the host DDX3 protein by the vaccinia virus K7R protein revealed that the DDX3 is part of the TBK1/IKKε complex (179). The interaction of K7R with DDX3 prevented activation of IRF3 by TBK1/IKKε. Finally for the inhibition on the IRFs, the Kaposi sarcoma-associated herpesvirus LANA-1 protein binds IRF-E in the IFNβ promoter, outcompeting the binding of IRF3 (171). The Kaposi sarcoma-associated herpesvirus thus has two different proteins using two different strategies in inhibiting the same part of the RLR pathway.

The classical swine fever virus Npro targets both the IRF and the NFκB branch of the RLR pathway. Npro promotes proteasomal degradation of IRF3, thereby inhibiting IFN production. It can also bind IκBα, however the significance of this interaction is not clear (185). Rotavirus NSP1 protein targets multiple steps of the RLR pathway. It promotes proteasomal degradation of IRFs (180-182). It also promotes proteasomal
degradation of a subunit of the ubiquitin E3 ligase responsible for ubiquitination of IκBα, a ubiquitination necessary for the release of NFκB (183). NSP1 is also a down-regulator of RIG-I in a proteasome-independent manner (184).

Of the IFN antagonistic proteins targeting the RLR pathway identified so far, one has been identified in a fish pathogen. The Infectious spleen and kidney necrosis virus (ISKNV) ORFIIIL protein is a TRAF protein that can replace the cell’s TRAF2 or TRAF3 proteins in interaction with TRADD (165).

In addition to finding viral antagonistic proteins with multiple targets in the RLR pathway listed in table 3, some of the viral proteins with antagonistic effects on the RLR pathway are also antagonistic on other parts of the IFN system. The Influenza virus NS1 protein is for instance well known for its antagonistic properties both in IFN induction through blocking RIG-I activation, and in directly inhibiting antiviral proteins such as PKR and OAS/RNaseL. NS1 also inhibits export of mRNA from the nucleus (187). The NS3/4A from Hepatitis C virus in addition to cleaving IPS-1 also cleaves TRIF, a signal mediator in the TLR3/TLR22 mediated activation of IFN (188).

So far, virtually every step in the RLR signaling cascade has been identified as a target for viral antagonism. Some viruses targets the main members of the signaling cascade, but also targeting the more peripheral modifiers of the RLR cascade is a frequently used antagonistic property.
Infectious pancreatic necrosis virus

Infectious pancreatic necrosis virus (IPNV) is the causative agent of infectious pancreatic necrosis (IPN), one of the most common viral diseases of farmed salmonids worldwide. IPN as a disease infecting salmonids has been known since the 1940s (189). In 1955, it was established that the disease was caused by a virus, and the disease was named IPN based on the lesions observed on the pancreas (190). It was the first fish virus isolated and characterized in cell culture (191).

Susceptibility and pathogenesis may vary greatly dependent on the host infected, the virus strain and the environmental conditions (192). IPNV has a wide host range, and has been identified in more than 80 different species including shellfish, and a wide range of marine and freshwater fish species (193). Most of the organisms show no sign of disease after infection with IPNV.

IPNV causes mortality in Atlantic salmon juveniles during the hatchery period and on postsmolts shortly after transfer to the sea, resulting in substantial losses for the salmon farming industry (192). Vaccines are commercially available (194), but do not appear to provide a high level of protection. The numbers of outbreaks and losses due to IPN have been high over many years despite the fact that the majority of farmed Atlantic salmons in Norway have been vaccinated against IPNV (1, 195). A well-known feature of IPNV is its ability to establish a carrier state in salmonids, which makes it difficult to remove the virus from affected fish stocks. The impact of the IPNV carrier state on the health status of salmon is still unknown. Recent years there has been a decline in the number of IPN outbreaks in Norway that may in part be contributed to the expanding use
of fish with increased resistance to infection, so called quantitative trait loci (QTL)-fish (1, 196, 197). Elucidation of the molecular mechanisms underlying the infectivity of IPNV is important to further understand its pathogenic properties. At present, the selective breeding of QTL-fish might seems to be a successful method to combat the problems of IPNV infection, but the selection of one trait could cause the loss of other traits. A too homogenous population might also be less suited to deal with potential new emerging viral diseases currently not present in the IPN problem areas.

IPNV is an aquatic dsRNA virus belonging to the genus *Aquabirnavirus* of the *Birnaviridae* family. Birnaviruses are non-enveloped, single-shelled icosahedrical RNA viruses with a genome consisting of two segments dsRNA (198-200). Infectious bursal disease virus is the most extensively studied virus of the *Birnaviridae* family. It causes immunosuppression in infected birds by specifically targeting B-cells in the immune organ bursa for destruction (201).
Figure 6. IPNV. A. IPNV is a virus about 60 nm in diameter. The virus has no outer membrane, and consists of an icosahedral capsid of 60-70 nm protecting the dsRNA genome. B. The two genomic segments encode five proteins. The polyprotein encoded by segment A is cotranslationally cleaved by the cis-acting viral protease VP4 to yield VP4, and the structural proteins VP3 and preVP2. A separate reading frame of segment A encodes the non-structural protein VP5. Segment B encodes the viral RNA-dependent RNA polymerase.

The IPNV genome consists of two double-stranded (ds) RNA segments, packed in a non-enveloped single-shelled icosahedral capsid (198-200) (figure 6). The genome encodes five proteins termed VP1, VP2, VP3, VP4 and VP5 depending on the size. Segment B encodes VP1, which is a 94 kDa RNA dependent RNA polymerase (RdRp) found both in a free and a genome-linked form in the virion (202). The genome-linked
form of VP1 is covalently linked to both genome segments at the 5’ end by a phosphodiester bond (202). The rest of the viral genes are located on segment A, where the VP2 (51 kDa), VP4 (24 kDa) and VP3 (27 kDa) are encoded as a polyprotein, whilst VP5 is encoded in a second overlapping ORF. The polyprotein is co-translationally cleaved into the separate viral proteins by VP4, which is a serine-lysine protease (203, 204). The site of the lysine general base, K674, and the reactive serine residue, S633, are vital for the protease activity (205). The crystal structure of VP4 identified the substrate binding pockets and other substrate-VP4 interactions (206). VP2 is released from the polyprotein as a preprotein, and is further processed to yield the mature outer capsid protein VP2. Neutralizing antibodies against IPNV are mainly targeting the variable region and C-terminal end of VP2 (207). VP3 is a structural protein found in the inner surface of the viral capsid. VP3 has been shown to interact with VP1 and also with double-stranded RNA (208). Segment A contains a small overlapping ORF encoding VP5 is encoded by ORF in segment A overlapping the ORF of the polyprotein. It is a non-structural protein. The role of VP5 is yet undefined, and the previously proposed anti-apoptotic function is disputed as recombinant viruses deficient in VP5 expression has been shown to induce apoptosis to a similar degree as viruses encoding full-length and truncated forms of VP5 (209, 210). The size of VP5 may vary depending on the isolate, but some isolates lack the VP5 reading-frame altogether (211, 212).

Unlike DNA viruses, which for a large part rely on the host polymerases for replication, dsRNA viruses harbor their own RNA-dependent RNA polymerase (RdRp). For most dsRNA viruses, replication is monocistronic. After the viral particle is internalized in the cell, the genomic dsRNA is transcribed to mRNA shed from the virus
particle. The viral mRNA serves as the basis for both translation and replication. To avoid detection by the host antiviral machinery, many dsRNA viruses are replicating their genome inside the viral capsids (213).

IPNV enters cells through receptor-mediated endocytosis (214-216). The molecule responsible for cell attachment has not been identified, but is proposed to be VP2 (217). The replication cycle of IPNV takes place in the cytoplasm, and takes about 24 h at 15°C (218). At cell entry, the virus can immediately start the replication cycle, as the viral RdRp is active without any proteolytic alterations (219). During the replication cycle, two different viral particles are formed. First an uninfected, immature viral particle is formed, whilst an infective mature virion can be detected at a later timepoint (220). The majority of viral RNA in an infective cycle is not associated with viral particles, but rather with a viral ribonucleoprotein complex which mainly contains viral RNA species and VP1 (221). Of the IPNV-encoded proteins, only VP1 could be detected in the immature particle, although trace amounts of other proteins could be present without being detected. At early timepoints, only negative strand RNA is present, this likely reflects that the negative strand is synthesized first and used as a template for the synthesis of the positive strand. The positive strand is synthesized by a semi-conservative strand-displacement mechanism primed by the viral VP1, while the mechanism of synthesis of the negative-strand is unknown (202, 222). VP3 synthesis can be detected at 6 hours post infection, and VP2 after 8 hours. Early in the infection cycle it appears that VP2 and VP3 are synthesized in free polyribosomes, and when the majority of viral RNA has been synthesized, assembly of the viral particle takes place near the nucleus of the cell (223).
There are multiple strains of IPNV. The strains can be divided into nine subtypes in serogroup A and one in serogroup B (224). The subtypes display markedly different virulence (225). Comparison of isolates from the strains shows that the viral genome contains hyper variable regions (HVR) in the VP2 gene, and these regions are associated with pathogenicity. The HVR can be recognized by neutralizing antibodies, and the VP2 has been targeted for vaccine development (207, 226-228). Comparing sequences of IPNV from disease outbreaks shows that IPNV exists as mixed populations with differences in amino acid positions 217, 221 and 247 of VP2. There are four common combinations of amino acids in these positions, TAT, TTT, PAA and PTA. It has been shown that the different amino acid combinations play an important role for immunogenicity of IPNV vaccines, where the vaccines based on the virulent strains displaying the TAT-motif give a higher response in virus neutralization assays, ELISA antibody titers and less pathological signs in vaccinated fish after challenge (229). Development of new live vaccines based on the virulent phenotypes displaying the TAT motif in VP2 could be promising, and would lessen some of the concerns of the possible over-dependency of the use of QTL-fish.

**IFN antagonism by IPNV**

IPNV is sensitive to the antiviral action of IFN, as demonstrated by the efficient inhibition of virus growth in cell cultures pretreated with exogenous IFNa1 (14). In addition to the antagonistic effects by IPNV in the IFN system prior to transcription of IFN, antagonistic effects downstream of IFN induction have also been demonstrated. If
IFN treatment succeeds IPNV infection, IPNV is able to inhibit IFN signaling in the cells (230-232). The induction of transcription of ISGs by administration of exogenous IFN to cells is down-regulated by the IPN virus. Also, the salmon Mx protein have been shown to directly inhibit viral protein synthesis (129). The viral and host proteins directly involved in these actions have yet to be determined. Screening attempts in a yeast two-hybrid assay did not show any positive interactions between the IPNV proteins and one of the Atlantic salmon STAT1 isotypes (233).
Aims of study

The purpose of this project was to study the regulation of the Atlantic salmon type I IFN system with special focus on IFNa and viral antagonism by IPNV.

The major aims were

- to study the promoter regions of the IFNa1 and a2 genes
- to study the cytoplasmic IFNa induction pathway
- to study the IPNV antagonism on IFNa induction

General summary (of papers)

Paper I

Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions

In paper I we cloned the promoter regions from two type I IFN genes from Atlantic salmon. Both genes contained two promoter regions, and had the potential to encode IFN transcripts with either a long or a short 5’-untranslated regions, apparently controlled by two distinct promoter regions. The distal promoter regions contained IRF binding sites and a putative ATF-2/c-Jun element. The proximal promoter region contained a TATA-box, two IRF binding sites and a putative NFkB binding site. Both complete and several truncated promoter constructs were fused to a gene encoding firefly luciferase as a reporter. The different promoter reporter constructs were analyzed for activity in salmonid cell lines. Constructs containing the distal promoter were only poorly inducible by the synthetic dsRNA poly (I:C). The proximal promoter was highly inducible by poly
(I:C), and the induction was apparently NFκB -dependent. We concluded that the proximal promoter is the main promoter region in inducing transcription of the examined IFNs in Atlantic salmon. IPNV and ISAV were tested for their ability to induce the proximal promoter, and only ISAV were able to induce transcription before the cells died from the infection.

**Paper II**

**Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-kappaB and localizes to mitochondria**

RLR being identified as the main type I IFN inducing pathway in most cells of higher vertebrates, we wanted to examine whether it was also present in Atlantic salmon. A putative IPS-1 homologue from Atlantic salmon, AsIPS-1, was cloned and characterized. The AsIPS-1 had a relative low amino acid sequence identity to the described mammalian counterparts, only 18% identity to human IPS-1, but it contained the same characteristic domain structure found in mammalian IPS-1; a CARD-domain, a proline-rich region and a transmembrane domain. Functional studies showed that AsIPS-1 was expressed in all examined tissues, and overexpression of AsIPS-1 in cell culture induced an antiviral state. Deletion of either the CARD-domain or transmembrane domain abolished the induction of an antiviral state, and also only the full length protein was able to induce the IFNa1 promoter and an NF-κB binding promoter. Taken together, we identified AsIPS-1 as the Atlantic salmon IPS-1 homologue, with comparable functions to the mammalian IPS-1.
Paper III

Infectious Pancreatic Necrosis Virus Proteins VP2, VP3, VP4 and VP5 Antagonize IFNa1 Promoter Activation while VP1 induces IFNa1

Using the tools obtained in the previous studies, we wanted to identify possible IFN antagonistic genes in infectious pancreatic necrosis virus (IPNV). Among the many microbial threats to fish, IPNV is one of the major viral pathogens causing disease in the aquaculture industry worldwide. The IPNV is highly sensitive to IFNa, yet cannot mount an efficient IFNa response in infected cells. It was therefore very relevant to study the effect the separate IPN proteins had on IFNa induction. In paper III we show that several of the IPN proteins have powerful IFN antagonistic properties. Each of the separate IPNV genes cloned into an expression vector were tested for the ability to influence activation of the Atlantic salmon IFNa promoter or an NFκB-driven promoter mediated by the Atlantic salmon IPS-1 or interferon regulatory factors (IRF). This showed that preVP2, VP3 and VP5 have antagonistic effects on the activation of both promoters, whilst VP4 only had antagonistic effects on the IFNa promoter. The viral protease VP4 was the most potent inhibitor of IFN induction apparently targeting the IRF1 and IRF3 branch of the signaling cascade. The antagonism of VP4 is independent of the protease activity since the catalytically dead mutant VP4K674A inhibited activation of the IFNa1 promoter to a similar extent as wild type VP4. In contrast to the other IPNV proteins, the viral RNA-dependent RNA polymerase, VP1 activated the IFNa promoter in absence of the antagonistic viral proteins. VP1 also showed synergistic effects with IRF1 and IRF3 in inducing an IFNa-dependent antiviral state in cells. Taken together these results suggest
that IPNV has developed multiple IFN antagonistic properties to prevent IFN-induction by VP1 and IPNV’s dsRNA genome.
General discussion

The results are discussed in detail in the respective papers. Here some selected topics are further discussed, especially for the two first papers where new insight in the field has emerged after publication.

Governing the IFN production –IFN promoter elements

The research in paper I focus on characterizing the IFNa1/a2 promoters. Previous studies where IFN cDNA from Atlantic salmon was cloned, had shown that several transcript variants of IFNa existed, some containing a long 5’-UTR, some with a short 5’-UTR (14). These genes were classified as type I IFN genes based on that they were acid stable, could be induced by viral infection, had antiviral activity, and shared sequence similarities with other type I IFNs. In the initial studies, the Atlantic salmon IFNa was termed SasaIFNα due to similarities with the mammalian IFNα in a BLASTX analysis, and the presence of a cysteine residue as the first amino acid in the putative mature protein, similar to the mammalian IFNα (14). Also similarities with human IFNβ were present, and like IFNβ, the Atlantic salmon sequences contained mRNA destabilization elements in the 3’-UTR region of the cDNAs (14). The small cDNA was named SasaIFNα1 and the large SasaIFNα2.

In paper I, the promoter regions of SasaIFNα1 and SasaIFNα2 were cloned from bacterial artificial chromosome (BAC) clones. It was initially thought that SasaIFNα1 only existed as a short transcript and 2 only as a long transcript. However, the promoter sequences of the two genes suggested that both genes had the potential to produce
transcripts with both a short and a long 5’-UTR. The promoter regions from both genes have both a proximal and a distal promoter region, where the proximal promoter region includes position -202 to +26 relative to the SasaIFN-A1 transcription start site. This region was determined to be the main control region, as experiments with the distal promoter region showed that it was hardly inducible by the synthetic dsRNA poly(I:C) used to mimic a viral infection. The minimal promoter region of the proximal promoter was highly inducible.

In mammals, the main difference of the promoter region of IFNα and IFNβ is the presence or absence of an NFκB-binding element. The IFNβ promoter contains an NFκB-binding element essential for an immediate early response to viral infection (234, 235). The IFNα promoter contains no NFκB-binding element, but has multiple IRF binding sites, and is generally induced at a later timepoint than IFNβ. The structure of the proximal promoter region PR-I for both SasaIFNα1 and SasaIFNα2 and the sensitivity of PR-I to NFκB inhibitors, suggests that they have the capacity to govern an immediate early response, and that their induction properties are more related to the mammalian IFNβ. The long transcript produced from the distal promoter region PR-II was mainly present in lymphoid tissue, as the transcript levels in TO cells were very low compared with the transcript levels previously seen in head kidney (14). It is possible that the distinct PR-II responds to other stimuli than poly (I:C).

The presence of mRNA transcript variants of different length of the same IFN gene has been found in Atlantic salmon, zebrafish, channel catfish and rainbow trout (Paper I, (236-238)). It has been speculated that the long transcripts from Atlantic
salmon, channel catfish and rainbow trout that lack a classical signal peptide might be a way of diversifying the IFN functions, but more studies are needed to examine this possibility (239).

It has after publication of paper I been established that Atlantic salmon has additional IFN genes. The SasaIFNα1 is present in a multigene cluster with seven other IFN genes (25). As the salmon IFNs were found not to be orthologues of the mammalian IFNs, the salmon IFNs were given a new nomenclature (25). The former salmon IFNα1 and IFNα2 are now called IFNa1 and IFNa2. The other IFNs identified were classified based on sequence and expression properties. In Atlantic salmon at least 13 different type I IFNs exist. The fish type I IFNs can be subdivided into at least four different classes, termed IFNa, IFNb, IFNc and IFNd based on phylogenetic relationships (24). They can also be separated in two groups according to the presence of two or four cysteins (240). IFNa and IFNd contain 2 cysteins (2C IFNs) while IFNb and IFNc contain 4 cysteins (4C IFNs) (26). So far, Atlantic salmon is the only fish species where IFNs of all subtypes has been identified (24, 25). Like the mammalian IFNs, fish IFN genes can be found in multiple copies linked in the genome. The classes of IFNs present, and the number of copies of IFN genes can vary extensively depending on the fish species (239). The Atlantic salmon IFNs differ in promoter structure, where only the IFNa genes have an NFκB-binding site flanked by two IRF binding sites, characteristic of the immediate early mammalian IFNs induced by the RNA-binding pattern recognition receptors (PRRs) RIG-I/MDA5 and TLR3 (Paper I, (25)).
The RLR pathway in Atlantic salmon

In 2005, four separate groups identified IPS-1 (also known as MAVS/VISA/Cardif) in mammals as a key adaptor protein leading to IFNβ-induction by activating IRF3 and NFκB (67-70). Overexpression of IPS-1 triggered an antiviral state without further stimuli. In paper II we describe the cloning and characterization of an IPS-1-like molecule from Atlantic salmon. The Atlantic salmon (As) IPS-1 showed only 18% amino acid identity with the human IPS-1, and a multiple alignment analysis of putative IPS-1 genes from a range of different species showed that the amino acid identity was low, ranging from 22% for green spotted pufferfish to as little as 14% identity with chicken IPS-1.

Even though the amino acid identity with mammalian IPS-1 was low, AsIPS-1 contained all three characteristic domains found in mammalian IPS-1, the CARD-domain, a proline-rich region and a transmembrane (TM) domain was identified. The most conserved region of the protein was found in the CARD domain, with 25 – 43% amino acid identity to the other species. In co-transfection assays, we found that AsIPS-1 was able to stimulate the minimal Atlantic salmon IFNα1 promoter studied in paper I. Deletion of either the CARD-domain or the TM abolished the promoter activation. AsIPS-1 could, like the mammalian IPS-1, activate NFκB. Transfection of a Chinook salmon-derived cell line (CHSE214) with the AsIPS-1 mediated antiviral activity against IPNV, this in accordance with the results showing antiviral activity of overexpressed IPS-1 in mammalian studies. Adding a neutralizing antibody against IFNα reduced the antiviral potential of the transfected AsIPS-1. The cells still retained some resistance to
the viral infection even in the presence of the IFNa antibody. This might be an artifact of the method used, where the transfected cells will continue to produce IFNs due to the overexpression of IPS-1. Other possibilities are that it reflects the presence of other IFNs. Another potential reason is that the overexpressed IPS-1 might induce antiviral genes directly, without the need of the presence of IFN. The discovery of peroxisome-anchored IPS-1 in mammals that can directly induce the expression of the antiviral ISG viperin provides the rationale for this possibility (72).

The same year as paper II was published, another paper concerning fish IPS-1 was published, where IPS-1 orthologues from Atlantic salmon, zebrafish and cyprinids were cloned (111). This paper also showed antiviral activity of overexpressed IPS-1 in cell lines against several RNA viruses of the rhabdoviridae family and also a DNA virus of the iridoviridae family. Almost complete inhibition of the negative-stranded RNA virus replication was observed, and a marked decline in CPE caused by the dsDNA virus. They also cloned RIG-I and demonstrated its involvement in induction of IFN. Taken together, the results from paper II and Biacchesi et al were the first to demonstrate that IPS-1 plays similar roles in fish and mammals, and despite the sequence divergence, the function is conserved. These findings further supports that IFNa has similar roles as IFNβ.
**IFN antagonism by IPNV**

Considering the wide host range and the abundant and widespread distribution of IPNV, the most likely counter-measure to fight the disease in the salmon aquaculture industry is to develop efficient vaccines against the disease. So far, despite the extensive use of vaccination in Norway, IPNV remains responsible for large losses every year. As one of the main functions of the IFN system is to fight viral infections, we wanted to investigate the interaction between IPNV and the induction of IFNa. The cloning and characterization of the Atlantic salmon IRF transcription factors provided an additional tool for studying the IFN induction pathway (87, 241). In mammals it was recently shown that IPS-1 can also be found on the surface of peroxisomes. It seems that this peroxisomal localization can induce ISGs directly by activation of IRF1, whilst the mitochondrial localization activates IRF3, leading to induction of type I IFNs (72). In Atlantic salmon, it seems that IRF3 is the main regulator of IFNa induction, but also IRF1 and IRF7 are potent activators of the IFNa1 promoter (87).

In paper III, we found that four out of five genes from IPNV could antagonize the induction of IFNa. The most potent antagonist was the viral protease VP4. VP4 was also the viral protein with the most specific effect on the RLR signaling cascade, acting on the IRF branch and not the NFκB branch. A schematic overview of the results obtained in paper III is shown in figure 7. We did not succeed in finding the specific molecular mechanism underlying the antagonistic effects observed, and this would be an interesting topic for further studies. Especially looking into the modifications by the avian birnavirus VP4 on the GILZ-protein could be an interesting candidate for further studies on IPNV.
VP4 (242). However, the complexity of the RLR-mediated signaling cascade makes the identification of the specific proteins difficult, as far from all the proteins identified as members of the RLR signaling cascade have been identified in Atlantic salmon. And not only the central members of the signaling cascade, but also the positive and negative regulators of the signaling members, are possible targets of viral IFN-antagonism.

The inhibitory effects observed for VP2, VP3 and VP5 seemed to be of a more general nature than that of VP4, negating induction of both the IFN promoter and an
NFκB-driven promoter. The structural protein VP3 has previously been shown to interact with dsRNA, and also to bind to the viral RdRp VP1, potentially mechanisms to hide the viral RNA from detection (208). No consistent impact was observed on the levels of the transfection control gene used, Renilla luciferase, so the inhibition seemed not to be a general decline in cellular transcription and translation. The RdRp VP1 could by itself trigger the activation of the IFNa1 promoter construct used, providing a rationale for the necessity of the powerful IFN antagonistic properties observed by the other viral proteins.

**Concluding remarks and future perspectives**

The IFN system is highly complex, with a multitude of different receptors and intermingling signaling pathways resulting in the activation of different – or the same – IFN. The different IFNs involved in antiviral activity have somewhat separate but very powerful antiviral profiles. The presence of a multitude of different IFNs have been proposed to be due to two different reasons, that they have different properties and that they are produced at different times and places (243). In addition to that, it is possible that the IFNs reflect the redundancy in induction pathways and in antiviral protein profiles that may have co-evolved with viral disease to make it harder for viral antagonistic mechanisms to negate the antiviral response, or the different responses may have adapted to specifically combat different viral pathogens. When one part of the interferon system is attacked and brought down by a virus, another part might be ready to take its place.

It has been shown that IFNa, IFNb, IFNc and IFNd show differences in that they are induced in different cells, by different stimuli, in different organs and in different
amounts (26). In such a perspective, the characterization of the other IFN promoters would be a very interesting topic for further studies.

The presentation of the establishment of an antiviral state as a linear process is a highly simplified version. In reality multiple feedback mechanisms and loops in the presented pathways exist. It is possible for ISGs to be induced directly by infecting viruses, and the ISGs and IFNs have negative feedback regulatory mechanisms. An excessively elevated level of IFN induction can have deleterious effects on the organism, so a tight and carefully adjusted control regime is expected.

Our and other studies have found that IFNa from Atlantic salmon has functional similarities with the mammalian IFNβ. This considering antiviral activity, what cell-types are expressing IFNa and the signaling pathway leading to induction of IFNa expression. Even if the IFNs from fish and mammals are not evolutionary orthologues, the co-evolution of IFNs with similar induction pathways and antiviral properties shows the importance of the IFN system as a countermeasure for viral infections.

Studying viral-host interactions on the level of specific protein-protein interactions might potentially also reveal host proteins previously unknown to be involved in innate immunity.
The IFN promoter constructs from Atlantic salmon along with the expression constructs for the proteins involved in IFN induction provide tools to further elucidate potential and specific antagonistic properties of fish pathogenic viruses. Knowing more about the host-virus relationship for each virus will lead to better understanding of viral disease.

**Main conclusions**

- The promoter structure of IFNa1/2 is reminiscent of the promoter structure of human IFNβ.
- Members of the signaling pathway leading to type I IFN induction are conserved through evolution.
- IFNa is induced through the RLR-mediated pathway with the conserved central adaptor IPS-1.
- Most of the IPNV proteins have IFN-antagonistic properties, where VP4 is the most potent antagonist on the RLR-mediated pathway.
- The IFN reporter construct along with the members of the signaling pathway can be used for initial testing of antagonism in other viruses.
References


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Paper I
Paper II
Paper III