UNIVERSITY OF TROMSØ UIT

FACULTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
NORWEGIAN COLLEGE OF FISHERY SCIENCE

# Atlantic salmon type I interferons

## Induction and antiviral activity

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# Summary

Type I interferons (IFNs) are pivotal factors of the antiviral defense system of vertebrates. Following virus recognition most cells can secrete type I IFN, which upon binding to its receptor on the surface of other cells triggers the production of antiviral proteins. Collectively, these antiviral proteins can inhibit or delay viral replication. A total of 13 type I IFN genes have been identified in the Atlantic salmon genome, and these fall into four different subgroups named IFNa (three genes), IFNb (four genes), IFNc (five genes) and IFNd (one gene). The overall objective of the present work was to gain more knowledge about the distinct roles of IFNs of subgroup IFNa, IFNb, IFNc and IFNd in innate antiviral defense of Atlantic salmon.

The antiviral activity of one member from each of the four Atlantic salmon IFN subgroups was investigated (paper III). IFNa and IFNc showed a similar antiviral potency against infection pancreatic necrosis virus (IPNV) in TO cells, the antiviral activity of IFNb was lower than that of IFNa and IFNc, whereas no antiviral activity was detected for IFNd. The variable antiviral activity was also reflected in the ability of the distinct IFNs to stimulate transcription of antiviral genes. IFNa, IFNb and IFNc were furthermore for the first time firmly established to exhibit antiviral activity against ISAV, although the effect seemed to be transient (paper IV).

The induction of the IFNa, IFNb, IFNc and IFNd subgroups were studied in paper I, II and III. In mammals, type I IFNs (IFNαs and IFNβ) display different induction patterns in different cell types. The viral ssRNA mimic R848 is known to rapidly induce large amounts of IFNα through activation of TLR7 expressed in plasmacytoid dendritic cells (pDCs), whereas the viral dsRNA mimic polyinosinic-polycytidylic acid (poly (I:C)) is known to trigger an initial wave of IFNβ in multiple cell types through the activation of melanoma

differentiation-associated gene 5 (MDA5) and toll-like receptor (TLR) 3. The induction of IFNa, IFNb, IFNc and IFNd were studied in cells and various organs of Atlantic salmon treated with R848 and/or poly (I:C). In accordance with earlier data, we found that Atlantic salmon IFNa1/2 are the predominant IFNs induced in cell lines and tissues by the mammalian MDA5/TLR3 ligand poly (I:C) (paper I and III). The induction of Atlantic salmon IFNa1/2 thus shares some similarities with that of mammalian IFNβ. We also showed that IFNa1/2 is produced via an Atlantic salmon ortholog of interferon-beta promoter stimulator 1 (IPS-1) (paper II). IPS-1 is in mammals a key adaptor protein in the retinoic acid-inducible gene I (RIG-I)/MDA5 pathways. IFNb and IFNc were shown to somewhat resemble the IFNαs as they were relatively highly induced in cells present in immunological organs (head kidney and spleen) by the TLR7 ligand R848 (paper III). *In vivo,* IFNc was additionally induced by poly (I:C), possibly through receptors different from TLR3 and MDA5. IFNd expression was not triggered by either ligand. The duplication of the mammalian IFN genes is thought to have occurred after the divergence of birds and mammals, and the distinct type I IFN genes of fish are thus not direct orthologs of the distinct type I IFN genes of mammals. This suggests that fish and mammals have evolved certain similar IFN-induction mechanism through convergent evolution. The results further imply that the induction of mammalian IFNs occur at least partly through pathways that already existed in an ancestor fish.

The present work contributes to the characterization Atlantic salmon type I IFNs and shows that the IFNs display differences both in antiviral activity and in induction patterns.

# List of papers

**I: Kristel Berg, Tina Svingerud, Baojian Sun, Børre Robertsen.** An antiserum against Atlantic salmon IFNa1 detects IFN and neutralizes antiviral activity produced by poly I: C stimulated cells. Developmental & Comparative Immunology. Volume 33, Issue 4, April 2009, Pages 638-645.

**II: Silje Lauksund, Tina Svingerud, Veronica Bergan, Børre Robertsen.** Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-κB and localizes to mitochondria. Developmental & Comparative Immunology. Volume 33, Issue 11, November 2009, Pages 1196-1204.

**III: Tina Svingerud[*], Terese Solstad[*], Baojian Sun, May Liss J. Nyrud, Øyvind Kileng, Linn Greiner-Tollersrud, Børre Robertsen.** Atlantic Salmon Type I IFN Subtypes Show Differences in Antiviral Activity and Cell-Dependent Expression: Evidence for High IFNb/IFNc–Producing Cells in Fish. The Journal of Immunology. Volume 189, Issue 12, December 2012, Pages 5912-5923. [*] The authors contributed equally.

**IV: Tina Svingerud, Jenni Kristin Holand, Børre Robertsen.** Infectious salmon anemia virus (ISAV) is transiently inhibited by Atlantic salmon type I interferon. Manuscript. 2013

# Abbreviations

| | | | |
|---|---|---|---|
| AsIPS-1 | Atlantic salmon IPS-1 | MAPK | Mitogen-activated protein kinase |
| CARD | Caspase activation and recruitment domain | MDA5 | Melanoma differentiation-associated gene 5 |
| cDC | Conventional dendritic cell | MyD88 | Myeloid differentiation primary response gene 88 |
| CPE | Cytopathic effect | NEMO | NF-κB modulator |
| CPG | Cytosine phosphate guanine motifs | NF-κB | Nuclear factor kappa B |
| CRFB | Cytokine receptor family B | PAMP | Pathogen associated molecular pattern |
| eIF2α | Eukaryotic translation initiation factor 2A | pDC | Plasmacytoid dendritic cells |
| ER | Endoplasmic reticulum | PKR | Double stranded RNA-dependent protein kinase |
| FADD | Fas-associated death domain | PKZ | Protein kinase containing Z-DNA binding domains |
| FISH | Fluorescent in situ hybridization | Poly (I:C) | Polyinosinic-polycytidylic acid |
| IFN | Interferon | PRR | Pathogen recognition receptor |
| IKK | IκB kinase | RIG-I | Retinoic acid-inducible gene I |
| IPNV | Infectious pancreatic necrosis virus | RIP1 | Receptor interacting protein 1 |
| IPS-1 | Interferon-beta promoter stimulator 1 | RLR | RIG-I-like receptor |
| IRAK | IL-1 receptor-associated kinase | STAT | Signal transducer and activator of transcription |
| IRF | Interferon regulatory transcription factor | STING | Stimulator of IFN genes |
| ISA | Infectious salmon anemia | TANK | TRAF-family member associated NF-κB activator |
| ISAV | Infectious salmon anemia virus | TBK1 | TANK-binding kinase 1 |
| ISG | Interferon stimulated gene | TIR | Toll/IL-1 receptor |
| ISG15 | Interferon stimulated gene 15 | TLR | Toll-like receptor |
| ISGF3 | Interferon stimulated gene factor 3 | TRADD | Tumor necrosis factor receptor type 1-associated DEATH domain |
| ISRE | Interferon-stimulated response elements | TRAF | Tumor necrosis factor receptor-associated factor |
| IκB | Inhibitor of kappa *B* | TRIF | TIR-domain-containing adaptor inducing IFN-β |
| JAK | Janus kinase | TYK2 | tyrosine kinase 2 |
| LGP2 | Laboratory of genetics and physiology 2 | UBL | Ubiquitin-like |
| LRR | Leucine-rich repeat | | |

# 1. Introduction

Norway has for many years been a large scale producer and exporter of Atlantic salmon (*Salmo salar* L). In 2008, Norway accounted for 36.4 percent of the total salmonid production in the world (844.000 metric tonnes) [1]. Three years later, the production of Atlantic salmon alone exceeded 1.000.000 metric tonnes [2]. Despite high production levels there is still an estimated production loss of 15 to 20 percent during the sea water phase [3], and a large part of this loss is linked to viral diseases [3].

The type I IFNs are the major components of innate immunity that protects the host against viral infections [4]. Given the ongoing threat of viral infections in the fish farming industry, research on the immune system of fish in general and the type I IFNs in particular could provide valuable information. Increased knowledge of the immune system can for example potentially aid in the development of more efficient vaccines. Studies on fish IFNs are also interesting from an evolutionary view point.

The studies encompassing this doctoral thesis have explored the induction and antiviral activity of type I IFNs from Atlantic salmon. To give a background, I will first provide a detailed description of mammalian and fish IFNs. Then I will specifically review the different receptors the cell utilizes for recognition of viruses to induce type I IFN, their signaling pathways and their cell-dependent expression. Next I will refer to the IFN-signaling pathway that controls the induction of interferon stimulated genes (ISGs), before I describe in detail the nature of some of the antiviral proteins which are induced by IFN. Finally, before proceeding to the specific aims and discussing the findings of this work, I will give a short description of the two viruses used, IPNV and infectious salmon anemia virus (ISAV).

Although many studies have focused on fish type I IFNs in the last decade, many facets are still unexplored. Throughout the introduction I will thus in each section first give an

overview of the current knowledge in mammals followed by a summary of what is known on the same topic in the piscine world.

### 1.1 Interferons

**Mammals.** IFNs are cytokines which were originally named for their ability to *interfere* with viral replication [5]. Structurally the IFNs can be divided into three main groups or "types": type I, type II and type III. The human type I IFN family consists of 13 IFN-alpha (α) subtypes, and one subtype each of IFN-beta (β), IFN-epsilon (ε), IFN-kappa (κ) and IFN-omega (ω) [6]. Of the multiple type I IFNs, the IFNαs and IFNβ are considered to be the main antiviral IFNs. The more recently discovered type III IFNs also exhibit antiviral activity, and consist of three members: IFNλ1 (IL-29), IFN λ2 (IL-28A) and IFNλ3 (IL-28B) [7, 8]. A single type II IFN (also called IFNγ) is found in humans. Unlike the type I and type III IFNs which can be expressed by most cell types, IFNγ is mainly produced by T cells and natural killer cells [9]. The mammalian type I IFNs are encoded by a single exon, the type II IFN gene contains four exons and three introns and the type III IFN genes contain five exons and four introns [6]. The type I, II and III IFNs signal through distinct receptor complexes composed of IFNAR1/IFNAR2, IFNLR1/IL-10R2 and IFNGR1/IFNGR2, respectively [6]. Because the subject of this work is type I IFNs of Atlantic salmon, if the term "IFN" is used, it refers to type I IFNs and in particular IFNα/β.

**Fish.** In 2003, the first type I IFN genes from fish were cloned from Atlantic salmon, zebrafish (*Danio rerio*) and green spotted puffer (*Tetraodon nigroviridis*) [10-12]. Since then, type I IFN from several fish species including catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus* carpio) and sea bass (*Dicentratchus labrax*)

have been cloned and studied [13-16]. The fish type I IFNs are broadly classified into two groups: group I contains IFNs with two conserved cysteine residues, whereas group II contains IFNs with four conserved cysteine residues [14]. Phylogenetic analyses show that the group I IFNs can be further divided into IFNa and IFNd subgroups, whereas the group II IFNs can be divided into IFNb and IFNc subgroups (Fig. 1) [17, 18].

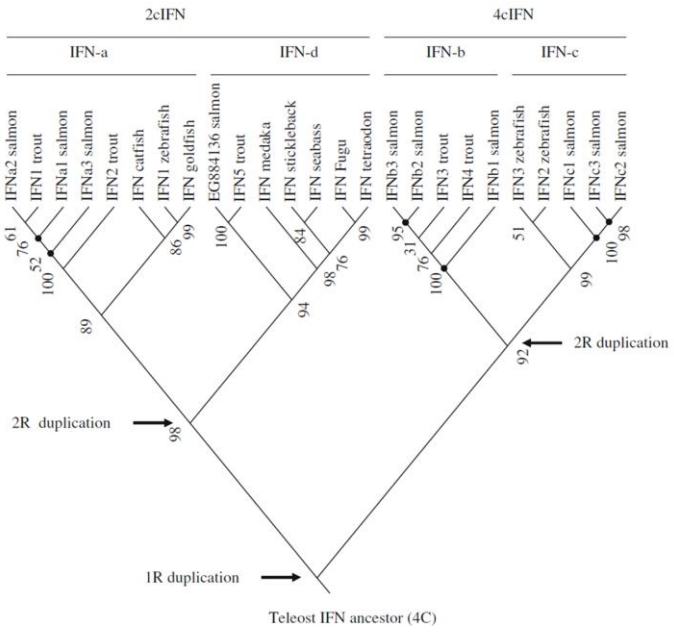


**Fig. 1. Classification and phylogenetic relationships of a selection of fish type I IFNs.** The figure was originally published as Fig. 3 in [17], and is reproduced with kind permission from Springer Science and Business Media ©. 2cIFN: interferons with two conserved cysteine residues, also called group I IFNs; 4cIFN, interferons with four conserved cysteine residues, also called group II IFNs.

A total of 13 type I IFN genes have been reported in Atlantic salmon, and eleven of these are found in the same genomic cluster [10, 17, 18]. For comparison, five type I IFN genes have been identified in rainbow trout [14, 17, 19], four in zebrafish [11, 12, 14, 20, 21] and one in green spotted puffer [11]. Group II IFNs have only been found in Atlantic salmon, rainbow trout and zebrafish so far, and these species belong to the superorders *Protacanthopterygii* and *Ostariophysi* [17]. Atlantic salmon is (at present) the only species where IFNs of all four subgroups (IFNa, IFNb, IFNc and IFNd) have been reported [17, 18]. Fish belonging to the superorder *Acanthopterygii* such as medaka (*Oryzias latipes*),

stickleback (*Gasterosteus aculeatus*) and green spotted puffer seem to only have IFNs of the group I IFNd subgroup [17, 22]. Table I lists the type I IFN identified in Atlantic salmon, rainbow trout, zebrafish and green spotted puffer and states the most common naming of these IFNs in the literature.

**Table I: Classification and naming of type I IFNs from Atlantic salmon, rainbow trout, zebrafish and green spotted puffer**

| | | **Group I** | | **Group II** | |
|---|---|---|---|---|---|
| **Superorder** | **Species** | **IFNa** | **IFNd** | **IFNb** | **IFNc** |
| **Protacanthopterygii** | **Atlantic salmon** | IFNa1<br>IFNa2<br>IFNa3 | IFNd | IFNb1<br>IFNb2<br>IFNb3<br>IFNb4 | IFNc1<br>IFNc2<br>IFNc3<br>IFNc4<br>IFNc5 |
| **Protacanthopterygii** | **Rainbow trout** | IFN1<br>IFN2 | IFN5 | IFN3<br>IFN4 | - |
| **Ostariophysi** | **Zebrafish** | IFNΦ1 | IFNΦ4 | - | IFNΦ2<br>IFNΦ3 |
| **Acanthopterygii** | **Green spotted puffer** | - | IFN | - | - |

-: IFN subtype not identified in this species

Since the discovery of fish virus-induced IFNs, there has been a debate as to whether these are homologs of mammalian type I or type III IFNs. On the one hand, the amino acid sequences of fish type I IFNs are more similar to mammalian type I IFNs than to type III IFNs [4]. The Atlantic salmon IFNa1 protein for example displays 27% and 20% sequence identity to human IFNα2b and IFNλ1, respectively [4]. On the other hand, the fish type I IFN genes contain four introns and the gene structure hence resemble that of mammalian type III IFNs [10, 11]. In addition, the structure of zebrafish IFN receptors is somewhat more similar to mammalian type III IFN receptors [21, 23]. Recent studies have, however, revealed that both type I and type III genes from amphibians (*Xenopus tropicalis*) contain introns, which implies

that the type I IFN ancestor gene contained introns [24]. Finally, when the crystal structures of zebrafish IFNΦ1 (IFNa subgroup) and IFNΦ2 (IFNc subgroup) were resolved in 2011, it became clear that both IFNs belong to the type I IFN group [25]. To date, no type III IFNs have been described in fish. Type II IFNs have been identified in several fish species [26], but since they are not the topic of this thesis they will not be further discussed. At the onset of this project, antiviral activity of type I IFNs from fish had only been demonstrated for the IFNa subgroup [4].

### 1.2 Type I interferons

Type I IFNs play a critical role in the innate antiviral immunity. They are produced and secreted by host cells in response to recognition of viral (and bacterial) infections by various PRRs. The IFN-receptor (IFNAR1/IFNAR2) is widely expressed on the surface of most cells, and activation of the IFN-receptor triggers a signaling cascade that results in induction of several hundred ISGs where many encode proteins with indirect or direct antiviral activity. Together these antiviral effectors establish an antiviral state in the cells which helps to limit viral replication and spread. The type I IFNs are multifunctional cytokines, and in addition to their antiviral effects they display antiproliferative and immunomodulatory activities [27]. As illustrated in Fig. 2, the production and antiviral function of type I IFNs can be divided into three steps, which will be discussed in detail in the following sections:

- Step I: Induction of type I IFNs following recognition of viruses
- Step II: IFN-mediated signaling
- Step III: The antiviral state

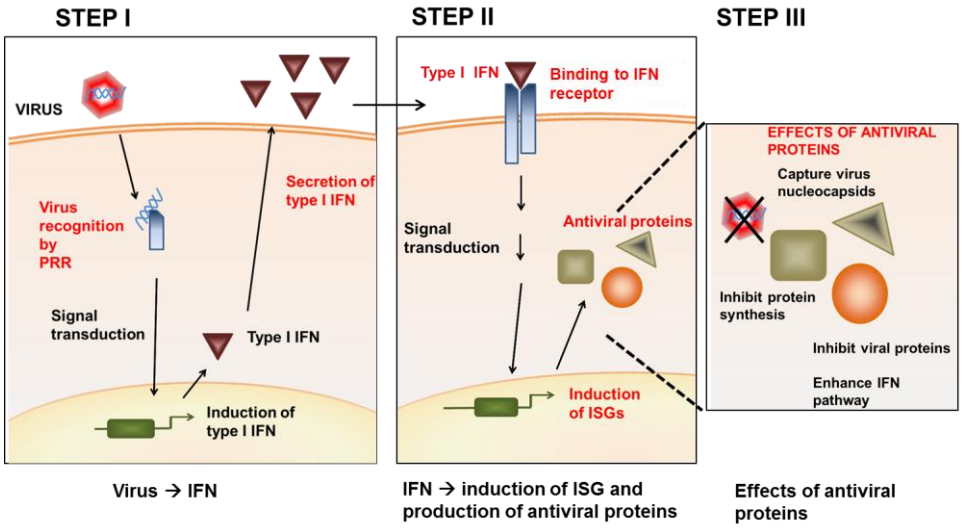


**Fig 2. Schematic overview of the IFN-circuit.** Step I: Recognition of virus by host pathogen recognition receptors (PRR) leads to signal transduction that culminates in the induction of type I IFN gene expression. Following protein synthesis, type I IFNs are secreted. Step II: Type I IFNs bind the IFN-receptor on the surface of the same or nearby cells. This start a signal transduction cascade that leads to the induction of interferon stimulated genes. Some of these genes encode proteins with antiviral properties. Step III. The antiviral proteins inhibit virus propagation by different mechanisms, these include (but are not limited to) capturing of viral nucleocapsids, inhibition of viral and cellular protein synthesis, inhibition of viral proteins and positive regulation of the IFN pathway. Collectively the antiviral proteins induce an antiviral state in the cell.

### 1.2.1 Step I: Induction of type I interferons following recognition of viruses

Type I IFNs can be secreted by all nucleated cells shortly after detection of virus. IFNα/β is produced in response to activation of several different receptors, but the transcription factor families that activate the IFNα/β promoters are in general shared. In the following two sections the transcription factors and the IFN promoter regions will thus be described in detail prior to reviewing the PRRs involved in recognition of viral nucleic acid.

#### Transcription factors involved in induction of type I IFN

**Mammals.** Three families of transcription factors are involved in the induction of type I IFNs: the interferon regulatory transcription factor (IRF) family, nuclear factor kappa B (NF-

κB) and c-jun/ATF2 (Fig. 3). IRF3 and IRF7 are considered to be the main regulators of IFNα/β induction, whereas NF-κB together with c-jun/ATF2 enhance the induction of IFNβ and additionally induce genes that encode proinflammatory cytokines [28, 29]. Upon activation of the correct PRR, IRF3 and IRF7, which are present in the cytosol in a latent form, are phosphorylated followed by dimerization and transport to the nucleus [30-32]. NF-κB is indirectly activated through removal of its inhibitor (IκB; inhibitor of kappa B). In unstimulated cells IκB blocks the nuclear translocation signal of NF-κB and the transcription factor is thus retained in the cytosol. Following phosphorylation of IκB, the inhibitor is ubiquitinylated and degraded by the proteasome, which frees NF-κB for translocation to the nucleus [33]. Activation of c-jun/ATF2 is controlled by the mitogen-activated protein kinase (MAPK) pathway [34]. In the nucleus, the IRFs, NF-κB and c-jun/ATF2 bind the promoter region of IFN genes to induce transcription (Fig. 3).

Expression of IFNα/β occurs in two waves in most tissues where IFNβ together with IFNα1 (mice) or IFNα4 (humans) are induced early after virus infection, followed by a second wave of the full range of IFNα subtypes [35]. The biphasic nature of IFNα/β induction is a consequence of differential expression of IRF3 and IRF7. More specifically, activated IRF3 together with activated NF-κB and c-jun/ATF2 induce the first wave of IFNβ (and IFNα1/4) [29, 30, 35]. IRF7, which is weakly expressed in most cell types, also participates in induction of the first-phase IFNs [36]. The second wave of IFN production is caused by a positive feedback system where IRF7 is upregulated by the newly synthesized IFNs [35]. Activated IRF7 promotes the expression of the full repertoire of IFNαs after transport to the nucleus [35]. Other IRFs have additionally been implicated in the induction of IFNα/β, but these IRFs seem to be dispensable for IFN-induction [36].

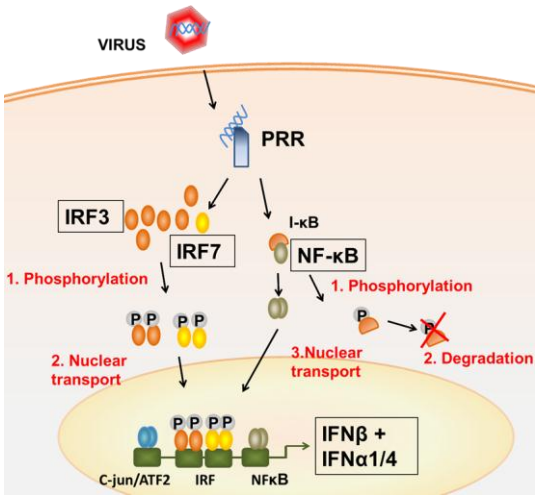


**Fig. 3. Activation of the transcription factors involved in induction of type I IFNs**. Prior to recognition of virus by PRRs, IRF3 and IRF7 are resting in the cytoplasm in a latent state. Binding of viral nucleic acid to PRRs leads to the activation of kinases that phosphorylates the IRFs. This leads to dimerization and translocation to the nucleus. NF-κB is activated by phosphorylation of its inhibitor (IκB), which is degraded by the proteasome. C-jun/ATF2 is activated through the MAPK-pathway (not shown).

**Fish.** IRF orthologs have been identified in fish [26]. Like their mammalian counterparts, IRF3 and IRF7 from rainbow trout, goldfish (*Carassius auratus*) and orange-spotted grouper (*Epinephelus coioides*) translocate to the nucleus after virus infection and/or stimulation with synthetic dsRNA (poly (I:C)) [37-39]. IRF3 from goldfish has furthermore been confirmed to be phosphorylated after poly (I:C) stimulation [38]. Overexpression of IRF3 and IRF7 leads to activation of type I IFN promoters and/or IFN gene transcription in several fish species including Atlantic salmon [38-43]. There are, however, some differences between the piscine and mammalian IRF system; in contrast to the static expression of mammalian IRF3s, IRF3 from several fish species is induced by type I IFN, virus infection and poly (I:C) [37, 38, 40, 44]. In addition, IRF3 from goldfish can be activated (phosphorylated) by recombinant IFN, whereas mammalian IRF3 is exclusively activated by virus infection or viral mimics [38].

### Type I interferon promoter regions

**Mammals.** The employment of different transcription factors for the induction of IFNβ and IFNαs is reflected in their promoter regions. While the IFNβ promoter contains two IRF

binding sites and one binding site each for NF-κB and c-jun/ATF2, the IFNα promoters do not contain NF-κB binding domains but have multiple IRF binding sites [45, 46].

**Fish.** Fish type I IFN genes also display differences in their promoter regions. The promoter of IFNa genes from several fish species resemble that of mammalian IFNβ containing predicted NF-κB, IRF, and in some cases c-jun/ATF2-binding motifs in the promoter proximal regions [17-19, 38, 47, 48]. By contrast, the promoter regions of group II IFN genes (i.e. IFNb and IFNc subgroups) from Atlantic salmon possess IRF binding motifs but not NF-κB motifs in the promoter proximal region (up to -500 nt), thus resembling the human IFNα promoter [18].

### Pathogen recognition receptors (PRRs)

The detection of virus and the subsequent production of IFN are mediated by cellular PRRs. A collective trait of the PRRs is that they recognize pathogens via conserved microbial traits named pathogen associated molecular patterns (PAMPs). The most common viral PAMPs are different forms of nucleic acids (RNA and DNA). Two key groups of PRRs are involved in sensing nucleic acid PAMPs: cytosolic RIG-I-like receptors (RLRs) which recognize RNA, and membrane-bound TLRs which respond to RNA and DNA [49]. The following sections will focus on the description of the nucleic acid binding RLRs and TLRs and their signaling pathways.

### RIG-I-like receptors (RLRs)

**Mammals.** The RLRs represent a group of cytosolic viral sensors that includes RIG-I, MDA5 and laboratory of genetics and physiology 2 (LGP2) [50]. All three members contain a DEDxD/H box helicase domain and a C-terminal domain responsible for RNA binding. RIG-I

and MDA5 additionally hold two N-terminal caspase activation and recruitment (CARD) domains which are required for signaling transduction (see below) [51]. LGP2 was initially proposed to be a negative regulator of RIG-I through sequestration of RNA [52-54], but later studies have uncovered that LGP2 in certain cases positively regulates the RLR pathway [55-57]. LGP2 also plays a role in regulation of the adaptive immune response [57].

Signaling through RIG-I is initiated by recognition of RNA in the form of short dsRNA, or uncapped ssRNA containing a 5`-triphosphate group [58-60], whilst MDA5-signaling is mainly elicited through recognition of long stretches of dsRNA (≥2 kbp) [61]. RNA polymerase III can synthesize 5`-triphosphate RNA from cytosolic DNA, and RIG-I can thus indirectly also respond to DNA [62, 63]. The choice of ligands efficiently discriminates between self- and non-self RNA: dsRNA is in general not found in the cytosol of healthy cells but is a common intermediate of viral replication, whereas the 5`-triphosphate group of cellular RNA is normally capped (mRNA) or removed (tRNA and rRNA) in the nucleus prior to cytosolic translocation [64]. In addition to the natural viral ligands, synthetic *in vitro* transcribed 5`-triphosphate dsRNA can be used to trigger RIG-I signaling, whilst synthetic dsRNA in the form poly (I:C) predominantly activates MDA5 [65, 66].

**Fish.** The RLR pathway seems to be well conserved across vertebrate species [67], and in the last few years RIG-I, MDA5 and LGP2 have been cloned from various fish species [68-73]. RIG-I has also been cloned from Atlantic salmon [68], and MDA5 and LGP2 from rainbow trout have been demonstrated to bind poly (I:C) [70].

### RLR signaling

**Mammals.** Following binding of RNA ligands, RIG-I and MDA5 associate with the adaptor protein IPS-1 (also known as MAVS, CARDIF and VISA) through CARD-CARD interactions (Fig. 4) [74-77]. IPS-1 is linked to the mitochondria through a C-terminal transmembrane domain, a localization that is critical for signal transduction [76]. The signaling downstream of IPS-1 is not fully characterized, but some important proteins include TRADD (tumor necrosis factor receptor type 1-associated DEATH domain), TANK (TRAF-family member associated NF-κB activator), TRAF3 (tumor necrosis factor receptor-associated factor 3), TRAF6, RIP1 (receptor interacting protein 1), FADD (fas-associated death domain) and NEMO (NF-κB modulator) [74, 77-84]. Recruitment of these proteins by IPS-1 activates the two kinases TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε; also known as IKKi) which phosphorylate and activate IRF3 and IRF7 [85]. The IKK complex (IKKα, IKKβ and NEMO) is also activated by the IPS-1 signaling complex, and IKK is in turn responsible for activation NF-κB through phosphorylation of its inhibitor IκB [86]. Once activated, IRF3, IRF7 and NF-κB translocate to the nucleus where they turn on the transcription of genes encoding IFNα/β and proinflammatory cytokines. Recent studies have uncovered that STING (Stimulator of IFN genes; also known as MITA), which is a transmembrane protein linked to the endoplasmic reticulum (ER), also plays an important role in the RIG-I pathway [87, 88]. Fig. 4 shows a simplified schematic illustration of the RIG-I/MDA5 signaling pathway.

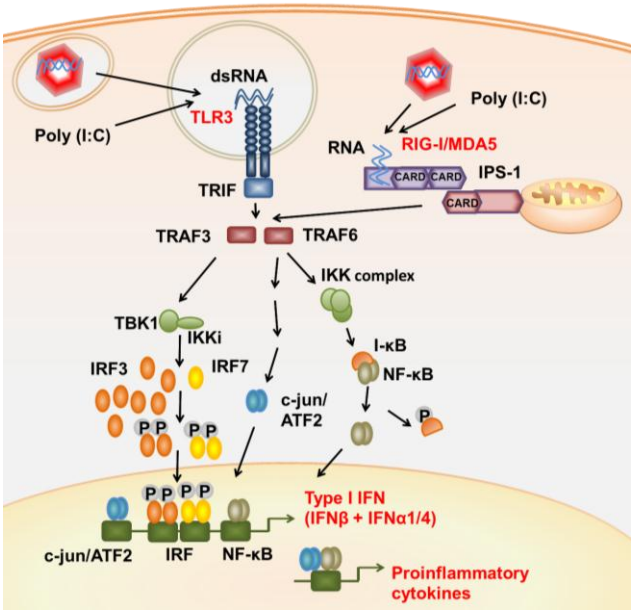


**Fig 4. RLR and TLR3 signaling. RIG-I and MDA5** are RNA sensors which are expressed in the cytosol. Upon substrate binding the CARD domains of RIG-I and MDA5 interact with the CARD domain of the mitochondria-linked adaptor protein IPS-1. Interaction with the adaptor protein leads to a signaling cascade with two main outcomes: (I) activation of TBK1 via TRAF3 and subsequent phosphorylation of IRF3 and IRF7 and (II) activation of the IKK complex through TRAF6 followed by phosphorylation of the inhibitor of NF-κB (I-κB). C-jun/ATF2 activation is also mediated through TRAF6. **TLR3** is normally present in the endosomes and interacts with TRIF following activation by dsRNA. TRIF in turn interacts with TRAF3 and TRAF6 and the subsequent signaling steps converge with that of the RLRs. The transcription factors IRF3, IRF7, NF-κB and c-jun/ATF2 translocates to the nucleus where they induce gene transcription of type I IFN (IFNβ and IFNα1/4) and proinflammatory cytokines. The IFNα family is induced following upregulation of IRF7 by newly synthesized IFN (not shown).

**Fish.** There have been published several studies on the RLR signaling pathway of fish in the last few years [22]. Paper II focuses on IPS-1 from Atlantic salmon, and the fish RLR signaling pathway will hence be examined in the general discussion (Chapter 4).

### Toll-like receptors (TLRs)

**Mammals.** Toll receptors were first identified in *Drosophila*, but homologs have later been identified in numerous species where they are named Toll-*like* receptors. The TLRs comprise a family of membrane-bound proteins which respond to pathogen-recognition by inducing various cytokines and promoting innate defense mechanisms. In terms of structure, the TLRs

consist of an N-terminal leucine-rich repeat (LRR) domain which recognizes PAMPs, a transmembrane domain that links the proteins to the plasma membrane or to endosomal membranes, and a Toll/IL-1 receptor (TIR) domain which is responsible for downstream signaling [89].

Collectively, 13 different TLRs have been identified in mammals. Four of these (TLR3, TLR7, TLR8 and TLR9) are well known to specifically induce type I IFNs and proinflammatory cytokines in response to recognition of viral nucleic acids [90]. Murine TLR13 has recently been shown to induce type I IFNs after recognition of a yet uncharacterized viral PAMP [91] and by bacterial RNA [92-94].

TLR3, TLR7, TLR8 and TLR9 are predominantly expressed in endosomal compartments, a characteristic that is thought to limit the recognition of self nucleic acids [95]. In healthy individuals host DNA and RNA are exclusively found in the nucleus or cytoplasm where they are unavailable for interaction with the TLRs. Viral nucleic acids, on the other hand, can reach the endosomes after viral entry by endocytosis, phagocytosis of apoptotic virus infected cells or autophagy-mediated engulfment of cytoplasmic material [89, 96, 97].

The viral nucleic PAMPs recognized by the TLRs are dsRNA (TLR3), ssRNA (TLR7/8) and unmethylated DNA containing cytosine-guanine (CpG) motifs (TLR9) [98]. TLR7 and TLR8 are closely related, and in humans both receptors react to ssRNA and synthetic analogs thereof (e.g. imidazoquinolines such as R848) [99-103]. However, only TLR7 responds to the synthetic ligand imiquimod (R837) [104]. In addition to being a substrate for MDA5, poly (I:C) can also be recognized by TLR3 [105].

**Fish.** At least 17 different TLRs have been identified collectively in fish, including homologs of the mammalian nucleic acid sensing TLRs (i.e. TLR3 and TLR7-9) in addition to some fish

specific TLRs such as TLR21 and TLR22 [106]. Structure-wise, the fish TLRs are conserved and resemble their orthologs from other species [26]. TLR22 has been identified in several fish species [107-112], and has in fugu (*Takifugu rubripes*) been demonstrated to bind dsRNA and activate type I IFN gene induction [112]. The spatial organization of some of the fish TLRs has been studied. Fugu TLR3 is found close to the ER in unstimulated fish [112], whereas rainbow trout TLR3 seems to require endosomal localization to be functional [113]. TLR22 from fugu is embedded in the plasma membrane and might functionally resemble mammalian TLR3, which is occasionally found at the cell surface [112].

### TLR3 (and TLR22) signal transduction

**Mammals**. Signal transduction by TLR3 (Fig. 4) is dependent on the adaptor protein TIR-domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM1) [114-116]. Both TLR3 and TRIF contain TIR domains and following ligand binding, the two proteins associate through TIR-TIR interactions [116]. To activate NF-κB, TRIF recruits TRAF6 which in a multistep process facilitates the activation of the IKK complex responsible for phosphorylation of IκB and subsequent activation of NF-κB [86, 117, 118]. TRAF6 is also involved in the stimulation of the MAPK pathway that activates c-jun/ATF2 [119]. Phosphorylation of IRF3 and IRF7 are mediated by TBK1 and IKKε, two kinases which are activated after recruitment of TRAF3 by TRIF [78, 85, 120]. Following phosphorylation, the IRFs translocate to the nucleus to activate transcription of IFNα/β.

**Fish.** TLR3 homologues have been identified in a variety of fish species including fugu, zebrafish and rainbow trout [112, 121, 122]. Like mammalian TLR3, fugu and zebrafish TLR3 have been shown to interact with TRIF [112, 123]. Zebrafish TRIF has further been

demonstrated to interact with the downstream kinase TBK1 [123] and to activate the NF-κB- and type I IFN-promoters [123, 124]. Although TRAF6 from zebrafish is involved in stimulation of the NF-κB promoter [121, 124], the protein does not seem to interact with TRIF [123]. Similar to TLR3, TLR22-mediated signaling is dependent on the adaptor protein TRIF [112].

### TLR7 and TLR9 signal transduction

**Mammals.** TLR7, TLR8 and TLR9 signal through the adaptor protein myeloid differentiation primary response gene 88 (Myd88). pDCs express particular high levels of TLR7 and TLR9, and in these cells, activation of the receptors leads to expression of particular large amounts of IFNα (but also IFNβ) [125]. Upon ligand binding, TLR7 and TLR9 interact with Myd88 followed by recruitment of IL-1 receptor-associated kinase (IRAK)-4, IRAK-1 and TRAF6 [64, 126]. IRF7, which is constitutively expressed at a high level in pDCs [127], interacts with Myd88 and TRAF6, and is subsequently phosphorylated by IRAK-1 [128-130]. Activated IRF7 travels to the nucleus where it preferentially induces the IFNα subtypes [31, 131]. Unlike other cell types, pDCs can hence rapidly induce high amounts of IFNα without the need for initial upregulation of IRF7. Fig.5 displays the TLR7 and TLR9 signaling pathways in pDCs. TLR8 is mainly found in conventional DCs (cDCs) and monocytes where it upon activation mainly induces proinflammatory cytokines and chemokines [132].

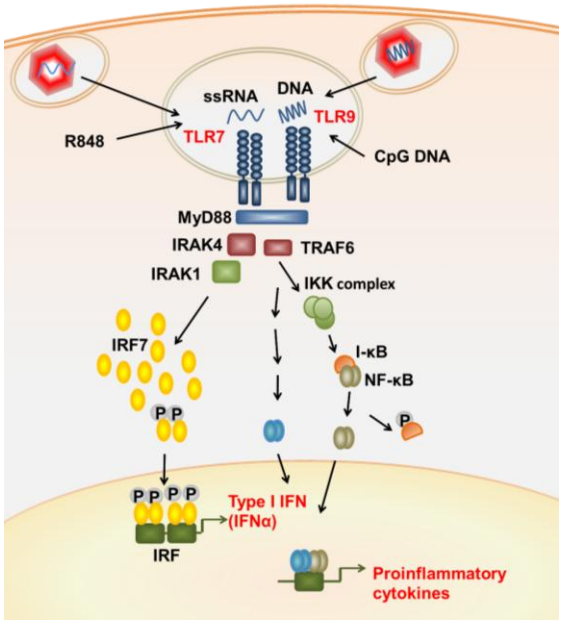


**Fig 5. Schematic simplified overview of TLR7/TLR9 signaling.** TLR7 and TLR9 are predominantly expressed in pDCs, and in these cells recognition of ligands (dsRNA and CpG DNA, respectively) leads to a rapid production of high levels of IFNα. Activated TLR7 and TLR9 interact with the adaptor protein MyD88 which in turn recruits signaling molecules including IRAK-1, IRAK-4 and TRAF6. This signaling complex mediates phosphorylation of IRF7. IRF7 shows a high constitutive expression in pDCs, a trait that aids the rapid induction of the IFNα subfamily. Proinflammatory cytokines are additionally induced in a process that involves the IKK complex.

**Fish.** MyD88 has been identified in fish and the structure is well conserved [133]. The MyD88-dependent TLR signaling pathway seems to be similar to that of mammals as overexpression of zebrafish MyD88 activates the human NF-κB and IFNβ promoters [134], whereas overexpression of Atlantic salmon MyD88 activates the salmon IFNa1 promoter in addition to a human NF-κB promoter [43, 135]. Atlantic salmon MyD88 has also been shown to be induced in leukocytes stimulated with CpG DNA (TLR9 ligand) [43]. Homologs of several IRAK sequences have further been identified in fish, including IRAK-1 from mandarin fish (*Siniperca chuatsi)* and grass carp (*Ctenopharyngodon idellus*) [136, 137] and IRAK-4 from zebrafish and tongue sole (*Cynoglossus semilaevis*) [121, 138].

### Cell-dependent expression of PRRs

**Mammals.** The RLR system is the key surveillance system responsible for inducing type I IFN in response to recognition of viral nucleic acids in most cell types including fibroblasts, macrophages and cDCs [139]. In these cells, the typical biphasic expression of type I IFNs is observed, with initial high levels of IFNβ and IFNα1/4 and a subsequent induction of the whole range of IFNα subtypes following IRF7 upregulation. TLR3 is also widely expressed in different cell types including cDCs, fibroblasts, epithelial cells and cells of the central nervous system [140]. By contrast, TLR7, TLR8 and TLR9 expression appears to be restricted to immune cells such as monocytes, DCs and B-cells [49, 89].

pDCs show a high expression of TLR7 and TLR9, but do not express other TLRs [141-143]. These cells are well known for their ability to rapidly produce vast amounts of IFNα (and IFNβ) following virus recognition by TLR7 or TLR9, and are considered to be the main producers of IFNα/β during viral infections [144]. The pDCs do not seem to use the RLRs for recognition of viral nucleic acid and induction of IFNs [139, 144-146]. The particular ability of pDCs to rapidly induce large amounts of IFNα is partially caused by a high basal level of IRF7 [127, 147]. Additionally, a unique mode of TLR ligand trafficking seems to contribute to the high IFN-producing capacity of pDCs; while certain TLR ligands are retained in the endosomes of pDCs for an extended period of time, other DCs rapidly shuttle the same ligands to the lysosomes where they cannot promote IFN production [148]. Fig. 6 illustrates the cell-dependent expression of TLRs and RLRs, and induction of IFN.

**Fish.** Like in mammals, fish MDA5, RIG-I and LGP2 have been found to be widely expressed in various tissues [69, 73, 149-151], which implies that these receptors operates in many cell types. Studies in rainbow trout and Atlantic salmon suggest that TLR7 has a more restricted expression: the transcript levels of TLR7 are higher in rainbow trout spleen and

head kidney than in other organs [113], whereas TLR7 transcripts are undetectable in Atlantic salmon TO cells, but present in Atlantic salmon head kidney leukocytes [18]. Dendritic-like cells have recently been characterized in rainbow trout and zebrafish [152, 153], but it is still an open question if fish have cells that resembles pDCs.

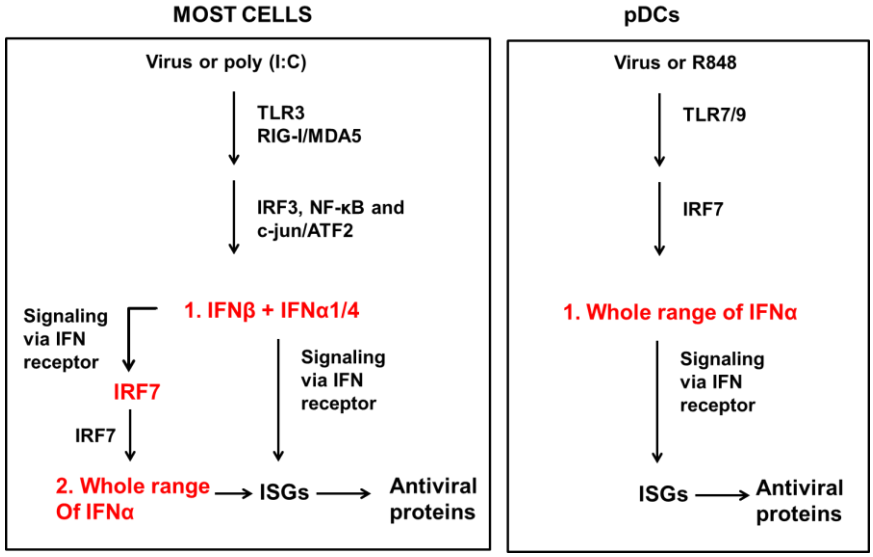


**Fig. 6. Cell-dependent expression of TLRs and RLRs, and induction of IFN.** In most mammalian cell types virus is recognized by TLR3 or RIG-I/MDA5. In these cells, IRF3 is strongly expressed and available in a latent form in the cytosol. IRF7 is weakly expressed (not shown). Binding of poly (I:C) or viral nucleic acid to TLR3/RIG-I/MDA5 leads to activation of IRF3, IRF7, NF-κB and c-jun/ATF2 which induce the first wave of IFNβ and IFNα1/4. The remaining IFNα subtypes are induced following upregulation of IRF7 by newly synthesized IFNβ and IFNα1/4. pDCs express unusual high levels of IRF7. In these cells, the whole family of IFNα subtypes and IFNβ (not shown) is rapidly produced upon activation of TLR7 or TLR9 without the need for IFN-priming.

### 1.2.2 Step II: Interferon-mediated signaling

#### Type I interferon receptors

**Mammals.** In mammals, all the type I IFNs signal through a heterodimeric receptor composed of two proteins named IFNAR1 and IFNAR2 [154].

**Fish.** In contrast to the mammalian type I IFNs, fish type I IFNs seem to exert their function through (at least) two different receptors. Studies in zebrafish have uncovered that IFNΦ1 (IFNa subgroup) and IFNΦ4 (IFNd subgroup) of group I IFNs utilize a receptor composed of cytokine receptor family B (CRFB)1 and CRFB5, while IFNΦ2 (IFNc subgroup) and IFNΦ3 (IFNc subgroup) of the group II IFNs signal through a heterodimer composed of CRFB2 and CRFB5 [21, 23]. So far, no functional studies on type I IFN receptors from other fish species have been published. The differential receptor usage of the human and zebrafish type I IFNs is illustrated in Fig. 7.

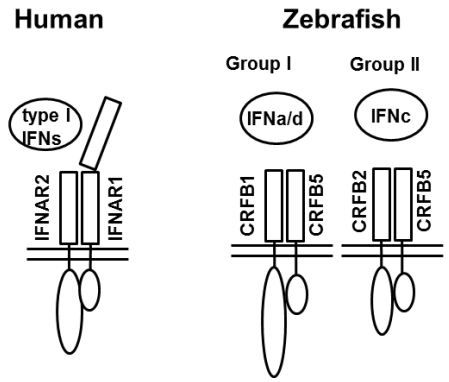


**Fig. 7. Receptor usage of human and zebrafish type I IFN**. All the type I IFNs of mammals signal through one shared receptor composed of IFNAR1 and IFNAR2. In zebrafish, IFNΦ1 and IFNΦ4 (i.e. group I IFNs of IFNa and IFNd subtype, respectively) and IFNΦ2 and IFNΦ3 (i.e. group II IFNs of IFNc subtype) signal through CRFB1/CRFB5 and CRFB2/CRFB5, respectively. The figure is inspired by [21].

### IFN-mediated signaling (JAK-STAT pathway)

**Mammals.** The IFN-receptor is widely expressed, and most cells are thus susceptible for the antiviral action of type I IFNs [154]. Recognition of IFN by the IFNAR heterodimer leads to signaling via the janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway, which results in the upregulation of numerous ISGs in the given cell [155]. Microarray analyses show that more than 1600 genes can be regulated by type I IFN, albeit only a few of the proteins encoded by these genes display direct antiviral effects [156].

As illustrated in Fig. 8, binding of type I IFN to its receptor leads to phosphorylation of the intracellular regions of IFNAR1 and IFNAR2 by two receptor-associated kinases named tyrosine kinase 2 (TYK2) and JAK1. Subsequently, the transcription factors STAT1 and STAT2 are recruited and subjected to phosphorylation by JAK1 and TYK2. This modification leads to dimerization of STAT1 and STAT2 which in turn interacts with IRF9 to form a complex known as interferon stimulated gene factor 3 (ISGF3). Following nuclear translocation, ISGF3 binds interferon-stimulated response elements (ISREs) present in the promoters of ISGs to initiate their transcription. Some of these ISGs encode antiviral proteins [155].

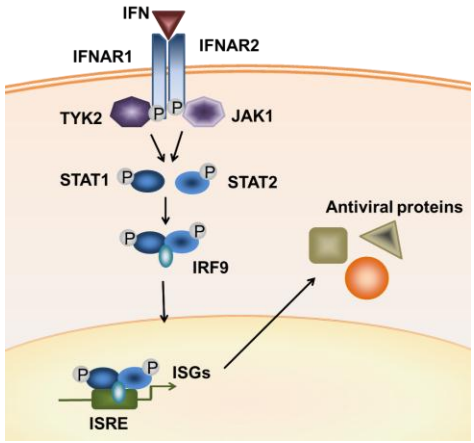


**Fig. 8: Type I IFN-signaling through the JAK/STAT pathway**. Upon binding of IFN to IFNAR1/IFNAR2 the receptor is phosphorylated by TYK2 and JAK1, which in turn also phosphorylates STAT1 and STAT2. These two transcription factors associates with IRF9 and forms the ISGF3 complex which binds the promoter regions of ISGs to elicit their transcription. Some ISGs encode antiviral proteins.

**Fish.** All the components of the IFN-signaling pathway including TYK2, JAK1, STAT1, STAT2 and IRF9 have collectively been identified in fish [20]. Two studies in goldfish indicate that the IFN-signaling pathway is conserved between fish and higher vertebrates. First, goldfish IRF9 and STAT1 have been shown to individually induce ISRE-elements and ISG transcription upon overexpression [157, 158]. Second, overexpressed goldfish STAT2 displays a synergistic effect on the IRF9-mediated ISG-induction [157]. STAT1 homologs

from several other fish species including zebrafish and Atlantic salmon have been studied [159-161], and Atlantic salmon STAT1 has been shown to be subjected to phosphorylation, to dimerize and to translocate to the nucleus in response to IFNa1-treatment [160]. A STAT2 ortholog has also been identified in Atlantic salmon [162]. Unlike mammalian type I IFN, some fish type I IFNs are themselves ISGs, meaning that they are upregulated following IFN-stimulation [17, 38, 163]

### 1.2.3 Step III: The antiviral state

Of the many genes upregulated by type I IFN, some encode proteins with direct antiviral activities. Many of these antiviral proteins can individually inhibit one or several stages of the viral life cycle. Collectively they promote a cellular antiviral state that is dedicated to fighting viral attacks. Interferon-stimulated gene 15 (ISG15), Mx and double stranded RNA-dependent protein kinase (PKR) represent some of the best characterized antiviral effectors identified in both fish and mammals, and these will thus be described in the following sections.

#### ISG15

**Mammals.** ISG15 is a small IFN-inducible protein of ~15 kDa composed of two ubiquitin-like (UBL) domains and a conserved LRLRGG sequence in the C-terminal [164]. The antiviral role of ISG15 has been confirmed in ISG15$^{-/-}$ knockout mice, which are more susceptible to RNA and DNA viruses such as Influenza A and Herpes Simplex Virus than wild type mice [165].

ISG15 is an ubiquitin-like protein that can conjugate to lysine residues of other proteins in a process called ISGylation. The cellular proteins targeted for ISGylation are diverse and includes protein involved a range of cellular processes [164]. The functional consequences of protein modification by ISGylation have been determined for some

individual proteins. Of the proteins involved in the IFN induction pathways, ISGylation increases the half-life of IRF3 by preventing ubiquitination and subsequent degradation [166, 167], whereas ISGylation negatively regulates RIG-I [168]. Some viral proteins are also targeted by ISGylation. For instance, the replication of influenza A virus is inhibited by ISGylation of the IFN-antagonistic NS1A protein [169, 170]. Certain viruses have evolved mechanisms to evade or block ISG15, and the NS1 protein of influenza B viruses has for example been shown to inhibit ISGylation by binding to ISG15 [171].

**Fish.** ISG15 homologs have been identified in a variety of fish species including goldfish, rainbow trout, black rockfish (*Sebastes schlegeli*), channel catfish (*Ictalurus punctatus*), Japanese flounder (*Paralichthys olivaceus*), Atlantic salmon, Atlantic cod (*Gadus morhua*) , tongue sole and red drum (*Sciaenops ocellatus*) [172-182]. The fish ISG15 homologs share many similarities with the mammalian counterparts. On the genetic level, the promoter regions of goldfish, Atlantic cod and Japanese flounder ISG15 have been confirmed to contain ISRE elements [172, 177, 179] and ISG15 is accordingly induced by viruses, poly (I:C) and type I IFN in many fish species [173-176, 178-182]. The overall protein structure is conserved with two UBL domains and a C-terminal LRGG sequence [172, 174, 175, 177-182] and ISG15 homologs from goldfish, Atlantic salmon and Atlantic cod have been confirmed to conjugate to an array of proteins both *in vitro* and *in vivo* [172, 178, 180].

### MX

**Mammals.** Mx proteins belong to the family of high-molecular-weight dynamin-like GTPases [183]. Two Mx proteins are expressed in humans, MxA and MxB, but only the former has been shown to have antiviral activity [183, 184]. MxA is localized to the smooth ER where it forms large aggregates that increase the half-life of the protein [185-187]. The

protein is produced following type I and type III IFN-stimulation and shows broad antiviral activity against a range of RNA viruses and a few DNA viruses including viruses replicating in the cytosol and in the nucleus [188, 189].

The antiviral mechanism of MxA is not fully determined, but a current hypothesis is that MxA inhibits viral propagation by forming oligomeric ring structures around the viral nucleocapsids (i.e. viral genome and associated proteins) to block their function [189, 190]. MxA has been shown to inhibit transport of thogotovirus nucleocapsids from the cytoplasm to the nucleus [191], and to bind nucleocapsid proteins of Influenza A virus and LaCrosse virus [192, 193]. MxA has additionally been suggested to modify host responses, exemplified by a recent study that showed interaction between MxA and two cellular helicases needed for efficient replication of influenza virus [194].

Two Mx proteins with antiviral effects have been identified in mice where Mx1 shows a nuclear expression while Mx2 is expressed in the cytosol [186]. Mx1 inhibits viruses replicating in the nucleus (i.e. influenza viruses), whereas Mx2 inhibits viruses replicating in the cytoplasm (i.e. vesicular stomatitis virus) [183].

**Fish.** The first fish Mx gene was cloned from common perch (*Perca fluviatilis*) more than 20 years ago [195]. Since then, Mx has been cloned and studied in a variety of fish species including rainbow trout, zebrafish and Atlantic salmon [196-198]. Three Mx-encoding cDNA sequences have been identified in Atlantic salmon (Mx1, Mx2 and Mx3) [197], where Mx1 has been shown to be induced by poly (I:C) and type I IFN, but not by LPS [10, 197, 199]. The salmon Mx1 protein is further localized to the cytoplasm and has been shown to confer antiviral activity against IPNV [200]. In agreement with the mode of action of mammalian Mx, Mx homologs from orange-spotted grouper and barramundi (*Lates calcarifer*) bind to nucleocapsid proteins of nodavirus [201, 202].

### PKR

**Mammals.** PKR is an IFN-induced serine threonine kinase composed of two N-terminal RNA binding motifs and a C-terminal kinase domain. PKR plays an antiviral role in the cell by regulating the translational machinery and thereby also the production of new virus proteins [203]. Recognition of dsRNA leads to activation of latent cytosolic PKR by dimerization and autophosphorylation, followed by inhibition of cellular and viral protein synthesis by PKR-mediated phosphorylation of the eukaryotic translation initiation factor 2A (eIF2α) [204, 205]. PKR additionally enhances IPS-1-mediated induction of IFNβ [206], and a recent study suggests that this can (at least partly) be attributed to PKR-mediated activation of NF-κB through inhibition of IκB protein synthesis [207].

The importance of PKR in antiviral immunity has been confirmed in $PKR^{-/-}$ mice, which are more vulnerable to vesicular stomatitis virus and influenza virus than wild-type mice [208, 209]. In addition to its antiviral role, PKR affects cellular processes such as apoptosis and cell growth [203].

**Fish.** PKR homologs have been cloned from several fish species including zebrafish, Japanese flounder, rock bream (*Oplegnathus fasciat*us) and goldfish [210-213]. Fish PKR seems to play a similar role as mammalian PKR, as overexpression of PKR from Japanese flounder and goldfish leads to phosphorylation of eIF2α and inhibition of virus replication [210, 213], while knockdown of PKR renders goldfish more susceptible to virus infection [213]. Prior to the identification of the fish PKR homologs, a PKR-like gene were the two dsRNA binding domains were replaced by two Zα domains (i.e. domains that bind DNA/RNA in the left-handed Z-conformation) was identified in goldfish [214]. An ortholog was subsequently found in zebrafish which was given the name PKZ (protein kinase containing Z-DNA binding domains) [215]. While Atlantic salmon PKZ has been shown to

phosphorylate eIF2α in response to Z-DNA and to inhibit protein translation [216], a study comparing PKR and PKZ from goldfish showed that both proteins can phosphorylate eIF2α and inhibit virus replication [213].

### 1.3 IPNV

IPN is a contagious disease that mainly affects salmonids. The disease has for many years caused considerable losses in the Norwegian salmonid farming industry and is still considered to be a threat with 154 registered outbreaks in 2011 [2]. The number of outbreaks and the IPNV-associated wastes seem to have declined the last few years, a trend that has partly been ascribed to the introduction of salmon breeding lines with increased resistance against IPNV and vaccine improvements [2].

IPN is caused by IPNV, which is a nonenveloped Aquabirnavirus that belongs to the *Birnaviridae* family. The virus has a bi-segmented genome of ~5kB which give rise to five viral proteins (VP1 – VP5) [217].

The virus is sensitive to the antiviral effectors induced by IFN. Pretreatment of salmonid cell lines with Atlantic salmon IFNa or constitutive expression of Atlantic salmon Mx1 inhibit the production of viral proteins and render cells less vulnerable to IPNV infection [10, 200, 218]. Although IPNV is sensitive to an already induced antiviral state, the virus has been shown to inhibit activation of the Mx promoter and production of Mx protein if cells are infected prior to treatment with Atlantic salmon IFNa1 [218]. IPNV thus seems to have evolved mechanisms to antagonize the induction of Mx1 and thereby possibly the JAK/STAT signaling pathway.

### 1.4 ISAV

Infectious salmon anemia (ISA) is a multisystemic disease that mainly affects farmed Atlantic salmon. The first ISA outbreak was observed in Norway in 1984, but the disease has later been detected in several countries including Canada, Scotland, the USA, Faroe Islands and Chile [219]. ISA caused large losses in the Atlantic salmon fish farming industry in Norway in the late 80s and early 90s with a peak of 80 reported incidents in 1990 [220]. From 2000 to 2011, there has been an average of 11 outbreaks per year and the disease now seems to be under reasonable control with only one registered outbreak in 2011 [2].

ISA is caused by an enveloped virus of the *Isavirus* genus called ISAV, which belongs to the same family as mammalian influenza and thogoto viruses (i.e. *Orthomyxoviridae*). The genome is composed of eight ssRNA segments of negative polarity which encodes at least 10 proteins [219].

ISAV has seemingly evolved strong mechanisms to counteract or evade the type I IFN system [221]. Although Atlantic salmon IFNa is highly induced in Atlantic salmon cell lines infected with ISAV, IFNa1 pretreatment gives little protection against ISAV-induced CPE [199, 221]. Two proteins encoded by segment 7 (s7ORF1) and segment 8 (s8ORF2) antagonize the poly (I:C)-triggered activation of the Atlantic salmon IFNa promoter [222]. s7ORF1 can additionally inhibit activation of the rainbow trout Mx promoter [223].

# 2. Aims of the study

A total of 13 type I IFN genes have been identified in the genome of Atlantic salmon and these IFNs are divided into four different subgroups: IFNa, IFNb, IFNc and IFNd [10, 17, 18]. The discovery of the multiple type I IFN genes raised the question as to why Atlantic salmon possess multiple type I IFNs and whether IFNs from the different subgroups play distinct antiviral roles in the fish. The overall aim of this project was to gain more knowledge about the distinct roles of IFNs of subgroup IFNa, IFNb, IFNc and IFNd in innate antiviral defense. To achieve this, we investigated (i) the antiviral activity of salmon IFNs against IPNV and ISAV, and (ii) the induction of the IFNs in cells and *in vivo* in response to poly (I:C) and R848, and in response to overexpression of Atlantic salmon IPS-1 (AsIPS-1) in CHSE cells. The specific aims were to:

* Produce an antibody against IFNa1 for use as a tool to study the Atlantic salmon IFNs (paper I)

* Examine the antiviral activity of IFNs of subgroup b, c and d against IPNV, and compare the antiviral potency of IFNs of subgroups a, b, c and d (paper III)

* Examine if the type I IFNs show antiviral activity against ISAV, and whether there are differences in potency among IFNs of subgroups a, b and c (paper IV)

* Determine whether IFNa1 is the main IFN produced by poly (I:C)-treated cells and if leukocytes can produce additional IFNs (paper I)

* Clone AsIPS-1 to study the RLR-pathway, and determine if IPS-1 is involved in induction of IFNa1/2 (paper II)

* Examine if IFNs of subgroup a, b, c and d are differentially induced by poly (I:C) and R848 in different cells and organs (paper III)

# 3. Abstracts of papers

**I: An antiserum against Atlantic salmon IFNa1 detects IFN and neutralizes antiviral activity produced by poly I: C stimulated cells.** Kristel Berg, Tina Svingerud, Baojian Sun, Børre Robertsen. Developmental & Comparative Immunology. Volume 33, Issue 4, April 2009, Pages 638-645.

Type I interferons (IFNs) play a crucial role in innate immune responses against virus infections in vertebrates. Two IFNs (IFNa1 and IFNa2) have previously been cloned from Atlantic salmon. In the present work a polyclonal antiserum, which was generated against salmon IFNa1 was used to study its production in cells by immunoblot detection and neutralization of antiviral activity. The antiserum was first confirmed to detect and neutralize the antiviral activity of recombinant salmon IFNa1 produced in HEK293 cells. The antiserum also detected IFNa1 and neutralized 95-98% of the antiviral activity in supernatants of poly I:C stimulated salmon TO cells. This suggests that IFNa1/IFNa2 are the major IFNs produced by poly I:C stimulated TO cells. The antiserum neutralized most of the IFN activity in poly I:C stimulated head kidney leucocytes from three of five individuals, but in stimulated leucocytes from the other two individuals only 75% of the antiviral activity was neutralized. This shows that although IFNa1/IFNa2 are major IFNs secreted by poly I:C stimulated leucocytes, these cells can also produce additional molecules with IFN-like activity.

**II: Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-κB and localizes to mitochondria.** Silje Lauksund, Tina Svingerud, Veronica Bergan, Børre Robertsen.Developmental & Comparative Immunology. Volume 33, Issue 11, November 2009, Pages 1196-1204.

The striking difference in evolution of type I IFN genes of fish and mammals poses the question of whether these genes are induced through similar or different signalling pathways in the two vertebrate groups. Previous work has shown that expression of both Atlantic salmon (Salmo salar) IFNa1 and mammalian IFN-beta genes is dependent on IRF and NF-kappaB elements in their promoters. In mammals, IFN-beta transcription is induced through the RIG-I/MDA5 pathway where the adaptor protein IPS-1 plays a key role in the signal transduction. In this work we show that an Atlantic salmon homologue of IPS-1 (AsIPS-1) mediates activation of the salmon IFNa1 promoter and an NF-kappaB driven promoter. AsIPS-1 shares only 18% identity in amino acid sequence with human IPS-1, but possesses the CARD, proline-rich and transmembrane domains found in mammalian IPS-1. Overexpression of AsIPS-1 resulted in induction of an antiviral state in the cells apparently due to induction of IFN. Deletion of the CARD and transmembrane domains of AsIPS-1 abolished its ability to activate the IFNa1 promoter and the NF-kappaB driven promoter, and thus its ability to induce an antiviral state. AsIPS-1 is located to mitochondria similar to human IPS-1. Taken together, IPS-1 plays a key role in the induction of Atlantic salmon IFNa1, which appears to be the first and major IFN induced in host cells upon recognition of viral dsRNA.

**III: Atlantic Salmon Type I IFN Subtypes Show Differences in Antiviral Activity and Cell-Dependent Expression: Evidence for High IFNb/IFNc–Producing Cells in Fish.** Tina Svingerud, Terese Solstad, Baojian Sun, May Liss J. Nyrud, Øyvind Kileng, Linn Greiner-Tollersrud, Børre Robertsen. The Journal of Immunology. Volume 189, Issue 12, December 2012, Pages 5912-5923.

This work reveals distinct roles of the two-cysteine-containing type I IFNs, IFNa and IFNd, and the four-cysteine-containing IFNb and IFNc in antiviral immunity of Atlantic salmon. IFNa and IFNc showed similar antiviral activities and ability to induce antiviral genes, IFNb was less active, and IFNd showed no activity. Expression of IFNs was compared by treatment of cells or fish with the dsRNA polyinosinic-polycytidylic acid (poly(I:C)), which induces IFNs via the viral RNA receptors MDA5 and TLR3/TLR22 and with the imidazoquinoline R848, which induces IFNs via TLR7. Poly(I:C) strongly induced IFNa in cell lines, whereas the other IFNs showed little response, indicating that IFNa is the main IFN subtype induced through the RIG-I/MDA5 pathway. In contrast, IFNb and IFNc are the main IFNs induced through the TLR7 pathway because R848 induced high transcript levels of IFNb and IFNc and low transcript levels of IFNa in the head kidney and spleen. IFNd was constitutively expressed in cells and organs but showed no response to poly(I:C) or R848. Fluorescence in situ hybridization studies showed that poly (I:C) induced IFNa and IFNc in a variety of cells in the head kidney, spleen, gills, liver, and heart, whereas R848 induced coexpression of IFNb and IFNc in distinct cells in head kidney and spleen. These cells are likely to be specialized high IFN producers because they were few in numbers despite high IFNb/IFNc transcript levels in the same organs. High IFN expression in response to TLR7 ligation is a feature shared by mammalian plasmacytoid dendritic cells.

**IV: Infectious salmon anemia virus (ISAV) is transiently inhibited by Atlantic salmon type I interferon.** Tina Svingerud, Jenni Kristin Holand, Børre Robertsen. Manuscript. 2012

In the present work we have investigated the antiviral activity of Atlantic salmon type I interferons (IFNs) against infectious salmon anemia (ISAV), which is an orthomyxovirus that causes a multisystemic disease in farmed salmon. Previous studies have shown that salmon IFNa1 provided little protection against ISAV-induced cytopathic effect (CPE). The present work demonstrates, however, that salmon IFNa1 induces antiviral activity in ASK cells against both the high virulent strain ISAV4 and the low virulent strain ISAV7, measured by qPCR of ISAV segment 6 RNA, Western blot analysis of the encoded protein hemagglutinin-esterase (HE) and reduction in viral titers. The antiviral effect lasted approximately 72 h after which virus replication increased in the IFNa1-stimulated cells and approached that observed in unstimulated ISAV4-infected cells. This thus most likely explains the lack of antiviral activity of salmon IFN against ISAV measured by the CPE reduction assay. A comparative study showed that IFNa1 and IFNc displayed comparable antiviral activity against ISAV4 while IFNb had less antiviral activity. This is in agreement with what was previously observed in studies of antiviral activity of IFNa1, IFNb and IFNc against IPNV. IFNa1 seemed to inhibit replication of ISAV7 somewhat more than of ISAV4, but both strains seemed able to overcome the antiviral state induced by IFNa1. On the other hand, ISAV7 induced the two IFN-inducible antiviral effector proteins, Mx and ISG15, to a higher degree than ISAV4 in untreated ASK cells, which suggests that the two strains may differ in their ability to promote production of IFN and/or IFN-induced antiviral proteins.

# 4. Discussion

The results of this work have been discussed in detail in the enclosed papers. In the following sections the general aspects of the discussions will be placed into context with each other, and some updated issues will be presented.

## 4.1 Antiviral activity of IFNa, IFNb, IFNc and IFNd

### IPNV

Atlantic salmon IFNa1 has previously been shown to possess antiviral activity against IPNV and salmonid alpha virus 3 [10, 218, 221, 224, 225]. The antiviral activity of Atlantic salmon IFNs of the IFNb, IFNc and IFNd subgroups was, however, unknown at the onset of this project. Paper I indicated that other IFNs than IFNa play an antiviral role in Atlantic salmon. Initially we observed that an antibody raised against IFNa1 could neutralize virtually all (> 99%) of the antiviral activity of recombinant IFNa1 and 95-98% of the antiviral activity produced by Atlantic salmon TO cells transfected with the viral mimic poly (I:C). Subsequent experiments showed that the same antibody in some cases neutralized only about 75% of the antiviral activity released from poly (I:C) stimulated primary head kidney leukocytes. This led us to hypothesize that leukocytes could produce other IFNs (e.g. IFNb and/or IFNc) in addition to IFNa when stimulated with poly (I:C), and that these IFNs also display antiviral activity. At the time of publication of paper I, genes encoding IFNb and IFNc had been detected in the Atlantic salmon genome [18], but nothing was known regarding their biological activity. In paper III we cloned and expressed recombinant IFNb and IFNc, and it was finally confirmed that also these IFNs display antiviral activity against IPNV.

Comparative studies of the salmon IFNs revealed that IFNa and IFNc display similar antiviral activity against IPNV, and that they also induced similar levels of ISGs such as PKR,

Mx and ISG15 (paper III). Although IFNb also inhibited propagation of IPNV, the effect was lower and the induction of the ISGs was delayed compared to IFNa and IFNc. Zebrafish IFNa (IFNΦ1) and IFNd (IFNΦ4) signal through a receptor composed of CRFB1 and CRFB5, whereas IFNc (IFNΦ2 and IFNΦ3) signal through a complex of CRFB2 and CRFB5 [21, 23]. In that context, the lower antiviral activity of IFNb could be due to a lower expression of a putative "IFNb-receptor" in the tested cell lines. Multiple CRFB genes have been discovered in the Atlantic salmon genome (unpublished data), but it is presently not known if Atlantic salmon like zebrafish expresses two (or more) type I IFN receptors. The lower activity of IFNb could also be due to a lower affinity and/or differential interaction with a receptor shared by all the IFNs. It is well known that the different mammalian type I IFNs show variable affinity for the IFNAR receptor, and that the antiviral activity the elicit is of variable potency [226].

IFNd displayed no antiviral activity against IPNV in TO and CHSE cells regardless of the type of expression system used (human, bacterial and salmonid cells; paper III). The lack of antiviral activity suggests that the cell lines either do not express the putative "IFNd-receptor", or that IFNd does not have inherent antiviral activity. In accordance with our results, zebrafish IFNΦ4 (which forms a clade with the IFNd subgroup [26]) does not protect zebrafish larva against infectious hematopoietic necrosis virus, and overexpression of IFNϕ4 in zebrafish embryos gives only a weak induction of viperin [21]. The induction was besides reported as "less reproducible" than that seen for the other IFNs [21].

### ISAV

Paper IV investigated the antiviral activity of IFNa, IFNb and IFNc against the *Orthomyxovirus* ISAV. Although the first mammalian type I IFN was identified by its ability to inhibit the *Orthomyxovirus* Influenza A [5], previous studies have failed at firmly establishing an antiviral role of salmon IFNa1 against ISAV using the CPE-reduction assay [199, 221]. In paper IV we observed that pretreatment of ASK cells with IFNa1 gave a slight inhibition of ISAV-induced CPE in experiments performed at the viral optimum temperature (i.e. 15°C). Western blot and qPCR studies further showed that the antiviral effect of IFNa1 was transient and that the effect declined four to five days after infection. This suggests that ISAV have evolved strategies to counteract the IFN-induced antiviral state and possibly that IFN-antagonistic proteins are accumulating during virus replication. Alternatively, the expression of antiviral proteins could be naturally decreasing during this time period. The temporary antiviral effects of the IFNs most likely explain previous difficulties to show inhibition of ISAV, as the ISAV CPE-reduction assay typically takes more than 5 days to complete. Similar to that observed for IPNV, IFNb did not give as strong protection against ISAV as IFNa and IFNc.

### IFN evasion strategies of ISAV

ISAV express two proteins with putative IFN antagonistic properties named s7ORF1 and S8ORF2 [222, 223]. The latter has been shown to inhibit activation of the salmon IFNa promoter, whereas the former has been shown to inhibit activation of both the salmon IFNa promoter and the rainbow trout Mx promoter [222, 223]. In paper IV, we compared the sensitivity of a high (ISAV4) and a low (ISAV7) virulent ISAV strain [227] to the IFN-induced antiviral state. Both strains seemed to be transiently inhibited by IFNa1, but the inhibition of ISAV7 was slightly higher than ISAV4 at five days after infection. ISAV7-

infection further induced higher levels of Mx and ISG15 protein than ISAV4 in unstimulated cells, which implicates that ISAV4 may have evolved more refined mechanisms than ISAV7 to evade or counteract the IFN system. Many viruses antagonize the IFN-system at one or multiple levels, and Randall et al. (2008) describe five main mechanisms that viruses use to escape the inhibitory effects of IFN: (i) inhibition of host gene expression or protein synthesis; (ii) to replicate in a manner that is unaffected by the antiviral actions of IFN; (iii) to evade recognition by PRR or their IFN-induction pathways; (iv) to block the IFN-signaling pathway or (v) to block the functions of antiviral protein [27]. Both ISAV4 and ISAV7 can thus potentially have developed mechanisms falling into class (v) and (iv), as they are only transiently inhibited by the IFN-induced antiviral state. ISAV4 additionally seems to evade/inhibit the production of Mx proteins more efficiently than ISAV7, which could be the outcome of any of the four first mechanisms.

### 4.2 Induction of IFNa, IFNb, IFNc and IFNd

The induction of the salmon type I IFNs were studied by different approaches. In paper I the secretion of IFNa by poly (I:C) stimulated TO cell and freshly isolated leukocytes was studied by antibody neutralization assays. In paper II, we cloned and studied the MDA5/RIG-I adaptor IPS-1 from Atlantic salmon, and investigated its capacity to induce IFNa. Paper III compared the induction patterns of IFNa, IFNb, IFNc and IFNd in cells and fish in response to poly (I:C) and the TLR7/8 ligand R848. The primers and probes used in the latter paper were designed to recognize (i) IFNa1/a2, (ii) all the IFNs of the IFNb subgroup (i.e. IFNb1 to IFNb4) and (iii) all the IFNs of the IFNc subgroups (i.e. IFNc1 to IFNc5). In the following sections, the term “IFNa” thus refers to IFNa1 and IFNa2, “IFNb” refers to all the IFNb members and the term “IFNc” refers to all the IFNc members.

### IFNa is the main IFN induced in poly (I:C) *in vitro*

It has been known for many years that Atlantic salmon cell lines secrete factors with antiviral activity after stimulation with poly (I:C). Gene expression studies have implicated that IFNa1 and/or IFNa2 are the key factors responsible for this antiviral activity [18]. As mentioned above (section 4.1), an antibody against Atlantic salmon IFNa1 could neutralize virtually all the antiviral activity released by poly (I:C) transfected TO cells (paper I). This strongly supports that IFNa1 and/or IFNa2 are the main antiviral factors released by these cells. Since IFNa1 and IFNa2 show 95% amino acid sequence identity [48], the assay could not distinguish the one IFN from the other. In agreement with the data from paper I, paper III showed that TO cells transfected with poly (I:C) massively upregulate IFNa (>4000-fold), whereas a minor upregulation was seen for IFNb (~30-fold) and IFNc (~150-fold). A similar induction pattern has been reported in rainbow trout RTG-2 cells, where IFN1 and IFN2 (both IFNa subgroup) are induced by poly (I:C), while IFN3 (IFNb subgroup) is not [14].

Transfection of mammalian cells with poly (I:C) predominantly activates MDA5 [65, 66], which through NF-κB and IRF3 induces an initial wave of IFNβ. mRNA transcripts of MDA5 are found in TO cells [18] and rainbow trout MDA5 bind poly (I:C) [70], which supports that fish and mammalian MDA5 play similar roles. As transfection of TO cells with poly (I:C) predominantly induced IFNa, it thus seems that this is the main IFN induced upon activation of Atlantic salmon MDA5. Analogous to IFNβ, the Atlantic salmon IFNa1 and IFNa2 promoters contain NF-κB and IRF binding sites [48] which further supports induction of these IFNs through the RLR pathway.

When poly (I:C) was added extracellularly to TO cells a slight induction of IFNa transcripts (~60-fold) was detected (paper III). TO cells showed detectable transcripts levels of TLR3, but not of TLR22 (paper III). The lack of TLR22 transcript in these cells suggests that IFNa here may be induced through TLR3. Freshly isolated head kidney leukocytes also

produced IFNa after addition of poly (I:C), but some cells seemed to additionally produce IFNb and/or IFNc (paper I). As both TLR3 and TLR22 transcripts were detected in leukocytes (paper III), the production of IFNa could possibly be initiated by activation of both receptors.

### IFNa is induced via IPS-1 and the RLR pathway

To study the RLR pathway and its role in induction of IFN, an Atlantic salmon IPS-1 (AsIPS-1) ortholog was cloned and studied in paper II. In mammals, IPS-1 is the key adaptor protein of the RLR pathway [74-77]. The mammalian IPS-1 sequence is composed of a C-terminal CARD domain, which interacts with the CARD domains of RIG-I/MDA5, a proline rich region and an N-terminal transmembrane domain that anchors the protein to the mitochondria [76]. The cloned AsIPS-1 sequence showed an overall low sequence identity with mammalian IPS-1 (18 to 21%), but all the three characteristic domains were identified. Overexpression of AsIPS-1 led to activation of both a NF-κB promoter and the salmon IFNa1 promoter and inhibited IPNV-induced CPE. This is in accordance with mammalian studies where overexpression of IPS-1 promotes antiviral immunity by induction of type I IFN through activation of NF-κB and IRF3 [74-77]. The inhibition of IPNV-induced CPE was further reduced (but not abolished) in the presence of an antibody against Atlantic salmon IFNa1, which supports that AsIPS-1 is involved in the production of IFNa-like molecules in CHSE cells. Since CHSE cells are derived from Chinook salmon, the lack of complete neutralization could be caused by sequence differences between Atlantic salmon and Chinook salmon IFNa.

The same year, Biacchesi and coworkers (2009) cloned IPS-1 orthologs from zebrafish, Atlantic salmon and EPC cells [68]. They presented additional phylogenetic and gene synteny data which supported that piscine IPS-1 sequences are orthologous to mammalian IPS-1. They also demonstrated that fish RIG-I is involved in induction of IFN and antiviral immunity [68]. Paper II together with the study of Biacchesi et al. represented the

two first studies to show functional conservation of the RLR-pathway from fish to higher vertebrates. Later studies have extended this knowledge, and MDA5, TBK1, STING and IRF3 have been confirmed to play important roles in the RLR signaling pathway also in fish [70, 71, 149, 228].

### Both IFNa and IFNc are induced by poly (I:C) in vivo

Paper I suggested that freshly isolated leukocytes stimulated with poly (I:C) produce IFNa but in certain cases also other IFNs. To study the induction pattern of the distinct IFN subgroups *in vivo,* poly (I:C) was injected into live fish (i.p.), organs were harvested after 12 h and samples were studied by qPCR and fluorescent in situ hybridization (FISH) (paper III). The qPCR analysis showed that IFNa was induced by poly (I:C) in in all the studied organs (i.e. head kidney, spleen, gills, liver, heart, brain, skin and ovaries). The FISH studies were in agreement with the qPCR data, and IFNa was found widely expressed in multiple cells in all the studied organs (head kidney, spleen, gills and liver). In the head kidney and spleen, IFNa transcripts were detected in cells in close proximity to the sinusoids (i.e. small blood vessels) and in endothelial cells lining the blood vessels.

Although IFNc only showed a minor upregulation in poly (I:C) transfected TO cells, a distinct upregulation was observed *in vivo* in several organs (head kidney, spleen and heart). In contrast to IFNa, IFNc showed a minor expression in liver and was not detected in endothelial cells of head kidney and spleen.

The differential expression of IFNa and IFNc in TO cells, endothelial cells, and liver tissue suggests that IFNa and IFNc can be induced by different cells and possibly through different PRRs. The observation that poly (I:C) induces high levels of IFNc *in vivo,* but not in the TLR22-negative TO cells, suggests that TLR22 is potential candidate.

### IFNb and IFNc are induced by R848 in vivo

In paper III we also studied the induction of Atlantic salmon IFNs in TO cells and fish in response to the TLR7/8 ligand R848. No upregulation of IFN was observed in TO cells treated with R848, which is in agreement with earlier work using the TLR7 ligand S-27609 [18, 229] and with the fact that no TLR7 transcripts were detected in TO cells (paper III). *In vivo,* R848 induced relatively high transcript levels of IFNb and IFNc in head kidney and spleen of R848-injected Atlantic salmon. Despite the relative high upregulation seen in the qPCR analysis, FISH analyses showed that only a few cells in head kidney and spleen were IFNb and IFNc positive. From this we deduced that that IFNb and IFNc are relatively highly upregulated by R848 in a few cells restricted to immunological organs. These cells seem to share certain similarities to the mammalian pDCs which are (i) few in numbers, (ii) which enter lymphoid tissues from the blood where they (iii) induce large amount of IFNα upon activation of TLR7 [125]. If the high IFNb/c-producing salmon cells are homologous to pDCs, represents a pDC precursor cell, or have a completely different origin is at present unknown. Dendritic cells have only recently been identified in rainbow trout and zebrafish [152, 153], and the scarcity of cell-specific markers in fish hampers the characterization of distinct cell types.

### What about IFNd?

Atlantic salmon IFNd was neither induced by poly (I:C) nor R848 in TO cells or in fish (paper III). In agreement with our results, the zebrafish IFNϕ4 (IFNd subgroup) promoter was not activated in poly (I:C) transfected CAB cells [71]. The results are, however, somewhat in conflict with a study by Chang et al. (2009), where rainbow trout IFN5 (IFNd subgroup) was induced in RTG-2 cells stimulated with poly (I:C) [17]. Differences in the IFNd promoter regions could possibly explain the variable induction properties; while the rainbow trout IFNd

promoter contains two IRF binding sites [17], the salmon and zebrafish IFNd promoters contain only a single IRF binding site ([71]; paper III).

The apparent lack of antiviral activity of Atlantic salmon IFNd and the absence of induction in response to viral mimics, suggests that this IFN might have evolved an atypical role in Atlantic salmon or alternatively that it is a nonfunctional gene. Induction wise, there is, however, still a possibility that Atlantic salmon IFNd is induced through a receptor that recognizes other ligands than poly (I:C) and R848. Since no antiviral activity of IFNd has been confirmed in species that belong to the superorders *Ostariophysi* (e.g. zebrafish) and *Protacanthopterygii* (e.g. Atlantic salmon and rainbow trout) the role of IFNd from these species will be an interesting topic for future studies.

Fish species that belong to the superorder *Acanthopterygii* (e.g. Japanese flounder, medaka, stickleback, sea bass, green spotted puffer and rock bream) seem to only possess IFNs of the IFNd subgroup [11, 16, 230, 231]. Antiviral activity of these IFNs has not yet been confirmed, but recombinant IFNs from green spotted puffer and rock bream induce a moderate upregulation of Mx [11, 231]. IFNd from Japanese flounder has further been shown to be induced by poly (I:C) and by overexpression of MDA5, which suggests that this IFN plays a role in antiviral immunity [230].

It is estimated that the split between the Acanthopterygii/Ostariophysi and the Protacanthopterygii superorders occurred 217 million years ago [232]. During this time, IFNs of subgroup IFNd thus seem to have evolved to be “the” antiviral IFNs in *Acanthopterygii* species, while the role of IFNd is more unclear in fish species of the *Protacanthopterygii* and *Ostariophysi* superorders.

**Summary - induction of IFNa, IFNb and IFNc**

We found that IFNa was widely induced in all the studied cells (TO, ASK, primary leukocytes) and organs in response to the MDA5/TLR3/TLR22 ligand poly (I:C), and that IFNa was also induced by overexpression of the RLR adaptor IPS-1. IFNb and IFNc, on the other hand, were relatively highly induced by the TLR7/8 ligand R848 in distinct cells present in immunological organs. IFNc additionally showed high transcript levels in certain organs of fish injected with poly (I:C), which was in contrast to the marginal expression seen in TO cells.

Although the ligand specificity of the fish RLRs and TLRs have been little studied, some results indicate that the fish receptors have similar specificities as those of mammals. Fugu TLR3 has been shown to induce IFN in rainbow trout RTG-2 cells upon recognition of poly (I:C) [112], while rainbow trout MDA5 has been confirmed to bind poly (I:C) [70]. The qPCR data from paper III further supports that R848 signals through TLR7 in Atlantic salmon since R848-responsive primary head kidney leukocytes displayed detectable levels of TLR7 transcripts, whereas the R848-unresponsive TO cells did not. A recent study further revealed that out of fifteen different TLRs (TLR1-TLR14, TLR21 and TLR22/23) the ligand binding domains (i.e. LRR) of TLR3 and TLR7 are the most highly conserved throughout vertebrate evolution [233], which supports that ligands specificity of these receptors are conserved.

Assuming that TLR3, TLR7 and MDA5 have a similar ligand specificity in fish and mammals, a possible scenario for induction of Atlantic salmon IFNs is as follows: IFNa is widely induced in many cell types by activation of the TLR3 and/or RLR pathways, IFNb and IFNc are relatively highly induced by a few immune cells through the TLR7 pathway, while IFNc is induced by yet another pathway, possibly TLR22 (Fig. 9). This has been deduced from the observation that TO cells, which predominantly induce IFNa upon poly (I:C) stimulation (paper I, paper III), show detectable transcript levels of MDA5 [18] and TLR3,

but not of TLR22 (paper III). And further by the observation that poly (I:C) induces relative high transcript levels of IFNc *in vivo*, but not in the TLR22-negative TO cells. This model has, however, not taken into account that activation of the same PRR might induce variable responses in different cell types due to differences in the intracellular signaling pathways from one cell type to another.

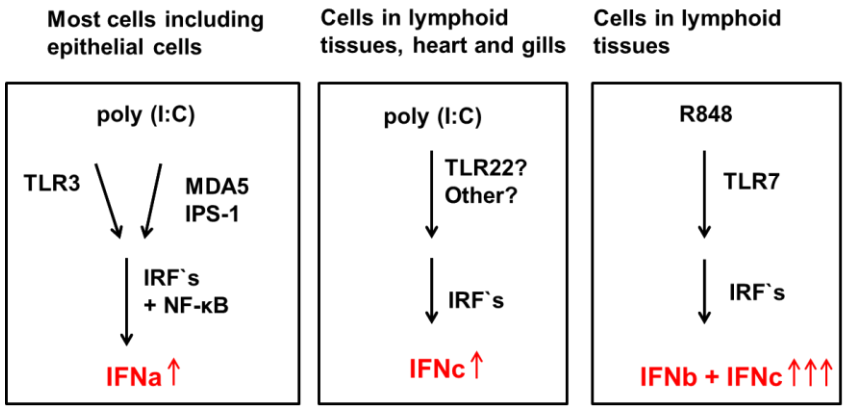


Fig. 9. Proposed simplified model of pathways that controls the induction of the distinct Atlantic salmon IFN subgroups.

### 4.3 Concluding remarks

This work suggests that Atlantic salmon IFNa, IFNb, IFNc and IFNd play distinct roles in Atlantic salmon since the IFNs (i) display variable antiviral potency and gene-inducing capacity and (ii) are induced at different levels and in different cells and organs in response to dsRNA and ssRNA mimics.

The induction of Atlantic salmon type I IFNs somewhat resembles the induction pattern of mammalian IFNα and IFNβ. One the one hand, IFNa is like IFNβ induced widely in many cell types by the TLR3/MDA5/TLR22 ligand poly (I:C) (paper I and paper III) and NF-κB seems to play a role in the induction (paper II). On the other hand, IFNb and IFNc are similarly to mammalian IFNαs induced relatively highly in a few cells in response to the TLR7 ligand R848 (paper III). The expansion of the mammalian IFN genes is thought to have

occurred after the divergence of birds from mammals [234], and the mammalian IFNα and IFNβ genes thus do not have direct orthologs in fish. That fish and mammals independently have evolved certain similar mechanisms for induction of distinct IFN genes, suggests that it is highly important to have a complex type I IFN system that can be induced by different viral ligands in different cells. It also suggests the induction of mammalian IFNs occur at least partly through pathways that already existed in an ancestor fish. Hertzog (2012) has proposed that multiple type I IFNs exists for two reasons: "first, that they have different properties; and second, that they are produced at different times and in different places" [235]. The data from the present work fits well with this hypothesis.

### 4.4 Future perspectives

There are many opportunities to characterize the Atlantic salmon IFNs further. Cloning and functional studies of putative Atlantic salmon IFN receptor genes could possibly explain the cause of the lower antiviral potency of IFNb compared to IFNa and IFNc, and clarify whether the lower activity is due to usage of an IFNb-specific receptor. Other interesting aspects could be to elucidate the role of TLR22 in induction of IFNc, and to determine the ligand specificity of distinct PRRs. The induction of the distinct salmon IFNs could also be further studied by cloning the IFNb and IFNc promoters for use in reporter gene assays. From an evolutionary view point, it would particularly interesting to characterize the R848-responsive IFNb/IFNc producing cells in head kidney and spleen. To do this, it would be invaluable to develop antibodies against cell-specific surface components. Finally, it would be appealing to study the potential use of TLR/RLR ligands and type I IFNs as vaccine adjuvants in fish. Studies in mouse and humans suggest that IFNα and R848 have the potential to act as antiviral vaccine adjuvant [236, 237], and this would be a most exciting field to investigate further in fish.

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# PAPER I

# PAPER II

# PAPER III

# PAPER IV